THE EFFECTS OF CHRONIC ETHANOL INTAKE ON THE ALLOSTERIC INTERACTION BETWEEN GABA AND BENZODIAZEPINE AT THE GABAA RECEPTOR

THESIS

Presented to the Graduate Council of the University of North Texas in Partial Fulfillment of the Requirements For the Degree of

MASTER OF SCIENCE

By

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May, 1992
Chen, Jianping, The Effects of Chronic Ethanol Intake on the Allosteric Interaction Between GABA and Benzodiazepine at the GABAA Receptor. Master of Science (Biomedical Sciences/Pharmacology), May, 1992, 133 pp., 4 tables, 30 figures, references, 103 titles.

This study examined the effects of chronic ethanol intake on the density, affinity, and allosteric modulation of rat brain GABA_A receptor subtypes. In the presence of GABA, the apparent affinity for the benzodiazepine agonist flunitrazepam was increased and for the inverse agonist RO15-4513 was decreased. No alteration in the capacity of GABA to modulate flunitrazepam and RO15-4513 binding was observed in membranes prepared from cortex, hippocampus or cerebellum following chronic ethanol intake or withdrawal. The results also demonstrate two different binding sites for [^3H]RO 15-4513 in rat cerebellum that differ in their affinities for diazepam. Chronic ethanol treatment and withdrawal did not significantly change the apparent affinity or density of these two receptor subtypes.
ACKNOWLEDGEMENT

I would like to express my sincere thanks to my major professor, Dr. Michael W. Martin. I deeply appreciate his guidance and direction which initiated this study, and his kindness in sharing his laboratory facilities with me. His suggestions, patience, encouragement and support in the laboratory have contributed significantly to my understanding of the receptor mechanism of drug action. The friendship he has extended to me during the past two years is highly valued and greatly appreciated.

I would also like to thank Drs. Lal, Yorio and Leudtke for their diverse assistance which aided me in the completion of this thesis. A special thanks to Dr. Wallis for her help in animal treating and technical support in laboratory work.

My sincere thanks to Drs. Prather and Razazadeh for their assistance in supplying rat brain membranes and some chemicals which enabled completion of the study.

Finally, I would like to acknowledge all of the assistance and support I have received from the faculty and especially the staff of the Pharmacology Department at Texas College of Osteopathic Medicine, Fort Worth, Texas.
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INTRODUCTION

Abuse of alcohol ranks as one of the top health problems of the world. Yet, the mechanism by which alcohol acts is not completely understood. Based on recent evidence that the receptor for gamma-aminobutyric acid (GABA) is an important site of alcohol action, the present investigation was designed to test if chronic ethanol intake induces any changes in the ligand binding properties of the GABA receptor complex, especially the allosteric binding interactions between GABA and benzodiazepine agonists and inverse agonists. The principle hypothesis is that prolonged ethanol administration reduces the functional capacity of GABA to modulate the GABA receptor-coupled chloride channel as measured by ligand binding. This reduced functional capacity of the GABA receptor should be evident during the early stages of ethanol withdrawal and could contribute to the central nervous system dysfunction evident during ethanol withdrawal.

Role of GABA as a Major Inhibitory Neurotransmitter in the Mammalian CNS

The activity of the nervous system is not only a pattern of excitation; it is a balance of both excitation
and inhibition. There is an entire class of operations in the nervous system that is inhibitory rather than excitatory. These operations are mediated by a special network of inhibitory neurons that, rather than exciting their target cells, depress the firing of target cells or eliminates their firing entirely. The function of inhibitory neural networks is becoming clearer. These networks act as brakes on the entire nervous system to prevent a runaway spree of neuronal firing and help to adjust the specific responsiveness of the excitatory networks that convey and interpret information about the external world. The inhibitory effects are accomplished by the release of specific molecules called inhibitory neurotransmitters.

The most prevalent inhibitory neurotransmitter in the mammalian central nervous system is gamma-aminobutyric acid, or GABA. Neurons that secrete GABA are referred to as GABAergic. GABA was first discovered in the mammalian central nervous system early in the 1950's. At that time many believed that this substance was merely a metabolic byproduct. During later years, it has become apparent that this chemically simple substance serves an important neurotransmitter function (Enna and Gallagher, 1983).
The synthesis, storage, release, reuptake, and metabolism of GABA generally satisfy necessary criteria for a neurotransmitter substance. As showed in figure 1, GABA is synthesised in the nerve terminal and the majority of GABA found in brain is derived from glutamic acid (Yoneda and Roberts, 1982). The conversion of glutamate to GABA is catalyzed by the enzyme glutamic acid decarboxylase and GABA is converted primarily to succinic semialdehyde by the action of GABA transaminase, though other pathways have been suggested. Both of these enzymes are found in association with GABAergic cells. A significant amount of GABA is stored in nerve terminals and numerous studies have shown that it is released by a calcium-dependent process upon action potential. Like many other neurotransmitters, the major portion of GABA released following depolarization is actively and efficiently taken up and reaccumulated in the nerve terminal, though a significant amount may also be transported into glia. Observations supporting a neurotransmitter role for GABA were crucial for the development of the general concept of amino acid transmission. That is, because of the impetus provided by studies on GABA, extensive research now suggests that other amino acid derivatives, such as glutamic acid, glycine, and taurine may also act as neurotransmitters in the central nervous system.
Figure 1. Schematic illustration of a GABAergic neuron indicating the major pathway for the synthesis and degradation of GABA. The conversion of the glutamate to GABA is catalyzed by the enzyme glutamic acid decarboxylase (GAD), and GABA is converted primarily to succinic semialdehyde (SSA) by the action of GABA transaminase (GABA-T). A significant amount of GABA is stored in nerve terminals, and released following nerve stimulation. The major portion of GABA released following depolarization is in the nerve ending by an active transport process. Bicuculline is a GABA antagonist at the receptor.
Although early biochemical studies supported a neurotransmitter role for GABA, the evidence of such a function in mammalian systems were not compelling until electrophysiological techniques demonstrated a specific response to this substance (Curtis and Watkins, 1965). Taken together, the binding of GABA to the receptor activated the chloride channel and caused an influx of Cl⁻. This chloride ion influx causes hyperpolarization of the nerve cell and thus produces inhibition of cell activity.

The GABA receptor is a ligand-gated chloride ion channel that mediates the majority of inhibitory synapses in the central nervous system. The GABA receptor-chloride ionophore complex contains binding domains for GABA, benzodiazepines, barbiturates and picrotoxin with each recognition site contributing to the control of chloride flux through the channel (fig.2). Receptor activation changes the intracellular concentration of chloride and thus alters the polarity of the neuron. In general, cellular activity diminishes following GABA receptor activation, leading to the designation of GABA as an inhibitory neurotransmitter. The most important finding in this regard was the discovery of picrotoxin and bicuculline, agents that could rather selectively block the electrophysiological actions of GABA (Curtis et al, 1971).
Figure 2. Proposed model of the GABA-benzodiazepine-chloride channel receptor complex. GABA binding to its receptor activates the chloride channel through a coupling mechanism involving GABA-modulin and the benzodiazepine agonist (BZ) receptor protein (1). Binding of benzodiazepine (BZ) agonist enhances this coupling function (2) and increases GABA binding in a reciprocal relationship (3). Binding of barbiturates (BARB) also enhances the coupling function (4), increases the affinity of GABA for its receptor (5), and may directly activate the chloride channel at high concentrations (6). (Adapted From Katzung, B.G., Basic and Clinical Pharmacology, pp270, 1989.)
These data indicated the presence of a physiologically active receptor on mammalian neurons, thus firmly establishing GABA as a neurotransmitter.

**Drug Interaction at the GABA Receptor-ionophore Complex**

Since GABAergic inhibitory neurons are so widely distributed in the CNS, it is understandable that manipulation of the GABA receptor in the mammalian central nervous system has major effects on animal behavior (Arnt and Scheel-Kruger, 1979; Scheel-Kruger et al, 1980). In general, GABA receptor activation results in sedation and a decrease in muscle tone and motor activity (Blavet et al, 1982). Blockade of the central nervous system GABA receptors by either bicuculline or picrotoxin leads to excitation and generalized seizures (Enna et al, 1981). This response illustrates the importance of the GABAergic system in exerting a tonic inhibitory effect on central nervous system activity. These observations also led to the belief that GABA receptor agonists may be useful in the treatment of some types of epilepsy (Meldrum, 1975).

