EXPERIMENTAL SEIZURE MODELS AND NEW ANTIEPILEPTIC DRUGS

A thesis by

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SUMMARY

The objective of this programme of work was to study experimental seizure models and new antiepileptic drugs. Initial investigations addressed the contribution of basic animal models of epilepsy to its experimental study. Next, an attempt was made to emphasise the crucial role of neuronal inhibition and excitation in epileptogenesis and to relate these phenomena to the study of novel antiepileptic agents. Finally, the future of epilepsy research, in terms of appropriate strategies for AED development and innovative experimental paradigms was examined.

Investigation of basic animal seizure models

Of all the experimental seizure models in current laboratory employment, the pentylenetetrazol (PTZ) test, and the maximal (MES) and minimal (Min-ES) electroshock tests are among the most popular by virtue of their simplicity and economy. The primary aim of these studies was to afford a familiarity with these three basic animal seizure models and to validate them as techniques for subsequent use. These studies also incorporated an investigation of concentration-effect relationships with PTZ which attempted to delineate previously observed efficacy problems with this compound in our laboratory. Validation of all three experimental models was satisfactory, with results reflecting those reported in the literature. Although the concentration-effect studies with PTZ afforded a degree of insight into its pharmacokinetics, attempts to provide a suitable explanation for its lack of convulsant action in some animals proved unsuccessful.

Antiepileptic drug enhancement of neuronal inhibition

Impairment of γ-aminobutyric acid (GABA)-mediated neuronal inhibition is believed to be one of the fundamental aetiological mechanisms of epileptogenesis. These investigations compared and contrasted the experimental anticonvulsant profiles and mechanisms of action of vigabatrin (VGB) and tiagabine (TGB), two novel AEDs which have been proposed to enhance GABA-mediated inhibition. VGB raised the threshold for induction of tonic seizures, determined by the Min-ES test, but was without effect in the PTZ and MES tests. TGB, in contrast, exhibited anticonvulsant effects against both PTZ- and MES-induced seizures. Drug mechanisms were investigated in isolated brain tissue and in primary cultures of cerebral cortical astrocytes and neurones. Previously reported mechanisms of action of the two drugs were confirmed, with VGB inhibiting GABA metabolism by an action on GABA-aminotransferase (GABA-T), and TGB blocking GABA uptake in a non-cell-specific manner. An inhibitory effect of VGB on glutamic acid decarboxylase (GAD) was also verified, and an additional, previously unreported action of the drug on GABA uptake was proposed.

Antiepileptic drug attenuation of neuronal excitation

Glutamate-induced neuronal excitability and voltage-sensitive calcium influx are believed to be inexorably entwined at all stages of epileptogenesis. These studies compared and contrasted the experimental anticonvulsant profiles and mechanisms of action of nimodipine (NMD) and amlodipine (AML), members of the dihydropyridine (DHP) class of calcium channel blockers which have been proposed as putative AEDs. In single dose, NMD was effective against MES-induced seizures and also raised the tonic seizure threshold, determined by the Min-ES test. Its effects in the MES test appeared to extend

beyond the time suggested by its pharmacokinetic profile, determined by concomitant analysis of drug concentrations in the plasma and brain. NMD exhibited a substantial loss of anticonvulsant efficacy following repeated administration. Single dose AML protected against PTZ induced seizures but was essentially without effect in the MES test. Studies of drug mechanisms employed primary cultures of cerebellar granule cell neurones and cerebral cortical astrocytes. NMD reduced depolarisation-induced calcium influx in a non-cell-specific manner, but was without effect on neuronal glutamate release. AML limited neuronal calcium entry induced by depolarising concentrations of potassium alone.

Combinations of novel antiepileptic drugs

Previously unexplored combinations of novel antiepileptic agents are now under investigation in an attempt to address the significant clinical problem of drug-resistant epilepsy. This study explored the nature and extent of any interaction between VGB and lamotrigine (LTG), both recently licensed for the treatment of refractory seizures. The experimental parameters chosen for investigation were the MES test, brain GABA-T and GAD activities, and brain GABA and glutamate concentrations. LTG effectively reduced MES-induced seizures, but was without effect on any of the selected neurochemical parameters. In contrast, VGB was devoid of anticonvulsant activity, while exhibiting effects on the GABAergic system consistent with its putative mechanism of action. The effects of experimental combination therapy did not differ from those observed with the respective monotherapies. Thus, it was concluded that there was no experimental interaction between LTG and VGB on the parameters selected for evaluation.

Novel strategies for antiepileptic drug development

It has been proposed that to satisfactorily address the problem of refractory epilepsy the development of novel antiepileptic agents with similarly novel mechanisms of action is required. Nicotinylalanine (NA) is a newly-synthesised neuroactive compound which is believed to exert its effects by inhibition of the kynurenine pathway, resulting in increased brain concentrations of kynurenic acid, an endogenous antagonist at the glycine recognition site on the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor. This study explored the anticonvulsant profile of NA in three standard animal models of seizure. NA protected against PTZ induced seizures in mice in a dose and time dependent manner and was also active in the maximal and minimal electroshock tests. These preliminary results would suggest that NA warrants further investigation as a putative AED.

Development of a novel animal model of epilepsy

It is now recognised that few, if any, of the existing "animal models of the epilepsies" mirror the condition of chronically recurrent spontaneous seizures which is characteristic of human epilepsy. This study followed the preliminary development of an innovative model of partial epilepsy, proposed to more closely mimic the human condition. This model was characterised by a laser-induced lesion in the rat somatosensory cortex. Production of cortical laser lesions in the rat proved to be a feasible procedure. Histological investigation proposed the lesions to be highly reproducible and to possess cellular characteristics similar to those of disruptive brain insults in man. The lesion did not appear to be intrinsically epileptogenic, nor did the procedure influence the latency to generalised PTZ-induced seizures. Preliminary autoradiographical studies suggested that

brain damage associated with the procedure was confined to the lesion tract itself, and that cerebral glucose metabolism was additionally altered in adjacent, otherwise healthy, tissue. Despite possessing several attractive features, the full potential of this model for use in epilepsy research remains to be evaluated.

DECLARATION

I declare that this thesis was composed by myself and, except where acknowledged, is a record of work performed by myself. It has not been submitted previously for a higher degree.

This research was conducted in the Department of Medicine and Therapeutics, University of Glasgow, under the supervision of Dr M.J. Brodie.

Graeme J. Sills.
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PEER REVIEWED PUBLICATIONS

Nicotinylalanine increases cerebral kynurenic acid content and has anticonvulsant activity.
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ABBREVIATIONS

A (mA)	amperes (milliamperes)
AED	antiepileptic drug
AML	amlodipine
4-AP	4-aminopyridine
ATP	adenosine triphosphate
BZD	benzodiazepine
CA	calcium antagonist
cAMP	cyclic adenosine monophosphate
CBZ	carbamazepine
CC_{50}	convulsive current in 50% of animals
CD_{97}	convulsive dose in 97% of animals
Ci (mCi, μCi)	Curie (millicurie, microcurie)
CNS	central nervous system
CSF	cerebrospinal fluid
DHP	dihydropyridine
DMCM	methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
EAA	excitatory amino acid
ECS	electroconvulsive shock
ECT	electroconvulsive therapy
ED_{50}	effective dose in 50% of animals
EEG	electroencephalogram

ESM	ethosuximide
ETB	eterobarb
FBM	felbamate
FNZ	flunarizine
g (kg, mg, μg, ng)	gramme (kilogramme, milligramme, microgramme, nanogramme)
GABA	γ-aminobutyric acid
GABA-T	GABA-aminotransferase
GAD	glutamic acid decarboxylase
GBP	gabapentin
GEPR	genetically epilepsy-prone rat
GHB	γ-hydroxybutyrate
HeNe	helium/neon (laser)
HPLC	high performance liquid chromatography
IC_{50}	inhibitory concentration reducing response by 50%
ILAE	International League Against Epilepsy
IPSP	inhibitory post-synaptic potential
JME	juvenile myoclonic epilepsy
l (ml, μl)	litre (millilitre, microlitre)
LCGU	local cerebral glucose utilisation
LCZ	loreclezole
LSM	losigamone
LTG	lamotrigine
M (mM, μM, nM)	molar (moles per litre; millimolar, micromolar, nanomolar)
MES	maximal electroshock

Min-ES	minimal electroshock
MMP	N-monomethoxymethyl-phenobarbital
mol (mmol, μmol, nmol, pmol)	moles (millimoles, micromoles, nanomoles, picomoles)
3-MPA	3-mercaptopropionic acid
NA	nicotinylalanine
NAD	nicotinyl adenine dinucleotide
NFD	nifedipine
NIH	National Institutes of Health
NINCDS	National Institute of Neurological and Communicative Disorders and Stroke
NMD	nimodipine
NMDA	N-methyl-D-aspartate
NMDLA	N-methyl-D,L-aspartate
OXC	oxcarbazepine
PB	phenobarbitone
PCP	phencyclidine
PDS	paroxysmal depolarising shift
PHT	phenytoin
PRIM	primidone
PTZ	pentylenetetrazol
RLT	ralitoline
RMD	remacemide
rpm	revolutions per minute
SD	Sprague Dawley
SEM	standard error of the mean

SSAD	succinic semialdehyde dehydrogenase
STP	stiripentol
TGB	tiagabine
THE	tonic hind-limb extension
TPM	topiramate
VGB	vigabatrin
VPA	sodium valproate
ZNS	zonisamide

CHAPTER ONE

GENERAL INTRODUCTION

1.1 INTRODUCTION TO EPILEPSY

1.1.1 Definition of epilepsy

Epilepsy is one of the most common afflictions of man. With a prevalence of around 0.5 - 1.0 %, it is estimated that 50 million persons world-wide may have the disorder (Rogawski and Porter, 1990). Epilepsy is not a disease, but rather a collection of diverse syndromes, some of which are secondary to brain derangements, and some of which seem primarily genetic (Fisher, 1989). John Hughlings Jackson, one of the founders of modern neurology, defined epilepsy as "an episodic disorder of the nervous system arising from the excessively synchronous and sustained discharge of a group of neurones" (Jackson, 1870). Such a definition, unprecedented in its time, successfully encompasses the diverse clinical manifestations of the disease including disturbance of both motor and cognitive function. It also conforms to the precept that a single seizure is not epilepsy and emphasises the crucial role of neuronal excitation and inhibition in epileptogenesis (Meldrum, 1989). It remains the conventional wisdom, as borne out in a recent review by Meyer (1989), in which he proposes that "the excessive repetitive discharge of neurones is the basic dysfunction at the cellular level in clinical epilepsy." Thus, epilepsy is a disorder intrinsic to the brain: either "nature" or "nurture" has rendered a portion of the brain electrically unstable.

1.1.2 History of epilepsy

Epilepsy has been a recognised disorder for approximately 4 millennia. It is from the Greek word *epilambanein* from which "epilepsy" and "epileptic" are derived. It means "to seize" or "to attack" and was used to describe the ictal event and the afflicted person because seizures were noted to "seize" the body and/or the senses (Gross, 1992). Such

nomenclature remains in use today, but the use of the word "seizure" also suggests a cause of the ictus, namely, that one could be "seized" from without. The Egyptians and Babylonians recognised seizures as being associated with physical ills, such as head trauma, but, like the Greeks and Romans, sometimes ascribed their occurrence to causes more magical or occult than physical. Epileptics were thus often considered unclean or evil and Pliny advised persons to spit upon seeing an epileptic, "to throw back the contagion" (Temkin, 1971). Such magical thinking about seizures persists, in altered forms, and is part of the stigma attached to epilepsy today (Gross, 1992).

In many societies, the aetiology of epilepsy was attributed to the work of evil spirits. The oldest written account of epilepsy so far known - that of a Babylonian treatise discovered on a stone tablet in South Turkey (Kinnier Wilson and Reynolds, 1990) - is adamant: "If epilepsy falls once upon a person....it is the result of possession by a demon or departed spirit" and goes on to describe which demons cause which type of seizure. Hippocrates (c. 400 B.C.) was the first to attempt to dispel the prevalent thinking of the time that epilepsy was a sacred disease, that is, of divine or demonic cause. He defined this concept of "The Sacred Disease" as "a shelter for ignorant and fraudulent practices" (Temkin, 1971). He suggested that epilepsy was inherited and his suspicions centred upon the brain from where, he felt, an excess of phlegm overspilled into the bloodstream, the symptoms of the attack being dependent on the organs affected (Gross, 1992). Widespread belief in the epileptogenic effect of possession by spirits or demons, however, were slow to disappear.

In Biblical times it remained the conventional wisdom, as witness the Evangelist Mark's description of "the epileptic demoniac": "there is a spirit of dumbness in him, and when it takes hold of him it throws him to the ground, and he foams at the mouth and grinds his teeth and goes rigid" (Mark 9: 18). To this Luke adds: "it is slow to leave him, but when it does it leaves the boy worn out" (Luke 9: 37).

Progress in understanding the "falling sickness" (attributed to Paracelsus; cf. Esquirol, 1845) was slow. Galen's idea (130 - 200 A.D.) of "animal spirits" in body tubes was still being quoted by Willis in the late 17th century (Stevens, 1973). Social attitudes were also slow to progress. Although Aristotle had long before espoused an association between epilepsy and genius (still contemplated to this day cf. Caesar, Petrarch, Napoleon, Moliere, Van Gogh, Dostoevsky), Albich in the 18th century warned "neither talk nor bathe with them, since by their mere breath they infect people" (Temkin, 1971).

During the more rational approach of the "Enlightenment" period of the 18th and 19th centuries, attempts were made to classify epilepsy into idiopathic and secondary epilepsies, whose causes ranged from the respectably traumatic, to the witnessing of a seizure while pregnant (Esquirol, 1845). The association with the phases of the moon was scientifically laid to rest by Leuret (Temkin, 1971), notwithstanding today's interest in catamenial epilepsy, which was also noted in the early 18th century by Maisonneuve (Esquirol, 1845).

Modern epilepsy may have begun with John Hughlings Jackson who linked new electrophysiological evidence discovered by Fritsch and Hitzig (1870) with clinical

observations in his "Investigations of Epilepsies" (1873). Jackson believed that epilepsy was due to a discharge, a paroxysmal, excessive discharge of neurones within a local area that could then affect more normal areas of the brain. Here, finally, was a statement that linked the diverse symptomatology of seizures with their physiology: "According as the seat of the discharge varies, the symptoms of the paroxysm vary." The distinction between idiopathic and symptomatic epilepsy was thus mooted and the hypothesis raised that all seizures have a similar pathophysiology - a position still actively investigated (Gross, 1992).

Objective evaluations of the epilepsies ensued. Gowers' elegant descriptions of seizure states around the turn of the century were remarkable in their detail and depth. He discussed his cases in light of recent physiological advances and emphasised the relationship of focal seizures to focal lesions and the lack of focal pathology in cases of generalised seizures (Gowers, 1881). This, together with the work of Penfield and Jasper (1954), who reported a relationship between the functional anatomy of the brain and the specific nature of ictal activity, laid the foundations of the present day understanding of the epilepsies.

1.1.3 Classification of seizures

The characteristic event in epilepsy is the seizure which is associated with the episodic high frequency discharge of impulses by a group of neurones in the brain. What starts as a local abnormal discharge may then spread to other areas of the brain. The site of the primary discharge and the speed and extent of its spread confer the specificity of seizure type produced. The clinically-observed symptoms, ranging from a brief lapse of

concentration to a full-blown convulsive fit lasting minutes, are ultimately dependent upon the function of the brain region involved. Thus, involvement of the motor cortex causes convulsions, involvement of the hypothalamus results in peripheral autonomic discharge, and involvement of the reticular formation of the brainstem leads to loss of consciousness (Rang and Dale, 1987).

There are many types of epileptic seizures and a detailed description and classification of these is required. Individual seizures can be classified in many ways, by the clinical expression of the seizure, the characteristic electroencephalographic (EEG) changes, cellular aetiology, pathophysiology, anatomy, or age (Shorvon, 1990). Since epilepsy is often best regarded as a symptom rather than as a condition *per se* and since the aetiology, pathophysiology, and anatomy are often obscure, classification is inevitably arbitrary.

The most practical classifications are based on the clinical expression of seizures (Meldrum, 1989). This is exemplified by the classification drawn up by the Commission on Terminology of the International League Against Epilepsy (ILAE) in 1969. This scheme, revised in 1981 and widely adopted, is a classification of seizure type in which EEG data are taken into account, whereas aetiology, age, and anatomical site are ignored (Shorvon, 1990). The key factor is the local or generalised onset of the seizure as evaluated from clinical observation and electroencephalography. The major categories of this classification are illustrated in table 1.

Partial seizures are those in which the discharge remains localised. Simple partial seizures may have relatively modest symptoms without loss of consciousness. They

1. Partial seizures (seizures beginning locally)

   A. Simple partial seizures (consciousness not impaired)

      1. with motor symptoms

      2. with somatosensory or special sensory symptoms

      3. with autonomic symptoms

      4. with psychic symptoms

   B. Complex partial seizures (with impairment of consciousness)

      1. simple partial onset followed by impairment of consciousness

      2. with impairment of conciousness at onset

2. Generalised seizures (convulsive or non-convulsive)

   A. Absence

      1. Typical absences

      2. Atypical absences

   B. Myoclonic seizures

   C. Clonic seizures

   D. Tonic seizures

   E. Tonic-clonic seizures

   F. Atonic seizures

3. Unclassified epileptic seizures

Table 1:- Simplified version of the International classification of epileptic seizures (Commission on classification and terminology of the ILAE, 1981).

may be manifested as involuntary muscle contractions, abnormal sensory experiences, or autonomic discharge, dependent upon the brain region in which the abnormal focus is localised (Rang and Dale, 1987). Complex partial seizures are often associated with a focus in the temporal lobe (Meldrum, 1990) and are manifested by a loss of sensory awareness and/or the precipitation of stereotyped behaviour. Such behavioural patterns may consist of rubbing or patting movements or more elaborate movements such as dressing, walking, or hair-combing (Rang and Dale, 1987). Secondary generalised partial seizures have an initial focal component, either simple or complex, characterised by early unilateral EEG abnormalities, but propagate to fully generalised bilateral seizures (Meldrum, 1990). The underlying mechanism of this progression is not understood.

Generalised seizures almost certainly begin locally, but spread rapidly to involve the reticular system thus producing abnormal electrical activity throughout both hemispheres. Immediate loss of consciousness is characteristic of generalised seizures (Rang and Dale, 1987). The main categories are tonic-clonic seizures (grand mal), absences (petit mal), and myoclonic seizures. A tonic-clonic seizure consists of an initial strong contraction of the musculature causing a rigid extensor spasm. Respiration may cease and micturition and salivation often occur. The tonic phase lasts for about 1 minute and is followed by a series of violent synchronous jerks which gradually cessate over 2 - 4 minutes. The patient may remain unconscious for a few more minutes and gradually recovers although confusion may ensue (Rang and Dale, 1987). Absence seizures are most common in children and are much less dramatic than tonic-clonic seizures but tend to occur more frequently. The patient abruptly ceases whatever he or

she is doing and stares vacantly for a few seconds, with little or no motor disturbance. The patient is unaware of his or her surroundings. Recovery occurs suddenly, often with no after-effects (Rang and Dale, 1987). Myoclonic seizures are also more prevalent among children and infants. They are characterised by brief jerks of the arms or legs lasting 1 - 5 seconds. These events often occur in series (Gram, 1990). Juvenile myoclonic epilepsy (JME) is a genetically determined syndrome which has an onset around puberty (Janz, 1985). It is a common syndrome, presenting in about 5% of all cases of epilepsy. JME is characterised by phases of all three previously discussed generalised events. Initially, myoclonic jerks are observed and these are followed by sporadic periods of tonic-clonic seizures and, less frequently, absence attacks (Gram, 1990).

Recently, in recognition of the fact that a seizure-type classification does not account for other aspects of the heterogeneity of epilepsy, the ILAE devised a new scheme - the Classification of the Epilepsies and Epileptic Syndromes and related seizure disorders - which is now widely employed and is an attempt to categorise the epilepsies more comprehensively (Shorvon, 1990). This classification takes into account seizure type, EEG characteristics and prognostic, pathophysiological, and aetiological data. It retains the old division of epilepsy into partial and generalised (now called localisation-related) categories, with each category subdivided into symptomatic (cause known) and idiopathic (cause unknown) varieties. Two new categories are added; epilepsies and syndromes undetermined, whether focal or generalised, and special syndromes.

1.1.4 Cellular neurophysiology of epilepsy

The hypothesis of Hughlings Jackson that the critical abnormality responsible for epileptic phenomena is the excessive and synchronous discharge of groups of neurones has been amply confirmed. However, the cellular mechanisms which underlie epileptogenesis remain conjectural and the precise neuronal networks and mechanisms responsible for the spread of seizures have not been unequivocally identified. In a sense, this lack of knowledge has impeded advances in antiepileptic therapy, just as lack of anatomical knowledge slowed the advances in thinking about epileptic syndromes, physiology, and aetiology in past centuries (Gross, 1992).

Just as seizure activity has diverse clinical manifestations, then it is likely that different seizure types have differing neurophysiological bases. Indeed, studies on feline generalised epilepsy have suggested that the underlying mechanisms might be different from those of focal, or partial, epilepsy (Gloor and Fariello, 1988). At present, the basis of focal epilepsy is better understood than that of the generalised epilepsies. The primary cellular events in focal epileptogenesis can be divided into two distinct phenomena, interictal and ictal discharges (Dingledine et al, 1990). The concept of interictal and ictal events derives from electrographic-clinical correlations which contrast brief events often occurring focally (spikes, sharp waves, spikes and waves) without clinical motor manifestations with sustained paroxysmal activity in association with clinical seizures (Meldrum, 1988).

Interictal events are considered to be initially characterised by burst firing in single neurones (Meyer et al, 1986a). This phenomenon is almost universal, having been

observed in acute *in vitro* models, as well as in chronic seizure foci in animals and man. The cellular mechanisms proposed to underlie the initiation of neuronal burst firing are discussed in section 1.1.5. Burst firing is recorded intracellularly as a distinctive paroxysmal depolarising shift (PDS) in the resting membrane potential. It is characterised by a short but sustained period of depolarisation accompanied by a burst of action potentials (Ayala et al, 1970). The PDS is followed by a phase of hyperpolarisation, which is believed to prevent the discharge of closely recurrent PDSs. This hyperpolarisation is thought to be mediated, at least in part, by the input of inhibitory interneurones (Meldrum, 1988). The PDS is the contribution of the individual neurone to the extracellular field potential recorded as the interictal spike on EEG tracings (Matsumoto and Ajmone-Marsan, 1964).

Interictal events can spread horizontally within the same class of neurones and, hence, burst firing in a single neurone can precipitate synchronous burst firing of other neurones within the same neuronal aggregate (Meyer et al, 1986a). While recruitment occurs within a single neuronal aggregate, it invariably remains localised due to inhibitory mechanisms that limit the spread of the discharge both spatially and temporally (Meldrum, 1988). The exact mechanisms synchronising interictal events in individual neuronal aggregates are poorly understood but they are certainly synapse-mediated (Traub et al, 1987), and may also involve electrical connections (gap junctions) between neurones, extraneuronal ionic changes, and ephaptic spread (Dichter and Ayala, 1987).

Synaptic spread of interictal firing to other neuronal aggregates occurs to a variable extent and increases as the abnormal events become more intense and rhythmically recurrent (Meldrum, 1988). The extent of the spread of the interictal discharge may be conferred by the strength of local inhibitory control mechanisms. Interictal discharges are initially associated with enhanced inhibitory activity in projection areas. However, with interictal repetition, inhibitory activity fades and excitatory neurotransmission predominates, leading to synchronised burst firing in subsequent, synaptically-linked neuronal aggregates (Meldrum, 1990). This spread of epileptic activity is likely to rely on the well documented phenomenon of frequency-dependent inhibitory fade (Collingridge and Singer, 1990).

By their definition, interictal discharges fade out or are subject to inhibition before being transmitted to the descending motor neurones. Termination of the interictal discharge is associated with arrest of burst firing and replacement of the PDS with sustained hyperpolarisation, probably as a consequence of active inhibitory processes (Meldrum, 1990).

The transition from the interictal to ictal electrical event is poorly understood, but like the spread of interictal events might rely on the frequency-dependent depression of local inhibitory control mechanisms (Prince, 1985). Under the conditions of disinhibition, not only is the interictal discharge propagated to the subsequent neuronal aggregate but the phase of hyperpolarisation associated with the termination of the interictal burst is also greatly reduced. Thus, interictal discharges become even more frequent and are propagated to a greater extent. Ictal events, or electrographic seizures, have indeed been

likened to successive fusions of PDSs without intervening repolarisation (Meldrum, 1988). They are characteristically long-lasting depolarisations of the neuronal membrane with accompanying bursts of action potentials (Ayala et al, 1970). Again, under the conditions of disinhibition, it is proposed that this abnormal electrical activity is sufficiently sustained to propagate via excitatory circuits to the descending motor neurones and that this results in seizure generation.

1.1.5 Cellular mechanisms of spontaneous neuronal discharge

As discussed in section 1.1.4, the common initiator of spontaneous neuronal discharges is believed to be burst firing of single neurones. It has been proposed that this phenomenon can occur in several ways. It may arise spontaneously as an intrinsic abnormality in an "epileptic" neurone. It may also generate in the soma of a normal neurone in response to an abnormal synaptic input at the nerve terminal-dendrite interface or as a function of a defect in regulation of the local ionic micro-environment (Meldrum, 1989). Each of these phenomena could, in turn, be precipitated by innumerable cellular and/or molecular abnormalities. Some of the more commonly reported aetiological mechanisms proposed to underlie neuronal burst firing are now discussed.

Biochemical defects have no apparent origin and thus might underlie the more idiopathic epilepsies. Such defects include abnormalities in membrane ion transport and conductance, metabolic derangements of endogenous convulsant compounds, and functional impairment of primary neurotransmitter systems (Meldrum, 1989). Their

ability to be manifested in otherwise normal brain tissue might suggest them to be genetically determined defects.

Certain neurones, most notably the pyramidal cells of the hippocampus and neocortex, have the ability to exhibit spontaneous paroxysmal depolarisations, with accompanying bursts of action potentials, under normal conditions (Meyer et al, 1986a). The biochemical abnormalities that may predispose such neurones to generate abnormal electrical activity are, however, poorly understood. One of the most frequently reported defects in epileptic brain tissue is that associated with the cell membrane ionic pumps driven by adenosine triphosphate (ATP) hydrolysis (Grisar et al, 1992). Membrane-bound enzyme systems, such as the Na^+-K^+ ATPase and the Ca^{2+}-dependent ATPase, have frequently been reported to be abnormal in human epileptogenic tissue and in both induced and genetic animal models of epilepsy (Meldrum, 1989). A reduction in Na-K dependent ATPase has been reported in human focal epileptogenic tissue (Grisar and Delgado-Escueta, 1986) and at experimental seizure foci (Grisar et al, 1992). In DBA/2 mice, susceptible to seizures induced by audiogenic stimulation, an analogous reduction in the activity of Na-K dependent was initially reported and subsequently a selective reduction in the activity of Ca^{2+}-activated ATPase was also described (Palayoor et al, 1986). Other models of epilepsy, including chicks and gerbils with a genetic propensity to reflex epilepsy, show abnormalities in the ATPase systems (Rosenblatt et al, 1977). In addition to abnormalities in membrane ATPases, defects in potassium conductance and in voltage-sensitive calcium channels have also been reported in epileptic tissue (Meldrum, 1990). Any one of these biochemical abnormalities might confer the "inherently epileptogenic" nature of spontaneously

bursting neurones and thus could underlie, or at least contribute to, the paroxysmal neuronal events that are characteristic of seizure discharge.

The remainder of the proposed cellular mechanisms of epileptogenesis appear to rely on some prior brain insult. The neurodegeneration believed to underlie the precipitation of spontaneous burst discharges in each of these cases is commonly observed in pathological studies of epileptic brain (Meldrum, 1989) and could be viewed as the causative factor in symptomatic epilepsy. Examples of focal pathologies include those arising as a result of cellular microdysgenesis, congential vascular malformations, neoplasms, traumatic brain lesions, infarcts, abscesses, and cysts (Meldrum, 1989). Specific neurodegeneration following an episode of hypoxia/ischaemia (section 5.1.7) and the diffuse degeneration associated with diverse neurological disorders, such as Huntington's chorea and Alzheimer's disease, have also been implicated (Meldrum, 1989).

Syndromes of spasticity following ischaemic lesions of the spinal cord have been related to the selective loss of inhibitory glycinergic interneurones (Meldrum, 1989). Similarly, a selective loss of inhibitory γ-aminobutyric acid (GABA) interneurones in the hippocampus or neocortex may lead to a failure of recurrent inhibition and a predisposition to burst firing. Potential mediators of this specific cell loss have been associated with epileptic pathologies and include cellular microdysgenesis, episodes of hypoxia, traumatic brain injury, focal neoplasia, and cerebrovascular infarction. No matter the mechanism of neuronal loss, when inhibition is thus impaired, recurrent excitation predominates and can initiate dendritic burst firing in the postsynaptic cell

(Miles and Wong, 1987). Under normal conditions, the hyperpolarisation mediated by GABAergic interneurones prevents invasion of the soma by any abnormal event occurring at the nerve terminal-dendritic interface (Meldrum, 1989). When the hyperpolarisation is inadequate, bursting and PDSs can be observed in soma records (Schwartzkroin and Wyler, 1980). In support of this theory is experimental evidence from animals suggesting that hypoxia in infancy can selectively damage GABA neurones in the cortex (Sloper et al, 1980). In addition, there is a selective loss of GABAergic nerve terminals around an epileptic focus induced in the monkey cortex by local application of alumina (Ribak et al, 1979). Unfortunately, the characteristic neuronal loss in patients with epilepsy is not restricted to inhibitory interneurones. There is a similarly prominent reduction in the number of excitatory hippocampal pyramidal cells (Meldrum, 1989). It is possible, however, that the loss of excitatory neurones in human epilepsy is a consequence of seizure activity, as observed in electrically kindled animals (Sutula, 1990), rather than a precipitator of it. Thus, it is feasible and indeed much mooted, that selective degeneration of inhibitory GABAergic interneurones may predispose the affected brain region to the spontaneous neuronal discharge that is proposed to initiate epileptiform activity.

Supersensitivity and dendritic sprouting are two secondary phenomena which are often associated with the cell loss induced by local neuronal degeneration and which might contribute to the initiation of spontaneous neuronal discharge (Meldrum, 1989). Neurones immediately post-synaptic to the degenerating cells show supersensitivity to the neurotransmitter concerned. This phenomenon can be observed experimentally in the hippocampus of kindled rats where the kindling-induced loss of excitatory

pyramidal neurones is followed by supersensitivity of the CA1 dendritic zones and the dentate molecular layer to excitatory amino acid (EAA) neurotransmitters (Mody and Heinemann, 1987). Theoretically, supersensitive neurones would respond in an exaggerated manner to normal extracellular concentrations of the relevant neurotransmitter and that this exaggerated response may be sufficient to precipitate neuronal burst firing. This theory is supported by experimental evidence where neocortex de-afferented by under-cutting showed an enhanced tendency to fire epileptiform "afterdischarges" following electrical stimulation (Meldrum, 1989). Similarly, studies of animal seizure models, such as the monkey-alumina gel model, have suggested that the time-course of development of supersensitivity is comparable to that of the development of focal epilepsy. Unfortunately, clinical epileptogenesis following focal pathology has a much slower and variable time course (Meldrum, 1989). In addition to neurodegeneration-induced supersensitivity, dendrites adjacent to regions of synaptic degeneration sometimes show "sprouting" and grow to form new synapses in the space left vacant (Meldrum, 1989). Such synapses are sometimes functionally inappropriate and as such could also contribute to the initiation of the abnormal discharge. The cytological processes of supersensitivity and sprouting typically follow every pathology involving neuronal degeneration and, in relation to seizure disorders, might not only underlie the initiation of paroxysmal neuronal discharge, but also help to explain the relative lack of specificity in the primary pathologies of epilepsy (Meldrum, 1989).

The use of Golgi and related silver staining techniques has revealed a type of neuronal change that is universally observed in epileptic pathology and may relate to the

initiation of spontaneous neuronal discharge. It consists of an apparently progressive simplification or degeneration of the complex dendritic trees of pyramidal neurones in the hippocampus and neocortex, with a loss of spinous processes. The mechanism of dendritic degeneration is poorly understood although speculation has suggested it to be related to the supersensitivity of the N-methyl-D-aspartate (NMDA) receptor system observed in electrophysiological studies (Meldrum, 1989). What is then unclear is how this specific pathology might correlate with the supersensitivity-associated and seemingly opposite phenomenon of dendritic sprouting. Although proposed as a potential contributory factor in the initiation of burst discharges, dendritic degeneration is not specifically restricted to epileptic pathologies and has been reported in a variety of other neurodegenerative disorders (Meldrum, 1989). Nevertheless, such simplification has been observed experimentally, in the region of an alumina-induced epileptic focus in the monkey cortex (Ribak et al, 1979). Loss of spinous processes and dendritic simplification could be the ultrastructural correlate of an enhanced tendency to paroxysmal burst discharges, both because the degenerating dendrite might itself be more prone to burst firing and because such firing might more easily invade the soma due to electrotonic shortening (Meldrum, 1989).

Like dendritic degeneration, an increase in the number of fibrous or reactive astrocytes is also universally characteristic of epileptic pathology and has been proposed as an aetiological mechanism in epileptogenesis. Such gliosis has been observed in the region of focal epileptic lesions, in diffuse degenerative disorders, and in the secondary pathology associated with seizure activity. It has been suggested that some failure in the capacity of astrocytes to regulate the extracellular environment could be responsible for

the locally enhanced tendency to epileptiform burst firing (Meldrum, 1989). Of particular pertinence would be the widely reported capacity of the astrocyte to act as a "potassium-sink" (Walz, 1989), functional impairment of which could significantly contribute to spontaneous neuronal discharge. However, experimental investigations of astrocytic function in the region of focal epileptic lesions have demonstrated an enhanced, rather than impaired, ability to regulate the extracellular potassium ion concentration (Meldrum, 1989). Thus, the potential role of gliosis in the generation of spontaneous neuronal discharges remains to be evaluated.

1.2 TREATMENT OF EPILEPSY

1.2.1 Historical perspectives

Since the Ancients attributed epilepsy to demons, phlegms, and the phases of the moon, it is hardly surprising that the remedies of the time were equally bizarre. Dietary measures were legion and ranged from the abstention from meat to the absolute avoidance of all food and drink. Phlebotomy or the drinking of urine or vinegar were other simple measures, while more peculiar procedures included the provocation of sneezing last thing at night or the rubbing of affected limbs with the genitals of a seal (Temkin, 1971). The most direct approach was "trephining" - the production of a large hole in the skull, usually by cautery. Its versatility clearly suited all aetiologies as it could be used to allow the escape of phlegm or vapours - or indeed evil spirits - as required.

While Jackson's observations with regard to the physiological basis of the epilepsies were elegant and indeed surprisingly accurate for their time, he continued to claim that

"there can be no question that the ligature is a most valuable means of arresting such fits" (Jackson, 1873). Some attempts at scientific evaluation of therapies were, however, proceeding. Around 1840, Esquirol twice yearly submitted 30 female patients to a different traditional or novel remedy. A fine demonstration of the placebo response ensued: "A new medicine invariably suspended the attacks with some, for fifteen days; for a month, two months, and even three months, with others" (Esquirol, 1845).

Nitrate of silver remained the thinking man's drug of choice (Pereira, 1839) until 1857, when the first mention of potassium bromide was made during a paper at a meeting of the Royal Medical and Chirurgical Society in London (Sieveking, 1857). Bromide, the first genuinely effective therapy, was championed by Locock and was used by Hammond in 288 patients, producing an improvement in 243, with total seizure control in 91 (Hammond, 1871). The related potassium iodide was used by others including Charcot (Charcot, 1881).

Response to the availability of these drugs was perhaps over-enthusiastic. Overdosing was common, as Hammond reported: "As you see, he is broken down in appearance, has large abscesses in his neck, and is altogether in bad condition. But this is better than to have epilepsy" (Temkin, 1971). This, however, has to be viewed alongside contemporary practices. A series of 72 cases of trephining was published as late as 1861 and in 1866 cliteroidectomy was still being performed by Baker (Duffy, 1963). By the 1880s, however, surgery aimed at removing the affected parts of the brain was meeting with more conventional success. The foundations of modern day therapy had been laid.

1.2.2 Drug treatment of epilepsy

Antiepileptic drugs (AEDs) are the mainstay of epilepsy management and may have to be taken for life. Over the past 100 years development of suitable pharmacotherapies for epilepsy has been slow and the majority of compounds in clinical use today were discovered serendipitously. In recent years however, drug discovery has quickened with many new drugs now in, or entering, clinical deployment. These will be discussed in section 1.4. The following is a chronological summary of what shall be classed hereafter as the "traditional" or "established" AEDs, in terms of their clinical effectiveness and major side-effects.

The barbiturate compound, phenobarbitone (PB), was the first of the established AEDs to enter regular clinical use. Barbiturates produce a reversible depression of the central nervous system (CNS) that can range from mild sedation to general anaesthesia depending on dose (Rogawski and Porter, 1990). However, certain barbiturates, such as PB, have the capacity to exert maximal anti-seizure activity at doses below those that are markedly sedative or hypnotic. The antiepileptic properties of PB were discovered by Loewe (1912), and after its introduction, PB largely replaced the toxic bromide salts which had been the mainstays of epilepsy therapy for nearly 50 years (Rogawski and Porter, 1990). PB is highly sedative (Schmidt, 1985) and can also cause depression, hyperactivity and irritability. The long-term sequelae of its undoubted effects on higher cortical function (Guest et al, 1970) are not yet clear. Tolerance occurs (Gallagher and Freer, 1985) and withdrawal often provokes seizures (Buchthal et al, 1968). However, it lacks the added complication of a highly active metabolite and has been shown to be effective against generalised tonic-clonic, secondary generalised, and neonatal seizures

(Mattson et al, 1985; Rogawski and Porter, 1990). While it is no longer a first-line drug in developed countries, many patients continue to be well controlled on PB and the problems of withdrawal often argue against stopping the drug entirely. It remains a possible second-line drug and continues to play a major role in antiepileptic therapy in developing countries as it is readily available, inexpensive, and easy to use.

Phenytoin (PHT) was the first of the current three front-line AEDs to come into regular clinical use. It was discovered as a result of the search by Merritt and Putnam (1938) for a non-sedatory analogue of PB, capable of suppressing electroshock seizures in animals. Although PHT is much less sedative than PB (Jones and Wimbish, 1985), it has shown some impairment of psychomotor and cognitive processing (Thompson et al, 1981; Andrewes et al, 1986). The chronic side effects of PHT include hirsuitism, gingival hyperplasia, and facial coursening, making it a cosmetically displeasing drug. PHT is effective against generalised tonic-clonic and partial seizures (Wilder and Rangel, 1989).

Primidone (PRIM), first reported in 1953 (Bogue and Carrington, 1953), has anticonvulsant activity in both animals (Frey, 1985) and man (Gruber et al, 1957) but suffers from important drawbacks. These include having PB as a major metabolite (Butler and Waddell, 1956) which makes the antiepileptic effects of PRIM alone more difficult to assess. In a recent large controlled clinical trial of 4 major AEDs (Mattson et al, 1985), PRIM was easily the least effective and least well-tolerated drug. Marked sedation has long been recognised as a side-effect of PRIM. This, and other side-effects, such as gastrointestinal intolerance and psychosis, often lead to discontinuation of the

drug (Mattson et al, 1985). Physical dependence (Frey, 1985) and withdrawal seizures (Norton, 1970) are other problems. Antiepileptic efficacy is similar to that of PB, but with more severe side-effects there is little reason to employ this drug in preference to PB in the few patients for whom barbiturate treatment is now contemplated (Brodie, 1990).

Ethosuximide (ESM) became the drug of choice for the treatment of absence seizures shortly after its introduction in 1958 (Brodie and Dichter, in press). Although largely superseded by sodium valproate in this respect, it retains its place as a valuable drug in the antiepileptic armamentarium. It is relatively non-toxic and non-sedative (Rogawski and Porter, 1990). Acute toxicity with ESM is dose-related (Brodie, 1990) and usually involves the gastrointestinal tract (nausea, vomiting, and abdominal pain) and the central nervous system (lethargy, dizziness, ataxia, and psychosis). Unlike other AEDs, ESM does not induce or inhibit hepatic drug metabolism (Brodie, 1990). As a result of its very narrow spectrum of activity, ESM is not used except for the treatment of absence seizures.

Carbamazepine (CBZ), a drug chemically related to the tricyclic antidepressants, was introduced in 1963 (Theobald and Kunz, 1963). Its efficacy was found to be similar to that of PRIM (Rodin et al, 1976) and it has become a major antiepileptic, particularly for partial seizures (Cereghino et al, 1974). It has generally low toxicity, although rashes occur in more than 5% of patients (Pellock et al, 1984) and hyponatraemia is a recognised side-effect (Gram and Jensen, 1989). As with the other major AEDs, CBZ does have neurological side-effects, mainly nausea, dizziness, headache, and diplopia

(Brodie, 1990). It is arguable that CBZ is one of the least sedative AEDs (Andrewes et al, 1986), although deleterious effects on psychomotor function have been demonstrated in both healthy volunteers (Macphee et al, 1986a) and patients (Macphee et al, 1986b). CBZ represents a first-line treatment for partial and generalised tonic-clonic seizures (Mattson et al, 1985).

The antiepileptic properties of sodium valproate (VPA) were first discovered accidentally in 1963 when valproic acid was being used in animal studies as a solvent for the drugs under formal investigation (Meunier et al, 1963). As the most recent, major addition to the clinical treatment of epilepsy (Editorial, 1988), VPA has a wide background of successful trials in various animal models of epilepsy (Löscher, 1985). Clinical studies have confirmed its effectiveness against generalised tonic-clonic seizures (Gram and Bentsen, 1984) and partial epilepsy (Shakir et al, 1981), as well as other epilepsies (Gram and Bentsen, 1985). Common side-effects with VPA include tremor, weight gain, hair loss, and ankle swelling (Brodie, 1990). It is an acceptable alternative to CBZ and PHT in both partial and secondary generalised tonic-clonic epilepsy and is the treatment of choice for primary generalised syndromes, including tonic-clonic, myoclonic, and absence seizures (Brodie, 1990). VPA shares with CBZ a reputation for causing less sedation than other AEDs (Gillham et al, 1991) and is also notable for its lack of enzyme induction (Brodie, 1990).

The benzodiazepines (BZDs) are the most recent additions to the clinician's antiepileptic armamentarium. While diazepam remains the drug of choice for status epilepticus (Simon, 1985), its derivatives clonazepam and clobazam are most often used

in epilepsy on a chronic basis (Caccia and Garattini, 1985). Both drugs are effective adjuvant therapy in controlled studies (Mikkelsen et al, 1981; Allen et al, 1983) and clobazam in particular has markedly reduced sedative properties compared to typical BZDs (Trimble and Robertson, 1986). Clonazepam is effective in the treatment of myoclonic and generalised tonic-clonic seizures (Naito et al, 1987), whereas clobazam is more commonly employed as adjuvant therapy in refractory epilepsy (Robertson, 1986). Withdrawal seizures can be a problem with both drugs (Fialip et al, 1987) and loss of efficacy after variable periods of time has been widely reported (Trimble and Robertson, 1986). Commonly observed side-effects of the BZDs include depression, irritability, and tiredness (Brodie, 1990).

1.2.3 Surgical treatment of epilepsy

Epilepsy surgery is over 100 years old (Horsley, 1886) and most of the operations have a long and reliable track record (Jensen, 1975). Estimates suggest that in the USA there are about 360,000 patients with uncontrolled complex partial seizures, at least 75,000 of whom would be suitable for surgery (Dreifuss, 1987); for the UK there might be 2000 patients who would benefit, very few of whom are clinically assessed and treated (Polkey, 1990).

There are two methods of surgical treatment. In the first, one tries to find a focal origin for the epilepsy, usually with an underlying lesion; the aim of the surgery is to remove the lesion and the accompanying focus. In the second, a surgical manoeuvre is used to alter brain activity and so modify the effect or the spread of the seizure. In general, surgical procedures that aim to remove epileptogenic tissue together with any

identifiable lesion are more common and more successful than those that attempt to modify brain activity (Polkey, 1990).

Most resections are temporal (Rasmussen, 1987), although the precise operative technique in the temporal lobe depends on the findings of pre-surgical investigation and on the nature of the lesion (Polkey, 1988). Resective surgery is usually highly successful. In a review published in 1987 (Engel, 1987) of 3245 operations conducted at 40 centres, it was shown that 80% of epileptic patients experienced a degree of relief following resective temporal lobe surgery, with 55% of patients having their epilepsy relieved completely. For extratemporal resections, 71% of patients were improved with 43% experiencing total seizure relief and the figures for hemispherectomy were 96% and 77% respectively (Polkey, 1990).

Operations to change brain function are less common and less successful than resective surgery. Stereotactic lesions in the brain produce an improvement rate of about 30% (Spencer, 1987). With modern neurosurgical techniques callostomy has become much safer; seizure control is improved in up to 50% of patients, but patients are seldom rendered seizure free (Spencer et al, 1987). Additionally, multiple sub-pial transection, pioneered by Morrell and associates (Morrell et al, 1989), has been used in eloquent areas such as the motor cortex; horizontal fibres are divided to reduce seizures while the vertical fibres are spared, thus preserving function.

1.3 TRADITIONAL ANTIEPILEPTIC DRUGS

As described in section 1.2.2, there are a limited number of AEDs available for current monotherapy in patients with epilepsy. While the use of animal seizure models has provided information regarding the likely spectrum of clinical efficacy of these compounds, such investigations have afforded little insight into the cellular mechanisms responsible for their anticonvulsant effects. For these established AEDs, only three basic mechanisms of action have been proposed (Macdonald and Kelly, 1993); blockade of neuronal voltage-activated sodium channels, blockade of neuronal voltage-sensitive calcium channels, and facilitation of the inhibitory action of the neurotransmitter GABA at its receptor sites. Much of this mechanistic evidence has been gleaned from *in vitro* studies employing electrophysiological techniques in isolated cells and tissue slice preparations (section 1.5.5). The following compares and contrasts the experimental anticonvulsant profiles of the established AEDs and the mechanisms by which they are proposed to exert their pharmacological effects.

1.3.1 Phenytoin and carbamazepine

PHT and CBZ have very specific effects on the pattern of electroshock-induced seizures in mice. Both drugs completely abolish the tonic phase of the convulsions (usually scored as hind-limb extension) but may enhance or prolong the clonic phase of the seizure (Swinyard et al, 1989; Rogawski and Porter, 1990). In contrast, PHT is ineffective against seizures induced by a variety of chemoconvulsants, including pentylenetetrazol (PTZ), bicuculline, picrotoxin, penicillin, and strychnine (Eadie and Tryer, 1989; Swinyard et al, 1989). Similarly, CBZ has been shown to be ineffective against tonic seizures induced by PTZ (Rogawski and Porter, 1990), although there have

been reports of efficacy of the drug against PTZ-induced clonic seizures (Larkin et al, 1992b). PHT is weakly active against myoclonic seizures in photosensitive baboons (Meldrum et al, 1975) and generalised seizures in alumina cream lesioned cats (Majkowski et al, 1976). The effects of both PHT (Callaghan and Schwark, 1980; McNamara et al, 1989) and CBZ (Wada, 1977; Hönack and Löscher, 1989) have been reported to be variable in nature against seizures induced by amygdaloid kindling in the rat. Both drugs have been shown to limit the propagation of paroxysmal activity from regions of epileptic cortex, even though they may actually increase the frequency of spiking in such foci (Morrell et al, 1959; David and Grewal, 1976). As a consequence, it is often stated that PHT and CBZ inhibit seizure spread, but do not halt the initiation of epileptic discharges.

In recent years it has been elegantly demonstrated that both PHT and CBZ can interact with the voltage-activated Na^+ channels responsible for the action potential upstroke in a highly specific voltage- and frequency-dependent manner (Macdonald, 1989; Rogawski and Porter, 1990). Both of these drugs have been shown to reduce the frequency of sustained repetitive firing of action potentials in cultured neurones (McLean and Macdonald, 1983; McLean and Macdonald, 1986b). The characteristic property of these AEDs was that they did not reduce the amplitude or duration of single action potentials, but reduced the ability of neurones to fire trains of action potentials at high frequency (Macdonald and Kelly, 1993). The observed limitation of high-frequency repetitive firing was voltage-dependent, being increased after depolarisation and reduced after hyperpolarisation. This action of PHT and CBZ appeared to be mediated by a shift of sodium channels to an inactive state that was similar to the normal inactive state but

recovery from which was slowed. These effects have been observed in a variety of preparations including mouse spinal cord and central neurones in culture (McLean and Macdonald, 1983; McLean and Macdonald, 1986b), rat myelinated nerve fibres (Schwarz and Grigat, 1989), rat hippocampal pyramidal neurones (Wakamori et al, 1989), and recombinant human sodium channels expressed in *Xenopus* oocytes (Tomaselli et al, 1989). Thus, evidence from voltage clamp experiments has confirmed the basic mechanism of action of PHT and CBZ. This is the only known action of the drugs that can adequately explain their ability to suppress seizures without causing generalised depression of the nervous system (Rogawski and Porter, 1990). Both drugs appear to stabilise the inactive form of the sodium channel in a voltage-dependent manner, to slow the rate of recovery from sodium channel inactivation, and to shift the steady-state sodium inactivation curve to more negative voltages (Macdonald and Kelly, 1993). Of interest is the finding that PHT has a stronger slowing effect than CBZ (Macdonald and Kelly, 1993), which may result in slightly different actions of these AEDs under different conditions of repetitive firing and help to may explain the subtle clinical differences between them.

Other proposed mechanisms for PHT include an interaction with voltage-dependent Ca^{2+} channels of the T-type (Twombly et al, 1988) and non-T-type varieties (Greenberg et al, 1984; Crowder and Bradford, 1987). The blockade of non-T-type calcium channels by PHT may also be related to its reported depression of excitatory neurotransmitter release (Gage et al, 1980). In general, these effects of PHT have been observed at doses higher than those required to block sodium channels and so their relevance to the antiepileptic effects of the drug are unclear (Rogawski and Porter,

1990). PHT has also been suggested to enhance GABA-mediated synaptic inhibition (Raabe and Ayala, 1976), to potentiate responses to exogenously applied GABA (Deisz and Lux, 1977), and to inhibit the post-synaptic actions of EAA neurotransmitters (Sastry and Phillis, 1976). Again these additional mechanisms have been largely dismissed due to the high drug concentrations required to elicit these effects. Like PHT, CBZ has been shown to reduce voltage-dependent non-T-type calcium influx and to reduce excitatory neurotransmitter release (Crowder and Bradford, 1987). These drug effects were also observed at concentrations higher than those required to block sodium channels. CBZ has been proposed to be an antagonist at A_1 and A_2 adenosine receptor sites in the brain (Marangos et al, 1987). This is somewhat surprising since adenosine has been proposed as an endogenous anticonvulsant compound (Dunwiddie and Worth, 1982). While CBZ has a relatively high affinity for these sites compared to its affinity for the voltage-dependent sodium channel (Rogawski and Porter, 1990), extensive investigations have failed to find a link between the effects of the drug at adenosine receptors and its antiepileptic activity (Marangos et al, 1983; Weiss et al, 1985). Other proposed mechanisms for CBZ include effects on catecholamine systems (Purdy et al, 1977) and an interaction with peripheral-type BZD receptors (Marangos et al, 1983; Weiss et al, 1985).

1.3.2 Barbiturates and benzodiazepines

The barbiturates, PB and PRIM and the BZDs, diazepam, clobazam, and clonazepam, have been classed together here because, as with PHT and CBZ above, they are also proposed to possess a degree of similarity in their mechanisms of action.

PB is active in most experimental seizure models and as such has a broader spectrum of activity than either PHT or CBZ. It is able to protect against both electroshock- and PTZ-induced seizures (Swinyard et al, 1952; Craig and Shideman, 1971) and is also effective in the amygdaloid kindling model in the rat (Callaghan and Schwark, 1980). The antiepileptic effects of PRIM have been at least partly attributed to its primary metabolism to PB (Butler and Waddell, 1956), although their experimental anticonvulsant profiles differ in that PRIM has little activity against PTZ-induced seizures (Bourgeois et al, 1983). Selective metabolism to phenylethylmalonamide, the other primary metabolite of PRIM, which has a similar, but significantly less potent, spectrum of experimental anticonvulsant activity to PB (Leal et al, 1979; Bourgeois et al, 1983) might confer this inactivity. Nevertheless, the role of the parent compound and the two active metabolites in the experimental, or indeed clinical, anti-seizure activity of PRIM is difficult to determine (Bourgeois, 1989).

More than 50 chemically distinct BZDs are marketed world-wide. Although pharmacokinetic differences exist among individual drugs and relative potencies may vary in the different anticonvulsant screens, they all exhibit a similar anticonvulsant profile which is presumed to relate to a common mechanism of action. The BZDs are potent anticonvulsants in a wide variety of animal seizure models (Rogawski and Porter, 1990). They are particularly effective against PTZ-induced seizures but also protect against seizures induced by other chemoconvulsants including picrotoxin and flurothyl. The BZDs are active against focal seizures induced by local application of alumina cream and strychnine, in reflex epilepsies such as those occurring in the photosensitive *Papio papio* baboon and DBA/2 mouse, in kindled seizures, and in absence-like

seizures in the *tottering* mutant mouse (Swinyard and Castellion, 1966; Reinhard and Reinhard, 1977; Heller et al, 1983; Swinyard et al, 1989). At higher doses these drugs block maximal electroshock (MES) seizures and those induced by systemic strychnine (Rogawski and Porter, 1990).

It has been proposed that the sedative/hypnotic and anticonvulsant effects of the barbiturates may be mediated by different mechanisms of action. The traditionally held view that the barbiturates exert their effects at the $GABA_A$ receptor - chloride ionophore complex (section 4.1.1) has been elegantly confirmed using single channel recordings in cultured mouse spinal cord neurones (Mathers and Barker, 1980; Twyman et al, 1989). The barbiturates pentobarbital (Macdonald et al, 1989) and phenobarbital (Twyman et al, 1989) increased the probability of opening of the intrinsic chloride ion channel of the $GABA_A$ receptor complex and prolonged the duration of opening, without affecting the single channel conductance. The open period of the channel may correspond to the period in which the GABA receptor is occupied by an agonist (Rogawski and Porter, 1990). From radioligand binding studies it has been shown that barbiturates shift the GABA receptor to a state in which the affinity for GABA is increased and the rate at which GABA dissociates is decreased (Yang and Olsen, 1987). It is plausible that, in the presence of barbiturates, the prolonged occupancy of the GABA receptor allows a greater number of channel openings to occur prior to dissociation of the agonist.

While an effect on the $GABA_A$ receptor - chloride ionophore complex is likely to play a role in mediating the anticonvulsant effects of PB, additional mechanisms might confer the relative anticonvulsant potency of PB over other more sedative barbiturates. The

barbiturates are able to reduce depolarisation-evoked neurotransmitter release from a wide variety of preparations (Richter and Waller, 1977; Potashner et al, 1980; Crowder and Bradford, 1987), albeit at concentrations higher than therapeutic brain levels. It has been proposed that this effect is mediated by blockade of voltage-sensitive calcium influx into nerve terminals (Leslie et al, 1980; Heyer and Macdonald, 1982). At the molecular level, the barbiturates appear to selectively block calcium influx through N- and L- but not T-type channels (Gross and Macdonald, 1988). Single channel recordings from rat dorsal root ganglion neurones (Gross and Macdonald, 1988) and recombinant human brain calcium channels expressed in *Xenopus* oocytes (Gundersen et al, 1988) have suggested that the barbiturates bind to the inactivated state of the channel to depress the Ca^{2+} current amplitude, speed its decay, and enhance its steady state inactivation.

Other proposed mechanisms of barbiturate action include an enhancement of voltage-dependent potassium currents (Huguenard and Wilson, 1985) and blockade of non-NMDA type EAA receptors (Miljkovic and Macdonald, 1986). The relevance of any or all of these proposed mechanisms of action to the antiepileptic effects of PB remains to be evaluated. It must also be noted that the mechanism of PRIM action has largely been considered to mirror that of its metabolite PB, particularly while the cellular effects of its other active metabolic product, phenylethylmalonamide, remain unclear. Whether this general assumption is well-founded remains to be determined.

As previously alluded to, the BZDs possess a degree of similarity to the barbiturates in their mechanism of action. In electrophysiological studies BZDs potently enhance

synaptically mediated GABAergic inhibition as well as responses to exogenously applied GABA (Haefely, 1983). It is believed that, as for the barbiturates, the $GABA_A$ receptor - chloride ionophore complex possesses a binding site for the BZDs (Ehlert, 1986). Binding of GABA to its recognition site on this complex stimulates an increase in Cl^- permeability that is mediated by an intrinsic chloride ion channel (Barker and Ransom, 1978). BZDs reliably enhance this response to GABA without affecting responses to other inhibitory amino acids such as glycine, β-alanine, or taurine that are also mediated by Cl^- selective channels (Macdonald and Barker, 1978). The enhancement of GABA mediated effects is believed to be due to a shift in the GABA dose-response curve; there is no alteration in maximal GABA response, indicating that the sensitivity to GABA is increased without altering the total number of available Cl^- channels (Choi et al, 1981). While BZD-induced augmentation of GABA responses occurs at relatively low drug concentrations, the maximal potentiation observed is modest and substantially less than that produced by the barbiturates (Rogawski and Porter, 1990). Studies of fluctuation analysis (Study and Barker, 1981) and single channel, patch-clamp recording (Twyman et al, 1989) have proposed the BZDs to increase the frequency of Cl^- channel opening, without affecting the single channel conductance or the open time. These proposed effects of the BZDs are in contrast to those proposed for the barbiturates, which are believed to prolong the channel open time without increased its frequency of opening.

The observation that the BZDs have the capacity to abolish absence-like manifestations in rats with spontaneously occurring spike-wave seizures has suggested that these drugs may possess more than one mechanism of action (Fariello and Ticku, 1983). Several

studies have demonstrated that drugs which potentiate GABAergic systems can worsen absence-like seizures or even induce epileptiform activity in these animals (Marescaux et al, 1985). The BZDs however, can abolish these spontaneous seizures and may also completely eliminate the aggravated seizures in animals treated with other compounds proposed to facilitate GABAergic function (Marescaux et al, 1985). These results suggest that the anti-absence activity of the BZDs, at least in animal models, is not dependent on their ability to augment GABA-mediated inhibition. Non-BZD receptor mediated mechanisms, proposed to play a part in the antiepileptic properties of these drugs and perhaps to confer their unusual anti-absence capacity, include an inhibition of adenosine uptake and blockade of neuronal sodium and calcium channels (Haefely, 1989).

1.3.3 Ethosuximide

The narrow range of clinical activity of ESM, against generalised absence seizures alone, is reflected in its unique anticonvulsant profile in animal seizure models. ESM specifically blocks PTZ- and bicuculline-induced clonic seizures in mice (Swinyard et al, 1989), but fails to have any activity against tonic seizures induced by MES except at anaesthetic doses (Reinhard and Reinhard, 1977). The drug is effective in a variety of animal models of spontaneous generalised absence seizures. These include the mutant *tottering* mouse (Heller et al, 1983), the Wistar rat (Marescaux et al, 1984), and the genetically epilepsy-prone Kyoto-Wistar rat (Sasa et al, 1988). ESM is also effective in reducing the number of absence-like events induced by systemic administration of γ-hydroxybutyrate (GHB; Snead, 1978; Snead, 1988).

The unique profile of activity exhibited by ESM would suggest that it possesses a novel and distinct mechanism of action, at least in comparison to drugs like PHT and CBZ which are inactive against absence seizures or may even worsen them (Marescaux et al, 1984). Until recently, however, it was unclear what this mechanism might be. ESM fails to limit sustained high-frequency repetitive firing of cultured neurones at therapeutic concentrations (McLean and Macdonald, 1986a). This would suggest that ESM does not possess a PHT-like action on Na^+ channels and this is reflected in its inability to influence experimental seizures induced by electroshock. Similarly, ESM does not augment post-synaptic GABA responses (Barnes and Dichter, 1984), although it may interact with the picrotoxin site on the $GABA_A$ receptor complex (Coulter et al, 1989b). This is not surprising considering that the majority of compounds, with the exception of the BZDs, which facilitate GABAergic function are inactive against, or may even worsen, absence seizures (Marescaux et al, 1985). Recently it has been demonstrated that ESM, unlike any other established AED, selectively blocks voltage-sensitive calcium influx through T-type channels in thalamic neurones (Coulter et al, 1989c).

The expression of generalised absence seizures, either experimentally or clinically, is believed to rely on a unique low-threshold activated, Ca^{2+}-mediated burst firing in thalamocortical relay neurones (Vergnes et al, 1987). Voltage-clamp recordings from acutely isolated thalamic neurones suggest that these cells express both L- and T-type voltage-sensitive calcium channels (Hernández-Cruz and Pape, 1989) and that they are unusual within the CNS in having a relatively large T-type current (Rogawski and Porter, 1990). This observation may, at least in part, account for their unique and characteristic firing properties. Recently, Coulter and colleagues (1989c) have reported

that ESM, at therapeutically relevant concentrations, produces up to a 40% reduction in the amplitude of this T-type calcium current in thalamic neurones. The ESM block of T-type current was voltage-dependent and was markedly reduced at depolarised potentials. The drug did not influence the time course of activation or inactivation of the current, its voltage-dependency for activation or steady-state inactivation, or its recovery from inactivation. Thus, unlike PHT's action on voltage-activated sodium channels, ESM fails to alter the gating of the T-type channel (Rogawski and Porter, 1990).

The proposal that the antiepileptic actions of ESM are mediated specifically by effects on T-type current in thalamic neurones were further confirmed by the lack of effect, in this respect, of succinimide, a structurally related but pharmacologically inactive compound (Coulter et al, 1989a). Similarly, therapeutically relevant concentrations of dimethadione (Coulter et al, 1989c) and desmethsuximide (Coulter et al, 1990), active metabolites of the more obsolete anti-absence drugs trimethadione and methsuximide respectively, also selectively reduced T-type calcium channel current in thalamic neurones. Thus, drugs that block T-type calcium channels at clinically relevant concentrations appear to be effective anti-absence agents.

1.3.4 Sodium valproate

Although VPA was initially identified on the basis of its ability to protect against PTZ-induced seizures, the drug was subsequently shown to possess a wide spectrum of anticonvulsant activity in animal seizure models (Chapman et al, 1982). VPA is active against tonic and clonic seizures induced by the systemic administration of a wide variety of chemoconvulsants including PTZ, 3-mercaptopropionic acid (3-MPA),

bicuculline, picrotoxin, and strychnine (Bartoszyk et al, 1986b). It is also active in the MES test (Löscher and Schmidt, 1988), the photosensitive *Papio papio* baboon (Löscher and Schmidt, 1988), audiogenically-susceptible DBA/2 mice (Schechter et al, 1978), and in the kindling model (Löscher et al, 1986b). VPA is, however, only weakly active against seizures induced by EAAs (Chapman et al, 1982). Like PHT, VPA prevents the spread of abnormal discharges from a focal cortical lesion or from a site of kindling but has little or no effect on the primary generation of these epileptiform events (Fariello and Smith, 1989). Nevertheless, with a far broader spectrum of experimental anticonvulsant activity than PHT, VPA has been proposed to exert its effects by a different mechanism, or mechanisms.

The mechanism of the antiepileptic action of VPA remains to be definitively determined. As the drug has such a wide spectrum of anticonvulsant activity, it has been attractive to speculate that it may exert its effects by a combination of mechanisms. While experimental evidence exists for such a multifactorial mechanism of action, the precise role of individual components of this proposal have yet to be determined and, indeed, each of them in turn has been called into question.

Acute administration of VPA to mice produces an increase in whole brain and synaptosomal GABA concentration (Löscher, 1981) which has, on occasion (Anlezark et al, 1976; Nau and Löscher, 1982), been correlated with the drug's anticonvulsant activity. Similarly, increases in plasma (Löscher and Schmidt, 1980) and cerebrospinal fluid (CSF; Löscher and Siemes, 1984) GABA levels have been reported in patients being treated with clinically effective doses of VPA. The mechanism by which the drug

increases brain GABA concentrations is not well understood. It has been shown to reduce the activity of several enzymes involved in the metabolic degradation of GABA, including GABA-aminotransferase (GABA-T; Löscher, 1980), succinic semialdehyde dehydrogenase (SSAD; Harvey et al, 1975), and aldehyde reductase (Whittle and Turner, 1978). However, the effect of VPA on GABA-T is weak when compared to prototypic inhibitors such as vigabatrin (VGB; Larsson et al, 1986) and inhibition of enzyme activity is not observed *in vivo* after administration of anticonvulsant doses of the drug (Nau and Löscher, 1982), or *in vitro* in intact cells (Gram et al, 1988). VPA has also been shown to increase the activity of glutamic acid decarboxylase (GAD), the enzyme responsible for GABA synthesis in the brain, following acute administration (Phillips and Fowler, 1982). The increase in GAD activity was found to parallel the elevation of brain GABA levels produced by a single dose of the drug (Nau and Löscher, 1982) and to remain elevated with chronic administration (Löscher and Nau, 1982). In subsequent *in vivo* experiments however, Löscher (1989) found the GABA synthesis rate to be enhanced only in the substantia nigra and to a lesser extent in the striatum. Which, if any, of the actions of VPA on the enzymes of the GABAergic pathway might be responsible for its effect on brain GABA levels and the extent to which such an effect contributes to its antiepileptic action, is unclear.

Like PHT and CBZ, VPA has the ability to limit the sustained repetitive firing of Na^+-dependent action potentials in cultured cortical and spinal cord neurones at therapeutically relevant concentrations (McLean and Macdonald, 1986a). It remains uncertain, however, whether it has the same mechanism as PHT and CBZ in this respect. Although VPA has been shown to exert a use- and voltage-dependent limitation

of sustained repetitive firing, the evidence linking these observations to effects at the sodium channel are indirect (Rogawski and Porter, 1990). Indeed, biochemical studies employing a batrachotoxin-stimulated $^{22}Na^{+}$ flux assay have failed to demonstrate an interaction between VPA and Na^{+} channels (Willow et al, 1984). In addition, detailed voltage-clamp studies with VPA have not been performed in preparations of central neurones (Macdonald and Kelly, 1993), and those which have been conducted, in peripheral neurones, suggest that the effects of the drug on Na^{+} currents are only observed at supra-therapeutic concentrations (VanDongen et al, 1986). Moreover, 2-en-valproate, a metabolite of VPA that is as potent an anticonvulsant as its parent compound in several animal seizure models, was without effect on sustained repetitive firing in cultured mouse central neurones (McLean and Macdonald, 1986a), raising further questions about the Na^{+} channel hypothesis.

The observation that VPA is one of the most effective drugs against generalised absence seizures has raised speculation that it may interact with the voltage-sensitive T-type calcium channel in a manner analogous to ESM. Initial studies with VPA failed to demonstrate any effect of the drug on the low threshold calcium current in thalamic neurones (Coulter et al, 1990). However, in a subsequent investigation, VPA was shown to reduce T-type calcium current in rat primary afferent neurones in culture (Kelly et al, 1990). This effect was modest and the maximal reduction (16%) in T-type current was observed only at supra-therapeutic concentrations. Whether such a modest effect would be sufficient to explain the anti-absence actions of VPA is unclear. Furthermore, the basis for the discrepancy in the results obtained in rat thalamic neurones and those in rat

primary afferent neurones remains uncertain. Whether voltage-sensitive T-type calcium channel blockade is a relevant mechanism for VPA action remains to be determined.

Acute doses of VPA have been consistently found to decrease brain levels of the EAA aspartate, without affecting those of glutamate or GABA (Schechter et al, 1978). This decrease in aspartate concentration has been shown to correspond with the period of anticonvulsant action of the drug (Chapman et al, 1983). Furthermore, there is a stronger correlation between the anticonvulsant potencies of a series of VPA analogues and their ability to reduce brain aspartate concentration than with their effects on GABA levels (Chapman et al, 1984). In addition to effects on whole brain aspartate levels, therapeutically relevant concentrations of VPA have been shown to selectively reduce veratrine-stimulated aspartate release from isolated rat cortical slices (Crowder and Bradford, 1987). The drug was without effect on evoked release of glutamate or GABA from this preparation. The mechanism by which VPA might interfere with aspartatergic systems in the brain is unknown.

Other potential contributors to the mechanism of VPA action include antagonism of picrotoxin binding (Ticku and Davis, 1981) and facilitation of BZD binding (Koe, 1983) at the $GABA_A$ receptor complex, enhancement of the post-synaptic action of GABA at this receptor (Macdonald and Bergey, 1979; Schmutz et al, 1979), and a pre-synaptic effect resulting in enhancement of K^+-stimulated GABA release (Gram et al, 1988). The relevance of any one of the above mechanisms to the clinical antiepileptic actions of VPA remains to be determined.

1.4 NOVEL ANTIEPILEPTIC DRUGS

Partly as a result of the endeavours of the Antiepileptic Drug Development programme of the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) at the National Institutes of Health (NIH), USA, numerous novel AEDs have been developed over the last 10 - 15 years. A number of these compounds have since entered, or are now entering, regular clinical use for the treatment of convulsive disorders. Similarly, a significant number of new drugs are currently undergoing clinical efficacy and safety trials to evaluate their potential for the treatment of epilepsy. Finally, the continued search for novel therapies has identified a range of promising experimental stage compounds.

1.4.1 Novel antiepileptic drugs in clinical use

VGB was the first AED to enter regular clinical use that was designed specifically for the treatment of epilepsy (Grant and Heel, 1991). The drug has been proposed to exert its antiepileptic effect by irreversible inhibition of GABA-T, the enzyme directly responsible for the metabolic degradation of the inhibitory neurotransmitter GABA (Lippert et al, 1977). As such, the drug has been shown to elevate GABA levels in the plasma, CSF, and brain of experimental animals (Böhlen et al, 1979) and the plasma and CSF in man (Ben-Menachem et al, 1989). VGB has an unusual experimental anticonvulsant profile in that it may not be active in either the PTZ or MES test following systemic administration (Myslobodsky et al, 1979; Bernasconi et al, 1988). The drug is, however, effective against seizures induced by picrotoxin (Bernasconi et al, 1988), 3-MPA (Liu et al, 1990), and amygdaloid kindling (Löscher et al, 1989) and in the experimental reflex epilepsies (Schechter et al, 1977; Meldrum and Horton, 1978).

Clinically, VGB has been employed as add-on therapy for the treatment of drug-resistant epilepsy in the UK since 1989 (Reynolds et al, 1991). Aspects of experimental neuropathology have prevented licencing of the drug in the USA (Butler et al, 1987). VGB has been shown to produce a significant (>50%) reduction in seizure frequency in around 50% of patients (Grant and Heel, 1991) and appears to possess particular efficacy against complex partial seizures (Ring et al, 1990), the most refractory form of chronic epilepsy. The pharmacology and biochemistry of VGB action are discussed in detail in section 4.1.2.

Lamotrigine (LTG) is a novel antiepileptic agent which was discovered during the mass screening of anti-folate compounds for anticonvulsant activity (Yuen, 1991). LTG is chemically unrelated to existing AEDs and its weak anti-folate effects appear unrelated to its anticonvulsant activity (Rogawski and Porter, 1990). Its ability to limit sustained repetitive firing in cultured mouse spinal cord neurones at therapeutically relevant concentrations (Cheung et al, 1992) has proposed LTG to possess a mechanism of action similar to that of PHT and CBZ, namely use- and frequency-dependent blockade of voltage-sensitive Na^+ channels in the neuronal membrane (Macdonald and Kelly, 1993). Nevertheless, LTG possesses a distinct experimental anticonvulsant profile when compared to PHT and CBZ which may reflect subtle mechanistic differences between the compounds. LTG is active against the tonic phase of both PTZ- and MES-induced seizures (Lamb et al, 1985; Yuen, 1991), blocks the development and expression of amygdaloid kindled seizures (Miller et al, 1986), and reduces electrically-evoked afterdischarge duration in the rat, dog, and marmoset (Wheatley and Miller, 1989). LTG is, however, ineffective against both threshold and clonic seizures induced by PTZ

(Rogawski and Porter, 1990). The extensive pre-clinical pharmacology of the drug is discussed in section 6.1.6. Clinical experience with LTG has suggested the drug to be active against partial seizures and generalised tonic-clonic, myoclonic, and absence events (Yuen, 1991). It has demonstrated efficacy in ten double-blind, placebo-controlled trials with 30% of patients experiencing a 50% reduction in the incidence of these seizure types (Leach and Brodie, in press). LTG was licenced for use in the UK as add-on treatment for refractory epilepsy in 1992.

Gabapentin (GBP) is a chemically novel compound, related in structure to GABA, which was designed as a GABAmimetic that could freely cross the blood-brain barrier (Ojemann et al, 1988). Despite its structural similarity to GABA, GBP has often failed to influence GABA pharmacology, although it has been shown to potentiate responses to this inhibitory neurotransmitter in the rat neonatal optic nerve (Kocsis and Honmou, in press). At present, its precise mechanism of action remains unclear. GBP failed to demonstrate any effect on sustained repetitive firing in cultured mouse spinal cord neurons (Taylor et al, 1988), although prolonged drug exposure did limit high frequency action potential firing in mouse central neurons in culture (Wamil and McLean, 1994). It was without effect on $GABA_A$ or $GABA_B$ receptor binding, strychnine-sensitive glycine receptor binding, GABA metabolism (Bartoszyk et al, 1986b), or on voltage-sensitive calcium influx (Kelly et al, 1991). It was similarly devoid of effect on single channel GABA, glycine, NMDA, non-NMDA, and voltage-dependent calcium conductances in a range of cultured rodent neurons (Rock et al, 1993). Recent reports have suggested that GBP may increase GABA turnover in certain regions of the brain (Löscher et al, 1991c) and that it binds with high affinity to a site, proposed to be the

plasma membrane L-amino acid transporter (Stewart et al, 1993), where it is potently displaced by the anticonvulsant compound 3-isobutyl GABA (Taylor et al, 1993). Investigations are ongoing to characterise this interaction more fully (Taylor, in press) and to determine its relevance to the antiepileptic effects of the drug. GBP has been shown to have an experimental anticonvulsant profile similar to that of VPA (Rogawski and Porter, 1990). The drug is effective against tonic seizures induced by a variety of chemoconvulsants including PTZ, bicuculline, picrotoxin, and strychnine (Bartoszyk et al, 1986b). It is also active in the MES test in rats (Rogawski and Porter, 1990) and against reflex seizures in DBA/2 mice, Mongolian gerbils (Bartoszyk and Hamer, 1987), and genetically epilepsy-prone rats (GEPRs; Naritoku et al, 1988). GBP has shown only weak activity in the photosensitive *Papio papio* baboon and was without effect in the kindling model (Foot and Wallace, 1991). Clinically, the drug has demonstrated efficacy against both partial and generalised tonic-clonic seizures (Crawford et al, 1987; Sivenius et al, 1989). GBP was licenced for use, in the UK, as add-on treatment for refractory epilepsy in 1993.

Felbamate (FBM) is a dicarbamate compound that is structurally related to the anti-anxiety agent meprobamate (Brodie and Porter, 1990). The mechanism by which FBM exerts its antiepileptic effects remains unclear. The drug has failed to demonstrate any effect on ligand-binding to the GABA, BZD, or picrotoxin recognition sites on the $GABA_A$ receptor complex (Macdonald and Kelly, 1993). Its potentiation of diazepam effects against both PTZ- and MES-induced seizures (Gordon et al, 1991) and its facilitation of GABA responses in cultured hippocampal neurones under voltage-clamp conditions (Rho et al, 1994) has, however, suggested some indirect action at this

receptor site. An action at the NMDA receptor complex has also been proposed for FBM. It has been shown to attenuate NMDA responses in cultured hippocampal neurones by a channel blocking mechanism (Rho et al, 1994) and to inhibit binding of 5,7-dichlorokynurenic acid at the associated strychnine-insensitive glycine site (McCabe et al, 1993). FBM is effective in blocking experimental seizures induced by PTZ, MES, bicuculline, and picrotoxin (Swinyard et al, 1986) but not those precipitated by strychnine (Perhach et al, 1986). It has also been found to be effective in the prevention of seizures following cortical application of alumina gel in rhesus monkeys (Leppik and Graves, 1989). These experimental results suggest that FBM has the capacity to both increase seizure threshold and to limit the spread of abnormal electrical discharge (Sofia et al, 1991). In clinical studies, FBM has been found to be effective in complex partial seizures in adults and in the Lennox-Gastaut syndrome in children (Macdonald and Kelly, 1993) and was licenced for use in the treatment of these disorders in the USA in 1993. It has recently been approved for similar indications in several European countries, but not the UK.

Oxcarbazepine (OXC), the 10-keto analogue of CBZ, is a prodrug which is selectively metabolised to the active compound 10-hydroxy-carbazepine (Jensen et al, 1991). Although structurally similar to CBZ, the drug was designed to bypass the inherently toxic metabolite, carbamazepine-10,11-epoxide, which has been proposed to mediate the majority of the CNS side-effects of CBZ and its induction effect on hepatic drug metabolism (Rogawski and Porter, 1990). Although little data exists on the mechanism of OXC action, similar experimental and clinical anticonvulsant profiles to CBZ would suggest that it also exerts its effects by blockade of voltage-sensitive Na^+ channels. Its

ability to limit sustained repetitive firing in cultured mouse central neurones (McLean et al, 1994) would support this hypothesis. The drug has also demonstrated 4-aminopyridine (4-AP) - sensitive effects on penicillin-induced epileptiform discharges in the hippocampal slice (McLean et al, 1994), suggesting an additional action on neuronal potassium channels which might have clinical relevance. OXC has been shown to reduce tonic seizures induced by MES in both rats and mice (Jensen et al, 1991) and to prevent partial seizures in rhesus monkeys induced by cortical application of alumina gel (Rogawski and Porter, 1990). The drug was less active against seizures induced by a variety of chemoconvulsants, including PTZ, picrotoxin, and strychnine (Dam and Jensen, 1989). The majority of clinical studies of OXC have compared it with CBZ (Reinikainen et al, 1987). In general, these studies have concluded that both drugs possess similar efficacy against partial and generalised tonic-clonic seizures and that OXC may precipitate fewer adverse events (Dam et al, 1989). Recently, Aikia and colleagues (1989) also found OXC and PHT to be similarly effective after 1 year of treatment. OXC was recently licenced for the treatment of partial and tonic-clonic seizures in 12 countries.

Flunarizine (FNZ) is a piperazine derivative, originally proposed to exert its antiepileptic effects by blockade of voltage-sensitive calcium entry (Binnie, 1989) through both L- and T-type channels (Tytgat et al, 1988). Despite its ability to bind with high affinity to the proposed "dihydropyridine" receptor in the brain (Gould et al, 1984), there is little evidence to suggest that this effect mediates its anticonvulsant action. Indeed, the experimental anticonvulsant profile of FNZ bears more resemblance to that of PHT than that of the organic calcium antagonists. In addition, FNZ has been shown

to reduce sustained repetitive firing in cultured central neurones (McLean, 1987) and to inhibit [^{3}H]-batrachotoxin binding to neuronal sodium channels in rat brain synaptosomes (Grima et al, 1986). Thus, it has been proposed that FNZ might exert its antiepileptic effects by blockade of voltage-sensitive sodium channels, in a manner analogous to PHT and CBZ (Rogawski and Porter, 1990). The drug has demonstrated efficacy against both MES- and PTZ-induced tonic seizures (Desmedt et al, 1975; 1976) and against reflex seizures in DBA/2 mice and *Papio papio* baboons (De Sarro et al, 1986). FNZ is also active against seizures induced by systemic bicuculline (Wauquier et al, 1986), focal D,L-allylglycine (Ashton and Wauquier, 1979a), and amygdaloid kindling (Ashton and Wauquier, 1979b). Clinically, FNZ is marketed primarily for the treatment of migraine and vertigo although it has demonstrated efficacy against intractable complex partial seizures (Alving et al, 1989). The drug is marketed in 38 European, Latin American, Asian, and African countries for the treatment of refractory epilepsy.

Zonisamide (ZNS) is a member of a novel class of benzisoxazole anticonvulsants which have similar spectra of experimental anticonvulsant activity to PHT (Rogawski and Porter, 1990). In accordance with a PHT-like mechanism of action, ZNS blocked sustained repetitive firing in cultured mouse central neurones at therapeutically relevant concentrations (Rock et al, 1989). However, ZNS has also demonstrated the capacity to inhibit ligand binding to both $GABA_A$ and the associated BZD receptors in rat brain membranes (Mimaki et al, 1988) and to exert a VPA-like effect in studies of excitatory and inhibitory mechanisms in the cat spinal trigeminal nucleus (Fromm et al, 1987). These additional components to its mechanism of action may confer its ability to

suppress cortical seizure foci in cats (Ito et al, 1980) and its activity against clinical myoclonic seizures (Henry et al, 1988). ZNS was active against experimental seizures induced by MES (Masuda et al, 1980) and amygdaloid kindling (Kamei et al, 1981). It was also effective against reflex seizures in Mongolian gerbils (Bartoszyk and Hamer, 1987) and chemically-induced cortical seizure discharges (Ito et al, 1986). ZNS has been marketed for the treatment of epilepsy in Japan since 1989. It has demonstrated particular efficacy against myoclonic seizures (Henry et al, 1988) and is also active against refractory partial seizures (Sachellares et al, 1985). Clinical trials with ZNS in the USA were suspended until recently, following observations relating the drug to the increased incidence of renal calculi (Brodie and Porter, 1990). European studies with the drug are ongoing (Schmidt et al, 1993).

1.4.2 Novel antiepileptic drugs undergoing clinical trial

Tiagabine (TGB) is a novel antiepileptic agent currently in clinical trial for the treatment of epilepsy. It is a centrally active nipecotic acid derivative, proposed to exert its anticonvulsant effects by inhibition of GABA uptake into both neurones and glia (Nielsen et al, 1987). The pharmacology and biochemistry of the drug are discussed in detail in section 4.1.3. It only very weakly interacts with the BZD receptor and the chloride ion channel of the $GABA_A$ receptor and does not appreciably bind to other neurotransmitter receptors (Rogawski and Porter, 1990). Experimentally, TGB has demonstrated efficacy against audiogenic seizures in DBA/2 mice (Nielsen et al, 1991), the motor manifestations of amygdaloid kindled seizures (Pierce et al, 1991), and the tonic and clonic components of PTZ-induced seizures in both rats and mice (Nielsen et al, 1991). It afforded only partial protection against photically-induced seizures in *Papio*

papio baboons and in bicuculline- and MES-induced seizures in mice (Nielsen et al, 1991). It was without effect against 4-AP (Yamaguchi and Rogawski, 1992) and dendrotoxin induced seizures (Coleman et al, 1992). Although many of the clinical studies with TGB remain in their infancy, promising initial reports suggest that the drug is active against both partial and secondary generalised seizures (Leach and Brodie, in press).

Remacemide (RMD) is a novel antiepileptic agent which emerged from a drug discovery programme aimed at creating a molecule with a 3-dimensional structure similar to that of PHT (Rogawski and Porter, 1990). It has been shown to limit sustained repetitive firing in cultured mouse central neurones in a manner analogous to that of PHT and CBZ (Macdonald, 1989) and, indeed, the experimental anticonvulsant profile of the drug is also similar to that of PHT. In addition, it has been shown to interact with the MK-801 site on the NMDA receptor complex (Garske et al, 1991), although the relevance of this comparatively weak effect to its precise mechanism of action remains to be determined. RMD has demonstrated efficacy against electroshock-induced seizures in mice and rats, but was inactive in the PTZ test (Muir and Palmer, 1991). It protected against audiogenically-induced seizures in DBA/2 mice and NMDA-induced seizures and lethality in mice (Rogawski and Porter, 1990), but was without effect on the development of kindling and the expression of fully kindled seizures in rats (Kupferberg, 1989). The drug was similarly inactive against seizures induced by the systemic administration of picrotoxin, bicuculline, and strychnine (Muir and Palmer, 1991). RMD has been proposed to have clinical efficacy against partial and generalised

tonic-clonic seizures (Muir and Palmer, 1991) and is currently undergoing clinical trials for these disorders.

Stiripentol (STP), chemically unrelated to existing AEDs (Vincent, 1991), is the most promising compound of a series of ethylene alcohols that have demonstrated anticonvulsant activity in animal seizure models (Brodie and Porter, 1990). Like VPA and VGB, the drug has been shown to increase whole brain GABA levels following systemic administration (Poisson et al, 1984). Neurochemical studies have proposed the anticonvulsant effects of STP to be mediated by an inhibition of both GABA metabolism (Wegmann et al, 1978) and uptake (Poisson et al, 1984). STP has demonstrated efficacy against experimental seizures induced by PTZ, MES, and bicuculline (Poisson et al, 1984) and has been shown to delay the onset of seizures induced by 4-deoxypyridoxine hydrochloride in alumina gel-treated rhesus monkeys (Lockard et al, 1985). Controlled clinical trials with STP are ongoing and initial reports have suggested that the drug is active in both refractory partial seizures (Rascol et al, 1989) and absence seizures (Farwell et al, 1989).

Topiramate (TPM) is a sugar sulphamate compound, structurally distinct from other anticonvulsant drugs (Rogawski and Porter, 1990). Little data exists on the proposed mechanism of action of TPM, although with a similar experimental and clinical anticonvulsant profile to PHT (Rogawski and Porter, 1990), blockade of voltage-sensitive Na^+ channels might contribute to its antiepileptic effects. The drug has demonstrated efficacy against MES-induced seizures in mice and rats, but was without effect in the PTZ test (Maryanoff et al, 1987). In clinical trial, TPM has been shown to

protect against drug-resistant partial seizures and to be devoid of interaction with PHT, CBZ, or VPA (Floren et al, 1989).

Eterobarb (ETB) is a barbituric acid derivative with attenuated sedative and hypnotic activity compared to PB (Rogawski and Porter, 1990). ETB itself does not enter the brain and thus can be considered as a prodrug for its active metabolites, N-monomethoxymethyl-phenobarbital (MMP) and PB. The mechanism of action of ETB has been presumed to be identical to that of PB (section 1.3.2), although MMP may also contribute in this respect. Indeed, ETB has a distinct experimental anticonvulsant profile to PB, being more active in the MES test than in the PTZ test (Gallagher, 1989) and this may reflect the alternate properties of MMP (Rogawski and Porter, 1990). The majority of clinical studies with ETB have compared it to PB and, as such, have suggested it to possess a far greater therapeutic ratio than its metabolite (Wolter, 1991). ETB has demonstrated efficacy against partial and generalised tonic-clonic seizures in adults (Wolter, 1991) and has been proposed to precipitate fewer episodes of hyperactivity in children currently treated with barbiturate anticonvulsants (Bernardina et al, 1989).

Ralitoline (RLT) is a structurally novel anticonvulsant which, based on its pharmacological properties, is expected be a potent and broad spectrum AED (Anhut et al, 1991). RLT has been shown to displace [^{3}H]-batrachotoxin binding from isolated rat brain membranes and to reduce sustained repetitive firing in cultured mouse spinal cord neurones (Anhut et al, 1991), suggesting that the drug exerts its anticonvulsant effects by blockade of voltage-dependent sodium channels in an analogous manner to PHT and

CBZ. RLT has demonstrated efficacy against tonic seizures induced by MES and a variety of chemoconvulsants, including PTZ, 3-MPA, bicuculline, picrotoxin, and strychnine. It has also been shown to be effective against threshold clonic seizures induced by intravenous, but not subcutaneous, administration of PTZ (Anhut et al, 1991). In addition, the drug has shown efficacy against hippocampal kindled seizures (Bartoszyk et al, 1986a) and reflex seizures in various genetically epilepsy-prone animal models including the DBA/2 mouse, photosensitive *Papio papio* baboon (Anhut et al, 1991), and Mongolian gerbil (Bartoszyk and Hamer, 1987). Clinical studies with RLT remain in their infancy. However, it has demonstrated a beneficial effect on EEG spike-wave discharges in patients with refractory partial seizures (Anhut et al, 1991).

Losigamone (LSM) is a product of structure-activity screening for anticonvulsant activity of a series of analogues and derivatives of the naturally occurring 5- and 6-membered lactones (Stein et al, 1991). The mechanism of action of LSM remains to be elucidated. It does not bind to GABA, picrotoxin, or BZD receptor sites and, although it has been shown to reduce adenosine uptake in mouse brain synaptosomes, this effect was only observed at high concentration (Stein et al, 1991). In lower concentrations the drug potentiated GABA-induced chloride conductance in primary cultured spinal cord neurones, an effect blocked by both picrotoxin and bicuculline (Stein et al, 1991). These observations have suggested that LSM might exert its anticonvulsant action via effects at the $GABA_A$ receptor complex (Stein et al, 1991). In mice, the drug has been shown to be effective against tonic seizures induced by MES, PTZ, bicuculline, and 4-AP as well as clonic seizures induced by PTZ, bicuculline, and picrotoxin (Stein et al, 1991). It afforded no protection against tonic strychnine and

picrotoxin seizures in mice, clonic NMDA seizures in mice, or clonic PTZ seizures in rats (Stein et al, 1991). Phase I clinical trials with LSM have been completed and phase II studies are now underway.

1.4.3 Promising experimental stage compounds

With greater understanding of the cellular mechanisms proposed to underlie the seizure discharge (section 1.1.5), rational drug design for epilepsy has progressed greatly in the last 10 years. There is a large body of evidence to suggest that the paroxysmal neuronal discharges which typify epileptic activity can be generated under three main conditions: i) impairment of GABA-mediated inhibition; ii) enhancement of glutamatergic excitation; iii) endogenous bursting characteristics of inherently "epileptogenic" neurones of the hippocampus and neocortex (Mutani et al, 1991). Clearer understanding of these processes has provided much of the basis for the informed design of the novel antiepileptic therapies described above. There is, however, a continued requirement for the development of novel agents with potentially more pharmacological selectivity, better pharmacokinetics, more benign side effects, fewer drug interactions, and wider spectra of clinical activity.

