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# MECHANISMS OF RELEASE AND REUPTAKE OF AMINO ACID CENTRAL NEUROTRANSMITTERS

Michael James Williams Brennan

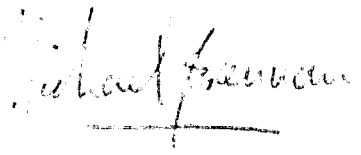
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for the Degree of Doctor of Philosophy.

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Johannesburg

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DECLARATION

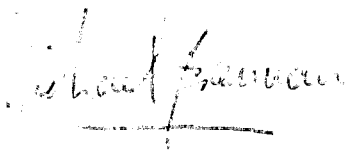
I hereby declare that the work presented in this thesis is my own and has not previously been submitted for a degree at this or any other university.

Signed: 

M J W Brennan

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Signed: 

M J W Brennan

to Jenny

PREFACE

An understanding of the stereospecificity of the transport processes for amino acid neurotransmitters is essential to the development of techniques for labelling specific pools of transmitters in heterogeneous nervous tissues. This study was initiated with the purpose of establishing the stereochemical criteria for substrates for amino acid transport processes in nerve endings. Using analogues of the inhibitory amino acid neurotransmitter γ-aminobutyric acid (GABA) some of the limitations imposed on substrates for the high affinity GABA uptake mechanism were established.

During the course of this study it was found that the porphyrin precursor δ-aminolaevulinic acid (δ-ALA) exhibited potent effects on GABA and glutamate uptake and efflux in rat brain synaptosomes. δ-ALA is a 5-carbon chain analogue of GABA. The demonstration that δ-ALA inhibited stimulated GABA release and that this effect was prevented by the GABA receptor antagonists bicuculline and picrotoxin, led to the suggestion that GABA release is subject to negative feedback control via GABA autoreceptors. This hypothesis has now been confirmed by several investigators.

The role of δ-ALA in the pathogenesis of the neural dysfunction in the acute porphyric attack is discussed.

Since the pharmacology of the GABA autoreceptor is completely unknown, it was decided to investigate the effect of several known GABA agonists on stimulated GABA release. These studies have suggested that the autoreceptors are pharmacologically identical to the postsynaptic GABA receptors and, further, that release of GABA is regulated directly via activation of these receptors. This is the first time that such a relationship has been demonstrated for any GABA receptor-mediated effect.

ABSTRACT

The effect of a number of GABA analogues on the release of preloaded $^{3}$H-GABA from rat cortical synaptosomes was examined. Of these only L-2,4-diaminobutyric acid (DABA) and δ-ALA stimulated efflux to any significant extent. β-Alanine was a weak stimulant of release while L-ornithine (the 5-carbon chain analogue of DABA), L-homocysteine and L-methionine were inactive. These results confirm the idea that DABA exchanges primarily with neuronal pools of GABA and β-alanine with glial pools. In addition, it appears that the length of the hydrocarbon chain is of importance in determining the extent to which a GABA analogue interacts with the neuronal high affinity GABA transport site. The upper limit of separation of zwitterionic centres is higher at neuronal than at glial sites.

δ-ALA at concentrations above $10^{-4}$M exhibited marked effects on high affinity GABA and glutamate transport in nerve endings. Since influx of δ-ALA into the mammalian brain is known to be limited, these effects are unlikely to be responsible for the neural dysfunction characterising acute attacks of the hereditary hepatic porphyrias.

At low concentrations ($10^{-6}$M), δ-ALA was found to inhibit the $K^+$-stimulated release of preloaded $^3$H-GABA from rat cortical synaptosomes. This effect was prevented by the GABA receptor antagonists bicuculline and picrotoxin. These results suggest the existence of bicuculline-sensitive inhibitory GABA autoreceptors and imply that δ-ALA is a potent agonist at these receptors. Inhibition of stimulated GABA release is evident at δ-ALA concentrations well below those already recorded in the CSF of patients during acute porphyric attacks. Thus, interference with GABA function in the brain provides a tenable explanation for the neuropsychiatric manifestations of the acute porphyric attack.

The pharmacological specificity of the GABA autoreceptors remains unknown. In the final part of this study, the effect of a number of known GABA agonists (muscimol, PSA, isoguvacine and THIP) on stimulated GABA release was examined. A good correlation between the potency of the drugs at autoreceptors and their potency in inhibiting specific $^3$H-GABA binding to plasma membranes was found. These suggest that the autoreceptor is pharmacologically identical to

the site labelled with $^{3}$H-GABA in conventional binding studies. In addition, it was shown that inhibition of GABA release is correlated directly with GABA receptor occupancy. This implies a close coupling of the autoreceptors to the effector mechanism and suggests a lack of "spare" auto-receptors. Such a relationship between a receptor - mediated effect and receptor occupancy has not previously been demonstrated for any GABA-mediated effect.

PUBLICATIONS

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Articles in International Journals:

1. The effect of DABA and β-alanine on the efflux of [$^3$H] GABA from preloaded rat brain synaptosomes.
   M.J.W. Brennan and R.C. Cantrill (1978)
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   M.J.W. Brennan and R.C. Cantrill (1979).
   J. Neurochem. 33, 721-725.

5. δ-Aminolaevulinic acid is a potent agonist for GABA autoreceptors.
M.J.W. Brennan and R.C. Cantrill (1979).
Nature 280, 514-515.

6. Neurotoxicity of delta-aminolaevulinic acid re-evaluated.
M.J.W. Brennan (1979).
S. Afr. J. Sci. 75, 335.

7. A simple and versatile apparatus for the continuous superfusion of nervous tissues preparations.
R.C. Cantrill and M.J.W. Brennan (1980).
Experientia 36, 141-142.

8. An *in vitro* technique for the superfusion of tissue preparations.
M.J.W. Brennan and R.C. Cantrill (1980).
S. Afr. J. Sci. (in the press).

9. GABA autoreceptors and porphyria.
M.J.W. Brennan and R.C. Cantrill (1980).
S. Afr. J. Sci. (in the press).

10. Effect of delta-aminolaevulinic acid on GABA receptor binding in synaptic plasma membranes.
M.J.W. Brennan, R.C. Cantrill and S. Kramer (1980).
Int. J. Biochem (in the press).

11. Pharmacology of GABA autoreceptors.
M.J.W. Brennan, R.C. Cantrill and P. Krogsgaard-Larsen (1980).
Proc. Europ. Soc. Neurochem. (in the press).

Invited Articles in International Publications:

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M.J.W. Brennan and R.C. Cantrill (1980).
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2. Potentiated activity of delta-aminolaevulinic acid.
M.J.W. Brennan and R.C. Cantrill (1979).
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3. Evidence for presynaptic GABA receptors.
M.J.W. Brennan, R.C. Cantrill and H. Epstein (1979).
4th Natn. Congr. S. Afr. Biochem. Soc.
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1. The effect of hypothyroidism on the development of neuronal high affinity GABA uptake in rat brain.
   M.J.W. Brennan, R.C. Cantrill and A.J. Bentley (1979). 4th Natn. Congr. S. Afr. Biochem. Soc. (Grahamstown). 101.

2. The role of presynaptic membrane protein phosphorylation in modulating neurotransmitter release.
   M.J.W. Brennan and R.C. Cantrill (1980). Biochem. Soc. Trans. 8, 60-61.

3. Methionine-enkephalin is a potent stimulant for GABA release.
   M.J.W. Brennan, R.C. Cantrill and B.A. Wylie (1980). Biochem. Soc. Trans. 8, 61-62.

4. Presynaptic membrane protein phosphorylation modulates the release of GABA from preloaded synaptosomes.
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5. Control of transmitter release by synaptic membrane protein phosphorylation.
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6. Interaction of cholinergic and adrenergic systems in the rabbit hypothalamus.
K.P. Klugman, M.J.W. Brennan and R.C. Cantrill (1980).
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7. Effects of adenosine derivatives on the uptake and release of L-glutamate in rat brain synaptosomes.
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8. The effect of ethanol on the high affinity GABA transport system in rat brain synaptosomes.
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S. Afr. Med. J. (in the press).

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R.C. Cantrill, M.J.W. Brennan, J.L. McLoughline and B.C. Shanley (1980).
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# CHAPTER 1

# INTRODUCTION

## 1.1 Amino acid neurotransmitters : general considerations.

Extensive study in recent years has suggested that several amino acids might serve as neurotransmitters in the mammalian CNS. Amino acid transmitter candidates include the inhibitory compounds $\gamma$-aminobutyric acid (GABA) and glycine, and the excitatory amino acids L-glutamate and L-aspartate (for a review see Curtis and Johnston, 1974). In the present study, aspects of the uptake of GABA and glutamate into nerve terminals and their release from the nerve endings on various stimuli were examined.

GABA is regarded as an important inhibitory neurotransmitter functioning throughout the mammalian CNS (see Roberts et al., 1976). Small interneurons, such as Golgi-type-II neurons and basket cells, in the neocortex, cerebellum, hippocampus and thalamus appear to be the major categories of nerve cells which release GABA (Bloom and Iversen, 1971; Iversen and Bloom, 1972; Sotelo et al., 1972; Ljungdahl and Hokfelt, 1973; Ljungdahl et al., 1973).

In addition, GABA has been localised in the spinal cord of the rat (Iversen and Bloom, 1972) and the cat (Ljungdahl and Hokfelt, 1973) by electron-microscopic autoradiography. GABA is synthesized in GABA ergic nerve terminals by the enzyme glutamate decarboxylase (E.C. 4.1.1.15)(Salganicoff and De Robertis, 1965; Balazs et al., 1966; Fonnum et al., 1970; Fonnum and Walberg, 1973; McLaughlin et al., 1974; Sellstrom et al., 1975); it is released in response to nerve stimulation, and removed from the synaptic cleft by a high affinity uptake system present in the nerve terminals, synapse-associated glial cells and post-synaptic neuronal perikarya (Iversen and Neal, 1968; Hokfelt and Ljungdahl, 1970; Bloom and Iversen, 1971; Henn and Hamberger, 1971; Storm-Mathisen, 1975). Although the point is contra-versial, this high affinity uptake system is thought to be the major mechanism whereby the postsynaptic action of GABA is terminated (Iversen and Johnston, 1971). Finally, GABA metabolism to succinic semialdehyde is catalysed by the enzyme GABA:α-ketoglutamate transaminase (GABA-T, and the oxidation of succinic semi-aldehyde to succinic acid is catalysed by succinic semialdehyde dehydrogenase (McIlwain and Bachelard, 1971).

The pathway: α-ketoglutarate → glutamate → GABA → succinic semialdehyde → succinate is known as the GABA shunt, and accounts for some 10% of cerebral oxidative metabolism (McIlwain and Bachelard, 1971).

The most convincing evidence that GABA is an inhibitory transmitter in the mammalian brain has been obtained through microiontophoretic experiments and intracellular recording studies. Administered from micropipettes, GABA depresses the firing of cortical neurons of several species (Krnjevic and Phillis, 1963; Crawford and Curtis, 1964; Biscoe et al., 1972; Clarke and Hill, 1972; Sillitto, 1975). Depression of firing of cortical neurons by GABA is accompanied by membrane hyperpolarization and an increase in membrane conductance, principally to $Cl^-$ (Krnjevic and Schwartz, 1967; Dreifuss et al., 1969). In these respects the action of GABA resembles that of the transmitter released by inhibitory neurons activated by a stimulus to the cortical surface (Krnjevic and Schwartz, 1967).

The specificity and pharmacological relevance of these phenomena were demonstrated in other electrophysiological experiments in which it was found that the convulsants picrotoxin and bicuculline, could selectively block neuronal responses to GABA at concentrations which have little or no effect on inhibition induced by other amino acids (Curtis et al., 1971). In addition, GABA is strongly implicated as the transmitter mediating presynaptic inhibition in the vertebrate spinal cord (Roberts, 1975); it is thought to act through a depolarisation of primary afferent terminals thus reducing the probability of release of quanta of excitatory transmitter (Takeuchi and Takeuchi, 1966; Roberts, 1975).

The theory of chemical transmission, first suggested by Elliot in 1904 and established by the investigations of Loewi (1921) and Dale et al., (1936), presupposes the existence of receptive sites on or in postsynaptic membranes which bind neurotransmitters released from presynaptic terminals.

The interaction of transmitter with receptor triggers the events leading to the membrane changes underlying nervous transmission. In recent years, ligand binding studies have facilitated the direct biochemical demonstration of receptor sites for a variety of neurotransmitters in the mammalian brain (Snyder, 1978). Using radiolabelled GABA, GABA binding sites having receptor-like properties have been measured in membranes prepared from rat and bovine brains (Zukin et al., 1974; Young et al., 1976; Wong and Horng, 1977; Lester and Peck, 1978).



The binding of $^{3}H$ GABA to its effector receptor site differs from binding to the transport carrier in that it is $Na^{+}$-independent (Enna and Snyder, 1977) and is masked in freshly prepared rat brain membranes by an acidic protein assumed to be an endogenous modulator of the GABA receptor (Guidotti et al., 1978; Toffano et al., 1978). Extensive washing of the membrane preparations or treatment with the detergent Triton X-100 removes this endogenous inhibitor and exposes the population of high affinity GABA binding sites (Enna and Snyder, 1977; Wong and Horng, 1977).

Careful biochemical studies have revealed that this inhibitory or modulator substance modifies the affinity of the GABA receptor recognition site in a non-competitive fashion, suggesting that the modulator interacts with a membrane component to produce an allosteric change in the conformation of the receptor site (Toffano et al., 1978). It is interesting to note that drugs of the benzodiazepine class competitively inhibit the binding of this modulator to its site and thus facilitate GABA ergic transmission (Guidotti et al., 1978).

Heuristically, the GABA receptor may be conceptualized as possessing at least two functional sites. One is the recognition site to which the neurotransmitter attaches and the other is the ionophore which, when activated, allows for the passage of $Cl^-$ ions (De Feudis, 1977; Horng and Wong, 1979; Olsen et al., 1979). The existence of separate sites is suggested by the fact that convulsants such as picrotoxin, tutin and tetramethylenedisulfotetramine, which are known to antagonise the action of GABA on neuronal membranes do not interfere with the attachment of GABA to the recognition site, but rather appear to compete

with $Cl^-$ at the ionophore. (Takeuchi and Takeuchi, 1969; Zukin et al., 1974; Enna et al., 1977; Olsen et al., 1979). Recently, Olsen et al., (1979) have reported a biochemical method for labelling the GABA receptor ionophore in brain tissue using $^3H$-dihydropicrotoxinin as a ligand. Using this assay, these investigators examined a series of barbiturates and found that these agents are capable of interacting with the dihydropicrotoxinin binding site and might potentiate GABA responses by facilitating ion conductance through the ionophore (Ticku and Olsen, 1978). If this hypothesis is correct, it would provide a biochemical mechanism of action for the electrophysiological findings indicating that the barbiturates prolong GABA ergic responses in a variety of neuronal systems (Nicoll, 1975; Ransom and Barker, 1976; Bowery and Dray, 1978; Tsuchiya and Fukushima, 1978). Despite these detailed studies, the molecular mechanisms whereby the interaction of GABA with its receptor site opens the $Cl^-$ ionophore remain unknown.

Although the evidence for a transmitter role of L-glutamate is not nearly as convincing as that supporting the role of GABA in transmission,

there is sufficient information to make such a function seem probable (for a review see Curtis and Johnston, 1974).

Glutamate is incorporated into proteins and peptides, is involved in fatty acid synthesis, contributes (along with glutamine) to the regulation of ammonia levels and the control of osmotic or anionic balance, serves as a precursor for GABA and for various Krebs cycle intermediates, and is incorporated as a constituent of at least two important cofactors (glutathione and folic acid). In view of these many roles, it is not surprising that glutamate is the most abundant amino acid in the adult mammalian CNS and shows a fairly even distribution (Costa et al., 1979). These considerations, plus the fact that glutamate excites virtually all neurones so far tested in vivo (Johnson, 1972), have made the definition of specific glutamergic pathways in the mammalian CNS extremely difficult. In the spinal cord of the cat, the high levels of glutamate in dorsal roots and dorsal horn relative to those ventrally, suggest that glutamate might be the transmitter of some primary afferents (Davidoff et al., 1967; Johnson, 1972; 1977). Dorsal root section, however, had little effect on dorsal horn glutamate levels (Johnson, 1977).

It is possible that glutamergic primary afferent terminals contain only a very small fraction of the total glutamate within he cord; ir this is the case, one would not expect a significant decrease in dorsal horn glutamate levels on sectioning of the dorsal roots.

Intracerebral infection of neonatal hamsters with a specific rat parvovirus results in the selective loss of more than 90% of the cerebellar granule cells (Young et al., 1974). This is associated with a marked fall in cerebellar glutamate levels and a reduction in the uptake of glutamate by cerebellar homogenates (Young et al., 1974). Cerebellar glutamate levels in the rat are significantly reduced by X-irradiation which is associated with a selective reduction in the number of granule cells (Valcana et al., 1972), and cerebellar glutamate levels are also low in mutant mice having a reduced number of granule cells (Hudson et al., 1976; Roffler-Tarlov and Sidman, 1978). These observations all suggest that the granule cell synapses with Purkinje cell dendrites may be glutamergic.

In the rat, lesions of corticostriatal pathways produce a significant reduction in the uptake of glutamate by tissue from the caudate-putamen (Mc Geer et al., 1977), and frontal cortical ablation results in a decrease in striatal glutamate levels (Kim et al., 1977). This suggests the existence of a glutamergic corticostriatal pathway.

In the hippocampal formation, evidence has been obtained suggesting that the perforant pathway, the granule cells and the pyramidal cells of areas CA3 and 4 may utilize glutamate as a transmitter substance (Nadler et al., 1976; Storm-Mathisen, 1977; Hamberger et al., 1978). In addition, the substantial reduction in the levels of glutamate in the guinea pig olfactory cortex following removal of the olfactory bulb suggests the presence of glutamergic fibres in the lateral olfactory tract (Harvey et al., 1975), and, similarly, changes in the glutamate content of the cerebral cortex after undercutting may be associated with the destruction of afferent glutamergic pathways (Koyama and Jasper, 1977).

In common with other amino acid transmitter candidates, glutamate can be shown to enter neural tissue by both a high affinity and a low affinity transport system (Cox and Bradford, 1978). The characteristics and possible roles of these transport systems will be discussed later.

Several investigators have tried to demonstrate the existence of specific receptor sites for glutamate by using ligand binding techniques (Johnston, 1979). Until recently, however, the probable existence of multiple binding sites, which may be either postsynaptic receptors or uptake sites, and the lack of specific agents to inhibit one or the other class of sites, prevented a clear biochemical demonstration of specific glutamate receptors. In the past few years several reports have appeared describing $Na^+$-independent binding of $^3H$-glutamate to brain membranes (Foster and Roberts, 1978; Baudry and Lynch, 1979a; b). This binding appears to exhibit several characteristics of binding to postsynaptic receptor sites and, as with other neurotransmitters (Young and Snyder, 1974; Enna and Snyder, 1977; Mohler and Okada , 1978; Tsai

and Lefkowitz, 1978) is regulated by several ions; monovalent cations decrease binding, while certain divalent cations enhance binding (Baudry and Lynch, 1979b).

There are major technical difficulties in investigating the ionic mechanism of the synaptic excitation of central neurones *in vivo*, largely because of the somatic and dendritic location of excitatory synapses (Curtis, 1979). Consequently, the ionic basis of glutamate-induced excitation is not fully elucidated. The reversible depolarisation of spinal, cortical and caudate neurones by glutamate is accompanied by an increase in membrane conductance, and the measured reversal potential is consistent with an increased membrane permeability to both $Na^+$ and $K^+$ ions (Curtis *et al.*, 1960a; Curtis, 1965; Krnjevic and Schwartz, 1967; Zieglgansberger and Puil, 1973; McLennan, 1975; Bernardi *et al.*, 1976). The involvement of $Cl^-$ ions seems unlikely (Curtis, 1979), and, since tetrodotoxin does not block the depolarisation, the sodium permeability increase differs from that associated with action potentials (Curtis *et al.*,

1972; Zieglgansberger and Puil, 1972). Further support for the participation of $Na^+$ ions is provided by the finding that the depolarisation of cultured human and rat spinal neurones by glutamate can be abolished reversibly by replacement of extracellular sodium with choline (Hosli et al., 1976). The excitatory effect of glutamate on spinal neurons is enhanced by p-mercuriphenylsulfonate, an inhibitor of the uptake of glutamate into cerebrocortical slices (Curtis et al., 1970; Balcar and Johnston, 1972a, 1973). This would tend to suggest that the depolarization is not generated by a carrier-linked transport of $Na^+$ and amino acids (Curtis et al., 1970) andthat uptake is, in fact, a major mechanism terminating the effect of glutamate (Cox and Bradford, 1978).

A number of agents which are powerful calcium chelators fail to excite neurons, suggesting that the excitation produced by glutamate is not merely a consequence of a lowering of the extracellular $Ca^{2+}$ concentration due to chelation of $Ca^{2+}$ by glutamate (Curtis et al., 1960b). The interaction between glutamate and external membrane receptors may, however, initiate a $Na^+$ permeability increase by displacing $Ca^{2+}$ from critical membrane sites (Curtis and Watkins, 1960).

Thus, many experimental results suggest that the ionic mechanism of the depolarisation of neurones by glutamate *in vivo* may be identical to that produced by a synaptically released transmitter. In one recent study however, a discrepancy between the reversal potentials for synaptic and glutamate depolarisations of cultured mouse spinal neurones suggests different ionic mechanisms (Ransom *et al.*, 1977).

## 1.2 Uptake of GABA and glutamate into nervous tissue.

Amino acids likely to have a transmitter function in the CNS can usually be shown to enter nervous tissues by both a high affinity (apparent Km = 1-50μM) and a low affinity (apparent Km = 0.1-1.0mM) transport system, whereas non-transmitter amino acids appear to possess only the low affinity system (Iversen and Neal, 1968; Johnston and Iversen, 1971; Logan and Snyder, 1971; 1972; Balcar and Johnston, 1972). Such high affinity systems have been described for GABA (Iversen and Neal, 1968), glutamate (Logan and Snyder, 1971; 1972), glycine (Neal and Pickles, 1969; Logan and Snyder, 1971; 1972), aspartate (Logan and Snyder, 1972) and taurine (Kaczmarek and Davison, 1972).

The transport systems for glycine are of particular interest. Glycine is an inhibitory transmitter in the mammalian spinal cord but not in the cerebral cortex (Curtis and Johnston, 1974) and is accumulated by a low affinity transport system in both tissues (Johnston and Iversen, 1971). A high affinity system for glycine is detectable only in the spinal cord (Johnston and Iversen, 1971). It therefore seems reasonable to connect high affinity amino acid uptake with some specific role related to the transmitter function of these compounds (Cox and Bradford, 1978). The characteristics and possible roles of the high affinity transport systems for GABA and glutamate will be discussed in some detail.

### 1.2.1. Dependence of high affinity uptake on $Na^+$ : the role of other ions.

The high affinity uptake of GABA and glutamate is absolutely dependent on the presence of $Na^+$ in the external medium (Iversen, 1971; 1973; Kuhar, 1973; Bennett et al., 1974; Kanner, 1978; Kanner and Sharon, 1978).

