## *Probing novel compound classes & a new interacting protein for the Mammalian G(_A) receptor*

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A thesis submitted to the University of Durham in accordance with the requirements for the degree of Doctor of Philosophy

# Probing Novel Compound Classes & a New Interacting Protein for the Mammalian GABA$_A$ Receptor

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Durham University

School of Health

2006

# Abstract

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the vertebrate brain mediating its fast inhibitory action via $GABA_A$ receptors. These receptors are implicated in a number of neurological diseases, making $GABA_A$ receptor ligands interesting as potential therapeutic agents.

The aims of this research project were two-fold: identifying leads for the discovery of new chemical entities that modify $GABA_A$ receptor function. The second aim was to increase the understanding of GABAgeric transmission by studying the pharmacological influence of a new interacting protein for the mammalian $GABA_A$ receptor, GRIF-1.

In the search for novel ligands for $GABA_A$ receptor, the pharmacology of three structurally distinct compound classes was investigated. The first class was the NSAID, Mefenamic acid (MFA) and a group of analogues. Results showed that MFA and a series of analogues selectively modulate $GABA_AR$ at the agonist binding site, but did not interact with either the picrotoxin or the benzodiazepine sites. Indeed the most significant result of this study was the identification of common active conformers of MFA compound and the differentiation of two analogues based on MFA structure, with an improvement in apparent efficacy. The second compound studied was Octyl-β-D-glucoside, a small molecule congener of a natural fungal metabolite, Caloporoside. These studies demonstrated that Octyl-β-D-glucoside is a positive modulator of $GABA_A$ receptor at the channel site demonstrated by its stimulation of specific [$^{35}$S] TBPS binding. The level of stimulation was similar to that elicited by diazepam and was occluded by GABA. Preliminary structure-activity study showed that the β-glycosidic linkage and chain length are crucial for the positive modulation of [$^{35}$S] TBPS binding to the $GABA_AR$ by this novel chemical class. The third compound series were essential oils derived from *Melissa officinalis* and *Lavendula angustifolia.* These two oils either singly or in combination have been reported to have a significant benefit in the treatment of agitation in dementia. The purpose of this study was to clarify the sedative and calming mechanisms of these two common essential oils by investigating their effects on the $GABA_AR$ complex. Melissa and Lavender both singly and in combination inhibit [$^{35}$S] TBPS binding to the channel site of $GABA_AR$. Melissa oil displayed the higher affinity. Melissa oil alone also showed a stimulatory effect on [$^{3}$H] muscimol binding. Interestingly, a combination effect on the inhibition of [$^{3}$H] flunitrazepam binding to the $GABA_AR$ has been shown when Lavender and Melissa oils are applied together (50:50), with no effect when applied alone. Neither Melissa nor Lavender oils

demonstrated any effect on the binding of [$^3$H] MK-801 to NMDA receptors, or [$^3$H] nicotine to nicotinic acetylcholine receptors. Furthermore, functional studies have demonstrated that both oils (0.01 mg/ml) applied to rat primary cortical neuron cultures, results in a significant reduction in both inhibitory and excitatory transmission, with a net depressant effect on neurotransmission. These data suggests that the calming/sedative effects of Melissa are mediated by multiple mechanisms in the CNS; the net effect is depressant on the overall neuronal network.

Finally, a pharmacological study was performed on GRIF-1a, a novel $GABA_A$ receptor β2 subunit trafficking protein, to gain further insights into the potential role of this novel protein at the inhibitory synapse. In the present work, evidence was provided that GRIF-1a does not increase α1β2γ2 receptor complex numbers, but appears importantly to stabilise the $GABA_AR$ in a conformation which facilitates binding to both GABA and benzodiazepines. These findings suggest that GRIF-1 protein may be a novel means of modifying the efficacy of synaptic inhibition.

In summary, this thesis provides a clear picture about four novel ways for the modulation of the $GABA_A$ receptor inhibitory transmission.

# Candidates Declaration

I confirm that no part of the materials presented has previously been submitted for a degree in this or any other University. If materials have been generated through joint work, my independent contribution has been clearly indicated. In all other cases, materials from the work of others has been clearly indicated, acknowledged and quotations and paraphrases indicated.

# Acknowledgements

I am grateful to Dr. Paul. L.Chazot for giving me the opportunity to carry out my PhD in his laboratory, for his academic supervision of this project and for his unfailing help, support and friendship over the last three years.

I wish to express my gratitude to Prof. George Lees (Otago School of Medical Sciences, Dunedin, NZ) and to Dr. Abdel Ennaceur (School of Pharmacy, Sunderland University) for their academic advice and support.

Many thanks are also due to Prof. Maher Salim (President, of Al-Ahliyya Amman University) and to Prof.Fatma Afifi (Faculty of Pharmacy, Jordan University) without their support, I would never have reached my ambition of completing a doctorate.

Thanks must also go to Islamic Development Bank for their financial support.

Thank you for every one else I've worked with in Lab 18, Rebecca Sheahan, Fiona Shenton, Andrea Bradford and Heather Chaffey, for their invaluable assistance and friendships.

Finally, I am indebted to my family, my great parents, wonderful brothers and sisters who have supported me in every thing I've ever achieved, helped me to keep a good perspective and have always kept me smiling.

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# Abbreviations

| | |
|---|---|
| AD | Alzheimer's disease |
| APS | Ammonium persulphate |
| $B_{max}$ | Maximum number of receptors per mg protein |
| BSA | Bovine serum albumin |
| $BS^3$ | bis (sulfosuccinimidyl) suberate |
| BPSD | Behavioral and Psychological Symptoms of Dementia |
| BZ | Benzodiazepine |
| cDNA | Complementary DNA |
| CNS | Central nervous system |
| $CO_2$ | Carbon dioxide |
| $^oC$ | Degrees centigrade |
| $Cl^-$ | Chloride ions |
| DMEM/F12 | Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 Ham |
| DMSO | Dimethyl sulphoxide |
| DNA | Deoxyribonucleic acid |
| DPM | Disintegrations per minute |
| dsDNA | Double stranded DNA |
| $dH_2O$ | Distilled water |
| DTT | Dithiothreitol |
| *E. coli* | *Escherichia coli* |
| $EC_{50}$ | Concentration of competitor that competes with half of the specific binding |
| $E_{max}$ | Maximum response |
| EDTA | Ethylenediaminetetracetic acid |
| EGTA | Ethylenebis(oxyethylenenitrilo)tetracetic acid |
| FCS | Foetal calf serum |
| Fm | Femto moles |
| FRET | Fluorescence Resonance Energy Transfer Technique |
| FLIPR | Fluorescence Imaging Plate Reader |
| GABA | γ-aminobutyric acid |
| $GABA_AR$ | γ-aminobutyric acid type receptor type A |
| GAD | Glutamic acid decarboxylase |
| GAT | GABA transporters |
| GF/B | Glass fibre filters |
| GFP | Green Fluorescent Protein |
| GRIF-1 | GABA receptor interacting factor-1 |
| $H_2O_2$ | Hydrogen peroxide |
| HB101 | Strain of E. coli competent cells |
| HCL | Hydrochloric acid |
| HD | Huntingdon's disease |
| HEK 293 | Human embryonic kidney 293 cells |
| HEPES | N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid |
| HRP | Horseradish peroxidase |
| 5-HT | 5-hydroxy-tryptamine |
| $IC_{50}$ | Concentration of the ligand giving 50% inhibition of specific binding |
| $K_D$ | Equilibrium Dissociation constant |
| kDa | Kilodaltons |
| $K_I$ | Inhibition constant |
| KCC1 | $K^+$/ $Cl^-$ co-transporter (1) |
| KCC2 | $K^+$/ $Cl^-$ co-transporter (2) |

| | |
|---|---|
| LGIC | Ligand gated ion channel |
| M | Molar |
| mA | Milli amps |
| MFA | Mefenamic Acid |
| MK-801 | (+)-5-Methyl-10,11-dihydr-5*H*-dibenzo[a,d]cyclohepten-5,10-imine |
| ml | Milli-litre |
| mM | Milli-molar |
| $M_1$ | Muscarinic acetylcholine receptors subtype1 |
| NaCl | Sodium chloride |
| $NaHCO_3$ | Sodium hydrogen carbonate |
| NaOH | Sodium Hydroxide |
| Na-Az | Sodium azide |
| nAChR | nicotinic acetylcholine receptor |
| $n_H$ | Hill coefficient |
| nM | Nano Molar |
| NSAIDs | Non-steroidal anti-inflammatory drugs |
| NMDA | *N*-methyl-D-aspartate |
| O.D. | Optical density |
| 8-OH-DPAT | 8-hydroxy-2-(di-n-propylamino) tetralin |
| PAGE | Polyacrylamide gel electrophoresis |
| PBS | Phosphate buffered saline |
| PH | Potential of Hydrogen |
| QIAGEN | Plasmid DNA Maxi Kit |
| RNA | Ribonucleic acid |
| SAR | Structure Activity Relationship |
| SSRI | Selective Serotonin Reuptake Inhibitor |
| SDS | Sodium dodecyl sulphate |
| Sulfo-NHS-SS-biotin | Sulfosuccinimidyl-2-(biotinamido) ethyl-1, 3-dithiopropionate |
| TBS | Tris-buffered saline |
| TBPS | t- butylbicyclophosphorothionate |
| TBOB | t-butylbicycloorthobenzoate |
| TE buffer | Tris-HCL, EDTA buffer |
| TEMED | N,N,N,′N′-Tetramethyletylenediamine |
| THDOC | 5α-pregnane-3 α, 21-diol-20-one |
| Tris | Tris (hydroxymethyl) methylamine |
| V/v | Volume per volume |
| U.V | Ultra-violet |
| V | Volts |
| VGAT | Vesicular GABA transporter |
| w/v | Weight per volume |
| μg | Microgram |

# Chapter 1

# Introduction

## 1.1 Central Nervous System Drug Discovery Challenges

The population of the world is getting older. During the first 50 years of this millennium, the worldwide population aged over 65 years is projected to increase from 6.9% of the total population to 15.9%, which constitutes an extra billion elderly individuals (Alavijeh *et al.*, 2005). This is attributable to a combination of a progressive increase in life expectancy and elevated fertility in many countries during the two decades after World War II.

This growing number of older adults will increase the demands on both public health system and on medical and social services, particularly for chronic neurological disorders such as stroke, Alzheimer's disease and Parkinson's disease. Such disorders affect older adults disproportionately and contribute to disability, diminish quality of life and increased healthcare costs. Thus, stroke afflicts 30% of persons aged over 65 years (fatally in10%), and its incidence doubles during successive decades. Alzheimer's disease affects 10% of the population aged over 65 years and rises to 49% of those age 80 years or more. Parkinson's disease affects 1% of persons aged 60 or older and 2.6% of those over the age of 85 years (Hurok *et al.*, 2005).

Many thousands of compounds undergo the early stages of the process, but very few achieve drug status. Successful candidates have to fulfil the essential criteria of potency, selectivity, oral bioavailability (for orally administered drugs), therapeutic efficacy, along with an acceptable side effect profile. Therefore, CNS research and development are associated with significant challenges: it takes longer to get a CNS drug to market (12–16 years) compared with a non-CNS drug (10–12 years) and there is a higher attrition rate for CNS drug candidates than for non-CNS drug candidates. This is attributable to a variety of factors, including the complexity of the brain, the liability of CNS drugs to cause CNS side effects and the requirement of CNS drugs to cross the blood-brain barrier (BBB) (Alavijeh *et al.*, 2005; Hurok *et al.*, 2005; Hilbush *et al.*, 2005).

The major mechanism for neuronal inhibition in the adult mammalian central nervous system utilizes γ-aminobutryric acid $GABA_A$ receptors to reduce and control cells excitability. Given the important role of these receptors in neuronal inhibition, they are prime targets of many therapeutic agents and are the object of intense studies aimed at correlating their structure and function.

## 1.2 Glutamate& GABA Centric View of CNS Function

Glutamate- and GABA-releasing neurons form the basis for neurotransmission in the mammalian central nervous system (CNS). The co-ordination of these excitatory and inhibitory systems, together with intrinsic voltage-gated ion channels and G-protein-coupled receptor modulation, provides the diverse neuronal firing patterns, network activity and synaptic plasticity that are required for the complexity of CNS function. Major excitatory and inhibitory inputs onto neurons release glutamate and γ-aminobutyric acid (GABA), respectively. These inputs are usually paired to achieve a coordinated balance between excitatory and inhibitory events (Foster & Kemp, 2006).

Glutamate and GABA are released from nerve terminals in high concentration to activate postsynaptic ionotropic receptors that directly modify the membrane potential of the receptive neuron (generating an excitatory (EPSP) or inhibitory (IPSP) postsynaptic potential); the sum of these inputs determines the threshold for firing of the receptive neuron and the propagation of information through neuronal networks. This basic system is modulated through G-protein-coupled receptors (GPCRs) for a variety of neuroactive substances, including monoamines, neuropeptides, locally produced neuromodulators (e.g. adenosine and anandamide), and glutamate and GABA themselves. GPCRs for glutamate and GABA exist on their respective synaptic terminals (autoreceptors) and on the terminals of each other and of other neurotransmitters (heteroreceptors) to regulate neurotransmitter release. In addition, GPCRs for both glutamate and GABA are present on the postsynaptic membrane and can modify membrane properties through an influence on both glutamate and GABA-gated ion channels, G-protein-coupled potassium channels and voltage-gated ion channels. Both neurons and glial cells that surround the synapse have specialized transporters that efficiently remove glutamate and GABA from the extracellular space, whose primary role is to maintain the fidelity of synaptic transmission (Bowery & Smart, 2006).

In addition to the generation of the multiple patterns of neuronal activity that are observed in CNS function, these basic systems also exhibit plastic changes that appear to be fundamental to both the development and the maintenance of complex cognitive functions, such as learning and memory. Long-term potentiation and depression (LTP and LTD, respectively) result from significant changes in synaptic strength that can be long lasting or even permanent, and are primarily a phenomenon of glutamate-mediated neurotransmission involving both ligand-gated ion channel and GPCR components Related phenomena, such as the enhanced response of spinal neurons to repeated noxious stimuli, or 'wind-up', appear to be the basis of pathological conditions such as neuropathic pain ((Foster & Kemp, 2006; Bowery & Smart, 2006).

Given the fundamental involvement of the glutamate and GABA neurotransmitter systems in CNS function and the evidence for their malfunction in disease states, practically every molecular component of these neurotransmitter systems has been examined as a potential target for novel therapeutic agents. The purpose of this study is to provide a novel ways of the therapeutic approaches that are based on modifying GABA mediated neurotransmission. We hope that the study represents a primary screening step in the process of the discovery and development of safe and effective medicines for CNS disorders. Although GABA-based therapeutics has been in clinical use for some time, effective and safe drugs targeting the GABA system have been slower to emerge. The major current trend is to pursue approaches based on allosteric modulation and subtype selectivity to achieve therapeutic efficacy with reduced side effect potential.

## 1.3 γ-Aminobutyric acid (GABA)

γ-Aminobutyric acid (GABA) is the most prevalent inhibitory neurotransmitter in the mammalian central nervous system (CNS). It was independently identified and reported to be present in the vertebrate brain by Roberts and Frankel and by Awapara and collaborators in the1950 (Awapara *et al.*, 1950, Robert & Frankel, 1950). However, a further twenty years was required before GABA was shown to satisfy all the classical criteria of a neurotransmitter (Krnjevic, 1974, Roberts, 1986). Upon the discovery of glutamic acid decarboxylase and its employment as a marker for GABAgeric neurons, the studies revealed that many, if not most, GABAergic neurons in the brain are interneurons, and it has been estimated that 30-40% of all CNS neurons utilize GABA as their primary neurotransmitter (Hendry *et al.*, 1987, Roberts, 1986, Bloom *et al.*, 1971).

The major pathway for GABA synthesis involves the decarboxylation of L-glutamate by glutamic acid decarboxylase (GAD), an enzyme whose brain distribution shows a direct correlation to the concentration of GABA; it exists in two isoforms, referred as to GAD 65 and GAD 67 (Tian *et al.*, 1999; Whiting, 1999).

The mechanism by which GABA exerts its effect involves binding of GABA to specific chloride ($Cl^-$) ion channel proteins in the post-synaptic membrane; GABA binding causing a conformational change in these channel proteins, opening a central ionic pore that allows an influx of ($Cl^-$) anions into the post-synaptic neurons. This hyperpolarises and inhibits action potentials, thus preventing signal transmission (Sakmann *et al.*, 1983, Bormann *et al.*, 1987). Once used, GABA is removed from the synapse by high affinity sodium ($Na^+$) dependent GABA transporters (GAT [1-4]) that are present in the pre-synaptic terminal and surrounding glial cells (Soudijn & Wijingaarden, 2000, Foster & Kemp, 2006). GABA is metabolised by GABA transaminase to form succinic acid semialdehyde which then is oxidised to form succinic acid and re-cycled via the citric acid cycle (Iverson & Neal, 1968) Figure 1.1.

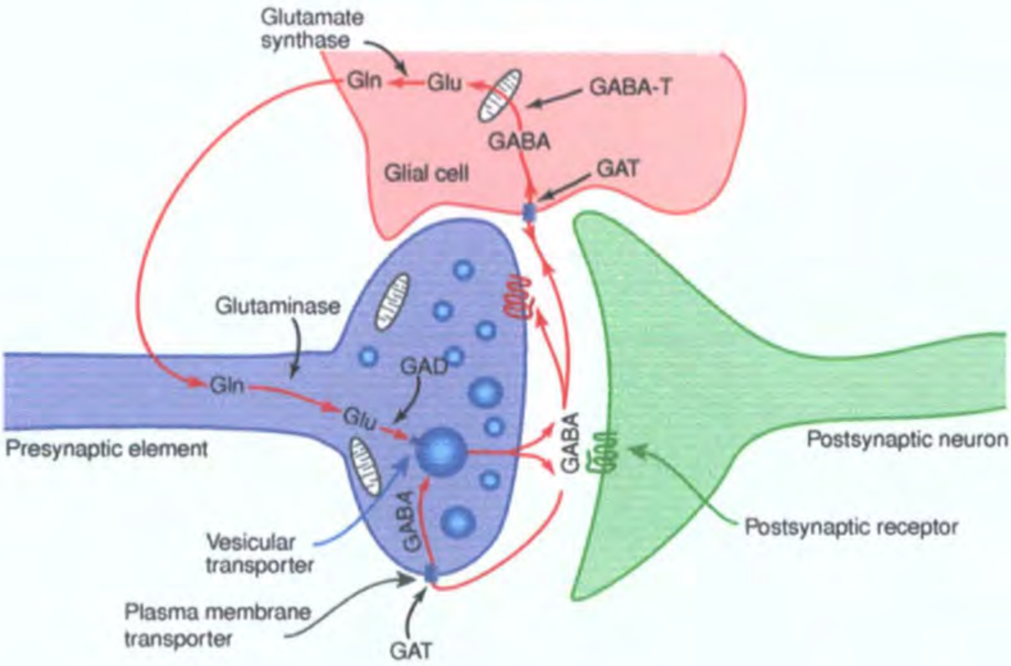



**Figure 1.1:** Schematic depiction of the life cycle of a GABAgeric neuron (Zigmond *et al.*, 2003)

In addition to its role in inhibitory neurotransmission in the adult brain, GABA also acts as a trophic factor during neural development. During the early stages of neural development, the effects of GABA are excitatory (Cherubini *et al.*, 1991). This may be due to a reversed transmembrane ($Cl^-$) gradient, resulting in a higher intracellular than extracelluar concentration of ($Cl^-$) ions and the net efflux of ($Cl^-$) ions upon the opening of $GABA_AR$ channels, thus depolarising the post synaptic neurons (Misgeld *et al.*, 1986). The shift in ion gradient during early postnatal development that leads to the switch from excitatory to inhibitory effects of GABA activation, is due to an increase in expression levels of the ion extruding neuronal $K^+$/ $Cl^-$ co-transporters 2 (KCC2) ( Rivera *et al.,* 1999). KCC2 counters the effects of the ($Cl^-$) accumulating KCC1 which is strongly expressed from birth. GABA transmission thus begins to display its characteristic inhibitory effects.

## 1.4 GABA Receptors

Once released into synaptic cleft, GABA acts through specific receptors located in pre- and postsynaptic membranes. Three different receptor classes for GABA have been defined in terms of physiology and pharmacology namely, $GABA_A$, $GABA_B$ and $GABA_C$ receptors. The $GABA_AR$ has been the most extensively characterised.
The first GABA receptor subtype to be described was later defined as the $GABA_A$ receptor. $GABA_AR$ are integral ($Cl^-$) channels (Bormann, 1988, Silvilotti & Nistri, 1991) and are located pre- and post-synaptically throughout the CNS. GABA influences neuronal excitability and affects glial cells at $GABA_AR$ by increasing permeability to chloride ions (Curtis *et al.*, 1968, Krnjevic, 1974; Olsen, 1982), usually causing membrane hyperpolarization in neurons and depolarisation in glial cells. $GABA_AR$ are activated by GABA, muscimol and isoguvacine, inhibited competitively by bicuculline and non-competitively by picrotoxin and are subject to allosteric modulation by a number of chemically diverse allosteric modulators.

The $GABA_B$ receptors are G protein-coupled receptors. Activation of $GABA_B$ receptors leads to a slow-acting inhibition of neurons by inhibition of voltage gated $Ca^{+2}$ channels and stimulation of G protein-coupled $K^+$ channels. $GABA_B$ receptors are insensitive to bicuculline, but are sensitive to the $GABA_B$ receptor agonist, baclofen (Bowery *et al.*, 1980; Hill & Bowry, 1981), and the antagonist, phaclofen (Kerr *et al.*, 1987). These are seven transmembrane proteins which function as heterodimers at both pre- and post-synaptic sites (Kaupmann *et al.*, 1997, 1998).

$GABA_C$ receptors are a separate sub-class of ionotropic GABA receptor channel. They are composed of the subunits ρ1-3 which has not been shown to associate with $GABA_AR$ subunits (Hackman *et al.*, 1998; Enz & Cutting, 1998). $GABA_C$ receptors also have a distinct pharmacological profile to the $GABA_A$ receptors (Bormann, 2000). They are stimulated by GABA, muscimol, cis-4aminocrotonic acid (CACA, a conformationally restricted analogue of GABA) and its trans-isomer (trans-4-aminocrotonic acid , TACA) ( Sivilotti & Nistri, 1989, 1991, Feigenspan *et al.*, 1993, Lukasiewicz *et al.*, 1994; Dong *et al.*, 1994), but is insensitive to baclofen and bicuculline ( Quian & Dowling 1993, Feigenspan *et al.,* 1993, Dong *et al.*, 1994). Unlike $GABA_A$ receptors, $GABA_C$ receptors are not modulated by benzodiazepines, barbiturates (Sivilotti & Nistri, 1991; Bormann & Fiegenspan, 1995) or neurosteriods (Feigenspan *et al.*, 1993). $GABA_C$ receptors are mainly found in the retina, where they are expressed 10-fold higher than $GABA_A$ receptors and do not desensitise on prolong activation (Cutting *et al.*, 1991, Feigenspan *et al.,* 1993, Quian & Dowling, 1993, Lukasiewicz *et al.*, 1994, Dong *et al.*, 1994).
Thus, a diversity of receptors classes exists for which GABA is the endogenous ligand, this diversity is further increased by the existence of a number of subtypes of the GABA receptor which differ in terms of physiology and pharmacology.

# 1.5 $GABA_A$ Receptors

## 1.5.1 $GABA_A$ Receptors Structure

$GABA_A$ receptors are members of a superfamily of ligand-gated ion channel (LGIC) that includes nicotinic acetylcholine (nACh) receptors, glycine receptors, 5-hydroxytryptamine type 3 ($5\text{-}HT_3$) receptors and invertebrate glutamate-gated chloride channels (Cully *et al.*, 1994, Karlin & Akabas, 1995). nACh receptors have been extensively characterised and are often used as a prototype for the whole (LGIC) superfamily. Evidence suggests that these receptors are comprised of five individual subunits with each subunit having similar membrane topology (Anand *et al.,* 1991, Cooper *et al.*, 1991, Unwin, 1993, 1995, 1996, Nayeem *et al.,* 1994) Figure1.2.

The subunits of the $GABA_AR$ are usually between 400 to 500 amino acid in length, comprising of a large hydrophilic extracelluar N-terminal domain (Approximately 200 amino acids) that contains 2-3 N-glycosylation sites and a 15-residue cysteine loop. This domain is followed by four hydrophobic transmembrane domains (4TM) of approximately 20 amino acid each and a large intracellular loop of ~120-150 residues between TM domains 3 and 4 (Schofield *et al.,* 1987, Olsen & Tobin, 1990, Brut & Kamatchi, 1991, Wisden & Seeburg, 1992). The loop domain contains several regulatory sequences, including phosphorylation sites for protein kinase A (PKA), protein kinase C (PKC) and tyrosine kinase (Moss & Smart, 1996). $GABA_AR$ subunits have a very short extracelluar C-terminal with few or no residues protruding from the outer face of the membrane (Figure1.3). The subunits share ~30-40% amino acid sequence identity between subunits of the same class (Macdonald & Olsen, 1994). The large intracellular loop of each subunit is the most divergent region with little or no identity between subunits of the same or different classes. The second transmembrane domains of subunits are thought to form the lining of the ion channel pore (Xu & Akabas, 1992, 1994) (Figure 1.4). Evidence from nACh receptors suggests that this pore is narrowest in the middle with the extracellular region and intracellular region widening out (Unwin, 1993). Based on the permeabilities of large polyatomic anions the pore diameter of $GABA_AR$ is 5.6 Å (Bormann *et al*, 1987).

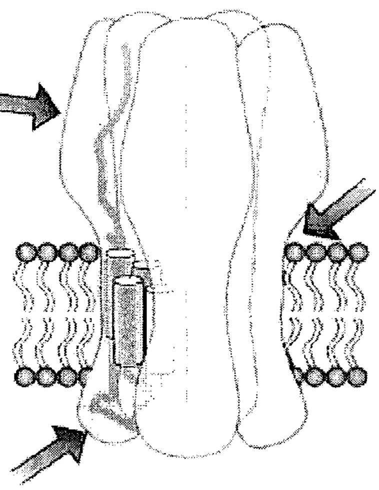


**Figure 1.2:** Schematic representation of $GABA_AR$ structure

Arrows illustrate binding sites for allosteric ligands

(Hogg *et al.*, 2005)

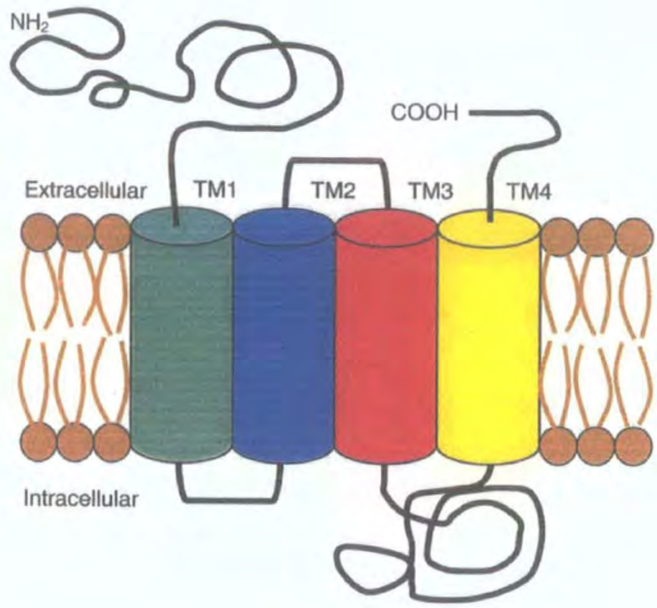



**Figure 1.3:** Structural membrane topology of a typical $GABA_AR$ subunit

(Whiting, 2003)


**Figure 1.4:** A schematic view of the $GABA_AR$ channel pore, as viewed perpendicular to the plane of the membrane, showing the second membrane spanning domain (TM2) lining the pore of the ion channel

(Bormann, 2000)

## 1.5.2 $GABA_A$ Receptors Subunit Composition & Stoichiometry

The first two GABA receptor proteins were isolated in the early 1980's (Sigel *et al*, 1983, Sigel & Barnard, 1984) followed by the cloning and expression of these subunits in 1987 (Schofield *et al.*, 1987). Since this time additional subunits have been isolated, sequenced, cloned and expressed, bringing the total number of human $GABA_AR$ subunits to 19 (Johnston, 2005, Korpi *et al.*, 2006). These subunits have been subclassified according to their degree of amino acid identity as α, β, γ, δ, ρ, θ, ε and π. To date for human there are 6α subunits (α1-6), three β subunits (β1-3), three γ subunits ( γ1-3), three ρ subunits (ρ 1-3), one δ subunit, one θ subunit, one ε subunit and one π subunit. The predominant location of ρ subunits within the retina and the different pharmacological properties of the receptors, has led some authors to classify these subunits as a distinct group($GABA_C$), however it has recently been suggested that the ρ subunits be classified as a subset of the $GABA_A$ receptors ( Chebib, 2004).

A number of splice variants have been described, which in the human include the long and short from of the γ2 subunit (Whiting *et al.*, 1990, Kofuji *et al.*, 1991) and a splice variant of the β3 subunit (Kirkness & Fraser, 1993). Additional splice variants have been identified in other species namely rat α6 (Korpi *et al.*, 1994) and chicken β2 and β4 (Bateson *et al.*, 1991, Harvey *et al.*, 1994). The γ2L isoform has an insert of eight amino acids between TM3 and TM4 which provide additional phosphorylation sites and has an influence on ethanol modulation (Wafford *et al.*, 1991). The ε subunit may be substituted for a γ subunit or a δ subunit and confer insensitivity to anaesthetics (Davies *et al.*, 1997). The π subunit and θ subunits show greatest amino acid sequence identity with the β subunits. The π subunit is only found outside of the CNS (Hedblom & Kirkness, 1997), while θ will assemble with α, β and γ subunits in the brain to form receptors with a 4-fold reduction in the sensitivity to GABA (Bonnert *et al.*, 1999). Combination of these $GABA_AR$ subunits associate to form pentameric integral membrane proteins, arranged to form a central ionic pore. Each subunit confers particular molecular and pharmacological properties to the fully assembled receptor. This allows for the formation of a range of receptor subtypes (Mckernan & Whiting, 1996). The large number of subunits provide hundreds of thousands of possible combinations, although the actual number of $GABA_AR$ is many fewer, the main subunit combination is the α1β2γ2 receptor that account for about 40% of all $GABA_A$ receptors. It should be noted that the relative and absolute amounts of receptor subtypes are not precisely known (Mckernan & Whiting, 1996, Korpi *et al.*, 2006). The approximate abundance of $GABA_A$ receptors subtypes in the rat brain are summarised as a pie chart, Figure1-5.

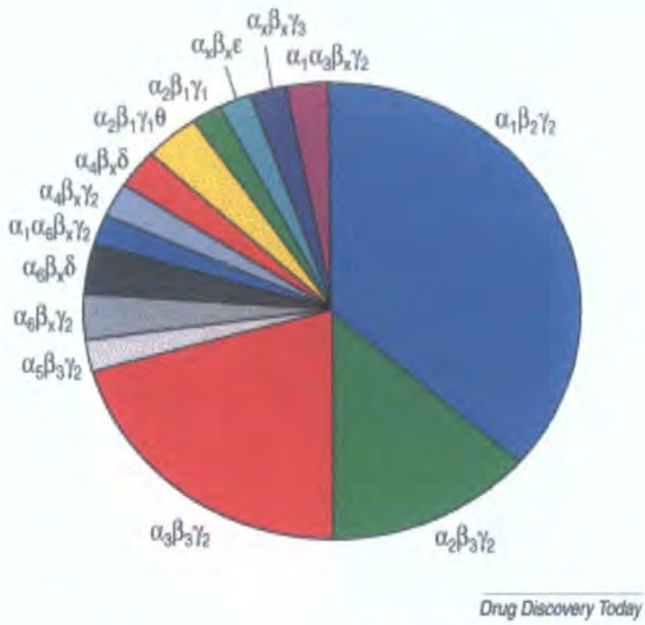



**Figure 1.5** A pie chart representing the approximate abundance of $GABA_AR$ subtypes in the rat brain, subscript x indicates the type α, β or γ subunit is not known, this is a broad stroke representation and other receptor subunit combinations probably exist (Whiting, 2003)

## 1.5.3 Regional and Developmental Changes in the Expression of $GABA_A$ Receptor in the Brain

There is a large developmental and regional heterogeneity of $GABA_AR$ expression in the brain. The distribution pattern of $GABA_AR$ subunit has been studied using subtype selective probes (Wisden *et al.*, 1992, Laurie *et al.*, 1992, Davies *et al.,* 1997, Hedblom & kirkness, 1997) and by detecting subunit immunoreactivities in particular neurons (Fritschy & Mohler, 1995, Pirker *et al.*, 2000, Bonnert *et al.*, 1999). The α1 subunit is by far the most widely expressed of the α subunits. The α2 and α3 subunits show a more localised distribution i.e.α2 is found mainly in the forebrain and cerebellum and α3 subunits are mainly found in the olfactory bulb and layers V and VI of the cerebral cortex (Wisden *et al.*, 1992). The α4 subunit shows the weakest expression of the α subunits, followed by α5, which also shows a low overall distribution. The α6 subunit is localized almost exclusively to cerebellar granule cells.

There are 3 β subunits. The β2 subunit is the most abundantly expressed β isoform in the brain, closely followed by β3. β1 is less common. The β subunits have also been implicated in targeting of receptor to distinct subcellular location (Connolly *et al.*, 1996 a). The γ2 subunit is the most abundantly expressed γ isoform, followed by γ1. The γ3 subunit has a low overall distribution in adult brain. Expression of the δ subunit is mainly restricted to the cerebellum and frequently co-localise with the α4 and α6 subunits. The ε subunit is found in the amygdala, thalamus and subthalamic nucleus (Davies *et al.*, 1997a). The θ subunit is distributed through out the striatum, hypothalamus, amygdala, hippocampus, substantia nigra and regions of the hind brain (Bonnert *et al.*, 1999). The π subunit is only found outside the brain, in lung, prostate, thymus and showing most prominent expression in the uterus (Hedblom & Kirkness, 1997).

A developmental shift in subunit expression may contribute towards subunit availability and therefore $GABA_AR$ subunit composition. Studies of the $GABA_AR$ subunit mRNA expression show a change in subunit composition between embryonic and adult receptors (Araki *et al.*, 1992, Laurie *et al.,* 1992). In these studies the α1 subunit showed an expression pattern consistent with an adult form of subunit, being expressed in neonatal neurons, but not during any stage of embryonic development. The α2, α3, and α5 subunits were shown to be strongly expressed during embryonic and early postnatal development, but to decrease in levels at later stages. This pattern was particularly pronounced for the α5 subunit. α4 was found in both differentiated and undifferentiated cells. The α6 and δ subunits showed no mRNA expression during embryonic development, appearing in early postnatal brain. The β1 subunit was present in the undifferentiated neuroepithelium and expression increased during development and postnatally. β2 and β3 both appeared during cortical development. β3 expression was strongest perinatally, while β2 expression was highest in adult brain. Expression of γ1 and γ3 subunits showed stronger expression at earlier stages of development, but neither was widely expressed at any stage. The γ2 subunit showed strong expression at all stages of development and adulthood. These changes in subunit subtype expression reflect the change in function of $GABA_A$ receptors during neuronal development.

Potential receptor subtype composition can be predicted by the co-expression of subunits in the same neuronal population. A major subtype is thought to be consisting of α1, β2 and γ2 subunits due to their expression in many brain regions.

## 1.5.4 Receptor Assembly and Anchoring

Expression studies which have focused on α1, β1-2 and γ2 subunits have revealed that access to the cell surface is limited to the combination αβ and αβγ2 subunits (Aggelotti & Macdonald, 1993, Macdonald & Olsen, 1994; Rabow *et al.*, 1995, Connolly *et al.*, 1996). Most single subunits, with the exception of β3, β1, α1/γ2 and β2/γ2 combination, are retained in the endoplasmic reticulum where they are degraded (Connolly *et al.*, 1996 b). A recent study has identified the importance of residue 58-67 in the subunit isoforms in the assembly of receptors composed of αβ and αβγ subunits (Taylor *et al.*, 2000). Deletion of these residues within α1 or α6 prevented cell surface expression with the β3 subunit implicating the importance of these residues in mediating $GABA_AR$ assembly.

Expression of receptor c-DNAs has been used to determine the minimal subunit requirement for the production of GABA-gated chloride channels which show the full pharmacological repertoire of neuronal $GABA_A$ receptors. It is generally accepted that the expression of single subunits alone does not lead to the formation of functional channel (Macdonald & Olsen, 1994, Rabow *et al.*, 1995), although the β1 and β3 subunits are able to form spontaneous open chloride channels that are insensitive to GABA but can be blocked by picrotoxin and enhanced by propofol and pentobarbital (Connolly *et al.*, 1996 b, Krishek *et al.*, 1996, Wooltorton *et al.*, 1997, Davies *et al.*, 1997 b). Expression of α and β subunits produces GABA-gated currents which are modulated by barbiturates, inhibited by GABA antagonist and zinc ions , but are not enhanced by benzodiazepine ( Levitan *et al.*, 1988, Macdonald & Olsen 1994). Co-expression of a γ subunit with α and β subunit is necessary for the formation of a benzodiazepine binding site and also sensitivity to $Zn^{2+}$ antagonism (Pritchett *et al.*, 1989, Draguhn *et al.*, 1990, Smart *et al.*, 1991). Replacement of the γ subunit with δ or ε subunits results in benzodiazepine insensitivity of the expressed receptor. Contradictory reports on the function of receptors consisting of α, β and ε subunits have been made with respect to their response to a range of anaesthetics (Davies *et al.*, 1997a, Whiting *et al.*, 1997). Therefore the consensus of opinion from these experiments is that in vivo most $GABA_A$ receptors consist of α, β and γ.

## 1.5.5 Targeted Disruption of $GABA_A$ Receptors, Subunit Gene

The contribution of individual receptor subunits to $GABA_AR$ function in the brain has been studied by the deletion of defined subunits by homologous recombination. A number of transgenic lines have now been produced lacking a single $GABA_AR$ subunit by the targeted disruption of $GABA_A$ receptor subunit genes. Deletion of the γ2 subunit causes a 94% reduction in the number of benzodiazepine binding sites, with a reduction in the single channel conductance and Hill Coefficient to a level consistent with those measured for an αβ recombinant receptor (Gunther *et al.*, 1995). The loss of the γ2 subunit was paralleled by a loss of the protein gephyrin and $GABA_AR$ clusters, thus suggesting a role of this protein in the aggregation of γ2 subunit-containing receptors .The phenotype of the mice lacking the γ2 subunit is characterized by retarded growth, sensory motor dysfunction, and reduced life span. Follow-up studies showed disruption of the normal clustering of $GABA_A$ receptors in the cultured cortical and hippocampal neurons and brain slices from γ2-/-mice, accounting for the observed phenotype (Essrich *et al.,* 1998, Kneussel *et al.*, 1999). Deletion of the β3 subunits causes the density of $GABA_A$ receptor to be approximately halved and resultant $GABA_A$ mediated transmission is severely impaired (Homancis *et al.,* 1997). The mice that survive to adulthood are hyperactive, have poor co-ordination and suffer epileptic seizures. Loss of the α5 gene causes the specific loss of zolpidem insensitive benzodiazepine binding sites but with no obvious phenotype defects (Fritschy *et al.*, 1997). Disruption of the α6 subunit results in a loss of diazepam insensitive Ro 15-4513 binding in the cerebellar granule cell layer, and a selective degradation of the δ subunit (Jones *et al.,* 1997). The latter suggests that α6 and δ subunits specifically associate during receptor assembly. Finally, disruption of the δ-subunit gene associated with an attenuated sensitivity to neuroactive steroids with multiple defects in behavioural response to ethanol (Mihalek *et al.,* 1999, 2001). Over all these transgenic studies show how the α and β subunits are required for the efficient assembly and cell surface assembly of $GABA_AR$ in vivo and the γ2 subunit is critical in the targeting /clustering of the final receptor complex.

## 1.6 $GABA_A$ receptor Pharmacology

The $GABA_AR$ possesses binding sites for many chemically diverse compounds. Included amongst these are sites for agonists, partial agonists, competitive-antagonists and positive and negative allosteric modulators. Literature prompted a conclusion that there appeared to be at least 11 distinct sites on $GABA_A$ receptors for interactions with specific ligands (Johnston, 2005). The likely sites were: (1) agonist/ partial agonist/competitive agonist recognition sites (2) picrotoxinin sites (3)sedative-hypnotic barbiturate sites (4) neuroactive steroid sites (5) benzodiazepine sites (6) ethanol sites (7) sites for inhalation anaesthetics (8) sites for furosemide associated with α6 subunits (9) sites for $Zn^{2+}$ (10) sites for a variety of divalent cations, such as $Ca^{2+}$, $Sr^{2+}$, $Ba^{2+}$, $Cd^{+2}$, $Mn^{2+}$ and $Mg^{2+}$ (11) sites for Lanthanum ions ($La^{3+}$) (Johnston, 2005) Figure 1.6

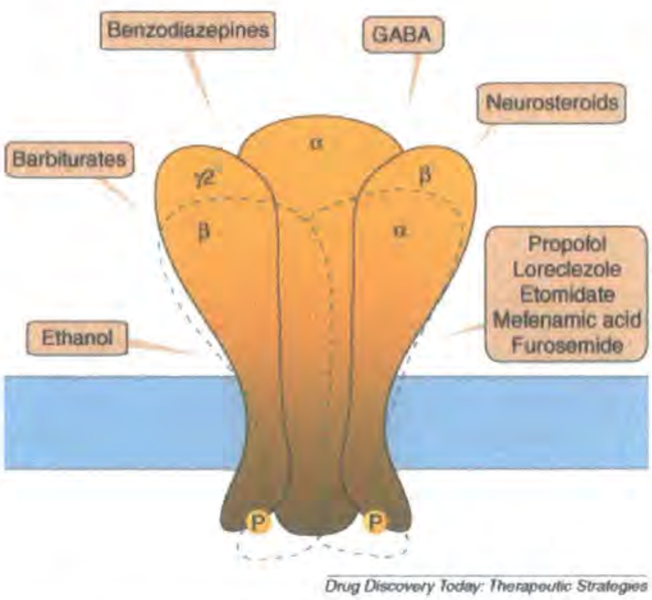



**Figure 1.6** Schematic representation of a typical $GABA_AR$ ion channel illustrating pentameric assembly of α, β and γ subunits with 2:2:1 stoichiometry. Also shown allosteric binding sites for some compound classes. (The location of the binding sites in the graph is arbitrary).

(Möhler & Rudolph, 2004)

A description of some of the important agonists and antagonists which have contributed to the characterisation of the $GABA_A$ receptor is presented below:

## 1.6.1 $GABA_A$ Receptor Agonists

GABA is the primary endogenous ligand for the $GABA_AR$. It is a flexible molecule which can adopt a number of low energy conformations allowing it to interact with different GABA receptors, enzymes and transporters (Johnston *et al.*, 1978). Another $GABA_A$ agonist, which is more potent than GABA itself, is muscimol, the naturally occurring isoxazole analogue obtained from the hallucinogenic mushroom *Amanita muscaraia*.

Curtis *et al* (1971) demonstrated that GABA (0.5M) and imidazole acetic (0.5 M) acid were approximately equipotent as depressants of cat spinal cord interneurons when applied ionotophoretically, whereas muscimol (0.5 M) was much more potent. The potency ratio was reflected by the current required to produce equal diminution of neuronal firing (20nA for GABA compared to 1nA for muscimol in one cell and 2nA for GABA comparing to 3 nA imidazole acetic in a second cell. Similar experiments by (Krogsgaard-Larsen *et al.*, 1977) have shown that THIP (4, 5, 6, 7-tetrahydroisoxazolo-[4,5-c] pyridine-3-ol, a conformationally restricted, bicyclic synthetic analogue of muscimol, is more selective for GABA receptors than muscimol or GABA, but is equipotent with GABA and less potent than muscimol in terms of stability to inhibit neuronal activity in cat spinal cord in vivo (Krogsgaard-larsen *et al.*, 1977).

Isoguvacine (1,2,3,6 tetrahydropyridine-4-carboxylic acid), a compound where the isoxazole of THIP has been substituted by a carboxyl group, however, is equipotent with muscimol in its ability to inhibit neuronal activity in the cat spinal cord in vivo (Krogsgaard-larsen *et al.*, 1977) and demonstrate some selectivity for β subunits (Bureau & Olsen, 1990). Studies in rat cerebral cortex slices demonstrate that neither THIP nor isoguvacine (0.5-1mM) affect GABA transaminase activity or GABA uptake (Krogsgaard-larsen *et al.*, 1977). ZAPA (Z-3-[(aminoiminomethyl) thio] prop-2-enoicacid) a conformationally restricted isothiouronium analogue of GABA facilitates the binding of diazepam $EC_{50}$ 0.19 μM for ZAPA and 0.46 μM for GABA (Allan *et al.,* 1986) and displaces the low affinity binding of GABA to rat brain membranes $IC_{50}$ in washed synaptosomal membranes for inhibition of [$^3$H] GABA binding, GABA 70 μM and ZAPA 46μM (Allan *et al.*, 1991). (+)-TACP(+)-trans-(1S,3S)-3aminocyclopentane-1-carboxylic acid), is a stereoisomer of a cyclopentane analogue of GABA (Allan *et al.*, 1979). (+)–TACP is a potent $GABA_A$ agonist, which does not interact with GABA enzymes or transport system. The structures of representative $GABA_A$ receptors agonist are shown in Figure 1.7

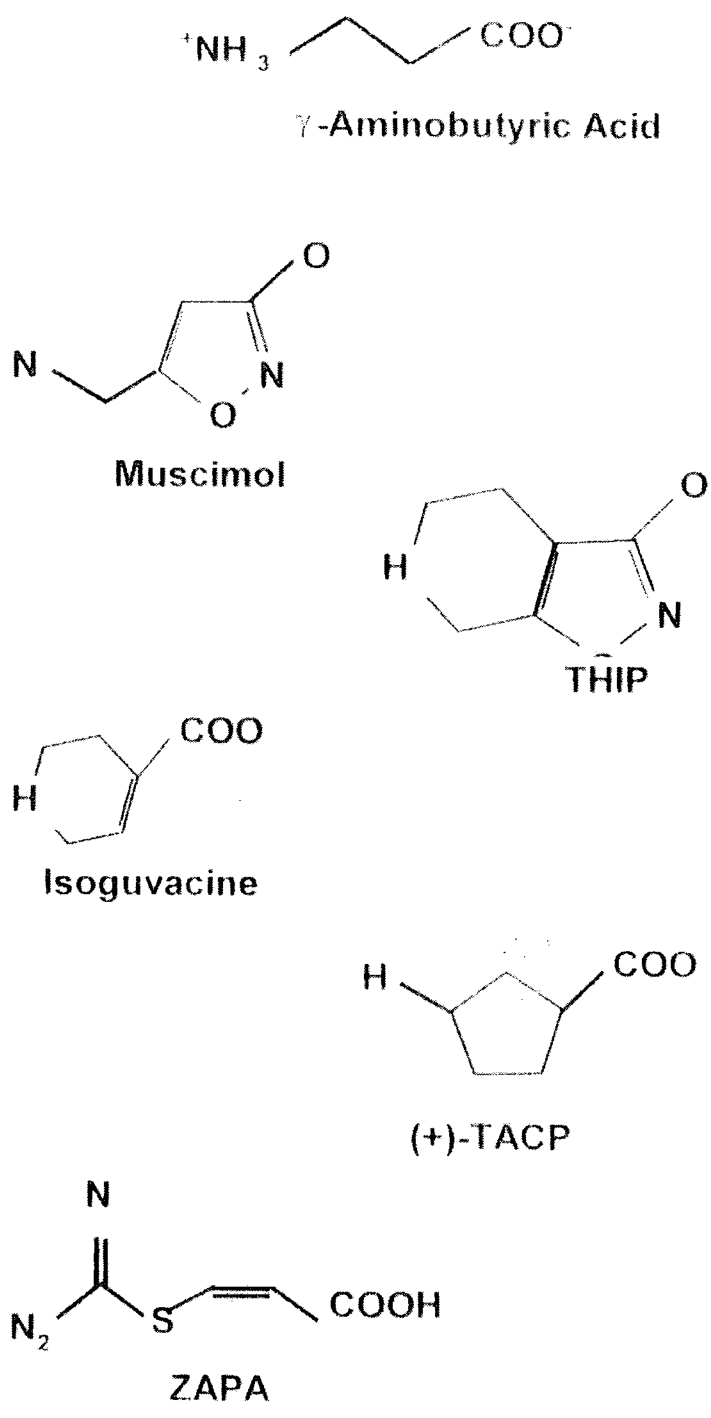
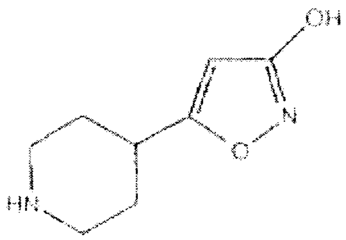


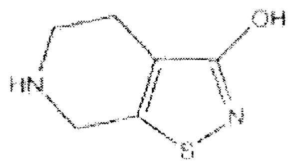

**Figure 1.7** $GABA_A$ receptor agonists

## 1.6. 2 $GABA_A$ Receptor Partial Agonists

A number of partial agonists also exist, for example, 4 PIOL (5-(4-piperidyl) isoxazol-3-ol), thio-THIP, (Krogsgaard-Larsen *et al.*, 1994), piperidine-4-sulphonic acid and other related compounds (Falch *et al.,* 1985) Figure 1.8. 4-PIOL is a "non-fused" THIP analogue which is approximately 200 times less potent than isoguvacine as an agonist, with an $EC_{50}$ 91 µM in whole-cell voltage–clamped hippocampal neurons, and 30 times less potent than bicuculline methochloride as an antagonist (Kristiansen *et al.,* 1991). Thio-THIP appears to be a low-efficacy partial agonist in human brain recombinant receptors expressed in oocytes (Krogsgaard-Larsen *et al.*, 1994), but a full agonist in cat spinal dorsal horn interneurons where it has half the potency of THIP or GABA when these agonists are applied electrophoretically at concentrations of 0.2 M (Krogsgaard-Larsen *et al.*, 1983).




4-PIOL




THIO-THIP

**Figure 1.8:** $GABA_A$ receptor partial agonists

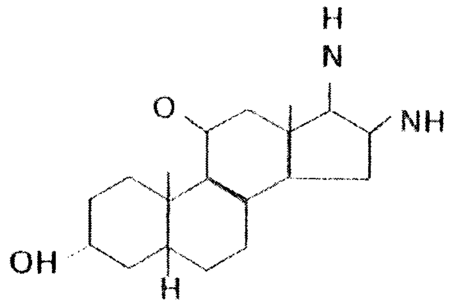
## 1.6.3 $GABA_A$ Receptor Competitive Antagonists

Competitive antagonists of $GABA_AR$ are thought to act at the GABA recognition sites. In 1970, bicuculline, a convulsant compound from the plant *Dicentra cucullaria*, was found to antagonise the inhibitory actions of GABA in cat spinal cells, whereby bicuculline (10mM) was found to considerably reduce the depressant action of elecrophoretically-applied GABA on neuronal excitability (Curtis *et al.*, 1970). Bicuculline is a phthalide isoquinoline alkaloid and structurally similar to the $GABA_A$ receptor agonist muscimol (Andrew & Johnston, 1979). In addition, other convulsant isoquinoline alkaloids, such as (+) hydrastine and corlumine have been associated with antagonism (Curtis, 1974).

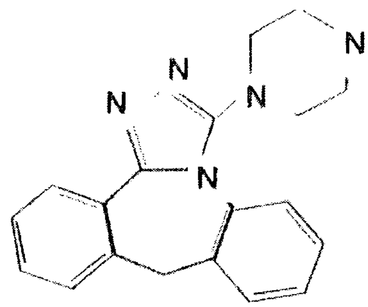
Securinine, from the plant *Securinega suffructicosa,* is a convulant indolizidine alkaloid which is selective $GABA_AR$ antagonist. Securinine induced tonic seizure in mice with a dose four times less potent than that of bicuculline ( 28 ± 3 mg/Kg versus 8 ± 4 mg/kg), but was approximately 7 times less potent than bicuculline in inhibiting $[^3H]$ GABA binding to rat brain membrane (Beutler *et al.*,1985). In the same study, electrophysiological experiments conducted in cat spinal neurons revealed that securinine blocked the inhibitory action of GABA but not glycine. A series of pyridazinyl derivatives of GABA are potent competitive antagonists of $GABA_AR$ (Wermuth *et al.*, 1987). The most widely used is SR95531 (Gabazine), which is selective $GABA_A$ antagonist in the spinal cord in vivo (Gynther & Curtis, 1986). Binding studies using $[^3H]$ GABA and GABA stimulated $[^3H]$ diazepam binding to rat brain membrane indicated that SR95531 is a competitive inhibitor of high affinity GABA binding sites and a non-competitive inhibitor of low affinity binding sites (Heaulme *et al.,* 1986). This indicates a difference between SR95531 and bicuculline in their relative potencies for high and low affinity $GABA_A$ binding sites with SR95531 were being more potent at high affinity sites and bicuculline being more potent at low affinity sites (Johnston, 1991).

RU135 (3-α-hydorxy-16-imino-5β-17-aza-androstan-11-one) is an aminidine steroidal compound which is the most potent competitive antagonist of $GABA_A$ receptors described. It is 500 times more potent than bicuculline in inhibiting GABA enhancement of diazepam binding (Hunt & Clements-Jewery, 1981).

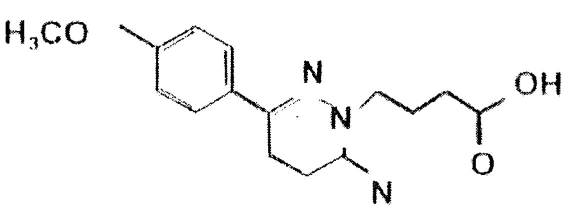
Pitrazepin ( 3-( piperazinyl-1)-9H-dibenz(c,f) triazolo (4,5-a)azepine) ( and a number of other N-aryl piperazines ) is known to be potent, but not selective in $GABA_AR$ antagonist (Braestrup & Nielsen, 1985), 3-10 times more potent than bicuculline, depending on the test preparation ( Johnston, 1991). Pitrazepine however, is not specific for $GABA_A$ receptor since it inhibits the binding of the glycine antagonist, strychnine, at the same concentration as it inhibits $GABA_A$ receptors (Braestrup & Nielsen, 1985). Other competitive $GABA_A$ antagonists include (+) –tubocurarine, which apart from being an acetylcholine nicotinic antagonist, also weakly antagonised cortical $GABA_A$ and glycine receptors (Hill *et al.*, 1972). The structures of competitive $GABA_A$ antagonist are shown in Figure 1.9.



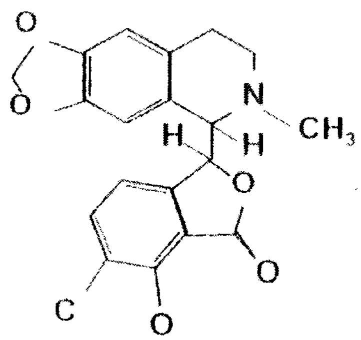
RU5135



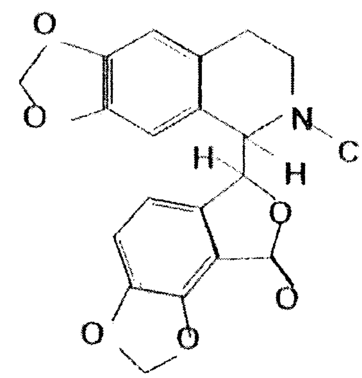
Pitrazepine



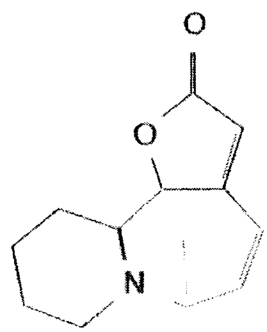
SR95531



(+) Hydrastine



Bicuculline



Securinine

**Figure 1.9** Competitive antagonists of $GABA_A$ receptors

## 1.6.4 $GABA_A$ Receptor Non-competitive Antagonists

A wide range of compounds antagonise $GABA_A$ receptors in a non-competitive manner. The structures of representative non-competitive $GABA_A$ antagonists are shown in Figure 1-10. Of major interest are the so called (cage) convulsants, such as picrotoxin and TBPS, which act at sites closely, associated with the chloride ion channel of $GABA_A$ receptors. Their antagonist action is directed towards the $GABA_A$ activated channel chloride rather than the GABA recognisition site on $GABA_AR$ complexes. Picrotoxin obtained from the poisonous plant *Anamirta cocculus,* is an equimolar mixture of a potent convulsant, picrotoxinin and less potent convulsant, picrotin (Curtis, 1974). Picrotoxin has been reported to antagonise the neuronal effects of 5-HT (Mayer & Straughan, 1981) and glycine (Curtis *et al.,* 1969). Picrotoxin does not inhibit the binding of GABA agonist or benzodiazepine to $GABA_AR$. Picrotoxin binding sites identified with [$^3$H]-dihydropicrotoxinin (DHP) or preferably with [$^{35}$S] TBPS, which gives a better signal to noise ratio than [$^3$H] DHP, are closely associated with the chloride channel of $GABA_A$ receptor complexes. $GABA_A$ agonist and positive modulators, such as barbiturates, benzodiazepines and steroids, allosterically inhibiting TBPS binding by reducing its affinity. Some $GABA_AR$ negative modulators such as convulsant β-carboline and γ-butyrolactone, enhance TBPS binding affinity, suggesting that high TBPS binding might be associated with a (closed) confirmation of the chloride channel (Gee, 1988, Sieghart, 1992). A very wide range of compounds seems to bind to sites that influence picrotoxinin binding sites that are clearly central to the activation of $GABA_A$ receptors (Kerr & Ong, 1992).

Furosemide, is a ($Cl^-$) transport blocker used as a diuretic. Furosemide (0.1-1mM) antagonised muscimol-evoked response in rat cuneate nucleus slices in a non-competitive manner. In addition, furosemide antagonises recombinant $GABA_AR$ expressed in oocytes in a subunit-selective manner. Electrophysiological experiments have shown that furosemide potently antagonise α6β2γ2S ($IC_{50}$≈10μM), but not α1β2γ2S containing receptor ($IC_{50}$>3mM) (Korpi *et al.*, 1995). Binding studies also indicated that furosemide was selective for β2/3 γ2-containing receptors and was ineffective at α1/6β1γ2S- containing receptors (Korpi *et al.*, 1995).

$Zn^{2+}$ (50-300μM) has also been shown to inhibit GABA-evoked responses in rat neurons (Smart & Constanti, 1990, Smart, 1992). Patch clamping studies of embryonic and adult sympathetic neurons performed by Smart, 1992 revealed that antagonism of GABA evoked currents by $Zn^{2+}$ was subject to developmental influence, whereby embryonic neurons were much more sensitive to inhibition than adult neurons. This study demonstrated that $Zn^{2+}$ did not affect the main single channel conductance mean open and shunt time, but rather reduced the opening frequency of the GABA-gated ($Cl^-$)

channel. Moreover, recombinant studies have shown that only hetero-oligomer recombinant $GABA_A$ receptors, devoid of a γ-subunit are sensitive to $Zn^{2+}$ inhibition (Draguhn *et al.*, 1990, Smart *et al.*, 1991; Hosie *et al.*, 2003).

Other non-competitive $GABA_AR$ antagonists or more correctly, negative allosteric modulators (for review see Johnston, 1996), include convulsant β-carboline pentylenetetrazole, quinolone antibiotic together with NSAIDs.

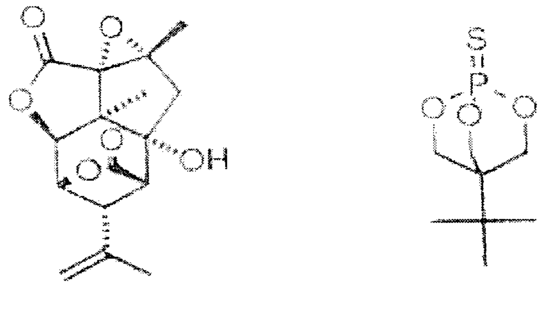



**Figure 1.10:** Non-competitive antagonists of $GABA_A$ receptors

## 1.6.5 GABA Transporters & Reuptake Inhibitors

An alternative approach to increasing GABA receptor activation is to inhibit GABA transport process (Iversen *et al.*, 1967, 1968). Four subtypes of cell surface transporters for GABA, GAT (1–4), have been identified (Schousboe *et al.*, 2004). GAT-1 and GAT-4 have the highest expression and are widely distributed in the mammalian CNS, being located predominantly on GABA nerve terminals. GAT-2 is present primarily on glia and GAT-3 is densely expressed on leptomeninges and the choroid plexus. Thus, GAT-1 and GAT-4 are thought to play a major role in regulating GABAergic neurotransmission. The potent inhibition of GAT-4 by zinc has led to the proposal that co-release of this divalent cation with glutamate from glutamatergic nerve endings (e.g. CA3 region of the hippocampus) serves to increase adjacent GABA levels to control excessive glutamate-mediated excitation of neurons (Cohen-Kfir *et al.*, 2005). A single vesicular transporter for GABA (VGAT) also provides the same function to package glycine in glycinergic neurons. Indeed, some neurons have been demonstrated to release both GABA and glycine from the same vesicles (Foster *et al.*, 2006). Superficially, inhibition of GABA transport would be expected to produce similar therapeutic effects to those of the benzodiazepines, as the end result is enhancement of the effects of endogenous GABA following its release. Tiagabine is a selective GAT-

1 inhibitor that is approved for use in epilepsy and is being tested in Phase II clinical trials for anxiety, neuropathic pain and insomnia (Bowery & Smart 2006). It is clear, however, that the side effect profile of Tiagabine is less favourable than for the $GABA_A$ positive allosteric modulators and includes tremor, ataxia, dizziness and somnolence, features also seen in the GAT-1 null mouse ( Chiu *et al.*, 2005), suggesting that these are directly linked to transporter inhibition. Compounds with some selectivity towards GAT-3 and -4 (SNAP-5114) and broad-spectrum inhibitors (e.g. NNC 05-2045) also display anticonvulsant activities, but are weaker than Tiagabine and correspondingly less potent at elevating extracellular GABA (Dalby, 2000, Foster *et al.*, 2006). The enzyme aminotransferase (GABA-T) is the only molecular component of GABA synthesis/degredation to be targeted. Vigabatrin (i.e γ-Vinyl GABA) is an inhibitor of GABA-T that leads to elevations in extracellular GABA levels and is used clinically as anticonvulsant (Foster *et al.*, 2006).

# 1.7 $GABA_A$ Receptors Allosteric Modulators

A large number of pharmacologically and clinically important drugs e.g. benzodiazepines, general anaesthetic agents, anticonvulsant and ethanol exert their effect mainly or exclusively via interaction with $GABA_A$ receptors (Sieghart, 1995, Stephenson, 1995). In addition, a number of other substances have been shown to interact with $GABA_A$ receptors e.g. loreclezole, steroids, avermectin, furosemide, zinc, picrotoxin and lanthanum ($La^{3+}$). Binding studies, electrophysiological and behavioural experiments have shown that these compounds allosterically interact with the $GABA_A$ receptor via a number of binding sites (Barnard *et al.*, 1998, Johnston, 2005). Over the last 25 years a significant efforts has focused on examining receptor subtype selectivity for a number of agents and identifying the amino acid residue that form the binding site or are involved in the transduction mechanism of these ligand to better understand their mechanism of action. The properties of a number of these allosteric sites are outlined below:

## 1.7.1 GABA Binding Site:

GABA, the endogenous ligand for $GABA_A$ receptors, causes inhibition of post-synaptic action potentials by binding to specific interaction sites on $GABA_AR$. There are two low affinity binding sites for GABA located at the interphase between the α and β subunits. These sites are activated by μM concentrations of GABA and are important for channel gating. Studies with rat and human recombinant $GABA_AR$ have shown that the identity of the α subunit influences the apparent GABA affinity. Receptors containing the α3 subunit are generally the least sensitive with $EC_{50}$ values ranging 11-487μM (Sigel *et al.*, 1990, Ebert *et al.*, 1994, Smith *et al.*, 2001). Whereas receptors containing the α6 subunit are generally the most sensitive with $EC_{50}$ values ranging between 0.2-1.5 μM (Korpi & Lüddens, 1993, Korpi *et al.*, 1995, Ebert *et al.*, 1997). Studies using photoaffinity labelling, site–directed mutagenesis, or the substituted cysteine accessibility method (SCAM) have been used to locate residues involved in the GABA binding domain on α1 and β2 subunits (Amin & Wiess, 1993, Smith & Olsen, 1994, Boileau *et al.*, 1999, Wagner & Czaikowski, 2001). The two homologous domains of the β subunit, Y157/T160 and T202/Y205, were found to be involved in GABA binding (Smith & Olsen, 1994). The distance between the Y157/T160 and T202/Y205 binding domain suggested that a loop structure may be necessary to bring these domains together. The α1 subunit residue F64, R66 and S68 (Boileau *et al.,* 1999) and the β2 subunit residues S204,Y205,R207 and S209 (Wagner & Czajkowski, 2001) were all shown to line the GABA binding pocket, while β2 F200, S201,T202 and G203 do not line the pocket , but affect the affinity of GABA binding. The high affinity binding sites for GABA are thought to form from conformational variants of the low affinity binding sites (Baur & Sigel, 2003) and involved in the stabilisation state of $GABA_AR$ (Newell & Dunn, 2002). δ subunit-containing $GABA_AR$ subtypes have a higher affinity for GABA than those containing the γ subunit. They do not show desensitisation on prolong activation (Nusser *et al.,* 1998). This is consistent with their role in tonic inhibition. These receptors are found mainly in cerebellar granule cells on extra-synaptic somatic and dendritic membranes, where GABA is found at low, but constant, concentrations. γ-Subunit containing receptors have a lower sensitivity to GABA than δ-subunit containing receptors and are desensitised on prolonged activation, therefore they are more suited to phasic inhibition (Nusser *et al.*, 1998) these receptors are mainly localized to synaptic sites where GABA is found at higher concentration but for a short period of time.

## 1.7.2 Benzodiazepine Binding Site:

Benzodiazepines, whose actions include sedation, anxiolysis, anticonvulsant, hypnosis and muscle relaxation, have been in clinical practice for 40 years. Benzodiazepines enhance the actions of GABA at the $GABA_AR$ by increasing the frequency of ($Cl^-$) channel opening. The binding site for the benzodiazepine is found at the α-γ interface. It is the γ subunit that confers benzodiazepine sensitivity to the receptor, while the particular effect of a benzodiazepine are modulated by the α subunit subtype associated with the $GABA_AR$. There are 3 γ subtypes. The γ2 and γ3 subunits confer benzodiazepine sensitivity to fully assembled αβγ receptors. γ2 subunits are widespread throughout the brain and mediate most of the effects of benzodiazepine, while γ3 is only weakly distributed throughout the brain (Pirker *et al.*, 2000). The γ1 subunit is widely distributed throughout the brain, but confers benzodiazepine sensitivity 10 times weaker than that of the γ2 subunit (Ymer *et al.*, 1990). The α subunit variant receptor subtypes α1βγ2, α2βγ2, α3βγ2 and α5βγ2 are benzodiazepine-sensitive. The α1 subunit confers benzodiazepine type I pharmacology in that it displays a high affinity for the non-benzodiazepine agonist, CL 218 872, 2-oxoquazepam and the inverse agonist, methyl-β-carboline-3-carboxylate (β-CCM)) (Pritchett *et al.*, 1989). α Variant receptor subtypes α2βγ2, α3βγ2 and α5βγ2 are relatively insensitive to these compounds, i.e. display benzodiazepine type II pharmacology. These subunits potentially mediate the anxiolytic effects of benzodiazepines. In particular, α5 is thought to be involved in regulating the amnesic effects as it is found mainly in hippocampus, as detected by radioligand binding (Sur *et al.,* 1999). Knockout mice for the α5 gene have been shown to possess an enhanced ability to remember the location of a hidden platform in a water maze test (Collinson *et al.,* 2000). The α4βγ2 and α6βγ2 receptor subtypes are benzodiazepine insensitive. Studies have been carried out to identify amino acids that are important for the binding of benzodiazepines. Site-directed mutagenesis of amino acid in the γ2 subunit have implicated M57, M58, M130 (Kucken *et al.*, 2000, Buhr & Sigel, 1997) F77, A79 and T81 (Teissere & Czajkowski, 2001) as components of the benzodiazepine binding pocket.

### 1.7.3 Channel Binding Site (Picrotoxinin / TBPS site):

Picrotoxin and some bicyclic cage compounds are convulsants which antagonize GABA-induced ($Cl^-$) conductance responses. These compounds, however, did not inhibit GABA receptor binding and did not displace benzodiazepines from their high affinity binding sites (Olsen,1982). Binding sites identified by [$^3$H]α–dihydropicrotoxinin(DHP)or the cage convulsant [$^{35}$S] t-butylbicyclophosphorothinate (TBPS) which exhibit a better signal-to-noise ration rather than [$^3$H] DHP, seem to be closely associated with the ($Cl^-$) ion channel of $GABA_AR$ ( Squires *et al.,* 1983). Convulsant compounds that bind to the DHP/TBPS site seem to reduce directly ($Cl^-$) conductance by sterically hindering the entry of ($Cl^-$) across the ion channel. GABA and compounds which mimic or facilitate the effect of the GABA receptor (e.g. benzodiazepines, barbiturates, steroids) allosterically inhibited [$^{35}$S] TBPS binding by reducing its binding affinity. Compounds reducing the efficacy of GABA at $GABA_A$ receptors, such as some convulsant α-carboline enhanced [$^{35}$S] TBPS binding affinity through specific interactions with the benzodiazepine receptors. Thus, the high affinity

TBPS binding might be associated with the "closed" conformation of the ($Cl^-$) ion channel (Gee, 1988).

## 1.7.4 Other Binding Sites.

A number of other compounds have been shown to have modulatory effect on $GABA_AR$, these include:

### 1.7.4.1 Barbiturates

The allosteric modulatory site by which barbiturates affect the function of $GABA_A$ receptor is less well defined, barbiturate potentiation is seen on recombinant receptors even when γ subunit , which is a prerequisite for the presence of the benzodiazepine site, is absent, and it is found even in homomeric receptors containing exclusively α1 or β1 subunit (Pritchett *et al.*, 1988). Barbiturates such as pentobarbital or secobarbital enhance GABA responses and mimic GABA by opening the integral ion channel in the absence of GABA (Macdonald & Olsen, 1994). They increase the average open duration of $GABA_AR$ single channel currents without altering channel conductance (Barker *et al.,* 1979, Study *et al.*, 1981). It is thought that they alter the intrinsic gating of the channel once GABA is bound, so increasing the proportion of channel opening of longer duration (Macdonald *et al.*, 1989).

### 1.7.4.2 Steroids

Various synthetic and natural steroids (e.g., alphaxolone, androsterone, pregnanolone) act as allosteric modulators of $GABA_AR$ (Harrison & Simmonds, 1984, Lambert *et al.*, 1990, 1995). GABA potentiation by steroids is found in either homomeric (β1) or heteromeric (α1β1, α1β1γ2) recombinant receptors (Puia *et al.*, 1990; Zaman *et al.,* 1992), where the type of α subunit influences the degree of potentiation (Lan *et al.*, 1991, Shingai *et al.*, 1991). The steroid site, which is likely to be different from the barbiturate site, may be of particular physiological significance, since the brain is capable of synthesizing steroids that affect $GABA_AR$ function (neurosteroids) (Baulieu & Robel, 1990).

### 1.7.4.3 General Anaesthetic

A large number of structurally diverse agents such as isoflurane, enflurane, barbiturates, etomidate, propofol and steroid anaesthetics have been reported to interact with the GABA receptors (reviewed in Franks, 2006, Franks& Lieb, 1998; Krasowski & Harrison, 1999, Thompson & Wafford, 2001, Yamakura *et al.*, 2000). At therapeutic concentrations this interaction is primarily potentiation of the GABA response. It must be noted however that many of these agents, at therapeutic concentrations, also modulate other ligand gated ion channel such as nACh, glycine, AMPA and kainate receptors.

#### 1.7.4.4 Ethanol

Ethanol and other alcohols have been shown to have a potentiation effect on $GABA_A$ receptors (Deitrich *et al.*, 1989). For example ethanol enhances the response of cultured spinal cord neurons to GABA (Celentano *et al.*, 1988). The effects of ethanol at low concentrations were thought to be dependent on phosphorylation of a specific residue on an intracellular domain of the $GABA_AR$ (Wafford *et al.*, 1991, 1992). More recently it has been shown that targeted deletion of this region has no effect on the ethanol response (Homanics *et al.*, 1999) while sites within the transmembrane domain of the receptor have been shown to be important for modulation seen at higher concentration of ethanol (Mihic *et al.*, 1997).

#### 1.7.4.5 Loreclezole

Loreclezole is an effective anticonvulsant agent which has been shown to potentiate the opening of GABA gated chloride channel in cultured rat cortical neurons and xenopus oocyte expressing recombinant $GABA_AR$ (Wafford *et al.*, 1994). Unlike benzodiazepines, whose affinity or efficacy is not influenced by the type of β subunit (Hadingham *et al.*, 1993) loreclezole displayed clear selectivity for β2/3 containing receptor over β1. This selectivity was subsequently identified to be due to the presence of an Asn residue at position 265 of β2/3 of TM2 (Wingrove *et al.*, 1994), however it has not been demonstrated if this amino acid forms part of the binding site for loreclezole.

#### 1.7.4.6 Zinc

The divalent cation zinc is a non-competitive antagonist of $GABA_AR$ whose sensitivity depends on the receptor subunit composition (Draguhn *et al.*, 1990, Smart *et al.*, 1991, Chang *et al.*, 1995). Heat inactivation studies (Squires *et al.*, 1982) and radioligand binding studies (Mackerer *et al.*, 1978) suggested the presence of a $Zn^{2+}$ binding site at some, but not all $GABA_AR$ subtypes. This site seems to be localized extracellularly and to be distinct from the GABA, the benzodiazepine, barbiturate, picrotoxin and steroids site (Celentano *et al.*, 1991).

#### 1.7.4.7 Negative Allosteric Modulators of $GABA_A$ Receptor

A number of negative allosteric modulators also exist for the $GABA_AR$ including β-carboline, DMCM, Diazepam-binding inhibitor and β-substituted γ-butyrolactone (Johnston, 1996).

# 1.8 Allosteric Modulation of Ligand–Gated Ion Channels

Binding of the endogenous ligand (neurotransmitter) to an LGIC causes opening of the channel. At least two models have been proposed to describe this. The induced fit model (Koshland *et al.,* 1966), predicts that the binding of an agonist molecule to the receptor protein induces a conformational change in the region of the ligand-binding site, which propagates to the pore region and causes opening of the ion-conducting pore. The second, allosteric model, predicts that the receptor protein constantly undergoes spontaneous changes between distinct conformational states. These transitions between states have different "energy barriers". Each of these conformational states has a different affinity for the ligand and the binding of a ligand to the LGIC preferentially stabilizes the receptor in a given state. Typically, the binding of an agonist molecule stabilizes the channel in the open state. This model was first proposed by Monod et al. to explain the observed behavior of proteins like haemoglobin (Monod *et al.*, 1965), and was extended to LGICs by Karlin, who proposed that it could describe the functioning of nAChRs ( Karlin, 1967). The high density of nAChRs found in the electric organ of the Electrophorus electricus (eel), and the discovery of the selective antagonist α-bungarotoxin, resulted in these receptors being extensively studied and becoming the basis for the first models to describe LGIC function. The binding site for the natural ligand, that activates the receptor, is known as the orthosteric-binding site. In addition the binding of ligands at other sites on the receptor surface can modify the functioning of the receptor. This concept of modulation of LGIC activity by the binding of a second ligand, or allosteric modulator, was introduced by Karlin (Karlin, 1967) and termed, allosteric modulation. The binding site for an allosteric modulator is an allosteric-binding site; each receptor may have several allosteric-binding sites which are selective for different ligands. An elegant illustration of the conformational changes affecting a LGIC at rest, following agonist binding or in the presence of an allosteric modulator has been recently illustrated, using electron microscopy, for AMPA receptors (Nakagawa *et al.,* 2005). Importantly the allosteric concept introduces another determining notion that is: binding of a molecule at any location on the protein complex can affect the stability of the complex and/or the energy barrier(s) for conformational changes. This is the principle of allosteric modulation. Typically, molecules that cause allosteric modulation are termed allosteric effectors and are divided in two classes. **Positive allosteric effectors** enhance the agonist-induced response whereas **negative allosteric effectors** reduce receptor function. In addition to modulating LGICs allosteric modulation can affect the function of a wide range of cellular proteins including enzymes and metabotropic membrane receptors (Soudijn *et al.*, 2002, Christopoulos *et al.*, 2002).

## 1.9 Importance of Allosteric Modulators asTherapeutics

Searches of current literature and patents reveal that several pharmaceutical companies are developing positive and negative allosteric effectors for different LGIC families. In such a quest, we shall keep in mind that screening strategies will determine, right from the beginning, the type of allosteric modulators that could be identified. Namely, protocols that use short-term agonist exposure will help to characterize non-competitive antagonists and/or positive allosteric modulators of resting and open states. While protocols based on long-term agonist exposure could lead to the identification of allosteric modulators of some desensitized states. Typical examples of allosteric effectors include negative allosteric effectors of the NMDA receptors such as ifenprodil or compounds which act similarly (Rachline *et al.*, 2005, Perin-Dureau *et al.,* 2002). The therapeutic target is to reduce the glutamate cytotoxicity observed following cerebrovascular injuries. By contrast with competitive antagonists that, at saturating concentrations, could totally inhibit LGIC functions, negative allosteric modulators are fine-tuning tools that could have a neutral behaviour in normal physiological conditions but that could be very active in pathophysiological situations, without leading to complete receptor inhibition. But why should allosteric effectors be more suitable as drugs for blocking LGICs than competitive or non-competitive inhibitors? A first distinction between these classes of molecules is that a larger repertoire of allosteric effectors is expected than for the other inhibitors. The reason for this difference is that the binding of allosteric effectors is not restricted to the ligand-binding site or the ion-conducting pore, as in the case of competitive or open channel blockers. This advantage should allow the design of compounds that are more specifically targeted to particular receptor subtypes. In addition, it should be remembered that binding of a negative allosteric effector which affects the isomerization coefficient causes both a reduction of the receptor agonist sensitivity and activity. Positive outcomes of these advantages have already been observed and use of negative allosteric effectors of neuronal nicotinic receptors have been proposed for smoking cessation while negative effectors acting at the $GABA_A$ or $5HT_3$ receptors have been proposed for reduction of alcohol dependence. Wide spectrum of actions ranging from pain, to epilepsy to schizophrenia, etc., has been proposed for neurosteroids that increase or reduce $GABA_AR$ activity (Hogg *et al.*, 2005).

Positive allosteric effectors also show promise as therapeutically compounds. While benzodiazepines and their broad clinical use have already paved the way for positive allosteric effectors, newcomers include neuronal nicotinic acetylcholine modulators such as galanthamine. The positive clinical outcomes reported in the treatment of neurodegenerative cholinergic diseases such as Alzheimer's is attributed, at least in part, to the allosteric effects of galanthamine. (Albuquerque *et al.*, 2001, Maelicke *et al.*, 2000). Alternatively, talampanel (a negative allosteric modulator of AMPA receptors) was developed to treat some epilepsies but is also being assessed for protection of brain injuries and therapeutics for Parkinson's disease. In contrast, positive allosteric effectors of the AMPA such as Org 24448 have been proposed to treat schizophrenia (Quirk *et al.*, 2003, Hogg *et al.*, 2005). In view of the very broad range of allosteric effector applications an important future can be seen for these types of molecules that should provide additional benefits to the already know spectrum of compounds that are targeting LGICs.

Orthostatic agonists provoke sustained activation of the receptors even in the absence of a physiological activity of the corresponding neuronal network. For calcium permeable channels, such as NMDA or the nicotinic α7 receptors such activation can result in cytotoxic effects. In addition, sustained exposure can cause receptor desensitization that may result in the opposite of the desired effects. Thus, administration of orthosteric agonists needs very precise control of dosage and pharmacokinetic monitoring. Use of orthosteric antagonists can lead, as a function of the drug concentration, to an insurmountable blockade of the receptors and therefore complete inhibition of the physiological response. Use of open channel blockers is associated with a use dependent effect that can also be insurmountable. Moreover, open channel blockers have a poor selectivity and are difficult to target to a precise receptor subtype. In contrast, the effects of an allosteric effector are limited by the nature of the receptor modulation. Once all the effector-binding sites are saturated the receptor is maximally modulated and presence of a higher concentration of the modulator will not result in further effects. This ceiling effect has important advantages because it offers a much larger safety margin in drug administration and patient compliance. Overall, allosteric modulators offer several advantages over classic orthosteric compounds or open channel blockers and can be expected to have a bright future in drug discovery.

# 1.10 $GABA_A$-based Therapeutic Approaches

GABA pharmacology has already yielded many important drugs that are widely used in the treatment of anxiety and panic disorders, epilepsy, muscle spasticity, sleep disorders and as anaesthetics. There is every hope that the new understanding of the molecular pharmacology of GABAergic transmission will lead to a new generation of more selective drugs with improved safety profiles and entirely new indications will be discovered. Future possibilities in the development of drugs acting on $GABA_A$ receptor hold great interest; there is an urgent need for therapeutic improvement over existing therapies and drugs for currently untreatable diseases. A large number of compounds based on three therapeutic strategies are currently in clinical trials for diseases that span a wide range of CNS disorders these strategies are:

1. **Positive Allosteric Modulator of $GABA_AR$.**
2. **Subtype Selective $GABA_AR$ Modulators.**
3. **Naturally Occurring Alternatives.**

A summary of the principles of these strategies will be discussed below:

## 1.10.1 Positive Allosteric Modulator of $GABA_A$ receptor

Many allosteric-binding sites have been identified on the $GABA_AR$ highlighting one of the important advantages of the search for allosteric effectors as compared to conventional agonists and antagonists (Whiting *et al.*, 2006, Rudolph & Möhler, 2006). This multiplicity of potential-binding sites greatly increases the probability of finding a molecule that is selective in activity at a particular site of the receptor, however, the binding of an allosteric modulator to diverse sites on the receptor can be difficult to detect if using screening techniques which detect changes in the binding of labelled ligands (Hogg *et al.*, 2005, Olsen *et al.*, 2004). The increasing availability of high throughput screening on receptor function, such as FLIPR, site-directed mutagenesis, photoaffinity labelling and cysteine scanning allows us to map binding sites at the amino acid level at any location on the receptor (Smith *et al.*, 2003, Hogg *et al.*, 2005). These studies illustrate that a specific protein residue must be present for each allosteric effector to bind and exert its action (Hogg *et al.*, 2005). For example these studies have elegantly shown that benzodiazepines bind at the interface between the α1 and γ2 subunits in the receptor complex and thereby explain why only receptors containing the γ subunit are modulated by this class of allosteric effector. Binding of different ligands to this site can either potentiate the $GABA_A$ receptors or block the benzodiazepine effect (e.g.flumazenil = Ro 15-1788 is an antagonist of the benzodiazepine-binding site) (Sigel *et al.*, 1997, Berezhnoy *et al.*, 2005). A combination of mutagenesis and photoaffinity labelling fishing for allosteric sites on $GABA_A$ receptors, suggests that anaesthetic modulators of $GABA_AR$ bind directly to the protein and that certain domains are most likely points to contact. These include, firstly the ion channel TM2, especially the extra-cellular portion, secondly the agonist binding sites and homologous pockets at other subunit interfaces of the pentameric receptor, the third site is on the linker region stretching from the agonist loop C to the top of the TM1 region ( Olsen *et al.*, 2004). The continuing evolution of novel technologies and assay approaches with appropriate sensitivity and resolution to measure subtle modulation of GABA (A) ion channels will facilitate ongoing investigation of the physiological functions and mechanisms of these positive allosteric modulators (Smith *et al.,* 2003, Kardos, 1999).

### 1.10.2 Subtype Selective $GABA_A$ Receptor Modulators

Historically, the $GABA_AR$ has been the target of many drug treatments. The earliest compounds were ions like bromide, followed by barbiturates and finally, from 1960s onwards, a number of benzodiazepines. The benzodiazepines were considered, at the time of their introduction, as very efficient and safe "minor tranquillizers", but more recently, their use has been criticized because of the dependence-producing effects. This concerns the prolonged use of especially the long-acting anxiolytic compounds rather than the short-acting sedative and sleep-inducing ones. This is also accompanied by clear tolerance development that has limited their use, for example, in epilepsy (Korpi *et al.*, 2006). However, several efficient benzodiazepines are in use, and a clinician can select a benzodiazepine agonist in relation to its length of action, dosage form, metabolic interactions and other drug safety features. Pharmacologists do not see any strong need to develop further standard non-selective benzodiazepine-site agonist drugs, since there is a good selection of effective compounds for insomnia, anxiety and sedation. In addition, there is a selection of α1 subunit-preferring hypnotics (Zolpidem, Zopiclone and Zaleplon), and this category might not need any more members for the clinical use (Korpi *et al.*, 2006, Whiting *et al.*, 2006, Rudolph & Mohler, 2006).

With the improved knowledge of the subtypes of $GABA_A$ receptors and their putative functions, subtype selective positive allosteric modulators could provide equivalent efficacy to the older benzodiazepines with fewer side effects. $GABA_AR$ subtypes, through their specific regional, cellular and subcellular localization, are linked to distinct neuronal circuits and consequently serve distinct functions, $GABA_AR$ subtype-selective drugs are therefore expected to provide novel pharmacological profiles (Korpi *et al.*, 2006, Whiting *et al.*, 2006, Rudolph & Mohler, 2006, Dawson *et al.,* 2005). Receptors containing the $\alpha_1$ subunit mediate sedation and serve as targets for sedative hypnotics.

Agonists selective for α2- and/or α3-containing $GABA_A$ receptors have been shown to provide anxiolysis without sedation in preclinical models, whereas inverse agonists selective for α5-containing $GABA_A$ receptors provide memory enhancement. Agonists selective for α3-containing $GABA_A$ receptors may be suitable for the treatment of deficits in sensorimotor processing in psychiatric disorders. (Korpi *et al.*, 2006, Whiting *et al.*, 2006, Rudolph & Mohler, 2006, Sieghart *et al.*, 2002, Johnston, 2005). There are two approaches for developing a receptor subtype-selective modulator Figure 1-11.

The most obvious approach is to develop a compound with binding selectivity-that is, with higher affinity for one receptor subtype than for another. For GABA receptors, the clear example is zolpidem, which has higher affinity for α1 subunit-containing receptors. The alternative approach is to develop compounds with efficacy selectivity-that is, compounds which might bind with equal affinity to several receptor subtypes but will selectively modulate the activity of one or some of them. Given this approach, the potential opportunities to develop compounds with different efficacy profiles are, in theory, significant (Whiting *et al.*, 2006). Thus, at one extreme, one could consider developing a compound with absolute efficacy selectivity, potentiating the activity at only a single $GABA_AR$ subtype and having no efficacy at any of the others. At the other extreme, in theory, one could develop a compound with a predefined spectrum of efficacies at the different receptor subtypes (e.g. an agent that is a full agonist at α1βxγ2, a weak partial agonist at α2βxγ2, a strong partial agonist at α3βxγ2 and a silent antagonist at α5βxγ2 receptor subtypes).

