Modulation of Adenylyl Cyclase by $GABA_B$ Receptors ~~in Rat Brain~~ *In Vitro* and *In Vivo*

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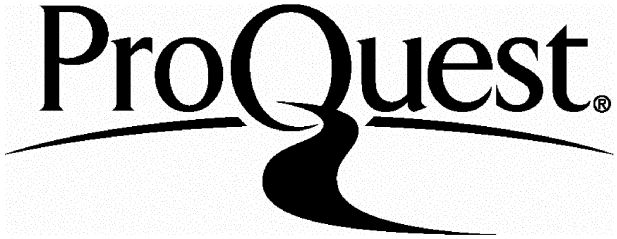
Antony R. Knight B.Sc.

Department of Pharmacology,
The School of Pharmacy,
29-39 Brunswick Square,
London, WC1N 1AX



Abstract

In previous studies it has been shown that forskolin and noradrenaline stimulate cAMP production in *in vitro* preparations of rat brain tissue, and that baclofen can inhibit the forskolin stimulation, and augment the noradrenaline stimulation. The purpose of the present study was to determine the pharmacological profile of this $GABA_B$ receptor mediated response, and investigate its physiological relevance by using *in vivo* microdialysis in awake freely moving rats, and by determining the class of cells in which the response occurs.

Thus, in a cross-chopped rat cortical slice preparation, it was confirmed that (-)Baclofen inhibited forskolin stimulated adenylyl cyclase activity and augmented noradrenaline stimulated adenylyl cyclase activity. The potency of five further agonists was investigated (SKF97541, CGP47656, CG44533, 3-APA and CGP44532). Of these agonists two compounds were significantly more potent as inhibitors of forskolin stimulated adenylyl cyclase than as augmenters of noradrenaline stimulated adenylyl cyclase activity, these were (-)baclofen (pEC_{50} of 6.07±0.29 and 5.04±0.17 respectively ($p<0.01$)), and CGP47656 (pEC_{50}=6.44±0.05 and 4.08±0.26 respectively ($p<0.01$)). The specificity of (-)baclofen and CGP47656 may be due to the low intrinsic activity of these compounds, and a relatively high receptor reserve for the $GABA_B$ receptor mediated inhibition of forskolin stimulated adenylyl cyclase activity. Six antagonists (CGP49311A, CGP46381, CGP45024, CGP35348, CGP45397, CGP36742) were also tested for their ability to antagonize 30 μM (-)baclofen. These ranged in potency in the forskolin assay from CGP49311A (pEC50 5.4±0.30) to CGP36742 (pEC50 3.87±0.16). Each compound had similar potency in the two assays.

In vivo microdialysis was used to investigate the ability of forskolin, noradrenaline and (-)baclofen to modulate adenylyl cyclase activity in the frontal cortex of rats. Forskolin (100 μM) administered through the dialysis probe (flow rate 2μl.min^{-1}, 30 min fractions). caused a significant ($p<0.01$) elevation in cAMP in dialysates. A two pulse protocol gave a control S2/S1 ratio of 7.3±0.25. (-)Baclofen (10 μM, 100 μM, 1 mM and 10 mM) was administered through the probe during the second forskolin pulse, however, the S2/S1 ratios in the presence of (-)baclofen did not differ significantly from control (1.38±0.37, 1.27±0.52, 1.14±0.13 and 3.66±1.92 respectively). Using different conditions (flow rate 1.2 μl min^{-1}, 10 min fractions) noradrenaline administered through the probe also caused a significant increase in cAMP concentration in dialysates ($p<0.05$), however a second pulse was ineffective. The inclusion of (-)baclofen in the perfusion medium during the first pulse increased the concentration of cAMP in dialysates, but the difference was not significant.

Experiments were also conducted to determine the relative contribution made to forskolin and noradrenline stimulated adenylyl cyclase activity by glial cells and neurons. Flourocitrate, a glial cell specific metabolic inhibitor, reduced forskolin stimulated adenylyl cyclase activity by 78% and eliminated the stimulation caused by noradrenaline in cross chopped cortical slices. In cultured glial cells both forskolin and noradrenaline caused large increases in cAMP production (306 fold and 93 fold respectively). However, these responses were not modulated by 100 μM (-)baclofen. These data suggest that most noradrenaline, and a proportion of forskolin and noradrenaline stimulated adenylyl cyclase activity may be associated with glial cells.

In addition, the long term effects of $GABA_B$ receptor activation and antagonism were investigated. Thus rats were dosed with (-)baclofen and the $GABA_B$ antagonists CGP46381 and CGP36742 for three weeks ((-)baclofen, 10 $mg.kg^{-1}day^{-1}$; antagonists 100 $mg.kg^{-1}day^{-1}$, IP). No regulation of $GABA_B$ receptor function, as assayed by modulation of adenylyl cyclase activity in frontal cortex, was detected. Autoradiography detected an up regulation of radioligand receptor binding in the lateral thalamic nucleus (115 % increase) in response to CGP46381, and CGP36742 caused an up regulation in the lateral dorsal thalamic nucleus (103 %), the substantia nigra (156%) and the CA3 pyramidal cell layer of the hippocampus (68%). (-)Baclofen caused a complex spectrum of up and down regulation in different brain regions which requires confirmation in a more detailed study.

Genetic Absence Epilepsy Rats from Strasbourg are a strain of rats susceptible to absence seizures. An autoradiographic study of the brains of these animals revealed no differences in the levels of radioligand binding to $GABA_A$ and $GABA_B$ receptors between these animals and controls. This suggests that an alteration in these receptor populations might not underlie this genetic condition.

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Table of Abbreviations.

3-APA	3-aminopropyl phosphinic acid
5-HT	5-hydroxytryptamine
ACSF	artificial cerebrospinal fluid
ANOVA	analysis of variance
B_{max}	total number of drug binding sites
CNS	central nervous system
cAMP	cyclic adenosine 3',5'-monophosphate
EPSP	excitatory post-synaptic potential
pEC_{50}	negative logarithm of the half maximally effective molar concentration
EEG	electro-encephalograph
G	constant of gravitation
g	gram
G-protein	GTP binding protein
GABA	gamma aminobutyric acid
GAD	L-glutamate decarboxylase
GAERS	genetic absence epilepsy rats from Strasbourg
GDP	Guanosine diphosphate
GDP-β-S	Guanosine 5,-O-(2-thiodiphosphate)
GTP	Guanosine triphosphate
GTP-γ-S	Guanosine 5'-0-13-thiotriphosphate
Hz	Hertz
id	internal diameter
ip	intra-peritoneal
IPSP	inhibitory post-synaptic potential

IBMX	3-isobutyl-1-methylxanthine
K_d	dissociation rate constant at equilibrium
KRB	Krebs Ringer buffer
M	molar
m	meters
MW	molecular weight
mCi	millicurie
min	minutes
ml	millilitres
mol	mole
mV	millivolt
od	outer diameter
°C	degrees Centigrade
pA_2	negative logarithm of the the molar concentration of antagonist required to give a two fold shift in the agonist dose response curve
pH	negative logarithm of the molar concentration of hydrogen ions
rpm	revolutions per minute
s	second
SEM	standard error of the means
THIP	4,5,6,7-tetrahydro isoxazolo[4,5-c]pyridin-3-ol

Publications Derived Form This Work

Papers

Knight, A.R. and Bowery, N.G. (1992) $GABA_B$ receptors in rats with spontaneous generalized non-convulsive epilepsy. J. Neural. Transm. 35 (Suppl.), 189-196.

Bowery , N.G., Knight, A.R. and Malcangio, M. (1993) $GABA_B$ receptors as therapeutic targets, in *Advances in Neuropharmacology* (F.C. Rose, ed) Smith Gordon, London. pp 217-233

Abstracts

Knight, A.R. and Bowery, N.G. (1992) Nitric oxide is unlikely to be involved in mediating $GABA_B$ responses in the rat CNS. J. Pharmacy Pharmacol. 44 (Suppl.), 1093.

Knight, A.R., and Bowery, N.G. (1992) The effect of fluorocitrate on $GABA_B$ modulation of forskolin and noradrenaline stimulated adenylyl cyclase activity. Pharmacol. Communications 2, 144-145.

Knight, A.R. and Bowery, N.G. (1992) *In vitro* and *in vivo* effects of baclofen on cAMP production in rat cerebral cortex. Soc. Neurosci. Abstr. 486.2.

Bowery, N.G., Knight, A.R. and Malcangio, M. (1993) $GABA_B$ receptor function. J. Neurochem. S, 235 C.

1
Introduction

1.1. General Introduction

The neurotransmitter, γ-aminobutyric acid (GABA), is concentrated in central nervous system (CNS) tissue where it is found in concentrations 200-1000 fold greater than other classical neurotransmitters such as dopamine, noradrenaline, acetylcholine and 5-hydroxytryptamine (5-HT) (McGeer and McGeer, 1978). GABA appears to function as a neurotransmitter at up to 30% of all brain synapses (Bloom and Iversen, 1971), and almost all mammalian neurones are sensitive to its actions. GABAergic neurones are widely distributed, comprising both local neural networks and inter-regional pathways (Chase and Tamminga, 1979). GABA is thus a good candidate for the principal inhibitory neurotransmitter in the mammalian brain.

GABA in the CNS

GABA is the most abundant amino acid in brain tissue, were it is found in concentrations very much higher than elsewhere in the body (1 to 10 μmol/g wet weight (Baxter, 1970)). Despite being an amino acid, GABA is not incorporated into proteins, but nevertheless appears to have a role in cerebral metabolism. It is an intermediary in the "GABA shunt" which is linked to the tricarboxylic acid cycle. Thus GABA within in the CNS, is synthesised from L-glutamate, which in turn is produced from glucose via the tricarboxylic acid cycle. GABA is synthesized from L-glutamate by the enzyme L-glutamate decarboxylase (EC 4.1.1.15, GAD). This enzyme is

located almost exclusively in the CNS (Wu et al., 1978), with the highest levels being found in axon terminals. GABA is stored in synaptic vesicles, in axon terminals, and is released into the synaptic cleft under depolarizing conditions. It is removed from the synaptic cleft by an active transport system, which mediates its entry into presynaptic terminals, postsynaptic cells and surrounding glial cells. GABA is catabolized by GABA transaminase (4-aminobutyrate: 2 oxoglutarate transaminase; EC 2.6.1.19), which appears to be the key enzyme regulating the levels of GABA in the brain. Inhibitors of this enzyme, such as γ–vinyl-GABA, have been used both clinically and experimentally to raise GABA levels in the CNS. The product of GABA transaminase is succinic semialdehyde, which is in turn oxidized by succinic semialdehyde dehydrogenase (EC 1.2.1.24). This enzyme is located in mitochondria, where the resultant succinate can re-enter the tricarboxylic acid cycle (Cash et al., 1979).

It was first proposed that GABA might be an inhibitory neurotransmitter in the central and peripheral nervous systems of animals by Elliot (1959). This proposition, however, did not receive widespread support until the mid 1960's, when it was demonstrated that GABA hyperpolarized cortical neurones by virtue of an increase in their permeability to chloride ions (Krnjevic and Schwartz, 1966, 1967). This chloride current was found to have a similar reversal potential to electrically evoked inhibitory postsynaptic potentials (IPSPs)(Dreifuss et al., 1969; Krnjevic and Schwartz, 1966, 1967). Thus, it was proposed that naturally occurring IPSPs were mediated by endogenous GABA acting at a new class of cell surface receptor (Diamond, 1968). This determination of the mechanism of action of GABA, coupled to its abundance and

distribution, lead ultimately to the appreciation that GABA is a very widely used inhibitory neurotransmitter in the vertebrate CNS.

Multiple Types of GABA Receptor

Whilst GABA is of major significance within the brain, it is also present in the periphery. Organs where GABA and GAD have been found include the female reproductive system (Celotti et al., 1986), intestinal myenteric plexus (Miki et al., 1983), gallbladder (Saito et al., 1985), blood vessels (Hamel et al., 1981), urinary bladder (Kusunoki et al., 1984), kidney (Whelan et al., 1969) and pancreas (Okada et al., 1976). In the periphery, functional responses apparently due to GABA receptor activation, were demonstrated as long ago as 1958 (Hobbiger, 1958a; 1958b). Subsequent investigations into the presynaptic actions of GABA in isolated atria (on the terminals of postganglionic sympathetic nerves deriving from the superior cervical ganglion) revealed that stimulation of a population of GABA receptors inhibited the release of [^{3}H]-noradrenaline. Whilst this response was mimicked by GABA, it displayed a different pharmacological profile to classical GABA responses. For instance, it was insensitive to the classical GABA antagonists bicuculline (Bowery et al., 1981.), 4,5,6,7-tetrahydro isoxazolo[4,5-c]pyridin-3-ol (THIP) , isoguvacine and piperidine-4-sulphonic acid (Bowery and Hudson, 1979). This observation initiated the characterisation of two GABA receptor subtypes: the classical GABA receptor and the so-called bicuculline insensitive GABA receptor. Other seminal early findings concerning this bicuculline-insensitive GABA receptor were that functional responses were not dependent on the presence

of chloride (Bowery et al., 1981), and that these receptors were sensitive to the agonist (-)baclofen (4-amino-3-(p-chlorophenyl)-butyric acid) (Bowery et al., 1981), which was inactive at the classical GABA receptor (Curtis et al., 1974). The role of this new receptor as a neuromodulator affecting neurotransmitter release was confirmed in several isolated tissue preparations, including transmurally stimulated rabbit basilar artery (Anwar and Mason, 1982) and guinea pig ileum (Kleinrock and Kilbinger, 1987; Ong and Kerr, 1983). In each of these studies, (-)baclofen was able to modulate electrically-evoked functions, but not the application of exogenously applied agonists; this indicated a presynaptic action.

Bicuculline-insensitive GABA receptors were also demonstrated to have a neuromodulatory role in the CNS where the release of potassium-stimulated [^{3}H]-noradrenaline, [^{3}H]-dopamine and [^{3}H]-5-HT were all attenuated by (-)baclofen (Bowery et al., 1980; Schlicker et al., 1984; Gray and Green, 1987).

[^{3}H]-GABA and [^{3}H]-baclofen have been used for radioligand receptor binding assays in rat brain homogenates. These studies detected the presence of two GABA receptors, with different pharmacological specificities in the CNS (Hill and Bowery, 1981). These authors also designated the term $GABA_A$ for the bicuculline-sensitive receptor and $GABA_B$ for the bicuculline-insensitive site. The finding that (-)baclofen was consistently active at the $GABA_B$ receptor in both functional and radioligand binding studies has resulted in it becoming established as the prototypic agonist for $GABA_B$ receptor-mediated responses. In addition to possessing a different pharmacological profile, radioligand binding of

tritiated agonists to the $GABA_B$ receptor differed from binding to the $GABA_A$ receptor in a number of other ways. These included a sensitivity to the divalent cations, Mg^{2+} and Ca^{2+} (Bowery et al., 1983), Guanosine triphosphate (GTP) (Hill et al., 1984) and triton (Bowery et al., 1983). In a series of classic studies, radioligand binding to GABA receptors was achieved in slide mounted sections of rat brain. Autoradiographic images were thus produced, and the distribution of subtypes of GABA receptor within the brain was established (Bowery et al., 1984, 1987). These experiments highlighted a dramatic spatial separation between the two receptors. The clearest difference in the distribution of the subtypes of receptor was seen in the cerebellum, where $GABA_B$ receptors are found almost exclusively in the molecular layer and $GABA_A$ receptors are present predominantly in the granule cell layer (Wilkin et al., 1981). In the forebrain, both types of receptor were widely distributed, with $GABA_B$ receptors being particularly abundant in the thalamus and the superior colliculus (Bowery et al., 1984, 1987).

$GABA_A$ Receptors

Unlike $GABA_B$ receptors, $GABA_A$ receptors gate chloride channels (Adams and Brown, 1975; Blaxter and Carlen, 1987). Much information about the $GABA_A$ receptor has been accumulated, partially due to the early availability of naturally occurring drugs that act specifically and with high affinity at this receptor. These include compounds such as muscimol, a nanomolar potency agonist from the mushroom *Amanita muscaria* (Krogsgaard-Larsen et al., 1979), bicuculline, an alkaloid derived from

plants of the genera *Dicentra* and *Corydalis*, and the convulsant picrotoxin from the plant species *Anamirta cocculus*.

Molecular biological studies have shown that $GABA_A$ receptors are composed of 5 protein subunits arranged in the cell membrane to form a central ion pore. There are at least three classes of subunit, designated α, β and γ. Each of these exists in a number of different forms. Variation in the type and relative number of each subunit in the heteromeric receptor makes subcategories of $GABA_A$ receptor possible and the total number of variants may be vast.* The pharmacology of these receptors has been well described and includes not only the agonist binding site but also modulatory sites for benzodiazepines, barbiturates, convulsants such as picrotoxin, and anaesthetic steroids such as alphaxalone. The binding of GABA to the agonist recognition site causes the ionophore to open and this allows chloride ions to cross the cell membrane.

$GABA_A$ receptors are widely distributed, both centrally and peripherally (Bowery and Brown, 1974; Adams and Brown, 1975). They are found both pre- and postsynaptically, and in both locations $GABA_A$ receptors are considered to be inhibitory. Postsynaptically, they mediate a fast inhibitory postsynaptic potential (IPSP) by gating the chloride current. This current has a reversal potential of around -39 mV and may cause hyperpolarization, or depolarization depending upon the resting potential of the activated cell. Under physiological conditions, the $GABA_A$ receptor mediated IPSPs are often seen to follow a glutamate mediated excitatory postsynaptic potential (EPSP), and it may function to rapidly terminate that response. $GABA_A$ receptors also occur on the terminals of

* for review see Macdonald, R.L. and R.W. Olsen 1994, $GABA_A$ Receptor Channels, Annu. Rev. Neurosci. 17,569-602.

excitatory neurones. Such receptors are presumably innervated by GABAergic interneurones, which are thus capable of influencing the extent of depolarization in the terminals, thereby modulating neurotransmitter release. This mechanism has been used to account for presynaptic inhibition in spinal cord, the cuneate and gracile nuclei and the thalamus (Bradford, 1986).

$GABA_B$ Receptors

$GABA_B$ receptors also occur centrally and peripherally (Bowery et al., 1981). They are found in pre- and postsynaptic locations, and in both situations they exert an inhibitory influence. Postsynaptically, for instance in hippocampal pyramidal cells, they gate a potassium current. The result is a relatively slow, long lasting, IPSP which hyperpolarizes the cell (Andrade et al., 1986; Gahwiler and Brown, 1985; Nicoll and Newberry, 1984). $GABA_B$ receptors are also coupled to a calcium channel in such a way that agonist binding can cause inhibition of a calcium current. The reduction in intracellular calcium caused by the blockade of this current is a likely mechanism for the inhibition of neurotransmitter release (Bowery et al., 1980). Where neurotransmitter release is inhibited at excitatory synapses, $GABA_B$ receptor stimulation results in net inhibition (Potashner, 1979; Johnston, 1980; Bonanno and Raiteri, 1992). Net excitation, however, will occur where $GABA_B$ receptors exist on inhibitory terminals, for instance $GABA_B$ autoreceptors. The existence of such autoreceptors has been confirmed, not only in rodent brain (Waldmeier et al., 1988; Giralt et al., 1990) but also in human cerebral cortex (Bonanno et al., 1989).

$GABA_B$ Receptors and Second Messengers

Neurotransmitter receptors can be divided into two broad categories: ionotropic, or ligand gated ion channels, and metabotropic, in which agonist binding results in a change in the metabolic processes within the cell. In this second class of receptor, the initial intracellular changes are brought about by a GTP-binding protein (G-protein). That $GABA_B$ receptors belong to the latter category has been established with a variety of experimental evidence: (1) radioligand binding to $GABA_B$ receptors is sensitive to guanyl nucleotides, which promote the association of G-proteins to receptors, (Hill et al., 1984); (2) divalent cations are required for agonist binding (Bowery et al., 1983), as they are for other receptors now known to be linked to G-proteins (Williams et al., 1978); (3) GABA receptor agonists increase GTP-ase activity in brain homogenates (Ohmori et al., 1990); (4) responses to $GABA_B$ receptor activation, such as the slow IPSPs in hippocampal CA3 neurones, can be inhibited by pertussis toxin (a G-protein inhibitor), and can be mimicked by guanosine 5'-0-13-thiotriphosphate (GTP-γ–S) (Thalmann, 1988), a non-hydrolyzable analogue of GTP that enhances G-protein function by promoting subunit dissociation (Gilman, 1987); (5) antibodies raised to G-proteins inhibit (-)baclofen-stimulated GTP-ase activity (Dolphin et al., 1992); (6) [^{3}H]-GABA binding to $GABA_B$ receptors in rat brain membrane fragments is enhanced by the addition of several purified G-protein species (Morishita et al., 1990); (7) purified $GABA_B$ receptors can mediate the inhibition of adenylyl cyclase activity when they are reconstituted in an artificial phospholipid membrane in the

presence of adenylyl cyclase and a partially purified mixture of G-proteins (Nakayasu et al., 1993). No doubt future reconstitution studies with purified proteins will yield an exhaustive list of the species of G-protein which interact with $GABA_B$ receptors.

In order to determine the physiological roles for $GABA_B$ receptors it is necessary to know something of how G-protein linked receptors function. The sequence of events believed to follow agonist binding to a G-protein linked receptor is shown schematically in Figure 1.1. The components of the G-protein system are shown arranged on the intracellular face of the cell surface membrane, although their precise organization is not known (1). Briefly, an agonist binds to the receptor to which the heterotrimer G-protein is already bound (2). Gaunosine diphosphate (GDP) which is bound to the α subunit of the G-protein is exchanged for GTP in the presence of magnesium (3). The G-protein then dissociates from the receptor and itself dissociates into α and $\beta\gamma$ subunits (4). The α subunit causes a physiological response by interacting with one of a number of effectors (5,6). Initially the α subunit was considered to be the principal intracellular messenger, however, the $\beta\gamma$ subunit is also able to influence biochemical events within the cell, possibly by binding other available α subunits and thereby regulating the active species (8) or by interacting with specific effectors itself (7) (Lefkowitz, 1992). Following activation of the effector the α subunit is again freed and GTP is hydrolysed to GDP. The α subunit is now available to re-associate with a $\beta\gamma$ unit (9) and to bind to an unoccupied receptor (for review see Birnbaumer, 1990; Gilman, 1987; Casey and Gilman, 1988).

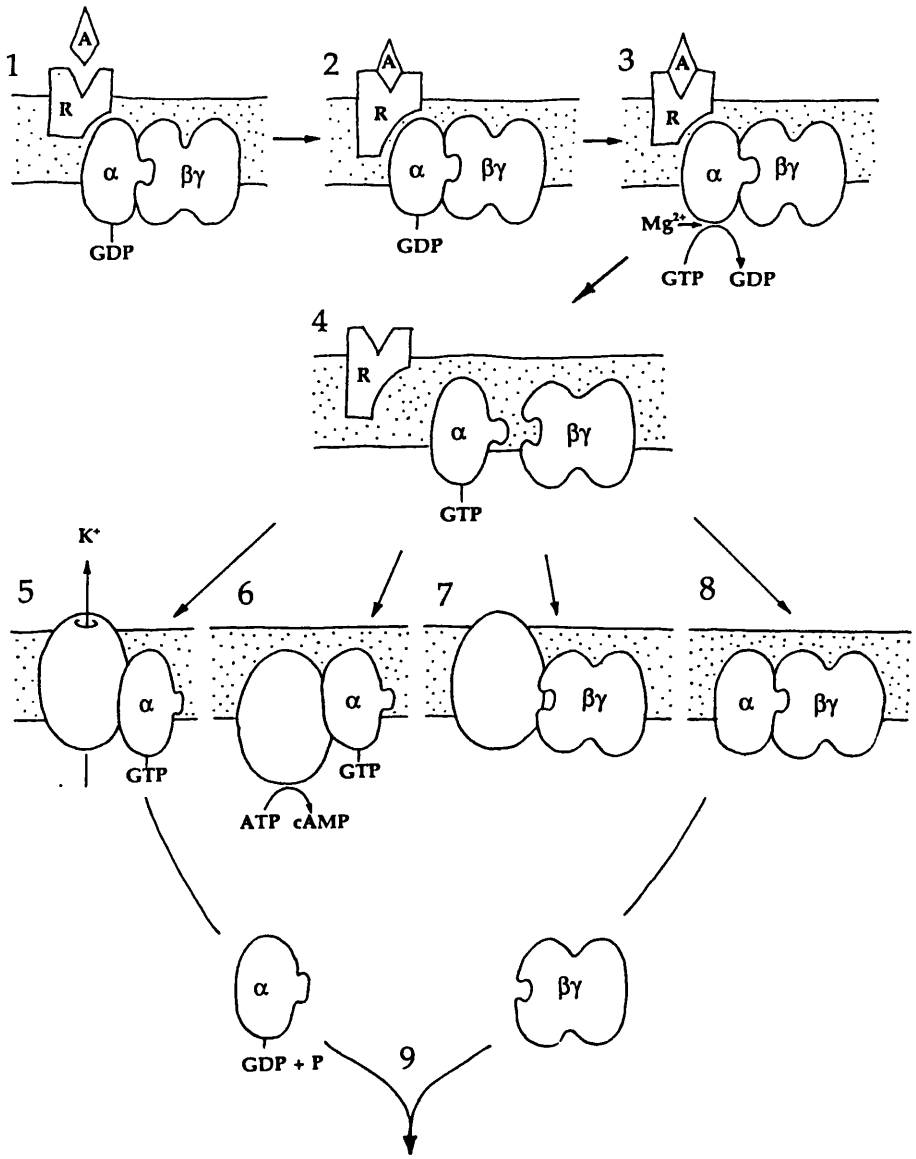


Figure 1.1. Receptor mediated G-protein activation. Schematic diagram representing the sequence of events that take place during signal transduction following the binding of an agonist to a G-protein linked receptor A=agonist, R=receptor, α and βγ =components of the G-protein, E=effector, GDP=guanosine diphosphate, GTP=guanosine triphosphate, ATP=adenosine triphosphate, P=inorganic phosphate. For an explanation of the sequence of events see text.

The mechanism of signal transduction is complicated by the variety of receptors which bind to G-proteins with varying degrees of specificity, the variety of G-proteins, many of which interact with several receptors, and a multiplicity of effectors, each of which may be under the influence of several G-proteins.

Classification of G-proteins

Many species of G-protein apparently exist. These were originally categorised into 3 principal types, on the basis of the action of their α subunit and their sensitivity to bacterial toxins. G_i (inhibitory) inhibits the production of cyclic adenosine 3',5'-monophosphate (cAMP) by adenylyl cyclase, and is sensitive to toxins derived from Bordatella pertussis. G_s (stimulatory) increases the production of cAMP by adenylyl cyclase, and is sensitive to toxins from Vibrio cholera. G_o (other) is used to describe G-proteins that do not fit into the first two categories. These categories are no longer strictly accurate, and multiple types of each of the α subunits of G_i, G_s and G_o have now been characterised (see Birnbaumer, 1990; Gilman, 1987; Casey and Gilman, 1988). Nevertheless, the classification remains useful in describing G-protein mediated events.

$GABA_B$ receptors have been demonstrated to couple pertussis toxin-sensitive G-proteins in pharmacological studies (pertussis toxin ADP-ribosylates G_i and G_o, Andrade et al., 1986). Reconstitution studies have been used to demonstrate that $GABA_B$ receptors couple to several specific forms of G-protein (Morishita et al., 1990). These include G_o, G_o* (a novel

form of G_o purified from brain (Goldsmith et al., 1988)) and G_{i1} (one of three forms of G_i identified by molecular cloning (Neer and Clapham, 1988)). The interaction of $GABA_B$ receptors specifically with Gi and Go has been corroborated with antibody studies (Dolphin et al., 1992) and reconstitution studies using purified $GABA_B$ receptors (Nakayasu et al., 1993). These G-proteins (G_i and G_o) have been shown to interact with the proteins shown in Table 1.1, $GABA_B$ receptor stimulation is therefore likely to include these effects. Indeed, the modulation of adenylyl cyclase and alterations in potassium channel activity are classic $GABA_B$ receptor mediated responses (Bowery, 1989).

In addition, other second messengers have been linked to the activation of $GABA_B$ receptors. One of these is phospholipase A2 (Duman et al., 1986), the activation of which leads ultimately to the formation of arachidonic acid, and the activation of subtypes of protein kinase C (Axelrod, 1988). $GABA_B$ receptor activation has also been shown to inhibit 5-HT- and histamine-stimulated inositol phospholipid metabolism in mouse cerebral cortex (Godfrey et al., 1988; Crawford and Young, 1990).

When interpreting the actions of $GABA_B$ receptor drugs, it is important to remember that the G-protein signal transduction system is shared by many neurotransmitter receptor systems. The activation of G-protein linked systems within the cell may therefore be dependant upon a variety of agonists, causing both stimulatory and inhibitory influences. For instance, $\beta\gamma$ freed by $GABA_B$ receptor stimulation may cause α subunits of an unrelated G-protein type to be recycled faster, or $GABA_B$ receptors may share the same effector with other neurotransmitters (Wojcik et al., 1985.

G-protein subunit	Effector
α_i	adenylyl cyclase catalytic unit
	cAMP phosphodiesterase
	potassium channel
α_o	potassium channel
$\beta\gamma$	unstimulated adenylyl cyclase
	αs stimulated adenylyl cyclase
	cAMP phosphodiesterase
	phospholipase A2
	potassium channel

Table 1.1. Specificity of G-protein subunits. The major targets of dissociated G-protein subunits believed to be activated by the $GABA_B$ receptor. Table adapted from Neer and Clapham (1988).

This could effect the tone in G-protein mediated effects not directly linked to the $GABA_B$ receptor. These interactions are intriguing in that the effects of activated receptors are integrated intracellularly. In addition several receptors may bind to one G-protein type e.g. 5-HT and $GABA_B$ (Andrade et al., 1986), so again activation of other systems will influence the response to $GABA_B$ receptor activation. These interactions extend the influence of $GABA_B$ receptors as neuromodulators.

1.2. Electrophysiological Considerations

Potassium Currents

Baclofen has been noted to induce a membrane hyperpolarization in a number of cells types (e.g. rat hippocampal cell bodies (Newberry and Nicoll, 1984a; 1984b; Gahwiler et al., 1984) and guinea pig hypothalamic neurons (Ogata and Abe, 1982)). This has been confirmed as an outward potassium current, since the reversal potential shows the expected dependence on external potassium concentration and is inhibited by external barium ions and internal cesium ions (Gahwiler and Brown, 1985; Newberry and Nicoll, 1984b; Hablitz and Thalmann, 1987; Inoue et al., 1985). Baclofen continues to cause this hyperpolarization in the presence of tetrodotoxin or cadmium (Newberry and Nicoll, 1985) and in calcium free medium (Ogata, 1990), suggesting that this effect is postsynaptic.

It was initially difficult to imagine a physiological role for this current. This was because at physiological resting membrane potentials

the $GABA_A$ receptor mediated change in Cl^- conductance caused a much greater hyperpolarization than the $GABA_B$ receptor mediated potassium current (Gahwiler and Brown, 1985). Since the endogenous agonist would be expected to activate both receptor types, the more modest $GABA_B$ receptor mediated hyperpolarization would be inconsequential. This however seems not to be the case, since the two currents appear to be temporally and spatially separated (Newberry and Nicoll, 1985). Thus, intracellular recordings made from hippocampal pyramidal cells revealed hyperpolarizations in response to ionophoretic application of GABA. The features of these hyperpolarizations differed when GABA was applied to different regions of the cell. The application of GABA to the cell body caused a hyperpolarization with a fast onset and decay. This hyperpolarization is due to a bicuculline-sensitive Cl^- current, and is therefore due to $GABA_A$ receptor activation. The application of GABA to the dendrites of these cells also produces a hyperpolarizaton which has a slower onset and decay (which is consistent with a G-protein mediated effect) and is resistant to bicuculline (Alger and Nicoll, 1982). This second current is therefore likely to be mediated by $GABA_B$ receptor activation. Since these two responses occur differentially, depending on the placement of the of the ionophoresis electrode, it was concluded that $GABA_A$ receptors were concentrated on the cell body and $GABA_B$ receptors were on the dendrites. Where these populations of receptors overlap, a biphasic response may occur, since the $GABA_B$ response occurs so much more slowly than the $GABA_A$ response. Biphasic IPSPs which fit this description have been recorded in many species and cell types: in

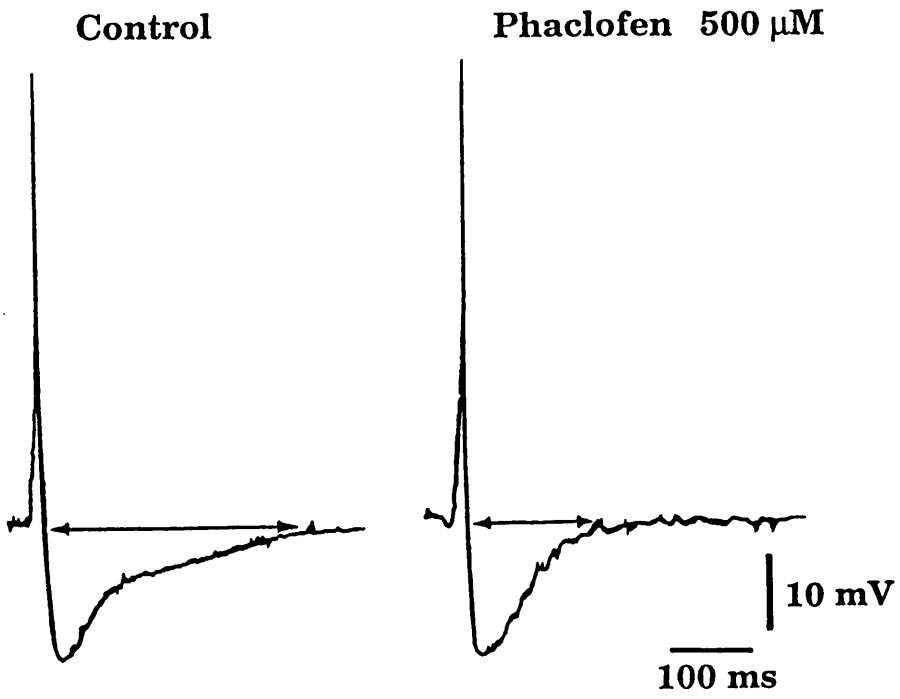


Figure 1.2. Postsynaptic potentials in a rat cortical slice. A typical e.p.s.p. followed by a biphasic, GABA mediated i.p.s.p. recorded intracellularly in a frontal cortical slice following electrical stimulation of superficial cortical layers. Bath application of the $GABA_B$ antagonist phaclofen (500 uM for 3 min) eliminates the late, long lasting hyperpolarization. Taken from Karlsson et al., (1988).

experimental preparations where excitatory afferent pathways are stimulated, the biphasic IPSP is seen to follow a glutamate mediated excitatory postsynaptic potential (EPSP) (Alger, 1984; Crunelli et al., 1988; Hirsch and Burnod, 1987; Lancaster and Wheal, 1981; Newberry and Nicoll, 1984b; Satou, et al., 1982; Stevens et al., 1985; Thalmann and Ayala, 1982). In some of these preparations, the involvement of $GABA_B$ receptors in the late long lasting hyperpolarization has been demonstrated by selective antagonism with the $GABA_B$ receptor antagonist phaclofen (Figure 1.2). This antagonist blocks the effects of $GABA_B$ receptor agonists in hippocampal and thalamic neurons (Soltesz et al., 1988), in neocortical cells (Karlsson et al.,1988) and in septal cells (Hasuo 1988). The physiological role of the long lasting hyperpolarization in the pyramidal cells appears to be the inhibition of repetitive firing, causing synaptic communication to fail during high frequency transmission (Davies et al., 1989).

Calcium Currents

There also appears to be an intimate relationship between $GABA_B$ receptors and membrane calcium conductances. The interaction between GABA receptors and calcium channels, however, appears to be complex and involve more than one type of calcium channel.

Initial, electrophysiological, studies upon the effect of $GABA_B$ receptor agonists on membrane currents were undertaken in primary afferent nerves, where the presence of $GABA_B$ receptors had already been demonstrated. In dorsal root ganglion cells, the activation of $GABA_B$

receptors was shown to decrease the duration of calcium dependent action potentials (Dunlap and Fischbach 1981, Desarmenien et al., 1984). In biochemical studies in central nervous system tissue, $GABA_B$ receptor activation has been shown to inhibit potassium-stimulated calcium influx into synaptosomes using the fluorescent dye Quin 2 (Bowery and Williams, 1986; Stirling et al., 1989). The modulation of calcium currents by $GABA_B$ receptors has been shown to be sensitive to the G-protein inhibitor Guanosine 5'-O-(2-thiodidiphosphate (GDP-β-s) in both chick (Holz et al., 1986; Rane and Dunlap, 1986) and rat dorsal root ganglion cells (Scott and Dolphin, 1986; Dolphin and Scott, 1986; Dolphin et al., 1989). In addition, $GABA_B$ receptor mediated inhibition of these currents has been shown to be reduced by pertussis toxin and enhanced by GTP (Scott and Dolphin, 1986; Dolphin and Scott, 1987), suggesting that this action is indeed mediated by a G-protein.

There are a number of possible mechanisms for the influence of G-proteins over calcium channels. For example, the activated components of the dissociated G-protein (either α or βγ subunits) might have a direct effect on the calcium channel. Alternatively, a soluble second messenger might be involved. Dolphin et al., (1989) and Green and Cotterell (1988) found no evidence for the presence of a soluble second messenger, favouring the direct action of a G-protein similar to that described for the Gs influence over L-type calcium channels (Yatani et al., 1988). Several other authors favour this close coupling hypothesis (Dolphin et al., 1989; Robertson and Taylor, 1986). However, the $GABA_B$ receptor mediated inhibition of calcium influx could be an indirect response, mediated through cyclic 3',5'adenosine monophosphate (cAMP) dependent phosphorylation. Such

cAMP dependent phosphorylation has been demonstrated with some voltage sensitive calcium channels, specifically the β-subunit of calcium antagonist receptors (Curtiss and Catterall, 1985). An investigation into the modulation of neuronal L-type calcium channels by $GABA_B$ receptors has suggested a complex effect, since the action of GTP-γ–S (a G-protein activator) does not accurately mimic the action of baclofen. Thus, GTP-γ-S inhibits only one phase of the calcium current, whereas baclofen causes a total inhibition. This may be evidence that calcium channels are under the influence of several G-proteins, like the adenylyl cyclase catalytic unit (Dolphin et al., 1989). Although both G_i and G_o have been demonstrated to inhibit calcium currents (Hescheler et al., 1986; Dolphin et al., 1989), the class of G-protein responsible for $GABA_B$ receptor mediated inhibition has yet to be established.

A further mechanism through which $GABA_B$ receptor activation could influence calcium channel activity is through a change in membrane permeability to another ion, such as potassium. This would induce effects in voltage-dependant calcium channels. Indeed, there is evidence for this in cultured hippocampal CA3 cells, in which the effect of baclofen on the calcium current is inhibited when the potassium current is blocked (Gahwiler and Brown, 1985).

Other evidence, however, points to a direct effect on the inward calcium current. In voltage clamped cat dorsal root ganglion cells, Robertson and Taylor (1986) reported a reduction in the peak amplitude of calcium currents which occurred in the absence of any effects on potassium current. This work has been corroborated in cultured dorsal

root ganglion neurons by Dolphin and Scott, (1986) and receives further support from other studies (Dunlap and Fischbach, 1981; Deisz and Lux, 1985). Moreover, dihydropyridine calcium ligands modulate $GABA_B$ radioligand binding (Al-Dahan and Thalmann, 1989), suggesting the possibility of an intimate relationship between the dihydropyridine sensitive, high voltage activated L-type calcium channel and the $GABA_B$ receptor protein.

The modulation of calcium influx by (-)baclofen has been cited as a possible mechanism by which neurotransmitter release is influenced in the terminals of primary afferent cells (Curtis et al., 1981; Henry, 1982; Lanthorne and Cotman, 1981). Indeed, this could provide a mechanism for the inhibition of neurotransmitter release by $GABA_B$ receptors at all synaptic terminals where these receptors are situated. However, in the hippocampus, Cd^{2+}, a calcium channel blocker, is markedly less efficient than (-)baclofen as an inhibitor of synaptic responses. This evidence suggests that (-)baclofen might have a direct effect upon neurotransmitter release, at least in the hippocampus (Thompson et al., 1992). Indeed, the importance of extracellular calcium in neurotransmitter release remains equivocal (Zucker and Haydon, 1988)

In summary, $GABA_B$ receptor agonists activate two membrane currents. Firstly an outward potassium current which is responsible for IPSPs (Gahwiler and Brown, 1984; Nicoll and Newberry, 1984). Secondly, in dorsal root ganglion cells $GABA_B$ receptor agonists inhibit an inward calcium current, reducing periods of depolarization (Dolphin and Scott, 1986; Robertson and Taylor, 1986). It has been proposed that the *direct* effect

(as opposed to voltage dependant, secondary effects) of $GABA_B$ receptor mediated modulation of calcium currents is exclusively presynaptic and the modulation of potassium currents is exclusively postsynaptic (Dolphin and Scott, 1986). However, no direct evidence for this exists (Stirling et al., 1989).

1.3. Agonists and Antagonists at the $GABA_B$ Receptor

Research into the role of $GABA_B$ receptors in the central nervous system was initially hampered by lack of compounds that act selectively and potently at this receptor. This paucity is reflected in the first descriptions of $GABA_B$ receptor mediated events as bicuculline-insensitive GABA responses. The first selective $GABA_B$ receptor agonist to be identified was baclofen. This was produced initially as a lipophilic analogue of GABA so that it would cross the blood brain barrier. Baclofen soon became established as centrally acting muscle relaxant and antispasticity agent (see Bowery and Pratt, 1992). The pharmacological specificity of baclofen was determined by Bowery et al., (1983) using radioligand receptor binding experiments. These experiments also determined the chiral specificity of the novel receptor. The higher potency of the (-)isomer for $GABA_B$ receptors added credence to the hypothesis that the therapeutic actions of baclofen are mediated through this receptor, since it is the (-) isomer which is most effective at inhibiting spinal reflexes in spasticity (Olpe et al., 1978). This isomer is also 20 times more potent than the (+)isomer at depressing synaptic activity in the immature rat isolated spinal cord (Ault and Evans, 1978) and two orders of magnitude more potent as an antinociceptive in rats

(Wilson and Yaksh, 1978), confirming these responses as mediated by $GABA_B$ receptors. For many years then, (-)baclofen has been the drug of choice for demonstrating a $GABA_B$ receptor mediated effect. However, care must be taken when interpreting the effects of baclofen, since at very high concentrations baclofen has agonist effects at α2-adrenoceptors (Fung et al., 1985).

More recently, the phosphinic analogue of GABA, 3-aminopropyl phosphinic acid (3-APA), has been demonstrated to be more potent than (-)baclofen in binding studies, displacing radioligand binding to $GABA_B$ receptors with an IC_{50} of 1 nM (Pratt et al., 1989). In functional assays, it is 10 to 100 times more potent than (-)baclofen (Hills et al., 1989). The methylated analogue of this compound, SKF97541 (3 aminopropyl(methyl)phosphinic acid), is also a $GABA_B$ receptor agonist. Although it is rather less potent than 3-APA as a displacer of $GABA_B$ receptor radioligand binding, it is approximately 10 times more potent than 3-APA in electrophysiological studies (Seabrook et al., 1990; 1991)(Figure 1.3).

Selective antagonists for the $GABA_B$ receptor have also been difficult to obtain. GABA itself is a flexible molecule which can adopt a wide variety of conformations. Despite the observation that the $GABA_A$ receptor preferring and the $GABA_B$ receptor preferring conformations of GABA represent two extremities of these folding capabilities (Kerr et al., 1990), initial attempts to produce a specific $GABA_B$ antagonist were flawed by activity at the $GABA_A$ receptor. For instance delta-amino valeric acid was

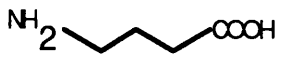
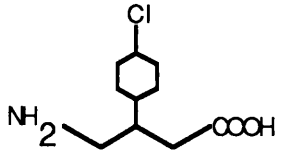
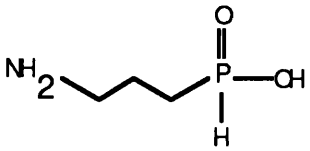
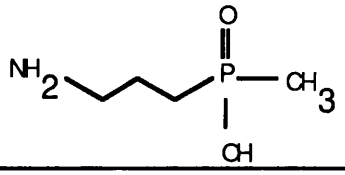
GABA 40 nM $	
baclofen 30 nM *	
3APA 1 nM +	
SKF97541 14 nM *	

Figure 1.3. The stuctures and binding potency of $GABA_B$ receptor agonists. These compounds represent published structures which were either used in the present study or mentioned in the text. Data was taken from the following sources (compounds in brackets represent the radioligand against which the displacers were tested): ($)Bowery et al., 1983 ([^{3}H]-baclofen); (*) Froestl et al., 1992 ([^{3}H]-3-APA); (+) Pratt et al., 1992 ([^{3}H]-GABA).

identified as a $GABA_B$ receptor antagonist in peripheral tissues such as the rat annococcygeus muscle, (Muhyaddin et al., 1982) the guinea pig ileum (Sawynok, 1986) and the gastrocnemius and plantar foot muscles of the rat (Schwartz et al., 1988). However delta-amino valeric acid was found to be active at $GABA_A$ receptors with almost equal binding potency (Bowery et al., 1983). The first really selective antagonist was the phosphono- analogue of baclofen, β-(p-chlorophenyl)-3-aminopropyl phosphonic acid (phaclofen), synthesized by Cheifari et al. (1987). Phaclofen was subsequently demonstrated to antagonise the action of baclofen in a number of peripheral preparations, including the baclofen-induced depression of twitch responses in ileum (Kerr et al., 1987; Ong et al., 1987). Phaclofen also antagonized the ability of baclofen to cause presynaptic inhibition of excitation in spinal interneurons (Kerr et al., 1987; Dutar and Nicoll, 1988a). However, phaclofen did not penetrate the blood brain barrier well and was of relatively low potency (pA_2= 4, Kerr et al., 1990). The ß-hydroxy substituted sulphonic analogue of baclofen, 3-amino-2-(4-chlorophenyl)-2-hydroxypropane sulphonic acid (2-hydroxysaclofen) has subsequently proved to be of higher potency (pA_2= 5.6, Kerr et al., 1990). Phaclofen and 2-hydroxysaclofen have entered the literature as "classical" antagonists of $GABA_B$ receptor mediated responses.

Very recently, however, these two compounds have been superceded by a selection of compounds from Ciba-Geigy. The first exciting development was the publication of CGP35348, (3-aminopropyl-(dioxymethyl)-phosphinic acid) with a binding affinity of 35 μM (Bittiger et al., 1990). This first compound was soon followed by CGP36742 (Bittiger et al., 1992) and CGP46381 (Olpe et al., 1993), which are the first $GABA_B$

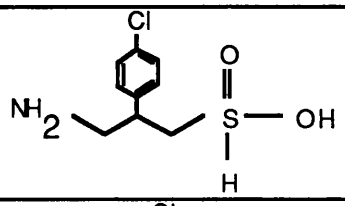
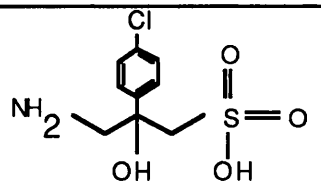
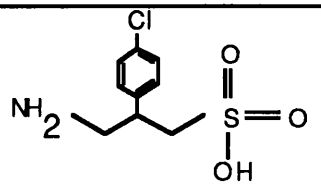
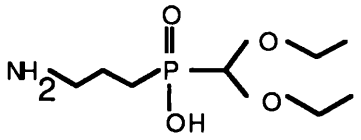
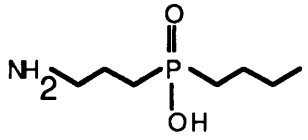
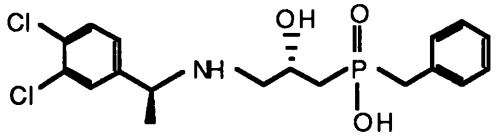
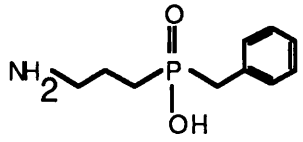
phaclofen 130 μM *	
2-0H-saclofen 12 μM *	
saclofen 26 μM *	
CGP35348 35 μM #	
CGP36742 35 μM #	
CGP54626 6 nM #	
CGP46381 5 μM **	

Figure 1.4. The stuctures and binding potency of GABA$_B$ receptor antagonists. These compounds are all published structures which were either used in the present study or are mentioned in the text. Data was taken from the following sources: (#)Bittiger et al., 1988 ; (*) Froestl et al., 1992; (**)Olpe et al 1993. All compounds were tested as displacers of [^{3}H]-3-APA

receptor antagonists to readily cross the blood brain barrier, although they still lack dramatic potency (35 and 5 μM respectively, Bittiger et al., 1990; Olpe et al., 1990). As one of the earliest orally active compounds, CGP36742 is the first $GABA_B$ receptor antagonist to be used in clinical trials where it is being assessed as an antiepileptic (Bittiger et al., 1992). In addition, CGP54626 is currently the most potent $GABA_B$ receptor antagonist available, with an IC_{50} against $GABA_B$ receptor radioligand binding of 4.4 nM (Froestl et al., 1992). (Figure 1.4).

1.4. Molecular Biology of the $GABA_B$ Receptor

Many insights into the mechanism of receptor activation and its involvement in cellular processes are gained when the primary, secondary and tertiary structure of the receptor under consideration is elucidated. This can only occur when the protein has been cloned and purified in sufficient quantities to allow investigation. Although this work has not yet been completed for the $GABA_B$ receptor, the initial steps of solubilization and purification have been achieved. Solubilization of the $GABA_B$ receptor is possible with a variety of detergents, and has been accomplished with porcine brain (Facklam and Bowery, 1993) and bovine brain (Kuriyama et al., 1990). Using bovine brain and optimal solubilization conditions with sodium deoxycholate, some of the properties of the $GABA_B$ receptor are lost. These properties include the sensitivity of [^{3}H]-GABA binding to calcium channel blockers, the ability of $GABA_B$ receptor agonists to stimulate GTPase activity, and the ability of $GABA_B$ receptor agonists to inhibit forskolin-stimulated adenylyl cyclase activity (Ohmori et al., 1990;

Kuriyama et al., 1990). This deficit may be the result of damage to the receptor, selective solubilization of the components of the relevant signal transduction system, or simply the loss of the membrane organization of these components. This is an interesting finding because some receptors such as the D2 dopamine receptor exhibit a similar loss of function (Ohara et al., 1988), whilst others, such as the A1 adenosine receptor (Nakata, 1989) and opioid receptors (Wong et al., 1989), do not. This might indicate that $GABA_B$ receptors share specific molecular characteristics with the D2 receptor. However, the concentration and type of detergent are critical for the maintenance of G-protein coupling during solubilization. The loss of function by some receptors may merely represent poorly optimized solubilization conditions.

Following solubilization, purification was initially attempted with affinity resin chromatography using several different ligands, including GABA, 3-aminopropyl phosphonic acid, and (-)baclofen attached by either the amino or the carboxyl group. The baclofen affinity resins proved to be the most efficient, both of these achieved a purification of around 22 fold. A higher degree of purification (140 fold) was achieved by performing gel filtration chromatography on this extract. GABA binding proteins with two different molecular weights (80 and 240 kDa) were eluted using this procedure. The higher molecular weight protein may be the consequence of extensive glycosylation, or of a proportion of the receptors still being complexed to another protein, such as a G-protein (Kuriyama, 1990). More recently, a definitive purification has been achieved using immuno-affinity beads. These are prepared with a monoclonal antibody raised to the partially purified receptor (Nakayasu et al., 1993; 1992). This study resulted

in an 8000-fold purification of the receptor, and confirmed a molecular weight of 80 kDa for the uncomplexed protein.

Over 70 G-protein linked receptors have been cloned and sequenced to date. These display a wide range of sequence homology (6 to 93%) (Hibert et al., 1993), yet they do have a number of highly conserved features. Thus, it is possible to deduce some of the likely characteristics of the $GABA_B$ receptor protein structure. For instance, other G-protein linked receptors are single proteins with seven hydrophobic domains which are proposed to be membrane spanning regions. These hydrophobic domains are interspersed with hydrophilic loops (Bunzow et al., 1988; Dixon et al., 1986; Kobilka et al., 1988; Bonner et al., 1987). The loops on the cytoplasmic side of the cell membrane have phosphorylation sites which may be responsible for desensitization, and these loops presumably form a G-protein binding site. The transmembrane spanning regions are folded into α helices, and are arranged in a tight bundle. The agonist binding site of most G-protein linked receptors is located deep in the centre of this bundle. This is a highly conserved feature; a recent survey of six different G-protein linked receptors revealed that each retained an aspartate residue on the third membrane spanning domain. This residue formed an ion pair with a cationic amino group in the agonist. This ion pairing was the principal ionic interaction in agonist binding (Hibert et al., 1993). It is interesting to note in this respect, that substitution of the amino group of $GABA_B$ receptor agonists always leads to a dramatic loss in affinity (Froestl et al.,1992); this finding may indicate the presence of such an ionic pairing system in the $GABA_B$ receptor. However the confirmation of these structural features as

characteristics of the $GABA_B$ receptor awaits a more detailed investigation of the molecular biology of this protein.

1.5. Aims of the Present Study

Overview

The range of agonists and antagonists available for use in the investigation of $GABA_B$ receptor mediated responses has increased dramatically in the last few years. In the present study, a number of these compounds were tested to characterize in detail the pharmacological profile of $GABA_B$ receptors in two different functional assays. Experiments were also conducted to investigate the role played by glial cells in one of the classic functional assays of $GABA_B$ receptor activity, namely the modulation of cAMP production in rat cortical slices. In addition, two studies of more direct clinical relevance were conducted. In the first, rats were chronically dosed with compounds acting at $GABA_B$ receptors to determine their long term effects upon $GABA_B$ receptor populations. In the second, possible pathological alterations in $GABA_B$ receptors were investigated in an animal model of epilepsy.