In a variety of brain preparations, this transport appears to be active, temperature dependent and inhibited by various metabolic poisons, such as 2,4-dinitrophenol and cyanide, which interfere with intracellular ATP synthesis, as well as by the inhibitor of the $(Na^+ + K^+)$-ATPase ouabain (Varon et al., 1964; Weinstein et al., 1965; Strasberg and Elliott, 1967; Iversen and Neal, 1968; Kuriyama et al., 1969; White and Keen, 1970; Balcar and Johnston, 1972; Martin and Smith, 1972). It has been suggested that the participation of the $(Na^+ + K^+)$-ATPase in transport is indirect and that, in fact, the $Na^+$ and also $K^+$ gradients across the membranes of presynaptic nerve terminals and glial cells may represent the immediate driving forces for active neurotransmitter uptake (Bogdanski et al., 1968; Martin and Smith, 1972; Martin, 1973; Holtz and Coyle, 1974). This is in accordance with the general hypothesis that solute accumulation can be achieved by co-transport with ions which move down their electrochemical potential gradients into the cell or cell organelle (Riggs et al., 1958).

Recently, studies on the active uptake of GABA and glutamate into membrane vesicles

have lent strong support to this concept of neurotransmitter transport in the mammalian CNS (Kanner, 1978; Kanner and Sharon, 1978). Active transport of both GABA and glutamate was absolutely dependent on external $Na^+$ and could be driven by artificially imposed $Na^+$ gradients (out > in) and $K^+$ gradients (in > out)(Kanner, 1978; Kanner and Sharon, 1978). Surprisingly, GABA transport was also absolutely dependent on the presence of external small monovalent anions (Kanner, 1978) while glutamate transport had no such requirement (Kanner and Sharon, 1978). The sole source of energy in these experiments was that of the artificially imposed transmembrane ion gradients and under these conditions, transport was not inhibited by ouabain. These results imply that the role of the $(Na^+ + K^+)$-ATPase in high affinity amino acid transport in intact synaptosomes is indirect; namely, it creates transmembrane ion gradients. Further support for a secondary role of the $(Na^+ + K^+)$-ATPase stems from the finding that $K^+$ is not necessary for GABA or glutamate transport in membrane vesicles in which artificial $Na^+$ gradients have been created (Kanner, 1978; Kanner and Sharon, 1978).

In studies with intact synaptosomes, a $K^+$ requirement was reported (Martin and Smith, 1972; Martin, 1976). These results suggest that $K^+$ is required in the process by which the $Na^+$ gradient is created, but not in the actual translocation of GABA or glutamate.

The precise role of $Na^+$ in amino acid transmitter transport remains unknown. In the case of GABA, there is strong evidence for a mechanism in which the binding of GABA to the transport carrier is activated by $Na^+$ (Weinstein, et al., 1965). The saturation kinetics of the system with respect to $Na^+$ and GABA suggest that the transport carrier may contain two or more $Na^+$ sites which interact cooperatively while involving only one site for GABA (Martin, 1973; 1976; Blaustein and King, 1976). The role of $Cl^-$ in GABA transport remains entirely speculative (Kanner, 1978).

1.2.2. Homo- and heteroexchange.

High-affinity uptake of released neuroactive amino acids has been interpreted as the means whereby the postsynaptic action of these compounds is terminated (Iversen and Johnston, 1971).

In fact the discovery of a high affinity transport system for an amino acid in nervous tissue has been taken as supporting a neurotransmitter function for the compound. However, since the discovery by Levi and Raiteri (1974) that unlabelled transmitter amino acid can exchange with labelled transmitter across the neuronal membrane, the physiological significance of high affinity uptake and its role in synaptic transmission processes have been the subject of much controversy (Raiteri et al., 1975; Benjamin and Quastel, 1976; Cox and Bradford, 1978; Okamoto and Namina, 1978). If synaptosomes are prelabelled with low concentrations of $^{3}$H-GABA or $^{3}$H-glutamate and superfused, the addition of cold GABA or glutamate respectively to the superfusion medium will increase efflux of label from the synaptosomes in a saturable, concentration-dependent manner (Levi and Raiteri, 1974; Raiteri et al., 1975; Levi et al., 1976). This suggests that exogenous amino acid is able to exchange with an endogenous pool of the compound. Only in the case of glycine (Aprison and McBride, 1973; Levi and Raiteri, 1975) has net uptake of amino acid been demonstrated at low concentrations of substrate (in the high affinity range).

Net uptake of GABA has been observed in rat brain slices exposed to 200μM GABA (Iversen and Neal, 1968), but, at this concentration, low affinity transport could probably account for the uptake. In synaptosomes depleted of endogenous GABA, net high affinity uptake of labelled GABA may occur (Ryan and Roskoski, 1977); however, since estimates of the concentration of GABA in GABAergic terminals vary from 50-150mM (Fonnum and Walberg, 1973), it is very doubtful whether significant depletion of presynaptic GABA stores could occur as a result of physiological depolarisation - consequently this situation is rather artificial.

Heteroexchange between endogenous and exogenous pools of different amino acids may also occur through the high affinity carriers for GABA and glutamate (Raiteri et al., 1975; Levi et al., 1976; Okamoto and Namima, 1978). As will be discussed later, this mechanism provides a useful experimental tool for examining the stereospecificity of the amino acid binding site on the transport carrier.

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In addition, this has a bearing on inhibition studies where apparent inhibition of high affinity GABA (Johnston et al., 1975; 1976a, b; 1977; Krogsgaard-Larsen, et al., 1975; Krogsgaard-Larsen, 1978) and glutamate (Balcar and Johnston, 1972a, b; 1973; Roberts and Watkins, 1975) uptake by analogues may merely represent competition for the exchange mechanism between the apparent inhibitor and the exogenous labelled substrate.

It is conceivable in the case of glutamate that the stimulation of efflux of synaptosomal labelled transmitter by cold amino acid could be explained by glutamate-induced synaptosomal depolarisation (Cox and Bradford, 1978). The minimum concentration of glutamate required to induce endogenous glutamate release (10μM)(Levi et al., 1976) is, however, far lower than the threshold of neuronal depolarisation of cerebrocortical slices by glutamate (about 200μM)(Gibson & McIlwain, 1965). Thus, this mechanism seems unlikely.

Recently, a theoretical consideration of the forces controlling the distribution of GABA across the synaptosomal membrane has suggested that, in intact nervous tissue, concentrative uptake of GABA begins in the range of $10^{-6}$M external GABA (Sellstrom et al., 1976).

This is in the range of the apparent Km for high affinity GABA transport (Henn and Hamberger, 1973; Somoza et al., 1977; Hitzemann and Loh, 1978). In addition, compounds which apparently inhibit the uptake of GABA under in vitro conditions enhance the inhibitory action of electrophoretically administered GABA and GABA agonists on spinal neurones and cerebellar Purkinje cells in anaesthetised cats (Curtis et al., 1970; 1976; 1977; Krogsgaard-Larsen, et al., 1975; Lodge et al., 1977; 1978). Similarly, p-chloromercuriphenylsulfonate, which is known to block uptake of glutamate into cerebrocortical slices (Balcard and Johnston, 1972a; 1973; Curtis et al., 1970) enhances the excitatory action of glutamate on cat Renshaw cells and spinal interneurones (Cox and Bradford, 1978). The conclusion to be drawn from these results is that high affinity transport processes are important for the maintenance of low extraneuronal concentrations of transmitter amino acids. This does not necessarily imply a role for high affinity uptake in limiting the action of synaptically released transmitter in vivo.

If homoexchange does occur *in vivo*, it is difficult to ascribe a function to what seems a wasteful process. A purely hypothetical explanation might invoke some metabolic role for the phenomenon. Thus, homoexchange might provide a mechanism for the shunting of transmitter from a releasable transmitter pool to a non-releasable metabolic pool; presumably the homoexchange process would have to be energetically more favourable than the direct intrasynaptosomal transfer from one pool to the other. Interestingly, it has been demonstrated that exogenous glutamate will exchange with preloaded endogenous glutamate in the presence of ouabain which specifically inhibits high affinity glutamate uptake (Benjamin and Quastel, 1976). This suggests the existence of two separate systems which can be activated independently. Clearly one could mediate a metabolic role and the other a role related to synaptic transmission. However, Raiteri *et al.*, (1975) have reported that ouabain does affect homoexchange.

### 1.2.3. Stereospecificity of high affinity amino acid transport.

The high affinity uptake mechanisms which exist in neurones and glial cells for amino acid transmitters are characterised by a marked degree of substrate specificity. The specificities of the neuronal transport systems for GABA and glutamate have been elucidated largely by examining the effects of various analogues on (a) high affinity neuronal transmitter uptake and (b) release of transmitter from various brain preparations by heteroexchange diffusions. This aspect of amino acid transmitter transport will be discussed in detail in a later section of this thesis.

## 1.3. Release of GABA and glutamate from nervous tissues.

Although much is known about the release of neurotransmitters from peripheral nerves, very little by comparison is known about the release process in brain.

This has been due, in part, to technical difficulties inherent to the study of transmitter release in intact perfused brain or brain slices. In such preparations the temporal collection of released substances is limited by the long distances over which the substances must pass before collection, during which catabolism or reuptake could take place. In addition, manipulations of the immediate extracellular environment cannot be achieved rapidly or accurately making detailed kinetic analysis almost meaningless. Many of these difficulties have been obviated by the use of synaptsomes as an experimental system for the study of neurotransmitter release in brain (Blaustein, et al., 1972; De Belleroche and Bradford, 1972a, b; Bradford, et al., 1973; Levy et al., 1973). Release from synaptosomes can be studied independently of multineuronal or neural-glial interactions, substances can be rapidly applied without intervening diffusional barriers, and released transmitter can be collected quickly and directly. Thus, both the transmitter substance and the release process can be quantitatively analysed.

### 1.3.1. Depolarisation-induced release of GABA.

The release of previously accumulated GABA from a number of brain preparations shows many of the characteristics associated with the physiological release of neurotransmitters. Release can be evoked by increasing the external $K^+$ concentration, by veratridine and, in some cases, by electrical stimulation, and has a very marked, but not absolute, requirement for $Ca^{2+}$ (Machiyama, et al., 1967; Srinivasan, et al., 1969; Benjamin and Quastel, 1972; Blaustein, et al., 1972; De Belleroche and Bradford, 1972a, b; Levy, et al., 1973; Tappaz and Pacheco, 1973; Mulder and Snyder, 1974; Davies, et al., 1975; Holz, 1975; Raiteri, et al., 1975; Cotman, et al., 1976; Redburn, et al., 1976; Olsen, et al., 1977; Vargas, et al., 1977). When efflux is stimulated by increased external $K^+$ concentration, veratridine or the calcium ionophore A23187, the process is strongly $Ca^{2+}$-dependent ( Srinivasan, et al., 1969; Benjamin and Quastel, 1972; Mulder and Snyder, 1974; Davies, et al., 1975; Raiteri, et al., 1975; Redburn, et al., 1976).

These three agents ($K^+$, veratridine and A23187) act by distinctly different mechanisms, but lead to a common action: an increase in membrane permeability to $Ca^{2+}$. High concentrations of $K^+$ depolarise the membrane of the nerve ending by reducing the electrochemical gradient, and this in turn increases the permeability to $Ca^{2+}$ (Goldring and Blaustein, 1973). Veratridine increases permeability to $Na^+$, leading to membrane depolarisation and hence increased $Ca^{2+}$ permeability (Ulbricht, 1969; Goldring and Blaustein, 1973), and A23187 inserts into the membrane and creates new $Ca^{2+}$ channels (Pressman, 1973). Thus, these three treatments increase $Ca^{2+}$ permeability and all enhance the rate of efflux of pre-loaded GABA. Current evidence supports the theory that an increase in the intracellular concentration of $Ca^{2+}$ is a necessary and sufficient requisite for inducing transmitter secretion (Llinas and Nicholson, 1975).

Consistent with a release process limited by a $Ca^{2+}$ influx controlled by membrane depolarisation is the finding that increases in $Ca^{2+}$ concentration appear to saturate the release process in the presence of 55mM $K^+$ (Cotman, et al., 1976)

When the depolarisation-limited influx of $Ca^{2+}$ is by-passed with A23187, release increases linearly with the log $[Ca^{2+}]$ (Cotman, et al., 1976; Redburn, et al., 1976). This situation may represent a relatively direct $Ca^{2+}$-release site interaction. The notion of a release site (X) with which $Ca^{2+}$ interacts

$$(Ca^{2+} + X \rightleftharpoons Ca^{2+}\ X)$$

has been applied in analogy to a Michaelis-Menton analysis of steady state enzyme kinetics (Jenkinson, 1957; Dodge and Rahamimoff, 1967). For a reaction

$$nCa^{2+} + X \rightleftharpoons Ca_n^{2+}\ X$$

the maximum slope of the relationship ln release vs ln $[Ca^{2+}]_o$ estimates the parameter n (Cotman, et al., 1976). Linearity of release with log $[Ca^{2+}]_o$ has been described for other systems (Hubbard, 1961; Kirkepar and Misu, 1967) and probably represents a receptor mediated event.

The effect of $Ca^{2+}$ on the release of GABA is antagonised by $Mn^{2+}$ and $Mg^{2+}$ (Levy, et al., 1973; 1974; Cotman, et al., 1976; Redburn, et al., 1976) while the alkaline earths

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$Sr^{2+}$ and $Ba^{2+}$ substitute for $Ca^{2+}$ in stimulating secretion (Cotman, et al., 1976; Redburn, et al., 1976). This feature appears to be a characteristic of many stimulus-secretion coupling systems (Douglas and Rubin, 1964; Elmqvist and Feldman, 1965). The ordering of alkaline earth efficacy in supporting GABA release is $Mg^{2+} < Ca^{2+} < Sr^{2+} < Ba^{2+}$ which is in general agreement with that seen for peripheral stimulus-secretion coupling systems (Douglas and Rubin, 1964). The differences in divalent cation release-stimulating efficacy are probably based on differences in membrane permeation and intracellular binding site affinities (Blioch, et al., 1968).

Thus, in situations where $Mn^{2+}$ and $Mg^{2+}$ antagonize the effect of $Ca^{2+}$ on GABA release in depolarising solutions, they are thought to compete with $Ca^{2+}$ for presynaptic permeation sites (Balnave and Gage, 1973; Cooke, et al., 1973).

Assessment of the role of $Ca^{2+}$ in GABA release is further complicated by the observation that, while for a given $[Ca^{2+}]_o$ increases in $[K^+]_o$ always increase release of GABA, GABA release in the presence of $[K^+]_o$ lower than 55mM apparently decreases, rather than reaches an asymptote, with higher $[Ca^{2+}]_o$ (Levy et al., 1974). This phenomenon has been termed "$Ca^{2+}$-autoinhibition". Three possible mechanisms can be suggested to explain $Ca^{2+}$-autoinhibition of GABA release:

a) $Ca^{2+}$ may decrease its own membrane permeability;

b) $Ca^{2+}$ once having permeated, may decrease its own ability to promote release, and

c) $Ca^{2+}$ may partially reverse the depolarizing effects of $K^+$ thereby indirectly decreasing its own membrane permeability.

It is noteworthy here, that $Ca^{2+}$-autoinhibition has been observed for GABA release, but not for the noradrenaline system (Cotman et al., 1976), a distinction not accomodated a priori by any of these alternatives.

In addition, it cannot be assumed that the effect of $K^+$ is mediated via depolarisation since other depolarising agents like veratridine do not stimulate $Ca^{2+}$-independent release (Redburn, 1978). It is probable that the $Ca^{2+}$-independent component of $K^+$-stimulated synaptosomal GABA release stems from glial contamination of the preparation, or from vesicles derived from non-synaptic areas of axons. Calcium-independent release of other neurotransmitters has been reported from such areas (Weinreich and Hammerschlag, 1975).

The profile of $Ca^{2+}$-dependent release of GABA appears to be biphasic, characterised by a peak seen at the earliest times resolvable (0.3-0.6 s) followed by a sustained plateau (Redburn et al., 1976). This is reminiscent of the synaptic fatigue observed when the neuromuscular junction (Elmqvist and Feldman, 1966) or adrenal medulla (Lastowecka and Trifaro, 1974) is chemically depolarised and during electrical stimulation of brain slices or motoneurons (Srinivasan et al., 1969).

Recently, data have been obtained which suggest that there is a small readily releasable transmitter pool of GABA which is rapidly depleted by $Ca^{2+}$-dependent $K^{+}$-stimulation (Levy et al., 1976). The remaining transmitter does not redistribute freely from storage pools into the readily releasable pool (Levy et al., 1976). It is conceivable that the biphasic profile of GABA release is due to a rapid depletion of the transmitter pool followed by slow redistribution of GABA from the storage pool to the transmitter pool from where it is released.

Another line of evidence links $Ca^{2+}$ transport intimately with GABA stimulus-secretion coupling processes. The inorganic dye ruthenium red is a specific inhibitor of $Ca^{2+}$ transport and binding in isolated subcellular particles including mitochondria (Moore, 1971; Vasington, 1972; Reed and Bygrave, 1974), synaptosomes (Swanson et al., 1974; Goddard and Robinson, 1976; Kamino et al., 1976; Vickers and Dowdall, 1976) and synaptic plasma membranes (Madeira and Antunes-Madeira, 1973).

Ruthenium red produces a significant inhibition of the $Ca^{2+}$-stimulated release of GABA in $K^{+}$-depolarised synaptosomes almost certainly by blocking the uptake of $Ca^{2+}$ into the synaptosomes (Tapia and Meza-Ruiz, 1977). Also, several drugs such as the benzodiazepines (Olsen et al., 1977), phenothiazines and tricyclic antidepressants (Moss et al., 1974; Lahdesmaki et al., 1975) are potent inhibitors of $Ca^{2+}$-dependent GABA release. The inhibition of GABA release by chlorpromazine occurs at fairly low concentrations (Olsen et al., 1977) similar to concentrations previously observed to inhibit catecholamine release (Seeman and Lee, 1975). Thus, the inhibitory effects of such agents on the release of transmitters are not limited to, nor specific for, any one transmitter and therefore may involve general mechanisms common to many stimulus-secretion coupling processes. This inhibition of release is likely to occur at the level of a) the regulation of membrane potential (and indirectly $Ca^{2+}$ permeability), or b) direct regulation of $Ca^{2+}$ permeability (Hubbard, 1970).

It is noteworthy that similar concentrations of these drugs may inhibit $Na^+$-dependent GABA transport in various neural tissues (Iversen and Johnston, 1971; Olsen et al., 1977).

The association of a $Ca^{2+}$-dependent stimulus-secretion coupling process with a high affinity $Na^+$-dependent uptake system is well established for a number of neurotransmitters including [illegible] (Mulder and Snyder, 1974; Mulder et al., 1974). There is, however, no evidence for a tight coupling of the uptake and release mechanisms. Release is inhibited by $Mn^{2+}$ which has little effect on uptake and the absence of $Na^+$ in the medium inhibits uptake, but has little effect on the release of previously accumulated GABA (Redburn, 1978). These data seem to suggest a lack of simultaneous control over both processes. The two processes must be indirectly linked, however, in that they share a common transmitter pool and the respective rates of uptake or release must be ultimately dependent upon the size of that pool.

In summary: it is envisaged that the entry of $Ca^{2+}$ from the medium into the cytoplasm of nerve endings is the inital event triggering the release of GABA, and it is possible that the intraterminal mitochondria might play a role in the removal of excess cytoplasmic $Ca^{2+}$ when the depolarisation ceases (Alnaes and Rahamimoff, 1975; Parducz and Joo, 1976; Vickers and Dowdall, 1976).

Unfortunately, there are some discrepancies in the $Ca^{2+}$-dependency of GABA efflux when the results of several electrical stimulation studies are considered. Controlled electrical stimulation of rat brain cortex slices has been shown to release, by a process that has absolute dependency on $Ca^{2+}$, radioactive noradrenaline, acetylcholine, 5-hydroxytryptamine, p-tyramine, dopamine and octopamine but not GABA, glutamate, aspartate, glycine, serine, α- and β-alanine, histidine, homocarnosine, β-phenylethylamine or α-aminoisobutyrate (Somogyi and Szerb, 1972; Orrego et al., 1974; 1976; Orrego and Miranda, 1976).

In addition, Redburn et al., (1976) were unable to evoke $Ca^{2+}$-dependent release of GABA from either rat or rabbit synaptosomes, although Bradford et al., (1973) have reported $Ca^{2+}$-dependent release with electrical stimulation from rabbit but not from rat synaptosomal suspensions. Beds of rat cortical synaptosomes incubated in grid electrodes did, however, demonstrate $Ca^{2+}$-dependent release of GABA on electrical stimulation (De Belleroche and Bradford, 1972a). Labelled GABA is not released by mild electrical stimulation of brain cortex slices in the absence of aminooxyacetic acid (AOAA)(Orrego and Miranda, 1976), and when release is induced by such stimuli in the presence of AOAA there is no $Ca^{2+}$-dependency (Srinivasan et al., 1969). As mentioned above, the accurate assessment of release phenomena in brain slices is confounded by the relatively great distances over which the released compound must diffuse before measurement is possible - this entails the possibility of significant reuptake of transmitter. In addition, the presence of glial cells further complicates interpretation of release data.

Glia do respond to $K^+$-depolarisation, the release of GABA showing only a low $Ca^{2+}$-dependency (Bowery and Brown, 1972; Minchin and Iversen, 1974; Minchin, 1975; Minchin and Nordmann, 1975). It is entirely conceivable that $Ca^{2+}$-independent release of GABA elicited by electrical stimulation of slices originates from non-neuronal stores.

Data derived from $K^+$-depolarisation experiments should, however, also be interpreted with caution. Elevated extracellular $K^+$, aside from increasing the efflux of putative transmitters such as GABA, glutamate, aspartate and glycine, has been shown to release amino acids such as glutamine, alanine, leucine and α-aminoisobutyrate for which no transmitter role seems plausible (Roberts, 1974; Vargas and Orrego, 1976). In addition, $K^+$ has also been reported to increase α-aminoisobutyrate efflux from rat striated muscle (Kipnis and Parrish, 1965). These results indicate that, although $K^+$ is a powerful stimulus for inducing transmitter secretion, it has a multiplicity of other effects that considerably reduce its specificity.

As discussed by Douglas (1968) and Rubin (1970) an absolute requirement for $Ca^{2+}$ has been described for all vesicle-containing exocytotic secretion systems. Subcellular distribution studies, however, suggest that GABA is present in the soluble cytoplasmic compartment and is not released from vesicles within the nerve terminal (Mangan and Whittaker, 1966; De Belleroche and Bradford, 1973). The question of why a soluble cytoplasmic constituent such as GABA requires $Ca^{2+}$ for its release under certain circumstances, but not in others, remains to be answered.

### 1.3.2. Depolarisation-induced release of glutamate.

Many of the general points discussed above in relation to GABA release studies apply equally to glutamate release. Neuronal depolarisation, induced by increased extracellular $K^{+}$ or electrical pulses, causes release of preloaded glutamate from slices of corpus striatum, cerebral cortex and olfactory cortex (Katz et al., 1969; Arnfred and Hertz, 1971; Matsui and Yamamoto, 1975).