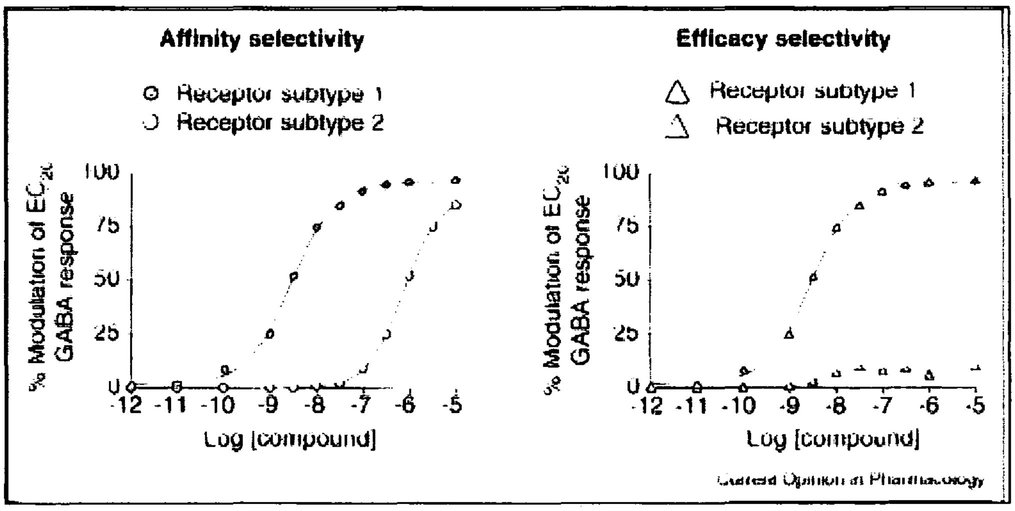



**Figure 1.11:** Achieving $GABA_AR$ subtype selectivity through selective affinity or selective efficacy

(Whiting, 2006)

In the graph on the left, the hypothetical $GABA_A$ modulator has greater than 200-fold higher affinity for receptor subtype 1, compared with receptor subtype 2, although at sufficiently high concentrations it reaches full potentiation at both subtypes. In the graph on the right, the hypothetical $GABA_A$ modulator has approximately the same affinity ($EC_{50}$) at both receptor subtypes but exhibits subtype-selective efficacy, exhibiting full agonism at subtype 1 but minimal efficacy at subtype 2.

Both genetic and medicinal chemistry approaches have been used to identify the pharmacological relevance of $GABA_AR$ subtypes. The genetic approach involved rendering individual $GABA_AR$ subtypes insensitive to the benzodiazepine diazepam by introducing a point mutation (Rudolph & Mohler, 2004). The respective behavioural deficit was attributed to the respective $GABA_AR$ subtype. Studies with point-mutated mice have revealed that the sedative action of diazepam is mediated by α1-containing $GABA_A$ receptors (Rudolph *et al.*, 1999, Mckernan *et al.*, 2000), whereas the anxiolytic-like action is mediated by α2-containing $GABA_A$ receptors (Low *et al.*, 2000). Studies on mice lacking, either partially or completely, the α5 subunit have revealed a role for α5-containing $GABA_A$ receptors in trace fear conditioning and in water maze learning with improvements in performance (Crestani *et al*, 2002, Collinson *et al*, 2002). In the medicinal chemistry approach, ligands with selective affinity or efficacy for particular $GABA_AR$ subtypes were developed. Ligands with selective efficacy for α2- and/or α3-containing $GABA_A$ receptors were found to display anxiolytic activity (Atack *et al.*, 2005), whereas partial inverse agonists acting at α5-containing receptors enhanced memory performance (Sternfeld *et al.*, 2004, Chambers *et al.*, 2004). The medicinal chemistry approach largely supported the results from the genetic studies (Rudolph & Mohler, 2006). Recent neurobiological studies on new animal models and receptor subunit mutations have revealed novel aspects of the $GABA_A$ receptors, which might allow selective targeting of the drug action in receptor subtype-selective fashion, either on the synaptic or extra-synaptic receptor populations. In table1.1, there is a brief overview of some of the compounds presently under development for the $GABA_A$ system, their interaction with the receptor subunit and psychiatric disease associations.

**Table 1.1:** $GABA_A$ receptor subtype ligands (Rudolph & Mohler, 2006)

| Drug | Main activity | Interaction with recombinant $GABA_A$ receptors | References |
|---|---|---|---|
| **Benzodiazepine site ligands** | | | |
| Zolpidem<br>Zaleplone | Hypnotics | Preferential affinity for $\alpha1$ | (Dämgen *et al.*,1999) |
| Indiplon | Hypnotic | Preferential affinity for $\alpha1$ | (Foster *et al.*,2004) |
| L-838 417 | Anxiolytic | Comparable affinity at $\alpha1$, $\alpha2$, $\alpha3$ and $\alpha5$ subtypes. Partial agonist at $\alpha2$, $\alpha3$ and $\alpha5$ (but not $\alpha1$) subtypes | (Mckeran *et al.*, 2000) |
| Ocinaplon | Anxiolytic | Comparable affinity at $\alpha1$ $\alpha2$, $\alpha3$ and $\alpha5$ subtypes. Partial agonist at $\alpha2$, $\alpha3$ &$\alpha5$ subtypes. Nearly full agonist at $\alpha1$ | (Lippa *et al.*, 2005) |
| SL 651 498 | Anxiolytic | Agonist at $\alpha2$ and $\alpha3$. Partial agonist at $\alpha1$ and $\alpha5$ subtypes | (Griebel *et al.*, 2003) |
| TPA 023 | Anxiolytic | Partial agonist at $\alpha2$ and $\alpha3$subtypes. Antagonist at $\alpha1$ and $\alpha5$ subtypes | (Atack *et al.*, 2006) |
| TP003 | Anxiolytic (at high receptor occupancy) | Comparable affinity at $\alpha1$, $\alpha2$, $\alpha3$ and $\alpha5$ subtypes. Selective agonist efficacy at $\alpha3$ subtype | (Dias *et al.*, 2005) |
| ELB139 | Anxiolytic | Selective receptor profile uncertain | (Langen *et al.*, 2005) |
| L-655 708 | Memory enhancer, anxiogenic | Partial inverse agonist, with preference for $\alpha5$ subtype | (Sterfeld *et al.*, 2004, Chamber *et al.*, 2004, Navarro *et al.*, 2002, Lippa *et al.*, 2005) |
| $\alpha3$ IA | Anxiogenic | Weak inverse agonist at $\alpha3$ | (Atack *et al.*, 2005 ) |
| **Ligands at modulatory sites other than the benzodiazepine site** | | | |
| Ethanol | Anxiolytic, sedative | High sensitivity ($\geq$3 mM) at $\alpha4(\alpha6)\beta3\delta$, medium sensitivity ($\geq$30 mM) at $\alpha4(\alpha6)\beta2\delta$ and low sensitivity ($\geq$100 mM) at $\alpha4(\alpha6)\beta3\gamma2$ | (Wallner *et al.*, 2003) |
| Neurosteroids (e.g. 3$\alpha$, 5$\alpha$-THDOC) | Anxiolytic, sedative Anaesthetic | High sensitivity at $\delta$-containing subtypes and at $\alpha1$ and $\alpha3$ receptors in combination with $\beta1$ | (Belelli *et al.*, 2005) |
| Intravenous anaesthetics (etomidate propofol) | Sedative, anaesthetic | Act on receptor subtypes containing $\beta3$ (i.e. mainly $\alpha2$ and $\alpha3$ subtypes) | (Rudolph *et al.*, 2004) |
| Dihydroquinoline (compound 4) | Anxiolytic | Agonist efficacy at $\alpha2$ not $\alpha1$subtype | (Johnston *et al.*, 2004) |
| **GABA site** | | | |
| Gaboxadol | Hypnotic | Partial agonist at $\alpha1$ and $\alpha3$ subtypes. Full agonist at $\alpha5$ subtype. Agonist at $\alpha4\beta3\delta$ receptors | (Storustovu *et al.*, 2003) |

### 1.10.3 Naturally Occurring Alternatives

Ancient pharmacopoeias from different regions of the world have recorded numerous herbal medicines purported to have psychotropic potential. These offer a vast repertory of potential substances that can be developed into modern psychiatric pharmaceuticals. Indeed, nearly 25% of today's conventional drugs originated directly or indirectly from plants; many valuable psychoactive drugs, such as yohimbine, ephedrine, tubocurarine and galanthamine, were discovered through the study of indigenous remedies (Carlini, 2003, De Smet, 1997, Houghton & Seth, 2003). An increasing number of herbal products, represented by St. John's Wort, Ginseng, kava, and Ginkgo biloba, have been introduced into psychiatric practice in the past decade. There are also a large number of herbal medicines whose therapeutic potential has been assessed in a variety of animal models and whose mechanisms of actions have been investigated through neurochemical approaches. These studies have provided useful information for the development of new pharmacotherapies from medicinal plants for use in clinical psychiatry (Beaubrun & Gray, 2000, Desai & Grossberg, 2003, Fugh-Berman & Cott, 1999, Lake, 2000, Walter & Rey, 1999, Wong *et al.*, 1998).

There are currently few plant-derived drugs approved for clinical use. This is largely because most herbal medicines are complex mixtures of chemical components and have diverse biological and pharmacological actions. The herbal constituents for which behavioral effects and pharmacological properties have been well characterized may be good candidates for further investigations that may ultimately result in clinical use. These categories of herbal constituents include: the anxiolytic agents, honokiol, magnolol ( Kuribara *et al.,* 2001, Maruyama *et al*, 2001), several flavonoids such as Amentoflavone , Apigenin , Gensitein, hispidulin ( De Feo & Faro, 2003, Marder *et al.*, 2003, Goutman *et al.*, 2003), several terpeniods such as picrotoxinin (Chebib & Johnston, 2000), Bilobalide ( Sasaki *et al.*, 1999), Thymol (Mohammadi *et al.,* 2001) , α-Thujone ( Deiml *et al.*, 2004) , (+) Borneol ( Granger *et al.*, 2005) , Valerenic acid (Yuan *et al.*, 2004) others like kavactones (Singh & Singh, 2002, Wong *et al.,* 1998) and glycowithanolides (Bhattaacharya *et al.*, 2002); the antidepressant agents, the oligosaccharide MW-97 (Zhang *et al.,* 2002) , rosmarinic acid, caffeic acid and apigenin ( Nakazawa *et al.*, 2003, Takeda *et al.*, 2002); the neuroleptic agents: asarone (Cho *et al.,* 2002, Koo *et al.,* 2003), reticuline (Morais *et al.*, 1998) and polygalasaponins (PGS) (Chung *et al.*,1995, 2002) and the antidementia preparations, ferulic acid (Irie & Keung *et al.,* 2003, Lee *et al.,* 2003), dehydroevodiamine (DHED) (Park *et al.,* 2003), galanthamine ( Woodruff-pak *et al.,* 2003) , gastrodin (An *et al.*, 2003) , Huperzine A (Cheng *et al*, 1996), hyperforin ( Khalifa *et al.,* 2001) and paeoniflorin ( Ohta *et al.*, 1993) (All reviewed in Zhang, 2004, Johnston, 2005).

Also, some of these constituents with well-defined chemical structures may offer templates and models for synthesis of analogue drugs with higher efficacy and less adverse effects. However, although these herbal preparations have shown therapeutic potential in animal models, the clinical science of most herbal extracts and herbal mixtures is in its infancy. Interestingly, the pharmacological actions of many herbal agents involve to some extent the mechanisms known to be responsible for conventional psychotherapeutic actions. For instance, several anxiolytic constituents have the capacity to enhance the inhibitory function of central $GABA_A$/BDZ receptor complex. Like classical antidepressants, many herbal antidepressant agents inhibit MAO activity and modulate monoaminergic neurotransmission. Similar to donepezil and tacrine, which have been approved for the treatment of patients with Alzheimer's disease, anti-dementia effects of many herbal agents are related to the inhibition of AChE activity. On the other hand, a considerable number of herbal extracts and constituents, most notably antidepressant and anti-dementia agents, possess antioxidant and neuroprotective actions, as evidenced by protection against neuronal cell death induced by exposure to excessive free radicals, excitatory toxins, toxic derivatives of amyloid precursor protein and other neurotoxins. There is increasing evidence that free radical-mediated CNS neuronal dysfunction is involved in the pathophysiology of AD, schizophrenia and psychosis. Free radicals (oxyradicals, such as superoxide, hydroxyl ions, and nitric oxide) cause cell injury when they are generated in excess or the antioxidant defense is impaired. Both of these processes seem to be affected in these disorders. Therefore antioxidant and neuroprotective agents could have therapeutic potential in AD, mood disorders and schizophrenia (Mahadik & Mukherjee, 1996, Lee *et al.,* 2002, Perry *et al.*, 2002 and Yao *et al.,* 2001).

Considering the limitations of the available conventional pharmacotherapeutic agents for psychiatric illnesses, particularly the treatment refractoriness, high relapse rates and diverse adverse side effects that occur with long-term treatments, herbal remedies may provide an alternative for patients, especially for those with lingering conditions and intolerance to adverse effects. In fact, some clinical studies have demonstrated the beneficial effects of herbal remedies in the treatment of certain psychiatric conditions, most notably depression, anxiety, insomnia and dementia (Desai & Grossberg, 2003, Fugh-Berman & Cott, 1999, Lake, 2000, Perry *et al.*, 2003 and Wong *et al.,* 1998). Some herbal preparations used as an adjunctive therapy are also effective in treating refractory schizophrenia (Zhang *et al.*, 2001) the extrapyramidal symptoms induced by antipsychotics (Ishikawa *et al.*, 2000) and management of agitation in severe dementia (Ballard *et al.*, 2002). Collectively, behavioural studies of herbal remedies have created a unique opportunity for the development of new pharmacotherapies for psychiatric

MEMBERS OF THE CORPORATION

The following persons shall be members of the Corporation, that is to say – (1) The President for the time being; (2) the Vice-Presidents for the time being; (3) the Members of the Council for the time being; (4) the Director and Members of the Board of Professors for the time being; (5) the Graduates; (6) the Donors.

Any body of persons, corporate or unincorporate, contributing such money as would cause such body of persons if they were an individual to be deemed a donor of the Corporation, may from time to time, in such manner as they think expedient, and as may be approved of by the council, nominate any person belonging to their body to be a donor of the corporation, and to represent them in all matters relating to the Corporation.

GENERAL MEETINGS

A general meeting of the Corporation shall be held once at the least in every year at such time as may be fixed by the Council.

Special general meetings shall be held whenever summoned by the President or the Council.

If at any meeting of the Corporation neither the President nor a Vice-President is present at the time appointed for holding the same, or within a quarter of an hour afterwards, the members present shall choose some one of their number to preside at such meeting.

The Council shall present to the general meeting an account of the condition of the Corporation, with such particulars as the Council may think requisite.

On the occasion of any such vacancies having occurred in the Council as are by this Our Charter required to be filled by the Corporation in general meeting assembled, the general meeting shall proceed to fill up such vacancies by election.

A general meeting shall transact any such business not in this Our Charter specially mentioned as may be laid before them by the Council.

Ten members, personally present, shall be a quorum at any general meeting of the Corporation.

If at any general meeting of the Corporation ten members are not present within an hour after the time appointed for holding the same, the meeting shall stand adjourned to the same day in the next week, and if at such adjourned meeting ten members are not present within an hour after the time appointed for holding the meeting, the meeting shall stand adjourned *sine die.*

Every member of the Corporation present at the general meeting shall be entitled to one vote and no more, with this exception, that if at any meeting, or upon the taking of a poll, the number of votes given against and in favour of any matter are equal, the person presiding may give a second or casting vote.

Subject to such provision of this Our Charter as defines the purposes of the Corporation, the Corporation may in general meeting from time to time, by passing a special resolution in manner herein-after mentioned, alter any of the provisions of this Our Charter, and make new provisions in place thereof or in addition thereto, and any provisions so made by special resolution shall be deemed to be provisions of this Our charter of the same validity as if they had been originally contained therein, and shall be subject in like manner from time to time to be altered or modified by any subsequent special resolution: Provided always, that such alterations and provisions shall not be of any force unless the same have been recommended by the Council, nor until they have been approved by Us, or other the Sovereign for the time being.

A resolution of the Corporation shall be deemed to be special which has been passed at a general meeting of the Corporation, and confirmed at a subsequent general meeting held after an interval of not less than thirty days nor more than two months from the date of the meeting at which such resolution was first passed, subject to the condition following:-

Notice of both meetings and of the object for holding the same, mist be given according to the mode in which notices of general meetings are required to be given by the regulations of the Corporation for the time being in force.

steadily (Kneussel & Betz, 2000, Luscher & Fritschy, 2001, Moss & Smart, 2001, Beck *et al.*, 2002).

In this section, the current knowledge is summarised about the mechanisms governing the sorting, targeting and clustering of $GABA_A$ receptors and associated-proteins and the dynamic regulation of $GABA_AR$ subtypes in GABAergic synapses. Sorting and targeting mechanisms determine the subcellular compartment in which receptors are localized and clustering refers to the aggregation at synaptic sites. Figure 1- 12

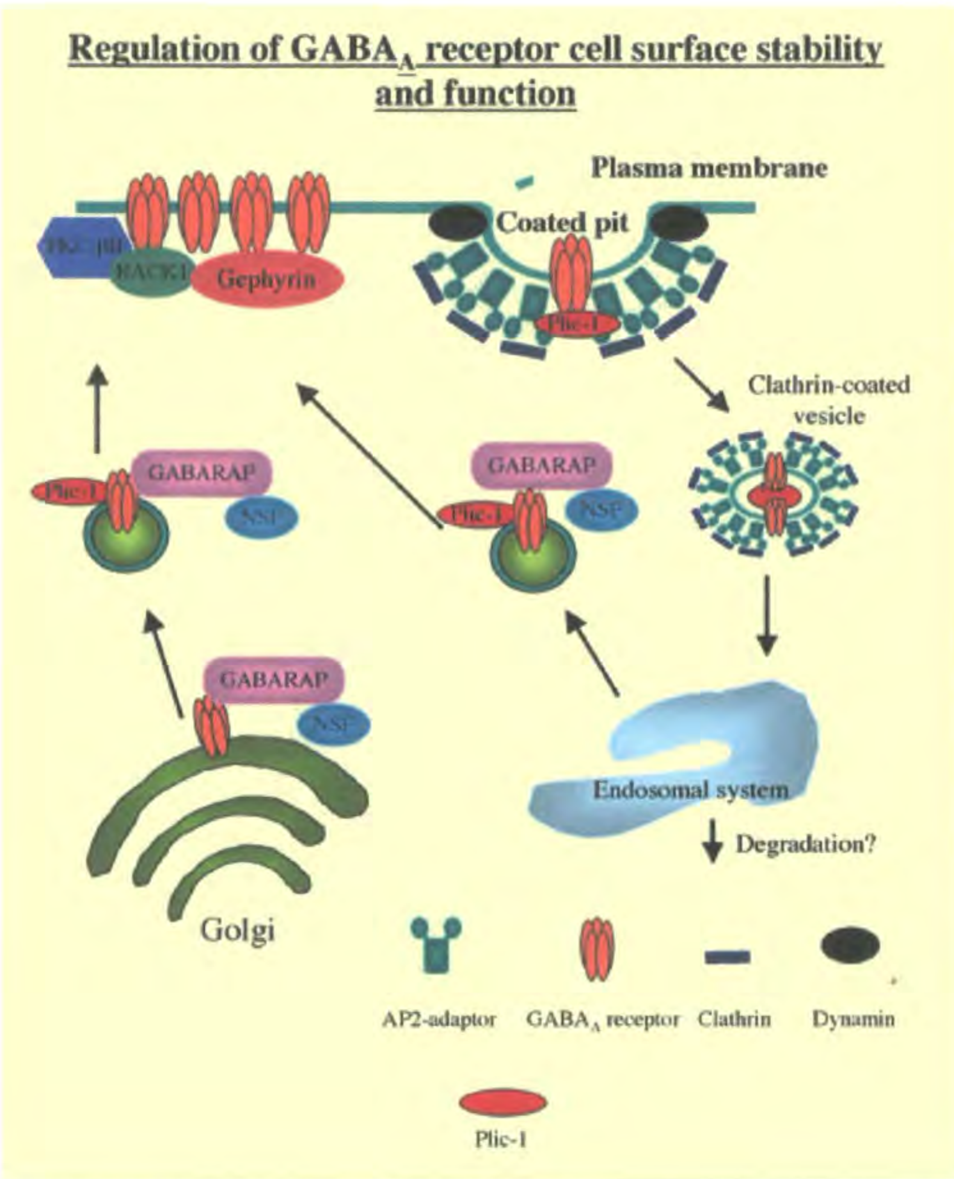



**Figure 1.12:** Regulation of $GABA_AR$ cell stability and function.

(Kittler & Moss, 2001)

### 1.11.1 Multiple Roles of $GABA_A$ Receptor-Associated Proteins

$GABA_AR$ assembly, processing, trafficking, surface expression, recycling and activity are regulated by a range of interacting proteins. Table 1.2 shows a summary list of the proteins that have been identified with $GABA_AR$ ion channel super family and their regulatory functions.

**Table 1.2:** A table of the known $GABA_AR$ interacting proteins

| No | Interacting Protein | Subunit Specificity | References | Function |
|---|---|---|---|---|
| 1. | Gephyrin | Indirect interaction with γ2 | Barnes 2000 , Brünig *etal.*2002, Christie *et al*, 2002a, b , Danglot *et al.*, 2003 , Fritschy *et al.*, 2003, Kneussel *et al.*, 2000 a,b , Levi et al., 2004 , Meier *et al.*, 2004 , Sassoe-Pogentto *et al.*, 2000 , Wang *et al.*, 1999 , Paarmann *et al.*, 2005. | Clustering $GABA_AR$ by indirect association |
| 2. | GABARAP $GABA_AR$ associated protein | γ2 394-411 | Wang *et al.*, 1999, Kneussel *et al.*, 2000, Kitter *et al.*,2001, Kanematsu *et al.*, 2002 | Intracellular $GABA_AR$ trafficking, membrane targeting, receptor degradation |
| 3. | Rapsyn | β1-β3 & γ2 | Ebert *et al.*, 1999, Yang *et al*, 1997 | Clustering |
| 4. | GRIF-1 $GABA_AR$ interacting factor (1) | B2 subunit ( 324-394) | Beck *et al.*, 2002 Brickley *et al.*, 2005 | $GABA_AR$ trafficking factor |
| 5. | Plic-1 $GABA_AR$ – associated ubiquitin-like protein | α1-α3 (346-355) α6,β1-β3 | Bedford *et al.*, 2001 | Increase surface expression and trafficking factor |

Continue...

Table 1.2

| No | Interacting Protein | Subunit Specificity | References | Function |
|---|---|---|---|---|
| 6. | gC1q-R | B1-β3 (399-413) | Schaerer *et al.*, 2001 | Multifunctional protein |
| 7. | AP2 Adaptin | Interact with β3, β2-IL (343/433) γ2S and γ2L | Kittler *et al.*, 2000, 2005; Herring *et al.*, 2003 | Regulation of endocytosis |
| 8. | GTAP-34 $GABA_AR$ tubulin complex associated protein | α1 subunit containing receptor | Kannenberg *et al.*, 1997,1999 | Phosphorylation of β2 subunit |
| 9. | BIG2 Brefeldin A-inhibited GDP/GTP exchange factor 2 | Interacts with all β subunits | Charych *et al.* 2004 | $GABA_AR$ trafficking factor recruitment of clathrin/AP-1 coat complex for receptor endocytosis |
| 10. | GODZ Golgi apparatus-specific protein with DHHC zinc finger domain. | Palmitoylates the γ2 subunit | Keller *et al.*, 2004 | $GABA_AR$ trafficking factor |
| 11. | D5-dopamine receptors | γ2 subunit | Liu *et al.*, 2000, Yan *et al.*, 1997 | Receptor cross-inhibition |
| 12 | GRAMP-1 γ-aminobutryric acid A receptor-associated membrane proteins | γ2 subunit | Fritschy *et al.*, 2003 | Unknown |

### 1.11.2 Trafficking and Internalization of $GABA_A$ Receptors

Regulation of the number of neurotransmitter receptors inserted in the postsynaptic membrane represents a powerful mechanism for rapid and transient changes in synaptic strength (Turrigiano, 2000; Kittler & Moss, 2001, Sheng & Lee, 2001). In principle, membrane receptor density at any given time point is the result of three major components: rate of membrane insertion, rate of endocytosis and speed of lateral mobility. There is increasing evidence for the various members of the family of ligand-gated channels for dynamic regulation of synaptic and extrasynaptic receptor density, with endocytosis followed by recycling or degradation, representing, perhaps, the major factors for short-term regulation of neuronal function (Carroll *et al.,* 1999; Kittler *et al.*, 2000, Lin *et al.*, 2000 and Barnes, 2001). The analysis of the function of Plic-1 also has emphasized the importance of the dynamic regulation of cell surface expression of $GABA_A$ receptors, and has suggested an alternative hypothesis to the concept of clustering (Bedford *et al.*, 2001). Indeed, assuming that $GABA_A$ receptors are shuffled continuously between the postsynaptic membrane and a subsynaptic compartment, shifting this equilibrium in one or the other direction is likely to have a major influence on the number of receptors available for synaptic transmission at a given time-point. Furthermore, the importance of other mechanisms should not be underestimated, as underscored by the recent demonstration that glycine receptors and AMPA receptors are highly mobile in the plasma membrane and can reversibly enter or leave zones of confinement most likely corresponding to postsynaptic sites, in which they stay immobile only for short periods of time (Meier *et al.*, 2001, Borgdorff & Choquet, 2002). The "release" of receptors from zones of confinement stresses the importance of considering receptor clustering as a dynamic process, and not as a permanent anchoring and immobilization in the postsynaptic density. Clathrin-dependent endocytosis is a major mechanism for recycling and degradation of membrane proteins, and it plays an essential role in desensitization of G-protein-coupled receptors (Ferguson, 2001, Tsao & Von Zastrow, 2001). For $GABA_A$ receptors, it has been suggested to occur constitutively in A293 cells expressing recombinant $\alpha1\beta3\gamma2$ receptors, in cultured hippocampal neurons, and in rat cerebral cortex (Connolly *et al.,* 1999; Kittler *et al.,* 2000, Kittler & Moss, 2001, Kumar *et al.*, 2003). In addition, clathrin-independent endocytosis has also been reported (Cinar & Barnes, 2001). A putative interaction between the clathrin adaptor protein AP2 and the intracellular domains of the $\beta1$-, $\beta3$- and $\gamma2$-subunits, but not of any of the $\alpha1$-$\alpha6$-subunit, was identified using pull-down assays (Kittler *et al.*, 2000). Most importantly, blockade of clathrin-dependent endocytosis with a peptide interfering with the association between amphiphysin and dynamin resulted in a large increase in the amplitude of miniature inhibitory postsynaptic currents (IPSCs) in cultured neurons (Kittler *et al.*, 2000, 2005). These results support the conclusion that the number of $GABA_A$ receptors at the cell surface depends on a dynamic equilibrium between insertion and removal.

### 1.11.3 Lateral Movement of $GABA_A$ Receptor in the Plasma Membrane.

The strength of inhibitory synaptic currents is directly correlated with the number of synaptic $GABA_A$ receptors (Otis *et al.*,1994, Nusser *et al.*, 1998). Thus, any mechanism that regulates the expression, lateral mobility, or rate of endocytosis or reinsertion of $GABA_A$ receptors into the postsynaptic plasma membrane is predicted to have profound effects on neural excitability. Lateral mobility of glycine and AMPA receptors has been addressed using video tracking of antibody-coated fluorescent particles that bind to these receptors on the surface of neurons. These experiments have established that glycine receptors alternate between diffusive and stationary behaviours, which correlate with extrasynaptic and synaptic localization of these receptors, respectively (Meier *et al.*, 2001). Similar studies on $GABA_A$ receptors using low-resolution fluorescence photo-bleach recovery measurements indicate that $GABA_A$ receptors behave similar to glycine and AMPA receptors (Perez-Velazquez & Angelides, 1993). In particular, these experiments showed that $GABA_A$ receptors on spinal cord neurons are organized into relatively immobile and mobile receptor pools that move in the plane of the plasma membrane. Thus, lateral movement in the plasma membrane represents a mechanism by which the local $GABA_AR$ concentration might be regulated to adjust the efficacy of synaptic inhibitory transmission (Lüscher *et al.*, 2004).

### 1.11.4 Extra-Cellular Signals

It has been demonstrated that the number of cell surface $GABA_AR$ at synapses can be regulated by a number of diverse factors, such as insulin and BDNF (Wan *et al.*, 1997; Henneberger *et al.*, 2002, Brünig *et al.*, 2001), via activation of tyrosine kinase receptors, in particular, ligand-activated receptors. The regulation is bi-directional, with insulin treatment leading to a rapid recruitment of cell-surface $GABA_A$ receptors (Wan *et al.*, 1997) and brain-derived neurotrophic factor (BDNF) application having the opposite effect (Brünig *et al.*,2001). The effects of BDNF are mediated postsynaptically, since application of this neurotrophin leads within minutes to a decrease in amplitude, but not in frequency or kinetic, of miniature IPSCs in cultured hippocampal neurons (Brünig *et al.*, 2001, Jovanovic *et al.*, 2000, 2004). Furthermore, this effect is paralleled by a decreased cell surface immunoreactivity of $GABA_AR$ subunits that can be prevented by blockade of tyrosine kinase signalling. It has also been shown that BDNF application leads to a rapid and transient phosphorylation of the β3-subunit, but it is not established which kinase is involved, nor whether this effect relates to changes in cell surface expression (Jovanovic *et al.*, 2000). However, Tanaka *et al.* (1997) showed that the reduction of $GABA_AR$ function by BDNF is prevented by postsynaptic $Ca^{2+}$ chelation or of phospholipase C inhibition, suggesting a possible involvement of PKC.

## 1.11.5 Functional Regulation of $GABA_A$ Receptor by Phosphorylation

Many of the $GABA_AR$ subunits contain within their respective loop consensus amino acid sequences for phosphorylation by the serine/threonine protein kinases protein kinase A (α6,β1,β2, β3, γ2L/S, γ3), protein kinase C (β1,β2, β3, γ2L/S, γ3 ), cGMP-dependant protein kinase (α6,β1,β2, β3, γ2L/S, γ3) and CaM kinase II (α6,β1,β2, β3, γ2L/S, γ3), as well as consensus sequences for phosphorylation by tyrosine kinases (γ1,γ2L/S) and src ((γ1,γ2L/S) ( Moss & Smart 1996). The eight amino acid insert of the γ2 splice variant contains an additional consensus sequence for protein kinase C (Whiting *et al.,* 1990; Kofuji *et al.,* 1991). Protein phosphorylation mechanisms have been shown to modulate $GABA_AR$ function (Brandon *et al.*, 2001, 2002 a, b, Balduzzi *et al.,* 2002). In addition to direct effects on channel-gating properties, phosphorylations of $GABA_AR$ subunits have multiple effects related to cycling between synaptic sites and intracellular compartments. In recombinant expression systems, for example, activation of PKC increases the rate of $GABA_AR$ internalization (Connolly *et al.*, 1999, Filippova *et al.*, 2000). It should be emphasized, however, that a direct phosphorylation of $GABA_AR$ subunits does not appear to regulate cell surface stability. Intermediate substrates, possibly including $GABA_AR$-interacting proteins, could be involved (Brandon *et al.*, 2000). In addition, it is not known whether PKC activation stimulates the rate of endocytosis or the rate of degradation of internalized receptors in vivo (Brandon *et al.,* 2000). Recent evidence indicates that PKC and its anchoring protein, receptor for activated C kinase (RACK) 1, directly bind to specific sites on the $GABA_AR$ β-subunits and that RACK1 potentiates $GABA_AR$ phosphorylation by PKC (Brandon *et al.*, 2002 a,b). RACK1 binding is also important for modulation of $GABA_AR$ function upon activation of metabotropic serotonin and acetylcholine receptors that are positively coupled to phospholipase C and PKC (Feng *et al.*, 2001, Brandon *et al.,* 2002 a, b). Suppression of $GABA_AR$ signalling by PKC phosphorylation, therefore, might represent an important, novel mechanism for serotonergic and cholinergic modulation of neuronal activity in vivo.

The β1–3 subunits are of particular interest in the context of phosphorylation-mediated modulation of $GABA_A$ receptors as they contain conserved serine residues (Ser409 in β1, Ser410 in β2, and Ser408/Ser409 in β3) that can be differentially phosphorylated by multiple serine/threonine kinases in vitro and in heterologous expression systems (McDonald & Moss, 1997). The β3 subunit Ser408/Ser409 is subject to phosphorylation by PKA, and phosphorylation of this site in recombinant receptors results in potentiation of the GABA response (McDonald *et al.*, 1998). In contrast, phosphorylation of the β1 subunit at Ser409 results in inhibition of the GABA response, while the β2 subunit is not a PKA substrate (McDonald *et al.*, 1998, Brandon *et al.*, 2003). Thus, the β subunit variant appears to contribute to differential effects of PKA activation on $GABA_AR$ currents observed in different types of neurons. PKA-dependent phosphorylation of $GABA_A$ receptors is also evident in cultured neurons but only in the presence of PKC inhibitors (Brandon *et al.*, 2000).
Phosphorylation by PKA is facilitated by A-kinase anchoring protein (AKAP), a PKA adaptor protein that selectively interacts with the β1 and β3 but not β2 subunit of $GABA_A$ receptors and thereby contributes to the target specificity of PKA (Brandon *et al.*, 2003).

The large intracellular loop domain of the γ2S/L subunit of $GABA_A$ receptors contains multiple sites (Ser327, Ser348 and Thr350 in γ2S and an extra-phosphorylation site at Ser343 of γ2L) that can be phosphorylated by different serine/threonine kinases in vitro (Moss *et al.*, 1992). Functional analyses of $GABA_A$ receptors containing point-mutated γ2 subunits and expressed in heterologous cells indicates that the phosphorylation state of these sites can have profound effects on $GABA_AR$ function. Evidence for a critical role of the phosphostate of Ser327 of the γ2 subunit in vivo is provided by Wang *et al.*, (2003). Table 1.3 shows a summary list of kinases and phosphatases that have been identified with $GABA_AR$ ion channel superfamily, their regulatory functions and proposed mechanisms (taken from Lüscher *et al.*, 2004)

**Table 1.3** Kinases, phosphatases

| No. | name | Effect (s) | Proposed mechanism/function(s) |
|---|---|---|---|
| 1. | AKT (also known as PKB), serine/ threonine kinase | Increases surface expression | Phosphorylates the β2 subunit (S410) in response to insulin signalling (Wan *et al.*, 1997; Wang *et al.*, 2003b). |
| 2. | Calcineurin | Modulate receptor activity | Dependant on NMDA receptor function, tetanus-driven Ca2+ influx directs calcineurin binding to the γ2 subunits, resulting in selective phosphorylation of the basally phosphorylated γ2 subunit ( Ser327) ( Wang *et al.*, 2003a). Thus site can be phosphorylated by PKC but not PKA in vitro (Moss *et al.*, 1992). |
| 3. | PKC Protein kinase C | Decrease surface expression | Stimulates endocytosis of $GABA_AR$ in heterologous cells in the absence of receptor phosphorylation (Chapell *et al.*, 1998; Connolly *et al.*, 1999; Kittler *et al.*, 2000b; Cinar & Barns, 2001). PKC phosphorylates the β2, β3 and γ2 subunits in vitro. |
| 4. | PKC/ PP2A Protein kinase C/protein phosphatase A | Modulate receptor activity | Modulates phosphorylation state specifically of the β3 subunit (Ser408/Ser409) in response to BDNF and implicated in phosphorylation-dependent modulation of $GABA_AR$ channel function (Jovanovic *et al.*, 2004). |
| 5. | PKA/PP1c Protein kinase A/protein phosphatase 1c | Modulate receptor activity | Phosphorylate and dephosphorylate selectively the β1 (Ser409) and β3 subunits (Ser408/Ser409) with subunit-specific effects on receptor activity. Phosphorylation of β1 and β3 results in inhibition and potentiation of GABA response, respectively (Macdonald *et al.*, 1998). |
| 6. | Src Tyrosine kinase | Modulate receptor activity | Binds to the intracellular loop of β and γ2 subunits .Phosphorylates the γ2 subunit (Y365/Y367) in vitro (Moss *et al.*, 1995; Brandon *et al.*, 2001). In neurons, unspecified tyrosine phosphatases maintain the γ2 subunit mostly in dephosphorylated state. |

## 1.12 Alteration of $GABA_A$ Receptor Expression and Function in Developmental, Neurological and Psychiatric Disorders.

It is difficult to demonstrate a causative role for disturbed $GABA_A$ receptor function in the pathophysiology of any disorder, but a connection may be surmised on the basis of the following findings: (1) a genetic linkage between disorder incidence and for example, subunit mutation or polymorphism in humans, (2) altered $GABA_AR$ function (due to changes in receptor subunit composition or subunit expression) in patients with the disorder, (3) mouse models with selective $GABA_AR$ alterations displaying similarities to human disorder, and (4) good clinical efficacy of $GABA_A$ergic drugs in the treatment of the disorder (Korpi *et al.*, 2006). Based on these criteria, a role for the $GABA_A$ergic system may be proposed in a variety of developmental and neuropsychiatric disorders (Smith *et al.*, 1998; Benes, 1999; DeLorey & Olsen, 1999, Lancel, 1999, Buxbaum *et al.*, 2002, Brambilla *et al.*, 2003, Davies, 2003). Disorders linked with an altered $GABA_A$ergic system, or which can be efficiently treated with $GABA_A$ergic drugs will be discussed below, these include:

### 1.12.1 Developmental Disorders

$GABA_A$ receptors have been associated with three types of developmental disorders, **Rett syndrome, Autism** and **Angelman syndrome.**

**1.12.1.1 Rett syndrome (RTT):** is a severe neurodevelopment disorder, genetic in origin which was first described by Austrian doctor, Andrea Rett, in 1966. It is a dominant X-linked pathology and is the second leading cause of mental retardation in females, with an incidence of 1 in 10,000 (Perini *et al.*, 2006, Pelka *et al.*, 2006). The onset of the disease occurs in early childhood between 6 and 18 months of age. (RTT) is characterized by the progressive loss of intellectual function, fine and gross motor skills and communicative abilities and the development of stereotypic hand movements, all of which occur after a period of normal development. Mutations in the methyl-CpG binding protein 2 (MECP2) genes, located at Xq28, account for 75% of RTT patients. Symptoms associated with the failure of mutated MECP2 to regulate transcription of a specific gene, DLX5, one allele of which is normally imprinted. Without the (MECP2) protein, production of the Dlx5 protein is increased, which is likely to influence production of the neurotransmitter GABA and may also affect the expression of other, related genes in the DLX family with consequences for the development of the brain (LaSalle *et al.*, 2005, Tejada, 2006).

#### 1.12.1.2 Autism

Autism is another genetic neurodevelopment disorder characterized by impairments in reciprocal social interaction and communication and the presence of restricted and repetitive patterns of interest or behaviour. These impairments are apparent in the first 3 years of life and persist into adulthood. About 75% of affected individuals also have some intellectual impairment (Ma *et al.,* 2005). The most common cause is due abnormalities or deletion of 15q11-q13 chromosome. Several lines of research indicate that there are abnormalities in the GABA system that may lead to developmental changes similar to those observed in Autism. The evidence implicates $GABA_AR$ subunit genes as functional candidates for Autism (Blatt *et al.* 2001, Hussman 2001 and Aldred *et al.* 2003). During development, GABA acts as an excitatory neurotransmitter because of the high intracellular chloride concentration in immature neurons (Jentsch *et al.*, 2002). It is notable that the studies found a significant decrease in GABAR density in Autism (Blatt *et al.*, 2001) and an elevated plasma GABA level in autistic children (Dhossche *et al.*, 2002). The most promising region identified by Autism association studies is on chromosome 15q11-12, which harbors a set of three GABAR subunit genes *GABRB3, GABRA5* and *GABRG3* (encoding the $GABA_A$ receptor's β3, α5 and γ3 subunits, respectively), all are clustered within 15q11-q13 (Cook *et al.,* 1998; Martin *et al.*, 2000, Wolpert *et al.* 2000, Boyar *et al.*, 2001, Buxbaum *et al.*, 2002). Chromosome 15q11-q13 duplications and deletions have also been documented in children with Autism (Smith *et al.*, 2000). In addition, several groups have identified this region as an area of interest through linkage studies (Philippe *et al.*, 1999, Liu *et al.* 2001). All of these findings from direct or indirect mapping studies strongly suggest that the $GABA_AR$ subunit genes may play an important role, both independently and interactively, in the etiology of Autism.

#### 1.12.1.3 Angelman Syndrome (AS)

AS is an inherited disorder that includes severe mental retardation and epilepsy. Patients have no speech, puppet-like gait with jerky movements, hyperactivity, disturbed sleep, bouts of inappropriate laughter, a pronounced jaw and widely spaced teeth (lalande *et al.*, 1999, Delorey *et al.*, 1999, Handforth *et al.*, 2005). The syndrome results from deletion or mutation within maternal chromosome 15q11-q13. Considerable evidence suggests that the gene or genes responsible for AS are expressed only from the maternal chromosome 15, a situation known as parental imprinting (lalande *et al.*, 1999, Delorey *et al.*, 1999). This epigenetic marking of certain regions of the parental genomes is characterized by parent-of-origin-specific allelic DNA methylation, allele-specific DNA replication timing and physical pairing of the two chromosome 15 homologues. Imprinting is important for normal development, and its disregulation causes several human disorders. The epilepsy of AS has been studied and indicates a rather typical electroencephalographic abnormality with slowing and notched wave and spikes (lalande *et al.*, 1999, Jiang *et al.*, 1999). Various types of seizures occur, usually including myoclonus and atypical absence. Variable severity among patients suggests potential molecular diversity in the genetic mechanism, possibly the involvement of more than one gene. AS can arise from the following molecular genetic defects: a deletion in 15q11-q13 that covers the Angelman gene or genes, mutations that alter imprinting and paternal uni-parental disomy for the region. Another 20% or so of patients with clinical symptoms of AS have none of these three defects but are believed to have mutations in one or more genes in the region, and this may be familial. The UBE3A gene, which codes for the enzyme ubiquitin protein ligase involved in protein degradation and processing, has been found to be mutated in many but not all of patients with AS and can be considered a major Angelman candidate gene (lalande *et al.*, 1999). Other potential candidate genes in the region include a cluster of three $GABA_AR$ subunits, which are involved in inhibitory synaptic transmission in the brain. The GABRB3 gene, which codes for the beta 3 subunit, is deleted in most persons with AS (Saitoh *et al.*, 1992, Knoll *et al.*, 1993, Sinnett *et al.*, 1993). The absence of this gene in mice causes craniofacial abnormalities and neurological impairment with seizures. The exact role of UBE3A and GABRB3 in the syndrome and their imprinting status still needs further investigation (lalande *et al.*, 1999; Delorey *et al.*, 1999).

## 1.12.2 Neurological Disorders

Changes of $GABA_AR$ function may be relevant for the pathophysiology of different neurological diseases.

### 1.12.2.1 Epilepsy

The "GABA hypothesis" of seizure disorders (Meldrum 1979; Olsen *et al.*, 1986) suggests that a deficiency in GABAgeric inhibitory synaptic transmission may contribute to the synchronous hyperexcitable activity of epileptic brain. This hypothesis is supported by the effectiveness of anticonvulsant drugs which enhance GABAergic transmission (Fritschy, 2004). Genetic evidence that $GABA_A$ receptors are involved in human idiopathic epilepsy has been provided for three distinct mutations in the γ2-subunit gene and one mutation in the α1-subunit gene. The $\gamma2^{K289M}$ point mutation in the extracellular loop between TM2 and TM3 of the γ2-subunit was reported in a family with generalized epilepsy with febrile seizures (Baulac *et al.*, 2001). In recombinant α1β2γ2 receptors expressed in *Xenopus* oocytes, this mutation reduced the amplitude of GABA-induced currents. However, potentiation by diazepam was not affected. Two other mutations, γ2$^{R43Q}$ (Wallace *et al.*, 2001) and a single nucleotide exchange at the splice donor site of intron 6 (Kananura *et al.*, 2002) were reported in two families with childhood epilepsy and febrile seizures. The γ2$^{R43Q}$ mutation, when expressed in *Xenopus* oocytes, did not affect GABA-gating of recombinant α1β2γ2 receptors, but suppressed diazepam potentiation. The splice-donor site mutation most likely results in a non-functional allele. Finally, a loss-of-function mutation of α1-$GABA_A$ receptors (α1$^{A322D}$) was detected in a family with an autosomal dominant form of juvenile myoclonic epilepsy (Cossette *et al.*, 2002). A possible contribution of $GABA_A$ receptors to other forms of epilepsy, notably to temporal lobe epilepsy, is suggested by the profound changes in expression that have been reported in patients and in various rodent models of temporal lobe epilepsy with hippocampal sclerosis (Duncan, 1999; Olsen *et al.*, 1999, Coulter, 2001) In patients, the extensive neuronal loss in CA1, which is one of the characteristic features of hippocampal sclerosis, is accompanied by a marked decrease in benzodiazepine-binding sites (Savic *et al.*, 1988, Debets *et al.*, 1997). However, a detailed examination at the cellular and subcellular level, using immunohistochemistry with subunit-specific antibodies, revealed a complex pattern of changes, characterized above all by an increased staining intensity on surviving neurons and by subtype-specific changes in subcellular distribution of $GABA_A$ receptors in epileptic tissue (Loup *et al.*, 2000). Among the most pronounced and consistent changes was the increased α1- and α2-subunit immunoreactivity in the soma and apical dendrites of the dentate gyrus granule cells and an apparent translocation of the α3-subunit immunoreactivity from the somatic region to the distal dendrites in CA2 pyramidal cells (Loup *et al.*, 2000). In experimental temporal lobe epilepsy, changes in $GABA_AR$ subunit expression have been analyzed in several animal models, with largely convergent results. The main observation was increased expression of $GABA_A$ receptors in the dentate gyrus granule cells, with changes in pharmacological properties, suggesting aberrant expression of $GABA_A$ receptors in these cells. Notably, an increase in α3-, α4- and δ-subunit expression has been reported in rats experiencing chronic recurrent seizures following i.p. injection of the muscarinic agonist pilocarpine or the glutamate receptor agonist kainic acid (Schwarzer *et al.*, 1997, Brooks-Kayal *et al.*, 1998, Fritschy *et al.*, 1999). However, chronic recurrent seizures induced by i.p. injection of kainic acid or pilocarpine do not mimic the complex partial seizures experienced by most patients with temporal lobe epilepsy. Also the pattern of neuronal loss is significantly different from that reported in neuropathological studies of hippocampal sclerosis. These limitations are partially overcome in a mouse model of temporal lobe epilepsy, in which spontaneous recurrent partial seizures are induced following unilateral injection of kainic acid into the dorsal hippocampus (Bouilleret *et al.*, 1999, Riban *et al.*, 2002). In these mice, the dentate gyrus granule cells become hypertrophic and undergo a prominent dispersion. Although the α4- and δ-subunits were not analyzed, a marked increase in α1-, α2-, α5-, and γ2-subunit immunoreactivity was observed in the epileptic dentate gyrus (Bouilleret *et al.*, 2000) which corresponded to an increase in the size and density of postsynaptic clusters co-localized with gephyrin and dystrophin (Kneussel *et al.,* 2001). These findings are strongly suggestive of the formation of novel GABAergic synapses, possibly reflecting sprouting of GABAergic axons in the epileptic dentate gyrus. Furthermore, they show that $GABA_AR$-associated proteins increase in parallel with $GABA_A$ receptors at postsynaptic sites. In addition to the well-known recurrent mossy fiber sprouting in the dentate gyrus and CA3 area, the formation of aberrant GABAergic connections might thus also be considered as a contributing factor to temporal lobe epilepsy. A direct demonstration for a prominent increase in the density of GABAergic axons in the dentate gyrus has been provided in the pilocarpine model, using dual labelling for GAD and GAT-1 (André *et al.*, 2001). These findings suggest that reactive sprouting of GABAergic axons in response to lesion might be a common response in the dentate gyrus. In addition, they indicate that increased, rather than decreased, GABAergic inhibition might be a key feature of epileptognesis and seizure expression in the dentate gyrus (Fritschy *et al.*, 2003).

#### 1.12.2.2 Huntington's Disease (HD)

HD is an inherited neurodegenerative disease characterized by progressive in voluntary choreiform movements, psychopathological changes and dementia (Fritschy *et al.*, 2004). The gene deficit involved in this disease is known but pathogenesis is still unknown. The abnormal gene responsible for disease is located near the end of the short arm of chromosome 4. It normally contains 11-34 cytosine adenine-guanine (CAG) repeats, each coding for glutamine. In patients with HD, the trinucleotide repeat increased to 42-86 or more copies and the greater the number of repeats, the earlier the age of onset and the more the progression of the disease. The gene codes for Huntingtin, a protein of unknown function (Ganong, 2005). Poorly soluble protein aggregates, which are toxic, form in the cell nuclei and elsewhere. However, the correlation between aggregates and symptoms is less than perfect. It appears that a loss of the function of Huntingtin occurs that is proportionate to the size of the (CAG) insert. At present no effective treatment is clinically available, and the disease is uniformly fatal (Ganong, 2005). Degeneration of GABAergic neurons is one of the hallmarks of HD. In the caudate nucleus and putamen, it is accompanied by a profound reduction in benzodiazepine-binding sites and $GABA_AR$ subunit immunoreactivity, corresponding to the loss of neurons (Faull *et al.*, 1993 , Kunig *et al.*, 2000). However, in the globus pallidus, a major target of striatal neurons, $GABA_A$ receptors are increased, suggesting compensatory up-regulation in the remaining synapses. Experimentally, following quinolinic acid-induced lesions of the striatum to mimic the pattern of neuronal degeneration of HD increased $GABA_AR$ β2/3-subunit immunoreactivity has been demonstrated in the substantia nigra pars reticulata (Brickell *et al.*, 1999). A detailed electron microscopic analysis, using post-embedding techniques, revealed a selective increase of $GABA_AR$ labeling in symmetric synapses, but not of AMPA receptors in asymmetric synapses, in lesioned animals (Fujiyama *et al.*, 2002). Most strikingly, the increased expression of $GABA_A$ receptors is long-lasting (>15 months), but it is induced very rapidly, being detectable by autoradiography within 2 hr following intrastriatal quinolinic acid injection (Brickell *et al.*, 1999). The signals involved in this rapid induction have not been investigated. While increasing the number of postsynaptic $GABA_A$ receptors might represent a compensatory response to the loss of GABAergic afferent, such a response can also occur in response to a loss of glutamatergic terminals. Thus, the number of postsynaptic $GABA_AR$ clusters associated with gephyrin increases in the molecular layer of the dentate gyrus following entorhinal cortex lesions (Simburger *et al.*, 2001). Deafferentation of excitatory input to the dentate gyrus, therefore, appears to induce a profound synaptic remodelling on dendrites of granule cells, possibly affecting GABAergic circuits. Interestingly, the effect was cell-specific, since $GABA_AR$ clusters on interneurons, distinguished by the presence of the α1-subunit, were not affected in this experimental paradigm (Simburger *et al.*, 2001). To understand the significance of this remodelling, it will be necessary to determine whether novel GABAergic synapses are formed and which neurons respond to the lesion by reactive sprouting ( Fritschy *et al.*, 2003).

#### 1.12.2.3 Alzheimer's Disease (AD)

AD is the most common type of dementia and is characterized by cognitive deficits and behavioural and psychological symptoms (Lanctot *et al.*, 2004). These behavioural and psychological symptoms of dementia (BPSD) include delusion, hallucinations, aggression, aberrant motor behaviour, sleep disruptions, agitation, depression and apathy (Devanand *et al.*, 1997; Cummings *et al.*, 1998). Research in the pathobiology of AD has revealed a gross disruption of neurotransmitter systems (Hardy *et al.*, 1996), including the cholinergic (Cummings *et al.*, 1998) and serotonergic systems

(Reinikainen *et al.,* 1988) in both cortical and subcortical areas of the brain. Although deficits in the cholinergic system have been associated with both cognitive changes (Whitehouse *et al.*, 1981) and BPSD (Cummings *et al.,* 1996), manipulation of the cholinergic system has limited effectiveness. Hence attention has turned to other possible therapeutic targets for patients with AD. Evidence suggests that the GABA system may play a supplementary role in other brain diseases by modulating dopamine and serotonin. GABA's association with such neuropsychiatric symptoms as anxiety, aggression and psychosis (Keverne *et al.*, 1999), as well as its ability to regulate acetylcholine, dopamine and serotonin (Decker *et al.,* 1991, Zorumski *et al.,* 1991), make it a therapeutic target for controlling BPSD. Moreover, potentiating GABAergic inhibition can potentially counteract elevated glutamate excitation and decrease excitotoxicity in cortical circuits (Gu *et al.*, 2003). While each of the study designs has limitations, post-mortem studies, ante-mortem studies, neuroimaging studies and markers of CNS GABA function, provide converging evidence that GABA is decreased in AD. Post-mortem studies on cortical areas have, for the most part, shown reduced frontal, temporal and parietal GABA concentration in AD (Lanctot *et al.*, 2004). When combined with GABA and benzodiazepine binding studies, it seems that the temporal region is most affected in AD patients. Interestingly, neuroimaging studies have not identified either in the frontal or temporal region as having altered GABA among AD patients. Rather it is the parietal cortex that seems to have reduced GABAeric binding. It can be expected that many of the post-mortem studies were performed on subjects with advanced AD; while the in vivo neuroimaging studies were performed on subjects with less advanced disease (Lanctot *et al.*, 2004).Thus widespread involvement of the cortical regions may occur with advanced AD or reflect lack of correction for cerebral atrophy. GABA within the limbic system, which is the primary area for emotion and behaviour in the human brain, has not been shown to have ubiquitous GABA reduction. In summary, rather than defining the pathology of AD, the presence of GABAergic reduction may reflect subtypes of the disease, which is consistent with a role in BPSD that accompany AD (Lanctot *et al.*, 2004). Few studies attempt to link BPSD with GABAergic disruption. Clinical experience supports GABA as a therapeutic target for symptomatic treatment of BPSD. Aggression and agitation are drug-responsive symptoms. Benzodiazepines, for example, are commonly used in clinical practice to reduce agitation and aggression exhibited by patients with dementia (Conn *et al.,* 1999). Although benzodiazepines such as lorazepam have been reported to work in some cases (Fritz *et al.*, 1990), the potential therapeutic effect may be outweighed by adverse events, such as sedation (Ancill *et al.*, 1991, Sunderland *et al.*, 1989). Other GABA therapies including anti-convulsants, show greater promise in managing behaviours such as aggression but are limited by toxicity, while antiepileptic drugs such as vigabatrin, tiagabine and topiramate offer novel mechanisms of action that involve the GABAergic system. These have not been evaluated among patients with AD (Lanctot *et al.*, 2004).

#### 1.12.2.4 Stiff-Person Syndrome (SPS)

SPS is a rare disorder of the central nervous system that is characterized by rigidity and episodic spasms of musculature (Stayer *et al.*, 1998). Spasms can be precipitated by audiogenic stimuli, stress or fear. The pathogenesis of SPS is believed to be of an autoimmune origin with the GABAergic system highly implicated (Solimena *et al.*, 1991). Approximately 60% of all patients with SPS have high titers of autoantibodies directed against the two isoforms of GAD, GAD 65 and GAD 67 (Ellis *et al.*, 1996, Dinkel *et al.*, 1998). Moreover, anti-GAD-positive sera and cerebrospinal fluid from SPS patients inhibit GABA synthesis in rat cerebellar tissue in vitro (Dinkel *et al.*, 1998). Magnetic resonance analysis of SPS patients with a high titer of anti-GAD antibodies showed lower levels of GABA in motor cortex and posterior occipital cortex, suggesting that the antibodies are directed against GAD lower levels of GABA in these regions (Dalakas, 2001). Drugs that enhance GABAergic activity, such as high-dose diazepam and vigabatrin, which decreases GABA catabolism, improve the symptoms of SPS (Cohen *et al*, 1966, Prevett *et al.*, 1997).

### 1.12.3 Psychiatric Disorders

GABA systems have been implicated in the pathogenesis of major psychiatric disorders such as schizophrenia, sleep disorders, anxiety and premenstrual Dysphoric disorder.

#### 1.12.3.1 Schizophrenia

Overactivity of the dopaminergic system in the brain is considered to be a contributing factor to the symptomatology of schizophrenia. Morphological studies have shown that the dopaminergic system receives GABAergic inhibitory input mainly via α3-containing $GABA_A$ receptors (Fritschy & Möhler, 1995). α3-Knockout mice displayed no adaptive changes in the expression of α1, α2 and α5 subunits and anxiety-related behaviour was normal. However, the mice displayed a marked deficit in prepulse inhibition of the acoustic startle reflex, pointing to a deficit in sensorimotor information processing (Yee *et al.*, 2005). This deficit in prepulse inhibition was normalized by administration of the antipsychotic dopamine D2 receptor antagonist haloperidol, suggesting that the phenotype is caused by hyperdopaminergia (Yee *et al.*, 2005). Attenuation of prepulse inhibition is a frequent phenotype of psychiatric conditions, including schizophrenia.

These results suggest that α3-selective agonists may constitute an effective treatment for sensorimotor gating deficits in various psychiatric conditions. This view is supported by the observation that the benzodiazepine site partial agonist bretazenil in earlier open clinical trials displayed antipsychotic activity similar to that of neuroleptic drugs (Delini-Stula., 1996). It is conceivable that α3-selective agonists would lack the sedative or extrapyramidal side effects of classical neuroleptics and would thus be valuable agents for various psychiatric conditions. Numerous theories abound regarding the pathophysiology of schizophrenia, one of which is a GABA hypothesis that postulates that perturbations in GABAergic neurotransmission underpin the basic pathophysiological mechanism in schizophrenia (Benes & Berretta, 2001).The evidence to support this hypothesis, however, is primarily descriptive in nature. Changes in gene expression of $GABA_AR$ subunits (Akbarian *et al.,* 1995) and glutamic acid decarboxylase (Akbarian *et al.*, 1995, Volk, 2002) have been reported in postmortem brains of schizophrenic patients. However, this may not be a specific finding because changes in gene expression for other major neuroreceptors also have been observed in schizophrenic brain. Another line of evidence to support the GABA hypothesis of schizophrenia is that treatment of schizophrenia with antiepileptic drugs that target GABAergic transmission has shown positive results (Hosak *et al.*, 2002). Benzodiazepines given in conjunction with neuroleptics may help ameliorate positive symptoms of schizophrenia, anxiety and agitation. In addition, valproate co-administered with neuroleptics appears to be effective in treating positive symptoms, irritability, hostility and violent behaviour. Alterations in several biochemical and anatomical markers of GABAergic transmission have been reported in schizophrenic patients, including changes in GAD expression, muscimol binding and number of interneurons ( Lewis, 2000, Benes & Berretta, 2001, Nutt & Malizia, 2001, Blum & Mann, 2002). The regions affected include the hippocampus, anterior cortex and medial prefrontal cortex. In most cases, the specificity of these alterations with regard to the disease type, medication and brain region affected has not been established. One study has reported, however, a selective reduction in the number of GABAergic axon terminals formed by chandelier neurons onto the axon initial segment of pyramidal cells in areas 9 and 46 of the prefrontal cortex, labelled with antibodies to GAT-1 (Woo *et al.*, 1998). This decrease was not seen in age-matched, non-schizophrenic psychiatric patients and was independent of antipsychotic medication at the time of death. Since the number and size of parvalbumin-positive neurons, which include chandelier neurons, was not affected, these results were taken as evidence for an alteration in GAT-1 expression and not for a decrease in the number of axon terminals. A recent study by the same group demonstrates compensatory up-regulation of α2-$GABA_AR$ immunoreactivity in the axon initial segment, again occurring selectively in schizophrenic patients independently of antipsychotic medication (Volk *et al.*, 2002). These findings, therefore, support the hypothesis of a disturbed GABAergic transmission in the prefrontal cortex of schizophrenic subjects due to a selective alteration of GABAergic function in the synapses formed by chandelier cells (Fritschy *et al.*, 2003).

### 1.12.3.2 Sleep Disorders

GABA systems are known to play important role in sleep and positive allosteric modulators of $GABA_A$ receptors are widely used to promote restful sleep (Gottesmann, 2002). Two observations indicate the importance of β3 $GABA_A$ receptor subunits in sleep. Oleamide, an endogenous sleep promoting fatty acid, is inactive in β3 $GABA_AR$ subunit knockout mice (Laposky *et al.,* 2001). A mutation in β3 $GABA_A$ subunits has been described in a patient with chronic insomnia. Functional characterization of this mutant showed a slower rate of desensitisation compared with normal $GABA_A$ receptors (Buhur *et al.*, 2002). The treatment of insomnia is regarded as a developing market for agents acting on $GABA_A$ receptors. The first and second generation of hypnotics (barbiturates and benzodiazepines respectively) decrease waking, increase slow-wave sleep and enhance the intermediate stage situated between slow-wave sleep and paradoxical sleep, at the expense of this last sleep stage. The third generation of hypnotics (Zolpidem, Zaleplon & Zopiclone) act similarly on waking and slow-wave sleep but the slight decrease of paradoxical sleep during the first hours does not result from an increase of the intermediate stage. These drugs show some selectivity for α1 subunit containing $GABA_A$ receptors, acting as allosteric modulators. A structurally related compound called Indiplon is in phase III clinical trials for insomnia and acts in a similarly selective manner (Smith *et al.*, 2001, Sanna *et al.*, 2002, Petroski *et al.*, 2006, Rudolph & Möhler, 2006). Also in phase III clinical trials is gabaxadol (THIP) , a directly acting $GABA_AR$ partial agonist, that interacts with the $GABA_AR$ population that is insensitive to benzodiazepines (Stroustovu *et al.*, 2003, Rudolph & Möhler, 2006). Many herbal preparations are used to promote sleep. For example, chamomile tea and valerian contains flavonoids which have been shown to enhance the positive allosteric modulation of benzodiazepine on $GABA_AR$ (Johnston *et al.,* 2005).

### 1.12.3.3 Anxiety Disorders

Anxiety disorders are a prevalent and disabling set of diseases which continue to represent a significant disease burden (Whiting, 2006). They can be categorized further into several distinct subgroups, including generalised anxiety disorder (the largest group), panic disorder, social anxiety and various phobias. For about 30 years from the 1960s, the gold standard treatment of anxiety disorders were the benzodiazepines (BZs). Benzodiazepine (BZ) anxiolytics mediate their clinical effects by enhancing the effect of GABA at the $GABA_AR$. Classical BZ full agonists such as diazepam had an improved safety profile over the barbiturate drugs that they largely replaced and had a rapid onset of efficacy much valued by the patient. However, BZs were not perfect drugs (Woods, 1998) and their sedative properties, cognitive impairing effects and perhaps most importantly of all, dependence and abuse liability has generated a significant negative perception in the eyes of the regulatory agencies, prescribing clinicians and the general public. As such, in recent years, anxiety disorders have frequently been treated with the antidepressant selective serotonin reuptake inhibitors (SSRIs) (Rickels *et al.*, 2002). This is in large part because the SSRIs lack the side effects that beset the BZs and also because anxiety is often comorbid with depressive disorders. The major disadvantage of SSRIs is their speed of onset of efficacy (Whiting, 2006). An important unmet medical need and a significant commercial opportunity, exists for a novel, fast-acting anxiolytic agent lacking the unwanted side effects of classical, full agonist, non selective BZs. To date, the 'second generation' partial agonist approach has not achieved this goal, with encouraging preclinical data failing to translate into a clear clinical advantage; however, ocinaplon (developed by DOV Pharmaceutical) might paradoxically prove to be the exception, although further clinical data are required. A more recent approach, to develop receptor subtype-selective modulators, holds some promise but has yet to demonstrate translation of the encouraging preclinical data into the clinic (Whiting, 2006).

#### 1.12.3.4 Premenstrual Dysphoric Disorder (PMDD)

Changes in cognition, mood and drug sensitivity across the menstrual cycle may be attributed to hormonal regulation of GABAergic transmission (Sundstrom *et al.*, 1998, Wong *et al.*, 2003). PMDD occurs during the luteal phase of the menstrual cycle and is characterized by severe alterations in mood, behaviour and physical well-being that significantly compromise the individual's ability to function in personal, professional, and social situations. In healthy control females, plasma GABA levels increase from the follicular to the luteal phases, whereas GABA levels are reduced in women with PMDD (Halbreich *et al.*, 1996). Brain GABA declines from follicular to the luteal phases in healthy controls, whereas levels increase across the cycle in women with PMDD (Epperson *et al.*, 2002). Despite the differing results when comparing plasma and brain GABA levels, there is nevertheless a striking difference in phase-specific GABA levels when comparing women with PMDD with controls. An increase in cortical inhibition during the luteal phase was reported in controls, but not women with PMDD (Wong *et al.*, 2003).

In summary, direct evidence for morphological alterations in GABAergic circuits and distribution of $GABA_A$ receptors is available for major neurological and psychiatric disorders.

## 1.13 Aims of the Thesis

The overall objective of this thesis was pharmacological characterisation of three structurally distinct $GABA_AR$ compound classes, Mefenamic acid, Caloporoside and essential oil natural products of Melissa & lavender, and to examine in detail the pharmacological effects of a novel $GABA_AR$ interacting protein GRIF-1.

# Chapter 2

## Material and General Methods

### 2.1 Source of Materials

#### 2.1.1 Sigma-Aldrich Chemical Company (Poole, Dorset, UK):

(-)-Nicotine hydrogen tartrate salt, β-actin, GABA ,Calcium chloride, Diazepam, Dithiothreitol (DTT), Dulbecco's modified eagle medium/F12 containing L-glutamine, Dulbecco's modified eagle medium/F12, Ethylene diamine tetra acetic acid (EDTA), Ethylenebis(oxyethylenenitrilo)tetracetic acid (EGTA), Foetal calf serum, Folin-ciolcalteau phenol reagent,Glutamate, Hexyl-β-D-glucoside , Hydrogen peroxide (30% v/v), Ketamine ,Kodak D-19 developer, Kodak fixer, Agar, Luminol, Methotrexate, Octyl-α-D-glucoside, p-coumaric acid, Penicillin (500IU/ml)/ streptomycin (500μg/ml) solution, Phosphate buffer saline, Picrotoxinin, Pre-stained molecular weight markers (molecular weight range 200-2.5Kd), Sodium azide, Sodium bicarbonate 7.5% (w/v), Sodium dodecyl sulphate (SDS), Sodium hydroxide, Sodium phosphate, Streptavidin beads, Triton X-100, Trypsin EDTA, Tween-20, α-Chymotrypsin.

#### 2.1.2 BDH Laboratory Supplies (Leicetershire, UK):

Acrylamide, Ammonium persulphate, Chloroform, Dimethyl sulphoxide (DMSO), Ethanol, Hydrochloric acid, Isopropanol, Lactose, Methanol, N,N,N',N'-teramethylethylenediamine (TEMED), Potassium phosphate, Sodium chloride, Sodium hydrogen carbonate.

#### 2.1.3 Amersham International (Aylesbury, Bucks, UK):

[$^{3}$H] Flunitrazepam, specific activity (91.0 Ci/mmole), [$^{3}$H] Nicotine, specific activity (77.0 Ci/mmole), Blotting paper, Nitrocellulose, Hyperfilm™, HRP linked secondary antibody- rabbit, HRP linked secondary antibody-mouse, Binding filters.

#### 2.1.4 Perkin Elmer Life Science (U.S.A):

[$^{35}$S]-*t*-butylbicyclophosphorothionate (TBPS), specific activity (80 Ci/mmole).

**2.1.5 American Radiolabel Chemicals (ARC), (U.S.A):**

[$^3$H] Muscimol, specific activity (36.5 Ci/mmole), [$^3$H] MK-801, specific activity (25.0 Ci/mmole).

**2.1.6 Promega Ltd (Southampton, UK):**

HB101 Competent *E.coli* cells.

**2.1.7 GIBCO, Invitrogen (Life Technologies, U.S.A):**

Reagent plus, Lipofectamine, Optimum I essential media with Glutamax-1, Geneticin / G -418 sulphate.

**2.1.8 Chemicon International, Inc (Temecula, CA):**

Rabbit Anti-GABA$_A$R β2 and Rabbit anti-GABA$_A$ $\gamma_2$ affinity purified polyclonal antibodies.

**2.1.9 QIAGEN Ltd (Dorking, Surry, UK):** QIAGEN® plasmid maxi kit.

**2.1.10 Pierce (Rockford, UK):** EZ-Sulfo-NHS-SS-Biotin.

**2.1.11 Calbiochem (Darmstadt, Germany):**

Protease inhibitor cocktail, Heptyl-β-D-glucoside, Nonyl-β-D-glucoside.

**2.1.12 Avocado's, Alfa Aesar Chemical Company (Ward Hill, U.S.A):**

D-Glucose, D-Mannose.

**2.1.13 National Diagnostic Ltd (Hull, UK):**

Ecoscint, Decon 90.

**2.1.14 Miscellaneous**

- r-GRIF-1a-cDNA and Anti-GRIF antibody 8-633, were generous gifts from Professor F.A Stephenson, School of Pharmacy, London.
- GABA$_A$R α1β2γ2 cell line was a kind gift form Dr David Graham (Sanofi Aventis, France).
- Human embryonic kidney (HEK) 293 cells were from European collection of cell culture, Salisbury, Wilts.
- Rabbit Anti-GABA$_A$R α1 affinity purified polyclonal antibody was a gift from Dr Chris Thompson (Durham).

## 2.2 Instruments and Equipments

- **Spectrophotometry:** Jenway Genova spectrophotometer.
- **Centrifuges**: Bench-top refrigerated Biofuge fresco Heraeus, Beckmann J2 series
- **Incubators:** Sanyo cell incubator.
- **Orbital shaker:** Stuart scientific 505.
- **Water bath:** Stuart scientific,Nüve.
- **Balances:** Milligram amounts were weighed using Mettler Toledo balance. All other amount were weighed using Scout Pro Ohaus balance.
- **Electrophoresis equipment:** Polyacrylamide gels were cast in Biotech gel caster using gel plates of 10x8cm, electrophoresis was performed using a Hoefer Mighty small II vertical slab SE250 unit and transferred using a Hoefer TE series Transphor tank, all supplied by Life Technologies.
- **Radioligand binding equipment**: Bound radioactivity was collected using a Brandel cell harvester. Radioactivity was counted using a Beckman scintillation counter.
- **Confocal microscope**: Laser-scanning confocal microscope (Zeiss LSM 510 META).
- **Other equipment:** Immunoblotting cassette, pH meter was a Mettler Toledo MP220.
- **Glassware, plastics and disposables:** Hamilton syringe. Dounce glass/glass homogeniser. Cell scrapers, 250ml sterile cell culture flaks from Greiner. Sterile filters: 0.2μm Sartorius Sartolab-V150 filter unit. Cryogenic vials. Sterile pipettes and 250ml sterile filter lid cell culture flasks from Bibby sterilin. Falcon tubes. Filters for radioligand binding, Whatman GF/B filters.

## 2.3 Preparation of Standard Solutions

**2.3.1 [$^3$H] Flunitazepam Binding Assay Buffer:**

50mM Tris-HCl, 5mM EDTA, 5 mM EGTA, pH 7.4

**2.3.2 [$^3$H] Muscimol Binding Assay Buffer:**

50mM Tris-HCl, pH 7.4

**2.3.3 [$^{35}$S] TPBS Binding Assay Buffer:**

50mM Tris-HCl, containing 0.2 M NaCl, pH 7.4

**2.3.4 [$^3$H] MK-801 Binding Assay Buffer:**

25 mM sodium phosphate buffer pH 7.4

**2.3.5 [$^3$H] Nicotine Binding Assay Buffer:**

50 mM Tris buffer containing 8 mM $CaCl_2$ PH 7.4

**2.3.6 Radioligand Binding Wash Buffer:**

10mM sodium phosphate pH 7.4

**2.3.7 Homogenization Buffer:**

50 mM Tris–HCl, pH 7.4, containing 5 mM EDTA, 5 mM EGTA and 320 mM sucrose.

**2.3.8 Lowry Reagent A:**

2% (w/v) sodium carbonate, 0.1M sodium hydroxide and 5% (w/v) SDS.

**2.3.9 Lowry Reagent B:**

2% (w/v) sodium potassium tartrate.

**2.3.10 Lowry Reagent C:**

1% (w/v) copper sulphate.

**2.3.11 Stacking Gel Buffer:**

0.5M Tris-glycine, pH 6.8, containing 8mM EDTA and 0.4% (w/v) SDS.

**2.3.12 Resolving Gel Buffer:**

50mM Tris, 384mM glycine, 1.8mM EDTA and 0.1% (w/v) SDS pH 8.8.

**2.3.13 Stock Acrylamide:**

30% (v/v) acrylamide and N,N'-methylenebisacrlyamide.

**2.3.14 Electrode Buffer:**

50mM Tris, 384mM glycine, 1.8mM EDTA and 0.1% (w/v) SDS pH 8.8.

**2.3.15 Sample Buffer:**

30mM sodium hydrogen phosphate, pH 7.0, 30% (v/v) glycerol, 0.05% (v/v) bromophenol blue and 7.5% (w/v) SDS.

**2.3.16 Pre-stained Molecular Weight Markers:**

Pre-stained standards (protein molecular weight range 6.5-200 KDa, Sigma), stored in sample buffer, section 2.3.15.

**2.3.17 Transfer Buffer:**

25mM Tris, pH 8.4, 192mM glycine and 20% (v/v) methanol.

**2.3.18 Phosphate Buffered Saline (PBS):**

4mM sodium hydrogen phosphate, 1.7mM potassium hydrogen phosphate, pH 7.4, 137mM sodium chloride, 107mM potassium chloride.

**2.3.19 Tris Buffered Saline (TBS):**

50mM Tris-HCl, 0.9% NaCl, pH 7.4

**2.3.20 Loading Buffer:**

0.25% (w/v) bromophenol blue, 30% (v/v) glycerol and 60mM EDTA pH 8.0

**2.3.21 Biotinylation Wash Buffer:**

PBS containing 4% sucrose

**2.3.22 Quenching Buffer:**

192mMglycine, 4% sucrose in TBS

**2.3.23 Lysis Buffer:**

50mM Tris-HCl, 0.9% NaCl, pH 8.0, 2 mM EDTA+ 500µl Protease inhibitor cocktail.

### 2.3.24 Iso-osmotic Saline Solution (SS) Buffer:

137mM NaCl, 5.3mM KCl, 0.17 $Na_2HPO4$, 0.22 mM $KH_2PO4$, 10mM HEPES, 33mM glucose, 44mM sucrose, pH 7.4

### 2.3.25 TEE Buffer:

50 mM Tris-citrate, pH 7.1 containing 5mM EDTA and 5mM EGTA.

## 2.4 General Methods

### 2.4.1 P2 Membrane Preparation:

Adult male rats (200–300 g), Wistar strain, were maintained under a 12 h light, 12 h dark cycle at temperature of 23 °C and 65% humidity, with water and standard laboratory food available ad libidum. Animal treatment and husbandry were in accordance with approved use of animals in scientific procedures regulated by the Animals (Scientific Procedures) Act 1986, UK. The animals were killed humanely using a Schedule 1 procedure. The animals were sacrificed by stunning followed by decapitation. The Head of the rat was removed and placed on ice. Excess tissue from neck, up to the base of the skull was trimmed with a pair of large scissors. Using a scalpel or blade, a midline skin incision was made along the top of the skull (from between the eyes to the base of the skull) and pulled back to expose the bone. With the points of the small scissors two lateral cuts in the bone at the base of the skull were made to enable this (flap) to be removed with the forceps. The small scissors were then used to carefully cut up the side of the skull, removing the one covering the top of the brain with forceps. Again, using the small scissors, a midline cut was made through the nasal sinuses and this bone was removed. With a small spatula, the brain was carefully scooped out rinsed with sucrose, to remove any remaining blood, hair, meningeal or bone fragment, the required tissue (forebrain) dissected immediately and kept frozen at -20 until use.

The tissue was then homogenized in ice-cold homogenisation buffer (section 2.3.7) containing 320 mM sucrose, using a dounce glass/glass homogenizer. The homogenate was centrifuged at 1000 × *g*, 4 °C for 10 min, the supernatant was stored in ice, and the pellets was re-homogenized in ice-cold buffer again, re-centrifuged at 1000 × *g*, 4 °C for 10 min. The supernatant from the first and second centrifugation steps were pooled together and centrifuged at 12,000 × *g*, 4 °C for 30 min. The supernatant was discarded and the pellet resuspended in 50 mM Tris containing 5 mM EDTA and 5 mM EGTA (5 ml/g of original tissue), and frozen at −20 °C until use.

### 2.4.2 Freezing/thawing Protocol for the Preparation of Well-Washed Rat Membranes:

The $GABA_AR$ binding assays were performed with well-washed rat membranes prepared by a five-step freeze-thaw protocol (Enna *et al.*, 1975; Rezai *et al.*, 2003). Briefly the unwashed membranes, prepared as described above, were thawed , resuspended in 50 volumes of homogenization buffer (pH 7.4) (section 2.3.7) and were snap frozen in liquid nitrogen, centrifuged 12,000 × *g*, 4 °C for 30 min. The pellets were washed four additional times by resuspension in 50 volumes of ice-cold homogenization buffer, snap frozen in liquid nitrogen followed by centrifugation at 12,000 × *g*, 4 °C for 30 min. Finally, the pellets were suspended in homogenization buffer (pH 7.4). The tissue was then homogenized using a dounce glass/glass homogenizer, aliquots into (1 ml) samples and were then frozen and stored at −20 °C.

### 2.4.3 Determination of Protein Concentration:

The protein concentration was determined using the method of Lowry *et al.* (1951) employing Bovine Serum Albumin as the standard protein. A stock solution of BSA (1mg/ml) was serially diluted in water, to give a range of standard BSA concentrations from 0 to 100 µg/ml. Lowry reagent A (section 2.3.8), Lowry reagent B (section 2.3.9) and Lowry reagent C (section 2.3.10) were mixed in a volume ratio of A (50): B (1): C (1). To both the BSA standards and the unknown protein samples (5µl protein + 95µl $dH_20$, and 10µl protein + 90µl $dH_20$) 0.5 ml of the mixture of reagent A, B and C was added, each sample was vortexed and incubated at room temperature for 10 minutes. All samples were assayed in triplicate. On the addition of 50 µl of Folin-Ciocalteau phenol reagent (1 M, 1:1 mix of Folin reagent and water) each sample was mixed and incubated at room temperature for 30 minutes. The reaction was terminated by the addition of 500µl of water. The O.D. at $\lambda$ = 750 nm was determined for each sample using a Jenway Genova spectrophotometer. A calibration curve was plotted of O.D. at $\lambda$ = 750 nm for the BSA samples. This was then used to determine the unknown protein concentration for any prepared well washed rat membranes samples.

### 2.4.4 Cell Culture

#### 2.4.4.1 Preparation of DMEM/F12 Medium + L-Glutamine:

All procedures were performed using sterile conditions. Powdered Dulbecco's Modified Eagle Medium/F12 (DMEM/F12 1: 1 mixture) (15 g/l) containing, L-glutamine (0.0365 g/l) and 15 mM HEPES was mixed with sterile water (800 ml). The mixture was supplemented with 10 % (v/v) FCS, 40 ml of 7.5 % (w/v) $NaHCO_3$ (final 3.0 g/l) and penicillin (5001 U/ml) / streptomycin (500 µg/ml) solution (20 ml). The final volume was made up to 1litre with sterile water and the pH of the medium was adjusted to pH 7.6

using NaOH (2 M). The medium was filter-sterilised using a 0.2 μm Sartorius Sartolab-V150 filter unit and stored at 4°C until use.

### 2.4.4.2 Cell Cultivation of $GABA_AR$ Cell Line:

Human embryonic Kidney (HEK) 293 cell line expressing $\alpha_1\beta_2\gamma_2L$ subunits of $GABA_AR$ was a kind gift from Dr. David Graham (Sanofi-Aventis Research, France). Procedure for the development of this stable cell lines was described previously (Besnard *et al.*, 1997). Briefly rat α1, β2, γ2 subunits of $GABA_A$ receptor were expressed in human embryonic kidney cell lines (HEK 293), the cells were transfected with plasmid containing α1, β2 cDNA and plasmid encoding G418 resistance. G418 resistant colonies were screened for [$^3$H] muscimol binding, the best α1, β2 subunit expressing colony was then super-transfected with a plasmid coding for the γ2 rat subunit and a mutant DHFR gene. After a second round of selection, this time in the presence of methotrexate, those colonies that co-expressed ternary α1, β2, γ2 subunits of $GABA_A$ receptor combination were distinguished using [$^3$H] flumazenil as a probe.

For the preparation of a new culture, a single cryogenic vial of frozen $GABA_AR$ cell line was thawed at 37°C. Cells were pelleted by centrifugation at 200xg for 5 minutes at 4°C and resuspended in 15 ml of sterile DMEM/F12 media containing G418 at a concentration of 1mg/ml and methotrexate 100nM. The cells were added to a tissue culture flask, which was incubated at 37°C in 5 % $CO_2$ and cultured.

### 2.4.4.3 Sub-Culturing of $GABA_AR$ Cell Line:

$GABA_AR$ cell line were grown in 250 ml culture flasks at 37°C in 5% $CO_2$ in DMEM/F12 media containing, L-glutamine in an incubator. Every 1-2 week cells were sub-cultured by the removal of the old media and then washed with pre-warmed sterile PBS solution (10 ml). Following 1 minute incubation in trypsin-EDTA (2 ml) at 37°C, DMEM/F12 media containing L-glutamine (12 ml) was added to the cells. The cells were then separated by gentle pipetting. Finally, the cell suspension (2 ml) was added to a fresh flask and a further 15 ml of sterile DMEM/F12 media containing G418 at a concentration of 1mg/ml and methotrexate 100nM and was added to the new flask, which was incubated at 37°C in 5% $CO_2$.

### 2.4.4.4 Harvesting & Cell Homogenate Preparation of $GABA_AR$ Cell Line:

Membranes from control $GABA_AR$ cell line were prepared as described by Fuchs *et al.* (1995). The cells were harvested at 90-95% confluent growth. The culture media were removed; the cells were washed once with (10 ml) PBS, followed by 15ml of ice-cold homogenisation buffer (section 2.3.7). Cells were scraped off the bottom of the flask using Greiner cell scrapers. Cell suspensions were centrifuged at 3000 X g for 5 min at 4C°. The cells pellet collected and homogenised with glass/glass homogeniser for 30 strokes in ice–cold homogenisation buffer. The homogenate was re-centrifuged at 30,000 X g for 30 min at 4C°. The cell homogenate was re-homogenised, centrifuged and the final cell pellet resuspended in (7ml) buffer and assayed immediately for radioligand binding assay.

#### 2.4.4.5 Preparation of New Stocks of GABA Cell Line:

$GABA_AR$ cell line stocks were prepared by subjecting the cells to trypsin-EDTA (4 ml) dissociation for 1 minute at 37°C and 20 ml of DMEM/F12 medium containing L-glutamine was added. The cells were centrifuged at 200xg for 5 minutes at 4°C. The pellet was resuspended in DMEM/F12 medium containing L-glutamine (4.8ml) supplemented with, FCS (0.6ml) and DMSO (0.6ml). The cell suspension was immediately divided into three cryogenic vials and stored at –80°C for 34 hours and then transferred to liquid nitrogen.

### 2.4.5 Radioligand Binding Assays

#### 2.4.5.1 [$^3$H] Flunitrazepam Binding Assay:

[$^3$H] flunitrazepam binding assays were performed as previously described Rezai *et al.* (2003) and Thomas *et al.* (1997). Briefly, well-washed rat forebrain membranes prepared by five-step freeze-thaw protocol were thawed, centrifuged and the supernatant was discarded. The pellets were resuspended again in fresh buffer (section 2.3.1) to yield a final protein concentration in the assay of 1 mg/ml. An amount of 100 µg membrane protein was incubated with [$^3$H] flunitrazepam (approximately 1 nM) for 1 h at 4 °C with a range of test concentrations ($10^{-11}$ to $10^{-4}$ M). Non-specific binding was defined in the presence of 100 µM diazepam.

#### 2.4.5.2 [$^3$H] Muscimol Binding Assay:

[$^3$H] muscimol binding assays were performed as previously described in Böhme *et al.* (2004). Briefly, well-washed rat forebrain membranes prepared by five-step freeze-thaw protocol were thawed, centrifuged and the supernatant was discarded. The pellets were resuspended again in fresh buffer (section 2.3.2.) to yield a final protein concentration in the assay of 1 mg/ml. An amount of 100 µg membrane protein was incubated with [$^3$H] muscimol (approximately 10 nM) for 1 h at 4 °C with a range of test concentrations ($10^{-11}$ to $10^{-4}$ M). Non-specific binding was defined in the presence of 100 µM GABA.

### 2.4.5.3 [$^{35}$S]-*t*-butylbicyclophosphorothionate (TBPS) Binding Assay:

[$^{35}$S] TBPS binding was performed essentially as described in Im *et al.* (1994). Briefly, well-washed rat membranes prepared by a five-step freeze-thaw protocol were, on the day of experiment, centrifuged and the supernatant was discarded. The pellets were resuspended in fresh buffer (section 2.3.3) to yield a final protein concentration in the assay of 1 mg/ml. An amount of 100 µg membrane protein was incubated with [$^{35}$S] TBPS (approximately 20 nM) for 90 min at 25 °C with a range of test concentrations ($10^{-11}$ to $10^{-4}$ M). Non-specific binding was defined in the presence of 100 µM picrotoxinin.

### 2.4.5.4 [$^{3}$H] MK-801 Binding Assay:

[$^{3}$H] MK-801 binding assays were performed as previously described Chazot *et al.* (1993). Briefly, well-washed rat forebrain membranes prepared by five-step freeze-thaw protocol were thawed, centrifuged and the supernatant was discarded. The pellets were resuspended again in fresh buffer (section 2.3.4) to yield a final protein concentration in the assay of 1 mg/ml. An amount of 100 µg membrane protein was incubated with [$^{3}$H] MK-801 (approximately 1 nM) and 10 µM glutamate for 2 h at 22 °C with a range of test concentrations ($10^{-11}$ to $10^{-4}$ M). Non-specific binding was defined in the presence of 10 mM ketamine.

### 2.4.5.5 [$^{3}$H] Nicotine Binding Assay:

[$^{3}$H] Nicotine binding assays were performed as previously described in Wake *et al.* (2000). Briefly, well-washed rat forebrain membranes prepared by five-step freeze-thaw protocol were thawed, centrifuged and the supernatant was discarded. The pellets were resuspended again in fresh buffer (section 2.3.5) to yield a final protein concentration in the assay of 1 mg/ml. An amount of 150 µg membrane protein was incubated with [$^{3}$H] Nicotine (approximately 4 nM) for 1 h at 25 °C with a range of test concentrations ($10^{-11}$ to $10^{-4}$ M). Non-specific binding was defined in the presence of 100 µM (-)-Nicotine hydrogen tartrate salt.