Pharmacologically Defined Subtypes of $GABA_B$ Receptor

There have been a number of reports of sub-populations of $GABA_B$ receptors with differing pharmacologies. Much of the initial speculation in

these reports was based on the activity, or lack of activity, of the low potency antagonist phaclofen. For instance, it was reported that baclofen acts at two populations of receptors in the spinal cord; one of these receptors, on afferent terminals, was sensitive to phaclofen and the other was insensitive (Curtis et al., 1986; Curtis et al., 1981). Phaclofen was also used, in a study by Stirling et al. (1989). These authors reported that this compound, along with β-phenyl-GABA and δ–aminovaleric acid, was unable to antagonise the inhibition of calcium influx caused in synaptosomes by (-)baclofen. This was in spite of the fact that all three compounds had previously been demonstrated to be $GABA_B$ receptor antagonists in other systems (Kerr et al., 1987; Ong et al., 1987; Muhyaddin et al., 1982; Sawynok, 1986). Stirling et al. therefore proposed that the $GABA_B$ receptors in their synaptosomal preparation had a different pharmacology to that previously described for $GABA_B$ receptors in the periphery. The specificity of phaclofen for certain populations of $GABA_B$ receptor has received corroboration from a number of authors. For instance Dutar and Nicoll (1988b) reported that the postsynaptic action of (-)baclofen (a K^+ dependent IPSP) was antagonized by phaclofen, but the presynaptic action of (-)baclofen (inhibition of an afferent stimulated, glutamate mediated EPSP) was not; Harrison (1989) reported that the presynaptic inhibition of GABA release by (-)baclofen was insensitive to phaclofen in central neurons, and Wang and Dun (1990) reported similar findings for the release of excitatory and inhibitory neurotransmitters at afferent terminals in the spinal cord. These studies all agree that phaclofen is able to antagonize the postsynaptic, but not the presynaptic, actions of baclofen.

Some compounds, such as carbachol apparently distinguish between the activity of GABA and the activity of baclofen, blocking hyperpolarizations only in response to the latter (Muller and Misgeld, 1989). This specificity is shared by phaclofen in some preparations. For instance, in hippocampal pyramidal cells, phaclofen appears to block a baclofen induced K^+ dependent IPSP more potently than it blocks a similar response induced by GABA (Dutar and Nicoll 1988a). Other antagonists display a similar specificity, for instance 4-aminopyridine in guinea-pig hippocampal pyramidal cells (Inoue et al., 1985,) and in guinea-pig hypothalamic neurons (Ogata et al., 1987). Another kind of specificity is shown by the $GABA_B$ receptor antagonist 2-OH-saclofen. This failed to completely inhibit hyperpolarizations induced by topical application of GABA in the hippocampus, though the same concentration of 2-OH-saclofen completely antagonised electrically evoked GABA mediated IPSPs (Segal, 1990). In this case the electrically evoked IPSPs are presumably mediated exclusively by synaptic receptors while the effects of topical application may be mediated by extrasynaptic receptors as well. Thus the differential sensitivity of the two responses to 2-OH-saclofen may indicate a pharmacological difference between synaptic and extra synaptic receptor populations (Segal, 1990).

A large literature supports the contention that post-, but not presynaptic $GABA_B$ receptors are sensitive to G-protein inhibitor, pertussis toxin. In these experiments pertussis toxin is applied by intraventricalar injection prior to the start of the experiment (Colmers and Williams, 1988; Dutar and Nicoll, 1988b; Colmers and Pittman, 1989; Gallagher et al., 1990), or bath applied to cultured neurons (Harrison, 1989). These data may

merely indicate that subpopulations of $GABA_B$ receptors are coupled to different signal transduction pathways, rather than indicating the occurrence of subtypes of receptor.

The most recent, and convincing, work on $GABA_B$ receptor subtypes has been by Bonanno and Raiteri (1992, 1993), who have proposed three classes of $GABA_B$ receptor on the basis of differential sensitivity to phaclofen, CGP35348 and the novel antagonist CGP52432. These authors studied potassium-stimulated release of GABA, glutamate and somatostatin in rat brain synaptosomes. The release of all three neurotransmitters was inhibited by (-)baclofen with similar potency. (-)Baclofen-inhibited GABA release was insensitive to CGP35348; (-)baclofen-inhibited glutamate release was insensitive to phaclofen, and (-)baclofen-inhibited somatostatin release was sensitive to both, suggesting three subtypes of receptor. The highly potent antagonist, CGP52432 was two orders of magnitude more potent as an antagonist of (-)baclofen-inhibited GABA release than it was in the other two assays, confirming that pharmacological differences exist.

This plethora of experimental data has produced many suggestions that subtypes of $GABA_B$ receptor exist. However, conclusive experiments have yet to be performed, especially since some of the the initial findings have not been corroborated by subsequent work. For instance, Solis and Nicoll (1992) failed to demonstrate the specificity of phaclofen for postsynaptic $GABA_B$ receptor mediated responses. In addition these authors found that the more recently available $GABA_B$ receptor antagonist CGP35348 inhibited both pre- and postsynaptic responses with similar

potency. This adds weight to the argument that pre- and postsynaptic receptors are similar. Also, unlike phaclofen, CGP35348 and 2-OH-saclofen are able to antagonize both bicuculline-insensitive GABA IPSPs as well as baclofen induced IPSPs (Solis and Nicoll, 1992). Indeed pharmacological specificity in this assay is difficult to interpret since electrically evoked, GABA-mediated, IPSPs represent a considerable methodological difference to topically applied baclofen. A similar criticism may be levelled at the study by Muller and Misgeld (1989) in which carbachol blocked the postsynaptic action of bath applied baclofen but not ionophoretically applied GABA. This was taken to imply that there are different mechanisms involved in the postsynaptic actions of GABA and baclofen. It is essential that this work is corroborated, since the data are incompatible with the well established hypothesis that GABA is the endogenous neurotransmitter which mediates the slow IPSP (Solis and Nicoll, 1992). Indeed more recent work in this field has demonstrated that baclofen responses are not inhibited by carbachol under similar conditions to those in which it fails to block GABA responses (Bijak et al., 1991).

Recent work also indicates that the specificity of pertussis toxin as a postsynaptic inhibitor of $GABA_B$ receptor mediated responses may well be the result of problems of access to axon terminals. Intra-hippocampal injections of the toxin appear to be effective on both pre- and postsynaptic actions (Stratton et al., 1991).

The work by Bonnano and Raiteiri remains the most interesting of these studies. however, whilst CGP35348 and CGP52432 are specific for sub-populations of $GABA_B$ receptor in this functional study, they

apparently displace $GABA_B$ receptor radioligand binding by 100% and complex displacement kinetics have not been reported for these compounds (Froestl et al., 1992). This work therefore requires corroboration to the extent that $GABA_B$ receptors with similar specificities have yet to be identified in other preparations or by the use of different techniques.

In summary, subtypes of $GABA_B$ receptor have been proposed on a variety of experimental data, however none of these studies has yet been conclusive so there remains a need for tests with new antagonists to identify compounds which are selective for one sub-population versus another. A convincing demonstration of $GABA_B$ receptor subtypes using pharmacological agents awaits the development of high potency antagonists that reflect the specificity of compounds such as Phaclofen and CGP35348.

Identification of compounds that are specific for sub-populations of $GABA_B$ receptors must be a primary objective in the development of therapeutic $GABA_B$ receptor drugs. This is because the extensive neuromodulatory role of $GABA_B$ receptors makes it difficult to predict the effects of systemic, or even locally applied $GABA_B$ receptor drugs at the level of the whole animal. $GABA_B$ receptors interact extensively with other neurotransmitters at a number of different functional levels. Firstly, they modulate release of neurotransmitter presynaptically and thus alter the levels of extracellular neurotransmitter impinging upon a specific target cell. At the level of the cell surface receptor, $GABA_B$ receptors influence the actions of other neurotransmitters where different signal transduction pathways converge on the same G-protein, and where different G-proteins

converge on the same effector (G-protein activated enzyme or ion channel). Finally, integration of neurotransmitter actions can occur at the level of the cell membrane, where the degree of polarization and ion flux is a property of all the channels present. Compounds which act at specific sub-populations of $GABA_B$ receptors would represent valuable research tools and possibly therapeutically effective drugs. It was therefore decided to investigate the potency of a selection of recently available $GABA_B$ receptor agonists and antagonists upon two functional assays of $GABA_B$ receptor activation. For this purpose the $GABA_B$ receptor mediated modulation of cAMP production in CNS tissue was selected.

Pharmacology of the Modulation of Adenylyl Cyclase by $GABA_B$ Receptors

The effects of $GABA_B$ receptor stimulation on adenylyl cyclase (ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1) in cortical tissue are two-fold: an inhibition of forskolin-stimulated adenylyl cyclase activity, and an augmentation of β-adrenoceptor-stimulated adenylyl cyclase activity. $GABA_B$ receptor activation alone has no effect on adenylyl cyclase activity (Karbon and Enna, 1985; Hill, 1985). The $GABA_B$ receptor mediated inhibition of forskolin-stimulated adenylyl cyclase is a well documented phenomenon (Karbon and Enna, 1985; Hill, 1985), and indicates that the $GABA_B$ receptor is linked to the G_i class of G-proteins. The $GABA_B$ receptor mediated augmentation of noradrenaline-stimulated adenylyl cyclase has also been well characterized (Hill, 1985; Karbon and Enna, 1985). $GABA_B$ receptors are also able to augment adenylyl cyclase activity stimulated by vasoactive intestinal peptide (Watling and Bristow, 1986),

adenosine A2, and prostaglandin E1 (Karbon and Enna, 1985). The ability to augment adenylyl cyclase activity is not confined to $GABA_B$ receptors; H1 histamine receptor agonists potentiate H2 receptor-stimulated cAMP (Al-Gadi and Hill, 1985), and α2–adrenoceptor agonists potentiate β-adrenoceptor activation (Leblanc and Ciaranello, 1984). Both inhibitory and augmenting responses have been confirmed in human brain. although the modulatory effects of $GABA_B$ receptor activation have not (Kendall et al., 1992).

It was initially proposed that the ability of $GABA_B$ receptors to both inhibit and stimulate adenylyl cyclase activity was due to the presence of two pools of cAMP. This hypothesis suggested that the two cAMP pools were compartmentalized in different cells, possibly representing a presynaptic and a postsynaptic response (Hill, 1985); one pool was under the influence of a stimulatory second messenger and one pool was under the influence of an inhibitory second messenger. Thus there might be two functionally discrete populations of $GABA_B$ receptors in the brain (Karbon et al., 1990). The inhibitory response was clearly mediated by G_i. Two candidates for mediating the stimulatory response have been investigated, these are phospholipase A2 and phospholipase C. Evidence has been presented to support this hypothesis (for review see Enna and Karbon, 1987). However, it is now considered likely that the augmentation response is a consequence of the activity of dissociated βγ G-protein subunits (Lefkowitz, 1992).

Two things make detailed investigations into the pharmacological profiles of $GABA_B$ receptor mediated responses especially pertinent at the

Glial Cells in the Central Nervous System

Neuroglia have been recognized as a distinct class of cells in the CNS since the late 1800's. They can be broadly divided into two categories: macroglia and microglia. The first of these two subdivisions contains astrocytes and oligodendrocytes. Each of these classes of cells contians further subdivisions of morphologically and functionally distinct types (Montgomery, 1994)

Astrocytes are estimated to comprise 20-25% of whole brain tissue, with densities of up to 50% of the total volume in some brain regions. Approximately 50% of glial cells in the mammalian brain are astrocytes. Astrocytes seem to have three major functions: the guidance and support of neurons during migration and growth; the miantianance of the chemical composition of the extracellular fluid around neurons (including uptake mechanisms for important neurotransmitters) and the modulation of immune reactions by the production of antigens (for review see Montgomery, 1994).

The principal function of oligodendrocytes appears to be the formation of myelin sheaths around axons. As such they are the counter part of Schwann cells in the peripheral nervous system. They are found in high densities in white matter in the CNS (for review see Miller et al., 1989).

Microglia are usually present in the adult mammalian CNS as "ramified microglia", they are small cells, 5-10 μm in diameter, with fine radiating processes. During tissue infection they convert to "reactive microglia", these are phagocytic amoeboid cells, which remove redundant nerve processess and cell debris resulting from cell death. They appear to behave as macrophages do in the periphery. They represent 5 to 15% of the volume of brain tissue and are evenly distributed throughout the CNS. (for review see Davies et al., 1994).

Davis, E.J., Foster , T. D, and Thomas, W.E., (1994) Cellular forms and functions of brain microglia, Brain Res. Bull. 34, 73-78.

Miller, R.H., ffrench-Constant, C. and Raff, M.C. (1989) The macroglial cells of the rat optic nerve. Ann. Rev. Neurosci. 12, 517-534.

Montgomery, D.L. (1994) Astrocytes: form, functions and roles in disease, Vet. Pathol. 31, 145-167.

present time. Firstly, the increasing number of reports of pharmacologically distinct sub-populations of $GABA_B$ receptors, and secondly the availability of a wider range of $GABA_B$ receptor agonists and antagonists than was hitherto the case. To this end, the modulation of adenylyl cyclase by $GABA_B$ receptors would seem to represent an ideal model in which to determine the potency of new compounds, not least because two apparently distinct functional assays can be undertaken in the same tissue. The two responses may be mediated by different receptors populations, and should specific compounds be identified, the basis of the specificity is unlikely to be due to differential tissue penetration or other experimental variables. Thus in the present study a range of agonists and antagonists was used to determine the pharmacological profile of the $GABA_B$ receptors augmenting and inhibiting stimulated adenylyl cyclase activity.

Adenylyl Cyclase in Glial Cells

If data concerning the modulation of adenylyl cyclase activity are to be of use in predicting the neuronal activity of $GABA_B$ receptor drugs, then we must presuppose that the receptors under consideration are specifically located on neurons. In this light, it is worth mentioning glial cells, which may make up the bulk of brain tissue and are sensitive to most neurotransmitters through receptors which appear to be identical to those found on the neurons themselves. In addition, glial cells use cAMP as an intracellular messenger. Of particular relevance to this study is the observation that between 75% and 95% of the β–adrenoceptor-stimulated

Additional evidence that adenylyl cyclase, and indeed, β–adrenoceptor stimulated adenylyl cyclase is located in glia cells has come from immunocytochemical studies. For instance, it has been reported that cAMP assayed by immunocytochemistry is located in glial cells in the superior cervical ganglion, (Ariano et al., 1982) this study also demonstrated an increase in this pool of cAMP in response to β-adrenoceptor stimulation. An initial study into the localization of cAMP in the caudate nucleus was conducted using light microscopy. This revealed cAMP staining apparently located in neurons (Ariano and Matus, 1981), however, a subsequent ultrastructural investigation by the same group revealed that most staining was in fact in astrocytic processes (Ariano et al., 1982). A glial reactive component to cAMP staining has also been identified (Stone and Ariano, 1989).

Ariano, M.A., Butcher, L.L. and Appleman, M.M. (1980) Cyclic nucleotides in the rat caudate-putamen complex: histochemical characterization and effects of deafferentation and effects of deafferentation and kainic acid linfusion, Neuroscience , 5 1269-1276.

Stone, E.A. and Ariano, M.A. (1989) Are glial cells targets of the central noradrenergic system? A review of the evidence. Brain Research Reviews, 14 297-309.

Ariano, M.A., Briggs, C.A. and McAfee. D.A., (1982) Cellular localization of cyclic nucleotide changes in rat superior cervical ganglion, Cell. Mol. Neurobiol., 2, 143-155.

Ariano, M.A. and Matus, A.I. (1981) Ultrastructural localization af cyclic GMP and cyclic AMP in rat striatum, J. Cell Biol., 91 287-292.

cAMP in rat forebrain slices derives from glial cells (Stone et al., 1990)? This is important since the augmentation of β–adrenoceptor-stimulated cAMP by $GABA_B$ receptors has been proposed to occur as a result of an intracellular interaction. This information, together with the recent description of $GABA_B$ receptors on glial cells, (Hosli and Hosli, 1992; 1990), suggested that the augmentation of noradrenaline-stimulated cAMP production in rat cortical slices might occur at least in part in glial cells. We therefore employed fluorocitrate, a selective inhibitor of glial cell respiration and ATP production (Stone et al., 1990; Paulsen et al., 1987; Hassel, 1992) to eliminate glial cell activity in rat cortical slices in which the modulation of adenylyl cyclase by neurotransmitter receptors was investigated.

In addition the ability of forskolin and noradrenaline to stimulate adenylyl cyclase in glial cells was investigated using glial cells in culture. The ability of (-)baclofen to modulate these responses was also investigated.

Dialysed cAMP as an Index of $GABA_B$ Receptor Function *In Vivo*

The physiological relevance of the *in vitro* modulation of adenylyl cyclase activity was investigated using the technique of *in vivo* microdialysis. Most microdialysis work has concentrated on monitoring neurotransmitters and their metabolites, which has allowed the general levels of activity in neurotransmitter systems to be correlated with behavioral state and to the application of drugs systemically. In addition, levels of neurotransmitter can be determined in the presence of locally

applied pharmacological agents, and the resultant changes in neurotransmitter levels can be interpreted as effects upon the release of relevant neurotransmitters. Because microdialysis is used principally for measuring neurotransmitter release its value has almost exclusively been in the investigation of presynaptic mechanisms. Since neuronal cAMP is believed to be derived predominantly from postsynaptic cells (Onali et al., 1988), this raises the possibility that that it can be used as an index of activation for postsynaptic receptors *in vivo* (Hutson and Suman-Chauhan, 1990).

cAMP is produced intracellularly as a second messenger in response to to a variety of neurotransmitter receptors, (Stoof and Kebabian, 1981; Lazareno et al., 1985). It is also found extracellularly. For instance, *in vivo*, it has been demonstrated to occur in the cerebrospinal fluid of *Rhesus* monkeys (Post et al., 1979) and in the lateral ventricle of the rat (Korf et al., 1976). *In vivo* studies have also demonstrated that the concentration of this extracellular pool of cAMP is dependent on the activation of cell surface receptors (Hutson and Suman-Chauhan, 1990; Stone and John, 1990), and as such it is believed to be derived from the intra-cellular pool, by a poorly described "overflow" or "egress". The concentration of extracellular cAMP has been demonstrated to reflect the intracellular concentration of cAMP in a number of *in vitro* studies (Stoof and Kebabian, 1981; Barber and Butcher, 1983; Lazareno et al., 1985; Egawa et al., 1988). The present study investigated the effect of $GABA_B$ receptor stimulation upon extracellular levels of cAMP in the frontal cortex of rat using the technique of intracerebral microdialysis. Two lines of thought prompted this study. Firstly, $GABA_B$ receptor stimulation has no intrinsic effect upon baseline

cAMP levels *in vitro* (Hill et al., 1985; Enna and Karbon, 1984). This is presumably because adenylyl cyclase is inactive in an extensively washed, cross chopped cortical slice preparation. To predict the effect of a $GABA_B$ receptor agonist on adenylyl cyclase *in vivo* it is necessary to know something about adenylyl cyclase *in vivo*, in particular, whether there is any tonic activity, and if there is, is it receptor/Gs mediated, or does it mimic the effect of forskolin. Secondly, this technique would provide an index of the activity of $GABA_B$ receptor drugs *in vivo*, providing information on their potency, brain penetration, and perhaps their half life.

1.6. Clinical Implications of the Present Study

The extensive neuromodulatory role of $GABA_B$ receptors both in the CNS and in the periphery means that the therapeutic potential for $GABA_B$ receptor drugs is vast (see Bowery and Pratt, 1992). Clinical and experimental data have revealed a number of the most promising therapeutic strategies. These include the use of $GABA_B$ receptor drugs in depression, epilepsy, spasticity and pain.

Depression

Because of their slow onset of action the clinical effectiveness of antidepressant drugs is believed to be caused by a physiological change in the brain brought about by long term use. One such change, which is consistently observed following the chronic administration of these drugs,

is down-regulation of β–adrenoceptors and 5-HT receptors (for review see Pratt and Bowery, 1992). Interestingly, increases in $GABA_B$ receptor radioligand binding to rat cerebral cortical membranes have also been described. This change follows chronic treatment with a number of different antidepressant therapies. These include both drug administration and electroshock therapy and were first described by Lloyd et al., (1985). Despite the fact that some authors have failed to reproduce these findings in toto (Cross and Horton, 1988; McManus and Greenshaw, 1991; Szekely et al., 1987) this effect remains an interesting lead. Indeed, a growing literature supports the involvement of $GABA_B$ receptor up-regulation in antidepressant treatment (see Pratt and Bowery, 1990; 1993; Lloyd, 1990), and a wide variety of antidepressant drugs have now been reported to increase $GABA_B$ receptor numbers. These include tricyclic antidepressants (amitriptyline, desipramine), 5-HT uptake inhibitors (fluoxetine, citalopram), an α2-adrenoceptor antagonist (idazoxan), antidepressant benzodiazepines (adinazolam alprazolam), compounds which increase GABA activity (progabide, fengabine), dopaminergic compounds (bupropion, nomifensine), mixed uptake blockers and monoamine oxidase inhibitors (trazodone and iprindole), and a phosphodiesterase inhibitor (rolipram). In contrast reserpine, which depletes monoamines and induces depression in humans (Goodwin et al., 1972), reduced radioligand binding to $GABA_B$ receptors, following chronic administration (for review see Lloyd, 1990).

Two studies have been performed in which a functional assay of $GABA_B$ receptor activation was investigated following antidepressant treatment. The first of these was undertaken in mouse brain following the

chronic administration of imipramine. It was found that the ability of (-)baclofen to augment β-adrenoceptor-stimulated cAMP production was enhanced (Suzdak and Giantusos 1986). The second study was undertaken in rats following chronic administration of imipramine, desmethylimipramine or maprotiline (Szekely et al., 1987). These authors reported no effect on the ability of (-)baclofen to modulate forskolin-stimulated adenylyl cyclase activity. These data suggest that up-regulation may occur in a sub-population of $GABA_B$ receptors associated with enhancement of β–adrenoceptor activity.

If the upregulation of $GABA_B$ receptors seen in these studies underlies the therapeutic activity of antidepressant drugs, then it might suggest that depressive illness was associated with decreased GABAergic tone. Indeed, Berrettini et al. (1982), observed decreased GABA concentrations in cerebrospinal fluid of untreated, depressed patients, and Petty and Sherman (1984), observed a lower than normal plasma concentration of GABA in similar patients.

More direct evidence that decreased GABAergic tone is associated with depression comes with the observation that the pro-GABA drugs, progabide and fengabine are antidepressant (Lloyd et al., 1983; 1987; Morselli et al., 1980; Musch and Garreau 1986). However, the role of GABA in depression appears to be complex since the $GABA_B$ agonist (-)baclofen has been shown to exacerbate depression in depressed patients (Post et al., 1991). Thus a number of studies have directed attention towards GABAergic systems as targets for antidepressant drugs, even though the

significance of changes in GABA levels, and regulation of receptor numbers, has yet to by established.

For many years, it was believed that the primary effect of antidepressant drugs was to cause an increase in the synaptic concentration of monoamines by blocking their uptake (Khun, 1958). It is conceivable that an up-regulation of presynaptic $GABA_B$ receptors would represent a compensatory response to this. That is to say a feedback mechanism may exist whereby elevated synaptic levels of monoamine cause a decrease in monoamine released from axon terminals. This feedback mechanism could be mediated by the inhibitory action of GABA at $GABA_B$ receptors on monoaminergic terminals. Nevertheless, the increase in monoaminergic tone caused by chronic antidepressant treatment apparently remains sufficiently great to cause down-regulation in β–adrenoceptors and 5-HT_2 receptors. The pharmacological profile of chronic antidepressant administration is thus an increase in monoamine availability, a down-regulation of postsynaptic monoamine receptors and an up-regulation of $GABA_B$ receptors, possibly located presynaptically. It is possible to propose that a $GABA_B$ receptor antagonist might induce a similar spectrum of effects. Such a compound would antagonise the action of endogenous GABA at $GABA_B$ heteroceptors. This would initially enhance monoamine release, which would in turn cause a down regulation of β-adrenoceptors and 5-HT_2, receptors. Finally, chronic administration of a $GABA_B$ antagonist might cause an upregulation in the $GABA_B$ receptors. This raises the possibility that a $GABA_B$ antagonist might possess antidepressant activity. This novel therapeutic strategy might repay investigation, since current antidepressant therapy is limited

by parasympathetic and cardiovascular side effects, and of course a delayed onset of action (Lloyd, 1990). In the present study levels of radioligand binding to GABA receptors was assessed following chronic administration of (-)baclofen, and the antagonists CGP36742 and CGP46381 to determine if the antagonists mirrored the effects of antidepressants. The ability of $GABA_B$ receptors to modulate adenylyl cyclase activity was also investigated after these chronic dosing regimes.

Epilepsy

The clinical syndrome of epilepsy is characterized by a sudden and excessive burst of activity by neurons in a particular part of the brain (Meldrum, 1990). The locality of this seizure activity within the brain gives rise to a number of different syndromes which can be differentiated clinically (for review see Meldrum, 1990). Thus, the epilepsies can be classified into either focal epilepsy (also called partial epilepsy) or generalized epilepsy. Focal epilepsy may be associated with dramatic convulsions, but generalized epilepsy is characterized by periods of complete behavioural arrest. In a few patients, a focal seizure may progress to a generalized seizure, but in general the syndromes are distinct. Focal epilepsy can be further subdivided into two categories: simple partial seizures and complex partial seizures. Simple partial seizures (also called focal cortical seizures) are cortical in origin, and, depending upon their precise location, patients may have motor, sensory, autonomic, or psychic symptoms. These seizures may spread to the hippocampus, the basal ganglia, the thalamus and brain stem. Complex

partial seizures (also called temporal lobe or limbic seizures) typically start in the temporal lobe, but usually involve other limbic structures such as the hippocampus and the amygdala.

Generalized seizures, also called absence seizures because of the absence of behavioral activity, are characterized by rhythmic and symmetrical cortical discharges. The involvement of the thalamus as well as the cortex in these discharges has been known for some time (William, 1953; 1965). The principal pathophysiology of this condition appears to involve an oscillating signal between these two structures. The site of origin of this rhythm is contested. One possibility is that a central nucleus situated in the lower brain stem is involved. This would satisfy two essential criteria, firstly, a centrally located nucleus is in an ideal situation to send signals to both hemispheres of the higher brain simultaneously. This would account for the bilateral synchronisation observed in electroencephalograph (EEG) recordings. Secondly, central, lower brain stem nuclei are involved in maintaining and adjusting states of arousal. It is therefore intriguing that the characteristics of the EEG during periods of epilepsy resemble those seen in certain states of sleep (Penfield and Jasper, 1954). However, the first of these two arguments has been countered by the observation that the bilateral synchronization might depend upon transmission through the corpus callosum rather than a common brainstem source (Meldrum, 1990). In addition Bancaud et al., (1974) has shown that absence seizures can be triggered by unilateral electrical stimulation of the frontal cortex. This led Gloor (1979) to suggest that the trigger sites are located in the cortex, with the seizure spreading subsequently to the thalamus. Nevertheless, a primary role for the

thalamus in this seizure condition is likely (Meldrum 1990), not least because of its powerful rhythm generating capabilities (Steriade and Llinas, 1988).

As the principle inhibitory neurotransmitter in the mammalian central nervous system is GABA, and as epilepsy can be viewed as hyperexcitability of central neurons, GABA has long been a focus of research for antiepileptic drugs. Interference with $GABA_A$ receptors is likely to play an important role in the initiation and spread of seizure activity and it is well known that $GABA_A$ agonists, e.g. muscimol (Enna and Beutler, 1985) and compounds that increase GABA concentration, e.g. γ–vinyl GABA, (Meldrum and Horton, 1978) or which enhance GABA function, e.g. benzodiazepines (Meldrum and Braestrup, 1984), are all anticonvulsant. Conversely $GABA_A$ antagonists such as picrotoxin and bicuculline are all convulsant (Enna and Beutler, 1985). In addition, GABA has been found to be elevated in patients with epilepsy (Woods et al., 1979), which may represent activation of an endogenous feedback mechanism, to compensate for neuronal hyperexcitability.

$GABA_B$ receptor agonists, as inhibitors of glutamate release and membrane hyperpolarizing agents, might be predicted to have anticonvulsant activity. Indeed baclofen has been shown to be anticonvulsant against a number of chemically induced seizures in rats, for example isoniazid and strychnine induced seizures (Niak et al., 1976) and kainic acid induced seizures (Ault et al., 1986). However, baclofen appears to be pro-convulsant in other models, for instance it exacerbates epileptic myoclonus in in kindled rats (Cotterell and Robertson, 1987). This

may be due to the relative importance of postsynaptic inhibition and presynaptic disinhibition in different models. These discrepancies are paralleled by electrophysiological findings, in which baclofen can inhibit epileptiform activity (Swartzwelder et al., 1986), but also can decrease inter-ictal (between seizure) events which indirectly causes an increase in ictal (seizure) events (Swartzwelder et al., 1987). Thus, the ultimate effectiveness of present $GABA_B$ receptor agonists as anticonvulsants in focal epilepsy is still unknown (Karlsson et al., 1990). This is, however, one field where the development of compounds which are specific for sub-populations of $GABA_B$ receptor may yet yield dramatic results.

(a) The Function of $GABA_B$ Receptors in the Thalamus

One of the most exciting recent developments in the field of $GABA_B$ receptor research concerns the apparent function of $GABA_B$ receptors in the thalamus. $GABA_B$ receptors apparently 'prepare' thalamocortical cells for burst firing by activating calcium currents. In such a case, GABA could no longer be considered simply an inhibitory neurotransmitter, but must be considered as an essential component in the generation of bursts of excitation. To understand how this can happen it is necessary to consider the physiology of the thalamus. Early mechanistic studies in the thalamus utilized the lateral geniculate slice, in which electrical stimulation of sensory afferent fibers (the optic tract) gave rise to an excitatory, followed by an inhibitory potential in postsynaptic cells within the lateral geniculate nucleus (Hirsch, and Burnod, 1987; Crunelli et al., 1988; Soltesz, et al., 1989). This sequence of postsynaptic potentials is essentially similar to that

which has previously been described in hippocampal pyramidal cells (Alger and Nicoll, 1982; Newberry and Nicoll, 1985). The EPSP in thalamic cells was mediated by an excitatory amino acid, probably L-glutamate. The IPSP was biphasic, a characteristic of IPSPs in many preparations. The initial phase was a dramatic hyperpolarization, which was likely to be mediated via $GABA_A$ receptors, since it had a reversal potential consistent with a chloride current (Kelly et al., 1979, Crunelli et al.,1988). This initial hyperpolarization was also reversibly blocked by bicuculline and picrotoxin at concentrations that are equally effective at inhibiting the hyperpolarization that arises from the ionophoretic application of GABA (Hirsch and Burnod, 1987; Crunelli et al., 1988; Soltesz et al.,1989). In contrast, the later and longer lasting phase of the IPSP is dependent upon potassium and was blocked by the $GABA_B$ receptor antagonists phaclofen and 2-OH-saclofen (see Bowery, 1989; Soltesz et al., 1989; 1988; Kerr et al., 1987). It also had a long latency to onset (20-50 ms) which is consistent with a G-protein activated response. The late phase of the IPSP is thus likely to be mediated by the action of GABA upon $GABA_B$ receptors. This sequence of postsynaptic potentials, an excitatory response followed by a fast $GABA_A$, and a slow $GABA_B$ receptor mediated IPSP, may be a common feature in thalamic physiology. The sequence occurs in thalamocortical cells (Soltesz et al., 1989; Hu et al., 1989), and in a high proportion of all thalamic cells (Hirsch and Burnod, 1987; Crunelli et al., 1988; Soltesz et al., 1989, Hu et al., 1989) upon electrical stimulation of the relevant cortical afferents.

(b) Voltage Dependant Effects of $GABA_B$ Receptor Stimulation Upon Calcium Channels

It is possible to ascribe a number of functions to $GABA_B$ receptors in regulating the flow of sensory information into the thalamus; $GABA_B$ receptor mediated IPSPs hyperpolarize a postsynaptic cell for periods of 50-400 msec, and thus may inhibit repetitive firing as they do in the hippocampus. Secondly, presynaptic $GABA_B$ receptors presumably exhibit a neuromodulatory role on excitatory and inhibitory neurotransmitter release. Finally, it seems that the long lasting IPSP is well suited to activate a particular type of calcium channel (for review see Steriade and Llinas, 1988). This has suggested another physiological role for $GABA_B$ receptors which is described here.

There are a variety of different calcium channels and these have been classified into two broad groups on the basis of their electrophysiological properties (Bean, 1989). These are, high voltage activated calcium channels (which include N, L and P type channels) which activate at high thresholds (positive to -20 mV), and low voltage activated calcium channels which are relevant to the present study.

Low voltage activated calcium channels are responsible for the "T" type calcium current. Prior to opening these channels need to be de-inactivated by membrane potentials greater than -75 mV, after which they become activated at potentials less than -70 mV. At resting potentials of -60 mV a single $GABA_B$ receptor-mediated, potassium dependent, IPSP will have sufficient amplitude, and duration to allow de-inactivation of these

channels (Crunelli et al., 1989). The $GABA_B$ receptor mediated IPSP also decays sufficiently rapidly to activate the calcium current, both *in vitro*, (Crunelli et al., 1989) and *in vivo* (Roy et al., 1984). Under physiological conditions, the result of the calcium dependent depolarization is often a high frequency burst of action potentials (Roy et al., 1984; Crunelli et al., 1988; Soltesz et al., 1989). This raises the intriguing possibility that these action potentials may travel in a circuit which ultimately sends a signal back to the thalamus, (Such a circuit could exist entirely within the thalamus or extend to the cortex (Morison and Basset, 1945). Indeed, systems of reciprocal innervation between the cortex and the thalamus are well described (Jones, 1983)). It is conceivable that when the signal returns to the thalamus it might generate another sequence of postsynaptic potentials similar to the first. If this is the case, then here might be the mechanism by which rhythms of activity are generated within the thalamus. Thus, areas of the thalamus might show repetitive, rhythmic activity, consisting of successive EPSPs, IPSPs, calcium depolarizations, and action potentials. An essential link in these oscillations of activity is the activation of $GABA_B$ receptors which, therefore, have a unique and important function in the thalamus. Rhythmic bursts of activity which fit this description have been recorded in many parts of the brain (Figure 1.5), they are termed spike and wave discharges and indeed they appear to emanate from the thalamus. The specific structures or cells which release the GABA responsible for activating the IPSPs have yet to be identified. GABAergic cells in the nucleus reticularis have been proposed as a key site (Steriade and Llinas, 1988). Indeed, lesions of the nucleus reticularis eliminate spontaneous long lasting hyperpolarizations in the cat

Rhythmic oscillations in membrane potential in the thalamus.

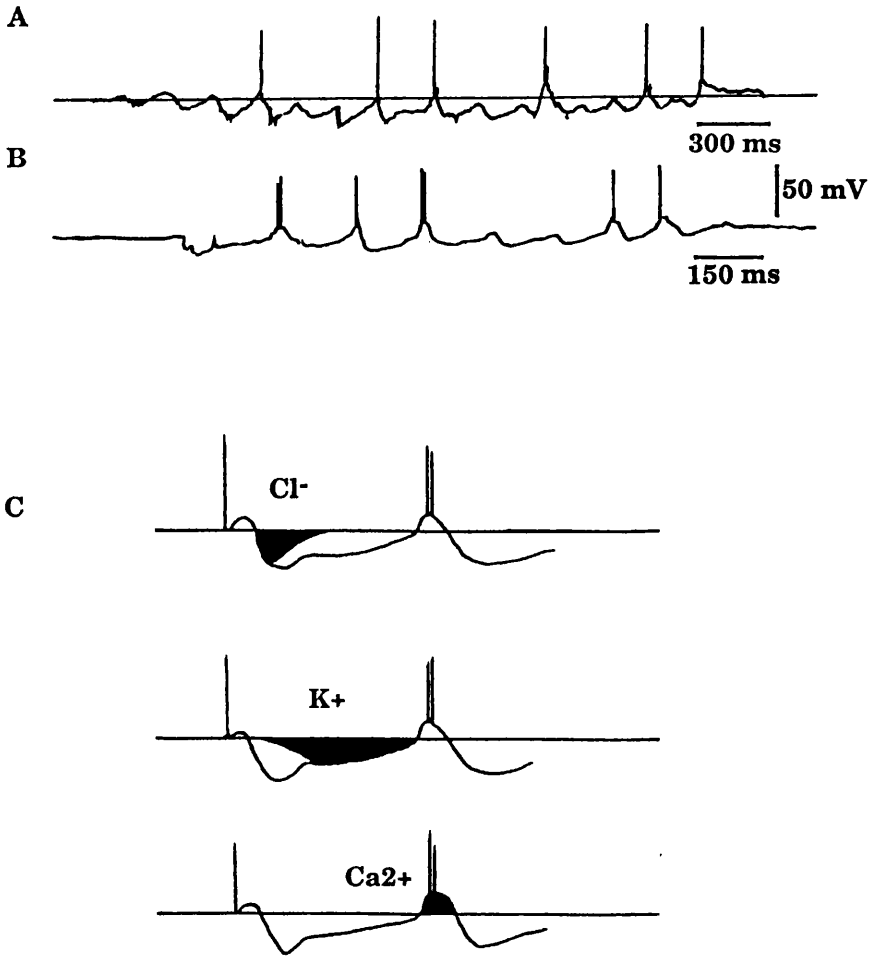


Figure 1.5. Rhythmic oscillations in membrane potential in the thalamus. (A) spontaneously occuring oscillations in membrane potential in a relay neuron from the ventral lateral thalamic nucleus of an anaesthetized cat (intracellular recording). (B) Stimulation of the motor cortex can induce a similar pattern of bursting in thalamic relay neurons. (C) interpretation of the response shown in (B) in terms of the ions responsible for the changes in potential. Diagram taken from Roy et al., 1984.

ventroposterior nuclei *in vivo* (Steriade and Llinas, 1988). In addition, cells of the nucleus reticularis are reciprocally connected to thalamocortical relay neurons of the dorsal thalamic nuclei, where powerful rhythms have been recorded. These thalamocortical cells could also provide the link by which rhythms are transmitted to the cortex. Further evidence suggesting that the nucleus reticularis is the principle site of rhythm generation includes the observation that EEG synchronization is absent in the the anterior thalamic nuclei that do not have connections with the nucleus reticularis, (Mulle et al., 1985), and EEG synchronization is also absent in relay nuclei that have had their connections with the nucleus reticularis lesioned (Steriade et al., 1985). The rhythms also persist in the nucleus reticularis after it has been isolated from other thalamic nuclei. However, $GABA_B$ receptor mediated IPSPs can be recorded in vitro in the absence of the nucleus reticularis (Hirsch and Burnod, 1987; Crunelli et al., 1988; Soltesz et al.,1989). Thus, it is also possible that the GABA responsible for IPSPs derives at least in part from local interneurons.

(c) Physiological Relevance of Spike and Wave Discharges

Spike and wave discharges have been demonstrated both in physiological and pathological conditions. For instance, they are believed to play an important role in slow wave sleep. During waking hours the rhythms are suppressed by several possible mechanisms. Such mechanisms include an increased cholinergic input from the brain stem (Hu et al., 1989; Crunelli and Leresche, 1991), increased noradrenergic tone which depolarizes the cell to around -55 mV, and thus reduces the

amplitude of the $GABA_B$ receptor mediated hyperpolarization (McCormick 1989; Crunelli and Leresche 1991), and increased intra-thalamic $GABA_B$ receptor mediated inhibition. The involvement of $GABA_B$ receptors in sleep is backed up by the observation that intravenous baclofen increases burst firing in thalamocortical cells of the ventrobasal complex (Clarke, 1983) and causes decreased arousal. Baclofen administration in humans results initially in EEG synchronization, drowsiness and sedation (see Bowery 1989; Young and Delwaide 1981b).

(d) Absence Epilepsy

Abnormal burst firing has been associated with at least one pathological condition and that is absence, or petit mal epilepsy (Huguenard and Prince, 1992). This is a type of generalized non-convulsive epilepsy that occurs mostly in children, and presents itself as momentary lapses in consciousness, during which the characteristic EEG pattern can be recorded. This EEG pattern consists of bilaterally synchronous spike and wave discharges which occur with a frequency around 3 Hz. Again it is the thalamus that plays an important role in the generation of these discharges, both in animal models (Avoli and Gloor, 1982a; 1982b; Buzsaki et al., 1988; Vergnes et al., 1987; 1990) and in human petit mal patients (William 1953). The involvement of $GABA_B$ receptors in in this type of epilepsy is confirmed by the fact that progabide, a pro GABA drug, and baclofen cause these attacks to occur more frequently in patients already susceptible to them (Gloor and Fariello, 1988).

There are a number of models of absence epilepsy, one of the most recently described is an inbred strain of rats: the Genetic Absence Epilepsy Rat from Strasbourg (GAERS). These animals express an autosomal dominant characteristic which gives rise to the symptoms of absence epilepsy (Marescaux et al., 1992c). Characteristic bilaterally synchronous spike and wave discharges, occur spontaneously when the animals are awake but quiescent. The attacks occur at a rate of about 1 per minute and have a mean duration of 17 s. During these discharges the animals become immobile yet display rhythmic twitches of their vibrissae (Lannes et al., 1988; Marescaux et al., 1984a). The brain structures involved in the generation of these spike and wave discharges are the lateral thalamic relay, the reticular nuclei and the frontoparietal cortex, (Vergnes et al., 1987; 1990; Lui et al., 1992).

These animals appear to represent a good model of petit mal epilepsy, not only in the similarity of the symptoms with the human disease but also in the types of drugs that effect the seizure condition (Marescaux et al., 1992b; 1992c). Thus, drugs such as benzodiazepines, sodium valproate, ethosuximide and trimethadone, all of which suppress petit mal seizures in humans, are also effective in this model. Anticonvulsants that specifically suppress focal or convulsive epilepsy such as carbamazepine, phenytoin and phenobarbital have little effect or aggravate the seizures. In addition, drugs that induce seizures that have been used in animal models of petit mal epilepsy all enhance the expression of seizures. These drugs include pentelenetetrazole, penicillin, γ-hydroxy butyrate, and THIP (Depaulis et al.,1988; Marescaux et al., 1984; 1987; 1984b; 1992c; Vergnes, 1982;). The most pertinent observation for the present study is that the novel

$GABA_B$ receptor antagonist CGP35348 is a potent inhibitor of seizures in these animals (Marescaux, et al., 1992a). The crucial role played by $GABA_B$ receptors in the generation of spike and wave discharges and the effectiveness of CGP35348 suggests that an enhancement of $GABA_B$ receptor function might be the primary physiological, change underlying the seizures seen in these animals. One way in which cells might become supersensitive to $GABA_B$ receptor stimulation is by over expression of this receptor. To investigate this possibility the density of GABA receptors in specific brain nuclei was investigated using receptor autoradiography.

1.7. Other Therapeutic Targets

Spasticity

The racemic mixture of baclofen (Lioresal) is already used extensively in the clinic as an antispasticity agent (Haas et al., 1985). Spasticity is a disorder of muscle tone which consists of an increase in excitability of the segmental spinal reflex arc. Reflexes in this system are initiated when skeletal muscle is stretched and the muscle spindles within it are activated. These muscle spindles activate primary 1a afferent nerves, which enter the spinal cord via the dorsal root and synapse with alpha motor neurons in the ventral horn. The alpha motor neurons innervate the same muscle from which the afferent signal is derived, when activated they cause its contraction. Physiologically, hyperexcitability of these alpha motor neurons results in an increase in tonic stretch reflexes and exaggerated tendon jerks. Clinically it results in painful and disturbing

flexor and extensor spasms which can occur either spontaneously or as a result of cutaneous stimuli. Such hyperexcitability can result from from one of a number of lesions, such as traumatic injury or multiple sclerosis. This latter disease often results in damage to a number of neuronal systems, in particular, the descending corticospinal, vestibulospinal and reticulospinal pathways. Abnormal muscle tone which does not involve this spinal reflex, such as dystonia and Parkinsonian rigidity, is specifically excluded from this definition of spacticity. The effectiveness of baclofen in spasticity is due primarily to its effect on this spinal reflex. This is demonstrated by the fact that it is as effective in patients with complete spinal transections as it is in patients with less severe lesions (Young and Delwaide, 1981a). Its mode of action appears to be the inhibition of the release of excitatory neurotransmitters (Fox et al., 1978; Ault and Evans, 1981) which presumably impinge upon the alpha motor neuron.

Pain

Systemically applied baclofen is antinociceptive and this might also be due to decreased neurotransmitter release in the spinal cord. However central administration also produces antinociception, suggesting that higher centres may be involved (Cutting and Jordan 1975; Levy and Proudfit 1977; Bartolini et al., 1981). Finally, (-)baclofen is used clinically in the treatment of trigeminal neuralgia (Terrence et al., 1985).

1.8. Summary of the Aims of the Present Study

The aims of the present study are primarily to investigate in detail several aspects of the $GABA_B$ receptor modulation of adenylyl cyclase. These include the use of newly available agonists and antagonists to characterize in detail the pharmacological profile of populations of $GABA_B$ receptors which inhibit forskolin stimulation, and augment noradrenaline-stimulated adenylyl cyclase activity. Experiments were also conducted to investigate the possible mechanisms by which this dual effect might occur, in the same sample of tissue, in particular to investigate the possibility that different cell types, such as glia and neurons, might be involved. This entailed the use of a glial cell toxin (fluorocitrate) in rat cortical slices, and performing the assay in glial cells in culture. The technique of intracerebral microdialysis was used to investigate the physiological relevance of the modulation of adenylyl cyclase activity by $GABA_B$ receptors *in vivo*.

The recent availability of orally active antagonists for the $GABA_B$ receptor prompted an investigation into changes in $GABA_B$ receptor radioligand binding and function brought about by chronic administration of these drugs.

The effectiveness of $GABA_B$ receptor antagonists in alleviating seizures in an animal model of absence epilepsy (GAERS) suggested that an enhancement in $GABA_B$ receptor function might be the primary physiological change underlying their pathology. As a consequence

receptor autoradiography for $GABA_A$ and $GABA_B$ receptors was performed on the brains of GAERS to investigate this possibility.

2
Materials and Methods

Determination of the Effects of $GABA_B$ Receptor Ligands Upon cAMP Production in Rat Cortical Slices

Male Wistar rats (body weight 250-300g) were killed by cervical dislocation and their brains rapidly removed. The cerebral cortex was dissected and cross chopped (250 x 250 μm at 90°) using a McIlwain tissue chopper. Tissue slices were suspended in Krebs Ringer Buffer (KRB) containing 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 11 mM glucose and 0.28 mM ascorbate. This suspension was gassed continuously with 95% oxygen/5% carbon dioxide, and maintained at 37°C. Cortical slices were suspended in the proportion of one cortex to 100 ml KRB, and were washed for 1 hour and 30 min in 3 changes of KRB. Prior to their addition to assay tubes, the slices were decanted into a glass test tube and allowed to settle. 50 μl aliquots (containing approximately 1 mg of protein) of the sedimented slices were then added to polyethylene tubes containing 400 μl of KRB or 350 μl KRB and 50 μl of KRB containing dissolved $GABA_B$ receptor agonist. Antagonists were tested in the presence of 30 μM (-)baclofen. Concentration/response curves for agonists and antagonists were defined with at least 6 concentrations each separated by half a log unit. All determinations were carried out in triplicate. After addition to the assay tubes, the slices were agitated briefly with a stream of 95% oxygen and allowed to equilibrate for 10 min at 37°C in the presence the test drug. Three 50 μl aliquots of the cortical slice preparation were reserved for protein determination.

The assay was commenced by the addition of 50 μl of 100 μM forskolin or 50 μl of 1 mM noradrenaline (Final concentration 10 μM and 100 μM

respectively, final assay volume 500 µl), immediately followed by agitation with a stream of 95% oxygen. Assays were routinely incubated for 10 min for pharmacological studies, and for intervals between 2 and 14 minutes for time course experiments. Each determination was carried out in triplicate. Basal cAMP levels were measured in tubes containing only tissue slices and KRB. The assay was terminated by immersing the assay tubes in a near boiling waterbath for 3 min. The contents of the cooled assay tubes were transferred to 1.5 ml Eppendorf microfuge tubes. These were centrifuged for 3 min at 3000 revolutions per minute (rpm) in an Eppendorf bench top centrifuge to remove tissue debris. Aliquots of the supernatant were stored at -20°C until assayed for cAMP content.

2.2. Assay of cAMP in Supernatant From Cortical Slices

The following assay procedure is based on the method of Brown et al., (1971)

Preparation of cAMP Binding Protein

Twenty bovine adrenal glands were collected from a local slaughter house and placed on ice. Throughout the preparation procedure, this tissue was maintained at 4 °C. The cortical tissue was dissected from the adrenal glands and added to 1.5 volumes of ice cold 50 mM Tris HCl buffer containing 0.25 M sucrose, 25 mM KCl and 5 mM $MgCl_2$ (4 °C, pH 7.4). This mixture was homogenised using a Waring blender (maximum speed for 2 min). The homogenate was centrifuged at 2000 G for 5 min in 50 ml

centrifuge tubes using a Beckman J2-21M/E refrigerated centrifuge. The supernatant was collected, pooled and redispensed into clean 50 ml centrifuge tubes, then recentrifuged at 5000 G for 15 min. This supernatant, which contained the cAMP binding protein, was pooled, thoroughly mixed and dispensed into microfuge tubes in 500 µl aliquots. These were stored at -20 °C prior to use.

Titration of Binding Protein With [^{3}H]-cAMP

To determine the concentration of binding protein at which the cAMP assay was maximally sensitive, 7 dilutions (between 5 fold and 120 fold) of binding protein were prepared in duplicate. 50 µl of these dilutions were incubated with 150 µl aliquots of 2.5 nM [^{3}H]-cAMP (adenosine 3',5'-cyclic phosphate[2,8-^{3}H] ammonium salt, 28.9 Ci.mmol^{-1}) in 50 mM Tris HCl (pH 7.4, 4°C). The final assay volume was made up to 350 µl. Under these assay conditions the optimal concentration of cAMP binding protein was considered to be a 1:40 dilution (Figure 2.1). This concentration was chosen because it was sufficiently low to allow the occupation of a relatively small proportion of the binding sites, thus conferring maximum sensitivity upon the assay (Cheng and Prussoff, 1973). This concentration of [^{3}H]-cAMP and binding protein also provided a sufficiently high number of DPM to accurately plot a standard curve

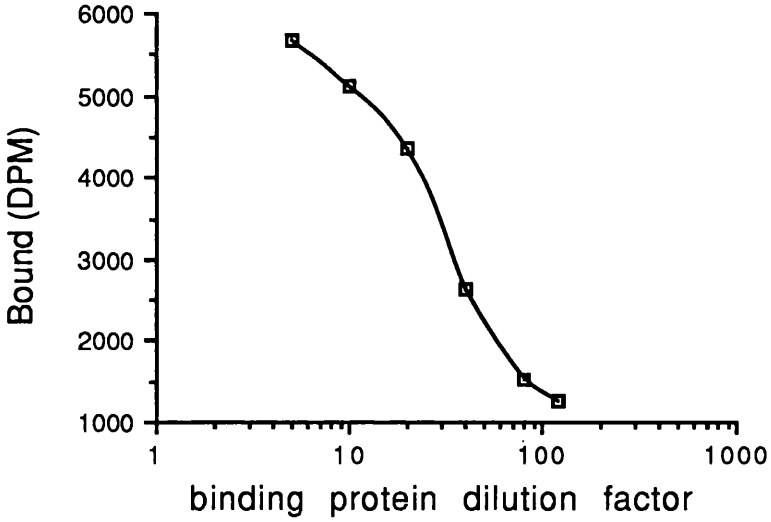


Figure 2.1. cAMP binding protein titration curve. 150 µl of 2.5 nM [^{3}H]-cAMP was incubated with increasing dilutions of cAMP binding protein in a final assay volume of 350 µl to determine a suitable dilution for routine assay conditions.

Binding Protein Assay for cAMP

Nine standard concentrations of cAMP were prepared in the concentration range 0.3 nM - 3·0 µM in distilled water. These were stored at -20 °C prior to use. The assay was set up on ice, in 1.5 ml microfuge tubes to which were added either 50 µl of standard cAMP or 50 µl of unknown sample. Standard concentrations were assayed in duplicate. Unknown samples were assayed in triplicate. 150 µl aliquots of 50 mM Tris HCl (pH 7.4) containing 2 mM theophylline (assay buffer) and 2.5 nM [^{3}H]-cAMP were dispensed into each assay tube. The binding reaction was commenced by the addition of 100 µl of binding protein (diluted 1:40 in assay buffer). Total [^{3}H]-cAMP binding was determined in the absence of cold cAMP (standard or unknown) and non specific binding was determined in the absence of binding protein. The tubes were vortex mixed and incubated overnight at 4 °C.

On the following day a 2% (wt/vol) solution of bovine serum albumin was prepared in assay buffer. Activated charcoal was suspended in this in the proportion of 1g in 10 ml. cAMP bound to the binding protein was separated from unbound cAMP by dispensing 100 µl aliquots of this suspension into all assay tubes. The tubes were rapidly vortex mixed and centrifuged for 5 min at 3000 rpm in an Eppendorf bench top centrifuge. The supernatant was decanted into 20 ml scintillation vials, to which 8 ml of Optiphase scintillation fluid was added. The scintillation vials were vortex mixed. The amount of radioactivity in the supernatant (bound) was quantified by liquid scintillation spectrophotometry for 3 min in an LKB

Standard curve for the assay of cAMP.