Impairment of GABAergic inhibition has been mooted as one of the most likely contributors to epileptic discharge for many years. As a result, potential pharmacological manipulation of GABAergic mechanisms has been exhaustively studied in the search for novel antiepileptic therapies. Several of the traditional AEDs, including PB, VPA, and the BZDs, appear to exert their effects, at least in part, by potentiation of GABA-mediated inhibition (Macdonald and Barker, 1979). Indeed,

some of the novel antiepileptic agents discussed above, such as VGB, TGB, and STP, have also been proposed to enhance GABAergic neurotransmission and with potentially more selectivity than their predecessors. These compounds are beginning to make their mark in the pharmacotherapy of epilepsy and initial reports suggest that they may represent a small but significant improvement on existing treatment. Although much of the emphasis in the search for novel AEDs has now turned to potential modulators of excitatory neurotransmission, some promising anticonvulsant drugs with selective GABAergic mechanisms of action remain in development. Included in this group are the anticonvulsant β-carbolines which potentiate the action of GABA at the $GABA_A$ receptor complex (Meldrum, 1984) and derivatives of the neurosteroids, which are also endogenous modulators of GABA action at this receptor site (Kokate et al, 1993).

The discovery of $GABA_A$ receptor heterogeneity, in terms of sub-unit composition, and the subsequent demonstration of the diverse anatomical distribution of these receptor sub-types (Macdonald, 1993), may have implications for the development of selective GABA-enhancing anticonvulsants in the longer term. Loreclezole (LCZ), recently withdrawn from clinical development, was the first compound to demonstrate anticonvulsant activity through a proposed sub-unit selective action (Wingrove et al, 1994). LCZ has demonstrated anticonvulsant efficacy in a wide range of experimental seizure models (De Beukelaar and Tritsmans, 1991) and recent data suggests that its effects are mediated via an action at β_2 and β_3 but not β_1 sub-units of the pentameric $GABA_A$ receptor complex (Wingrove et al, 1994). Development of LCZ was recently suspended owing to a pharmacokinetic profile incompatible with clinical investigation. Analogues with improved pharmacokinetics may, however, represent the first clinically

useful drugs of what promises to be a long line of selective GABA facilitating compounds, site-directed to individual receptor sub-units and thus to particular regions of the brain.

With the ever increasing awareness of the role of excitatory neurotransmission in epileptogenesis, more and more emphasis is now being placed on the development of novel anticonvulsant agents with direct effects on excitatory processes. As described in sections 1.4.1 and 1.4.2, many existing pharmacotherapies of epilepsy rely on a non-specific attenuation of neuronal excitation, predominantly through an action on voltage-dependent sodium channel conductance. More recently, the search for novel antiepileptic therapies has been directed towards direct modulators of glutamatergic neurotransmission (Chapman and Meldrum, 1991; Meldrum, 1992).

The discovery of selective antagonists of the NMDA receptor (Davies and Watkins, 1982; Wong et al, 1986), demonstration of their anticonvulsant effects (Croucher et al, 1982; Sato et al, 1988), and greater awareness of the role of this receptor in epileptic processes (Dingledine et al, 1990), has led to several of these compounds being proposed as putative AEDs. The majority of glutamate receptor antagonists remain at an early experimental stage in their development as AEDs, although one or two such compounds are now entering clinical trial in this respect.

Of the original competitive NMDA receptor antagonists, the most selective compounds, AP5 and AP7, were systemically active in a variety of models of reflex epilepsy including audiogenic seizures in DBA/2 mice and GEP rats and photically-induced

myoclonus in *Papio papio* baboons (Meldrum, 1992). These compounds were also active against chemically-induced seizures and those precipitated by electroshock (Chapman, 1991). The more recently developed competitive NMDA antagonists, CPPene and CGS 37849, which are structurally related to AP5 and AP7, afford very long lasting protection against photically-induced myoclonic seizures in the *Papio papio* baboon following oral administration (Chapman et al, 1991a).

Non-competitive NMDA antagonists, which act on the open channel of the receptor-ionophore complex, such as phencyclidine (PCP) and MK-801 are also anticonvulsant in animal seizure models but produce motor toxicity and/or cognitive impairment at doses equal to, or less than, those required to elicit an anticonvulsant response (Meldrum, 1992). Some related compounds such as remacemide (section 1.4.2) and ADCI (Rogawski et al, 1991) are, however, weakly active at this site and, as such, produce anticonvulsant effects at doses that do not precipitate motor toxicity.

Compounds active at the strychnine-insensitive glycine recognition site on the NMDA receptor complex, in a manner similar to that proposed for FBM (section 1.4.2), have also demonstrated anticonvulsant activity in animal seizure models and may hold promise for future development as AEDs. Of these, HA-966 (Meldrum, 1992) and L-687,414 (Smith and Meldrum, 1992), have been shown to protect against evoked seizures in a variety of models of reflex epilepsy.

Recent evidence linking not only the NMDA sub-type of glutamate receptor, but also the AMPA (α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid) subtype to

epileptic processes has opened another potential avenue of drug development (Dingledine et al, 1990). Despite being in their infancy, in developmental terms, when compared to antagonists at the NMDA receptor, several AMPA receptor antagonists have demonstrated anticonvulsant effects in animal seizure models. Of these, NBQX and GYKI 52466 have shown significant but short-lived anticonvulsant activity in rodent and primate models of epilepsy at doses not producing motor side effects (Chapman et al, 1991b; Smith et al, 1991). A recent report has also suggested that co-administration of NMDA and AMPA antagonists might elicit synergistic anticonvulsant effects on experimental seizures induced by kindling (Löscher et al, 1993).

A recent clinical evaluation of the competitive NMDA antagonist CPPene in patients with intractable complex partial seizures proved disappointing, with all subjects withdrawing prematurely from the trial as a result of neurological side effects (Sveinbjornsdottir et al, 1993). Nevertheless, these authors insisted that continued investigation of related compounds was warranted. Thus, the full potential of EAA antagonists in the treatment of epilepsy remains to be realised.

Members of the dihydropyridine (DHP) class of calcium channel blockers have been proposed as putative AEDs by virtue of their ability to limit neuronal excitation (Meyer, 1989). These compounds have been the subject of extensive experimental and clinical, investigation into their anticonvulsant properties. They have an impressive spectrum of activity against chemically-induced seizures in animals (Karler et al, 1991; Larkin et al, 1992b; Palmer et al, 1993) and they protect against seizures induced by electrocortical

shock (ECS; Meyer et al, 1990), ischaemia (Meyer et al, 1986a), and ethanol withdrawal (Little et al, 1986). They are also active in animal models of reflex epilepsy (De Sarro et al, 1988; 1990). These compounds and nimodipine (NMD) in particular, are discussed in detail in section 5.1.9. Unfortunately, the DHPs have often failed to reproduce their experimental efficacy in clinical studies. Their lack of effect, in this respect, has been suggested to be related to low circulating drug concentrations (Larkin et al, 1991). The inevitable cardiovascular side-effects of these drugs has thus far prevented evaluation of higher DHP doses. Future development of novel, centrally-specific calcium channel blockers that can extrapolate their experimental efficacy into the clinical arena may represent a significant advance in the treatment of epilepsy.

The abnormalities in ionic homeostasis which may confer the "spontaneously epileptogenic" nature of certain neurones and thus might underlie idiopathic epileptogenesis (section 1.1.5) are likely to be too general in nature to serve as potential targets for therapeutic manipulation (Meldrum, 1989). Other potential contributors to spontaneous neuronal discharge, which might more easily be pharmacologically targeted, include genetically determined defects in neurotransmitter and endogenous anticonvulsant function. It has been proposed that such abnormalities could be compensated for, or corrected by, specific pharmacotherapy (Meldrum, 1989). Defects in primary neurotransmitter function could, theoretically, be remedied by the rational employment of any one of the GABA-enhancing or excitation-attenuating compounds discussed above. In contrast, functional potentiation of one or more of the variety of endogenous anticonvulsant compounds thus far discovered might represent a more subtle approach in the suppression of spontaneous neuronal discharge (Mutani et al,

1991). Endogenous anticonvulsants currently of interest include adenosine (Dragunow et al, 1985) and kynurenic acid (section 7.1.2).

1.5 EXPERIMENTAL MODELS OF EPILEPSY

The study of the epilepsies has been dependent on the use of model systems. Much of what is now known about seizures has been derived directly, or indirectly, from animal models (Fisher, 1989). There are countless numbers of animal models of epilepsy (Löscher and Schmidt, 1988), which may reflect the diversity of the clinical syndromes they attempt to mirror, however, they all have significant limitations for their use (section 8.1.1). The most commonly reported of these is the observation that many of the proposed animal models of the epilepsies are, in fact, models of a single epileptic seizure and thus do not mirror the condition of chronically recurrent spontaneous seizures which is characteristic of human epilepsy (Löscher and Schmidt, 1988). Nevertheless, they remain in continual employment for the investigation of epilepsy and the identification of novel AEDs and will do so until they are superseded by more clinically relevant experimental paradigms.

1.5.1 Genetic animal models of epilepsy

Animals with a genetically-determined susceptibility to seizures can be roughly divided into two groups, those in which the seizures are spontaneously recurrent and those in which some form of sensory stimulation is required to elicit a seizure response.

Animals which exhibit spontaneously recurrent seizures, by their very nature, should represent ideal experimental models of epilepsy. However, these genetically

compromised species are rarely used in the pre-clinical testing of AEDs (Löscher and Schmidt, 1988). Their main disadvantage is that in most of these animals the naturally occurring seizures cannot be precipitated at will by the investigator, making drug-efficacy studies time-consuming, especially when seizure frequency is low. Among the most useful genetic animal models of spontaneous seizure are epileptic dogs and rats with absence epilepsy.

Epileptic dogs have proved useful as models for focal and primary and/or secondary generalised seizures (Löscher et al, 1985). In addition to idiopathic syndromes, some dogs have also demonstrated symptomatic epilepsy, usually in response to brain tumours (Löscher et al, 1985). Veterinary experience with epileptic dogs has suggested that they respond to drug treatment in a similar manner to patients with epilepsy, with focal seizures apparently more resistant to treatment than generalised tonic-clonic seizures (Löscher and Schmidt, 1988). Although these animals represent an ideal source for the investigation of idiopathic and intractable seizures, they are not suitable for drug screening programmes due to their low seizure frequency (Edmonds et al, 1978) and the logistical problems associated with maintaining sufficient numbers of dogs in long-term studies (Löscher and Schmidt, 1988).

Rats with spontaneous generalised absence seizures have a sufficiently high seizure frequency to allow their use in drug screening programmes. Approximately 15 - 30 % of Sprague-Dawley and Wistar rats exhibit spontaneous spike-wave discharges which are associated with absence seizure-like behavioural arrest and twitching of the vibrissae (Vergnes et al, 1982). Susceptible animals may experience up to 100 absence-like

events per day. Age appears to be a critical factor in their precipitation, with seizure onset at some time after 14 - 18 weeks and persisting for life (Van Luijtelaar and Coenen, 1986). Seizure incidence increases in these animals when two such rats are used for breeding, demonstrating the genetic nature of the abnormality. The pharmacological sensitivity of these seizures is similar to that of human absence epilepsy (Löscher and Schmidt, 1988). These rats with spontaneous non-convulsive epilepsy, although not widely employed, represent perhaps the most closely relevant animal model to human absence epilepsy presently available.

Other animal models with spontaneously recurrent seizures include the *tottering* mouse, the AE mouse, and the Syrian golden hamster. *Tottering* mice, which exhibit focal motor seizures, have a high seizure frequency which would be compatible with long-term AED testing (Noebels and Sidman, 1979). However, breeding difficulties with this strain have precluded its extensive use (Löscher and Schmidt, 1988). The generalised tonic-clonic seizures observed in AE mice are relatively insensitive to treatment with traditional AEDs and thus might represent a suitable animal model of intractable seizures (Löscher et al, 1986a). The drug-resistant seizures in this strain are not, however, sufficiently frequent to contemplate inclusion of these animals in drug screening programmes (Löscher and Schmidt, 1988). The inbred BIO 86.93 strain of Syrian golden hamsters exhibits myoclonic and generalised tonic seizures both spontaneously (Löscher and Schmidt, 1988) and in response to mild stress (Yoon et al, 1976). These events are also relatively resistant to antiepileptic therapy. However, the seizure characteristics of these animals bear no resemblance to those of human epilepsy

and, furthermore, these animals are not available commercially (Löscher and Schmidt, 1988).

Genetically compromised animal strains which exhibit seizures in response to sensory stimulation are far more widely employed in AED evaluation than their purely spontaneous counterparts. This is a reflection of the readily evokable nature of their seizures. The major drawback of these genetic animal models with reflex seizures is that such modes of seizure precipitation are unusual in humans and so they bear little relevance to the clinical syndromes they are proposed to represent. Nevertheless, these animals provide the researcher with model systems of reproducible seizures that do not require electrical or chemical induction and which have a similar phenomenology, if not basis, to those occurring in human epilepsy.

The photosensitive baboon, *Papio papio*, which is perhaps the most clinically relevant model of primary generalised epilepsy in current laboratory use (Fisher, 1989), was first reported by Killam and colleagues (1966). These animals, indigenous to the Casamance region of Senegal, typically exhibit myoclonic seizures, although a small percentage also show full tonic-clonic events (Naquet and Meldrum, 1972). Seizure characteristics include facial and body clonus with a concomitant 3 Hz spike-wave record on the EEG and occasional progression to tonic-clonic discharge (Fisher, 1989). While these primates occasionally exhibit spontaneous myoclonic activity, seizures are more commonly precipitated by intermittent light stimulation at frequencies of around 25 Hz. Although the tonic-clonic EEG sequence in *Papio papio* is similar to that observed in humans, seizures in these animals have a different sensitivity to anticonvulsant

treatment than their proposed clinical correlates (Löscher and Schmidt, 1988). In addition, this species does not lend itself to humanitarian or economical investigation.

The most popular genetic model of reflex epilepsy is the DBA/2 strain of mouse which, between the age of 2 and 4 weeks, exhibits generalised seizures upon audiogenic stimulation (Seyfried and Glaser, 1985). At 8 weeks of age, when these mice are free from audiogenic susceptibility, they continue to show an increased sensitivity to seizures induced by MES and EAAs (Engstrom and Woodbury, 1988). The characteristic sequence of audiogenically-induced seizure activity begins with a phase of wild running, followed by clonic convulsions, tonic extension, and then either respiratory arrest (in around 60% of animals) or complete recovery (Löscher and Schmidt, 1988). The clonic phase is generally used for anticonvulsant drug evaluations. The majority of AEDs in clinical use are active against audiogenic seizures in DBA/2 mice and while this may restrict their use in the search for novel therapies of drug-resistant epilepsy, it unlikely than any potential anticonvulsant compound would be rejected by this model (Löscher and Schmidt, 1988). Although the DBA/2 mouse has no direct counterpart in clinical epilepsy, it is relatively inexpensive and is gaining popularity in anticonvulsant drug screening programmes.

Another popular rodent model of genetic reflex epilepsy is the GEPR, studied extensively by Laird and Jobe (1987). These animals are vulnerable to audiogenically induced seizures from around 2 - 4 weeks after birth. Two distinct colonies of these animals exist, GEPR-3s and GEPR-9s, characterised by the stimulus-induced seizure severity. Only the GEPR-9 strain exhibits the tonic-clonic phase of the response (Fisher,

1989). The characteristic sequence of seizure activity, wild running, leading to clonic convulsions, and tonic extension in GEPR-9s, is similar to that of the DBA/2 mouse although these animals rarely experience respiratory arrest (Löscher and Schmidt, 1988). For anticonvulsant drug evaluation, the tonic-clonic component of the GEPR-9 response pattern is commonly employed, pharmacological sensitivity of which compares with the efficacy of drugs in the MES test (Löscher and Schmidt, 1988). Again, this rodent model has no counterpart in clinical epilepsy and in contrast to the DBA/2 mouse, breeding difficulties with this strain (Consroe et al, 1979) preclude its more extensive use.

The Mongolian gerbil is another genetic animal model of reflex epilepsy, first described by Thiessen and co-workers in 1968. The most common method of seizure induction in this species is an air blast of 5 - 10 bars pressure, directed at the back, which results in seizure precipitation in around 98% of the animals (Löscher and Frey, 1984). Seizure susceptibility to other stimuli can be increased by selective breeding of responsive animals (Loskota et al, 1974). An age-dependent seizure severity is expressed in the Mongolian gerbil, with young animals (7 - 10 weeks) experiencing facial myoclonic seizures, while older animals exhibit extensive myoclonic or tonic-clonic convulsions (Löscher and Frey, 1984). Maximal seizure severity is generally reached at about 7 months of age and does not change thereafter. Anticonvulsant sensitivity of the two different seizure types differs in that clinical anti-absence agents are effective against the focal myoclonic seizures in young animals whereas PHT, CBZ, and PB are more active against the generalised motor seizures in older animals (Frey et al, 1983). The progression of seizure severity reported in this species has been proposed as a suitable

model for investigation of the mechanisms of the age-dependent onset of generalised seizures and of secondary generalisation in patients (Löscher and Schmidt, 1988). Unfortunately, these animals have significant drawbacks which has limited their experimental use. These include a particularly long post-ictal refractory period which restricts their testing to once per week and a sensitivity to handling which may result in seizure precipitation during systemic drug administration (Löscher and Schmidt, 1988).

1.5.2 Animal models with electrical seizure induction

That electrical stimulation of the brain can precipitate seizure activity has been recognised for over a century (Fritsch and Hitzig, 1870; Albertoni, 1882). Today, several animal models of epilepsy rely on electrical stimulation of the brain to induce characteristic patterns of seizure activity. These are widely employed in investigations of epileptogenesis and in the evaluation of AED action.

The MES test is arguably the best-studied and most useful seizure model (Fisher, 1989). This model is discussed in detail in section 3.1.2. It relies on administration of a supramaximal electrical stimulus, via ear-clip or corneal electrodes, to elicit a tonic flexion of the fore-limbs and, more commonly, the hind-limbs of both mice and rats (Löscher and Schmidt, 1988). Anticonvulsant drug efficacy is determined by the ability of a drug to reduce the incidence of the "all or none" hind-limb response in individual groups of animals (Löscher and Schmidt, 1988). The MES test is probably the most useful animal model for the prediction of the likely clinical efficacy of drugs against generalised tonic-clonic seizures. Thus, PHT, CBZ, PB, and PRIM are all highly active in this test, whereas ESM is devoid of activity (Löscher and Schmidt, 1988). One of the

major drawbacks of the MES test is that it occasionally yields false negative results as a function of its supramaximal nature (Löscher and Schmidt, 1988).

Another commonly employed electroshock seizure model which overcomes the insensitivity problem of the MES test is the minimal, or threshold, electroshock (Min-ES) test (Löscher and Schmidt, 1988). This test, again discussed in detail in section 3.1.2, has been advocated for use in conjunction with its supramaximal counterpart (Löscher et al, 1991a). The stimulating current, greatly reduced in comparison to the MES test, is again administered via ear-clip or corneal electrodes. Sub-threshold seizures in this model are characterised by a face and fore-limb clonus, progressing to a "running-bouncing" clonus (Racine, 1972). AEDs can be evaluated by their ability to suppress 5 seconds of sustained fore-limb clonus (Fisher, 1989), although such seizure manifestations can only be precipitated by the use of corneal electrodes (Browning and Nelson, 1985). More commonly, investigators employ the Min-ES test to evaluate the effects of AEDs on the threshold for induction of tonic seizures by the "up-down" method (Löscher and Schmidt, 1988; section 3.1.2). Like the MES test, this model predicts the effectiveness of drugs against generalised seizures of the grand mal type, but is much more sensitive in this respect.

ECS seizures are commonly precipitated in anaesthetised rabbits and are quantified in terms of EEG paroxysms rather than motor manifestations. The procedure involves implantation of bipolar stimulating electrodes into the frontoparietal cortex with EEG recording electrodes applied to the cortical surface (Meyer et al, 1990). Seizure activity is induced, in the presence of a quaternary ammonium muscle blocker to abolish motor

activity, by electrical impulses at 15 minute intervals and is recorded as the duration of the associated paroxysmal electrical activity on the EEG trace (Meyer et al, 1990). Control responses and those in the presence of the test substance can be gleaned from the same animal and these are compared in terms of the discharge duration. Although this technique is not widely employed for the evaluation of AED action, perhaps due to its inherent complexity, it has implications for the investigation of the mechanisms of epileptogenesis.

The kindling model of partial epilepsy is one which is rapidly gaining popularity for the investigation of both epileptogenic mechanisms and AED action (McNamara, 1984; McIntyre and Racine, 1986). Kindling is a manifestation of the fact that "seizures beget seizures" (Gowers, 1881) and is a phenomenon by which repeated electrical stimulation of a particular brain region results in an enhanced excitability and the subsequent precipitation of epileptic seizures (Fisher, 1989).

The amygdala is usually the most responsive structure to kindling stimuli, although most regions of the forebrain can be kindled (Goddard et al, 1969). Every species so far studied is subject to kindling, although most investigators employ the rat for such experimentation. Unilateral implantation of bipolar stimulating and recording electrodes is followed, approximately 2 weeks later (Löscher and Schmidt, 1988), by daily electrical stimulation with an initially sub-convulsive current (Fisher, 1989). After a few days of stimulation the train of shocks precipitates electrical afterdischarges which become progressively more complex and prolonged with each kindling stimulus.

The behavioural response to kindling evolves through 5 stages (Class I - V) of epileptiform activity (Racine, 1972). Classes I and II, characterised by immobility and twitching of the vibrissae followed by facial clonus and head nodding, are proposed to mimic human complex partial seizures. The class III kindled response is characterised by unilateral fore-limb clonus (contralateral to the focus) and has been suggested to mimic the clinical profile of a simple partial seizure. Classes IV and V are associated with rearing and bilateral fore-limb clonus followed by loss of the righting reflex and generalised clonic seizures. These may be considered as models of secondarily generalised partial seizures (McNamara, 1984; Löscher and Schmidt, 1988; Fisher, 1989). Once animals reach stage 5 they can be considered to be fully kindled and the increased sensitivity to electrical stimulation prevails in these animals for several months, reflecting persistent changes in brain function.

Most kindling experiments are terminated after elicitation of one or more class V motor seizures. However, if stimulation is continued for a few weeks, rodents exhibit spontaneous epileptic seizures in the absence of their priming shocks (Pinel and Rovner, 1978). Among those animals exhibiting at least three spontaneous stage 5 motor seizures, the occurrence of spontaneous seizures persisted for up to 7 months in the absence of electrical stimulation (Löscher and Schmidt, 1988).

Kindling is a relatively time consuming procedure as it requires chronic implantation of electrodes and regular electrical stimulation (once daily for up to 15 days) to establish the fully kindled seizures that are generally employed for AED evaluation (Löscher and Schmidt, 1988). If animals are kindled too rapidly the post-ictal refractory period

interferes with kindling acquisition (Goddard et al, 1969) and if the recovery period after electrode implantation is too short the sensitivity of the animals to kindling is lowered (Löscher and Schmidt, 1988).

Kindling has advantages over other models of partial seizures in that the effects of AEDs can be evaluated against not only the fully kindled state, but also against the development of kindled seizures (Löscher and Schmidt, 1988). Similarly, drug effects on the threshold current required to elicit seizures in fully kindled animals can be compared to the supramaximal currents required to precipitate seizures in naive animals. Thus, the researcher can discriminate between drugs that increase the seizure threshold and those active against seizure spread (Löscher and Schmidt, 1988). In addition, four different measures of anticonvulsant efficacy can be measured in a kindled animal: (1) seizure latency (time from stimulation to the first sign of seizure activity), which is a measure of the rate of seizure spread from a focus, (2) seizure severity, graded according to the five classes described above, (3) seizure duration and (4) afterdischarge duration, which is a measure of the limbic electrographic activity recorded from a focus.

The majority of established AEDs, with the possible exception of ESM, are active against the development of kindling and/or the expression of fully kindled seizures (Rogawski and Porter, 1990). In general, the traditional AEDs, including PHT, CBZ, and PB, are significantly less potent in blocking kindling-induced focal motor seizures (classes I - III) than generalised motor seizures (classes IV and V; Albright and Burnham, 1980; Löscher and Schmidt, 1988). Such data are consistent with the clinical

observation that partial seizures are more resistant to AED treatment than are generalised seizures (Brodie, 1990).

1.5.3 Animal models with systemic chemical seizure induction

Innumerable chemical compounds, at toxic doses, can induce seizures following systemic administration (Fisher, 1989). Only those which have a relevance to the investigation of the epilepsies will be discussed here.

PTZ is probably the most commonly employed chemoconvulsant in the laboratory evaluation of AED action (Löscher and Schmidt, 1988). PTZ is a tetrazol derivative (Stone, 1970) which produces consistent convulsive actions in rodents, cats, and primates following systemic administration. PTZ seizure models, maximal and threshold, are discussed in detail in section 3.1.1. Briefly, the maximal PTZ test involves subcutaneous (s.c.) administration of a species-dependent dose of the drug (70 - 100 mg/kg) to induce sustained myoclonic seizures, generalised clonic seizures and, at higher dose, a tonic extensor response within 3 - 5 minutes. This test is often referred to as the CD_{97} PTZ test, as it employs a convulsant dose (CD) sufficient to induce seizures in 97% of naive animals. The latency to the precipitation of individual seizure states is recorded and compared in drug evaluation studies (Löscher et al, 1991b). Graduated intravenous administration of the drug is employed in the PTZ threshold test. This is a sensitive measure of the clonic, and tonic, seizure threshold and is often more responsive to AED treatment than its maximal counterpart (Löscher et al, 1991b). The mechanism of the convulsant action of PTZ is believed to be related to an inhibitory effect at the $GABA_A$ receptor complex (sections 3.1.1). PTZ-induced tonic seizures can

be blocked by most drugs effective in the traditional MES test (Löscher and Schmidt, 1988), whereas PTZ-induced clonic seizures have been proposed as a predictor for likely anti-absence activity in man (Eadie, 1985). While this criterion holds true for the efficacy of ESM (Swinyard and Woodhead, 1982) and the inactivity of PHT (Krall et al, 1978) against PTZ-induced clonic seizures, its rather loose correlation with the clinical syndrome has been repeatedly questioned. The consensus of opinion would now suggest that the PTZ seizure tests have no direct parallel in the clinical arena (Löscher and Schmidt, 1988).

There are several other chemoconvulsant compounds that have been proposed to exert their effects by antagonism at the $GABA_A$ receptor complex (Löscher and Schmidt, 1988). Those most commonly used in experimental studies include bicuculline and picrotoxin. Although they differ in their precise mechanisms of convulsant action, with bicuculline competitively antagonising GABA binding and picrotoxin irreversibly interacting with the chloride ionophore of the $GABA_A$ receptor complex (Barolet et al, 1985), they tend to have parallel sensitivities to traditional AEDs (Fisher, 1989). The only exception to this rule is CBZ which, unexplainedly, protects against picrotoxin- but not bicuculline-induced clonic convulsions (Porter et al, 1984). The characteristic seizures precipitated by these compounds are in most instances similar to those induced by PTZ and so do not offer any significant advantage in this respect (Löscher and Schmidt, 1988). Similarly, the motor manifestations produced by systemic bicuculline and picrotoxin administration have no direct counterpart in clinical epilepsy (Löscher and Schmidt, 1988).

Strychnine is a chemoconvulsant compound which precipitates strong tonic extensor spasms following systemic administration (Fisher, 1989). Although this drug also interacts with the $GABA_A$ receptor (Braestrup and Nielsen, 1980), it is believed to exert its proconvulsant action by an irreversible antagonism of glycine binding (Curtis et al, 1971). Glycine is an important inhibitory neurotransmitter in the brainstem and spinal cord (Probst et al, 1986) and has also been proposed as a co-agonist at the NMDA receptor via an action on strychnine-insensitive binding sites (section 7.1.2). The lack of cortical EEG seizure activity associated with strychnine-induced convulsions (Stone, 1972) might reflect its predominant action on inhibitory glycinergic interneurones, termed Renshaw cells, in the spinal cord. Thus, strychnine-induced seizures may have little or no relation to clinical epilepsy syndromes (Löscher and Schmidt, 1988). Furthermore, the tonic seizures precipitated by strychnine are not fully relieved by doses of common AEDs that are active in other chemoconvulsant seizure models (Fisher, 1989).

The EAA receptor agonists, NMDA and kainic acid, are now regularly employed in the production of experimental seizures in animals. Systemic kainic acid administration results in the acute or sub-acute production of seizures which may be manifested for several hours or days (Fisher, 1989). It is predominantly used as a model of partial seizures arising in the hippocampus although these can sometimes progress to readily evaluable generalised tonic-clonic convulsions (section 8.1.2). In higher dose, kainate has been employed as a model for the limbic cell damage which can occur with clinical status epilepticus (Sloviter, 1987). NMDA-induced seizures are more conventional in chemoconvulsant terms. Systemic administration of 100 - 200 mg/kg NMDA to mice

precipitates a chain of events characterised by initial behavioural abnormalities, followed by clonic and then tonic seizures, and eventually death within 60 minutes (Fisher, 1989; Palmer et al, 1993). The clonic, tonic, and lethality aspects of the NMDA response have all been employed for comparative drug evaluations. Like strychnine-induced seizures, those precipitated by NMDA are relatively resistant to conventional AED treatment. Weak protection against NMDA-induced seizures and lethality is afforded by VPA, the BZDs, and specific EAA antagonists alone (Czuczwar et al, 1985; Leander et al, 1988b). While the kainic acid model has a degree of relevance to clinical epileptic syndromes (section 8.1.2), no such correlation has been described for NMDA-induced seizures (Löscher and Schmidt, 1988).

Other systemic chemoconvulsants employed in the laboratory evaluation of AED action include the GABA synthesis inhibitors, 3-MPA and D,L-allylglycine (Horton and Meldrum, 1973), the potassium channel inhibitor, 4-AP (Yamaguchi and Rogawski, 1992), the cholinomimetic, pilocarpine (Turski et al, 1983), and the inverse BZD receptor agonist, methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM; Petersen, 1983). All of these compounds induce generalised motor seizures in laboratory animals following systemic administration (Löscher and Schmidt, 1988; Fisher, 1989). An unusual addition to this list is the anaesthetic compound flurothyl which induces tonic-clonic seizures upon inhalation (Biossier et al, 1968). Also meriting special consideration here are GHB (Snead, 1988) and penicillin (Fisher and Prince, 1977) which have been proposed to precipitate specific absence-like events following systemic administration.

1.5.4 Animal models with focal chemical seizure induction

The capacity of several chemical compounds to precipitate electrical seizure activity and/or convulsions following direct administration into the brain has led to their wide-spread use in epilepsy research. Such animal models have often been proposed to be suitable experimental paradigms for the study of the human partial epilepsies (Fisher, 1989) although their exact relevance remains unclear. The cat - focal penicillin model (Matsumoto and Ajmone-Marsan, 1964), in particular, has proved invaluable in the investigation of the mechanisms of focal epileptogenesis (Prince, 1968).

Intra-muscular injection of penicillin in the cat gives rise to generalised absence-like events (Fisher and Prince, 1977), whereas direct administration of penicillin onto, or into, the cortex of cats and rats, results in the acute precipitation of focal seizure activity (Prince, 1978). Application of a cotton wool plug, soaked in 1.7 - 3.4 mM penicillin, to the exposed cortex results in the generation of interictal spikes on the surface EEG within a few minutes (Fisher, 1989). These discharges often resemble human interictal spikes recorded from the cortex at corticography (Fisher, 1989). The prevailing motor manifestations of this paroxysmal activity are invariably suppressed under the conditions of general anaesthesia. This model has been extensively employed in investigations of the neuronal basis of focal epileptogenesis and the synchronisation and spread of the associated seizure activity (Prince, 1972; 1978) but is rarely used in the evaluation of AED action (Golden and Fariello, 1984). The mechanism by which penicillin exerts its convulsant effects appears unrelated to the antibiotic actions of the drug. It is believed to induce seizure activity by blockade of GABA-mediated inhibitory post-synaptic potentials (IPSPs; Wong and Prince, 1979) via an action at the $GABA_A$

receptor complex (Twyman et al, 1992). At higher doses, penicillin's effects appear less specific (Fisher, 1989).

Other focal chemical convulsants proposed to have specific GABAergic mechanisms include bicuculline (Campbell and Holmes, 1984), picrotoxin (Usunoff et al, 1969), and the cephalosporin compound, cefazolin (Morocutti et al, 1986). Strychnine has also been suggested to exert its focal convulsant effects by blockade of GABA-mediated inhibition (Knopman, 1975) although its systemic convulsant action is believed to be anti-glycinergic in origin (Curtis et al, 1971). Cholinergic (Turski et al, 1987) and anticholinergic (Daniels and Spehlman, 1973) compounds have also been used to produce focal seizure discharges, as have the specific EAA agonists kainic acid and quinolinic acid (Craig, 1984; Vezzani et al, 1986).

Animal models employing application or implantation of particular metals onto, or into, the cerebral cortex have been described as the most realistic experimental representations of the human partial epilepsies (Fisher, 1989). These models are discussed in detail in section 8.1.2. The aluminium hydroxide gel model in the monkey (Ward, 1972) is the best validated of these, although specific salts of iron, tungsten, zinc, and cobalt have also been used to produce experimental epileptic foci (Löscher and Schmidt, 1988). Neurochemical studies have suggested that these metals exert their convulsant effects by impairment of GABAergic inhibition (Craig, 1984). The seizures precipitated by cortical application of convulsant metals are chronically recurrent (Fisher, 1989) and relatively resistant to AED treatment (Löscher and Schmidt, 1988). Thus, they have been proposed as suitable experimental media for investigation of the

intractable partial epilepsies (Fisher, 1989). While they have been employed in the study of the mechanisms of focal epileptogenesis (Craig, 1984), their inherent complexity has restricted their use in AED evaluations (Gladding et al, 1985).

Other, more loosely classed, focal "chemical" convulsants include tetanus toxin and the cryogenic substances liquid nitrogen and ethylchloride. These are also discussed in detail in section 8.1.2. Tetanus toxin induces limbic seizures following intra-hippocampal injection (Mellanby et al, 1977), whereas liquid nitrogen and ethylchloride precipitate focal epileptic discharges upon cortical application (Loiseau et al, 1987). Both of these models, tetanus toxin and cryogenic injury, have been proposed to represent human partial epilepsies (Fisher, 1989).

1.5.5 Neurophysiological seizure models

Although not strictly animal models of epilepsy, these diverse experimental paradigms do employ animal and in some cases, human tissues. These models allow the study of seizure discharge and AED actions at the cellular and molecular level and are used to further the understanding of epileptogenesis and to identify the mechanisms of new and established AEDs. Although in no way advocated as a substitute for whole animal models, these preparations provide information inaccessible by more traditional methods.

The rat hippocampal slice preparation (Laursen, 1984) has been especially productive for understanding of the epilepsies. It has been employed principally in the investigation of seizure discharges, in terms of their ionic (section 5.1.2) and physiological (section

1.1.4) bases, but has also provided insight into the specific mechanisms of AED action. Hippocampal slices are prepared from rodent sources by decapitation followed by rapid dissection of the whole brain. Both hippocampi are removed and sliced, perpendicular to the long axis, into approximately 500 μm sections (Fisher, 1989). This method preserves an intact three neurone circuit and any additional associated recurrent circuitry within each slice. Isolated hippocampal slices can be maintained for up to 18 hours. Recording, intra- or extra-cellular, is performed in a small chamber, oxygenated and perfused with artificial CSF (Fisher, 1989). Since brain slices do not move, epileptiform activity is an electrical phenomenon. Both PDSs and ictal discharges (section 1.1.4) can be observed in the isolated hippocampal slice. Such seizure-like activity may arise spontaneously, although it is more commonly evoked by electrical stimulation, chemical challenge, or ionic manipulation (Fisher, 1989). The advantages of the hippocampal slice preparation in neurophysiological and/or neurochemical studies include no blood-brain barrier, a lack of interference of anaesthetic compounds, and mechanical stability (Stone, 1989). Its disadvantages include uncertainty regarding its precise neuronal composition and potential interference of partial mechanical- or hypoxia-induced injury (Fisher, 1989). The hippocampal slice model is useful for screening putative anticonvulsant compounds, although it does not necessarily afford a correlation with efficacy or toxicity in the whole animal (Oliver et al, 1977).

Isolated human brain epileptic foci, resected at surgery (section 1.2.3) have been employed for neurohistological (Babb, 1986), neurophysiological (Schwartzkroin and Haglund, 1986), and neuropharmacological (van Gelder, 1987) study. While this tissue has the advantage over model systems in allowing direct investigation of the human

condition (Fisher, 1989), significant drawbacks have restricted its contribution in all but the neuropathological field (Sherwin, 1988). Resected human epileptic foci, being the primary causes of secondary epilepsy, are invariably regions of non-specific brain damage and are thus heterogeneous in nature. Similarly, optimal control tissue is impossible to acquire, the closest approximation being that obtained at post mortem where changes in neurophysiology and neurochemistry may further complicate interpretation (Fisher, 1989).

Primary cultures of both neurones and glial cells are now extensively employed in epilepsy research (Crain, 1972). Much of what is known about AED mechanisms of action has been derived directly from studies of cultured cells (Macdonald and Kelly, 1993). Primary culturing is a technique which is very much cell-specific (sections 2.9, 2.10 and 2.11). It requires an animal source of brain tissue, which is dissociated by specific enzymatic or mechanical methods. Cells, thus isolated, are maintained under physiological conditions for specific periods of time. Cultured neurones and astrocytes can be used for neurochemical (Gallo et al, 1982), neurophysiological (White et al, 1992), and neuropharmacological (Carboni and Wojcik, 1988) investigations. Such studies may incorporate whole cell recordings (McLean and Macdonald, 1986a; 1986b) or patch clamp techniques (Twyman et al, 1992). The advantages of using primary cultured cells include a lack of interference of neighbouring cells, the ability to deliver known concentrations of drug to the cell membrane, and the visibility of cells allowing direct electrophysiological recording (Stone, 1989). Disadvantages of such preparations include a lack of indication regarding the functional appropriateness of cells and/or

synapses, little or no correlation with whole animal studies (Stone, 1989), and possible inter-assay heterogeneity.

Other neurophysiological preparations employed in epilepsy research include the neocortical slice, which has similar uses to its hippocampal counterpart (Fisher, 1989) and techniques such as recombinant deoxyribonucleic acid (DNA) implantation into egg cells (oocytes) of the toad *Xenopus laeus*, facilitating study of AED action on single receptors or ion channels (Gundersen et al, 1988; Tomaselli et al, 1989; Wafford et al, 1990) and single cell recording from individual neurones *in vivo* in the anaesthetised animal (Löscher and Schmidt, 1988).

CHAPTER TWO

MATERIALS AND METHODS

2.1 MATERIALS AND EQUIPMENT

2.1.1 Materials

Radioisotopes: γ-[2,3,-^{3}H]-aminobutyric acid, $^{45}CaCl_2$, and [N-methyl-^{3}H]-PK-11195 were obtained from DuPont (New England Nuclear). 2-Deoxy-D-[1-^{14}C]-glucose was from Amersham.

Chemicals: Ammonium dihydrogen orthophosphate ($NH_4H_2PO_4$), ammonium sulphate ($(NH_4)_2SO_4$), calcium chloride ($CaCl_2$), cresyl violet, eosin, glacial acetic acid, glial fibrillary acidic protein (GFAP), D-glucose, haemotoxylin, hydrochloric acid (HCl), hystomount mounting medium, magnesium sulphate ($MgSO_4$), 2-mercaptoethanol, neutral buffered formalin, osmic acid, paraffin wax, perchloric acid, phosphoric acid, potassium chloride (KCl), potassium dihydrogen orthophosphate (KH_2PO_4), sodium bicarbonate ($NaHCO_3$), sodium chloride (NaCl), di-sodium hydrogen orthophosphate (Na_2HPO_4), sodium hydroxide (NaOH). and sodium dihydrogen orthophosphate (NaH_2PO_4) were all obtained from Merck.

Para-aminobenzoic acid (PABA), γ-aminobutyric acid (GABA), 2-aminoethylisothironium bromide (AET), boric acid, bovine serum albumin (BSA), cytosine arabinoside (ARA-C), 3'5'-dibutyryl cyclic adenosine monophosphate (cAMP), dithiothreitol, ethylene diamine tetra-acetic acid (EDTA), DNase I, gabaculline, L-glutamic acid, glutaraldehyde, heparin, HEPES, gold, insulin, α-ketoglutaric acid (α-KG), 3-mercaptopropionic acid (3-MPA), D,L,-norvaline, pentylenetetrazol (PTZ), *o*-phthalaldehyde (OPA), poly-D-lysine, pyridoxal-5'-phosphate (PLP), soya bean trypsin

inhibitor (SBTI), TRIZMA hydrochloride (Tris/HCl), Tween 80, trypsin, and veratridine were all obtained from Sigma Chemical Company.

Dulbecco's modified Eagle's medium (DMEM), Earle's balanced salt solution (EBSS), foetal calf serum (FCS), L-glutamine, horse serum (HS), minimal essential medium (MEM), penicillin, phosphate buffered saline (PBS), sterile culture water, and streptomycin were all obtained from Gibco BRL.

Acetonitrile, dichloromethane, diethylether, ethanol, hexane, isopentane, and methanol were obtained from Rathburn Chemicals Ltd.

Coomassie Brilliant Blue G-250 protein assay dye reagent and Dowex AG50Wx8 ion exchange resin were from BIORAD; nitrendipine was from Bayer; *p*-methylphenobarbital (p-MPB) from Aldrich; xylocaine from Astra Pharmaceuticals; Picofluor 40 scintillation fluid from Packard; unlabelled PK-11195 from Research Biochemicals; and D-19 developer and Kodafix were from Kodak GRL.

Pharmaceuticals: Amlodipine (2-[(2-amino-ethoxyl)methyl]-4(2-chlorophenyl)-3-ethoxycarbonyl-5-methoxycarbonyl-6-methyl-1,4-dihydropyridine) was obtained from Pfizer Central Research; lamotrigine (3,5-diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine, isethionate) from the Wellcome Foundation; nicotinylalanine (γ-(3-pyridyl)-γ-oxo-α-aminobutyrate) from Professor T.W. Stone, Department of Pharmacology, University of Glasgow; nimodipine (isopropyl-(2-methoxyethyl)-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate) from Bayer; tiagabine ((R-)-

(-)-1-[4,4-Bis(3-methyl-2-thienyl)-3-butenyl]-3-piperidine-carboxylic acid, hydrochloride) from Novo Nordisk A/S; and vigabatrin (D,L-4-aminohex-5-enoic acid) from Marion Merrell Dow.

2.1.2 Equipment used in analyses

Autoradiography: A Cambridge Instruments Quantimet 970 computer-assisted image analysis system was employed for densitometric analysis. X-ray plates were developed using a standard Kodak automatic processor. Tritium-sensitive film (Hyperfilm B_{max}) was from Amersham and ^{14}C-sensitive X-ray plates were from Kodak GRL.

Blood monitoring: A Gould Stratham P231D (model 2202) pressure transducer was used for blood pressure recording, a Corning blood gas analyzer was employed for blood gas analysis, and a semi-automated Beckman glucose oxidase enzyme assay analyzer was used for plasma glucose determination. Polyethylene cannulae (external diameter 0.96 mm; internal diameter 0.58 mm; 15 cm long) were obtained from Portex.

Centrifugation: A Wifug haemicrofuge was employed for small volume samples and a refrigerated MSE Mistral 2L centrifuge for all other samples.

Electroshock stimulation: A Ugo Basile 7801 electroconvulsive therapy (ECT) unit was used.

High performance liquid chromatography: Three systems were used. For determination of amino acid concentrations, a Waters model 510 pump, a Waters WISP 710B injector, a

Perkin Elmer LS-5 luminescence spectrophotometer, and a Shimadzu C-R1B integrator were used. Excitation and emission wavelengths were 330 and 440 nm respectively, with bandpasses of 20 nm. For PTZ measurement, a Waters model 501 pump, a Gilson model 231 injector incorporating a Gilson model 401 dilutor, a Kontron Instruments UVIKON 735LC ultraviolet detector, and a computer-based Jones Chromatography JCL-6000 integration package were used. Detection wavelength was 202 nm. For determination of NMD concentrations, a Hewlett Packard 1084B liquid chromatography system incorporating a Hewlett Packard 79850B integrator and a Philips Pye Unicam PU-4020 ultraviolet detector were used. Detection wavelength was 235 nm.

Homogenisation: Where preservation of an enzyme was important a glass Potter Elvehjam vessel and motor powered teflon pestle were used. The alternative method employed a polytron homogeniser and included sonication in the homogenisation process. Small volume samples were homogenised by sonication alone in a MSE Soniprep 150.

Scanning electron microscopy: Prepared cell cultures were coated with gold using a Polaron splitter-coater and screened with a Jeol T-300 scanning electron microscope.

Scintillation counting: A Canberra Packard 2000CA TRI-CARB liquid scintillation counter was used.

Sonication: Plasma samples for drug assay were sonicated in a MSE Soniprep 150.

Spectrophotometry: Protein concentrations were assayed with a Philips Pye Unicam PU-8600 UV/vis spectrophotometer incorporating a PU-8605 cell programmer.

Statistical analysis: Statistical analysis was performed using the MINITAB statistical package (release 8.21) on an Amstrad ALT- 386SX microcomputer.

2.2 GENERAL METHODS

2.2.1 Animal use

Two strains of mice were employed for investigation, CF1 mice (Bantin and Kingman) and latterly ICR mice (Harlan Olac). Studies involving rats used the out-bred Sprague Dawley (SD) strain, which were supplied by Bantin and Kingman and again latterly by Harlan Olac. Neonatal and foetal animals required for cell culture techniques were supplied by the Joint Animal Facility at the University of Glasgow from a breeding colony of SD rats. Experimental animals were housed in the departmental animal unit and for a short period within the animal facility at Gartnavel General Hospital, Glasgow. Animals were exposed to a controlled temperature and humidity environment throughout with a 12 hour light/dark cycle and had access to food and water *ad libitum*.

2.2.2 Blood sampling

Plasma drug levels in mice were determined where appropriate. Experimental animals were stunned by a blow to the head and a truncal blood sample obtained, into an Eppendorf tube, following decapitation. Fresh blood samples were centrifuged in a haemicrofuge at 2000 rpm for 5 minutes and the resultant supernatant plasma removed and stored at -20^{0}C until required.

2.2.3 Brain tissue removal

In general, animals were sacrificed by a blow to the head followed by decapitation. On occasion decapitation was performed under terminal halothane or ether anaesthesia. In a few instances rats were euthanised by carbon dioxide (CO_2) asphyxiation prior to removal of the head. The skin and tissues overlying the skull were incised and then removed. The point of a pair of bone cutters or scissors was inserted into the foramen magnum and the occipital bone incised, in either direction, in the dorsal plane. The occipital bone was then prised free of the underlying cerebellum. Next, the parietal bones of the skull were incised down either side, again in the dorsal plane, roughly at the level of the base of the brain. The parietal bones were prised clear of the brain surface taking care not to damage the underlying tissues. The meningeal membranes were cleared from the brain surface. Finally, the frontal bones were removed by a sharp fracture in the coronal plane just anterior to the olfactory lobes of the brain. Following severance of the optic nerves, the intact brain was removed with the aid of spatulas, weighed where applicable, and stored appropriately until required. In studies involving analysis of discrete brain regions, the brain was carefully dissected in accordance with the method of Glowinski and Iversen (1966) and each region weighed prior to storage.

2.2.4 Determination of protein concentrations

Many of the assays reported in this thesis required accurate and reproducible analysis of sample protein content to enable quantifiable calculation of experimental results. The recently developed BIORAD method is a sensitive test of protein concentration particularly when small volume samples with low protein content are under investigation. The method relies on the colour change of a dye (Coomassie Brilliant Blue G-250) in

response to protein concentration. Standards were prepared, in duplicate, over the range 0.5 - 2.0 μg/ml BSA. Samples of unknown protein concentration, also analysed in duplicate, were diluted into this range. BIORAD protein assay dye reagent was diluted 1:1 with water and added, in equal volume, to standards and samples alike. Following vortex-mixing, tubes were incubated at room temperature for 5 minutes and then read at 595 nm in a spectrophotometer. The intensity of the colour obtained was proportional to the amount of protein present and after construction of a standard curve, the protein content of samples could be determined. Results were corrected for dilution, averaged, and expressed in mg/ml.

2.2.5 Sample storage

Plasma and brain samples for drug assay were stored at -20^{0}C until analysis. Brain samples for the study of enzyme activities and neurotransmitter levels were stored at -70^{0}C until required. Brains for [^{14}C]-2-deoxyglucose and [^{3}H]-PK-11195 autoradiography were immersed in isopentane chilled to -45^{0}C with dry ice immediately following removal. Whole brains for histological investigations were immersed in neutral buffered 10% formalin solution immediately on removal. Samples of cultured cells were stored at -20^{0}C prior to liquid scintillation counting and protein determination. Cell cultures employed for the investigation of enzyme activities were stored at -70^{0}C. All samples known to contain light-sensitive drugs were wrapped in aluminium foil prior to freezing.

2.3 EXPERIMENTAL SEIZURE TESTS

2.3.1 Pentylenetetrazol test

This test was performed in accordance with the method described by Löscher and Schmidt (1988). PTZ, warmed to 37^0C, was administered to mice and rats subcutaneously (s.c.) in the back of the neck, in a dose of 85 mg/kg in 0.9% saline. The injection volumes did not exceed 0.3 ml (mice) and 1.0 ml (rats). On occasion, administration of PTZ to rats was carried out by the intraperitoneal (i.p.) route in a dose of 85 mg/kg in 0.9% saline. Again, injection volume did not exceed 1.0 ml. The latency to the first observed generalised seizure with loss of the righting reflex was recorded. Whenever possible the studies were performed in a single-blind fashion. An arbitrary cut-off time of 15 - 30 minutes was established prior to commencing each study. Animals were sacrificed within 30 seconds of the seizure or at the cut-off time if no seizure occurred.

2.3.2 Maximal electroshock test

This test was performed in accordance with the method described by Löscher and Schmidt (1988). Constant current electroshock stimuli were delivered to mice via auricular electrodes from an ECT unit. Electrodes were moistened with 0.9% saline to improve conductance. MES stimuli consisted of 0.2 seconds of rectangular positive pulses (pulse width = 0.4 ms) at a frequency of 60 Hz. The stimulation current required to induce tonic hind-limb extension (THE) in 100% of control animals in anticonvulsant tests was determined prior to each study using a group of naive animals.

2.3.3 Minimal electroshock test

This test was performed in accordance with the method described by Löscher and Schmidt (1988). Min-ES seizures were induced, via 0.9% saline moistened auricular electrodes, by an ECT unit. Stimuli consisted of 0.2 seconds of rectangular positive pulses of varying current (frequency = 60 Hz, pulse width = 0.4 ms) to examine tonic seizure threshold. The variable current allowed threshold determination by the "up-down" method (Kimball et al, 1957). The starting stimulus current was 6 mA in all cases. The subsequent stimulus intensities were determined by the response of the previous animal. If the previous animal failed to exhibit a tonic seizure the current was increased by one unit (mA) and, conversely, if the previous animal did exhibit a tonic seizure the subsequent current was decreased by a similar amount.

2.4 DETERMINATION OF GABA-AMINOTRANSFERASE ACTIVITY

This method was devised from modifications of the methods of White and Sato (1978) and Larsson and co-workers (1986).

2.4.1 Reagents

All solutions required for enzyme assay were prepared in deionised water. An EDTA buffer was prepared weekly for sample preparation and stored at 4^0C. The buffer consisted of 0.1 mM EDTA, 0.5 mM dithiothreitol, and 0.1 mM KH_2PO_4. PLP was added daily as required (final concentration = 0.2 mM) and the buffer adjusted to pH 8.0 with 4 M NaOH. A [^{14}C]-GABA incubation medium was prepared every 2 - 3 months and stored at -20^0C. The incubation medium consisted of 0.68 mM GABA (specific activity = 1.46 mCi/mmol), 1.8 mM EDTA, and 200 mM K H_2PO_4 with the pH adjusted to 6.9 with 1 M NaOH.

2.4.2 Sample preparation

Whole brains, stored at -70^{0}C for up to one month, were thawed and homogenised in 4 volumes (v/w) of EDTA buffer. Cultured cells for analysis, which had been scraped and stored at -70^{0}C in EDTA buffer, were freeze/thawed 3 times and then homogenised in their existing solutions. All samples were centrifuged at 2000 rpm for 20 minutes at 4^{0}C. The resultant supernatant was decanted and its protein content determined, in duplicate, by the BIORAD method (section 2.2.4). The volume of the remaining supernatant was adjusted with EDTA buffer to give a final protein concentration of 1 mg/ml.

2.4.3 Assay for enzyme activity

A 50 µl volume of the adjusted supernatant was added to 25 µl of 0.68 mM α-KG and 25 µl of the [^{14}C]-GABA incubation medium to give a final assay volume of 100 µl. Assays were performed in duplicate with a blank assay included for each sample by replacing the α-KG with 25 µl water. All samples were vortex-mixed for 10 seconds and then incubated for 60 minutes at 37^{0}C. The reaction was terminated by the addition of 10 µl 2M HCl followed immediately by vortex-mixing for 10 seconds. The incubation mixtures were transferred to the surface of a resin in small disposable ion-exchange columns (Dowex AG50Wx8, pre-washed with deionised water, 0.5 x 3.0 cm, in 9 inch glass Pasteur pipettes plugged with a glass bead). Radioactive products were eluted directly into glass scintillation vials using 3 portions of 0.5 ml water. Each portion was placed in the original incubation tube and transferred to the column with the same pipette used to transfer the incubation mixture. Twelve ml of Picofluor 40 scintillation fluid was added to each vial and the disintegrations per minute (dpm) were counted for 10 minutes by liquid scintillation counting.

2.4.4 Calculations

The radioactive content of samples was analysed in comparison to the dpm obtained from standard solutions containing known amounts of radioligand. Results were corrected for background and blank sample counts and quantified in relation to protein content and reaction time. Enzyme activities were expressed as nmol/min/mg protein.

2.5 DETERMINATION OF GLUTAMIC ACID DECARBOXYLASE ACTIVITY

This method was devised from modifications of the methods of Kocchar and colleagues (1989), Wolf and Klemisch (1991), and Chakraborty and co-workers (1991).

2.5.1 Reagents

A sodium phosphate-AET buffer was prepared weekly for sample preparation and stored at 4^0C. The buffer consisted of 0.1 M Na_2HPO_4 and 1 mM AET. The buffer pH was adjusted to 7.0 with 0.1 M NaH_2PO_4. An incubation medium was prepared daily as required and consisted of 50 mM L-glutamic acid, 250 μM PLP, 0.4% 2-mercaptoethanol, and 57 μM gabaculline.

2.5.2 Sample preparation

Whole brains, stored at -70^0C for up to one month, were thawed and homogenised in 10 volumes (v/w) sodium phosphate-AET buffer. Samples were then centrifuged at 2000 rpm for 10 minutes. The supernatant was decanted and an aliquot taken for determination of protein content by the BIORAD method (section 2.2.4).

2.5.3 Assay for enzyme activity

Incubation medium (100 µl) was added to each of two 100 µl aliquots of supernatant per sample. The reaction in one aliquot (blank) was terminated immediately while the other (test) was allowed to continue for a period of 60 minutes at 37^0C. Termination was performed in both cases by the addition of 100 µl 1% perchloric acid. Terminated blank and test reaction mixtures were diluted 1/10 with water and assayed for GABA content by high performance liquid chromatography (HPLC; section 2.6.5).

2.5.4 Calculations

Enzyme activity was calculated by subtraction of the blank GABA concentration from the test GABA concentration to give a value for GABA production during the reaction period. Results were quantified in relation to both reaction time and protein concentration and were expressed as nmol/min/mg protein.

2.6 DETERMINATION OF AMINO ACID NEUROTRANSMITTER CONCENTRATIONS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

This method was devised from a modification of the method of Durkin and colleagues (1988).

2.6.1 Reagents

Stock solutions of amino acid standards (1 mg/ml in distilled water) were prepared monthly, stored at 4^0C, and diluted to working standard solutions daily as required. The derivatization reagent mixture (OPA - 3-MPA) was prepared weekly by dissolving 50 mg OPA in 4.5 ml methanol and 0.5 ml borate buffer then adding 50 µl 3-MPA with the

mixture being stored at 4^0C in the dark. The borate buffer was made weekly by adjusting 0.5 M boric acid to pH 9.5 with 1 M NaOH.

2.6.2 Mobile phase

Glutamate concentrations were determined in mobile phase consisting of 75:25 (v/v) 0.57 M acetate buffer (pH 3.75, containing 100 mg/l EDTA) / acetonitrile. For GABA analysis the mobile phase was 60:40 (v/v) 0.2 M acetate buffer (pH 3.80, containing 100 mg/l EDTA) / acetonitrile. Acetate buffers were prepared by adding 32.8 ml (0.57 M) or 11.5 ml (0.2 M) glacial acetic acid to 925 ml of water, adjusting the pH with 3 M NaOH and diluting to 1 litre with water. Flow rates were 1.0 ml/min throughout.

2.6.3 Calibration

Calibration curves (in water) were constructed for glutamate (0.5 - 5.0 μg/ml) and GABA (2.0 - 20 μg/ml) and were seen to be linear ($r \geq 0.930$) in all cases. Limits of detection for both glutamate and GABA were found to be 5 ng/ml in a 50 μl sample. Intra- and inter-assay variations were calculated at 3.4% and 8.7% for glutamate respectively and 2.8% and 7.9% for GABA respectively.

2.6.4 Sample preparation

Whole brains, previously frozen and stored at -70^0C, were homogenised in 10 volumes (v/w) of 1% perchloric acid. Samples obtained from cell cultures, also stored at -70^0C, were homogenised in the 1 M NaOH solution in which they had been stored and an aliquot taken for determination of protein content by the BIORAD method (section 2.2.4).

All samples were centrifuged at 2000 rpm for 5 minutes, the supernatant decanted and diluted 1/10 with water prior to derivatization.

2.6.5 Derivatization

A 50 µl aliquot of the diluted supernatant was reacted with 200 µl methanol, 200 µl borate buffer, and 50 µl OPA - 3-MPA solution. D,L-norvaline (50 ng) was added as an internal standard to give a total reaction volume of 550 µl. Reaction mixtures were vortexed and allowed to stand at room temperature for 4 minutes prior to injection of 10 µl onto the column.

2.6.6 Calculations

Concentrations of amino acids were calculated by comparison of peak height ratios of analyte to internal standard and quantified in relation to the wet weight of tissue for brain samples and the protein concentration for cell culture samples. Results were expressed as µg/g and µg/mg protein for brain and culture samples respectively.

2.7 DETERMINATION OF PENTYLENETETRAZOL CONCENTRATIONS IN PLASMA AND BRAIN BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

This method was devised from modifications of the methods of Soto-Otero and co-workers (1987) and Ramzan (1988).

2.7.1 Reagents

Stock solutions of PTZ and p-MPB (internal standard) were prepared monthly as 1 mg/ml solutions in methanol and stored at -20^{0}C. Working standard solutions were prepared daily as required.

2.7.2 Mobile phase

PTZ concentrations were determined in mobile phase consisting of 60:35:5 0.1 M phosphate buffer (pH 6.9) / methanol / acetonitrile. The phosphate buffer was prepared by adjusting a 0.1 M Na_2HPO_4 solution to pH 6.9 with 0.1 M NaH_2PO_4. Flow rate was 2.0 ml/min throughout.

2.7.3 Calibration

Calibration curves (in blank plasma) were constructed for PTZ in the range 5 - 100 μg/ml were seen to be linear ($r \geq 0.930$) in all cases. Limits of detection were found to be 2 μg/ml in a 200 μl sample and intra- and inter-assay variations were calculated at 6.1% and 11.7% respectively.

2.7.4 Sample preparation

Brain homogenate for analysis was obtained by homogenisation of whole and dissected brain, previously frozen and stored at -20^{0}C, in 5 volumes (v/w) of distilled water. Plasma samples for analysis, also frozen and stored at -20^{0}C, were sonicated prior to use. Internal standard (p-MPB, 1 μg) was added to all samples prior to extraction.

2.7.5 Extraction procedure

PTZ was extracted from 1 ml of brain homogenate or 200 μl of plasma in screw-capped test tubes. Addition of 4 ml and 7 ml dichloromethane to plasma and brain samples respectively was followed by vortex-mixing for 30 seconds. An excess of crystalline $(NH_4)_2SO_4$ was added, samples were vortex-mixed for a further 30 seconds and then centrifuged at 2000 rpm for 10 minutes. The aqueous supernatant was discarded and the organic phase transferred to a clean conical test-tube and evaporated to dryness at 37^0C under nitrogen in a fume cupboard. The residue was reconstituted in 400 μl mobile phase, transferred to an Eppendorf tube, vortexed for 10 seconds and centrifuged at 2000 rpm for 5 minutes. A 100 μl volume of the resultant supernatant was injected onto the column.

2.7.6 Calculations

PTZ concentrations were determined by comparison of peak height ratios of analyte to internal standard and quantified in relation to volume for plasma samples and wet weight of tissue for brain samples. Results were expressed as μg/ml and μg/g for plasma and brain samples respectively.

2.8 DETERMINATION OF NIMODIPINE CONCENTRATIONS IN PLASMA AND BRAIN BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

This method was devised from modifications of the methods of Waller and co-workers (1984) and Larkin and colleagues (1992b).

2.8.1 Reagents

Stock solutions of NMD standard (100 μg/ml) in 50:50 methanol / water were prepared monthly and diluted to a working standard solution daily as required. All procedures were

performed under sodium light because NMD rapidly breaks down under other artificial light sources and sunlight.

2.8.2 Mobile phase

NMD concentrations were determined in mobile phase consisting of 50:50 acetonitrile / 8.7 mM $NH_4H_2PO_4$ solution (pH adjusted to 3.5 with 1 M phosphoric acid). Flow rate was 1.5 ml/min.

2.8.3 Calibration

Calibration curves (in blank plasma) were constructed for NMD in the range 0.2 - 0.5 μg/ml and were shown to be linear ($r \geq 0.930$) in all cases. Limits of detection were calculated at 10 ng/ml in a 200 μl sample and intra- and inter-assay variations calculated at 4.8% and 8.2% respectively.

2.8.4 Sample preparation

Brain homogenate for analysis was obtained by homogenisation of whole brain, previously frozen and stored at -20^{0}C, in 5 volumes (v/w) distilled water. Plasma samples for analysis, also frozen and stored at -20^{0}C, were sonicated prior to use. Nitrendipine (400 ng) was added as an internal standard to all samples prior to extraction.

2.8.5 Extraction procedure

NMD was extracted from 1 ml of brain homogenate or 200 μl of plasma by the following method. Addition of 300 μl of 1 M NaOH and 5 ml of 50:50 hexane/diethylether was followed by 10 seconds of vortex-mixing and transfer to a clean conical test-tube. Samples

were then centrifuged at 2000 rpm for 10 minutes. The upper organic layer was decanted into a clean conical test-tube and evaporated to dryness at 50^0C under nitrogen in a fume cupboard. Samples were reconstituted in 250 µl of 70:30 methanol/water and 100 µl injected onto the column.

2.8.6 Calculations

NMD concentrations were determined by comparison of peak height ratios of analyte to internal standard and quantified in relation to volume for plasma samples and wet weight of tissue for brain samples. Results were expressed as µg/ml and µg/g for plasma and brain samples respectively.

2.9 PRIMARY CULTURE OF CEREBELLAR GRANULE CELLS

This method was devised from modifications of the methods of Meier and Schousboe (1982) and Courtney and colleagues (1990).

2.9.1 Reagents

A poly-D-lysine solution, for culture dish coating, was prepared by dissolving 1.5 mg poly-D-lysine in 100 ml sterile culture water. Solution B, for tissue transfer and cell isolation, consisted of 0.25% D-glucose, 0.3% BSA, and 0.0382% $MgSO_4$ in PBS. A trypsin solution, for cell dissociation, was prepared by dissolving 50 mg of trypsin in 20 ml of solution B. A concentrated DNAse/SBTI solution for counteracting the enzymatic effect of trypsin and to break down any DNA released from cells, consisted of 2.8% DNAse I, 5% SBTI, and 0.0382% $MgSO_4$ in solution B. A weak DNAse solution was prepared by diluting 3.2 ml of the concentrated DNAse/SBTI solution to 20 ml with

solution B. An underlying solution for cell decontamination consisted of 4% BSA and 0.0382% $MgSO_4$ in EBSS. Finally, a culture medium was prepared which consisted of MEM supplemented with 10% (v/v) FCS, 50 I.U./ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 0.6% D-glucose, and 0.14% KCl. The culture medium was further supplemented with 10 μM ARA-C, where indicated, to remove all replicating, non-neuronal cells. All solutions were prepared under sterile conditions, using sterile water where required and filter sterilised through a 0.2 μm pore filter prior to use and/or storage. Solutions were stored sterile at 4^0C for up to 5 days.

2.9.2 Tissue preparation

Tissue for cell isolation was removed under aseptic conditions. Seven day old rat pups were decapitated and the skin overlying the skull was peeled away. The entire skull surface was removed by inserting the point of a pair of scissors into the foramen magnum and incising the skull down either side in the dorsal plane. The cerebellar cortex was removed with a sharp pinch between the points of a pair of curved watchmaker's forceps. The removed cerebellar cortices were placed in a sterile universal tube containing 6 ml of solution B prior to cell isolation.

2.9.3 Cell isolation

Cerebella were chopped into small cubes (0.375 mm^3) by two passes (at 90^o) in a McIlwain tissue chopper. Chopped tissue was transferred to a sterile 50 ml Falcon tube containing 20 ml trypsin solution and gently agitated to reduce clumping. A sterile plastic pipette was used to aid this process. Tubes were capped, shaken gently, and incubated for 20 minutes at 37^0C. After incubation, 20 ml of weak DNAse/SBTI solution was added and

tubes mixed gently. Cells were pelleted by gentle centrifugation (2000 rpm for 1 minute). The supernatant was discarded and 1 ml concentrated DNAse/SBTI solution added. Cells were resuspended by trituration of the cell pellet suspension through a sterile glass pipette. The cell suspension was transferred to a 15 ml Falcon tube, a further 1ml of concentrated DNAse/SBTI solution added and trituration repeated through a sterile glass pipette with a narrowed end. Underlying the cell suspension with 2ml of 4% BSA using a "kwill" filling tube facilitated decontamination of the cells. Cells were again pelleted by centrifugation at 2000 rpm for 5 minutes. The supernatant was discarded and the cells resuspended in 2ml culture medium. A ten-fold dilution of this cell suspension (90 µl culture medium + 10 µl cell suspension) was prepared and 10 µl employed for cell counting in a haemocytometer. The total number of cells was calculated and the volume of the cell suspension adjusted with culture medium to give 1.25 million cells per ml. Falcon Primaria culture dishes (35 mm^2) had been pre-coated with 2 ml poly-D-lysine solution for 1 hour at room temperature and allowed to air dry. A 2 ml volume of cell suspension was plated on each dish and plates were incubated at 37^0C for 30 minutes. Thereafter the medium was replaced with a further 2 ml of culture medium to remove all non-attached cells.

2.9.4 Culture maintenance

The cultures were maintained at 37^0C in an environment of 95% oxygen (O_2) / 5% CO_2 with a humidity of $\geq$ 90%. After 36 to 48 hours in culture, the medium was replaced with 2 ml culture medium containing 10 µM ARA-C to eliminate all non-neuronal cells. On the sixth day in culture the medium was replaced with a further 2 ml of culture medium without ARA-C. Cultures were seen to be fully mature and ready for use between day 8 and day 10 and were viable for up to 15 days. A scanning electron micrograph of typical

cerebellar granule cells in primary culture, obtained by the method described in section 2.15, is illustrated in plate 1.

2.10 PRIMARY CULTURE OF CEREBRAL CORTICAL ASTROCYTES

This method was devised from modifications of the methods of Larsson and co-workers (1981) and Bender and Hertz (1984).

2.10.1 Reagents

A culture medium was prepared which consisted of DMEM supplemented with 20% (v/v) HS, 2.5 mM L-glutamine, 50 I.U./ml penicillin, and 50 μg/ml streptomycin. The medium was further supplemented with 0.25 mM cAMP, where indicated, to facilitate differentiation of the cells. The medium was prepared under sterile conditions and filter sterilised through a 0.2 μm pore filter prior to use and/or storage. Media were stored sterile at 4^0C for up to 5 days.

2.10.2 Tissue preparation

Tissue for cell isolation was removed under aseptic conditions. One day old rat pups were decapitated and the skin overlying the skull was peeled away. The entire skull surface was removed by inserting the point of a pair of scissors into the foramen magnum and incising the skull down either side in the dorsal plane, taking care not to damage the underlying cortex. The cerebral cortices were removed from either hemisphere with a sharp pinch between the points of a pair of curved watchmaker's forceps. The removed tissue was placed in a 55 mm^2 culture dish containing DMEM and, with the aid of a dissecting microscope, the olfactory bulbs, basal ganglia, hippocampal formations and meninges

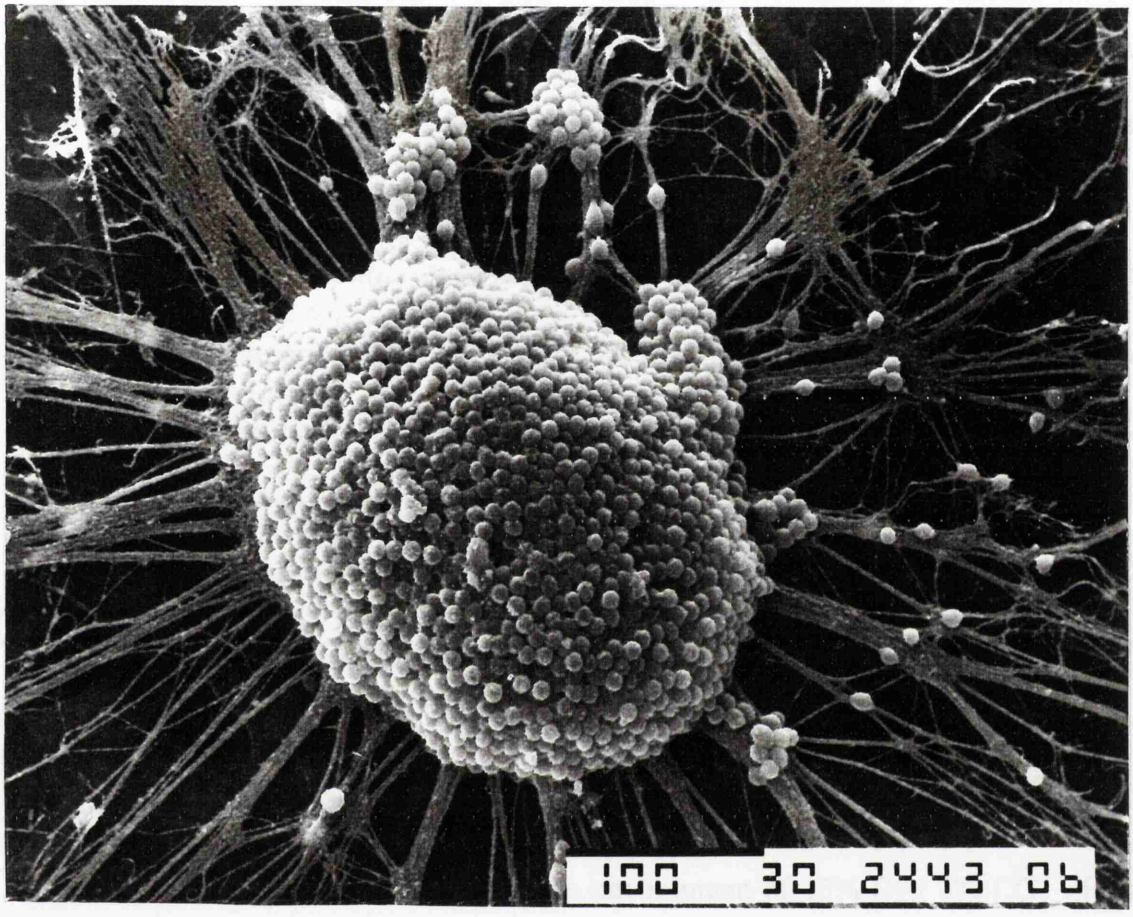

PLATE 1:- Scanning electron micrograph of typical cerebellar granule cells after 10 days in primary culture. Black bar in exposure code box represents 100 μm.

were removed. The dissected neopallia were then transferred to a sterile universal tube containing 6 ml of DMEM prior to cell isolation.

2.10.3 Cell isolation

The dissected neopallia were cut into small cubes (0.5 mm^3) by two passes (at 90^o) in a McIlwain tissue chopper. The chopped tissue was transferred to a sterile glass filter (80 μm nylon mesh) and the filtrate collected in a sterile beaker. The chopped material was washed through the filter with culture medium to give a final volume of 3 ml per brain. A sterile plastic pipette was used to aid this process. The filtrate was passed through a sterile needle (BD Microlance 21G 0.8 x 40) three times to separate the cells. The volume of the resulting suspension was adjusted with culture medium to allow a 3 ml aliquot per petri dish with a ratio of 1 brain to 3 dishes. A 3 ml volume of the final cell suspension was plated onto 55 mm^2 Falcon Primaria culture dishes.

2.10.4 Culture maintenance

The cultures were maintained at 37^0C in an environment of 95% O_2 / 5% CO_2 with a humidity of $\geq$ 90%. The culture medium (3 ml) was replaced every 3 - 4 days throughout. The HS concentration was reduced to 10% at the first medium change with a final reduction to 5% at the second change. The HS concentration remained at 5% thereafter. Once the cells reached confluence (or after 14 days in culture) the medium was supplemented with 0.25 mM cAMP. At this stage penicillin and streptomycin were omitted from the culture medium due to possible interference with subsequent experimental procedures. Cultures were seen to be fully mature and ready for use between day 21 and day 24 and were viable for up to 42 days. A scanning electron micrograph of

typical cerebral cortical astrocytes in primary culture, obtained by the method described in section 2.15, is illustrated in plate 2.

2.11 PRIMARY CULTURE OF CEREBRAL CORTICAL NEURONES

This method was devised from modifications of the methods of Larsson and co-workers (1981), Yu and co-workers (1984), and Courtney and colleagues (1990).

2.11.1 Reagents

All of the reagents used for primary culture of cortical neurones are listed in section 2.9.1 with the following exceptions. A poly-D-lysine solution, for culture dish coating, was prepared by dissolving 1.5 mg poly-D-lysine in 100 ml borate buffer (pH 8.4). The borate buffer was prepared by adjusting the pH of 15 μM boric acid to 8.4 with 1 M NaOH. The culture medium consisted of MEM supplemented with 20% (v/v) HS, 2 mM L-glutamine, 7 μM PABA, 100 μI.U./litre insulin, 0.6% D-glucose, and 0.14% KCl. The culture medium was further supplemented with 40 μM ARA-C, where indicated, to remove all replicating, non-neuronal cells. All solutions were prepared under sterile conditions, using sterile water where required and filter sterilised through a 0.2 μm pore filter prior to use and/or storage. Solutions were stored sterile at 4^0C for up to 5 days.

2.11.2 Tissue preparation

Tissue for cell isolation was removed under aseptic conditions. Pregnant female rats, at 15 days post-conception, were sacrificed by a blow to the head followed by cervical dislocation. The abdomen was opened and all foetuses removed into a culture dish containing MEM. The membraneous-like skull surface was removed with forceps and the

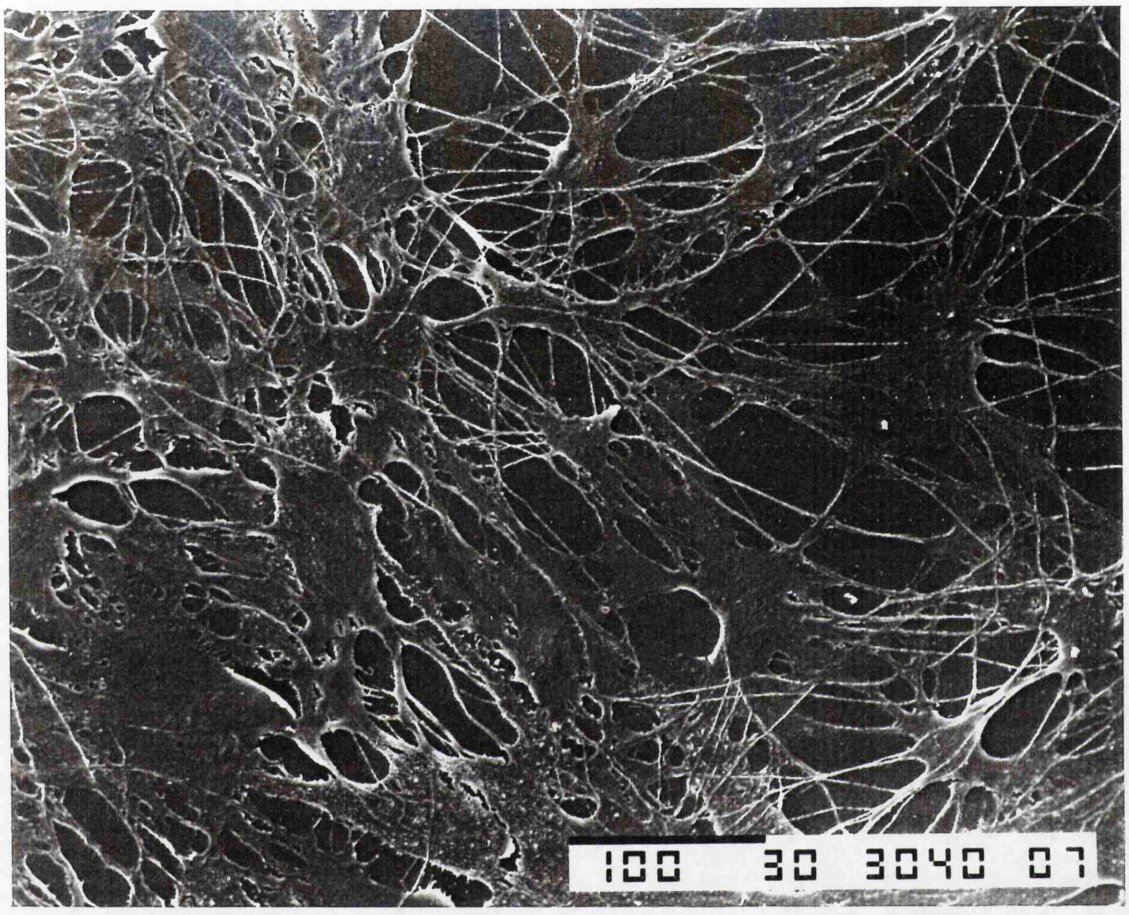

PLATE 2:- Scanning electron micrograph of typical cerebral cortical astrocytes after 21 days in primary culture. Black bar in exposure code box represents 100 μm.

cerebral cortices removed with a delicate pinch between the points of a pair of straight watchmaker's forceps. The dissected cortices were transferred to a sterile universal tube containing 6 ml of MEM prior to cell isolation.

2.11.3 Cell isolation

The cerebral cortices were treated in accordance with the cell isolation procedure described in section 2.9.3 with the following modification. Following the cell count, the total number of cells was calculated and the volume of the cell suspension adjusted with culture medium to give 0.75 million cells per ml.

2.11.4 Culture maintenance

The cultures were maintained at 37^0C in an environment of 95% O_2 / 5% CO_2 with a humidity of $\geq$ 90%. After 48 hours in culture the medium was replaced with 2 ml culture medium containing 40 μM ARA-C to eliminate all non-neuronal cells. At this point the HS concentration was reduced to 10%. The HS concentration remained at 10% thereafter. Twenty four hours later (day 3) and again on day 6 the medium was again replaced with 2 ml culture medium without ARA-C. Cultures were seen to be fully mature and ready for use between day 8 and day 10 and were viable for up to 15 days. A scanning electron micrograph of typical cerebral cortical neurones in primary culture, obtained by the method described in section 2.15, is illustrated in plate 3.

2.12 [^{14}C]-GABA UPTAKE INTO CULTURED ASTROCYTES AND NEURONES

This method was devised from modifications of the methods of Larsson and co-workers (1981) and Yu and colleagues (1984).

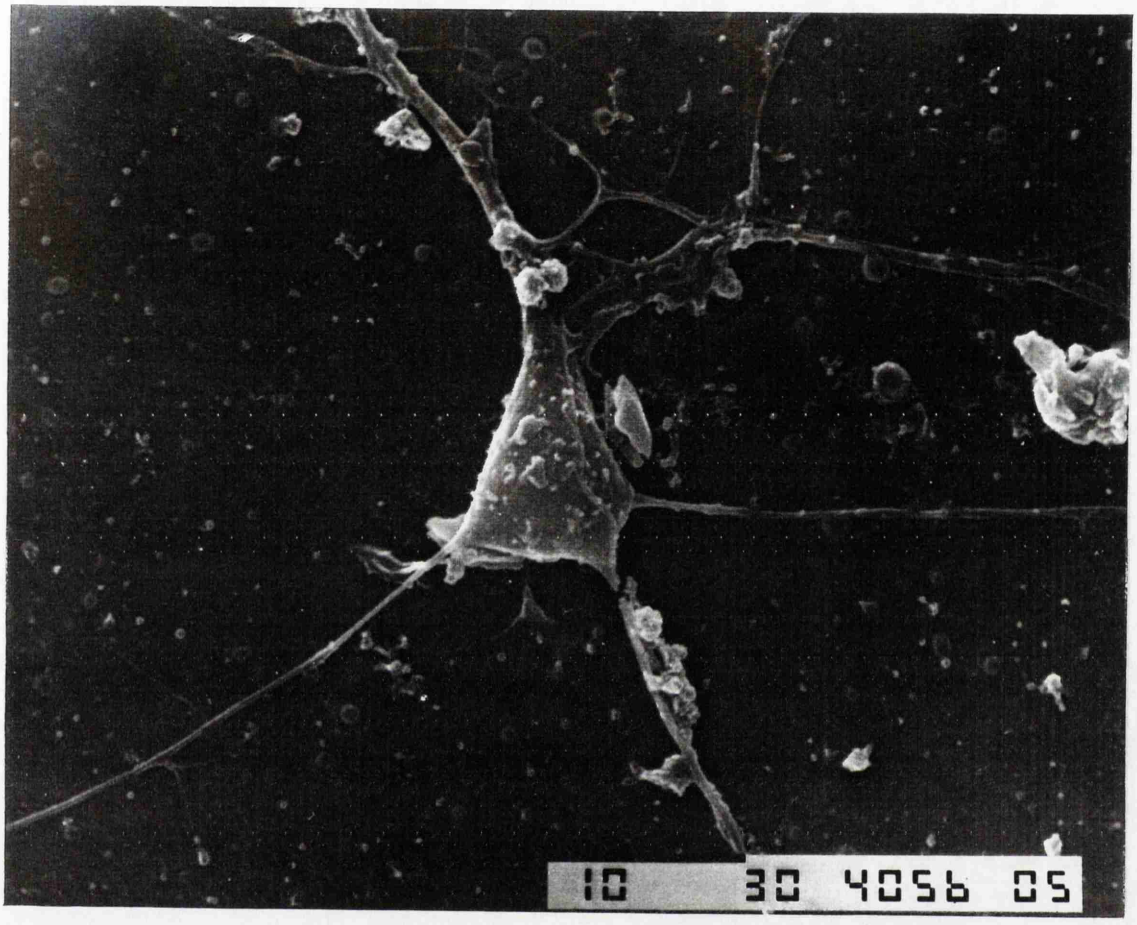

PLATE 3:- Scanning electron micrograph of typical cerebral cortical neurones after 10 days in primary culture. Black bar in exposure code box represents 10 μm.

2.12.1 Reagents

A standard balanced salt solution (BSS) was used throughout the investigations of [^{14}C]-GABA uptake. Its composition was as follows: 136 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4$, 2.6 mM $NaHCO_3$, 0.4 mM KH_2PO_4, 0.34 mM Na_2HPO_4, 1.3 mM $CaCl_2$, 5.6 mM D-glucose and 15 mM HEPES. The solution was adjusted to pH 7.4 with 1 M NaOH and stored, at 4^{0}C, for up to 1 week. BSS was warmed to 37^{0}C prior to use.

2.12.2 Culture preparation

Cultures for investigation were removed from the incubator and the existing culture medium aspirated. Cultures were washed twice (2 x 1 ml neurones, 2 x 2 ml astrocytes) with BSS (37^{0}C) before being returned to the incubator in a further volume of BSS (2 ml neurones, 3 ml astrocytes) for an equilibration period of 20 minutes.

2.12.3 [^{14}C]-GABA uptake procedure

The pre-washed cultures were removed from the incubator and the existing BSS aspirated. This solution was replaced by BSS (1 ml neurones, 2 ml astrocytes) containing the drug concentrations appropriate to the individual experiment. Control plates received BSS alone. All culture plates were returned to the incubator for a further period of 1 hour. After the incubation period, a further 1 ml of BSS (with appropriate control/drug treatment) containing 100 μM [^{14}C]-GABA (specific activity = 1 mCi/mmol) for neurones and 150 μM [^{14}C]-GABA (specific activity = 1 mCi/mmol) for astrocytes was added to each plate. Incubation was allowed to continue for 5 minutes before the cultures were washed with 5 volumes (1 ml neurones, 2 ml astrocytes) of warmed BSS. Cells were finally removed from the plates by scraping in 1 M NaOH (0.5 ml neurones, 1.0 ml astrocytes). Aliquots

were taken for protein determination by the BIORAD method (section 2.2.4) and liquid scintillation counting in 8 ml of Picofluor 40 scintillation fluid.

2.12.4 Calculations

Liquid scintillation counting was employed to analyse GABA uptake in individual cultures in comparison to the dpm of standard solutions containing known amounts of radioligand. Results were quantified by the relation of GABA uptake to the protein concentration and expressed as pmol/min/mg protein in individual cultures.

2.13 [$^{45}Ca^{2+}$]-INFLUX INTO CULTURED ASTROCYTES AND NEURONES

This method was devised from modifications of the methods of Carboni and colleagues (1985), Carboni and Wojcik (1987), and Hertz and co-workers (1989).

2.13.1 Reagents

A standard BSS was used throughout these investigations. The composition of this solution is described in section 2.12.1. A "high K^+" BSS was employed for some studies, the salt composition of which differed from "normal" BSS as follows: 26 mM NaCl, 115 mM KCl. The concentrations of all other constituents remained unchanged. In studies involving the use of veratridine, a 10 mM stock solution was prepared in ethanol with a subsequent dilution to a working concentration in BSS. Care was taken to ensure that all cultures employed in veratridine studies were exposed to the same ethanol concentration.

2.13.2 Culture preparation

Cultures for investigation were prepared in accordance with the method described in section 2.12.2.

2.13.3 Potassium-stimulated [$^{45}Ca^{2+}$]-influx procedure

Pre-washed cultures were divided into two groups. The existing BSS was aspirated and replaced (1 ml neurones, 1.5 ml astrocytes) by drug-containing BSS in one group and BSS alone (control) in the other. All culture plates were returned to the incubator for a further period of 1 hour. After the incubation period, control and drug-treated plates were further divided into 2 sub-groups each. One control and one drug-treated sub-group was supplemented with "normal" BSS (1 ml neurones, 1.5 ml astrocytes) containing 1 μCi $^{45}Ca^{2+}$. The remaining two sub-groups were supplemented with "high K^{+}" BSS (1 ml neurones, 1.5 ml astrocytes) also containing 1 μCi $^{45}Ca^{2+}$. Drugs were included in these solutions where appropriate. Following a reaction time of 60 seconds, the plates were washed with 5 volumes (1 ml neurones, 2 ml astrocytes) of "normal" BSS ($37^{0}C$). Cells were finally removed from the plates by scraping in 1 M NaOH (0.5 ml neurones, 1.0 ml astrocytes). Aliquots were taken for protein determination by the BIORAD method (section 2.2.4) and liquid scintillation counting in 8 ml of Picofluor 40 scintillation fluid.

2.13.4 Veratridine-stimulated [$^{45}Ca^{2+}$]-influx procedure

Pre-washed cultures were divided into two groups. The existing BSS was aspirated and replaced by drug-containing BSS (1 ml) in one group and BSS (1 ml) alone (control) in the other. All culture plates were returned to the incubator for a further period of 1 hour. After the incubation period, control and drug-treated plates were further divided into 2

sub-groups each. One control and one drug-treated sub-group was supplemented with BSS (1 ml) containing 1 μCi $^{45}Ca^{2+}$. The remaining two sub-groups were supplemented with BSS (1 ml) containing 50 μM veratridine and 1 μCi $^{45}Ca^{2+}$. Drugs were included in these solutions where appropriate. Following a reaction time of 60 seconds, the plates were washed with 5 volumes (1 ml) of BSS (37^0C). Cells were finally removed from the plates by scraping in 1 M NaOH (0.5 ml). Aliquots were taken for protein determination by the BIORAD method (section 2.2.4) and liquid scintillation counting in 8 ml of Picofluor 40 scintillation fluid.

2.13.5 Calculations

Liquid scintillation counting was employed to analyse [$^{45}Ca^{2+}$]-influx in comparison to the dpm of standard solutions containing known amounts of radioligand. Results were quantified by the relation of calcium influx to the protein concentration and expressed as nmol/min/mg protein in individual cultures.

2.14 GLUTAMATE RELEASE FROM CULTURED NEURONES

This method was devised from modifications of the methods of Gallo and colleagues (1982) and Philibert and Dutton (1989).

2.14.1 Reagents

The compositions of "normal" BSS and a "high K^+" BSS used in these studies are described in sections 2.12.1 and 2.13.1 respectively.

2.14.2 Culture preparation

Cultures for investigation were prepared in accordance with the method described in section 2.12.2.

2.14.3 Potassium-stimulated glutamate release procedure

Pre-washed cultures were divided into two groups. The existing BSS was aspirated and replaced by drug-containing BSS (1 ml) in one group and BSS (1 ml) alone (control) in the other. All culture plates were returned to the incubator for a further period of 1 hour. After the incubation period, control and drug-treated plates were further divided into 2 sub-groups each. One control and one drug-treated sub-group was supplemented with "normal" BSS (1 ml) while the remaining two sub-groups were supplemented with "high K^+" BSS (1 ml). Drugs were included in these solutions where appropriate. Following a reaction time of 60 seconds, a 500 µl sample of the bathing medium was removed from each plate for analysis of glutamate content by HPLC (section 2.6). The cells were removed from the culture dishes by scraping in 1 M NaOH (0.5 ml) for analysis of protein content.

2.14.4 Calculations

Glutamate release from individual cultures was determined by HPLC (section 2.6), quantified in relation to the protein concentration, and expressed as ng/min/mg protein.