Selective activation or inhibition of GABA receptors in the basal ganglia can dramatically alter motor activity and seizure threshold. This suggests that the receptors in this brain region may play an important role in
extrapyramidal function. Indeed, abnormalities in basal ganglia GABA activity are thought to be caused by motor impairments associated with some neurological disorders (Enna et al, 1981).

GABA receptors may also influence sensory responses (Kendall et al, 1982). It has been observed that the activation of central GABA receptors causes analgesia similar to that observed with opiates (Andree et al, 1983). Furthermore, animals tolerant to opiate analgesia are cross tolerant to GABA receptor agonists, suggesting that the two system are indirectly linked (Andree et al, 1983).

As described above, one of the distinctive characteristics of the GABA receptor is their wide spread distribution throughout the CNS (Schofield et al, 1987). Both GABA-containing neurons and GABA binding sites are found in all regions of this system suggesting that GABA is one of the most important neuroregulators of brain function. Thus arises a problem with regard to the development of GABAergic drugs. That is, are all GABA receptors pharmacologically and functionally identical?

Increasing evidence supports that a role for the GABAergic system in the action of many central nervous system depressant and excitatory drugs. The major type of inhibitory synaptic transmission mediated by GABA involves a rapid increase in the postsynaptic membrane conductance.
to chloride ion (Nistri et al, 1980) following the interaction of GABA with its recognition sites, the receptor. The rapid time course indicates that the neurotransmitter receptor is tightly coupled with the ion channel, with opening and closing of the ion channel being determined by the binding of the neurotransmitter at a regulatory site. Modulation of the postsynaptic GABA receptor-chloride ionophore complex appears to account for many of the sedative, anticonvulsant, anxiolytic, and muscle relaxant actions of benzodiazepines (Haefelt et al, 1979; Simmonds, 1981) and also the sedative-hypnotic and anticonvulsant actions of barbiturates (Simmonds, 1981; Andrews and Johnston, 1979), as well as the convulsant action of picrotoxin and related substances (Simmonds, 1981).

The role of GABA/BZ receptor complex in mediating the action of anxiolytic, sedative-hypnotic, and depressant drugs has been studied using electrophysiological and radioligand binding techniques. Electrophysiological studies have shown that benzodiazepines and barbiturates decrease neuronal excitability by enhancing GABA-mediated opening of the chloride ion channel, resulting in membrane hyperpolarization. Further, benzodiazepine inverse agonists which produce anxiogenic and convulsant behavior, diminish GABA's inhibition action. Similarly, the GABA
antagonist bicuculline and chloride channel blocker picrotoxin decrease GABA-mediated chloride conductance and thus increase excitability. Radioligand binding studies have demonstrated that benzodiazepines and barbiturates bind to distinct recognition sites on the GABAA receptor complex. These sites are also distinct from the GABA recognition site but interact allosterically to influence GABA receptor binding. Thus, benzodiazepines and barbiturates enhance GABA's action in a chloride dependent manner.

Various types of agents have been developed as indirectly acting GABA agonists (Enna et al., 1981), including substances that inhibit GABA transaminase, such as γ-acetylenic GABA, and inhibitors of high affinity GABA transport, such as nipecotic acid ethyl ester. Although neither of these drugs has any appreciable affinity for the GABA receptor recognition site, both activate this system by increasing the synaptic content of the amino acid.

In regard to GABA antagonists, bicuculline and picrotoxin are the most extensively studied. Bicuculline appears to act directly at the GABA receptor recognition site as a competitive antagonist for GABA binding whereas picrotoxin seems to interact at the ion channel (Enna and Maggi, 1979). This picrotoxin site may also be the receptor region at which certain culvulsants act to block
GABA receptor activity (Ticku et al, 1978).

Since GABA receptor antagonists are all potent convulsants, these drugs have no obvious clinical utility. On the other hand, GABA receptor agonists such as muscimol and progabide may be potentially useful for the treatment of a variety of neuropsychiatric illnesses, including Huntington's disease, epilepsy, anxiety, and depression (Enna et al, 1981).

The GABA Receptor Complex

GABA initiates its effects by binding to a specific receptor protein complex on the extracellular surface of the target cell. Great strides were made in understanding the nature of the GABA receptor following the introduction of radiolabeled ligands that specifically bind to the receptor. Numerous studies have been performed since Peck et al. in 1973 (Peck et al.), Zukin et al. (1974), and Enna and Snyder (1975) first demonstrated the saturable binding of $^3$H-GABA to brain synaptic membranes. Further crucial evidence for a specific receptor has come from studies with the selective GABA antagonist bicuculline and its methohalide salts (Curtis et al, 1970; Curtis et al, 1971).

For the GABA receptor assay, small amounts of tissue are incubated with a radioactive ligand, most typically $^3$H-
GABA or \(^{3}\text{H}\)-muscimol, a GABA receptor agonist, and the amount of radioligand bound to the receptor recognition site is quantified by inhibiting isotope attachment with a high concentration of unlabeled ligand. The amount of radioactivity that remains attached to the tissue in the presence of the unlabeled species represents nonspecific binding, and is subtracted from the total binding to calculated the amount of bound radioligand associated with the receptor recognition site.

One radioactive antagonist that has been employed to assay the GABA receptor is \(^{3}\text{H}\)-bicuculline methiode. This compound is able to bind to the receptor recognition site and competitively inhibit \(^{3}\text{H}\)-GABA binding but it has no intrinsic activity of itself.

GABA initiates its inhibitory effects by binding to a postsynaptic GABA receptor complex which is thought to consist of three functional components, namely the GABA, the benzodiazepine, and the TBPS (t-butylbicyclophorothionate) "receptors" (Haefely, 1984; Olsen et al, 1984). The TBPS receptor probably represents a site within or near the chloride channel whose opening is triggered by the binding of GABA to the receptor. Channel opening is modulated by the benzodiazepine receptor which, depending on the type of ligand, either enhances or reduces GABA receptor function (Mohler 1984). Modulation of this type
of GABA receptor by numerous CNS depressant drugs appears to mediate their actions as anxiolytics, anticonvulsants, sedative-hypnotics, and muscle relaxants (Haefely and Polc, 1983). The GABA hypothesis of anxiety control is supported by the finding that receptor sites for two classes of anxiolytic drugs, the benzodiazepines and barbiturates, are physically associated with the postsynaptic membrane GABA receptor–chloride ion channel complex.

GABA receptors are widely distributed in the CNS and are involved in modulating CNS excitability both pre- and post-synaptically. Historically, GABA receptors have been classified into two types: GABAA and GABAB. GABAA receptors are activated by GABA agonists like muscimol and open chloride channels that are antagonized by picrotoxin and bicuculline. Benzodiazepine agonists and barbiturates facilitate GABAA responses. GABAB receptors can be selectively activated by the antispastic drug baclofen and their responses are blocked by phaclofen. These receptors appear to be coupled to voltage-gated calcium channels, potassium channels and/or G-proteins in the postsynaptic membrane.

Radioligand binding studies have demonstrated that the subunits, which form the GABAA complex, have multiple allosteric binding sites for GABA agonists, benzodiazepines, picrotoxin-like convulsants, barbiturates,
and steroids (Enna, 1979). When benzodiazepines bind to its binding sites, the binding affinity for GABA is increased therefore chloride channel activity is increased. GABA enhance benzodiazepine agonist binding vice versa.

