EFFECTS OF ANTIEPILEPTIC DRUGS IN RODENT AND HUMAN ASTROCYTE CULTURES, RODENT BRAIN AND PENTYLENETETRAZOL-INDUCED SEIZURES IN MICE

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ABSTRACT

There is increasing evidence to suggest that most antiepileptic drugs (AEDs) have multiple mechanisms of action which may contribute to their efficacy and/or toxicity. Of particular current interest is the effects of AEDs on the excitatory and inhibitory amino acid systems regulated by glutamate and γ-aminobutyric acid (GABA), respectively.

Primary cultures of rat cortical astrocytes were employed to examine the effects of a range of AEDs on the uptake of GABA and glutamate and on the activity of the GABA-metabolizing enzyme GABA-transaminase (GABA-T). One hour exposures to sodium valproate (SVP), vigabatrin (VGB), felbamate (FBM) and tiagabine (TGB) significantly reduced GABA uptake into primary cultures of rat cortical astrocytes over a broad range of concentrations examined. Phenobarbitone (PB), phenytoin (PHT), carbamazepine (CBZ), lamotrigine (LTG), gabapentin (GBP), topiramate (TPM), levetiracetam (LEV) and desglycinyl-remacemide (DGR) were without effect.

A one hour exposure to PB resulted in a reduction in glutamate uptake into primary cultures of rat cortical astrocytes with the lowest three concentrations examined, with no effect on glutamate transport at the highest concentrations. TGB and DGR were also found to reduce glutamate uptake, however, the effects appeared to concentration-independent and not evident over the dose range. PHT, CBZ, SVP, VGB, LTG, FBM, GBP, TPM and LEV were without effect.

One hour exposures to VGB and DGR consistently reduced the activity of GABA-T in primary cultures of rat cortical astrocytes. SVP and TGB were also found to decrease

GABA-T activity, however, the effect was only evident at the highest concentration examined for each of the drugs. PB, PHT, CBZ, LTG, FBM, GBP, TPM and LEV were without effect.

Glutamine synthetase (GS) is a key enzyme in the regulation of glutamate neurotransmission in the central nervous system (CNS). The effects of single and repeated intraperitoneal (i.p.) administration of a range of AEDs on GS activity were investigated in mouse brain. Four hours after the final dose the animals were sacrificed and their brains removed for analysis of GS activity. Single doses of PHT and CBZ were found to dose-dependently reduce enzyme activity. Repeated doses of PB, PHT, CBZ, FBM and TPM dose-dependently reduced the activity of GS. SVP, VGB, LTG, GBP, TGB, LEV and DGR were without effect on enzyme activity.

In addition, due to the glial location of the enzyme, the effects of a range of AEDs on GS activity were examined in primary cultures of rat cortical astrocytes. A one hour exposure to PB reduced GS activity in primary cultures of rat cortical astrocytes. PHT, CBZ, SVP, VGB, LTG, FBM, GBP, TPM, TGB, LEV and DGR were without effect following a one hour exposure.

These results suggest that effects of these AEDs on the transport and metabolism of GABA and glutamate may contribute to the clinical effects of the drugs, and appear to support the observation that many AEDs have multiple mechanisms of action.

FBM, TPM and LEV are three new AEDs whose precise mechanism of action remains to be fully elucidated. Each has reported effects on amino acid neurotransmitter

systems. In light of this, the effects of single and repeated administration of FBM, TPM and LEV were investigated on GABA- and glutamate-related neurochemistry in mouse brain. Both single and repeated treatments with FBM, TPM and LEV were without significant effect on the concentrations of GABA, glutamate and glutamine. Similarly, single and repeated treatments with all three drugs were without significant effect on the activities of GABA-T or glutamic acid decarboxylase (GAD). These results suggest that effects on GABAergic and glutamatergic systems are not involved in the mechanisms of action of these drugs.

Approximately 30% of patients with epilepsy do not respond to monotherapy and require treatment with two or more AEDs. However, there is very little scientific or clinical evidence highlighting the efficacy of particular combinations. Three of the new AEDs, LTG, GBP and TPM are commonly used as add-on therapy for refractory partial seizures. The effects of these drugs in single dose and in combination were examined on experimental seizures induced by the chemoconvulsant pentylenetetrazol (PTZ) in mice, in an attempt to identify efficacious combinations of new AEDs.

When administered alone, only the highest dose of LTG examined significantly increased the latency to the first PTZ-induced generalised seizure. Both GBP and TPM alone were without effect. Five of the nine combinations of LTG with GBP significantly increased the seizure latency in mice. In addition, a combination of GBP with TPM was found to have a significant anticonvulsant effect when compared to control. All other combinations of AEDs examined were without effect on PTZ-induced generalised seizures at one hour post-administration. This preliminary study woud suggest that the combination of LTG with GBP, and possibly TPM with GBP,

may be an effective polytherapy regimen and require further detailed experimental and clinical investigation.

SVP, VGB and TGB were all found to exert effects on the GABAergic system when investigated in primary cultures of rat cortical astrocytes. It was of interest to examine the effects of these drugs on the uptake of GABA into primary cultures of human adult and foetal astrocytes. SVP, VGB and TGB significantly reduced the uptake of GABA into adult human astrocytes in primary culture following a one hour exposure. SVP and VGB also significantly reduced GABA uptake into primary cultures of human foetal astrocytes. However, TGB was without effect in the foetal astrocytes following a one hour exposure. The lack of effect of TGB in the human foetal astrocyte cultures may indicate the incomplete development of the GABA transporter GAT-1 in foetal brain where TGB is known to act. The reduction in GABA uptake exerted by SVP and VGB in human foetal astrocytes may reflect a different mechanism of action of GABA uptake inhibition from that of TGB, possibly not involving GAT-1.

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DECLARATION

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

Caroline M. Fraser

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ABBREVIATIONS

AED	antiepileptic drug
AET	2-aminoethylisothironium bromide
cAMP	3'5'-dibutyryl cyclic adenosine monophosphate
AMPA	α-amino-3-hydroxy-5-methyl-isoxazole propionic acid
ATP	adenosine triphosphate
BSA	bovine serum albumin
BSS	balanced salt solution
BZD	benzodiazepine
$CaCl_2$	calcium chloride
CBZ	carbamazepine
CD_{97}	dose causing clonic seizures in 97% of animals
CLB	clobazam
CNS	central nervous system
cpm	counts per minute
CRF	central research facility
CZP	clonazepam
DGR	desglycinyl-remacemide
DMEM	Dulbecco's modified eagle medium
DMEM-F12	Dulbecco's modified eagle medium - Hams F-12
DTT	dithiothreitol
dpm	disintegrations per minute
DZP	diazepam
EDTA	ethylene diamine tetra-acetic acid
EEG	electroencephalogram

ESM	ethosuximide
FBM	felbamate
FCS	foetal calf serum
GABA	γ-aminobutyric acid
GABA-T	GABA-transaminase
GAD	glutamic acid decarboxylase
GAERS	genetic absence epilepsy rat from strasbourg
GBP	gabapentin
GFAP	glial fibrillary acidic protein
GS	glutamine synthetase
HBSS	Hanks' balanced salt solution
HCl	hydrochloric acid
$HClO_4$	perchloric acid
HPLC	high performance liquid chromatography
HS	horse serum
ILAE	international league against epilepsy
i.p.	intraperitoneal
i.v.	intravenous
KCl	potassium chloride
α-KG	α-ketoglutaric acid
KH_2PO_4	potassium phosphate
LEV	levetiracetam
LOS	losigamone
LTG	lamotrigine
MES	maximal electroshock

$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
3-MPA	3-mercaptopropionic acid
MSO	methionine sulphoximine
NaCl	sodium chloride
NAD	nicotinamide adenine dinucleotide
$NaHCO_3$	sodium bicarbonate
NaH_2PO_4	sodium di-hydrogen orthophosphate
Na_2HPO_4	di-sodium hydrogen orthophosphate
NaOH	sodium hydroxide
NH_4Cl	ammonium chloride
NMDA	N-methyl-D-aspartate
OPA	*o*-phthalaldehyde
OXC	oxcarbazepine
PB	phenobarbitone
PBS	Dulbecco's phosphate buffered saline
PDC	L-*trans*-pyrollidine-2,4-dicarboxylic acid
PEG	polyethylene glycol 400
PHT	phenytoin
PLP	pyridoxal-5'-phosphate
PRM	primidone
PTZ	pentylenetetrazol
RMD	remacemide hydrochloride
s.c.	subcutaneous
SD	Sprague Dawley

S.E.M.	standard error of the mean
SSAD	succinate semialdehyde dehydrogenase
SVP	sodium valproate
TGB	tiagabine
TPM	topiramate
TWEEN 80	polyoxyethylene sorbitan monooleate
VGB	vigabatrin
ZNS	zonisamide

CHAPTER ONE

INTRODUCTION

1.1 EPILEPSY

Epilepsy is one of the most common serious neurological disorders in man, affecting around 50 million people world-wide (Shorvon, 1990). Approximately 0.5 - 1% of the population are thought to be affected (Rogawski and Porter, 1990). The incidence varies with age, with rates greatest in childhood, falling to low levels in adult life and rising again among the elderly (Shorvon, 1996).

1.1.1 Definition of epilepsy

The term seizure refers to a transient alteration of behaviour due to abnormal synchronized and repetitive burst firing of neuronal populations in the central nervous system (CNS). Epilepsy is a syndrome of episodic brain dysfunction characterized by recurrent unpredictable spontaneous seizures (Shin and McNamara, 1994). A local abnormal discharge may spread to other areas of the brain. The site of the primary discharge and the speed and extent of its spread determines the symptoms that are produced. These range from a brief lapse of attention to a full-blown convulsive fit lasting for several minutes. The particular symptoms produced depend on the function of the affected brain region. In general, involvement of the motor cortex causes convulsions and involvement of the hypothalamus causes peripheral autonomic discharges. Loss of consciousness occurs if the reticular formation in the upper brainstem is affected (Rang and Dale, 1991).

1.1.2 Seizure classification

There are many different types of epileptic seizure. In 1969, the International League Against Epilepsy (ILAE) introduced a scheme for the classification of epileptic seizures. Since this publication, sophisticated techniques such as electroencephalogram

(EEG) recording and radiotelemetry have become available, therefore, several other commissions on classification and terminology have convened to update, amend and improve the classification scheme (Commission on Classification and Terminology of the International League Against Epilepsy, 1981). There is a need for classification, as the choice of antiepileptic treatment will depend on the seizure type (Brodie and Dichter, 1996).

Seizures are divided fundamentally into two groups, namely partial and generalised. This concept dates back to Hughlings Jackson, but did not become common usage until developed by the Commission on Classification of the International League Against Epilepsy almost 40 years later (Commission on Classification and Terminology of the International League Against Epilepsy, 1981).

1.1.3 Partial (focal, local) seizures

Those seizures in which the first clinical and electroencephalographic changes indicate initial activation of a system of neurones limited to one part of the cerebral hemisphere are generally termed partial seizures. A partial seizure is classified primarily on the basis of whether or not consciousness is impaired. When consciousness is not impaired the seizure is classified as a simple partial seizure. When consciousness is impaired the seizure is classified as a complex partial. Simple partial seizures may evolve into complex partial seizures. A partial seizure may also progress to a generalised motor seizure (Commission on Classification and Terminology of the International League Against Epilepsy, 1981).

1.1.4 Generalised (convulsive or non-convulsive) seizures

Those seizures in which the first clinical changes indicate initial involvement of both hemispheres are referred to as generalised seizures. Consciousness may be impaired. The ictal electroencephalographic patterns initially are bilateral and are thought to reflect neuronal discharge which is widespread in both hemispheres (Commission on Classification and Terminology of the International League Against Epilepsy, 1981). The generalised seizures include tonic-clonic (previously known as *grand mal*), absence (previously known as *petit mal*), myoclonic, atonic, clonic and tonic seizures.

1.1.5 Other seizure types

A third category of seizure types also exists. This group is referred to as unclassified seizure types, and includes all seizures which cannot be classified because of inadequate or incomplete data, and some which defy classification. Recently, a fourth category was added to the classification, which incorporates epileptic syndromes. This classification takes into account such factors as the age of the patient, type of seizure, presence or absence of an underlying neurologic lesion and a presence or absence of a family history (Brodie and Dichter, 1996). An epilepsy or epileptic syndrome is either idiopathic, which is virtually synonymous with genetic epilepsy, or symptomatic, i.e. due to structural lesion or major identifiable metabolic derangement (Commission on Classification and Terminology of the International League Against Epilepsy, 1989).

1.2 NEUROTRANSMITTERS AND EPILEPSY

The normal functioning of the CNS is thought to be maintained by a fine balance between excitation and inhibition. Disruption of this balance may result in the generation of abnormal electrical activity (Schwartzkroin, 1993). A deficiency in

inhibitory γ-aminobutyric acid (GABA) neurotransmission (Meldrum, 1984) or a disorder in glutamate-mediated excitatory neurotransmission (Bradford, 1995) have been proposed to contribute to seizure initiation.

1.2.1 Inhibitory neurotransmission

The neurotransmitters GABA, glycine, β-alanine and taurine play a role in inhibitory neurotransmission in the brain (Meldrum, 1984). GABA is the major inhibitory transmitter in the CNS of vertebrates, and plays a central role in neuronal function. It was first identified in the brain in 1950 (Awapara *et al,* 1950; Roberts and Frankel, 1950).

GABA is synthesized, *de novo,* entirely from glutamate. This reaction is catalyzed by glutamic acid decarboxylase (GAD) in the presence of pyridoxal phosphate as a cofactor (Meldrum, 1975). GAD is localized exclusively in GABAergic neurones, and antibodies to this enzyme are used as a marker for GABAergic neurones in the CNS (Snead, 1983).

Traditionally GABA was thought to act on two subtypes of receptor, $GABA_A$ and $GABA_B$. More recently several lines of evidence have indicated the existence of a third subtype of GABA receptor, $GABA_C$ (Bormann and Feigenspan, 1995). $GABA_A$ receptors consist of five variable subunits forming a ligand-gated chloride permeable ion channel and are located mainly postsynaptically. Activation of $GABA_A$ receptors leads to an increase in chloride permeability and thus hyperpolarization. GABA and muscimol act as agonists at this receptor. The $GABA_A$ receptor has modulatory sites for benzodiazepines, barbiturates, neurosteroids and ethanol. Antagonists at $GABA_A$

receptors, such as bicuculline and picrotoxin, increase excitability in the CNS and may cause convulsions (Stone, 1995).

$GABA_B$ receptors are G-protein linked receptors, located both pre- and postsynaptically. Activation of $GABA_B$ receptors usually leads to an increase in potassium conductance, resulting in hyperpolarization of neurones. GABA and baclofen act as agonists, and phaclofen as an antagonist at this receptor (Stone, 1995).

The $GABA_C$ receptor is pharmacologically distinct from $GABA_A$ and $GABA_B$, and is located predominantly in the vertebrate retina. It is a chloride pore which is insensitive to both bicuculline and baclofen, and is activated selectively by *cis*-4-aminocrotonic acid (Bormann and Feigenspan, 1995).

Following neuronal release and receptor activation, GABA is removed from the synaptic cleft into both nerve terminals and astrocytes by several high affinity uptake carriers. Molecular cloning has revealed the existence of four GABA transporters named GAT-1, 2 and 3 and BGT-1 (Borden *et al*, 1994). Following uptake GABA is metabolized by two mitochondrial enzymes. GABA-transaminase (GABA-T) catalyzes the metabolism to succinic semialdehyde in the presence of α-ketoglutarate (α-KG) as a cofactor, and succinic semialdehyde dehydrogenase (SSAD) completes the metabolism to the inactive compound succinate in the presence of nicotinamide adenine dinucleotide (NAD; Meldrum, 1975).

1.2.2 Evidence for the role of GABA in epilepsy

There is substantial evidence to indicate that a decreased inhibition by GABA may contribute to seizure generation and epilepsy.

- GABAergic impairment has been described as the basis for many of the chemically-induced seizures models (Fisher, 1989).
- GABA levels in the cerebrospinal fluid have been found to be reduced in patients with chronic epilepsy (Maynham *et al*, 1980).
- In surgical human epileptogenic tissue a decrease in GAD activity as well as an increase in GABA-T activity has been demonstrated (Lloyd *et al*, 1985).
- A 30-50% decrease in GABAergic neurones or nerve terminals has been observed in the amygdala of kindled rats (Loscher and Schwark, 1987).
- A 60-70% decrease in extracellular GABA levels in microdialysates from kindled rat amygdala has recently been reported (Kaura *et al*, 1995).
- A reduction in glutamate-induced GABA release has been observed in rat and human epileptic tissue, and has been postulated to be due to a significant reduction in the number or efficiency of GABA transporter proteins (During *et al*, 1995).

Manipulation of GABAergic neurotransmission remains one of the most attractive targets in the experimental and clinical investigation of epilepsy. Antiepileptic drugs (AEDs) can increase the inhibitory actions of GABA by decreasing GABA metabolism by GABA-T, decreasing the reuptake of GABA into neurones and glia, or by increasing the synthesis of GABA by GAD (Schachter, 1995).

1.2.3 Excitatory neurotransmission

Glutamate is the predominant excitatory neurotransmitter in mammalian brain, proposed to be released at a large proportion of synapses in the CNS. It is present in the brain in a higher concentration than any other amino acid (Greenamyre and Porter, 1994).

Glutamate has a number of synthetic pathways. It can be synthesized *de novo* from glucose; by the deamination of glutamine which is catalyzed by glutaminase; or by the transamination of 2-oxoglutarate catalyzed by aspartate aminotransferase (Fonnum, 1984).

Glutamate release causes depolarization and thus excitation of neurones, by acting on a variety of receptors. The N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-isoxazole propionic acid (AMPA), and kainate are known as the ionotropic receptors. They form ion channels which are permeable to sodium, potassium and calcium ions, and are named according to the preferred analogue of glutamate to which they respond. As well as a binding site for NMDA or glutamate, the NMDA receptor has binding sites for glycine and polyamines. The ion channel of the NMDA receptor is subject to a voltage-dependent block by magnesium. A fourth type of glutamate receptor known as the metabotropic receptor also exists. This is a G-protein linked receptor coupled to effector systems which generate second messengers, and selectively responds to the agonist *trans*-1-amino-cyclopentyl-1,3-dicarboxylate (*trans*-ACPD; Stone, 1995).

Inactivation of glutamate following release occurs via a high affinity sodium- and energy-dependent uptake system present in nerve endings and glia. It is thought that glial uptake is physiologically more important than neuronal uptake (Schousboe, 1981). Three different glutamate transporters have been identified as GLAST, GLT-1 and EAAC-1 (Lehre *et al*, 1995).

Glutamate which is taken up into neurones can be metabolized by GAD to form GABA, or by glutamate dehydrogenase to from α-KG. Released glutamate, which is accumulated in astrocytes, is metabolized to glutamine via the enzyme glutamine synthetase (GS). This glutamine which is synthesized in astrocytes serves as a precursor for the formation of glutamate and GABA (Waniewski, 1992).

1.2.4 Evidence for the role of glutamate in epilepsy

Evidence also exists which indicates that an increase in excitatory glutamate neurotransmission may be involved in the aetiology of epilepsy.

- Glutamate is documented to be epileptogenic when applied directly to mammalian brain (Stone and Javid, 1983) or when administered systemically (Bradford and Dodd, 1975).
- Kainic acid and NMDA are used as chemoconvulsants in animals (Fisher, 1989).
- The phenomenon of kindling is reported to be highly dependent on NMDA-receptor activation (Cain *et al*, 1988).
- Antagonism of NMDA receptors has been shown to block the development of kindling (McNamara *et al*, 1988).
- An increase in glutamate receptor density has been demonstrated in children with

generalised seizures (Represa *et al*, 1989), and in adults with temporal lobe epilepsy (Geddes *et al*, 1990).

- Patients with primary generalised or focal epilepsy are reported to have elevated plasma levels of glutamate (Janjua *et al*, 1982).

AEDs may diminish excitatory transmission by decreasing the rate of synthesis of glutamate, decreasing the synaptic release of glutamate or by blocking the post-synaptic actions of excitatory amino acids (Meldrum, 1984).

1.3 ASTROCYTES

Neuroglia were first recognised as distinct cellular elements in the CNS in 1846, but it took until the 1920's for all of the major glial populations, namely astrocytes, oligodendrocytes, Schwann cells and microglial cells to be recognised. The term neuroglia (meaning "nerve glue") was first used by the German pathologist Rudolf Virchow to describe regions between neurones that he considered analogous to the cementing connective tissue seen in other bodily organs (Kimelberg and Norenberg, 1989).

In the late 1940's neurones were clearly identified as the elements in the brain associated with electrical activity and the ability to transfer information via chemical synapse. The glial cells failed to generate this electrical excitability, and so were regarded as the silent elements of the brain (Kettenmann, 1996).

Astrocytes account for 50% of the total glial population (Kimelberg, 1983) and are estimated to comprise as much as 20 to 50% of the total volume in some brain areas.

They are up to ten times more numerous in the CNS than neurones. Astrocytes, in general, are process-bearing cells, and a characteristic of all types is a large surface area owing to the extensive branching of the cellular processes (Juurlink and Hertz, 1992). Astrocytes have long been considered as playing structural and supporting roles, and reacting in a stereotypical manner to injury and disease. However, mainly due to advances in cell culture and specific identification of these cells, knowledge of astrocytic development, form and function, and roles in disease has increased substantially in recent years (Montgomery, 1994).

1.3.1 Types of astrocytes

There are two major types of astrocytes in the CNS; fibrous, which are found in the white matter, and protoplasmic which predominate in the grey matter (Fedoroff *et al*, 1984). Protoplasmic and fibrous astrocytes are now more commonly classified as type I and type II, respectively. Fibrous astrocytes are small process-bearing cells, stellate in appearance, and characterized as having more intermediate filaments than protoplasmic astrocytes. A single acidic protein, namely glial fibrillary acidic protein (GFAP) has been reported to be the major component of the astrocytic filaments. Histological and immunofluorescence studies have confirmed that GFAP is selectively located in astrocytes, making it an unequivocal astrocytic marker in the CNS (Eng *et al*, 1971; Bignami and Dahl, 1977).

In addition to the type I and type II astrocytes, a few more specialized cells have been identified, such as the Bergman fibres in the cerebellum, Muller fibres in the retina and radial glia which are involved in brain development (Juurlink and Hertz, 1985; Kimelberg and Norenberg, 1989).

1.3.2 Development of type I and type II astrocytes

Type I and type II astrocytes have been shown to be derived from separate glial progenitors. Their development in rat optic nerve cultures has been studied using immunofluorescence techniques. Type I astrocytes were found to differentiate on the 16th embryonic day of prenatal development from a committed precursor cell, whereas type II astrocytes did not differentiate until the second postnatal week, and it was from a bipotential precursor cell that can also give rise to oligodendrocytes. This bipotential precursor cell is known as the O-2A progenitor (Raff *et al*, 1983). It is thought that progenitor cell differentiation into type I is triggered by the culture medium, but that the differentiation of type II from progenitor cells requires a type I secreted factor (Tabernero *et al*, 1996). The O-2A progenitor has been located *in vivo* in the optic nerve, cerebellar cortex and cerebral cortex (Juurlink and Hertz, 1992). In cell culture experiments it has been demonstrated that the O-2A progenitor can differentiate into a type II astrocyte when cultured in medium containing 10% foetal calf serum. However, when the progenitors were cultured in serum-free medium they differentiated into oligodendrocytes (Temple and Raff, 1985).

Although the type II astrocyte has been identified in culture, its existence *in vivo* has not yet been fully established and remains controversial. However, several lines of evidence exist which favour the existence of type II astrocytes *in vivo*. The timing of the generation of type II astrocytes in culture is in agreement with the timing of the appearance *in vivo* of small numbers of cells with the type II astrocytic antigenic phenotype - monoclonal antibody A2B5 positive and GFAP positive (Noble, 1991). In addition, cells with the antigenic phenotype of type II astrocytes exist in freshly dissociated neonatal white matter but not grey, and in frozen sections of adult brain

most astrocytes in white matter but not grey are A2B5 positive (Raff *et al*, 1983). The production of type II astrocytes in dissociated culture has a very specific and reproducible timing, which suggests that if the generation of type II *in vitro* is an artefact, then it is extremely well regulated. Type I and type II are not interconvertable which further suggests the existence of two distinct astrocyte types (Skoff and Knapp, 1991). It remains to be clarified whether GFAP positive O-2A lineage cells in the developing CNS are stable type II astrocytes or alternatively O-2A progenitors which have been induced to express GFAP transiently before going on to become oligodendrocytes, as can happen in culture (Noble, 1991).

1.3.3 Role of astrocytes in the central nervous system

The astrocytes in the CNS are intimately associated with other cells. Astrocyte processes surround cell bodies and processes of neurones, and form a dense subpial network of fibres known as the glia limitans. Astrocyte processes completely encircle blood vessels, an observation which led some earlier investigators to wrongly identify this association as the site of the blood brain barrier. It is now known that astrocytic end feet are not the structural or anatomical site of the blood brain barrier as once believed (Kimelberg and Norenberg, 1989). Astrocytes do, however, play a role in the induction and maintenance of the blood brain barrier by forming tight junctions between the endothelial cells and modifying the transport properties of the cerebral endothelium (Montgomery, 1994).

Astrocytes are thought to be responsible for the maintenance of the extracellular environment in the brain, and regulate the physiological conditions at the synaptic cleft and the microenvironment around the neurones (Kimelberg, 1983). During

neurotransmission there is a flux of potassium into the extracellular space. Astrocytes accumulate this potassium and are responsible for its movement away from areas of high neuronal activity to areas of low. This concept is known as spatial buffering (Montgomery, 1994). Glial cells have also been found to contain the enzyme carbonic anhydrase, suggesting an involvement in pH regulation (Church *et al*, 1980).

Astrocytes are regarded as actively participating in neurotransmission. Termination of transmission requires uptake of the neurotransmitters from the synaptic cleft, where the transmitter may be reused or metabolized. Astrocytes have been shown to possess mechanisms for the uptake and metabolism of neurotransmitters including GABA and glutamate (Schousboe, 1981). In addition, the uptake and metabolism of glutamate and ammonia by astrocytes via the enzyme GS represents an important detoxification mechanism in the brain (Meister, 1974). The finding that astrocytes express a wide variety of receptor types adds to the accumulating evidence indicating that they are responsive to neuronal signals and participate in neurotransmission (Murphy and Pearce, 1987).

The radial glia have been shown to play a key role in the guidance of neuronal migration during embryonic development of the brain. Neurones are thought to migrate from their points of origin in the CNS along the processes of glial cells to their final destination in the brain (Kimelberg and Norenberg, 1989).

The CNS has no lymphoid population or lymphatic drainage and was once viewed as being isolated from the body's immune system. However, it is now recognised that astrocytes function as a liaison between the immune system and the CNS, behaving as

macrophages, by functioning as antigen presenting cells and being capable of phagocytosis (Montgomery, 1994).

1.3.4 Functional differences between type I and type II astrocytes

As well as differing morphologically, type I and type II astrocytes differ functionally. Type I are the most common type of astrocyte in the CNS and actively proliferate in culture and respond to growth factors. It is type I astrocytes which undergo reactive gliosis in response to injury. In contrast, type II astrocytes do not proliferate actively in culture and fail to respond to growth factors. Type I astrocytes are involved in potassium homeostasis and accumulation of glutamate and other amino acid neurotransmitters, and express a wide variety of receptors for neurotransmitters. While type I astrocytes affect neurones in a mainly inhibitory manner, type II exert mainly excitatory effects on neurones. Type II astrocytes exhibit GABA uptake and release, but not metabolism. In contrast, type I astrocytes are incapable of GABA release, but release glutamate via a potassium-induced calcium-independent mechanism. Type II astrocytes have a greater basal activity of GS and a higher density of sodium channels than type I (Juurlink and Hertz, 1992).

1.3.5 Role of astrocytes in disease

In addition to roles in normal CNS functioning, astrocytes have also been shown to play a role in disease. In disease states cellular swelling and hypertrophy of astrocytes occur. Astrocyte swelling is thought to be due to an influx of potassium or glutamate. Head trauma, hypoxia-ischaemia and status epilepticus are accompanied by astrocytic swelling (Kimelberg and Norenberg, 1989). Astrocytes proliferate in response to injury. This phenomenon is known as reactive gliosis. Injured neurones release a

substance which triggers astrocytic proliferation and it is thought that both mature astrocytes as well as precursor cells are involved in this response (Fedoroff *et al*, 1984).

1.3.6 Astrocytes and epilepsy

Theories linking astrocytes to seizure activity are based largely on their involvement in the regulation of the neural microenvironment. Failure in the buffering mechanisms by astrocytes has been proposed to be a significant factor in the aetiology of epilepsy (Castiglioni *et al*, 1990; Nilsson *et al*, 1992). Astrocytes from epileptic foci display morphologic changes such as an increase in orthogonal arrays at the pial surface compared to normal brain, and exhibit a decrease in extracellular potassium stimulated sodium-potassium ATPase (Montgomery, 1994).

Astrocytes cultured from audiogenic seizure-prone mice or from epileptic foci display an increase in carbonic anhydrase activity, and an increase in the uptake of glutamate. This is thought to be a protective mechanism, as the overall concentration of glutamate has been shown to be elevated in the cerebral cortex of audiogenic seizure-prone mice (Tiffany-Castiglioni and Castiglioni, 1986).

High levels of GS have been reported in glial cells of the CNS. Inhibition of this enzyme by methionine sulfoximine (MSO) leads to seizure generation in mice (Tiffany-Castiglioni and Castiglioni, 1986).

In recent years, a number of studies have been undertaken to assess the contribution of astrocytes to seizure mechanisms. Bordey and Sontheimer (1998) have recently

characterized the properties of human glial cells associated with epileptic seizure foci. They reported that seizure-associated astrocytes had complex highly branched processes, were stellate in appearance and stained intensely for GFAP, as would be expected for reactive astrocytes. Glia associated with seizure foci were also found to express sodium channels at densities sufficient to generate slow action potentials in current clamp studies. In addition, these cells displayed a lack of inwardly rectifying potassium currents, which in normal astrocytes are implicated in the control of potassium levels. The results of this study suggest that astrocytes from seizure foci differ in morphological and physiological properties, and that glial potassium buffering could be impaired, thus contributing to the pathophysiology of seizures. Furthermore, impaired potassium homeostasis and permeability through glial inwardly rectifying potassium channels has been reported in a rodent experimental model of reactive gliosis (D'Ambrosio *et al*, 1998).

In support of the theories linking astrocytes to the pathology of epilepsy, O'Connor and colleagues (1998) demonstrated that hippocampal astrocytes from seizure foci have an impaired ability to clear the extracellular space of glutamate, an effect which has been proposed to contribute to increased neuronal excitability. In addition, it has been shown that fluorocitrate, a reversible and specific inhibitor of astrocytic metabolism, can precipitate seizures (Willoughby *et al*, 1998).

1.4 TREATMENT OF EPILEPSY

1.4.1 History of antiepileptic drug treatment

Epilepsy has been known since the beginning of recorded history, and has been referred to as "the dread disease", "the sacred disease" and "the falling sickness". The word 'epilepsy' is derived from a Greek word meaning "to throw oneself" (Patsalos and Duncan, 1994). The victims of this disease were thought to be "possessed", "bewitched" or "inflicted by the Gods" (Swinyard, 1980). The treatment of epilepsy emerged from ignorance, superstition and religious beliefs. Until the 19th century, patients with epilepsy were subjected to prejudice, and treated with remedies of antiquity which often resulted in death.

During the 19th century, the understanding of the neuropathological basis of epilepsy, together with serendipitous drug discovery, and the development of synthetic chemistry and experimental animal models resulted in the first effective antiepileptic therapy (Patsalos and Sander, 1994). The introduction of bromide in the 1857 marked the first effective treatment for epilepsy (Harden, 1994). The drug was, however, associated with significant side effects, and is no longer used routinely in the treatment of epilepsy (Patsalos and Sander, 1994).

The sedative action of bromide correlated with seizure reduction, and led in 1912 to the discovery of the barbiturate phenobarbitone (PB), another sedative with efficacy in seizure disorders. The continued search for similar molecules with less sedative effects resulted in the development of numerous other barbiturates (Harden, 1994). Phenytoin (PHT) was introduced in 1938, followed by primidone (PRM) in 1952, ethosuximide

(ESM) in 1958 and carbamazepine (CBZ) in 1967. In 1963, sodium valproate (SVP) was serendipitously discovered as having antiseizure properties when it was used as a solvent in a drug screening programme and was approved for use in Europe in 1974 (Brodie and Dichter, 1997).

After a hiatus of nearly 20 years, no fewer than eight new AED's; vigabatrin (VGB), lamotrigine (LTG), felbamate (FBM), gabapentin (GBP), oxcarbazepine (OXC), topiramate (TPM), zonisamide (ZNS) and tiagabine (TGB) have received a licence in at least one country during the 1990's. More new agents, including levetiracetam (LEV), remacemide (RMD) and losigamone (LOS) are in development, with more than a few 'numbered' compounds undergoing phase one evaluation (Wilson and Brodie, 1996).

1.4.2 Strategies for the drug treatment of epilepsy

The past decades have witnessed an increase in our knowledge and understanding of the pathophysiology of brain diseases and basic mechanisms of drug activity. This knowledge has generated several rational strategies for drug development, aimed at identifying new anticonvulsant drugs with high specificity and/or selectivity of action. The most important strategies of rational design of anticonvulsant drugs have been enhancement of GABA-mediated neuronal inhibition, diminution of glutamate-mediated neuronal excitation and modulation of sodium, potassium and particularly calcium ion channels (Loscher, 1998). All of these targets for anticonvulsant drug development are thought to be critically involved in the pathophysiology of epileptic processes (Dichter, 1994).

1.4.3 Established antiepileptic drugs

PHENOBARBITONE

PB is a barbiturate which has considerably greater anticonvulsant effect in relation to its sedative effect than other barbiturates. It is effective in the treatment of partial and generalised seizures, but its clinical usefulness in the developed world is limited by sedation. Other side effects of PB include alterations in mood, cognition and behaviour and memory impairment. The drug is an enzyme inducer so can accelerate the metabolism of many lipid soluble drugs (Brodie and Dichter, 1996).

PB is effective in both the maximal electroshock (MES) and pentylenetetrazol (PTZ) animal seizure models (Schachter, 1995). It binds to an allosteric regulatory site on the $GABA_A$ receptor and produces an increase in the affinity for GABA and a decrease in the rate of GABA dissociation (Yang and Olsen, 1987). This causes an increase in the mean $GABA_A$ receptor chloride channel opening time without affecting single channel conductance (Twyman *et al*, 1989). This effect is thought to contribute to the antiepileptic effects of PB, but additional mechanisms have been proposed to account for the relative antiepileptic potency of this drug over other more sedative barbiturate compounds. Such additional mechanisms include an inhibitory action on calcium influx through voltage-sensitive N- and L-type calcium channels (Gross and Macdonald, 1988), blockade of non-NMDA type excitatory amino acid receptors (Miljkovic and Macdonald, 1986) and enhancement of voltage-dependent potassium currents (Huguenard and Wilson, 1985).

PHENYTOIN

PHT was the result of a search to find a non-sedating analogue of PB (Rogawski and Porter, 1990). It is effective for the treatment of partial and tonic-clonic seizures. Gum hypertrophy, acne, hirsuitism, drowsiness, ataxia and cognitive difficulties are some of the side effects experienced with the drug (Brodie and Dichter, 1996).

PHT can abolish the tonic phase, but may enhance or prolong the clonic phase of MES-induced seizures in animals. It has been shown to be ineffective against seizures induced by PTZ, bicuculline, picrotoxin, penicillin and strychnine (Rogawski and Porter, 1990).

PHT has been proposed to exert its antiepileptic effects by interacting with the voltage-activated sodium channels that are responsible for the action potential upstroke in a highly specific voltage- and frequency-dependent manner. It has been shown to stabilize 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).

In addition to effects on sodium channels, PHT has been proposed to induce voltage- and frequency-dependent block of T-type calcium channels (Twombly *et al*, 1988), inhibitory effects on non-T-type calcium channels (Crowder and Bradford, 1987) and a potentiation of GABA-mediated synaptic inhibition (Raabe and Ayala, 1976; McLean and Macdonald, 1983).

CARBAMAZEPINE

CBZ has a tricyclic structure similar to some of the standard antidepressant drugs. Pharmacologically and clinically its actions resemble those of PHT (Rang and Dale, 1991). It is effective for the treatment of partial and generalised tonic-clonic seizures, but is ineffective, and can be deleterious, in patients with absence or myoclonic seizures. Adverse effects most often associated with CBZ therapy are diplopia, headache, dizziness and nausea (Brodie and Dichter, 1996).

CBZ and PHT have a similar spectrum of activity in animal seizure models. Both drugs selectively prevent tonic hindlimb extension in MES-induced seizures, and both are ineffective against seizures induced by PTZ. CBZ does, however, have a higher protective index than PHT.

CBZ has an active metabolite, carbamazepine-10,11-epoxide, which has a similar spectrum of anticonvulsant activity as the parent compound (Rogawski and Porter, 1990). Like PHT, CBZ has been proposed to exert its anticonvulsant effects by an inhibition of voltage-dependent sodium channels. However, PHT has been shown to have a more pronounced frequency-dependent block than CBZ, which may help to explain the subtle clinical differences between the drugs (Macdonald and Kelly, 1993).

Other proposed mechanisms of CBZ action include an inhibition of voltage-dependent non-T-type calcium influx (Crowder and Bradford, 1987) and antagonistic effects at brain adenosine A_1 and A_2 receptors. However, as adenosine is a putative endogenous anticonvulsant this would question the relationship between adenosine receptor antagonism and the antiepileptic effects of the drug (Rogawski and Porter, 1990).

SODIUM VALPROATE

SVP is a broad spectrum anticonvulsant agent, with efficacy in many types of seizures, and especially in those with idiopathic generalised epilepsy. Treatment with SVP has been associated with a number of adverse effects including dose-related tremor, weight gain, thinning or loss of hair and menstrual irregularities. In addition, hepatotoxicity has also been observed, particularly in children under three years old who are receiving polytherapy (Brodie and Dichter, 1996).

In animal seizure models, SVP is effective against a wide variety of chemoconvulsants including PTZ-induced seizures, and is also effective in the MES test (Rogawski and Porter, 1990). Its mechanism of action is yet to be fully elucidated, but it is thought to exert its pharmacological effects by a combination of mechanisms.

SVP has been shown to increase GABA levels in animal brain (Loscher, 1981) and in the plasma and cerebrospinal fluid of treated patients (Loscher and Schmidt, 1980; Loscher and Siemes, 1984). The increase in GABA levels has been proposed to be due to an increased synthesis of GABA via a stimulatory action on GAD. Inhibitory actions of SVP on the GABA-degrading enzymes GABA-T, SSAD and aldehyde reductase have also been reported to contribute to the increase in GABA concentration (Rogawski and Porter, 1990). However, the blockade of GABA-T is thought to be weak and reversible, and may occur at doses above those therapeutically relevant (Nilsson *et al* 1992).

In a similar manner to PHT and CBZ, SVP has also been shown to exert a use- and voltage- dependent limitation of sustained repetitive firing of sodium dependent action

potentials (McLean and Macdonald, 1986).

The observation that SVP is effective against generalised absence seizures led to suggestions that, like ESM, it may interact with voltage-sensitive calcium channels. Initial studies with SVP failed to demonstrate an effect on the low-threshold calcium current. A later study, however, demonstrated that SVP modestly reduced T-type calcium currents in primary afferent neurones. The discrepancy in effects on calcium channels and the relevance of them require clarification (Macdonald and Kelly, 1993).

In addition to these effects, SVP has been consistently found to decrease levels of the excitatory amino acid aspartate in rodent brain (Schechter *et al*, 1978). The mechanism by which SVP reduces aspartate is at present unknown. The extent to which each of these proposed mechanisms contributes to the antiepileptic efficacy of SVP remains to be clarified.

The established AEDs PRM, ESM and the benzodiazepines (BZDs) are licenced for use in the UK, but were not investigated in this thesis.

PRIMIDONE

PRM is often considered as a pro-drug for its active metabolites PB and phenylethylmalonamide. The mechanism of action of PRM is less well documented than that of other barbiturate compounds. The actual role of the parent compound and the active metabolites in the clinical efficacy of the drug remain to be determined, but it is thought to act at the $GABA_A$ receptor in a similar manner to PB. (Rogawski and Porter, 1990). The efficacy of PRM is similar to that of PB, but it is less well tolerated

(Brodie and Dichter, 1996).

ETHOSUXIMIDE

ESM was developed empirically by modification of the barbituric acid ring structure. The drug is efficacious in the treatment of absence seizures, but is inactive against other seizure types. Side effects of ESM mainly involve gastrointestinal disturbances such as nausea and vomiting, and CNS related effects such as lethargy, dizziness and ataxia. There are also rare reports of blood dyscrasias, some of which have been fatal (Brodie and Dichter, 1996).

In animal models, ESM specifically blocks PTZ- and bicuculline-induced clonic seizures, but fails to exert any activity against tonic seizures induced by MES (Rogawski and Porter, 1990). ESM acts by reducing low-threshold, transient, voltage-dependent T-type calcium channel conductance in thalamic neurones (Coulter *et al*, 1989).

BENZODIAZEPINES

There are over 50 chemically distinct BZDs marketed worldwide. They are potent anticonvulsants in a wide variety of animal seizure models including PTZ- and picrotoxin-induced seizures, and at higher doses block MES- and strychnine-induced seizures. Although pharmacokinetic differences exist and relative potencies vary between the BZDs, they all exhibit a similar anticonvulsant profile which is thought to relate to their common mechanism of action (Rogawski and Porter, 1990).

A receptor for BZDs is thought to exist on the $GABA_A$ receptor complex, where they

act to enhance inhibitory responses to GABA. The BZDs have been shown to increase the frequency of the $GABA_A$ receptor chloride channel opening, without affecting single channel conductance or open time. This effect is in contrast to the barbiturates which prolong the channel opening time without increasing the frequency of opening (Twyman *et al*, 1989). Other mechanisms of BZD action include an inhibition of adenosine uptake and blockade of voltage-dependent sodium and calcium channels (Rogawski and Porter, 1990).

Diazepam (DZP) was introduced in 1968, and is used in the acute treatment of status epilepticus. The sedative effect and development of tolerance with most BZDs limits their use as maintenance anticonvulsant therapy (Rang and Dale, 1991). Clobazam (CLB) was introduced as an anxiolytic in 1975, and its anti-seizure activity was recognised soon after. It is a useful adjunctive drug in refractory epilepsy (Brodie and Dichter, 1997). Clonazepam (CZP) is thought to be relatively more selective as an anticonvulsant, and has limited use in the treatment of refractory myoclonic seizures (Brodie and Dichter, 1996).

1.4.4 Novel antiepileptic drugs

VIGABATRIN

VGB was the first AED to be developed on the basis of a targeted mechanism of action. It is a synthetic derivative of the inhibitory neurotransmitter GABA. The drug has two enantiomers, only one of which, the S-enantiomer, is pharmacologically active (Hannah *et al*, 1995). VGB was licenced for use in the UK in 1989 as add-on treatment for partial seizures (Reynolds *et al*, 1991). The drug is also useful in the treatment of infantile spasms (Wilson and Brodie, 1996). Sedation, dizziness and

headache are the most frequently reported adverse events with VGB. Other reported problems include fatigue, weight gain, depression and behavioural problems (Hannah *et al*, 1995).

VGB has demonstrated efficacy against a range of experimental seizures, including those induced by picrotoxin (Bernasconi *et al* 1988) and amygdaloid-kindling (Loscher *et al*, 1989) and is also effective in genetic models of reflex epilepsy (Schechter *et al*, 1977).

VGB exerts its antiepileptic effects by an enzyme-activated suicide inhibition of GABA-T, the enzyme directly responsible for the metabolic degradation of GABA (Lippert *et al*, 1977; Jacob *et al*, 1990). The drug has been shown to elevate GABA levels in plasma, cerebrospinal fluid and brain of experimental animals (Bohlen *et al*, 1979), and in the plasma and cerebrospinal fluid in man (Ben-Menachem *et al* 1989). VGB has also been found, in our laboratory, to have an inhibitory effect on the uptake of GABA into astrocytes (Leach *et al*, 1996).

LAMOTRIGINE

LTG is a phenyltriazine with weak anti-folate properties. It emerged from a mass screening of anti-folate compounds for anticonvulsant activity. Structure-activity studies have failed to show a correlation between its anti-folate activity and anticonvulsant potency. It has been licenced as add-on therapy for refractory partial seizures with or without secondary generalisation in more than 70 countries, with approval for use as monotherapy and for use in children in many of these. LTG was licenced for use in the UK in 1991. There is also evidence to suggest that the drug may

be effective for absence and myoclonic seizures (Wilson and Brodie, 1996). Adverse events most frequently reported for LTG include headache, nausea and vomiting, diplopia, dizziness, ataxia and tremor (Brodie, 1992b). The development of rash complicates the initial management of 3-5% of patients, but in mild cases it may subside spontaneously without withdrawal of the drug. However, in rare cases the Stevens-Johnson syndrome may develop. There is evidence to suggest that gradual introduction of LTG lessens the risk of rash (Dichter and Brodie, 1996).

In animal models LTG exhibits high potency in the MES test, and can also block the development of kindled seizures in rats. Like PHT, LTG is ineffective against both threshold and clonic seizures induced by PTZ, suggesting a similar mechanism of action (Rogawski and Porter, 1990).

In rat cerebral cortical slices LTG potently inhibited veratrine-evoked glutamate and aspartate release (Leach *et al*, 1986). It has been shown to limit the high frequency firing of sodium-dependent action potentials in cultured mouse spinal cord neurones (Cheung *et al*, 1992), therefore, suggesting a use- and frequency-dependent blockade of voltage-sensitive sodium channels similar to PHT and CBZ (Macdonald and Kelly, 1993). More recently, a number of studies have also indicated that LTG exhibits blockade of calcium currents (Wang *et al*, 1996; vonWegerer *et al*, 1997).

FELBAMATE

Felbamate (FBM) was approved for use in the United States in 1993, as monotherapy and adjunctive therapy for partial seizures with or without secondary generalisation. In children it is effective as adjunctive therapy in seizures of the Lennox-Gastaut

syndrome. It is a dicarbamate, structurally related to, but less sedative than, the anxiolytic agent meprobamate (Pellock and Boggs, 1995). FBM is a broad spectrum anticonvulsive drug, but the occurrence of aplastic anaemia and hepatic failure (Pellock and Brodie, 1997) have limited its use to those patients with intractable epilepsy who have been unresponsive to alternative therapies (Leppik and Wolff, 1995). Other adverse events associated with FBM treatment include gastrointestinal and neurological symptoms such as anorexia, nausea and vomiting, weight loss, headache, dizziness and diplopia (Wilson and Brodie, 1996).

In animal models it has demonstrated efficacy against seizures induced by several chemoconvulsants including PTZ, picrotoxin, NMDA and quisqualate, and MES (Swinyard *et al*, 1986; White *et al*, 1992). It has been shown to be ineffective against bicuculline- and strychnine-induced seizures (Swinyard *et al*, 1986). FBM has been proposed to possess a broader spectrum of activity than the conventional AEDs PHT and CBZ (Rho *et al*, 1994). Its effectiveness in both chemically- and electrically-induced seizures has suggested that the drug exerts its anticonvulsant activity by increasing seizure threshold and preventing seizure spread (Swinyard *et al*, 1986). In addition to its antiepileptic activity, FBM has been shown to have a neuroprotective effect in animal models of hypoxic-ischaemic injury (Wasterlain *et al*, 1992; Chronopoulos *et al*, 1993; Wasterlain *et al*, 1994; Wallis and Panizzon, 1995; Wasterlain *et al*, 1996).

As yet, the precise mechanism of action of FBM remains to be determined. In ligand binding studies, FBM failed to interact with the $GABA_A$ receptor complex (Ticku *et al*, 1991). The drug has, however, been shown to enhance $GABA_A$ receptor-mediated

chloride currents in rat hippocampal neurones (Rho *et al*, 1994). In addition, Gordon and colleagues (1991) reported that a sub-protective dose of FBM could potentiate the anticonvulsant effects of diazepam against seizures induced by MES, PTZ and isoniazid. The drug has also been shown to interact with the strychnine-insensitive glycine modulatory site of the glutamate NMDA receptor (McCabe *et al*, 1993; White *et al*, 1995b).

In addition to its effects on amino acid systems, FBM has been reported to exert blockade of voltage dependent sodium channels (White *et al*, 1992), and inhibition of dihydropyridine-sensitive calcium channels (Stefani *et al*, 1996). It is likely that FBM possesses multiple mechanisms of action, but the exact contribution of each of these effects to the antiepileptic effects of the drug remain to be evaluated.

GABAPENTIN

GBP is a hydrophilic analogue of GABA which can freely cross the blood-brain barrier. It was licenced for use in the UK in 1993 as add-on therapy for the treatment of refractory partial seizures, with or without generalised tonic-clonic seizures. Side effects of GBP include somnolence, dizziness, ataxia, fatigue, nystagmus, headache, tremor, and less commonly, diplopia, nausea and vomiting (Wilson and Brodie, 1996).

GBP has an experimental and clinical profile similar to that of SVP. In animal experimental models GBP is effective against tonic seizures induced by a variety of chemoconvulsants and is also active in the MES test (Rogawski and Porter, 1990). At present the precise mechanism of action of GBP remains unclear. It has been suggested that it may interact specifically with a plasma membrane site proposed to be the system

L-amino acid transporter (Stewart *et al*, 1993).

GBP has demonstrated only limited effects on the GABAergic system, and has been reported to exert no effects at GABA receptors or uptake carriers in the brain (Taylor *et al*, 1998). In animal studies the drug has been shown to increase GABA turnover in isolated regions of the brain (Loscher *et al*, 1991b), and there is evidence to suggest that GABA levels were elevated in the occipital cortex of patients undergoing a clinical trial (Petroff *et al*, 1996). GBP has been reported to increase the activity of GAD, suggesting an increase in the synthesis of GABA from glutamate (Taylor *et al*, 1998). In addition, at high concentrations it has been shown to inhibit GABA-T. However, this effect is not thought to be clinically relevant (Goldlust *et al*, 1995). The drug has been reported to increase electrophysiological responses to GABA in the rat optic nerve (Kocsis and Honmou, 1994).

A recent study in our laboratory (Leach *et al*, 1997a) demonstrated that repeated GBP administration reduced whole brain glutamate levels. The mechanism by which the drug exerts this action is unclear. Goldlust and colleagues (1995) reported that GBP inhibited branched-chain amino acid aminotransferase, and this effect may contribute to the increased glutamate concentrations observed.

In addition to effects on amino acid systems, GBP has been reported to reduce sustained repetitive firing of neurones in a manner similar to PHT (Wamil and McLean, 1994), and has been demonstrated to bind with high affinity to a calcium channel subunit in the brain (Gee *et al,* 1996). Recently the drug was also shown to exert inhibitory effects on L-type voltage-gated calcium channels (Stefani *et al*, 1998).

TOPIRAMATE

TPM, a sulphamate-substituted monosaccharide compound (Langtry *et al*, 1997), was licenced in the UK in 1995 for use as add-on therapy in refractory patients. It is effective in the treatment of partial-onset seizures with or without secondary generalisation. TPM can produce CNS-related side effects including ataxia, confusion, dizziness, fatigue, paraesthesia, somnolence and cognitive impairment. Less common problems are amnesia, anorexia and weight loss, diplopia, nausea and vomiting (Wilson and Brodie, 1996).

TPM prevents seizures in a number of animal models including tonic seizures induced by MES (Shank *et al*, 1994), amygdala-kindled rats (Wauquier and Zhou, 1996) and genetic models of epilepsy (Nakamura *et al*, 1994). It is ineffective in blocking chemically-induced seizures (Patsalos and Duncan, 1994). TPM is thought to act by blocking the spread of seizure discharges.

It appears likely that TPM possesses multiple mechanisms of action. It has been reported to exert blockade of voltage-activated sodium channels (Coulter *et al*, 1993; Kawasaki *et al*, 1996; Zona *et al*, 1997; Wu *et al*, 1998) and inhibitory effects on kainate-induced responses (Coulter *et al*, 1995). In addition, TPM reportedly potentiates responses to GABA at the $GABA_A$ receptor (White *et al*, 1995a; White *et al*, 1997) and may exert a weak carbonic anhydrase inhibition. Its antiepileptic properties are, however, thought to be independent of the carbonic anhydrase inhibition (Shank *et al*, 1994). It has also been reported that TPM may reduce abnormally high levels of glutamate and aspartate in the hippocampus of spontaneously epileptic rats (Kanda *et al*, 1996).

TIAGABINE

TGB is a nipecotic acid analogue into which a lipophilic anchor has been incorporated to facilitate crossing of the blood-brain barrier after oral administration. It has proven efficacy as add-on therapy for patients with partial onset seizures including secondarily generalised tonic-clonic seizures, and was licenced for use in the UK in 1998. TGB appears to be well tolerated. Side effects of the drug include tiredness, dizziness, nervousness, tremor, confusion and gastrointestinal disturbances (Leach and Brodie, 1998).

TGB is effective 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 rodents. It is said to be ineffective against MES-induced seizures (Nielsen *et al*, 1991).

TGB is mechanistically the most precise AED in clinical use. It is a potent and selective inhibitor of GABA uptake into neurones and glia, consequently increasing extracellular GABA levels (Suzdak and Jansen, 1995). It has been shown to specifically inhibit the GAT-1 GABA transporter, but is not a substrate for it (Borden *et al*, 1994). TGB is the first GABA uptake inhibitor to be introduced into clinical practice. It has no significant affinity for other uptake sites and does not affect sodium or calcium channels (Schachter, 1998). To date no other mechanism has been proposed to explain its antiepileptic activity.

1.4.5 Novel antiepileptic drugs undergoing clinical evaluation

LEVETIRACETAM

LEV is the *S* enantiomer of the ethyl analogue of the nootropic agent piracetam (Loscher *et al*, 1996). It is currently undergoing clinical trial for the treatment of epilepsy. Initial reports are encouraging and indicate that the drug has efficacy against a wide range of seizure types (Stables *et al,* 1995). LEV has demonstrated a significant efficacy in patients with refractory complex partial seizures with or without secondary generalisation (Sharief *et al*, 1996). The drug is reported to be well tolerated with only mild or moderate adverse effects reported. These include drowsiness, memory impairment, dizziness, headache, depression and mood changes (Patsalos and Duncan, 1994; Wilson and Brodie, 1996).

LEV has demonstrated a broad spectrum of activity in experimental animal models. It is reported to be effective against threshold and maximal PTZ- and electroshock-induced convulsions, and also protects against seizures induced by amygdaloid kindling and those precipitated by bicuculline, picrotoxin and NMDA (Gower *et al*, 1992; Loscher and Honack, 1993; Loscher *et al*, 1998). LEV has also demonstrated efficacy in two genetic models of epilepsy, namely the genetic absence epilepsy rat from strasbourg (GAERS) and the audiogenic-seizure prone rat (Gower *et al*, 1995).

As with many of the other novel AEDs, the precise mechanism of action of LEV remains to be determined. The drug fails to interact with classical receptor and ion channel sites in the brain. LEV has, however, been proposed to bind in a stereospecific manner to a specific binding site in CNS membranes, where it is weakly displaced by PB and PTZ, suggesting that it may be GABA-related (Noyer *et al*, 1995). A study by

Loscher and colleagues (1996) reported that treatment with LEV induced alterations in GABA metabolism and turnover in discrete areas of rat brain.

REMACEMIDE HYDROCHLORIDE

RMD is a novel AED which emerged from a drug discovery programme aimed at developing a novel compound with a three-dimensional structure similar to that of PHT (Rogawski and Porter, 1990). RMD is experimentally and clinically active, but undergoes significant metabolism to the desglycinyl derivative ARL 12495AA (DGR). This metabolite has a longer half-life than RMD (Stables *et al*, 1995) and a more potent pharmacological profile. It is currently undergoing clinical evaluation for the treatment of complex partial seizures with or without secondary generalisation. Initial studies show that the drug is well tolerated. The most common adverse effects reported are dose-dependent light-headedness, dizziness and gastrointestinal upset (Patsalos and Duncan, 1994).

Both RMD and DGR are effective against a range of experimental seizures, including those induced by MES, hippocampal kindling, NMDA, kainate and 4-aminopyrine (Stagnitto *et al*, 1990; Garske *et al*, 1991; Palmer *et al*, 1992; Cramer *et al*, 1994).

The precise mechanism of action of RMD remains to be determined. Both RMD and DGR prevent sustained repetitive firing of cultured spinal cord neurones (Wamil *et al*, 1996) and hippocampal CA1 neurones (Norris and King, 1997) via a blockade of voltage-sensitive sodium channels. DGR has also been shown to decrease glutamate and aspartate release from cortical slices (Srinivasan *et al*, 1995). There is evidence to suggest that the drug may also exert its antiepileptic effects by an action at the NMDA

subtype of glutamate receptor (Hu and Davies, 1995; Subramaniam *et al*, 1996). RMD has been shown to weakly interact with the NMDA receptor. However, DGR reportedly displaces [^{3}H]-dizocilpine binding to the NMDA ion channel at clinically relevant concentrations (Subramaniam *et al*, 1996). A recent study conducted in our laboratory demonstrated that repeated administration of DGR at high dose significantly increased GABA-T activity and decreased GAD activity (Leach *et al*, 1997b). These effects have been postulated to contribute to the proconvulsant action of the drug seen clinically at higher doses.

A number of other novel compounds are in the early stages of development. These include LOS (Leppik, 1994); retigabine, a potassium channel opener (Rundfelt, 1997); LY 300164, an antagonist at glutamate AMPA / kainate receptors (Czucwar *et al*, 1998); ganaxolone, a neurosteroid which modulates the $GABA_A$ receptor (Carter *et al*, 1997); and SB 204269, an AED with a proposed novel mechanism of action (Herdon *et al*, 1997).

AIMS

It would appear from the literature that the precise mechanism of action of many AEDs remains to be determined, and that most have multiple mechanisms of action. Many of these AEDs exert effects on the GABAergic and glutamatergic systems. The effects of AEDs on these amino acid neurotransmitter systems is of particular interest at present. In view of this, the aim of this study was to examine the effects of a range of established, novel and experimental AEDs on GABA and glutamate neurochemistry in primary cultures of rat cortical astrocytes and in mouse brain. As the vast majority of studies on neurochemical effects of AEDs employ rodent brain tissue, it was also of interest to investigate the effects of several of these drugs on GABA transport in primary cultures of human astrocytes. In addition, as many patients receiving therapy for epilepsy require two or more drugs, a preliminary study was undertaken in an attempt to identify potentially useful combinations of new AEDs in mice using the PTZ seizure model.

CHAPTER TWO

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals: adenosine triphosphate (ATP), Amberlite CG-50 hydrogen form resin, γ-aminobutyric acid (GABA), 2-aminoethylisothironium bromide (AET), ammonium chloride (NH_4Cl), boric acid, bovine serum albumin (BSA), calcium chloride ($CaCl_2$), collagenase, 3'5'-dibutyryl cyclic adenosine monophosphate (cAMP), dithiothreitol (DTT), DNase I, ethylene diamine tetra-acetic acid (EDTA), gabaculline, gentamycin, D-glucose, L-glutamic acid, L-glutamine, HEPES, HEPES sodium salt, homoserine, imidazole-HCl, α-ketoglutaric acid (α-KG), magnesium sulphate ($MgSO_4$), 2-mercaptoethanol, 3-mercaptopropionic acid (3-MPA), methionine sulfoximine (MSO), D,L-norvaline, ouabain, papain, paraformaldehyde, pentylenetetrazol (PTZ), *o*-phthalaldehyde (OPA), polyethylene glycol 400 (PEG), poly-L-lysine, polyoxyethylene sorbitan monooleate (Tween 80), potassium chloride (KCl), potassium phosphate (KH_2PO_4), pyridoxal-5'-phosphate (PLP), sodium azide, sodium bicarbonate ($NaHCO_3$), sodium chloride (NaCl), di-sodium hydrogen orthophosphate (Na_2HPO_4), sodium di-hydrogen orthophosphate (NaH_2PO_4), soya bean trypsin inhibitor, trypan blue, trypsin, were all obtained from the Sigma Chemical Company.

