EFFECTS OF SODIUM VALPROATE ON RELEASE OF NEUROTRANSMITTERS AND THEIR METABOLITES <u>IN</u> <u>VIVO</u>: RELATIONSHIP TO MOTOR SEIZURES IN THE RAT.

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Abstract

The effects of the anticonvulsant sodium valproate (VPA) on central neurotransmitter release and metabolism, during acute and sub-chronic administration, were investigated using in vivo microdialysis in conscious rats. In addition, an attempt was made to relate these neurochemical effects to the modulation of seizure activity in two rodent models of convulsive epilepsy. Neurotransmitters and metabolites were quantified using high performance liquid chromatography.

1. VPA dose-dependently increased extracellular (EC) levels of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxytryptamine (5-HT) and γ-aminobutyric acid (GABA) in the ventral hippocampus (VH). VPA differentially affected levels of DA and 5-HT measured in the anterior and posterior striatum.

2. Prolonged dosing of rats with VPA (2 x 200 mg/kg/day for 2, 4, 7 and 14 days) resulted in alterations of basal and high potassium-evoked release of hippocampal aspartate (ASP), glutamate (GLU), taurine (TAU), glutamine (GLN), GABA, DA, 5-HT and their major metabolites.

3. Studies utilizing tetrodotoxin (TTX) in conjunction with in vivo microdialysis assessed the effects of blocking voltage-dependent neurotransmitter release in the VH on the acute pharmacodynamic actions of VPA previously observed (1.). Under these conditions, the effects of VPA (400 mg/kg) on EC accumulation of DA, 5-HT, and GABA were differentially attenuated, whilst EC levels of ASP were increased.

4. Seizures induced in rats using the convulsant isonicotinic acid hydrazide (INH) were observed concurrently with measurement of VH amino acid levels in vivo. INH administration alone resulted in reductions of EC GABA levels, deficits which correlated closely with seizure progression. ASP and GLU levels increased non-significantly during these episodes. Co-administration of INH with VPA (400 mg/kg) resulted in complete reversal of both behavioural and neurochemical deficits.

5. The possible involvement of DA and 5-HT in the anticonvulsant action of VPA against seizures induced in rats with pilocarpine was investigated. Blockade of central 5-HT_{1A} receptors and stimulation of D_1 receptors was found to severely impair the anticonvulsant effects of VPA in this model.

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2-DG	2-deoxyglucose
2-en-VPA	2-en-valproate
3-MPA	3-mercaptopropionic acid
5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine
5-HTP	5-hydroxytryptophan
6-OH-DA	6-hydroxydopamine
8-OH-DPAT	8-hydroxy-di-(n-propylamino) tetralin
ACP	anterior caudate putamen
ACSF	artificial cerebrospinal fluid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPH	amphetamine
AMPT	α-methyl-p-tyrosine
ANOVA	analysis of variance
AP-5	D(-)-2-amino-5-phosphopentanoic acid
ASP	aspartate
BBB	blood brain barrier
cGMP	cyclic guanosine 3',5' monophosphate
cont	control ("pilocarpine only" group)
CSF	cerebrospinal fluid
DA	dopamine
DMCM	methyl-6,7-dimethoxy-4-ethyl-β-carboline-carboxylate
DMSO	dimethyl sulphoxide
DOI	(±)-2,5-dimethoxy-4-iodoamphetamine
DOPAC	3,5-dihydroxyphenylacetic acid
DRN	dorsal raphé nucleus

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EAA	excitatory amino acid
EC	extracellular
ECD	electrochemical detector
EDTA	ethylenediaminetetra-acetic acid
EEG	electroencephalograph
FPIA	fluorescence polarization immunoassay
GABA	γ-aminobutyric acid
GABA-T	GABA-transaminase
GAD	glutamate decarboxylase
GHB	γ-hydroxybutyrate
GLN	glutamine
GLU	glutamate
GLY	glycine
HPLC-ED	high performance liquid chromatography with electrochemical detection
HPLC-FD	high performance liquid chromatography with fluorometric detection
HVA	homovanillic acid
Hz	hertz
INH	isonicotinic acid hydrazide
IPSP	inhibitory post synaptic potential
ket	ketanserin
LTP	long-term potentiation
MAO	monoamine oxidase
MES	maximal electroshock
met	metergoline
NA	noradrenaline
NAN-190	1-(2-methoxyphenyl)-4-[4-(2-

	phthalimido)butyl] piperazine
	hydrobromide
NMDA	N-methyl-D-aspartate
NMDLA	N-methyl-D,L-aspartate
OD	outer diameter
ODS	octadecylsilicate
OPA	o-pthaldialdehyde
PC	personal computer
*p*CPA	*p*-chlorophenylalanine
PLP	pyridoxal-5-phosphate
p,p'-DDT	1,1,1-trichloro-2,2-bis
	(*p*-chlorophenyl) ethane
PTZ	pentylenetetrazol
rac	raclopride
sch	SCH 23390
skf	SKF 38393
SSADH	succinic semi-aldehyde
	dehydrogenase
SSAR	succinic semialdehyde reductase
SWD	spike wave discharge
SWG	standard wire gauge
TAU	taurine
TFMPP	1-(3-trifluoromethylphenyl)
	piperazine
TSC	thiosemicarbazide
TTX	tetrodotoxin
VH	ventral hippocampus

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VOC	voltage-operated channel
VPA	sodium valproate

Publications

The following publications have resulted from original work presented in this thesis.

Published papers.

Biggs, C.S., Pearce, B.R. Fowler, L.J. and Whitton, P.S. (1992) Regional effects of sodium valproate on extracellular concentrations of 5-hydroxytryptamine, dopamine and their metabolites in rat brain: an in vivo microdialysis study. *J. Neurochem.*, **59**, 1702-1708.

Biggs, C.S., Pearce, B.R., Fowler, L.J. and Whitton, P.S. (1992) The effect of sodium valproate on extracellular GABA and other amino acids in the rat ventral hippocampus: an in vivo microdialysis study. *Brain Res.*, **594**, 138-142.

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....For Naomi and My Family....

Chapter One

General Introduction

1.Sodium valproate; a brief therapeutic overview.

Sodium valproate (sodium di-n-propylacetate; VPA) is amongst the most widely used antiepileptic drugs in current clinical use. It has achieved this as a consequence of two major pharmacological and clinical attributes: Firstly, it has one of the widest ranges of activity amongst any of the available medications in treating the aetiologicaly diverse group of neurological disorders known collectively as the epilepsies. Secondly and possibly more importantly, VPA is remarkably free of severe side effects when compared to many other similarly prescribed antiepileptics (Pinder et al, 1977). Coupled with simplicity of chemical synthesis and consequently, low production costs these factors have culminated in the drugs current status as the "all-round" antiepileptic medication.

The modern use of VPA in the clinic dates back to 1962 when valproic acid was employed as a lipophilic vehicle for dissolving candidate anticonvulsants for experimental use (Meunier et al, 1963). When it was discovered that the vehicle itself was effective in reducing the severity of seizures induced in rodents with the chemiconvulsant pentylenetetrazol (PTZ), development of the original lead compounds as antiepileptics ceased in favour of the vehicle Subsequently the sodium salt of valproic acid was the subject of extensive clinical trials and originally licensed as an antiepileptic in France in 1967. The drug was first released for clinical use in the UK by Sanofi Pharma as *Epilim* in 1974 and has remained a valuable asset for treament of the epilepsies ever since.

1.1 Use of sodium valproate as an antiepileptic.

VPA is used widely in the treatment of human epilepsies and related disorders, although as will be discussed later, the drug has a number of other pharmacological properties and clinical uses.

Myoclonic Seizures.

This group of disorders is of the primary generalised type and are characterised by involuntary and repetitive clonic contraction of somatic muscle groups ('jerking'). VPA (as monotherapy and as part of a combined regime) has proved to be highly effective in ameliorating myoclonus. Clinical studies have confirmed that the drug is particularly effective against childhood myoclonic epilepsy (Delgado-Escueta and Enrile-Bacsal, 1984) with an average efficacy of 75% in reducing seizure incidence in all myoclonic epilepsy catagories (Pinder et al, 1977).

Petit mal or Absence Seizures.

Seizures of the absence type are typically characterised by brief periods of unconciousness, motor arrest, myoclonic 'jerks' with blinking and often 3 Hz spike wave discharges (SWD) during diagnostic EEG. VPA alone or more often in combination with ethosuximide (Rowan et al, 1983) has proved invaluable in controlling absences among patients of all age groups. Covanis et al (1982) reported on a remarkable study in which 90% of their patients were seizure-free within two weeks of commencing treatment.

Photosensitive Epilepsy.

Photosensitive or 'reflex' epileptic patients have recurrent motor seizures that can be triggered by flickering or stroboscopic light, flashing at a particular

frequency. As with other forms of epilepsy, these episodes are associated with abnormal EEG activity. VPA as monotherapy has been repeatedly demonstrated to be highly efficacious in the treatment of this condition. For example, in a study of 24 drug-naive patients and 18 patients refractory to previous therapy, VPA alone abolished all photic seizing in 75% of the former and 78% of the later group (Covanis et al, 1982).

Primary Generalised Tonic-Clonic ('Grand Mal') Epilepsy.

Patients experiencing seizures of this type generally display a characteristic symptomatology with tonic somatic muscular hyperflexion (accompanied by generalised low voltage fast discharges detected on EEG), evolving into periods of clonic seizing with consecutive high amplitude spike discharges and EEG suppressionThere is usually a loss of consciousnessfor the duration of the seizure. Again, as is typical with regard to generalised convulsive and non-convulsive (absence) epilepsy, VPA appears highly effective in reducing the incidence of these most debilitating episodes. Although less commonly used as monotherapy, overall figures from a collection of studies suggest approximately 70 % success for complete control of seizures (Covanis et al, 1982; Callaghan et al, 1985; Turnbull et al, 1985).

Partial Seizures.

Partial epilepsy is an 'umbrella' term used to categorise seizures with accompanying EEG disturbances originating in a particular brain locus. Symptoms depend critically upon the location of the abnormal neuronal activity within the brain and its level of containment or spread to other structures (*secondary generalisation*). This broad category may be further

subdivided into *simple partial seizures,* during which motor seizing, auditory, visual and olfactory hallucinations are commonly experienced and *complex partial seizures,* which are often ~~secondary~~ generalised to tonic-clonic seizures with psychological derangements. In contrast with the obvious merits of VPA when used in the management of generalised epilepsy, partial seizures respond less consistently to the antiepileptic and it is almost always given as part of a polytherapeutic regimen (Richens and Ahmad, 1975; Simon and Penry, 1975). Athough these authors reported reductions in seizure frequency of 33-75% in 40 % of their combined test groups, approximately one third of these patients failed to respond significantly to VPA. In contrast, Covanis et al (1982) found 64% of patients presenting with secondary generalised tonic-clonic seizures to be completely controlled during a four-year follow up period. These and other findings suggest that VPA is substantially more effective in ameliorating seizures where EEG disturbances (and possibly, the precipitating pathological factors) encompass the forebrain as a whole, rather than those which appear to originate from a defined CNS locus.

1.2 Other clinical uses for sodium valproate.

Consistent with its wide range of activity against various forms of epilepsy, VPA has been used, although less routinely in the treatment of other, mainly neurological conditions. Favourable results following VPA treatment of anxiety disorders (Roy-Byrne et al, 1989) and the alcohol withdrawal syndrome (Littleton and Griffiths, 1975; Lambe, 1980) have been described, with mixed effects on neuroleptic-induced dyskinesias (Linnoila, M., 1976; Gibson, 1979). Conversely, a frank deterioration in type 1 schizophrenic symptoms (i.e.,

paranoid delusional psychosis) has been observed following even limited treament with VPA (Lautin et al, 1980) and use of the anticonvulsant is limited in schizophrenic patients presenting with seizures.

2. Activity of sodium valproate in animal seizure models.

Following the initial findings of Meunier et al (1963), suggesting that VPA was active against rodent PTZ-induced seizures, much work has since revealed VPA to possess anticonvulsant activity in a wide range of animal seizure models. These include models of both focal and generalised seizures provoked by administration of chemoconvulsant agents, by direct electrical induction of abnormal neuronal discharges and in genetically seizure-prone animals, in addition to models of non-convulsive absence epilepsy (for review and explanation of animal seizure paradigms see Fisher, 1989). Examples of this wide ranging efficacy following acute administration of VPA are provided in table 1. As a measure of the likely aetiological similarities between certain animal models of epilepsy and human seizure disorders, one can see that VPA is particularly effective against rat absence seizures (Löscher et al, 1984) and this accords with the remarkable effectiveness of the drug in treating human absences (Covanis et al, 1982). Similarly, VPA has been found to inhibit generalised tonic-clonic convulsions induced by the benzodiazepine receptor inverse agonist DMCM at relatively low doses (Petersen, 1983). As previously outlined, VPA seems to be more effective in the treatment of generalised, rather than partial epilepsies. This has been borne out to some extent by studies utilizing animal models of partial (focal) seizures, since EEG evidence suggests that VPA does not inhibit abnormal focal discharges (induced by

Model			Valproate dosing		
Convulsant (mg/kg) or stimulus amplitude	Seizure type	Species	Route of administration	ED_{50}[a] (mg/kg)	Sample references
PTZ[b] 70	Clonic	Rat	i.p.	74-260	Swinyard (1964); Kupferberg (1980)
Picrotoxin 3.2	Clonic	Mouse	i.p.	390	Kupferberg (1980)
3-MPA[c] 66	Clonic	Mouse	i.p.	290	Löscher (1980)
INH[d] 200	Clonic	Mouse	p.o.	280	Löscher and Frey (1977)
NMDLA[e] 340	Clonic	Mouse	i.p.	340	Czuczwar et al (1985)
DMCM[f] 15	Clonic/Tonic	Mouse	i.p.	60	Petersen (1983)
Alumina gel	Clonic	Cat	i.p.	200	Fariello and Mutani (1970)
MES[g] 150 mA	Tonic	Rat	i.p.	140-170	Kupferberg (1980)
Amygdaloid kindling 500 μA	Complex partial	Rat	i.p.	220-300	Löscher et al (1986)
Epileptic rats	Absence 3Hz SWD[h]	Rat	i.p.	81	Löscher et al (1984)
Photosensitive Baboons	Photically-induced myoclonus	Baboon	i.v.	200	Löscher and Meldrum (1984)

a Dose which protects 50% of animals from seizures.   b Pentylenetetrazol. c 3-Mercaptopropionic acid d Isonicotinic acid hydrazide e N-methyl-D,L-aspartate
f Methyl-6,7-dimethoxy-4-ethyl-β-carboline-carboxylate g Maximal electroshock h Spike wave discharge.

Table 1. Anticonvulsant efficacy of sodium valproate in a variety of animal seizure models.

alumina gel or cobalt implants), but instead limits the spread of seizure activity away from the focus (Fariello and Mutani, 1970). This may be a contributory factor to the ability of VPA to prevent secondary generalisation of partial seizures observed clinically (Covanis et al, 1982).

Although sharing a relatively wide range of activities across the spectrum of human epilepsy and animal seizure models, a consistent and striking dissimilarity is found with the high doses of drug often required to achieve anticonvulsant effects during animal studies (Chapman et al, 1982). These differences are possibly due, in part, to wide cross-species variation in pharmacokinetic parameters associated with disposition and plasma protein binding of VPA (Löscher, 1978), rather than fundamental differences in pharmacodynamic actions of the drug. Of the relatively few studies that have investigated the effects of more prolonged VPA administration on animal seizure parameters, most have noted a persistence of anticonvulsant action following termination of treatment (Leviel and Naquet, 1977; Pellegrini et al, 1978; Walter et al, 1980). It is possible that these longer term actions of the anticonvulsant might be mediated by an active metabolite such as trans-2-en-VPA, since this species is known to persist in rodent brain tissue far longer than the parent drug (Löscher and Nau, 1983). Accumulation of an active metabolite in the brain may also explain why monitoring plasma VPA levels in patients is not a useful predictive index of therapeutic efficacy .

3. Involvement of neurotransmitter systems in seizure mechanisms.

This section reviews the possible involvement of amino acid utilizing neurotransmitter systems in the genesis and propagation of seizure activity in

the genetically epileptic brain and following seizure induction with chemoconvulsants. In addition, the probable importance of monoaminergic neurotransmission in modulating seizure activity is also discussed.

3.1 Amino acids and seizures

Considerable research effort has been made with the aim of identifying a primary neurochemical deficit responsible for abnormal neuronal firing in the CNS and, hence the expression of convulsive and non-convulsive seizures. Given the diversity of epileptic and related disorders, it is probably unwise to implicate a single common factor in their overall aetiology. However, a consistent finding in many neurochemical studies has been that altering inhibitory neurotransmission in the CNS, particularly that associated with γ-aminobutyric acid (GABA) can have a profound effect upon susceptibility to seizures. This was initially demonstrated by Hayashi (1954) who found that topical application of GABA onto canine motor cortex prevented the electroencephalographic spread of focal ictal activity. In addition, drugs which are known to lead to inhibition of the GABA-synthetic enzyme glutamic acid decarboxylase (EC 4.1.1.15; GAD) including hydrazides (Killam and Bain, 1957) and allylglycine (Horton et al, 1978) are also potent convulsants in rodents. Inhibition of the post-synaptic actions of GABA with $GABA_A$ receptor antagonists (Meldrum and Braestrup, 1984) and β-carbolines such as DMCM (Petersen, 1983) invariably has a similar proconvulsant action in experimental animals. An abnormality in central GABAergic neurotransmission as a central causative factor in human epilepsies has been implied from knowledge of dysfunction of central GABAergic systems in genetically epilepsy prone

rodents. For example, in seizure-prone gerbils there is evidence for increased GABA-containing neuronal numbers (Lomax et al, 1986), decreased tissue GABA levels compared with non-epileptic strains (Löscher, 1985) and a decrease in post-synaptic receptor density (Petersen et al, 1984). Despite this compelling evidence, attempts to uncover similar faults of GABAergic neurotransmission in human epilieptic subjects have been less successful. This may in part be limited by the variable levels of GABA measured in ex vivo tissue samples excised during neurosurgical interventions, compared with those measured in control samples (Van Gelder et al, 1972; Perry et al, 1975; Perry and Hansen, 1981). Thus, although there is considerable evidence suggesting that many antiepileptic drugs, including VPA alter central GABAergic neurotransmission (reviewed by Rogawski and Porter, 1990), no consistent defect involving this system has yet been identified in human epileptics.

In addition to the considerable body of neurochemical evidence suggesting an involvement of central inhibitory neurotransmission in many seizure models and genetically acquired animal epilepsies, manipulation of central excitatory amino acid (EAA) neurotransmitter systems greatly affects seizure parameters in many in vivo and in vitro models of epilepsy. In vitro preparations such as cortical and hippocampal slices, stimulated with elevated potassium (K^+) ion concentrations, exposed to hypoxia (Chamberlin et al, 1990) or in the presence of a reduced extracellular magnesium (Mg^{2+}) ion concentration, generate electrographic seizure activity. Seizure discharges can be blocked by EAA receptor antagonists, particularly those active at the N-methyl-D-aspartate

(NMDA) receptor complex (Coan and Collingridge, 1985; Traynelis and Dingledine, 1988; Kleckner and Dingledine, 1989). There is also extensive evidence to suggest that both non-competitive and competitive NMDA receptor antagonists are active against a range of experimental seizures in vivo. For example, dizocilpine (MK-801) reduces the frequency of absence seizures (Peeters et al, 1989) and severity of rodent electroshock seizures (Clineschmidt et al, 1982), whilst D (-)-2-amino-5-phosphonopentanoic acid (AP-5) is effective in reducing cortical focal seizures induced by cobalt (Coutinho-Netto et al, 1981) and protecting DBA/2 mice against audiogenic seizures (Croucher et al, 1982). Effectiveness of these drugs against such a variety of experimental seizures is indicative of the pivotalrole that this neurotransmitter system plays in regulating CNS excitability. Significantly, human studies have been moderately successful in identifying a possible defect involving EAA neurotransmission in partial epilepsy. Excised epileptic foci from patients with drug-refractory complex partial epilepsy have been demonstrated to contain greater numbers of NMDA receptors and NMDA receptor-associated glycine modulatory sites compared with healthy tissue, with an indication that α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor density may also be elevated (McDonald et al, 1991). Of particular interest are the recent studies of Carlson et al (1991) using in vivo microdialysis to sample the extracellular environment of an epileptic focus in the cortex of a patient undergoing elective neurosurgery. These researchers found that basal extracellular levels of glutamate (GLU), aspartate (ASP) and glycine (GLY) were greatly elevated within the focus immediately before and during partial

seizure activity detected by concurrent EEG measurement. Although this represents data from one patient only, it suggests that an exaggerated neuronal release of amino acid neurotransmitters may underlie seizure induction in this form of epilepsy. Further, lamotrigine, a drug particularly effective in the control of partial seizures (Richens and Yuen, 1989) is thought to exert a proportion of its anticonvulsant effects through an inhibition of neuronal GLU release (Leach et al, 1986).

3.2 Monoamines and seizures.

A large body of information detailing the possible involvement of monoamine neurotransmitter systems in mechanisms of seizure induction and propagation has been accumulated, both as a result of animal studies and clinical findings. The possible importance of these systems was first inferred from studies in which central stores of monoamines had been chemically depleted. These treatments resulted in a lowering of PTZ seizure thresholds in rodents (Chen et al, 1954) and abolition of the effects of amphetamine (AMPH) in raising electroconvulsive seizure thresholds in rabbits (DeSchaepdryver et al, 1962). Later experiments sought to define more clearly the role of individual monoaminergic systems in control of seizures, following chemical manipulation of these systems. Central noradrenergic transmission was found to be an important modulatory influence on the progression of electroshock seizures (Jobe et al, 1974), those induced by PTZ (Doteuchi and Costa, 1973) and audiogenic seizures (Horton et al, 1980). In the latter study, it was found that the α_2-adrenoceptor agonist clonidine protected against seizures, whilst α_1-adrenoceptor antagonists (prazosin and phentolamine) exacerbated seizure

activity. More recently, abnormalities in the forebrain noradrenergic system of genetically epileptic rats have been found, particularly a decrease in frontal α_1 sites compared to non-epileptic Sprague-Dawley rats (Nicoletti et al, 1986). Since earlier studies of the involvement of catecholamines in experimental seizures utilized the neurotoxin 6-hydroxydopamine (6-OH-DA) to non-selectively lesion central catecholaminergic pathways (Doteuchi and Costa, 1973; Jobe et al, 1974), it was not possible on this basis to determine if dopaminergic systems were involved in these seizure mechanisms. As an alternative to chemical depletion as a means of compromising dopamine (DA) release, a selection of DA agonists and antagonists were assessed in animal models for pro- or anticonvulsant activity. Using this approach, it was found that the non-selective DA receptor agonist apomorphine was anticonvulsant against photic seizures induced in the Senagelese Baboon *Papio papio* (Meldrum et al, 1975), DBA/2 mouse audiogenic seizures (Anlezark and Meldrum, 1975) and reduced firing of cobalt-induced epileptic foci (Dow et al, 1974). In the PTZ model of generalised epilepsy, apomorphine has an inconsistent influence on seizure thresholds, with reports of a null effect on PTZ-induced convulsions (Kleinrok et al, 1978) and an anticonvulsant effect (Löscher and Czuczwar, 1986). More recently, with the development of specific DA receptor agonists and antagonists, the D_1 agonist SKF 38393 was found to be highly proconvulsant in a model of limbic motor seizures provoked by the cholinomimetic pilocarpine (Al-Tajir et al, 1990a). In this study, SKF 38393 treatment resulted in seizures in all animals administered a dose of pilocarpine not normally noted to induce convulsions. In contrast, pretreatment of rats

with the D_2 receptor agonist quinpirole protected against seizures normally

induced following a fully convulsant dose of pilocarpine. From these and other

studies it is clear that the dopaminergic system can exert opposing influences

on seizures induced by cholinomimetics, although stimulation of D_1 receptors

is convulsant in its own right following depletion of central catecholamine

stores (Starr et al, 1987). Neurochemical changes to dopaminergic systems in

vivo following induction of seizures are documented: Evidence exists for

reduced tissue DA levels in kindled rats (Engels and Sharpless, 1977) and

increased D_2 receptor density in the nucleus accumbens of rats following

hippocampal kindling (Csernansky et al, 1988), although *total* central DA

binding site density may be reduced following amygdaloid kindling (Ashton

et al, 1980). Clinical information has provided some indications for the likely

involvement of central dopaminergic systems in human epilepsies. Much of

this evidence is by necessity "circumstantial" in that it relies on findings

associated with dopaminergic drug administration in patients predisposed to

seizures, since biochemical studies of excised epileptic foci and CSF taken from

epileptic patients have proved largely inconclusive. AMPH has been noted to

control generalised convulsions and absence seizures (Livingston et al, 1973),

whilst apomorphine has been used with some success in the treatment of

photic seizures in patients with progressive myoclonic epilepsy (Mervaala et

al, 1990). Another monoamine neurotransmitter 5-hydroxytryptamine (5-HT)

has long been thought to play a role in seizure mechanisms, with indications

of this possible involvement from both human and animal studies. Kilian and

Frey (1973) found that depleting the brain of its 5-HT stores with the trytophan

hydroxylase inhibitor *p*-chlorophenylalanine (pCPA) lowered the threshold for electrically and chemically induced convulsions in rodents, although a similar action was not evident in the L-allylglycine induced model of generalised epilepsy (Ashton and Waquier, 1981). Conversely, enhancing central synthesis of the monoamine with the 5-HT precursor, 5-hydroxytryptophan (5-HTP) was found to protect against photically-induced seizures in *Papio papio* (Meldrum et al, 1972). Genetically epilepsy prone rats (GEPRs) have reduced central levels of 5-HT compared to other strains (Daily and Jobe, 1986) and administration of the 5-HT reuptake inhibitor fluoxetine significantly reduced audiogenic seizures in these animals, correlating temporaly with enhanced accumulation of 5-HT in the thalamic extracellular enviroment (Daily et al, 1991). Fluoxetine is also anticonvulsant against seizures in rats initiated by focal injection of bicuculline and central injection of the drug indicates that the substantia nigra may be an important structure for expression of the anticonvulsant effect (Pasini et al, 1992). Recently the involvement of specific 5-HT receptor subtypes in the control of seizure activity has become clearer: Kindled seizures in a feline model were potently suppressed by the selective 5-HT_{1A} receptor partial agonist 8-hydroxy-2-(di-n-propylamino) tetralin (8-OHDPAT; Wada et al, 1993), whilst the 5-HT_2 receptor agonist (±)-2,5-dimethoxy-4-iodoamphetamine (DOI) resulted in a potentiation of such seizures (Wada et al, 1992a). Using a range of serotonergic antagonists, Semenova and Ticku (1992) were able to identify a role for 5-HT_{1C} ,5-HT_3 and 5-HT_4 receptors in the DBA/2 mouse audiogenic seizure model. The most consistent finding from human studies has been the discovery of the likely involvement

of central serotonergic mechanisms in the aetiology of myoclonic epilepsy. Reduced CSF levels of the 5-HT metabolite, 5-hydroxyindoleacetic acid (5-HIAA) have been recorded for a variety of myoclonic disorders (Deahl and Trimble, 1991), suggesting that central serotonergic neuronal activity may be compromised in these patients. In post-anoxic myoclonus, treatment of patients with 5-HTP has been shown to correct CSF 5-HIAA levels and reduce myoclonic episodes (Chadwick et al, 1975).

4. Neuroanatomical structures of importance in epilepsy.

Having illustrated the importance of central neurotransmission in regulating seizure activity in the epileptic brain, it is necessary to give an overview of the involvement of specific brain regions in the genesis and propagation of seizures. Investigation of neurotransmitter function in these structures and how anticonvulsant drugs interact with these systems will provide valuable information regarding the likely mechanisms of action through which such drugs operate. The review is not comprehensive and is limited to a brief discussion of the literature regarding the likely involvement of the hippocampus and striatum in seizure mechanisms.

4.1 The hippocampus.

Considerable evidence exists which indicates that the hippocampus is a structure critical for the generation of abnormal electrographic activity underlying the clinical manifestations of many (focal) epileptic disorders (Bradford and Peterson, 1987). In juvenile rats, there is some evidence to suggest that an overabundance of recurrent excitatory synapses can develop in area CA3 of the hippocampus, leading to excessive and abnormal neuronal

discharges and electrographic spread of seizure activity (Swann et al, 1992), such miswiring resulting in increased seizure susceptibility in adulthood. This may result from the observation that relatively brief seizure episodes can result in long lasting alterations of hippocampal excitability, similar in character to the phenomenon of long-term potentiation (LTP; Ben-Ari and Rerpressa, 1990). It is also thought that the exceptionally dense packing of cell bodies in hippocampal cell layers (particularly within the CA1 field) reduces the ability of the structure to buffer changes in EC ionic composition and hence, modulate neuronal excitability (McBain et al, 1990) Thus, there may be an inherent structural propensity within the neural networking of this structure which renders it prone to seizure generation. The hippocampus appears to be particularly susceptible to neuronal damage induced by chemoconvulsants. Seizure activity, reflected by enhanced regional glucose metabolism, was greatest in this structure following seizure induction with L-allylglycine and kainic acid (Evans and Meldrum, 1984). Loss of specific hippocampal neurones is evident histologically following seizure induction with L-allylglycine in baboons (Meldrum et al, 1974). A similar pattern of neuronal loss (confined to the hippocampus) was found in the brains of cats receiving focal septal nuclei injections of ouabain(Baldy-Moulinier et al, 1973). In both cases, the pattern of hippocampal cell loss and reactive glial cell growth was identical to that seen in temporal lobe epilepsy (Meldrum et al, 1974). Clinically, hippocampal resection in patients with evidence of focal seizure initiation and hippocampal cellular reorganization, results in excellent control of temporal lobe seizures (de Lanerolle et al, 1992). In the E1 mouse (a genetic variant that exhibits

temporal lobe seizures confirmed by EEG) immunohistochemistry has identified a variety of hippocampal neurochemical abnormalities (King and LaMotte, 1989), involving GABAergic, monoaminergic and peptidergic neurotransmitter systems. Recently, Zis et al (1992) reported that hippocampal concentrations of extracellular 5-HT and HVA in rats rose sharply in response to seizures induced electrically or with flurothyl. Whether this represents a non-specific response to abnormal neuronal firing or is part of aninnatecontrol mechanism to contain the seizure within the hippocampus remains to be determined. Thus, the hippocampus may play a critical role in initiating seizure discharges, due to innate neuronal hyperexcitability and possibly neurochemical abnormalities associated with hippocampal neurotransmission.

4.2 The striatum.

Since the basal ganglia form a major portion of the extrapyramidal motor system it is perhaps not surprisingthat they are involved in the propagation of motor seizures. The caudate nucleus and putamen, collectively termed the striatum, has received particular attention from researchers investigating control of seizure spread throughout the epileptic brain. In contrast with the amygdala and hippocampus, electrical stimulation of the striatum actually inhibits the expression of seizure activity induced by cortical application of alumina gel (Oakley and Ojemann, 1982) and in human patients with partial epilepsies (Sramka et al, 1980). Furthermore, bilateral electrolytic destruction of the striatum enhances the establishment of seizures induced by PTZ (Kryzhanovski et al, 1985). Taken together, these findings suggest that the striatum has a net negative modulatory influence over electrographic spread

of seizures and that mechanical damage or neurochemical abnormalities within the structure impede this function. The functioning of the dopaminergic system within the striatum has received extensive attention in relation to the control of seizure propagation (reviewed by Starr, 1993) and seems to be of pivotal importance in this regard. It has been shown that focal stimulation of D_2 receptors in the anterior portion of the caudate in rats protects against pilocarpine-induced limbic seizures (Al-Tajir and Starr, 1990), whereas selective stimulation of D_1 receptors in the substantia nigra exerts a proconvulsant effect (Al-Tajir et al, 1990b). These mechanisms are discussed in greater detail in chapter 8. Little evidence currently exists to suggest that human epileptic patients have striatal neurochemical abnormalities, although it could be inferred from animal studies that cortical damage may impede the ability of the striatum to limit seizure spread (Warencyia et al, 1986; Al-Tajir and Starr, 1991). In the latter study it was demonstrated that lesioning corticostriatal glutamatergic afferents abolished the anticonvulsant action of quinpirole when focally applied to the anterior striatum.

5. Neurochemical actions of VPA.

Since a large part of the original findings presented in this thesis have been concerned with *in vivo* neurochemical measurements following VPA treatment, it is necessary to present an overview of the known effects of the drug on CNS neurochemistry with special emphasis placed on possible interactions with neurotransmitter amino acids and monoamines.

5.1 Interaction of VPA with central GABAergic neurotransmission.

Given the manifest anticonvulsant actions associated with inhibitory

neurotransmission (see section 3.1), it is perhaps inevitable that a majority of

neurochemical investigations of the central actions of VPA have focussed on

the GABAergic system. Initial reports suggested that acute administration of

VPA to rodents raised whole brain GABA levels (Godin et al, 1969),

concurrently protecting against audiogenic seizures (Simler et al, 1968; Schecter

et al, 1978). Thus, the hypothesis was promptly formed that VPA might exert

its anticonvulsant action via augmentation of brain GABA levels and therefore,

central inhibitory neurotransmission. Despite extensive evidence suggesting an

involvement of brain GABA in seizure mechanisms (see section 3.1), this

hypothesis has been repeatedly tested and challenged. Of particular contention

is the finding by some groups that doses of VPA insufficient to elevate whole

tissue GABA levels remain potently anticonvulsant in animal seizure models

(Anlezark et al, 1976; Morre et al, 1984). This discrepancy has been partially

resolved as a result of studies investigating the effects of VPA on subcellular

and brain regional GABA accumulation. Thus, doses as low as 125 mg/kg

have been found to increase GABA levels in synaptosomes prepared from

mouse whole brain (Löscher, 1981) and those prepared from several regions

of rat brain (Löscher and Vetter, 1985). These latter workers found that the

increase in synaptosomal GABA, which was predominantly that of the

neuronal compartment (Iadorola and Gale, 1979; 1981) was evident within 5

min of VPA injection and that these effects were temporally related to the

anticonvulsant and antinoci ceptive actions of the drug.

Although it is now generally accepted that VPA enhances brain GABA

accumulation, the precise mechanisms through which it exerts these actions

and the biochemical substrates on which it acts are still a matter of debate. Extensive research has highlighted the likely interaction of VPA with enzymes of the GABA synthetic and catabolic pathways. As is widely known, GABA is formed in nerve terminals via the decarboxylation of GLU by GAD. Following reuptake into neuronal terminals, postsynaptic elements and glial cells, GABA is degraded firstly to succinic semialdehyde (SSA), a step catalysed by GABA-transaminase (GABA-T; E.C. 2.6.1.19). SSA may then be reduced to γ-hydroxybutyrate (GHB) by aldehyde reductase (SSAR; E.C. 1.1.1.2) or reenter central oxidative metabolism via oxidation to succinate, a step catalysed by succinic semialdehyde dehydrogenase (SSADH; E.C. 1.2.1.16). It is thought that this latter route of metabolism represents the more important pathway <u>in vivo</u> (Chapman et al, 1982; Whittle and Turner, 1982).

Godin et al (1969) attributed the anticonvulsant properties of VPA <u>in vivo</u> to an inhibition of GABA-T activity, with a consequent elevation of brain GABA levels. Following this initial investigation however, it became clear on the basis of numerous studies that this enzyme was significantly inhibited by VPA only at very high drug concentrations, equivalent to doses outside the therapeutic range for treatment of both human and chemically-induced seizures. For example, K_i values of 40 mM have been recorded for inhibition of the enzyme, prepared from human brain homogenates (Maitre et al, 1978) and similar or even higher values recorded for inhibition of rabbit brain GABA-T (Fowler et al, 1975) and that assayed in mouse brain homogenates (Löscher, 1980). More recently however, synaptosomal GABA-T has been demonstrated to be significantly inhibited by doses of VPA within the therapeutic range for

seizure protection in animals (Löscher, 1981; 1993). The failure of previous studies to detect an inhibitory effect of the anticonvulsant may be attributable to differing sensitivities between glial cell GABA-T and the neuronal enzyme. Indeed, Larsson et al (1986) have demonstrated that the latter enzyme (assayed in cultured cortical neurones) is particularly sensitive to inhibition by VPA (K_i= 0.63 mM). However, since the glial cell enzyme is the predominant form found in whole tissue (Löscher, 1993),inhibition of the relatively sparsely distributed neuronal isozyme at anticonvulsant doses is unlikely to directly account for the central GABA-elevating actions of the drug. Other enzymes in the GABA-catabolic pathway (namely SSADH and SSAR) have consistently been shown to be sensitive to inhibition by VPA (Whittle and Turner, 1978; Van der Laan et al, 1979) and at doses active in animal seizure models (Chapman et al, 1982). The inhibitory effect of VPA against SSADH is more potent than for that against GABA-T <u>in vitro</u> (typical K_i values of 0.5-5 mM) and it has been suggested that inhibition of this enzyme <u>in vivo</u> may in turn stimulate a feed-forward inhibition of GABA-T, since SSA has a potent inhibitory action on the forward transamination step catalysed by this enzyme (Van der Laan et al, 1979). Doubts were later raised concerning this theory since Simler et al (1981) could not detect a significant |susceptibility of GABA-T activity to raised levels of SSA induced by inhibition of SSADH with VPA. The enzyme responsible for converting SSA to GHB, 'specific' SSAR is probably inhibited by VPA, although this has been disputed (Vayer et al, 1988). However, GHB formation is inhibited in rat brain homogenates (Whittle and Turner, 1978), indicating that the 'non-specific' SSAR (potently inhibited

by VPA, K_i =0.07-0.08 mM) is likely to contribute to the overall formation of

GHB. A reduction of GHB formation in vivo may in itself significantly

contribute to the anticonvulsant action of VPA, since GHB is known to possess

pro-convulsant actions (Marcus et al, 1967) and in particular, can induce

generalized absence seizures (Snead, 1988).

If the effects of VPA administration on the catabolism of GABA are less than

conclusive, then what of its interaction with the GABA synthetic pathway?

This question has been addressed using several different approaches. In early

studies, VPA was found to increase incorporation of ^{14}C into rodent brain

GABA pools (assayed following injection of [^{14}C] glucose) by 30-90% (Godin

et al, 1969; Taberner et al, 1980). Activation of GAD following VPA treatment

is thought to underlie this effect (Löscher, 1993). Such activation is rapid in

onset, with concurrent elevation of mouse whole brain GABA (Nau and

Löscher, 1982) and rat synaptosomal GABA (Löscher and Vetter, 1985),

together with seizure protection. Further, GAD is thought to be associated

with a releaseable neuronal GABA pool (Abe and Matsuda, 1983) and this

suggests that VPA enhances accumulation of GABA in this compartment

rather than metabolic pools of the amino acid (Iadorola and Gale, 1979; 1981).

GAD exists in an active holoenzyme form and an inactive apoenzyme form

which is present in large reserve (see Martin and Rimvall, 1993). It is possible

that VPA induces conversion of apoGAD to holoGAD, thereby increasing the

synthetic capacity for GABA and this possibility is discussed in greater detail

in chapter 7. Interestingly, at high doses of VPA (>400 mg/kg), GAD activity

is depressed (Löscher, 1985).

In addition to the effects of VPA on GABA synthesis and catabolism, the drug

is known to exert effects on GABA release mechanisms. VPA has been shown

to enhance release of GABA from cultured cortical neurones (Gram et al, 1988)

and rat cortical slices (Ekwuru and Cunningham, 1990) in the presence of

raised external K^+ concentrations as a depolarising stimulus. This represents

a genuine facilitatory action of VPA on GABA release since the drug does not

alter reuptake of the amino acid from the synaptic space (Ross and Craig,

1981). VPA also increases inhibitory post synaptic potential recording (IPSP)

from hippocampal neurones (Preisendorfer et al, 1987), although there is little

evidence to suggest that the anticonvulsant interacts with the $GABA_A$ receptor-

chloride ionophore complex (Löscher, 1980; Olsen, 1981; Squires et al, 1983).

An indirect indication that GABAergic neurotransmission may be enhanced in

human patients receiving VPA as antiepileptic medication comes from the

finding that CSF GABA levels are increased in these subjects (Löscher and

Siemes, 1985).

5.2 Interaction of VPA with excitatory amino acid neurotransmission.

The possible interaction of VPA with central EAA neurotransmitter systems

and its relevance to the anticonvulsant mode of action of the drug has received

comparatively little attention. In vitro studies have revealed that VPA affects

rodent brain levels of ASP and, less consistently, GLU (see Chapman et al,

1982) within a dose range active in animal seizure paradigms. Additionally,

VPA may reduce release of neurotransmitter ASP in vivo, since the drug

inhibits release of this EAA from rat cortical slices (Crowder and Bradford,

1987). A strong correlation between anticonvulsant potency against audiogenic seizures in DBA/2 mice and reduced cerebral ASP levels has been noted following treatment with VPA (Schecter et al, 1978) and VPA analogues (Chapman et al, 1983). In line with the finding that VPA is active against clonic seizures induced by NMDA (Czuczwar et al, 1985), the drug inhibits NMDA-evoked transient depolarization of cortical slice neurones at relatively low doses (0.1-1.0 mM; Zeise et al, 1991).

5.3 Interaction of VPA with central monoaminergic neurotransmission.

Ever since it was noted that VPA was active against a variety of myoclonic seizure types (Pinder et al, 1977) and that central serotonergic systems may be dysfunctional in these conditions (Chadwick et al, 1975; Van Woert and Hwang, 1981), the possibility of an interaction of the anticonvulsant with this neurotransmitter system has been suspected. Limited clinical studies revealed that VPA elevated CSF 5-HIAA levels in two patients receiving the drug during the successful treatment of post-anoxic myoclonus (Fahn, 1978) and it was concluded that VPA acted in this manner by blocking the active transport of the 5-HT metabolite from the brain (Hwang and Van Woert, 1979; MacMillan et al, 1979). Later studies however, suggested that VPA does indeed induce an increase in central 5-HT turnover in pargyline-pretreated rodents (Whitton et al, 1985) and that serotonergic receptor antagonists and 5-HT reuptake blockers respectively decrease or enhance the action of VPA in experimental myoclonus (Van Woert and Hwang, 1981). Recently, it was shown that acute VPA injection in rats resulted in an increase of central 5-HT release in rodents as measured by in vivo microdialysis (Whitton and Fowler,

1991). Further discussion of the possible role of central 5-HT systems in the anticonvulsant action of VPA can be found in chapters 3, 5, 7 and 8.

An involvement of dopaminergic neurotransmission in mediating a proportion of the anticonvulsant action of VPA has been difficult to demonstrate using in vitro and ex vivo techniques. Horton et al (1977) noted that rodent brain levels of the dopamine metabolite, homovanillic acid (HVA) were increased following VPA treatment, but this did not correlate temporally with seizure protection. As in the case of 5-HIAA, it was concluded that VPA raised brain HVA concentrations by blocking active transport of this metabolite from the brain. In addition, these same authors did not find the anticonvulsant efficacy of VPA to be compromised when animals were pretreated with the catecholamine synthesis inhibitor α-methyl-*p*-tyrosine (AMPT). Given that VPA has been shown to increase CSF HVA concentrations in schizophrenic men presenting with epilepsy (Zimmer et al, 1980), Löscher (1993) concluded that central dopaminergic neurotransmission might mediate the antipsychotic and antidystonic actions reported following VPA administration, but did not contribute to the anticonvulsant action of the drug. In whole brain ex vivo studies, VPA was not shown to significantly elevate central DA accumulation (Horton et al, 1977; Whitton, P.S., unpublished data). This does not rule out an effect of the drug on brain regional DA turnover and data presented in chapters 3, 5, 7 and 8 of this thesis may prompt a reexamination of the possible involvement of central DA systems in the anticonvulsant action of VPA. There is no evidence to date which suggests that VPA increases central noradrenergic function in patients receiving the drug, although this has been

demonstrated in rodents (Whitton et al, 1984). These authors found that acute

administration of 200 mg/kg VPA resulted in an enhanced accumulation of

NA in whole brain ex vivo, following inhibition of monoamine oxidase (MAO)

with pargyline. This finding may have some connection with the overall

anticonvulsant action of VPA both in animal models and clinical epilepsy, but

clearly more data is required to elucidate this.

Most of the non-human studies reviewed in this section have referred to

neurochemical findings following acute treatment with VPA. It should be

noted that the effect of the drug on neurotransmitters and metabolites in

animals receiving VPA for a prolonged period has also been investigated and

these findings are discussed in some depth in chapter 5.

5.4 Neurophysiological and neurochemical actions of VPA not directly

associated with central neurotransmission.

VPA is known to have pharmacodynamic actions on systems associated with

pre- and postsynaptic neuronal function other than those directly concerned

with central neurotransmitter synthesis and metabolism. Interaction of VPA

with these systems may be of equal importance with the aforementioned

actions of the drug when attempting to delineate its anticonvulsant mode of

action. The probable importance of each of these actions should, however, be

weighed against the concentration of VPA required to elicit them and its

relation to the dose range for anticonvulsant activity. A summary of these

known neurophysiological and neurochemical actions is presented in table 2.

Findings	Species	Effective dose or concentration	Sample references
Lipophilic disordering of neuronal membranes.	Mouse	mMol range	Perlman and Goldstein, (1984)
Reduction in sustained repetitive firing of action potentials recorded from cultured cortical neurones	Mouse	6-200 µM	McLean and Macdonald, (1986)
Decrease in firing rate of SNR[a] neurones in vivo	Rat	100-200 mg/kg	Farrant and Webster, (1989)
Activation of K^+-conductance in neurones.	Aplysia	5-30 mM	Slater and Johnston, (1978)
Use-dependent reduction of inward sodium current measured in cultured cortical neurones.	Mouse	6-200 µM	McLean and Macdonald, (1986)
No effect on low-threshold (T) calcium current recorded from thalamic neurones in vitro.	Rats, Guinea pigs	up to 1 mM	Coulter et al, (1989)
Alteration of central cGMP[b] levels in vivo.	Mouse	400 mg/kg	Lust et al, (1978)

a Substantia nigra pars reticulata. b Cyclic guanosine 3',5'-monophosphate. cGMP levels are sharply elevated in cerebellar neurones at the onset of experimental seizures and might play a role in maintaining seizure activity via control of Purkinje cell firing (Lust etal, 1978).

Table 2. Summary of the actions of VPA on pre- and postsynaptic neuronal elements.

6.Aims of this work and techniques used.

Epilepsies comprise some of the commonest neurological conditions encountered in medicine. Epilepsy affects between 0.04% and 0.1% of the global population and in many cases, continues to be highly debilitating despite the advent of modern pharmacotherapy and neurosurgery. For this reason, it is important that anticonvulsant therapy is continually improved in an attempt to control incidence of refractory seizures and improve often unacceptable drug side effect profiles. Informatiom regarding the manner in which existing anticonvulsant drugs such as VPA alter brain neurochemistry is therefore of fundamental importance and may aid in future design of novel compounds.

In the preceding sections of this introduction, experimental and clinical evidence for the involvement of central excitatory and inhibitory neurotransmission in the pathophysiology of epilepsies has been presented, together with a brief review of the known pharmacodynamic actions of VPA on these systems. Pharmacological agents that alter brain biochemistry might be expected to exert a proportion of their action at the cellular level by altering *extracellular* concentrations of neurotransmitters. Demonstration of such phenomena would have important implications for the assumed mode of action attributed to such agents since it would provide a likely indication of changes in neurotransmitter activity at the synaptic level. Such data would therefore provide a 'missing link' in a chain of events from primary biochemical changes induced by the drug through to its ultimate neurophysiological and behavioural outcome.

Given the lack of data currently available regarding the effect of VPA (and other anticonvulsants) on central neurochemistry <u>in vivo</u>, it was decided that this should be investigated in relation to the known neurochemical actions of the drug. The work presented in this thesis falls into two major categories, based empirically on the techniques used: Thus in chapters 3-6, the effects of acute and prolonged VPA administration on extracellular neurotransmitter and metabolite levels in rat brain were extensively studied using the techniques of <u>in vivo</u> microdialysis and high performance liquid chromatography (HPLC). In the penultimate chapter (8), interactions between VPA and monoaminergic neurotransmitters were studied inferentially using an established model of intractable epilepsy (pilocarpine-induced limbic seizures; Turski et al, 1989). These two <u>in vivo</u> pharmacological approaches were bought together in chapter 7, in which the effects of VPA and a chemoconvulsant on extracellular excitatory and inhibitory amino acid neurotransmitters were monitored concurrently with behavioural changes.

<u>6.1 In vivo microdialysis studies.</u>

The relatively new technique of <u>in vivo</u> microdialysis has become an established and reliable method of monitoring extracellular levels of a variety of endogenous and exogenous substances. The brain is particularly amenable to study using this technique (reviewed by Benveniste, 1989) and it is now often the method of choice for <u>in vivo</u> monitoring of the extracellular (EC) space. The technique has a number of advantages over other systems used for studying the brain EC space and these are summarised in table 3. Detailed information regarding microdialysis procedures is provided in chapter 2.

As indicated in sections 4.1 and 4.2, the hippocampus and striatum are considered to play crucial roles in the generation and spread of seizure activity. In addition, both structures are richly innervated by monoaminergic and amino acid neurotransmitter pathways, allowing the collection and analysis of a large number of neurotransmitters and metabolites, using the microdialysis technique. Since many of these substances share roles in controlling neuronal excitability via interaction with receptors, activation or blocking of which can profoundly alter the character, duration and intensity of seizures, it seemed logical to examine the neurochemical effects of VPA in these structures. In addition, VPA may be selectively accumulated in the hippocampus, compared to the cortex and striatum (Löscher and Nau, 1983) suggesting that the drug might effect these structures differently. Furthermore, there is some evidence to suggest that the hippocampus is a specific site for the anticonvulsant action of VPA. In rabbits, subjected to electrically induced focal seizures in neocortex, visual cortex and hippocampus, VPA (but not carbamazepine or phenobarbital) strongly suppressed seizure after-discharges (AD) recorded in the hippocampus, but not cortical regions (Kubota et al, 1990). The converse was apparent for the other anticonvulsants. These findings do not exclude the possibility that the striatum may be an important site for the anticonvulsant mechanism of action of VPA. In the pilocarpine model of limbic motor seizures, the striatum appears to play a critical role in modulating seizure threshold and intensity (Turski et al, 1988; Al-Tajir and Starr, 1990) and VPA is highly effective in preventing these seizures (Turski et al, 1989). Additionally, VPA is thought to exert part of its anticonvulsant

	Advantages	Disadvantages
In situ Ion-selective microelectrodes	a. All brain regions can be examined. b. Time resolution <1s. c. Tip of electrode <1µm.	a. Only detects ions
Carbon fibre microelectrode	a. All brain regions can be examined. b. Can be used in conscious animals. c. Time resolution < 1min. d. Tip of electrode < 100µm	a. Only detects oxidisable compounds. b. Selectivity poor in absence of previous HPLC analysis. c. Drainage depletion. d. Some electrodes have short working life in vivo
Ex situ Push-pull cannula	a. All brain regions can be examined. b. Can be used in conscious animals. c. BBB[†] intact following implantation.	a. Time resolution >10 min b. Drainage depletion. c. Enzymatic degradation of collected compouns. d. Deproteinisation of sample prior to HPLC. e. Tissue trauma following and during implantation. f. Cannula diameter >1mm
Microdialysis probe	a. All brain regions can be examined. b. Can be used in conscious animals. c. BBB intact following implantation. d. Minute tissue trauma within the first 48 hours. e. No need for deproteinisation of samples. g. No enzymatic degradation of samples.	a. Time resolution > 5 min b. Drainage depletion. c. Probe diameter approximately 0.6mm

† Blood brain barrier.