Benzodiazepines are among the most widely prescribed drugs in the world. A unifying hypothesis for their action in the body has been developed based upon the ability of the benzodiazepines to interact with GABAergic mechanisms. The relationship between GABA and the benzodiazepines had been predicted on the basis of electrophysiological experiments. These studies indicated that in the presence of benzodiazepines, the response of neuronal cells to GABA was enhanced. Generally, the response of GABA is inhibitory on neuronal activity and is the result of its hyperpolarization caused by the opening of an ion channel leading to increased chloride flux. Intracellular studies have indicated that the frequency of transition from a closed channel to a open state is increased in the presence of benzodiazepines (Study and Barker, 1981). A simple model of the putative mechanism of benzodiazepine action on the GABAA receptor is shown in fig. 3. In the first step, benzodiazepines bind to a specific recognition site (1), causing a change in receptor protein conformation (2). This structural change induces a change in the conformation of a separate and distinct GABA binding site (3),
Figure 3. A model of the putative mechanism of benzodiazepine action on GABAA receptors. The first step is benzodiazepine binding to the receptor binding sites (1), causing changes in the receptor protein conformation (2). This conformational change induces specific rearrangement of portions of the molecule that form the GABA binding site (3), thus allowing GABA to bind to the receptor with higher affinity (4). When GABA binds to the receptor, the chloride channel is opened and Cl\textsuperscript{-} flows into the cell (5). This Cl\textsuperscript{-} influx cause hyperpolarization and inhibition of cellular excitability.
increasing the receptor affinity for GABA (4). When GABA binds to the receptor, chloride channels are opened and Cl- ions flow into the cell (5), leading to hyperpolarization and inhibition of neuronal excitability. Three types of responses can occur when benzodiazepines or their competitive inhibitors bind to the GABA receptor. The binding can elicit what is termed an agonist response, positively modulating the opening of the GABA-chloride channel. A second class of interaction is exemplified by the classical benzodiazepine receptor antagonist RO15-1788, flumazenil, an imidazobenzodiazepine which binds to the receptor, blocks binding and effects, but has no intrinsic activity of its own. Finally, binding of certain ligands such as DMCM, RO15-4513, F67143, and β-CCE can elicit a negative response, in which the opening of the GABA-Cl- channel is negatively modulated. Compounds with this efficacy are called inverse agonists. These kinds of compounds are anxiogenic and can be either proconvulsant or convulsant.

The GABA Shift Test A biochemical test for the pharmacological and behavioral properties of compounds that interact with benzodiazepine receptors has been developed based on the effects of GABA described above. This test has become known as the "GABA shift". Benzodiazepine agonists show an increase in their affinity for the
receptor in the presence of GABA. In contrast, compounds without dominant intrinsic activity, i.e., antagonists, show no change in receptor affinity in the presence of GABA (Mohler and Richards, 1981). This is consistent with their possessing almost no activity beyond the ability to block the actions of benzodiazepine agonists. Compounds with intrinsic activity opposite to anxiolytic benzodiazepine agonists, i.e., inverse agonists, actually show a decrease in the affinity for receptor in the presence of GABA (Braestrup and Nielsen, 1981). Thus, the GABA shift predicts generally, although not perfectly, the behavioral properties of compounds that interact at this site. Partial agonists with some benzodiazepine-like activities are also predicted by the GABA shift. Some of these compounds may turn out to be very useful in therapy since these compounds may have significant anxiolytic activity while not producing some of the more severe sedative side effects associated with full agonists.

**Benzodiazepines as Agonists** Benzodiazepines are widely used as anxiolytics, hypnotics, and anticonvulsants. They are considered positive modulators of GABAA receptor function in that they enhance opening of Cl− channels and therefore hyperpolarizing the cell and reducing spontaneous firing. The discovery of specific high affinity binding sites for 3H-diazepam (Squires and Braestrup, 1977; Mohler
and Okada, 1977) or $^3$H-flunitrazepam (Steth et al, 1978) in brain membranes has stimulated research on the molecular mechanism of action of benzodiazepines. Radiolabeled benzodiazepines bind to brain membranes with high affinity in a rapid, reversible, and saturable manner. There is an excellent correlation between the clinical potency of a series of benzodiazepines and their ability to displace $^3$H-diazepam or $^3$H-flunitrazepam from their binding sites on rat membrane preparations. Furthermore, a close association of these binding sites with a GABA receptor, a chloride ion channel, and several drug binding sites has been demonstrated (Olsen, 1982; Squires, 1984). Because these binding sites have been found in the CNS of all vertebrate species and most of the actions of the classical benzodiazepines seem to be centrally mediated, (Haefely et al, 1981) it is generally assumed that the specific binding sites for $^3$H-diazepam or $^3$H-flunitrazepam are the receptors through which the benzodiazepines exert their pharmacological and clinical actions.

When the potentiating action of benzodiazepine tranquilizers on GABAergic synaptic transmission was discovered, the exact site and mechanism of these drugs was unknown. The finding that GABA enhances benzodiazepine binding, and that benzodiazepine agonists enhance GABA binding, strongly suggested a bidirectional, allosteric
interaction between GABA and the benzodiazepine binding sites on the same supramolecular complex. Numerous studies using radioligand binding and antibodies strongly suggested the existence of a receptor-channel complex consisting of different subunits carrying GABA, benzodiazepine and other drug binding sites, respectively, as shown in the hypothetical model in Figure 2.

Three Classes of Ligands for the Benzodiazepine Receptor

The benzodiazepine receptor is the first receptor known to mediate two diametrically opposite effects, namely facilitation and inhibition of GABA receptor function, which both can be blocked by a third class of ligand. This situation raises problems of mechanistic interpretation. Since the benzodiazepine tranquilizers were the first compounds used to identify the binding site that mediates most, if not all, of the relevant effects of benzodiazepine, it was logical to call it the benzodiazepine receptor. Flumazenil (RO 15-1788), a compound was found which binds to the same binding site as classical benzodiazepine tranquilizers without inducing an easily detectable effect by itself, but selectively inhibiting that of the former agents. The positive allosteric modulators of GABAA receptor function were called benzodiazepine agonists, and ligands acting like flumazenil were called benzodiazepine antagonists. The
problem became more complicated when β-carbolines were discovered and shown to produce effects diametrically opposite to those of benzodiazepine agonist (depression of GABAA receptor function) at a site indistinguishable from benzodiazepine agonists, which accordingly were blocked by benzodiazepine antagonists. Terms such as convulsive or anxiogenic benzodiazepine ligands were used at the beginning. Later the term "inverse agonist" was introduced to indicate those compounds have a intrinsic activity at the GABAA receptor-ionophore. Compounds that exhibit the entire spectrum of activity between zero and maximal negative allosteric modulation have been described. In behavioral tests, benzodiazepine agonists relieve anxiety and show sedative and anticonvulsant properties. In contrast, the inverse agonists such as DMCM, βCCE, βCCM, FG 7142 and RO 15-4513 have a profile of pharmacological activity opposite to that of agonists. In fact they are proconvulsants or convulsants and anxiogenic in animals and in humans. The effects of both agonist and inverse agonists are antagonised by a third class of ligands, such as RO 15-1788 and ZK 93426, which are not able to modify the function of the GABA receptor complex by themselves and therefore are classified as antagonists.

**RO 15-4513 as an inverse agonist** The imidazodiazepine, RO 15-4513 (ethyl 8-amino-5-methyl-6-oxo-4H-imidazo [1,5-
[1,4] benzodiazepine-3-carboxylate) has been reported to have inverse agonist activity at the GABA receptor. It can reverse most of the behavioral effects involving the chloride channel. RO 15-4513 came into prominence because it was active in blocking some of the effects of ethanol (Bonetti et al, 1985; Suzdak et al, 1986). Structurally, RO 15-4513 is a analogue of the benzodiazepine receptor antagonist RO 15-1788 (fig. 4). Both drugs compete for the same binding sites in brain tissue. Under physiological conditions, RO 15-4513 binds reversibly to central benzodiazepine receptors. When exposed to ultraviolet radiation, RO 15-4513 forms covalent bonds with these receptors. Because of this property, it has been used as a photoaffinity ligand for the benzodiazepine receptor (Mohler et al, 1984). These binding studies have been interpreted to indicate that RO 15-4513 binds to all conformational states of central benzodiazepine receptors and it also binds to substrates in the cerebellum that are not displaced by diazepam or βCCE. These cerebellar sites have been described as "non specific" (Sieghart et al, 1987), but they may be relevant to some of the effects of RO 15-4513 such as its ability to block the motor incoordinating effects of ethanol.