Glacial acetic acid, hydrochloric acid (HCl), magnesium chloride ($MgCl_2$), perchloric acid ($HClO_4$) and sodium hydroxide (NaOH) were obtained from Merck.

Dulbecco's Modified Eagle Medium (DMEM), Dulbecco's Modified Eagle Medium-Hams F-12 (DMEM-F12), Dulbecco's phosphate buffered saline (PBS), foetal calf serum (FCS), fungizone, L-glutamine, Hanks' Balanced Salt Solution 10X (HBSS),

horse serum (HS), new-born calf serum and penicillin-streptomycin were all obtained from Gibco BRL.

Picofluor 40 scintillation fluid and Ultima Gold XR scintillation fluid were purchased from Canberra Packard.

Coomassie Brilliant Blue G-259 protein assay dye reagent, Dowex AG1-X8 acetate form ion exchange resin, and Dowex AG50W-X8 hydrogen form ion exchange resin were obtained from BIORAD.

High performance liquid chromatography (HPLC) grade acetonitrile and methanol were obtained from Rathburn Chemicals Ltd.

L-*trans*-pyrollidine-2,4-dicarboxylic acid (PDC) was purchased from Research Biochemicals International.

Citifluor AF1 antifadent solution was obtained from Citifluor Products.

2.1.2 Radioisotopes: γ-[^{14}C(U)]-aminobutyric acid and L-[^{14}C(U)]-glutamic acid were purchased from NEN Research Products.

2.1.3 Antiepileptic drugs: Carbamazepine (5H-dibenz[b,f]azepine-5-carboxamide), phenobarbitone (5-ethyl-5-phenyl-2,4,6-trioxohexahydropyrimidine), phenytoin (5,5-diphenyl-2,4-imidazolidinedione), and sodium valproate (2-propylpentanoic acid) were all purchased from Sigma Chemical Company; desglycinyl-remacemide (±)-1-methyl-

1,2-diphenylethylamine) was a gift from Astra Charnwood; felbamate (2-phenyl-1,3-propanediol dicarbamate) from Schering-Plough Research Institute; gabapentin (1-(aminomethyl)-cyclohexaneacetic acid) from Parke-Davis Pharmaceutical Research; lamotrigine (6-(2,3-dichlorophenyl)-1,2,4-triazine-3,5-diamine) from GlaxoWellcome Research and Development; levetiracetam ((S)-α-ethyl-2-oxo-pyrrolidine acetamide) from UCB Pharma; tiagabine ((R)-(-)-1-[4,4-bis(3-methyl-2-thienyl)-3-butenyl]-3-piperidine-carboxylic acid, hydrochloride) from Novo Nordisk A/S; topiramate (2,3:4,5-bis-O-(1-methylethylidene)-β from the R.W. Johnson Pharmaceutical Research Institute; and vigabatrin (D,L-4-aminohex-5-enoic acid) from Hoechst Marion Roussel.

2.1.4 Antibodies: Rabbit Anti-Cow Polyclonal glial fibrillary acidic protein (GFAP) was purchased from DAKO Ltd., and Goat Anti-Rabbit IgG (γ heavy chain specific) RITC-labelled from EURO-PATH Ltd.

2.1.5 Animals: ICR strain mice supplied by Harlan Olac were used for all neurochemical studies. Neonatal rat pups for astrocyte cultures were supplied by the Central Research Facility (CRF) at the University of Glasgow from a breeding colony of Sprague Dawley (SD) rats. Animals were housed in the CRF in a controlled temperature and humidity environment with day / night cycle conditions and access to food and water *ad libitum*. Experimental animals were kept for a minimum period of seven days prior to use to allow for acclimatisation. All experimental work was governed by the Animals (Scientific Procedures) Act, 1986 (UK).

2.2 REMOVAL AND STORAGE OF BRAIN TISSUE

For radiochemical assays of glutamine synthetase (GS) and GABA-transaminase (GABA-T) activities, and determination of amino acid concentrations and glutamic acid decarboxylase (GAD) activity by HPLC, mouse brain tissue was removed in the following manner. Animals were sacrificed by a blow to the head and cervical dislocation, followed by decapitation. Brains were then carefully removed and placed in eppendorf tubes on dry ice until storage at -70^{0}C. For some studies, the left and right hemispheres of the brain were separated by a midline incision and stored as described until required for assay. For cell culture [^{14}C]-GABA and [^{14}C]-glutamate uptake studies, cells were scraped and stored at -20^{0}C prior to liquid scintillation counting and protein concentration determination. Cells for GS and GABA-T assays were scraped and stored at -70^{0}C until required.

2.3 PRIMARY CULTURE OF RAT CORTICAL ASTROCYTES

This method was a modification of the methods of Larsson and colleagues (1981) and Bender and Hertz (1984).

2.3.1 Culture medium

Media were prepared under sterile conditions, and were filter sterilised through a 0.2 μm pore filter prior to use. Media were stored sterile at 4^{0}C for up to five days. Dissection medium consisted of DMEM supplemented with 20% HS (v/v), 2.5 mM L-glutamine, 100 I.U./ml penicillin, 100 μg/ml streptomycin, 0.1 mg/ml gentamycin and 25 μg/ml fungizone. Culture medium consisted of DMEM supplemented with 20, 10 or 5% (v/v) HS, 2.5 mM L-glutamine and 0.1 mg/ml gentamycin.

2.3.2 Isolation of cells

One-day-old rat pups were decapitated, the cerebral cortices removed under aseptic conditions, and placed in a culture dish containing dissection medium. With the aid of a dissecting microscope, the cortices were carefully cleared of the olfactory bulbs, basal ganglia, hippocampal formations and meninges and placed in a sterile universal tube containing 6 ml dissection medium. The dissected neopalia were then cut into 0.5 mm^3 cubes by two passes (at 90^0) in a McIlwain tissue chopper. The chopped tissue was transferred to a sterile glass filter containing 80 μm nylon mesh and the filtrate collected in a sterile beaker. The material was washed through the filter with 20% HS-DMEM culture medium to give a final volume of 3 ml/brain. The filtrate was then passed through a sterile needle (BD Microlance 21G 0.8 x 40) three times to disperse cells. The volume of the resulting suspension was adjusted appropriately with 20% HS-DMEM culture medium. For [^{14}C]-GABA and [^{14}C]-glutamate uptake, and GABA-T activity studies, cells were plated onto 60 x 15 mm Falcon Primaria culture dishes allowing a 3 ml aliquot of adjusted cell suspension per culture dish in a ratio of 1 brain to 3 dishes. For GS activity studies, cells were plated onto 24 well Sarstedt tissue culture dishes allowing a 500 μl aliquot of adjusted cell suspension per well in a ratio of 1 brain to each 24 well dish. For GFAP staining, cells were plated onto glass coverslips (Chance No.1 13 mm round) coated with 150 μl of poly-L-lysine solution and placed in 24 well Sarstedt tissue culture dishes allowing a 500 μl aliquot of adjusted cell suspension per well in a ratio of 1 brain to each 24 well dish .

2.3.3 Culture maintenance

The cultures were maintained at 37^{0}C in an environment of 95% air / 5% CO_2 with a humidity of ≥90%. The culture medium 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. Once the cells reached confluence (around day 14 in culture), the medium was supplemented with 0.25 mM cAMP to induce cell differentiation. Cultures were ready for use seven days after the addition of cAMP (around day 21). At this stage, the astrocytes were identified as positive for glial fibrillary acidic protein (GFAP) (section 2.5).

2.4 PRIMARY CULTURE OF HUMAN ASTROCYTES FROM FOETAL POST-MORTEM AND ADULT SURGICAL TISSUE

This method was a modification of the method of O'Connor and co-workers (1998).

2.4.1 Culture medium

Media were prepared under sterile conditions, and were stored sterile at 4^{0}C for up to five days. Isolation medium consisted of DMEM-F12 supplemented with 100 I.U./ml penicillin, 100 μg/ml streptomycin, 0.1 mg/ml gentamycin and 25 μg/ml fungizone. Plating medium consisted of DMEM-F12 supplemented with 15% (v/v) FCS, 20 mM D-glucose, 100 I.U./ml penicillin, 100 μg/ml streptomycin, 0.1 mg/ml gentamycin and 25 μg/ml fungizone, and the solution was filter sterilised through a 0.2 μm pore filter prior to use.

2.4.2 Enzymatic digestion solutions

Each of these solutions was prepared fresh and filter sterilised through a 0.2 μm pore filter immediately prior to use. Solution 1 consisted of DMEM-F12 containing 0.125% (v/v) trypsin, 20 μg/ml DNase, 20 U/ml papain and 0.02% (v/v) collagenase. Solution 2 consisted of DMEM-F12 containing 0.05% (v/v) trypsin, 20 μg/ml DNase, 0.02% (v/v) EDTA and 0.02% (v/v) collagenase. Solution 3 consisted of DMEM-F12 containing 0.004% (v/v) DNase, 0.05% (v/v) soya bean trypsin inhibitor, and 0.16% (v/v) BSA.

2.4.3 Human brain tissue

Adult brain tissue was obtained from the temporal lobes of patients undergoing surgery for intractable epilepsy. Foetal tissue was obtained from the temporal lobes of spontaneously aborted foetuses (16 - 24 weeks gestation). Samples ranging from 7 to 36 hours post-mortem were received and could be successfully cultured. Approval to work with the adult surgical and foetal post-mortem tissue was granted from the Ethics committees of the Southern General Hospital NHS Trust, Glasgow and Yorkhill NHS Trust, Glasgow, respectively.

2.4.4 Isolation of cells

All brain samples were collected in 10 ml of sterile isolation medium and kept on ice. Adult and foetal tissue were treated in an identical manner, and unless otherwise stated, all brain tissue was maintained on ice throughout the entire procedure. The tissue was cleaned of blood vessels, meninges and other debris, and cut into 1 mm^3 cubes by two passes (at 90^0C) in a McIlwain tissue chopper. The chopped tissue was transferred to a sterile tube containing 10 ml of fresh isolation medium, mixed gently to

wash, and centrifuged (Heraeus Biofuge 15R) at 560 x *g* for 5 minutes at 4^0C. The supernatant was discarded and the tissue was then subjected to a series of enzymatic digestions. The tissue was first digested for 20 minutes at 37^0C in 2 ml of Solution 1. Following a 20 minute incubation the solution was centrifuged at 560 x *g* for 5 minutes at 4^0C and the supernatant decanted into a sterile tube containing 10 ml of plating medium. The remaining pellet was digested for a further 20 minutes at 37^0C in 2 ml of Solution 1 and centrifuged at 560 x *g* for 5 minutes at 4^0C. The supernatant was decanted into a second sterile tube containing 10 ml of plating medium. The resulting pellet was digested for 20 minutes at 37^0C in 2 ml of Solution 2, centrifuged at 560 x *g* for 5 minutes at 4^0C, and the supernatant decanted into a third sterile tube containing 10 ml of plating medium. The remaining pellet underwent a final digestion for 5 minutes at 37^0C in 1 ml of Solution 3. The digested pellet was then gently triturated 15 times using a sterile glass pipette and centrifuged at 560 x *g* for 5 minutes at 4^0C. The supernatant from this digestion was pooled with those from the previous three digestions and centrifuged at 315 x *g* for 10 minutes at 4^0C. The supernatant was discarded and the pellet resuspended in 2 ml of isolation medium. 20µl of the resuspended cells were diluted in 30 µl serum free DMEM plus 50 µl trypan blue and vortex mixed. 10 µl of the diluted cell suspension was counted in a haemocytometer and the total number of cells was calculated. The volume of cell suspension was adjusted with plating medium to give a density of 80, 000 cells/cm^2 in a final volume of 200 µl per well in Primaria 24 well culture dishes. The cells were left in the incubator for 1 - 4 hours to allow for attachment to wells, then the existing medium was aspirated and cultures re-fed with 500 µl fresh plating medium.

2.4.5 Culture maintenance

The cultures were maintained at 37^{0}C in an environment of 95% air / 5% CO_2 with a humidity of ≥90%. The culture medium was replaced every 3 - 4 days throughout, until the cells reached confluence (around day 21). At this stage, the astrocytes were identified as positive for GFAP (section 2.5).

2.5 GLIAL FIBRILLARY ACIDIC PROTEIN IMMUNOFLUORESCENCE STAINING

2.5.1 Paraformaldehyde solution

A 4% paraformaldehyde solution was prepared by adding 4 g of paraformaldehyde to 100 ml of sterile PBS and heated to 56^{0}C. A few drops of 1 M NaOH were added to dissolve the paraformaldehyde. The solution was placed on ice to cool and the pH adjusted to 7.4 with 1 M NaOH. This was then aliquoted and stored at -20^{0}C for up to six months.

2.5.2 Staining medium

Staining medium consisting of 20 ml new-born calf serum, 50 ml 10X HBSS, 2.5 ml of 10% (v/v) sodium azide solution, 2.6 g HEPES sodium salt, 2.38 g HEPES and 427.5 ml of sterile distilled water was prepared and stored at 4^{0}C for three months.

2.5.3 Staining procedure

Astrocytes which had been grown on glass coverslips (section 2.3.2) were removed from 24 well culture dishes and placed on a petri dish to dry. A drop of the 4% paraformaldehyde solution was added to each coverslip and left for 30 minutes at room

temperature to fix the cells. The coverslips were then rinsed in staining medium (by carefully holding the edge of the coverslips with watchmaker's forceps and dipping into a vial of the medium). The primary antibody (Rabbit Anti Cow Polyclonal GFAP) was diluted 1 in 100 and 50 µl was added to each coverslip and left at room temperature for one hour, in a petri dish with the lid on to prevent drying. This antibody was removed by rinsing thoroughly in staining medium. The secondary antibody which carried the fluorescent tag (Goat Anti-Rabbit IgG γ heavy chain specific RITC labelled) was then diluted 1 in 100 and 50 µl added to each coverslip and left as before for one hour. At the end of the incubation, the antibody was removed by rinsing in staining medium, followed by rinsing in distilled water (to prevent crystal formation). Each coverslip was then mounted face-down onto a glass slide containing a drop of Citifluor AF1 antifadant solution and left to dry.

2.5.4 Examination of slides

The stained astrocytes were stored at 4^0C until required for examination (within 3 months of staining) using a fluorescent microscope (Zeiss Axioplan with epi-illumination and phase contrast optics) and selected areas of the coverslips were photographed (MC 100 camera control unit and 35 mm film cassette).

2.6 DETERMINATION OF PROTEIN CONCENTRATION IN CULTURED RAT AND HUMAN ASTROCYTES AND MOUSE BRAIN

Protein concentrations were determined by the BIORAD method, which relies on the colour change of Coomassie Brilliant Blue G-250 dye. A duplicate set of standards were prepared over the range 0.25 - 2 µg/ml with BSA. Samples of unknown protein

concentration were diluted within this range. BIORAD protein assay dye reagent was diluted 1 : 1 with water and an equal volume was added to standards and samples. Tubes were vortex mixed, incubated for 5 minutes at room temperature, then read at 595 nm in a spectrophotometer (Dynex MR5000). Results were corrected for dilution and expressed in mg/ml.

2.7 [^{14}C]-GABA UPTAKE INTO CULTURED RAT AND HUMAN ASTROCYTES

This method was a modification of the methods of Larsson and co-workers (1981) and Yu and colleagues (1984).

2.7.1 Balanced salt solution (BSS)

The BSS used throughout the [^{14}C]-GABA uptake experiments consisted of 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 five days.

2.7.2 [^{14}C]-GABA uptake procedure

The cultures were removed from the incubator and the existing medium aspirated. Individual plates (rat astrocytes) or wells (human astrocytes) were washed twice (2 x 2 ml plates; 2 x 200 µl wells) with BSS (which had been warmed to 37^{0}C prior to use), and 2 ml (plates) or 200 µl (wells) of BSS containing the appropriate drug solution was added to each culture. Control groups received BSS alone. All cultures were returned to the incubator for one hour. After the incubation period a further 1 ml (plates) or 100 µl (wells) of BSS (with the appropriate drug or control treatment)

containing 150 μM [^{14}C]-GABA (specific activity = 1 mCi / mmol) was added to each plate or well. The cultures were incubated for a further five minutes, the BSS was aspirated and cultures washed (5 x 2 ml plates; 5 x 200 μl wells) with fresh BSS. Cells were removed from plates by scraping in 1 ml of ice cold 1 M NaOH, and from wells by scraping in 250 μl of ice cold I M NaOH. Aliquots were taken for protein concentration determination by the BIORAD method (section 2.6) and liquid scintillation counting in 6 ml of Picofluor 40 scintillation fluid.

2.7.3 Liquid scintillation counting

Samples were counted for ^{14}C in a liquid scintillation counter (Canberra Packard 2000CA TRI-CARB), which had previously been programmed to convert counts per minute (cpm) into disintegrations per minute (dpm), based on the counting efficiency. Counting efficiency in liquid scintillation counting is dependent upon the composition of the sample / scintillator complex. Efficiency may vary due to phenomena known as "quenching" which interfere either with the scintillation process itself or with the transmission of light to the photomultiplier.

The most commonly used standardisation method in liquid scintillation counting is the external standards ratio, whereby the sample is irradiated with a high activity external gamma source to superimpose a Compton spectrum on to that of the sample isotope. The ratio of the net Compton count rate can then be calculated by subtracting the sample counts from the gamma source counts. The counter is programmed by preparing a series of reference standards with the same composition as the samples and "quenched" to different degrees, to cover the quench range of the experimental samples. A calibration curve relating % efficiency to the external standards ratio was

constructed and stored in the counter. Experimental samples were then automatically converted from cpm to dpm using the formula : $dpm = \frac{\text{Net cpm} \times 100}{\% \text{ Efficiency}}$

The radioactive content of individual cultures was compared to known radioactive standards and the results quantified by the relation of GABA uptake to the protein concentration and expressed as pmol / minute / mg protein.

2.8 [^{14}C]-GLUTAMATE UPTAKE INTO CULTURED RAT ASTROCYTES

This method was a modification of the method of Yu and colleagues (1984).

2.8.1 [^{14}C]-Glutamate uptake procedure

BSS was prepared as described in section 2.7.1. The cultures were removed from the incubator and the existing medium aspirated. Individual plates were washed twice (2 x 2 ml) with BSS (which had been warmed to 37^{0}C prior to use) and 2 ml of BSS containing the appropriate drug solution was added to each culture. Control groups received BSS alone. All cultures were returned to the incubator for one hour. After the incubation period a further 1ml of BSS (with the appropriate drug or control treatment) containing 150 μM [^{14}C]-glutamate (specific activity = 1 mCi / mmol) was added to each plate or well. The cultures were incubated for a further five minutes, the BSS was aspirated and cultures washed (5 x 2 ml) with BSS. Cells were removed from plates by scraping in 1 ml of ice cold 1 M NaOH. Aliquots were taken for protein concentration determination by the BIORAD method (section 2.6) and liquid scintillation counting (section 2.7.3) in 6 ml of Picofluor 40 scintillation fluid. The radioactive content of individual cultures was compared to known radioactive

standards and the results quantified by the relation of glutamate uptake to the protein concentration and expressed as pmol / minute / mg protein.

2.9 DETERMINATION OF GABA-TRANSAMINASE ACTIVITY IN CULTURED RAT ASTROCYTES AND IN MOUSE BRAIN

This method was a modification of the methods of White and Sato (1978) and Larsson and colleagues (1986).

2.9.1 Reagents

All solutions were prepared using deionised water. An EDTA buffer was prepared weekly and stored at 4^0C. It consisted of 0.1 mM EDTA, 0.5 mM DTT, and 0.1 mM KH_2PO_4. 0.2 mM PLP was added daily as required and the pH was adjusted to 8 with 1 M NaOH. A [^{14}C]-GABA incubation medium, prepared monthly and stored at -20^0C, consisted of 2.7 mM GABA (specific activity = 0.37 mCi / mmol), 1.8 mM EDTA and 200 mM KH_2PO_4 with the pH adjusted to 8 with 1 M NaOH.

2.9.2 Preparation of ion exchange resin

Dowex AG50W-X8 resin was pre-washed five times with deionised water before use.

2.9.3 Sample preparation

Whole mouse brains, which had been stored at -70^0C for up to one month, were thawed and homogenised (polytron PT 1200) in 4 volumes (v/w) of EDTA buffer. Cultured rat astrocytes which had been scraped and stored at -70^0C in 500 µl EDTA buffer, were freeze / thawed three times then homogenised. All samples were then centrifuged (Heraeus Biofuge 15R) at 600 x *g* for 20 minutes at 4^0C and the

supernatant decanted and its protein content determined by the BIORAD method (section 2.6). The remaining supernatant was adjusted with EDTA buffer to give a final protein concentration of 1 mg/ml for whole brain, or 0.2 mg/ml for astrocyte cultures.

2.9.4 GABA-transaminase assay

To a 50 µl volume of the adjusted supernatant, 25 µl of 0.68 mM α-KG and 25 µl of the [^{14}C]-GABA incubation medium was added. Assays were performed in duplicate with a blank assay included for each sample by replacing the α-KG with 25 µl water. Samples were vortex mixed and incubated for 60 minutes at 37^{0}C. The reaction was terminated by the addition of 10 µl 2 M HCl. The incubated samples were mixed and applied to single use ion exchange columns (9 inch glass Pasteur pipettes plugged with a glass bead) containing 0.5 x 3 cm Dowex resin. Radioactive products were eluted directly into glass scintillation vials using 3 x 0.5 ml aliquots of deionised water. Twelve ml of Picofluor 40 scintillation fluid was added to each vial and radioactive content of the samples counted in a liquid scintillation counter (section 2.7.3). The radioactive content of samples was compared to known radioactive standards, corrected for background and blank sample counts, and related to protein content and reaction time. GABA-T activity was expressed as nmol / minute / mg protein.

2.10 DETERMINATION OF GLUTAMINE SYNTHETASE ACTIVITY IN CULTURED RAT ASTROCYTES AND MOUSE BRAIN

This method was a modification of the method of Pishak and Phillips (1979).

2.10.1 Reagents

All solutions were prepared using deionised water. Imidazole-HCl buffers were prepared monthly and stored at 4^0C. The 100 mM and 5 mM buffers consisted of 100 mM and 5 mM imidazole-HCl, respectively, and were adjusted to pH 7.2 with 1 M NaOH. A test assay solution consisting of 4 mM 2-mercaptoethanol and 40 mM ATP in 100 mM imidazole-HCl buffer was prepared daily. A blank assay solution was prepared daily by omitting ATP from the test solution. A [^{14}C] L-glutamic acid incubation medium, prepared monthly and stored at -20^0C, consisted of 40 mM L-glutamic acid (specific activity = 0.12 mCi / mmol), 60 mM $MgCl_2$, 16 mM NH_4Cl and 4 mM ouabain in 100 mM imidazole-HCl buffer.

2.10.2 Preparation of ion-exchange resins

The Amberlite CG-50 hydrogen form resin was pre-washed five times with 1 M NaOH, then five times with 1 M HCl, followed by five washes with deionised water before use. The Dowex AG1-X8 acetate form resin was pre-washed five times with 1 M acetic acid, followed by five washes with deionised water before use.

2.10.3 Sample preparation

Whole mouse brains, which had been stored at -70^0C for up to three months, were thawed and homogenised (polytron PT 1200) in 4 volumes (v/w) of 5 mM imidazole-HCl buffer. Cultured rat astrocytes which had been scraped and stored at -70^0C in 250

µl 5 mM imidazole-HCl buffer, were freeze / thawed three times then vortex mixed. All samples were then centrifuged (Heraeus Biofuge 15R) at 800 x *g* for 10 minutes at 4^0C and the supernatant decanted and its protein content determined by the BIORAD method (section 2.6). The remaining supernatant was adjusted with 5 mM imidazole-HCl buffer to give a final protein concentration of 2 mg/ml for whole brain, or 0.1 mg/ml for astrocyte cultures.

2.10.4 Glutamine synthetase assay

To a 50 µl volume of the adjusted supernatant, 25 µl of the test assay solution and 25 µl of the [^{14}C] L-glutamic acid incubation medium was added. Assays were performed in duplicate with a blank assay included for each sample. Samples were vortex mixed and incubated for 15 minutes at 37^0C. The reaction was terminated by the addition of 1 ml of ice cold deionised water. The incubated samples were mixed and applied to single use tandemly arranged chromatography columns (BIORAD). The first column contained 0.8 x 3.5 cm Dowex resin and was placed directly over a second column containing 0.8 x 3.5 cm Amberlite resin. One ml of ice cold deionised water was added to each column, but was not collected. Newly synthesised glutamine was eluted into plastic scintillation vials using 5 x 1 ml aliquots of deionised water. Ten ml of Ultima Gold XR scintillation fluid was added to each vial and the radioactive content of the samples determined in a liquid scintillation counter (section 2.7.3). The radioactive content of samples was compared to known radioactive standards, corrected for background and blank sample counts, and related to protein content and reaction time. GS activity was expressed as nmol / minute / mg protein.

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

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

2.11.1 Equipment

All HPLC analyses were performed at room temperature on a Beckman Ultrasphere 25 cm x 4.6 mm internal diameter, 5 μm reversed phase column. The system consisted of a Shimadzu LC-10AT pump, a Shimadzu SIL-9A auto-injector and a Perkin-Elmer LS-5 fluorescence spectrophotometer. The excitation and emission wavelengths were 330 and 440 nm, respectively, with slit widths set at 15 and 20 nm, respectively. Data was recorded and integrated on a Jones JCL6000 chromatography data system.

2.11.2 Reagents

Stock solutions of amino acid standards (GABA, glutamate and glutamine) and internal standards (homoserine and D,L-norvaline) - 1 mg/ml in distilled water, were prepared monthly and stored at -20^{0}C. All standards were diluted daily as required. A borate buffer was prepared monthly (and stored at room temperature), by adjusting 0.5 M boric acid to pH 9.5 with 3 M NaOH. The derivatization reagent (OPA - 3-MPA) was prepared weekly (and stored dark at 4^{0}C) by dissolving 50 mg OPA in 4.5 ml methanol and 0.5 ml borate buffer then adding 50 μl 3-MPA.

2.11.3 Mobile phase

GABA was determined in a mobile phase consisting of 60 : 40 (v/v) 0.2 M acetate buffer (pH 3.7, containing 100 mg/l EDTA) / acetonitrile. Acetate buffer (0.2 M) was

prepared by diluting 11.5 ml glacial acetic acid to 1 litre with water, adding 100 mg EDTA and adjusting the pH to 3.7 with 3 M NaOH. Glutamate and glutamine were determined simultaneously in a mobile phase consisting of 80 : 20 (v/v) 0.57 M acetate buffer (pH 3.7, containing 100 mg/l EDTA) / acetonitrile. Acetate buffer (0.57 M) was prepared by diluting 32.8 ml glacial acetic acid to 1 litre with water, adding 100 mg EDTA and adjusting the pH to 3.7 with 3 M NaOH. Flow rates were 1 ml/min throughout.

2.11.4 Calibration

Standard curves were constructed for GABA (2 - 20 μg/ml), glutamate (15 - 150 μg/ml) and glutamine (5 - 50 μg/ml) and were found to be linear ($r \geq 0.970$) in all cases. The limits of detection in a 50 μl sample were found to be 5 ng/ml, 25 ng/ml and 12.5 ng/ml for GABA, glutamate and glutamine, respectively. Intra- and inter-assay variations were calculated at 2.8% and 7.9% for GABA (5 μg/ml), 4.7% and 9.2% for glutamate (30 μg/ml) and 4.1% and 8.5% for glutamine (10 μg/ml), respectively.

2.11.5 Extraction

Mouse brain samples (previously stored at -70^{0}C) were weighed and homogenised (polytron PT 1200) in 10 volumes (v/w) of 1% perchloric acid. Samples were then centrifuged (Heraeus Biofuge 15R) at 1440 x *g* for 5 minutes at 4^{0}C and the supernatant decanted and diluted 1 in 10 with water prior to derivatization.

2.11.6 Derivatization

A 50 µl aliquot of the diluted supernatant was reacted with 400 µl methanol, 400 µl borate buffer and 100 µl OPA - 3-MPA solution. 50 µl of 1 µg/ml D,L-norvaline was added to samples as an internal standard for GABA samples, and 50 µl of 1 µg/ml homoserine was added to samples as an internal standard for glutamate and glutamine. Reaction mixtures were vortex mixed and allowed to stand at room temperature for 5 minutes prior to a 10 µl injection onto the column. Amino acid concentrations were calculated by comparison of peak height ratios of analyte to internal standard, quantified in relation to wet weight of tissue, and expressed as µg/g.

2.12 DETERMINATION OF GLUTAMIC ACID DECARBOXYLASE ACTIVITY BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

This method was a modification of the methods of Kochhar and co-workers (1989) and Wolf and Klemisch (1991).

2.12.1 Reagents

A sodium phosphate-AET buffer was prepared weekly (and stored at 4^0C) and consisted of 0.1 M Na_2HPO_4 and 1 mM AET. The buffer was adjusted to pH 7 with 0.1 M NaH_2PO_4. An incubation medium was prepared daily and consisted of 50 mM L-glutamic acid, 250 µM PLP, 0.4% 2-mercaptoethanol and 57 µM gabaculline in sodium phosphate-AET buffer.

2.12.2 Sample preparation

Mouse brain samples (previously stored at -70^{0}C for up to 1 month) were thawed and homogenised (polytron PT 1200) in 10 volumes (v/w) of sodium phosphate-AET buffer. Samples were then centrifuged (Heraeus Biofuge 15R) at 1440 x *g* for 10 minutes at 4^{0}C, the supernatant decanted and its protein content determined by the BIORAD method (section 2.6). Remaining supernatant was then adjusted to 1 mg/ml with sodium phosphate-AET buffer.

2.12.3 Glutamic acid decarboxylase assay

100 µl of incubation medium was added to each of two 100 µl aliquots of adjusted supernatant per sample. The reaction in one (blank) was terminated immediately by the addition of 100 µl 1% perchloric acid, while the other (test) was allowed to continue for 30 minutes at 37^{0}C. This was also terminated by the addition of 100 µl 1% perchloric acid. Terminated blank and test reaction mixtures were stored at -20^{0}C until required for assay. Samples were thawed and centrifuged (Heraeus Biofuge 15R) at 1440 x *g* for 10 minutes at 4^{0}C and the resulting supernatant diluted 1 in 10 with water prior to GABA content measurement by HPLC (section 2.11). GAD activity was calculated by subtraction of the blank GABA concentration from the test GABA concentration, giving a value for GABA production during the incubation period. Results were quantified in relation to reaction time and protein concentration, and expressed as nmol / min / mg protein.

2.13 PENTYLENETETRAZOL TESTING

This test was performed by the method of Loscher & Schmidt (1988). PTZ was prepared for injection in 0.9% saline. 85 mg/kg PTZ was warmed to 37^0C and 0.3 ml administered subcutaneously (s.c.) in the back of the neck. The time to the first generalised seizure with loss of the righting reflex was recorded in individual mice. A cut-off time of 15 minutes was chosen. Animals were sacrificed immediately after the seizure or after the 15 minute cut-off time if no seizure had occurred.

2.14 DATA ANALYSIS AND STATISTICAL METHODS

Statistical analysis was performed using MINITAB for Windows statistical package (version 10.1) on an Elonex PC-5120/1 microcomputer. All results were calculated as the percentage of mean control values. Group results were then expressed as the mean percentage ± the standard error of the mean (S.E.M.). Drug treated groups were compared to control using one-way analysis of variance with Dunnett correction for multiple comparisons.

CHAPTER THREE

EFFECTS OF ANTIEPILEPTIC DRUGS ON THE TRANSPORT OF GABA AND GLUTAMATE AND THE METABOLISM OF GABA IN PRIMARY CULTURES OF RAT CORTICAL ASTROCYTES

3.1 INTRODUCTION

3.1.1 Astrocytes

Astrocytes account for more than half of the glial cell population in the brain and are estimated to comprise as much as 20 to 50% of the total volume in some brain areas. They are up to ten times more numerous in the CNS than neurones (Kimelberg, 1983; Kimelberg and Norenberg, 1989). Astrocytes were once regarded as playing only structural and supporting roles in the nervous system, but are now widely accepted to be responsible for the maintenance of the extracellular environment in the brain, regulating the microenvironment around the synaptic cleft and neurones (Montgomery, 1994). Failure in this regulation by astrocytes has been proposed to be a significant factor in the aetiology of epilepsy (Tiffany-Castiglioni and Castiglioni, 1986; Castiglioni *et al*, 1990; Nilsson *et al*, 1992).

3.1.2 Uptake and metabolism of neurotransmitters in astrocytes

There is substantial evidence to suggest that the principal mode of inactivation of amino acid neurotransmitters released from nerve endings is by reuptake (Snyder *et al*, 1970; Iversen, 1972; De Feudis, 1975). Studies using synaptosomes, brain slices and cultured cells have shown that the inhibitory and excitatory amino acid neurotransmitters are taken up by high affinity transport systems (Borg *et al*, 1979; Hertz, 1979; Schousboe, 1981; Fonnum, 1984). Both neurones and glial cells have been demonstrated to be involved in the uptake of amino acids (Hamberger, 1971; Henn and Hamberger, 1971; Iversen and Kelly, 1975). It is thought that GABA is released predominantly from neurones (Hertz and Schousboe, 1980) and studies on its transport have shown that most synaptically released GABA is reaccumulated into

presynaptic nerve endings, and that a smaller fraction is taken up into surrounding astrocytes. In contrast to this, the rate and capacity of astrocytic accumulation of glutamate is significantly higher than of the uptake into neurones (Schousboe *et al*, 1992). GABA-T, the only GABA metabolizing enzyme of physiological importance, is located intracellularly with approximately equal activities in neurones and glial cells (Schousboe, 1981). The intracellular location of GABA-T indicates the importance of the uptake of GABA into cells in the termination of its actions (Hertz and Schousboe, 1980). Glutamate which is taken up into neurones is largely converted to GABA via the neuronal-specific enzyme GAD, and that which is taken up into astrocytes is metabolized to glutamine by the glial-specific enzyme GS (Schousboe, 1981).

3.1.3 History of brain cell culture

The first nerve tissue cultured successfully was by Harrison in 1907, where a small portion of early frog neural tube was grown in clotted lymph as an explant culture. The earliest documented primary cultures enriched in astroglial cells were obtained by Shein (1965), and later by Varon and Raiborn (1969). However, it was Booher and Sensenbrenner (1972) who were the first to use the developmental characteristics of different neural cell types to achieve separation between cultures consisting almost exclusively of glial cells and those highly enriched in neurones. Today, virtually all of the techniques available for the preparation of astrocyte cultures are based on modifications of this method. Obtaining cultures enriched in astrocytes is dependent on selecting a time in the development of a particular brain region where neuronogenesis is complete and there are a large number of proliferating immature astroglial cells. In the rat and mouse this stage is new-born, where the neopallium (cortex and underlying intermediate and subventricular zones) contains many proliferating astroglial

precursors (Juurlink and Hertz, 1985; 1992). In the human brain, immature glia can be identified by GFAP in the spinal cord by eight weeks, and in the cerebrum by ten weeks gestation (Rutka *et al*, 1987).

3.2 AIMS

GABA and glutamate are the major inhibitory and excitatory, respectively, neurotransmitters in the brain and the effect of AEDs on these systems is of particular importance in epilepsy research (discussed in chapter 1). Primary cultures of astrocytes from the cerebral cortex have been proposed as a model system for studies on the transport of GABA, glutamate and glutamine (Schousboe *et al*, 1983a). To date, the precise mechanisms of action of many of the frequently used AEDs remains to be elucidated. PB, PHT, CBZ, SVP, LTG, FBM, GBP, TPM and DGR would all appear to possess multiple mechanisms of action (Hannah *et al*, 1995; Dichter and Brodie, 1996; Leach *et al*, 1997b). In contrast, VGB and TGB are two new AEDs which are reported to exert their anticonvulsant actions via single and specific effects on the GABA system, VGB by an irreversible inhibition of the enzyme GABA-T, and TGB by blockade of neuronal and glial GABA uptake (Lippert *et al*, 1977; Nielsen *et al*, 1991). However, a recent study performed in our laboratory employing primary cultures of rat cortical astrocytes suggested that VGB may additionally block the uptake of GABA as well as inhibiting GABA-T (Leach *et al*, 1996). Despite numerous studies, the precise mechanism of action of the experimental anticonvulsant LEV (Margineanu and Wulfert, 1995; Noyer *et al*, 1995; Loscher *et al*, 1996) remains to be characterized. The aim of this study was, therefore, to examine the actions of these established, new, and experimental AEDs on the uptake and metabolism of GABA. In addition, validation of the glutamate uptake assay was performed and the effects on glutamate

uptake were also examined. The effects of AEDs on the metabolism of glutamate is not included here as it will be examined in detail in chapter 4.

3.3 EXPERIMENTAL PROTOCOLS AND RESULTS

3.3.1 Effects of new and established antiepileptic drugs on the uptake of [^{14}C]-GABA into primary cultures of rat cortical astrocytes

EXPERIMENTAL PROTOCOL: Rat brain tissue was isolated and cultured as described in section 2.3. Astrocytes were identified as positive for GFAP, and photographed as described in section 2.5 (photograph 1). For each of the 12 drug experiments, cultures were randomly separated into six (SVP, VGB, GBP, TGB and LEV) or seven (PB, PHT, CBZ, LTG, FBM, TPM and DGR) groups (n = 8 / group), and exposed to the following drugs in BSS (stock solutions of CBZ, PHT and FBM were dissolved in 500 µl ethanol prior to dilution with BSS):

PB:- 1, 3, 10, 30, 100, 300 and 1000 µM

PHT:- 1, 3, 10, 30, 100, 300 and 1000 µM

CBZ:- 1, 3, 10, 30, 100, 300 and 1000 µM

SVP:- 50, 250, 500, 1250, 2500 and 5000 µM

VGB:- 10, 50, 100, 250, 500 and 1000 µM

LTG:- 0.3, 1, 3, 10, 30, 100 and 300 µM

FBM:- 3, 10, 30, 100, 300, 1000 and 3000 µM

GBP:- 10, 50, 100, 250, 500 and 1000 µM

TPM:- 1, 3, 10, 30, 100, 300 and 1000 µM

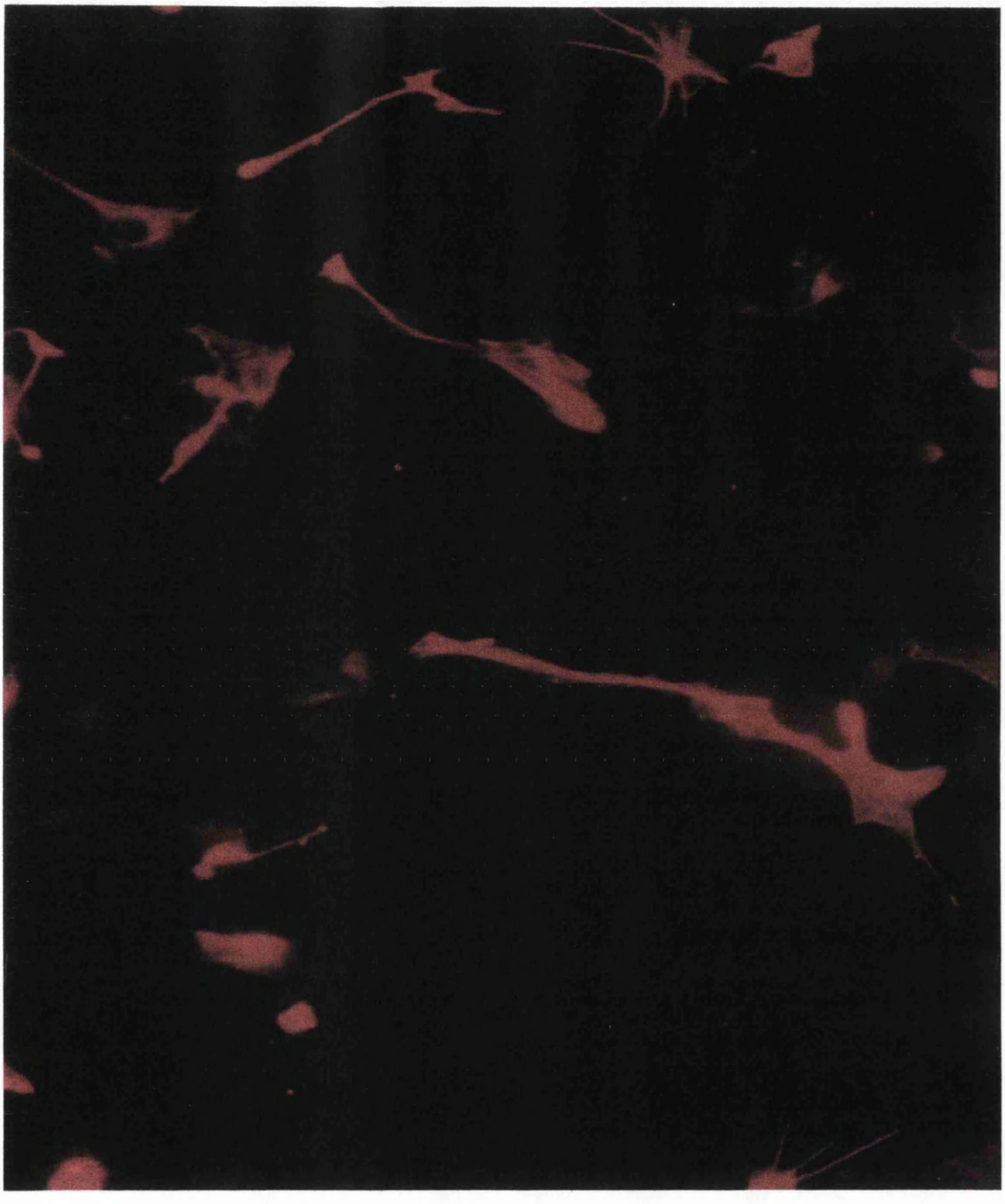
TGB:- 10, 50, 100, 250, 500 and 1000 nM

LEV:- 10, 50, 100, 250, 500 and 1000 μM

DGR:- 1, 3, 10, 30, 100, 300 and 1000 μM

Drug concentrations used were relative to therapeutic doses, and were based on effective concentrations stated in the literature. For each drug experiment, a control group (n = 8) was exposed to BSS alone. After a one hour incubation, cells were assayed for GABA uptake as described in section 2.7.

RESULTS: All results are summarised in table 1. SVP (50, 250, 500, 1250, 2500 and 5000 μM; figure 4), VGB (100, 250, 500 and 1000 μM; figure 5), FBM (3, 10, 30, 100, 300, 1000 and 3000 μM; figure 7) and TGB (10, 50, 100, 250, 500 and 1000 nM; figure 10) all significantly ($P < 0.05$) reduced the uptake of GABA into primary cultures of rat cortical astrocytes. PB (figure 1), PHT (figure 2), CBZ (figure 3), LTG (figure 6), GBP (figure 8), TPM (figure 9), LEV (figure 11) and DGR (figure 12) were found to have no significant effect on the transport of GABA.



PHOTOGRAPH 1. Rat cortical astrocytes in primary culture (day 21) stained with glial fibrillary acidic protein (GFAP) and examined using a fluorescent microscope (x 450).

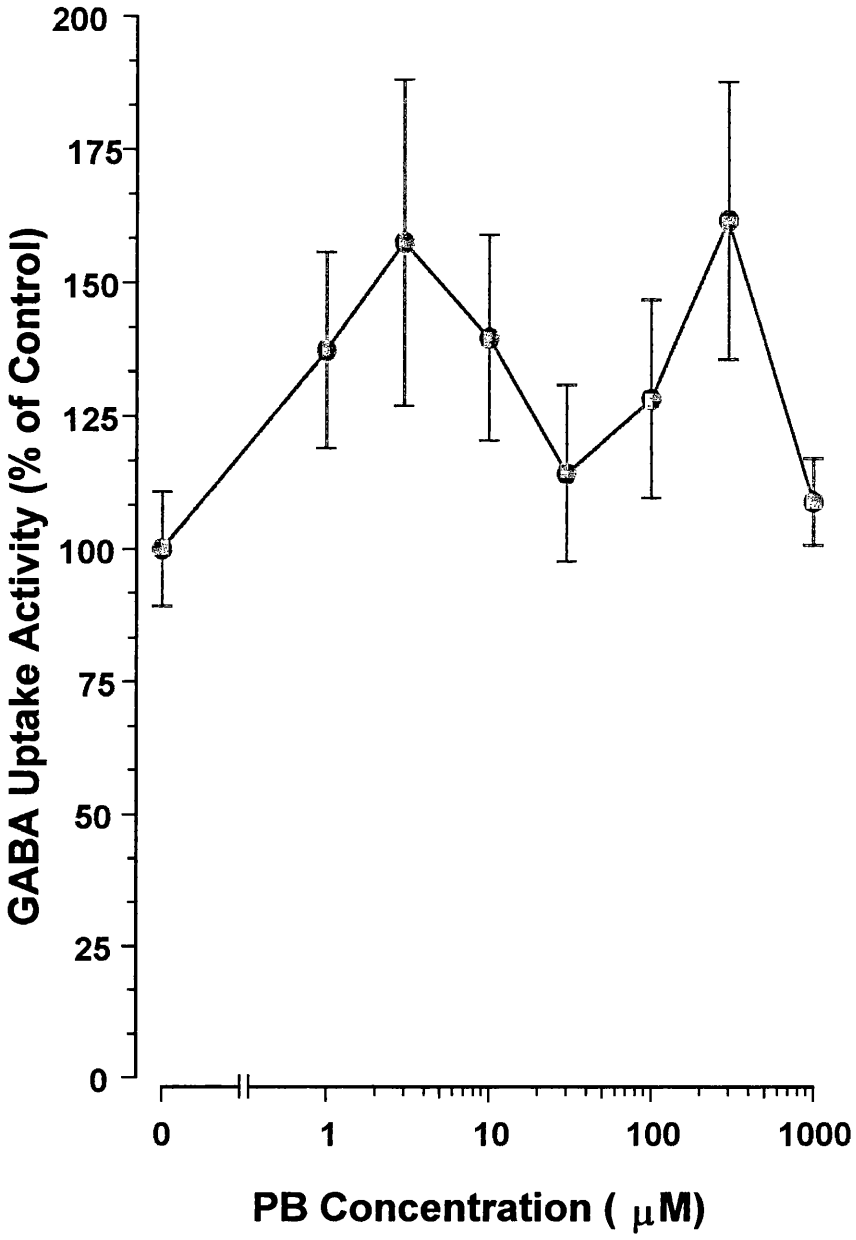


FIGURE 1. Effect of phenobarbitone (PB; 0 - 1000 μM) on the uptake of GABA into primary cultures of rat cortical astrocytes. Results (n = 8) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

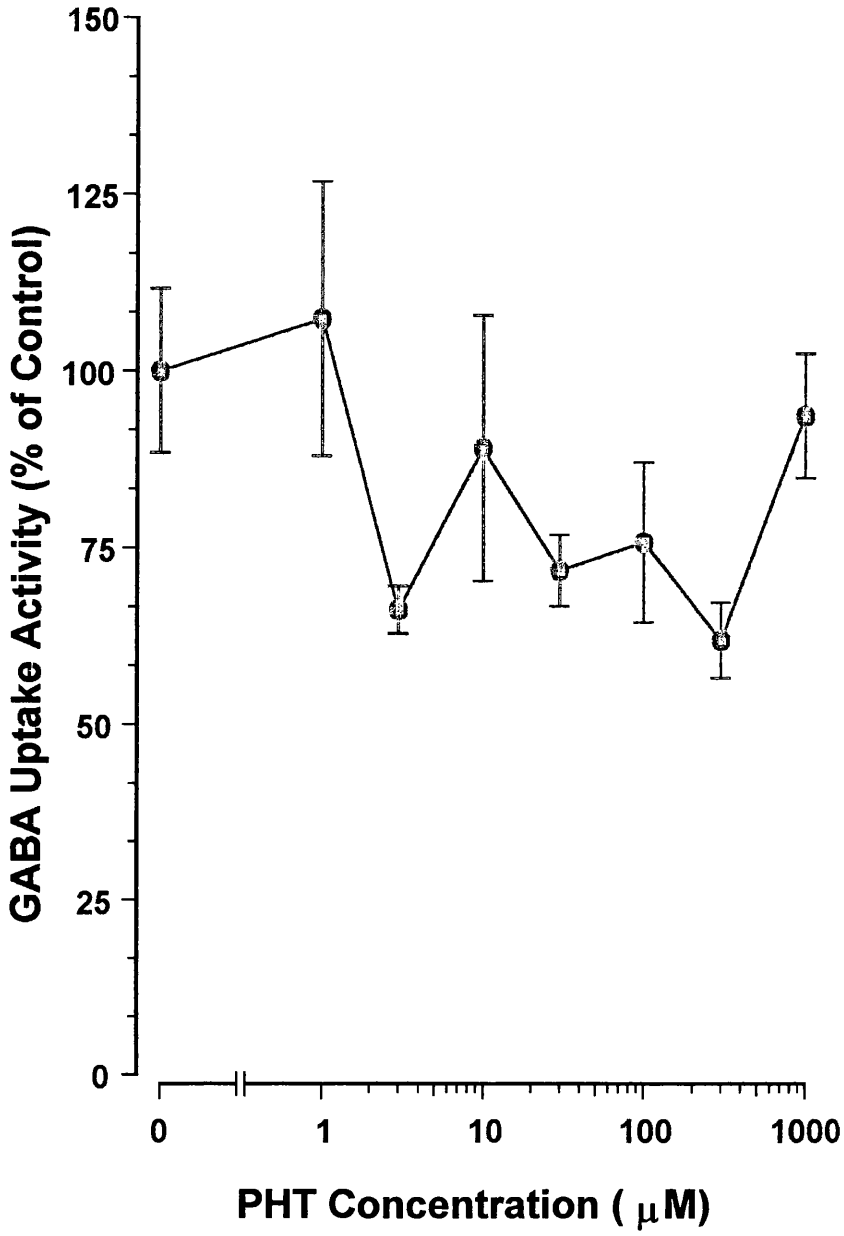


FIGURE 2. Effect of phenytoin (PHT; 0 - 1000 μM) on the uptake of GABA into primary cultures of rat cortical astrocytes. Results (n = 8) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

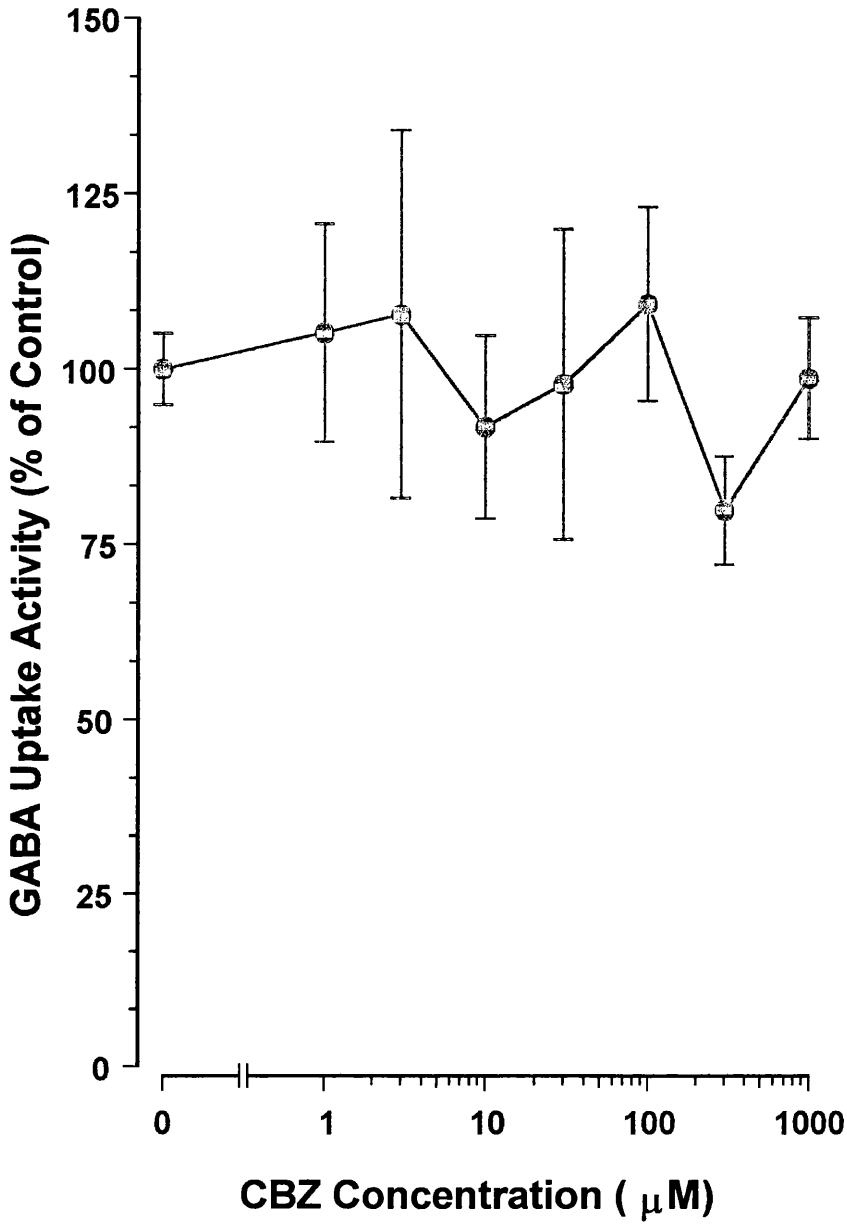


FIGURE 3. Effect of carbamazepine (CBZ; 0 - 1000 μM) on the uptake of GABA into primary cultures of rat cortical astrocytes. Results (n = 8) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

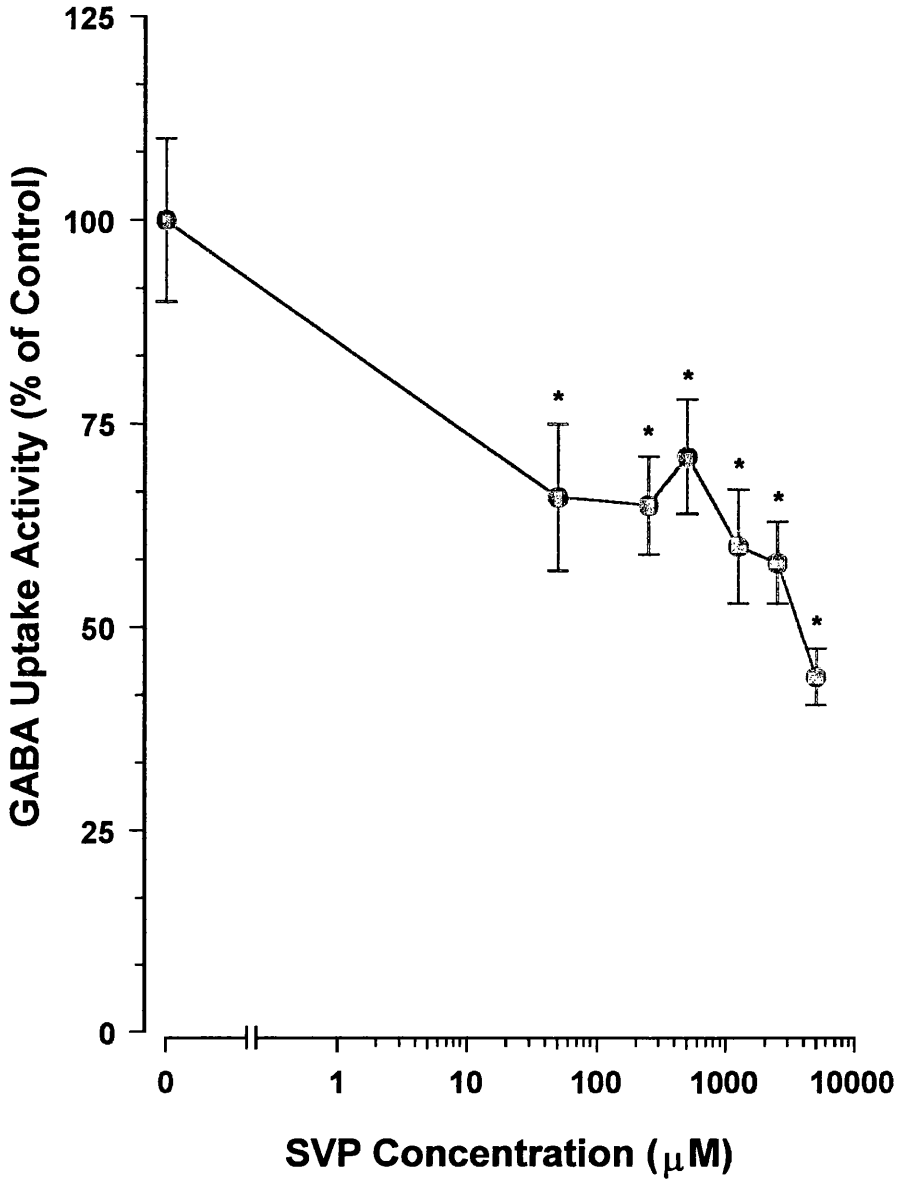


FIGURE 4. Effect of sodium valproate (SVP; 0 - 5000 μM) on the uptake of GABA into primary cultures of rat cortical astrocytes. Results (n = 8) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.). Statistical significance (* $P < 0.05$) was determined by one way analysis of variance with Dunnett correction for multiple comparisons.

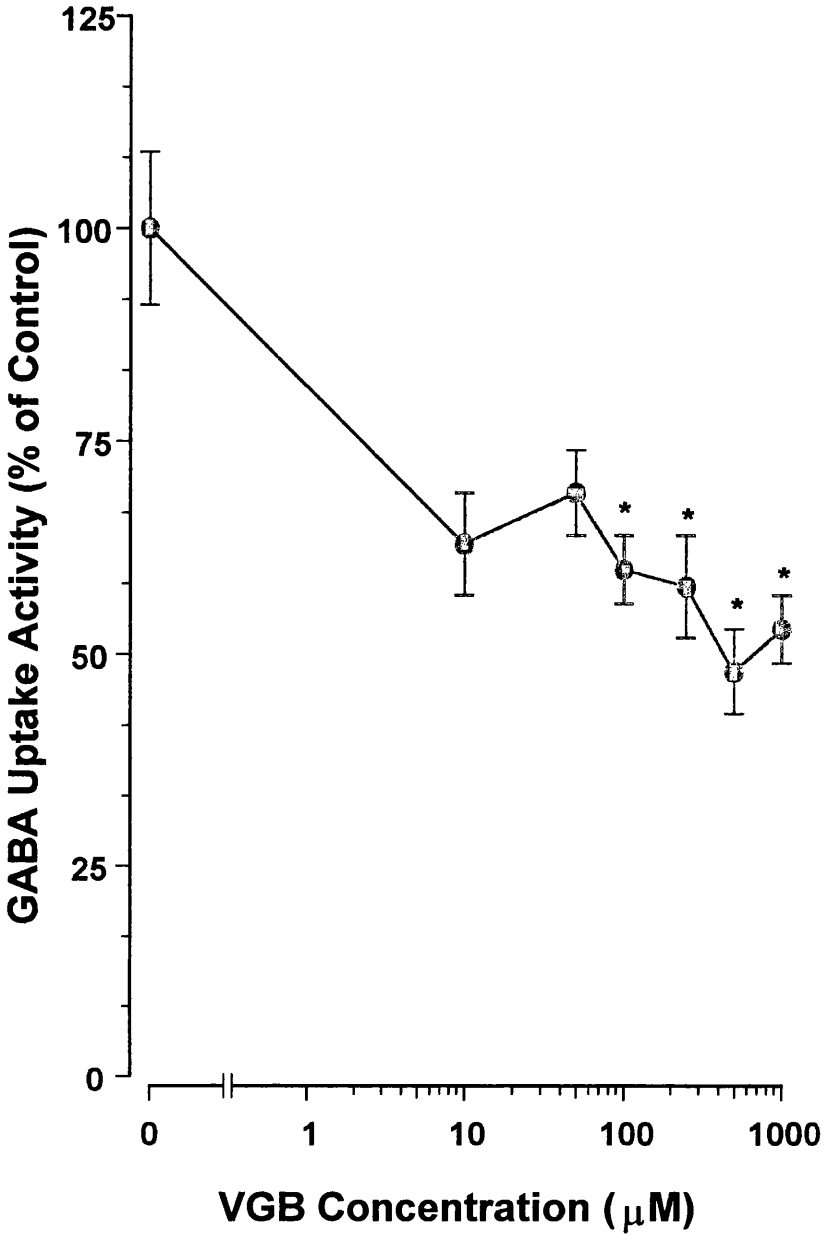


FIGURE 5. Effect of vigabatrin (VGB; 0 - 1000 μM) on the uptake of GABA into primary cultures of rat cortical astrocytes. Results (n = 8) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.). Statistical significance (* $P < 0.05$) was determined by one way analysis of variance with Dunnett correction for multiple comparisons.