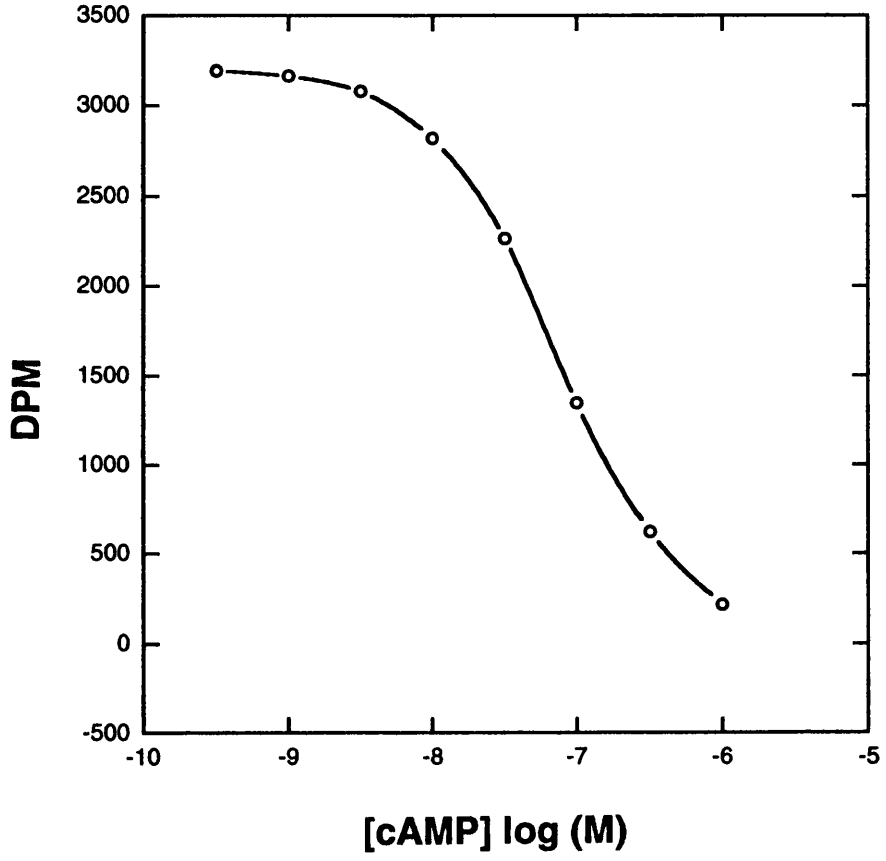


Figure 2.2. Standard curve for the assay of cAMP. A typical log concentration/inhibition curve plotted for known concentrations of cold cAMP. against 1 nM [^{3}H]-cAMP. Standard concentrations were assayed in duplicate, data are from a single experiment.

1219 Rackbeta liquid scintillation counter (42% efficiency). The "radioimmunoassay" iterative curve fitting program on the Rackbeta counter was utilised to convert disintegrations per minute (DPM) in the unknown samples to concentration of cAMP. This was achieved by reference to the standard concentrations. A typical standard curve is shown in Figure 2.2.

Results were initially expressed as pmol of cAMP per mg protein (pmol mg^{-1}). The half maximally effective molar concentration (EC_{50}) was estimated for each of the drugs tested by fitting an asymmetric sigmoid curve to the data using a least squares curve fitting function in a commercially available data analysis package ("Fig P", Fig. Perfect Research Software, Nashville) for an IBM PC. The EC_{50} was converted to a pEC_{50} (negative logarithm of the EC_{50}). All data represents the mean of at least 3 separate experiments. When expressed in graph form, data from pharmacological studies were transformed to percent inhibition of the forskolin response or percent enhancement of the noradrenaline response. This was to normalise variation between assays.

Protein Assay

The amount of cortical tissue used in the cAMP assay was estimated by protein determination. The method used was that of Bradford (1976). This assay is based on the binding of an azo dye "Coomassie Brilliant Blue G-250" to protein, causing a shift in the absorbance maximum of the dye from 465 nm to 595 nm. Briefly, 100 mg of Coomassie Blue dye was

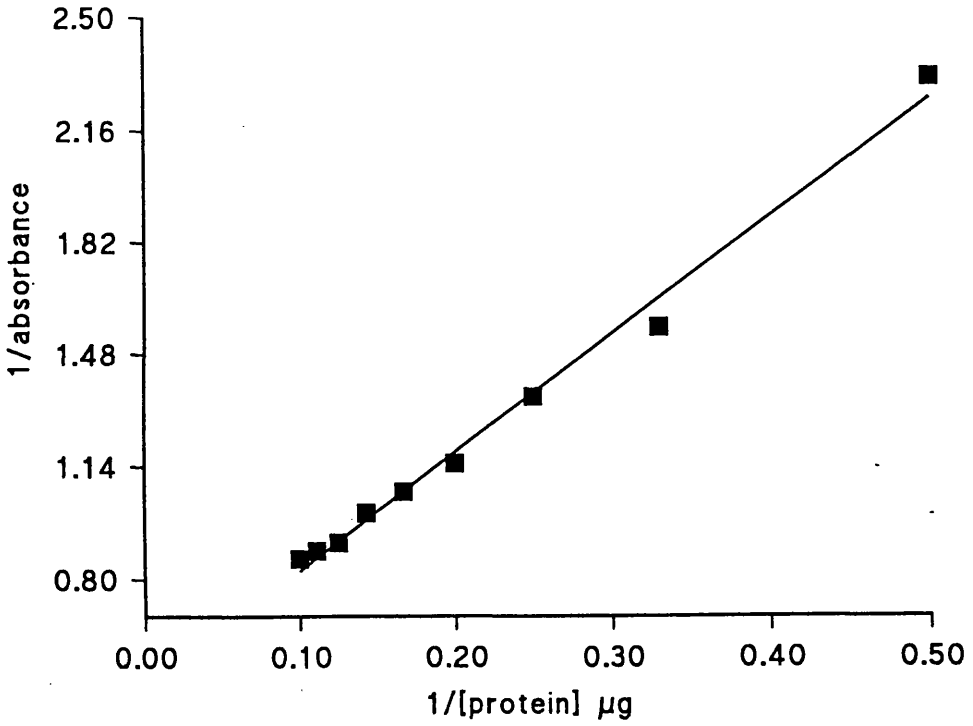


Figure 2.3. Standard curve for the assay of protein concentrations. This double reciprocal plot was prepared using known concentrations of bovine serum albumin, and was used to assay the concentraion of protein in rat cortical slice preparations. Data represents triplicate determinations from a single typical assay.

dissolved in 50 ml of 95% ethanol. 100 ml of 85% (w/v) phosphoric acid was added to this and the solution was ultimately diluted to a final volume of 1 litre with water. For the assay, 1 ml aliquots of this solution were added to either 100 µl of unknown sample or 100 µl of a standard concentration of protein. Absorbance was read at 595 nM using a Cecil CE505 double beam ultraviolet spectrophotometer. The concentration of protein in unknown samples was estimated by comparison with a standard curve prepared from 10 concentrations of bovine serum albumin ranging from 10 to 100 µg ml^{-1}. A typical standard curve is shown in Figure 2.3.

The Involvement of Glial Cells in cAMP Overflow

(a) Experiments with Fluorocitrate

Cross chopped cortical slices were prepared from rat cerebral cortex, as described previously. These were washed for 10 minutes in KRB in the proportion of 1 cortex in 100 ml. The KRB was aspirated and replaced, whilst the suspension was turbulent it was divided into 2 aliquots. Fluorocitrate (final concentration 0.1 mM) was added to one aliquot, whilst the second aliquot (control) received an equivalent quantity of KRB. The cortical slices were maintained in these solutions, at 37 °C with continuous oxygenation for 2 hours. After this period the slices were rinsed in 3 changes of KRB. This was followed by another 30 min incubation period. The slices were further rinsed in 3 changes of KRB after which they were allowed to sediment and dispensed into assay tubes as before.

(b) cAMP Production in Cultured Glial Cells

Cultures of astrocytes were grown to confluency (approximately 4 days) in 24 well "multiwell" plates. On the day of the experiment the growing medium was aspirated from the wells and the cells were rinsed in three changes of KRB (37 °C). After the addition of 2 ml of KRB, the multiwell plates were returned to the incubator. After a 2 hour incubation period the plates were removed and the incubation medium aspirated. The cells were washed in three changes of KRB. KRB (2 ml) was again pipetted into each of the wells. On this occasion, the wells where the effect of $GABA_B$ receptor stimulation was to be investigated received KRB containing 0.1 mM (-)baclofen. This was followed by a 10 min pre-incubation. The cAMP generating stage of the assay was commenced by the addition of forskolin (final concentration 10 μM) or noradrenaline (final concentration 100 μM). Each of these compounds was added to wells containing (-)baclofen and wells without. Basal cAMP was determined in the remaining wells. The plate was incubated under these conditions for 10 minutes after which the incubation was terminated by floating the multiwell plate on the surface of a near boiling water bath. Up to this point the plates had been maintained at 37 °C throughout the experiment. Cells were then lysed by floating the plate in a bath sonicator for 3 min. Finally the contents of the wells were vigorously mixed with a pipette before being transferred to microfuge tubes. These were centrifuged for 5 minutes at 3000 rpm in a bench top centrifuge to remove tissue debris. The supernatant from this was assayed for cAMP using the binding protein assay.

Materials

The following materials were obtained from the specialist suppliers in brackets: Rats (Bantin Kingman); [^{3}H]-cAMP (New England Nuclear); Charcoal "Norit GSX" (BDH); bovine serum albumin "fraction V" (Sigma, Cat No. a-4503) and Optiphase (LKB Scintillation Products). All of the $GABA_B$ receptor active material used in this study ((-)baclofen, SKF97541, 3-APA, CGP47656, CGP44533, CGP44532, CGP49311A, CGP46381, CGP45025, CGP35348, CGP45397, CGP36742) was obtained from Ciba Geigy. Astrocyte cell cultures were provided by Dr Brian Pearce of this department. All other materials were obtained from commercial sources.

2.4. Intra Cerebral Microdialysis Methods

Dialysis Probe Construction

The shaft of the probe was constructed from a 20 mm length of 24 gauge stainless steel tubing. Into this was inserted two lengths of fused silica tubing (internal diameter (id) 40 μm, outer diameter (od) 140 μm). At the lower (dialysing) end of the probe, one length of this tubing terminated 10 mm short of the end of the steel tube, the other terminated 4 mm beyond. At the upper (external) end of the probe, inlet and outlet ducts were created by individually inserting the two fused silica tubes into each of two 10 mm lengths of 27 gauge stainless steel tubing. The two pieces of fused silica tubing were sealed into the three pieces of stainless steel tubing at this "Y"

junction with epoxy resin, (Figure 2.4). This junction was given structural rigidity with dental acrylic. The dialysing membrane was constructed from an 10 mm length of cuprophan capillary membrane (MW cut off at 10,000). This was threaded over the exposed length of fused silica tubing at the lower end of the probe, and into the lumen of steel tubing constituting the shaft. Epoxy resin was used to seal this membrane to the steel shaft, and to plug its lower end. Perfusion lines were made from 100 mm lengths of polythene tubing (id 0.28 mm, od 0.61 mm), these were pushed onto the inlet and outlet ducts, and the junctions were sealed with hot melt adhesive. A schematic diagram of the completed probe is shown in Figure 2.5.

Dialysis Probe Implantation

All dialysis probes were implanted into the frontal cortex.

Male wistar rats of 250-300g body weight were anaesthetised with 400 mg kg^{-1} chloral hydrate i.p. and placed in a stereotaxic frame. A short sagittal incision was made in the skin above the dorsal surface of the cranium, and the skin and superficial tissue were reflected to reveal the bones beneath. Apertures were made in the right parietal and left frontal bones using a dentist's burr. Stainless steel screws were driven into these openings to assist in securing the dialysis probe. Stereotaxic co-ordinates (obtained initially from Paxinos and Watson (1986), and later confirmed by dissection) were used to locate a spot on the right frontal bone vertically above the right frontal cortex and anterior to the caudate nucleus. An aperture was opened at this point using the burr, taking care not to damage the tissue immediately beneath. The membranes enclosing the brain (dura

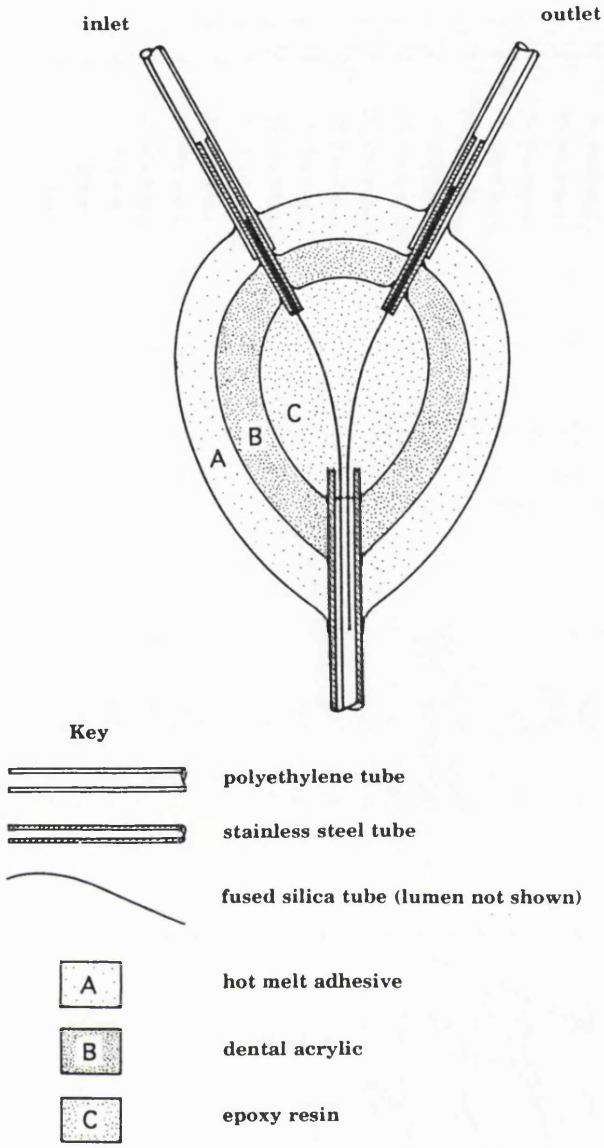


Figure 2.4. Construction of dialysis probe. Longitudinal section through a dialysis probe showing the method of construction. Joints between the fine bore silica tubing and the stainless steel tube are sealed using epoxy resin (C). Dental acrylic gives the whole junction structural rigidity (B) and hot melt adhesive seals the junction between the stainless steal tubing and the polyethylene perfusion lines (A).

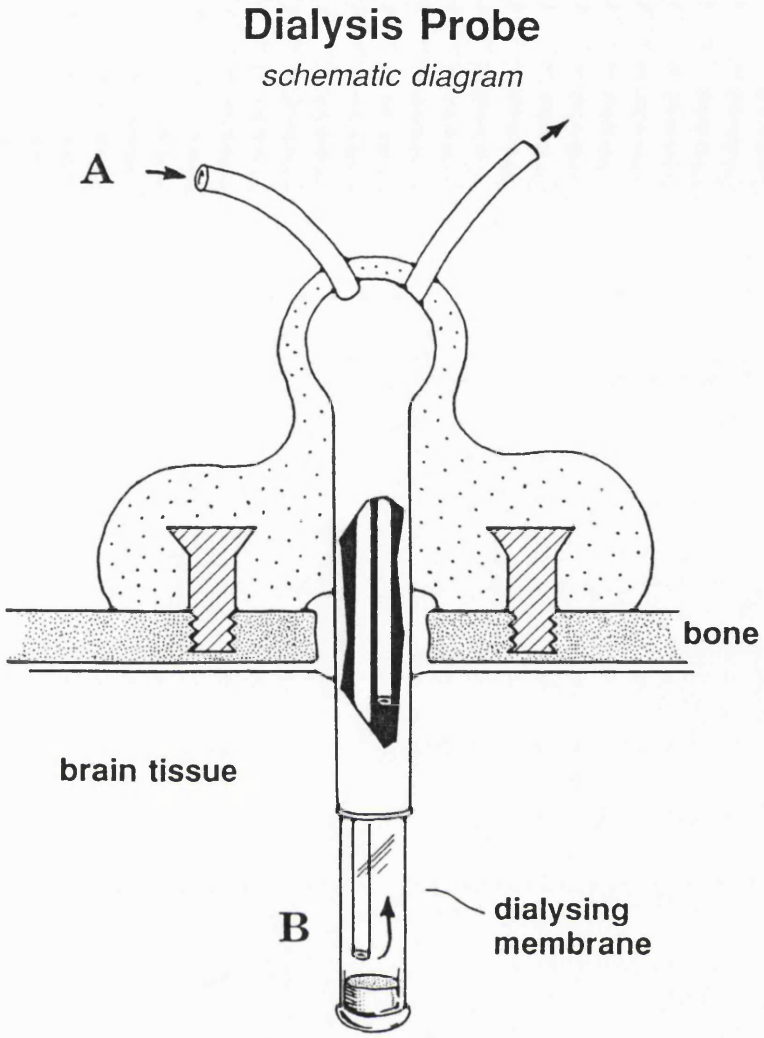


Figure 2.5. Schematic diagram of a dialysis probe. The perfusing solution enters the probe at (A) and flows through the lumen of the silica glass tubing to the tip of the probe. Upon leaving the silica glass tube (B) it flows back up the outside of the silica glass tubing, wetting the inside of the dialysing membrane. It proceeds into the lumen of the steel shaft of the probe from whence it is collected by the shorter length of silica glass tubing and delivered to the outflowing perfusion line.

mater and pia mater) were cut through at this point using the sharp tip of a hypodermic needle.

A new dialysis probe was filled with artificial cerebro-spinal fluid (ACSF) containing NaCl 124 mM, KCl 5.4 mM, $MgSO_4$ 0.6 mM, $NaHCO_3$ 25 mM, KH_2PO_4 1 mM, Glucose 6 mM and $CaCl_2$ 1.3 mM, pH 7.4 and filtered through a sterile single use filter unit (pore size 0.2 μm) immediately prior to use. Each probe was checked for leaks and blockages before implantation. The tip of the probe was then inserted vertically into the rat brain, 3.9 mm anterior to bregma, 1.5 mm right of centre and 5 mm below dura, using the stereotaxic frame. The probe was secured in this position using dental acrylic, which also assisted in closing the wound. The openings in the polythene tubing used to perfuse the dialysis probe were temporarily plugged. The rat was removed from the stereotaxic frame and allowed to recover overnight in the cage in which the experiment would subsequently be performed. The rats were constrained in cubic perspex cages (side length 260 mm) in which they were given free access to food and water. The animals were admitted to the cage via a hinged lid, in the centre of which was a circular hole 25 mm in diameter through which the perfusion lines were threaded. The lines were kept taught by means of counter weights.

Forskolin-Stimulated Adenylyl Cyclase *in Vivo*

A 500 μl gas tight Hamilton syringe was filled with ACSF and attached to a Harvard Apparatus syringe infusion pump "22". This was

used to perfuse the implanted dialysis probe via 500 mm of fine bore polythene tubing (i.d. 0.28 mm, o.d. 0.61 mm). This tubing was connected to the inlet tube of the dialysis probe with a 10 mm length of wider diameter tubing (i.d. 0.58 mm, o.d. 0.96 mm). Experimental manipulations in the composition of the perfusate were made by changing over perfusion systems at this point. The probe was perfused at a rate of 2 μl min^{-1}, and the rat remained unanaesthetized, and restricted only by the confines of the experimental cage for the duration of the experiment. Fractions were collected for 30 min periods, in 1.5 ml microfuge tubes via a 300 mm length of carnegie perfusion line. The initial 30 min fraction was collected and analysed for cAMP, but it gave high and variable results and was not used in subsequent analysis. The second fraction was designated "fraction 1", and subsequent fractions were numbered sequentially. Fractions 1 to 3 were used to establish a baseline level of cAMP in the dialysate. At the beginning of the fraction 4, adenylyl cyclase was stimulated by changing the perfusate from ACSF to ACSF containing 100 μM forskolin. After 30 min the perfusate was changed back to control ACSF. This initial period of stimulation (S1) was used to normalise the experimental data. The second period of stimulation (S2) was commenced at the beginning of fraction 9 and again lasted for 30 min. For this period the ACSF contained either 100 μM forskolin (control experiments) or 100 μM forskolin plus one of a range of concentrations of (-)baclofen (0.01 - 10.0 mM). The experiment was terminated after fraction 11 and the animal destroyed. The placement of the probe was confirmed by dissection. The effect of (-)baclofen alone was determined by introducing the highest concentration used (10 mM) into the probe at fraction 4, and continuing to perfuse with this concentration of (-)baclofen until fraction 6 when the experiment was terminated. The

fractions of dialysate were stored at -20 °C until assayed for cAMP content, using a binding protein assay (Brown et al., 1971, described in a previous section.). Each 60 µl fraction allowed a single cAMP determination. The results were expressed graphically either as the concentration of cAMP (nM) in each fraction or as a percentage of baseline cAMP. The effect of (-)baclofen upon forskolin-stimulated cAMP was determined by summing the molar concentrations of cAMP in those fractions in which cAMP was elevated (fraction 4 and 5 for S1 and fractions 9 and 10 for S2), and subtracting the baseline cAMP levels (taken as the mean of the two fractions preceding the period of stimulation) the elevation in cAMP concentration for the S2 stimulation was then divided by the elevation in cAMP concentration for the S2 stimulation (S2/S1 ratio). All experiments were conducted at least in triplicate.

Noradrenaline-Stimulated Adenylyl Cyclase *in Vivo*

To determine the effect of (-)baclofen on noradrenaline-stimulated cAMP production the following modifications were made to the forskolin method: the dialysis probe was perfused at a rate of 1.2 µl min^{-1} and fractions were collected for 10 min periods; adenylyl cyclase was stimulated with 10 mM noradrenaline by changing over the perfusion system at the beginning of fraction 6 for 10 min. The slow perfusion rate and the short periods of fraction collection however meant that the new ACSF did not reach the dialysing membrane until the beginning of fraction 7. The effect of (-)baclofen on noradrenaline-stimulated adenylyl cyclase activity was investigated by perfusing with ACSF containing 10 mM noradrenaline and

10 mM (-)baclofen, the effect of (-)baclofen alone was determined by perfusing the probe with 10 mM (-)baclofen for fractions 6 to 12. The pooled samples from 5 individual rats allowed a single piont determination of cAMP concentration. Experiments were conducted in triplicate (15 rats). The results were expressed graphically as concentration of cAMP (nM) in each fraction.*

Materials

The following items mentioned in the methods section were obtained from the specialist suppliers in brackets: 24 gauge stainless steel tubing and 27 gauge stainless steel tubing (Tomlinson Tube and Instrument Ltd.); fused silica tubing (Scientific Glass Engineering), dental acrylic "Duralay" (Health Co.), cuprophan capillary membrane (Armstrong Bradley), polythene tubing (Portex Ltd.), hot melt adhesive (RS Components), skull screws, 1/4x1B (the Clerckenwell Screw Co), filters for sterilising ACSF (Sartorius "minisart"), Hamilton gastight syringes (V.A. Howe), Carnegie perfusion line (Carnegie Medicine Inc). Rats (Bantin Kingman) and (-)baclofen (Ciba Geigy). The cages in which the dialysis was performed were made up by Dave Tullet on site.

* The effects of (-)baclofen upon noradrenaline-stimulated cAMP were assessed by comparing the levels of cAMP at each time point with ANOVA followed by Dunetts multiple range test, and by estimating the area under the elevated portion of each curve, comparing these means with a two way Students t-test.

2.5. Autoradiography Methods

Tissue Preparation

Male wistar rats (250 to 300 body weight) were anaesthetized with sodium pentobarbitone (0.4 $mg.kg^{-1}$) and perfuse/fixed with 200 ml of 0.1% paraformaldehyde in 0.01 M phosphate buffered saline (4 °C), administered transcardially. Those parts of the brain contained within the cranium (from the olfactory bulb to the brain stem) were dissected on ice, trimmed and mounted on a cryostat chuck using O.C.T. mounting medium. The brain and chuck were then rapidly frozen by immersion in isopentane (-35 °C) before being transferred to a -70 °C freezer, where they were stored for at least 18 hours.

New microscope slides were cleaned by soaking in 0.2 M hydrochloric acid in 95% industrial methylated spirits for 5 min. The slides were thoroughly dried in an oven. Thus cleaned the microscope slides were submersed in 0.5% (w/v) gelatine solution containing 0.05% chromic potassium sulphate for 1 min and dried in a warm oven.

Brain tissue and microscope slides were placed into the cryostat chamber 30 min before section cutting commenced to allow their temperature to equilibrate to -20 °C. Successive 10 μm thick sections were cut from the tissue either in the parasagittal or transverse plane and thaw mounted onto microscope slides, one per slide. The mounted sections were allowed to dry at room temperature for one hour before storage at -20 °C.

Binding Experiments.

The binding experiments were carried out as described by Bowery et al. (1987; 1984) and Chu et al. (1987) who have determined optimal conditions for the binding of [^{3}H]-GABA binding to sections of rat brain. This technique is used routinely in this laboratory. Sections of brain tissue were thawed at room temperature and washed for 60 min in two changes of 50 mM Tris HCl (pH 7.4, wash and assay buffer), after which the sections were dried at room temperature. Each section was encircled with a line from a "Pap pen" to retain the incubation medium, after which they were incubated with radioligand. This was achieved by pipetting 100 µl of assay buffer containing 50 nM [^{3}H]-GABA (96.4 Ci/mmol) onto the section. $GABA_B$ receptors were labelled in the presence of 2.5 mM $CaCl_2$ and 40 µM isoguvacine, non specific binding was determined on an adjacent section in the presence of 100 µM (-)baclofen. $GABA_A$ receptors where labelled in the presence of 100 µM (-)baclofen and absence of $CaCl_2$, non-specific binding was determined in the presence of 100 µM isoguvacine.

After 20 min the incubation was terminated by aspirating the incubation medium and washing away non-specific binding. For $GABA_B$ receptor radioligand binding the sections were agitated for 3s in each of 2 baths of ice cold assay buffer (1, 2, 3 and 5 s were used for preliminary "off rate" studies, Figure 2.6) and 2 x 15 s for $GABA_A$ binding (2.5, 5, 7.5, 10 and 15 s were used in preliminary "off rate" studies, Figure 2.7). The sections were then rinsed briefly in ice cold distilled water, excess liquid was again

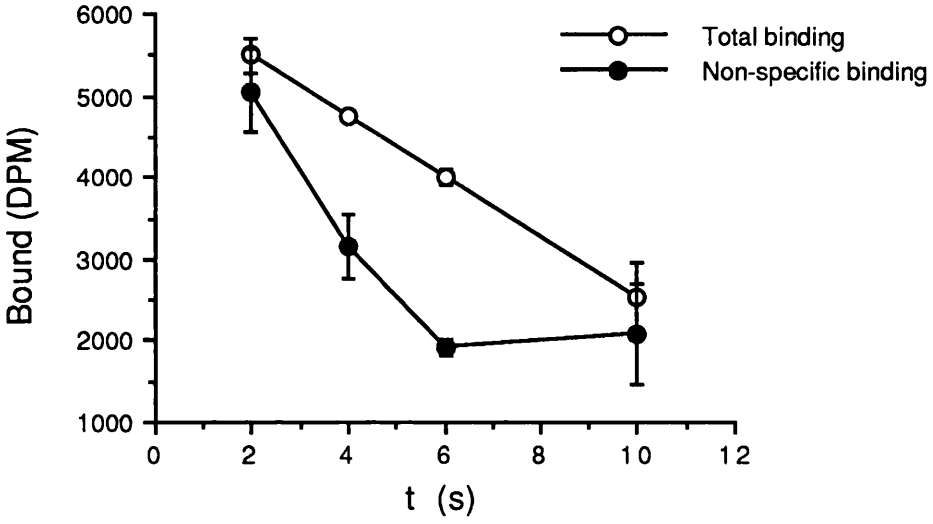


Figure 2.6. The effect of washing upon $GABA_B$ receptor radioligand binding in sections. [^{3}H]-GABA was applied to sections in the presence of 100 μM isoguvacine and in the presence and absence of 40 μM (-)baclofen. Slides were incubated for 20 minutes before being washed for 2, 4, 6 and 10 s. Slides were subsequently immersed in scintillation fluid in 20 ml scintillation vials, soaked over night and vortex mixed, before the radioactivity in each vial was estimated in a Beta-counter. Experiments were performed in triplicate, this graph represents the data from a single typical experiment.

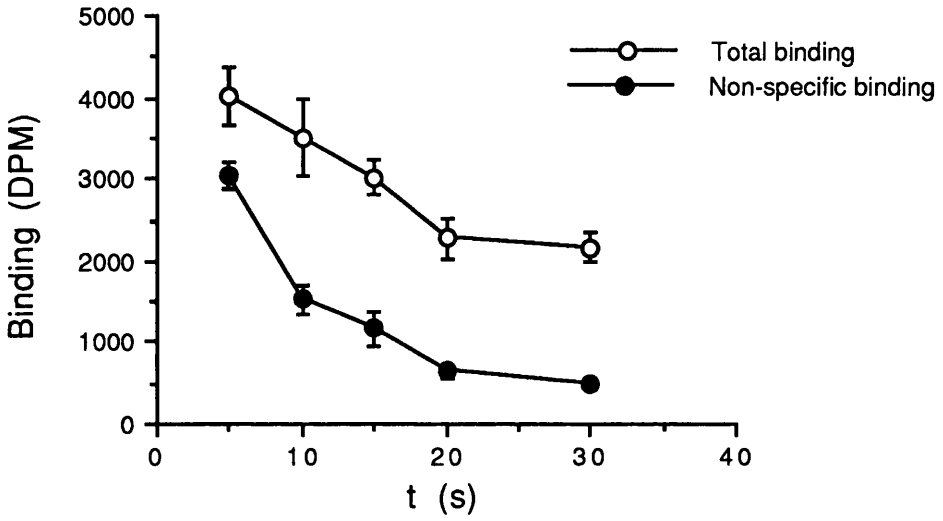


Figure 2.7. The effect of washing upon $GABA_A$ receptor radioligand binding in sections. [^{3}H]-GABA was applied to sections in the presence of 100 μM (-)baclofen and in the presence and absence of 40 μM isoguvacine. Slides were incubated for 20 minutes before being washed for 5, 10, 15, 20 and 30 s. Slides were subsequently immersed in scintillation fluid in 20 ml scintillation vials, soaked over night and vortex mixed, before the radioactivity in each vial was estimated in a Beta-counter. Experiments were performed in triplicate, this graph represents the data from a single typical experiment.

aspirated from the surface of the sections, before they were laid in a stream of warm air to dry.

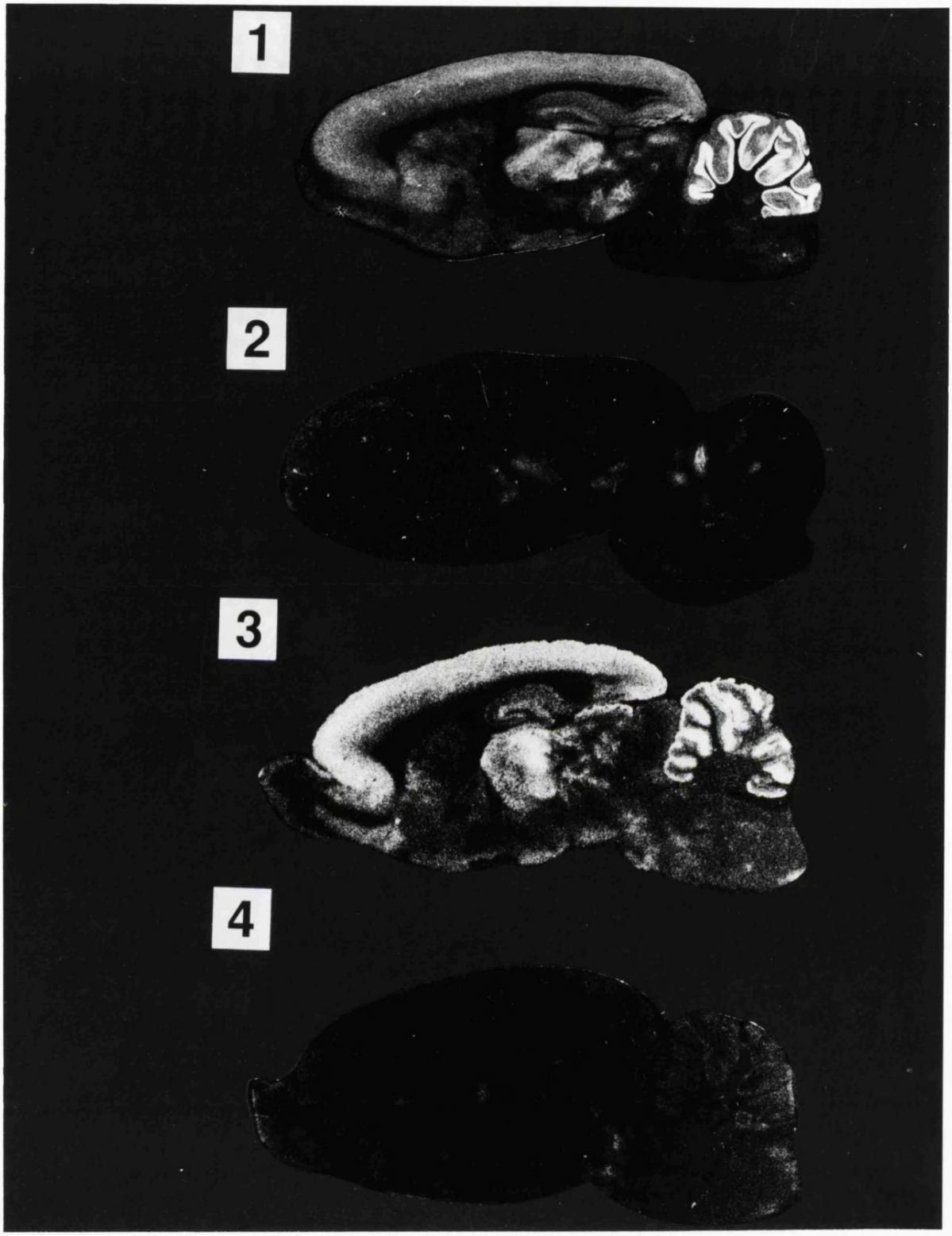
Bound radioactivity was visualised by apposing the sections to tritium sensitive Hyperfilm-^{3}H for 3 to 4 weeks. The latent images thus produced were developed in Kodak D-19 for 2 min at 20 °C and fixed in Kodak Unifix for 4 min.

The optical density of specific areas of the radiographic images were quantified using a Quantimet 970 image analyser. These readings were converted to mCi of radio activity per mg of protein by reference to polymer standards.* Specifically bound radiolabel was calculated by subtracting non-specific binding. The non-specific value was determined from the relevant section in precisely the same location as the total. Data were ultimately expressed as fmol specific [^{3}H]-GABA binding per mg of protein. The anatomical location of brain regions selected for study were identified using "The rat brain in stereotaxic co-ordinates" (Paxinos and Watson, 1986). Each data point represents the mean of three densitometer readings taken from each structure, from each of three rat brains (9 readings, n=3).

Dark field images of representative autoradiographs were produced by photographically "printing" the autoradiographs. To do this the tritium sensitive film was used as a negative and printed onto Kodak resin coated photographic paper. This was processed with Ilford "Ilfospeed" chemicals. Typical images for total and non-specific binding for $GABA_A$ and $GABA_B$ binding are shown in Figure 2.8.

* Tritium micro-scales (Amersham)

2.8. Dark field autoradiographs of [^{3}H]-GABA binding to $GABA_A$ and $GABA_B$ receptors. Binding was performed in parasagittal sections of rat brain approximately 1.8 mm from the midline. The binding reaction was carried out at room temperature for 20 min, using 50 nM [^{3}H]-GABA. $GABA_B$ receptors were labelled selectively in the presence of 40 µM isoguvacine (3), non-specific binding was defined in the presence of 100 µM (-)baclofen (4). $GABA_A$ receptors were labelled selectively in the presence of 100 µM (-)baclofen (1), non-specific binding was defined with 40 µM isoguvacine (2).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2
3
4

2.6. Preparation of the Brains of Genetic Absence Epilepsy Rats

The brains from three rats which *in vivo* had shown the characteristic spike and wave discharges of absence epilepsy, and three animals from the same strain which did not show this characteristic, were obtained from Dr. C. Marescaux. These brains had been rapidly dissected from animals after decapitation and were stored at -70 °C in transit and storage. 10 μm thick parasagittal sections of these rat brains were cut by cryostat, and $GABA_A$ and $GABA_B$ receptor autoradiography was performed as previously described.

Materials

The following items mentioned in the text were obtained from the specialist suppliers in brackets: pentobarbital (Sagatal) 60 mg/ml (RMB Animal Health Ltd); O.C.T mounting compound (TissueTek); premium microscope slides, 76x26x1.2 mm (BDH); the Pap pen was a gift of Miles Ltd. (UK); [^{3}H]-GABA (4-animo-n-[2,3-^{3}H]butyric acid), radioactive polymer standards and Hyperfilm-^{3}H (Amersham International plc., Amersham, UK); isoguvacine (Cambridge Research Biochemicals); baclofen (CGP11973A) was a gift from Ciba Geigy; D-19 developer, photographic fixative, and Photographic paper (Kodabrom II RC) were manufactured by Kodak and obtained from local suppliers, Ilfospeed photographic chemicals were manufactured by Ilford, and obtained from the same supplier. The frozen Genetic Absence Epilepsy Rat brains were the kind gift of Dr C. Marescaux at the Clinique Neurologique, C.H.U. in Strasbourg. All other

items are commercially available and were purchased from suppliers of laboratory materials.

2.7. Chronic Dosing of Rats with $GABA_B$ Receptor Active Compounds.

Twenty four rats (male, wistar, body weight 230-250g) were randomly assigned to four experimental groups. Each experimental group received either (-)Baclofen, CGP36742 or CGP46381. These compounds were dissolved in sterile physiological saline solution and injected intraperitoneally daily. (-)Baclofen was administered at a dose of 10 mg kg^{-1} day^{-1}, whilst CGP36742 and CGP46381 were administered at a dose of 100 mg kg^{-1} day^{-1}. The fourth group of animals received injections of the saline vehicle alone. This treatment regime was continued for a period of three weeks. Twenty four hours after the last injection the animals were killed. From each experimental group, three of the animals were processed to provide brain tissue for $GABA_B$ receptor autoradiography, and three of the animals were processed to provide cortical slices for the cAMP overflow assay (both of these procedures have been described previously).

3

The Pharmacology of $GABA_B$ Receptor Mediated Modulation of Forskolin- and Noradrenaline-Stimulated Adenylyl Cyclase Activity *In Vitro*

3.1. Introduction

In recent years, the number of available drugs with which to investigate $GABA_B$ receptor mediated responses has increased dramatically. It was thus considered apposite to investigate the potency of a variety of these new compounds in biochemical assays of $GABA_B$ receptor function. Two closely related biochemical correlates of $GABA_B$ receptor activation were selected for this study, namely the inhibition of forskolin-stimulated adenylyl cyclase activity, and the augmentation of noradrenaline-stimulated adenylyl cyclase activity. The compounds selected for this study included $GABA_B$ receptor agonists, such as the classical agonists (-)baclofen, SKF97541 and 3-APA, as well as several novel compounds (CGP47656, CGP44533 and CGP44532). $GABA_B$ receptor antagonists were also tested for their ability to antagonise the modulatory responses caused by (-)baclofen. The antagonists selected for study included recently published compounds (CGP46381, CGP35348 and CGP36742) as well as novel compounds (CGP49311A, CGP45025 and CGP45397). A detailed pharmacological profile, allowing a comparison of these 2 responses, was thus undertaken for the first time.

3.2. Results

Characterisation of Forskolin- and Noradrenaline-Stimulated Adenylyl Cyclase Activity in Rat Cortical Slices

Both forskolin and noradrenaline were found to concentration dependently stimulate cAMP production in rat cortical slices (Figure 3.1). Forskolin had little or no effect on cAMP production at 1 μM, but at higher concentrations had a dramatic effect; 0.1 mM produced the highest levels of adenylyl cyclase stimulation (1750 % of basal levels). This appeared to be a maximally effective concentration. Noradrenaline was ineffective at 1 μM and 10 μM, however 100 μM produced over 80% of the maximum response to noradrenaline, which was seen at 1 mM (480% above basal levels).

Time Dependence of cAMP Production

Time course studies were undertaken to investigate the linearity and rate of production of cAMP in this assay system. In the presence of 10 μM forskolin, the rate of cAMP production was found to be 36.9 ± 14.0 pmol mg protein^{-1} min^{-1} (mean ± standard error of the means (SEM), n=10). In the presence of 100 μM noradrenaline, the rate of cAMP production was 13.5 ± 7.9 pmol mg protein^{-1} min^{-1} (n=13). The rate of cAMP production in the presence of forskolin and noradrenaline proved to be linear under the these assay conditions for at least the first 12 minutes of the incubation

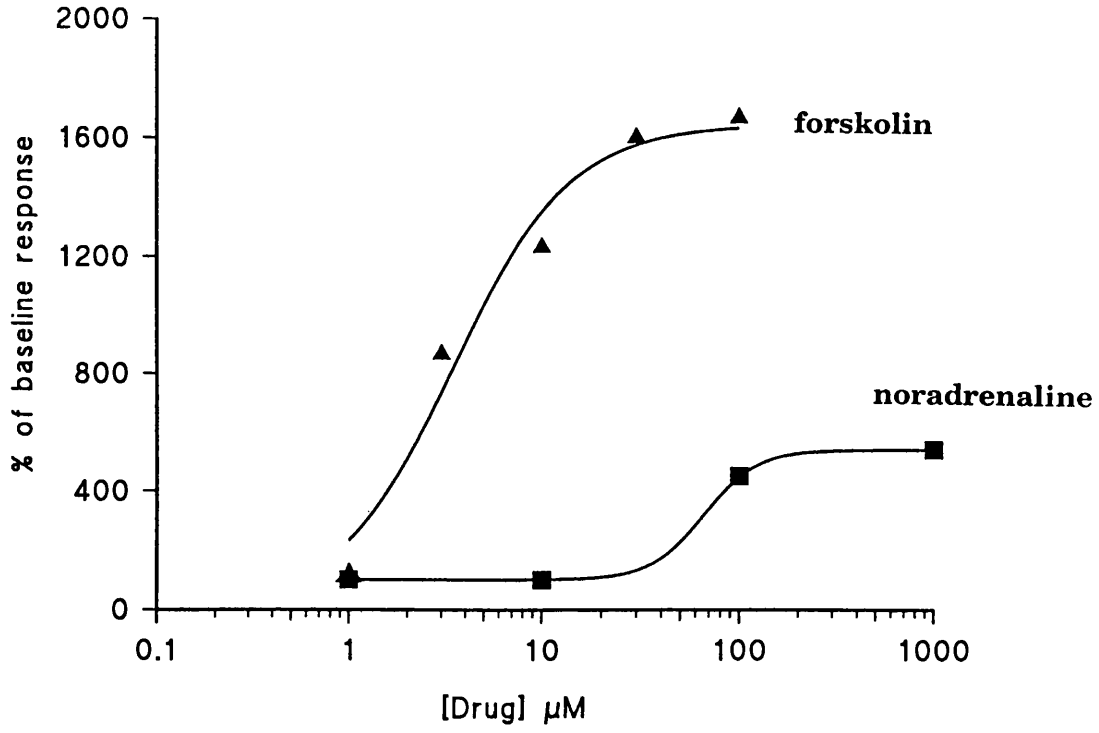


Figure 3.1. Dose dependency of forskolin- and noradrenaline-stimulated cAMP production. These concentration/response curves were obtained by incubating aliquots of rat cortical slices with increasing concentrations of forskolin or noradrenaline for 10 min. Both compounds showed a concentration dependent increase in the amont of cAMP recorded in the supernatant. Graphs represent results from a single typical experiment.

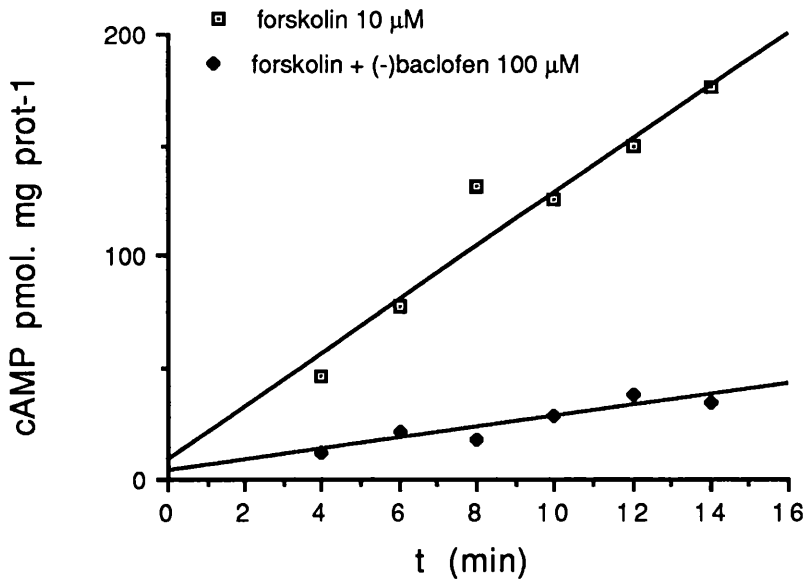


Figure 3.2. Time course for the generation of forskolin-stimulated cAMP in rat cortical slices. Adenylyl cyclase was stimulated by the addition of 10 μM forskolin to the incubation medium. Under these conditions the production of cAMP was linear for at least 14 min. The rate of cAMP production was reduced, but remained linear, in the presence of 100 μM (-)baclofen. Time points were assayed in triplicate, the graph represents data from a single typical experiment.

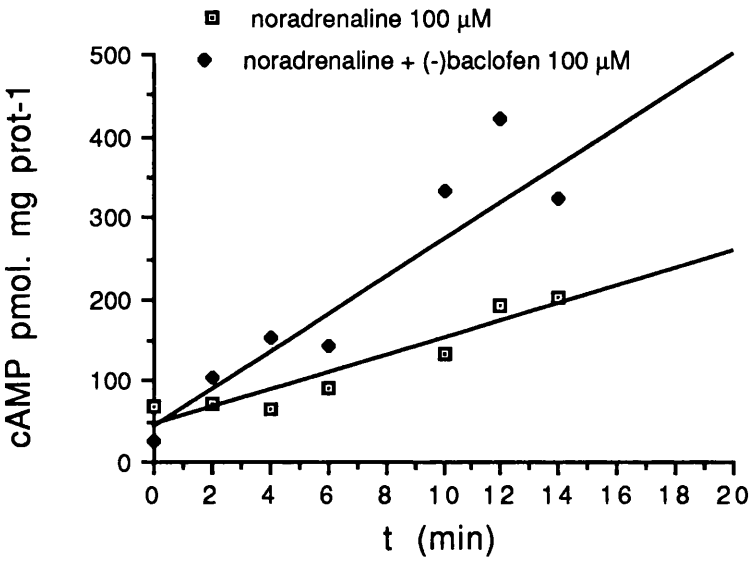


Figure 3.3. Time course for the generation of noradrenaline-stimulated cAMP in rat cortical slices. Adenylyl cyclase was stimulated by the addition of 100 µM noradrenaline to the incubation medium. Under these conditions the production of cAMP was linear for at least 14 min. The rate of cAMP production was enhanced, but remained linear, in the presence of 100 µM (-)baclofen. Time points were assayed in triplicate, the graph represents data from a single typical experiment.

period (Figure 3.2 and 3.3). Basal levels of cAMP were 18.2 ± 2.2 pmol mg protein^{-1} and did not increase significantly during the period of the incubation. This value represents approximately 5% of the cAMP assayed in tissue slices after incubation with 10 μM forskolin for 10 min (normal assay conditions).

Characterisation of the Modulation of Adenylyl Cyclase Responses by (-)Baclofen

Concentrations of forskolin (10 μM) and noradrenaline (100 μM) which produced near maximal stimulation of cAMP levels were selected as standard assay conditions. These conditions were used to investigate a range of concentrations of (-)baclofen to characterise the modulation of these two responses. (-)Baclofen was found to concentration dependently inhibit forskolin-stimulated adenylyl cyclase activity with a pEC_{50} of 6.07 ± 0.29 (mean, ± SEM, n=3, Figure 3.4), and to augment noradrenaline-stimulated adenylyl cyclase activity with a pEC_{50} of 5.04 ± 0.17 (n=3, Figure 3.5). Time course experiments were conducted in which a near maximally effective concentration of (-)baclofen (100 μM) was included in the incubation medium. (-)Baclofen was thus seen to have a similar modulatory effect at all the time points at which it was tested (Figure 3.2 and 3.3). The rate of production of cAMP remained linear in the presence of (-)baclofen for the duration of the experiment. The inclusion of 100 μM (-)baclofen in the incubation medium reduced forskolin-stimulated cAMP production by 40.3 ± 4.57 % (n=10). 100 μM (-)Baclofen increased noradrenaline-stimulated cAMP by 119 ± 28.5 % (n=13).

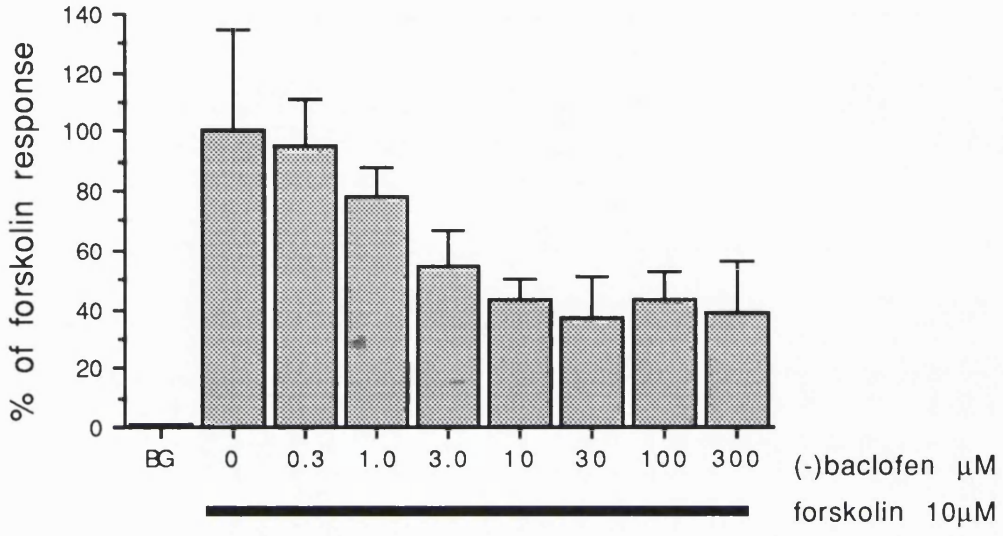


Figure 3.4. The effect of (-)baclofen upon forskolin-stimulated cAMP production in rat cortical slices. This concentration response curve was obtained by incubating slices for 10 min with increasing concentrations of (-)baclofen in the presence of 10 μM forskolin. The maximum inhibition caused by (-)baclofen occurred at around 10 μM, after which there was no further inhibition. Graph represents the mean data from 3 separate experiments, which have been normalised by expressing results as the percentage of the response elicited by 10 μM forskolin. BG = background.

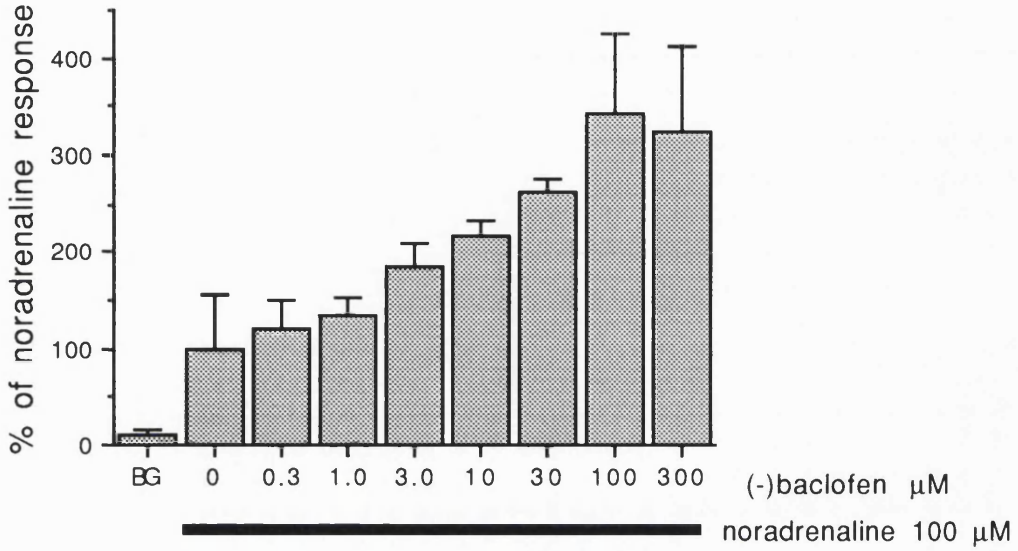


Figure 3.5. The effect of (-)baclofen upon noradrenaline-stimulated cAMP production in rat cortical slices. This concentration response curve was obtained by incubating slices for 10 min with increasing concentrations of (-)baclofen in the presence of 100 μM noradrenaline. The maximum effect caused by (-)baclofen occured at 100 μM, after which there was no further enhancement. Graph represents the mean data from 3 separate experiments, which have been normalised by expressing results as the percentage of the response elicited by 100 μM noradrenaline. BG = Background.

Characterisation of $GABA_B$ Receptor Agonists as Modulators of Adenylyl Cyclase Activity

Five $GABA_B$ receptor agonists (SKF97541, 3-APA, CGP44532, CGP44533 and CGP47656) were tested for their ability to mimic (-)baclofen as modulators of adenylyl cyclase activity. All of the compounds were able to inhibit forskolin-stimulated adenylyl cyclase activity with a similar maximum response to 100 μM (-)baclofen (which was included as a control in each assay). The potency of these agonists spanned approximately one order of magnitude, the highest potency was displayed by SKF97541 (pEC_{50} = 6.66), and the lowest potency by CGP44532 (pEC_{50} = 5.63)(Table 3.1, Figures 3.6 to 3.10).