In addition, the release of endogenous glutamate from superfused cortex during electrical stimulation of the reticular formation has been demonstrated *in vivo* (Jasper and Koyama, 1969) and release from olfactory cortex slices during electrical stimulation of the lateral olfactory tract, has been shown *in vitro* (Bradford and Richards, 1976). Also, electrical stimulation of the brachial plexus in anaesthetised rats has been shown to release glutamate and glutamine from the contra-lateral sensorimotor cortex, while photic-stimulation of the eyes specifically increased glutamate release from the visual cortex in the same preparation (Abdul-Ghani *et al.*, 1979). Release of endogenous glutamate from synaptosomes can be induced by exposure to electrical stimulation, high extracellular $K^+$, or veratrine (Bradford, 1970; De Belleroche and Bradford, 1972a; Bradford *et al.*, 1973; Wedege *et al.*, 1977). As in the case of GABA, stimulated glutamate release shows a marked $Ca^{2+}$-dependence. Thus, $K^+$-evoked release of endogenous glutamate from cortical, spinal or hypothalamic

synaptosomes is abolished in $Ca^{2+}$-deficient medium containing EGTA (De Belleroche and Bradford, 1972a; Bradford et al., 1973; Osborne et al., 1973). However, electrically stimulated glutamate release is not abolished even in the presence of EGTA (De Belleroche and Bradford, 1972a) suggesting that some of the electrically induced release occurs by a $Ca^{2+}$-independent mechanism. Again, such release probably occurs from non-neuronal elements. Release of preloaded (Matsui and Yamamoto, 1975) and endogenous (Bradford and Richards, 1976) glutamate induced by electrical stimulation of the lateral olfactory tract is strongly $Ca^{2+}$-dependent as is the $K^+$- and veratrine-induced release of glutamate from slices of dentate gyrus (Nadler et al., 1977).

The glutamate released by high $K^+$ or veratrine appears to be derived from the cytoplasmic glutamate pool (Cox and Bradford, 1978). Again, the precise role of $Ca^{2+}$ in supporting the non-vesicular release of neurotransmitter is unclear.

Recently it has been reported that synaptically released glutamate is derived primarily from glutamine rather than glucose (Bradford et al., 1978; Cotman and Hamberger, 1978; Hamberger et al., 1978). This suggests that glutamine might be useful in labelling the transmitter pool of glutamate in neurones and synaptosomes (Cox and Bradford, 1978). Hopefully, studies of release from specific transmitter pools will yield some information on the function of $Ca^{2+}$ in amino acid transmitter stimulus-secretion coupling processes.

### 1.3.3. Regulation of release via auto-receptors.

There is considerable evidence, in both the peripheral and central nervous systems that presynaptic α-adrenoceptors modulate noradrenaline release induced by both electrical and $K^+$ stimulation; α-agonists inhibit and α-antagonists facilitate this release (Farnebo and Hamberger, 1971; Langer et al., 1972; Starke and Montel, 1973; De Potter et al., 1974; Dismukes and Mulder, 1976; Langer, 1978).

Similar control mechanisms also occur in dopaminergic (Starke and Montel, 1973; Carlsson, 1975; Roth et al., 1975; Seeman and Lee, 1975; Iversen et al., 1976) and cholinergic (Szerb and Somogyi, 1973) neurones. Recently, strong evidence has been provided for the existence of bicuculline-sensitive GABA autoreceptors which exert negative feedback control over GABA release from GABA-ergic nerve terminals (Mitchell and Martin, 1978; Snodgrass, 1978). Based on differences in affinity for α-receptor agonists and antagonists a subclassification of α-adrenoreceptors into $\alpha_1$ (post-synaptic) and $\alpha_2$ (presynaptic) has been postulated and generally accepted (Langer, 1974). To date the pharmacology of GABA autoreceptors remains unknown. Elucidation of any differences in the affinities of GABA autoreceptors and postsynaptic receptors for GABA agonists and antagonists is of prime importance both in delineating the function of the autoreceptors and in the manipulation of GABA systems in the brain for therapeutic reasons.

### 1.3.4. Possible role of presynaptic membrane phosphorylation in regulating release.

It is well established that synaptic plasma membranes from mammalian brain contain a bound protein kinase which catalyses the phosphorylation of endogenous membrane proteins and which is stimulated by cyclic-AMP (Weller and Rodnight, 1970; 1973; Ueda et al., 1973). Recently it has been shown that the kinase activity is associated largely with the presynaptic plasma membrane (Weller, 1977) and that cyclic-AMP stimulated phosphorylation of presynaptic membrane proteins results in a decreased membrane permeability to $Ca^{2+}$ without affecting the permeability to $Na^{+}$ or $K^{+}$ (Weller and Morgan, 1977). As discussed above, release of a variety of neurotransmitters is known to be dependent on $Ca^{2+}$ influx (Hubbard, 1970). This suggests that membrane protein phosphorylation might play an important role in regulating the release of transmitters from nerve endings.

Recently, Brennan and Cantrill (1980a, b) have reported that incubation of synaptosomes under phosphorylating conditions markedly reduces the $Ca^{2+}$-dependent component of the $K^{+}$-stimulated release of preloaded GABA; the accumulation of $^{3}H$-GABA by the nerve endings is not significantly affected by phosphorylation. These results imply that the $Ca^{2+}$ channel affected by membrane phosphorylation is the one concerned with regulation of GABA release on depolarisation of the nerve terminal, and that phosphorylation and dephosphorylation of presynaptic membrane components is a potential mechanism for the control of transmitter release. Such a mechanism would be affected by agents and treatments known to alter intracellular cyclic-AMP concentrations. Preliminary data suggest that ATP and its metabolites may also alter the high affinity uptake of glutamate in synaptosomes from whole rat brain (Warner et al., [illegible]).

Finally, $Ca^{2+}$ itself is known to regulate the activity of a protein kinase in brain membranes (Greengard, 1978). If the $Ca^{2+}$-dependent phosphorylation of membrane components alters membrane permeability to $Ca^{2+}$ this would provide a mechanism for $Ca^{2+}$-autoinhibition of GABA release since $Ca^{2+}$ would effectively be controlling its own permeation.

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# CHAPTER 2

# MATERIALS AND METHODS

## 2.1. Chemicals

The following compounds were obtained from the Sigma Chemical Company, St. Louis, MO, U.S.A.

β-Alanine

γ-Aminobutyric acid (GABA)

δ-Aminolaevulinic acid (δ-ALA)

Aminooxyacetic acid (AOAA)

(+)-Bicuculline

(+)-2,4-Diaminobutyric acid (DABA)

L-Glutamic acid

L-Homocysteine

L-Methionine

L-Ornithine

Picrotoxin

The following GABA agonists were gifts from Dr. P. Krogsgaard-Larsen, Copenhagen.

5-Aminomethyl-3-isoxazolol (muscimol)

Piperidine-4-sulphonic acid (PSA)

4,5,6,7-Tetrahydroisoxazolo-(5,4,-C)pyridin-3-ol (THIP),

1,2,3,6-Tetrahydropyridine-4-carboxylic acid (isoguvacine).

All other chemicals were of Analar reagent grade obtained commercially.

## 2.2. Radiolabelled compounds

[2,3-$^{3}$H] GABA, specific activity 54 Ci/mmol; L-[U-$^{14}$C] glutamic acid, specific activity 290mCi/mmol; [4-$^{14}$C]-δ-ALA, specific activity 54mCi/mmol were obtained from the Radiochemical Centre, Amersham, Bucks, U.K.

## 2.3. Preparation of synaptosomes.

Synaptosomes were prepared from the cerebral cortices of adult male and female Wistar rats (fed on ordinary laboratory diet, Epol) by the method of Gray and Whittaker (1962) as modified by Marchbanks (1975). All procedures were carried out at 4°C. Tissue was homogenised with 30 strokes in a hand glass homogeniser (radial clearance 0.25mm) in 10 volumes of ice-cold 0.32M sucrose. The homogenate was centrifuged at 1000g x 10 minutes in a Beckman SW 27.1 swing-out centrifuge rotor. The pellet was discarded and the supernatant centrifuged at 10,000g x 20 minutes. The resulting mitochondrial-synaptosomal pellet was resuspended in 2ml 0.32M sucrose per g original tissue and layered on a discontinuous density gradient of 0.8M sucrose (5ml) on 1.2M sucrose (5ml).

Gradients were centrifuged at 57,000g x 120 minutes. Synaptosomes harvested from the 0.8M - 1.2M interface were diluted slowly 1 : 1 with 0.32M sucrose, giving an osmolarity of 0.6-0.8M and centrifuged at 20,000g x 20 minutes. The pellets were then dispersed in 0.32M glucose to give a protein concentration of about 5mg/ml (Lowry et al., 1951) and allowed to recover at 4°C for 15 minutes.

Sucrose solutions having a density equal to that of synaptosomes (1.0-1.2M) are hyperosmolar. In order to prevent exposure to hyperosmolarity a number of workers have used the polydextran Ficoll as a substitute for sucrose (Kurokawa et al., 1965; Abdel-Latif, 1966; Potter, 1968; Garey et al., 1972). It has been claimed that the metabolic properties and morphology of the particles are superior after isolation on a Ficoll gradient (Potter, 1968). Although few systematic studies have been done, Kurokawa et al., (1965) noted a 20% loss of acetylcholine as a result of exposure to high sucrose concentrations.

As far as metabolism is concerned, protein synthesis (compare Morgan and Austin, 1968 with Antilio et al., 1968) and carrier mediated choline transport (Marchbanks, 1968) in synaptosomes are little affected by exposure to hyper-osmotic conditions. In addition, Ficoll solutions must be predialysed, or precipitated with ethanol to remove salt. They are more viscous than sucrose solutions of equivalent density and thus the time taken in the centrifuge to approach sedimentation equilibrium is much lengthened. In the absence of any proven benefit in the use of Ficoll, the preparation of synaptosomes by all established techniques utilising sucrose was preferred.

## 2.4. Uptake of radiolabelled amino acids by synaptosome preparations.

### 2.4.1. Uptake of $^{3}$H-GABA by synaptosome suspensions.

For the preloading of synaptosomes prior to superfusion studies, the method of Raiteri et al., (1975) was used. Portions of fresh synaptosomal pellets resuspended in 0.32M glucose (protein concentration about 5mg/ml) were diluted 1:10 with ice-cold incubation medium (final concentrations : 128mM NaCl;

5mM KCl; 2.7mM $CaCl_2$; 1.2mM $MgSO_4$; 10mM Tris-HCl buffer at pH 7.35). Aminooxyacetic acid was added to a final concentration of 0.1 mM to prevent GABA catabolism (Iversen and Johnston, 1971; Levy et al., 1973; Minchin and Iversen, 1974; Hammerstad and Lytle, 1976). One-ml aliquots of the suspension were preincubated at 37°C for 15 minutes in a shaking water bath in an air atmosphere. [2,3-$^3$H] GABA of specific activity 54 Ci/mmol was diluted with unlabelled GABA to give a specific activity of 10 Ci/mmol and a small volume (1/100 of the final volume) added to the incubation tubes to a final concentration of 0.5µM. Incubation was continued for a further 10 minutes. The percentage uptake of $^3$H-GABA by the synaptosomes was determined as follows: incubation was carried out in centrifuge tubes. At the end of the 10 minute period, the suspension was spun at 60,000g x 10 minutes and the ratio of radioactivity in the synaptosomal pellet to that free in the supernatant, was calculated.

5mM KCl; 2.7mM $CaCl_2$; 1.2mM $MgSO_4$; 10mM Tris-HCl buffer at pH 7.35). Aminooxyacetic acid was added to a final concentration of 0.1 mM to prevent GABA catabolism (Iversen and Johnston, 1971; Levy et al., 1973; Minchin and Iversen, 1974; Hammerstad and Lytle, 1976). One-ml aliquots of the suspension were preincubated at 37°C for 15 minutes in a shaking water bath in an air atmosphere. [2,3-$^3$H] GABA of specific activity 54 Ci/mmol was diluted with unlabelled GABA to give a specific activity of 10 Ci/mmol and a small volume (1/100 of the final volume) added to the incubation tubes to a final concentration of 0.5µM. Incubation was continued for a further 10 minutes. The percentage uptake of $^3$H-GABA by the synaptosomes was determined as follows: incubation was carried out in centrifuge tubes. At the end of the 10 minute period, the suspension was spun at 60,000g x 10 minutes and the ratio of radioactivity in the synaptosomal pellet to that free in the supernatant, was calculated.

Then the synaptosomes were lyzed by resuspending the pellet in distilled water; the tubes were again spun and the ratio of radioactivity in the membrane pellet to that free in the supernatant determined. Approximately 90% of the supplied radiolabelled GABA was found associated with the synaptosomal pellet after the preloading incubation. Of this, over 95% could be recovered in the supernatant after hypotonic lysis of the synaptosomes while less than 5% remained associated with the membranes. Although it is possible that some redistribution of GABA between membrane and solvent occurred during the period of osmotic lysis, these results indicated that the accumulated GABA was localised predominantly in the cytoplasm.

In experiments examining the uptake of GABA by the nerve endings, aliquots of synaptosomes were preincubated as described above. $^{3}$H-GABA at a specific activity of 2Ci/mmol was added to the tubes to a concentration of 0.5μM.

Incubation was continued for a 10 minute period after which the aliquots were poured onto Millipore filters (0.45µm pore) resting on the bottoms of the wells of a Millipore 3025 sampling manifold. The incubation medium was drawn off under vacuum and the filters washed with 2 x 3ml of incubation medium within 30 seconds. Control experiments to account for radioactivity trapped non-specifically in the pores of the filters were carried out. The filters were solubilized in 10ml of Aquagel I liquid scintillant (Chemlab) and counted in a Packard Tri-Carb Spectrometer at 37% efficiency. Radioactivity remaining on the filters minus the control was defined as $^{3}$H-GABA taken up.

The technique of filtration as a method for separating synaptosomes from the incubation medium may be criticised, since uptake is likely to be underestimated if the system possesses a rapid dissociation rate. Results obtained using this method are however in close accordance with those published using centrifugation techniques (Somoza et al., 1977).

### 2.4.2. Effect of δ-ALA on synaptosomal $^{3}$H-GABA uptake.

In experiments examining the effect of the porphyrin precursor δ-aminolaevulinic acid (δ-ALA) on $^{3}$H-GABA uptake, δ-ALA, at various concentrations, was added to the incubation medium and the synaptosomes equilibrated for 15 minutes in the presence of δ-ALA before the addition of $^{3}$H-GABA. Uptake was terminated after 10 minutes by filtration (as described in 2.4.1.).

### 2.4.3. Uptake of $^{14}$C-glutamate by synaptosome suspensions.

For the preloading of synaptosomes prior to superfusion studies, 1 ml aliquots of synaptosome suspensions were preincubated for 15 minutes at 37$^{o}$C as described above for preloading with $^{3}$H-GABA. A small volume (1/100 of the final volume) of a solution containing a known concentration of L-[U-$^{14}$C] glutamate at specific activity 290mCi/mmol was added to the incubation tubes to give a final concentration in the tube of 0.5μM. Incubation was continued for a further ten minutes.

In experiments examining the uptake of glutamate by the nerve endings, aliquots of synaptosomes were preincubated as described above. $^{14}$C-glutamate at a specific activity of 290mCi/mmol was added to the tubes to a concentration of 0.5µM $^{14}$C-glutamate and unlabelled glutamate was added to give a final concentration of glutamate in the range 0.5-50.0µM. Incubation was continued for a 10 minute period after which the reaction was terminated by rapid filtration on Millipore filters as described above. Again, control experiments to account for radioactivity trapped non-specifically in the pores of the filters were carried out. The filters were solubilized in 10ml of Aquagel I and counted in a Packard Tri-Carb spectrometer at 74% efficiency. Radioactivity remaining on the filters minus the control was defined as $^{14}$C-glutamate taken up.

### 2.4.4. Metabolism of $^{14}$C-glutamate by synaptosomal suspensions.

After the 10 minute loading incubation, aliquots of synaptosomes were centrifuged at 60000g X 10 min and the pellets lyzed by resuspending in 2ml distilled water.

The suspension was again centrifuged, and 50µl aliquots of the supernatant applied to silica gel TLC plates which were developed in n-butanol : acetic acid : water, 80:20:20 (by volume). Under these conditions, 93.5% of the recovered radioactivity co-chromatographed with authentic $^{14}$C-glutamate.

### 2.4.5. Effect of δ-ALA on synaptosomal $^{14}$C-glutamate uptake.

In some experiments examining the effect of δ-ALA on $^{14}$C-glutamate uptake, δ-ALA at various concentrations, was added to the incubation medium and the synaptosomes equilibrated for 15 minutes in the presence of δ-ALA before the addition of $^{14}$C-glutamate. In other experiments, δ-ALA was added together with $^{14}$C-glutamate after the preincubation period. Uptake was terminated after 10 minutes by filtration.

### 2.4.6. Uptake of $^{14}$C-δ-ALA by synaptosome suspensions.

Aliquots of synaptosome suspensions were preincubated for 15 minutes at 37$^{o}$C as described above.

The suspension was again centrifuged, and 50µl aliquots of the supernatant applied to silica gel TLC plates which were developed in n-butanol : acetic acid : water, 80:20:20 (by volume). Under these conditions, 93.5% of the recovered radioactivity co-chromatographed with authentic $^{14}$C-glutamate.

2.4.5. Effect of δ-ALA on synaptosomal $^{14}$C-glutamate uptake.

In some experiments examining the effect of δ-ALA on $^{14}$C-glutamate uptake, δ-ALA at various concentrations, was added to the incubation medium and the synaptosomes equilibrated for 15 minutes in the presence of δ-ALA before the addition of $^{14}$C-glutamate. In other experiments, δ-ALA was added together with $^{14}$C-glutamate after the preincubation period. Uptake was terminated after 10 minutes by filtration.

2.4.6. Uptake of $^{14}$C-δ-ALA by synaptosome suspensions

Aliquots of synaptosome suspensions were preincubated for 15 minutes at 37°C as described above.

A small volume (1/100 of the final volume) of a solution containing a known concentration of [4-$^{14}$C] δ-ALA at specific activity 54mCi/mmol was added to the incubation tubes to a concentration of 0.5μM $^{14}$C-δ-ALA and unlabelled δ-ALA was added to give a final concentration of δ-ALA in the range 0.5-100μM. Incubation was continued for a time period in the range 10-30 minutes. The reaction was terminated by rapid filtration on Millipore filters as described above. Again, control experiments to account for radioactivity trapped non-specifically in the pores of the filters were carried out. The filters were solubilized in 10ml of Aquagel I and counted in a Packard Tri-Carb spectrometer at 74% efficiency. Radioactivity remaining on the filters minus the control was defined as $^{14}$C-δ-ALA taken up.

## 2.5. Superfusion studies.

### 2.5.1. The apparatus.

The measurement of the release of neuro-transmitter substances from slices or sub-cellular fractions of nervous tissue has been confounded by the presence of very active transport systems.

These uptake processes are responsible for the removal of the released substance from the surrounding medium before an accurate measurement of release can be made. Thus, the appearance of transmitter in the medium after a specific treatment may be interpreted as release; alternatively, inhibition of the reuptake of spontaneously released substance would produce the same net effect. Recently this problem has been overcome by the development of a superfusion apparatus shown to minimize reuptake of released transmitter by the continuous removal of the medium together with the released transmitter (Raiteri et al., 1974; 1975). This apparatus consists of a chamber at the bottom of which rests a Millipore filter onto which the synaptosomes are bedded by filtration. Batches of medium are then passed over the synaptosome beds and fractions collected. This system involves several difficulties. Firstly, the volume of medium in the chamber is constantly changing; secondly, the introduction of a new batch of medium into the chamber would be expected to disrupt the synaptosome bed and so produce inconsistent results.

The alternative of replenishing medium when the chamber is not quite empty would result in the mixing of control and test media with irreproducible results. (This latter problem can evidently be overcome if the synaptosome beds are packed tightly by vigorous filtration - Bradford, H.F. personal communication).

The apparatus used in the experiments described here was designed for the superfusion, at constant volume of subcellular particles or whole brain slices. The chamber depicted in Fig.1 consists of a central barrel of machined perspex which is screwed into two identical perspex endpieces. Each end piece is fitted with a Millipore 25mm filter support frit and a rubber "O" ring (Millipore) to seal the connection. The outlet of the endpiece is machine to give minimum dead volume. The apparatus can be used in the conventional manner to superfuse a bed of subcellular particles (such as synaptosomes) resting on the lower Millipore filter. In this mode, an appropriate volume of medium can be introduced into the chamber and the volume thereafter kept constant since the apparatus is

FIGURE 1.

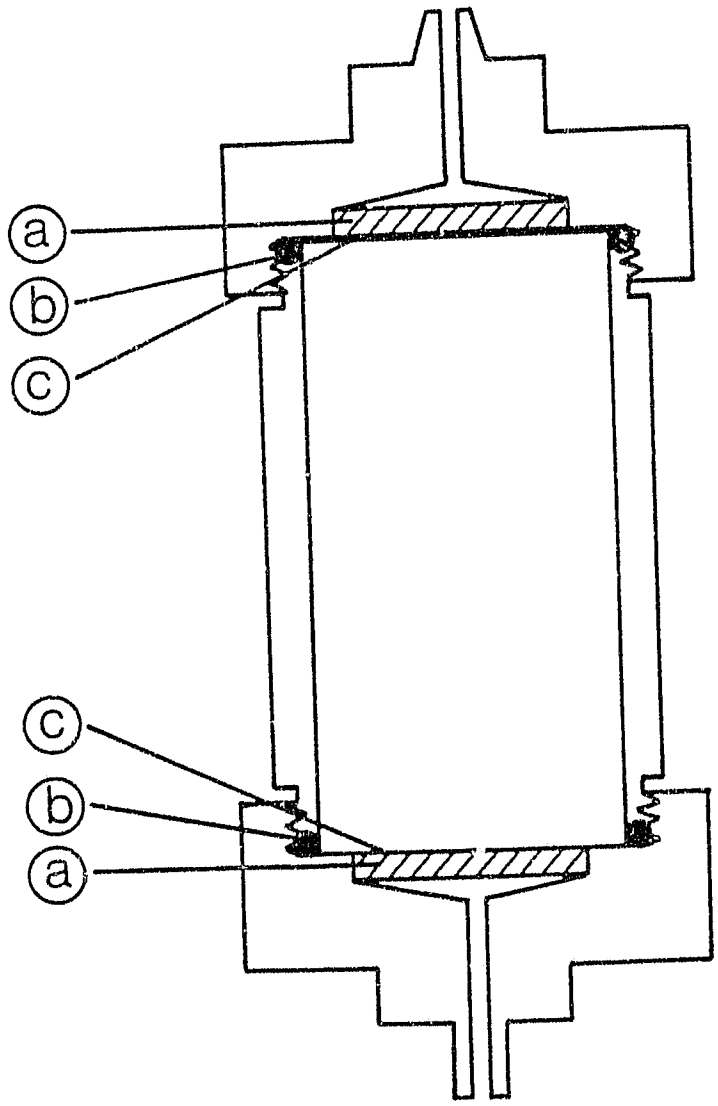


FIGURE 1.