Researchers at Hoffmann-La Roche reported several years ago that RO 15-4513 was capable of antagonizing some of the
Figure 4. Chemical structure of the excitatory/convulsant imidazodiazepine RO 15-4513 and the benzodiazepine receptor antagonist flumazenil (RO 15-1788).
electrophysiological and behavioral effects of ethanol. Suzdak and coworkers (Suzdak et al, 1986) confirmed these findings and reported that RO 15-4513 not only reversed ethanol behavioral effects but also antagonized ethanol stimulated $^{36}\text{Cl}^-$ uptake into isolated brain vesicles. These effects seemed to be mediated by the benzodiazepine receptor, since they were prevented by the benzodiazepine receptor antagonist RO 15-1788. The capacity of RO 15-4513 to reduce GABA binding is probably due to allosteric coupling between the benzodiazepine and GABA receptors. Thus, the antagonism of ethanol by RO 15-4513 may involve RO 15-4513 binding to the benzodiazepine receptor, consequent lowering of the affinity of the GABA receptor for GABA, and resultant prevention of the GABA-mediated effects of low doses of ethanol (Ticku et al, 1988). RO 15-4513 also antagonizes the direct effects of ethanol, that is, direct effects on synaptosomal chloride conductance produced by high concentration of ethanol in the absence of GABA (Suzdak et al, 1986). So it was initially hoped that RO 15-4513 might help reveal the biochemical basis for ethanol's effects. However, this antagonism is not specific to ethanol and its proconvulsant properties make it unsuitable for use in humans. But it is a potentially powerful tool with which to investigate the neuropharmacology of ethanol and other CNS depressants.
Ethanol and the Benzodiazepine-GABA Receptor Ionophore Complex

Ethanol is perhaps the most widely used and abused psychotropic drug known to mankind. The molecular mechanism by which ethanol produces its effects and the neuronal components involved in ethanol action have been a matter of debate for many years. Ethanol has many properties in common with benzodiazepines and barbiturates such as anxiolytic, anticonvulsant and muscle relaxant actions. Large doses of ethanol cause behavioral depression and anaesthesia, an action also shared by benzodiazepines and barbiturates. All these facts indicate that a common modulatory pathway may be involved in the central action of these three categories of drugs. Because benzodiazepines are known to act at the GABA receptor and barbiturates have a binding site on the GABA-benzodiazepine receptor complex, most investigations have focused on studying the effects of ethanol on GABAergic pathways. Recent neurophysiological and several behavioral observations indicate that ethanol has a pharmacological profile similar to that of classes of drugs like benzodiazepine and barbiturates, which enhance GABAergic transmission in the mammalian CNS. Behavioral,
electrophysiological, and biochemical studies suggest that ethanol may exert some of its effects by enhancing GABAergic transmission. However, the ability of ethanol to potentiate GABA-mediated responses in electrophysiological experiments has been controversial.

Behavioral studies using diverse paradigms like ethanol-induced motor incoordination, loss of righting reflex, the anticonvulsant effect of ethanol, and ethanol-induced withdrawal symptoms, have also implicated GABAergic pathways in the actions of ethanol. Thus, the GABA antagonist bicuculline attenuates ethanol induced motor impairment (Hakkinen and Kulonen 1976). Further, agents which elevate GABA levels in the CNS are reported to enhance ethanol-induced motor incoordination (Frye et al, 1983). Drugs which binds to the picrotoxin site of the GABA receptor complex, like picrotoxin and isopropylbicycloclophosphate, decrease ethanol-induced loss of the righting reflex (Mendelson et al, 1985). Ticku et al. demonstrated that ethanol exhibited an anticonvulsant effect against bicuculline, picrotoxin and strychnine (Rostogi and Ticku, 1986). These investigators also observed that combining subeffective doses of the other facilitators of GABAergic transmission, producing potentiated effects in vivo (Rostogi and Ticku, 1986). Similar results were also observed against electroshock-
induced seizures. An anticonflict effect of ethanol and an anticonvulsant effect against chemoconvulsants also have been reported (Liljequist and Eagel, 1984). Frye and co-workers have implicated the GABA system in audiogenic seizures associated with ethanol withdrawal. These observations, coupled with the fact that ethanol withdrawal symptoms can be prevented by GABA mimetics, suggests the involvement of the GABAergic system in the pharmacological effects of the ethanol, and at least in part, in ethanol withdrawal.

Studies conducted in the past to explore the possible mechanism of action of ethanol on $^{36}$Cl$^{-}$ flux suggest that ethanol could cause perturbation of membrane lipids, leading to increased membrane fluidity (Strong et al. 1984). Therefore, an effect of ethanol on membrane lipids may alter the microenvironment of GABA/benzodiazepine receptor coupled Cl$^{-}$ channel, resulting in an increase in Cl$^{-}$ conductance (Suzdak et al, 1986). Another in vitro model system to study GABA synaptic pharmacology utilizes intact cultured spinal cord neurons. These neurons have GABA-gated Cl$^{-}$ channels and central-type benzodiazepine receptors that are coupled to the GABA, picrotoxin, and barbiturate receptor sites. In mouse spinal cord cultured neurons ethanol potentiated GABA-mediated Cl$^{-}$ influx and at higher concentrations it directly activated Cl$^{-}$ channels in
these neurons (Ticku and Mehta, 1988). Both the potentiating and direct effects of ethanol were blocked by bicuculline and picrotoxin, suggesting the involvement of the same GABA-gated Cl⁻ channels in the action of ethanol. Further, the effect of ethanol was specific on GABA-gated Cl⁻ channels, since ethanol did not effect glycine-induced ³⁶Cl⁻-influx in the same cell. Regardless of the exact molecular mechanism of how ethanol stimulated ³⁶Cl⁻-transport, whether GABA receptor mediated or directly, a number of electrophysiological and biochemical studies suggest that the pharmacological profile of ethanol action, i.e., antianxiety, hypnotic, anticonvulsant, and withdrawal responses centers around the GABA/BZ mediated Cl⁻-transport mechanism, and as a possible chloride channel modulator.

Effects of ethanol on the binding to various sites on the GABA receptor complex have been investigated in vitro following acute and/or chronic administration of ethanol. In vitro, using membrane homogenates, it was found that ethanol does not effect the binding of GABA and benzodiazepine agonists to their binding sites (Greenberg et al, 1984). However ethanol was reported to enhance the binding of [³⁵H]diazepam to a lubrol-solubilized fraction in vitro (Davis and Tickeu 1981). The reason for this discrepancy is not clear and is possible that the effect observed in a detergent-solubilized preparation could be
nonspecific or artifactual, since no reproducible effect could be observed in membrane homogenates. Ethanol does inhibit the binding of \(^{35}\text{S}\)t-butylbicyclophosphorothionate (TBPS) to the picrotoxin site \textit{in vitro} (Liljequist et al., 1986). This interaction of ethanol with TBPS binding sites appears to be allosteric, since it accelerates the dissociation kinetics of TBPS. Since TBPS binding is also inhibited by barbiturates, it was suggested that ethanol may modulate GABAergic transmission via the picrotoxin site (Maksay and Ticku, 1985). However, the concentration of ethanol that inhibited TBPS binding was very high. The importance of ethanol inhibition of TBPS binding is not clear, but it could be involved in ethanol intoxication.