As augmenters of noradrenaline-stimulated adenylyl cyclase activity, the agonists ranged in potency approximately 1.5 orders of magnitude, from SKF97541 (pEC_{50} = 6.11) to CGP47656 (pEC_{50} = 4.48) (Table 3.1). One compound (CGP47656) produced a maximum response that was significantly smaller than the response to 100 μM (-)baclofen. Thus, CGP47656 produced a 1.39-fold increase in noradrenaline-stimulated cAMP production, whereas baclofen produced a 2.36-fold increase (ANOVA followed by Duncans multiple range test, $p<0.05$, Figure 3.7). Nevertheless CGP47656 gave a similar maximum response to (-)baclofen as an inhibitor of forskolin-stimulated adenylyl cyclase activity (Figure 3.7). The remaining compounds (SKF97541, 3-APA, CGP44532 and CGP44533) all produced similar maximum responses to 100 μM (-)baclofen as

	Forskolin		Noradrenaline	
Compound	pEC_{50}	SEM	pEC_{50}	SEM
SKF97541	6.66	0.52	6.11	0.32
CGP47656	6.44	0.05	4.48*	0.26
(-)baclofen	6.07	0.29	5.04*	0.17
CGP44533	5.67	0.45	4.94	0.32
3-APA	5.66	0.38	5.01	0.20
CGP44532	5.63	0.03	5.54	0.32

Table 3.1. The potency of $GABA_B$ receptor agonists as modulators of adenylyl cyclase activity. $GABA_B$ receptor agonists were tested for their ability to modulate forskolin and noradrenaline-stimulated adenylyl cyclase activity. Concentration response curves were defined with at least 7 concentrations in duplicate at half decade intervals. Parameters for the concentration response curve were estimated by fitting a logistic equation to the data from each experiment using a least squares curve fitting program in "Fig-P" on an IBM compatible PC. The geometric mean of the half maximally effective concentration is expressed as a pEC_{50} and is shown in the left hand column for each assay condition, the SEM for these values is reported in the right hand column. * significantly different from potency from the forskolin assay $p<0.05$ two way Students t-test).

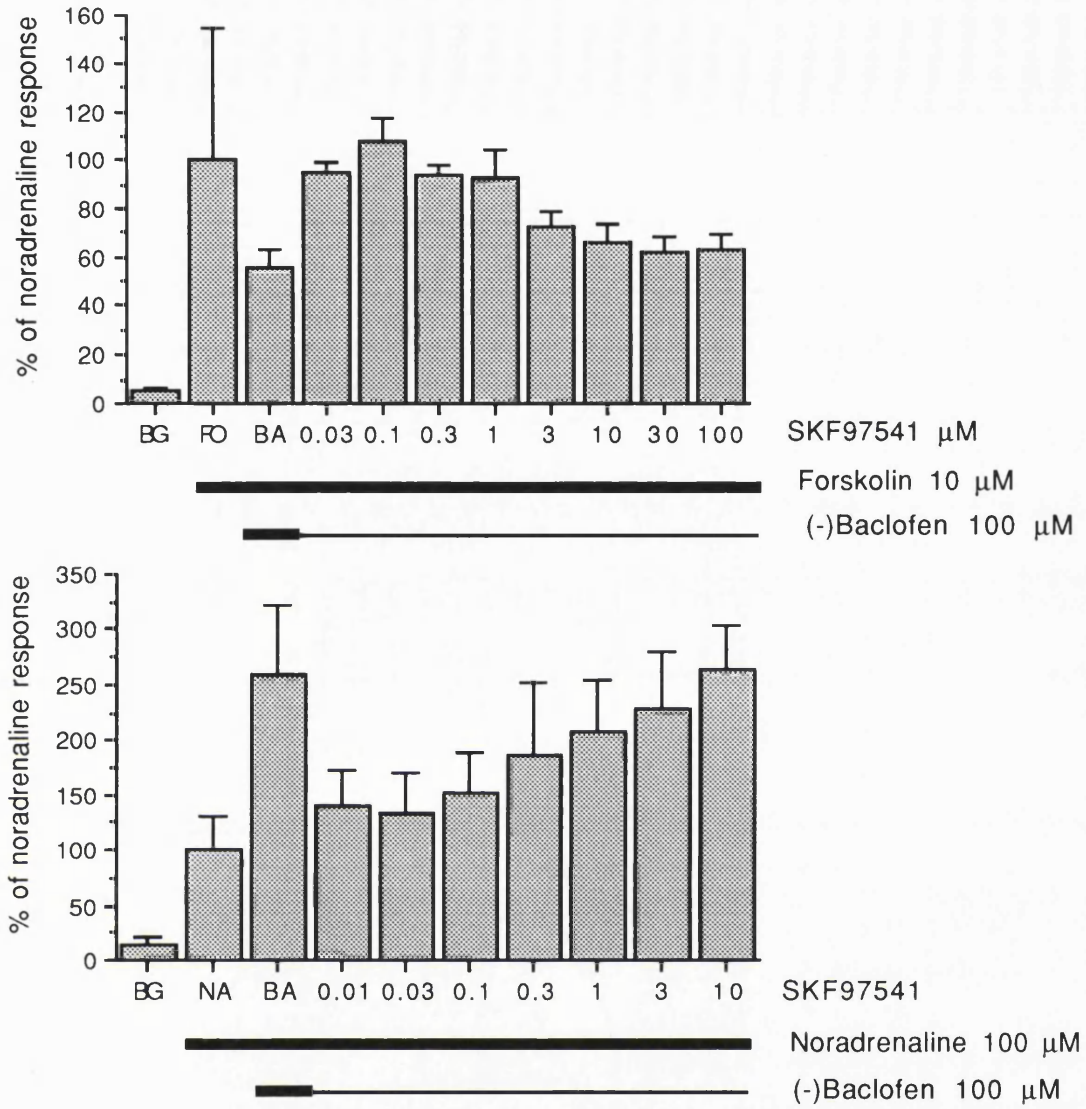


Figure 3.6. Modulation of forskolin- and noradrenaline-stimulated adenylyl cyclase activity by SKF97541. This concentration/effect curve was obtained by incubating increasing concentrations of SKF97541 with rat cortical slices in the presence of forskolin (10µM)(upper graph, n=7) and noradrenaline (100µM)(lower graph, n=7). BG = background, FO=forskolin, NA = noradrenaline, BA = (-)baclofen in the presence of either forskolin or noradrenaline.

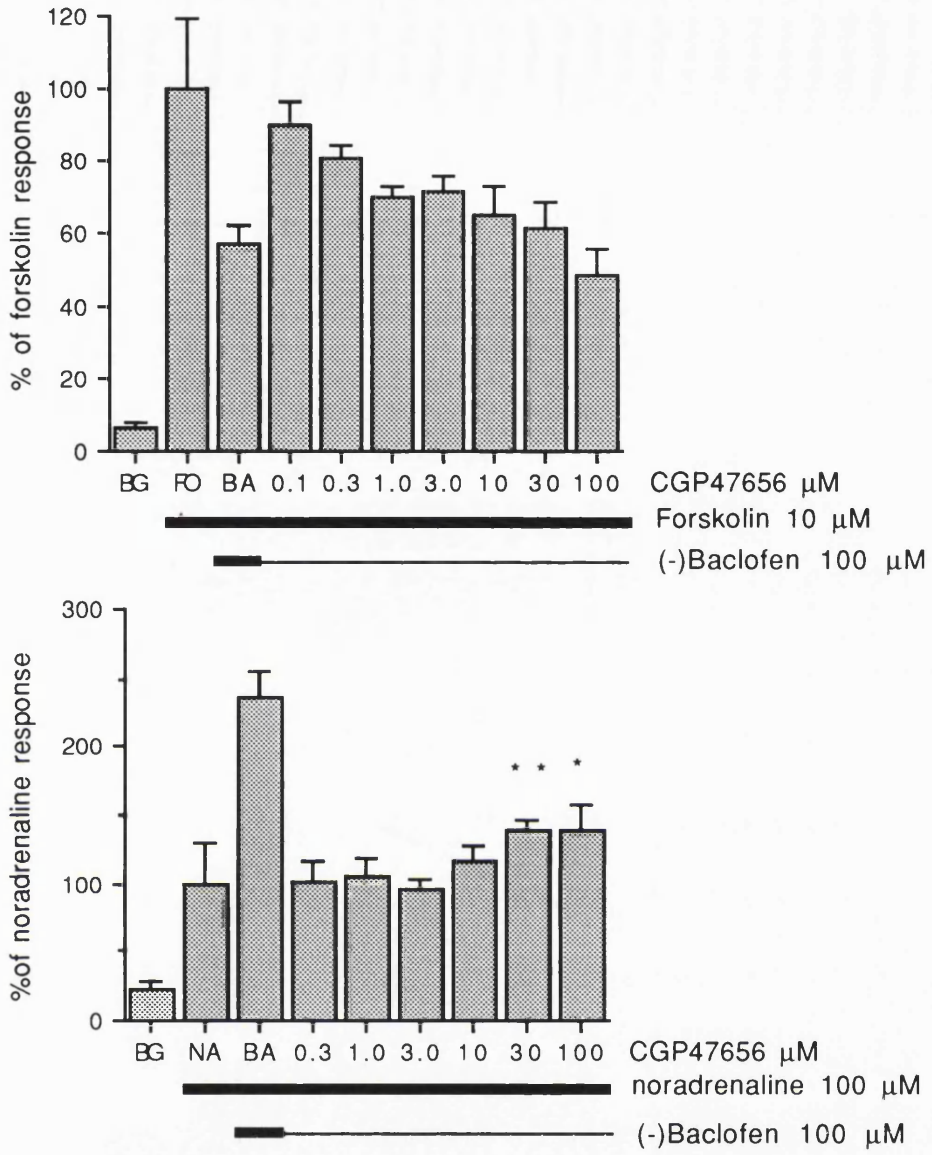


Figure 3.7. Modulation of forskolin- and noradrenaline-stimulated adenylyl cyclase activity by CGP47656. This concentration/effect curve was obtained by incubating increasing concentrations of CGP47656 with rat cortical slices in the presence of forskolin (10µM)(upper graph, n=3) and noradrenaline (100µM)(lower graph, n=3). BG = background, FO=forskolin, NA = noradrenaline, BA = (-)baclofen in the presence of either forskolin or noradrenaline.

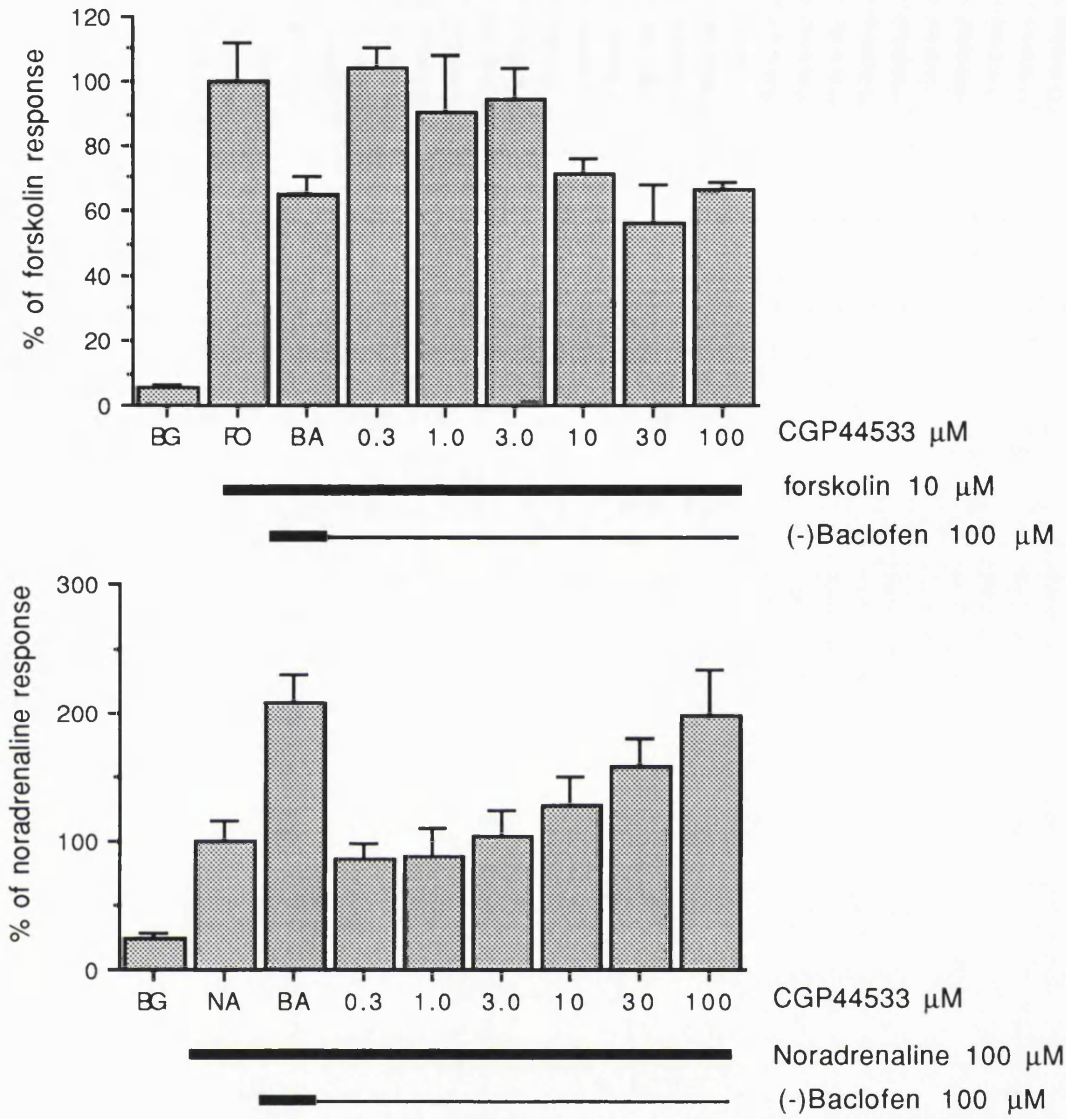


Figure 3.8. Modulation of forskolin- and noradrenaline-stimulated adenylyl cyclase activity by CGP44533. This concentration/effect curve was obtained by incubating increasing concentrations of CGP44533 with rat cortical slices in the presence of forskolin (10µM)(upper graph, n=3) and noradrenaline (100µM)(lower graph, n=4). BG = background, FO=forskolin, NA = noradrenaline, BA = (-)baclofen in the presence of either forskolin or noradrenaline.

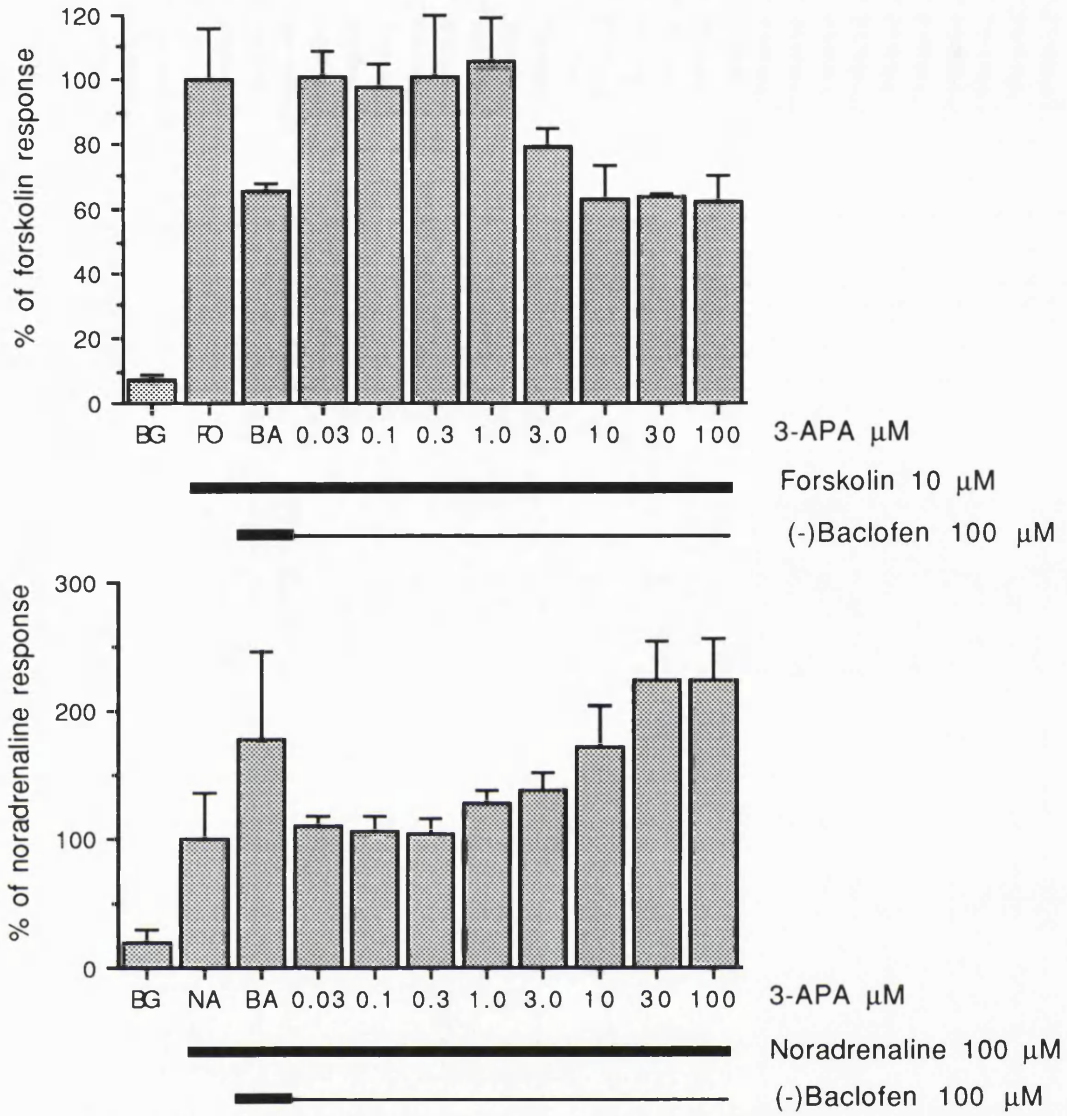


Figure 3.9. Modulation of forskolin- and noradrenaline-stimulated adenylyl cyclase activity by 3-APA. This concentration/effect curve was obtained by incubating increasing concentrations of 3-APA with rat cortical slices in the presence of forskolin (10μM)(upper graph, n=3) and noradrenaline (100μM)(lower graph, n=4). BG = background, FO=forskolin, NA = noradrenaline, BA = (-)baclofen in the presence of either forskolin or noradrenaline.

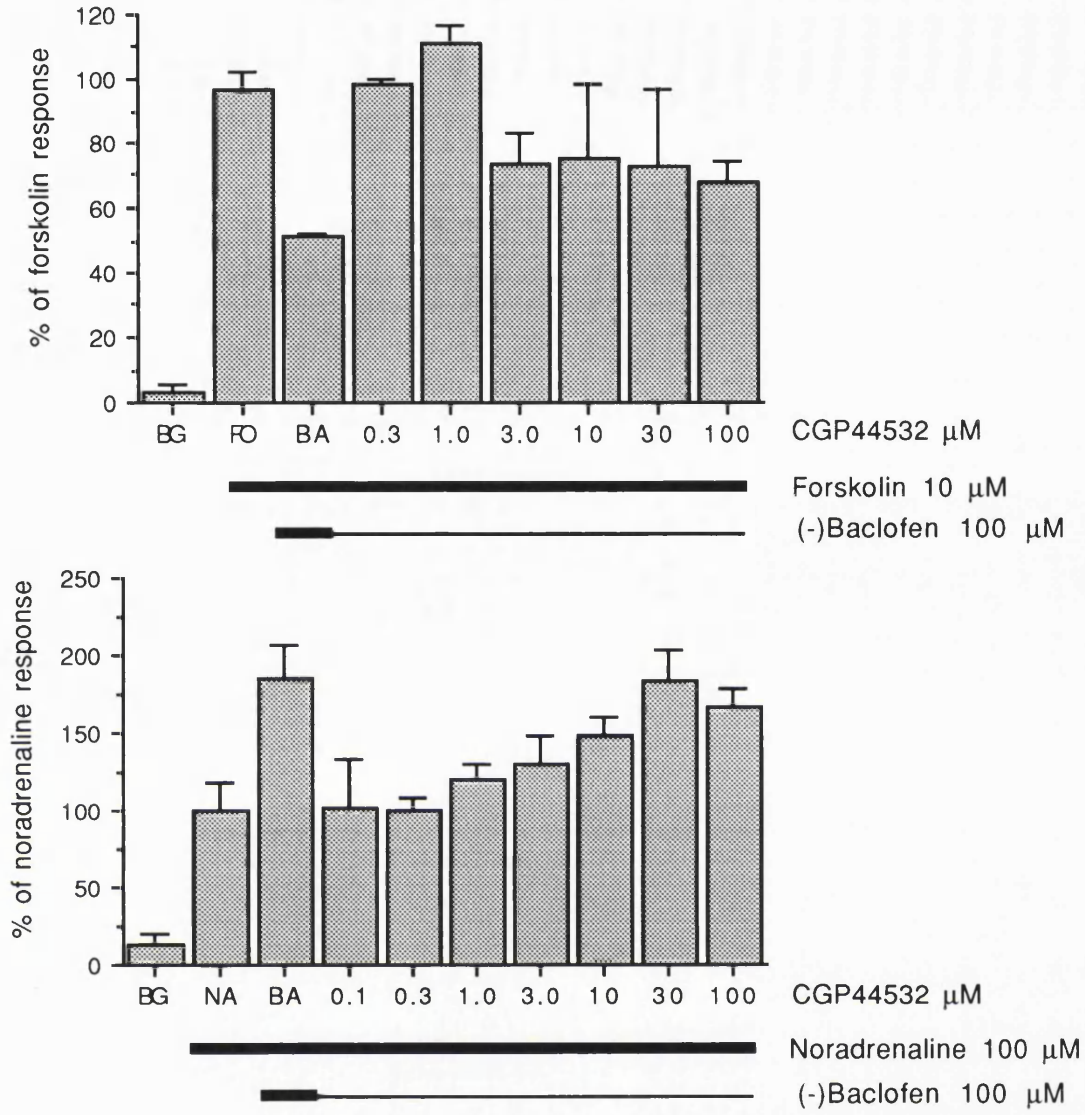


Figure 3.10. Modulation of forskolin- and noradrenaline-stimulated adenylyl cyclase activity by CGP44532. This concentration/effect curve was obtained by incubating increasing concentrations of CGP44532 with rat cortical slices in the presence of forskolin (10µM)(upper graph, n=3) and noradrenaline (100µM)(lower graph, n=3). BG = background, FO=forskolin, NA = noradrenaline, BA = (-)baclofen in the presence of either forskolin or noradrenaline.

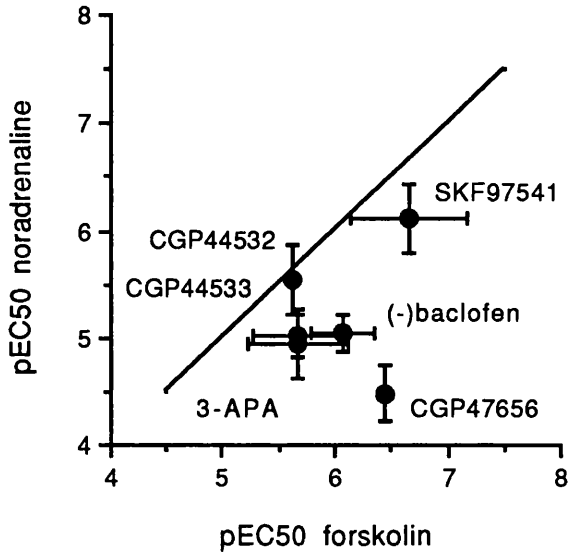


Figure 3.11. Correlation graph of the potency of $GABA_B$ receptor agonists as modulators of forskolin- and noradrenaline-stimulated adenylyl cyclase activity. Data represents the mean of at least three separate experiments. The line of identity is indicated on the graph.

augmenters of noradrenaline-stimulated adenylyl cyclase activity (Figures 3.6 to 3.10).

All of the $GABA_B$ receptor agonists were less potent in their ability to enhance noradrenaline-stimulated cAMP production than in their ability to inhibit forskolin-stimulated cAMP production (Figure 3.11). This difference achieved statistical significance (ANOVA followed by Duncans multiple range test, $p<0.05$) for two of these compounds. Thus, (-)baclofen enhanced noradrenaline-stimulated cAMP production with a potency approximately one order of magnitude lower than it inhibited forskolin-stimulated cAMP production. Similarly, CGP47656 augmented noradrenaline-stimulated cAMP production nearly two orders of magnitude less potently than it inhibited forskolin-stimulated cAMP production (Table 3.1).

Characterization of $GABA_B$ Receptor Antagonists on $GABA_B$ Receptor Mediated Modulation of Adenylyl Cyclase

A range of $GABA_B$ receptor antagonists were tested for their ability to antagonise the effects of 30 μM (-)baclofen under these assay conditions. The highest concentration of each of the compounds tested gave inhibition of the (-)baclofen response which was not significantly different from 100% inhibition. The pEC_{50} for each of these compounds at inhibiting the (-)baclofen mediated inhibition of forskolin-stimulated adenylyl cyclase assay ranged from 5.45 for CGP49311A to 3.87 for CGP36742. Concentraton response curves for these $GABA_B$ receptor antagonists, are illustrated in

	Forskolin		Noradrenaline	
Compound	pEC_{50}	SEM	pEC_{50}	SEM
CGP49311A	5.45	0.30	5.55	0.32
CGP46381	4.98	1.02	4.95	0.53
CGP45024	4.54	0.30	5.31	0.65
CGP35348	4.49	0.48	4.70	0.27
CGP45397	4.12	0.23	4.96	0.33
CGP36742	3.87	0.16	4.81	0.55

Table 3.2. Potency of $GABA_B$ receptor antagonists against the (-)baclofen modulation of adenylyl cyclase activity. $GABA_B$ receptor antagonists were tested for their ability to inhibit the modulation of forskolin- and noradrenaline-stimulated adenylyl cyclase brought about by (-)baclofen. Dose response curves were defined with at least 7 concentrations in duplicate at half decade intervals. Parameters for the dose response curves were estimated by fitting a logistic equation to the data from each experiment using a least squares curve fitting program in Fig-P on an IBM compatible PC. The geometric mean of the half maximally effective concentration is expressed as a pEC_{50} and is shown in the left hand column for each assay condition, the SEM for these values is reported in the right hand column.

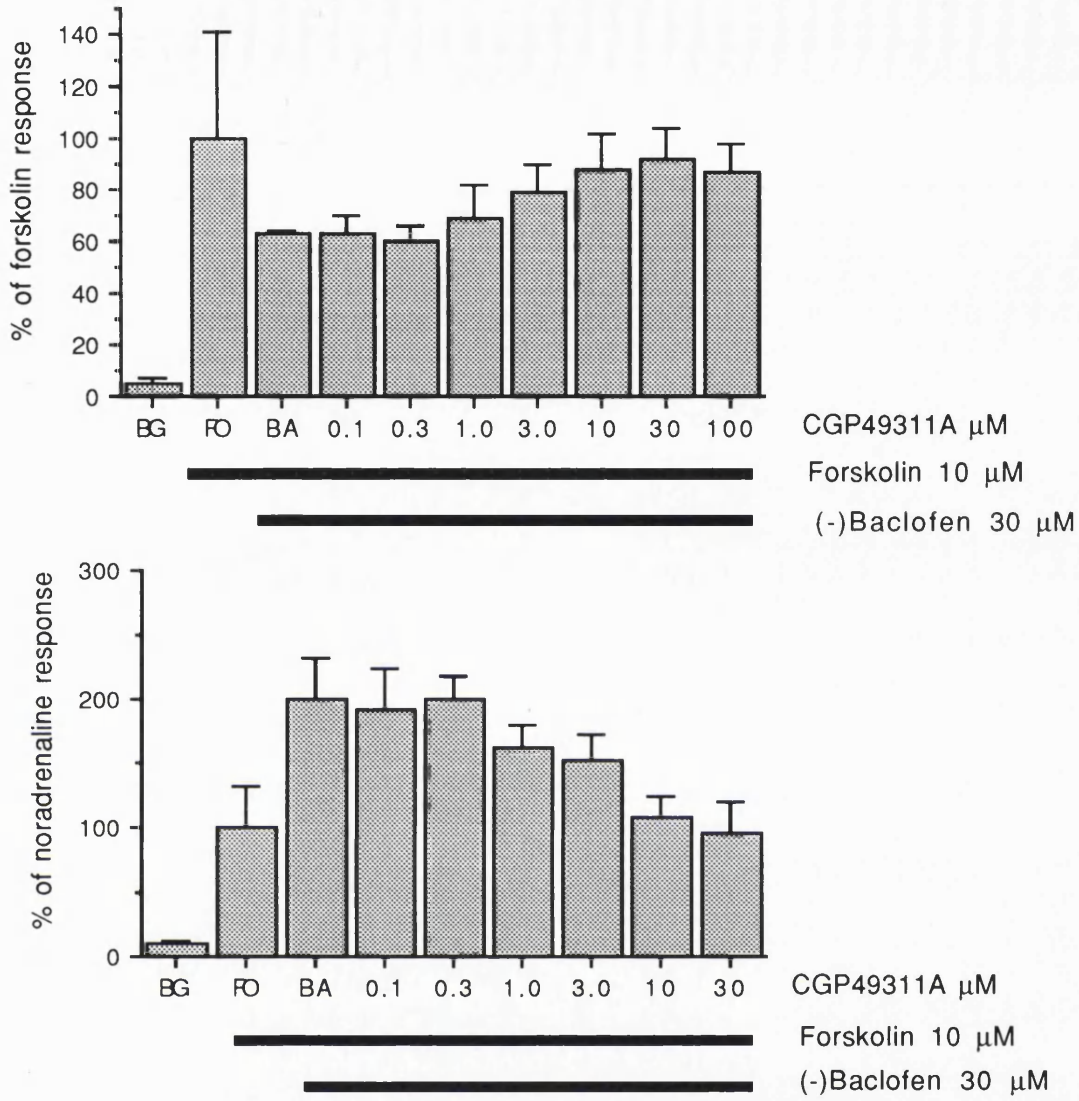


Figure 3.12. Antagonism by CGP49311A of the modulation adenylyl cyclase by (-)baclofen. Antagonism of (-)baclofen-inhibited forskolin-stimulated adenylyl cyclase activity (upper graph), and (-)baclofen augmented noradrenaline-stimulated adenylyl cyclase activity (lower graph), by increasing concentrations of CGP49311A. Graphs represent the mean of three separate experiments. Data has been normalised by expressing the data as % of the forskolin or % of the noradrenaline response. The error bars on the forskolin and noradrenaline columns represent variation between assays.

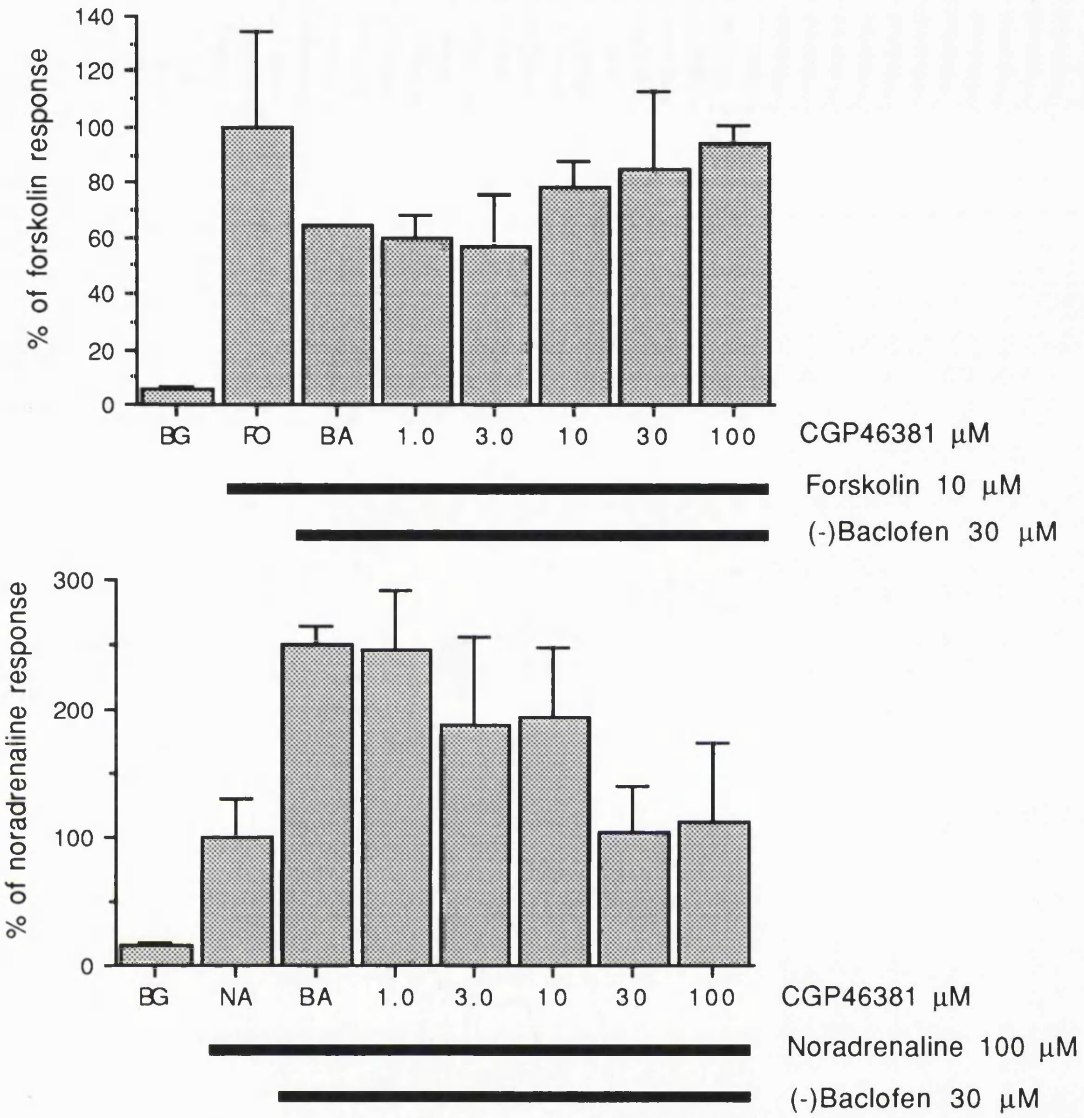


Figure 3.13. Antagonism by CGP46381 of the modulation adenylyl cyclase by (-)baclofen. Antagonism of (-)baclofen-inhibited forskolin-stimulated adenylyl cyclase activity (upper graph), and (-)baclofen augmented noradrenaline-stimulated adenylyl cyclase activity (lower graph), by increasing concentrations of CGP46381. Graphs represent the mean of three separate experiments. Data has been normalised by expressing the data as % of the forskolin or % of the noradrenaline response. The error bars on the forskolin and noradrenaline columns represent variation between assays.

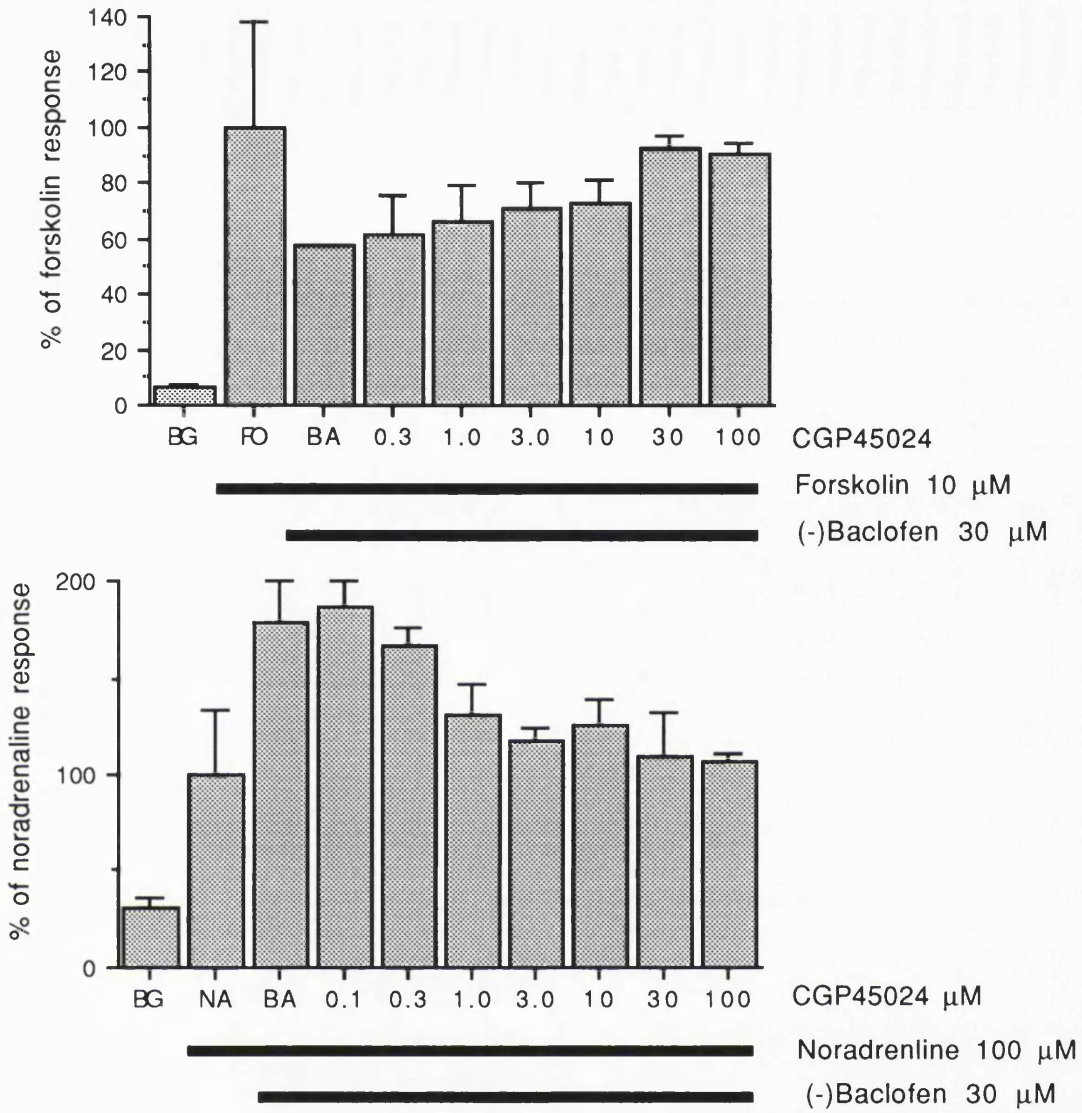


Figure 3.14. Antagonism by CGP45024 of the modulation adenylyl cyclase by (-)baclofen. Antagonism of (-)baclofen-inhibited forskolin-stimulated adenylyl cyclase activity (upper graph), and (-)baclofen augmented noradrenaline-stimulated adenylyl cyclase activity (lower graph), by increasing concentrations of CGP45024. Graphs represent the mean of three separate experiments. Data has been normalised by expressing the data as % of the forskolin or % of the noradrenaline response. The error bars on the forskolin and noradrenaline columns represent variation between assays.

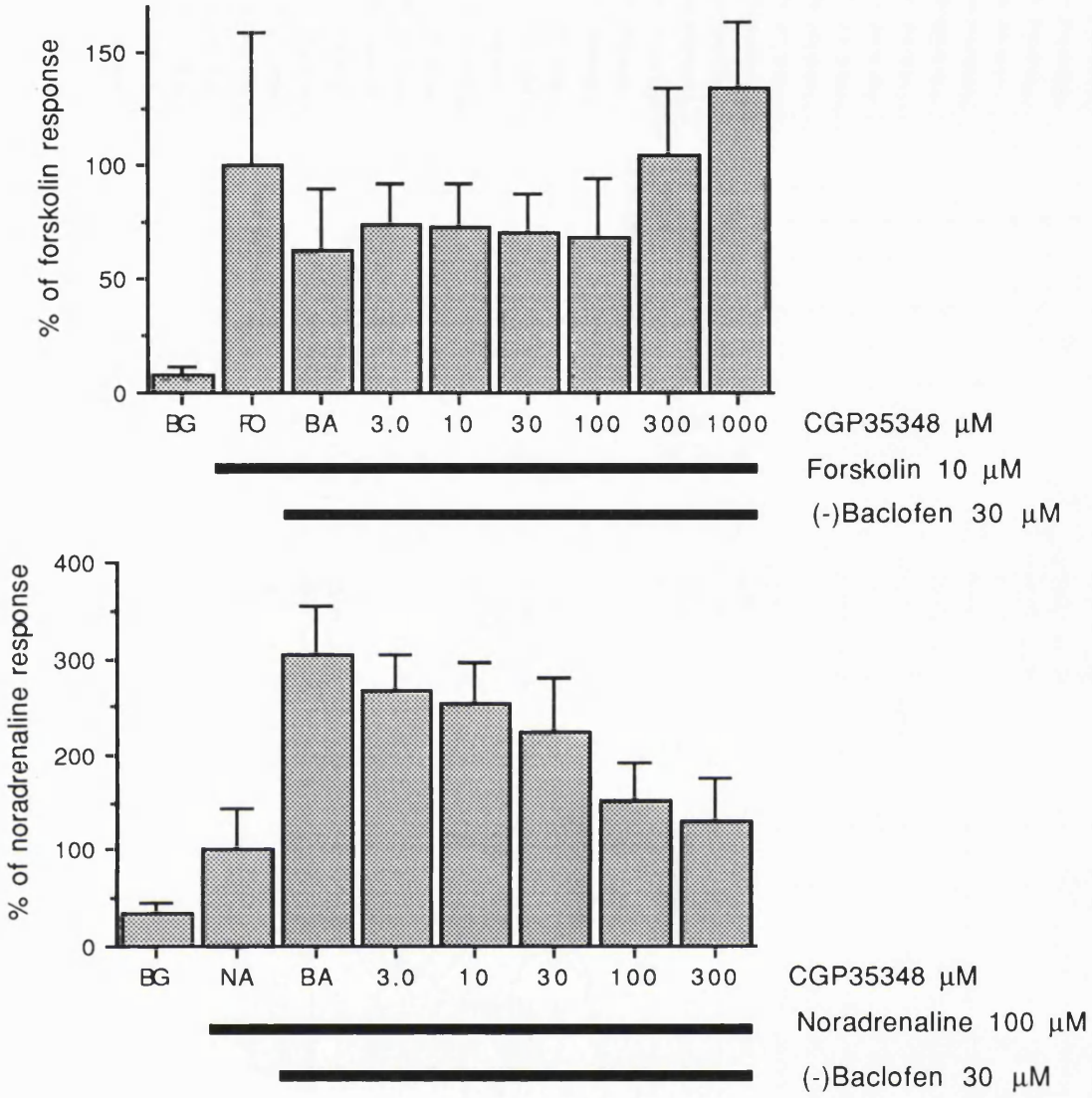


Figure 3.15. Antagonism by CGP35348 of the modulation adenylyl cyclase by (-)baclofen. Antagonism of (-)baclofen-inhibited forskolin-stimulated adenylyl cyclase activity (upper graph), and (-)baclofen augmented noradrenaline-stimulated adenylyl cyclase activity (lower graph), by increasing concentrations of CGP35348. Graphs represent the mean of three separate experiments. Data has been normalised by expressing the data as % of the forskolin or % of the noradrenaline response. The error bars on the forskolin and noradrenaline columns represent variation between assays.

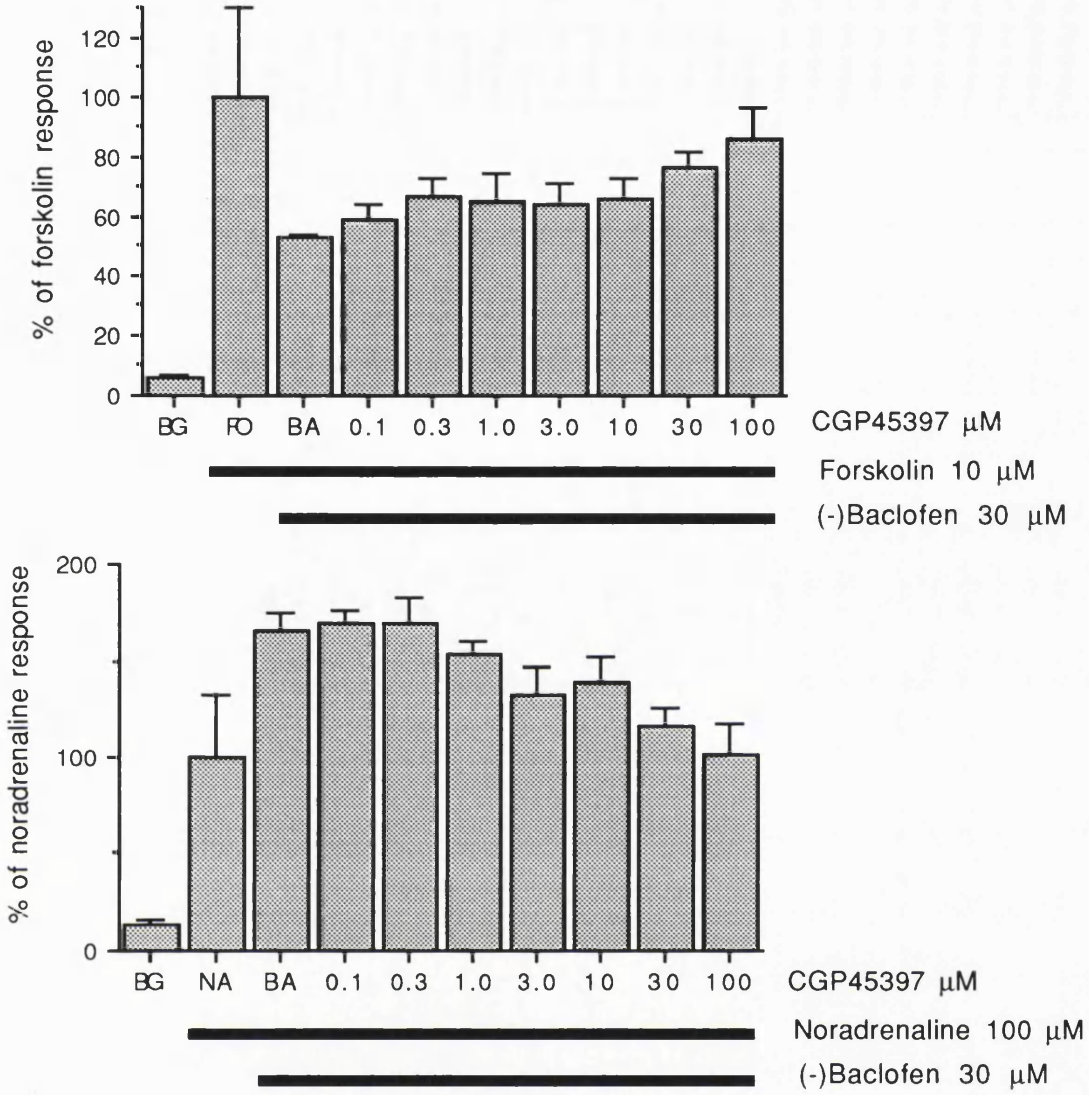


Figure 3.16. Antagonism by CGP45397 of the modulation adenylyl cyclase by (-)baclofen. Antagonism of (-)baclofen-inhibited forskolin-stimulated adenylyl cyclase activity (upper graph), and (-)baclofen augmented noradrenaline-stimulated adenylyl cyclase activity (lower graph), by increasing concentrations of CGP45397. Graphs represent the mean of three separate experiments. Data has been normalised by expressing the data as % of the forskolin or % of the noradrenaline response. The error bars on the forskolin and noradrenaline columns represent variation between assays.

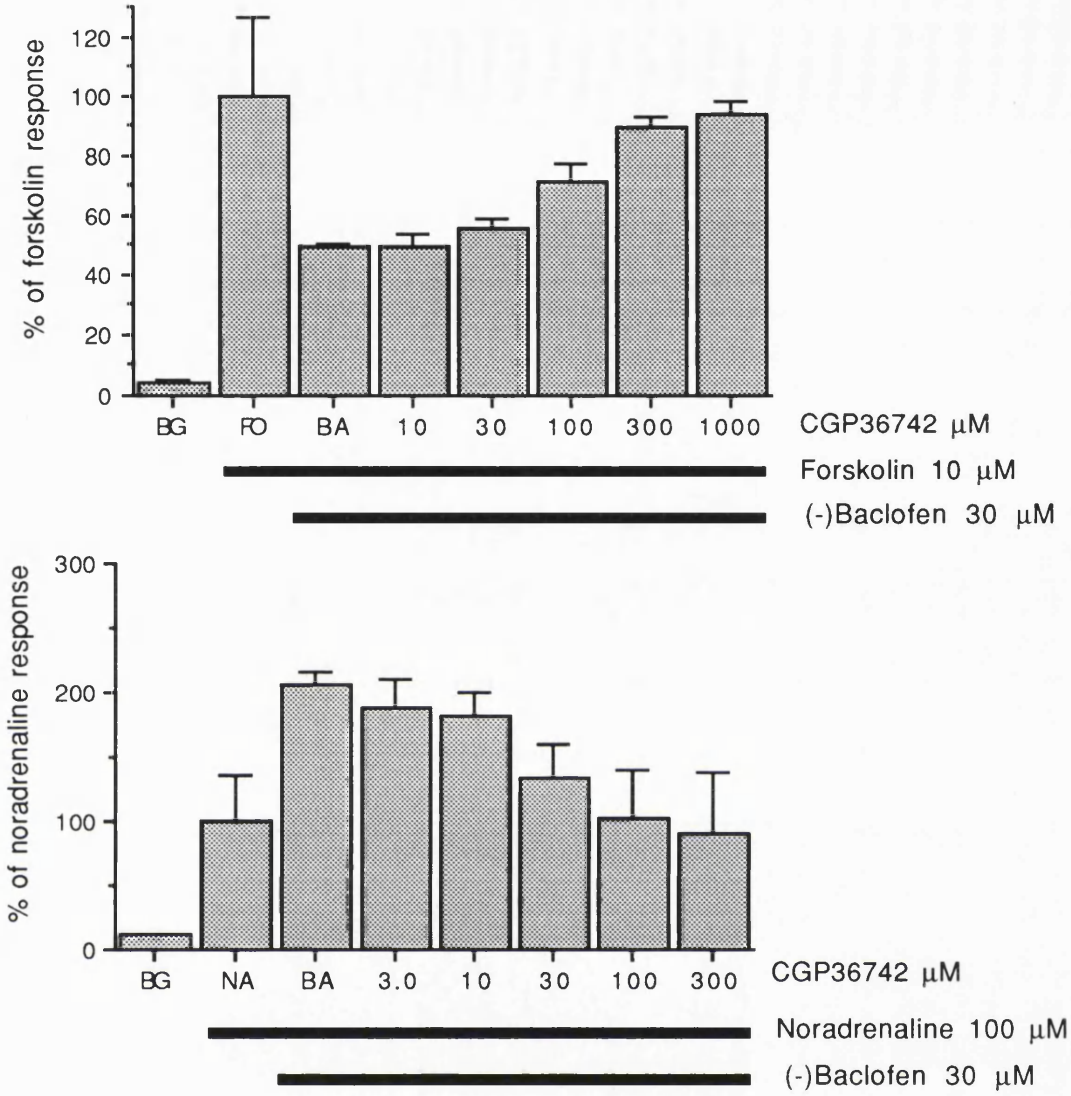


Figure 3.17. Antagonism by CGP36742 of the modulation adenylyl cyclase by (-)baclofen. Antagonism of (-)baclofen-inhibited forskolin-stimulated adenylyl cyclase activity (upper graph), and (-)baclofen augmented noradrenaline-stimulated adenylyl cyclase activity (lower graph), by increasing concentrations of CGP36742. Graphs represent the mean of three separate experiments. Data has been normalised by expressing the data as % of the forskolin or % of the noradrenaline response. The error bars on the forskolin and noradrenaline columns represent variation between assays.

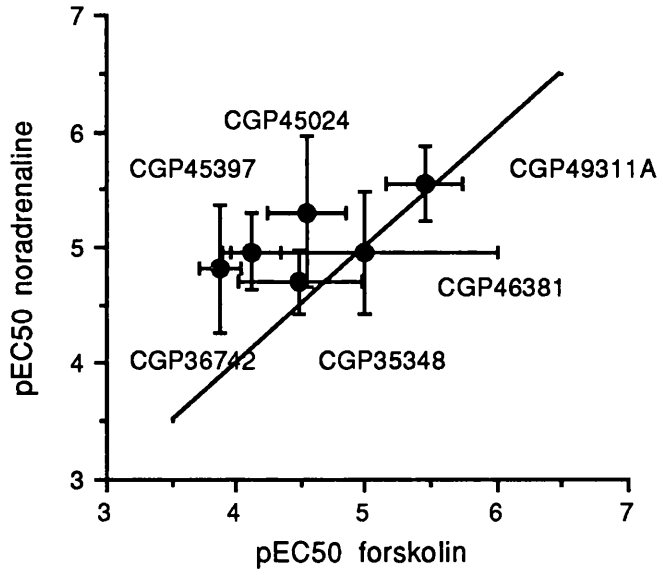


Figure 3.18. Correlation graph of the potency of $GABA_B$ receptor antagonists. Potency was assessed as the ability to inhibit 100 μM (-)baclofen as a modulator of either forskolin or noradrenaline-stimulated adenylyl cyclase activity. Data represents the mean of at least three separate experiments. The line of identity is indicated on the graph.

Figure 3.12 to 3.17. No statistical difference was found in the potency of any of the compounds as the antagonists of (-)baclofen-inhibited, forskolin-stimulated adenylyl cyclase activity and as antagonists of (-)baclofen-augmented noradrenaline-stimulated adenylyl cyclase activity (ANOVA followed by Duncans multiple range test, $p>0.10$) The potency of these antagonists is shown in Table 3.2. However, with the exception of CGP46381 all of these compounds were slightly more potent at antagonising the augmentation of noradrenaline-stimulated adenylyl cyclase activity than they were at antagonising the inhibition of forskolin-stimulated adenylyl cyclase activity (Figure 3.18).

3.3. Discussion

The present investigation was concerned with the modulation of adenylyl cyclase via the activation of cell surface $GABA_B$ receptors. In order to see this modulation, the enzyme was stimulated with either forskolin or noradrenaline. Forskolin is a novel diterpene derived from the Indian plant *Coleus forskolii* (Bhat et al., 1977), which is believed to stimulate adenylyl cyclase by an action at the catalytic subunit itself (Seamon and Daly, 1981; Seamon et al., 1981). It is a useful pharmacological tool with which to investigate the modulation of adenylyl cyclase because it allows the expression of G_i mediated inhibition, such as that produced by $GABA_B$ receptor activation. The other effect of $GABA_B$ receptor activation upon adenylyl cyclase is stimulatory. This effect is seen when the enzyme is already stimulated by α subunits derived from the G_s class of G-protein (Hill, 1985; Watling and Bristow, 1985; Lefkowwitz, 1992). In the present study this was achieved by stimulating β-adrenoceptors with

their natural agonist noradrenaline. Compounds such as isoprenaline are more selective β-adrenoceptor agonists than noradrenaline. However, noradrenaline was selected for use in the present study for two reasons. Firstly so that direct comparisons could be made with previous work in which noradrenaline had been used, and secondly so that the study would remain physiologically relevant. It should be born in mind that at high concentrations of noradrenaline an interaction with α2-adrenoceptors might in itself augment the actions of noradrenaline at β-adrenoceptors. In the present study 100 μM noradrenaline gave a similar maximum response to isoprenaline (data not shown) suggesting that this effect was negligible. Concentration response curves to forskolin and noradrenaline allowed the selection of a just maximally effective concentration of each of these compounds (100μM noradrenaline and 10μM forskolin) with which to investigate the effect of $GABA_B$ receptor agonists.