Diagram of the construction of the perspex superfusion apparatus in cross section. All dimensions are based on the 25mm Millipore filter shown at (c). Rubber "O" rings are shown (b) together with the Millipore support frits (a).

airtight. It is also possible to superfuse whole tissue slices against gravity by pumping the medium into the chamber from the bottom of the apparatus and drawing it cut of the top. Such upward displacement of the medium keeps the tissue preparation in suspension and prevents blockage of the lower filter, a problem often encountered in superfusion of tissue slices by conventional techniques. Since it is necessary to fill the chamber completely with medium when operating in this mode, the volume of the chamber can be varied by altering the length of the barrel.

An apparatus consisting of four chambers of the type described above arranged in parallel (Plate 1) has been used extensively in the investigation of the efflux of GABA and glutamate from synaptosomes. In addition, the apparatus has been used to monitor the release of labelled noradrenaline from hypothalamic slices (Klugman et al., unpublished results).

PLATE 1.

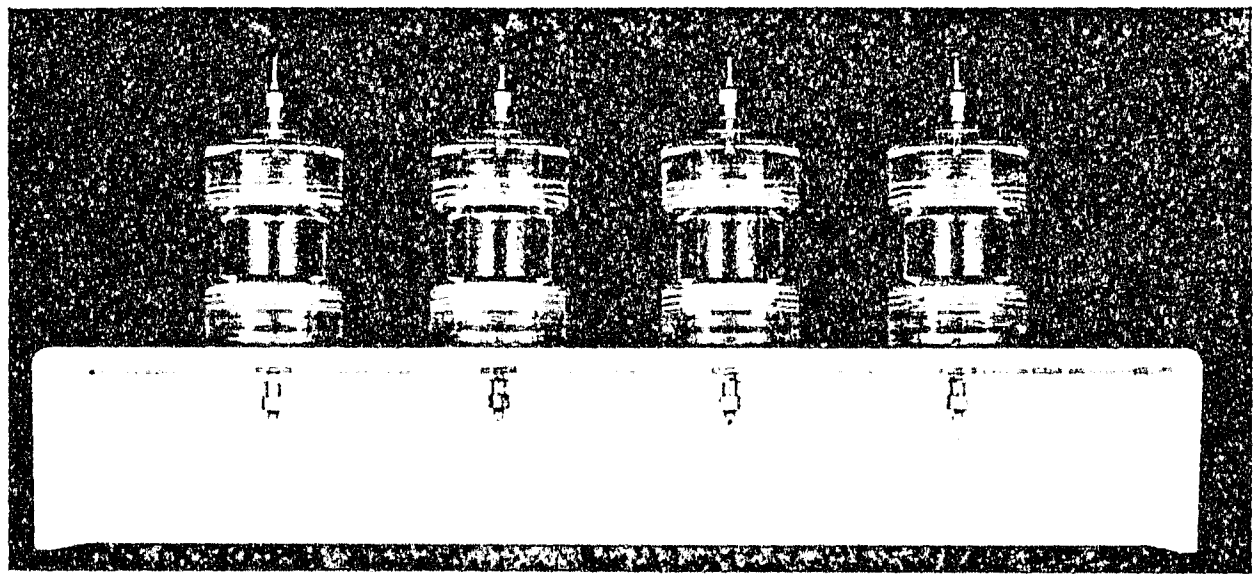


PLATE 1.

Complete superfusion apparatus consisting of 4 chambers arranged in parallel, as used in experiments to examine neurotransmitter release.

2.5.2. The technique.

Aliquots of preloaded synaptosomes were layered on Millipore filters (0.45µm pore) resting on filter supports constituting the bottoms of four parallel superfusion chambers of the type described above, and maintained at 37°C in a precision incubator. The chambers were connected to a multichannel peristaltic pump, the excess medium drawn off at maximum flow rate, and the filters washed with 10ml of control superfusion medium (concentrations : 128mM NaCl; 5mM KCl; 2.7mM $CaCl_2$; 1.2mM $MgSO_4$; 30mM glucose; 10mM Tris-HCl buffer at pH 7.35). In experiments involving GABA, the medium also contained 0.1mM aminooxyacetic acid to prevent GABA catabolism (Iversen and Johnston, 1971; Levy et al., 1973; Minchin and Iversen, 1974; Hammerstad and Lytle, 1976). The flow rate was adjusted to 0.5ml/minute and 1-minute fractions were collected directly into mini-scintillation counting vials. Superfusion was continued for 10 minutes with control medium to establish a baseline rate of labelled amino acid efflux; the control

medium was then exchanged for the test medium, and superfusion continued for a further 15 minutes. Aquagel I liquid scintillant (Chem-Lab), 5ml, was added to each vial and the radioactivity of the vials counted in a Packard Tri-Carb spectrometer ($^{3}H$, 37% efficiency; $^{14}C$, 74% efficiency). Filters were solubilised in 10ml of Aquagel I and counted similarly. Stimulation of release was calculated as the percentage increase in efflux over the base-line instimulated level.

It was considered that superfusion under the conditions described above minimized reuptake of released transmitter since increasing the flow rate through the chamber to 1.0ml/minute did not increase the radioactivity captured per unit time.

## 2.6. Preparation of whole brain synaptic plasma membranes.

Synaptic membranes were prepared from the whole brains of adult male and female Wistar rats (fed on an ordinary laboratory diet, Epol) by the method of Enna and Snyder (1975).

Rats were killed by stunning and the whole brains removed into 15 volumes of ice-cold 0.32M sucrose and homogenized with 30 strokes in a hand glass homogenizer (radial clearance, 0.25mm). All procedures were carried out at 4°C. The homogenate was centrifuged at 1000g x 10 minutes; the pellet was discarded and the supernatant fluid was centrifuged at 20,000g x 20 minutes. The crude mitochondrial-synaptosomal pellet was resuspended in distilled water and dispersed with a Brinkman Polytron PT-10 (setting 6) for 30 seconds. The suspension was centrifuged at 8000g x 20 minutes. The supernatant fluid was collected and the pellet, a bilayer with a soft buffy coat, was rinsed carefully with the supernatant fluid to collect the upper layer. This suspension was then centrifuged at 48000g x 20 minutes. The final crude synaptic membrane pellets were resuspended in distilled water and recentrifuged at 48000g x 20 minutes. The membranes were then stored at -20°C for at least 18 hours, but not longer than 30 days.

2.7. GABA receptor binding studies.

Frozen pellets of synaptic membranes were

resuspended in distilled water, maintained at 2°C for 10 minutes and centrifuged at 48000g x 20 minutes. The pellets were then suspended in 50mM Tris-citric acid buffer (pH 7.1) to give a protein concentration of 0.4-0.6mg/ml (Lowry et al., 1951). Two ml aliquots of the suspeusionwere incubated in triplicate at 4°C for 5 minutes in the presence of $^{3}$H-GABA (specific activity 54 Ci/mmol) in the concentration range 2-10nM, and in the presence or absence of unlabelled GABA or bicuculline at concentrations up to 1mM. The reaction was terminated by rapid filtration through Whatman GF/B glass microfibre filters and the filters washed within 10 seconds with 2 x 5ml aliquots of ice-cold buffer. Filters were disintegrated by vigorous shaking in 10ml Aquagel I liquid scintillant (Chemlab) and counted in a Packard Tri-Carb 2660 spectrometer with automatic quench correction. Specific $^{3}$H-GABA binding was calculated as the difference between total binding and binding in the presence of 1mM unlabelled GABA or bicuculline. The effect of δ-ALA on $Na^{+}$-independent $^{3}$H-GABA binding was determined using δ-ALA at concentrations up to 5mM as the displacer.

# CHAPTER 3

# RESULTS

## 3.1. Effect of GABA analogues on the efflux of preloaded $^{3}$H-GABA from synaptosomes.

### 3.1.1. Unlabelled GABA (Fig.2).

Unlabelled GABA at a concentration of 1mM in the test superfusion medium stimulated the efflux of $^{3}$H-GABA from preloaded rat cortical synaptosomes by 705 $\pm$ 60% (mean $\pm$ s.e.m. n = 3). The homoexchange mechanism was maximally stimulated by 1mM GABA since increasing the concentration to 5mM did not elicit a great stimulation of efflux of label. These results were in accordance with those of Raiteri et al., (1975).

### 3.1.2. β-Alanine (Fig.2).

β-Alanine at a concentration of 1mM stimulated $^{3}$H-GABA release from the preloaded synaptosomes by 210 $\pm$ 40% (mean $\pm$ s.e.m. n = 3). Thus, at a concentration at which exchange with unlabelled GABA was maximal, β-alanine stimulated exchange to about 38% of maximum.

FIGURE 2.

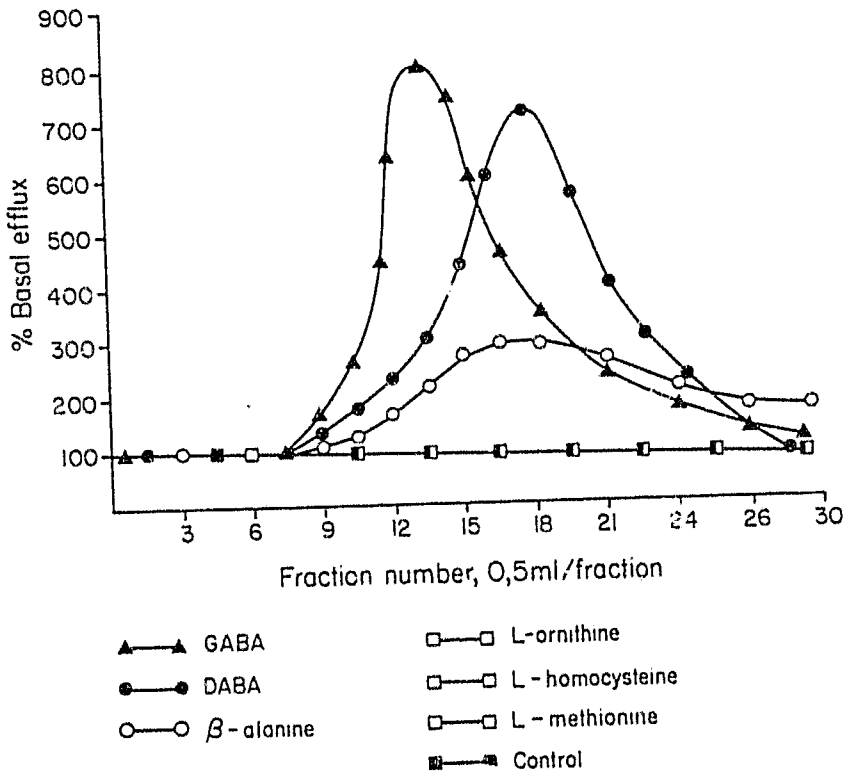

900
800
700
600
500
400
300
200
100
% Basal efflux
3
6
9
12
15
18
21
24
26
30
Fraction number, 0,5ml/fraction
GABA
DABA
β-alanine
L-ornithine
L-homocysteine
L-methionine
Control

FIGURE 2.

Stimulation of $^{3}$H-GABA efflux by GABA analogues. Shown is a plot of percentage change in efflux (baseline efflux = 100%) against fraction number. The arrow represents the change-over from plain medium to the test medium containing the GABA analogues (1mM) listed below. The first few fractions were very variable in each case and are not shown. Each curve is an average of 3 superfusion experiments. Variation between individual curves was less than 13% of the mean. ▲, GABA; ●, DABA; ○, β-alanine; □, L-ornithine; □, L-homocysteine, □, L-methionine; ■, control.

Superfusion rate : 0.5ml/minute.

FIGURE 2.

Stimulation of $^{3}$H-GABA efflux by GABA analogues. Shown is a plot of percentage change in efflux (baseline efflux = 100%) against fraction number. The arrow represents the change-over from plain medium to the test medium containing the GABA analogues (1mM) listed below. The first few fractions were very variable in each case and are not shown. Each curve is an average of 3 superfusion experiments. Variation between individual curves was less than 13% of the mean. ▲, GABA; ●, DABA; O, β-alanine; □, L-ornithine; □, L-homocysteine, □, L-methionine; ■, control. Superfusion rate : 0.5ml/minute.

This finding supported that of Raiteri et al., (1975).

3.1.3. DABA (Fig.2).

(+)-2,4-Diaminobutyric acid (DABA) at a concentration of 1mM stimulated $^{3}$H-GABA release by 655 ± 55% (mean ± s.e.m., n = 3). By contrast with β-alanine, DABA at 1mM produced a striking stimulation of $^{3}$H-GABA efflux; about 94% of the maximal stimulation.

3.1.4. L-Ornithine, L-homocysteine and L-methionine (Fig.2).

L-Ornithine, an amino acid one methylene group longer than DABA, the excitatory amino acid L-homocysteine and its analogue L-methionine produced no stimulation of $^{3}$H-GABA efflux from preloaded synaptosomes at a concentration of 1mM.

3.1.5. δ-ALA

δ-ALA is an omega amino acid with a 5 carbon chain similar in structure to GABA, and fits within the guidelines of McGeer et al., (1961) as a GABA analogue.

High concentrations of δ-ALA (0.75-5.0mM) in the superfusion medium produced very marked stimulation of $^{3}$H-GABA efflux from preloaded synaptosomes (Fig.3). The maximum stimulation of $^{3}$H-GABA release obcainable by superfusion with unlabelled GABA is 705 ± 60% (mean ± s.e.m., n = 3) at a concentration of 1mM (Fig.2). δ-ALA at concentrations of 1mM and 5mM stimulated $^{3}$H-GABA efflux by 826 ± 61.5% and 1356 ± 32.9% respectively (mean ± s.e.m., n = 3). However, low concentrations of δ-ALA (0.1-0.5mM) produced very little stimulation of $^{3}$H-GABA efflux (Fig.3), considerably less than that produced by similar concentrations of unlabelled GABA (Raiteri et al., 1975).

## 3.2 Potassium-stimulated release of GABA from synaptosome suspensions

### 3.2.1. Effect of raised extracellular $K^{+}$ on $^{3}$H-GABA release.

Membrane depolarisation induced by 55mM $K^{+}$ (using KCl) in the superfusion medium

FIGURE 3.

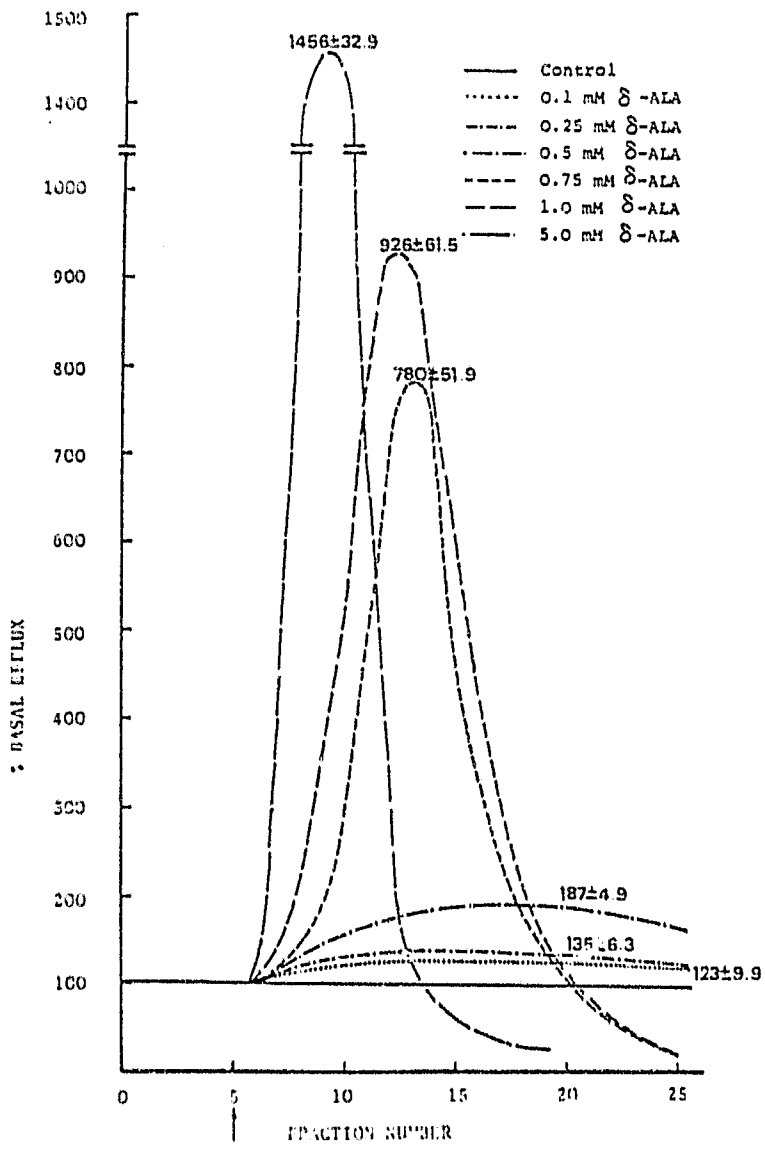

1500
1400
1000
900
800
700
600
500
400
300
200
100
% BASAL EFFLUX
0
5
10
15
20
25
FRACTION NUMBER
1456±32.9
926±61.5
780±51.9
187±4.9
135±6.3
123±9.9
Control
0.1 mM δ -ALA
0.25 mM δ-ALA
0.5 mM δ-ALA
0.75 mM δ-ALA
1.0 mM δ-ALA
5.0 mM δ-ALA

FIGURE 3.

Stimulation of $^{3}$H-GABA efflux by δ-ALA. Shown is a plot of percentage change in efflux (baseline efflux = 100%) against fraction number. The arrow represents the change-over from plain medium to the test medium containing : ———, control; ....., 0.1mM δ-ALA; —·—·—, 0.25mM δ-ALA; — - —— - ——, 0.5mM δ-ALA; -----, 0.75mM δ-ALA, ___ ___ ___, 1.0mM δ-ALA; ____ ____, 5.0mM δ-ALA.

The first fractions were very variable in each case and are not shown. Each curve is an average of 3 experiments, and the values are the maximum stimulation (mean ± s.e.m., n = 3) elicited by the test medium. Superfusion rate was 0.5ml/minute and 0.5ml fractions were collected. The curves pass through every experimental point; however, these have not been shown for reasons of clarity.

stimulated $^{3}$H-GABA release by 188 ± 26.9% (mean ± s.e.m., n = 6)(Fig.4).

### 3.2.2. Calcium-dependence of $K^{+}$-stimulated $^{3}$H-GABA release.

Omission of $Ca^{2+}$ from the medium or substitution of $Mg^{2+}$ for $Ca^{2+}$in the medium did not significantly affect basal release of radioactivity calculated as % total tissue stores released per one minute fraction (0.57 ± 0.068%, mean ± s.e.m., n = 7). Potassium (55mM)-stimulated release of $^{3}$H-GABA was, however, reduced by 76 ± 2.2% (n = 4) in $Ca^{2+}$- free medium (Fig.4). This indicated that some 76% of $K^{+}$-stimulated release was $Ca^{2+}$-dependent. These results are in close accordance with those of Okamoto and Namima (1978).

### 3.2.3. Effect of δ-ALA on $K^{+}$-stimulated $^{3}$H-GABA release.

δ-ALA reduced the $K^{+}$ (55mM)-stimulated release of $^{3}$H-GABA from the nerve endings in a dose-dependent fashion (Fig.5).

FIGURE 4.

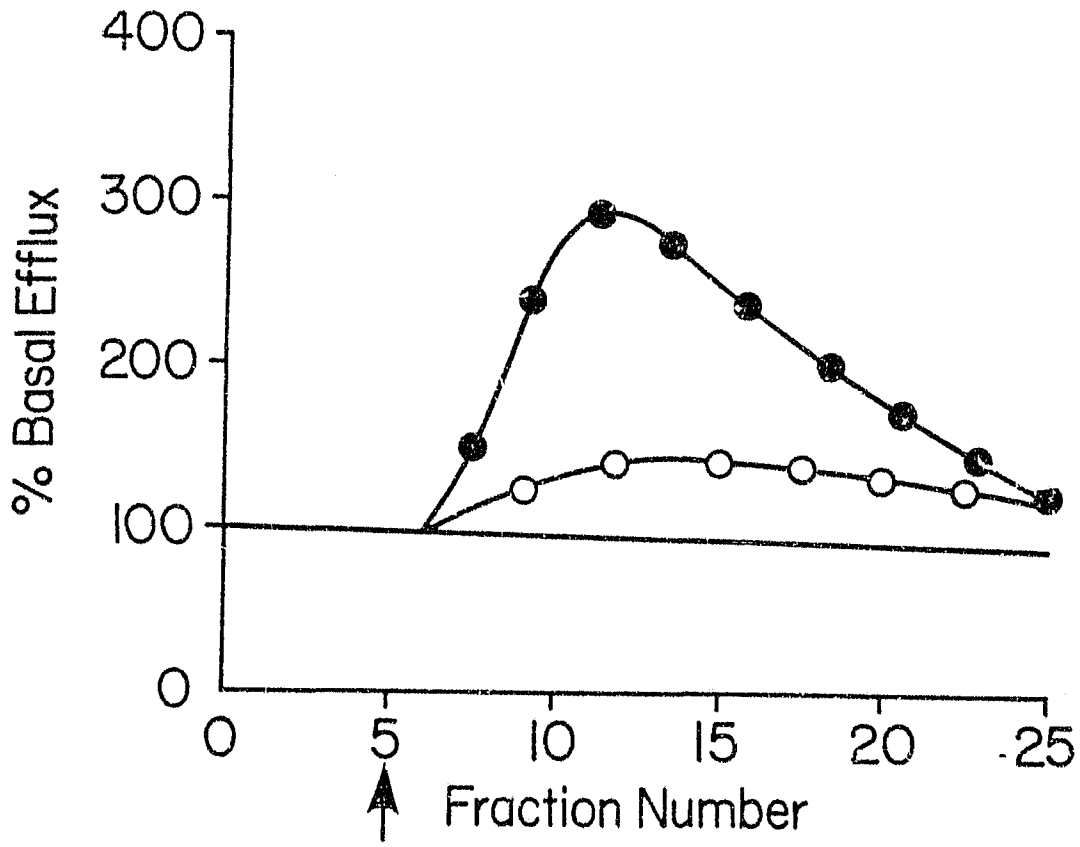


FIGURE 4.

Stimulation of $^{3}$H-GABA release elicited by 55mM $K^{+}$. The arrow represents the change-over from plain medium to test medium containing 55mM $K^{+}$ in the presence (●) or absence (O) of 2.7mM $Ca^{2+}$. Curves are the mean of 6 (●) or 4 (O) experiments. Standard errors less than 10% of the mean.