Despite the many pharmacological similarities between benzodiazepines, barbiturates, and ethanol it is still unclear whether ethanol has a similar action on GABA receptor function. While behavioral and electrophysiological studies suggest that ethanol potentiates GABAergic neurotransmission, there is conflicting evidence for a direct action at the GABA receptor. The addition of ethanol to brain membranes \textit{in vitro} has been reported to have no effect on either \(^3\text{H}\) diazepam (Greenberg et al., 1984) or \(^3\text{H}\)-muscimol binding (Greenberg et al., 1984).
Recently, a great deal of attention has been made of the observation that the benzodiazepine receptor inverse agonist, RO 15-4513, could block many of the effects of ethanol, including effects on the GABA receptor-gated Cl⁻ channel (Lister, 1987). RO 15-4513 reverses the behavioral effects of ethanol, which were blocked by Ro 15-1788. RO 15-4513 has also been reported to reverse intoxication due to large doses of ethanol (Nutt et al, 1988), but others have have failed to detect such a protective effect. However, these investigators used massive doses (7.5-15g/kg) of ethanol. It is feasible that such high doses of ethanol effect a variety of biological processes in addition to GABAergic transmission. The consensus seems to be that RO 15-4513 reverses most of the effects of ethanol which may be related to the GABA system. Responses to ethanol such as hypothermia, which apparently is not mediated by GABAergic transmission, are not blocked by RO 15-4513. Kulkarni and Ticku (1989) observed that RO 15-4513 reversed the inhibition by ethanol of convulsions and mortality due to bicuculline better than those due to picrotoxin, and FG-7142 was less effective. Further, RO 15-4513 was not as effective against the inhibition by pentobarbital of convulsions due to bicuculline or picrotoxin. Ticku et al (1988) also observed that chronic ethanol treatment (intragastric method) produced a
selective increase in the number of binding sites for 
$[^3H]RO$ 15-4513 in cortex and cerebellum, but not in 
hippocampus or striatum (Mhatre et al, 1988).

From all these reports, it could be concluded that 
many of the pharmacological effects of ethanol can be 
related to its ability to facilitate GABAergic transmission 
in the CNS. This is mainly based on behavioral and 
electrophysiological studies and on recent biochemical 
assays measuring GABA-induced $^{36}$Cl flux. The results of 
these studies are consistent with the hypothesis that 
ethanol tolerance and dependence results from a 
compensatory decrease in GABAergic transmission as a 
consequence of chronic enhancement of activity of ethanol. 
Chronic ethanol administration results in the development 
of the tolerance to the behavioral actions of ethanol and 
attenuates ethanol enhancement of GABA activated chloride 
flux (Allen and Harris, 1987). In addition, the signs 
associated with withdrawal from ethanol are diminished by 
treatments which increase GABAergic transmission and 
increased by treatments that reduce GABA mediated 
transmission (Fadda et al 1985).

Ethanol administration results in enhancement of GABA 
stimulated Cl$^-$ flux both by a direct (Mehta et al. 1988; 
Suzdak et al. 1986), and/or indirect potentiation of GABA 
effects (Allan et al. 1987; Ticku et al. 1986). These
actions of ethanol can be prevented or reversed by benzodiazepine inverse agonists that inhibit GABA-stimulated Cl⁻ flux (Mehta et al. 1988). In contrast, it appears that chronic ethanol treatment results in tolerance that may be due to a compensatory decrease in GABA-mediated inhibition in the brain (Buck et al. 1990; Morrow et al. 1988).

Anxiety is an important symptom of ethanol withdrawal because it may motivate resumptive ethanol intake in chronic alcoholics (Meyer 1986). Idemudia et al (1989) have demonstrated in an animal model, that measures of anxiety were enhanced in response to antagonists acting selectively at GABA, but not glycine receptor sites during withdrawal (Idemudia et al. 1989). More recently it has been demonstrated that during the protracted period of ethanol withdrawal, tolerance developed to the anxiolytic effect of drugs in direct relation to their ability to enhance GABAergic action (Lal et al. 1990). This evidence indicates that the symptoms of withdrawal might be due to an unmasking of an ethanol-induced deficit in the activity of the GABA receptor coupled chloride channel.

The GABA-chloride ionophore complex contains binding domains for GABA, benzodiazepines, barbiturates and picrotoxin, with each recognition site contributing to the control of chloride flux through the channel (Maksay et al.
If prolonged administration of ethanol reduces the functional capacity of the GABA receptor coupled chloride channel, this might be reflected by a decreased capacity of GABA to modulate the binding of benzodiazepine agonists and inverse agonists. Therefore, the present studies were designed to examine the effect of chronic ethanol exposure or withdrawal on the capacity of GABA to modulate benzodiazepine receptor agonist and inverse agonist binding to the receptor.

Molecular Biology of GABAA Receptors

Considerable understanding has been gained regarding the structure and function of the GABA receptor using both biochemical and molecular cloning approaches. In 1987, Schofield et al. (Schofield et al., 1987) reported the identification and sequences of cDNAs for the \( \alpha \) and \( \beta \) subunits of the GABAA receptor. Since then, several groups, especially Seeburg and colleagues (Pritchett et al., 1988), have reported the cloning of multiple types and subtypes of GABAA receptor subunits, leading to an explosion of information about the possible molecular structure of the GABA receptor. Cloning data indicate that the GABAA receptor composition is highly diverse in terms of subunit composition and distribution in the CNS.
Recently, the GABA receptor has been cloned and is believed to be composed of two $\alpha$ (benzodiazepine-receptor) and two $\beta$ (GABA-receptor) subunits. More recently, this receptor has been indicated as a heterooligomeric protein composed of several distinct polypeptide types, $\alpha$, $\beta$, $\gamma$, and $\delta$. Cloning of these polypeptides revealed that they show 20-40\% identity with each other (Olsen and Tobin, 1990).

The rapid development of molecular biology data has made the composition of GABA/BZ receptors clearer. Luddens et al. (1990) have recently cloned a novel subunit ($\alpha_6$) of the GABAA receptor complex localized to cerebellar granule cells. This unique localization in rat cerebellum together with the similarity between the pharmacological profile of a reconstituted receptor expressing this subunit (Luddens et al. 1990) and diazepam-insensitive (DZ-IS) benzodiazepine receptors described by Korpi and associates (Unsi-Oukari and Korpi, 1990) strongly suggest that the $\alpha_6$ protein is a component of DZ-IS benzodiazepine receptors. The ability of RO 15-4513 to antagonize some of the neurochemical, electrophysiological, and behavioral effects of ethanol (Ticku et al. 1988; Morrosu et al. 1989) coupled with the report that an alcohol non-tolerant line of rats has a higher density of DZ-IS sites than a corresponding tolerant line (Unsi-Oukari et al. 1990) led to the proposal
that the antagonism of alcohol-induced motor impairment by RO 15-4513 was affected through an action at the DZ-IS benzodiazepine receptor.

RO 15-4513 is a partial inverse agonist at central GABAA receptors that antagonizes the behavioral effects of ethanol and the ethanol induced enhancement of Cl\(^-\) flux through the GABA\(\alpha\) receptor/Cl\(^-\)-channel complex. This drug is also capable of decreasing the intake of ethanol by alcohol preferring or operant trained rats. Understanding the site of action of RO 15-4513 might provide clues to the mechanisms underlying the acute effects of ethanol and the adaptive changes responsible for the development of ethanol tolerance and dependence. The second part of the present study was designed to test the hypothesis that chronic ethanol intake may induce changes in the expression of GABA\(\alpha\) receptor subtypes in the cerebellum and thus the binding properties of one or both of these putative receptors.

METHODS

Animals

This study used male hooked rats of the Long-Evans strain (Charles River Breeding Laboratories, Wilmington, MA)
which were housed individually. Water and food (Purina Rodent Chow) were freely available in the home cages and the room lights remained on from 7AM to 7PM each day and under a 12 hours light-dark cycle. The room was maintained at a constant temperature between 21-23° C.

Materials

Gamma-amino-n-butyric acid HCl, flunitrazepam, diazepam, DMCM and bicuculline methiodide were obtained from Sigma Chemical Co., St. Louis, MO. RO 15-4513 was generously provided by Hoffman La-Roche, Nutley, NJ. \(^3\)H-flunitrazepam (specific activity 85.2 Ci/mmol) and \(^3\)H-RO 15-4513 (specific activity 24.3 Ci/mmol) were obtained from Dupont - NEN Research Products, Boston, MA. Filters Schleicher & Schuell #30 were obtained from Keene, Inc., NH.