(-)Baclofen was found to dose dependently inhibit the forskolin response and to dose dependently augment the noradrenaline response. This is a well characterised profile, and the present findings are in agreement with those of other authors (Enna and Karbon, 1984; Karbon and Enna, 1985; Hill, 1985 and Wojcik and Neff, 1984). (-)Baclofen had no discernible effect upon baseline cAMP production. This in keeping with the findings of Hill (1985) and Karbon and Enna (1985). However, Wojcik and Neff observed an inhibition of basal cAMP levels in the presence of (-)baclofen. They recorded their baseline cAMP levels in the presence of added GTP which in itself promotes G-protein subunit dissociation, and hence artificially raises levels of adenylyl cyclase activity.

Time course experiments demonstrated that under these assay conditions, when cAMP was stimulated with 10 μM forskolin or 100 μM noradrenaline, the production of cAMP was linear for the duration of the standard incubation period (10 min). When 100 μM (-)baclofen was included in the incubation medium, forskolin-stimulated cAMP production was inhibited and noradrenaline-stimulated cAMP was augmented. In each case the linearity of cAMP production was maintained. Since (-)baclofen showed a similar degree of modulation at all time points at which it was tested, this indicated that the effects of (-)baclofen were due to a change in the rate of cAMP production and not a plateauing of the response. This is consistent with the hypothesis that the modulation of these responses by $GABA_B$ receptor stimulation is due to an allosteric modulation of the enzyme by a second messenger, and not due to some other effect such as depletion of precursors.

The potency of six $GABA_B$ receptor agonists were detemined as modulators of adenylyl cyclase activity. These included (-)baclofen, SKF97541, 3-APA and three novel compounds, CGP47656, CGP44533 and CGP44532. The structures of the novel compounds is given in figure 3.11. All of the agonists tested were more potent as inhibitors of forskolin-stimulated adenylyl cyclase activity than they were as agents augmenting the noradrenaline response. Moreover, two of the agonists tested exhibited statistically significant selectivity for modulating forskolin-stimulated adenylyl cyclase activity over modulating noradrenaline-stimulated adenylyl cyclase activity. These were (-)baclofen and CGP47656. There are a number of possible explanations for this difference in potency of (-)baclofen and CGP47656 in the two assays. One possibility is that there are

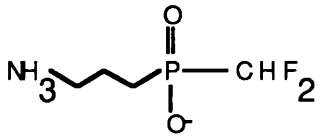
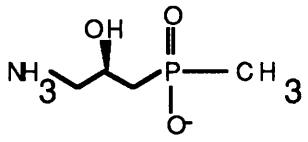
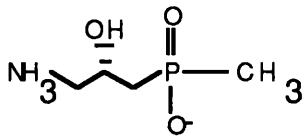
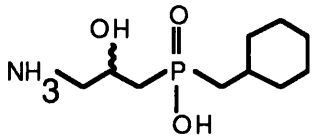
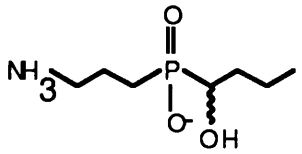
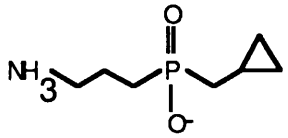
CGP47656 AGONIST	
CGP44533 AGONIST	
CGP44532 AGONIST	
CGP49311A ANTAGONIST	
CGP45024 ANTAGONIST	
CGP45397 ANTAGONIST	

Figure 3.19. Structures of novel GABA$_B$ receptor ligands used in the present study. Six of the compounds used to investigate the pharmacology of GABA$_B$ receptor mediated modulation of adenylyl cyclase activity were recently synthesized by Ciba-Geigy. No previous biological data have been reported for these compounds, although their effectiveness as GABA$_B$ receptor agonists and antagonists was confirmed by Ciba-Geigy.

two pharmacologically distinct types of $GABA_B$ receptor with different affinities for these agonists. One receptor, for which (-)baclofen and CGP47656 had high affinity, would be responsible for mediating an inhibitory action on adenylyl cyclase and a second receptor, for which these compounds had a lower affinity would be responsible the stimulatory action. Evidence for pharmacologically distinguishable subtypes of $GABA_B$ receptor has been described by Bonnano and Raiteri (1992; 1993). These authors initially divided $GABA_B$ receptors into baclofen sensitive and baclofen insensitive receptors. This later class of receptor is, at present, represented only by an autoreceptor in the spinal cord. The present study describes a difference in potency for (-)baclofen at inhibiting forskolin-stimulated adenylyl cyclase activity and at augmenting noradrenaline-stimulated adenylyl cyclase activity. It is possible that the difference in potency for (-)baclofen reflects this classification of $GABA_B$ receptors. However, two important factors must be considered when using agonists to classify receptors. Firstly, there is the possibility that agonists might display different affinities for the same receptor when that receptor is coupled to different G-proteins (Kenakin and Morgan, 1988). That is to say, all G-proteins may not induce the optimal conformational change for high affinity agonist binding. In the present case, $GABA_B$ receptors coupled to the inhibitory response are likely to be bound to G_i, whereas the receptors mediating the augmentation response may be linked to either G_i or G_o. These are the two classes of G-protein which have been demonstrated to bind to $GABA_B$ receptors (Morishita et al., 1990). However, this may not be the explanation for the differences in agonist potency observed in the present study, since only two of the six agonists showed selectivity for inhibition of forskolin-stimulated adenylyl cyclase activity. G-protein

modulation of agonist affinity would be likely to affect all of the compounds tested.

The second important consideration when using agonists to classify receptors involves intrinsic efficacy and receptor reserve. The potency of an agonist is determined by its binding affinity and its intrinsic efficacy. The binding affinity of a drug is a measure of the number of receptors to which it binds at a given concentration. The intrinsic efficacy is the proportion of receptors, to which the drug is bound, which become activated. An agonist with low intrinsic efficacy must bind to a larger number of receptors than an agonist of high intrinsic efficacy in order to elicit a response of similar magnitude. If a full response can be elicited by a high efficacy agonist binding to only a proportion of the receptors it may not be possible to distinguish between a high affinity, low efficacy compound and a high efficacy, low affinity compound.

According to classical receptor theory, a single agonist may have different potencies in two different functional assays. This is possible when a large proportion of the available receptors need to be occupied in one functional assay to produce a maximum response (low receptor reserve), but occupation of a small proportion of the available receptors elicits a maximum response in the other assay (large receptor reserve). Agonists of high intrinsic efficacy (full agonists) are relatively insensitive to differences in receptor reserve, but dose response curves to agonists with lower intrinsic efficacy (which may be partial agonists in some functional models) will be shifted to the right in systems with a low receptor reserve. A number of examples of this have been described in the literature. For

instance the D2 dopamine receptor partial agonist 3-PPP displays apparent selectivity for presynaptic D2 receptors which have a high receptor reserve, over postsynaptic D2 receptors, which have almost no receptor reserve (Meller et al., 1987; Meller et al., 1988). Receptor reserve has also been used to explain discrepancies in the pharmacology of D-LSD (Hoyer and Boddeke, 1993) which has been described variously as a $5HT_2$ receptor agonist (Glennon, 1990) or antagonist (Pierce and Peroutka, 1990) in different models. Recently, cell lines expressing the same cloned receptor in differing numbers have been used to demonstrate that the potency of β2-adrenoceptor agonists (George et al., 1988) and 5-HT receptor agonists (Fargin et al., 1988; Fargin et al., 1989) increase with higher levels of receptor expression. Indeed shifts in potency of two orders of magnitude have been recorded with increased levels of receptor expression (Fargin et al., 1989). Interestingly, different receptor reserves, and hence agonist potencies may occur where a single population of receptors interacts with two different second messenger systems. Thus when a single population of receptors is linked to two different effectors by the same G-protein a "reserve" may occur at the level of the effector. Similarly, different reserves may occur at the level of the G-protein, where the same receptor population is linked to two G-proteins which are present in different numbers (Hoyer and Boddeke, 1993).

In this context it is worth reviewing the mechanisms by which $GABA_B$ receptors might influence adenylyl cyclase activity. It was initially suggested that one way to account for the duplex effect of $GABA_B$ receptor activation upon stimulated adenylyl cyclase activity was to propose the presence of two pools of adenylyl cyclase. These pools could be

compartmentalized, possibly in different cells, one under the influence of a stimulatory second messenger and one under the influence of an inhibitory second messenger. Thus there might be two functionally discrete $GABA_B$ receptors in the brain (Karbon et al., 1990). Initially there was some debate as to which second messenger, activated by $GABA_B$ receptors, caused the augmentation response. Two candidates were phospholipase A2 and phospholipase C. Indeed, evidence has been presented to suggest that these enzymes might be involved to some extent (for review see Enna and Karbon, 1987). However, a number of features of the G-protein signal transduction system have recently been described, which allow a more likely mechanism for this duplex response to be proposed. Of relevance in this context is the cloning of four types of adenylyl cyclase (Krupinski et al., 1989; Feinstein et al., 1991; Bakalyar and Reed, 1990; Gao and Gilman, 1991) termed types I to IV. The sensitivity of these enzymes to G-protein modulation has been investigated by expressing them in cell lines (SF9 cells) along with purified G-protein subunits. As predicted, type I and type III were both stimulated by the α subunit of G_s (α_s) subunits and slightly inhibited by $\beta\gamma$ subunits (presumably the $\beta\gamma$ subunit was complexing the activated α_s subunit). Types II and IV adenylyl cyclase, however, gave surprising results. Like types I and III, they were stimulated by α_s, but this response was dramatically enhanced by the presence of the $\beta\gamma$ subunit (Tang and Gilman, 1991; Gao and Gilman, 1991). This action of $\beta\gamma$ could clearly provide an explanation for the augmentation of β-adrenoceptor-stimulated adenylyl cyclase by $GABA_B$ receptor activation. Indeed, it may be a common mechanism for all other such responses, such as the $\alpha 2$-adrenoceptor mediated potentiation of β-adrenoceptor stimulation (Leblanc and Ciaranello, 1984), the H1 histamine receptor mediated potentiation of

H2 receptor stimulation (Al-Gadi and Hill, 1985), and the effects of $GABA_B$ receptor activation upon vasoactive intestinal peptide stimulation (Watling and Bristow, 1986). These effects of $\beta\gamma$ have been further confirmed by Federman et al. (1992) as follows: mammalian HEK293 cells were transfected with α2-adrenoceptors. Initially, activation of these receptors inhibited cAMP production, presumably via G_i. When co-transfected with type II adenylyl cyclase α2-adrenergic stimulation was now found to stimulate adenylyl cyclase activity. This appeared to be due to the action of $\beta\gamma$ from G_i since the response was blocked by co-transfection with the α subunit of transducin, which scavenges $\beta\gamma$ subunits.

It is relevant to the present study that a higher number of free $\beta\gamma$ subunits are required to maximally enhance the activity of α_s activated adenylyl cyclase than α_s subunits are required to initially stimulate adenylyl cyclase activity (Tang and Gilman, 1991; Federman et al., 1992). This is understandable, if it were not the case then presumably the dissociation of G_s would provide sufficient α and $\beta\gamma$ subunits to maximally stimulate the enzyme. Thus, the α subunit mediated effects of $GABA_B$ receptor activation upon adenylyl cyclase require the activation of fewer receptors than the $\beta\gamma$ mediated effects. That is to say, the α subunit mediated effect upon adenylyl cyclase will have a relatively high receptor reserve and the $\beta\gamma$ mediated effects will have a relatively low receptor reserve. Thus we might expect a low efficacy agonist to be selective for the forskolin (α_i) actuated $GABA_B$ receptor mediated response rather than the noradrenaline ($\beta\gamma$) actuated response. This argument will only apply if the rate of signal transduction is not limited by a low density of G-proteins. G-proteins, however, appear to be present in cells in in concentrations

perhaps two orders of magnitude greater than β-adrenoceptors and adenylyl cyclase, indicating that this is unlikely to be the case (Aluosi et al., 1991). To explain the results of the present study it is neccesary to propose that (-)baclofen, which had significantly lower potency as an augmenter of noradrenaline-stimulated adenylyl cyclase activity, has lower intrinsic efficacy than agonists whose potency remains more similar in the two assay conditions (SKF97541, CGP44533, 3-APA and CGP44532). CGP47656 appears to be even more selective for the inhibition of forskolin-stimulated adenylyl cyclase activity than is (-)baclofen (two orders of magnitude rather than one). This would suggest that it is a compound with even lower intrinsic efficacy. Indeed the dose response curve to this compound as an augmenter of noradrenaline-stimulated shows another related phenomenon. The maximum effect elicited by this compound was lower than that elicited by (-)baclofen, i.e. it is a partial agonist. Thus it is possible to hypothesize that CGP47656 has such low intrinsic efficacy that it is unable to activate sufficient numbers of receptors to cause a maximal effect. Thus, the maximum response is limited by the number of receptors in the preparation.

In the present study, the agonist 3-APA was half a log unit less potent at augmenting noradrenaline-stimulated adenylyl cyclase than it was at modulating forskolin-stimulated adenylyl cyclase, though this difference was not statistically significant. Scherer et al., (1988) also tested a single concentration of 3-APA as a modulator of forskolin and noradrenaline-stimulated cAMP. They reported that 3-APA was an agonist in its ability to inhibit forskolin-stimulated adenylyl cyclase activity, but was ineffective as an augmenter of noradrenaline-stimulated adenylyl

cyclase activity. These authors proposed two pharmacologically distinct $GABA_B$ receptors on the basis of these data. There is, then a discrepancy in the selectivity of 3-APA between the study by Scherer (completely selective) and the present study (poorly selective). This might now be explained in terms of different numbers of viable receptors in the preparation used in the present study and that used by Scherer et al. (1988). A reduced number of viable receptors would decrease the receptor reserve. If the number of viable receptors were sufficiently reduced it might cause a low efficacy agonist to become specific for the functional response with the highest receptor reserve, in this case inhibition of forskolin-stimulated adenylyl cyclase. This hypothesis would also explain why Ferkany et al. (1988) observed pharmacological specificity in functional assays, but not in the binding experiments reported in the same study. Intrinsic efficacy might also explain why Stirling et al. (1980) found that phaclofen, δ-aminovaleric acid and β-phenyl GABA, possessed agonist activity whilst subsequent studies have described them as antagonists (Kerr et al., 1987; Ong et al., 1987; Muhyaddin et al., 1983 and Sawynok1986) . Thus, these compounds may be very weak partial agonists; in most functional assays they occupy receptors, but fail to cause a measurable response. In the synaptosomal preparation used by Stirling et al. they may be able to occupy sufficient numbers of receptors for their agonist activity to be expressed. These observations therefore, may support the involvement of receptor reserve in $GABA_B$ receptor pharmacology.

In summary, it is possible to propose pharmacologically distinct $GABA_B$ receptors on the basis of agonist potency (Scherer et al., 1988), however, alternative hypotheses exist, and for the time being Occams razor

demands that we accept differing intrinsic efficacies as the reason why some agonists show selectivity in these two assay systems.

In contrast to agonists, the potency of antagonists is defined only by their binding affinity. Thus a selection of antagonists were tested in these assay systems to establish their ability to antagonize the action of 30 μM (-)baclofen. These compounds included the first of a new generation of $GABA_B$ receptor antagonists produced by Ciba Geigy (CGP35348) as well as two compounds which are of interest because they have been demonstrated to cross the blood brain barrier (CGP46381 and CGP36742). A number of other novel antagonists were provided by Ciba Geigy, (CGP49311A, CGP45024, and CGP45397, the structures for which are given in figure 3.19). The pEC_{50} for these compounds as antagonists of (-)baclofen-inhibited, forskolin-stimulated adenylyl cyclase activity ranged from 3.87 to 5.45. However, none of these antagonists showed any statistically significant selectivity (ANOVA followed by Duncans multiple range test) for one assay system over the other. There is evidence from previous work that some antagonists can distinguish sub-populations of $GABA_B$ receptors. For instance, phaclofen has been demonstrated to be a $GABA_B$ antagonist in a number of assay systems. These include peripheral tissues such as the rat annococcygeus muscle (Muhyaddin et al., 1982), and the guinea pig ileum (Sawynok, 1986; Ong et al., 1987; Kerr et al 1987) as well as central nervous system tissue such as hippocampal and thalamic neurons (Soltesz et al., 1988), neocortical cells (Karlsson et al.,1988) and in septal cells (Hasuo 1988). However, of the $GABA_B$ receptors in the spinal cord, phaclofen is selective for those on the terminals of primary afferents (Curtis et al., 1986; Curtis et al 1981). Similarly, in the hippocampus presynaptic,

but not postsynaptic $GABA_B$ receptors are sensitive to phaclofen (Dutar and Nicoll 1988b). Phaclofen is also ineffective against baclofen induced calcium influx in synaptosomes (Stirling et al., 1989). Thus it is possible to present a case for phaclofen sensitive and phaclofen insensitive $GABA_B$ receptors (Dutar and Nicholl, 1988; Harrison, 1989; Wang and Dun 1990; Williams and Lacaille; 1990 and Curtis et al., 1981 and 1986) However, phaclofen is a relatively weak antagonist at $GABA_B$ receptors, (binding potency 130 μM (Froestl et al., 1992)) and may itself possess some agonist properties (Stirling et al., 1980). As such, reports of phaclofen sensitive and insensitive $GABA_B$ receptors must be interpreted with care until the work is repeated with higher potency antagonists.

$GABA_B$ antagonists of higher potency than phaclofen have recently become available and have been demonstrated to be selective for sub-populations of $GABA_B$ receptors in at least one study. Thus, release regulating $GABA_B$ receptors have been classified by Bonanno and Raiteri (1993a; 1993b) on the basis of their sensitivity to two antagonists. Receptors regulating GABA release were relativly insensitive to CGP35348, receptors regulating glutamate release were relatively insensitive to phaclofen and receptors regulating somatostatin release were highly sensitive to both of these antagonists. In the present study, CGP35348 showed no specificity for either forskolin or noradrenaline-stimulated adenylyl cyclase activity. The antagonist phaclofen was not used because of its low potency. Thus the pharmacological divisions proposed by Bonanno and Raiteri have not been reflected in the present work.

With the exception of CGP46381 all of the antagonists used in the present study were marginally more potent as inhibitors of (-)baclofen as an augmenter of noradrenaline-stimulated adenylyl cyclase activity (Figure 3.7). The explanation for this may be the lesser potency of (-)baclofen in this assay.

In Summary, data from the present study does not suggest that the inhibition of forskolin-stimulated adenylyl cyclase and the augmentation of noradrenaline-stimulated adenylyl cyclase are mediated by pharmacologically distinguishable receptors. These data do not of course preclude the possibility that a newly developed antagonist will distinguish between these two responses. However, it should be borne in mind in future studies that recent advances in the field of molecular biology have elucidated the mechanisms by which the inhibition and augmentation responses may occur, and that these mechanisms may involve only a single populaton of receptors. Intriguingly, this study has highlighted the fact that, under certain circumstances it is possible to develop, agonists with apparent specificity for sub-populations of receptors in the absence of confirmed receptor subtypes.

4

The Effects of $GABA_B$ Receptor Activation Upon Basal, Forskolin- and Noradrenaline-Stimulated Adenylyl Cyclase Activity *In Vivo*

4.1. Introduction

This study investigated the possibility of using the technique of microdialysis to assess $GABA_B$ receptor activation *in vivo*. It was performed to allow a comparison to be made between the *in vivo* pharmacological modulation of adenylyl cyclase activity and that previously described *in vitro*. In addition, since no *in vivo* assay has been developed to demonstrate that $GABA_B$ receptor drugs cross the blood brain barrier, this study was undertaken to examine whether *in vivo* microdialysis could be used in this capacity.

Several differences between *in vivo* and *in vitro* preparations may influence the ability of drugs to modulate adenylyl cyclase activity. One important difference between these preparations is the influence of endogenous neurotransmitters. Since *in vitro* preparations are extensively washed, endogenous neurotransmitters are largely removed from the system, while these neurotransmitter influences would be fully functional in a dialysis experiment. Such endogenous influences could markedly influence the effects of $GABA_B$ receptor agonists. For example, *in vitro,* (-)baclofen has no influence on basal cAMP levels, but augments noradrenaline-stimulated cAMP levels. In the presence of endogenous, tonic, activation of β-adrenoceptors *in vivo*, (-)baclofen administration alone might influence the levels of cAMP. Another major difference between *in vitro* slice preparations and the microdialysis preparation is that the integrity of neuronal circuits is largely maintained in the dialysis preparation. Thus, drugs may have effects *in vivo* which cannot be

observed *in vitro* since they are dependent upon neuronal circuitry destroyed in the *in vitro* slice preparation.

The first experiments described here examine the ability of (-)baclofen to influence the basal levels of cAMP *in vivo*. This was investigated by administering a high concentration of (-)baclofen through the dialysis probe. In addition, in order to demonstrate that the adenylyl cyclase activity measured *in vivo* was sensitive to modulation by pharmacological agents, the effects of forskolin and noradrenaline were examined. Since these two compounds have previously been demonstrated to stimulate adenylyl cyclase activity *in vivo* (Hutson, and Suman-Chauhan, 1990; Stone and John 1990) these experiments were performed to validate our measurement of cAMP efflux. Finally, the effects of (-)baclofen upon these two responses were examined, to see if the *in vivo* effects of (-)baclofen mimicked those seen *in vitro*.

4.2. Results

In Vitro Recovery of cAMP

To test the validity of the microdialysis method, experiments were performed to investigate the *in vitro* recovery of cAMP. Dialysis probes were immersed in a solution of 2.5 nM [^{3}H]-cAMP in artificial cerebro-spinal fluid (ACSF) maintained at 37 °C. The probes were perfused with ACSF at 1.2 and 2 μl min^{-1}. The amount of radioactivity in the dialysate was then quantified using liquid scintillation spectrophotometry and

expressed as a percentage of the radioactivity outside the probe. The in vitro recovery of cAMP was thus determined to be 26 ± 3.2 % (n = 4) at a flow rate of 2 μl min^{-1} and 35 ± 3.6 % (n = 4) at a flow rate of 1.2 μl min^{-1}. The *in vivo* results were not corrected for the recovery rate.

The ability of (-)baclofen to diffuse out of the probe was determined by a similar method. Dialysis probes were immersed in a small volume of ACSF (200 μl), maintained at 37 °C. The probes were perfused with ACSF containing 10 mM (-)baclofen, at a rate of 1.2 and 2 μl min^{-1} for 1 hour. The concentration of (-)baclofen in the bathing ACSF was then bioassayed by determining the ability of aliquots of this liquid to inhibit forskolin-stimulated adenylyl cyclase activity in a rat cortical slice preparation. At both flow rates samples of the bathing medium gave maximum inhibition of the forskolin response. When compared to responses from standard concentrations of (-)baclofen, this suggested that the concentration of (-)baclofen outside the probe was almost equivalent to the concentration inside.

Forskolin-Stimulated cAMP Production *In Vivo*

The effect of (100 μM) forskolin upon dialysed cAMP levels was initially investigated using a perfusion rate of 2 μl min^{-1} and collecting 30 minute fractions. However, in the absence of phophodiesterase inhibitors no discernible alteration in cAMP levels were observed in response to the forskolin pulse (Figure 4.1).

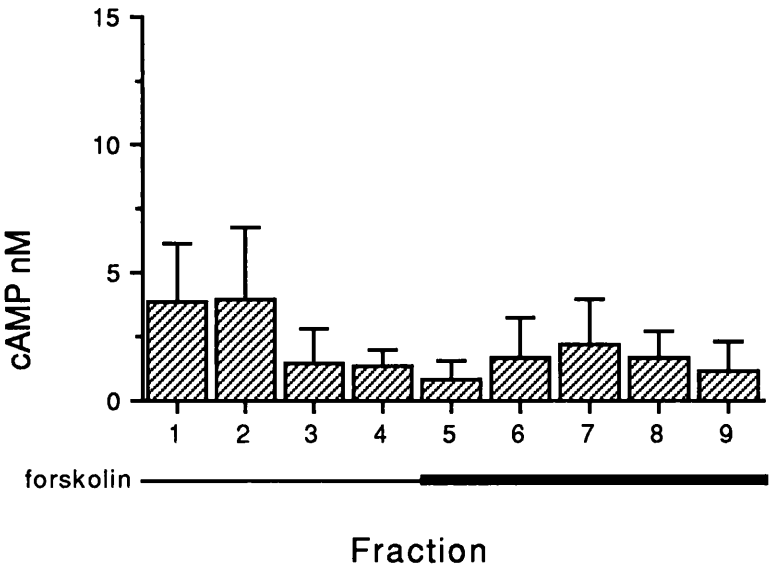


Figure 4.1. The effect of forskolin upon dialysed cAMP in the absence of phosphodiesterase inhibitors. Dialysis was performed as described in the text, 100 μM forskolin was administered in the dialysing medium from the beginning of fraction 5 until the end of the experiment. Data is expressed as concentration (nM) of cAMP in the dialysate. No phosphodiesterase inhibitors were included in this protocol. (n=3).

A robust effect of forskolin upon baseline cAMP levels was established in experiments in which the dialysis probe was perfused at a rate of 2 μl min^{-1}, and the ACSF used contained the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) at a concentration of 1 mM. Forskolin (100μM) was administered through the probe for the duration of two separate fractions: 4 and 9. This caused a significant increase in the basal concentration of cAMP in the dialysate in the fraction during which the forskolin was administered, and the subsequent fraction (fractions 4 and 5, and 9 and 10)(Figure 4.2). Due to a high degree of variability between animals, a two-pulse protocol was chosen to investigate the effects of (-)baclofen. The first stimulation was used to normalise the data and the second stimulation was used to examine the effects of drugs on the forskolin response. For each animal, the increase in cAMP caused by the first pulse of forskolin (S1) was determined by calculating a mean basal level from fractions 2 to 3, subtracting this baseline value from the mean stimulated levels observed during fractions 4 and 5. The increase in cAMP caused by the second pulse (S2) was calculated in a similar manner, using fractions 7 and 8 for basal release, and 9 and 10 for stimulated release. A ratio between the effects of forskolin during these two pulses (S2/S1) was then calculated for each experiment. The S2/S1 ratio under control conditions was 0.73 ± 0.25.

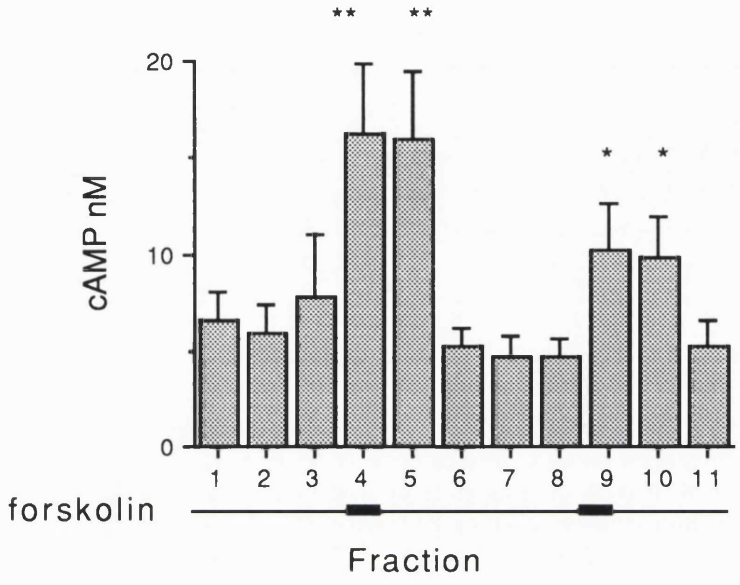


Figure 4.2. The effect of forskolin upon dialysed cAMP in the presence of IBMX. Data is expressed as concentration (nM) of cAMP in the Dialysate. Normalized data (percent of baseline) for these experiments is shown in Figure 4.4. Forskolin (100 μM) was administered through the probe during fractions 4 and 9. The phosphodiesterase inhibitor IBMX (1 mM) was present in the dialysis probe perfusing medium throughout the experiment. Significant elevations in cAMP were recorded in fractions 4 and 5, and 9 and 10 (* $p<0.05$ and ** $p<0.01$, one way Students t test)($n = 9$).

The Effect of (-)Baclofen Upon Baseline Levels of cAMP

To investigate the effect of 10 mM (-)baclofen on baseline cAMP levels, dialysis probes were perfused at a rate of 2 μl min^{-1}, and fractions were collected for 30 min periods. 10 mM (-)Baclofen was included in the dialysing medium at the beginning of fraction 5, and remained until the end of the experiment. Basal cAMP levels were very stable, at levels near 3 nM. The introduction of 10 mM (-)baclofen into the dialysing medium failed to influence this level of cAMP (Figure 4.3).

The Effect of (-)Baclofen Upon Forskolin-Stimulated cAMP *In Vivo*

In order to investigate the effect of (-)baclofen upon forskolin-stimulated adenylyl cyclase activity *in vivo,* a series of experiments were conducted which were similar to those described for the determination of the action of forskolin. Thus, the dialysis probe was perfused at a rate of 2 μl min^{-1} with ACSF containing 1 mM IBMX and fractions were collected for periods of 30 min. Two pulses of forskolin (100 μM) were administered, at fractions 4 and 9. During the first of these pulses (S1) forskolin was administered alone. During the second of these pulses (S2) one of a range of concentrations of (-)Baclofen (10 μM to10 mM) was added to the perfusing medium along with forskolin. Both pharmacological agents were removed from the dialysing medium at the beginning of fraction 10.

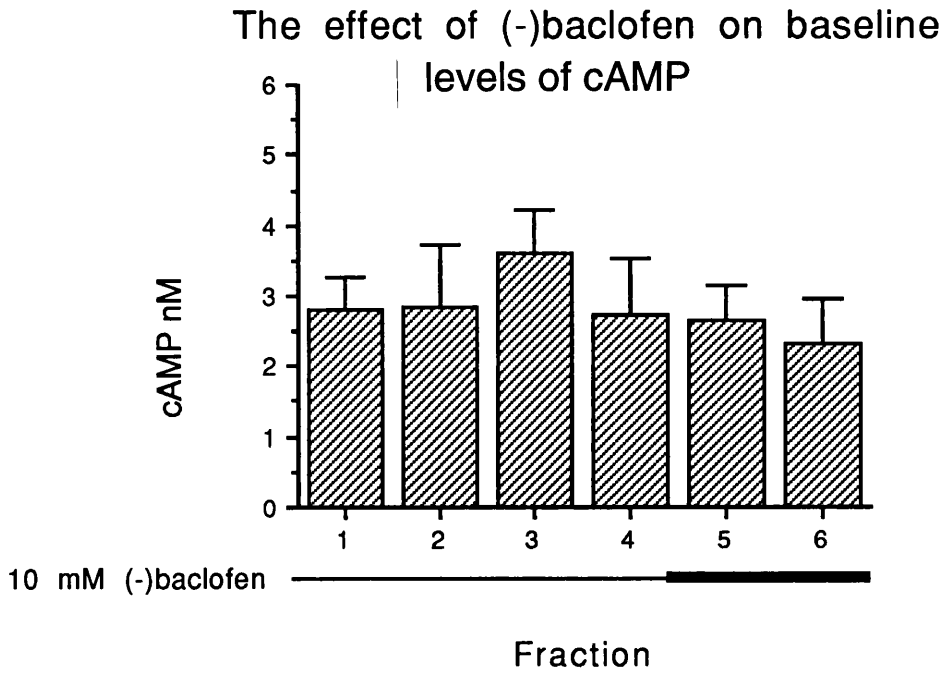


Figure 4.3. The effect of (-)baclofen upon baseline levels of cAMP in dialysates The data is expressed as the concentration of cAMP (nM) in dialysate derived from the frontal cortex of rats. The probes were perfused at a rate of 2µl min^{-1}, the duration of each fraction was 30 min. Data shows a stable baseline for the first 4 fractions (2 hours), and the lack of effect of 10 mM (-)baclofen, which was administered via the probe for the duration of fraction 5 and 6. Data represent the mean values from 3 rats.

(-)Baclofen administration had no influence on forskolin-stimulated cAMP efflux *in vivo* at concentrations up to 10 mM (Figures 4.5 to 4.8). The data are summarized in S2/S1 ratios in Figure 4.9. Though there was a tendency for S2/S1 ratios to increase in the presence of (-)baclofen, these differences were not statistically significant (ANOVA followed by Dunnetts multiple range test, $p>0.10$).

Noradrenaline-Stimulated cAMP Production *In Vivo*

The effect of 1 mM noradrenaline upon basal cAMP levels was initially investigated using a similar protocol to that designed to investigate the effect of forskolin. 1 mM ascorbate was included in the ACSF used for perfusing the probe to prevent oxidation of the noradrenaline. Using this protocol, no stimulation of cAMP by noradrenaline was observed (Figure 4.10).

The failure to observe an effect of noradrenaline in this assay might be due to the rapid desensitisation of β-adrenoceptors. This can cause agonist induced elevation of of cyclic nucleotides to return to basal levels within 30 minutes, despite the continued presence of neurotransmitter (Harden, 1983). The protocol was re-designed to enable examination of cAMP efflux over a shorter time frame than that used for forskolin. Thus, dialysate fractions were collected for periods of 10 minutes. In addition, to increase the cAMP concentration in each sample, the perfusion rate through the dialysis probe was reduced to 1.2 $\mu l\ min^{-1}$ (Ungerstedt, 1984)

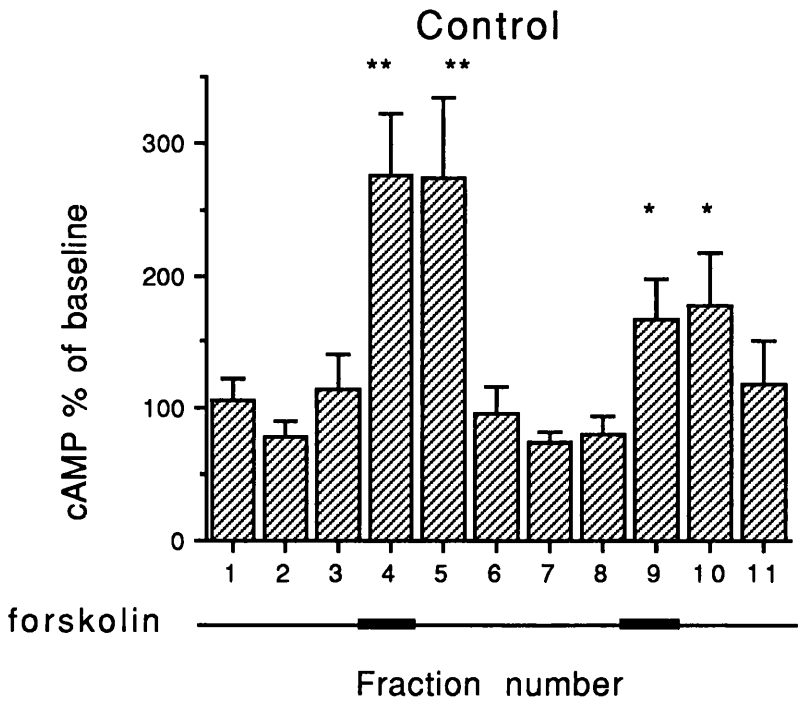


Figure 4.4. The effect of two pulses of forskolin upon the level of cAMP in dialysate from rat frontal cortex. 100 μM forskolin was administered through the probe during fractions 4 and 9. Numbers along the abscissa represent fraction number. Data are expressed as percent of baseline levels (mean of the concentration in the first three fractions). This figure represents the same data as that shown in figure 4.2 (* $p<0.05$, ** $p<0.01$, one way Students t-test. n = 9).

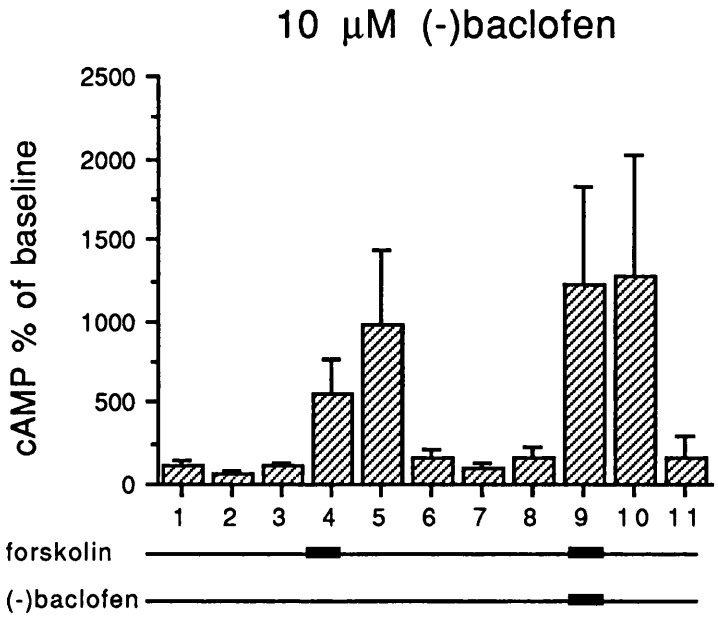


Figure 4.5. The effect of 10 μM (-)baclofen upon the increase in dialysed cAMP caused by 100 μM forskolin. Forskolin was included in the perfusion buffer for the duration of fraction 4 and fraction 9. (-)Baclofen was included in the perfusion buffer for the duration of fraction 9. Numbers on the abscissa represent fraction number (n=3).

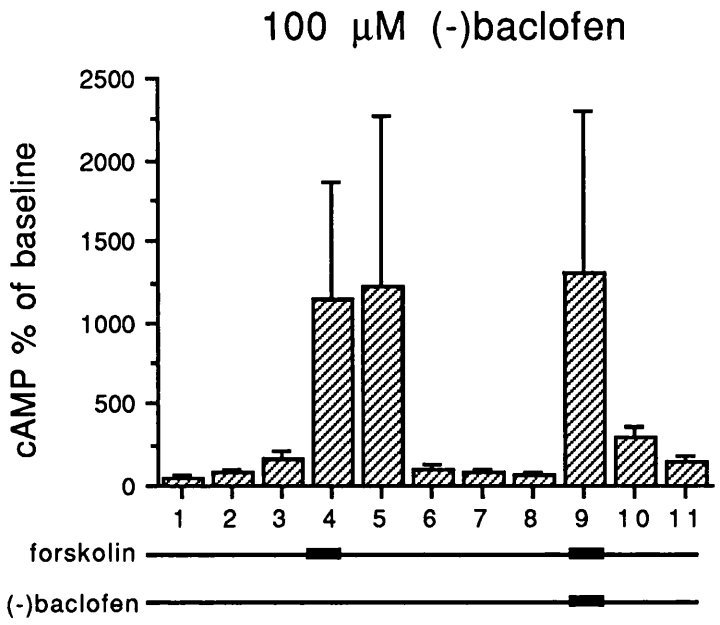


Figure 4.6. The effect of 100 μM (-)baclofen upon the increase in dialysed cAMP caused by 100 μM forskolin. Forskolin was included in the perfusion buffer for the duration of fraction 4 and fraction 9. (-)Baclofen was included in the perfusion buffer for the duration of fraction 9. Numbers on the abscissa represent fraction number (n=3).

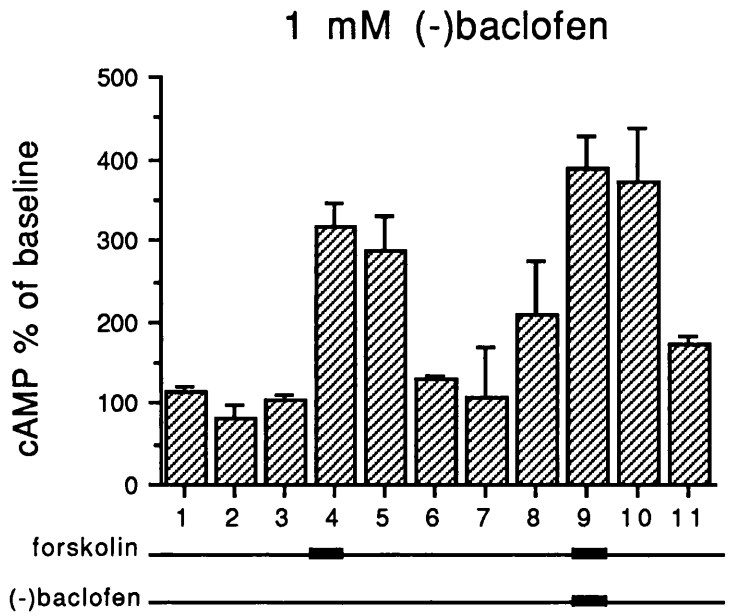


Figure 4.7. The effect of 1 mM (-)baclofen upon the increase in dialysed cAMP caused by 100 μM forskolin. Forskolin was included in the perfusion buffer for the duration of fraction 4 and fraction 9. (-)Baclofen was included in the perfusion buffer for the duration of fraction 9. Numbers on the abscissa represent fraction number. (n=3).

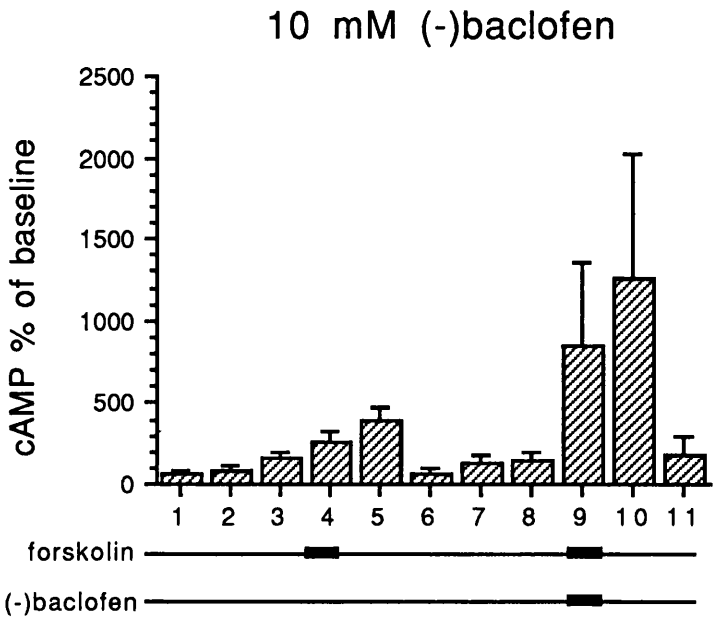


Figure 4.8. The effect of 10 mM (-)baclofen upon the increase in dialysed cAMP caused by 100 μM forskolin. Forskolin was included in the perfusion buffer for the duration of fraction 4 and fraction 9. (-)Baclofen was included in the perfusion buffer for the duration of fraction 9. Numbers on the abscissa represent fraction number. (n=3).

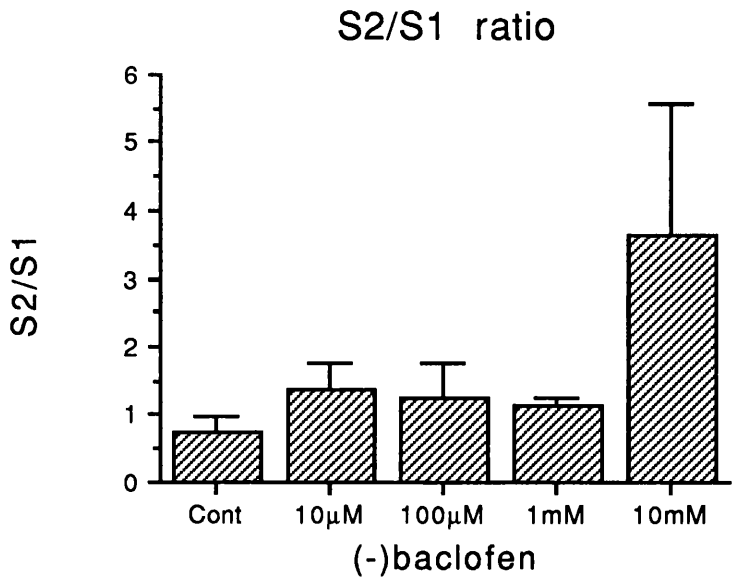


Figure 4.9 Graphic representation of the S2/S1 ratios for (-)baclofen. Four concentrations of (-)baclofen were assessed for their ability to inhibit forskolin-stimulated cAMP in rat frontal cortex dialysates. (-)Baclofen was applied during the second (S2) period of stimulation, The effect of (-)baclofen is represented by expressing this response as a ratio of the control (S1) response (S2/S1 ratio). (Control, n = 9, others n = 3).

In order to collect sufficient dialysate to assay the concentration of cAMP using the binding protein method, the samples from 5 individual animals were pooled. The results of an experiment performed using this protocol are shown in Figure 4.11. This figure shows a gradual decline in the quantity of cAMP recovered in dialysate during the first four samples, until a steady baseline is reached at around fraction 4. A dramatic rise in the levels of cAMP was recorded during the first period of noradrenaline stimulation, but no response resulted from the second period of stimulation. Again this was assumed to be due to desensitization. Accordingly, future experiments utilized a one-pulse protocol, and direct comparisons were made between animals.

The Effect of (-)Baclofen Upon Baseline Levels of cAMP

Since a different protocol (perfusion rate, fraction length) was used to assay the effects of noradrenaline on cAMP in dialysates, it was necessary to re-determine the effect of (-)baclofen alone using this protocol. The result of these experiments are shown in Figure 4.12. (-)Baclofen (10 mM) neither stimulated nor inhibited baseline levels of cAMP when used in this manner.

The Effect of a Single Pulse of Noradrenaline Upon cAMP Levels *In Vivo*

Using the slower flow rate (1.2 $\mu l\ min^{-1}$), and taking fractions at more frequent intervals than in the case of forskolin, it was possible to

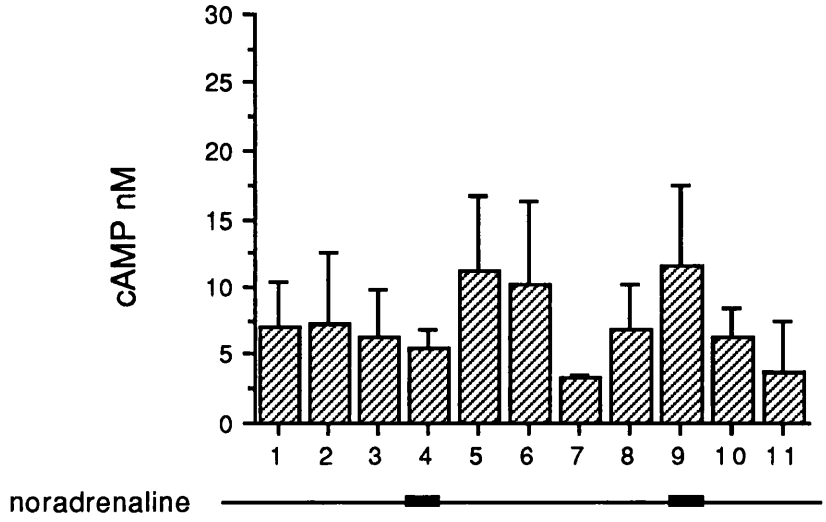


Figure 4.10. The effect of 1 mM noradrenaline upon basal levels of cAMP in dialysates (30 min fractions, flow rate=2 µl.min $^{-1}$). The probes implanted into the frontal cortex of rats, and experimental conditions similar to those used to analyse the effect of forskolin were used. Noradrenaline was administered at fractions 4 and 9, for the duration of the fraction. (n = 3).

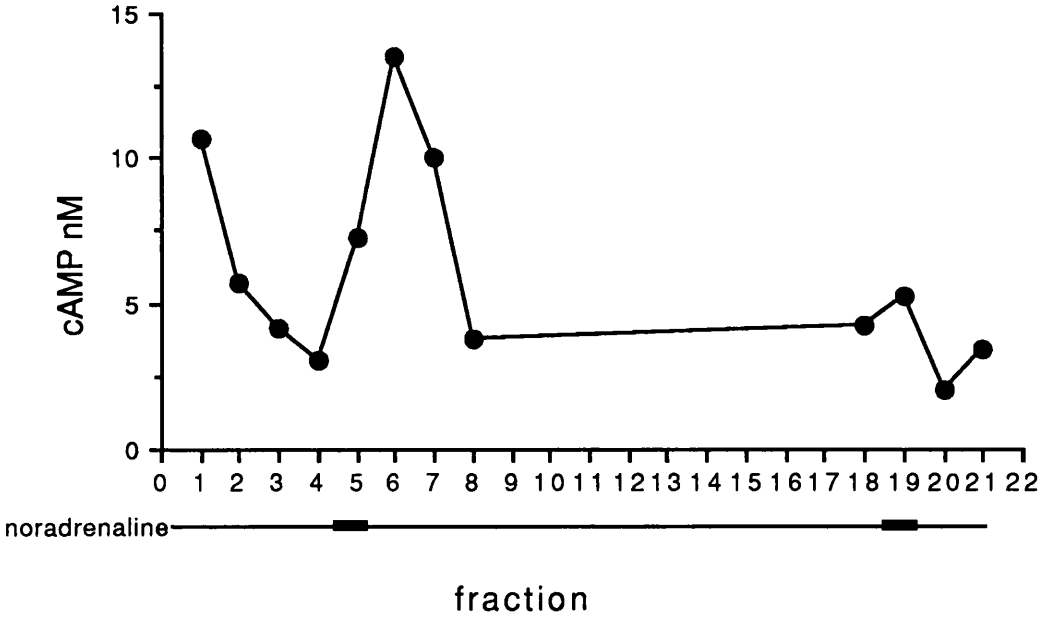


Figure 4.11. The effect of two pulses of noradrenaline upon cAMP level in dialysates (10 min fractions, flow rate=1.2 µl.min $^{-1}$) Probes were implanted into the frontal cortex of rats. In order to improve the sensitivity and temporal resolution of the dialysis procedure, probes were perfused at a rate of 1.2 µl.min^{-1}, fractions were collected for 10 minute time periods. Noradrenaline was present in the perfusing medium for the duration of fractions 5 and 19. Fractions 9 to 17 were not analysed. Graph represents data obtained from the pooled samples derived from 5 individual animals, each point represents a single cAMP measurement.

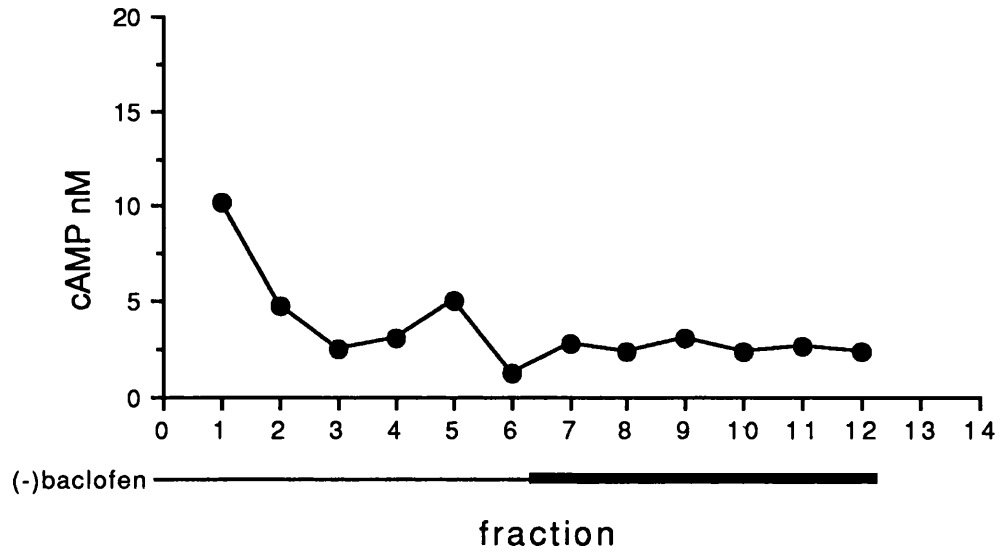


Figure 4.12 The effect of 10 mM (-)baclofen upon baseline levels of cAMP (10 min fractions, flow rate=1.2 μl.min $^{-1}$). An initial decline in the level of cAMP in the dialysate in the first three fractions followed by a steady baseline. 10 mM (-)baclofen was introduced into the perfusing medium at fraction 7 and remained until the end of the experiment, it did not appear to influence the level of cAMP. Data represents the pooled samples from 5 animals.

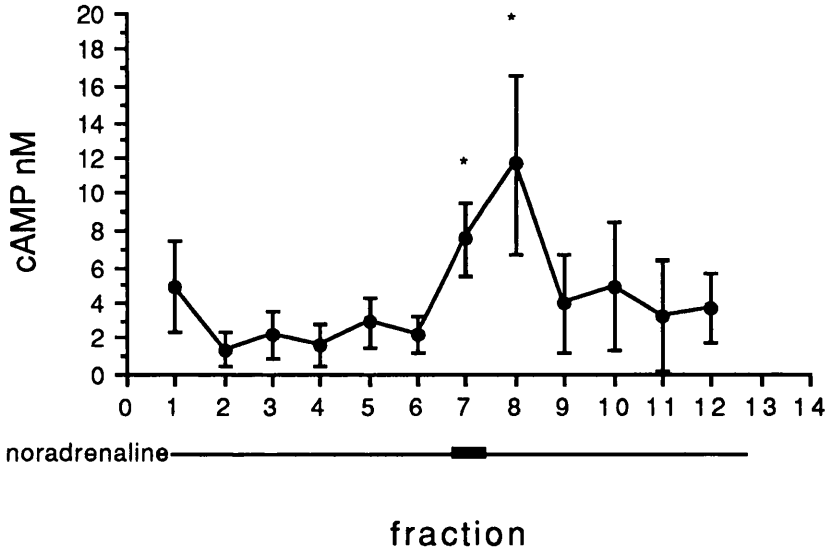


Figure 4.13. Stimulation of cAMP by noradrenaline (1mM) in dialysates. Probes were implanted in rat frontal cortex and were perfused at a rate of 1.2 μl min^{-1}. Fractions were collected for 10 min periods. Data points are the mean of three experiments in which the samples were derived from the pooled dialysate of 5 animals (15 animals, n = 3) * $p < 0.05$ one tailed Students t-test.