### 2.4.5.6 Binding Assay Protocol

The following protocol was used for the binding assays:

- Each assay condition was performed in triplicate.
- All binding assays were carried out in 4 ml polystyrene tubes.
- Ligand stock was diluted in assay buffer to a concentration 10 x times the desired final concentration.
- Solutions of test compounds were prepared at 10 x times the desired final concentration.
- 80 µl assay buffer were added to the first three tube set ( which represents total binding) and 60 µl assay buffer for all other tubes in the experiment.
- 20 µl of the 10xtest compound were added to the tubes, except the total and non-specific binding sets.
- 20 µl of the 10x non-specific agent were added to the second three set of tubes for determination of non-specific binding.
- 100 µl of membrane preparation or cell homogenate were added to all the tubes.
- 20 µl of the 10 x [$^3$H] or [$^{35}$S] ligand were added to all the tube .
- The samples were vortexed and incubated until binding equilibrum was achieved. For many ligand , 1 hr at room temperature is sufficent to react equilibrium.
- At the end of the incubation , the assay mixtures were harvested onto pre-soaked (0.2% polyethyleneimine) GF/B filters using Brandel cell harvester. Washed with 3 quick washes with ice-cold 10 mM sodium phosphaste buffer pH 7.4.
- The filters were transfered into 3 ml scintillation vials, each incubated with 1 ml scintillation cocktail, left for 16-24 hr at room temperature. Membrane bound readioactivity was determined by liquid scintillation counter ( Beckman LS 500 CE).

All tested compounds were, dissolved at $10^{-1}$ M in DMSO and serial dilutions were made with respective assay buffer. GABA, (-)-Nicotine hydrogen tartrate salt, and Ketamine stocks ($10^{-2}$ M) were made in assay buffer. Diazepam stocks ($10^{-2}$ M) were prepared in absolute ethanol. Picrotoxinin stocks ($10^{-2}$ M) were prepared in DMSO. No effect of solvents on radioligand binding assays was seen at concentrations below 0.1% (v/v) DMSO or 0.1% (v/v) ethanol.

### 2.4.6 Analysis of Radioligand Binding Assay

#### 2.4.6.1 Data Analysis for Competition Studies:

Results from the radioligand binding assays were analysed using GraphPad Prism 4 Software program (GraphPad Software, San Diego, CA). For competition assays non-linear least squares regression was used, Curves were best fitted to a one- or two-site binding model. The $EC_{50}$ and $IC_{50}$ values are the concentrations for half-maximal enhancement and displacement, respectively. Data were analysed using a Student's unpaired *t*-test, with levels of significance set at $p < 0.05$.

The $IC_{50}$ values for competition curves fitted to a one-site competition model, were calculated from the following equation,

$$y = \frac{A + (B - A)}{1 + 10^{(x - \log IC_{50})}}$$

Where:

A and B = the minimum and maximum percentage specific binding respectively.

Y = specific binding at a fixed concentration of displacing drug

X = $\log_{10}$ concentration of the displacer

$IC_{50}$ = concentration of the displacer which inhibits 50% of the specific binding of the radioligand.

The $IC_{50}$ values for competition curves fitted to a two-site competition model were calculated from,

$$Y = \frac{A + (B - A)}{\left(\frac{Fraction1}{1 + 10^{(x - \log IC_{50}1)}}\right) + \left(\frac{1 - Fraction1}{1 + 10^{(x - \log IC_{50}2)}}\right)}$$

Where: A, B, X and Y are as above, (1) and (2) = the high and low affinity sites for the one-site and two-site binding models, the apparent inhibition constants ($K_I$) were calculated using the Cheng-Prusoff equation (Cheng-Prusoff, 1973),

$$K_I = \frac{IC_{50}}{\left(1+\left(\frac{L}{K_D}\right)\right)}$$

Where:

$IC_{50}$ = concentration of ligand giving 50% of the specific binding.

[L] = [$^3$H] Radioligand concentration.

$K_D$ = dissociation constant from saturation binding of [$^3$H] radioligand to the $GABA_A$ receptors.

### 2.4.7 $GABA_AR$ Binding Sites:

Three major sites present on $GABA_A$ receptors that can be directly investigated by binding studies, which include the GABA-, the benzodiazepine- and the picrotoxinin/ t-butylbicyclophosphorothionate (TBPS) binding sites, Figure 2.1, A.

Several ligands can be used for the pharmacological characterization of $GABA_AR$ by radioligand binding techniques. The following ligands were used in the present study: [$^3$H] muscimol to examine the affinity for the GABA binding site, [$^3$H] flunitrazepam to examine the affinity for the benzodiazepine site of the receptor and the convulant [$^{35}$S] TBPS to assess the site inside the chloride channel.

In this study, the pharmacology of three structurally distinct compound classes was investigated using radioligand binding techniques on native and recombinant α1β2γ2L model of $GABA_AR$ stably expressed in HEK293 cells. The molecular structure of α1β2γ2 $GABA_AR$, the most common type found in the brain, the major binding domain and ligand utilized in this study are shown in Figure 2.1, B.

### 2.4.8 GABA$_A$R Radioligand Binding, Positive & Negative Controls:

In order to validate our binding assays, a series of control compounds were tested for each binding protocol using adult rat forebrain membranes (native) and HEK293 cell homogenates expressing α1β2γ2L subunits (Recombinant).

#### 2.4.8.1 The Effect of Picrotoxin and Diazepam on [$^{35}$S] TBPS Binding to Native and Recombinant α1β2γ2L GABA$_A$R:

Two control compounds were used to validate [$^{35}$S] TBPS binding assay, picrotoxinin in native preparation and diazepam in both native and recombinant preparations. Picrotoxinin displayed a steep monophasic inhibition of [$^{35}$S] TBPS binding with Hill slope close to unity $n_H = 1.3 \pm 0.2$ with an apparent $IC_{50} = 309 \pm 1$ nM. Figure 2.2 shows the results. The observed $IC_{50}$ appears to be in close agreement with the published literature (257± 12 nM) (Wong *et al*, 1983).

Consistent with pervious published data (Concas *et al.*, 1990, Ghiani *et al.*, 1996), diazepam significantly enhanced specific binding of [$^{35}$S] TBPS to both native and recombinant preparations in a concentration-dependent manner, data were best fit to sigmoidal model, yielding a mean $E_{max} = 157 \pm 3\%$, $E_{max} = 133 \pm 4\%$ and apparent $EC_{50} = 4.3 \pm 0.5$ nM, $EC_{50} = 38 \pm 2$ nM respectively. Figure 2.3 shows the results from three independent experiments, each performed in triplicate for each membrane preparation. However, it is known from the literature that diazepam had a bidirectional modulatory effect on [$^{35}$S] TBPS binding. At low concentrations diazepam enhanced the specific binding of [$^{35}$S] TBPS whereas, at high concentrations it elicited an inhibitory effect. This effect is clearer in the recombinant preparation which is devoid of GABA, since the pharmacological action of diazepam seems to be strictly dependent on the presence of GABA at the receptor site. The observed apparent $EC_{50}$ values obtained are similar to the literature (eg. Concas *et al.*, 1990, Ghiani *et al.*, 1996).

#### 2.4.8.2 The Effect of GABA & Diazepam on [$^3$H] Flunitrazepam Binding to Native and Recombinant α1β2γ2L GABA$_A$R:

To validate the [$^3$H] flunitrazepam binding assay, two control compounds were used GABA & diazepam in native and recombinant cell membrane preparations. It is known that binding sites for GABA and benzodiazepines are allosterically coupled; this is demonstrated by the ability of GABA to enhance the binding of [$^3$H] flunitrazepam as shown in Figure 2.4. GABA significantly enhanced specific binding of [$^3$H] flunitrazepam to native and recombinant preparations in a concentration-dependent manner, yielding a mean $E_{max} = 153 \pm 4\%$, $161 \pm 8\%$ and apparent $EC_{50} = 31 \pm 2$ nM, $3 \pm 2$ μM, respectively. GABA enhancement of [$^3$H] flunitrazepam to native and recombinant preparations observed in our study was in the range of that observed for well-washed rat cerebral cortical membranes by Ghiani *et al.*, (1996 ) and membranes from HEK cells stably transfected with α1β2γ2S subunits of $GABA_AR$ reported by Pericic *et al.*,(2001).

Diazepam significantly displaced specific binding of $[^3H]$ flunitrazepam in a concentration-dependent manner in both native and recombinant preparations. Data were best fit to sigmoidal model yielding a mean apparent $IC_{50}$ = 19.4 ± 1.5 nM, 18.3 ± 1.6 nM respectively. Figure 2.5 shows the results from three independent experiments, each performed in triplicate for each membrane preparation. The potency of diazepam to displace $[^3H]$ flunitrazepam is in good agreement with the literature data obtained for the displacement of the same radioligand from cerebellar membranes (Massotti *et al*, 1991) and from HEK 293 cells transfected with α1β2γ2s subunits of $GABA_AR$ (Pritchett *et al.*, 1989, Pericic *et al.*, 2001).

**2.4.8.3 The Effect of GABA on $[^3H]$ Muscimol Binding to Native and Recombinant α1β2γ2L $GABA_AR$:**

A control experiment of $[^3H]$ muscimol binding to native and recombinant preparations, in the presence of different concentrations of GABA was carried out to validate the assay. GABA significantly displaced specific binding of $[^3H]$ muscimol in a concentration-dependent manner in both native and recombinant preparations. Data were best fit to sigmodial model variable slope with a pseudo-Hill coefficient, close to unity in both preparations ($n_H$ = 0.72 ± 0.06, 1.0 ± 0.2) with an apparent $IC_{50}$ = 106 ± 1 nM and 191 ± 1 nM respectively. Figure 2.6 shows the results from three independent experiments, each performed in triplicate for each membrane preparation. The observed potency of GABA for inhibiting $[^3H]$ muscimol binding is also in good agreement with the binding affinity of this drug for wild type α1β2γ2 membranes reported by Baur *et al* ., (2003).

**2.4.9 NMDA Receptor, Radioligand Positive Control:**

**2.4.9.1 The Effect of Ketamine on $[^3H]$ MK-801 Binding to Adult Rat Forebrain.**

A control experiment of $[^3H]$ MK-801 binding to adult rat forebrain in the presence of different concentrations of Ketamine was carried out to validate the assay. Ketamine significantly displaced specific binding of $[^3H]$ MK-801 in a concentration-dependent manner in native preparation. Data were best fit to one site model yielding a mean of apparent $IC_{50}$ = 2 ± 1 mM. Figure 2.7 shows the results. The observed $IC_{50}$ value appears to be lower than the literature value for some studies (Wang *et al.*, 1999).

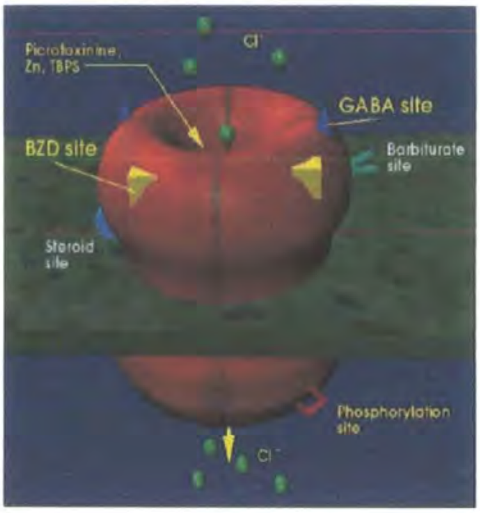
### 2.4.10 Nicotinic Acetylcholine Receptor, Radioligand Binding, Positive Control:

#### 2.4.10.1 The Effect of Nicotine on [$^3$H] Nicotine Binding to Adult Rat Forebrain.

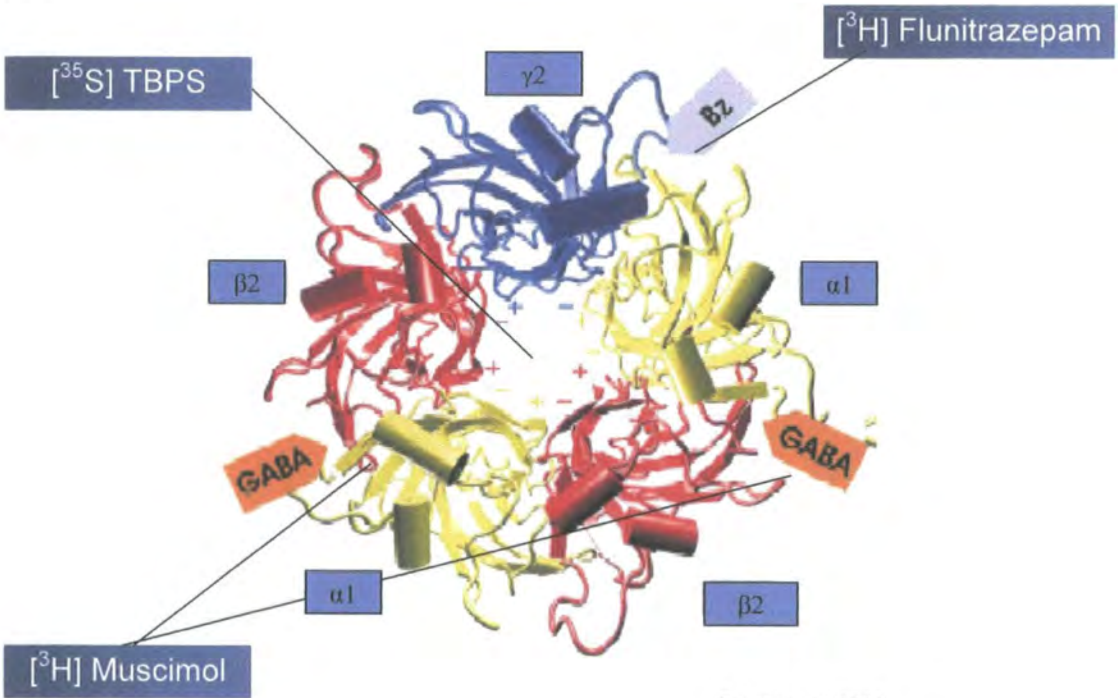
A control experiment of [$^3$H] nicotine binding to adult rat forebrain, in the presence of different concentrations of nicotine, was carried out to validate the assay. Figure 2.8 shows the results. Nicotine produced a concentration dependent inhibition of [$^3$H] nicotine binding, data was best fit to two site competition model, comprising high and low affinity binding sites in the ratio 74: 26 ( high: Low % , SD ± 6). Site one apparent $IC_{50}$ 4.5 nM, site two apparent $IC_{50}$= 3.5 µM. Nicotine binds to the α4β2 nAChR with high affinity (4.5 nM) whereas the α7 is 1000-fold less sensitive (3.5 µM). This selectivity profile is consistent with the published literature (Peng *et al*, 1994, Xiao *et al*, 1998).

Overall, the standard compounds displayed the appropriate pharmacological properties based on the published literature. Any small discrepancies in affinities were probably due to differences in experimental protocols and tissue preparations used.

**(A)**




**(B)**




Ernest *et al*, 2003

**Figure 2.1:** Model structure of $GABA_A$ receptor showing

**(A)** Three binding site domains (GABA, benzodiazepine and picrotoxin / TBPS).

**(B)** The absolute arrangement for α1, β2 and γ2 containing $GABA_A$ with the major binding sites and radioligand used in this study.

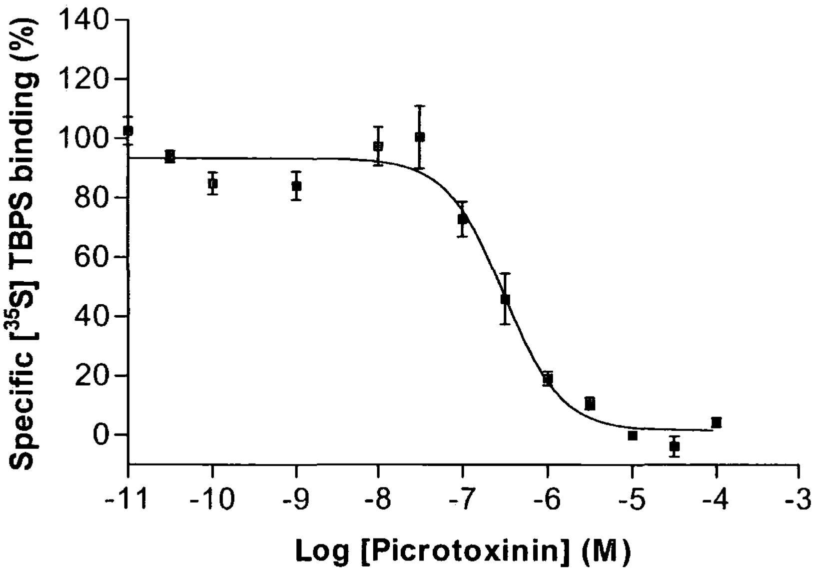
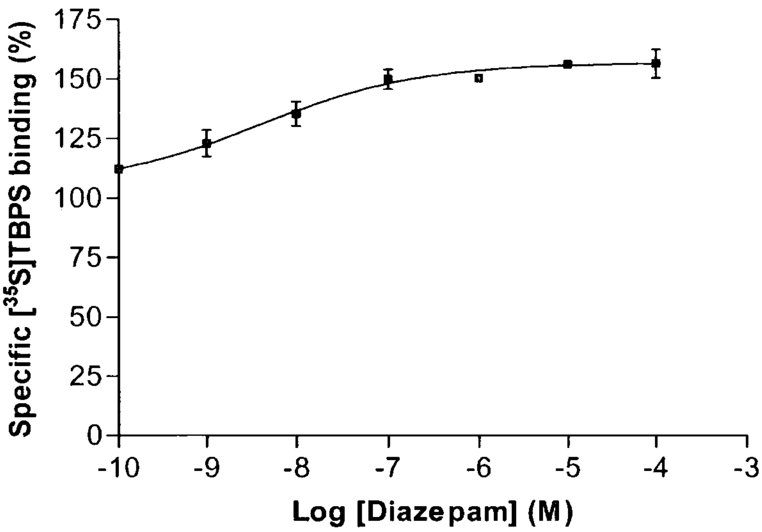



**Figure 2.2:** [$^{35}$S] TBPS competition binding to well-washed adult rat forebrain membranes by picrotoxinin. Results are mean ± S.D for three independent experiments each performed in triplicate.

A.




B.

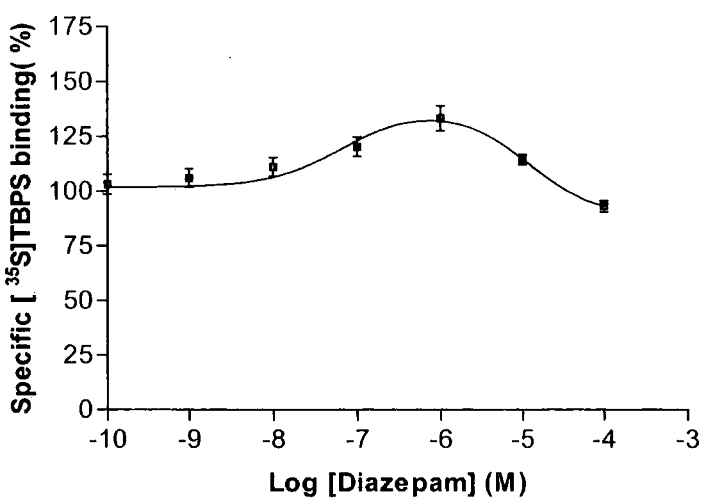
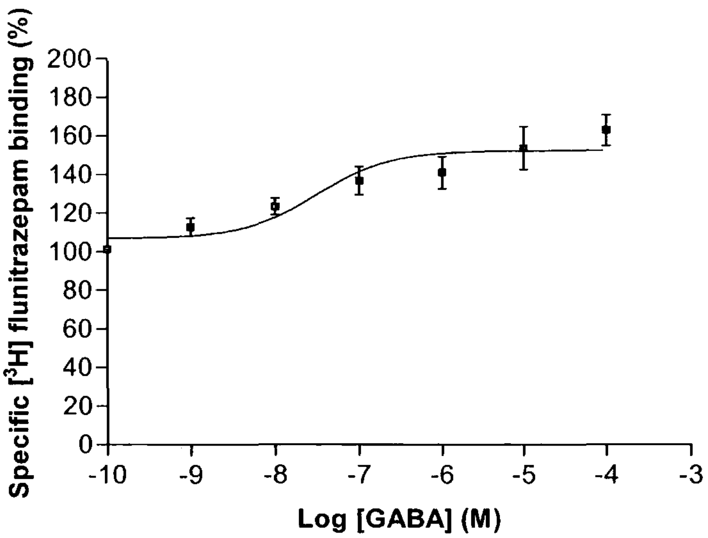



**Figure 2.3**: [$^{35}$S] TBPS competition binding to **(A)** well-washed adult rat forebrain membranes **(B)** $GABA_AR$ cell line by diazepam. Results are mean ± S.D for three independent experiments each performed in triplicate.

A.




B.

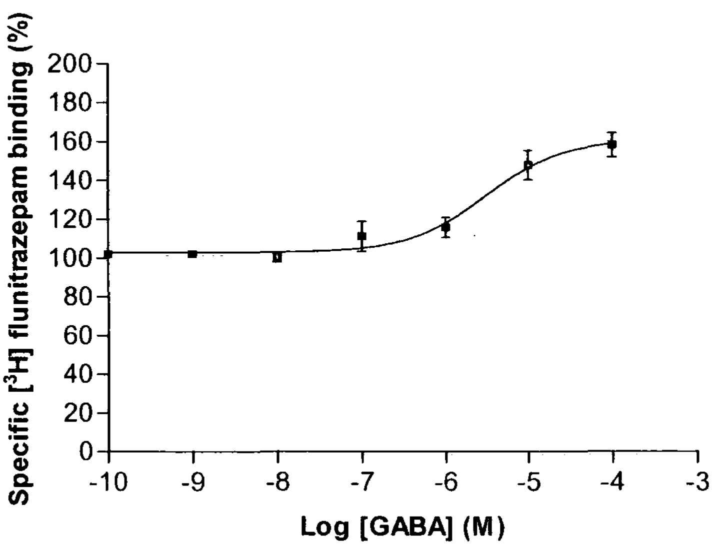
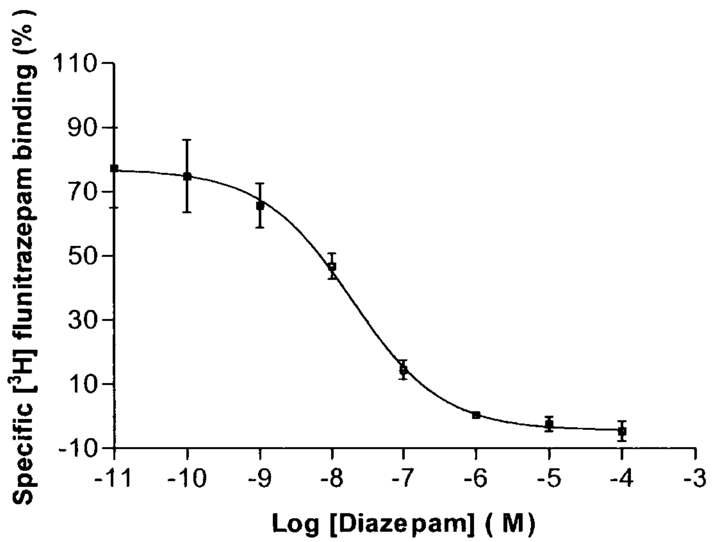



**Figure 2.4:** Effect of GABA upon [$^3$H] flunitrazepam binding to **(A)** adult rat forebrain membranes **(B)** GABA$_A$R cell line. Results are mean ± S.D for three independent experiments each performed in triplicate.

A.




B.

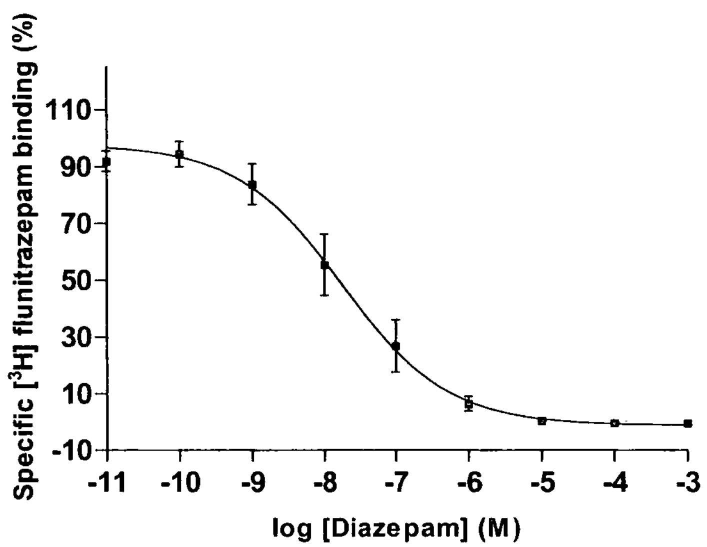
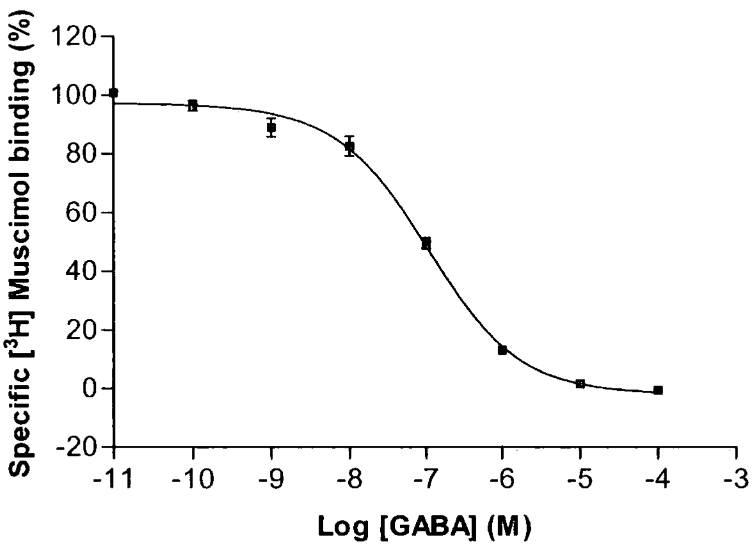



**Figure 2.5:** Effect of Diazepam upon [$^3$H] flunitrazepam binding to **(A)** adult rat forebrain membranes **(B)** $GABA_AR$ cell line. Results are mean ± S.D for three independent experiments each performed in triplicate.

A.




B.

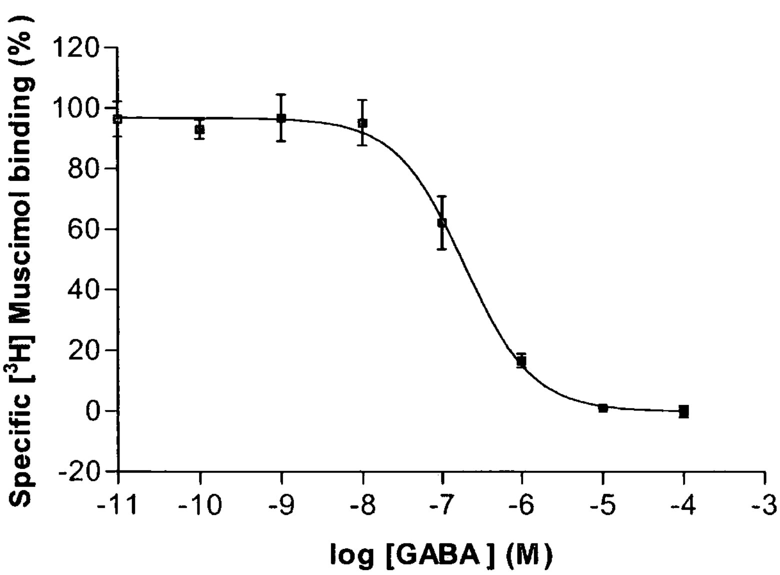



**Figure 2.6:** [$^3$H] muscimol competition binding to **(A)** adult rat forebrain membranes. **(B)** $GABA_AR$ cell line by GABA. Results are mean ± S.D for three independent experiments each performed in triplicate.

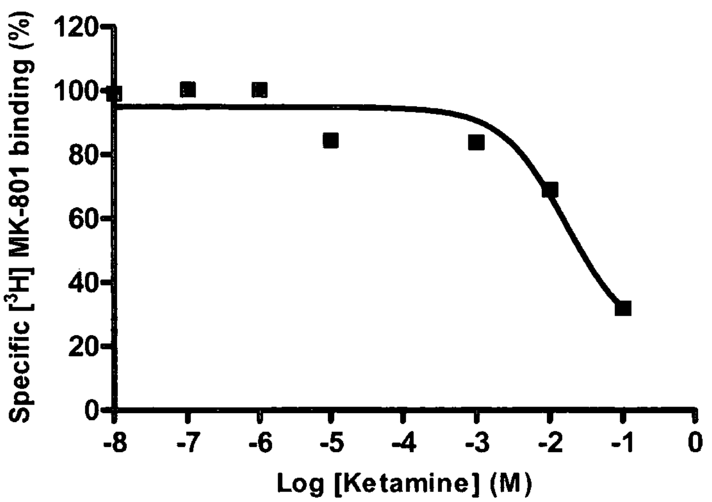



**Figure 2.7:** [$^3$H] MK-801 competition binding to adult rat forebrain membranes by ketamine.

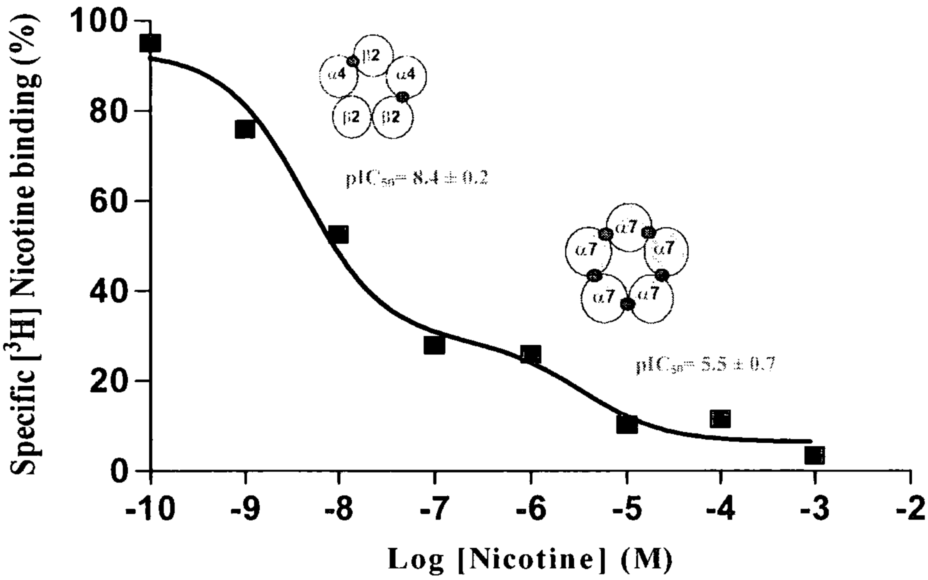



**Figure 2.8:** [$^3$H] nicotine competition binding to adult rat forebrain membranes by nicotine. Results are mean ± S.D for three independent experiments each performed in triplicate.

# Chapter 3

# Detailed $GABA_A$ Receptor Pharmacological Characterization of Non-steroidal Anti-inflammatory Drug, Mefenamic acid

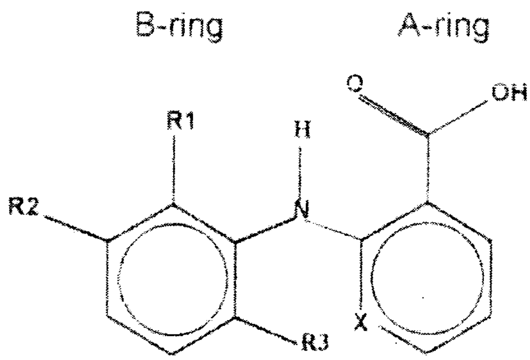
## 3.1 Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are prescribed for their analgesic, anti-inflammatory and anti-pyretic properties (Rang *et al.*, 2003). Due to their efficacy and relative lack of toxicity, NSAIDs are among the most widely used therapeutic agents worldwide (Kean *et al.*, 2005).

Fenamates are a class of NSAIDs derived from N-phenyl-anthranilic acid. The most common ones are Mefenamic, Flufenamic, Meclofenamic and Niflumic acids. The therapeutic activity of these compounds is believed to be due their ability to reduce prostaglandin synthesis by inhibiting the cyclo-oxygenase pathway (Flower, 1974, Ham *et al.*, 1972). Fenamate NSAIDs commonly prescribed for the relief of mild to moderate pain including that experienced during dysmenorrhoea and rheumatoid arthritis (Fang *et al.*, 2004). The most common side effects observed with their treatment, like other NSAIDs, generally pertain to gastrointestinal disturbances. However, other adverse effects observed include headache, visual disturbances, dizziness, drowsiness and anxiety (Kean *et al.*, 2005).

Each fenamate molecule is essentially made up of three planar groupings: two six-membered rings A, B bridged by an imino N atom and a carboxyl group attached ortho to the imino N atom on ring A, Figure 3.1. The differences in fenamates are due to different substituents on the second six-membered ring. A further difference between niflumic acid and flufenamic acid is the replacement of a CH group by an N atom in ring A of the former.

Almost thirty years ago, Vane (1971) demonstrated that aspirin like-drugs namely NSAIDs, inhibited prostaglandin synthesis, thus reducing inflammation and thereby symptoms of pain. Subsequently, over 50 NSAIDs used in clinical practice have been found to inhibit prostaglandin synthesis (Rang *et al.*, 2003). In addition to inhibition of prostaglandin synthesis in the periphery, NSAIDs are known to penetrate the central nervous system (CNS) (Bannwarth *et al.* 1989), suggesting that they may have direct effects on neuronal function. They are analgesic even when administered directly into the CNS (Malmberg & Yaksh 1992, McCormack 1994). Indeed, the fenamate NSAIDs, MFA prevents convulsions and protects rats from seizure-induced forebrain damage evoked by pilocarpine (Ikonomidou-Turski *et al.* 1988). MFA is also anti-epileptogenic against pentylenetetrazol (PTZ)-induced seizure activity at low doses, but will potentiate PTZ-evoked seizures at higher doses (Wallenstein, 1991). In humans, MFA is associated with coma (Gossinger *et al.* 1982, Hendrickse, 1988) and convulsions in over a third of all cases of overdose (Smolinske *et al.*, 1990).




| Fenamate | R1 | R2 | R3 | X |
|---|---|---|---|---|
| Mefenamic Acid | $CH_3$ | $CH_3$ | H | C |
| Meclofenamic acid | Cl | $CH_3$ | Cl | C |
| Flufenamic Acid | H | $CF_3$ | H | C |
| Tolfenamic Acid | Cl | $CH_3$ | H | C |
| Niflumic Acid | H | $CF_3$ | H | N |

**Figure 3.1**: General structure representation of fenamates and a table of group substitutions for a series of Fenamates.

## 3.2 Physiological Effects of NSAIDs on Ion Channel Function:

A number of studies have demonstrated that NSAIDs modulate ion channel function on various neuronal and non-neuronal ion channels including non-selective cation channels (Lerma & Del Rio, 1992, Shaw *et al.*, 1995) and chloride channels (White & Aylwin, 1990, McCarty *et al.*, 1993).

### 3.2.1 Non-Neuronal Preparations:

Several studies have shown that certain fenamates induce intracellular $Ca^{2+}$ release. McDougall *et al.* (1988) demonstrated that Flufenamic acid and MFA uncoupled oxidative phosphorylation causing an inhibition of calcium uptake with $IC_{50}$s of 7 μM and 68 μM (respectively) and thereby increased cystosolic $Ca^{2+}$ levels in mitochondria isolated from rat liver.

Later, Northover *et al.* (1990) showed that Flufenamate (5μM) induced intracellular release in isolated myocardial cells. Poronnik *et al.* (1992) showed that Flufenamic (with $EC_{50}$ of 100 μM), MFA and Niflumic acid (in descending order of potency) induced intracellular $Ca^{2+}$ release in a mouse mandibular cell line. In addition, Flufenamic acid (between 37-500 μM) and MFA (with less potency) have been shown to directly activate potassium channel in human jejunum (Farrugia *et al.,* 1993a) similar results were obtained in canine jejunum (Farrugia *et al.*, 1993b).

Niflumic acid ( $K_d$ of 261 μM), Flufenamic acid and MFA (although less potently than flufenamic acid) also potentiated calcium-activated $K^+$ channels in plasma membrane vesicles from pig coronary smooth muscle by increasing open channel probability (Ottolia & Toro, 1994). Fenamate have also been shown to inhibit non-selective cation channels in non-neuronal preparations. For example, Flufenamic, MFA (both with an $IC_{50}$ of 10 μM) and Niflumic acid ($IC_{50}$ of 50 μM) inhibited non-selective cation channels in rat exocrine pancreas (Gögelein *et al.*, 1990). Fenamates have also been shown to block non-selective cation channels in rat distal colon cells (Siemer & Gögelein, 1992) in murine L cells (Jung *et al.,* 1992) and in mouse mandibular cell line (Poronnik *et al.*, 1992).

An early study by Cousin & Motais (1979) demonstrated that Niflumic and Meclofenamic acid with ($IC_{50}$ of 0.63 μM and 0.75 μM respectively) non-competitively inhibited anion transport in human erythrocyte. More recently, it has been shown that fenamate inhibit $Ca^{2+}$ activated chloride channels in certain epithelial cell types (Chao & Mochizuki, 1992). For example Niflumic and Flufenamic acid inhibit chloride conductance in the basolateral membrane lining the ascending loop of Henle in rabbit kidney (Wangemann *et al.*, 1986). In addition, Flufenamic and Niflumic acid have been shown to inhibit $Ca^{2+}$ activated chloride conductance in Xenopus oocytes (White & Aylwin, 1990, Woodward *et al.*, 1994). McCarty *et al.* (1993) reported that Flufenamic acid (200 μM) inhibits the cystic fibrosis transmembrane conductance regulator chloride channel expressed in Xenopus oocytes by a voltage dependant mechanism, suggesting open-channel blockade.

### 3.2.2 Neuronal Preparations:

There have been few studies investigating the action of NSAIDs on ion channel function in neuronal preparation. However, early studies demonstrated that salicylic acid at millimolar (1-30 mM) concentrations, inhibited chloride channel ion permeability and increased potassium ion permeability in buccal ganglion neurons of the marine mollusc, Navanax inermis (Barker & Levitan, 1971).

Neto (1980) later demonstrated that salicylic acid (2-5 mM) reduced the spike amplitude, and at higher concentrations (10-20 mM), blocked conduction of the compound action potential recorded in rabbit vagus and frog sciatic nerve. Interestingly, Shaw *et al.*, (1995) demonstrated non-selective cation channel block with Flufenamic acid (300-500 μM) but not MFA in molluscan neurons. Lerma & Martin del Rio (1992) reported that Niflumic and Flufenamic inhibited NMDA-gated cation channel in mouse spinal cord neurons, with $IC_{50}$ values of ~ 350 μM. Chen *et al.* (1998) reported an inhibition of NMDA, but not kainate-mediated responses, by MFA, Meclofenamic and Flufenamic acid (all at 1mM), recorded in salamander retinal ganglion neurons. The studies reviewed above clearly reveal that NSAIDs influence the behaviour of a variety of ion channel.

#### 3.2.2.1 Fenamate & $GABA_AR$:

Three studies demonstrated that fenamates modulate neuronal $GABA_AR$. A radio-ligand binding study by Evonuik & Skolnick (1988) demonstrated that Niflumic acid inhibited ($Cl^-$) modulated [$^{35}S$] TBPS binding to rat neuronal $GABA_AR$ and suggested that Niflumic acid acts at or near a binding site within the GABA gated ($Cl^-$) channel. Woodward *et al.*, (1994) demonstrated that fenamate modulated rat cortical $GABA_AR$ expressed in Xenopus oocyte. Furthermore, a study by Halliwell *et al.*, (1999) demonstrated that MFA modulate human recombinant $GABA_AR$ function expressed in both Xenopus oocyte and HEK 293 cells.

## 3.3 Novel Clinical Application of Fenamate NSAIDs:

### 3.3.1 Subunit-Selective Modulation of $GABA_AR$:

The Fenamate group of NSAIDs have been proposed to exhibit receptor subtype-dependant positive and negative modulation of the $GABA_A$ receptor. Halliwell *et al.,* (1999) demonstrated that MFA potentiated GABA-activated currents for $\alpha1\beta2\gamma2_S$ ($EC_{50} = 3.2 \pm 0.5$ μM), but not for $\alpha1\beta1\gamma2_S$ receptors. MFA also enhanced GABA-activated responses and directly activated $\alpha1\beta2/\beta3$ $GABA_A$ receptors, but inhibited responses to GABA on $\alpha1\beta1$ constructs ($IC_{50} = 40 \pm 7.2$ μM). A comparison of β1, β2 and β3 subunits suggested that the positive modulatory action of MFA involved asparagine (N) 290 in the second transmembrane domain (TM2) of the β2 and β3 subunits. Mutation of N290 to serine (S) markedly reduced modulation by MFA in $\alpha1\beta2$ (N290S) $\gamma2_S$ receptors, whereas $\alpha1\beta1$ (S290N) $\gamma2_S$ constructs revealed potentiated responses to GABA $EC_{50} = 7.8 \pm 1.7$ μM and direct activation by MFA. The potentiation by MFA displayed voltage sensitivity. The direct activation, potentiation and inhibitory aspects of MFA action were predominantly conferred by the β subunits as the spontaneously active homomeric β1 and β3 receptors were susceptible to modulation by MFA. Molecular comparisons of MFA, loreclezole and etomidate agents which exhibit similar selectivity for $GABA_AR$, revealed their ability to adopt similar structural conformations. This study indicates that N290 in TM2 of β2 and β3 subunits is important for the regulation of $GABA_AR$ function by MFA (Halliwell *et al.,* 1999).

By using quantitative autoradiography with $GABA_AR$-associated ionophore ligand [$^{35}S$] TBPS on rat brain sections, Sinkkonen *et al.* 2003 demonstrated that one of the fenamates, Niflumate, at micromolar concentration was found to potentiate GABA actions in most brain areas, whereas being in the cerebellar granule cell layer an efficient antagonist similar to Furosemide. With recombinant $GABA_A$ receptors expressed in *Xenopus laevis* oocytes, they found that Niflumate potentiated 3 μM GABA responses up to 160% and shifted the GABA concentration-response curve to the left in $\alpha1\beta2\gamma2$ receptors, the predominant $GABA_AR$ subtype in the brain. More recently Smith *et al.* (2004) examined the influence of different β subunit expression on the potency and efficacy of a variety of ligands to modulate and activate the ion channels, using FRET technique. Several compounds discriminated $\beta_2/\beta_3$ from $\beta_1$-containing receptors including the anticonvulsant loreclezole, the anaesthetic etomidate, and a group of anti-inflammatory agents including MFA.

### 3.3.2 Ischaemia & Neuroprotection:

Post-ischaemic inflammation has been implicated in playing an important role in the delayed progression of damage to brain tissue (Kochanek & Hallenbeck, 1992). Following cerebral ischaemia, local expression of a cascade of inflammatory protein is induced which includes COX-2, a mediator of the cytotoxic effects of inflammation (Seibert *et al.*, 1995, Smith & Dewitt, 1995). Nogawa *et al.* (1997) have demonstrated in rats that cerebral ischaemia leads to the up-regulation of COX-2 (but not COX-1) expression, protein and reaction products ($PGE_2$) within the injured site. The selective COX-2 inhibitor, NS-398, attenuated the ischaemic damage, suggesting that selective COX-2 inhibitors may be protective during the post-ischaemic period.

Chen *et al.* (1998) reported that fenamate NSAIDs (and the NMDA receptor antagonist MK-801) protect embryonic chick retinal neurones against glutamate-induced damage and ischaemia-induced injury. In particular, fenamates were protective against NMDA and kainate–induced excitotoxicity. The authors suggested that although inhibition of prostaglandin synthesis by NSAIDs undoubtedly plays a role in their neuroprotective effects during post-ischaemic inflammation, other mechanisms may contribute to these effects.

Asanuma *et al.* (2001, 2004) demonstrated that NSAIDs aspirin, MFA, indomethacin and ketoprofen directly and dose-dependently scavenged generated nitric oxide radicals. Results suggest that the protective effects of these four non-steroidal anti-inflammatory drugs against apoptosis might be mainly due to their direct nitric oxide radical scavenging activities in neuronal cells. These direct nitric oxide quenching activities represent novel effects of NSAIDs for neuroprotective effects.

### 3.3.3 Alzheimer's Disease:

Recent data also suggests that the neurodegeneration associated with AD involves COX enzymes. AD lesions are characterized not only by the presence of amyloid plaques and neurofibrillary tangles, but also by the accumulation of many inflammatory proteins, such as inflammatory cytokines, complement proteins and their regulators, which may be promote neuronal death (McGeer *et al.*, 1994, 1995, Asanuma *et al.*, 2001, Cacquevel *et al.*, 2004). These pro-inflammatory cytokines cause a marked induction of COX-2 enzyme level (Hamplel & Müller, 1995, Cochran & Vitek, 1996). These data have led to the hypothesis that patients taking NSAIDs to control other anti-inflammatory diseases, such as arthritis, may also have a reduced chance of developing AD. Although there is no direct evidence to-date, a number of epidemiological studies have indicated that NSAIDs (and other anti-inflammatory treatments) may indeed delay the onset and slow the progression of neurodegenerative disorders such as AD (McGeer *et al.*, 1996, McGeer & McGeer 2003, Sugaya *et al.*, 2000, Yan *et al.*, 2003). In addition, Breitner *et al.* (1995) have reported a delay onset of AD with NSAIDs and histamine $H_2$ blocking drugs and suggest that the actions of these very different drugs may be linked to the action of COX on the NMDA pathway to reduce NMDA-mediated glutamatergic excitotoxicity.

Together, the data above strongly indicate that NSAIDs exert analgesic and anti-inflammatory effects not only in periphery, but also within the CNS. The mechanisms underlying these actions may involve targets additional to COX enzyme inhibition.

Given the pharmacological importance of fenamates and their potential subtype selective modulation of $GABA_AR$ this suggests that the structure of fenamate might be a useful template for the design of a novel anti-epileptic and /or neuroprotective drugs. Further insights into the mechanisms of potentiation and inhibition will require additional types of study, particularly binding assays.

In the present study, a number of analogues of MFA, were synthesized and tested on $GABA_ARs$. Pharmacological characterization of the synthesized compounds was carried out using receptor binding assays. In addition, a molecular modelling study based on MFA was performed to explore the dimensions and properties of different size substituents on the structural flexibility and molecular geometry in this chemical series.

## 3.4 Materials & Methods

### 3.4.1 Materials:

A series of MFA derivatives, substituted with different groups on the second six-membered ring, were synthesized by our collaborator Dr.Patrick Steel (Chemistry Department, Durham University). Chemical structures of these compounds, their chemical formulas and molecular weights are shown in Table 3.1.

### 3.4.2 Methods:

A series of dose-response competition binding experiment were performed with [$^{35}$S] TBPS, [$^{3}$H] Muscimol and [$^{3}$H] Flunitrazepam using well-washed adult rat forebrain and HEK 293 cells stably expressing recombinant α1β2γ2L $GABA_A$R subunits.

Well-washed adult rat forebrain membrane preparation, Lowry assay protein concentration determination , cell culture and radioligand binding assays were all performed as described in Chapter 2, [section 2..4.2., 2.4.3, 2.4.4 and 2.4.5 respectively].

### 3.4.3 Molecular Modelling:

MFA and 14 analogues substituted with different groups on the second six-membered ring have been included in a chemical comparative analysis, in collaboration with Dr.Colin James (School of Pharmacy, London University). The molecules were built and the geometry of the structures has been optimized to minimum energy using Open Eye Scientific software for Molecular Modelling (http://www.eyesopen.com).

MFA (Compound 6) can be considered in at least four conformations which have been called C6_symm1, C6_symm 2, C6_symm3 and C6_symm 4, each with about the same potential energy (when considered as isolated molecules). Conformer 1 & 3 are related by symmetry as is 2 &4 since these pairs have closer energy to each other.

Each of these four conformations of C6 was then used in separate comparisons, comparing each in turn with all the conformations of all analogues. Measurement of chemical similarity was carried out using two programmes; one called "ROCS" which is (a shaped–based superposition method). The other one called "EON" which calculates the electrostatic property similarities between query molecule (MFA, conformers) and a set of conformers of the 14 analogues.

# 3.5 Results

## 3.5.1 Effect of MFA & Analogues on the Binding of Ligands to the $GABA_AR$ complex:

### 3.5.1.1 Effect of MFA & Analogues on the Picrotoxin Binding Site of the $GABA_AR$ Complex labelled by [$^{35}$S] TBPS:

In the initial studies, we examined the effect of MFA on the binding of [$^{35}$S] TBPS to well-washed adult rat forebrain and $GABA_AR$ cell line membranes. Figure 3.2 shows that MFA did not alter the binding of TBPS ligand to either rat forebrain or $GABA_AR$ cell line membranes, across the full range of concentrations of MFA in both preparations in at least three independent experiments. There was a decrease in the binding at 100 µM for the MFA in both preparations; this was likely due to the presence of 0.1% DMSO (solvent used to dissolve the drug).

In order to examine whether these effects were shared by all MFA derivatives, the 14 analogues were examined for their abilities to inhibit [$^{35}$S] TBPS binding to adult rat forebrain membranes, at two different concentrations (10 and 30 µM). Figure 3.3 shows the results. None of the tested compounds showed any activity on specific [$^{35}$S] TBPS binding in three independent experiments at both tested concentrations. Results suggest a lack of interaction of these compounds with picrotoxin site of the $GABA_AR$.

### 3.5.1.2 Effect of MFA & Analogues on the Benzodiazepine Binding Site of the $GABA_ARs$ Complex labelled by [$^3$H] Flunitrazepam:

Since MFA and analogues did not affect the picrotoxin site of $GABA_AR$ within the concentration range tested, further binding experiments were performed on other binding sites of the $GABA_AR$. Figure 3.4 shows that no significant effect (positive or negative) was observed with MFA upon [$^3$H] flunitrazepam binding in three independent experiments in both preparations. This suggests a lack of interaction (either directly or allosterically) of this compound with the benzodiazepine site of the $GABA_AR$.

To confirm our results, [$^3$H] flunitrazepam binding assay was carried out for selected analogues of MFA, each with different substituted groups; these were compound 3, compound 8, compound 12 and compound 15. All selected compound failed to have any inhibitory or stimulatory effects in comparison with MFA. A decrease in the specific binding at 100 µM concentrations of all tested compounds was likely due to the presence of 0.1% DMSO (solvent effect). Results strongly suggested a lack of allosteric or competitive linkage of MFA and analogues with the benzodiazepine binding site of the $GABA_AR$. Figure 3.5 shows the results.

#### 3.5.1.3 The Effect of MFA on the Agonist Binding Site of the $GABA_AR$ Complex labelled by [$^3$H] Muscimol:

Binding results demonstrated that MFA and analogues did not affect the picrotoxin binding site, or the benzodiazepine site. In order to assess whether MFA directly binds, or allosterically modulates muscimol binding to the agonist binding site, a range of concentrations of MFA were tested upon [$^3$H] muscimol binding to a well-washed rat forebrain and $GABA_AR$ cell line preparations. Figure 3.6 show that MFA produced a concentration dependant inhibition of specific [$^3$H] muscimol binding to well washed rat forebrain membranes. Data was best fit to sigmodial model variable slopes with apparent $IC_{50}$= 5 ± 1 µM, $n_H$= -0.89 ± 0.2. In contrast, MFA stimulated the specific binding of [$^3$H] muscimol to agonist site in a concentration dependant manner in $GABA_AR$ cell line, yielding a mean of $E_{max}$ = 175 ± 5% and apparent $EC_{50}$ = 5 ± 1 µM. Figure 3.6 shows the results.

#### 3.5.1.4 The Effect of MFA analogues on the Agonist Binding Site of the $GABA_AR$ Complex labelled by [$^3$H] Muscimol:

Results showed that MFA exhibit selective efficacy at $GABA_AR$ agonist binding site. We next examined other MFA analogues, to confirm the modulatory activity at this site of $GABA_AR$ and to investigate which structural features of this group of compounds determine efficacy and affinity as preliminary structure-activity studies in this chemical series. Interestingly, many of the MFA analogues selectively stimulated [$^3$H] muscimol binding to $GABA_AR$ cell line in a concentration dependant manner, exhibiting varying levels of efficacy and affinity. Figure 3.7 shows the results. A summary of [$^3$H] muscimol binding affinities and efficacies of all tested compounds to membranes of $GABA_AR$ cell line are shown in Table 3.2

### 3.5.2 Molecular Modelling Analysis:

Structural comparison of MFA and 14 analogues molecules were performed using "Pymol" software. Molecular conformations were energy minimized using MacroModel. The molecules were then re-assigned charges using AMIBCC (part of QuACPAC).

MFA (Compound 6) can be considered in at least four conformations which have been called C6_symm1, C6_symm 2, C6_symm3 and C6_symm 4, each with about the same potential energy (when considered as isolated molecules). Conformer 1 & 3 are related by symmetry as is 2 &4 since these pairs have closer energy to each other. The four possible conformations of MFA are shown in Figure 3.8.

Each of these four conformers of C6 was then modelled independently, comparing each in turn with all the conformations of all molecules. Comparative chemical analysis was carried out using "ROCS" (Rapid Overlay of Chemical Structures) a shape –based superposition method. A ROCS tanimoto value (0-1) ordered the results. This is shown in the column headed EON_shape. Once aligned the program called "EON" was used to compare the electrostatic properties which is dependent on conformational changes rather than shape, placing a new order on the results; this is shown in the column headed ET_pb. In addition the pharmacological binding parameters ($EC_{50}$ & $E_{max}$) for [$^3$H] muscimol binding to membranes of $GABA_AR$ cell line by MFA and analogues are also shown. Table 3.3, 3.4, 3.5 and 3.6 summarizes the parameters for modelling of all compounds conformations with MFA conformers 1, 2, 3 and 4 as query molecules.

Interestingly, structural similarity modelling of MFA (4 conformers) with all conformers of analogues showed a high structural similarity between MFA conformer 1 and 3 (conformers with the same potential energy) with a group of same compounds but with different conformers matching , detected from Eno_shape and ET_pt numbers closer to a value of unity , in comparion with MFA conformers giving the values of Eno_shape and ET_pt equals to unity as the query molecule.

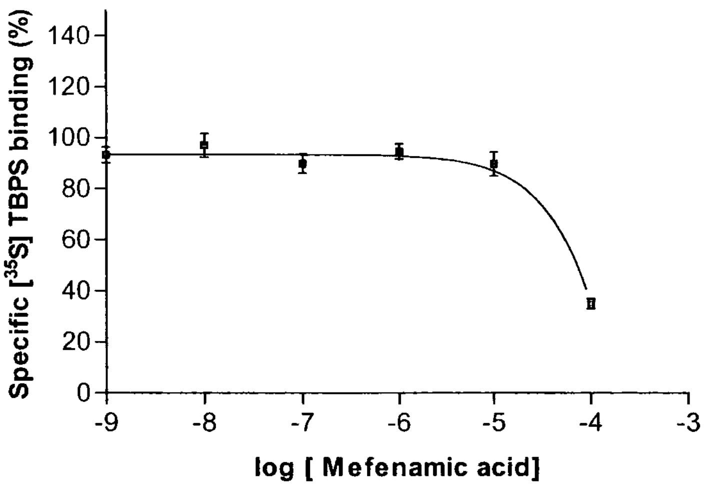
**Conformer 1 MFA** : showed high structural similarility with C1_symm 1, C2 _symm 1, C3_symm 1, C4_symm 1 ,C5_symm 4, C10_symm 1, C10_sym 4, C15_symm 6 and C15_symm 7. **Conformer 3 MFA:** showed high structural similarility with C1_symm 2, C2 _symm 2, C3_symm 2, C4_symm2 ,C5_symm 2, C10_symm 2, C10_sym 3, C15_symm 5 and C15_symm 8.

In contrast, weak structural similarity link between MFA conformer 2 and 4 (conformers having the same potential energy) and all analogue conformers. High structural similarity was only detected with compound 10, again with different conformers matching. **Conformer 2 MFA** showed high chemical similarility with C10_symm 6 and **Conformer 4 MFA** showed high chemical similarility with C10_symm 5.

These Molecular modelling data suggest that substituted $R_1$ and $R_2$ groups on the ring B of fenamate structure plays a significant role in the binding activity of these compounds , high chemical similarity between MFA (conformer 1 & 3) and C1, C2, C3, C4, C5, C10 and C15 suggesting that introduction of alkyl group subsitiution ( methyl or ethyl) on $R_1$ or $R_2$ position results in enhancement of the activity in this chemical series. Replacement of the $R_1$ group with H, Cl or OMe are tolerated but with a significant reduction of binding efficacy for the $GABA_AR$ agonist site. Compounds 2, 3, 4 and 15 showed an increase in efficacy for $GABA_AR$ agonist binding sites with an affinity that is comparable to MFA except compound 4 which showed a decrease in the binding affinity. In contrast compound 1, 5, and C10 showed a reduced binding efficacy for $GABA_AR$ agonist binding sites, with a binding affinity that is comparable to MFA. Figure 3.9 shows conformers structure of compound 2 and 15 both with high binding affinity for $GABA_AR$ agonist site, and compound 8 with low binding affinity.

In view of the above , we can conclude that subsitution on $R_1$ or $R_2$ appears to be an important determinant for intermolecular interactions in this chemical series. No subsitution on $R_1$ , methyl or ethyl subsitution on $R_1$ or R2 , methyl group at the meta position of ring B, OMe or $Cl^-$ at R1 are all possible structural features which appear to be critical for the activity of MFA on $GABA_AR$.

A.




B.

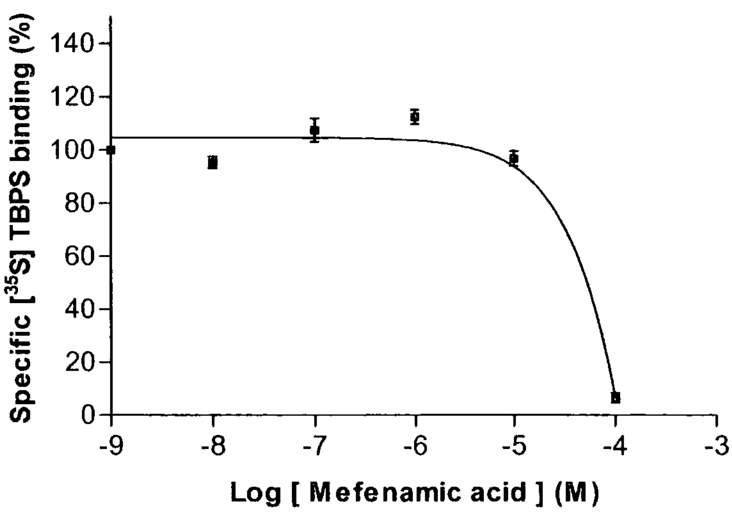
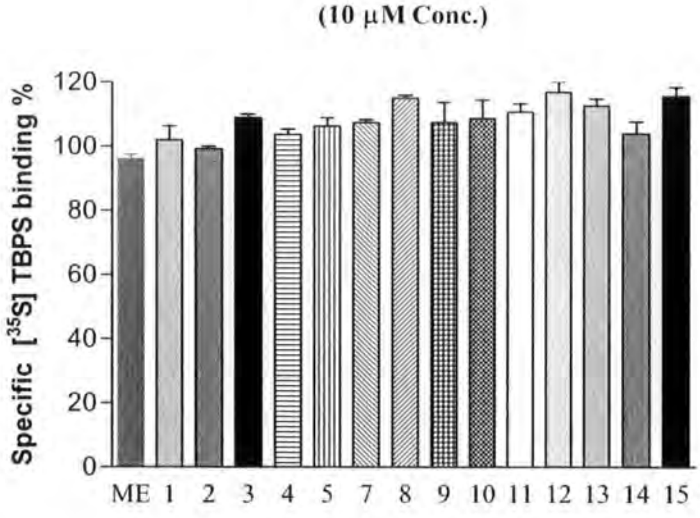



**Figure 3.2**: [$^{35}$S] TBPS competition binding to **(A)** well-washed adult rat forebrain membranes **(B)** $GABA_AR$ cell line by MFA. Results are expressed as percentages mean ± S.D. for three independent experiments for each.

A.

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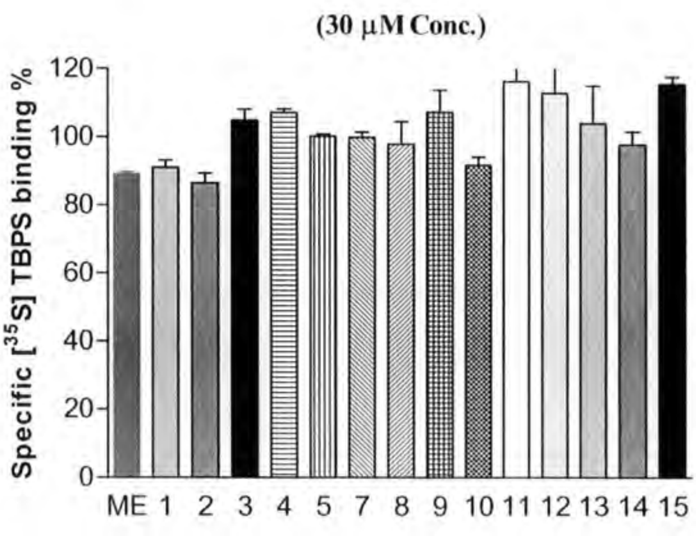



**Figure 3.3**: Effects of MFA & analogues on specific [$^{35}$S] TBPS binding to well-washed adult rat forebrain membranes at **(A)** 10 μM, **(B)** 30 μM. Results are expressed as percentages mean ± S.D. for three independent experiments for each.

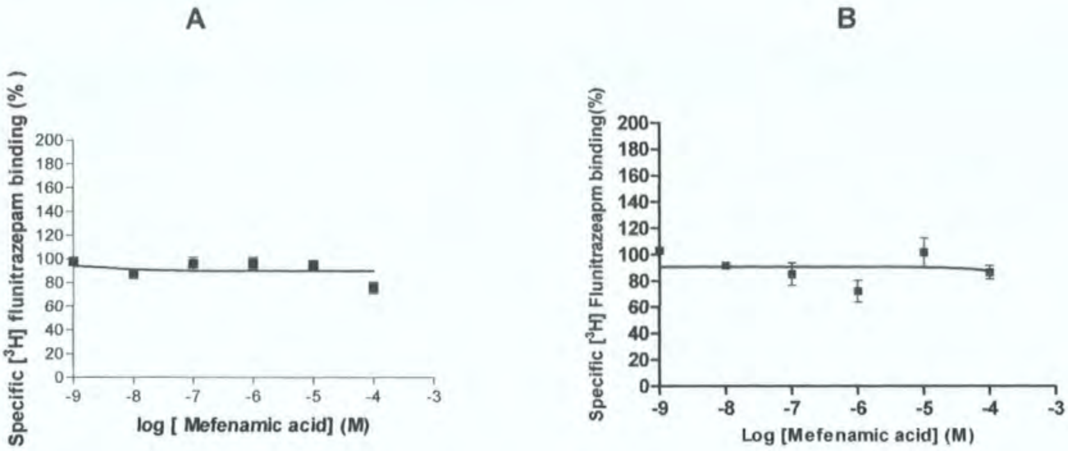



**Figure 3.4:** [$^3$H] flunitrazepam competition binding to **(A)** adult rat forebrain membranes **(B)** $GABA_AR$ cell line by MFA. Results are expressed as percentages mean ± S.D. for three independent experiments for each.

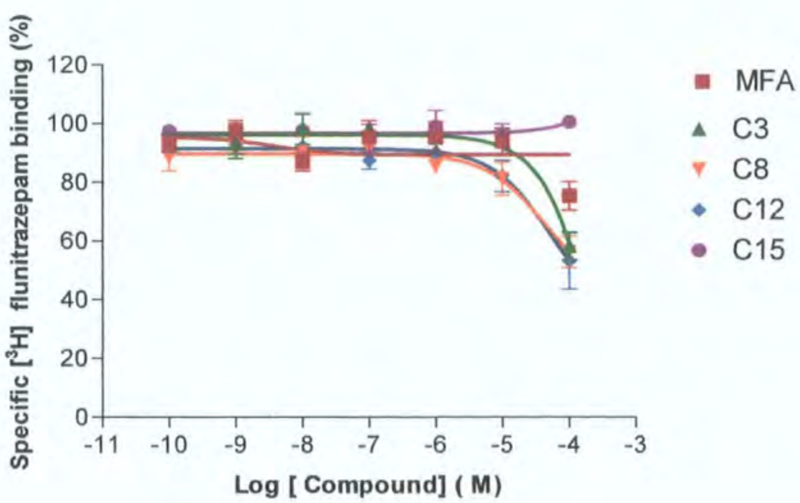
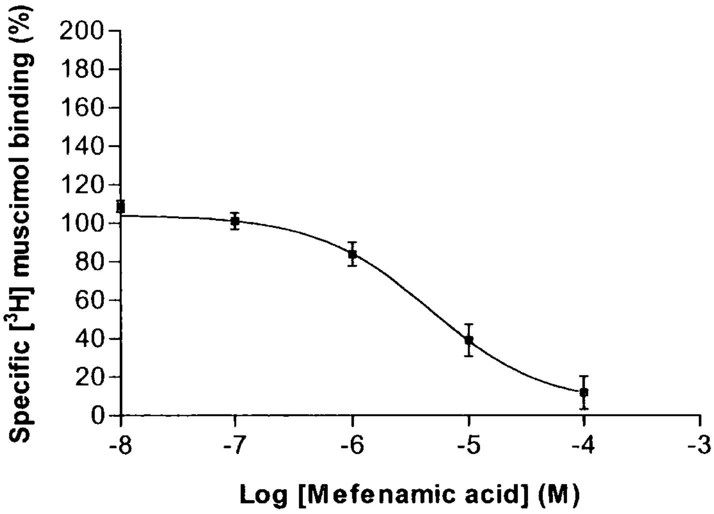



**Figure 3.5:** [$^3$H] flunitrazepam competition binding to adult rat forebrain membranes, by MFA, compound 3, compound 8, compound 12 and compound 15. Results are expressed as percentages mean ± S.D. for three independent experiments for each.

A.




B.

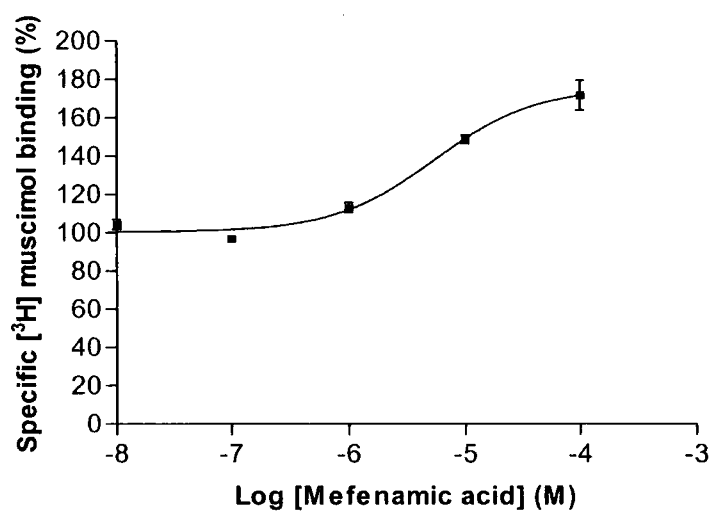



**Figure 3.6:** [$^3$H] Muscimol competition binding to **(A)** adult rat forebrain membranes **(B)** $GABA_AR$ cell line by MFA. Results are expressed as percentages mean ± S.D. for three independent experiments for each.

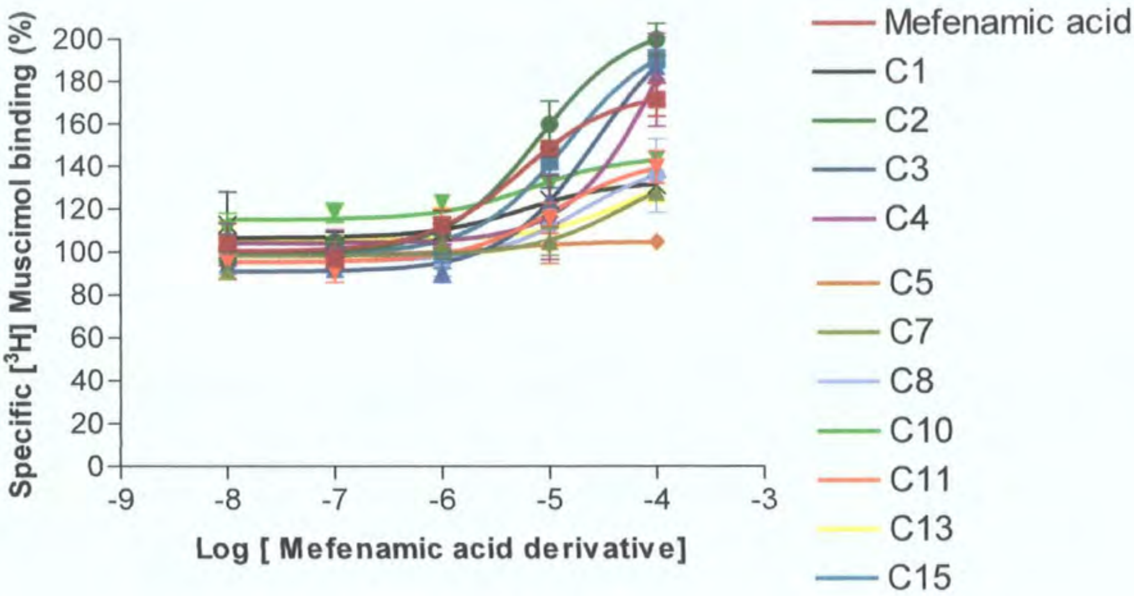



**Figure 3.7:** [$^3$H] Muscimol competition binding to $GABA_AR$ cell line by MFA & analogues. Results are expressed as percentages mean ± S.D. for three independent experiments for each.

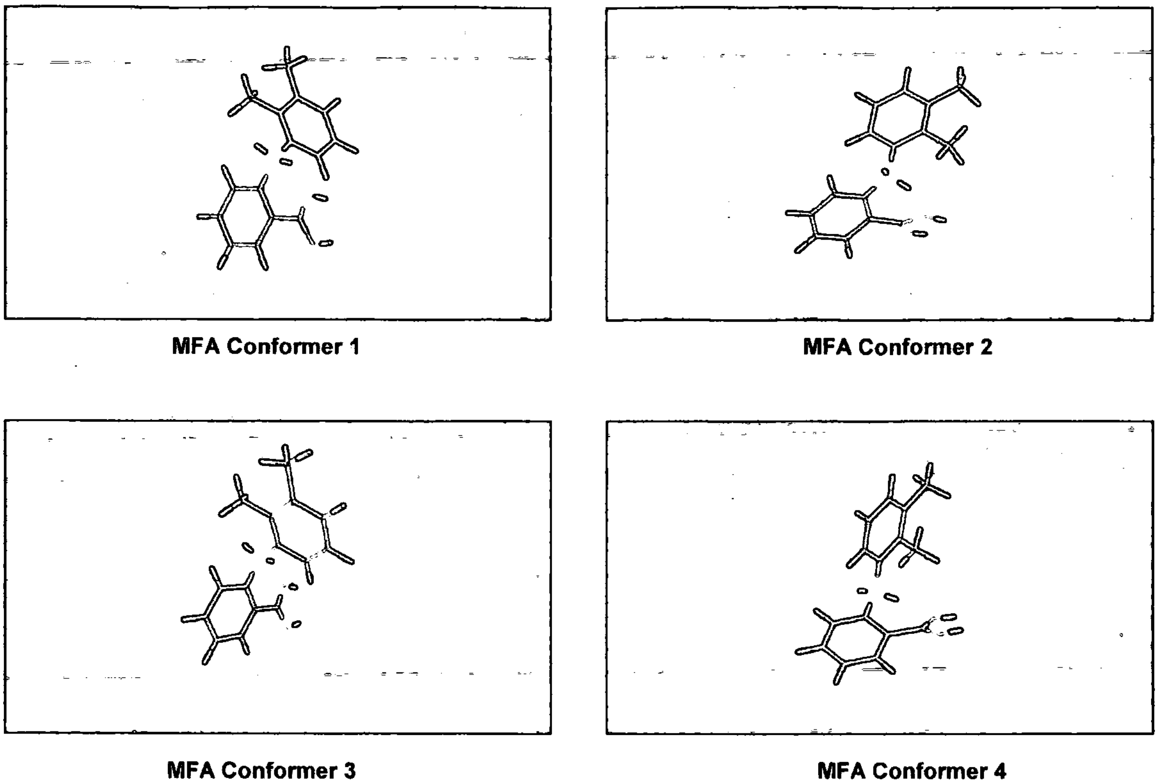
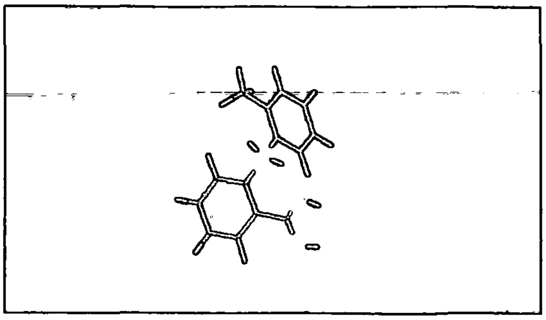
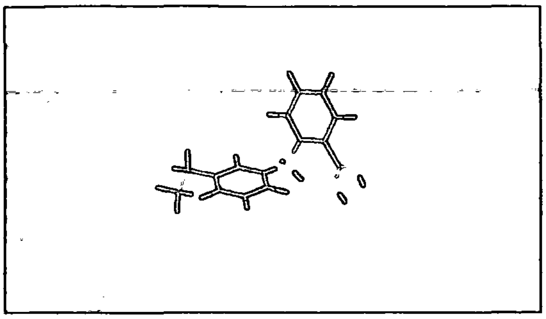


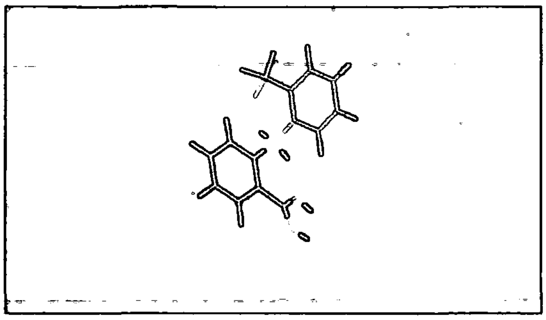

**Figure 3.8:** Four possible conformations of Mefenamic acid.



**Compound 2 , Conformer 1**



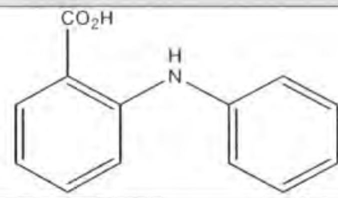
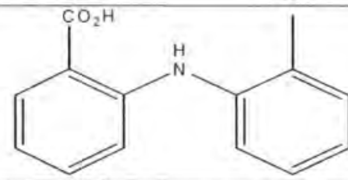
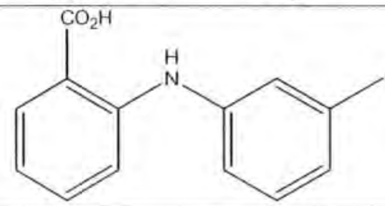
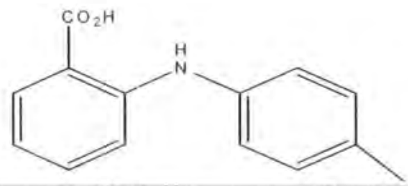
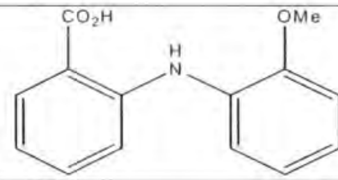
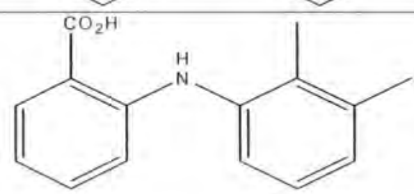
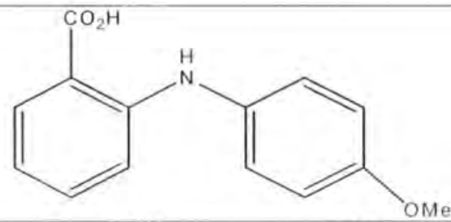
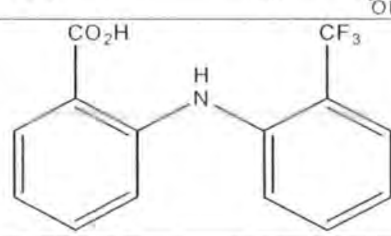
**Compound 15 , Conformer 7**



**Compound 8 , Conformer 1**

**Figure 3.9:** Conformations of selected MFA analogues: Compound 2: Conformer 1, Compound 15: Conformer 7 and Compound 8: Conformer 1.

**Table 3.1:** Chemical Structure of MFA & analogues examined with various sites of $GABA_AR$ complex.

| Compound name | Molecular Formula | Molecular weight ( g/L) | Chemical Structure |
|---|---|---|---|
| Compound 1 | $C_{13} H_{11}NO_2$ | 213.23 |  |
| Compound 2 | $C_{14}H_{13}NO_2$ | 227.26 |  |
| Compound 3 | $C_{14}H_{13}NO_2$ | 227.26 |  |
| Compound 4 | $C_{14}H_{13}NO_2$ | 227.26 |  |
| Compound 5 | $C_{14}H_{13}NO3$ | 243.26 |  |
| Compound 6 Mefenamic acid | $C_{15}H_{15}NO2$ | 241.29 |  |
| Compound 7 | $C_{14}H_{13}NO3$ | 243.26 |  |
| Compound 8 | $C_{14} H_{10} F_3 NO_2$ | 281.23 |  |

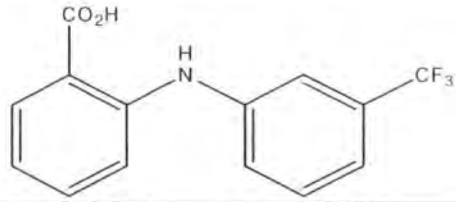
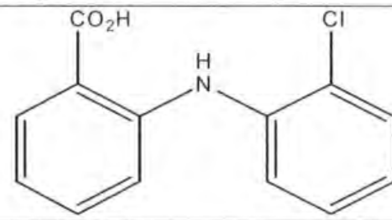
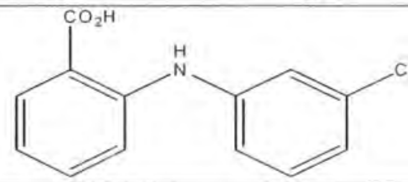
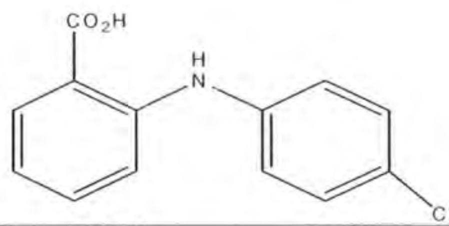
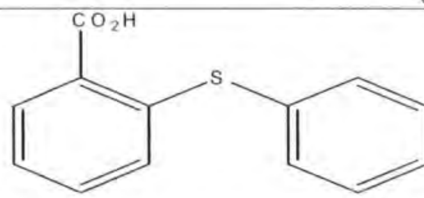
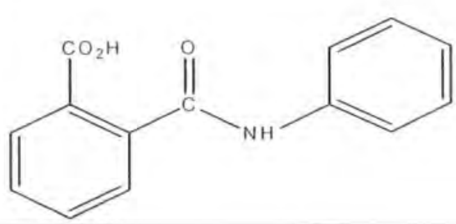
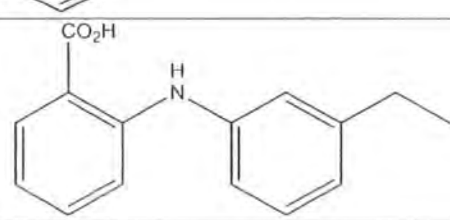
| Compound name | Molecular Formula | Molecular weight ( g/L) | Chemical Structure |
| --- | --- | --- | --- |
| Compound 9 | $C_{14}H_{10}F_3NO_2$ | 281.23 |  |
| Compound 10 | $C_{13}H_{10}NO_2Cl$ | 247.68 |  |
| Compound 11 | $C_{13}H_{10}NO_2Cl$ | 247.68 |  |
| Compound 12 | $C_{13}H_{10}NO_2Cl$ | 247.68 |  |
| Compound 13 | $C_{13}H_{10}O_2S$ | 230.28 |  |
| Compound 14 | $C_{14}H_{11}NO_3$ | 241.24 |  |
| Compound 15 | $C_{15}H_{15}NO_2$ | 241.29 |  |

**Table 3.2**: Summary of the pharmacological binding parameters ($EC_{50}$ & $E_{max}$) for [$^3$H] muscimol binding to membranes of $GABA_AR$ cell line.

| Compound name | $EC_{50}$ (µM) | $E_{max}$ |
|---|---|---|
| 1 | 6 ± 1 | 133 ±11 |
| **2** | **8 ± 1** | **208 ± 9** |
| 3 | 27± 1 | 214 ± 13 |
| 4 | 127 ± 2 | 278 ±32 |
| 5 | 3 ± 3 | 105 ± 4 |
| 6 **(MFA)** | **5 ± 1** | **175 ± 5** |
| 7 | 51 ± 6 | 143 ± 28 |
| 8 | 21 ± 4 | 144 ±17 |
| 9 | not tested | not tested |
| 10 | 6 ± 3 | 145 ± 6 |
| 11 | 11 ± 2 | 144 ±10 |
| 12 | not tested | not tested |
| 13 | 62 ± 4 | 144 ± 15 |
| 14 | not tested | not tested |
| 15 | **15 ± 2** | **204 ± 10** |

Data are means ± SD of three independent experiments, each performed in triplicate.