Solutions

The composition of buffer A was as follows: 50mM Tris HCl, 120mM NaCl\(_2\), 5mM KCl, 2mM CaCl\(_2\), 1mM MgCl\(_2\), the pH was 7.4 at 30°C. Buffer B contains 20mM KH\(_2\)PO\(_4\), 100mM KCl, pH 7.4 at 4°C. Washing buffer consisted of 10mM Tris-HCl and 150mM of NaCl, pH 7.4 at 4°C. Sucrose buffer contains 0.32M sucrose, 10mM Hepes, pH 7.4 at 4°C.
Experimental Procedures

Ethanol Administration-Protocol 1: Normal laboratory chow was removed and rats (Long-Evans strain) were given free access to 100 ml/day of a nutritionally complete control (ethanol free) or ethanol diet (with ethanol concentration of 0.98 umol/ml or 4.5 % w/v ) daily for 4 days as required by the experiments. On the morning of the next day, 50ml of the diet was presented to the rats, and 12 hours later 10ml of a more concentrated (2.6mmol/ml) form of the diet, resulting in a dose of 78 mmol/kg, was administered by gavage.

Ethanol Administration-Protocol 2: Male hooded Long-Evans rats received ethanol in a liquid diet (4.5% ethanol, 38% calories) that served as their sole source of food and water. 100ml of diet was made available daily to ethanol treated animals and a second group of control animals received the same volume of dextrin diet that was consumed by the ethanol animals. On the seventh day of the treatment, animals were given 50ml of the appropriate diet in the morning. At 8:45 pm the diet bottles were removed, the animals was gavaged with a 3g/Kg dose of ethanol in liquid diet and given free access to solid rat chow and water overnight. At 8:45am of the following morning the animals were removed from their home cages and killed by decapitation. Collecting tissue at 12 hours after the last
dose of ethanol, tissue and blood ethanol concentrations were at zero.

Membrane Preparation

Method 1: Rats were decapitated and whole rat brain were dissected and placed in ice cold 10mM Tris-HCl buffer at pH 7.4, homogenized, centrifuged at 40000xg, 20 min, washed with Tris-HCl for 3 times. No hypotonic buffer incubation and no freeze/thaw processing were used in this method.

Method 2: Rats were decapitated and brains dissected with cerebral cortex or other brain area placed in 100 volumes of ice cold 10 mM Tris HCl, pH 7.4. The buffer used throughout the membrane preparation procedure was 10mM ice-cold Tris HCl, pH 7.4. The tissue was homogenized using a Brinkman polytron at a setting of 7 for 16 sec and then centrifuged at 40,000 x g for 20 min. The pellet was resuspended in the buffer, allowed to stand for 30 min at 4°C (hypotonic lysis) and subsequently centrifuged at 40,000 x g for 20 min. The pellet was resuspended in 100 volumes of the buffer for storage at -80°C overnight. Upon thawing, the suspension was washed three more times as described above, and refrozen in the buffer for storage at -80°C. On the day of assay, the tissue was thawed,
centrifuged at 40,000 x g for 20 min and resuspended to 100 volume of buffer A. This extensive washing protocol was utilized to completely remove endogenous GABA (see figure 10).

Method 3: Following chronic treatment with ethanol, rats were decapitated, cerebellums and whole brain of the rats were dissected and placed in 10mM Hepes/0.32 M sucrose buffer. The tissue was homogenized by 15 hand strokes in 20 volumes of cold 10mM Hepes/0.32 M sucrose buffer with a Teflon glass homogenizer and centrifuged at 1000 x g for 10 min. The supernatant was centrifuged at 40,000 x g for 15 min to obtain the mitochondrial plus microsomal (P2) fraction. This fraction was dispersed in cold distilled water and homogenized with a Brinkman Polytron at a setting of 7-16 seconds. The suspension was centrifuged at 50,000 g for 15 min, and the pellet resuspended in buffer contain 20mM KH₂PO₄, 100 mM KCl at pH 7.4, centrifuged at 40,000 g and resuspended in the same buffer, aliquoted and frozen at -80°C until use. On the day of the assay, the tissue was thawed, similarly centrifuged, washed once more and resuspended in buffer B.

Binding Studies

³H-Flunitrazepam Binding Assay: Aliquots of washed membrane suspension (0.5 ml =approximately 0.1-0.15 mg
protein) were incubated for 30 min at 30°C and then 30 min at 4°C in triplicate with 0.3 nM \(^3\)H-Flunitrazepam for competition curves or 0.2 to 10 nM \(^3\)H-flunitrazepam for saturation experiments in the total volume of 1.0 ml of buffer A. Incubation was terminated by the addition of 5 ml of ice-cold 10 mM Tris HCl, pH 7.4 and rapid filtration over the premoistened glass fiber filter (Schleicher and Schuell, No. 30). The filter were washed twice with an additional 5 ml ice cold wash buffer. Nonspecific binding was determined in the presence of 10 \(\mu M\) flunitrazepam and represented less than 0.5% of the added radioactivity. Competitive displacement of \(^3\)H-flunitrazepam binding was determined using nine concentrations \((10^{-10} \text{ to } 10^{-7} M)\) of nonradioactive flunitrazepam or RO 15-4513 or other ligands, in the absence or presence of 0.1 mM GABA. The efficiency of the removal of endogenous GABA from each membrane preparation was measured by the capacity of \(10^{-5} M\) bicuculline methiodide to inhibit \(^3\)H-flunitrazepam binding. Bicuculline had no significant effect on \(^3\)H-flunitrazepam binding in any of the membrane preparations used.

\[ ^3H \text{RO 15-4513 binding assay:} \] Rat brains without cerebellum or cerebellum membrane preparations were thawed and centrifuged at 40,000 x g for 15 min and resuspended in buffer B. The procedure for \[^3H\]RO 15-4513 saturation and
competition binding were similar to the $[^3H]$flunitrazepam binding assay described above except buffer B were used instead of buffer A. All tubes were placed on ice and ligands were added as indicated in the figure legend. 0.1 nM $[^3H]$RO 15-4513 were added for competition curve, 0.2-35 nM $[^3H]$RO 15-4513 for saturation curves. $10^{-5}$ M Diazepam was added to measure only diazepam insensitive binding sites. Diazepam-sensitive (DZ-S) binding were defined by substracting diazepam-insensitive (DZ-IS) binding from total binding. Non specific binding was determined in the presence of $10^{-5}$M of RO 15-4513. After adding ligands, $[^3H]$RO 15-4513 and the membrane suspension, the samples were incubated at 30°C for 30min and on ice for 30 min, 5ml of 10mM ice cold Tris HCl was added, sample was filtered and washed with 2 x 5 ml washing buffer. The filters were put in scintillation vials and 10 ml of scintillation fluid was of added. Samples were counted on a Packhard scintillation counter with an efficiency was 40%. Each reaction conditions was performed in triplicate.

Protein Determination

Assay of protein concentration utilized the Lowry assay method (Lowry et al., 1951) and bovine serum albumin as a standard after precipitating protein with 12% TCA. After
adding foling reagent, the absorbance were measured at 660 nM with a spectrophotometer. Protein were plotted versus absorbance and a standard curve generated. The concentration of the protein of unknown samples from binding assay were determined from its absorbance and the standard curve.

Statistical Analysis

A one-way analysis of variance (ANOVA) was used to analyze the effect of treatments on IC50 values, GABA-ratio values and percent change in total $^3$H-flunitrazepam binding. Individual comparisions were subsequently detected utilizing post hoc contrasts.

Curve Fitting

Binding data were analyzed using the Marquardt method. The model used for competition curves was that for law of mass action binding of the competing ligand with either a single site (n=1) or two independent sites (n=2) as follows:

$$B_s = \frac{\sum_{i=1}^{n} B_i}{\sum_{i=1}^{n} \frac{B_i S}{1+S}}$$
in which $S$ is the concentration of competing ligand, $B_s$ is the $[^{3}H]$ ligand bound at a given concentration of $S$, $B_i$ is the number of specific binding sites, $I_i$ is the IC50 for the competing ligand at each binding site. A similar model was applied to $[^{3}H]$flunitrazepam and $[^{3}H]$RO 15-4513 saturation isotherms, as follows:

$$B_s = \sum_{i=1}^{n} \frac{B_i Q}{K_i + Q}$$

where $K_i$ is the apparent $K_d$ for $[^{3}H]$ ligand at each binding site and $Q$ is the concentration of free $[^{3}H]$ligand.