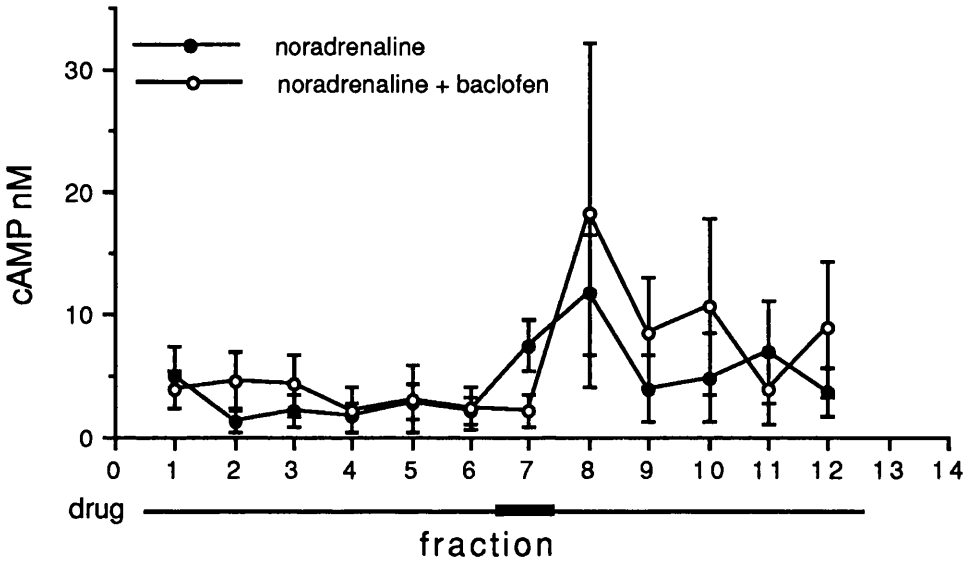


Figure 4.14. The effect of (-)baclofen upon noradrenaline-stimulated cAMP in dialysates. Probes were implanted into the frontal cortex and perfused at a rate of 1.2 $\mu l.min^{-1}$. Fractions were collected for 10 min periods. During fraction 7 animals received either noradrenaline (1 mM) or noradrenalin plus (-)baclofen (10 mM). Data represents the mean of three experiments in which the samples were derived from the pooled dialysate of 5 animals (15 animals, n = 3).

record an increase in cAMP levels in dialysates as a consequence of a 10 min pulse of noradrenaline. Significant increases in cAMP concentration were recorded during the pulse and in the fraction immediately following it (Figure 4.13).

The effect of (-)Baclofen upon Noradrenaline Stimulated cAMP *In Vivo*

The influence of (-)baclofen upon this response was investigated by perfusing the dialysis probe with ACSF containing 1 mM noradrenaline and 10 mM (-)baclofen for the duration of one fraction. The results are shown in Figure 4.14. Although (-)baclofen and noradrenaline appeared to produce a greater maximal response than noradrenaline alone, and the levels of cAMP remained more elevated for a longer period of time, these effects were not statistically significant (ANOVA followed by Dunnetts multiple range test, $p>0.10$).

The effects of (-)baclofen upon noradrenaline-stimulated adenylyl cyclase activity were also analysed by comparing the area under the elevated portion of each curve. No statistically significant difference was found (Students t-test, n=3).

4.3. Discussion

Experiments were conducted to explore the feasibility of measuring cAMP in dialysates derived from rat frontal cortex. The initial studies were concerned with the measurement of baseline concentrations of cAMP, and utilized a flow rate of 2 $\mu l\ min^{-1}$, fractions were collected for periods of 30 min. The baseline concentration of cAMP was determined to be approximately 3 nM in the dialysing medium. This baseline was similar to that recorded by Egawa et al. (1988) in the frontal cortex, but was lower than

that detected by Hutson and Suman-Chauhan (1990) in the striatum. This suggests that regional variations in the concentration of extracellular cAMP occur.

Previous studies have attempted to calculate the extracellular concentrations of cAMP in brain tissue by evaluating the *in vitro* recovery efficiency of the dialysis probe and correcting for this factor *in vivo* (Stone and John, 1990). However, this method is subject to a number of unknown variables. Firstly, the rate of diffusion of cAMP through brain tissue is unknown. This will affect the extent to which cAMP is depleted in the vicinity of the probe. It is also probable that the phosphodiesterase inhibitor IBMX diffuses out of the probe, artificially raising cAMP concentration in the vicinity of the probe. Thus, in the present study, no estimate was made of the extracellular concentration of cAMP in the brain.

Experiments were performed to determine the effect of (-)baclofen on baseline cAMP levels. The administration of 10 mM (-)baclofen through the dialysis probe did not alter baseline levels of cAMP. This is consistent with *in vitro* studies where (-)baclofen also did not alter unstimulated levels of cAMP. However, since *in vitro* (-)baclofen influenced noradrenaline or forskolin-stimulated adenylate cyclase activity, the failure to observe any effect of (-)baclofen *in vivo* suggests that the endogenous tone in the adenylyl cyclase system is relatively low. In addition, since (-)baclofen did not influence basal cAMP levels, it is unlikely that this compound influenced regional activation of β-adrenoceptors *in vivo* by activating $GABA_B$ receptors on noradrenergic terminals (Bowery et al., 1980). The lack of effect of (-)baclofen in these assay conditions might also be due to low levels of

(-)baclofen actually reaching $GABA_B$ receptors. This could occur if (-)baclofen failed to diffuse out of the dialysis probe. Experiments conducted here suggest that this is not the case, indeed (-)baclofen appears to diffuse out of the probe relatively freely. However, these experiments do not determine the extent to which (-)baclofen diffuses through brain tissue, or the rate at which it is cleared from the brain. Both of these could be expected to dramatically effect the concentration of (-)baclofen in brain tissue. Another explanation for the lack of effect of (-)baclofen is that the $GABA_B$ receptors in frontal cortex might already be fully occupied by endogenous agonist.

The effects of forskolin, by contrast with the effects of (-)baclofen, were relatively dramatic. A concentration of forskolin was selected to be 10 fold greater than that which gave a near maximal stimulation of cAMP in the *in vitro* experiments. This was to allow for the efficiency with which compounds diffuse through the dialysing membrane and through the surrounding tissue. This concentration of forskolin, administered through the probe for 30 min caused an elevation in cAMP to around 275% of basal level, and cAMP remained elevated for 1 hour. It was noted that whilst the basal level of cAMP recorded in these experiments was relatively stable in each animal, a high degree of variation was found in basal levels between animals, a feature of cAMP dialysis that was noted by Hutson and Suman-Chauhan (1990). These variations might be due to differences in the micro environment around the tip of the dialysis probe. Such differences might include the extent of gliosis around the probe, the precise location of the probe within the cortical laminae and small variations in the specification of the dialysis probe.

In order to investigate the effect of $GABA_B$ receptor stimulation upon this response it was necessary to use an internal control in each experiment. It was decided to include two periods of stimulation with forskolin in each experiment (S1 and S2). The first period of stimulation was used to standardise the preparation and the second period of stimulation was used to investigate the effects of (-)baclofen. In control experiments the S2 stimulation was smaller than the S1 stimulation (S2:S1=0.73 ± 0.25). Although in this case the S2:S1 ratio was not significantly different from unity, in many release experiments it is less than one. This is likely to be due to fatigue, either pharmacological desensitisation, or depletion of precursors. (-)Baclofen was included, during the second period of stimulation at 4 concentrations at decade intervals between 10 μM and 10 mM. Whilst all of these gave apparently larger S2:S1 ratios than the control response, none was significantly greater than the control ratio. This was particularly interesting since from the *in vitro* data it was expected that $GABA_B$ receptor stimulation would exert an inhibitory effect upon forskolin-stimulated adenylyl cyclase activity.

There are a number of possible explanations for this lack of effect of (-)baclofen in addition to those already discussed in the context of baseline cAMP. For instance, it is possible that forskolin and baclofen have different diffusion rates through the tissue. Thus (-)baclofen might reach only a small proportion of the adenylyl cyclase that is stimulated by forskolin. It is also important to appreciate that there are fundamental differences between the *in vivo* microdialysis method and the *in vitro* cortical slice

assay. One of the most striking differences is that the cortical slice assay is terminated by near boiling temperatures, a process which presumably lyses many of the cells in the preparation. The subsequent assay then detects cAMP which is derived from both intracellular and extracellular pools. In microdialysis experiments in which animals are allowed to recover over night, much of the tissue damage is repaired, and cAMP is derived exclusively from the extracellular space. This raises the possibility that baclofen does modulate cAMP production *in vivo,* but extracellular cAMP concentrations do not reflect intracellular concentrations.

Experiments were also performed to assess the possibility of using the technique of microdialysis, under these experimental conditions, to investigate the effects noradrenaline upon cAMP production. However, no response to 1 mM noradrenaline was recorded. It is possible that the reason for this lack of response was desensitisation of β-adrenoceptors, such that elevated cAMP levels only occurred during the first few minutes of the 30 minute fraction. To counter this, fractions were collected for 10 minute periods. In addition, to increase the concentration of cAMP in the dialysate a slower perfusion rate (1.2 μl min^{-1}) was used. To compensate for these two changes fractions from five individual animals were pooled for the assay of cAMP at each time point. Using these parameters a noradrenaline-stimulated increase in the cAMP was recorded for a period of 20 min. However, even after a lapse of 100 minutes a second pulse of 1 mM noradrenaline was ineffective, suggesting again that the receptors had desensitised. This made the two pulse method used to determine the effcts of (-)baclofen upon forskolin-stimulated adenylyl cyclase activity unusable in the present case. Thus, the most viable way to assess the effect of

$GABA_B$ receptor stimulation upon noradrenaline-stimulated cAMP production was to make a direct comparison of the absolute values of cAMP in a single pulse. When this was done the addition of 10 mM (-)baclofen appeared to cause a greater maximal response than was seen in the presence of noradrenaline alone, and the levels of cAMP remained elevated for a longer period of time. However these differences were not statistically significant. Again there are several possibilities why $GABA_B$ receptor stimulation failed to enhance the levels of cAMP seen in the presence of noradrenaline. It is possible that the release of endogenous noradrenaline has been inhibited by the presence of the $GABA_B$ receptor agonist working at terminal autoreceptors. Alternatively the $GABA_B$ receptors responsible for modulating adenylyl cyclase may be fully occupied by endogenous GABA.

Conclusions

In summary, the administration of various concentrations of (-)baclofen through the probe did not appear to modulate baseline levels of cAMP dialysed from rat frontal cortex. In contrast, both forskolin and noradrenaline (under different conditions) caused an elevation of dialysed cAMP. However, neither of these responses was modified in the presence of (-)baclofen. The reason why $GABA_B$ receptor stimulation failed to modulate forskolin and noradrenaline-stimulated cAMP awaits further experiments. Of particular value would be the determination of the effect of a $GABA_B$ receptor antagonist to investigate any interference of endogenous GABA at the relevant $GABA_B$ receptors.

5
The Involvement of Glial Cells in Receptor Mediated Adenylyl Cyclase Activation in Rat Cortical Slices.

5.1. Introduction

Brain slices are frequently used as an experimental preparation of neuronal tissue in neurophysiological experiments. In many cases, such as in neurotransmitter release studies or electrophysiological studies, it is legitimate to assume that the neurotransmitter receptors under investigation are neuronal. However, in these preparations glial cells are present in greater numbers than the neurons themselves (Hertz and Richardson, 1984). Glial cells are known to possess adenylyl cyclase (for review see Hertz and Richardson, 1984), β-adrenoceptors (Hosli and Hosli, 1982) and $GABA_B$ receptors (Hosli and Hosli, 1990). Indeed, it has been reported that up to 95% of cAMP produced as a result of β–adrenoceptor activation in rat forebrain slices is derived from glial cells (Stone et al., 1990). This raises the possibility that $GABA_B$ receptor mediated augmentation of β-adrenoceptor-stimulated cAMP actually occurs in glial cells. This hypothesis is supported by the observation that the $GABA_B$ receptor mediated modulation of β–adrenoceptor-stimulated cAMP is absent in a preparation of synaptosomes (Hill and Dolphin, 1984). Since the $GABA_B$ receptor mediated inhibition of forskolin-stimulated cAMP production is intact in synaptosomal preparations (Wojcik and Neff, 1984; Hill and Dolphin, 1984) it suggests that this response, in contrast, may be present in a purely neuronal preparation. In the present study two experiments were under taken to examine the role of glial cells in the $GABA_B$ receptor mediated modulation of cAMP production. In the first, the specific glial cell toxin fluorocitrate was used to eliminate glial cell metabolism from rat cortical slices. The second experiment involved the

investigation of forskolin and noradrenaline-stimulated adenylyl cyclase activity in glial cells in culture.

5.2. Results

cAMP Overflow in Control and Fluorocitrate Treated Rat Cortical Slices

Control rat cortical slices were incubated with various agents to stimulate cAMP production. Relatively low levels of cAMP were detected in unstimulated slices (1.3 ± 0.6 pmol mg protein^{-1} min^{-1}, n=3). In the presence of 10 μM forskolin the production of cAMP was enhanced approximately 12 fold (15.2 ± 3.27 pmol mg protein^{-1} min^{-1}, ANOVA, followed by Duncans multiple range test $p<0.01$, n=3). In order to compare the effects of other treatments the remaining data was normalized by expressing it as the percentage of the forskolin response in these untreated slices. Thus, in control slices (-)baclofen (100 μM) inhibited the response to forskolin to $62.3 \pm 3.6\%$ of the response to forskolin alone. Noradrenaline (100 μM) caused an enhancement of cAMP production which was rather less dramatic than that caused by 10 μM forskolin ($24.3 \pm 0.33\%$ of the forskolin response), but which nevertheless represented a significant increase over unstimulated cAMP levels (ANOVA, followed by Duncans multiple range test, $p<0.01$). In the presence of (-)baclofen the response to noradrenaline was increased to $43.3 \pm 11.2\%$ of the forskolin response. This represented a significant increase over the response to noradrenaline alone (ANOVA, followed by Duncans multiple range test, $p<0.01$)(Figure 5.1).

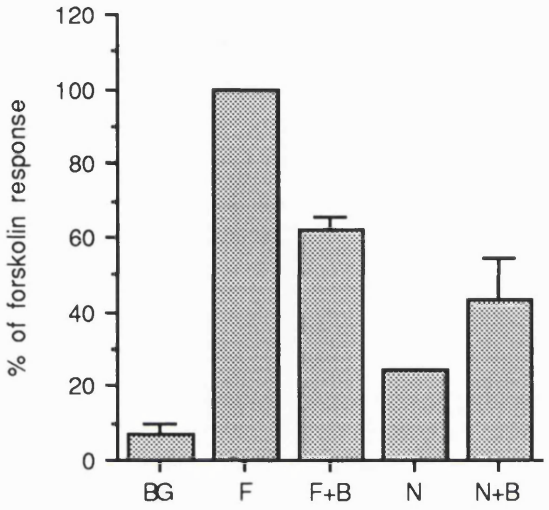


Figure 5.1. Forskolin and noradrenaline-stimulated adenylyl cyclase activity in control rat cortical slices. Levels of cAMP in control rat cortical slices assayed under the following conditions: background (BG), 10 μM forskolin (F), in the presence and absence of 100 μM (-)baclofen (B), and 100 μM noradrenaline (N) in the presence and absence of 100 μM (-)baclofen (B). Data are expressed as a percentage of the forskolin response. (n = 3).

In fluorocitrate-treated slices, forskolin-stimulated cAMP overflow was reduced to 22.0 ± 3.2% of the level achieved in control slices (Figure 5.2). Noradrenaline (100 μM) was unable to enhance cAMP production in these slices. In contrast to the control slices, (-)baclofen did not inhibit forskolin-stimulated adenylyl cyclase activity or influence the levels of cAMP produced in the presence of noradrenaline (Figure 5.3).

cAMP Production in Glial Cell Culture

The background levels of cAMP detected in cultured glial cells was very low (3.7 ± 0.7 pmol ml^{-1}). Incubating the cells with forskolin (10 μM) for 10 min produced a 306 fold increase in the level of cAMP (1132 ± 109 pmol ml^{-1}). However, (-)baclofen (100 μM) did not modulate this response. Similarly, noradrenaline (100 μM) alone produced a 93 fold stimulation of cAMP overflow, but again this response was not influenced by the presence of (-)baclofen (Figure 5.4).

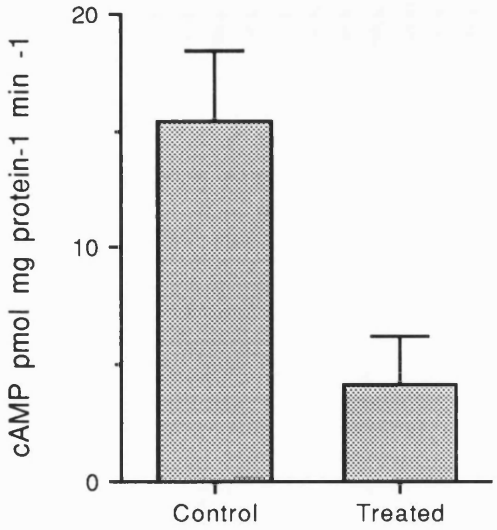


Figure 5.2. The effect of fluorocitrate upon forskolin-stimulated adenylyl cyclase activity in rat cortical slices. Levels of cAMP in control rat cortical slices and slices pretreated with fluorocitrate were assayed following incubation for 10 min with 10 μM forskolin. Pretreatment with fluorocitrate under the present conditions causes a reduction in adenylyl cycalase activity of 78% (n = 3).

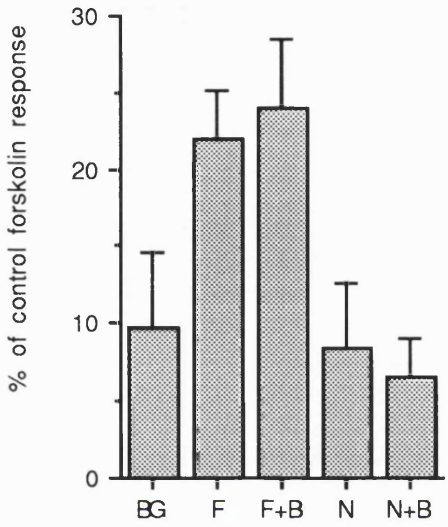


Figure 5.3. The effect of fluorocitrate upon the ability of (-)baclofen to modulate forskolin and noradrenaline-stimulated adenylyl cyclase activity. Levels of cAMP in control rat cortical slices and slices pretreated with fluorocitrate were assayed under the following conditions: background (BG), 10 μM forskolin (F) in the presence and absence of 100 μM (-)baclofen (B), and 100 μM noradrenaline (N) in the presence and absence of 100 μM (-)baclofen (B). Data are expressed as a percentage of the forskolin response in control slices. (n = 3).

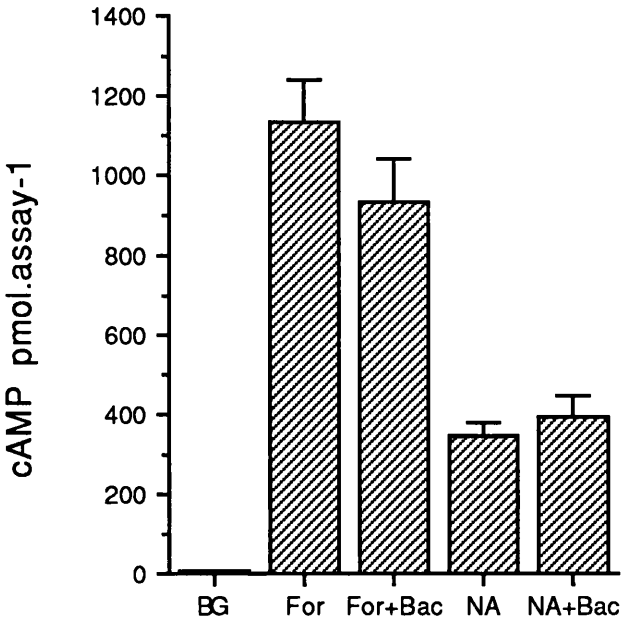


Figure 5.4. The GABA$_B$ modulation of forskolin- and noradrenaline-stimulated adenylyl cyclase in glial cells. The concentration of cAMP in cultured glial cells was assayed after stimulation of adenylyl cyclase under various pharmacological conditions. The conditions represent background (BG), 10 μM forskolin (For), in the presence and absence of 100 μM (-)baclofen (Bac), and 100 μM noradrenaline (NA) in the presence and absence of 100 μM (-)baclofen. (n = 4).

5.3. Discussion

The experiments described in this chapter were designed to examine the role of glial cells in the $GABA_B$ receptor mediated modulation of adenylyl cyclase activity in two different ways. The first used the glial cell toxin fluorocitrate to produce a slice preparation containing only neuronal elements. Fluorocitrate functions as an inhibitor of aconitase (EC 4.2.1.3.)(Peters, 1957), halting the tricarboxylic acid cycle at this point. This stops metabolic processess within the cell, halting the production of ATP, the essential precursor of cAMP (Fonnum, 1985; Muir et al., 1986). Fluorocitrate is reported to be specific for glial cells by virtue of a selective uptake process, which is considered to be the same mechanism which allows glial cells to selectively take up unfluorinated citrate and acetate (O'Neal and Koeppe, 1966; Fonnum, 1985). In the present study rat cortical slices were pre-incubated with fluorocitrate, using identical conditions to those found by Stone et al., (1990) to selectively eliminate glial cell activity from slices. These authors found that this treatment regime left neuronal activity intact. Thus, intracellular recordings from "bursting" cortical neurons revealed that resting membrane potentials were not altered by fluorocitrate treatment, and neither was the ability of these neurons to generate action potentials in response to depolarizing currents. In addition these authors used a preparation of heart tissue to demonstrate that pretreatment with fluorocitrate did not directly influence the β-adrenoceptor mediated activation of adenylyl cyclase. they also found that fluorocitrate was equally effective in the presence and absence of phosphodiesterase inhibitors, indicating that its effects were not a consequence of phosphodiesterase activation (Stone et al., 1990).

In the present study, pretreatment of brain slices with fluorocitrate eliminated a large proportion of forskolin-stimulated adenylyl cyclase activity. This result is consistent with the idea that much of the adenylyl cyclase activity in brain slices is derived from glial cells. However, a small pool of adenylyl cyclase was resistent to the actions of fluorocitrate, and this may well be located in neuronal cells. Pretreatment with fluorocitrate abolished the elevation in cAMP production mediated by noradrenaline. This is in broad agreement with Stone et al. (1990), who found that up to 95% of the adenylyl cyclase activity stimulated by β-adrenoceptor activation emanated from glial cells. Data from the present study supports the idea that adenylyl cyclase activity is present in both neuronal and glial elements in rat brain slices, and that almost all of the adenylyl cyclase activity that responds to β–adrenoceptor activation is located in glial cells.

In the second series of experiments in this study, cell culture was used to create a preparation containing only glial cells. Noradrenaline and forskolin produced a relatively large stimulation of cAMP production in these cultures, suggesting that glial adenylyl cyclase makes an important contribution to the cAMP production in rat brain slices. The localization of β-adrenoceptor-stimulated cAMP in glial cells is an important finding since it indicates that glial cells are a target for the neuronal adrenergic system in rat brain, Indeed some authors have proposed that they may be the main target (Minneman et al., 1978; Nahorski et al., 1979). This is interesting since it suggests that the trophic, metabolic and uptake functions performed by glia may be under neuronal control.

In the present study it was found that the modulation of adenylyl cyclase activity by $GABA_B$ receptors was abolished in both of the above preparations. There are a number of possible explanations for this. For instance, the modulation of cAMP overflow by $GABA_B$ receptors might rely upon the presence of both glial cells and neurons. Such a relationship might involve a soluble intercellular messenger which communicates the activation of one cell type with the other. Alternatively the $GABA_B$ receptor mediated modulation of adenylyl cyclase may be absent from one or both preparations for other reasons. For instance, the inhibition of glial cell metabolism in a slice preparation may inhibit the activity of neurons by removing the trophic and supportive functions attributed to glial cells. This, however, is unlikely considering the short periods of time that brain slices are maintained *in vitro*. The $GABA_B$ receptor mediated modulation of adenylyl cyclase activity may also be absent from cultured cells for other reasons. For instance, cells in culture are known to be biochemically different, and to express different genes to the same cells in intact CNS tissue. It is therefore possible that the ability of $GABA_B$ receptors to mediate the modulation of adenylyl cyclase activity is lost during the process of cell culture.

In summary the glial cell toxin, fluorocitrate, and cultured glial cells were used to investigate the possibility that a proportion of the $GABA_B$ receptor mediated modulation of cAMP production occurs in glial cells. The ability of forskolin and noradrenaline to stimulate cAMP production was markedly reduced in brain slices treated with fluorocitrate. Both forskolin and noradrenaline were able to stimulate cAMP production in a

glial cell culture. These data confirm previous suggestions (Stone et al., 1990) that a large proportion of cAMP is derived from glial cells. $GABA_B$ receptor mediated modulation of adenylyl cyclase was absent in both preparations raising the possibility that both cell types are required.

Problems associated with the interpretation of slice data

Whilst the stimulation of cAMP production, and indeed many neurotransmitter release assays have been well characterized in cross chopped cortical slices, the interpretation of these data in a physiological context contains a number of pitfalls. Some of these are inherent in the nature of the slice preparation itself (for instance the removal of axonal input from other brain regions) others are due to the preparation techniques often used to prepare slices. Of major consideration in this context is tissue damage. Light microscopy of mechanically chopped slices has shown many pathological changes, for instance, large necrotic nerve cells may be characterized by swollen and vacuolated cell bodies and small necrotic neurons may be characterized by condensed, nuclei, expanded cytoplasm and swollen structures witihin the neuropil. Shrunken nerve cell dendrites also occur). Transmission electron microscopy of similar preparations has revealed the disruption of many cellular components (nuclei, mitochondria, golgi apparatus and endoplasmic reticulum) as well as swollen glial cell processses and neuronal fibres. Tissue damage of this kind is particularly extensive in mechanically chopped slices, such as those used in the present study. For a comprehensive review see Alger et a.l (1984). A rigorous interpretation of the slice data presented here, in particular with reference to the cell types involved in the production of cAMP, would depend upon undertaking a histological examination of the slices used for these experiments and determining the levels of preservation of the cell types involved.

Alger, B.E., Dhanjal, S.S., Dingledine, R., Garthwaite, J, Henderson, G., King, G.L., Lipton, P., North, A., Schwartzkroin, P.A., Sears, T.A., Segal, M., Whittingham, T.S. and Williams, J. (1984) Brain Slice Methods: Evaluating Slice Data: Histology, in *Brain Slices*, (Dingledine R., ed) pp 413-421 Plenum. New York.

6
The Effect of Chronic Administration of $GABA_B$ Receptor Antagonists and (-)Baclofen Upon $GABA_B$ Receptors

6.1. Introduction

CGP46381 and CGP36742 are the first orally active $GABA_B$ receptor antagonists. They have been used in the present study along with (-)baclofen to investigate the possible effects of long term administration of $GABA_B$ receptor drugs, upon populations of $GABA_B$ receptors. These effects have been assayed using two techniques. Firstly, the ability of $GABA_B$ receptors to modulate adenylyl cyclase activity in cerebral cortex was determined in brains of chronically dosed animals. Secondly, autoradiography was used to assess local changes in [^{3}H]-GABA binding to $GABA_B$ receptors.

6.2. Results

Adenylyl Cyclase Modulation in Chronically Dosed Animals

In saline treated animals, 10 μM forskolin cause a dramatic 18 fold stimulation of adenylyl cyclase activity (ANOVA, followed by Duncan's multiple range test, $p<0.01$). In the same group of animals 100 μM isoprenaline caused a 2.8 fold increase in the basal level of cAMP production. (ANOVA, followed by Duncan's multiple range test, $p<0.01$, Figure 1), Because of the wide variation between experimental animals, the ability of $GABA_B$ receptor stimulation to modulate these responses was expressed as percent inhibition of forskolin response (Figure 6.2), and

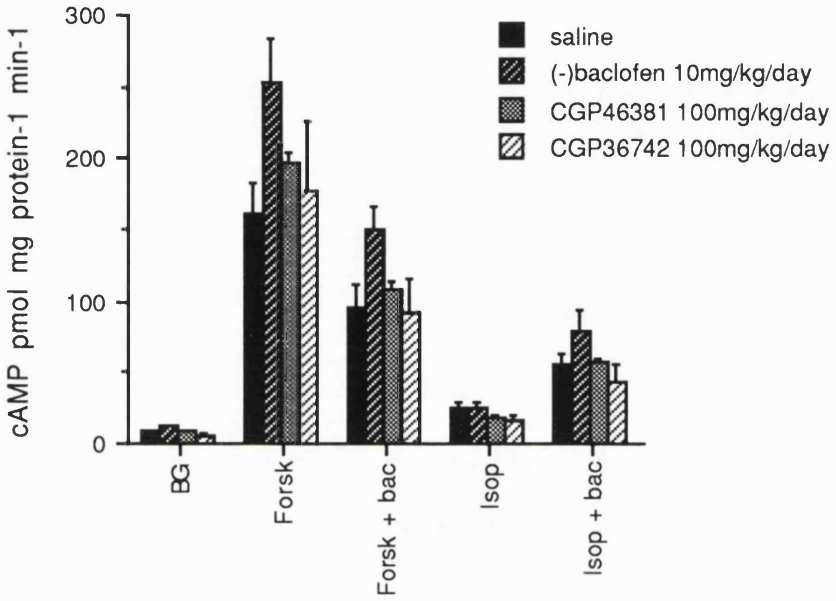


Figure 6.1. The effect of chronic dosing upon forskolin- and isoprenaline-stimulated adenylyl cyclase in rat cortical slices. The concentration of cAMP in supernatant of slices was determined after stimulation of adenylyl cyclase with either 10 μM forskolin (forsk.) or 100 μM isoprenaline (isop), both in the presence or absence of 100 μM (-)baclofen (bac). The responses in slices derived from animals that had been treated with (-)baclofen, CGP46381 or CGP36742 did not differ from saline treated animals (ANOVA followed by Duncans multiple range test $p>0.05$).

All drugs were administered I.P.

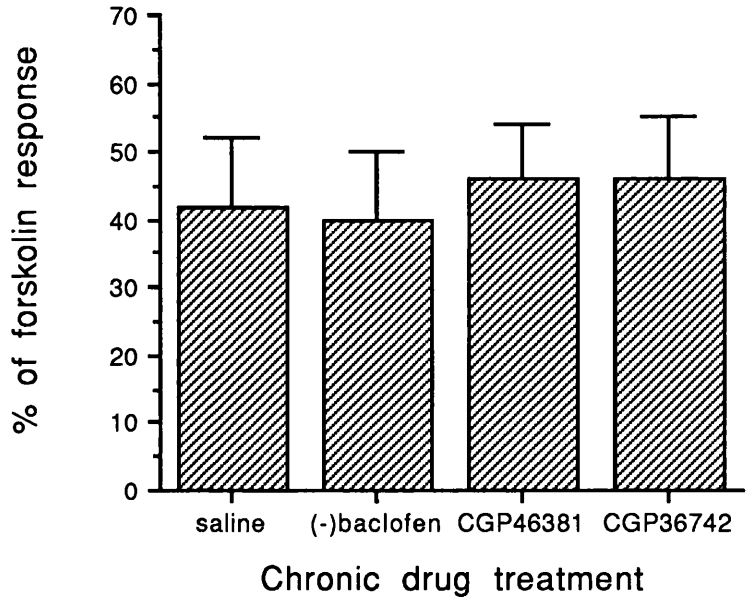


Figure 6.2. $GABA_B$ receptor mediated modulation of forskolin-stimulated adenylyl cyclase activity in chronically dosed animals. The modulation of forskolin-stimulated adenylyl cyclase activity by 100 μM (-)baclofen was expressed as a percentage of the response to 10 μM forskolin. None of the responses to (-)baclofen in drug treated animals differed from saline treated controls ($p>0.05$, ANOVA, followed by Duncans multiple range test).

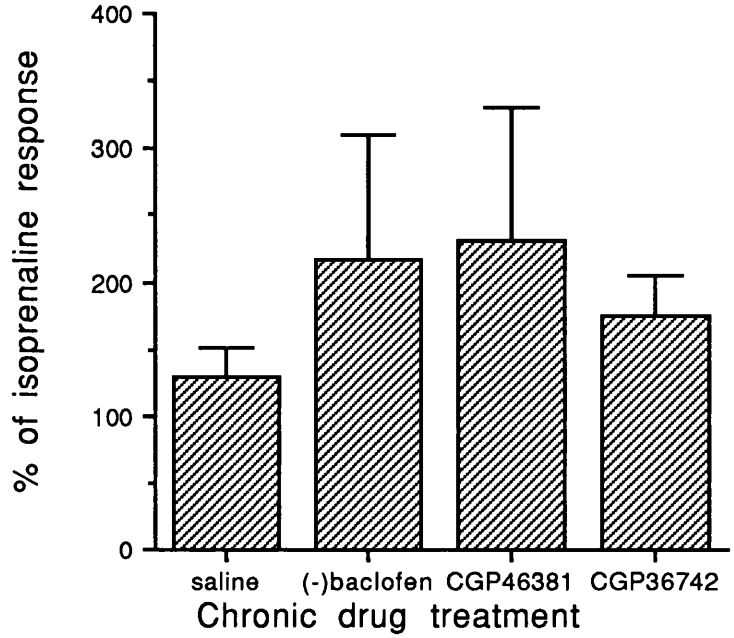


Figure 6.3. $GABA_B$ receptor mediated modulation of isoprenaline-stimulated adenylyl cyclase activity in chronically dosed animals. Data for the modulation of isoprenaline-stimulated adenylyl cyclase activity by 100 μM (-)baclofen is expressed here as percent enhancement of the of the response to 100 μM isoprenaline. None of the responses to (-)baclofen in drug treated animals differed from that in saline treated controls ($p>0.05$, ANOVA, followed by Duncans multiple range test).

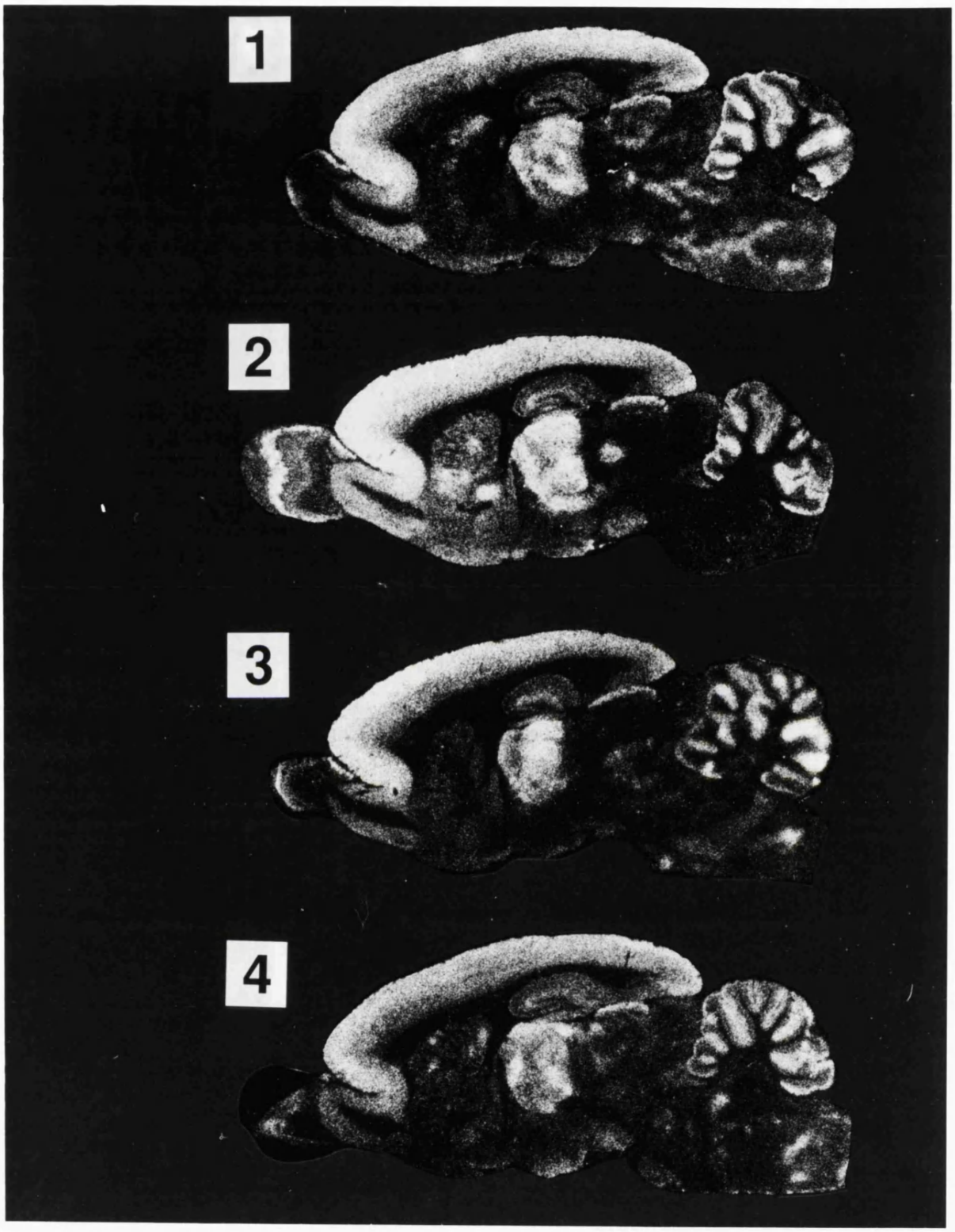
percent augmentation of the isoprenaline response (Figure 6.3). In control samples the presence of 100 μM (-)baclofen caused a 40% inhibition of the forskolin response (ANOVA followed by Duncan's multiple range test, $p<0.01$) and a 129% increase in the isoprenaline response (ANOVA followed by Duncan's multiple range test, $p<0.01$) in the saline treated control animals.

Chronic administration of CGP46381, CGP36742 (100 mg kg^{-1} $day^{-1)}$ or (-)baclofen (10 mg kg^{-1} day^{-1}) did not significantly alter the absolute rates of cAMP production in the presence of forskolin or isoprenaline, nor did it significantly alter the absolute rate of cAMP production in the presence of 100 μM (-)baclofen (ANOVA followed by Duncan's multiple range test, $p>0.05$)(Figure 6.1). The ability of (-)baclofen to modulate forskolin and isoprenaline-stimulated adenylyl cyclase also remained unaltered in chronically dosed animals (ANOVA followed by Duncan's multiple range test, $p>0.05$)(Figures 6.2 and 6.3).

Autoradiography of $GABA_B$ Receptors in Chronically Dosed Animals.

Dark field images of representative sections of brain from each group of animals are shown in Figure 6.4. An anatomical description of these sections, showing the structures selected for quantitative analysis is shown in Figures 6.5 and 6.6. No gross changes in the distribution of $GABA_B$ receptors, caused by drug administration was apparant in these images. The results of quantitative analysis of the autoradiographs is shown in Figures 6.7 to 6.9. For each reading, specific binding was calculated by

Figure 6.4. Distribution of $GABA_B$ receptors in chronically dosed animals. Dark field autoradiographs of the distribution of [^{3}H]-GABA binding to $GABA_B$ receptors in parasagittal sections of rat brain approximately 1.8 mm from the midline. Binding was performed in: 1, saline treated control animals; 2, Baclofen (10 mg.kg^{-1}day^{-1}); 3, CGP36742 (100 mg kg^{-1} day^{-1}); 4, CGP46381 (100 mg kg^{-1} day^{-1}). The binding reaction was carried out at room temperature for 20 min, using 50 nM [^{3}H]-GABA. $GABA_B$ receptors were labelled selectively in the presence of 40 μM isoguvacine and non-specific binding (not shown) was defined in the presence of 100 μM (-)baclofen.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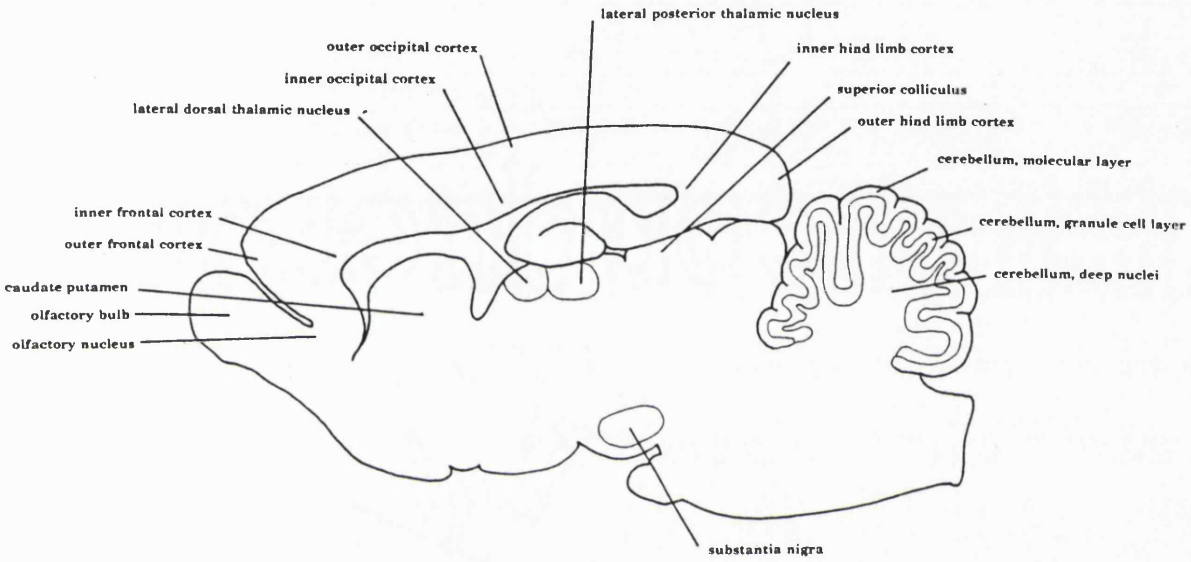


Figure 6.5. Anatomy of the rat brain. An anatomic description of the regions seen in a parasagittal section of rat brain approximately 1.8 mm from midline (cf. Figure 6.4). The labelled areas were those selected quantitative analysis in the present study. The outline and nomenclature are taken from Paxinos and Watson (1986).

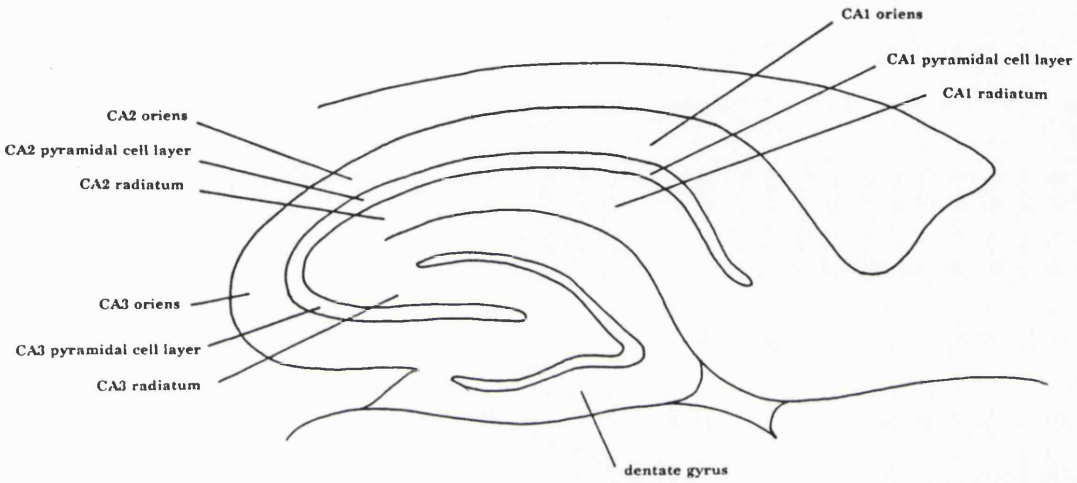


Figure 6.6. Anatomy of the rat hippocampus An anatomic description of the regions of the hippocampus seen in a parasagittal section of rat brain approximately 1.8 mm from midline (cf. Figure 6.4). The labelled areas were those chosen for quantitative analysis in the present study. The outline and nomenclature are taken from Paxinos and Watson (1986).

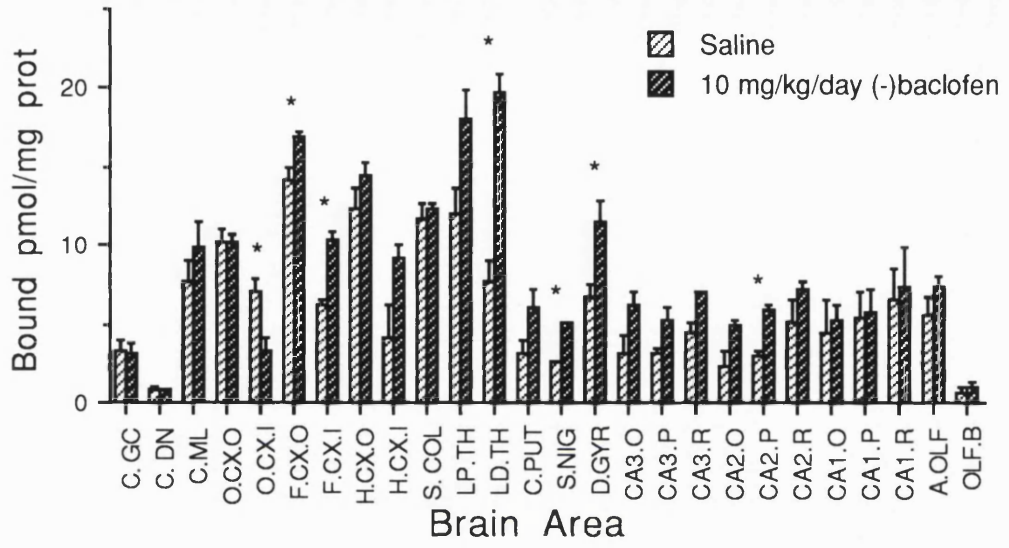


Figure 6.7. The effect of chronic administration of (-)baclofen upon $GABA_B$ receptor radioligand binding. Quantitative analysis of levels of [^{3}H]-GABA binding to $GABA_B$ receptors in autoradiographs of brains of rats dosed with saline or (-)baclofen (10 mg $kg^{-1}day^{-1}$) for three weeks. The areas analysed are: cerebellum granule cell layer (C.GC), cerebellum deep nuclei (C.DN), cerebellum molecular layer (C.ML), outer occipital cortex (O.CXP), inner occipital cortex (O.CXO), outer frontal cortex (F.CXO), inner frontal cortex (FCXI), outer hind limb cortex (H.CXO), inner hind limb cortex (H.CXI), superior colliculus (S.C.), lateral posterior thalamus nucleus (LP.TH), lateral dorsal thalamic nucleus (LD.TH), caudate putamen (C.PUT), substantia nigra (S.NIG), dentate guyrs (D.GYR), stratum oriens (O), pyramidal cell layer (P) and stratum radiatum (R) of the hippocampus have been divided into CA1, CA2 and CA3 zones, anterior olfactory nucleus (A.OLF) and olfactory bulb (OLF.B). (*= significantly different from saline $p < 0.05$, Students t-test).

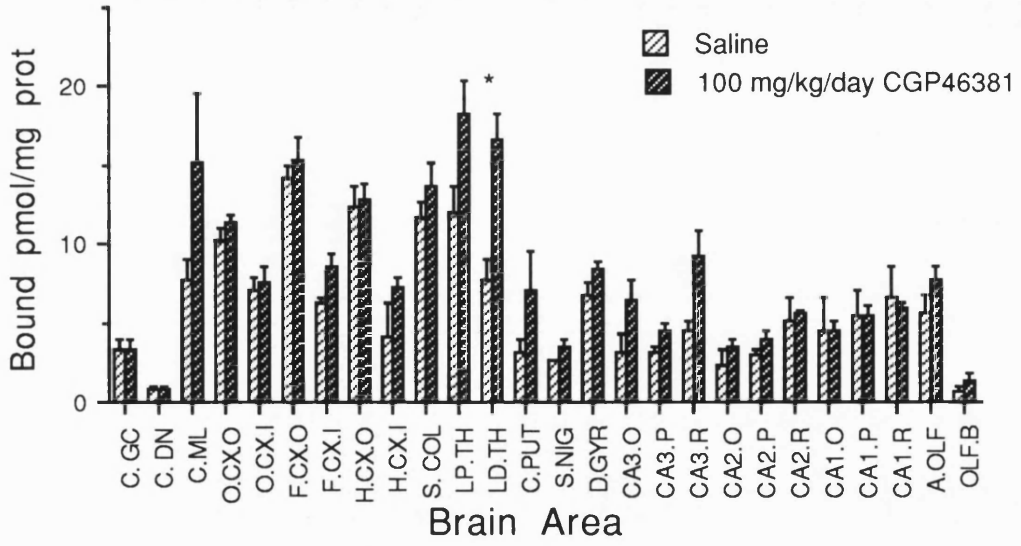


Figure 6.8. The effect of chronic administration of CGP46381 upon $GABA_B$ receptor radioligand binding. Quantitative analysis of levels of [^{3}H]-GABA binding to $GABA_B$ receptors in autoradiographs of brains of rats dosed with saline or CGP46381 (100 mg $kg^{-1}day^{-1}$) for three weeks. The areas analysed are: cerebellum granule cell layer (C.GC), cerebellum deep nuclei (C.DN), cerebellum molecular layer (C.ML), outer occipital cortex (O.CXP), inner occipital cortex (O.CXO), outer frontal cortex (F.CXO), inner frontal cortex (FCXI), outer hind limb cortex (H.CXO), inner hind limb cortex (H.CXI), superior colliculus (S.C.), lateral posterior thalamus nucleus (LP.TH), lateral dorsal thalamic nucleus (LD.TH), caudate putamen (C.PUT), substantia nigra (S.NIG), dentate guyrs (D.GYR), stratum oriens (O), pyramidal cell layer (P) and stratum radiatum (R) of the hippocampus have been divided into CA1, CA2 and CA3 zones, anterior olfactory nucleus (A.OLF) and olfactory bulb (OLF.B). (*= significantly different from saline $p < 0.05$, Students t-test).

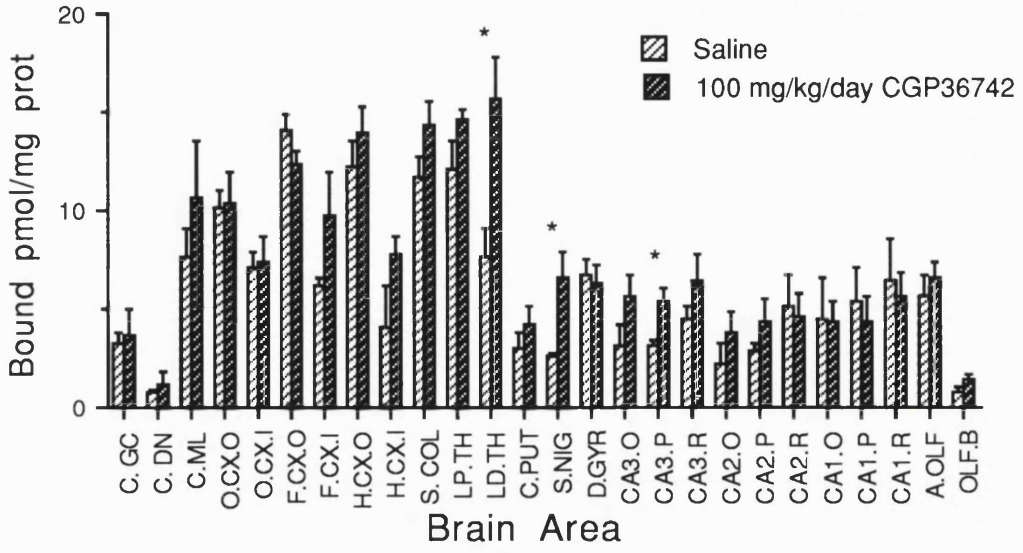


Figure 6.9. The effect of chronic administration of CGP36742 upon $GABA_B$ receptor radioligand binding. Quantitative analysis of levels of [^{3}H]-GABA binding to $GABA_B$ receptors in autoradiographs of brains of rats dosed with saline or CGP36742 (100 mg $kg^{-1}day^{-1}$) for three weeks. The areas analysed are: cerebellum granule cell layer (C.GC), cerebellum deep nuclei (C.DN), cerebellum molecular layer (C.ML), outer occipital cortex (O.CXP), inner occipital cortex (O.CXO), outer frontal cortex (F.CXO), inner frontal cortex (FCXI), outer hind limb cortex (H.CXO), inner hind limb cortex (H.CXI), superior colliculus (S.C.), lateral posterior thalamus nucleus (LP.TH), lateral dorsal thalamic nucleus (LD.TH), caudate putamen (C.PUT), substantia nigra (S.NIG), dentate guyrs (D.GYR), stratum oriens (O), pyramidal cell layer (P) and stratum radiatum (R) of the hippocampus have been divided into CA1, CA2 and CA3 zones, anterior olfactory nucleus (A.OLF) and olfactory bulb (OLF.B). (*= significantly different from saline $p < 0.05$, Students t-test).

subtracting non-specific binding from a total binding value. Results represent the mean of three such readings. Each value was taken from one of three separate sections, each of which was derived from a different animal of the same experimental group (n=3). The difference between the mean density of binding in the drug treated animal was compared to the density of binding in the equivalent brain area in the saline treated animal using a Students t test. This revealed statistically significant ($p<0.05$) differences in the levels of binding in treated animals in several areas. Chronic administration of (-)baclofen (10 mg/kg) caused a down-regulation of 54% in [^{3}H]-GABA binding to $GABA_B$ receptors in the inner layers of the occipital cortex, and an up-regulation of $GABA_B$ receptors in 6 out of the 27 areas analysed. Areas showing an up-regulation in $GABA_B$ receptors included the outer layers of the frontal cortex (20%), the inner layers of the frontal cortex (68%) and the lateral dorsal thalamic nucleus (159%), the substantia nigra (96%), the dentate gyrus (72%) and the CA2 pyramidal cell layer of the hippocampus (50%).