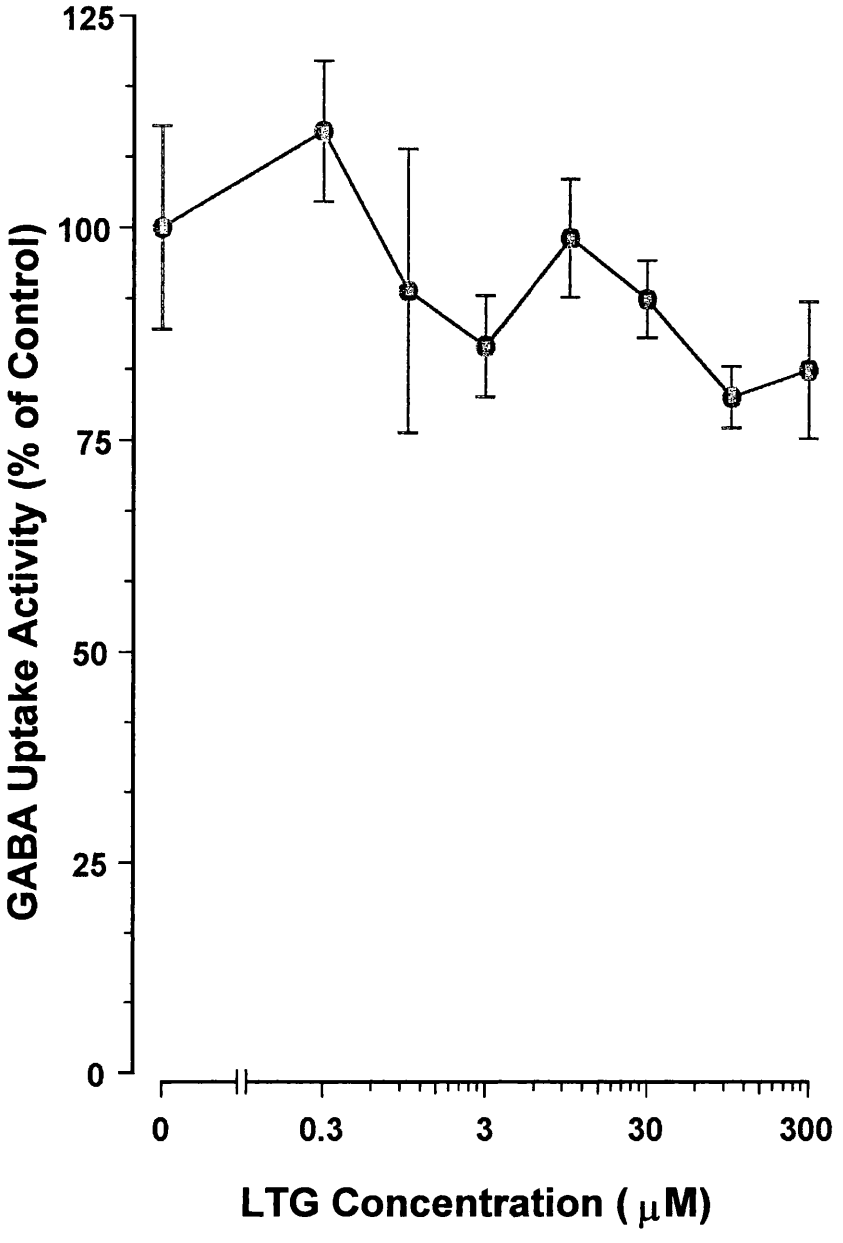


FIGURE 6. Effect of lamotrigine (LTG; 0 - 300 μM) on the uptake of GABA into primary cultures of rat cortical astrocytes. Results (n = 8) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

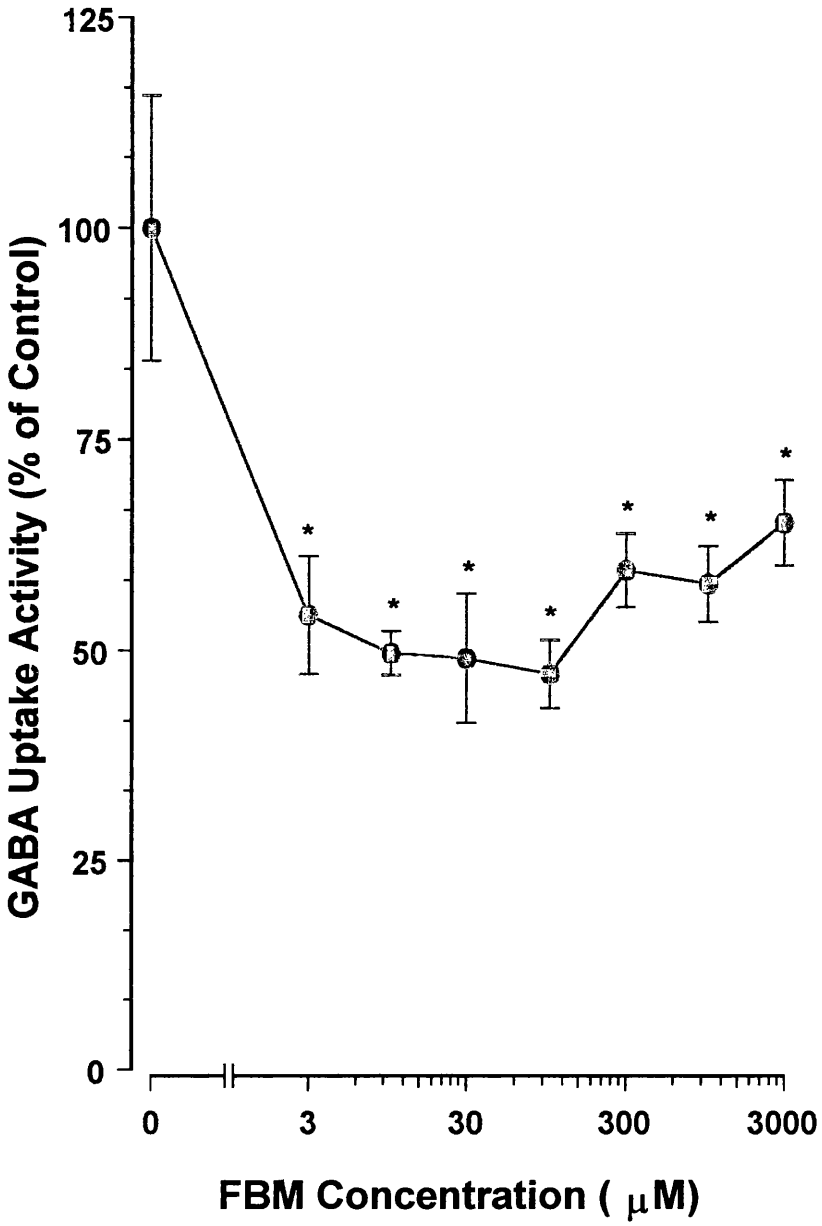


FIGURE 7. Effect of felbamate (FBM; 0 - 3000 μM) on the uptake of GABA into primary cultures of rat cortical astrocytes. Results (n = 8) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.). Statistical significance (* $P < 0.05$) was determined by one way analysis of variance with Dunnett correction for multiple comparisons.

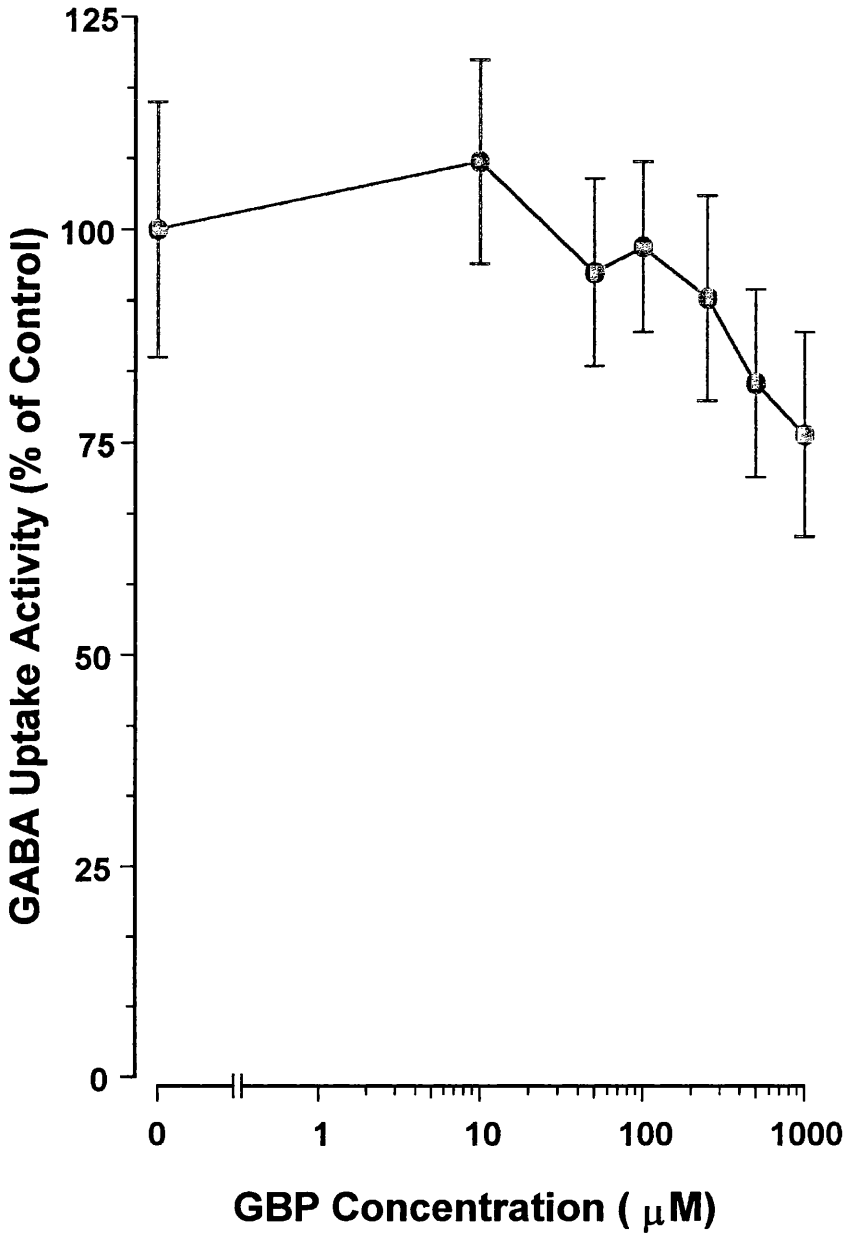


FIGURE 8. Effect of gabapentin (GBP; 0 - 1000 μM) on the uptake of GABA into primary cultures of rat cortical astrocytes. Results (n = 8) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

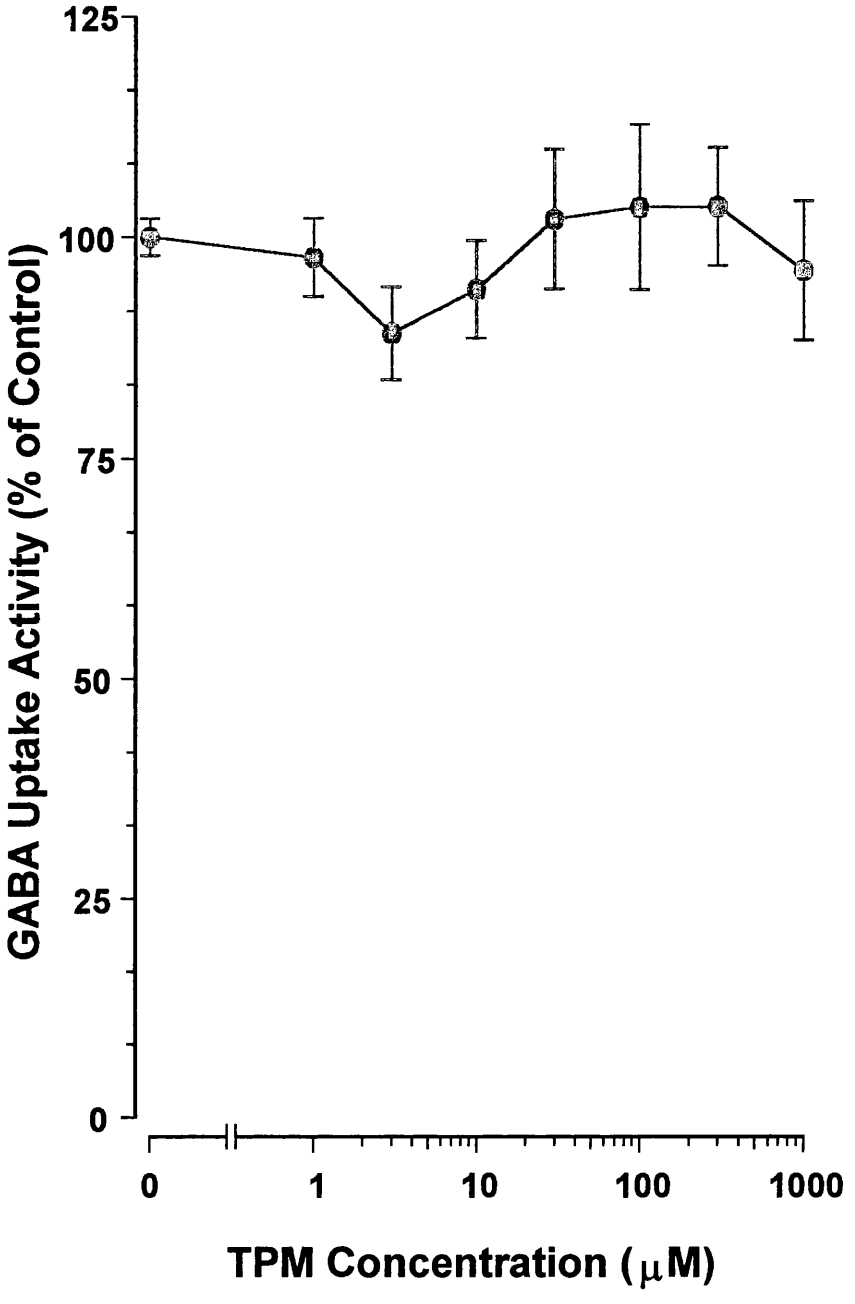


FIGURE 9. Effect of topiramate (TPM; 0 - 1000 μM) on the uptake of GABA into primary cultures of rat cortical astrocytes. Results (n = 8) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

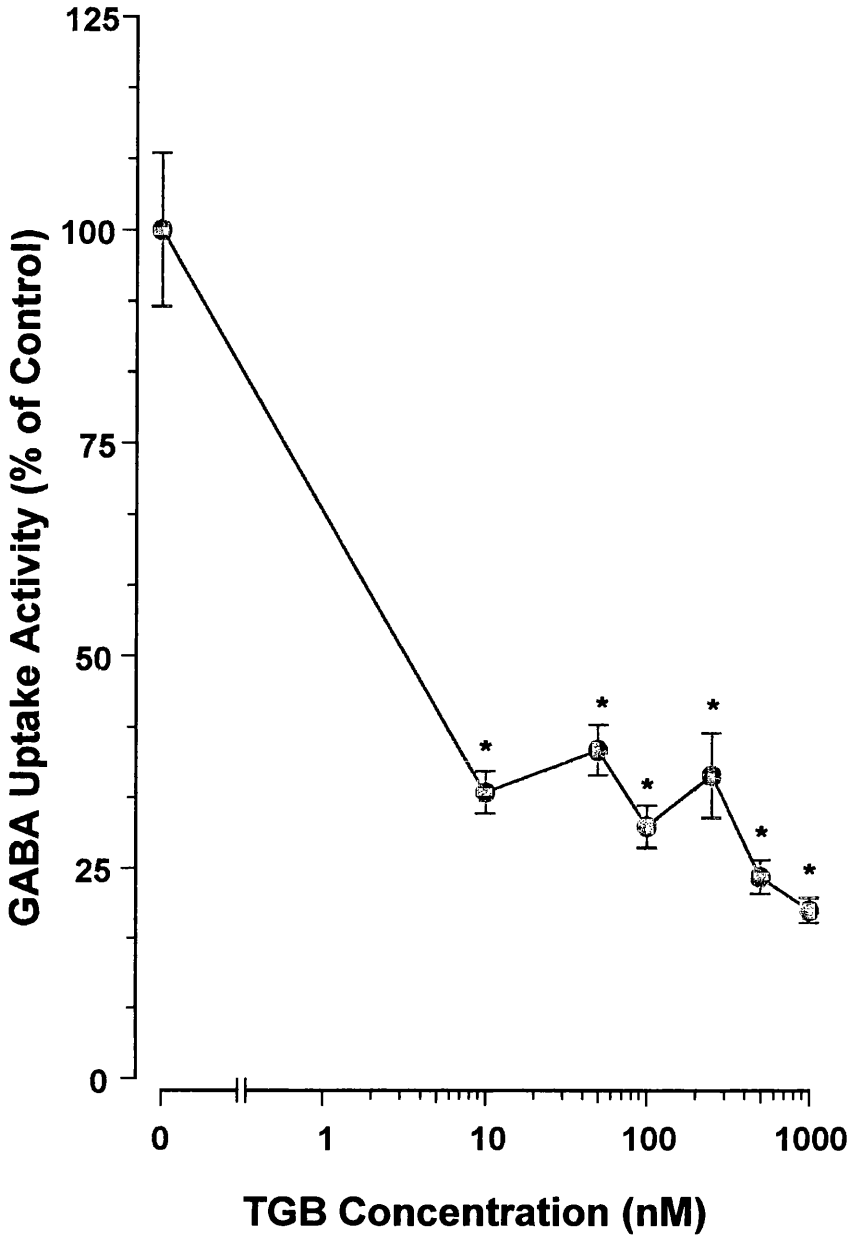


FIGURE 10. Effect of tiagabine (TGB; 0 - 1000 nM) on the uptake of GABA into primary cultures of rat cortical astrocytes. Results (n = 8) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.). Statistical significance (* $P < 0.05$) was determined by one way analysis of variance with Dunnett correction for multiple comparisons.

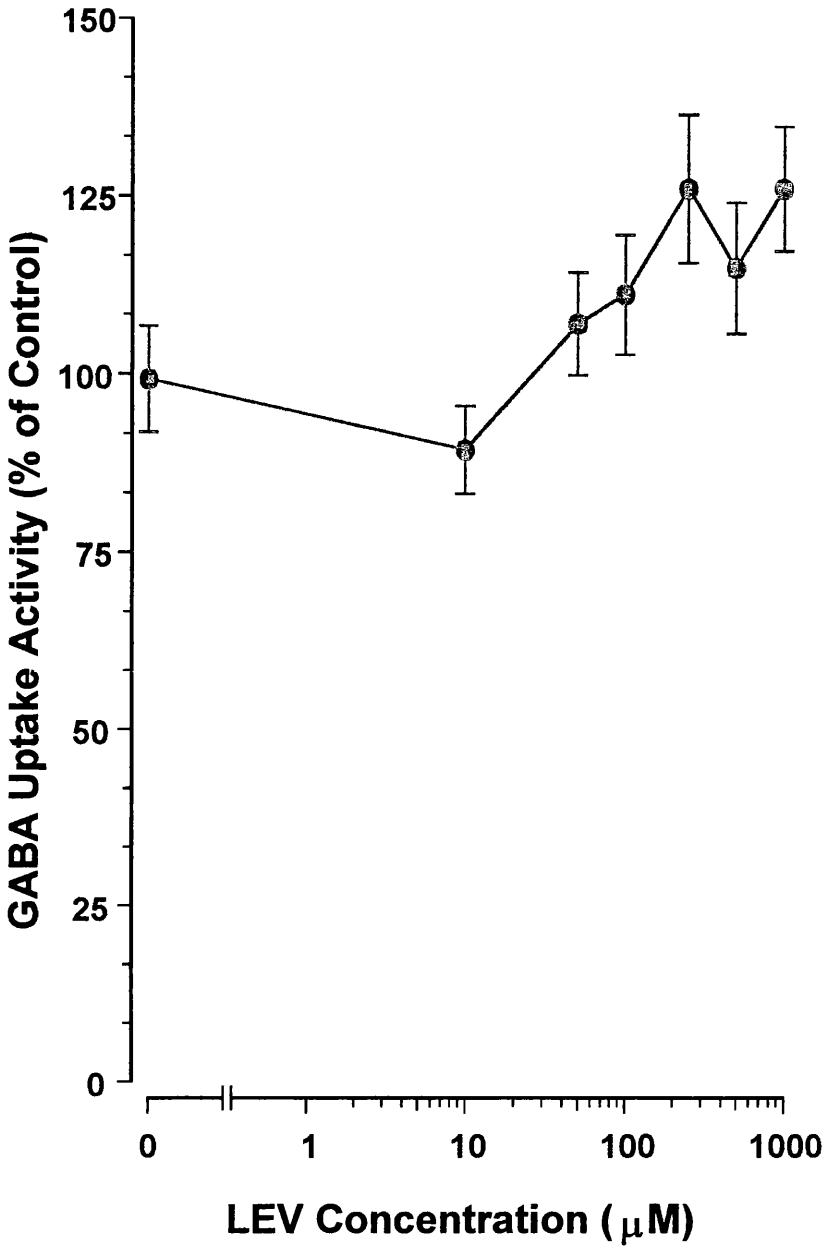


FIGURE 11. Effect of levetiracetam (LEV; 0 - 1000 μM) on the uptake of GABA into primary cultures of rat cortical astrocytes. Results (n = 8) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

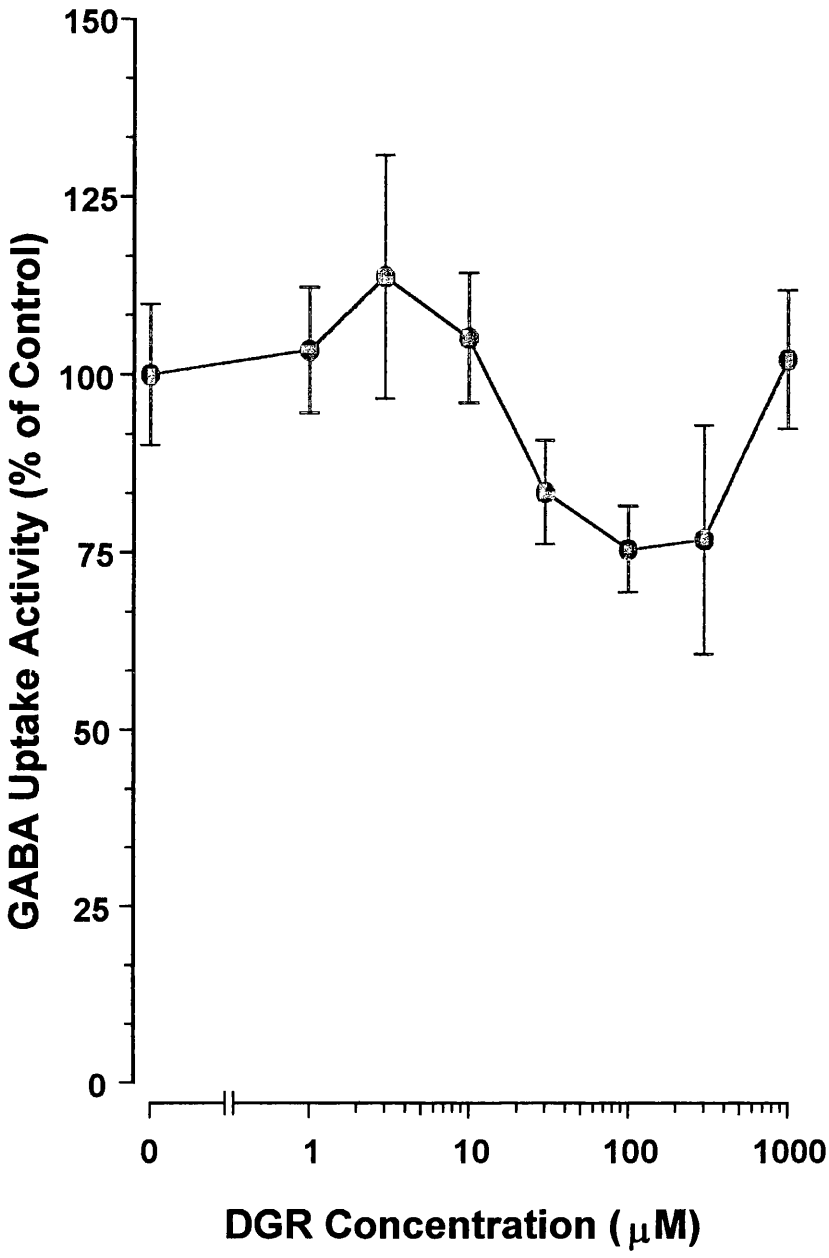


FIGURE 12. Effect of desglycinyl-remacemide (DGR; 0 - 1000 μM) on the uptake of GABA into primary cultures of rat cortical astrocytes. Results (n = 8) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

3.3.2 Validation of the [^{14}C]-glutamate uptake assay in primary cultures of rat cortical astrocytes

EXPERIMENTAL PROTOCOL: Rat brain tissue was isolated and cultured as described in section 2.3. Cultures were randomly separated into six groups (n = 5 / group) and exposed to the glutamate uptake blocker PDC (1, 3, 10, 30, 100 and 300 μM) in BSS. A control group (n = 5) was exposed to BSS alone. After a one hour incubation, cells were assayed for glutamate uptake as described in section 2.8.

RESULTS: PDC (10, 30, 100 and 300 μM; figure 13) was found to significantly ($P < 0.05$) reduce the uptake of [^{14}C]-glutamate (to 20% of control value) into primary cultures of rat cortical astrocytes. 1 and 3 μM PDC were found to have no effect. The reduction in glutamate uptake exhibited by the glutamate uptake blocker PDC confirms the validity of this assay for use in the following AED experiments.

3.3.3 Effects of new and established antiepileptic drugs on the uptake of [^{14}C]-glutamate into primary cultures of rat cortical astrocytes

EXPERIMENTAL PROTOCOL: Rat brain tissue was isolated and cultured as described in section 2.3. For each of the 12 drug experiments, cultures were randomly separated into seven groups (n = 8 / group) and exposed to the following drugs in BSS (stock solutions of CBZ, PHT and FBM were dissolved in 500 μl ethanol prior to dilution with BSS):

PB:- 1, 3, 10, 30, 100, 300 and 1000 μM

PHT:- 1, 3, 10, 30, 100, 300 and 1000 μM

CBZ:- 1, 3, 10, 30, 100, 300 and 1000 μM

SVP:- 3, 10, 30, 100, 300, 1000 and 3000 μM

VGB:- 1, 3, 10, 30, 100, 300 and 1000 μM

LTG:- 0.3, 1, 3, 10, 30, 100 and 300 μM

FBM:- 3, 10, 30, 100, 300, 1000 and 3000 μM

GBP:- 1, 3, 10, 30, 100, 300 and 1000 μM

TPM:- 1, 3, 10, 30, 100, 300 and 1000 μM

TGB:- 0.1, 0.3, 1, 3, 10, 30 and 100 μM

LEV:- 1, 3, 10, 30, 100, 300 and 1000 μM

DGR:- 1, 3, 10, 30, 100, 300 and 1000 μM

For each drug experiment, a control group (n = 8) was exposed to BSS alone. After a one hour incubation, cells were assayed for glutamate uptake as described in section 2.8.

RESULTS: PB (1, 3 and 10 μM; figure 14), TGB (1 and 30 μM; figure 23) and DGR (10, 300 and 1000 μM; figure 25) all significantly ($P < 0.05$) reduced the uptake of glutamate into primary cultures of rat cortical astrocytes. PHT (figure 15), CBZ (figure 16), SVP (figure 17), VGB (figure 18), LTG (figure 19), FBM (figure 20), GBP (figure 21), TPM (figure 22) and LEV (figure 24) were found to have no significant effect on the transport of glutamate.

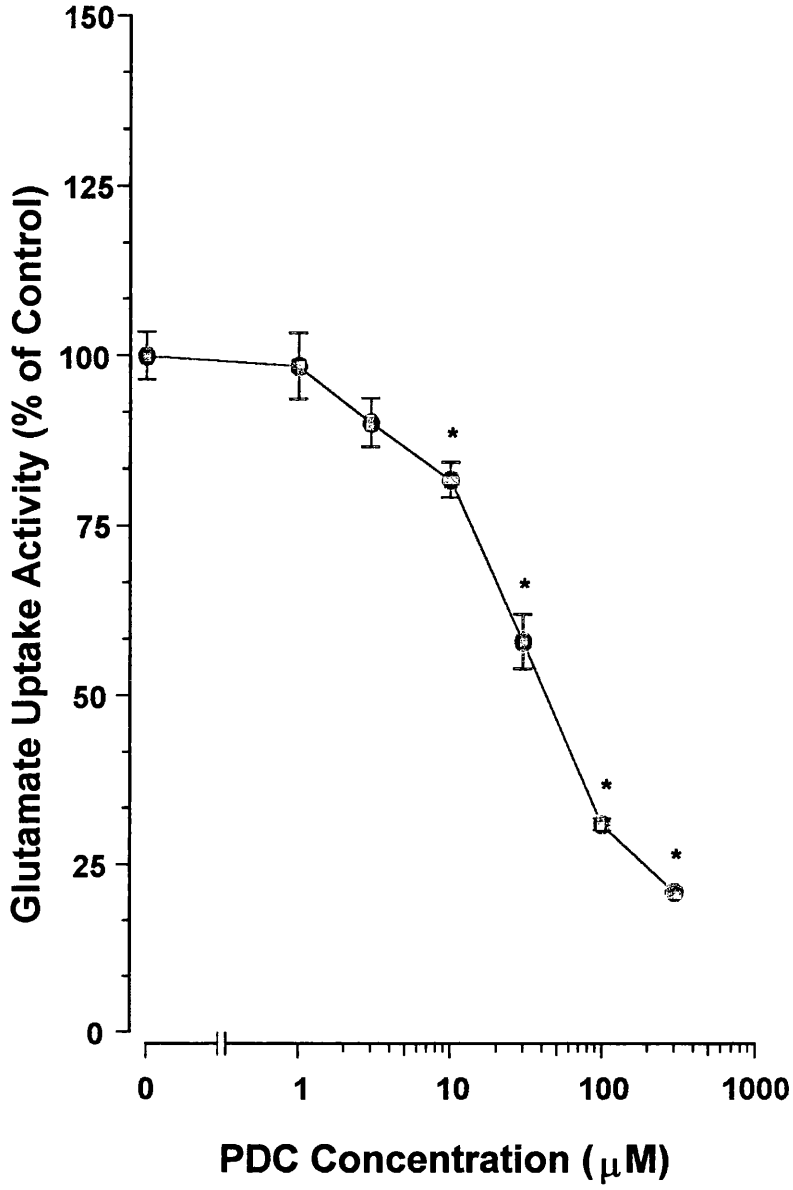


FIGURE 13. Effect of L-*trans*-pyrollidine-2,4-dicarboxylic acid (PDC; 0 - 300 μM) on the uptake of glutamate into primary cultures of rat cortical astrocytes. Results (n = 8) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.). Statistical significance (* $P < 0.05$) was determined by one way analysis of variance with Dunnett correction for multiple comparisons.

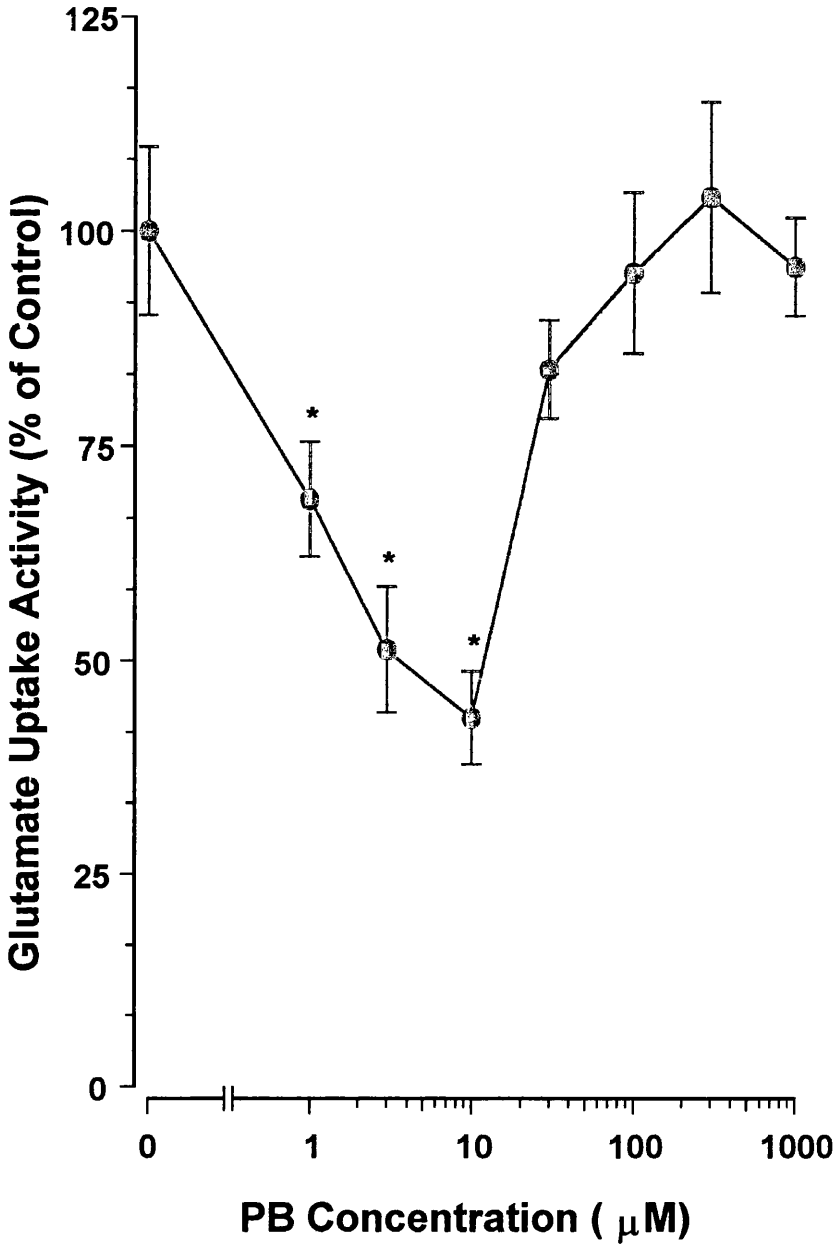


FIGURE 14. Effect of phenobarbitone (PB; 0 - 1000 μM) on the uptake of glutamate into primary cultures of rat cortical astrocytes. Results (n = 8) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.). Statistical significance (* $P < 0.05$) was determined by one way analysis of variance with Dunnett correction for multiple comparisons.

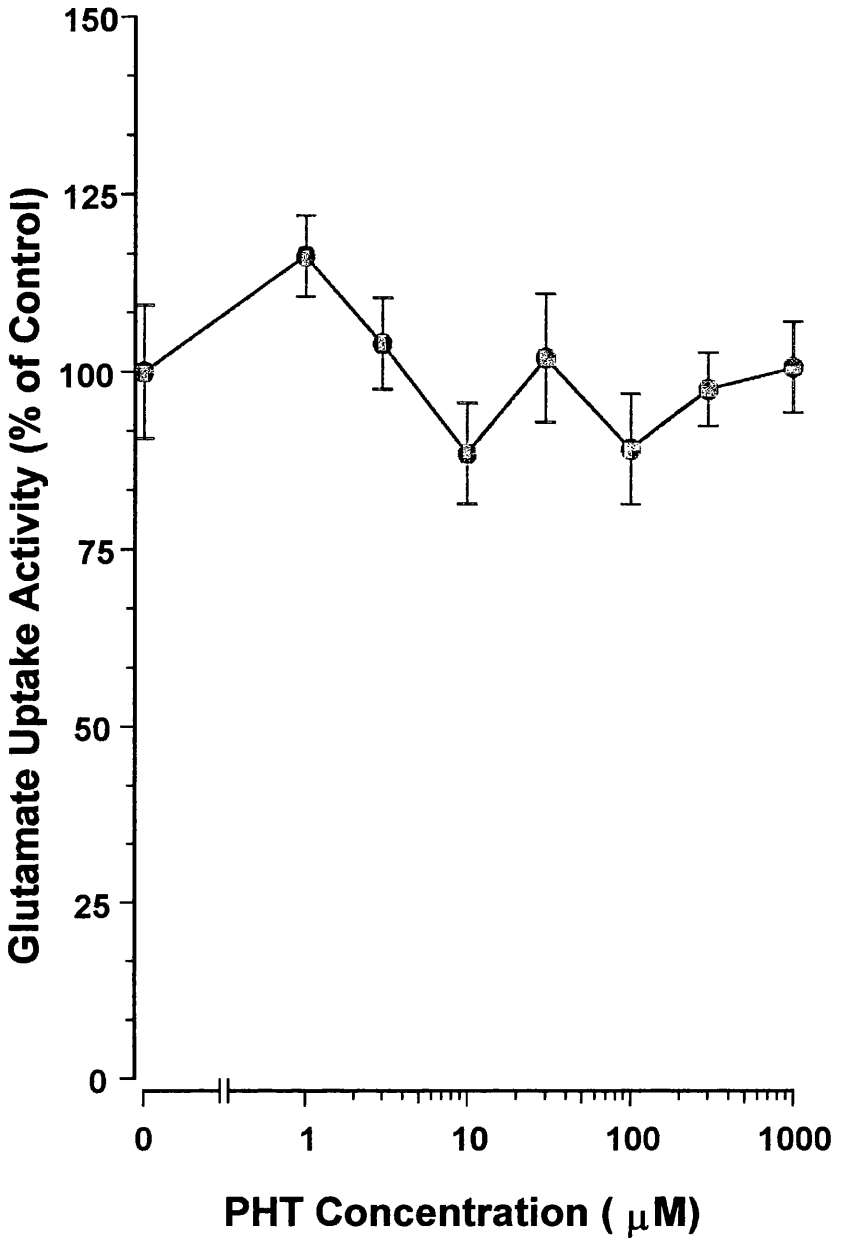


FIGURE 15. Effect of phenytoin (PHT; 0 - 1000 μM) on the uptake of glutamate into primary cultures of rat cortical astrocytes. Results (n = 8) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

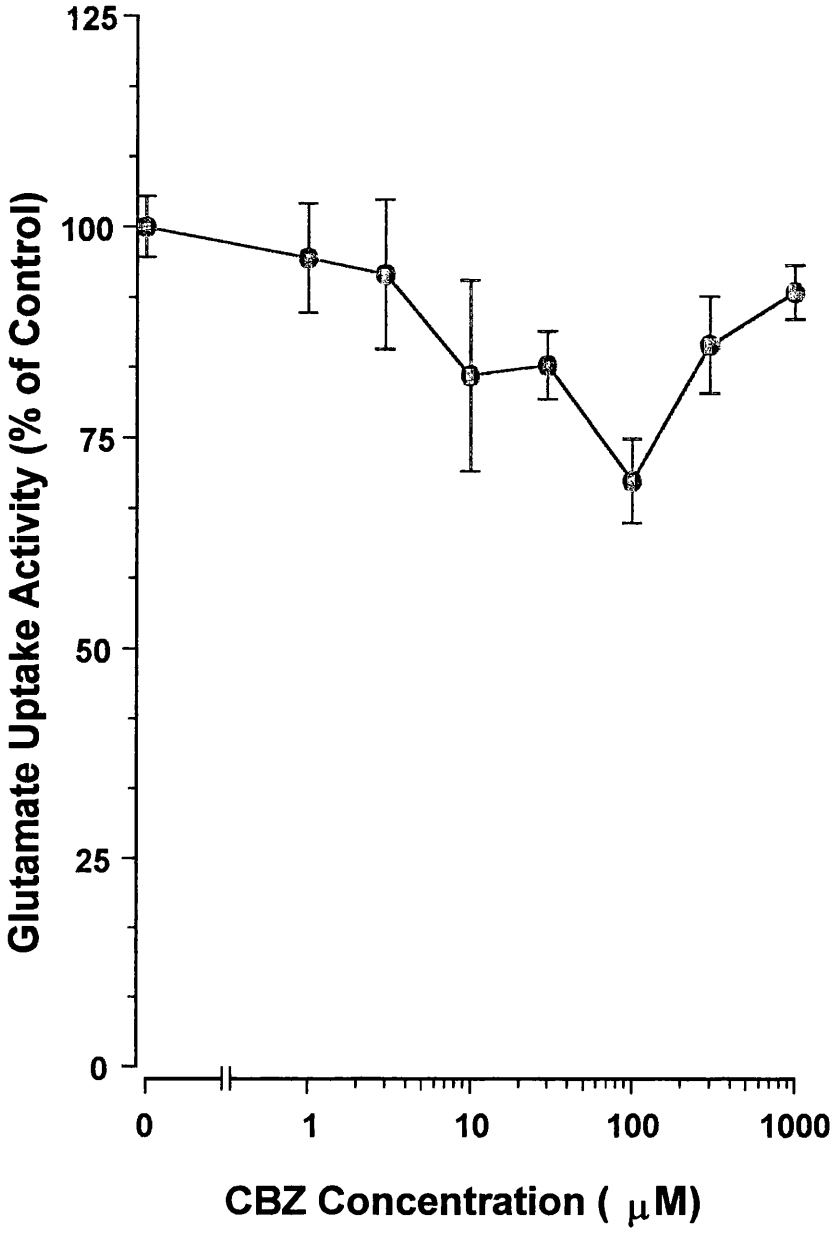


FIGURE 16. Effect of carbamazepine (CBZ; 0 - 1000 μM) on the uptake of glutamate into primary cultures of rat cortical astrocytes. Results (n = 8) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

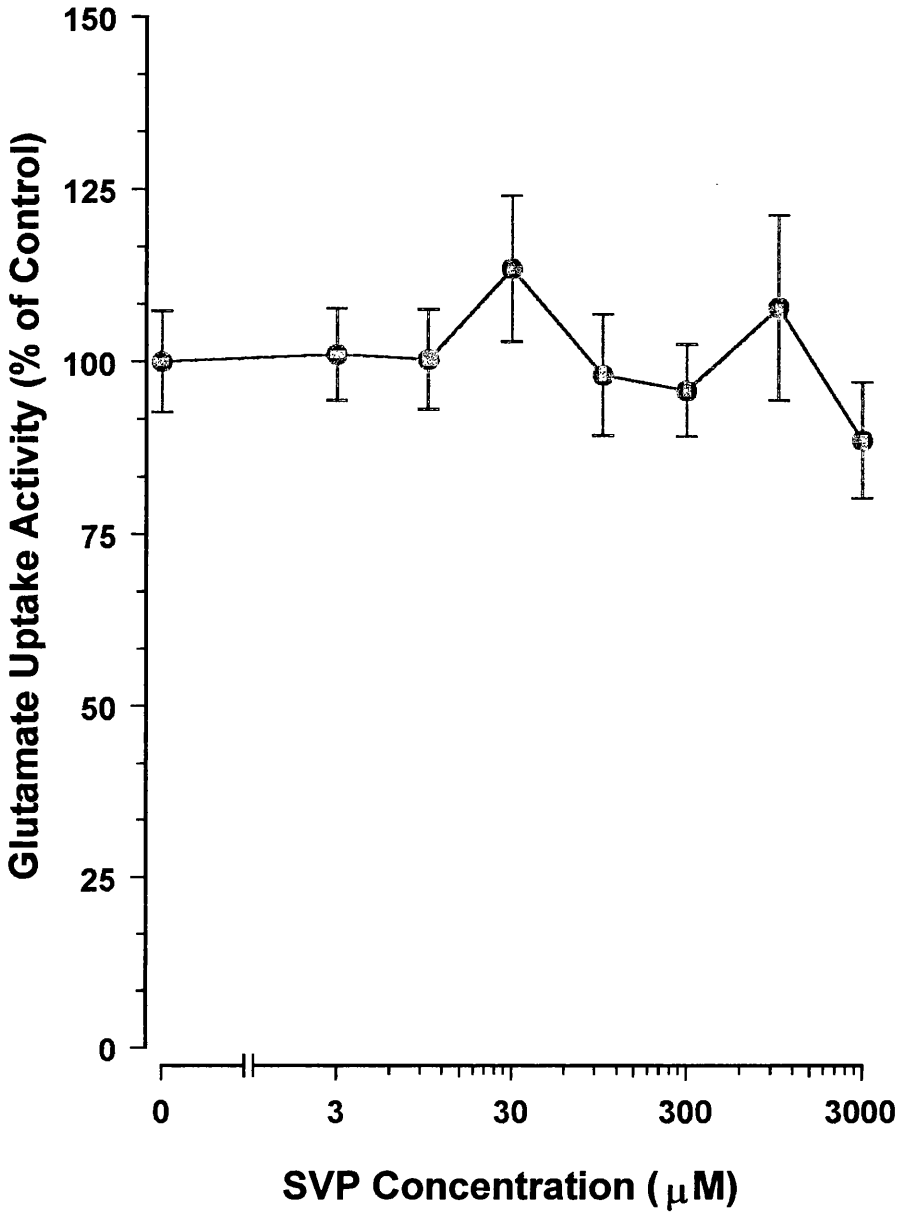


FIGURE 17. Effect of sodium valproate (SVP; 0 - 3000 μM) on the uptake of glutamate into primary cultures of rat cortical astrocytes. Results (n = 8) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

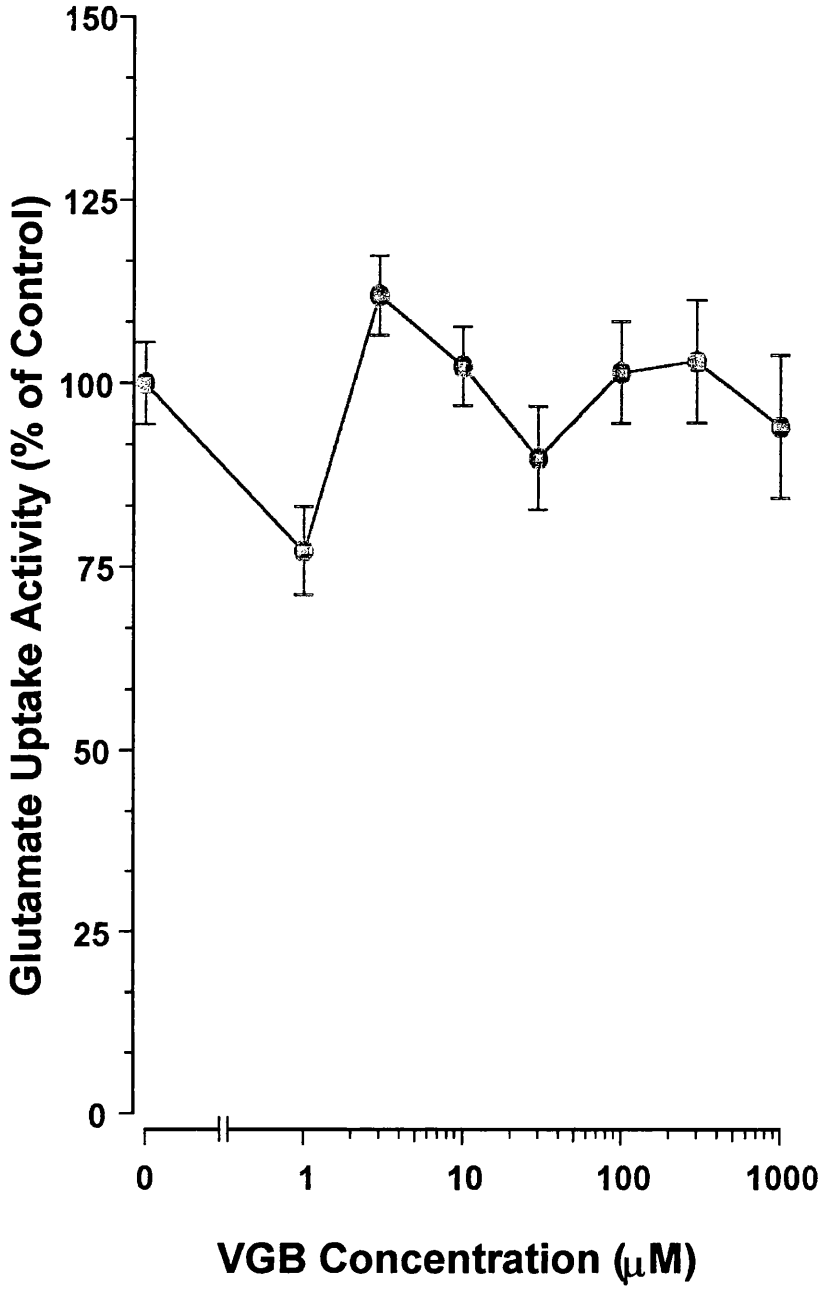


FIGURE 18. Effect of vigabatrin (VGB; 0 - 1000 μM) on the uptake of glutamate into primary cultures of rat cortical astrocytes. Results (n = 8) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

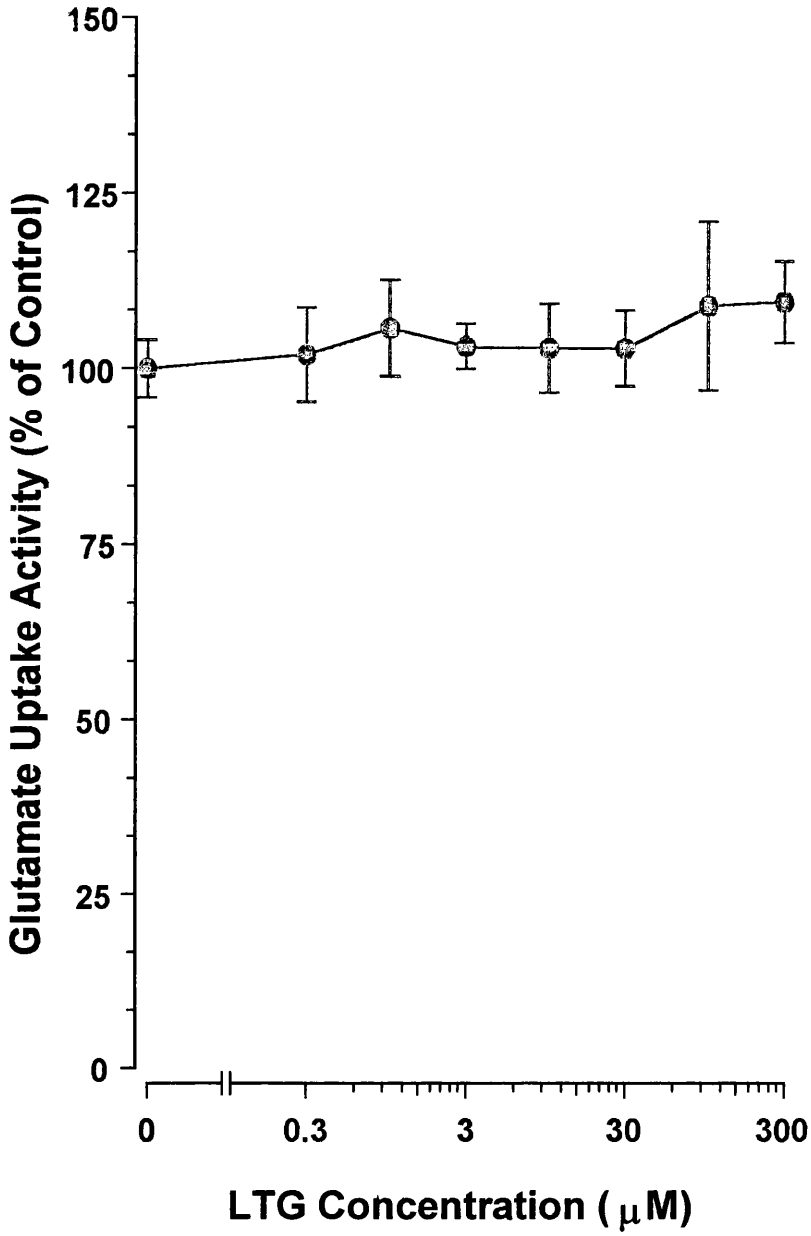


FIGURE 19. Effect of lamotrigine (LTG; 0 - 300 μM) on the uptake of glutamate into primary cultures of rat cortical astrocytes. Results (n = 8) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

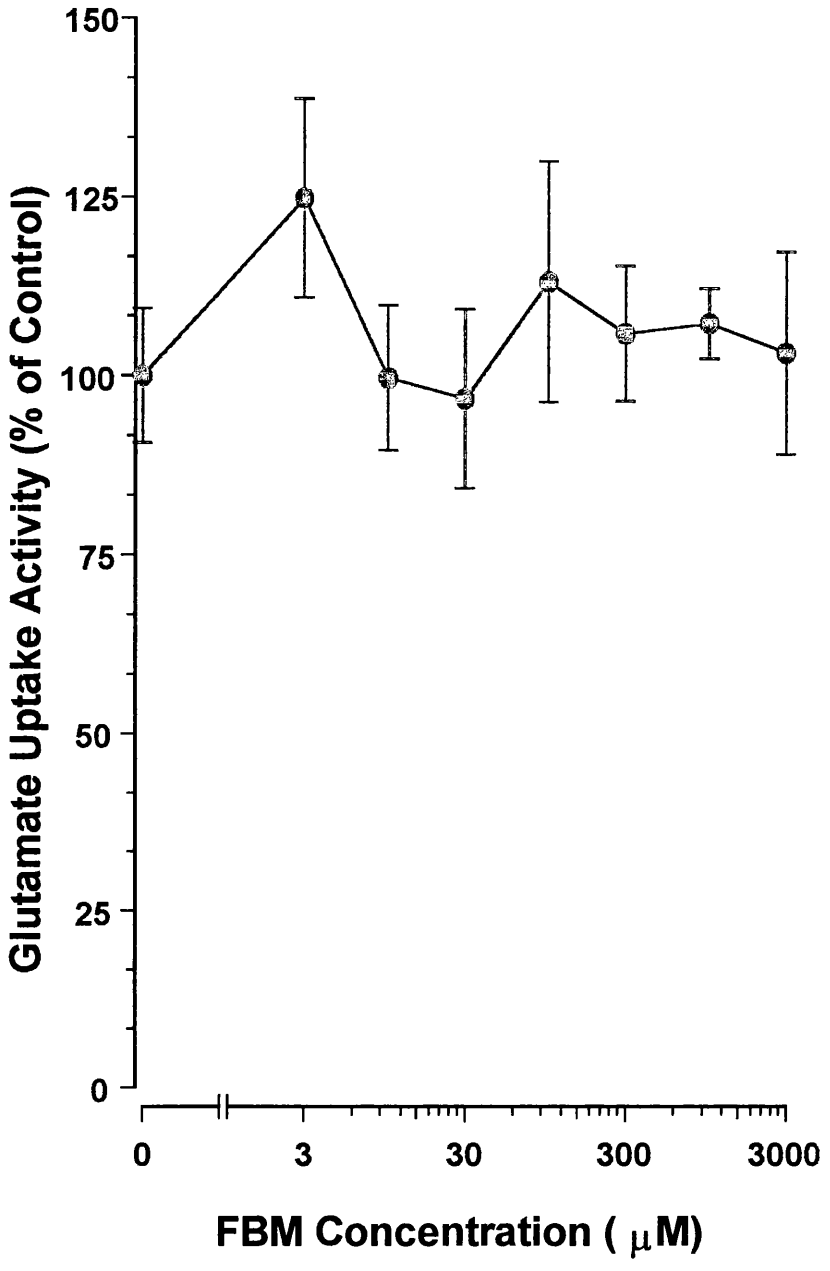


FIGURE 20. Effect of felbamate (FBM; 0 - 3000 μM) on the uptake of glutamate into primary cultures of rat cortical astrocytes. Results (n = 8) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

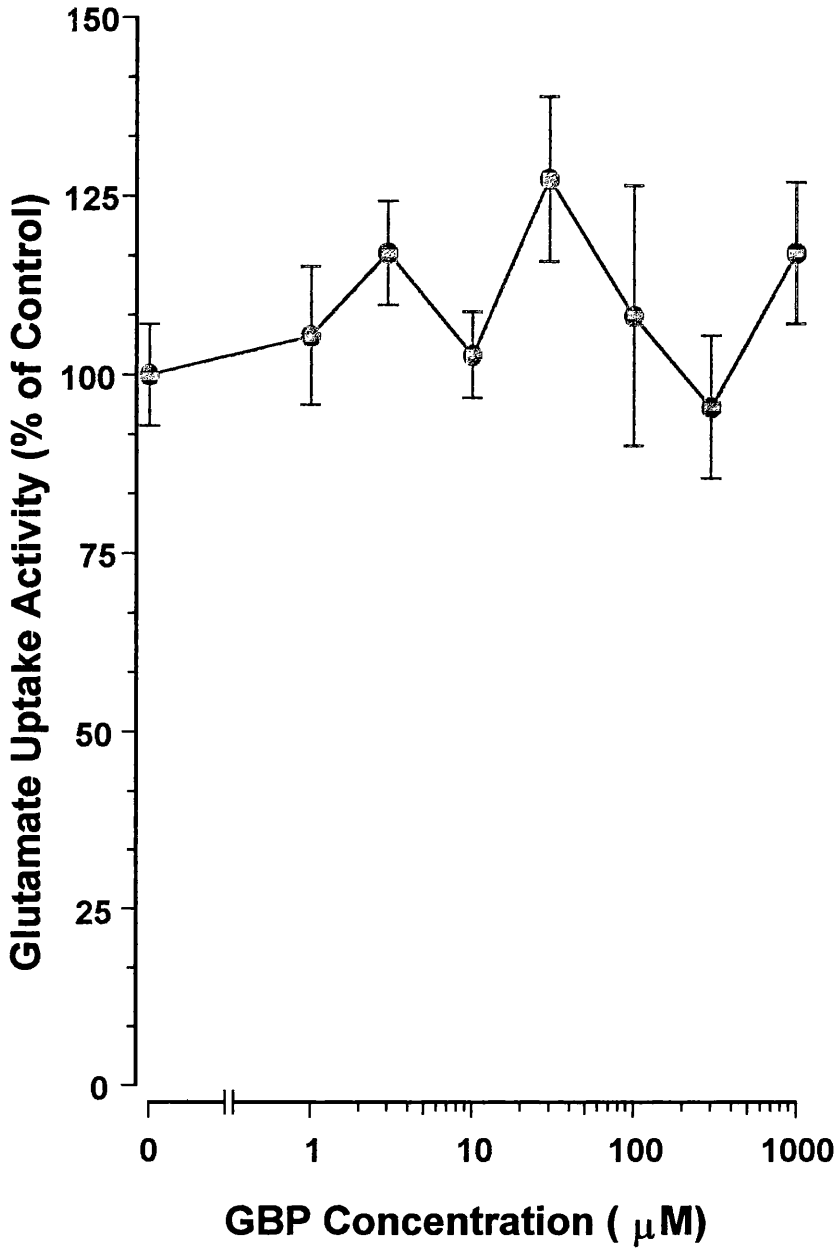


FIGURE 21. Effect of gabapentin (GBP; 0 - 1000 μM) on the uptake of glutamate into primary cultures of rat cortical astrocytes. Results (n = 8) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

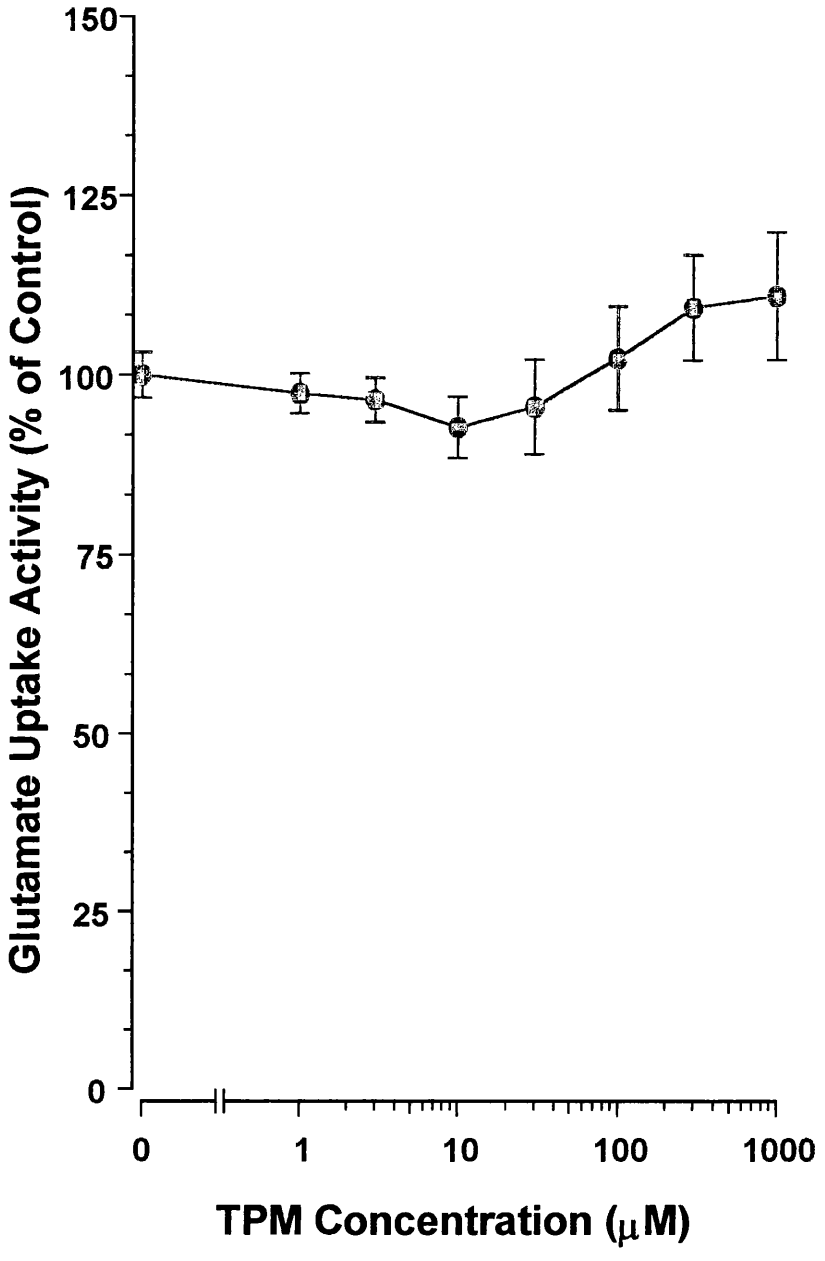


FIGURE 22. Effect of topiramate (TPM; 0 - 1000 μM) on the uptake of glutamate into primary cultures of rat cortical astrocytes. Results (n = 8) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

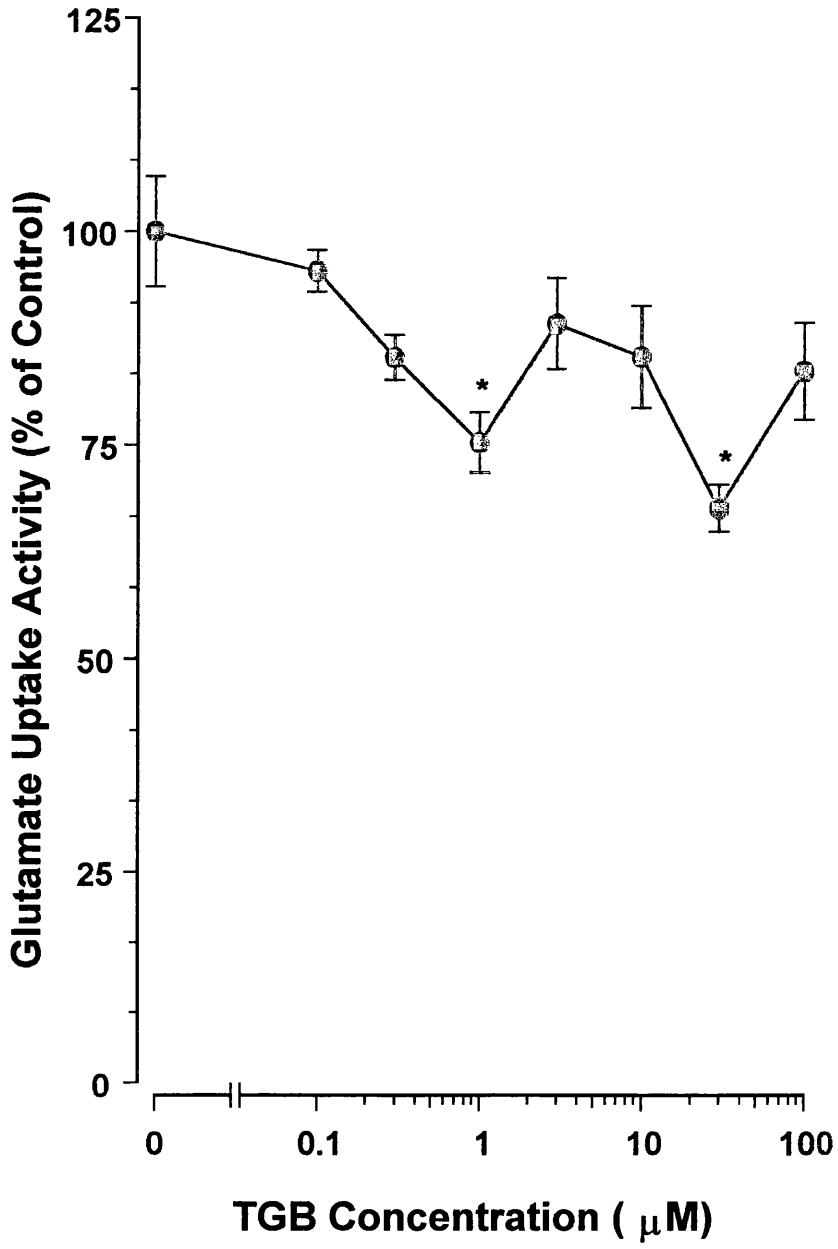


FIGURE 23. Effect of tiagabine (TGB; 0 - 100 μM) on the uptake of glutamate into primary cultures of rat cortical astrocytes. Results (n = 8) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.). Statistical significance (* $P < 0.05$) was determined by one way analysis of variance with Dunnett correction for multiple comparisons.