2.15 SCANNING ELECTRON MICROSCOPY OF CELL CULTURES

2.15.1 Reagents

A 0.2 M phosphate buffer was prepared by adjusting the pH of 0.2 M Na_2HPO_4 to 7.4 with 0.2 M NaH_2PO_4. A buffered 3% glutaraldehyde solution was prepared in phosphate buffer, supplemented with 5.4% D-glucose and adjusted to pH 7.4 with 1 M NaOH. A buffered 1% osmic acid solution was prepared in phosphate buffer and adjusted to pH 7.4 with 1 M NaOH.

2.15.2 Fixation and dehydration of cells

Cells were fixed by bathing the culture dishes in 3% glutaraldehyde solution for 15 minutes. Plates were rinsed in several washings of phosphate buffer for 15 minutes. Osmic impregnation was then performed by exposure of the culture dishes to 1% osmic acid for 30 minutes. Plates were again rinsed in several washings of phosphate buffer for 15 minutes. Finally, cells were dehydrated by sequential 15 minute exposures to 70%, 90%, and 100% ethanol.

2.15.3 Critical point drying

Prepared culture dishes were arranged in baskets and then placed in 100% ethanol-filled boats. Boats were then placed inside a small, perfusible hyperbaric chamber with a temperature regulating water jacket. The chamber door was tightly sealed and the chamber flooded with liquid CO_2. The outlet valve was opened and the ethanol allowed to run off leaving the chamber filled with liquid CO_2 for approximately 4 minutes. At this point all of the chamber valves were closed and the boats left covered with liquid CO_2 for a further 30 minutes. The contents of the chamber were flushed twice more with liquid CO_2 to

remove any remaining ethanol. The level of the CO_2 was then allowed to drop, the valves were closed and the chamber heated to 35^0C. Opening the outlet valve allowed the now gaseous CO_2 to escape and once the chamber pressure had returned to 1 atmosphere the culture dishes were removed.

2.15.4 Cell preparation and microscopy

Prepared culture dishes were sectioned and mounted on aluminium stubs with double sided tape. Cells were then coated with gold and screened in a scanning electron microscope.

2.16 *In vivo* [^{14}C]-2-DEOXYGLUCOSE AUTORADIOGRAPHY

This method was performed in exact accordance with that first described by Sokoloff and colleagues (1977).

2.16.1 Preparation of animals

On the day of the experiment each animal was anaesthetised with 1% halothane in 70:30 nitrous oxide (N_2O) / O_2. Femoral vessels were exposed bilaterally by blunt dissection via small incisions through the skin in each side of the animal's groin. Polyethylene cannulae, containing heparinised saline (10 IU/ml) were inserted 2 cm into both femoral arteries and veins and tied off using silk thread. The incision site was infiltrated with a local anaesthetic gel (Xylocaine 2%) and closed. A loose fitting plaster cast was applied around the pelvis and lower abdomen, with care taken not to restrict thoracic movements. The plaster was taped to a lead weight and, with the animals thus restrained, the anaesthesia was discontinued. The animals were allowed to recover for a minimum period of 2 hours

prior to further intervention. In all animals, arterial blood pressure was measured throughout using a pressure transducer connected to one femoral artery cannula. Core temperature was measured by means of a rectal temperature probe and the rats maintained normothermic by heating lamps.

2.16.2 Measurement of local cerebral glucose utilisation

The measurement of local cerebral glucose utilisation (LCGU) was initiated by the intravenous (i.v.) administration, over 30 seconds, of 50 μCi [^{14}C]-2-deoxyglucose (specific activity = 50 mCi/mmol) in 0.7 ml 0.9% saline. Fourteen timed arterial blood samples were taken throughout the following 45 minutes. Blood was centrifuged immediately and plasma was assayed for ^{14}C activity, by liquid scintillation counting in 4 ml of Picofluor 40 scintillation fluid, and for the concentration of glucose by glucose analyser. Approximately 35 minutes after 2-deoxyglucose administration a sample of arterial blood was taken for analysis of pCO_2 and pH by a blood gas analyser. At 45 minutes after isotope administration, the rats were killed by decapitation, their brains removed as described in section 2.2.3 and frozen in isopentane chilled with dry ice at -45^{0}C.

2.16.3 Preparation and processing of brain slices

Frozen brains were cut into 20 μm coronal sections at -22^{0}C in a cryostat and 3 in every 10 sections were mounted on glass cover slips, then dried rapidly on a hot plate at 60^{0}C. The brain sections were placed against X-ray plates along with precalibration plastic standards (concentration range 44 - 1475 nCi/g) for 28 days.

2.16.4 Analysis of results

At the end of the film exposure period, autoradiograms were developed and local tissue concentrations of ^{14}C were determined by quantitative densitometric analysis. For each anatomical region of interest, the image analyser calculated the mean of 6 optical density values taken in 3 sections per animal. Structures in the rat brain were defined anatomically with reference to the stereotaxic atlas of Paxinos and Watson (1982). LCGU was calculated using the operational equation for the technique from the plasma history of ^{14}C and glucose levels (Sokoloff et al, 1977).

2.17 *In vitro* [^{3}H]-PK-11195 AUTORADIOGRAPHY

This method was performed in accordance with that of Benavides and colleagues (1983).

2.17.1 Tissue preparation

Rats were killed by decapitation. Their brains were dissected out, frozen by immersion in isopentane at -45^{0}C, and cut into 20 μm-thick coronal sections using a cryostat microtome. Triplicate tissue sections (2 for total, 1 for non-specific ligand binding), taken at 200 μm intervals throughout the brain and adjacent to those sections used in contemporaneous [^{14}C]-2-deoxyglucose studies, were thaw-mounted onto gelatin-subbed slides and stored at -20^{0}C.

2.17.2 [^{3}H]-PK-11195 ligand binding procedure

Slide-mounted sections were washed twice for 30 seconds in Tris/HCl buffer (170 mM, pH 7.4) at 4^{0}C to remove any ^{14}C remaining in the tissue from *in vivo* 2-deoxyglucose autoradiography which had been performed in the same animals. Sections were then

incubated in Tris/HCl buffer containing 1 nM [^{3}H]-PK-11195 (specific activity = 75.2 Ci/mmol) for 30 minutes at room temperature (total binding). Non-specific binding was determined by incubating adjacent sections with [^{3}H]-PK-11195 (1 nM) in Tris/HCl buffer containing an excess of unlabelled PK-11195 (3 μM), to competitively displace the isotopically-labelled ligand, for 30 minutes at room temperature. After incubation, sections were washed twice for 5 seconds in Tris/HCl buffer (4^{0}C), dipped in distilled water (4^{0}C), and dried rapidly in a stream of air. The brain sections and a set of precalibrated tritium standards (concentration range = 1060 - 17720 nCi/mg) were then apposed to tritium-sensitive film for 14 days.

2.17.3 Development of autoradiograms

At the end of exposure time films were manually developed in D-19 developer for 5 minutes at 17^{0}C. Development was terminated by a 30 second rinse in deionised water at 20^{0}C. After fixing for 10 minutes at 20^{0}C in Kodafix, films were washed in running filtered water for 40 mins, rinsed in deionised water, and then suspended in a drying cabinet overnight.

2.17.4 Analysis of results

Areas of increased [^{3}H]-PK-11195 binding were identified, corrected for non-specific binding, and quantitified in terms of ligand binding area in individual sections through the use of a densitometry system. The image analyser calculated the mean area of 3 regions of increased binding in each section. Structures in the rat brain were defined anatomically with reference to the stereotaxic atlas of Paxinos and Watson (1982).

2.18 HISTOLOGICAL INVESTIGATIONS

2.18.1 Brain preparation

Brains, removed in accordance with the method described in section 2.2.3, were immediately immersed in a neutral buffered 10% formalin solution prior to tissue section preparation.

2.18.2 Tissue section preparation

The brain was dissected into several blocks which were then processed overnight into paraffin wax. Cryostat sections were cut at 4 μm and three sections in ten from each block were taken and mounted on glass slides for further investigation.

2.18.3 Staining techniques

Brain sections for further investigation were de-waxed. One section from each group of ten was stained with haemotoxylin/eosin, one section stained with cresyl violet, and the final section processed immunohistochemically with GFAP. Slices were then mounted in Hystomount mounting medium and cover slips applied.

2.18.4 Observations

Observations were made both macro- and microscopically in all prepared sections. Sections of interest were photographed.

2.19 STATISTICAL METHODS

The Mann Whitney U test was employed for two sample results analysis. This non-parametric test was chosen to compare two independent sample groups because of the

large variations and abnormal distributions which often arose. Although not applicable when a non-parametric test is in use, means and standard errors of the mean (± SEM) are quoted almost exclusively for ease of comprehension. MES-induced seizures, being of an "all or none" nature, were compared by the Chi square method. Min-ES seizure thresholds, quoted as median values, were determined by regression analysis of individual results and compared by the Mann Whitney U test. Finally, Pearson's product moment correlation coefficients are reported throughout.

CHAPTER THREE

INVESTIGATION OF BASIC ANIMAL SEIZURE MODELS

3.1 INTRODUCTION

The AED development programme of the NINCDS at the NIH, USA, is based, primarily, on the use of two standard animal models of seizure, namely the s.c. CD_{97} PTZ test and the MES test (Gladding et al, 1985). Although the predictive value of the PTZ and MES tests together contributes significantly to the identification and classification of novel antiepileptic compounds, these procedures are not intended to be employed universally without exception. Often drugs, ineffective against both PTZ- and MES-induced seizures, will exhibit potent anticonvulsant effects in other animal seizure models. This situation is highlighted clearly in the case of VGB, a drug devoid of effect in both the PTZ and MES tests (Löscher and Schmidt, 1988), yet active against amygdala kindled seizures (Shin et al, 1986) and possessing efficacy against both partial and secondary generalised seizures in man (Grant and Heel, 1991). The PTZ and MES tests are, therefore, by no means infallible, however, they do provide the researcher with simple and economic methods of screening potential AEDs.

3.1.1 Pentylenetetrazol seizure models

PTZ (leptazol, metrazol) is a tetrazol derivative (Stone, 1970) which produces consistent convulsive actions in rodents, cats, and primates following systemic administration. It is probably the most extensively used chemoconvulsant for investigation of the anticonvulsant effects of novel drugs. Since its introduction into clinical practice as a substitute for camphor in the supposedly therapeutic generation of seizures (Meduna and Friedman, 1939) it has been widely employed as the primary screen for anticonvulsant compounds (Hahn, 1960; Stone, 1972). At present, PTZ is employed in two distinct animal seizure models, the maximal, or s.c. $CD_{97,}$ test and the threshold, or i.v. infusion, test.

The universally recognised s.c. CD_{97} PTZ test involves the administration of between 70 and 100 mg/kg PTZ to rodents. Precise dose levels are pre-determined to ensure a convulsive action in 97% of animals. Such seizures are characterised by initial myoclonic jerks with Straub tail, followed by sustained myoclonus, and finally generalised clonic seizures with loss of the righting reflex (Fisher, 1989). Systemic administration of higher PTZ doses can result in tonic extension of the hind-limbs, a phenomenon more often associated with electroshock stimuli. Evaluation of s.c. PTZ-induced seizures is performed by recording the latency to the particular end-point of interest. In this respect, some researchers choose 5 seconds of sustained myoclonus, while others select the first generalised seizure with loss of the righting reflex. This latter end-point is possibly more reliable and easier to recognise in drug screening programmes. Individual PTZ seizure latencies are dependent upon dose, injection site, and administration vehicle (Löscher et al, 1991b) and care should be taken to incorporate these variables when assimilating data from several different laboratory sources.

Although less commonly employed, the i.v. PTZ infusion model, described by Orloff and colleagues (1949) and Hint and Richter (1958), is gaining popularity among researchers. Graduated i.v. PTZ infusion allows determination of individual seizure thresholds. It has the advantage over the s.c. test in that thresholds for both clonic and tonic seizures can be separately established in the same animals. Thus, it represents a sensitive system for the evaluation of anticonvulsant drugs on distinct seizure types in relatively small numbers of animals. However, the major disadvantage of this threshold test is its more complex methodology, making it a less attractive option in large drug screening programmes.

PTZ-induced seizures, in a variety of species, exhibit an EEG profile characterised by spike-wave complexes and sharp hypersynchronized poly-spikes (Huot et al, 1973). Based on these observations and the investigation of established AEDs (Porter et al, 1984), loose parallels have been drawn between the PTZ test and human absence (petit mal) epilepsy. ESM, a drug with particular efficacy against clinical absence episodes, selectively inhibits PTZ-induced clonic seizures (Swinyard and Woodhead, 1982), is an effective anticonvulsant in rats with a genetic tendency to absence events (Marescaux et al, 1984), but is without effect on tonic seizures induced by MES (Reinhard and Reinhard, 1977). Conversely, PHT, ineffectual against human absence epilepsy (Penry and Newmark, 1979), is devoid of action in the PTZ test (Krall et al, 1978). Thus, the assumption was made that the PTZ test would provide a model system with which to predict the efficacy of novel anticonvulsant drugs in absence seizures in man (Eadie, 1985). It would appear, however, that this criterion, while possessing a degree of accuracy, is an excessive over-simplification (Löscher and Schmidt, 1988).

The mechanism of the convulsant action of PTZ is not completely understood, but is thought to relate to the inhibitory function of the neurotransmitter GABA (Olsen, 1981a; section 4.1.1). PTZ appears to interact with the $GABA_A$ receptor - chloride ionophore complex to reduce GABA mediated inhibition (Wilson and Escueta, 1974). There is evidence to suggest that this receptor complex contains a specific binding site for convulsant compounds (Olsen, 1981a), although whether PTZ exerts its effects at this particular site remains unclear.

Early anatomical studies suggested that PTZ exerted its actions primarily on the cortex (Ajmone-Marsan and Marossero, 1950) whereas more recently the mesencephalic reticular formation (Velasco et al, 1975; Faingold, 1977) and mammillothalamic tracts (Mirski and Ferrendelli, 1987) have been implicated as potential sites of PTZ action with regard to seizure generation.

As described in section 1.5.3, several other compounds that act to reduce GABAergic neurotransmission have been employed as chemoconvulsants in experimental animals. The $GABA_A$ receptor antagonists, bicuculline and picrotoxin (Olsen, 1981a), and the GABA synthesis inhibitors, thiosemicarbazide and allylglycine (Meldrum, 1979; Meldrum, 1985), are all active convulsants following systemic administration. While these compounds may be useful in the identification of novel AEDs with specific GABAergic mechanisms of action, the seizure profiles observed with them are often very similar to that seen with PTZ and, as such, none possess any significant advantage over PTZ as a front-line seizure model.

Thus, PTZ appears to be a useful chemoconvulsant compound for the laboratory evaluation of AED action. It can be employed to investigate a variety of seizure states, dependent on administration route, dose, and end-point selection. PTZ appears to rely on reducing GABA-mediated inhibition for its convulsant mechanism. It may highlight compounds with a specific influence on the GABAergic neurotransmitter system and perhaps predict the efficacy of drugs in human absence seizures. Although the i.v. PTZ infusion test may afford a more sensitive measure of drug-induced alterations in seizure thresholds, the inherent simplicity of the maximal test confers its superiority in drug

evaluations. Ultimately, the s.c. CD_{97} PTZ model provides the researcher with a quick, simple, and economical method of screening potential AEDs.

3.1.2 Electroshock seizure models

Together with the maximal PTZ test, the MES test represents one of the most popular seizure models in present use (Löscher et al, 1991a). That electrical stimulation of the brain can result in seizure generation has been recognised for over a century (Fritsch and Hitzig, 1870; Albertoni, 1882). Quantification of these observations into a readily usable laboratory technique (Spiegel, 1937) facilitated the use of the MES test in anticonvulsant drug screening for the first time. In 1938, Merritt and Putnam described the identification of PHT following use of the MES test to evaluate the anticonvulsant efficacy of a series of phenyl derivatives.

The full potential of electroshock stimulation as an experimental model of epilepsy became apparent with the intensive studies of Goodman and co-workers in the 1940s (Toman et al, 1946; Swinyard, 1949; Swinyard et al, 1952). These investigators revealed a similarity between the nervous systems of rodents and humans in response to electrical stimulation and established the electroshock method as a useful technique for the laboratory screening of anticonvulsant drugs.

In the MES test, animals receive an electrical stimulus of sufficient intensity to induce maximal (tonic extensor) seizures of the hind limbs. An apparatus similar to the one described by Woodbury and Davenport (1952) is generally used to deliver the stimulus. Electrical stimuli are applied through either ear-clip (auricular) or corneal electrodes and

delivered by either constant current or constant voltage systems. Typical stimulation parameters are 0.2 seconds of rectangular positive (or square-wave) pulses at 60 Hz with a pulse width of 0.4 ms. Stimulating currents of 50 mA (mice) or 150 mA (rats), delivered by constant current systems, are generally sufficient to induce tonic extension of the hind-limbs in naive animals (Löscher and Schmidt, 1988). This end-point is employed universally following MES stimulation and is an "all or none" phenomenon. Results arising from such studies are evaluated by calculating the percentage of animals, within the study group, exhibiting THE. The supramaximal stimulus allows the researcher to delineate all extrinsic factors which may influence day to day fluctuations in the seizure threshold and to over-ride any inter-animal variations which may be present (Löscher et al, 1991a). The disadvantage of the supramaximal MES test is that drugs which possess significant anticonvulsant activity yet do not raise the seizure threshold sufficiently may be dismissed as ineffective (Löscher and Schmidt, 1988).

Another commonly employed electroshock seizure model which overcomes this insensitivity problem and which has been advocated for use in conjunction with the MES test is the Min-ES test (Löscher et al, 1991a). This is a more sensitive test which determines a drug's ability to alter the threshold for induction of tonic seizures. The delivery systems and the stimulation parameters used in this model are identical to those used in the supramaximal test. The current applied is, however, greatly reduced in comparison. The most common application of this model evaluates the minimum current required to induce tonic seizures in 50% of animals. This is determined by sequential application of small currents to large groups of animals where the stimulus intensity applied to individual animals is determined by the response of the previous one (the

"up-down" method; Kimball et al, 1957). If the previous animal fails to exhibit a tonic seizure then the current is subsequently increased by one unit (mA). Conversely, if the previous animal does exhibit a tonic seizure the subsequent current is decreased by a similar amount. Each animal is used only once and the threshold can be determined mathematically from the positive and negative responses within each group. While the MES test produces a single seizure type and thus a single experimental end-point, the Min-ES test can be used to investigate several seizure profiles. Low intensity stimulation precipitates face and fore-limb clonus, with subsequent increases in current leading to "running-bouncing" clonus and eventually tonic seizures of the hind-limbs (Racine, 1972). Unlike the PTZ infusion test, these individual seizure types must be precipitated in separate animals due to post-ictal rises in seizure threshold. Nevertheless, the effects of AEDs on these distinct seizure states can be investigated by an experienced researcher. It should be noted that the face and fore-limb clonus cannot be precipitated via auricular stimulation (Browning and Nelson, 1985) and so it appears that the seizure profile is dependent on stimulation site.

In contrast to the PTZ test, electroshock seizure models, both supramaximal and threshold, have been proposed to predict the likely clinical efficacy of drugs against generalised tonic-clonic seizures (Swinyard, 1972). Laboratory evaluations of existing AEDs, such as PHT and CBZ, have supported this proposal (Swinyard et al, 1989). Further attempts at clinical correlations have suggested that the MES test predicts a drug's ability to prevent seizure spread whereas the PTZ test gauges the capacity of a drug to increase the threshold for induction of seizure activity (Löscher et al, 1991a).

Early anatomical studies suggested that sub-threshold electroshock stimulation preferentially activated a region of forebrain resulting in face and fore-limb clonus. Successive increases in stimulus intensity resulted in spread of this excitation throughout the brain until a point where stimulation became sufficient (threshold) to recruit the brainstem reticular formation and produce maximal tonic seizures in the hind-limbs (Woodbury and Esplin, 1959). While this depiction of events has a degree of accuracy, in that clonic seizures do appear to be forebrain mediated (Browning, 1985) and tonic seizures controlled by brainstem centres (Chui and Burnham, 1982; Browning and Engel, 1984), it is now becoming apparent that MES stimulation activates the forebrain and brainstem simultaneously and that the brainstem overrides the forebrain in taking control of the spinal cord (Kreindler et al, 1958; Browning, 1987). It is interesting, then, that the physical manifestations of maximal seizures induced by both PTZ and MES are mediated through the mesencephalic reticular formation, yet these tests are proposed to mirror entirely separate clinical disorders and the majority of AEDs in use today have differing affinities in these two models.

While there are several models of maximal seizures, purported to predict the efficacy of drugs in human tonic-clonic epilepsy (section 1.5; Fisher, 1989), none surpass the MES test, particularly when employed in conjunction with the Min-ES method. The electroshock tests provide the researcher with well established, simple, and economical techniques with which to investigate the efficacy of novel anticonvulsant compounds. They rely on electrical activation of the brain to produce seizures and may highlight drugs which reduce the spread of epileptiform activity. In so doing they may predict the effectiveness of compounds against generalised tonic-clonic seizures, one of the most common forms of human epilepsy.

3.2 EXPERIMENTAL AIMS

The aims of the following studies were to develop a greater understanding of the seizure tests employed in this thesis and to perform some of the background analysis essential to evaluation of experimental results in subsequent chapters. Although systemic administration of PTZ is widely employed to identify potential AEDs, little is known about this compound's pharmacokinetic or pharmacodynamic properties. Failure of individual animals to respond to PTZ is not uncommon and often creates difficulties in data analysis. At present there appears to be no valid explanation for this phenomenon. In this respect, a quantitative study of PTZ concentration-effect relationships was undertaken. Two further qualitative studies are described in this chapter. An initial investigation of the current required to consistently elicit tonic seizures in control animals by MES was undertaken in an attempt to set a standard on which all future work could be designed. Secondly, it was hoped that determination of the starting current and subsequent increments or decrements thereof, would give a background knowledge of the Min-ES test and facilitate accurate evaluation of the seizure threshold in later studies.

3.3 EXPERIMENTAL PROTOCOLS AND RESULTS

3.3.1 Concentration-effect relationships with pentylenetetrazol in the mouse - 1

PROTOCOL:- Mice were separated into six groups (n=6/group) and administered PTZ (s.c.) by the following regimen. Groups 1 - 3 received 85 mg/kg PTZ and were sacrificed 1 and 5 minutes post-dosing and at seizure onset respectively. Groups 4 - 6 were given 50 mg/kg PTZ and were also sacrificed at 1 and 5 minutes post-dosing and at seizure onset respectively. Plasma and whole brain samples were obtained as described in sections 2.2.2

and 2.2.3 respectively. They were stored in accordance with section 2.2.5 prior to analysis of PTZ content by the method outlined in section 2.7.

RESULTS:- None of the animals in groups 1 (85 mg/kg PTZ; 1 minute), 2 (85 mg/kg PTZ; 5 minutes), 4 (50 mg/kg PTZ; 1 minute), 5 (50 mg/kg PTZ; 5 minutes), or 6 (50 mg/kg PTZ; seizure onset) experienced a seizure prior to sacrifice. Those in group 6 were sacrificed at an arbitrary time of 15 minutes post-dosing. All of the animals in group 3 (85 mg/kg PTZ; seizure onset) experienced a generalised seizure, with a mean (± SEM) time to seizure onset of 216 (± 19) seconds. In groups 1 - 3, PTZ concentration in both plasma and brain increased in relation to the time of exposure (figure 1). Following administration of 50 mg/kg PTZ both plasma and brain (figure 2) concentrations increased initially with time (groups 4 and 5) but had begun to drop off by 15 minutes post-dosing (group 6).

3.3.2 Concentration-effect relationships with pentylenetetrazol in the mouse - 2

PROTOCOL:- Mice were separated into eight treatment groups (n=6/group) and exposed to varying concentrations of PTZ (s.c.) for different periods of time. Groups 1 - 4 received 25, 50, 85, and 100 mg/kg PTZ respectively and were sacrificed at 2 minutes post-dosing. Groups 5 and 6 were administered 25 mg/kg PTZ and were sacrificed at 10 and 30 minutes post-dosing respectively. Groups 7 and 8 were given 85 mg/kg PTZ and were sacrificed at 10 minutes and at seizure onset respectively. Samples were obtained, stored, and analysed as described in section 3.3.1. Mouse brains were dissected by the method of Glowinski and Iversen (1966) prior to storage. Cortex, cerebellum, and brainstem regions were identified macroscopically and dissected away. The tissue remaining upon the removal of these three areas was designated midbrain for the purposes of this study.

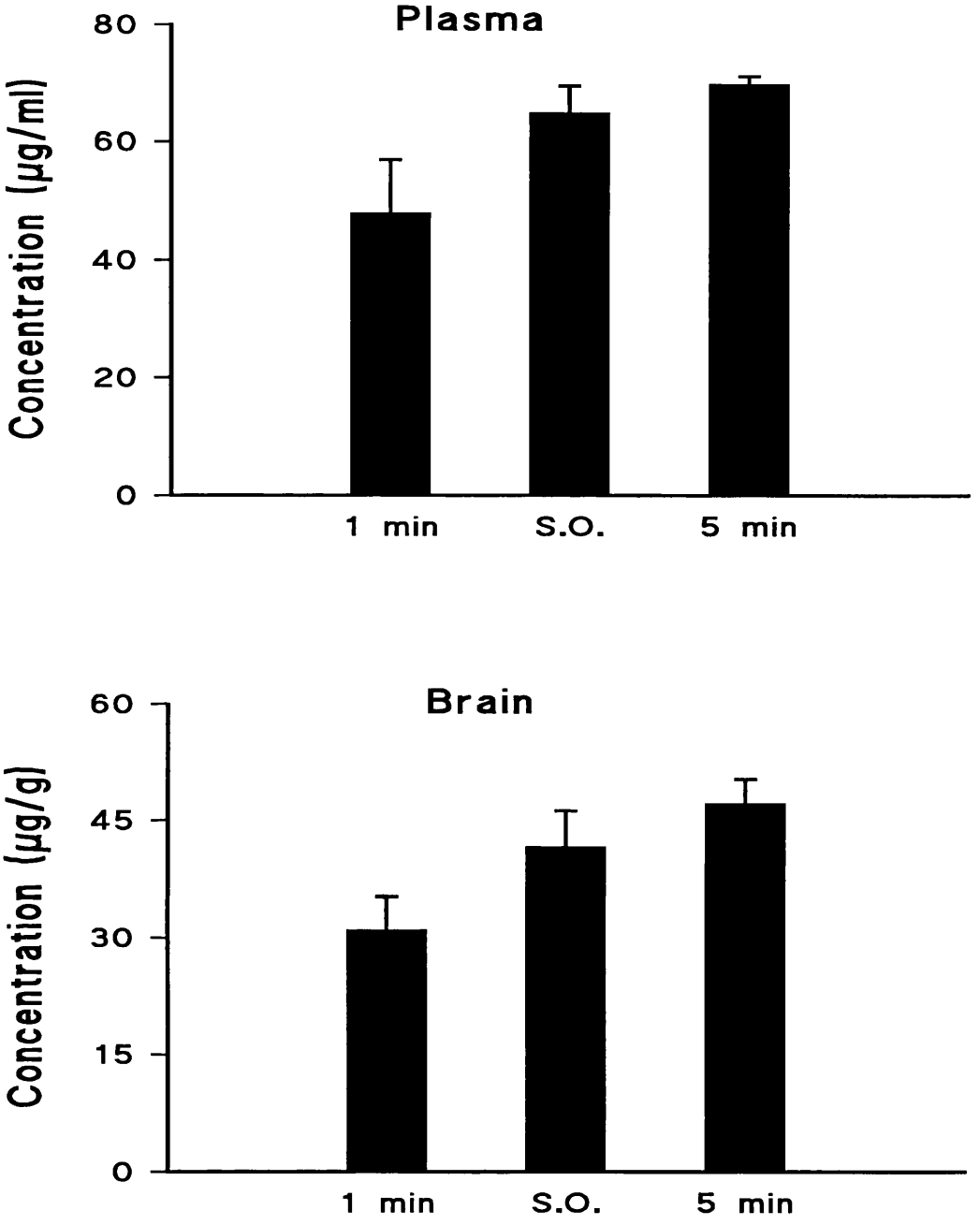

FIGURE 1:- Plasma (upper; µg/ml) and brain (lower; µg/g) pentylenetetrazol (PTZ) concentrations at 1 and 5 minutes post-dosing and at seizure onset (S.O.) following a single subcutaneous administration of 85 mg/kg PTZ. Results are expressed as mean (± SEM) concentrations in groups of 6 mice. The mean (± SEM) time to seizure onset was 216 (± 19) seconds. Animals depicted at 1 and 5 minutes post-administration did not experience seizures prior to sacrifice.

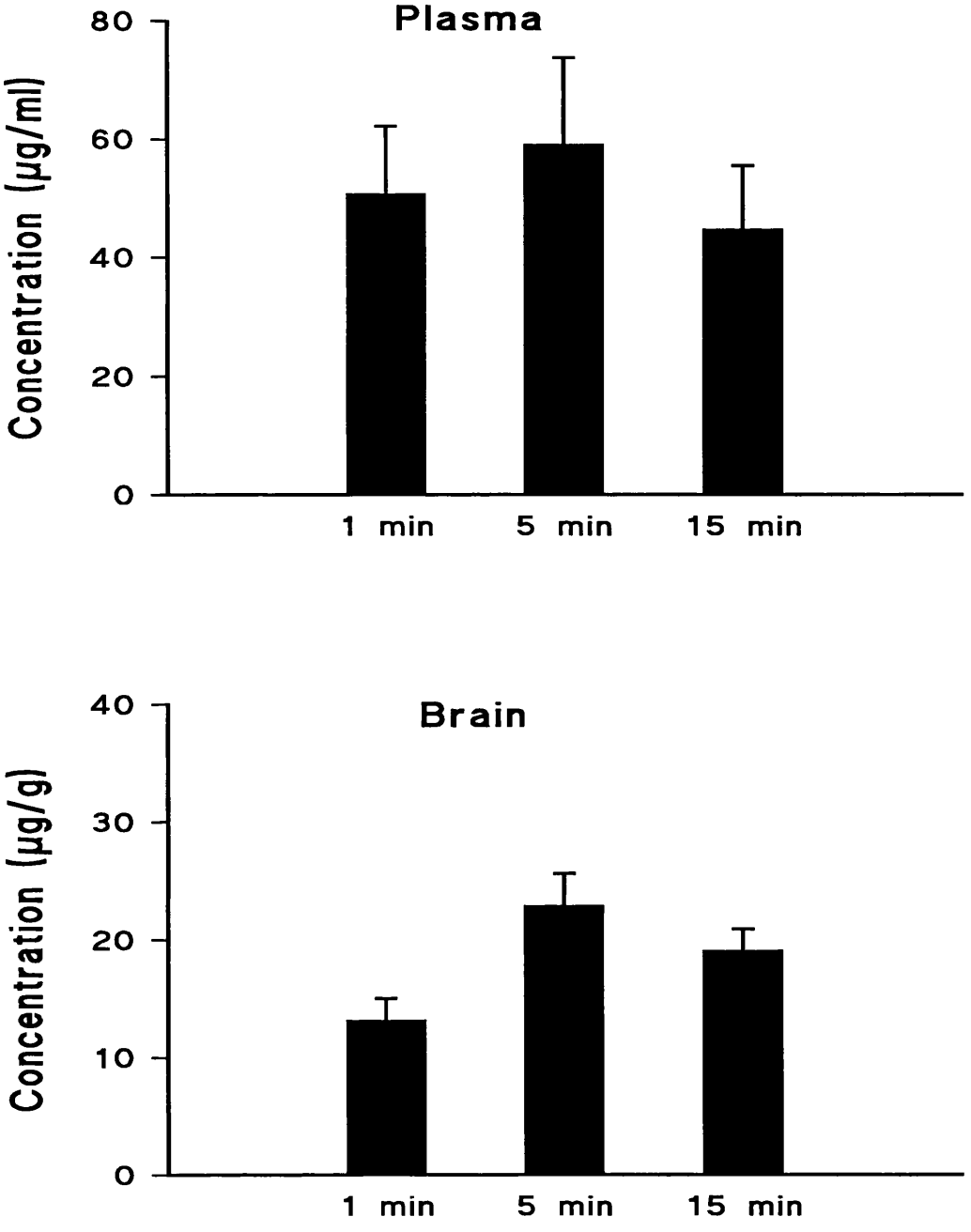

FIGURE 2:- Plasma (upper; µg/ml) and brain (lower; µg/g) pentylenetetrazol (PTZ) concentrations at 1, 5, and 15 minutes after a single subcutaneous administration of 50 mg/kg PTZ. Results are expressed as mean (± SEM) concentrations in groups of 6 mice. None of the animals experienced a seizure prior to sacrifice.

RESULTS:- None of the animals in groups 1 (25 mg/kg PTZ; 2 minutes), 2 (50mg/kg PTZ; 2 minutes), 3 (85 mg/kg PTZ; 2 minutes), 4 (100 mg/kg PTZ; 2 minutes), 5 (25 mg/kg PTZ; 10 minutes), 6 (25 mg/kg PTZ; 30 minutes), or 7 (85 mg/kg PTZ; 10 minutes) experienced a seizure prior to sacrifice. All of the animals in group 8 (85 mg/kg PTZ; seizure onset) experienced a generalised seizure, with a mean (± SEM) time to seizure onset of 231 (± 26) seconds. Concentration data for group 6 are not available as the PTZ concentration in the plasma and all areas of the brain were below the detectable limits of the assay (section 2.7.3). In groups 1 - 4 the PTZ concentration increased with dose in the plasma (figure 3) and all brain areas (figure 4) with one exception only (cerebellum, 85mg/kg). Comparison of groups 1 and 5 suggested that the plasma concentration of PTZ peaked prior to 10 minutes (figure 5) while the concentrations in all brain areas continued to increase up to this point (figure 6). The inability to detect PTZ in samples taken from animals in group 6 indicated only that the brain convulsant levels peaked at some point between 2 and 30 minutes (figure 6). By comparing the results from groups 3, 7, and 8 it appeared that while PTZ concentrations in the plasma, cortex, and midbrain peaked around the time of seizure onset, those in the cerebellum and brainstem rose in relation to exposure time (figures 7 and 8).

3.3.3 Preliminary evaluation of the maximal electroshock test

PROTOCOL:- Naive animals (mice) were divided into seven groups (n=10/group) and received transauricular electroshock using currents of 41, 44, 47, 50, 53, 56, and 59 mA respectively. The other stimulation parameters employed for the induction of MES seizures remained constant throughout (section 2.3.2). The incidence of THE within each group was recorded.

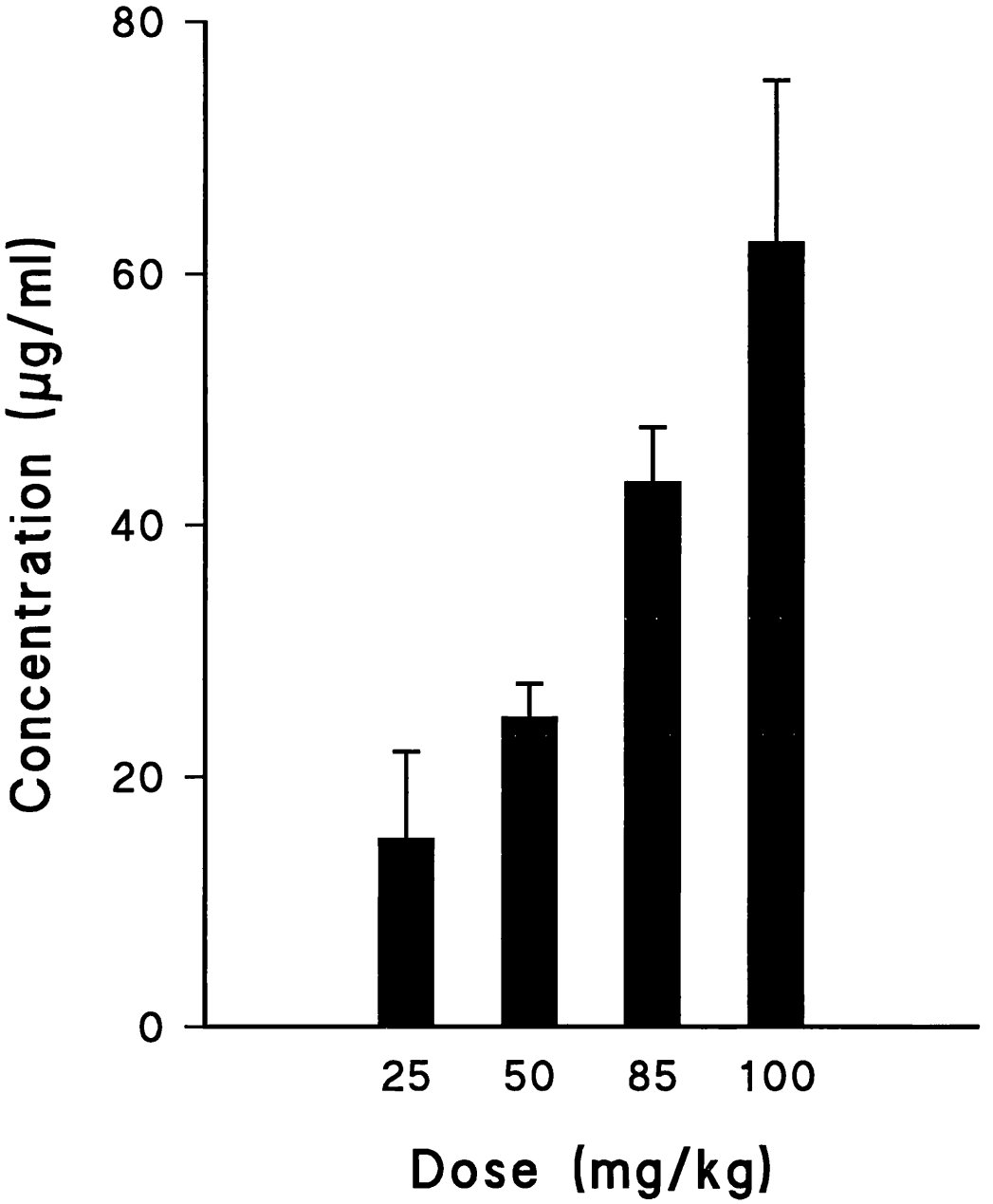

FIGURE 3:- Effects of subcutaneous pentylenetetrazol (PTZ) dose (25 - 100 mg/kg) on plasma PTZ concentrations at 2 minutes post-administration. Results are expressed as mean (± SEM) concentrations (µg/ml) in groups of 6 mice. None of the animals experienced a seizure prior to sacrifice.

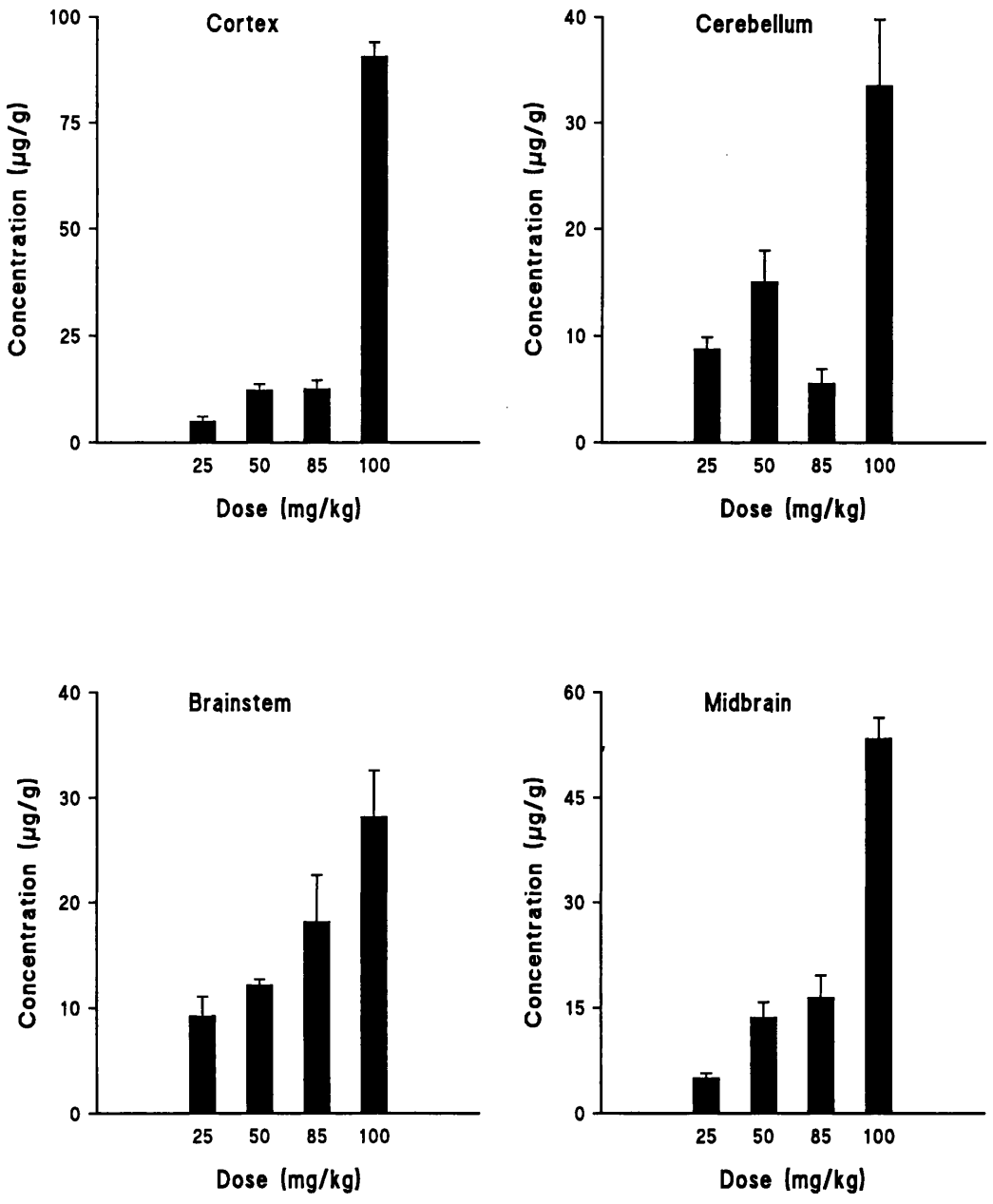

FIGURE 4:- Effects of subcutaneous pentylenetetrazol (PTZ) dose (25 - 100 mg/kg) on cortex (upper left), cerebellum (upper right), brainstem (lower left), and midbrain (lower right) PTZ concentrations at 2 minutes post-administration. Results are expressed as mean (± SEM) concentrations (µg/g) in groups of 6 mice. None of the animals experienced a seizure prior to sacrifice.

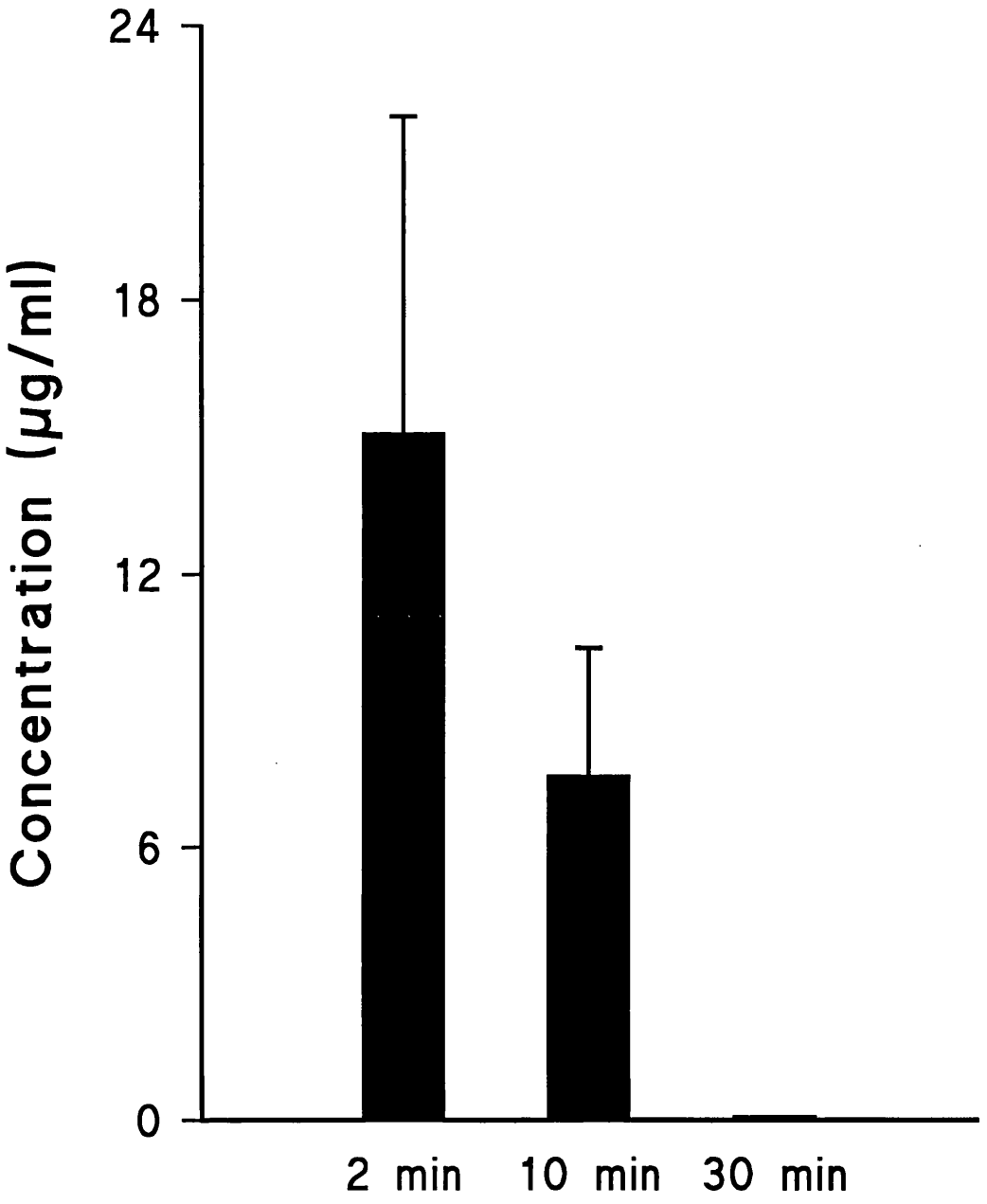

FIGURE 5:- Plasma pentylenetetrazol (PTZ) concentrations at 2, 10, and 30 minutes after a single subcutaneous administration of 25 mg/kg PTZ. Results are expressed as mean (± SEM) concentrations (µg/ml) in groups of 6 mice. None of the animals experienced a seizure prior to sacrifice.

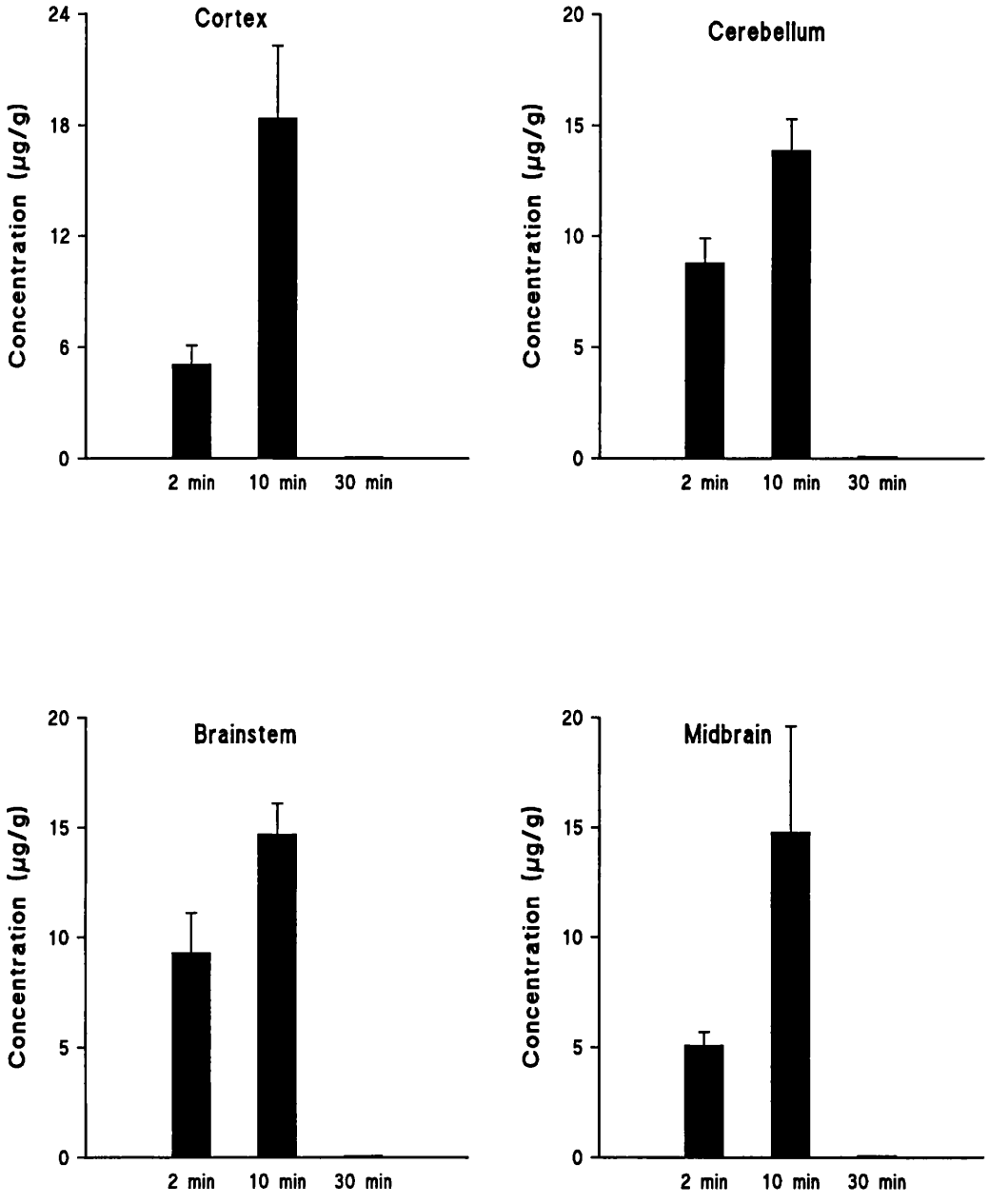

FIGURE 6:- Cortex (upper left), cerebellum (upper right), brainstem (lower left), and midbrain (lower right) pentylenetetrazol (PTZ) concentrations at 2, 10, and 30 minutes after a single subcutaneous administration of 25 mg/kg PTZ. Results are expressed as mean (± SEM) concentrations (µg/g) in groups of 6 mice. None of the animals experienced a seizure prior to sacrifice.

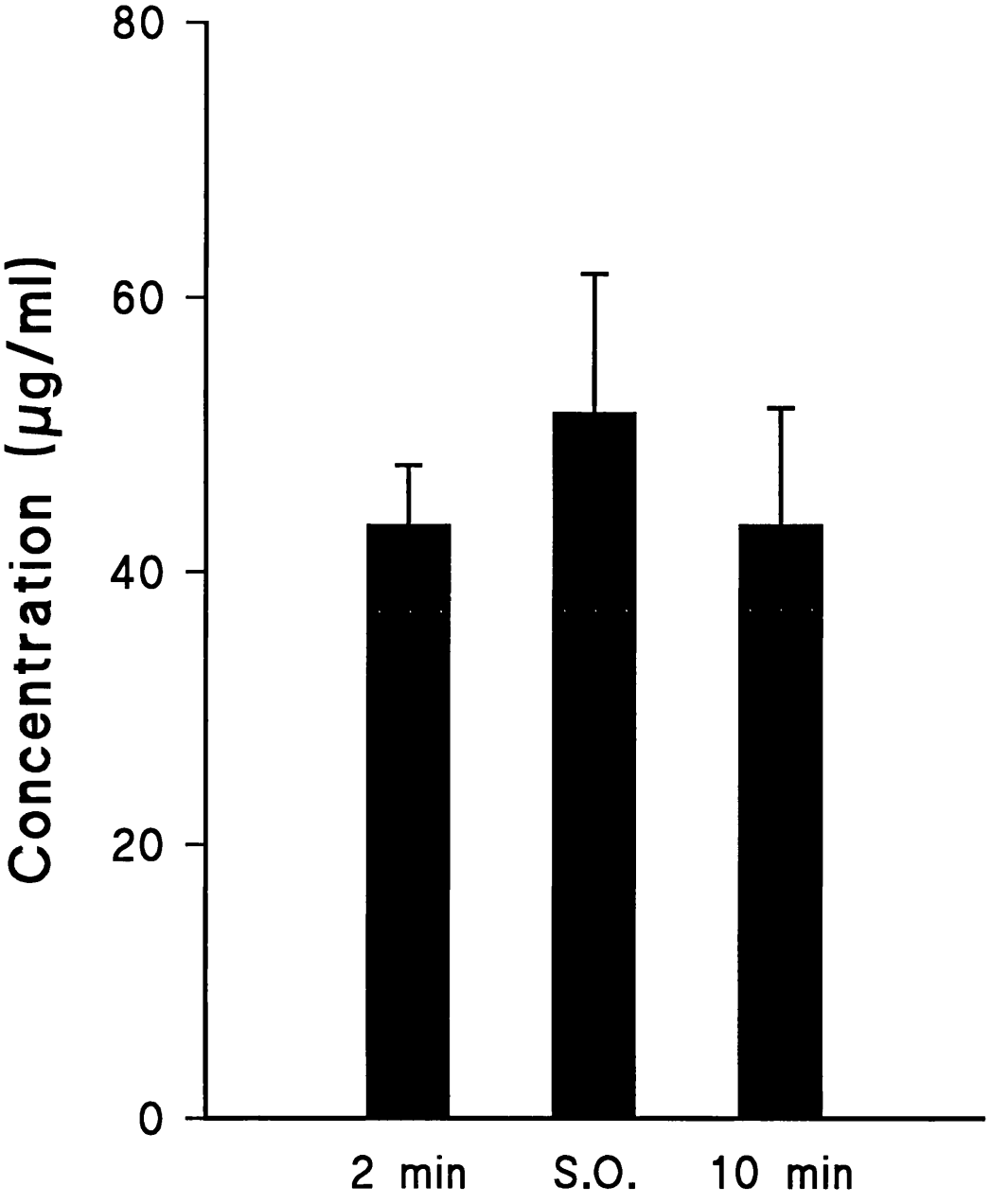

FIGURE 7:- Plasma pentylenetetrazol (PTZ) concentrations at 2 and 10 minutes post-dosing and at seizure onset (S.O.) following a single subcutaneous administration of 85 mg/kg PTZ. Results are expressed as mean (± SEM) concentrations (µg/ml) in groups of 6 mice. The mean (± SEM) time to seizure onset was 231 (± 26) seconds. Animals depicted at 2 and 10 minutes post-administration did not experience a seizure prior to sacrifice.

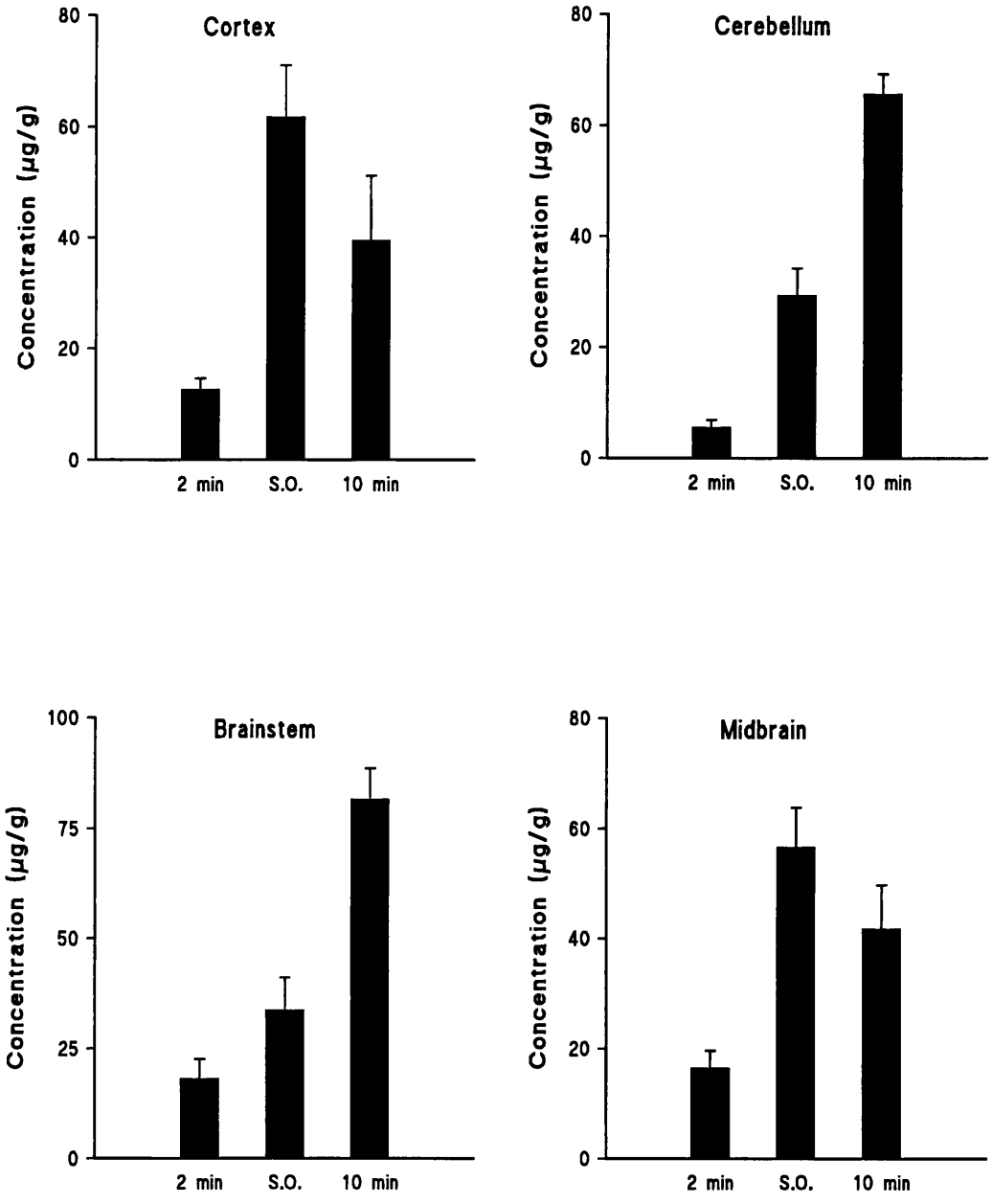

FIGURE 8:- Cortex (upper left), cerebellum (upper right), brainstem (lower left), and midbrain (lower right) pentylenetetrazol (PTZ) concentrations at 2 and 10 minutes post-dosing and at seizure onset (S.O.) following a single subcutaneous administration of 85 mg/kg PTZ. Results are expressed as mean (± SEM) concentrations (µg/g) in groups of 6 mice. The mean (± SEM) time to seizure onset was 231 (± 26) seconds. Animals depicted at 2 and 10 minutes post-administration did not experience a seizure prior to sacrifice.

RESULTS:- The effect of altering the stimulus intensity (current) on the incidence of THE following MES stimulation is illustrated in figure 9. The occurrence of THE appeared to increase with current up to 100% at 50 mA. Successive increases in current beyond 50 mA were without effect. The CC_{50} was observed to be 47 mA.

3.3.4 Preliminary evaluation of the minimal electroshock test

PROTOCOL:- A group (n=30) of naive mice were sequentially subjected to threshold electroshock stimuli as described in section 2.3.3. The arbitrary starting current for investigation of the tonic seizure threshold was set at 6 mA and subsequent increments and decrements at 1 mA. The number of positive (tonic seizure) and negative (no tonic seizure) responses was recorded.

RESULTS:- A starting stimulus of 6 mA and increments and decrements of 1 mA thereafter gave a satisfactory determination of the tonic seizure threshold. The results of this evaluation are represented graphically in figure 10. The median threshold value, determined by regression analysis of positive and negative responses, was calculated at 7.00 mA.

3.4 DISCUSSION

The aims of these preliminary studies were to explore the standard animal seizure models employed in the remainder of this thesis and to afford familiarity with these techniques for their subsequent use.

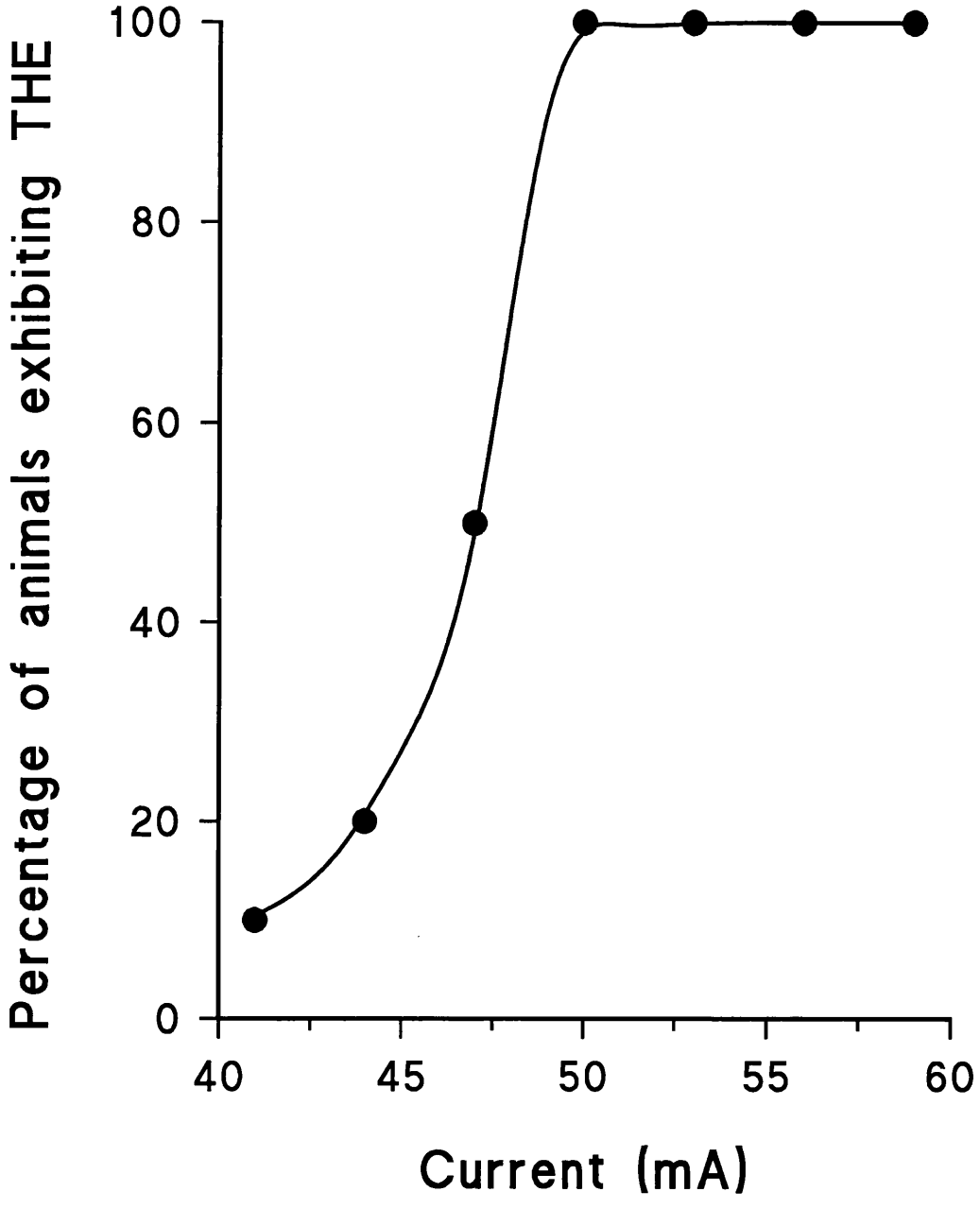

FIGURE 9:- Effects of stimulus intensity (current) on the incidence of tonic hind-limb extension (THE) in control animals. Results are expressed as the percentage of animals (mice) in groups of 10 exhibiting THE.

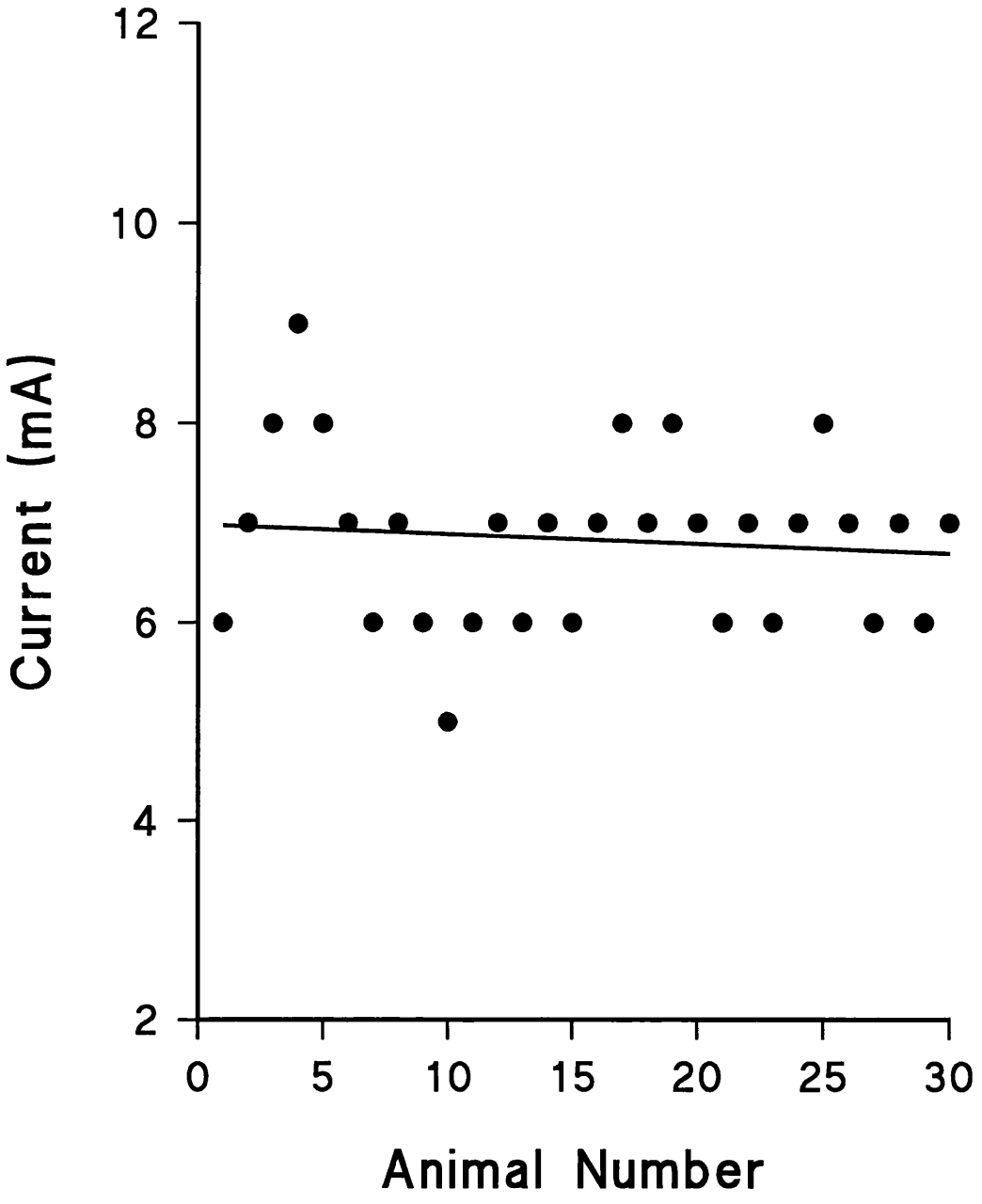

FIGURE 10:- Evaluation of the threshold current for induction of tonic seizures by the "up-down" method (Kimball et al, 1957). The seizure threshold was calculated by regression analysis (solid line) of the results obtained from a group of 30 control mice.

The fact that PTZ fails to induce convulsions in a significant proportion of laboratory animals has not only precipitated disputes over its integrity as an experimental seizure model, but often makes data analysis problematic. Although the emergence of a clear explanation for the apparent lack of PTZ effect in certain animals was not forthcoming from these experiments, the results did provide a degree of insight into the pharmacokinetics and pharmacodynamics of PTZ in the rodent. The concepts of interest are described below.

PTZ study - 1

In the first study, there appeared to be no relationship between the PTZ concentration and the latency to seizure onset. Animals treated with 85 mg/kg PTZ and devoid of seizure activity had higher plasma and whole brain convulsant levels than those experiencing seizures at the same PTZ dose (figure 1). Possible explanations for this disparity included inter-animal variations in seizure susceptibility and/or $GABA_A$ receptor function, although such influences should have been delineated by the use of genetically identical animals. Although this phenomenon might underlie the lack of response of certain animals to convulsant doses of PTZ, it was proposed that seizure onset was more likely to be dependent upon PTZ concentration in a discrete brain region than in whole brain. This proposition precipitated the second PTZ study.

PTZ study - 2

From the second study, analysis of PTZ concentrations in discrete brain regions suggested that cortical and midbrain convulsant levels peaked at or around the time of seizure onset, whereas those in the brainstem and cerebellum increased in relation to exposure period

(figure 8). Definitive correlations between PTZ concentrations in discrete brain regions and the latency to seizure onset could not be drawn from these studies. However, the lack of motor manifestations in those animals exhibiting the highest brainstem convulsant concentrations (figure 8) would seem to contradict the proposal of this brain region as one of the primary sites of PTZ action (Faingold, 1977). Thus, this study proposed a differential distribution of PTZ in the brain, but offered no explanation of the lack of effect of PTZ in some animals. Similarly, information regarding the primary site of PTZ action was not forthcoming.

An additional observation from the second PTZ study suggested that at higher doses (85 mg/kg) plasma PTZ concentrations were relatively constant (figure 7) whereas brain concentrations varied greatly with exposure time (figure 8). From these observations it was proposed that the pharmacokinetic profile of PTZ was characterised by an initial saturation of the plasma compartment, followed by a seemingly slower entry into the brain.

The recurrent problem with drawing valid conclusions from the PTZ studies appeared to be one of resolution. The incorporation of *in vivo* microdialysis techniques might help to clarify any future studies in this respect. Nevertheless, this investigation yielded some novel data on the PTZ seizure model and also facilitated a familiarity and validation of it for subsequent use in our laboratory.

Maximal and minimal electroshock studies

Both electroshock studies facilitated a confident validation of these procedures for use in our laboratory. Investigation of the current required to induce THE revealed results bearing similarity to previously published data. The minimum current required to induce maximal tonic seizures in 100% of control animals was found to be 50 mA, a figure frequently reported by other researchers (Fisher, 1989). Similarly, Löscher and Schmidt (1988) have quoted tonic seizure thresholds in naive mice of between 6 and 9 mA, dependent on age, strain, and stimulation site, with those determined by auricular stimulation generally lower than those employing corneal electrodes. Thus, a preliminary evaluation of the Min-ES test yielding a median threshold value of 7.00 mA was deemed satisfactory. Finally, comparison of these precursory electroshock studies proposed the supramaximal current to be within the often cited range of 5 - 10 times greater than threshold (Löscher and Schmidt, 1988).

3.5 CONCLUSIONS

In conclusion, the investigations described in this chapter provided a background knowledge of the principal seizure tests employed throughout the remainder of this thesis and thus precipitated informed experimental design thereafter. Qualitatively, all of the studies performed were of long-term benefit, however the expectation of accomplishing sound quantitative analysis of PTZ effects in the brain was, in retrospect, naive.

CHAPTER FOUR

ANTIEPILEPTIC DRUG ENHANCEMENT OF NEURONAL INHIBITION

4.1 INTRODUCTION

GABA is established as the major inhibitory neurotransmitter in the mammalian brain. There is evidence to suggest that the nervous system is held in a state of perpetual restraint, "wired" with circuits which tonically inhibit pacemaker neurons and feedforward and/or feedback cell discharges, surround and dampen the output of spontaneously discharging neurones, and influence pre-synaptic inhibition and facilitation. The CNS operates by relaxing these restraints (i.e. disinhibition) via excitatory inputs. The majority of this inhibitory "wiring" is comprised of neurones which release GABA as their neurotransmitter.

4.1.1 GABA as an inhibitory neurotransmitter in the brain

GABA was first identified in the brain in 1950 (Awapara et al, 1950; Roberts and Frankel, 1950; Udenfriend, 1950) and shortly afterwards its involvement in the mediation of seizure activity became apparent. Hayashi observed that direct application of GABA onto the motor cortex of the dog attenuated local epileptic discharge (Hayashi and Nagai, 1956; Hayashi, 1959) and Killam and Bain (1957) reported inhibition of GABA synthesis to be the principle mechanism of action of the convulsant hydrazides. Understanding of the synthesis (Weinstein et al, 1963), release (Jasper et al, 1965), post-synaptic effects (Krnjevic and Schwartz, 1967), specific antagonism (Galindo, 1969), and inactivation (Roberts and Kuriyama, 1968) of GABA followed and precipitated its proposal as an inhibitory neurotransmitter in the mammalian brain.

GABA is formed, *de novo*, entirely from L-glutamic acid. This reaction is catalysed by the enzyme GAD in the presence of pyridoxal phosphate as a cofactor (Meldrum, 1975). GAD

is exclusively localised to GABAergic neurones in the vertebrate CNS and can be employed as a definitive marker for these cells (Snead, 1983).

Following synaptic release, GABA exerts its actions via specific cell membrane receptors. At present two specific receptor sites for GABA have been identified in the mammalian brain and have been designated $GABA_A$ and $GABA_B$.

The $GABA_A$ receptor has been the subject of many studies (Olsen, 1982; Levitan et al, 1988; Olsen and Tobin, 1990) and is now well characterised. Extensive research has shown the $GABA_A$ receptor to be a macromolecular protein, composed of five variable subunits, forming a chloride ion-selective channel and possessing additional binding sites for BZDs (Ehlert, 1986), barbiturates (Willow and Johnston, 1983), picrotoxin (Olsen, 1981a), penicillin (Twyman et al, 1992), and neurosteroids (Majewska et al, 1986). Binding of GABA to this receptor subtype leads to opening of the channel, chloride ion entry into the cell, hyperpolarisation, and inhibition of action potential generation (Enna and Gallagher, 1983).

Thirteen different $GABA_A$ receptor subunits have been identified (Wingrove et al, 1994) and the composition of individual receptors, with respect to these subunits, is thought to confer the presence and/or affinity of the various binding sites contained within the intact receptor complex (Verdoorn et al, 1990; Sigel et al, 1990). Although $GABA_A$ receptors *per se* are distributed widely, determination of the regional distribution of receptor subunits, by *in situ* hybridization, has confirmed that many of the subunits are highly localised within the brain (Macdonald, 1993) and may be responsible for the heterogeneity

of $GABA_A$ receptors previously reported in binding studies (Olsen et al, 1984; McCabe et al, 1988).

The less well characterised $GABA_B$ receptor was originally considered to be predominantly an autoreceptor, mediating feedback inhibition of GABA release. It now appears to be widely distributed in the brain on both nerve terminals and apical dendrites (Bowery, 1989). Agonist binding to $GABA_B$ receptors in the brain results in the intracellular accumulation of cAMP by activation of adenylate cyclase (Pratt et al, 1989). The physiological role of the $GABA_B$ receptor remains unclear, however studies performed in hippocampal slices have suggested that receptor activation may play a role in long-term potentiation (Dutar and Nicholl, 1988; Davies et al, 1991) and may also be epileptogenic (Swartzwelder et al, 1987).

Following receptor activation, GABA is removed from its site of action, in the synaptic cleft, by a high affinity uptake mechanism (Logan and Snyder, 1971). This process, together with direct diffusion of GABA into post-synaptic nerve cell bodies (Hydén et al, 1986), appears to be one of the most significant mechanisms of GABA inactivation and can be observed in both neurones and glial cells (Iversen and Kelly, 1975).

Upon cellular uptake, GABA is successively metabolised by the actions of two mitochondrial enzymes. GABA-T catalyses the conversion of GABA to succinic semialdehyde in the presence of α-KG as a cofactor and SSAD completes the metabolism to succinate in the presence of nicotinyl adenine dinucleotide (NAD; Meldrum, 1975).

Regional distribution studies, employing GAD immunocytochemistry, have revealed the hypothalamus, globus pallidus, and substantia nigra to be the brain areas of highest GABA concentration (Fahn, 1976; Enna, 1981). Major GABAergic pathways in the brain include projections from the caudate nucleus, putamen, and globus pallidus to the substantia nigra (Gale et al, 1977; Roberts, 1978; Gale and Iadarola, 1980) and extensive networks of inhibitory interneurones in the cortex (Sloper et al, 1980) and hippocampus (Miles and Wong, 1987).

Although it is widely reported that reduction of GABAergic inhibition produces seizures and that enhanced inhibition is anticonvulsant, this criterion is not unequivocal. Gale (1989; 1992) has elegantly demonstrated that, while universal GABA augmentation may be anticonvulsant, localised GABA enhancement may be proconvulsant. Inhibition of GABA metabolism by VGB in the pontine reticular formation and certain areas of the thalamus results paradoxically in seizure generation. There are also some regions of the brain, such as the substantia nigra, in which an increase in GABA transmission is anticonvulsant but a reduction in GABA transmission does not induce seizures.

Since the identification of GABA as the major inhibitory neurotransmitter in the brain much interest has focused on impaired GABA function as an aetiological factor in epileptogenesis (Olsen et al, 1986). This has led to the existence of a greater volume of literature on the relationship between GABA and the pathogenesis of epilepsy than for any other neuroactive substance (Snead, 1983).

Decreased GAD activity, decreased GABA concentrations, and decreased GABA receptor binding have all been observed in experimentally induced epileptic foci in animals (Ribak et al, 1979; Bakay and Harris, 1981). Similarly, reduced GAD activity, reduced GABA receptor binding, and increased GABA-T activity have been reported in post-surgical human epileptogenic tissue (Lloyd et al, 1985). Coursin (1964) reported seizures in children with a dietary deficiency of vitamin B_6 (pyridoxine) and attributed this disorder to impaired GABA synthesis in the absence of sufficient concentrations of pyridoxal phosphate, the essential cofactor for GAD-mediated catalysis. Measurement of CSF GABA concentrations has provided further support for the role of GABA dysfunction in epileptogenesis. Reduced GABA levels in the CSF have been reported in clinical studies of chronic epilepsy (Manyam et al, 1980) and in febrile convulsions (Löscher et al, 1981). Finally, not only do many of the proposed animal models of epilepsy employ GABA impairment in the production of seizures (Fisher, 1989), but augmentation of GABA function has been proposed as one of the principle mechanisms of action of a wide range of chemically unrelated, clinically-effective AEDs (Macdonald and Barker, 1979; Rogawski and Porter, 1990). Manipulation of GABAergic neurotransmission remains one of the most attractive targets in the experimental and clinical investigation of epilepsy.

4.1.2 Inhibition of GABA metabolism as an antiepileptic mechanism

An increase in the amount of GABA in the pre-synaptic compartment of neurones may result in augmented release at the synapse and enhanced GABA-mediated inhibition in the brain. Inhibition of GABA-T, the rate-limiting enzyme in what is the only pathway for catabolism of brain GABA, is the most effective method of increasing pre-synaptic GABA concentrations (Meldrum, 1979).

VGB is a structural analogue of GABA which was rationally designed for the treatment of epilepsy as a selective, enzyme-activated, suicide inhibitor of GABA-T which covalently binds to the enzyme at its site of action (Lippert et al, 1977). VGB has been shown to reduce GABA-T activity in a dose-dependent manner in cultured cortical astrocytes and neurones (Larsson et al, 1986) and in a time- and dose-dependent manner following systemic administration to rodents (Jung et al, 1977). A cumulative effect on brain GABA metabolism with repetitive VGB dosing was also demonstrated by Jung and colleagues (1977).

In whole animal studies, pronounced accumulation of GABA has been reported in glial cells, in addition to increases in the neuronal pool, following systemic administration of VGB (Neal et al, 1989). There is evidence to suggest that the increased GABA in the neuronal compartment is in a readily releasable pool (Gram et al, 1988) and that the increased astrocytic GABA may also be released under depolarising conditions, such the accumulation of extracellular K^+ observed during seizure activity (Neal et al, 1989). The existence of two functionally distinct compartments in which VGB increases GABA levels, namely glial cells and GABAergic neurones, may underlie the rather complex relationship between elevation of whole brain GABA and anticonvulsant efficacy (Gale, 1989; Gale, 1992).

Early experimental studies with VGB suggested that following a single 1500 mg/kg dose (i.p.) in mice there was a rapid fall in brain GABA-T activity which remained below control values for up to 5 days. This enzyme inhibition was accompanied by a concomitant increase in whole brain GABA concentration which reached a plateau after 4

hours. At 48 hours post-administration, brain GABA levels began to descend. However, they remained significantly higher than control values for up to 120 hours after VGB administration (Schechter et al, 1977).

Although VGB has been shown to have little or no effect on other transaminases or on SSAD (Jung and Seiler, 1978), it appears to have a significant inhibitory effect on *in vivo* GAD activity. Tissue dependent decreases in GABA synthesis of between 25 and 50% have been reported in both rats (Neal and Shah, 1990) and mice (Jung et al, 1977) following chronic VGB treatment. This inhibitory effect was proposed to have a slower rate of onset than that seen with GABA-T and was attributed to feedback inhibition by the high levels of GABA attained (Porter and Martin, 1984; Löscher and Frey, 1987).

An inhibitory effect on GABA synthesis might help to explain the tolerance to the anticonvulsant effects of VGB reported in experimental studies (Löscher, 1982; Löscher and Frey, 1987; Rundfeldt and Löscher, 1992). Although loss of efficacy during long-term clinical treatment with VGB is relatively uncommon (Sander et al, 1990), reduced GABA synthesis has been reported in resected brain tissue from epileptic foci in humans (Lloyd et al, 1986). A ceiling to effective dosage with the drug reported for individual patients (McKee et al, 1993) may also be a manifestation of reduced GAD activity.

Dose-related increases in CSF GABA levels have been observed following VGB administration to both animals (Böhlen et al, 1979) and man (Ben-Menachem et al, 1989), leading to suggestions that CSF GABA levels may be employed as a marker for the effects of VGB in the brain. Similarly, analysis of platelet GABA-T activity has been

proposed as a method of therapeutic monitoring of the drug (Valdizán and Armijo, 1991). As yet, no consistent correlation has been observed between these proposed GABAergic markers and the antiepileptic effects of the drug (Riekkinen et al, 1989; Ben-Menachem, 1989).

The anticonvulsant profile of VGB in animal seizure models has been investigated extensively. VGB has demonstrated efficacy against audiogenic seizures in DBA/2 mice (Schechter et al, 1977), photogenic epilepsy in baboons (Meldrum and Horton, 1978), the development (Shin et al, 1986) and motor manifestations (Löscher et al, 1989) of amygdaloid kindling, and the seizures induced by a variety of chemoconvulsants such as picrotoxin (Bernasconi et al, 1988), 3-MPA (Liu et al, 1990), and the inverse BZD ligands (Chapman et al, 1987). VGB was without effect on the EEG seizure activity recorded from rats with spontaneous nonconvulsive epilepsy (Marescaux et al, 1985), a syndrome generally exacerbated by compounds that facilitate GABAergic transmission. Controversy surrounds the anticonvulsant effects of VGB in both of the standard seizure models (section 3.1), the CD_{97} PTZ test (Myslobodsky et al, 1979; Bernasconi, et al, 1988) and the MES test (Iadarola and Gale, 1982; Bonhaus and McNamara, 1988; Bernasconi et al, 1988). Conflicting reports, in this respect, may be related to differences in species and administration site.

In chronic animal toxicity studies, a consistent, dose-dependent, reversible microvacuolation and intramyelinic oedema was observed with VGB in the white matter tracts of non-primate brain (Hammond and Wilder, 1985; Butler et al, 1987). Although no such lesions have been observed following long-term treatment with VGB in man (Agosti

et al, 1990; Hammond et al, 1992), these observations have slowed the clinical development of the drug, particularly in North America.

Clinically, VGB has proven effective as adjuvant therapy in patients with refractory epilepsy (Reynolds et al, 1991) with a 50% reduction in seizure frequency in around 50% of patients (Grant and Heel, 1991). VGB appears to possess particular efficacy against complex partial seizures (Ring et al, 1990), the most common drug-resistant form of chronic epilepsy.

4.1.3 Inhibition of GABA uptake as an antiepileptic mechanism

The specific sodium- and energy-dependent uptake of synaptically released GABA can be observed in pre-synaptic nerve terminals and glial cells (Iversen and Kelly, 1975) and represents one of the most significant mechanisms of rapid GABA inactivation in the brain (Meldrum, 1975). Non-specific inhibition of carrier-mediated GABA transport may enhance GABAergic transmission, by blockade of its rapid inactivation and provide a novel approach to the manipulation of GABA-mediated inhibition in the pharmacological amelioration of seizures (Krogsgaard-Larsen et al, 1987).

TGB is a recently developed compound which has been shown to be a potent and selective inhibitor of GABA uptake into neurones and glial cells (Nielsen et al, 1987). TGB consists of a nipecotic acid moiety linked by an aliphatic chain to a lipophilic anchor and, unlike its parent compound, nipecotic acid and other predecessors, is centrally active following systemic administration.

TGB inhibits the uptake of GABA into rat forebrain synaptosomal preparations and cultured cortical neurones and glial cells with IC_{50}'s of 75, 446, and 182 nM respectively (Pierce et al, 1991). It binds to the GABA uptake carrier with a K_D of 18 nM (Braestrup et al, 1990) but does not appear to be a substrate for the transporter, nor does it stimulate release of GABA from neurones (Pierce et al, 1991). The drug only very weakly interacts with the BZD receptor and the chloride channel of the $GABA_A$ receptor and does not appreciably bind to other neurotransmitter receptors (Rogawski and Porter, 1990).

In the CA1 region of the rat hippocampal slice, TGB increases the amplitude and duration of both depolarising and hyperpolarising responses to iontophoretically applied GABA and increases the antidromic IPSP amplitude (Rekling et al, 1990). In pyramidal cells of the CA3 layer of the rat hippocampal slice, TGB prolongs the duration of monosynaptic IPSPs elicited in the presence of EAA antagonists and increases the mean decay time constant of $GABA_A$-mediated synaptic currents (Thompson and Gähwiler, 1992). Systemic administration of anticonvulsant doses of TGB significantly increases extracellular GABA concentrations in rat globus pallidus, ventral pallidum, and substantia nigra (Fink-Jensen et al, 1992).

Evaluation of the anticonvulsant profile of TGB, following systemic administration, in animal seizure models has revealed the drug to be effective against audiogenic seizures in DBA/2 mice (Nielsen et al, 1991), the motor manifestations of amygdaloid-kindled seizures (Pierce et al, 1991), the tonic and clonic components of PTZ-induced seizures in both rats and mice (Nielsen et al, 1991), and seizures induced by DMCM (Nielsen et al, 1991). TGB afforded only partial protection against photically-induced seizures in *Papio*

papio baboons and bicuculline- and MES-induced seizures in mice (Nielsen et al, 1991). It was without effect against dendrotoxin- and 4-AP-induced seizures (Coleman et al, 1992; Yamaguchi and Rogawski, 1992).

Psychomotor side effects were observed following TGB administration in several studies. These adverse events, evaluated using the rotarod, traction, and exploratory locomotor tests, were observed at doses 12 - 31 times greater than the dose required to protect against DMCM-induced seizures in mice. In rats, the ratio of anticonvulsant activity against PTZ-induced seizures to sedatory effects was 2.5 and impaired motor coordination, diffuse tremor, and slow abnormal movements of the limbs were unquantified observations following administration of 4 times the anticonvulsant dose to photosensitive *Papio papio* baboons (Nielsen et al, 1991).

Based on the preclinical results, TGB was selected as a candidate for further development in the treatment of epilepsy. The drug has now entered clinical trial (section 1.4.2) and although studies remain in their infancy, promising initial reports suggest that TGB affords significant protection against partial and secondary generalised seizures (Leach and Brodie, in press).

4.2 EXPERIMENTAL AIMS

The aims of the following studies were to investigate the experimental anticonvulsant profiles of VGB and TGB and to explore their mechanisms of action. Despite the successful use of VGB in the clinical treatment of epilepsy, controversy continues over its efficacy in animal seizure models and the precise mechanism by which it exerts its

pharmacological effects. TGB, by comparison, is a relatively novel antiepileptic compound, less well characterised both in terms of experimental anticonvulsant profile and mechanism of action. The standard seizure models described in section 3.1 were employed to investigate the *in vivo* actions of these drugs, while rodent brain tissue and primary cultures of cortical astrocytes and neurones facilitated *in vitro* exploration of their mechanisms of action.

4.3 EXPERIMENTAL PROTOCOLS AND RESULTS

4.3.1 Dose and time dependent effects of single vigabatrin administration on pentylenetetrazol-induced seizures in mice

PROTOCOL:- Mice were separated into 5 treatment groups (n=20/group) and VGB was administered (i.p.) in doses of 25, 50, 100, and 200 mg/kg. One group (control) received vehicle (0.9% saline) alone. At 24 and 48 hours post-administration 10 animals from each group were subjected to the s.c CD_{97} PTZ test (section 2.3.1) and the latency to the first generalised seizure in individual animals was recorded.

RESULTS:- Single doses of VGB (25 - 200 mg/kg) were ineffectual in lengthening the time to the first generalised seizure induced by PTZ at 24 and 48 hours post-administration (figure 11).