Chronic administration of CGP46381 (100 mg/kg) caused a statistically significant up-regulation of $GABA_B$ receptor binding in only one area, the lateral dorsal thalamic nucleus (115%). Chronic administration of CGP36742 (100 mg/kg) caused a significant up-regulation in 3 of the areas that were investigated, these were the lateral dorsal thalamic nucleus (103%) the substantia nigra (156%) and the CA3 pyramidal cell layer in the hippocampus (68%).

6.3. Discussion

In the present study rats where chronically dosed with the $GABA_B$ receptor antgonists CGP35348 or CGP36742, or the $GABA_B$ agonist (-)baclofen. The effects of these treatments upon $GABA_B$ receptors was investigated using two biochemical assays. Firstly, to investigate the functionality of $GABA_B$ receptors following chronic administration of drugs, cross chopped cortical slices were prepared from the experimental animals and the ability of (-)baclofen to modulate forskolin and isoprenaline-stimulated adenylyl cyclase was investigated. The treatments used in this study did not alter the ability of forskolin or isoprenaline to stimulate adenylyl cyclase activity, or the ability of 100 μM (-)baclofen to modulate these two responses. These data are in broad agreement with that of Pratt and Bowery (1993), however, these authors found an up-regulation of forskolin-stimulated adenylyl cyclase activity as a result of chronic CGP36742 treatment, a finding which has not been repeated in the present study.

To examine the possibility that these chronic treatments induced localized changes in $GABA_B$ receptor density that could not be detected in the cortical slice preparation an autoradiographic study of $GABA_B$ receptors was undertaken. The saline treated controls revealed high levels of [^{3}H]-GABA binding to $GABA_B$ receptors in the molecular layer of the cerebellum, in the cortex (in particular the outer layers), the superior colliculus and in the thalamus (in particular the lateral dorsal and lateral posterior nuclei). This is in agreement with previous studies on the distribution of $GABA_B$ receptors (Bowery et al., 1984; 1987).

All of the compounds tested had some effect on the levels of radioligand binding to $GABA_B$ receptors in at least one of the areas under investigation. The compound which had the most wide ranging effects was the specific agonist (-)baclofen. Chronic treatment with (-)baclofen induced a down-regulation in radioligand binding in the inner layers of the occipital cortex. This finding can be explained as a classical effect of chronic dosing with an agonist. Thus, a compensatory down regulation is induced through over activation of receptors. Chronic treatment with (-)baclofen also induced up-regulation of $GABA_B$ receptors in some areas. These areas included the inner and outer layers of the frontal cortex, the lateral dorsal thalamic nucleus, the substantia nigra, the dentate gyrus and the CA2 pyramidal cell layer of the hippocampus. This new finding is less easy to explain.

One way to explain the ability of (-)baclofen to induce an up regulation of $GABA_B$ receptors is to hypothesize that (-)baclofen is acting preferentially at presynaptic receptors. This action would reduce the amount of endogenous GABA released into the synaptic cleft at GABAergic synapses. This reduction in release might cause a compensatory up-regulation in the number of postsynaptic $GABA_B$ receptors. This hypothesis presupposes that the down-regulation in the synaptic concentration of GABA must more than compensate for the increased concentration of (-)baclofen in the brain. Thus the efficacy with which (-)baclofen reduced neurotransmitter release must be greater than its ability to stimulate (and cause down-regulation of) postsynaptic receptors. Since there is no evidence that (-)baclofen acts preferentially at presynaptic

receptors, this would require the presynaptic $GABA_B$ receptor to be more efficiently coupled to its effectors than the postsynaptic receptor. Whilst this assertion is purely speculative, there may be good physiological reasons for it being true. For instance, if both pre- and postsynaptic receptors desensitised to the same extent then the following scenario might prevail. A rise in the synaptic concentration of GABA would cause both pre- and postsynaptic receptors to desensitise to compensate. The desensitisation of presynaptic receptors however would allow yet more GABA to be released causing further postsynaptic desensitisation. Equilibrium might never be attained. If presynaptic receptors did not show desensitisation then a rise in released GABA would cause presynaptic inhibition, and postsynaptic down-regulation of receptors. Both of these might be regarded as compensatory mechanisms. Thus it is conceivable that dosing with an agonist might cause an up-regulation in radioligand receptor binding under these circumstances.

An alternative explanation for the up-regulation in $GABA_B$ receptor radioligand binding seen following chronic dosing with (-)baclofen is related to the novel findings described in chapter 3, from which it was proposed that (-)baclofen might have partial agonist properties. If (-)baclofen has a lower intrinsic efficacy than the endogenous agonist GABA, and if $GABA_B$ receptors are highly saturated with GABA *in vivo*, then (-)baclofen might show antagonist properties. These antagonist properties could reveal themselves as an up-regulation in $GABA_B$ receptors following chronic dosing.

Whilst such a detailed study of the effects of chronic dosing with (-)baclofen has not been reported before, Suzdak and Giantusos (1986) have reported a down-regulation in high affinity binding of [^{3}H]-GABA binding to $GABA_B$ receptors, and an up-regulation of low affinity binding sites in mouse brain homogenates, suggesting that the effects of chronic administration of (-)baclofen may indeed be complex.

Chronic dosing with the two novel antagonists, CGP46381 and CGP36742, produced less wide spread effects, both of these compounds produced an up-regulation in radioligand receptor binding in the lateral dorsal thalamic nucleus. In addition CGP36742 produced an up-regulation in radioligand receptor binding in the substantia nigra and the CA3 pyramidal cell layer. Up-regulation of receptors is the classical response to chronic treatment with antagonists, and is thus easier to explain than the up-regulation seen as a result of chronic (-)baclofen administration. The reason why these effects were specific for discrete brain nuclei may reflect the degree of activation of $GABA_B$ receptors in these nuclei. Thus, antagonists are only effective in regions where receptors are endogenously activated. So we can speculate that in the lateral dorsal thalamic nucleus in particular the $GABA_B$ receptors are more frequently activated than those elsewhere. Alternatively, the $GABA_B$ receptors in this region may be unusually sensitive to desensitisation by the action of agonists.

It is interesting to observe that chronic treatment with antagonists or the agonist (-)baclofen all induced an up-regulation of $GABA_B$ receptors in the lateral dorsal thalamic nucleus. The similarity of effects of (-)baclofen with those of the antagonists may be explained either as the activation of the

presynaptic receptors decreasing the concentration of endogenous agonist, or as (-)baclofen expressing antagonist properties.

It is interesting to compare the present data with those obtained from studies in which animals have been chronically dosed with antidepressants. A number of these studies have demonstrated up-regulation of $GABA_B$ receptors in the frontal cortex. For instance Pilc and Lloyd (1984) acheived an increase in the total number of binding sites (B_{max}) of between 39% and 66% using amytriptaline, desipramine, citalopram, viloxazine and pargyline. These findings were extended to include nomifensine and zimeldine (16% and 34% increase respectively) by Lloyd et al., (1985).

This is an interesting comparison, the present study has shown $GABA_B$ receptor antagonists can up-regulate $GABA_B$ receptors, a feature of many antidepressants. In addition $GABA_B$ agonists are capable of inhibiting monoamine release (Bowery et al., 1980), and it is therefore likely that an antagonist would increase monoamine release. Increasing the synaptic concentration of monoamines is believed to be the mechanism by which antidepressants work. These comparisons suggest the theoretical possibility that $GABA_B$ receptor antagonists have antidepressant activity.

However, there are a number of features relating to the up-regulation of $GABA_B$ receptors by chronic administration of antidepressants that remain to be explained. For instance, the effect of desipramine on $GABA_B$ receptors appears not to be dose dependant (Lloyd et al., 1985). Some workers report different responses to chronic dosing when binding is

performed with [^{3}H]-GABA or [^{3}H]-baclofen (Szekley et al., 1987). Indeed, two studies have found no effect of chronic antidepressant treatment upon $GABA_B$ receptor radioligand binding (Cross and Horton, 1988; McManus and Greenshaw, 1991). Importantly Cross et al., (1988) found no change in $GABA_B$ receptors in either drug free or antidepressant treated suicide victims. Pratt and Bowery (1993) may have resolved some of the discrepancies in earlier studies by demonstrating very localized up-regulation of $GABA_B$ receptors in lamina I of the frontal cortex. This was in response to chronic desipramine, and chronic CGP36742. However, such an up-regulation was not seen in the present study, suggesting the presence of an as yet undescribed variable and indicating that this effect of chronic $GABA_B$ receptor activation requires further investigation.

The involvement of GABAergic systems in depressive illness remains a complex phenomenon. Some studies suggest that a decrease in GABAergic tone may be involved in the primary pathology of this disorder (Berrettini et al., 1982). If it is possible to deduce the mechanisms underlying affective disorders by studying changes brought about by the chronic administration of effective treatments to normal animals, then the up-regulation of $GABA_B$ receptors seen in most studies (for review see Prat and Bowery, 1993) suggests that GABA and $GABA_B$ receptors are involved in depressive illness. However the most likely cause of this up-regulation in receptors is a down-regulation in the concentration of GABA in the brain. Thus it is not possible to say whether depression is associated with enhanced, or reduced $GABA_B$ receptor activity. In the present study it is not possible to resolve the relative importance of these two effects. The administration of a $GABA_B$ receptor antagonist is capable of causing either

of these possibilities depending upon the relative importance of its pre- and postsynaptic activity. Thus the value of $GABA_B$ receptor antagonists in affective disorders awaits a more detailed study of the role of GABAergic systems in this disease, and investigations into the relative potencies of $GABA_B$ receptor antagonists at pre- and postsynaptic receptors.

7
GABA Receptors in Genetic Absence Epilepsy Rats, an Autoradiographic Study

7.1. Introduction

$GABA_B$ receptors are thought to play an important role in the generation of rhythmic activity by the thalamus. They mediate a long lasting K^+ dependent hyperpolarization which prepares the thalamic cells to fire a burst of action potentials (Crunelli and Leresche, 1991). It is hypothesized that some of these action potentials travel in a thalamocortical loop, returning to the thalamus to stimulate a further $GABA_B$ receptor mediated hyperpolarization. This mechanism may underly the the "spike and wave" discharges seen in generalized non-convulsive, or petit mal epilepsy. Thus $GABA_B$ receptors may play a fundamental role in petit mal epilepsy.

A rat model of petit mal epilepsy has recently been described (Marescaux et al., 1984a; 1984b; 1987; Vergnes et al., 1982; 1987). This is an inbred strain of rat termed Genetic Absence Epilepsy Rats from Strasbourg (GAERS). Both the symptoms and pharmacology of the seizure condition in these animals resembles human petit mal epilepsy. One of the primary symptoms in these animals is the generation of bilaterally synchronous spike and wave discharges.

Seizures in these animals can be manipulated by a variety of drugs, most interestingly they are ameliorated by the $GABA_B$ receptor antagonist CGP35348 (Marescaux et al., 1992a). This finding, coupled to the important role apparently played by $GABA_B$ receptors in the generation of spike and wave discharges in the thalamus (Crunelli and Leresche, 1991) led to the hypothesis that a pathological alteration in

$GABA_B$ receptors might underlie this seizure condition. To investigate this possibility, autoradiography was used to compare the amount of radioligand binding to $GABA_B$ and $GABA_A$ receptors in GAERS and similar rats that did not show the symptoms of generalized non-convulsive epilepsy.

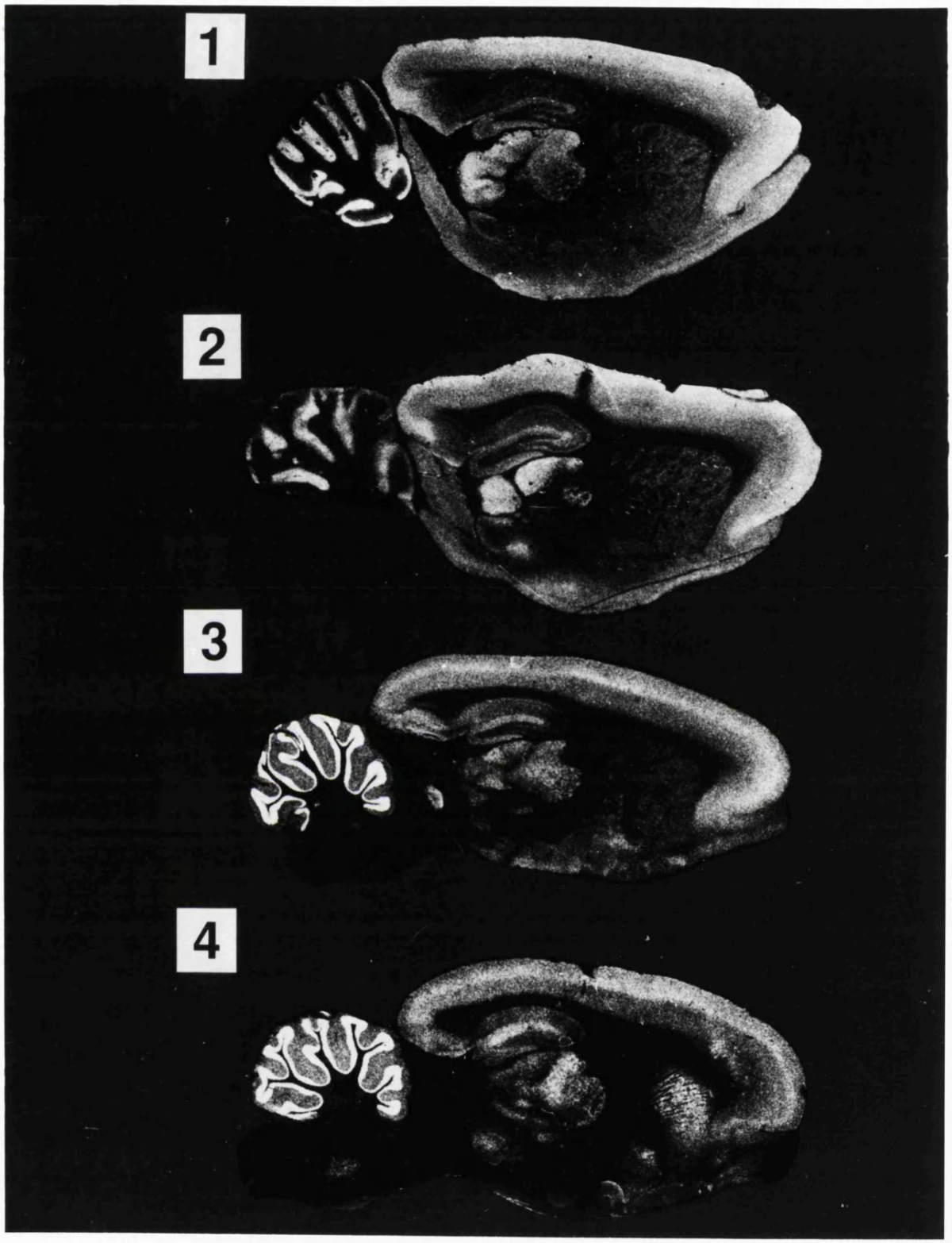
The areas of the brain selected for study were chosen either for their implication in epilepsy, or because they were areas where $GABA_A$ or $GABA_B$ receptors are particularly dense, and thus GABAergic mechanisms are likely to be important. Areas included in the first category include cortex, the thalamic nuclei and hippocampus. Of these, the cortex and the thalamic nuclei have been most clearly implicated in absence epilepsy. They represent areas where spike and wave discharges have been recorded. The lateral dorsal, lateral posterior and ventral posterior nuclei and the posterior thalamic nuclear group are relay nuclei which are likely to be included in the neuronal circuits responsible for abnormal oscillatory rhythms (Mclachan et al., 1984; Vergnes et al., 1987; 1982). The hippocampus was selected as it has frequently been used as an *in vitro* preparation in which to study epileptiform activity (Ault et al., 1983; Swartzwelder et al., 1986), although it appears not to be involved in spike and wave discharges. The molecular layer and the granular layer of the cerebellum were selected since they have the highest levels of GABA binding in the brain, and also provide a good separation of $GABA_A$ sites from $GABA_B$ sites. The granular layer has a high ratio of $GABA_A$ sites to $GABA_B$ sites (5:1) and the molecular layer has a high ratio of $GABA_B$ sites to $GABA_A$ sites (2:1).

7.2. Results

Dark field images of the distribution of [^{3}H]-GABA binding to $GABA_A$ receptors in parasagittal sections of rat brain is shown in Figure 7.1.(1 and 2). The sections come from one brain which showed the genetic absence epilepsy syndrome, and one that did not. The sections represent total binding in each of these brains. [^{3}H]-GABA binding to $GABA_B$ receptors is shown in two similar sections in Figure 7.1. (3 and 4). The amount of specifically bound radioactivity was estimated in the following regions: inner fronto-parietal cortex (laminae V-VI); outer fronto-parietal cortex (laminae I-IV); hippocampus (CA1 Oriens); lateral thalamic nucleus; latero-posterior thalamic nucleus; ventral posterior thalamic nucleus; striatum; and the molecular and granule cell layer of the cerebellum. The anatomical location of these regions is illustrated in Figure 7.2. The data from the quantitative analysis are shown in Figures 7.3 to 7.4.

There was no significant difference in the distribution of binding to $GABA_A$ receptors (ANOVA, $F_{(1,4)}<0.10$, $p=0.76$) or $GABA_B$ receptors (ANOVA, $F_{(1,4)}<0.10$, $p=0.98$) between the control and epileptic brains. Neither was there any difference in the levels of specific binding in any of the individual structures from which density values where taken, either in the case of $GABA_A$ receptors (ANOVA, followed by Duncans multiple range test, $p>0.05$) or $GABA_B$ receptors (ANOVA, followed by Duncans multiple range test, $p>0.05$).

7.1. The distribution of $GABA_A$ and $GABA_B$ receptors in GAERS. Dark field autoradiographs of the distribution of radioligand binding to $GABA_A$ and $GABA_B$ receptors in parasagittal sections of rat brain approximately 1.8 mm from the midline. Binding was performed for $GABA_B$ receptors in: 1) control and 2) seizure prone rats, Binding was performed for $GABA_A$ receptors in: 3) control and 4) seizure prone rats. The binding reaction was carried out at room temperature for 20 min, using 50 nM [^{3}H]-GABA. $GABA_B$ receptors were labelled selectively in the presence of 40 μM isoguvacine and non-specific binding (not shown) was defined in the presence of 100 μM (-)baclofen. $GABA_A$ receptors were labelled selectively in the presence of 100 μM (-)baclofen, and non-specific binding (not shown) was defined with 40 μM isoguvacine.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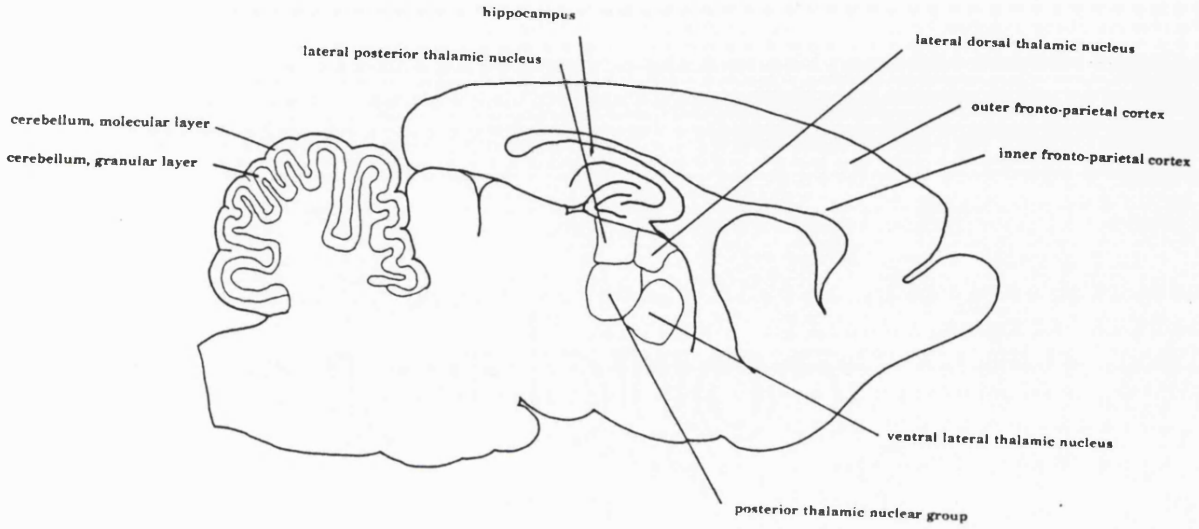

Figure 7.2. Areas of rat brain selected for quantitative analysis An anatomic description of the regions seen in a parasagittal section of rat brain approximately 1.8 mm from midline (cf. Figure 7.1). The labelled areas are those chosen for quantitative analysis in the present study. The outline and nomenclature are taken from Paxinos and Watson (1986).

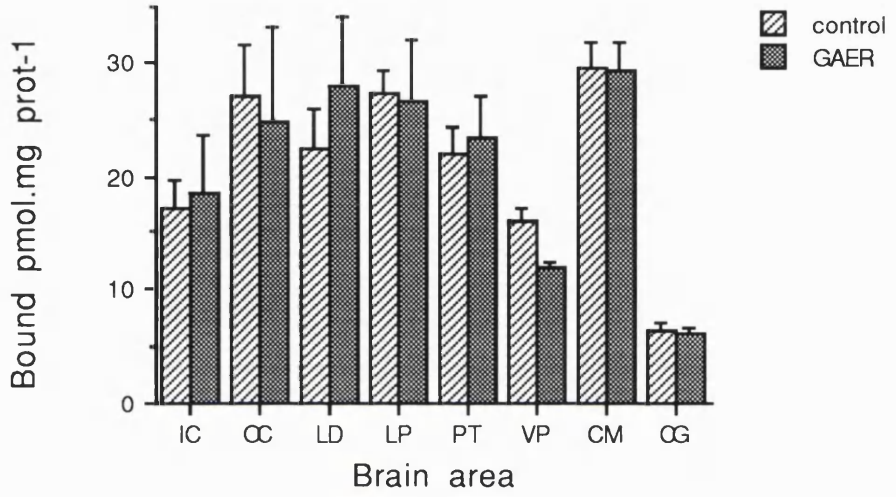

Figure 7.3. Comparison of the levels of radioligand binding to $GABA_B$ receptors in GAERS and control rat brain. Density readings were taken from autoradiographs prepared from parasagittal sections of rat brain. Binding was performed with 50 nM [^{3}H]-GABA as described in the text. The brain areas are abbreviated as follows: inner fronto-parietal cortex (IC); outer fronto-parietal cortex (OC); lateral dorsal thalamic nuclei (LD); lateral posterior thalamic nucleus (LP); posterior thalamic nuclear group (PT); venteral posterior thalamic nucleus (VP); cerebellum, molecular layer (CM); cerebellum granular layer (CG) and hippocampus (HP).

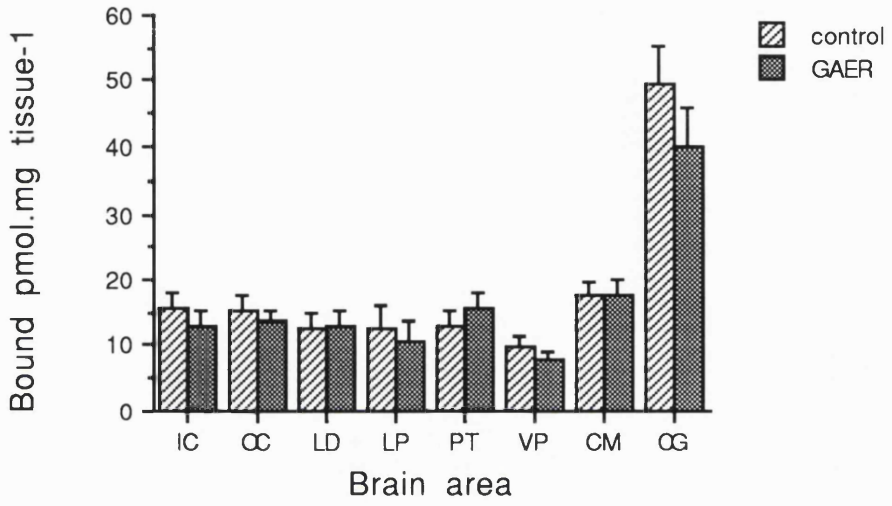

Figure 7.4. Comparison of the levels of radioligand binding to $GABA_A$ receptors in GAERS and in control rat brain. Density readings were taken from parasagittal sections of GAERS and control rat brain. Radioligand binding was performed with 50 nM [^{3}H]-GABA as described in the text. The brain areas are abbreviated as follows: inner fronto-parietal cortex (IC); outer fronto-parietal cortex (OC); lateral dorsal thalamic nuclei (LD); lateral posterior thalamic nucleus (LP); posterior thalamic nuclear group (PT); venteral posterior thalamic nucleus (VP); cerebellum, molecular layer (CM); cerebellum granular layer (CG) and hippocampus (HP).

7.3. Discussion

The distribution of $GABA_A$ receptor radioligand binding in control sections was in broad agreement with that reported by Bowery et al. (1987). High levels of radioligand binding were recorded in the granule cell layer of the cerebellum, while lower levels were recorded in the cortex and hippocampus, and molecular layer of the cerebellum.

The distribution of $GABA_B$ receptor radioligand binding in control sections was also in agreement with that described by Bowery et al. (1987) and Chu et al. (1990). The highest levels of $GABA_B$ receptor radioligand binding were present in the molecular layer of the cerebellum, the zonal layer of the superior colliculus, the outer layers of the cortex and the thalamus.

A visual comparison of the autoradiographs derived from control and epilepsy prone rats did not reveal any gross structural changes, or differences in the distribution of $GABA_A$ or $GABA_B$ receptor radioligand binding. Quantitative analysis of the optical density of areas of the film representing specific brain nuclei was undertaken.

When levels of binding in sections from epileptic animals GAERS brains were compared to levels of binding in sections from non-epileptic animals, no differences were found in the amount of [^{3}H]-GABA binding to $GABA_A$ receptors or $GABA_B$ receptors in any of the areas investigated. The concentration of [^{3}H]-GABA used for these studies (50

nM) was close to the value for the dissociation rate constant (K_d) of GABA for $GABA_A$ and $GABA_B$ receptors. This has been determined to be approximately 110 nM (Zukin et al., 1974; Enna and Snyder, 1975; Krogsgaard-Larsen et al., 1981) and 70 nM (Hill and Bowery, 1981, Hill et al., 1984) respectively. Thus the assay should have been sensitive both to changes in affinity (K_d) and also to changes in the total number of receptors in the preparation (B_{max}). The present data therefore suggests that it is unlikely that the primary pathology in this seizure condition is an over abundance or a change in affinity of either of these two receptors.

The receptor of primary interest in this study is the $GABA_B$ receptor. It is implicated in absence epilepsy both by the crucial role played by $GABA_B$ receptors in the thalamus in generating the characteristic spike and wave discharges, and also by the ability of CGP35348 the $GABA_B$ receptor antagonist to reduce the duration of these seizures. These two powerful pieces of evidence suggest that GABA-ergic neurotransmission mediated by $GABA_B$ receptors might still underly the seizure condition. For instance there might be abnormal receptors situated outside the areas investigated here or altered sub-populations of $GABA_B$ receptors which represent too small a fraction of the total receptor density to be characterized accurately in the present study. Also, the present study does not eliminate the possibility that there might be alterations in $GABA_B$ receptor mediated neurotransmission caused by irregularities in the receptor at locations other than the agonist recognition site. For instance in the efficiency of signal transduction and G-protein coupling. Increases in GABAergic

tone caused by elevated neurotransmitter levels would not be detected in this study.

$GABA_A$ receptors have not been implicated in absence epilepsy to the same extent as $GABA_B$ receptors. Nevertheless some $GABA_A$ receptor drugs do effect spike and wave discharges in GAERS. Some agonists, such as THIP, increase the duration of these discharges, whilst antagonists at the $GABA_A$ receptor are ineffective. Benzodiazepine agonists suppress spike and wave discharges, and inverse agonists increase their duration. Pathological changes in this receptor are thus a possibility, although it is not possible to predict the nature of these changes since benzodiazepines seem to have the opposite effects to competitive $GABA_A$ receptor agonists. The lack of change in $GABA_A$ receptor radioligand binding is also interesting to the extent that it may highlight a possible dichotomy in the pathology of convulsive epilepsy and non-convulsive epilepsy. Previous studies have demonstrated changes in $GABA_A$ receptor radioligand binding associated with convulsive epilepsy. In particular, down regulation of receptors has been noted in DBA/2 mice (Horton et al., 1982) and in humans with convulsive epilepsy (Lloyd et al., 1981).

The lack of changes in $GABA_B$ receptor radioligand binding in the present study is in contrast to studies that have been performed in the lethargic mouse model of petit mal epilepsy. This mouse has an autosomal recessive mutation which causes spontaneous absence seizures. It is reported that these animals have reduced numbers of $GABA_B$ receptors in the cerebral cortex, and reduced $GABA_B$ receptor

function in the hippocampus (Hosford et al., 1992). This is an interesting finding in that it further implicates GABAB receptors in petit mal epilepsy. However, the lack of change in $GABA_B$ receptors in GAERS raises the possibility that petit mal epilepsy is a clinically defined syndrome which results from one of several possible lesions.

In conclusion, the present study suggests that it is unlikely that major alterations in $GABA_A$ or $GABA_B$ receptor expression are responsible for the seizures displayed by GAERS. However these data do not exclude the possibility that a change in the levels of endogenous agonist, or a change in signal transduction are responsible for the susceptability to seizures.

8
Summary and Future Directions

Summary

The present study was concerned primarily with aspects of the modulation of adenylyl cyclase activity by $GABA_B$ receptors. *In vitro,* $GABA_B$ receptor activation inhibits forskolin-stimulated adenylyl cyclase activity, but augments noradrenaline-stimulated adenylyl cyclase activity. The physiological explanation for this duplex effect has been one of the puzzles of $GABA_B$ receptor pharmacology since it was first described (Hill, 1985; Karbon and Enna, 1984 and Wojcik and Neff, 1984).

This bi-directional effect of $GABA_B$ receptor stimulation has given rise to two hypotheses which have been investigated in the present study. Firstly, it has been postulated that the stimulatory and inhibitory effects upon adenylyl cyclase may be caused by the activation of two separate populations of $GABA_B$ receptors. The present study has investigated the potency of a range of $GABA_B$ receptor agonists and antagonists on these two responses to build up a pharmacological profile of each postulated population of receptors. Four of the six agonists tested (SKF97541, CGP44533, 3-APA and CGP44532) showed no statistical difference in potency as inhibitors of forskolin-stimulated adenylyl cyclase activity or as augmenters of noradrenaline-stimulated adenylyl cyclase activity. (-)Baclofen and CGP47656, however, were at least one order of magnitude more potent at inhibiting forskolin-stimulated adenylyl cyclase activity than they were at augmenting noradrenaline-stimulated adenylyl cyclase activity. This was interpreted as meaning that these two compounds had a relatively low intrinsic efficacy, and that the augmentation of

noradrenaline had a lower receptor reserve than the inhibition of forskolin-stimulated activity. The observation that CGP47656 was a partial agonist in the augmentation of noradrenaline-stimulated adenylyl cyclase activity can also be explained in this way.

An important development in resolving the effects of $GABA_B$ receptor modulation on adenylyl cyclase activity has been the cloning of four different types of adenylyl cyclase. This has allowed the modulatory effects of purified G-protein subunits to be investigated with each of these isoforms (see Lefkowitz, 1992)). The allosteric enhancement by $\beta\gamma$ of α_s stimulated activity in types II and III of the enzyme seems currently to be the most likely explanation for the augmentation of β-adrenoceptor-stimulated adenylyl cyclase activity by $GABA_B$ receptor agonists.

The relative potencies of agonists in the present study is in keeping with the stoichiometry of the interaction of α and $\beta\gamma$ subunits with adenylyl cyclase (reviewed by Hoyer and Boddeke, 1993). Thus, a relatively large number of $\beta\gamma$ subunits are required to maximally express the augmentation response, but a relatively small number of G-proteins need to be activated to maximally express α subunit modulation. If the G-protein subunits which mediate the $GABA_B$ receptor modulation of adenylyl cyclase activity are derived from the same population of receptors and G-proteins, this would indicate a large receptor reserve for the inhibition of forskolin-stimulated adenylyl cyclase activity and a small receptor reserve for the augmentation of noradrenaline-stimulated adenylyl cyclase activity. Confirmation of this interpretation of the present results would come most convincingly from cultured cells in which the level of expression of $GABA_B$ receptors could be

controlled. Alternatively, reconstitution studies, could by performed, using cloned adenylyl cyclase, purified α and βγ G-protein subunits and purified $GABA_B$ receptors.

None of the antagonists tested showed specificity for either of the two effects of $GABA_B$ receptor stimulation. This indicates that, with the presently available compounds, the $GABA_B$ receptors in these two assays are pharmacologically identical. A corollary of the finding that the α and βγ subunits from the same G-protein could be responsible for inhibition and augmentation of adenylyl cyclase is that both responses may be accommodated within the same cell. However, it remains possible that the differential expression of the four types of adenylyl cyclase in sub-populations of cells may mean that the inhibitory and stimulatory effects are in fact mediated by sub-populations of $GABA_B$ receptors. Whenever such divisions exist it is possible that pharmacologically distinct receptors might be identified. Thus, the assessment of $GABA_B$ antagonists on each of these responses should continue.

The second hypothesis tested in the present study concerns the cell types in which the $GABA_B$ receptor modulation of adenylyl cyclase takes place. It has been shown that $GABA_B$ receptors occur on a variety of cells within CNS tissue. These can be distinguished pharmacologically, for example dopaminergic, adrenergic, serotonergic (Bowery et al., 1980) and GABAergic (Waldmeier et al., 1988), or anatomically, for example cerebellar granule cells, (Wojcik and Neff, 1984) hippocampal pyramidal cells (Newberry and Nicol, 1985) and Glial cells (Hosli and Hosli, 1990). In order to give physiological meaning to the modulation of adenylyl cyclase by

$GABA_B$ receptor activation it is necessary to discover which cells are thus affected . In addition, it is possible that the inhibitory and the stimulatory effects upon adenylyl cyclase occur in separate populations of cells. The modulatory responses might thus be mediated via different intracellular signal transduction mechanisms (e.g. different G-proteins or different isoforms of adenylyl cyclase). It has previously been demonstrated that β-adrenergic agonists are unable to stimulate adenylyl cyclase in a synaptosomal preparation, whilst a response to forskolin remains. In synaptosomes also, (-)baclofen fails to modulate adenylyl cyclase activity in the presence of a β-adrenoceptor agonist, but nevertheless inhibits forskolin-stimulated adenylyl cyclase activity (Hill and Dolphin, 1984). This suggested that the cellular structures involved in the interaction of β-adrenoceptors with adenylyl cyclase, and possibly $GABA_B$ receptors, might be destroyed in synaptosomal preparations. Such structures might, for instance, be neuronal cell bodies or glial cells. Credence is given to the glial cell hypothesis by the discovery that up to 95% of β-adrenoceptor-stimulated cAMP in rat cortical slices is derived from glial cells (Stone et al., 1990). The hypothesis that a proportion of the adenylyl cyclase activity recorded in the present study is derived from glial cells, was tested with two series of experiments. The first involved the use of the glial cell toxin, fluorocitrate. This compound eliminated noradrenaline-stimulated adenylyl cyclase activity, and a large proportion of forskolin-stimulated adenylyl cyclase activity in rat cortical slices. It also eliminated the modulatory effect of (-)baclofen in both assay conditions. This would seem to confirm that much of the adenylyl cyclase activity recorded in cortical slices originates from glial cells. It also suggests that the $GABA_B$ receptor mediated modulation of adenylyl cyclase activity is also located in glial cells.

In the second series of experiments adenylyl cyclase activity was investigated in a glial cell culture. Both noradrenaline and forskolin produced dramatic increases in adenylyl cyclase activity in this preparation. However, (-)baclofen was ineffective as a modulator. Thus the modulation of adenylyl cyclase activity was not seen in cortical slices which had been treated with a glial cell toxin, or in glial cell cultures. This leaves us with the speculative conclusion that both neurons and glia are required for the expression of the complete profile of adenylyl cyclase modulation. Before this conclusion can be drawn, a number of further experiments should be performed. Firstly, the specificity of fluorocitrate has been demonstrated under the conditions in which it is used in the present study by (Stone et al., 1990)). Nevertheless, this specificity should be confirmed. $GABA_B$ receptors have been demonstrated on astrocytes by Hosli and Hosli (1990; 1992) however, the ability of (-)baclofen to interact with $GABA_B$ receptors on glial cells in the present study should be confirmed, either by the use of radioligand binding studies or some other electrophysiological or biochemical assay. Establishing such protocols for the investigation of populations of $GABA_B$ receptors on different cell types in primary culture and cell lines may well prove instrumental in elucidating the specific effects of $GABA_B$ receptor mediated modulation of adenylyl cyclase activity.

Intracranial microdialysis was performed to determine the effect of drugs upon adenylyl cyclase activity *in vivo*. The analysis of cAMP in perfusates derived from dialysis probes implanted in rat frontal cortex revealed that cAMP levels were enhanced by both forskolin (100 μM) and noradrenaline (1 mM). This confirmed the findings of previous studies

(Hutson and Suman-Chauhan, 1990; Egawa et al., 1988). Since $GABA_B$ receptor activation modulates forskolin and noradrenaline-stimulated adenylyl cyclase activity *in vitro*, experiments were also conducted to determine the effect of $GABA_B$ receptor activation upon these responses *in vivo*. Thus, (-)baclofen (10mM) had no effect upon baseline levels of cAMP. Also (-)baclofen did not significantly alter the elevated levels of cAMP caused by the administration of forskolin or noradrenaline. It is thus unlikely that the present protocol can be used as an index of activity of agonist drugs at central $GABA_B$ receptors *in vivo*. However, it is possible that the $GABA_B$ receptors being investigated were already fully occupied by endogenous GABA. Thus, it is a priority to investigate the effects of an antagonist such as CGP54626 using this protocol. If such a compound is ineffective, then an alternative explanation for the lack of $GABA_B$ receptor modulation *in vivo* must be sought. One possibility is that *in vivo* microdialysis detects only extracellular cAMP, whilst *in vitro* intracellular cAMP is released when cortical slices are boiled to terminate the experiment. Thus it is conceivable that extracellular cAMP does not accurately reflect intracellular cAMP concentrations. This finding prompts an investigation into the transport or diffusion mechanisms by which cAMP leaves cells, and questions the relevance of using cAMP efflux as an index of intracellular adenylyl cyclase activity.

Data are presented here upon the effects of chronic administration of $GABA_B$ receptor drugs upon $GABA_B$ receptor function and [^{3}H]-GABA binding to $GABA_B$ receptors. Neither of the two antagonists, CGP35348 or CGP36742 (100 mg kg^{-1} day^{-1}), or the agonist (-)baclofen (10 mg kg^{-1} day^{-1}) caused a change in the responsiveness of adenylyl cyclase to forskolin or

noradrenaline, or the ability of (-)baclofen to modulate these two responses. However, a series of changes in radioligand binding to $GABA_B$ receptors was detected using autoradiography. For (-)baclofen these changes included both up- and down-regulation of binding, suggesting a complex mode of action involving pre- and postsynaptic receptors. Both of the antagonists produced localized up-regulations in the substantia nigra, thalamus and hippocampus. In view of the complex and rather localized changes revealed here, this study should be regarded as preliminary work. Further studies should concentrate on the areas highlighted here by changes in radioligand receptor binding. In particular, the complex profile of (-)baclofen should be confirmed. If the relative efficacies of CGP35348 and CGP36742 are to be determined, then the doses of each should be modified in the light of recent data to account for differences in metabolism, brain penetration, bioavailability or binding potency. As the two drugs have different structures, this would confirm any effects as being caused by occupation of $GABA_B$ receptors.

Up-regulation of $GABA_B$ receptors has been linked to antidepressant therapy (Lloyd et al., 1985). To give weight to the argument that $GABA_B$ receptor antagonists could be antidepressant, it would be appropriate to measure levels of other receptors known to be regulated by antidepressant treatment, in particular β-adrenoceptors (Banerjee et al., 1977) and 5-HT_2 receptors (Peroutka and Snyder, 1980). In a further study using radioligand binding, saturation analysis should be used to determine receptor numbers. In addition the newly developed antagonists should be tested in animal models of depression.

Autoradiography was also employed to investigate pathological changes in GABA receptors in genetic absence epilepsy rats. Similar levels of radioligand binding to $GABA_A$ and $GABA_B$ receptors were found in normal and pathological brains. This indicates that the biological basis of this seizure condition must either reside in minute changes, beyond the sensitivity of the present study, or lie elsewhere. In view of the crucial role played by $GABA_B$ receptors in the generation of seizures, and the effectiveness of the $GABA_B$ antagonists CGP 35348 as a therapy, future experiments should still concentrate on this neurotransmitter system. In this light, it would be interesting to assess the level of activity at $GABA_B$ receptors by determining the concentration of endogenous GABA within relevant areas of the thalamus. It would also be interesting to determine the sensitivity of functional assays of $GABA_B$ receptor activation, such as modulation of adenylyl cyclase activity.

The Future

Recent years have seen several dramatic developments in the field of $GABA_B$ receptor pharmacology. Firstly, the availability of compounds (particularly antagonists) with activity at this receptor has made the detailed investigation of the pharmacological profiles of $GABA_B$ receptors possible for the first time. This work may eventually lead to the definition of pharmacologically distinguishable subtypes of $GABA_B$ receptor.

Secondly, the demonstration that $GABA_B$ receptors are crucial to the generation of absence seizures and the development of an antagonist which

crosses the blood brain barrier promises a new generation of therapeutically effective $GABA_B$ receptor drugs. These discoveries give a certain indication that the coming years will be an exceptionally fruitful period in $GABA_B$ receptor research.

9
References

Adams, P.R. and Brown, D.A. (1975) Actions of γ-aminobutyric acid in sympathetic ganglion cells. J. Physiol. 250, 85-120.

Al-Dahan, M.I. and Thalmann, R.H. (1989) Effects of dihydropyridine calcium channel ligands on rat brain gamma-aminobutyric acid B receptors. J. Neurochem. 53, 982-985.

Al-Gadi, M. and Hill, S.J. (1985) Characterization of histamine receptors mediating the stimulation of cyclic AMP accumulation in rabbit cerebral cortical slices. Br. J. Pharmacol. 85, 877-888.

Alger, B.E. (1984) Characteristics of a slow hyperpolarizing synaptic potential in rat hippocampal pyramidal cells. J. Neurophysiol. 52, 892-910.

Alger, B.E. and Nicoll, R.A. (1982) Pharmacological evidence for two kinds of GABA receptor in rat hippocampal pyramidal cells studied in vitro. J. Physiol. 305, 279-296.

Alousi, A.A., Jasper, J.E., Insel, P.A. and Motulsky, H.J. (1991) Stoichiometry of receptor-Gs-adenylate cyclase interactions. FASEB J. 5, 2300-2303.

Andrade, R., Malenka, R.C. and Nicoll, R.A. (1986) A G-protein couples serotonin and $GABA_B$ receptors to the same channels in hippocampus. Science 234, 1261-1265.

Anwar, N. and Mason, D.F.J. (1982) Two actions of γ-amino butyric acid on the responses of the isolated basilar artery from the rabbit. Br. J. Pharmacol. 75, 177-188.

Ault, B. and Evans R.H. (1978) Central depressant actions of baclofen. J. Physiol. 284, 131P.

Ault, B. and Evans, R. (1981) The depressant action of baclofen on the isolated spinal cord of the neonatal rat. Eur. J. Pharmacol. 71, 357-364.

Ault, B. and Nadler, J.V. (1983) Anticonvulsant like actions of baclofen in the rat hippocampal slice. Br. J. Pharmacol. 78, 701-708.

Ault, B., Gruenthal, M., Armstrong, D.R., and Nadler, J.V. (1986) Efficacy of baclofen and phenobarbital against the kainic acid limbic seizure-brain damage syndrome. J. Pharmacol. Exp. Ther. 239, 612-617.

Avoli, M. and Gloor, P. (1982a) Interaction of cortex and thalamus in spike and wave discharges of feline generalized penicillin epilepsy. Exp. Neurol. 76, 196-217.

Avoli, M. and Gloor, P. (1982b) Role of the thalamus in generalized penicillin epilepsy: observations on decorticated cats. Exp. Neurol. 77, 386-402.

Axelrod, J., Burch, R.M. and Jelsema, C.L. (1988) Receptor-mediated activation of phospholipase
via GTP-binding proteins: arachidonic acid and its metabolites as second messengers. Trends Neurosci. 11, 117-123.

Bakalyar, H.A. and Reed, R.R. (1990) Identification of a specialized adenylyl cyclase that may mediate odorant detection . Science, 250, 1403-1406.

Bancaud, J., Talairch, J., Morel, P., Bresson, M., Bonis, A., Geier, S., Hemon, E. and Biser, P. (1974) "Generalized" epileptic seizures elecited by electrical stimulation of the frontal lobe in man. Electroenceph. Clin Neurophysiol. 37, 275-282.

Banerjee, S.P., Kung, L.S., Riggi, S.J. and Chanda, S.K. (1977) Development of β-adrenergic receptor subsensitivity by antidepressants. Nature 268, 455-456.

Barber, R. and Butcher, R.W. (1983) The egress of cyclic AMP from metazoan cells, Adv. Cycl. Nucleot. Res. 15, 119-138.

Bartolini,A., Bartolini, R., Biscini, A., Giotti, A. and Malmberg, P. (1981) Investigations into baclofen analgesia: effect of naloxone, bicuculline, atropine and ergotamine. Br. J. Pharmacol. 72, 156-157P.

Baxter, C.F. (1969) Changes in gamma-aminobutyric-acid-shunt enzymes and substrates after administration of carbonyl reagents and vitamin B6 *in vivo*: an apparent discrepancy in assay techniques. Ann. N.Y. Acad. Sci. 166, 267-280.

Bean, B.P. (1989) Classes of calcium channels in vertebrate cells. Ann. Rev. Physiol. 51, 367-384.

Berrettini W.H., Nurnberger, J.I., Hare, T., Gershon, E.S. and Post R.M. (1982) Plasma and CSF GABA in affective illness. Br. J. Psychiatr. 141, 483-487.

Bhat, S.V., Bajwa, B.S., Dornauer, H. and De Souza, N.J. (1977) Structures and stereochemistry of new labdane diterpenoids from Coleus forskolii briq. Tetrahedron lett. 19, 1669-1692.

Bijak, M., Misgeld, U. and Muller, W. (1991) Interaction of noradrenergic and cholinergic agonists with ligands increasing K^+-conductance of guinea pig hippocampal neurons in vitro. Eur. J. Neurosci. 3, 473-479.

Birnbaumer, L. (1990) G-Proteins in signal transduction. Ann. Rev. Pharmacol. Toxicol. 30, 675-705.

Bittiger, H., Bernasconi., R., Froestl, W., Hall, R., Jaekel, J., Klebs, K., Krueger, L., Mickel, S. J., Mindadori, C., Olpe, H.-R., Pfannkuch, F., Pozza, M., Probst, A., van Riozen, H., Schmutz, M., Schuetz, H., Steinmann, M.W., Vassout, A., Waldemeier, P., Beick, P., Farger, G., Gleiter, C., Schmidt, E.-K., and Marescaux, C. (1992) $GABA_B$ antagonists: potential new drugs. Pharmacol. Commun. 23, 70-74.

Bittiger, H., Froestl, W., Hall, R., Karlsson, G., Klebs, K., Olpe, H-R., Pozza, M.F., Steinmann, M.W. and Van Riezen, H. (1990) Biochemistry, electrophysiology and pharmacology of a new $GABA_B$ antagonist: CGP 35348 in, *$GABA_B$ receptors in Mammalian Function* (Bowery, N.G., H. Bittiger, H. and Olpe, H.-R., eds), pp. 47-60. John Wiley and Sons, Chichester.

Blaxter, T.J. and Carlen, P.L. (1987) GABA responses in rat dentate granule neurons are mediated by chloride. Can. J. Physiol. Pharmacol. 66, 637-642.

Bloom, F. and Iversen, L. (1971) Localizing ^{3}H-GABA in nerve terminals of rat cerebral cortex by electron microscopic autoradiography. Nature 229, 628-630.

Bonanno, G. and Raiteri, M. (1992) Functional evidence for multiple $GABA_B$ receptor subtypes in the rat cerebral cortex. J. Pharmacol. Exp. Ther. 262, 114-118.

Bonanno, G. and Raiteri, M. (1993) Multiple $GABA_B$ receptors. Trends Pharmacol. Sci. 14, 259-261.

Bonanno, G., Cavazzani, P., Andrioli, G.C., Asaro, D., Pellegrini, G. and Raiteri, M. (1989) Release regulating autoreceptors of the $GABA_B$ type in human cerebral cortex. Br. J. Pharmacol. 96, 341-346.

Bonner, T.I., Buckley, N.J., Young, A.C. and Brann, M.R. (1987) Identification of a family of muscarinic acetylcholine receptor genes. Science 237, 527-532.

Bowery N.G., Price, G.W., Hudson, A.L., Hill, D.R., Wilkin, G.P. and Turnbull, M.J. (1984) GABA receptor multiplicity, visualization of different receptor subtypes in the mammalian CNS. Neuropharm. 23, 219-231.

Bowery, N.G. (1989) $GABA_B$ receptors and their significance in mammalian pharmacology. Trends Pharmacol. Sci. 10, 401-407.

Bowery, N.G. and Hudson, A.L. (1970) Gamma-aminobyturic acid reduces the evoked release of ^3H-noradrenaline from sympathetic nerve terminals. Br. J. Pharmacol. 66, 108P

Bowery, N.G. and Brown, D.A. (1974) Depolarizing actions of γ-amino butyric acid and related compounds in rat superior cervical ganglia in vitro. Br. J. Pharmacol. 50, 205-218.

Bowery, N.G. and Pratt, G.D. (1992) $GABA_B$ receptors as targets for drug action. Arnzeim. Forsch. Drug Res. 42, 215-223.

Bowery, N.G. and Williams, L.C. (1986) $GABA_B$ receptor activation inhibits the increase in nerve terminal $[Ca^{++}]$ induced by depolarization. Br. J. Pharmac. 87, 37P

Bowery, N.G., Doble, A., Hill, D.R., Hudson, A.L., Shaw, J.S., Turnbull, M.J. and Warrington, R. (1981) Bicuculline-insensitive GABA receptors on peripheral autonomic nerve terminals. Eur. J. Pharmacol. 71, 53-70.

Bowery, N.G., Hill, D.R. and Hudson, A.L. (1983) Characteristics of $GABA_B$ receptor binding sites on rat whole brain synaptic membranes. Br. J. Pharmacol. 78, 191-206.

Bowery, N.G., Hill, D.R., Hudson, A.L., Doble, A., Middlemiss, D.N., Shaw, J. and Turnbull, M. (1980) (-)Baclofen decreases neurotransmitter release in the mammalian CNS by an action at a novel GABA receptor. Nature 283, 92-94.

Bowery, N.G., Hudson, A.L. and Price, G.W. (1987) $GABA_A$ and $GABA_B$ receptor site distribution in the rat central nervous system. Neuroscience 20, 365-383.

Bradford, H.F. (1986) Chemical Neurobiology: an introduction to neurochemistry. pp. 507-515. Freeman, New York.

Bradford, M.M. (1976) A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analyt. Biochem. 72, 248-254.

Brown, B.L., Albano, J.D.M., Ekins, R.P., Sgherzi, A.M. and Tampion, U. (1971) A simple and sensitive saturation assay method for the measurement of adenosine 3'5'-cyclic monophosphate. Biochem. J. 121, 561-562.

Bunzow, J.R., Van Tol, H.H.M., Grandy, D.K., Albert, P., Salon, J., Christie, M., Machida, C.A., Neve, K.A. and Civelli, O. (1988) Cloning and expreession of a rat D2 dopamine receptor cDNA. Nature 336, 783-787.

Buzsaki, G., Bickford, R.G., Ponomareff, G., Thal, L.K., Mandel, D. and Gage, F.H. (1988) Nucleus basalis and thalamic control of neocortical activity in the freely moving rat. J. Neurosci. 8, 4007-4026.

Casey, P.J. and Gilman, A.G. (1988) G-Protein involvement in receptor-effector coupling. J. Biol. Chem. 263, 2577-2580.

Cash, C., Maitre, M., Ciesielski, L. and Mandel, P. (1979) Brain Succinic Semialdehyde Dehydrogenase, in: *GABA-Biochemistry and CNS Functions* (Mandel, P and DeFeudis, F.V., eds), pp. 93-109. Plennum Press, New York.

Celotti, F., Apud, J.A., Rovescalli, A.C., Melcangi, R.C., Negri-Cesi, P. and Racagni, G. (1986) The GABAergic extrinsic innervation of the rat fallopian tubes: Biochemical evidence and endocrine modulation, in *GABA and Endocrine Function* (Racagini, G. and Donoso, A.O., eds), pp. 251-264. Academic Press. New York.

Chase, N.C. and Tamminga, C.A. (1979) GABA system participation in human motor, cognitive and endocrine function, in *GABA neurotransmitters, pharmacochemical, Biochemical and pharmacological aspects.* (Krogsgaard-Larsen, P., Scheel-Kruger, J. and Kofod, H, eds) pp. 283-294 Academic Press. New York.

Chiefari, J., Galanopoujos, S., Janowski, W.K., Kerr, D.I.B. and Prager, R.H. (1987) The synthesis of phosphonobaclofen, an antagonist of baclofen. Aust. J. Chem. 40, 1511-1518.

Cheng, Y.C. and Prussoff W.H. (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (IC_{50}) of an enzymatic reaction. Biochem. Pharmacol, 3099-3108.

Chu, D.C., Albin, R.C., Young, A.B. and Penny, J.B. (1990) Distribution and kinetics of $GABA_B$ binding sites in rat central nervous system: a quantitative study. Neuroscience 34, 342-357.

Clarke, K.A. (1983) Effect of baclofen on sensory transmission through the ventrobasal thalamic nucleus of the rat. Neuropharm. 22, 1231-1235.