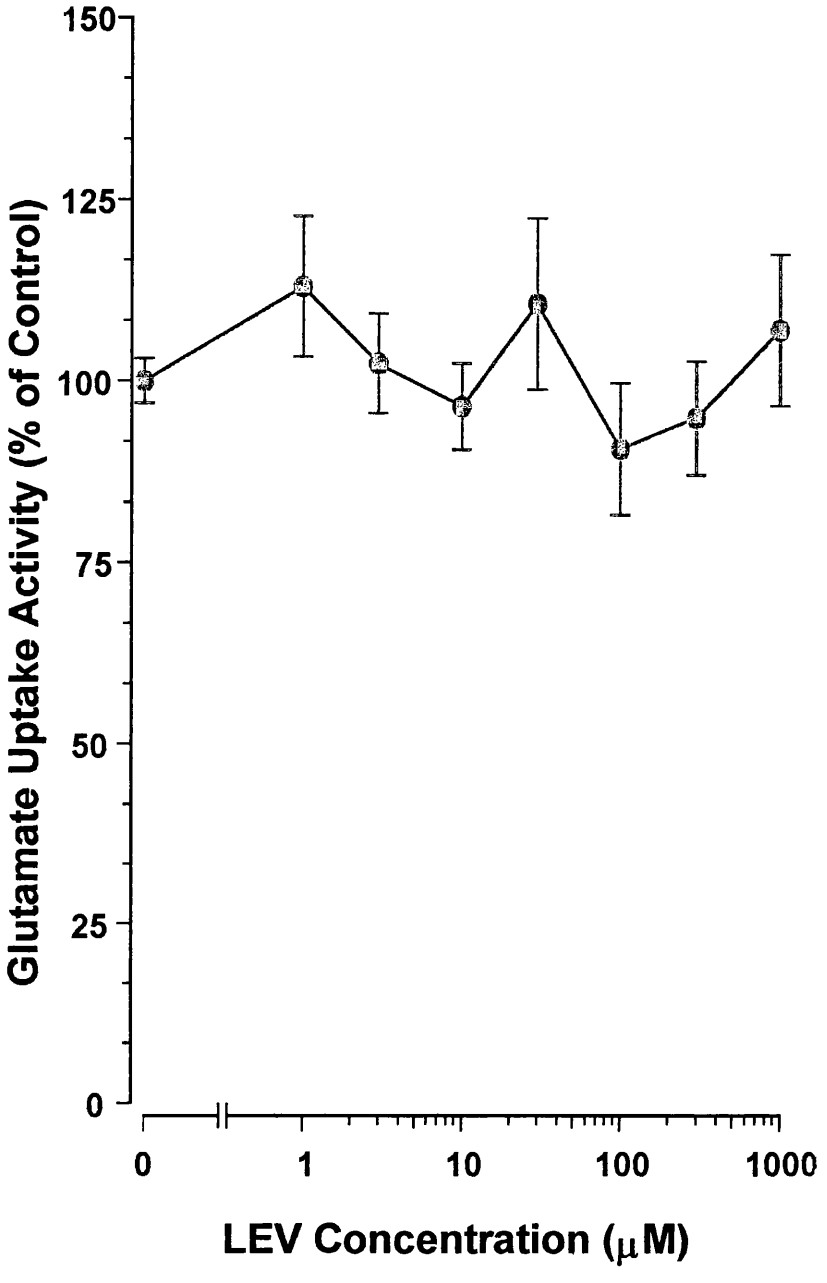


FIGURE 24. Effect of levetiracetam (LEV; 0 - 1000 μM) on the uptake of glutamate into primary cultures of rat cortical astrocytes. Results (n = 8) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

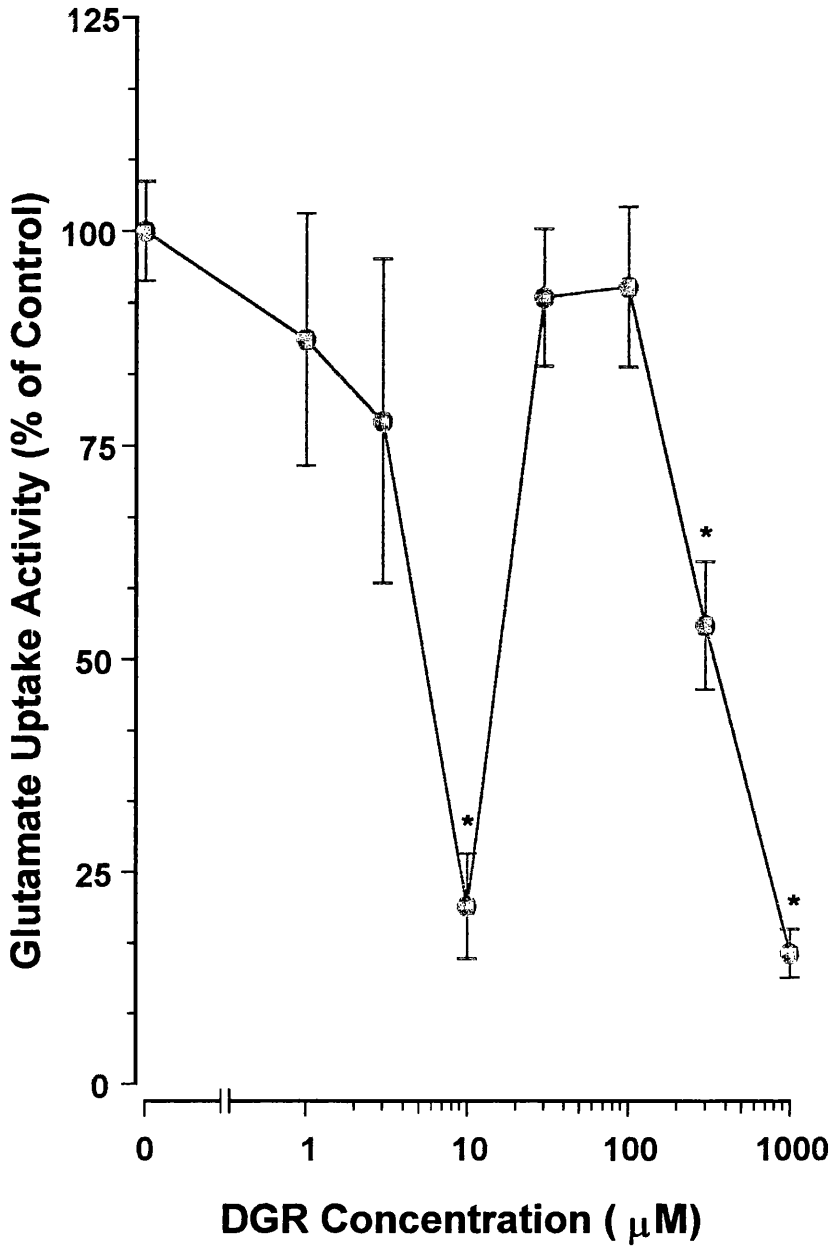


FIGURE 25. Effect of desglycinyl-remacemide (DGR; 0 - 1000 μM) on the uptake of glutamate into primary cultures of rat cortical astrocytes. Results (n = 8) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.). Statistical significance (* $P < 0.05$) was determined by one way analysis of variance with Dunnett correction for multiple comparisons.

3.3.4 Effects of new and established antiepileptic drugs on the metabolism of GABA in primary cultures of rat cortical astrocytes

EXPERIMENTAL PROTOCOL: Rat brain tissue was isolated and cultured as described in section 2.3. For each of the 12 drug experiments, cultures were randomly separated into six (SVP, VGB, GBP, TGB and LEV) or seven (PB, PHT, CBZ, LTG, FBM, TPM and DGR) groups (n = 8 / group), and exposed to the following drugs in BSS (stock solutions of CBZ, PHT and FBM were dissolved in 500 µl ethanol prior to dilution with BSS):

PB:- 1, 3, 10, 30, 100, 300 and 1000 µM

PHT:- 1, 3, 10, 30, 100, 300 and 1000 µM

CBZ:- 1, 3, 10, 30, 100, 300 and 1000 µM

SVP:- 50, 250, 500, 1250, 2500 and 5000 µM

VGB:- 10, 50, 100, 250, 500 and 1000 µM

LTG:- 0.3, 1, 3, 10, 30, 100 and 300 µM

FBM:- 3, 10, 30, 100, 300, 1000 and 3000 µM

GBP:- 10, 50, 100, 250, 500 and 1000 µM

TPM:- 1, 3, 10, 30, 100, 300 and 1000 µM

TGB:- 10, 50, 100, 250, 500 and 1000 nM

LEV:- 10, 50, 100, 250, 500 and 1000 µM

DGR:- 1, 3, 10, 30, 100, 300 and 1000 µM

For each drug experiment, a control group (n = 8) was exposed to BSS alone. After a one hour incubation, cells were assayed for GABA-T activity as described in section 2.9.

RESULTS: SVP (5000 μM; figure 29), VGB (50, 100, 250, 500 and 1000 μM; figure 30), TGB (1000 nM; figure 35) and DGR (30, 100, 300 and 1000 μM; figure 37) all significantly ($P < 0.05$) reduced the activity of GABA-T in primary cultures of rat cortical astrocytes. PB (figure 26), PHT (figure 27), CBZ (figure 28), LTG (figure 31), FBM (figure 32), GBP (figure 33), TPM (figure 34) and LEV (figure 36) were found to have no significant effect on the metabolism of GABA.

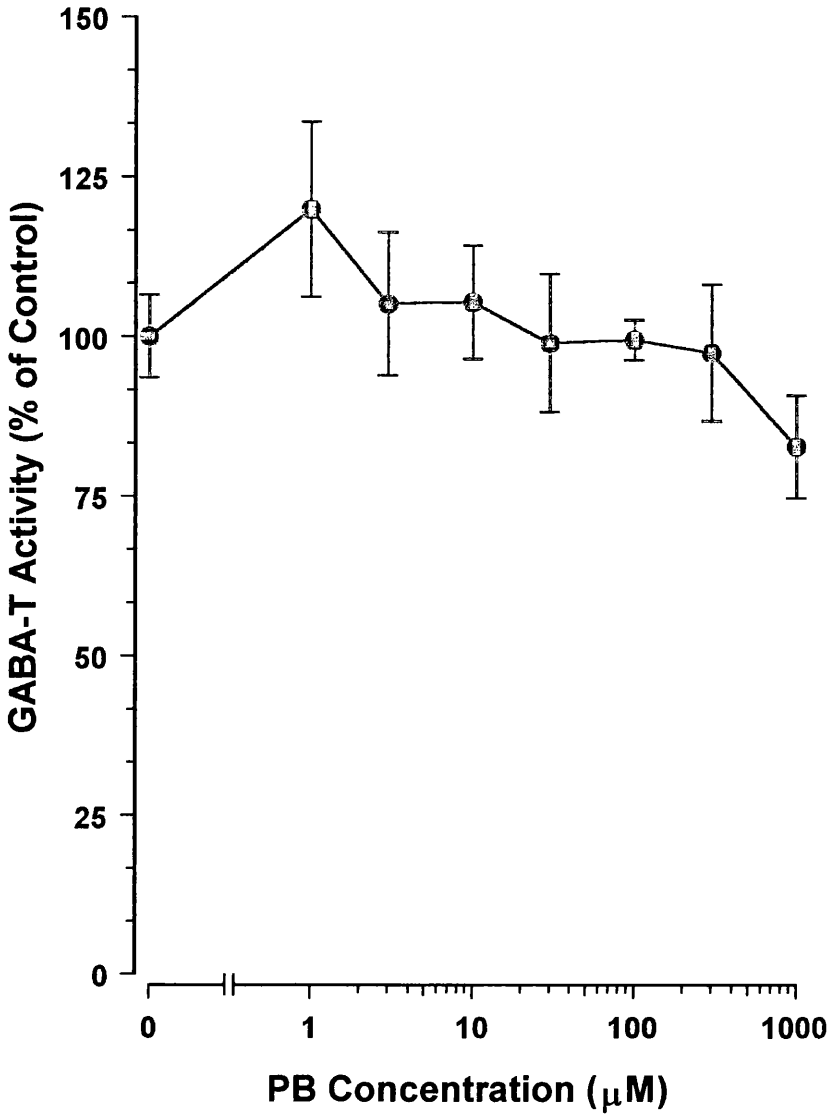


FIGURE 26. Effect of phenobarbitone (PB; 0 - 1000 μM) on the activity of GABA-transaminase (GABA-T) in primary cultures of rat cortical astrocytes. Results (n = 8) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

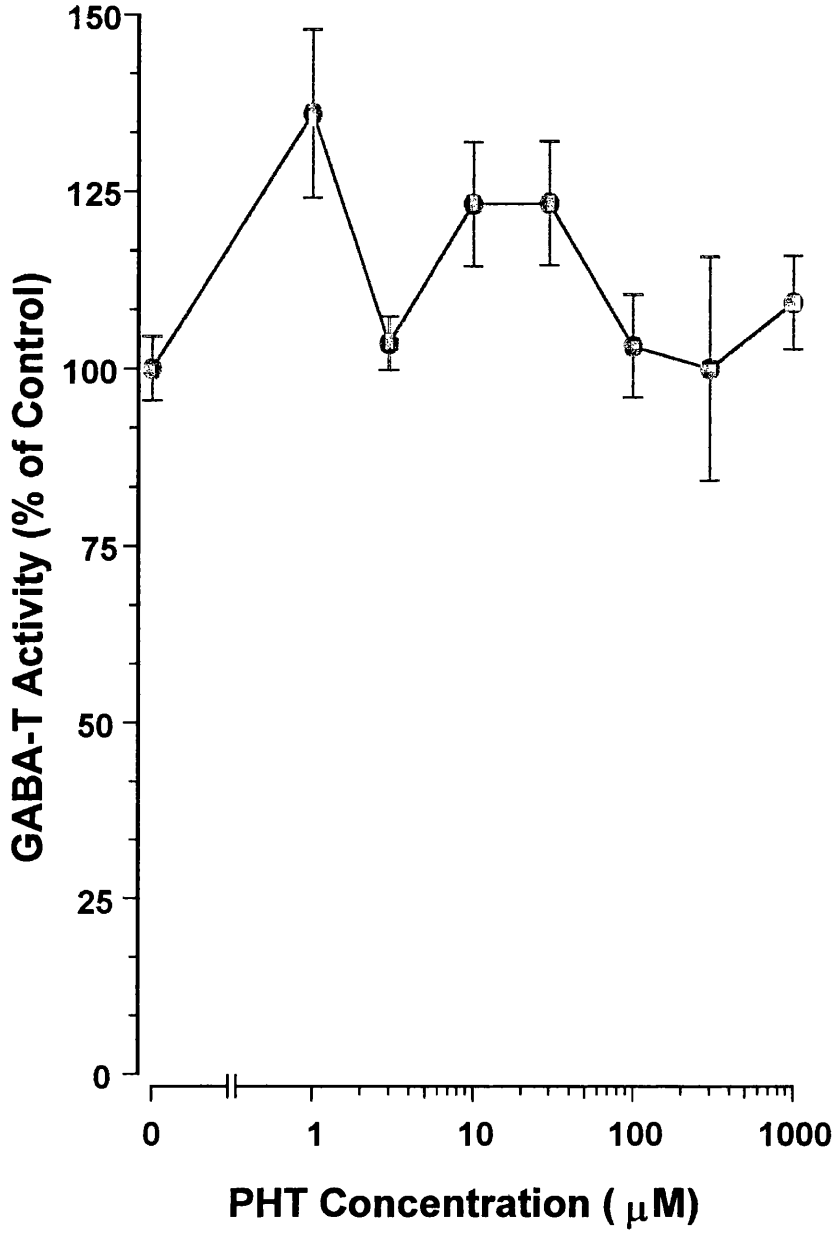


FIGURE 27. Effect of phenytoin (PHT; 0 - 1000 μM) on the activity of GABA-transaminase (GABA-T) in primary cultures of rat cortical astrocytes. Results (n = 8) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

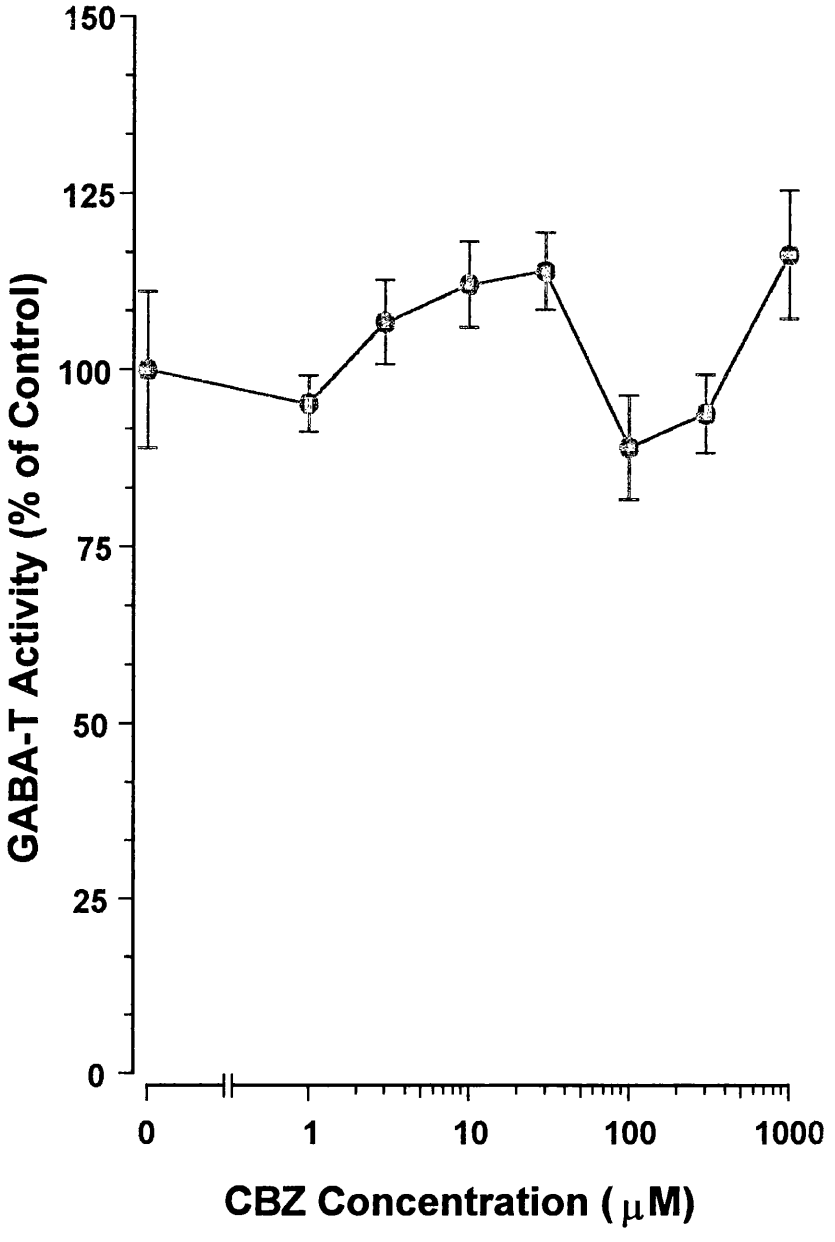


FIGURE 28. Effect of carbamazepine (CBZ; 0 - 1000 μM) on the activity of GABA-transaminase (GABA-T) in primary cultures of rat cortical astrocytes. Results (n = 8) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

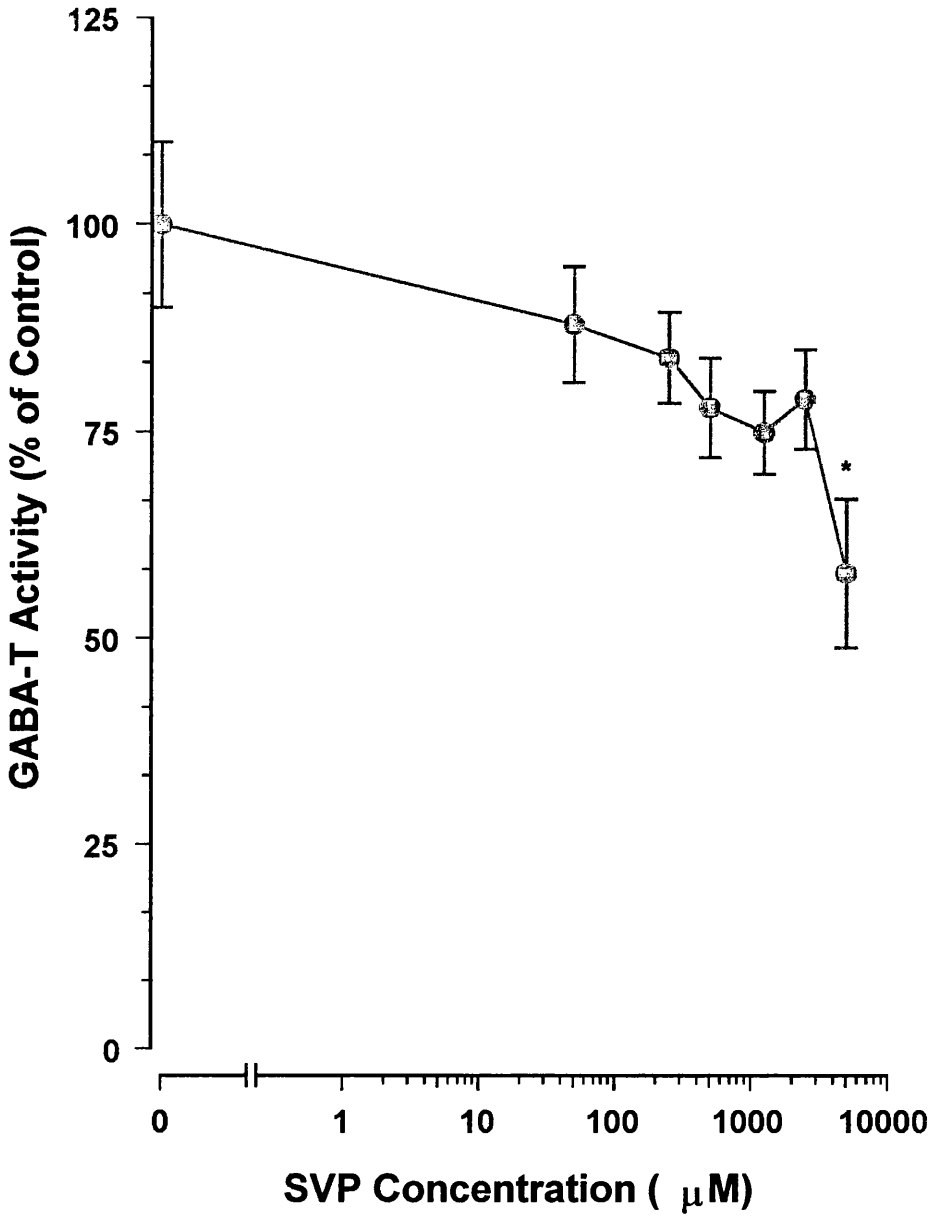


FIGURE 29. Effect of sodium valproate (SVP; 0 - 5000 μM) on the activity of GABA-transaminase (GABA-T) in primary cultures of rat cortical astrocytes. Results (n = 8) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.). Statistical significance (* $P < 0.05$) was determined by one way analysis of variance with Dunnett correction for multiple comparisons.

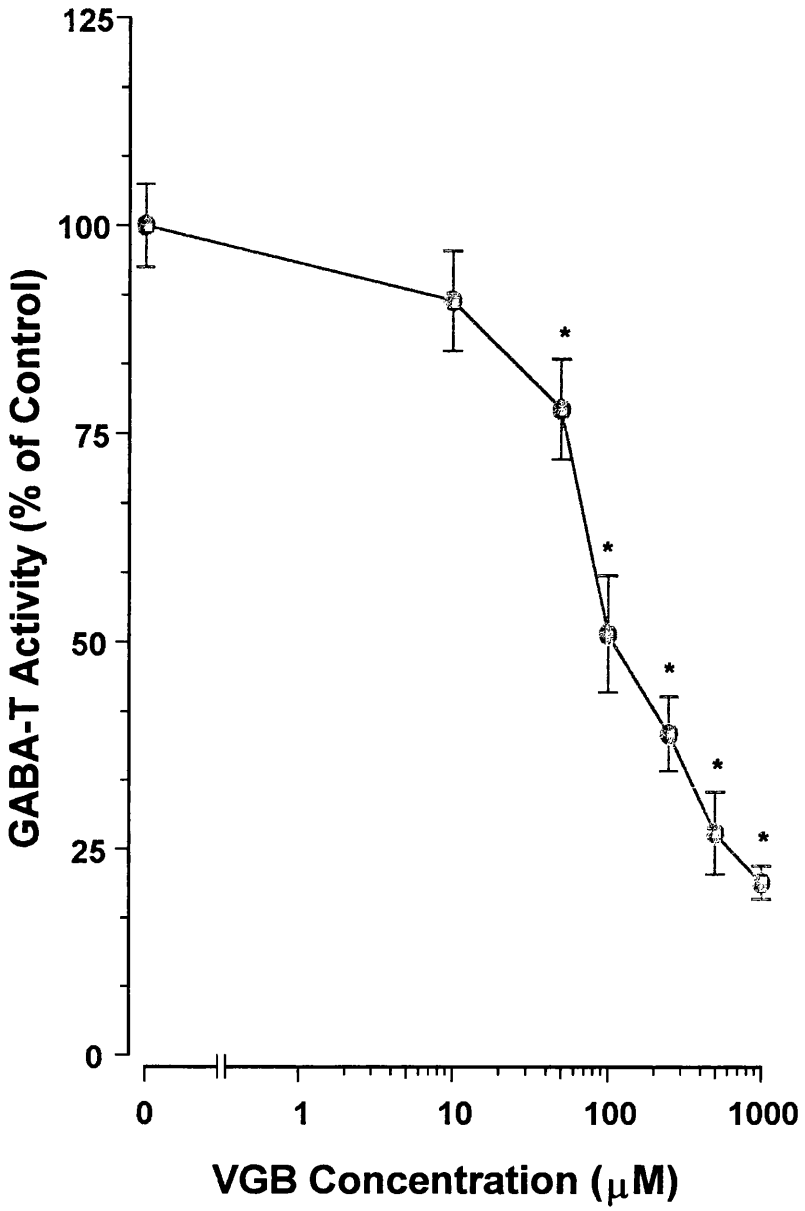


FIGURE 30. Effect of vigabatrin (VGB; 0 - 1000 μM) on the activity of GABA-transaminase (GABA-T) in primary cultures of rat cortical astrocytes. Results (n = 8) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.). Statistical significance (* $P < 0.05$) was determined by one way analysis of variance with Dunnett correction for multiple comparisons.

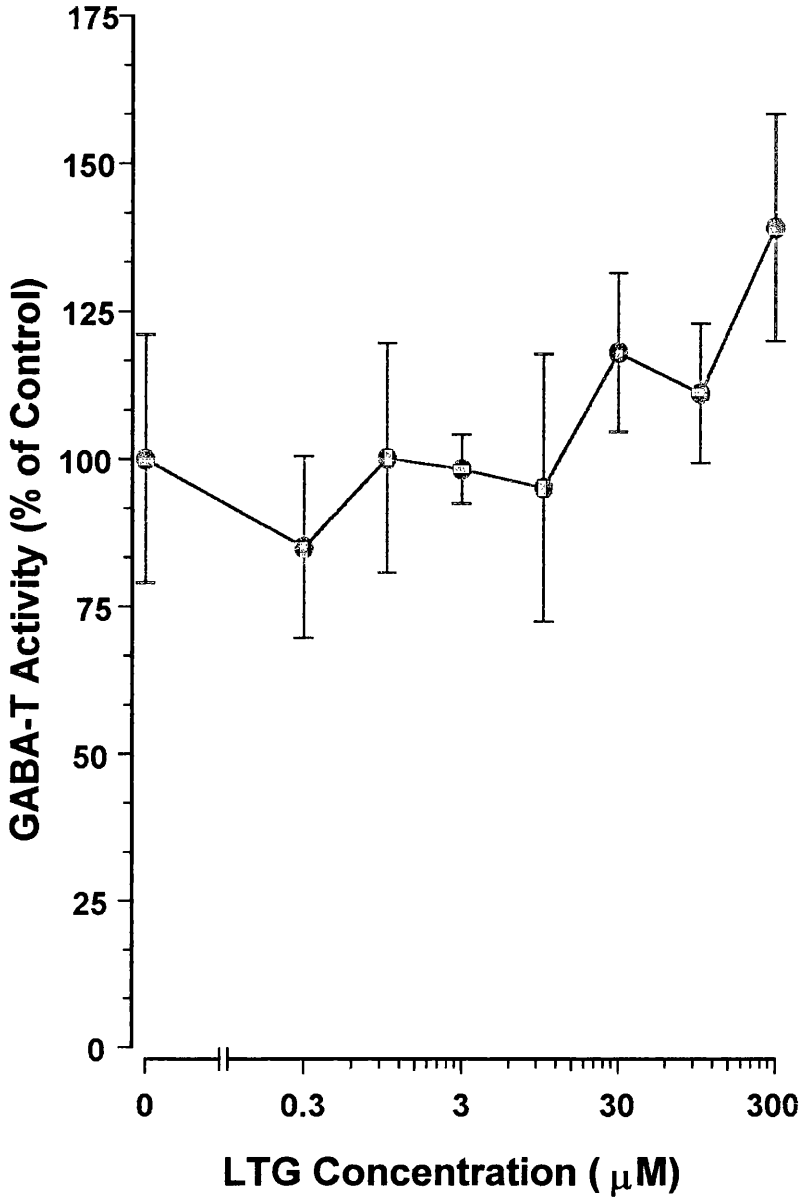


FIGURE 31. Effect of lamotrigine (LTG; 0 - 300 μM) on the activity of GABA-transaminase (GABA-T) in primary cultures of rat cortical astrocytes. Results (n = 8) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

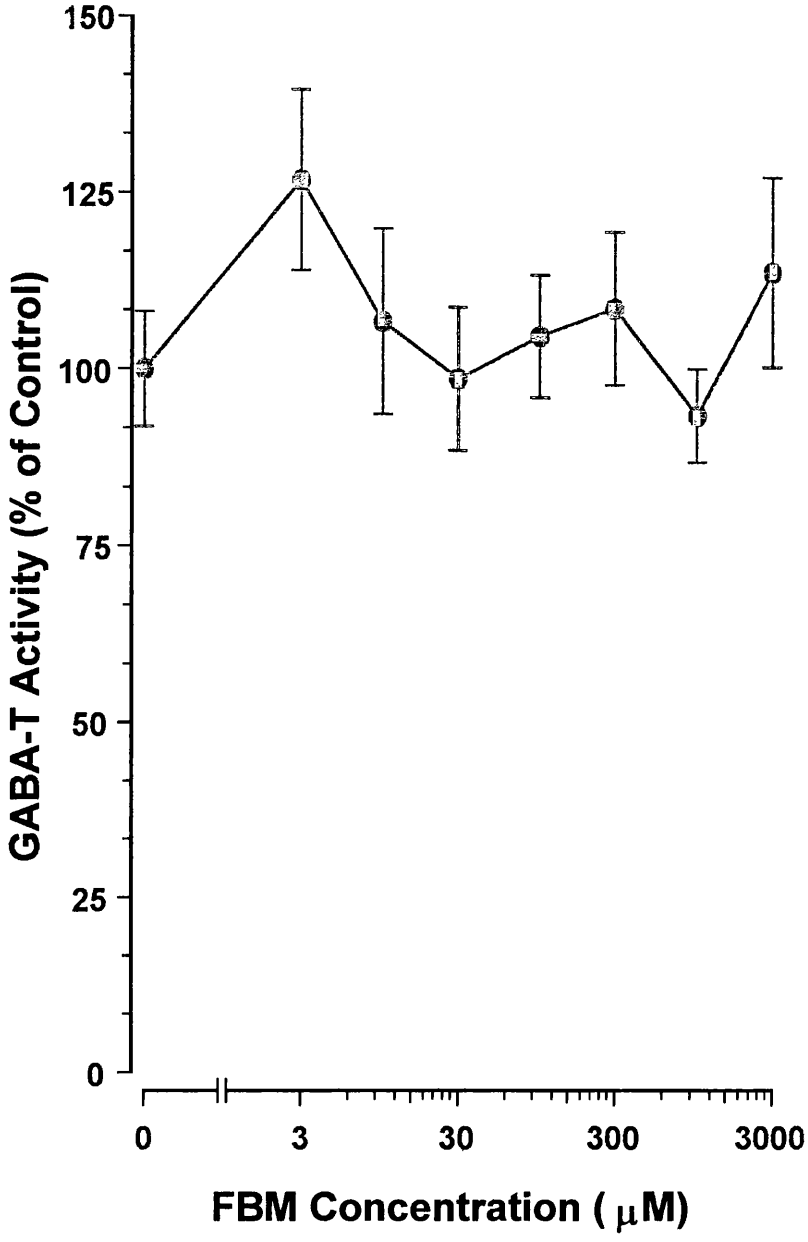


FIGURE 32. Effect of felbamate (FBM; 0 - 3000 μM) on the activity of GABA-transaminase (GABA-T) in primary cultures of rat cortical astrocytes. Results (n = 8) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

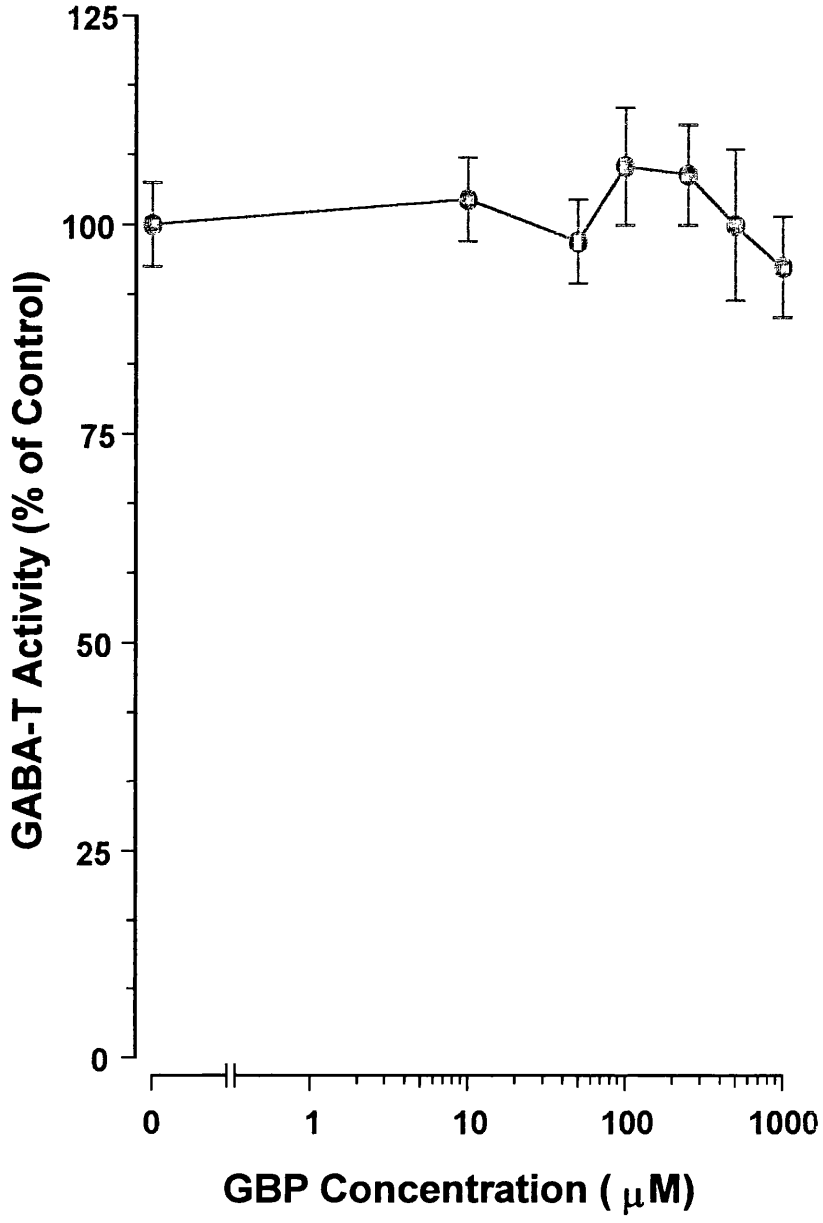


FIGURE 33. Effect of gabapentin (GBP; 0 - 1000 μM) on the activity of GABA-transaminase (GABA-T) in primary cultures of rat cortical astrocytes. Results (n = 8) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

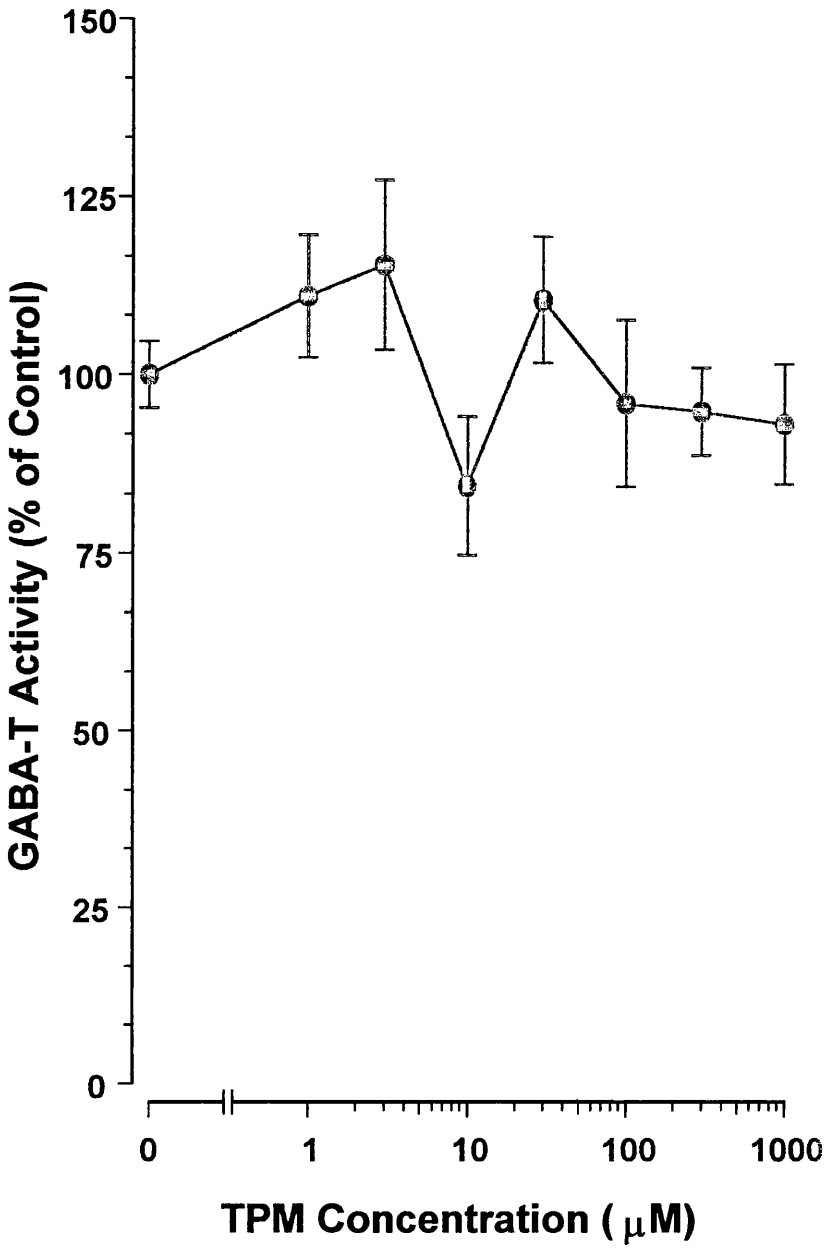


FIGURE 34. Effect of topiramate (TPM; 0 - 1000 μM) on the activity of GABA-transaminase (GABA-T) in primary cultures of rat cortical astrocytes. Results (n = 8) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

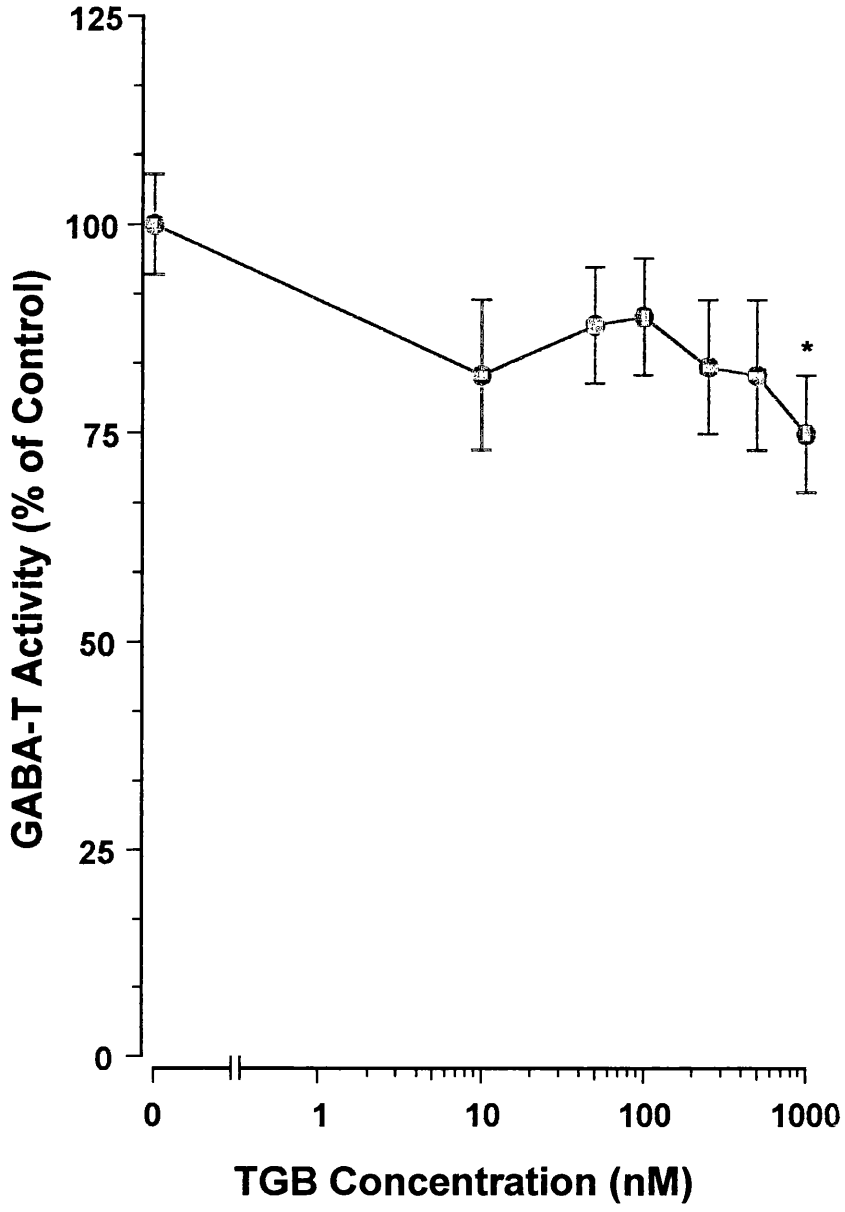


FIGURE 35. Effect of tiagabine (TGB; 0 - 1000 nM) on the activity of GABA-transaminase (GABA-T) in primary cultures of rat cortical astrocytes. Results (n = 8) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.). Statistical significance (* $P < 0.05$) was determined by one way analysis of variance with Dunnett correction for multiple comparisons.

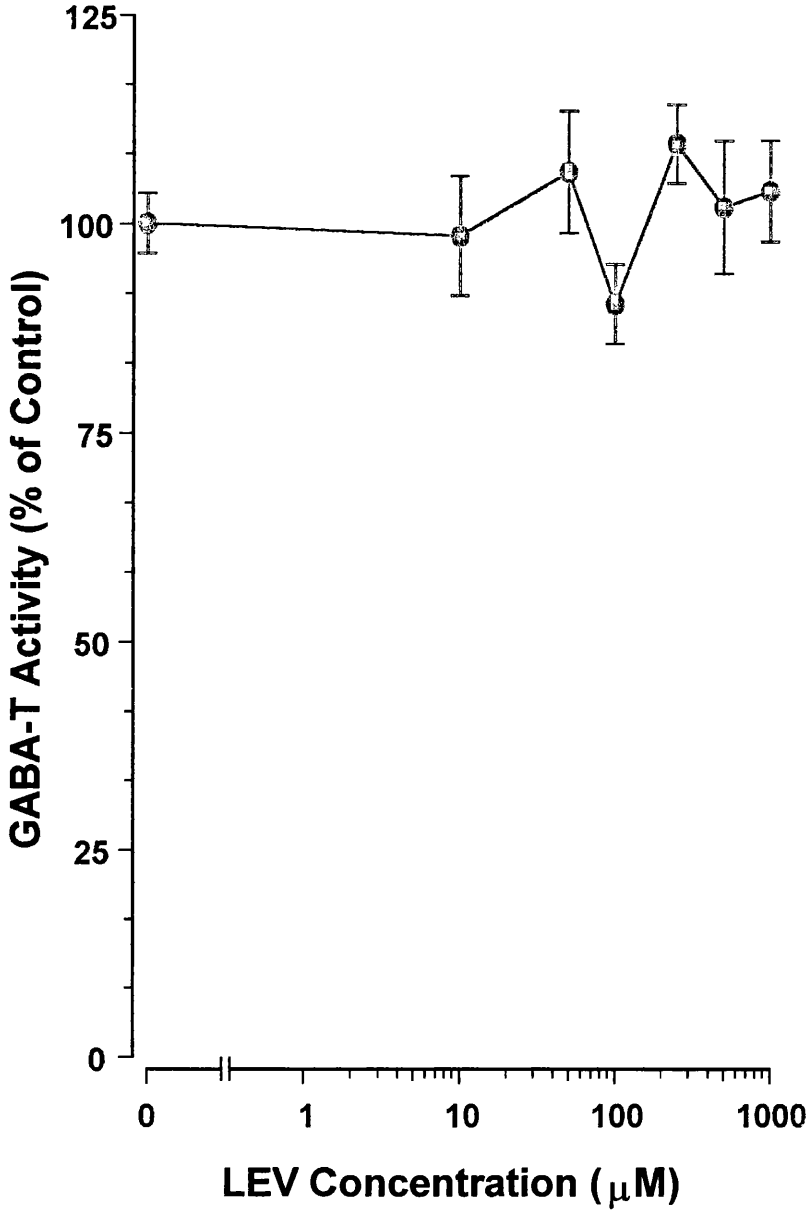


FIGURE 36. Effect of levetiracetam (LEV; 0 - 1000 μM) on the activity of GABA-transaminase (GABA-T) in primary cultures of rat cortical astrocytes. Results (n = 8) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

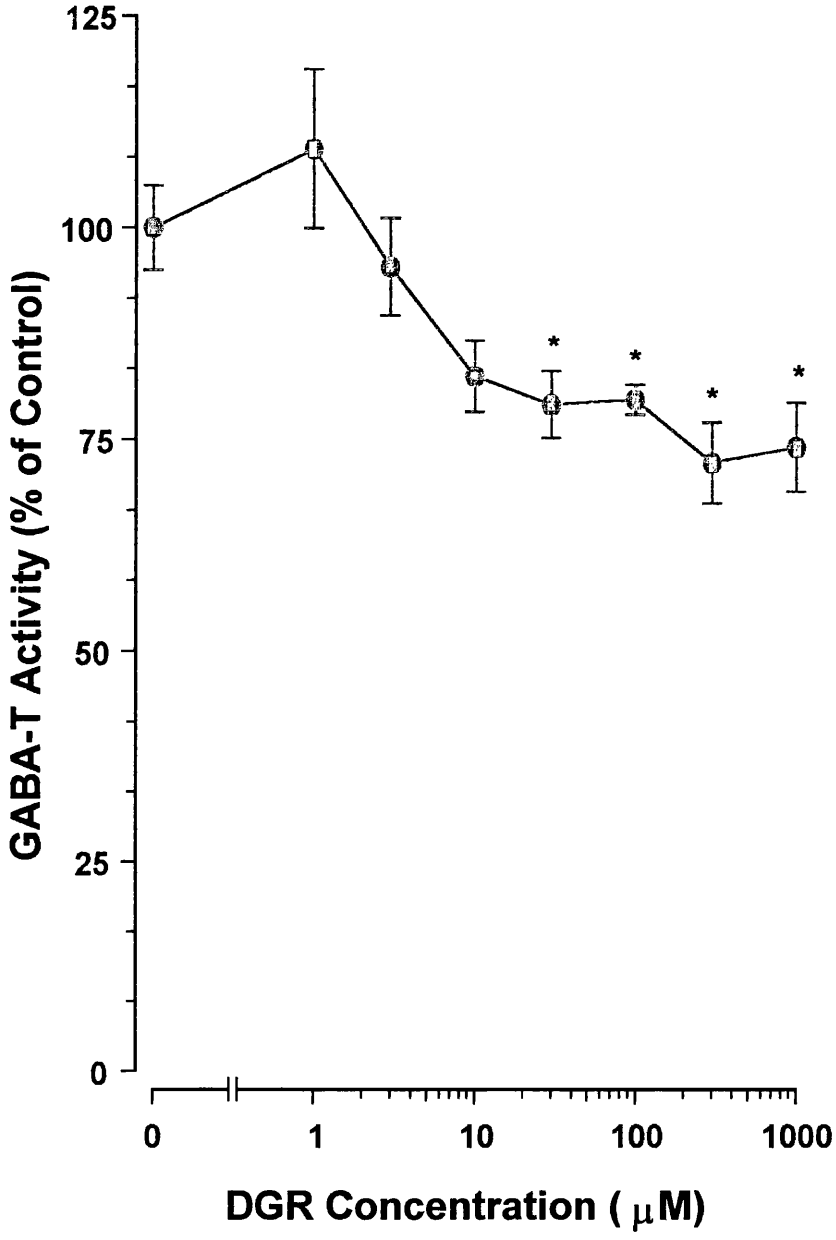


FIGURE 37. Effect of desglycinyl-remacemide (DGR; 0 - 1000 μM) on the activity of GABA-transaminase (GABA-T) in primary cultures of rat cortical astrocytes. Results (n = 8) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.). Statistical significance (* $P < 0.05$) was determined by one way analysis of variance with Dunnett correction for multiple comparisons.

TABLE 1. Effects of antiepileptic drugs on the uptake of GABA and glutamate and GABA-transaminase activity in primary cultures of rat cortical astrocytes following a one hour exposure - summary of results (NS = no significant effect, SIG ↓ = significant reduction $p < 0.05$).

AED	GABA uptake	Glutamate uptake	GABA-transaminase
Phenobarbitone (1 - 1000 μM)	NS	1 – 10 μM SIG ↓	NS
Phenytoin (1 - 1000 μM)	NS	NS	NS
Carbamazepine (1 - 1000 μM)	NS	NS	NS
Sodium valproate (3 - 5000 μM)	50 – 5000 μM SIG ↓	NS	5000 μM SIG ↓
Vigabatrin (1 - 1000 μM)	100 – 1000 μM SIG ↓	NS	50 – 1000 μM SIG ↓
Lamotrigine (0.3 - 300 μM)	NS	NS	NS
Felbamate (3 - 3000 μM)	3 – 3000 μM SIG ↓	NS	NS
Gabapentin (1 - 1000 μM)	NS	NS	NS
Topiramate (1 - 1000 μM)	NS	NS	NS
Tiagabine (0.01 - 100 μM)	0.01 – 1 μM SIG ↓	1 & 30 μM SIG ↓	1 μM SIG ↓
Levetiracetam (1 - 1000 μM)	NS	NS	NS
Desglycinyl remacemide (1 - 1000 μM)	NS	10, 300 & 1000 μM SIG ↓	30 – 1000 μM SIG ↓

3.4 DISCUSSION

The aim of these studies was to determine the effects of a range of AEDs on the transport of GABA and glutamate, and the metabolism of GABA, in primary cultures of rat cortical astrocytes.