Table 3. Advantages and disadvantages of various in vivo monitoring techniques.

Reproduced with modifications from Benveniste, 1989.

effect by enhancing GABAergic neurotransmission in the substantia nigra (Löscher, 1989), a structure intimately associated with striatal function (Graybiel and Ragsdale, 1979). Methodologically, the hippocampus and striatum in the rat lend themselves to in vivo microdialysis. Reference to figure 2 (chapter 2) illustrates the relative ease with which microdialysis probes can be be implanted into these structures. All microdialysis experiments presented in successive chapters used conscious animals, since anaesthesia is thought to interfere with the effect of VPA on brain EC monoamine levels, as estimated using in vivo differential pulse voltametry (Crespi et al, 1986).

6.2 Pilocarpine seizure model.

The pilocarpine model of limbic motor seizures was chosen to investigate the role of monoaminergic neurotransmission in the mechanism of action of VPA on the basis of findings presented in chapter 3. and due to the established role of monoamines in the aetiology of seizures induced by the cholinomimetic (Turski et al, 1988; Janusz and Kleinrok, 1989) The neurochemical consequences of pilocarpine administration and subsequent seizures in rodents are discussed in chapter 8. The reader is referred to Turski et al (1989) for a detailed review of this model.

Chapter Two

Materials and Methods

1. Animals

Male albino Wistar rats, weighing 220-350g were used for all experiments. Prior to experiments animals were group housed under conditions of constant humidity and temperature ($22 \pm 1°C$). Animals were conditioned to a light-dark cycle, with light from 07:00 to 17:00 every day. Access to food (standard rodent diet) and water was *ad libitum*.

2. Microdialysis

2.1 Details of concentric microdialysis probe construction

Microdialysis probes were constructed in the laboratory using a design outlined by Whitton et al. (1990). Construction details are as follows: A steel cannula (24 swg stainless steel, 2 cm length, 0.52 mm internal diameter) formed the body of the probe, through which two lengths of fine fused silica tubing were inserted. One length of tubing emerged from the opposite end of the cannula whilst the other was fed approximately half the distance through the cannula. A drop of araldite adhesive was used to secure the two lengths of silica in position at the appropriate end of the cannula. Once the adhesive had dried, a smaller cannula (27 swg stainless steel, 1 cm length, 0.38 mm internal diameter) was slipped over each of the protruding lengths of silica tubing and secured with araldite. At this stage, the probe under construction resembled a " Y ". The junction between the three steel cannulae was coated in quick setting dental acrylic (Duralay, Reliance Dental MFG. Company, U.S.A.) to make the structure more robust. After trimming any excess slilica tubing protruding beyond the ends of the 27 swg cannulae, the single portion of silica protruding from the opposite end of the 24 swg cannula was trimmed

to 4 mm. To complete the probe, a hollow fibre dialysis membrane (10 Kdalton cut-off point; Cuprophan fibre, ENKA, AG, Germany) was fed over the trimmed silica tube such that when cut, the dialysis fibre*extended 0.5 mm beyond the end of this tube. The cut end of the dialysis fibre was carefully sealed with a minute drop of araldite and the opposite end of the fibre secured to the inner circumference of the 24 swg cannula in the same manner. Owing to the small scale of the work, a binocular microscope was used as a visual aid at each stage of construction. Figure 1. illustrates diagrammatically the structure of the completed probe. Examination of each completed probe verified that the *active* membrane length was set at 4 mm. On completion of the probe, approximately 5 cm of polythene tubing (pp10 tubing, OD 0.68 mm; Portex Ltd, U.K.) was pushed tightly over the end of each 27 swg cannula to allow the probe to be perfused with fluid. After allowing the araldite to cure, probes were gently perfused with saline to test for patency. Any probe that failed to allow passage of saline or displayed an inordinately high resistance to the flow of saline was rejected at this point. For all experiments, both <u>in vivo</u> and recovery, very low internal volume tubing (1.2 µl/100 mm; FEP tubing, Carnegie Medicin, Sweden) was fitted to the probe output tube. This facilitated the rapid transit of substances collected in the perfusate via the dialysis membrane to a collecting vial. Consequently, a more accurate temporal correlation between collection of substances at the dialysis membrane/brain tissue or dialysis membrane/bathing fluid interfaces and their appearance in the collecting vial could be made. This was deemed particularly important in the case of monoamine neurotransmitters and their metabolites since these are

✱ O.D. 0.6 mm
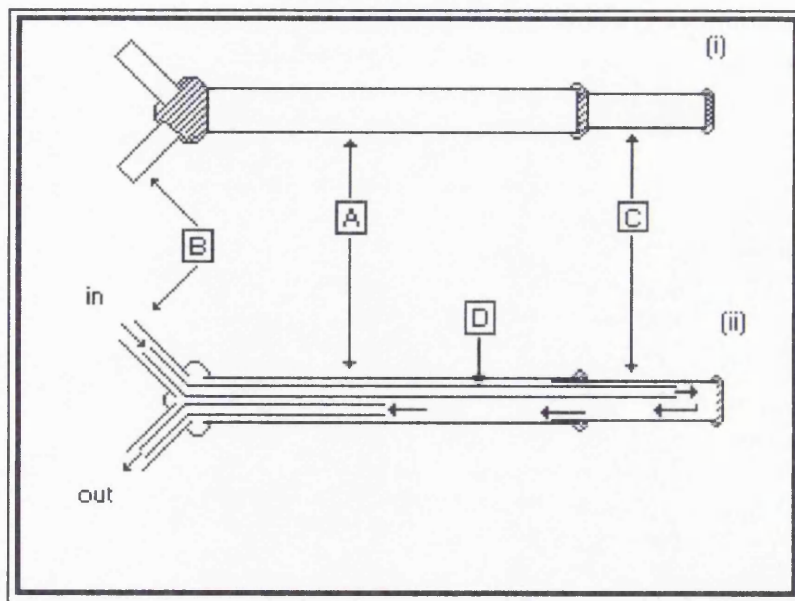
Figure 1 Diagramatic representation of a dialysis probe (i) and (ii) cross sectional view showing silica tubing. Key:- (A) 24 swg steel cannula. (B) 27 swg steel tubes. (C) dialysis membrane. (D) fused silica tubing. Dialysis membrane was sealed at its open end and also to the 24 swg cannula with araldite (shaded areas, right). Steel cannulae junction was sealed with araldite and dental acrylic (shaded area, left). Refer to text for further construction details.

often subject to rapid oxidation.

2.2 In vitro recoveries for monoamines and amino acids

To ascertain how faithful an estimate of EC monoamine or amino acid concentrations could be obtained from dialysis experiments, it was necessary to calculate recovery of these species from a bathing medium by probes perfused at an appropriate flow rate. To this end DA, 5-HT, DOPAC, 5-HIAA HVA were dissolved in distilled double-deionised water to 1 pmol/μl, with L-cysteine added (20 mg/100 ml) to limit oxidation (Chai and Meltzer, 1992). Probes were suspended in this bathing medium and perfused with artificial cerebrospinal fluid (ACSF) at a flow rate of 0.5 μl/min (model 22 infusion pump, Harvard Apparatus, U.S.A.) and four 30 min. fractions collected. ACSF composition was as follows:- KCl 2.5 mM, NaCl 125 mM, MgCl$_2$ 1.18 mM and CaCl$_2$ 1.26 mM, pH 7.0. In all experiments, ACSF was delivered to the probe input tubing via an appropriate length of pp10 tubing fitted to a gas-tight 500μl microsyringe (Hamilton,). Fractions were immediately analysed for monoamine and metabolite content using HPLC with electrochemical detection (HPLC-ED; see 3.1). All monoamines and metabolites were obtained from Sigma U.K. The collated results of these experiments are presented in table 4 (a). Estimation of recovery of amino acids from the EC enviroment was carried out in a similar manner as that for the monoamines. ASP, GLU, L-glutamine (GLN), taurine (2-aminoethanesulphonic acid; TAU) and GABA were all dissolved to a concentration of 1pmol/μl in distilled double-deionised water without the addition of L-cysteine. As previously, probes were suspended in this solution and perfused with ACSF at a flow rate of 0.5 μl/min. Again, four

Analyte	% Recovery (range; n=5)
DA	26-27
DOPAC	25-27
HVA	18-20
5-HT	19-21
5-HIAA	18-20

(b)

Analyte	% Recovery (range; n=5)
ASP	37-41
GLU	35-39
GLN	49-53
TAU	47-53
GABA	43-51

Tables 4 (a) and (b). % Recovery estimates for dialysate monoamines (a) and amino acids (b) in vitro.

30 min. fractions were collected. Fractions were analysed for amino acid content using HPLC with fluorimetric detection (HPLC-FD; see 3.2) All amino acids were obtained as solids from Sigma, U.K. Collated results are presented in table 4 (b).

From monoamine and amino acid recovery experiments, % relative recovery of analyte from the bathing medium was calculated using the relationship overleaf.

(i)   **Recovery**$_{invitro}$= C_o / C_i[1]

where C_o is the analyte concentration in probe outflow and C_i is the analyte concentration present in the bathing medium. Accepting that conditions in vitro can only approximate to those in vivo, estimates of actual brain EC levels of monoamines and amino acids were made in subsequent microdialysis experiments on the basis of the relationship:-

(ii) C_i = C_o / **recovery in vitro**[1]

where C_i is the estimated EC analyte concentration and C_o is the concentration of analyte measured from probe outflow in vivo.

On completion of all microdialysis experiments, raw data were adjusted using the above scheme in order that a more accurate estimate of analyte concentrations in the brain extracellular compartment could be made.

2.3 Stereotaxic surgery and probe implantation.

All rats used in microdialysis experiments were subjected to essentially the same surgical techniques for implantation of microdialysis probes. Surgical procedures were as follows: Male Wistar rats, previously maintained under

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[1]   [1] equations reproduced from Benveniste 1989.

conditions described in section 1. were anaesthetised with chloral hydrate (Sigma, U.K.) dissolved in saline at a dose of 400 mg/kg i.p. Anaesthetised rats were secured in a stereotaxic frame (David Kopf, U.S.) and the incisor bar set according to the size of animals used. Typically, for rats in the size range 220-350g this was approximately -3.3 to -2.5 mm. Once the incisor bar had been correctly adjusted, ensuring that the surface of the animal's head was parallel with the frame base plate, surgery was commenced. A medial incision of the scalp was made and the two skin flaps reflected laterally to expose the skull surface. Any membranous material was removed by blunt dissection and bleeding controlled with cotton wool. Having exposed the skull bone sutures, bregma was located and carefully marked with pencil. At this point, stereotaxic coordinates (Paxinos and Watson, 1982) were used to position a dental drill (tungsten carbide burr, tip 2 mm) above the skull surface dependent on the brain area to be studied. Coordinates for the three areas studied during the course of the research are given in table 5. A burr hole was carefully drilled in the skull at the appropriate coordinates and the dura mater exposed. Two additional burr holes were drilled in positions on the skull distant from the initial hole and a 2 mm stainless steel grub screw fastened into each with a jeweller's screwdriver. Typically, one screw would be placed anterior and to the left of bregma, whilst the other would be placed posterior and to the right of bregma.

	ANTERIOR*	LATERAL*	VERTICAL†
VH	-5 mm	5 mm	8 mm
ACP	1.6 mm	2.6 mm	8 mm
PCP	0.2 mm	3 mm	8 mm

Table 5 Stereotaxic coordinates for probe implantation sites.

*Key:- VH ventral hippocampus, ACP anterior caudate putamen, PCP posterior caudate putamen. * positions reletive to bregma. †taken from surface of dura.*

Probes were implanted unilaterally into the brain substance without prior insertion of guide cannulae. The probe was mounted in a holder (in-house design), which itself was secured by means of a bolt to the frame. With the aid of a hand magnifying lens, the probe was positioned over the remaining burr hole and lowered until the tip made visible contact with the dura. The graduated thumbwheel of the instrument manipulator was used to gently lower the probe the required depth into the brain. During this process the probe was gently perfused with ACSF in order to limit compression of the dialysis membrane by the tissues. This was considered important in preventing collapse of the membrane *in situ*, a phenomenon which may have accounted for apparent probe blockage or high hydrostatic resistance.

Having successfully implanted the probe, this was secured rigidly in place by flooding the operation site with quick-drying dental acrylic, the scull screws

anchoring the whole assembly tightly to the skull. Additionally, the tubing junction of the probe was also encased in dental acrylic to provide durability. The surgery completed, animals were removed from the stereotaxic frame and wrapped in paper towels to limit post-surgical hypothermia. Animals were singly housed in purpose built perspex cages with food and water and allowed an overnight recovery period (18.0±2.0 hours). This extended period of recovery was allowed in the light of evidence suggesting that acute implantation results in tissue damage, principally oedema. (Edvinsson et al, 1971). Thus acutely implanted microdialysis probes often recover excessive quantities of analytes which do not reflect true estimates of EC basal levels. It has been shown that dialysate neurotransmitters recovered following acute probe implantation are largely derived from damaged nerve terminals rather than steady-state release from neuronal stores. This is illustrated by the work of Westerink and de Vries (1988) concerning the origin of dopamine collected in dialysates from probes following acute implantation into the rat striatum.

2.4.1 Microdialysis; general procedures.

This section details experimental protocol common to all microdialysis studies performed during the period of research. Following the standard recovery period (see above), rats were prepared for dialysis. All experiments were performed using freely moving animals. A gas-tight microsyringe was filled with ACSF and the needle connected to a length of pp10 tubing (typically 20-30 cm). Once flushed with liquid, the other end of this tubing was connected via a short length of wider bore tube to the probe inlet and the syringe plunger gently depressed to assess the patency of the system. Problems

occasionally encountered at this point included high hydrostatic resistance or frank blockage of the probe. Reasons for these failures are speculative, but may be accounted for by the displacement and subsequent lodging of particles of grit or salts in the silica tubing. In view of this inconvenience, the upmost care was taken during probe construction to eliminate possible sources of particulate contamination. ACSF used in all experiments contained the 5-HT reuptake inhibitor citalopram ($1\mu M$) to aid recovery of 5-HT. Once found to be patent, the probe outlet was connected to a length of FEP tubing (20 ± 3 cm) and the entire system thoroughly flushed with ACSF. ACSF was perfused via the probe at a rate of $0.5\mu l/min$ using a Harvard infusion pump and in all cases, a 30 min fraction collected and discarded prior to the collection of further dialysates. This procedure was adopted in order to eliminate abnormally high levels of analytes which would have accumulated in the vicinity of the dialysis membrane during the post-operative period. Methodological differences between separate studies that employed microdialysis are described in detail in the appropriate results chapters.

3. Histology

As a means of examining the consistency of accurate probe placement in the brain area under study, rats had their brains removed for gross histological examination following the completion of microdialysis experiments. Brains were perfused with a fixative solution prior to removal as follows: Animals were deeply anaesthetised with 80 mg/kg pentobarbitone sodium (Expiral; Ceva) and the thoracic cavity opened. The left cardiac ventricle was located and approximately 50 ml heparinised saline perfused in order to flush as much

blood as possible from the vasculature. A fixative solution (4% phosphate-buffered paraformaldehyde saline, pH 7.4) was then perfused via the same route. After 120 mins., probes were carefully removed from the skull with the use of pliers and brains removed. Coronal brain sections were cut with a razor blade and examined under a binocular light microscope. The location of each probe tract in relation to the target area was recorded and where an obvious deviation from this site was noted, data obtained from the corresponding experiment was rejected. Typical probe placements are illustrated in figure 2.

4. High performance liquid chromatography.

All dialysates obtained from <u>in vitro</u> recovery and <u>in vivo</u> experiments were analysed for monoamine and amino acid content using HPLC. Two systems were available for this purpose; a system utilizing electrochemical detection (HPLC-ED) and a system utilizing fluorimetric detection of derivatised amino acids (HPLC-FD). Dialysis samples were not subject to pre-analysis treatments such as acidification or deproteination. As a general procedure, dialysates to be analysed for monoamine content were injected into the HPLC system on the day of collection or were stored at -80°C for a period not exceeding 1 week. Samples to be analysed for amino acid content were stored at -80°C for a period not exceeding 2 months prior to HPLC.

4.1 Analysis of monoamines using HPLC with electrochemical detection.

4.1.1 System configuration and chromatographic conditions.

The HPLC system for detection and quantification of dialysate monoamines and their metabolites consisted of the following component parts:- Gilson solvent delivery module (model 303), a C18 reverse-phase column (3 μm
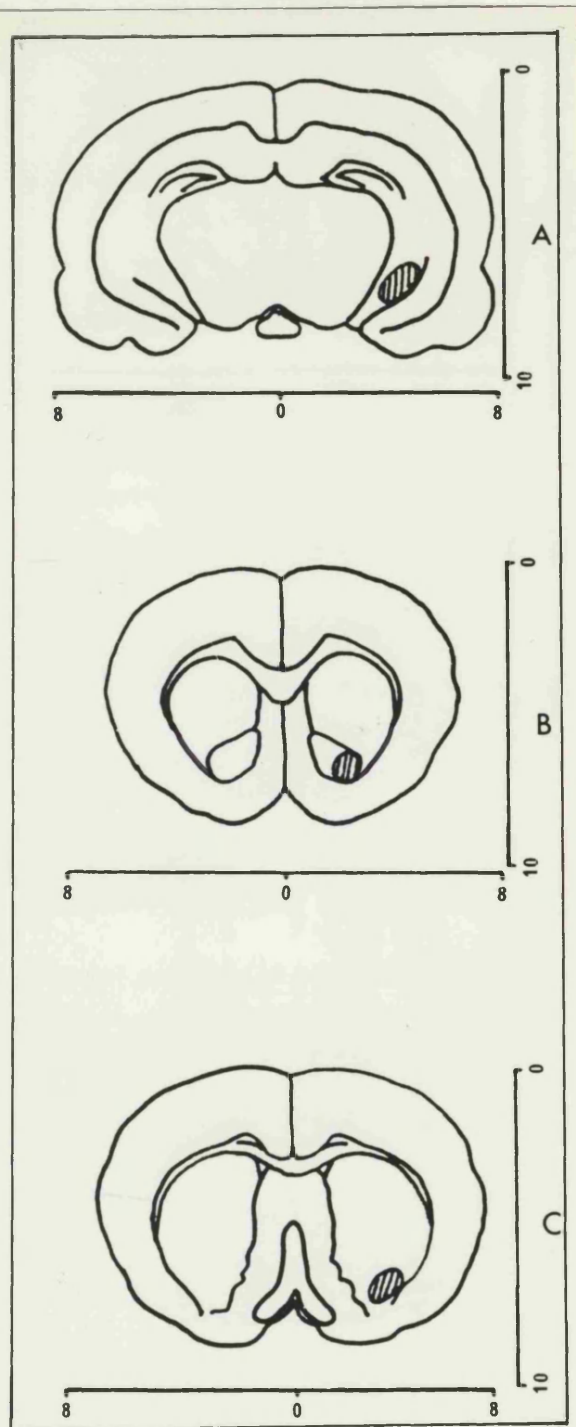
Figure 2 Diagrams to illustrate probe positioning in the rat brain during microdialysis experiments. Coronal sections were made to verify correct placement of microdialysis probes in VH (A), ACP (B) and PCP (C). Hatched areas indicate range of actual probe tip placements within the structure of interest. See text for details of histology and stereotaxic coordinates. Scale in mm.

particle size ODS) and an ESA Coulochem model 5100A electrochemical detector with model 5011 porous carbon electrode analytical cell (Severn Analytical Ltd., UK.) Data were collected and processed using a PC system (Dell Corporation, USA) interfaced to the electrochemical detector (ECD) via a data collection unit (Drew Ltd, UK). Additionally, a BBC Goerz Metrawatt SE 120 chart recorder was coupled directly to the ECD output. This latter facility allowed a further 10-fold amplification of the ECD output. At the time of writing, no reports of DA detection in ventral hippocampal dialysates were known to exist, other than those from this laboratory (Chapters 3, 5 and 6, this thesis). With the aid of this additional recording facility, it has been possible to detect very low (<10 fmol) levels of DA, the peaks of which would otherwise appear indistinct from the baseline noise on chromatograms generated using the PC software (Drew Roseate software) alone. The chart recorder facility also proved valuable in resolving 5-HT, which was also found to be present in all dialysates at a very low level (see results sections, chapters 3, 5 and 6). Figures 3 b,c and d illustrate representative separations for dialysate monoamines. The separation technique used was based on that of Hutson et al. (1989) with modifications to allow concurrent detection of DA and its metabolites. All separations were isocratic and the mobile phase had the following composition: Sodium acetate 90 mM, citric acid 35mM, EDTA 0.34 mM and sodium octylsulphonic acid 0.06 mM (as ion pairing reagent) with 12% methanol, pH 4.2. Helium-degassed mobile phase was pumped at a flow rate of 1.0 ml/min. The ECD settings were; guard cell +0.5 V, electrode 1 +0.01 volts and electrode 2 +0.45 V (analytical cell) during all separations.
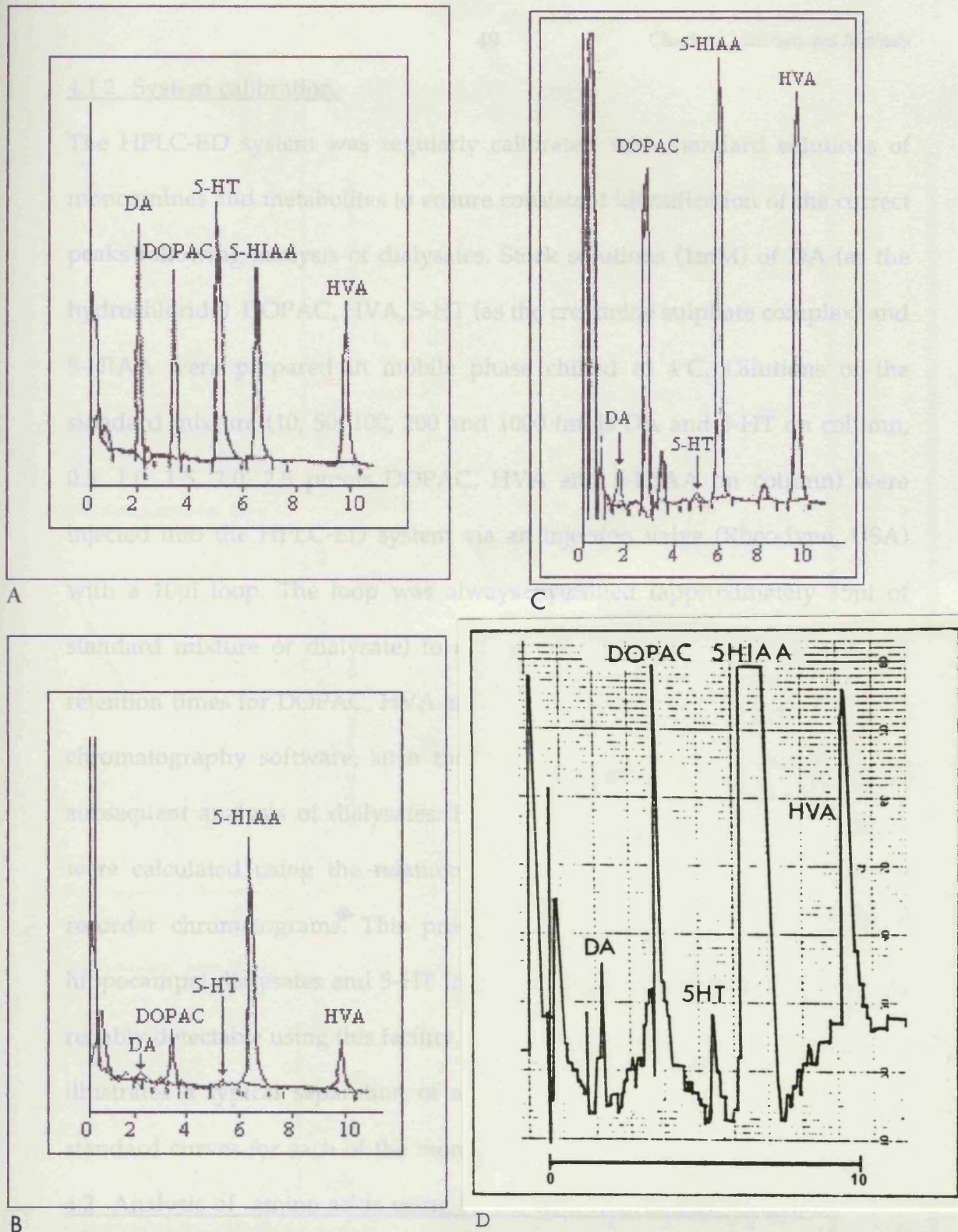
Figure 3. Typical chromatograms obtained following separation of monoamines using HPLC with ED. (A) shows a representative standard mixture (1 pmol). (B) and (C) are typical representations of basal monoamine output obtained in the ventral hippocampus and striatum respectively. Horizontal axis represents elution time in min. (D) is the same separation as (B) using the chart recording facility (see text).

4.1.2 System calibration.

The HPLC-ED system was regularly calibrated with standard solutions of monoamines and metabolites to ensure consistant identification of the correct peaks following analysis of dialysates. Stock solutions (1mM) of DA (as the hydrochloride) DOPAC, HVA, 5-HT (as the creatinine sulphate complex) and 5-HIAA were prepared in mobile phase chilled to 4°C. Dilutions of the standard mixture (10, 50, 100, 200 and 1000 fmols DA and 5-HT on column, 0.5, 1.0, 1.5, 2.0, 2.5 pmols DOPAC, HVA and 5-HIAA on column) were injected into the HPLC-ED system via an injection valve (Rheodyne, USA) with a 10μl loop. The loop was always overfilled (approximately 15μl of standard mixture or dialysate) to ensure reproducibility. Peak areas and retention times for DOPAC, HVA and 5-HIAA were calculated using the PC chromatography software, such that peak recognition was established for subsequent analysis of dialysates. For DA and 5-HT calibrants, peak areas were calculated using the relationship area= ½ base x height from chart recorder chromatograms.* This procedure was adopted so that DA from hippocampal dialysates and 5-HT from all dialysates, which were often only reliably detectable using this facility, could be accurately quantified. Figure 3a illustrates a typical separation of a monoamine standard mixture. Sample standard curves for each of the monoamines are provided (figure 4).

4.2 Analysis of amino acids using HPLC with fluorometric detection.

4.2.1 System configuration

HPLC separation and quantification of amino acids was achieved using a system consisting of the following components; two Gilson solvent delivery

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✱ measurements were made using a ruler.
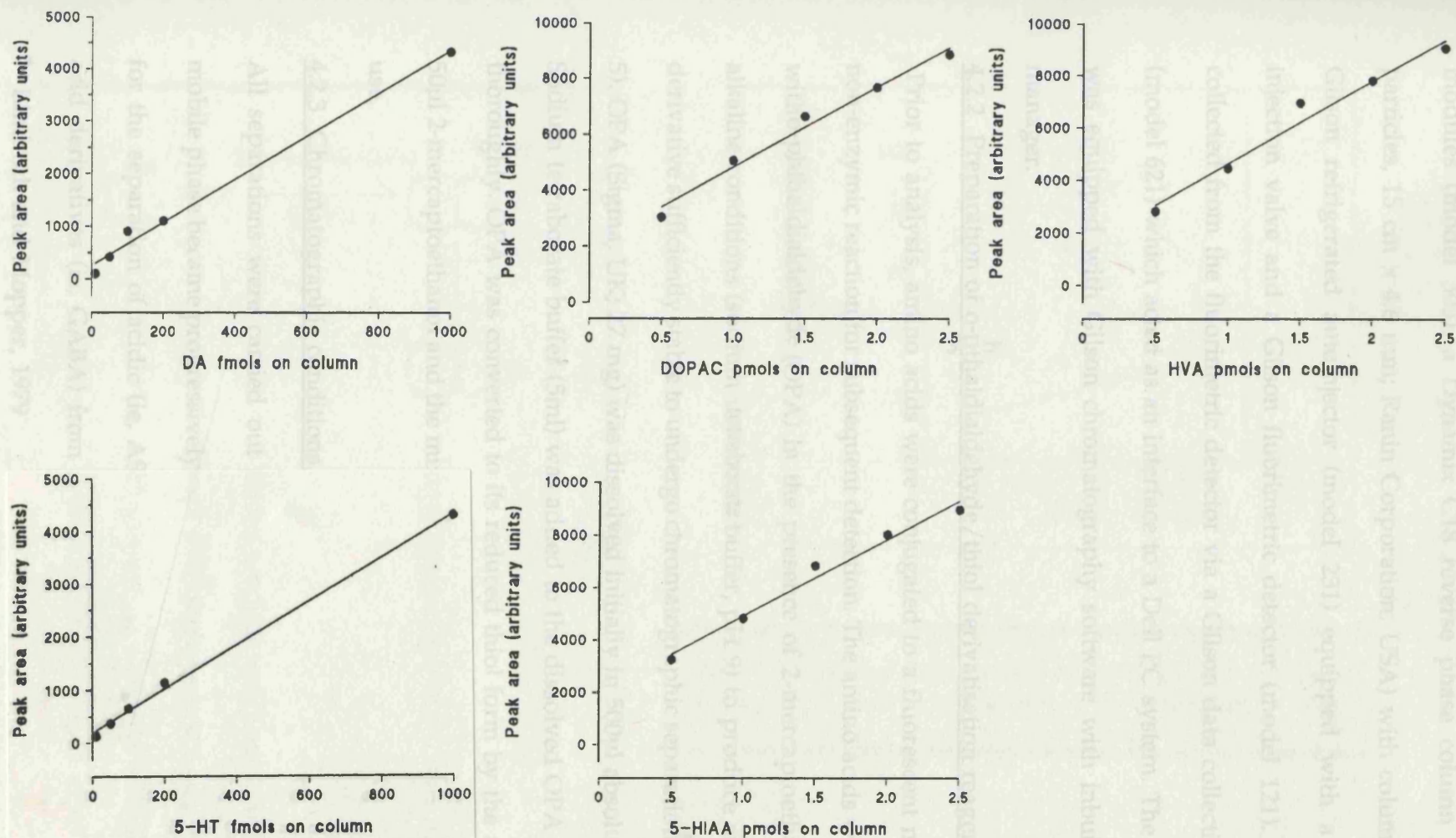
Figure 4 Typical calibration plots for monoamines obtained using HPLC with EC detection. Refer to text for details of chromatographic techniques.

modules (model 303), a Dynamix C18 reverse phase column (5µm ODS particles, 15 cm x 4.6 mm; Ranin Corporation, USA) with column heater, a Gilson refrigerated autoinjector (model 231) equipped with a Rheodyne injection valve and a Gilson fluorimetric detector (model 121). Data were collected from the fluorimetric detector via a Gilson data collection module (model 621) which acted as an interface to a Dell PC system. The PC system was equipped with Gilson chromatography software with inbuilt gradient manager.

4.2.2 Preparation of o-phthaldialdehyde/thiol derivatisation reagent.

Prior to analysis, amino acids were conjugated to a fluorescent moiety by a non-enzymic reaction for subsequent detection. The amino acids were reacted with o-phthaldialdehyde (OPA) in the presence of 2-mercaptoethanol under alkaline conditions (sodium tetraborate buffer, pH 9) to produce an isoindole derivative sufficiently stable to undergo chromatographic separation (see figure 5). OPA (Sigma, UK; 27 mg) was dissolved initially in 500µl absolute ethanol. Sodium tetraborate buffer (5ml) was added to the dissolved OPA and mixed thoroughly. OPA was converted to its reduced thiol form by the addition of 50µl 2-mercaptoethanol and the mixture refrigerated at 4°C overnight prior to use.

4.2.3 Chromatographic conditions.

All separations were carried out under gradient conditions, such that the mobile phase became progressively less polar with elution time. This allowed for the separation of acidic (ie, ASP) through to relatively non-polar amino acid derivatives (ie, GABA) from a mixture over a period of 40 min. Two

* Lindroth and Mopper, 1979
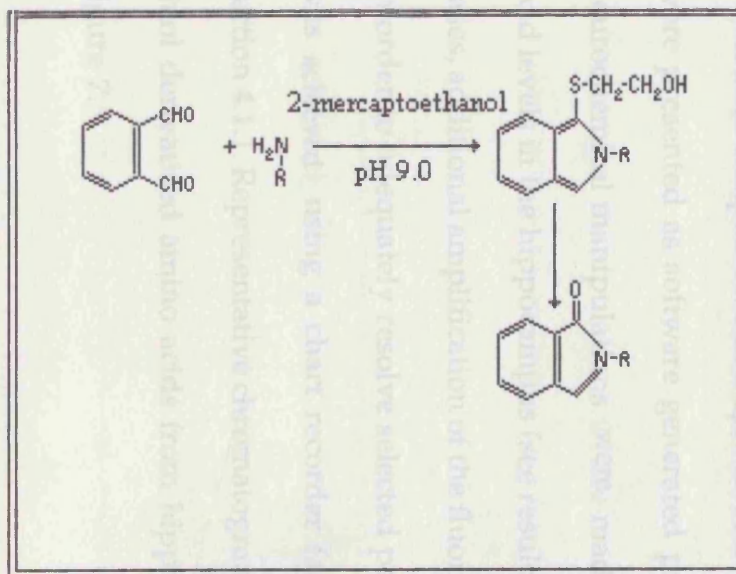
Figure 5 Reaction of OPA with amino acid to form a fluorescent isoindole derivative. Further details of sample preparation can be found in the text.
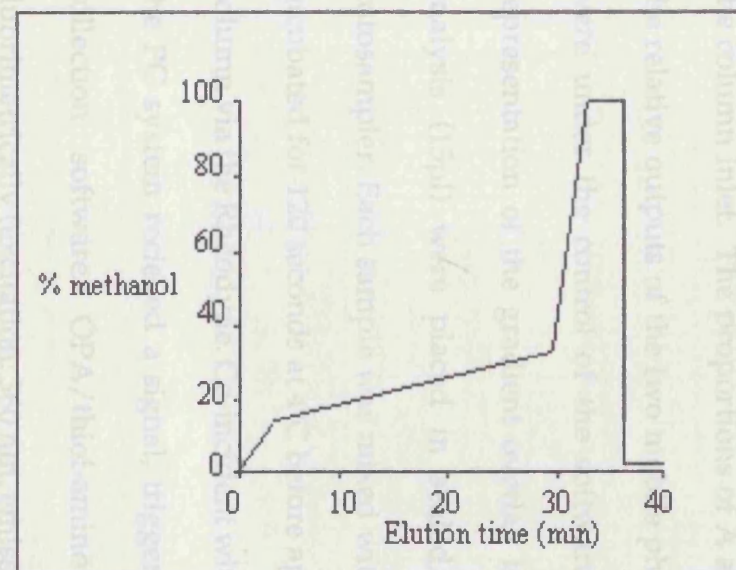
Figure 6 Graphical representation of mobile phase composition during HPLC separation of OPA-thiol derivatised amino acids.

mobile phase components, A (50 mM sodium dihydrogen orthophosphate, pH

5.5, 20 % methanol) and B (100% methanol) were mixed prior to delivery to

the column inlet. The proportions of A and B in the mix were determined by

the relative outputs of the two mobile phase delivery modules, both of which

were under the control of the software gradient manager. A diagrammatic

representation of the gradient overlay is provided in figure 6. Samples for

analysis (15µl) were placed in sealed, opaque vials in the rack of the

autosampler. Each sample was mixed with 15µl of the OPA/thiol reagent and

incubated for 120 seconds at 4°C before application of the mixture (20µl) to the

column via the Rheodyne. Co-incident with injection of the derivatised sample,

the PC system recieved a signal, triggering the gradient manager and data

collection software. OPA/thiol-amino acid conjugates were detected

fluorimetrically (excitation: 360 nm, emission: 455 nm). Upon completion of the

separation, the entire cycle was repeated automatically until the desired

number of samples had been processed. Chromatograms and peak area data

were presented as software generated printouts. During some experiments,

neurochemical manipulations were made in an attempt to reduce EC amino

acid levels in the hippocampus (see results sections, chapters 6 and 7). In these

cases, additional amplification of the fluorimetric detector output was required

in order to adequately resolve selected peaks (namely, ASP and GABA). This

was achieved using a chart recorder facility identical to that described in

section 4.1.1. Representative chromatograms illustrating the separation of OPA-

thiol derivatised amino acids from hippocampal dialysates are presented in

figure 7.
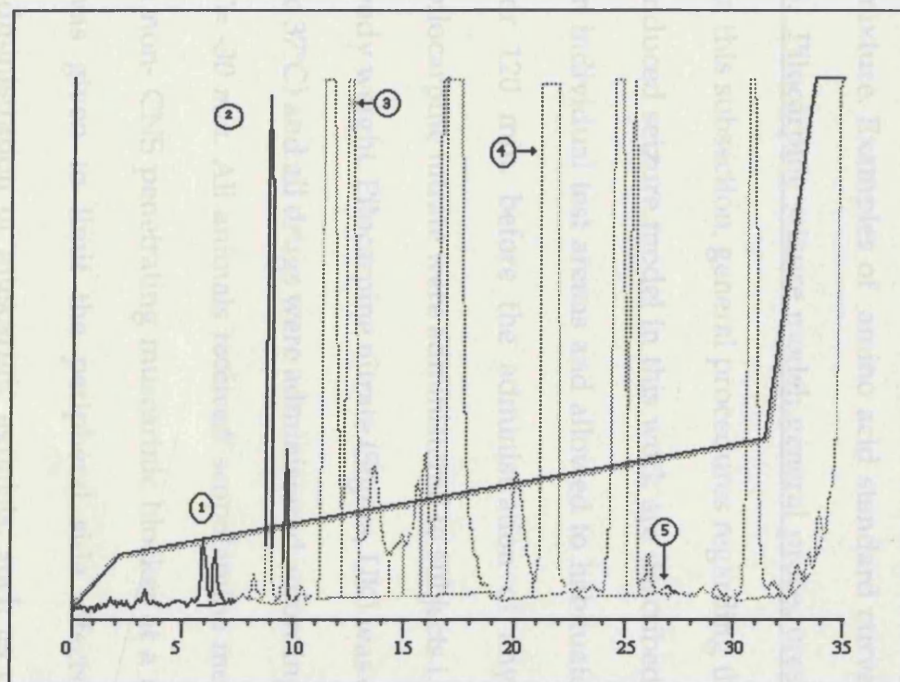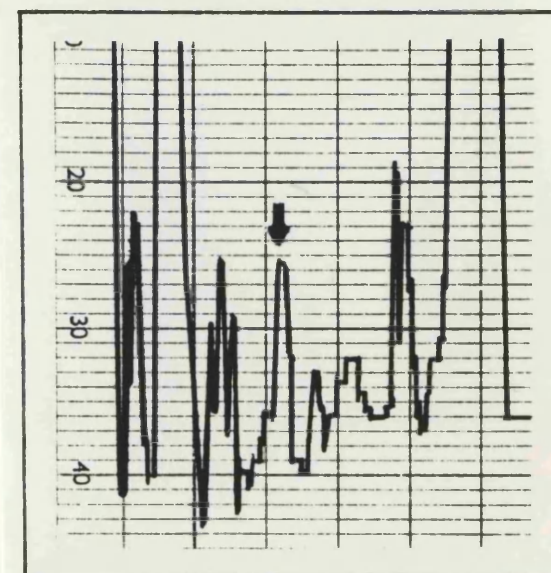
Figure 7 Separation of OPA-thiol amino acid derivatives from hippocampal dialysates using HPLC with fluorimetric detection. (A) Typical chromatogram obtained under conditions of basal amino acid output. Peaks denoted are:- (1) ASP, (2) GLU, (3) GLN, (4) TAU and (5) GABA. Horizontal scale represents elution time in min., whilst the black line shows gradient buffer conditions (see figure 6) superimposed on the chromatogram. (B) Representative chart recording of hippocampal dialysate OPA-thiol amino acid derivatives separated using HPLC with fluorimetric detection. Such recordings were made when it was deemed likely that drug treatments would result in lowered EC GABA levels. Peaks are analogous to those of (A). The chart recorder facility allowed a further ten-fold amplification of detector output (see text). Only a portion of an entire recording is shown, since the peak of interest (GABA) was resolved towards the end of the elution period and is denoted by an arrow.

4.2.4 System calibration.

Standard curves for amino acid quantification in samples were routinely obtained. Stock solutions (1mM) of ASP, GLU, GLN, TAU and GABA were prepared in mobile phase A. Dilutions of a mixture of the above amino acids (0.15, 0.31, 0.625, 1.25 2.5, 5 and 25 pmols final quantity on column) were dispensed in 15μl aliquots into sealed, opaque vials and analysed. The resultant chromatograms were used to establish a quantity/peak area relationship for each species. In the case of GABA, the lowest two calibrant peak areas were obtained from the chart recorder to allow the very low levels of this amino acid found in some dialysates (< 1 pmol) to be estimated with some accuracy. Figure 8 shows a typical separation for an amino acid standard mixture. Examples of amino acid standard curves are provided (figure 9).

5. Pilocarpine seizure model; general procedures.

In this subsection, general procedures regarding the use of the rat pilocarpine-induced seizure model in this work are described. Rats (see 1.) were housed in individual test arenas and allowed to habituate to their new surroundings for 120 min. before the administration of any drugs. Drugs other than pilocarpine nitrate were administered to subjects i.p. in volumes of 0.1ml/100g body weight. Pilocarpine nitrate (Sigma, UK) was dissolved in saline (warmed to 37°C) and all drugs were administered according to the following schedule:-

T= -30 min. All animals received scopolamine methyl bromide (Sigma, UK) , a non- CNS penetrating muscarinic blocker at a dose of 1 mg/kg This drug was given to limit the peripheral side effects associated with systemic administration of muscarinic agonists, such as cardiovascular collapse and
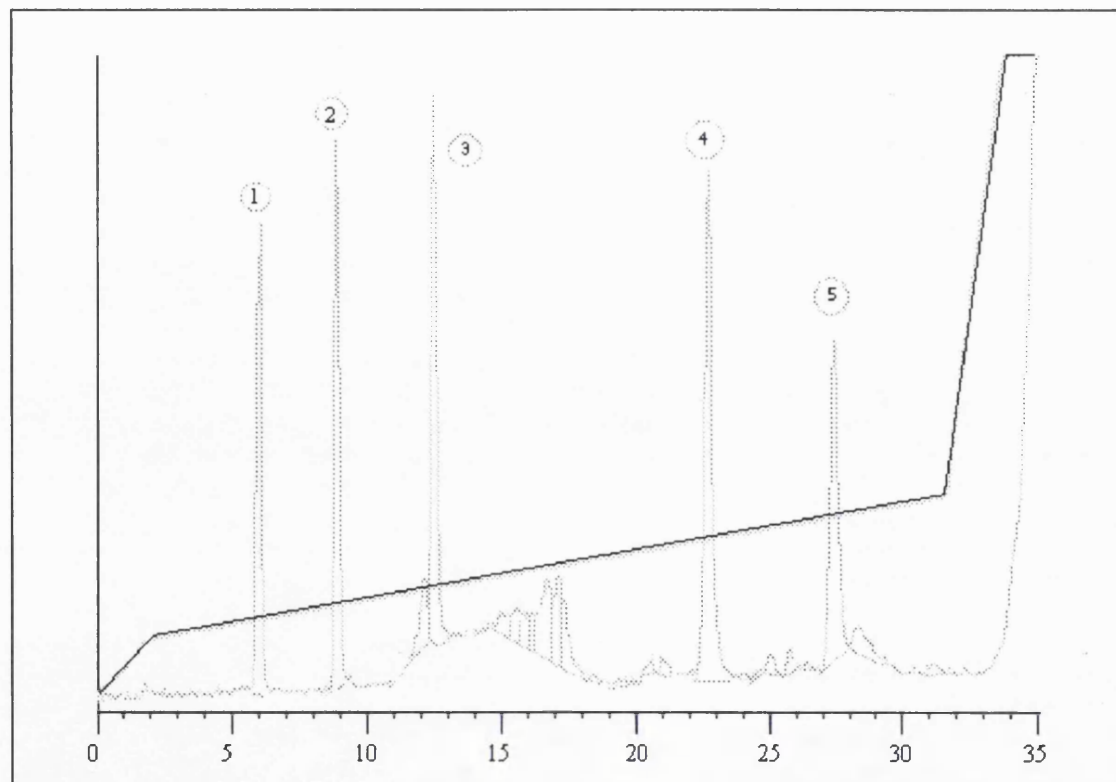
Figure 8 Typical chromatogram representing separation of a standard mixture (25 pmols) of five OPA-thiol derivatised amino acids using HPLC with fluorimetric detection. Peaks are:- (1) ASP, (2) GLU, (3) GLN, (4) TAU and (5) GABA. Horizonatal scale represents elution time in min. Black line shows gradient buffer conditions (see figure 6) superimposed on the chromatogram.
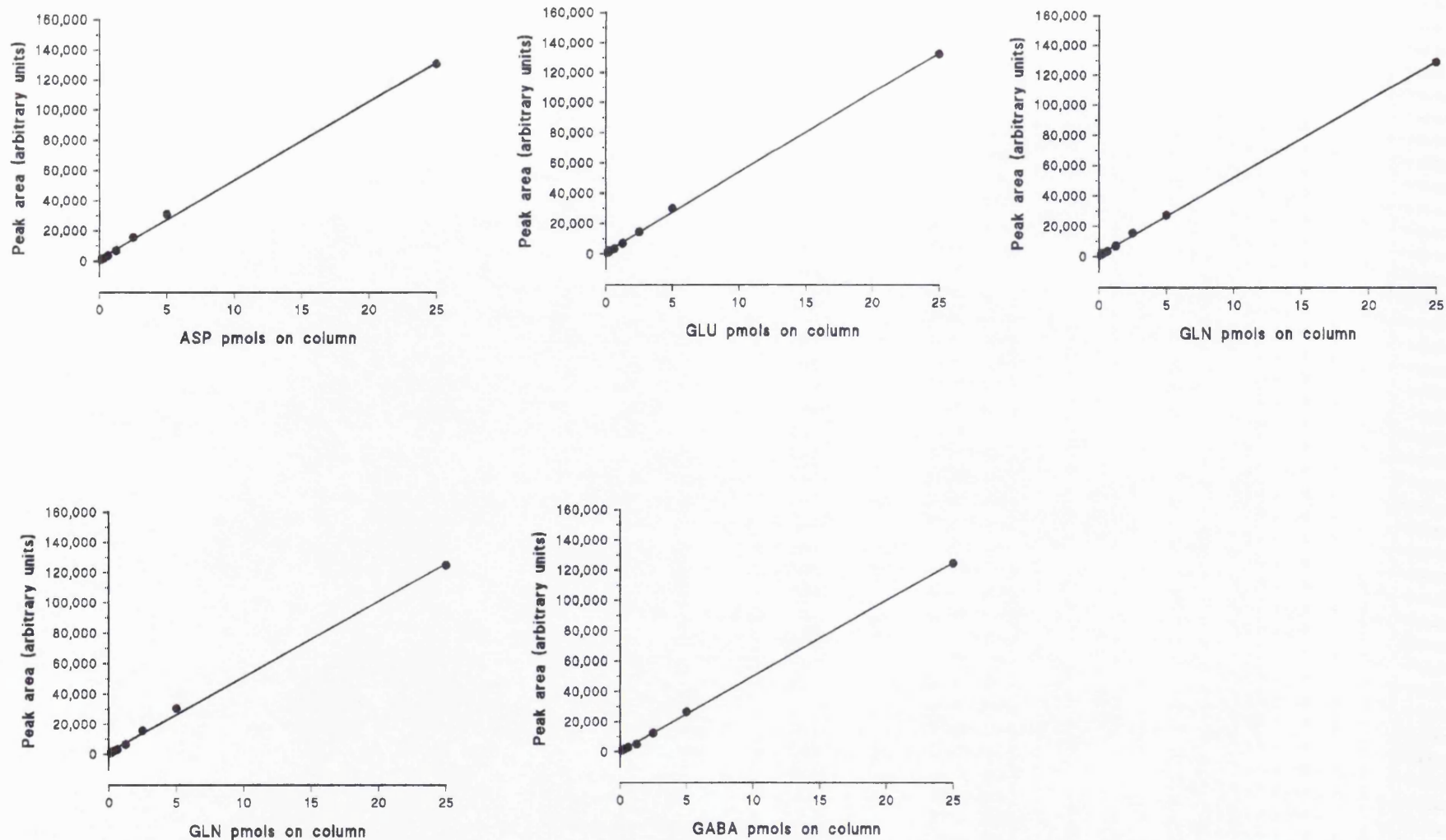
Figure 9 Typical calibration plots for amino acids obtained using HPLC with fluorimetric detection. Refer to text for details of chromatographic techniques.

hypersecretion of mucus and saliva. Additionally, a dose of the appropriate test drug or saline (control animals) was administered i.p. at this time.

T= 0 mins. Animals received a convulsant dose (400 mg/kg; Turski et al, 1988) of pilocarpine nitrate and an anticonvulsant dose of VPA (300 mg/kg) or saline.

T= 0-180 mins. Animals were observed for signs of seizure activity. Seizures were scored on a point system at 30 min intervals (see table 6).

On completion of experiments, raw data were processed in the following manner: The average seizure rating for each treatment was obtained by calculating the mean of seizure scores recorded at each 30 min observation interval. The timepoint at which individual subjects experienced their initial tonic-clonic seizure with rearing ('level 3 seizure') was taken as a reference point for seizure onset. For each animal, this timepoint was calculated as a percentage of the entire observation period ('mean latency'). This form of data presentation was chosen since in some cases, no level 3 seizures were recorded. Thus, in these cases, a mean latency of 100% was used to denote a failure to reach the level 3 seizure threshold, rather than presenting the data as "180 min" which risked misleading the reader. Mean seizure score and mean latency were both presented as bar charts (see results section, chapter 8). In addition, two other seizure parameters (mortality ratio and seizure ratio) were recorded and presented as tables (see results section, chapter 8).

6. Statistics.

Details of statistical analysis of data are presented in the 'Data analysis' section of individual chapters.

Score	Behaviour
0	Normal behaviour
1	'Limbic' automatisms and facial tremor
2	Head nodding and/or wet dog shakes
3	Limbic forelimb clonic seizures with rearing[†]
4	Status epilepticus (continuous seizing for at least 60 min) and or clonic-tonic seizures
Aditional points	
+2	Death within 30 min following pilocarpine administration.
+1	Death within 31-180 min following pilocarpine administration

† referred to in chapter 8 as "stage 3" seizing or seizures.