Colmers, W.F. and Pittman, Q.J. (1989) Presynaptic inhibition by neuropeptide toxin treatment. Brain Res. 489, 99-104.

Colmers, W.F. and Williams, J.T. (1988) Presynaptic inhibition by neuropeptide toxin treatment discriminates between pre- and post synaptic actions of baclofen in rat dorsal raphe nucleus *in vitro*. Neurosci. Lett. 933, 300-306.

Cotterel, G.A. and Robertson, H.A. (1987) Baclofen exacerbates epileptic myoclonus in kindled rats. Neuropharmacology, 26, 645-648.

Crawford, M.L.A. and Young, J.M. (1990) Modulation by GABA of agonist-induced inositol phospholipid metabolism in guinea-pig and rat brain, in *$GABA_B$ Receptors in Mammalian Function* (Bowery, N.G., Bittiger, H. and Olpe, H.-R., eds), pp. 161-182. John Wiley and Sons, Chichester.

Cross, J.A. and Horton, R.W. (1988) Effects of chronic oral administration of the antidepressants, desmethylimipramine and zimelidine on rat cortical $GABA_B$ binding sites: a comparison with 5-HT_2 binding site changes. Br. J. Pharmacol. 93, 331-336.

Cross, J.A., Cheetham, S.C., Crompton M.R., Catona, C.L.E. and Horton, R.W. (1988) Brain $GABA_B$ binding sites in depressed suicide victims. Psychiatr. Res. 26, 119-129.

Crunelli, V. and Leresche, N. (1991) A role for $GABA_B$ receptors in excitation and inhibition of thalamocortical cells. Trends Neurosci. 14, 16-21.

Crunelli, V., Haby, M., Jassik-Gerschenfeld, D., Leresche, N. and Pirchio, M. (1988) Cl^- and K^+ dependent inhibitory postsynaptic potentials evoked by interneurones of the rat lateral geniculate nucleus. J. Physiol. 399, 153-176.

Crunelli, V., Lightowler, S. and Pollard, C.E. (1989) A "T" type Ca^{2+} current underlies low-threshold Ca^{2+} potentials in cells of the cat and rat lateral geniculate nucleus. J. Physiol. 413, 543-561.

Curtis, B.M. and Catterall, W.A. (1985) Phosphorylation of the calcium antagonist receptor of the voltage sensitive calcium channel by cAMP-dependent protein kinase. Proc. Natl. Acad. Sci. 82, 2528-2532.

Curtis, D.R., Game, C.J.A., Johnston, G.A.R. and McCulloch, R.M. (1974) Central effects of β-(p-chlorophenyl)-γ-aminobutyric acid. Brain Res., 70, 493-499.

Curtis, D.R., Gynther, B.D., and Malik, R. (1986) A pharmacological study of group I muscle afferent terminals and synaptic excitation in the intermediate nucleus and Clarkes column of the cat spinal cord. Exp. Brain. Res. 64, 105-113.

Curtis, D.R., Lodge, D., Bornstein, J.C. and Peet, M.J. (1981) Selective effects of (-)baclofen on spinal synaptic transmission in the cat. Exp. Brain Res. 42, 158-170.

Cutting, D.A. and Jordan, C.C. (1975) Alternative approaches to analgesia: baclofen as a model compound. Br. J. Pharmacol. 54, 171-179.

Davies, C.H., Davies, S.N. and G.L. Collingridge (1989) Paired pulse depression of monosynaptic GABA mediated inhibitory post synaptic responses in rat hippocampus. J. Physiol. 424, 513-531.

Deisz, R.A. and Lux, H.D. (1985) γ-aminobutyric acid induced depression of calcium currents of chick sensory neurons. Neurosci. Lett. 5, 205-210.

Depaulis, A., Bourguignon, J.J., Marescaux, C., Vergnes, M., Schmitt, M., Micheletti, G., Waster, J.M. (1988) Effects of gamma-hydroxybutylactone derivatives on spontaneous generalized nonconvulsive seizures in the rat. Neuropharmacology 27, 683-689.

Desarmenian, M., Feltz, P., Occhipinti, G., Santangelo, F. and Schlochter, R. (1984) Coexistince of $GABA_A$ and $GABAB_B$ receptors on Aδ and C primary afferents. Br. J. Pharmacol. 81, 327-333.

Diamond, J.A. (1968) The activation and distribution of GABA and L-glutamate receptors on Goldfish Mauthner neurons: an analysis of dendritic remote inhibition. J. Physiol. 194, 669-723.

Dixon, R.A.F., Kobilka, B.K., Strader, D.K., Benovic, K., Dohlman, H.G., Frielle, T., Bolanowski, M.A., Bennett, C.D., Rands, E., Diehl, R.E., Mumford, R.A., Slater, E.E., Sigal, I.S., Caron, M.G. and Lefkowitz, R.J. (1986) Cloning of the gene and cDNA for mammalian beta-adrenergic receptor and homology with rhodopsin. Nature 321, 75-79.

Dolphin, A.C. and Scott, R.H. (1986) Inhibition of calcium currents in cultured rat dorsal root ganglion neurones by (-)baclofen. Br. J. Pharmacol. 88, 213-220.

Dolphin, A.C. and Scott, R.H. (1987) Calcium channel currents and their inhibition by (-)baclofen in rat sensory neurones: Modulation by Guanine nucleotides. J. Physiol. 386, 1-17.

Dolphin, A.C., McGuirk, S.M. and Scott, R.H. (1989) An investigation into the mechanisms of inhibition of calcium channel currents in cultured sensory neurons of the rat by guanine nucleotide analogues and baclofen. Br. J. Pharmacol. 97, 263-273.

Dolphin, A.C., Sweeny, M.I., Pearson, H.A., Silver, M. and Menoe-Johansson, A.S. (1992) An electrophysiological and biochemical study of the interaction between $GABA_B$ receptors and voltage-dependant Ca^{2+} channels. Pharmacology Communications, 2, 89.

Dreifuss, J.J., Kelly, J.S. and Krnjevic, K. (1969) Cortical inhibition and γ-aminobutyric acid. Exp. Brain Res. 9, 137-154.

Duman, D.S., Karbon, E.W., Harrington, C. and Enna, S.K. (1986) An examination of the involvement of phospholipases A2 and C in the α-adrenergic and γ-aminobutyric acid receptor modulation of cyclic AMP accumulation in rat brain slices. J. Neurochem. 47, 800-810.

Dunlap, K. and Fischbach, G.D. (1981) Neurotransmitters decrease the calcium conductance activated by depolarization of embryonic chick sensory neurons. Neurosci. Letts. 47, 265-270.

Dutar, P and Nicoll, R.A. (1988a) A physiological role for $GABA_B$ receptors in the central nervous system. Nature 332, 156-158.

Dutar, P. and Nicoll, R.A. (1988b) Pre- and postsynaptic $GABA_B$ receptors in the hippocampus have different pharmacological properties. Neuron 1, 585-591.

Egawa, M., Hoebel, B.G. and Stone, E.A. (1988) Use of microdialysis to measure brain noradrenergic receptor function *in vivo*. Brain Res. 458, 303-308.

Elliot, K.A.C. (1959) .Is gamma-aminobutyric acid a neurotransmitter? Proc. 4th Int. Congr. Biochem. 3, 251.

Enna, S.J. and Beutler, S.A. (1985) GABA receptors as a site for antiepileptic drug action, in *Epilepsy and GABA Receptor Agonists: Basic and Therapeutic Research* (Bartholini, G. Bossi, L ., Lloyd, K.G. and Morselli, P.L., eds), pp. 195-201. Raven Press, New York.

Enna, S.J. and Karbon, E.W. (1984) $GABA_B$ receptors and transmitter-stimulated cAMP accumulation in rat brain. Neuropharmacology 23, 821-822.

Enna, S.J. and Karbon, E.W. (1987) Receptor regulation: evidence for a relationship between phospholipid metabolism and neurotransmitter receptor-mediated cAMP formation in brain. Trends Pharmacol. Sci. 8, 21-24.

Enna, S.J. and Snyder, S.H. (1975) properties of gamma-amino butyric acid (GABA) receptor binding to rat brain synaptic membrane fractions. Brain Res. 100, 81-97.

Facklam, M. and Bowery, N.G. (1992) Solubilization and characterization of $GABA_B$ receptor binding sites from porcine brain synaptic membranes. Br. J. Pharmacol. 110, 1291-1296.

Fargin, A., Raymond, J.R., Lohse, M.J., Kobilka, B.K., Caron, M.G. and Lefkowitz, E.J. (1988) The genomic clone G-21 which resembles a β-adrenergic receptor sequence encodes the $5\text{-}HT_{1A}$ receptor. Nature 335, 358-360.

Fargin, A., Raymond, J.R., Regan, J.W., Cotecchia, S., Lefkowitz, R.J. and Caron, M.G. (1989) Effector coupling mechanisms of the cloned $5\text{-}HT_{1A}$ receptor. J. Biol. Chem. 264, 14848-14852.

Federman, A.D., Conklin, B.R., Schrader, K.A., Randall, R.R. and Bourne H.R. (1992) Hormonal stimulation of adenylyl cyclase through Gi protein βγ subunits. Nature 356, 159-161.

Feinstein, P.G., Schrader, K.A., Bakalyar, H.A., Tang, W.J., Krupinski, J., Gilman, A.G. and Reed, R.R. (1991) Molecular cloning and characterizationof a Ca^{++}/Calmodulin sensitive adenylyl cyclase from rat brain. Proc. Natl. Acad. Sci. 88, 10173-10177.

Fonnum, F. (1985) Determination of transmitter amino acid turnover, in *Neuromethods, Vol 3: Amino Acids* (Boulton,, A.A., Baker, G.B. and Wood, J.D., eds), pp. 201-237. Humana Press, Clifton, New Jersey.

Fox, S., Krnjøvic, K., Morris, M., Puil, E.,Werman, R., (1978) Action of baclofen on mammalian synaptic transmission. Neurosci. 3, 495-515.

Froestl, W., Mickel, S.J., von Sprecher, G., Bittiger, H., and Olpe H.-R. (1992) Chemistry of new $GABA_B$ antagonists. Pharmacol. Commun. 2, 52-56.

Fung, S.C., Swarbrick, M.J. and Fillenz., M. (1985) Effects of baclofen on in vitro noradrenaline release from rat hippocampus and cerebellum in action at an α2-adrenergic receptor. Neurochem Int. 7, 155-159.

Gahwiler, B.H. and Brown D.A., (1985) $GABA_B$ receptor activated K^+ current in voltage clamped CA3 pyramidal cells on hippocampal cultures. Proc. Natl. Acad. Sci. 82, 1558-11562.

Gahwiler, B.H., Maurer, R. and Wuethrich, H.J. (1984) Pitrazepin, a novel $GABA_A$ antagonist. Neurossci lett. 45, 311-316.

Gallagher, J.P., Phelan. K.D., Twery, M.J., Hasuo, H., (1990) Pertussis toxin blocks post- but not presynaptic actions of baclofen in the rat dorsolateral septal nucleus, in GABAB Receptors in Mammalian Function (Bowery, N.G., Bittiger, H. and Olpe, H.-R., eds). pp. 127-140. John Wiley and Sons, Chichester.

Gao, B. and Gilman, A.G. (1991) Cloning and expression of a widely distributed (type IV) adenylyl cyclase. Proc. Natl. Acad. Sci. 88, 10178-10182.

George, S.T., Berrios, M., Hadcock, J.R., Wang, H.Y. and Malbon, C.C. (1988) Receptor density and cAMP accumulation: analysis in CHO cells exhibiting stable expression of a cDNA that encodes the beta$_2$ adrenergic receptor. Biochem. Biophys. Res. Comm. 150, 665-672.

Gilman, A.G. (1987) G-proteins: transducers of receptor-generated signals. Ann. Rev. Biochem. 56, 615-649.

Giralt, M.T. , Bonanno, G. and Raiteri, M., (1990) GABA terminal autoreceptors on the pars compacta and in the pars reticulata of the rat substantia nigra are $GABA_B$. Eur. J. Pharmacol. 175, 137-144.

Glennon, R.A. (1990) Do classical hallucinogens act as $5HT_2$ agonists or antagonists? Neuropsychopharmacology. 3, 509-517.

Gloor, P. (1979) Generalised epilepsy with spike and wave discharge: a reinterpretation of its electrographic and clinical manifestations. Epilepsia 20, 571-588.

Gloor, P. and Fariello, R.G. (1988) Generalized epilepsy: some of its cellular mechanisms differ from those of focal epilepsy. Trends Neurosci. 1, 63-68.

Godfrey, P.P., Grahame-Smith, D.G. and Gray J.A. (1988) $GABA_B$ receptor mediated inhibition of 5-HT stimulated phoshatidylinositol turnover in mouse cerebral cortex. Br. J. Pharmacol. (Suppl.) 251P.

Goldsmith, P., Backlund, Jnr. P.S., Rossiter, K., Carter, A., Milligan, G., Unson, C.G. and Speigel, A. (1988) Purification of heterotrimeric GTP binding Protiens from brain: identification of a novel from of G_o. Biochemistry 27, 7085-7090.

Goodwin. F.K., Ebert, M.H.,and Bunney, W.E. Jr. Mental effects of reserpine in man: a review, in, *Psychiatric Complications of Medicinal Drugs* (Shader, R.I., ed), pp. 73-101 Raven press, New York.

Gray J.A. and Green A.R. (1987) $GABA_B$ mediated inhibition of potassium evoked release of endogenous 5-hydroxytryptamine from mouse frontal cortex. Br. J. Pharmacol. 91, 517-533.

Green, K.A. and Cotterell, G. A. (1988) Actions of baclofen on components of the Ca^{++}-current in rat and mouse DRG ganglion neurones in culture. Br J. Pharmacol. 94, 235-246.

Haas, H.L., Greene, R.W. and Olpe, H.-R. (1985) Stereoselectivity of L-baclofen in hippocampal slices of rat Neurosci Lett. 55, 1-4.

Hablitz, J.J. and Thalmann, R.H. (1987) Conductance changes underlying a late synaptic hyperpolarization in hippocampal CA3 neurons. J. Neurophysiol. 58, 160-171.

Hamel, E., Krause, D.N. and Roberts, E. (1981) Specific cerebrovascular localization of glutamate decarboxylase activity. Brain Res. 223, 199-204.

Harden, T.K., (1983) Agonist induced desensitization of the β-adrenergic receptor-linked adenylyl cyclase. Pharmacol. Reviews 35, 5-32.

Harrison, N.L. (1989) On the presynaptic action of baclofen at inhibitory synapses between cultured rat hippocampal neurones. J. Physiol. 422, 433-446.

Hassel, B., Paulsen, R., Johnsen, A. and Fonnum, F. (1992) Selective inhibition of glial cell metabolism *in vivo* by fluorocitrate. Brain Res. 576, 120-124.

Hasuo, H. and Gallagher, J.P. (1988) Comparison of antagonism by phaclofen of baclofen-induced hyperpolarizating potentials recorded intracellulary from rat dorsolateral septal neurons. Neurosci. Lett. 86, 77-81.

Henry, J.L. (1982) Pharmacological studies on the prolonged depressant effects of baclofen on lumbar dorsal horn units in the cat. Neuropharm. 21, 1085-1093.

Hertz, L. and Richardson, J.S. (1984) Is neuropharmacology merely the pharmacology of neurons - or are actrocytes important too? Trends Pharmacol. Sci. 5, 272-276.

Hescheler, J., Rosenthal, W., Trautweunm, W. and Schultz, G. (1986) The GTP-binding protein, N_o, regulates neuronal calcium channels. Nature 325, 445-447.

Hibert, M.F., Trumpp-Kallmeyer, S., Hoflack, J. and Bruinvels, A. (1993) This is not a G-protein coupled receptor. Trends Pharmacol. Sci. 14, 7-12.

Hill, D.R. (1985) $GABA_B$ receptor modulation of adenylate cyclase activity in rat brain slices. Br. J. Pharmacol. 84, 249-257.

Hill, D.R. and Bowery N.G. (1981) ^{3}H-baclofen and ^{3}H-GABA bind to bicuculline-insensitive $GABA_B$ sites in rat brain. Nature 209, 149-152.

Hill, D.R. and Dolphin, A.C. (1984) Modulation of adenylate cyclase activity by $GABA_B$ receptors. Neuropharm. 23, 829-830.

Hill, D.R., Bowery, N.G. and Hudson, A.L. (1984) Inhibition of $GABA_B$ receptor binding by guanyl nucleotides. J. Neurochem. 42, 652-657.

Hills, J.M., Dingsdale, R.A. and Howson, E. (1989) 3-Aminopropylphosphinic acid inhibits the cholinergic twitch in the guinea-pig ileum through $GABA_B$ receptor agonist activity. Br. J. Pharmacol. 96, 51P.

Hirsch, K. and Burnod, Y. (1987) A synaptically evoked late hyperpolarization in the rat dorsolateral geniculate neurons in vitro Neurosci. 23, 457-468.

Hobbiger, F. (1958a) Effects of gamma-aminobutyric acid in the isolated mammalian ileum. J. Physiol. 142, 147-164.

Hobbiger, F. (1958b) Antagonism by gamma-aminobutyric acid to actions of 5-hydroxytryptamine and nicotine in isolated organs. J. Physiol. 144, 349-360.

Holz, G.G., Rane, S.G. and Dunlap, K. (1986) GTP-binding proteins mediate transmitter inhibition of voltage-dependent calcium channels. Nature 319, 670-672.

Horton R.W., Prestwich, S.A. and Meldrum, B.S. (1982) γ-Aminobutyric acid and benzodiazepine binding sites in audiogenic seizure susceptible mice. J. Neurochem. 39, 864-870.

Hosli, E. and Hosli, L. (1982) Evidence for the existence of α and β-adrenoceptors on neurons and glial cells of cultured rat central nervous system-an autoradiographic study. Neurosci. 7, 2873-2881

Hosli, L. and Hosli, E. (1990) Evidence for $GABA_B$ receptors on cultured astrocytes of rat CNS: autoradiographic binding studies. Exp. Brain Res. 80, 621-625.

Hosli, L. and Hosli, E. (1992) $GABA_B$-receptors on astrocytes: electrophyiological and autoradiographic evidence. Phamacol. Comm. 2, 47-48.

Hosford, D.A., Clark, S., Cao, Z., Wilson,.W.A., Lin, F.H., Morisett, R.A. and Huin, A. (1992) The role of $GABA_B$ receptor activation in absence seizures of lethargic (lh/lh) mice Science, 257, 398-401

Hoyer, D. and Boddeke, H.W.G.M. (1993) Partial agonists, full agonists and antagonists: dilemmas of definition. Trends Pharmacol. Sci. 14, 270-275.

Hu, B., Steriade, M. and Deschenes, M. (1989) The effects of peribrachial stimulation on neurons of the lateral geniculate nucleus Neurosci. 31, 13-24.

Huguenard, J.R. and Prince, D.A. (1992) A novel T-type current underlies prolonged Ca^{2+}-dependent burst firing in GABAergic neurons of rat thalamic reticular nucleus. J. Neurosci. 12, 3804-3817.

Hutson, P.H. and Suman-Chauhan, N. (1990) Activation of post synaptic striatal dopamine receptors monitored by efflux of cAMP *in vivo.* Neuropharm. 29, 1011-1016.

Inoue, M., Matsuo, T. and Ogata, N. (1985) Baclofen activates voltage-dependant and 4-aminopyridine sensitive K^+ conductance in guinea pig hippocampal pyramidal cells maintained *in vitro.* Br. J. Pharmacol. 84, 833-841.

Johnston, G.A.R., Hailstone, M.H. and Freeman. C.G. (1980) Baclofen: stereoselective inhibition of excitant amino acid release. J. Pharm. Pharmacol. 32, 230-231.

Jones, E.G. (1983) The thalamus, in *Chemical Neuroanatomy* (Emson, P.C. ed) Raven, New York.

Karbon, E.W. and Enna, S.J. (1985) Characterization of the relationship between γ-aminobutyric acid B agonists and transmitter-coupled cyclic nucleotide-generating systems in rat brain. Mol. Pharmacol. 27, 53-59.

Karbon, E.W., Zorn, S.H., Newland, R.J. and Enna, S.J. (1990) Evidence for the existence of $GABA_B$ receptor subpopulations in mammalian central nervous system, in *$GABA_B$ Receptors in Mammalian Function* (Bowery, N.G., Bittiger, H. and Olpe, H.-R., eds). pp. 127-140. John Wiley and Sons, Chichester.

Karlsson, G., Pozza, M. and Olpe, H.R. (1988) Phaclofen: a $GABA_B$ blocker reduces long-duration inhibition in the neocortex. Eur. J. Pharmacol. 148, 485-486.

Karlsson, G., Schmutz, M., Kolb, C., Bittiger, H. and Olpe, H.-R. (1990) $GABA_B$ receptors and experimental models of epilepsy, in *$GABA_B$ Receptors in Mammalian Function* (Bowery, N.G., Bittiger, H. and Olpe, H.-R., eds). pp. 349-365 John Wiley and Sons, Chichester.

Kelly, J.S., Godfraind, J.-M. and Maruyama, S. (1979) The presence and nature of inhibition in small slices of dorsal lateral geniculate nucleus of rat and cat incubated *in vivo.* Brain Res. 168, 388-392.

Kenakin, T.P. and Morgan, P. (1988) Theoretical effects of single and multiple transducer receptor coupling proteins on estimates of the relative potency of agonists. Mol. Pharmacol. 35, 214-222.

Kendall, D.A., Millns, P.J. and Frith, J.L. (1992) Direct and indirect stimulations of cyclic AMP formation in human brain. Br. J. Pharmacol. 105, 899-902.

Kerr, D.I.B., Ong J., Prager, R.H., Gynther, B.D. and Curtis, D.R. (1987) Phaclofen: a peripheral and central baclofen antagonist. Brain Res. 405, 150-154.

Kerr, D.I.B., Ong, J. and Prager, R.H. (1990) $GABA_B$ receptor agonists and antagonists. in *$GABA_B$ Receptors in Mammalian Function* (Bowery, N.G., Bittiger, H. and Olpe, H.-R., eds). pp. 29-45 John Wiley and Sons, Chichester.

Kleinrok, A., and Kilbinger, H. (1983) γ-aminobutyric acid and cholinergic transmission in the guinea pig ileum. Naunyn Schmiedebergs Arch. Pharmacol. 323, 216-220.

Kobilka, B.K., Matsui, H., Kobilka, T.S., Yang-Feng, T.L., Francke, U., Caron, N.G., Lefkowitz, R.J. and Regan, J.W. (1987) Cloning, sequencing and expression of the gene coding for the human platelet α_2-adrenergic receptor. Science 238, 650-656.

Korf, J., Boer, P.H. and Fekkes, D. (1976) Release of cerebral cyclic AMP into push-pull perfusates in freely moving rats. Brain Res. 113, 551-561.

Krnjevic, K. and Schwartz, S. (1966) Is γ-amino butyric acid an inhibitory neurotransmitter? Nature 211, 1372-1374.

Krnjevic, K. and Schwartz, S. (1967) The action of γ-aminobutyric acid on vertebrate neurones. Exp. Brain. Res. 3, 320-336.

Krogsgaard-Larsen, P., Hjeds, J., Curtis, D.R., Lodge, D. and Johston, G.A.R. (1979) Dihydromuscimol, thiomuscimol and related heterocyclic compounds as GABA analogues. J. Neurochem. 32, 1717-1724.

Krogsgaard-Larsen, P., Snowman, A., Lummis, S.C. and Olsen R.W. (1981) Charactisation of the binding of the GABA agonist [^{3}H]-piperidine-4-sulphonic acid to bovine brain synaptic membranes. J. Neurochem. 37, 401-409.

Krupinski, J., Coussen, F., Bakalyar, H.A., Tang, W.J., Feinstein, P.G., Orth, K., Slaughtr, C., Reed, R.R. and Gilman, A.G. (1989) Adenylyl cyclase amino acid sequence: possible channel or transporter-like structure. Science 244, 1558-1564.

Kuhn, R. (1958) The treatment of depressive states with G 22355 (imipramine hydrochloride). Am. J. Psychiatry 115, 459-469.

Kuriyama, K., Ohmori, Y. and Taguchi, J.I. (1990) Solubilization and partial purification of cerebral $GABA_B$ receptors, in *$GABA_B$ Receptors in Mammalian Function* (Bowery, N.G., Bittiger, H. and Olpe, H.-R., eds). pp. 183-193 John Wiley and Sons, Chichester.

Kusunoki, M., Taniyama, K. and Tanaka, C. (1984) Neuronal $GABA_B$ release and GABA inhibition of ACh release in guinea pig urinary bladder. Am. J. Physiol. 246, R502-R509.

Lancaster, B. and Wheal, H.V. (1981) The synaptically evoked late hyperpolarization in hippocampal CA1 pyramidal cells is resistant to intracellular EGTA. Neurosci. 12, 267-275.

Lannes, B., Micheletti, G., Vergnes, M., Marescaux, C., Depaulis, A. and Warter, J.M. (1988) Relationship between spike and wave discharges and vigilance levels in rats with spontaneous petit mal-like epilepsy. Neurosci. Lett. 94, 187-191.

Lanthorn, T.H. and Cotman, C.W. (1981) Baclofen selectively inhibits excitatory synaptic transmission in the hippocampus. Brain Res. 225, 171-178.

Lazareno, S., Marriott, D.B. and Nahorski, S.R. (1985) Differential effects of selective and non-selective neuroleptics on intracellular and extracellular cyclic AMP accumulation in rat striatal slices. Brain Res. 361, 91-98.

Leblanc, G.G. and Ciaranello, R.D. (1984) α-Noradrenergic potentiation of neurotransmitter stimulated cAMP in rat striatal slices. Brain Res. 293, 57-65.

Lefkowitz, R.J. (1992) The subunit story thickens. Nature 358, 372.

Levy, R.A. and Proudfit, H.K. (1977) The analgesic action of baclofen β-(4-chlorophenyl)-aminobutyric acid]. J. Pharmacol. Exp. Ther. 202, 437-445.

Liu, Z., Vergnes, M., Depaulis, A. and Marescaux, C. (1992) Involvement of intrathalamic $GABA_B$ neurotransmission in the control of abscence seizures in the rat. Neurosci. 48, 87-93.

Lloyd, K.G. (1990) Antidepressants and $GABA_B$ site up regulation, in *$GABA_B$ Receptors in Mammalian Function* (Bowery, N.G., Bittiger, H. and Olpe, H.-R., eds), pp. 297-307John Wiley and Sons, Chichester.

Lloyd, K.G., Morselli, P.L., Depoortere, H., Fournier, V., Zivkovic, R., Scatton, B., Broekkamp, C.L., Worms, P. and Bartholini, G. (1983) The potential use of GABA agonists in psychiatric disorders: evidence from studies with progabide in animal models and clinical trials. Pharmacol. Biochem. Behav. 18, 957-966.

Lloyd, K.G., Munari, C., Bossi, L., Stoeffels, C, Talairach, J. and Morselli, P.L. (1981) Biochemical evidence for the alterations of GABA-mediated synaptic transmission in pathological brain tissue (stereo EEG or morphological definition) for epileptic patients, in *Neurotransmitters Seizures and Epilepsy.* (Morselli, PL., Lloyd, K.G., Loscher, W., Meldrum, B., Reynolds, E.H., eds), pp. 325-338, Raven press, New York.

Lloyd, K.G., Thuret, F. and Pilc, A. (1985) Upregulation of γ-aminobutyric acid (GABA) B binding sites in rat frontal cortex: a common action of repeated administration of different classes of antidepressants and electroschock. J. Pharmacol. Exp. Ther. 235, 191-190.

Lloyd, K.G., Zivkovic, R., Sanger, D., Koly, D., Depootere, H. and Bartholini, G. (1987) Fengabine, a novel antidepressant GABAergic agent: I. Activity in models for antidepressant drugs and psychopharmacological profile. J. Pharmacol. Exp. Ther. 241, 245-250.

Marescaux, C., Micheletti, G., Vergnes, M., Depaulis, A., Rumbach, L. and Warter, J.M. (1984a) A model of chronic spontaneous petit mal like seizures in the rat: comparison with pentelenetetrazole-induced seizures. Epilepsia 25, 326-331.

Macdonald, R.L. and Olsen, R.W. (1994) $GABA_A$ Receptor Channels, Ann. Rev. Neurosi. 17: 569-602.

Marescaux, C., Vergnes, M. and Bernasconi, R. (1992a) $GABA_B$ receptor antagonists: potential new antiabsence drugs. J. Neural. Transm. (Suppl.) 35, 179-188.

Marescaux, C., Vergnes, M. and Depaulis, A. (1992b) Genetic absence epilepsy in rats from Strasbourg - A review. J. Neural. Transm. (Suppl.) 35, 37-70.

Marescaux, C., Vergnes, M. and Micheletti, G. (1984b) Antiepileptic drug evaluation on a new animal model: spontaneous petit mal epilepsy in the rat. Fed. Proc. 43, 280-281.

Marescaux, C., Vergnes, M., Depaulis, A., Micheletti, G., and Warter, J.M. (1992c) Neurotransmission on rats' spontaneous generalized nonconvulsive epilepsy, in *Neurotransmitters in Epilepsy* (Avanzini, G., Fariello, R., Heinneman, U. and Engel, J. eds), pp. 453-464 Elsevier, Amsterdam.

Marescaux, C., Vergnes, M., Jensen, L.H., Petersen, E., Depaulis, A., Micheletti, G. and Waster, J.M. (1987) Bidirectional effects of beta-carbolines in rats with spontaneous petit mal-like seizures. Brain. Res. Bull. 19, 327-335.

McCormick, D.A. (1989) Cholinergic and noradrenergic modulation of thalamocortical processing. Trends Neurosci. 12, 215-221.

McGeer, E.G. and McGeer, P.L. (1978) GABA-containing neurons in schizophrenia, huntington's chorea and normal ageing, in *GABA neurotransmitters, pharmacochemical, Biochemical and pharmacological aspects.* (Krogsgaard-Larsen, P., Scheel-Kruger, J., Kofod, H., eds), Academic Press, New York.

McLachan, R.S., Gloor, P. and Avoli, M. (1984) Differential participation of some "specific" and "non-specific" thalamic nuclei in generalized spike and wave discharges of feline generalized penicillin epilepsy. Brain Res. 307, 277-287.

McManus, D.J. and Greenshaw, A.J. (1991) Differential effects of antidepressants on $GABA_B$ and β-adrenergic receptors in rat cereberal cortex. Biochem. Pharmacol. 42, 1525-1528.

Meldrum B and Braestrup C (1984) GABA and the anticonvulsant action of benzodiazepines and related drugs, in *Actions and Interactions of GABA and Benzodiazepines* (Bowery, N.G., ed), pp133-153. Raven Press. New York

Meldrum, B.S. (1990) Epilepsy octet, anatomy, physiology and pathology of epilepsy. Lancet 336, 231-234.

Meldrum, B.S. and Horton, R. (1978) Blockade of epileptic responses in the photosensitive baboon, *Papio papio* by two irrevesible inhibitors of GABA-transaminase, γ-acetylenic GABA (4-amino-hex-5-ynoic acid) and γ-vinyl GABA (4-amino-hex-enkoic acid). Psychopharmacol. 5, 47-50.

Meller, E., Bohmaker, K., Namba, Y., Friedhoff, A.J. and Goldstein, M. (1987) Relationship between receptor occupancy and response at striatal dopamine autoreceptors. Mol. Pharmacol. 31, 592-598.

Meller, E., Enz., A. and Goldstein, M. (1988) Absence of receptor reserve at striatal dopamine receptors regulating cholinergic neuronal activity. Eur. J. Pharmacol. 155, 151-154.

Miki, Y., Taniyama, K., Tanaka, C. and Tobe, T. (1983) GABA, glutamic acid decarboxylase and GABA transaminase levels in the myenteric plexus in the intestine of humans and other mammals. J. Neurochem. 40, 861-865.

Minneman, K.P., Quik, M. and Emson, P.C. (1978) Receptor linked cyclic AMP systems in rat neostriatum: differential localization revealed by kainic acid injections, Brain Res. 151, 507-521.

Morishita, R., Kato, K. and Asano, T. (1990) $GABA_B$ receptors couple to G-proteins G_o, G^*_o and G_{i1} but not G_{i2}. Fed. Exp. Biol. Sci. 271, 231-235.

Morison, R.S. and Basset, D.L. (1945) Electrical activity of the thalamus and basal ganglia in decorticate cats. J. Neurophysiol. 8, 309-314.

Morselli, P.L., Bossi, L., Henry, J.F., Zarifian, J.E. and Bartholini, G. (1980). On the therapeutic action of SL 76 002, a new GABAmimetic agent: preliminary observations in neuropsychiatric disorders. Brain Res. Bull. 5, (Suppl.) 411-414.

Muhyaddin, M., Roberts, P.J. and Woodruff, G.N. (1982) Presynaptic gamma-aminobutyric acid receptors in the rat anococcygeus muscle and their antagonism by 5-aminovaleric acid. Br. J. Pharmacol. 77, 163-168.

Muir, D., Berl, S. and Clarke, D.D. (1980) Acetate and fluoroacetate as possible markers for glial metabolism *in vivo*. Brain Res. 380, 336-340.

Mulle, C., Madariaga, A. and Deschenes, M. (1986) Morphology and electrophysiological properties of reticularis thalamic neurons in cat: *in vivo* study of a thalamic pacemaker. J. Neurosci. 6, 2134-2145.

Muller, W. and Misgeld, U. (1989) Carbachol reduces I_K, baclofen, but not I_K, GABA in guinea pig hippocampal slices. Neurosci. Lett. 102, 229-234.

Musch, B. and Garreau, M. (1986) An overview of the antidepressant activity of fengabine in open clinical trials, in *Biological Psychiatry,* (Shagass, C. ed) pp. 920-922. Elsevier, New York.

Nahorski, S.R., Howlett, D.R. and Redrave, P. (1979) Loss of β-adrenoceptor binding sites in rat striatum following kainic acid lesions. Eur. J. Pharmacol. 60, 249-252.

Nakata, H. (1989) Affinity chromatography of A1 adenosine receptors of rat brain membranes. Mol. Pharmacol. 35, 780-786.

Nakayasu, H., Mizutani, H., Hanai, K., Kimura, H. and Kuriyama, K. (1992) Monoclonal antibody to GABA binding protein, a possible $GABA_B$ receptor Biochem. Biophys. Res. Commun. 182, 722-726.

Nakayasu, H., Nishikawa, M., Mizutani, H., Kimura, H. and Kuriyama, K. (1993) Immunoaffinity purification and charaterization of γ-aminobutyric acid (GABA) B receptor from bovine cerebral cortex. J. Biol. Chem. 268, 8658-8664.

Neer, E.J. and Clapham, D.E. (1988) Roles of G-protein subunits on transmembrane signalling. Nature 333, 129-134.

Newberry, N.R. and Nicoll, R.A. (1984a) Direct hyperpolarizing action of baclofen on hippocampal pyramidal cells. Nature 308, 450-452.

Newberry, N.R. and Nicoll, R.A. (1984b) A bicuculline-resistant inhibitory post synaptic potential in rat hippocampal pyramidal cells *in vitro.* J. Physiol. 348, 239-254.

Newberry, N.R. and Nicoll, R.A. (1985) Comparison of the action of baclofen with γ-aminobutyric acid on rat hippocampal pyramidal cells in vitro. J. Physiol. 360, 161-185.

Niak, S.R., Guidotti, A. and Costa, R. (1976) Central GABA receptor agonists: comparison of muscimol and baclofen. Neuropharmacology 15, 479-484.

Nicoll, R.A. and Newberry, N.R. (1984) A possible postsynaptic inhibitory action for $GABA_B$ receptors on hippocampal pyramidal cells. Neuropharmacology. 23, 849-850.

O'Neal, R.M. and Koeppe, R.E. (1966) Precursors *in vivo* of glutamate, aspartate, and their derivatives in rat brain. J. Neurochem. 13, 835-847.

Ogata, N. (1990) Physiological and pharmacological characterization of $GABA_B$ receptor-mediated potassium conductance, in *$GABA_B$ receptors in Mammalian Function* (Bowery, N.G., H. Bittiger, H. and Olpe, H.-R., eds), pp. 273-291. John Wiley and Sons, Chichester.

Ogata, N. and Abe, H. (1982) Neuropharmacology of the brain slice: effects of substance-P on neurons in the guinea pig thalamus. Comp. Biochem. Physiol. 72, 171-178.

Ogata, N. Inoue, M. and Matsuo, T. (1987) Contrasting properties of K^+ conductances induced by baclofen and γ-aminobutyric acid in slices of the guinea pig hippocampus. Synapse 1, 62-69.

Ohara, K., Haga, K., Berstein, G., Haga, T., Ichiyama, A. and Ohara, K. (1988) The interaction between D2 dopamine receptor and GTP-binding protein. Mol. Pharmacol. 33, 290-296.

Ohmori, Y., Hirouchi, N., Taguchi, J. and Kuriyama, K. (1990) Functional coupling of the γ-aminobutyric acid B receptor with calcium ion channels and GTP-binding proteins and its alteration following solubilization of the γ-aminobutyric acid receptor. J. Neurochem. 54, 80-85.

Okada, Y., Taniguchi, H. and Shimada, C. (1976) High concentrations of GABA and high glutamate decarboxylase activity in rat pancreatic islets and human insulinoma. Science 194, 620-622.

Olpe, H.-R., Demieville, H., Valtzer, V., Bencze, W.L., Koella, W.P., Wolf, P. and Haas H.L. (1978) The biological activity of *d*- and *l*-baclofen (Lioresal). Eur. J. Pharmacol. 52, 133-136.

Olpe, H.-R., Karlsson, G., Pozza, M.G., Brugger, F., Steinmann, M., Van Riezen, H., Fagg, G., Hall, R.G., Froestl, W. and Bittiger, H. (1990) CGP35348: a centrally active blocker of $GABA_B$ receptors. European J. Pharmacol. 187, 27-38.

Olpe, H.-R., Steinmann, M.W., Ferrat, T., Pozza, M.F., Greiner, K., Brugger, F., Froestl, W., Mickel, S.K. and Bittiger, H. (1993) The actions of orally active $GABA_B$ receptor antagonists on GABAergic transmission *in vivo* and *in vitro*. Eur. J. Pharmacol. 223, 179-186.

Onali, P., Olianas, M.C., Bunse, B. and Gessa, G.L. (1988) Bimodal regulation of striatal adenylate cyclase by dopamine, in *Central and peripheral Dopamine Receptors: Biochemistry and Pharmacology* (Spamo P.F., Biggio, G., Toffano, G. and Gessa, G.L., eds), pp. 201-215, Fidia Res. Series, Liviana Press, Padova.

Ong, J., Kerr, D.I.B. and Johnson, G.A.R. (1987) Differing actions of β-chlorophenyl-GABA and GABA in the guinea-pig isolated ileum. Neurosci. Lett. 77, 109-113.

Ong, J. and Kerr, D.I.B. (1983) $GABA_A$ and $GABA_B$ receptor mediated modification of intestinal motility. Eur. J. Pharmacol. 86, 9-17.

Paulsen, R.W., Contstabile, A., Villani, L. and Fonnum, F. (1987) An *in vivo* model for studying function of brain tissue temporarily devoid of glial cell metabolism: the use of fluorocitrate. J. Neurochem. 48, 1377-1385.

Paxinos, G. and Watson, C. (1986) The Rat Brain in Stereotaxic Co-ordinates. Academic Press, London.

Penfield, W and Jasper, H. (1954) Epilepsy and the Functional Anatomy of the Human Brain. Little Brown, Boston.

Petera, R.A. (1957) Mechanisms of the toxicity of the active constituent of Dichapetalum cymosum and related compounds. Adv. Enzymol. 18, 113-159.

Petty, F. and Sherman, A.D. (1985) Plasma GABA levels in psychiatric illness. J. Affective Disord. 6, 131-138.

Pierce, P.A. and Peroutka, S.G. (1990) Antagonist properties of d-LSD at 5-hydroxytriptamine-2 receptors Neuropsychopharmacology 3, 503-508.

Pilc, A. and Lloyd, K.G. (1984) Chronic antidepressants and $GABA_B$ receptors: a hypothesis of antidepressant drug action. Life Sci. 35, 2149-2154.

Post, R.M., Smith, C.C., Squillace, K.M. and Tallman, J.F. (1979) Effect of chronic cocaine on behaviour and cyclic AMP in cerebrospinal fluid in rhesus monkeys. Commun. Psychopharm. 3, 1143-152.

Post, RM., Ketter, T.A., Joffe, R.T. and Kramlinger, K.L. (1991) Lack of beneficial effect of *l*-baclofen in affective disorder. Internat. Clinical Psychopharmacol. 6, 197-207.

Potashner, S.J. (1979) Baclofen: effects on amino acid release and metabolism in slices of guinea pig cerebral cortex. J. Neurochem. 32, 103-109.

Pratt, G.D. and Bowery, N.G. (1993) Repeated administration of desipramine and a $GABA_B$ receptor antagonist, CGP36742, discretely up-regulates $GABA_B$ receptor binding sites in rat frontal cortex. Br. J. Pharmacol. 110, 724-735.

Pratt, G.D., Knott, C., Davey, R. and Bowery, N.G. (1989) Characterizaton of 3-aminopropylphosphinic acid as a $GABA_B$ agonist in rat brain tissue. Br. J. Pharmacol. 96, (Suppl.) 141P.

Rane, S.O. and Dunlap, K. (1986) Kinase C activator 1,2-oleylacetylglycerol attenuates voltage dependent calcium current in sensory neurons. Proc. Natl. Acad. Sci. 83, 184-188.

Robertson, B. and Taylor, W.R. (1986) Effects of γ-aminobutyric acid and (-)baclofen on calcium and potassium currents in cat dorsal root ganglion neurones *in vitro*. Br. J. Pharmacol. 89, 661-615.

Roy, J.P., Clerq, M., Steriade, M. and Deschenes, M. (1984) Electrophysiology of neurons of lateral thalamic nuclei in cat: mechanisms of long lasting hyperpolarizations. J. Neurophysiol. 51, 1220-1235.

Saito, N., Taniyama, K. and Tanaka, C. (1985) Uptake and release of gamma-aminobutyric acid in guinea pig gallbladder. Am. J. Physiol. 249, G192-G196.

Satou, M., Mori, K., Tazawa, Y. and Takagi, S.F., (1982) Two types of postsynaptic inhibiton in pyriform corrtex of the rabbit: fast and slow inhibitory postsynaptic potentials. J. Neurophyisiol. 48, 1142-1156.

Sawynok, J. (1986) Baclofen activates two distinct receptors in the rat spinal cord and the guinea pig ileum. Neuropharmacology 25, 795-798.

Scherer, R.W., Ferkany, J.W. and Enna, S.J. (1988) Evidence for pharmacologically distinct subsets of $GABA_B$ receptors. Brain Res. Bull. 21, 439-443.

Schlicker, E., Classen, K., and Gothert, M. (1984) GABA receptor-mediated inhibition of serotonin release in the rat brain. Naunyn-Schmeidbergs Arch. Pharmacol. 326, 99-105.

Schwartz, M., Klockgether, T., Wullner, U., Turski, L. and Sontag, K.-H. (1988) δ-aminovaleric acid antagonizes the pharmacological actions of baclofen in the central nervous system. Exp. Brain. Res. 70, 618-626.

Scott, R.H., and Dolphin, A.C. (1986) Regulation of calcium currents by GTP analogue: Potentiation of (-)baclofen-mediated inhibition. Neurosci. Lett. 56, 59-64.

Seabrook, G.R., Howson, W. and Lacey, M.G. (1990) Electrophysiological characterization of potent agonists and antagonists at pre- and postsynaptic $GABA_B$ receptors on neurones in rat brain slices. Br. J. Pharmacol. 101, 949-957.

Seabrook, G.R., Howson, W. and Lacey, M.G. (1991) Subpopulations of GABA-mediated synaptic potentials in slices of rat dorsal striatum are differentially modulated by presynaptic $GABA_B$ receptors. Brain Res. 562, 332-334.

Seamon, K.B. and Daly, J.W. (1981) Activation of adenylate cyclase by the diterpene forskolin does not require the guanine nucleotide regulator protein. J. Biol. Chem. 256, 9799-9801.

Seamon, K.B., Padgett, S. and Daly, J.W. (1981) Forskolin: unique diterpene activator of adenylate cyclase in membranes and intact cells. Proc. Nat. Acad. Sci. 78, 3363-3367.

Segal, M. (1990) A subset of local interneurons generate slow inhibitory postsynaptic potentials in hippocampal neurons. Brain Res. 511, 163-164.

Solis, J.M. and Nicoll, R.A. (1992) Pharmaclogical characterization of $GABA_B$-mediated responses in the CA1 region of the rat hippocampal slice. J. Neurosci. 12, 3466-3472.

Soltesz, I., Haby, M., Leresche, N., and Crunelli, V. (1988) The $GABA_B$ antagonist phaclofen inhibits the late K^+-dependent IPSP in cat and rat thalamic and hippocampal neurones. Brain Res. 448, 351-354.

Soltesz, I., Lightowler, S., Leresche, N. and Crunelli, V. (1989) On the properties and origin of the $GABA_B$ inhibitory postsynaptic potential recorded in morphologically identified projection cells of the cat dorsal lateral geniculate nucleus. Neurosci. 33, 23-33.

Southam, E., Morris, R., and Garthwaite, J. (1992) Sources and targets of nitric oxide in rat cerebellum. Neurosci. Lett. 137, 241-244.

Steriade, M., Deschenes, M., Domich, L. and Mulle, C. (1985) Abolition of spindle oscillations in thalamic nuerons disconnected from nucleus reticularis thalami. J. Neurophysiol. 54, 1473-1497.

Steriade, M. and Llinas, R.R. (1988) Functional states of the thalamus and associated neuronal interplay. Physiol. Rev. 68, 649-742.

Stevens, D.R., Gallagher, J.P., and Shinick-Gallagher, P. (1985) Intracellular studies *in vitro* of inhibitory synaptic events and the action of GABA agonists in the dorsolateral septal nucleus (DLSN) of the rat. Soc. Neurosci. Abstr. 11, 281.

Stirling, J.M., Cross, A.J., Robinson, T.N. and Green, A.R. (1989) The effects of $GABA_B$ receptor agonists and antagonists on potassium-stimulated $[Ca^{2+}]_i$ in rat brain synaptosomes. Neuropharmacology 28, 699-704.

Stone, E.A. and John, S.M. (1990) *In vivo* measurement of extracellular cyclic AMP in the brain: use in studies of β-adrenoceptor function in nonanesthetized rats. J. Neurochem. 55, 1942-1949.

Stone, E.A., Sessler, F.M. and Weimin, L. (1990) Glial localization of adenylate-cyclase-coupled β-adrenoceptors in rat forebrain slices. Brain Res. 530, 295-300.

Stoof, J.C. and Kebabian, J.W. (1981) Opposing roles for D-1 and D-2 dopamine receptors on efflux of cyclic AMP from rat neostriatum. Nature 294, 336-368.

Stratton, K.R., Cole A.J., Pritchett, J. Eccles, C.U., Worley, P.F. and Baraban, J.M. (1989) Intrahippocampal injections of pertussis toxin blocks adenosine suppression of synaptic responses. Brain Res. 494, 359-364.

Suzdak, P.D. and Gianutsos, G. (1986) Effect of chronic imipramine or baclofen on GABA-B binding and cyclic AMP production in cerebral cortex. Eur J. Pharmacol. 131, 129-133.

Swartzwelder, H.S., Bragdon, A.C., Sutch, C.P., Ault, V. and Wilson, W.A. (1986) Baclofen supresses hippocampal epileptiform activity at low concentrations without supressing synaptic transmission. J. Pharmacol. Exp. Ther. 237, 881-887.

Swartzwelder, H.S., Lewis, D.V., Anderson, W.W. and Wilson, W.A. (1987) Seizure like events in brain slices: suppression by interictal activity. Brain Res. 410, 362-366.

Szekely, A.M., Barbaccia, M.L. and Costa, E. (1987) Effect of a protracted antidepressant treatment on signal transduction and [^{3}H]-(-)baclofen binding at $GABA_B$ receptors. J. Pharmacol. Exp. Ther. 243, 155-159.

Tang, W.J. and Gilman, A.J. (1991) Type specific regulation of adenylyl cyclase by G protein $\beta\gamma$ subunits. Science 254, 1500-1503.

Terrence, C.F., From, G.H. and Tenicela, R. (1985) Baclofen as an analgesic in chronic peripheral nerve disease. Eur. Neurol. 24, 380-385.

Thalmann, R.H. (1988) Evidence that guanosine triphosphate (GTP)-binding proteins control a synaptic response in brain: effect of pertussis toxin and GTP-γ-S on the late inhibitory postsynaptic potential of hippocampal CA3 neurons. J. Neurosci. 8, 4589-4602.

Thalmann, R.H. and Ayala, G.F. (1982) A late increase in potassium conductance follows synaptic stimulation of granule neurons of the dentate gyrus. Neurosci. Lett. 29, 243-248.

Thompson, M.S., Scanziani, M., Capogna, A. and Gahwiler, B.H. (1992) The effects of adenosine, baclofen and Cd^{2+} on miniature EPSCs and IPSCs in the hippocampus reveal novel mechanism of presynaptic action. Soc. Neurosci. Abstr. 334.7.

Ungerstedt, U. (1984) Measurement of neurotransmitter release by intracranial microdialysis, in *Measurement of Neurotransmitter Release* (Marsden, C.A., ed), pp. 81-105 John Wiley and Sons, Chichester.

Vergnes, M., Marescaux C., Depaulis, A., Micheletti, G. and Warter, J.M. (1987) Spontaneous spike and wave discharges in the thalamus and cortex in a rat model of genetic petit mal-like seizures. Exp. Neurol. 96, 127-136.

Vergnes, M., Marescaux, C. and Depaulis, A. (1990) Mapping of spontaneous spike and wave discharges in Wistar rats with genetic generalized non-convulsive epilepsy. Brain Res. 593, 87-91.

Vergnes, M., Marescaux, C., Micheletti, G., Reis, J., Depaulis, A., Rumbach, L. and Warter, J.M. (1982) Spontaneous paroxysmal electroclinical patterns in rat: a model of generalized non-convulsive epilepsy. Neurosci. Lett. 33, 97-101.

Waldmeier, P.C., Wicki, P., Feldtrauer, J.-J. and Baumann, P.A. (1988) Potential involvement of a baclofen-sensitive autoreceptor in the modulation of the release of endogenous GABA from rat brain slices *in vitro*. Naunyn-Schmiedeberg's Arch. Pharmocol. 337, 289-295.

Wang, M.Y. and Dun, N.J. (1990) Phaclofen-insensitive presynaptic inhibitory action of (+-)Baclofen in neonatal rat motoneurones in vitro. Br. J. Pharmacol. 99, 413-421.

Watling, K.J. and Bristow, D.R. (1986) $GABA_B$ receptor-mediated enhancement of vasoactive intestinal prptide-stimulated cyclic AMP production in slices of rat cerebral cortex. J. Neurochem. 46, 1756-1762.

Whelan, D.T., Scriver, S.R. and Mahyuddin, F. (1969) Glutamic acid decarboxylase and gamma-aminobutyric acid in mammalian kidney. Nature 244, 916-917.

Wilkin, G.P., Hudson, A.L., Hill, D.R. and Bowery, N.G (1981) Autoradiographic localization of $GABA_B$ receptors in rat cerebellum. Nature 294, 584-587.

William, D. (1953) A study of thalamic and cortical rhythms in petit mal. Brain 76, 50-69.

William, D. (1965) The thalamus and epilepsy. Brain 88, 539-556.

Williams, L.T., Mullikin, D. and Lefkowitz, R.J. (1978) Magnesium dependence of agonist binding to adenylate cyclase-coupled hormone receptors. J. Biol. Chem. 253, 2984-2989.

Williams, S. and Lacaille, J.C. (1990) Bicuculline- and phaclofen-resistant hyperpolarizations evoked by glutamate applications to striatum laconosum moleculare in CA1 pyramidal cellsof the rat hippocampus in vitro. Eur. J. Neurosci. 2, 993-1003.

Wilson, P.R. and Yaksh, T.L. (1978) Baclofen is antinociceptive in the spinal intrathecal space of animals. Eur. J. Pharmacol. 51, 323-330.

Wojcik, W.J. and Neff, N.H. (1984) γ-Aminobutyric acid B receptors are negatively coupled to adenylate cyclase in brain, and in the cerebellum these receptors may be associated with granule cells. Mol. Pharmacol. 25, 24-28.

Wojcik, W.J., Cavalla, D. and Neff, N.H. (1985) Co-localized adenosine A1 and γ-amino acid B ($GABA_B$) receptors of cerebellum may share a common adenylate cyclase catalytic unit. J. Pharmacol. Exp. Ther. 232, 62-66.

Wong, Y.H., Demolinou-Mason, C.D. and Barnard, E.A. (1989) Opioid receptors in magnesium-digitonin-solubilized rat brain membrane are tightly coupled to a pertussus toxin-sensitive guanine nucleotide-binding protein. J. Neurochem. 52, 999-1009.

Woods, J.H., Hare, T.A., Glaeser, B.S., Bellenger, JC. and Post, R.M. (1979) Low cerebral spinal fluid γ-amino butyric acid content in seizure patients. Neurology 29, 1203-1208.

Wu, J.Y., Chude, O., Wein, J., Roberts, E., Saito, K. and Wong, E. (1978) Distribution and tissue specificity of glutamate decarboxylase (RC 4.1.1.15). J. Neurochem. 30, 849-857.

Yatani, A., Codina, J., Imoto,Y., Reeves, J.P., Birnbaumer, L. and Brown, A.M. (1988) A G-protein directly modulates mammalian cardiac calcium currents. Science 238, 1288-1291.

Young, R.R. and Delwaide, P.J. (1981a) Drug Therapy: Spasticity, part one. New England J. Med. 304, 28-33.

Young, R.R. and Delwaide, P.J. (1981b) Drug Therapy: Spasticity, part two. New England J. Med. 304, 96-99.

Zucker, R.S. and Haydon, P.G. (1988) Membrane potential has no direct role in evoking neurotransmitter release. Nature 335, 360-362.

Zukin, S.R., Young, A.B. and Snyder, S.H. (1974) Gamma aminobutyric acid binding to receptor sites in rat central nervous system. Proc. Natn. Acad. Sci. 71, 4802-4807