3.4.1 Effects of antiepileptic drugs on the transport of GABA in primary cultures of rat cortical astrocytes

SVP, VGB, FBM and TGB were all found to significantly reduce the uptake of GABA into primary cultures of rat cortical astrocytes. SVP, at all concentrations examined was found to reduce the transport of GABA into these cells. This effect is in agreement with a previous study, where Nilsson and colleagues (1992) found that acute treatment of rat cortical astrocytes with SVP resulted in a decreased affinity for the transport of GABA. In addition to the documented inhibition of GABA-T (Lippert *et al*, 1997; Jacob *et al*, 1990), VGB was also found to reduce the transport of GABA into cultured rat astrocytes. This result is consistent with the earlier study carried out in our laboratory (Leach *et al*, 1996), and adds further weight to the proposal that VGB does not exert its effects via a single mechanism of action. At all doses examined, FBM significantly reduced the uptake of GABA following a one hour exposure. This effect has not previously been reported and is in addition to several other possible mechanisms of action of the drug. These include enhancement of $GABA_A$ receptor-mediated responses (Rho *et al*, 1994), interaction with the strychnine-insensitive glycine modulatory site of the NMDA receptor (McCabe *et al*, 1993) and inhibitory effects on voltage-dependent sodium channels (White *et al*, 1992) and dihydropyridine-sensitive calcium channels (Stefani *et al*, 1996). The reduction in GABA uptake exhibited by TGB in this study is in agreement with its documented mechanism of

action (Suzdak and Jansen, 1995). All other drugs examined were found to have no significant effect on the transport of GABA following a one hour exposure. The failure to observe significant effects with PB, PHT and CBZ may possibly be due to the large standard errors present. This may be a result of batch variation in cultures as all graphed results in this chapter are pooled from two separate batches of cultured cells. The failure of GBP to exert an effect on GABA uptake in this study is in keeping with the experiments of Su and colleagues (1995), who reported that GBP exerted no effect on the uptake of GABA in glial or neuronal cultures. The lack of effect of TPM on GABA uptake in this study is not in agreement with a recent study conducted in our laboratory, where TPM was found to significantly reduce the uptake of GABA into primary cultures of rat cortical astrocytes (Sills *et al*, 1998). The reason for this discrepancy is unknown, but may be due to heterogeneity between batches of cultured astrocytes. Further work is required to clarify the actions of TPM on GABA uptake.

3.4.2 Effects of antiepileptic drugs on the transport of glutamate in primary cultures of rat cortical astrocytes

The lowest three concentrations of PB (1, 3 and 10 μM) examined in this study significantly reduced glutamate uptake into cultured rat cortical astrocytes. This effect was not, however, evident at the higher concentrations of 30 – 1000 μM. The reason for the lack of effect at higher concentrations is unknown, but may indicate that at higher concentrations PB is exerting additional effects which may be acting in a compensatory manner. It remains possible that a PB-induced *e.g.* reduction in the synthesis or an increase in the metabolism of glutamate would mask any reduction in glutamate uptake exerted by the drug. Concentrations of 1 and 30 μM TGB were found to exert inhibitory effects on the transport of glutamate into cultured rat

astrocytes. This effect has not been reported previously. All other concentrations (0.1, 0.3, 3, 10 and 100 μM) of TGB examined in this study failed to exert a significant effect on glutamate transport. Further work is required to clarify the actions of TGB on glutamate transport and to determine the clinical relevance of the effect. Concentrations of 10, 300 and 1000 μM DGR exerted inhibitory effects on the transport of glutamate into cultured rat astrocytes, whereas 1, 3, 30 and 100 μM were without effect. The reduction in glutamate uptake induced by DGR has not previously been reported. Due to the lack of concentration-dependency of the effect and the large standard errors present, it appears unlikely that this effect would contribute to the clinical efficacy of the drug. It is possible that this effect is an artefact of the culture process and not an AED-induced effect. However, no conclusions can be made without further work. All other drugs examined had no significant effect on the transport of glutamate following a one hour exposure.

3.4.3 Effects of antiepileptic drugs on the metabolism of GABA in primary cultures of rat cortical astrocytes

SVP, VGB, TGB and DGR all significantly reduced the activity of GABA-T in primary cultures of rat cortical astrocytes. The reduction induced by SVP has been reported previously (Larsson *et al*, 1986). However, as it occurred only at the highest concentration (5000 μM), it may not contribute to the clinical efficacy of the drug. The reduction in GABA-T exhibited by VGB reproduced the recognised mechanism of action of the drug (Lippert *et al*, 1977; Jacob *et al*, 1990). At the highest concentration examined (1000 nM), TGB was also found to reduce the activity of the enzyme. This effect has not previously been reported. TGB reportedly exhibits its antiepileptic

actions via a single action, namely inhibition of GABA uptake (Suzdak and Jansen, 1995). This study would suggest that the drug does not act via a single effect, and that it may too possess multiple mechanisms of action. The reduction in enzyme activity induced by the experimental drug DGR is a previously unreported effect. DGR reduced GABA-T activity at concentrations of 30, 100, 300 and 1000 μM, suggesting that this effect may contribute to the clinical actions of the drug. All other AEDs examined had no effect on GABA-T activity following a one hour exposure.

3.5 CONCLUSIONS

In conclusion, several of the AEDs examined in this study had previously unreported effects on the uptake of GABA and glutamate and on the metabolism of GABA. Inhibition of GABA uptake is the reported mechanism of action of TGB, but additionally may contribute to the clinical efficacy of SVP, VGB and FBM. It is unknown if the reduction in glutamate uptake induced by PB, TGB and DGR is involved in the mechanism of action of the drugs. More work is required before any firm conclusions can be drawn from this study. The inhibition of GABA-T exerted by VGB confirms its recognised mechanism of action, but this effect may also contribute to the anticonvulsant effects of DGR. As the reduction in GABA-T activity exerted by SVP occurred only at the highest concentration examined, the clinical relevance of this effect remains to be determined. The TGB-induced inhibition of GABA-T activity suggests that, at higher doses, this may be an additional mechanism of action of the drug. This study would suggest that VGB and TGB may not give rise to their antiepileptic effects via a single mechanism of action as previously thought, and adds to the increasing evidence suggesting that most AEDs have multiple mechanisms of action.

CHAPTER FOUR

EFFECTS OF ANTIEPILEPTIC DRUGS ON GLUTAMINE SYNTHETASE ACTIVITY IN MOUSE BRAIN AND PRIMARY CULTURES OF RAT CORTICAL ASTROCYTES

4.1 INTRODUCTION

4.1.1 Glutamine synthetase

GS is a key enzyme in the regulation of glutamate neurotransmission in the CNS. It catalyzes the synthesis of glutamine from glutamate, and is responsible for the detoxification of ammonia in the brain (Meister, 1974). GS is crucial for nitrogen homeostasis, as the glutamine formed by this enzyme is a constituent of proteins and serves as a nitrogen source for a number of biosynthetic pathways (Meister, 1980). It has been demonstrated to be specifically located in the glial fraction of the brain, primarily in astrocytes (Martinez-Hernandez *et al*, 1977; Norenberg, 1979; Norenberg and Martinez-Hernandez, 1979). Glutamine which is synthesized in astrocytes via GS is then transported into neurones, where it serves as a precursor for the formation of the neurotransmitters glutamate and GABA (Waniewski and Martin, 1986; Waniewski, 1992). GS is said to be of particular importance in the brain, as although glutamate has a number of metabolic enzymes, the only known pathway for the synthesis of glutamine is via GS (Cooper *et al*, 1983). However, the direct uptake of glutamine from the blood would provide a non-enzymatic source of glutamine independent of GS.

4.1.2 Glutamine synthetase and epilepsy

It is well documented that systemic administration of MSO, the classical inhibitor of GS, precipitates seizures in many species (Peters and Tower, 1959; Sellinger *et al*, 1984; Swanson *et al*, 1990). The convulsant activity of MSO has been attributed to its inhibition of GS (Meister, 1980), where an increase in glutamate and enhanced excitation at neuronal sites is thought to be responsible for seizure activity. It has been

demonstrated that genetically epilepsy-prone rats have a significantly lower brain GS activity than control rats, and this has been proposed to result in the increased seizure susceptibility exhibited by these animals (Carl *et al*, 1993). Seizure-prone gerbils have also been shown to exhibit a reduced brain GS activity compared to normal gerbils (Laming *et al*, 1989), which adds further support to the link between a reduction in GS and seizure activity.

4.2 AIMS

It has been reported that the AED SVP stimulates GS activity in rat cortical and cerebellar homogenates (Nolan *et al*, 1985; Phelan *et al*, 1985). In light of the finding that GS may be involved in AED action, and the evidence suggesting a role in seizure generation, the aim of this study was to investigate the effects of single and repeated administration of a range of established, new and experimental AEDs on enzyme activity in mouse brain. In addition, due to the glial location of the enzyme, the effects of these drugs on GS activity was determined in primary cultures of rat cortical astrocytes.

4.3 EXPERIMENTAL PROTOCOLS AND RESULTS

4.3.1 Validation of the glutamine synthetase assay in mouse brain

EXPERIMENTAL PROTOCOL: The brains were removed from two adult male ICR mice, and samples prepared for assay as described in section 2.10.3. Samples were randomly separated into five groups (n = 4 / group) and MSO (0.1, 0.3, 1, 3 and 10 mM) was incorporated into the reaction mixture. A sixth group (control; n = 4) of samples had no MSO added. GS activity was determined as described in section 2.10.

RESULTS: All concentrations (0.1, 0.3, 1, 3 and 10 mM) of MSO examined were found to significantly ($P < 0.05$) reduce the activity of GS in mouse brain (figure 38).

4.3.2 Glutamine synthetase activity versus protein concentration in mouse brain

EXPERIMENTAL PROTOCOL: The brains were removed from two adult male ICR mice, and samples prepared for assay as described in section 2.10.3. Protein levels were determined as described in section 2.6, and diluted appropriately to give the following concentrations: 0.5, 1, 1.5, 2, 2.5, 3 and 6 mg/ml (n = 4 / concentration) GS activity was determined as described in section 2.10.

RESULTS: A protein concentration of 2 mg/ml was found to be the optimal for GS activity in mouse brain (figure 39).

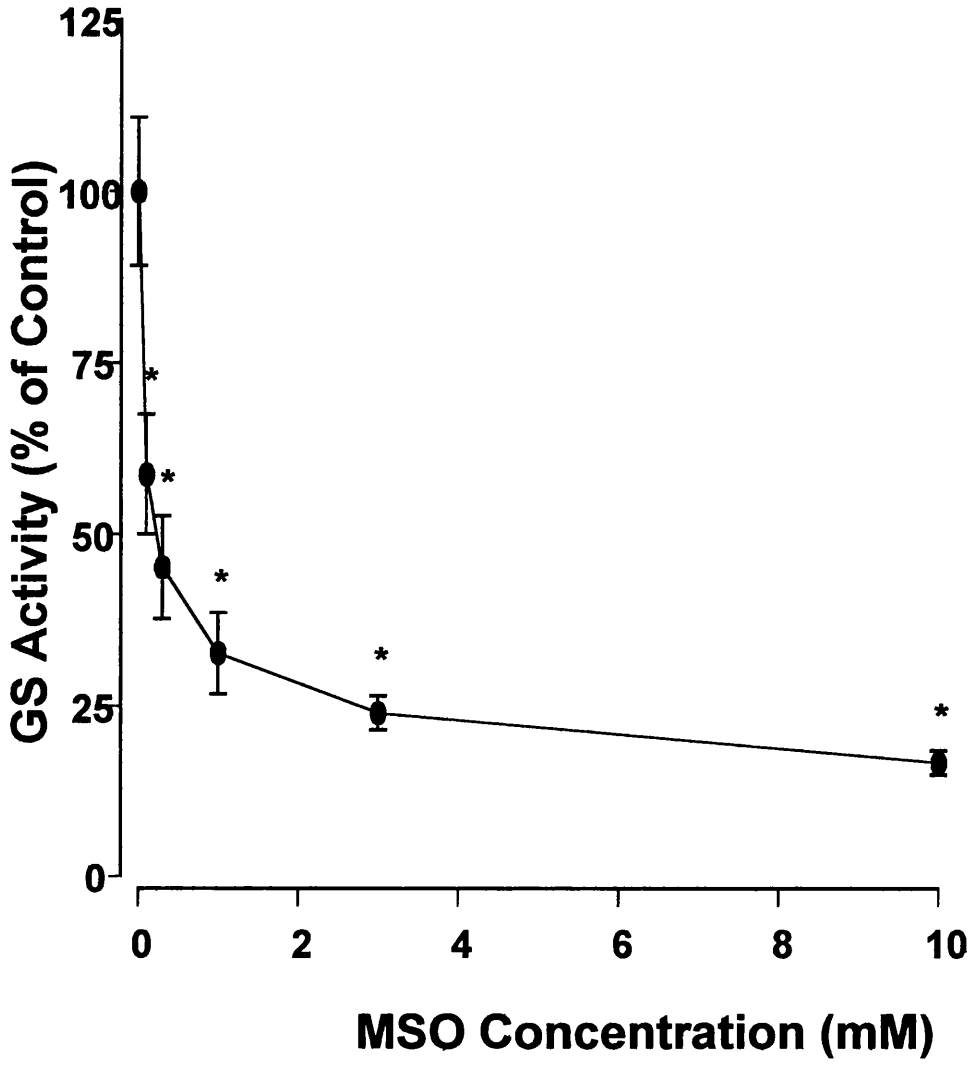


FIGURE 38. Effect of methionine sulfoximine (MSO; 0.1 - 10 mM) on the activity of glutamine synthetase (GS) in mouse brain. Results (n = 4) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.). Statistical significance (* $P < 0.05$) was determined by one way analysis of variance with Dunnett correction for multiple comparisons.

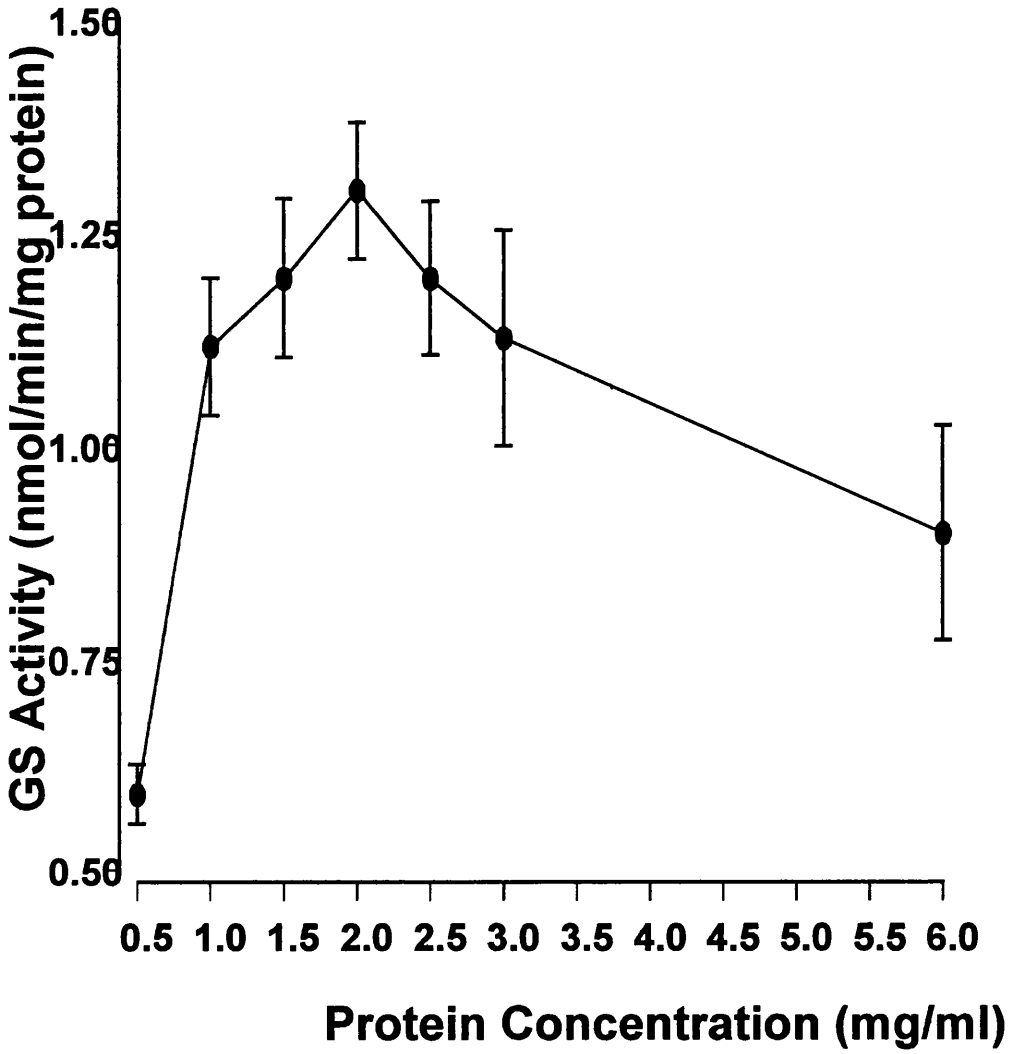


FIGURE 39. Effect of protein concentration on the activity of glutamine synthetase (GS) in mouse brain. Results (n = 4) are expressed as nmol / min / mg protein and error bars denote the standard error of the mean (S.E.M.).

4.3.3 Effects of antiepileptic drugs on glutamine synthetase activity in mouse brain

EXPERIMENTAL PROTOCOL: All drugs were prepared daily for i.p. injection. SVP, VGB, LTG, GBP, TGB, LEV and DGR were dissolved in 0.9% saline. PB, PHT, CBZ and TPM were prepared as a suspension in 0.5% TWEEN 80. FBM was suspended in 30 / 70 (v/v) PEG 400 / water. For each of the 12 drug studies, adult male ICR mice were randomised into six groups (n = 12 / group). The following doses of drugs were administered:

PB:- 0.1, 0.3, 1, 3, 10 and 30 mg/kg

PHT:- 0.3, 1, 3, 10, 30 and 100 mg/kg

CBZ:- 0.1, 0.3, 1, 3, 10 and 30 mg/kg

SVP:- 3, 10, 30, 100, 300 and 1000 mg/kg

VGB:- 3, 10, 30, 100, 300 and 1000 mg/kg

LTG:- 0.3, 1, 3, 10, 30 and 100 mg/kg

FBM:- 1, 3, 10, 30, 100 and 300 mg/kg

GBP:- 1, 3, 10, 30, 100 and 300 mg/kg

TPM:- 0.3, 1, 3, 10, 30 and 100 mg/kg

TGB:- 0.1, 0.3, 1, 3, 10 and 30 mg/kg

LEV:- 1, 3, 10, 30, 100 and 300 mg/kg

DGR:- 0.3, 1, 3, 10, 30 and 100 mg/kg

For each drug experiment, a control group (n = 12) received the appropriate vehicle alone. Four hours after the first dose, six animals from each group were sacrificed, and their brains removed and stored at -70^{0}C until required for assay. In the six remaining mice from each group, treatment was continued twice daily (8 am and 4 pm) for five days, with the exception of VGB which was administered once daily. At four hours

after the final dose of the repeated treatment regimen, the remaining animals were sacrificed and their brains removed and stored at -70^{0}C until required for assay. A general four hour time period between injecting and sacrificing was chosen to allow time for the drug to be absorbed, enter the brain and an effect on enzyme levels to be seen. GS activity was determined as described in section 2.10. TPM doses of 300 and 1000 mg/kg were not examined in this study due to adverse effects (see chapter 5).

RESULTS: Results are summarised in table 2. Both single and repeated treatments with PHT (figure 41) and CBZ (figure 42) significantly ($P < 0.05$) reduced the activity of GS four hours after the final dose. No results are shown for repeated 100 mg/kg PHT, as treatment was discontinued in this group due to adverse effects. Single doses of PB (figure 40), FBM (figure 46) and TPM (figure 48) were without effect on enzyme activity, although repeated administration of these drugs significantly ($P < 0.05$) reduced GS activity at 4 h post-administration. Single and repeated treatments with SVP (figure 43), VGB (figure 44), LTG (figure 45), GBP (figure 47), TGB (figure 49), LEV (figure 50), and DGR (figure 51) were without effect on mouse brain GS activity at four hours post-administration.

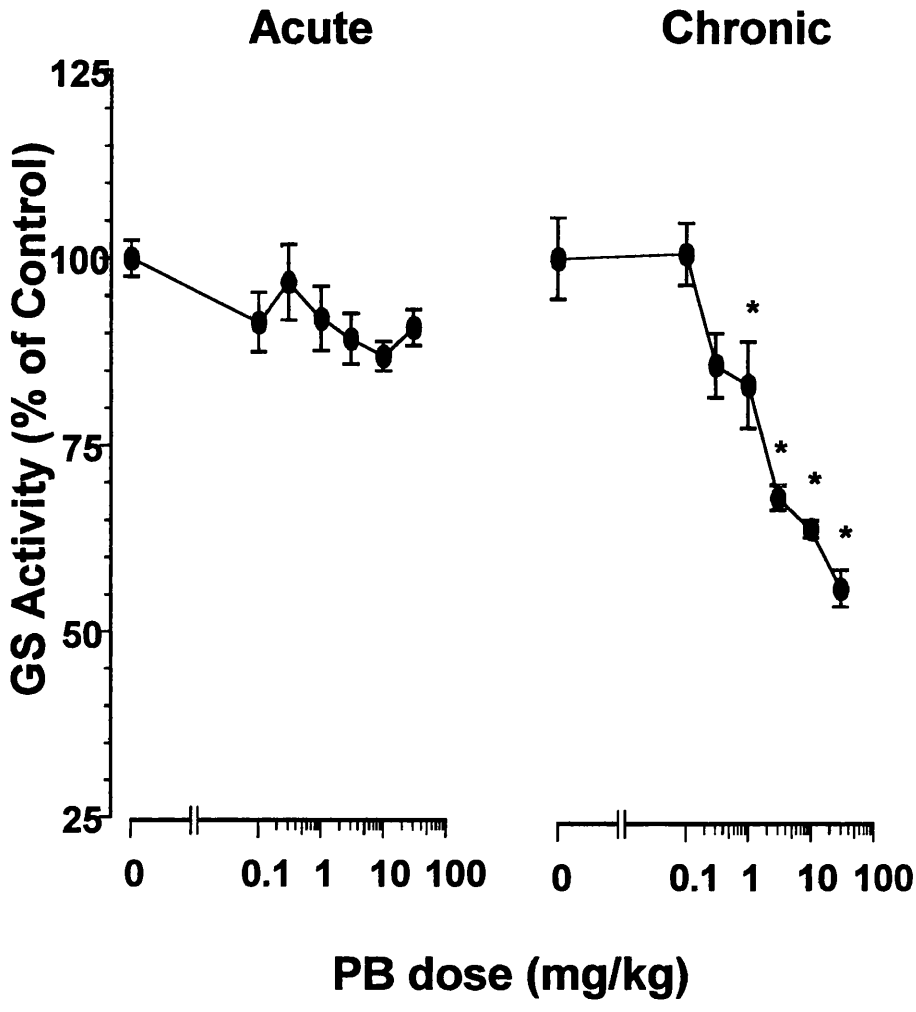


FIGURE 40. Effect of phenobarbitone (PB; 0 - 30 mg/kg) on mouse brain glutamine synthetase (GS) activity at four hours after acute (single dose; left) and chronic (twice daily for five days; right) administration. Results (n = 6) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.). Statistical significance (*$P < 0.05$) was determined by one way analysis of variance with Dunnett correction for multiple comparisons.

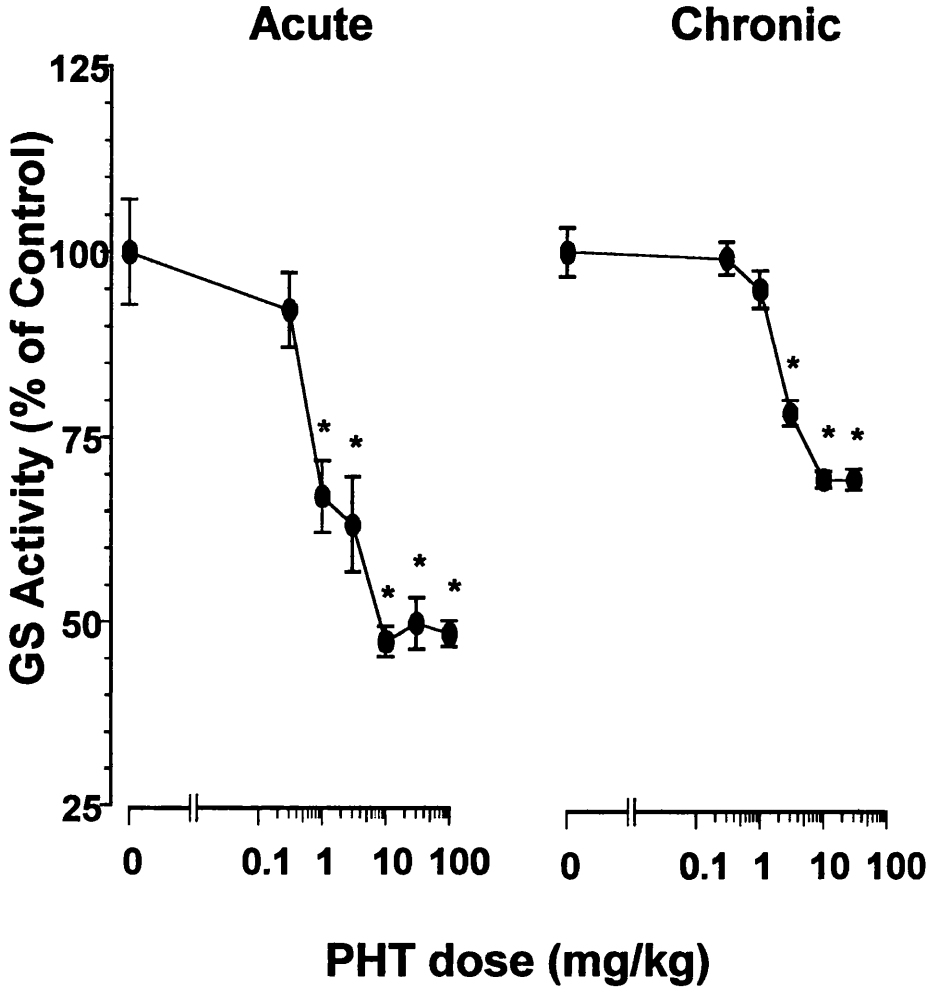


FIGURE 41. Effect of phenytoin (PHT; 0 - 100 mg/kg) on mouse brain glutamine synthetase (GS) activity at four hours after acute (single dose; left) and chronic (twice daily for five days; right) administration. Results (n = 6) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.). Statistical significance (*$P < 0.05$) was determined by one way analysis of variance with Dunnett correction for multiple comparisons.

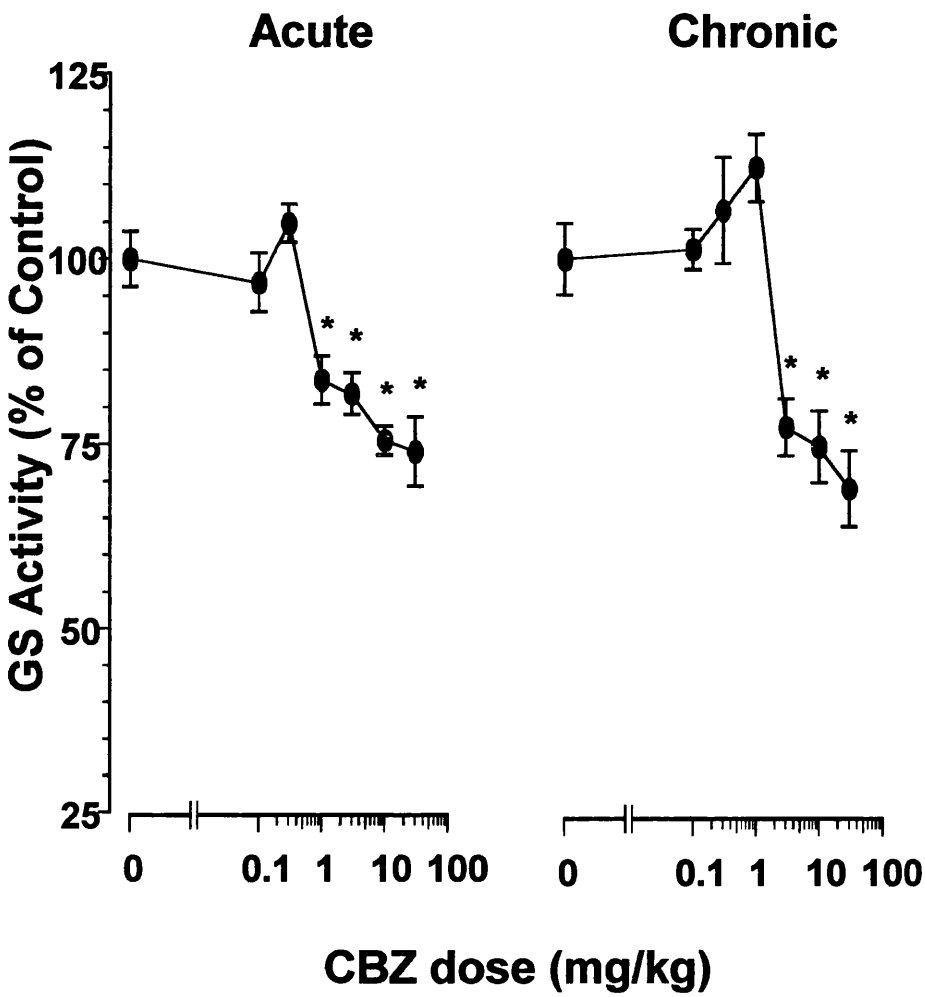


FIGURE 42. Effect of carbamazepine (CBZ; 0 - 30 mg/kg) on mouse brain glutamine synthetase (GS) activity at four hours after acute (single dose; left) and chronic (twice daily for five days; right) administration. Results (n = 6) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.). Statistical significance (*$P < 0.05$) was determined by one way analysis of variance with Dunnett correction for multiple comparisons.

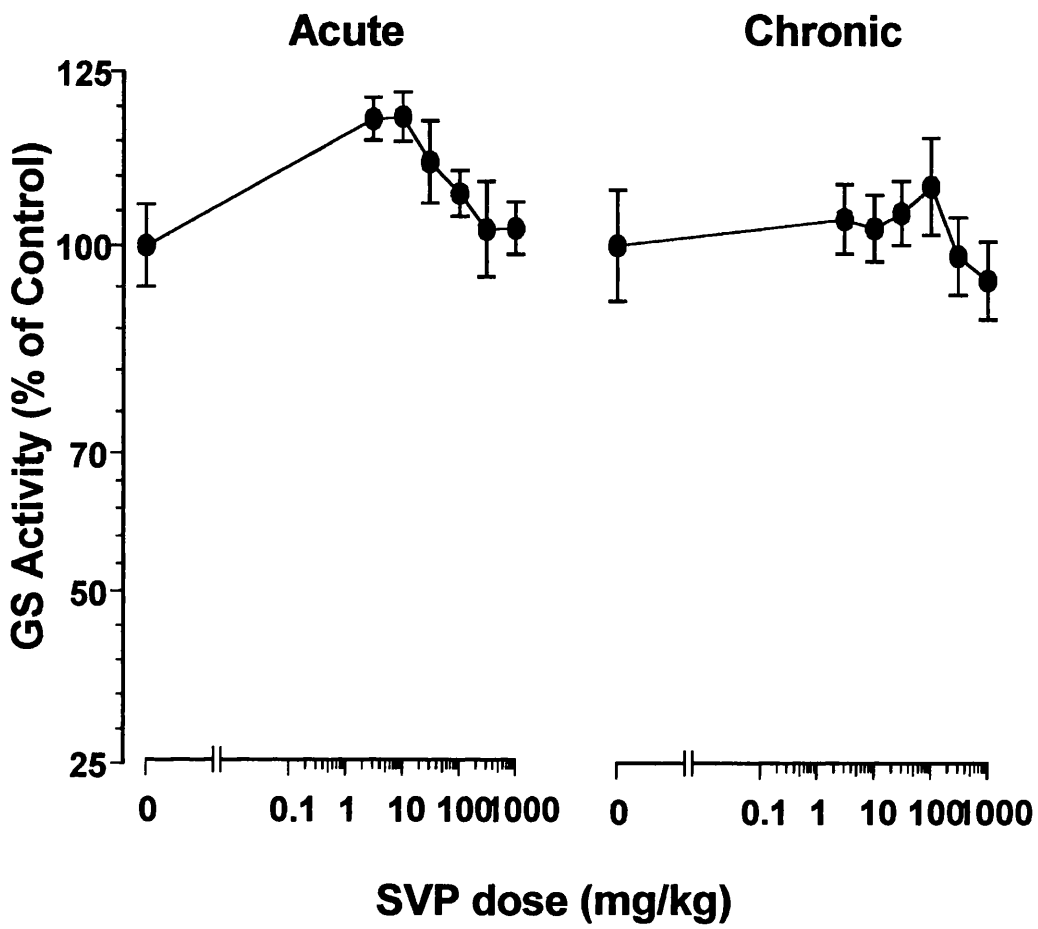


FIGURE 43. Effect of sodium valproate (SVP; 0 - 1000 mg/kg) on mouse brain glutamine synthetase (GS) activity at four hours after acute (single dose; left) and chronic (twice daily for five days; right) administration. Results (n = 6) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

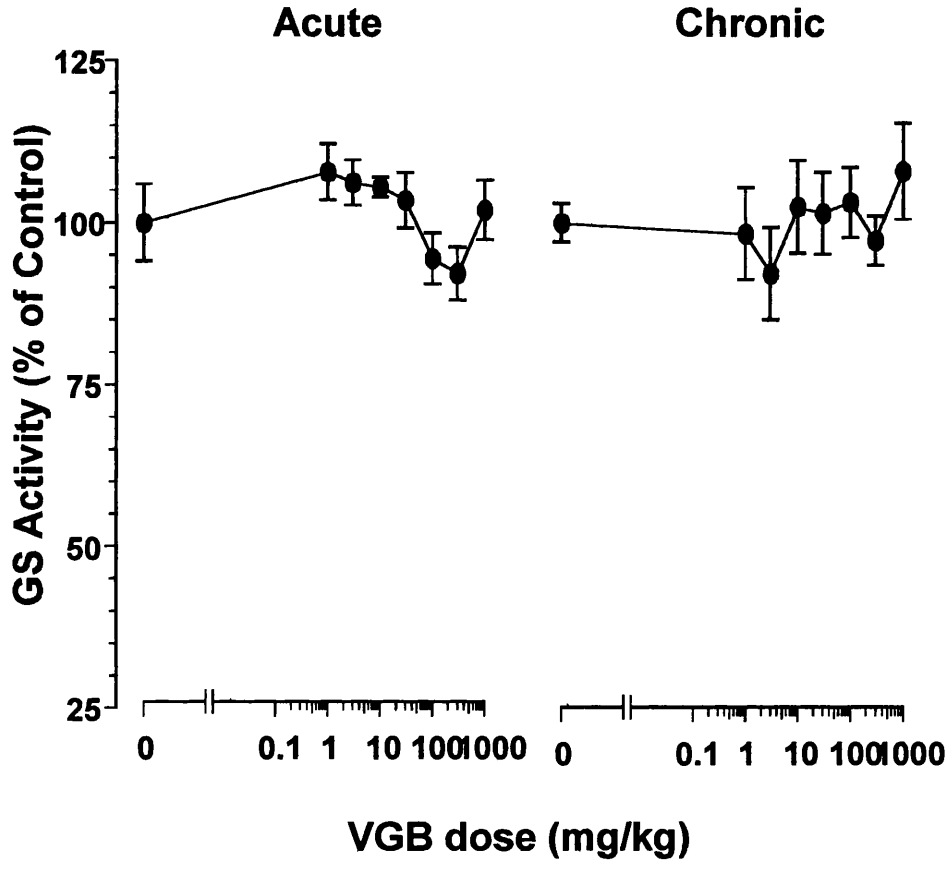


FIGURE 44. Effect of vigabatrin (VGB; 0 - 1000 mg/kg) on mouse brain glutamine synthetase (GS) activity at four hours after acute (single dose; left) and chronic (once daily for five days; right) administration. Results (n = 6) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

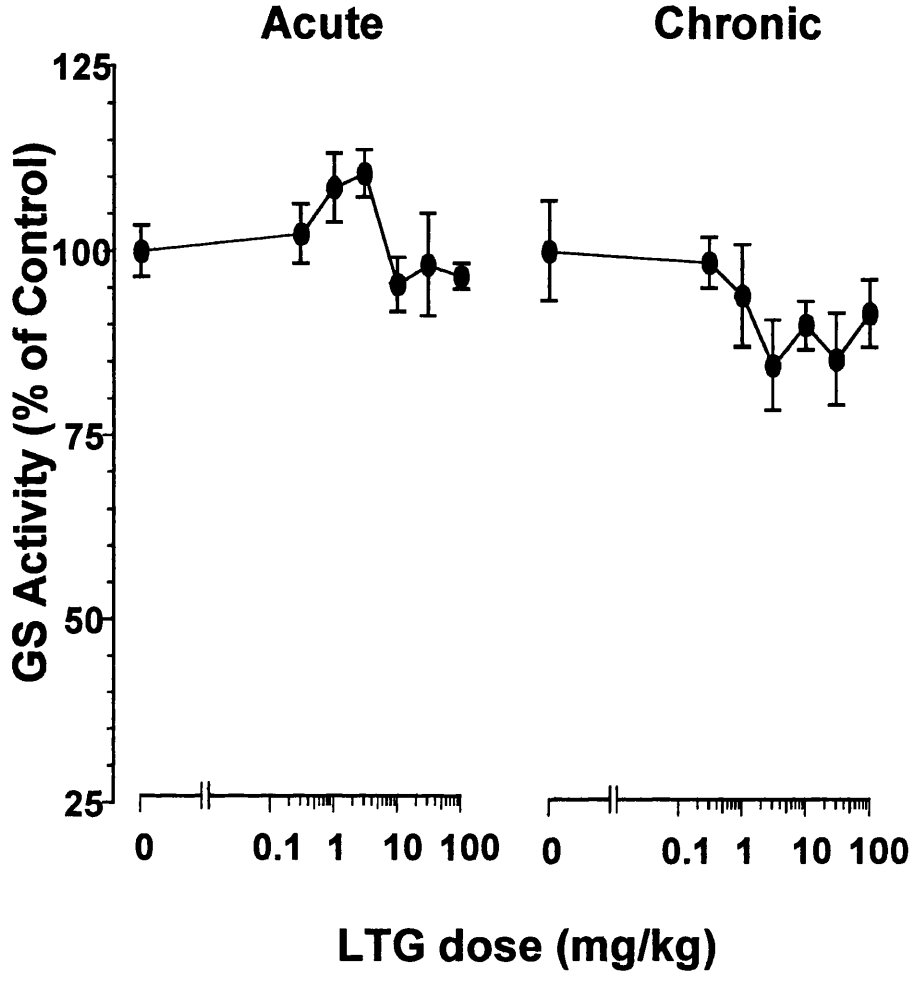


FIGURE 45. Effect of lamotrigine (LTG; 0 - 100 mg/kg) on mouse brain glutamine synthetase (GS) activity at four hours after acute (single dose; left) and chronic (twice daily for five days; right) administration. Results (n = 6) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

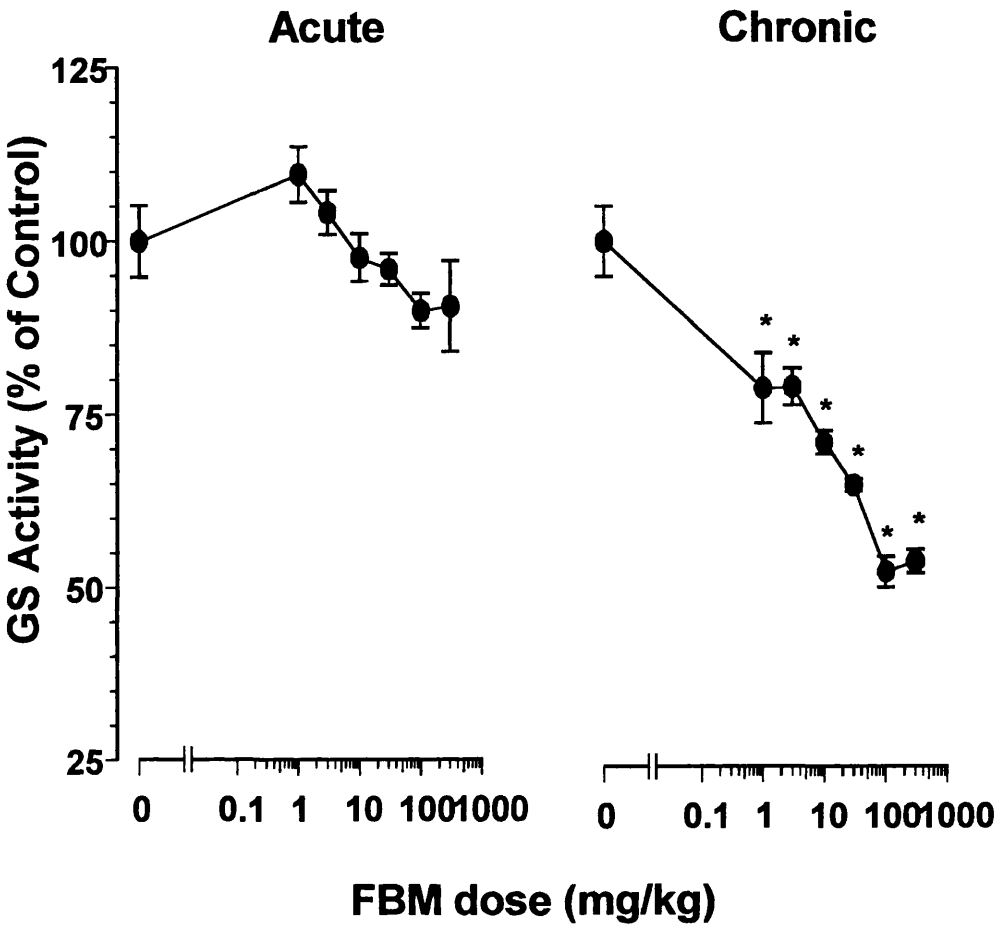


FIGURE 46. Effect of felbamate (FBM; 0 - 300 mg/kg) on mouse brain glutamine synthetase (GS) activity at four hours after acute (single dose; left) and chronic (twice daily for five days; right) administration. Results (n = 6) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.). Statistical significance (*$P < 0.05$) was determined by one way analysis of variance with Dunnett correction for multiple comparisons.

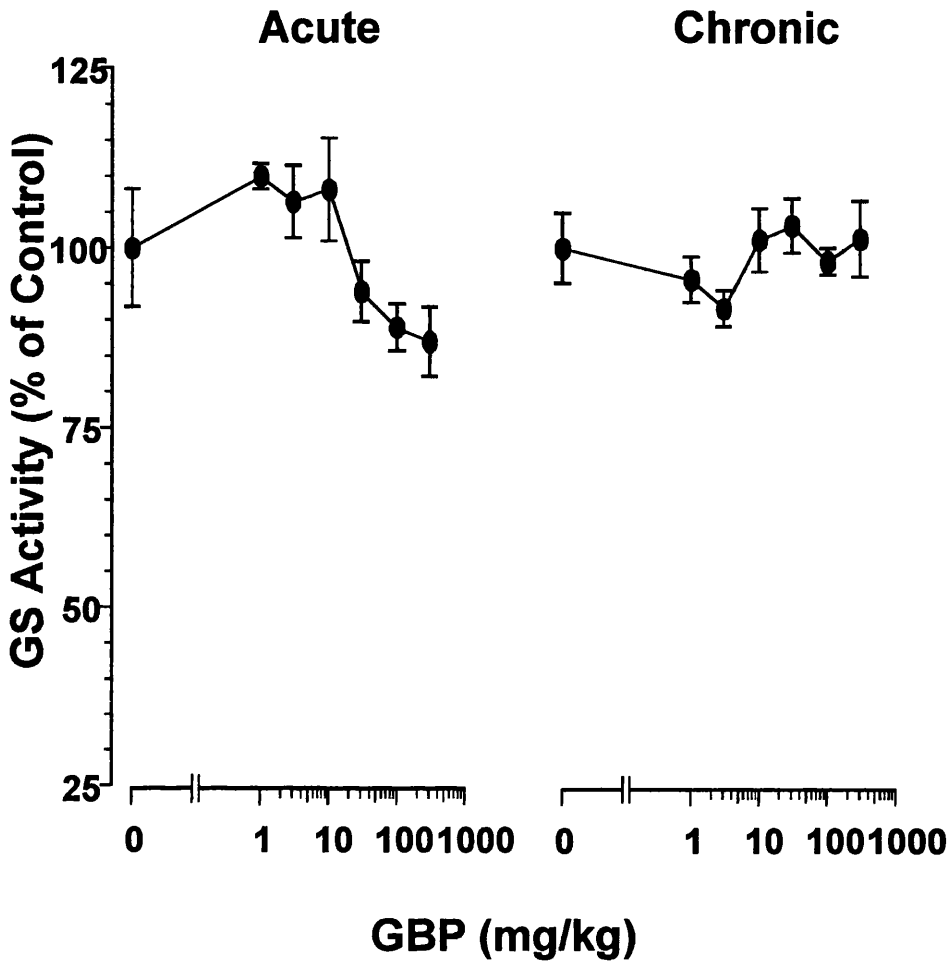


FIGURE 47. Effect of gabapentin (GBP; 0 - 300 mg/kg) on mouse brain glutamine synthetase (GS) activity at four hours after acute (single dose; left) and chronic (twice daily for five days; right) administration. Results (n = 6) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

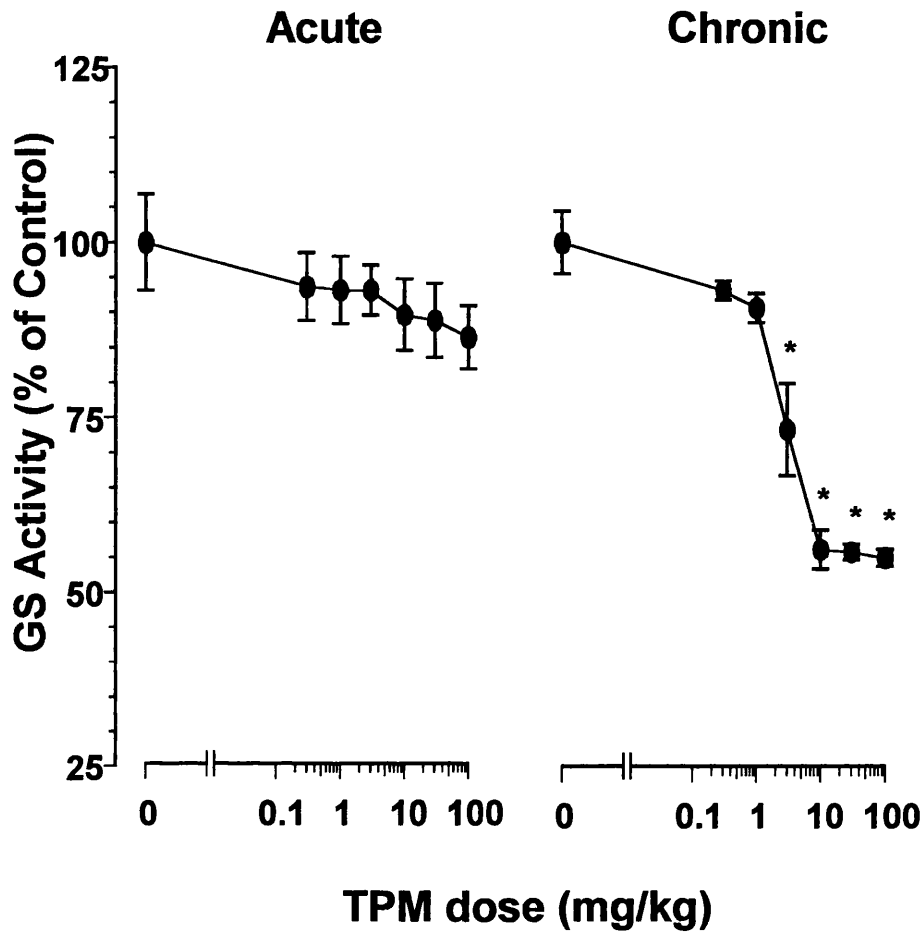


FIGURE 48. Effect of topiramate (TPM; 0 - 100 mg/kg) on mouse brain glutamine synthetase (GS) activity at four hours after acute (single dose; left) and chronic (twice daily for five days; right) administration. Results (n = 6) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.). Statistical significance (*$P < 0.05$) was determined by one way analysis of variance with Dunnett correction for multiple comparisons.

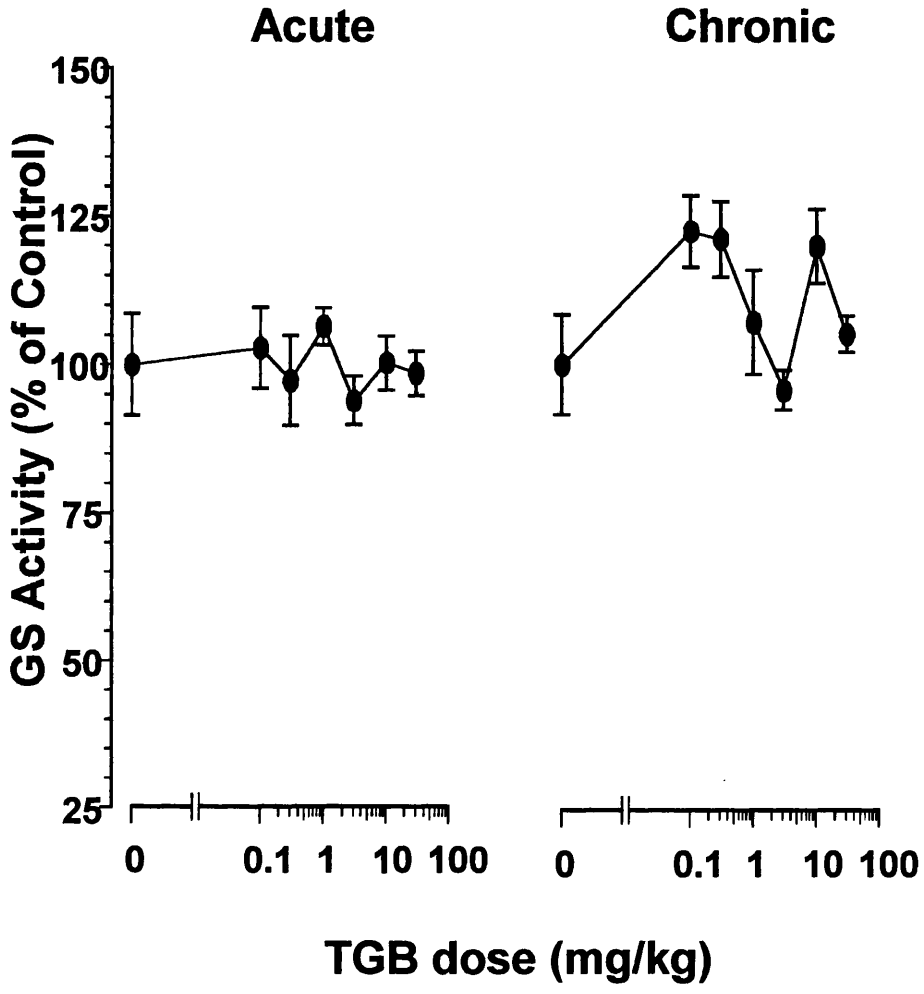


FIGURE 49. Effect of tiagabine (TGB; 0 - 30 mg/kg) on mouse brain glutamine synthetase (GS) activity at four hours after acute (single dose; left) and chronic (twice daily for five days; right) administration. Results (n = 6) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

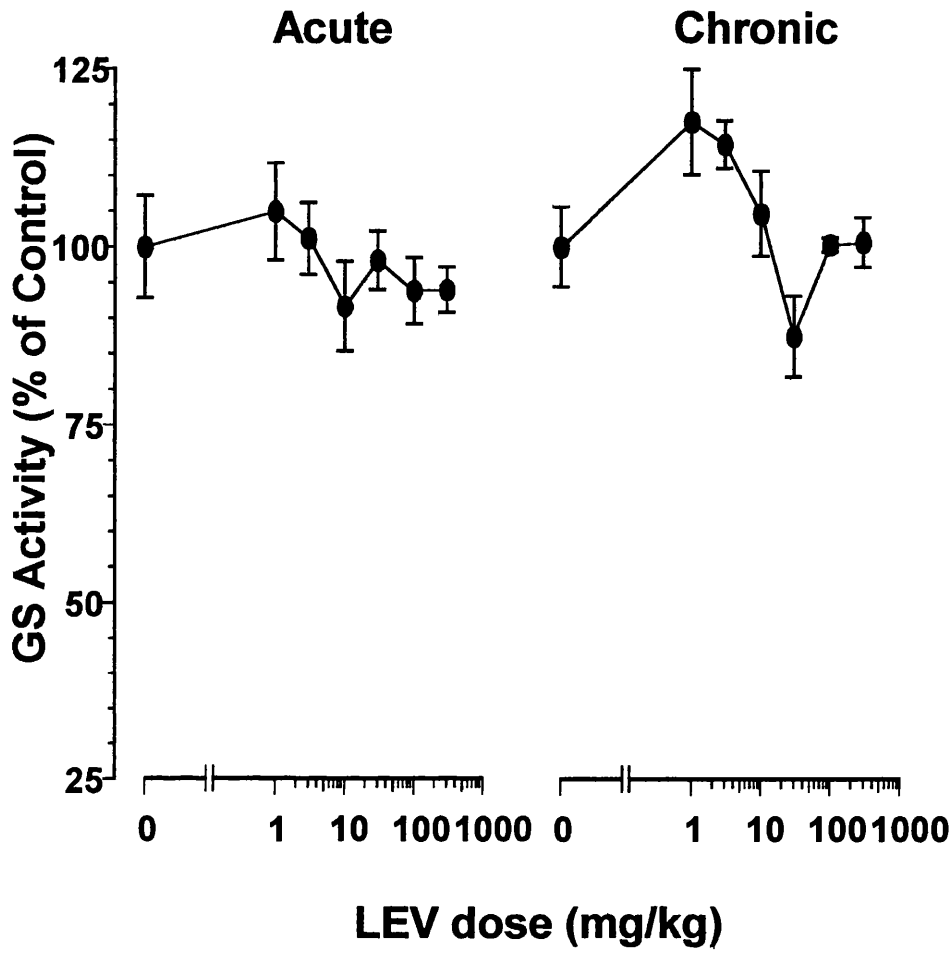


FIGURE 50. Effect of levetiracetam (LEV; 0 - 300 mg/kg) on mouse brain glutamine synthetase (GS) activity at four hours after acute (single dose; left) and chronic (twice daily for five days; right) administration. Results (n = 6) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

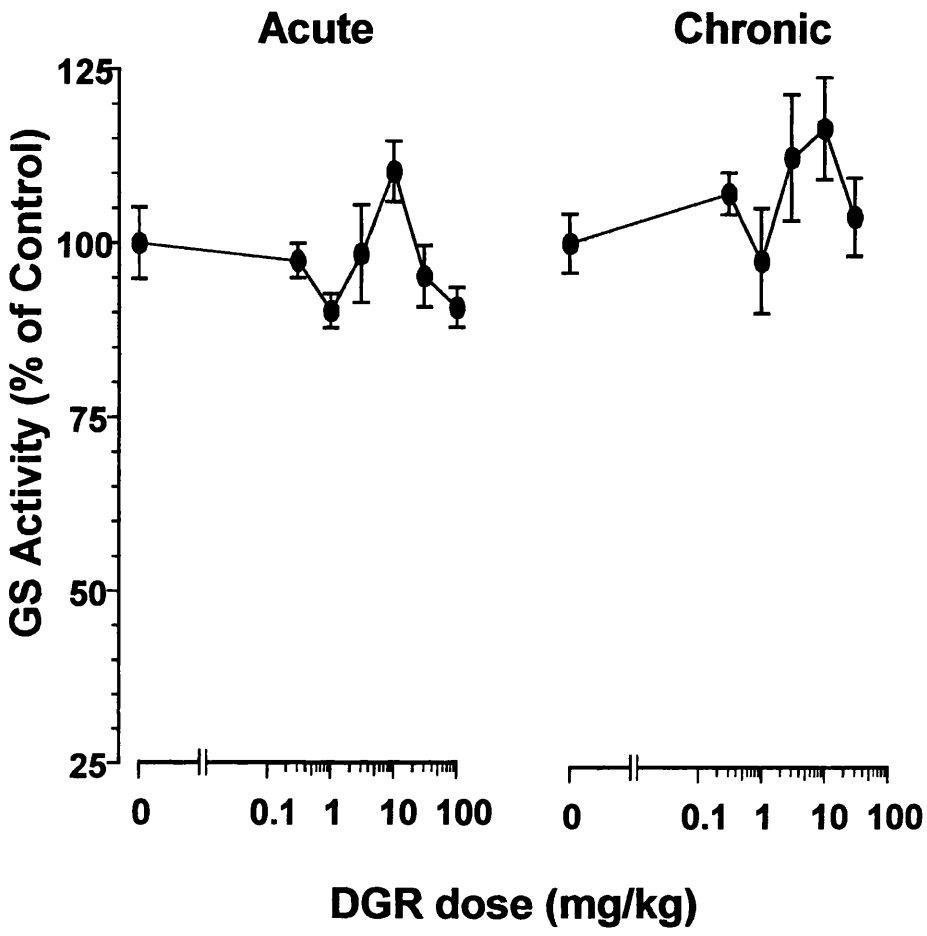


FIGURE 51. Effect of desglycinyl-remacemide (DGR; 0 - 100 mg/kg) on mouse brain glutamine synthetase (GS) activity at four hours after acute (single dose; left) and chronic (twice daily for five days; right) administration. Results (n = 6) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

4.3.4 Validation of the glutamine synthetase assay in primary cultures of rat cortical astrocytes

EXPERIMENTAL PROTOCOL: Rat brain tissue was isolated and cultured as described in section 2.3. Astrocytes were randomly separated into five groups (n = 4 / group) and exposed to 0.1, 0.3, 1, 3 and 10 mM of MSO (the glutamine synthetase inhibitor) in BSS. A sixth group (control; n = 4) of astrocytes were exposed to BSS alone. After a one hour incubation, cells were assayed for GS activity as described in section 2.10.

RESULTS: All concentrations (0.1, 0.3, 1, 3 and 10 mM) of MSO examined were found to significantly ($P < 0.05$) reduce the activity of GS in primary cultures of rat cortical astrocytes (figure 52).

4.3.5 Glutamine synthetase activity versus protein concentration in primary cultures of rat cortical astrocytes

EXPERIMENTAL PROTOCOL: Rat brain was isolated and cultured as described in section 2.3. The existing medium was aspirated and cultures were washed three times with 500 µl BSS. Individual wells were scraped and stored at -70^{0}C in 250 µl 5 mM imidazole buffer. Cultures were then freeze / thawed three times, and vortex mixed. Protein levels were determined as described in section 2.6, and diluted appropriately to give the following concentrations: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7 mg/mg (n = 4 / concentration). GS activity was determined as described in section 2.10.

RESULTS: A protein concentration of 0.1 mg/ml was found to be optimal for GS activity in primary cultures of rat cortical astrocytes (figure 53).