Table 6 Scale for behavioural changes observed in rats following i.p. administration of pilocarpine. Reproduced from Janusz and Kleinrok (1989) with modifications.

Chapter Three

Regional effects of sodium valproate on EC levels of 5-HT,

DA and their major metabolites in the rat brain.

Introduction

Research has indicated a possible role for monoamines in the aetiology of certain seizure disorders and epilepsies (see general introduction). Since VPA is a widely used and highly efficacious anticonvulsant, both in the clinic and the laboratory, it seems possible that its mode of action might involve an action on brain monoaminergic systems. There is some evidence for an action of VPA on central serotonergic neurotransmission; Chadwick et al (1975) reported on the use of the 5-HT precursor, 5-hydroxytryptophan in the successful . treatment of post-anoxic myoclonus and noted that CSF 5-HIAA levels normalised concurrently with clinical improvement. In addition, another clinical study reported 5-hydroxyindoleacetic acid (5-HIAA) levels to be increased in the CSF of a patient receiving VPA treatment for this disorder (Fahn, 1978). Lazarova et al (1983) demonstrated a synergistic effect between the 5-HT releasing agent *d*-fenfluramine and VPA against the tonic component of PTZ induced seizures in rats. The doses of both drugs used were found to be ineffective if administered alone. Subsequent investigation has revealed VPA to enhance 5-HT synthesis and turnover (Kempf et al., 1982; Whitton et al., 1983; 1985) and these changes showed some correlation with protection against seizures induced by picrotoxin. MacMillan et al (1987) suggested that increases of 5-HIAA concentrations in the CSF of rats treated with VPA reflected blockade of egress of this metabolite from the brain via inhibition of membrane acid uptake sites, in a similar fashion to probenecid. Horton et al (1977), found that compromising central serotonergic and dopaminergic neurotransmission with the   drugs ;, *p*-CPA and α-methyl-p-tyrosine

respectively, did not alter the anticonvulsant effect of VPA against sound induced seizures in DBA/2 mice. Both the previously cited studies did however acknowledge that VPA treatment in rodents resulted in enhanced CNS accumulation of HVA and 5-HIAA, possibly reflecting increased monoaminergic neuronal activity. In this section, an attempt has been made to discover if the reported changes in tissue monoamine content following VPA treatments are reflected as changes in neurotransmitter release and EC metabolite accumulation, with possible implications for the functional significance of these observations.

In vivo microdialysis was used to monitor changes in EC monoamines from the VH, ACP and PCP of rats acutely treated with VPA. These brain regions were chosen for investigation on the basis of their proposed importance in the genesis and modulation of seizure activity in the epileptic brain (see general introduction, section 4).

Experimental details.

Microdialysis.

Experiments were carried out with animals implanted with probes in VH, ACP and PCP. In all experiments, 11 consecutive 30-min dialysates were collected. Sodium valproate (Reckitt and Colman, Hull, U.K.) at doses of 100, 200, 400 mg/kg was administered i.p. in a volume of 0.1ml saline per 100g body weight after 120 min to rats in the three VH and ACP treatment groups. Animals with probes implanted in the PCP recieved VPA doses of 200, 400 and 800 mg/kg. Control animals received i.p. injections of 0.9% saline at the same time point. A separate group of animals, implanted with probes in the VH received a 30 min infusion of ACSF containing 60 mg/ml VPA, 120 min from the commencement of dialysis. Dialysates were analysed by HPLC-ED soon after collection or following a maximum post-experimental period of 1 week. In the latter case, dialysates were rapidly frozen (-80°C). All data obtained from HPLC were corrected for in vitro recovery of monoamines (see chapter 2 section 2.2).

Estimation of VPA concentration in the extracellular space.

In vitro recovery experiments were performed to estimate the recovery of VPA from bathing medium and hence, assuming that the drug diffused across the dialysis membrane in the reverse direction, the quantity of drug reaching the EC space when infused via microdialysis probes. Microdialysis probes (n=5) were suspended in a bathing medium containing 50 mg/ml VPA. All other experimental details were as before (section 2.2). Dialysates were analysed for VPA content using fluorescence polarization immunoassay (FPIA; Sigma

diagnostic kit, Sigma UK). Fluorescein-labelled VPA and VPA containing dialysate or calibrant samples were incubated with VPA-specific monoclonal antibody. The amount of VPA in either dialysate or calibrant was inversely proportional to light polarization, as measured by an Abbott TDx polarizing fluorimeter. Using data obtained from these experiments, it was possible to estimate the concentration of VPA that would be delivered to the EC space via dialysis probes. This permitted calculation of the amount of drug to be added to ACSF in order to acheive brain EC concentrations of VPA in the range likely to be observed following i.p. administration of the drug.

Data analysis

For each experiment, levels of monoamines in the four dialysates collected prior to saline or VPA treatments were averaged and used as a measure of basal output. Quantities of analytes in each dialysate, pre- and post-treatment were then calculated as a percentage of this value. Statistical comparisons were made between drug treatments and saline controls for each of the collection periods using a Mann-Whitney U test.

Animals

Rats weighing 240-265g were used in this investigation.

Results

Ventral hippocampus (VH).

Basal levels (M x 10^{-10} ,n=24) of DA, DOPAC, 5-HT, 5-HIAA and

HVA were determined as ranges of 47.7-48.3, 530-636, 97-109, 9660-10,520 and

880-1,120 respectively. These ranges were derived from all experiments

performed and were calculated from the 4 pre-treatment samples taken from

each animal. Basal levels of 5-HT and 5-HIAA were consistent with previous

measurements (Hutson et al., 1989; Sharp et al., 1989; Whitton and Fowler

1991). VPA was found to elevate EC levels of DA, DOPAC and 5-HT in a

dose-dependent fashion (figures 10a,b and 11a), whilst HVA and 5-HIAA

concentrations remained unchanged (figures 10c and 11b). As can be seen in

figures 10a and 11a, DA and 5-HT levels were increased only transiently

following VPA injection (approximately 30 min), however, at a VPA dose of

400 mg/kg, DOPAC levels remained elevated for 90 min post-injection (figure

10b). Both the striking effect of VPA upon hippocampal 5-HT level and the the

concomitant lack of change in basal 5-HIAA level are consistent with

observations made by Whitton and Fowler (1991).

Posterior caudate putamen (PCP).

In contrast with those measured in the ventral hippocampus, EC basal level

of DA, DOPAC and HVA were found to be markedly greater in this region,

most notably DA which was present at 9-fold higher concentrations here than

in the hippocampus. Ranges were calculated as described above, from 32

animals as 279-341, 12,370-13,870 and 19,538-21,538 M x 10^{-10}

respectively. Low basal levels of 5-HT were noted in dialysates collected from

66
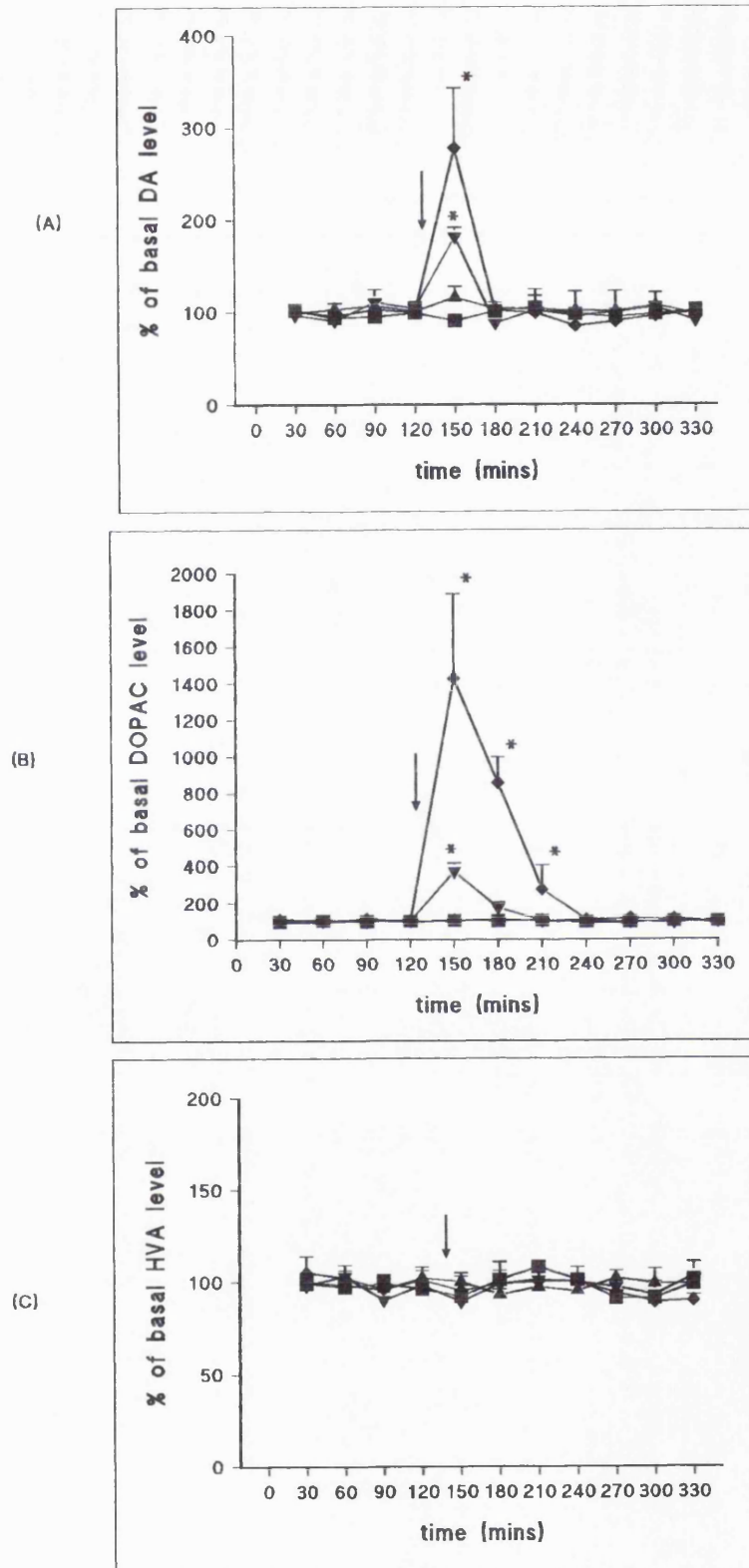
Figure 10 Effect of acute VPA injection on ventral hippocampal dialysate DA (A), DOPAC (B) and HVA (C). Arrow denotes VPA or saline injection. Key:- filled squares saline, triangle 100 mg/kg, inverted triangle 200 mg/kg, diamond 400 mg/kg. All values are mean + s.e.m. of 6 rats per group. * indicates data points significantly different from saline controls in each group (p<0.05).
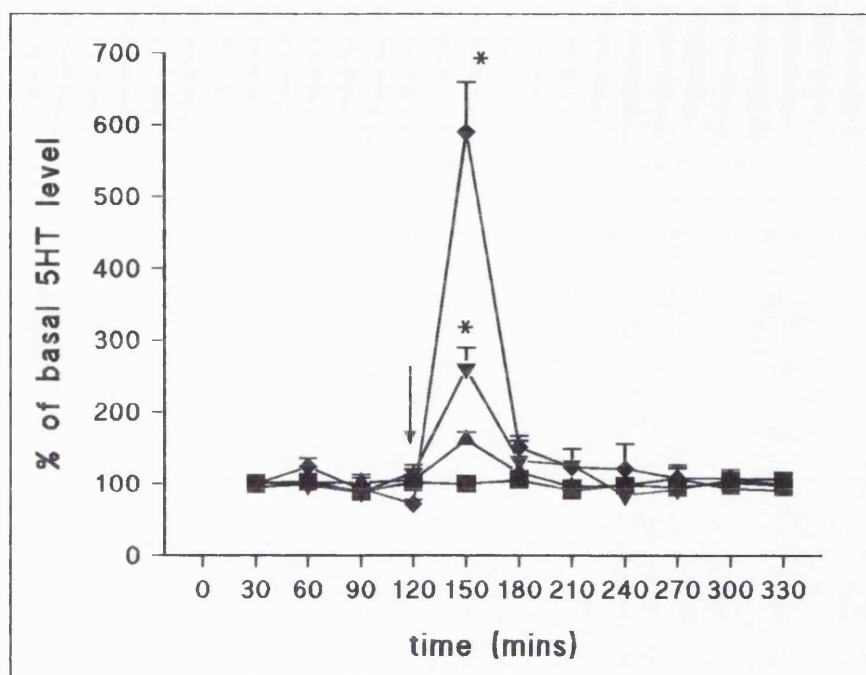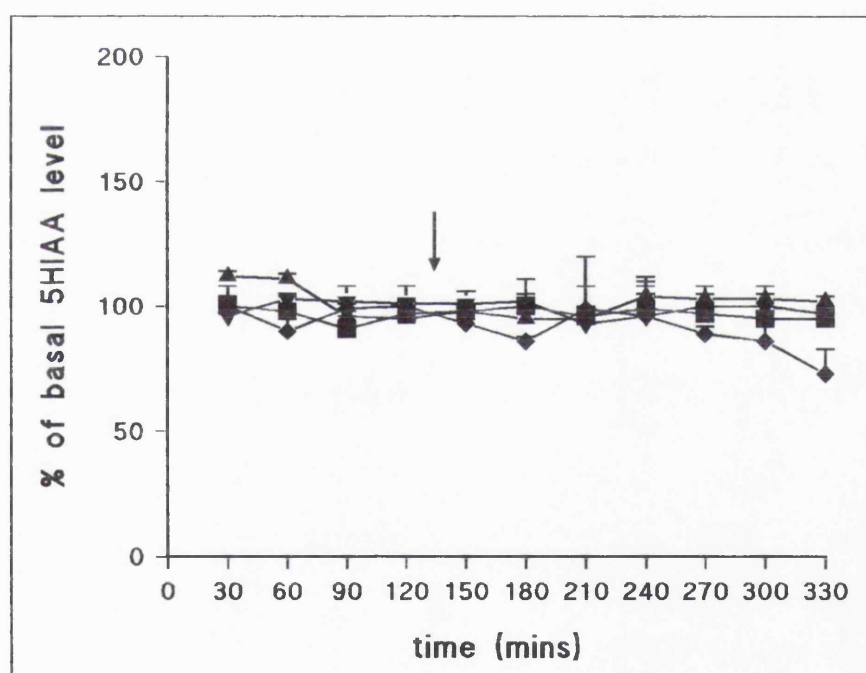
Figure 11 Effect of acute VPA injection on ventral hippocampal dialysate 5-HT (A) and 5-HIAA (B). Arrow denotes VPA or saline injection. Key:- filled squares saline, triangle 100 mg/kg, inverted triangle 200 mg/kg, diamond 400 mg/kg. All values are mean + s.e.m. of 6 rats per group. * indicates data points significantly different from saline controls in each group (p<0.05).
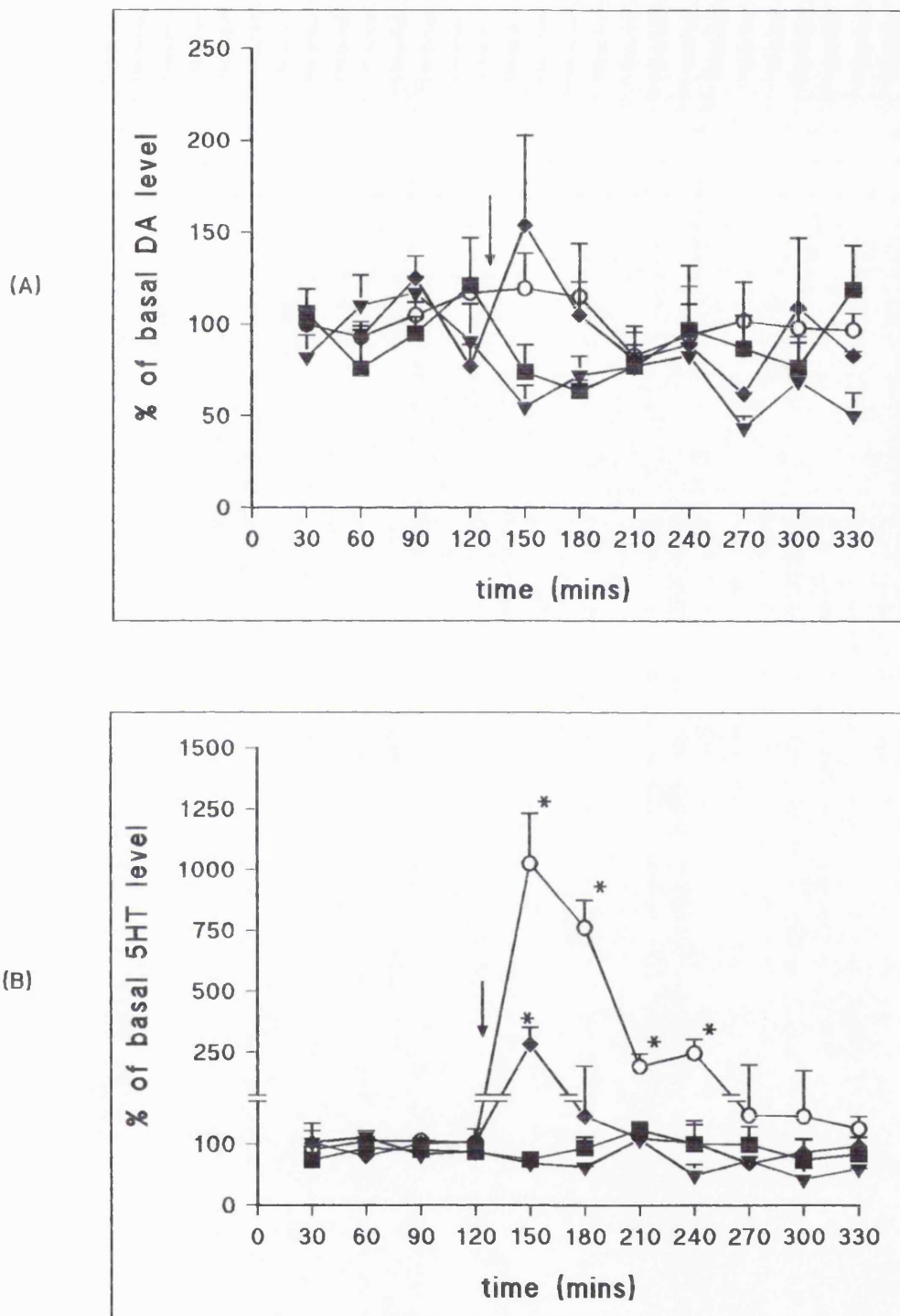
Figure 12 Effect of acute VPA injection on posterior caudate putamen dialysate DA (A) and 5-HT (B). Arrow denotes VPA or saline injection. Key:- filled squares saline, inverted triangle 200 mg/kg, diamond 400 mg/kg, open circle 800 mg/kg. All values are mean + s.e.m. of 8 rats per group. * indicates data points significantly different from saline controls in each group (p<0.05).

posterior caudate putamen implanted rats (range 29-45   M x 10^{-10}),

although its metabolite 5-HIAA was present at a relatively high concentration

(range 21,660-22,760   M x 10^{-10}). These measurements are

comparable with those determined in other laboratories (Westerink and De

Vries, 1988;1989; Kalen et al., 1988).

Even at VPA doses as high as 800 mg/kg, dialysate levels of DA were not

greatly elevated above basal concentrations and no significant differences

existed between drug treated animals and saline controls (figure 12a).

Similarly, levels of DOPAC, HVA and 5-HIAA were all unchanged with

respect to those found in saline treated animals (data not shown). VPA did

however induce a significant increase in the concentration of 5-HT in the PCP

(figure 12b). Large doses of the drug were required to elicit changes in

dialysate 5-HT and at the highest dose of 800 mg/kg, 5-HT levels in the first

post injection sample (t=150 min) were approximately four-fold higher than

that after injection of 400 mg/kg VPA.

Anterior caudate putamen (ACP).

Basal EC levels of DA, DOPAC and HVA (ranges 311-368, 11,420-13,320 and

23,480-25,080   M x 10^{-10} respectively, n=24) were comparable with

those determined in the PCP. 5-HT and 5-HIAA concentrations were found to

be in the ranges 41-51 and 24,080-27,480   M x 10^{-10} respectively.

Again, all measurements were consistent with those determined in the

previously cited studies. Levels of 5-HT and DA in samples taken following

injection of VPA were increased above baseline, reaching maxima 30 and 60

min post-injection respectively (figures 13a and b). Most notably, dialysate DA
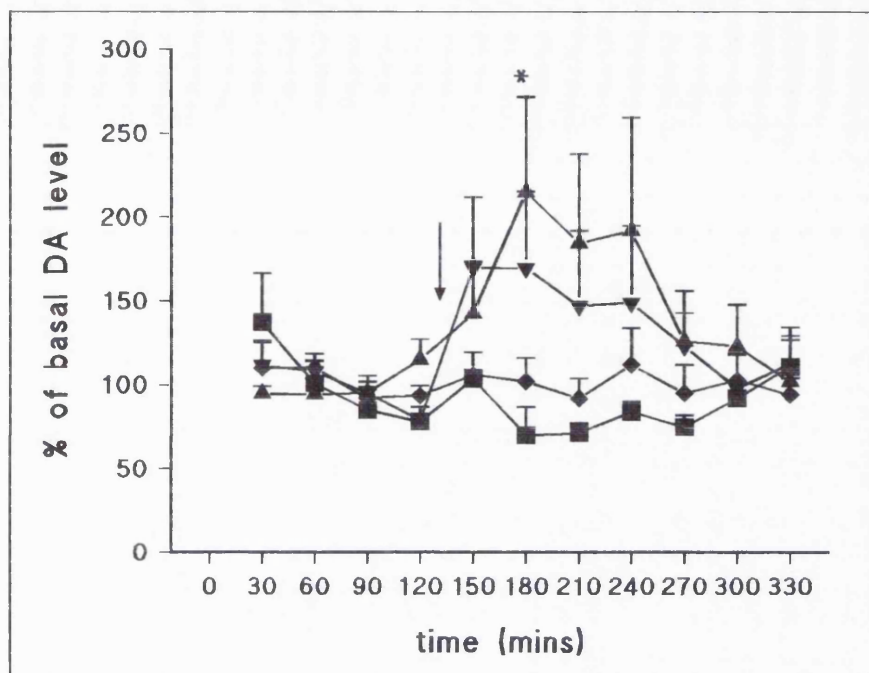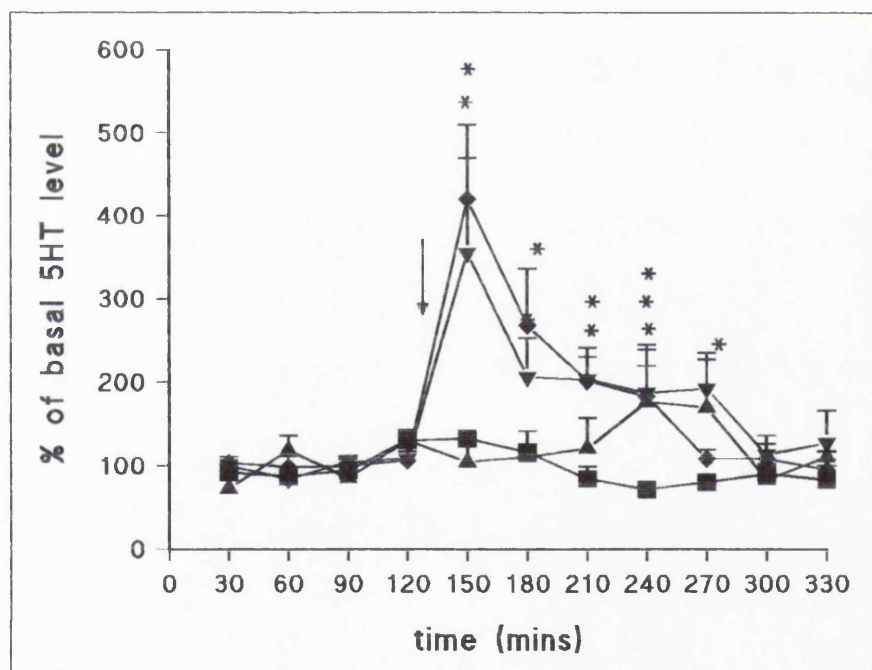
Figure 13 Effect of acute VPA injection on anterior caudate putamen dialysate DA (A) and 5-HT (B). Arrow denotes VPA or saline injection. Key:- filled squares saline, triangle 100 mg/kg, inverted triangle 200 mg/kg, diamond 400 mg/kg. All values are mean + s.e.m. of 6 rats per group. * indicates data points significantly different from saline controls in each group (p<0.05).

levels appeared to be inversely correlated with the dose of VPA administered, with the 100 mg/kg dose inducing the largest post-injection increase. Conversely, 5-HT levels post-injection were found to be dose dependently elevated by the drug. Levels of DOPAC, HVA and 5-HIAA all remained unaltered by VPA treatments (data not shown).

VPA infusion experiments.

The results obtained from <u>in vitro</u> recovery experiments indicated that the fraction of VPA likely to diffuse from a microdialysis probe (at a flow rate of 0.5μl/min) into the interstitial space surrounding the dialysis membrane was in the range 26.0-36.5 %. This figure was used to calculate the concentration of VPA in ACSF to be given as a 30 min infusion via microdialysis probes. Brain concentrations of VPA only reach 10-20 % of those in plasma following acute i.p. or oral dosing (Aly and Abel-Latif 1980), possibly due to the extensive plasma protein binding of the drug in this species (Löscher 1978). Given these data, it was felt that a concentration of 60 mg/ml VPA applied to the brain in ACSF as a 30 min infusion would achieve approximately the same local tissue concentration as that observed following an i.p. dose of 200-300 mg/kg.

As can be seen from figures 14a and b, VPA applied as an infusion via microdialysis probes resulted in consistent increases in hippocampal EC DA and 5-HT. The effects of VPA on monoamine output under these conditions appeared to be more gradual in onset, with the peak increases in dialysate DA and 5-HT achieved 60 min following the commencement of infusion. Additionally, the responses to the drug were longer lasting than those seen

72
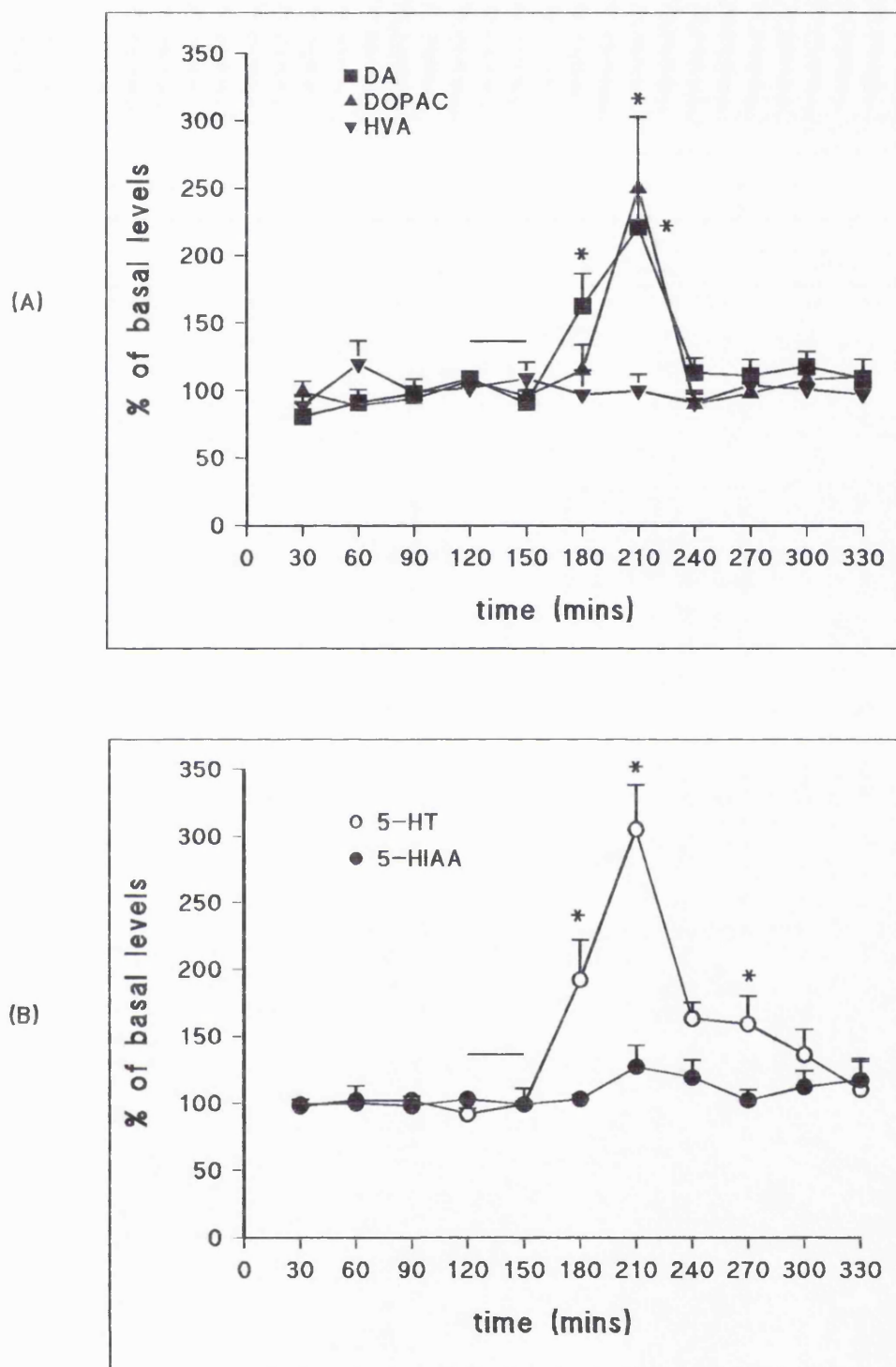
Figure 14 Effect of VPA (60 mg/ml) infused into the ventral hippocampus via microdialysis probes. DA and its major metabolites (A), 5-HT and 5-HIAA (B). Bar denotes duration of VPA infusion. All values are mean + s.e.m. of 6 rats per group. * indicates data points significantly different from the pre-infusion basal level of each monoamine. (p<0.05).

following i.p. injection, suggesting that washout of VPA from the brain is prolonged if applied via microdialysis probes. As with i.p. dosing, the present treatment resulted in a substantial increase in EC DOPAC, concomitant with the increase in DA output (figure 14a). EC HVA and 5-HIAA levels were not significantly increased by this treatment.

Discussion

The results presented in this chapter suggest that VPA treatments have multiple effects upon dialysate levels of DA and 5-HT. In the ventral hippocampus, elevation of both DA and 5-HT levels occur only transiently following injection of VPA, whilst in the anterior caudate putamen, a more sustained increase above basal level of 5-HT was observed. Anterior caudate putamen dialysate DA levels were found to be inversely related to VPA dose. However, in the posterior caudate putamen, VPA was essentially devoid of effect upon EC levels of DA and induced major elevations of 5-HT concentration only at the higher doses of 400 and 800 mg/kg.

Rats were observed to display intense "wet dog" shake behaviour (de Boer, 1977) commencing 1-2 min following VPA injection. VPA-induced "wet dog" shakes appear to be 5-HT dependent, since pretreatment of rats with L-tryptophan exacerbates this condition, whilst depletion of central 5-HT with p-CPA ameliorates it (Fletcher and Harding, 1981). These data serve to corroborate the present observation of a rapid VPA-induced increase in EC 5-HT by providing a behavioural correlate. Moreover, pharmacokinetic data have shown both CNS and plasma concentrations of single i.p. doses of VPA to be maximal within 15 min of administration (Nau and Löscher, 1982). These rapid changes in EC monoamine levels were unlikely to be secondary to stress caused by injection of the drug since both dialysate DA and 5HT are significantly elevated following infusion of 60 mg\kg VPA via dialysis probes placed in the ventral hippocampus (figure 14). Hwang and Van Woert (1979) found that VPA potentiated the anti-myoclonic activity of chlorimipramine in

a rat model of myoclonic epilepsy induced by the insecticide, 1,1,1-trichloro-

2,2-bis (p-chlorophenyl) ethane (p,p'-DDT). They attributed this to the ability

of VPA to displace tryptophan from its plasma protein binding sites, hence

increasing the fraction of free amino acid available for transport into the CNS,

possibly resulting in enhanced 5-HT synthesis (Gal et al, 1978). The present

data challenge this view, since the direct delivery of VPA into the hippocampal

EC microenviroment is likely to circumvent any effect of the drug on

displacement of plasma tryptophan, whilst clearly resulting in increased 5-HT

output in this region. Moreover, VPA appears to enhance synthesis of this

neurotransmitter, since Whitton et al (1985) found whole brain 5-HT levels to

be increased by the drug in pargyline treated rats.

As is evident from figure 11, VH dialysate levels of 5-HIAA were unaffected

by VPA at any of the doses used. This is consistent with the findings of a

previous microdialysis study (Whitton and Fowler, 1991) and also applies to

both striatal areas examined in the present study. The reasons for the lack of

VPA induced elevation of EC 5-HIAA concentrations both in this and the

previously cited study are unclear. These observations are particularly

interesting in the light of evidence suggesting that the drug increases 5-HIAA

levels in whole brain (Whitton et al., 1985) and CSF (MacMillan et al., 1987).

The inclusion of citalopram in the dialysate might have been a factor, but

subsequent investigation in the absence of the reuptake inhibitor failed to

demonstrate any change in 5-HIAA accumulation (data not shown). Since the

EC 5-HIAA/5-HT was found to be very high (approximately 98 to 1 in

hippocampal dialysates), it is possible that a relatively modest increase in 5-HT

output would not significantly contribute to the total 5-HIAA pool, following deamination. This phenomenon would be further compounded by the blockade of 5-HT reuptake.

Ventral hippocampal DOPAC levels were greatly increased following injection of VPA, although HVA levels were unchanged. It is not clear why VPA should preferentially increase DOPAC concentration in the ventral hippocampus, but the substantially lower basal levels of DOPAC determined in dialysates from this region compared to those found in striatal dialysates could be significant. Thus, it could be that a relatively large drug-induced increase in EC DA concentration might be reflected in a substantial and concomitant change in DOPAC level if the basal level of that metabolite were relatively low (ie., a low DA/DOPAC ratio). An alternative view is that VPA might exert a *regionally specific* action on synthesis of DA. Previous reports would suggest that the EC pool of DOPAC, as estimated by microdialysis, may be largely derived from newly synthesised DA and not from the deamination of re-uptaken DA (Zetterström et al, 1988; Fairbrother et al, 1990; Soares-da Silva and Garrett, 1990). A substantial proportion (40%) of hippocampal DA is thought to be derived from noradrenergic terminals (Scatton et al, 1980) and both α_2-adrenergic and D_2-dopaminergic agonists reduce DOPAC output from hippocampal monoaminergic terminals as measured by microdialysis (Vahabzadeh and Fillenz, 1991). It therefore follows that the substantial increases in hippocampal dialysate DA noted in the present study could have been brought about by an enhancement of synthesis of the monoamine in hippocampal dopaminergic and noradrenergic terminals. Indeed, there is some

evidence suggesting that turnover of central NA is increased following acute

VPA treatment (Whitton et al, 1984). Increased accumulation of DA in

catecholaminergic terminals might result in an increased intraneuronal

conversion of the parent amine to the DOPAC. From the present data, since

VPA also appears to enhance output of parent monoamines in the VH, it

seems likely that the drug affects neurotransmitter release processes in

addition to a possible action on transmitter synthesis. In chapter 6, an attempt

was made to determine whether the effects of VPA on hippocampal dialysate

levels of DA and 5-HT were dependent upon ongoing action potential traffic

and the Na^+-sensitive component of neurotransmitter release.

Measurements made during the present study revealed basal DOPAC levels

to be approximately 40-fold higher than that of DA in the striatum. Thus it is

possible that the relatively small VPA-induced increases in DA noted here

would not significantly contribute to the overall level of DOPAC present in the

EC space. Zetterström et al (1985), using intrastriatal microdialysis, showed

that administration of neuroleptics (previously shown to increase striatal

dopamine synthesis; Westerink and Korf, 1976) increased dialysate DOPAC

concentrations. In the present study, where anterior caudate putamen dialysate

concentrations of DA were increased modestly following VPA treatments

although not accompanied by an increased output of DOPAC, it would seem

unlikely that VPA increases turnover and synthesis of the monoamine in this

area. Overall, some evidence would suggest that VPA does not increase

whole brain DA synthesis (Whitton et al, unpublished data), unless given at

very high doses (>600 mg/kg) with resultant severe motor toxicity (Horton et

al, 1977).

With regard to the present study, the effects of VPA upon levels of DA in anterior caudate putamen dialysates were notable. Here, with increasing dose of VPA, a clear trend towards a decrease in dialysate DA was observed (figure 13a). The converse applied to levels of 5-HT in the equivalent dialysates, although the overall magnitude of change was greater (figure 13b). At present, the neuropharmacological basis of these observations is unclear, although it seems possible that the two monoamines might modulate each others release in the anterior striatum, dependent, perhaps, upon the dose of VPA administered. Some in vitro data supporting these assertions exist. 5-HT has been shown to dose-dependently decrease [^{3}H]DA release from superfused rat striatal slices (Westfall and Tittermary, 1982) and more recently, this phenomenon was shown to be blocked by the 5-HT$_2$ antagonist ketanserin (Muramatsu et al .,1988).

Regionally specific effects of VPA upon tissue levels of 5-HT and 5-HIAA have been reported (Shukla, 1985; Hwang and Van Woert, 1979) and the present data would suggest that VPA produces qualitatively different neurochemical profiles in discrete rat brain regions. A study of regional rat brain levels of VPA and its major metabolite 2-en-VPA following acute and chronic dosing with the parent anticonvulsant revealed tissue concentrations of VPA to be 22% lower in the striatum than in the hippocampus (Löscher and Nau, 1983). Thus, VPA would appear to be more selectively accumulated in the hippocampus than in the striatum, which could contribute to the different regional neurochemical changes observed following microdialysis.

Turski et al (1988) located the site of apomorphine-mediated protection against pilocarpine induced seizures to the ACP in rats. Dopamine D_2 receptors, which are thought to mediate the anticonvulsant effects of dopaminergic agonists, are most densely situated in this area (Richfield et al., 1987). It is perhaps of significance that in the present study, DA was found to be preferentially (although modestly) increased in dialysates collected from this area of the striatum, thus correlating with a high density of dopamine-sensitive anticonvulsant sites.

Summary

1. VPA was found to dose-dependently increase dialysate 5-HT in both the VH and the ACP. DA levels were dose-dependently elevated by VPA in hippocampal dialysates, whilst in the anterior striatum, with increasing dose of VPA a clear trend towards a decrease in EC DA was observed. VPA produced little effect upon equivalent PCP dialysates, with the exception that 5-HT output appeared to be greatly increased at the 400 and 800 mg/kg doses.

2. VH dialysate levels of DOPAC were substantially elevated in the presence of VPA, this possibly reflecting an enhancement of intraneuronal turnover in DA.

3. Other metabolite measurements failed to disclose similar effects. The inclusion of citalopram in the ACSF might in part account for a lack of effect on EC 5-HIAA level.

4. These differing effects on regional monoamine output and possibly, metabolism are typical of the diverse and numerous neurochemical data accumulated regarding VPA. The present findings extend the possibility that VPA may produce a component of its anticonvulsant action by interacting with brain monoaminergic systems.

Chapter Four

Effects of VPA on EC levels of GABA and other

amino acids in the ventral hippocampus.

Introduction

As reviewed in the general introduction, it is well documented that VPA interacts with central GABAergic mechanisms. Acute treatment of animals with VPA is known to increase GABA concentrations in whole brain (Godin et al, 1969) and discrete brain regions (Iadorola and Gale, 1979; Philips and Fowler, 1982; Löscher, 1989). In addition, the drug enhances accumulation of GABA in the CNS of experimental animals and in the CSF of patients receiving the drug for the long term management of epilepsy (Löscher and Siemes, 1985). It was previously thought that relatively low doses of the anticonvulsant stimulated no net increase in whole tissue GABA, whilst still exerting an anticonvulsant effect (Morre et al, 1984). Closer scrutiny of the effects of VPA on GABA accumulation in subcellular fractions have revealed that doses as low as 125 mg/kg can increase GABA concentrations in the synaptosomal compartment (Löscher, 1981) Significantly, GABA turnover in the neuronal compartment seems to be preferentially stimulated by VPA (Iadorola and Gale, 1981). Given these data, it would seem reasonable to propose that VPA might exert a proportion of its anticonvulsant action by increasing central synaptic output of GABA. A recent in vitro study suggested that VPA increased K^+-stimulated release of endogenous and $[^3H]$-GABA from cultured cortical neurones (Gram et al, 1988) although Farrant and Webster (1989) failed to observe any change in spontaneous nigral GABA release by VPA using push-pull cannulae. There is, however, evidence that VPA enhances IPSP's recorded from hippocampal neurones in vitro (Preisendorfer et al, 1987). Due to the proposed importance of the hippocampus as a structure involved

in seizure generation (Bradford and Peterson, 1987), it was decided that this area should be investigated with <u>in vivo</u> microdialysis to establish if acute VPA treatments resulted in alterations of EC GABA concentration. In addition, other amino acids important in regulating neuronal excitability (ASP and GLU), involved in GABA synthesis (GLN) and putative neuromodulation (TAU) were also monitored for changes in their respective EC concentrations following VPA treatments.

Experimental details

Methodologically, this series of experiments were carried out in a similar fashion to those for collection of VH dialysates described in chapter 3. The protocol differed only in that 12 consecutive dialysates were collected from each animal, since a preliminary experiment had shown the action of VPA on dialysate GABA levels to be more protracted than effects on dialysate monoamines. Dialysates were analysed for ASP, GLU, GLN, TAU and GABA content using HPLC-FD. Data obtained from chromatograms were corrected for in vitro recovery of amino acids.

Data analysis

Data were calculated as a percentage of basal amino acid levels, on the same basis as the monoamine data presented in chapter 3. Data were analysed for statistical differences between drug treatments and saline controls at each time point using a Mann-Whitney U test.

Animals

Rats weighing 220-300g were used in this investigation.

Results

Basal amino acid levels in dialysates.

Basal levels of amino acids determined in VH dialysates ($M \times 10^{-7}$ l

n=32) were; 2.65-3.35 (GABA), 29.7-37.1 (GLU), 99.3-104.3 (TAU), 2.5-

3.7 (ASP) and 38.1-48.5 (GLN) These values are in close accordance with those

recorded in other laboratories (Lehmann et al, 1983; Tossman et al, 1987).

GABA

VPA treatments altered basal GABA levels in a complex fashion (figure 15).

At the highest dose of 400 mg\kg, peak dialysate GABA concentrations (200%

of basal level) were reached approximately 60 min following i.p. dosing. This

dose of VPA has been demonstrated to be active in chemiconvulsant seizure

paradigms, but only weakly active against maximal electroconvulsions (Frey

and Löscher, 1976). The lower dose of 200 mg/kg did not significantly alter

dialysate GABA, whilst the lowest dose administered significantly *decreased*

dialysate GABA, an effect which persisted 90 min post injection.

Other amino acids

VPA treatments (at any dose tested) failed to significantly alter basal levels of

hippocampal dialysate ASP, GLU (figure 16) and GLN, TAU (figure 17).
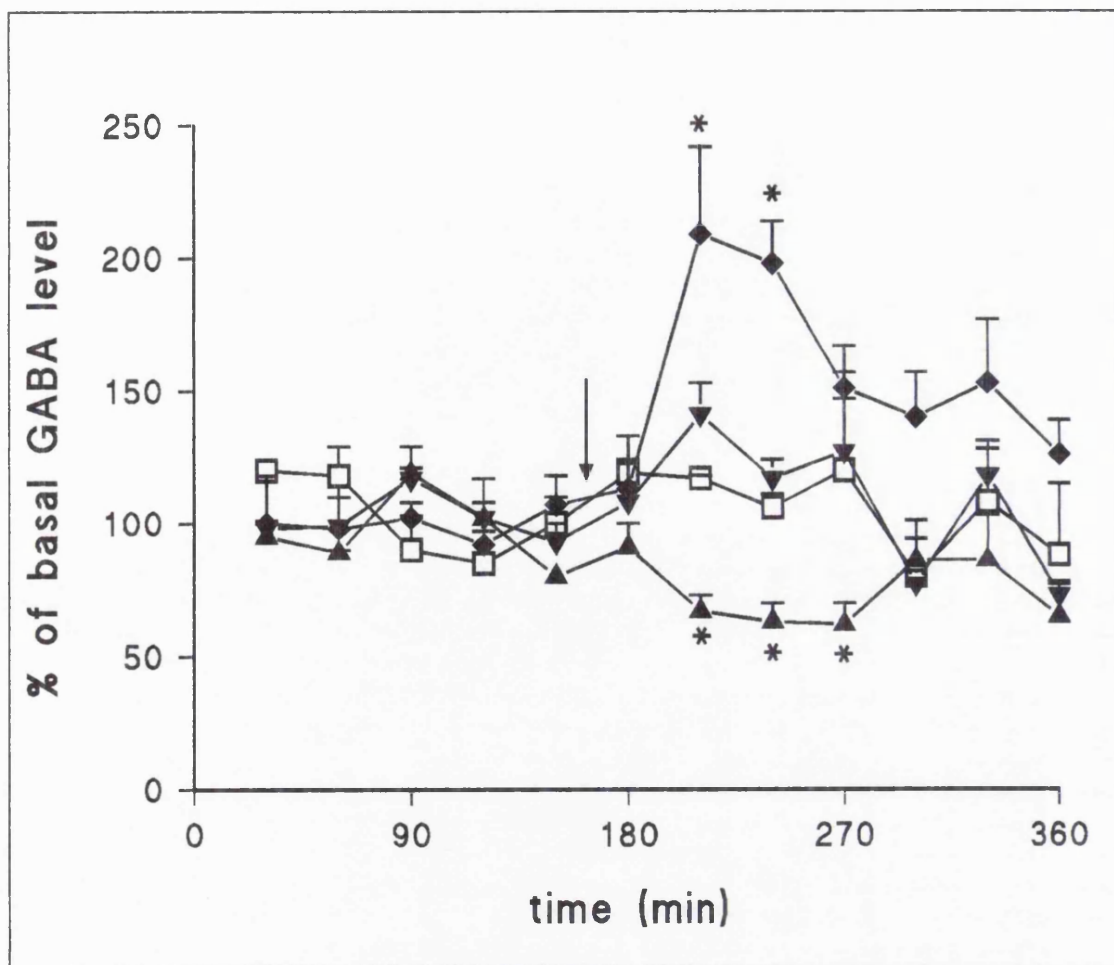
Figure 15 Effect of VPA treatments on hippocampal dialysate levels of GABA. Arrow denotes VPA or saline injection. Key:- open square saline, triangle 100 mg/kg VPA, inverted triangle 200 mg/kg VPA, diamond 400 mg/kg VPA. All values are mean + s.e.m., n=8 rats per treatment group. * indicates data points significantly different from saline controls in each group (p<0.05).
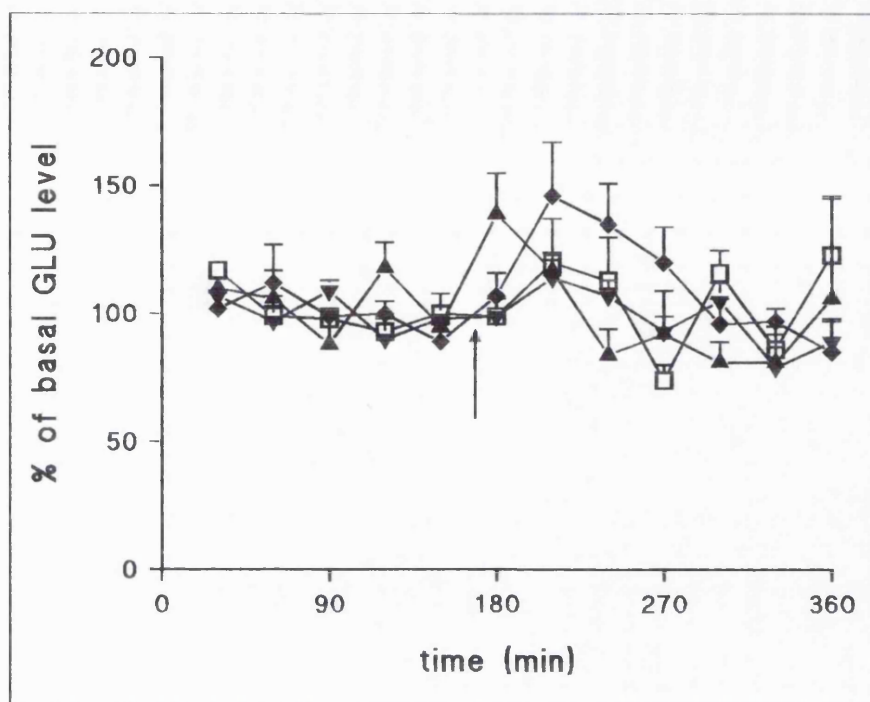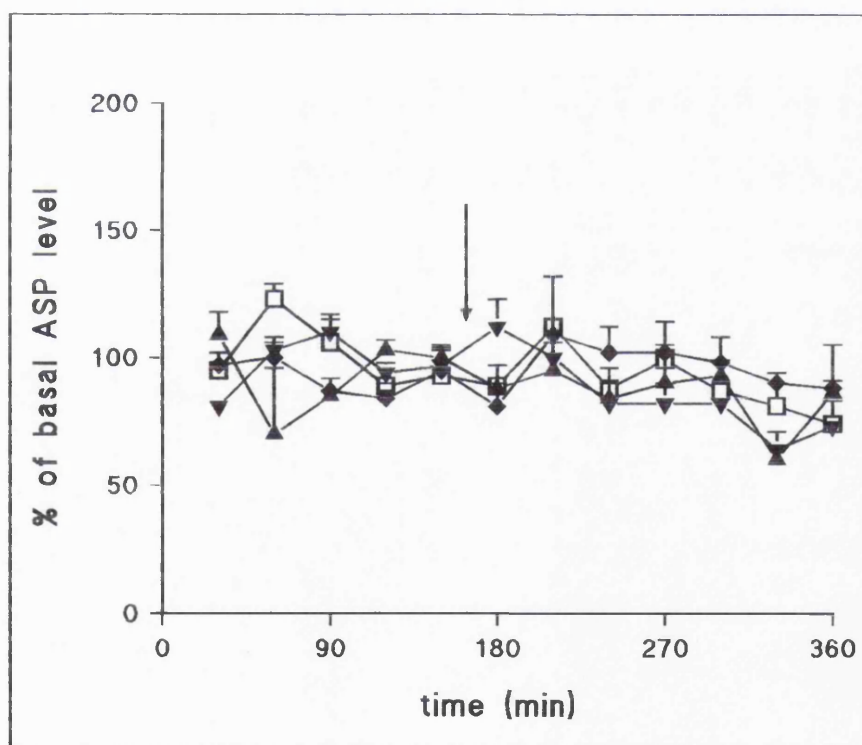
Figure 16 Effect of VPA treatments on hippocampal dialysate levels of (A) GLU and (B) ASP. Arrow denotes VPA or saline injection. Key:- open square saline, triangle 100 mg/kg VPA, inverted triangle 200 mg/kg VPA, diamond 400 mg/kg VPA. All values are mean + s.e.m., n=8 rats per treatment group.
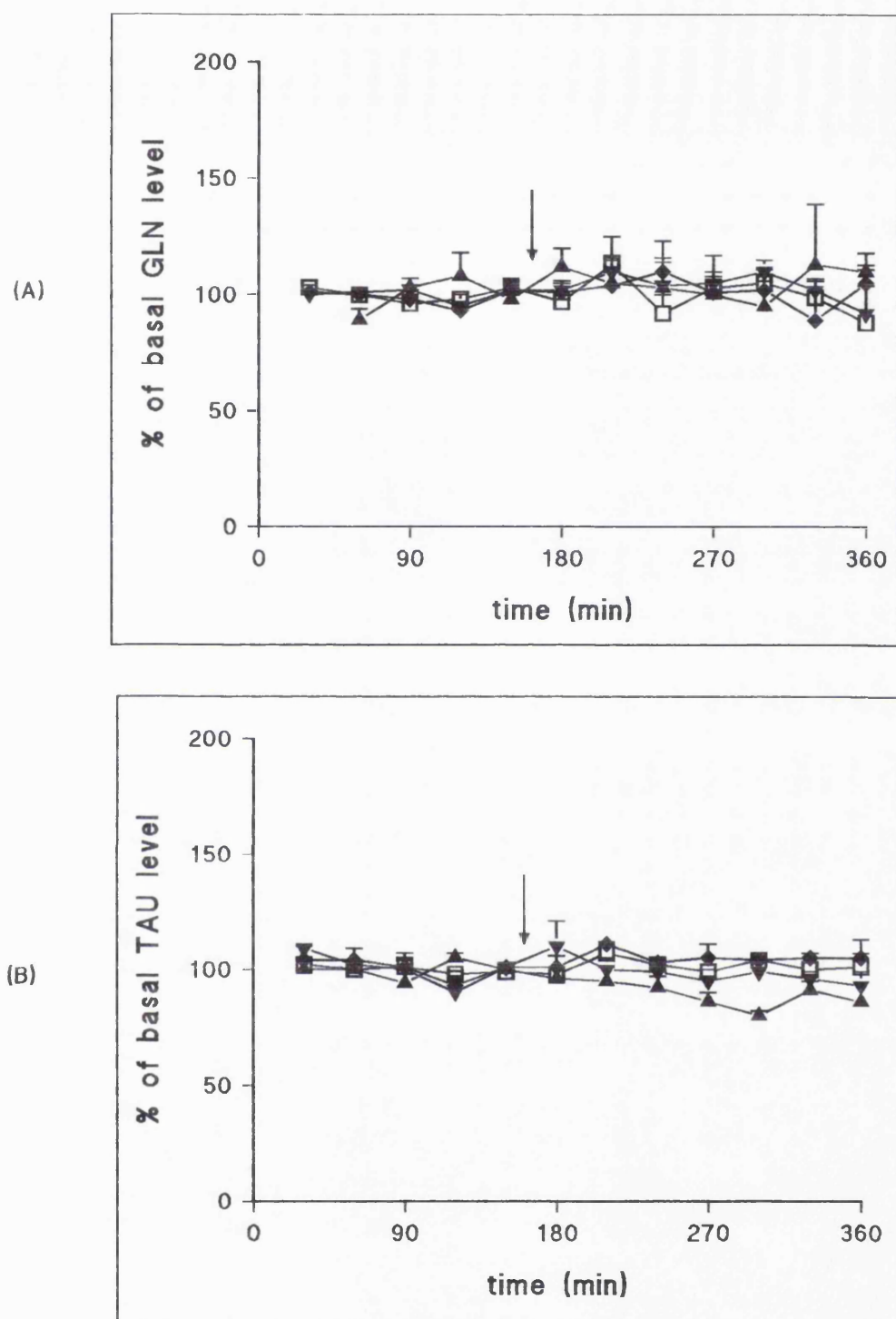
Figure 17 Effect of VPA treatments on hippocampal dialysate levels of (A) GLN and (B) TAU. Arrow denotes VPA or saline injection. Key:- open square saline, triangle 100 mg/kg VPA, inverted triangle 200 mg/kg VPA, diamond 400 mg/kg VPA. All values are mean + s.e.m., n=8 rats per treatment group.