4.3.2 Dose and time dependent effects of single vigabatrin administration on maximal electroshock-induced seizures in mice

PROTOCOL:- Mice were separated into 5 treatment groups (n=20/group) and VGB was administered (i.p.) in doses of 25, 50, 100, and 200 mg/kg. One group (control) received vehicle (0.9% saline) alone. At 24 and 48 hours post-administration 10 animals from each

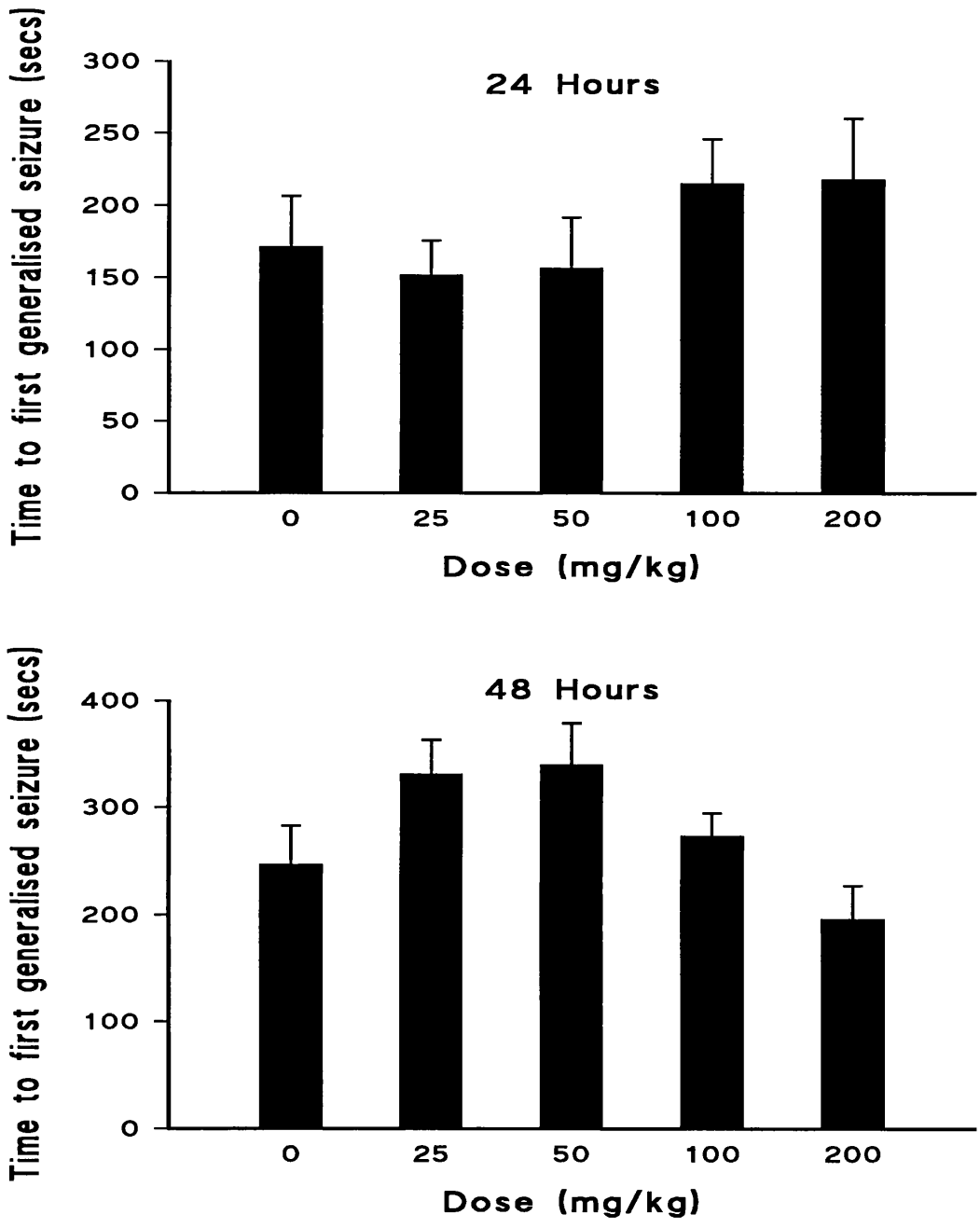

FIGURE 11:- Effect of single vigabatrin doses (25 - 200 mg/kg) on the latency to the first generalised seizure induced by 85 mg/kg PTZ at 24 (upper) and 48 (lower) hours post-administration. Results are expressed as mean (± SEM) time (seconds) in groups of 10 mice.

group were subjected to the MES test (section 2.3.2) using a stimulus current of 50 mA. The incidence of THE within each group was recorded.

RESULTS:- Single doses of VGB (25 - 200 mg/kg) were without effect on the incidence of THE induced by MES at 24 and 48 hours post-administration (figure 12).

4.3.3 Dose dependent effects of repeated vigabatrin treatment on pentylenetetrazol-induced seizures in mice

PROTOCOL:- Mice were separated into 5 treatment groups (n=10/group) and VGB was administered (i.p.) in doses of 25, 50, 100, and 200 mg/kg. One group (control) received vehicle (0.9% saline) alone. Treatment was continued once daily for 7 days. At 24 hours after the final dose the animals were subjected to the s.c CD_{97} PTZ test (section 2.3.1) and the latency to first the generalised seizure in individual animals was recorded.

RESULTS:- Repeated treatment with VGB (25 - 200 mg/kg) was ineffectual in lengthening the time to the first generalised seizure induced by PTZ at 24 hours after the final dose (figure 13).

4.3.4 Dose dependent effects of repeated vigabatrin treatment on maximal electroshock-induced seizures in mice

PROTOCOL:- Mice were separated into 5 treatment groups (n=10/group) and VGB was administered (i.p.) in doses of 25, 50, 100, and 200 mg/kg. One group (control) received vehicle (0.9% saline) alone. Treatment was continued once daily for 7 days. At 24 hours after the final dose the animals were subjected to the MES test (section 2.3.2) using a stimulus current of 50 mA. The incidence of THE within each group was recorded.

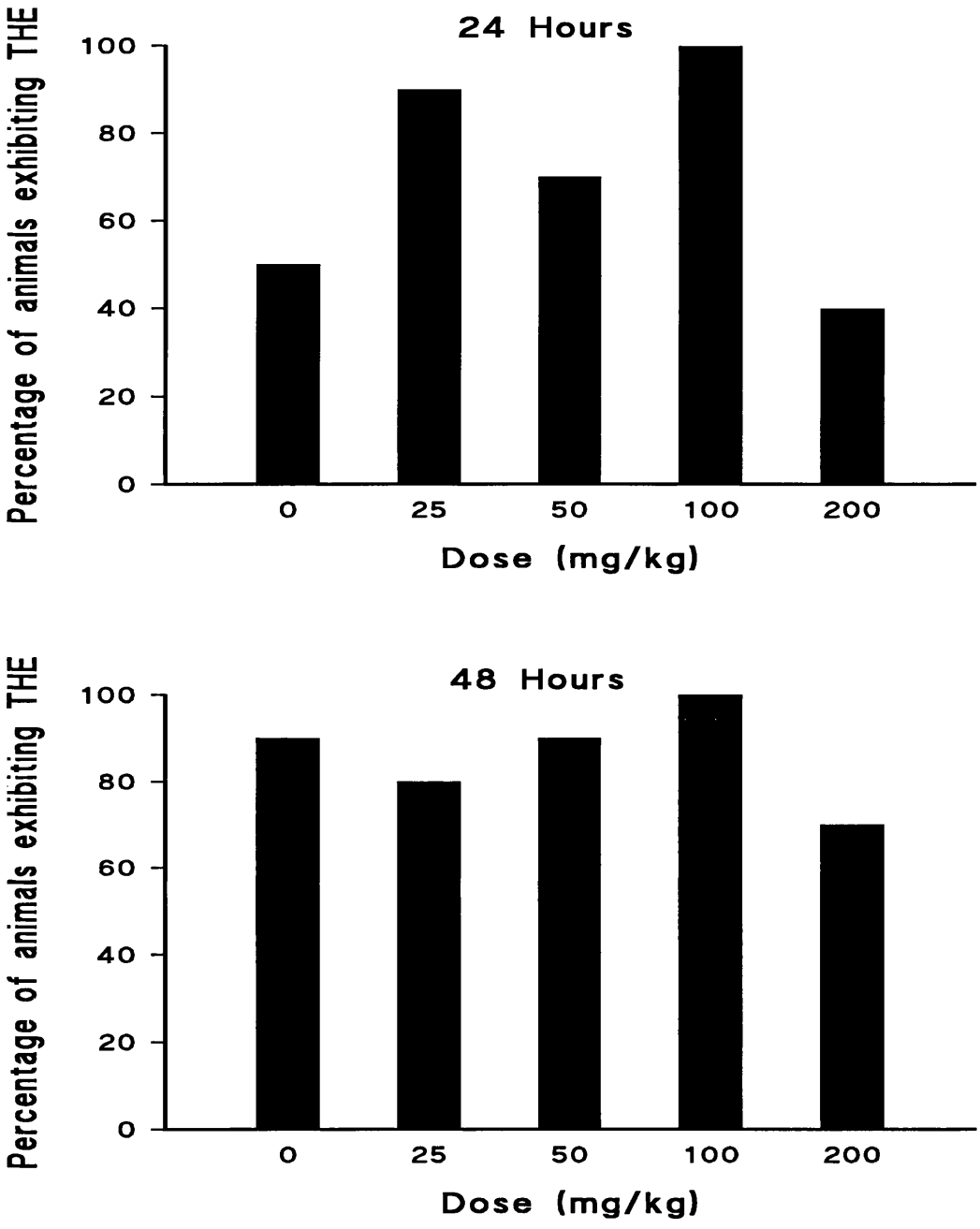

FIGURE 12:- Effect of single vigabatrin doses (25 - 200 mg/kg) on the incidence of tonic hind-limb extension (THE) induced by MES at 24 (upper) and 48 (lower) hours post-administration. Results are expressed as the percentage of animals (mice) in groups of 10 exhibiting THE.

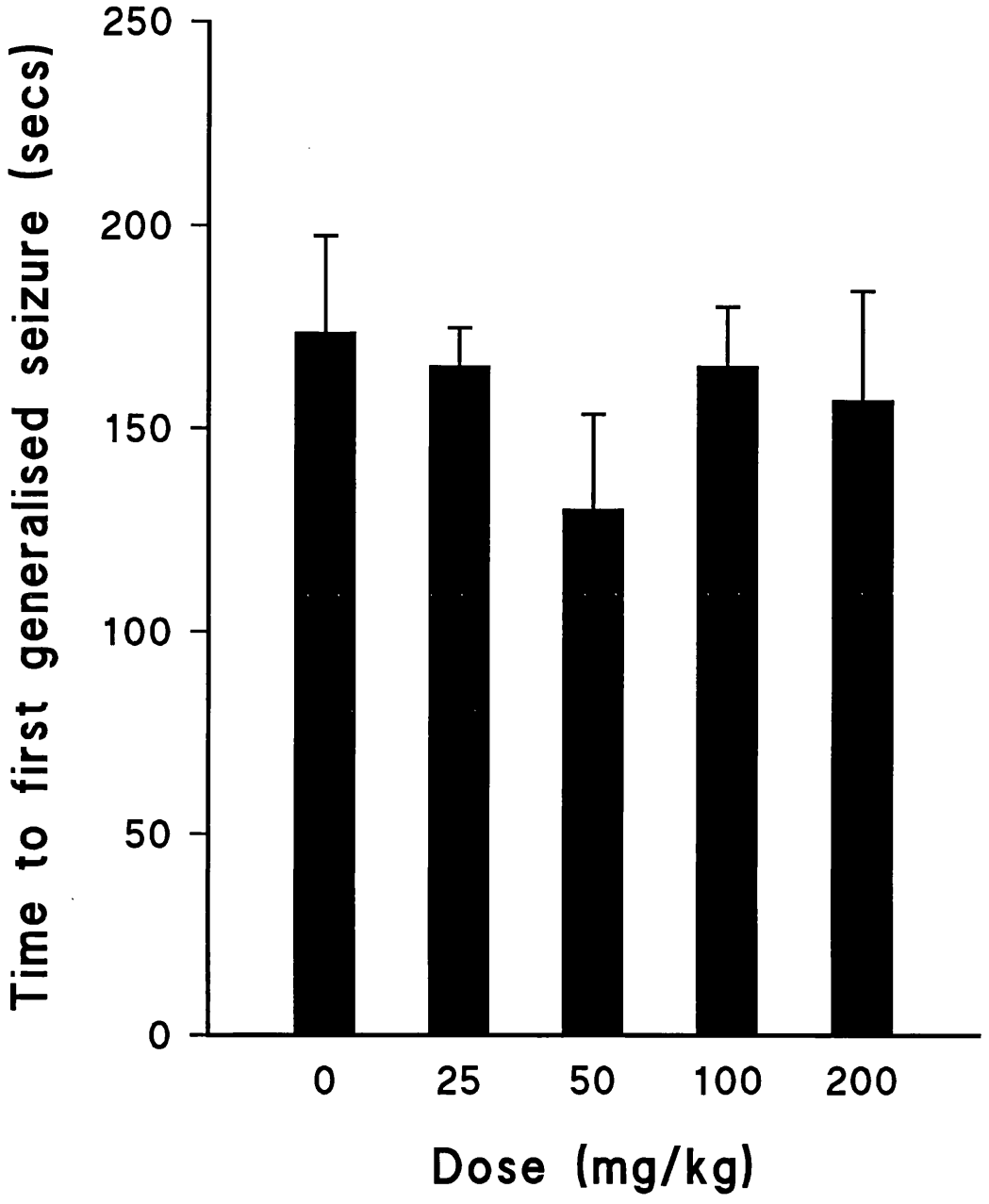

FIGURE 13:- Effect of repeated vigabatrin dosing (25 - 200 mg/kg; once daily for 7 days) on the latency to the first generalised seizure induced by 85 mg/kg PTZ at 24 hours after the final dose. Results are expressed as mean (± SEM) time (seconds) in groups of 10 mice.

RESULTS:- Repeated treatment with 50 mg/kg VGB significantly ($p < 0.01$) reduced the incidence of THE induced by MES at 24 hours after the final dose (figure 14). VGB was without significant effect, compared to control, at all other doses investigated.

4.3.5 Effect of single vigabatrin administration on the threshold for induction of tonic seizures in mice

PROTOCOL:- Mice were separated into two groups (n=30/group) and treated with either 50 mg/kg VGB (i.p.) or vehicle (0.9% saline) alone. At 24 hours post-administration each animal was subjected to the Min-ES test (section 2.3.3). The number of positive (tonic seizure) and negative (no tonic seizure) responses within each group was recorded.

RESULTS:- Compared to control, single dose VGB (50 mg/kg) significantly ($p < 0.05$) increased the tonic seizure threshold determined by Min-ES at 24 hours post-administration (figure 15). Median threshold values, determined by regression analysis of positive and negative responses, were 4.00 mA (control) and 5.00 mA (VGB-treated).

4.3.6 Dose and time dependent effects of repeated vigabatrin treatment on pentylenetetrazol-induced seizures in rats

PROTOCOL:- Rats were separated into 5 treatment groups (n=12/group) and VGB was administered (i.p.) in doses of 25, 50, 100, and 200 mg/kg. One group (control) received vehicle (0.9% saline) alone. Treatment was continued once daily for 7 days. At 2, 24, and 48 hours after the final dose 4 animals from each group were subjected to the s.c CD_{97} PTZ test (section 2.3.1) and the latency to first the generalised seizure in individual animals was recorded.

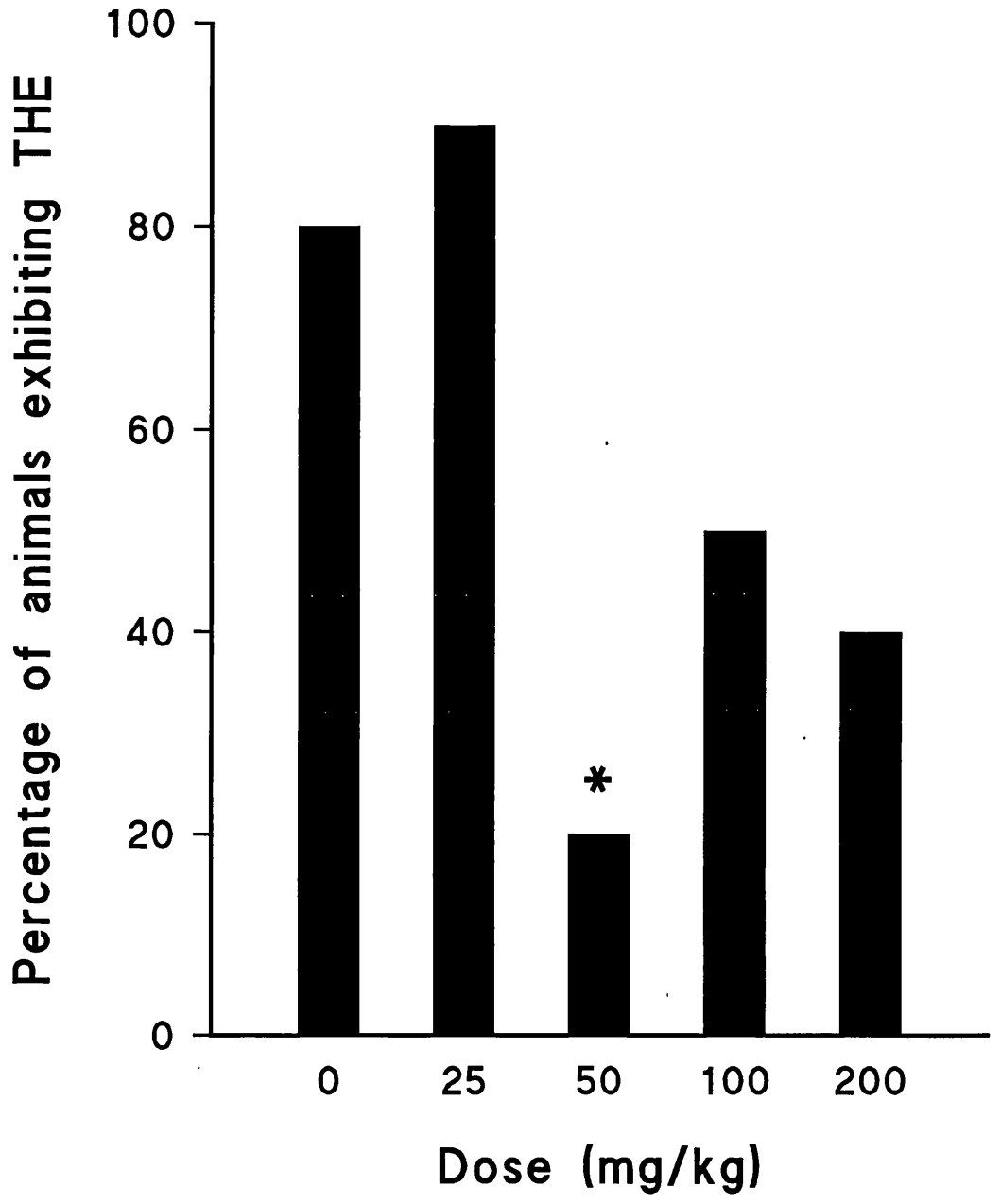

FIGURE 14:- Effect of repeated vigabatrin dosing (25 - 200 mg/kg; once daily for 7 days) on the incidence of tonic hind-limb extension (THE) induced by MES at 24 hours after the final dose. Results are expressed as the percentage of animals (mice) in groups of 10 exhibiting THE. Statistical significance (*$p < 0.01$) was determined by the Chi square test.

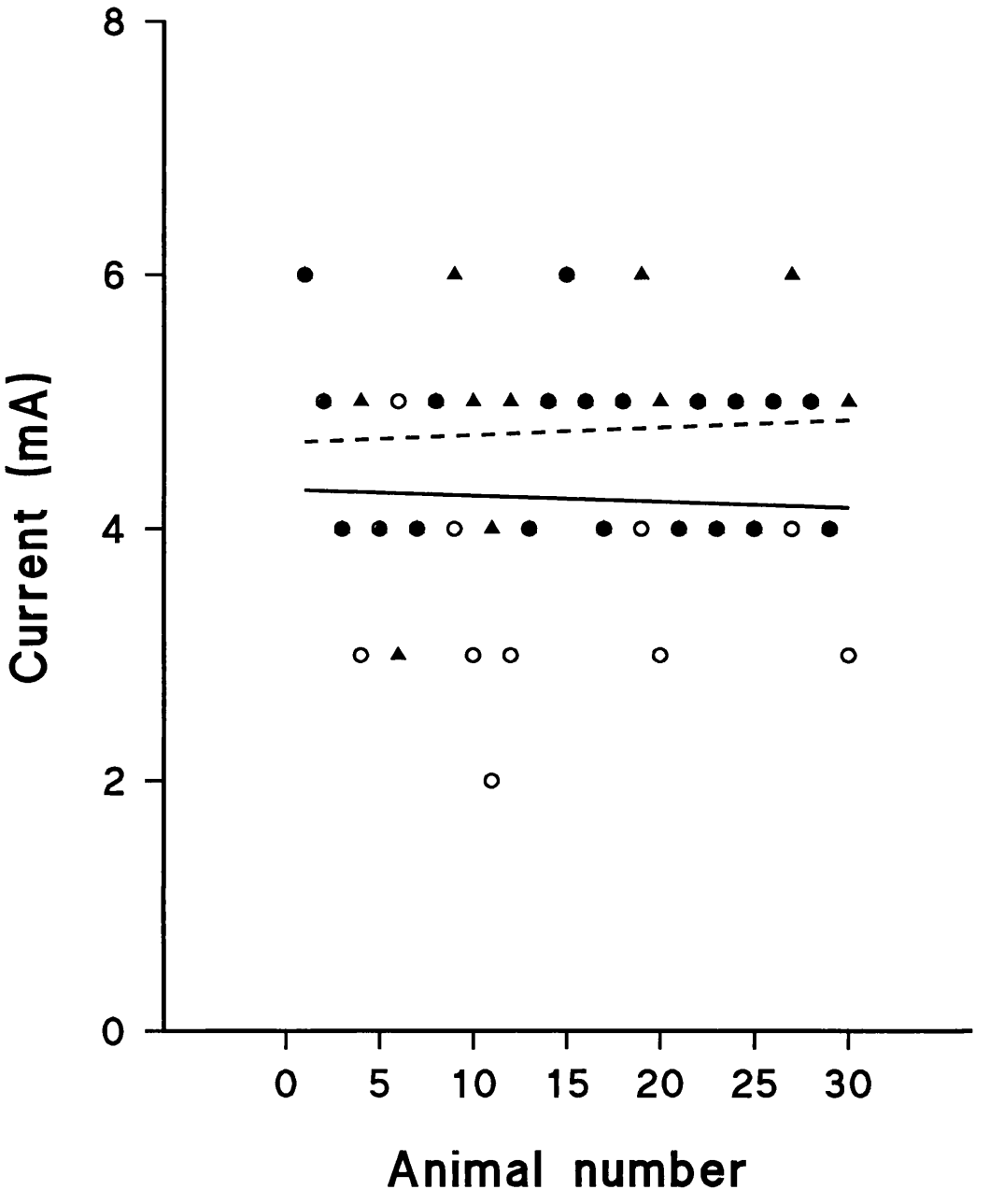

FIGURE 15:- Effect of vigabatrin treatment (50 mg/kg; closed triangles, broken line) vs control (open circles, solid line) on the tonic seizure threshold determined by Min-ES at 24 hours post-dosing. Closed circles represent data points at which animals from opposing groups (control and vigabatrin-treated) coincide. Seizure thresholds were calculated by regression analysis of the results obtained from groups of 30 mice and statistical significance ($p < 0.05$) was determined by the Mann Whitney U-test.

RESULTS:-Repeated treatment with VGB (25 - 200 mg/kg) appeared to elicit a dose dependent increase in the time to the first generalised seizure induced by PTZ at 24 and 48 hours after the final dose (figure 16). This apparent trend was, however, disregarded as the numbers of animals employed were too small for statistical significance to be inferred.

4.3.7 Dose dependent effects of single tiagabine administration on pentylenetetrazol-induced seizures in mice

PROTOCOL:- Mice were separated into 6 treatment groups (n=12/group) and TGB was administered (i.p.) in doses of 0.1, 0.5, 1.0, 2.0, and 5.0 mg/kg. One group (control) received vehicle (0.9% saline) alone. At 6 hours post-administration each animal was subjected to the s.c CD_{97} PTZ test (section 2.3.1) and the latency to the first generalised seizure in individual animals was recorded.

RESULTS:- Single doses of TGB (2 and 5 mg/kg) significantly (2 mg/kg, $p < 0.005$; 5 mg/kg, $p < 0.05$) lengthened the time to the first generalised seizure induced by PTZ at 6 hours post-dosing (figure 17). TGB was without significant effect, compared to control, at all other doses investigated.

4.3.8 Dose dependent effects of single tiagabine administration on maximal electroshock-induced seizures in mice

PROTOCOL:- Mice were separated into 6 treatment groups (n=20/group) and TGB was administered (i.p.) in doses of 0.1, 0.5, 1.0, 2.0, and 5.0 mg/kg. One group (control) received vehicle (0.9% saline) alone. At 6 hours post-administration each animal was subjected to the MES test (section 2.3.2) using a stimulus current of 54 mA. The incidence of THE within each group was recorded.

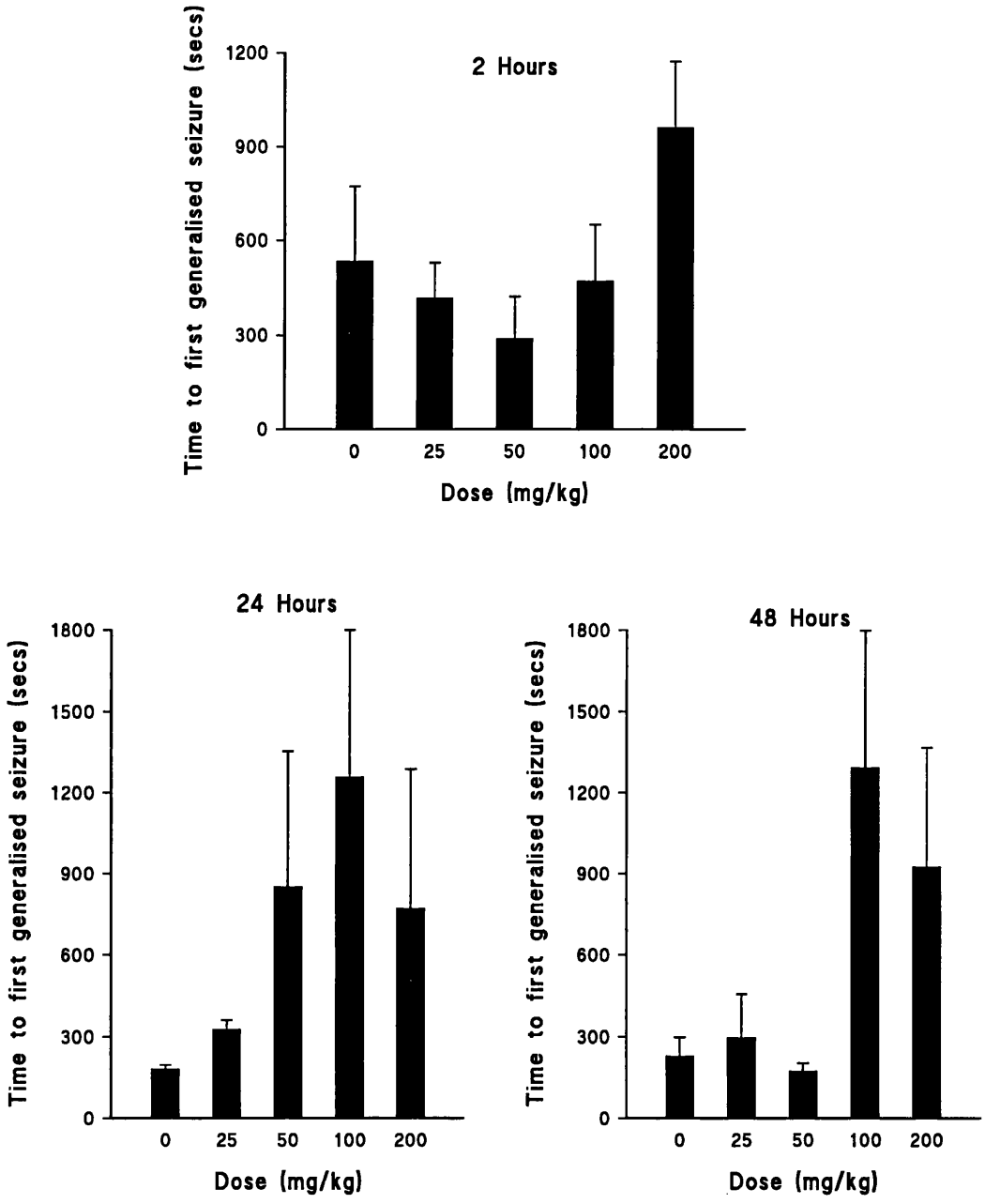

FIGURE 16:- Effect of repeated vigabatrin dosing (25 - 200 mg/kg; once daily for 7 days) on the latency to the first generalised seizure induced by 85 mg/kg PTZ at 2 (upper), 24 (lower left), and 48 (lower right) hours after the final dose. Results are expressed as mean (± SEM) time (seconds) in groups of 4 rats.

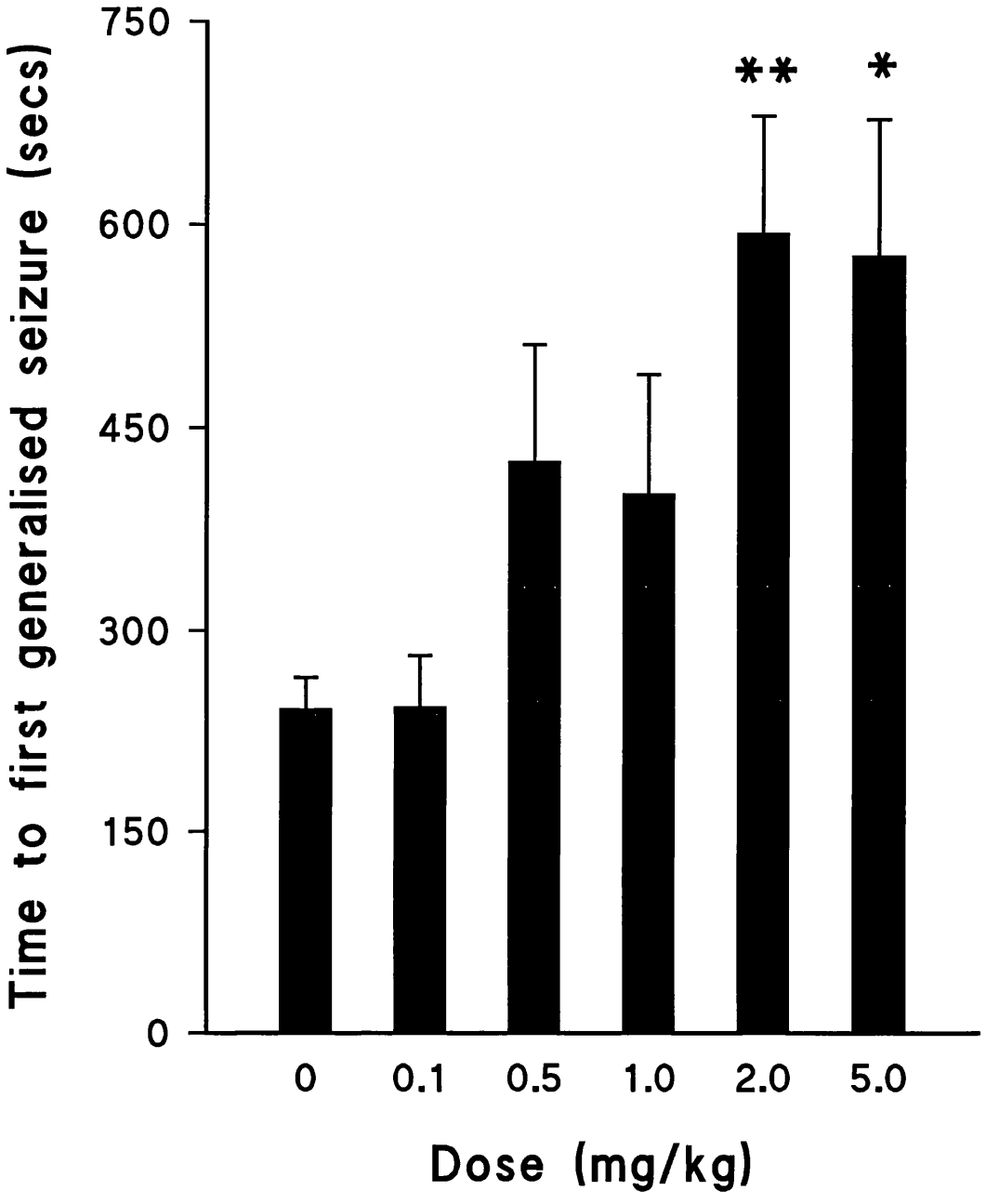

FIGURE 17:- Effect of single tiagabine doses (0.1 - 5.0 mg/kg) on the latency to the first generalised seizure induced by 85 mg/kg PTZ at 6 hours post-administration. Results are expressed as mean (± SEM) time (seconds) in groups of 12 mice. Statistical significance (*$p < 0.05$; **$p < 0.005$) was determined by the Mann Whitney U-test.

RESULTS:- Single doses of TGB (2 and 5 mg/kg) significantly ($p < 0.05$) reduced the incidence of THE induced by MES at 6 hours post-dosing (figure 18). TGB was without significant effect, compared to control, at all other doses investigated.

4.3.9 Time dependent effects of single tiagabine administration on pentylenetetrazol-induced seizures in mice

PROTOCOL:- Mice were separated into two treatment groups (n=48/group) and were treated with either 2 mg/kg TGB (i.p.) or vehicle (0.9% saline) alone. Six animals from each group were sequentially subjected to the s.c. CD_{97} PTZ test at 1, 2, 4, 8, 12, 18, 24, and 36 hours post-administration. The latency to the first generalised seizure in individual animals was recorded.

RESULTS:- A single dose of TGB (2 mg/kg) significantly (2 and 8 hours, $p < 0.05$; 4 hours, $p < 0.01$) lengthened the time to the first generalised seizure induced by PTZ at 2, 4, and 8 hours post-administration (figure 19). TGB was without significant effect, compared to control, at all other time points investigated.

4.3.10 Time dependent effects of single tiagabine administration on maximal electroshock-induced seizures in mice

PROTOCOL:- Mice were separated into two treatment groups (n=80/group) and were treated with either 2 mg/kg TGB (i.p.) or vehicle (0.9% saline) alone. Ten animals from each group were sequentially subjected to the MES test at 1, 2, 4, 8, 12, 18, 24, and 36 hours post-administration using a stimulus current of 54 mA. The incidence of THE within each group was recorded.

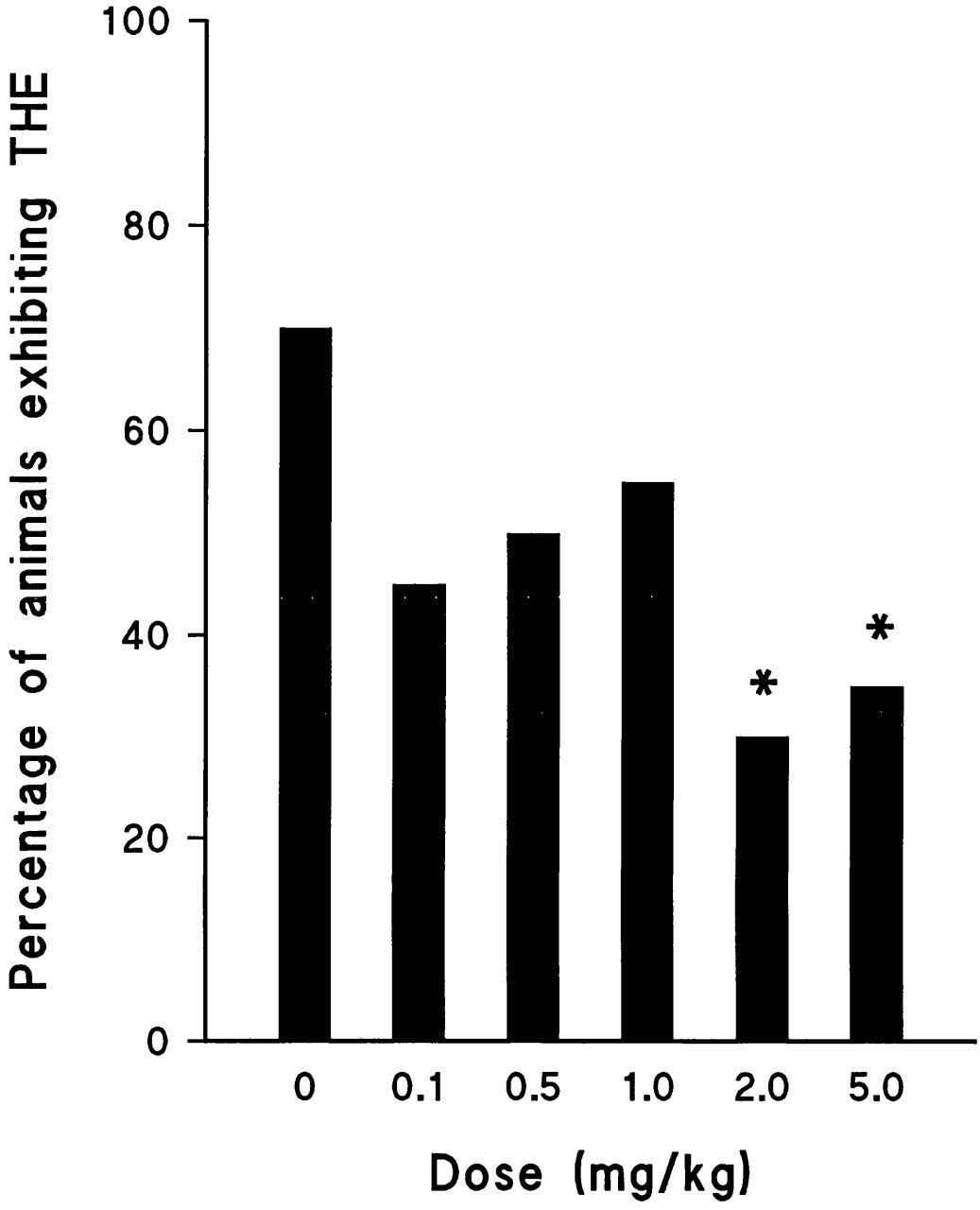

FIGURE 18:- Effect of single tiagabine doses (0.1 - 5.0 mg/kg) on the incidence of tonic hind-limb extension (THE) induced by MES at 6 hours post-administration. Results are expressed as the percentage of animals (mice) in groups of 20 exhibiting THE. Statistical significance (*$p < 0.05$) was determined by the Chi square test.

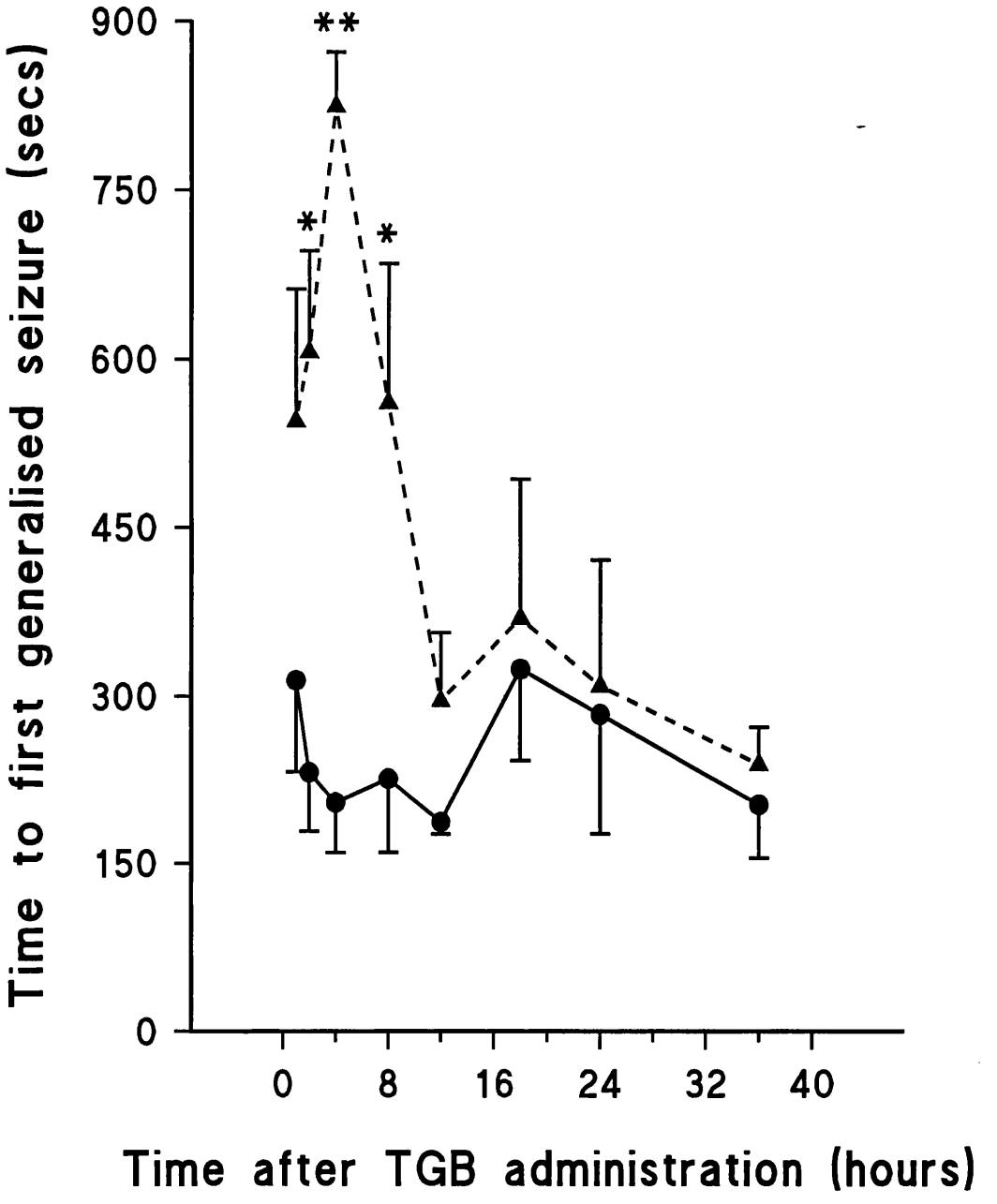

FIGURE 19:- Effect of tiagabine (TGB) treatment (2 mg/kg; triangles, broken line) vs control (circles, solid line) on the latency to the first generalised seizure induced by 85 mg/kg PTZ at 1 - 36 hours post-administration. Results are expressed as mean (± SEM) time (seconds) in groups of 6 mice. Statistical significance (*$p < 0.05$; **$p < 0.01$) was determined by the Mann Whitney U-test.

RESULTS:- A single dose of TGB (2 mg/kg) significantly ($p < 0.005$) reduced the incidence of THE induced by MES at 1, 2, and 4 hours post-administration (figure 20). TGB was without significant effect, compared to control, at all other time points investigated.

4.3.11 Dose and time dependent effects of single vigabatrin administration on brain GABA-aminotransferase activity in mice

PROTOCOL:- Mice were separated into 5 treatment groups (n=20/group) and VGB was administered (i.p.) in doses of 25, 50, 100, and 200 mg/kg. One group (control) received vehicle (0.9% saline) alone. At 24 and 48 hours post-administration 10 animals from each group were sacrificed by cervical dislocation and their brains removed by the procedure described in section 2.2.3. Samples were stored in accordance with section 2.2.5 prior to analysis of GABA-T activity (section 2.4).

RESULTS:- Single doses of VGB (50, 100, and 200 mg/kg) produced a significant ($p < 0.05$), dose-related decrease in whole brain GABA-T activity, compared to control, at 24 and 48 hours post-administration (figure 21). A single dose of 25 mg/kg VGB was without significant effect at either time point. Administration of 200 mg/kg VGB resulted in a significantly ($p < 0.05$) greater inhibition of GABA-T activity at 24 hours than at 48 hours. Differences in enzyme inhibition, in relation to incubation time, were not significantly different at any other dose investigated.

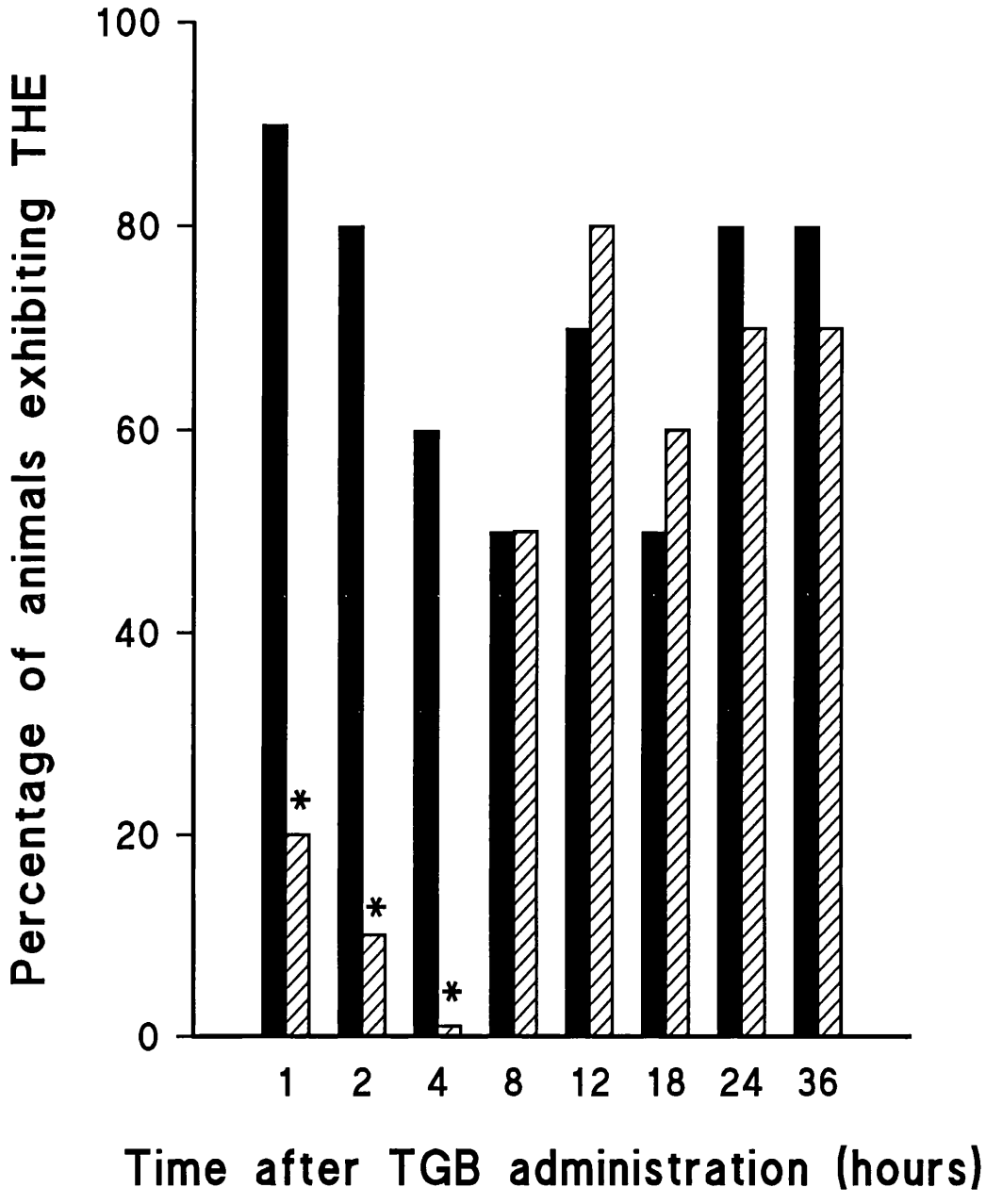

FIGURE 20:- Effect of tiagabine (TGB) treatment (2 mg/kg; hatched bars) vs control (solid bars) on the incidence of tonic hind-limb extension (THE) induced by MES at 1 - 36 hours post-administration. Results are expressed as the percentage of animals (mice) in groups of 10 exhibiting THE. Statistical significance (*$p < 0.005$) was determined by the Chi square test.

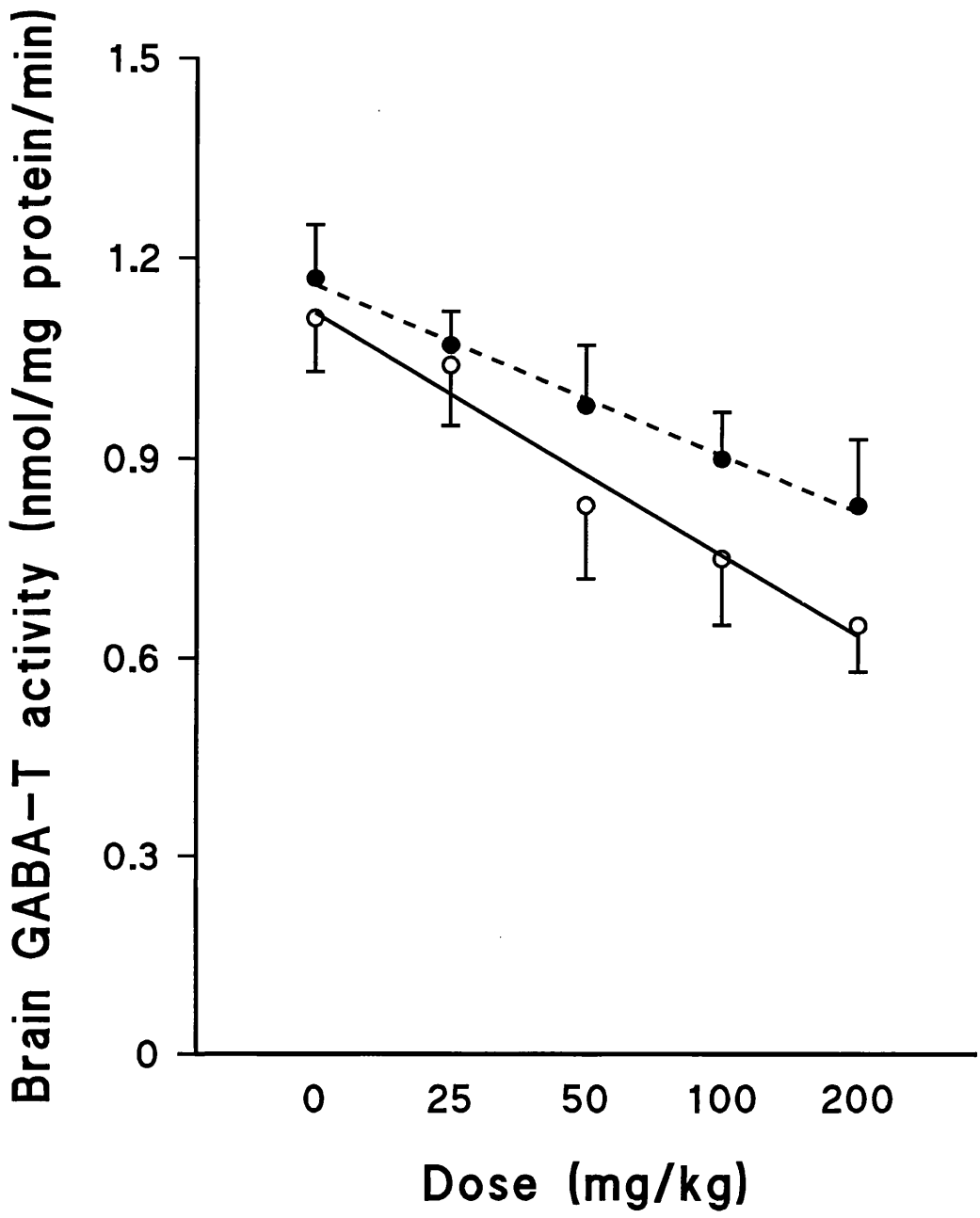

FIGURE 21:- Effect of single vigabatrin doses (25 - 200 mg/kg) on brain GABA-aminotransferase (GABA-T) activity at 24 (open circles, solid line) and 48 (closed circles, broken line) hours post-administration. Results are expressed as mean (± SEM) activity in groups of 10 mice.

4.3.12 Dose and time dependent effects of single vigabatrin administration on brain GABA-aminotransferase activity in rats

PROTOCOL:- Rats were separated into 7 treatment groups (n=12/group) and VGB was administered (i.p.) in doses of 100, 200, 300, 400, 800, and 1600 mg/kg. One group (control) received vehicle (0.9% saline) alone. At 2, 24, and 48 hours post-administration 4 animals from each group were sacrificed by cervical dislocation and their brains removed by the procedure described in section 2.2.3. Samples were stored in accordance with section 2.2.5 prior to analysis of GABA-T activity (section 2.4).

RESULTS:- Single dose VGB treatment (300 - 1600 mg/kg) produced a significant ($p < 0.05$), dose-related decrease in the activity of whole brain GABA-T activity, compared to control, at 2 and 24 hours post-administration (figure 22). At 48 hours, only 1600mg/kg VGB significantly ($p < 0.05$) inhibited the enzyme. The inhibition of GABA-T observed following treatment with 100 and 200 mg/kg VGB did not reach significance at any time point.

4.3.13 Comparison of single and repeated vigabatrin administration on brain GABA-aminotransferase activity in mice

PROTOCOL:- Mice were separated into 10 treatment groups (n=10/group) and VGB was administered (i.p.) to the first 5 groups in single doses of 25, 50, 100, and 200 mg/kg. One group (control) received vehicle (0.9% saline) alone. The remaining 5 groups received identical doses of VGB (i.p.), once daily for 7 days. At 24 hours post-administration, or 24 hours after the final dose in the repeatedly treated group, the animals were sacrificed by cervical dislocation and their brains removed by the procedure described in section 2.2.3.

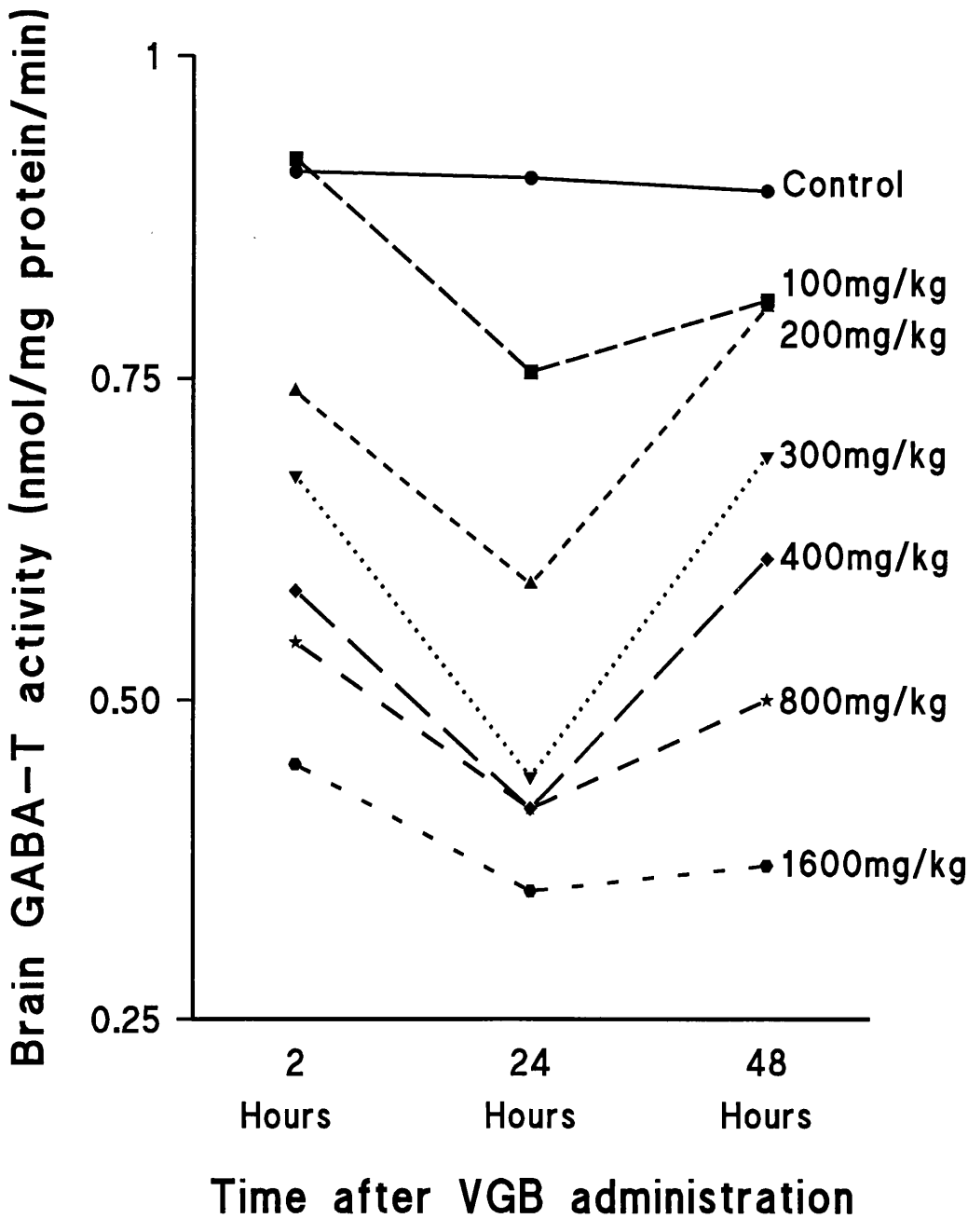

FIGURE 22:- Effect of single vigabatrin (VGB) doses (100 - 1600 mg/kg) on brain GABA-aminotransferase (GABA-T) activity at 2, 24, and 48 hours post-administration. Results are expressed as mean activity in groups of 4 rats. Error bars have been omitted for ease of comprehension.

Samples were stored in accordance with section 2.2.5 prior to analysis of GABA-T activity (section 2.4).

RESULTS:- Single dose and repeated VGB treatment (25 - 200 mg/kg) significantly (single, $p < 0.05$; repeated, $p < 0.005$) reduced brain GABA-T activity, compared to control, at 24 hours post-dosing at all of the doses investigated (figure 23). Enzyme inhibition appeared independent of dose following single administration, while repeated VGB treatment reduced GABA-T activity in a dose-related manner. GABA-T activity was significantly ($p < 0.005$) lower in repeatedly treated animals than in singly dosed animals at all dose levels.

4.3.14 Time dependent effect of vigabatrin on GABA-aminotransferase activity in cerebral cortical astrocytes in primary culture

PROTOCOL:- Cultures of cerebral cortical astrocytes, isolated and maintained as described in section 2.10, were separated into 4 groups (n=8/group) and exposed to 100 μM VGB in BSS for 30, 60, and 120 minutes. One group (control) was exposed to blank BSS. Cultures were then washed in BSS and scraped in EDTA buffer (section 2.4.1) prior to storage. Samples were stored in accordance with section 2.2.5 and assayed for GABA-T activity by the method described in section 2.4.

RESULTS:- A single dose of VGB (100 μM) significantly ($p < 0.01$) reduced GABA-T activity in primary cultures of cerebral cortical astrocytes following exposure times of 60 and 120 minutes (figure 24). VGB was without significant effect, compared to control, following a 30 minute exposure.

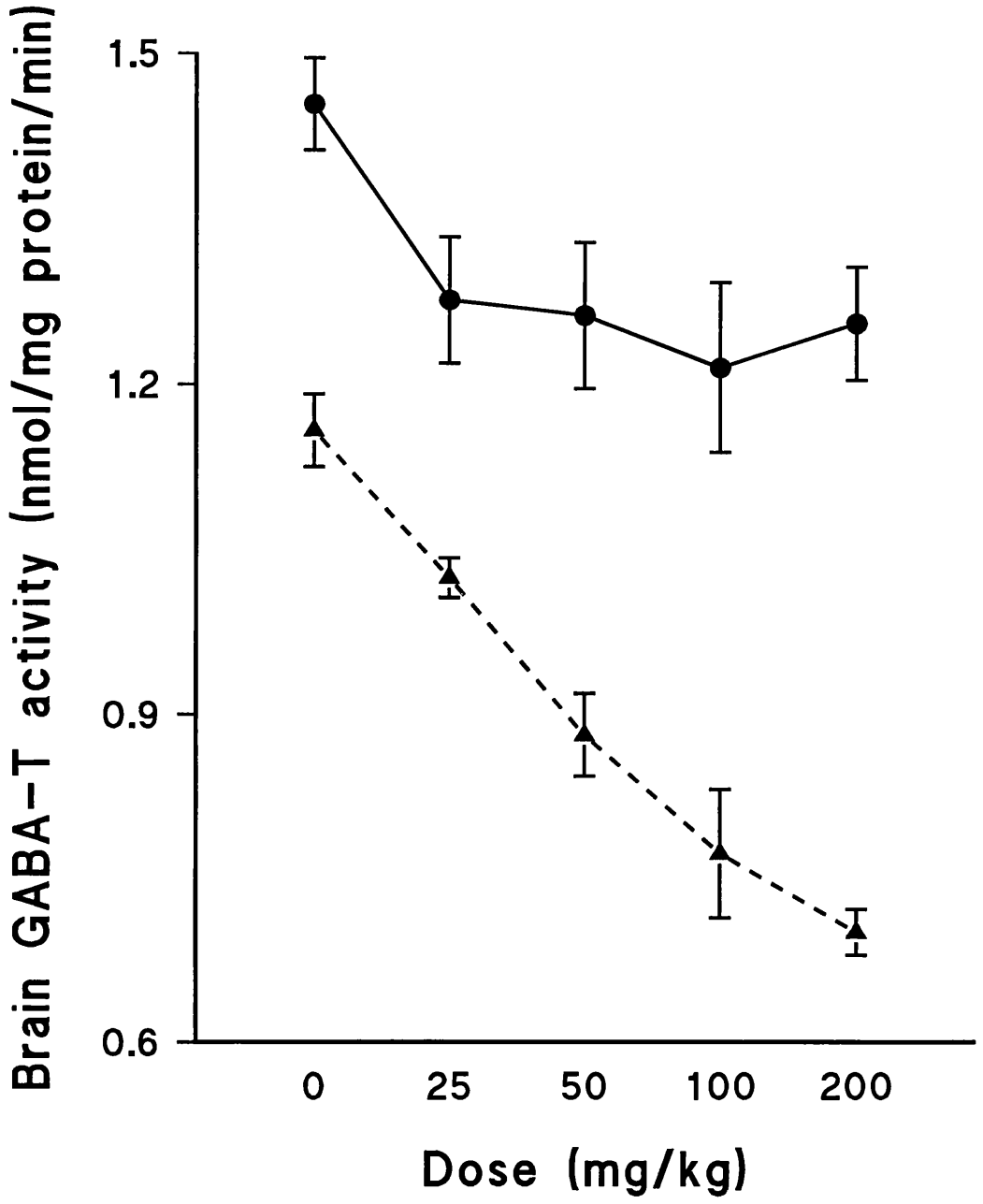

FIGURE 23:- Effect of single (circles, solid line) vs repeated (once daily for 7 days; triangles, broken line) vigabatrin treatment (25 - 200 mg/kg) on brain GABA-aminotransferase (GABA-T) activity at 24 hours post-dosing. Results are expressed as mean (± SEM) activity in groups of 10 mice.

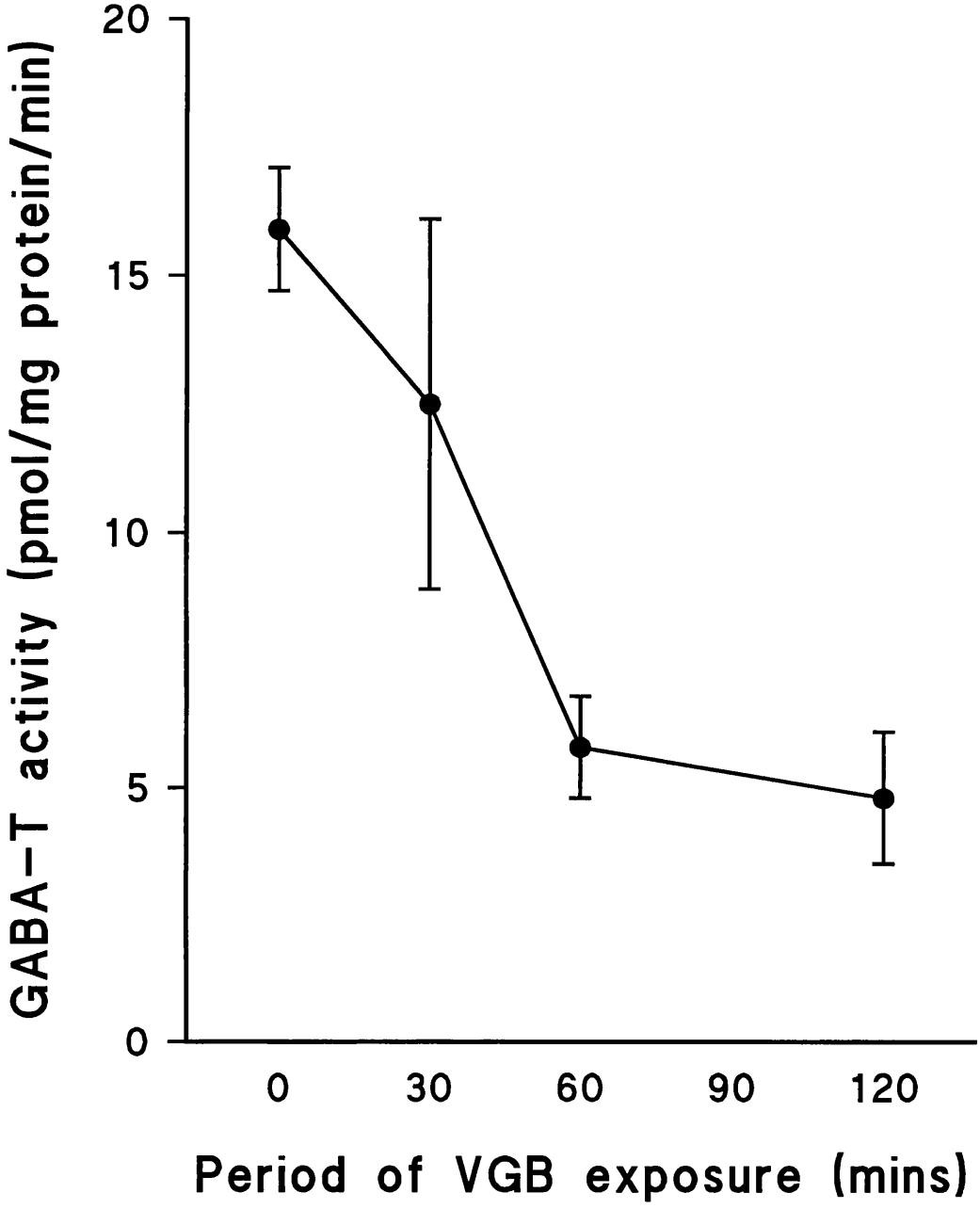

FIGURE 24:- Time dependent effects of single dose vigabatrin (VGB; 100 μM) on the activity of cerebral cortical astrocyte GABA-aminotransferase (GABA-T). Results are expressed as the mean (± SEM) activity in groups of 8 plates.

4.3.15 Dose and time dependent effects of single vigabatrin administration on brain GABA concentrations in mice

PROTOCOL:- Mice were separated into 5 treatment groups (n=12/group) and VGB was administered (i.p.) in doses of 25, 50, 100, and 200 mg/kg. One group (control) received vehicle (0.9% saline) alone. At 24 and 48 hours post-administration 6 animals from each group were sacrificed by cervical dislocation and their brains removed by the procedure described in section 2.2.3. Samples were stored in accordance with section 2.2.5 prior to analysis of whole brain GABA concentration (section 2.6).

RESULTS:- Single doses of VGB (25 - 200 mg/kg) were without significant effect on whole brain GABA concentrations, compared to control, at 24 and 48 hours post-administration (figure 25).

4.3.16 Effect of repeated vigabatrin treatment on brain GABA-aminotransferase and glutamic acid decarboxylase activities and GABA concentrations in rats

PROTOCOL:- Rats were separated into two groups (n=6/group) and treated with either 100 mg/kg VGB (i.p.) or vehicle (0.9% saline) alone once daily for 7 days. At 4 hours after the final dose each animal was sacrificed by cervical dislocation and their brains removed by the procedure described in section 2.2.3. Samples were stored in accordance with section 2.2.5 prior to homogenisation and concomitant analysis of whole brain GABA-T (section 2.4) and GAD activities (section 2.5) and GABA concentration (section 2.6).

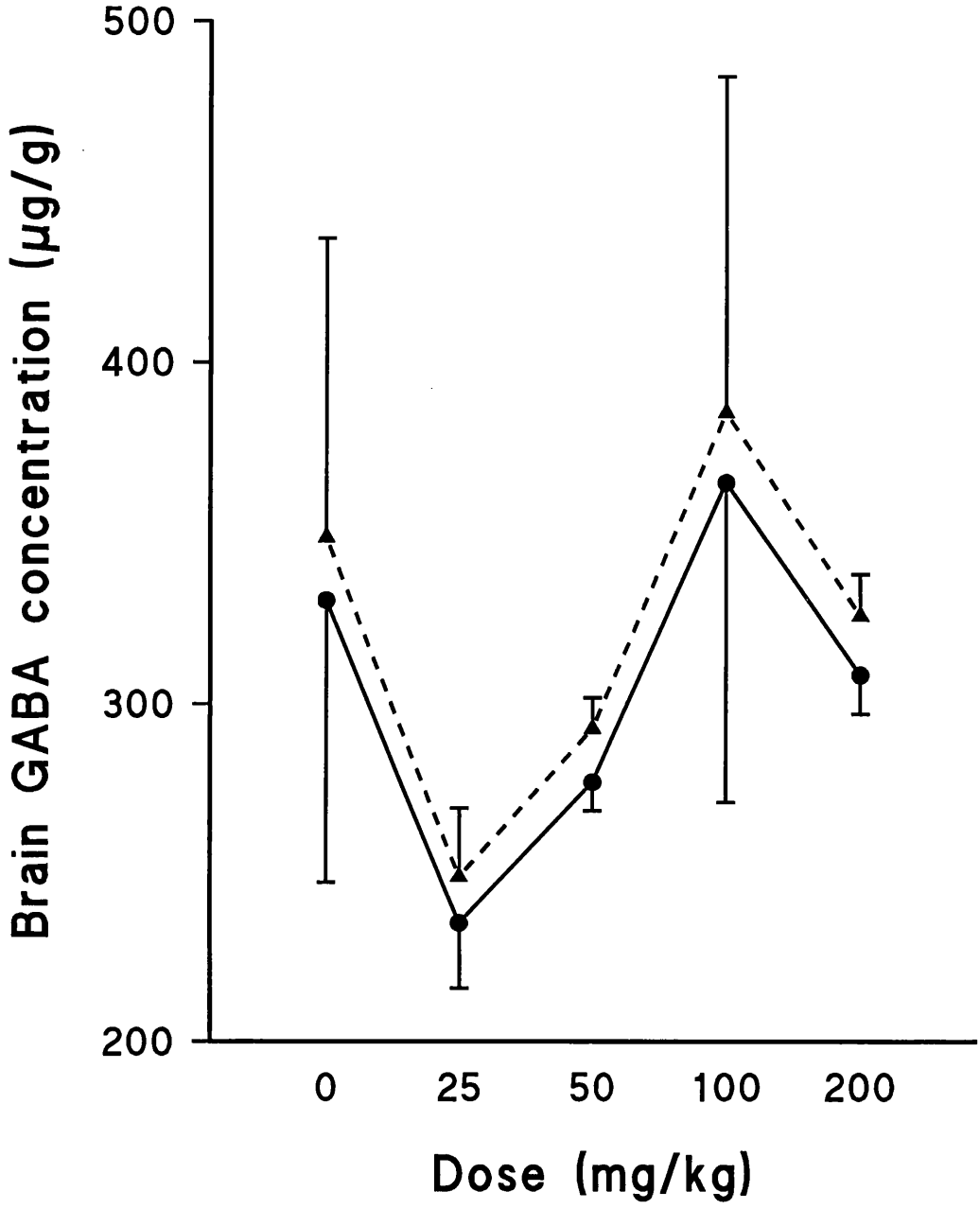

FIGURE 25:- Effect of single vigabatrin doses (25 - 200 mg/kg) on brain GABA concentration at 24 (circles, solid line) and 48 (triangles, broken line) hours post-administration. Results are expressed as mean (± SEM) concentration in groups of 6 mice.

RESULTS:- Repeated VGB treatment (100 mg/kg) significantly reduced mean brain GABA-T activity by 41% ($p < 0.005$) and GAD activity by 40 % ($p < 0.005$), compared to control, but was without effect on whole brain GABA concentration at 4 hours after the final dose (figure 26).

4.3.17 Effects of vigabatrin and tiagabine on GABA uptake into cerebral cortical astrocytes in primary culture

PROTOCOL:- Cultures of cerebral cortical astrocytes, isolated and maintained as described in section 2.10, were separated into 4 groups (n=12/group) and exposed to 200 nM TGB, 100 μM VGB, and a combination of both drugs in BSS. One untreated group (BSS alone) was employed as a control. After a 1 hour incubation cultures were assayed for GABA uptake by the procedure described in section 2.12.

RESULTS:- Single doses of TGB (200 nM) and VGB (100 μM), and a combination (COMB) of both drugs, significantly (TGB, $p < 0.001$; VGB, $p < 0.005$; COMB, $p < 0.05$) reduced GABA uptake, compared to control, into primary cultures of cerebral cortical astrocytes following a one hour exposure (figure 27). Combination treatment resulted in an inhibition of GABA uptake that was significantly ($p < 0.05$) less than that observed with either drug alone.

4.3.18 Effects of vigabatrin and tiagabine on GABA uptake into cerebral cortical neurones in primary culture

PROTOCOL:- Cultures of cerebral cortical neurones, isolated and maintained as described in section 2.11, were separated into 4 groups (n=8/group) and exposed to 200 nM TGB, 100 μM VGB, and a combination of both drugs in BSS. One untreated group (BSS alone)

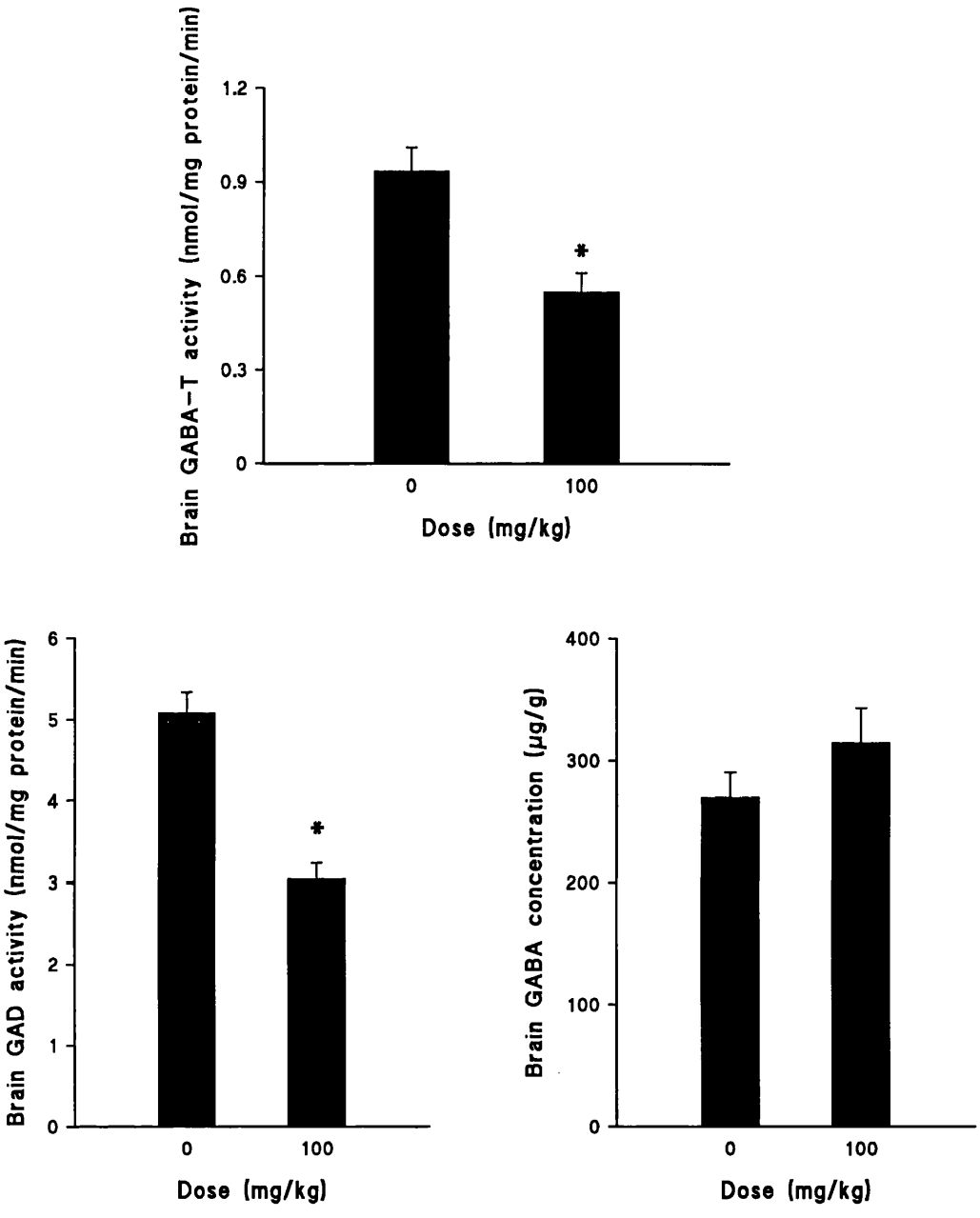

FIGURE 26:- Effect of repeated vigabatrin dosing (100 mg/kg; once daily for 7 days) on brain GABA-aminotransferase (GABA-T) activity (upper), glutamic acid decarboxylase (GAD) activity (lower left), and GABA concentration (lower right) at 4 hours after the final dose. Results are expressed as means (± SEM) in groups of 6 rats. Statistical significance (*$p < 0.005$) was determined by the Mann Whitney U-test.

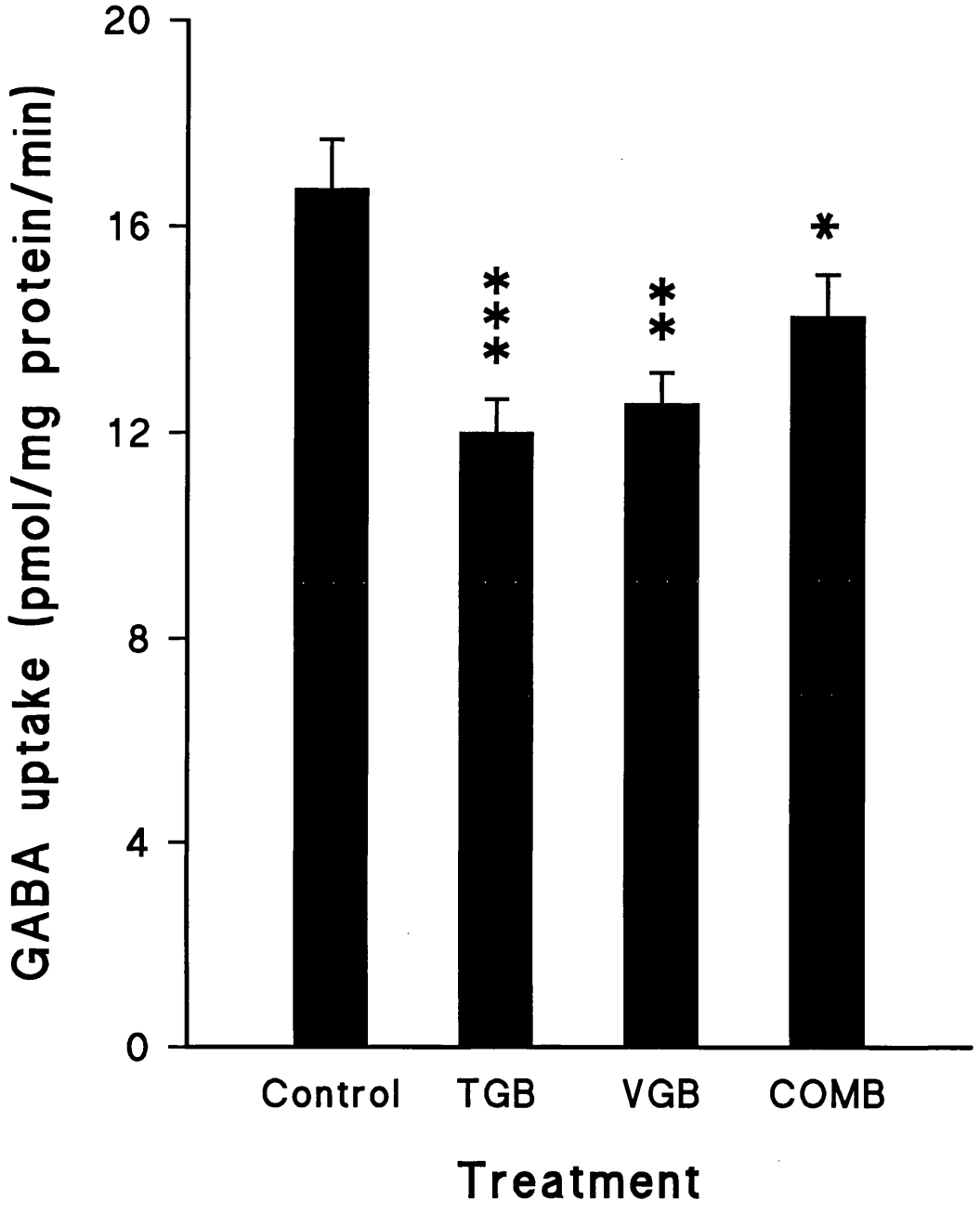

FIGURE 27:- Effect of 1 hour exposures to tiagabine (TGB; 200 nM), vigabatrin (VGB; 100 μM), and a combination (COMB) of both drugs on GABA uptake into cerebral cortical astrocytes in primary culture. Results are expressed as the mean (± SEM) uptake in groups of 12 plates. Statistical significance (*$p < 0.05$; **$p < 0.005$; ***$p < 0.001$) was determined by the Mann Whitney U-test.

was employed as a control. After a 1 hour incubation cultures were assayed for GABA uptake by the procedure described in section 2.12.

RESULTS:- Single doses of TGB (200 nM) and VGB (100 μM), and a combination (COMB) of both drugs, significantly ($p < 0.05$) reduced GABA uptake, compared to control, into primary cultures of cerebral cortical neurones following a one hour exposure (figure 28). The effects of combination treatment did not differ significantly from those observed with either drug alone.

4.4 DISCUSSION

VGB and TGB are novel antiepileptic agents, proposed to exert their pharmacological effects by different mechanisms. The aims of these studies were to explore the experimental anticonvulsant profiles of VGB and TGB in the available animal seizure models and to investigate their mechanisms of action using *in vitro* techniques.

Effects of VGB on PTZ-induced seizures

Single doses of VGB at 24 and 48 hours post-administration (figure 11) and repeated drug treatment at 24 hours after the final dose (figure 13), were ineffectual in lengthening the time to first generalised seizure induced by PTZ in mice. The drug was similarly without effect at 2, 24, and 48 hours post-dosing in rats (figure 16). Although these results appear conclusive and confirm the findings of Myslobodsky and colleagues (1979), they are not entirely consistent with previously published data. Bernasconi and co-workers (1988) reported single dose VGB to be effective against PTZ-induced seizures in the mouse for up to 24 hours post-administration. The negative findings of the present studies could,

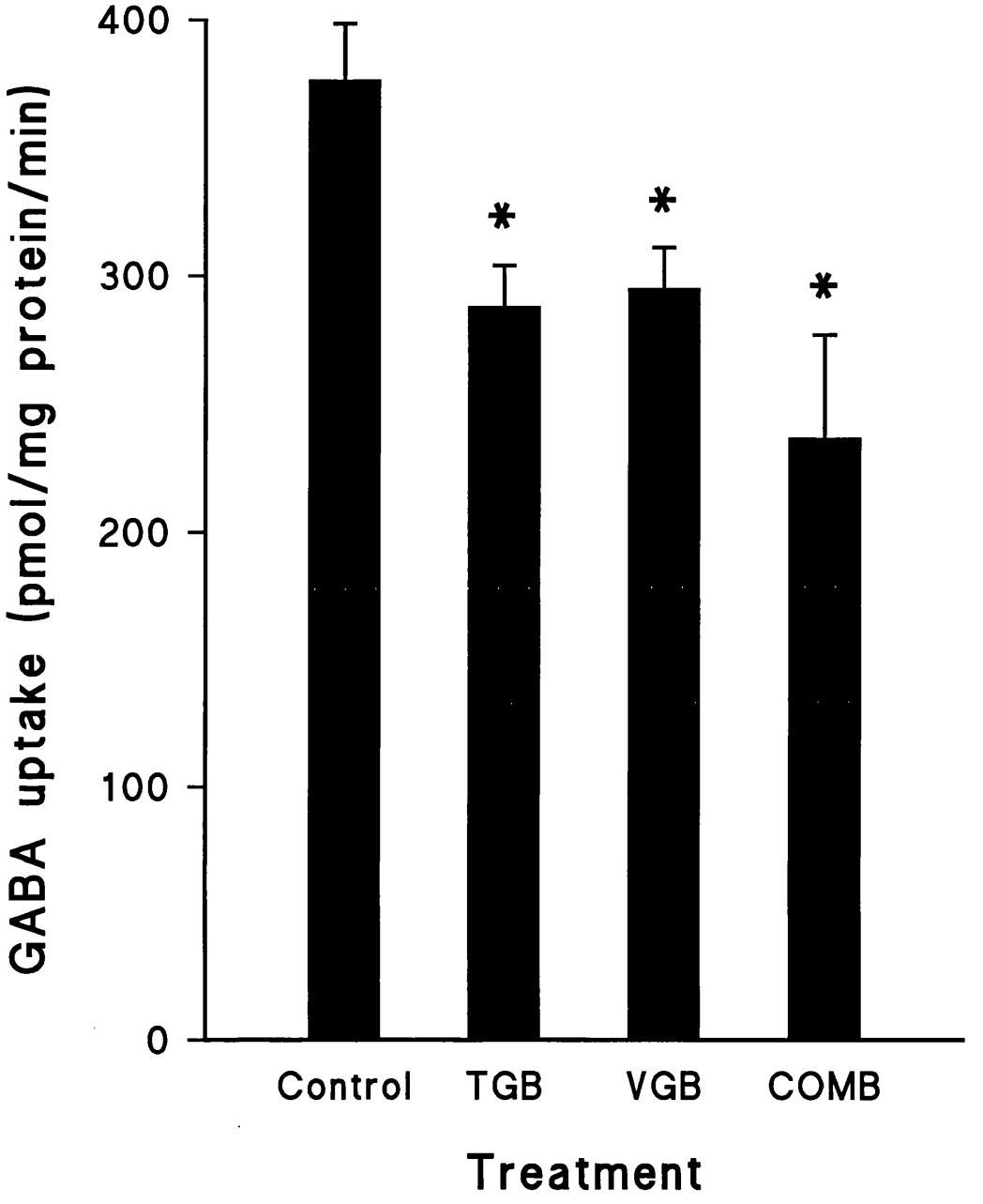

FIGURE 28:- Effect of 1 hour exposures to tiagabine (TGB; 200 nM), vigabatrin (VGB; 100 μM), and a combination (COMB) of both drugs on GABA uptake into cerebral cortical neurones in primary culture. Results are expressed as the mean (± SEM) uptake in groups of 8 plates. Statistical significance (*$p < 0.05$) was determined by use of the Mann Whitney U-test.

thus, be explained in terms of a time-dependency to the anticonvulsant effects of VGB, where the drug is active against PTZ-induced generalised seizures for up to 24 hours only. In this respect, the only comparable study reported here was performed in rats at 2 hours post-administration (figure 16) and did not employ sufficient numbers of animals to allow definitive statistical evaluation.

Effects of VGB on MES-induced seizures

In studies of MES-induced seizures, only repeatedly dosed 50 mg/kg VGB reduced the occurrence of THE (figure 14). All other doses of the drug were without effect in this experimental paradigm. Thus, VGB was construed as ineffectual against seizures induced by MES, a proposal consistent with previously published data. Although anticonvulsant actions of the drug against MES-induced seizures have been reported following direct micro-injection into discrete brain regions (Iadarola and Gale, 1982; Bonhaus and McNamara, 1988), no such effect has been observed following systemic VGB administration (Bernasconi et al, 1988).

Effects of VGB on Min-ES seizures

Single dose VGB elevated the threshold for induction of tonic seizures at 24 hours post-administration (figure 15). This is a previously unreported action of the drug, although it has demonstrated efficacy in the Min-ES test following repeated administration (Löscher, 1982). Such an effect might correlate with VGB's proposed ability to suppress generalised motor seizures in man (Leach and Brodie, in press) and justifies further evaluation of its dose- and time-related effects in sub- rather than supra-maximal seizure models. This observation, together with the lack of effect of VGB in the MES test, also

supports the proposal (Löscher et al, 1991a) that the Min-ES test should be employed in conjunction with the MES test in the evaluation of anticonvulsant drug efficacy against electroshock-induced seizures.

Effects of TGB on PTZ-induced seizures

Single doses of TGB (2 and 5 mg/kg) significantly lengthened the time to the first generalised seizure induced by PTZ at 6 hours post-administration (figure 17). A subsequent time-dependent study proposed TGB (2 mg/kg) to afford a similar protection against PTZ-induced generalised seizures at 2, 4, 6, and 8 hours post-dosing (figure 19). These observations were essentially consistent with previously published data in this respect. Nielsen and colleagues (1991) reported TGB to protect against PTZ-induced clonic seizures (ED_{50} = 1.3 mg/kg) at 30 minutes post-dosing. A possible explanation for the lack of effect of TGB at 1 hour post-administration (figure 19) was, however, not forthcoming.

Effects of TGB on MES-induced seizures

Single doses of TGB (2 and 5 mg/kg) significantly reduced the incidence of THE following MES stimulation at 6 hours post-dosing (figure 18). A subsequent time-dependent study proposed TGB (2mg/kg) to be effective against MES-induced seizures at 1, 2, 4, and 6 hours after single administration (figure 20). These findings were in contrast to those of Nielsen and colleagues (1991), who reported TGB to afford only partial protection against MES-induced seizures (ED_{50} = 40 mg/kg) at 30 minutes post-administration. Differences in electrical stimulus period and stimulus site, known to

influence the outcome of electroshock studies (Löscher et al, 1991a), might explain this apparent disparity in experimental results.