**Table 3.3** : Data summary for modelling of all conformations of analogues with **MFA conformer 1**, as a query molecule

| Compound | Potential Energy (KJ/mol) | Data | | | |
|---|---|---|---|---|---|
| | | ET_Pt | ENO_shape | $E_{Max}$ | $EC_{50}$ (μM) |
| c1_symm1 | 161.6691 | 0.852 | 0.924 | 133 ± 11 | 6 ± 1 |
| c1_symm2 | 161.6575 | 0.527 | 0.625 | 133 ± 11 | 6 ± 1 |
| | | | | | |
| c2_symm1 | 179.5447 | 0.976 | 0.974 | 208 ± 9 | 8 ± 1 |
| c2_symm2 | 179.5346 | 0.284 | 0.616 | 208 ± 9 | 8 ± 1 |
| | | | | | |
| c3_symm1 | 169.7678 | 0.849 | 0.900 | 214 ± 13 | 27 ± 1 |
| c3_symm2 | 169.8828 | -0.136 | 0.618 | 214 ± 13 | 27 ± 1 |
| c3_symm3 | 176.9317 | 0.831 | 0.949 | 214 ± 13 | 27 ± 1 |
| c3_symm4 | 186.4429 | 0.560 | 0.628 | 214 ± 13 | 27 ± 1 |
| | | | | | |
| c4_symm1 | 170.4539 | 0.843 | 0.905 | 278 ± 32 | 127 ± 2 |
| c4_symm2 | 170.6340 | 0.215 | 0.615 | 278 ± 32 | 127 ± 2 |
| | | | | | |
| c5_symm1 | 210.8171 | -0.083 | 0.663 | 105 ± 4 | 3 ± 3 |
| c5_symm2 | 216.2418 | 0.306 | 0.573 | 105 ± 4 | 3 ± 3 |
| c5_symm3 | 211.0686 | 0.576 | 0.888 | 105 ± 4 | 3 ± 3 |
| c5_symm4 | 216.2568 | 0.860 | 0.947 | 105 ± 4 | 3 ± 3 |
| | | | | | |
| c6_symm1 | 199.2714 | 1.000 | 1.000 | 175 ± 5 | 5 ± 1 |
| c6_symm2 | 206.0885 | 0.591 | 0.694 | 175 ± 5 | 5 ± 1 |
| c6_symm3 | 199.3423 | 0.167 | 0.624 | 175 ± 5 | 5 ± 1 |
| c6_symm4 | 206.0769 | -0.112 | 0.648 | 175 ± 5 | 5 ± 1 |
| | | | | | |
| c7_symm1 | 206.0769 | 0.760 | 0.867 | 143 ± 28 | 51 ± 6 |
| c7_symm2 | 211.1574 | 0.768 | 0.870 | 143 ± 28 | 51 ± 6 |
| c7_symm3 | 211.1547 | 0.171 | 0.597 | 143 ± 28 | 51 ± 6 |
| c7_symm4 | 211.0981 | 0.199 | 0.609 | 143 ± 28 | 51 ± 6 |
| | | | | | |
| c8_symm1 | 179.3534 | 0.302 | 0.521 | 144 ± 17 | 21 ± 4 |
| c8_symm2 | 179.8903 | 0.034 | 0.905 | 144 ± 17 | 21 ± 4 |
| c8_symm3 | 184.4028 | 0.198 | 0.628 | 144 ± 17 | 21 ± 4 |
| c8_symm4 | 186.1835 | 0.716 | 0.834 | 144 ± 17 | 21 ± 4 |
| c8_symm5 | 189.4554 | 0.519 | 0.614 | 144 ± 17 | 21 ± 4 |
| c8_symm6 | 189.0596 | -0.201 | 0.694 | 144 ± 17 | 21 ± 4 |

Continue…

| Compound | Potential Energy (KJ/mol) | | Data | | | |
|---|---|---|---|---|---|---|
| | | | ET_Pt | ENO_shape | $E_{Max}$ | $EC_{50}$ (μM) |
| c9_symm1 | 176.5413 | | 0.692 | 0.776 | ------------ | ---------- |
| c9_symm2 | 176.6899 | | -0.172 | 0.569 | ------------ | ---------- |
| c9_symm3 | 177.3631 | | 0.628 | 0.864 | ------------ | ---------- |
| c9_symm4 | 177.3817 | | 0.140 | 0.540 | ------------ | ---------- |
| | | | | | | |
| c10_symm1 | 170.3101 | | 0.826 | 0.970 | 145 ± 6 | 6 ± 3 |
| c10_symm2 | 170.1837 | | 0.362 | 0.610 | 145 ± 6 | 6 ± 3 |
| c10_symm3 | 176.7758 | | -0.095 | 0.664 | 145 ± 6 | 6 ± 3 |
| c10_symm4 | 177.1844 | | 0.815 | 0.942 | 145 ± 6 | 6 ± 3 |
| c10_symm5 | 178.9231 | | -0.137 | 0.684 | 145 ± 6 | 6 ± 3 |
| c10_symm6 | 178.9671 | | 0.586 | 0.716 | 145 ± 6 | 6 ± 3 |
| | | | | | | |
| c11_symm1 | 163.4483 | | 0.776 | 0.940 | 144 ± 10 | 11 ± 2 |
| c11_symm2 | 163.4725 | | 0.424 | 0.613 | 144 ± 10 | 11 ± 2 |
| c11_symm3 | 163.1615 | | -0.162 | 0.615 | 144 ± 10 | 11 ± 2 |
| c11_symm4 | 163.1210 | | 0.781 | 0.901 | 144 ± 10 | 11 ± 2 |
| | | | | | | |
| c12_symm1 | 164.0460 | | 0.755 | 0.904 | ----------- | ---------- |
| c12_symm2 | 164.2854 | | 0.163 | 0.612 | ----------- | ---------- |
| | | | | | | |
| c13_symm1 | 188.9787 | | 0.027 | 0.898 | 144 ± 15 | 62 ± 4 |
| c13_symm2 | 188.8703 | | 0.026 | 0.715 | 144 ± 15 | 62 ± 4 |
| | | | | | | |
| c14_symm1 | 223.0535 | | 0.029 | 0.503 | ----------- | ---------- |
| c14_symm2 | 222.9294 | | 0.014 | 0.483 | ----------- | ---------- |
| | | | | | | |
| c15_symm1 | 168.5379 | | 0.845 | 0.863 | 204 ± 10 | 15 ± 2 |
| c15_symm2 | 168.4214 | | -0.0137 | 0.596 | 204 ± 10 | 15 ± 2 |
| c15_symm3 | 168.3518 | | 0.849 | 0.861 | 204 ± 10 | 15 ± 2 |
| c15_symm4 | 168.3961 | | -0.137 | 0.596 | 204 ± 10 | 15 ± 2 |
| c15_symm5 | 168.3945 | | 0.516 | 0.592 | 204 ± 10 | 15 ± 2 |
| c15_symm6 | 168.4469 | | 0.851 | 0.907 | 204 ± 10 | 15 ± 2 |
| c15_symm7 | 168.5190 | | 0.862 | 0.938 | 204 ± 10 | 15 ± 2 |
| c15_symm8 | 168.7777 | | 0.513 | 0.589 | 204 ± 10 | 15 ± 2 |

**Table 3.4 :** Data summary for modelling of all conformations of analogues with **MFA conformer 2,** as a query molecule

| Compound | Potential Energy (KJ/mol) | Data | | | |
|---|---|---|---|---|---|
| | | ET_Pt | ENO_shape | $E_{Max}$ | $EC_{50}$ (μM) |
| c1_symm1 | 161.6691 | 0.613 | 0.764 | 133 ± 11 | 6 ± 1 |
| c1_symm2 | 161.6575 | 0.224 | 0.634 | 133 ± 11 | 6 ± 1 |
| | | | | | |
| c2_symm1 | 179.5447 | 0.595 | 0.727 | 208 ± 9 | 8 ± 1 |
| c2_symm2 | 179.5346 | -0.117 | 0.651 | 208 ± 9 | 8 ± 1 |
| | | | | | |
| c3_symm1 | 169.7678 | 0.620 | 0.746 | 214 ± 13 | 27 ± 1 |
| c3_symm2 | 169.8828 | 0.223 | 0.655 | 214 ± 13 | 27 ± 1 |
| c3_symm3 | 176.9317 | 0.576 | 0.702 | 214 ± 13 | 27 ± 1 |
| c3_symm4 | 186.4429 | 0.517 | 0.623 | 214 ± 13 | 27 ± 1 |
| | | | | | |
| c4_symm1 | 170.4539 | 0.612 | 0.743 | 278 ± 32 | 127 ± 2 |
| c4_symm2 | 170.6340 | 0.234 | 0.623 | 278 ± 32 | 127 ± 2 |
| | | | | | |
| c5_symm1 | 210.8171 | 0.206 | 0.823 | 105 ± 4 | 3 ± 3 |
| c5_symm2 | 216.2418 | -0.151 | 0.609 | 105 ± 4 | 3 ± 3 |
| c5_symm3 | 211.0686 | 0.530 | 0.691 | 105 ± 4 | 3± 3 |
| c5_symm4 | 216.2568 | 0.537 | 0.695 | 105 ± 4 | 3 ± 3 |
| | | | | | |
| c6_symm1 | 199.2714 | 0.593 | 0.695 | 175 ± 5 | 5 ± 1 |
| c6_symm2 | 206.0885 | 1.000 | 1.000 | 175 ± 5 | 5 ± 1 |
| c6_symm3 | 199.3423 | -0.114 | 0.648 | 175 ± 5 | 5 ± 1 |
| c6_symm4 | 206.0769 | -0.104 | 0.630 | 175 ± 5 | 5 ± 1 |
| | | | | | |
| c7_symm1 | 206.0769 | 0.515 | 0.715 | 143 ± 28 | 51 ± 6 |
| c7_symm2 | 211.1574 | 0.580 | 0.713 | 143 ± 28 | 51 ± 6 |
| c7_symm3 | 211.1547 | 0.211 | 0.608 | 143 ± 28 | 51 ± 6 |
| c7_symm4 | 211.0981 | 0.195 | 0.601 | 143 ± 28 | 51 ± 6 |
| | | | | | |
| c8_symm1 | 179.3534 | 0.045 | 0.597 | 144 ± 17 | 21 ± 4 |
| c8_symm2 | 179.8903 | -0.021 | 0.631 | 144 ± 17 | 21 ± 4 |
| c8_symm3 | 184.4028 | 0.485 | 0.863 | 144 ± 17 | 21 ± 4 |
| c8_symm4 | 186.1835 | 0.743 | 0.619 | 144 ± 17 | 21 ± 4 |
| c8_symm5 | 189.4554 | 0.733 | 0.879 | 144 ± 17 | 21 ± 4 |
| c8_symm6 | 189.0596 | 0.160 | 0.499 | 144 ± 17 | 21 ± 4 |

Continue...

| Compound | Potential Energy (KJ/mol) | | Data | | | |
|---|---|---|---|---|---|---|
| | | | ET_Pt | ENO_shape | $E_{Max}$ | $EC_{50}$ (μM) |
| c9_symm1 | 176.5413 | | 0.678 | 0.654 | ------------ | ----------- |
| c9_symm2 | 176.6899 | | 0.184 | 0.629 | ------------ | ----------- |
| c9_symm3 | 177.3631 | | 0.465 | 0.628 | ------------ | ----------- |
| c9_symm4 | 177.3817 | | -0.053 | 0.539 | ------------ | ----------- |
| | | | | | | |
| c10_symm1 | 170.3101 | | 0.495 | 0.732 | 145 ± 6 | 6 ± 3 |
| c10_symm2 | 170.1837 | | -0.094 | 0.644 | 145 ± 6 | 6 ± 3 |
| c10_symm3 | 176.7758 | | 0.464 | 0.798 | 145 ± 6 | 6 ± 3 |
| c10_symm4 | 177.1844 | | 0.751 | 0.653 | 145 ± 6 | 6 ± 3 |
| c10_symm5 | 178.9231 | | -0.152 | 0.612 | 145 ± 6 | 6 ± 3 |
| c10_symm6 | 178.9671 | | 0.877 | 0.964 | 145 ± 6 | 6 ± 3 |
| | | | | | | |
| c11_symm1 | 163.4483 | | 0.559 | 0.735 | 144 ± 10 | 11 ± 2 |
| c11_symm2 | 163.4725 | | 0.188 | 0.616 | 144 ± 10 | 11 ± 2 |
| c11_symm3 | 163.1615 | | 0.208 | 0.651 | 144 ± 10 | 11 ± 2 |
| c11_symm4 | 163.1210 | | 0.698 | 0.745 | 144 ± 10 | 11 ± 2 |
| | | | | | | |
| c12_symm1 | 164.0460 | | 0.569 | 0.745 | ----------- | ---------- |
| c12_symm2 | 164.2854 | | 0.178 | 0.623 | ----------- | ---------- |
| | | | | | | |
| c13_symm1 | 188.9787 | | 0.034 | 0.733 | 144 ± 15 | 62 ± 4 |
| c13_symm2 | 188.8703 | | 0.012 | 0.755 | 144 ± 15 | 62 ± 4 |
| | | | | | | |
| c14_symm1 | 223.0535 | | 0.041 | 0.692 | ----------- | ---------- |
| c14_symm2 | 222.9294 | | 0.024 | 0.775 | ----------- | ---------- |
| | | | | | | |
| c15_symm1 | 168.5379 | | 0.494 | 0.664 | 204 ± 10 | 15 ± 2 |
| c15_symm2 | 168.4214 | | 0.225 | 0.653 | 204 ± 10 | 15 ± 2 |
| c15_symm3 | 168.3518 | | 0.625 | 0.723 | 204 ± 10 | 15 ± 2 |
| c15_symm4 | 168.3961 | | 0.226 | 0.647 | 204 ± 10 | 15 ± 2 |
| c15_symm5 | 168.3945 | | -0.030 | 0.855 | 204 ± 10 | 15 ± 2 |
| c15_symm6 | 168.4469 | | 0.619 | 0.700 | 204 ± 10 | 15 ± 2 |
| c15_symm7 | 168.5190 | | 0.619 | 0.700 | 204 ± 10 | 15 ± 2 |
| c15_symm8 | 168.7777 | | -0.137 | 0.597 | 204 ± 10 | 15 ± 2 |

**Table 3.5** : Data summary for modelling of all conformations of analogues with **MFA conformer 3**, as a query molecule

| Compound | Potential Energy (KJ/mol) | Data | | | |
|---|---|---|---|---|---|
| | | ET_Pt | ENO_shape | $E_{Max}$ | $EC_{50}$ (μM) |
| c1_symm1 | 161.6691 | 0.530 | 0.625 | 133 ± 11 | 6 ± 1 |
| c1_symm2 | 161.6575 | 0.853 | 0.924 | 133 ± 11 | 6 ± 1 |
| | | | | | |
| c2_symm1 | 179.5447 | 0.287 | 0.616 | 208 ± 9 | 8 ± 1 |
| c2_symm2 | 179.5346 | 0.977 | 0.974 | 208 ± 9 | 8 ± 1 |
| | | | | | |
| c3_symm1 | 169.7678 | -0.139 | 0.618 | 214 ± 13 | 27 ± 1 |
| c3_symm2 | 169.8828 | 0.846 | 0.900 | 214 ± 13 | 27 ± 1 |
| c3_symm3 | 176.9317 | 0.263 | 0.635 | 214 ± 13 | 27 ± 1 |
| c3_symm4 | 186.4429 | 0.828 | 0.934 | 214 ± 13 | 27 ± 1 |
| | | | | | |
| c4_symm1 | 170.4539 | 0.518 | 0.615 | 278 ± 32 | 127 ± 2 |
| c4_symm2 | 170.6340 | 0.848 | 0.905 | 278 ± 32 | 127 ± 2 |
| | | | | | |
| c5_symm1 | 210.8171 | 0.566 | 0.888 | 105 ± 4 | 3 ± 3 |
| c5_symm2 | 216.2418 | 0.859 | 0.947 | 105 ± 4 | 3 ± 3 |
| c5_symm3 | 211.0686 | -0.089 | 0.664 | 105 ± 4 | 3 ± 3 |
| c5_symm4 | 216.2568 | 0.312 | 0.573 | 105 ± 4 | 3 ± 3 |
| | | | | | |
| c6_symm1 | 199.2714 | 0.169 | 0.624 | 175 ± 5 | 5 ± 1 |
| c6_symm2 | 206.0885 | -0.075 | 0.892 | 175 ± 5 | 5 ± 1 |
| c6_symm3 | 199.3423 | 1.000 | 1.000 | 175 ± 5 | 5 ± 1 |
| c6_symm4 | 206.0769 | 0.598 | 0.649 | 175 ± 5 | 5 ± 1 |
| | | | | | |
| c7_symm1 | 206.0769 | 0.175 | 0.597 | 143 ± 28 | 51 ± 6 |
| c7_symm2 | 211.1574 | 0.204 | 0.609 | 143 ± 28 | 51 ± 6 |
| c7_symm3 | 211.1547 | 0.762 | 0.867 | 143 ± 28 | 51 ± 6 |
| c7_symm4 | 211.0981 | 0.768 | 0.870 | 143 ± 28 | 51 ± 6 |
| | | | | | |
| c8_symm1 | 179.3534 | 0.028 | 0.905 | 144 ± 17 | 21 ± 4 |
| c8_symm2 | 179.8903 | 0.294 | 0.521 | 144 ± 17 | 21 ± 4 |
| c8_symm3 | 184.4028 | 0.671 | 0.834 | 144 ± 17 | 21 ± 4 |
| c8_symm4 | 186.1835 | 0.228 | 0.628 | 144 ± 17 | 21 ± 4 |
| c8_symm5 | 189.4554 | -0.203 | 0.694 | 144 ± 17 | 21 ± 4 |
| c8_symm6 | 189.0596 | 0.519 | 0.614 | 144 ± 17 | 21 ± 4 |

Continue...

| Compound | Potential Energy (KJ/mol) | | Data | | | |
|---|---|---|---|---|---|---|
| | | | ET_Pt | ENO_shape | $E_{Max}$ | $EC_{50}$ (μM) |
| c9_symm1 | 176.5413 | | -0.176 | 0.570 | ------------- | --------- |
| c9_symm2 | 176.6899 | | 0.693 | 0.775 | ------------ | ----------- |
| c9_symm3 | 177.3631 | | 0.337 | 0.540 | ------------ | ----------- |
| c9_symm4 | 177.3817 | | 0.627 | 0.865 | ------------ | ----------- |
| | | | | | | |
| c10_symm1 | 170.3101 | | 0.371 | 0.610 | 145 ± 6 | 6 ± 3 |
| c10_symm2 | 170.1837 | | 0.829 | 0.970 | 145 ± 6 | 6 ± 3 |
| c10_symm3 | 176.7758 | | 0.800 | 0.942 | 145 ± 6 | 6 ± 3 |
| c10_symm4 | 177.1844 | | -0.105 | 0.664 | 145 ± 6 | 6 ± 3 |
| c10_symm5 | 178.9231 | | 0.592 | 0.716 | 145 ± 6 | 6 ± 3 |
| c10_symm6 | 178.9671 | | -0.138 | 0.684 | 145 ± 6 | 6 ± 3 |
| | | | | | | |
| c11_symm1 | 163.4483 | | 0.425 | 0.613 | 144 ± 10 | 11± 2 |
| c11_symm2 | 163.4725 | | 0.775 | 0.940 | 144 ± 10 | 11± 2 |
| c11_symm3 | 163.1615 | | 0.779 | 0.901 | 144 ± 10 | 11± 2 |
| c11_symm4 | 163.1210 | | -0.162 | 0.615 | 144 ± 10 | 11± 2 |
| | | | | | | |
| c12_symm1 | 164.0460 | | 0.171 | 0.612 | ----------- | ---------- |
| c12_symm2 | 164.2854 | | 0.755 | 0.904 | ----------- | ---------- |
| | | | | | | |
| c13_symm1 | 188.9787 | | 0.027 | 0.715 | 144 ± 15 | 62± 4 |
| c13_symm2 | 188.8703 | | 0.030 | 0.898 | 144 ± 15 | 62 ± 4 |
| | | | | | | |
| c14_symm1 | 223.0535 | | 0.012 | 0.483 | ----------- | ---------- |
| c14_symm2 | 222.9294 | | 0.032 | 0.503 | ----------- | ---------- |
| | | | | | | |
| c15_symm1 | 168.5379 | | -0.138 | 0.596 | 204 ± 10 | 15 ± 2 |
| c15_symm2 | 168.4214 | | 0.847 | 0.863 | 204 ± 10 | 15 ± 2 |
| c15_symm3 | 168.3518 | | -0.140 | 0.620 | 204 ± 10 | 15 ± 2 |
| c15_symm4 | 168.3961 | | 0.849 | 0.861 | 204 ± 10 | 15 ± 2 |
| c15_symm5 | 168.3945 | | 0.851 | 0.907 | 204 ± 10 | 15 ± 2 |
| c15_symm6 | 168.4469 | | -0.137 | 0.584 | 204 ± 10 | 15 ± 2 |
| c15_symm7 | 168.5190 | | 0.517 | 0.589 | 204 ± 10 | 15 ± 2 |
| c15_symm8 | 168.7777 | | 0.861 | 0.938 | 204 ± 10 | 15 ± 2 |

**Table 3.6 :** Data summary for modelling of all conformations of analogues with **MFA conformer 4**, as a query molecule

| **Compound** | **Potential Energy** (KJ/mol) | | **Data** | | | |
|---|---|---|---|---|---|---|
| | | | **ET_Pt** | **ENO_shape** | $E_{Max}$ | $EC_{50}$ (μM) |
| c1_symm1 | 161.6691 | | 0.223 | 0.634 | 133 ± 11 | 6 ± 1 |
| c1_symm2 | 161.6575 | | 0.614 | 0.764 | 133 ± 11 | 6 ± 1 |
| | | | | | | |
| c2_symm1 | 179.5447 | | -0.113 | 0.651 | 208 ± 9 | 8 ± 1 |
| c2_symm2 | 179.5346 | | 0.603 | 0.727 | 208 ± 9 | 8 ± 1 |
| | | | | | | |
| c3_symm1 | 169.7678 | | 0.216 | 0.655 | 214 ± 13 | 27 ± 1 |
| c3_symm2 | 169.8828 | | 0.614 | 0.746 | 214 ± 13 | 27 ± 1 |
| c3_symm3 | 176.9317 | | -0.0.63 | 0.899 | 214 ± 13 | 27 ± 1 |
| c3_symm4 | 186.4429 | | 0.594 | 0.726 | 214 ± 13 | 27 ± 1 |
| | | | | | | |
| c4_symm1 | 170.4539 | | 0.216 | 0.623 | 278 ± 32 | 127 ± 2 |
| c4_symm2 | 170.6340 | | 0.611 | 0.743 | 278 ± 32 | 127 ± 2 |
| | | | | | | |
| c5_symm1 | 210.8171 | | 0.527 | 0.691 | 105 ± 4 | 3 ± 3 |
| c5_symm2 | 216.2418 | | 0.536 | 0.695 | 105 ± 4 | 3 ± 3 |
| c5_symm3 | 211.0686 | | 0.211 | 0.823 | 105 ± 4 | 3 ± 3 |
| c5_symm4 | 216.2568 | | -0.147 | 0.609 | 105 ± 4 | 3 ± 3 |
| | | | | | | |
| c6_symm1 | 199.2714 | | -0.113 | 0.648 | 175 ± 5 | 5 ± 1 |
| c6_symm2 | 206.0885 | | -0.098 | 0.630 | 175 ± 5 | 5 ± 1 |
| c6_symm3 | 199.3423 | | 0.597 | 0.694 | 175 ± 5 | 5 ± 1 |
| c6_symm4 | 206.0769 | | 1.000 | 1.000 | 175 ± 5 | 5 ± 1 |
| | | | | | | |
| c7_symm1 | 206.0769 | | 0.198 | 0.608 | 143 ± 28 | 51 ± 6 |
| c7_symm2 | 211.1574 | | 0.192 | 0.601 | 143 ± 28 | 51 ± 6 |
| c7_symm3 | 211.1547 | | 0.523 | 0.715 | 143 ± 28 | 51 ± 6 |
| c7_symm4 | 211.0981 | | 0.585 | 0.713 | 143 ± 28 | 51 ± 6 |
| | | | | | | |
| c8_symm1 | 179.3534 | | 0.021 | 0.631 | 144 ± 17 | 21 ± 4 |
| c8_symm2 | 179.8903 | | 0.051 | 0.597 | 144 ± 17 | 21 ± 4 |
| c8_symm3 | 184.4028 | | 0.720 | 0.619 | 144 ± 17 | 21 ± 4 |
| c8_symm4 | 186.1835 | | 0.478 | 0.863 | 144 ± 17 | 21 ± 4 |
| c8_symm5 | 189.4554 | | 0.167 | 0.498 | 144 ± 17 | 21 ± 4 |
| c8_symm6 | 189.0596 | | 0.731 | 0.879 | 144 ± 17 | 21 ± 4 |

Continue...

| Compound | Potential Energy (KJ/mol) | ET_Pt | ENO_shape | $E_{Max}$ | $EC_{50}$ (μM) |
|---|---|---|---|---|---|
| | | Data | | | |
| c9_symm1 | 176.5413 | 0.172 | 0.629 | ----------- | ----------- |
| c9_symm2 | 176.6899 | 0.675 | 0.652 | ------------ | ----------- |
| c9_symm3 | 177.3631 | 0.151 | 0.539 | ------------ | ----------- |
| c9_symm4 | 177.3817 | 0.474 | 0.630 | ------------ | ----------- |
| | | | | | |
| c10_symm1 | 170.3101 | -0.092 | 0.645 | 145 ± 6 | 6 ± 3 |
| c10_symm2 | 170.1837 | 0.506 | 0.733 | 145 ± 6 | 6 ± 3 |
| c10_symm3 | 176.7758 | 0.753 | 0.655 | 145 ± 6 | 6 ± 3 |
| c10_symm4 | 177.1844 | 0.458 | 0.799 | 145 ± 6 | 6 ± 3 |
| c10_symm5 | 178.9231 | 0.871 | 0.964 | 145 ± 6 | 6 ± 3 |
| c10_symm6 | 178.9671 | -0.148 | 0.612 | 145 ± 6 | 6 ± 3 |
| | | | | | |
| c11_symm1 | 163.4483 | 0.187 | 0.616 | 144 ± 10 | 11 ± 2 |
| c11_symm2 | 163.4725 | 0.568 | 0.735 | 144 ± 10 | 11 ± 2 |
| c11_symm3 | 163.1615 | 0.697 | 0.746 | 144 ± 10 | 11 ± 2 |
| c11_symm4 | 163.1210 | 0.201 | 0.651 | 144 ± 10 | 11 ± 2 |
| | | | | | |
| c12_symm1 | 164.0460 | 0.179 | 0.623 | ----------- | ---------- |
| c12_symm2 | 164.2854 | 0.568 | 0.744 | ----------- | ---------- |
| | | | | | |
| c13_symm1 | 188.9787 | 0.008 | 0.755 | 144 ± 15 | 62 ± 4 |
| c13_symm2 | 188.8703 | 0.032 | 0.733 | 144 ±15 | 62 ± 4 |
| | | | | | |
| c14_symm1 | 223.0535 | 0.021 | 0.776 | ----------- | ---------- |
| c14_symm2 | 222.9294 | 0.039 | 0.692 | ----------- | ---------- |
| | | | | | |
| c15_symm1 | 168.5379 | 0.214 | 0.653 | 204 ± 10 | 15 ± 2 |
| c15_symm2 | 168.4214 | 0.480 | 0.664 | 204 ± 10 | 15 ± 2 |
| c15_symm3 | 168.3518 | 0.221 | 0.647 | 204 ± 10 | 15 ± 2 |
| c15_symm4 | 168.3961 | 0.616 | 0.723 | 204 ± 10 | 15 ± 2 |
| c15_symm5 | 168.3945 | 0.624 | 0.700 | 204 ± 10 | 15± 2 |
| c15_symm6 | 168.4469 | -0.022 | 0.855 | 204 ± 10 | 15.± 2 |
| c15_symm7 | 168.5190 | -0.133 | 0.596 | 204 ± 10 | 15 ± 2 |
| c15_symm8 | 168.7777 | 0.622 | 0.700 | 204 ± 10 | 15 ± 2 |

## 3.6 Discussion

MFA [(2-[2,3-dimethylphenyl]amino)benzoic acid], an anthranilic acid derivative, is a non steroidal anti-inflammatory, antipyretic and analgesic agent that is used for post operative and traumatic inflammation, analgesic for rheumatoid arthritis and antipyretic in acute respiratory tract infection ( Winder *et al.*, 1962, Fang *et al.*, 2004).

There is scientific evidence that fenamates such as MFA modulates GABAgeric transmission in the central nervous system. Many studies have demonstrated that NSAIDs could affect neuronal function by directly modulating ligand-gated ion channel function.

The rational behind selecting these compounds in our study was based upon a number of observations from different studies. Briefly these were, firstly, NSAIDs can produce analgesic effects even when administered directly into the CNS of rodents (Malmberg & Yaksh, 1992, McCormack, 1994). Secondly, in humans, NSAIDs induce complex behavioural effects especially when taken in overdose and this appeared to be particularly true for the fenamate NSAID, MFA (Wallenstein, 1991). Thirdly, three studies had demonstrated that fenamate, such as MFA could, modulate the function of $GABA_A$ receptors (Evonuik & Skolnick, 1988, Woodward *et al.*, 1994, Halliwell *et al.*, 1999). Fourthly, MFA shows neuroprotective effects and improves cognitive impairment in vitro and in vivo AD models (Hee Kang *et al.*, 2003, Etminan *et al.*, 2003). These findings suggest that MFA may serve as a lead structure in the development of novel therapeutic agents for AD and brain ischemia.

In the present study, therefore the pharmacological characterization of MFA & 14 analogues as a positive modulator for $GABA_A$Rs were investigated in both native and recombinant membrane preparations using radioligand binding techniques. We studied the interaction of MFA and analogues with various sites of the $GABA_A$R complex in rat brain and stable $GABA_A$R cell line membranes, in order to identify their potential site of action, to check their selective modulation effect and to further characterise the mechanism of action of these compounds in the CNS.

To investigate the pharmacological effect of MFA in further detail, we synthesised and pharmacologically tested a small group series of MFA analogues substituted with different groups on the ring B of the fenamate structure. Furthermore a molecular modelling study based on MFA was performed to explore the effect of different substituent sizes on the affinity, efficacy and structural flexibility.

Results demonstrated that MFA and analogues did not interact with picrotoxin or benzodiazepine binding sites of the $GABA_AR$ expression in either rat forebrain membranes or $GABA_AR$ cell line. This suggests a lack of interaction (either directly or allosterically) of the compounds with these two sites of $GABA_AR$. In contrast, MFA showed a clear activity at the agonist site $GABA_AR$ in native and recombinant receptor preparations. MFA displaced [$^3$H] muscimol binding in well washed membranes in concentration dependent manner; on the other hand, MFA stimulated [$^3$H] muscimol binding to $GABA_AR$ cell line, in concentration dependant manner. The different pharmacological profile displayed by MFA on both preparations is probably attributable to the lack of ability of MFA to activate the $GABA_AR$ in the presence of GABA. The enhancement of [$^3$H] muscimol binding by MFA must be observed only in the complete absence of GABA (recombinant prep). In the presence of endogenous GABA and other modulators (unwashed or incomplete washed membranes) the same concentration of MFA inhibited [$^3$H] muscimol binding. This GABA sensitivity is shared by a number of other $GABA_A$ modulators such as diazepam, loreclezole, propofol and lactose (Ghiani *et al.*, 1996, Rezai *et al.*, 2003). These results reveal the exceptionally complex system of allosteric modulation in this ligand gated channel representing the $GABA_AR$.

Data show that MFA modulate recombinant $GABA_AR$ in a highly selective manner, positive modulation detected at the agonist site of $\alpha_1\beta_2\gamma_2L$ $GABA_AR$ model, these observations are consistent with the pervious study (Halliwell *et al.*, 1999) demonstrating subunit selective modulation of $GABA_AR$ by the NSAIDs, MFA. Indeed, their data have shown that MFA modulates $GABA_AR$ in a highly receptor β subunit-dependent manner: heteromeric recombinant receptor containing $\beta_1$ subunits are relatively insensitive to MFA, whereas $\beta_2$ or $\beta_3$ subunit-containing receptors are enhanced and directly activated by MFA using whole–cell patch clamping technique. Smith *et al.* (2004) has also reported several allosteric modulators for which β subunits are important determinants of efficacy and potency using FRET technique, notably for loreclezole, etomidate and a group of anti-inflammatory agents including MFA.

Crystallographic and theoretical studies performed by Dhanaraj and Vijayan (1988) demonstrated that fenamates are composed of two 6 membered rings which are linked by an imino bridge. For most fenamates, the ring A (see introduction, Figure 3.1) carboxyl group is co-planar with the imino bridge and is stabilized by an internal hydrogen bond. Rotation of the B-ring is possible, but is limited by the steric hindrance occurring between the A-ring hydrogen ortho to the imino linkage and the substituted $R_1$ and $R_2$ groups on the ring-B, such that the 2 rings have non-planner orientations.

This appears to be especially true for MFA ($R_1$ and $R_2$ =$CH_3$) and Meclofenamic acid ($R_1$ = $Cl^-$ , $R_2$ =$CH_3$) which have relatively bulky $R_1$ and $R_2$ groups compared to Flufenamic acid ($R_1$=H; $R_2$= $CF_3$). In case of Niflumic acid, replacement of a carbon atom with hydrogen on the A-ring, results in a loss of steric hindrance enabling the molecule to adopt an almost planner conformation. The ability of fenamate to potentiate or inhibit GABA-mediated responses being dependant upon conformation of the molecule was also suggested by Woodward *et al* (1994). Planar conformations, such as MFA, Meclofenamic acid, Flufenamic acid and Tolfenamic acid were found to be effective modulators, the degree of this modulation depended on phenyl-ring substitution at the $R_2$ group on the B-ring.

The modulatory effect of MFA and 14 analogues on the agonist binding site of $GABA_AR$ labelled by [$^3$H] muscimol binding to $GABA_AR$ cell line was carried out as a preliminary structure activity study for this compound series. The first structure-activity question we addressed was the effect of substitutions on ring B of MFA ( $R_1$,$R_2$= $CH_3$), if it has any effect on the modulatory activity of $GABA_AR$ , so we tested compound 1 with no substitution ( $R_1$, $R_2$=H) , Compound 2 ( $R_1$=$CH_3$, $R_2$=H), Compound 3 ( $R_1$=H, $R_2$=$CH_3$), compound 4 ( $R_1$= H, and $CH_3$ substituted at the para position , different position of $R_1$ & $R_2$) and compound 15 ( $R_1$=H, $R_2$= $CH_2CH_3$). Data suggested that substitutions on the $R_1$, $R_2$ had a clear effect on the modulatory activity. Compound 2 and 15 were the most efficacious of these compounds, with similar affinity but greater efficacy than MFA (208 ± 9, 204 ±10 respectively compared with 175 ± 5 for MFA). Compound 1 exhibited similar affinity as MFA but lower efficacy. In contrast, compound 3 and 4 showed higher modulatory effect but lower affinity compared to MFA. These data suggest that one alkyl group substitution on the ring B either on $R_1$ or $R_2$ enhanced the modulatory activity.

Next, we investigated the effect of bulky group substitution of the phenyl ring of MFA, so we compared 3 sets of compound groups, group 1 substituted with (OMe) these were compound 5 and 7, group 2 substituted with trifluoromethyl group ($CF_3$), these were compound 8 & 9, Group 3 were substituted with chlorine atom ($Cl^-$) these were compound 10, 11 and 12. Results showed that introduction of bulky group substitution [(OMe), ($CF_3$), and ($Cl^-$)] on the B-ring of MFA resulted in a significant reduction of the affinity for the $GABA_AR$ agonist site compared to that of MFA.

Finally we investigated the importance of the imino bridge between the ring A & B. Replacement of this linker group with sulphur (S) such as compound 13, or amino group ( C=ONH) such as compound 14 ,abolished the modulatory activity in both cases.

These data suggest that the imino linker group is an important determinant of activity in these compounds.

Structural similarities of MFA conformers (1,2,3 & 4) with all tested analogues by molecular modelling, are in agreement with what was found experimentally, suggesting that substituted $R_1$ and $R_2$ groups on the ring B of fenamates structure, plays a significant role in the activity of these compound , high chemical similarity between MFA (conformer 1 & 3) and C1, C2, C3, C4, C5, C10 and C15 indicate that the introduction of alkyl group subsitiution ( methyl or ethyl) on $R_1$ or $R_2$ position result in enhancement of the activity in this chemical series. Replacement of the $R_1$ group with H, Cl or OMe are tolerated but with a significant reduction in the binding efficacy for the $GABA_A$ receptor agonist site.

Above all, results of the present study show a selective modulation of MFA at the $GABA_AR$ agonist site. Small changes in the structure of MFA can affect the efficacy and potency of modulation. This is consistent with the idea that fenamates have specific interactions with $GABA_AR$. Modulation is strongly dependant on more than just conformation and suggests that substitution at $R_1$ and $R_2$ plays a role in determining levels of activity at $GABA_AR$. Clearly, these substitutions have pronounced effects on modulation even though they have little effect on the conformational mobility of ring B, but it seems they play a role in stabilizing the imino linkage between the two rings.

Evidence suggests that NSAID can effectively cross the blood-brain barrier (Bannwarth *et al.,* 1989). This, together with the relatively high therapeutic plasma concentrations that are achieved in the region of (80 μM , 20- 70 μM) for MFA and Niflumic acids, respectively (Halliwell *et al.*, 1999, Sinkkonen *et al.*, 2003), suggests that appreciable brain levels might be achieved. Indeed, observations of anti-epileptogenic effects (Wallenstein, 1991) and adverse events associated with anti-inflammatory overdose are consistent with activity at central $GABA_AR$. Given that low micromolar concentrations are sufficient to potentiate β2/3-containing $GABA_AR$; this suggests that modulatory effects could occur at clinically relevant concentrations. In addition, NSAID sensitive $\alpha_1\beta_2\gamma_{2L}$ receptor subtype is the largest $GABA_AR$ population in mammalian brain (McKernan and Whiting, 1996).

In summary, the experiments described in this thesis add to the understanding of MFA pharmacology. We have performed a comprehensive radioligand binding characterization of the effect of MFA on $GABA_AR$. We demonstrate the selective allosteric modulation at the agonist site of the receptor. Our preliminary SAR was restricted to a comparison of MFA and 14 structurally related analogues. This initial study shows that small changes in the structure of MFA can affect the affinity and efficacy of modulation. This sensitivity to alterations in the structure should be addressed in more detail by testing new analogues with other possible substitutions on the ring A and B of the fenamate structure. Any potential therapeutic value as GABAergic drugs in this chemical series will be dependent on improving affinity and efficacy.

# Chapter 4

# Detailed Pharmacological Characterization of a Positive Allosteric Modulator of $GABA_A$ Receptor, Octyl-β-D-Glucoside

## 4.1 Introduction

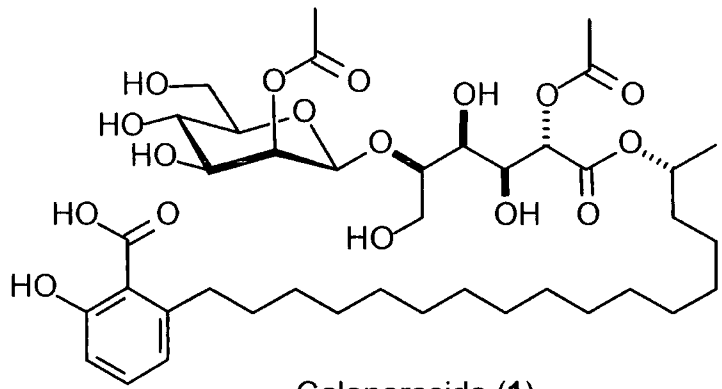
Caloporoside is a natural active fungal metabolite, which was isolated several years ago from fermentation of *Caloporous dichrous*, and was originally described to exhibit weak antibacterial and antifungal activity, as well as selective phospholipase C inhibitory activity (Weber *et al.*, 1994). In the same year, two similar secondary metabolites were isolated from the same fungus species, and were reported in a preliminary study to act as inhibitors of [$^{35}$S]TBPS binding to the $GABA_A$/benzodiazepine chloride channel receptor complex *in vitro* (Shan *et al.,* 1994).

Synthesis and biological evaluation of the Caloporoside analogue, Deacetyl caloporoside, has been reported (Tatsuta *et al.*, 1996). The compound appeared to display modest binding affinity for the $GABA_A$ receptor channel (cited $IC_{50}$ = 40-60 μM) (Tatsuta *et al.*, 1994; 1996). Detailed pharmacological analyses were lacking in these reports. The chemical structure of Caloporoside was elucidated by combination of chemical and spectroscopic methods (Weber *et al.*, 1994, Shan *et al.*, 1994, Eder *et al.*, 2002). Caloporoside consists of salicylic acid and a β-D-mannopyranosyl-d-mannonic acid moiety which are linked by an alkyl chain; the sugar part carries two acetyl residues at the 2- and 2'-position. Analogues of this compound have been described (Eder *et al.*, 2002) which differ from the natural product in the aldohexose and the aldonic acid moiety. For example, the sugar moiety may be D-mannopyrannosyl-D-mannoic acid, which can be un-substituted or substituted (Eder *et al.*, 2002).

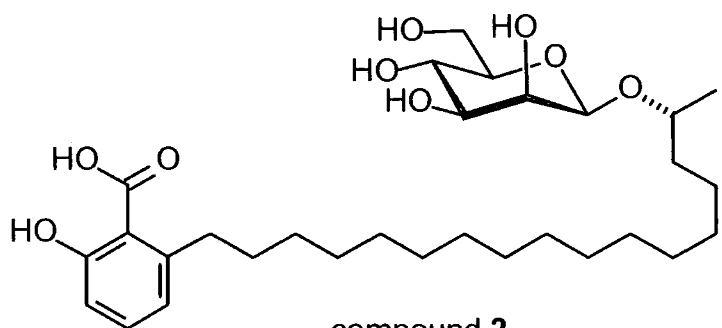
Successful chemical synthesis of the physiologically active fungal metabolite Caloporoside has been described by our collaborator (Fürstner *et al.*, 1996, 1998). The published procedure permits the synthesis of Caloporoside and other closely related analogues, which may prove to be promising compounds for further biological evaluation. The other interesting issue relates to the sugar moiety of Caloporoside, which is characterized by the highly unusual β-(1 → 5) linkage of d-mannopyranoside unit to a d-mannonate ester. The stereoselective chemical synthesis of the β-mannopyranosidic linkage is not a trivial issue in carbohydrate chemistry; however, practical synthesis of β-mannopyranosides has been described (David *et al.*, 1998, Fürstner *et al.*, 1996, 1998). Recently, a new strategy for the synthesis of mannopyranoside was reported (Benjamin, 2000, Singh *et al.*, 2000).

Our laboratory showed that a simple polar deaceylated Caloporoside derivative is a positive functional modulator of the $GABA_A$ chloride channel. Octyl-β-D mannopyransoside (100 μM) significantly and reversibly increased the magnitude of $GABA_A$ currents evoked in the cultured rat cortical pyramidal neurons (Lees *et al.*, 2000). A subsequent study demonstrated that a simple β-linked disaccharide, lactose, but not the α-linked disaccharides maltose or sucrose, can bind the $GABA_AR$ channel, detected by positive modulation of [$^3$H] TBOB binding to the rodent $GABA_AR$ (Rezai *et al.*, 2003.)

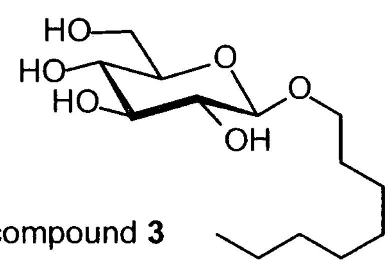
Here, we extend the pharmacological profile of this new class of $GABA_AR$ ligand, using the radioligand binding approach with the high specific activity channel radioligand [$^{35}$S] TBPS. Three compounds, with the chemical structures shown in Figure 4.1, were studied in the first instance: the synthetic parent molecule Caloporoside, 2-Hydroxy-6-{[(16R)- β-D-mannopyranosyloxy) heptadecyl]} benzoic acid ( HMHB), which lacks the mannonic acid ester segment (compound **2**) and Octyl-β-D-glucoside (compound **3**).




Caloporoside (**1**)




compound **2**




compound **3**

**Figure 4.1:** Chemical structures of novel $GABA_AR$ compounds. Compound **1**, Caloporoside; compound **2**, 2-hydroxy-6-{[(16R)-(β-d-mannopyranosyloxy) heptadecyl]} benzoic acid (HMHB); compound **3**, Octyl-β-D-glucoside.

## 4.2 Materials & Methods

### 4.2.1Materials:

Synthetic Caloporoside, and two further congeners, 2-hydroxy-6-([(16R)-(beta-d-mannopyranosyloxy) heptadecyl]) benzoic acid (HMHB) and Octyl-β-D-glucoside were chemically synthesised by our collaborator Professor Fürstner, A. (Max Plank Director, Mulheim, Germany), all other compounds were obtained from commercial sources.

### 4.2.2 Methods:

A series of dose–response competition binding experiment were performed with [$^{35}$S] TBPS, [$^{3}$H] Muscimol, [$^{3}$H] Flunitrazepam, [$^{3}$H] MK-801 and [$^{3}$H] Nicotine using well-washed adult rat forebrain membranes.

Well-washed adult rat forebrain membrane preparation, Lowry assay protein concentration determination and radioligand binding assays were all performed as described in Chapter 2 [section 2.4.2., 2.4.3 and 2.4.5 respectively].

## 4.3 Results

### 4.3.1 The Effect of the Three Compounds on the Binding of [$^{35}$S] TBPS to the Picrotoxin Site of the $GABA_AR$ Complex:

The effect of the three compounds on [$^{35}$S] TBPS binding was examined. Caloporoside completely displaced specific [$^{35}$S] TBPS binding to well-washed membranes in a concentration-dependant manner. Data were best fit to a one-site binding model, with a pseudo Hill coefficient close to unity, yielding a mean apparent $IC_{50}$ = 14.7 ± 0.1 μM. HMHB also completely displaced specific [$^{35}$S] TBPS binding to well-washed membranes in a concentration dependant manner. Data were best fit to a one-site binding model, yielding a mean apparent $IC_{50}$ = 14.2 ± 0.1 μM. Figure 4.2, A& B. In contrast, Octyl-β-D-glucoside stimulated [$^{35}$S] TBPS binding in a similar fashion to diazepam, yielding a mean $E_{max}$ = 144 ± 4% and apparent $EC_{50}$ = 39.2 ± 22.7 nM respectively, Figure 4.2, C.

### 4.3.2 Sensitivity to GABA:

In order to confirm that the stimulatory response was GABA-sensitive, 0.3 μM GABA was applied to the well-washed membranes. The presence of GABA partially reduced the overall [$^{35}$S] TBPS binding (by approximately 20%), and completely abolished the stimulation of [$^{35}$S] TBPS binding by both diazepam and Octyl-β-D-glucoside, Figure 4.3 A&B. Octyl-β-D-glucoside failed to have any inhibitory or stimulatory effects in the presence of GABA.

### 4.3.3 The Effect of Octyl-β-d-glucoside on the Agonist Binding Site of the GABA$_A$R labelled by [$^3$H] Muscimol:

In order to assess whether Octyl-β-D-glucoside directly binds, or allosterically modulates muscimol binding to the agonist binding site, a range of concentrations of Octyl-β-D-glucoside was tested upon [$^3$H] muscimol binding to a well-washed rat forebrain preparation. Specific binding was defined using 100 μM GABA. No significant effect (positive or negative) was detected across the full range of concentrations of Octyl-β-D-glucoside in at least three independent experiments. Figure 4.4 shows the results.

### 4.3.4. The Effect of the Three Compounds on the Benzodiazepine Binding Site of the GABA$_A$R labelled by [$^3$H] Flunitrazepam:

In order to investigate whether caloporoside and the congeners are binding to the benzodiazepine site itself, a [$^3$H] flunitrazepam binding assay was used. Specific [$^3$H] flunitrazepam binding was defined using diazepam (100 μM) and represented >90% of the total binding (not shown). In contrast, no significant effect (positive or negative) was observed with caloporoside, HMHB or Octyl-β-D-glucoside upon [$^3$H] flunitrazepam binding in at least five independent experiments Figure 4.5 shows the results. This suggests a lack of interaction (either directly or allosterically) of these three compounds with the benzodiazepine site of the GABA$_A$R. A small reduction in binding observed at a test concentration of 100 μM for the three compounds was due to the presence of 0.1% DMSO (solvent effect).

### 4.3.5 Influence of the Side Chain Carbon Length and Stereochemistry:

Results showed that Octyl-β-D-glucoside produced enhancement of [$^{35}$S] TBPS binding characteristic to GABA-gated chloride channels, it appeared to be a promising compound. So we carried out preliminary structure activity studies, to determine the basic core structure required for optimal activity.

The first structure activity question we addressed was whether the ring structure alone, and without substituents, had a modulatory effect on GABA$_A$R. The core monosaccharide glucose and analogues, galactose and mannose were tested. All three common sugars were found to have no significant effect upon [$^{35}$S] TBPS binding up to concentration of 10 mM, Figure 4.6, A&B shows the results.

We then investigated the effect of α and β conformation of the bond on the modulatory activity of the compound (stereochemistry effect), so we compared the activity of Octyl-α-D-glucoside to Octyl-β-D-glucoside using similar assay conditions of [$^{35}$S] TBPS. Octyl-α-D-glucoside appeared to be inactive, Figure 4.7, A &B showed the results. This indicates that the β-linkage is important for the interaction with the $GABA_AR$.

The influence of different substitution on position-1 of Octyl-β-D-glucoside ring system on the binding of $GABA_AR$ was further investigated. Four compounds with different carbon chain length were tested. These were Hexyl- β-D-glucoside, Heptyl-β-D-glucoside, Nonyl-β-D-glucoside and Methoxy-β-D-glucoside. The first three compounds were obtained from commercial sources and compound 4 was produced as a side product of a synthesis trial for Octyl-β-D-mannopyranoside, the positive modulator that modulate the function of $GABA_A$ chloride channel complex functionally in cortical mammalian neurons ( Lees *et al.*, 2000). An attempt to synthesise Octyl-O-β-D-mannopyransoside, was made using a method previously described by Singh (Singh *et al.*, 2000). This method was performed by the activation of the anomeric center of 2, 3, 4, 6-tetra-O-benzyl-1-O-D-mannopyranosyl propane-1, 3-diyldioxyphosphate in the presence of TMSOTf (Trimethylsilyl triflate). The desired compound was obtained by debenzylation, using palladium hydroxide [$Pd(OH)_2$] on carbon under nitrogen atmosphere. Unfortunately debenzylation step was carried out in the presence of ethanol and the substituted group (Octyl) was replaced by ($OCH_3$) group during the last step of the reaction confirmed by NMR analysis. A summary of the experimental protocol and synthesis steps are shown in appendix I. This compound was also used as a part of our structure activity study, since substitution on position-1 of Octyl-β-D-glucoside ring is reduced to one carbon length. Results are shown in Figure 4.8, A&B.

Our results showed that the length of carbon chain on position-1 of Octyl-β-D-glucoside ring is an important determinant for the affinity of this compound for the $GABA_A$ receptor. Introduction of a C6, C7, C9 or $OCH_3$ substituent on the position -1 of the compound Octyl-β-D-glucoside resulted in complete loss of activity, compared with Octyl-β-D-glucoside. This indicates that the length of carbon chain on this position is important for interaction with the $GABA_AR$.

#### 4.3.6 Does Lactose Bind to the Same Site as Octyl-β-D-glucoside?

Previously, we showed that lactose potentiated [$^{3}$H] TBOB binding to the channel site of the $GABA_A$ receptor, with a maximal effect observed at 10 μM. In contrast, interestingly, lactose has no effect upon [$^{35}$S] TBPS binding up to 100 μM. However, we showed that lactose (10 μM) completely occluded the potentiation by Octyl-α-d-glucoside of [$^{35}$S] TBPS binding. Octyl-β-D-glucoside failed to have any inhibitory or stimulatory effects in the presence of lactose, Figure 4.9.

### 4. 3.7. Selectivity of Action of Octyl-β-D-glucoside upon $GABA_AR$:

The action of Octyl-β-D-glucoside on a number of other common neuronal ligand gated ion channels was determined to confirm selectively of this compound for the $GABA_AR$. Thus, the effect of Octyl-β-D-glucoside was investigated at the excitatory ligand gated ion channels gated by NMDA, using [$^{3}$H] MK-801 binding assay. Additionally, the effect of Octyl-β-D-glucoside, was also determined on neuronal nicotinic receptors ($\alpha_4\beta_2$, $\alpha_7$ nAChRs) using [$^{3}$H] nicotine binding assay. Octyl-β-D-glucoside had no effect (positive or negative) upon [$^{3}$H] MK-801 or [$^{3}$H] nicotine binding up to a 100 μM, figure 4.10 & 4.11 respectively. Results showed that Octyl-β-D-glucoside does have selectively of action upon $GABA_A$ receptors and does not modulate all ligand gated ion channels.

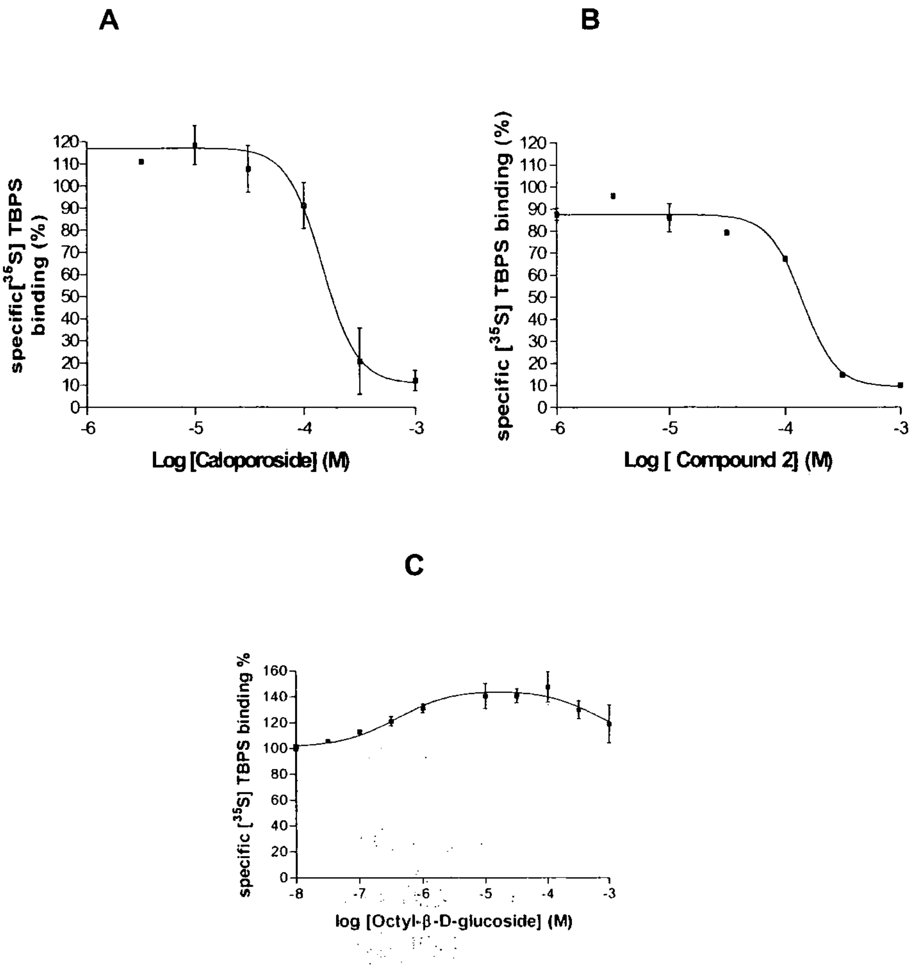
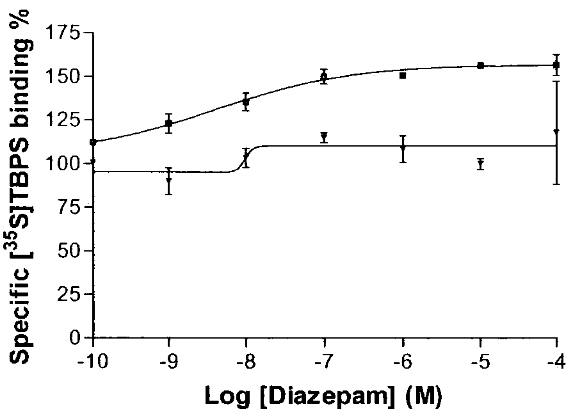



**Figure 4.2:** Effect of Caloporoside and congeners upon [$^{35}$S] TBPS binding to rat forebrain membranes. Effects of the compounds (**A**= Caloporoside; **B** = HMHB; **C**= Octyl-β-D-glucoside) on specific [$^{35}$S] TBPS binding to well-washed adult rat forebrain membranes. Results are expressed as mean percentage values ± S.D. for three independent experiments.

A




B

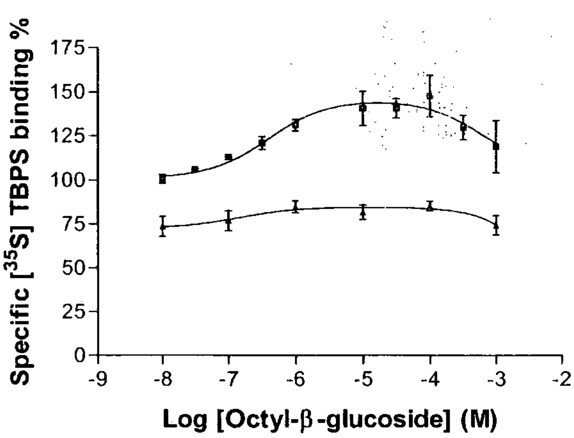



**Figure 4.3**: Effect of Diazepam **(A)** and Octyl-β-D-glucoside **(B)** on specific [$^{35}$S] TBPS binding to well-washed adult rat forebrain membranes, in the absence (■) and presence of GABA (0.03 μM) (▼). Results are expressed as mean percentage values ± S.D. for three independent experiments.

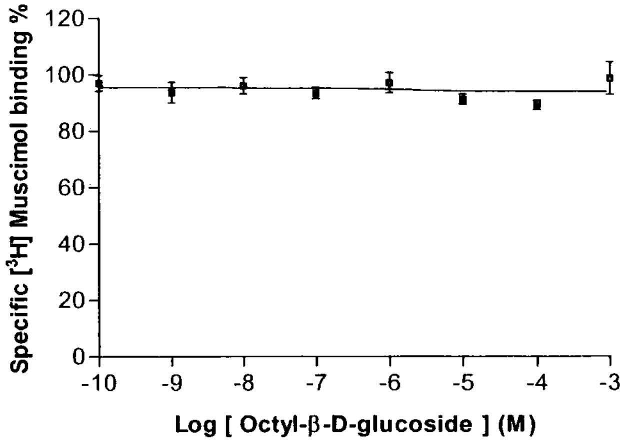



**Figure 4.4:** [$^{3}$H] Muscimol competition binding to adult rat forebrain membranes by Octyl-β-D-glucoside. Results are expressed as mean percentage values ± S.D. for three independent experiments.

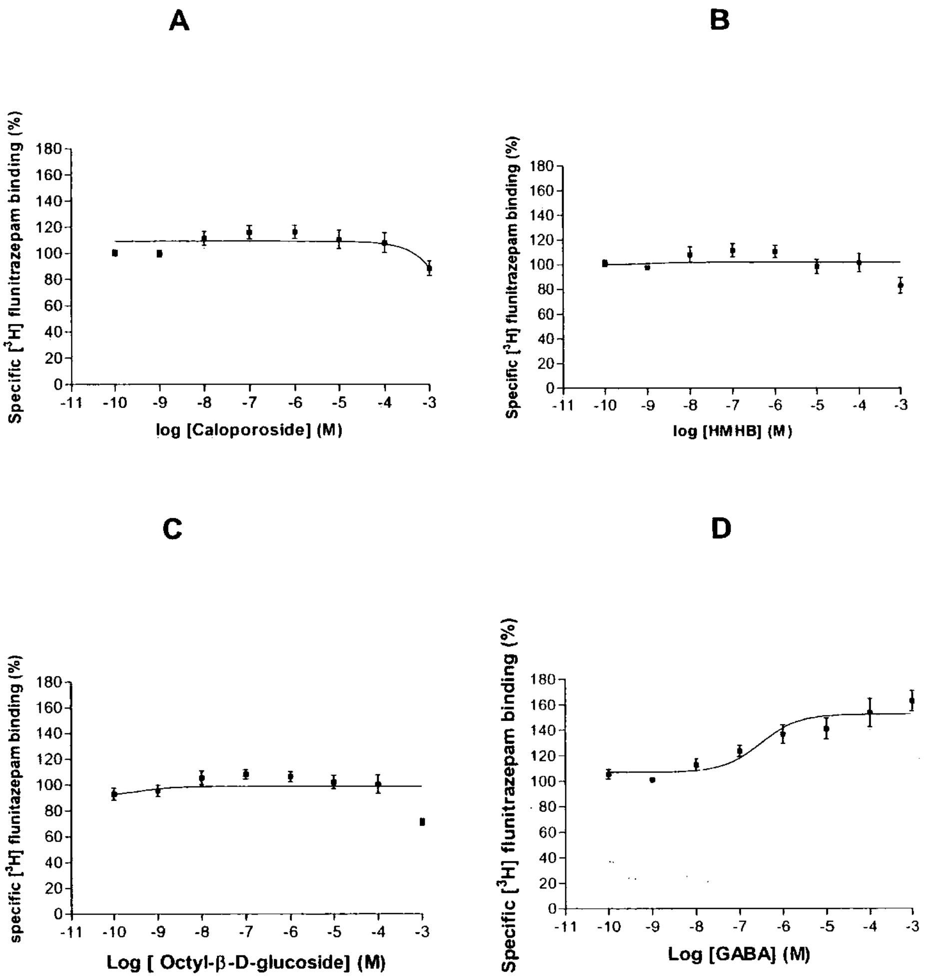



**Figure 4.5**: Effects of compounds (**A**= Caloporoside; **B** = 2-Hydroxy-6-{[(16R)- β-D-mannopyranosyloxy) heptadecyl]} benzoic acid; **C** = Octyl-β-D-glucoside, **D**= GABA) on [$^3$H] flunitrazepam binding to well-washed adult rat forebrain membranes. Results are expressed as mean percentage values ± S.D. for three independent experiments.

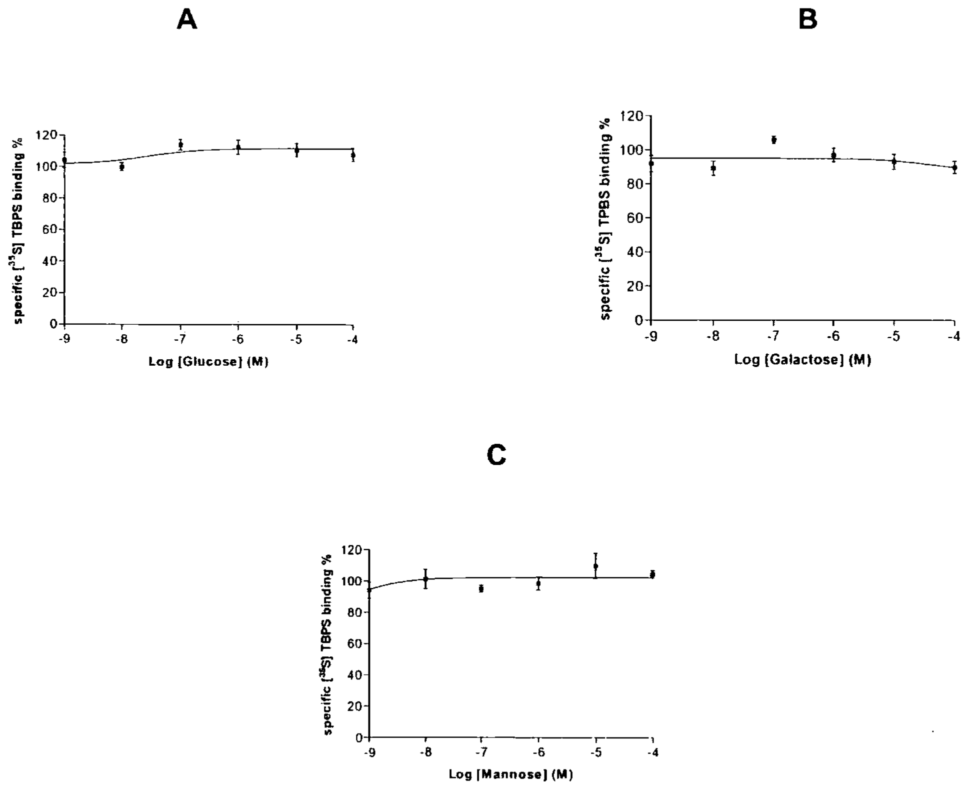
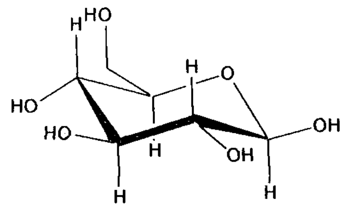
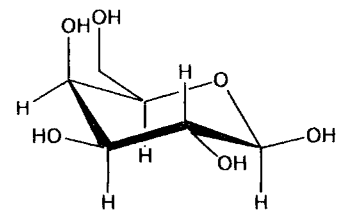


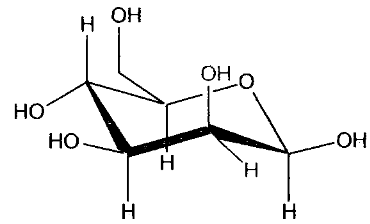

**Figure 4.6 (A):** Effect of **(A)** Glucose, **(B)** Galactose and **(C)** Mannose upon [$^{35}$S] TBPS binding to rat forebrain membranes. Results are expressed as mean percentage values ± S.D. for three independent experiments.



**β-D-Glucose**



**β-D-Galactose**



**β- D-Mannose**

**Figure 4.6 (B):** Structures of monosaccharide assayed on [$^{35}$S] TBPS binding to rat forebrain membranes $GABA_AR$.

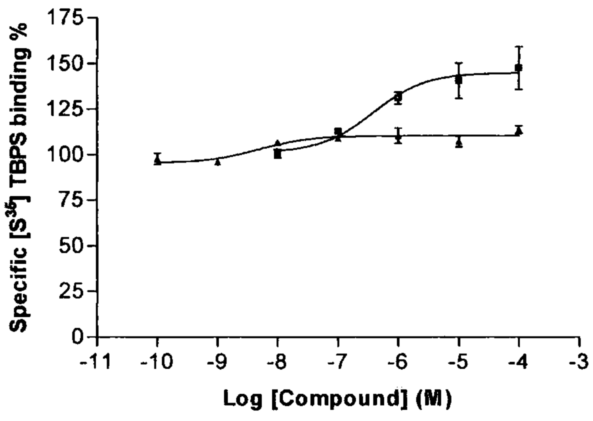
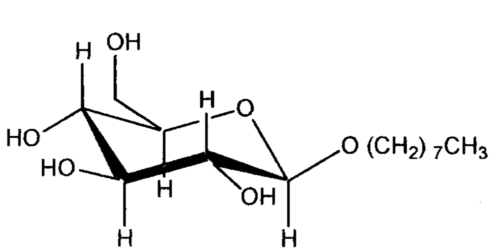
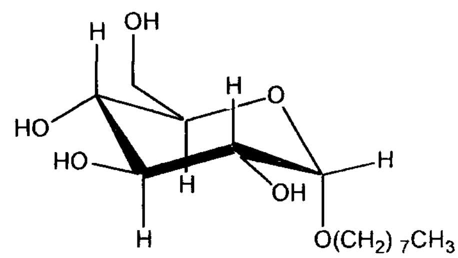



**Figure 4.7 (A):** Effects of Octyl-α-D-glucoside (▲) in comparison to Octyl-β-D-glucoside (■) on specific [$^{35}$S] TBPS binding to well-washed adult rat forebrain membranes. Results are expressed as mean percentage values ± S.D. for three independent experiments.



**Octyl-β-D-glucoside**



**Octyl-α-D-glucoside**

**Figure 4.7 (B):** Comparison of Octyl-α-D-glucoside and Octyl-β-D-glucoside structures.

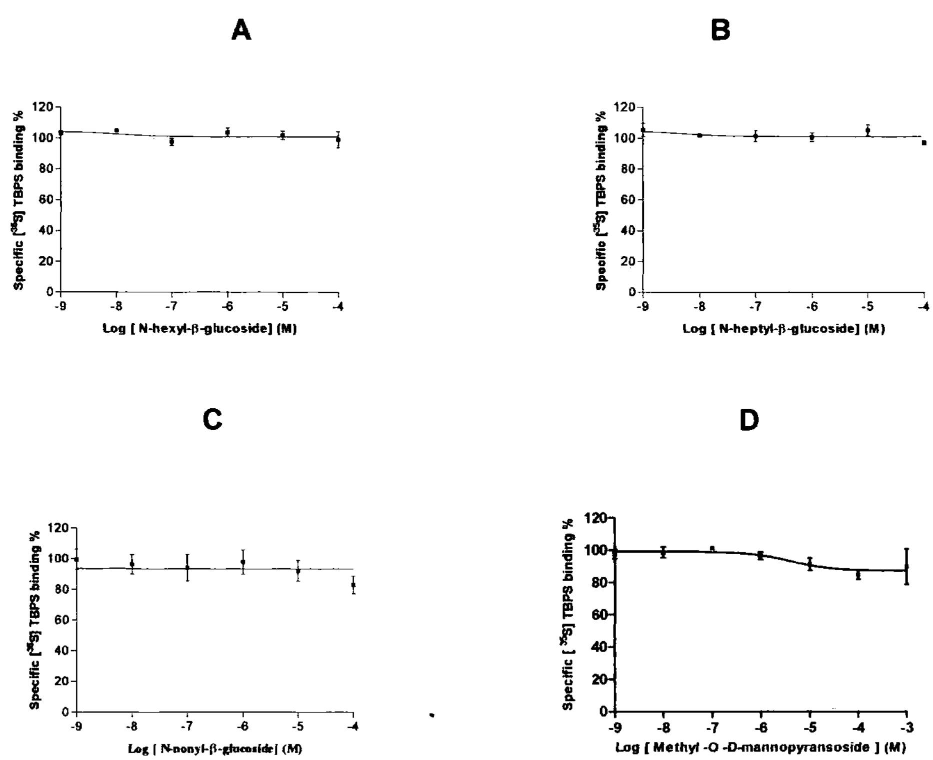
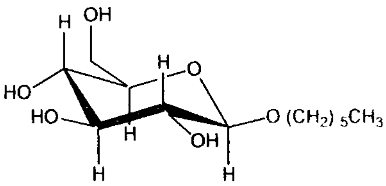
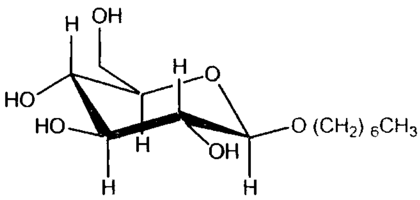


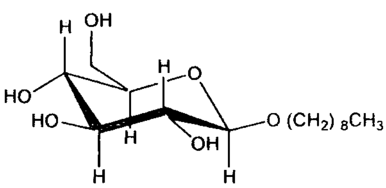

**Figure 4.8 (A):** Effect of **(A)** hexyl-β-D-glucoside, **(B)** Heptyl-β-D-glucoside **(C)** Nonyl-β-D-glucoside and **(D)** Methyl-O-β-D-mannopyransoside upon [$^{35}$S] TBPS binding to rat forebrain membranes.



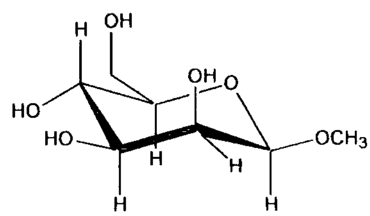
**Hexyl-β-D-glucoside**



**Heptyl-β-D-glucoside**



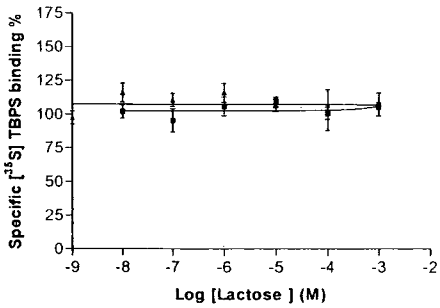
**Nonyl-β-D-glucoside**



**Methyl -O-β-D-mannopyransoside**

**Figure 4.8 (B):** Structures of compounds with different side chain carbon length assayed on [$^{35}$S] TBPS binding to rat forebrain membranes $GABA_AR$.

A




B

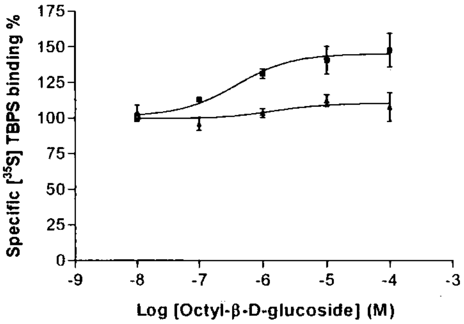



**Figure 4.9:** Effect of lactose upon Octyl-β-D-glucoside modulation of [$^{35}$S] TBPS binding to rat forebrain membranes.

**A.** Effect of lactose upon [$^{35}$S] TBPS binding to well-washed adult rat forebrain membranes, in the absence (■) and presence of Octyl--β-D-glucoside (10 μM) (▼)

**B.** Effect of Octyl-β-D-glucoside on specific [$^{35}$S] TBPS binding to well-washed adult rat forebrain membranes, in the absence (■) and presence of lactose (10 μM) (▼).

Results are expressed as percentages (mean values ± SD for three independent experiments) of control specific [$^{35}$S] TBPS binding in the absence of test compounds.

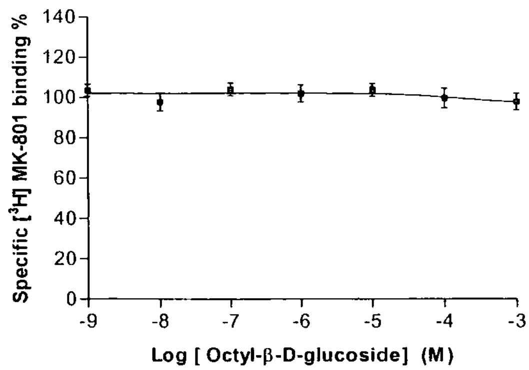



**Figure 4.10**: [$^3$H] MK-801 competition binding to adult rat forebrain membranes by Octyl-β-D-glucoside. Results are expressed as percentages mean ± S.D. for three independent experiments for each.

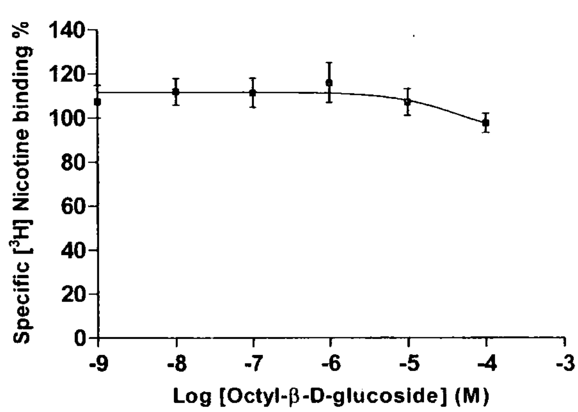



**Figure 4.11**: [$^3$H] Nicotine competition binding to adult rat forebrain membranes by Octyl-β-D-glucoside. Results are expressed as percentages mean ± S.D. for three independent experiments for each.

## 4.4 Discussion

The effects of Caloporoside and two smaller congeners were assayed using a [$^{35}$S] TBPS binding assay on adult rat forebrain membranes. These data suggest that Caloporoside and HMHB are low affinity inhibitory $GABA_AR$ channel ligands, while, in contrast, Octyl-β-D-glucoside is a relatively high affinity positive $GABA_AR$ channel modulator. The positive modulatory effect of Octyl-β-D-glucoside was occluded in the presence of GABA, in a similar fashion to benzodiazepines, indicating that the modulatory action of Octyl-glucoside is related to the conformational state of the chloride channel (Xue *et al.*, 1996). GABA sensitivity is shared by a number of other $GABA_AR$ modulators, as well as benzodiazepines, including loreclezole, propofol and lactose (Xue *et al.*, 1996; Ghiani *et al.,* 1996, Rezai *et al.,* 2003).The lack of inhibitory action of Octyl-β-D-glucoside at high concentrations is a property shared by diazepam, but not loreclezole or propofol. This property has been previously attributed to the lack of ability of diazepam to activate $GABA_AR$ channel in the absence of GABA (Ghiani *et al.*, 1996).

The lack of effect of Octyl-β-D-glucoside upon [$^{3}$H] muscimol binding demonstrated that Octyl-β-d-glucoside does not directly bind to the agonist binding site. Based on shared properties of Octyl-β-D-glucoside and diazepam in modulating [$^{35}$S] TBPS, we also directly investigated the effect of Octyl-β-D-glucoside upon [$^{3}$H] flunitrazepam binding, using well-washed membranes. Neither Caloporoside, HMHB nor Octyl-β-D-glucoside displayed any significant (positive or negative) effect upon [$^{3}$H] flunitrazepam binding, which strongly suggested a lack of allosteric or competitive linkage with the benzodiazepine site. This property is in marked contrast to other ligands tested, such as GABA and diazepam, respectively. GABA positively modulates and diazepam competitively inhibited [$^{3}$H] flunitrazepam binding, consistent with previous studies. These data confirm that the novel compound class binds to a unique site on the $GABA_AR$. It should be noted that Octyl-β-D-glucoside has been previously used as a detergent for the solubilisation of $GABA_A$ receptors, but at high mM concentrations (e.g. Hammond *et al.,* 1986). However, the propensity of Octyl-β-D-glucoside to bind to membranes indicates that it may bind within the membrane spanning channel domain of the $GABA_AR$. The lack of effect of Octyl-β-D-glucoside upon channel binding of [$^{3}$H] MK-801 to another common ligand gated channel, namely the NMDA glutamate receptor, and neuronal nicotinic receptor suggests that Octyl-β-D-glucoside does not bind non-selectively and indiscriminately modulate all ligand-gated channels in the membrane.

Interestingly, the monosaccharide present in compound 3, glucose and other analogues galactose and mannose had no significant effect upon [$^{35}$S] TBPS indicating that the presence of the extended side chain was absolutely necessary for $GABA_AR$ modulation. In order to investigate whether the nature of the glycosidic linkage is important, we compared, in parallel, the effects of Octyl-α-D-glucoside, and Octyl-β-d-glucoside over the same concentration range. In contrast to Octyl-β-D-glucoside, Octyl-α-D-glucoside appeared to be inactive.

Our experiments imply that modulation depends on more than just the bond conformation, and suggest that carbon chain length also plays a role in determining the activity on [$^{35}$S] TBPS binding. Results indicated that both the β-linkage and an alkyl side-chain of 8-C in length, was crucial for the positive modulation of [$^{35}$S] TBPS binding. These results extend upon our previous observations with β- and α-linked disaccharides, which showed that β-glycosidic linkage yielded higher affinity $GABA_A$ receptor binding than α-glycosidic linkage (Rezai *et al.*, 2003).

Interestingly, lactose had no affect upon [$^{35}$S] TBPS which is in contrast to its affect upon [$^{3}$H] TBOB binding (Rezai *et al.*, 2003). The differences in salt concentration in the two assays may explain this difference. Furthermore, the expanded structure of TBOB in comparison to TBPS may account for the differential allosteric influence of lactose and warrants further study. However, lactose (10 μM) completely blocked the positive modulation of [$^{35}$S] TBPS, which provides evidence for a shared binding site between these two β-glycosidic linked ligands.

In conclusion, this study has delineated clear differences in the pharmacological binding properties of the large natural product Caloporoside and the small polar congener, Octyl-β-D-glucoside. The findings reported in this study also provides evidence, firstly that Octyl-β-D-glucoside binding is independent of the benzodiazepine and agonist binding sites, secondly, that the side chain is absolutely required for activity, and thirdly that glycosidic linkage and side chain length are important determinants of the modulatory activity. This present study has provided a clearer picture of the SAR of this novel class of $GABA_AR$ modulator, which warrants further elucidation using $GABA_AR$ electrophysiological and behavioural studies (Lees *et al.*, 2000, Ennaceur *et al.*, 2006).

The work presented in this chapter has been published in *Biochem.Pharmacol. Journal*, 2005.

**Abuhamdah, S, Fuerstner, A, Lees, G and Chazot, PL (2005). Pharmacological binding studies of caloporoside and novel congeners with contrasting effects upon [$^{35}$S] TBPS binding to mammalian GABA-A receptor, *Biochem.Pharmacol.* 70(9):1382-8.**

# Chapter 5

## Natural Products & $GABA_A$ Receptors Elucidation of the Pharmacological Mechanisms of Melissa & Lavender Essential Oils

### 5.1 Introduction

The importance of natural products in the future of drug discovery is clear. Novel biologically active natural products will continue to serve as lead compounds for drug development and as biochemical probes for the discovery of pharmacological and biochemical processes (Jones *et al.*, 2006). Clearly, the natural products discovered to date have played a vital role in improving the human condition, and this role will continue as long as there are unexplored sources of novel natural products.

Aromatherapy using extracts of selected plant species offers one possible alternative to pharmacotherapy (Diamond, 2003). Knowledge of the distillation of essential oils and their application to improve health and well-being was introduced into science in the 10th century (Ballard *et al.*, 2002). Aromatherapy is currently used worldwide in the management of chronic pain, depression, anxiety, some cognitive disorders, insomnia and stress-related disorders (Perry & Perry, 2006). However, there is still not sufficient evidence to recommend widespread use in clinical practice and a key question of whether these treatments can provide a viable alterative to existing pharmacological agents needs to be addressed (Diamond, 2003).

Clear scientific evidence in psychiatric disorders and the effects of essential oils in vitro and in vivo models have been published (Beaubrun & Gray, 2000, Howes *et al.,* 2003, Diamond, 2003). It is concluded that aromatherapy provides a potentially effective treatment for a range of psychiatric disorders. In addition, taking into account the available information on safety, aromatherapy appears to be without the adverse effects of many conventional psychotropic drugs. Investment in further clinical and scientific research is clearly warranted (Perry & Perry, 2006).

## 5.2 Aromatherapy for Dementia

Dementia is increasingly an important management problem as the elderly population increases. Although attention is usually focused on cognitive deficits, more than 50% of people with dementia experience behavioural or psychiatric symptoms, by convention referred to as "Behavioural and Psychological Symptoms in Dementia" BPSD which include aggression, agitation, screaming, wandering, hallucination and delusion. They are distressing for the patients and problematic for their carers, and are frequently the trigger for placement in residential or nursing home care (Ballard *et al.*, 2002, Sato *et al.*, 2006, Lanari *et al.,* 2006).

Pharmacological treatment with neuroleptic agents is often the first line treatment for these symptoms. There are no trials specifically in people with severe dementia, although placebo-controlled trials have demonstrated moderate efficacy for the treatment of BPSD with neuroleptic agents in people with mild/moderate dementia (Daiello *et al.*, 2003, Kurz *et al.*, 2005, Omelan, 2006). Neuroleptics are often poorly tolerated by people with dementia, particularly those with severe dementia, and there is a high risk of adverse events (e.g. Parkinsonism, drowsiness, falls, accelerated cognitive decline and increased mortality) and a detrimental impact on key indicators of quality of life, including activities, well-being and social interaction (Lanari *et al.,* 2006). However, even neuroleptics have been the best-studied class of drugs to date, modest efficacy and significant potential side effects often limit their use (Ballard *et al.*, 2002, Carson *et al.*, 2006).

The most frequent and persistent BPSD syndrome in patients with severe dementia is agitation, usually characterized by a combination of aggression (Verbal and/or physical) restlessness, and abnormal vocalization in the context of subjective anxiety. Therefore, particularly for those with severe dementia, there is an urgent need to identify safer and better tolerated treatment paradigms for behavioural disturbance, especially for the management of agitation (Herrmann *et al.*, 1997, Lanari *et al.*, 2006).

"Complementary" or "alternative" therapies have become more popular and commonly used over the last decade and have been applied to a wide range of health problems, including people with dementia (Diamond *et al.*, 2003). Therapies have included massage (e.g. Kim *et al*, 1999), aromatherapy (e.g. Vance, 1999) and herbal medicine (e.g Perry *et al*, 1999). Of these aromatherapy is reported to be the most commonly used in the world, and is possibly the most widely used complementary therapy for people with dementia (Perry & Perry, 2006).

Aromatherapy is a part of the discipline of phytotherapy (the use of the whole plants or parts of plants for medicinal purposes) and uses pure essential oils from fragrant plants (such as Peppermint, Sweet Marjoram, Lavender and Rose) to help relieve health problems and improve quality of life in general (Perry *et al.*, 1998, Howes *et al.,* 2003, Houghton & Howes 2005).

Essential oils have been defined as non-oily, highly fragrant essences extracted from plants by distillation, which evaporate readily (Tisserand, 1988) and have been used by health care professionals all over the world for their antibiotic and antiviral properties for many years (Newall *et al.*, 1996, Price & Price, 1999). They are most commonly used in oil burners, in bath water, or massaged into the skin, thus the aroma of the essential oil evaporates and stimulates the olfactory sense. The healing properties of aromatherapy are claimed to include promotion of relaxation and sleep, relief of pain, and reduction of depressive symptoms (Newall *et al.,* 1996, Buckle, 2003), the rationale being that the essential oils have a calming and de-stressing effect. As such, aromatherapy might be of use as an intervention for people who have little or no preserved language function, are confused or for whom verbal interaction is difficult and conventional medicine is seen as of only marginal benefit. Aromatherapy has therefore been used for people with dementia to reduce disturbed behaviour, to promote sleep and to stimulate motivational behaviour (Price & Price, 1999, Buckle, 2003).

Despite its frequent use, the rationale for aromatherapy is based on anecdotal rather than scientific evidence. Moreover, aromatherapy does impose a cost on consumers. It is also frequently used in combination with other therapeutic approaches, such as massage, which adds to the cost, is more intrusive and increases the vulnerability of the recipients. Additionally, there remain some concerns regarding the safety of aromatherapy, as some essential oils have been found to have a significantly toxic effect on rodents (Newall *et al.*, 1996). Aromatherapy is currently not under any licensing restrictions and is easily accessible from pharmacies and health product stores. There is therefore a need for the effects of aromatherapy to be adequately documented (Buckle, 2003, Diamond *et al.*, 2003)

## 5.3 Neuronal System Dysfunction in Dementia

There is mounting evidence that links BPSD to specific alterations in neurochemistry, which may provide the basis of pharmacological manipulation. Dementia is associated with dysfunction in multiple neurotransmitter systems. Although the most well-studied neuronal system dysfunction is in the cholinergic system, there is also evidence supporting dysfunction in the serotonergic, noradrenergic, dopaminergic and GABA systems. Since these neurotransmitters are known to regulate behaviours and also are amenable to pharmacological intervention, research attention has recently focused on the possible relation between these neurotransmitter dysfunctions and behavioural disorders seen in dementia (Garcia-Alloza *et al.*, 2005, Lanari *et al.*, 2006).

The neurotransmitter GABA is reported to be involved in behaviours such as aggression. Animal studies have shown that increasing GABA can decrease aggression (Eichelman, 1987). Deficits in the central GABA system have been demonstrated in the brains of patient with dementia (Elisson *et al.,* 1986, Hardy *et al.*, 1987). Some indirect evidence is provided by some of the drugs that are effective in the treatment of agitation like benzodiazepines (Herrmann *et al.,* 1997). Furthermore, valproic acid, which is also effective in aggressive behaviours associated with dementia, is also believed to increase GABA (Mellow *et al.*, 1993). Clearly, direct evidence is required before any link between disruptions in the GABA system and specific behaviour is demonstrable (Herrmann *et al.,* 1997, Garcia-Alloza *et al.*, 2005, Lanari *et al.*, 2006).

## 5.4 Medicinal Plants for Dementia Therapy

The most commonly used essential oils for dementia therapy in controlled trials have been Lavender (*Lavendula augustifolia*) and lemon balm(*Melissa officinalis*), singly or in combination. The trials have involved people with advanced dementia in residential care and have generally assessed behavioural symptoms, particularly agitation as outcome measures. The trials divide equally between inhalation and dermal application, with duration up to 4 weeks. What is remarkable, given the diversity of trial design and the type of aromatherapy, is that all the treatment have resulted in significant benefits include reduction of agitation, insomnia, wandering, difficult behaviour and social withdrawal (Perry & Perry, 2006).

A series of case reports has indicated some potential benefits of aromatherapy in dementia pilot placebo-controlled trials, limitation of these studies were the small number of patients and a relatively short period of follow up assessment. According to literature data only one comprehensive study demonstrated benefit for people with severe dementia (Ballard *et al.,* 2002). Ballard *et al.* (2002) reported a double blind placebo-controlled trial with Melissa oil for treatment of agitation in 71 patients suffering from severe dementia, results indicate that aromatherapy with Melissa essential oil is safe, well tolerated and highly efficacious with additional benefits on key quality of life parameters. These findings highlight the need for longer term multi-center trials investigating the role and mechanisms of action of aromatherapy as an alternative to psychotropic medication for the treatment of agitation in people with severe dementia (Perry & Perry, 2006).

## 5.5 Melissa & Lavender

Two plant essential oils were selected for the present study, these were Melissa and Lavender. Melissa oil is the essential oil extracted from the leaves of *Melissa officinalis* L. (Lamiaceae), Figure 5.1 A. This plant has been used as a medicinal plant for more than 2000 years. In traditional medicine *M.officinalis* was used as a calming and strengthening remedy, to treat migraines, neuroses and hysteria. The plant has been claimed for promoting long life and for restoring memory (Howes *et al.,* 2003). The Commission E Monograph in Germany approves the use of *M.officinalis* for nervous insomnia. In modern alternative medicine *M.officinalis*, essential oil is used in aromatherapy to alleviate depression, anxiety, stress and insomnia (McVicar, 1994). In addition, the safety of treatment with balm essential oil has been well established in clinical populations (Price & Price, 1999).

Main constituents: 0.01%- 0.20% essential oil, at least 70 components including: monoterpenes > 60%, mainly aldehydes, citronellal (30%-40%), citral (20%-30%), citronellal, nerol, gerniol and β-ocimene. Sesquiterpene> 35%, β-carophyllene and germacrene D (Bisset, 1994, Tittle *et al.*, 1982, Wagner, 1996, Newall *et al.*, 1996).

*M.officinalis* leaf was reported to alleviate mild anxiety and nervousness in a double-blind study alone and in combination with *Valeriana officinalis* root. It was also reported to be as effective as triazolam, but did not cause drowsiness or impair concentration (Yarnell, 1998). A hydroalcohol (30% ethanol) extract of *M.officinalis* leaf was sedative in mice and potentiated barbiturate induced sleep, but *M.officinalis* essential oil did not demonstrate these sedative effects (Soulimani *et al.,* 1991).

Kennedy *et al.* (2004, 2006) reported attenuation of laboratory-induced psychological stress in human after acute administration of Melissa extracts. Other activities of *M.officinalis* extracts that may be useful for dementia therapy include antioxidant effects (Howes *et al.,* 2003) and binding to muscarinic and nicotinic receptors in vitro (Perry *et al.*, 1998, Wake *et al.*, 2000, Kennedy *et al.*, 2002, 2003), which suggests that favorable effects on cholinergic function may occur in patients with dementia.

Lavender oil is the essential oil obtained from the aerial part of *Lavendula angustifolia* Mill (Lamiaceae). Figure 5.1, B. The plant is used in traditional and folk medicines in different parts of the world for the treatment of several gastrointestinal, nervous and rheumatic disorders. It is also used as an anti-bacterial, anti-fungal, anti-depressant carminative, smooth muscle relaxing agent and sedative (Duke, 1989, Evans, 1989, Leung & Foster, 1996).

Main constituents: 1%- 3% essential oil, at least contains 150 components mainly linalyl acetate (30 - 55%) & linalool (20-35%), with small quantities of nerol, borneol, β-ocimen, geraniol cineole, caryophyllene-epoxide and camphene (Wagner, 1996).

The pharmacological profile of Lavender essential oil has been the most widely investigated and provides a model for the pharmacological activity of essential oil and its individual constituents (Basch *et al.*, 2004, Cavanagh & Wilkinson, 2002, Perry & Perry, 2006). The action of Lavender may be significant in the quest for novel anxiolytic agents that lack the dependency issues associated with current therapies such as benzodiazepines (Betts, 2003). Inhalation of the essential oil of (*L.angustifolia*) has been found to block pentetrazol, nicotine and electroshock-induced convulsions (Yamada, 1994) and exhibit dose-dependant anti-conflict effects in mice similar to those of diazepam (Umezu, 2000). The main constituents of Lavender, the monoterpenoid linalool, possess anticonvulsant properties in glutamate related seizure models and effects on NMDA receptor binding (Elisabetsky *et al.,* 1995, 1999, Brum *et al,* 2001). It also inhibited potassium-stimulated glutamate release (Silva *et al.*, 2001) and modified the kinetics of the nicotine receptor ion channel at the mouse neuromuscular junction (Re *et al.*, 2000). Available data suggest that the anticonvulsant and CNS depressant effects of (*L. angustifolia*) and its main constituent linalool are likely to occur via modulation of components of the glutamateric system (i.e.NMDA receptor subtype), although more direct cellular mechanisms such as inhibition of adenylate cyclase and ion channel activity (affecting neurotransmitter release) may be relevant to its clinical effect (Lis-Balchin, 1999, Elisabetsky *et al.*, 1995, 1999, Brum *et al,* 2001, Re *et al.*, 2000). Such physiological mechanisms are consistent with the extensive use of Lavender as a sedative/CNS depressant and anti-anxiety agent in aromatherapy and herbal medicine (for reviews see Basch *et al.*, 2004, Cavanagh & Wilkinson, 2002, Perry & Perry, 2006).