Discussion

Whole brain and cortical studies have consistently shown GABA concentrations and GAD activity to be maximally elevated 30 min following acute i.p. dosing of VPA in rats (Iadorola et al, 1979; Philips and Fowler, 1982) and mice (Nau and Löscher, 1982). Regarding the hippocampus, VPA has been reported to elevate whole tissue GABA concentrations, but only following a treatment period of ten days (Patsalos and Lascelles, 1981). More recently it has been demonstrated that although VPA (200 mg/kg) may not significantly increase whole tissue hippocampal GABA levels acutely, GABA accumulation in the synaptosomal compartment is enhanced by approximately 80% and shows some correlation with seizure protection (Löscher and Vetter, 1985). The authors found that these effects were rapidly evident following i.p. injection of VPA, an increase in synaptosomal GABA occuring within 5-15 min. These data would suggest that acute VPA dosing selectively enhances GABA accumulation in a releasable pool, probably associated with the GABA-synthetic apparatus (Abe and Matsuda, 1983). Indeed, in vitro evidence suggesting that VPA may, in part, produce its anticonvulsant effects via enhancement of cerebral GABA synthesis was recently provided by Silverman et al (1991). These workers found the drug to increase the activity of purified porcine brain GAD at concentrations likely to occur in rodent brain and that would confer seizure protection. In the present study, 200 mg/kg VPA did not increase EC GABA concentrations significantly. This suggests that although this dose of drug subtantially increases synaptosomal GABA levels, it fails to elevate levels of the neurotransmitter in the hippocampus. The possibility

remains that increased EC levels of GABA may result in other brain regions following treatment with 200 mg/kg. Indeed, Löscher and Vetter (1985) found that VPA, at this dose, significantly increased synaptosomal GABA concentrations in striatum, frontal cortex, hypothalamus and tectum. Unfortunately, time constraints prevented a regional study of the effects of VPA on EC amino acids being incorporated into the present findings. Only at a higher dose (400 mg/kg) was an increase in hippocampal EC GABA level noted. No data currently exists regarding the effects of 400 mg/kg VPA on synaptosomal GABA concentration, so it is matter of conjecture to assume that this higher dose of drug should result in GABA overflow from nerve terminals due to enhancement of presynaptic GABA synthesis and accumulation. The data do however accord with the findings of Gram et al (1988), who measured increased release of GABA from cultured cortical neurones in response to VPA. It is of course, possible that another mechanism, not directly linked to biosynthetic or neurotransmitter release processes could account for these observations; for example, some evidence exists suggesting that VPA treatment can lower uptake affinity for [^{3}H]-GABA into cultured astrocytes (Nilsson et al, 1992). It could therefore be argued that VPA might elevate hippocampal dialysate levels of GABA partly by interacting with glial cells in the region of the dialysis membrane.

VPA (100 mg/kg) produced a sustained decrease in hippocampal EC GABA in the present study. It is currently uncertain as to the mechanisms responsible for the reduction in EC GABA concentration observed in the hippocampus following this treatment. However, Wolf et al (1988) have also reported a

significant reduction in EC GABA following local application of 40-200 µg/ml

VPA into the rat preoptic area via push-pull cannullae. The authors attributed

this effect to a feedback inhibition of GABA release, following an enhancement

of post-synaptic GABAergic function by VPA. Little evidence exists to support

this theory however, and most studies have failed to demonstrate an

interaction of VPA with post-synaptic GABAergic elements, such as $GABA_A$

receptors (Löscher 1980), benzodiazepine (Olsen 1981) or chloride ionophore

(Squires et al, 1983) binding sites. The effects noted by Wolf et al (1988) were

elicited by doses of VPA said to be within and above the therapeutic plasma

concentration range encountered in the clinic (equivelent to 40-100 µg\ml in

plasma). This led the authors to conclude that the relatively high doses of the

drug used in other rodent studies (200-600 mg\kg) resulted in data which

were not neurochemically relevant with regard to human therapeutics.

Although improvements are seen in epileptic patients receiving lower doses

(up to 45 mg\kg orally) of VPA, rodents are considerably less sensitive to this

agent (Chapman et al, 1982). In general, it should be noted that rodents

eliminate VPA approximately 10 times more rapidly than humans (Löscher

1993) and therefore require considerably larger doses of drug in order to

acheive anticonvulsant concentrations in the CNS. The reasons for this are not

entirely clear, but differences in dosing regimens, different plasma protein

binding profiles of VPA between man and rodents could contribute to the

apparent discrepancies between clinical and experimental data (Löscher 1978;

Meinardi et al, 1974).

The lack of effect of VPA on EC levels of the other four amino acids

measured in this study was to some extent unexpected when viewed against existing whole tissue ex vivo data. For example, ASP levels have been found to be significantly reduced in whole brain (Schechter et al, 1978) and hippocampus (Chapman et al, 1982) following acute i.p. injections of 400 mg\kg VPA. Schecter and co-workers (1978) found this decrease to correlate closely with both increased whole brain GABA and protection against audiogenic seizures in DBA\2 mice. Other workers have provided corroborative data, demonstrating a reduction of ASP release from rodent brain slices by VPA, applied to the preparation at concentrations active against seizure precipitants (IC_{50}=100µM; Crowder and Bradford, 1987). Chapman et al (1982) proposed that VPA might have an effect on ASP synthesis, possibly interacting with enzymes in the aspartate-oxaloacetate shuttle, possibly aspartate aminotransferase. VPA has been shown to substantially increase flux of substrates through the tricarboxylic acid cycle. [^{14}C]-Glucose incorporation into GABA was found to be increased by 90% 5 min following intraventricular injection of the label in VPA treated rats (Taberner et al, 1980). Thus it is possible that the VPA-stimulated increase in requirement of TCA substrates for conversion to GABA, via the GABA shunt pathway, could decrease the availability of substrates required for ASP synthesis. One study, (Patsolos and Lascelles, 1981) found hippocampal ASP to be markedly elevated following repeat i.p. doses of 300 mg\kg VPA, although a similar study found no changes in tissue ASP levels following 7-day treatment of rats with equivalent oral dosing (Perry and Hansen, 1978). In dialysates, rat hippocampal EC levels of ASP were found to be unaffected at all three doses of VPA tested (figure

16). Given that most of the available data would suggest that acute VPA treatments reduce tissue aspartate levels and release from <u>ex vivo</u> tissues, it could be argued that the lack of change in EC ASP noted here reflects factors unique to the <u>in vivo</u> nature of study: Specifically, since VPA has been shown to reduce [^{3}H] L-ASP high affinity uptake by cultured astrocytes (Nilsson et al, 1992), the reduction in tissue ASP could be offset by an effective increase in EC accumulation of the amino acid if VPA were to be active in this manner <u>in vivo</u>. Thus, in theory, one might expect to observe no net change in EC ASP if reduced tissue aspartate were to be reflected in reduced output of the amino acid.

Hippocampal dialysate GLU and GLN concentrations were found to be largely unaltered by all doses of VPA administered to rats in the present study. However, EC GLU was elevated modestly following 400 mg\kg VPA, a result which was not statistically significant (figure 16). Interestingly, this effect followed a similar temporal profile to that seen for dialysate GABA at the same VPA dose. This contrasts with findings demonstrating either a 10% decrease in tissue hippocampal GLU (Chapman et al, 1982) or no effect on whole brain GLU following dosing with VPA (Godin et al, 1969; Cremer et al, 1978). Levels of GLN have been shown to be increased in mouse brain following acute VPA treatment (Kapetanovic et al, 1988) and also in the CSF of children following prolonged treatment with the drug (Yaeken et al, 1987). These data are interesting when it is considered that GLN derived from glial cells (Norenberg and Martinez-Hernandez, 1979) may be an important determinant of EC GABA concentration in the rat brain (Paulsen and Fonnum,

1989). Since VPA may increase glial cell glutamine synthetase activity (Phelan et al, 1985;Nilsson 1992) and an elevated concentration of the amino acid is noted in CSF, this may stimulate an increase in GABA turnover and subsequent release, although possibly only following prolonged treatment with the drug.

VPA failed to alter dialysate TAU in the hippocampus (figure 17). This accords with the work of Miyazaki et al (1988) which indicated that acute administration of VPA induced no changes in whole brain TAU levels. However, it has been shown that VPA elevates TAU levels in most brain regions (including the hippocampus) when given chronically (Patsalos and Lacelles, 1981). Since it is thought that TAU may function as a modulator of the $GABA_A$-receptor complex (De Robertis, 1986; Horikoshi et al, 1988) it would be interesting to know whether chronic VPA dosing elevates EC TAU, thereby providing a possible functional correlate for the ex vivo findings.

Summary

1. Acute doses of VPA alter EC GABA concentrations in the ventral hippocampus and within a dose range that confers protection in rodent seizure paradigms.

2. At present it is not possible to explain the neurochemical basis of these findings, although whole tissue and synaptosomal studies suggest that VPA increases synthesis of GABA. No discrimination between the relative contributions of neuronal and non-neuronal cell types to the observed effects on EC GABA could be made at this stage, given that the uptake of GABA into glial cells may be altered by VPA.

3. Present data suggest that the effects of acute VPA doses on EC hippocampal amino acids may be confined to the GABAergic system, despite conflicting ex vivo data.

Chapter Five

Rat ventral hippocampal amino acid and monoamine levels

monitored in dialysates during a sub-chronic treatment period

with sodium valproate.

Introduction

Despite an extensive literature describing the effects of acute VPA treatments on brain neurotransmitter metabolism in experimental animals, comparatively little is known of the effects on these systems following *prolonged* treatment with the drug. Most reports have studied the effects of repeated VPA administration on whole brain (Kukino and Deguchi, 1977; Perry and Hansen, 1978) or brain regional (Williams et al, 1980; Patsolos and Lascelles, 1981; Simler et al, 1982) excitatory and inhibitory amino acid metabolism. The predominant finding from these studies suggests that cerebral amino acid levels are elevated following such treatments with VPA. Additionally, the magnitude of increase in amino acid accumulation measured following either acute doses of drug or the last injection in a repeated treatment regime differ. For example, cortical GABA levels determined 30 min after a single dose of 400 mg/kg VPA were moderately elevated (40%) above control values (Iadarola et al, 1979), whilst those determined in cortex removed 60 min after a final injection of 150 mg/kg VPA (150 mg/kg i.p. twice daily for 10 days) were substantially elevated (145%) above control values (Patsolos and Lascelles, 1981). These findings would appear to contradict those methodological criticisms of Simler et al (1982) which stated that "...because of (typically) the 1 or 2hr. interval between the last injection and sacrifice, ... results do not represent a chronic effect but rather that of the last injection." Of special interest with regard to the present work is the finding that tissue hippocampal GABA levels are only modestly elevated (10-20%) following acute VPA administration in rodents (Simler et al, 1978), but are substantially

increased (up to 160%) when animals are treated with VPA over a 10-14 day period (Patsolos and Lascelles, 1981; Simler et al, 1982).

Research using human subjects regarding changes in central amino acid neurotransmitter turnover during long term VPA therapy are even less numerous than animal studies and reports are conflicting: Some reports have demonstrated an increase in GABA levels measured in CSF (Zimmer et al, 1980; Löscher and Siemes, 1985) while another study found CSF GABA levels to remain unchanged following chronic therapy (Neophytides et al, 1978).

Information relating to responses of brain monoaminergic systems to prolonged administration of VPA is sparse given the probable importance of monoaminergic mechanisms in epilepsy. Data is limited to human studies carried out with the participation of mainly non-epileptic subjects (Zimmer et al, 1980; Nagao et al, 1979). The former study reported levels of HVA to be significantly elevated in CSF sampled from schizophrenic patients receiving 900-1200 mg VPA per day, whilst Nagao et al (1979) observed CSF HVA levels to be decreased during a similar treatment programme. Fahn (1978) presented results from two case studies of patients recieving VPA for the treatment of post anoxic action myoclonus and observed CSF levels of 5-HIAA to return to normal levels following extended periods of therapy (16-18 months). In this condition, CSF 5-HIAA concentrations seem to be consistently below control values (Guilleminault et al, 1973; Van Woert et al, 1977).

In all the studies previously outlined there has been a failure to address an important question: Does repeated dosing with VPA result in changes in EC (and presumably synaptic) neurotransmitter and metabolite levels which can

be monitored for the duration of such treatment? Clearly, such information

would be valuable in attempts to define more clearly CNS mechanisms

responsible for mediating the anticonvulsant actions of this agent, given that

it is frequently prescribed for the long term management of epilepsies (Pinder

et al, 1977).

Work presented in this chapter was carried out in an attempt to clarify

these points, using in vivo microdialysis to sample the EC enviroment of rat

hippocampal tissue for monoamines and amino acids over ; a 14 day

treatment period with VPA. In , addition, differences in the response of

hippocampal tissue to stimulation of neurotransmitter release evoked by high

EC levels of potassium ions (K^+), between treated and control animals were

assessed.

Experimental details

Two groups of rats (n=16 each) were initially group housed and their body weights recorded throughout the period of study. A treatment regime was established such that all animals in the test group received VPA twice daily at a dose of 200 mg/kg i.p. at approximately 08.00 h and 20.00 h. Control animals received saline injections each day at the equivalent time points. Both test and control rats were sub-grouped into sets of 4 animals on day 1 (ie., 4 x n=4 test, 4 x n=4 control) and during the hours of 13.00 and 18.00 (day 1), a test and control subgroup were implanted with microdialysis probes into the ventral hippocampus as previously described. All animals were allowed to recover from anaesthesia and received either a VPA dose or saline at 20.00 h. On the following day, all animals received their appropriate treatments at 08.00 h. Microdialysis was commenced at approximately 13.00 h and proceeded as follows: After the standard 30 min. stabilisation period, 7 consecutive 60 min dialysates were collected from each rat. Between dialysate 3 and 4, all rats received a 30 min infusion of high (100 mM) K^+ ACSF in order to estimate any difference in evoked release of neurotransmitters between test and control groups. Representative chromatograms illustrating K^+-evoked overflow of monoamines and amino acids, measured in hippocampal dialysates are provided in figure 18. All dialysates were divided into two equal portions and analysed for selected amino acids and monoamines using HPLC-FD or HPLC-ED respectively. The entire procedure was repeated so that animals exposed to 4, 7 and 14 days of VPA or saline treatment were subject to the above microdialysis procedure.

Data analysis

All values obtained from HPLC analysis were corrected for in vitro recovery

of amino acids and monoamines as previously described (see methods and

materials, section 2.2). Data are presented in tabular and bar chart form with

all values recorded as absolute quantity | as a molarity | rather

than as percentage of mean basal levels. This scheme was adopted in order to

illustrate any real variation in basal monoamine and amino acid release

between VPA and vehicle treated animals. Data were analysed for statisticaly

significant differences between control and treatment groups at each timepoint

using a student's t-test for unpaired data.

<u>**Animals**</u>

Rats weighing 230-260g were used in this investigation.

Results

Examination of data obtained during prolonged treatment of rats with VPA revealed a number of differences in basal and K^+-evoked neurotransmitter release measured in hippocampal dialysates when compared with saline treated animals. These findings are summarised below.

Dialysate monoamines: DA and its metabolites.

Data presented in table 7 and figure 19 suggest that prolonged VPA dosing did not result in any significant alteration in basal DA levels in hippocampal dialysates compared to control animals on days 2 and 14. There was however a trend towards elevated basal DA ouput on day 4 of treatment, although these values failed to reach statistical significance. In contrast, K^+-evoked release of DA was enhanced in VPA treated animals on day 14 and less consistently on day 4. Unfortunately, data relating to treatment day 7 was lost due to technical difficulties with the HPLC system. The evident lack of effect of VPA on basal DA levels measured during the period of treatment was reflected in a similar failure of the drug to significantly modify EC levels of DOPAC and HVA (figures 20, 21 and table 7) Basal levels of the metabolites recorded in treated animals did however appear to follow an upward trend during all microdialysis sessions, especially those carried out on days 7 and 14, when compared with equivalent control values. On day 14 and less consistently day 4, EC levels of both metabolites showed a slight downward trend in VPA treated animals following high K^+ infusion. These changes were delayed and corresponded to an increased output of DA compared to control as previously described (figure 19).

5-HT and 5-HIAA.

Basal dialysate 5-HT levels were not significantly elevated over control values during days 2, 7 and 14 microdialysis sessions, but were significantly increased on day 4 (table 7). In addition, during days 4 and 7 a substantial K^+-evoked 5-HT release with respect to that of control animals was observed, an apparent peak effect observed following 7 days treatment (figure 22). The VPA mediated potentiation of K^+-evoked 5-HT release did however decline following 14 days of treatment, suggesting that tolerance to this action of the anticonvulsant had developed. This was in striking contrast to the effects of VPA on dialysate 5-HIAA levels which were significantly elevated with respect to control values following 14 days of treatment in both basal and post K^+-stimulation dialysates (table 7 and figure 23). Basal 5-HT metabolism was markedly less affected by repeat VPA dosing on days 2, 4 and 7 although a significant reduction in dialysate 5-HIAA was noted 90 min following high K^+ infusion on day 4 and a non-significant reduction in the level of the metabolite noted at the same timepoint on day 7. Interestingly, this apparent delayed decrease in dialysate 5-HIAA was secondary to the primary effect of high K^+-stimulation on 5-HT release (figure 22 days 4 and 7) and the fall in metabolite levels tended to be more pronounced in VPA treated animals.

Dialysate amino acids: ASP and GLU.

Figure 24 and table 8 clearly show that basal ASP levels in the ventral hippocampal EC space were generally reduced compared to those of control animals. The mean reduction in basal ASP was most pronounced following 2 and 7 days VPA treatment (approximately 47% and 43% of control levels
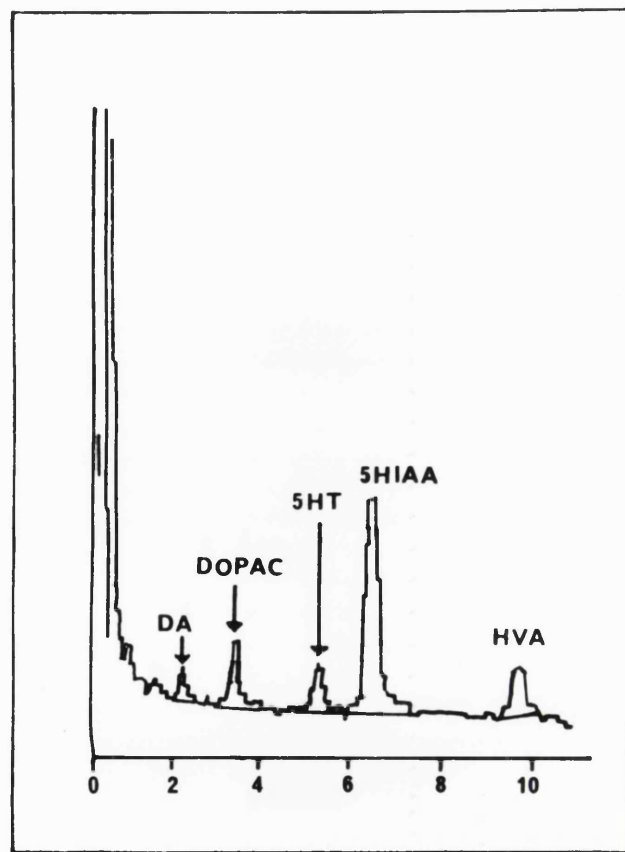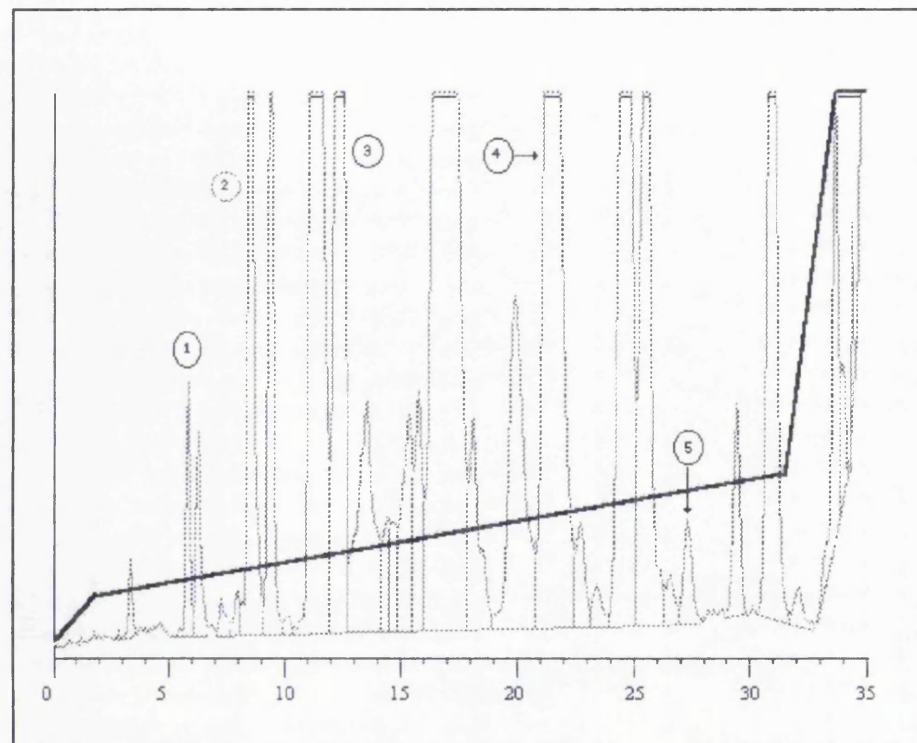
Figure 18 Representative chromatograms illustrating high K$^+$-evoked overflow of (A) monoamines and (B) amino acids in hippocampal dialysates. Key for panel (B):- ASP (1), GLU (2), GLN (3), TAU (4) and GABA (5). Horizontal axes represent elution time in min.

		Day 2	Day 4	Day 7	Day 14
DA	C	40±7.0	48±9.3	- *	36±6.0
	T	44.3±5.0	60.6±14	- *	46±5.0
DOPAC	C	833±102	578±162	1,056±250	1,500±408
	T	1,004±96	661±146	1,260±267	1,860±476
5HT	C	84±18	106±16	113±26	118±19
	T	82±17	186±25† (↑75%)	158±59	160±42
5HIAA	C	8,786±1,000	9,376±1,330	8,787±2,060	9,047±672
	T	11,113±1,266	9,496±1.023	10,540±2,503	13,043±975† (↑44%)
HVA	C	843±156	913±158	1,067±125	1,500±408
	T	1,015±263	838±173	1,370±401	1,741±477

Table 7 Mean basal levels of extracellular monoamines recovered from hippocampal dialysates during prolonged treatment with VPA (2x200mg/kg/day) or saline. Data relating to control (C) and treated (T) animals was obtained by averaging the first 3 basal monoamine values recorded from each animal prior to high potassium pulsing. * data unobtainable. All values represent mean $M \times 10^{-10}$ ± s.e.m. † denotes significant differences between control and treated animals at the $p<0.05$ level, n=4 animals per group.
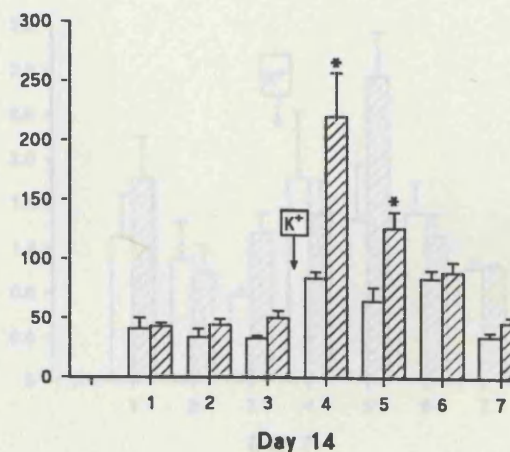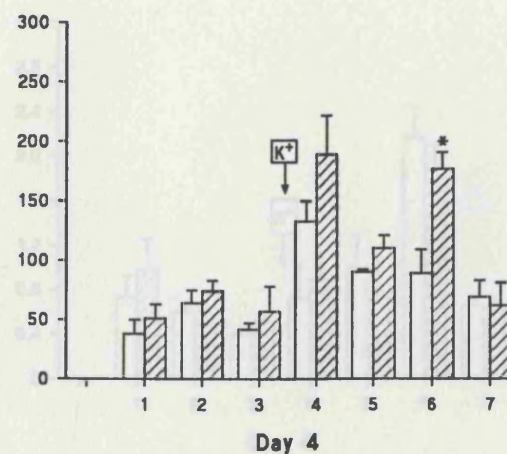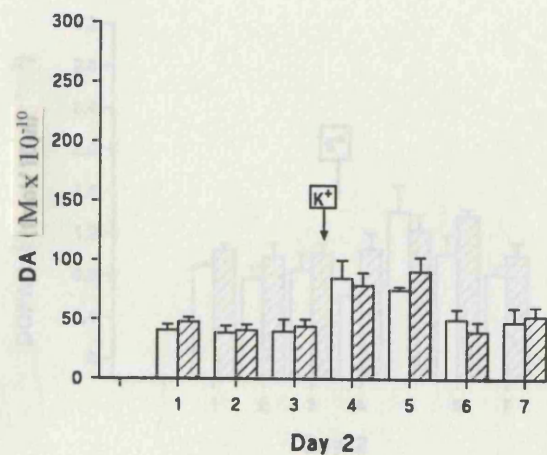
Figure 19 Bar charts showing effects of prolonged VPA treatments on extracellular levels of DA recovered from hippocampal dialysates. Individual timepoints on each chart represent dialysate collection periods in hours. Open bars and cross hatched bars represent saline treated and VPA treated animals respectively. Boxed K^+ indicates commencement of 30 min infusion of high potassium-containing ACSF. All values are $M \times 10^{-10}$ + s.e.m. * denotes significant differences between control and treated animals at the $p<0.05$, $n=4$ animals per group.
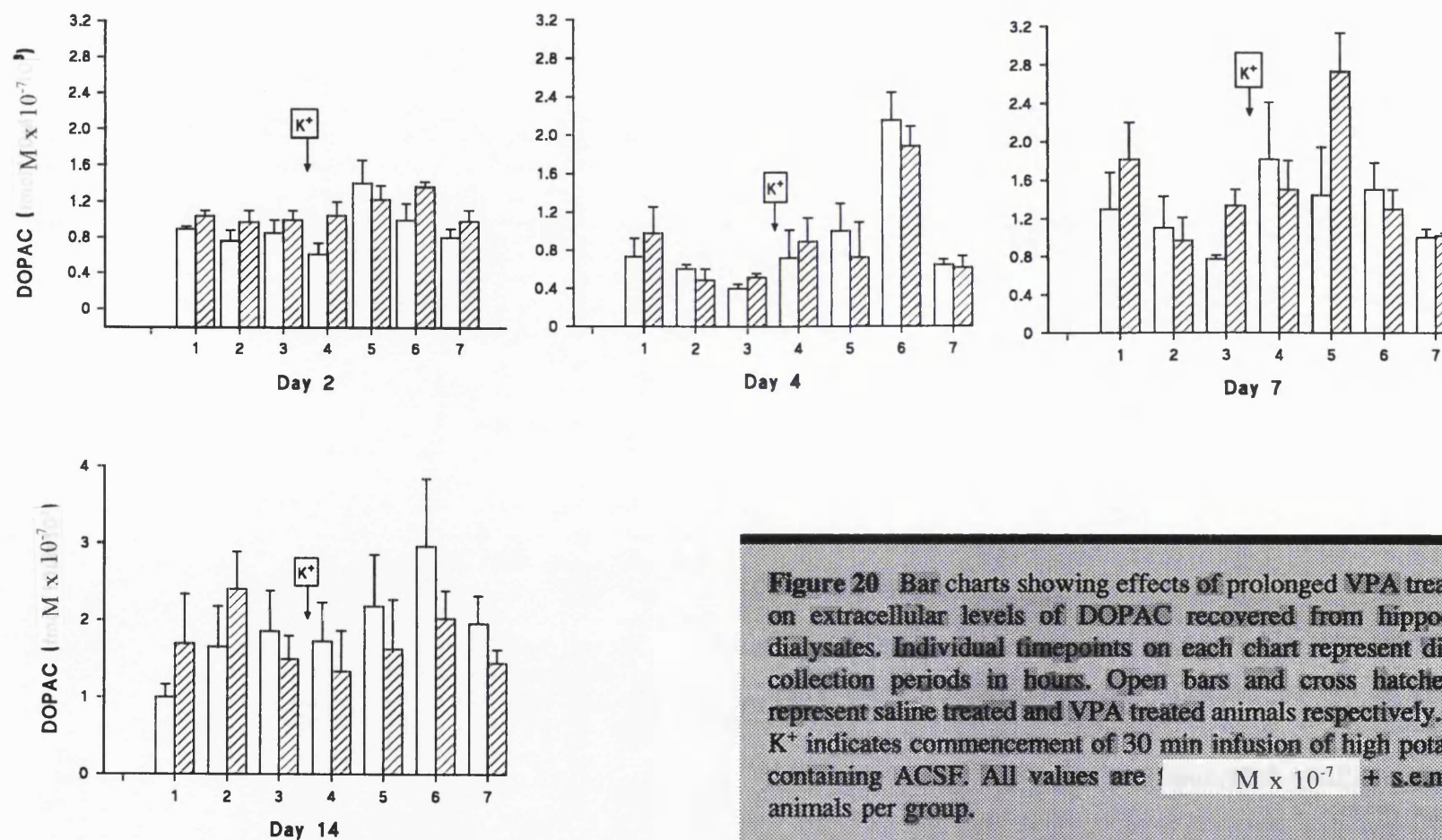
Figure 20 Bar charts showing effects of prolonged VPA treatments on extracellular levels of DOPAC recovered from hippocampal dialysates. Individual timepoints on each chart represent dialysate collection periods in hours. Open bars and cross hatched bars represent saline treated and VPA treated animals respectively. Boxed K^+ indicates commencement of 30 min infusion of high potassium-containing ACSF. All values are $M \times 10^{-7}$ + s.e.m., n=4 animals per group.
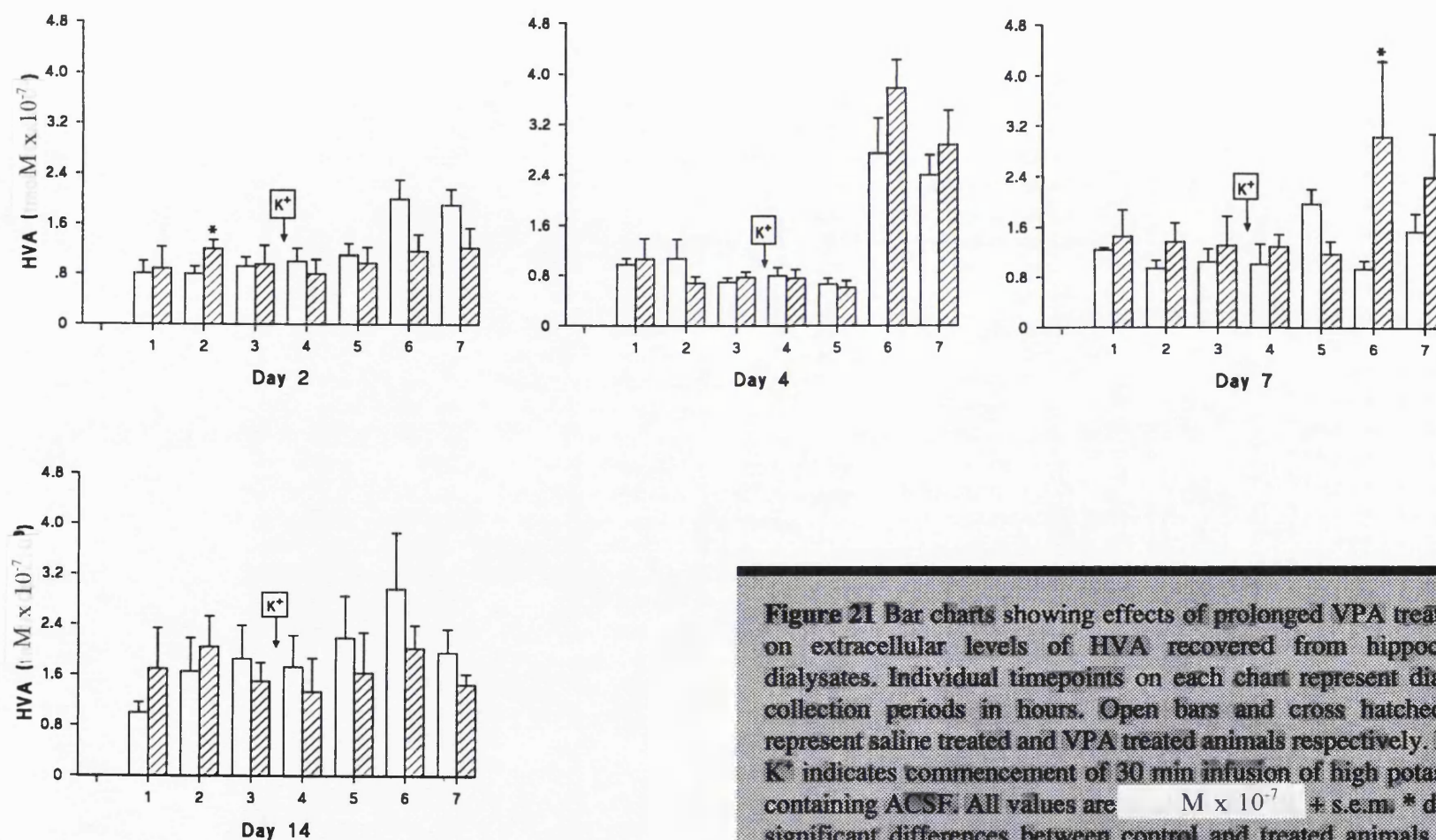
Figure 21 Bar charts showing effects of prolonged VPA treatments on extracellular levels of HVA recovered from hippocampal dialysates. Individual timepoints on each chart represent dialysate collection periods in hours. Open bars and cross hatched bars represent saline treated and VPA treated animals respectively. Boxed K^+ indicates commencement of 30 min infusion of high potassium-containing ACSF. All values are $M \times 10^{-7}$ + s.e.m. * denotes significant differences between control and treated animals at the $p < 0.05$ level, n=4 animals per group.
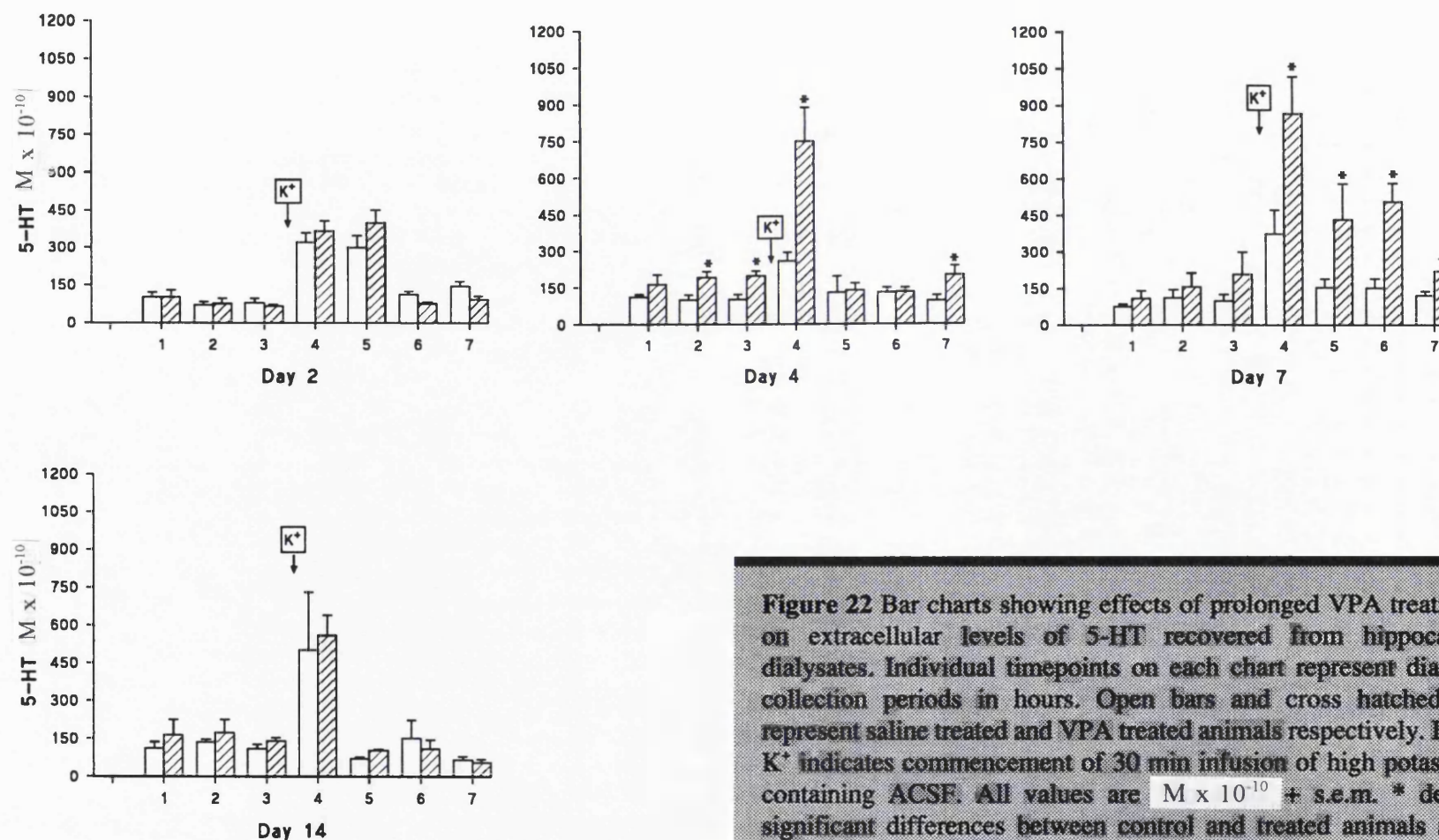
Figure 22 Bar charts showing effects of prolonged VPA treatments on extracellular levels of 5-HT recovered from hippocampal dialysates. Individual timepoints on each chart represent dialysate collection periods in hours. Open bars and cross hatched bars represent saline treated and VPA treated animals respectively. Boxed K^+ indicates commencement of 30 min infusion of high potassium-containing ACSF. All values are $M \times 10^{-10}$ + s.e.m. * denotes significant differences between control and treated animals at the $p < 0.05$ level, n=4 animals per group.
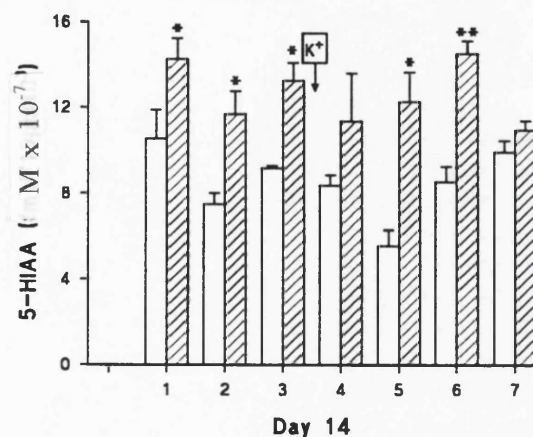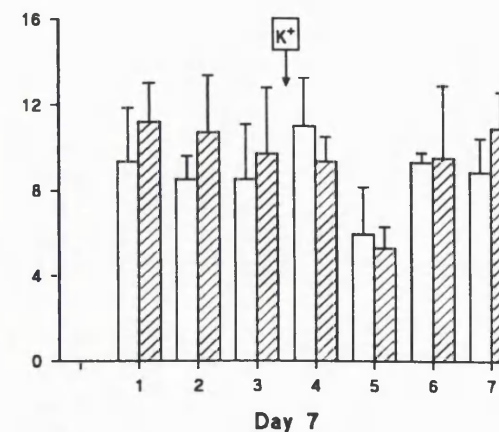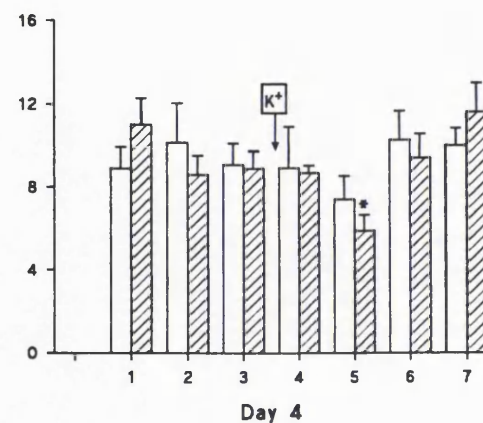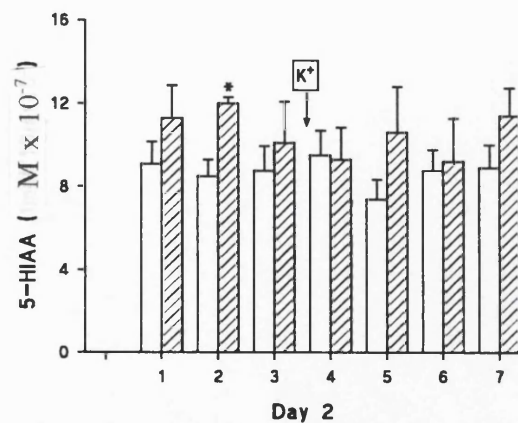
Figure 23 Bar charts showing effects of prolonged VPA treatments on extracellular levels of 5-HIAA recovered from hippocampal dialysates. Individual timepoints on each chart represent dialysate collection periods in hours. Open bars and cross hatched bars represent saline treated and VPA treated animals respectively. Boxed K^+ indicates commencement of 30 min infusion of high potassium-containing ACSF. All values are $M \times 10^{-7}$ + s.e.m. * and ** denote significant differences between control and treated animals at the $p<0.05$ and $p<0.01$ levels respectively, n=4 animals per group.

respectively; p<0.01). In contrast, during the subsequent 7 days treatment, tolerance to this effect had apparently developed (figure 24, day 14). ASP levels were markedly elevated above basal values following high K^+ infusion in both treated and control animals. On days 2 and 7 levels of ASP in dialysates collected from treated animals were significantly lower compared to equivalent control dialysates 90 min following high K^+ stimulation.

Basal GLU accumulation in hippocampal dialysates following prolonged treatment with VPA was somewhat erratic when compared to the relatively consistent steady-state concentrations of amino acid measured in control dialysates (table 8 and figure 25). On days 2 and 14, mean basal GLU concentrations were not significantly different between both groups, whereas a substantial fall was noted in the VPA treated group over mean control concentration (approximately 33% of control value; p<0.05) on day 4. An upward trend for basal GLU in treated animals was noted on day 7, although no values where significantly different from their equivalent controls. High K^+-infusion resulted in a variable enhancement of dialysate GLU levels (figure 25) over the 14 day experimental period, although this was perhaps to be expected given likely individual neurochemical variations between animals. Dialysate GLU levels from VPA treated animals subject to microdialysis on day 4 were generally lower than their equivalent control values, suggesting that the reduced basal output of GLU from hippocampal tissue represented a partial depletion of releasable GLU pools following VPA treatment. This trend was apparently reversed on day 7 when high K^+ infusion resulted in a net increase in GLU outflow measured in VPA treated animals compared to controls. The

longevity of this response was also increased compared to that monitored on all other days, with statistically significant differences between treatment and control values apparent 180 and 240 min following high K^+ infusion.

GLN.

Basal dialysate levels of GLN tended to be higher in treated animals on all but day 14 compared to those measured in control animals (table 8), although this was significantly different on day 2 only (figure 26; $p<0.05$). Although not apparent on day 2, high K^+ infusion generally resulted in a delayed decline in basal GLN concentrations particularly in control animals. On day 7 dialysate GLN levels estimated in VPA treated animals were significantly greater than those in control animals (samples 5 and 7, $p<0.05$ and $p<0.01$ respectively) following high K^+ infusion.

TAU and GABA.

Basal TAU levels in ventral hippocampal dialysates collected from VPA treated rats were found to be inconsistently elevated compared to those estimated in control animals (table 8). In contrast, during all microdialysis sessions high K^+ infusion resulted in a greater evoked release of the amino acid measured in some VPA treated animals when compared to those measured in control rats (figure 27). These values were found to be significantly different form control values on day 4 (samples 5 and 6; $p<0.01$ and $p<0.05$ respectively).

Basal GABA output was found to be similarly affected by prolonged VPA treatment since dialysate concentrations of the neurotransmitter were only found to be significantly higher than those of control animals on day 2 (table 8 and figure 28). Responses to high K^+ infusion were inconsistent between

microdialysis sessions and experimental groups: Only on day 4 were any

significant differences in evoked GABA release between control and treated

animals noted, although a more prolonged response to high K^+ in both groups

was evident on day 7.