Effects of VGB on brain GABA-T activity

Initial investigation of the mechanism of action of VGB involved characterisation of its effects on GABA-T, the reported molecular target of the drug in the brain (Lippert et al, 1977). Single doses of VGB reduced mouse brain GABA-T activity in a dose-related manner at 24 and 48 hours post-administration (figure 21). At higher drug doses this effect was observed to be significantly greater at 24 hours than at 48 hours post-dosing. Single doses of VGB also reduced rat brain GABA-T activity in a dose- and time-dependent manner (figure 22). Again the drug appeared to be most effective at 24 hours post-dosing, with only higher drug doses reducing enzyme activity within 2 hours. At 48 hours after a single VGB administration some recovery of enzyme activity, dependent on dose, was noted. Repeated VGB treatment appeared to have a cumulative effect on mouse brain GABA-T activity, when compared to single drug administration (figure 23). Repeated treatment also resulted in a more pronounced dose-dependency of enzyme inhibition. Finally, single dose VGB reduced GABA-T activity, in primary cultures of cerebral cortical astrocytes, in a time-dependent manner (figure 24). Maximal enzyme inhibition was observed within 1 hour in this experimental paradigm. All of these observations were consistent with previously published data (Jung et al, 1977; Larsson et al, 1986).

Effects of VGB on brain GABA concentration

While it was concluded that VGB had an undoubted inhibitory effect on rodent brain GABA-T, subsequent investigations attempted to further characterise this previously

reported action of the drug in terms of its effects on brain GABA concentrations. Single doses of VGB had no effect on mouse brain GABA levels at 24 and 48 hours post-administration (figure 25). The drug was similarly without effect on rat brain GABA levels at 4 hours after the final dose of a repeated drug treatment regimen (figure 26). Although these results were in direct contrast to those reported by Schechter and colleagues (1977), the VGB doses employed here were significantly lower. With such low doses, it is possible that analysis of mouse brain GABA concentrations at 24 and 48 hours post-dosing was performed too late to allow detection of any significant change in neurotransmitter levels. The lack of effect of repeated VGB treatment on rat brain GABA levels at four hours after the final dose, however, could not be explained by this theory. It is more likely that the observed inhibition of GABA synthesis, together with the inhibition of GABA metabolism, might underlie the lack of net change in brain GABA concentration in this case.

Effects of VGB on brain GAD activity

The effect of repeated VGB treatment on brain GAD activity (figure 26), proposed to mask the GABA-elevating effects of the drug, is consistent with that reported in the literature (Jung et al, 1977; Neal and Shah, 1990). This *in vivo* effect, more pronounced following repeated administration (Neal and Shah, 1990), might be responsible for the tolerance to the anticonvulsant effects of VGB observed in animals (Rundfeldt and Löscher, 1992) and the ceiling to effective dosage with the drug reported for individual patients (McKee et al, 1993). The hypothesis that reduced GAD activity is a result of feedback inhibition by increased GABA levels (Löscher and Frey, 1987) appears inconsistent here, where GABA levels are unaltered. However, in the absence of a clear

understanding of the time-dependency of this putative negative feedback, the implications of these results remain inconclusive.

Effects of TGB on GABA uptake

The mechanism of action of TGB was not as extensively investigated and the results obtained should be classed as preliminary. The drug appeared to block GABA uptake into primary cultures of cerebral cortical astrocytes (figure 27) and neurones (figure 28), confirming previous reports in this respect (Pierce et al, 1991). In the absence of dose-response studies, correlations in the potency of these effects with existing data could not be determined. Although these preliminary studies support the putative mechanism of action of TGB, further investigations of its dose- and time-dependent effects, cell specificity, and possible intracellular actions are anticipated to characterise the drug mechanism more fully.

Effects of VGB on GABA uptake

Although the effects of VGB on brain GABA-T appear conclusive, several experimental and clinical phenomena have been reported which suggest that VGB may exert additional molecular effects and that these may contribute to the drug's pharmacological profile. The diverse range of experimental anticonvulsant profiles exhibited by a variety of neuroactive compounds, all of which are proposed to act as inhibitors of brain GABA-T (Schechter et al, 1979), might suggest the contribution of secondary mechanisms of action. Similarly, the above conclusions and those of other workers (Bernasconi et al, 1988), suggest that the anticonvulsant effects of VGB in animal seizure models are not strictly related to the time of maximal GABA-T inhibition. Another and perhaps the most pertinent observation is

one of rebound seizures immediately upon clinical withdrawal of the drug (Tassinari et al, 1987; Reynolds et al, 1988). This would not be consistent with a drug which irreversibly inhibits an enzyme in the brain. The discontinuation of a pure enzyme inhibitor would be unlikely to result in any withdrawal syndrome until the drug was largely excreted from the body and new enzyme protein had been synthesised.

Several hypotheses to explain these phenomena exist. Regional drug distribution in the brain may underlie the diverse anticonvulsant profiles of a range of GABA-T inhibitors and possibly explain the relationship between enzyme inhibition and anticonvulsant efficacy. In addition, a sharp rise in CSF levels of the EAAs, glutamate and aspartate, has been observed in rats 96 hours after a single VGB dose, when only trace amounts of the drug itself were detectable (Halonen et al, 1990). These authors speculated that this observation might manifest itself clinically as a withdrawal hyperexcitability, possibly in the form of rebound seizures.

The experimental studies reported here have revealed preliminary data on the existence of an additional, hitherto unreported, mechanism of VGB action which could be employed to explain all of the intriguing phenomena described above. VGB in a dose of 100 μM, the approximate IC_{50} for inhibition of GABA-T in cultured cortical astrocytes (Larsson et al, 1986), significantly blocked GABA uptake into primary cultures of cerebral cortical astrocytes (figure 27) and neurones (figure 28). These observations, perhaps unsurprising when one considers the striking structural similarity between VGB and GABA, may be the result of a simple competitive reaction between the two molecules at the uptake carrier site. The interaction of VGB with TGB on GABA uptake into cortical astrocytes (figure

27), resulting in a lesser degree of blockade than with either drug given alone, would suggest similar sites of action for the two drugs and further support the postulated blockade of carrier mediated GABA transport as an additional mechanism of VGB action.

Although previous studies have suggested that VGB is not a substrate for the GABA transporter (Schousboe et al, 1986), the drug is believed to enter cells via some high affinity uptake system (Grant and Heel, 1991). The hypothesis that VGB may block GABA uptake simply by increasing intracellular GABA concentrations and thus reducing the involvement of any concentration gradient would not be consistent with an energy-dependent (i.e. concentration-independent) uptake system.

Further evaluation of this novel effect of VGB is required. Investigations of its reproducibility, dose- and time-dependency, and cell specificity, together with evaluation of the relationship between this and the intracellular effects of VGB are anticipated. These should conclude the characterisation of GABA uptake blockade and determine its relative importance as an additional mechanism of VGB action.

4.5 CONCLUSIONS

In conclusion, VGB and TGB exhibit different anticonvulsant profiles in experimental seizure models. VGB raised the threshold for induction of tonic seizures, determined by the Min-ES test, but was without effect in the PTZ and MES tests. TGB, in contrast, exhibited similar dose- and time-dependent anticonvulsant effects against both PTZ- and MES-induced seizures. Reported mechanisms of action of the two drugs were confirmed, VGB inhibiting brain GABA-T and TGB blocking non-cell-specific, carrier-mediated

GABA transport. Additional effects were observed with VGB which supported a previously reported inhibition of GAD but also suggested a hitherto undocumented blockade of non-cell-specific GABA uptake.

CHAPTER FIVE

ANTIEPILEPTIC DRUG ATTENUATION OF NEURONAL EXCITATION

5.1 INTRODUCTION

5.1.1 Role of excitation and inhibition in epileptogenesis

There is evidence to suggest the existence of an equilibrium between excitation and inhibition which maintains normal function in the brain and that disruption of this balance may result in the generation of abnormal electrical activity (Schwartzkroin, 1993). The aberrant electrical events we associate with epileptiform discharges are generally considered to be a manifestation of an atypical increase in neuronal excitability. Whether this increased excitability arises as a result of enhanced excitation or reduced inhibition is unclear. It is most likely that both of these phenomena are inextricably linked in the underlying pathogenesis of seizure generation and propagation.

Since the identification of GABA as the principal inhibitory neurotransmitter in the brain (section 4.1.1), the possible role of reduced inhibition in epileptogenesis and investigations of abnormalities in GABAergic function have provided a cornerstone of epilepsy research (Meldrum, 1975; Bradford and Dodd, 1976). Many of the AEDs in clinical use have been loosely characterised in terms of their action on the GABAergic system (Macdonald and Barker, 1979) and the selective blockade of GABA-mediated inhibition has been the principal on which many of the animal seizure models in present use have been based (Fisher, 1989). While interest in enhancing GABAergic inhibition in the pharmacological amelioration of seizures continues to thrive, much attention has turned to the role of increased excitation in the cellular aetiology of epilepsy and its potential for pharmacological manipulation.

5.1.2 Glutamate as an excitatory neurotransmitter in the brain

The dicarboxylic amino acid, glutamate, is now recognised as the major excitatory neurotransmitter in the mammalian brain and has been proposed to be released at a large proportion of all synapses in the CNS (Fonnum, 1984). The first reports of the endogenous EAAs as agents capable of mediating seizures arose when Hayashi (1954) observed that the inclusion of glutamate in cerebral ventricle superfusates initiated seizure activity. During the intervening years a wealth of information pertaining to the EAAs, and glutamate in particular, has been generated. Much is now known about the synthesis (Peng et al, 1991), release (Nicholls and Attwell, 1990), receptor-mediated effects (Monaghan et al, 1989), and inactivation (Schousboe et al, 1988) of neurotransmitter glutamate.

Glutamate can be synthesised, *de novo*, by transamination of α-ketoglutarate catalysed by aspartate aminotransferase, by direct amination of α-ketoglutarate catalysed by glutamic acid dehydrogenase, and by the deamination of glutamine catalysed by glutaminase. All of these mechanisms have been implicated in the synthesis of neurotransmitter glutamate (Peng et al, 1991). Astrocytes are believed to play a principal role here by synthesising and supplying the necessary precursors (Sonnewald et al, 1991). Synaptic release of glutamate is a calcium-dependent phenomenon, can be induced by potassium, veratridine, or 4-AP depolarisation, and can be blocked by magnesium (Horton, 1989; Nicholls and Attwell, 1990).

Following neuronal release, glutamate exerts its effects, pre- and post-synaptically, via specific, cell membrane receptors. At present, five types of glutamate receptors, termed NMDA, AMPA, kainate, L-AP4 (L-2-amino-4-phosphonobutyrate), and trans-ACPD

(trans-1-amino-cyclopentyl-1,3-dicarboxylate), have been identified and classed according to their preferential activation by a variety of agonists (Monaghan et al, 1989). The NMDA, AMPA, and kainate receptor subtypes are believed to form ionophore complexes which, when activated by agonist binding, become permeable to Na^+, K^+, and Ca^{2+} ions (Mayer and Miller, 1990). The NMDA receptor is, reportedly, the most abundant and widely distributed subtype in the mammalian CNS (Monaghan and Cotman, 1985), and differs from the AMPA and kainate receptors by possessing a far greater propensity for Ca^{2+} conductance (Mayer and Westbrook, 1987) and by being subject to a voltage dependent Mg^{2+} blockade (Mayer et al, 1984). The rapid activation characteristics of these receptor subtypes, particularly AMPA, predisposes them to be prime candidates in the mediation of fast excitatory neurotransmission (Collingridge and Singer, 1990). The less-well characterised L-AP4 receptor is thought to be a pre-synaptic autoreceptor, providing a negative feedback mechanism to reduce further neuronal glutamate release (Scatton, 1993). The trans-ACPD, or metabotropic, glutamate receptor subtype has been identified as a G-protein linked receptor, coupled to effector systems that generate second messengers in the target cell. Its physiological role remains unclear (Schoepp et al, 1990).

Inactivation of glutamate following its synaptic release occurs via a high affinity, sodium- and energy-dependent uptake system present in both nerve endings and glia (Drejer et al, 1982). Recent evidence suggests that glial uptake is more important than neuronal uptake in the rapid removal of glutamate from the synaptic cleft (Schousboe et al, 1988).

Autoradiographic measurement of glutamate uptake, and lesion studies of anatomically defined pathways, have facilitated identification of numerous glutamatergic pathways in

the mammalian brain (Fagg and Foster, 1983; Storm-Mathisen and Ottersen, 1988). The majority of glutamate releasing pathways appear to be descending, originating in the neo- and allo-cortex and innervating many sub-cortical regions and the spinal cord. Many cortico-cortical projections also employ glutamate as a neurotransmitter.

5.1.3 Evidence for a role of glutamate in epileptogenesis

Glutamate is universally excitatory when iontophoretically applied to the mammalian CNS (Curtis and Johnston, 1974). The evidence for glutamate as an important excitatory factor in epileptogenesis is well established. Glutamate precipitates seizures when given focally into the brain (Stone and Javid, 1983) or systemically in high dosage (Johnston, 1973; Bradford and Dodd, 1975). The selective glutamate receptor agonists, kainic acid and NMDA, are also convulsant following systemic administration and have been employed as chemoconvulsants (section 1.5.3) in the laboratory evaluation of anticonvulsant drugs (Lothman et al, 1981; Czuczwar et al, 1985). The kindling phenomenon, also employed as an animal model of epilepsy (section 1.5.2), appears to rely, at least in part, on induction of NMDA receptor-mediated synaptic transmission for the primary generation of motor seizures (Sutula, 1990). NMDA receptor antagonists have been shown to block the development of kindling (McNamara et al, 1988), although they afford only partial protection against fully kindled seizures (Löscher et al, 1988).

Further evidence to support a role of glutamatergic excitation in epileptogenesis comes from investigations of biochemical alterations in experimental seizure models and in man. Preliminary radioligand binding studies have suggested increases in glutamate receptor density in children with generalised seizures (Represa et al, 1989) and in adults with

temporal lobe epilepsy (Geddes et al, 1990). Release of glutamate into cortical superfusates is observed during experimentally-induced seizures (Dodd et al, 1980) and subsequent reductions in the concentration of glutamate in the underlying epileptic foci have been reported in animals (Koyama, 1972) and in man (Van Gelder et al, 1972). While all of the above observations are likely, if not certainly, to be manifestations of the epileptic activity itself rather than precipitators of it, additional studies have revealed underlying glutamatergic abnormalities which may participate in seizure susceptibility. Oguro and co-workers (1990) have demonstrated a significant reduction in NMDA receptor density, compared to control, in the cerebral cortex of naive, seizure-prone El mice and Janjua and colleagues (1992) have reported a significant increase in plasma glutamate levels in non-epileptic blood relatives of patients with hereditary primary generalised and partial seizures.

5.1.4 Inhibition of glutamate-induced neuronal excitability as an antiepileptic mechanism

Pharmacological interference with the glutamatergic system would appear to be an attractive option for a novel anticonvulsant drug mechanism. Inhibition of glutamate synthesis and/or release, blockade of glutamate receptors, and attenuation of post-synaptic effects could all, speculatively, be exploited in the pharmacological treatment of seizures. As described in sections 1.3.1 and 1.4.1, several existing AEDs exert their effects via an inhibitory action on excitatory neurotransmission. PHT, CBZ, and LTG all reduce glutamate release by blockade of voltage-sensitive sodium channels in the neuronal membrane (Rogawski and Porter, 1990). Several less well established drugs, including ZNS, FNZ, and TPM, may also rely on sodium channel blockade for their anticonvulsant effects (Rogawski and Porter, 1990). Specific antagonists of the NMDA receptor, AP5

(Croucher et al, 1982) and MK-801 (Sato et al, 1988), and the AMPA receptor, NBQX and GYKI 52466 (Smith et al, 1991), have demonstrated significant anticonvulsant efficacy in animal seizure models. Potential manipulation of the post-synaptic effects of glutamate would appear to centre on the inherent calcium dependence of all glutamate mediated effects. In this regard, many calcium entry blockers have demonstrated anticonvulsant efficacy in animal seizure models (De Sarro et al, 1988; Meyer et al, 1990) and some established AEDs appear to exert their effects, at least in part, by blockade of neuronal calcium influx (Ferrendelli and Daniels-McQueen, 1982; Crowder and Bradford, 1987).

5.1.5 Calcium dependence of glutamate effects

Calcium influx has been implicated in the post-synaptic, receptor-mediated effects of glutamate (Meyer, 1989), just as it is evidently a pre-requisite for the neuronal release of glutamate (Katz and Miledi, 1969). Excitatory neurotransmission, mediated by NMDA, AMPA, and kainate receptors, and synaptic differentiation, long-term potentiation, and neurodegeneration, mediated by NMDA receptors, are all thought to be dependent on neuronal calcium entry. It has also been suggested that glutamate-mediated excitation and calcium influx are inexorably entwined at all stages of epileptogenesis (Bradford and Peterson, 1987).

5.1.6 Role and regulation of calcium in the central nervous system

Intracellular calcium is involved in the control of a variety of processes in the brain including second messenger activation and membrane permeability, in addition to a role in neuronal excitability and the release of neurotransmitters. Calcium concentrations in the

extracellular fluid are in the millimolar range compared to nanomolar in the neuronal cytoplasm (Greenberg, 1987) and so subtle alterations in calcium homeostasis will have profound effects on neuronal function. Thus, the regulation of calcium entry and extrusion in the cytosol, is of primary importance in the maintenance of normal brain function (Meyer, 1989).

Extracellular calcium can enter neurones through two major classes of Ca^{2+} channels: voltage-dependent channels, sensitive to alterations in membrane potential, and receptor-operated channels, ligand-gated through cell membrane receptor site occupation. Calcium can also be released from intracellular stores, generally by a second messenger-sensitive activation (Meyer, 1989).

Neuronal voltage-dependent Ca^{2+} channels can be subdivided into N-, L-, and T-types, according to their characteristic conductances, time- and voltage-dependencies, and sensitivities to pharmacological blockade (Fox et al, 1987a; 1987b). N-type channels are a heterogeneous group of Ca^{2+} channels that are activated by strong depolarisations (Anwyl, 1991) and are substantially, but not completely, inactivated at positive holding potentials (Plummer et al, 1989). N-type channels, subject to blockade by ω-conotoxin GVIA (Bean, 1991), may be neurone-specific (Hess, 1990) and located pre-synaptically, regulating neurotransmitter release (Meyer, 1989). L-type channels are another heterogeneous group of high-voltage activated channels with a large single channel conductance (Anwyl, 1991) and almost no inactivation (Hess, 1990). These Ca^{2+} channels, subject to blockade by the DHP compounds (Triggle and Janis, 1987), are distributed widely in many tissues (Bean, 1991), and neuronally may be located predominantly post-synaptically on the cell soma

(Meyer, 1989), although there is evidence to suggest an additional role of the L-type channel pre-synaptically, controlling neurotransmitter release (Lemos and Nowycky, 1989). T-type channels are characterised by a low threshold of activation (Anwyl, 1991), a low single channel conductance (Hess, 1990), and a rapid and complete inactivation at negative holding potentials (Triggle, 1991). These channels are subject to blockade by amiloride (Triggle, 1991) and ESM (Coulter et al, 1989c) and are less densely distributed than N- or L-type channels (Bean, 1991). Recent evidence has suggested the existence of a further voltage-sensitive Ca^{2+} channel, the P-type channel, with a high threshold of activation and subject to blockade by the spider toxin, ω-Aga-IVA (Llinas et al, 1989). The functional significance of this channel remains unclear. Although the voltage-sensitive Ca^{2+} channels are primarily regulated by membrane potential, their activity may also be modulated by protein phosphorylation. A variety of intracellular second messengers, including cAMP, inositol trisphosphate, protein kinase C, and diacylglycerol, have been implicated in this process (Osterrieder et al, 1982; Putney Jr, 1986).

A host of neuromodulators, including substance P (Murase et al, 1986) and calcitonin gene-related peptide (Nohmi et al, 1986), can stimulate Ca^{2+} entry through receptor-operated channels. The most pertinent receptor-mediated calcium influx, to the present discussion, is that exerted by glutamate acting at the NMDA receptor subtype (Mayer and Miller, 1990). As described above, synaptic release of glutamate results in the activation of five different subtypes of glutamate receptor, three of which, AMPA, kainate, and NMDA, are ionotropic and directly regulate ion conductances in the post-synaptic cell (Monaghan et al, 1989). Of these three, only the NMDA receptor channel is significantly

permeable to Ca^{2+} ions (Mayer and Miller, 1990), however, under normal conditions, this channel is subject to a voltage-dependent blockade by Mg^{2+} ions (Mayer et al, 1984). During low frequency activation, the majority of post-synaptic current is carried by Na^{+} ions, mediated through AMPA and kainate receptors (Collingridge and Singer, 1990). During sustained pre-synaptic depolarisation, post-synaptic AMPA and kainate receptor activation results in a Na^{+}-dependent depolarisation of sufficient intensity to remove the voltage dependent Mg^{2+} blockade of the NMDA receptor (Collingridge and Singer, 1990) and, possibly, to open voltage dependent calcium channels, predominantly of the L-type, in the post-synaptic membrane (Meyer, 1989). Thus, calcium influx through NMDA-type glutamate receptors, does not exhibit classical receptor-operated characteristics, requiring prior activation of AMPA and/or kainate receptors and possessing a degree of voltage dependency. Whether this glutamate-mediated calcium entry is additionally influenced by protein phosphorylation, as observed with pure voltage dependent calcium channels, is presently unclear.

Extrusion of cytosolic calcium, to maintain low concentration, is regulated by a variety of pumps and sequestration processes. The Na^{+}/Ca^{2+} antiport pump (Reeves and Hale, 1984) restores intracellular calcium concentrations following neuronal depolarisation. The Ca^{2+}-ATPase pump undergoes a calcium dependent phosphorylation to extrude calcium ions from the cytosol (Penniston, 1983). Endoplasmic reticular uptake of calcium, dependent on the actions of another Ca^{2+}-ATPase pump, allows this sub-cellular structure to accumulate large amounts of calcium (Inesi, 1985). High capacity mitochondrial uptake, via both Ca^{2+}/Na^{+} and Ca^{2+}/H^{+} exchange, facilitates calcium removal from the cytosol, particularly during stimulation when there is a high calcium load (Borle, 1981).

Finally, calmodulin, a large intracellular protein, appears to possess the ability to bind free cytosolic calcium ions (Cheung, 1980).

5.1.7 Glutamate-induced excitability and calcium influx in epileptogenesis

The interactive involvement of glutamate-induced neuronal excitability and calcium influx in epileptogenesis falls into, perhaps, three categories, the establishment of the epileptic focus, the initiation of the primary cellular events of epileptogenesis at the epileptic focus and the propagation and eventual spread of the abnormal electrical activity from such a focus.

With the explosion of knowledge in the field of EAAs has come the observation that these compounds not only participate, to a large extent, in normal, excitatory synaptic transmission in the brain but that they are also neurotoxic in high concentration (Olney, 1978). Under anoxic conditions (Rothman, 1984), or in the absence of effective inactivation mechanisms (McBean and Roberts, 1985), synaptically released glutamate causes extensive neurodegeneration. The cell loss associated with glutamate-induced neurotoxicity is believed to arise as a result of NMDA receptor activation and a subsequent neurodegeneration in two phases. The first phase of neurotoxicity is believed to be a manifestation of a large Na^+ entry, and a concomitant, charge-balancing Cl^- entry, through ionotropic glutamate receptors. This results in an osmotic gradient within the cell and the subsequent entry of water facilitates cell swelling and osmolysis (Olney, 1990). The late, irreversible phase of neurotoxicity is believed to be entirely dependent on an excessive Ca^{2+} entry, through NMDA receptor channels, and possibly through voltage-sensitive calcium channels (Meyer, 1989). This calcium entry sets in motion a

host of enzyme cascades, involving protein kinases, phospholipases, endonucleases, and calpains, all of which may contribute to the ultimate death of the cell (Meldrum and Garthwaite, 1990). The late phase of experimental, hypoxia-induced neurodegeneration can be significantly reduced by the administration of both NMDA receptor antagonists (Gill et al, 1987) and blockers of the L-type calcium channel (Abele et al, 1990; Sucher et al, 1991). The selective loss of inhibitory interneurones, believed to underlie the establishment of epileptic foci in animal seizure models (Ribak et al, 1979), may be dependent on hypoxia-induced glutamate neurotoxicity (Sloper et al, 1980). Whether glutamate excitotoxicity, and the associated calcium influx, play a role in the underlying pathology of human epileptic foci remains to definitively evaluated.

Glutamate-induced neuronal excitability, and the associated calcium influx, have also been proposed to play a central role in the primary cellular events of epileptogenesis (Bradford and Peterson, 1987). These events are described in detail in section 1.1.4. Intrinsic burst firing, believed to underlie the phenomenon of interictal discharge, has been proposed to be a function of normal afferent excitatory input to abnormal neurones at the epileptic focus (Meldrum, 1988). The resultant depolarisation, Na^+-dependent via AMPA receptors, leads to an atypically sustained calcium influx (Meyer, 1989). This calcium entry substantially contributes to the depolarisation and, when of sufficient duration, precipitates a train, or burst, of action potentials before repolarisation, via Ca^{2+}-driven K^+ efflux and the Na^+/K^+ pump, can occur (Meyer, 1989). The aetiological mechanisms believed to predispose certain neurones to exhibit such paroxysmal discharges are discussed in section 1.1.5.

The atypically sustained calcium influx observed during paroxysmal depolarisations at the epileptic focus may be dependent on calcium entry through voltage-sensitive channels. A decrease in extracellular free Ca^{2+} levels (Heinemann and Louvel, 1983), with a concomitant increase in cytosolic Ca^{2+} concentration (Pumain et al, 1983), has been reported during synchronised burst firing at experimentally-induced epileptic foci. The majority of this calcium influx appeared to be mediated by the post-synaptic cell body and, as such, was shown to exhibit a substantial degree of voltage dependency (Pumain and Heinemann, 1985). In addition, experimentally-induced burst firing and PDSs in the hippocampal slice preparation can be blocked by the administration of voltage-sensitive calcium channel blockers (Witte et al, 1987; Bingmann and Speckmann, 1989), while calcium channel agonists have demonstrated exacerbation of these events (Walden et al, 1986). Thus, it appears that interictal discharges in the post-synaptic membrane are dependent upon voltage-sensitive calcium influx.

There is now substantial experimental evidence to suggest an additional role of NMDA receptor-mediated calcium influx in the generation of interictal epileptiform discharges (Bradford and Peterson, 1987). Activation of NMDA receptors in the caudate nucleus of anaesthetised cats (Herrling et al, 1983) precipitates periodic waves of depolarisation, with associated bursts of action potentials, which closely resemble the PDSs observed at experimental epileptic foci (Ayala et al, 1970). Low frequency activation of the Schaffer collateral - commisural pathway in the isolated hippocampal slice preparation, in the absence of Mg^{2+}, results in the generation of PDS-like discharges in the CA1 region (Walther et al, 1986). Similarly, spontaneous discharges, of sufficient frequency, in the CA3 region of the hippocampus, also in the absence of Mg^{2+}, may precipitate spontaneous

epileptiform activity in the CA1 region that can be equated with both interictal-like (Mody et al, 1987) and full blown ictal-like (Anderson et al, 1986) events. All of these experimentally-induced epileptiform discharges are susceptible to blockade by antagonists of the NMDA receptor (Herron et al, 1985; Peet et al, 1986). Thus, it seems that both voltage-sensitive calcium influx and NMDA receptor activation are inextricably involved in the primary generation of epileptiform discharges.

The final association between glutamate-induced excitability and calcium influx involves the spread of epileptiform activity from its site of primary generation. The observations that extensive glutamatergic pathways appear to course between various brain structures, and between the cerebral cortex and several sub-cortical brain regions (Fagg and Foster, 1983; Storm-Mathisen and Ottersen, 1988) have led to the suggestion that the spread of seizure activity from one cortical area to another, or to deep nuclei, relies, at least in part, on calcium-dependent EAA neurotransmission (Meldrum, 1984). The potent anticonvulsant actions of a variety of specific EAA receptor antagonists (Croucher et al, 1982; Patel et al, 1990; Chapman et al, 1991b) and calcium channel blockers (De Sarro et al, 1988; 1990) may, in part, be due to blockade of these widespread glutamatergic pathways, thus limiting the spread of hyperactivity.

5.1.8 Voltage-sensitive calcium entry blockade as an antiepileptic mechanism

As described above, neuronal voltage-sensitive calcium entry is of primary importance in glutamate-induced excitability in the nervous system and is inextricably involved at all stages of epileptogenesis. Calcium influx, through voltage-sensitive channels, may be a pre-requisite for the establishment of an epileptic focus, the initiation of epileptiform

activity, and the spread of the associated abnormal electrical events. As a result, the potential of voltage-sensitive calcium entry blockade as an antiepileptic mechanism has been investigated extensively in recent years.

There are many calcium antagonist (CA) drugs that selectively inhibit calcium entry, mostly by voltage-sensitive L-type channel blockade (Greenberg, 1987). These CAs have been employed for many years in the treatment of cardiovascular disorders (Godfraind et al, 1986) and, as such, have been classified, according to their structure, into four main groups, the piperazines, the phenylalkylamines, the benzothiazepines, and the DHPs (Vanhoutte and Paoletti, 1987).

The piperazine compound FNZ was the first CA to exhibit significant anticonvulsant activity in animal seizure models (section 1.4.1). It has demonstrated efficacy against both MES- and PTZ-induced tonic seizures (Desmedt et al, 1975; 1976) and against reflex seizures in DBA/2 mice and *Papio papio* baboons (De Sarro et al, 1986). FNZ is also active against seizures induced by systemic bicuculline (Wauquier et al, 1986), focal D,L-allylglycine (Ashton and Wauquier, 1979a), and amygdaloid kindling (Ashton and Wauquier, 1979b), and protects against epileptiform bursting in the *in vitro* hippocampal slice (Ashton et al, 1986; Bingmann and Speckmann, 1989). Investigations of the mechanism of FNZ action have, however, suggested that the drug exerts its anti-seizure effects by blockade of voltage-sensitive sodium channels, in a PHT-like manner, rather than by calcium entry blockade (Grima et al, 1986; McLean, 1987). This observation was consistent with the reported similarities in experimental anticonvulsant profiles of the two drugs (Rogawski and Porter, 1990). Other non-specific actions of FNZ include significant

antihistaminic, antiserotonergic, and antidopaminergic activity (Brodie and Porter, 1990). It is currently marketed in 38 countries for the treatment of refractory epilepsy (section 1.4.1).

The phenylalkylamine compound, verapamil, while exhibiting extensive inhibitory effects on *in vitro* epileptiform discharges (Bingmann and Speckmann, 1989; Pohl et al, 1992), and possessing a degree of anticonvulsant activity (Walden and Speckmann, 1988), has proven relatively ineffective in the majority of whole animal seizure models (Meyer et al, 1986; De Sarro et al, 1988; Wong and Rahwan, 1989). In contrast, the benzothiazepine compound, diltiazem, has been shown to reduce PTZ- and MES-induced seizures (Kamal et al, 1990), to attenuate audiogenic seizures in both mice (De Sarro et al, 1988) and rats (De Sarro et al, 1990), and to potentiate the anticonvulsant actions of NMD (Morón et al, 1990) and PHT (De Sarro et al, 1992).

5.1.9 Dihydropyridines as novel antiepileptic drugs

The rather non-specific effects of flunarizine (Brodie and Porter, 1990), and the relative lack of effects of verapamil, together with the potential of verapamil and diltiazem to inhibit the hepatic metabolism of existing AEDs (Macphee et al, 1986c; Brodie and Macphee, 1986), has limited the use of these classes of calcium entry blocker in the treatment of epilepsy. The DHP compounds, on the other hand, have shown promise as novel antiepileptic agents. The DHPs, including NMD and nifedipine (NFD), are selective blockers of calcium entry through L-type channels and have the advantage over other classes of calcium entry blocker in that they do not interfere with hepatic drug metabolism. Despite being without effect on epileptiform bursting in the *in vitro*

hippocampal slice (Ashton et al, 1986), the DHPs have demonstrated efficacy in an impressively wide range of animal seizure models.

The DHPs, and NMD in particular, have been shown to protect against seizures induced by audiogenic stimulation in DBA/2 mice (De Sarro et al, 1988) and GEP rats (De Sarro et al, 1990), ECS (Meyer et al, 1986b: 1990), high pressure syndrome (Dolin et al, 1988), ethanol withdrawal (Little et al, 1986), ischaemia (Meyer et al, 1986a), and topical applications of cefazolin (Morocutti et al, 1986), bicuculline, and PTZ (Meyer et al, 1986a). They have also demonstrated significant anticonvulsant effects against seizures induced by the systemic administration of a variety of chemoconvulsants, including PTZ (Meyer et al, 1987; O'Neill and Bolger, 1990; Larkin et al, 1992b), kainic acid (Paczynski et al, 1990; Karler et al, 1991), picrotoxin (Thomas, 1990), N-methyl-D,L-aspartate (NMDLA; Karler et al, 1991; Palmer et al, 1993), and the calcium channel agonist BAY K 8644 (Palmer et al, 1993). The DHPs were without effect on amygdala kindled seizures (Mack and Gilbert, 1992) and seizures induced by systemic bicuculline (Karler et al, 1991; Palmer et al, 1993) and strychnine (Dolin et al, 1988; O'Neill and Bolger, 1989).

The effect of the DHPs against MES-induced seizures is controversial. Early reports, employing nose and forehead electrodes, suggested a lack of effect (Hoffmeister et al, 1982) and these results were supported by further studies, employing corneal electrodes (Wong and Rahwan, 1989; Palmer et al, 1993). However, Kamal and colleagues (1990) demonstrated a small but significant effect of NMD against MES-induced seizures administered via ear-clip electrodes. Thus, DHP effects against electroshock seizures may be dependent on stimulation site as proposed by Löscher and co-workers (1991a).

Despite impressive experimental credentials, initial clinical observations with the DHPs have been somewhat disappointing. NFD has demonstrated only minimal benefits in the treatment of patients with severe refractory epilepsy (Sander and Trevisol-Bittencourt, 1990; Larkin et al, 1992a). In a recent controlled clinical trial, NMD also failed to exhibit any significant antiepileptic activity, an effect attributed to low concentrations of circulating drug (Larkin et al, 1991). However, not all reports, in this respect, have reached such pessimistic conclusions (Pelliccia et al, 1990; de Falco et al, 1992).

5.2 EXPERIMENTAL AIMS

The aims of the following studies were to investigate the experimental anticonvulsant profiles of the DHP compounds, NMD and amlodipine (AML), using the standard animal seizure models described in chapter 3. Electroshock models were employed to complete the characterisation of acute NMD effects, the initial studies being reported by Larkin and colleagues (1992b). This work also facilitated exploration of the efficacy of NMD against transauricular MES as proposed by Kamal and co-workers (1990). Clinical observations suggesting that NMD failed to exhibit significant antiepileptic effects due to low concentrations of circulating drug (Larkin et al, 1991) precipitated studies of the concentration- and time-dependency of any anticonvulsant effects. The effects of repeated NMD administration were also evaluated, allowing a more direct comparison between experimental and clinical results. AML was included in the investigations to allow a comparison with a longer-acting, non-photolabile DHP (Meredith and Elliot, 1992). Finally, the use of primary cultures of cerebellar granule cells and cerebral cortical astrocytes facilitated study of the mechanisms of DHP action with regard to blockade of calcium influx and any potential interaction with excitatory neurotransmitter release.

5.3 EXPERIMENTAL PROTOCOLS AND RESULTS

5.3.1 Dose dependent effects of single nimodipine administration on maximal electroshock-induced seizures in mice

PROTOCOL:- Mice were separated into 5 treatment groups (n=10/group) and NMD was administered (i.p.) in doses of 10, 40, 70, and 100 mg/kg. One group (control) received vehicle (0.5% Tween 80) alone. NMD suspensions were sonicated immediately prior to administration to ensure homogeneity. At one hour post-dosing the animals were subjected to the MES test (section 2.3.2) using a stimulus current of 50 mA. The incidence of THE within each group was recorded.

RESULTS:- NMD (10 - 100 mg/kg) produced a dose-dependent reduction in the incidence of THE induced by MES at 1 hour post-administration (figure 29). The ED_{50} for NMD against MES-induced seizures, calculated from this experiment, was 87 mg/kg.

5.3.2 Time and concentration dependent effects of single nimodipine administration on maximal electroshock-induced seizures in mice

PROTOCOL:- Control animals were given vehicle (0.5% Tween 80) alone and subjected to the MES test (section 2.3.2), using a stimulus current of 50 mA, at one and six hours post-administration to confirm the effectiveness of the stimulation parameters in producing THE. Experimental mice were administered 75 mg/kg NMD at time zero. The NMD suspension was sonicated immediately prior to administration to ensure homogeneity. The animals were separated into 6 groups (n=10/group) and sequentially subjected to MES at 0.5, 1, 2, 4, 6, and 12 hours after dosing using a stimulus current of 50 mA. The incidence of THE within each group was recorded. Following sacrifice, truncal blood and brain samples were obtained by the methods described in sections 2.2.2

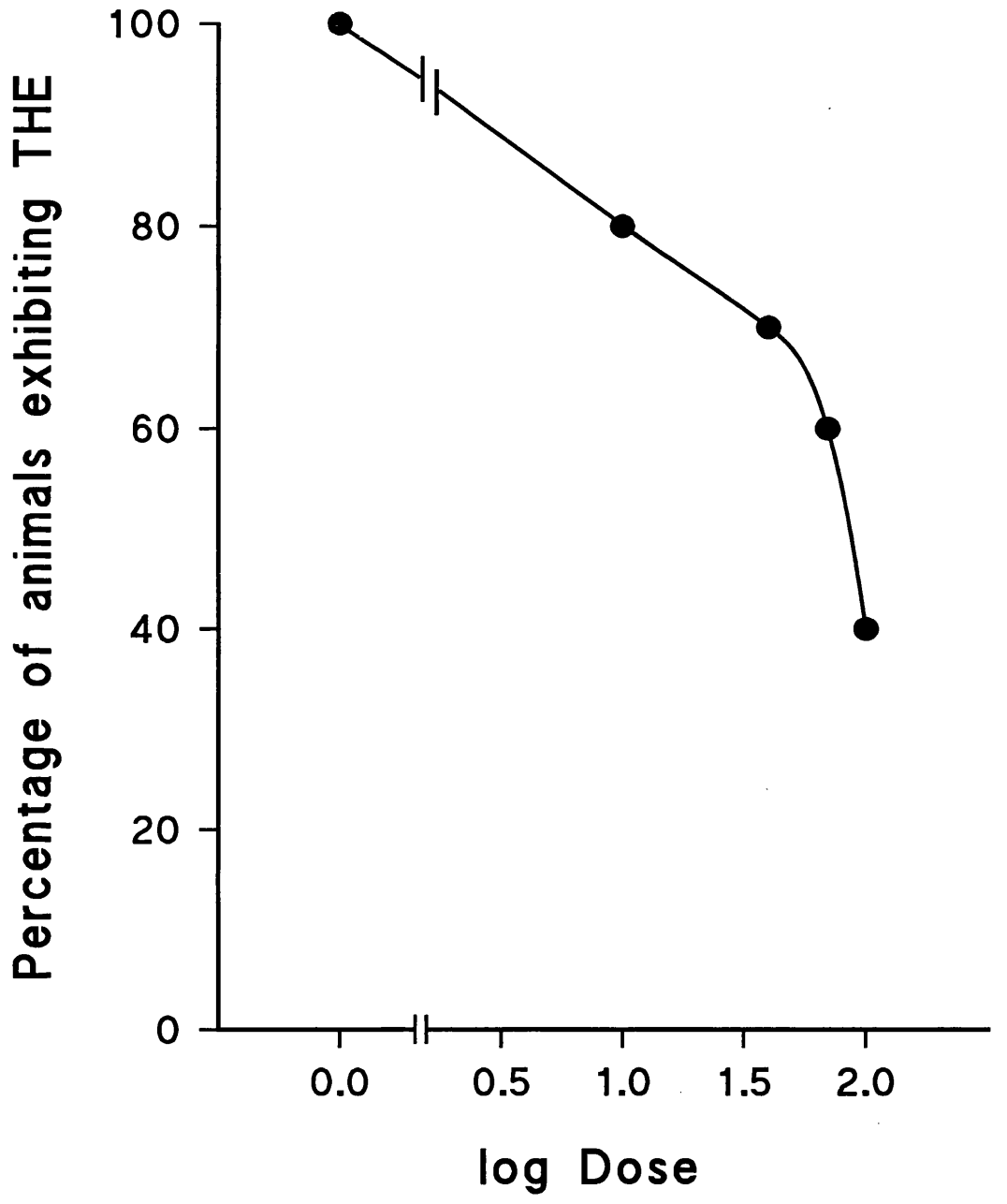

FIGURE 29:- Effect of single nimodipine doses (10 - 100 mg/kg), expressed logarithmically, on the incidence of tonic hind-limb extension (THE) induced by MES at 1 hour post-administration. Control animals are denoted by log Dose = 0.0 for ease of comprehension. Results are expressed as the percentage of animals (mice) in groups of 10 exhibiting THE.

and 2.2.3 respectively. Samples were stored in accordance with section 2.2.5 prior to analysis of NMD content by HPLC (section 2.8).

RESULTS:- THE was observed in 100% of control animals tested at 1 and 6 hours post-dosing, confirming the effectiveness of the stimulation parameters. Single dose NMD (75 mg/kg) produced a reduction in the occurrence of THE for up to 12 hours. This effect was significant at all time points (0.5 - 6 hours, $p < 0.01$; 12 hours, $p < 0.05$) and is illustrated in figure 30. Peak plasma and brain NMD concentrations were observed at 0.5 hours post-dosing and declined exponentially thereafter. NMD levels in plasma and brain correlated in a positive manner ($r = 0.677$, $p < 0.01$) and were seen to be below detectable limits by 12 hours post-administration (figure 31).

5.3.3 Effect of single nimodipine administration on the threshold for induction of tonic seizures in mice

PROTOCOL:- Mice were separated into 2 groups (n=30/group) and treated with either 75 mg/kg NMD (i.p.) or vehicle (0.9% saline) alone. At one hour post-administration each animal was subjected to the Min-ES test (section 2.3.3). The number of positive (tonic seizure) and negative (no tonic seizure) responses within each group was recorded.

RESULTS:- Compared to control, single dose NMD (75 mg/kg) significantly ($p < 0.001$) increased the tonic seizure threshold determined by Min-ES at 1 hour post-administration (figure 32). Median threshold values, determined by regression analysis of positive and negative responses, were 5.00 mA (control) and 7.00 mA (NMD-treated).

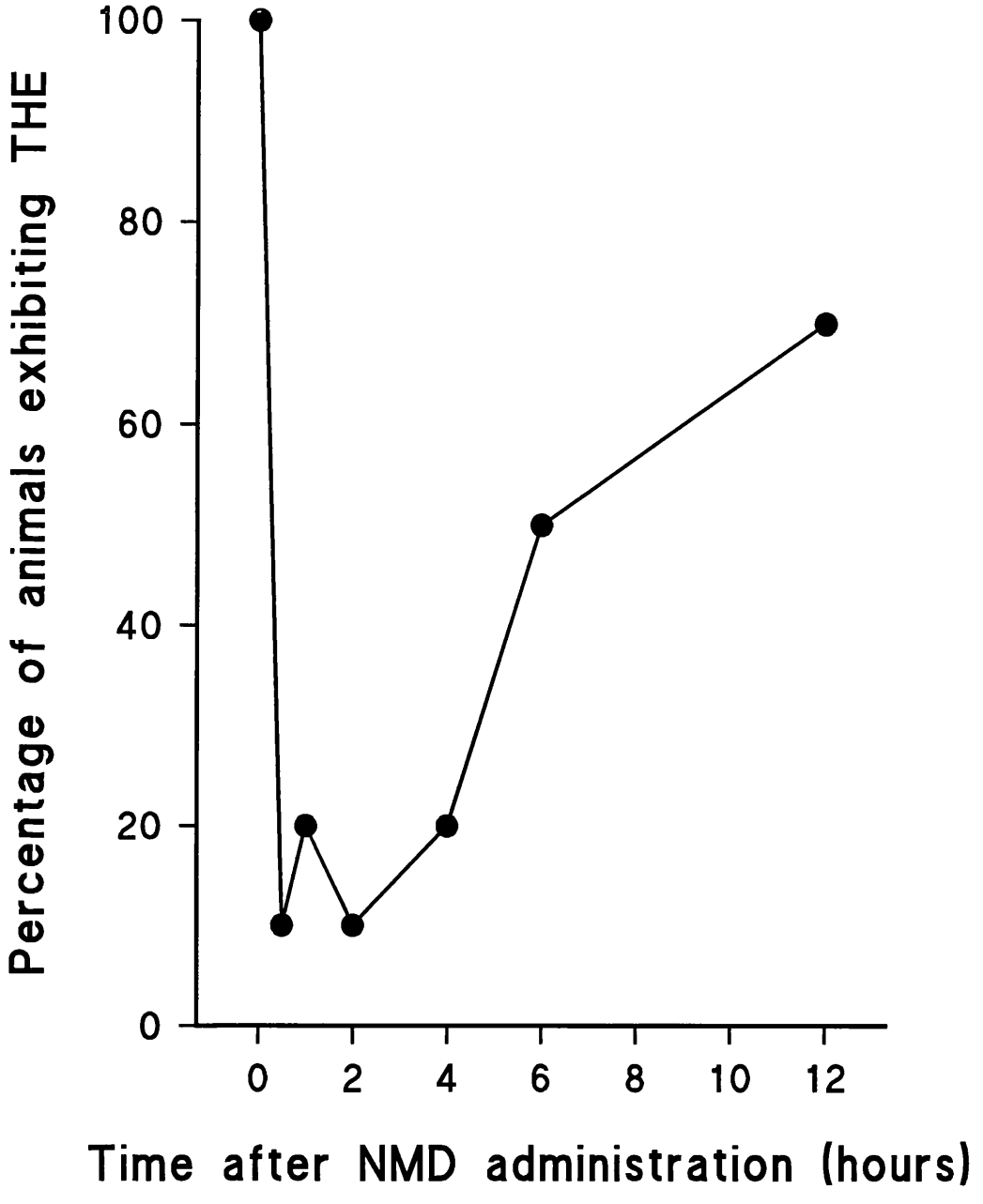

FIGURE 30:- Effect of a single nimodipine (NMD) dose (75 mg/kg) on the incidence of tonic hind-limb extension (THE) induced by MES at 1 - 12 hours post-administration. Results are expressed as the percentage of animals (mice) in groups of 10 exhibiting THE.

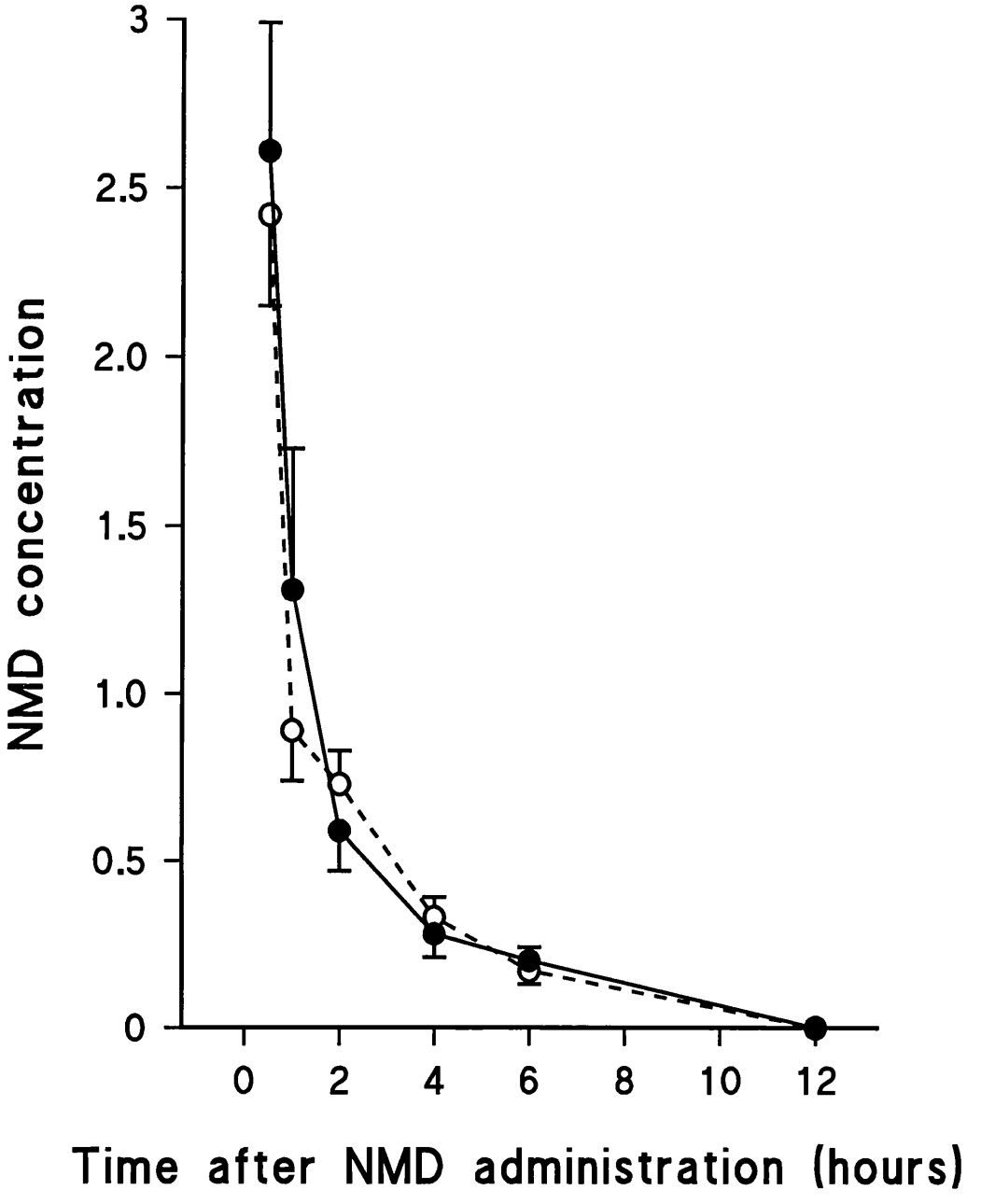

FIGURE 31:- Nimodipine (NMD) concentration-time curves in mouse plasma (μg/ml; open circles, broken line) and brain (μg/g; closed circles, solid line) following a single intraperitoneal dose (75 mg/kg). Results are expressed as the mean (± SEM) concentrations in groups of 10 animals.

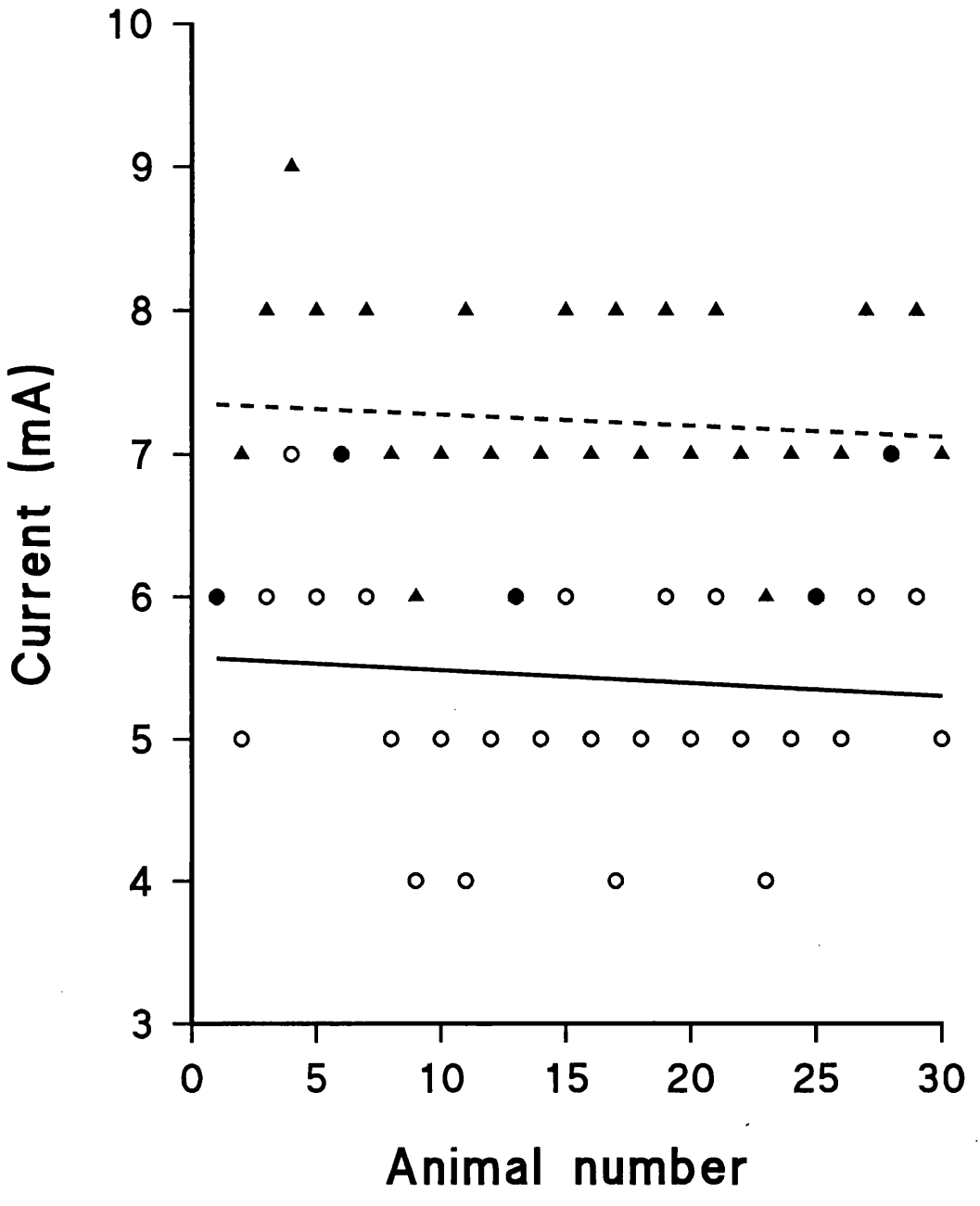

FIGURE 32:- Effect of nimodipine treatment (75 mg/kg; closed triangles, broken line) vs control (open circles, solid line) on the tonic seizure threshold determined by Min-ES at 1 hour post-dosing. Closed circles represent data points at which animals from opposing groups (control and nimodipine-treated) coincide. Seizure thresholds were calculated by regression analysis of the results obtained from groups of 30 mice and statistical significance ($p < 0.001$) was determined by the Mann Whitney U-test.

5.3.4 Dose dependent effects of repeated nimodipine treatment on pentylenetetrazol-induced seizures in mice

PROTOCOL:- Mice were separated into 5 treatment groups (n=6/group) and NMD was administered (i.p.) in doses of 25, 50, 75, and 100 mg/kg. One group (control) received vehicle (0.5% Tween 80) alone. Treatment was continued once daily for 8 days. NMD suspensions were sonicated immediately prior to administration to ensure homogeneity. At four hours after the final dose the animals were subjected to the s.c. CD_{97} PTZ test (section 2.3.1) and the latency to the first generalised seizure in individual animals was recorded.

RESULTS:- Repeated treatment with NMD (25 - 100 mg/kg) was ineffectual in lengthening the time to the first generalised seizure induced by PTZ at 4 hours after the final dose (figure 33).

5.3.5 Dose dependent effects of repeated nimodipine treatment on maximal electroshock-induced seizures in mice

PROTOCOL:- Mice were separated into 5 treatment groups (n=10/group) and NMD was administered (i.p.) in doses of 25, 50, 75, and 100 mg/kg. One group (control) received vehicle (0.5% Tween 80) alone. Treatment was continued once daily for 8 days. NMD suspensions were sonicated immediately prior to administration to ensure homogeneity. At four hours after the final dose the animals were subjected to the MES test (section 2.3.2) using a stimulus current of 50 mA. The incidence of THE within each group was recorded.

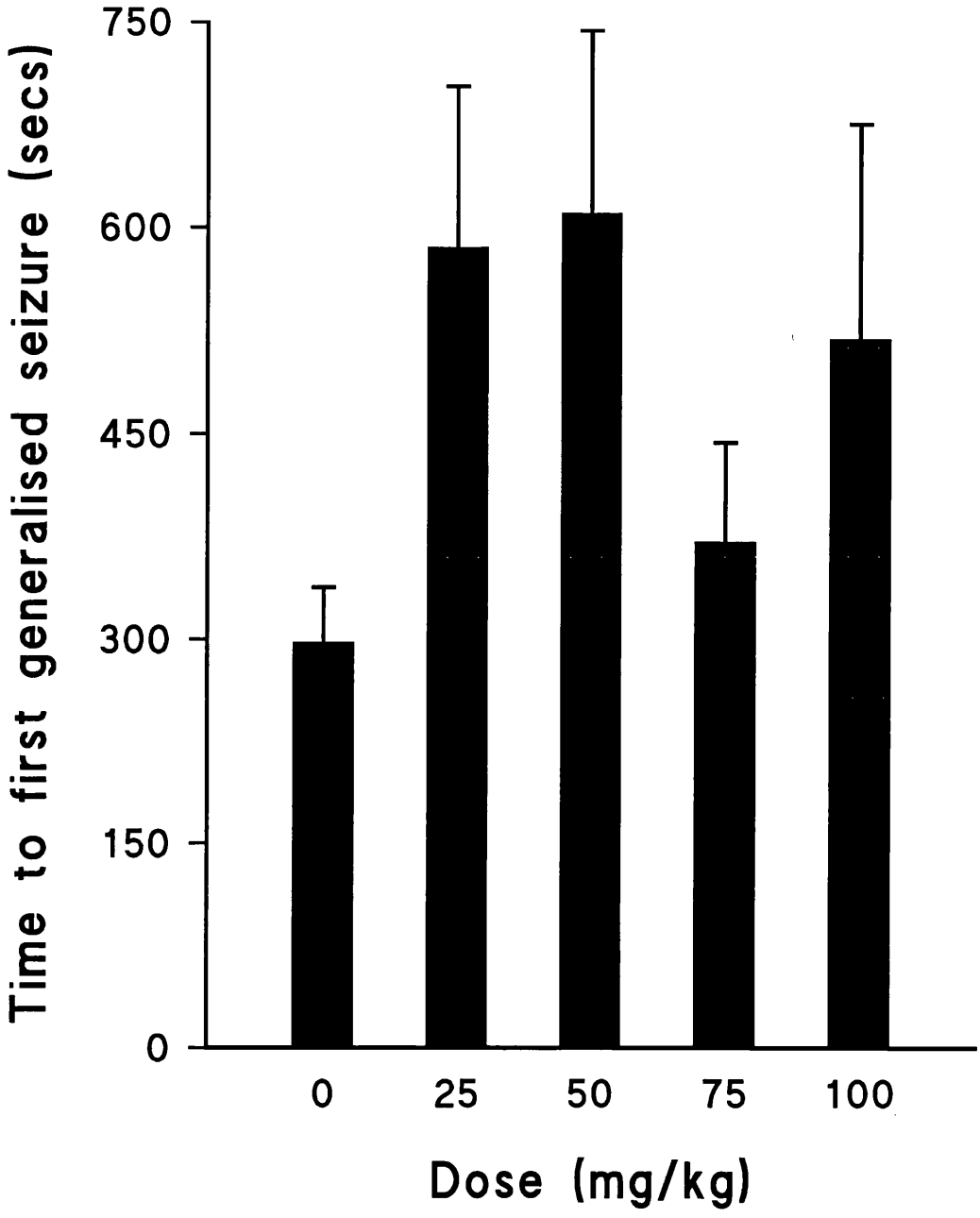

FIGURE 33:- Effect of repeated nimodipine dosing (25 - 100 mg/kg; once daily for 8 days) on the latency to the first generalised seizure induced by 85 mg/kg PTZ at 4 hours after the final dose. Results are expressed as mean (± SEM) time (seconds) in groups of 6 mice.

RESULTS:- Repeated treatment with NMD (25, 50, and 100 mg/kg) was without effect on the incidence of THE induced by MES at 4 hours after the final dose (figure 34). Repeated treatment with 75 mg/kg NMD significantly ($p < 0.05$) reduced the incidence of THE, compared to control, at 4 hours post-dosing.

5.3.6 Time and concentration dependent effects of repeated nimodipine treatment on pentylenetetrazol-induced seizures in mice

PROTOCOL:- Mice were separated into two treatment groups (n=48/group) and were given either 50 mg/kg NMD (i.p.) or vehicle (0.5% Tween 80) alone. Treatment was continued once daily for 8 days. The NMD suspension was sonicated immediately prior to administration to ensure homogeneity. Six animals from each group were sequentially subjected to the s.c. CD_{97} PTZ test at 1, 2, 4, 6, 8, 12, 24, and 36 hours after the final dose. The latency to the first generalised seizure in individual animals was recorded. Following sacrifice, truncal blood and brain samples were obtained by the methods described in sections 2.2.2 and 2.2.3 respectively. Samples were stored in accordance with section 2.2.5 prior to analysis of NMD content by HPLC (section 2.8).

RESULTS:- Repeated treatment with NMD (50 mg/kg) significantly ($p < 0.05$) lengthened the time to the first generalised seizure induced by PTZ at 1, 12, and 36 hours after the final dose (figure 35). NMD was without significant effect, compared to control, at all other time points investigated. Peak plasma and brain NMD concentrations were observed at 1 hour post-dosing and declined exponentially thereafter. NMD levels in plasma and brain correlated in a positive manner ($r = 0.753$, $p < 0.01$) and were seen to be below detectable limits by 12 hours after the final dose (figure 36).

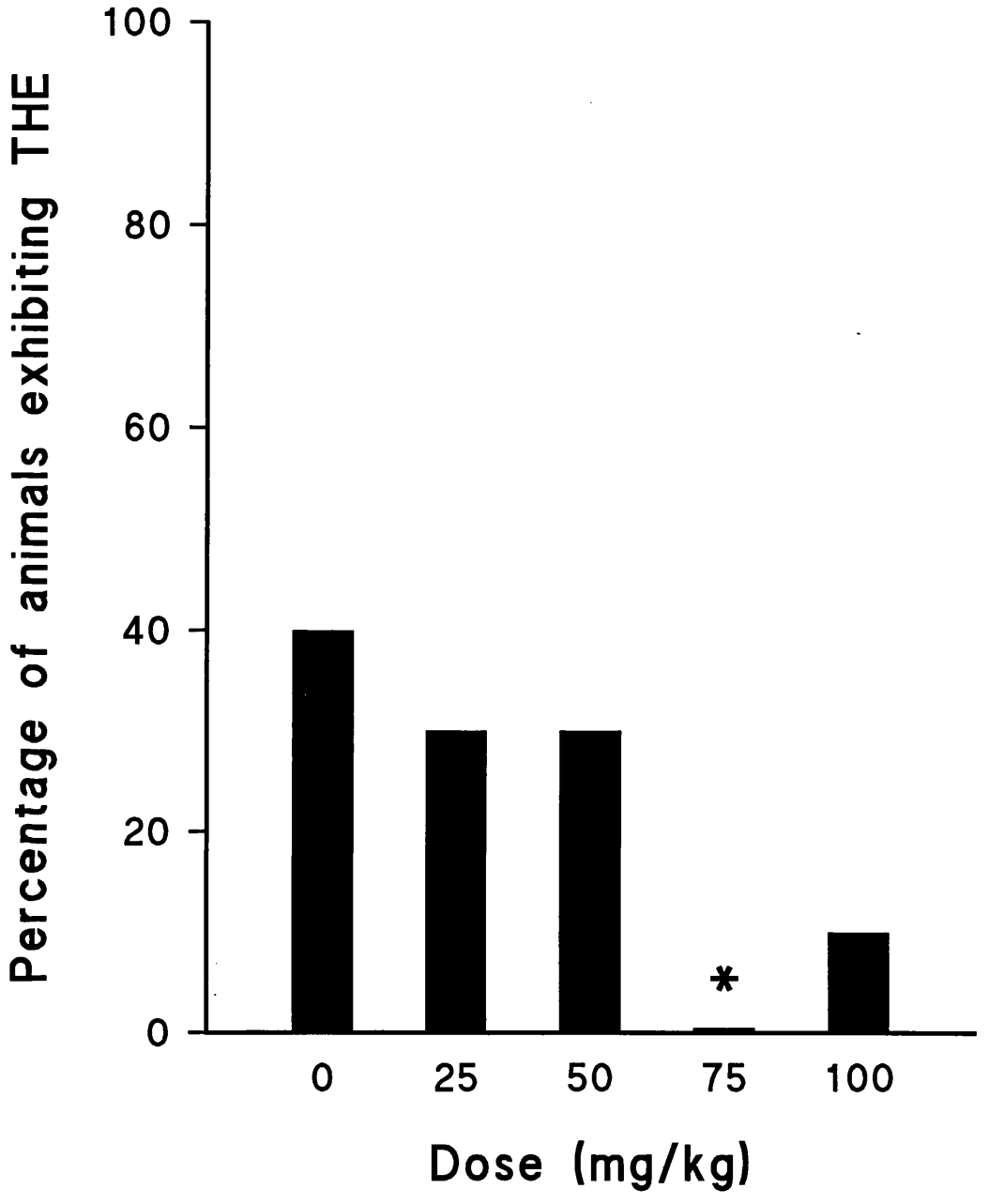

FIGURE 34:- Effect of repeated nimodipine dosing (25 - 100 mg/kg; once daily for 8 days) on the incidence of tonic hind-limb extension (THE) induced by MES at 4 hours after the final dose. Results are expressed as the percentage of animals (mice) in groups of 10 exhibiting THE. Statistical significance (*$p < 0.05$) was determined by the Chi square test.

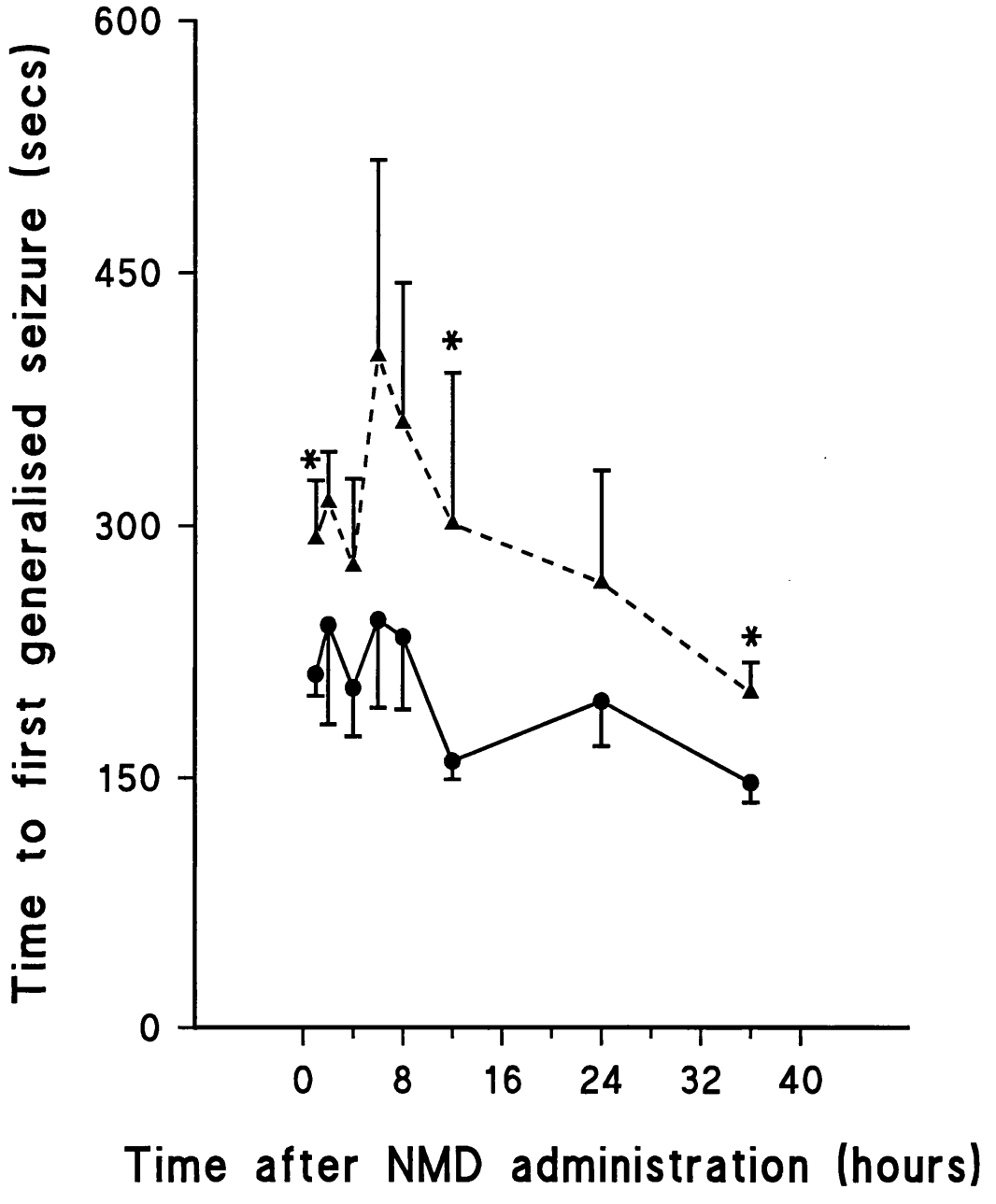

FIGURE 35:- Effect of repeated nimodipine (NMD) dosing (50 mg/kg; once daily for 8 days; triangles, broken line) vs control (circles, solid line) on the latency to the first generalised seizure induced by 85 mg/kg PTZ at 1 - 36 hours after the final dose. Results are expressed as the mean (± SEM) time (seconds) in groups of 6 mice. Statistical significance (*$p < 0.05$) was determined by the Mann Whitney U-test.

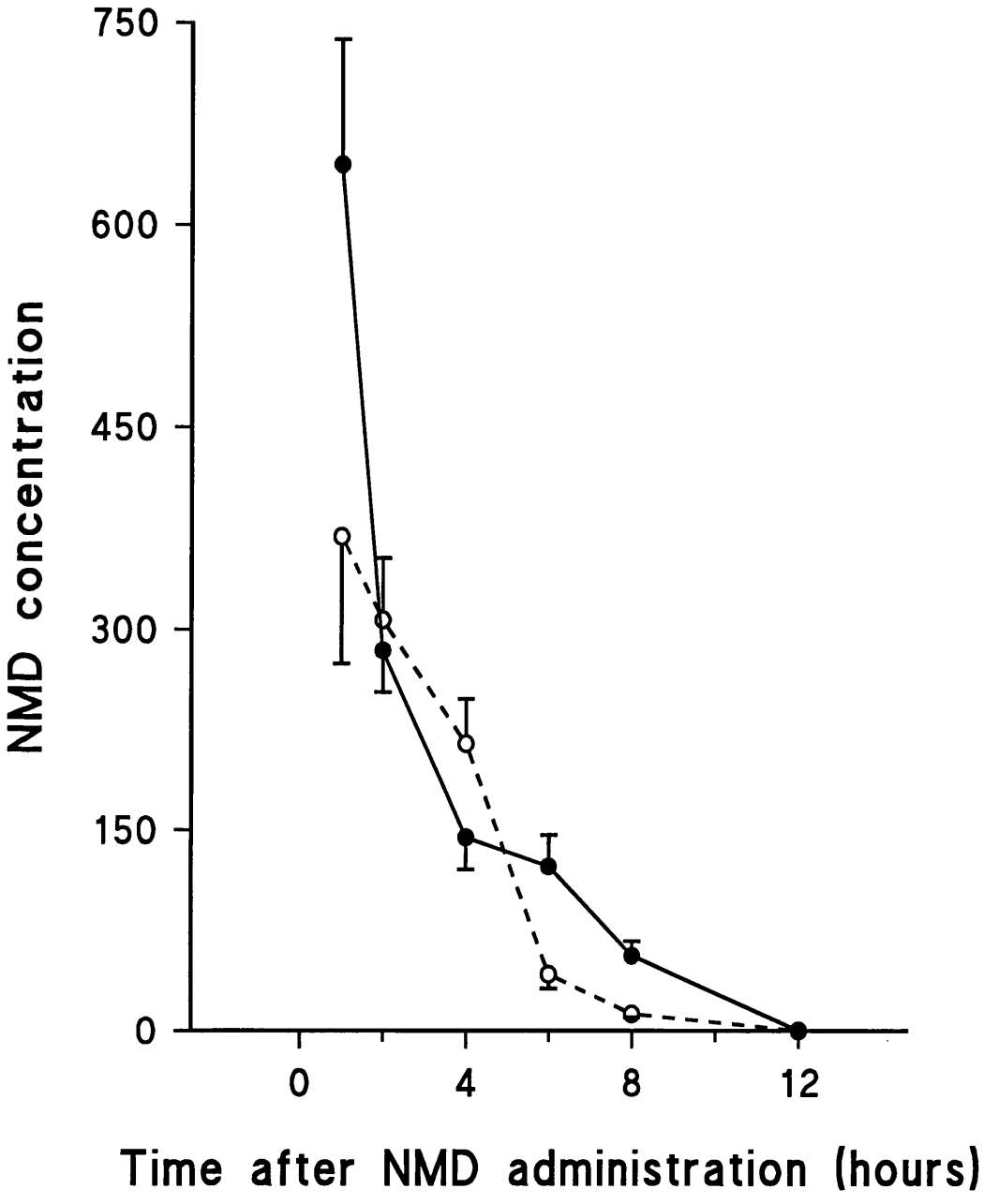

FIGURE 36:- Nimodipine (NMD) concentration-time curves in mouse plasma (ng/ml; open circles, broken line) and brain (ng/g; closed circles, solid line) following repeated administration (once daily for 8 days) of a 50 mg/kg dose. Results are expressed as the mean (± SEM) concentrations in groups of 6 animals.

5.3.7 Time and concentration dependent effects of repeated nimodipine treatment on maximal electroshock-induced seizures in mice

PROTOCOL:- Mice were separated into two treatment groups (n=80/group) and were given either 75 mg/kg NMD (i.p.) or vehicle (0.5% Tween 80) alone. Treatment was continued once daily for 8 days. The NMD suspension was sonicated immediately prior to administration to ensure homogeneity. Ten animals from each group were sequentially subjected to the MES test at 1, 2, 4, 6, 8, 12, 24, and 36 hours after the final dose using a stimulus current of 50 mA. The incidence of THE within each group was recorded. Following sacrifice, truncal blood and brain samples were obtained by the methods described in sections 2.2.2 and 2.2.3 respectively. Samples were stored in accordance with section 2.2.5 prior to analysis of NMD content by HPLC (section 2.8).

RESULTS:- Repeated treatment with NMD (75 mg/kg) significantly ($p < 0.01$) reduced the incidence of THE induced by MES at 2, 8, and 12 hours after the final dose (figure 37). NMD was without significant effect, compared to control, at all other time points investigated. Peak plasma and brain NMD concentrations were observed at 1 hour post-dosing and declined exponentially thereafter. NMD levels in plasma and brain correlated in a positive manner ($r = 0.740$, $p < 0.01$) and were seen to be below detectable limits by 24 hours after the final dose (figure 38).

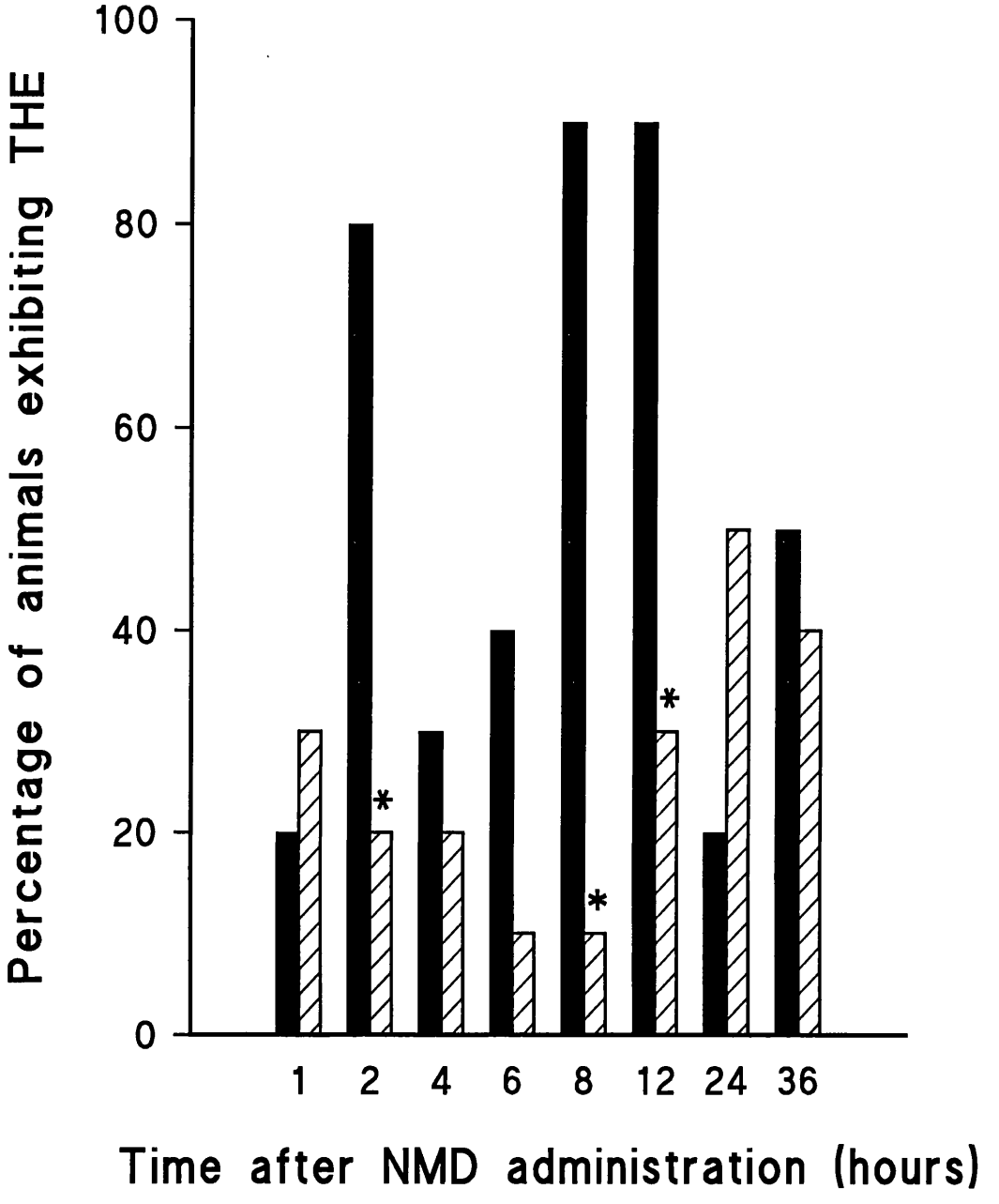

FIGURE 37:- Effect of repeated nimodipine (NMD) treatment (75 mg/kg; once daily for 8 days; hatched bars) vs control (solid bars) on the incidence of tonic hind-limb extension (THE) induced by MES at 1 - 36 hours after the final dose. Results are expressed as the percentage of animals (mice) in groups of 10 exhibiting THE. Statistical significance (*$p < 0.01$) was determined by the Chi square test.

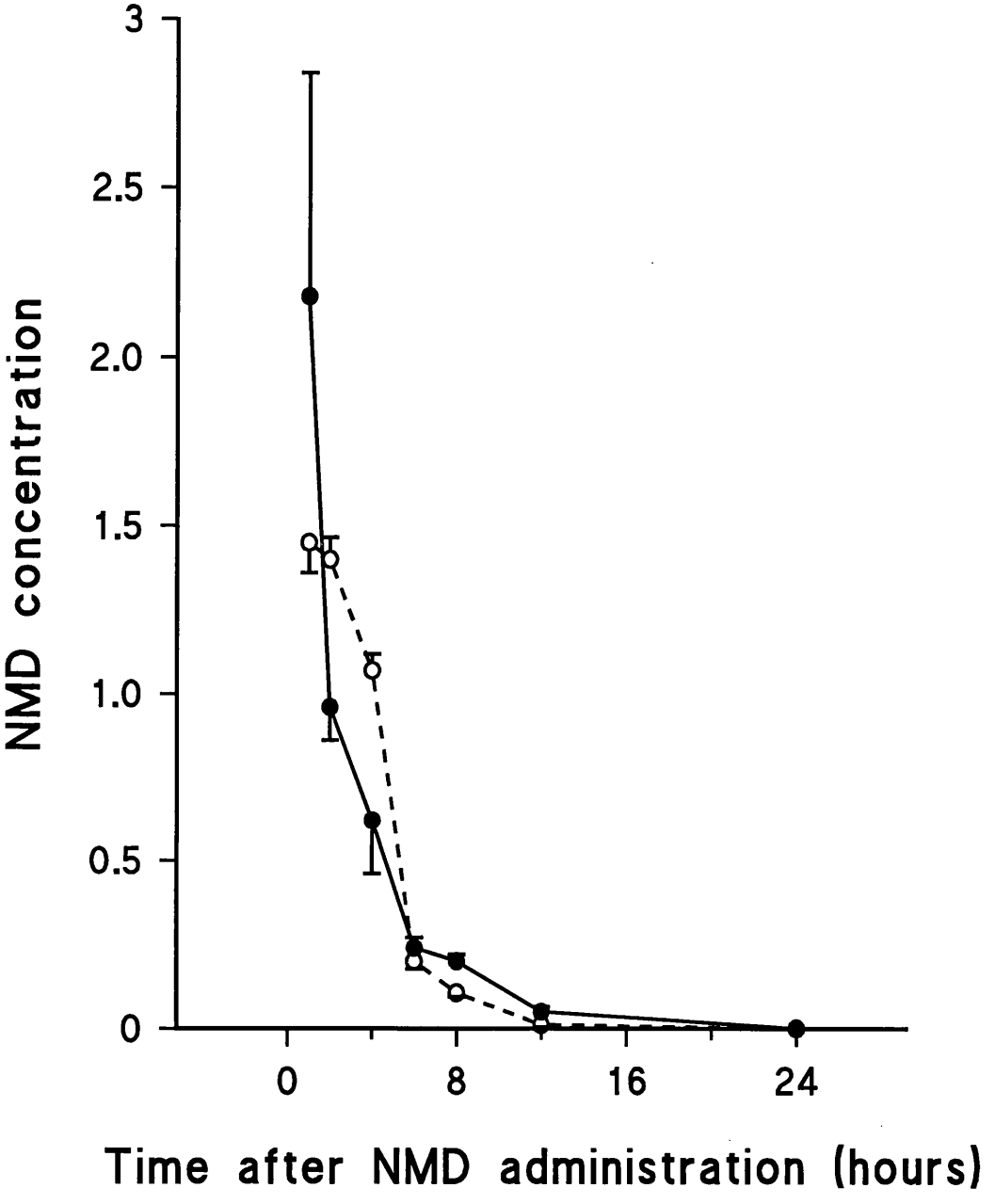

FIGURE 38:- Nimodipine (NMD) concentration-time curves in mouse plasma (μg/ml; open circles, broken line) and brain (μg/g; closed circles, solid line) following repeated administration (once daily for 8 days) of a 75 mg/kg dose. Results are expressed as the mean (± SEM) concentrations in groups of 10 animals.

5.3.8 Dose dependent effects of single amlodipine administration on pentylenetetrazol-induced seizures in mice

PROTOCOL:- Mice were separated into 6 treatment groups (n=6/group) and AML was administered (i.p.) in doses of 1, 5, 10, 20, and 30 mg/kg. One group (control) received vehicle (0.9% saline) alone. At six hours post-dosing the animals were subjected to the s.c. CD_{97} PTZ test (section 2.3.1) and the latency to the first generalised seizure in individual animals was recorded.

RESULTS:- Single doses of AML (10 and 20 mg/kg) significantly ($p < 0.05$) lengthened the time to the first generalised seizure induced by PTZ at 6 hours post-administration (figure 39). AML was without significant anticonvulsant effect, compared to control, at all other doses investigated. One animal given 20 mg/kg AML and all 6 animals given 30 mg/kg AML died prior to PTZ administration.

5.3.9 Dose dependent effects of single amlodipine administration on maximal electroshock-induced seizures in mice

PROTOCOL:- Mice were separated into 6 treatment groups (n=10/group) and AML was administered (i.p.) in doses of 1, 5, 10, 15, and 20 mg/kg. One group (control) received vehicle (0.9% saline) alone. At six hours post-dosing the animals were subjected to the MES test (section 2.3.2) using a stimulus current of 56 mA. The incidence of THE within each group was recorded.

RESULTS:- Single doses of AML (1 - 20 mg/kg) were without effect on the incidence of THE induced by MES at 6 hours post-administration (figure 40).

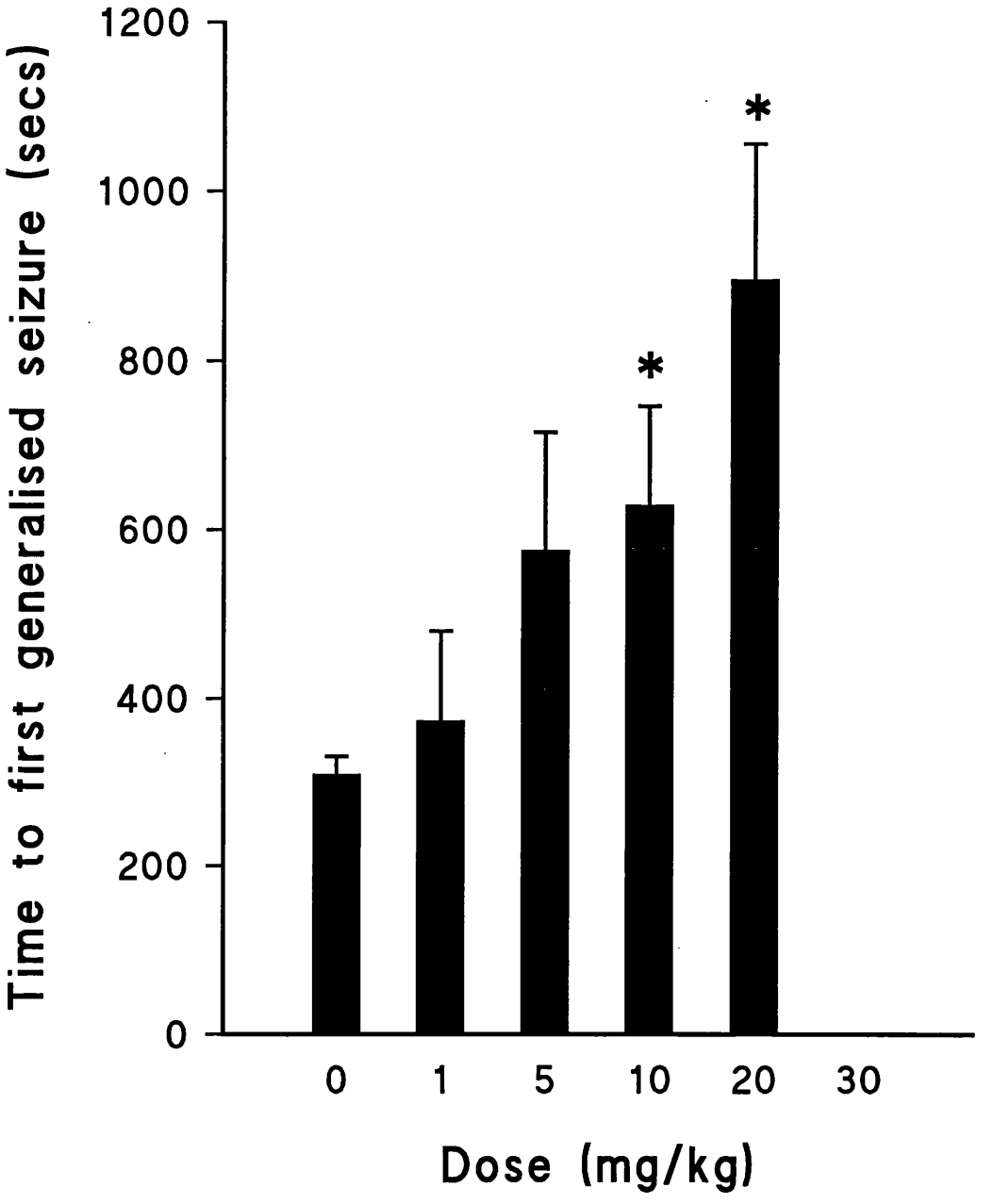

FIGURE 39:- Effect of single amlodipine doses (1 - 30 mg/kg) on the latency to the first generalised seizure induced by 85 mg/kg PTZ at 6 hours post-administration. Results are expressed as mean (± SEM) time (seconds) in groups of 6 mice. Statistical significance (*$p < 0.05$) was determined by the Mann Whitney U-test.