In summary, literature data demonstrated that Melissa and Lavender are used in a wide range of both cosmetic and therapeutic settings and the oils have been demonstrated to have a range of biological activities and they have a great therapeutic potential for treatments of agitation in severe dementia, while displaying minimal side effects.

Selection of the most appropriate aromatic oil/combination of oils for the reduction of agitation should be based on relevant pharmacological activity. It is important to determine the mechanism of action of the aromatic essential oil to be tested, not only to provide rational for treatment selection, but also so that treatment effects can be reproduced and optimized in future study and clinical practice, and to identify key chemical constituents that are active in scientific–based investigations. There is limited information about the pharmacological effect of these plants since most studies were placebo-controlled trials which use ethno-pharmacological approaches for selecting several plant species for their effect on symptoms such as anxiety, restlessness, excitability and depression.

In order to elucidate the pharmacological basis for the actions of the Melissa and Lavender essential oils, a pharmacological screen was carried out using radioligand binding in native and recombinant preparations. Lavender and Melissa oils were sourced from four separate authenticated suppliers. Interactions of the oils with both ligand-gated ion channel receptors (NMDA, nicotinic and $GABA_AR$) and G-protein coupled receptors (5-$HT1_A$, 5-$HT2_A$, muscarinic M1 and histamine $H_3$) implicated in agitation in severe dementia (Garcia-Alloza *et al.*, 2005, Lanari *et al.*, 2006) have been examined. This study was a part of project grant funded by the Alzheimer's Society (U.K), carried out at the medicinal plant research center (MPRC, Newcastle) including many collaborators. My part of the study was the effect of these oils on ligand–gated ion channels which will be represented in this chapter.

A.


B.


**Figure 5.1:**

**(A)** Leaves of *Melissa officinalis* L. (Lamiaceae), taken from www.accentbotanical.com

**(B)** Aerial parts of *lavandula angustifolia* Mill (Lamiaceae), taken from www.toptropical.com

## 5.6 Materials & Methods

### 5.6.1 Materials:

Melissa & Lavender essential oils were sourced from four separate authenticated suppliers (Baldwin's, Pranarom, Quintessence and Fytosan). An analysis of the terpene constituents based on gas chromatography mass spectroscopy (GCMS) was carried out at Royal Botanic Garden Kew, using Perkin-Elmer Autosystem XL (GC) coupled to a Perkin-Elmer TurboMass (quadrupole) MS, [DB-5MS column (30 m x0.25mm; film thickness, 0.25µm, Helium as carrier gas and temperature programming from 40°C to 300°C @ (3°C/min, injection temp 220 °C).

Identification of the substances was carried out by comparison of their retention indices (RI) with literature values and their mass spectral data with those from NIST/EPA/MSDC MASS Spectral Database. The Gas chromatography profiles showing the main constituents of the two oils from the four suppliers are shown in Appendix II.

GC analysis demonstrated that the principle monoterpenes detected in all Lavender oil samples were linayl acetate (36.7%, 41.6, 39.7% and 39.4%, respectively) and linalool (30.8%, 27.3%, 30.1% and 33.3%, respectively). The percentage composition of linayl acetate and linalool and other components detected comply with the percentage composition of *Lavandula angustifolia* oil described in the British Pharmacopeia, 2002.

The principle monoterpenes detected in all Melissa oil samples, were geranial and neral (citral). The percentage composition of citral in the samples was (54.9%, 27.3%, 38.7% and 49.7% respectively). The principle sesquiterpene detected in all oils was (E) caryophyllene, detected at (12.3%, 24.7%, 12.2% and 9.5% respectively). These compounds are reported to be some of the major components of *M.officinalis* essential oil (Bisset, 1994, Adams, 2001).

### 5.6.2 Methods:

A pharmacological screen has been conducted for Melissa & Lavender essential oils, either alone or in combination using radioligand binding techniques on rat adult forebrain membranes and recombinant $GABA_AR$ stably expressed in HEK293 cells.

Interactions of the oils have been examined focusing on the three major binding sites of $GABA_AR$: the benzodiazepine site, the GABA site and the channel site, to detect any $GABA_A$ modulatory activity. To confirm selectivity, interactions with other common ligand-gated ion channel receptors such as NMDA and neuronal nicotinic receptor were also investigated.

A series of dose–response competition binding experiments were performed with [$^{35}$S] TBPS, [$^{3}$H] Muscimol, [$^{3}$H] Flunitrazepam, [$^{3}$H] MK-801 and [$^{3}$H] Nicotine. Both oils were examined at four different concentrations [0.001, 0.01, 0.1 and 1 mg/ml]. Solutions of Melissa & Lavender oils were prepared on the day of the experiment in serial dilutions using assay buffer and oil stock of 100mg/ml in DMSO. No effect of solvents on radioligand binding assays was seen at concentrations below 0.1% (v/v) DMSO.

Well-washed adult rat forebrain membrane preparation, Lowry assay protein concentration determination, cell culture and radioligand binding assays were all performed as described in Chapter 2, [section 2.4.2., 2.4.3, 2.4.4 and 2.4.5 respectively].

## 5.7 Results

### 5.7.1 Effects of Melissa & Lavender Essential Oils on the Channel Binding Site of the $GABA_AR$ labelled by [$^{35}S$] TBPS:

To investigate the effect of Melissa and Lavender oils on the channel site of $GABA_A$ receptor [$^{35}S$] TBPS binding activity was carried to well-washed to adult rat forebrain, using four different oil concentrations [0.001, 0.01, 0.1 and 1 mg/ml] of Melissa and Lavender either alone or in combination. Specific binding was defined using 100 µM picrotoxinin (chapter 2, section 2.4.5.3). Figure 5.2 A, B & C shows the results. The binding of [$^{35}S$] TBPS decreased with increasing concentrations of the oils. The inhibition of [$^{35}S$] TBPS binding by increasing concentrations of the oils was dose dependent, attaining 50% inhibition between 0.01-0.1 mg/ml conc. for Melissa ($IC_{50}$ 0.040 mg/ml ± 0.001, correlation coefficient = 0.99), between 0.1-1 mg/ml conc. for Lavender ($IC_{50}$ 0.300 mg/ml ± 0.001, correlation coefficient = 0.99) and for the combination ($IC_{50}$ 0.070 mg/ml ± 0.001, correlation coefficient = 0.88) respectively.

GC analysis demonstrated that the principle monoterpenes constituent detected in the oil samples of Lavender were highly similar from the four suppliers (Baldwin's, Pranarom, Quintessence and Fytosan). In contrast, the principle monoterpenes constituents detected in oil samples of Melissa from the four suppliers showed some differences. The Baldwin's and Fytosan monoterpenes constituent were highly similar, while in the Pranarom and Quintessence samples ,traces of other monoterpenes were detected (see Appendix II).

In order to examine whether these differences have an effect on the activity of the oils, we tested the effect of Melissa from the four authenticated oil samples suppliers for their ability to inhibit [$^{35}S$] TBPS binding. Figure 5.3 shows the results. Melissa essential oils from the four suppliers showed similar dose-dependent inhibition on [$^{35}S$] TBPS binding, without significant differences in the affinity between the oils; suggested that the additional monoterpenes did not count for this pharmacological property.

### 5.7.2 Effects of Melissa & Lavender Essential oils on the Benzodiazepine Binding Site of the $GABA_AR$ labelled by [$^3H$] Flunitrazepam:

The effects of Melissa and Lavender oils on radioligand binding to benzodiazepine site of $GABA_AR$ were studied using [$^3H$] flunitrazepam binding assay (chapter 2, section 2.4.5.1). Specific [$^3H$] flunitrazepam binding was defined using diazepam (100 µM).

Melissa and Lavender oils alone did not alter the equilibrium binding of [$^3$H] flunitrazepam to $GABA_A$ receptors in adult rat forebrain membranes. Figure 5.4 A&B shows the results. Interestingly, an additive effect on the inhibition [$^3$H] flunitrazepam binding has been shown when Lavender and Melissa are applied in combination. Figure 5.4, C shows the results. The binding of [$^3$H] flunitrazepam decreased with increasing concentrations of the oil mixtures. The inhibition of [$^3$H] flunitrazepam binding by increasing concentrations of the oils was dose dependent, attaining maximum inhibitory effect at 0.1 mg/ml conc. with an apparent ($IC_{50}$<0.001 mg/ml).

### 5.7.3 Effects of Melissa & Lavender Essential Oils on the Agonist Binding Site of the $GABA_A$R labelled by [$^3$H] Muscimol:

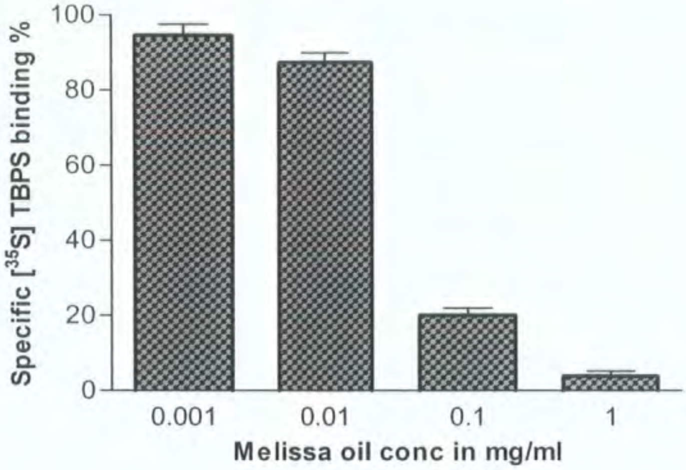
The effects of Melissa and Lavender oils on radioligand binding to agonist binding site of $GABA_A$R were studied using [$^3$H] muscimol binding assay (chapter 2, section 2.4.5.2). Specific binding was defined using 100 μM GABA. Lavender oil alone and combination did not alter the equilibrium binding of [$^3$H] muscimol to $GABA_A$ receptors in adult rat forebrain membranes. Figure 5.5 B&C shows the results. In contrast, Melissa essential oil alone enhanced the specific binding [$^3$H] muscimol to well-washed adult rat forebrain membranes in a concentration dependant manner, with a maximum enhancement at a concentration of (1 mg/ml) and apparent ($EC_{50}$ 0.099 mg/ml ± 0.001) Figure 5.5,A. The presence of Lavender oil in the mixture of oil sample abolishes the increase in [$^3$H] muscimol binding induced by Melissa oil alone.

### 5.7.4 Selectivity of Action of Melissa & Lavender Essential oils upon $GABA_A$R:

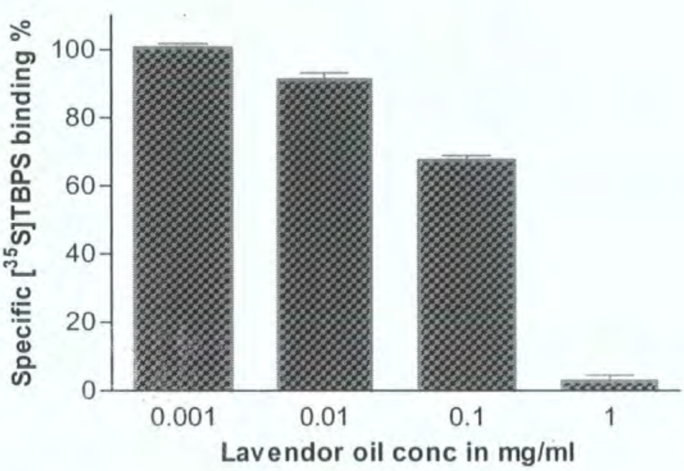
In order to assess the pharmacology of these oils in more detail, the effects of both oils either singly or in combination were determined on a number of other common neuronal ligand gated ion channels. Thus, the effect of Melissa and Lavender oils were investigated at the excitatory ligand gated ion channels gated by NMDA using [$^3$H] MK-801 binding assay (chapter 2, section 2.4.5.4). Additionally the effects of the oils were also determined on neuronal nicotinic receptors (α4β2, α7 nAChRs) using [$^3$H] nicotine binding assay (chapter 2, section 2.4.5.5). Results shows that Melissa and Lavender oils, singly or in combination, had no effect (positive or negative) upon [$^3$H] MK-801 or [$^3$H] nicotine binding up to a 1mg/ml conc. Figure 5.7 and 5.8 A, B & C shows the results.

### 5.7.5 Effects of Melissa & Lavender Essential Oils on the Three Binding Sites of the $GABA_AR$ Complex in $GABA_AR$ Cell Line:

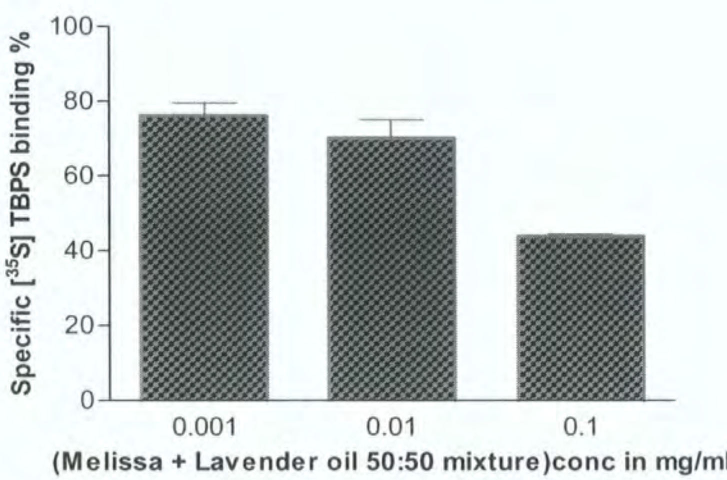
The effects of Melissa & Lavender oils on the three binding site of $GABA_AR$ labelled by $[^{35}S]$ TBPS , $[^{3}H]$ Muscimol and $[^{3}H]$ Flunitrazepam were carried out using $\alpha1\beta2\gamma2L$ $GABA_AR$ stable cell line (The most abundant subunit combinations in the brain). Figure 5.9 A, B. Similar results observed in $GABA_AR$ cell line preparation but with the effect being less pronounced than that of the rat brain data. These results provide evidence to the fact that these oils are much more effective in native tissue than in recombinant cell line.




A




B




C

**Figure 5.2:**

Effect of Melissa oil **(A),** Lavender oil **(B)** and Melissa + Lavender (50:50) mixture **(C)** upon [$^{35}$S] TBPS binding to rat forebrain membranes. Results are expressed as percentages (mean values ± SD for three independent experiments each performed in triplicate).

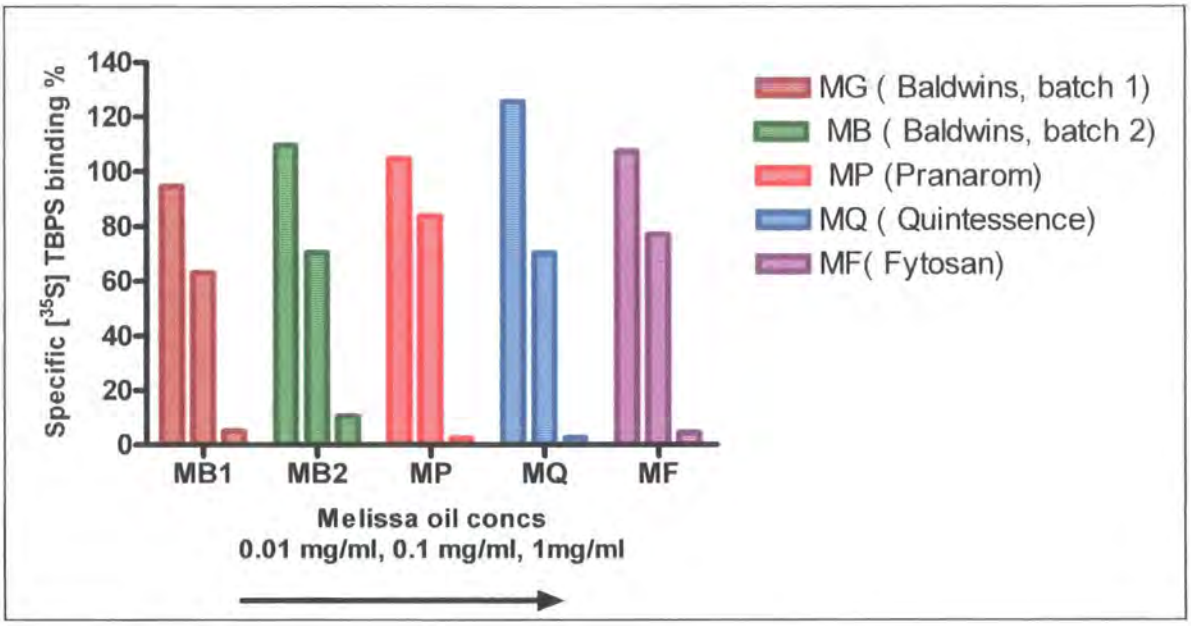
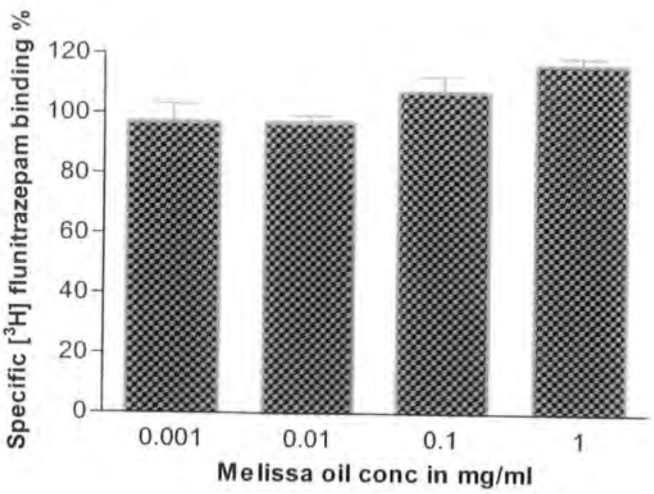
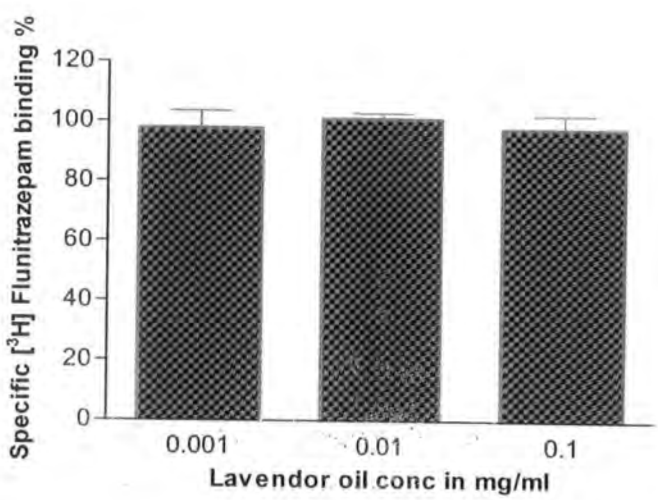



**Figure 5.3:**

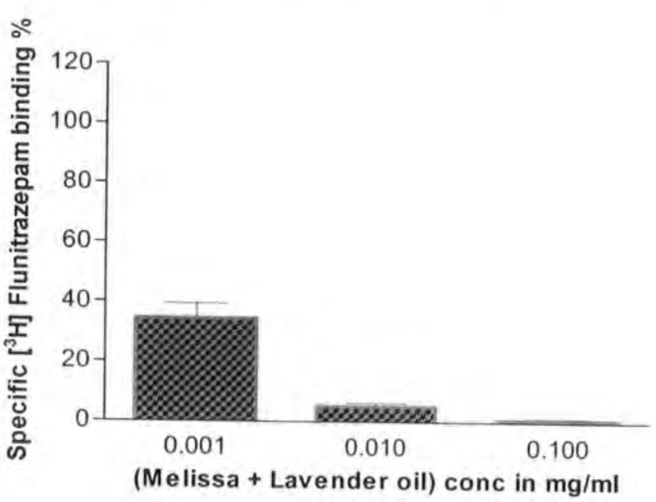
Effect of Melissa oil form four separate authenticated suppliers (Baldwin's, Pranarom, Quintessence and Fytosan) upon [$^{35}$S] TBPS binding to rat forebrain membranes. Results are expressed as percentages (mean values ± SD for three independent experiments).




A



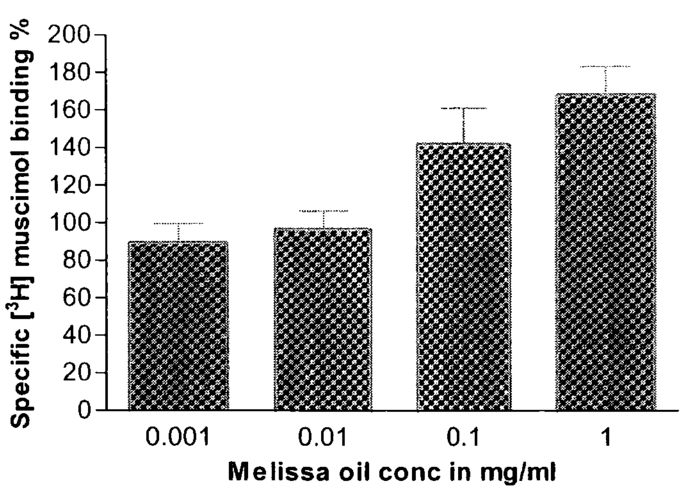

B



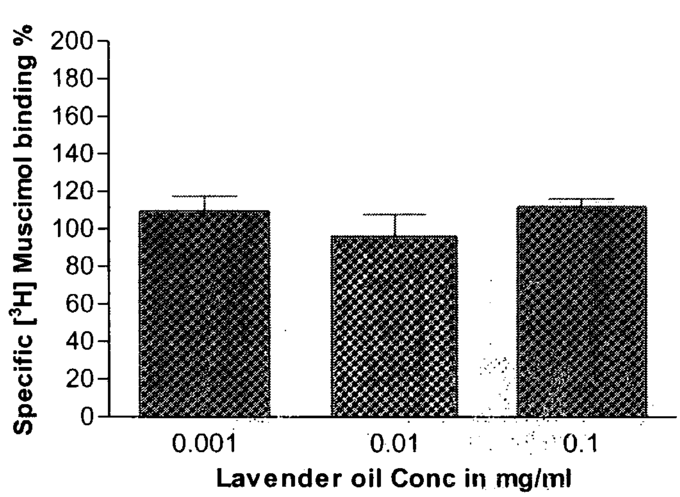

C

**Figure 5.4:**

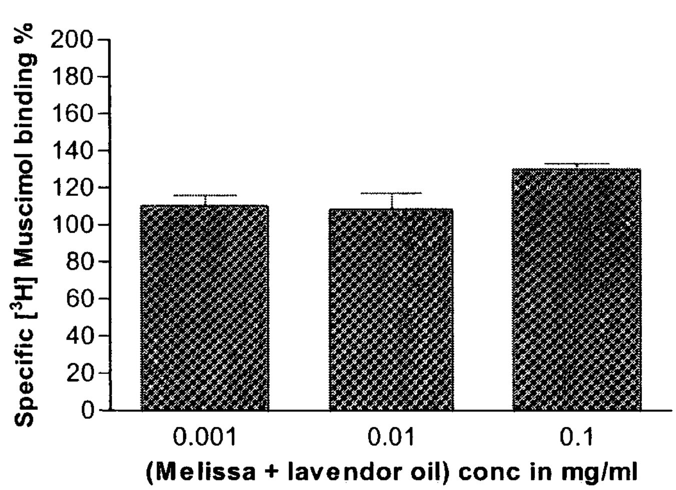
Effect of Melissa oil **(A)**, Lavender oil **(B)** and Melissa + Lavender (50:50) mixture **(C)** upon [$^{3}$H] flunitrazepam binding to rat forebrain membranes. Results are expressed as percentages (mean values ± SD for three independent experiments).




A



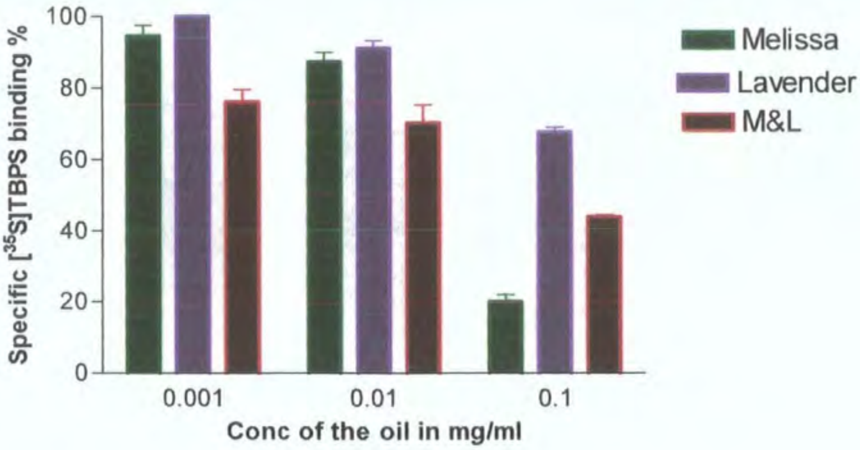

B



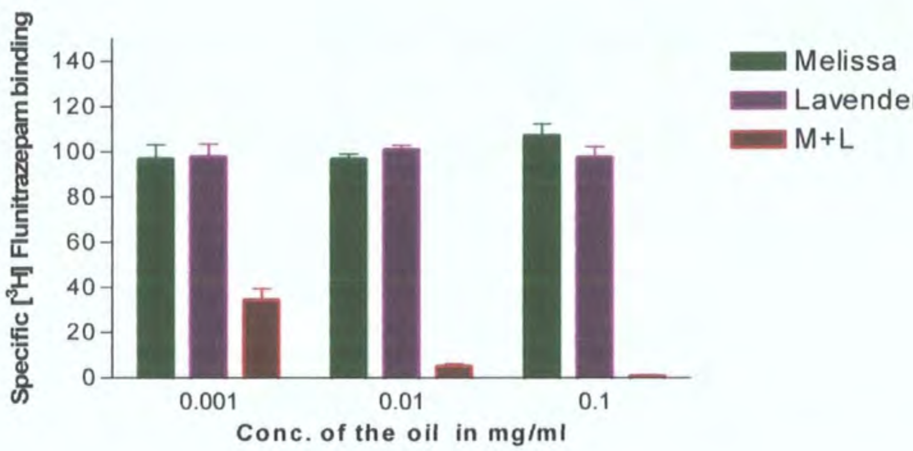

C

**Figure 5.5:**

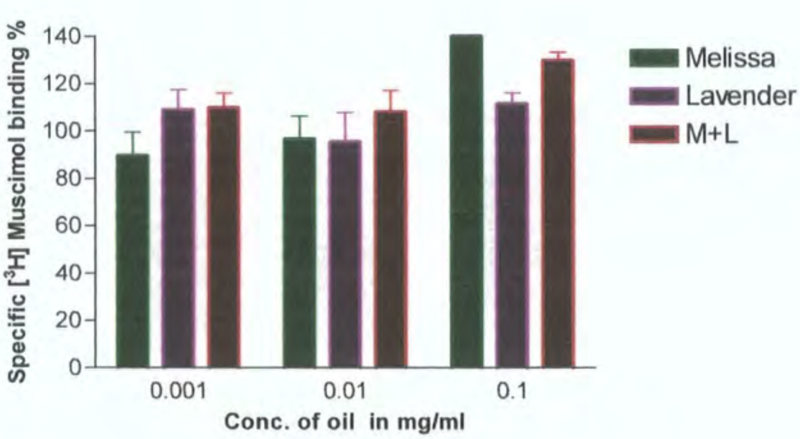
Effect of Melissa oil **(A)**, Lavender oil **(B)** and Melissa + Lavender (50:50) mixture **(C)** upon [$^3$H] muscimol binding to rat forebrain membranes. Results are expressed as percentages (mean values ± SD for three independent experiments).




A




B




C

**Figure 5.6:**

Effect of Melissa, Lavender and Melissa + Lavender (50:50) mixture upon $[^{35}S]$ TBPS binding **(A)**, $[^{3}H]$ Flunitrazepam binding **(B)** and $[^{3}H]$ Muscimol binding **(C)** to rat forebrain membrane. Results are expressed as percentages (mean values ± SD for three independent experiments)

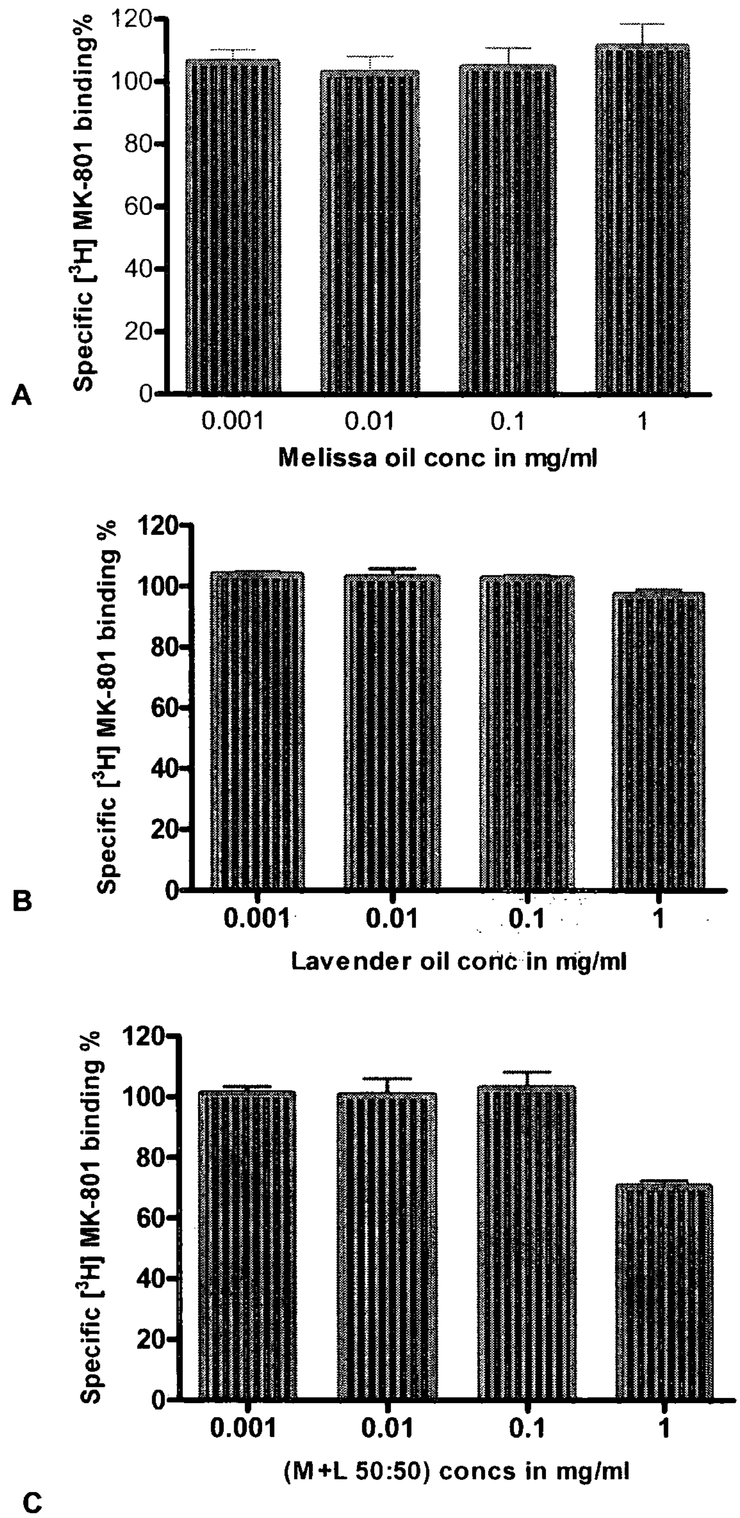



**Figure 5.7:**

Effect of Melissa oil **(A)**, Lavender oil **(B)** and Melissa + Lavender (50:50) mixture **(C)** upon [$^3$H] MK-801 binding to rat forebrain membranes. Results are expressed as percentages (mean values ± SD for three independent experiments).

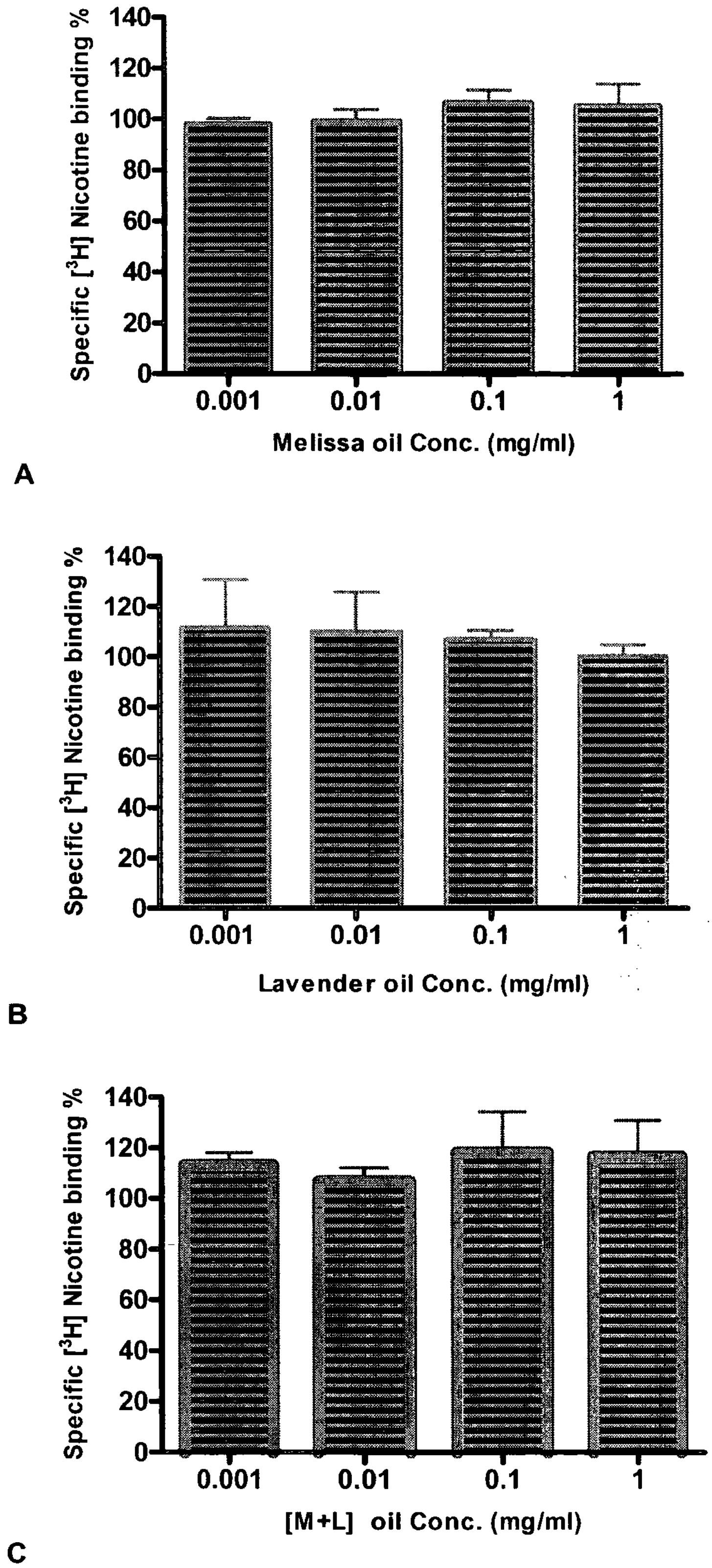



**Figure 5.8:**

Effect of Melissa oil **(A)**, Lavender oil **(B)** and Melissa + Lavender (50:50) mixture **(C)** upon [$^3$H] nicotine binding to rat forebrain membranes. Results are expressed as percentages (mean values ± SD for three independent experiments).

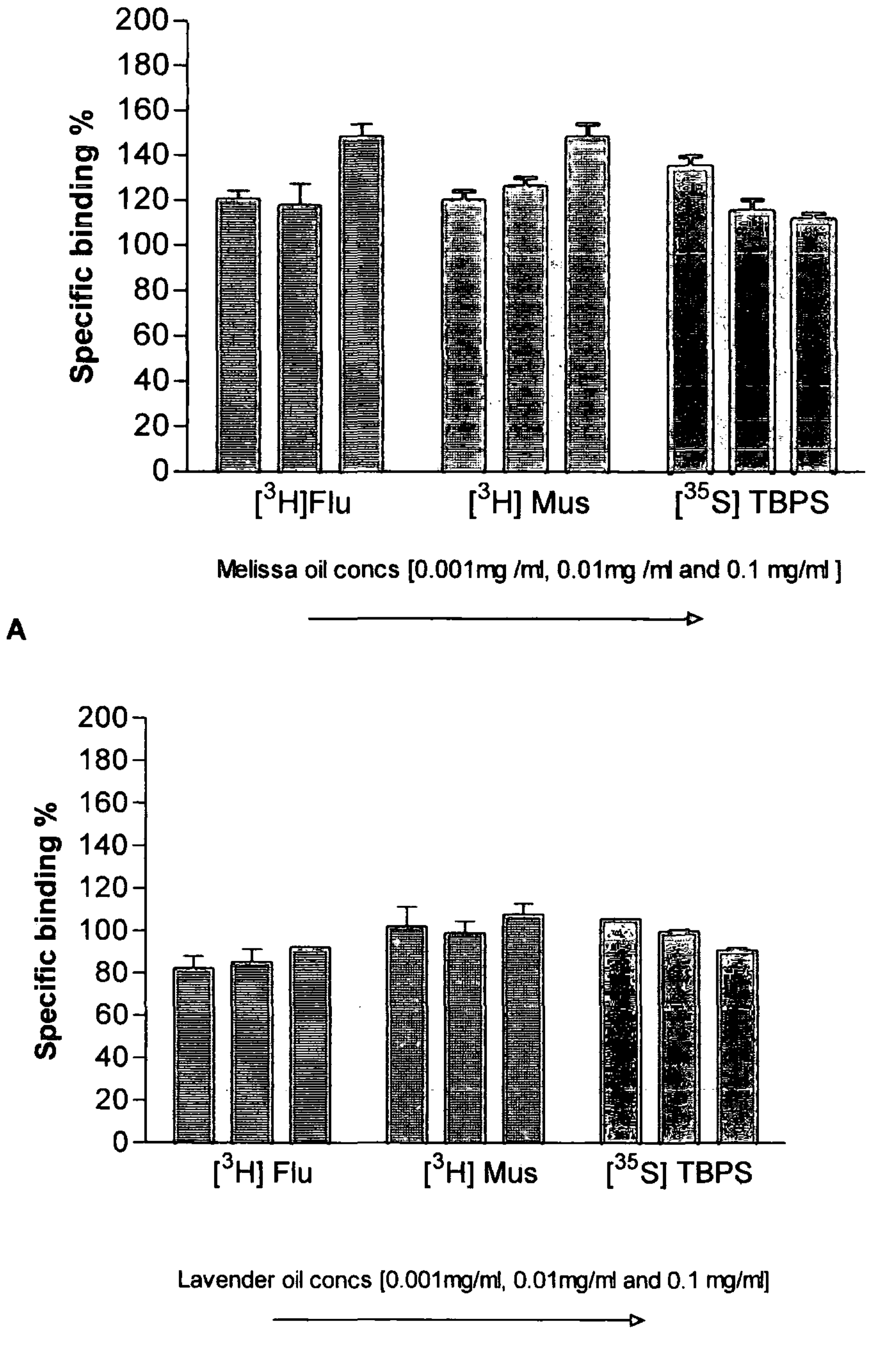



**Figure 5.9:**

Effects of Melissa oil **(A)**, Lavender oil **(B)** upon [$^3$H] flunitrazepam, [$^3$H] muscimol and [$^{35}$S] TBPS binding to $GABA_AR$ cell line. Results are expressed as percentages (mean values ± SD for three independent experiments).

## 5.8 Discussion

The behavioural and psychological symptoms of dementia (BPSD), which include aggression, agitation, screaming, wandering, hallucination and delusion have a negative impact on patient's activities of daily living and on caregiver's quality of life. Among the BPSD, aggression and agitation are especially serious and problematic symptoms for family caregivers and these symptoms are often the primary cause of hospital admission or institutional care (Lanari *et al.,* 2006, Sato *et al.,* 2006). In addition, it is reported that aggression and agitation occur in about 20–80% of patients with AD (Lanari *et al.*, 2006, Sato *et al.*, 2006). Imbalances of different neurotransmitters (acetylcholine, dopamine, noradrenaline, GABA and serotonin) have been proposed as neurobiological causes of BPSD (Lanari *et al.,* 2006, Herrmann *et al.*, 1997). Although non-pharmacological interventions, such as the verbal environmental intervention, should be first-line for milder BPSD (Rojas-Fernandez *et al.,* 2001) many psychotropic agents (e.g. conventional antipsychotics, benzodiazepines, antidepressants, anticonvulsants and beta-blockers) have been used to manage aggressive behaviour. However, their efficacy is insufficient (Carson *et al.,* 2006) and their use has been limited because of adverse effects such as orthostatic hypotension, arrhythmia, extra-pyramidal symptoms (EPS), urinary retention, constipation, sedation and delirium (Lanari *et al.,* 2006, Sato *et al.*, 2006).

Recently, newer atypical antipsychotics characterized by the serotonin ($5\text{-}HT_2$) and dopamine ($D_2$) antagonists have been used for the treatment of aggression in demented patients. Double-blind, placebo-controlled trials have demonstrated that some atypical neuroleptics, such as risperidone and olanzapine have beneficial effects and are well tolerated (Katz *et al.,* 1999, Brodaty *et al.*, 2003, De Deyn *et al.*, 1999, Schneider *et al.*, 1999, Street *et al.*, 2000) in the treatment of aggression and agitation in demented patients. Prescribed cautiously, psychotropic drugs may enhance the physical and psychological well-being of elderly patients. However, this age group is particularly sensitive to undesirable drug effects, which can lead to a decline in medical and functional status or to the use of additional prescriptions and an increased risk for drug interactions (Gurvich & Cunningham, 2000). Recently, an increased risk of cerebrovascular disease related to the use of risperidone and olanzapine was reported (Brodaty *et al.,* 2003, Wooltorton, 2000 & 2004). These warnings have led to controversy among clinicians (Mowat *et al.*, 2004, Smith & Beier, 2004). A increased risk of cerebrovascular disease after administration of atypical antipsychotics was not confirmed in a recent controlled trial (Moretti *et al.*, 2005) and in a population based retrospective study comparing the incidence of stroke in older adults (≥65 years) with dementia receiving atypical (olanzapine, risperidone and quetiapine) or typical antipsychotics (Gill *et al.,* 2005). Although atypical antipsychotic drugs are being used with increasing frequency, only a few randomized trials have evaluated their use in BPSD.

In summary, treatment of BPSD has not been standardised and currently entails various pharmacological and non-pharmacological approaches (Diamond *et al*, 2003, Perry & Perry, 2006). For the non-pharmacological treatment several interventions were identified, of these aromatherapy is reported to be the most widely used. Literature data have indicated positive effects of aromatherapy using selected essential oils. A series of case reports has indicated potential benefit of Melissa and Lavender oil supported by the finding of a pilot placebo control trials in dementia patients (Ballard *et al.*, 2002, Akhondzadeh *et al.*, 2003, Snow *et al.*, 2004).

Pharmacological targets for the reduction of agitation and accompany or underlying aggression or anxiety include the neurotransmitter systems serotonin (5HT), dopamine, acetylcholine (Via nicotinic and muscarinic receptors) and GABA. The serotonergic system is particularly implicated in agitation on the basis of genetic linkage data, agitation–reducing effects of SSRI antidepressant drugs and potent antipsychotic effects of antipsychotic drugs with high affinity for the 5-HT2 receptors, with specific involvement of the $5HT_{2A}$ subtypes. The dopamine $D_2$ receptor is implicated as the most consistent target of narcoleptic medication although clinico-pathological evidence implicating dopaminergic dysfunction in neuropsychiatric or behaviour symptoms in dementia far less convincing than that for the cholinergic system including both muscarinic and nicotinic cholinergic receptors . The nicotinic receptor is also associated with anxiolysis and the $GABA_A$ is the site action of anxiolytic (benzodiazepine) agents (Rojas-Fernandez *et al.*, 2001).

The primary selected target for the bioactivity of an essential oil relevant to the treatment of agitation is therefore be the $5\text{-}HT_2$ receptor; the secondary target sites will include the $GABA_A$, nicotinic ( high affinity binding site, alpha4/beta2) and muscarinic M1 receptors.

In this work, we have characterized the effect of Melissa and Lavender essential oils, singly or in combination towards the three major binding sites of the $GABA_AR$, the benzodiazepine site, the GABA site and the channel site, to detect any $GABA_A$ modulatory activity. To confirm selectivity, interactions with other common ligand-gated ion channel receptors NMDA and neuronal nicotinic receptor were also investigated.

We have shown that Melissa and Lavender essential oils either alone or in combination inhibit [$^{35}$S] TBPS binding in a concentration dependent manner in both native and recombinant preparations. The inhibitory effect on [$^{35}$S] TBPS binding was greater in affinity in native tissue than in recombinant cell line. The inhibitory effect of Melissa oil on [$^{35}$S] TBPS binding was consistent between the four separate authenticated European suppliers (Baldwins, Pranarom, Quintessence and Fytosan) even though extra monoterpenes constituents were detected in two of them.

Lavender oil alone and in combination showed no effect on [$^{3}$H] muscimol binding in both native and recombinant preparations. Interestingly, Melissa oil alone showed stimulatory effect on [$^{3}$H] muscimol binding. The different profile displayed by these oils is probably attributed to the interaction between the oil constituents; a constituent in Lavender oil blocks the Melissa oil activity when used in combination.

Moreover, our observation that the combination of the two oils resulted in an additive effect on [$^{3}$H] flunitrazepam binding suggests synergistic bioactivity occurs when the two oils are mixed. This finding highlights the importance of appreciating the issue of synergy, although isolating and identifying individual chemical constituents with relevant bioactivity provides a rational scientific basis for the medicinal use of the plant or essential oils. Synergistic bioactivity due to mixing different constituents is common (Evans, 1989; Williamson, 2001; Spinella, 2002).

Melissa oil alone also inhibits binding of [$^{3}$H]-8-OH-DPAT to 5-HT$1_A$ receptors and [$^{3}$H] pirenzepine to M1 receptors (results not shown). Neither Melissa, nor Lavender oils demonstrated any effect on the binding of [$^{3}$H]-MK-801 to NMDA receptors, or [$^{3}$H] nicotine to nicotinic acetylcholine receptors. Overall, therefore, Melissa oil appears to have a broad pharmacological profile.

To confirm our finding, patch clamping in cultured rat cortical neurons & electrophysiological testing was carried out in collaboration with Prof. George Lees (Department of Pharmacology and Toxicology, Dunedin, NZ). Data showed that both Melissa & lavender oils interacted with neurotransmission in rat cortical neurons in reversible and concentration dependent manner. 0.1mg/ml Melissa profoundly reduced GABA evoked-current on cultured neurons and silenced both inhibitory and excitatory traffic in neuronal networks; it also showed inhibitory effect on spontaneous activity. Results are shown in Appendix III, Figure 1. 1mg/ml Melissa profoundly inhibited GABA induced current and excitatory/inhibitory synaptic activity in neuronal networks (we can see that these "depressant effects) are probably mediated by an interaction with membrane excitability (pure $GABA_AR$ antagonists usually evoke epileptiform activity) via a different target site. Results are shown in Appendix III, Figure 2. Very similar result was observed with Lavender oil, 0.1mg/ml Lavender strongly reduced GABA-evoked currents. It also consistently prolonged currents evoked by exogenous GABA. Again the depressant effect on traffic is unlikely to reflect the net reduction in synaptic inhibition. Results are shown Appendix III, Figure 3.

In conclusion, it is apparent that the pharmacogical activities of Melissa and Lavender appear to reflect their uses in traditional medicine as sedative and anxiolytic. The ethno-pharmacological approach for selecting plants to investigate for the treatment of a particular disease is a relatively successful method for identification of plants and compounds that may be exploited for use therapeutically in neurodegenerative and other cognitive disorders.

A multi-centre, placebo-controlled clinical trial involving 150 people will follow this pharmacological study based on our findings. This will be the first study carried out based on clear pharmacological data. A group decision has been made for the use of Melissa oil alone from (Fytosan) supplier in the next step of the study plan. This selection was based on the fact that Melissa shows broad spectrum of pharmacological activity, results can be viewed as largely consistent with both the contemporary use of Melissa as a calming agent and mild sedative. The combination effect of Melissa and Lavender oils, although worthy of further investigation precluded the use of the mixture in the clinical trial as this effect may be detrimental to outcome. A standardised, commercial oil of *M.officinalis* prepared by Fytosan Company will be used in the clinical study, where the routine use of fertilizers and pesticides are prohibited. Standardisations and conformity of the extract will be assured by strict-in process controls during manufacture and complete GC/analysis of the resulting oil extract.

# Chapter 6

# Pharmacological Characterization of the Role of a Novel $GABA_A$ Receptor Associated Protein GRIF-1a

## 6.1 Introduction

A novel 913-amino acid protein, γ-aminobutyric acid type A ($GABA_A$) receptor interacting factor-1 (GRIF-1) has been cloned and identified as a $GABA_AR$ associated protein by virtue of its specific interaction with the $GABA_AR$ $\beta_2$ subunit intracellular loop in a yeast two-hybrid assay (Beck *et al.*, 2002). GRIF-1 has no homology with proteins of known function, but it is the rat orthologue of the human ALS2CR3/KIAA0549 gene. GRIF-1 is expressed as two alternative splice forms, GRIF-1a and a C-terminally truncated form, GRIF-1b. GRIF-1a mRNA has a wide distribution with a major transcript size of 6.2 kb.

GRIF-1a protein is only expressed in excitable tissues, *i.e.* brain, heart, and skeletal muscle as major immunoreactive bands of $M_r$ ~ 115 and 106 kDa and, in muscle and heart only, an additional 88-kDa species. When expressed in HEK 293 cells, GRIF-1a yielded three immunoreactive bands with $M_r$ ~ 115, 106 and 98 kDa. Co-expression of GRIF-1a and α1β2γ2 $GABA_AR$ in mammalian cells revealed some co-localization in the cell cytoplasm. Anti-FLAG-agarose specifically precipitated GRIF-$1_{FLAG}$ and $GABA_AR$ β2 subunits from HEK 293 cells co-transfected with GRIF-$1a_{FLAG}$ and β2 subunit clones. Further, immobilized GRIF-1-(8-633) specifically precipitated *in vitro* $GABA_AR$ α1 and β2 subunit immunoreactivities from detergent extracts of adult rat brain. The respective $GABA_AR$ β2 subunit/GRIF-1a binding domains were mapped using the yeast two-hybrid reporter gene assays. A possible role for GRIF-1a as a $GABA_AR$ β2 subunit trafficking factor was proposed (Beck *et al.*, 2002).

GRIF-1 was also identified as a protein that interacts with the enzyme uridine diphospho-N-acetylglucosamine: O-GlcNAc transferase (OGT) (Iyer *et al.*, 2003). OGT catalyzes the post-translational modification of proteins by β-O-linked *N*-acetylglucosamine (GlcNAc) in the cell cytoplasm. GRIF-1a thus has the alternative name OGT-interacting protein 98 (OIP98). GRIF-1a (OIP98) shares ~44% amino acid sequence identity over the full-length sequence with the human protein, OIP106, also known as KIAA1042. OIP106 was also shown to associate with OGT; both proteins contain predicted coiled-coil domains suggesting that GRIF-1 (OIP98) and OIP106 form a new gene family (Iyer *et al.*, 2003, Brickley *et al.*, 2005). The *Drosophila* orthologue of this family of proteins was found to be Milton. Milton shares ~44% amino acid homology with GRIF-1a. Milton is proposed to function in kinesin-mediated transport of mitochondria to nerve terminals (Brickley *et al.*, 2005). GRIF-1 and OIP106 also found to be associate with kinesin and mitochondria (Brickley *et al.*, 2005). Following expression in HEK 293 cells, both GRIF-1 and OIP106 were shown by co-immunoprecipitation to be specifically associated with an endogenous kinesin heavy chain species of 115 kDa and exogenous KIF5C. Association of GRIF-1a with kinesin was also evident in native brain and heart tissue. In the brain, anti-GRIF-1a, antibodies specifically co-immunoprecipitated two kinesin-immunoreactive species with molecular masses of 118 and 115 kDa and in the heart, one kinesin-immunoreactive species, 115 kDa, was immunoprecipitated. Further studies revealed that GRIF-1a was predominantly associated with KIF5A in the brain and with KIF5B in both the heart and in HEK 293 cells. Yeast two-hybrid interaction assays and immunoprecipitations showed that GRIF-1 associated directly with KIF5C with the GRIF-1/KIF5C interaction domain localized to GRIF-1 (124–283). These results further support a role for GRIF-1a and OIP106 in protein and/or organelle transport in excitable cells. GRIF-1 suggested functioning as adaptors in the anterograde trafficking of organelles, utilizing the kinesin-1 motor proteins, to synapses (Brickley *et al.*, 2005).

Recently a confocal microscopy study was carried out to investigate GRIF-1-kinesin-1 interactions in more detail (Pozo & Stephenson, 2006). Molecular interaction between GRIF-1 and the kinesin-1 family member KIF5C, was carried out using fluorescent yellow- and fluorescent cyan-tagged GRIF-1, KIF5C, the KIF5C MD (motor domain) and the KIF5C NMD (non-motor domain) fusion proteins. Each was characterized with respect to size and ability to co-associate by immunoprecipitation following expression in HEK-293 cells. Further, their distribution in transfected HEK-293 and transformed African green monkey kidney (COS-7) cells was analysed. The fluorescent GRIF-1 and KIF5C fusion proteins were all found to behave as wild-type. Double GRIF-1a/KIF5C transfectants revealed co-localization. The GRIF-1a/KIF5C and GRIF-1a/KIF5C NMD double transfectants showed different subcellular distributions compared with single GRIF-1, KIF5C or KIF5C NMD transfections. The study confirms the association between GRIF-1 and kinesin-1 NMDs (Pozo & Stephenson, 2006).

Fransson *et al.*, (2006) reported that the atypical Rho GTPases Miro-1 and Miro-2 have essential roles in mitochondrial trafficking. These proteins have tandem GTP-binding domains separated by a linker region with putative calcium-binding motifs. In addition, the Miro GTPases have a C-terminal transmembrane domain, which confers targeting to the mitochondria. In this study they showed that Miro interacts with the Kinesin-binding proteins, GRIF-1a and OIP106, suggesting that the Miro GTPases form a link between the mitochondria and the trafficking apparatus of the microtubules.

In the present study, we revisit the role of GRIF in $GABA_AR$ molecular pharmacology to provide further information about the possible multiple roles of GRIF-1a protein in the mammalian brain.

## 6.2 Materials & Methods

### 6.2.1 Preparation of GRIF-1a, c-DNA.

#### 6.2.1.1 Transformation of Competent *E.Coli* Cells:

This method was performed essentially as described by Dagert & Ehrlick (1979). For the transformation of competent *E.coli* cells, a frozen aliquot (100µl) of HB101 competent cells were removed from -80°C and thawed on ice for 5 minutes. GRIF-1a plasmid c-DNA was added to the competent cells (100µl) and gently mixed. The cell mixture was then incubated on ice for 30 minutes and heat shocked by placing in water bath at 42°C for 60 seconds. After 2 minutes incubation on ice, terrific broth (900 µl) was added to transformed cells. Following 1 hr incubation in an orbital shaker at 250xg, 37°C the cell suspension (100µl) was plated onto culture plates prepared with 1.5 % agar in terrific broth containing ampicillin (50 ug/ml). The culture plates were incubated at 37°C for 18-20 h in an inverted position.

#### 6.2.1.2 Glycerol Stocks of Transformed Competent *E.Coli* Cells:

Transformed competent *E.Coli* cell stocks were prepared by mixing 500 µl of terrific broth supplemented with 50% (v/v) sterile glycerol and 50 ug/ml ampicillin with 500 µl of the small overnight culture. The cell culture mixture was immediately added to cryogenic vials and stored at -80°C until use.

### 6.2.1.3 Amplification and Preparation of Plasmid DNA:

#### 6.2.1.3.1 Preparation of Small-Scale Culture of Plasmid DNA:

Terrific broth (10 ml) containing ampicillin (50 ug/ml) was added to a sterile 50 ml falcon tube and inculcated with one isolated colony from the culture plate using a sterile loop. The small culture was incubated for 18-20 hour in an orbital shaker at 250xg, 37°C.

#### 6.2.1.3.2 Preparation of Large-Scale Culture of Plasmid DNA:

Terrific broth (500 ml) containing ampicillin (50 ug/ml) was inoculated with 3 ml of the small overnight culture in a sterile 500 ml flask. The large culture was incubated for 18-20 hours in an orbital shaker at 250xg, 37°C.

#### 6.2.1.3.3 Harvesting the Large-Scale Culture and Purification of Plasmid DNA using QIAGEN™ plasmid Maxi-Kit:

*E.Coli* cells were harvested by transferring the large overnight culture into two ice-cold centrifuge tubes, and centrifuged at 6500xg for 10 minutes at 4°C. The supernatant was discarded and the remaining pellet was resuspended in ice-cold P1 buffer (10 ml). Bacteria containing plasmid were then lysed by the addition of P2 buffer (10 ml) mixed by gentle inversion and incubated at room temperature for 5 minutes. The mixture was then neutralised with chilled P3 buffer (10 ml) mixed by gentle inversion and incubated on ice for 20 minutes. The solution was then centrifuged at 14000xg for 30 minutes at 4°C and clear lysate was removed into fresh tube. A QIAGEN 500 tip was equilibrated with QBT buffer (10 ml). The lysate was gently poured onto the column and allowed to pass through the column under gravity flow. The column was washed twice with QC buffer (30 ml) then QF buffer (15 ml) was added to the column to elute the plasmid DNA. Ice-cold isopropanol (10.5 ml) was added to the eluted DNA and the solution centrifuged at 14000xg for 30 minutes at 4°C. The remaining pellet was carefully washed with ice-cold ethanol (1ml) and air dried for approximately 30 minutes. The purified DNA was dissolved in TEE buffer and stored at 4°C until the purity and yield of the DNA was calculated.

#### 6.2.1.3.4 Quantification and Determination of the Purity of the DNA yield:

The purity and concentration of plasmid DNA was determined by measuring the O.D at $\lambda$= 260 nm and at $\lambda$= 280 nm (Sambrook *et al*, 1989). The ratio of the optical densities at $\lambda$= 260 nm and at $\lambda$= 280 nm (O.D $_{\lambda}$= 260/O.D $_{\lambda}$=280) should be within the range 1.8-2.0. Plasmid DNA concentration at $\lambda$= 260 nm. An O.D. =1 corresponds to ~50 µg/µl for the double strand DNA (dsDNA). The DNA was then diluted to a final concentration of 1 µg/ml in TE buffer and stored in 100 µl aliquots at -20°C until use. Once thawed DNA was stored at 4°C.

## 6.2.2 Cell Culture

#### 6.2.2.1 Preparation of DMEM/F12 Medium + L-Glutamine:

As described in Chapter 2 section 2.4.4.1

#### 6.2.2.2 Cell Cultivation of GABA$_A$R Cell Line:

As described in Chapter 2 section 2.4.4.2

#### 6.2.2.3 Sub-culturing of GABA$_A$R Cell Line:

As described in Chapter 2 section 2.4.4.3

#### 6.2.2.4 Harvesting & Cell Homogenate Preparation of GABA$_A$R Cell Line:

As described in Chapter 2 section 2.4.4.4

#### 6.2.2.5 Preparation of New Stocks of GABA$_A$R Cell Line:

As described in Chapter 2 section 2.4.4.5

#### 6.2.2.6 Cell Cultivation of HEK 293 Cells:

For the preparation of a new culture of HEK 293 cells, a single cryogenic vial of frozen HEK 293 cells was thawed at 37°C. The cells were centrifuged at 200xg for 5 minutes at 4°C and resuspended in DMEMF/12 medium containing L-glutamine (15 ml). The cells were added to a tissue culture flask, which was incubated at 37°C in 5 % $CO_2$ and cultured.

#### 6.2.2.7 Sub-culturing of HEK 293 Cells:

HEK 293 cells were grown in 250 ml Greiner culture flasks at 37°C in 5% $CO_2$ in DMEM/F12 medium containing, L-glutamine in an Sanyo incubator. Every two to three days the cells were subcultured by the removal of the old medium and then washed with pre-warmed phosphate buffer salt solution (PBS) (10 ml). Following 1 minute incubation in trypsin-EDTA (2 ml) at 37°C, DMEM/F12 medium containing L-glutamine (10 ml) was added to the cells. The cells were then separated by gentle pipetting. Finally, the cell suspension (2 ml) was added to a fresh flask and a further 10 ml of DMEM/F12 medium containing L-glutamine was added to the new flask, which was incubated at 37°C in 5% $CO_2$ .

#### 6.2.2.8 Harvesting & Cell Homogenate Preparation HEK293 Cells:

Membranes from mock and GRIF-1a transfected HEK 293 cells were prepared as described by Fuchs *et al.* (1995). The cells were harvested at 24-36 h post-transfection. The culture media were removed; the cells were washed once with (10 ml) PBS, followed by 15ml of ice-cold homogenisation buffer (section 2.3.7). Cells were scraped off the bottom of the flask using Greiner cell scrapers. Cell suspensions were centrifuged at 3000 X g for 5 min at 4°C. The cells pellet collected and homogenised with glass/glass homogeniser for 30 strokes in ice–cold homogenisation buffer. The homogenate was re-centrifuged at 30,000 X g for 30 min at 4C°. The cell homogenate was re-homogenised, centrifuged and the final cell pellet resuspended in homogenisation buffer (section 2.3.7) (7ml). (~50-100µg protein) and was either assayed immediately for radioligand binding activity or alternatively were stored in 50µl aliquots at –20°C until use for immunoblotting.

#### 6.2.2.9 Preparation of New Stocks of HEK293 Cells:

HEK 293 cell stocks were prepared by subjecting the cells to trypsin-EDTA (4ml) dissociation for 1 minute at 37°C and 20 ml of DMEM/F12 medium containing L-glutamine was added. The cells were centrifuged at 200xg for 5 minutes at 4°C. The pellet was resuspended in DMEM/F12 medium containing L-glutamine (4.8ml) supplemented with, FCS (0.6ml) and DMSO (0.6ml). The cell suspension was immediately divided into three cryogenic vials and stored at –80°C for 34 hours and then transferred to liquid nitrogen.

### 6.2.2.10 Lipofetamine Plus Method of Transfection:

$GABA_AR$ cell line or HEK 293 cells were cultured and transiently transfected with GRIF-1a plasmid c-DNA using Lipofectamine Plus method as previously described by Tucker *et al.* (2003). Briefly a 1.5 ml eppendroff tube was used to mix 1.5 µg (GRIF-1a) c-DNA and 6µl of PLUS reagent in 150µl of Optimem-I Media (Gibco). In a separate 1.5 eppendroff tube, 5µl lipofectamine reagent was mixed with 150µl of Optimem-I Media. The two tubes were incubated separately at room temperature for 15 minutes. The contents of lipofectamine reagent tube were then transferred and mixed with the c-DNA and PLUS reagent. The single tube containing both lipids and the c-DNA was incubated at room temperature for another 15 min. In the meantime the cells at 50-70% confluence were washed three times with Optimum-I Media. At the end of the second incubation period the contents of tube 1 were made up to 1.5 ml with Optimum-I media and added to the washed cells. The cells were incubated at 37°C for 6 hours. The transfection mixture was then removed and replaced with ordinary growth media.

### 6.2.3 Confocal Microscopy Images:

$GABA_AR$ cells were seeded onto glass cover slips and grown to 40-60% confluence prior transfection with GRIF-1a-GFP (1µl) plasmid. 24-36 hour post transfection GABA cells were rinsed three times with PBS and then fixed in 4% paraformaldehyde in PBS for 10 min at room temperature. Cells were rinsed with PBS and permeabilized in PBS-1% bovine serum albumin (1 % PBS-BSA) containing Na-Az at room temperature for 3 min. Cover slips were mounted on glass slides, and images were captured by using a Zeiss LSM 510 laser scanning confocal microscope.

### 6.2.4 Cell Surface Biotinylation:

Surface biotinylation experiments were performed essentially as described previously by Archibald *et al.* (1998). Cells on tissue culture dishes were washed three times with ice-cold PBS containing 4% sucrose, and incubated with 0.5 ml of (1mg/ml) Sulfo-NHS-SS-Biotin (Pierce) in ice-cold PBS for 20 min at 4°C with gentle shaking. Excess biotin was removed by rapid washing twice in ice-cold PBS. The reaction was quenched for 10 min on ice with 192 mM glycine dissolved in TBS, pH 8. Next, cells were rinsed twice and scraped in ice-cold PBS, pelleted down, and homogenized in 166 µl of solubilization buffer (TBS, pH 8, 2 mM EDTA, protease inhibitor cocktail set III) containing 1% SDS. The samples were made up to 1 ml in eppendroff tubes with 833 µl of ice-cold solubilization buffer containing 1% Triton X-100, sonicated to ensure solubilization. The samples were then incubated with 20 µl of 50% slurry of streptavidin beads for 2 hr at 4°C. Beads were pelleted by centrifugation and aliquots of the supernatant were taken to represent the unbound intracellular pool. Beads were then washed twice with (1ml) of solubilisation buffer containing 1% Triton X-100, and twice with (1ml) solubilization buffer alone. SDS-PAGE sample buffer containing 50mM DTT (60 µl) was added to the beads; the samples were vigorously vortexed and heated to 50°C for 30 min. The tubes were then cooled, centrifuged and biotinylated proteins were eluted. Samples were prepared as aliquots of (100 µl) intracellular fractions and (12 µl) (biotinylated, surface fractions), all samples were frozen and stored at -20°C until use. To ensure that biotin was only labelling surface proteins, the integrity of the cell membrane during biotinylation was tested in each experiment by immunoblotting with an anti-β-actin antibody. In all experiments, β-actin immunoreactivity was not detected in the biotinylated fractions, even though abundant actin immunoreactivity was detected in the whole cell lysate.

#### 6.2.4.1 Chloroform/Methanol Method for Protein Precipitation:

Intracellular protein fractions were precipitated using chloroform/methanol precipitation described as follows. To the protein samples (100 µg), methanol (4 vol) was added and the samples were vortexed and centrifuged at room temperature at 18,000xg for 1 minute. Chloroform (1 vol) was added to the samples, which were vortexed and centrifuged at 18,000xg at room temperature for 1minute. To each of the samples water (3 vol) was added which were again vortexed and centrifuged at room temperature at 13000 rpm for 1 minute. The upper layer was carefully discarded and methanol (1 vol) was added to each of the samples. The samples were centrifuged at 18,000xg at room temperature for 4 minutes. The supernatant was removed and the samples were air-dried. The dried protein pellet was resuspended by vortexing in sample buffer (section 2.3.8), 100mM DTT (1.5 µl) and water to a final volume of 15 µl. The samples were boiled in a water bath for 5 minutes and then centrifuged at 18,000xg for 30 seconds at room temperature before analysis by SDS- PAGE.

### 6.2.5 Surface Expression via Proteolysis Technique:

Chymotrypsin-treatment was essentially performed as previously described by Hall & Soderling (1997). Briefly, following two washes with (SS) buffer (section 2.3.24), cultures were incubated with chymotrypsin (2 mg/ml in SS buffer) for 10 min at 37°C. Cultures were subsequently washed, once with (1 ml SS buffer), followed by adding (2ml) normal tissue culture media to the plates for 10 min, to quench the enzyme activity. Media was removed and the plates were washed three times with ( 1 ml SS buffer), after the third wash , (1.0 ml ) of fresh ice-cold homogenization buffer was added and the cells were scraped, centrifuged and re-dissolved in (1.0 ml) homogenizer buffer, homogenized using dounce glass/glass homogenizer, aliquots into (50 µl) samples and were stored at -20°C until use.

### 6.2.6 SDS-PAGE & Western Blotting

Immunoblotting was carried out essentially as described by Duggan *et al.* (1991), using SDS/PAGE in 7. 5% polyacrylamide mini-slab gels under reducing conditions.

#### 6.2.6.1 Preparation of Resolving Gel:

The resolving gel (7.5 %) was prepared by mixing water (6 ml) with resolving gel buffer (section 2.3.12), TEMED (6 µl), stock acrylamide (section 2.3.13) (3 ml), and 10 % (w/v) APS (60µl). The polyacrylamide solution was immediately poured into a Biotech gel caster holding 2 gels, using gel plates of 10 x 8 cm and spacers of 1 mm width. Saturated water/butanol solution (100 µl) was added over the top of each gel. The gels were covered with parafilm and were allowed to polymerise for 60 minutes at room temperature. Gels were individually wrapped in tissue and stored in electrode buffer (section 2.3.14) at 4°C until use.

#### 6.2.6.2 SDS-Polyacrylamide Gel Electrophoresis:

The resolving mini-slab gel was clamped into a Hoefer Mighty Small II vertical slab SE250 unit. The stacking gel was prepared by mixing water (2.3 ml) with stacking gel buffer (section 2.3.11) (1 ml), stock acrylamide (section 2.3.13) (650 µl) and TEMED (5 µl) and 10% (w/v) ammonium persulphate (80 µl) was added to the stacking gel solution and this was immediately poured into the mini-slab gel above the resolving gel. A welled comb was inserted into the stacking gel. After the polymerisation of the gel, the comb was carefully removed and the wells were washed with water. Electrode buffer (section 2.3.14) (~ 300 ml) was poured into the wells and into the base of the electrophoresis unit. Protein samples (15 µl) and pre-stained standards (protein molecular weight range of 200-6.5 kDa) (15 µl) were loaded into the wells of the stacking gel using a Hamilton syringe. Electrophoresis was carried out at a constant current of 15 mA for ~2 h until the appropriate pre-stained molecular weight marker (25 kDa) was at the bottom of the gel.

#### 6.2.6.3 Immunoblotting:

After SDS-PAGE (section 6.2.6.2), the proteins from the gels were transferred to nitrocellulose membranes. A transfer cassette sandwich was constructed with the following order of components each of which had been pre-equilibrated in transfer buffer (section 2.3.17) sponge, two sheets of blotting paper and nitrocellulose membrane. The SDS-PAGE gel, two sheets of blotting paper and a final piece of sponge were added to the transfer cassette sandwich. On the addition of each component to the transfer cassette air bubbles were carefully removed by pressing each layer with a test tube. Proteins were transferred at a constant voltage of 50 V for 2.5 hours using a Hoefer TE series transfer tank containing transfer buffer at room temperature. Following the transfer of the proteins, the nitrocellulose membrane was briefly rinsed with TBS (section 2.3.19) and incubated with blocking buffer which was TBS, containing 5 % (w/v) dried milk and 0.02 % (v/v) Tween-20 (15 ml) for 1 hour at room temperature with gentle shaking. After blocking of the non-specific antibody sites the nitrocellulose membranes were washed with ~10 ml of TBS. The appropriate affinity-purified primary antibodies were diluted in incubation buffer, which was TBS, pH 7.4 containing 2.5 % (w/v) dried milk to working concentrations (0.25-5 μg/ml). The nitrocellulose membranes were incubated with the diluted primary antibody solution (10 ml) for 1 hour at room temperature, or overnight at 4°C with gentle shaking.

After incubation with the primary antibody the nitrocellulose membranes were washed four times in wash buffer containing, TBS, containing, 2.5 % (w/v) dried milk and 0.2 % (v/v) Tween-20 (10 ml) at 10 minute intervals with gentle shaking at room temperature. Nitrocellulose membranes were then incubated with horseradish peroxidase (HRP) labelled secondary antibody, either anti-rabbit or anti-mouse depending on what the primary antibody was raised in, at a dilution of 1/2000 in incubation buffer (10 ml). The membrane was incubated for 1 hour at room temperature with gentle shaking. The unbound secondary antibody was removed by washing the membrane as described above. The nitrocellulose membrane was drained of excess wash buffer and briefly rinsed in TBS. Immunoreactive bands on the nitrocellulose membranes were developed by processing in a solution containing, 68 mM p-coumaric acid (100 μl), 1.25 mM luminol (10 ml) and 30 % $H_2O_2$ (6 μl) for 1 minute at room temperature. After removal of the reagents the immunoblot was wrapped in cling film, and placed in a film cassette. The immunoblot was exposed to Hyperfilm™ for various times (1-5 minutes). The film was then developed in Kodak D-19 Developer until the immunoreactive bands were visible and fixed in Kodak Unifix for 5 minutes at room temperature. The films were scanned into a computer and the immunoreactive bands were quantified using (Computer-assisted densitometry using microcomputer Imaging Device (MCID) version 7 software from Imaging Research Inc., Ontario, Canada. Image J programme. The significance of the effects of treatments was assessed using the student's t-test (Graph Pad Prism, Graph Pad, San Diego, CA). The 95% confidence level ($p < 0.05$) was considered statistically significant.

### 6.2.7 Radioligand Binding Assays

Equilibrium saturation binding of [$^3$H] Flunitrazepam and competitive inhibition experiments of [$^{35}$S] TBPS and [$^3$H] Muscimol binding were performed using $GABA_AR$ cell line, cell homogenate of mock and GRIF-1a transfected cells.

#### 6.2.7.1 [$^3$H] Flunitrazepam & Saturation Curve:

[$^3$H] Flunitrazepam saturation binding assays were performed as previously described by Thomas *et al.* (1997). Briefly, (~50-100 µg protein) (100 µl) $GABA_AR$ cell homogenate of mock and GRIF-1a transfected cells were incubated in 50 mM Tris buffer containing 5mM EDTA and 5 mM EGTA (pH=7.4) at 4 °C for 1h with a range of concentrations of [$^3$H] flunitrazepam [0.2–18 nM] (20 µl). The total assay volume was (200 µl). All concentration points were performed in triplicate. Non-Specific binding was defined in the presence of 100 µM Diazepam. Radioactivity bound to membranes was determined after rapid filtration on Whatman GF/C filters.

#### 6.2.7.2 Data Analysis for Saturation Studies:

Results from saturation studies were analysed by non-linear least square regression using GraphPad Prism. The saturation data were analysed by either the one-site or two-site binding hyperbola. The F-test was used to assess whether the one-site or the two-site model fit the data best ($P<0.05$ was deemed significant). The $K_D$ values for saturation curves fitted to a one-site hyperbola were calculated from the following equation,

$$Y = \frac{B_{max}\, X}{K_D + X}$$

Where:

Y = specific bound [$^3$H] Flunitrazepam bound.

X = concentration of [$^3$H] Flunitrazepam.

$B_{max}$ = maximum number of binding sites

Saturation data was fitted to the line by linear regression using GraphPad Prism for the Rosenthal transformations,

$$F(x) = ax + b$$

Where:

F(x) = specific [$^3$H] Flunitrazepam bound/ [$^3$H] Flunitazepam free,

a = slope – ($1/K_D$)

x = specific $[^3H]$ Flunitrazepam bound.

b = x-axis intercept ($B_{max}$)

### 6.2.7.3 $[^3H]$ Muscimol Binding Assay:

As described in Chapter 2 section 2.4.5.2

### 6.2.7.4 $[^{35}S]$-*t*-butylbicyclophosphorothionate (TBPS) Binding Assay:

As described in Chapter 2 section 2.4.5.3

### 6.2.7.5 Data Analysis for Competition Studies:

As described in Chapter 2 section 2.4.6.1

## 6.3 Results

### 6.3.1 Expression of GRIF-1a in $GABA_AR$ Cell Line:

To examine the expression of GRIF-1a protein in our stable $GABA_AR$ cell line system both confocal microscopy and immunoblotting were carried out. $GABA_AR$ cells adhered to poly-L-lysine-coated coverslips were transiently transfected with (GRIF-1a-GFP plasmid) using lipofetamine Plus method. Cells were fixed 24-40 h after transfection, coverslips were mounted on glass slides and images of localization were captured by confocal microscopy. Images are shown in Figure 6.1. Results shows that GRIF-1a protein is localized predominantly in the cell cytoplasm, this finding is consistent with Beck *et al.* (2002) observations. Transfection efficiency from the cell images was approximately 35%, calculated from the ratio of the number of fluorecent labelled cells : the total number of cells in a randomly selected field, which is respectable for this type of tranfection methodology ( Sambrook *et al.*, 1989) and routinely observed in our lab using this protocol.

GRIF-1a protein expression was also confirmed using immunoblotting. $GABA_AR$ cells were transiently transfected with GRIF-1a plasmid, cell homogenates of mock and GRIF-1a transfected cells were prepared 48 hr post-transfection and analyzed by immunoblotting using an anti-GRIF-1a antibody. Figure 6.2 shows the results. Mock transfected $GABA_AR$ cells showed no anti-GRIF-1 immunoreactivity. In contrast, two immunoreactive bands with $M_r$ Values of 115,000 and 106,000 were detected in $GABA_AR$ cell line transfected with GRIF-1a; these two bands were also reported by Beck *et al.* (2002). Immunoblot with these two immunoreactive bands 115,000 and 106,000 were used as a control standard to confirm the expression of the GRIF-1a protein during the pharmacological studies.

### 6.3.2 Effect of GRIF-1a protein on $GABA_AR$ α1, β2 and γ2 Subunit Expression:

The effect of GRIF-1a protein on $GABA_AR$ α1, β2 and γ2 subunit expression and stability was carried out by immmunblotting. $GABA_AR$ cell line was transiently co-transfected with GRIF-1a plasmid, cell homogenates of mock and GRIF-1a transfected cells were prepared 48 hr post-transfection and analyzed by immunoblotting using anti-$GABA_AR$ $\alpha_1$, anti-$GABA_AR$ $\beta_2$, anti-$GABA_AR$ $\gamma_2$ and anti-β-actin antibodies. Figure 6.3 shows the results. Data showed that the presence of GRIF-1a protein increases α1, β2 and γ2 subunit protein expression in comparison with control. Quantitative analysis of the immunoreactive bands showed that GRIF-1a increases the stability of these subunits α1, β2 and γ2 by 20%, 30% and 40 % respectively in comparison with control. Figure 6.4 shows the results. Statistical significance of the data was determined using paired student's t-test. Data were considered significantly different if $p \le 0.05$.

### 6.3.3 Investigation of the Role of GRIF-1a Protein in the Trafficking of $GABA_AR$ to the Cell Surface:

GRIF-1a is a member of coiled–coil family of protein thought to function as adaptors in the anterogarde trafficking of organelles utilizing motor proteins (Kinesin-1) to the synapse. To investigate the possible role of GRIF-1a protein in regulating membrane trafficking of $GABA_ARs$ to the surface, a cell surface protein biotinylation experiment was carried out followed by precipitation with streptavidin beads and western blot analysis. Results showed no significant difference in molecular weights and expression levels between surface and intracellular protein fractions for both α1 and β2 subunits. Figure 6.5 A& B shows the results. In addition, in the immunoblot probed with anti-GRIF-1a antibody, GRIF-1a protein was detected in the surface as well as in the intracellular fractions Figure 6.5, C. Therefore, from these experiments, it is apparent that GRIF-1a is a surface associated protein suggesting a role in controlling cell surface stability of $GABA_AR$. To ensure that biotin was only labelling surface protein fractions, the integrity of the cell membrane during biotinylation was tested in our experiment by re-probing the immunoblott with the anti-β-actin antibody. β-actin immunoreactivity was detected in the intracellular protein fractions only. Figure 6.5, D.

### 6.3.4 Characterisation of GRIF-1a Protein Expression in Control HEK 293 Cells:

Results suggest that GRIF-1a protein is present at the cell surface when expressed in the $GABA_AR$ cell line. To address whether the apparent surface expression of GRIF-1a protein was due to the presence of $GABA_ARs$, further experiments were carried out in control HEK293 cells lacking the $GABA_ARs$.

#### 6.3.4.1 Cell Surface Biotinylation in Control HEK293 Cells:

Cell surface biotinylation experiment was performed on control HEK 293 cells lacking $GABA_ARs$. Surface and Intracellular protein fractions were analyzed by western blotting and probed with the anti-GRIF-1a antibody. Results are shown in Figure 6.6. Interestingly, immunoblot observations were in agreement with $GABA_AR$ cell line results. GRIF-1a protein was clearly expressed in the surface protein fractions. As before to ensure that biotin was only labelling surface proteins, the integrity of the cell membrane during biotinylation was tested in our experiment by re-probing the immunoblott with the β-actin antibody. β-actin immunoreactivity was detected in the intracellular protein fractions only. Together, these results strongly suggest that GRIF1a is associated with a surface protein in HEK 293 cells; it performs its function on the surface even in absence of $GABA_ARs$.

### 6.3.4.2 Biotinylation Using Different Solubilization Reagents:

From the above results, it was apparent that GRIF-1 protein accesses the cell surface when expressed in the $GABA_AR$ cell line or control HEK 293 cells. Structure prediction analysis of GRIF-1a revealed a hydrophilic protein with no transmembrane domains and no hydrophobic signal peptide (Beck *et al.*, 2002). In addition, GRIF-1 protein hydropathy plot, Figure 6.7 showed no peaks with scores greater than 1.8, which indicates a lack of transmembrane regions (Kyte, Doolittle 1982). Taken together these data suggest that GRIF-1 is associated with surface proteins. To address the nature of association between the GRIF-1 and the surface proteins, we then performed the same biotinylation approach using different solubilisation conditions (1% SDS, 5% SDS and 100 mM DTT) to check any changes in the cell surface level expression of GRIF-1a protein. Subsequently, biotinylated and unbiotinylated fractions were subjected to immunoblotting using the anti-GRIF-1a and the anti-β-actin antibodies. Results are shown in Figure 6.8 A & B. No change was seen in surface expression of GRIF-1a protein with 100 mM DTT treatment. However, a significant reduction of GRIF-1a surface expression was seen with 5% SDS. To ensure that biotin was only labelling surface proteins and to demonstrate that an equal amount of protein was loaded, the transferred membranes were re-probed with the anti-β-actin antibody. Blots are shown in Figure 6.7 C & D. These results indicate that GRIF-1a is strongly associated with a surface protein in HEK 293. This association is disturbed by high concentrations of SDS but not affected by reducing agents such as DTT.

### 6.3.4.3 GRIF-1a Surface Expression via Proteolysis in Control HEK 293 Cells:

Three approaches can be used for detection of transmembrane proteins surface expression these include: Cross-linking method using $BS^3$, biotinylation using NH-SS-biotin or cleavage of surface receptor by proteolytic enzyme, chymotrypsin.

The biotinylation approach strongly suggests that GRIF-1a is associated with a surface protein in HEK 293 cells. To confirm our finding, we performed an alternative surface labelling approach, using proteolytic cleavage with the chymotrypsin enzyme in control HEK 293 cells. As shown in Figure 6.9, immunoreactivity for GRIF-1a protein was not significantly different in the chymotrypsin samples relative to the control samples, suggesting that GRIF-1a protein is not susceptible to cleavage by chymotypsin, and confirms that it is not a transmembrane protein.

In order to find the possible sites of interaction between GRIF-1a protein and chymotrypsin enzyme, we obtain the full length amino acid sequence GRIF-1a protein from NCBI/EMBL database, highlighting the sequence of GRIF/AB that we use in our experiment (GRIF-1a, $_{8\text{-}633}$) and predicted the possible sites of interaction with chymotryspsin enzyme. Figure 6.10 shows the possible sites of interaction in black arrows. These data suggest that brief periods of chymotrypsin treatment are not sufficient to proteolyse GRIF-1a protein, very minor breakdown products appears in the blots, indicating that GRIF-1a is not highly accessible to the enzyme at the surface.

### 6.3.5 Effect of GRIF-1a Protein on the Pharmacology of Rat Recombinant α1 β2 γ2L Model of GABA$_A$R:

To gain further insights into the potential role of this novel protein, we investigated the effect of transiently expressing GRIF-1a on the binding pharmacology of the rat recombinant α1β2γ2L GABA$_A$ receptor subtype stably expressed in HEK 293.

#### 6.3.5.1 [$^3$H] Flunitrazepam Saturation Binding in the Presence and Absence of GRIF-1a Protein.

Specific binding of [$^3$H]flunitrazepam to GABA$_A$R cell line membranes was saturable and best fit to a one-site binding hyperbola compared to a two-site model in both mock and GRIF-1a transfected cells ($P<0.05$). GRIF-1a protein produces a concentration dependant enhancement of [$^3$H] flunitrazepam binding activity in comparison to control cell homogenates. Figure 6.11, A shows the results. Analysis of the saturation binding isotherm using non-linear least square regression revealed that GRIF-1a enhanced the affinity (Kd) of [$^3$H] flunitrazepam binding without affecting the maximum no of binding sites ($B_{max}$). Figure 6.12, A&B shows the results. Rosenthal transformation of the saturation data was well approximated with a straight line. Figure 6.11, B.

Mean $K_D$ , $B_{max}$ values were:

| **Control** | **GRIF-1a** |
|---|---|
| $K_D$ = 8.32 ± 1.7 nM (n=3). | $K_D$ = 2.51 ±0.66 nM (n=3). |
| $B_{max}$ = 594 ±63 fmol/mg protein (n=3). | $B_{max}$ = 586 ±59 fmol/mg protein (n=3). |

#### 6.3.5.2 [$^3$H] Muscimol Competition Binding to GABA$_A$R Cell line by GABA in the Presence and Absence of GRIF-1a Protein.

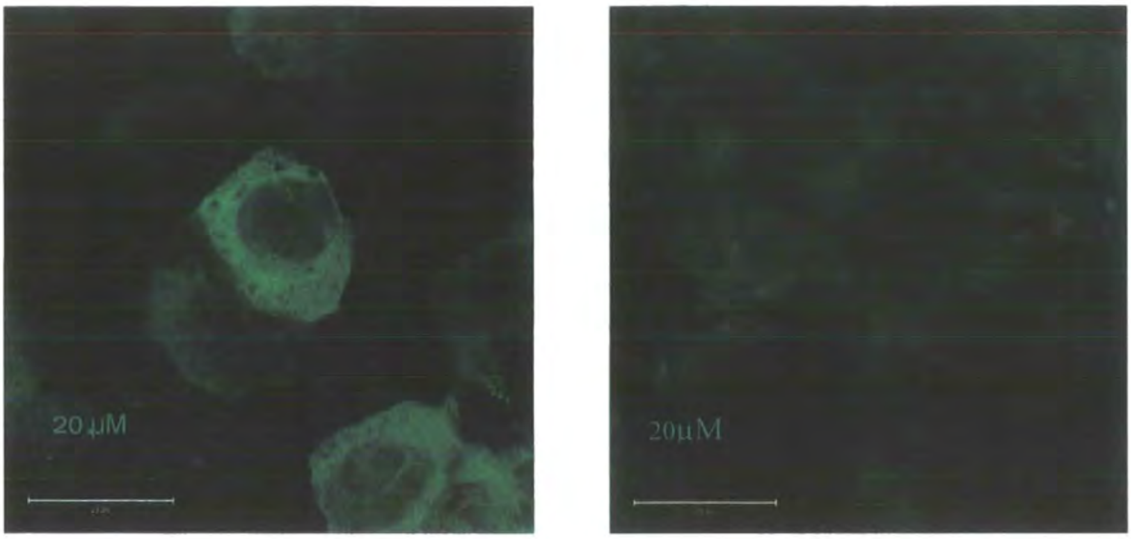
The effect of GABA on [$^3$H] muscimol binding to GABA$_A$R cell line in the presence and absence of GRIF-1a protein was investigated. Figure 6.13, A&B shows the results. In the absence of GRIF-1a protein, the GABA competition curve for [$^3$H] muscimol was best fit to a one site competition model with a pseudo-Hill coefficient, which was close to unity ($n_H$ = 1.0 ± 0.2) with an apparent $IC_{50}$ = 191 nM (100%). In the presence of GRIF-1a protein, the data were best fit to a two-site competition model comprising high- and low-affinity binding sites in the ratio (63:37) ( high: low%, SD ±12%), site one apparent $IC_{50}$ = 507 nM, site two apparent $IC_{50}$= 7 nM.