		Day 2	Day 4	Day 7	Day 14
ASP	C	3.18±0.36	2.6±0.42	3.0±0.5	2.8±0.5
	T	1.5±0.13†† (↓53%)	2.3±0.2	1.3±0.1††(↓57%)	2.2±0.9
GLU	C	32.8±1.2	31.7±7.0	36.0±7.0	36.6±9.0
	T	32.7±1.0	10.3±2.5†(↓66%)	54±17	29±7.0
GLN	C	81.3±13	91±11	116±50	93±13
	T	120±20	114±16	151±38	92±28
TAU	C	61.2±6.5	72.5±12.5	60±15	50±3.1
	T	80±15.4	77.5±8.5	52.5±15	57.5±8.75
GABA	C	3.28±0.13	3.3±0.16	2.4±0.4	3.6±0.38
	T	3.62±0.05†(↑10%)	3.6±0.36	2.1±0.5	3.3±0.3

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Table 8 Mean basal levels of extracellular amino acids recovered from hippocampal dialysates during prolonged treatment with VPA (2x200mg/kg/day) or saline. Data relating to control (C) and treated (T) animals was obtained by averaging the first 3 basal amino acid values recorded from each animal prior to high potassium infusion. All values represent mean $M \times 10^{-7}$ ± s.e.m. † and †† denote significant differences between control and treated animals at the $p<0.05$ and $p<0.01$ levels respectively, n=4 animals per group. Figures in parenthesis indicate percentage change.
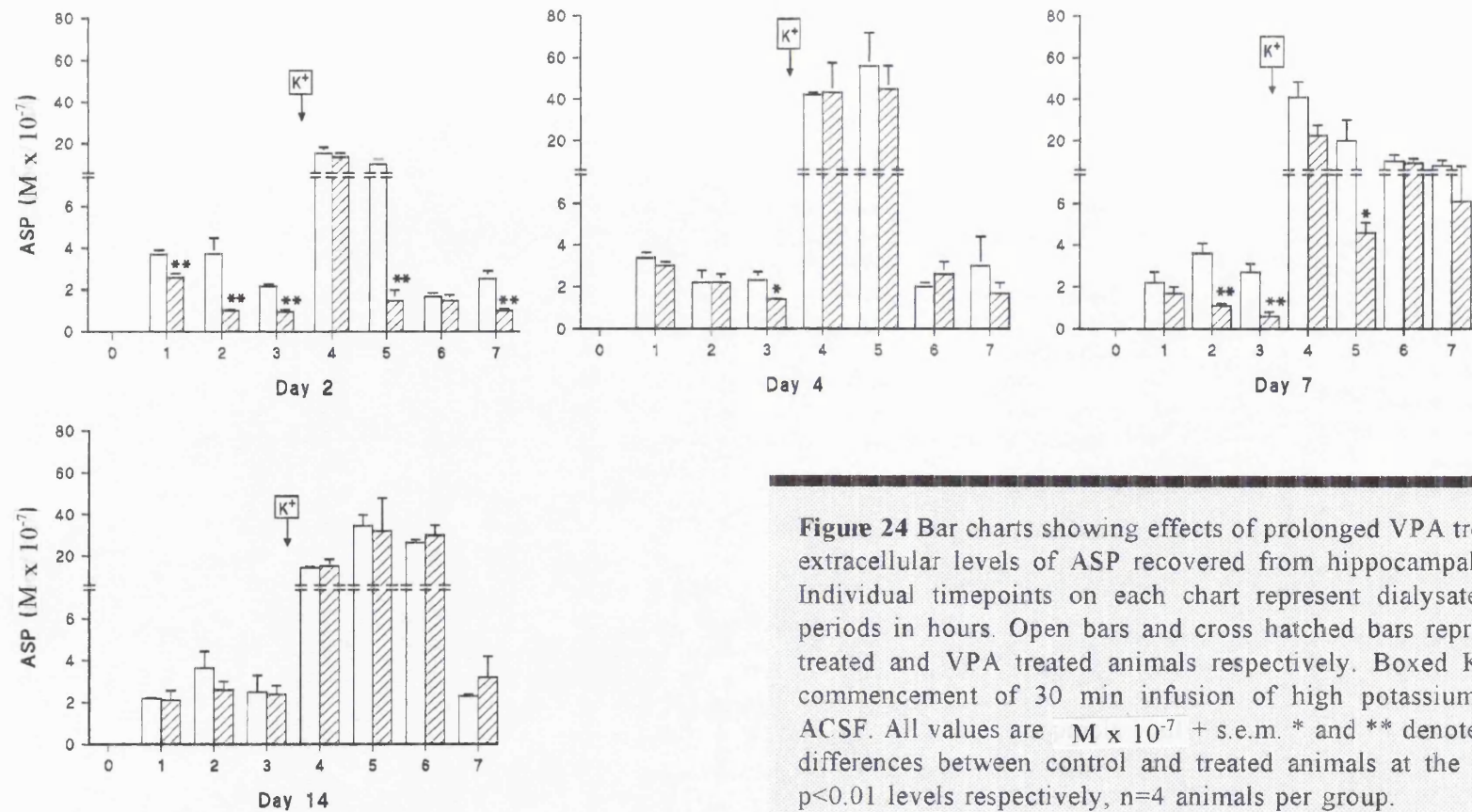
Figure 24 Bar charts showing effects of prolonged VPA treatments on extracellular levels of ASP recovered from hippocampal dialysates. Individual timepoints on each chart represent dialysate collection periods in hours. Open bars and cross hatched bars represent saline treated and VPA treated animals respectively. Boxed K^+ indicates commencement of 30 min infusion of high potassium-containing ACSF. All values are $M \times 10^{-7}$ + s.e.m. * and ** denote significant differences between control and treated animals at the $p<0.05$ and $p<0.01$ levels respectively, n=4 animals per group.
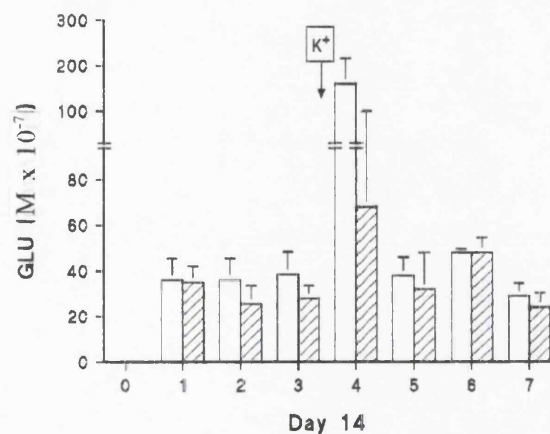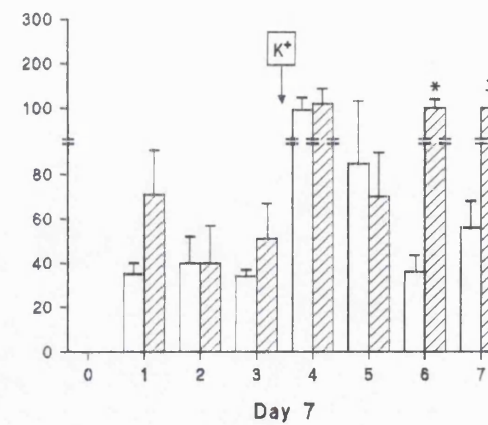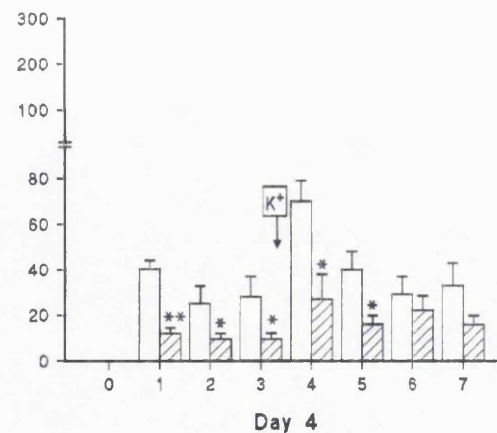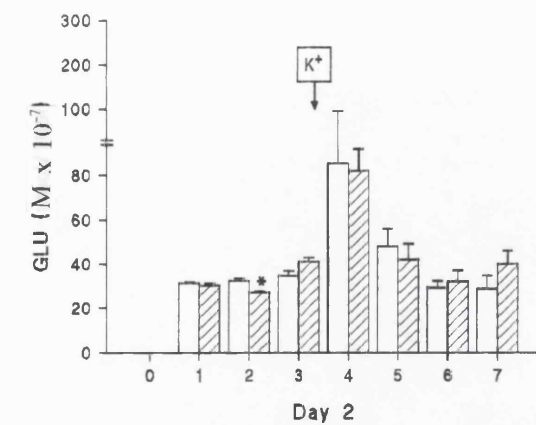
Figure 25 Bar charts showing effects of prolonged VPA treatments on extracellular levels of GLU recovered from hippocampal dialysates. Individual timepoints on each chart represent dialysate collection periods in hours. Open bars and cross hatched bars represent saline treated and VPA treated animals respectively. Boxed K^+ indicates commencement of 30 min infusion of high potassium-containing ACSF. All values are $M \times 10^{-7}$ + s.e.m. * and ** denote significant differences between control and treated animals at the $p<0.05$ and $p<0.01$ levels respectively, n=4 animals per group.
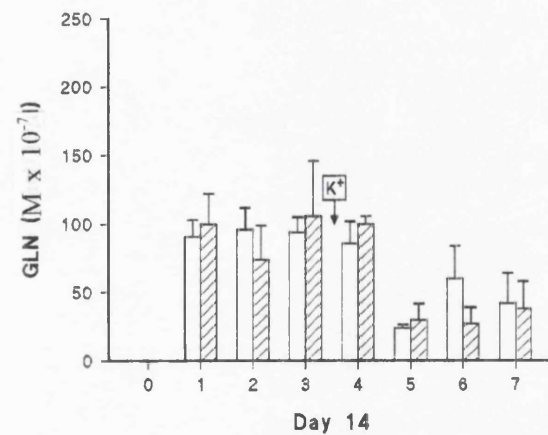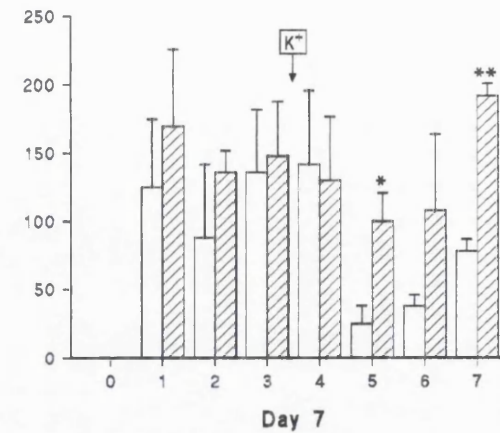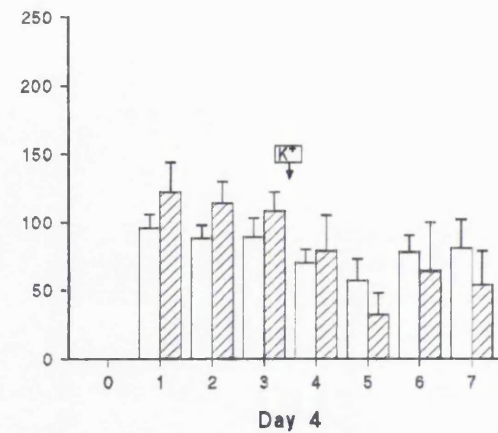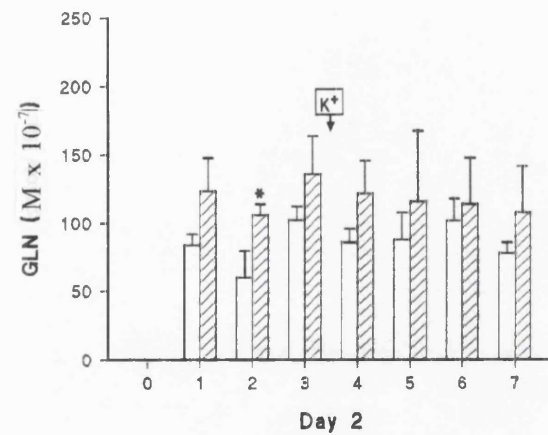
Figure 26 Bar charts showing effects of prolonged VPA treatments on extracellular levels of GLN recovered from hippocampal dialysates. Individual timepoints on each chart represent dialysate collection periods in hours. Open bars and cross hatched bars represent saline treated and VPA treated animals respectively. Boxed K^+ indicates commencement of 30 min infusion of high potassium-containing ACSF. All values are $M \times 10^{-7}$ + s.e.m. * and ** denote significant differences between control and treated animals at the $p<0.05$ and $p<0.01$ levels respectively, n=4 animals per group.
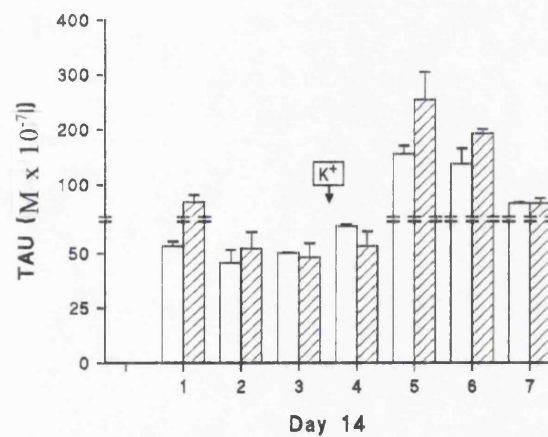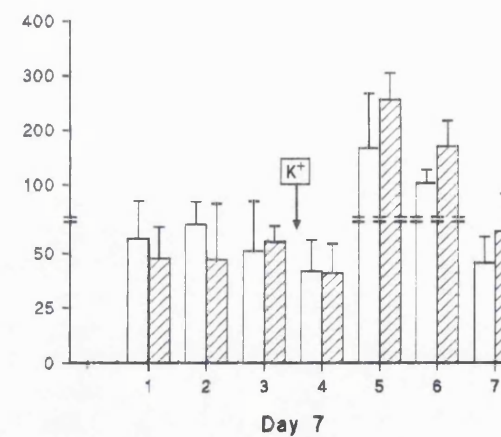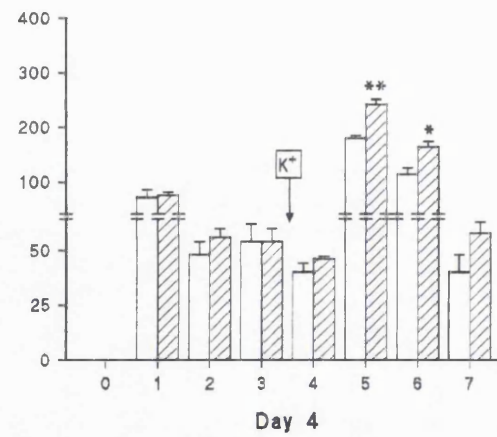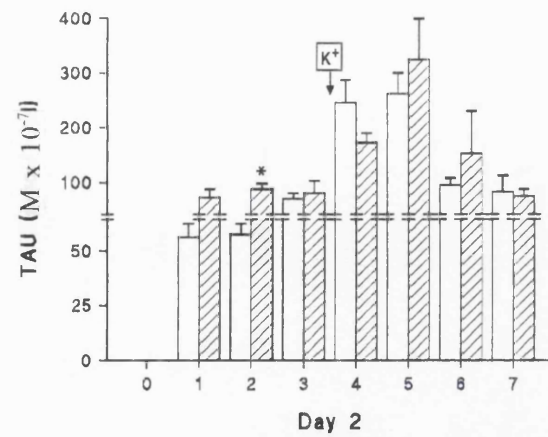
Figure 27 Bar charts showing effects of prolonged VPA treatments on extracellular levels of TAU recovered from hippocampal dialysates. Individual timepoints on each chart represent dialysate collection periods in hours. Open bars and cross hatched bars represent saline treated and VPA treated animals respectively. Boxed K^+ indicates commencement of 30 min infusion of high potassium-containing ACSF. All values are $M \times 10^{-7}$ l + s.e.m. * and ** denote significant differences between control and treated animals at the $p<0.05$ and $p<0.01$ levels respectively, n=4 animals per group.
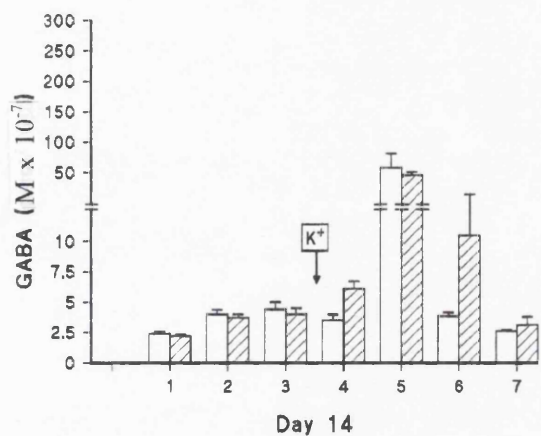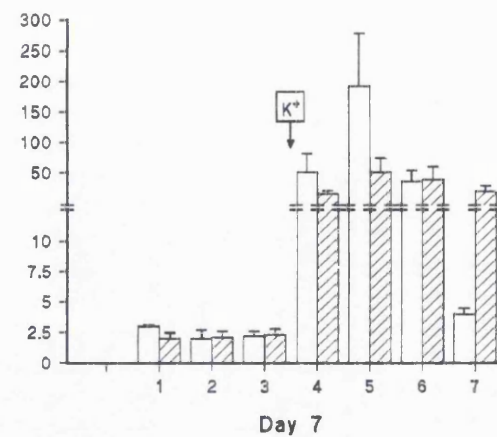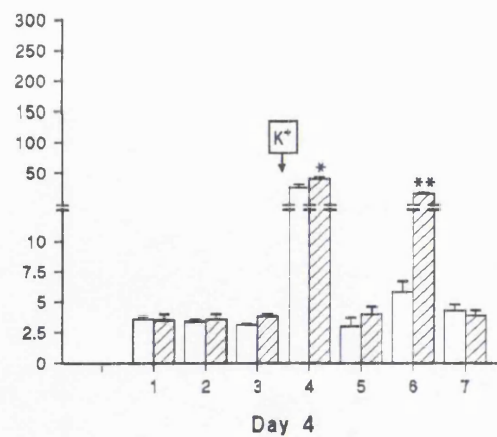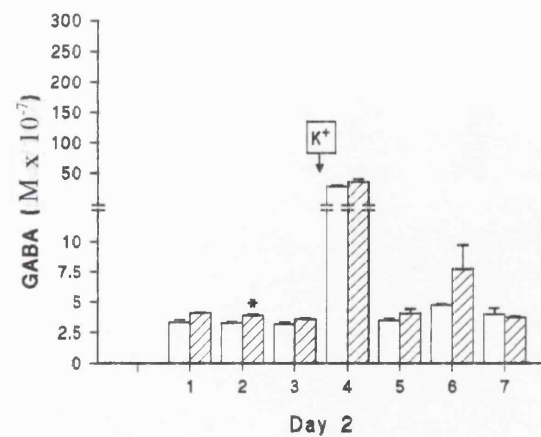
Figure28 Bar charts showing effects of prolonged VPA treatments on extracellular levels of GABA recovered from hippocampal dialysates. Individual timepoints on each chart represent dialysate collection periods in hours. Open bars and cross hatched bars represent saline treated and VPA treated animals respectively. Boxed K^+ indicates commencement of 30 min infusion of high potassium-containing ACSF. All values are $M \times 10^{-7}$ + s.e.m. * and ** denote significant differences between control and treated animals at the $p<0.05$ and $p<0.01$ levels respectively, n=4 animals per group.

Discussion

Overview.

Overall, the present data suggest that prolonged i.p. administration of VPA can alter EC levels of amino acids, monoamines and their metabolites associated with neurotransmission. However, these changes were rarely substantially different from control values and in many cases, only general trends could be identified. This observation alone serves to illustrate an inherent drawback of such studies in which it is not appropriate to normalise data on a 'percent of control' basis. Hence factors such as individual nutritional status, neurochemical variations between animals along with slight differences in microdialysis probe placements could all serve as sources of variation within and between groups, resulting in the relatively large standard errors prevalent here. Löscher (1982) encountered similar problems to those mentioned above during a study with oral VPA dosing of mice, despite careful monitoring to ensure a uniform intake of drug. In spite of these difficulties, the results of the present study have indicated that VPA may indeed differentially alter neurotransmitter output and metabolism during sub-chronic treatment of rats.

Monoamines; DA, DOPAC and HVA.

The apparent lack of effect of prolonged VPA treatment on basal dialysate DA levels indicates that the drug did not directly modify release of the neurotransmitter from monoaminergic neurones in the hippocampus. This is in contrast to the large, transient increase in EC concentration of the monoamine observed in microdialysed ventral hippocampus following acute

administration of 400 mg/kg VPA (see chapter 3), indicating that the acute

effects of VPA on release of this neurotransmitter are critically dependent

upon the brain concentration of the anticonvulsant. In the present study, basal

levels of the metabolites DOPAC and HVA were inconsistently and non-

significantly elevated compared to controls suggesting that prolonged dosing

with twice daily 200 mg/kg VPA only resulted in a marginal increase in

turnover of hippocampal DA. Depolarization of hippocampal tissues adjacent

to the dialysis membrane with an infusion of 100 mM K^+ containing ACSF did

induce an increased release of DA in both control and VPA treated animals

although the magnitude of this effect was notably lower than has been

observed previously; Fairbrother et al (1990) noted a maximal K^+-evoked

stimulation of DA release from nigrostriatal terminals of approximately 1500%

of basal efflux, a markedly greater response than that observed here. This

discrepancy is most likely to be the consequence of two main factors, namely

microdialysis in brain regions of widely differing dopaminergic terminal

density and the shorter sampling periods (20 min) employed by Fairbrother

et al (1990), rather than a fundamentally dissimilar neurochemical response of

cells from these brain regions to high K^+ depolarization. Figure 19 shows that

the evoked release of DA in VPA treated animals was progressively increased

with respect to that estimated in equivalent control dialysates during the

treatment period. It would be tempting to speculate that prolonged VPA

treatment (at the dose administered) may result in an enhanced intraneuronal

turnover and synthesis of DA in *some animals* without resulting in an elevation

of neurotransmitter output during steady-state conditions. However, the

pattern of metabolite accumulation in dialysates following depolarization of neurones with high K^+ is curious when compared to previous findings (Zetterström et al, 1988; Fairbrother et al, 1990). In these studies, similar depolarisation of striatal neurones led to a modest fall in the EC concentrations of both DOPAC and HVA, the authors concluding that the increased efflux of the parent amine reduced the presynaptic conversion of DA to DOPAC and ultimately, HVA extrasynaptically. The present results reveal no such consistent response of EC metabolite accumulation to depolarization, both in relation to control and VPA treated animals. Indeed, during days 4 and 14 (control animals) and day 7 (treated animals) DOPAC levels were actually elevated in dialysates collected from some animals. On the basis of these findings it could be argued that hippocampal dopaminergic terminals respond in a different manner to depolarizing stimuli, possibly due to their relatively sparse distribution within this structure (Scatton et al, 1980; Hörtangel et al, 1991). Thus, the effective concentrations of K^+ ions likely to be present in the vicinity of a given dopaminergic terminal could be somewhat lower than in the striatum, particularly if significant buffering of the excess electrolyte by interstitial cells were to take place. This scheme might also partially account for the modest effects of depolarization on DA efflux described above. Only on day 14 was a substantial increase in high K^+-evoked dialysate DA noted in VPA treated animals, but concomitant production of DOPAC and HVA was not modified compared to pre-stimulation levels. It is therefore not possible to conclude that prolonged VPA treatment resulted in an increased turnover of DA, although it seems likely that presynaptic accumulation of the

neurotransmitter was enhanced after 14 days of dosing. This conclusion is consistent with the tenuous evidence for the proposed action of VPA on DA synthesis (this thesis, chapters 3 and 6).

5-HT and 5-HIAA.

The present data indicate a tendency for prolonged VPA treatment to increase basal efflux of 5-HT as measured in hippocampal dialysates when compared with control rats, especially following 4 days of repeated dosing (table 7). The rapid return of 5-HT levels to 100% basal output observed following acute administration of the anticonvulsant (Whitton and Fowler, 1991; Chapter 3, this thesis) and the total lack of effect on basal 5-HT output noted on day 2 of the present study argue against the conclusion that the effects of prolonged treatment merely reflect a residual action of the final VPA dose before commencement of microdialysis. Prior to high K^+ infusion, EC 5-HIAA levels estimated in dialysates collected from day 2 VPA treated rats were noticeably higher than controls, suggesting that short-term dosing with the anticonvulsant may have resulted in enhanced intraneuronal turnover of 5-HT without stimulating additional release of the neurotransmitter. In contrast, after 4 days of repeated dosing with VPA, a significantly enhanced basal release of 5-HT was noted, with a correspondingly lower accumulation of metabolite (table 7 and figure 23). Following hippocampal tissue depolarization with high K^+ infusion, 5-HT overflow measured in VPA treated animals was significantly increased on days 4 and 7, suggesting that persistent dosing for this time period had led to greater presynaptic accumulation of 5-HT in a releaseable pool compared to that measured in control animals. No attempt to characterise

the possible Ca^{2+}- dependency of high K^+-evoked 5-HT overflow was made within the current study, but evidence suggests that a substantial proportion of basal and stimulated 5-HT release in spinal cord (Yaksh and Tyce, 1980) and striatum (Kalen et al, 1988) is derived from a Ca^{2+}-sensitive and therefore, presumably, a vesicular pool. K^+-mediated depolarization of microdialysed rat striatum has previously been shown to induce a decline in EC 5-HIAA, a response thought to reflect a shift of 5-HT from monoamine oxidase (MAO) associated pools to replenish vesicular pools following excessive 5-HT release (Kalen et al, 1988). A similar, if more modest response to this depolarizing stimulus was observed in the present study (figure 23). It is interesting to note that on days 4 and 7, the reduction in dialysate 5-HIAA tended to be more marked in VPA treated animals, possibly reflecting greater release of the parent amine following high K^+ infusion. Clearly by day 14, a further modification of serotonergic transmission had taken place in drug treated animals with a shift in favour of increased 5-HT catabolism. Evidence for this was provided by the detection of elevated 5-HIAA under basal conditions in the absence of significantly elevated basal 5-HT output (table 7). High K^+ infusion did not result in reduced 5-HIAA accumulation as previously observed suggesting that evoked 5-HT release in these animals had not caused partial depletion of presynaptic 5-HT pools available for catabolism to 5-HIAA. The relatively modest increase in 5-HT output evoked by high K^+ during this microdialysis session could have been as a result of an experimental factor (poor or incomplete penetration of K^+ into the tissues, etc.), but the relatively small standard error estimate would suggest that the reduction in evoked

release was genuine. Thus, treatment of rats with VPA for 14 days may have contributed to the induction of adaptive mechanisms, since on the present evidence neuronal 5-HT metabolism was enhanced without resulting in significantly increased basal neurotransmitter release. It is appropriate to state here that there is some direct evidence relating VPA administration (at least, acutely) to increased whole brain synthesis and turnover of 5-HT (Whitton et al, 1985).

From these studies it is clear that 5-HT output and metabolism under basal and depolarization-evoked conditions can be altered by VPA administered during a sub-chronic period, reinforcing the possibility that serotonergic neurotransmission might have role in mediating a component of the drugs anticonvulsant effects. Löscher et al (1988) proposed that the central mechanisms mediating anticonvulsant actions and the "wet dog" shake (WDS) component of the quasi-morphine absteinence behavioural syndrome (de Boer et al, 1977) observed following acute VPA administration were not related, since WDS became progressively less intense and anticonvulsant efficacy enhanced during the treatment period. WDS are commonly observed in rodents following the administration of the 5-HT precursor 5-hydroxytryptophan (5-HTP), can be abolished by pretreatment with pCPA (Fletcher and Harding, 1981) and comprise an element of the so-called serotonin behavioural syndrome (Green, 1984). That the anticonvulsant activity of VPA assessed during prolonged treatment is not in part related to changes in serotonergic neurotransmission, as previously asserted (Löscher et al, 1988; Löscher 1993) must be questioned for a number of reasons: Firstly, these

authors noted a significant reduction in WDS intensity and an increase in kindled seizure threshold after only 1 day of treatment with 3 x 200 mg/kg VPA, whereas in the present study basal EC 5-HT levels were elevated with respect to control values after 4 days of VPA dosing, with <u>in vivo</u> evidence for an up-regulation of 5-HT synthesis and metabolism throughout the treatment period. In addition, prolonged treatment of rats with 5-HTP which itself increases electroconvulsive seizure thresholds, progressively decreased the response to maximal electroconvulsions whilst the intensity of 5-HTP-induced WDS decreased sharply (Pagliusi and Löscher, 1985). There is evidence that the WDS component of the serotonin behavioural syndrome may be mediated by serotonergic neurones in the brain stem (Bedard and Pycock, 1977) and that 5-HT$_2$ receptor activation is involved (Peroutka et al, 1981; Leysen et al, 1984). 5-HT$_{1A}$ receptor stimulation has been most reliably associated with an anticonvulsant action in a variety of seizure models (Janusz and Kleinrok, 1989; Wada et al, 1992 a,b; Chapter 8, this thesis) and it is possible that VPA administration might result in the establishment of acute tolerance to WDS, whilst sparing 5-HT$_{1A}$-associated anticonvulsant activity. It is not possible at present to correlate VPA-induced increases in 5-HT output in structures associated with WDS generation and seizure propagation respectively, with the establishment of such tolerance.

No corroborative data regarding the effects of repeated VPA dosing on monoaminergic neurotransmitter release and metabolism are currently available, but the present data suggest that the neurochemical response to the drug is not uniform with respect to time. Indeed, it would seem on the basis

of these studies that both cumulative and adaptive changes may occur with regard to neuronal mechanisms responsible for synthesis, storage and metabolism of hippocampal 5-HT and possibly DA during prolonged treatment with VPA. The relevance of such changes to the long term anticonvulsant efficacy of this agent remains to be established and longer periods of dosing will be required to determine if chronic alterations to central monoaminergic neurotransmission occur.

Amino acids; ASP and GLU.

The effects of prolonged VPA administration in rodents on central amino acid metabolism ex vivo has been sparsely documented and no reports relating dosing with VPA to changes in brain EC amino acid levels are available. In view of this deficiency, the present study reports novel findings with implications for candidate neurochemical mechanisms through which this agent may exert its anticonvulsant action. Of particular interest is the observation that prolonged VPA administration results in reductions in basal ASP and less consistently, GLU concentrations during the period of treatment (table 8 and figures 24, 25). Since both amino acids have been consistently associated with epileptogenesis in vivo (see Choi, 1988 and general introduction for review) and massive elevation of EC ASP and GLU has been measured in human status epilepticus partialis (Carlson et al, 1992), reduction of basal excitatory amino acid efflux might be expected to reduce seizure activity in vivo. Paradoxically, existing ex vivo data suggests that prolonged administration of VPA (150 mg/kg/day) significantly *increased* whole tissue hippocampal accumulation of both the abovementioned EAAs, although

measurements were only made at a single timepoint following 10 days of dosing (Patsalos and Lascelles, 1981). These findings also differ from those following acute VPA dosing in which whole brain ASP concentrations were decreased in DBA/2 mice (Schecter et al, 1978) and GLU concentrations either modestly increased (Chapman et al, 1982) or unaffected (Godin et al, 1969) in rats (see chapter 4 for a fuller discussion of ASP data). These discrepencies could be due, in part to differing experimental design and the variable use of rodent species. Data presented in chapter 4 indicates that acute administration of 400 mg/kg VPA results in a modest elevation of EC GLU levels over basal output (figure 16) and the present data suggest that tolerance to this effect of the anticonvulsant may develop rapidly. Hence on day 2, minimal differences between basal GLU output in treated or control animals were evident, while day 4 experiments revealed basal GLU output estimated in treated animals to be significantly reduced with respect to control values (figure 25). However, following 7 days of VPA dosing, GLU output estimated in both basal dialysates and those collected following high K^+ infusion tended to be higher than in equivalent control samples. At the end of the 14-day treatment period, both basal and evoked GLU release followed a similar pattern to that measured in treated animals on day 2. This would suggest that prolonged administration of VPA may have resulted in a complex alteration of mechanisms influencing glutamatergic metabolism and release processes. No biochemical data supporting these in vivo findings are, as yet, available.

A similar apparent tolerance development to the effects of prolonged VPA administration on hippocampal ASP output estimated in dialysates was

evident on day 14, with basal release indistinguishable from that in control animals. Also of note here were the exaggerated responses to high K^+ depolarization on days 7 and 14. It would be tempting to conclude that this represented a compensatory increase in neuronal and/or glial ASP accumulation (or perhaps, increase in cellular sensitivity to depolarizing agents) following the evident down-regulation in basal ASP efflux induced by VPA treatment. Unfortunately, since control animals seemed to be similarly responsive to high K^+ on days 7 and 14, this phenomenon may merely represent the effects of a common enviromental or experimental factor.

GLN.

On all but day 14 of the present study, basal GLN efflux estimated in dialysates from treated animals tended to be higher than equivalent values estimated from control animals although variation was marked, especially on day 7 (figure 26). The apparent reduction of EC GLN levels following depolarization with high K^+ has been described previously and is thought to represent stimulation of GLN uptake by neurones requiring a source of precursor molecules for GLU and GABA synthesis (Tossman et al, 1986; Young and Bradford 1986). Of special interest with regard to the current study is the observation that high K^+ depolarization did not result in a substantial decrease of GLN levels estimated in treated animals on day 7 compared to control levels, which were lowered. It is possible that high K^+ stimulated uptake of GLN into adjacent cells was retarded due to the apparent increased accumulation of GLU and GABA in their releasable pools, evidenced by the prolonged increases in efflux of both amino acids evoked by this treatment

(see figures 25 and 28). Alternatively, since VPA is known to enhance the

activity of the GLN-synthetic enzyme, glutamine synthase (EC 6.3.1.2.; Phelan

et al, 1985), the present observations are consistent with the notion that

prolonged VPA treatment results in accumulation of GLN in its glial cell

synthetic compartment (Martinez-Hernandez et al, 1977). As previously noted

with regard to ASP and GLU in the present study, GLN levels estimated in

both treated and control animals were almost indistinguishable on day 14,

again suggestive of an adaptive response to repeated VPA administration.

TAU and GABA.

The lack of effect of prolonged VPA treatment on basal dialysate

concentrations of both TAU and GABA noted in this study was to some extent

unexpected: Previous ex vivo investigations have found that both whole brain

and hippocampal tissue levels of GABA and TAU were elevated following

prolonged treatment with VPA (Patsolos and Lascelles, 1981) and moreover,

these changes were effected at a lower daily dosage of the anticonvulsant (2

x 150 mg/kg/day). However, using the same treatment regime as that

employed in the present study, Simler et al (1982) were unable to demonstrate

a significant change in hippocampal GABA content with respect to control

values after 14 days, unless an additional acute dose of the drug was given 45

min prior to sacrifice. It is entirely possible that these apparent discrepancies

can be attributed to differences between the experimental protocols used by

the previously cited authors, namely the use of mice by Simler et al (1982),

sacrificed using focussed microwave irradiation versus the use of rats

sacrificed by decapitation (Patsolos and Lascelles, 1981). Major differences in

pharmacokinetic parameters with regard to VPA administration in mice and rats (Löscher 1978; J. Simiand, unpublished data) are most likely to effect the neurochemical outcome of prolonged dosing with the drug. In the present study, high K^+-evoked release of GABA estimated in dialysates collected from treated animals was only significantly higher than that of control animals on day 4 and even here, the augmentation of evoked GABA release was modest. These observations are of great interest when one considers the wealth of information strongly implicating central GABAergic mechanisms in the anticonvulsant mode of action of this agent (see general introduction). Although the vast majority of such studies have investigated acute anticonvulsant and neurochemical actions, prolonged administration of the drug can also significantly increase GABA accumulation in most rodent brain regions (see previous page and chapter introduction). Hypothetically, in order to convincingly demonstrate a functional link between changes in neuronal GABA turnover, receptor activation and ultimately anticonvulsant activity, an alteration of EC GABA concentrations in vivo following VPA administration must also be demonstrated. It would seem that at the dose used, VPA failed to significantly enhance dialysate GABA levels, a likely index of GABAergic neurotransmission in vivo. It may be that acute dosing only with the anticonvulsant raises tissue GABA concentrations sufficiently to result in a measurably enhanced output of the neurotransmitter. However, given the widely supported notion that VPA may exert a proportion of its anticonvulsant action by enhancing GABAergic neurotransmission, it probably unwise to dismiss a prolonged effect of the drug on GABA output in vivo on the basis

of this study alone. It is possible that a 'threshold' dose of VPA, administered

repeatedly, is required in order to elicit substantial changes in GABA release

and uptake <u>in vivo</u>.

It is possible that the variety of changes in both amino acidergic and

monoaminergic neurotransmitter output and metabolism could contribute to

an overall anticonvulsant action. However, a predominant finding of the

present study was that any previously recorded changes in neurotransmitter

levels (with respect to control animals) had subsided by the end of the

treatment period. This phenomenon was probably not attributable to an

experimental or enviromental factor peculiar to day 14, since levels of analytes

measured in control animals remained relatively stable. A more likely

explanation is that animals treated with the anticonvulsant for 14 days had

developed some form of tolerance in response to repeated VPA administration.

It is not possible on the basis of the present data alone to speculate on the

nature of such an adaptive mechanism, although there is evidence for

decreased VPA plasma levels (Löscher, 1985) in the rat and increased urinary

excretion of the drug following prolonged use in humans (Fisher et al, 1991).

Such pharmacokinetic changes following repeated administration of the drug

might be expected to result in a reduction of pharmacological efficacy, but this

possibility is further complicated by the likely envolvement of VPA

metabolites (such as 2-en-VPA) in the long-term pharmacodynamic and

neurochemical actions of the parent anticonvulsant (Löscher and Nau, 1982;

Nau and Löscher, 1984). Thus, the contribution of metabolites, principally 2-

en-VPA, to neurochemical effects during a prolonged treatment period with

the parent anticonvulsant may assume increasing importance with time. It could be argued that future studies of long-term effects on neurotransmitter release and metabolism should substitute VPA for 2-en-VPA, to assess whether similar tolerance phenomena (to those noted here with VPA) develop. In addition, it would be interesting to determine if the neurochemical changes reported here could be correlated with an anticonvulsant action over a similar treatment period, perhaps by using genetically seizure prone rats (see general introduction) as subjects.

Summary

1. Prolonged VPA administration, at least at a dose of 200mg/kg twice daily, did appear to result in some changes of basal neurotransmitter output measured in the rat hippocampus <u>in vivo</u>, most notably that of 5-HT, ASP and GLU.

2. High K^+-evoked efflux of DA, 5-HT, TAU and (possibly) GABA was also enhanced throughout the period of study, suggesting that such treatment had resulted in an accumulation of these substances in hippocampal cellular storage sites.

3. VPA-related changes in EC monoamines and amino acids largely subsided following the completion of treatment, suggesting that an adaptive response or tolerance to repeated administration had developed.

Chapter Six

Effects of tetrodotoxin pretreatment on monoamine and amino acid release in the ventral hippocampus of sodium valproate or saline treated rats

Introduction

Data presented in previous chapters suggest that VPA can induce changes in EC neurotransmitter and metabolite levels in the rodent brain following acute and prolonged treatment. Furthermore, the findings indicate that neurotransmitter systems thought to play a role in the aetiology of various epileptic conditions and experimental models of epilepsy are affected by the drug. Comparing these data with results obtained from ex vivo studies reveals that the anticonvulsant may alter EC monoamine and amino acid levels through a variety of neurochemical and neurophysiological mechanisms. For example, VPA enhances the synthesis and subsequent accumulation of both 5-HT (Whitton et al, 1985) and GABA (Nau and Löscher, 1982) in the rodent CNS, which appears to result in changes of output of both species, as measured by microdialysis. What is not clear however, is the nature of the mechanisms linking the observed changes in neurotransmitter turnover in vitro with neurotransmitter output quantified with in vivo techniques. Using microdialysis as a tool for studying changes in EC neurotransmitters in response to drug treatments provides us with phenomenological data, but tells us little of how the drug effects these changes. As was outlined in chapter 1, VPA is known to exert effects on cellular components other than enzyme systems coupled to neurotransmitter synthesis and catabolism. A starting point for examining the possible mechanisms whereby VPA induces apparent changes in neurotransmitter release would be to determine whether its effects are dependent upon ongoing neuronal activity. Using the classical blocker of voltage-operated sodium channels (Na^+-VOC's) tetrodotoxin (TTX; Narahashi,

1974) infused into the rat hippocampus via microdialysis probes, action potential dependent neurotransmitter release was reduced. VPA was administered to rats during this infusion period to determine if its acute effects on neurotransmitter output were dependent on action potential traffic to nerve terminals. It was reasoned that this investigation might clarify the following points: (i) Does VPA exert its effects on ventral hippocampal monoamine and amino acid levels by interacting with CNS elements remote from nerve terminal release sites or glial cells? (ii) Can the acute effects of VPA be related to an enhancement of Na^+-dependent depolarisation of neurones and / or glia and subsequent neurotransmitter release? (iii) Having effectively isolated neuronal terminals from their axons via block of voltage sensitive Na^+ channels, do the acute effects of VPA on EC monoamine and amino acid levels exhibit an absolute dependence upon action potential generation ?

Experimental details

Rats were implanted with microdialysis probes into the ventral hippocampus as detailed previously and allowed to recover overnight. Dialysis was commenced the following day and a total of 11 consecutive 30 min. dialysates collected from each animal. Animals were divided into 2 groups dependent on the treatments they received and these were as follows:-

Group 1. TTX only; Normal ACSF was infused from 0-120 min (dialysates 1-4) and then rats received a 60 min infusion of ACSF containing 1μM TTX. Animals recieved a saline injection i.p. 30 min. following the commencement of TTX infusion.

Group 2. TTX and VPA; Animals treated as above, but received VPA 400 mg/kg i.p. 30 min. following the commencement of TTX infusion.

The study was carried out in duplicate such that dialysates were available for quantification of both monoamines and amino acids. As before, dialysate monoamines were analysed using HPLC-ED and amino acids with HPLC-FD.

Data analysis

Data were analysed for statistically significant differences between animals receiving TTX/saline and those receiving TTX/VPA at each time point using a Mann-Whitney U test. Group sizes were n=8 it both cases.

Animals

Rats weighing 290-350g were used in this investigation.

Results

Basal levels of amino acids and monoamines measured in ventral hippocampal dialysates.

All estimates of basal amino acid and monoamine concentrations in ventral hippocampal dialysates were found to be within the same ranges as those calculated previously. These values may be found in the respective results sections of chapters 4 (monoamines) and 5 (amino acids).

Effects of TTX infusion on basal levels of VH monoamines.

Following infusion of TTX (1μM) for a period of 60 min, basal EC concentrations of monoamines were variously affected. Dialysate 5-HT and DA were rapidly and substantially lowered by the infusion of TTX (approximately 30 and 35% of basal levels prior to TTX infusion, respectively; figures 30a, 29a). The reduced output of both neurotransmitters was prolonged and accords with the findings of previous investigations (Sharp et al, 1990; Osborne et al, 1991). Metabolites of the two neurotransmitters were less affected by TTX infusion. EC levels of DOPAC were reduced to approximately 80% of control values 60 min following commencement of the infusion, whilst those of HVA were unaffected by the treatment (figures 29b, c). Previous investigation has similarly revealed a small effect on DOPAC levels and no change in HVA levels following TTX infusion into the dorsolateral striatum (Osborne et al, 1991). In the present study, 5-HIAA concentrations in dialysates were transiently, but not statistically significantly elevated in response to TTX (figure 30b).

Effect of TTX on basal levels of VH amino acids.

Ventral hippocampal amino acids were differentially affected by infusion of TTX. In contrast to monoamines, EC GABA accumulation was considerably less sensitive to TTX (figure 33), with the maximal reduction (approximately 50% of control level) delayed until 60 min post infusion. This finding further substantiates claims that only a proportion of brain EC GABA is derived from a TTX sensitive store (Osborne et al, 1991; Bourdelais and Kalivas, 1992), in contrast to another study that failed to detect any voltage dependency of GABA release in vivo (Timmerman et al, 1992). Dialysate GLU levels were unaffected by TTX infusion in the present study (figure 31a), a finding which is consistent with previous data (Drew et al, 1989), although in vivo K^+-evoked release of this amino acid from dialysed rat brain has also been demonstrated (Tossman and Ungerstedt, 1986; Westerink et al, 1987a; this thesis, chapter 5). In contrast, basal EC ASP levels were transiently *elevated* following TTX infusion, although this did not reach statistical significance (figure 31a). This is a curious finding, given the proposed neurotransmitter role attributed to brain ASP (Fonnum et al, 1986). Figure 32a demonstrates a lack of effect of TTX infusion on basal EC GLN in the rat hippocampus. This finding was expected since there exists little evidence suggesting that GLN fulfils a neurotransmitter role in the CNS. Jacobson and Hamberger (1984) found TTX to antagonise veratridine-induced decreases in olfactory bulb EC GLN concentrations and concluded that this was secondary to stimulation of ASP and GABA release, supporting a precursor role of GLN in the synthesis of these species (Bradford et al, 1983; Paulsen and Fonnum, 1989). In the present

(a)

(b)

142
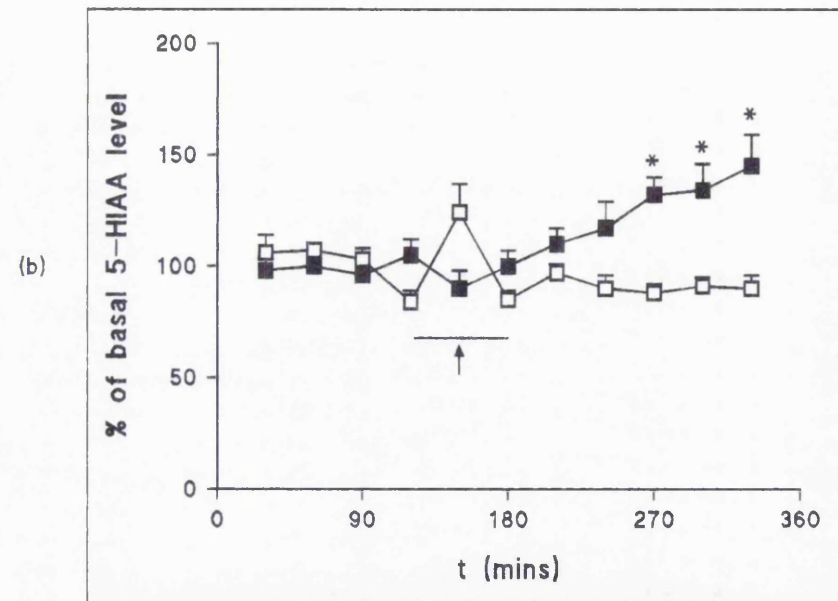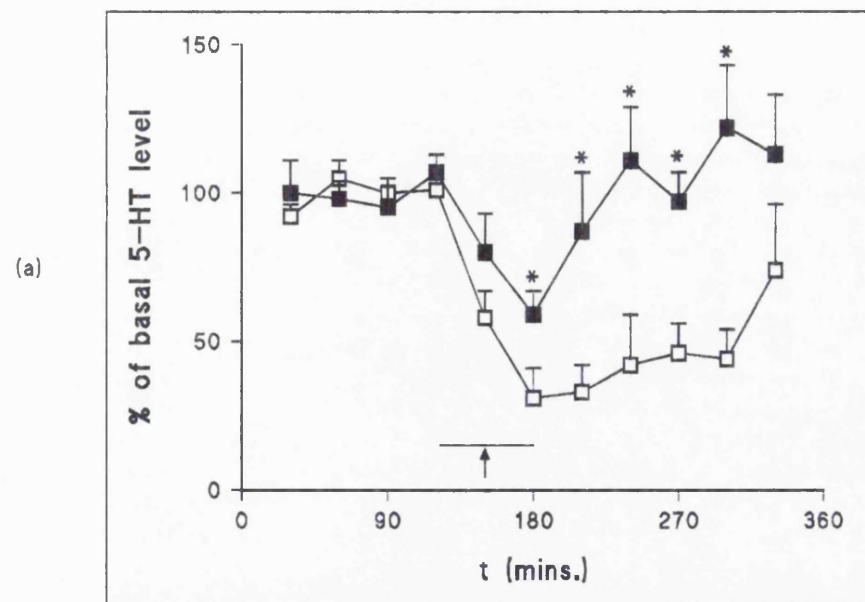
Figure 30 Effects of TTX infusion on dialysate 5-HT (a) and 5-HIAA (b) levels following administration of saline (open squares) or VPA 400 mg/kg (filled squares). Bar represents the period of TTX infusion, whilst the arrow denotes saline or VPA injection. * indicates values significantly different from their corresponding TTX/saline control values, p<0.05.

(a)

(b)

(c)
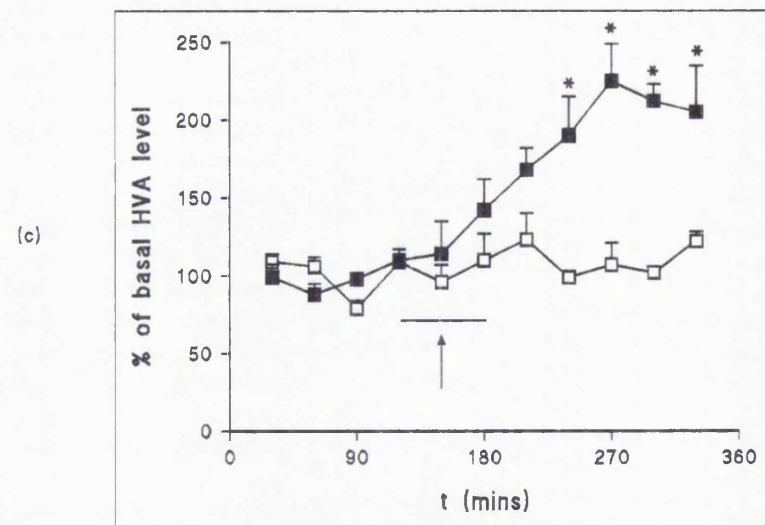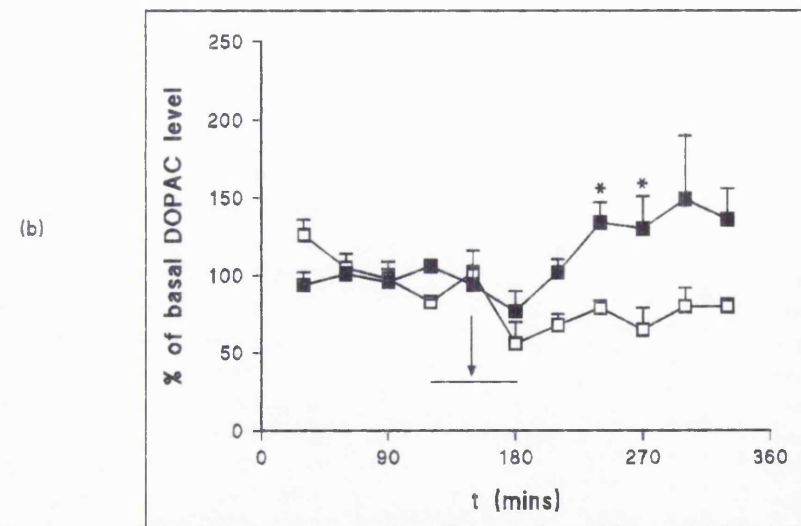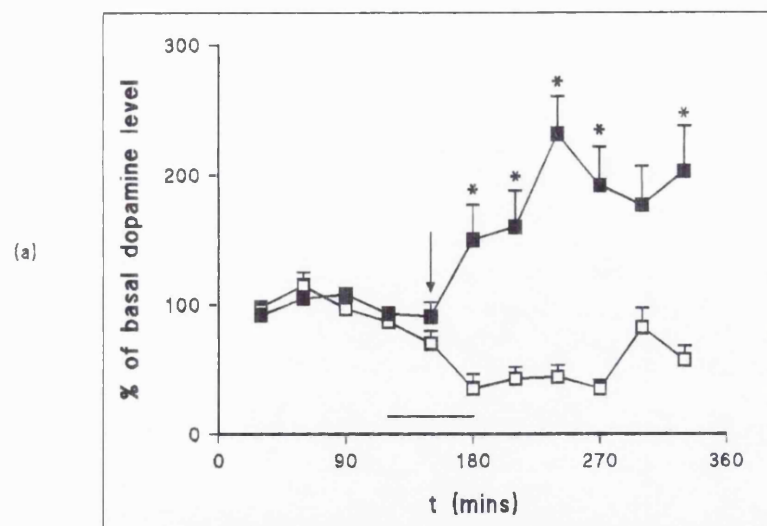
Figure 29 Effects of TTX infusion on dialysate DA (a), DOPAC (b) and HVA (c) levels following administration of saline (open squares) or VPA 400 mg/kg (filled squares). Bar represents the period of TTX infusion, whilst the arrow denotes saline or VPA injection.*indicates values significantly different from their corresponding TTX/saline control values, p<0.05.

study, EC hippocampal basal levels of the putative neurotransmitter TAU were found to remain unaffected by infusion of TTX (figure 32b), an observation consistent with those of other groups (Westerink et al, 1987b; Lehmann, unpublished data).