The statistical difference between one site and two site models was analyzed by comparing the residual variance between the predicted and actual data points according to the minimum sum of squares principle (Rodbard, 1974):

$$F = \left( \frac{(SS1 - SS2)/(dF1 - dF2)}{SS2/dF2} \right)$$

where $SS1$ and $SS2$ are the sum of squares of residuals for the one and two site fits, respectively, and $dF1$ and $dF2$ are the corresponding degrees of freedom. The $F$ statistic was evaluated on 2 $dF$ for the numerator and $dF2$ that ranged from 18 to 64 $dF$ for the denominator with probability levels of less than 0.05.
RESULTS

Characterization of [\(^3\)H]-Flunitrazepam Binding Properties

[\(^3\)H]-Flunitrazepam has been shown to bind to all subtypes of the GABA\(_A\)/benzodiazepine receptors expressed in brain and has the properties of high affinity and specificity that make it the radioactive ligand of choice for characterization of brain benzodiazepine receptors. The initial objective of this study was to develop and optimize a [\(^3\)H]-flunitrazepam binding assay using rat brain membrane fractions. Important considerations for performing receptor binding studies included: choice of tissue, method of membrane preparation, choice of radiolabeled ligand, nonradioactive displacer, incubation conditions including time, temperature, pH, buffer, and ionic environment, method of separating bound and free ligand, definition of nonspecific binding and demonstration of saturability and specificity. All of these factors were optimized in preliminary studies conducted in our laboratory and only the most pertinent of the results will be detailed here.

We established that [\(^3\)H]-flunitrazepam binds to brain membranes with high affinity (Kd approximately 1-3nM), exhibits low nonspecific binding, and that this binding is
saturable (Figure 5). Saturation binding data were further analyzed and plotted according to the method of Rosenthal (1967), i.e., as "popularized" Scatchard plots (Scatchard, 1949; Limbird, 1986) (Figure 6). This type of analysis allows the graphical determination of the number of binding sites, \( B_{\text{max}} = 1.52 \) pmol/mg protein. These estimates of receptor affinity and density are consistent with those reported in the literature for \([^3\text{H}]\)-flunitrazepam binding to crude rat brain membrane fractions. In addition, the binding data were analyzed by computer-assisted nonlinear regression analysis to models of one or two independent binding sites (see Methods). Estimates of the binding parameters, \( K_d \) and \( B_{\text{max}} \), reported here were obtained by this method. In general, \([^3\text{H}]\)-flunitrazepam binding to crude whole brain membrane fractions was best fit to a single population of binding sites.

Specific binding, as shown in Figure 5, was defined as the portion of ligand bound to membrane protein that is displacable by excess nonradioactive flunitrazepam (10 mM). The nondisplacable filter bound radioactivity was defined as nonspecific binding. This definition of nonspecific or nonreceptor binding activity was determined by assessing the capacity of unlabelled flunitrazepam to inhibit membrane binding of \([^3\text{H}]\)-flunitrazepam (Figure 7).
Figure 5: Saturation isotherm for $^3$H-flunitrazepam binding to rat brain membranes. Whole rat brain membranes prepared by Method I were incubated with increasing concentrations of $^3$H-flunitrazepam in the presence (Nonspecific) or absence (Total) of 10 μM unlabelled flunitrazepam as described in Methods. Specific binding (○) was defined as the difference between total and nonspecific binding. Filter bound radioactivity is plotted on the ordinate as cpm/assay and the free concentration of $^3$H-flunitrazepam is plotted on the abscissa. For total and specific binding the solid lines correspond to the computer-generated best fit to a single homogenous population of binding sites.
Figure 6: Scatchard Plot of $^3$H-Flunitrazepam Binding to Rat Whole Brain Membranes. The data shown in Figure 5 were transformed by the method of Rosenthal (1967) and plotted as the concentration of radioligand specifically bound per assay (pM) on the abscissa versus the ratio of specific bound to free radioligand on the ordinate. The solid line represents the predicted values for a single, homogeneous population of noninteracting sites with a $K_d = 0.8$ nM and $B_{\text{max}} = 68$ pM as determined by nonlinear regression analysis.
Unlabelled flunitrazepam competitively inhibited \[^3H\]-flunitrazepam binding with a Ki of approximately 3 nM which corresponds to the receptor affinity measured from saturation analysis (Figures 5 and 6). At concentrations of unlabelled ligand greater than $10^{-7}$ M, the amount of filter bound radioactivity reached a plateau and was not further inhibited in the presence of concentrations of flunitrazepam up to $10^{-4}$ M. Similar results were obtained with diazepam (data not shown). This non-inhibitable radioactivity was defined as nonreceptor binding, i.e., binding to the glass fiber filter, lipid, etc., and is designated as "nonspecific" binding. Nonspecific binding increased in a linear manner with increasing concentrations of \[^3H\]-flunitrazepam (Figure 5), was constant from assay to assay, represented less than 5-10% of the total binding (Figure 7) or less than 0.5% of total added radioactivity, and was linear with added membrane protein (data not shown).

The primary objective of the study was to determine allosteric interactions between GABA and benzodiazepine agonists and inverse agonists at the GABA/BZ receptor. We decided that the most efficient way to demonstrate this effect would be to analyze the competitive inhibition of \[^3H\]-flunitrazepam binding by a benzodiazepine agonist such as diazepam in the presence and absence of GABA. Using rat brain membrane fractions prepared by Method 1, our
Figure 7: Competitive Inhibition of $[^3\text{H}]$-Flunitrazepam Binding to Rat Brain Membranes by Unlabelled Flunitrazepam. Membranes prepared by Method 2 from whole rat brain were incubated with 0.3 nM $[^3\text{H}]$-flunitrazepam in the presence of increasing concentrations of nonradiolabelled flunitrazepam and total filter bound radioactivity determined as described in Methods. Each point is the mean ± S.D. of triplicate determinations. The data are representative of seven similar experiments.
Figure 8: Absence of Effect of GABA on Competitive Inhibition of [3H]-Flunitrazepam Binding by Diazepam. Rat cerebral cortical membranes prepared by Method 1 were incubated with 0.35 nM [3H]-flunitrazepam in the presence of increasing concentrations of diazepam either in the absence (○) or the presence (●) of 100 μM GABA. Specific filter-bound [3H]-flunitrazepam is shown on the ordinate. The solid lines represent the values predicted by a computer-generated best fit to a single population of homogeneous binding sites. Each point is the mean of triplicate determinations.
initial attempts to demonstrate an allosteric effect of GABA on 
$[^3H] $-flunitrazepam or diazepam binding were unsuccessful. 
As shown in Figure 8, diazepam inhibited the binding of 
$[^3H] $-flunitrazepam (0.3 nM) over a concentration range of 
$10^{-8}$M to $10^{-6}$ M. In the absence of added GABA, the IC$_{50}$ 
value, a measure of the apparent affinity of receptor for 
diazepam, was approximately 65 nM. Neither the shape of the 
inhibition curve nor the calculated IC$_{50}$ value, 51 nM, were 
substantially changed by the addition of a 100 μM GABA 
(Figure 8). Thus, the addition of exogenous GABA to the 
assay had no significant effect on benzodiazepine agonist 
binding. Similar results were obtained with different 
batches of GABA, different membrane preparations (all 
prepared by Method 1), and with the GABA receptor agonist 
muscimol (data not shown).