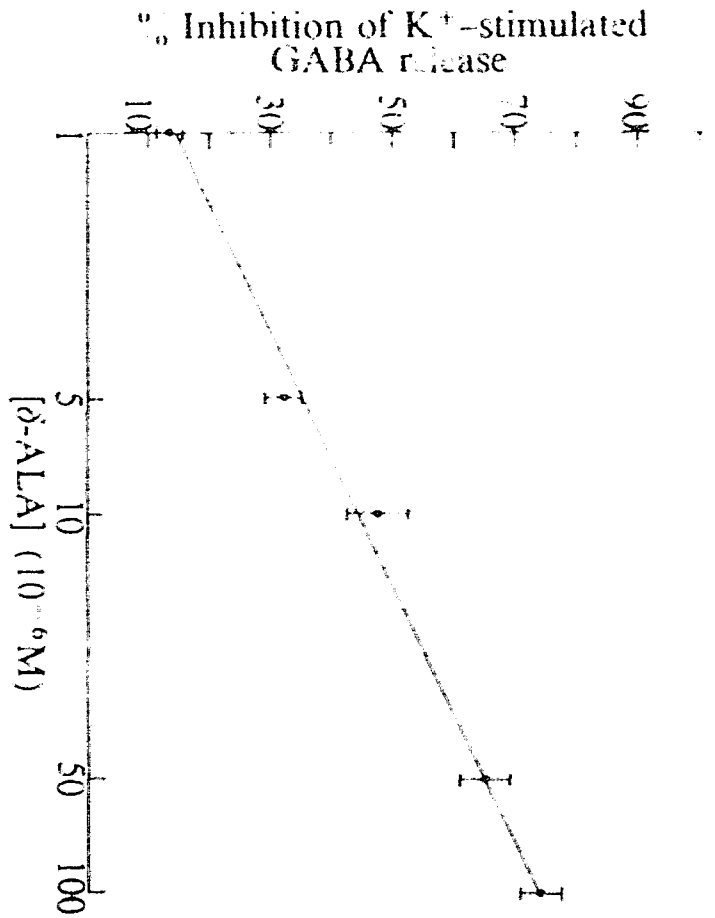

% Inhibition of K+-stimulated GABA release
1
10
30
50
70
90
5
10
50
100
[δ-AL] (10⁻⁶M)

FIGURE 5.

A dose-response curve for the inhibition of $K^+$ (55mM)-induced GABA release by δ-ALA. The horizontal axis gives concentration of δ-ALA (x $10^{-6}$M) on a logarithmic scale. Represented on the vertical axis is the % inhibition of $K^+$-stimulated GABA release. Each point is the mean % inhibition $\pm$ s.e.m. (n = 4). The curve is the best fit to the data by the method of least squares ( r = 0.97). It was not possible to produce 100% inhibition of the stimulated release , as δ-ALA alone at concentrations > 100μM caused increased efflux of radioactivity from the synaptosomes (Fig.3).

The reduction was significant at a δ-ALA concentration of 1μM, and at a concentration of 100μM, 75% of the stimulated release was abolished. In addition, it was found that a combination of 1mM δ-ALA and 55mM $K^+$ in the superfusion medium produced a 21.5% reduction in the stimulation due to 1mM δ-ALA alone and delayed the occurrence of the peak by about 4 fractions (Fig.6). The same effects were observed when the $K^+$ concentration was raised using potassium sulphate instead of potassium chloride and thus seemed to be independent of the external chloride concentration. The concentration of δ-ALA which inhibited 50% of the $K^+$-stimulated GABA release was 11.5 ± 0.87μM (determined by log-probit analysis of the best line fit shown in Fig.5).

### 3.2.4. δ-ALA-induced inhibition of GABA release prevented by GABA receptor antagonists.

δ-ALA has been shown to decrease the membrane resistance of the crayfish stretch receptor neuron, probably by increasing the $Cl^-$ conductance of the membrane (Dichter et al., 1977). In this respect it mimics the postsynaptic action of GABA (Takeuchi and Takeuchi, 1966).

FIGURE 6.

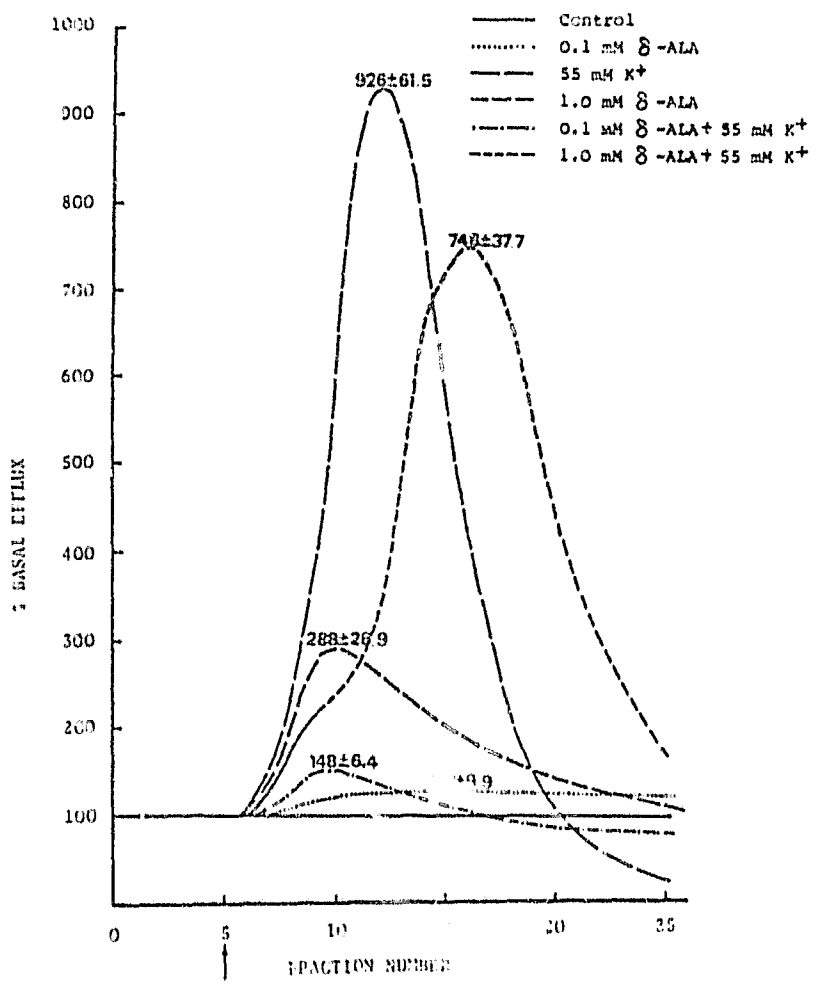

Control
0.1 mM δ-ALA
55 mM K+
1.0 mM δ-ALA
0.1 mM δ-ALA + 55 mM K+
1.0 mM δ-ALA + 55 mM K+
926±61.5
748±37.7
288±26.9
148±6.4
1000
900
800
700
600
500
400
300
200
100
0
5
10
20
35
% BASAL EFFLUX
FRACTION NUMBER

FIGURE 6.

Effects of δ-ALA on $K^+$-stimulated $^3H$-GABA release. Shown is a plot of percentage change in efflux (baseline efflux = 100%) against fraction number. The arrow represents the change-over from plain medium to the test medium containing : ———, control; ------, 55mM $K^+$; ....., 0.1mM δ-ALA; .——. ——., 0.1mM δ-ALA and 55mM $K^+$; — — —, 1.0mM δ-ALA; ----, 1.0mM δ-ALA and 55mM $K^+$. The first few fractions were very variable in each case and are not shown. Each curve is an average of 3 experiments, and the values are the maximum stimulation (mean ± s.e.m.) elicited by the test medium. Superfusion rate was 0.5ml/minute and 0.5ml fractions were collected. The curves pass through every experimental point; however, these have not been shown for reasons of clarity.

Clearly the effect on GABA release described above is explicable on the basis of δ-ALA interacting with presynaptic GABA receptors leading to an increased chloride conductance of the membrane. It was therefore decided to examine the effect of known GABA receptor antagonists on this phenomenon.

In all cases, the reduction of $K^+$-stimulated $^3H$-GABA release by δ-ALA was prevented by the GABA receptor antagonists bicuculline or picrotoxin ($10^{-6}$M)(Table 1). This demonstration that a compound can alter release of GABA in an appropriate fashion and that the effect is antagonized by known GABA receptor antagonists is strong evidence for the existence of GABA autoreceptors which can modulate GABA release. Recently, evidence supporting this concept has been presented (Mitchell and Martin, 1978; Snodgrass, 1978).

δ-ALA at 1μM or 10μM exhibited no effects on the basal release of GABA although, at 100μM, GABA efflux was slightly stimulated (Fig.3).

TABLE 1

Drug effects on the $K^+$-stimulated release of GABA from preloaded synaptosomes.

| Drug present | Induced release (% increase over baseline control) | Mean drug effect on induced release |
|---|---|---|
| 55mM $K^+$ alone (6) | 188 ± 26.9 | |
| $10^{-4}$M δ-ALA alone (4) | 23 ± 9.9 | |
| 55mM $K^+$ + $10^{-4}$M δ-ALA (4) | 48 ± 6.4* | -74.5% |
| 55mM $K^+$ + $10^{-4}$M δ-ALA + $10^{-6}$M bicuculline (4) | 191 ± 13.2 | + 1.6% |
| 55mM $K^+$ + $10^{-5}$M δ-ALA (4) | 92 ± 13.5* | -51.1% |
| 55mM $K^+$ + $10^{-5}$M δ-ALA + $10^{-6}$M picrotoxin (4) | 187 ± 10.2 | - 0.5% |
| 55mM $K^+$ + $10^{-6}$M δ-ALA (4) | 163 ± 7.5** | -13.0% |
| 55mM $K^+$ + $10^{-6}$M δ-ALA + $10^{-6}$M bicuculline (4) | 197 ± 13.1 | + 4.8% |

TABLE I

Release experiments were carried out as described in 2.5.2. The values in parentheses indicate the number of times the experimental conditions in the first column were repeated. Baseline efflux was calculated as % total tissue stores released per fraction ($0.57 \pm 0.068\%$, $n = 7$). The second column refers to the % increase in efflux over this control baseline level; in each case the $\pm$ s.e.m. is given. Statistical significance calculated using the two-sided Student t-t[illegible] The third column represents the average drug-induced change compared with the release caused by 55mM $K^+$ alone. δ-ALA at concentrations < $10^{-4}$M had no effect on the basal release of radioactivity from the synaptosomes. Bicuculline and picrotoxin alone ($10^{-6}$M or $10^{-5}$M) did not facilitate the release of GABA, presumably because the flow of superfusion fluid removed any GABA as it was released. In the absence of negative feedback effects to antagonise, the GABA receptor antagonists had no effect on basal efflux.

* $P < 0.001$, significant difference from 55mM $K^+$ alone.

** $P < 0.05$, significant difference from 55mM $K^+$ alone.

Neither bicuculline nor picrotoxin alone ($10^{-6}$M or $10^{-5}$M) modified the basal release of radioactivity in the conditions used here. This is expected since it is believed that any GABA released from the tissue is rapidly removed from the superfusion chamber by the flow of medium. The concentration of GABA in the medium does not therefore reach the levels necessary to stimulate presynaptic receptors and hence the bicuculline or picrotoxin has no negative feedback effects to antagonize.

### 3.3. Effect of GABA agonists on $K^+$-stimulated GABA release from synaptosomes; prevention by GABA receptor antagonists.

As discussed in the Introduction, there is now considerable evidence suggesting that the release of several neurotransmitters may be regulated via autoreceptors on presynaptic nerve terminals (Langer, 1978). Classical postsynaptic receptor agonists and antagonists have varying degrees of affinity for the corresponding autoreceptors; such pharmacological differences between presynaptic and postsynaptic receptor systems may open up the possibility of selective activation or blockade of one or other class of receptor by drugs.

Since the pharmacology of the GABA autoreceptor vis a vis the postsynaptic receptor is completely unknown, a collaborative study was started with Dr. P. Krogsgaard-Larsen (Copenhagen) to examine the effects of a range of known GABA agonists on $K^+$-stimulated GABA release and to compare their potency in inhibiting release with their potency in inhibiting specific $^3H$-GABA binding to rat cortical synaptic plasma membranes.

Muscimol, piperidine-4-sulphonic acid (PSA), isoguvacine and 4,5,6,7 - tetrahydroisoxazolo-(5,4-c) pyridin-3-ol (THIP) are potent GABA receptor agonists (Braestrup et al., 1979; Krogsgaard-Larsen et al., 1979). All reduced the $K^+$-stimulated release of preloaded $^3H$-GABA from rat cortical synaptosomes in a dose-dependent fashion (Fig.7). In every case, at every drug concentration tested, the reduction in $K^+$-stimulated release was prevented by bicuculline and picrotoxin ($10^{-6}$M)(Table 2). Again, the demonstration that agonist drugs modify GABA release appropriately and that the effect is prevented by specific receptor antagonists is evidence for a receptor-mediated phenomenon.

FIGURE 7.

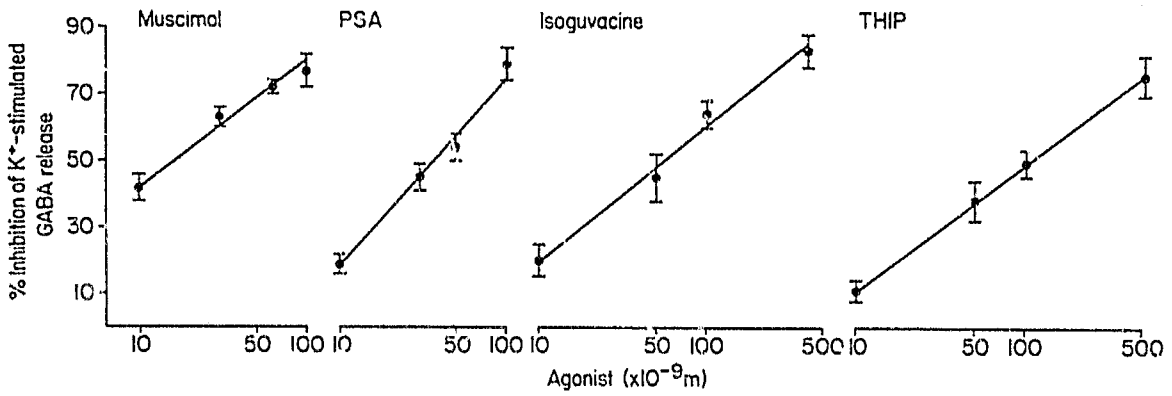

Muscimol
PSA
Isoguvacine
THIP
% Inhibition of K⁺-stimulated GABA release
90
70
50
30
10
10
50
100
500
Agonist (x10⁻⁹m)

FIGURE 7.

Dose-response curves for the inhibition of $K^+$(55mM)-induced GABA release by GABA agonists. The horizontal axes give concentrations of agonists (X $10^{-9}$M) on a logarithmic scale. Represented on the vertical axis is the % inhibition of $K^+$-stimulated GABA release. Each point is the mean % inhibition $\pm$ s.e.m. (n = 4). The curves are the best fits to the data by the method of least squares ( $r^2 > 0.98$).

TABLE 2

GABA agonist effects on the $K^+$-stimulated release of GABA from preloaded synaptosomes

| Drug present | Induced release (% increase over baseline control) | Mean drug effect on induced release |
|---|---|---|
| 55mM $K^+$ alone (6) | 188 ± 26.9 | |
| $10^{-7}$M muscimol alone (4) | 22 ± 4.9 | |
| 55mM $K^+$ + $10^{-8}$M muscimol (4) | 107 ± 11.1* | - 43% |
| 55mM $K^+$ + $10^{-8}$M muscimol + $10^{-6}$M bicuculline (2) | 194 ± 13.7 | + 3% |
| 55mM $K^+$ + $3 \times 10^{-8}$M muscimol (4) | 68 ± 7.6* | - 64% |
| 55mM $K^+$ + $3 \times 10^{-8}$M muscimol + $10^{-5}$M picrotoxin (2) | 197 ± 9.4 | + 5% |
| 55mM $K^+$ + $5 \times 10^{-8}$M muscimol (4) | 53 ± 4.9* | - 72% |
| 55mM $K^+$ + $5 \times 10^{-8}$M muscimol + $10^{-6}$M bicuculline (2) | 205 ± 19.6 | + 9% |

TABLE 2 (contd.)

| | Induced release (% increase over baseline control) | Mean drug effect on induced release |
|---|---|---|
| 55mM $K^+$ + $10^{-7}$M muscimol (4) | 47 ± 6.7* | - 75% |
| 55mM $K^+$ + $10^{-7}$M Muscimol + $10^{-6}$M picrotoxin (2) | 184 ± 7.2 | - 2% |
| 55mM $K^+$ + $10^{-8}$M PSA (4) | 154 ± 3.9*** | - 18% |
| 55mM $K^+$ + $10^{-8}$M PSA + $10^{-6}$M picrotoxin (2) | 190 ± 11.7 | + 1% |
| 55mM $K^+$ + $3 \times 10^{-8}$M PSA (4) | 100 ± 5.6* | - 47% |
| 55mM $K^+$ + $3 \times 10^{-8}$ M PSA + $10^{-6}$M bicuculline (2) | 199 ± 18.2 | + 6% |
| 55mM $K^+$ + $3 \times 10^{-8}$M PSA (4) | 86 ± 6.1* | - 54% |
| 55mM $K^+$ + $5 \times 10^{-8}$M PSA + $10^{-6}$M picrotoxin (2) | 179 ± 12.1 | - 5% |

TABLE 2 (contd.)

| | Induced release (% increase over baseline control) | Mean drug effect on induced release |
|---|---|---|
| 55mM $K^+$ + $10^{-7}$M PSA (4) | 42 ± 11.9* | - 78% |
| 55mM $K^+$ + $10^{-7}$M PSA + $10^{-6}$M bicuculline (2) | 192 ± 5.6 | + 2% |
| 55mM $K^+$ + $10^{-8}$M isoguvacine (4) | 150 ± 14.3*** | - 20% |
| 55mM $K^+$ + $10^{-8}$M isoguvacine + $10^{-6}$M bicuculline (2) | 175 ± 9.2 | - 7% |
| 55mM $K^+$ + $5 \times 10^{-8}$M isoguvacine (4) | 108 ± 16.2* | - 43% |
| 55mM $K^+$ + $5 \times 10^{-8}$M isoguvacine + $10^{-6}$M picrotoxin (2) | 194 ± 20.1 | + 3% |
| 55mM $K^+$ + $10^{-7}$M isoguvacine (4) | 68 ± 7.8* | - 64% |
| 55mM $K^+$ + $10^{-7}$M isoguvacine + $10^{-6}$M bicucullire (2) | 184 ± 12.0 | - 2% |
| 55mM $K^+$ + $5 \times 10^{-7}$M isoguvacine (4) | 32 ± 5.1* | - 83% |

TABLE 2 (contd.)

| | Induce release (% increase over baseline control) | Mean drug effect on induced release |
|---|---|---|
| 55mM $K^+$ + $5 \times 10^{-7}$M isoguvacine + $10^{-6}$M picrotoxin (2) | 169 ± 13.6 | - 10% |
| 55mM $K^+$ + $10^{-8}$M THIP (4) | 167 ± 14.6 | - 11% |
| 55mM $K^+$ + $10^{-8}$M THIP + $10^{-6}$M picrotoxin (2) | 196 ± 5.5 | + 4% |
| 55mM $K^+$ + $5 \times 10^{-8}$M THIP (4) | 118 ± 14.8** | - 37% |
| 55mM $K^+$ + $5 \times 10^{-8}$M THIP + $10^{-6}$M bicuculline (2) | 197 ± 9.6 | + 4% |
| 55mM $K^+$ + $10^{-7}$M THIP (4) | 98 ± 5.0* | - 48% |
| 55mM $K^+$ + $10^{-7}$M THIP + $10^{-6}$M picrotoxin (2) | 177 ± 8.4 | - 6% |
| 55mM $K^+$ + $5 \times 10^{-7}$M THIP (4) | 51 ± 9.1* | - 73% |
| 55mM $K^+$ + $5 \times 10^{-7}$M THIP + $10^{-6}$M bicuculline (2) | 197 ± 17.7 | + 5% |

TABLE 2.

Conditions as described in the legend to Table 1.

* P <0.001, significant difference from 55mM $K^+$ alone.

** P <0,01, significant difference from 55mM $K^+$ alone.

*** P <0.05, significant difference from 55mM $K^+$ alone.

The $IC_{50}$ values for the drugs in inhibiting $K^+$-stimulated GABA release are shown in Table 3.

PSA, isoguvacine and THIP at concentrations up to 1μM exhibited no effects on the basal release of $^3H$-GABA. However, muscimol, at concentrations of 0.1μM and above, stimulated GABA efflux in a dose-dependent fashion (Fig.8). The $IC_{50}$ value for inhibition of stimulated GABA release by muscimol is therefore likely to be overestimated, since it is difficult to correct for this combined effect. Again, neither bicuculline nor picrotoxin alone ($10^{-6}$M) modified basal release of radioactivity.

All the GABA agonists used in this study inhibited $^3H$-GABA binding to high affinity receptor sites in synaptic plasma membranes from rat cerebral cortex (Krogsgaard-Larsen et al., 1979; Krogsgaard-Larsen, unpublished results). The $IC_{50}$ values for the compounds are listed in Table 3. The order of potency of the compounds in inhibiting GABA receptor binding was the same as that for inhibiting $K^+$-stimulated GABA release.

TABLE 3.

Inhibition of $K^+$(55mM)-stimulated $^3$H-GABA release from synaptosomes and specific $^3$H-GABA binding in synaptic plasma membranes by GABA agonists.

| | $IC_{50}$ (nM) | |
|---|---|---|
| | GABA release | GABA binding |
| Muscimol | 16 ± 3 | 6 ± 0.2 |
| PSA | 36 ± 4 | 34 ± 2 |
| Isoguvacine | 49 ± 5 | 37 ± 3 |
| THIP | 110 ± 8 | 131 ± 5 |

TABLE 3.

$IC_{50}$ values for the inhibition of $K^+$-stimulated GABA release were calculated from a best fit line to the data (Fig.7) by log-probit analysis. Sodium-independent binding of $^3H$-GABA to membranes isolated from rat cerebral cortex was studied at $4^oC$ in Tris-citrate buffer (Krogsgaard-Larsen et al., 1979) The $IC_{50}$ was estimated by varying the concentration over a range of values using 4 data points for each of four inhibitor concentrations and calculating the line of best fit for a log-probit plot (Balcar et al., 1976). Results are means $\pm$ s.e.m. of 4 independent experiments.

FIGURE 8.