Effect of VPA adminstration during infusion of TTX on VH monoamines.

During and following infusion of TTX, acute administration of VPA affected EC monoamine accumulation rather differently than was previously demonstrated (Whitton and Fowler, 1991; this thesis, chapters 3 and 5). DA levels were elevated (maximal increase 250% of basal; figure 29a) following VPA administration, however the rate of increase was found to follow a slower time course than previously observed when animals were treated with VPA alone (Chapter 3, this thesis). In addition, EC DA remained elevated above basal output levels for the duration of microdialysis, a finding which is in sharp contrast with the transient response observed in the absence of TTX. Additionally, DOPAC accumulation in VH dialysates followed a similar timecourse to that of DA, post VPA administration (figure 29b). In contrast to previous observation (Chapter 3, this thesis), EC DOPAC levels were only modestly, although sustainedly elevated above basal values, reaching statistical significance 90 min following administration of VPA. Levels of HVA rose gradually in dialysates following VPA administration and the increases were sustained for the duration of the collection period (figure 29c). This contrasts with the null effect of the anticonvulsant on levels of this metabolite when administered in the absence of TTX.

Interestingly, in the present study the effects of acute VPA administration

on 5-HT output appeared to possess a greater dependency upon impulse flow

traffic to nerve terminals than in the case of DA output. Figure 30a clearly

shows that 5-HT output was biphasic following VPA injection. Thus, unlike

the output of DA following VPA treatment, dialysate 5-HT levels were

significantly reduced below basal values 30 min post VPA injection, although

the reduction was not as marked as in equivalent dialysates collected from

group 1 animals. In dialysates collected subsequent to this time point, levels

of 5-HT rose and were maintained at near basal values, in contrast to levels of

those measured in equivalent group 1 dialysates which remained significantly

below basal values for the duration of the experiment. Levels of the 5-HT

metabolite 5-HIAA exhibited a delayed and sustained increase in dialysates

collected 60 min post VPA treatment until the completion of microdialysis

(figure 30b).

Effect of VPA administration during infusion of TTX on VH amino acids.

Only GABA and ASP EC levels were significantly changed following acute

VPA treatments in the presence of intrahippocampal TTX. As was observed

with dialysate 5-HT during the present study, VPA altered EC GABA in a

biphasic manner. Initially, VPA induced only a non-statistically significant rise

in basal GABA (figure 33), suggesting that the initial increase in EC GABA

noted previously may be partially TTX sensitive (figure 15, chapter 4).

Subsequently dialysate GABA levels returned towards basal values and were

maintained at this level for the remaining period of microdialysis. Curiously,

EC ASP levels were substantially increased above basal values, reaching

statistical significance 60 min following VPA injection (figure 31a). Subsequent

to this time point, ASP levels fell to basal and remained so for the duration of the collection period. Both GLU and GLN were slightly elevated above basal levels (non significantly) following VPA injection, indicating that these small VPA-evoked changes were voltage independent (figures 31b, 32a). The magnitude and duration of the changes in basal GLU levels post VPA injection (given during TTX infusion) are similar to those observed previously in the absence of TTX (figure 16a, chapter 4). TAU levels were not altered following acute treatment with VPA and the presence of TTX in the ACSF did not appear to modify TAU ouput under these conditions (figure 32b).
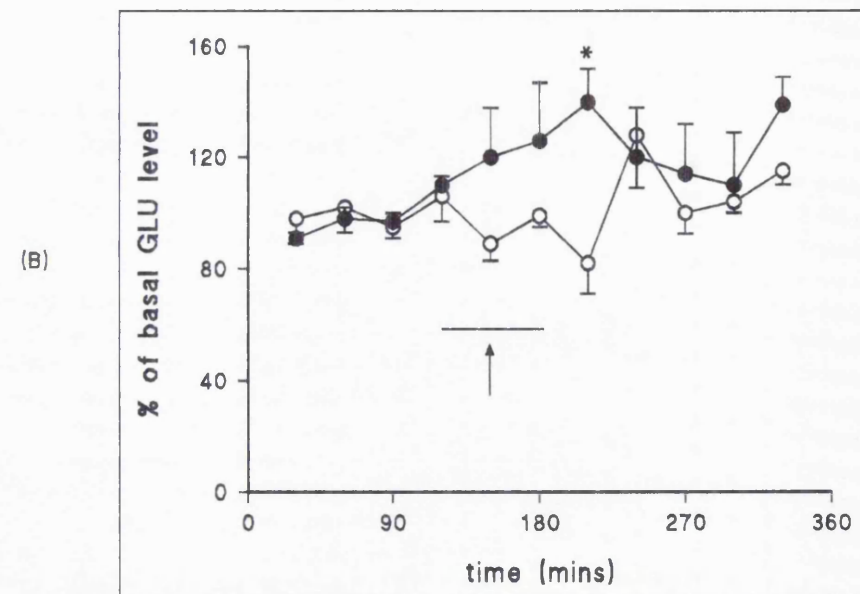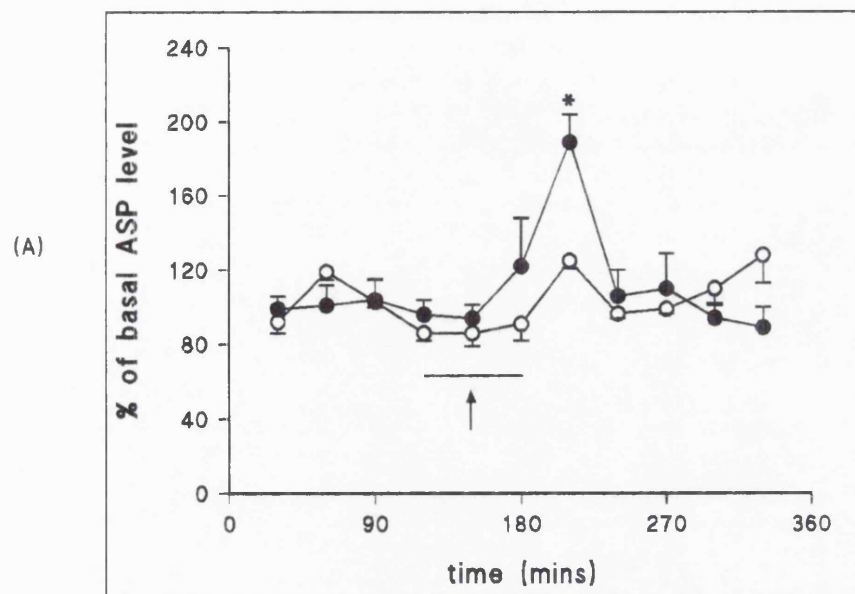
Figure 31 Effects of TTX infusion on dialysate ASP (A) and GLU (B) levels following administration of saline (open circles) or VPA 400 mg/kg (filled circles). Bar represents the period of TTX infusion, whilst the arrow denotes saline or VPA injection. * indicates values significantly different from their corresponding TTX/saline control values, p<0.05.
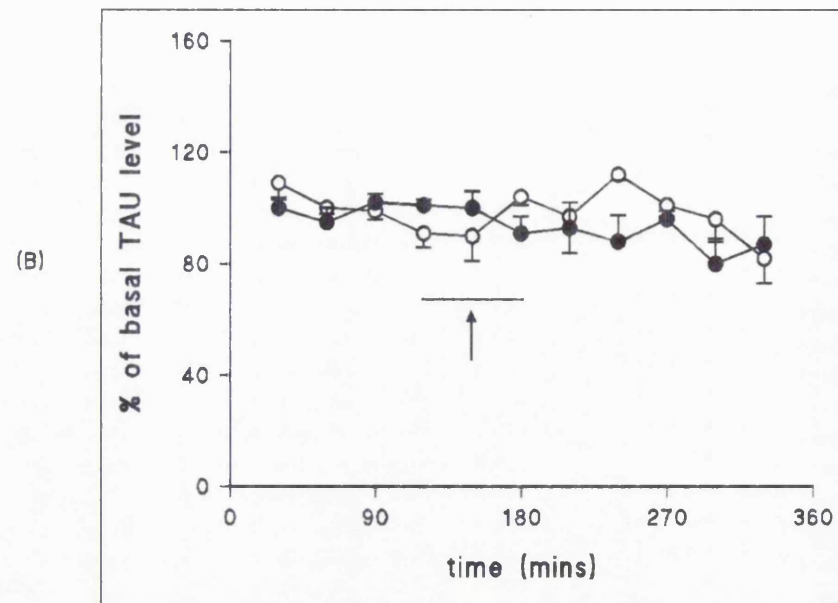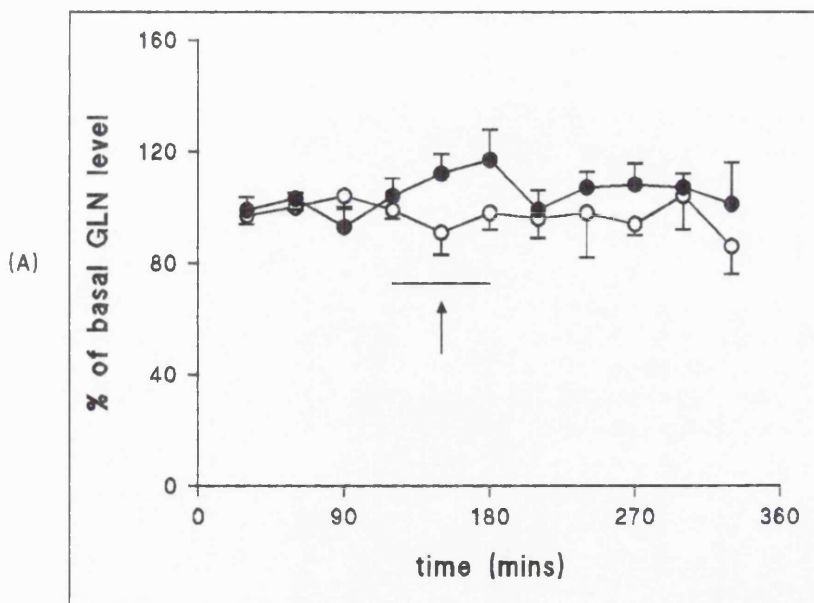
Figure 32 Effects of TTX infusion on dialysate GLN (A) and TAU (B) levels following administration of saline (open circles) or VPA 400 mg/kg (filled circles). Bar represents the period of TTX infusion, whilst the arrow denotes saline or VPA injection.
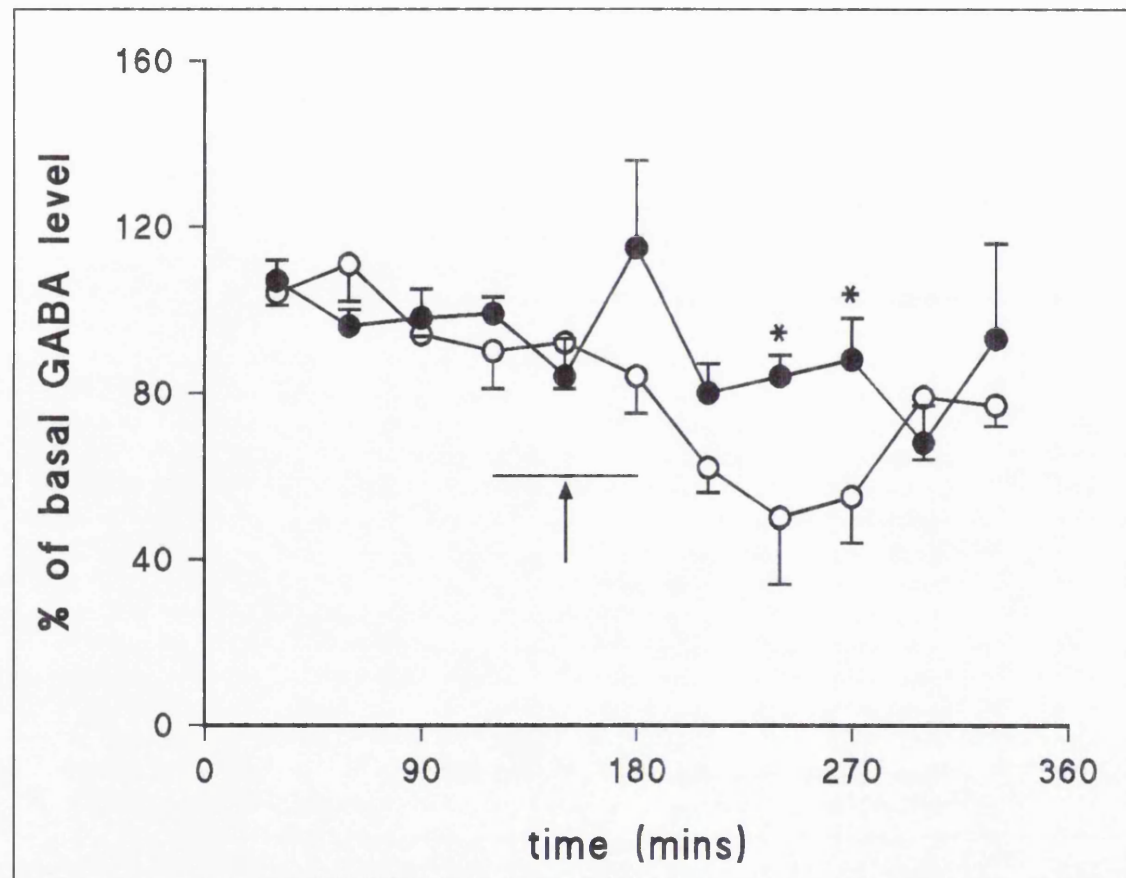
Figure 33 Effects of TTX infusion on dialysate GABA levels following administration of saline (open circles) or VPA 400 mg/kg (filled circles). Bar represents the period of TTX infusion, whilst the arrow denotes saline or VPA injection. * indicates values significantly different from their corresponding TTX/saline control values, p<0.05.

Discussion

The results obtained in this study suggest that the effects of acute VPA treatment on EC concentrations of monoamines and amino acids in the rat ventral hippocampus are, in part, mediated via mechanisms other than voltage dependent neurotransmitter release. However, infusion of TTX-containing ACSF did attenuate the responses to acute VPA with regard to output of GABA and 5-HT.

Monoamines.

The data presented in chapter 3 of this thesis indicate that the responses of both dopaminergic and serotonergic systems (especially in the VH) to acute administration of VPA are rapid in onset. Given the limited temporal resolution inherent in microdialysis, it was not possible to accurately determine the precise point during the 30 min post injection period corresponding to maximal accumulation of extracellular monoamines (see figures 29a, 30a). This fraction does however correspond with the time taken for peak brain levels of radiolabelled VPA to be reached following i.p. injection (30 min; Aly and Abdel-Latif, 1980), further strengthening the argument that VPA induces a rapid elevation in EC monoamines. At this stage it was felt that further investigation in an attempt to elucidate mechanisms by which VPA induces changes in EC monoamine levels was desirable.

With regard to DA, the effects of TTX infusion observed in the present study are of particular interest. DA has a controversial status as a neurotransmitter in the ventral hippocampus (Ishikawa et al, 1982; Hörtnagel et al, 1991) and it has been suggested that since the dopaminergic projection to the

hippocampal formation is relatively sparse, measurement of the monoamine

here may in part reflect its presence as a precursor for noradrenaline synthesis

and thus be derived principally from noradrenergic terminals (Scatton et al,

1980; Hörtnagel et al, 1991). However, selective lesioning of ascending

noradrenergic pathways does not reduce the DA content of ventral

hippocampal tissue (Bischoff et al, 1979) and the effects of TTX in the present

study strongly suggest that a substantial proportion of ventral hippocampal

DA is derived from ongoing dopaminergic neuronal activity. Evidence

presented by Vahabzadeh and Fillenz (1992) suggested that hippocampal

DOPAC was derived approximately equally from noradrenergic and

dopaminergic terminals (see chapter 3, discussion). TTX infusion did not result

in a substantial reduction in EC DOPAC accumulation (80% of basal level) and

no change in HVA levels (figs 29b, c). These findings receive support from

results of previous studies, which have consistently failed to observe a

significant coupling between DA release and metabolite accumulation

(Westerink et al, 1989; Osborne et al, 1991). However, Vahabzadeh and Fillenz

(1992) did record a significant reduction of EC DOPAC following infusion of

1µM TTX in VH dialysates, although the length of pulsing was longer than in

the present study (80 min vs 60 min). The present data reveal that VPA

treatment appears to result in an elevation of EC DA above basal

concentrations in the VH, despite the presence of TTX to reduce action

potential traffic to catecholaminergic terminals (figure 29a). However,

comparison of the respective responses of basal DA levels in the VH to

VPA/TTX in this study and that following administration of VPA alone

(Chapter 3, this thesis) reveal a marked difference in character. Figure 11a

(Chapter 3) indicates that VPA treatment alone results in a rapid and transient

(<30 min) increase in EC DA above basal release, whilst figure 29a indicates

that DA efflux follows a more gradual and sustained pattern of increase. The

mechanisms through which TTX pretreatment may affect DA release following

acute VPA are by no means clear. It is unlikely that VPA increases DA levels

through a carrier dependent mechanism in the same manner as the

psychostimulant amphetamine (AMPH), since the response of striatal DA

release to this drug was not TTX sensitive (Westerink et al, 1987b), whereas,

in the present study, TTX infusion markedly increased the time course of the

DA response to VPA. The TTX insensitive increase in dialysate DA following

VPA treatment may reflect an increase in intraneuronal DA synthesis and

turnover, since EC levels of DOPAC were concomitantly, although modestly

elevated above basal. As previously discussed, EC DOPAC collected in

dialysates is most likely to be primarily derived from newly formed DA

(Zetterström et al, 1988) and therefore an increased level of the metabolite

measured in the EC space might indicate increased intraneuronal turnover of

the neurotransmitter. Additionally, VPA treatment resulted in a sustained

increase of EC HVA levels above basal commencing during the TTX infusion,

an observation not made when VPA was administered in its absence.

Presumably, HVA levels rise under these circumstances because of the

persistence of DA and DOPAC in the EC space. Combining data from chapters

3 and 6, it could be argued that VPA induces a rapid release of the

monoamine which is followed swiftly by an equally rapid fall, a response that

may exhibit some voltage dependency. However, it should be noted that the character of the response is similar to that observed when neurotransmitter release is evoked with high K^+ ACSF (Kalen et al, 1988; Robertson et al, 1991), a phenomenon that is TTX insensitive (Fairbrother et al, 1990). The rapid termination of the response to VPA administered in the absence of TTX might be mediated by a presynaptic event such as stimulation of autoreceptors or, more generally, high affinity DA reuptake. When animals were treated with VPA in the presence of TTX, DA appeared to egress from neurones in an unregulated fashion, possibly reflecting a disablement of these presynaptic control mechanisms. Although brain VPA levels decline very rapidly following a single i.p. dose in rodents (Nau and Löscher 1982), this is unlikely to account directly for the transient rise in EC DA, since the response to the drug persists following TTX pretreatment. Alternatively, one might argue that TTX could in some way impede clearance of the anticonvulsant from the area directly adjacent to the microdialysis probe, although this seems unlikely.

In contrast to the response of DA containing neurones in the rat VH to combined TTX/VPA treatment, the release of 5-HT occured in a biphasic manner. The initial component (0-60 min post VPA) was TTX sensitive, VPA merely reducing the rate of decline in EC 5-HT levels. During the subsequent phase (60-210 min post VPA), 5-HT levels rose toward basal, a response which was TTX insensitive (note the persistent reduction in 5-HT output following cessation of TTX infusion in group 1 animals, possibly reflecting delayed washout of the neurotoxin). Interestingly, the duration of this secondary component closely followed the increase in rat whole brain 5-HT synthesis

noted by Orešković et al (1985) following VPA treatment. Therefore, it is possible that 5-HT output is depressed in a TTX sensitive manner until approximately 60 min following VPA treatment after which time, EC levels of the monoamine rise toward basal values, concomitant with VPA stimulated 5-HT synthesis. Under conditions of an apparently persistent blockade of serotonergic neuronal firing, it could be argued that newly synthesised 5-HT overflows from presynaptic transmitter pools, thereby passing into the EC space. However, it must be stressed that no attempt was made in the present study to characterise any Ca^{2+}-dependency of these acute responses to VPA and it is possible that the drug might interact with Ca^{2+}-linked vesicular release mechanisms in order to effect these changes. 5-HIAA levels rose in dialysates collected from group 2 animals, concomitant with the increase in 5-HT output, formerly depressed by TTX. 5-HIAA levels were not observed to rise following treatments with VPA alone (chapter 3, figure 11b). Since the presence of the 5-HT uptake inhibitor citalopram in ACSF would theoretically preclude a significant portion of EC 5-HIAA being derived from released 5-HT, it could be concluded that the current findings reflect increased intraneuronal turnover of the monoamine, stimulated by VPA. It is not clear why EC 5-HIAA accumulation fails to significantly increase in animals acutely treated with VPA only. One possible explanation is that the rapid elevation in EC 5-HT stimulated by VPA administration effectively inhibits enhanced turnover of the neurotransmitter, serotonergic neurones maintaining basal 5-HIAA output under these conditions. As was observed for DA output under the same conditions, the rapid termination of 5-HT overflow is consistent with

stimulation of presynaptic autoreceptors (5-HT$_{1A}$ or 5-HT$_{1B}$ subtypes) which are thought to regulate presynaptic serotonergic function (Hjorth and Tao, 1991; Lawrence and Marsden, 1992). This would explain the apparent lack of increase in 5-HIAA. Indeed a small, non-statistically significant *decrease* in EC 5-HIAA levels was consistently noted at time points following administration of 400 mg/kg VPA (chapter 3, figure 11b) and in some animals receiving repeated VPA doses, a significant decrease in the metabolite was noted following high K$^+$-infusion (Chapter 5, figure 23, days 4 and 7). In contrast the present study suggests that since 5-HT release was impeded by TTX, intraneuronal conversion of 5-HT to 5-HIAA might be expected to be enhanced in the presence of VPA. These findings share similarities with those presented in chapter 5, since basal 5-HIAA levels collected from animals receiving repeated VPA doses tended to be higher than those from control animals. It was concluded that prolonged treatment with the drug may have resulted in elevated intraneuronal turnover of the parent monoamine, since citalopram was again incorporated in ACSF used in these experiments.

Amongst the questions remaining to be addressed arising from the current findings are those relating to the apparent differential sensitivity of catecholaminergic and serotonergic neurones to combined TTX/VPA exposure: Why does 5-HT release following VPA administration remain at or below basal levels, whilst that of DA appears to be relatively unaffected in the presence of TTX? The reasons for these different responses are not clear on the basis of the current observations alone. However, there exist a number of possible explanations. (i) The two monoaminergic systems may possess

differing sensitivities to the biochemical changes induced by VPA. Horton et al (1977) did not find rodent whole brain DA and 5-HT synthesis to be differentially affected by 400-600 mg/kg VPA, although no attempt to characterise any brain regional heterogeneity of monoamine turnover was made. In the absence of any data of this nature, one might conclude that the drug affects monoaminergic terminals differentially at the level of the neuronal membrane and synaptic release mechanisms, rather than the presynaptic apparatus responsible for synthesis and catabolism of these species. (ii) VPA might influence the function of extraneuronal elements (such as astrocytes) with respect to buffering EC levels of monoamines. Rat primary astrocytes are thought to possess active uptake sites for monoamines and uptake of [^{3}H]5-HT into cultured cells seems to occur more readily than that of [^{3}H]DA (see Kimelberg, 1986 for review). It remains to be seen if VPA alters affinity or transport capacity of these glial monoamine selective uptake systems and data of this nature could prove valuable in delineating extraneuronal contributions to the effects of VPA on EC neurotransmitter levels. (iii) Serotonergic and catecholaminergic neuronal projections to the ventral hippocampus may differ in their sensitivities to TTX. Hence, recovery of action potential transmission to terminal release sites could occur selectively following TTX infusion, affecting the respective durations of reduced monoamine outputs. Unless this phenomenon occurs following VPA treatments only, this theory must be rejected, since EC levels of both monoamines recovered at approximately similar rates (figures 29a, 30a).

Amino acids

Data presented in figures 31b and 32 demonstrates the relative insensitivity of VH amino acid accumulation <u>in vivo</u> to TTX infusion. Only the effects of acute VPA treatments on dialysate levels of GABA and ASP (figures 31a and 33) were significantly modified by the neurotoxin.

A recurrent feature of many <u>in vivo</u> studies and also those utilizing <u>ex vivo</u> CNS tissues, is the inconsistency with which TTX sensitive amino acid release can be demonstrated. Use of <u>in vivo</u> microdialysis as a tool for investigating neurotransmitter release mechanisms is probably a more reliable approach than employing <u>ex vivo</u> tissues, since the functional integrity of neuronal circuits is preserved to a greater degree and mechanical damage to terminals is minimised (Bernath, 1992), providing an adequate post-operative recovery period is allowed. Using the microdialysis technique, central EC GABA has been shown to be partially derived from ongoing neuronal activity (Osborne et al, 1991; Bourdelais and Kalivas, 1992). However, this phenomenon has not been universally demonstrated (Westerink and de Vries, 1989; Timmerman et al, 1992). The results of the present study seem to support the findings of those conducted in the former two laboratories since a reduction of basal GABA output by approximately 50% was demonstrable following TTX infusion (figure 33). The lack of consistency between the results of these studies is probably attributable to differing experimental conditions, such as varying ACSF Ca^{2+} concentrations, TTX infusion duration, microdialysis probe design, brain region studied (Timmerman et al, 1992) and a wide range of perfusion rates (0.5-6µl/min). Since GAD is thought to be principally located in neurones

(Roberts, 1979) and Timmerman et al (1992) noted a reduction in substantia nigra EC GABA levels of only 50-60% following treatment with the irreversible GAD inhibitor, 3-MPA, it is likely that newly synthesised GABA (possibly representing 'transmitter' amino acid) contributes only a proportion of the entire EC pool. This is in contrast with monoamine neurotransmitters, EC levels of which may be almost totally derived from actively firing neurones (Westerink et al, 1987b; Sharp et al, 1990). A common feature of most studies investigating the cellular origin of GABA and other amino acids in vivo is that a significant proportion of basal efflux is not only TTX-insensitive, but is also independent of the presence of EC Ca^{2+}. Possible sources of this 'non-transmitter' GABA include a large neuronal cytoplasmic pool (Abe and Matsuda, 1983) which may reach the EC enviroment via Na^{+}-dependent bi-directional GABA carrier sites on neurones (Levi and Raiteri, 1978; Neal and Bowery, 1979) and from local glial cells (Pearce et al, 1981; Sarthy, 1983). It has also been proposed that the fail ure of microdialysis to consistently record GABA release representing ongoing neuronal activity may be due to restricted access of the active dialysis membrane area to neuronally-derived EC pools of the amino acid (Westerink and de Vries, 1989). These authors addressed the possibility that a reactive glial proliferation against the dialysis membrane in situ might result in dialysate GABA being largely representative of efflux from these cells, rather than local neurones. This hypothesis is not supported however, by data that suggest this tissue reaction is only initially evident 2-3 days following probe implantation (Hamberger et al, 1983; Hamberger and Nystrom, 1984).

With the exception of a small and transient increase in ASP, basal EC levels

of other amino acids of interest in the current work (GLU, GLN and TAU)

failed to disclose any significant changes following TTX infusion (figures 31b,

32a, b). This would suggest that a large proportion of the total hippocampal

EC amino acid pool is not derived from action potential evoked neuronal

release. Indeed, as with GABA, other workers have inconclusively

demonstrated impulse flow dependent amino acid release in vivo. The origins

of brain EC TAU are of particular interest with respect to the current study.

As has been demonstrated, prolonged treatment of rats with VPA resulted in

changes in basal and K^+-evoked TAU release (chapter 5, this thesis) and TAU

possesses anticonvulsant activity (Bonhaus and Huxtable, 1983; Bonhaus et al,

1983). Although Westerink et al (1987a) could not demonstrate any impulse

flow dependent release of the amino acid in vivo (an observation which

accords with the current data), Ca^{2+}-dependent TAU release from rabbit

hippocampus in response to NMDA infusion has been observed (Lehmann et

al, 1985). The same authors further suggested that TAU in this structure might

be released from somatodendritic sites, since NMDA was not found to

influence *synaptosomal* Ca^{2+} accumulation. Jacobson et al (1986), and Jacobson

and Hamberger (1984) found EC accumulation of ASP, GLU and TAU to be

ouabain and TTX sensitive during microdialysis of the rabbit olfactory bulb.

Paradoxically, Westerink et al (1989) found rat striatal 'neuroactive' amino acid

release to be stimulated by ouabain, whilst basal release was found to be both

TTX and Ca^{2+} independent. The authors considered it most likely that a major

portion of the extracellular pools of these species were not derived from classic

action potential-exocytotic coupled vesicular release. However, the possibility that neuronal terminals in this area could mobilise internal stores of Ca^{2+} to facilitate vesicular release of amino acids in response to an appropriate neurochemical or electrical stimulus was not dismissed. In addition, TTX-independent release of brain amino acids in vivo, evoked by ouabain or veratridine could theoretically arise through reversal of Na^+-driven amino acid carrier sites located in both neuronal and glial cell membranes, a mechanism that is also Ca^{2+}-independent (Levi and Riateri, 1993).

Acute VPA dosing did not modify the EC levels of GLN or TAU significantly when administered in the presence of TTX. The modest elevation in EC GLU and the small effect on GLN levels following VPA displayed no TTX sensitivity and may instead represent a metabolic response to the drug, reflecting increased supply of substrates for GABA synthesis (Paulsen and Fonnum, 1988). The lack of effect of acute VPA dosing on EC TAU levels is a consistent finding (see figure 17b, chapter 4) and suggests that only prolonged dosing with the drug can alter accumulation of this amino acid in the rat CNS (Patsolos and Lascelles, 1981; chapter 5, this thesis).

Dialysate GABA levels were altered by acute VPA administration in the presence of TTX (figure 33), with respect to GABA output measured in group 1 animals. Comparison with previously obtained data (figure 15, chapter 4) suggests that the initial increase in EC GABA in the rat VH is largely TTX sensitive, since the VPA-induced overflow of GABA was significantly reduced. Additionally, GABA levels during the period t=210 to 330 min inclusive remained at basal values, contrasting with a persistent (although non-

statistically significant) elevated efflux observed in non-TTX treated animals. This strongly implies that the acute effects of the drug on EC GABA accumulation may be, in part mediated via enhancement of neuronal depolarization. The relatively modest elevation of dialysate GABA following i.p. administration of 400 mg/kg VPA is in contrast to the massive effluxes of amino acid evoked by high K^+ ACSF (Tossman et al, 1986; Westerink and de Vries, 1989; this thesis, chapters 5 and 7). As has been discussed previously, high K^+ evoked release of amino acids both <u>in vitro</u> and <u>in vivo</u> is a relatively non-specific phenomenon, resulting in activation of Ca^{2+}-dependent transmitter release (Schousboe and Pasantes-Morales, 1989), inhibition of Na^+-dependent active uptake mechanisms (Drejer et al, 1982; Kramer and Baudry, 1984) and amino acid release from glial cells (Albrecht and Rafalowska, 1987). Unfortunately, time constraints have not permitted an examination of the Ca^{2+}-dependency of the initial (and rapid) elevation in EC GABA level following acute VPA administration. However, it seems likely that the drug may more selectively stimulate release of GABA from a 'transmitter pool', since it appears to readily enhance accumulation of the amino acid in synaptosomes, compared with whole tissue (Löscher and Vetter, 1985). The maintenance of basal EC GABA levels observed in the current study following combined VPA/TTX treatment and its comparison to equivalent data from group 1 animals would suggest that VPA may also exert some of its effects independently of an ongoing blockade of action potential traffic. It is of course possible that this TTX-insensitive efflux of GABA may require the presence of external Ca^{2+} (a theory yet to be tested) or alternatively reflects a less specific

action of the drug, that of presynaptic neuronal membrane disruption (Perlman and Goldstein, 1984). However, in the absence of any available in vivo data to support the former assertion and the observation by the above cited authors that such membrane disordering properties of the drug occur only in the mM concentration range, alternative explanations must be sought. It is perhaps significant that this TTX-insensitive component of in vivo GABA accumulation correlates well with the time-course for increased GABA synthesis observed in vitro following acute VPA treatments (Nau and Löscher, 1982). Löscher and Vetter (1985) found that 200 mg/kg VPA i.p. resulted in elevated hippocampal synaptosomal GABA concentrations (maximum increase, 180% of basal), although levels had fallen below statistical significance 120 min post treatment. However, it is certainly a possibility that the higher dose of anticonvulsant used in the present studies could result in a more persistant elevation of nerve terminal GABA accumulation and this might account for the currently observed TTX-insensitive egress of amino acid into the EC space. Lastly, a further possible source of TTX-insensitive GABA in dialysates associated with acute VPA treatment could represent efflux from local glial cells. Recent evidence presented by Nilsson et al (1992), using cultured neonatal rat astrocytes, suggests that short-term incubation with VPA can reduce uptake carrier affinity for GABA thereby providing a possible mechanism by which interaction of VPA with local glial cells could result in transient EC GABA elevations.

The enhanced (although transient) elevation of basal ASP levels following combined TTX and VPA treatments is both a curious and anomalous finding

(figure 31a). Other groups have failed to note any significant impulse flow dependency of basal ASP release in vivo (Butcher and Hamberger, 1987; Westerink et al, 1987a) or only a partial TTX sensitivity (Girault et al, 1986), suggesting that a proportion of basal EC excitatory amino acid release is as a result of spontaneous efflux from neuronal and non-neuronal elements (Bernath, 1991). Since a small, non-significant increase in dialysate ASP was observed following infusion of TTX, it is possible that the resultant action potential-linked neuronal inhibition could transiently *disinhibit* ASP release from excitatory terminals. No evidence currently exists to support this theory. However, in group 1 animals there seems to exist some temporal correlation with the onset of falling GABA levels noted following TTX infusion. Why the response should be enhanced in the presence of VPA is a matter of conjecture. This apparent enhancement of ASP accumulation in the hippocampal EC space following VPA administration is interesting since most of the related literature suggest that the drug decereases cerebral ASP turnover, at least following acute dosing (Schecter et al, 1978; Chapman et al, 1982). Perhaps the drug enhances local turnover of ASP (an effect reported during prolonged treatment with VPA, Patsolos and Lascelles, 1981) and following TTX infusion, results in a further enhancement of ASP release. The situation is further complicated by reports that acute VPA treatments *reduce* ASP release in vitro (Crowder and Bradford, 1987).

Clearly, using TTX in these experiments as a tool for investigating possible mechanisms by which VPA influences acute neurotransmitter release and metabolism in vivo has uncovered a complex neurochemical picture. The

anticonvulsant appears to affect local monoamine and amino acid release and

metabolism quite differently when neuronal firing is reduced by TTX, than in

the absence of the neurotoxin. Obviously, these interactions require further

characterisation and it is clear that VPA does not influence neurotransmitter

release via a single mechanism.

Summary

1. The initial rapid (but transient) release of DA <u>in vivo</u> previously observed following acute VPA injection was largely abolished during focal infusion of TTX into the VH. The response to VPA was more gradual, peak increases in dialysate DA occurred 120 min post injection.

2. Dialysate 5-HT fell sharply during TTX infusion and VPA injected 30 min from the start of infusion failed to reverse the decline in basal 5-HT output, merely reducing the rate of its decline. On cessation of TTX infusion, dialysate 5-HT rose swiftly towards basal levels in animals that had recieved VPA. These data indicate that the effect of acute VPA administration on 5-HT output <u>in vivo</u> consists of TTX- sensitive and - insensitive components.

3. At present, it is not possible to determine why output of DA and 5-HT are differentially affected by VPA during block of action potential traffic to hippocampal terminals.

4. Dialysate GLU, GLN and TAU were relatively unaffected following VPA administration, both in the presence and absence of TTX. In addition, TTX alone failed to affect output of the amino acids.This is consistent with previous data and suggests that release of these amino acids <u>in vivo</u> is not significantly voltage dependent. The small increase in GLU following VPA was probably as metabolic consequence of a stimulant action on GABA synthesis.

5. Dialysate ASP increased following VPA injection following TTX infusion. It is not clear as to the mechanism of this response, although disinhibition of ASP release is a possibility.

6. Both the initial rapid rise in dialysate GABA and the persistant elevation

above basal output observed when VPA was given in the absence of infused

TTX were depressed in the present study. This suggests that in addition to

effecting an increase in GABA synthesis and turnover <u>in vivo</u>, VPA may also

increase dialysate GABA by activating voltage-dependent release of the amino

acid.

Chapter Seven

The effect of isonicotinic acid hydrazide on extracellular amino

acids and convulsions in rats: Reversal of neurochemical and

behavioural deficits with sodium valproate.

Introduction

As demonstrated in chapter 4, hippocampal EC GABA levels were substantially increased following acute administration of VPA (400 mg/kg). Interestingly, this effect correlated temporally with enhanced GAD activity measured in rodent whole brain (Nau and Löscher, 1982) and GABA levels in synaptosomes (Löscher and Vetter, 1985). These authors also found a strong correlation between these parameters and protection against seizures induced by maximal electroshock and 3-MPA. In chapter 6, it was apparent that a proportion of hippocampal GABA release following acute treatment with 400 mg/kg VPA was TTX insensitive. Together with ex vivo findings of other studies extensively discussed in previous chapters, these data strongly suggest that VPA exerts a proportion of its effects on EC GABA by increasing synthesis and accumulation of the amino acid in the neuronal compartment.

The occurrence of convulsions in rodents following administration of hydrazides is well documented (Horton et al., 1979; Holdiness, 1987). Reduction of the brain concentration of GABA as a result of inhibition of GAD is considered to be the mechanism leading to seizures (Wood and Peesker, 1973). Pyridoxal-5-phospate (PLP) is a co-factor for GAD and other decarboxylases (: reviewed by Ebadi, 1981). Isonicotinic acid hydrazide (INH) and other hydrazides inhibit the enzyme pyridoxal kinase (ATP: pyridoxal 5-phosphotransferase, EC 2.7.1.35) which catalyses the formation of PLP (McCormick et al., 1960; Tapia and Awapara, 1967). PLP is derived from pyridoxine (vitamin B_6) and hydrazide-induced convulsions have a similar aetiology to those caused by dietary pyridoxine deficiency, particularly in

neonates (Guilarte et al., 1987; Guilarte, 1989). Given that administration of a convulsant dose of INH leads to a depletion of whole brain GABA (Wood and Pesker, 1973) and both hippocampal GABA and PLP (Horton et al, 1979), it would be of interest to determine whether these changes are reflected in reduced GABA output in vivo. VPA appears to enhance GABA release primarily via increasing its synthesis and INH depletes tissue GABA through an indirect inhibition of GAD. Hypothetically, reversal or prevention of INH-induced neurochemical deficits and convulsions with VPA, monitored in vivo would provide further insight into the interaction of VPA with the brain GABAergic system at the biochemical level. To this end the effect of acute INH administration, alone and in combination with VPA, on hippocampal EC amino acid concentrations was monitored using in vivo microdialysis. In addition, hippocampal amino acid release was evoked by high K^+ infusion, in order to give an indication of the relative sizes of releasable amino acid pools following treatment with INH alone or in combination with VPA.

Experimental details

Rats had microdialysis probes implanted into the VH as detailed in methods section 2.3 and allowed to recover overnight. In all cases, 12 consecutive dialysates were collected and were subsequently analysed for amino acid content using HPLC-FD. Animals were divided into 3 treatment groups on the following basis:-

Group 1. INH alone. Following the collection of 4 dialysates (0-120 min), rats in this group were given a convulsant dose of INH (250 mg/kg) and saline, both i.p.

Group 2. INH and VPA. Rats in this group received i.p. doses of VPA (400 mg/kg) and INH (250 mg/kg) following the collection of 4 dialysates (0-120 min.)

Group 3. Control group. Rats received i.p. injections of saline at the same time point for injection of drugs in the treatment groups.

Additionally, an attempt was made to estimate the relative sizes of the hippocampal amino acid pools available for release in all 3 groups, using high K^+ ACSF to evoke release of amino acids. In all cases, ACSF containing 100 mM K^+ was infused via the microdialysis probe for a period of 30 min following the collection of 7 dialysates (0-210 min). After collection of dialysate 8, high K^+ ACSF was replaced once more with normal ACSF for the remainder of the dialysis session.

<u>**Animals**</u>

Rats weighing 250-270g were used in this investigation.

Data analysis

Data were converted to percent of basal amino acid level on the same basis as those data presented in chapters 3, 4 and 6. Statistical comparisons between saline and drug treatments were made with a Mann-Whitney U test. Group sizes were n=8 in each case.

Results

Basal levels of amino acids in the rat VH determined in the present study were in close accordance with those previously observed (see chapter 4).

When given alone INH did not alter the basal level of any of the amino acids studied (figures 34, 35), with the exception of GABA. As shown in figure 36, after INH injection a rapid and sustained decrease in EC GABA was observed. During this period, approximately 30 min post drug, marked behavioural changes occurred in INH treated rats and these are summarised in table 9. It is notable that when VPA was co-administered with INH, no animals displayed convulsions at any stage (table 9). Neurochemically, VPA was found to completely reverse the decrease in dialysate GABA caused by INH, in fact a statistically significant elevation of basal GABA was observed (figure. 36). Of the amino acids studied, only GLN disclosed no increase following infusion of high K$^+$, the dialysate concentration of which was in fact decreased, irrespective of prior treatment, although this did not reach statistical significance (figure. 35a). This decrease may be associated with the evoked increases in GLU, which is in close metabolic relationship with GLN (Tossmann et al., 1986; discussion, chapter 5). Interestingly, evoked release of ASP and GLU both showed a tendency to be increased as a result of INH

treatment and in the case of ASP, this was statistically significant (Fig. 34a).

During the period of high K^+ stimulation the duration of seizures was observed to be increased and some of the animals exhibited periods of wild running (table 9). It seems possible that the worsened convulsions were the result of the increased EC EAA concentration, since these are considered to be proconvulsant (see general introduction).

When VPA was given with INH, the EAA dialysate concentation was the same as that seen in control animals (figure 34). In response to high K^+, the percentage increase in extracellular GABA was substantially greater than that seen for other amino acids (figures 34 and 35). In contrast to its effect on dialysate EAA level, INH caused a significant reduction in evoked GABA release compared with control (figure 36). VPA not only reversed the effect of INH on the evoked release of GABA but caused it to be substantially greater ($3,150\pm1,890\%$) than that occuring in control animals (figure 36). In the case of TAU, evoked release was less in those groups of animals which recieved INH alone or INH and VPA compared with controls (figure 35b), although in both groups this just failed to be statistically significant.

Time post-injection (min)	Treatments		
	INH (250 mg/kg) and saline	INH (250 mg/kg) and VPA (400 mg/kg)	Saline only
30	Initially, facial myoclonus (8/8), followed by forelimb clonic seizures (8/8)	Facial myoclonus (3/8) and WDS (8/8)	Normal behaviour
60	Full tonic-clonic convulsions (7/8)	Inactivity, sedation (7/8)	Normal behaviour
120	Status epilepticus (6/8)	Normal behaviour (8/8)	Normal behaviour
150	Wild running episodes observed following high K^+ infusion (4/6)†	Exploratory behaviour (8/8), intermittent WDS (5/8)	Mild facial myoclonus (4/8), exploratory behaviour (5/8) and intermittent WDS (4/8)

† 2 rats died in status epilepticus prior to high K^+ infusion.

Table 9 Summary of behavioural changes noted in animals following treatments. Figures in brackets indicate ratio of animals displaying particular behaviours. Note the seizures observed in INH/saline treated animals. These are more severe than previously observed using the same dose of convulsant (Horton et al, 1979). In the present study, it is possible that the presence of hippocampal damage due to microdialysis probe implantation could have served to exacerbate INH induced seizures.
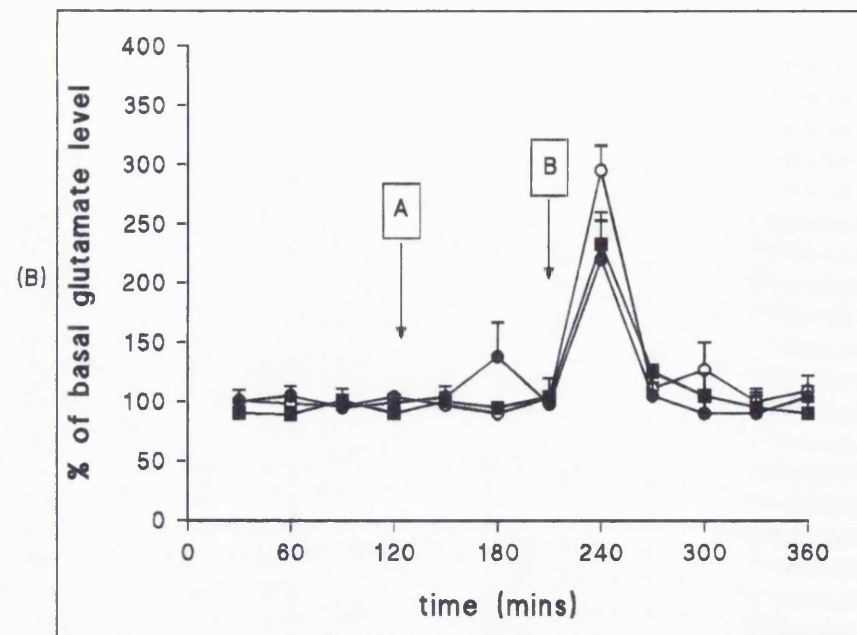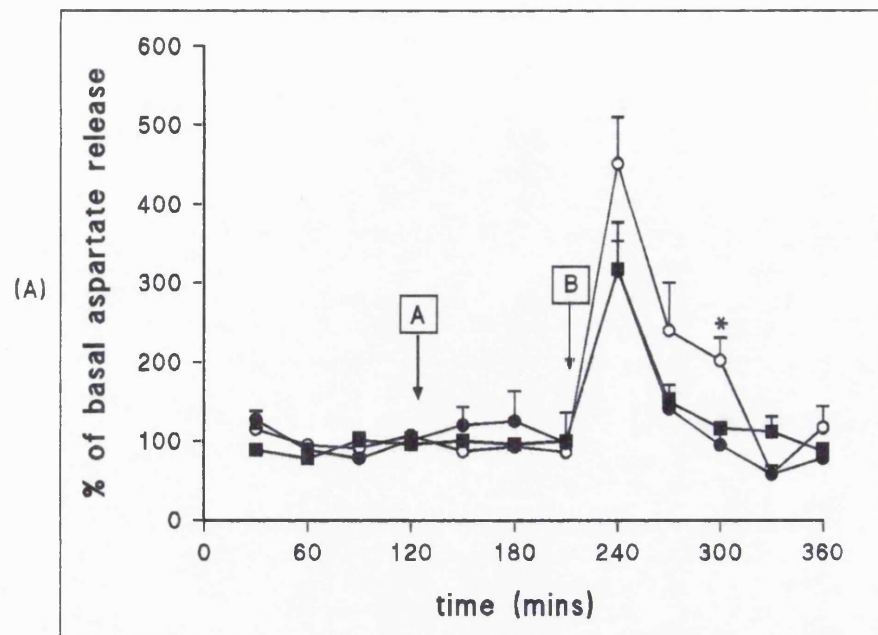
Figure 34 A and B. Effect of INH alone or in combination with VPA on ventral hippocampal dialysate levels of (A) ASP and (B) GLU. "A" denotes time at which drugs or saline were administered, and "B" the point at which 100mM K^+ was infused. Key:- open circles 250 mg\kg INH, filled squares saline, filled circles 250 mg\kg INH + 400 mg\kg VPA. All values mean ± SEM of 8 rats per group. * indicates points significantly different from controls (p<0.05).
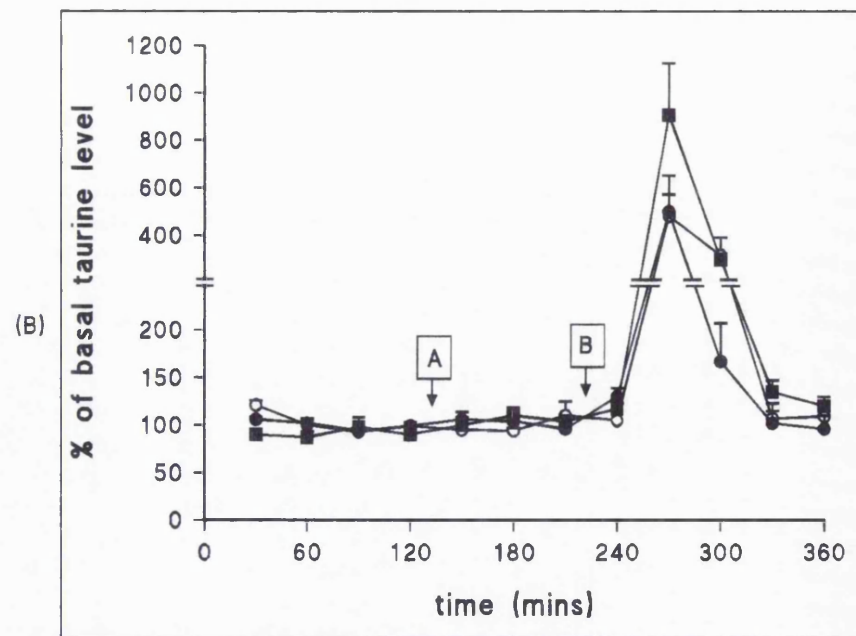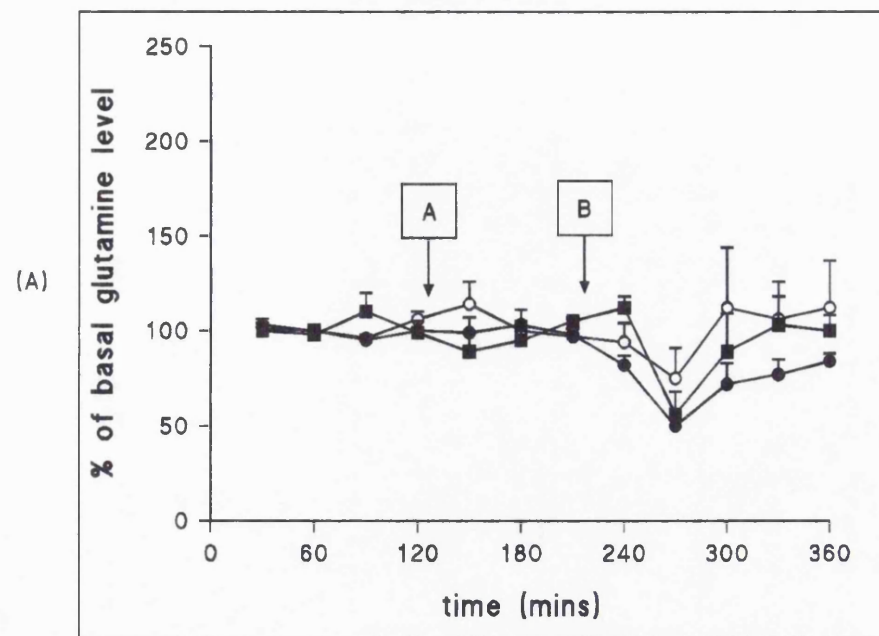
Figure 35 A and B. Effect of INH alone or in combination with VPA on ventral hippocampal dialysate levels of (A) GLN and (B) TAU . "A" denotes time at which drugs or saline were administered, and "B" the point at which 100mM K^+ was infused. Key:- open circles 250 mg\kg INH, filled squares saline, filled circles 250 mg\kg INH + 400 mg\kg VPA. All values mean ± SEM of 8 rats per group.
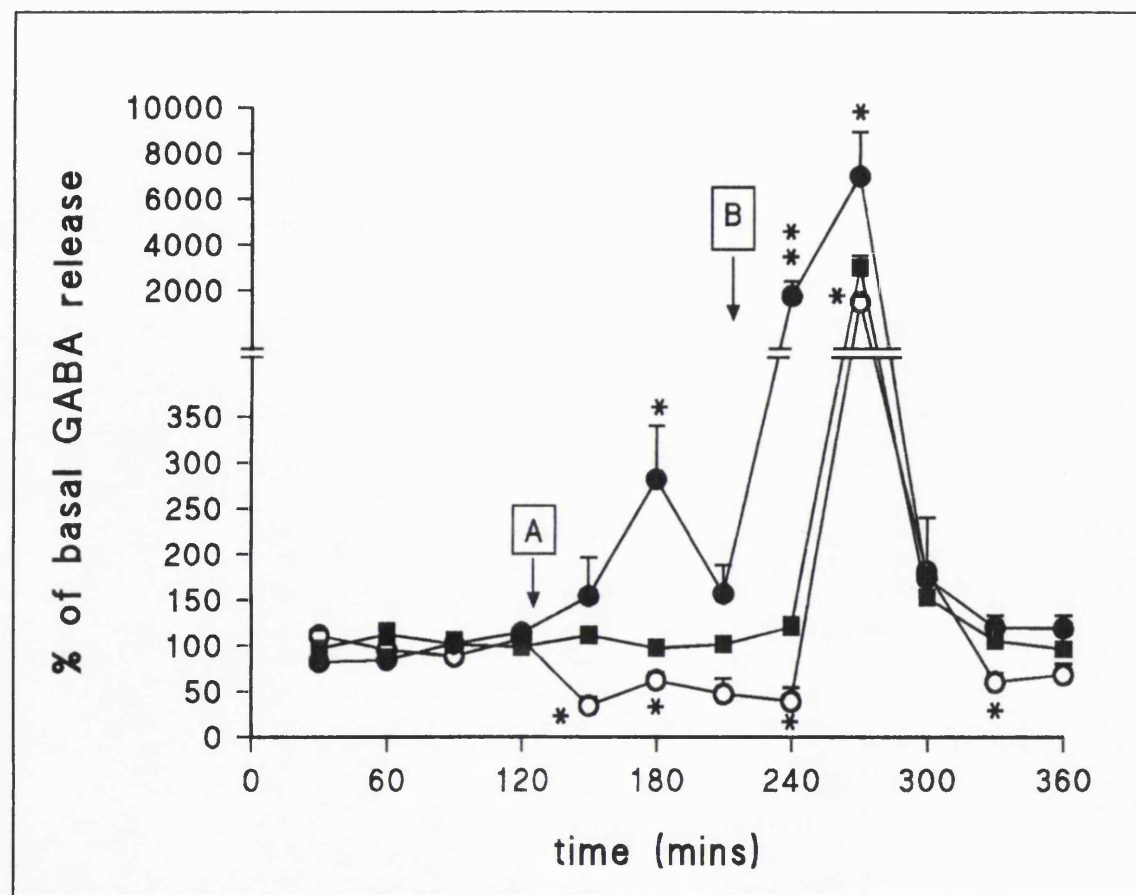
Figure 36 Effects of INH alone or in combination with VPA on basal and evoked GABA levels in ventral hippocampal dialysates. "A" denotes time at which drugs or saline were administered, and "B" the point at which 100mM K$^+$ was infused. Key:- open circles INH 250 mg/kg, filled squares saline, filled circles 250 mg\kg INH + 400 mg\kg VPA. All values mean ± SEM of 8 rats per group. * indicates data points significantly different from saline controls (p<0.05), ** (p<0.01).