Confocal microscopy images indicated 35% transfection efficiency, accordingly these results indicate that in the presence of GRIF-1a protein, we have two group of cells population, one group which expresses the GRIF-1a protein (37%), in these cells the affinity of binding was increased $IC_{50}$ = 7 nM, the remaining cell population (63%) does not express GRIF-1a protein, the affinity of these cell group was similar to control cells $IC_{50}$ = 507nM.

**6.3.5.3 [$^{35}$S]-TBPS Competition Binding to GABA$_A$R Cell Line by Picrotoxin in the Presence and Absence of GRIF-1a Protein.**

The effect of picrotoxin on [$^{35}$ S] TBPS binding to GABA$_A$R cell line in the presence and absence of GRIF-1a protein was also investigated. Figure 6.14 shows the results. Competition data for [$^{35}$S]TBPS binding by picrotoxin was best fit to sigmoidal model variable slope, with a pseudo-Hill coefficient, which was close to unity ($n_H$) = -1.12 ± 0.11, -0.9 ± 0.11 with an apparent $IC_{50}$ of (3.14 ± 0.04 μM , 2.88 ± 0.08 μM) in the absence and presence of GRIF-1a protein respectively. The presence of GRIF-1a protein had no significant effect on the competition curve of picrotoxin.

A B




GRIF-1a-GFP transfected cells Control

**Figure 6.1:** Confocal microscopy images of (A) $GABA_AR$ cell line transfected with GRIF-1a GFP (B) Control $GABA_AR$ cell line.

Transfection efficiency (35%)

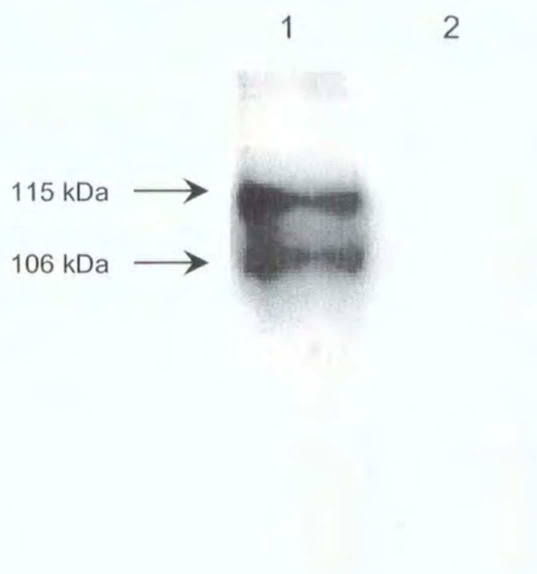



**Figure 6.2**: Immunoblot demonstrating the expression of GRIF-1a protein in stable $GABA_A$ R cell line.

Membranes were prepared from both mock and GRIF-1a transfected $GABA_AR$ cell line. Expression of GRIF-1a protein was analysed by immunoblotting using 7.5% SDS-PAGE under reducing conditions with 50µg of protein applied per gel lane.

The gel was probed with anti-GRIF-1a, antibody (1µg/ml).

Lanes:

Lane 1 GRIF-1a transfected cell homogenates.

Lane 2 Mock transfected cell homogenates.

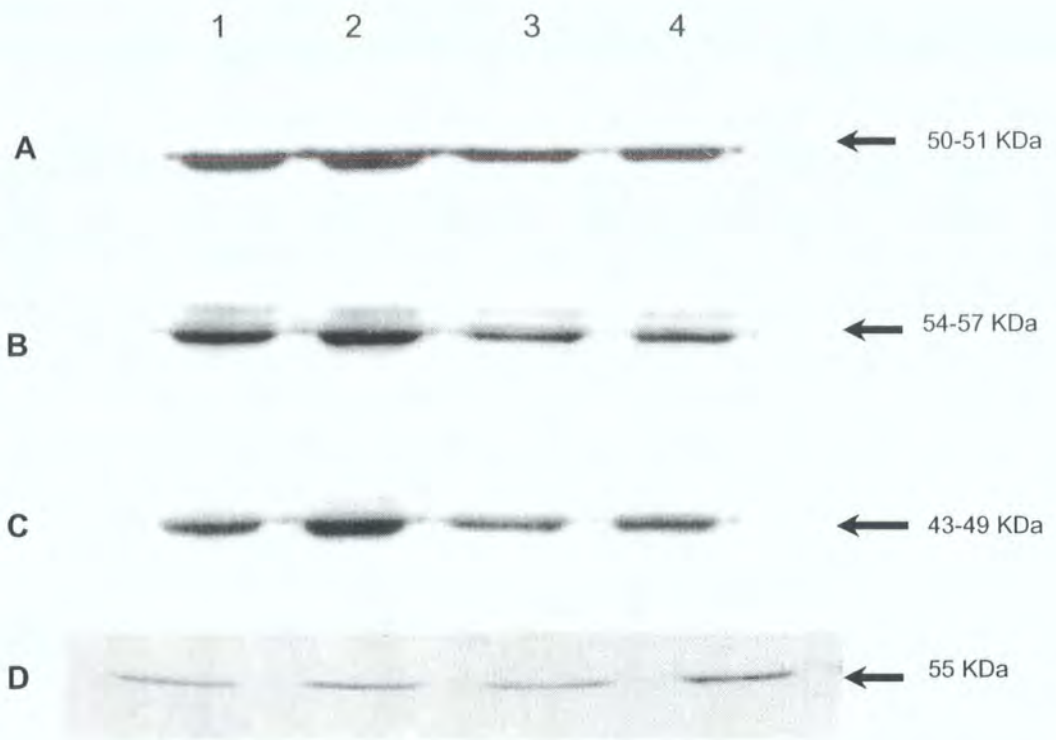



**Figure 6.3:** Immunoblots demonstrating the effect of GRIF-1a protein on $GABA_AR$ α1, β2 and γ2 subunit expression.

Membranes were prepared from both mock and GRIF-1a transfected cells. Effect of GRIF-1a protein on α1, β2 and γ2 subunits expression was analysed by immunoblotting using 7.5% SDS-PAGE under reducing conditions with 50μg of protein applied per gel lane.

**A** was probed with anti- $GABA_A$ receptor α1 antibody (2μg/ml).
**B** was probed with anti- $GABA_A$ receptor β2 antibody (1μg/ml).
**C** was probed with anti- $GABA_A$ receptor γ2 antibody (1μg/ml).
**D** was probed with anti- β-actin antibody (1μg/ml).

**A, B, C& D:**

Lane 1&2 GRIF-1a transfected cell homogenates.
Lane 3&4 Mock transfected cell homogenates.

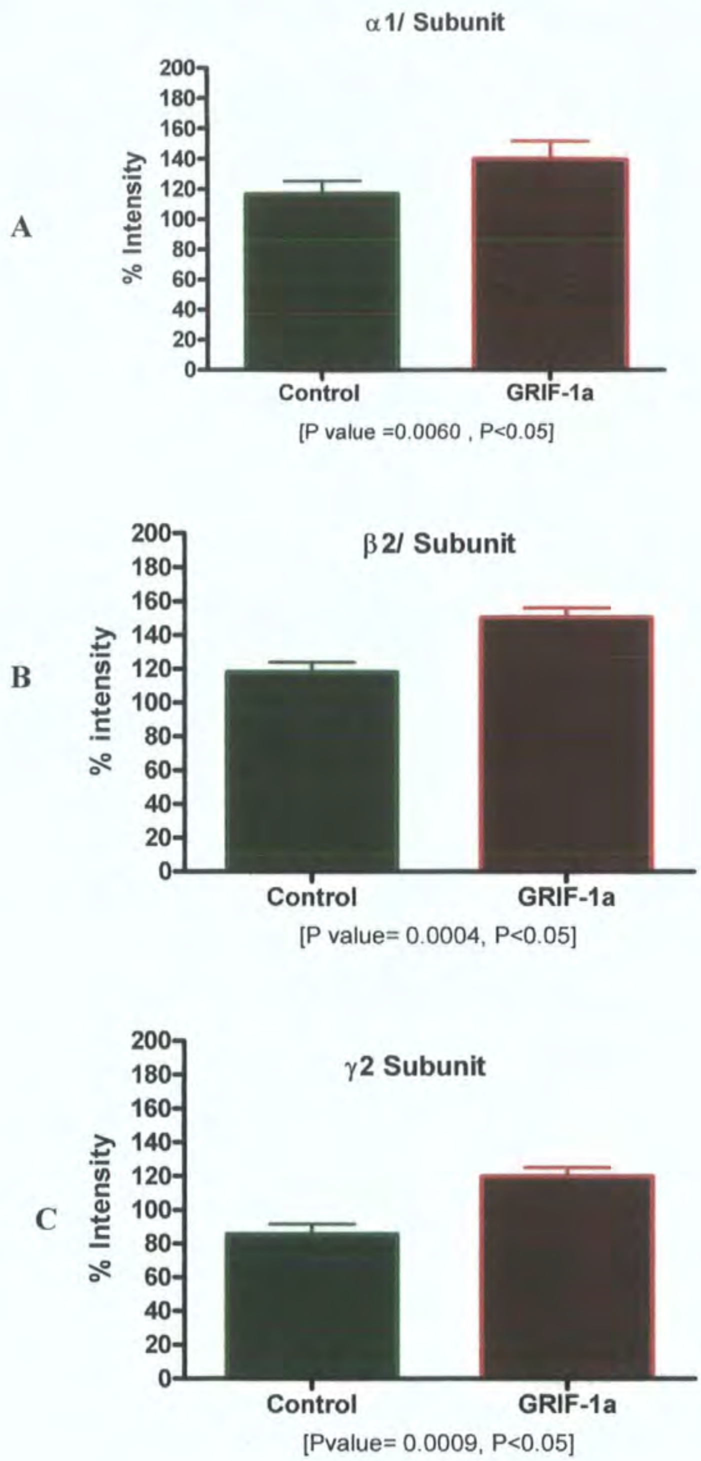
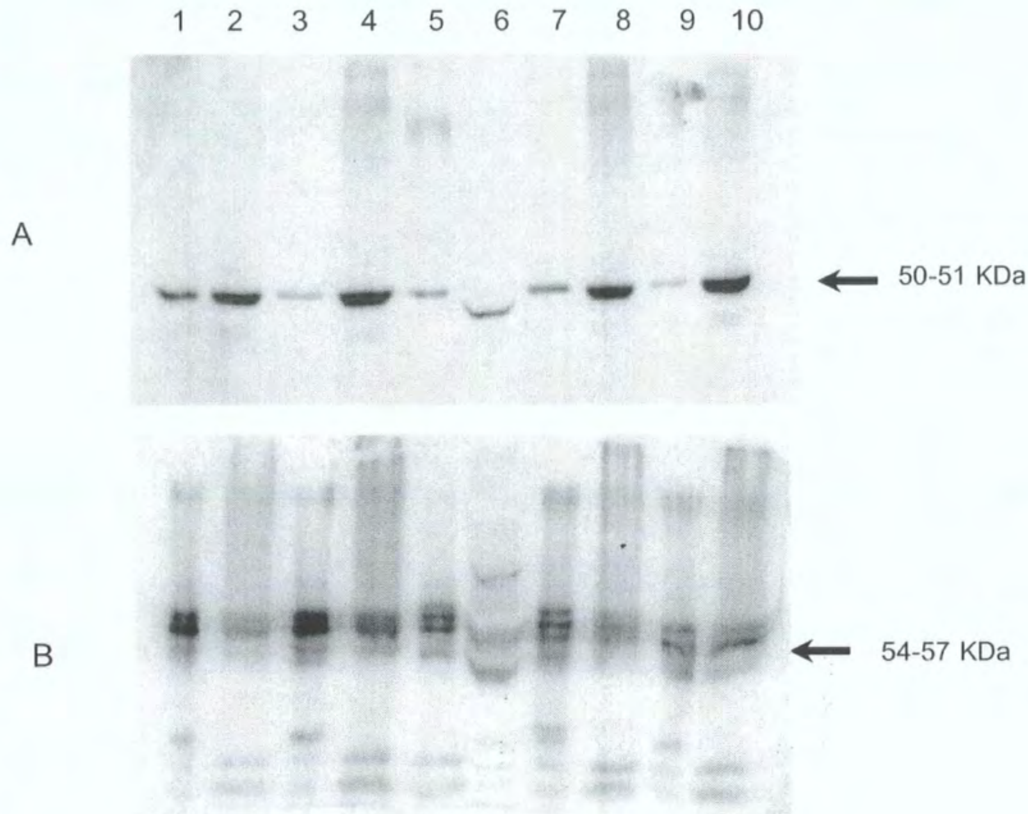



**Figure 6.4:** Quantitative analysis of the effect of GRIF-1a on $GABA_AR$ subunit protein expression **(A)** α1, **(B)** β2 and **(C)** γ2 in comparison with control cell homogenates.

The films were scanned into a computer and the immunoreactive bands were quantified using Computer-assisted densitometry using microcomputer Imaging Device (MCID) version 7 software from Imaging Research Inc., Ontario, Canada, Image J programme. Data was the analysed using the student's paired t-test (Graph Pad Prism, Graph Pad, San Diego, CA). The 95% confidence level $p < 0.05$ was considered statistically significant.


1 2 3 4 5 6 7 8 9 10
A
50-51 KDa
B
54-57 KDa

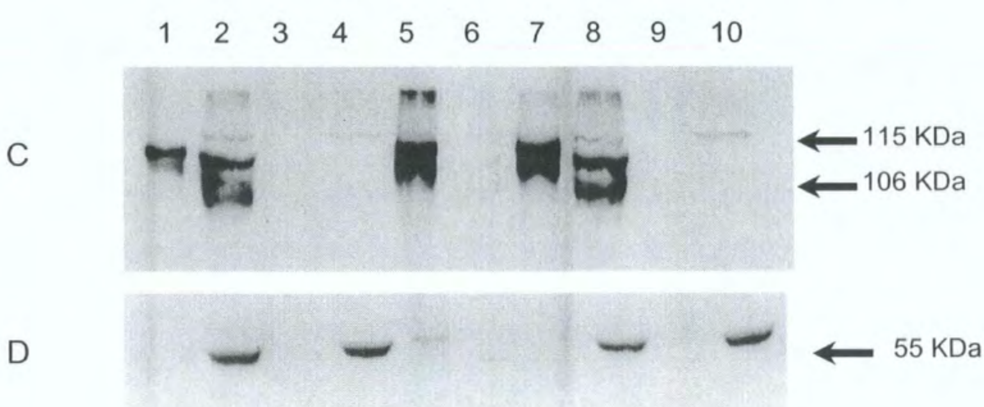

1 2 3 4 5 6 7 8 9 10
C
115 KDa
106 KDa
D
55 KDa

**Figure 6.5:** Immunoblots of cell surface protein biotinylation in $GABA_AR$ cell line.

**A** was probed with anti- $GABA_AR$ α1 antibody (2μg/ml).

**B** was probed with anti- $GABA_AR$ β2 antibody (1μg/ml).

**C** was probed with anti- GRIF-1a, antibody (1μg/ml).

**D** was probed with anti- β-actin antibody (1μg/ml).

Lanes

1&7 GRIF-1a transfected cells, surface protein fractions.

2&8 GRIF-1a transfected cells, Intracellular protein fractions.

3& 9 Mock transfected cells, surface protein fractions.

4&10 Mock transfected cells, Intracellular protein fractions.

5 Control $GABA_AR$ cell line cell homogenates.

6 Rat forebrain.

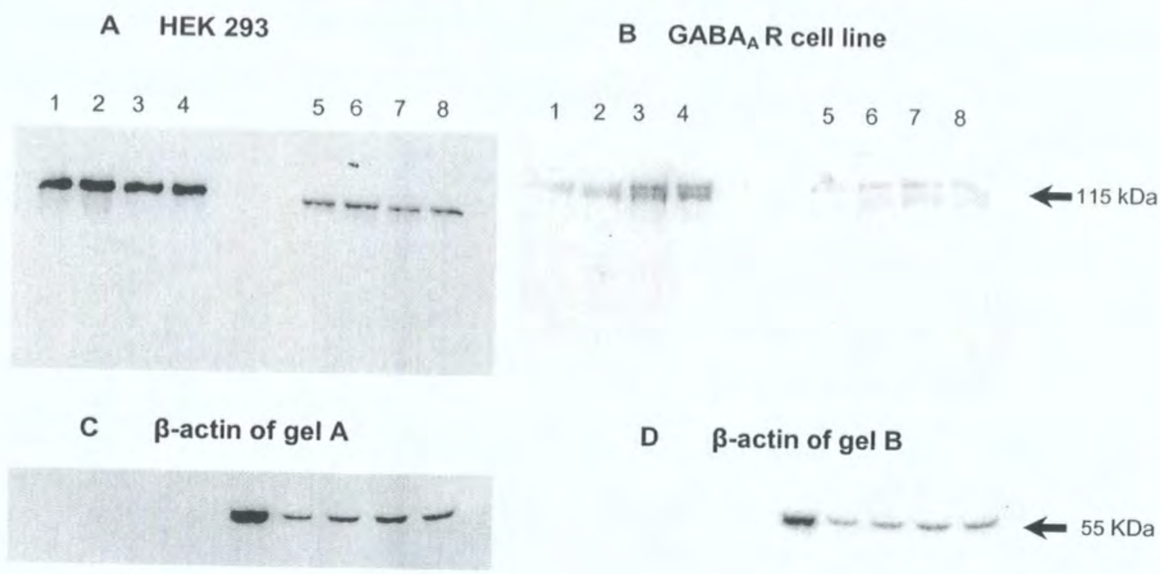



**Figure 6.6:** Immunoblots of cell surface protein biotinylation in (A) control HEK 293 and (B) GABA$_A$R cell line.

**A&C** Biotinylation in control HEK 293.

**B&D** Biotinylation in GABA$_A$R cell line.

**A &B** were probed with anti-GRIF-1, antibody (1μg/ml).

**C& D** were probed with anti- β-actin antibody (1μg/ml).

**Lanes in the A, B, C & D:**

1, 2, 3 & 4: GRIF-1a transfected cells, surface protein fractions.

5, 6, 7 & 8: GRIF-1a transfected cells, Intracellular protein fractions.

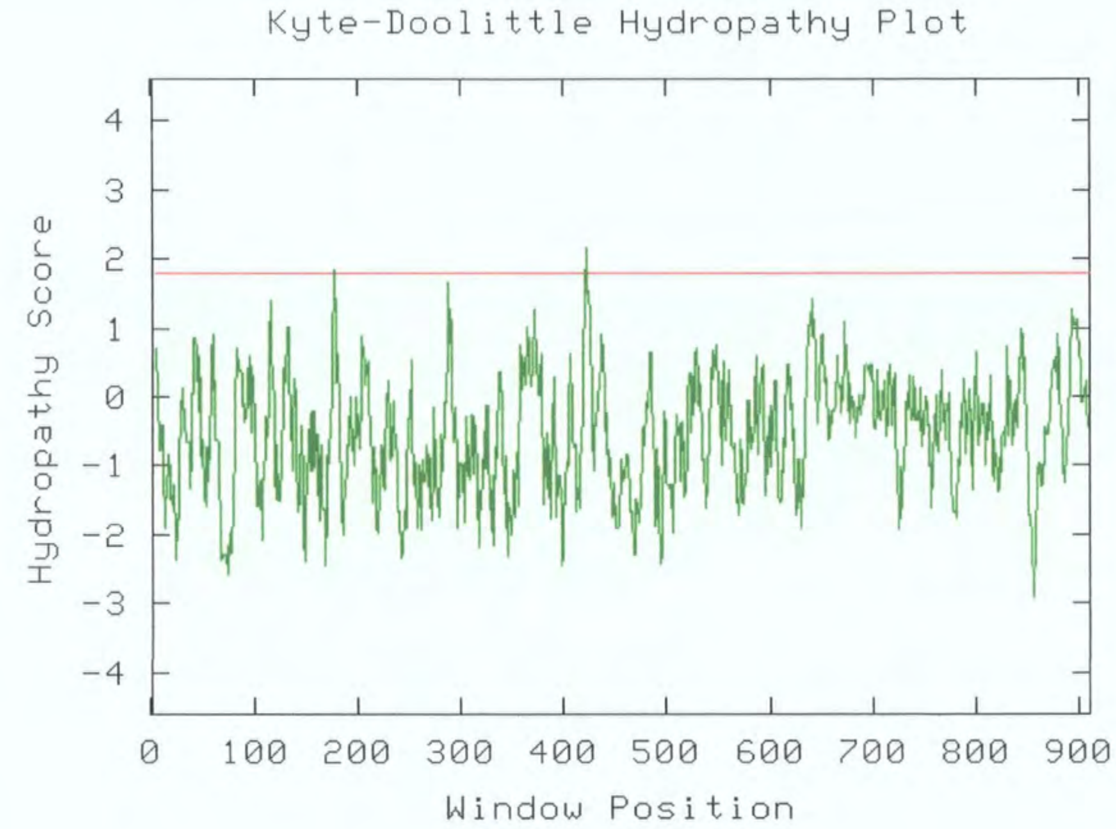



**Figure 6.7:** Kyte- Doolittle hydropathy plot of full length GRIF-1 protein

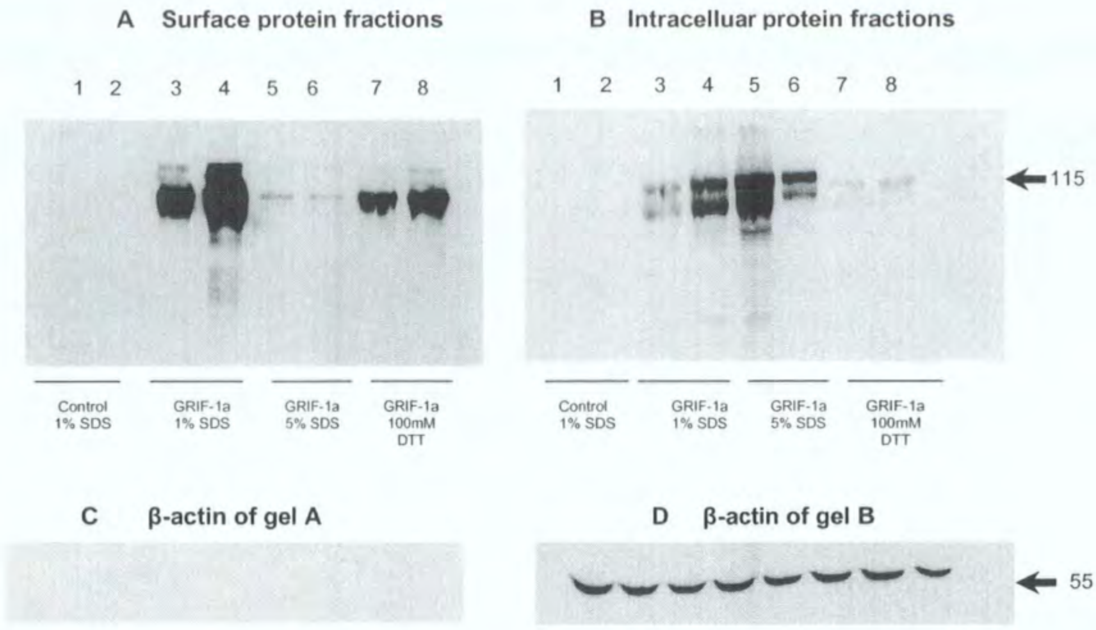



**Figure 6.8:** Immunoblots of cell surface biotinylation in control HEK 293 using different solubilization reagents.

**A& C** Surface protein fractions.

**B &D** Intracellular protein fractions.

**A &B** were probed with anti- GRIF-1a, antibody (1μg/ml).

**C& D** were probed with anti- β-actin antibody (1μg/ml).

**Lanes in the A, B, C & D:**

1&2 Mock transfected HEK 293 cells solubilized in 1% SDS.

3&4 GRIF-1a transfected HEK293 cells, solubilized in 1% SDS.

5&6 GRIF-1a transfected HEK293 cells, solubilized in 5% SDS.

7&8 GRIF-1a transfected HEK293 cells, solubilized in 100mM DDT.

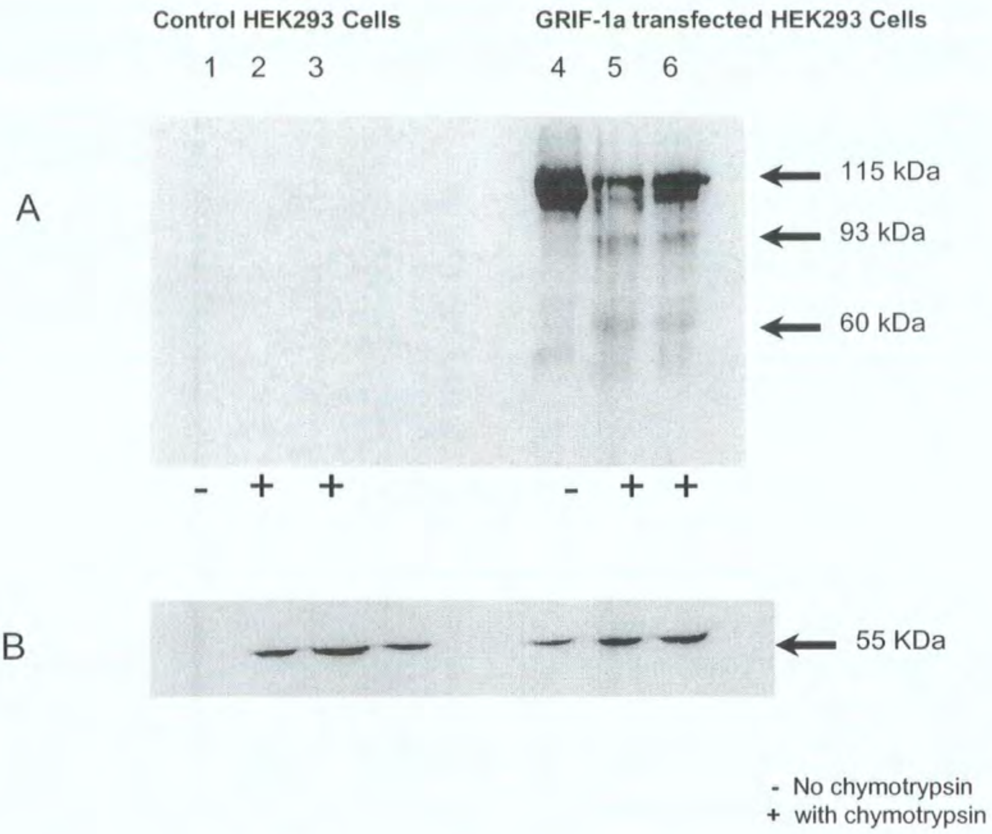



**Figure 6.9:** Effect of Chymotrypsin treatment on GRIF-1a expression in control HEK 293 cells.

**A** was probed with anti-GRIF-1a, antibody (1μg/ml).

**B** was probed with anti- β-actin antibody (1μg/ml).

**Lanes in A& B**

1 Mock transfected HEK293 cells, no chymotrypsin treatment.

2 & 3 Mock transfected HEK293 cells, Chymotrypsin (2mg/ml) for 10 min at 37°C.

4 GRIF-1a transfected HEK293 cells, no chymotrypsin treatment.

5 & 6 GRIF-1a transfected HEK293 cells, Chymotrypsin (2mg/ml) for 10 min at 37°C.

```
1   mslsqnai [ fk sqtgeeklms snhrdsesit dvcsnedlpe velvnlleeqlpqyklrvds
61  lflyenqdws qsshqqqdas etlspvlaee tfrymilgtd rveqmtktyn didmvthlla
121 erdrdlelaa rigqallkrn hvlseqnesl eeqlgqafdq vnqlqhelsk keellrivsi
181 aseesetdss cstplrfnes fslsqgllql dmmheklkel eeenmalrsk achiktetft
241 yeekeqklin dcvnelretn aqmsrmteel sgksdellry qeeissllsq ivdlqhklke
301 hviekeelrl hlqaskdaqr qltmelhelq drnmeclgml hesqeeikel rnkagpsahl
361 cfsqaygvfa geslaaeieg tmrkklslde esvfkqkaqq krvfdtvkva ndtrgrsvtf
421 pvllpipgsn rssvimtakp fesgvqqted ktlpnqgsst evpgnshprd ppglpedsdl
481 atalhrlslr rqnylsekqf faeewerklq ilaeqeeevs scealtenla sfctdqsett
541 elgsagclrg fmpeklqivk plegsqtlhh wqqlaqpnlg tildprpgvi tkgftqmpkd

601 avyhisdlee deevgitfqv qqplqleqkp app ] ppvtgif lppmtsaggpvsvatsnpgk

661 clsftnstft fttcrilhps ditqvtpssg fpslscgssa gsasntavns paasyrlsig
721 esitnrrdst itfsstrsla kllqergisa kvyhspasen pllqlrpkal atpstppnsp
781 sqspcsspvp feprvhvsen flasrpaetf lqemyglrps rappdvgqlk mnlvdrlkrl
841 giarvvktpv prengksrea emglqkpdsa vylnsggsll gglrrnqslp vmmgsfgapv
901 cttspkmgil ked
```

- Origin [Rattus norvegicus], 913 a. a.

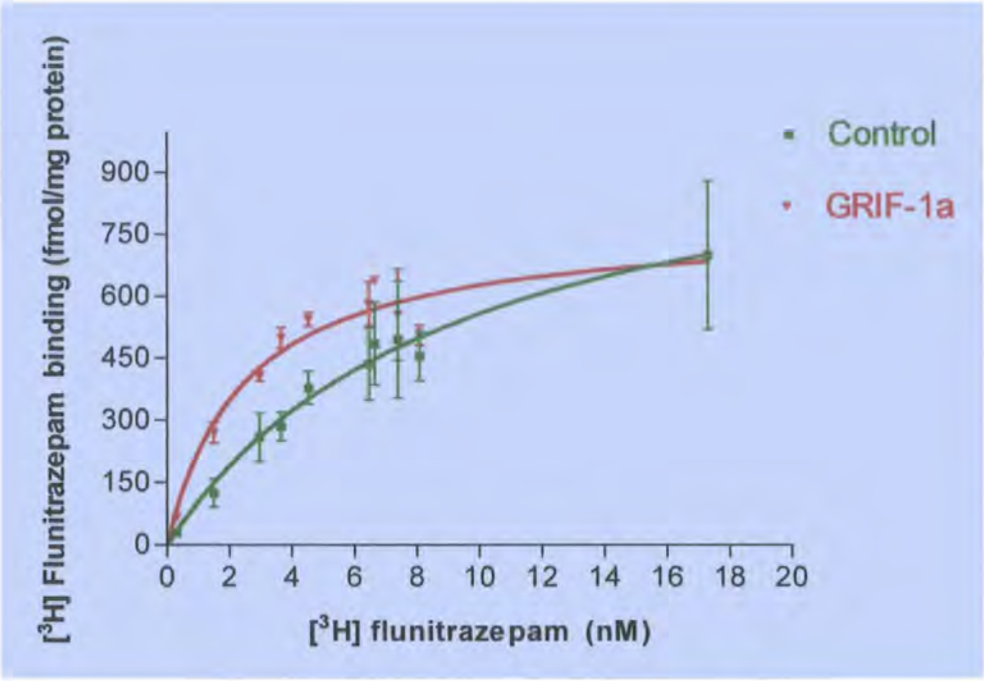
- Amino acids in [ ] represent the sequence of anti- GRIF/AB [8-633] used in the study.

- Main substrates for chymotrypsin enzyme are: Tryptophan (w), Tyrosine (Y), Phenylalanine (F), and Methionine (M).

-Amino acids in red represent possible sites of interaction of GRIF-1a with chymotrypsin enzyme, black arrows suggests 3 major sites of interaction

**Figure 6.10:** Amino acid sequence of full length GRIF-1.

A




B

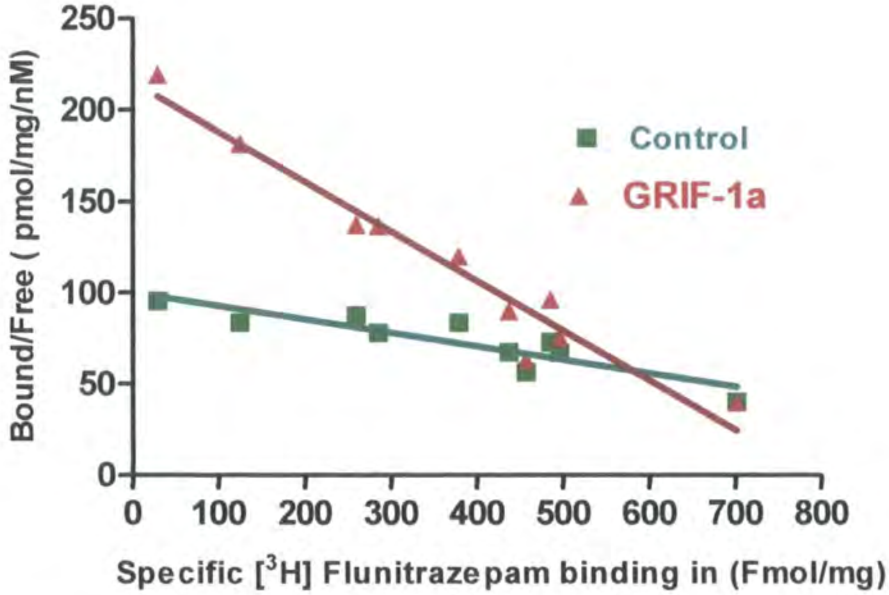


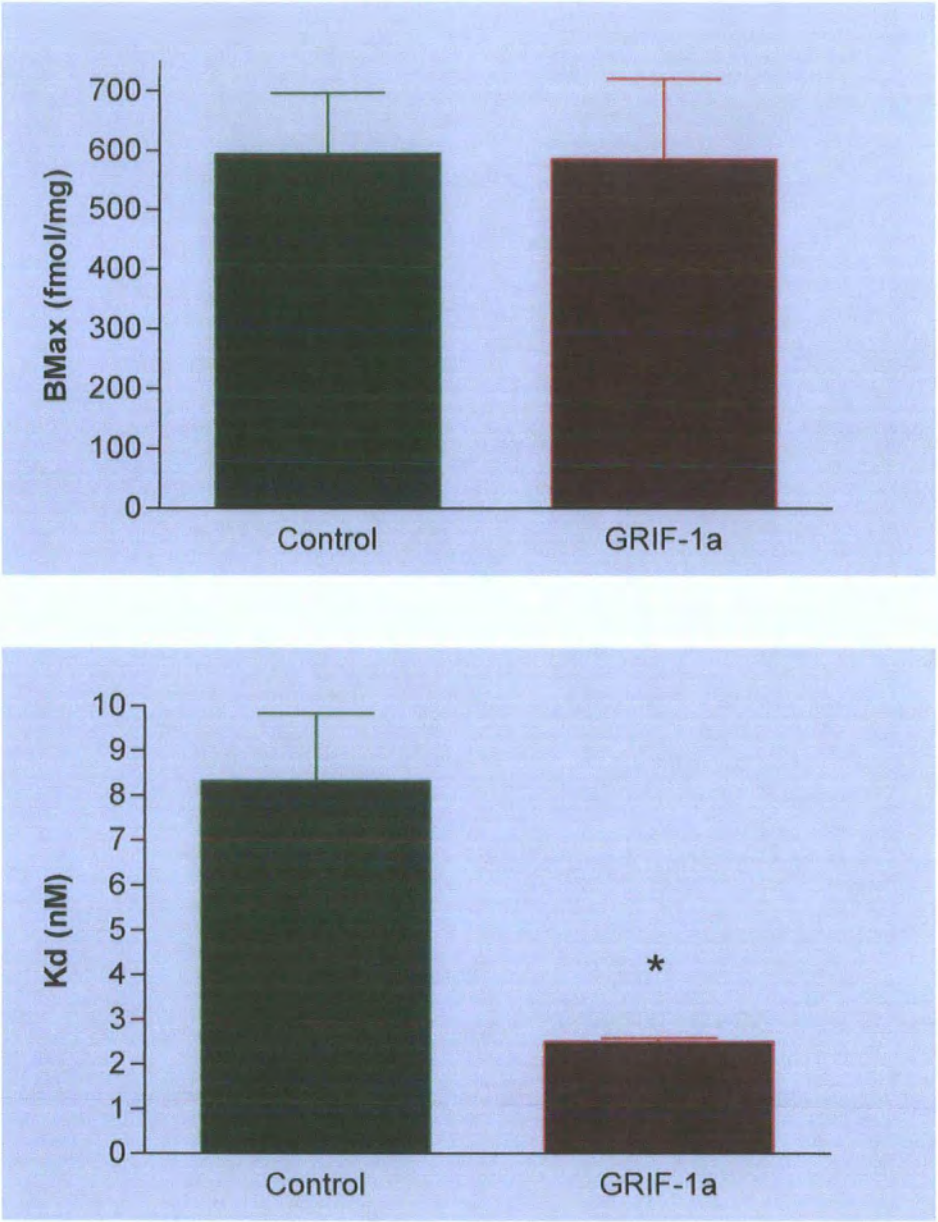

**Figure 6.11:** Saturation isotherm and Rosenthal transformation of [$^3$H] flunitrazepam binding to $GABA_AR$ cell line.

Data shown represents a mean ± SD for three separate experiments from three independent transfections.

**A** is the saturation isotherm of the saturation data.

**B** is the Rosenthal transformation of the saturation data.

A




B



Values shown are mean ± S.D for three independent transfections (* $p < 0.05$)

**Figure 6.12:** (A) Effect of GRIF-1a on $B_{max}$ values of [$^3$H] flunitrazepam binding to $GABA_AR$ cell line in (fmol/mg) protein, (B) Effect of GRIF-1a on $K_D$ values of [$^3$H] flunitrazepam binding to $GABA_AR$ cell line in (nM).

| **Control** | **GRIF-1a** |
|---|---|
| $K_D$ = 8.32 ± 1.7 nM (n=3). | $K_D$ = 2.51 ±0.66 nM (n=3). |
| $B_{max}$ = 594 ±63 fmol/mg protein (n=3). | $B_{max}$ = 586 ±59 fmol/mg protein (n=3). |

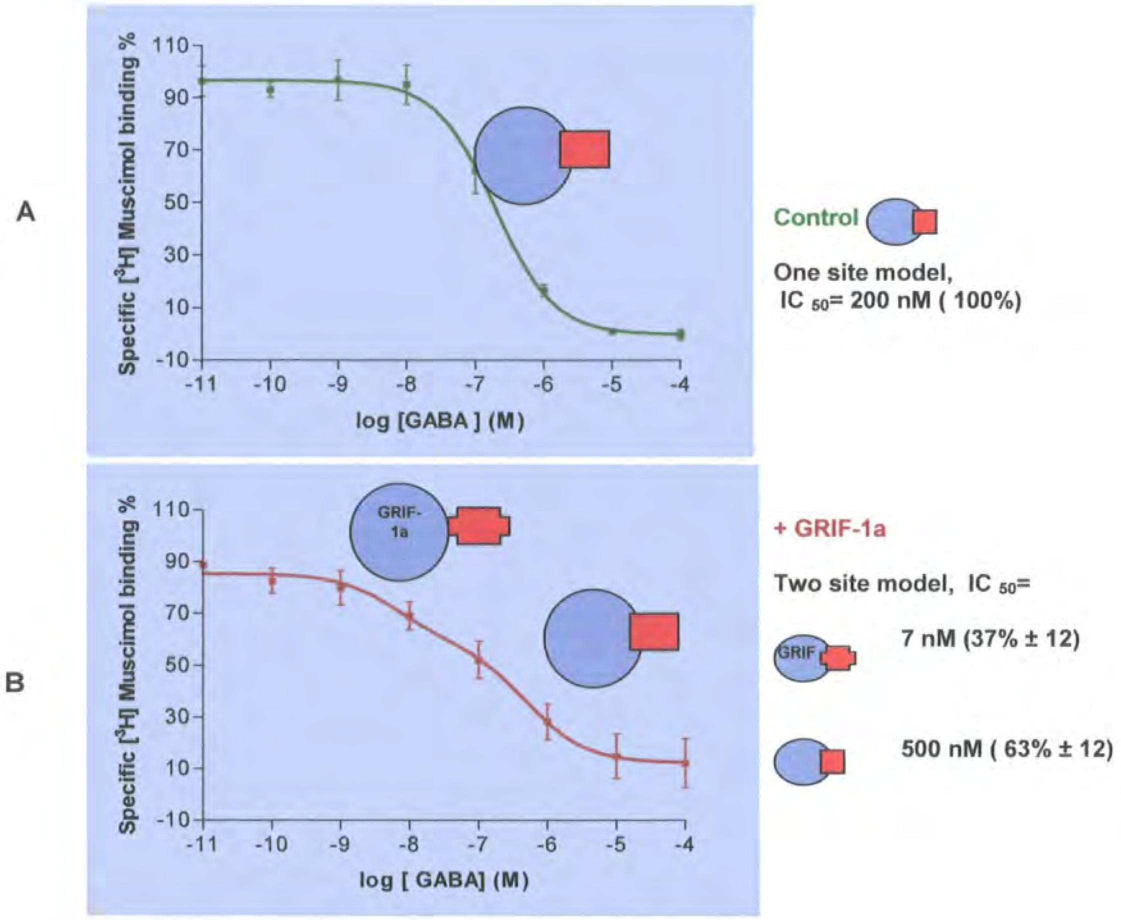
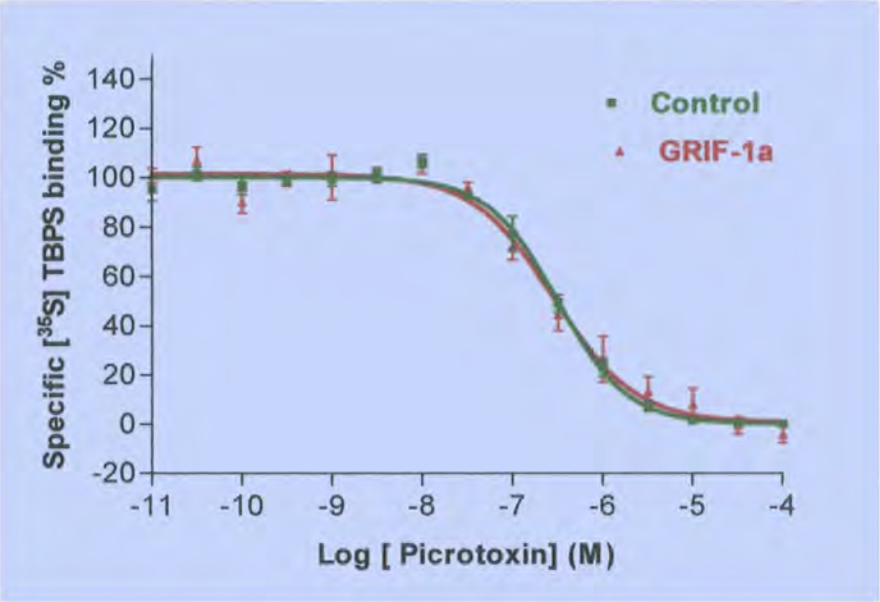



**Figure 6.13:** Effect of co-expression of GRIF-1a on the binding of [$^3$H] Muscimol to the GABA site of the $GABA_AR$ cell line. Data represents a mean ± SD for three separate experiments from three independent transfections.




| | Apparent $IC_{50}$ in ($\mu$M) | $n_H$ |
|---|---|---|
| **Control** | **3.14 ± 0.04** | **-1.12 ± 0.11** |
| **GRIF-1a** | **2.88 ± 0.08** | **-0.9 ± 0.11** |

**Figure 6.14:** Effect of co-expression of GRIF-1a on the binding of [$^{35}$S] TBPS to the picrotoxin site of the $GABA_AR$ cell line. Data represents a mean ± SD for three separate experiments from three independent transfections.

## 6.4 Discussion

GRIF-1 [$GABA_A$ (γ-aminobutyric $acid_A$) receptor interacting factor-1] was initially identified by a yeast two-hybrid screen searching for $GABA_AR$ clustering and trafficking proteins (Beck *et* al., 2002). GRIF-1 is the orthologue of the human protein, OIP98 [OGT (β-O-linked *N*-acetylglucosamine transferase) interacting protein 98] and it is the homologue of the protein OIP106. GRIF-1 is also probably the orthologue of the *Drosophila* protein Milton, a kinesin-associated protein that is involved in the transport of mitochondria to the synapses in retina. GRIF-1, OIP106 and Milton belong to a newly identified family of coiled-coil proteins. Although their function is not definitively established, it have been suggested that GRIF-1 is another example of an adaptor protein involved in motor-dependent trafficking of proteins (Brickley *et al.*, 2005). Recently GRIF-1 and OIP106 found to interact with Atypical Rho GTPases Miro-1& Miro-2 proteins; these are localized in the mitochondria and have been implicated in regulating mitochondrial homeostasis and plays essential roles in mitochondria trafficking (Fransson *et al.*, 2006).

The present chapter describes the pharmacological characterization of the role of this novel $GABA_AR$ associated protein (GRIF-1a) in mammalian brain. In this study, HEK 293 cells stably transfected with plasmids encoding for α1β2γ2L subunits of the rat $GABA_AR$ were our model system in the pharmacological studies, this subtype of $GABA_ARs$ is the most common type found in the brain.

The expression of GRIF-1a protein in the GABA cell line was confirmed by confocal microscopy images and by immunoblot probed with anti-GRIF-1a, antibody (Kindly provided by Prof. Anne Stephenson). Confocal microscopy imaging showed cytoplasm localization and in the immunoblot GRIF-1a immunoreactive protein band of Mr 115,000, 106,000 were detected in the GRIF-1a transfected cells. These results were consistent with what have been reported (Beck *et* al., 2002, Brickley *et al.*, 2005).

The first issue we addressed in the study was the effect of GRIF-1a on subunit protein expression α1, β2 and γ2. Data showed that the presence of GRIF-1a protein increases α1, β2 and γ2 subunit protein expression in comparison with control. Quantitative analysis showed that GRIF-1a increases the stability of these subunits by 20%-40% in comparison with control.

To investigate the possible role of GRIF-1a protein in regulating membrane trafficking of $GABA_AR$ to the surface, cell surface protein biotinylation was carried out. The experiment western blots showed no significant difference in the expression levels of α1 and β2 subunits, surface and intracellular protein fractions in the presence and absence of GRIF-1a protein. In the same assay, GRIF-1a protein was detected in the surface as well as in the intracellular fractions. These data indicate that GRIF-1a is a surface protein; it may be speculated to play a pivotal role in the transport, trafficking and assembly of $GABA_AR$ to the surface.

To explore whether the expression of GRIF-1a protein at the surface is dependent upon the presence $GABA_AR$, the same biotinylation experiment was carried out in control HEK293 cells lacking the $GABA_AR$. Surprisingly, GRIF-1a protein was clearly expressed in the surface protein fractions. These results implying that GRIF-1a is associated with a surface protein in HEK 293 cells; it performs its function on the surface even in absence of $GABA_AR$.

In order to study the interaction between GRIF-1a and surface proteins, we performed the biotinylation experiment under different solubilization conditions, 1% SDS, 5% SDS and 100 mM DTT. Data showed that 5% SDS reduced the level of surface expression of GRIF-1a protein to a negligible level. These results indicate that GRIF-1 is strongly associated with a surface protein in HEK 293. This association is disturbed by high concentrations of SDS but is not affected by 1% SDS or 100 mM DTT.

Next, we performed an alternative surface labelling approach, using proteolytic cleavage with the enzyme chymotrypsin in control HEK 293 cells. GRIF-1a protein was not affected by the chymotrypsin incubation, which indicates that GRIF-1a is not externally accessible protein.

Together, our biochemical and cellular observations suggest that GRIF-1a protein is strongly associated with surface proteins and it plays a role in the stabilization of $GABA_AR$ at the inhibitory synapse. These findings are in agreement with recent published data suggested GRIF-1 functioning as adaptors in the anterograde trafficking of organelles, utilizing the kinesin-1 motor proteins, to synapses (Brickley *et al.*, 2005). A schematic diagram showing a proposed function of GRIF-1 as an adaptor protein linking kinesin-1 to its cargo in anterograde trafficking mechanisms in neuron have been suggested recently Pozo & Stephenson, 2006. Figure 6.15. The authors suggested that GRIF-1 dimers attach a $GABA_ARs$-containing vesicle to kinesin. GRIF-1 is also attached to OGT trimers that have also been shown to be part of the transport complex.

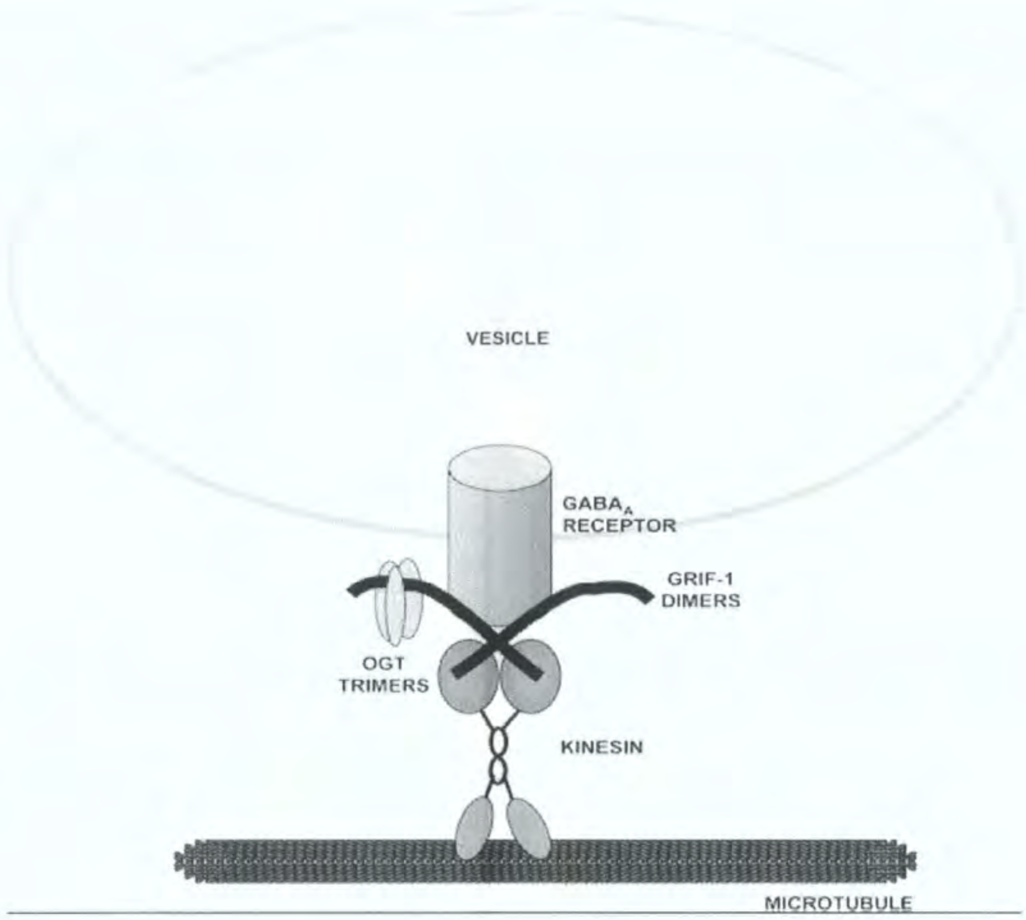



**Figure 6.15:** Schematic diagram showing the proposed function of GRIF-1 as an adaptor protein linking kinesin-1 to its cargo.

(From: Pozo & Stephenson, 2006).

In this study we also investigate the effect of transiently expressing GRIF-1a on the binding pharmacology of the rat recombinant α1β2γ2L model of $GABA_AR$ subtype stably expressed in HEK293 cells using radioligand binding assay.

Radioligand binding data showed that, firstly, co-expression of GRIF-1a enhanced, in a concentration-dependent manner, the apparent [$^3$H] Flunitrazepam binding to $GABA_A$ α1β2γ2 receptor. Saturation binding analysis showed this enhancement to be due to a 4-fold decrease in $K_D$ (increase in affinity) with little effect on the $B_{max}$ for [$^3$H] flunitrazepam (α1β2γ2 complexes). This was consistent with surface expression data demonstrating no significant difference between α1 and β2 subunit surface and intracellular expression in the presence and absence of GRIF-1a protein. Secondly, GRIF-1a protein increases affinity of GABA for the $GABA_A$ α1β2γ2L receptor (approx. 30-fold). Thirdly, GRIF-1a had no significant effect on picrotoxin competition binding for [$^{35}$S] TBPS.

Electrophysiological testing of GRIF-1a pharmacology in collaboration with Prof. George Lees (Department of Pharmacology and Toxicology, Dunedin, NZ) was in good agreement with the binding data. GRIF-1a induced a concentration-dependent increase GABA-induced chloride current at α1β2γ2L $GABA_AR$ using two electrodes, voltage-clamp electrophysiology. Figure 4-8 Appendix III, shows this positive modulatory effect of GRIF-1a protein on the GABA current.

Taken together, the present study demonstrate that GRIF-1a protein does not increase α1β2γ2 receptor complex numbers, but appears importantly to stabilise the $GABA_ARs$ in a conformation which facilitates binding to both GABA and benzodiazepines.

The physiological significance of this newly identified protein could be summarized in two major points: Firstly, if this protein enhances the affinity of binding at the benzodiazepine and GABA site, this protein could be a potential pharmacotherapy to enhance the activity of drugs that act at these sites of the receptor. Increasing affinity means reduction of the recommended doses and potentially fewer side effects with these drug treatments. Secondly, this protein could be a new therapeutic target for modulation of $GABA_A$ function, for treatment of numerous neurological conditions caused by deficits in GABA such as cerebral palsy, stroke, spinal cord injury, stiff-person syndrome and Parkinson disease. This idea is highly supported by what has been recently published by Gilbert *et al.* (2006) studying hypertonia in mice. Cloning the responsible gene for hypertonia identified a protein called Trak-1; this is found to be a trafficking and kinesin-1 binding protein and showed to interact with $GABA_ARs$ regulating the endocytic trafficking, targeting the receptor to the surface or block degradation. Hyrt mutant mice were found to have lower levels of γ-aminobutyric acid in the CNS, particularly the lower motor neurons than do wild type mice, indicating that the hypertonicity of the mutant is likely to be caused by deficits in GABA-mediated motor neuron inhibition (Gilbert *et al.*, 2006). Accordingly, GRIF-1 (highly related to Track-1) could be potential target for GABA deficits in CNS diseases.

In conclusion, this study provides a clear picture about the pharmacology of GRIF-1 protein, further investigation using electrophysiological and behavioural study could address its therapeutic efficiency as a novel means of modifying synaptic inhibition.

# Chapter 7

# Overall Discussion & Future Directions

## 7.1 Overview of Current Study

The main inhibitory neurotransmitter system in the brain, the gamma-aminobutyric acid (GABA) system, is the target for many clinically used drugs to treat, for example, anxiety disorders and epilepsy and to induce sedation and anesthesia. These drugs facilitate the function of pentameric A-type GABA (GABA (A)) receptors that are extremely widespread in the brain and composed from a repertoire of 19 subunit variants.

Direct evidence for alteration in GABAergic neuron morphology and in distribution of $GABA_AR$ is available for most prevalent neurological and psychiatric disorders, most likely reflecting changes in GABA circuits, including axonal sprouting and formation of novel synapses. While the analysis of causative mechanisms is impossible in human studies, some of these changes can be produced experimentally in animal models, and their molecular and cellular bases analyzed in vitro. The ultimate goals of these studies are to further our understanding of brain function and to provide effective treatments or relief of symptoms for neurological and psychiatric disorders.

$GABA_AR$ are the site of action of a number of clinically important drugs, many of which have been in use for several decades. However, it is only during the past 15 years that scientists began to uncover the structural and functional complexity of these receptors, due to the recent availability of the new tools of modern biology. Although there is already an appreciation and understanding of the diversity of the receptor family, the function of the plethora of subtypes (from synaptic level to their influence on animal behaviour) remains largely unexplored. It is clear, however, that these insights hold real opportunities for drug development. Importantly, this should be considered in terms of refining and improving upon existing medicines (which may be achieved, for example, by targeting BZs to defined receptor subtype) and in terms of developing novel drugs for alternative indications, targeted to the recently identified receptor subtypes.

The future of neurodegenerative therapeutic development depends upon two strategies: Screening and identifying new compounds which modify the GABA function. The alternative strategy is to increase the understanding of molecular pharmacology of

GABAgeric transmission; this will improve safety profiles and entirely new indications will be discovered.

In this thesis we have characterised the effect of three structurally distinct $GABA_AR$ compound classes, MFA, Caloporoside and essential oil natural products of Melissa & Lavender, and examined in detail the pharmacological effects of a novel $GABA_AR$ interacting protein, GRIF-1.

Pharmacological screening of the three compound classes and the molecular pharmacology of a new interacting protein for the mammalian $GABA_AR$ has been carried out using radioligand binding techniques on rat adult forebrain membranes and rat recombinant α1β2γ2L model of $GABA_AR$ stably expressed in HEK293 cells.

## 7.2 Several of the key Issues were Identified From This Study Outlined Below with Future Directions:

### 7.2.1 Pharmacological Characterization of NSAID, MFA:

Fenamates, a family of NSAIDs that inhibit the cyclo-oxygenase (COX) pathway, are N-arylated derivatives of anthranilic acid. The most common ones are mefenamate, flufenamate, meclofenamate, and niflumate. Recent studies have shown that fenamates are also capable of modulating a variety of ion channels. These modulatory effects include the inhibition of NMDA-gated cation channels; $Ca^{2+}$ activated non-selective cation channels and GABA-gated ($Cl^-$) channels.

MFA was shown to potentiate GABA on α1 and β2/β3 subunits containing receptors, but was inactive or inhibitory in β1 subunit-containing receptors (Halliwell *et al.*, 1999). Recently MFA was reported to have neuroprotective effects and improvement in cognitive impairment both in a vitro and in vivo in AD model (McGeer *et al.*, 2006, Joo *et al.*, 2006).

These data suggest that fenamates may serve as lead structures in the development of novel therapeutic agents with the potential for the treatment of anxiety, epilepsy or neuroprotective therapy. Given the pharmacological importance of MFA and its potential biological activity, it was thought of some interest to explore the chemistry of MFA compounds.

In the present study, a number of analogues of MFA, substituted with different groups on the aryl (phenyl) group were synthesized by our collaborator Dr.Patrick Steel and tested on $GABA_AR$.

Pharmacological characterization of the synthesized compounds was carried out using receptor binding assays in both native and recombinant preparations. In addition, a molecular modelling study based on MFA was performed together with our collaborator Dr.Colin James, to explore the dimensions and properties of different size substituents on the structural flexibility in this chemical series.

Our results showed that MFA and a series of analogues selectively modulates $GABA_AR$ at the agonist binding site, but did not interact with either the picrotoxin or the benzodiazepine sites. An examination of the SAR of MFA and a series of analogues substituted with different groups showed that the modulatory effect on the agonist binding sites of $GABA_AR$ labelled by [$^3$H] muscimol binding is in these compound series is highly affected by the size of substitution groups on the ring B of fenamate structure, modulation is affected by substitution at $R_1$ and $R_2$ of ring B. Introduction of alkyl group (methyl or ethyl) at $R_1$ or $R_2$ improve the modulatory activity. In contrast, bulky group substitution like (OMe, $CF_3$ or $Cl^-$ ) resulted in significant reduction of the affinity in comparison to MFA. The study also showed that the imino bridge between the ring A & B of fenamate structure is very important for activity; replacement of this group abolishes the modulatory activity.

A close agreement between results of molecular modelling and experimental observations, showing that MFA conformations 1 and 3 have a good structural similarity matching with C1, C2 , C3,C4, C5, C10 and C15. All differ in substitution on $R_1$ or $R_2$ of the phenyl ring. Indeed the most significant result of this study is perhaps the identifications of a common active conformer of MFA compound (Conformer 1& 3) and the differentiation of two compound analogues based on MFA structure, with improvement of efficacy, these were Compound 2 and compound C15, both showed promising results with pharmacological examination and molecular modelling analysis.

A number of questions however need to be answered before conclusions can be drawn about the $GABA_AR$ modulatory effect of MFA: (1) sensitivity, which ligand gated ion channels is sufficiently sensitive to clinically relevant concentration of MFA? Because at micromolar concentration these types of compounds reported to affect a wide range of ion channels. (2) Mechanisms, where does MFA bind? What is the exact mechanism by which MFA affect the function of ion channel? (3) In vivo importance: testing the specific behavioural action of MFA in animal models for anxiety and epilepsy. (4) Subunit dependency; the GABA potentiating action was dependent on the asparagine residue of TM2 in β2 and β3 subunits (Halliwell *et al.*, 1999) these needs further investigation, mutated or chimeric mammalian $GABA_AR$ subunit c-DNA could address whether the specific mutation in these subunits will affect the binding MFA to the $GABA_AR$. (5) Molecular modelling and QSAR analysis of the interaction of MFA derivatives with the agonist binding site of $GABA_AR$ complex in more detail, could clarify the potential activity of compound 2 and 15 in this chemical series. Future structure activity studies should address the effect of alkyl group length substitution at $R_1$ and $R_2$ positions, the role of carboxyl group on the first six-membered ring, the effect of replacement of CH group by N atom in the ring A as in Niflumic acid, the effect of replacement of the second six membered, ring B by alicyclic or aliphatic alternatives, and to examine the effect of different groups substitution on the ring A. Hopefully future studies will enhance our knowledge further and provide answers to these questions.

### 7.2.2 Positive Allosteric Modulator of $GABA_AR$, Octyl-β-D-Glucoside:

Caloporoside is a novel active fungal metabolite, isolated from culture filtrate of *Caloporus dichrous*, and have been reported to inhibit the binding of [$^{35}$S] TBPS in the $GABA_A$ channel receptor complex in vitro. Recently, our collaborator Prof. Fürstner has successfully synthesized Caloporoside and two analogues (Fürstner *et al.*, 1996, 1998). Herein, we have characterized the binding pharmacology of this synthetic caloporoside and two further congeners, 2-hydroxy-6-{[(16R)-(β-d-mannopyranosyloxy) heptadecyl]} benzoic acid and Octyl-β-D-glucoside on $GABA_AR$.

Caloporoside and 2-hydroxy-6-{[(16R)-(β-d-mannopyranosyloxy) heptadecyl]} benzoic acid produced a concentration-dependent complete inhibition of specific [$^{35}$S]TBPS binding. In contrast, Octyl-β-D-glucoside elicited a concentration-dependent stimulation of specific [$^{35}$S] TBPS binding. The level of stimulation was similar to that elicited by diazepam and was occluded by GABA (0.3 μM). However, the three test compounds failed to elicit any significant effect (positive or negative) upon [$^3$H] flunitrazepam or [$^3$H] muscimol binding, indicating that they did not bind directly, or allosterically couple, to the benzodiazepine or agonist binding site of the $GABA_AR$, respectively.

Preliminary structure-activity study showed that the constituent monosaccharide, glucose, and the closely related congeners Octyl-α-D-glucoside, Hexyl-β-D-glucoside, Heptyl-β-D-glucoside and Nonyl-β-D-glucoside have no significant effect upon [$^{35}$S] TBPS binding. These data together provide strong evidence that a β-glycosidic linkage and chain length are crucial for the positive modulation of [$^{35}$S] TBPS binding to the $GABA_AR$ by this novel chemical class.

The present study shows that Octyl -β-D-glycoside is a positive allosteric modulator of $GABA_AR$ in the binding assay. Our laboratory previously reported that Octyl-β-D-mannopyranoside, a simple polar deacetylated caloporoside derivative is a positive functional modulator of the $GABA_A$ chloride channels (Lees *et al.,* 2000). Octyl-β-D-mannopyranoside (100 µM) significantly and reversibly increased the magnitude of $GABA_A$ currents evoked in the cultured rat cortical pyramidal neurons. Chemical synthesis of Octyl-β-D-mannopyranoside to test its effect in the binding assay was not successful. The stereoselective chemical synthesis of the β-mannopyranosidic linkage poses a well–known problem in carbohydrate chemistry for two reasons first, the anomeric effect have been reported to afford the formation of the 1,2-trans-mannopyranosyl (α) linkage and not the β-linkage and second the 1,2-cis arrangements of the equatorial aglycone and the axial functionality at C-2 in β-mannopyranosides harbors repulsive steric effect (Barresi *et al.*, 1996, Ernst *et al.*, 2000, McCleary, 1988). Octyl-β-D-mannopyranoside was made using a method previously described by Singh *et al.*, (2000). However, we found that the procedure for the synthesis was not ideal and not an easy way to obtain the sugar. Unfortunately the final compound we obtain at the end of the reaction was the ($OCH_3$) substituted mannoside and not the Octyl-β-D-mannopyranoside, confirmed by NMR analysis; even though this product was useful in the pharmacological study, as a part of SAR of Octyl-β-D-glucoside.

In summary this study provides the most detailed characterization of this novel compound class. Further work will be needed to study the effect of these two sugars (Octyl-β-D-glycoside & Octyl-β-D-mannopyranoside) in more detail. Chemical synthesis of both sugars should be carried out following another published procedure (David *et al.*, 1998, Fürstner *et al.*, 1998, Benjamin, 2000, Abdel-Rahman *et al.*, 2002). Testing the biological activity towards the $GABA_AR$ by radioligand binding and patch clamping should be examined in parallel. Further structure activity studies are needed with the emphasis on the β forms and different size of substitution on position-1 of both sugar rings. Electrophysiological analysis should be able to clarify the mechanisms of action of these compounds on $GABA_AR$. Any promising activity with these compound series could be validated in an animal model as potential anticonvulsant or anxiolytic.

### 7.2.3 Natural products & $GABA_ARs$: Elucidation of the Pharmacological Mechanisms of Melissa and Lavender Essential oils:

Dementia is a serious public health problem currently affecting million people worldwide. Old people with dementia may display memory problems, negative emotion and agitated behaviours. Among these symptoms, agitated behaviours have been identified by caregivers as the most challenging care problems and often precipitate admission to residential facilities. The high prevalence of dementia and consequences of agitated behaviours caused by dementia highlight the importance of developing effective interventions for those with dementia, especially because the increased number of dementia is likely to place increasing burden on health care resources as our population ages. Agitated behaviours have traditionally been managed with the use of antipsychotic drugs or physical restraints; however, these treatments may cause adverse effects. The evidence for current pharmacological treatments for managing behavioural symptoms of people with dementia is still not ideal. Judicious use of antipsychotic drugs in those with dementia is recommended because these agents can cause many harmful side effects and lead to further decline. Use of physical restraint in those with dementia also increase the incidence of injuries and often leads to more agitation and is considered as indicator of poor quality of care in institutional settings. These concerns have led to research seeking alternative approaches to managing agitated behaviours of those with dementia and reduce the need for chemical or physical restraints. One such approach is the use of aromatherapy.

Aromatherapy is the therapeutic use of plant essential oils, to help relieve health problems and improve the quality of life in general. The healing properties of aromatherapy are claimed to include promotion of relaxation and sleep, relief of pain and reduction of depressive symptoms. Aromatherapy has become more common and has been applied to a wide range of health problems, including agitation in dementia. The most commonly used essential oils for dementia therapy in controlled trials have been Lavender and Melissa, singly or in combination. The trials have involved people with advanced dementia in residential care and have generally assessed behavioural symptoms, particularly agitation. As outcome measures, what is remarkable, despite the diversity of trials design, are that all treatments have resulted in significant benefit. The benefits include reductions in agitation, insomnia, wandering, difficult behaviour and social withdrawal (Perry & Perry, 2006).

The purpose of this study was to clarify the sedative and calming mechanisms of Melissa and Lavender essential oils in agitated patients, we studied the effect of these two plant essential oils either single or in combination by investigating their effects on $GABA_AR$ complex the major site of various anxiolytic, sedative–hypnotic and general anesthetic and on NMDA receptor and nicotinic acetylcholine receptor to address any possible specific CNS neurotransmitter effects.

Results have shown that Melissa and Lavender oils singly or in combination inhibit [$^{35}$S] TBPS binding on the channel site of $GABA_AR$. Melissa oil alone displayed the higher affinity. Melissa oil alone also showed stimulatory effect on [$^{3}$H] muscimol binding. Interestingly, an additive effect on the inhibition of [$^{3}$H]flunitrazepam binding to the $GABA_AR$ has been shown when Lavender and Melissa oils are applied in combination, with no effect when applied alone. Neither Melissa, nor Lavender oils demonstrated any effect on the binding of [$^{3}$H] MK-801 to NMDA receptors, or [$^{3}$H] nicotine to nicotinic acetylcholine receptors. In addition, Melissa oil alone also found to inhibit binding of [$^{3}$H]-8-OH-DPAT to 5-HT1A receptors and [$^{3}$H]-pirenzepine to M1 receptors (Mark S.J. Elliott, Kings College London). Overall, therefore, Melissa oil appears to have a broad pharmacological profile. Furthermore, functional studies have demonstrated that both oils (0.01 mg/ml) applied to rat primary cortical neuron cultures, results in a significant reduction in both inhibitory and excitatory transmission, with a net depressant effect on neurotransmission. These data suggests that the calming and sedative effect of Melissa mediated by multiple mechanisms on the CNS neurotransmitters; the net effect is a depressant on overall net work transmission in the neurons.

One major aim for future work is to identify the effect of Melissa on other possible target sites such as voltage gated sodium channels; this could define their functional roles in reduction of both inhibitory and excitatory transmission.

The data presented in this chapter illustrates the importance of highlighting three issues these include: Firstly, Melissa essential oil shows a broad pharmacological profile and significant numbers of controlled clinical trials demonstrated the therapeutic potential for treatment of severe dementia. Melissa extract was reported to be effective in modulation of mood and cognitive performance in AD (Wake *et al.*, 2000, Kennedy *et al.*, 2002, 2003). Recently ethanolic extract of *M.officinalis* was reported to have acetyl cholinesterase inhibition activity in vitro (Ferreira *et al.*, 2006). With these in mind, Melissa plant might potentially provide novel natural treatments for AD; accordingly this plant deserves further investigations. Secondly, plants selected for any pharmacological study should be well authenticated. Exact botanical species, plant part, extracting procedure, and dose level traditionally used should be well documented. It is essential that all future studies specify the exact derivation of the oils used and preferably, include a GC/MS profile of the oil and the percentage composition of the major constituents. Such variations are liable to lead to inconsistencies in reported bioactivities and efficacy. This is well demonstrated in our study; four different reputable suppliers for the oils showed differences in essential oil constituents and purity detected by GC/MS analysis. Adulteration by adding cheaper substitute is very common in many essential oils companies. Standardization of the essential oil in terms of chemical composition should be carried out before any research study. Thirdly, synergistic bioactivity due to mixing different constituents is common and thought to be an important contributor to the activity of many botanical medicines and natural product abstracts There are a number of examples of individual constituents showing synergistic activity after combination (Williamson, 2001; Spinella, 2002). A clear example is the effect of Melissa & Lavender oil mixtures on [$^3$H] flunitrazepam binding. This observation determined not to use the combination in the clinical trial as the therapeutic significance of this effect remain unclear.

In conclusion, natural products are excellent sources of chemically diverse, drug-like lead structures for drug discovery. Essential oil products are a significant part of modern medicine; investigation of their active principles and mechanisms of action is essential for these products to remain a part of the modern health care therapy.

### 7.2.4 Pharmacological Characterization of the Role of a Novel $GABA_AR$ Interacting Factor, GRIF-1a:

$GABA_AR$ are important key elements in setting the inhibitory tone of neurons in the brain, modulation of their expression, cellular distribution and function therefore has profound consequences for neural excitability under both physiological and pathological conditions (Lüscher *et al,* 2004). For efficient synaptic transmission, $GABA_AR$ need to be localized and anchored at postsynaptic site in precise opposition to pre-synaptic nerve terminal that release neurotransmitter GABA. It has become increasingly clear that receptors and ion channels in the central nervous system are not isolated entities but in fact form numerous interactions with other protein important to regulate membrane trafficking, plasma membrane insertion, synaptic clustering and turn over of the receptors (Kneussel *et al.*, 2002). A significant effort has been made to identify proteins that interact directly with the large intracellular domain (located between transmembrane domain TM3 and TM4) of the $GABA_AR$ subunits. This has revealed a number of receptor-associated proteins implicated in the regulation of phosphorylation, clustering and membrane trafficking of these ion channels (Lüscher *et al.*, 2004, Kneussel *et al.*, 2002).

The $GABA_AR$ interacting factor, GRIF-1 was identified as a $GABA_AR$ interacting protein in a yeast two hybrid screen of a rat brain c-DNA library, using β2 subunit as bait (Beck *et al.*, 2002). It is thus speculated from the homology found to known proteins that the neuronal protein, GRIF-1 which may fulfill a similar function in the transport of β2 subunit-containing $GABA_AR$ to inhibitory post synaptic membranes, may be a novel $GABA_AR$ trafficking factor.

To further assess the importance of this protein for $GABA_AR$ trafficking and function, the effect of this protein on the pharmacology of α1β2γ2L model of $GABA_AR$ stably expressed in HEK293 cells was investigated.

In the present work we provide clear evidence that GRIF-1a does not increase α1β2γ2 receptor complex numbers, but appears importantly to stabilise the $GABA_AR$ in a conformation which facilitates binding to both GABA and benzodiazepines, demonstrated by its ability to increase both ($Cl^-$) influx and [$^3$H] flunitrazepam binding, as well as by its tendency to increase [$^3$H] muscimol binding. Furthermore, our observations suggest that GRIF-1a is a tightly associated surface protein, it perform its function near the surface. These conclusions, contrast with the function of GABARAP, another GABA(A)R associated protein , which has been implicated in the intracellular membrane trafficking of $GABA_AR$ and not surface expression and synaptic localization (Kneussel *et al.*, 2000, Kittler *et al.*, 2001, O'Sullivan *et al.*, 2005) suggesting different regulatory functions of these associated proteins.

Several lines of evidence suggest that GRIF-1, OIP106, OIP 98, Milton and Trak-1 proteins are members of a coiled-coil family, associated with motor protein kinesin, and involved in the trafficking of mitochondria. These suggest that GRIF-1a has potentially multiple functions in the mammalian brain.

Further work will be needed to determine what role GRIF-1a may be playing at the inhibitory synapse. The physiological significance of this protein could be addressed by detailed electrophysiological analysis. The precise mechanism underlying the effect of GRIF-1 on surface trafficking of $GABA_AR$ remains to be determined. Transgenic and gene targeted mice would be particularly helpful in understanding the biological function of GRIF-1 protein. By similarity to Trak-1, GRIF-1 protein may be associated with any of GABA deficit CNS diseases; therefore regulating $GABA_AR$ function with GRIF-1 protein may be a novel means of modifying the efficacy of synaptic inhibition.

## 7.3 Concluding Remarks

Considering the compounds currently in clinical studies, the next few years hold great interest for those involved with the development of therapeutic strategies based on the GABA neurotransmitter systems. Will receptor subtype selective compounds and allosteric modulators provide efficacy without the unwanted side effects? If positive results are forthcoming, this could open the way for improvements over existing therapies and provide drugs for currently untreatable disease.

Much remains to be learned about the mechanisms controlling GABA release and clearance, neuron-glia interactions, trafficking and anchoring of $GABA_AR$, the associated proteins and transduction signaling. A greater understanding of the mechanisms controlling GABA system may also open up new therapeutic avenues which target the interacting protein rather than the receptor.

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# Appendix I

## Synthesis of Octyl-O-β-D-Mannopyransoside

### Introduction:

Octyl-O-β-D-mannopyransoside, was made using a method previously described by Singh (Singh *et al.*, 2000). The procedure was achieved by the activation of the anomeric center of 1-O-2,3,4,6-tetra-O-benzyl-D-mannopyranosylpropane-1,3-diylphosphate in the presence of TMSOTf (Trimethylsilyltriflate) and subsequent debenzylation.

### 2. Materials & Methods

#### 2.1 Materials:

**Sigma-Aldrich Chemical Company (Poole, Dorset, U.K):**
Acetonitril, Calcium hydride, Celite 521 (filter reagent), Chloroform, Cyclohexene, Diethyl ether, Iodine, Methanol, Octanol, Petroleum ether, Phosphorus oxychloride, Sodium carbonate, Sodium sulphate anhydrous.

**Lancaster Chemical Company (Lancashire, U.K):**
Acetyl chloride, Dichloromethane, Ethanol, Magnesium turning, Molecular sieves (3A, 1-2mm beads), N,N-dimethylformamide, N-methyl-imidazol, Palladium hydroxide on carbon, Sodium hydride, Tetra-butylammonium-iodide, Triethylamine, Trifluroacetic acid, Trimethylsilyl-trifluoro-methanesulphonate.

**Avocado's, Alfa Aesar Chemical Company (Ward Hill, U.S.A):**
Benzyl bromide, Calcium chloride, anhydrous, D-Mannose, Propane-1, 3 diol.

#### 2.2 General Methods:

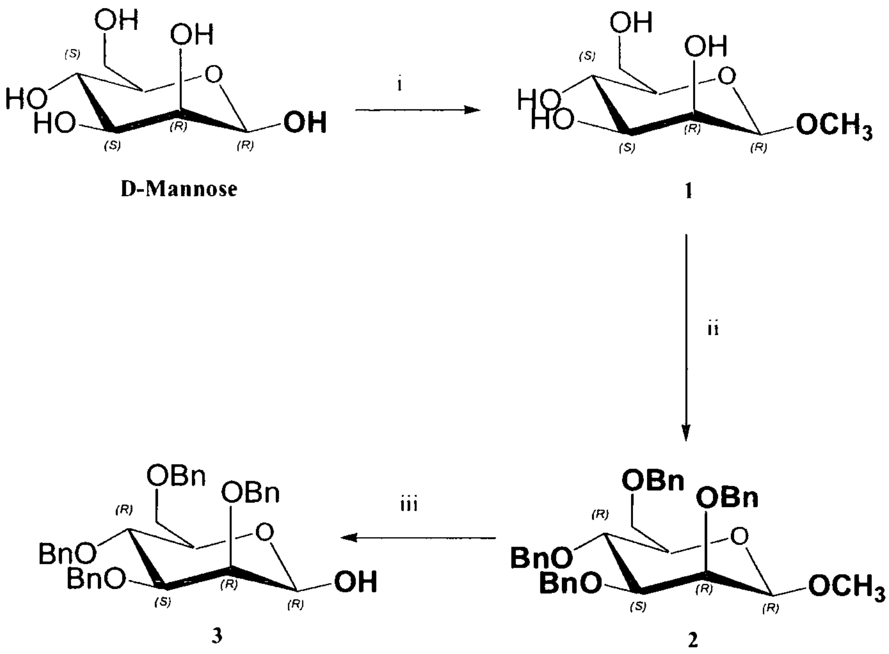
$^{1}H$ NMR spectra were measured at 400 MHz with a JEOL GSX 270 FT NMR spectrometer. Chemical shifts were measured relative to internal tetramethylsilane ($\delta$: 0). $^{13}C$ NMR spectra were recorded at 100 MHz on the same instrument with internal $(CH_3)_4Si$ ($\delta$: 0, $CDCl_3$). IR spectra were recorded on a UNICAM series FT- instrument. Flash chromatography was performed using Fluka silica gel 60 (230–400 mesh) and the solvent petroleum ether (boiling range 40–60°C) was distilled prior to use. Thin layer chromatography was carried out using pre-coated aluminium plates (Merck Kieselgel 60 $F_{254}$) which were visualised under UV light and then with either phosphomolybdic acid or basic aqueous potassium permanganate as appropriate. All anhydrous reactions were carried out under argon or nitrogen. Anhydrous transfers were done with standard syringe techniques; all glassware was pre-dried overnight. Methanol, N,N-dimethylformamide, and dichloromethane were all distilled prior to use and stored over activated 3 Å molecular sieves, as described by Perrin and Armarego (Perrin *et al*, 1988).

## 3. Experimental

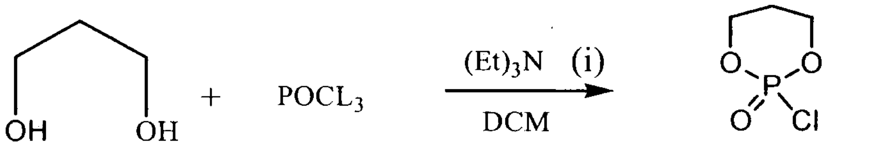
Octyl-O-β-D-mannopyransoside was made using a method previously described by Singh (Singh *et al.*, 2000). This method was achieved by the activation of the anomeric center of 2,3,4,6-tetra-O-benzyl-1-O-D-mannopyranosylpropane-1,3diyldioxyphosphate in the presence of TMSOTf (Trimethylsilyltriflate). The desired compound was obtained by debenzylation, using palladium hydroxide [$Pd(OH)_2$] on carbon under nitrogen atmosphere.

Synthesis steps are outlined in schemes 1, 2 and 3.

Treatment of D-Mannose in the presence of methanol and acetyl chloride generated the O-methoxy substituted derivative of mannose (**1**) this will provide protection of the anomeric center. Benzylation step was then carried out to in the presence of NaH and Tetrabutylammonium iodide in DMF to yields 2, 3, 4, 6-Tetra–benzyl-1-O-methyl-D-mannopyranose **(2)**. Methoxy group of benzylated product was hydoxylated again under reflux conditions for 48 hr to yield 2,3,4,6-Tetra–O-benzyl-1-α,β-D-mannopyranose **(3)**. Treatment of compound **(3)** with propane-1,3-diyldioxy-phosphylchloride in the presence of N-methyl imidazole resulted in the formation of the phosphonate **(4α, 4β)**. These were inseparable by flash chromatography. Phosphonate was then treated with Octanol in the presence of TMSOTf as catalyst at -78 °C for 30 min to yield 2, 3, 4, 6-Tetra–O-benzyl-α, β-octyl-D-mannopyranoside **(5α,β)**. Separation of the two products was carried out by short column chromatography using (petroleum ether: diethyl ether 1:1) solvent system and TLC monitoring, to yield α and β derivative of product **(5α,5β)**. N.M.R data shows that α fraction is not pure it still contains unreacted material, while β fraction was fairly pure. To obtain the desired product debenzylation step was carried out using palladium hydroxide on carbon, to replace all the benzyl groups into hydroxyl again to yield Octyl-O-β-D-mannopyransoside **(6, β)**.




**Scheme 1**: Reagents and conditions (i) MeOH, Acetyl chloride, reflux at 60 °C, 5 hr.
(ii) DMF, NaH, TBAI, Benzyl bromide, RT, 9 hr.
(iii) [$CH_3CN$:$CF_3COOH$:H2O(4:3:3)] reflux at 95 °C,48 hr.

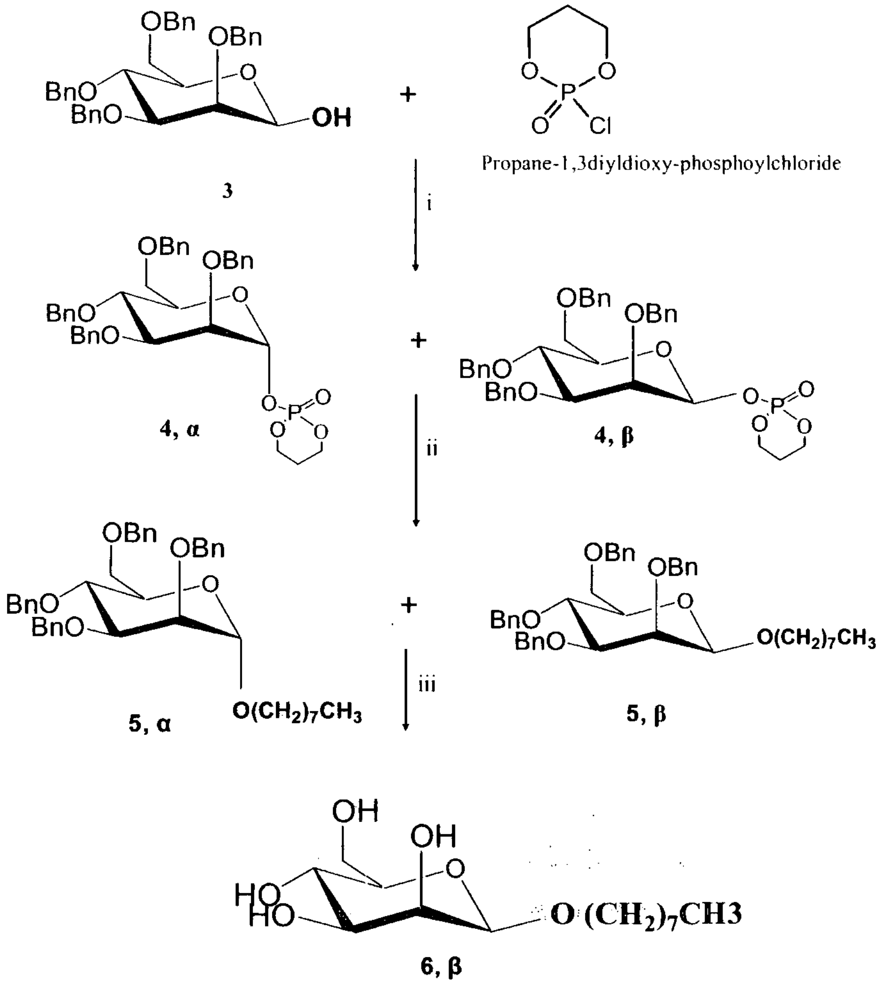



Propane-1, 3-diol    Phosphorus oxychloride    Propane-1,3diyldioxy-phosphoryl chloride

**Scheme 2:** Reagents and conditions (i) 0°C, 20 min then RT, 30 min

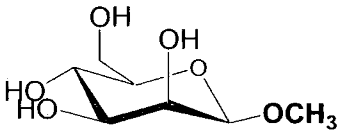
## Abbreviations

**MeOH**: Methanol, **DMF**: Dimethylformamide, **NaH**: Sodium hydride, **TBAI**: Tetrabutylammonium iodide, **RT**: Room temperature, **$CH_3CN$**: Acetonitril, **$CF_3COOH$**: Tri-fluoro-acetic acid. **$H_2O$**: Water, **DCM:** Dichloromethane, **$(Et)_3N$:** Triethylamine.



**Scheme 3:** Reagents amd conditions
(i) DCM, N-methylimidazole, 0 °C, 2hr, then RT, 16hr.
(ii) DCM, $CH_3(CH_2)_7OH$, TMSOTf (Cat.),-78°C, 1 hr.
(iii)Absoute ethanol, Pd $(OH)_2$, cyclohexene, 24hr.

**N.M.R analysis:**



## Reaction steps:

**Preparation of O-methyl-D-Mannose:** Portion of acetyl chloride (1.45g, 18.5mmol) were slowly added to (100 ml) of dry methanol and stirred at room temperature for 1 hour, then D-mannose ( 10 g, 55.5 mmol) was added and the reaction mixture was stirred at 60 C° under reflux for 5 hr. Upon cooling to room temperature, the mixture was stored at 0 C° for overnight. The obtained solid product was filtered, washed with cold methanol and dried to yield 13.5 g (92%) white crystals product (1). ($R_F$ value = 0.6, Chloroform: Methanol 9:1).