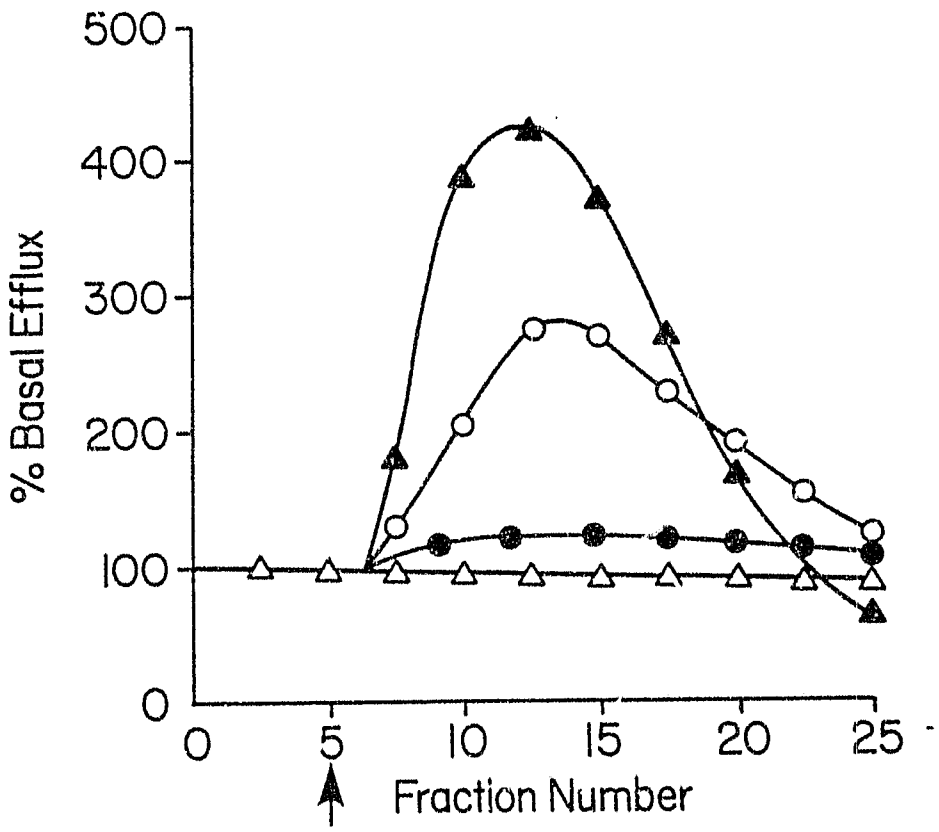

500
400
300
200
100
0
% Basal Efflux
0
5
10
15
20
25
Fraction Number

FIGURE 8.

Stimulation of $^{3}$H-GABA efflux by muscimol. Shown is a plot of percentage change in efflux (baseline efflux = 100%) against fraction number. The arrow represents the change-over from plain medium to the test medium containing: ●, 0.1μM muscimol; O, 0.5μM muscimol; ▲, 1.0μM muscimol; Δ, control. Each curve is an average of 4 experiments; variation between individual curves was less than 10%.

In addition, there was a good absolute correlation between receptor occupancy (ie. the concentration of agonist that displaces 50% of the specific GABA receptor binding) and inhibition of stimulated GABA release (Fig.9).

The data shown in Table 3 suggest that the GABA autoreceptors and the receptors labelled by $^{3}$H-GABA in synaptic plasma membranes are identical pharmacologically, at least as far as the compounds tested are concerned. This is of considerable significance since it implies that in binding studies using $^{3}$H-GABA or $^{3}$H-muscimol (Snodgrass, 1978) both pre- and postsynaptic GABA receptor sites are labelled. The interpretation of such studies must therefore take cognisance of this fact.

To date, a strict quantitative comparison between the results of binding studies and neurophysiological data has proved difficult. This is due to the impossibility of precise regulation of drug concentrations in the extracellular fluid during microelectrophoretic experiments.

FIGURE 9.

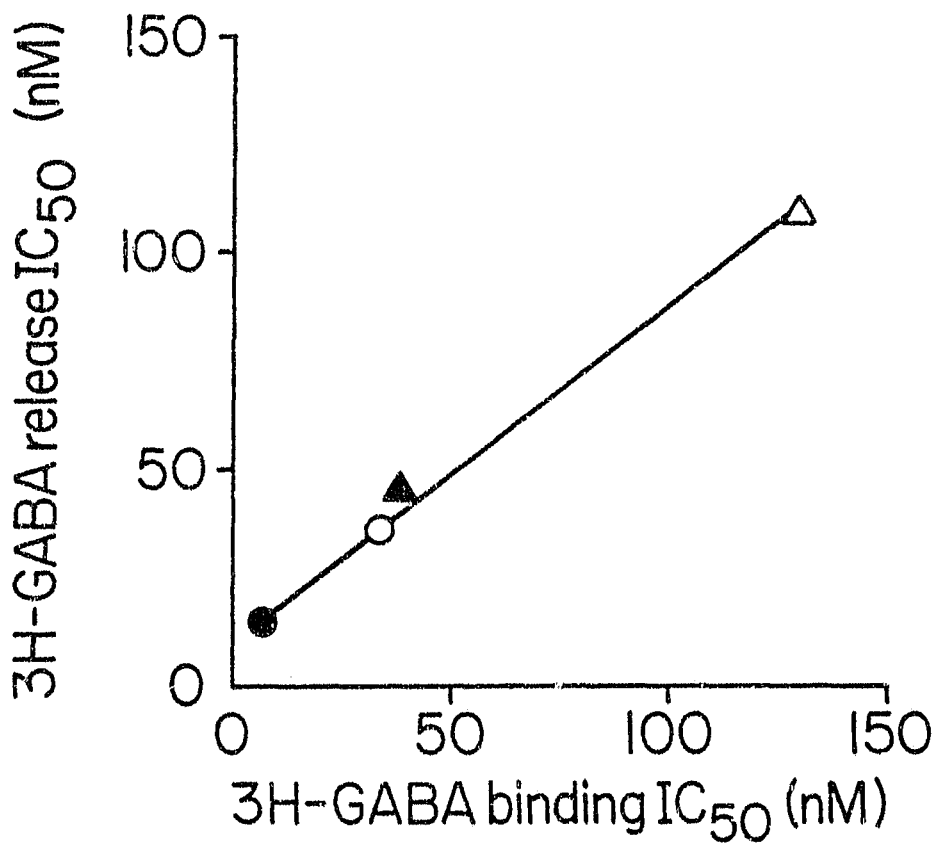

3H-GABA release IC50 (nM)
150
100
50
0
0
50
100
150
3H-GABA binding IC50 (nM)

FIGURE 9.

Correlation between GABA receptor occupancy and inhibition of $K^+$-stimulated GABA release for 4 GABA agonists. o, muscimol; O, PSA; ▲, isoguvacine; △, THIP. Line fitted to the data by regression analysis using the method of least squares. ( $r^2 = 0.99$).

Thus, the results described above provide the first direct correlation between a GABA receptor-mediated effect and receptor occupancy.

## 3.4. GABA receptor binding studies.

### 3.4.1. Sodium-independent binding of $^3$H-GABA to synaptic plasma membranes.

Binding of $^3$H-GABA to synaptic plasma membranes from whole rat brain was determined as described in 2.7.1.

Specific $^3$H-GABA binding was saturable with increasing $^3$H-GABA concentration (Fig.10). Binding was half-maximal at a $^3$H-GABA concentration of 2.7nM and maximal at 7.2nM. The concentration of unlabelled GABA that inhibited 50% of the specific binding ($IC_{50}$) was 0.25µM (Fig.11). Scatchard analysis (Fig.12) showed a single class of GABA receptors having a dissociation constant (Kd) of 7.2 $\pm$ 1.35nM (mean $\pm$ s.e.m., n = 3). The maximum binding value (B max) was 17.1 $\pm$ 0.81 fmol/mg protein (mean $\pm$ s.e.m., n = 3).

FIGURE 10.

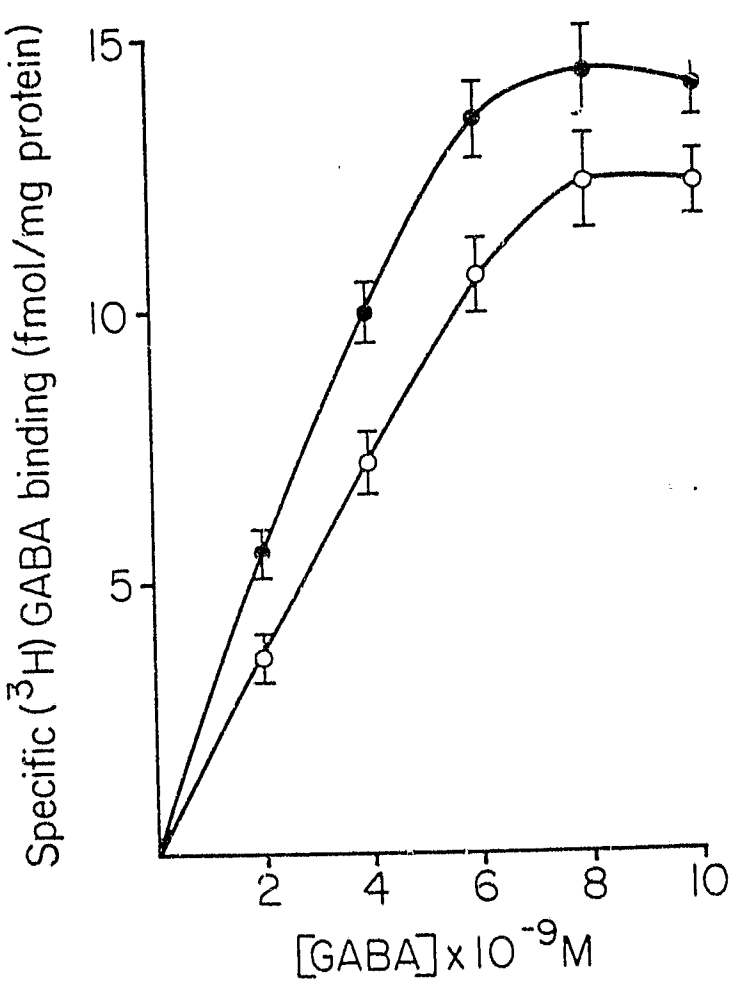

Specific ($^3$H) GABA binding (fmol/mg protein)
15
10
5
2
4
6
8
10
[GABA] x 10$^{-9}$M

FIGURE 10.

Saturability of $Na^+$-independent $^3H$-GABA binding to rat synaptosomal membranes. Frozen synaptosomal membranes were prepared and assayed for receptor binding as described in 2.7. The receptor binding assay was conducted in the presence of increasing concentrations of $^3H$-GABA using 1mM unlabelled GABA (●) or 1mM δ-ALA (O) as displacer. Each point is the mean ± s.e.m. of 3 experiments.

FIGURE 11.

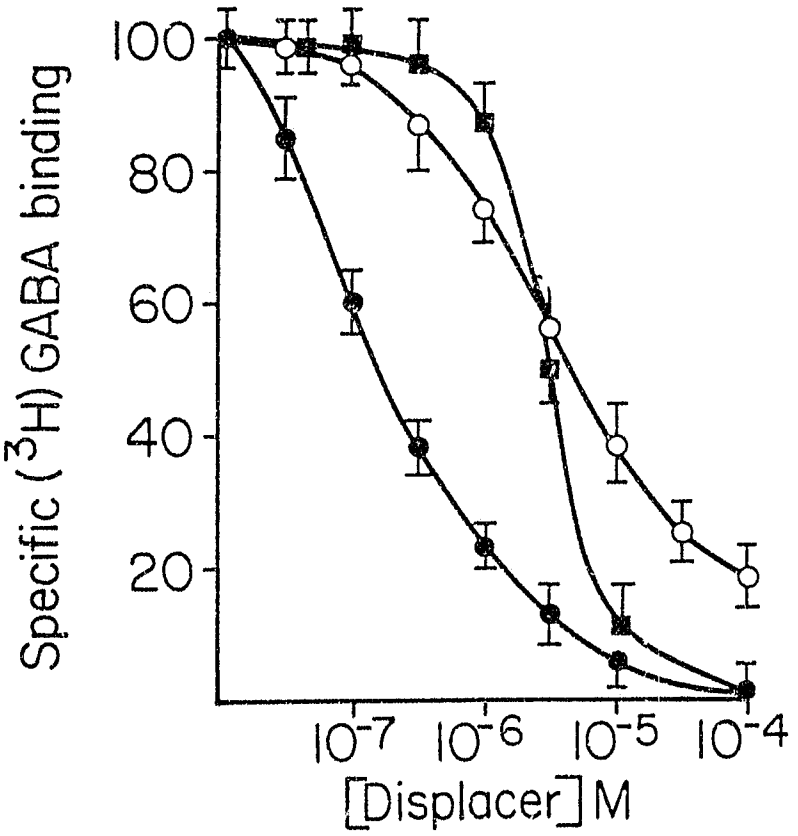

Specific ($^3$H) GABA binding
100
80
60
40
20
$10^{-7}$
$10^{-6}$
$10^{-5}$
$10^{-4}$
[Displacer] M

FIGURE 11.

Displacement of specific $^{3}$H-GABA binding by unlabelled GABA (●), (+) bicuculline (■) and δ-ALA (O). The standard receptor assay as described in 2.7. was carried out using 8nM $^{3}$H-GABA and varying concentrations of displacer. Each point is the mean ± s.e.m. of 3 experiments.

FIGURE 12.

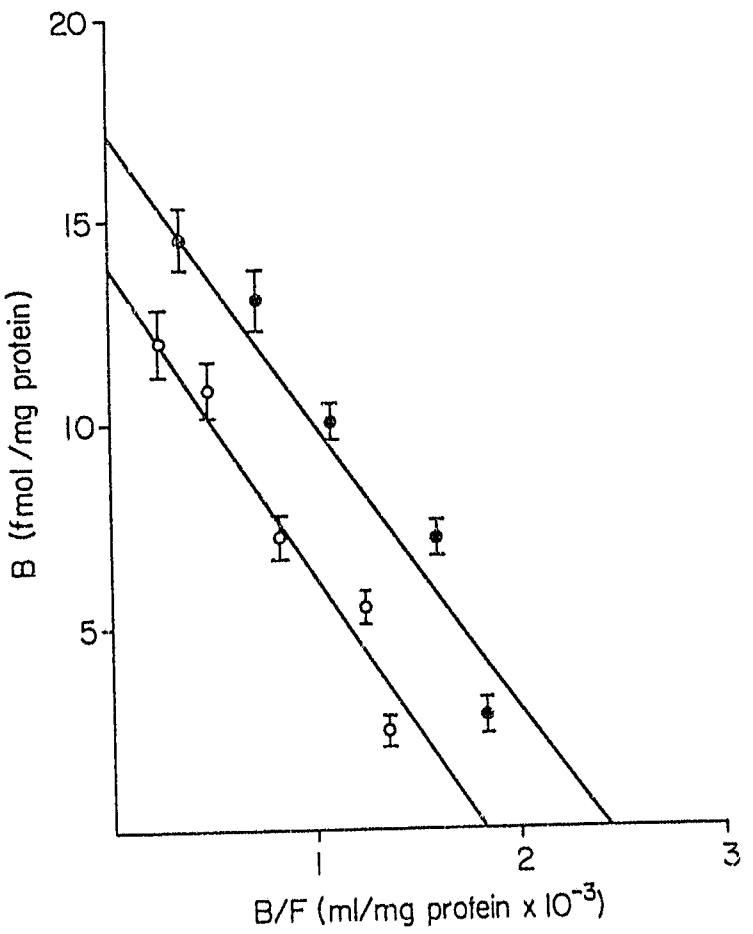


FIGURE 12.

Scatchard analysis of the binding of $^{3}$H-GABA to rat synaptosomal membranes. Binding was measured as described in 2.7. after a 5 minute incubation withvarying concentrations of $^{3}$H-GABA alone or with 1mM unlabelled GABA (●) or 1mM δ-ALA (O) as displacer. In the figure specifically bound ligand (B) is plotted as a function of bound ligand over free ligand ($^{B}/_{F}$). Each point is the mean ± s.e.m. of three experiments.

### 3.4.2. Effect of δ-ALA on GABA receptor binding.

When δ-ALA (1mM) was used to displace $^{3}$H-GABA bound to synaptic plasma membranes, specific $^{3}$H-GABA binding was saturable at a level of 15% lower than that obtained using unlabelled GABA (1mM)(Fig.10). δ-ALA was found to be quite a potent displacer of specific $^{3}$H-GABA binding, with an $IC_{50}$ value of 7.5μM (Fig.11). Scatchard analysis (Fig.12) of $^{3}$H-GABA binding using δ-ALA (1mM) as displacer revealed a single population of GABA binding sites with a Kd of 7.5 ± 1.10nM (mean ± s.e.m., n = 3) and a Bmax of 13.9 ± 0.50 fmol/mg protein (mean ± s.e.m., n = 3).

The $IC_{50}$ value of 7.5 ± 0.82μM (mean ± s.e.m., n = 3) for δ-ALA in inhibiting specific $^{3}$H-GABA binding correlates quite well with the $IC_{50}$ of 11.5 ± 0.87μM for δ-ALA in inhibiting $K^{+}$-stimulated GABA release (Fig.5). Thus, as far as five compounds are concerned, there appears to be no pharmacological difference between the GABA autoreceptor and the site labelled by $^{3}$H-GABA in synaptic membranes (Table 4).

TABLE 4.

Inhibition of $K^+$ (55mM)-stimulated $^3$H-GABA release from synaptsomes and specific $^3$H-GABA binding in synaptic plasma membranes by GABA agonists.

| | $IC_{50}$ (nM) | |
|---|---|---|
| | GABA release | GABA binding |
| Muscimol | 16 ± 3 | 6 ± 0.2 |
| PSA | 36 ± 4 | 34 ± 2 |
| Isoguvacine | 49 ± 5 | 37 ± 3 |
| THIP | 110 ± 8 | 131 ± 5 |
| β-ALA | 11500 ± 870 | 7500 ± 820 |

TABLE 4.

Legend as for TABLE 3.

FIGURE 13.

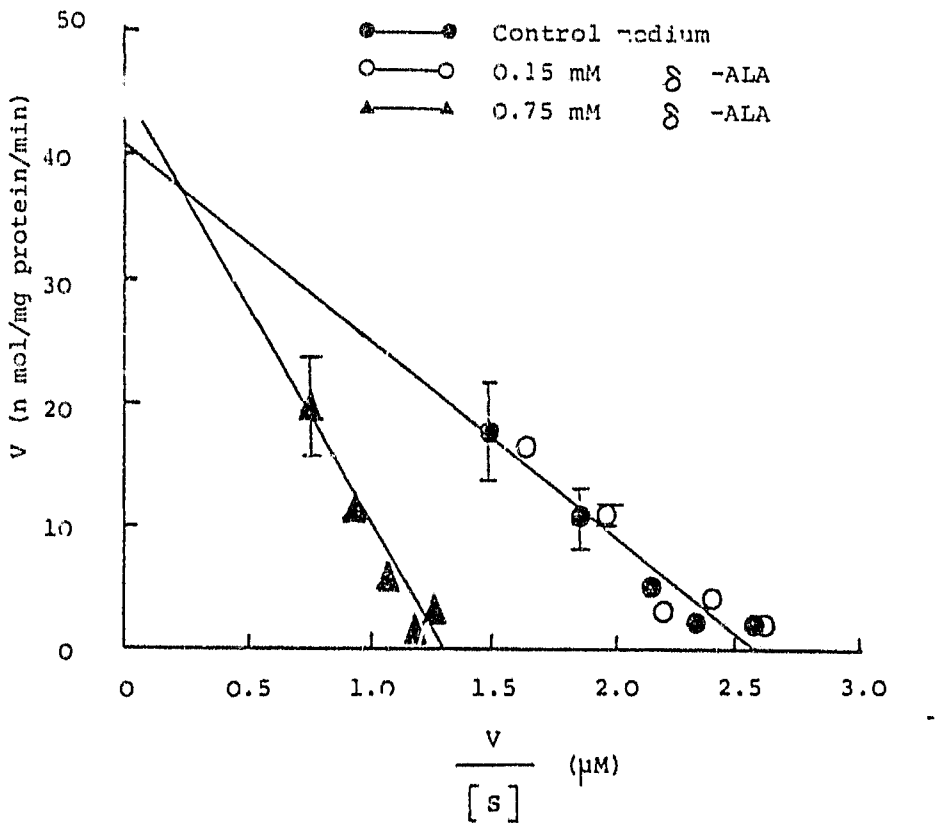

Control medium
0.15 mM δ -ALA
0.75 mM δ -ALA
v (n mol/mg protein/min)
50
40
30
20
10
0
0
0.5
1.0
1.5
2.0
2.5
3.0
v / [s] (μM)

FIGURE 13.

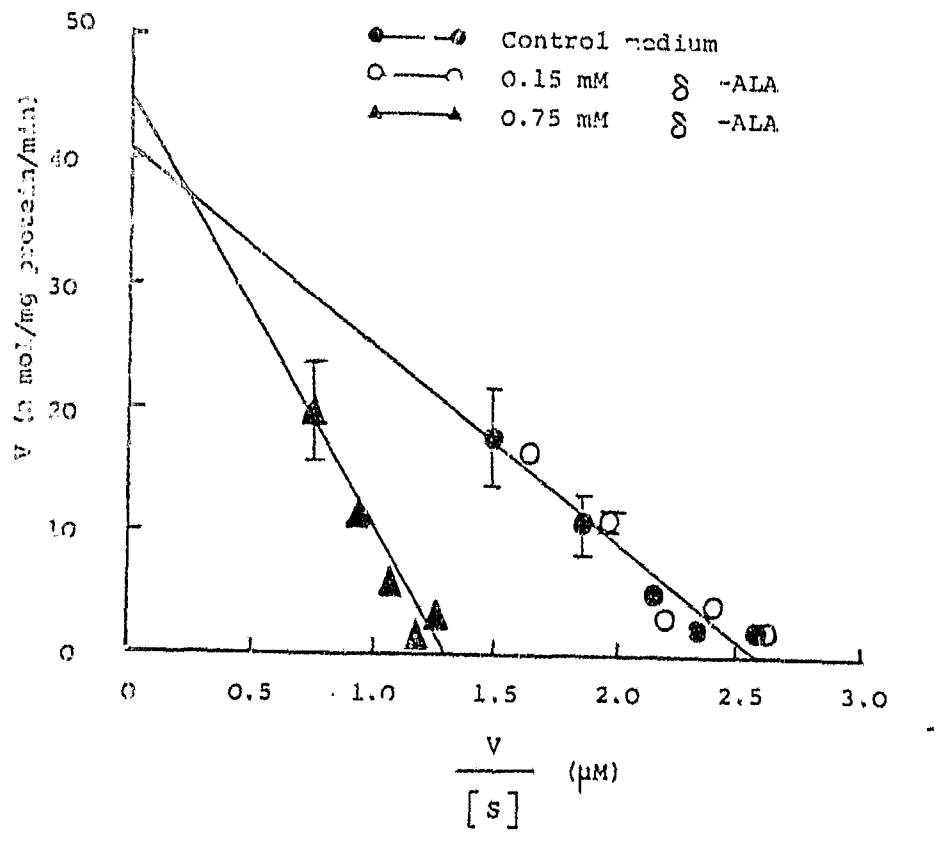

Control medium
0.15 mM δ -ALA
0.75 mM δ -ALA
V (n mol/mg protein/min)
50
40
30
20
10
0
0
0.5
1.0
1.5
2.0
2.5
3.0
V / [S] (μM)

FIGURE 13.

Kinetic analysis of the uptake of labelled GABA into rat cerebral synaptosomes. The uptake was measured as described in 2.4.1. after a 10 minute incubation with varying concentrations of $^{3}$H-GABA. In experiments examining the effects of δ-ALA on uptake of $^{3}$H-GABA, synaptosomes were preincubated in the presence of δ-ALA for 15 minutes before addition of 0.5μM $^{3}$H-GABA. In this Eadie-Hofstee plot, the uptake rate (v) is plotted as a function of the uptake rate over the GABA concentration ($^{v}/_{[s]}$). Each point is the mean of at least 8 experiments and the lines represent the best fit to the data by the method of least squares. Standard error bars are shown where these are greater than the size of the points. Key: ●, control medium; ○, 0.15 mM δ-ALA; ▲, 0,75mM δ-ALA.