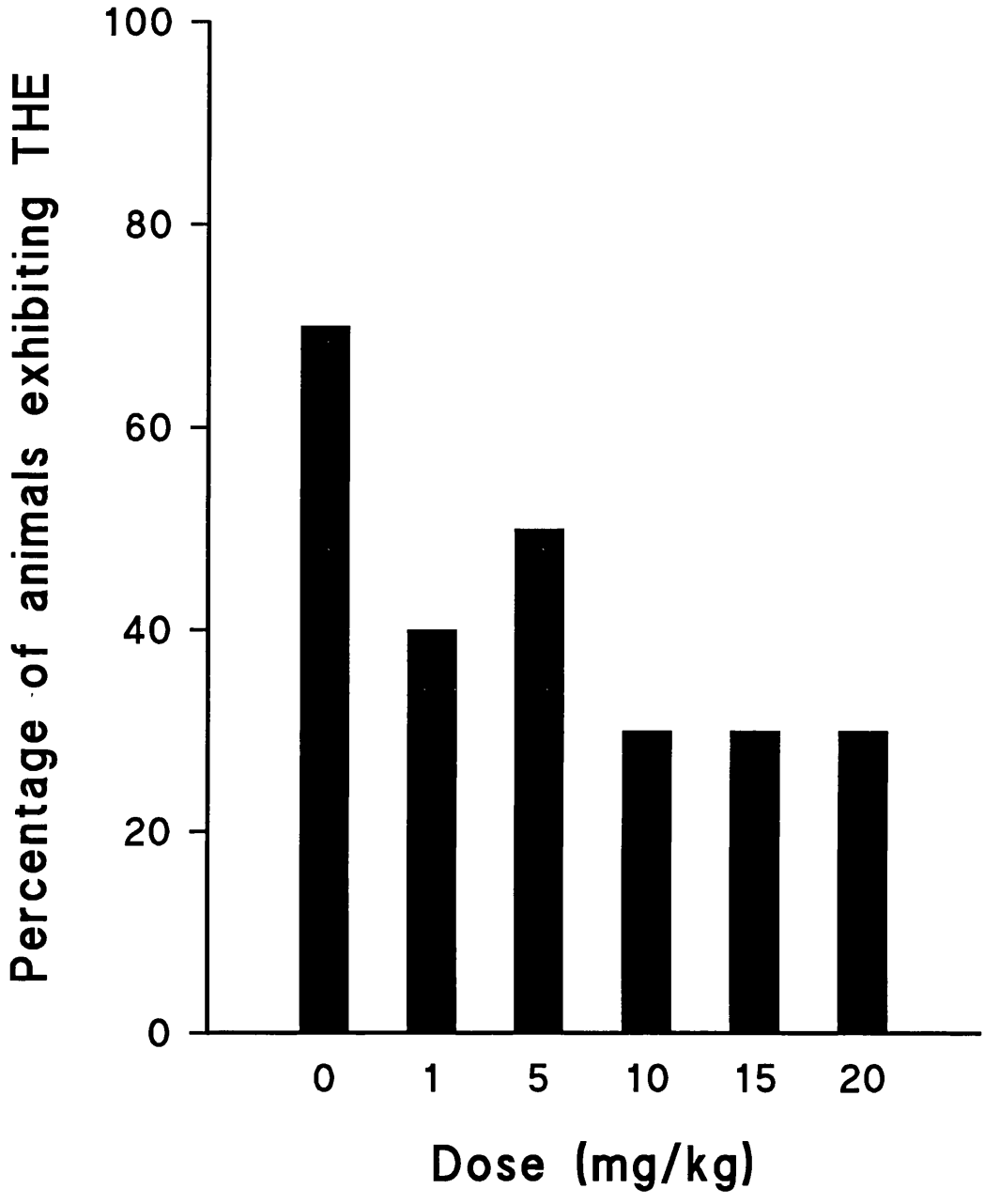

FIGURE 40:- Effect of single amlodipine doses (1 - 20 mg/kg) on the incidence of tonic hind-limb extension (THE) induced by MES at 6 hours post-administration. Results are expressed as the percentage of animals (mice) in groups of 10 exhibiting THE.

5.3.10 Time dependent effects of single amlodipine administration on pentylenetetrazol-induced seizures in mice

PROTOCOL:- Mice were separated into two treatment groups (n=80/group) and were given either 10 mg/kg AML (i.p.) or vehicle (0.9% saline) alone. Ten animals from each group were sequentially subjected to the s.c. CD_{97} PTZ test at 1, 2, 4, 6, 8, 12, 24, and 36 hours post-administration. The latency to the first generalised seizure in individual animals was recorded.

RESULTS:- Single dose AML (10 mg/kg) significantly (1, 4, and 6 hours, $p < 0.005$; 2, 8, and 12 hours, $p < 0.05$) lengthened the time to the first generalised seizure induced by PTZ at 1, 2, 4, 6, 8, and 12 hours post-administration (figure 41). AML was without significant effect at 24 and 36 hours post-dosing.

5.3.11 Time dependent effects of single amlodipine administration on maximal electroshock-induced seizures in mice

PROTOCOL:- Mice were separated into two treatment groups (n=80/group) and were given either 10 mg/kg AML (i.p.) or vehicle (0.9% saline) alone. Ten animals from each group were sequentially subjected to the MES test at 1, 2, 4, 6, 8, 12, 24, and 36 hours post-administration using a stimulus current of 54 mA. The incidence of THE within each group was recorded.

RESULTS:- Single dose AML (10 mg/kg) significantly reduced the incidence of THE induced by MES at 8 ($p < 0.005$) and 12 ($p < 0.01$) hours post-administration alone (figure 42). AML was without significant effect at all other time points investigated.

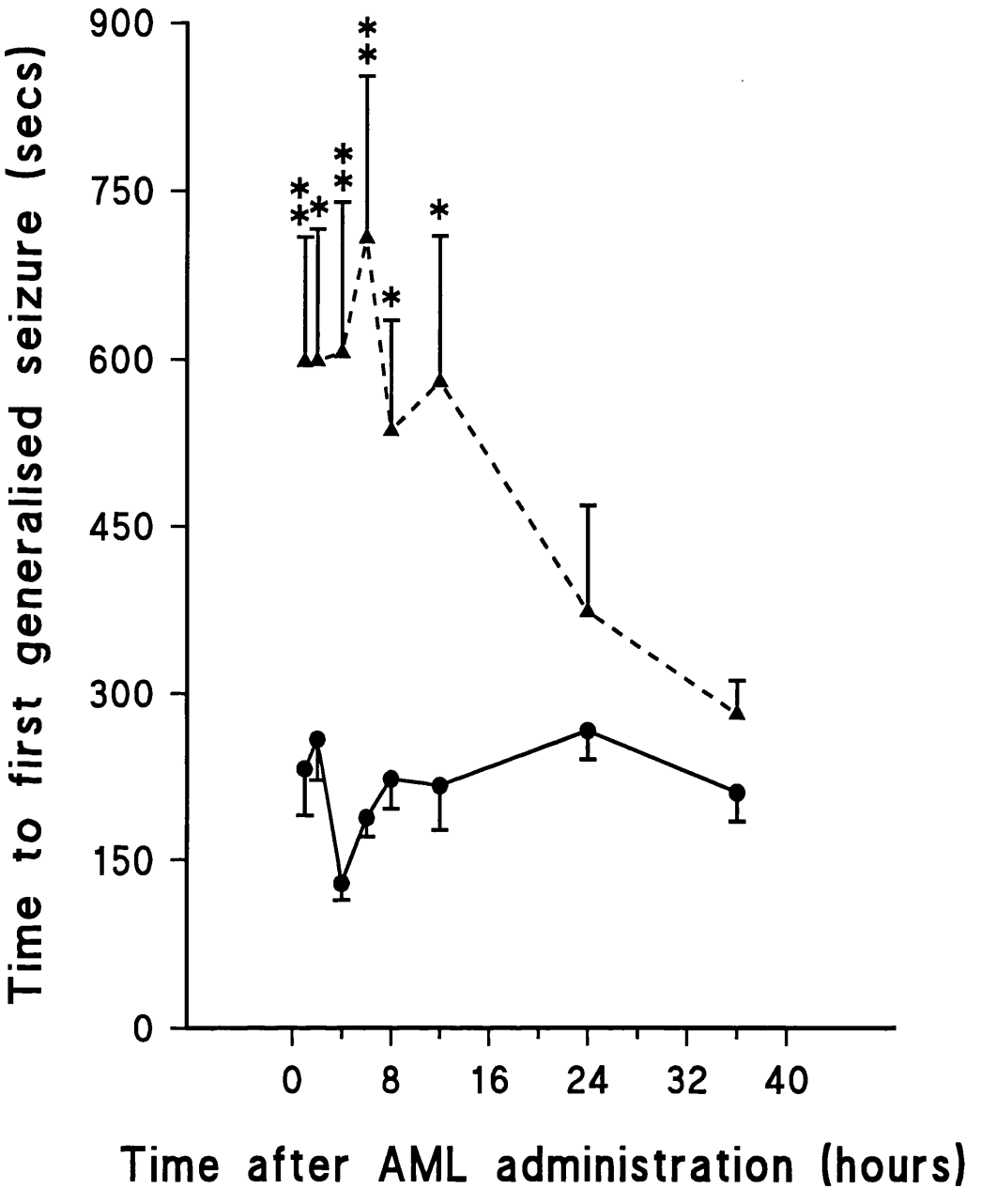

FIGURE 41:- Effect of a single amlodipine (AML) dose (10 mg/kg; triangles, broken line) vs control (circles, solid line) on the latency to the first generalised seizure induced by 85 mg/kg PTZ at 1 - 36 hours post-administration. Results are expressed as the mean (± SEM) time (seconds) in groups of 6 mice. Statistical significance (*$p < 0.05$; **$p < 0.005$) was determined by the Mann Whitney U-test.

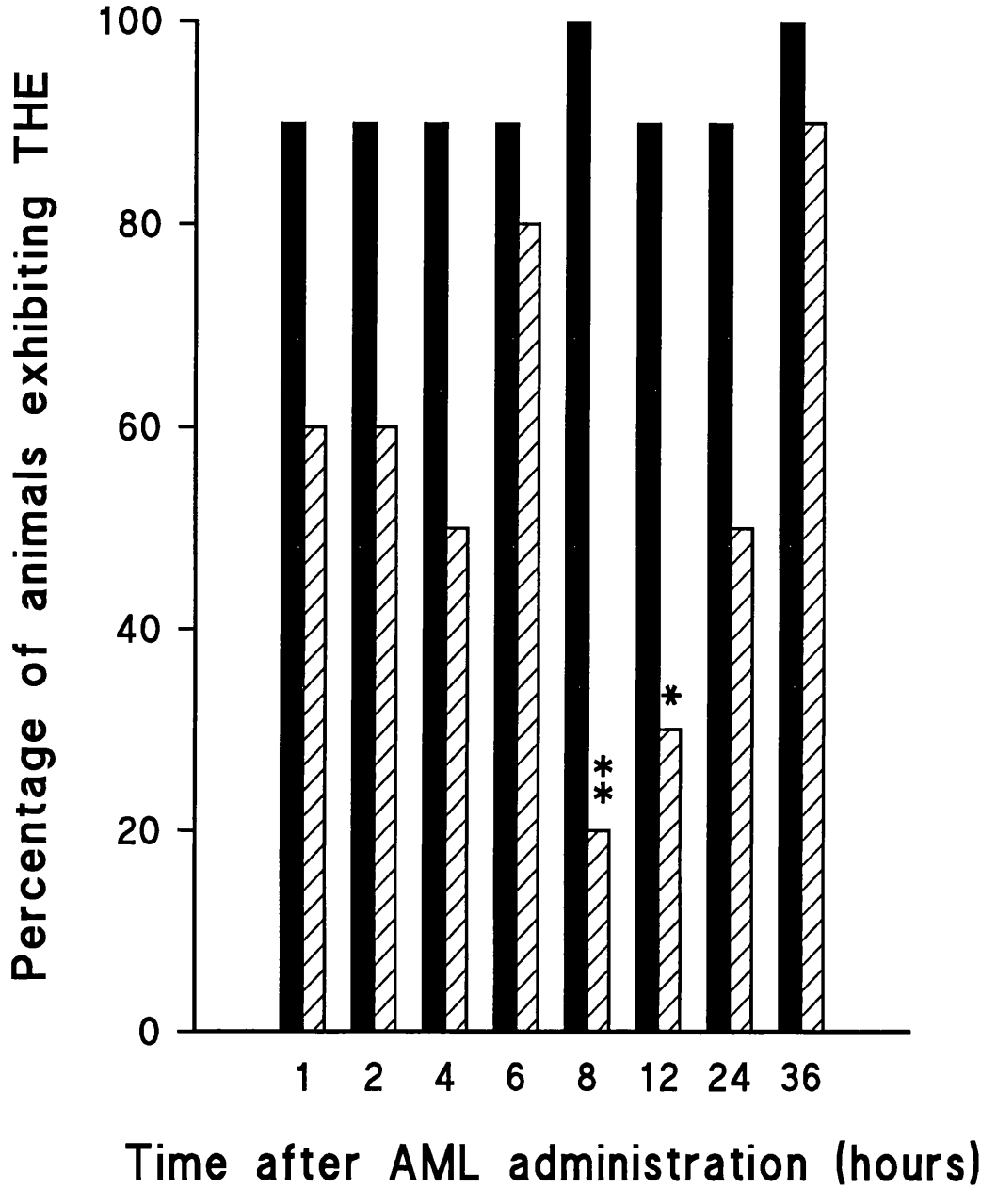

FIGURE 42:- Effect of single amlodipine (AML) treatment (10 mg/kg; hatched bars) vs control (solid bars) on the incidence of tonic hind-limb extension (THE) induced by MES at 1 - 36 hours post-administration. Results are expressed as the percentage of animals (mice) in groups of 10 exhibiting THE. Statistical significance (*$p < 0.01$; **$p < 0.005$) was determined by the Chi square test.

5.3.12 Effect of nimodipine on potassium-induced calcium influx into cerebellar granule cells in primary culture

PROTOCOL:- Primary cultures of cerebellar granule cells, isolated and maintained as described in section 2.9, were separated into 4 groups (n=10/group). Two groups were exposed to 1 μM NMD in BSS for 1 hour, while the remaining two groups were exposed to BSS alone. After the incubation period the cultures were assayed for calcium influx by the procedure described in section 2.13.3.

RESULTS:- NMD (1 μM) significantly ($p < 0.05$) reduced the calcium influx induced by 55 mM K^+ into primary cultures of cerebellar granule cells (figure 43). NMD was without effect on basal calcium influx into non-depolarised cultures.

5.3.13 Effect of nimodipine on veratridine-induced calcium influx into cerebellar granule cells in primary culture

PROTOCOL:- Primary cultures of cerebellar granule cells, isolated and maintained as described in section 2.9, were separated into 4 groups (n=8/group). Two groups were exposed to 1 μM NMD in BSS for 1 hour while the remaining two groups were exposed to BSS alone. After the incubation period the cultures were assayed for calcium influx by the procedure described in section 2.13.4.

RESULTS:- NMD (1 μM) significantly ($p < 0.05$) reduced the calcium influx induced by 50 μM veratridine into primary cultures of cerebellar granule cells (figure 44). NMD was without effect on basal calcium influx into non-veratridine challenged cultures.

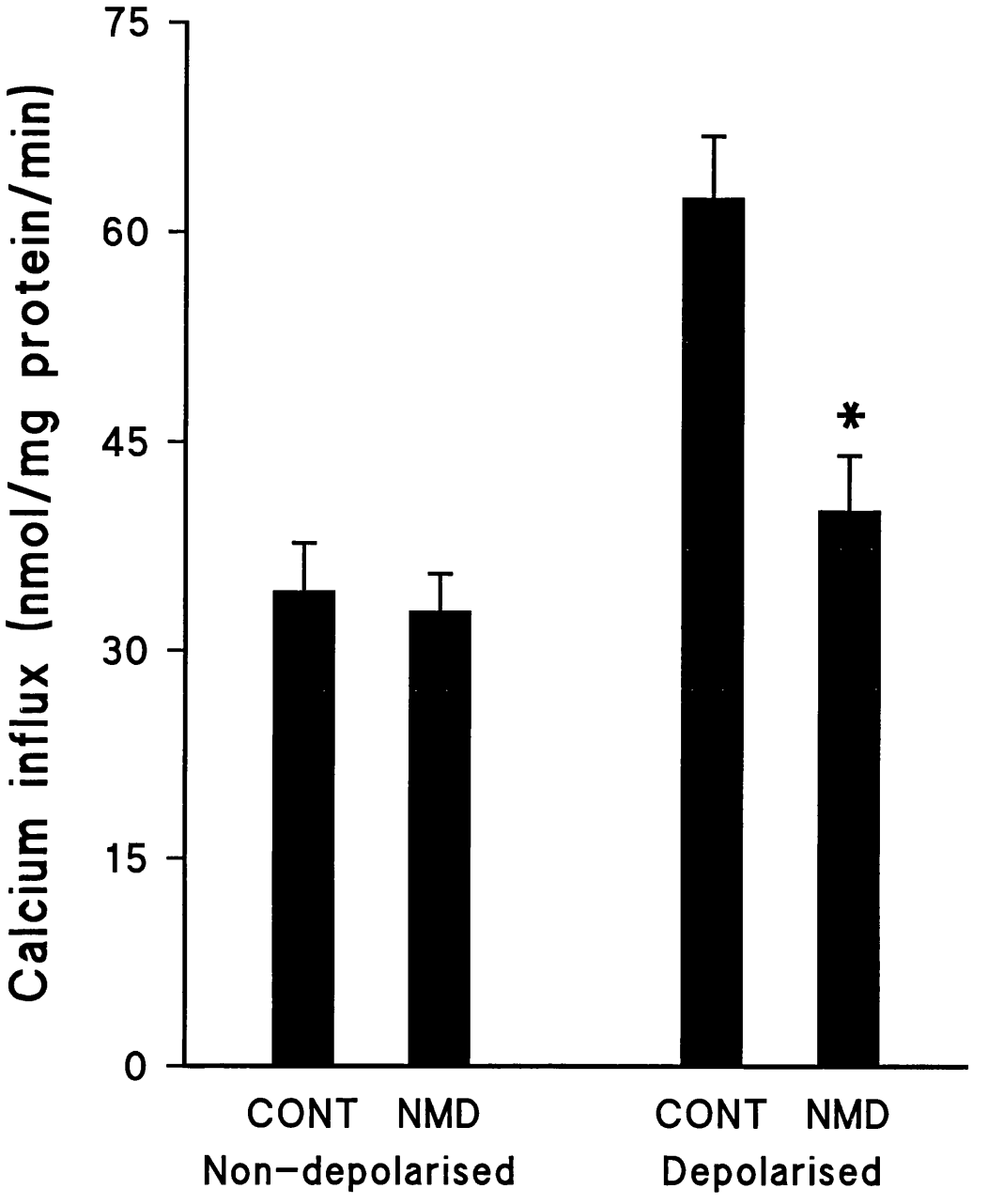

FIGURE 43:- Effect of 1 hour exposures to control (CONT) and nimodipine (NMD; 1 μM) treatments on calcium influx into non-depolarised and potassium-depolarised cerebellar granule cells in primary culture. Results are expressed as the mean (± SEM) calcium influx in groups of 10 plates. Statistical significance (*$p < 0.05$) was determined by the Mann Whitney U-test.

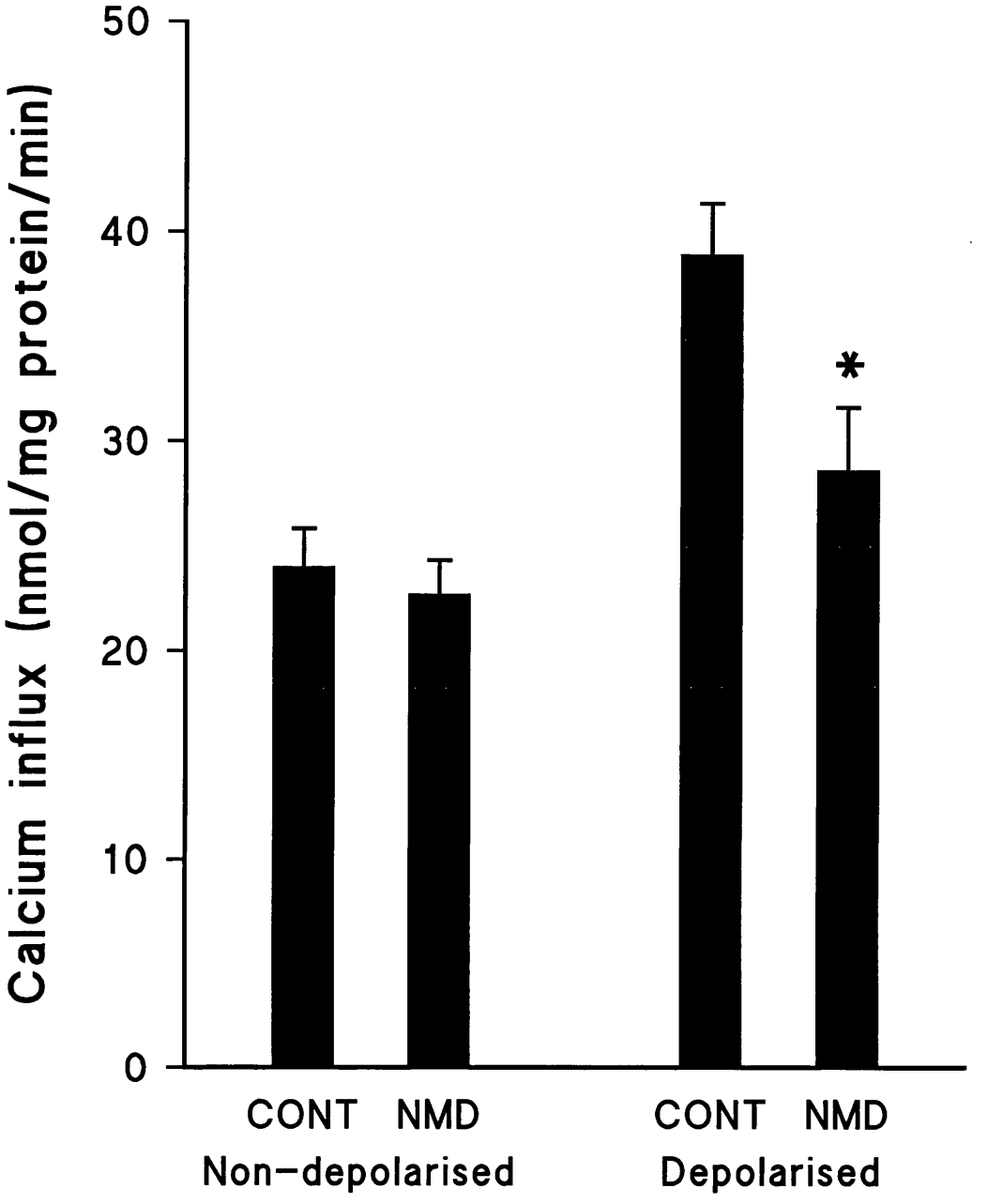

FIGURE 44:- Effect of 1 hour exposures to control (CONT) and nimodipine (NMD; 1 μM) treatments on calcium influx into non-depolarised and veratridine-depolarised cerebellar granule cells in primary culture. Results are expressed as the mean (± SEM) calcium influx in groups of 8 plates. Statistical significance (*$p < 0.05$) was determined by the Mann Whitney U-test.

5.3.14 Effect of nimodipine on potassium-induced calcium influx into cerebral cortical astrocytes in primary culture

PROTOCOL:- Primary cultures of cerebral cortical astrocytes, isolated and maintained as described in section 2.10, were separated into 4 groups (n=6/group). Two groups were exposed to 1 μM NMD in BSS for 1 hour while the remaining two groups were exposed to BSS alone. After the incubation period the cultures were assayed for calcium influx by the procedure described in section 2.13.3.

RESULTS:- NMD (1 μM) significantly ($p < 0.05$) reduced the calcium influx induced by 55 mM K^+ into primary cultures of cerebral cortical astrocytes (figure 45). NMD was without effect on basal calcium influx into non-depolarised cultures.

5.3.15 Effect of nimodipine on potassium-induced glutamate release from cerebellar granule cells in primary culture

PROTOCOL:- Primary cultures of cerebellar granule cells, isolated and maintained as described in section 2.9, were separated into 4 groups (n=8/group). Two groups were exposed to 1 μM NMD in BSS for 1 hour while the remaining two groups were exposed to BSS alone. After the incubation period the cultures were assayed for glutamate release by the procedure described in section 2.14.

RESULTS:- NMD (1 μM) was without effect on glutamate release induced by 55 mM K^+ from primary cultures of cerebellar granule cells (figure 46). NMD was similarly without effect on basal glutamate release from non-depolarised cultures.

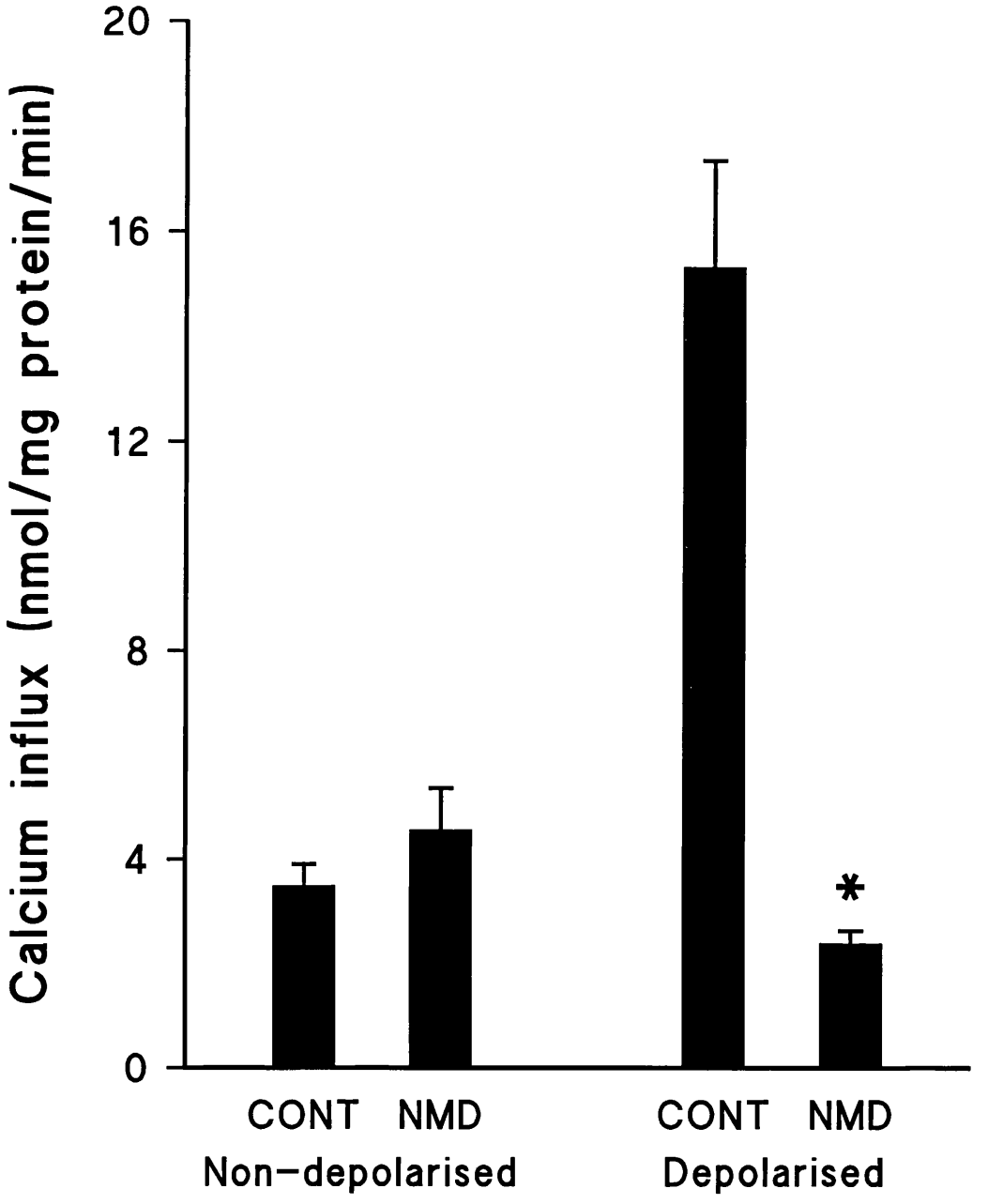

FIGURE 45:- Effect of 1 hour exposures to control (CONT) and nimodipine (NMD; 1 μM) treatments on calcium influx into non-depolarised and potassium-depolarised cerebral cortical astrocytes in primary culture. Results are expressed as the mean (± SEM) calcium influx in groups of 6 plates. Statistical significance (*$p < 0.05$) was determined by the Mann Whitney U-test.

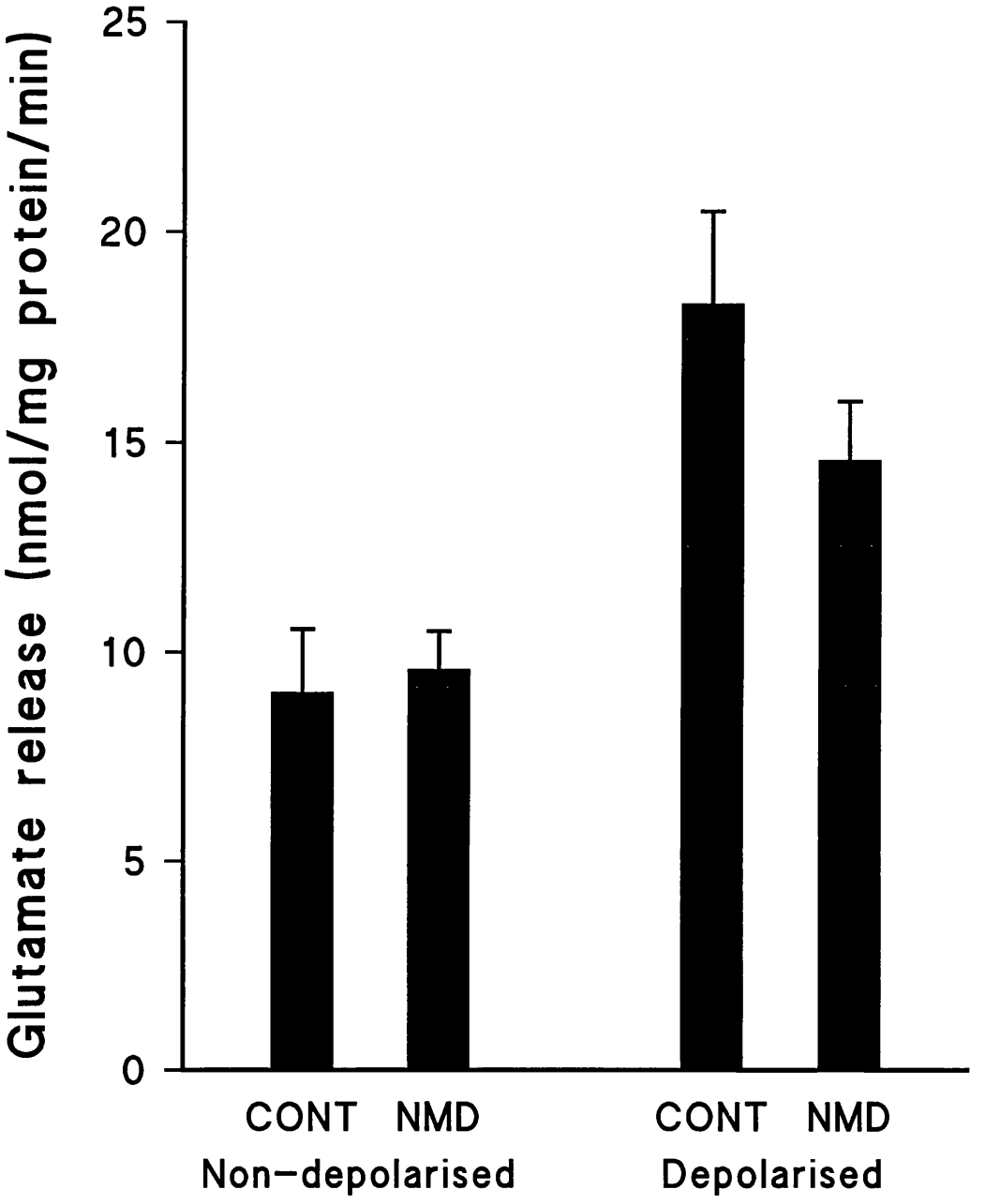

FIGURE 46:- Effect of 1 hour exposures to control (CONT) and nimodipine (NMD; 1 μM) treatments on glutamate release from non-depolarised and potassium-depolarised cerebellar granule cells in primary culture. Results are expressed as the mean (± SEM) glutamate release in groups of 8 plates.

5.3.16 Effect of amlodipine on potassium-induced calcium influx into cerebellar granule cells in primary culture

PROTOCOL:- Primary cultures of cerebellar granule cells, isolated and maintained as described in section 2.9, were separated into 4 groups (n=10/group). Two groups were exposed to 1 μM AML in BSS for 1 hour while the remaining two groups were exposed to BSS alone. After the incubation period the cultures were assayed for calcium influx by the procedure described in section 2.13.3.

RESULTS:- AML (1 μM) significantly ($p < 0.05$) reduced the calcium influx induced by 55 mM K^+ into primary cultures of cerebellar granule cells (figure 47). AML was without effect on basal calcium influx into non-depolarised cultures.

5.3.17 Effect of amlodipine on veratridine-induced calcium influx into cerebellar granule cells in primary culture

PROTOCOL:- Primary cultures of cerebellar granule cells, isolated and maintained as described in section 2.9, were separated into 4 groups (n=10/group). Two groups were exposed to 1 μM AML in BSS for 1 hour while the remaining two groups were exposed to BSS alone. After the incubation period the cultures were assayed for calcium influx by the procedure described in section 2.13.4.

RESULTS:- AML (1 μM) was without effect on calcium influx induced by 50 μM veratridine into primary cultures of cerebellar granule cells (figure 48). AML was similarly without effect on basal calcium influx into non-depolarised cultures.

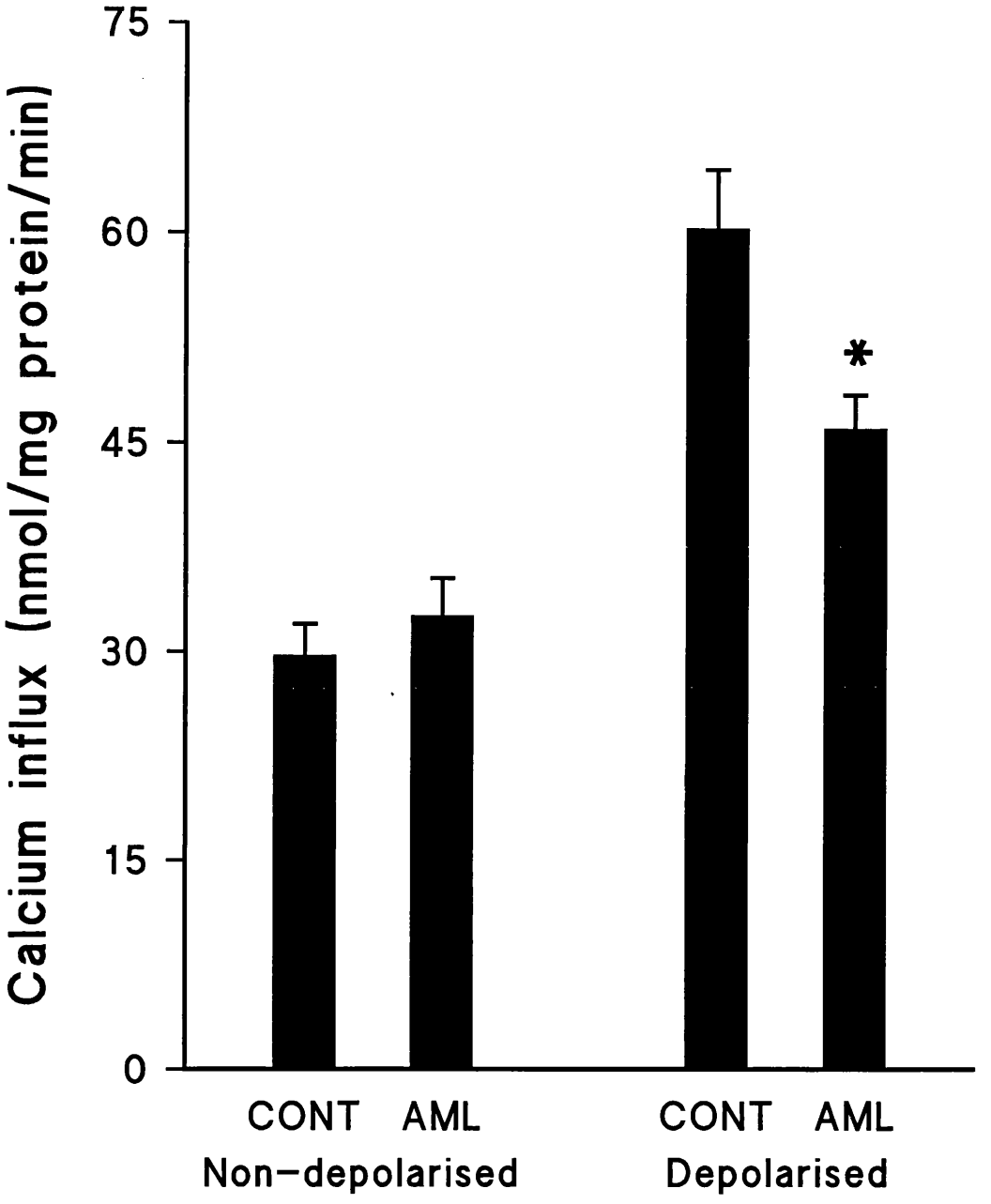

FIGURE 47:- Effect of 1 hour exposures to control (CONT) and amlodipine (AML; 1 μM) treatments on calcium influx into non-depolarised and potassium-depolarised cerebellar granule cells in primary culture. Results are expressed as the mean (± SEM) calcium influx in groups of 10 plates. Statistical significance (*$p < 0.05$) was determined by the Mann Whitney U-test.

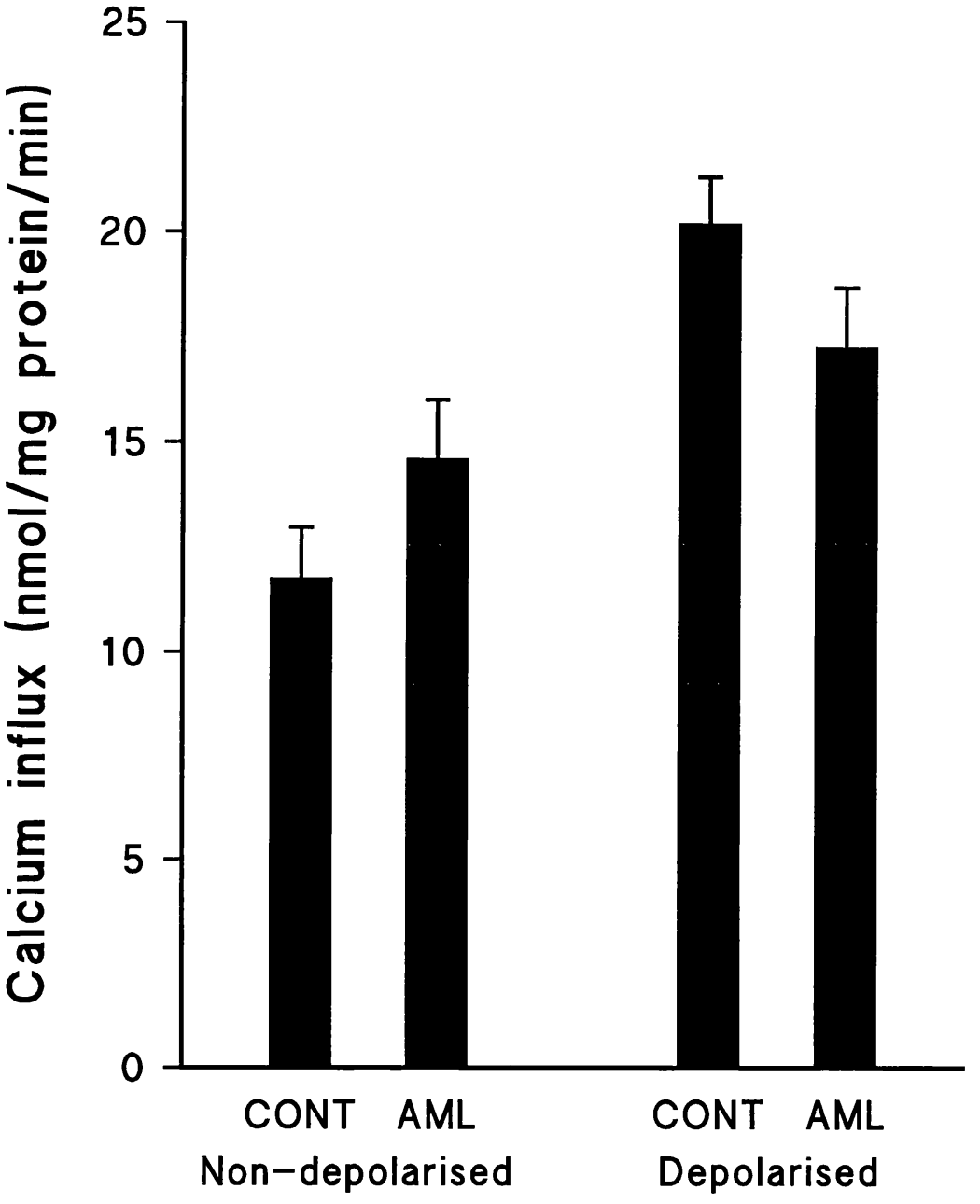

FIGURE 48:- Effect of 1 hour exposures to control (CONT) and amlodipine (AML; 1 μM) treatments on calcium influx into non-depolarised and veratridine-depolarised cerebellar granule cells in primary culture. Results are expressed as the mean (± SEM) calcium influx in groups of 10 plates.

5.4 DISCUSSION

With the overwhelming evidence for the involvement of voltage-sensitive calcium influx at all stages of epileptogenesis, the DHP compounds have been proposed as putative AEDs. The aims of these studies were to investigate the experimental anticonvulsant profile of the DHPs and to explore their mechanism of action.

Dose dependent effects of acute NMD on MES-induced seizures

NMD has probably been the most extensively investigated DHP with regard to potential anticonvulsant properties. It has demonstrated efficacy against a wide range of experimentally induced seizures (De Sarro et al, 1988; Meyer et al, 1990). Larkin and colleagues (1992b), in our laboratory, showed NMD to effectively increase the latency to PTZ-induced seizures following single administration. The studies with NMD described above were conducted as a continuation of these initial observations. Single dose NMD produced a dose-dependent attenuation of tonic seizures induced by MES with an ED_{50} of 87 mg/kg (figure 29). This result contrasted reports of a lack of effect of the drug in the MES test (Hoffmeister et al, 1982; Wong and Rahwan, 1989; Palmer et al, 1993) but supported the results of Kamal and co-workers (1990). The discrepancies in outcome of these different studies may reflect experimental differences in electrical stimulus site. The negative findings arose following nose and forehead and also corneal application of electrical stimulus whereas positive studies, including those reported here, employed ear-clip electrodes. Such observations would support the stimulus site dependency of drug effects against electroshock seizures proposed by Löscher and colleagues (1991a).

Time dependent effects of acute NMD on MES-induced seizures

A single 75 mg/kg dose of NMD was seen to reduce the incidence of THE induced by MES for up to 12 hours (figure 30). Concomitant analysis of plasma and brain drug levels revealed NMD to be below detectable limits by this time (figure 31). While the time-dependency of effect is not routinely determined in experimental studies of anticonvulsant drugs, one researcher (Thomas, 1990) has observed a significant effect of a similar dose of NMD against picrotoxin-induced seizures in female rats 24 hours after a single administration. Thomas also demonstrated an anticonvulsant effect of a much lower dose of NMD (5 mg/kg) at 72 hours after single administration. These data would suggest that the experimental anticonvulsant effects of NMD are both dose-dependent, as discussed above, and time-dependent. The pharmacokinetic data obtained here mirror previously published work by Larkin and colleagues (1992b), who reported the half-time of elimination of NMD from mouse brain to be 22.4 minutes. Thus, the pharmacodynamic effects of NMD may extend beyond the time suggested by its pharmacokinetic profile. However, even with the high resolution HPLC assay employed, it is possible that sufficient, undetectable amounts of NMD remain in the brain at 12 hours to produce an anticonvulsant effect. Clinical observations have led to the postulation that even in the presence of detectable drug levels in the plasma, NMD was devoid of a significant antiepileptic effect due to insufficient concentrations of circulating drug (Larkin et al, 1991). Although it is possible that NMD does not penetrate the brain as effectively in humans, the observations of Thomas (1990), and those reported here, do not support this theory. A detailed pharmacokinetic investigation of NMD in animals, and possibly in man, using a higher resolution assay is required to clarify these observations further.

Effects of acute NMD on Min-ES seizures

A single 75 mg/kg dose of NMD was observed to raise the threshold for the induction of tonic seizures by Min-ES (figure 32). Employing the loose parallels drawn by Löscher and colleagues (1991a), this outcome would suggest that NMD is capable of reducing seizure spread, and, as such, may be active against generalised motor seizures in man.

Dose dependent effects of chronic NMD on PTZ-induced seizures

Following repeated administration NMD was without effect on PTZ-induced seizures at any of the doses investigated (figure 33). These results support the findings of Jagiello-Wótowicz and co-workers (1991) who also reported a loss of effect following repeated administration of similar DHP doses. This apparent loss of anticonvulsant efficacy, when compared to acute studies (O'Neill and Bolger, 1990; Larkin et al, 1992b), at 4 hours after the final NMD dose may be explained in terms of the proposed time-dependency of NMD effect, discussed above. It may also reflect a down-regulation of DHP binding sites in the brain and, as such, might help to explain the lack of effect of the DHPs in some clinical studies (Larkin et al, 1991; Larkin et al, 1992a). Investigations of radioligand binding to the DHP-sensitive site on L-type calcium channels in the brain are required to clarify this latter hypothesis.

Dose dependent effects of chronic NMD on MES-induced seizures

Repeated administration of 75 mg/kg NMD reduced the incidence of tonic seizures induced by MES at 4 hours after the final dose (figure 34). All of the other doses investigated were without significant effect. Again these results do not correlate with those obtained from the single dose studies, described above. While this loss of efficacy may

again reflect the time-dependency of NMD effect and/or biochemical alterations in binding site characteristics, one would imagine that either or both of these explanatory phenomena would be dose-dependent.

Time dependent effects of chronic NMD on PTZ-induced seizures

Repeated administration of 50 mg/kg NMD, selected as the most effective, albeit non-significant, dose in experiment 5.3.4 (figure 33), resulted in an increased latency to PTZ-induced seizures at 1, 12, and 36 hours after the final dose (figure 35). NMD was ineffective at all of the other time points investigated. Concomitant analysis of drug levels in the plasma and brain revealed NMD concentrations to be below detectable limits by 12 hours post-administration (figure 36). These results again suggest that the drug continues to exert an anticonvulsant effect after it becomes undetectable in the brain. There also appears to be no consistent time-dependent pattern to NMD effects against PTZ-induced seizures. The proposed explanations for the absolute lack of effect of NMD in experiment 5.3.4 (figure 33) would appear to be contradicted by this intermittent effect, interpretation of which is difficult.

Time dependent effects of chronic NMD on MES-induced seizures

Repeated administration of 75 mg/kg NMD, selected as the only effective dose in experiment 5.3.5 (figure 34), produced a reduction in the incidence of THE induced by MES at 2, 8, and 12 hours after the final dose (figure 37). NMD was ineffective at all of the other time points investigated. Concomitant analysis of plasma and brain drug levels revealed NMD concentrations to be below detectable limits at 24 hours post-administration (figure 38). In this case, the pharmacodynamic effects of NMD in the

MES test did not appear to extend beyond the time suggested by its pharmacokinetic profile. Contrary to the results obtained in experiment 5.3.5 (figure 34), NMD failed to exhibit an anticonvulsant effect at 4 hours post-dosing. Again the effects of repeated NMD treatment appear to be intermittent and difficult to interpret.

Dose dependent effects of acute AML on PTZ-induced seizures

AML was employed as a comparator to the effects of NMD to determine whether this longer-acting, non-photolabile DHP (Meredith and Elliot, 1992) might exert a similar experimental anticonvulsant profile. It was proposed that the extended half-life of this drug would render it more suitable than the short-acting compounds, NMD and NFD, in the long-term treatment of epilepsy. Single doses of AML (10 and 20 mg/kg) increased the latency to PTZ-induced seizures at 6 hours post-administration (figure 39). Doses of 1 and 5 mg/kg AML were without significant anticonvulsant effect. An important observation was the death of one animal given 20 mg/kg AML and all six animals given 30 mg/kg. Following single administration, AML effects on PTZ-induced seizures did not exhibit a clear dose-dependency, and the anticonvulsant effects of the drug were observed at doses close to those producing lethality. While AML does attenuate PTZ-induced seizures in a similar manner to NMD (Larkin et al, 1992b), its narrow therapeutic window may preclude its further evaluation as a putative AED. The use of the rotarod test or a similar measure of experimental neurotoxicity might help to clarify the position of AML as a potential AED more definitively.

Dose dependent effects of acute AML on MES-induced seizures

Single doses of AML (1 - 20 mg/kg) were without significant effect on tonic seizures induced by MES at 6 hours post-administration (figure 40). No lethality was observed at any of the doses investigated. These results suggest that AML exhibits a different experimental anticonvulsant profile to NMD. Although the spectrum of anticonvulsant effect of AML may be less wide than that observed with NMD, one must be cautious in disregarding AML as a potential AED in light of the time-dependency of DHP effect proposed for NMD above.

Time dependent effects of acute AML on PTZ-induced seizures

Single dose AML (10 mg/kg), selected as the most effective, non-lethal, dose in experiment 5.3.8 (figure 39), significantly increased the time to first generalised seizure induced by PTZ for up to 12 hours post-administration (figure 41). AML was without effect at 24 and 36 hours post-dosing. These results suggest that acute AML has a similar anticonvulsant action to NMD against chemoconvulsant-induced seizures, as defined by the results of Thomas (1990) and Larkin and colleagues (1992b).

Time dependent effects of acute AML on MES-induced seizures

Single dose AML (10 mg/kg), selected as the most effective, albeit non-significant, dose in experiment 5.3.9 (figure 40), significantly reduced the incidence of THE induced by MES at 8 and 12 hours post-administration alone (figure 42). AML was without effect at all of the other time points investigated. These results appear to contradict those obtained in experiment 5.3.9 (figure 40), which suggested that AML was devoid of activity in the MES test. When evaluated together, the results of these two studies provide a degree of

support for the proposed time-dependency to the anticonvulsant effects of the DHPs. Nevertheless, the effects of AML in the MES test appear to be limited and to differ significantly from those of NMD.

Effects of NMD on calcium influx into neurones

A one hour exposure to NMD (1 μM) significantly reduced calcium influx into cerebellar granule cell neurones following potassium-induced depolarisation (figure 43). The drug was without effect on basal calcium entry into non-depolarised cultures. These results served to confirm the mechanism of DHP action as blockade of voltage-sensitive calcium entry and supported previous observations by Carboni and Wojcik (1988) in this respect.

NMD (1 μM) also reduced neuronal calcium influx into cerebellar granule cells following veratridine-induced depolarisation (figure 44). Again the drug was without effect on basal calcium entry into non-veratridine challenged cultures. These results further supported the putative mechanism of DHP action and suggested that these drugs may reduce calcium entry induced by non-specific depolarising stimuli. This study supported the findings of Carboni and colleagues (1985) in this respect.

Effects of NMD on calcium influx into astrocytes

NMD (1 μM) reduced calcium influx into primary cultures of cerebral cortical astrocytes following potassium-induced depolarisation (figure 45). NMD was without effect on basal calcium entry into non-depolarised cultures. These observations correlated with the reported mechanism of DHP action and supported the findings of Hertz and co-workers (1989) in this respect. These data also provided further evidence for the existence of

voltage-sensitive calcium channels on astrocytes, as proposed by MacVicar (1984). The physiological role of these channels remains unclear.

Effects of NMD on glutamate release from neurones

Glutamate is believed to be the predominant neurotransmitter released by cerebellar granule cell neurones in culture (Gallo et al, 1982). A one hour exposure to NMD (1 μM) failed to influence potassium-induced glutamate release from primary cultures of cerebellar granule cells (figure 46). NMD was also without effect on basal release from non-depolarised cultures. These results suggested that all of the depolarisation-induced calcium influx observed in the previous studies was mediated by the cell soma, with no involvement of the pre-synaptic terminals. Such a suggestion would be in keeping with the cellular distribution of calcium channel sub-types proposed by Meyer (1989). Voltage-sensitive calcium channels of the L- and T-types are believed to be located post-synaptically and involved in synaptic transmission, whereas pre-synaptic control of neurotransmitter release is thought to be mediated by the N-type channel alone. Although Lemos and Nowycky (1989) have suggested that the L-type channel may also have a pre-synaptic location, the present studies did not support this finding. It is possible, however, that the distribution of voltage-sensitive calcium channels on the dendrites, cell soma, and pre-synaptic nerve terminals may be dependent on cell type.

Effects of AML on calcium influx into neurones

AML (1 μM) significantly reduced calcium influx into primary cultures of cerebellar granule cell neurones following potassium-induced depolarisation (figure 47). The drug was without effect on basal calcium entry into non-depolarised cultures. These results

suggested that AML possessed a similar mechanism of action to NMD in this respect, and provided further evidence to support blockade of voltage-sensitive calcium entry as a predominant mechanism of DHP action.

A one hour exposure to AML (1 μM) failed to influence veratridine-induced calcium influx into primary cultures of cerebellar granule cells (figure 48). The drug was also without effect on basal calcium entry into non-veratridine challenged cultures. These results suggested subtle differences in the mechanisms of action of AML and NMD which might correlate with their distinct experimental anticonvulsant profiles, discussed above. Further studies are required to elucidate the precise mechanisms by which individual DHP compounds exert their effects.

5.5 CONCLUSIONS

In conclusion, the DHPs appear to be effective experimental anticonvulsants, although the effects of individual compounds may vary with drug incubation time and the seizure test employed. NMD was effective in transauricular electroshock seizure models following single administration, and the pharmacodynamic effects of the drug, in these models, were proposed to extend beyond the time suggested by its pharmacokinetic profile. NMD was much less effective against experimentally-induced seizures following repeated administration. The anticonvulsant effects of the drug in this respect were intermittent and unpredictable, and did not appear to be concentration- or time-dependent. AML appeared to possess a different experimental anticonvulsant profile to NMD, and to elicit its anticonvulsant effects at doses close to those producing lethality. Exploration of the mechanism of DHP action confirmed previous reports, which had suggested their capacity

to limit depolarisation-induced, voltage-sensitive calcium influx. The differential effects of individual compounds, in this respect, were proposed to confer their differing anticonvulsant effects in animal seizure models. Finally, NMD appeared unable to influence calcium-dependent glutamate release, an effect consistent with the reported differential cellular distribution of calcium channel subtypes.

CHAPTER SIX

COMBINATIONS OF NOVEL ANTIEPILEPTIC DRUGS

6.1 INTRODUCTION

6.1.1 Antiepileptic drug polypharmacy

It has been reported that between 70 and 80% of the epileptic population can be adequately controlled by existing pharmacological therapies (Meinardi, 1992). A large proportion of these patients can expect to experience complete abolition of seizures with antiepileptic monotherapy (Beghi et al, 1986). Patients whose seizures cannot be controlled by existing monotherapies are treated with combinations of two or more AEDs (Bourgeois, 1988). This group often includes the 20% or more of patients with refractory epilepsy, resistant to any form of pharmacological treatment (Brodie, 1990). There are two major indications for prescribing a combination of two or more AEDs (Bourgeois, 1988). Firstly, the combination protects against two or more different seizure types that cannot be controlled by one of the drugs alone (i.e. a broader antiepileptic spectrum). Secondly, the combination provides better seizure control at a given level of toxicity or a similar degree of seizure control with less toxicity than observed with either drug given alone (i.e. better protective index).

Clear clinical evidence supporting efficacy for particular AED combinations is scarce and difficult to obtain. It requires a quantitative assessment of the antiepileptic effect, and of dose-related side effects, of single drugs and combinations in relatively homogeneous populations of epileptic patients. While there is some evidence to suggest that 10 - 20% of patients with refractory epilepsy will benefit substantially from treatment with two drugs (Reynolds and Shorvon, 1981), there is much more evidence which suggests that polypharmacy produces cognitive impairment (Albright and Bruni, 1985; Trimble, 1987)

and complicated drug interactions (Brodie, 1992). Thus, until recently, treatment of epilepsy with two or more drugs has been frowned upon.

The fact that almost 20% of the epileptic population remain resistant to treatment despite recent additions to the clinician's armamentarium necessitates not only the development of further novel antiepileptic therapies, but also the design of a rational basis for combining established AEDs. Until the advent of new therapeutic agents, the problem of refractory epilepsy can be addressed by the use of experimental seizure models to determine the potential protective indices of combinations of existing AEDs (Millichap, 1969).

6.1.2 Experimental studies with combinations of established antiepileptic drugs

Most combinations of the conventional AEDs have been studied in experimental models (Bourgeois, 1988). The vast majority of pharmacodynamic interactions are characterised by a purely additive effect on experimental seizure protection (Leppik and Sherwin, 1977; Bourgeois, 1986; Morris et al, 1987). However, the neurotoxic interaction is infra-additive in approximately one half of the combinations studied (Bourgeois, 1988). This observation would allude to a potentially better therapeutic index for these combinations than for either component drug alone. Such combinations include PB with PHT (Bourgeois, 1986), VPA with CBZ (Bourgeois, 1988), and PB with CBZ (Kostadinova et al, 1992). One of the problems of developing a rational basis for AED polypharmacy from experimental studies with these established compounds arises from their ill-defined and multifactorial mechanisms of action (section 1.3). The potential of combining recently developed AEDs, with known mechanisms of action, either with themselves or with

existing pharmacotherapies in an attempt to achieve superior therapeutic indices remains to be evaluated.

6.1.3 Classification of antiepileptic drug mechanisms

From the preceding chapters it is evident that pharmacological enhancement of inhibitory function and/or attenuation of excitatory function in the brain has antiepileptic effects. The enhanced GABAergic inhibition observed with VGB and TGB (chapter 4) afforded protection against some experimentally-induced seizures and both of these compounds have known clinical efficacy in the treatment of epilepsy (Reynolds et al, 1991; Leach and Brodie, in press). Furthermore, several established AEDs in clinical use, including VPA and PB, have been characterised in terms of their ability to potentiate GABA-mediated inhibition (Macdonald and Bergey, 1979; Macdonald and Barker, 1979). The reduction in excitation observed with NMD (chapter 5) also afforded some protection against experimentally-induced seizures and this drug has shown some promise for development as a novel antiepileptic agent (Pelliccia et al, 1990; de Falco et al, 1992). Again several established AEDs, including PHT and CBZ, are believed to exert their antiepileptic effects by reducing central excitation (Rogawski and Porter, 1990). Thus, it appears that there not only exists two discrete approaches for the development of novel antiepileptic agents, but also two distinct sub-types of AED with which to evaluate optimal combination therapy.

6.1.4 Experimental studies with combinations of novel antiepileptic drugs

Several studies have been performed to evaluate the potential of novel anticonvulsant drugs, in combination with both established AEDs and with other experimental stage

compounds, in the treatment of drug-resistant epilepsy. The CAs, discussed at length in chapter 5, have emerged as putative add-on drugs for the treatment of refractory epilepsy by virtue of their ability to potentiate the experimental anticonvulsant effects of existing AEDs without influencing their pharmacokinetics. Several CA compounds, including FNZ, NMD, and diltiazem, have been shown to enhance the anticonvulsant action of established AEDs, such as PHT and CBZ, in seizures induced by MES (Czuczwar et al, 1990; Czuczwar et al, 1992) and in audiogenic seizures in DBA/2 mice (De Sarro et al, 1992). Simultaneous administration of the CAs did not affect the plasma concentrations of existing antiepileptic therapies nor did the combination treatments result in enhanced neurotoxic side effects. Similarly, the CAs have demonstrated the ability to enhance the actions of other experimental stage compounds. Diltiazem has been shown to potentiate the anticonvulsant effects of NMD (Morón et al, 1989) and NMD has been proposed to exhibit synergism with the non-competitive NMDA antagonist MK-801 (O'Neill and Bolger, 1989) in the PTZ test. Finally, ameltolide, a recently characterised anticonvulsant with a supposedly similar anticonvulsant spectrum to PHT (Leander et al, 1988a) has been reported to enhance the anticonvulsant effects of PHT and CBZ in the MES test (Leander, 1992).

In the studies of novel anticonvulsant drug combinations outlined above, all of the established and experimental stage drugs employed have been proposed to exert their effects by reducing central excitation. Few studies have investigated the potential of combining agents which enhance inhibition, either with each other or with those which attenuate excitation.

A recent report (Klitgaard et al, 1993) highlighted a specific investigation of the effects of combinations of 4 anticonvulsant drugs, with well-defined mechanisms of action, on audiogenic seizures in DBA/2 mice. Of the drugs employed, diazepam and NNC-05-0711 (a GABA uptake inhibitor) were considered to enhance GABAergic inhibition, whereas CGP 39551 (an NMDA receptor antagonist) and NBQX (an AMPA receptor antagonist) were considered to reduce glutamatergic excitation. The authors concluded that anticonvulsant synergism was observed only when two compounds with similar mechanisms of action (i.e. reduced excitation or enhanced inhibition) were co-administered. No such observations were made when two compounds with different mechanisms were given together. These preliminary results suggested that reinforcement on one neurotransmitter system may be more effective than a combined action on two different systems. These experimental observations served as one of the first insights into a rational basis for combining drugs with known mechanisms of action for the treatment of refractory epilepsy. Unfortunately, the lack of concomitant neurotoxicity testing, in addition to the use of mostly experimental stage compounds, prevented definitive evaluation of the potential protective indices of such combinations for the clinical treatment of epilepsy.

6.1.5 Rational basis for experimental combinations of antiepileptic drugs

All of the positive observations discussed above, including those of Klitgaard and colleagues (1993), and those pertaining to the CAs, suggest that experimental, and perhaps clinical, combinations of AEDs should comprise of drugs with similar mechanisms of action. However, with the exception of the preliminary report of Klitgaard and co-workers, there appear to be no comparative studies, in terms of drug mechanisms, to substantiate

this proposal. Furthermore, the use of mostly experimental stage compounds by Klitgaard and colleagues, in addition to their lack of data on the neurotoxicity of drug combinations, prevents a more definitive evaluation on the basis for combining AEDs being reached. Thus, whether we should choose combinations of drugs with similar or different mechanisms of action remains unclear. That combinations of agents with similar mechanisms may be synergistic (Klitgaard et al, 1993), whereas those with different mechanisms may be more broad spectrum (Stolarek et al, in press), is purely speculation. Further investigations of AED combinations, with regard to their potential therapeutic indices, are clearly required.

Current development of new drugs with well-defined, single mechanisms of action suggests a potential for combining these synergistically and holds promise for a more rational approach to the treatment of refractory epilepsy with two or more AEDs. Many of these compounds, such as LTG and VGB, are now entering regular clinical use. These drugs remain less-well characterised in terms of potentially beneficial interactions than the established AEDs, discussed in section 6.1.2. Direct extrapolation of the experimental results of interaction studies with these new antiepileptic therapies into the clinical arena gives them an additional advantage over experimental stage compounds.

6.1.6 Lamotrigine as a novel antiepileptic agent

The observation that chronic treatment with AEDs could lead to impairment of folate metabolism (Reynolds et al, 1966), and the further demonstration that folates could produce seizures in animals (Hommes and Obbens, 1972), proposed the antifolate effects of the major AEDs to relate to their anticonvulsant action. LTG is a novel antiepileptic

agent which was discovered during the resultant screening of a range of antifolate compounds for anticonvulsant activity (Yuen, 1991). LTG is chemically unrelated to existing AEDs and its weak antifolate activity appears unrelated to its anticonvulsant efficacy (Yuen, 1991). The drug has been proposed to possess a similar experimental spectrum of activity to PHT (Rogawski and Porter, 1990). Clinically, it appears to possess a wider range of antiepileptic efficacy, being active against primary generalised syndromes, in addition to partial and secondary generalised seizures (section 1.4.1). LTG has been shown to attenuate tonic seizures induced by MES in both mice (Lamb et al, 1985) and rats (Miller et al, 1986) and to block the development and expression of kindled seizures in the rat (Miller et al, 1986). Like PHT, LTG was inactive against PTZ-induced threshold and clonic seizures but did protect against the tonic component of these seizures (Yuen, 1991). It has also demonstrated efficacy against the electrically-evoked after-discharge duration in the rat and dog (Wheatley and Miller, 1989). LTG blocked veratridine-induced transmitter release from isolated rat neocortical slices but was without effect on potassium-induced release (Leach et al, 1986). It has also been shown to block sustained repetitive firing in cultured mouse spinal cord neurones in a dose-dependent manner (Cheung et al, 1992). As a result of these *in vitro* observations, LTG has been proposed to exert its anticonvulsant effects by blockade of neuronal voltage-sensitive sodium channels (Macdonald and Kelly, 1993). It has demonstrated efficacy in ten double-blind, placebo-controlled clinical trials giving a 50% reduction in the incidence of partial and primary generalised seizures in 30% of patients (Leach and Brodie, in press).

6.1.7 Vigabatrin as a novel antiepileptic agent

VGB has recently been introduced as a novel agent for the treatment of epilepsy. It has been proposed to exert its effects by enhancing centrally-mediated inhibition via a blockade of GABA-T, the enzyme responsible for the metabolism of brain GABA (Grant and Heel, 1991). The full range of pharmacological effects of the drug are discussed in section 4.1.2. VGB has demonstrated efficacy in several double-blind, placebo-controlled clinical trials, with 50% of patients experiencing a greater than 50% reduction in the incidence of partial seizures (Grant and Heel, 1991).

6.1.8 Clinical combination treatment with lamotrigine and vigabatrin

Anecdotal observations (Kirker and Reynolds, 1990; Stewart et al, 1992) and a recent report of a placebo-controlled, dose-ranging study (Stolarek et al, in press) have suggested a possibly beneficial interaction between LTG and VGB in the clinic. The latter authors observed 50% reduction in partial and secondary generalised seizure number in 45% of VGB-treated patients following add-on treatment with LTG. Further evaluation of this clinical interaction is anticipated once both drugs obtain a product licence for use as monotherapy.

6.2 EXPERIMENTAL AIMS

The aims of the following studies were to investigate and compare the effects of repeated administration of LTG and VGB, alone and in combination, on seizures induced by MES and on selected neurotransmitter concentrations and enzyme activities in the brain. These investigations attempted to determine the nature and extent of any interaction between these two drugs which may have relevance for their concomitant clinical use. The

experimental design facilitated quantification of potential drug interactions by the isobolographic method described by Leander (1992). Contrary to the proposals of Klitgaard and colleagues (1993), these drugs were specifically selected because they are proposed to exert their effects by distinct mechanisms; LTG by reducing excitation and VGB by enhancing inhibition. As such, it was hoped that this investigation would provide further insight into a rational basis for combining drugs with known mechanisms of action in the clinical treatment of epilepsy.

6.3 EXPERIMENTAL PROTOCOLS AND RESULTS

6.3.1 Investigation of experimental interaction between low dose lamotrigine and low dose vigabatrin

PROTOCOL:- Mice were separated into 4 treatment groups (n=22/group) and were administered (i.p.) 1.5 mg/kg LTG, 10 mg/kg VGB, or a combination (COMB) of both drugs. One group (control) received vehicle (0.9% saline) alone. Treatment was continued once daily for 15 days. Four hours after the final dose 10 animals from each group were subjected to the MES test (section 2.3.2) using a stimulus current of 50 mA. The incidence of THE within each group was recorded. The remaining 12 animals from each group were sacrificed at 4 hours after the final dose and their brains removed by the procedure described in section 2.2.3. The brain of each animal was divided into two hemispheres by an incision in the saggital plane and samples were stored in accordance with section 2.2.5 prior to further analysis. The left hemispheres of 6 animals from each group were analysed for GABA-T activity (section 2.4), while the corresponding right hemispheres were analysed for GAD activity (section 2.5). The GABA concentration (section 2.6) was determined in the remaining left hemispheres of 6 animals from each group, while glutamate levels (section 2.6) were determined in the corresponding right hemispheres.

RESULTS:- Repeated low dose treatments with LTG, VGB, and COMB were without effect on the incidence of THE induced by MES at 4 hours after the final dose (figure 49). The activities of brain GABA-T (figure 50) and GAD (figure 51) and the brain concentrations of GABA (figure 52) and glutamate (figure 53) were unaffected by any of the treatments.

6.3.2 Investigation of experimental interaction between medium dose lamotrigine and medium dose vigabatrin

PROTOCOL:- Mice were separated into 4 treatment groups (n=22/group) and were administered (i.p.) 7.5 mg/kg LTG, 50 mg/kg VGB, or a combination (COMB) of both drugs. One group (control) received vehicle (0.9% saline) alone. Treatment was continued once daily for 15 days. Four hours after the final dose 10 animals from each group were subjected to the MES test (section 2.3.2) using a stimulus current of 50 mA. The incidence of THE within each group was recorded. The remaining 12 animals from each group were sacrificed at 4 hours after the final dose and their brains removed by the procedure described in section 2.2.3. The brain of each animal was divided into two hemispheres by an incision in the saggital plane and samples were stored in accordance with section 2.2.5 prior to further analysis. The left hemispheres of 6 animals from each group were analysed for GABA-T activity (section 2.4), while the corresponding right hemispheres were analysed for GAD activity (section 2.5). The GABA concentration (section 2.6) was determined in the remaining left hemispheres of 6 animals from each group, while glutamate levels (section 2.6) were determined in the corresponding right hemispheres.

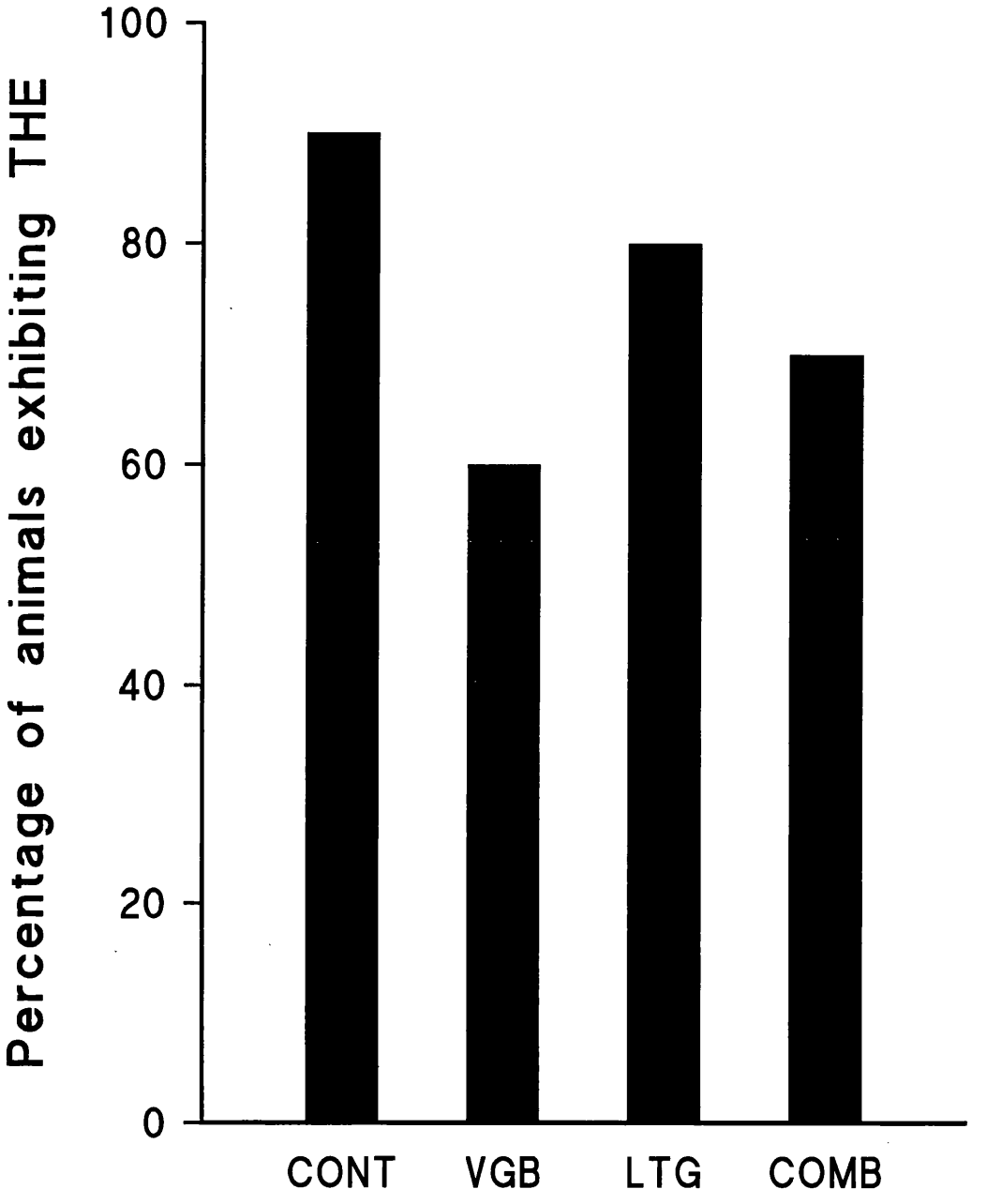

FIGURE 49:- Effect of repeated (once daily for 15 days) control (CONT), vigabatrin (VGB; 10 mg/kg), lamotrigine (LTG; 1.5 mg/kg), and combination (COMB) treatments on the incidence of tonic hind-limb extension (THE) induced by MES at 4 hours after the final dose. Results are expressed as the percentage of animals (mice) in groups of 10 exhibiting THE.

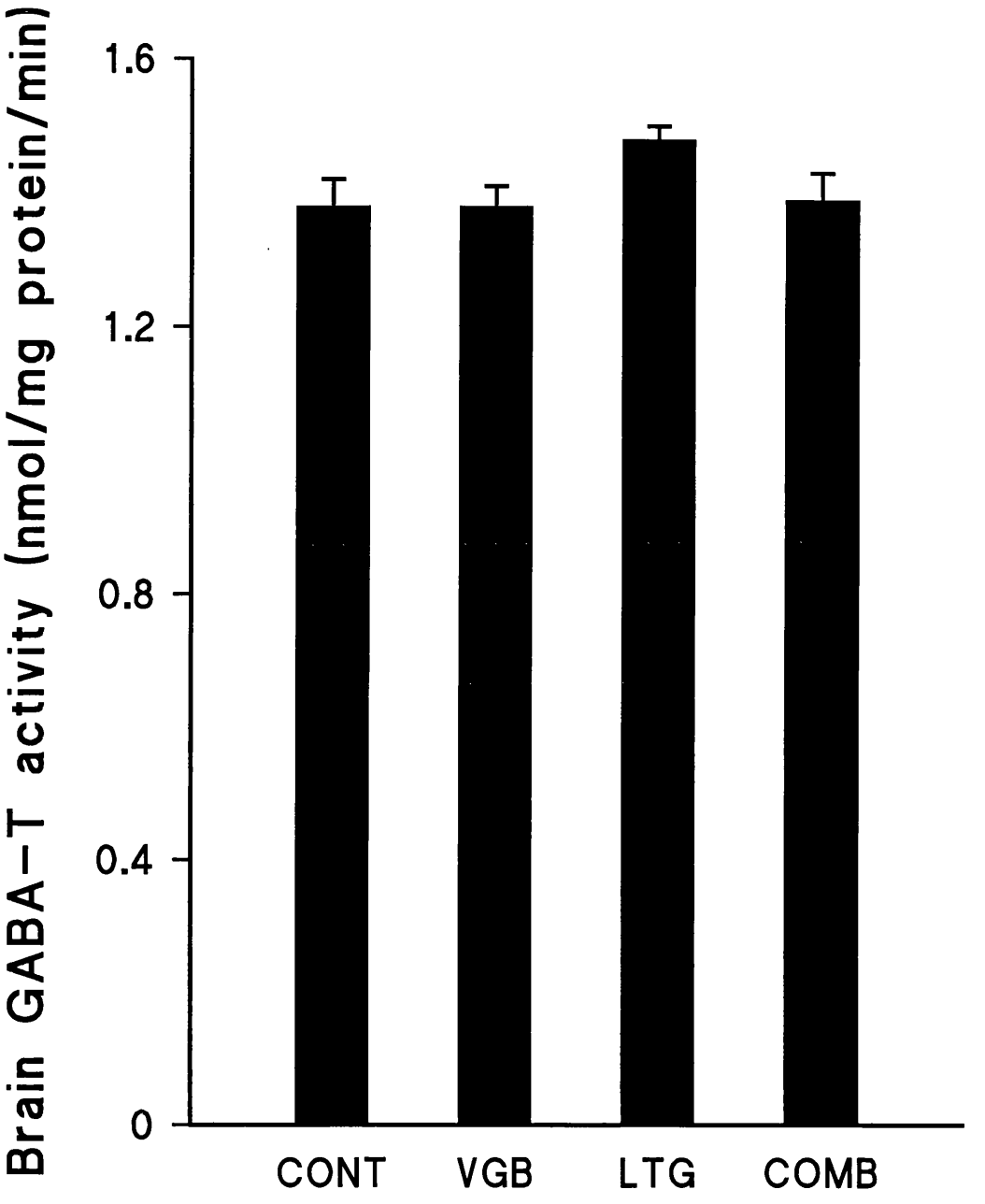

FIGURE 50:- Effect of repeated (once daily for 15 days) control (CONT), vigabatrin (VGB; 10 mg/kg), lamotrigine (LTG; 1.5 mg/kg), and combination (COMB) treatments on brain GABA-aminotransferase (GABA-T) activity at 4 hours after the final dose. Results are expressed as the mean (± SEM) activity in groups of 6 mice.

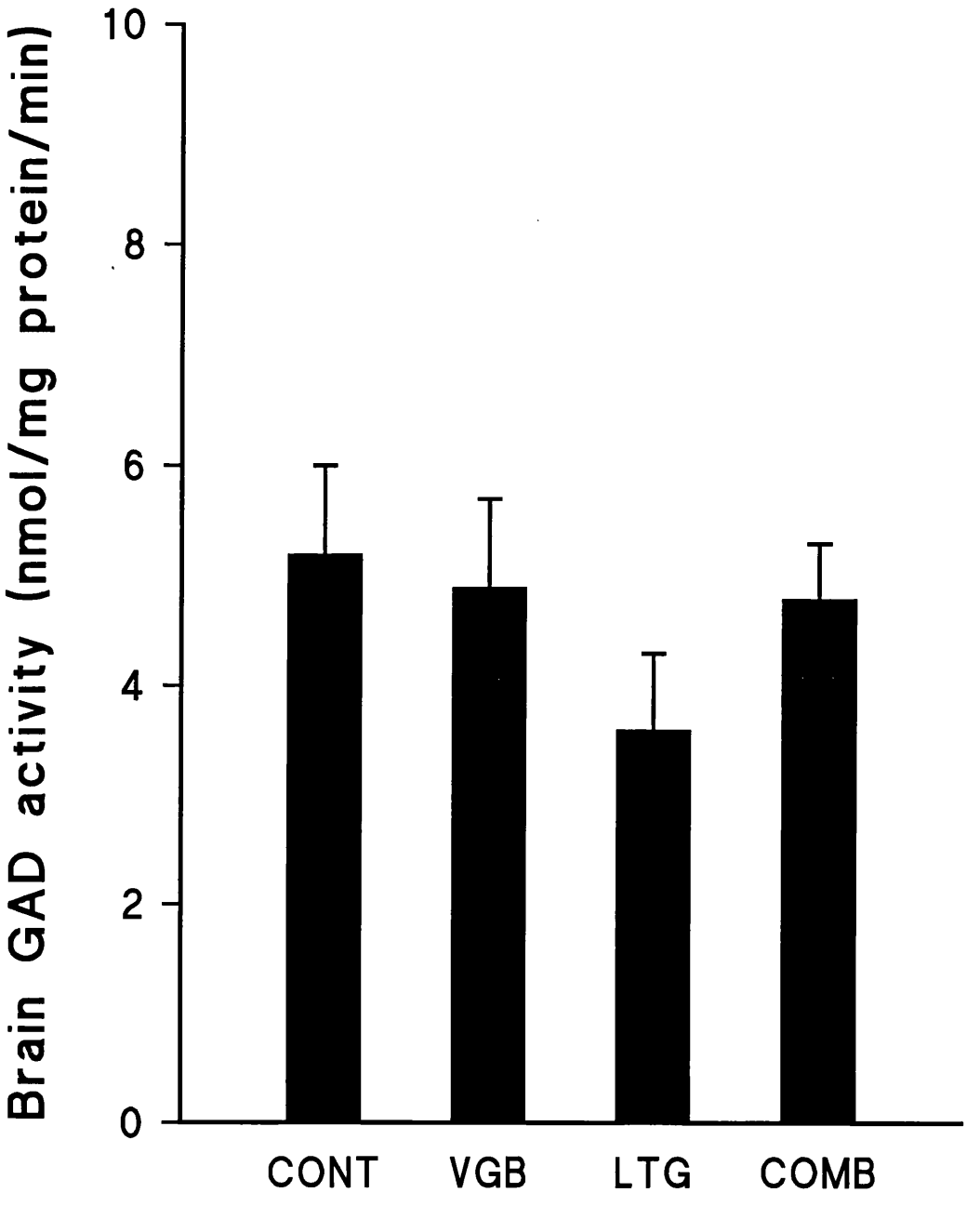

FIGURE 51:- Effect of repeated (once daily for 15 days) control (CONT), vigabatrin (VGB; 10 mg/kg), lamotrigine (LTG; 1.5 mg/kg), and combination (COMB) treatments on brain glutamic acid decarboxylase (GAD) activity at 4 hours after the final dose. Results are expressed as the mean (± SEM) activity in groups of 6 mice.

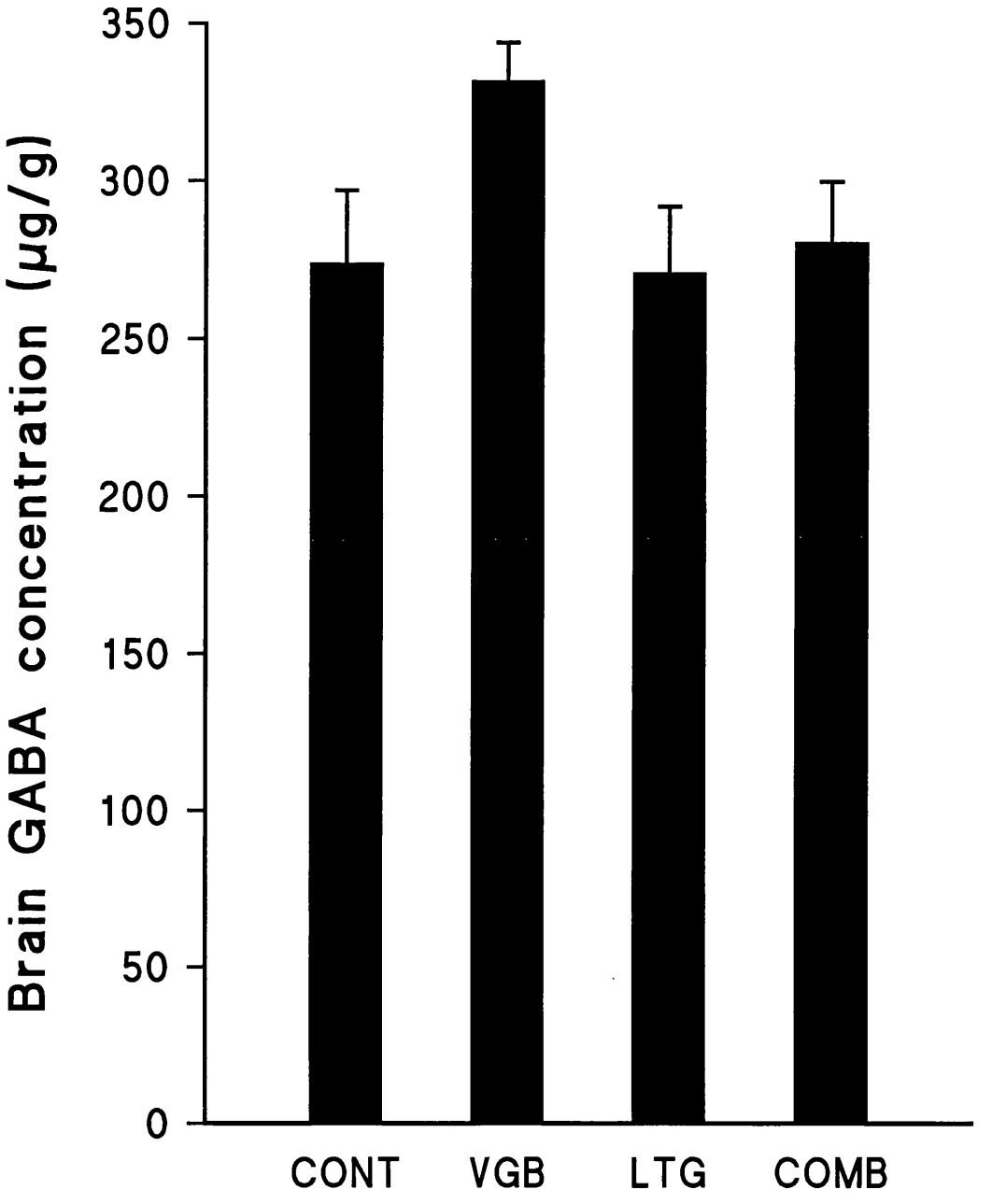

FIGURE 52:- Effect of repeated (once daily for 15 days) control (CONT), vigabatrin (VGB; 10 mg/kg), lamotrigine (LTG; 1.5 mg/kg), and combination (COMB) treatments on brain GABA concentration at 4 hours after the final dose. Results are expressed as the mean (± SEM) concentration in groups of 6 mice.

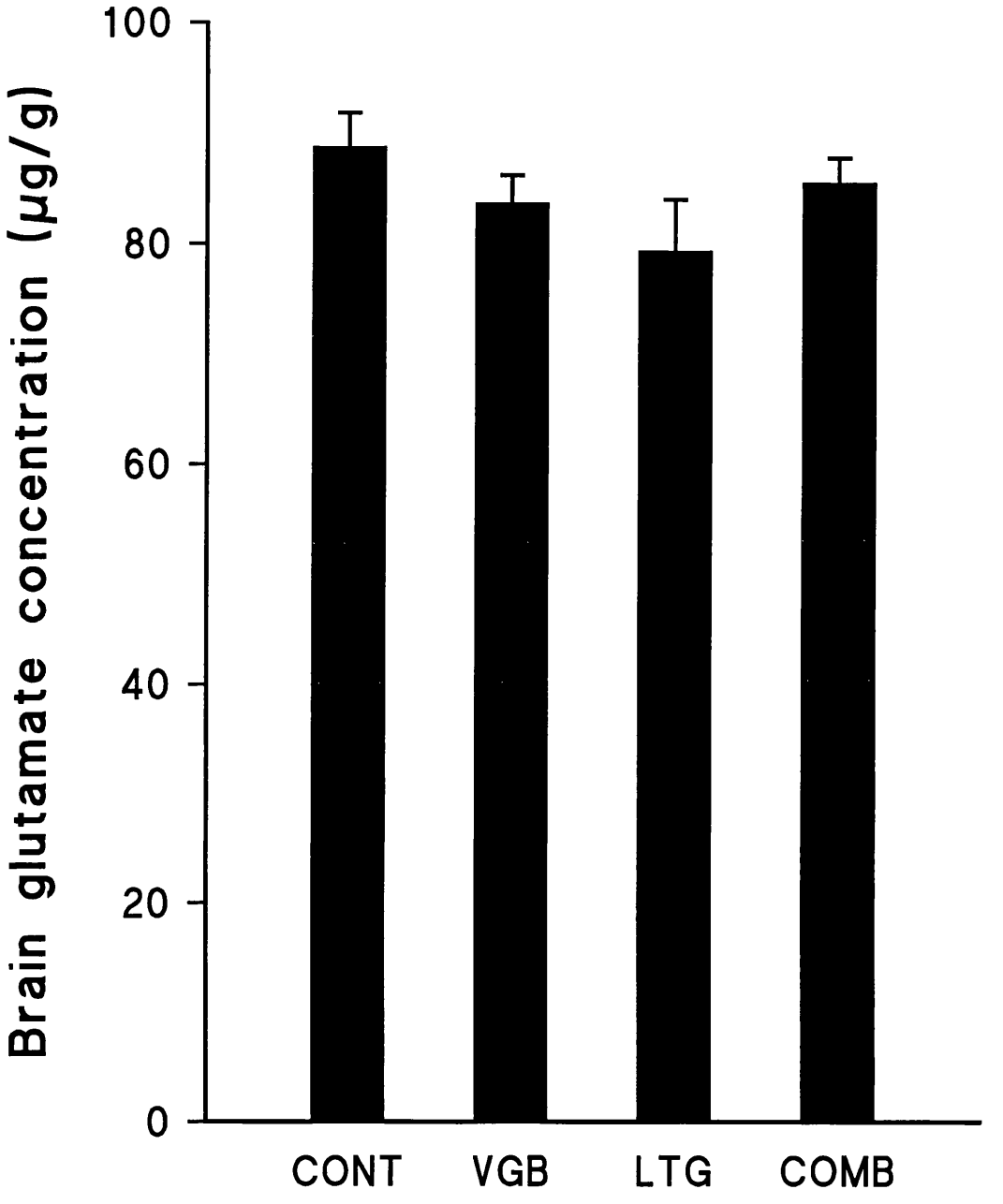

FIGURE 53:- Effect of repeated (once daily for 15 days) control (CONT), vigabatrin (VGB; 10 mg/kg), lamotrigine (LTG; 1.5 mg/kg), and combination (COMB) treatments on brain glutamate concentration at 4 hours after the final dose. Results are expressed as the mean (± SEM) concentration in groups of 6 mice.

RESULTS:- Repeated medium dose treatments with LTG and COMB significantly ($p < 0.05$) reduced the incidence of THE induced by MES at 4 hours after the final dose, while medium dose VGB was without effect (figure 54). Brain GABA-T activity was significantly ($p < 0.01$) lowered 4 hours after the final treatment with both VGB and COMB, while medium dose LTG was without effect (figure 55). The activity of brain GAD (figure 56) and the brain concentrations of GABA (figure 57) and glutamate (figure 58) were unaffected by any of the treatments.

6.3.3 Investigation of experimental interaction between high dose lamotrigine and high dose vigabatrin

PROTOCOL:- Mice were separated into 4 treatment groups (n=22/group) and were administered (i.p.) 37.5 mg/kg LTG, 250 mg/kg VGB, or a combination (COMB) of both drugs. One group (control) received vehicle (0.9% saline) alone. Treatment was continued once daily for 15 days. Four hours after the final dose 10 animals from each group were subjected to the MES test (section 2.3.2) using a stimulus current of 50 mA. The incidence of THE within each group was recorded. The remaining 12 animals from each group were sacrificed at 4 hours after the final dose and their brains removed by the procedure described in section 2.2.3. The brain of each animal was divided into two hemispheres by an incision in the saggital plane and samples were stored in accordance with section 2.2.5 prior to further analysis. The left hemispheres of 6 animals from each group were analysed for GABA-T activity (section 2.4), while the corresponding right hemispheres were analysed for GAD activity (section 2.5). The GABA concentration (section 2.6) was determined in the remaining left hemispheres of 6 animals from each group, while glutamate levels (section 2.6) were determined in the corresponding right hemispheres.