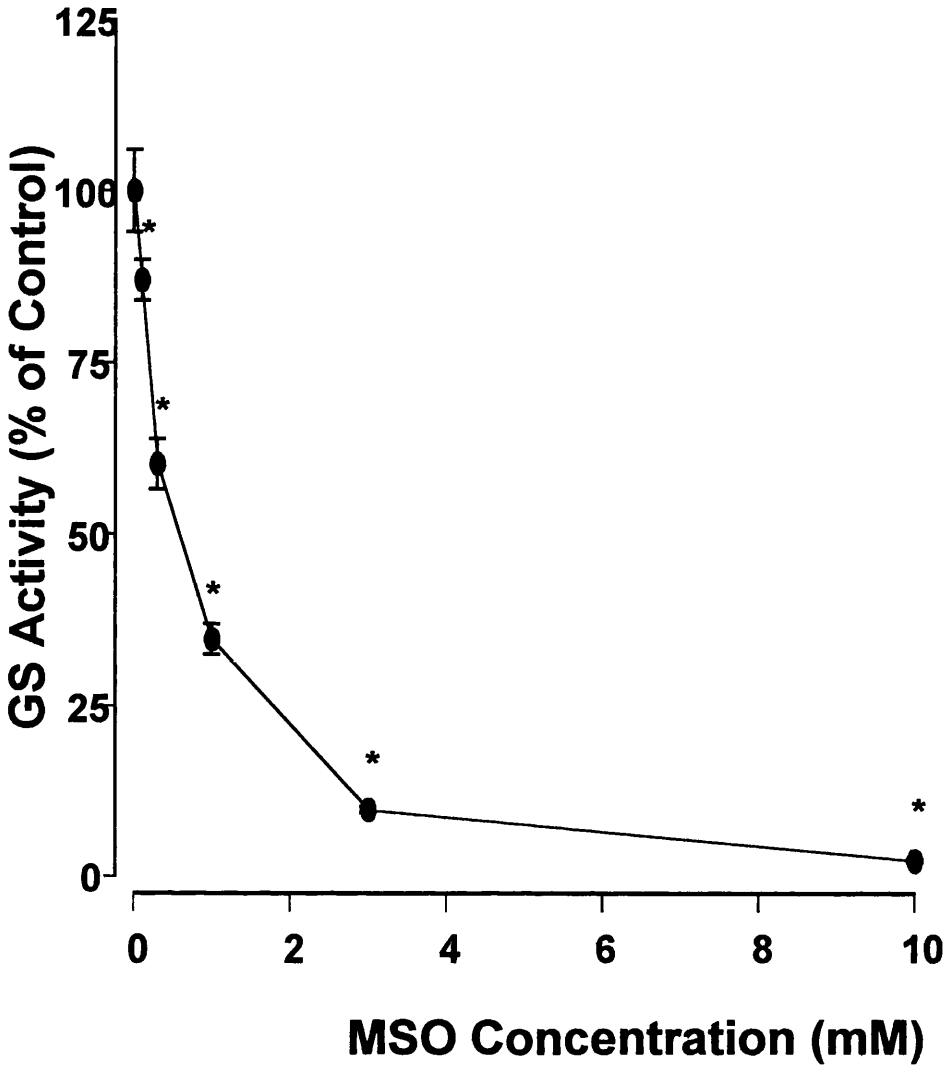


FIGURE 52. Effect of methionine sulfoximine (MSO; 0.1 - 10 mM) on the activity of glutamine synthetase (GS) in primary cultures of rat cortical astrocytes. Results (n = 4) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.). Statistical significance (* $P < 0.05$) was determined by one way analysis of variance with Dunnet correction for multiple comparisons.

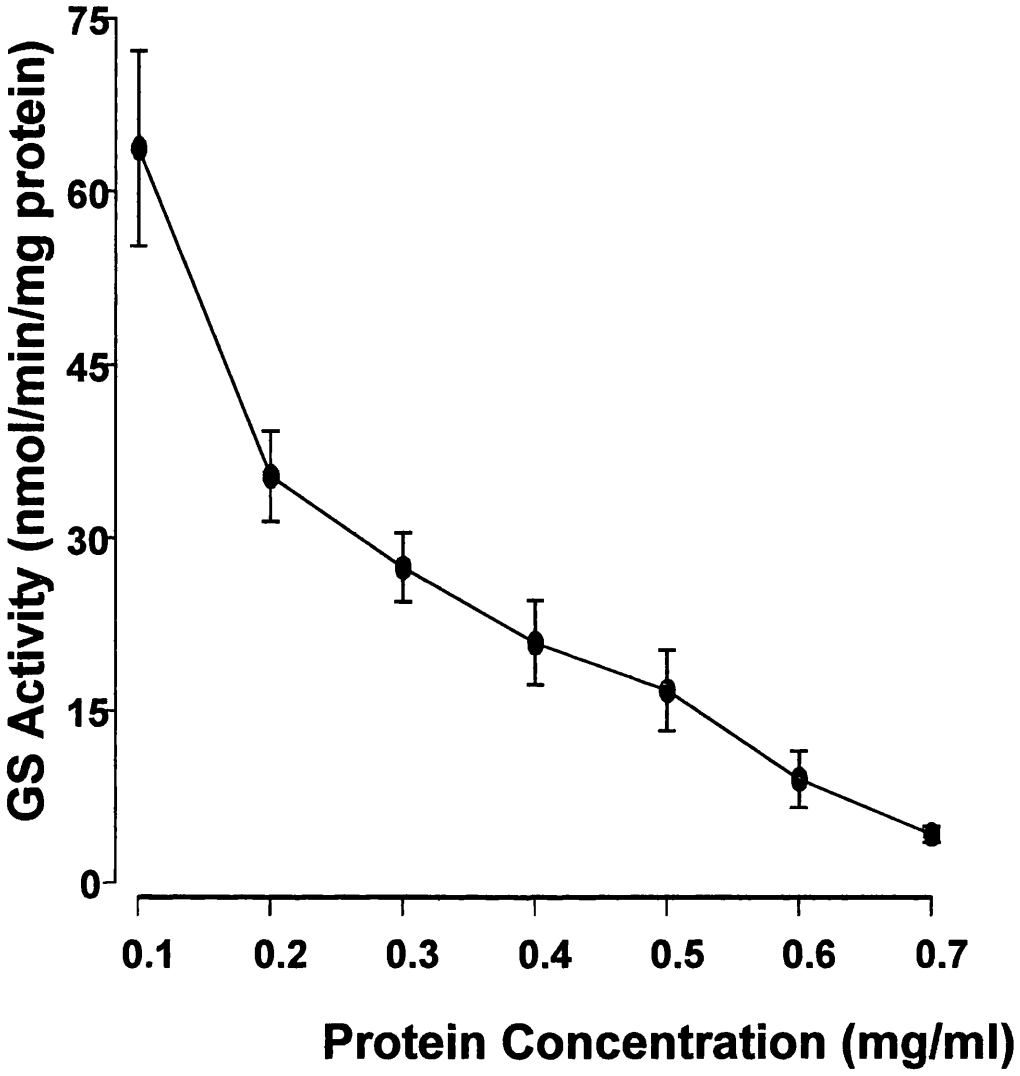


FIGURE 53. Effect of protein concentration on the activity of glutamine synthetase (GS) in primary cultures of rat cortical astrocytes. Results (n = 4) are expressed as nmol / min / mg protein and error bars denote the standard error of the mean (S.E.M.).

4.3.6 Effects of antiepileptic drugs on glutamine synthetase activity in primary cultures of rat cortical astrocytes

EXPERIMENTAL PROTOCOL: Rat brain tissue was isolated and cultured as described in section 2.3. For each of the 12 drug experiments, cultures were randomly separated into seven groups (n = 4 / group), and exposed to the following drugs in BSS (stock solutions of CBZ, PHT and FBM were dissolved in 500 µl ethanol prior to dilution with BSS):

PB:- 1, 3, 10, 30, 100, 300 and 1000 µM

PHT:- 1, 3, 10, 30, 100, 300 and 1000 µM

CBZ:- 1, 3, 10, 30, 100, 300 and 1000 µM

SVP:- 3, 10, 30, 100, 300, 1000 and 3000 µM

VGB:- 1, 3, 10, 30, 100, 300 and 1000 µM

LTG:- 0.3, 1, 3, 10, 30, 100 and 300 µM

FBM:- 3, 10, 30, 100, 300, 1000 and 3000 µM

GBP:- 1, 3, 10, 30, 100, 300 and 1000 µM

TPM:- 1, 3, 10, 30, 100, 300 and 1000 µM

TGB:- 0.1, 0.3, 1, 3, 10, 30 and 100 µM

LEV:- 1, 3, 10, 30, 100, 300 and 1000 µM

DGR:- 1, 3, 10, 30, 100, 300 and 1000 µM

For each drug experiment, a control group (n = 4) was exposed to BSS alone. After a one hour incubation, cells were assayed for GS activity as described in section 2.10. A one hour incubation period (as opposed to a four hour incubation as in the mouse brain experiments) was chosen as it was assumed that as the drugs were being applied directly to the cells that any effects on enzyme levels would be seen rapidly.

RESULTS: Results are summarised in table 3. PB (30, 100 and 300 μM; figure 54) significantly ($P < 0.05$) reduced the activity of GS in primary cultures of rat cortical astrocytes. PHT (figure 55), CBZ (figure 56), SVP (figure 57), VGB (figure 58), LTG (figure 59), FBM (figure 60), GBP (figure 61), TPM (figure 62), TGB (figure 63), LEV (figure 64) and DGR (figure 65) were all found to have no significant effect on the activity of GS in primary cultures of rat cortical astrocytes following a one hour exposure.

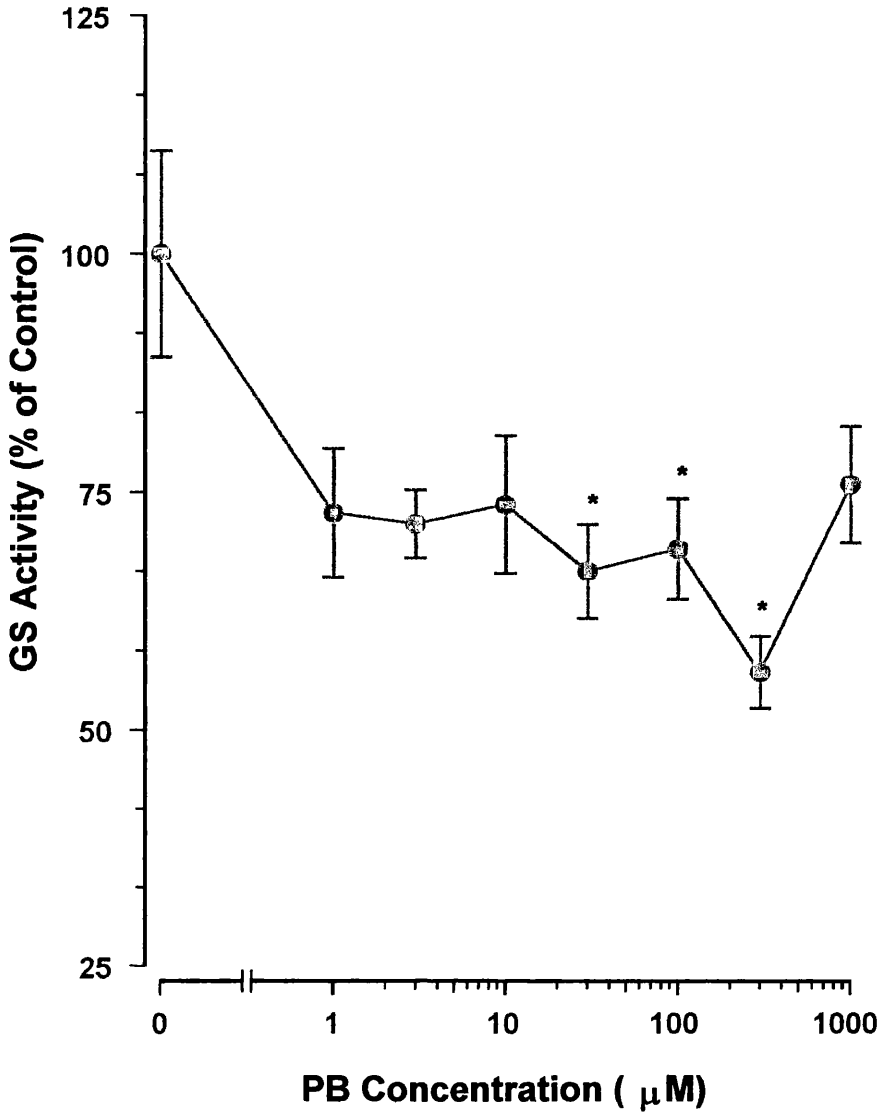


FIGURE 54. Effect of phenobarbitone (PB; 0 - 1000 μM) on the activity of glutamine synthetase (GS) in primary cultures of rat cortical astrocytes. Results (n = 4) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.). Statistical analysis was determined by one way analysis of variance with Dunnett correction for multiple comparisons.

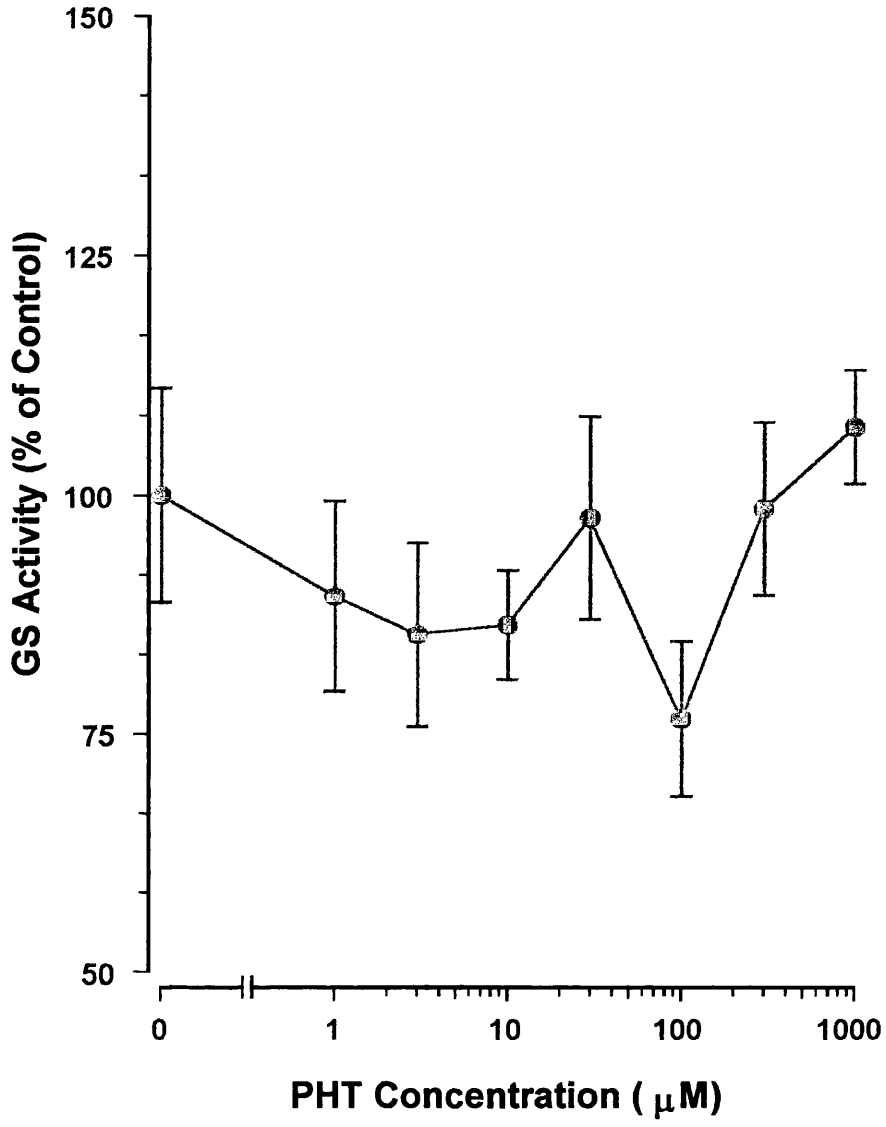


FIGURE 55. Effect of phenytoin (PHT; 0 - 1000 μM) on the activity of glutamine synthetase (GS) in primary cultures of rat cortical astrocytes. Results (n = 4) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

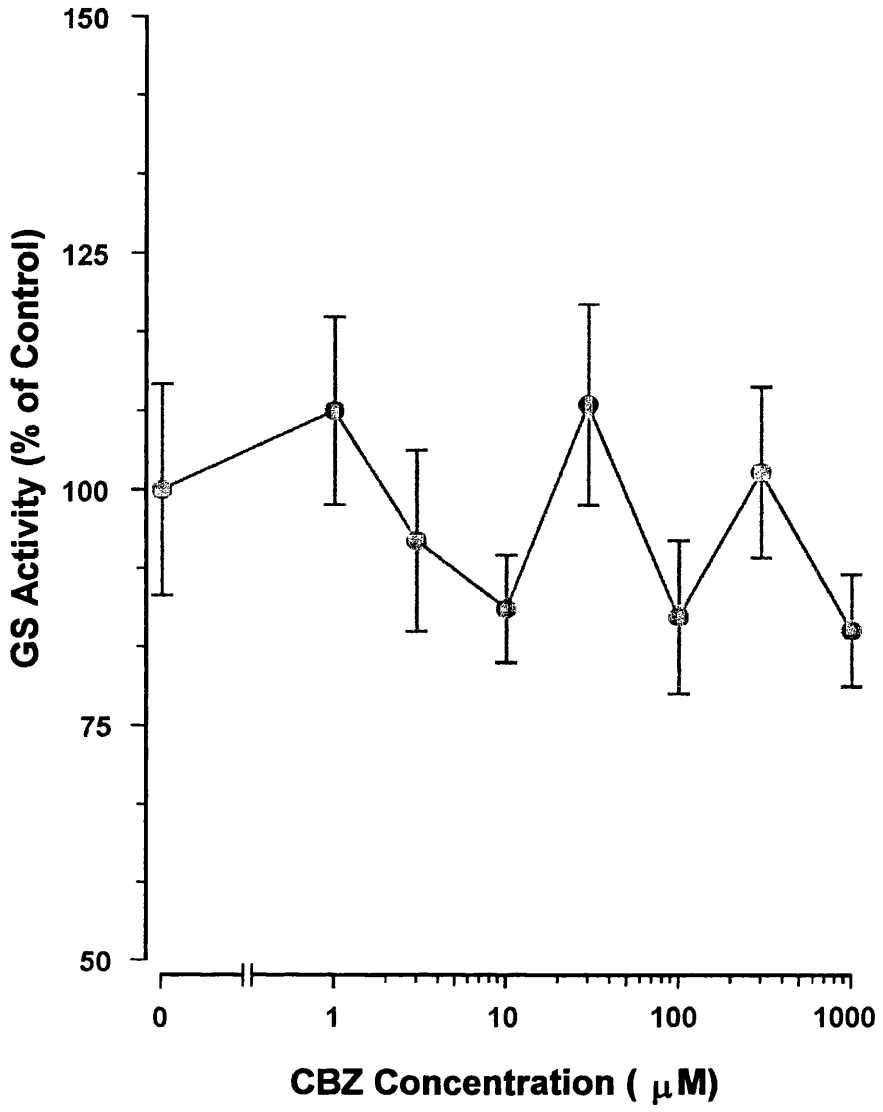


FIGURE 56. Effect of carbamazepine (CBZ; 0 - 1000 μM) on the activity of glutamine synthetase (GS) in primary cultures of rat cortical astrocytes. Results (n = 4) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

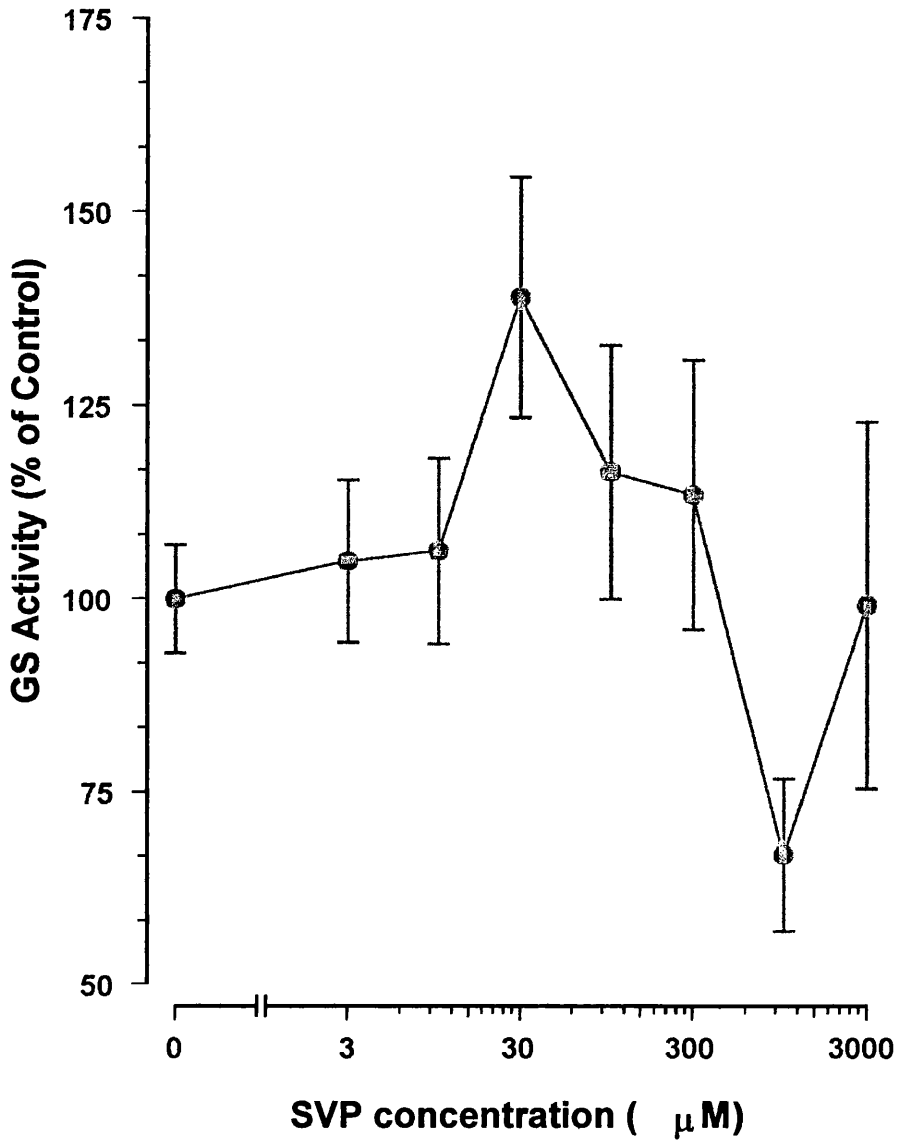


FIGURE 57. Effect of sodium valproate (SVP; 0 - 3000 μM) on the activity of glutamine synthetase (GS) in primary cultures of rat cortical astrocytes. Results (n = 4) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

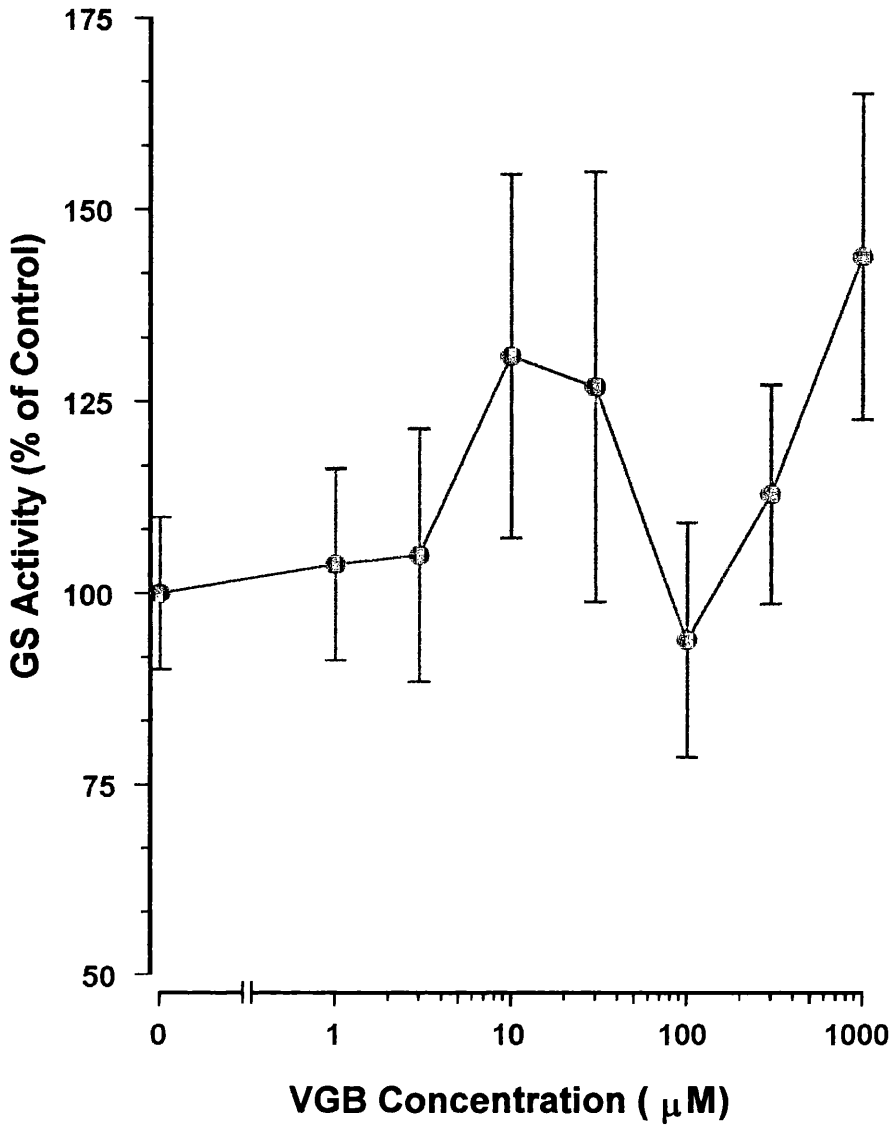


FIGURE 58. Effect of vigabatrin (VGB; 0 - 1000 μM) on the activity of glutamine synthetase (GS) in primary cultures of rat cortical astrocytes. Results (n = 4) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

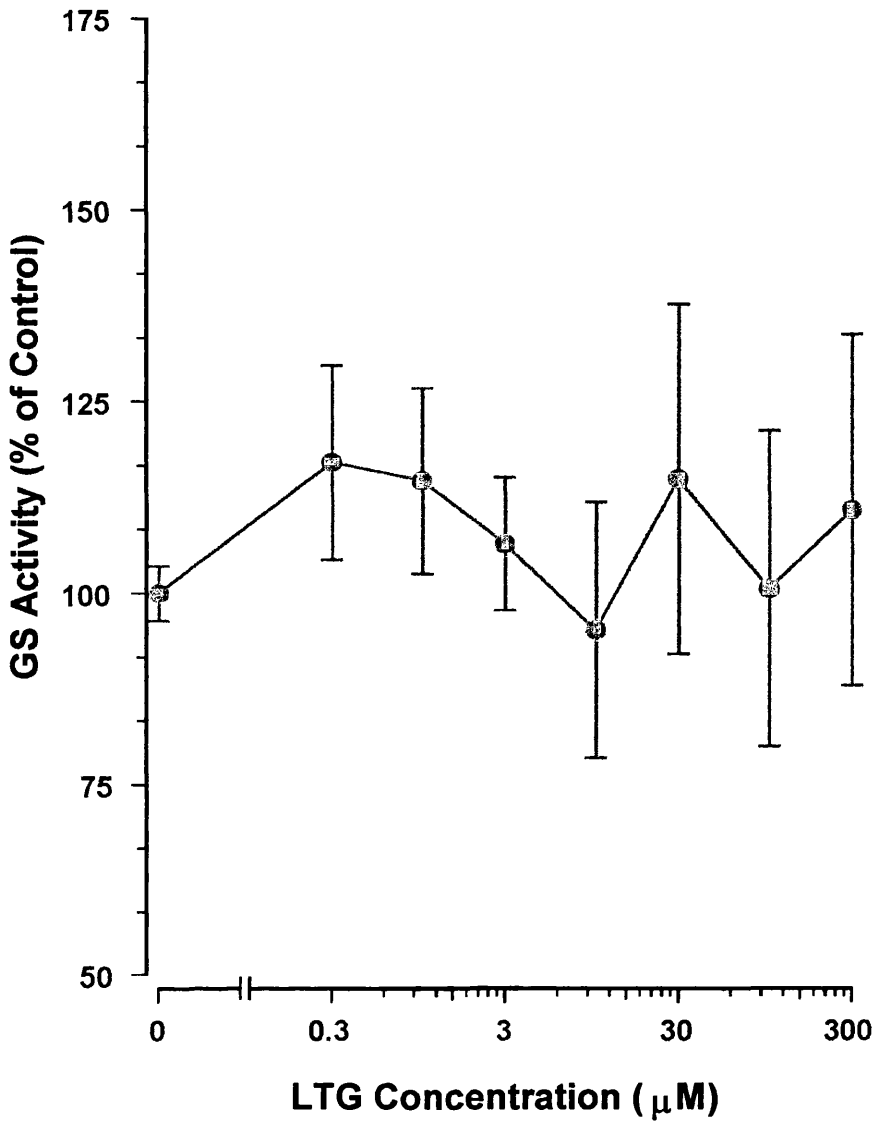


FIGURE 59. Effect of lamotrigine (LTG; 0 - 300 μM) on the activity of glutamine synthetase (GS) in primary cultures of rat cortical astrocytes. Results (n = 4) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

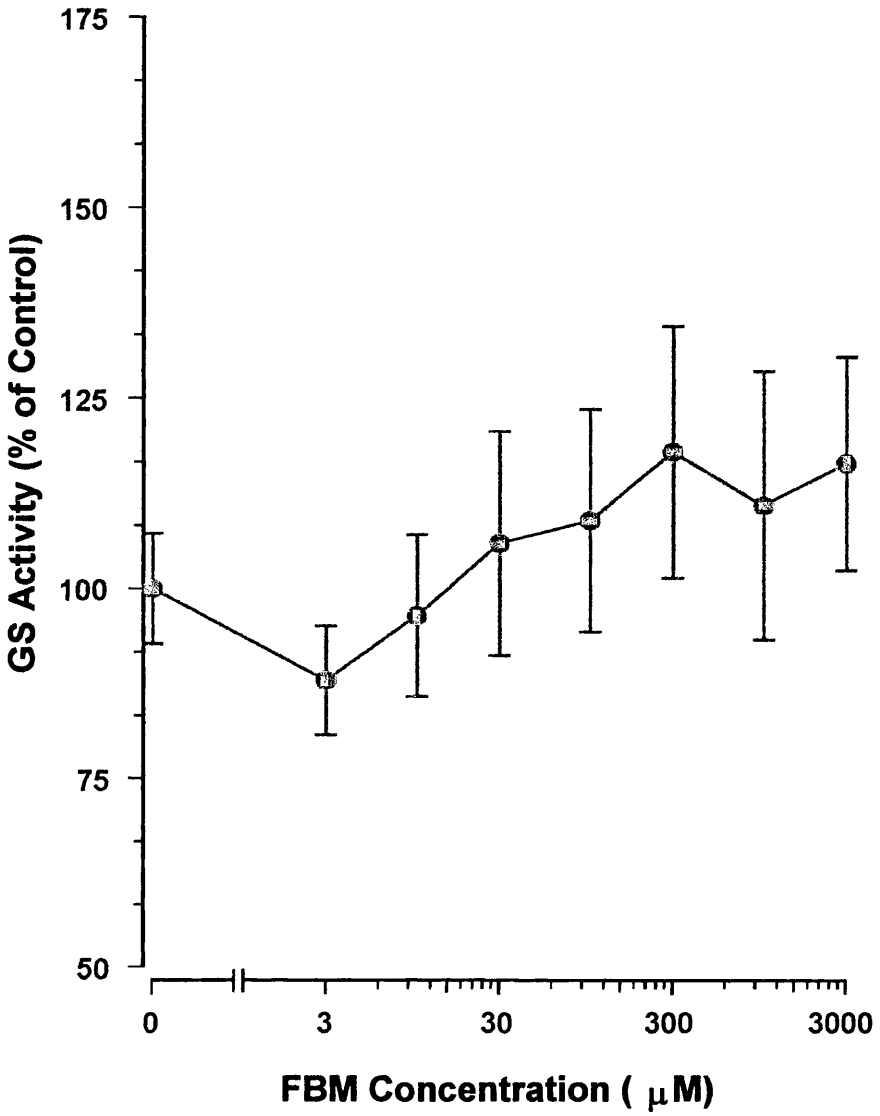


FIGURE 60. Effect of felbamate (FBM; 0 - 3000 μM) on the activity of glutamine synthetase (GS) in primary cultures of rat cortical astrocytes. Results (n = 4) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

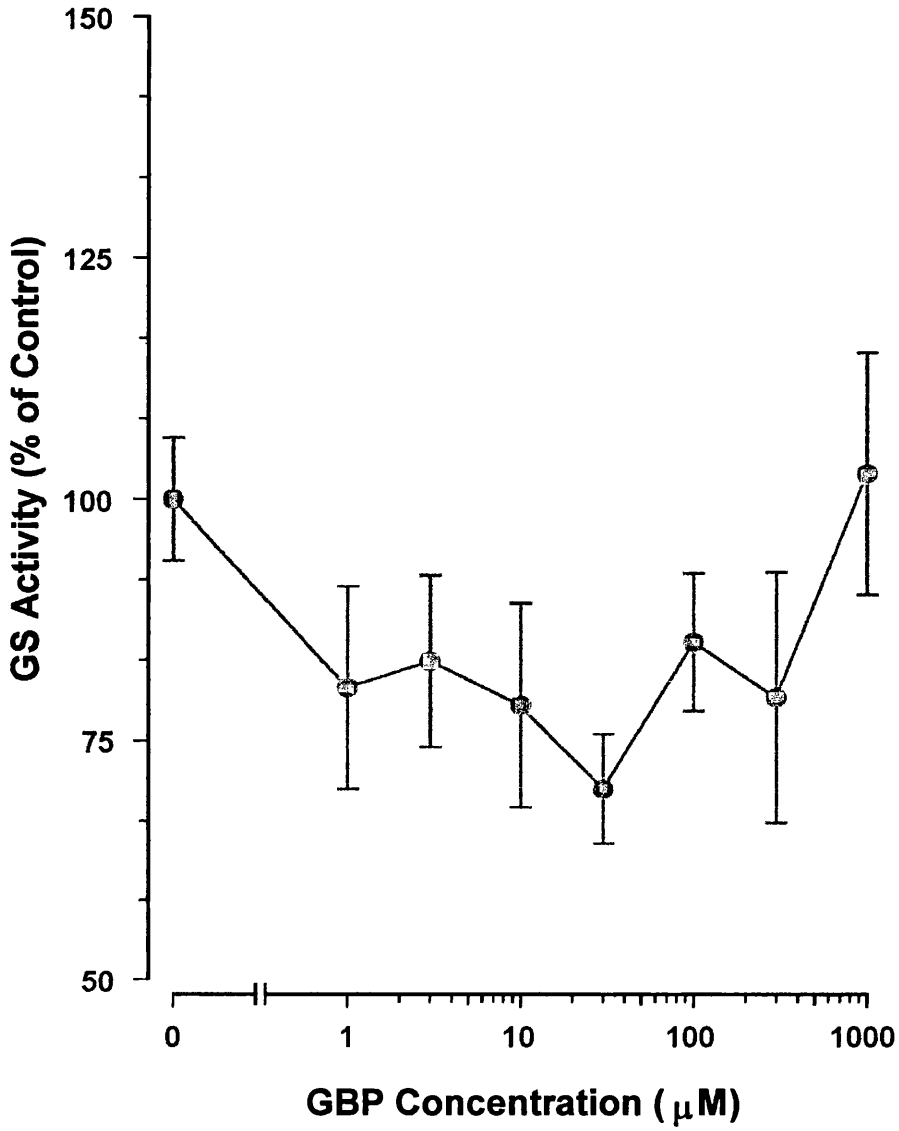


FIGURE 61. Effect of gabapentin (GBP; 0 - 1000 μM) on the activity of glutamine synthetase (GS) in primary cultures of rat cortical astrocytes. Results (n = 4) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

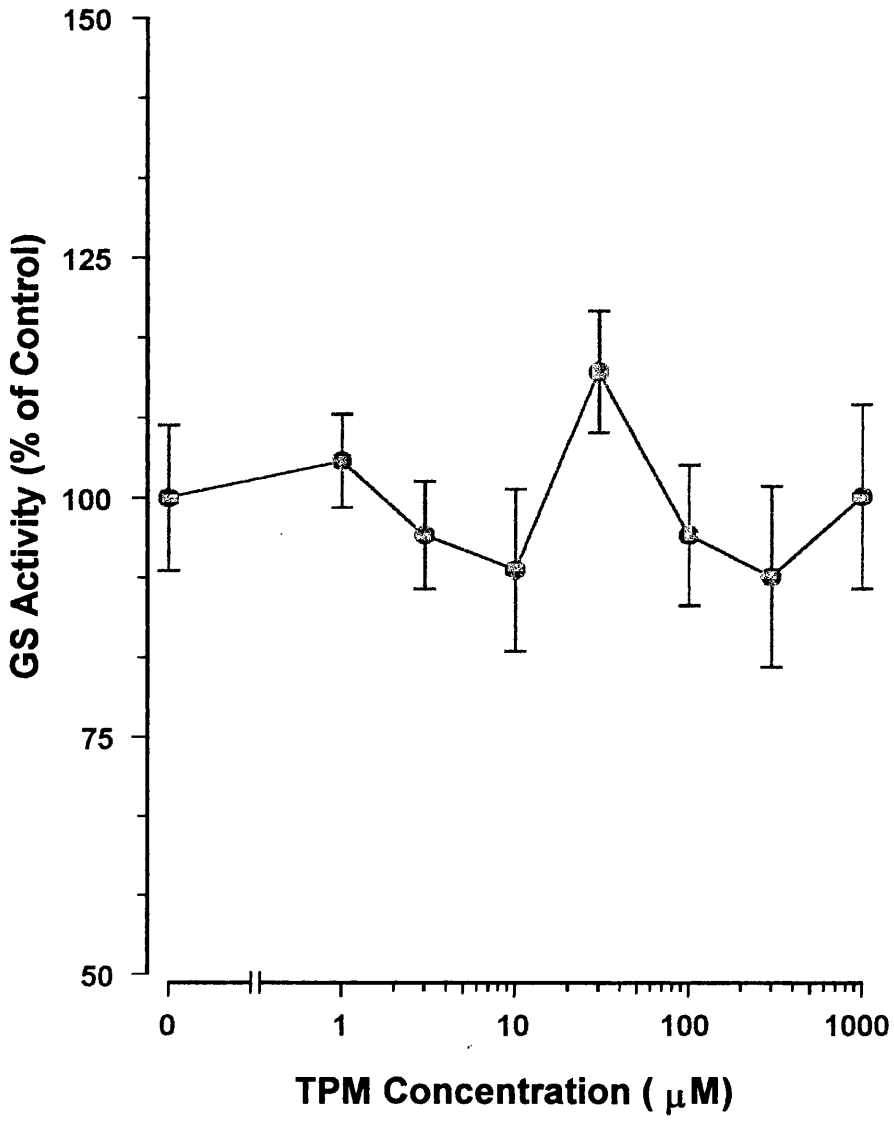


FIGURE 62. Effect of topiramate (TPM; 0 - 1000 μM) on the activity of glutamine synthetase (GS) in primary cultures of rat cortical astrocytes. Results (n = 4) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

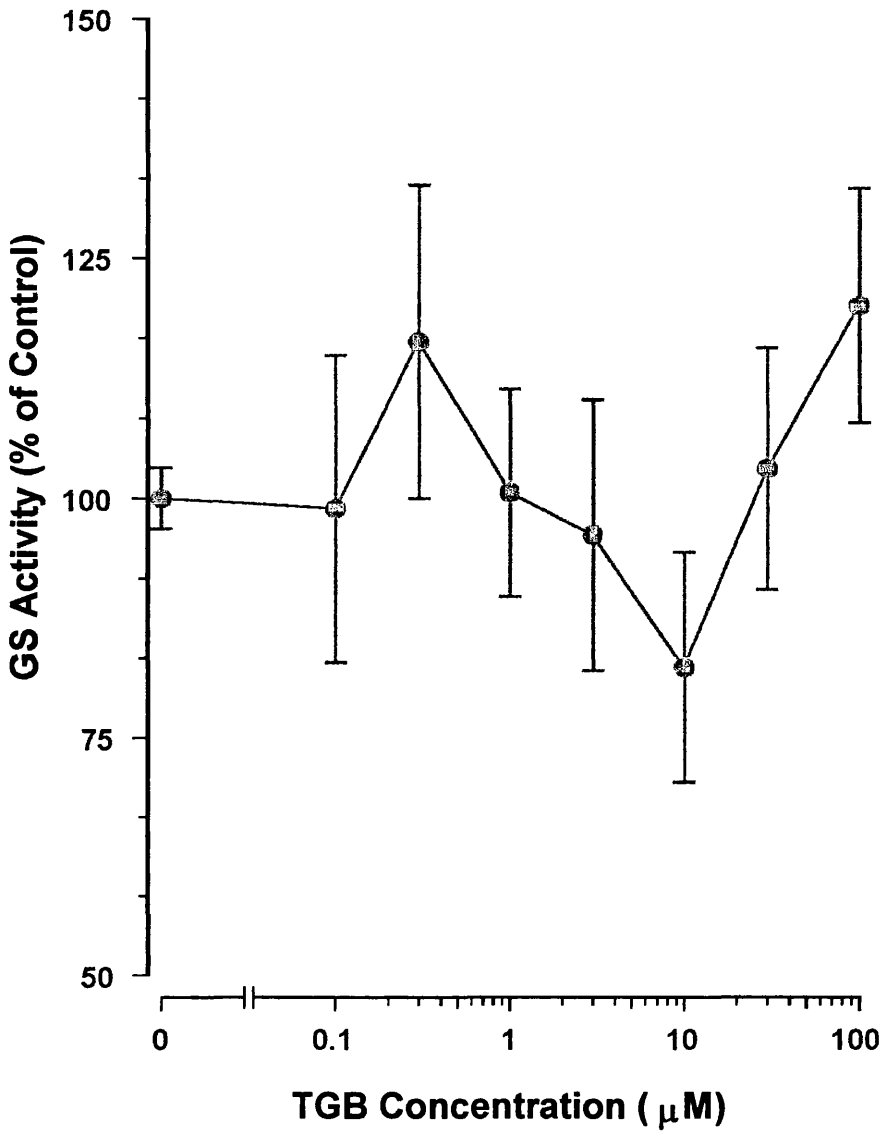


FIGURE 63. Effect of tiagabine (TGB; 0 - 100 μM) on the activity of glutamine synthetase (GS) in primary cultures of rat cortical astrocytes. Results (n = 4) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

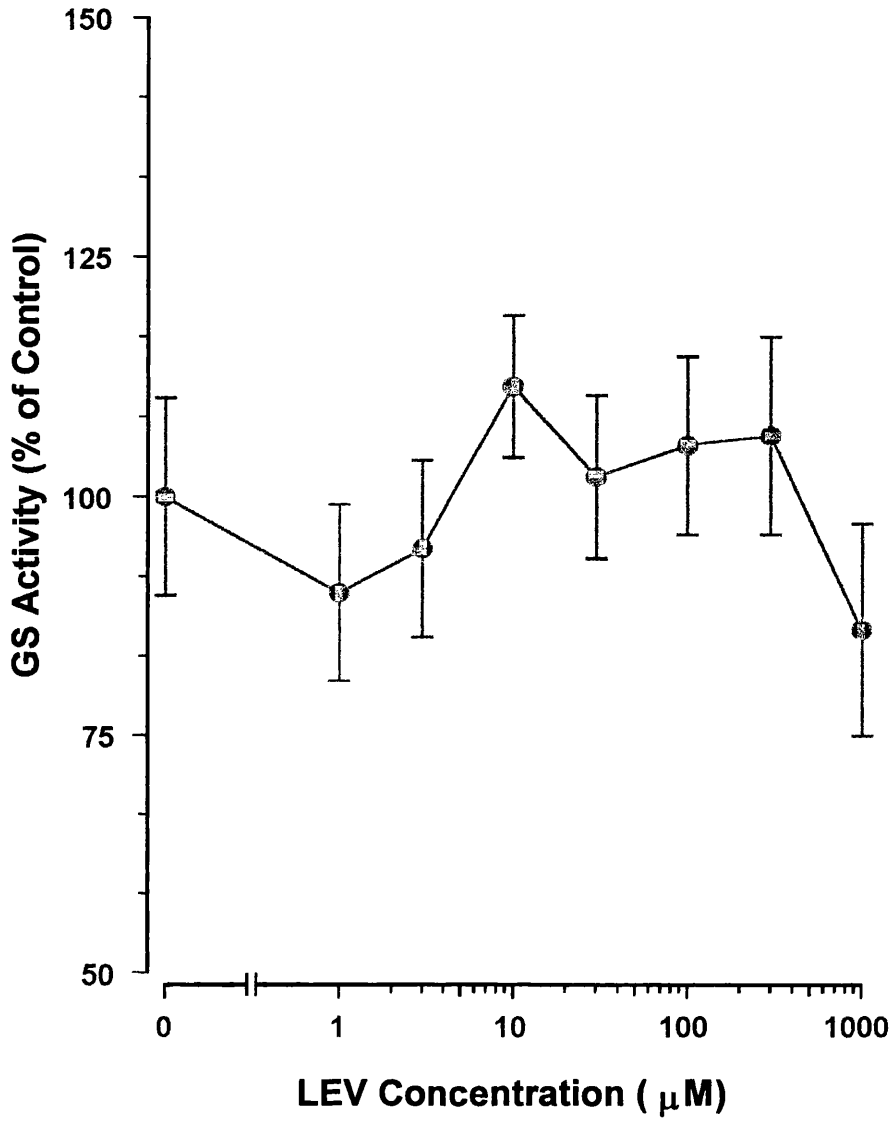


FIGURE 64. Effect of levetiracetam (LEV; 0 - 1000 μM) on the activity of glutamine synthetase (GS) in primary cultures of rat cortical astrocytes. Results (n = 4) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

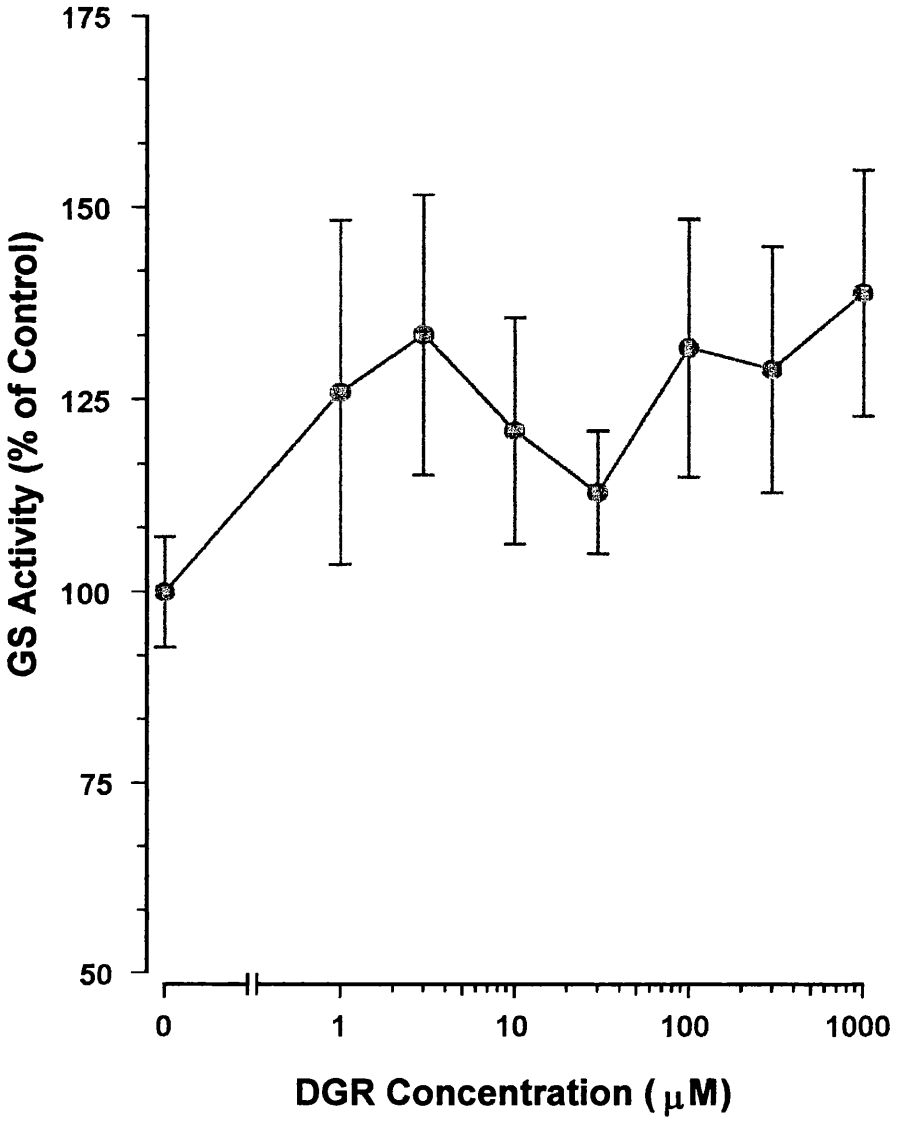


FIGURE 65. Effect of desglycinyl-remacemide (DGR; 0 - 1000 μM) on the activity of glutamine synthetase (GS) in primary cultures of rat cortical astrocytes. Results (n = 4) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

TABLE 2. Effects of antiepileptic drugs on the activity of glutamine synthetase in mouse brain following acute (single dose) and chronic (twice daily for five days) administration - summary of results (NS = no significant effect, SIG ↓ = significant reduction $p < 0.05$).

	Glutamine Synthetase Activity	
AED	Acute	Chronic
Phenobarbitone (0.1 - 30 mg/kg)	NS	1 – 30 mg/kg SIG ↓
Phenytoin (0.3 - 100 mg/kg)	1 – 100 mg/kg SIG ↓	3 – 30 mg/kg SIG ↓
Carbamazepine (0.1 - 30 mg/kg)	1 – 30 mg/kg SIG ↓	3 – 30 mg/kg SIG ↓
Sodium valproate (3 - 1000 mg/kg)	NS	NS
Vigabatrin (3 - 1000 mg/kg)	NS	NS
Lamotrigine (0.3 - 100 mg/kg)	NS	NS
Felbamate (1 - 300 mg/kg)	NS	1 – 300 mg/kg SIG ↓
Gabapentin (1 - 300 mg/kg)	NS	NS
Topiramate (0.3 - 100 mg/kg)	NS	3 – 100 mg/kg SIG ↓
Tiagabine (0.1 - 30 mg/kg)	NS	NS
Levetiracetam (1 - 300 mg/kg)	NS	NS
Desglycinyl-remacemide (0.3 - 100 mg/kg)	NS	NS

TABLE 3. Effects of antiepileptic drugs on the activity of glutamine synthetase in primary cultures of rat cortical astrocytes following a one hour exposure - summary of results (NS = no significant effect, SIG ↓ = significant reduction $p < 0.05$).

AED	Glutamine Synthetase Activity
Phenobarbitone (1 - 1000 μM)	30 – 300 μM SIG ↓
Phenytoin (1 - 1000 μM)	NS
Carbamazepine (1 - 1000 μM)	NS
Sodium valproate (3 - 3000 μM)	NS
Vigabatrin (1 - 1000 μM)	NS
Lamotrigine (0.3 - 300 μM)	NS
Felbamate (3 - 3000 μM)	NS
Gabapentin (1 - 1000 μM)	NS
Topiramate (1 - 1000 μM)	NS
Tiagabine (0.1 - 100 μM)	NS
Levetiracetam (1 - 1000 μM)	NS
Desglycinyl-remacemide (1 - 1000 μM)	NS

4.4 DISCUSSION

In light of the importance of GS in regulating glutamate neurotransmission, and it's possible involvement in seizure generation and AED action, the effects of single and repeated administration of a range of AEDs on its activity was determined in mouse brain. Additionally, as the enzyme is specifically located in astrocytes, the effects of these AEDs on GS activity was also examined in primary cultures of rat cortical astrocytes.

4.4.1 Effects of antiepileptic drugs on glutamine synthetase activity in mouse brain

Both single and repeated treatments with PHT and CBZ, and repeated administration of PB, FBM and TPM significantly and dose-dependently reduced the activity of GS. Given that an inhibition of this enzyme results in a diminished capacity for the brain to metabolize, and thereby detoxify glutamate and ammonia, it would appear unlikely that the reduction in activity demonstrated contributes to the antiepileptic actions of these drugs. Accumulation of glutamate and ammonia may be expected to facilitate neuronal excitation, and precipitate seizures, as seen with the GS inhibitor MSO. It is therefore possible that the inhibition of GS may decrease anticonvulsant effect or contribute to the toxicity of these compounds. Interestingly, those drugs with an inhibitory action on the enzyme are those which most frequently exhibit CNS-related toxicity, such as ataxia, sedation, dizziness and cognitive impairment (Rogawski and Porter, 1990; Dichter and Brodie, 1996).

Both single and repeated treatments with SVP, VGB, LTG, GBP, TGB, LEV and DGR were found to have no effect on the activity of GS, suggesting that an action on

the enzyme is not involved in the antiepileptic or toxic mechanisms of these drugs. The failure to demonstrate the SVP-induced potentiation reported by other groups (Nolan *et al*, 1985; Phelan *et al*, 1985) may have been due to differences in experimental design. The other groups added SVP directly to the enzyme incubation medium and observed an *in vitro* response, whereas in this study, I have examined the *ex vivo* effects of the AEDs on enzyme activity. In a study using primary cultures of rat cortical astrocytes, Collins and colleagues (1994) reported that when added to intact cultures, SVP significantly reduced that activity of GS. However, when incorporated directly into the assay tubes, SVP was found to stimulate enzyme activity.

4.4.2 Toxicity of antiepileptic drugs

It is well documented that AED treatment may worsen seizures, either by aggravating pre-existing ones, or by inducing new seizure types (Troupin and Ojemann, 1975; Osorio *et al*, 1989; Murphy *et al*, 1991; Loiseau, 1998; Perucca *et al*, 1998). This phenomenon is referred to as paradoxical intoxication and is most commonly reported in patients treated with PHT (Osorio *et al*, 1989; Murphy *et al*, 1991; Perucca, *et al*, 1998), and CBZ (Troupin and Ojemann, 1975; Perucca *et al*, 1998). Experiments on glial cell cultures have suggested that at higher PHT concentrations, the cells appear to be less able to regulate extracellular potassium (White *et al*, 1985). This has been proposed to partially explain the excitatory effects of the drug seen clinically at high therapeutic doses. It has also been reported that PB (Perucca *et al*, 1998), FBM (Wolff *et al*, 1994) and TPM (Elger *et al*, 1998) may increase seizure frequency in some patients. From this evidence, it is possible that the occurrence of paradoxical intoxication may be related to the reduction in GS activity exerted by these drugs, in particular CBZ and PHT, the two most commonly linked to paradoxical intoxication,

and the only AEDs found to have both acute and chronic effects on GS activity in this study.

As with the incidence of paradoxical intoxication, the tendency to reduce enzyme activity is more prominent in the established AEDs, with a reduction in GS only evident following repeated administration with two of the newer drugs, FBM and TPM. The most marked reduction in enzyme activity was evident following single and repeated dosing with two of the established AEDs, CBZ and PHT, which are known to exert their antiepileptic effects via blockade of voltage-dependent sodium channels (Rogawski and Porter, 1990). Interestingly, LTG, a new AED whose antiepileptic mechanism of action is also thought to be mediated via blockade of sodium channels (Cheung *et al*, 1992), had no effect on the activity of GS. Similarly, DGR, the experimental drug with proposed sodium channel blocking effects (Wamil *et al*, 1996) failed to influence the activity of the enzyme.

It is unclear why CBZ and PHT reduced GS activity following a single dose, while no other drug examined had acute effects. The mechanism of the inhibition may differ between the AEDs with acute effects and those with effects only after chronic treatment. It is possible that CBZ and PHT are interacting with the active site of the enzyme, resulting in direct inhibition evident following a single dose. PB, FBM and TPM, the AEDs which had effects following only repeated administration, may be reducing enzyme activity via an indirect mechanism, such as an effect on protein synthesis or gene expression. The possibility that these AEDs are actually reducing the amount of enzyme present and not simply inhibiting it cannot be excluded without further investigation. The inhibition may also be indirect, in that it is occurring

secondary to a change in the concentration of a neurotransmitter, or activity of another enzyme in the brain. Such effects may take time to develop, therefore only becoming apparent following repeated administration. Each of these possibilities require further detailed investigation in order to determine the nature of the inhibition of GS.

4.4.3 Effects of antiepileptic drugs on glutamine synthetase activity in primary cultures of rat cortical astrocytes

Of the twelve AEDs examined, only PB was found to significantly reduce the activity of GS in primary cultures of rat cortical astrocytes. This effect is consistent with the reduction in enzyme activity induced by PB in mouse brain. The effect of PB on the activity of GS in cultured astrocytes has been examined previously in other studies. In primary cultures of mouse cortical astrocytes, a seven day exposure to PB was found to have no effect on GS activity (Swaiman and Machen, 1991). In contrast to this, a more recent study examined the effects of PB in rat brain aggregate cultures, and reported a slight increase in the activity of GS following an eight day exposure to the drug (Schilter *et al*, 1995). More work is required to clarify the effects of PB on GS activity in cultured cells.

PHT, CBZ, FBM and TPM significantly reduced GS activity in mouse brain, but all failed to exert any significant effect on enzyme activity in the cultured cells. As GS is specifically located in glia, it is unclear why these AEDs which had effects in whole brain had no effect in the cultured cells. A previous study using primary cultures of rat cortical astrocytes found that when SVP (1.2 mM) was incorporated into the culture medium, there was no significant effect noted until day two of exposure, where the activity of GS was reduced. Enzyme activity was further reduced after three and four

days in culture (Collins *et al*, 1994). This would suggest that the one hour exposure of the AEDs in this study may not be sufficient to exert an effect, and that a longer incubation period should be examined. However, in primary cultures of mouse cortical astrocytes, a seven day exposure to PHT was still without effect on the activity of GS (Swaiman and Machen, 1991). Similarly, in rat brain aggregate cultures, eight day exposures to CBZ and SVP were without significant effect on the activity of the enzyme (Schilter *et al*, 1995). More work is therefore required to determine the exposure time of the drugs and enzyme activity.

Another possible explanation for the lack of effect of PHT, CBZ, FBM and TPM in the astrocyte cultures could be due to a species difference. It remains possible that the drugs are effective at reducing enzyme activity in mouse brain and that no effect is seen in the astrocyte cultures as they are derived from rat brain. It may be useful to carry out experiments using astrocytes cultured from mouse brain to investigate the significance of species difference. Alternatively, the lack of effect of these AEDs in the cultured astrocytes may simply be due to the large SEM values observed in these experiments. It is possible that variability between astrocyte cultures is preventing any true AED-induced effects from being observed.

4.5 CONCLUSIONS

In conclusion, the reduction in GS activity in mouse brain following both single and repeated treatments with PHT and CBZ, and repeated treatments with PB, FBM and TPM is unlikely to be related to the antiepileptic actions of these drugs. It is possible that inhibition of this enzyme may contribute to the toxicity of these drugs, particularly CNS-related adverse effects. It is also possible that this reduction in GS activity may be

contributing to the phenomenon of paradoxical intoxication seen clinically with these drugs in some patients. The inhibition in GS activity induced by PB in the astrocyte cultures further supports the suggestion that this effect may be implicated in its clinical toxicity. However, the results obtained from the cell culture experiments are inconclusive and further work is required.