## 3.6. Effect of δ-ALA on synaptosomal $^{3}$H-GABA uptake.

Preincubation of synaptosomes in the presence of low concentrations of δ-ALA (0.05-0.25mM) had no appreciable effect on $^{3}$H-GABA uptake (Fig.14). Concentrations in the range of 0.5-2.0mM, however, markedly inhibited $^{3}$H-GABA accumulation by the nerve endings. The inhibition was maximal at 0.5mM (63%) and a further increase in δ-ALA concentration up to 2mM was unable to effect greater inhibition. At a δ-ALA concentration of 0.75mM, the inhibition was of a competitive mode and the Eadie-Hofstee plot yielded a Ki for δ-ALA of 467μM (Fig.13).

## 3.7. Efflux of preloaded $^{14}$C-glutamate from synaptosomes.

### 3.7.1. Homoexchange between external unlabelled glutamate and the labelled pool.

Unlabelled glutamate in the test superfusion medium stimulated the efflux of preloaded $^{14}$C-glutamate in a dose-dependent fashion (Fig.15). Presumably this efflux of $^{14}$C-glutamate was mediated via a homoexchange mechanism (Levi and Raiteri, 1974).

FIGURE 14.

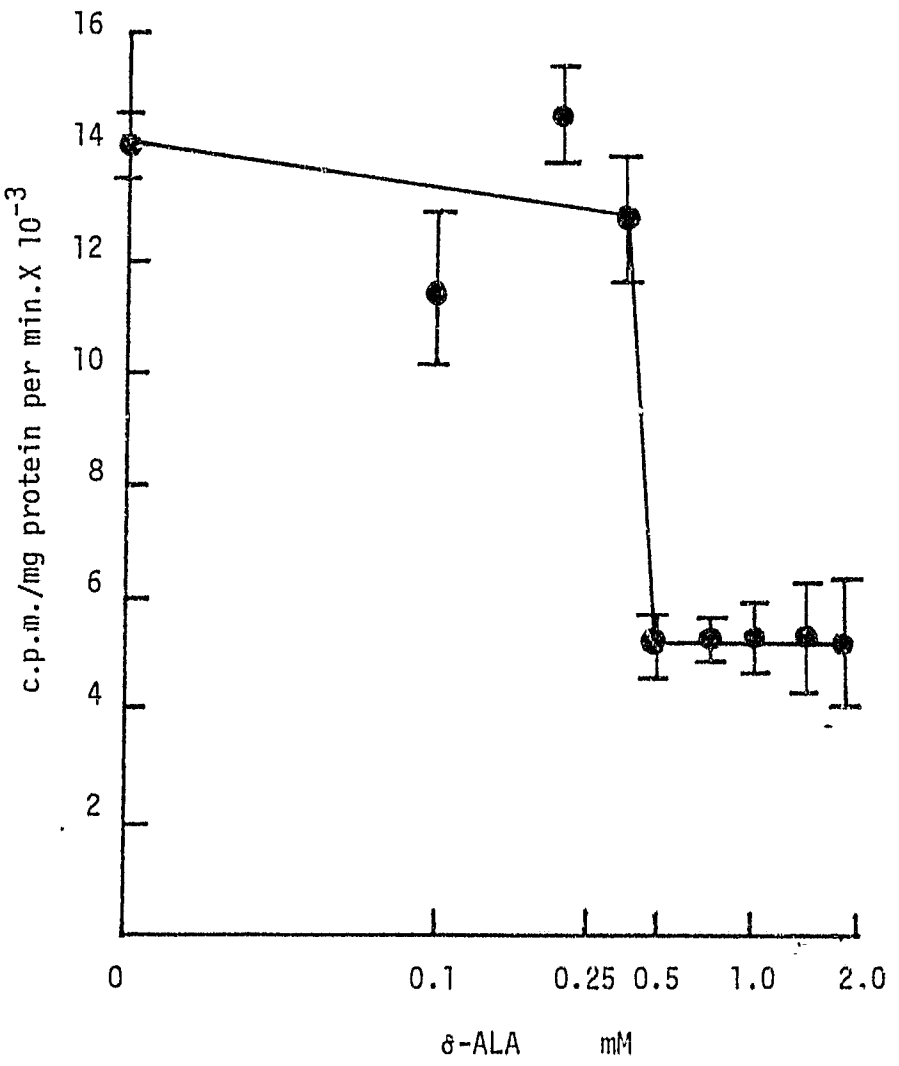

16
14
12
10
8
6
4
2
c.p.m./mg protein per min.X $10^{-3}$
0
0.1
0.25
0.5
1.0
2.0
δ-ALA mM

FIGURE 14.

Effect of eight increasing concentrations of δ-ALA on $^{3}$H-GABA uptake into the synaptosome fraction of rat cerebral cortex. Uptake was measured as described in 2.4.2. following preincubation in the presence of δ-ALA. The points are means ± s.e.m. of 4 independent experiments.

FIGURE 15.

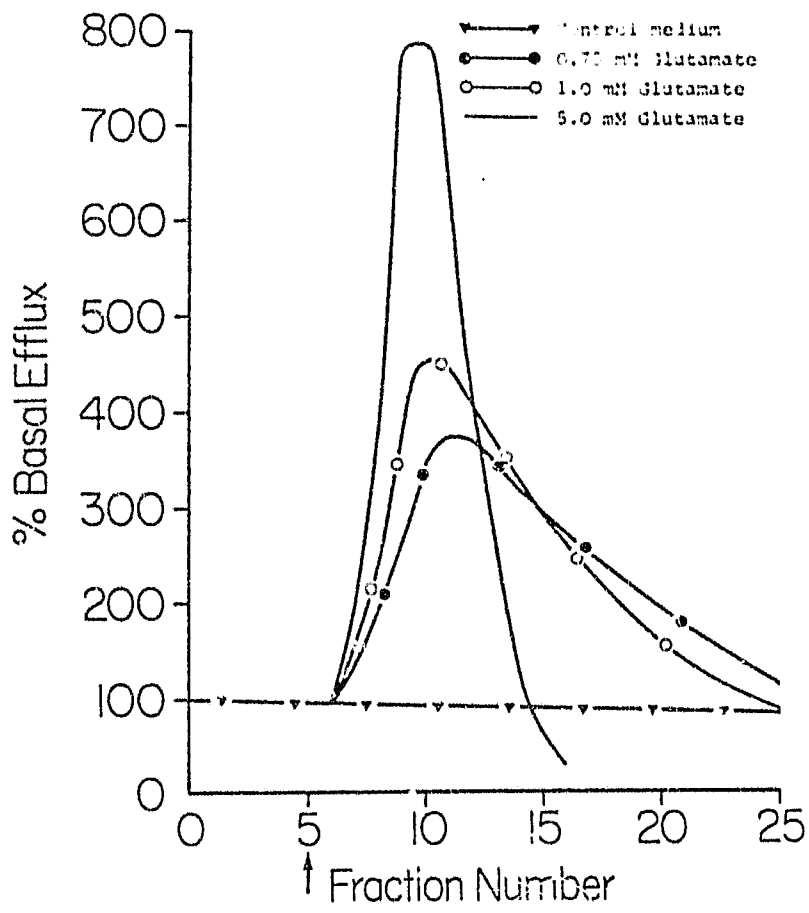

800
700
600
500
400
300
200
100
0
% Basal Efflux
0
5
10
15
20
25
Fraction Number
Control medium
0.75 mM Glutamate
1.0 mM Glutamate
5.0 mM Glutamate

FIGURE 15.

Stimulation of $^{14}C$-glutamate efflux from pre-loaded synaptosomes. Shown is the percentage change in efflux (baseline efflux = 100%) against fraction number. The arrow represents the change-over from plain medium to the test medium containing: ▼, control medium; ●, 0,75mM glutamate; ○, 1.0mM glutamate; —— 5.0mM glutamate. The first few fractions were very variable in each case and are not shown. Each curve is an average of 3 superfusion experiments. Variation between individual curves was less than 10% of the mean.

### 3.7.2. Effect of δ-ALA on $^{14}$C-glutamate efflux from preloaded synaptosomes.

High concentrations of δ-ALA (0.75-5.0mM) produced significant stimulation of $^{14}$C-glutamate efflux (Fig.16). No stimulation of efflux was obtained at δ-ALA concentrations of 0.5mM or lower. In addition, the presence of unlabelled glutamate in the superfusion medium elicited greater stimulation of efflux than did the corresponding concentrations of δ-ALA (compare Figs. 15 and 16). These findings contrast with the effects of δ-ALA on $^{3}$H-GABA efflux from preloaded synaptosomes (3.1.5.); here high concentrations of δ-ALA (0.75-5.0mM) produced very marked stimulation of $^{3}$H-GABA efflux, greater than that obtainable with similar concentrations of unlabelled GABA and the stimulation was still significant at a δ-ALA concentration of 0.1mM.

## 3.8. Potassium-stimulated release of glutamate from synaptosomes.

### 3.8.1. Effect of raised extracellular $K^+$ on $^{14}$C-glutamate release.

Membrane depolarisation induced by 55mM $K^+$ in the superfusion medium stimulated $^{14}$C-glutamate

FIGURE 16.

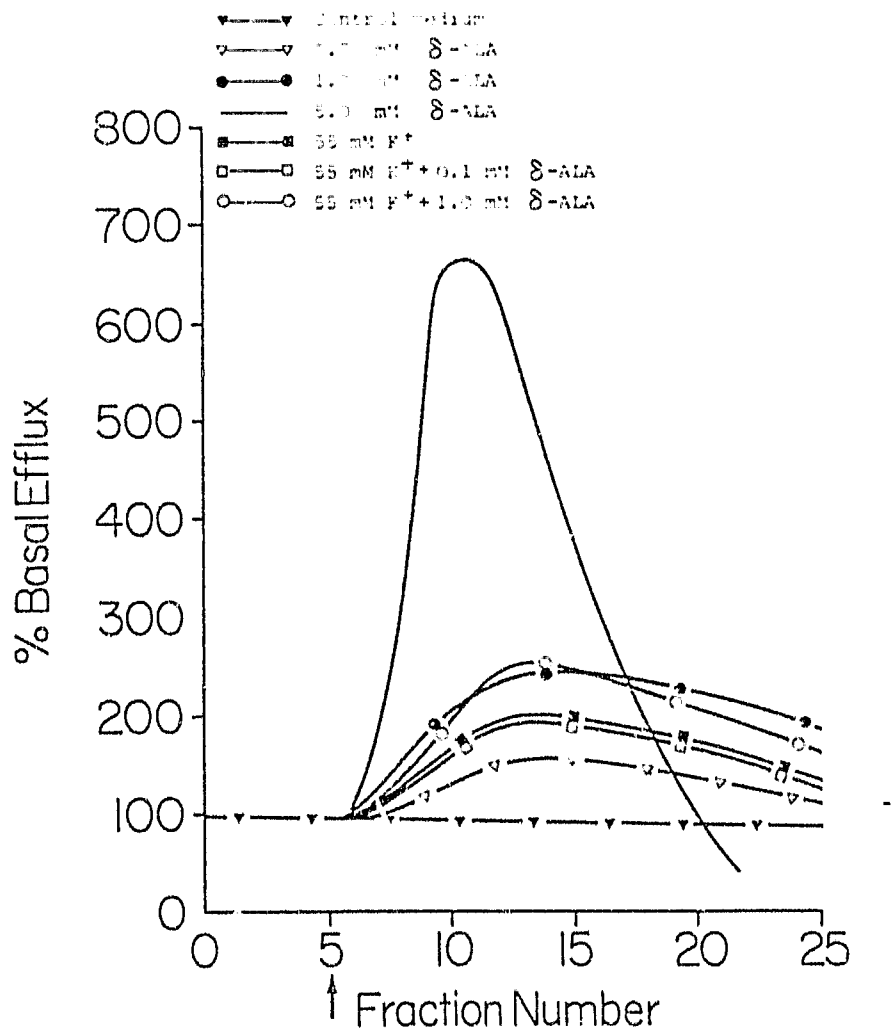

δ-ALA
δ-ALA
δ-ALA
55 mM K+
55 mM K+ + 0.1 mM δ-ALA
55 mM K+ + 1.0 mM δ-ALA
% Basal Efflux
800
700
600
500
400
300
200
100
0
0
5
10
15
20
25
Fraction Number

FIGURE 16.

Stimulation of $^{14}C$-glutamate efflux from preloaded synaptosomes. Shown is the percentage change in efflux (baseline efflux = 100%) against fraction number. The arrow represents the change-over from plain medium to the test medium containing: ▼, control medium; ▽, 0.75mM δ-ALA; ●, 1.0mM δ-ALA; —, 5,0mM δ-ALA; ■, 55mM $K^+$; □, 55mM $K^+$ and 0.1mM δ-ALA; ○, 55mM $K^+$ and 1.0mM δ-ALA. The first few fractions were very variable in each case and are not shown. Each curve is an average of 3 superfusion experiments. Variation between individual curves was less than 10% of the mean.

release by 105 ± 4% (mean ± s.e.m., n = 3) (Fig.16).

### 3.8.2. Effect of δ-ALA on $K^+$-stimulated $^{14}C$-glutamate.

δ-ALA at concentrations up to 1.0mM did not significantly affect the stimulation of $^{14}C$-glutamate release due to 55mM $K^+$ (Fig.16). This contrasts with the reduction of $K^+$-stimulated release of $^3H$-GABA produced by δ-ALA at low concentrations.

## 3.9. Uptake of $^{14}C$-glutamate by synaptosome suspensions.

Accumulation of $^{14}C$-glutamate by synaptosomes was measured over a range of concentrations from 0.5-50μM as described in 2.2.3. An apparent Km of 9.8μM was calculated from the data shown in the Eadie-Hofstee plot (Fig.17). There is some disparity in the Km values reported for glutamate transport in various nerve tissue preparations. Levi and Raiteri (1973) obtained only a low affinity component (Km = 0.4mM) for uptake into brain slices from adult rats, but observed a high affinity uptake process for glutamate (Km = 31 M) in 'mini-slices' or prisms.

FIGURE 17.

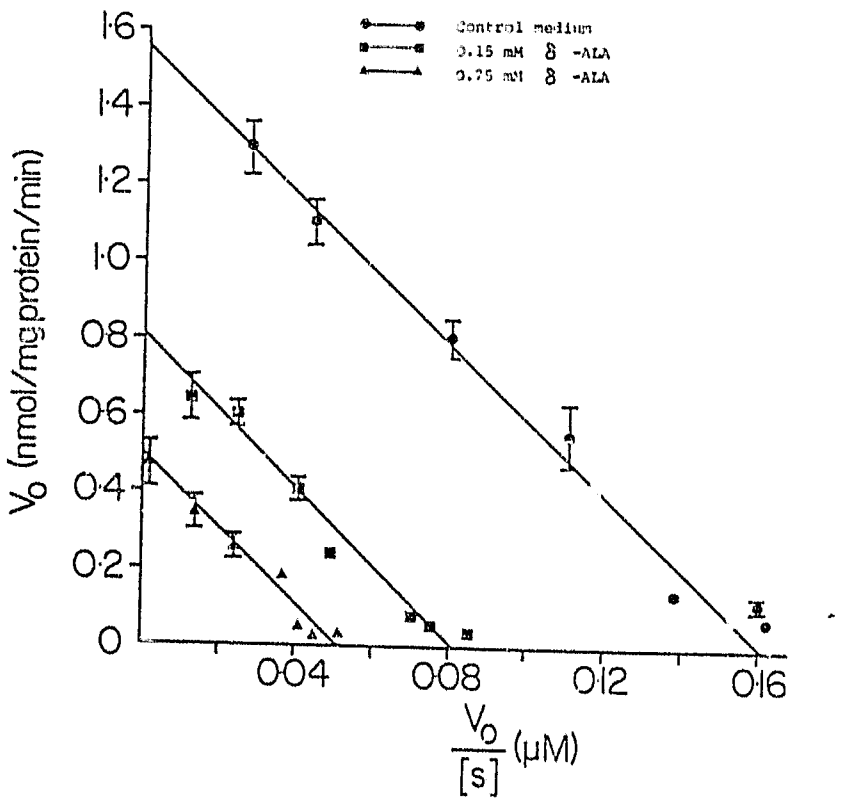

Control medium
0.15 mM δ -ALA
0.75 mM δ -ALA
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
V0 (nmol/mg protein/min)
0·04
0·08
0·12
0·16
V0/[s] (μM)

FIGURE 17.

Kinetic analysis of the uptake of labelled glutamate into rat cerebral synaptosomes. The uptake was measured as described in 2.4:3. after a 10 minute incubation with varying concentrations of $^{14}$C-glutamate. In experiments examining the effect of δ-ALA on uptake of $^{14}$C-glutamate the same effect was observed when synaptosomes were preincubated in the presence of δ-ALA and when δ-ALA was added simultaneously with $^{14}$C-glutamate after preincubation. In this Eadie-Hofstee plot, the uptake rate ($V$) is plotted as a function of the uptake rate over the glutamate concentration ($V/[s]$). Each point is the mean of at least 8 experiments and the lines represent the best fit to the data by the method of least squares. Standard error bars are shown where these are greater than the size of the points. ●, control medium; ■, 0.15mM δ-ALA; ▲, 0.75mM δ-ALA.

A Km value of 20μM has been reported for glutamate uptake into rat cortex slices (Balcar and Johnston, 1972a) and one of 29.8μM for uptake into the crude synaptosomal fraction from rat cortex (Takagaki, 1978).

### 3.10. Effect of δ-ALA on synaptosomal $^{14}$C-glutamate uptake.

The effect of δ-ALA on $^{14}$C-glutamate accumulation was examined over a range of concentrations up to 2.0mM (Fig.18). The presence of δ-ALA markedly inhibited uptake of $^{14}$C-glutamate, and the inhibition was maximal at a δ-ALA concentration of 2.0mM. The same effect was observed when synaptosomes were preincubated in the presence of δ-ALA and when δ-ALA was added simultaneously with $^{14}$C-glutamate after preincubation. Kinetic analysis (Fig.17) showed that the inhibition was of a non-competitive type. There is a discrepancy in the values of the inhibition constant (Ki) calculated from the data of Fig.17. At a δ-ALA concentration of 0.15mM, a Ki of 170 M was obtained, while at a δ-ALA concentration of 0.75μM a value of 350μM was obtained. The reasons for this difference are not clear.

FIGURE 18.

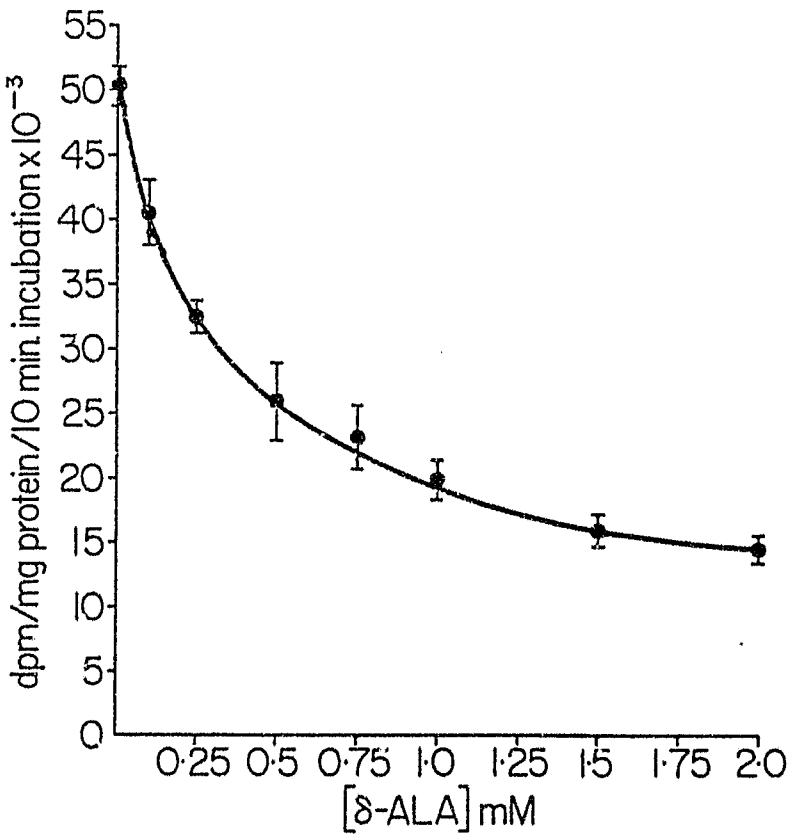

dpm/mg protein/10 min incubation x 10⁻³
55
50
45
40
35
30
25
20
15
10
5
0
0·25 0·5 0·75 1·0 1·25 1·5 1·75 2·0
[δ-ALA] mM

FIGURE 18.

Effect of seven varying concentrations of δ-ALA on $^{14}$C-glutamate uptake (0.5μM) into the synaptosome fraction of rat cerebral cortex. Uptake was measured as described in 2.4.3. following preincubation in the presence of δ-ALA. The points are means ± s.e.m. of 4 independent experiments.

## 3.11. Uptake of $^{14}$c-δ-ALA by synaptosome suspensions.

Synaptosomes were incubated in the presence of a range of concentrations of $^{14}$C-δ-ALA (0.5-100 M) for a range of time periods (10-30 minutes) as described in 2.4.6. There was no detectable accumulation of radioactivity by the synaptosomes when compared to control experiments accounting for nonspecific binding to the Millipore filters.

# CHAPTER 4

# DISCUSSION

## 4.1. Stereospecificity of the neuronal GABA transport mechanism.

An important problem in elucidating the precise roles of GABA in the mammalian brain is the existence of multiple pools of GABA which makes it difficult to determine the origin of GABA released during experiments designed to demonstrate transmitter release after nervous stimulation. For example , it is clear that, in many areas of the brain, GABA is accumulated by glial cells in addition to neurones, while in the peripheral nervous system, GABA uptake appears to be a property of glial cells exclusively (Schon and Kelly, 1974a,b). It is thus not possible to specifically label neuronal GABA pools utilizing uptake processes in heterogeneous tissues. Although the transport processes for GABA in neurones and glia have remarkably similar properties, the use of GABA analogues as probes for the stereospecificity of these processes has yielded valuable information regarding the constraints imposed on substrates for the systems.

Clear differences have been demonstrated between the neuronal and glial systems in the mammalian CNS and hopefully these will be useful in the labelling of specific pools of GABA.