Discussion

Brain GABA synthesis is rate limited by GAD activity for which PLP is an essential co-factor (reviewed by Martin and Rimvall, 1993). Studies indicate that GAD operates at a small proportion of its maximal capacity leaving a large potential reserve for GABA synthesis (Martin and Rimvall, 1993). However, GAD activity is rate limited by the availability of PLP (reviewed by Ebadi, 1981), and this is decreased by INH, leading to decreases in tissue GABA and PLP, measured in several brain regions (Horton et al., 1979). More recently it has been observed that two forms of GAD exist: GAD A, which does not require exogenous PLP for maximal activity, and GAD B, which does. These two froms of GAD are not homogeneously distributed throughout the brain, with GAD B being particularly enriched in hippocampus (reviewed by Ebadi et al., 1990). Significantly, INH has been found to decrease the content and release of GABA from synaptosomes (Wood et al., 1988). The present in vivo data are in agreement with the in vitro observations of Wood et al. (1988), and support the view that decreased GABA release plays a crucial role in INH-induced convulsions. Given the propensity of the hippocampus to act as a focus for seizure discharge generation (Bradford and Peterson, 1987; Ben-Ari and Repressa, 1990), reduction of EC GABA measured in this structure concurrent with seizures is of particular interest. It is not known if the hippocampus acts as specific target for the induction of INH-related seizures, although other regions (cerebellum, pons-medulla and striatum) have been proposed as possible sites for the expression of "wild running" seizures following convulsant doses of INH (Horton et al, 1979). The observation that evoked

release of ASP and to a lesser extent GLU tend to be greater in INH treated animals suggests that increased EEA accumulation may play a role in the generation or maintenance of seizures following INH administration. Such a role appears to be less important than loss of inhibitory transmission, since the basal release of these EAA's was not altered by INH (figure 34), whereas that of GABA was (figure 36), and convulsions occurred prior to high K^+ stimulation.

VPA was effective in reversing both the behavioural and neurochemical changes induced by INH treatment (table 9 and figures 34, 35 and 36). Both basal and evoked release of GABA were increased when VPA was given with INH compared with the reductions in these parameters caused by INH alone. In addition, the increase in hippocampal dialysate ASP by INH was reversed by VPA. These data suggest that an augmentation of GABA release and, perhaps to an extent, a decrease in evoked EAA release may combine to mediate the anticonvulsant action of VPA in this seizure model. Thus, the restoration of an overall balance of central excitatory and inhibitory neurotransmission appears to be a crucial factor in the suppression of epileptiform acivity.

As previously discussed, acute administration of VPA promotes a rapid elevation of brain GABA and GAD activity in rodents (Iadarola et al., 1979; Nau and Löscher, 1982; Phillips and Fowler, 1982). These changes are particularly marked in the synaptosomal fraction, with 80% increases in hippocampal synaptosome GABA content observed, an increase disclosing a temporal association with anticonvulsant action (Löscher and Vetter, 1985).

In vitro, VPA has been found to activate purified porcine GAD at concentrations which confer seizure protection in rodents (Silverman et al., 1991). This direct action of VPA on GAD activity suggests an interaction with the enzyme itself. In vivo, the situation may be more complex and it is possible that an indirect facilitatory effect of the drug, such as an increase in the availability of PLP to the GABA synthetic compartment may contribute to reversal of INH-induced deficits. The latter possibility is given some support by the observations with hydrazides that reduction of tissue PLP by these compounds decreases GABA synthesis (Horton et al., 1979), and the present data, in which the protective effect of VPA could result from a reversal of INH depletion of PLP and a subsequent increase in its cellular concentration. In this respect a study of a possible effect of VPA on pyridoxal kinase activity in vitro would be of great interest. The possibility remains that VPA may interact with GAD directly, maybe via an allosteric site (Silverman et al, 1991), since in GAD activation assays, the anticonvulsant did not alter K_m values for L-GLU, but increased V_{max} for the conversion of substrate to GABA.

As has been indicated previously, manipulation of brain monoamines can profoundly alter thresholds for expression of motor seizures (see general introduction). VPA has been observed to increase the synthesis of 5-HT (Whitton et al., 1985) and NA (Whitton et al., 1984), the precursors of which utilise PLP-dependent decarboxylases within their biosynthetic pathways. Thus there remains the possibility that alterations of EC monoamine concentrations (chapters 3, 5 and 6) could contribute to the anticonvulsant action of VPA against INH-induced convulsions. Unfortunately, since INH and related

compounds are MAO inhibitors, the evaluation of any data following use of INH to putatively decrease monoamine synthesis would be compromised as a result of this action. Furthermore, INH has been found to be highly electrochemically active and interferes with the detection of monoamines in dialysates using HPLC-ED (data not shown). However, thiosemicarbazide (TSC) a convulsant thought to mediate its action via GABA synthesis in a similar manner to INH (Horton et al, 1979) also inhibits the activity of L-aromatic amino acid decarboxylases responsible for 5-HT and DA synthesis in vitro (Sawaya et al, 1978). It may possibly be the case that activation of the appropriate decarboxylase is the focal point of the prosynthetic activity of VPA for GABA, 5-HT, NA (and possibly) DA, although the anticonvulsant probably exerts a proportion of its effects in vivo through promoting neurotransmitter release (Preisendorfer et al, 1987; Gram et al, 1988; see also chapters 3 and 6). The former hypothesis remains speculative at this time, but nonetheless, requires further investigation.

Summary

1. The present study has provided <u>in vivo</u> neurochemical evidence to support the view that hydrazide-induced convulsions in rodents (Horton et al., 1979), and possibly in humans following INH overdose (Holdiness, 1987), are the result of a decrease in synaptic GABA, possibly accompanied by enhanced EAA activity.

2. It is thought that this is the first study to demonstrate decreases in EC GABA concurrently with the incidence of seizures <u>in vivo</u>.

3. The present <u>in vivo</u> data are supportive of <u>in vitro</u> findings observing decreased GAD activity following INH treatment.

4. VPA is effective in reversing INH-induced convulsions and neurochemical deficits and may achieve this by promoting a direct or indirect activation of GAD, with the possibility that other decarboxylases may be similarly affected.

Chapter Eight

Involvement of dopamine and serotonin receptor subtypes in

the protective action of sodium valproate against pilocarpine-

induced seizures

Introduction

As has been discussed elsewhere in this thesis, relatively little is known of the possible involvement of monoaminergic neurotransmitter systems in the anticonvulsant action of VPA. Although there is clear evidence that these systems are involved in a variety of seizure models (Jobe et al, 1973; Anlezark and Meldrum et al, 1975; Turski et al, 1988; Wada et al, 1992 a,b) and human epilepsies (Fahn, 1978; Mervaala et al, 1990), Horton et al (1977) failed to demonstrate a link between monaminergic neurotransmission and VPA. This finding was not however, supported by other neurochemical studies which suggested that VPA synergistically enhanced the anticonvulsant effects of serotonergic drugs against chemoconvulsants (Hwang and Van Woert, 1979; Lazarova et al, 1983). Subsequent investigations into the role played by monoaminergic neurotransmission in experimental seizure development have revealed a complex picture, one in which the *balance* of 5-HT and DA receptor subtype stimulation may be of primary importance. Evidence presented in these investigations has suggested that at particular receptor subtypes both 5-HT and DA mediate an anticonvulsant action. Specifically stimulating 5-HT_{1A} receptors has been found to decrease epileptiform activity in the cat (Wada et al., 1992a, b) and rat (Janusz and Kleinrok, 1989), while the D_2 receptor has been shown to mediate an anticonvulsant action in rats (Al-Tajir and Starr, 1990; Wahnschaffe and Loscher, 1991). Conversely, selective stimulation of D_1 receptors following systemic administration of the partial agonist SKF 38393 was found to potently lower the threshold for pilocarpine-induced seizures (Al-Tajir et al, 1990b) and DOI, a 5-HT_2 agonist was found to facilitate

generalized convulsions in hippocampal kindled cats (Wada et al, 1992 a). Therefore, the failure of earlier studies to detect an involvement of these neurotransmitters in the anticonvulsant action of VPA could theoretically be accounted for by the lack of any specific pharmacological tools. In addition, given the diverse neurochemical actions attributed to VPA, choice of seizure model may be of considerable importance when attempting to characterise possible interactions between neurotransmitter systems and anticonvulsants. Importantly, VPA has been shown to differentially alter extracellular levels of DA and 5-HT centrally (Whitton and Fowler 1991; chapter 3, this thesis), therefore presumably altering monoaminergic receptor activation, at least in the striatum and hippocampus.

In the present study, using the pilocarpine model of limbic motor seizures (Turski et al., 1989), the effects of drugs acting at specific 5-HT and DA receptor sutypes on the anticonvulsant action of VPA has been investigated.

Experimental details

The reader is referred to chapter 2, section 5 for a description of the experimental procedures and conditions used to carry out work presented in this chapter. However, details of specific pharmacological agents used in this investigation for characterising monoamine neurotransmitter receptor involvement in the anticonvulsant action of VPA are given below. In addition, the dose of each drug administered is given in parenthesis.

1. 5-HT receptor antagonists.

The non-selective 5-HT receptor antagonist metergoline (3 mg/kg) was kindly donated from the Miriam Marks Department of Neurochemistry, Institute of Neurology, London. The relatively selective $5\text{-HT}_2/5\text{-HT}_{1c}$ receptor antagonist ketanserin tartrate (2mg/kg) and 1-(2-methoxyphenyl)-4-[4-(2-phthalimido)butyl] piperazine hydrobromide (NAN-190; 5 mg/kg) which has 5-HT_{1A} receptor antagonist properties, were both obtained from Research Biochemicals Incorporated, UK.

2. Dopamine receptor agonists.

The selective D_1 receptor agonist SKF 38393 (15 mg/kg) and the D_2 receptor agonist quinpirole (LY-171555; 2 mg/kg) were both gifts kindly donated by Dr Mike Starr, Department of Pharmacology, The School of Pharmacy, London.

3. Dopamine receptor antagonists.

Both SCH 23390 (0.25 mg/kg, an highly potent D_1 receptor antagonist) and raclopride (1 mg/kg), a drug with D_2 receptor antagonist selectivity were again gifts from Dr Mike Starr's laboratory.

All drugs, with the exception of raclopride, metergoline and NAN-190 were dissolved in saline and administered in a volume of 0.1ml per 100g animal body weight. Raclopride and metergoline could be dissolved in saline with prior acidification (glacial acetic acid) and gentle warming. To eliminate any possible false positive results obtained due to the likely irritant qualities of these drugs upon i.p. injection, appropriate control animals were injected with 5% glacial acetic acid/saline vehicle. NAN-190 was first lyophillised in dimethyl sulphoxide (DMSO) with sonification and diluted with saline to achieve the desired concentration (20% DMSO V/V final). All test drugs were administered 30 min prior to challenge with pilocarpine.

Data analysis

Data were analysed with one-way analysis of variance (ANOVA) with post testing (Dunnett multiple comparison test) for statistically significant variation from control seizure parameters. Group sizes were as follows:- pilocarpine control group n=18, pilocarpine/VPA control group n=8 and all other treatment groups n=5.

Animals

Rats weighing 280-300g were used in this investigation.

Results

All rats challenged with pilocarpine 400 mg/kg and saline convulsed tonically with a mean latency to seizure onset of $21.4\pm3.2\%$ of the observation period. As observed previously (Turski et al., 1989) VPA was effective in decreasing seizure intensity and mortality while markedly increasing the time to seizure onset (figures 37, 38, 39, and 40, tables 10 and 11). Drugs modifying 5-HT transmission had little significant effect on seizure parameters (figures 37, 38), although ketanserin did increase the time to seizure onset (figure 38a). When given in combination with VPA, neither metergoline nor ketanserin significantly modified the actions of the anticonvulsant (figures 37b, 38b and table 10). However, blockade of 5-HT_{1A} receptors by NAN-190 increased seizure intensity and decreased latency to seizure onset with respect to pilocarpine/VPA control animals (figures 37b, 38b), as well as increasing the ratio of animals displaying seizures and seizure related deaths (table 10).

Drugs interacting with dopamine receptors tended to modify basal seizure parameters to a greater extent than was the case with drugs affecting 5-HT receptors. Quinpirole significantly decreased seizure intensity, but had no effect on latency to seizure onset, whilst the D_1 receptor antagonist SCH 23390 showed a significant anticonvulsant effect in all parameters recorded (figures 39, 40 and table 11). In contrast, SKF 38393 was markedly proconvulsant, significantly increasing mean seizure score, decreasing latency to onset (figures 39a, 40a), and causing a small increase in the mortality ratio (table 11). When given in conjunction with VPA, raclopride significantly worsened the mean seizure score (figure 39b) while SKF 38398 completely reversed the

anticonvulsant effect of VPA (figures 39b, 40b and table 11). SCH 23390 appeared to synergise with VPA, reducing the mean seizure score to below that seen with VPA alone although this effect just failed to reach statistical significance (figure 39b). In addition, no animal ¡receiving SCH 23390 in conjunction with VPA displayed a level three seizure during the period of observation (figure 40b and table 11). Although quinpirole showed some anticonvulsant activity when given alone, it did not reduce the effects of pilocarpine challenge when given with VPA any more than VPA alone (figures 39b, 40b).

Treatment	Dose (mg/kg)	Seizure Parameter			
		Frequency of "level 3" seizures		Mortality	
		Pilocarpine[†]	Pilocarpine/VPA[*]	Pilocarpine[†]	Pilocarpine/VPA[*]
Saline	-	18/18	4/8	6/18	0/8
NAN-190	5.0	5/5	4/5	2/5	1/5
Metergoline	3.0	4/5	2/5	1/5	0/5
Ketanserin	2.0	5/5	2/5	0/5	0/5

Table 10 Effects of serotonergic drug pretreatment on seizure frequency and mortality in rats challenged with pilocarpine or pilocarpine with VPA. † dose fixed at 400 mg/kg. * dose fixed at 300 mg/kg.
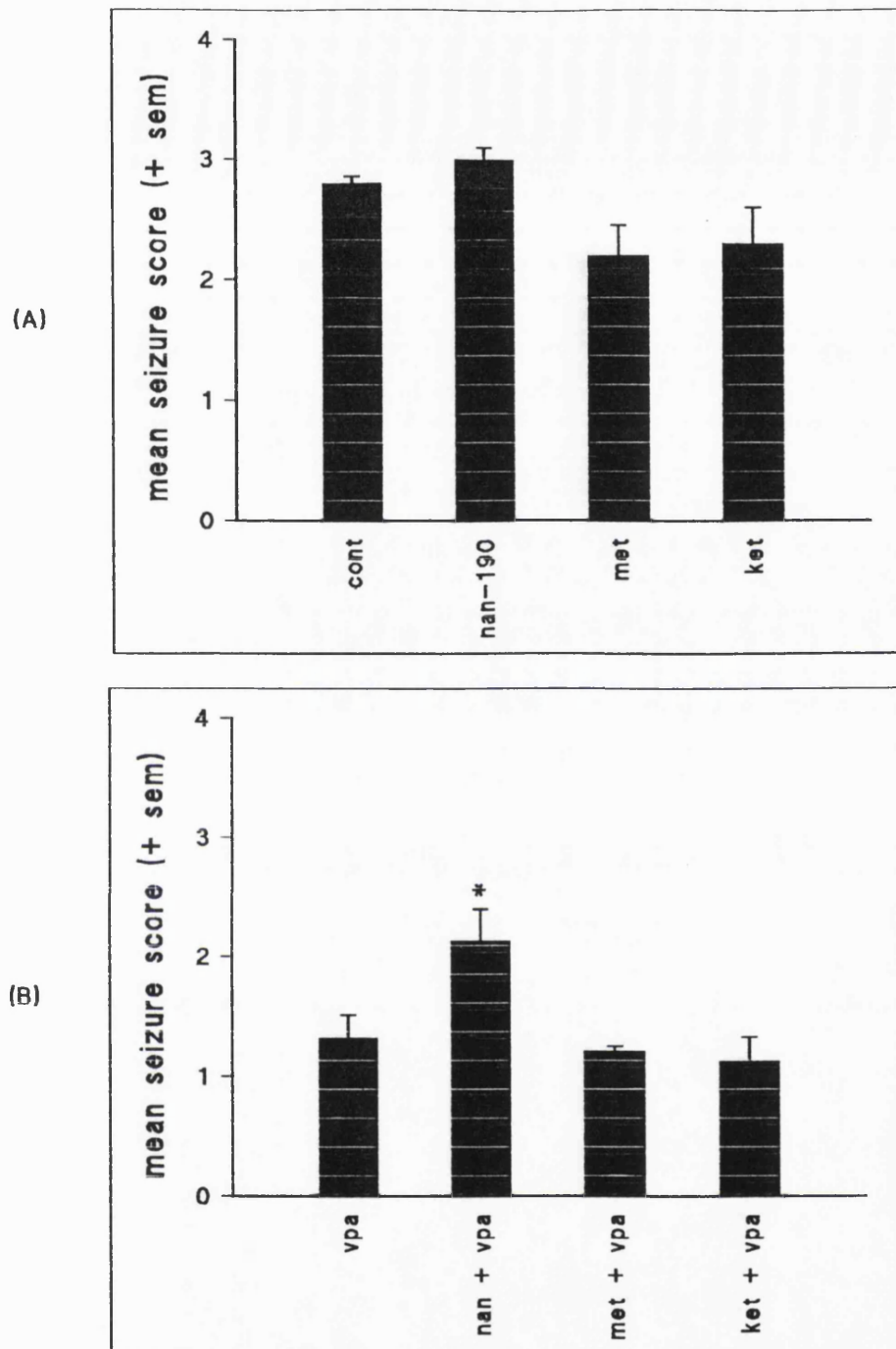
Figure 37 Modification of pilocarpine-induced seizure severity by pretreatment with serotonergic drugs. Influence of drugs on seizures induced by pilocarpine alone (400 mg/kg; panel A) or pilocarpine co-administered with VPA (300 mg/kg; panel B). All values represent mean seizure score + s.e.m. * denotes value significantly different from pilocarpine only controls (p<0.05). Abbreviations:- (cont) pilocarpine only, (vpa) pilocarpine+VPA, (nan) NAN-190, (met) metergoline and (ket) ketanserin.
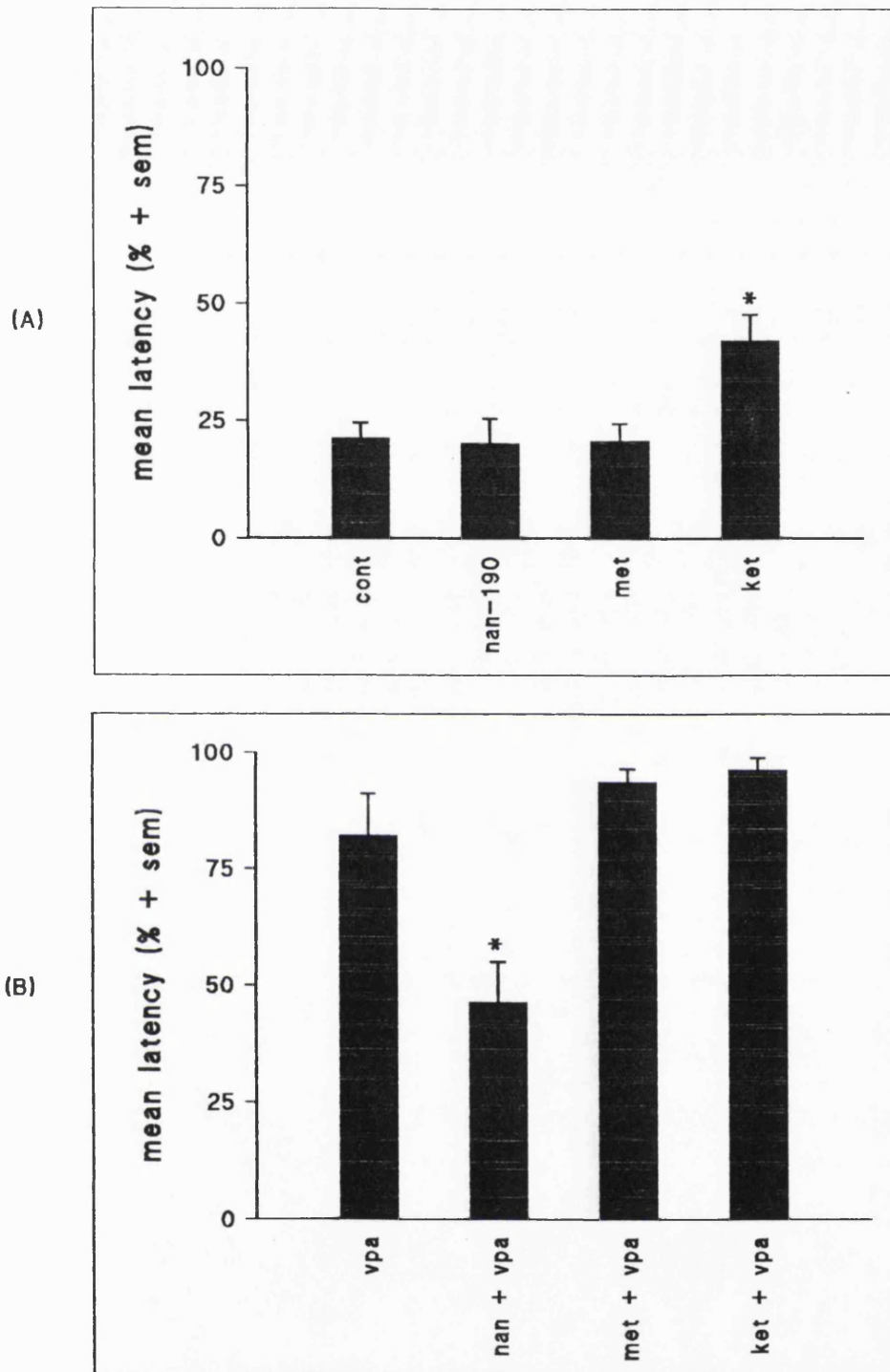
Figure 38 Alteration of latency to onset of "level 3" seizures following pretreatment with serotonergic drugs. Influence of drugs on seizures induced by pilocarpine alone (400 mg/kg; panel A) or pilocarpine co-administered with VPA (300 mg/kg; panel B). All values represent mean latency + s.e.m. * denotes values significantly different from pilocarpine only controls (p<0.05). Abbreviations:- (cont) pilocarpine only, (vpa) pilocarpine+VPA, (nan) NAN-190, (met) metergoline and (ket) ketanserin.

Treatment	Dose (mg/kg)	Seizure Parameter			
		Frequency of "level 3" seizures		Mortality	
		Pilocarpine[†]	Pilocarpine/VPA[*]	Pilocarpine[†]	Pilocarpine/VPA[*]
Saline	-	18/18	4/8	6/18	0/8
Raclopride	1.0	4/5	2/5	1/5	0/5
Quinpirole	2.0	4/5	3/5	1/5	0/5
SCH 23390	0.25	3/5	0/5	0/5	0/5
SKF 38393	15.0	5/5	5/5	3/5	1/5

Table 11 Effects of dopaminergic drug pretreatment on seizure frequency and mortality in rats challenged with pilocarpine or pilocarpine with VPA. † dose fixed at 400 mg/kg. * dose fixed at 300 mg/kg.
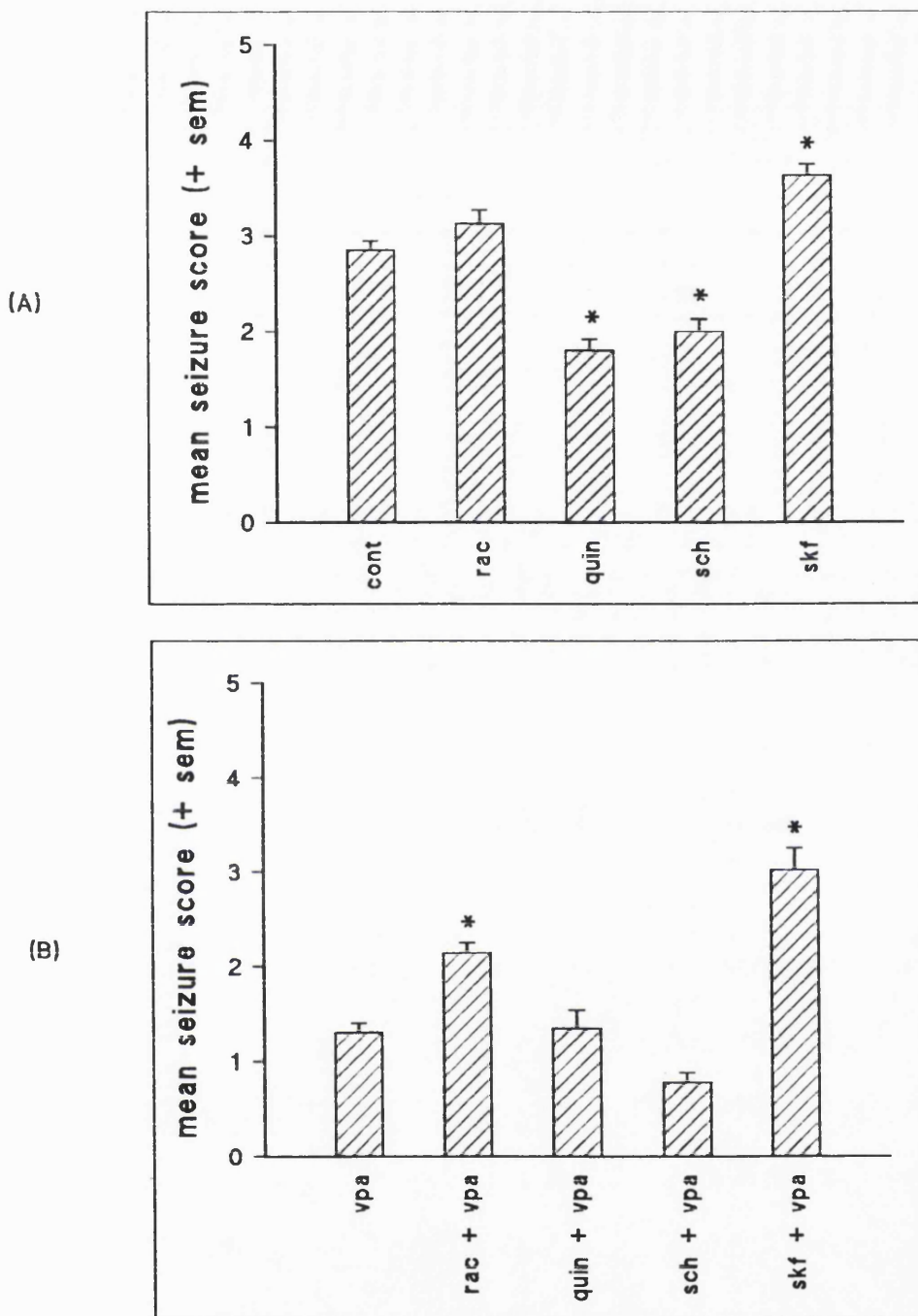
Figure 39 Modification of pilocarpine-induced seizure severity by pretreatment with dopaminergic drugs. Influence of drugs on seizures induced by pilocarpine alone (400 mg/kg; panel A) or pilocarpine co-administered with VPA (300 mg/kg; panel B). All values represent mean seizure score + s.e.m. * denotes value significantly different from pilocarpine only controls (p<0.05). Abbreviations:- (cont) pilocarpine only, (vpa) pilocarpine+VPA, (rac) raclopride, (quin) quinpirole, (sch) SCH23390 and (skf) SKF 38393.
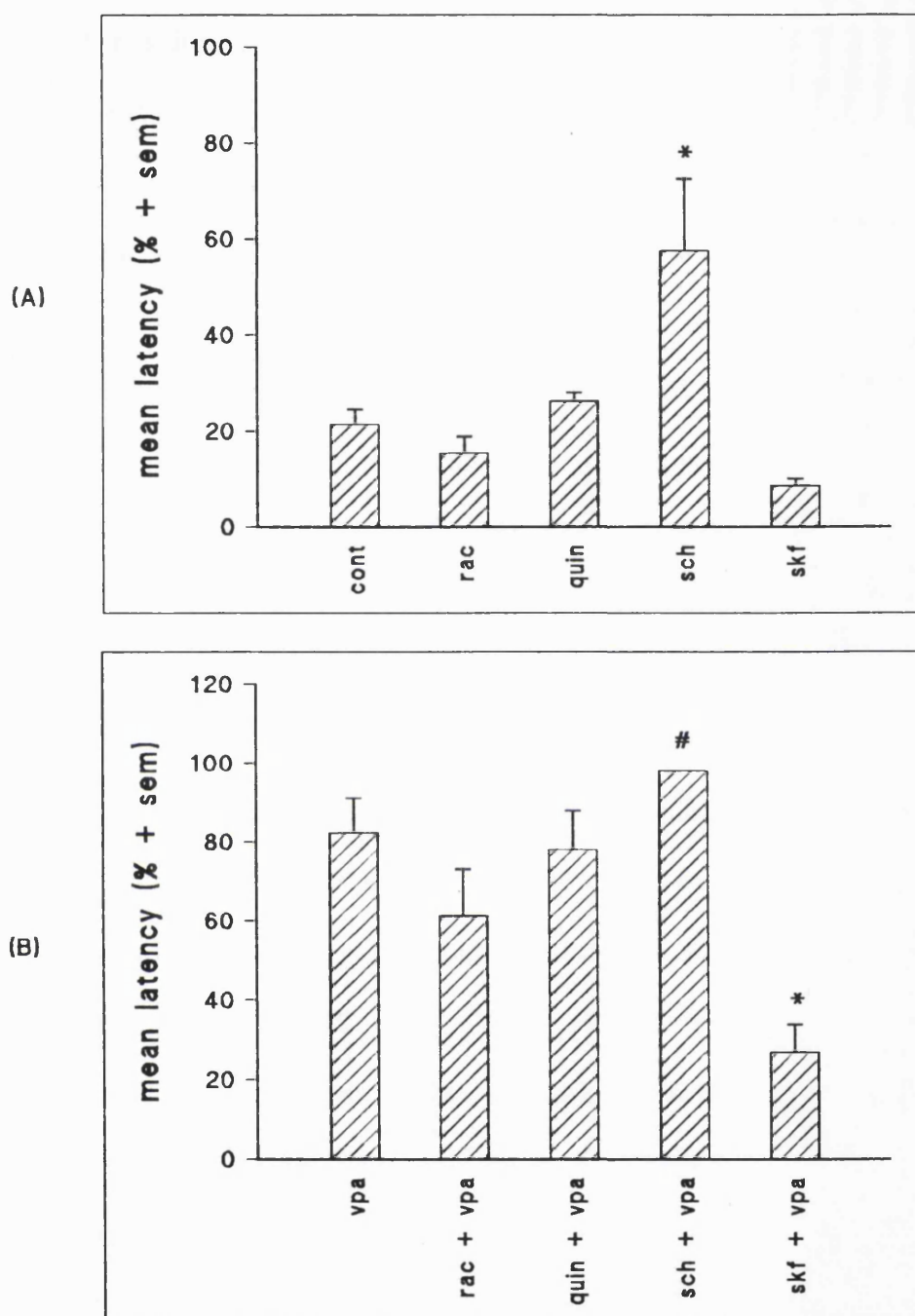
Figure 40 Alteration of latency to onset of "level 3" seizures following pretreatment with dopaminergic drugs. Influence of drugs on seizures induced by pilocarpine alone (400 mg/kg; panel A) or pilocarpine co-administered with VPA (300 mg/kg; panel B). All values represent mean latency + s.e.m. * denotes values significantly different from pilocarpine only controls ($p < 0.05$). # indicates failure of rats to reach seizure level 3. Abbreviations:- (cont) pilocarpine only, (vpa) pilocarpine+VPA, (rac) raclopride, (quin) quinpirole, (sch) SCH 23390 and (skf) SKF 38393.

Discussion

Neurochemistry of pilocarpine-induced ('limbic') seizures.

Pilocarpine-induced seizures are considered to be a model of intractable epilepsy (Turski et al., 1989) acute manifestations of which may be countered by a number of clinically used anticonvulsants, including VPA. The initial central response to pilocarpine challenge is activation of muscarinic receptors, which in turn is thought to enhance and maintain actions of the EAA's GLU and ASP (Olney et al, 1983;1986). These sequelae lead to development of 'limbic' seizures, so called due an electrographic spread of abnormal discharges through the hippocampus, then amygdala and cortex (Turski et al, 1983a). Following recovery from acute seizures induced by sub-lethal doses of the drug, recurrent spontaneous seizures often appear 5-10 days following initial challenge (Turski et al, 1983b). It is thought that this occurs as a consequence of ongoing damage to CNS structures (Clifford et al, 1987) and may reflect an activation of glutamate-related excitotoxic mechanisms (Olney et al, 1986). More recent studies have suggested involvement of dopaminergic (Turski et al., 1988; 1990; Al Tajir et al., 1990a,b) and serotonergic systems (Janusz and Kleinrok, 1989) in the development of pilocarpine-induced seizures, observations that are consistent with the findings of the present study.

Interaction of VPA with serotonergic agents.

In the present study an attempt was made to characterise the possible involvement of the 5-HT$_{1A}$ and 5-HT$_2$ receptor subtypes in the action of VPA against pilocarpine-induced seizures. Recent findings have suggested an

involvement of these receptors in modulating suceptibility to epileptiform activity in the cat (Wada et al., 1992a, b) and 'limbic' seizures induced by pilocarpine in rats (Janusz and Kleinrok, 1989). Additionally, ketanserin and the potent 5-HT$_{1A}$ agonist 8-hydroxy-2-(di-n-propylamino)tetralin (8-OHDPAT) have been shown to improve ischemia induced abnormalities (normalisation of field potentials and 2-deoxyglucose (2-DG) uptake) in the CA1 region of rat hippocampal slices (Shibata et al, 1992). These previously cited studies have supported an antiepileptic and neuroprotective role for central 5-HT$_{1A}$ receptor stimulation, and the converse following activation of 5-HT$_2$ receptors. In the present investigation, when pilocarpine challenged rats were given NAN-190 (5 mg/kg) no change in basal seizure parameters was observed (figures 37a, 38a). However when this drug was used to pretreat rats subsequently co-administered VPA and pilocarpine, the anticonvulsant efficacy of VPA was significantly compromised (figures 37b, 38b and table 10). Since it has been demonstrated that the anticonvulsant enhances release of 5-HT in the hippocampus in vivo (Whitton and Fowler, 1991; chapter 3, this thesis), phasic stimulation of post synaptic 5-HT receptors in this structure could play an important role in suppressing pilocarpine-induced seizures in the rat. Radioligand binding studies suggest that it is unlikely that VPA directly interacts with 5-HT receptors (Pranzatelli, 1988). However, an indirect action of VPA on 5-HT$_{1A}$ receptors is considered likely since binding and autoradiographic study has revealed this to be the predominant 5-HT receptor subtype located in the hippocampus (Patsos and Palacios, 1985). Indeed previously it was shown that 8-OHDPAT reduced the severity of pilocarpine-

induced seizures in the rat (Janusz and Klienrok, 1989) and suppressed

hippocampal kindled seizures in the cat (Wada et al, 1992 a). $5\text{-}HT_{1A}$ receptor

agonists may exert an anticonvulsant action in these seizure models by

inhibiting firing of hippocampal CA1 pyramidal cells (Ropert, 1988). Although

it has been argued that systemically administered $5\text{-}HT_{1A}$ agonists

preferentially activate modulatory $5\text{-}HT_{1A}$ somatodendrtic autoreceptors located

in the dorsal raphé nuclei (DRN; Hjorth and Magnusson, 1988), reducing

activity in 5-HT terminal fields from ascending serotonergic projections, the

doses of agonist used in this study were very low (10-300 µg/kg). Thus, the

higher doses of 8-OHDPAT used to elicit an anticonvulsant response (Janusz

and Kleinrok, 1989: 0.5 and 1.0 mg/kg; Wada et al, 1992 a: 0.1 and 1.0 mg/kg)

could have resulted in activation of hippocampal post-synaptic $5\text{-}HT_{1A}$ sites as

well as somatodendritic autoreceptors. Furthermore, the adverse effect of

NAN-190 against the anticonvulsant action of VPA noted in the present study

could have been bought about via a dual mechanism involving interactions

with both somatodendritic and post-synaptic $5\text{-}HT_{1A}$ receptors. This is borne

out by the findings of Hjorth and Sharp (1990) who noted that NAN-190

displayed mixed agonist/antagonist properties. Local application of the drug

into the hippocampus was without effect on 5-HT output in vivo, whilst

systemic administration resulted in a reduction of hippocampal 5-HT ouput,

apparently resulting from activation of somatodendritic $5\text{-}HT_{1A}$ sites. It should

be mentioned that the effects of NAN-190 noted in the present study represent

a genuine action involving $5\text{-}HT_{1A}$ receptors. This compound is known to

possess significant α_1-adrenoceptor antagonist activity (Glennon et al, 1988), a

property which may be in itself proconvulsant (Horton et al, 1980). However, as shall be discussed the 5-HT$_2$ antagonist ketanserin, a drug which is also known to block α-adrenoceptors (Hoyer, 1987; Schotte and Leysen, 1988) had the reverse effect to NAN-190 on a seizure parameter. Thus, both agents probably exerted their respective effects in the present study via an action independent of α-adrenoceptors.

Blockade of 5-HT$_2$ receptors with ketanserin indicated some anticonvulsant activity of the drug with respect to time of seizure onset, but no significant effect on the actions of VPA was observed (figures 37b, 38b and table 10). This accords with the findings of Wada et al (1992a) who observed that DOI shortened and ketanserin prolonged the latency to onset of generalised convulsions in hippocampal kindled cats, although VPA was not investigated in this study. Quipazine, a drug which displays 5-HT$_2$ agonist properties (Gardner, 1985) has also been found to shorten latency to seizure onset in the pilocarpine seizure model (Janusz and Kleinrok, 1989), although the effects of this drug could be partially accounted for by its cholinomimetic properties (Gillet et al, 1985). Taken together, these data suggest that selective stimulation of central 5-HT$_2$ receptors may lower the threshold at which pilocarpine induced 'limbic' motor seizures are expressed without affecting seizure intensity. The present data would suggest that antagonism of 5-HT$_2$ receptors by ketanserin did not significantly further enhance the VPA effect of increasing seizure latency. Under conditions of 5-HT$_2$ receptor blockade, VPA-induced release of 5-HT from central serotonergic neurones might be expected to shift the balance of opposing 5-HT$_{1A/2}$ receptor stimulation in favour of the 5-HT$_{1A}$-

mediated anticonvulsant component previously described. However, the modest augmentation seen (figures 37b, 38b) would suggest that removal of the putative proconvulsant component mediated by 5-HT$_2$ receptors does not result in a strong anticonvulsant effect associated with 5-HT$_{1A}$ receptor stimulation. It should be noted that this scheme may be further complicated by the possible involvement of 5-HT$_{1B}$ receptors in modulation of pilocarpine-induced seizures (Janusz and Kleinrok, 1989). No investigation into the possible role of this serotonergic receptor subtype in mediating the anticonvulsant action of VPA in this model was made due to unavailability of any selective ligands such as 1-(-3-trifluoromethylphenyl)piperazine (TFMPP). As shown, general blockade of 5-HT receptors with metergoline failed to significantly alter the activity of VPA (figures 37b, 38b) suggesting that global changes in serotonergic transmission may mask the involvement of 5-HT in the anticonvulsant effects of the drug. This accords with the findings of Janusz and Kleinrok (1989) who observed no modification of seizure parameters in pilocarpine challenged rats when pretreated with another non-specific 5-HT receptor antagonist methysergide. To what extent this feature is specific to the pilocarpine model of epilepsy remains to be determined.

Interaction of VPA with dopaminergic agents.

Much evidence exists to suggest that DA may have an important role in the modulation of seizure activity in various animal models of epilepsy (Dow et al, 1974; Anlezark and Meldrum, 1975; Meldrum et al, 1975; King and Burnham, 1980) and also human epilepsy (Livingston et al, 1973; Mervaala et al, 1990). Specifically, with regard to the pilocarpine model of epilepsy, D$_1$

receptor activation has generally been associated with a lowering of seizure thresholds, whilst D_2 activation the converse (Turski et al., 1989; Al Tajir et al., 1990a, b; Burke et al., 1990). In addition, the neuroanatomical sites at which modulation of DA receptors influences progression of these seizures have been located and the consequences of specific receptor manipulation pharmacologically characterised (Turski et al, 1988; Al-Tajir et al, 1990b; Al-Tajir and Starr, 1990;1991). Specifically, the opposing actions of DA in the basal ganglia are thought to be largely mediated by nigral D_1 receptors and striatal D_2 receptors respectively. Although it is recognised that the balance of dopaminergic tone *throughout* the CNS is likely to be critical in determining susceptability to convulsive stimuli, recent work characterising involvement of hippocampal DA recptors in this model (Alam and Starr, 1992;1993) is of particular interest here. This is because microdialysis studies have revealed the dose related effects of VPA on hippocampal EC DA levels to be most consistently observed in this structure (chapter 3, this thesis) and also due to the pivotal importance of the hippocampus in the genesis of pilocarpine-induced seizures (Turski et al, 1989). The present data are in general agreement with these previous observations in that systemic SKF 38393 was found to significantly increase mean seizure score, mortality and modestly reduce latency to seizure onset. However, the D_1 antagonist SCH 23390 had the opposite effect on these parameters in the current study (figures 39, 40 and table 11), an observation not made previously (Al-Tajir et al, 1990a). These authors found systemic administration of SCH 23390 (0.25 mg/kg) to reduce the severity of seizures but not the number of animals convulsing. This

apparent discrepency might be partially explained by different pilocarpine

doses used in the two studies. Microinjection of this agent into the rat caudate

nucleus (Al-Tajir and Starr, 1990) and substantia nigra (Al-Tajir et al, 1990b)

subsequently disclosed a full anticonvulsant effect in rats challenged with

convulsant doses of the cholinomimetic, suggesting that D_1 blockade

throughout the brain may produce only a partial anticonvulsant effect. Alam

and Starr (1992) found that blockade of hippocampal D_1 sites with SCH 23390

delayed onset and reduced the severity of pilocarpine-induced seizures, but

additional stimulation of these receptors with SKF 38393 did not further

augment seizure parameters. This is consistent with the present observation

that systemically applied SKF 38393 was unable to significantly reduce latency

to seizure onset (figure 40a) suggesting that D_1 receptors, at least in this

structure are maximally stimulated under conditions of tonic DA release. In

contrast, when given in conjunction with VPA, SKF 38393 completely reversed

the anticonvusant action of the drug (figure 40b) while VPA and SCH 23390

synergised to produce a more marked seizure protection than either drug

alone (figure 40b and table 11). This suggests that D_1 receptors possibly located

in the substantia nigra (Al-Tajir et al, 1990b) are not maximally tonically

stimulated and may in part be responsible for diminishing the effectiveness of

VPA. If VPA were to cause a release of DA from nigral terminals, a property

evident in other brain regions, the combination of increased D_1 stimulation by

DA itself and SKF 38393 might explain why the agonist promoted such a

profound reversal of the effects of the anticonvulsant. Using this scheme, it

follows that during D_1 receptor blockade the tone of the dopaminergic system

will be directed to relatively greater D_2 receptor activity, greatly enhancing the action of VPA against all seizure parameters measured. The precise ratio of D_1/D_2 tone appears to be crucial to the convulsant state of the animal (Barone et al., 1990; Burke et al., 1990), however the way in which this tone is maintained <u>in vivo</u> is still unclear. Interestingly, specific activation of D_2 receptors in the striatum (Al -Tajir and Starr, 1991) and in particular, the hippocampus (Alam and Starr, 1993) results in a substantial protective effect against seizures and as such, VPA-induced DA release (chapter 3, this thesis) in these structures would be expected to result predominantly in D_2 activation and a powerful anticonvulsant action. Alam and Starr (1992) revealed that the anticonvulsant action afforded by D_2 stimulation was predominant over the converse effects mediated by D_1 receptors in the hippocampus and taken with the present data, support the notion that VPA may exert an anticonvulsant action against pilocarpine by influencing dopaminergic neurotransmission in this structure. The D_2 agonist quinpirole had some anticonvulsant activity in the present study when administered systemically, but did not significantly augment the action of VPA against any parameter recorded (figure 39b, 40b and table 11). This is an anomalous result given the strong anticonvulsant effects previously attributed to the agonist when focally injected into the rat hippocampus (Alam and Starr, 1993) and may be explained by the apparently poor bioavailability of this drug following intraperitoneal injection (Turski et al, 1990). These authors did however observe suppression in all signs of electrographic spread of pilocarpine-induced seizure activity through the hippocampus and cortex following focal injection of quinpirole into the

striatum. In the current study, no significant worsening of basal seizure parameters was seen after systemic administration of the D_2 antagonist raclopride (figure 39a, 40a and table 11). This observation is consistent with other work investigating the consequences of D_2 anatagonism in this model (Burke et al, 1990), but it would seem that raclopride can significantly lower the threshold for pilocarpine-induced convulsions if central D_2 sites are presensitised with lithium (Barone et al, 1991). In addition, it was recently demonstrated that the level of *hippocampal* D_2 stimulation might play a crucial role in setting seizure thresholds since focally applied raclopride potently enhanced convulsions in animals recieving a usually sub-convulsant dose of pilocarpine (100mg/kg; Alam and Starr, 1993). In the present study, raclopride had a relatively modest deleterious effect upon the anticonvulsant properties exerted by VPA and these data serve to further illustrate that the degree of tonic D_2 receptor activation subserving an anticonvulsant action is probably not uniform throughout the brain. Hence, if VPA were to enhance the level of D_2 stimulation in structures such as the hippocampus and anterior striatum to a different extent, blockade of these receptors throughout the brain might be expected to cancel out the particularly potent effects noted in the hippocampus by Alam and Starr (1993) upon microinjection of quinpirole.

The present data strongly support a role for monoaminergic systems in regulating seizure activity <u>in vivo</u> induced by the cholinomimetic pilocarpine. The findings also suggest that the anticonvulsant action of VPA in this model is in part mediated by release of DA and 5-HT, acting at specific receptors mediating pro- and anticonvulsant actions respectively. Additionally, this work

reveals a link between <u>in vivo</u> neurotransmitter release patterns following VPA

treatments, behavioural responses to drugs which alter convulsive thresholds

via monoaminergic mechanisms and a possible indirect interaction of the

anticonvulsant with these regulatory systems at the receptor level.