CHAPTER FIVE

GABA- AND GLUTAMATE-RELATED NEUROCHEMICAL EFFECTS OF THE NEW ANTIEPILEPTIC DRUGS FELBAMATE, TOPIRAMATE AND LEVETIRACETAM IN MOUSE BRAIN

5.1 INTRODUCTION

In recent years, a significant number of new drugs for the treatment of epilepsy have entered clinical trials or have been licensed for use. FBM, TPM and LEV are three novel AEDs, at various stages in development, but all whose precise mechanism of action remains to be determined.

5.1.1 Felbamate

A number of mechanisms for the antiepileptic and neuroprotective effects of FBM have been proposed, but the precise action, or actions, of the drug remain to be determined. FBM has demonstrated potentiation of inhibitory GABA neurotransmission as well as attenuation of glutamate responses. FBM enhances $GABA_A$ receptor-mediated chloride currents in rat hippocampal neurones (Rho *et al*, 1994), and has been shown to interact with the strychnine-insensitive glycine modulatory site of the NMDA receptor (McCabe *et al*, 1993; McCabe *et al*, 1998). However, in ligand binding studies, FBM failed to interact with the $GABA_A$ receptor complex (Ticku *et al,* 1991). It has been reported that a sub-protective dose of FBM could potentiate the anticonvulsant effects of diazepam against seizures induced by MES, PTZ and isoniazid (Gordon *et al*, 1991). In addition to its effects on amino acid systems, FBM has been reported to block voltage-dependent sodium channels (White *et al*, 1992) and dihydropyridine-sensitive calcium channels (Stefani *et al*, 1996).

5.1.2 Topiramate

As yet the precise mechanism of TPM's anticonvulsant activity remains to be established. It appears to possess multiple mechanisms of action including blockade of voltage-activated sodium channels (Coulter *et al*, 1993; Kawasaki *et al*, 1996; Zona *et*

al, 1997; Wu *et al*, 1998) and inhibitory effects on kainate-induced responses (Coulter *et al*, 1995). In addition, TPM reportedly potentiates responses to GABA at the $GABA_A$ receptor (White *et al*, 1995a; White *et al*, 1997) and may exert a weak carbonic anhydrase inhibition (Shank *et al*, 1994). Recent evidence has also suggested that TPM may reduce abnormally high levels of glutamate and aspartate in the hippocampus of spontaneously epileptic rats (Kanda *et al*, 1996).

5.1.3 Levetiracetam

The precise mechanism of action of LEV remains to be determined. Its anticonvulsant activity is highly stereospecific. However, no significant affinity for known receptors has been found and the drug fails to interact with ion channel sites in the brain (Noyer *et al*, 1995). LEV has, however, been proposed to act via a specific binding site in CNS membranes, which has not yet been characterized, where it is weakly displaced by PB and PTZ, suggesting it may be GABA-related (Noyer *et al*, 1995). In addition, a recent study reported that treatment with LEV induced alterations in GABA metabolism and turnover in discrete areas of rat brain (Loscher *et al*, 1996).

5.2 AIMS

Due to lack of precise mechanism of action data and reported effects on GABA and glutamate neurotransmitter systems, the aim of this study was to investigate the effects of single and repeated administration of FBM, TPM and LEV on GABA- and glutamate-related neurochemistry in mouse brain.

5.3 EXPERIMENTAL PROTOCOLS AND RESULTS

5.3.1 Neurochemical effects of felbamate in mouse brain

EXPERIMENTAL PROTOCOL: FBM was prepared daily for i.p. injection as a suspension in 30 / 70 (v/v) PEG 400 / water. In both single and multiple dose studies, adult male ICR mice were randomised into five treatment groups (n = 12 / group) and administered FBM 1, 3, 10, 30 and 100 mg/kg. (The 300 mg/kg dose which was previously used in chapter 4 could not be used here as there was not enough drug left). A sixth group of 12 mice (control) received vehicle alone. In the multiple dose phase of the study, treatment was continued twice daily (8 am and 4 pm) for five days. (A five day study with twice daily dosing was chosen to avoid weekend injections). In both phases, four hours after the final dose, the animals were sacrificed and their brains removed. Brains were divided into two hemispheres by a midline incision and stored at -70^0C until required for assay. Six left hemispheres from each of the groups (single and multiple dose; control and drug treated) were assayed for GABA concentrations as described in section 2.11. Glutamate and glutamine were measured simultaneously in six right hemispheres from each group as described in section 2.11. The remaining six left hemispheres from each group were assayed for GABA-T activity as described in section 2.9., and the remaining six right hemispheres from each group were assayed for GAD activity as described in section 2.12.

RESULTS: At all doses examined, both single and repeated treatments with FBM were without significant effect on the concentrations of GABA (figure 66), glutamate

(figure 67) or glutamine (figure 68). Similarly, single and repeated treatments were without effect on the activities of GABA-T (figure 69) or GAD (figure 70).

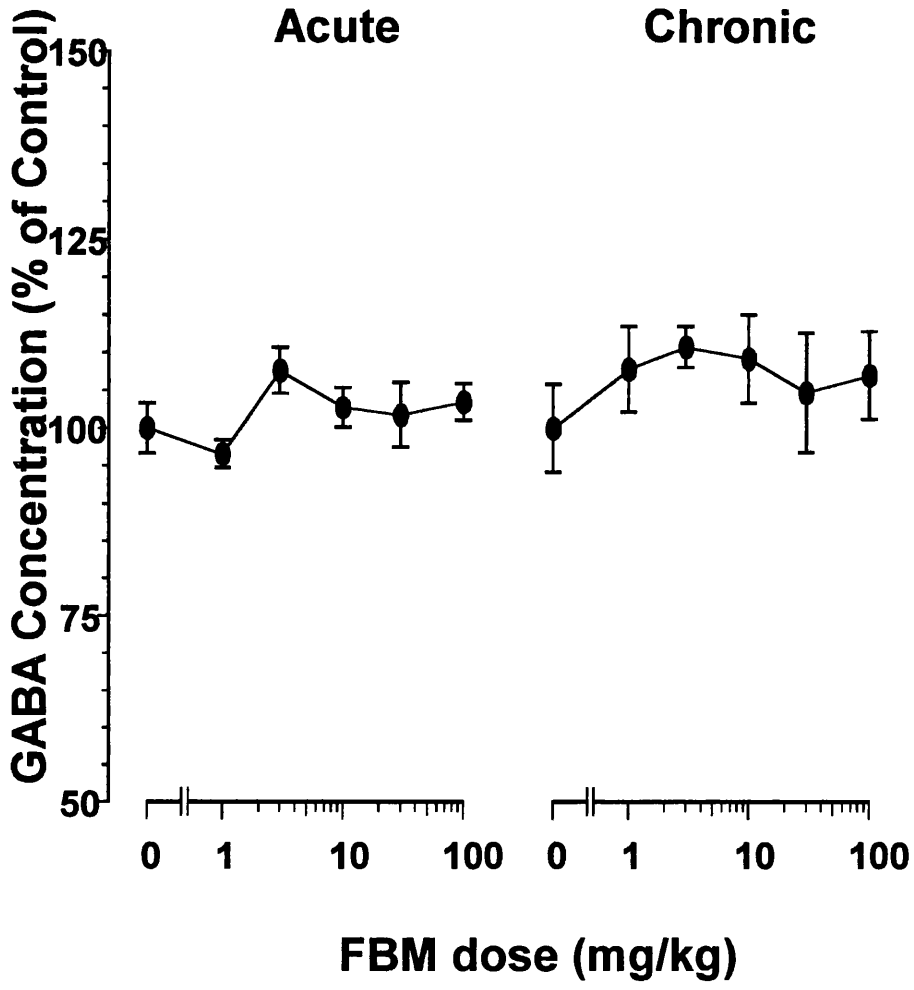


FIGURE 66. Effect of felbamate (FBM; 0 - 100 mg/kg) on mouse brain GABA concentration at four hours after acute (single dose; left graph) and chronic (twice daily for five days; right graph) administration. Results (n = 6) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

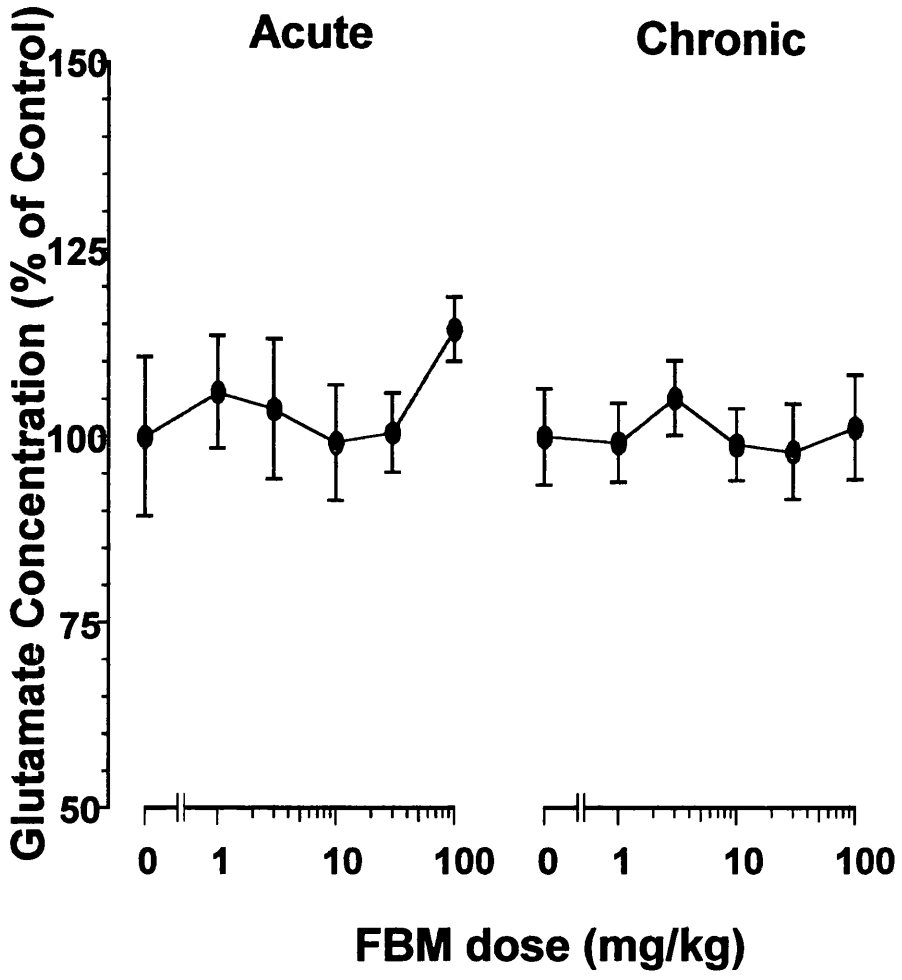


FIGURE 67. Effect of felbamate (FBM; 0 - 100 mg/kg) on mouse brain glutamate concentration at four hours after acute (single dose; left graph) and chronic (twice daily for five days; right graph) administration. Results (n = 6) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

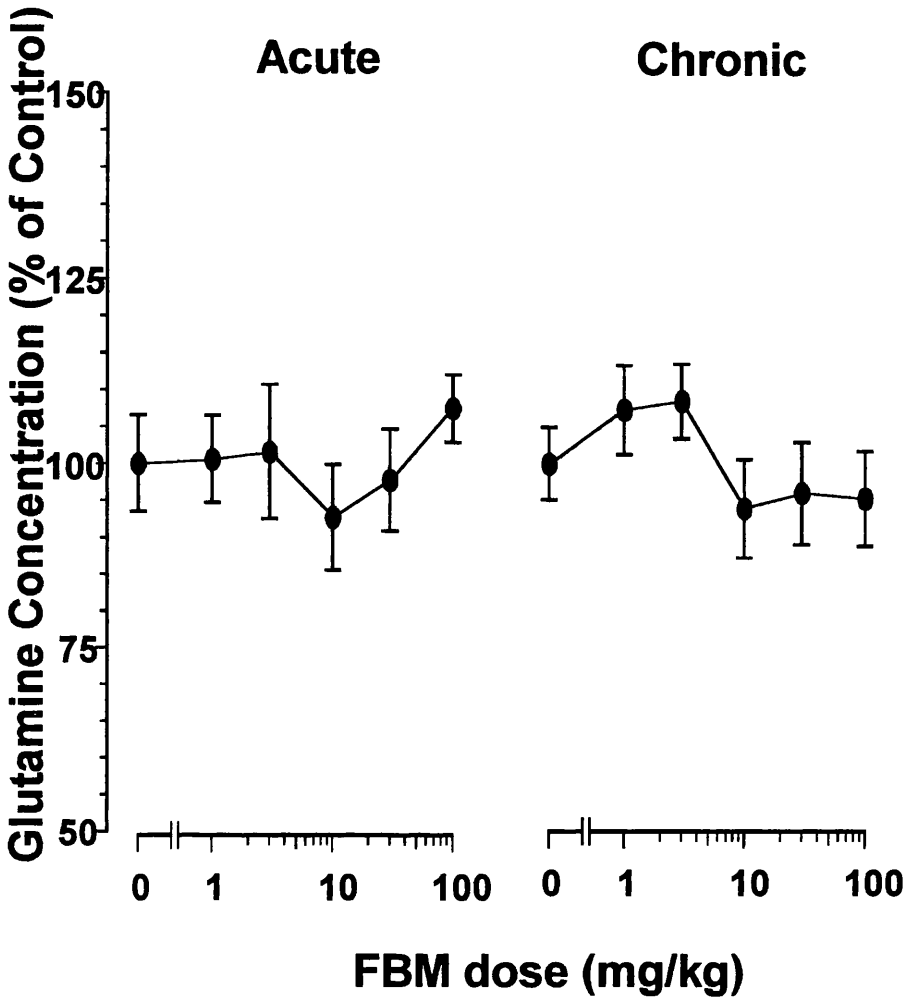


FIGURE 68. Effect of felbamate (FBM; 0 - 100 mg/kg) on mouse brain glutamine concentration at four hours after acute (single dose; left graph) and chronic (twice daily for five days; right graph) administration. Results (n = 6) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

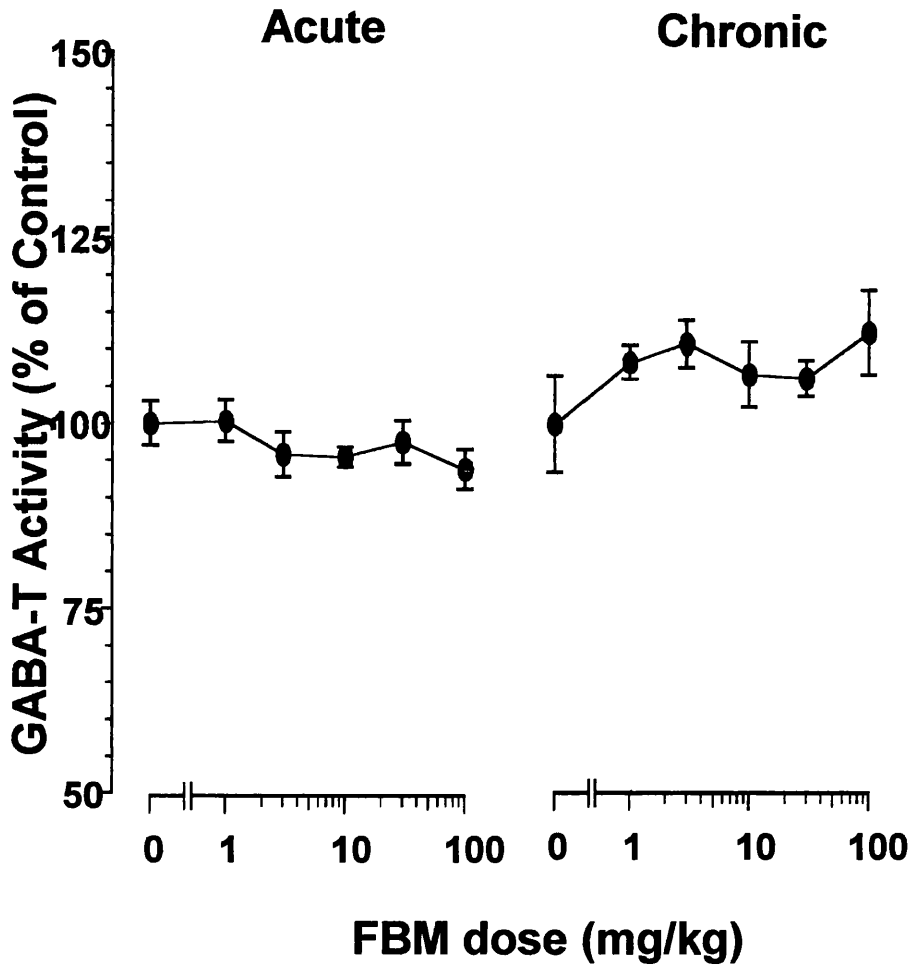


FIGURE 69. Effect of felbamate (FBM; 0 - 100 mg/kg) on mouse brain GABA-transaminase (GABA-T) activity at four hours after acute (single dose; left graph) and chronic (twice daily for five days; right graph) administration. Results (n = 6) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

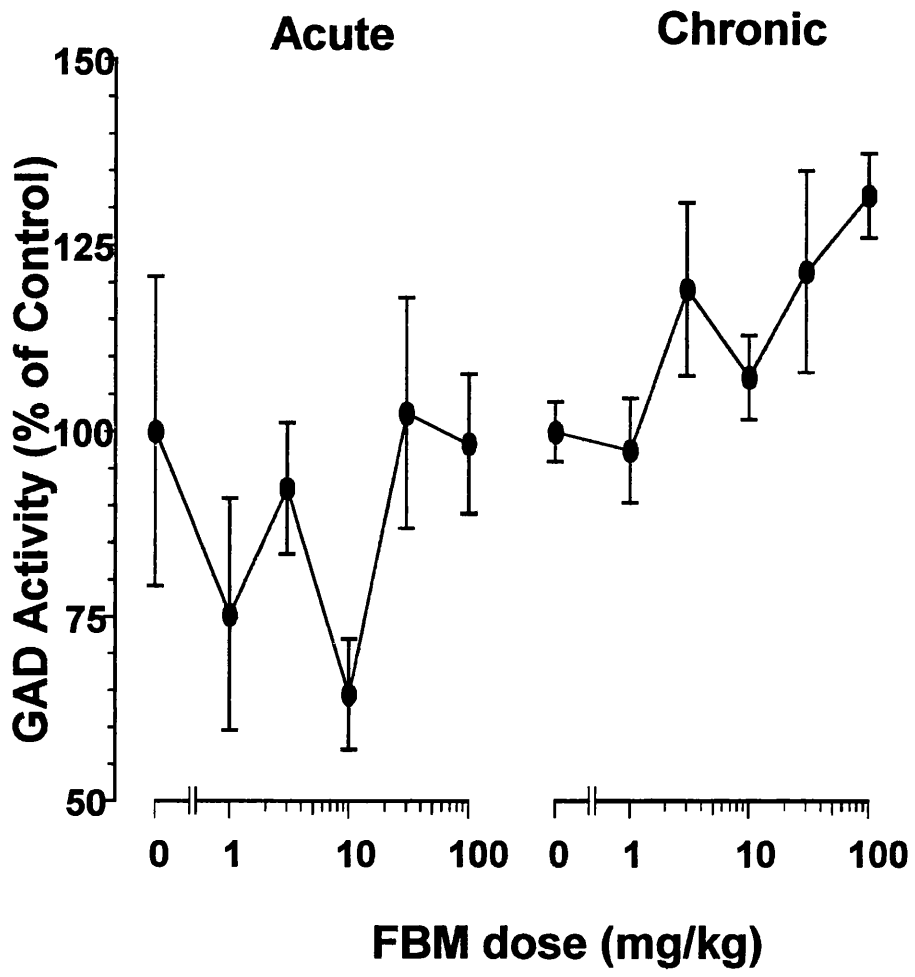


FIGURE 70. Effect of felbamate (FBM; 0 - 100 mg/kg) on mouse brain glutamic acid decarboxylase (GAD) activity at four hours after acute (single dose; left graph) and chronic (twice daily for five days; right graph) administration. Results (n = 6) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

5.3.2 Neurochemical effects of topiramate in mouse brain

EXPERIMENTAL PROTOCOL: TPM was prepared daily for i.p. injection as a suspension in 0.5% TWEEN 80. In both single and multiple dose studies, adult male ICR mice were randomised into nine treatment groups (n = 20 / group), and administered TPM 0.1, 0.3, 1, 3, 10, 30, 100, 300 and 1000 mg/kg. A tenth group of 20 mice (control) received vehicle alone. In the multiple dose phase of the study, treatment was continued once daily for eight days. In both phases, four hours after the final dose, the animals were sacrificed and their brains removed. Brains were divided into two hemispheres by a midline incision and stored at -70^{0}C until required for assay. Ten left hemispheres from each of the groups (single and multiple dose; control and drug treated) were assayed for GABA concentrations as described in section 2.11. Glutamate and glutamine were measured simultaneously in ten right hemispheres from each group as described in section 2.11. The remaining ten left hemispheres from each group were assayed for GABA-T activity as described in section 2.9., and the remaining ten right hemispheres from each group were assayed for GAD activity as described in section 2.12.

RESULTS: Repeated administration of 1000 mg/kg TPM resulted in the death of all 20 animals prior to the end of the eight day treatment period. At all doses examined, both single and repeated treatments with TPM were without significant effect on the concentrations of GABA (figure 71), glutamate (figure 72) or glutamine (figure 73). Similarly, single and repeated treatments were without effect on the activities of GABA-T (figure 74) or GAD (figure 75).

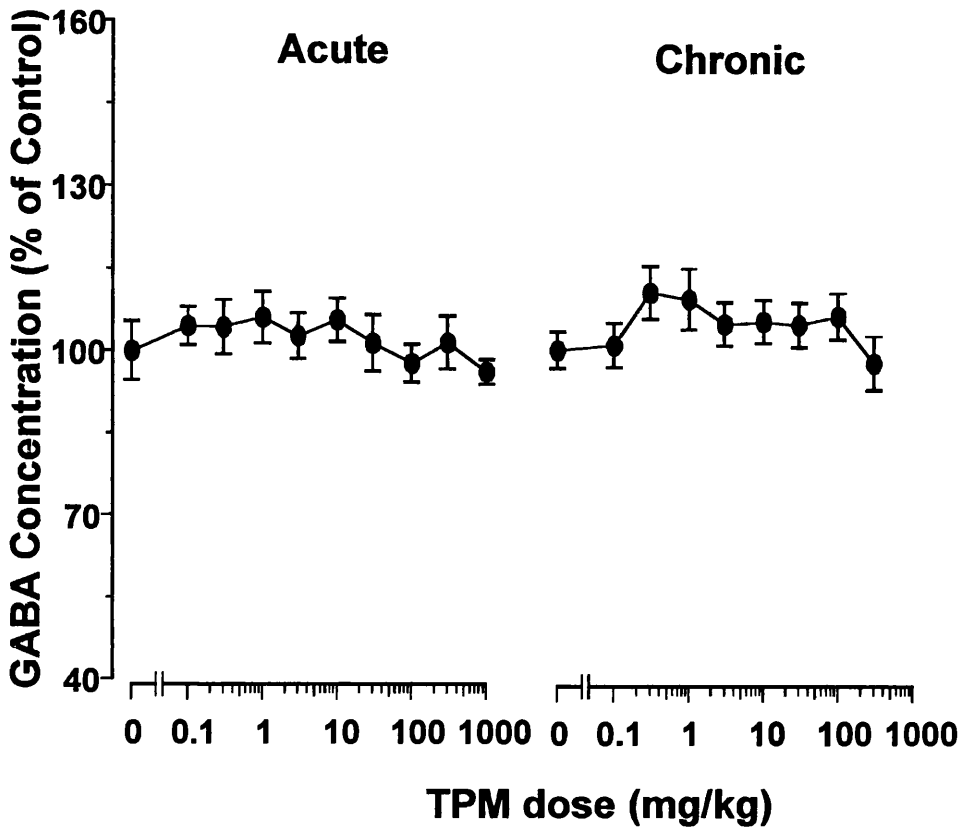


FIGURE 71. Effect of topiramate (TPM; 0 - 1000 mg/kg) on mouse brain GABA concentration at four hours after acute (single dose; left graph) and chronic (once daily for eight days; right graph) administration. Results (n = 10) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

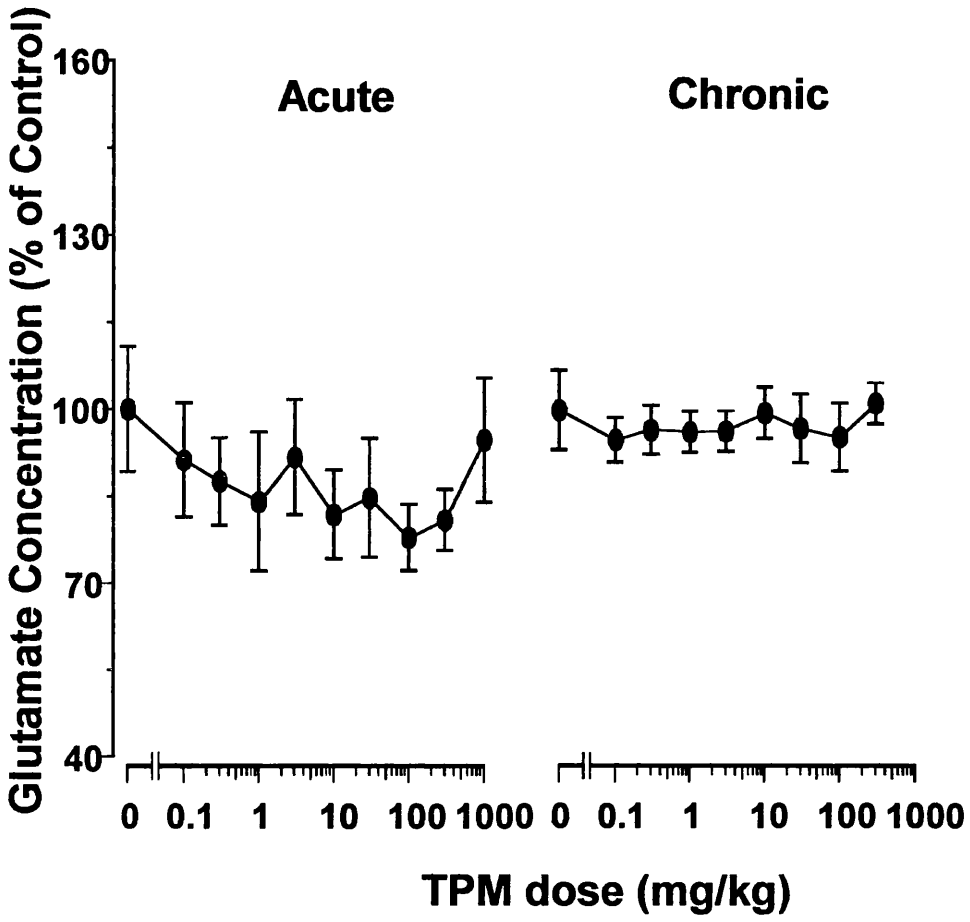


FIGURE 72. Effect of topiramate (TPM; 0 - 1000 mg/kg) on mouse brain glutamate concentration at four hours after acute (single dose; left graph) and chronic (once daily for eight days; right graph) administration. Results (n = 10) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

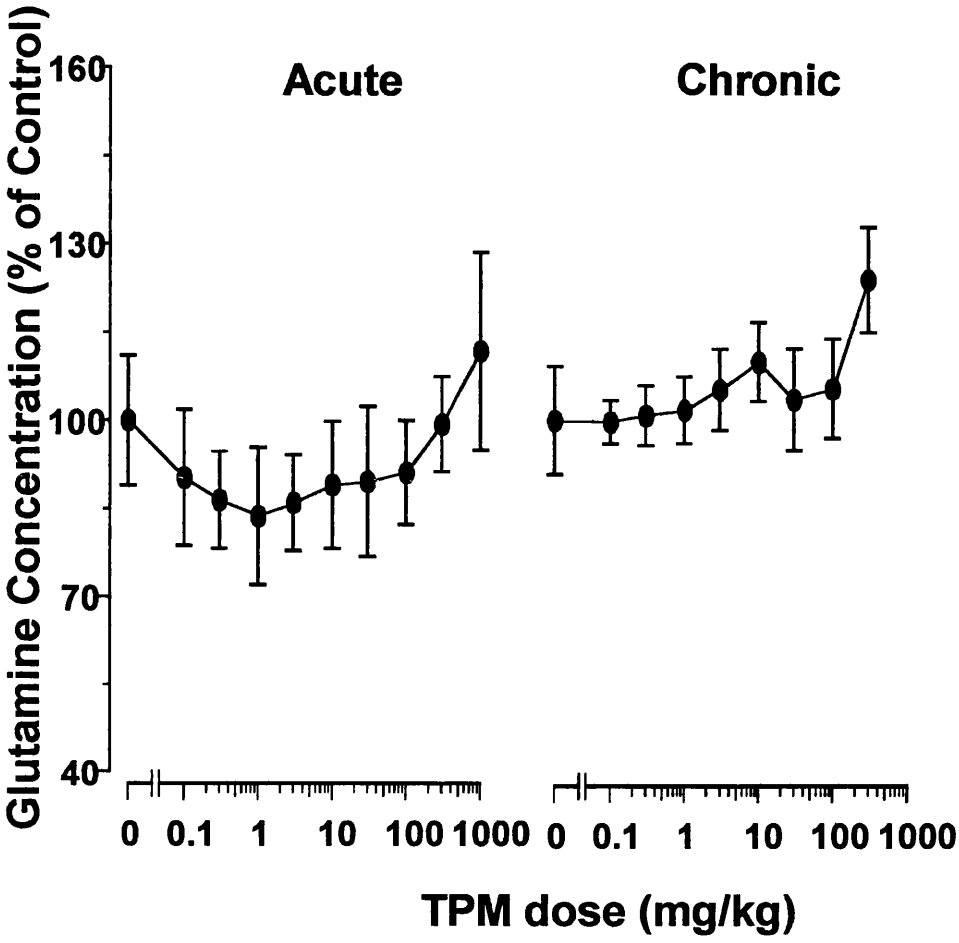


FIGURE 73. Effect of topiramate (TPM; 0 - 1000 mg/kg) on mouse brain glutamine concentration at four hours after acute (single dose; left graph) and chronic (once daily for eight days; right graph) administration. Results (n = 10) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

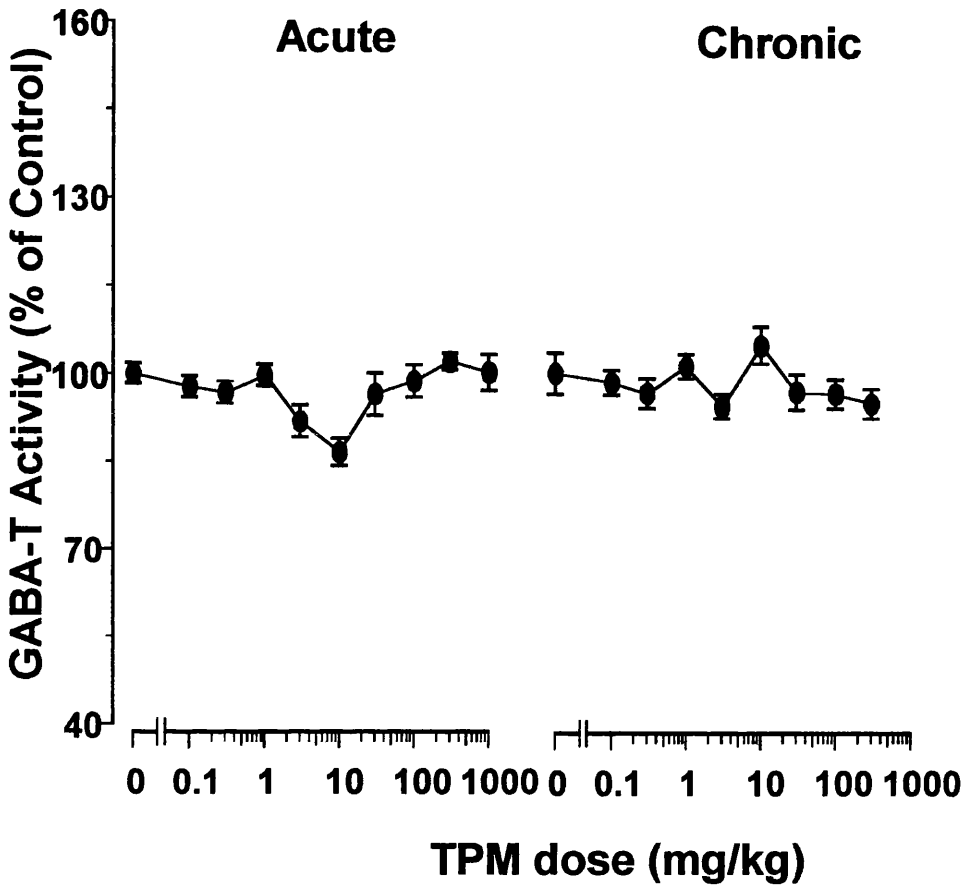


FIGURE 74. Effect of topiramate (TPM; 0 - 1000 mg/kg) on mouse brain GABA-transaminase (GABA-T) activity at four hours after acute (single dose; left graph) and chronic (once daily for eight days; right graph) administration. Results (n = 10) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

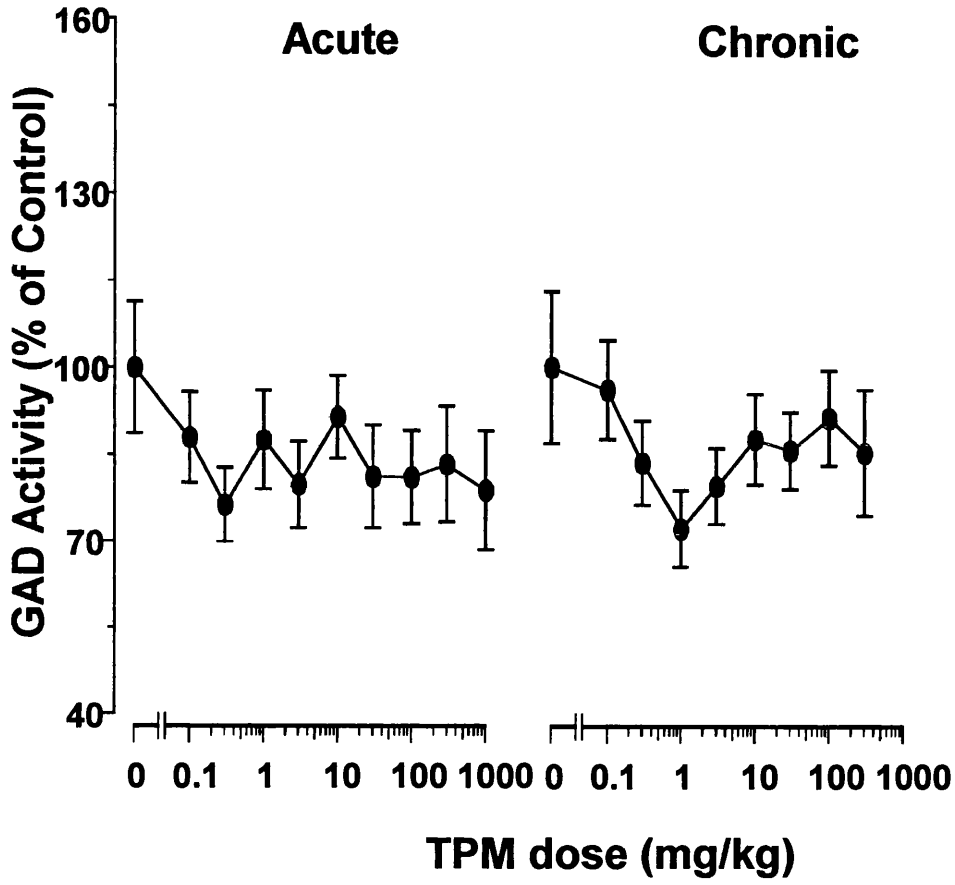


FIGURE 75. Effect of topiramate (TPM; 0 - 1000 mg/kg) on mouse brain glutamic acid decarboxylase (GAD) activity at four hours after acute (single dose; left graph) and chronic (once daily for eight days; right graph) administration. Results (n = 10) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

5.3.3 Neurochemical effects of levetiracetam in mouse brain

EXPERIMENTAL PROTOCOL: LEV was prepared daily for i.p. injection in 0.9% saline. In both single and multiple dose studies, adult male ICR mice were randomised into six treatment groups (n = 12 / group), and administered LEV 1, 3, 10, 30, 100 and 300 mg/kg. A seventh group of 12 mice (control) received vehicle alone. In the multiple dose phase of the study, treatment was continued twice daily (8 am and 4 pm) for five days. (A five day study with twice daily dosing was chosen to avoid weekend injections). In both phases, four hours after the final dose, the animals were sacrificed and their brains removed. Brains were divided into two hemispheres by a midline incision and stored at -70^{0}C until required for assay. Six left hemispheres from each of the groups (single and multiple dose; control and drug treated) were assayed for GABA concentrations as described in section 2.11. Glutamate and glutamine were measured simultaneously in six right hemispheres from each group as described in section 2.11. The remaining six left hemispheres from each group were assayed for GABA-T activity as described in section 2.9, and the remaining six right hemispheres from each group were assayed for GAD activity as described in section 2.12.

RESULTS: At all doses examined, both single and repeated treatments with LEV were without significant effect on the concentrations of GABA (figure 76), glutamate (figure 77) or glutamine (figure 78). Similarly, single and repeated treatments were without effect on the activities of GABA-T (figure 79) or GAD (figure 80).

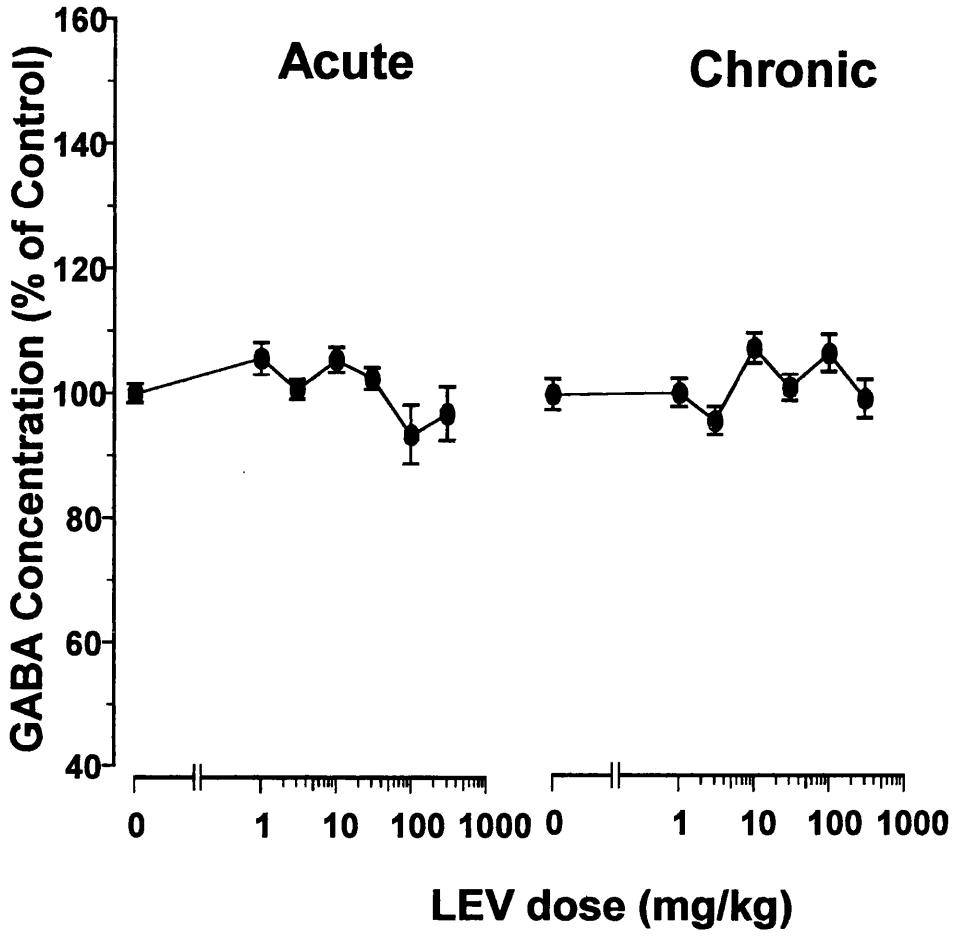


FIGURE 76. Effect of levetiracetam (LEV; 0 - 300 mg/kg) on mouse brain GABA concentration at four hours after acute (single dose; left graph) and chronic (twice daily for five days; right graph) administration. Results (n = 6) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

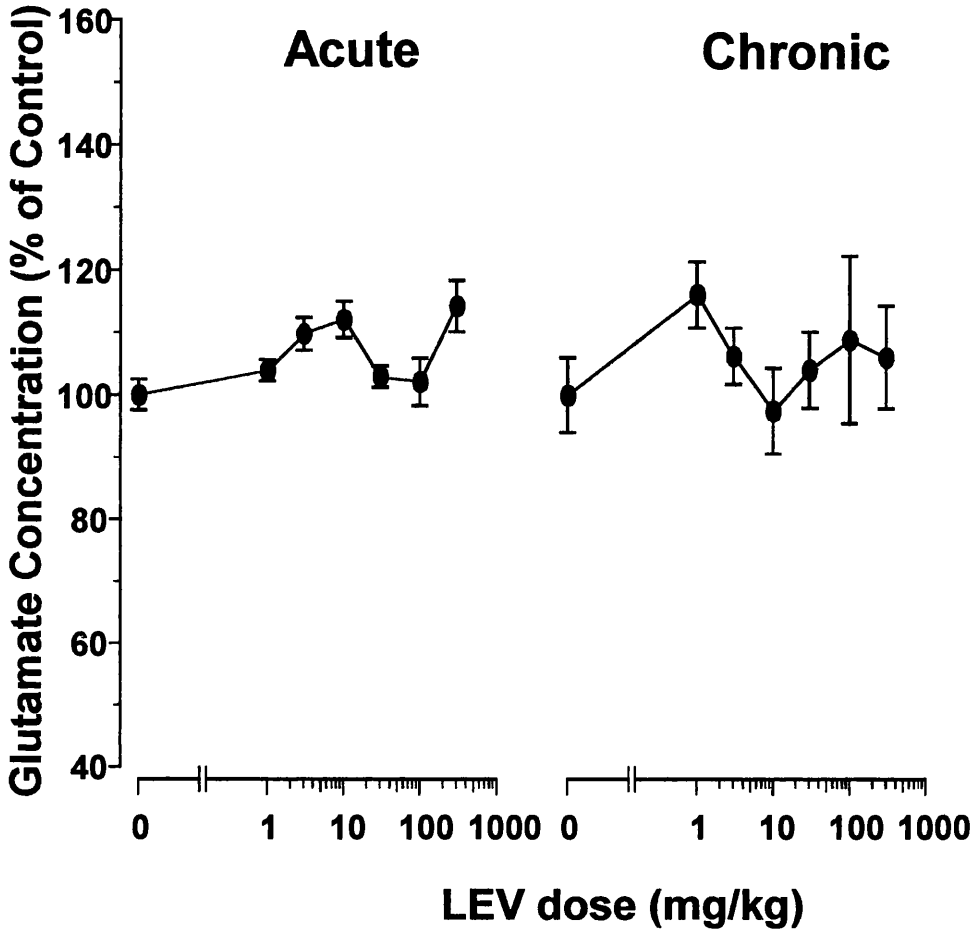


FIGURE 77. Effect of levetiracetam (LEV; 0 - 300 mg/kg) on mouse brain glutamate concentration at four hours after acute (single dose; left graph) and chronic (twice daily for five days; right graph) administration. Results (n = 6) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

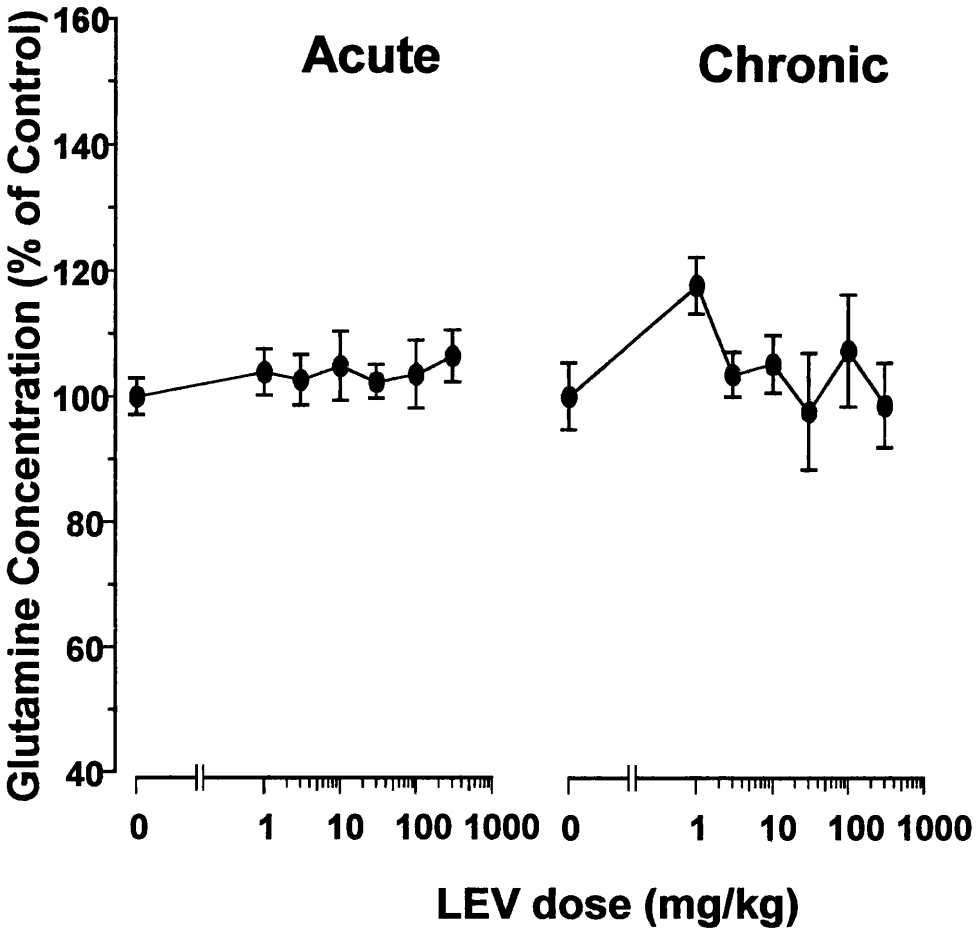


FIGURE 78. Effect of levetiracetam (LEV; 0 - 300 mg/kg) on mouse brain glutamine concentration at four hours after acute (single dose; left graph) and chronic (twice daily for five days; right graph) administration. Results (n = 6) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

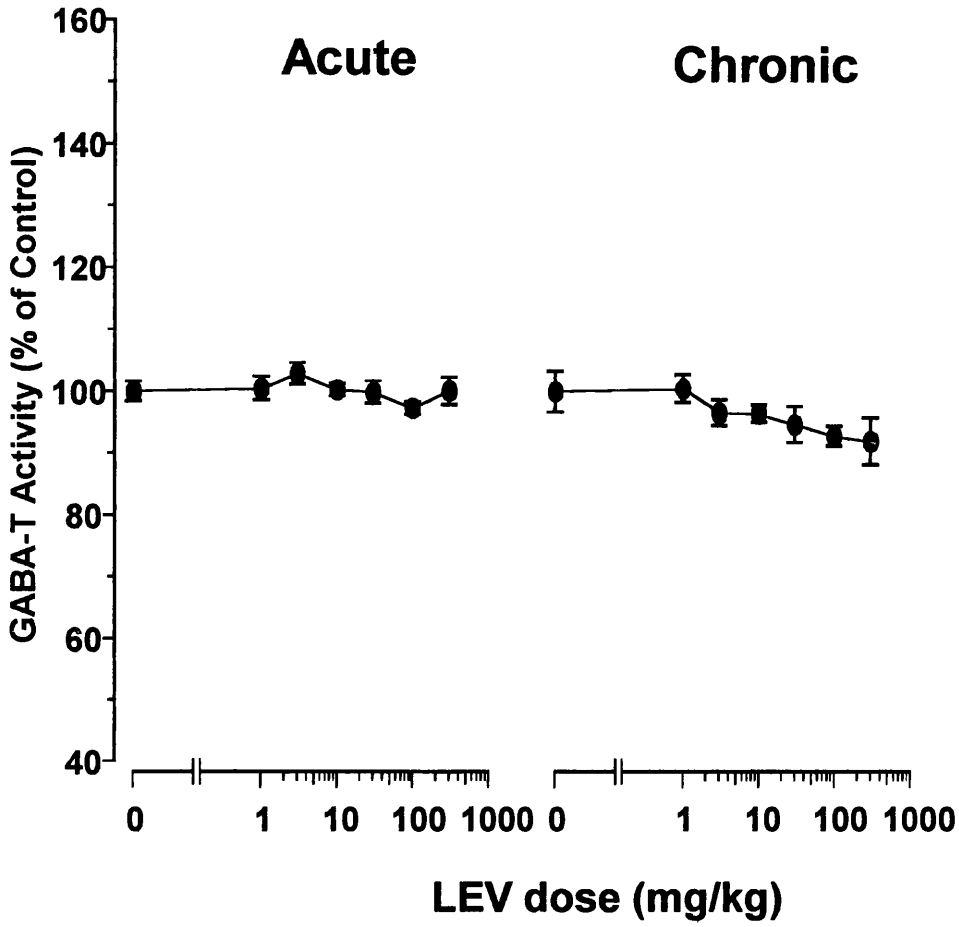


FIGURE 79. Effect of levetiracetam (LEV; 0 - 300 mg/kg) on mouse brain GABA-transaminase (GABA-T) activity at four hours after acute (single dose; left graph) and chronic (twice daily for five days; right graph) administration. Results (n = 6) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

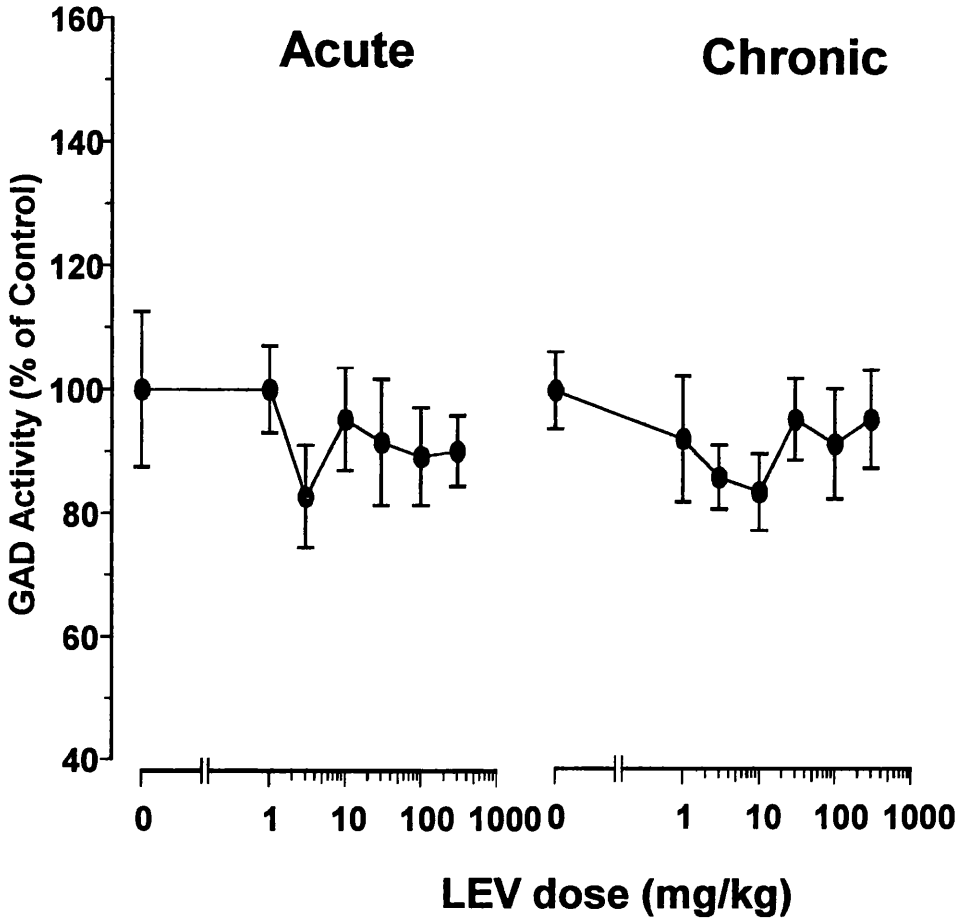


FIGURE 80. Effect of levetiracetam (LEV; 0 - 300 mg/kg) on mouse brain glutamic acid decarboxylase (GAD) activity at four hours after acute (single dose; left graph) and chronic (twice daily for five days; right graph) administration. Results (n = 6) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.).

5.4 DISCUSSION

As yet, the precise mechanisms of action of FBM, TPM and LEV remain to be determined. Several reports have suggested that actions on GABAergic and/or glutamatergic systems contribute to the antiepileptic effects of these drugs. The aim of this study was, therefore, to investigate the GABA- and glutamate-related neurochemical effects of the drugs in mouse brain.

5.4.1 Effects of single and repeated administration of felbamate on GABA- and glutamate-related neurochemistry in mouse brain

FBM was without significant effect on the concentrations of GABA, glutamate and glutamine following both single and repeated administration. Similarly, there was no effect on the activity of the enzyme GABA-T. Following repeated treatment with higher doses of FBM, there was a trend toward increased GAD activity. However, this effect failed to reach statistical significance and was observed at supra-therapeutic doses which would question its clinical relevance. Taken together, these results would suggest that the antiepileptic effects of FBM are not mediated by an action on the neurotransmitters and/or enzymes of the GABAergic and glutamatergic systems.

The lack of effect of repeated FBM administration on glutamate and glutamine concentrations in mouse brain would not appear to be in agreement with the results obtained with FBM in the GS study. Repeated administration of FBM (1 – 300 mg/kg) was previously shown (chapter 4) to significantly reduce the activity of GS. As GS is the enzyme responsible for the synthesis of glutamine from glutamate, such a reduction in its activity would possibly be expected to result in reduced glutamine and increased glutamate brain concentrations. However, this was not found to be the case, and

suggests that the decrease in GS activity is being compensated for by some other mechanism, such as an increase in the clearance of glutamate from the synapse or an increase in the synthesis of glutamine from *e.g.* glucose. Further work is required to clarify these conflicting results.

Despite the inability of FBM to influence GABAergic neurochemistry in this study, and its reported failure in radioligand binding studies to interact with the $GABA_A$ receptor complex (Ticku *et al*, 1991), voltage-clamp recordings from cultured rat hippocampal (Rho *et al*, 1994) and mouse cortical neurones (Kume *et al*, 1996) have clearly demonstrated a FBM-induced enhancement of GABA responses. In addition, Gordon and colleagues (1991) reported that a sub-protective dose of FBM could potentiate the anticonvulsant effects of diazepam against seizures induced by MES, PTZ and isoniazid.

In addition to effects on the GABA system, there is also evidence to suggest that FBM may interfere with glutamate-mediated neurotransmission. In mice, it effectively prevented seizures induced by NMDA and quisqualate (White *et al*, 1992), suggesting activity on the glutamatergic system. Although reports regarding the interaction of FBM within the ion channel of the NMDA receptor are conflicting (White *et al*, 1992; Subramaniam *et al*, 1995), the drug has been shown to inhibit the binding of 5,7-dichlorokynurenic acid to the strychnine-insensitive glycine site on the extracellular domain of this receptor (McCabe *et al*, 1993). White and colleagues (1995b) have similarly demonstrated a modulatory effect of the drug at this site. The effective concentrations of FBM were comparable in both studies and correlated closely with those proposed to evoke the antiepileptic and neuroprotective actions of the drug.

Thus, despite the failure of FBM to influence glutamate-related neurochemistry in this study, there is substantial evidence to suggest that it has the ability to modulate the activity of the NMDA sub-type of glutamate receptor and that such an effect may be relevant to its clinical actions.

The lack of effect of FBM on GABA- and glutamate-related neurotransmission in this study may be due to the limitations of the methods employed. It is possible that regional changes in amino acid concentrations or enzyme activities remained undetected due to the volume of tissue analyzed. Similarly, effects on neurotransmitter turnover cannot be excluded. It may, therefore, be beneficial to carry out a more detailed neurochemical study, employing techniques such as microdissection and microdialysis to clarify this issue. It is worth noting that the methods used in this study have been used in our laboratory to highlight previously unreported neurochemical effects of GBP (Leach *et al*, 1997a) and DGR (Leach *et al*, 1997b).

5.4.2 Effects of single and repeated administration of topiramate on GABA- and glutamate-related neurochemistry in mouse brain

TPM failed to influence the concentrations of GABA, glutamate or glutamine following both single and repeated treatment at all doses examined. Similarly, it was without significant effect on the activities of GABA-T and GAD. These results would suggest that an interaction with GABA- and glutamate-related neurochemical parameters is not involved in the mechanism of action of the drug.

TPM has recently been reported to increase brain GABA levels in healthy volunteers (Kuzniecky *et al*, 1998) and in patients with epilepsy (Petroff *et al*, 1999). This effect

has been proposed to contribute to the antiepileptic effects of the drug. However, the increase in brain GABA concentrations could not be demonstrated in mice treated with TPM in this study, and may reflect a species difference. As TPM has been shown to potentiate responses to GABA at the $GABA_A$ receptor (White *et al*, 1995a; White *et al*, 1997), and has inhibitory actions at the AMPA / kainate subtype of glutamate NMDA receptors (Coulter *et al*, 1995), effects on these amino acid systems cannot be excluded. As with the lack of effect of FBM in this study, it is possible that TPM may be exerting regional effects on the amino acid systems, and that these effects are being masked by the volume of tissue employed for analyses.

There are a number of studies demonstrating TPM-induced blockade of voltage-dependent sodium channels (Coulter *et al*, 1993; Kawasaki *et al*, 1996; Zona *et al*, 1996). In experimental animal models, TPM is effective against MES (Shank *et al*, 1994), audiogenic (Nakamura *et al*, 1994) and amygdaloid-kindled seizures (Wauquier and Zhou, 1996). It has only weak activity in the subcutaneous PTZ test (Shank *et al*, 1994), exhibiting a profile similar to that of PHT and CBZ, the AEDs which act at voltage dependent sodium channels. However, TPM clearly differs from these established compounds, in that it has a wider range of *in vitro* actions and a broader clinical spectrum of anticonvulsant activity. Therefore, despite the results of this study indicating that the drug does not act via effects on the amino acid systems, the suggestion that TPM has a multifactorial mode of action cannot be excluded.

5.4.3 Effects of single and repeated administration of levetiracetam on GABA- and glutamate-related neurochemistry in mouse brain

LEV failed to influence the concentrations of GABA, glutamate and glutamine in mouse brain following both single and repeated administration. It was similarly without significant effect on the activities of GABA-T and GAD. These results imply that the mechanism of action of the drug does not involve an action on these amino acid systems.

LEV has been proposed to act via a novel binding site in the brain where it is weakly displaced by several other AEDs and GABA-related substances (Noyer *et al*, 1995), and has been shown to reduce bicuculline-induced hyperexcitability in rat hippocampal CA3. Margineanu and Wulfert (1995) have, however, proposed that this effect is manifested via a non-GABAergic mechanism. In addition, the efficacy of the drug in GAERS genetic model of absence seizures (Gower *et* al, 1995) lends further weight to the non-GABAergic theory of action, as it is thought that drugs which exert their effects via a GABAergic mechanism of action exacerbate the spontaneous discharges observed in this model (Vergnes *et al*, 1984).