Much of the information on stereospecificity of transport has been derived by examining the effects of analogues on (a) high affinity GABA uptake, and (b) release of GABA from various brain preparations by heteroexchange diffusions. Raiteri et al., (1975) tested a wide range of substances including glutamate, taurine, phenylalanine, acetylcholine, noradrenaline and 5-hydroxytryptamine and found them to have no effect on the release of $^{3}$H-GABA from rat brain synaptosomes up to a concentration of $10^{-3}$M; only unlabelled GABA and γ-amino-β-hydroxybutyric acid stimulated release to any appreciable extent. To this list may be added the GABA analogue L-2,4-diaminobutyric acid (DABA) but not its 5-carbon chain analogue L-ornithine nor the sulphur containing amino acids L-homocysteine and L-methionine (Fig.2). DABA is almost as effective as unlabelled GABA itself in stimulating $^{3}$H-GABA release from rat brain synaptosomes, while β-alanine, a 3-carbon

chain analogue of GABA, has only a slight stimulatory effect on neuronal GABA release (Fig.2 and see also Raiteri et al., 1975).' DABA is a potent inhibitor of GABA uptake in rat cortical slices while β-alanine has little inhibitory effect · (Iversen and Johnston, 1971); the reverse is true in the case of GABA uptake into the glial cells of rat dorsal root ganglia, β-alanine being a much more potent inhibitor of this process than DABA (Schon and Kelly, 1974b). β-Alanine produces a marked stimulation of GABA efflux from peripheral glial cells, while DABA is less effective in stimulating release (Minchin, 1975). These results, plus the observation that DABA causes a marked efflux of GABA from rat cortical slices in which GABA is localised predominantly in nerve terminals (Bloom and Iversen, 1971; Iversen and Bloom, 1972) while β-alanine does not (Hammerstad and Lytle, 1976) provides strong evidence for the suggestion that DABA exchanges primarily with neuronal pools of GABA and β-alanine with the glial pool.

The above results suggest that the length of the hydrocarbon chain is of importance in determining the degree to which an amino acid analogue of

of GABA is exchanged with GABA by the neuronal transport system, since the reduction or elongation of the chain by one methylene group reduces or totally abolishes exchange.

Recently it has been shown that a conformationally restricted structural analogue of GABA, cis-1,3-aminocyclohexanecarboxylic acid (ACHC), which inhibits GABA transport in slices of cerebral cortex in a linearly competitive fashion (Beart et al., 1972) selectively inhibits neuronal transport of GABA and has little or no effect on transport of GABA into glial cells (Bowery et al., 1976; Neal and Bowery, 1977). It is also capable of producing a net increase in the efflux of GABA from neuronal but not glial GABA pools (Bowery et al., 1976). A similar picture has emerged with respect to (-)nipecotic acid and its analogues guvacine (1,2,5,6-tetrahydropyridine-3-carboxylic acid) and (±)-cis-4-hydroxynipecotic acid. These compounds are all potent and substrate-competitive inhibitors of GABA uptake in rat cortical slices (Johnston et al., 1975; Krogsgaard-Larsen and Johnston, 1975; Johnston et al., 1976 a,b; Krogsgaard-Larsen, 1978).

The mechanism of inhibition of GABA uptake by these compounds appears to be identical to that of DABA; all act as non-competitive inhibitors when preincubated with the tissue and as competitive inhibitors when added at the same time as GABA (Simon and Martin, 1973; Johnston et al., 1976a). Interestingly, there are no significant regional or species differences in the action of these inhibitors on GABA uptake (Lodge et al., 1976). The structural specificity of the GABA uptake system has been further elucidated via structure-activity studies on a number of compounds related to nipecotic acid. Very minor alterations in structure render the compounds inactive with respect to GABA uptake (Krogsgaard-Larsen, 1978). Based on these results, it may be concluded that ACHC, nipecotic acid, guvacine and cis-4-hydroxynipecotic acid reflect the conformation of GABA in which it is transported by its carrier. This conformation is clearly different to that in which GABA interacts with its receptors since muscimol, PSA, isoguvacine and THIP, all potent GABA receptor agonists, are very weak or inactive with respect to GABA uptake (Beart et al., 1972; Krogsgaard-Larsen 1978).

In addition, only muscimol stimulates $^{3}$H-GABA efflux from preloaded synaptosomes to any significant extent (Fig.8), PSA, isoguvacine and THIP being completely inactive.

In aqueous solution, ACHC is likely to exist as the zwitterionic form and, from pK measurements, it has been concluded that the molecule exists predominantly in the diequatorial conformation (Hewgill and Jeffries, 1955). In this conformation, the compound represents a partially restricted, partially folded analogue of GABA where the separation of zwitterionic centres (mean of the two N...O distances) is approximately 5.6A. The corresponding distance observed in the crystal structure of β-alanine is approximately 3.8A (Jose and Paut, 1965). As discussed above, β-alanine inhibits mainly glial uptake of GABA, while nipecotic acid is more than an order of magnitude more potent as an inhibitor of neuronal GABA uptake (Johnston et al., 1976a). It would seem therefore that the selectivity of inhibitors of neuronal GABA uptake is probably related to the separation of their zwitterionic centres and that the upper limit of this separation for

interaction at glial uptake sites is lower than that at neuronal uptake sites (Bowery et al., 1976). This is further supported by the fact that 4-aminotetrolic acid, in which the zwitterionic centres have a minimum separation of 5.5A, is also a selective inhibitor of neuronal GABA uptake (Bowery et al., 1976). Nipecotic acid, like ACHC, also adopts the chair conformation in both the solid state and aqueous solution, but has a smaller separation of charged centres of approximately 4.5 (Bowery et al., 1976). It interacts with both neuronal and glial transport sites for GABA, although much more strongly with the neuronal site (Johnston et al., 1976a). Whatever the lower limit of separation of zwitterionic centres may be, the upper limit imposes a very narrow range on the system since L-ornithine, the 5-carbon chain analogue of DABA, is completely inactive with respect to heteroexchange with $^{3}$H-GABA in synaptosomes (Fig.2).

## 4.2. Effect of δ-ALA on amino acid transmitter transport.

The porphyrin precursor δ-aminolaevulinic acid (δ-ALA) is an omega amino acid with a 5-carbon chain similar in structure to GABA.

interaction at glial uptake sites is lower than that at neuronal uptake sites (Bowery et al., 1976). This is further supported by the fact that 4-aminocrotonic acid, in which the zwitterionic centres have a separation of 5.5A, is also a selective inhibitor of neuronal GABA uptake (Bowery et al., 1976). Nipecotic acid, like ACHC, also adopts the chair conformation in both the solid state and aqueous solution, but has a smaller separation of charged centres of approximately 4.5A (Bowery et al., 1976). It interacts with both neuronal and glial transport sites for GABA, although much more strongly with the neuronal site (Johnston et al., 1976a). Whatever the lower limit of separation of zwitterionic centres may be, the upper limit imposes a very narrow range on the system since L-ornithine, the 5-carbon chain analogue of DABA, is completely inactive with respect to heteroexchange with $^{3}$H-GABA in synaptosomes (Fig.2).

## 4.2. Effect of δ-ALA on amino acid transmitter transport.

The porphyrin precursor δ-aminolaevulinic acid (δ-ALA) is an omega amino acid with a 5-carbon chain similar in structure to GABA.

It fits within the guidelines of McGeer et al., (1961) as a GABA analogue. Consequently, the effect of δ-ALA on GABA transport in rat brain synaptosomes was examined. At high concentrations (0.75mM and above) δ-ALA produced marked stimulation of $^{3}$H-GABA efflux from preloaded synaptosomes (Fig.3). This stimulation exceeded the maximum obtainable by homoexchange with unlabelled GABA (Fig.2). At lower concentrations of δ-ALA (0.1-0.5mM) the stimulation was lower than that elicited by similar concentrations of unlabelled GABA.

δ-ALA has been shown to inhibit the $(Na^{+}+ K^{+})$-ATPase isolated from red blood cells and from rabbit brain although it does not inhibit the $(Na^{+}+ K^{+})$-ATPase in intact red cell "ghosts" (Becker et al., 1971). Inhibition of the synaptosomal $(Na^{+}+ K^{+})$-ATPase by $10^{-4}$ M ouabain produces approximately a 100% increase in $^{3}$H-GABA efflux (Raiteri et al., 1975); consequently, it is not possible that the large efflux of $^{3}$H-GABA elicited by high concentrations of δ-ALA is due solely to inhibition of the $(Na^{+} + K^{+})$-ATPase.

δ-ALA produces maximum inhibition of $^{3}$H-GABA uptake at a concentration of 0.5mM (Fig.14) although the very strong stimulatory effect of δ-ALA on $^{3}$H-GABA release only becomes evident at concentrations of 0.75mM and above; there is thus some disparity between the concentration at which δ-ALA inhibits $^{3}$H-GABA uptake and that at which it markedly stimulates release. Nevertheless, the fact that δ-ALA competitively inhibits $^{3}$H-GABA uptake into synaptosomes (Fig.13) suggests that a part of its strong stimulatory effect might be mediated by an exchange between synaptosomal $^{3}$H-GABA and external δ-ALA, although the magnitude of the effect at high δ-ALA concentrations cannot be explained in terms of the GABA transport mechanism alone or by inhibition of synaptosomal $(Na^{+} + K^{+})$-ATPase or by a summation of the two.

The effects of δ-ALA on amino acid transport processes in nerve endings are not specific for the GABA system. Thus, high concentrations of δ-ALA (0.75mM and above) produced marked stimulation of $^{14}$C-glutamate efflux from preloaded synaptosomes (Fig.16). In this case however, the stimulation was lower than that produced by equivalent concentrations of unlabelled glutamate (Fig.15).

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The effects of δ-ALA on amino acid transport processes in nerve endings are not specific for the GABA system. Thus, high concentrations of δ-ALA (0.75mM and above) produced marked stimulation of $^{14}$C-glutamate efflux from preloaded synaptosomes (Fig.16). In this case however, the stimulation was lower than that produced by equivalent concentrations of unlabelled glutamate (Fig.15).

In addition, uptake of $^{14}$C-glutamate into nerve endings was inhibited by δ-ALA (Fig.18) in a non-competitive fashion (Fig.17).

The finding that δ-ALA itself is not accumulated by synaptosomes suggests that δ-ALA-stimulated release of transmitter amino acids is unlikely to be mediated via an exchange mechanism, since exchange of both GABA and glutamate is associated with the high-affinity transport systems for these compounds (Levi and Raiteri, 1974; Simon et al., 1974; Ryan and Roskoski, 1977).

In view of the relatively high concentrations of δ-ALA required to produce effects on transmitter uptake and release, and the lack of specificity of the compound with respect to the transmitter, it is possible that δ-ALA exerts its effects via some nonspecific metabolic activity. Alternatively, since δ-ALA resembles both GABA and glutamate structurally, it is possible that δ-ALA binds to or near the external components of the transport sites and inhibits amino acid transport but is not itself translocated across the membrane. Such suggestions are entirely speculative.

4.3. δ-ALA is an agonist at GABA autoreceptors.

δ-ALA has been shown to decrease the membrane resistance of the crayfish stretch receptor neuron, probably by increasing the $Cl^-$ conductance of the membrane (Dichter et al., 1977). In this respect it mimics the postsynaptic action of GABA (Takeuchi and Takeuchi, 1966). Since the state of depolarisation of the synaptosomal membrane can directly control the release of GABA in the presence of $Ca^{2+}$ by controlling $Ca^{2+}$ influx (Cotman et al., 1976), it was decided to investigate the effect of δ-ALA on $^3$H-GABA release induced by a depolarising concentration of $K^+$ (55mM). δ-ALA reduced the $K^+$-stimulated release of $^3$H-GABA from the nerve endings in a dose-dependent fashion (Fig.5). The reduction was significant at a δ-ALA concentration of 1μM, and at a concentration of 100μM, 75% of the stimulated release was abolished. This effect of δ-ALA was specific for GABA release, since δ-ALA at concentrations up to 1.0mM did not affect $K^+$-stimulated release of $^{14}$C-glutamate from the same preparation (Fig.16). In all cases the reduction of $K^+$-stimulated GABA release induced by δ-ALA was prevented by bicuculline and picrotoxin (Table 1).

This finding that a compound can alter GABA release in an appropriate fashion and that the effect is prevented by known GABA receptor antagonists is evidence for the existence of presynaptic GABA receptors (GABA autoreceptors) capable of regulating GABA release through a negative feedback control pathway. Similar control mechanisms are thought to occur in adrenergic (Farnebo and Hamberger, 1971; Langer et al., 1972; Starke and Montel, 1973; De Potter et al., 1974; Dismukes and Mulder, 1976; Langer, 1978), dopaminergic (Starke and Montel, 1973; Carlsson, 1975; Roth et al., 1975; Seeman and Lee, 1975; Iversen et al., 1976) and cholinergic (Szerb and Somogyi, 1973), neurones. Recently, evidence supporting the existence of GABA autoreceptors has been published (Mitchell and Martin, 1978; Snodgrass, 1978).

## 4.4. A mechanism for the neural dysfunction in acute porphyria.

Acute attacks of the hereditary hepatic porphyrias are frequently accompanied by neuropsychiatric symptoms.

The presentation of nervous system involvement varies; in decreasing order of frequency, there may be motor neuropathy, confusion, psychiatric manifestations, hyperexcitability and epileptic-type seizures (Eales, 1963). Psychiatric manifestations include depression, anxiety, insomnia and an organic brain syndromes (Tschudy, 1974). Although the actiology of the neural dysf[illegible] the acute attack is unknown, sever[illegible]nisms may be suggested at a neuro-chemical level. It is possible that porphyrin precursors, which are produced in excess by the liver during acute episodes, gain access to the nervous system and exert direct neurotoxic effects; alternatively, a metabolic defect in haem biosynthesis in nerve cells might result in a functional haemoprotein deficiency which becomes critical at the time of the acute attack.

In support of the first hypothesis, there is good evidence, from the case reports of many patients in acute porphyric episodes, that the onset of neurological symptoms is accompanied by raised excretion of δ-ALA (Becker, 1972). In addition, there appears to be a rough correlation between the level of precursor excretion and clinical severity (Ackner et al., 1961).

A striking feature of the hepatic porphyrias is the absence of porphyrin precursors during the latent phase of the condition as opposed to high levels of δ-ALA present in the blood and urine during the acute phase with decreased excretion always accompanying or preceding clinical improvement (Eales et al., 1963; 1971).

Early studies on the possible neurotoxicity of δ-ALA conducted in vivo or on isolated organs have been subject to some controversy. Several of these investigations have suggested that δ-ALA is pharmacologically inert (Goldberg et al., 1954; Jarrett et al., 1956; Marcus et al., 1970; Shanley et al., 1972). However, these studies can now be criticized on a number of points. In the experiments with isolated organs, very low levels of porphyrin precursors were tested against limited physiological parameters (Goldberg et al., 1954; Jarrett et al., 1956). The in vivo experiments (Marcus et al., 1970; Shanley et al., 1972) were conducted over a relatively short period of time, the levels of δ-ALA were not measured in the plasma, and neither the uptake into the CNS nor the rate of clearance by the kidneys was taken into account.

In other studies conducted *in vivo*, evidence for neurotoxic effects has been reported. Experiments in which the porphyrinogenic drug allylisopropylacetamide was administered to rats, resulting in increased excretion of δ-ALA, have shown effects such as deep sleep (Biempica *et al.*, 1967) weakness and ataxia involving the rear legs (Yuwiler *et al.*, 1970), increased susceptibility to isonicotinyl hydrazide-induced convulsions (Kosower and Rock, 1968) and acute flaccid paresis in pantothenate deficient rats (De Matteis and Rimington, 1962). In addition, recent work in which δ-ALA was administered chronically and acutely to rats and mice (McGillion *et al.*, 1973) or injected directly into the CNS of rats (Shanley et al., 1975) has demonstrated convincing behavioural effects including convulsions.

In addition to these behavioural effects, the mechanism of which is unknown, δ-ALA has been shown to have a wide variety of pharmacological actions *in vitro*. Thus, δ-ALA inhibits transmitter release at the neuromuscular junction (Feldman *et al.*, 1968; 1971), inhibits ventral and dorsal root responses and depresses dorsal root potentials in the frog spinal cord

(Loots et al., 1975), and depolarises frog muscle fibres (Becker et al., 1975). These findings may be explicable on the basis of a nonspecific metabolic action of δ-ALA such as inhibition of the $(Na^+ + K^+)$-ATPase (Becker et al., 1971).

In two recent independent studies, δ-ALA has been shown to mimic the postsynaptic action of GABA on the crayfish stretch receptor neurone (Dichter et al., 1977) and on motoneurons and primary afferents in the frog spinal cord (Nicoll, 1976). The spontaneous activity in the frog spinal cord was extremely sensitive to δ-ALA which was only slightly less effective than GABA in inhibiting spontaneous activity. To date, there has been no systematic study of the effects of δ-ALA on GABAergic and glutamergic systems in the mammalian CNS.

In this study, it has been shown that δ-ALA is capable of affecting the uptake and efflux of GABA and glutamate in nerve endings in vitro; these effects occur only at relatively high concentrations of δ-ALA (at least 100μM).

In an impressive series of experiments, Shanley et al., (1975) demonstrated that δ-ALA does not pass the blood-brain barrier of experimental animals in anything except trace amounts. The highest levels of δ-ALA recorded in the cerebrospinal fluid (CSF) of patients during acute porphyric attacks are in the range 16-20μM (Sweeney et al. 1970). At these low concentrations, δ-ALA could not be expected to produce any effects on amino acid transmitter transport systems.

Experiments described here have, however, demonstrated that δ-ALA can inhibit the stimulated release of GABA probably by acting as an agonist at GABA autoreceptors. This effect is evident at low concentrations of δ-ALA ($IC_{50}$ = 11.5μM) which are well within the range of concentrations obtainable in the CSF of experimental animals (McGillion et al., 1974) and those recorded in the CSF of patients during acute attacks of porphyria characterized by hyperexcitability and convulsions (Sweeney et al., 1970). In this regard, it is noteworthy that a functional deficiency of GABA has been implicated in the pathogenesis of epileptic convulsions (Van Gelder

Courtois, 1972; Van Gelder et al., 1972; Gibson and Joseph, 1975; Ribak et al., 1979).

Although the hyperexcitability and convulsions noted in patients with the highest levels of δ-ALA in the CSF are explicable solely on the basis of reduced GABA-mediated inhibition in the CNS, we cannot explain the entire specturm of nervous system involvement in porphyria on such a basis. The disease has features of a chronic degenerative disorder in which central demyelinating lesions (Gibson and Goldberg, 1956) and primary axonal damage (Sweeney et al., 1970) may be noted. I would like to propose that the symptomatology of the acute attack is explicable in some measure by a direct neurotoxic mechanism involving δ-ALA and that this is superimposed on an underlying metabolic defect (as yet undefined) which accounts for the degenerative features of the disease.

## 4.5. GABA autoreceptors.

### 4.5.1. Pharmacology of GABA autoreceptors.

As a result of the work described above and that of Mitchell and Martin (1978) and

Snodgrass (1978) the existence of receptors capable of exerting an inhibitory effect on GABA release has been firmly established. Since, in the adrenergic and dopaminergic systems, classical postsynaptic receptor agonists and antagonists have varying degrees of affinity for the corresponding autoreceptor, it is likely that similar differences in affinity for agonists and antagonists will differentiate pre- and post-synaptic GABA receptors. In the studies described here no differences could be detected in the potencies of five agonists in inhibiting GABA release and inhibiting high affinity $Na^+$-independent $^3H$-GABA binding to synaptic membranes (Table 4). In addition, GABA autoreceptors were shown to be bicuculline- and picrotoxin-sensitive (Tables 1 and 2). It would thus appear that the autoreceptors are identical pharmacologically with the sites labelled by $^3H$-GABA or $^3H$-muscimol (Snodgrass, 1978) in synaptic membranes, at least as far as the compounds tested are concerned.

Recently, it has been suggested that two classes of bicuculline-sensitive GABA binding sites exist in the CNS (Braestrup et al., 1979; Karobath et al., 1979). One of these is coupled to a benzodiazepine receptor and may differentiate a continuum of pure and partial GABA agonists, while the other, which does not activate benzodiazepine binding, possesses no such selectivity (Braestrup et al., 1979). It is noteworthy here that the inhibition of GABA release induced by GABA agonists is evident in fresh tissue; this implies that the site is free to interact with the ligands and is not masked by the endogenous inhibitor of GABA binding described by Guidotti et al., (1978) and Toffano et al., (1978).

Since benzodiazepines compete with the modulator for binding to its site, (Guidotti et al., 1978) it appears that those GABA receptors which are modulated by the inhibitor are also those linked to the benzodiazepine modulator binding activity. This would suggest that the GABA autoreceptors correspond to those GABA receptors not linked to benzodiazepine receptors and hence not subject to inhibitor modulation.

The situation is further complicated by a very recent description of bicuculline-insensitive GABA receptors present on the terminals of neurones in the peripheral nervous system (Bowery and Hudson, 1979; Brown and Higgins, 1979) and CNS (Bowery et al., 1980). Activation of these receptors results in an inhibition of the stimulated release of noradrenaline from rat atria and acetylcholine from preganglionic terminals in the rat superior cervical ganglion (Bowery and Hudson, 1979; Brown and Higgins, 1979) and of noradrenaline, dopamine and 5-hydroxytryptamine from brain slices (Bowery et al., 1980). These effects are not prevented by bicuculline or other GABA antagonists. In addition, the GABA agonist 3- aminopropane sulphonic acid which is at least as active as GABA at bicuculline-sensitive sites (Curtis et al., 1961; Bowery and Brown, 1974; Mohler and Okada, 1978) is inactive at this receptor (Bowery and Hudson, 1979; Brown and Higgins, 1979; Bowery et al., 1980);

by contrast, (-) baclofen, which is inactive at bicuculline-sensitive sites (Curtis et al., 1974; Fox et al., 1978), is as active as GABA in reducing st mulated transmitter output (Bowery et al., 1979).

Thus, there are two classes of bicuculline - sensitive GABA sites - the conventional post-synaptic receptor and the autoreceptor. As far as is known these are identical pharmacologically, although the former is probably coupled to a benzodiazepine receptor while the latter is not. In addition, there are presynaptic GABA receptors on adrenergic, dopaminergic, cholinergic and serotoninergic nerve terminals. These differ from the first two in that they are bicuculline-insensitive and show a different affinity for known GABA agonists to that shown by the conventional receptors.

### 4.5.2. Inhibition of GABA release is correlated with GABA receptor occupancy.

The data shown in Table 4 suggest a good absolute correlation between receptor occupancy by

agonist drugs and inhibition of stimulated GABA release. This is of great importance since it has not been possible previously to correlate an effect with occupancy of receptor sites mediating that effect. This demonstration provides evidence for the direct coupling of GABA autoreceptors to the effector mechanism - presumably a $Cl^-$ ionophore since the effect is blocked by picrotoxin - and shows further that this mechanism can be modified by drugs.

## 4.6. Conclusion

Strong evidence has been provided for the existence of GABA autoreceptors pharmacologically identical to the sites labelled by $^3$H-GABA in synaptic plasma membranes and widely assumed to represent the post-synaptic GABA receptor. The porhyrin precursor δ-ALA is a potent agonist at these autoreceptors and this mechanism may well explain some of the neural dysfunction characterising the acute porphyric attack. Other effects of δ-ALA on amino acid transport processes occur at concentrations outside

the range recorded in the CSF of patients during acute porphyric attacks. Further, it has been shown that there is a good correlation between receptor occupancy by a number of GABA agonists and inhibition of stimulated GABA release. This suggests that the receptor is directly linked to the effector mechanism - probably a $Cl^-$ ionophore.

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