**Preparation of 2, 3, 4, 6-Tetra–benzyl-1-O-methyl-D-mannopyranose:** O-methyl-D-Mannose **(1)** (10g, 51.5 mmol) was dissolved in dry DMF (100 ml) at room temperature and stirred under nitrogen for 1 hr. Sodium hydride (6.1g) and Tetrabutylammonium iodide (4.6g) were added to the solution and stirred under nitrogen for another 30min. After that Benzyl bromide (30.6 ml, 257.2 mmol) was added drop wise over 1 hr at room temperature, after completion of the addition the reaction mixture was stirred at room temperature for 8 hr. DMF was removed under reduced pressure and the residue extracted with ethyl acetate (2x100ml). Ethyl acetate layer was washed with water, dried ($Na_2SO_4$), concentrated and purified by silica gel flash chromatography using (Petroleum ether: Diethyl ether (2:1) solvent system to yield 13g ( 78%) yellow oily product **(2).** ($R_F$ value = 0.38, Petroleum ether: Diethyl ether 2:1).

**Preparation of 2, 3, 4, 6-Tetra–O-benzyl- α, β-D-mannopyranose:** 2, 3, 4, 6-Tetra–benzyl-1-O-methyl-D-mannopyranose **(2)** (5g) was dissolved in a mixture of (Tri-fluoro acetic acid: Acetonitril: water 3:4:3) refluxed with continuous stirring at 95 C° for 2 days. Solvents were removed under reduced pressure and residue was neutralized with sodium bicarbonate, extracted with ethyl acetate. Ethyl acetate layer was washed with water, dried on ($Na_2SO_4$), concentrated and purified by silica gel chromatography using (Petroleum ether: Diethyl ether (8:2) to yield 2.2g ( 74%) pale yellow product **(3)** ( $R_f$ value = 0.2 , Petroleum ether: Diethyl ether 8:2 ).

**Preparation of 2-Chloro-1, 3, 2-dioxaphosphacyclohexane-2-oxide or (propane-1,3 diyldioxy- phosphoryl chloride):** A solution of propane-1, 3-diol (5g, 65 mmol) and triethylamine (18 ml, 130 mmol) in dichloromethane (30 ml) and a solution of phosphorus oxychloride (10 g, 65mmol) in dichloromethane (35 ml) were added slowly and simultaneously with stirring to dichloromethane (35 ml) at 0 C° the reaction mixture was stirred at the same temperature for 20 min and a further 30 min at room temperature. The solvents were removed in vacuo, the solid was extracted with diethyl ether and filtered and the filtrate was evaporated to dryness to yield 10g (80%) colourless viscous liquid propane-1, 3-diyldioxyphosphoryl chloride (Scheme 2).

**Preparation of 2,3,4,6-Tetra-O- benzyl-1-O- 1',3', 2'-dioxaphosphacyclohexane- α, β-D-mannopyranosyl-2-oxide (Phosphonate):** 2,3,4,6-Tetra–O-benzyl-α,β-D-mannopyranose **(3)**(5g, 9.3mmol) was dissolved in dry dichloromethane (50 ml) at 0 C° under inert atmosphere, propane–1,3-diyldioxy-phosphoryl chloride (0.29gm,1.84 mmol) was added with continues stirring followed by 1-methylimidazole (0.15gm,1.84 mmol). Stirring was continued for 16 hour at room temperature. The solvent was then removed and the oily residue re-dissolved in dichloromethane and evaporated in order to remove traces of 1-methylimidazole. The resulting residue was dissolved in dichloromethane (50 ml) and washed with aqueous $NaHCO_3$ and water. The organic layer was dried ($Na_2SO_4$) and the solvent was removed under reduced pressure. The residue was purified by flash chromatography (Diethyl ether: Petroleum ether 5:1) to yield 623mg (68%), inseparable pale yellow oil (α, β isomers) **product 4 (α, β).**

**Preparation of 2, 3, 4, 6-Tetra-O- benzyl- α, β-octyl-D-mannopyranoside:** To a stirred solution of phosphonate (0.24gm, 0.36 mmol) in dichloromethane (5ml) at -78 C° under an inert atmosphere was added TMSOTf (Trimethyl silyl triflate) (cat 0.2 equiv) and after 2 min, a solution of octanol (0.047gm, 0.36 mmol) in dichloromethane (3ml) was added. The reaction mixture was stirred at -78 C° for 30 min and then allowed to warm up to 0 C° before being quenched with saturated aqueous NaHCO3 (10 ml) and extracted with dichloromethane (10 ml). The organic phase was dried ($Na_2SO_4$) concentrated in vacuo and the residue was purified by silica gel chromatography (Petroleum ether: Diethyl ether 1:1) to yield 400 mg (73%) **product 5 (α, β).**

**Separation of 2, 3, 4, 6-Tetra-O- benzyl- α-Octyl-D-mannopyranoside and 2, 3, 4, 6-Tetra-O- benzyl- β-Octyl-D-mannopyranoside:** Separation of the two isomers was carried out using a short column silica gel chromatography in ( petroleum ether: diethyl ether 1:1) solvent system and TLC monitoring of separation to yield Octyl-β-D-mannopyranoside (300mg) and Octyl-α- D-mannopyranoside (90 mg).

**Debenzylation**: Separated pure fraction of 2,3,4,6-Tetra-O-benzyl-β-Octyl-D-mannopyranoside was dissolved in absolute ethanol (10 ml) and refluxed with continuous stirring, palladium hydroxide on carbon (200 mg) was added, followed by the addition of cyclohexene (0.15 ml), reflux was continued up to 48 hour, the reaction mixture was then filtered through celite and evaporated under vacuo to yield the debenzylated product Octyl-β-D-mannopyranoside (230mg) (**product 6 β**).

## 4. Results:

### 4.1 Octyl-β-D-mannopyranoside Identification:

Identification of the final product was carried out using spectroscopic data: IR, $^1$HNMR and $^{13}$C NMR.

**IR Data:** 3413.40 cm$^{-1}$ due to OH, 3293.84 cm$^{-1}$ due to C-H bond of (CH), 2925.50 cm$^{-1}$ due to C-H bond of ($CH_2$), 2856.07 cm$^{-1}$ due to C-H bond of ($CH_3$), 1724.06 cm$^{-1}$ due to C-O-C bond, 1378.86, 1465.64 cm$^{-1}$ due to C-O bond, 1054.87, 1025.95 cm$^{-1}$ due to C-C bond.

**$^1$H NMR:** (400 MHz, $D_2O$): δ 3.32 (3H, s, $CH_3$), 3.52 (1 H, m, H5), 3.55 (1 H, m, H4) , 3.65 (1 H, m, H6b), 3.68 (1 H, m, H3), 3.81 (1 H, dd, J = 11.6, 1.6, H6a), 3.84 (1 H, dd, J = 3.2, 1.6, H2), 4.67 (1 H, d, J = 1.6, H2).

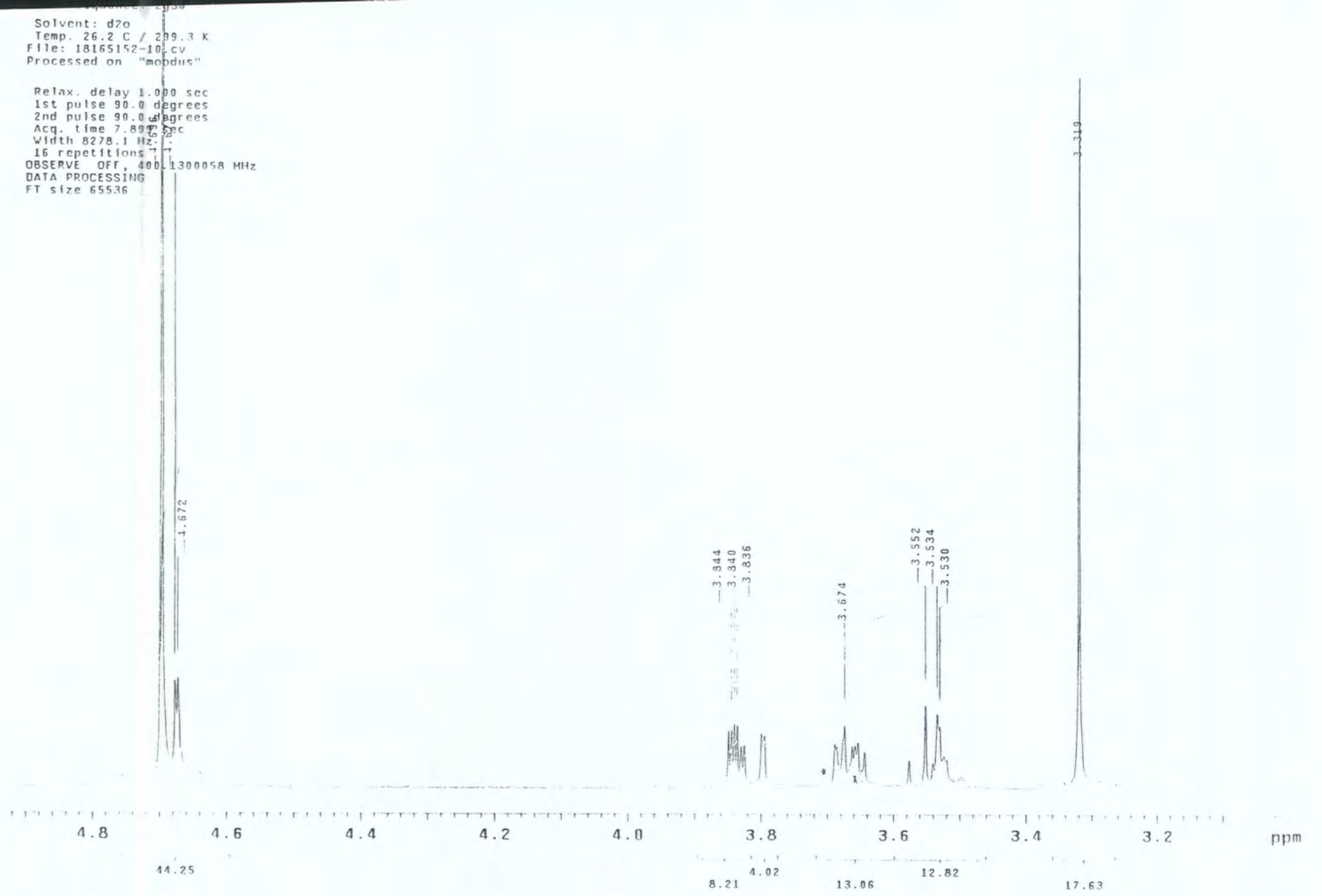
**$^{13}$C NMR:** (100 MHz, $CDCl_3$): δ 54.7 ($OCH_3$), 61.0 (C6), 66.8 (C4), 70.0 (C2), 70.6 (C3), 72.6 (C5), 100.9 (C1).

**Mass spectroscopy:** m/z ($ES^+$) 217 ($MNa^+$), 411 ($M_2Na^+$).

$^1$HNMR and $^{13}$C NMR analysis showed that the product we obtain in the last step of the reaction is the ($OCH_3$) substituted mannopyranoside and not Octyl-β-D-mannopyranoside the compound that we are looking forward to test the biological activity on $GABA_A$ receptor. Ethanol was used in the last step as a solvent; exchange of Octyl group with ethanol happen in the reaction mixture to give us the ($OCH_3$) substituted mannopyranoside. The compound product was unfortunately not our aim, even though we use the product compound as a part of SAR of Octyl-β-D-glucoside, in chapter 3.

## 5. Conclusion:

The use of another published procedure is highly recommended for future work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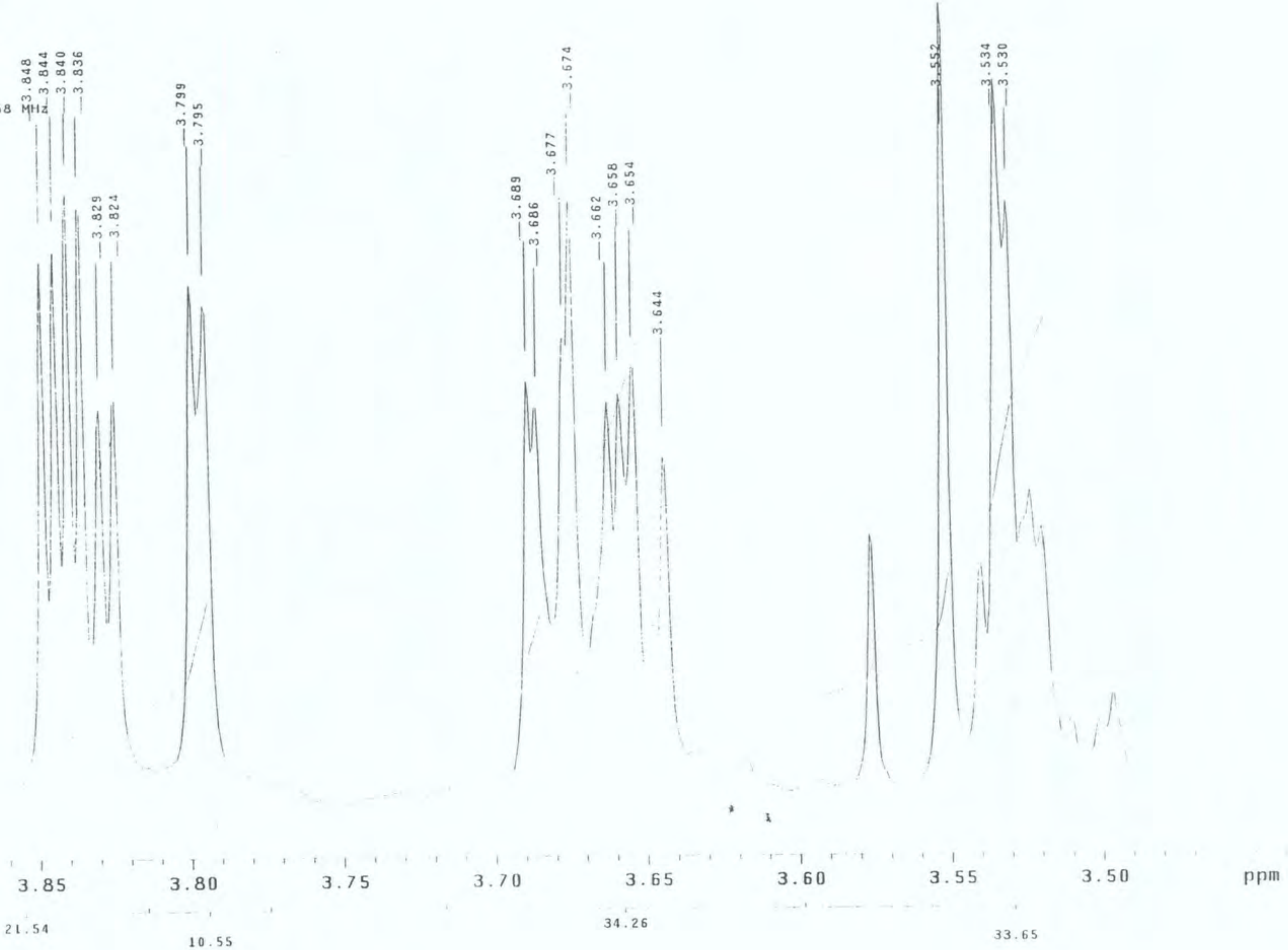

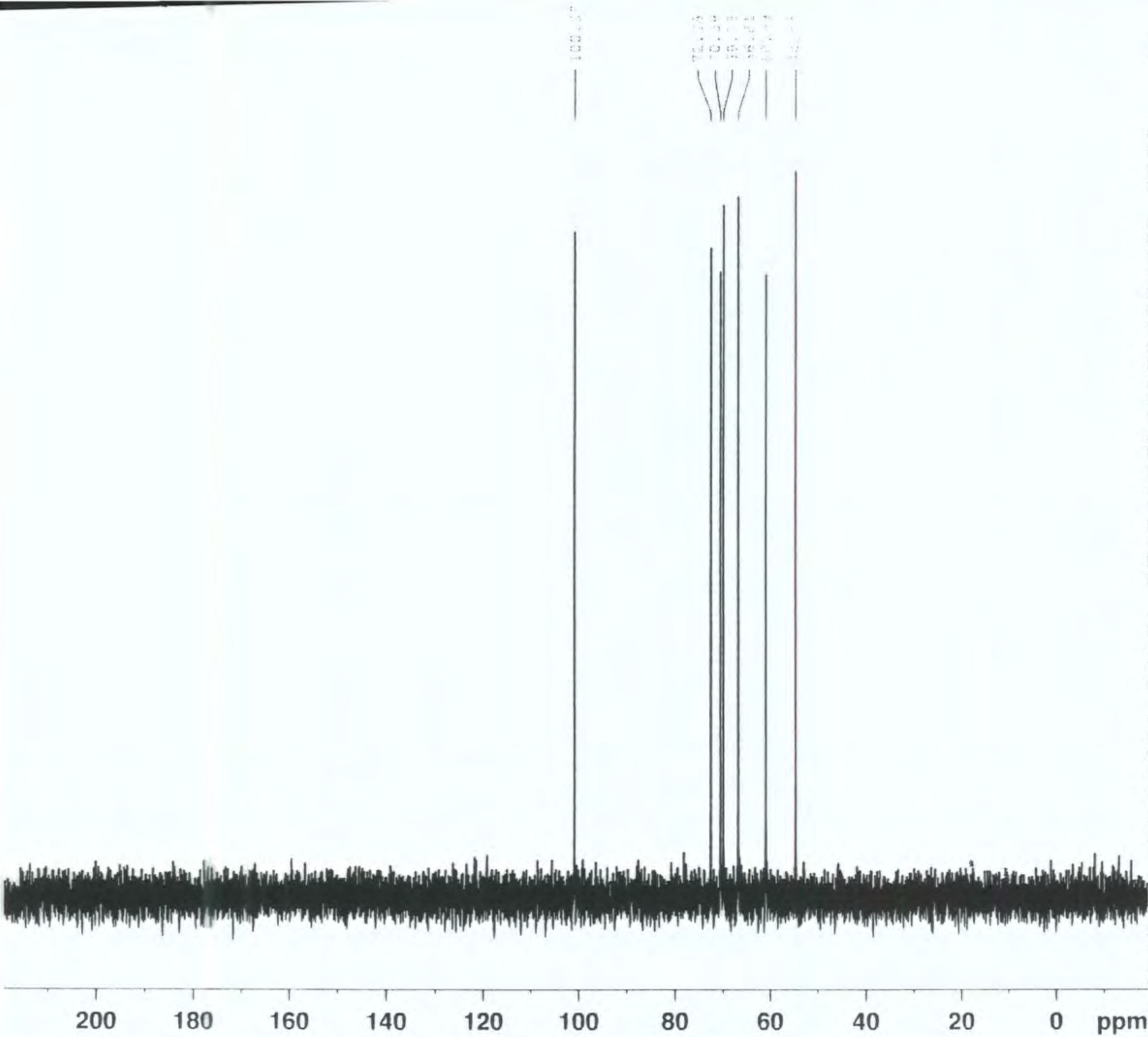


BRUKER

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F2 - Processing parameters

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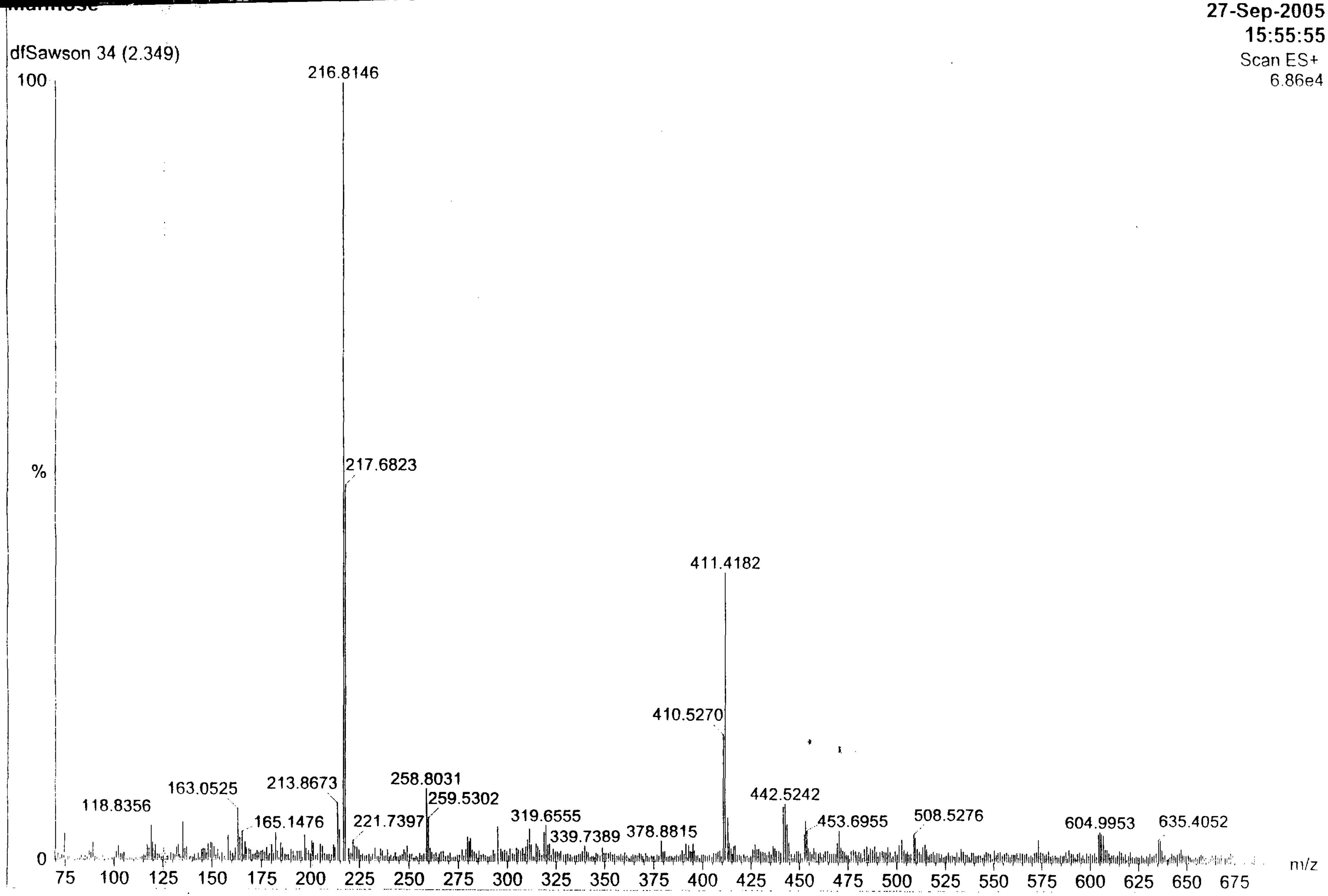
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m/z

# Appendix II

**Gas Chromatography - Mass spectroscopy (GC/MS) Profiles of the major constituents of *Melissa officinalis* L. and *lavandula angustifolia* Mill essential oils from four suppliers (Baldwin's, Pranarom, Quintessence and Fytosan) carried out at Royal Botanic Garden Kew**

ROYAL BOTANIC GARDENS KEW

# Melissa (*Melissa officinalis*) Essential Oils

## GC-MS Analysis [1]

### Sample preparation

1/100 dilution in diethyl ether.

### Method

GC: Perkin-Elmer AutoSystem XL
Column: 30 m x 0.25 mm i.d. x 0.25 µm DB-5MS (J. & W. Scientific)
Temp prog.: 40-300°C @ 3°C/min
Carrier gas: Helium (flow: 1ml/min)
Injection temp.: 220 °C
MS (quadrupole): Perkin-Elmer TurboMass (quadrupole)
Source: EI (70 eV)
Source temp.: 180 °C
Scan range: 38-600 *m/z*
Scan time: 0.50 s
Inter-scan delay: 0.20 s

### Analysis results summary

The principal monoterpenes detected in all oils (BI 14460, BI 14462, BI 14463, BI 14464) were geranial and neral (geranial + neral = citral). The percentage composition of citral in BI 14460, BI 14462, BI 14463 and BI 14464 was 54.9%, 27.3%, 38.7% and 49.7%, respectively. The principal sesquiterpene detected in all oils (BI 14460, BI 14462, BI 14463, BI 14464) was (*E*)-caryophyllene, detected at 12.3%, 24.7%, 12.2% and 9.5%, respectively. These compounds are reported to be some of the major components of *Melissa officinalis* essential oil[1].

### Results

| Compound | Retention time (min) | Baldwins (BI 14460)<br>Percentage composition (TIC) and (RI)* | Pranarom (BI 14462)<br>Percentage composition (TIC) and (RI)* | Quintessence (BI 14463)<br>Percentage composition (TIC) and (RI)* | Fytosan (BI 14464)<br>Percentage composition (TIC) and (RI)* | RI (published) |
|---|---|---|---|---|---|---|
| α-Pinene | 10.3 | Nd | Tr (928) | 0.2 (928) | Nd | 939 |
| Sabinene | 11.9 | Nd | Tr (966) | 0.1 (966) | Nd | 975 |
| β-Pinene | 12.2 | Nd | Nd | 0.9 (971) | Nd | 979 |
| 1-Octen-3-ol | 12.3 | 0.6 (974) | 0.2 (974) | 0.3 (974) | 0.7 (974) | 979 |
| 6-Methyl-5-hepten-2-one | 12.5 | 1.9 (979) | 0.8 (978) | 2.0 (979) | 2.1 (978) | 986 |
| Myrcene | 12.7 | Nd | Tr (983) | 0.2 (983) | Nd | 991 |
| 3-Octanol | 13.1 | 0.1 (991) | Tr (991) | 0.1 (991) | 0.1 (991) | 991 |
| *o*-Cymene | 14.3 | Tr (1019) | Tr (1019) | 0.4 (1019) | Tr (1019) | 1026 |
| Limonene | 14.5 | Tr (1024) | 0.3 (1024) | 5.4 (1024) | Tr (1024) | 1029 |
| (*Z*)-β-Ocimene | 14.8 | Tr (1032) | 0.1 (1032) | 0.1 (1032) | Tr (1031) | 1037 |
| (*E*)-β-Ocimene | 15.3 | 0.5 (1043) | 0.6 (1043) | 0.4 (1043) | 0.2 (1042) | 1050 |
| Bergamal | 15.6 | Tr (1050) | 0.1 (1050) | 0.1 (1050) | 0.1 (1049) | 1057 |
| γ-Terpinene | 15.9 | Nd | Nd | 0.3 (1055) | Nd | 1060 |
| Linalool | 17.8 | 0.8 (1100) | 0.3 (1100) | 0.6 (1100) | 0.8 (1100) | 1097 |
| Nonanal | 18.1 | Nd | 0.1 (1105) | 0.1 (1105) | Nd | 1101 |
| *cis*-Rose oxide | 18.3 | 0.1 (1111) | 0.1 (1111) | 0.1 (1110) | 0.2 (1110) | 1108 |
| *Trans*-Rose oxide | 19.1 | 0.1 (1128) | Tr (1128) | 0.1 (1128) | Tr (1127) | 1126 |

| Compound | Retention time (min) | Baldwins (BI 14460)<br><br>Percentage composition (TIC) and (RI)* | Pranarom (BI 14462)<br><br>Percentage composition (TIC) and (RI)* | Quintessence (BI 14463)<br><br>Percentage composition (TIC) and (RI)* | Fytosan (BI 14464)<br><br>Percentage composition (TIC) and (RI)* | RI (published) |
|---|---|---|---|---|---|---|
| Citronellal | 20.3 | 3.3 (1156) | 8.6 (1157) | 2.9 (1156) | 3.9 (1156) | 1153 |
| n-Nonanol | 21.2 | Nd | Tr (1176) | 0.1 (1176) | Nd | 1169 |
| α-Terpineol | 22.3 | 0.1 (1201) | Tr (1201) | 0.1 (1201) | 0.1 (1200) | 1189 |
| Nerol | 23.7 | 0.9 (1232) | 0.3 (1231) | 1.3 (1232) | 0.8 (1232) | 1230 |
| β-Citronellol | 23.9 | 0.4 (1236) | 1.5 (1235) | 3.0 (1236) | 0.6 (1235) | 1226 |
| Neral | 24.3 | 22.9 (1249) | 10.7 (1247) | 13.3 (1248) | 20.5 (1248) | 1238 |
| Geraniol | 24.8 | 1.8 (1260) | 0.6 (1259) | 2.5 (1259) | 1.5 (1259) | 1253 |
| Methyl citronellate | 25.2 | 0.3 (1266) | 1.2 (1266) | 1.0 (1266) | 0.4 (1266) | 1261 |
| Geranial | 25.7 | 32.0 (1281) | 16.6 (1279) | 25.4 (1280) | 29.2 (1280) | 1267 |
| Neryl formate | 26.0 | Tr (1286) | Tr (1285) | Tr (1285) | Tr (1285) | 1282 |
| Thymol | 26.6 | 0.1 (1300) | Nd | Nd | 0.1 (1299) | 1290 |
| Methyl geranate | 28.0 | 0.7 (1330) | 0.5 (1330) | 0.8 (1330) | 0.9 (1330) | 1325 |
| α-Copaene | 30.3 | 0.5 (1383) | 1.6 (1383) | 0.5 (1382) | 0.5 (1382) | 1377 |
| Geranyl acetate | 30.5 | 3.3 (1387) | 1.2 (1386) | 2.8 (1387) | 3.0 (1386) | 1381 |
| β-Bourbonene | 30.6 | 0.1 (1390) | 0.9 (1390) | 0.2 (1390) | 0.2 (1390) | 1388 |
| β-Elemene | 30.9 | 0.1 (1396) | 0.5 (1396) | 0.1 (1396) | 0.1 (1396) | 1391 |
| (*E*)-Caryophyllene | 32.2 | 12.3 (1426) | 24.7 (1427) | 12.2 (1426) | 9.5 (1425) | 1419 |
| β-Copaene | 32.6 | Tr (1434) | 0.2 (1434) | 0.1 (1434) | Tr (1434) | 1432 |
| α-Humulene | 33.6 | 0.9 (1458) | 2.0 (1458) | 0.9 (1458) | 0.7 (1458) | 1455 |
| Allo-aromadendrene | 33.8 | 0.1 (1462) | 0.2 (1462) | 0.2 (1462) | 0.1 (1461) | 1460 |
| γ-Muurolene | 34.4 | 0.1 (1476) | 0.2 (1476) | 0.1 (1476) | 0.1 (1476) | 1480 |
| Germacrene D | 34.7 | 1.8 (1482) | 7.0 (1483) | 1.6 (1482) | 0.4 (1481) | 1485 |
| (*Z*, *E*)-α-Farnesene[a] | 35.1 | 0.2 (1492) | 0.3 (1492) | 0.3 (1492) | 0.1 (1492) | - |
| α-Muurolene | 35.4 | 0.2 (1498) | 0.4 (1498) | 0.2 (1498) | 0.2 (1498) | 1500 |
| γ-Cadinene | 36.0 | 0.1 (1511) | 0.4 (1511) | 0.2 (1511) | 0.2 (1510) | 1514 |
| δ-Cadinene | 36.2 | 0.7 (1516) | 1.2 (1516) | 0.8 (1515) | 0.5 (1515) | 1523 |
| Germacrene-D-4-ol | 38.5 | 0.2 (1568) | 1.0 (1568) | 0.1 (1567) | 0.1 (1568) | 1576 |
| Caryophyllene oxide | 38.7 | 3.9 (1572) | 4.8 (1572) | 4.2 (1572) | 8.2 (1572) | 1583 |
| Humulene epoxide II | 39.7 | 0.2 (1596) | 0.3 (1596) | 0.2 (11596) | 0.4 (1595) | 1608 |
| τ- Cadinol[a] | 40.9 | Tr | 0.2 (1624) | 0.2 (1624) | Tr | - |
| α-Cadinol | 41.0 | 0.4 (1625) | 0.2 (1625) | 0.2 (1625) | 0.4 (1625) | 1654 |
| τ-Muurolol[a] | 41.4 | 0.7 (1635) | 0.5 (1635) | 0.4 (1635) | 0.8 (1634) | - |
| Oplopanone | 44.2 | 0.1 (1699) | 0.1 (1698) | Nd | 0.1 (1698) | 1740 |

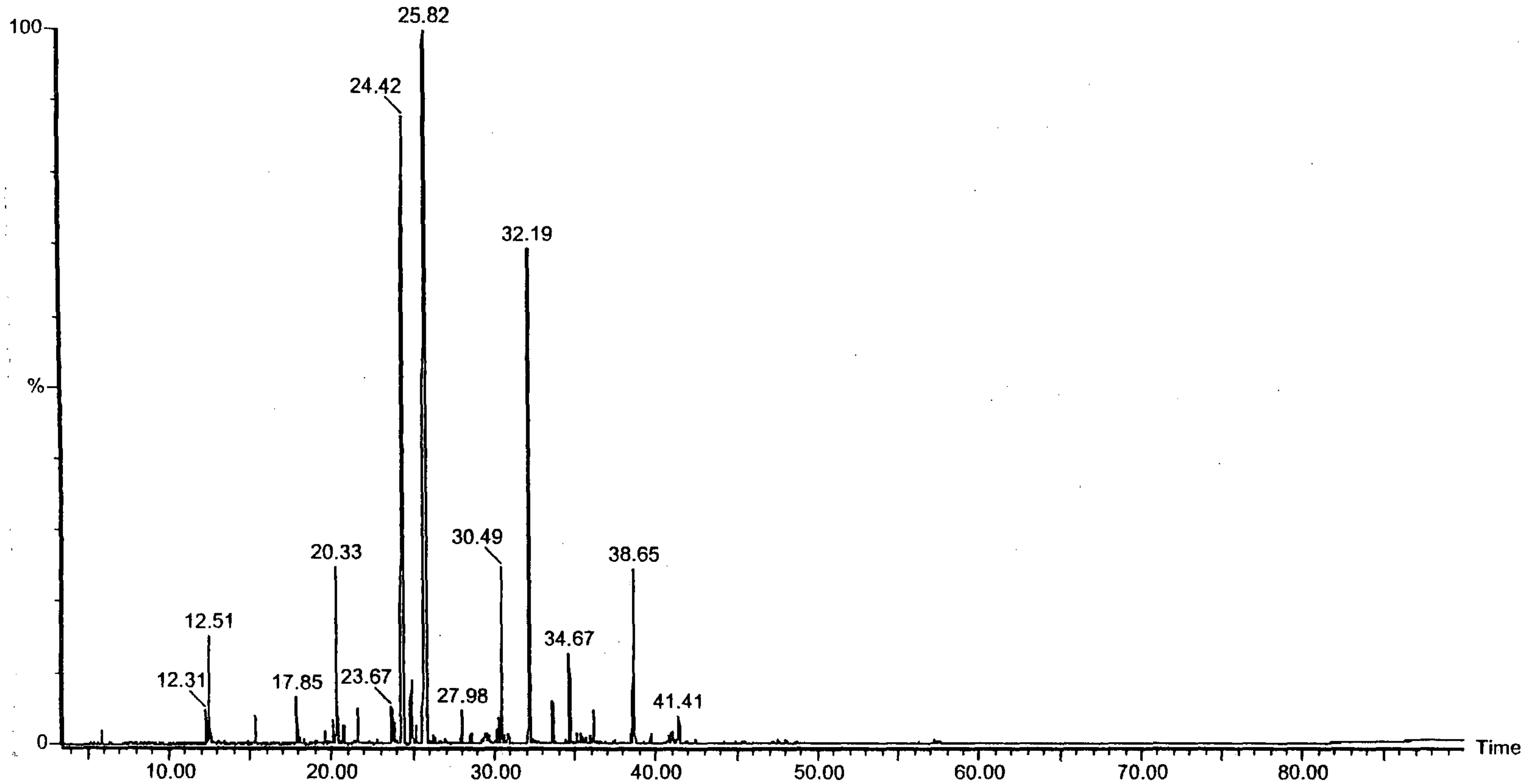
Tr: < 0.1 %; Nd: not detected; * Experimental RI value (in brackets).
All compounds identified by comparing retention indices (calculated against an *n*-alkane series) and by comparing mass spectra with published data[2,3], except: [a]compounds identified by comparing mass spectra with published data[2].

**References**

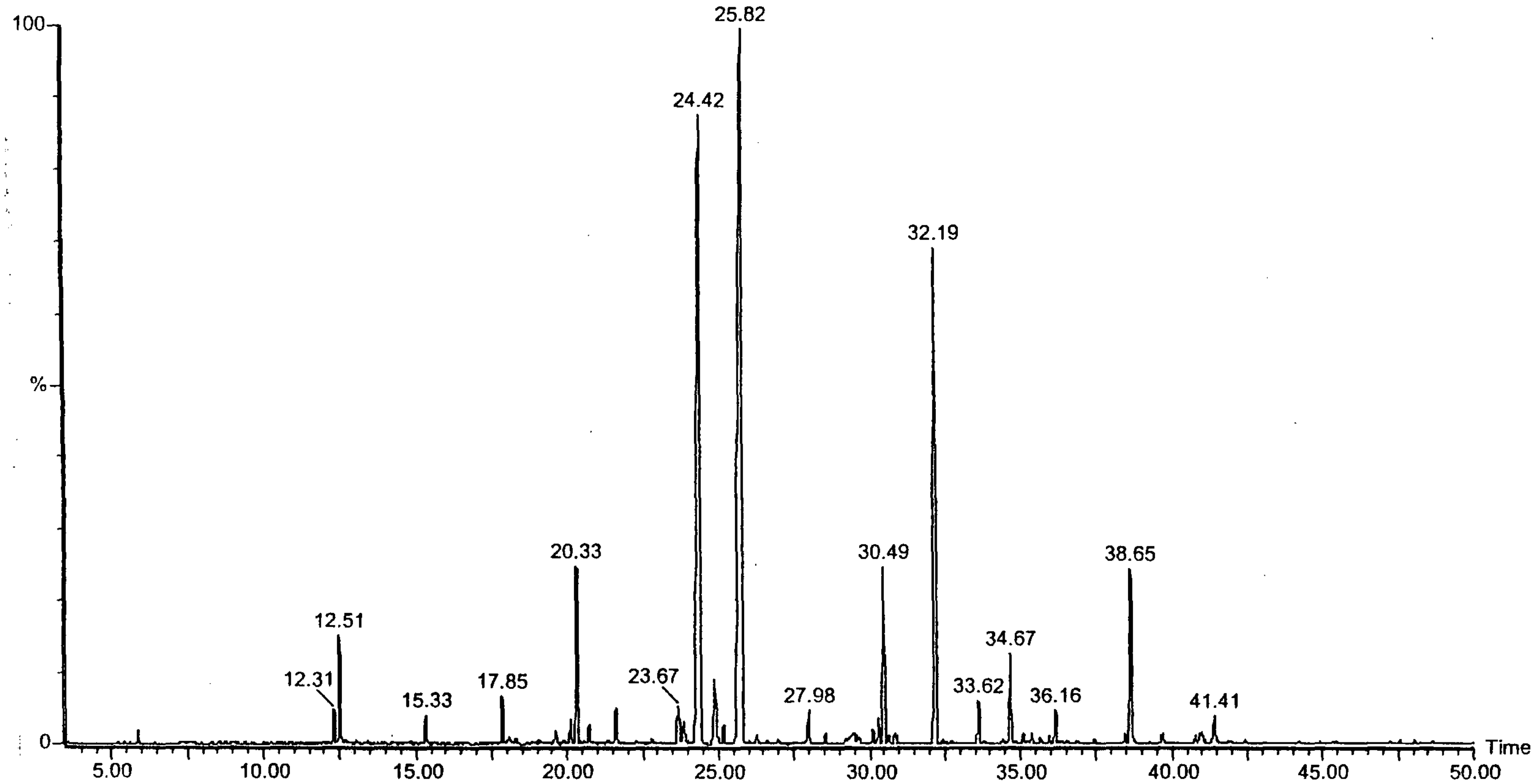

[1] N.G. Bisset (1994) Herbal Drugs and Phytopharmaceuticals. MedPharm GmbH Scientific Publishers, Stuttgart, Germany.

[2] P. Ausloos, C. Clifton, S.G. Lias, A. Shamim, S. Stein, NIST/EPA/NIH Mass Spectral Database (v. 4.0). US Department of Commerce, Gaitherburg, USA

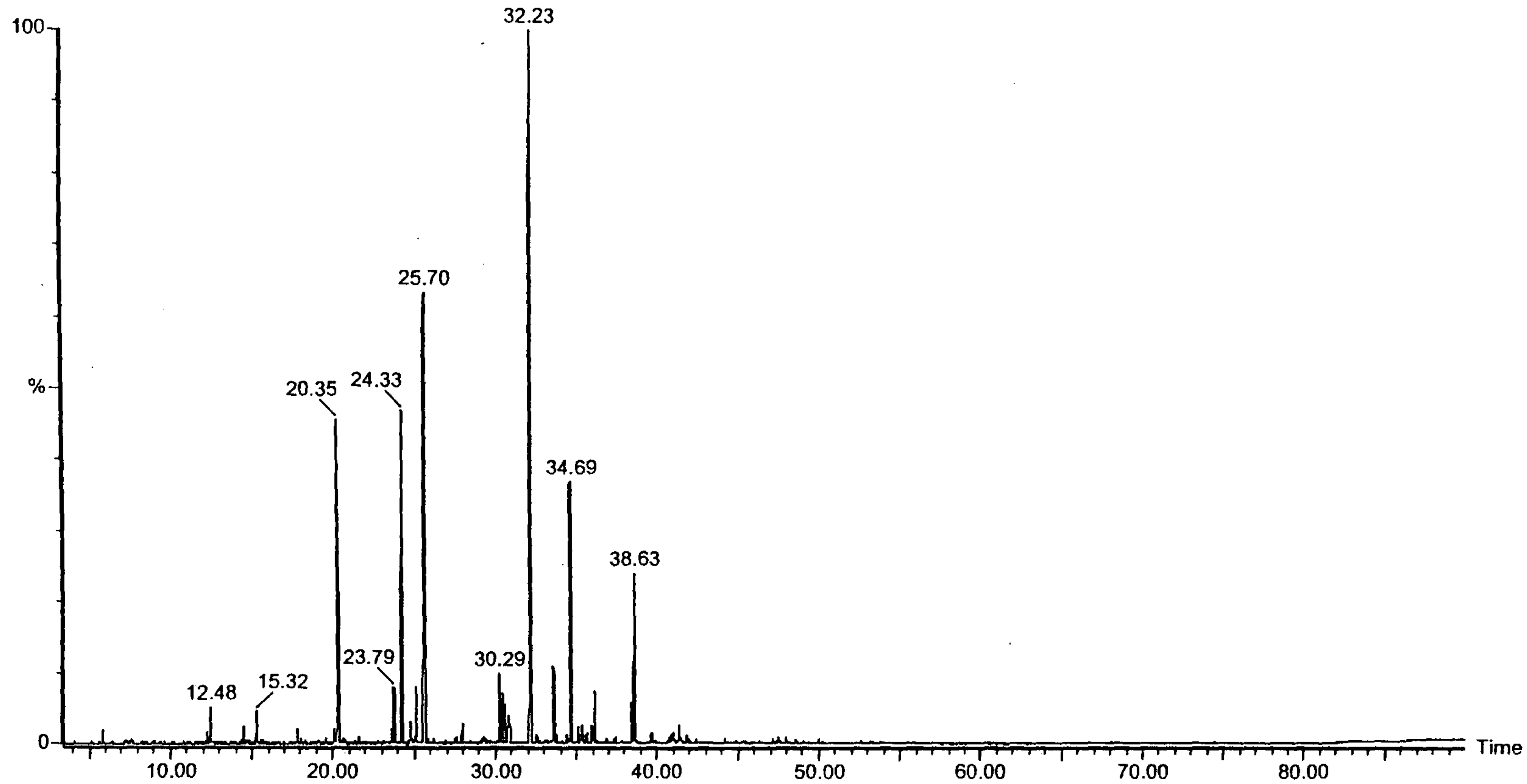
[3] R.P. Adams, Identification of Essential Oil Components by Gas Chromatography / Quadrupole Mass Spectroscopy. Allured Publishing Corporation, Illinois, USA, 2001.



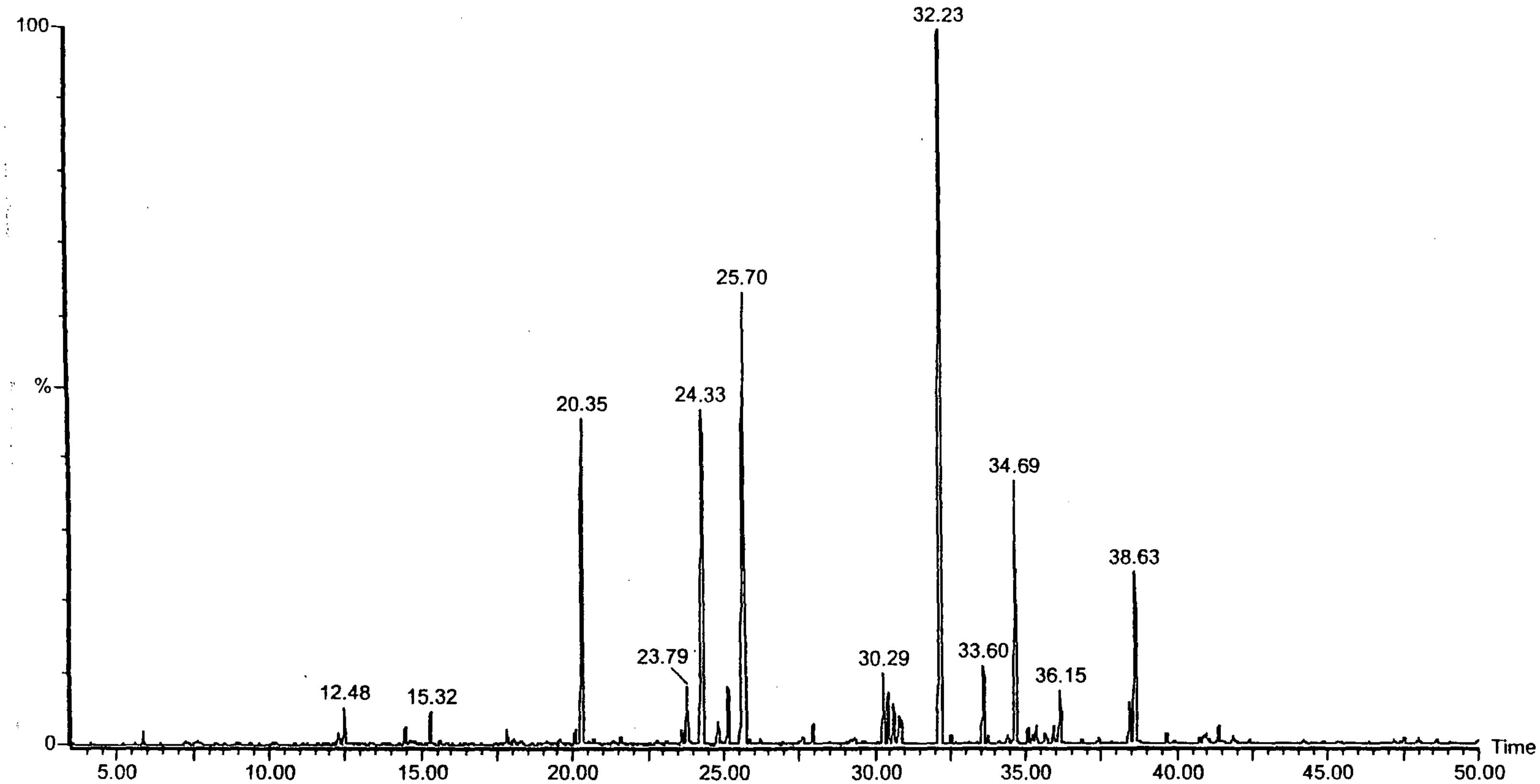

GC-MS total ion chromatogram of *Melissa officinalis* essential oil (Baldwins, BI 14460, diluted 1/100 in diethyl ether) using a 30 m x 0.25 mm i.d. x 0.25 µm DB-5MS column.



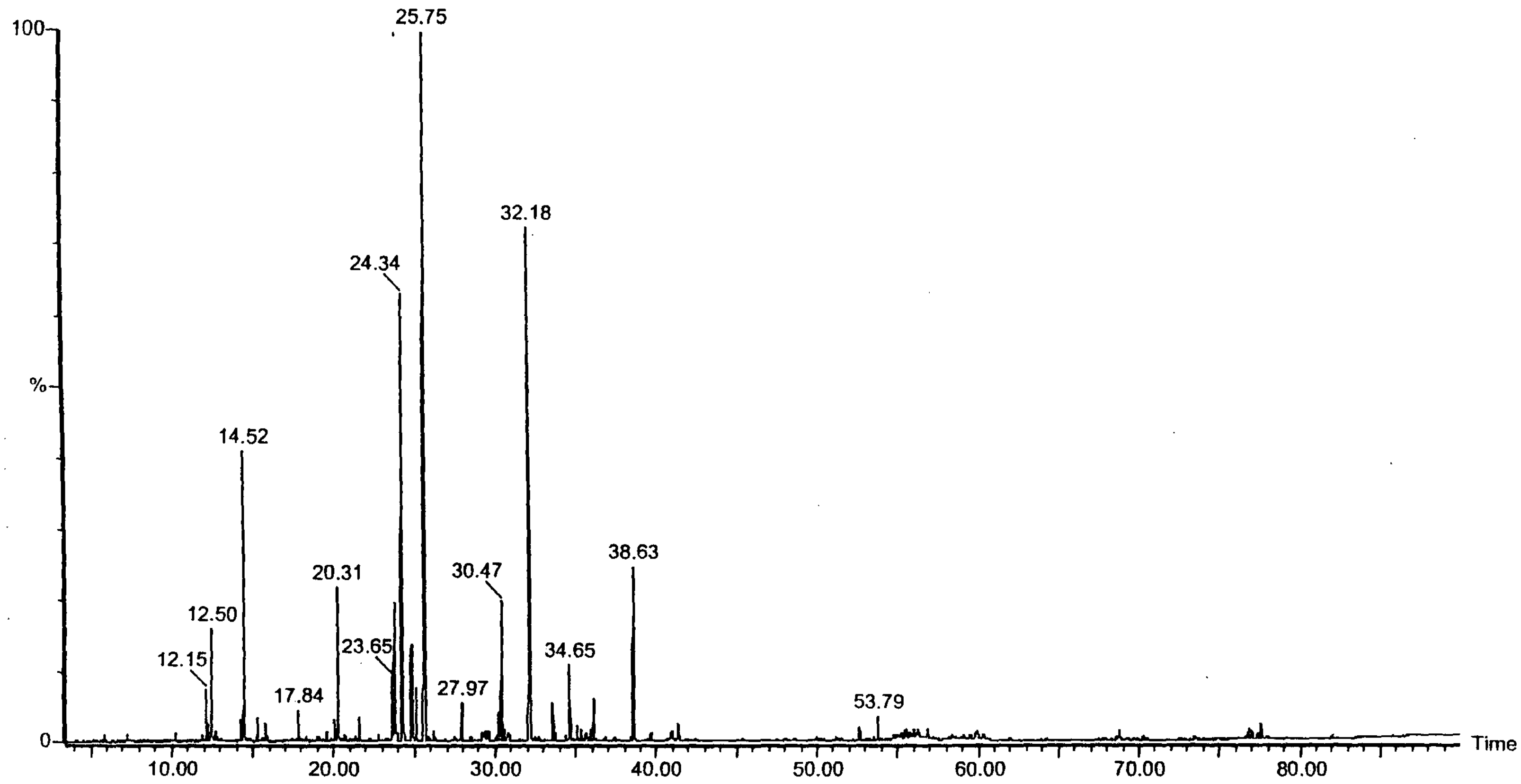

GC-MS total ion chromatogram (0 – 50 min) of *Melissa officinalis* essential oil (Baldwins, BI 14460, diluted 1/100 in diethyl ether) using a 30 m x 0.25 mm i.d. x 0.25 µm DB-5MS column.



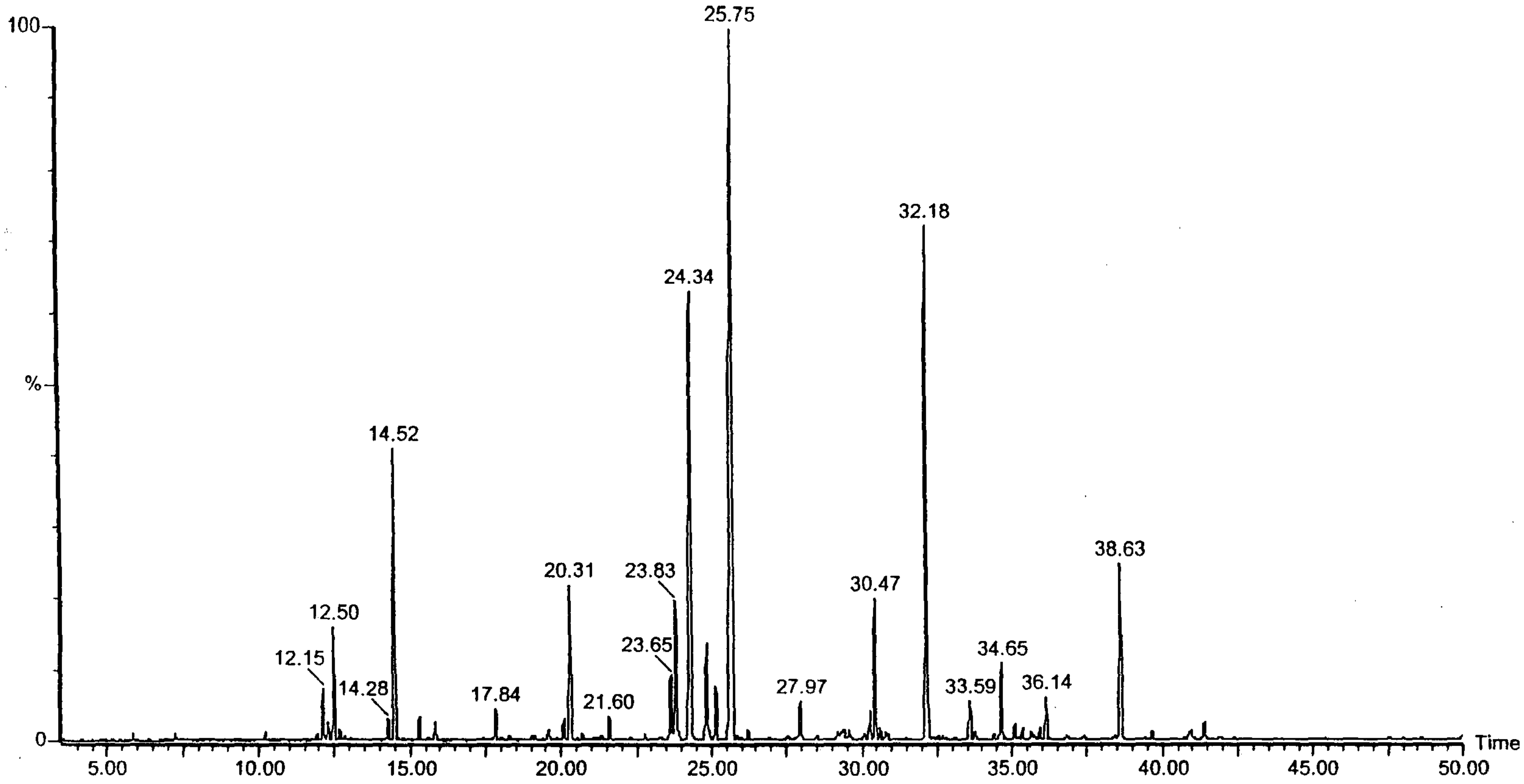

GC-MS total ion chromatogram of *Melissa officinalis* essential oil (Pranarom, BI 14462, diluted 1/100 in diethyl ether) using a 30 m x 0.25 mm i.d. x 0.25 µm DB-5MS column.




GC-MS total ion chromatogram (0 – 50 min) of *Melissa officinalis* essential oil (Pranarom, BI 14462, diluted 1/100 in diethyl ether) using a 30 m x 0.25 mm i.d. x 0.25 µm DB-5MS column.




GC-MS total ion chromatogram of *Melissa officinalis* essential oil (Quintessence, BI 14463, diluted 1/100 in diethyl ether) using a 30 m x 0.25 mm i.d. x 0.25 µm DB-5MS column.

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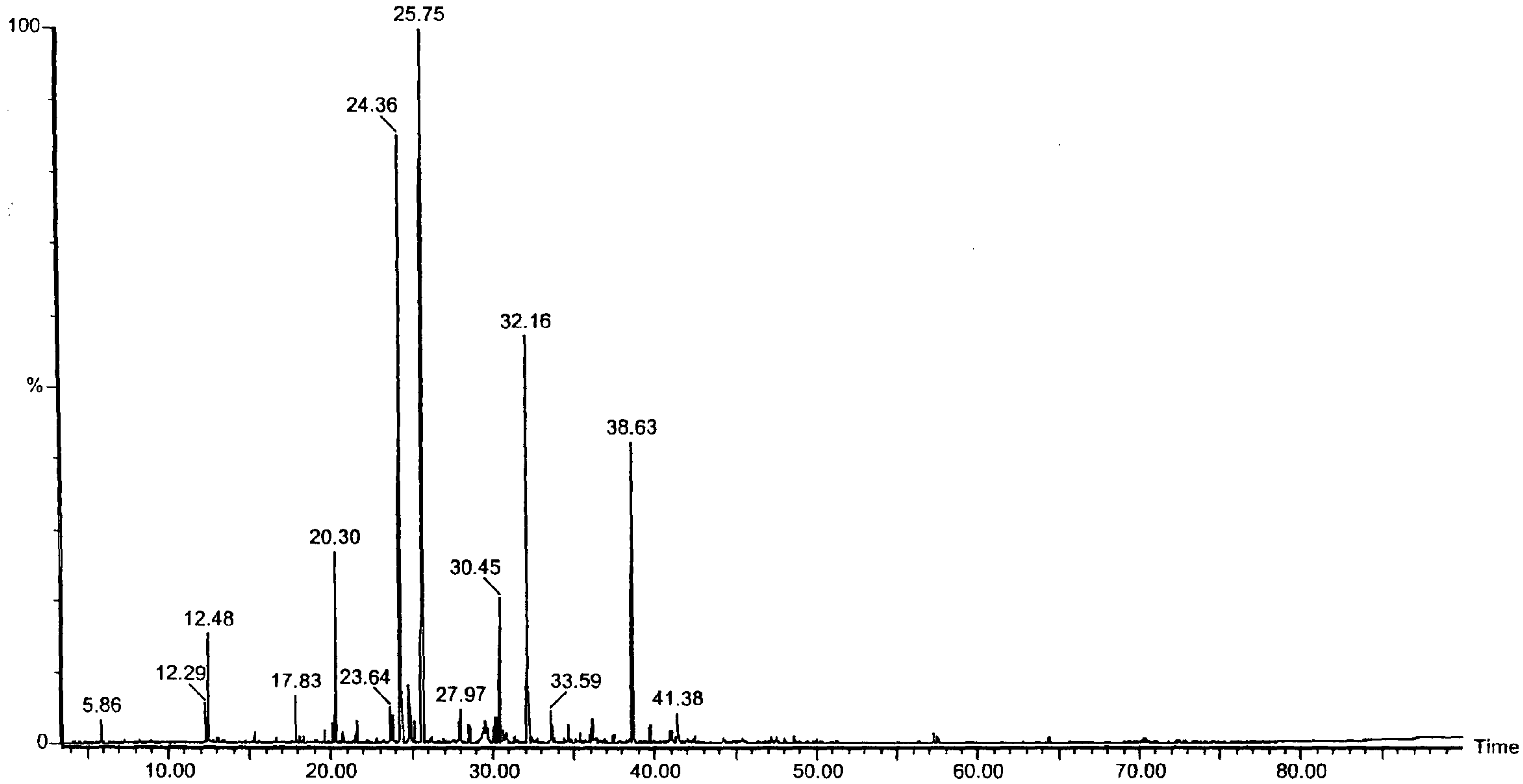
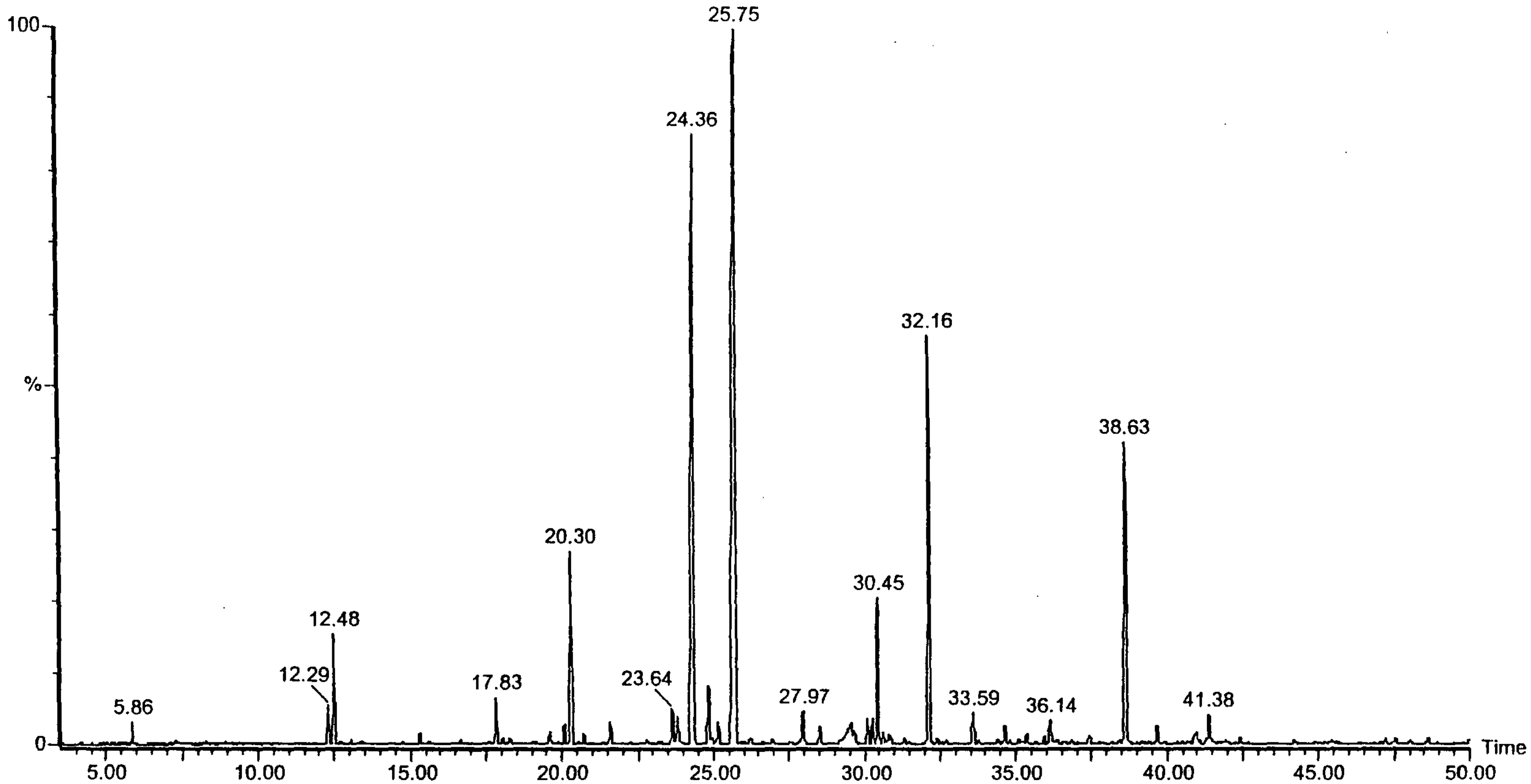



GC-MS total ion chromatogram of *Melissa officinalis* essential oil (Fytosan, BI 14464, diluted 1/100 in diethyl ether) using a 30 m x 0.25 mm i.d. x 0.25 µm DB-5MS column.




GC-MS total ion chromatogram (0 – 50 min) of *Melissa officinalis* essential oil (Fytosan, BI 14464, diluted 1/100 in diethyl ether) using a 30 m x 0.25 mm i.d. x 0.25 µm DB-5MS column.

ROYAL BOTANIC GARDENS KEW

# Lavender (*Lavandula angustifolia*) Essential Oils

## GC-MS Analysis [1]

### Sample preparation

1/100 dilution in diethyl ether.

### Method

| | |
|---|---|
| GC: | Perkin-Elmer AutoSystem XL |
| Column: | 30 m x 0.25 mm i.d. x 0.25 µm DB-5MS (J. & W. Scientific) |
| Temp prog.: | 40-300°C @ 3°C/min |
| Carrier gas: | Helium (flow: 1ml/min) |
| Injection temp.: | 220 °C |
| MS (quadrupole): | Perkin-Elmer TurboMass (quadrupole) |
| Source: | EI (70 eV) |
| Source temp.: | 180 °C |
| Scan range: | 38-600 *m/z* |
| Scan time: | 0.50 s |
| Inter-scan delay: | 0.20 s |

### Analysis results summary

The principal monoterpenes detected in all oils (BI 14450, BI 14451, BI 14458, BI 14459) were linalyl acetate (36.7%, 41.6%, 39.7% and 39.4%, respectively) and linalool (30.8%, 27.3%, 30.1% and 33.3%, respectively). The percentage composition of linalyl acetate, linalool and other components detected comply with the percentage composition of *Lavandula angustifolia* oil described in the British Pharmacopoeia (2002)[1].

### Results

| Compound | Retention time (min) | Baldwins (BI 14450) Percentage composition (TIC) and (RI)* | Pranarom (BI 14451) Percentage composition (TIC) and (RI)* | Quintessence (BI 14458) Percentage composition (TIC) and (RI)* | Fytosan (BI 14459) Percentage composition (TIC) and (RI)* | RI (published) |
|---|---|---|---|---|---|---|
| α-Thujene | 10.0 | Tr (921) | 0.1 (921) | Tr (921) | Tr (921) | 930 |
| α-Pinene | 10.3 | 0.1 (928) | 0.1 (928) | 0.1 (928) | 0.1 (928) | 939 |
| Camphene | 11.0 | 0.1 (944) | 0.1 (944) | 0.2 (944) | 0.2 (944) | 954 |
| Sabinene | 12.0 | Tr (967) | Tr (967) | Tr (966) | Tr (966) | 975 |
| β-Pinene | 12.2 | 0.1 (971) | 0.1 (971) | 0.1 (971) | 0.1 (971) | 979 |
| Oct-1-en-3-ol | 12.3 | 0.2 (975) | 0.2 (975) | 0.2 (974) | 0.3 (974) | 979 |
| 3-Octanone | 12.6 | 0.3 (980) | 0.5 (980) | 0.6 (980) | 0.5 (980) | 984 |
| Myrcene | 12.7 | 0.5 (984) | 0.3 (984) | 0.4 (984) | 0.5 (984) | 991 |
| Hexyl acetate | 13.8 | 0.3 (1008) | 0.5 (1008) | 0.4 (1008) | 0.3 (1008) | 1009 |
| *o*-Cymene | 14.3 | 0.2 (1020) | 0.1 (1020) | 0.2 (1020) | 0.2 (1020) | 1026 |
| Limonene | 14.5 | 0.3 (1025) | 0.1 (1025) | 0.2 (1024) | 0.3 (1024) | 1029 |
| 1, 8-Cineole | 14.7 | 0.6 (1028) | 0.5 (1028) | 0.8 (1028) | 1.0 (1028) | 1031 |
| (*Z*)-β-Ocimene | 14.9 | 3.3 (1033) | 4.2 (1033) | 2.6 (1033) | 2.1 (1032) | 1037 |
| (*E*)-β-Ocimene | 15.4 | 1.3 (1044) | 2.2 (1044) | 1.1 (1043) | 0.9 (1043) | 1050 |
| *trans*-Linalool oxide (furanoid) | 16.5 | 0.3 (1069) | 0.2 (1069) | 0.3 (1069) | 0.2 (1069) | 1073 |
| *cis*-Linalool oxide (furanoid) | 17.2 | 0.2 (1086) | 0.1 (1086) | 0.1 (1086) | 0.1 (1086) | 1087 |
| Linalool | 18.1 | 30.8 (1105) | 27.3 (1104) | 30.1 (1105) | 33.3 (1105) | 1097 |

| Compound | Retention time (min) | Baldwins (BI 14450)<br><br>Percentage composition (TIC) and (RI)* | Pranarom (BI 14451)<br><br>Percentage composition (TIC) and (RI)* | Quintessence (BI 14458)<br><br>Percentage composition (TIC) and (RI)* | Fytosan (BI 14459)<br><br>Percentage composition (TIC) and (RI)* | RI (published) |
|---|---|---|---|---|---|---|
| 1-Octen-3-yl acetate | 18.3 | 1.2 (1110) | 1.3 (1110) | 1.0 (1110) | 0.9 (1109) | 1113 |
| Camphor | 20.1 | 0.4 (1150) | 0.4 (1150) | 0.5 (1150) | 0.5 (1150) | 1146 |
| Lavandulol | 20.9 | 1.0 (1169) | 0.5 (1169) | 0.7 (1169) | 0.5 (1169) | 1181 |
| Borneol | 21.3 | 0.8 (1178) | 0.6 (1177) | 1.0 (1177) | 1.2 (1177) | 1169 |
| Terpinen-4-ol | 21.7 | 3.7 (1187) | 2.9 (1187) | 2.4 (1187) | 1.9 (1186) | 1177 |
| Cryptone | 21.9 | 0.2 (1192) | 0.1 (1192) | 0.3 (1192) | 0.3 (1192) | 1186 |
| α-Terpineol | 22.3 | 1.3 (1202) | 0.2 (1201) | 0.6 (1202) | 1.0 (1201) | 1189 |
| Nerol | 23.7 | 0.2 (1232) | Tr (1232) | 0.1 (1232) | 0.2 (1232) | 1230 |
| Isobornyl formate | 23.8 | 0.1 (1236) | 0.1 (1236) | 0.1 (1236) | 0.1 (1236) | 1239 |
| Linalyl acetate | 25.0 | 36.7 (1262) | 41.6 (1262) | 39.7 (1262) | 39.4 (1262) | 1257 |
| Lavandulyl acetate | 26.4 | 5.5 (1294) | 3.9 (1294) | 5.4 (1294) | 2.9 (1293) | 1290 |
| Hexyl tiglate | 28.4 | Tr (1339) | 0.1 (1339) | Tr (1339) | Tr (1339) | 1333 |
| Neryl acetate | 29.6 | 0.5 (1368) | 0.1 (1367) | 0.3 (1367) | 0.4 (1367) | 1362 |
| Geranyl acetate | 30.5 | 1.0 (1387) | 0.2 (1387) | 0.7 (1387) | 0.8 (1387) | 1387 |
| 7-*epi*-Sesquithujene | 31.5 | Tr (1410) | Tr (1410) | Tr (1410) | Tr (1409) | 1391 |
| *cis*-α-Bergamotene | 31.9 | Tr (1420) | Tr (1420) | Tr (1420) | Tr (1419) | 1413 |
| (*E*)-Caryophyllene | 32.2 | 3.6 (1425) | 4.9 (1426) | 4.4 (1425) | 4.2 (1425) | 1419 |
| *trans*-α-Bergamotene | 32.7 | 0.2 (1438) | 0.2 (1438) | 0.2 (1438) | 0.2 (1438) | 1435 |
| (*E*)-β-Farnesene | 33.6 | 1.7 (1457) | 2.2 (1458) | 1.3 (1457) | 1.6 (1457) | 1457 |
| Germacrene D | 34.7 | 0.2 (1483) | 0.3 (1482) | 0.2 (1482) | 0.3 (1482) | 1485 |
| β-Bisabolene | 35.8 | Tr (1508) | Tr (1507) | Tr (1507) | Tr (1507) | 1506 |
| γ-Cadinene | 36.0 | 0.1 (1512) | 0.1 (1512) | 0.2 (1511) | 0.1 (1511) | 1514 |
| Caryophyllene oxide | 38.7 | 0.6 (1572) | 0.5 (1572) | 0.6 (1572) | 0.7 (1572) | 1583 |

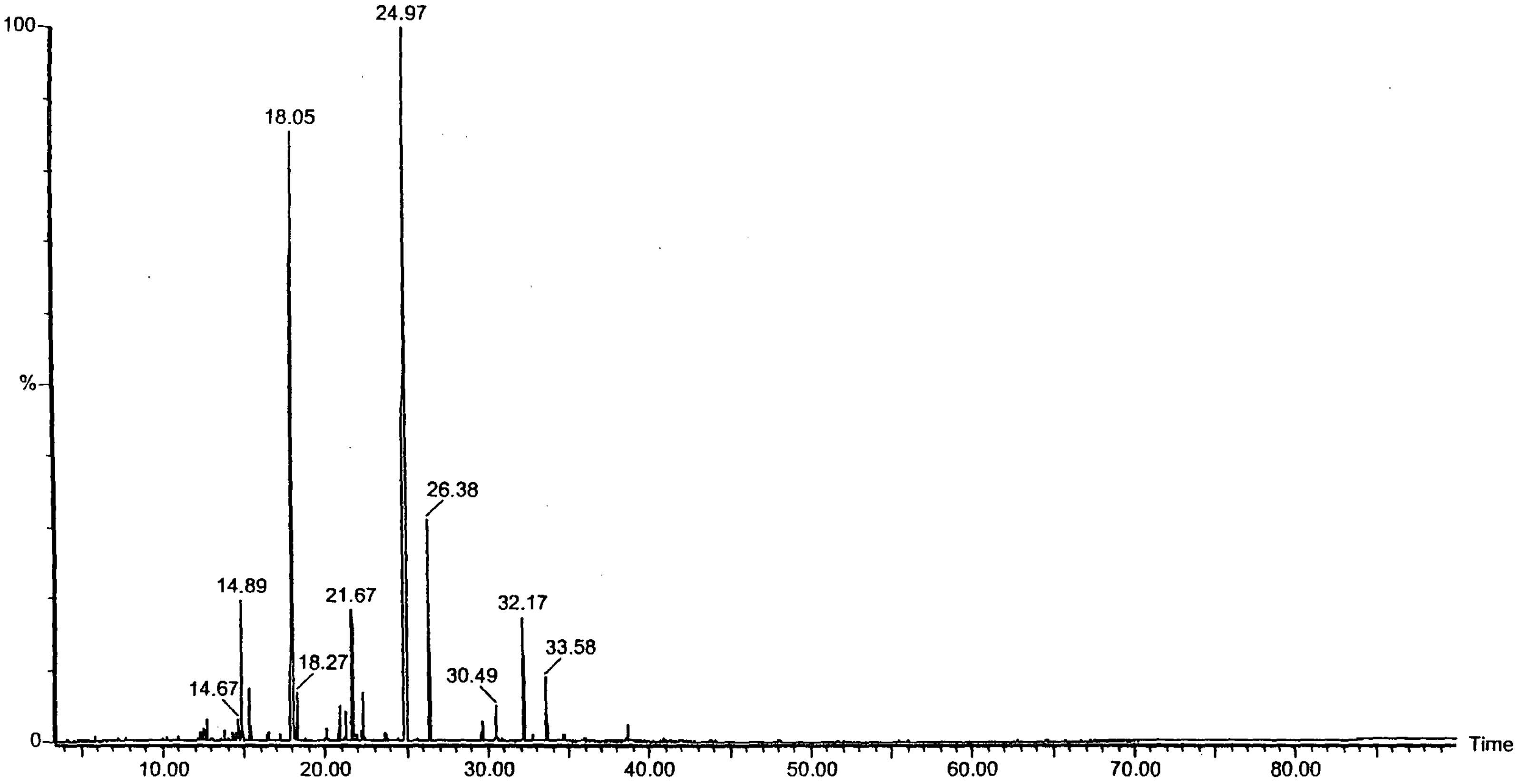
Tr: < 0.1 %; Nd: not detected; * Experimental RI value (in brackets).
All compounds identified by comparing retention indices (calculated against an *n*-alkane series) and by comparing mass spectra with published data[2, 3].

**References**

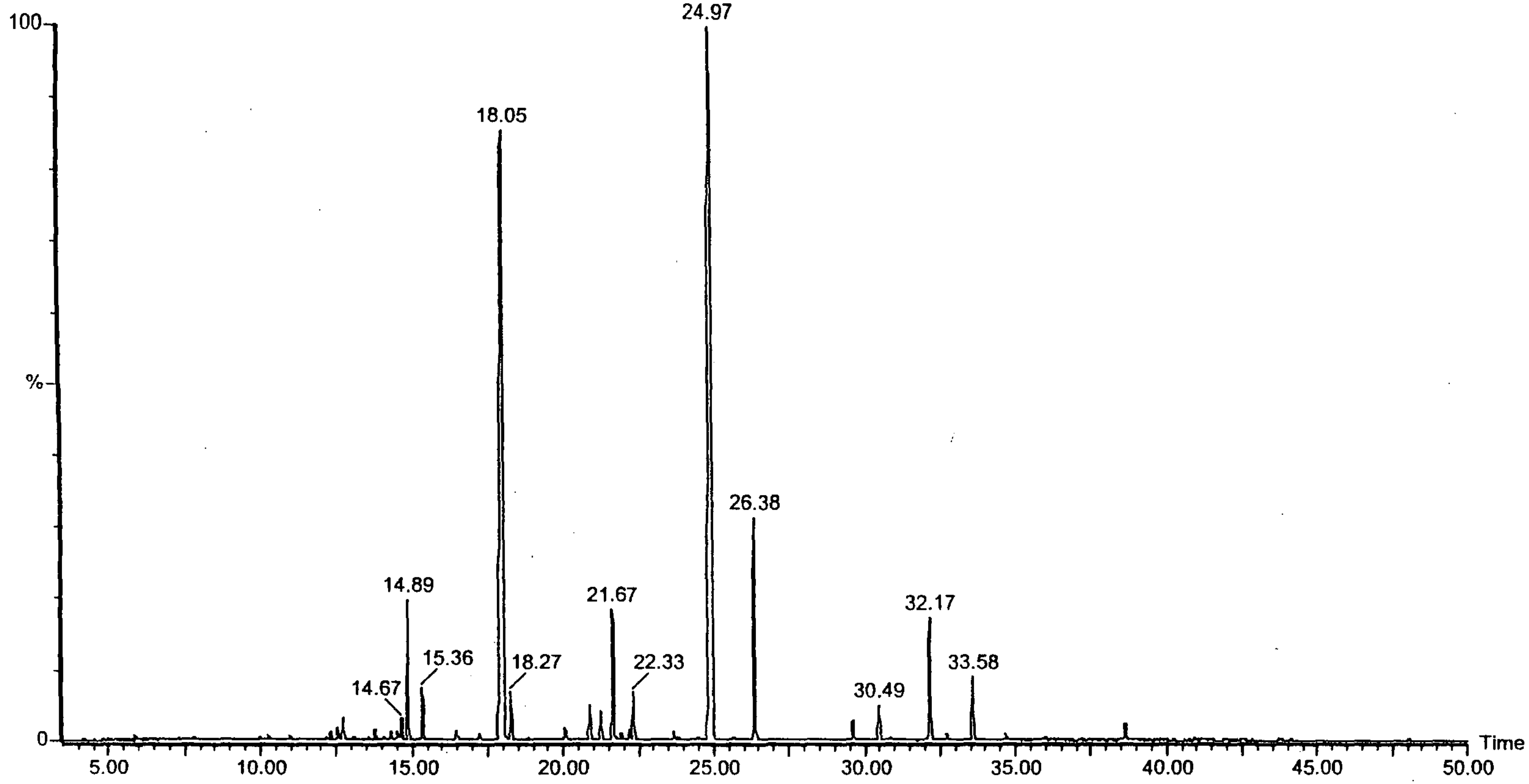

[1] British Pharmacopoeia. Volume I. (2002) The Stationery Office, London.

[2] P. Ausloos, C. Clifton, S.G. Lias, A. Shamim, S. Stein, NIST/EPA/NIH Mass Spectral Database (v. 4.0). US Department of Commerce, Gaitherburg, USA

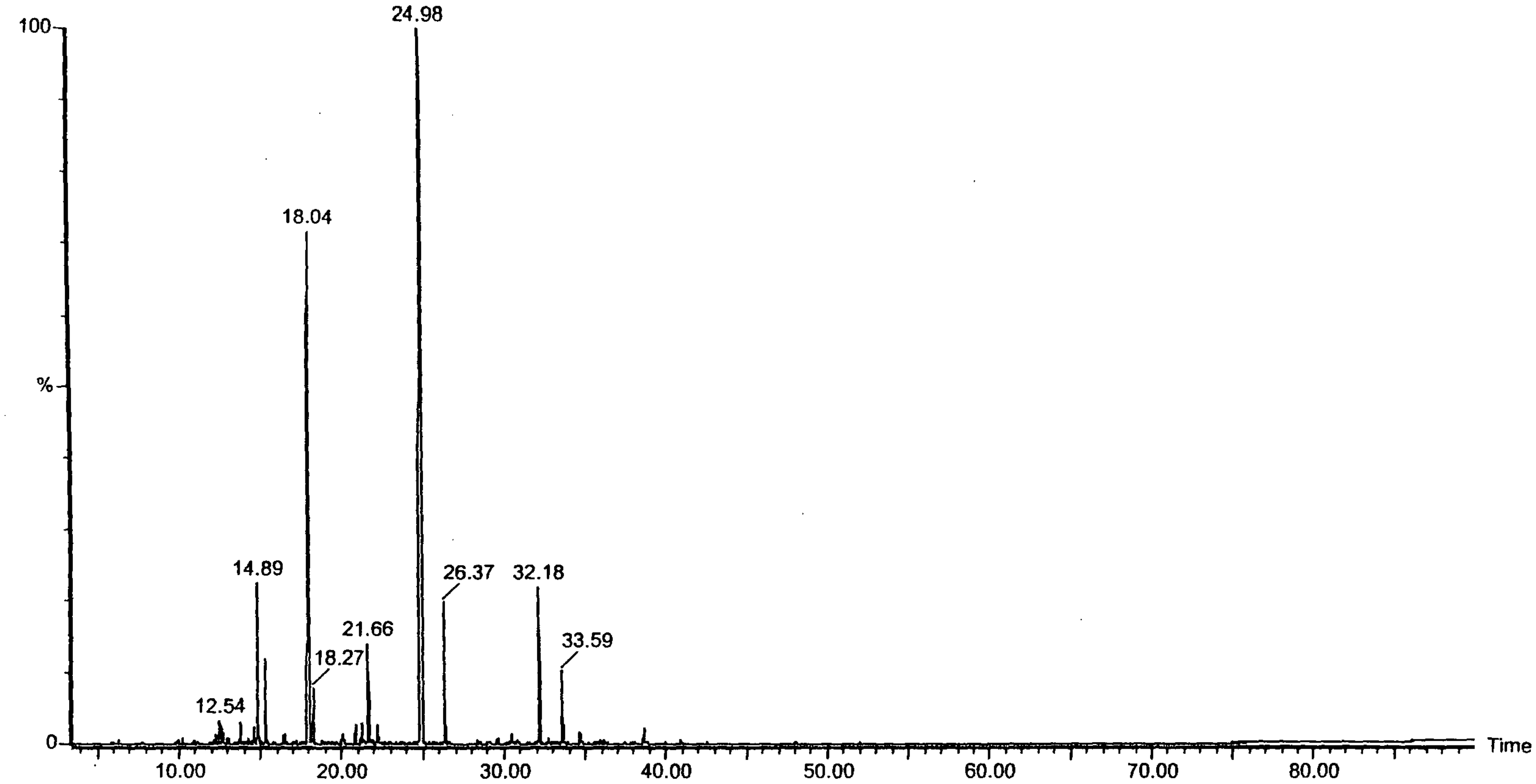
[3] R.P. Adams, Identification of Essential Oil Components by Gas Chromatography / Quadrupole Mass Spectroscopy. Allured Publishing Corporation, Illinois, USA, 2001.



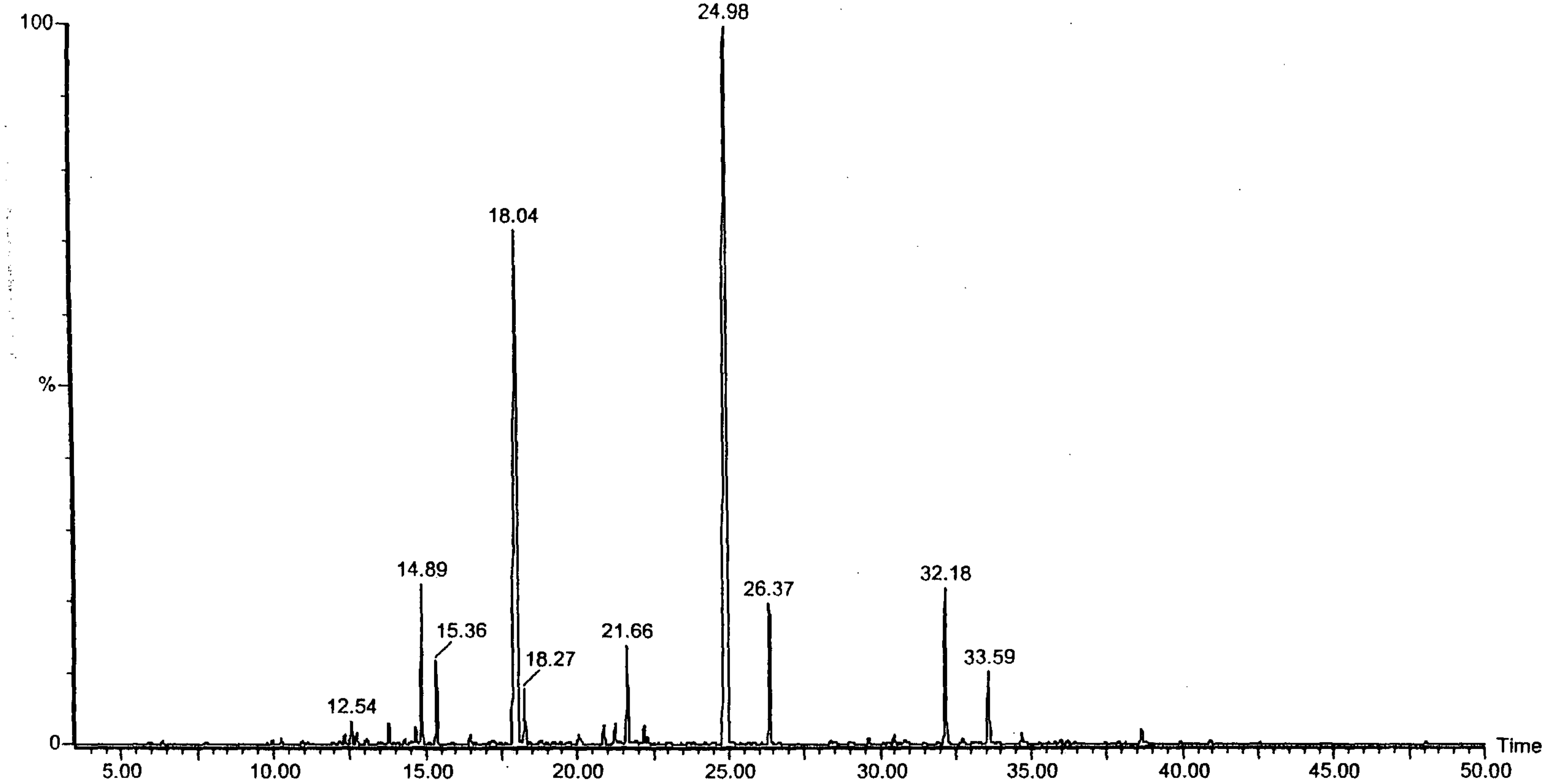

GC-MS total ion chromatogram of *Lavandula angustifolia* essential oil (Baldwins, BI 14450, diluted 1/100 in diethyl ether) using a 30 m x 0.25 mm i.d. x 0.25 µm DB-5MS column.