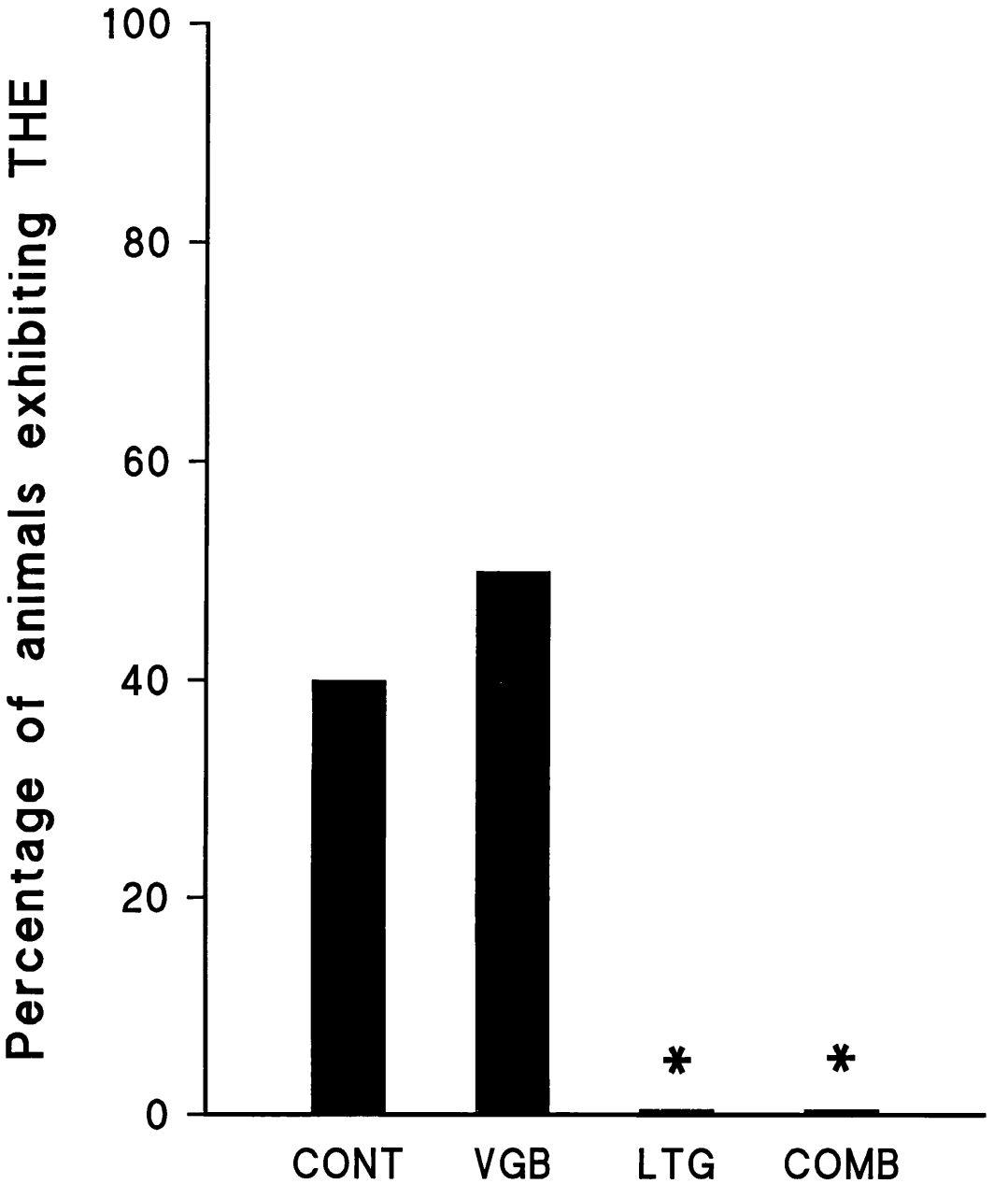

FIGURE 54:- Effect of repeated (once daily for 15 days) control (CONT), vigabatrin (VGB; 50 mg/kg), lamotrigine (LTG; 7.5 mg/kg), and combination (COMB) treatments on the incidence of tonic hind-limb extension (THE) induced by MES at 4 hours after the final dose. Results are expressed as the percentage of animals (mice) in groups of 10 exhibiting THE. Statistical significance (*$p < 0.05$) was determined by the Chi square test.

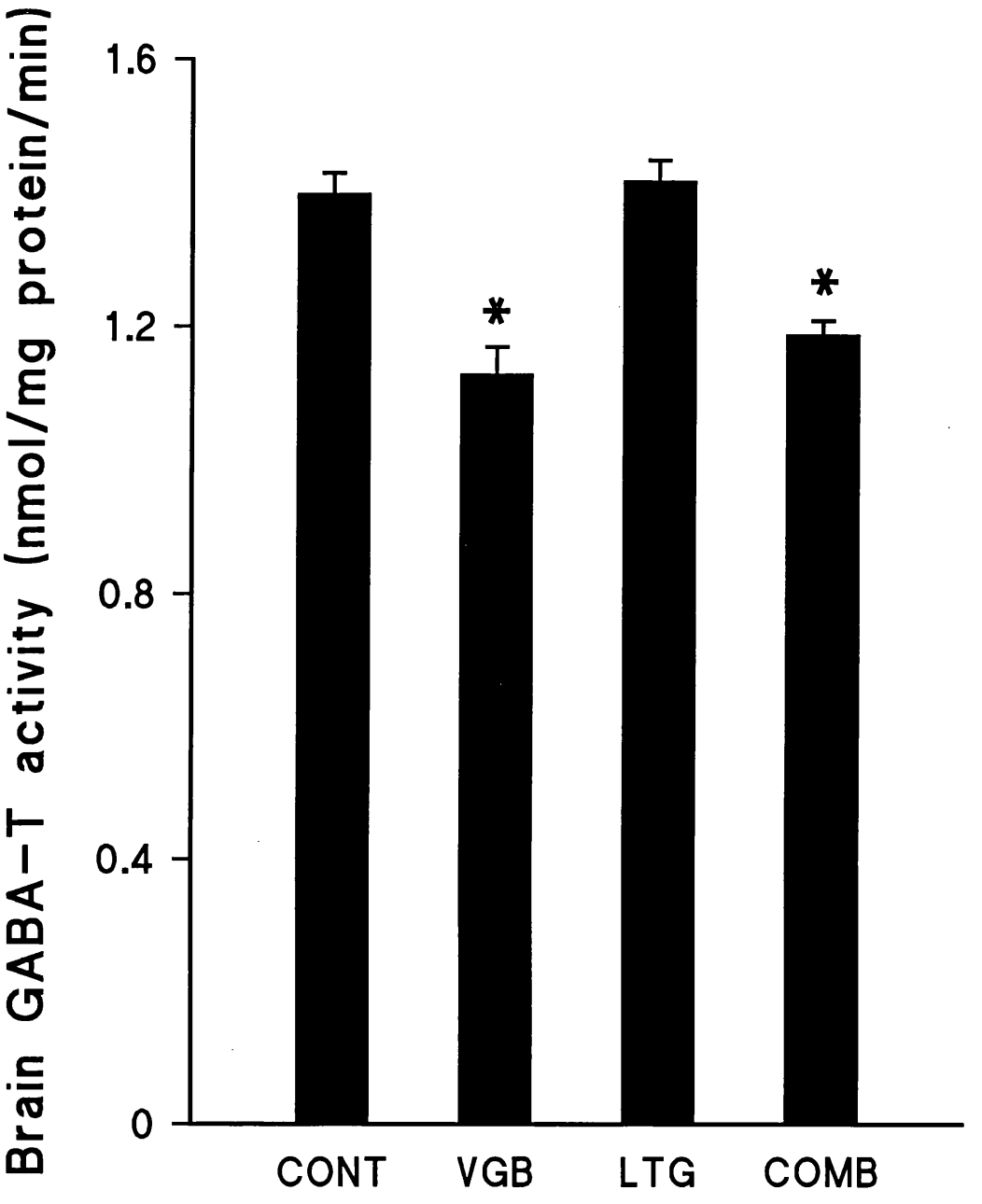

FIGURE 55:- Effect of repeated (once daily for 15 days) control (CONT), vigabatrin (VGB; 50 mg/kg), lamotrigine (LTG; 7.5 mg/kg), and combination (COMB) treatments on brain GABA-aminotransferase (GABA-T) activity at 4 hours after the final dose. Results are expressed as the mean (± SEM) activity in groups of 6 mice. Statistical significance (*$p < 0.01$) was determined by the Mann Whitney U-test.

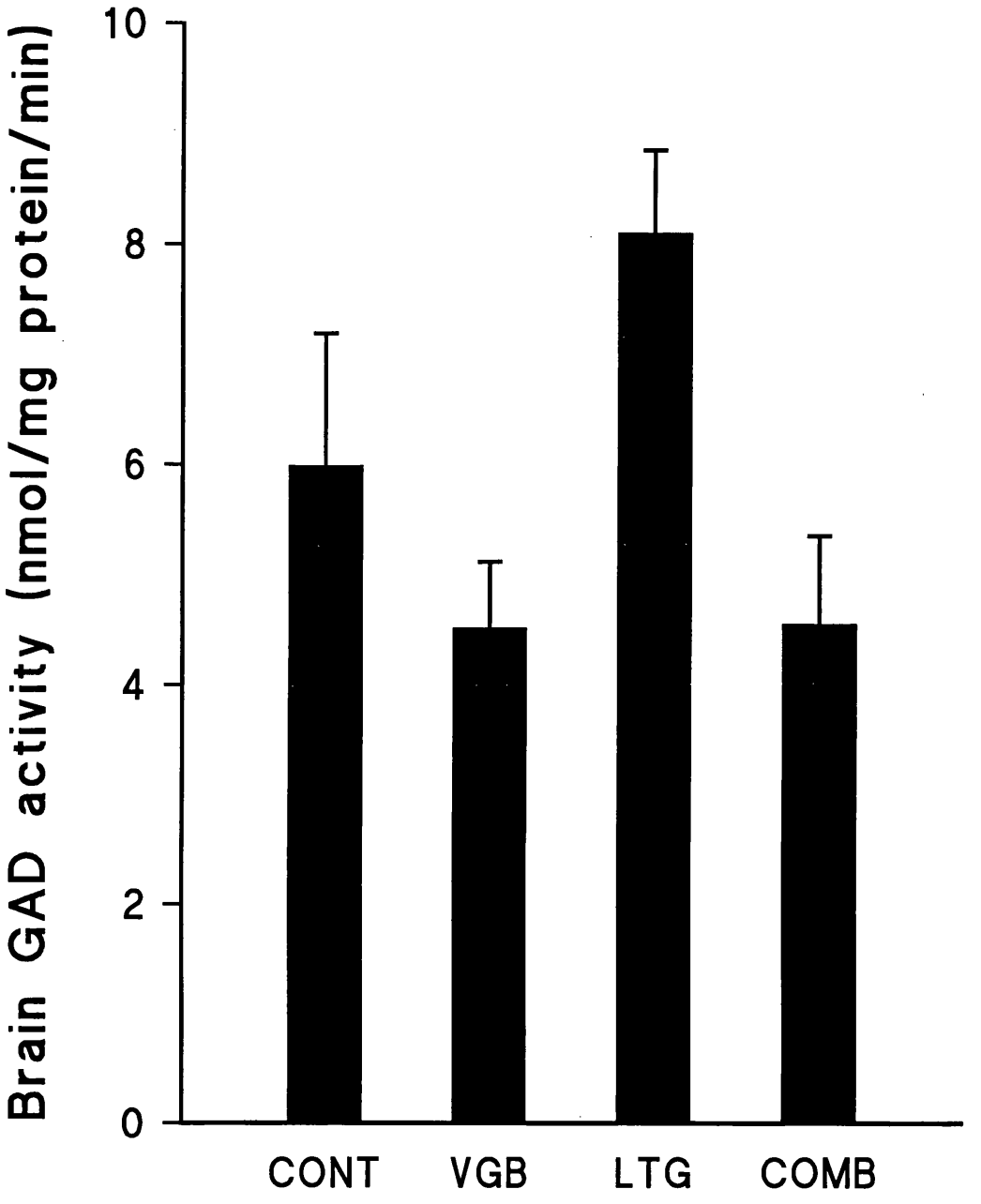

FIGURE 56:- Effect of repeated (once daily for 15 days) control (CONT), vigabatrin (VGB; 50 mg/kg), lamotrigine (LTG; 7.5 mg/kg), and combination (COMB) treatments on brain glutamic acid decarboxylase (GAD) activity at 4 hours after the final dose. Results are expressed as the mean (± SEM) activity in groups of 6 mice.

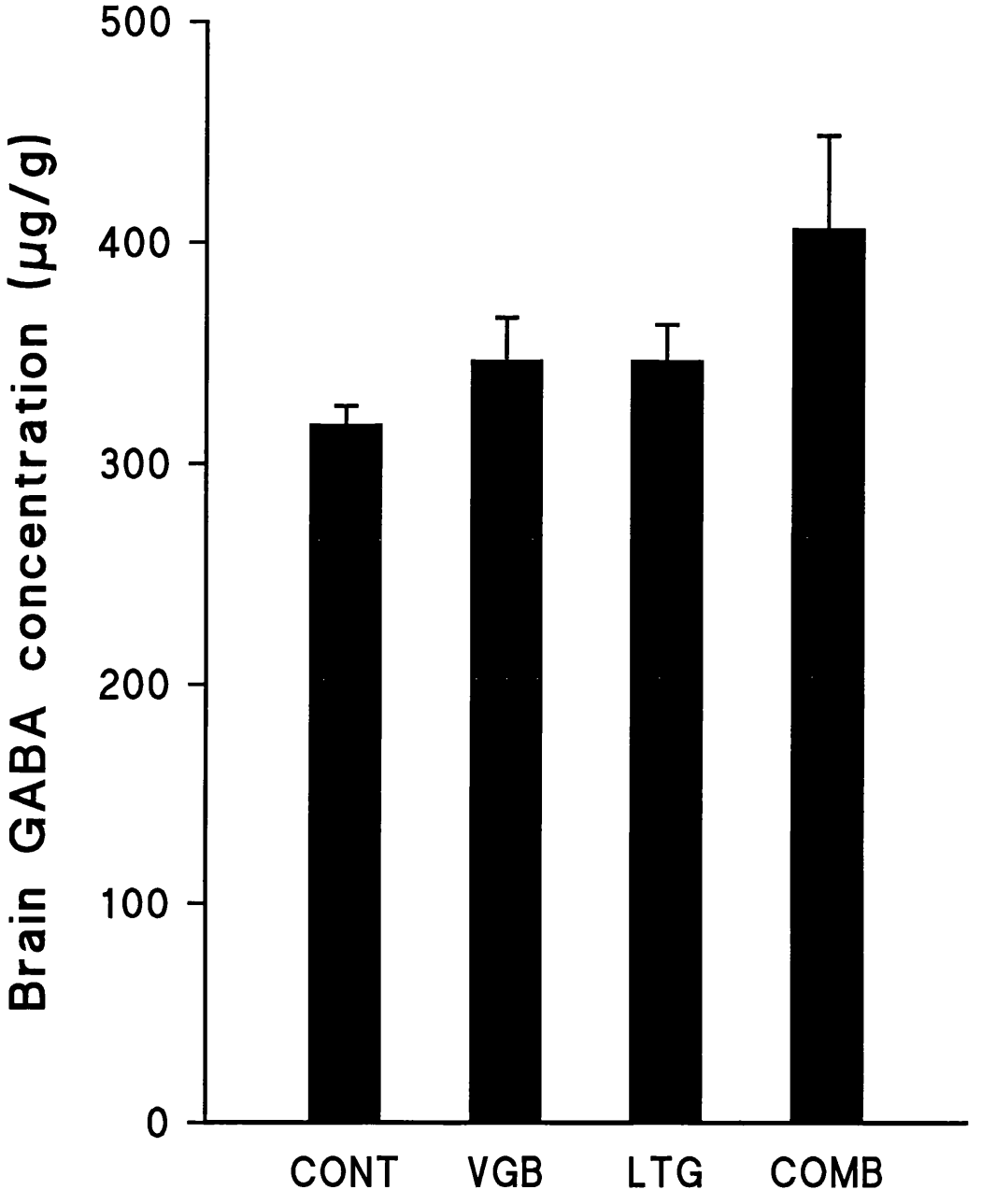

FIGURE 57:- Effect of repeated (once daily for 15 days) control (CONT), vigabatrin (VGB; 50 mg/kg), lamotrigine (LTG; 7.5 mg/kg), and combination (COMB) treatments on brain GABA concentration at 4 hours after the final dose. Results are expressed as the mean (± SEM) concentration in groups of 6 mice.

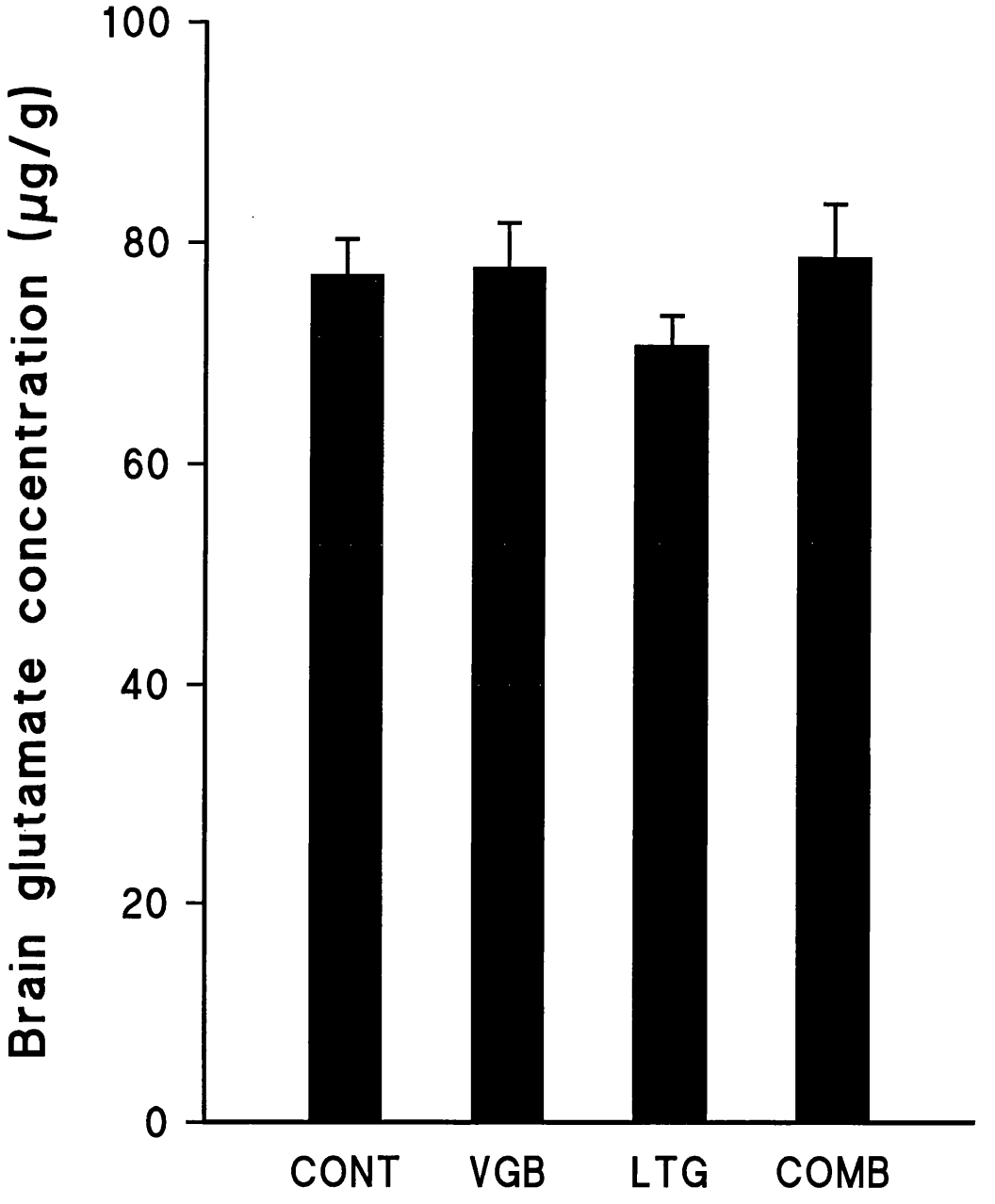

FIGURE 58:- Effect of repeated (once daily for 15 days) control (CONT), vigabatrin (VGB; 50 mg/kg), lamotrigine (LTG; 7.5 mg/kg), and combination (COMB) treatments on brain glutamate concentration at 4 hours after the final dose. Results are expressed as the mean (± SEM) concentration in groups of 6 mice.

RESULTS:- Repeated high dose treatments with LTG and COMB significantly ($p < 0.01$) reduced the incidence of THE induced by MES at 4 hours after the final dose, while high dose VGB was without effect (figure 59). Brain GABA-T activity was significantly ($p < 0.01$) lowered 4 hours after the final treatment with both VGB and COMB, while high dose LTG was without effect (figure 60). Brain GAD activity was unaltered by any of the treatments (figure 61). Brain GABA concentration was significantly ($p < 0.01$) increased, compared to control, following VGB and COMB treatments, while high dose LTG was without effect (figure 62). Brain glutamate concentration was unaltered by any of the treatments (figure 63).

6.4 DISCUSSION

The aims of these studies were to investigate and compare the effects of LTG and VGB, given alone and in combination, on tonic seizures induced by MES and on selected neurotransmitter concentrations and enzyme activities in the brain. These experimental parameters were chosen on the grounds of availability and were not intended to be representative of a definitive evaluation of all potential pharmacological or neurochemical interactions of these drugs with relevance to epilepsy.

Effects of drug treatment on MES-induced seizures

Tonic seizures induced by MES were unaffected by repeated treatment with VGB at all of the doses investigated (figures 49, 54, and 59). These findings were in keeping with those reported by Bernasconi and colleagues (1988), but contrasted those reported in section 4.3.4 (figure 14), where 50 mg/kg VGB significantly reduced THE following repeated administration. Differences in the VGB treatment period might explain this discrepancy.

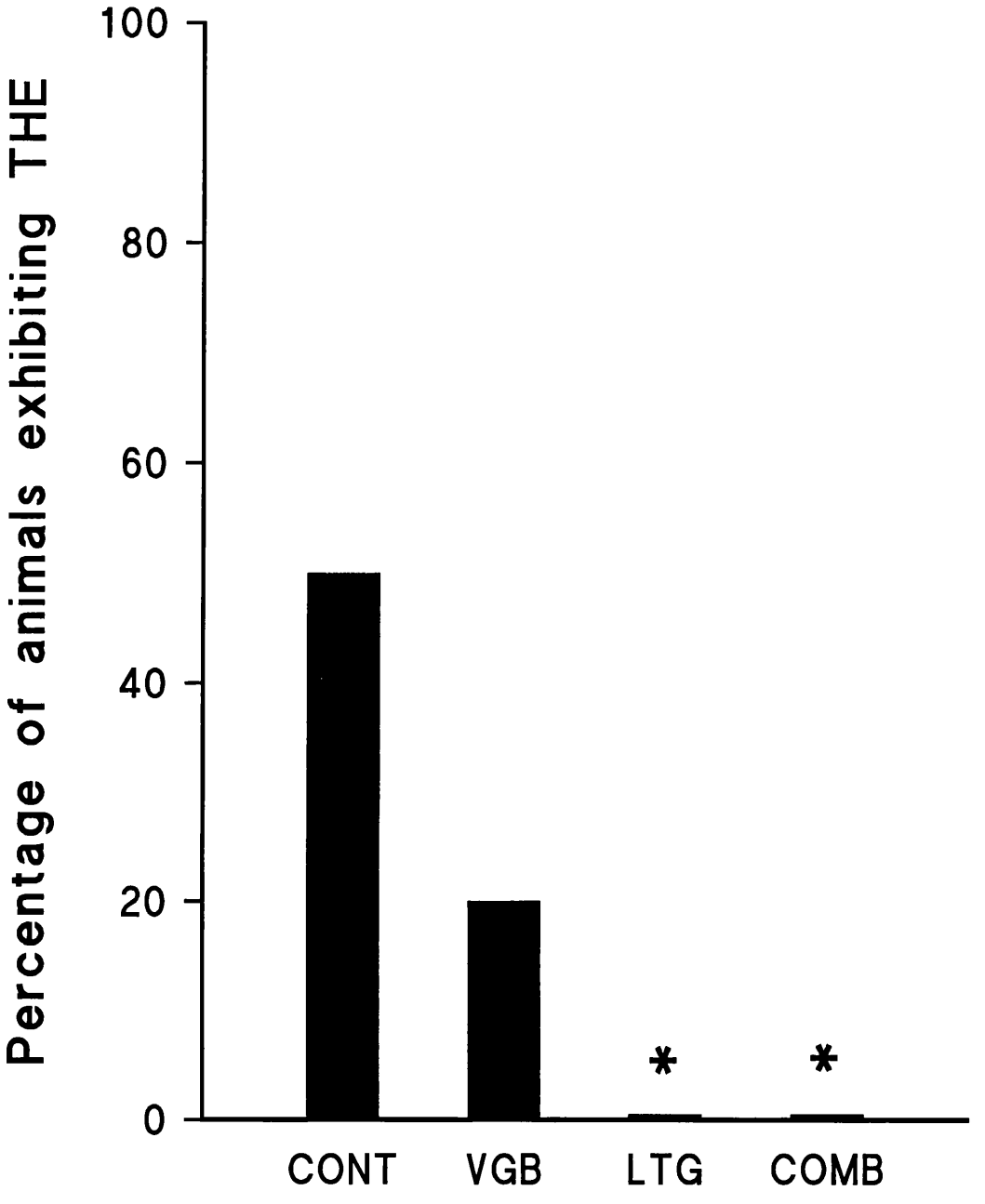

FIGURE 59:- Effect of repeated (once daily for 15 days) control (CONT), vigabatrin (VGB; 250 mg/kg), lamotrigine (LTG; 37.5 mg/kg), and combination (COMB) treatments on the incidence of tonic hind-limb extension (THE) induced by MES at 4 hours after the final dose. Results are expressed as the percentage of animals (mice) in groups of 10 exhibiting THE. Statistical significance (*$p < 0.01$) was determined by the Chi square test.

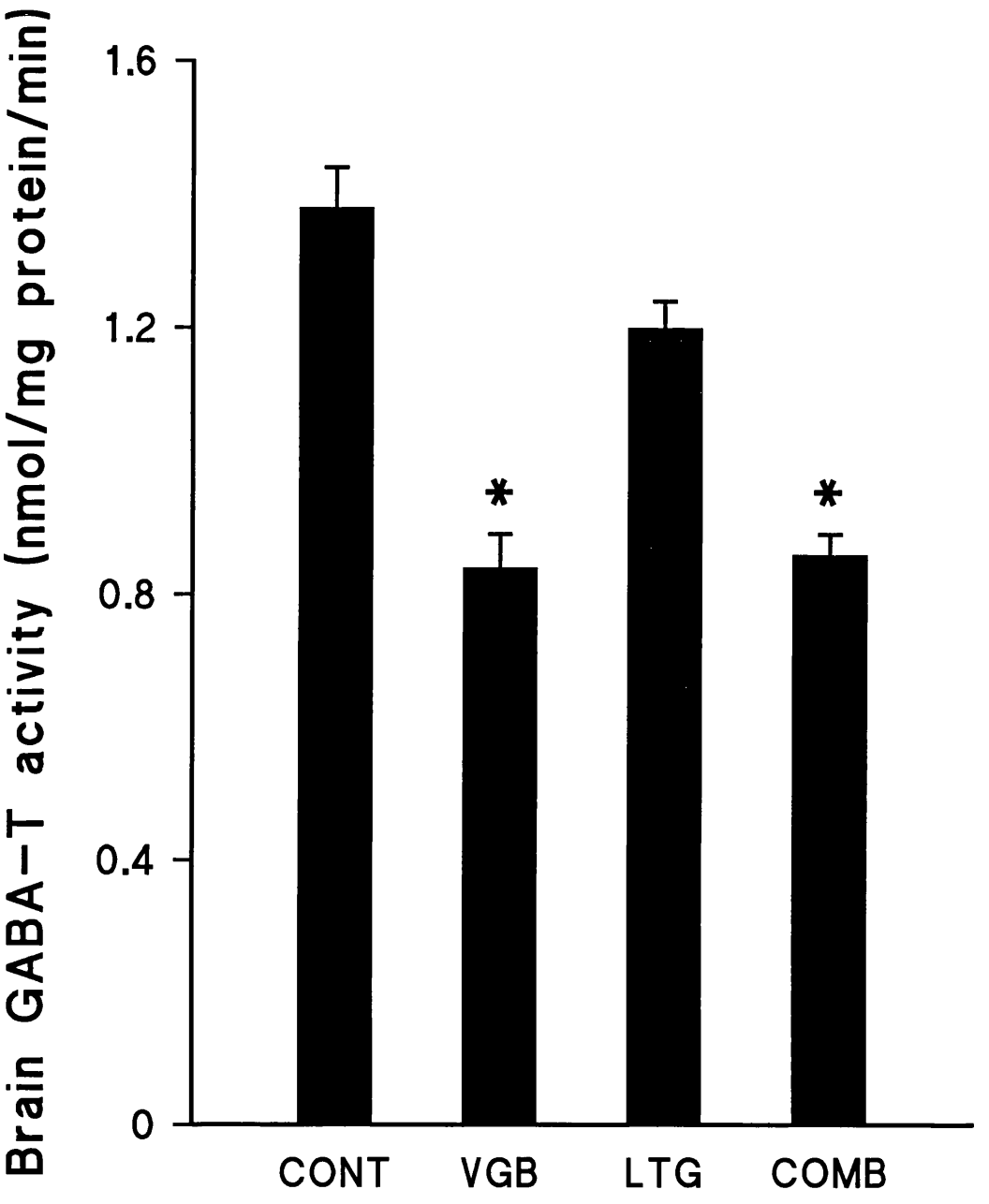

FIGURE 60:- Effect of repeated (once daily for 15 days) control (CONT), vigabatrin (VGB; 250 mg/kg), lamotrigine (LTG; 37.5 mg/kg), and combination (COMB) treatments on brain GABA-aminotransferase (GABA-T) activity at 4 hours after the final dose. Results are expressed as the mean (± SEM) activity in groups of 6 mice. Statistical significance (*$p < 0.01$) was determined by the Mann Whitney U-test.

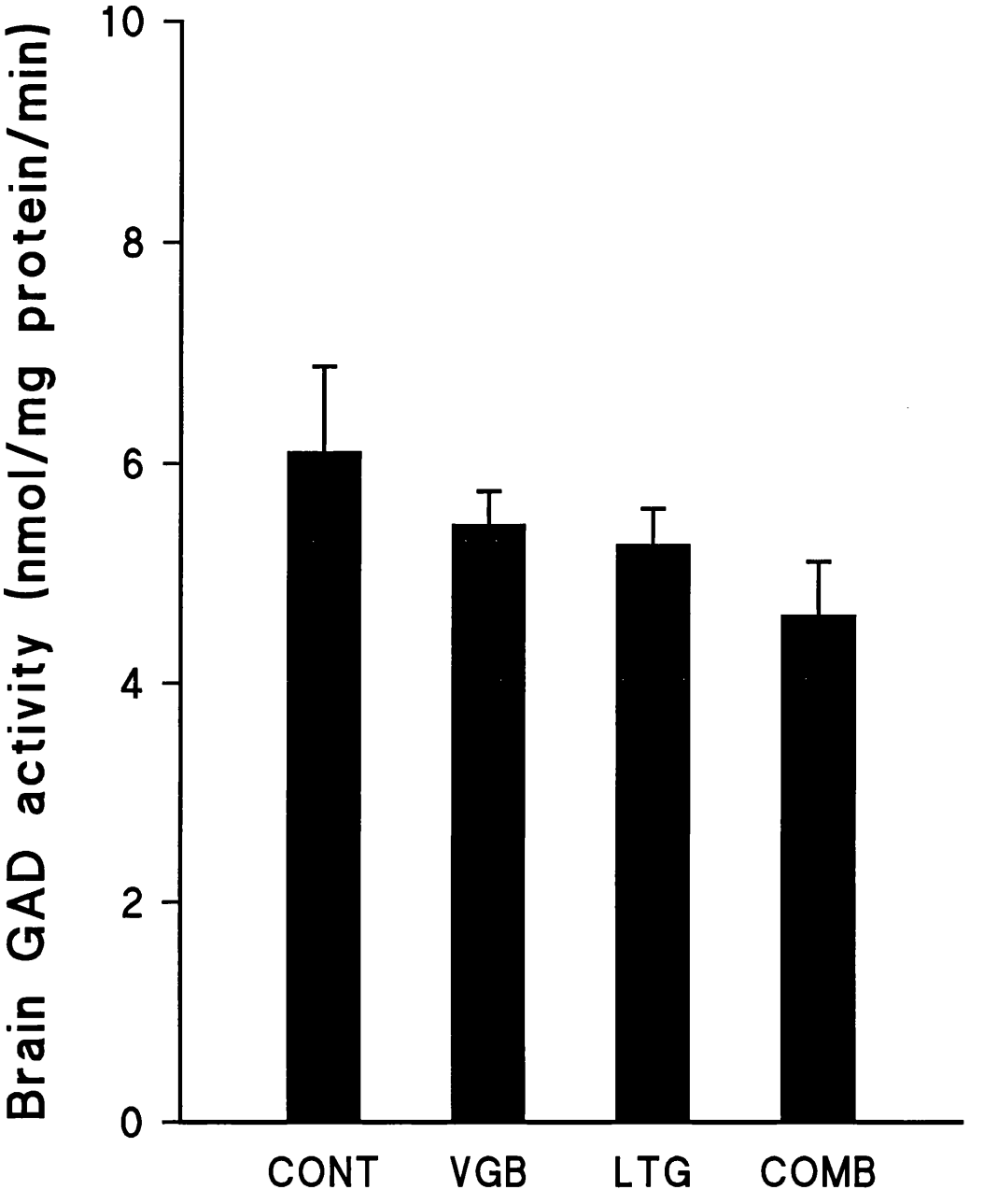

FIGURE 61:- Effect of repeated (once daily for 15 days) control (CONT), vigabatrin (VGB; 250 mg/kg), lamotrigine (LTG; 37.5 mg/kg), and combination (COMB) treatments on brain glutamic acid decarboxylase (GAD) activity at 4 hours after the final dose. Results are expressed as the mean (± SEM) activity in groups of 6 mice.

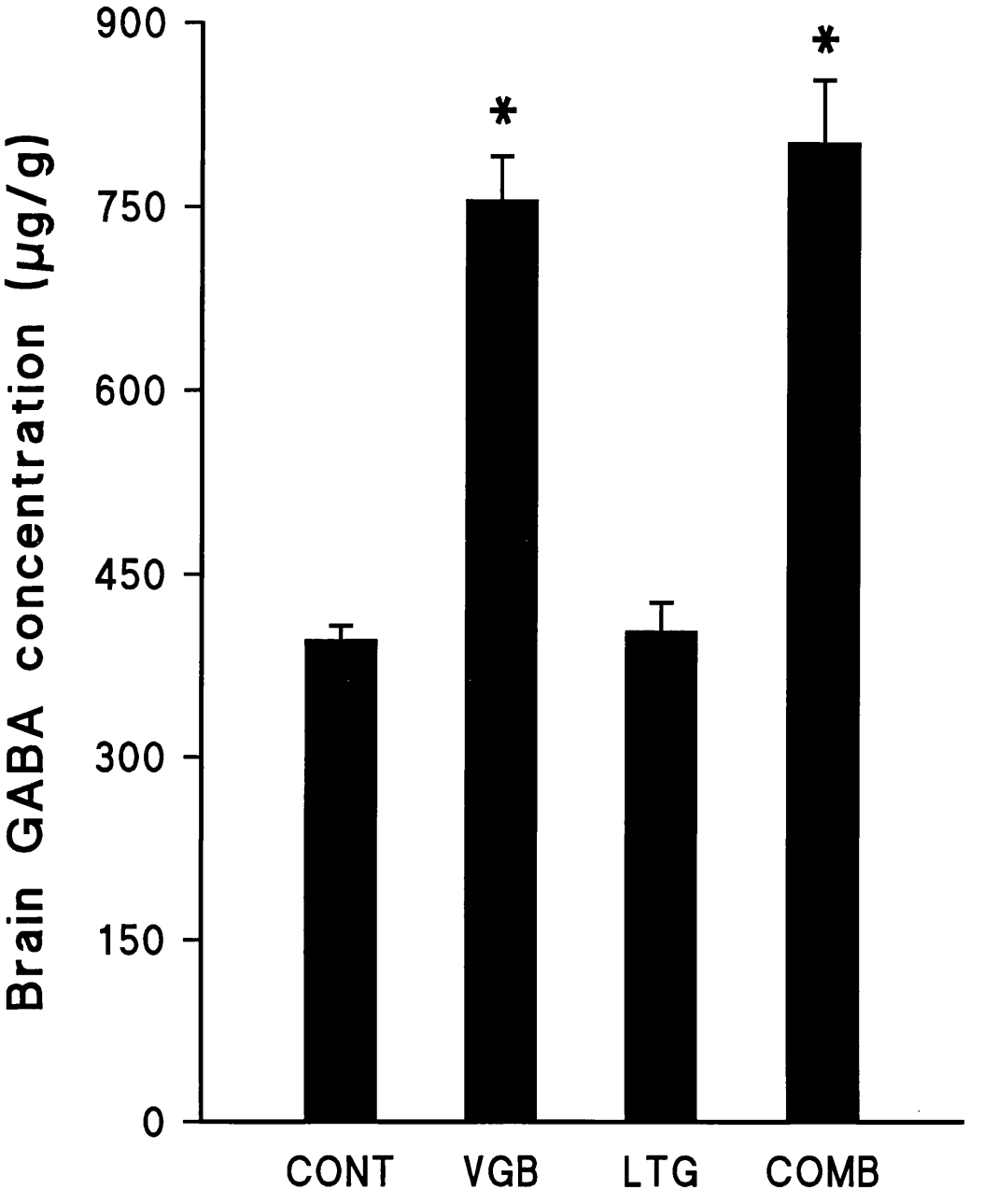

FIGURE 62:- Effect of repeated (once daily for 15 days) control (CONT), vigabatrin (VGB; 250 mg/kg), lamotrigine (LTG; 37.5 mg/kg), and combination (COMB) treatments on brain GABA concentration at 4 hours after the final dose. Results are expressed as the mean (± SEM) concentration in groups of 6 mice. Statistical significance (*$p < 0.01$) was determined by the Mann Whitney U-test.

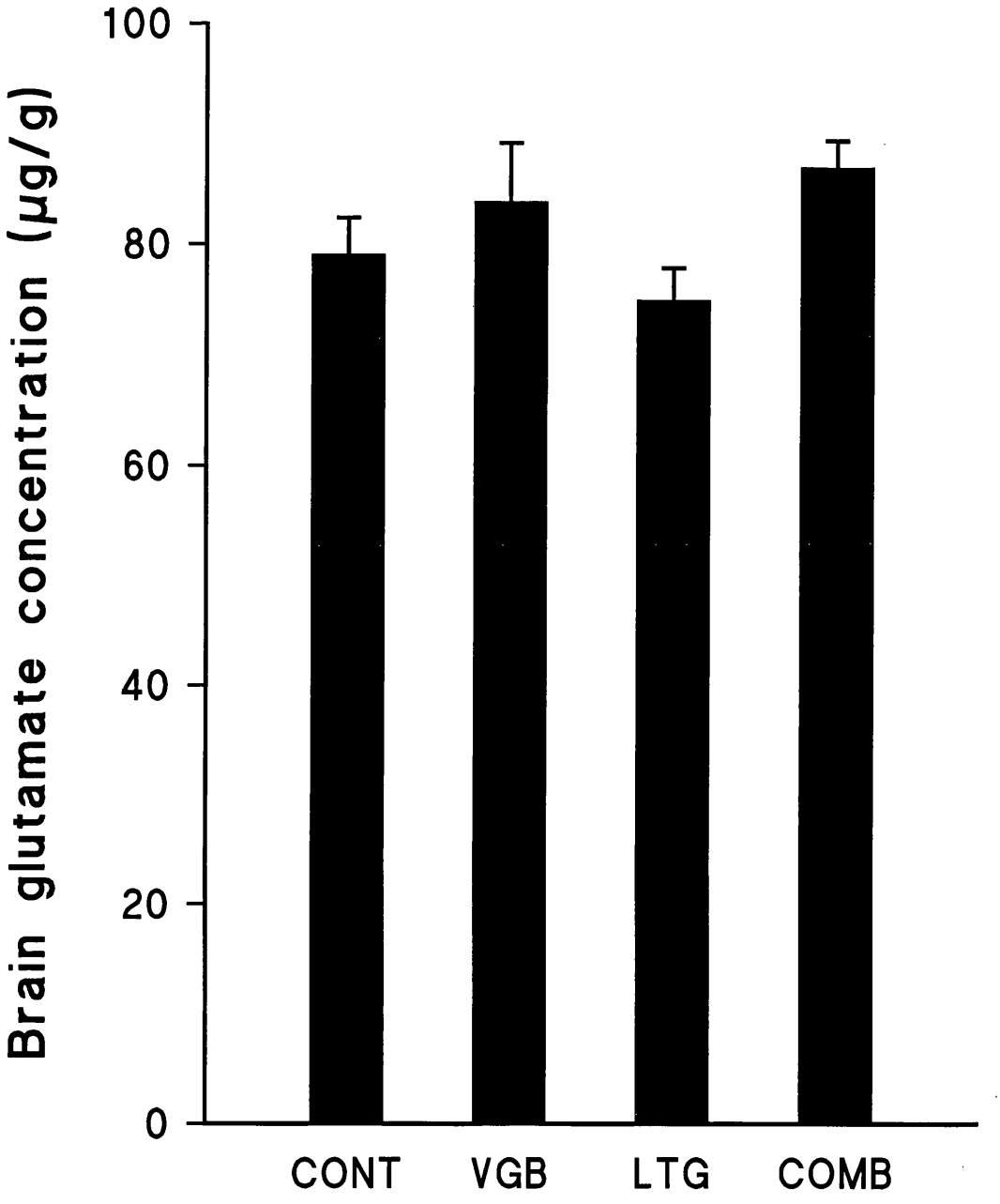

FIGURE 63:- Effect of repeated (once daily for 15 days) control (CONT), vigabatrin (VGB; 250 mg/kg), lamotrigine (LTG; 37.5 mg/kg), and combination (COMB) treatments on brain glutamate concentration at 4 hours after the final dose. Results are expressed as the mean (± SEM) concentration in groups of 6 mice.

Ineffectual in low doses (figure 49), LTG and COMB significantly reduced the incidence of MES-induced tonic seizures following repeated medium and high dose treatments (figures 54 and 59). LTG has previously been reported to attenuate electroshock-induced convulsions (Lamb et al, 1985), and present results serve to confirm these observations. Under the conditions of the protocol employed, the ability of LTG to afford complete protection against MES-induced tonic seizures following both medium and high dose treatments prevented the investigation of any potential interaction with VGB in this respect.

Effects of drug treatment on brain GABA-T activity

Repeated treatment with LTG was without effect on the activity of brain GABA-T at all of the doses investigated (figures 50, 55, and 60). While devoid of effect at low dose (figure 50), VGB and COMB significantly reduced the activity of brain GABA-T following repeated medium and high dose administration (figures 55 and 60). This effect appeared to exhibit a degree of dose-dependency. The observation that repeated VGB administration reduces mouse brain GABA-T activity in a dose-dependent manner is in keeping with that reported in section 4.3.13 (figure 23), and is consistent with the experimental results of Jung and co-workers (1977) and Schechter and colleagues (1977). The lack of a statistically significant difference between the enzyme inhibition observed following VGB and COMB treatments, together with the lack of effect of LTG, suggested that the inhibitory action of COMB treatment on brain GABA-T activity was mediated solely by VGB. Thus, LTG did not appear to influence the capacity of VGB to inhibit brain GABA-T.

Effects of drug treatment on brain GAD activity

Repeated treatments with LTG, VGB, and COMB were without significant effect on brain GAD activity at any of the doses investigated (figures 51, 56, and 61). The lack of effect of VGB in this respect was in contrast to that reported in section 4.3.16 (figure 26), where repeated administration of 100 mg/kg VGB reduced rat brain GAD activity by 40%. Although this discrepancy might be explained in terms of differing species, Jung and colleagues (1977) have reported an effect of VGB on brain GAD activity in mice. Other possible contributors to this experimental disparity included differing VGB doses and treatment periods. The lack of effect of LTG on brain GAD activity was unsurprising when one considers its proposed single mechanism of action. The absolute lack of effect of any treatment on brain GAD activity prevented evaluation of potential drug interactions on this experimental paradigm.

Effects of drug treatment on brain GABA concentration

Brain GABA concentration was unaffected by repeated LTG treatment at all of the doses investigated (figures 52, 57, and 62). This lack of effect was again unsurprising, with no documented evidence to suggest that LTG might interfere with the GABAergic system. Repeated treatments with VGB and COMB significantly increased brain GABA concentrations in high dose (figure 62), but were without effect at low and medium doses (figures 52 and 57). The lack of effect of repeated medium dose treatments with VGB and COMB on brain GABA levels was surprising when one considers the significant effect on brain GABA-T activity (figure 55), and the lack of effect on GAD activity (figure 56), exerted at these dose levels. This result was consistent, however, with that observed with VGB alone in section 4.3.16 (figure 26). Similarly, the increase in brain GABA

concentration observed following high dose treatment was in keeping with the results of Jung and colleagues (1977). The lack of a statistically significant difference in the increases in brain GABA concentration exerted by VGB and COMB treatments, together with the lack of effect of LTG in this respect, suggested that the GABA enhancing actions of the COMB treatment were mediated entirely by VGB. Thus, LTG did not appear to influence the ability of VGB to elevate brain GABA levels.

Effects of drug treatment on brain glutamate concentration

Repeated treatments with LTG, VGB, and COMB were without significant effect on brain glutamate concentrations at any of the doses investigated (figures 53, 58, and 63). The effects of anticonvulsant drugs on whole brain glutamate levels are not routinely evaluated and so no correlations could be drawn between these results and those in the literature. Although the metabolic pathways of GABA and glutamate are inextricably linked, without the ability to discriminate between a neurotransmitter or metabolic role for these amino acids in whole brain no comparison and/or contrast can be made with the effects of these treatments on brain GABA levels. Thus, the absolute lack of effect of any treatment on brain glutamate concentrations, together with the absence of comparable results, prevented evaluation of potential drug interactions on this experimental paradigm.

6.5 CONCLUSIONS

In conclusion, LTG and VGB exerted individual and distinct effects on the experimental paradigms chosen for investigation. LTG effectively reduced MES-induced seizures, but was without effect on any of the selected neurochemical parameters. In contrast, VGB was devoid of anticonvulsant action, while exhibiting effects on the GABAergic system

consistent with its putative mechanism of action. In contrast to the clinical observations of Stolarek and colleagues (in press), the effects of experimental combination therapy did not differ from those observed with the respective monotherapies. In patients, however, there may be more than one seizure type and/or more than one epileptogenic mechanism operant, whereas this study employed only a single seizure model in a genetically homogeneous population of rodents. This lack in correlation with the clinical situation is a recurrent problem in experimental epilepsy research. These results did not permit any form of isobolographic analysis and so it was concluded that there was no experimental interaction between LTG and VGB on the parameters selected for evaluation.

CHAPTER SEVEN

NOVEL STRATEGIES FOR ANTIEPILEPTIC DRUG DEVELOPMENT

7.1 INTRODUCTION

7.1.1 Novel mechanisms of antiepileptic drug action

As described in section 1.3, the established antiepileptic therapies are believed to exert their pharmacodynamic effects by one or more of three fundamental mechanisms. Blockade of neuronal voltage-activated sodium channels (PHT and CBZ), blockade of T-type voltage-sensitive calcium channels (ESM), and enhancement of the post-synaptic effects of GABA at the $GABA_A$ receptor (PB and BZDs) have been implicated as the predominant mechanisms of AED action (Macdonald and Kelly, 1993). Although a number of these established AEDs appear to possess more than one mechanism (section 1.3), their secondary actions are generally observed at drug concentrations higher than those producing their primary effects (Rogawski and Porter, 1990).

As discussed in section 6.1.1, treatment with these established AEDs adequately controls seizures in fewer than 80% of the epileptic population (Meinardi, 1992). It can be proposed, therefore, that pharmacological manipulation of one or more of the three primary mechanisms of AED action is inadequate for treatment of the epileptic population as a whole. Furthermore, present treatment with established AEDs results in a greater number of adverse side effects and complicated drug interactions than would be desired (Brodie, 1990). To satisfactorily address the problem of intractable epilepsy we must develop novel treatments with similarly novel mechanisms of action. These novel agents should also exhibit minimal side effects and drug interactions to maximise their potential as treatments for drug-resistant epilepsy.

Recent additions to the clinician's pharmacological armamentarium include TGB, GBP, FBM, and RMD. All of these drugs have been proposed to exert their antiepileptic effects by novel mechanisms (Rogawski and Porter, 1990; Macdonald and Kelly, 1993). TGB has been reported to selectively block GABA uptake into neurones and glia (Nielsen et al, 1991; section 4.1.3), whereas the precise mechanisms of GBP, FBM, and RMD action remain to be definitively elucidated. Recent observations have suggested that GBP may exert its effects by interacting with the plasma membrane L-amino acid transporter (Taylor, in press; section 1.4.1), FBM by antagonism at the strychnine-insensitive glycine site on the NMDA receptor complex (McCabe et al, 1993; section 1.4.1), and RMD by an interaction at the MK-801 site on the NMDA receptor (Muir and Palmer, 1991; section 1.4.2). Preliminary clinical evaluations of these drugs, particularly TGB, suggest that collectively they may provide a useful advance in the treatment of patients with refractory epilepsy (Macdonald and Kelly, 1993; Leach and Brodie, in press). However, until conclusive clinical studies are completed with these novel agents there remains a requirement for the development of further novel antiepileptic agents.

7.1.2 Novel approaches in the manipulation of excitatory amino acid neurotransmission

Established AEDs, such as PHT and CBZ, and recently developed agents such as LTG, undoubtedly interfere with EAA neurotransmission by blocking glutamate release through their actions on neuronal sodium channels (Rogawski and Porter, 1990). Similarly, physiological antagonism of the post-synaptic effects of glutamate, such as that observed with the DHP calcium channel blockers, discussed in chapter 5, will influence EAA neurotransmission (Meyer, 1989) and holds promise for development as a novel antiepileptic mechanism (de Falco et al, 1992). Evaluation of the potential of direct, or

indirect, pharmacological manipulation of the post-synaptic, receptor-mediated effects of glutamate as an antiepileptic mechanism remains in its infancy.

The NMDA subtype of glutamate receptor has been proposed as the most likely to be involved in epileptic processes (Dingledine et al, 1990). Advances in understanding of the pharmacology of this receptor-ionophore complex in the last decade (Foster and Fagg, 1987; Scatton, 1993) are now being exploited in all manner of neurological disorders (Olney, 1990), including epilepsy (Meldrum, 1992). Evaluation of selective glutamate receptor antagonists now provides much of the impetus in the search for anticonvulsant drugs with novel mechanisms of action (Chapman and Meldrum, 1991).

The NMDA receptor is believed to be a macromolecular protein (Scatton, 1993) comprised of several variable subunits (Nakanishi, 1992) forming an ion channel permeable to Na^+, K^+, and Ca^{2+} ions (Mayer and Miller, 1990). The intact receptor complex possesses a binding site for the endogenous agonist glutamate, in addition to a site for glycine binding (Johnson and Ascher, 1987), a polyamine site (Ransom and Stec, 1988), a Zn^{2+} site (Mayer et al, 1989), and binding sites for Mg^{2+} (Ascher and Nowak, 1987) and for PCP and the dissociative anaesthetics (Mayer et al, 1989) within the ion channel itself. The presence and/or affinity of each of these binding sites is thought to be conferred by the configuration of the variable subunits within individual intact receptor complexes (Nakanishi, 1992).

NMDA receptor activation can be blocked, to elicit an anticonvulsant effect, in many different ways. Potent anticonvulsant activity was first demonstrated by the competitive

antagonists of the glutamate recognition site on the NMDA receptor. The long chain phosphono analogues of D-glutamate, such as AP5 and AP7, were shown to be effective anticonvulsants, following parenteral and oral administration, in several models of reflex epilepsy, including audiogenic seizures in DBA/2 mice and GEP rats and photically induced myoclonus in *Papio papio* baboons (Croucher et al, 1982; Meldrum, 1984). These compounds have demonstrated similar efficacy against both electroshock- and chemically-seizures (Chapman, 1991). More recently synthesised NMDA receptor antagonists, such as D-CPPene and CGS 37849, have been shown to afford long-lasting protection against photically-induced myoclonus in *Papio papio* baboons following oral administration (Patel et al, 1990; Chapman et al, 1991a).

The non-competitive NMDA receptor antagonists, PCP and MK-801, act on the open state of the intrinsic ion channel of the receptor complex and as such possess anticonvulsant activity (Leander et al, 1988b; Chapman and Meldrum, 1989; Johnson and Jones, 1990). While these drugs precipitate motor side effects at doses equal to or less than the anticonvulsant dose (Meldrum, 1992), some related compounds such as RMD (Garske et al, 1991; section 1.4.2) and ADCI (Rogawski et al, 1991), which are weakly active at the PCP/MK-801 site, exhibit anticonvulsant effects at doses which show no motor toxicity.

The glycine recognition site on the NMDA receptor complex represents another potential target for the pharmacological amelioration of seizures. It has been proposed that binding of endogenous glycine to this strychnine-insensitive site modulates the physiological actions of glutamate, at its recognition site, in a positive manner (Scatton, 1993), and that glutamate and glycine binding may exhibit a positive co-operativity (Hood et al, 1990).

Glycine site activation may be an absolute requirement for NMDA receptor activation (Monaghan et al, 1989). In some preparations NMDA fails to elicit an electrophysiological response in the absence of glycine, or in the presence of glycine site antagonists such as 7-chlorokynurenic acid (Kleckner and Dingledine, 1988). Thus, glycine may play a co-agonist role at the NMDA receptor.

The strychnine-insensitive glycine site on the NMDA receptor complex is unusual in that it possesses an endogenous antagonist, kynurenic acid, a by-product of the kynurenine pathway of tryptophan metabolism (Stone and Connick, 1985), which has been identified in both rodent (Carla et al, 1988) and human (Turski et al, 1988) brain. Together with the fact that glycine site antagonists are anticonvulsant, established by intra-cerebroventricular administration of 7-chlorokynurenic acid (Singh et al, 1990), this discovery represents a significant advance in the search for novel anticonvulsant drug mechanisms. Unfortunately, the majority of the glycine site antagonists derived from kynurenic acid have little anticonvulsant activity when administered systemically (Meldrum, 1992). Most of the currently available kynurenic acid derivatives have a limited ability to penetrate the blood-brain barrier. HA-966, and its methyl derivative L-687,414, are, however, moderately active systemically in both rodent models of reflex epilepsy (Meldrum, 1992) and in photosensitive baboons (Smith and Meldrum, 1992).

Direct pharmacological modulation of the glycine site appears to be an attractive option in the search for novel anticonvulsant drug mechanisms. The anticonvulsant effects of 7-chlorokynurenic acid were observed at doses well below those precipitating motor toxicity (Singh et al, 1990). However, such derivatives of kynurenic acid and other

mechanistically related compounds have inappropriate pharmacokinetics and insufficient efficacy (Meldrum, 1992) to merit further evaluation as potential clinical AEDs. A series of novel compounds with glycine site antagonist activity are presently under development (Palfreyman and Baron, 1991).

Another possible pharmacological approach to attenuating glycine-induced co-operativity at the NMDA receptor would be to selectively increase the concentration of endogenous kynurenic acid. Although the concentration of kynurenic acid in normal brain is low (approximately 20 pmol/g; Carla et al, 1988), it remains the only endogenous EAA antagonist to be identified in the mammalian CNS. Increasing kynurenic acid concentration in the brain could be accomplished by selective inhibition of the enzymes involved in the kynurenine pathway of tryptophan metabolism (Decker et al, 1963). While endogenous kynurenic acid may not be as effective an antagonist at the glycine site as some of its synthetic analogues (Lodge and Johnson, 1990), development of selective, centrally-active inhibitors of the kynurenine pathway may abrogate the requirement for investigation of more effective antagonists. This proposal could be supported by the inversely analogous case of GABA, in terms of agonists and antagonists, where an enzyme inhibitor, such as VGB (Grant and Heel, 1991), is a much more effective anticonvulsant drug than compounds, such as muscimol (Meldrum, 1984), with selective activity at GABA receptors.

7.1.3 The kynurenine pathway and the effects of nicotinylalanine

Approximately two thirds of the tryptophan not used for protein synthesis is metabolised down the kynurenine pathway (figure 64). Tryptophan is successively metabolised to NAD and/or glutaryl CoA by enzymatic action. Pertinent neuroactive products of this metabolism include kynurenic acid, a selective blocker of the glycine site on the NMDA receptor as discussed above, and quinolinic acid, a potent excitotoxin with agonist effects at the glutamate recognition site of the NMDA receptor (Stone and Burton, 1988). Nicotinylalanine (NA) is an aminobutyrate analogue of kynurenine (figure 64) which has been shown to inhibit the enzymes kynurenine hydroxylase and kynureninase of the kynurenine pathway (Decker et al, 1963). The sequential inhibition of these enzymes results in a block of the metabolic pathway of tryptophan at this stage and a re-direction of kynurenine metabolism via the B6 transaminase enzymes, resulting in an increased production of kynurenic and xanthurenic acids (Decker et al, 1963). It is proposed that this subtle, indirect method of blockade of the glycine site on the NMDA subtype of glutamate receptor, by increasing brain kynurenic acid concentration, may have an anticonvulsant effect. If so, this would represent an entirely novel mechanism for anticonvulsant drug action.

7.2 EXPERIMENTAL AIMS

The aims of the following studies were to investigate the experimental anticonvulsant profile of NA, a putative anticonvulsant compound with a novel mechanism of action. The experimental seizure models described in chapter 3 were employed for this purpose.

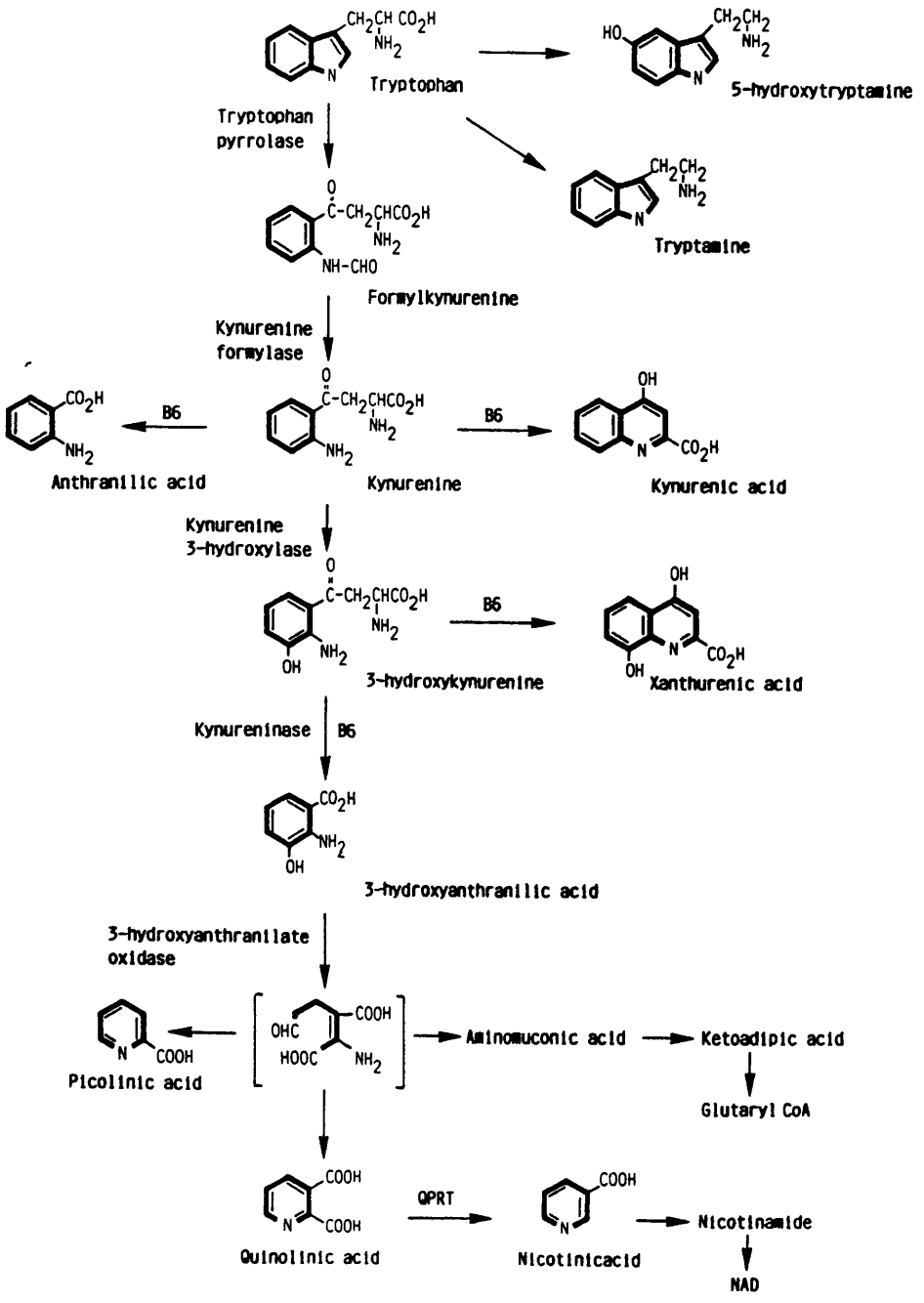

FIGURE 64:- Schematic representation of the kynurenine pathway of tryptophan metabolism. Figure was reproduced from Connick et al (1992), with permission.

7.3 EXPERIMENTAL PROTOCOLS AND RESULTS

7.3.1 Dose and time dependent effects of nicotinylalanine on pentylenetetrazol-induced seizures in mice

PROTOCOL:- Mice were separated into 4 groups (n=30/group) and 20 animals from each group were treated with NA (i.p.) in doses of 370, 740, 2220, and 4440 mg/kg. The remaining 10 animals in each group received vehicle (0.9% saline) alone. At one hour post-administration 10 drug-treated animals and all 10 control animals from each group were subjected to the s.c. CD_{97} PTZ test (section 2.3.1). At four hours post-administration the remaining 10 drug-treated animals in each group were also subjected to the s.c. CD_{97} PTZ test. The latency to the first generalised seizure in individual animals was recorded.

RESULTS:- Single doses of NA (370 and 740 mg/kg) significantly (370 mg/kg, $p < 0.001$; 740 mg/kg, $p < 0.05$) lengthened the time to the first generalised seizure induced by PTZ at 1 hour post-dosing (figure 65). A single 4440 mg/kg dose of NA exerted a proconvulsant effect by significantly ($p < 0.05$) reducing the time to the first generalised seizure at 1 hour post-administration (figure 65). A single 2220 mg/kg dose of NA at 1 hour (figure 65) and all doses of NA at 4 hours (figure 66) were without significant effect.

7.3.2 Dose dependent effects of nicotinylalanine on the threshold for induction of tonic seizures in mice

PROTOCOL:- Mice were separated into 3 groups (n=30/group) and treated with either 185 mg/kg NA, 370 mg/kg NA, or vehicle (0.9% saline) alone. At one hour post-administration each animal was subjected to the Min-ES test (section 2.3.3). The number of positive (tonic seizure) and negative (no tonic seizure) responses within each group was recorded.

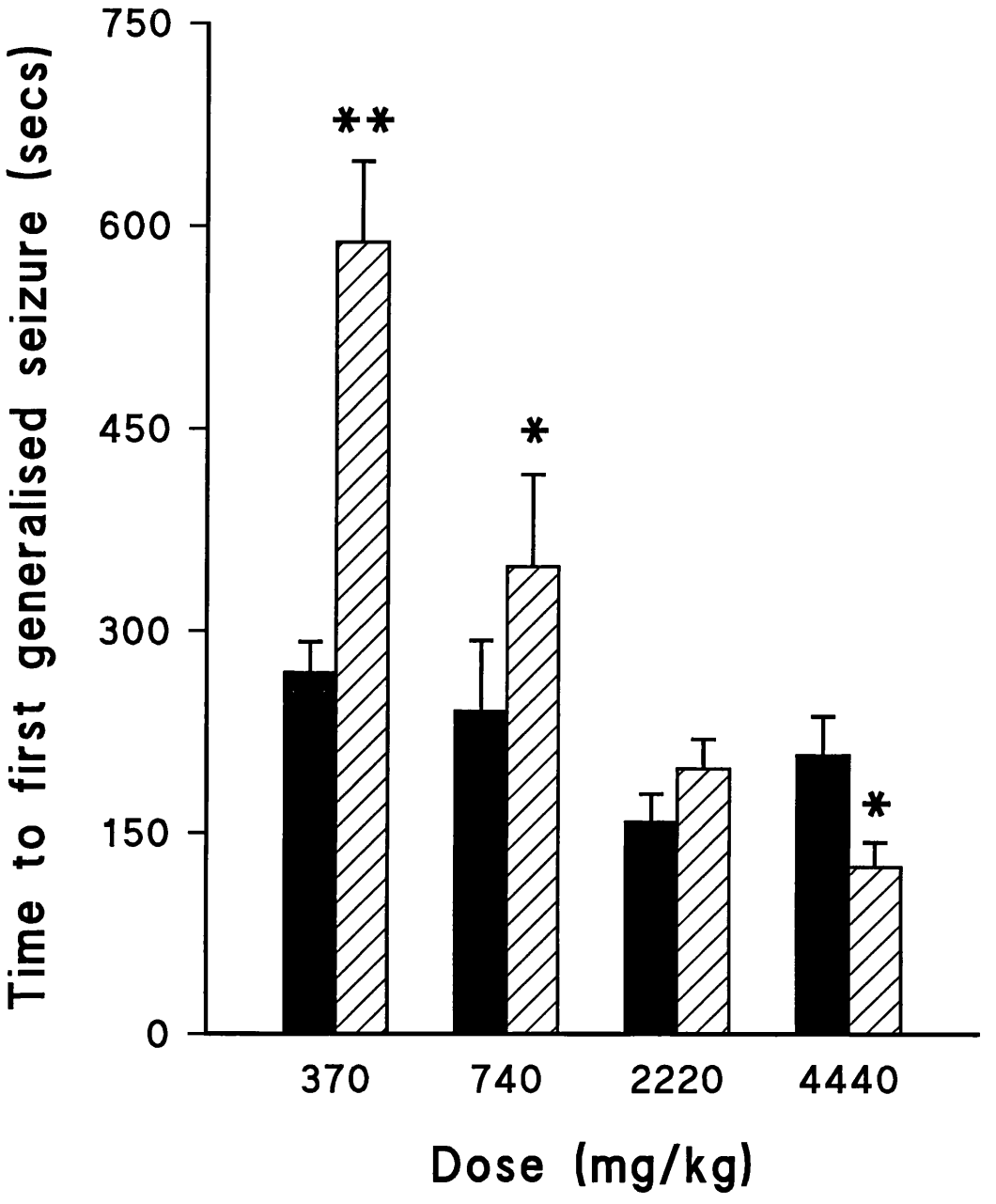

FIGURE 65:- Effect of single nicotinylalanine doses (370 - 4440 mg/kg; hatched bars) vs control (solid bars) on the latency to the first generalised seizure induced by 85 mg/kg PTZ at 1 hour post-administration. Results are expressed as the mean (± SEM) time (seconds) in groups of 10 mice. Statistical significance (*$p < 0.05$; **$p < 0.001$) was determined by the Mann Whitney U-test.

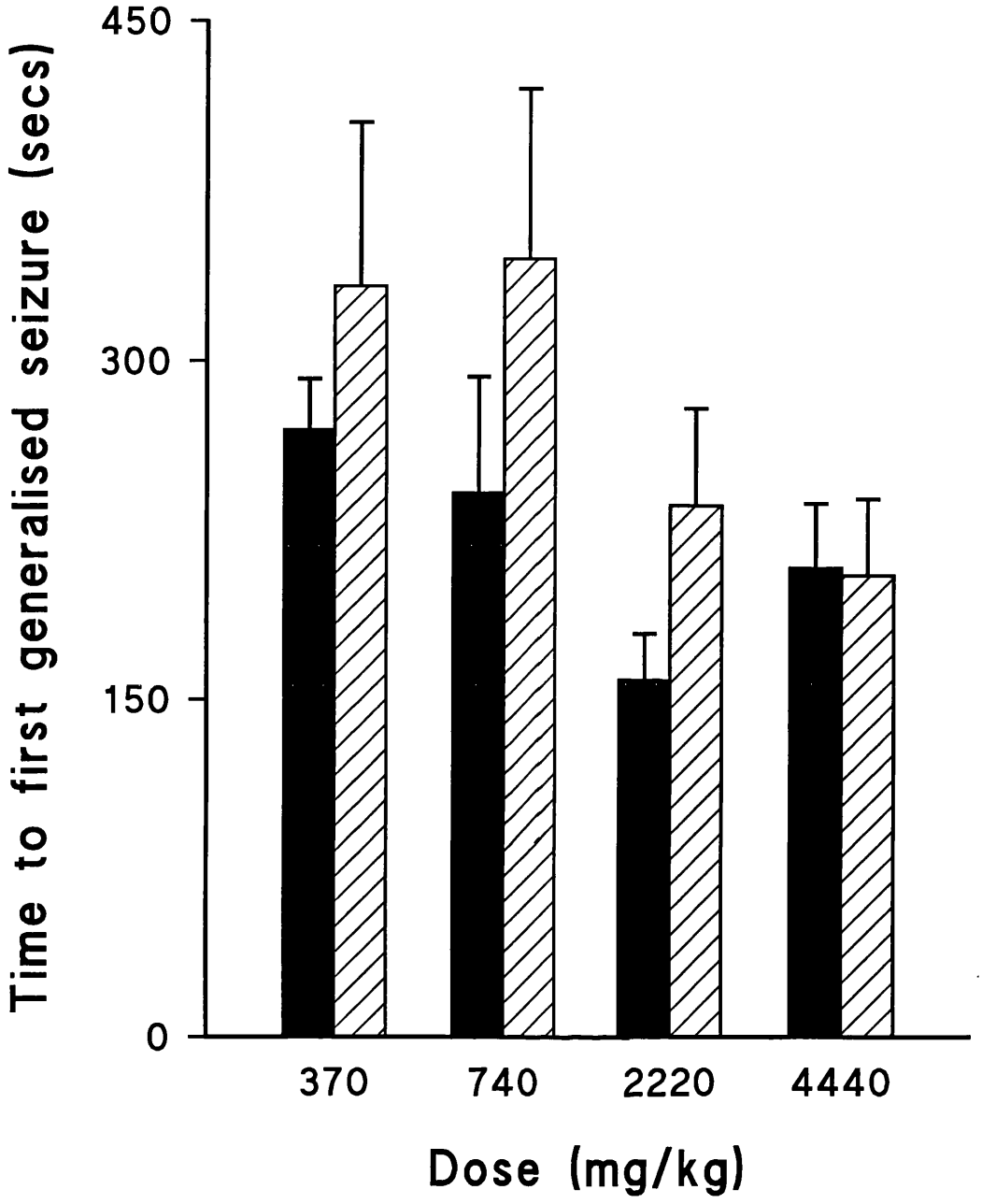

FIGURE 66:- Effect of single nicotinylalanine doses (370 - 4440 mg/kg; hatched bars) vs control (solid bars) on the latency to the first generalised seizure induced by 85 mg/kg PTZ at 4 hours post-administration. Results are expressed as the mean (± SEM) time (seconds) in groups of 10 mice.

RESULTS:- A single 185 mg/kg dose of NA was without significant effect on the tonic seizure threshold determined by Min-ES at 1 hour post-administration (figure 67). In contrast, a single 370 mg/kg dose of NA significantly ($p < 0.001$) increased the threshold for induction of tonic seizures at 1 hour post-dosing (figure 68). Median threshold values, determined by regression analysis of positive and negative responses, were 5.00 mA (control), 5.00 mA (185 mg/kg NA) and 7.00 mA (370 mg/kg NA).

7.3.3 Effect of nicotinylalanine on maximal electroshock-induced seizures in mice

PROTOCOL:- Mice were separated into two groups (n=10/group) and treated with either 370 mg/kg NA or vehicle (0.9% saline) alone. At one hour post-administration each animal was subjected to the MES test (section 2.3.2) using a stimulus current of 50 mA. The incidence of THE within each group was recorded.

RESULTS:- A single 370 mg/kg dose of NA significantly ($p < 0.001$) reduced the incidence of THE induced by MES at 1 hour post-administration (figure 69).

7.4 DISCUSSION

The aims of these studies were to determine the experimental anticonvulsant profile of NA, a putative anticonvulsant compound with a novel mechanism of action.

Effects of NA on PTZ-induced seizures

Single doses of NA (370 and 740 mg/kg) increased the latency to the first generalised seizure induced by PTZ at 1 hour post-administration (figure 65). This is a previously unreported finding for NA and may correlate with the optimal dose and incubation time

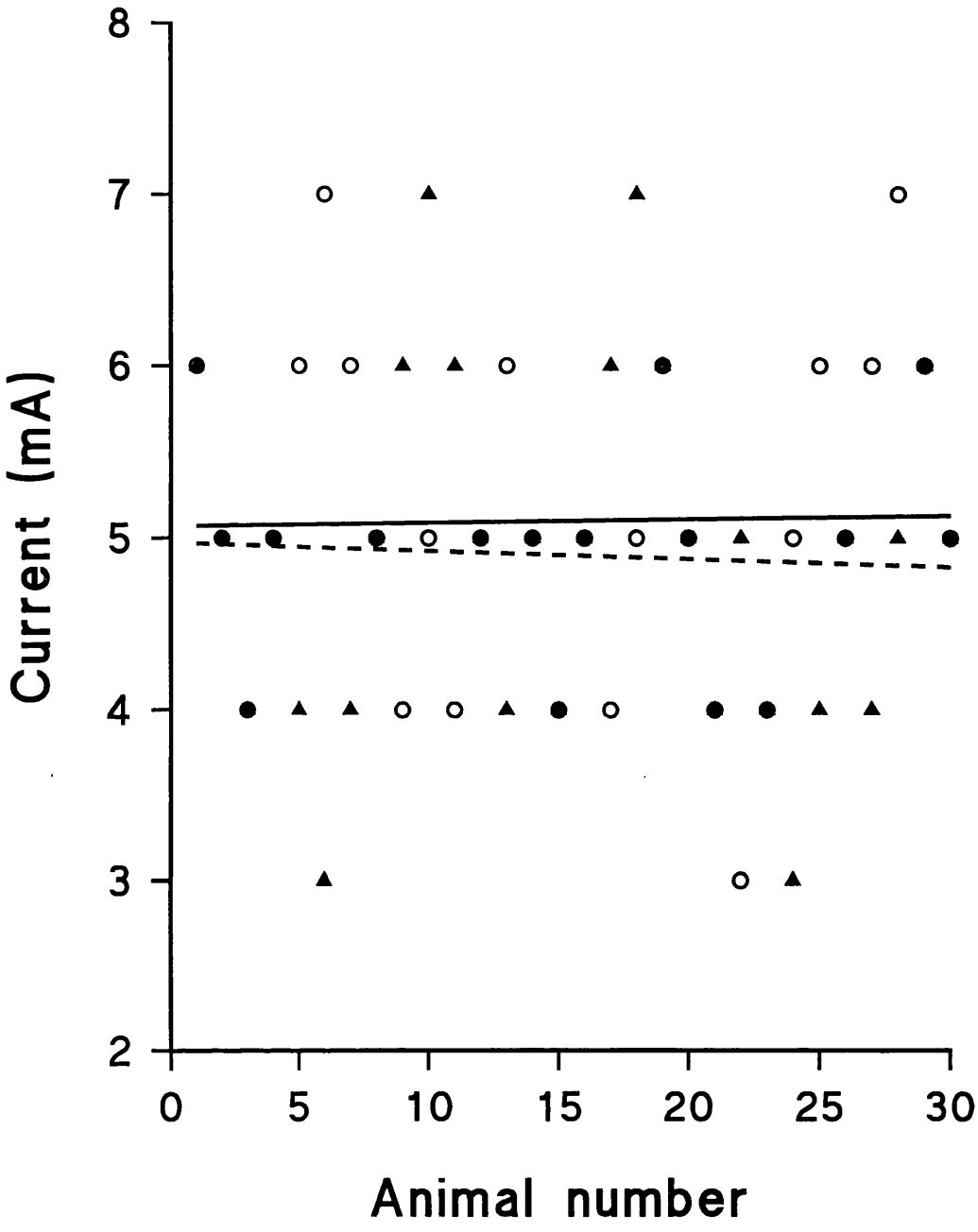

FIGURE 67:- Effect of nicotinylalanine treatment (185 mg/kg; closed triangles, broken line) vs control (open circles, solid line) on the tonic seizure threshold determined by Min-ES at 1 hour post-dosing. Closed circles represent data points at which animals from opposing groups (control and nicotinylalanine-treated) coincide. Seizure thresholds were calculated by regression analysis of the results obtained from groups of 30 mice.

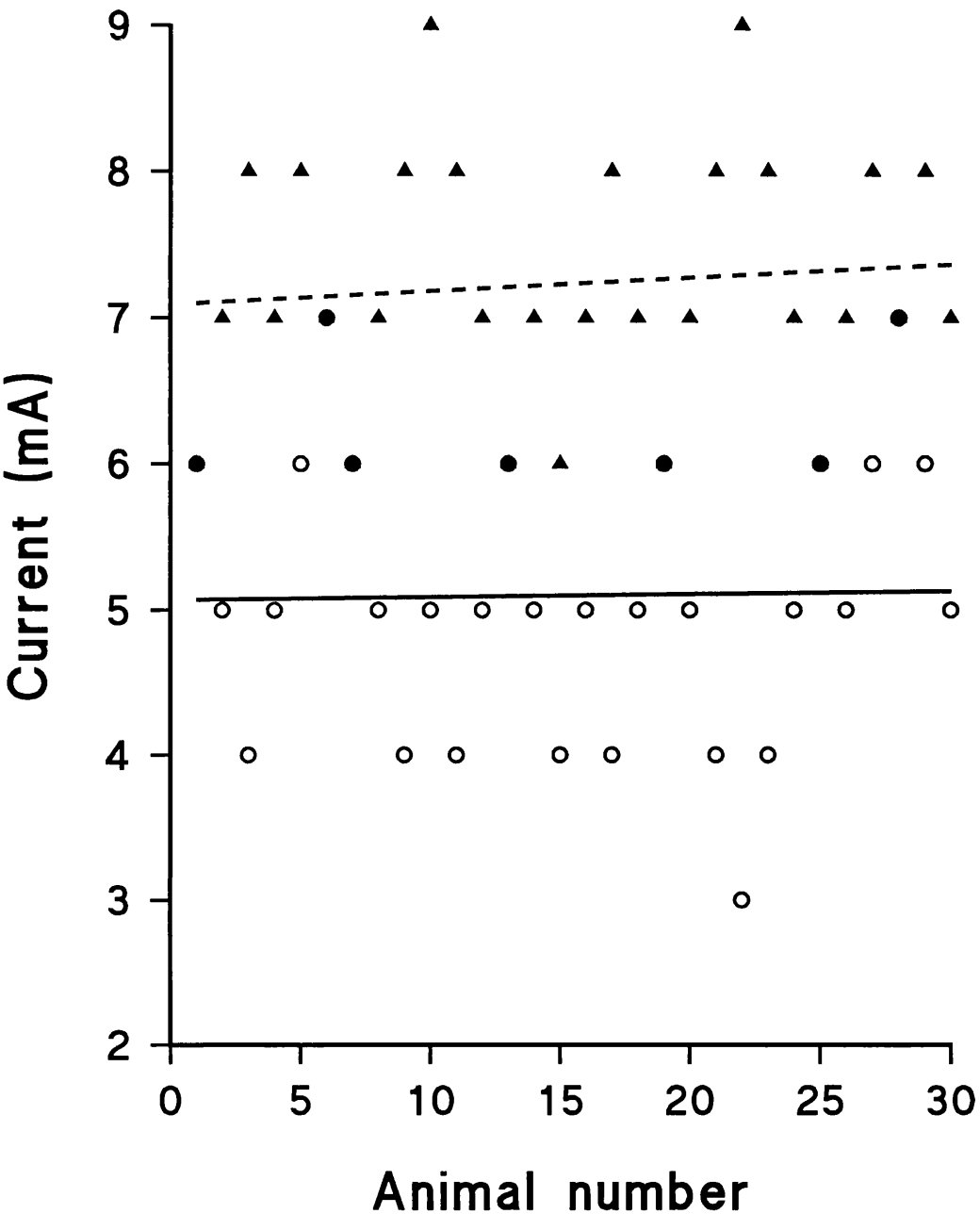

FIGURE 68:- Effect of nicotinylalanine treatment (370 mg/kg; closed triangles, broken line) vs control (open circles, solid line) on the tonic seizure threshold determined by Min-ES at 1 hour post-dosing. Closed circles represent data points at which animals from opposing groups (control and nicotinylalanine-treated) coincide. Seizure thresholds were calculated by regression analysis of the results obtained from groups of 30 mice and statistical significance ($p < 0.001$) was determined by the Mann Whitney U-test.

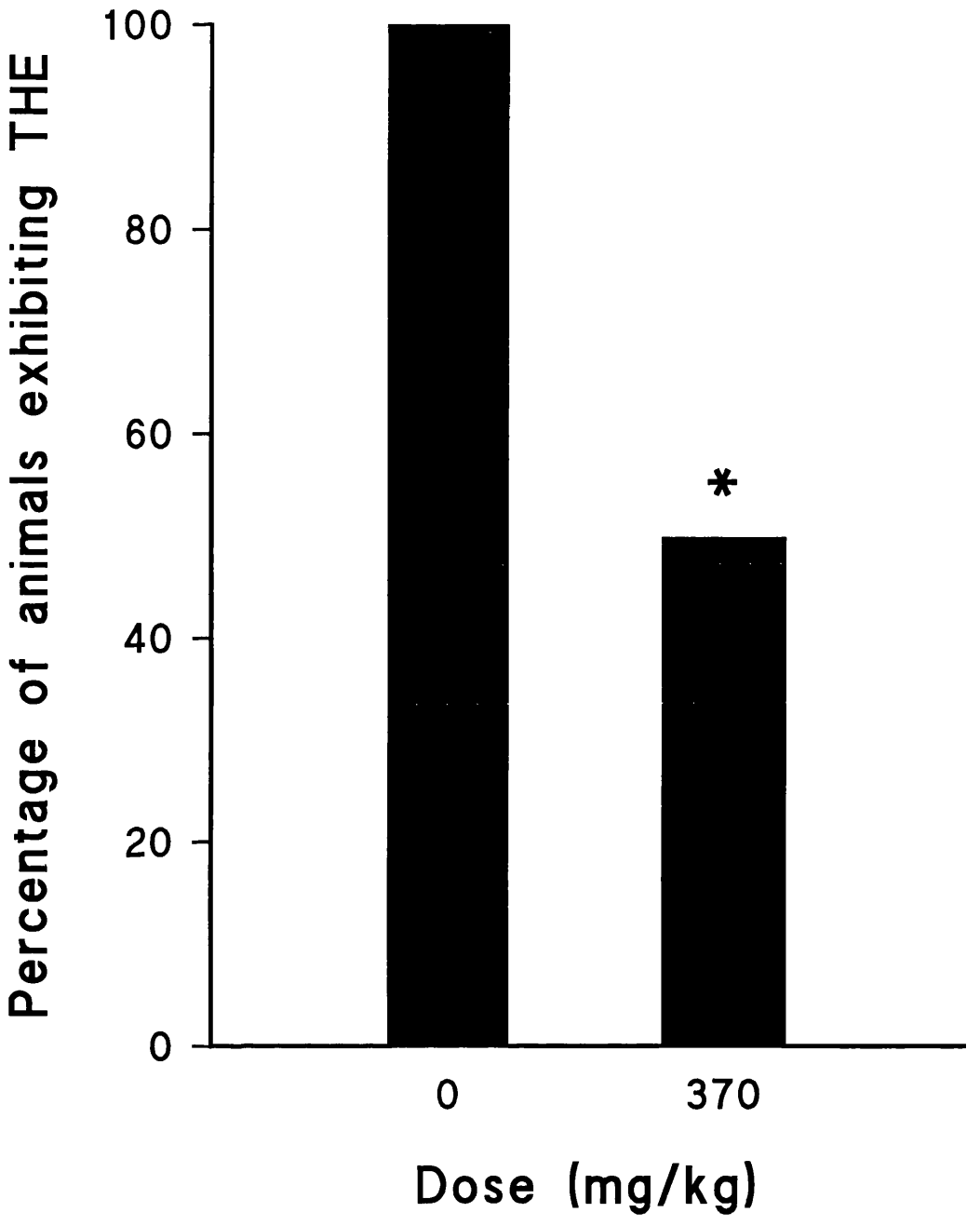

FIGURE 69:- Effect of a single nicotinylalanine dose (370 mg/kg) on the incidence of tonic hind-limb extension (THE) induced by MES at 1 hour post-administration. Results are expressed as the percentage of animals (mice) in groups of 10 exhibiting THE. Statistical significance (*$p < 0.001$) was determined by the Chi square test.

required for a maximal increase in brain kynurenic acid concentration. The highest dose of NA (4440 mg/kg) was seen to exert a proconvulsant effect by reducing the time to the first generalised seizure induced by PTZ at 1 hour post-dosing (figure 65). This is not an uncommon feature for an anticonvulsant drug. Many experimental and established AEDs have been shown to exhibit U-shaped dose-response curves (Nielsen et al, 1991). Whether this proconvulsant effect of NA is related to a neurotoxic action was not investigated although no ataxia or sedation was outwardly apparent in these animals. The lack of effect of any of the doses of NA at 4 hours post-administration (figure 66) suggested a relatively short half-life for this compound and/or a loss of efficacy by this time. In the absence of an assay to determine drug concentrations in the brain, this assumption was employed in the design of subsequent studies, all of which were performed at 1 hour post-dosing.

Effects of NA on Min-ES seizures

A single 370 mg/kg dose of NA increased the threshold for induction of tonic seizures, determined by Min-ES at 1 hour post-administration (figure 68). Concomitant analysis revealed a single 185 mg/kg dose of NA to be ineffective in this respect (figure 67). Again this anticonvulsant effect of NA is previously unreported. The effectiveness of 370 mg/kg NA in both the maximal PTZ test and the threshold electroshock test, with the relative lack of effect of both lower and higher doses in these experimental paradigms, suggested that this dose was close to optimal. As a result, this dose alone was selected for further investigation.

Effects of NA on MES-induced seizures

A single 370 mg/kg dose of NA significantly reduced the incidence of THE induced by MES at 1 hour post-administration (figure 69). Again this anticonvulsant effect of NA is previously unreported. This observation further supported the proposal that 370 mg/kg is, or is close to, the optimal anticonvulsant dose of NA.

7.5 CONCLUSIONS

In conclusion, NA appears to be an effective anticonvulsant against experimentally induced seizures. While these studies do not provide a definitive evaluation in this respect, they do suggest that the drug has efficacy against both PTZ- and MES-induced maximal seizures and against threshold electroshock seizures. Employing the loose parallels drawn by Löscher and colleagues (1991a), this evidence would propose that NA is not only capable of reducing seizure spread, as determined by the electroshock tests, but also of limiting seizure initiation. The drug appeared to be most effective at a dose of 370 mg/kg and to have a peak of effect some time before 4 hours. Further dose- and time-dependent studies are required to confirm this. Selective enzyme inhibition of the kynurenine pathway is a novel mechanism for anticonvulsant drug action and these results would suggest that it merits further investigation.

CHAPTER EIGHT

DEVELOPMENT OF A NOVEL ANIMAL MODEL OF EPILEPSY

8.1 INTRODUCTION

8.1.1 Animal models of the epilepsies

The study of the epilepsies has been dependent on the use of model systems. Much of what is now known about the epilepsies, and about AEDs, has been derived directly, or indirectly, from animal models (Fisher, 1989). Thus, it is appropriate to consider the value and limitations of the currently employed animal models of this disorder.

There are a large number of model systems in current use (section 1.5), firstly, because there are multiple types of clinical epilepsy to model (section 1.1.3) and, secondly, because none of the current models provides a definitive imitation in this respect. In addition, all of the existing models of epilepsy possess significant limitations.

The vast majority of the so-called "animal models of the epilepsies" are in fact models of a single seizure. Few, if any, mirror the condition of chronically recurrent spontaneous seizures which is characteristic of human epilepsy. Although some species, such as the beagle dog (Edmonds et al, 1978), do develop spontaneous seizures (Löscher and Schmidt, 1988; section 1.5.1), these cases are sporadic and are not usually observed in animals suitable for experimentation.

A further limitation of the various experimental epilepsy models in current use is the observation that they are models in which the traditional AEDs are active. Selective employment of these in the search for novel antiepileptic agents may preclude the identification of potentially useful compounds with new mechanisms of action (Löscher and Schmidt, 1988).

Finally, the most commonly employed animal models, the s.c. CD_{97} PTZ and MES tests (Gladding et al, 1985; section 3.1), are proposed to predict the effectiveness of drugs against absence and generalised tonic-clonic seizures respectively (Woodbury, 1972). However, most patients with these seizure types can be successfully treated with existing AEDs (Brodie, 1990) and there is a much more pressing clinical demand for novel treatments of the more intractable disorders, such as the partial epilepsies.

Thus, while existing models of epilepsy may represent the final common pathway for many kinds of epileptic seizures (Porter, 1990), it is also possible that potentially useful compounds have been discarded through their selective use. Few help to address the significant clinical problem of intractable seizures and fewer still mirror the chronic spontaneously recurrent nature of human epilepsy. Therefore, there is a continued requirement for the development of new animal models of epilepsy.

8.1.2 Existing animal models of the partial epilepsies

As discussed above, few animal models provide the researcher with a suitable experimental paradigm for the investigation of the more intractable epileptic syndromes, such as the partial epilepsies. Those which do afford a representation of the clinical disorders in this respect (Fisher, 1989) are not universally employed in epilepsy research and are rarely used in the routine screening of potential antiepileptic agents (Löscher and Schmidt, 1988).

The partial epilepsies can be roughly divided into two classes. Simple partial, or focal, epilepsy is believed to be essentially cortical in origin (Meldrum, 1990), whereas complex

partial seizures are thought to arise in the limbic system (Gloor et al, 1982). The following separation of models for simple partial and complex partial seizures follows this rather crude clinical distinction.