The efficacy of LEV against MES (Gower *et al,* 1992), audiogenic seizures in genetically epilepsy-prone rats (Gower *et al*, 1995) and amygdaloid-kindled seizures (Loscher and Honack, 1993) might suggest a similar profile to those AEDs which exhibit inhibitory actions at voltage-sensitive sodium channels (Loscher and Schmidt, 1988; Rogawski and Porter, 1990). There is, however, no evidence at present to suggest that LEV has any activity at this site (Saccan and Lloyd, 1994). In summary, it appears that LEV has a wide spectrum of anticonvulsant activity and a multifactorial

mode of action. However, the results of this study are inconclusive and the mechanism by which it exerts its anticonvulsant activity remains to be determined.

5.5 CONCLUSIONS

In conclusion, FBM, TPM and LEV failed to influence several GABA- and glutamate-related neurochemical parameters in mouse brain, suggesting that such actions do not contribute to the antiepileptic effects of these drugs. However, in light of their broad anticonvulsant profiles, it remains possible that all three possess multiple mechanisms of action and effects on GABA and glutamate neurochemistry cannot be ruled out without further more detailed investigations.

CHAPTER SIX

EFFECTS OF COMBINATIONS OF NEW ANTIEPILEPTIC DRUGS ON PENTYLENETETRAZOL-INDUCED SEIZURES IN MICE

6.1 INTRODUCTION

6.1.1 Monotherapy versus polytherapy

Management of seizures with a single AED is the treatment regimen of choice (Schmidt and Gram, 1995), and up to 70% of newly diagnosed patients can be effectively treated with a single AED (Perucca, 1997). However, it is estimated that in around 30% of patients suffering from epilepsy, treatment with a single AED does not provide satisfactory seizure control, and treatment with two or more AEDs is required - with many still not seizure free (Ferrendelli, 1995; Brodie and Dichter, 1996). In recent years, an increase in polypharmacy has been observed as many of the newer AEDs are introduced as add-on therapy (Genton and Roger, 1997). Although the practice of combining anticonvulsant medication is common, the theoretical basis for this practice has not been studied systematically (Chez *et al*, 1994). There is insufficient clinical data available to support the use of particular efficacious combinations of AEDs (Brodie, 1992a).

Many of the older drugs have previously been examined in combination using experimental animal models, such as PHT and VPA (Chez *et al*, 1994), VPA and PB (Bourgeois, 1988a), VPA and CBZ (Bourgeois, 1988a), VPA and ESM (Bourgeois, 1988b), and CBZ and PB (Bourgeois and Wad, 1988). Combinations of VPA and CZP (Mireles and Leppik, 1985), and VPA and ESM - for the treatment of absence seizures (Rowan *et al*, 1983) have been used clinically. However, the established AEDs are not ideal candidates for polytherapy (Schmidt and Gram, 1995), with complex and overlapping mechanisms of action, and frequent drug interactions (Brodie, 1992a). It is thought that combinations of the newer AEDs, with more predictable pharmacokinetics

and fewer drug interactions, may prove to be better tolerated (Leach, 1997). Recently it was noted that LTG with TPM appeared to be a useful combination in several patients in our research group. In support of this observation, a combination of LTG with TPM was also found to protect mice from PTZ-induced seizures (Stephen *et al*, 1998).

6.1.2 Pentylenetetrazol seizure model

Following systemic administration, there are numerous chemicals which can induce seizures at toxic doses. These include PTZ, GABA antagonists such as bicuculline, picrotoxin and penicillin, inhibitors of GABA synthesis, inverse benzodiazepine receptor agonists, the glycine antagonist strychnine, the cholinomimetic drug pilocarpine, and NMDA and kainate glutamate receptor agonists. Of these chemoconvulsants, PTZ is the most commonly employed (Loscher and Schmidt, 1988). The AED development program of the National Institutes of Health (NIH) USA, which since its initiation in 1975 has stimulated the development of various new AEDs, is primarily based on only two seizure models, namely, the MES test and the s.c. PTZ test (Loscher and Schmidt, 1988). PTZ is thought to produce myoclonic, generalised clonic and at higher doses tonic convulsions (Fisher, 1989), by interacting with the $GABA_A$ receptor - chloride ionophore complex, thereby reducing GABA mediated inhibition (Wilson and Escueta, 1974). There are two animal models which rely on PTZ to produce convulsions, namely the threshold for clonic seizures after i.v. infusion of PTZ, and the s.c. CD_{97} PTZ test (Loscher *et al*, 1991a). The s.c. CD_{97} is the most commonly used PTZ model and involves the administration of a dose of PTZ (usually between 80 and 100 mg/kg in mice) which induces generalised clonic seizures in all animals of a group. The seizure susceptibility of the particular strain can be

determined by dose-effect measurement, and the CD_{97} (i.e. the dose causing clonic seizures in 97% of the animals) can be calculated prior to AED evaluation by the test (Loscher *et al*, 1991a). The endpoints used in this model differ in that some researchers use the first episode of continuous generalised clonic activity of at least five seconds (Swinyard, 1949), whereas others have used the first generalised clonic seizure with loss of the righting reflex as the endpoint (Loscher and Schmidt, 1988). The s.c. PTZ test identifies compounds that are efficacious against generalised absence and myoclonic seizures, and is one of the most commonly used animal models in the search for effective AEDs (White, 1997). Due to the simplicity of the s.c. PTZ model, it was employed to evaluate the efficacy of combinations of new AEDs in the following studies. The loss of righting reflex was chosen as the endpoint, as it is possibly more reliable and easier to recognise.

6.2 AIMS

There are very few reports highlighting beneficial combinations of newer AEDs. Experimental studies may provide some insight into effective combinations of new AEDs with maximal efficacy and minimal toxicity. Three of the new AEDs, LTG, GBP and TPM are commonly used as add-on therapy for refractory partial seizures, with or without secondary generalisation (Dichter and Brodie, 1996). The aim of these studies was to investigate the effects of these drugs in single dose and in combination on experimental seizures induced by s.c. PTZ in mice, in an attempt to identify efficacious combinations of new AEDs.

6.3 EXPERIMENTAL PROTOCOLS AND RESULTS

6.3.1 Effects of lamotrigine and gabapentin in single dose and in combination on pentylenetetrazol-induced seizures in mice

DRUG ADMINISTRATION: LTG and GBP were prepared for injection in 0.9% saline. Mice were randomised into groups (n = 8) and administered (mg/kg): LTG (0.5, 2.5, 12.5); GBP (2, 10, 50); GBP (2) + LTG (0.5, 2.5, 12.5); GBP (10) + LTG (0.5, 2.5, 12.5); and GBP (50) + LTG (0.5, 2.5, 12.5) i.p. A control group (n = 8) received vehicle alone. At one hour post-AED administration, mice were subjected to the PTZ test (section 2.13). A one-hour period was chosen (as opposed to four hours) to allow a greater number of animals to be subjected to the test in the mornings, and to minimise the effect of circadian rhythms by avoiding afternoon testing. All drug doses examined in this chapter were based on previous studies carried out in the laboratory.

RESULTS: In single dose, only LTG (12.5 mg/kg) significantly ($P < 0.05$) increased the time to the first PTZ-induced generalised seizure. Combinations of LTG (2.5 mg/kg) with GBP (2 and 10 mg/kg) and LTG (12.5 mg/kg) with GBP (2, 10 and 50 mg/kg) all significantly ($P < 0.05$) increased the seizure latency in mice (figure 81).

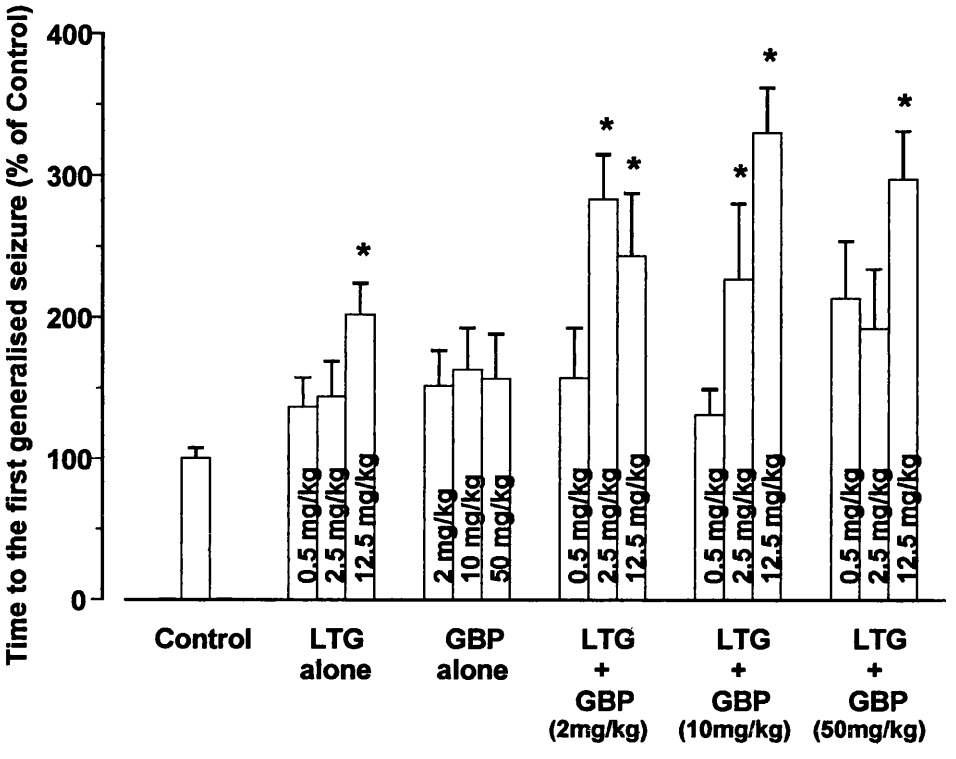


FIGURE 81. Effects of lamotrigine (LTG) and gabapentin (GBP) in single dose and in combination on the time to the first generalised seizure induced by 85 mg/kg pentylenetetrazol (s.c.) in groups of (n = 8 - 24) mice. Results are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.). Statistical significance (* $P < 0.05$) was determined by one way analysis of variance with Dunnett correction for multiple comparisons.

6.3.2 Effects of gabapentin and topiramate in single dose and in combination on pentylenetetrazol-induced seizures in mice

DRUG ADMINISTRATION: GBP and TPM were prepared for injection in 0.9% saline. Mice were randomised into groups (n = 8) and administered (mg/kg): GBP (2, 10, 50); TPM (5, 25, 125); TPM (5) + GBP (2, 10, 50); TPM (25) + GBP (2, 10, 50); and TPM (125) + GBP (2, 10, 50) i.p. A control group (n = 8) received vehicle alone. At one hour post-AED administration, mice were subjected to the PTZ test (section 2.13)

RESULTS: In single dose, GBP and TPM were without effect on PTZ-induced seizures in mice, at the doses examined. A combination of GBP (10 mg/kg) with TPM (25 mg/kg) was found to have a significant ($P < 0.05$) anticonvulsant effect when compared to control. All other combinations of GBP with TPM were without effect (figure 82).

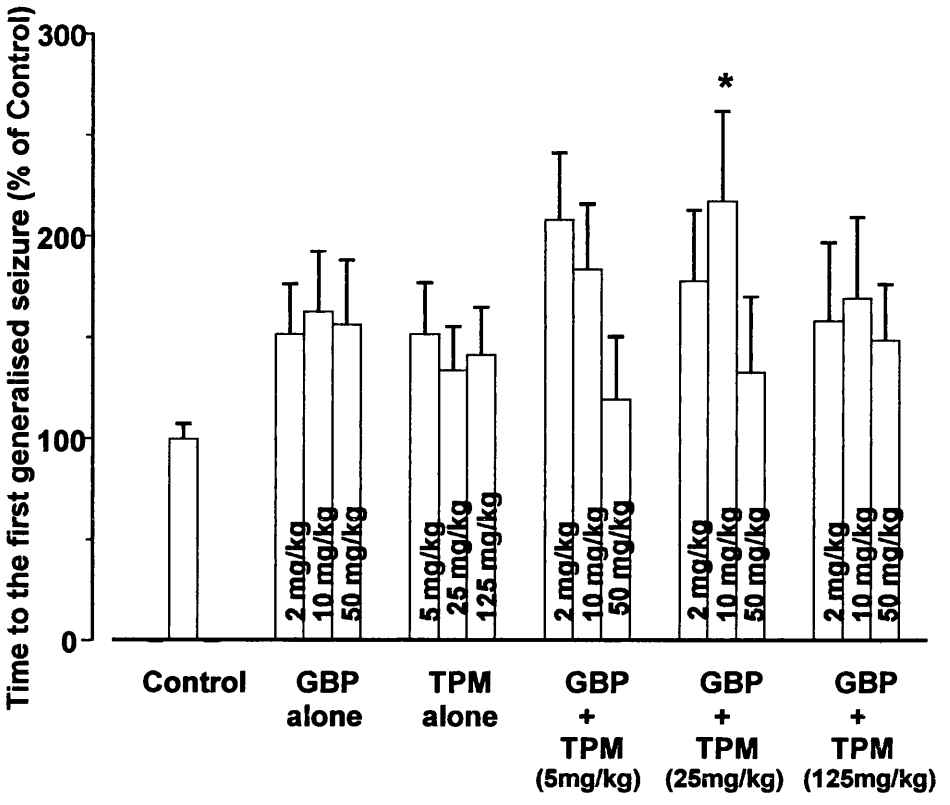


FIGURE 82. Effects of gabapentin (GBP) and topiramate (TPM) in single dose and in combination on the time to the first generalised seizure induced by 85 mg/kg pentylenetetrazol (s.c.) in groups of (n = 8 - 24) mice. Results are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.). Statistical significance (* $P < 0.05$) was determined by one way analysis of variance with Dunnett correction for multiple comparisons.

6.3.3 Effects of topiramate and lamotrigine in single dose and in combination on pentylenetetrazol-induced seizures in mice

DRUG ADMINISTRATION: Mice were randomised into groups (n = 8) and administered (mg/kg): TPM (5, 25, 125); LTG (0.5, 2.5, 12.5); LTG (0.5) + TPM (5, 25, 125); LTG (2.5) + TPM (5, 25, 125); and LTG (12.5) + TPM (5, 25, 125) i.p. A control group (n = 8) received vehicle alone.

RESULTS: In single dose, only LTG (12.5 mg/kg) significantly ($P < 0.05$) increased the time to the first generalised seizure. All combinations of TPM with LTG examined were found to have no effect on the latency to generalised seizures induced by PTZ, at one hour post-administration (figure 83).

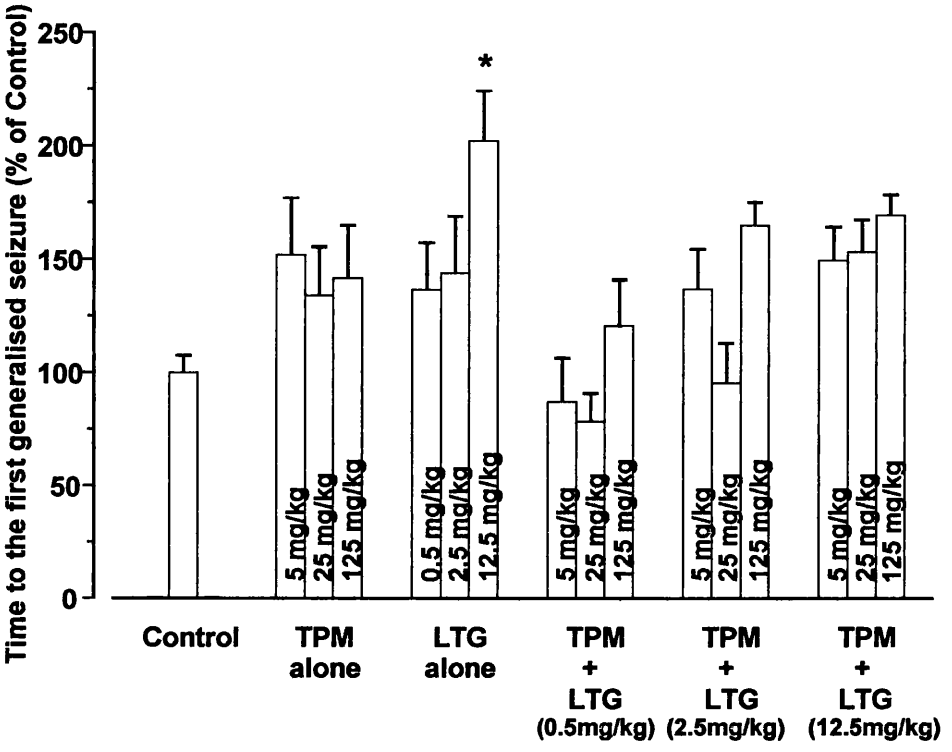


FIGURE 83. Effects of topiramate (TPM) and lamotrigine (LTG) in single dose and in combination on the time to the first generalised seizure induced by 85 mg/kg pentylenetetrazol (s.c.) in groups (n = 8 - 24) of mice. Results are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.). Statistical significance (* $P < 0.05$) was determined by one way analysis of variance with Dunnett correction for multiple comparisons.

6.4 DISCUSSION

The aim of these studies was to identify potentially effective combinations of new AEDs for use in the treatment of refractory epilepsy. Administration (s.c.) of the chemoconvulsant PTZ was chosen to evaluate the effects of three new AEDs LTG, GBP and TPM, in single dose, and in combination, on experimentally-induced seizures in mice.

6.4.1 Effects of single antiepileptic drugs on pentylenetetrazol-induced seizures

When administered alone, only the highest dose of LTG examined significantly increased the latency to the first generalised seizure with loss of the righting reflex. Both GBP alone and TPM alone were without effect.

6.4.2 Effects of lamotrigine and gabapentin in combination on pentylenetetrazol-induced seizures

Five of the nine combinations of LTG with GBP were found to significantly increase the time to PTZ-induced generalised seizures when compared to control, suggesting that there is an additive interaction occurring between the AEDs. However, when combinations of LTG and GBP were compared to LTG alone they were not significantly different. LTG 12.5 mg/mg in combination with GBP 2, 10 and 50 mg/kg all significantly increased the time to PTZ-induced seizures. Similarly, LTG 2.5 mg/kg in combination with GBP 2 and 10 mg/kg also increased the time to PTZ-induced seizures. However, LTG 2.5 mg/kg in combination with the highest dose of GBP examined (50 mg/kg) failed to increase the time to the first generalised seizure when compared to control, suggesting that this particular combination has an infra-additive effect. These results suggest that there is an interaction occurring between the drugs

which requires further investigation. In support of this, a recent study by De Sarro and colleagues (1998) reported that GBP potentiated the anticonvulsant activity of several AEDs including LTG, against audiogenic seizures in DBA/2 mice.

LTG is thought to exert its anticonvulsant effects by blockade of voltage-dependent sodium channels, thereby preventing the release of the excitatory neurotransmitters glutamate and aspartate (Leach *et al*, 1986). The mechanism of action of GBP is unclear (Wilson and Brodie, 1996), but in a similar manner to LTG, GBP has been shown to inhibit voltage-dependent sodium currents (Wamil and McLean, 1994). In addition to this, it has been reported to bind to a calcium channel in the brain (Gee *et al*, 1996), and recently it has been suggested that GBP may also exert effects on the GABAergic system (Leach *et al*, 1997a). In the search for pharmacological synergism in the management of refractory epilepsy, there is much speculation as to which AEDs should be examined in combination. It has been suggested that AEDs which target the same neurotransmitter system may be a logical approach in treating patients with a single seizure type, and those with differing modes of action may be beneficial in patients who experience a range of seizures (Leach and Brodie, 1994). With regard to this, the combination of LTG and GBP may be of value in treating patients with a single seizure type, as both drugs exert effects on voltage-dependent sodium channels. It may also be beneficial to patients with multiple seizure types, due to the additional reported effects of GBP on calcium channels and the GABAergic system.

6.4.3 Effects of gabapentin and topiramate in combination on pentylenetetrazol-induced seizures

A combination of GBP (10 mg/kg) with TPM (25 mg/kg) was found to significantly increase the latency to the first PTZ-induced seizure when compared to control. All other combinations were without effect. The observation that only one of the nine combinations examined was effective questions the clinical relevance of this finding, and requires further detailed work to evaluate the use of this combination as an effective polytherapy regimen.

The precise mechanism of action of TPM remains to be fully elucidated, but it is thought to possess multiple anticonvulsant effects (Dichter and Brodie, 1996). TPM has been reported to have inhibitory actions on voltage-dependent sodium channels (Coulter *et al*, 1993; Zona *et al*, 1997). There is also evidence to suggest that the drug has inhibitory effects on the AMPA / kainate subtype of glutamate receptor (Coulter *et al*, 1995), and it has been shown to potentiate GABAergic responses at the $GABA_A$ receptor (White *et al*, 1997). As both GBP and TPM appear to share a common effect on sodium channels, and both appear to possess multiple mechanisms of action, one could speculate that this combination may be of benefit in treating both patients with a single seizure type, and those experiencing a range of seizure types.

6.4.4 Effects of lamotrigine and topiramate in combination on pentylenetetrazol induced seizures

In this study, all nine combinations examined of LTG with TPM failed to exert any significant anticonvulsant effect in preventing PTZ-induced seizures when compared to control. These results fail to reproduce the protectant effect reported by Stephen *et al*

(1998), and suggest that the addition of TPM to LTG has a detrimental effect on seizure protection. LTG 12.5 mg/kg alone significantly increased the time to PTZ-induced seizures, however, when combined with TPM 5, 25 and 125 mg/kg the protective effect of LTG was lost, suggesting an infra-additive interaction. This study provides no evidence to suggest this combination may be of benefit in the treatment of refractory epilepsy. It is not known why the studies, which were conducted in the same laboratory under the same experimental conditions fail to agree. The only difference between the two studies is that different batches of TPM were employed in the investigations, and may account for the variation in results. No conclusions can therefore be drawn until further work is carried out to clarify the differences between the two studies.

6.4.5 Further work

For simplicity, this preliminary study examined only at a few doses for each drug, and combinations were examined in only the PTZ seizure model. Further combination studies are therefore required in additional seizure models. This study examined combinations of only three new AEDs, and as the newer drugs in general are reported to have fewer interactions than the older ones, future work should include combinations of the remaining new drugs. In support of this, a recent study has suggested that GBP may augment the antiepileptic effects of VGB in hippocampal slices (Lucke *et al*, 1998). It may also be of benefit to investigate combinations of the new drugs with the established ones. Indeed, in a recent clinical trial it has been observed that a combination of LTG with the established AED VPA may display synergism (Brodie *et al*, 1997). Finally, as this study only concentrated on acute administration, it would be of benefit to assess the effects of combinations of AEDs

following chronic treatment, to ensure that any potential therapeutic benefits are not accompanied by an increase in toxicity.

6.5 CONCLUSIONS

In conclusion, this study fails to conclusively identify effective combinations of new AEDs. However, the results observed for combinations of LTG with GBP suggest that this combination may be of interest clinically, and warrants further detailed experimental and clinical investigation. There is also a hint that the combination of GBP and TPM may be of some interest clinically, whereas the effects of the combination of LTG with TPM require clarification.

CHAPTER SEVEN

EFFECTS OF ANTIEPILEPTIC DRUGS ON THE TRANSPORT OF GABA IN PRIMARY CULTURES OF HUMAN CORTICAL ASTROCYTES

7.1 INTRODUCTION

Astrocytes account for more than half of the glial cell population in the brain and are actively involved in the uptake and metabolism of the major inhibitory neurotransmitter GABA (Kimelberg, 1983; Kimelberg and Norenberg, 1989). Primary cultures of astrocytes have been used extensively to study the transport of GABA (Schousboe *et al*, 1977; Larsson *et al*, 1981; Schousboe, 1981; Schousboe *et al*, 1983b; Hansson *et al*, 1985), and are ideal tools for investigating the mechanisms of action of AEDs.

SVP, VGB and TGB are three anticonvulsant drugs which exhibit effects on GABA neurotransmission. SVP is an established AED whose precise mechanism of action remains to be elucidated. It is thought to exert multiple actions, amongst which are several effects on the GABAergic system. These include an increase in the concentration of the GABA synthesizing enzyme GAD, and inhibitory effects on the GABA degrading enzymes GABA-T, succinic semialdehyde dehydrogenase and aldehyde reductase, leading to an increase in brain GABA concentration (Rogawski and Porter, 1990). VGB and TGB are two novel AEDs which exert their anticonvulsant actions via specific effects on the GABAergic system. VGB irreversibly inhibits the GABA degrading enzyme GABA-transaminase (Jacob *et al*, 1990) and TGB selectively blocks the glial and neuronal uptake of GABA (Suzdak and Jansen, 1995).

The vast majority of AED neurochemical studies are performed using rodent brain and do not employ human brain tissue. In recent years, the use of animals for experimental purposes has become morally less acceptable. Moreover, there are differences between animal brain and human brain, and it is difficult to determine if an AED will have the

same effect in humans as it has in an animal experiment. It is thought that the use of human brain tissue will afford a more representative insight into the clinical action of AEDs under investigation.

7.2 AIMS

The effects of AEDs have not previously been demonstrated in primary glial cultures from human brain. However, the supply of viable human tissue was not as abundant as hoped, and much of the tissue received was utilised in establishing the method. Therefore, only a limited number of AEDs were selected for investigation, and the only parameter examined was GABA uptake. The aim of this preliminary study was, therefore, to investigate the effects of SVP, VGB and TGB on the transport of GABA in cultured human astrocytes from adult and foetal brain, and to compare the results with those obtained from the rodent experiments (chapter 3).

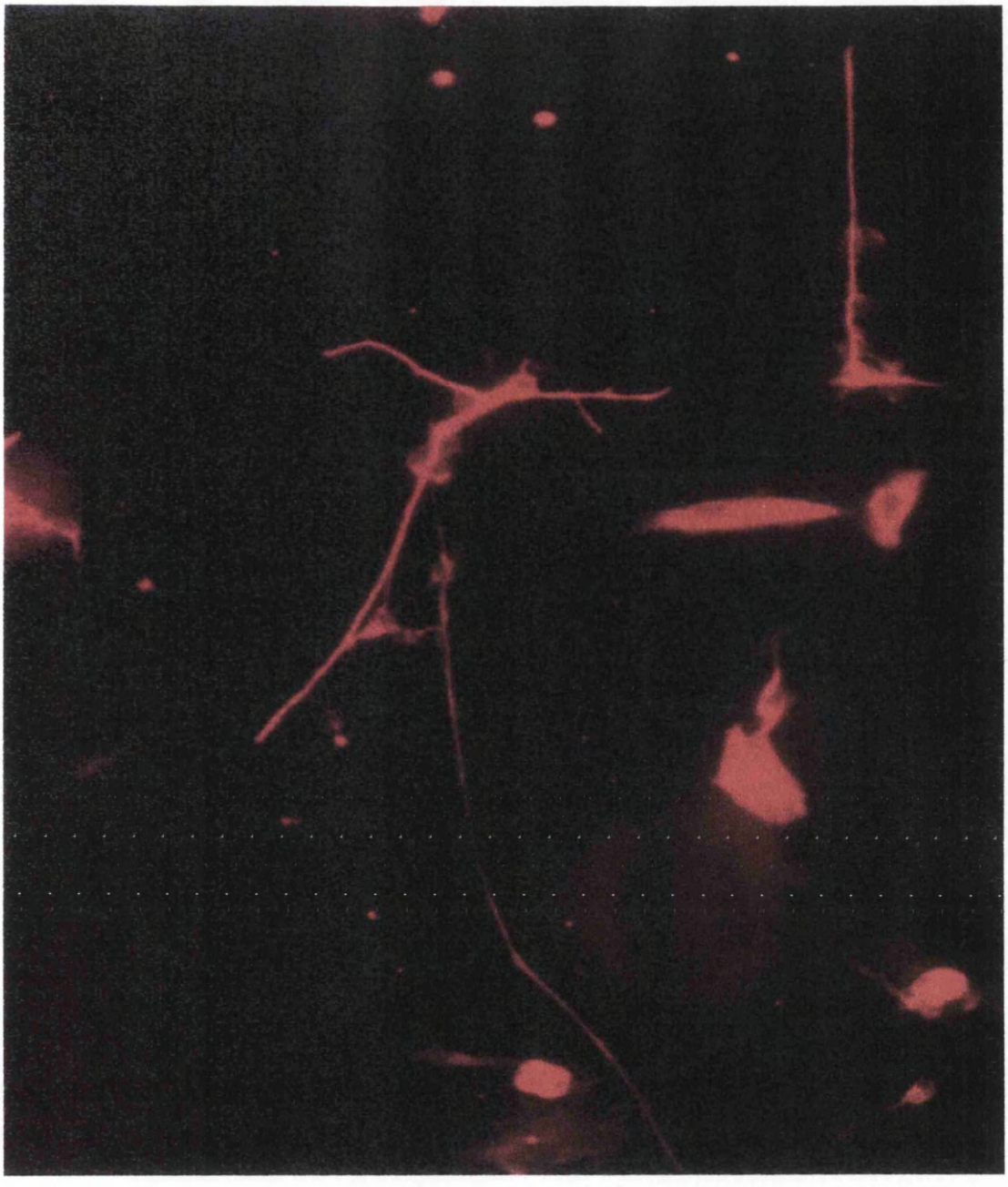
7.3 EXPERIMENTAL PROTOCOLS AND RESULTS

7.3.1 Effects of sodium valproate, vigabatrin and tiagabine on the uptake of [^{14}C]-GABA into primary cultures of human adult astrocytes

EXPERIMENTAL PROTOCOL: Adult brain tissue was obtained and cultured as described in section 2.4. Astrocytes were identified as positive for GFAP and photographed as described in section 2.5 (photograph 2). Dose / response curves could not be constructed due to insufficient amounts of tissue. Single drug concentrations which had a significant effect on GABA uptake in the rat cultures were therefore examined. Cultures were separated into three groups (n = 24) and exposed to SVP (1000 μM), VGB (100 μM) and TGB (200 nM) in BSS. A fourth group (control) was

exposed to BSS alone. After a one hour incubation, cells were assayed for GABA uptake as described in section 2.7.

RESULTS: SVP (1000 μM), VGB (100 μM) and TGB (200 nM) significantly ($P < 0.05$) reduced the uptake of [^{14}C]-GABA into primary cultures of human adult astrocytes following a one hour exposure (figure 84).



PHOTOGRAPH 2. Human adult cortical astrocytes in primary culture (day 21) stained with glial fibrillary acidic protein (GFAP) and examined using a fluorescent microscope (x 450).

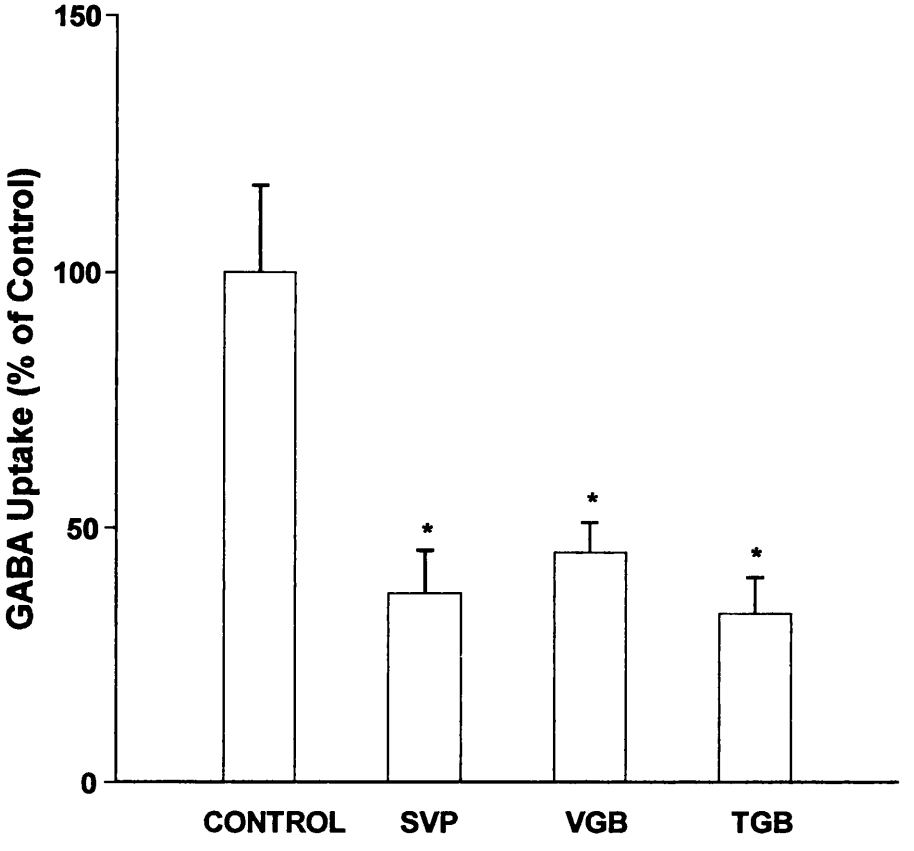
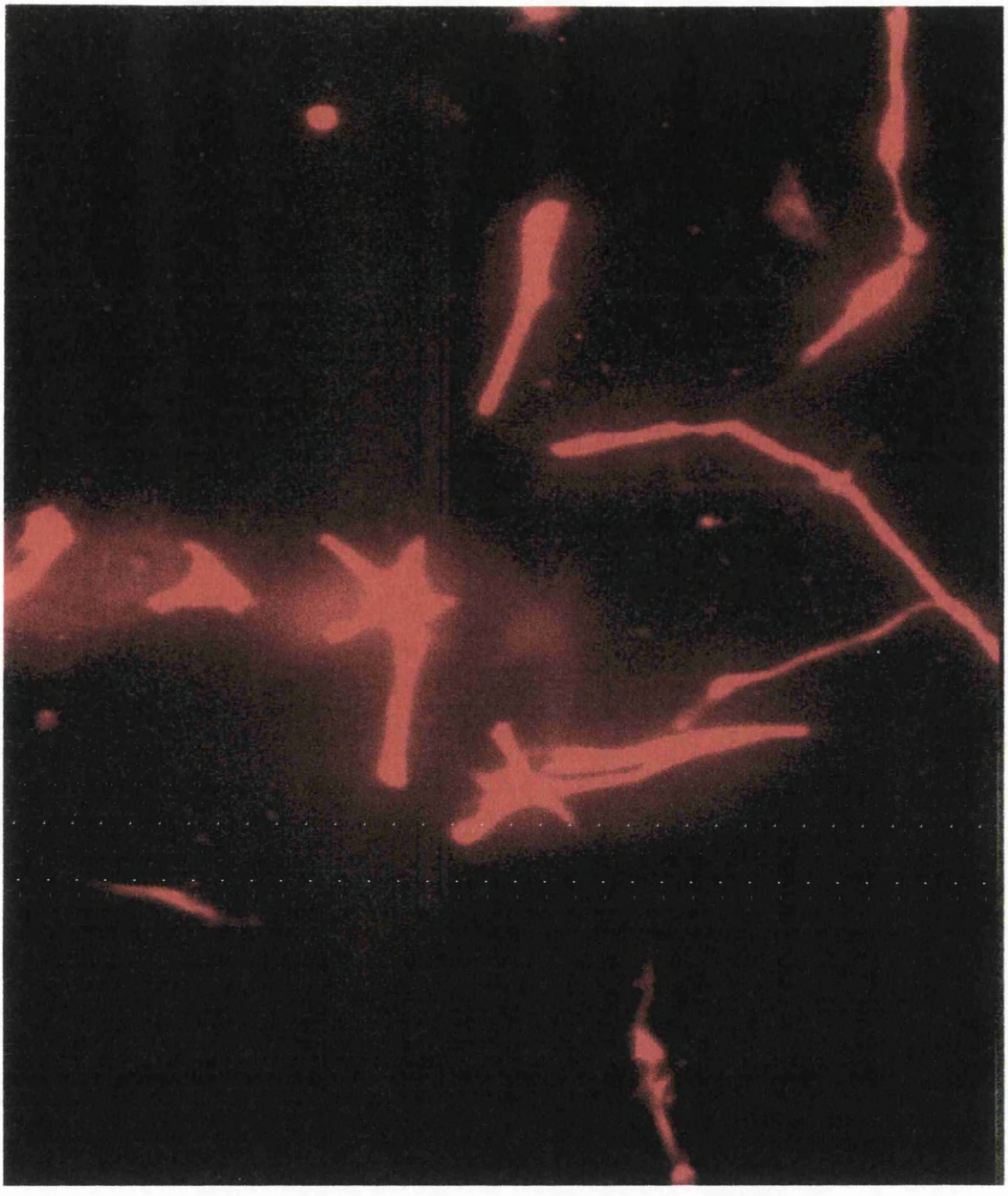


FIGURE 84. Effects of sodium valproate (SVP; 1000 μM), vigabatrin (VGB; 100 μM), and tiagabine (TGB; 200 nM) on the uptake of GABA into primary cultures of human adult cortical astrocytes. Results (n = 24) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.). Statistical significance (*$P < 0.05$) was determined by one way analysis of variance with Dunnett correction for multiple comparisons.

7.3.2 Effects of sodium valproate, vigabatrin and tiagabine on the uptake of [^{14}C]-GABA into primary cultures of human foetal astrocytes

EXPERIMENTAL PROTOCOL: Foetal brain tissue was obtained and cultured as described in section 2.4. Astrocytes were identified as positive for GFAP and photographed as described in section 2.5 (photograph 3). As in the foetal astrocyte experiments, drug concentrations which had a significant effect on GABA uptake in the rat cultures were chosen. An additional increased concentration of 500 nM of TGB was examined in this study as the 200 nM was without effect. Cultures were separated into four groups (n = 17) and exposed to SVP (1000 μM), VGB (100 μM), TGB (200 nM) and TGB (500 nM) in BSS. A fifth group (control) was exposed to BSS alone. After a one hour incubation, cells were assayed for GABA uptake as described in section 2.7.

RESULTS: SVP (1000 μM) and VGB (100 μM) significantly ($P < 0.05$) reduced the uptake of [^{14}C]-GABA into primary cultures of human foetal astrocytes following a one hour exposure. Both doses of TGB (200 and 500 nM) examined were without effect (figure 85) on the transport of GABA.



PHOTOGRAPH 3. Human foetal cortical astrocytes in primary culture (day 21) stained with glial fibrillary acidic protein (GFAP) and examined using a fluorescent microscope (x 450).

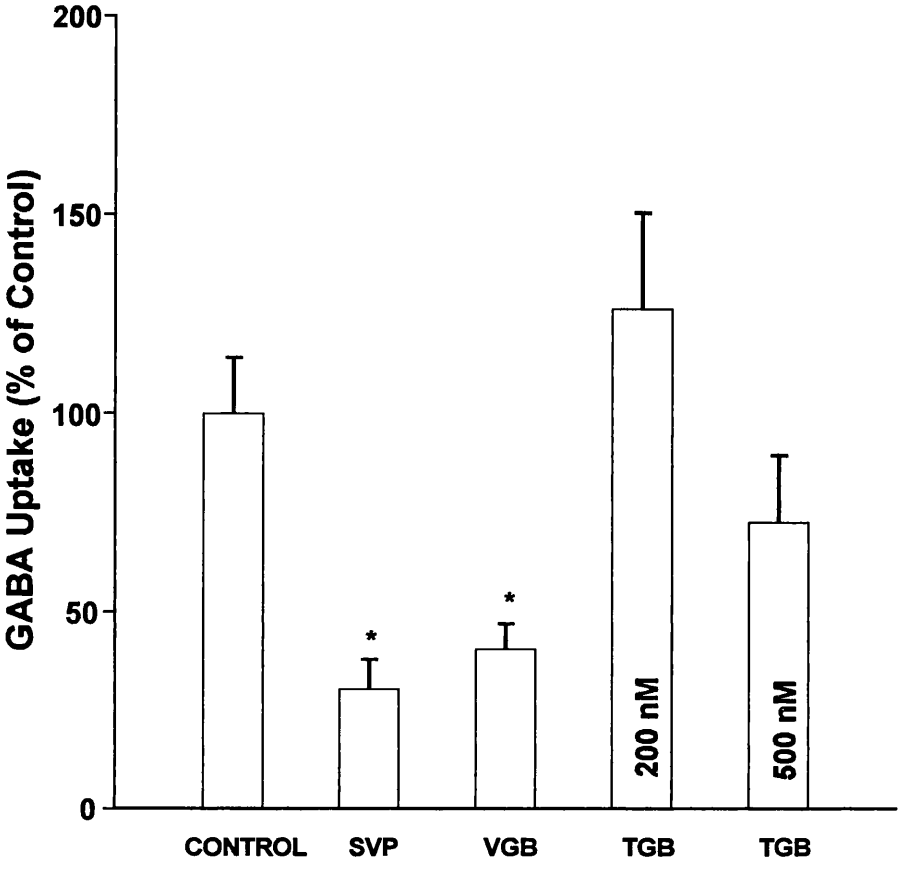


FIGURE 85. Effects of sodium valproate (SVP; 1000 μM), vigabatrin (VGB; 100 μM), and tiagabine (TGB; 200 and 500 nM) on the uptake of GABA into primary cultures of human foetal cortical astrocytes. Results (n = 17) are expressed as the mean percentage of mean control values and error bars denote the standard error of the mean (S.E.M.). Statistical significance (*$P < 0.05$) was determined by one way analysis of variance with Dunnett correction for multiple comparisons.

7.4 DISCUSSION

SVP, VGB and TGB are three AEDs which have previously been demonstrated to exert effects on the GABAeric system (Lippert *et al*, 1977; Larsson *et al*, 1986; Rogawski and Porter, 1990; Nielsen *et al*, 1991; Borden *et al*, 1994; Suzdak and Jansen, 1995). However, many studies employ rodent tissue to investigate AED mechanisms of action, and these drugs have not previously been examined in primary cultures of human astrocytes. The aim of this study was therefore to determine the effects of SVP, VGB and TGB on the uptake of [^{14}C]-GABA into primary cultures of both human foetal and adult brain.

7.4.1 Effects of sodium valproate on the uptake of GABA into primary cultures of human adult and foetal astrocytes

At the concentration examined, SVP was found to significantly reduce the uptake of GABA into both adult and foetal astrocytes. A reduction of GABA uptake into rat cortical astrocytes was also observed with a similar dose of SVP (chapter 3, figure 4). In addition, this effect is consistent with a previous study carried out using primary cultures from rodent brain, where Nilsson and colleagues (1992) found that acute treatment of rat cortical astrocytes with SVP resulted in a decreased affinity, but not transport capacity, for GABA. SVP has also been demonstrated to inhibit GABA uptake in frog dorsal root ganglion cells (Klee *et al*, 1985). These results would suggest that adult and foetal human tissue, rodent and frog brain respond in a similar manner to SVP.

7.4.2 Effects of vigabatrin on the uptake of GABA into primary cultures of human adult and foetal astrocytes

As well as the documented inhibition of GABA-T, work in our laboratory recently demonstrated that VGB reduced the uptake of GABA into primary cultures of astrocytes from rat neonatal tissue (Leach *et al*, 1996). This effect has again been demonstrated in primary cultures of rat cortical astrocytes in this thesis (chapter 3, figure 5). This study has confirmed that this additional effect of VGB also occurs in primary cultures of astrocytes from human adult and foetal brain, and further indicates that it may be involved in the antiepileptic mechanism of action of the drug.

7.4.3 Effects of tiagabine on the uptake of GABA into primary cultures of human adult and foetal astrocytes

It is well documented that TGB exerts its antiepileptic effects via inhibition of GABA uptake (Giardina, 1994; Mengel, 1994; Suzdak and Jansen, 1995), as demonstrated in rat glial and neuronal cultures and rat brain synaptosomes. It is thought to specifically inhibit the GABA transporter GAT-1 (Borden *et al*, 1994). Results obtained using primary cultures of rat cortical astrocytes in this thesis (chapter 3, figure 10) reproduce this TGB-mediated reduction in GABA uptake at doses of TGB ranging from 10 to 1000 nM. In contrast with the results obtained with SVP and VGB, TGB was found only to influence the transport of GABA in the adult human astrocytes, with no significant reduction in the uptake of GABA in the foetal cells at both doses of TGB examined. The apparent discrepancy in results between adult and foetal tissue may reflect incomplete development of the nervous system, in that the GAT-1 transporter, which TGB inhibits, may not be present in cells cultured from 16 - 24 week old foetal brain. Therefore, a lack of effect of TGB is observed. Further work is needed to clarify

this. It is, however, interesting to note that SVP and VGB do not appear to discriminate between foetal and adult tissue. This would suggest that these drugs may be reducing the uptake of GABA via a different mechanism, possibly not specific and not involving the GAT-1 transporter. Differences in the transport of these drugs into cells has previously been described. It is thought that SVP is itself transported by a carrier mediated uptake (Nilsson *et al*, 1990), whereas it has been shown that TGB is not a substrate for the GABA uptake carrier (Mengel, 1994).

7.5 CONCLUSIONS

In conclusion, this preliminary study has demonstrated inhibition of GABA uptake by SVP, VGB and TGB in adult astrocytes, and by SVP and VGB in foetal cultures. The lack of effect of TGB in the foetal cultures may highlight a lack of development of the GAT-1 transporter in 16 - 24 week foetal brain tissue, and suggests that SVP and VGB reduce GABA uptake by a mechanism distinct from that of TGB. The effects of SVP and VGB on GABA uptake appear to be consistent in rat and human tissue. The reduction in GABA uptake exhibited by TGB in the rat experiments is consistent with that in the adult human tissue, but not in the foetal human tissue. This study has therefore confirmed that the drugs examined have similar effects in animal and human tissue, but may highlight an important difference in the mechanism of GABA uptake inhibition exhibited by these drugs. This is, however, a preliminary study and further research of this nature is required in order to draw more definite conclusions on the responses of AEDs in different species.

CHAPTER EIGHT

GENERAL DISCUSSION AND CONCLUSIONS

8.1 MECHANISMS OF ACTION OF THE ESTABLISHED ANTIEPILEPTIC DRUGS

8.1.1 Phenobarbitone

In addition to the documented effects of PB on the $GABA_A$ receptor, and more recently, effects on voltage-sensitive calcium and potassium channels, and blockade of NMDA-receptors, this study has revealed previously unreported effects of the drug on glutamate neurotransmission. In primary cultures of rat cortical astrocytes PB, at low doses, was found to significantly reduce the transport of glutamate into the cells. This effect has not previously been reported and the contribution to its anticonvulsant efficacy remains to be determined. It may perhaps lead to a reduction in efficacy of the drug or may even contribute to the toxicity of PB. In support of this theory, PB was also found to significantly reduce the activity of the glutamate-metabolizing enzyme GS. This effect was observed in mouse brain following repeated administration of the drug and was consistent over a range of doses. Additionally, a single concentration of PB was found to inhibit the enzyme in primary cultures of rat cortical astrocytes, and was the only AED of the twelve examined to do so. This apparent reduction in glutamate uptake and inhibition of GS activity may account for the CNS-related adverse effects and increase in seizure frequency observed clinically with PB at high doses.

8.1.2 Phenytoin

PHT has been proposed to exert its anticonvulsant effects via several reported mechanisms, namely interaction with voltage-activated sodium channels, voltage- and frequency-dependent block of T-type calcium channels, inhibitory effects on non-T-

type calcium channels and potentiation of GABA-mediated synaptic inhibition. From experiments conducted in this thesis it would appear that like PB, PHT also exhibits inhibition of GS in mouse brain. However, whereas PB exerted effects following only chronic administration, PHT was found to decrease the activity of GS over a range of doses following both acute and chronic administration. PHT failed to exert the inhibition of GS demonstrated by PB in primary cultures of rat astrocytes. As with PB, it is unlikely that inhibition of this enzyme would contribute to the anticonvulsant effects of PHT, and may contribute to the phenomenon of paradoxical intoxication observed clinically with PHT at higher doses. PHT demonstrated no effects on GABA or glutamate uptake or on the activity of GABA-T in primary cultures of rat cortical astrocytes in this study.

8.1.3 Carbamazepine

A number of mechanisms are reported to be responsible for the anticonvulsant effects of CBZ. In a similar manner to PHT, CBZ exerts inhibition of voltage-dependent sodium channels and non-T-type calcium channels. In addition, it has also been reported to interact with brain adenosine A_1 and A_2 receptors, however, the clinical significance of this effect remains to be evaluated. CBZ is another of the established AEDs which exhibited a reduction in GS activity in this study. The reduction in enzyme activity was evident in mouse brain following both acute and chronic administration. Again, this effect is previously unreported, and may be related to the increase in seizure frequency seen clinically with CBZ at higher doses. No effects of CBZ on the transport of GABA and glutamate or the metabolism of GABA were noted in this study.

8.1.4 Sodium Valproate

SVP is a broad spectrum AED thought to exert its anticonvulsant effects by a combination of mechanisms. It has been shown to exert numerous effects on the GABAergic system, blockade of voltage-dependent sodium channels, and to decrease aspartate levels in rodent brain. The extent to which each of these proposed mechanisms contributes to the antiepileptic efficacy of SVP remains to be determined. Experiments employing primary cultures of rat cortical astrocytes in this thesis demonstrated that SVP, at all concentrations examined, inhibited the transport of GABA into these cells. These results confirm a previous group's findings. Additionally SVP inhibited the transport of GABA into primary cultures of both human foetal and adult cultured astrocytes adding to the increasing evidence that the GABAergic system is involved in the mechanism of action of SVP.

8.2 MECHANISMS OF ACTION OF THE NEW ANTIEPILEPTIC DRUGS

8.2.1 Vigabatrin

VGB was the first AED to be developed on the basis of a targeted mechanism of action, and is one of the few reported AEDs with a single specific mechanism of action. VGB exerts its antiepileptic effects by irreversible inhibition of the GABA degrading enzyme GABA-T. However, in a recent study in our laboratory VGB was found to inhibit the uptake of GABA into cultured astrocytes, suggesting that it also possesses multiple mechanisms of action. The experiments carried out in this thesis demonstrate a VGB-induced inhibition of GABA-T therefore confirming the documented mechanism of action of the drug. In addition, VGB demonstrated inhibition of the uptake of GABA into primary cultures of rat cortical astrocytes, and

also into primary cultures of human astrocytes from foetal and adult brain, further supporting the notion that like other AEDs VGB acts via multiple mechanisms of action.

8.2.2 Lamotrigine

In animal models LTG exhibits an experimental profile similar to that of PHT, suggesting a similar mechanism of action. In rat cerebral slices LTG potently inhibits veratrine-evoked glutamate and aspartate release. It has been shown to limit the high frequency firing of sodium-dependent action potentials in cultured mouse spinal cord neurones, suggesting a use- and frequency-dependent block of voltage sensitive sodium channels similar to PHT and CBZ. However, LTG possesses a broader anticonvulsant profile than the established AEDs with effects on sodium channels. More recently a number of studies have indicated that LTG also exhibits blockade of calcium channels. In this thesis, LTG, unlike PHT and CBZ, failed to reduce the activity of GS in mouse brain. This study highlights that although LTG has a similar profile to the established AEDs PHT and CBZ, differences in the drugs do exist. LTG was without effect on the transport and metabolism of GABA and glutamate in primary cultures of rat cortical astrocytes in this study.

8.2.3 Felbamate

As yet, the precise mechanism of action of FBM remains to be determined. It has been shown to enhance $GABA_A$ receptor-mediated chloride currents in rat hippocampal neurones, and to interact with the strychnine-insensitive glycine modulatory site of the glutamate NMDA receptor. In addition to effects on amino acid systems FBM is thought to block voltage-dependent sodium channels and to inhibit dihydropyridine-

sensitive calcium channels. It therefore remains likely that FBM possesses multiple mechanisms of action. Experiments in this study found that FBM at all concentrations examined significantly reduced the uptake of GABA into primary cultures of rat cortical astrocytes. This effect has not been reported previously, and may reflect an additional mechanism which contributes to the antiepileptic efficacy of the drug. FBM was without effect on the uptake of glutamate, the activity of GABA-T and the activity of GS in primary cultures of rat cortical astrocytes. Similarly, both single and repeated administration of FBM was without effect on the concentrations of GABA, glutamate and glutamine, and on the activities of GABA-T and GAD in mouse brain. Repeated administration of FBM was, however, found to significantly reduce the activity of GS in mouse brain in this thesis. A reduction in GS activity may be expected to lead to a decrease in brain glutamine concentrations. The reduction in GS activity in this study was not, however, accompanied by a decrease in brain glutamine or an increase in brain glutamate levels. The reason for the apparent lack of effect of FBM on mouse brain neurotransmitter concentrations is unknown, but may reflect a compensatory mechanism such as an increase in the clearance of glutamate from the synapse, an increase in the synthesis of glutamine from glucose, or an increase in the uptake of glutamine direct from the blood.

8.2.4 Gabapentin

GBP is a hydrophilic analogue of GABA which can freely cross the blood-brain barrier. It has, however, demonstrated only limited effects on the GABAergic system, and has been reported to exert no effects at GABA receptors or uptake carriers in the brain. GBP has been shown to increase GABA turnover in rodent brain, and there is evidence to suggest that GABA levels were elevated in the cortex of patients. The drug has been

reported to increase the activity of GAD, and in addition at high concentrations has been shown to inhibit GABA-T (an effect which is not thought to be clinically relevant). A recent study in our laboratory showed that repeated administration of GBP reduced whole brain glutamate levels in mice. In addition to effects on amino acids the drug has been reported to reduce sustained repetitive firing of neurones. GBP was recently reported to exert inhibitory effects on L-type voltage-gated calcium channels, and has been demonstrated to bind with high affinity to a calcium channel sub-unit in the brain. Despite the increasing evidence in the literature indicating multiple mechanisms of action of GBP, the results of this study failed to identify any novel or additional effects of the drug. GBP was without effect on the uptake of GABA and glutamate and on the activities of GABA-T and GS in primary cultures of rat cortical astrocytes. Similarly, the drug was without effect on the activity of GS in mouse brain.

8.2.5 Topiramate

TPM is thought to act by blocking the spread of seizure discharges. It appears likely that it too possesses multiple mechanisms of action. It has been reported to exert blockade of voltage-activated sodium channels and inhibitory effects on kainate-induced responses. In addition TPM reportedly potentiates responses to GABA at the $GABA_A$ receptor and may reduce abnormally high levels of glutamate and aspartate in the hippocampus of spontaneously epileptic rats. In this study, primary cultures of rat cortical astrocytes were employed to investigate the effects of TPM on the transport and metabolism of GABA and glutamate. TPM failed to exert any significant effect on the uptake or metabolism of GABA or glutamate in the cultured cells. Similarly, the chapter examining the GABA- and glutamate-related neurochemical effects of TPM

revealed no effect on mouse brain concentrations of GABA, glutamate and glutamine, and no significant effect on the activities of GABA-T and GAD. Repeated administration of TPM was, however, responsible for a significant decrease in the activity of GS in mouse brain. This effect has not previously been reported, and as discussed in chapter four, may indicate a toxic effect of the drug. However, as observed with FBM (which also reduced brain GS levels) the decrease in GS was not accompanied by a change in mouse brain concentrations of glutamate or glutamine. Again, it can only be speculated that compensatory changes are taking place, thereby preventing any alteration in neurotransmitter concentrations. Taken together, the results of this study and reports in the literature indicate that TPM does give rise to its effects by a multifactorial mechanism.

8.2.6 Tiagabine

TGB is said to be the most mechanistically precise AED in clinical use. It is a potent and selective inhibitor of the uptake of GABA into neurones and glia. It is thought to specifically inhibit the GAT-1 GABA transporter. It has no significant affinity for other uptake sites and does not affect sodium or calcium channels. To date no other mechanism has been proposed to explain its antiepileptic efficacy. TGB significantly inhibited the uptake of GABA into primary cultures of rat cortical astrocytes in this study, confirming the documented mechanism of action of the drug. However, at the highest concentration examined, TGB additionally reduced the activity of GABA-T in primary cultures of rat cortical astrocytes. The drug was also shown to reduce the transport of glutamate into the cells, however, as the effect appeared to be independent of dose and was evident following only two of the eight concentrations examined the clinical relevance is questionable. TGB had no significant effect on GS activity in

cultured rat astrocytes or in mouse brain in this study. Therefore, although the reduction in GABA-T activity occurred only at the highest concentration examined, this effect cannot be ignored and questions the thinking that TGB acts by a single mechanism of action. This requires further investigation. In primary cultures of human adult astrocytes, TGB significantly inhibited GABA uptake into the cells. This is in agreement with the data for the rat cultures. However, an interesting observation in this thesis was the failure of the drug to affect GABA transport in cultured human astrocytes from foetal brain. This observation suggests that the GAT-1 transporter in human foetal brain is not developed sufficiently for the drug to be able to act. Additionally, the ability of SVP and VGB to inhibit GABA uptake in both adult and foetal human astrocyte cultures suggests a difference in the mode of action of GABA uptake inhibition exerted by the AEDs.

8.3 MECHANISMS OF ACTION OF THE EXPERIMENTAL STAGE ANTIEPILEPTIC DRUGS

8.3.1 Levetiracetam

As with many of the other novel AEDs, the precise mechanism of action of LEV remains to be determined. The drug fails to interact with the classical receptor and ion channel sites in the brain. LEV has, however, been proposed to bind in a stereospecific manner to a specific binding site in CNS membranes, where it is weakly displaced by PB and PTZ, suggesting that it may be GABA-related. Treatment with LEV has been reported to induce alterations in GABA metabolism and turnover in discrete areas of rat brain. The work carried out in this thesis fails to clarify the mechanism of action of LEV. In primary cultures of rat cortical astrocytes LEV failed to exert a significant

effect on the uptake of GABA or glutamate, or on the activities of GABA-T or GS. Experiments carried out in mouse brain similarly failed to demonstrate a significant effect of LEV on the concentrations of GABA, glutamate or glutamine or on the activities of GS, GABA-T or GAD. These results would suggest that the mechanism of action of LEV is likely to exclude effects on GABA and glutamate neurotransmission, however, this remains to be determined.

8.3.2 Desglycinyl-remacemide

DGR is the desglycinyl metabolite of the novel AED RMD. This metabolite has a longer half-life and a more potent pharmacological profile than RMD. As with LEV, the precise mechanism of action of this novel compound remains to be determined. DGR has been shown to prevent sustained repetitive firing of cultured spinal cord and hippocampal CA1 neurones, and has also been shown to decrease the release of glutamate and aspartate from cortical slices. It is reported that DGR may also exert effects at the NMDA receptor, where it has been reported to displace [^{3}H]-dizocilpine binding. A recent study in our laboratory showed that repeated administration of DGR at high dose significantly increased GABA-T and decreased GAD activity. Both effects have been postulated to contribute to the proconvulsant action of the drug seen clinically at higher doses. Several of the other AEDs examined reduced GS activity and this effect may account for their proconvulsant properties seen clinically at high doses. In this thesis, DGR had no significant effect on the activity of GS in either primary cultures of rat cortical astrocytes or in mouse brain, therefore, contributing no further experimental evidence to explain the proconvulsant effects of DGR seen clinically at high doses. In studies employing primary cultures of rat cortical astrocytes DGR had no significant effect on the transport of GABA into the cells. However, a reduction in

glutamate uptake was evident following DGR treatment, suggesting that this effect may be involved in the clinical and/or toxic actions of the drug. DGR was found to significantly reduce the activity of GABA-T in the cultured astrocytes. This effect has not previously been reported and may indicate an additional mechanism of action contributing to the anticonvulsant effects of the drug.

8.4 CONCLUSIONS

From the literature, it is becoming increasingly evident that most AEDs have multiple mechanisms of action. The data presented in this thesis gained from experiments employing primary cultures of rat cortical astrocytes, human adult and foetal astrocytes, and mouse brain support the multiple mode of action theory, and have highlighted several previously unreported additional mechanisms for some of the AEDs. For the AEDs with reported single and specific mechanisms of actions this study has revealed that they too may possess multifactorial modes of action.

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