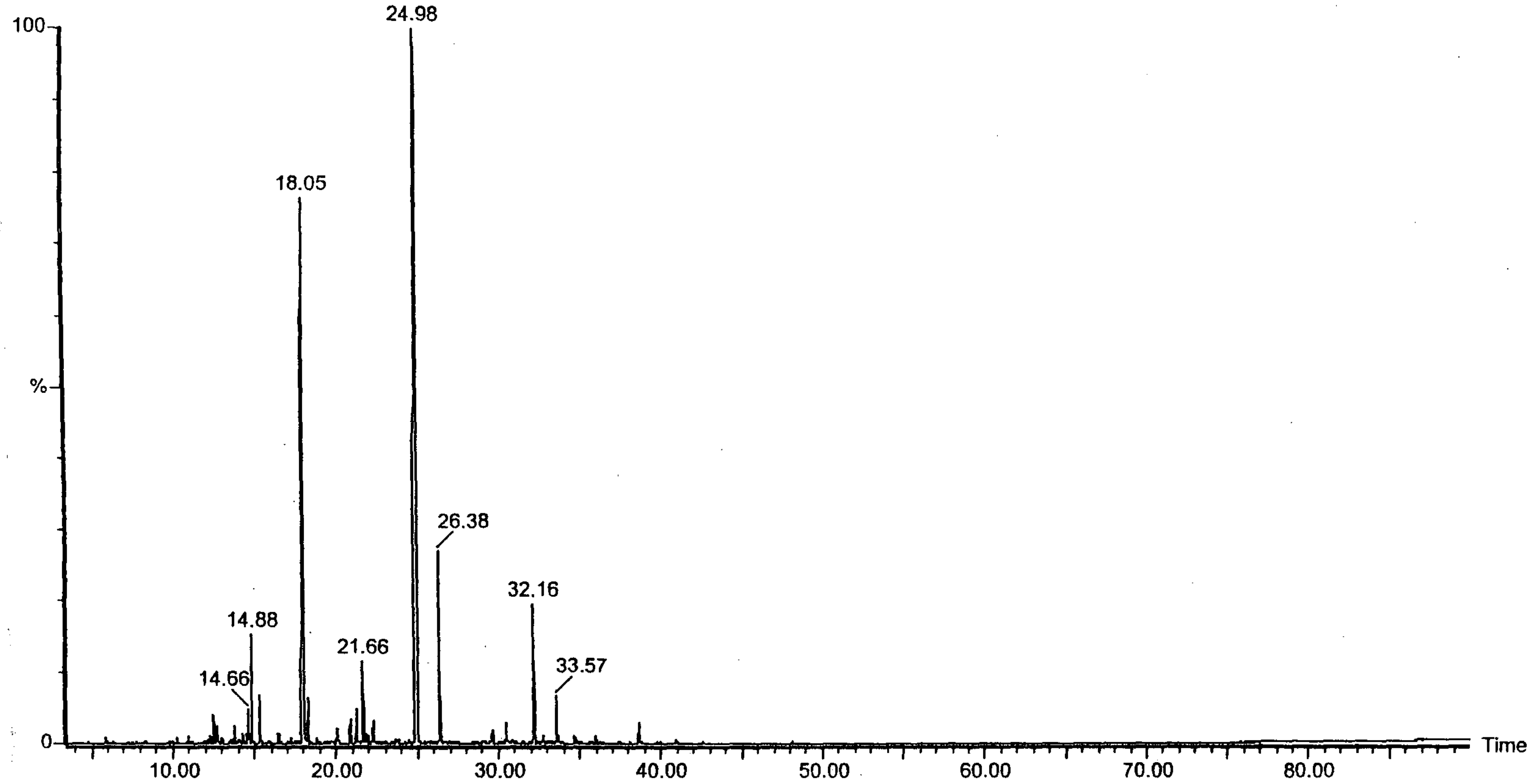

GC-MS total ion chromatogram (0 – 50 min) of *Lavandula angustifolia* essential oil (Baldwins, BI 14450, diluted 1/100 in diethyl ether) using a 30 m x 0.25 mm i.d. x 0.25 µm DB-5MS column.



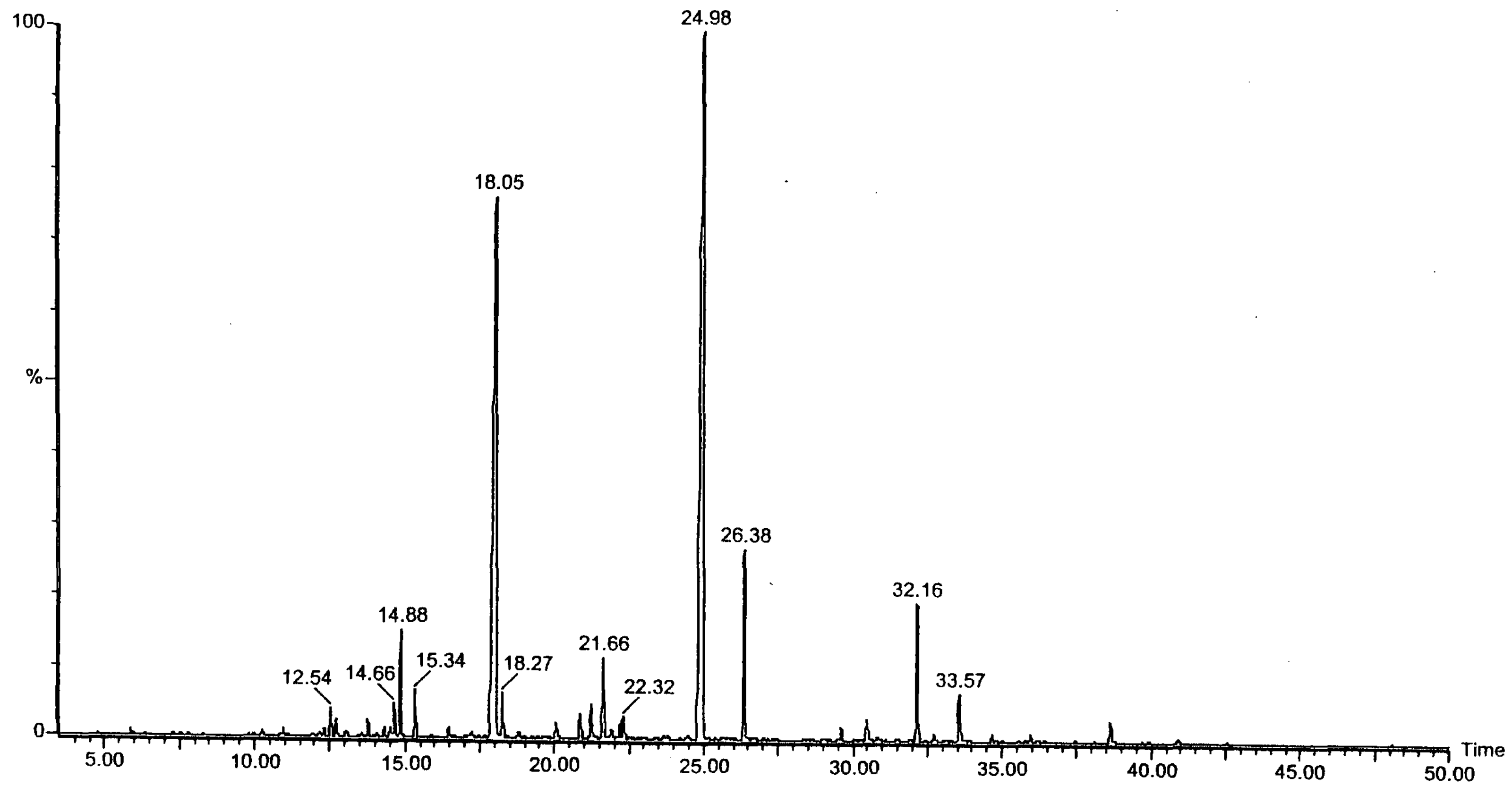

GC-MS total ion chromatogram of *Lavandula angustifolia* essential oil (Pranarom, BI 14451, diluted 1/100 in diethyl ether) using a 30 m x 0.25 mm i.d. x 0.25 µm DB-5MS column.



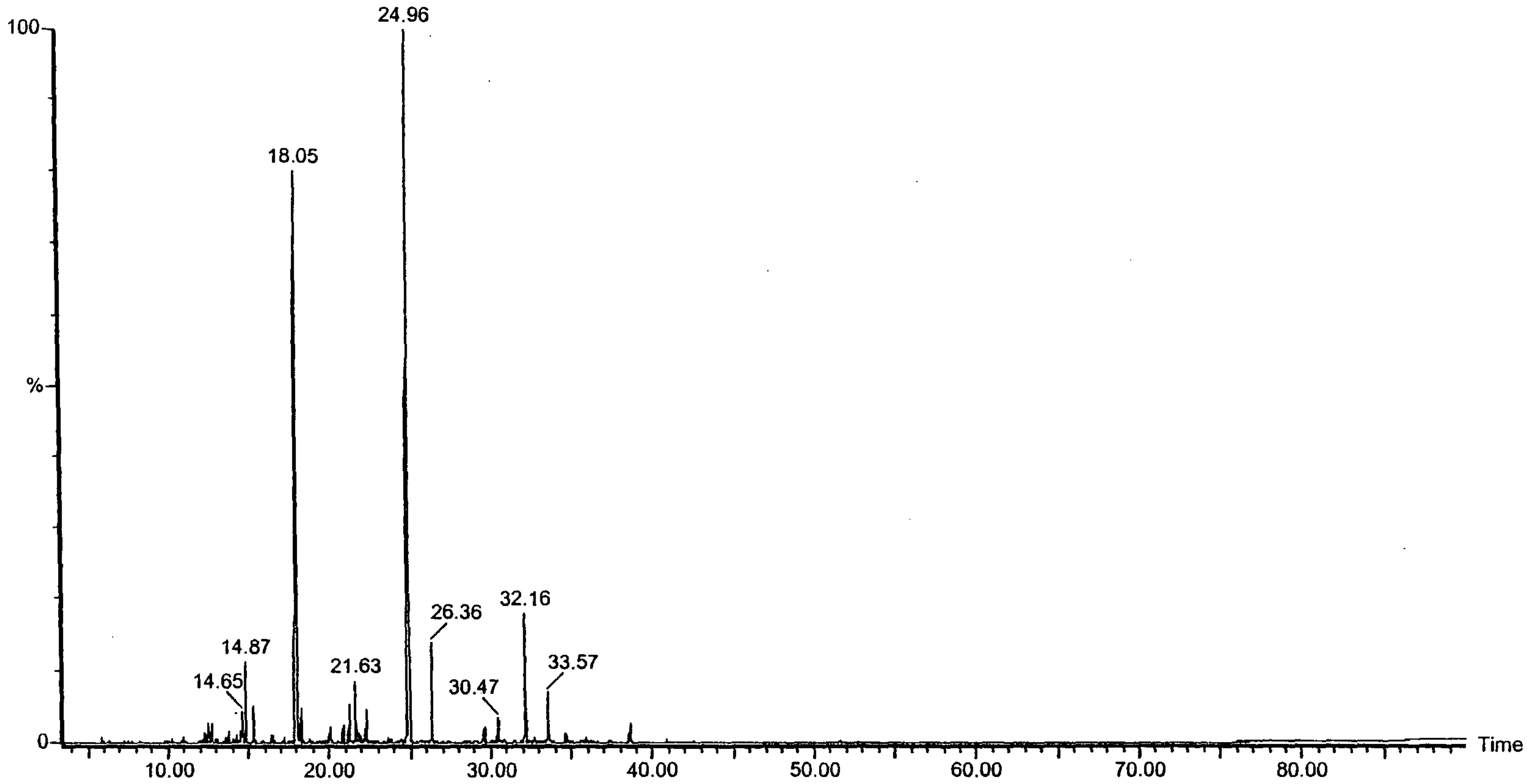

GC-MS total ion chromatogram (0 – 50 min) of *Lavandula angustifolia* essential oil (Pranarom, BI 14451, diluted 1/100 in diethyl ether) using a 30 m x 0.25 mm i.d. x 0.25 µm DB-5MS column.



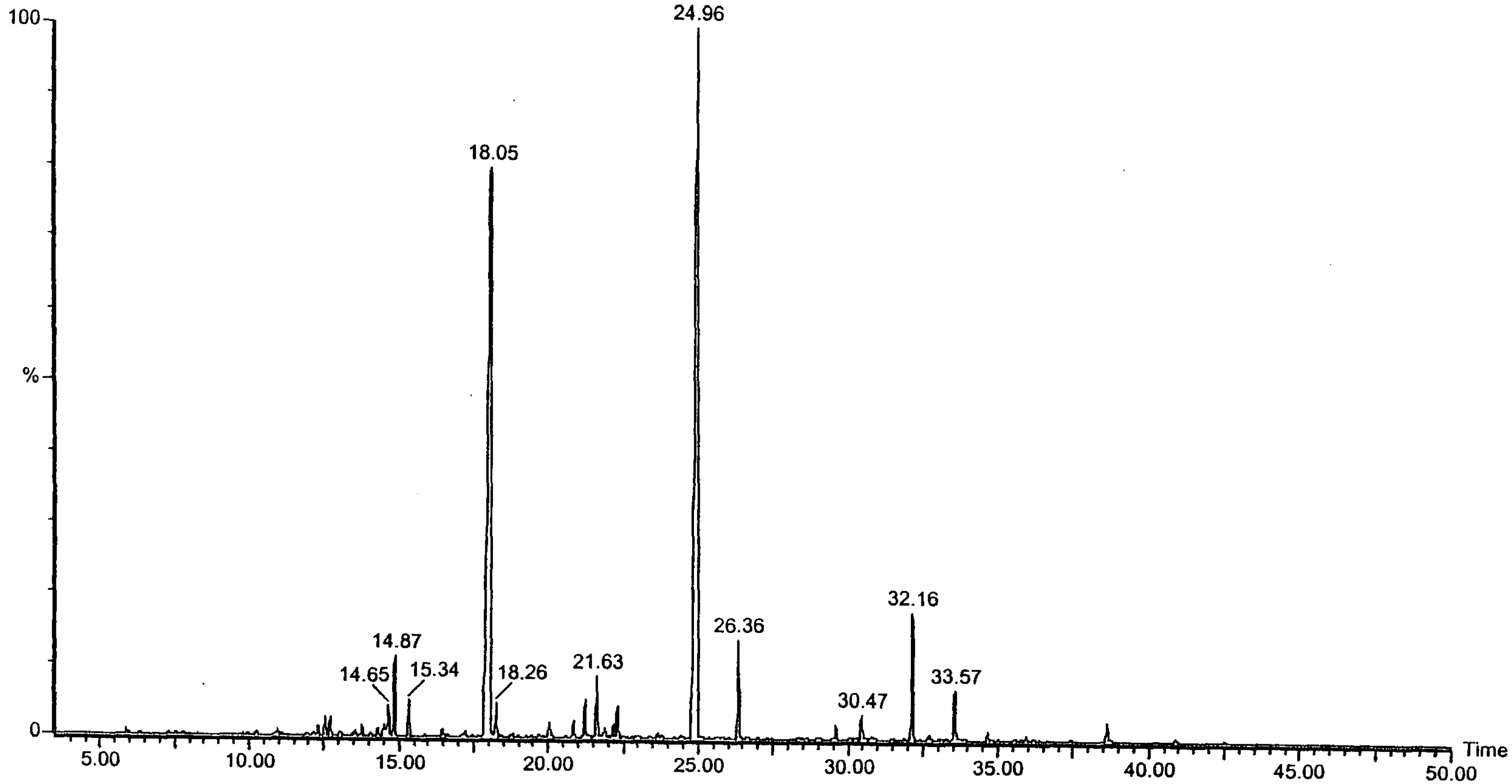

GC-MS total ion chromatogram of *Lavandula angustifolia* essential oil (Quintessence, BI 14458, diluted 1/100 in diethyl ether) using a 30 m x 0.25 mm i.d. x 0.25 µm DB-5MS column.




GC-MS total ion chromatogram (0 – 50 min) of *Lavandula angustifolia* essential oil (Quintessence, BI 14458, diluted 1/100 in diethyl ether) using a 30 m x 0.25 mm i.d. x 0.25 µm DB-5MS column.




GC-MS total ion chromatogram of *Lavandula angustifolia* essential oil (Fytosan, BI 14459, diluted 1/100 in diethyl ether) using a 30 m x 0.25 mm i.d. x 0.25 µm DB-5MS column.




GC-MS total ion chromatogram (0 – 50 min) of *Lavandula angustifolia* essential oil (Fytosan, BI 14459, diluted 1/100 in diethyl ether) using a 30 m x 0.25 mm i.d. x 0.25 µm DB-5MS column.

# Appendix III

## Electrophysiological Patch Clamping Testing

in collaboration with

Prof. George Lees & Liping Huang

(Department of Pharmacology and Toxicology, Otago School of Medical Sciences, Dunedin, NZ)

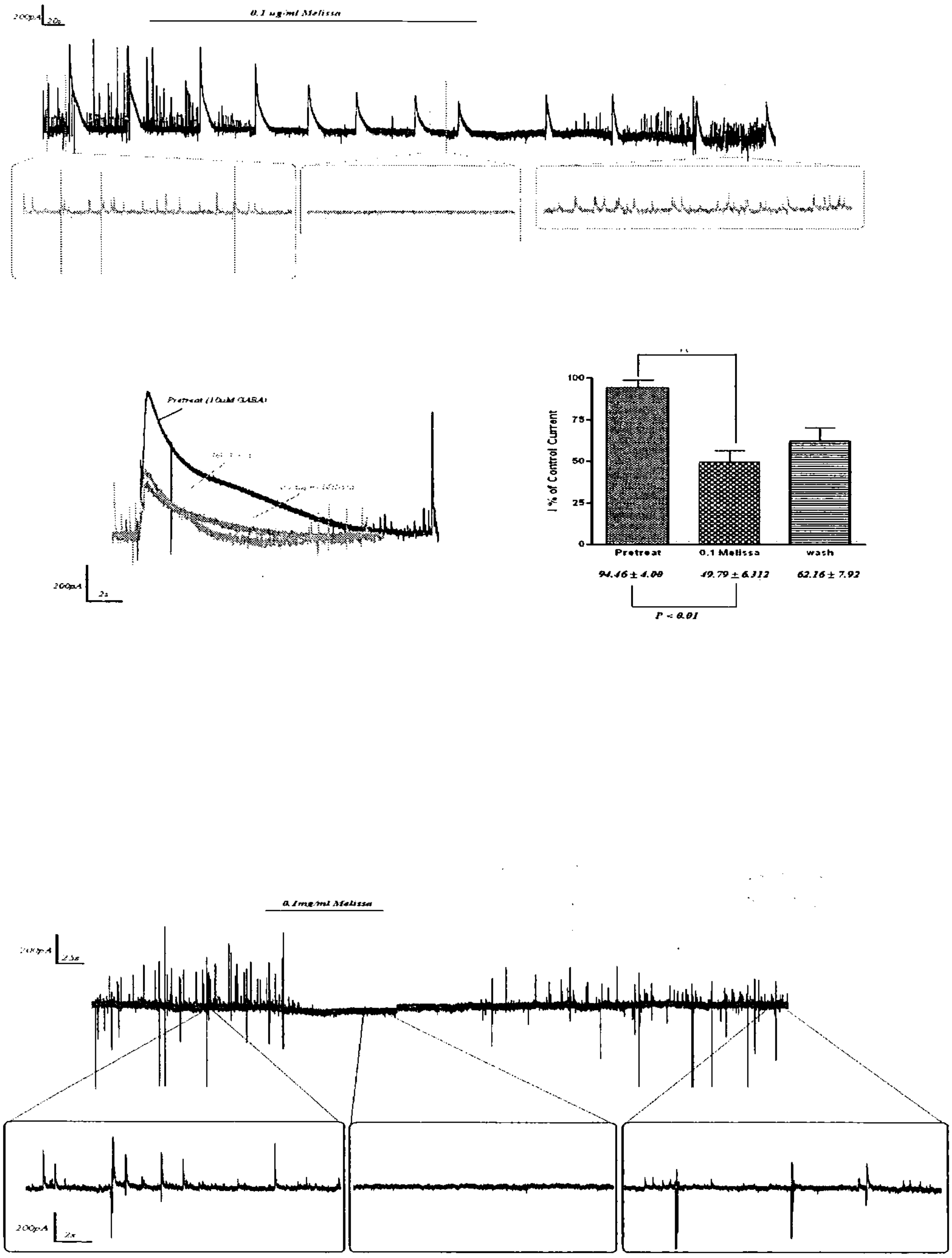



**Figure 1:** Melissa (0.1mg/ml) profoundly reduced GABA evoked-current, silenced both inhibitory and excitatory traffic in rat cultured cortical neurons, it also showed inhibitory effect on spontaneous activity.

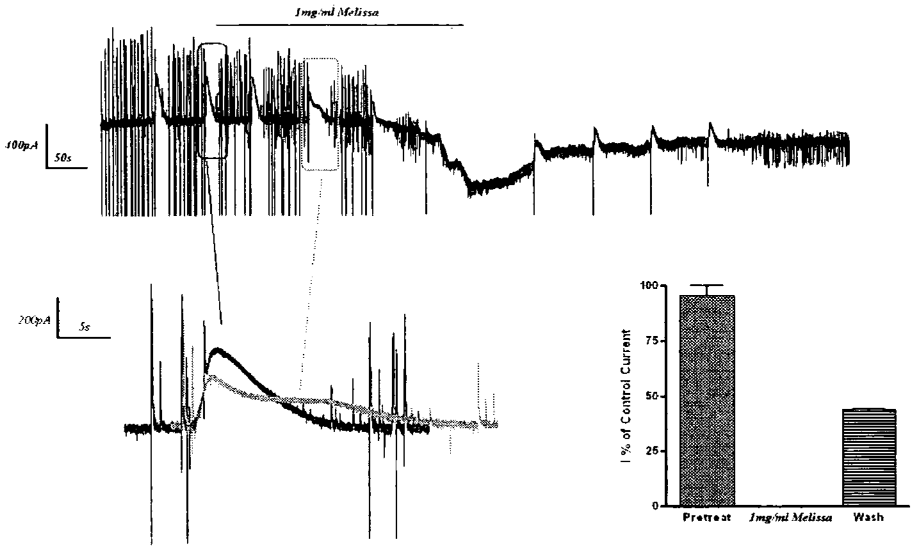



**Figure 2:** Melissa (1mg/ml) profoundly inhibited GABA induced current and excitatory/inhibitory synaptic activity in rat cultured cortical neurons.

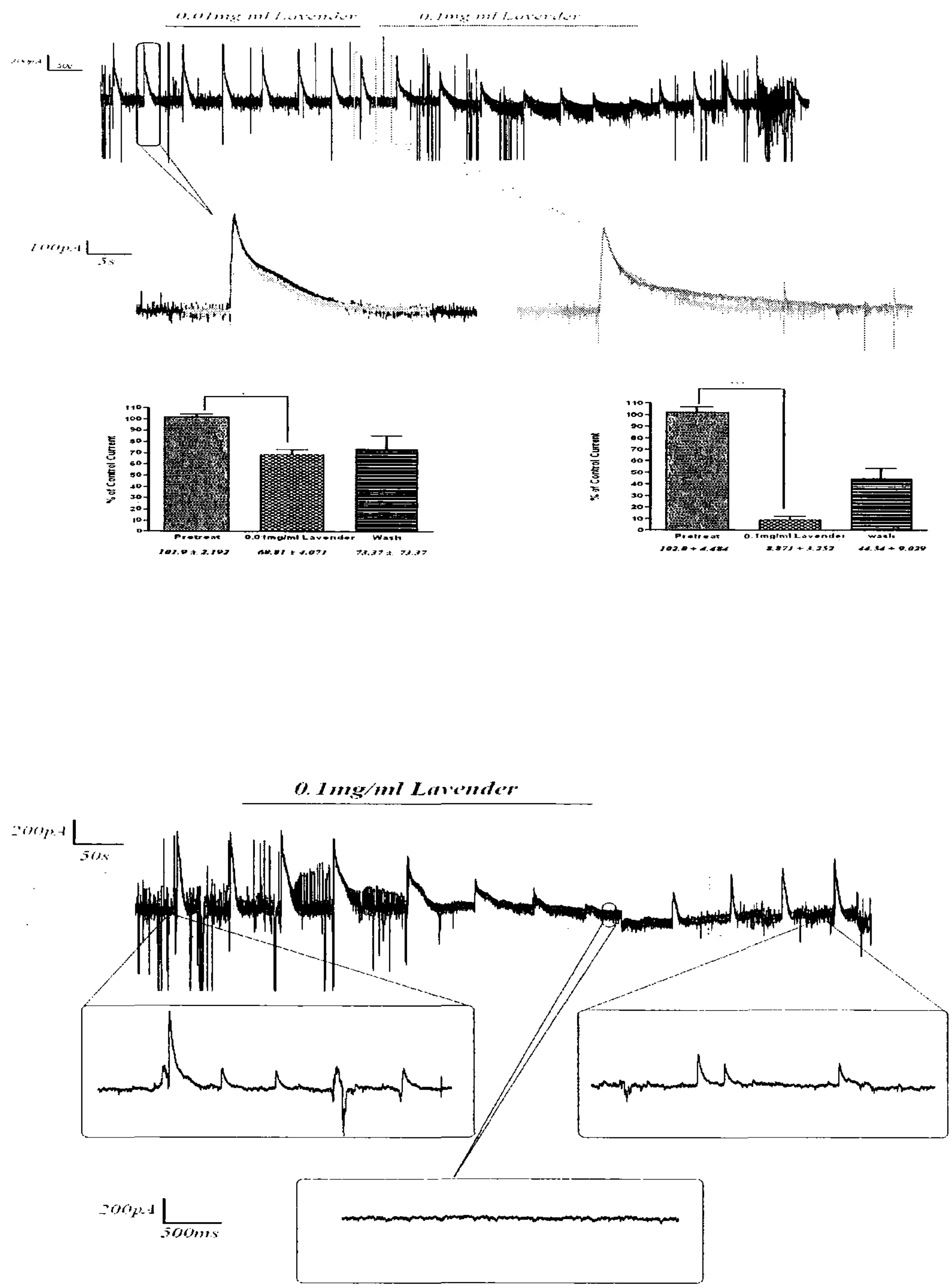



**Figure 3:** Lavender oil (0.1mg/ml) strongly reduced GABA-evoked currents. It also consistently prolonged currents evoked by exogenous GABA.

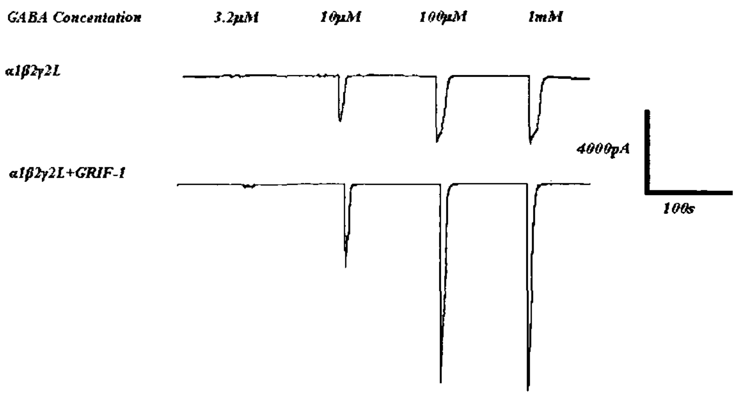



**Figure 4**: A representative trace of GABA activated ($Cl^-$) current in the presence and absence of GRIF-1 on $GABA_AR$ cell line.

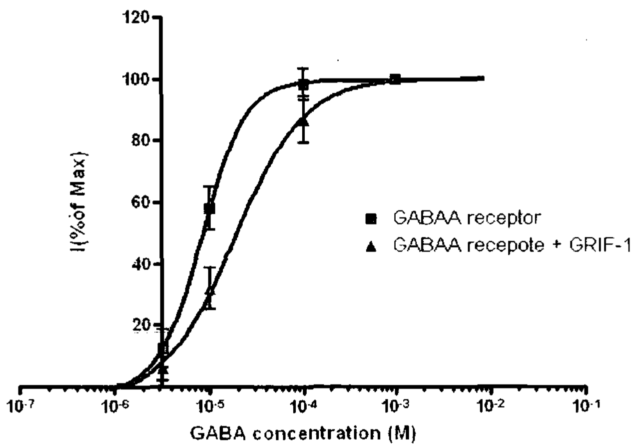
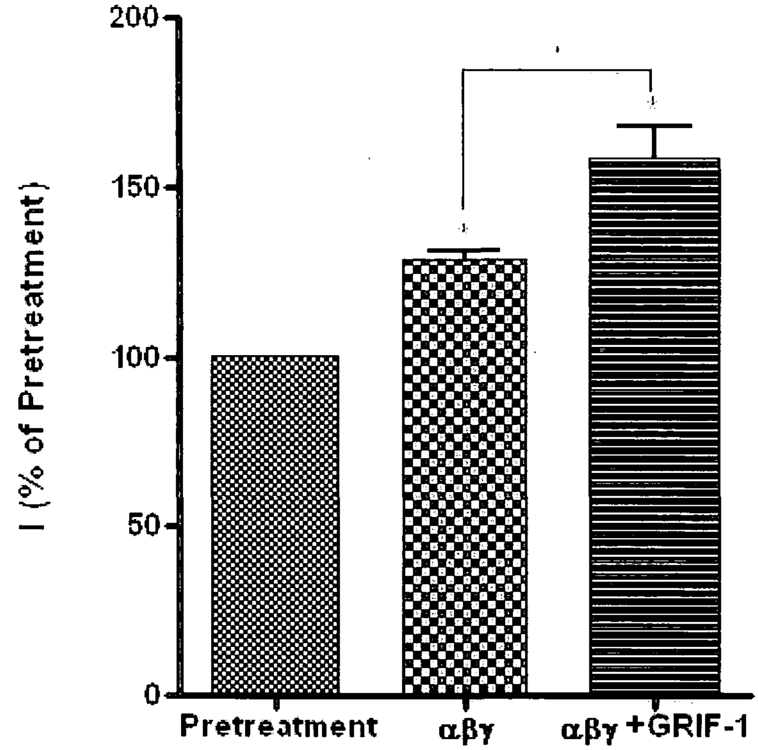



**Figure 5**: Concentration-response curves for GABA in the absence and presence of GRIF-1a protein on α1β2γ2L $GABA_AR$ cell line.




αβγ : 128.9 ± 2.466 % Pretreatment

αβγ + GRIF : 158.8 ± 9.339 % Pretreatment

**Figure 6:** Histograms shows the % of control current before and after application of GRIF-1a on α1β2γ2L $GABA_AR$ cell line. Each bar represent the mean ± SEM per group of cells (N=6-7 cells).

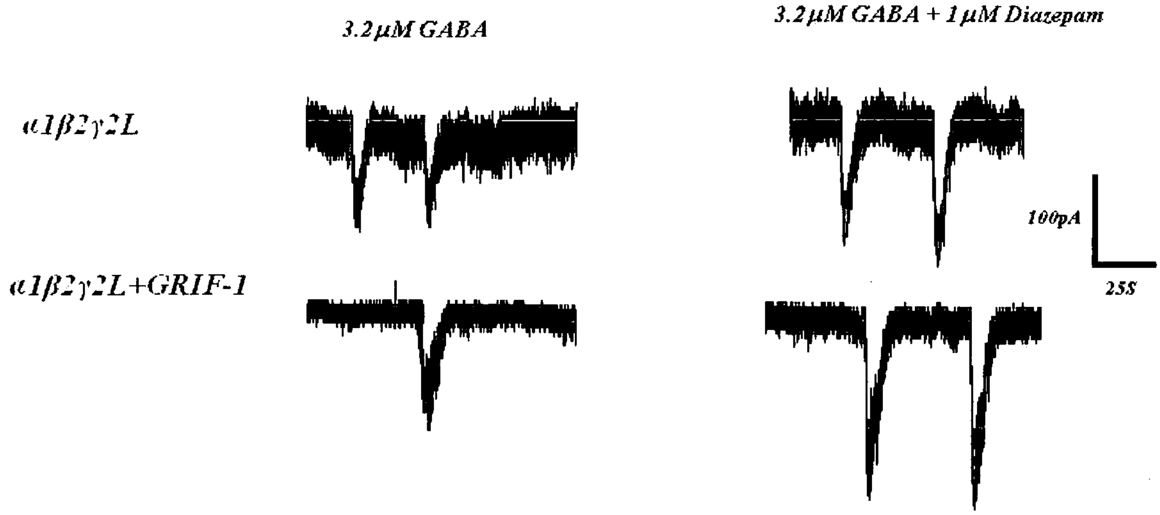



**Figure 7**: A representative trace of GABA activated ($Cl^-$) current by GABA, GABA + 1μM diazepam in the presence and absence of GRIF-1a on α1β2γ2L $GABA_AR$ cell line.

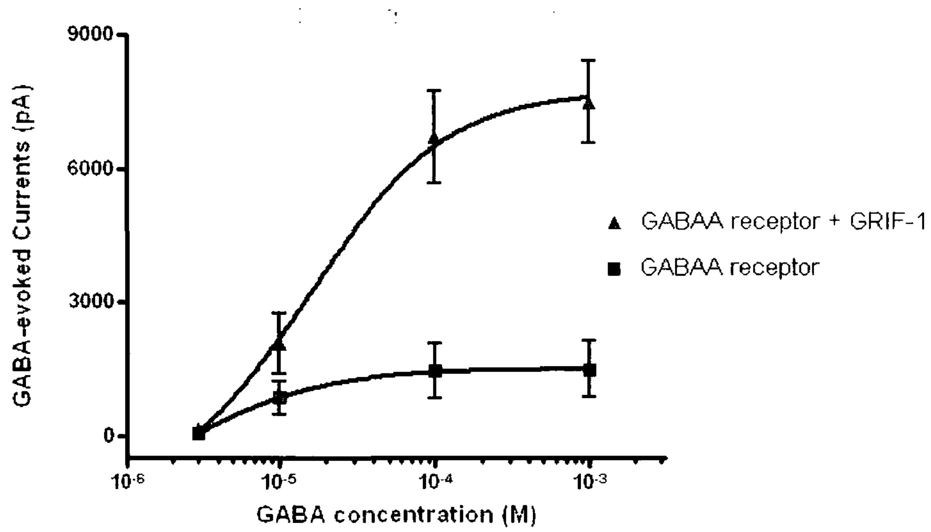



**Figure 8**: Concentration-response curves for (GABA + diazepam) in the absence and presence of GRIF-1a protein on α1β2γ2L $GABA_AR$ cell line.

# Publications

## Research articles:

**Abuhamdah, S**, Fuerstner, A, Lees, G and Chazot, PL (2005). Pharmacological binding studies of caloporoside and novel congeners with contrasting effects upon $[^{35}S]$ TBPS binding to mammalian $GABA_A$ receptor, *Biochem. Pharmacol.* 70(9), 1382-1388.

## In preparation:

**S Abuhamdah**, K Brickley, FA Stephenson, G Lees and PL Chazot The influence of rGRIF-1 upon rodent recombinant α1β2γ2 GABA-A receptor pharmacology

**S Abuhamdah**, L Huang, MSJ Elliott, EK Perry, C Ballard, & PT Francis, G Lees, PL Chazot. Elucidation of the Pharmacological Mechanism of Melissa and Lavender essential oils: correlation to anti-agitation effects

**S Abuhamdah**, L Huang, G Lees and PL Chazot. Preliminary SAR of Mefenamic acid for the α1β2γ2 GABA-A receptor

## Abstracts:

L Huang, G Lees, **S Abuhamdah** and PL Chazot (2006) *ASCEP conference* Australia. Seeking a mechanism of action for essential oils in the treatment of agitation in human dementia (poster presentation).

MSJ Elliott, **S Abuhamdah**, L Huang, EK Perry, C Ballard, G Lees, PL Chazot & PT Francis (2006) *International Conference on Alzheimer's Disease (ICAD),* Portugal. Essential Oils as Potential Treatment for Agitation in Severe Dementia: Elucidation of the Pharmacological Mechanism of Melissa and Lavender (poster presentation).

**S Abuhamdah**, K Brickley, FA Stephenson and PL Chazot (2005) *Biochem. Soc Trans. Molecular determinants of synaptic function: molecules and models* (Southampton University) The influence of rGRIF-1 upon rodent recombinant α1β2γ2 GABA-A receptor pharmacology (Poster communication)

**S Abuhamdah**, K Brickley, FA Stephenson and PL Chazot (2005) *BNA Postgraduate and Early Career Symposium* (Durham University). The influence of a novel $GABA_A$ receptor associated protein, GRIF-1a, upon $GABA_A$ receptor pharmacology (Oral communication)

**S Abuhamdah** and PL Chazot (2005) UK GRAD Programme, Getting your Message Across-Presenting to the Public, Leeds University. Probing new ways to modulate dysfunction of the $GABA_A$ receptor: resetting the balance in the brain (Poster communication)

**S Abuhamdah**, A Fuerstner, G Lees and PL Chazot (2004) *Br. J. Pharmacol. (Suppl.)* Newcastle University Meeting. Further characterization of Octyl-β-D-glucoside: a novel modulator of the rodent $GABA_A$ receptor (Poster communication)

**S Abuhamdah**, A Fuerstner, G Lees and PL Chazot (2004) *Br. J. Pharmacol. (Suppl.)* Bath University Meeting. Pharmacological characterization of caloporoside and analogues using [$^{35}$S] TPBS binding to adult rat forebrain membranes (Poster communication)

**BRITISH PHARMACOLOGICAL SOCIETY, SUMMER MEETING 5th -8th JULY 2004, BATH UNIVERSITY,
(POSTER COMMUNICATION)**

## PHARMACOLOGICAL CHARACTERISATION OF CALOPOROSIDE AND ANALOGUES USING [$^{35}$S] TBPS BINDING TO ADULT RAT FOREBRAIN $GABA_A$ RECEPTORS

S. Abuhamdah[1,2], A. Fürstner[3], G. Lees[2] and P.L. Chazot[1]
[1]School of Biological and Biomedical Sciences, Durham University, UK; [2]Sunderland Pharmacy School, University of Sunderland, UK; [3] Max-Planck-Institute, Mulheim, Germany

Caloporoside is a natural active fungal metabolite, which was isolated several years ago from fermentation of *Caloporous dichrous* and was described to exhibit antibacterial, antifungal and phospholipase C inhibitory activity (Weber *et al*, 1994). Chemical synthesis of caloporoside and a number of analogues has been described (Fürstner *et al*, 1996, 1998). We have previously reported evidence that related compounds, lactose and octyl-β-D-mannoside, bind and functionally modulate rodent $GABA_A$ receptors, respectively (Rezai *et al.*, 2003; Lees, Chazot *et al.*, 2000).

In this present study, we have characterized the pharmacology of caloporoside and two further congeners, 2-Hydroxy-6-{[(16R)-beta-D-mannopyranosyloxy) heptadecyl]} benzoic acid and octyl-beta-D-glucoside on $GABA_A$ receptors using [$S^{35}$]-t-butylbicyclophosphoorothionate radioligand binding assay. The assay was performed in 50 mM Tris-buffer supplemented with 0.2 M NaCl (pH 7.4). Well-washed adult rat Sprague Dawley strain forebrain membranes (100 μg) were incubated in the presence of 25 nM [$S^{35}$] TBPS radioligand, at 25 C° for 90 min. Non-specific binding was defined in the presence of 100 μM picrotoxinin. [$^{3}$H] flunitrazepam binding assays were performed as described in Rezai *et al.*, 2002. The reactions were terminated by rapid filtration using a Brandell cell harvester.

Caloporoside and 2-Hydroxy-6-{[(16R)-β-D-mannopyranosyloxy) heptadecyl]} benzoic acid produced concentration-dependent complete inhibition of specific [$S^{35}$] TBPS binding with overall apparent $IC_{50}$ values of (147 ± 1 μM) and (142 ± 1 μM) respectively, and steep pseudo Hill coefficients ($n_H$ = -2.78 ± 1.4 and -2.96 ± 0.84, respectively) (mean ± SD for three determinations). In contrast, octyl-β-D-glucoside elicited a concentration-dependent stimulation of specific [$S^{35}$] TBPS binding ($E_{max}$ = 125%; $EC_{50}$ = 390 ± 230 nM, mean ± SD for three independent experiments). The level of stimulation was similar to that elicited by flunitrazepam ($E_{max}$ = 120%; $EC_{50}$ = 4.3 ± 3.1 nM, mean ± SD for three independent experiments), determined in parallel experiments. However, the three test compounds elicited no significant effect (positive or negative) upon [$^{3}$H] flunitrazepam binding, indicating that these compounds did not bind directly, or allosterically couple, to the benzodiazepine site of the $GABA_A$ receptor. This study suggests that octyl-β-D-glucoside is a high affinity positive $GABA_A$ receptor channel modulator, and may act in a similar fashion to octyl-β-D-mannoside (Lees, Chazot *et al.*, 2000).

Lees G., Chazot, P.L. *et al.*, (2000) *Bioorg. Med. Chem. Letts.* 10, p1759-1761.
Fürstner, A. *et al.*, (1996) *Tetrahedron,* 52, p15071-15078.
Fürstner A. *et al.*, (1998) *J. Org. Chem.* 63, p3072-3080.
Rezai, N. *et al.*, (2003) *Biochem. Pharmacol.* 65, p619-623.
Weber, W. *et al.*, (1994) *J. Antibiotic*, 47, p1188-1194.

**BRITISH PHARMACOLOGICAL SOCIETY, WINTER MEETING 16th -8th DECEMBER 2004, UNIVERSITY OF NEWCASTLE, (POSTER COMMUNICATION)**

# FURTHER PHARMACOLOGICAL CHARACTERISATION OF OCTYL-β-D-GLUCOSIDE: A NOVEL MODULATOR OF THE RODENT $GABA_A$ RECEPTORS

S. Abuhamdah[1,2], A. Fürstner[4], G. Lees[3] and P.L. Chazot[1]
[1]School of Biological and Biomedical Sciences[2]School of Health, Durham University, UK; [3]Department of Pharmacology and Toxicology, University of Otago Medical School, Dunedin, NZ; [4] Max-Planck-Institute, Mulheim, Germany

γ-aminobutyric acid, (GABA) is the major inhibitory neurotransmitter in the vertebrate brain, and is still of great interest therapeutically because it comprises the myriad of binding sites for pharmaceutically important drugs that interact allosterically with GABA agonist site of the channel. However we still do not have ideal anxiolytic, sedative, or hypnotic drugs for chronic treatments (Korpi *et al.*, 2002). Octyl-O-β-D-glucoside represents a member of a novel class of $GABA_A$ receptor positive modulator. In this study, we have characterized further the $GABA_A$ receptor pharmacology of the octyl-O-β-D-glucoside class using an [$S^{35}$]-t-butylbicyclophosphoorothionate radioligand binding assay. The assay was performed in 50 mM Tris-buffer supplemented with 0.2 M NaCl (pH 7.4), and a test concentration range of $10^{-9}$M-$10^{-2}$M. Well-washed adult male rat Sprague Dawley strain forebrain membranes (100 μg) were incubated in the presence of 25 nM [$S^{35}$] TBPS radioligand, at 25 C° for 90 min. Non-specific binding was defined in the presence of 100 μM picrotoxinin. The reactions were terminated by rapid filtration using a Brandell cell harvester, and washed with 3 x 4ml washes with ice-cold sodium phosphate buffer, pH 7.4. Novel data cited are mean ± SD % stimulation of specific [$^{35}$S] TBPS binding for 3-5 separate experiments (GraphPad Prism 3).

As reported previously, octyl-O-β-D-glucoside elicited a concentration-dependant stimulation of specific [$^{35}$S] TBPS binding (mean $E_{max}$= 144 ± 4%; apparent $EC_{50}$ = 392 ± 227 nM) (Abuhamdah *et al.,* 2004). In this present study, the core monosaccharide glucose (107 ± 2% at 1mM), octyl-α-D-glucoside (110 ± 1% at 1mM), nor hexyl-β-D-glucoside (94 ± 2% at 1mM) had little or no effect upon control [$^{35}$S] TBPS binding. Previously, we showed that lactose potentiated [$^{3}$H] TBOB binding to the channel site of the $GABA_A$ receptor, with a maximal effect observed at 10 μM (Rezai *et al.*, 2003). Here we showed that lactose (10 μM) occluded the potentiation by 100 μM octyl-α-D-glucoside of [$^{35}$S] TBPS binding (144 ± 4% without lactose; 108 ± 9 % with 10 μM lactose), indicating a shared binding site.

This present study provides new evidence that the modulatory effect of octyl-O-β-D-glucoside upon GABA receptor is dependent on the presence of the side chain, the nature of the glycosidic linkage and the side chain length. This work provides a clearer picture of the SAR of this novel class of $GABA_A$ receptor modulator, which warrants further elucidation using electrophysiological and behavioural approaches (Lees, Chazot *et al.*, 2000).

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Lees G., Chazot, P.L. *et al.* (2000) *Bioorg. Med. Chem. Letts.* 10, 1759-1761.
Rezai, N. *et al.* (2003) *Biochem. Pharmacol.* 65, 619-623.
Korpi, E.R *et al.* (2002). *Prog. Neurobiol*, 67,113-159.

*This work is funded, in part, by an Islamic Bank Development scholarship.*

**UK GRAD PROGRAMME, GETTING YOUR MESSAGE ACROSS-PRESENTING TO THE PUBLIC, 26th MAY 2005, LEEDS UNIVERSITY, (POSTER COMMUNICATION)**

**PROBING NEW WAYS TO MODULATE DYSFUNCTION OF THE $GABA_A$ RECEPTOR: RESETTING THE BALANCE IN THE BRAIN.**

Sawsan Abuhamdah & Paul L Chazot
School of Biological and Biomedical Sciences, Durham University.

The brain is wonderfully complex organ which underpins how you function, who you are, and likely a more accurate answer the meaning of life than"42". Within the brain there is a balance between excitation and inhibition which is exquisitely controlled, and if this balance is lost, this can lead to serious neurological and psychological consequences for the individual. The major inhibitory transmitter in the human brain is a small molecule called GABA, which binds to receptor protein called $GABA_A$ receptors which are found on the receiving brain cell in specialist structures called synapse. Because of the importance of these receptors in the brain balancing act, they are one of the most important targets for treating serious brain disorders, such as epilepsy, anxiety and sleep disorders, suffered by millions worldwide. The drugs currently used although useful, still have serious drawbacks. My work is involved, firstly at the basic level, in dissecting out newly identified proteins which take $GABA_A$ receptors to the correct synapse in a brain cell and, secondly at the applied level, in developing new improved compounds which overcome the under-activity of the $GABA_A$ receptors, seen in many human brain disorders.

My poster will describe the importance of " electrical balance" in the brain (using a simple "see-saw' analogy) and highlight the serious effects on the individual if that balance is lost (brain disorders). I will describe the role of GABA in the brain balancing act, and why it is important drug target with examples of common drugs used to treat brain disorders. For this, I will focus on epilepsy, which gives a clear visual picture of "electrical imbalance" leading to over-excitability of the brain (seizures). This over-activity can be overcome and re-balanced by increasing the inhibitory system in the brain, which is controlled by GABA. Again I will use a see-saw analogy to explain this. I will the describe the problems examples, much discussed in the press. I will then briefly demonstrate my approach to develop new improved strategies to regulate the GABA system, to re-address the imbalance in brain disorders with less side-effect.

**BNA POSTGRADUATE AND EARLY CAREER SYMPOSIUM, 14th-15th SEPTEMBER 2005, DURHAM UNIVERSITY,**
**(ORAL COMMUNICATION)**

## THE INFLUNECE OF A NOVEL $GABA_A$ RECEPTOR ASSOCIATED PROTEIN, GRIF-1a, UPON $GABA_A$ RECEPTOR PHARMACOLOGY.

Sawsan Abuhamdah[1,2], Kieran Brickley[3], F Anne Stephenson[3] and Paul L Chazot[1]
[1]School of Biological and Biomedical Sciences, [2]School of Health, Durham University; [3]School of Pharmacy, University of London

γ-aminobutyric acid type A ($GABA_A$) receptor interacting factor (GRIF-1a) is a 913 amino acid protein previously proposed to function as $GABA_A$ receptor $\beta_2$ subunit trafficking protein. To gain further insights into the potential role of this novel protein, we investigated the effect of transiently expressing rGRIF-1a on the binding pharmacology of the rat recombinant (α1β2γ2) $GABA_A$ receptor subtype stably expressed in HEK 293. Firstly, co-expression of rGRIF-1a enhanced, in a concentration-dependent manner, the apparent [$^3$H] Flunitrazepam binding to $GABA_A$ α1β2γ2 receptor. Saturation binding analysis showed this enhancement to be due to a 4-fold decrease in $K_D$ (increase in affinity) with little effect on the $B_{max}$ for [$^3$H] Flunitrazepam (α1β2γ2 complexes). Secondly, in the absence of rGRIF-1a, the GABA competition curve for [$^3$H] muscimol was best fit to a single site (apparent $IC_{50}$ = 191 nM, nH = 1.0 $\pm$ 0.2), while in the presence of rGRIF-1a, the data were best fit to a two-site model (apparent $IC_{50}$ values: 7 nM (37 $\pm$ 12%) and 507 nM (63 $\pm$ 12%), respectively; nH = 0.5 $\pm$0.1). Thirdly, rGRIF-1a had no significant effect on picrotoxin competition binding for [$^{35}$S] TBPS. Taken together, these results suggest that GRIF does not increase α1β2γ2 receptor complex numbers, but appears to stabilise the $GABA_A$ receptor in a conformation which facilitates binding to both GABA and benzodiazepines. We are currently investigating the effect of GRIF-1a upon surface expression of the $GABA_A$ receptor complex.

M Beck *et al.* (2002). *J.Biol.chem*, 277, 30079-30090.

*The authors wish to thank Dr David Graham (Sonofi Aventis, France) for the kind gift of the α1β2γ2 cell line, and the Islamic Development Bank for funding this study.*

*Biochem. Soc Trans.* **MOLECULAR DETERMINANTS OF SYNAPTIC FUNCTION: MOLECULES AND MODELS, 22-23 SEPTEMBER 2005, SOUTHAMPTON UNIVERSITY,**
**(POSTER COMMUNICATION)**

# THE INFLUENCE OF GRIF-1a UPON RECOMBINANT RODENT α1β2γ2 $GABA_A$ RECEPTOR PHARMACOLOGY.

S Abuhamdah[1,2], K Brickley[3], FA Stephenson[3] and PL Chazot[1]
[1]School of Biological and Biomedical Sciences, [2]School of Health, Durham University; [3]School of Pharmacy, University of London

γ-aminobutyric acid type A ($GABA_A$) receptor interacting factor (GRIF-1a) is a 913 amino acid protein previously proposed to function as $GABA_A$ receptor $\beta_2$ subunit trafficking protein. To gain further insights into the potential role of this novel protein, we investigated the effect of transiently expressing rGRIF-1a on the binding pharmacology of the rat recombinant (α1β2γ2) $GABA_A$ receptor subtype stably expressed in HEK 293. Firstly, co-expression of rGRIF-1a enhanced, in a concentration-dependent manner, the apparent [$^3$H] Flunitrazepam binding to $GABA_A$ α1β2γ2 receptor. Saturation binding analysis showed this enhancement to be due to a 4-fold decrease in $K_D$ (increase in affinity) with little effect on the $B_{max}$ for [$^3$H] Flunitrazepam (α1β2γ2 complexes). Secondly, in the absence of rGRIF-1a, the GABA competition curve for [$^3$H] muscimol was best fit to a single site (apparent $IC_{50}$ = 191 nM, nH = 1.0 ± 0.2), while in the presence of rGRIF-1a, the data were best fit to a two-site model (apparent $IC_{50}$ values: 7 nM (37 ± 12%) and 507 nM (63 ± 12%), respectively; nH = 0.5 ±0.1). Thirdly, rGRIF-1a had no significant effect on picrotoxin competition binding for [$^{35}$S] TBPS. Taken together, these results suggest that GRIF does not increase α1β2γ2 receptor complex numbers, but appears to stabilise the $GABA_A$ receptor in a conformation which facilitates binding to both GABA and benzodiazepines.

*The authors wish to thank Dr David Graham (Sonofi Aventis, France) for the kind gift of the α1β2γ2 cell line, and the Islamic Development Bank for funding this study.*

**INTERNATIONAL CONFERENCE ON ALZHEIMER'S DISEASE (ICAD), Portugal, 2006.**
**(POSTER COMMUNICATION)**

# ESSENTIAL OILS AS POTENTIAL TREATMENT FOR AGITATION IN SEVERE DEMENTIA: ELUCIDATION OF THE PHARMACOLOGICAL MECHANISM OF MELISSA AND LAVENDER OILS.

Mark S.J. Elliott[1], Sawsan Abuhamdah[2], Liping Huang[3], Elaine K. Perry[1], Clive Ballard[1], George Lees[3], Paul L. Chazot[2], Clive Holmes[4], Alistair Burns[5] & Paul T. Francis[1]

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Key Words: Agitation, Severe dementia, Neuroleptics, Essential oils, Melissa, Lavender, Radioligand binding, Electrophysiology

Agitation is a frequent syndrome demonstrated by people with severe dementia, manifesting mainly in restlessness and verbal, but sometimes physical, aggression. Currently recommended treatments for agitation include the neuroleptic drugs, despite their modest efficacy and severe adverse effects. Alternative therapies for the management of agitation include the use of the essential oils *Melissa officinalis* and *Lavendula angustifolia*. Several previous studies have demonstrated that Melissa oil can diminish the symptoms of agitation, while displaying minimal side effects. In order to elucidate the pharmacological basis for the actions of the Melissa and Lavender oils, a pharmacological screen has been conducted using radioligand binding in rat cortical membranes. Lavender and Melissa oils were sourced from four separate authenticated suppliers. Interactions of the oils with both G-protein coupled receptors ($5\text{-}HT_{1A}$, $5\text{-}HT_{2A}$, muscarinic M1 and histamine $H_3$) and ligand-gated ion channel receptors (NMDA, nicotinic and $GABA_A$ channel, agonist and benzodiazepine sites) implicated in agitation in severe dementia have been examined.

The most potent effects of both Lavender and Melissa oils have been shown to occur at the $H_3$ ([$^3$H]-clobenpropit), $5\text{-}HT_{2A}$ ([$^3$H]-ketanserin) and $GABA_A$ receptors. Interestingly, particularly strong inhibition of [$^3$H]-flunitrazepam binding to the $GABA_A$ receptor ($IC_{50}$ < 0.001 mg/ml) has been shown when Lavender and Melissa oils are applied in combination, with no effect when applied alone. Melissa oil alone also inhibits binding of [$^3$H]-8-OH-DPAT to $5\text{-}HT_{1A}$ receptors and [$^3$H]-pirenzepine to M1 receptors. Neither Melissa, nor Lavender oils demonstrated any effect on the binding of [$^3$H]-MK-801 to NMDA receptors, or [$^3$H]-nicotine to nicotinic acetylcholine receptors. Overall, therefore, Melissa oil appears to have a broader pharmacological profile. Results were similar between oils from the four different sources.

Furthermore, functional studies have demonstrated that both oils (0.01 mg/ml) applied to rat primary cortical neurone cultures, results in a significant reduction in *both* inhibitory and excitatory transmission, with a net depressant effect on neurotransmission.

A multi-centre, placebo-controlled clinical trial involving 150 people will follow this pharmacological study based on its findings.

This work was funded by a grant from the Alzheimer's Society.

**AMERICAN SOCIETY OF CLINICAL EVOKED POTENTIALS (ASCEP), AUSTRALIA, 2006.**
**(POSTER COMMUNICATION)**

# SEEKING A MECHANISM OF ACTION FOR ESSENTIAL OILS IN THE TREATMENT OF AGITATION IN HUMAN DEMENTIA.

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Agitation is a severe and persistent feature of advanced dementia. Neuroleptic drugs are frequently used as treatments but cause over sedation, social withdrawal, enhanced risk of stroke and may accelerate cognitive decline. *Melissa officinalis* (Mo) has been used historically for its calming and attention maintenance properties and we are participating in a large multi-centre dementia trial to assess this essential oil in patients later this year. We have used electrophysiology and radioligand binding techniques to address the hypothesis that the (Mo) may be a $GABA_A$ receptor modulator. Increasing concentrations (0.01, 0.1 and 1.0 mg/ml) of (Mo) were incubated with cortical brain membrane homogenates from male adult Wistar rats or the rat $\alpha1\beta2\gamma2$ $GABA_A$ receptor subtype expressed in HEK 293 cells, and a fixed concentration of radioligand equal to the approx. $K_d$ for each ligand. (Mo) (approx. $IC_{50}$ = 0.03mg/ml) significantly displaced $[^{35}S]$-TBPS binding in both preparations, but had no effect upon $[^3H]$ MK801 or $[^3H]$ nicotine binding to native membranes up to 1mg/ml. Patch clamp experiments on primary cultures from rat cortex demonstrated that (Mo) (0.1mg/ml) reduced currents through the $GABA_A$ channel but concurrently blocked the spontaneous synaptic traffic in the cultured networks. We conclude that (Mo) does exert depressant effects on neural activity but that this is not a reflection of its disinhibitory effect on the $GABA_A$ complex.

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Biochemical Pharmacology 70 (2005) 1382–1388

Biochemical Pharmacology

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# Radioligand binding studies of caloporoside and novel congeners with contrasting effects upon [$^{35}$S] TBPS binding to the mammalian $GABA_A$ receptor

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Received 24 June 2005; accepted 26 July 2005

## Abstract

Caloporoside is a natural active fungal metabolite, which was isolated from *Caloporous dichrous* and was described to exhibit antibacterial, antifungal and phospholipase C inhibitory activity. We have previously reported evidence that related β-linked compounds, lactose and octyl-β-D-mannoside, bind and functionally modulate rodent $GABA_A$ receptors, respectively. We have characterized the binding pharmacology of synthetic caloporoside and two further congeners, 2-hydroxy-6-{[(16R)-(β-D-mannopyranosyloxy)heptadecyl]} benzoic acid and octyl-β-D-glucoside on $GABA_A$ receptors using a [$^{35}$S]-*t*-butylbicyclophosphoorothionate (TBPS) radioligand binding assay. Caloporoside and 2-hydroxy-6-{[(16R)-(β-D-mannopyranosyloxy)heptadecyl]} benzoic acid produced concentration-dependent complete inhibition of specific [$^{35}$S] TBPS binding with overall apparent $IC_{50}$ values of 14.7 ± 0.1 and 14.2 ± 0.1 μM, respectively. In contrast, octyl-β-D-glucoside elicited a concentration-dependent stimulation of specific [$^{35}$S] TBPS binding ($E_{max}$ = 144 ± 4%; $EC_{50}$ = 39.2 ± 22.7 nM). The level of stimulation was similar to that elicited by diazepam ($E_{max}$ = 147 ± 6%; $EC_{50}$ = 0.8 ± 0.1 nM), and was occluded by GABA (0.3 μM). However, the three test compounds failed to elicit any significant effect (positive or negative) upon [$^3$H] flunitrazepam or [$^3$H] muscimol binding, indicating that they did not bind directly, or allosterically couple, to the benzodiazepine or agonist binding site of the $GABA_A$ receptor, respectively. The constituent monosaccharide, glucose, and both the closely related congeners octyl-β-D-glucoside or hexyl-β-D-glucoside have no significant effect upon [$^{35}$S] TBPS binding. These data, together, provide strong evidence that a β-glycosidic linkage and chain length are crucial for the positive modulation of [$^{35}$S] TBPS binding to the $GABA_A$ receptor by this novel chemical class.


*Keywords:* $GABA_A$ receptor; Allosteric modulator; TBPS; SAR; Channel site; Antagonist; β-Linkage; Sugar

## 1. Introduction

γ-Aminobutyric acid (GABA) is the principal inhibitory neurotransmitter in mammalian central nervous system. It is involved in a wide spectrum of physiological functions and behaviours, through binding to the ionotropic $GABA_A$ and metabotropic $GABA_B$ receptors, respectively. Actions of several important classes of clinically used drugs, such as benzodiazepines, barbiturates and anaesthetics, are at least partly mediated by allosteric interactions at the $GABA_A$ receptors [1,2].

The great molecular diversity of the multisubunit hetero-oligomeric $GABA_A$ receptors provides opportunities to develop novel drugs, e.g. for anxiety, sleep disorders, alcoholism and epilepsy, by establishing the relevant molecular targets for receptor subtype-specific action [3–5].

The high-affinity binding displayed by cage convulsants, such as TBPS, have proven to be useful in the development of radioligands for $GABA_A$ receptors and for their subsequent in vitro biochemical and pharmacological char-

*Abbreviations:* GABA, γ-aminobutyric acid; TBPS, *t*-butylbicyclophosphoorothionate; SAR, structure activity relationship; TBOB, *t*-butylbicycloorthobenzoate; DMSO, dimethylsulphoxide

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0006-2952/$ – see front matter 
doi:10.1016/j.bcp.2005.07.026

acterization [5]. These studies have revealed that $GABA_A$ receptors have multiple allosteric binding sites for drugs which, when occupied, modulate (positively or negatively) the inhibitory actions of GABA [1,2,5,6].

Caloporoside is a natural active fungal metabolite, which was isolated several years ago from fermentation of *Caloporous dichrous*, and was originally described to exhibit weak antibacterial and antifungal activity, as well as phospholipase C inhibitory activity [7]. In the same year, two related secondary metabolites were isolated from the same fungus species, and were reported, in a preliminary study, to act as inhibitors of $[^{35}S]$ TBPS binding to the $GABA_A$/benzodiazepine chloride channel receptor complex in vitro [8]. Synthesis and biological evaluation of the caloporoside analogue, deacetylated caloporoside, has been reported [9,10]. The compound appeared to display modest binding affinity for the $GABA_A$ receptor channel (cited $IC_{50} = 40–60\ \mu M$) [9,10]. Detailed pharmacological analyses were lacking in these reports. The chemical structure of caloporoside was elucidated by combination of chemical and spectroscopic methods [7,8,11]. Caloporoside consists of salicylic acid and a β-D-mannopyranosyl-D-mannonic acid moiety which are linked by an alkyl chain; the sugar part carries two acetyl residues at the 2- and 2′-position. Analogues of this compound have been described [11] which differ from the natural product in the aldohexose and the aldonic acid part. For example, the sugar moiety may be D-mannopyrannosyl-D-mannoic acid, which can be unsubstituted or substituted [11].

Successful chemical synthesis of the physiologically active fungal metabolite caloporoside has been described by our group [12,13]. The published procedure permits the synthesis of caloporoside and other closely related analogues, which may prove to be promising compounds for further biological evaluation. The other interesting issue relates to the sugar moiety of caloporoside, which is characterized by the highly unusual β- (1 → 5) linkage of a D-mannopyranoside unit to a D-mannonate ester. The stereoselective chemical synthesis of the β-mannopyranosidic linkage is not a trivial issue in carbohydrate chemistry, however, practical syntheses of β-mannopyranosides have been described [14,15]. Recently, a new strategy for the synthesis of mannopyranoside was reported [16,17].

Our laboratory showed that a simple polar deacetylated caloporoside derivative is a positive functional modulator of the $GABA_A$ chloride channel. Octyl-β-D mannopyranoside (100 μM) significantly and reversibly increased the magnitude of $GABA_A$ currents evoked in the cultured rat cortical pyramidal neurons [18]. A subsequent study demonstrated that a simple β-linked disaccharide, lactose, but not the α-linked disaccharides maltose or sucrose, can bind the $GABA_A$ receptor channel, detected by positive modulation of $[^3H]$ TBOB binding to the rodent $GABA_A$ receptor [6].

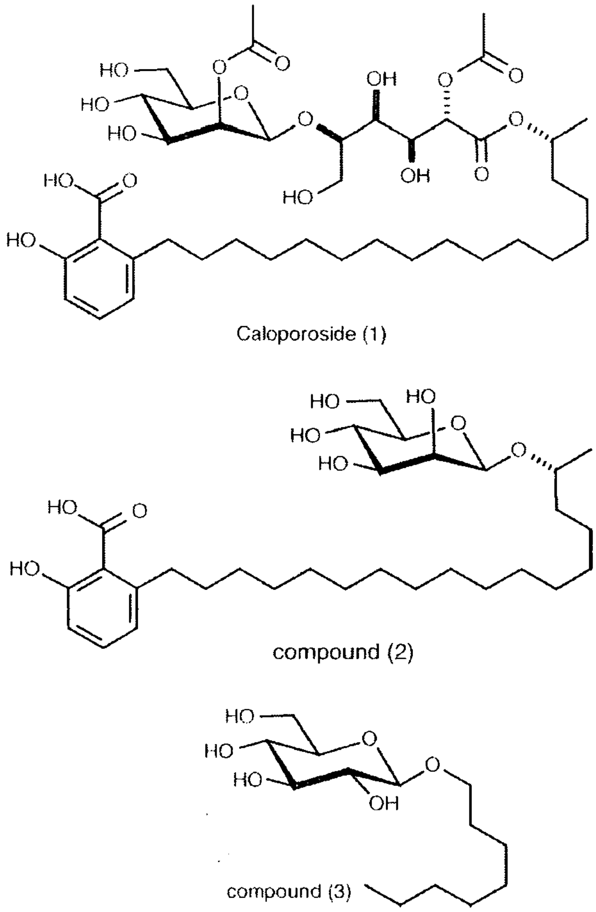


Fig. 1. Chemical structures of novel $GABA_A$ receptor compounds. Compound **1**, caloporoside; compound **2**, 2-hydroxy-6-{[(16R)-(β-D-mannopyranosyloxy)heptadecyl]} benzoic acid (HMHB); compound **3**, octyl-β-D-glucoside.

In this present report, we extend further the pharmacological binding profile of this new class of $GABA_A$ receptor ligand, using a radioligand binding approach with the high specific activity channel radioligand $[^{35}S]$ TBPS. Three compounds, with the chemical structures shown in Fig. 1, were studied in the first instance: the synthetic parent molecule Caloporoside, 2-hydroxy-6-{[(16R)-(β-D-mannopyranosyloxy)heptadecyl]} benzoic acid (HMHB), which lacks the mannonic acid ester segment (compound **2**), and octyl-β-D-glucoside (compound **3**). We provide new evidence that synthetic caloporoside is a low affinity $GABA_A$ receptor ligand and in contrast, that the small polar congener, octyl-β-D-glucoside is a high-affinity positive modulator of $[^{35}S]$ TBPS binding. Furthermore, we report that the modulatory activity of octyl-β-D-glucoside is dependent upon both the glycosidic linkage and length of the alkyl side chain.

A preliminary account of this work was reported recently in abstract form at the BPS conference in the University of Bath [19].

## 2. Materials and experimental procedures

### 2.1. Materials

[$^3$H] flunitrazepam, specific activity (91.0 Ci/mmol) was obtained from Amersham Biotech (Amersham), UK. [$^{35}$S]-*t*-butylbicyclophosphorothionate (TBPS), specific activity (80 Ci/mmol) from Perkin-Elmer Life Science, USA. [$^3$H] MK-801, specific activity (25 Ci/mmol) was obtained from ARC (USA). [$^3$H] muscimol, specific activity (36.5 Ci/mmol) was obtained from ARC (USA). Picrotoxinin, diazepam, γ-aminobutyric acid (GABA), octyl-α-D-glucoside, hexyl-β-D-glucoside, glutamate and ketamine were all obtained from Sigma Pharmaceuticals (Poole, UK). The three test compounds were synthesised in house, dissolved at $10^{-1}$ M in DMSO, and serial dilutions made with respective assay buffer. GABA stocks ($10^{-2}$ M) were made in assay buffer. Diazepam stocks ($10^{-2}$ M) were prepared in absolute ethanol. Picrotoxinin stocks ($10^{-2}$ M) were prepared in DMSO. Ketamine stocks ($10^{-2}$ M) were prepared in assay buffer. No effect of solvents on radioligand binding assays was seen at concentrations below 0.1% (v/v) DMSO or 0.1% (v/v) ethanol (data not shown).

### 2.2. Methods

A series of dose-response competition binding experiments were performed using [$^{35}$S] TBPS, [$^3$H] muscimol, [$^3$H] flunitrazepam and [$^3$H] MK-801 using well-washed adult rat forebrain membranes.

### 2.3. Tissue preparation

Adult male rats (200–300 g), Wistar strain, were maintained under a 12 h light, 12 h dark cycle at temperature of 23 °C and 65% humidity, with water and standard laboratory food available ad libidum. Animal treatment and husbandry were in accordance with approved use of animals in scientific procedures regulated by the Animals (Scientific Procedures) Act 1986, UK. The animals were killed humanely using a Schedule 1 procedure. The brains were rapidly removed, and the required tissue (forebrain) dissected immediately and kept cool on ice. The tissue was then homogenized in ice-cold homogenisation buffer (50 mM Tris–HCl, pH 7.4, containing 5 mM EDTA and 5 mM EGTA and 320 mM sucrose) using a dounce glass/glass homogenizer. The homogenate was centrifuged at 1000 × *g*, 4 °C for 10 min, the supernatant was stored in ice, and the pellets was re-homogenized in ice-cold buffer again, re-centrifuged at 1000 × *g*, 4 °C for 10 min. The supernatant from the first and second centrifugation steps were pooled together and centrifuged at 12,000 × *g*, 4 °C for 30 min. The supernatant was discarded and the pellet resuspended in 50 mM Tris containing 5 mM EDTA and 5 mM EGTA (5 ml/g of original tissue), and frozen at −20 °C.

### 2.4. Freeze-thaw protocol for the preparation of well-washed rat membranes

The $GABA_A$ receptor binding assays were performed with well-washed rat membranes prepared by a five-step freeze-thaw protocol [6]. The final aliquots (1 ml) were then frozen and stored at −20 °C.

### 2.5. Determination of protein concentration

The protein concentration was determined using the Lowry assay protocol [20] using Bovine serum albumin as the standard protein.

### 2.6. Radioligand binding assays

#### 2.6.1. [$^{35}$S]-t-butylbicyclophosphorothionate (TBPS) binding assay

[$^{35}$S] TBPS binding was performed essentially as described in [21]. Briefly, well-washed rat membranes prepared by a five-step freeze-thaw protocol were, on the day of experiment, centrifuged and the supernatant was discarded. The pellets were resuspended in fresh 50 mM Tris buffer containing 0.2 M NaCl, pH 7.4, to yield a final protein concentration in the assay of 1 mg/ml. An amount of 100 μg membrane protein was incubated with [$^{35}$S] TBPS (approximately 20 nM) for 90 min at 25 °C with a range of test concentrations ($10^{-11}$ to $10^{-4}$ M). This was sufficient incubation time to achieve equilibrium (data not shown). Non-specific binding was defined in the presence of 100 μM picrotoxinin.

#### 2.6.2. [$^3$H] muscimol binding assay

[$^3$H] muscimol binding assays were performed as previously described in [22]. Briefly, well-washed rat forebrain membranes prepared by five-step freeze-thaw protocol were thawed, centrifuged and the supernatant was discarded. The pellets were resuspended again in fresh 50 mM Tris buffer pH 7.4 to yield a final protein concentration in the assay of 1 mg/ml. An amount of 100 μg membrane protein was incubated with [$^3$H] muscimol (approximately 10 nM) for 1 h at 4 °C with a range of test concentrations ($10^{-11}$ to $10^{-4}$ M). Non-specific binding was defined in the presence of 100 μM GABA.

#### 2.6.3. [$^3$H] flunitrazepam binding assay

[$^3$H] flunitrazepam binding assays were performed as previously described [6,23]. Briefly, well-washed rat forebrain membranes prepared by five-step freeze-thaw protocol were thawed, centrifuged and the supernatant was discarded. The pellets were resuspended again in fresh 50 mM Tris buffer containing 5 mM EDTA and 5 mM EGTA to yield a final protein concentration in the assay of 1 mg/ml. An amount of 100 μg membrane protein was incubated with [$^3$H] flunitrazepam (approximately 1 nM) for 1 h at 4 °C with a range of test concentrations ($10^{-11}$ to

$10^{-4}$ M). Non-specific binding was defined in the presence of 100 μM diazepam.

### 2.6.4. [³H] MK-801 binding assay

[³H] MK-801 binding assays were performed as previously described [24]. Briefly, well-washed rat forebrain membranes prepared by five-step freeze-thaw protocol were thawed, centrifuged and the supernatant was discarded. The pellets were resuspended again in fresh 25 mM sodium phosphate buffer pH 7.4, to yield a final protein concentration in the assay of 1 mg/ml. An amount of 100 μg membrane protein was incubated with [³H] MK-801 (approximately 1 nM) and 10 μM glutamate for 2 h at 22 °C with a range of test concentrations ($10^{-11}$ to $10^{-4}$ M). Non-specific binding was defined in the presence of 10 mM ketamine.

All four binding assays were terminated by rapid filtration through Whatman GF/B filters pre-soaked in phosphate buffer, which were washed (3 ml × 3 ml) using ice-cold 10 mM sodium phosphate buffer (pH 7.4), using a Brandel cell harvester. Filters were transferred into scintillation vials, liquid scintillation fluid added and incubated for 16–24 h at room temperature. The bound radioactivity was quantified using Beckman LS 500 CE scintillation spectrophotometer with a counting time of 4 min per vial.

### 2.7. Data analysis

Results from the radioligand binding assays were analysed using non-linear least squares regression (GraphPad Prism 4 Software). Curves were best fitted to a one- or two-site binding model as described in [6]. The $EC_{50}$ and $IC_{50}$ values are the concentrations for half-maximal enhancement and displacement, respectively. Data were analysed using a Student's unpaired *t*-test, with levels of significance set at $p < 0.05$.

## 3. Results

### 3.1. The effect of the test compounds on [³⁵S] TBPS binding to the $GABA_A$ receptor

Two control compounds were tested in order to validate the assay and membrane preparation. Picrotoxinin displayed a steep monophasic inhibition of [³⁵S] TBPS binding with Hill slope close to unity (nH = 1.28 ± 0.22; apparent $IC_{50}$ = 0.33 ± 0.12 μM). In contrast, diazepam stimulated [³⁵S] TBPS binding with a mean $E_{max}$ = 147 ± 6%; apparent $EC_{50}$ = 0.80 ± 0.43 nM (mean ± S.D. for at least three individual experiments, Fig. 3B).

Caloporoside completely displaced specific [³⁵S] TBPS binding to well-washed membranes in a concentration-dependant manner. Data were best fit to a one-site binding model, with a pseudo Hill coefficient close to unity, yielding a mean apparent $IC_{50}$ = 14.7 ± 0.11 μM. HMHB also completely displaced specific [³⁵S] TBPS binding to well-washed membranes in a concentration dependant manner. Data were best fit to a one-site binding model, yielding a mean apparent $IC_{50}$ = 14.2 ± 0.1 μM (Fig. 2A and B). In contrast, octyl-β-D-glucoside stimulated [³⁵S] TBPS binding in a similar fashion to diazepam, yielding a mean $E_{max}$ = 144 ± 4% and apparent $EC_{50}$ = 39.2 ± 22.7 nM, respectively (Fig. 2C).

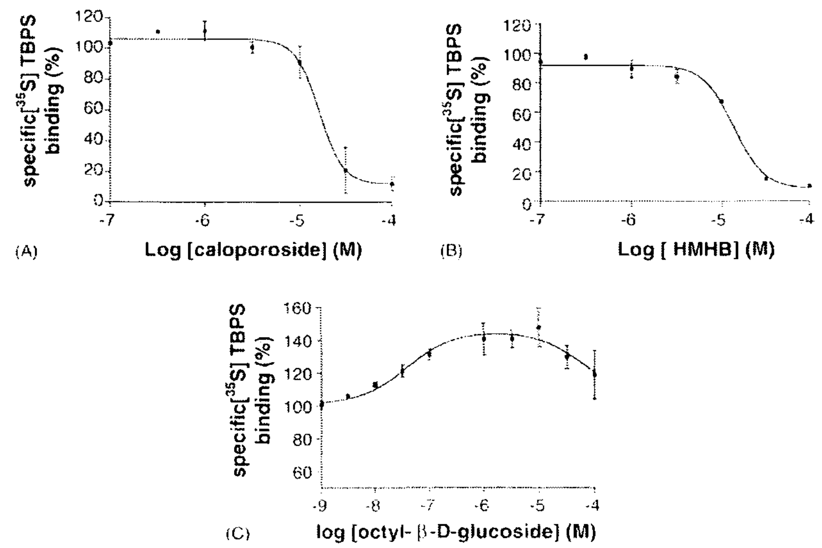


Fig. 2. Effect of caloporoside and congeners upon [³⁵S] TBPS binding to rat forebrain membranes. Effects of the compounds (A, caloporoside; B HMHB; C, octyl-β-D-glucoside) on specific [³⁵S] TBPS binding to well-washed adult rat forebrain membranes. Results are expressed as percentages (mean ± S.D. for three to six independent experiments) of control specific [³⁵S] TBPS binding in the absence of test compounds.

### 3.2. Sensitivity to GABA

In order to confirm that the stimulatory response was GABA-sensitive, 0.3 μM GABA was applied to the well-washed membranes. The presence of GABA partially reduced the overall [$^{35}$S] TBPS binding (by approximately 20%), and completely abolished the stimulation of [$^{35}$S] TBPS binding by both diazepam and octyl-β-D-glucoside (Fig. 3A and B). Octyl-β-D-glucoside failed to have any inhibitory or stimulatory effects in the presence of GABA.

### 3.3. The effect of octyl-β-D-glucoside on the agonist-binding site of the $GABA_A$ receptor labelled by [$^3$H] muscimol was investigated

In order to assess whether octyl-β-D-glucoside directly binds, or allosterically modulates muscimol binding to the agonist binding site, a range of concentrations of octyl-β-D-glucoside was tested upon [$^3$H] muscimol binding to a well-washed rat forebrain preparation. Specific binding was defined using 100 μM GABA. No significant effect (positive or negative) was detected across the full range of concentrations of octyl-β-D-glucoside in at least three independent experiments.

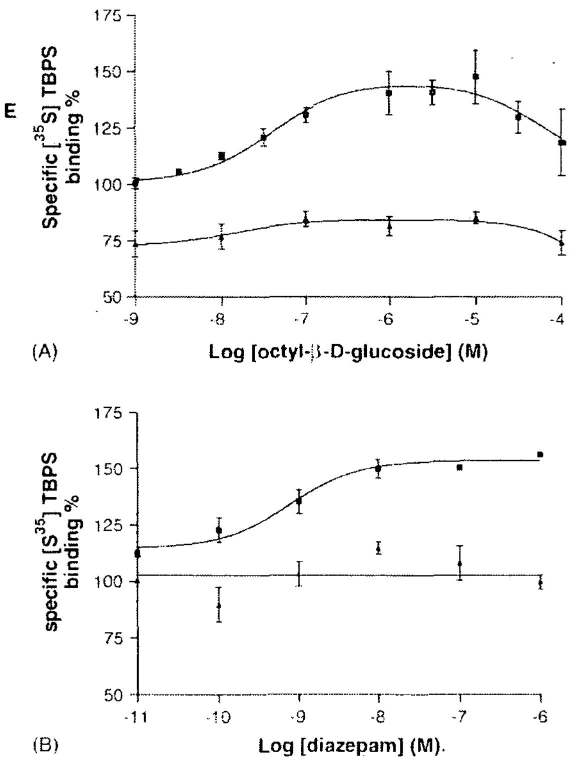


Fig. 3. Effect of GABA upon diazepam and octyl-β-D-glucoside modulation of [$^{35}$S] TBPS binding to rat forebrain membranes. Effect of diazepam or octyl-β-D-glucoside on specific [$^{35}$S] TBPS binding to well-washed adult rat forebrain membranes, in the absence (■) and presence of GABA (0.03 μM) (▼). Results are expressed as mean percentage values ± S.D. for three independent experiments.

### 3.4. The effect of the three compounds on the benzodiazepine-binding site of the $GABA_A$ receptor labelled by [$^3$H] flunitrazepam was investigated

In order to investigate whether caloporoside and the congeners are binding to the benzodiazepine site itself, a [$^3$H] flunitrazepam binding assay was used. Specific [$^3$H] flunitrazepam binding was defined using diazepam (100 μM) and represented >90% of the total binding (not shown). A control experiment of [$^3$H] flunitrazepam binding to rat forebrain, in the presence of different concentrations of GABA, was carried out to validate the assay. GABA significantly enhanced specific binding of [$^3$H] flunitrazepam to rat well washed forebrain in a concentration-dependent manner, yielding a mean $E_{max}$ = 153 ± 4% and apparent $EC_{50}$ = 31 ± 20 nM ($n$ = 3 independent experiments).

In contrast, no significant effect (positive or negative) was observed with caloporoside, HMHB or octyl-β-D-glucoside upon [$^3$H] flunitrazepam binding in at least five independent experiments. This suggests a lack of interaction (either directly or allosterically) of these three compounds with the benzodiazepine site of the $GABA_A$ receptor. A small reduction in binding observed at a test concentration of 100 μM for the three compounds was due to the presence of 0.1% DMSO (solvent effect).

### 3.5. Influence of the side chain carbon length and stereochemistry

The core monosaccharide of compound **3**, i.e. glucose, was tested and found to have no significant effect upon [$^{35}$S] TBPS binding up to concentration of 100 μM (Table 1). Furthermore, neither octyl-α-D-glucoside nor hexyl-β-D-glucoside elicited significant effects upon [$^{35}$S] TBPS binding up to concentration of 100 μM (Table 1).

### 3.6. Does lactose bind to the same site as octyl-β-D-glucoside?

Previously, we showed that lactose potentiated [$^3$H] TBOB binding to the channel site of the $GABA_A$ receptor,

Table 1
Pharmacological effect of a range of related compounds upon [$^{35}$S] TBPS binding to adult rat forebrain membranes

| Compound | Effect ($10^{-10}$ to $10^{-4}$ M) |
|---|---|
| Glucose | NE |
| Hexyl-D-β-glucoside | NE |
| Octyl-D-β-glucoside | $EC_{50}$ = 39 ± 23 nM, $E_{max}$ = 144 ± 4% |
| Octyl-D-α-glucoside | NE |
| Lactose (β-linked disaccharide) | NE |
| Octyl-D-β-glucoside + lactose ($10^{-5}$ M) | NE |

A series of related compounds were assayed for any potential effects upon [$^{35}$S] TBPS binding to well-washed adult rat forebrain membranes, over concentration range of $10^{-10}$ to $10^{-4}$ M ($n$ = 3–6 separate experiments). NE, no significant effect detected (positive or negative) ($p$ > 0.5).

with a maximal effect observed at 10 μM. In contrast, interestingly, lactose has no effect upon [$^{35}$S] TBPS binding up to 100 μM. However, we showed that lactose (10 μM) completely occluded the potentiation by octyl-α-D-glucoside of [$^{35}$S] TBPS binding. Octyl-β-D-glucoside failed to have any inhibitory or stimulatory effects in the presence of lactose (Table 1).

### 3.7. Selectivity of action of octyl-β-D-glucoside upon GABA$_A$ receptors

Octyl-β-D-glucoside had no effect (positive or negative) upon [$^3$H] MK-801 binding up to a 100 μM.

## 4. Discussion

The effects of caloporoside and two smaller congeners were assayed using a [$^{35}$S] TBPS binding assay on adult rat forebrain membranes. These data suggest that caloporoside and HMHB are low affinity GABA$_A$ receptor channel ligands, while, in contrast, octyl-β-D-glucoside is a relatively high affinity positive GABA$_A$ receptor channel modulator. The positive modulatory effect of octyl-β-D-glucoside was occluded in the presence of GABA, in a similar fashion to benzodiazepines, indicating that the modulatory action of octyl-glucoside is related to the conformational state of the chloride channel [25]. GABA sensitivity is shared by a number of other GABA$_A$ receptor modulators, as well as benzodiazepines, including loreclezole, propofol and lactose [6,25,26]. The lack of inhibitory action of octyl-β-D-glucoside at high concentrations is a property shared by diazepam, but not loreclezole or propofol. This property has been previously attributed to the lack of ability of diazepam to activate GABA$_A$ receptor channel in the absence of GABA [26].

The lack of effect of octyl-β-D-glucoside upon [$^3$H] muscimol binding demonstrated that octyl-β-D-glucoside does not directly bind to the agonist binding site. Based on shared properties of octyl-β-D-glucoside and diazepam in modulating [$^{35}$S] TBPS, we also directly investigated the effect of octyl-β-D-glucoside upon [$^3$H] flunitrazepam binding, using well-washed membranes. Neither caloporoside, HMHB nor octyl-β-D-glucoside displayed any significant (positive or negative) effect upon [$^3$H] flunitrazepam binding, which strongly suggested a lack of allosteric or competitive linkage with the benzodiazepine site. This property is in marked contrast to other ligands tested, such as GABA and diazepam, respectively. GABA positively modulates and diazepam competitively inhibited [$^3$H] flunitrazepam binding, consistent with previous studies. These data confirm that the novel compound class binds to a unique site on the GABA$_A$ receptor.

It should be noted that octyl-β-D-glucoside has been previously used as a detergent for the solubilisation of GABA$_A$ receptors, but at high mM concentrations (e.g. [27]). However, the propensity of octyl-β-D-glucoside to bind to membranes indicates that it may bind within the membrane spanning channel domain of the GABA$_A$ receptor. The lack of effect of octyl-β-D-glucoside upon channel binding of [$^3$H] MK-801 to another common ligand gated channel, namely the NMDA glutamate receptor suggests that octyl-β-D-glucoside does not bind non-selectively, and indiscriminately modulate all ligand-gated channels in the membrane.

Interestingly, the monosaccharide present in compound **3**, glucose had no significant effect upon [$^{35}$S] TBPS indicating that the presence of the extended side chain was absolutely necessary for GABA$_A$ receptor modulation. In order to investigate whether the nature of the glycosidic linkage is important, we compared, in parallel, the effects of octyl-α-D-glucoside, hexyl-β-D-glucoside and octyl-β-D-glucoside over the same concentration range. In contrast to octyl-β-D-glucoside, neither octyl-α-D-glucoside nor hexyl-β-D-glucoside significantly affected [$^{35}$S] TBPS binding. This strongly indicated that both the β-linkage and an alkyl side-chain in excess of 6-C in length, was crucial for the positive modulation of [$^{35}$S] TBPS binding. These results extend upon our previous observations with β- and α-linked disaccharides, which showed that β-glycosidic linkage yielded higher affinity GABA$_A$ receptor binding than α-glycosidic linkage [6].

Interestingly, lactose had no affect upon [$^{35}$S] TBPS which is in contrast to its affect upon [$^3$H] TBOB binding [6]. The differences in salt concentration in the two assays may explain this difference. Furthermore, the expanded structure of TBOB in comparison to TBPS may account for the differential allosteric influence of lactose and warrants further study. However, lactose (10 μM) completely blocked the positive modulation of [$^{35}$S] TBPS, which provides evidence for a shared binding site between these two β-glycosidic linked ligands.

In conclusion, this study has delineated clear differences in the pharmacological binding properties of the large natural product caloporoside and the small polar congener, octyl-β-D-glucoside. The findings reported in this study also provides evidence, firstly that octyl-β-D-glucoside binding is independent of the benzodiazepine and agonist binding sites, secondly, that the side chain is absolutely required for activity, and thirdly that glycosidic linkage and side chain length are important determinants of the modulatory activity. This present study has provided a clearer picture of the SAR of this novel class of GABA$_A$ receptor modulator, which warrants further elucidation using GABA$_A$ receptor electrophysiological and behavioural studies [18,28].

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