The best validated and most realistic models for the focal epilepsies are those employing application or implantation of metals onto, or into, the cerebral cortex. This procedure precipitates a state of "spontaneously" recurrent simple partial seizures, although the requirement for initial induction, by metallic implantation, would question this proposed spontaneity. Of this group, the aluminium hydroxide gel model in the monkey is the best characterised (Ward, 1972). Injection of 4% aluminium hydroxide into the monkey neocortex at a few adjacent sites results in the generation of partial seizures 1 to 2 months later. These seizures are recurrent, appear spontaneously, and persist for up to several years. The seizure characteristics are similar in type to human simple partial seizures and the respective electrophysiologies also correlate well. Neuropathological specimens excised from established alumina gel foci show extensive gliosis and dendritic degeneration of a nature similar to that observed at human neocortical foci (Westrum et al, 1964). Such spontaneously recurrent seizures can be precipitated by cortical application of other metals including cobalt (Dow et al, 1962), tungsten (Blum and Liban, 1960), zinc (Pei et al, 1983), and iron (Lange et al, 1980), although none of these models is documented as extensively as the alumina gel model.

Interestingly, the convulsant metal models, especially the tungstic acid model, are relatively resistant to AED therapy, proposing them as selective experimental paradigms for the investigation of intractable focal epilepsy in man. However, none of these models

is widely used in the screening of novel AEDs, although the monkey alumina gel model is now employed in the NINCDS programme to evaluate candidate drugs which have successfully passed all sequential test phases of the preclinical screening programme (Gladding et al, 1985). Thus, the metal deposition models are good models of chronic simple partial seizures which share several characteristics with their proposed clinical counterparts. The seizures observed are recurrent, essentially spontaneous in nature, and relatively resistant to treatment with established AEDs. Unfortunately these models are laborious and expensive to prepare, especially in monkeys, and involve injection of irritant compounds with little relevance to clinical epileptic foci into the cortex.

One model which does not require the injection of exogenous compounds into the brain is the cryogenic or freeze lesion model for simple partial seizures (Hanna and Stalmaster, 1973). This model involves application of liquid nitrogen or an ethylchloride spray directly onto an area of exposed neocortex in the rat, rabbit, or cat (Loiseau et al, 1987). This procedure, first described by Nims and colleagues (1941), results in the generation of spontaneous seizure activity within a few hours (Fisher, 1989). The peak of electroencephalographic abnormality is observed at 8 hours after freezing (Lewin and McCrimmon, 1968) and seizures persist for several days. Substantial cerebral oedema generally accompanies the lesion (Fisher, 1989). The cryogenic model is a relatively useful model of recurrent simple partial seizures although the period of ictal activity maybe classed as sub-chronic when compared to the metal deposition models and again the seizures are not strictly spontaneous. Together, these models represent suitable experimental paradigms for the investigation of the intractable focal epilepsies. One advantage of the cryogenic model is the lack of potential complications associated with

implantation of exogenous compounds into the brain, although the role of cerebral oedema in the generation of seizures in this model is unclear. Its ability to be manifested in small laboratory animals, such as rats, may also give it precedence over other such models.

Animal models for complex partial seizures, perhaps the most drug-resistant of the human epilepsies (Schmidt, 1984), have attracted much attention in recent years. Kainic acid is an analogue of the excitatory neurotransmitter glutamate which precipitates extensive hippocampal neuronal cell body degeneration following both focal and systemic administration (Olney et al, 1974). Kainic acid also produces seizure activity following systemic administration, generally at doses lower than those required to produce cell death (Lothman et al, 1981). Although kainic acid is a prototypic excitotoxin, the effects of threshold convulsant doses are probably mediated by synaptic disinhibition rather than excitotoxic cell death (Lancaster and Wheal, 1984). Kainic acid induces seizures which may be manifested for several days. The accompanying hippocampal lesions may also be employed to portray the pattern of limbic cell damage which can occur with clinical status epilepticus (Sloviter, 1987). Thus, systemic kainic acid administration results in a short period of recurrent seizures, the spontaneity of which is again questionable. The absolute value of this model of recurrent seizures in terms of investigation of intractable epilepsy, and in the evaluation of novel antiepileptic therapies, remains to be evaluated.

Another model of chronic recurrent complex partial seizures involves the injection of a small amount of tetanus toxin into the hippocampus of the rat or cat (Mellanby et al, 1977). Classification of this model as one for complex partial seizures results from the location of the injection site and the seizures observed rather than the properties of the

toxin itself (Fisher, 1989). Intra-hippocampal injection of a dose up to 10 times the mouse LD_{50} produces only local effects with no visible neuronal destruction even after seizures remit (Jefferys and Williams, 1987). Tetanus toxin-induced seizures are characterised by an initial arrest of motor activity, followed by myoclonic jerks of the forelimbs, and occasionally secondary generalisation to tonic-clonic seizures (Fisher, 1989). These seizures occur within one day of injection and recur over a one month period with animals exhibiting up to 100 such events every day. Whether or not the seizure generalises depends upon several factors, including spread to the cingulate area (Hawkins and Mellanby, 1987). Tetanus toxin-induced seizures are sensitive to treatment with some established AEDs, including CBZ (Hawkins et al, 1985). This model may be of benefit in the investigation of chronically recurrent complex partial seizures although the exact mechanism by which tetanus toxin precipitates seizures remains unclear. The relevance of this model to the intractable human epilepsies, and evaluation of novel treatments of them, also remains to elucidated.

The most commonly employed and best characterised of all models of the partial epilepsies is the kindling model (McNamara, 1984; McIntyre and Racine, 1986). However, for the most part this model, described in section 1.5.2, does not mirror the spontaneously recurrent nature of human complex partial seizures, with individual seizures being evoked in direct response to daily electrical stimulation. However, while most kindling experiments are terminated after elicitation of one or more class 5 motor seizures (Racine, 1972), if stimulation is continued for a few weeks thereafter, rodents begin to exhibit spontaneous epileptic seizures in the absence of priming shocks (Pinel and Rovner, 1978). Presumably, brain excitability has, at this point, reached a state in which

normal afferent activity can trigger epileptiform activity. Among those animals exhibiting at least three spontaneous stage 5 motor seizures, the occurrence of spontaneous seizures persisted for up to 7 months in the absence of electrical stimulation (Pinel and Rovner, 1978). Seizure characteristics included rearing, accompanied with bilateral forelimb clonus, followed by loss of balance and falling, and then generalised clonic seizures. Spontaneous seizures induced by kindling have also been identified in other species including baboons, cats, and dogs (McNamara, 1984). This prolonged period of kindling is a relatively time consuming procedure as it requires chronic implantation of electrodes and regular electrical stimulation (once daily for up to 4 weeks) to establish the state of recurrent spontaneous kindled seizures (Löscher and Schmidt, 1988). While the vast majority of established and experimental stage AEDs are active against one or both components of the traditional kindling response (Rogawski and Porter, 1990), whether such drugs are effective against these kindling-induced spontaneously recurrent seizures is unclear. Similarly, the absolute value of this model for investigation of chronically recurrent complex partial seizures is also unclear. It is possible that the secondary generalisation associated with this model may preclude the direct investigation of complex partial seizures and the evaluation of novel antiepileptic therapies of this seizure type.

8.1.3 Development of a novel animal model of partial epilepsy

None of the experimental models of the partial epilepsies discussed above is ideal. All have their limitations for the investigation of the most drug-resistant forms of human epilepsy. As a result, there is a continued requirement for the development of novel animal models of partial seizures. Several criteria exist for the embodiment of novel animal models into the laboratory investigation of the partial epilepsies and the routine evaluation

of novel AEDs effective against such intractable seizures. These novel models should be simple and economic to produce and maintain in the laboratory. They should also possess a degree of relevance to the clinical syndrome they are proposed to represent. The seizures observed should be recurrent and essentially spontaneous in nature. Finally, they should be relatively resistant to established AED treatment, and thus suitable experimental paradigms for the investigation of novel AEDs and their mechanisms of action.

With the explosion in the use of laser treatment in clinical medicine, particularly in corrective ophthalmic surgery, it was proposed that laser radiation could be exploited in a potential animal model of partial epilepsy. In a manner similar to that of the freeze lesion described above, it was hoped that a laser beam of sufficient intensity could be impinged upon a particular region of the cerebral cortex to induce tissue damage and produce a local epileptic focus. Such a procedure might have several advantages over existing animal models of partial seizures. Firstly, the procedure would not involve the application or implantation of exogenous compounds onto or into the brain. Secondly, the method could be designed for use in small animals, minimising the expense and husbandry difficulties associated with primate models. Thirdly, with the extensive use of similar procedures in clinical medicine, it could be anticipated that such a procedure would be devoid of secondary complications, such as cerebral oedema observed in the freeze lesion model, and perhaps also local inflammation. Finally, and most importantly, it was proposed this potential model might represent the first animal model of focal epilepsy in which the inducing stimulus was quantifiably variable and could be reproduced with a high degree of accuracy in large groups of animals.

8.1.4 Laser-induced lesions of the cerebral cortex

With no previously documented experimental blueprint for reference, the design and construction of the specialised apparatus required for this purpose was a laborious process. The complete experimental set-up is show in plate 4. A Synrad 10 watt CO_2 laser was chosen to deliver the lesioning radiation. An extensive beam delivery and alignment apparatus consisting of a zoom beam expander, a beam attenuator, a mirror block, a fine focus unit, and a magnifying lens were also incorporated. The invisible nature of CO_2 laser radiation necessitated the inclusion of a helium/neon (HeNe) laser whose red beam could be employed for aiming purposes. The HeNe laser was incorporated into the beam delivery assembly, adjusted to be in direct alignment with the main CO_2 beam, but had no ionising action of its own. Laser output could be controlled by various adjustments in the beam delivery apparatus. Absolute outputs ranged from 0.2 to 10.0 watts at intervals of approximately 0.1 watts. A foot pedal was incorporated to initiate the CO_2 beam, allowing the operator to conduct the lesioning procedure in a safe, "hands-free" manner. An intrinsic timing device controlled the CO_2 laser output and allowed timed exposures of between 1 and 60 seconds at 1 second intervals.

8.2 EXPERIMENTAL AIMS

The aims of the following preliminary studies were to explore the method of producing chronic laser lesions in the somatosensory cortex of rats and to determine the pathological reproducibility and seizure susceptibility of this procedure. Preliminary evaluations of the local biochemical changes arising as a result of this procedure were also conducted. Reproducibility, proposed to set this model apart from other models of chronic epilepsy, was determined by histological studies which compared the pathological nature and extent

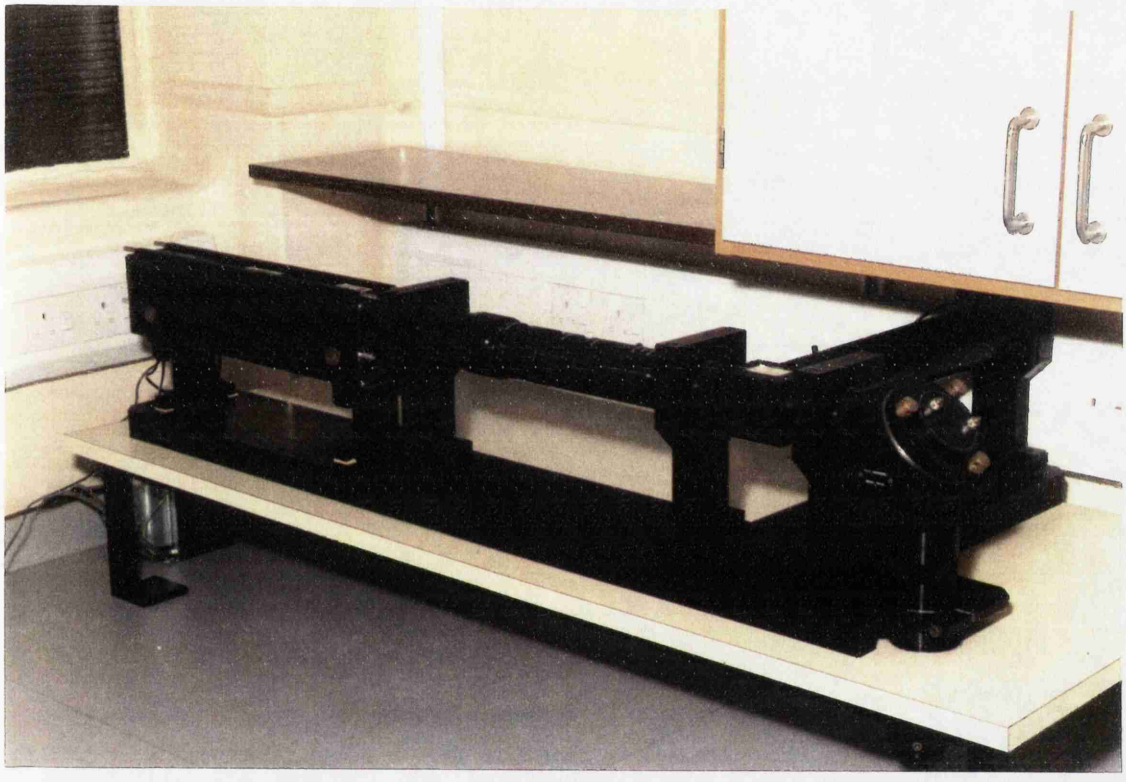

PLATE 4:- Experimental apparatus employed for the production of laser-induced lesions of the rat somatosensory cortex.

of a standardised lesion. Seizure susceptibility of the lesioning procedure was investigated by the use of the PTZ test which compared seizure latency in lesioned animals and in age and weight matched shams. Finally, preliminary investigation of the biochemistry of the lesion was performed by 2-deoxyglucose autoradiography, to determine the effects of the procedure on cerebral glucose metabolism (Sokoloff et al, 1977), and by PK-11195 autoradiography, which facilitated determination of the extent and volume of tissue damage imparted by the lesioning procedure (Benavides et al, 1983).

8.3 EXPERIMENTAL METHODS

8.3.1 Preparation of animals

SD rats (200 - 300g) were anaesthetised by placing them in a sealed chamber which was then flooded with 2% halothane in a 70:30 N_2O/O_2 mixture. The animals were allowed to remain in the chamber for up to 5 minutes to ensure anaesthesia had progressed to a sufficiently deep stage.

8.3.2 Surgical procedures

On removal from the chamber, animals were maintained under 1.5% halothane (in 70:30 N_2O/O_2) anaesthesia by application of a facemask. The scalp was cleaned with a disposable alcohol swab and a midline incision of approximately 1.5 cm in length was made with a scalpel. The skin was reflected in either direction and lightly clamped. The periosteal membranes overlying the right frontal and parietal bones of the skull were cleared with sharp forceps. A hand-held electric drill was used to fashion a small burr hole (2mm diameter) in the skull, over the somatosensory region of the cortex, and in alignment with the line of Bregma (Paxinos and Watson, 1982). The drilling time was

kept to a minimum (≤ 20 seconds) to prevent inadvertent heating of the skull. Care was also taken not to rupture the underlying dura mater.

8.3.3 Production of a cortical laser lesion

Animals were briefly removed from the anaesthetic apparatus and placed in the path of the HeNe aiming laser beam. The position of the animal was adjusted until the HeNe beam impinged directly on the centre of the circle of exposed dura mater. At this point the CO_2 laser beam was initiated. Animals were exposed to 0.5 watts of CO_2 laser radiation for a 10 second period. Movement of the head during the lesioning procedure was a fairly common problem, occurring in almost 10% of cases. Animals exhibiting such movements were sacrificed immediately. Those animals, surgically prepared as described in sections 8.3.1 and 8.3.2, but not exposed to laser radiation were classed as sham-operated.

8.3.4 Recovery of animals

Following the lesioning, or sham, procedure animals were re-fitted with the anaesthetic face mask and the scalp incision was closed with 4-5 cotton stitches. Animals were then placed in individual cages and monitored until consciousness was regained. On average the total procedure time, from initiation of anaesthesia to recovery, was ≤ 10 minutes. After a period of 5 days the animals were subjected to a light (1%) halothane anaesthesia, as described in section 8.3.1, to facilitate removal of the stitches. Animals were returned to their cages and monitored thereafter on a twice daily basis.

8.3.5 Long-term observations

In total, 126 animals underwent this procedure, 78 laser-treated and 48 sham-operated. Animals were kept for up to one year post-operative with few (≤ 5%) deviations from normal health. Scalp infection and self-removal of stitches were observed in ≤ 1% of animals and the incidence of unexpected mortality (≤ 1%) was similarly low. No extraneous motor or behavioural changes were observed in the post-operative survival period in any of the animals.

8.4 EXPERIMENTAL PROTOCOLS AND RESULTS

8.4.1 Pathological characteristics and reproducibility of the laser lesion

PROTOCOL:- At nine months post-operative the brains of 3 laser treated and 2 sham-operated animals were examined histologically. The animals were sacrificed by anaesthetic (6% halothane) overdose and their brains removed by the procedure described in section 2.2.3. The isolated brains were immersed in neutral buffered 10% formalin solution immediately on removal and the histological investigations described in section 2.18 were performed.

RESULTS:- Macroscopically, a well-circumscribed lesion was identified in the right parietal cortex of all three laser-treated animals. Microscopy of haemotoxylin/eosin stained sections revealed an area of old destruction of the cerebral cortex with a deficient centre and coagulative necrosis in the surrounding tissues. The lesion was approximately 1 mm in diameter at the cortical surface, tapering to around 60 μm in cortical layer six. The lesions of all three laser-treated animals were similar in nature. They transversed all six layers of the cortex with no damage to the underlying white matter. Cellular characteristics

of the lesion included a selective loss of neuronal cells, as determined by cresyl violet staining, and prominent gliosis and fibrosis, adjudged by GFAP immunohistochemistry. There was little or no infiltration of chronic inflammatory cells. No such lesions were identified either macro- or microscopically in the two sham-operated animals. Microscopic images of the laser lesion, stained with haemotoxylin/eosin, are illustrated in plates 5 (low power) and 6 (high power). Anatomically matched control sections, taken from sham-operated animals, are included for reference (plates 7 and 8).

8.4.2 Seizure susceptibility of the laser lesion

PROTOCOL:- At nine months post-operative 6 laser-treated and 6 sham-operated rats were evaluated for their susceptibility to chemically-induced seizures. Each animal was administered 85 mg/kg PTZ (i.p.) and the latency to the first generalised seizure in individual animals was recorded (section 2.3.1). The intraperitoneal route was chosen due to handling difficulties with animals whose average weight was 670 g.

RESULTS:- There was no significant difference in the time to the first generalised seizure induced by PTZ between the laser-treated and sham-operated groups (figure 70). Mean seizure latencies (± SEM) were 414 (± 131) seconds (laser-treated) and 549 (± 139) seconds (sham-operated).

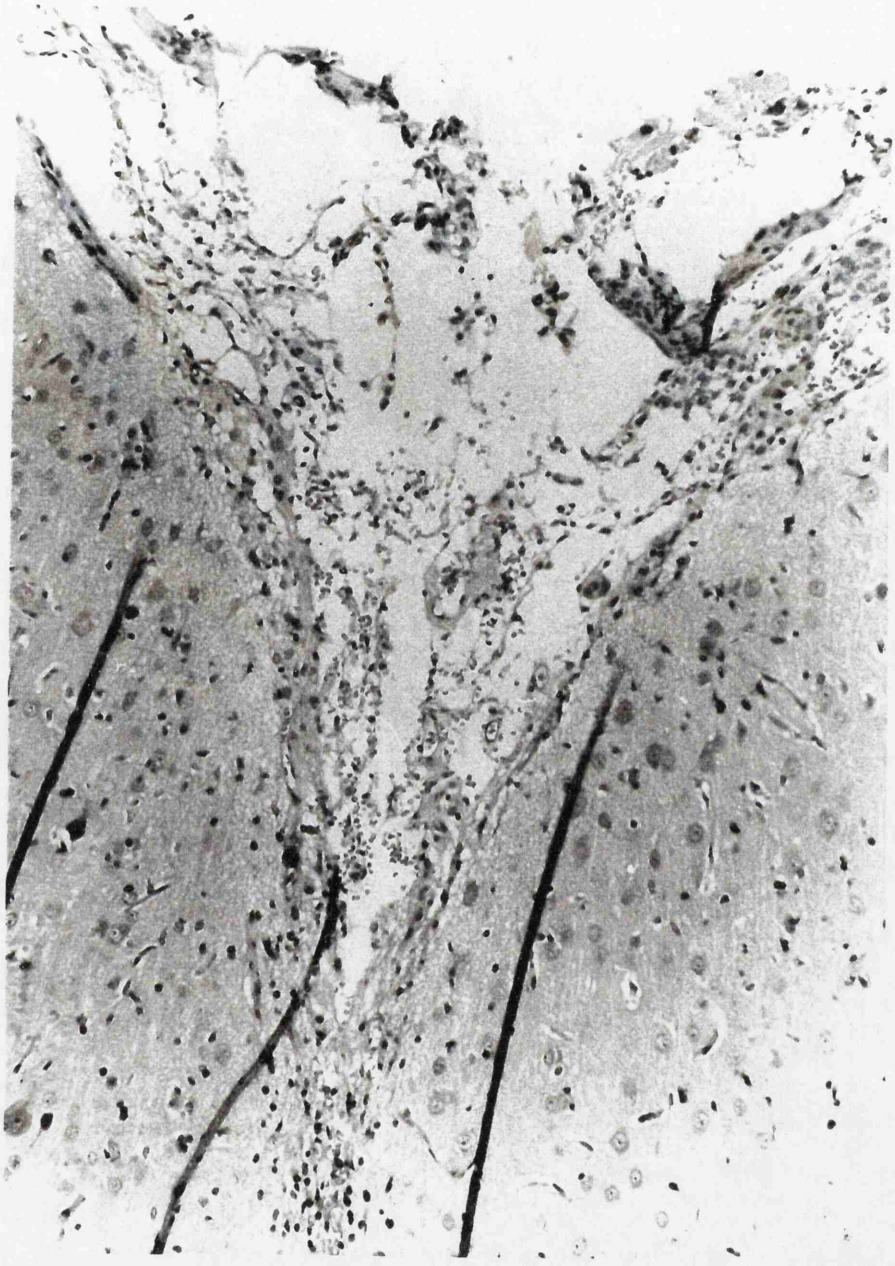

PLATE 5:- Low power (x 40) microscopic image of a laser lesion in the somatosensory cortex of the rat stained with haemotoxylin/eosin at nine months post-operative. The image encompasses cortical layers 1 - 4. Pronounced black lines are a result of a folding of the tissue section.

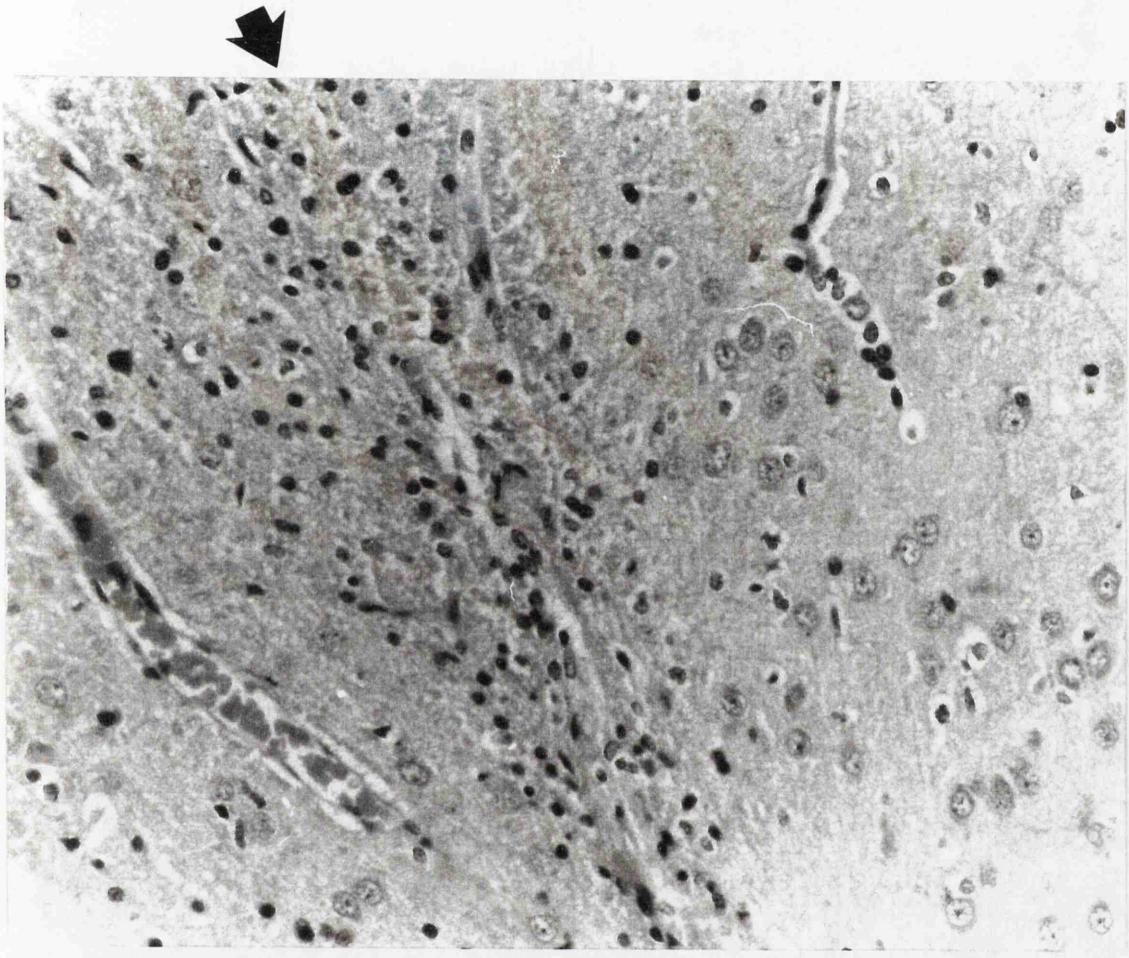

PLATE 6:- High power (x 100) microscopic image of a laser lesion in the somatosensory cortex of the rat stained with haemotoxylin/eosin at nine months post-operative. The image encompasses cortical layers 5 and 6. The lesion tract is indicated by the arrow.

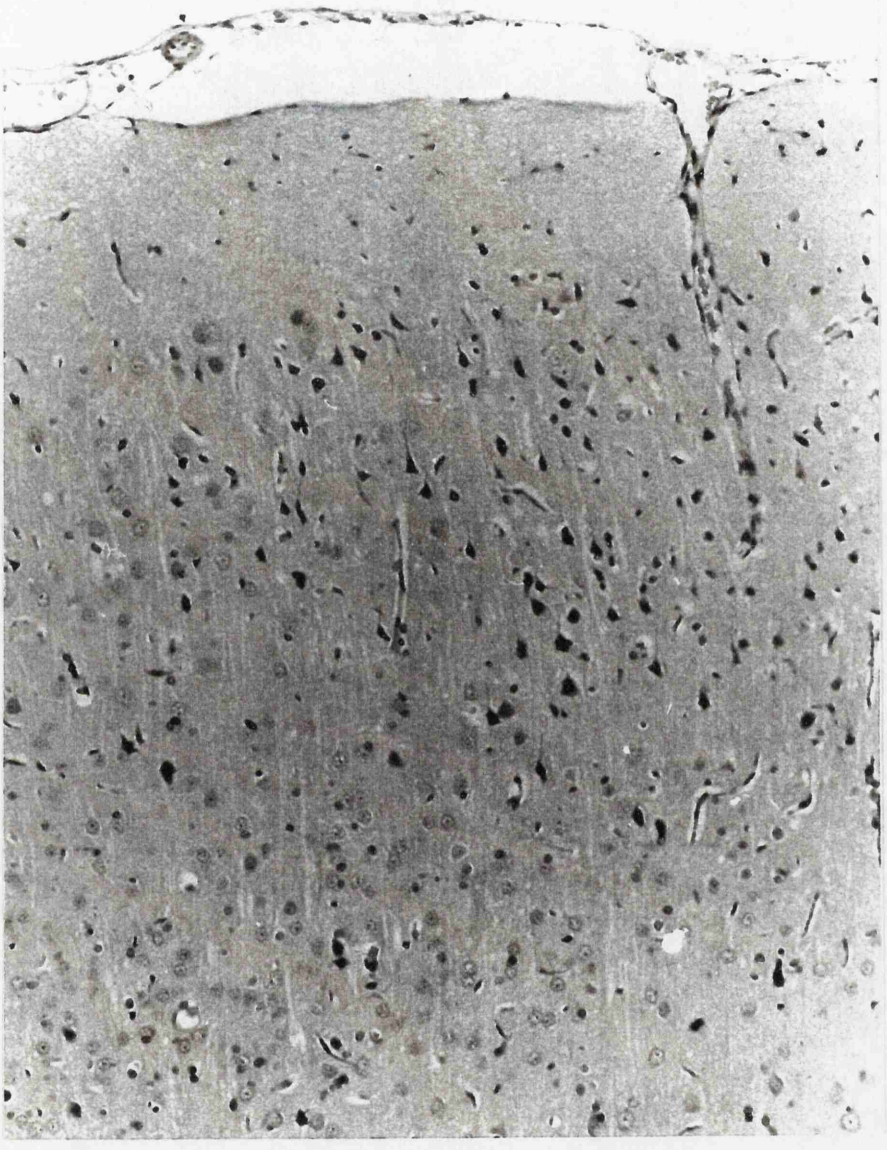

PLATE 7:- Low power (x 40) microscopic image of the somatosensory cortex of a sham-operated rat stained with haemotoxylin/eosin at nine months post-operative. The image encompasses cortical layers 1 - 4 and is anatomically matched to that in plate 5.

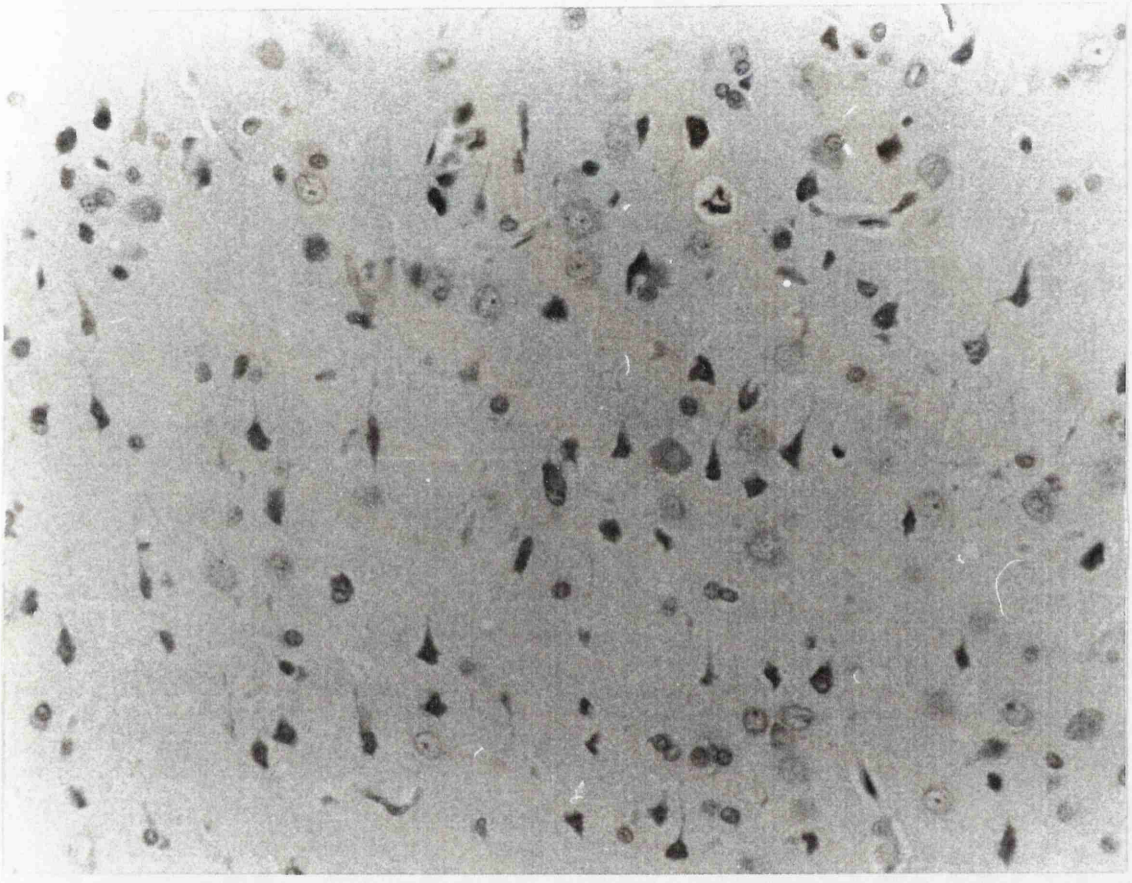

PLATE 8:- High power (x 100) microscopic image of the somatosensory cortex of a sham-operated rat stained with haemotoxylin/eosin at nine months post-operative. The image encompasses cortical layers 5 and 6 and is anatomically matched to that in plate 6.

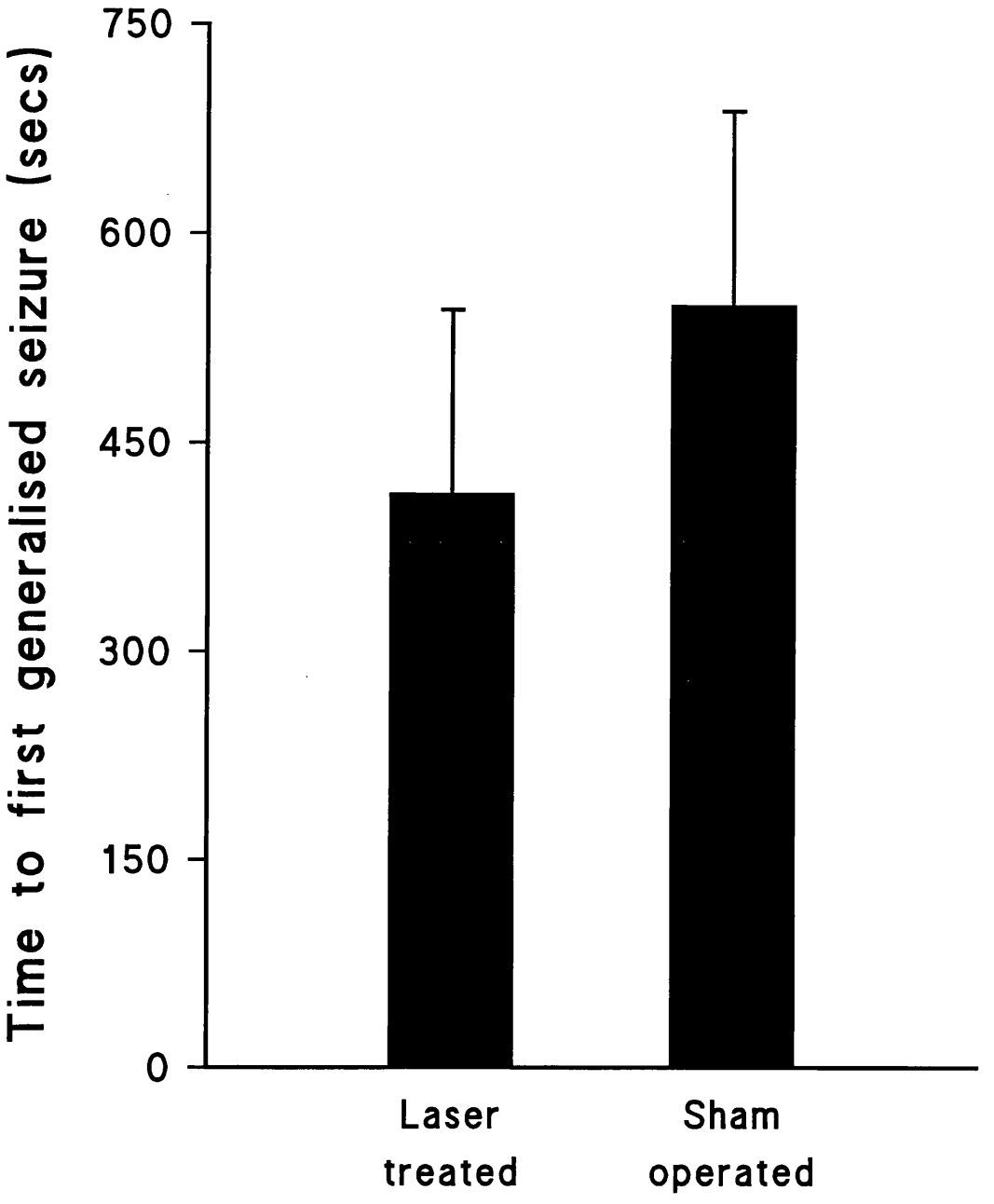

FIGURE 70:- Effects of laser-lesioning vs sham-operated treatment on the latency to the first generalised seizure induced by 85 mg/kg PTZ at nine months post-operative. Results are expressed as mean (± SEM) time (seconds) in groups of 6 rats.

8.4.3 Evaluation of cerebral glucose metabolism following the laser lesioning procedure

PROTOCOL:- At nine months post-operative 3 rats, 2 laser-treated (animals 1 and 2) and 1 sham-operated (animal 3), underwent assessment of cerebral function in terms of cellular glucose utilisation. This was performed by the *in vivo* [^{14}C]-2-deoxyglucose autoradiographical method described in section 2.16.

RESULTS:- The effects of the lesioning, and sham, procedures on LCGU were examined in eight discrete regions of the brain. These included the thalamus, globus pallidus, and caudate nucleus, major projection areas of the somatosensory cortex, the corpus callosum, a white matter tract, and the somatosensory cortex itself, both within the lesion and adjacent to it. The results for animals 1 - 3 are illustrated in tables 2 - 4 respectively. All of the structures were examined both ipsi- and contralaterally to the lesion. Contralateral values, and those obtained from the sham-operated animal (table 4), were employed as intra- and inter-animal controls respectively. With the exception of the ipsilateral somatosensory cortex, the lesioning procedure was without effect on LCGU in any of the regions investigated. In the laser-treated animals (tables 2 and 3), LCGU was reduced in the somatosensory cortex, both within the lesioned area and adjacent to it. The percentage reduction in LCGU, compared to contralateral control values, was greatest within the lesion itself. The area of reduced metabolism surrounding the lesion was seen to extend for approximately 0.5 mm in all directions. Autoradiographical images of the brain sections containing the lesions and of an anatomically matched control (sham-operated) are illustrated in plates 9 - 11.

	LCGU IPSILATERAL (μmoles/100g/min)	LCGU CONTRALATERAL (μmoles/100g/min)
Mediodorsal thalamus	85	88
Ventrolateral thalamus	74	77
Mediodorsal caudate	90	92
Ventrolateral caudate	97	98
Globus pallidus	47	50
Corpus callosum	40	40
Somatosensory cortex (within lesion)	72	116
Somatosensory cortex (adjacent to lesion)	87	116

TABLE 2:- Local cerebral glucose utilisation (LCGU) in eight distinct brain regions following the laser-lesioning procedure in animal # 1. Individual results are expressed as μmoles/100g/min and reflect the mean LCGU calculated from 6 optical density readings made in 3 sections per animal.

	LCGU IPSILATERAL (μmoles/100g/min)	LCGU CONTRALATERAL (μmoles/100g/min)
Mediodorsal thalamus	88	92
Ventrolateral thalamus	83	85
Mediodorsal caudate	104	101
Ventrolateral caudate	106	101
Globus pallidus	52	52
Corpus callosum	36	38
Somatosensory cortex (within lesion)	53	114
Somatosensory cortex (adjacent to lesion)	68	114

TABLE 3:- Local cerebral glucose utilisation (LCGU) in eight distinct brain regions following the laser-lesioning procedure in animal # 2. Individual results are expressed as μmoles/100g/min and reflect the mean LCGU calculated from 6 optical density readings made in 3 sections per animal.

	LCGU IPSILATERAL (μmoles/100g/min)	LCGU CONTRALATERAL (μmoles/100g/min)
Mediodorsal thalamus	88	89
Ventrolateral thalamus	80	77
Mediodorsal caudate	63	68
Ventrolateral caudate	72	70
Globus pallidus	38	44
Corpus callosum	26	26
Somatosensory cortex (within lesion)	92	88
Somatosensory cortex (adjacent to lesion)	87	94

TABLE 4:- Local cerebral glucose utilisation (LCGU) in eight distinct brain regions following the sham-operated procedure in animal # 3. Individual results are expressed as μmoles/100g/min and reflect the mean LCGU calculated from 6 optical density readings made in 3 sections per animal.

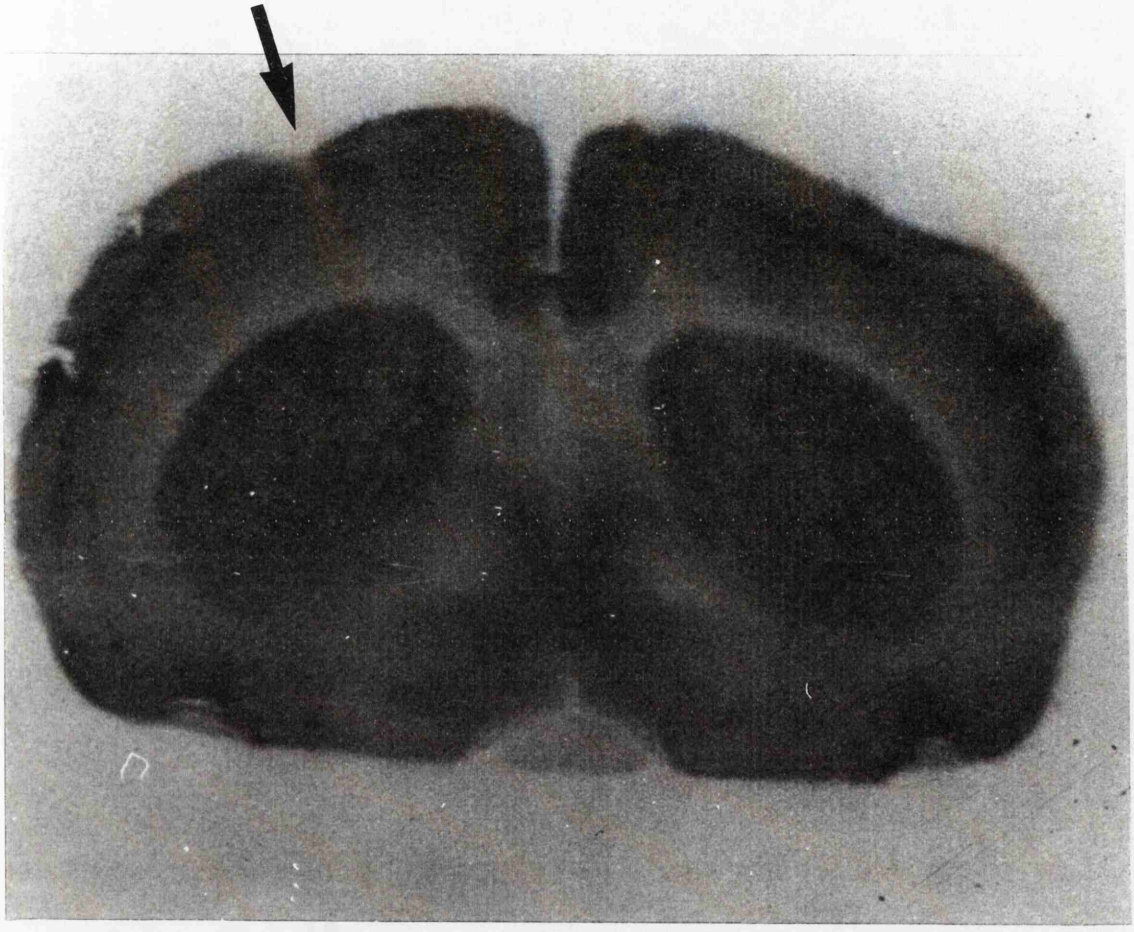

PLATE 9:- [^{14}C]-2-deoxyglucose autoradiographical image of a coronal rat brain section containing a laser lesion in the somatosensory cortex. This image was obtained from animal # 1 at nine months post-operative. The lesion tract is indicated by the arrow.

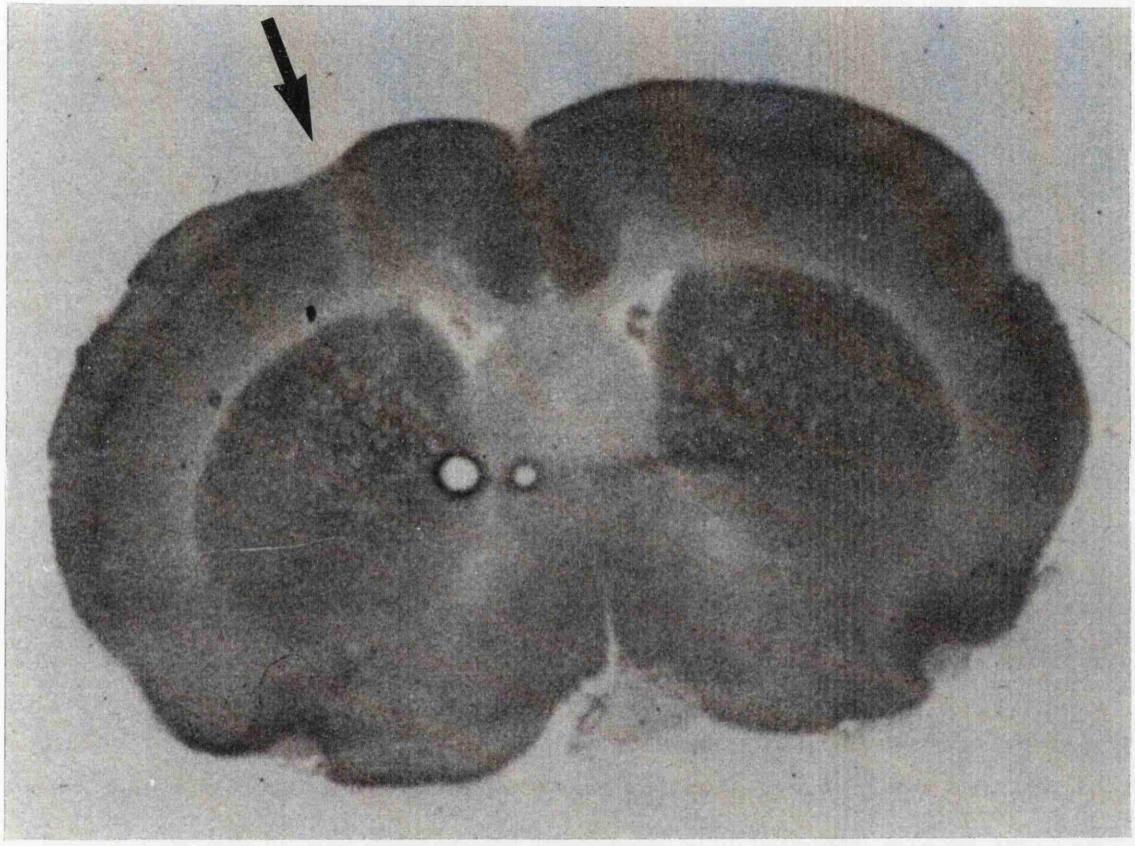

PLATE 10:- [^{14}C]-2-deoxyglucose autoradiographical image of a coronal rat brain section containing a laser lesion in the somatosensory cortex. This image was obtained from animal # 2 at nine months post-operative. The lesion tract is indicated by the arrow.

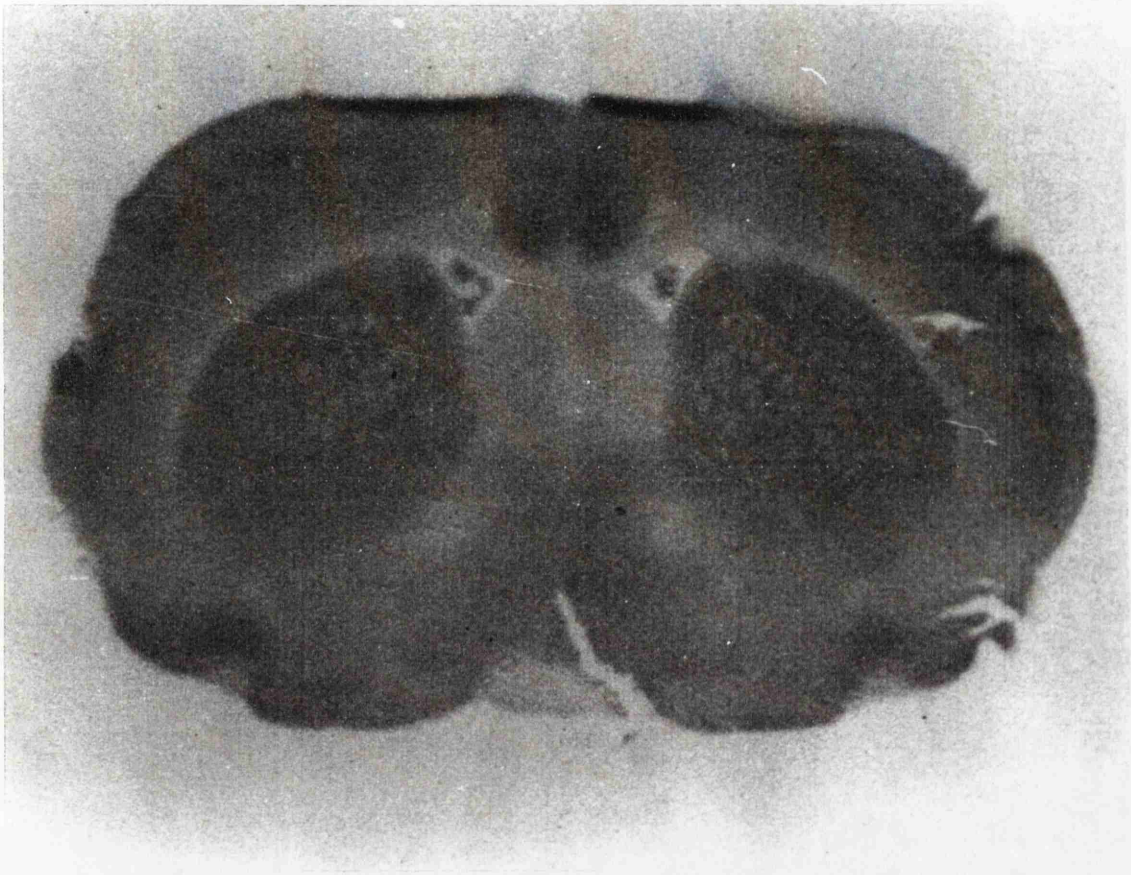

PLATE 11:- [^{14}C]-2-deoxyglucose autoradiographical image of a coronal rat brain section anatomically matched to those in plates 9 and 10. This image was obtained from the sham-operated rat (animal # 3) at nine months post-operative.

8.4.4 Determination of the extent of brain damage precipitated by the laser lesioning procedure

PROTOCOL:- At nine months post-operative 3 rats, 2 laser-treated (animals 1 and 2) and 1 sham-operated (animal 3), underwent assessment of brain damage indicated by peripheral-type BZD receptor binding. This was performed by the *in vitro* [^{3}H]-PK-11195 autoradiographical method described in section 2.17.

RESULTS:- Peripheral-type BZD receptor binding was employed as a marker for brain damage precipitated by the lesioning procedure. Extensive [^{3}H]-PK-11195 binding was evident in the region of the lesion in both laser-treated animals (animal 1, plate 12; animal 2, plate 13) and was absent in the sham-operated animal (animal 3, plate 14). There was no observable increase in [^{3}H]-PK-11195 binding in any other brain region. Analysis of the area of increased ligand binding in successive coronal sections allowed calculation of the lesion volume by plotting binding area against distance (animal 1, figure 71; animal 2, figure 72). The lesion volumes were calculated at 0.295 mm^3 and 0.254 mm^3 for animals 1 and 2 respectively.

8.5 DISCUSSION

The aims of these studies were to explore the method of producing laser lesions in the somatosensory cortex of the rat and to determine the pathological reproducibility and seizure susceptibility of this procedure. Preliminary evaluations of the local biochemical changes arising as a result of the procedure were also conducted.

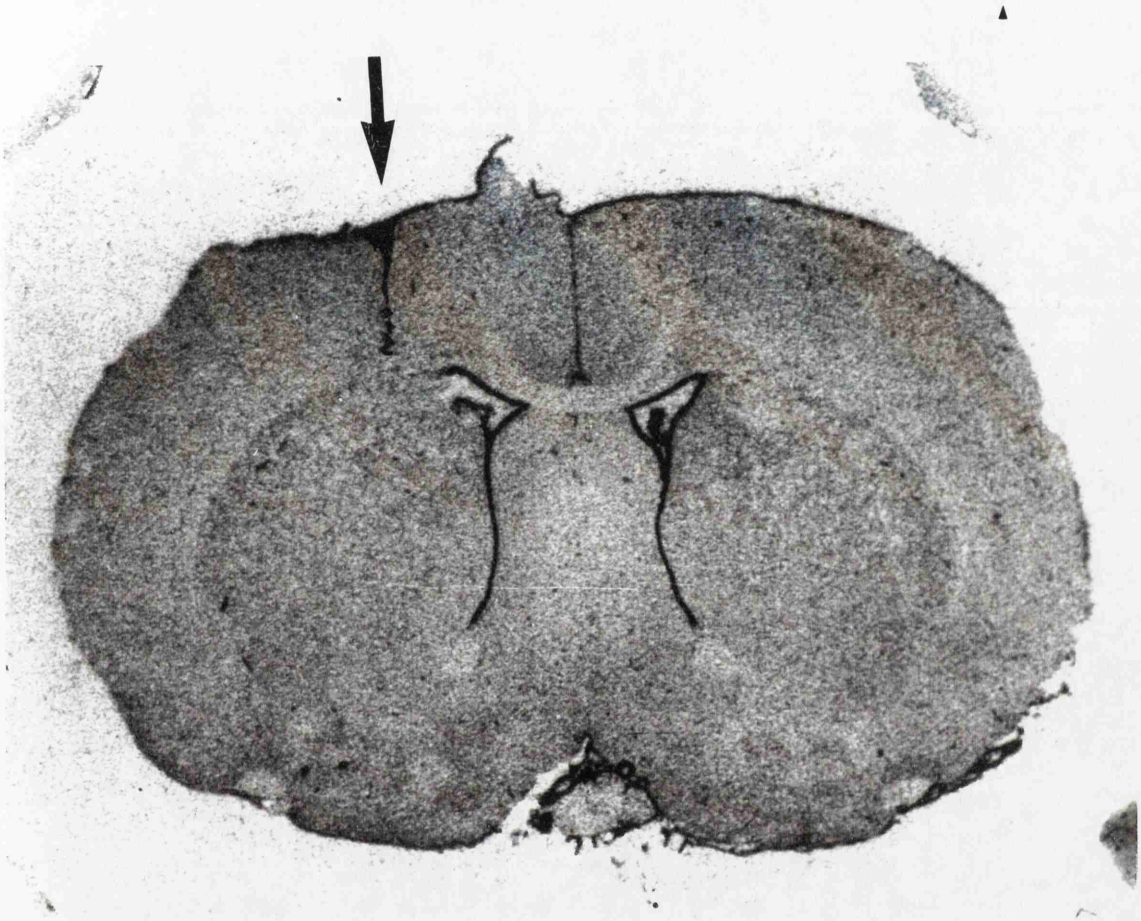

PLATE 12:- [^{3}H]-PK-11195 autoradiographical image of a coronal rat brain section containing a laser lesion in the somatosensory cortex. This image was obtained from animal # 1 at nine months post-operative. The darkened area of increased ligand binding corresponding to the lesion tract is indicated by the arrow.

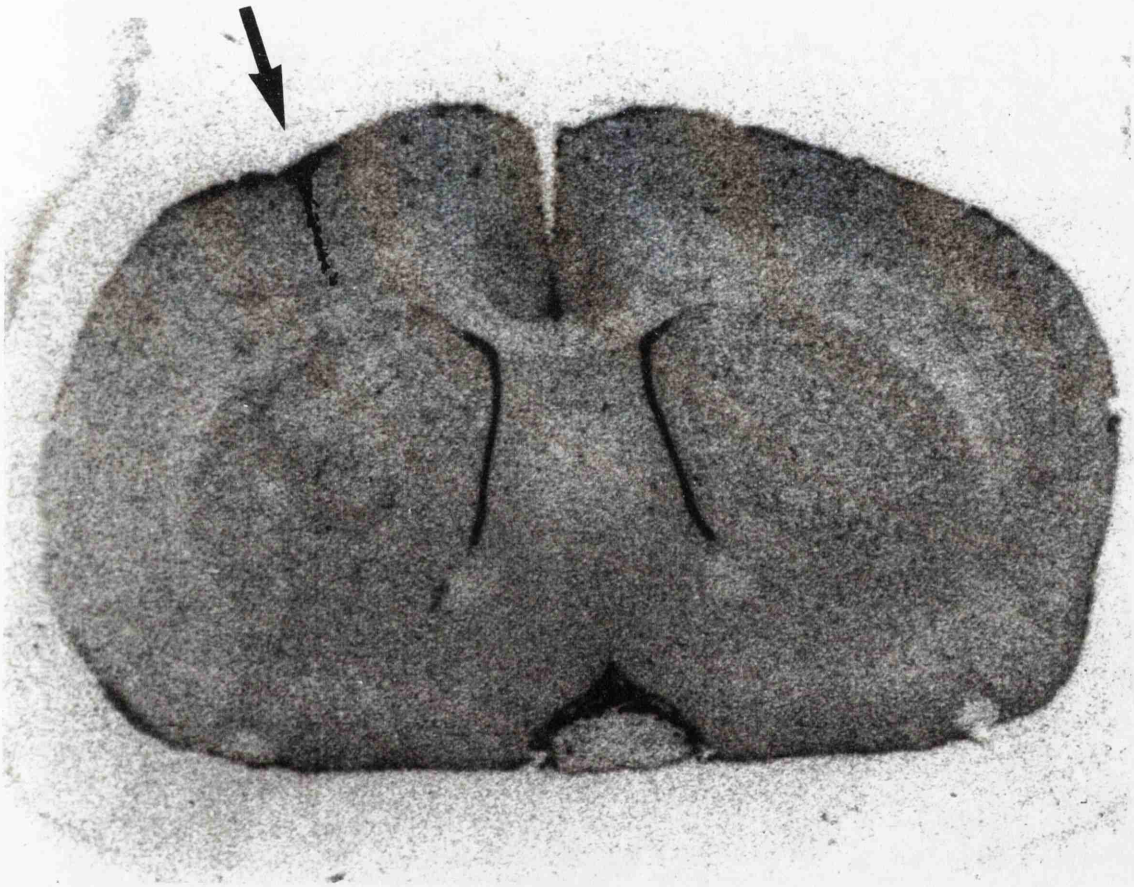

PLATE 13:- [^{3}H]-PK-11195 autoradiographical image of a coronal rat brain section containing a laser lesion in the somatosensory cortex. This image was obtained from animal # 2 at nine months post-operative. The darkened area of increased ligand binding corresponding to the lesion tract is indicated by the arrow.

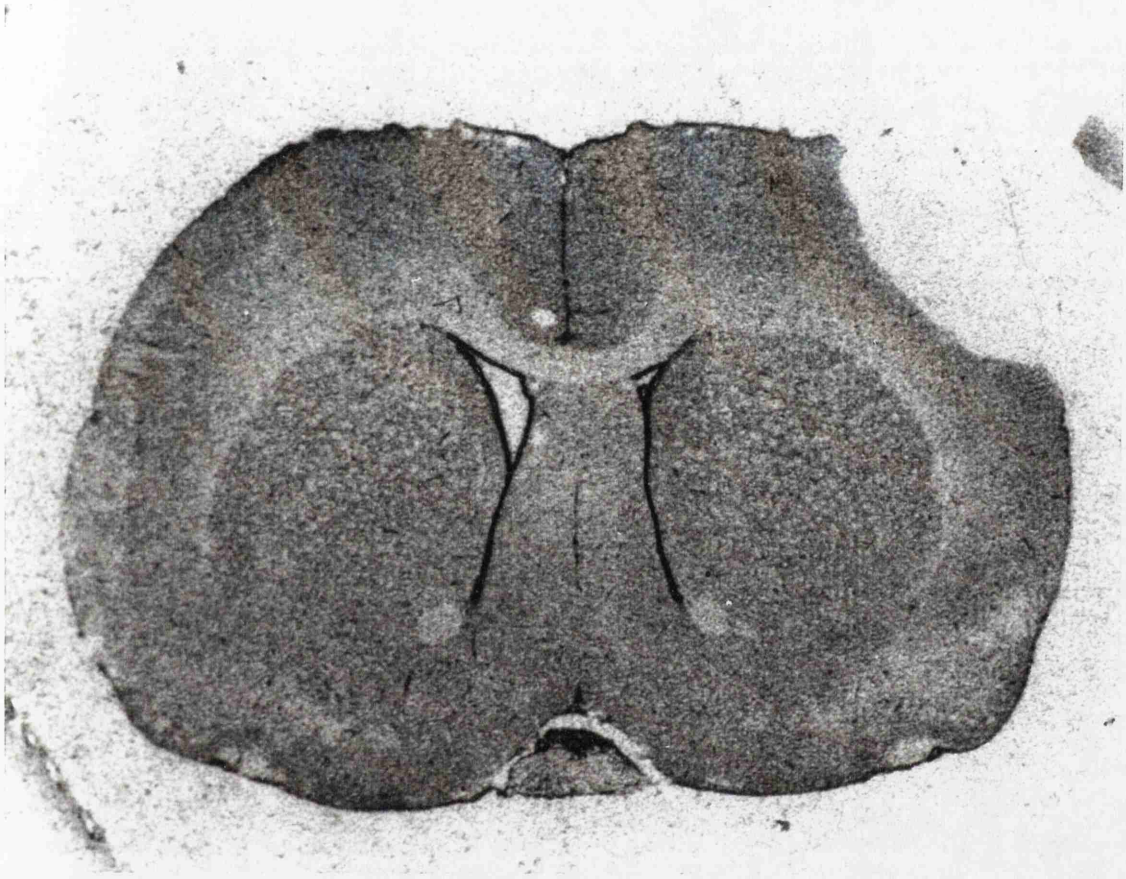

PLATE 14:- [^{3}H]-PK-11195 autoradiographical image of a coronal rat brain section anatomically matched to those in plates 12 and 13. This image was obtained from the sham-operated rat (animal # 3) at nine months post-operative.

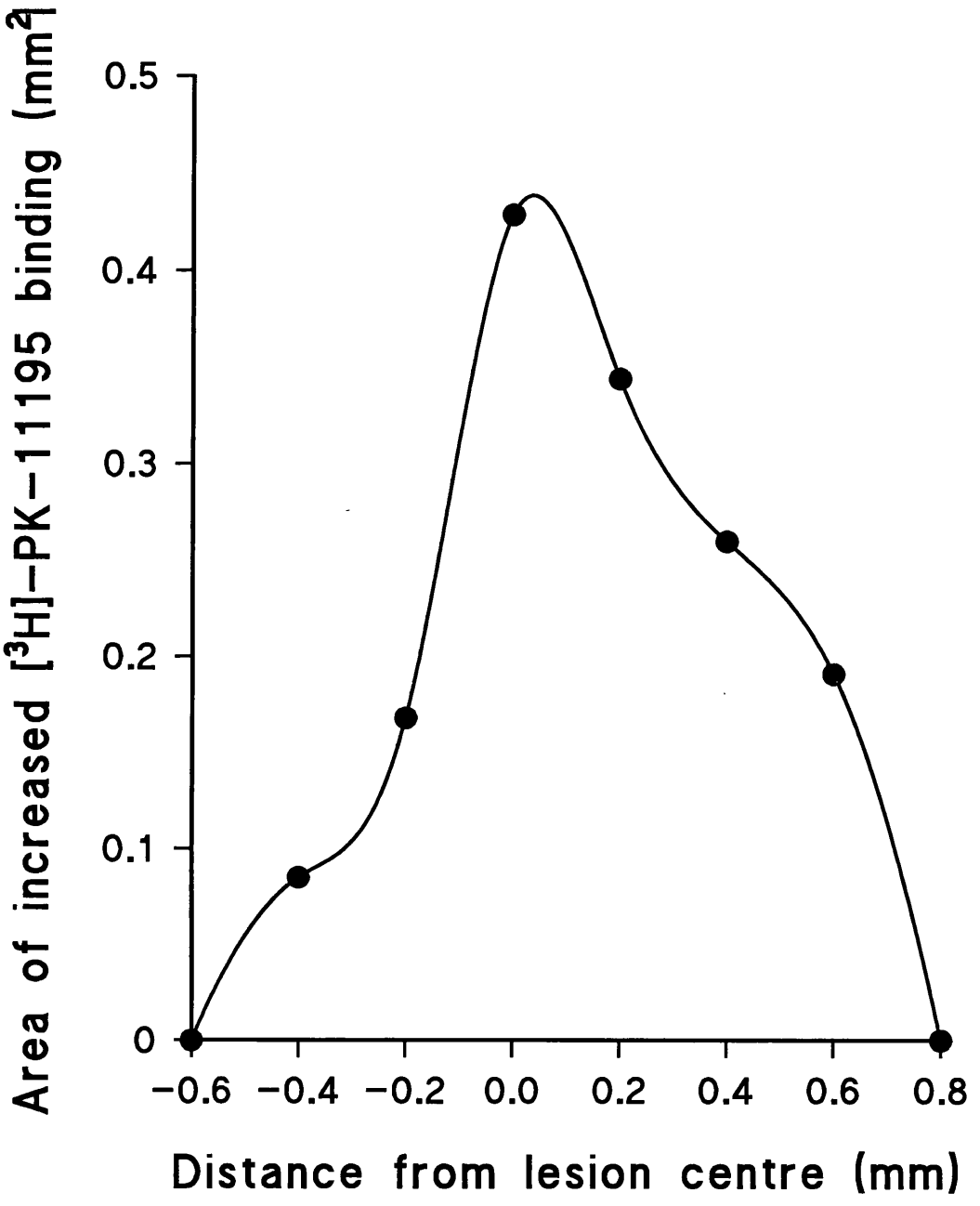

FIGURE 71:- Area of increased [^{3}H]-PK-11195 binding as a function of the distance (mm) from the observed centre of the laser lesion in animal # 1 at nine months post-operative. Results are expressed as mean area (mm^2) calculated from 3 regions of increased binding in each brain section.

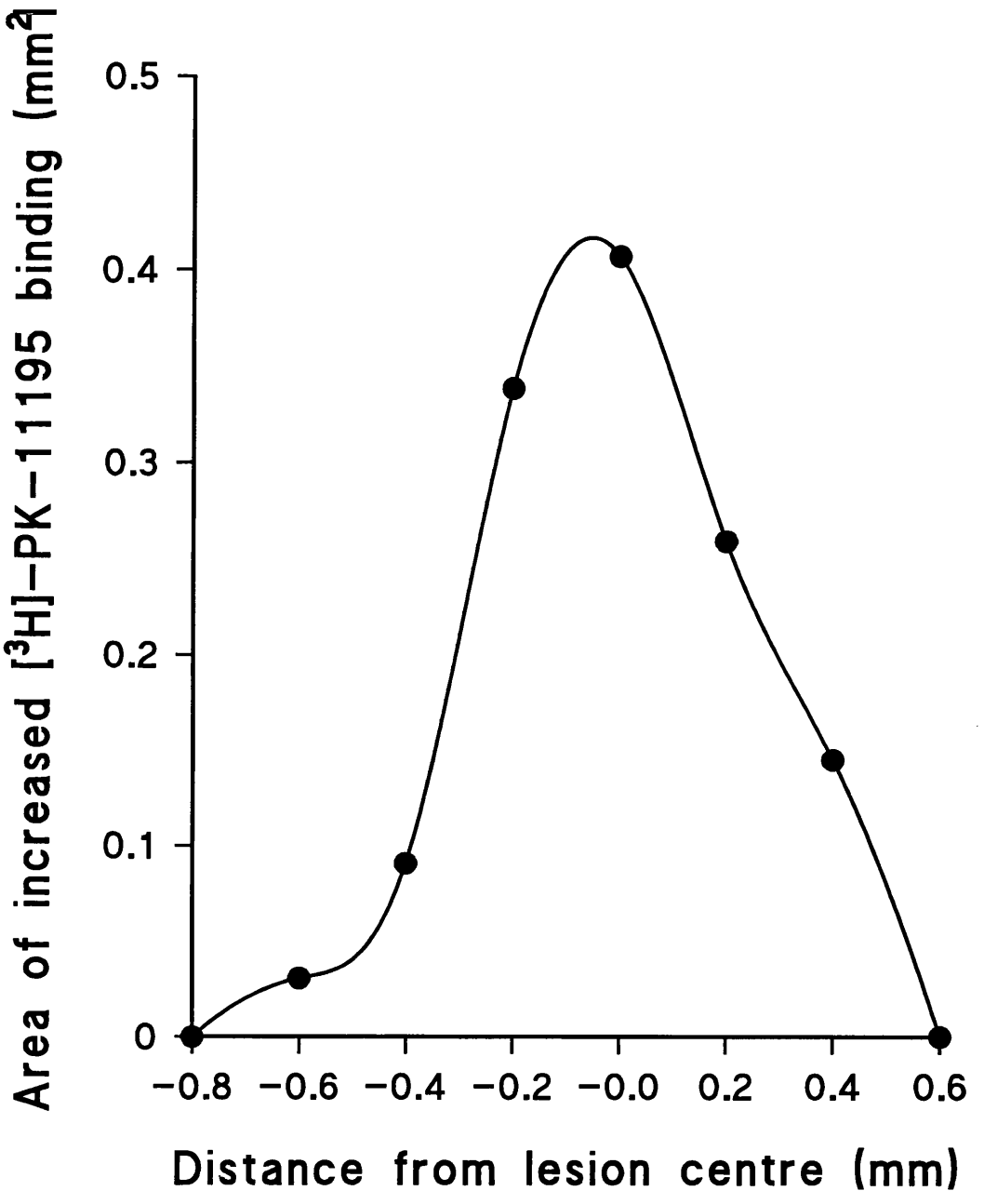

FIGURE 72:- Area of increased [^{3}H]-PK-11195 binding as a function of the distance (mm) from the observed centre of the laser lesion in animal # 2 at nine months post-operative. Results are expressed as mean area (mm^2) calculated from 3 regions of increased binding in each brain section.

Characterisation of the laser lesioning procedure

Exploration of the method of producing laser lesions, with a view to its further development in epilepsy research, revealed it to be a feasible procedure with a straight-forward methodology and high likelihood of success. The total procedure time was short enough to suggest that sufficient numbers of animals could be treated to allow quantifiable investigations in the future. The low incidences of unexpected mortality and adverse events resulting from the surgical procedures were encouraging. The post-operative survival period was sufficiently long to allow future investigations of the development and progression of pathological and local biochemical changes arising as a result of the lesioning procedure. Perhaps the most important observation was the lack of overt epileptic activity associated with the procedure. Problems associated with the technique included movement of the head during procedure and the precision of site-directed lesions. These could be addressed by incorporation of stereotaxic apparatus into the existing experimental set-up.

Pathological evaluation of the laser lesion

Histological investigations revealed both the nature and extent of the lesion to be reproducible. Inter-animal pathology was consistent and suggested that the lesion was tapered in shape (plate 5) and traversed all six layers of the cortex without damaging the underlying white matter. The lesion was deemed to be "clean", without infiltration of chronic inflammatory cells, and this suggested that secondary damage, arising from local inflammation, might not play a part in long-term development. The observation of selective neuronal loss (plate 6), together with prominent gliosis and fibrosis was consistent with that of an experimental epileptic lesion (Westrum et al, 1964), and also

proposed this model to have utility, not only for epilepsy, but for other fields of neurobiology. Future histological studies are planned and include an investigation of the pathological progression of the lesion from the time of initial insult.

Seizure susceptibility of the laser lesion

The maximal (CD_{97}) PTZ test was employed to evaluate the seizure susceptibility of a laser lesion in the somatosensory cortex. There was no significant alteration in the latency to the onset of generalised seizures induced by PTZ in the laser-treated animals when compared to sham-operated controls (figure 70). In hindsight, a more satisfactory conclusion may have been reached by the inclusion of a group of age and weight matched naive animals. Further investigation of the seizure susceptibility of this lesion with the supramaximal MES test, and particularly with the threshold PTZ and MES tests, described in sections 3.1.1 and 3.1.2 respectively, may yield more promising results.

Effects of the laser lesion on cerebral glucose metabolism

Preliminary investigations of the local biochemical changes precipitated by the lesioning procedure was conducted on two fronts. Examination of the LCGU of the lesioned area, and of major projection regions within the brain, suggested that the effects of the lesioning procedure were localised to the somatosensory cortex (tables 2 - 4). The cortical lesion tract exhibited a low LCGU when compared to both contralateral (intra-animal) and sham-operated (inter-animal) control values. This observation may be consistent with the selective loss of neuronal cells, reported histologically, within the lesion tract. Similarly, LCGU was depressed, compared to controls, in the immediate vicinity of the lesion. This area of reduced metabolism, which corresponded with apparently healthy tissue under

histological examination (plates 5 and 6), appeared to extend outwards from the lesion for approximately 0.5 mm in all directions. A suitable explanation for this additional phenomenon was not forthcoming. As with the histological investigations, studies of the progression and development of the lesion, with reference to local and distal biochemical alterations, are planned.

Evaluation of the brain damage associated with the laser lesion

Finally, the nature and extent of brain damage elicited by the lesioning procedure were examined using the well established technique of Benavides and colleagues (1983). Ligand binding to the peripheral-type BZD receptor is limited in normal brain tissue, but is greatly increased in damaged tissue and also in areas with a less potent blood-brain barrier (Benavides et al, 1983). Radiolabelled PK-11195 binding data suggested that the brain damage imparted by the lesioning procedure was confined to the somatosensory cortex itself. This finding was consistent with the studies of LCGU described above. However, the area of increased ligand binding was confined entirely to the lesion tract (plates 12 and 13), and, as such, correlated with the previous histological investigations. These observations allowed determination of the lesion volume by calculation of the area of increased ligand binding in successive coronal sections (figures 71 and 72). The lesion volumes were seen to differ only slightly between animals 1 and 2 and this supported the proposed pathological reproducibility of the lesion. Again, studies of the progression and development of the lesion, with reference to the nature and extent of tissue damage, are required to characterise this focal brain insult further.

8.6 CONCLUSIONS

In conclusion, production of cortical laser lesions in the rat is a feasible procedure which holds promise for laboratory investigations of disruptive brain insults. The potential of this model in epilepsy research remains to be evaluated and initial studies have suggested that it may have a wider applicability. The precise nature of the lesion, its apparent reproducibility, and the ability to quantifiably vary its extent are all features which would give it potential superiority over existing models of chronic epilepsy.

Future experimental directions with regard to this procedure include evaluation of the site-dependency of the lesion, investigation of the effects of animal age at the time of initial insult, and characterisation of the lesion in terms of *in vitro* and *in vivo* electrophysiology. The use of genetically epilepsy-prone animals is also envisioned in an attempt to explore the intrinsic epileptogenicity of the lesioning procedure.

CHAPTER NINE

GENERAL DISCUSSION AND CONCLUSIONS

9.1 GENERAL DISCUSSION

The objective of this programme of work was to study experimental seizure models and new antiepileptic drugs. The individual projects which comprise this thesis are to a certain extent diverse, although not unrelated. While the general introduction endeavours to summarise present understanding of epilepsy, its clinical treatment, and current laboratory practices employed in its investigation, the individual results chapters developed particular aspects of epilepsy research in more detail.

Initial investigations (chapter 3) addressed the contribution of basic animal models of epilepsy to its experimental study. Next, an attempt was made to emphasise the crucial role of neuronal inhibition and excitation in epileptogenesis and to relate these phenomena to the study of novel antiepileptic agents (chapters 4 - 6). Finally, the future of epilepsy research, in terms of appropriate strategies for AED development (chapter 7) and innovative experimental paradigms (chapter 8) was examined.

Individual results are discussed in detail in the relevant sections, both in relation to their particular drawbacks and in their consistency with previously published work. This general discussion attempts to summarise these individual aspects and to highlight those of particular interest.

Investigation of basic animal seizure models

Of all the experimental seizure models in current laboratory employment, the PTZ test and the maximal and minimal electroshock tests are among the most popular by virtue of their simplicity and economy. The primary aim of these studies was to afford a familiarity with

these three basic animal seizure models and to validate them as techniques for subsequent use. This objective was satisfactorily accomplished and the results obtained correlated closely with those reported in the literature. These studies also incorporated an investigation of concentration-effect relationships with PTZ. Although it would not be absurd to question the relevance of performing such a study with a purely experimental convulsant compound, the fact that it fails to induce convulsions in around 3 - 5 % of laboratory animals has precipitated in-house disputes over its integrity and reproducibility as an experimental indicator. Unfortunately, the resultant investigations, while providing a degree of insight into the pharmacokinetics of PTZ in the mouse, yielded little in the way of quantitative conclusions. In the absence of higher resolution analysis, an explanation for the lack of effect of PTZ in some animals was not forthcoming.

Antiepileptic drug enhancement of neuronal inhibition

Impairment of GABA-mediated neuronal inhibition is believed to be one of the fundamental aetiological mechanisms of epileptogenesis (Meldrum, 1989). Likewise, pharmacological enhancement of the inhibitory actions of GABA has been proposed as one of the primary mechanisms of action of several chemically unrelated AEDs (Macdonald and Barker, 1979). These investigations compared and contrasted the experimental anticonvulsant profiles and mechanisms of action of VGB and TGB, two recently developed AEDs which have been proposed to augment GABA-mediated inhibition.

VGB raised the threshold for induction of tonic seizures, determined by the Min-ES test, but was without effect in the PTZ and MES tests. TGB, in contrast, exhibited

anticonvulsant effects against both PTZ- and MES-induced seizures. Thus, VGB and TGB afforded differing degrees of protection against experimentally induced seizures. This would suggest that augmentation of GABA in the brain does not produce a universal pattern of seizure protection, but that anticonvulsant efficacy in individual seizure models may be related to particular drug mechanisms rather than physiological consequences. These results also appear to suggest that VGB is a relatively poor anticonvulsant in the models selected for evaluation. However, its efficacy against Min-ES seizures might propose its inactivity in the MES test to be a false negative observation. Such a proposition would support the reservations expressed by Löscher and Schmidt (1988) in employing only the PTZ and MES tests for identification of potential AEDs.

Mechanisms of action of VGB and TGB were investigated in isolated brain tissue and in primary cultures of cerebral cortical astrocytes and neurones. Previously reported mechanisms of action of the two drugs were confirmed, with VGB inhibiting GABA metabolism by an action on GABA-T, and TGB blocking GABA uptake in a non-cell-specific manner. An inhibitory effect of VGB on GAD was also verified and an additional, previously unreported action of the drug on GABA uptake was proposed. Surprisingly, and in contrast to previous reports (Schechter et al, 1977), VGB was without effect on whole brain GABA concentration. This apparent inconsistency was explained by confirmation of the drug's ability to reduce GABA synthesis in addition to its metabolism. The effect of VGB on GABA uptake was also found to contrast that reported by other investigators (Schousboe et al, 1986). Although no direct explanation for this apparent discrepancy could be offered, such an effect of VGB was proposed to correlate with some of its unexplained experimental and clinical actions. These observations have often led to

the suggestion that the pharmacological effects of VGB are not solely related to its inhibition of GABA-T.

Thus, VGB and TGB exert their anticonvulsant effects by distinct mechanisms although a degree of ubiquity may exist. The fact that these drugs possess entirely different experimental but similar clinical profiles of activity not only questions the exact relevance of any common mechanism to their anticonvulsant or antiepileptic effects, but might also challenge the predictive capacity of the standard animal seizure models employed.

Antiepileptic drug attenuation of neuronal excitation

There is substantial evidence to suggest that glutamate-induced neuronal excitability and voltage-sensitive calcium influx are inexorably entwined at all stages of epileptogenesis. Several existing AEDs have been proposed to exert their effects by reducing neuronal excitation (Rogawski and Porter, 1990) and calcium channel blockade has frequently been reported as an important secondary mechanism of AED action (Crowder and Bradford, 1987). These studies compared and contrasted the experimental anticonvulsant profiles and mechanisms of action of NMD and AML, members of the DHP class of calcium channel blockers which have been proposed as putative AEDs.

In single dose, NMD was effective against MES-induced seizures and also raised the tonic seizure threshold, determined by the Min-ES test. Although the activity of NMD in the MES test contrasted several previous reports (Hoffmeister et al, 1982; Wong and Rahwan, 1989), this disparity was satisfactorily delineated in terms of an electrical stimulus site dependency, known to influence the outcome of electroshock studies (Löscher et al,

1991a). Single dose AML protected against PTZ-induced seizures, but was essentially without effect in the MES test. The anticonvulsant effects of AML were, however, often observed at doses close to those producing notable animal fatalities. It was suggested that this lethality, associated with higher AML doses and presumably related to the potent cardiovascular actions of the drug, might preclude its further evaluation as a potential antiepileptic agent. The difference in anticonvulsant profiles of the two drugs was proposed to reflect possible differences in the precise molecular mechanisms of individual DHP compounds. Differing biophysical properties, such as lipid solubility and protein binding, might also have explained this discrepancy, although such unfounded suggestions would have been unlikely to concur with the similar activity of both drugs in the PTZ test. In the absence of experimental pharmacokinetic analysis of AML, to determine the extent of its penetration into the brain, a definitive judgement in this respect could not be discerned.

With drug concentration data mirroring previously published work (Larkin et al, 1992b) and pharmacodynamic effects which confirmed reports of prolonged seizure protection (Thomas, 1990), it was proposed that the anticonvulsant effects of NMD in the MES test might extend beyond the time suggested by its pharmacokinetic profile. Nevertheless, it was ultimately concluded that this hypothesis was derived from an inaccurate experimental pharmacokinetic profile rather than some unexplained pharmacodynamic effect. The fact that NMD is without proven antiepileptic action in man (Larkin et al, 1991), even in the presence of detectable drug levels, would support such a conclusion.

NMD exhibited a substantial loss of anticonvulsant efficacy following repeated administration, confirming previous reports in this respect (Jagiello-Wótowicz et al, 1991). This apparent tolerance was suggested to be mediated by a biochemical alteration at the DHP recognition site following prolonged exposure to the drug. Although unsubstantiated, this proposal might explain the lack of efficacy of the DHPs in the clinical treatment of epilepsy. Whether drugs which are believed to be antagonists, or in this case blockers, could precipitate such desensitisation is, however, unclear. Additionally, one would imagine that if this explanatory phenomenon were accurate, it would be subject to a degree of dose-dependency, a disposition not evident in these studies. Concomitant analysis of plasma and brain drug levels suggested a prevalence of NMD with repeated treatment, although, as discussed, this prolonged drug presence in the brain was not associated with extended seizure protection, but rather the opposite. Thus, with an anticonvulsant action of single dose NMD in the absence of detectable drug levels and a lack of effect of repeated NMD treatment in the presence of detectable drug levels, the relationship between concentration and anticonvulsant activity of NMD was difficult to interpret, if indeed one exists at all.

Studies of drug mechanisms employed primary cultures of cerebellar granule cell neurones and cerebral cortical astrocytes. NMD reduced depolarisation-induced calcium influx in a non-cell-specific manner, but was without effect on neuronal glutamate release. Although molecular discrimination was not anticipated, these observations suggested a preferential action of NMD on non-N-type calcium channels, assuming that the N-type channel alone is responsible for the pre-synaptic control of neurotransmitter release (Meyer, 1989). AML limited neuronal calcium entry induced by depolarising

concentrations of potassium but not veratridine. Such an effect is opposite to that observed with LTG, a recently developed AED, which reduces the calcium influx associated with veratridine, but not potassium, challenge via an action on neuronal sodium channels (Leach et al, 1986). Employing this inverse analogy, it could be proposed that an additional action on neuronal potassium channels might indirectly contribute to the calcium antagonist properties of AML.

Thus, NMD and AML appear to have subtle differences in their molecular mechanisms which might correlate with their differing spectra of activity in animal seizure models. Although the DHPs remain attractive experimental stage drugs, their full potential as antiepileptic agents may not be realised until the advent of more centrally selective compounds.

Combinations of novel antiepileptic drugs

Despite recent additions to the clinician's armamentarium, up to 30% of the epileptic population remain refractory to antiepileptic monotherapy. Extensive polypharmacy with established AEDs, both in combination with each other and with more recently developed compounds, has failed to address this significant clinical problem. Previously unexplored combinations of novel antiepileptic agents are now under laboratory investigation in an attempt to establish a more rational basis for polypharmacy in drug-resistant epilepsy. This study explored the nature and extent of any experimental interaction between repeatedly dosed VGB and LTG, both recently licenced for the treatment of refractory seizures.

LTG reduced the incidence of MES-induced seizures, but was without effect on any of the selected neurochemical parameters. These results reflected previous reports of the anticonvulsant efficacy of the drug (Lamb et al, 1985). They also suggested that LTG did not directly influence the GABAergic system, an observation consistent with its proposed cellular mechanism. In contrast, VGB was devoid of anticonvulsant activity, and exhibited effects on the GABAergic system in keeping with its putative mechanism of action. Several inconsistencies in the results obtained with VGB were, however, described. The lack of effect of VGB in the MES test, which contrasted the efficacy of the same drug dose in a previous study, was explained in terms of the drug treatment period. Similarly, its inability to influence brain GAD activity, which was again inconsistent with previous results, and those reported in the literature, was delineated in terms of drug doses and the treatment period employed. Finally, the lack of effect of medium dose VGB on whole brain GABA concentration, although consistent with a previous study, did not appear to concur with the observed inhibitory action on GABA metabolism at this dose level. Whereas previous results in this respect were explained by an additional inhibition of GABA synthesis, such a disposition was not evident in this study.

The effects of experimental combination therapy did not differ from those observed with the respective monotherapies. Combining VGB and LTG afforded a seizure protection similar to that observed with LTG alone and had neurochemical implications which correlated with the effects of VGB monotherapy. It was concluded that there was no experimental interaction between LTG and VGB on the parameters selected for evaluation. Thus, the basis of the clinically reported interaction between these two compounds could not be discerned from this study. It was likely that the limited number of

experimental parameters employed, selected on the grounds of availability rather than being representative of all potential sites of interaction, and their inherent lack of molecular resolution restricted more definitive investigation in this respect. Similarly, it was appreciated that this study, which employed a single seizure model and limited neurochemical parameters in a genetically homogeneous population of animals, did not reflect the diversity of clinical epilepsy in terms of seizure types and epileptogenic mechanisms.

Novel strategies for antiepileptic drug development

It has been proposed that to satisfactorily address the problem of refractory epilepsy, the development of novel antiepileptic agents with similarly novel mechanisms of action is required. NA is a newly-synthesised, neuroactive compound which is believed to exert its effects by selective enzyme inhibition of the kynurenine pathway of tryptophan metabolism. As such, it has been shown to increase brain concentrations of kynurenic acid, an endogenous antagonist at the glycine recognition site on the NMDA subtype of glutamate receptor (Connick et al, 1992). This study explored the anticonvulsant profile of NA in three standard animal models of seizure.

At one hour post-administration NA protected against PTZ-induced seizures in mice, and was also active in the maximal and minimal electroshock tests. It was without effect in the PTZ test at four hours post-dosing. These preliminary results would suggest that NA has a wide spectrum of experimental anticonvulsant activity and has the potential to reduce seizure spread, as determined by the electroshock tests, and also to limit seizure initiation, as determined by the PTZ test (Löscher et al, 1991a).

NA appeared to possess an unusual pharmacodynamic profile for a putative enzyme inhibitor, exhibiting anticonvulsant activity within one hour of administration and a subsequent loss of efficacy within four hours. However, it could be proposed that VGB is similar in this respect, with experimental anticonvulsant effects which appear unrelated to the time of maximal enzyme inhibition afforded by the drug (Bernasconi et al, 1988). Selective enzyme inhibition of the kynurenine pathway is a novel mechanism for anticonvulsant drug action and these results would suggest that it merits further investigation.

Development of a novel animal model of epilepsy

It is now recognised that few of the existing "animal models of the epilepsies" help to address the significant clinical problem of refractory partial seizures and fewer still mirror the condition of chronically recurrent spontaneous seizures which is characteristic of human epilepsy. This study followed the preliminary development of an innovative model of partial epilepsy, proposed to more closely mimic the human condition. This model was characterised by a laser-induced lesion in the rat somatosensory cortex.

Histological investigation proposed the lesion to be highly reproducible and to possess cellular characteristics similar to those of disruptive brain insults in man. The lesion did not appear to be intrinsically epileptogenic, nor did the procedure influence the latency to generalised PTZ-induced seizures. Preliminary autoradiographical studies suggested that the tissue damage associated with the procedure was confined to the lesion tract itself, but that cerebral glucose metabolism was additionally altered in an adjacent, otherwise healthy, region. It was concluded that the production of cortical laser lesions in the rat was

a feasible procedure which holds promise for laboratory investigations of disruptive brain insults. Although its full potential in epilepsy research remains to be evaluated, the explicit nature of the lesion, its apparent reproducibility, and the ability to quantifiably vary its extent were all features which suggested this model to have superiority over existing models of chronic epilepsy.

One of the initial concerns with this innovative experimental model was the lack of overt seizure activity, or even increased seizure susceptibility, associated with the lesioning procedure. It was, however, appreciated that the most useful animal models of epilepsy are those in which the investigator can precipitate a seizure at will, without having to rely purely on spontaneity (Löscher and Schmidt, 1988). Additional uncertainties over the precise anatomical location of the lesion, and its reproducibility in this respect, was satisfactorily addressed by the proposed incorporation of a stereotaxic frame into the existing experimental apparatus. Ultimately it was recognised that this putative model, although proposed to afford a more clinically relevant representation of refractory partial epilepsy than its predecessors, remains at an embryonic stage in its development and has the added disadvantage of being one which requires the use of sophisticated equipment of considerable expense.

9.2 FINAL CONCLUSIONS

In conclusion, the work contained within this thesis afforded investigation of several distinct fields of epilepsy research. The advantages and disadvantages of the traditional animal models of epilepsy were viewed alongside contemporary approaches in this respect. Recently developed antiepileptic agents and experimental stage compounds were

evaluated in the laboratory environment, with emphasis placed on the potential of enhancing inhibition and/or reducing excitation as basic mechanisms of AED action. This thesis attempted to explore small, selected areas of what is a burgeoning field of epilepsy research. It is hoped that at least some of this work will contribute to the increased understanding of the epilepsies, their experimental models, and their pharmacological amelioration.

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