It is recognised that this work to date represents a starting point for further

characterisation of neurotransmitter receptor involvement in anticonvulsant

pharmacology <u>in vivo</u>. The aim of the present study was to present a range of

arguments, formed on the outcome of investigations utilising a variety of

specific and non-specific pharmacological agents to manipulate monoaminergic

receptors, rather than to critically determine quantitative aspects of these

responses. This work also highlights the necessity for anticonvulsant

evaluation in a variety of animal seizure models, since the involvement of

monamines in the antiepileptic action of VPA has been disputed elsewhere

(see introduction).

Summary

1. Blockade of 5-HT$_{1A}$ or 5-HT$_2$ receptors did not subtantially alter intensity or time to onset of pilocarpine induced seizures in the absence of VPA. 5-HT$_{1A}$ receptor antagonism did however result in a significant diminution of the anticonvulsant action of VPA co-administered with pilocarpine.

2. Non-specific blockade of 5-HT receptors failed to alter basal seizure parameters and those following VPA co-administered with the convulsant.

3. A strong proconvulsant action was associated with stimulation of central D$_1$ receptors and the anticonvulsant action of VPA was completely reversed following pretreatment with a D$_1$ agonist. Conversely, antagonism of central D$_1$ receptors resulted in an enhancement of the efficacy of VPA against pilocarpine-induced seizures.

4. A weaker association between activation of D$_2$ receptors and anticonvulsant efficacy of VPA was observed, although blockade of these sites significantly worsened seizure intensity.

5. Taken as a whole, these data suggest that VPA may exert a proportion of its anticonvulsant action in this model via an indirect stimulation of 5-HT$_{1A}$ and D$_2$ receptors, located principally in the hippocampus and striatum.

Chapter Nine

Concluding remarks

Overview

During the course of these studies, much has been revealed concerning the

interaction of VPA with central neurotransmitter systems <u>in vivo.</u> A wealth of

data (reviewed in chapter 1) suggeststhat pharmacological manipulation of

amino acid and monoamine neurotransmitter systems can result in seizures in

experimental animals and VPA consistently ameliorates or prevents these

episodes. Additionally, VPA is also effective in treating seizures where there

is evidence of genetically acquired defects to neurotransmitter systems in

animals.

To some extent, the findings presented in this thesis have provided the

"missing link" mentioned in chapter 1, that of demonstrating changes in EC

neurotransmitter levels following both acute and prolonged treatments with

the anticonvulsant. In chapters 3, 4, 5 and 6, these actions were observed in the

brains of non-convulsing animals and are essentially phenomenological

findings. They are nevertheless of importance and adequately demonstrate the

ability of VPA to alter neurotransmitter release and metabolism in structures

thought to be intimately involved in the generation and propagation of seizure

activity. In a broader context, these findings may also explain why VPA

possesses anxiolytic (Roy-Byrne et al, 1989) and antidystonic (Linnoila, 1976;

Gibson, 1979) properties. As discussed earlier, increased brain EC levels of

amino acids and monoamines, notably GABA, 5-HT and DA alter thresholds

for propagation of seizure discharges throughout the brain or their generation

from a defined central locus. At the time of investigation, it was felt important

that such actions of the drug on basal EC neurotransmitter levels be

established in the absence of seizures, given the likely interference generated by abnormal neuronal discharges. Al-Tajir and Starr (1991b) noted disordering of striatal DA output measured using <u>in vivo</u> microdialysis, following challenge with pilocarpine and concluded that this probably represented a more widespread action of the convulsant on neuronal firing, rather than as a direct consequence of interaction with the dopaminergic system.

During the present studies, it was found that VPA (400 mg/kg) resulted in similar changes to hippocampal GABA output in animals challenged with the convulsant INH, compared with those observed in non-convulsing animals treated with the same dose of anticonvulsant (see chapters 7 and 4 respectively). Coupled with the apparent reduction in GABA output measured in hippocampal dialysates prior to the onset of INH-induced seizures, it is possible that these findings represent the first measurements of brain EC GABA <u>in vivo</u> which could be broadly correlated with the onset and maintainance of convulsions. These findings contrast with those obtained by Al-Tajir and Starr (1991b) in relation to the striatal dopaminergic system, where no such correlation was evident following seizure induction with pilocarpine. In addition, they add support to the notion that the hippocampus may represent a specific site of action for VPA (Löscher and Nau, 1983; Kubota et al, 1990).

All the studies previously mentioned investigated neurochemical changes following *acute* treatment with VPA. In chapter 5, it was discovered that prolonged administration of the drug resulted in some alterations of basal neurotransmitter output and in changes to releasable neurotransmitters pools.

Of course, many criticisms may be levelled at such a study (dose of drug and numbers of animals used, duration of study, etc.) but these findings establish the principle that VPA may influence EC neurotransmitter levels during a prolonged period of treatment. These are important findings, given the absence of equivalent clinical data. However, the lack of similar studies utilizing epileptic animals is unfortunate since this does not readily allow extrapolation of these data to epilepsy. It is not at present clear how VPA-induced changes in hippocampal neurotransmitter output relate to the long-term efficacy of the drug, but these studies represent a starting point for more a detailed evaluation of <u>in vivo</u> anticonvulsant pharmacology.

The findings presented in chapter 8 are of considerable interest since they extend the possibility that VPA may exert some of its anticonvulsant actions via an interaction with monoaminergic neurotransmission. This notion has recieved variable support (Horton et al, 1977; Chung Hwang and Van Woert, 1979; Lazarova et al, 1983). Critically, the use of relatively selective DA and 5-HT receptor ligands during the current research has allowed a more detailed investigation of how the anticonvulsant may affect the *balance* of pro- and anti-convulsant modulatory influences, associated with monoaminergic receptor activation within the CNS. Of greatest significance is the observation that changes in brain EC monoamines induced by VPA may be associated with a genuine anticonvulsant action, rather than simply motor toxicity evident in animals following large doses of the drug (Fletcher and Harding, 1981; Löscher, 1993). VPA-induced alterations in central monoamine output may play a role in the expression of the VPA behavioural syndrome (de Boer, 1977),

but, as discussed in chapter 5, tolerance to this action of the drug develops during subchronic treatment, whilst the anticonvulsant efficacy of the drug remains unaffected or even increases (Löscher et al, 1988). Since it has been established that anticonvulsant effects are associated with activation of particular monoaminergic receptor subtypes in several animal models of epilepsy (see general introduction 3.2 and chapter 8, discussion), it is possible that VPA may indeed interact with these systems to modulate seizure thresholds where previously no relation was thought to exist (Horton et al, 1977; Löscher et al, 1988; 1993).

A common mode of action of VPA on turnover and release of neurotransmitters?

On the basis of data presented in this thesis, it is clear that VPA affects neurotransmitter output in a number of ways: This is most convincingly illustrated by the differential actions of the drug on hippocampal amino acid and monoamine output when voltage dependent release processes where compromised with TTX (Chapter 6). In addition, there appears to exist a brain-regional heterogeneity in the effects of VPA on monoamine output (Chapter 3). Given that VPA is known to alter neuronal function in a number of different ways, it is probably unwise to suggest that the anticonvulsant alters neurotransmitter release via a *single* action. There are, however, grounds for suggesting that a *common* mechanism may exist through which this drug affects neurotransmitter synthesis: A consistent feature of in vitro studies investigating the biochemical mode of action of VPA is that the drug enhances GAD activity (see general introduction 5.1 for overview). Evidence suggests that at least part of this action may result from a direct activation the enzyme

(Silverman et al, 1991), although the possibility exists that the drug might promote an indirect activation of this enzyme by increasing availability of its cofactor PLP to the GABA-synthetic compartment (discussion, chapter 7). Since VPA also increase synthesis (Whitton et al, 1984; 1985) and release (Whitton and Fowler, 1991; Chapters 3, 5 and 6, this thesis) of monoamine neurotransmitters <u>in vivo</u>, it seems that the anticonvulsant may exert essentially similar actions on all these systems, possibly via a common interaction with elements of their synthetic pathways. Whether these apparently common effects of the drug on neurotransmitter synthesis relate to an action on biochemical substrates collectively involved in such processes (e.g., decarboxylase activity, cofactor availabilty) remains to be determined.

It is unlikely that a *definitive* mechanism through which VPA exerts an anticonvulsant action exists. However, work undertaken here has gone some way to delineating the role of central neurotransmitter release and metabolism in the mode of action of VPA. Clearly, there is enormous scope for future research and the investigations undertaken here provide starting points for more detailed studies.

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Regional Effects of Sodium Valproate on Extracellular Concentrations of 5-Hydroxytryptamine, Dopamine, and Their Metabolites in the Rat Brain: An In Vivo Microdialysis Study

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Abstract: The effects of sodium valproate (VPA; 100, 200, and 400 mg/kg, i.p.) on ventral hippocampal and anterior caudate putamen extracellular levels of dopamine (DA) and 5-hydroxytryptamine (5-HT) were examined using in vivo microdialysis. VPA induced dose-related increases in dialysate DA, 3,4-dihydroxyphenylacetic acid, and 5-HT in the ventral hippocampus. Anterior caudate putamen dialysate 5-HT was also dose dependently elevated by the drug, whereas DA levels tended to decrease with increasing VPA dose. In contrast, VPA (200, 400, and 800 mg/kg, i.p.) produced no significant elevation of DA in posterior caudate putamen dialysates, although 5-HT levels were significantly elevated at the 400- and 800-mg/kg doses. In all three regions studied, dialysate concentrations of 5-hydroxyindoleacetic acid and homovanillic acid remained at basal levels following VPA treatments. The results are discussed with regard to the possible anticonvulsant mode of action of VPA. **Key Words:** Sodium valproate—5-Hydroxytryptamine—Dopamine—In vivo microdialysis. **Biggs C. S. et al. Regional effects of sodium valproate on extracellular concentrations of 5-hydroxytryptamine, dopamine, and their metabolites in the rat brain: An in vivo microdialysis study.** J. Neurochem. **59,** 1702–1708 (1992).

Sodium valproate (VPA) is a widely used anticonvulsant agent, effective in the treatment of generalised tonic-clonic, partial, absence, and myoclonic seizures (Pinder et al., 1977). Despite the drug's extensive clinical use and its activity in many animal seizure models (Chapman et al., 1982; Löscher and Schmidt, 1988), a conclusive mode of action for VPA remains elusive. An extensive literature relating to the possible neurochemical effects of the drug has accumulated (for review, see Rogawski and Porter, 1990). Mechanisms of action involving GABAergic systems (Löscher, 1981; Phillips and Fowler, 1982), blockade of voltage-dependent Na^+ channels (McLean and Macdonald, 1986), and decreased brain aspartate levels (Schechter et al., 1978) have all been proposed. Research into the possible roles of monoamines in seizure disorders has proceeded concurrently with these areas of investigation, and both clinical and neurochemical evidence implicates 5-hydroxytryptamine (5-HT) in the anticonvulsant action of VPA. Chadwick et al. (1975) reported the use of the 5-HT precursor, 5-hydroxytryptophan, in the successful treatment of postanoxic myoclonus, and a clinical study reported 5-hydroxyindoleacetic acid (5-HIAA) levels to be increased in the CSF of patients receiving VPA treatment for this disorder (Fahn, 1978). Subsequent investigation has revealed VPA to enhance 5-HT synthesis and turnover (Kempf et al., 1982; Whitton et al., 1983, 1985). Recently, VPA was shown to increase extracellular concentrations of 5-HT in the rat ventral hippocampus using in vivo microdialysis (Whitton and Fowler, 1991).

In the light of these findings, we set out to investigate whether VPA altered extracellular levels of 5-HT and dopamine (DA) and to determine whether these effects were evident in brain regions such as the stria-

tum. Recent work has shown apomorphine (a dopamine agonist) to confer protection against pilocarpine-induced seizures in rats when injected into the anterior but not the posterior caudate putamen (Turski et al., 1988). We therefore decided to monitor extracellular monoamine levels in the ventral hippocampus and anterior and posterior caudate putamen following administration of VPA, using the technique of in vivo microdialysis.

MATERIALS AND METHODS

Animals

Male Wistar rats (Charles River, U.K.) weighing 260–300 g were housed individually in purpose-built Perspex experimental cages at room temperature (25°C). Access to food and water was ad libitum.

Materials

VPA (di-*n*-propyl acetate, sodium salt) was supplied by Reckitt and Colman Ltd. (Hull, U.K.). The 5-HT uptake inhibitor citalopram was obtained from Lundbeck and Co. (Copenhagen, Denmark). 5-HT maleate, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-HIAA, and DA hydrochloride were all supplied by Sigma.

Drug administration

VPA was dissolved in 0.9% saline and administered intraperitoneally in a volume of 0.1 ml per 100 g of body weight. Animals not receiving the drug were injected with saline and treated as controls. In all cases, injections were given 120 min following commencement of dialysate collection.

Microdialysis

Following anaesthesia with chloral hydrate (400 mg/kg, i.p.), rats were stereotaxically implanted with concentric microdialysis probes, constructed as described by Whitton et al. (1990). Three brain regions were studied: ventral hippocampus (A −5 mm, L 5 mm, V 8 mm below dura), posterior caudate putamen (A 0.2 mm, L 3 mm, V 8 mm below dura), and anterior caudate putamen (A 1.6 mm, L 2.6 mm, V 8 mm below dura). All coordinates were relative to bregma and were derived from the stereotaxic atlas of Paxinos and Watson (1982). Implanted animals were allowed a postoperative recovery period of approximately 18 h prior to dialysis. Artificial CSF (2.5 mM KCl, 125 mM NaCl, 1.18 mM MgCl$_2$, 1.26 mM CaCl$_2$) was perfused via the probe at a rate of 0.5 μl/min using a Harvard Apparatus syringe infusion pump 22, and following an initial 30-min stabilisation period, 11 consecutive 30-min samples were collected. In all experiments, the artificial CSF contained 1.0 μM citalopram in order to elevate basal 5-HT concentrations to detectable levels. Following the completion of each dialysis experiment, rats were anaesthetised with pentobarbitone sodium (Expiral; Ceva), and their brains were perfused-fixed via the left cardiac ventricle with buffered paraformaldehyde saline (4%; pH 7.4). After 120 min, brains were removed, coronal sections were cut, and correct probe placements were verified.

Sample analysis

Dialysates were analysed for monoamine and metabolite content using HPLC with electrochemical detection within 90 min of collection. The HPLC system consisted of a Gilson pump (model 303), a C 18 reverse-phase column, and an ESA electrochemical detector 5100A with analytical cell 5011. Data were collected via a Dell Corporation PC system 310 interfaced with the electrochemical detector via a Drew data collection unit or via an in-line BBC Goerz Metrawatt SE 120 chart recorder. This latter equipment allowed a further 10-fold amplification of the detector output. To our knowledge, no reports of DA detection in ventral hippocampal dialysates exist; however, in our system with its enhanced recording facilities, we were able to detect this species. All separations were isocratic and the mobile phase had the following composition: 90 mM sodium acetate, 35 mM citric acid, 0.34 mM EDTA, and 0.06 mM sodium octylsulphonic acid with 13% methanol, pH 4.2. The flow rate was set at 1.0 ml/min. Figure 1a represents a typical ventral hippocampal dialysate chromatogram generated by the PC software (Drew "Roseate" chromatography system), and Fig. 1b illustrates the same separation recorded through the chart recorder (see legend for peak identities). For each dialysis experiment, the peak areas for the monoamines and their metabolites were calculated and corrected for in vitro dialysis probe recoveries. These were determined as 40–41% (DA), 26–27% (DOPAC), 25–27% (5-HT), 19–21% (5-HIAA), and 18–20% (HVA). The values for the four preinjection samples were averaged and used as a measure of basal levels. Each sample, pre- and postinjection, was then calculated as a percentage of this value.

Statistics

Statistical comparisons were made between drug treatments and saline controls for each of the collection periods using a Mann–Whitney U test.

RESULTS

Ventral hippocampus

Basal levels of DA, DOPAC, 5-HT, 5-HIAA, and HVA were 47.7–48.3, 530–636, 97–109, 9,660–10,520, and 880–1,120 fmol/10 μl of dialysate, respectively (n = 24). These ranges were derived from all experiments performed and were calculated from the four pretreatment samples taken from each animal. Basal levels of 5-HT and 5-HIAA were consistent with previous measurements (Hutson et al., 1989; Sharp et al., 1989; Whitton and Fowler, 1991). VPA elevated extracellular levels of DA, DOPAC, and 5-HT in a dose-dependent fashion (Figs. 2a and b, and 3a), whereas HVA and 5-HIAA concentrations remained unchanged (Figs. 2c and 3b). As can be seen in Figs. 2a and 3a, DA and 5-HT levels increased only transiently following VPA injection (approximately 30 min); however, at a VPA dose of 400 mg/kg, DOPAC levels remained elevated for 90 min after injection (Fig. 2b). Both the striking effect of VPA on hippocampal 5-HT level and the concomitant lack of change in basal 5-HIAA level (see Figs. 3a and b) are consistent with recent observations by Whitton and Fowler (1991).

Posterior caudate putamen

In contrast with levels measured in the ventral hippocampus, extracellular basal levels of DA, DOPAC,
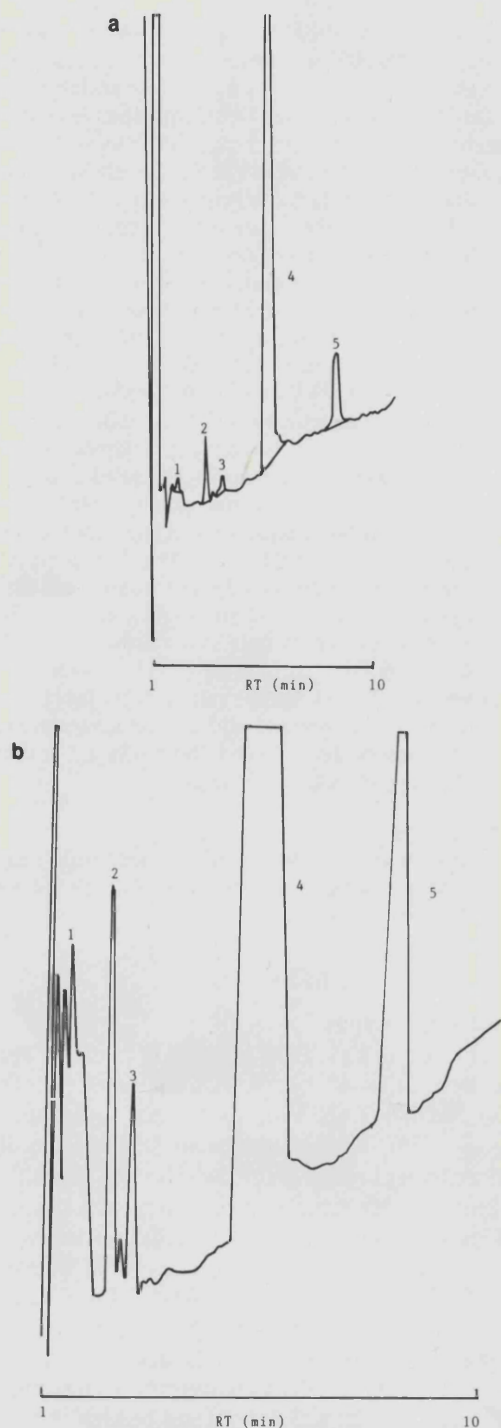
FIG. 1. Typical chromatograms for ventral hippocampal monoamines. Chromatogram generated by PC software (**a**). Same separation recorded using the chart recorder facility (**b**). 1, DA; 2, DOPAC; 3, 5-HT; 4, 5-HIAA; 5, HVA; RT, retention time.

basal levels of 5-HT were noted in dialysates collected from posterior caudate putamen-implanted rats (range 29–45 fmol/10 μl of dialysate), although the 5-HT metabolite 5-HIAA was present at a relatively high concentration (range 21,660–22,760 fmol/10 μl of dialysate). These measurements are comparable to those reported by other laboratories (Kalen et al., 1988; Westerink and de Vries, 1988, 1989).
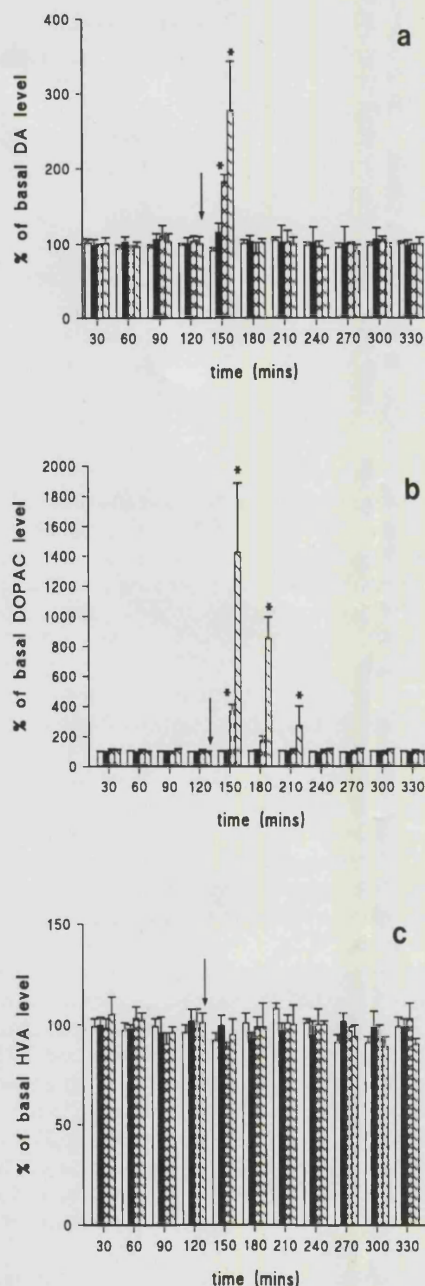
FIG. 2. Effect of VPA on ventral hippocampal dialysate DA concentration (**a**), DOPAC concentration (**b**), and HVA concentration (**c**). Arrows denote time of VPA or saline injection. Open bar, saline; filled bar, 100 mg/kg VPA; crossed bar, 200 mg/kg VPA; hatched bar, 400 mg/kg VPA. All values are means ± SEM of six rats per group. *Significantly different from saline controls in each group ($p < 0.05$).

and HVA were markedly greater in the caudate putamen, most notably DA, which was present at ninefold higher concentrations there than in the hippocampus. Levels in 32 animals ranged from 279 to 341, 12,370 to 13,870, and 19,538 to 21,538 fmol/10 μl of dialysate for DA, DOPAC, and HVA, respectively. Low

FIG. 3. Effect of VPA on ventral hippocampal dialysate 5-HT concentration (**a**) and 5-HIAA concentration (**b**). Arrows denote time of VPA or saline injection. Open bar, saline; filled bar, 100 mg/kg VPA; crossed bar, 200 mg/kg VPA; hatched bar, 400 mg/kg VPA. All values are means ± SEM of six rats per group. *Significantly different from saline controls in each group ($p < 0.05$).
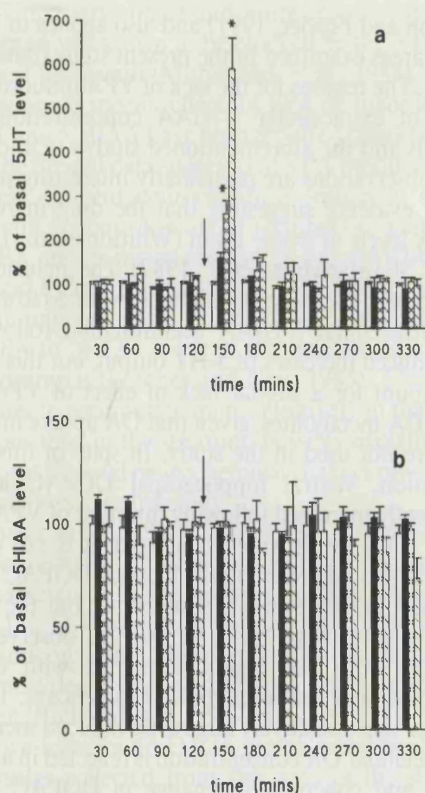
Even at VPA doses as high as 800 mg/kg, dialysate levels of DA were not greatly elevated above basal concentrations and no significant differences existed between drug-treated animals and saline controls (Fig. 4a). Similarly, levels of DOPAC, HVA, and 5-HIAA were all unchanged with respect to those found in saline-treated animals (data not shown). VPA did, however, induce a significant increase in the concentration of 5-HT in the posterior caudate putamen (Fig. 4b). Large doses of the drug were required to elicit changes in dialysate 5-HT, and at the highest dose of 800 mg/kg, 5-HT levels in the first postinjection sample (t = 150 min) were approximately fourfold higher than levels after injection of 400 mg/kg VPA.

Anterior caudate putamen

Basal extracellular levels of DA, DOPAC, and HVA (ranges 311–368, 11,420–13,320, and 23,480–25,080 fmol/10 μl of dialysate, respectively; n = 24) were comparable to those determined in the posterior striatal area. 5-HT and 5-HIAA concentrations were found to be in the ranges 41–51 and 24,080–27,480 fmol/10 μl of dialysate, respectively. Again, all measurements were consistent with those reported previously. Levels of 5-HT and DA in samples taken follow-

ing injection of VPA were increased above baseline, reaching maxima 30 and 60 min postinjection, respectively (Fig. 5a and b). Most notably, dialysate DA levels appeared to be inversely correlated with the dose of VPA administered, with the 100 mg/kg dose inducing the largest postinjection increase. Conversely, 5-HT levels postinjection were found to be elevated dose dependently by the drug. Levels of DOPAC, HVA, and 5-HIAA all remained unaltered by VPA treatments (data not shown).

DISCUSSION

The results of the present study suggest that VPA treatment has multiple effects on dialysate levels of DA and 5-HT. In the ventral hippocampus, elevation of both DA and 5-HT levels occurs only transiently following injection of VPA, whereas in the anterior caudate putamen, a more sustained increase above basal levels of 5-HT was observed. Anterior caudate putamen dialysate DA levels were inversely related to VPA dose. However, in the posterior caudate putamen, VPA was essentially devoid of effect on extracellular levels of DA and induced major elevations of 5-HT concentration only at the higher doses of 400

FIG. 4. Effect of VPA on posterior caudate putamen dialysate DA concentration (**a**) and 5-HT concentration (**b**). Arrows denote time of VPA or saline injection. Open bar, saline; crossed bar, 200 mg/kg VPA; hatched bar, 400 mg/kg VPA; horizontal striped bar, 800 mg/kg VPA. All values are means ± SEM of eight rats per group. *Significantly different from saline controls in each group ($p < 0.05$).
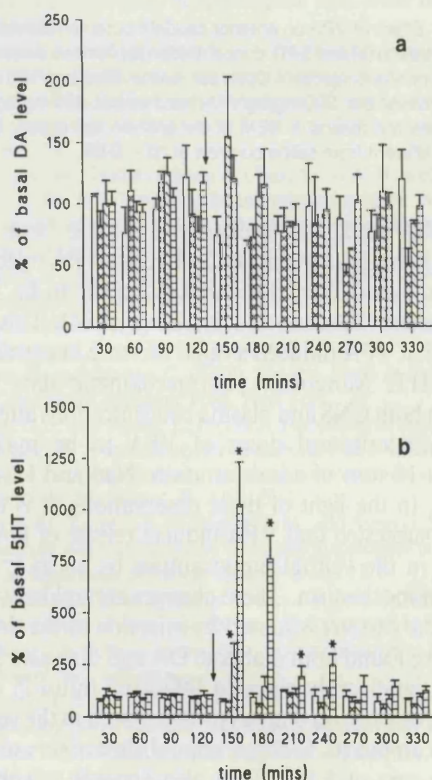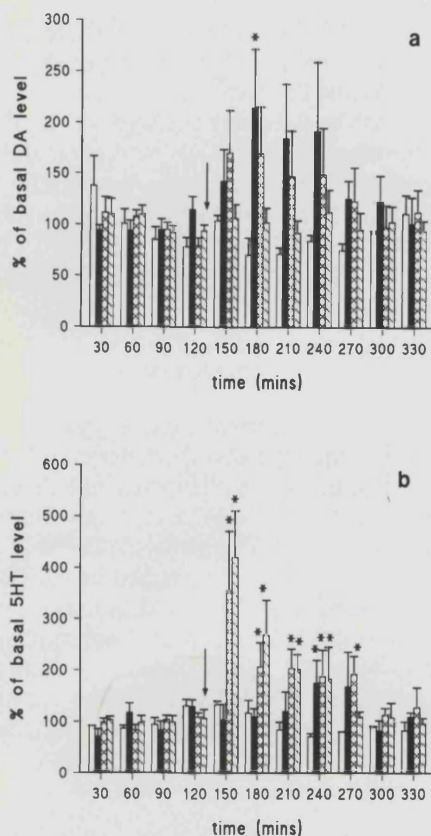
FIG. 5. Effect of VPA on anterior caudate putamen dialysate DA concentration (a) and 5-HT concentration (b). Arrows denote time of VPA or saline injection. Open bar, saline; filled bar, 100 mg/kg VPA; crossed bar, 200 mg/kg VPA; hatched bar, 400 mg/kg VPA. All values are means ± SEM of six animals per group. Significantly different from saline controls at *$p < 0.05$.

and 800 mg/kg. Rats displayed intense "wet dog" shake behaviour within 1–2 min after VPA injection. VPA-induced "wet dog" shakes appear to be 5-HT dependent (Fletcher and Harding, 1981). This suggests that VPA induced a rapid increase in extracellular 5-HT. Moreover, pharmacokinetic data have shown both CNS and plasma concentrations after single intraperitoneal doses of VPA to be maximal within 15 min of administration (Nau and Löscher, 1982). In the light of these observations, it is tentatively suggested that VPA induces release of DA and 5-HT in the ventral hippocampus by an as yet unknown mechanism. These changes are unlikely to be secondary to stress caused by injection of the drug as we have found both dialysate DA and dialysate 5-HT to be significantly elevated following infusion of 60 mg/kg of VPA via dialysis probes placed in the ventral hippocampus (C. S. Biggs, unpublished observations). In the case of 5-HT, VPA also appears to enhance synthesis, as Whitton et al. (1985) found whole brain 5-HT levels to be increased by the drug in pargyline-treated rats. As is evident in Fig. 3b, ventral hippocampal dialysate levels of 5-HIAA were not affected by VPA at each of the doses used. This is consistent with the findings of a previous microdialysis study

(Whitton and Fowler, 1991) and also applies to both striatal areas examined in the present study (data not shown). The reasons for the lack of VPA-induced elevation of extracellular 5-HIAA concentrations in both this and the aforementioned study are unclear. These observations are particularly interesting in the light of evidence suggesting that the drug increases 5-HIAA levels in whole brain (Whitton et al., 1985) and in CSF (MacMillan et al., 1987). The inclusion of citalopram in the dialysate might be expected to attenuate extracellular 5-HIAA accumulation following drug-induced increases in 5-HT output, but this does not account for a similar lack of effect of VPA on striatal DA metabolites, given that DA uptake inhibitors were not used in the study. In spite of this last observation, ventral hippocampal DOPAC levels were greatly increased following injection of VPA, although HVA levels were unchanged. It is not clear why VPA should preferentially increase DOPAC concentration in the ventral hippocampus, but the substantially lower basal levels of DOPAC observed in dialysates from this region compared with those found in striatal dialysates could be significant. Thus, it may be that a relatively large drug-induced increase in extracellular DA concentration is reflected in a substantial and concomitant change in DOPAC level when the basal level of that metabolite is relatively low. However, we observed the basal DOPAC level to be approximately 40-fold higher than the basal level of DA in the striatum, suggesting that the relatively small VPA-induced increases in DA noted here did not significantly contribute to the overall level of DOPAC present in the extracellular milieu. Zetterström et al. (1985), using intrastriatal microdialysis, showed that administration of neuroleptics (previously shown to increase striatal DA synthesis; Westerink and Korf, 1976) increased dialysate DOPAC concentrations. In the present study, where increased anterior caudate putamen dialysate concentrations of DA were not accompanied by increased output of DOPAC following VPA treatments, it would seem unlikely that VPA increased turnover and synthesis of the monoamine. Indeed, a previous investigation indicated that VPA failed to increase whole brain DA synthesis (P. S. Whitton, unpublished observations). In the present study, the effects of VPA on levels of DA in anterior caudate putamen dialysates were notable. Here, with increasing dose of VPA, a clear trend toward a decrease in dialysate DA was observed (Fig. 5a). The converse applied to levels of 5-HT in the equivalent dialysates, although the overall magnitude of change was greater (Fig. 5b). At present, the basis of this result is unclear, although it seems possible that the two monoamines might modulate each other's release in the anterior striatum, quantitatively dependent on the dose of VPA administered. Some in vitro data supporting these suggestions exists. 5-HT has been shown to decrease [^{3}H]DA release from superfused rat striatal slices in a dose-dependent manner (Westfall

and Tittermary, 1982) and more recently, this phenomenon was shown to be blocked by the 5-HT$_2$ antagonist ketanserin (Muramatsu et al., 1988).

Regionally specific effects of VPA on tissue levels of 5-HT and 5-HIAA have been reported (Shukla, 1985) and the present data suggest that VPA produces qualitatively different neurochemical profiles in discrete rat brain regions. A study of regional rat brain levels of VPA and its major metabolite 2-propyl-2-pentenoic acid following acute and chronic dosing with the parent anticonvulsant revealed tissue concentrations of VPA to be 22% lower in the striatum than in the hippocampus (Löscher and Nau, 1983). Thus, VPA appears to accumulate more selectively in the hippocampus than in the striatum, possibly explaining the different regional neurochemical changes observed by microdialysis in this study.

Turski et al. (1988) located the site of apomorphine-mediated protection against pilocarpine-induced seizures in the anterior caudate putamen in rats. DA D$_2$ receptors, which are thought to mediate the anticonvulsant effects of dopaminergic agonists, are most densely situated in the anterior caudate putamen (Richfield et al., 1987). It is perhaps significant that we have found DA to increase preferentially in dialysates collected from this area of the striatum, thus correlating with a high density of DA-sensitive anticonvulsant sites. No data are presently available concerning the efficacy of VPA in preventing pilocarpine-induced seizures.

To summarise, VPA dose dependently increased dialysate 5-HT in both the ventral hippocampus and the anterior caudate putamen. DA levels were dose dependently elevated by VPA in hippocampal dialysates, whereas in the anterior striatum, a clear trend toward decreasing extracellular DA levels with increasing dose of VPA was observed. VPA produced little effect on equivalent posterior striatal dialysates, with the exception that 5-HT levels appeared to increase greatly at the 400 and 800 mg/kg doses. Ventral hippocampal dialysate levels of DOPAC were substantially elevated in the presence of VPA, but all other metabolite measurements failed to disclose similar effects.

These observations may be significant in explaining the mode of anticonvulsant action of this drug in the light of evidence implicating monoamines in the aetiology of certain seizure disorders. Further work aims to examine the effects of chronic VPA dosing on dialysate concentrations of monoamines and their metabolites, as repeated administration of the drug is more pertinent to the clinical situation.

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The effect of sodium valproate on extracellular GABA and other amino acids in the rat ventral hippocampus: an in vivo microdialysis study

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We report the effects of i.p. administration of sodium valproate (VPA) on extracellular concentrations of various amino acids in the rat ventral hippocampus studied using in vivo microdialysis, followed by HPLC with fluorometric detection. At the doses used (100, 200 and 400 mg/kg), VPA had no effect on extracellular aspartate, glutamine and taurine, whilst inducing a small, but not statistically significant increase in glutamate at 200 and 400 mg/kg. In contrast, VPA administration produced a biphasic effect on extracellular GABA levels which was dependent on the dose used. At 100 mg/kg, VPA reduced GABA concentrations by 50% when compared to basal. 200 mg/kg VPA had virtually no effect, whilst 400 mg/kg VPA raised extracellular GABA levels to 200% of basal. The results are discussed in relation to the known pharmacological and anticonvulsant actions of VPA.

Sodium valproate (VPA) is an effective anticonvulsant agent for the management of most forms of epilepsy[25]. Despite an extensive literature, the precise mode of action of VPA remains uncertain. VPA increases GABA concentrations in whole brain[10], descrete brain regions[18,24] and synaptosomes[16,17.] Significantly, VPA appears to preferentially enhance GABA turnover in the neuronal compartment[13] and thus might be expected to increase GABAergic transmission. Despite these findings little data currently exists supporting a role for VPA in increasing GABA release centrally. A recent report suggested that VPA increased K$^+$-stimulated release of endogenous and [^{3}H]GABA from cultured cortical neurones[11], although Farrant and Webster[8] failed to observe any change in spontaneous nigral GABA release by VPA using push–pull cannulae. There is, however, evidence that VPA enhances inhibitory post-synaptic potentials recorded from hippocampal neurones in vitro[26].

We present data here which suggests VPA alters extracellular GABA, in ventral hippocampal dialysates collected from concious, freely moving rats. Since the hippocampus is a structure known to be particularly susceptible to epileptogenic phenomena[2] these data might be significant in explaining aspects of VPA efficacy in ameliorating some types of epilepsy.

Male Wistar rats (240–300 g) were housed at room temperature and allowed free access to food and water. Rats were anaesthetised with chloral hydrate (400 mg/kg, i.p.; BDH, UK) and stereotaxically implanted with concentric microdialysis probes which were constructed as previously described[30] into the ventral hippocampus using coordinates A −5 mm from bregma, L +5 mm, V 8 mm below dura. Coordinates were taken from the stereotaxic atlas of Paxinos and Watson[23]. Following a post-operative recovery period of approximately 18 h, dialysis was commenced with an artificial cerebrospinal fluid (ACSF) of the following composition: KCl 2.5 mM, NaCl 125 mM, MgCl$_2$ 1.18 mM and CaCl$_2$ 1.26 mM. ACSF was perfused at a rate of 0.5 μl/min using a Harvard Apparatus infusion pump (model 22), and following an initial 30 min equilibration period, 12 consecutive 30 min dialysates were collected. Injections of either saline or VPA (dissolved in 0.9% saline; VPA obtained from Reckitt and Colman, UK) were administered i.p. to rats 150 min fol-

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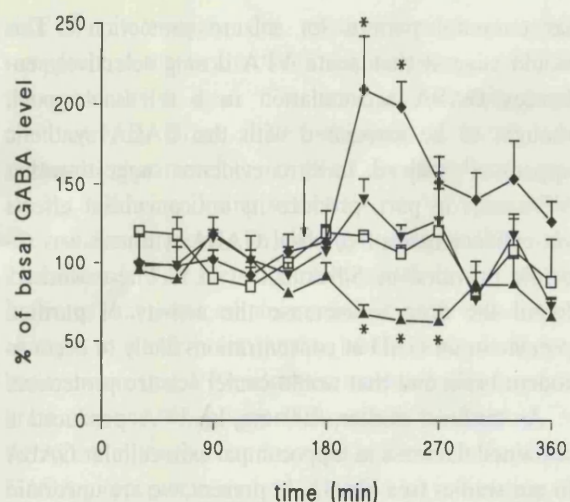
250
200
150
100
50
0
% of basal GABA level
0
90
180
270
360
time (min)
*
*
*
*
*

Fig. 1. Effects of VPA upon GABA levels in ventral hippocampal dialysates. The arrow denotes the time at which the VPA dose or saline was administered. (□) Saline; (▲) 100 mg/kg VPA; (▼) 200 mg/kg VPA; (◆) 400 mg/kg VPA. All values are mean ± S.E.M. of 8 rats per group. * indicates data points significantly different from saline controls in each group ($P < 0.05$).

lowing commencement of dialysis, in a volume of 0.1 ml per 100 g body weight. On completion of each experiment, rats were anaesthetised with pentobarbitone sodium (Expiral; Ceva) and their brains perfused-fixed via the left cardiac ventricle with heparinised paraformaldehyde saline (4%, pH 7.4). Brains were removed 120 min following fixation, and coronal sections cut in order to verify probe placements.

Dialysates were analysed for amino acid content using an HPLC method as follows:

The system comprised two Gilson pumps (model 303), a C18 reverse-phase column with column heater, a Gilson refrigerated autoinjector (model 231) and a Gilson fluorimetric detector (model 121). Dialysates (10μl) were pre-column derivatised with *o*-pthaldialdehyde (OPA) and amino acid conjugates resolved using a gradient separation. The mobile phase consisted of two components (A) 50 mM sodium dihydrogen orthophosphate, pH 5.5, with 20% methanol and (B)

(a)
200
150
100
50
0
% of basal aspartate level
90
180
270
360
time (min)
(b)
200
150
100
50
0
% of basal glutamate level
0
90
180
270
360
time (min)
(c)
200
150
100
50
0
% of basal glutamine level
0
90
180
270
360
time (min)
(d)
200
150
100
50
0
% of basal taurine level
0
90
180
270
360
time (min)

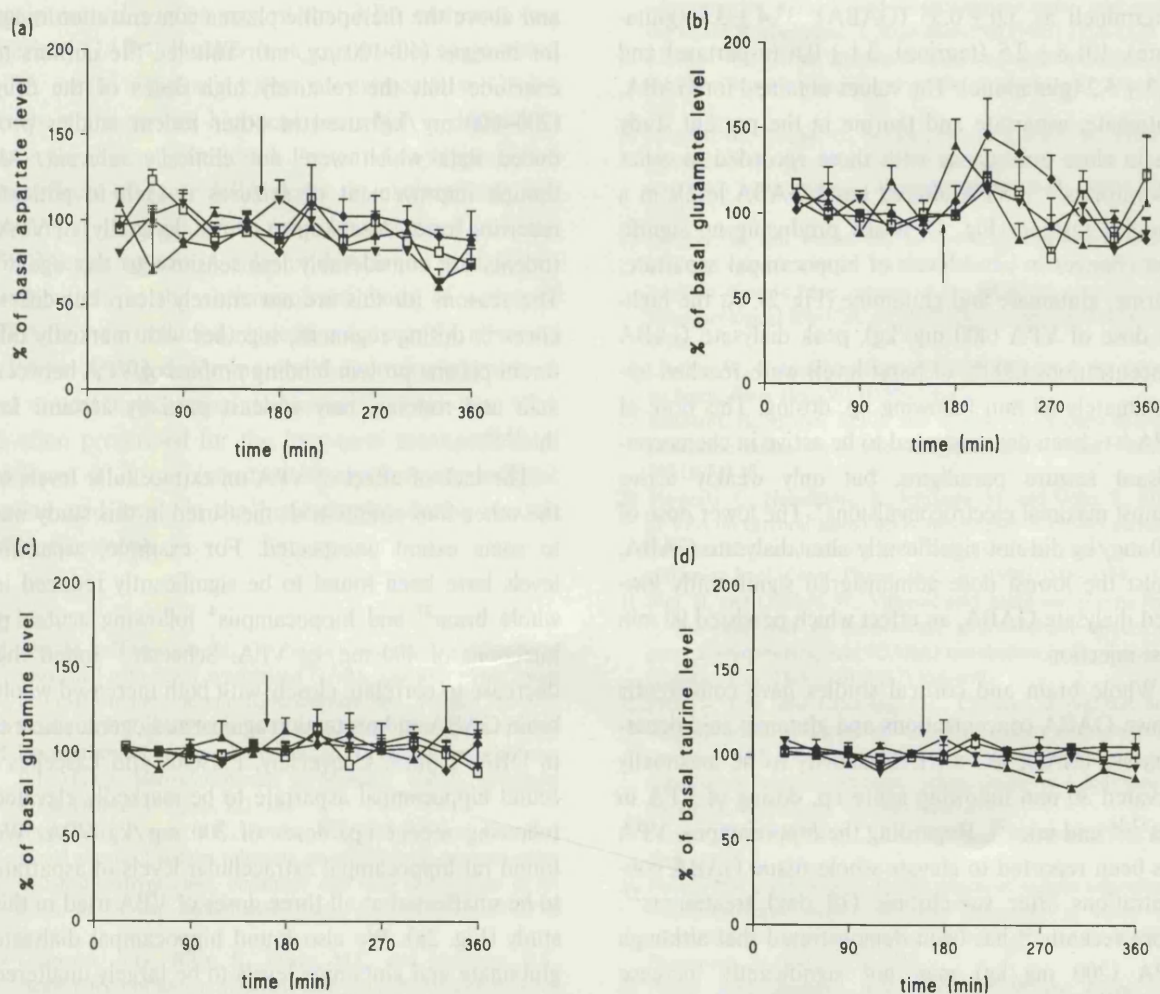

Fig. 2. Effect of VPA on ventral hippocampal dialysate levels of (a) aspartate, (b) glutamate, (c) glutamine and (d) taurine. The arrow denotes the time at which the VPA dose or saline was administered. (□) Saline; (▲) 100 mg/kg VPA; (▼) 200 mg/kg VPA; (◆) 400 mg/kg VPA. All values are mean ± S.E.M. of 8 rats per group.

100% methanol; gradient composition was determined by an Apple microcomputer installed with Gilson gradient management software, and mobile-phase flow rate was maintained at 1.0 ml/min. Data were collected by a Dell Corporation PC system 310 interfaced to the detector via a Drew data collection unit. All data obtained from the chromatograms were corrected for in vitro dialysis probe recovery of amino acids and these were found to be 47 ± 4 (GABA), 37 ± 2 (glutamate), 50 ± 3 (taurine), 39 ± 2 (aspartate) and 51 ± 2 (glutamine); all values mean% recovery $\pm$ S.E.M. at a flow rate of 0.5 μl/min; $n = 6$. For each experiment, levels of amino acids in the five dialysates collected prior to saline or VPA injection were averaged and used as a measure of basal levels. Quantities of amino acids in each dialysis sample, pre- and post-injection, were then calculated as a percentage of this value. Stastistical comparisons between saline and VPA treatments at each of the twelve collection periods were made with a Mann–Whitney U test.

Rat ventral hippocampal basal levels of amino acids (mean pmol $\pm$ S.E.M./10 μl dialysate; $n = 32$) were determined as 3.0 ± 0.35 (GABA), 33.4 ± 3.7 (glutamate), 101.8 ± 2.5 (taurine), 3.1 ± 0.6 (aspartate) and 43.3 ± 5.2 (glutamine). The values obtained for GABA, glutamate, aspartate and taurine in the present study are in close accordance with those recorded in other laboratories[14,29]. VPA altered basal GABA levels in a complex fashion (Fig. 1), whilst producing no significant changes in basal levels of hippocampal aspartate, taurine, glutamate and glutamine (Fig. 2). At the highest dose of VPA (400 mg/kg), peak dialysate GABA concentrations (200% of basal level) were reached approximately 60 min following i.p. dosing. This dose of VPA has been demonstrated to be active in chemoconvulsant seizure paradigms, but only weakly active against maximal electroconvulsions[9]. The lower dose of 200 mg/kg did not significantly alter dialysate GABA, whilst the lowest dose administered significantly lowered dialysate GABA, an effect which persisted 90 min post injection.

Whole brain and cortical studies have consistently shown GABA concentrations and glutamic acid decarboxylase (GAD, EC 4.1.1.15) activity to be maximally elevated 30 min following acute i.p. dosing of VPA in rats[12,24] and mice[21]. Regarding the hippocampus, VPA has been reported to elevate whole tissue GABA concentrations after sub-chronic (10 day) treatments[22]. More recently it has been demonstrated that although VPA (200 mg/kg) may not significantly increase whole-tissue hippocampal GABA levels acutely, GABA accumulation in the synaptosomal compartment is enhanced by approximately 80%, and this follows a simi-

lar temporal pattern for seizure protection[17]. This would suggest that acute VPA dosing selectively enhances GABA accumulation in a releasable pool, thought to be associated with the GABA synthetic apparatus[1]. Indeed, in vitro evidence suggesting that VPA may, in part, produce its anticonvulsant effects via enhancement of cerebral GABA synthesis was recently provided by Silverman et al.[28]. These workers found the drug to increase the activity of purified porcine brain GAD at concentrations likely to occur in rodent brain and that would confer seizure protection.

As outlined earlier, 100 mg/kg VPA produced a sustained decrease in hippocampal extracellular GABA in our studies (see Fig. 1). At present, we are uncertain as to the mechanisms responsible for the reduction in extracellular GABA concentration observed in the hippocampus following acute treatment with 100 mg/kg VPA. However, Wolf et al.[32] have also reported a significant reduction of extracellular GABA following local application of 40–200 μg/ml VPA into the rat preoptic area via push–pull cannullae. The effects noted were elicited by doses of VPA said to be within and above the therapeutic plasma concentration range for humans (40–100 μg/ml). This led the authors to conclude that the relatively high doses of the drug (200–600 mg/kg) used in other rodent studies produced data which were not clinically relevant. Although improvement of seizures is seen in patients receiving lower doses (up to 45 mg/kg orally) of VPA, rodents are considerably less sensitive to this agent[5]. The reasons for this are not entirely clear, but differences in dosing regimens, together with markedly different plasma protein binding profiles of VPA between man and rodents, may at least partially account for this[15,19].

The lack of effect of VPA on extracellular levels of the other four amino acids measured in this study was to some extent unexpected. For example, aspartate levels have been found to be significantly reduced in whole brain[27] and hippocampus[4] following acute i.p. injections of 400 mg/kg VPA. Schecter[27] found this decrease to correlate closely with both increased whole brain GABA and protection against audiogenic seizures in DBA/2 mice. Conversely, Patsolos and Lascelles[22] found hippocampal aspartate to be markedly elevated following repeat i.p. doses of 300 mg/kg VPA. We found rat hippocampal extracellular levels of aspartate to be unaffected at all three doses of VPA used in this study (Fig. 2a). We also found hippocampal dialysate glutamate and glutamine levels to be largely unaltered by all doses of VPA administered to rats, although glutamate was elevated following 400 mg/kg VPA, a result which was not statistically significant (Fig. 2b

and c). Interestingly, this effect followed a similar temporal profile to that seen for dialysate GABA at the same VPA dose. This contrasts with findings demonstrating either a 10% decrease in tissue hippocampal glutamate[5] or no effect on whole-brain glutamate following dosing with VPA[6,10]. VPA failed to alter dialysate taurine in the hippocampus (Fig. 2d). This accords with the work of Miyazaki et al.[20] which indicates that acute administration of VPA induces no changes in whole-brain taurine levels, however, it has been shown that VPA elevates taurine levels in most brain regions when given chronically[22]. Since it is thought that taurine can function as a modulator of the $GABA_A$ receptor complex[7], it would be interesting to know whether chronic VPA dosing elevates extracellular taurine.

In summary, it would seem that acute doses of VPA do alter extracellular GABA concentrations, at least in the ventral hippocampus and within a dose range that confers protection in rodent seizure paradigms. At present it is not possible to explain the neurochemical basis of these findings, although whole tissue and synaptosomal studies suggest that VPA increases synthesis of GABA. Furthermore, our studies suggest that VPA exerts a biphasic effect on extracellular GABA levels in the ventral hippocampus which is dependent upon the dose administered. Present data suggests that the effects of acute VPA doses on extracellular hippocampal amino acids may be confined to the GABAergic system. A role for non-amino acid neurotransmitters in the anticonvulsant mode of action of VPA is not excluded, indeed we recently observed VPA to elevate dopamine and 5-hydroxytryptamine in rat ventral hippocampal dialysates[3,31]. Given that VPA is often prescribed for the long-term management of epilepsy, it is our intention to monitor extracellular amino acid levels in the hippocampus during chronic VPA treatment.

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