We reasoned that if endogenous GABA had not been 
effectively washed out of the membrane preparation, then 
this would obscure any effect of added GABA on the binding 
data. To test for the presence of endogenous GABA in the 
membrane preparation, the GABA antagonist bicuculline 
methiodide was used. If there were endogenous GABA in the 
membrane preparations, the introduction of bicuculline and 
blocking of the endogenous GABA should result in inhibition 
of $[^3H] $-flunitrazepam binding. In initial experiments, 
bicuculline did inhibit $[^3H] $-flunitrazepam binding in a
concentration-dependent manner. Up to 60% inhibition of ligand binding was observed at concentrations of $10^{-4}$ M bicuculline in some experiments (Figure 9). Such a significant inhibition of binding was not anticipated and made us consider the possibility that bicuculline was capable of inhibiting $^3$H-flunitrazepam binding by a mechanism other than antagonism of GABA. We therefore tested whether the bicuculline-dependent inhibition was competitive or noncompetitive. Membranes (Method 1) were incubated with three different concentrations of $^3$H-flunitrazepam in the presence of increasing concentrations of bicuculline, but without added GABA, and the amount of specifically bound ligand determined as usual. The results of this experiment (Figure 9) show that $^3$H-flunitrazepam binding was inhibited in a dose-dependent manner by bicuculline methiodide (Figure 9, top). Two conclusions can be made from these results. First, there existed significant amounts of endogenous GABA in the membrane preparation; and second, since no rightward shift in the bicuculline inhibitory curve was observed as the concentration of $^3$H-flunitrazepam was increased, the inhibitory effect of bicuculline is noncompetitive in nature (Figure 9, bottom).

From this point, we had evidence that endogenous GABA was present in our crude, but thoroughly washed, membrane
Figure 9: Noncompetitive Inhibition of [³H]-Flunitrazepam Binding by Bicuculline Methiodide. Rat cerebral cortex membranes prepared by Method 1 were incubated with either 0.18 nM (▼), 0.36 nM (▼), or 0.83 nM (●) [³H]-flunitrazepam alone or in the presence of increasing concentrations of bicuculline methiodide as shown on the abscissa. The top panel shows the actual specific filter-bound [³H]-flunitrazepam; the bottom panel shows the same data plotted as the percent of binding observed at each ligand concentration in the absence of bicuculline. Each point is the mean of triplicate determinations.
preparations. Apparently, enough endogenous GABA was present to effect $^3$H-flunitrazepam binding to its receptor. It suggested that our membrane washing procedure was incapable of removing all of the GABA, and that perhaps during homogenization broken membrane fragments sealed and endogenous GABA was trapped inside and could not be eliminated by simple washing.

So, the next step was to develop a new method for preparing membranes to get rid of the endogenous GABA before binding studies could be successfully processed. Subsequent studies were made in which rat brain membranes were homogenized with a Brinkman polytron, centrifuged, and then resuspended in a hypotonic buffer and incubated to achieve a hypotonic lysis of any sealed membrane vesicles. Following this step, membrane suspensions were frozen and thawed to make the sealed synapsomes break. Finally, the membranes were washed by resuspension and centrifugation with ice-cold hypotonic buffer up to six times. (See Method 2 in Methods section.) The membrane preparation was tested for inhibition of $^3$H-flunitrazepam binding with bicuculline again and it was found to no longer have any significant effect on ligand binding (see Figure 10). Because it is very important to wash endogenous GABA away, the efficiency of removal of endogenous GABA from each membrane preparation was assessed by measuring the capacity of $10^{-5}$ M bicuculline
methiodide to inhibit benzodiazepines binding in the later studies. The membrane preparation was used only when bicuculline had no significant effect on benzodiazepine binding. This test for the presence of endogenous GABA by the addition of bicuculline was used on every membrane preparation used throughout the rest of the study.

Effects of GABA on Rat Brain Benzodiazepine Agonist Binding

Using membrane suspensions which had been washed free of endogenous GABA (see Method 2), a significant effect of added GABA on benzodiazepine agonist binding was readily observed. The key aspects of the allosteric effect of GABA are illustrated in Figures 10 through 13. At subsaturating concentrations of $^3$H-flunitrazepam, the addition of GABA increases radioligand binding 160% (Fig 10). Unlabelled flunitrazepam (Figure 10), or diazepam and other benzodiazepine full agonists (data not shown), are capable of completely inhibiting specific binding of $^3$H-flunitrazepam both in the presence and absence of GABA. Bicuculline methiodide (10 μM) had no effect on specific binding over the entire range of unlabelled flunitrazepam (only one point illustrated in Figure 10), but was capable of antagonizing the stimulation of $^3$H-flunitrazepam binding produced by added GABA (data not shown). Antagonism by bicuculline of GABA-stimulated binding could be overcome by
Figure 10: Stimulatory Effect of GABA on Benzodiazepine Agonist Binding. Rat brain membranes prepared by Method 2 were incubated with 0.3 nM [3H]-flunitrazepam and the indicated concentrations of unlabelled flunitrazepam either alone (○), with 100 μM GABA (●), or with 10μM bicuculline methiodide (▲). Each point is the mean ± S.D. of triplicate determinations. The IC50 values determined by nonlinear regression analysis (solid lines) were 4.9 nM (-GABA) and 2.6 nM (+GABA) resulting in a GABA ratio of 1.9.
increasing the concentration of added GABA (data not shown). Thus, the new method of preparing membranes allowed us to observe a significant effect of exogenously added GABA on benzodiazepine agonist binding.

GABA's stimulatory effect on binding could be due to either an increase in the number of binding sites or to an increase in the receptor affinity for \(^3\)H-flunitrazepam. This question was addressed by transforming the data such that binding curves observed in the absence and presence of GABA could be analyzed using the same ordinate scale (Figure 11). Specific binding data were normalized to the percent of total specific binding measured in the absence of competing drug. As shown in Figure 11, GABA causes a leftward shift in the inhibition curve indicating an increased receptor affinity for flunitrazepam. This phenomenon is the so-called "GABA shift". To better quantitate this effect, the data were subjected to a linear transformation and plotted as pseudo or indirect Hill plots (Figure 12) (Limbird, 1986). Hill plots allow for linear regression analysis of the data, estimation of the IC\(_{50}\) value as the x-axis intercept, comparison of the parallel nature of the two linearized curves, and derivation of a "Hill coefficient" as a measure of the homogeneity of the binding sites. For the data shown in Figure 12, the IC\(_{50}\) values were found to be 4.9 nM in the absence and 2.6 nM in
the presence of GABA. A "GABA ratio" was defined as a parameter to measure the magnitude of the allosteric effect of GABA and was obtained by dividing the IC$_{50}$ without GABA by the IC$_{50}$ with GABA. For the data shown in Figure 12 the GABA ratio was calculated to be 1.9 and over a series of more than 50 similar experiments ranged from 1.3 to 2.2.

Using whole brain (with or without cerebellum) as a source of tissue, flunitrazepam competition curves were generally found to have Hill coefficients approaching unity both in the presence and absence of GABA. This suggests that any heterogeneity in the central nervous system receptors that bind this benzodiazepine agonist are not readily revealed by this technique.

GABA, over the range of $10^{-7}$ to $3 \times 10^{-5}$ M, increased $^{3}$H-flunitrazepam binding in a concentration-dependent manner (Figure 13). The EC$_{50}$ for GABA-mediated stimulation of binding was estimated to be approximately 2-3 $\mu$M and maximal activation was achieved at 30 $\mu$M. Under the conditions used in this experiment, GABA increased specific binding of $[^{3}$H$]$-flunitrazepam by 242% (Figure 13). Concentrations of GABA greater than $3 \times 10^{-3}$ M produced only 40-60% of the maximal stimulation probably because these high concentration begin to lower the pH of the assay reaction mixture (data not shown). In all subsequent studies the maximally effective concentration of 100 $\mu$M GABA was used.
Figure 11: Demonstration of the Positive "GABA Shift" with Normalized Flunitrazepam Competition Curves Measured in the Presence and Absence of GABA. The data shown in Figure 10 (in absence and presence of 10μM GABA) are plotted as the percent of the binding observed in the absence of competing drug which for the -GABA condition was 1065 cpm/assay (14.1 fmol) and 1875 cpm/assay (24.8 fmol) for the +GABA condition.
Figure 12: Indirect Hill Plots of Competitive Inhibition of $[^3H]$-Flunitrazepam Binding with and without GABA. The data shown in Figure 10 (in absence and presence of 10μM GABA) are replotted as indirect Hill plots. Only the linear portions of the competition curves are used in this analysis, i.e., binding between 9% and 90% of maximum. $B$ = specific binding at each drug concentration; $B_0$ = maximal binding in the absence of competing drug. The solid lines are the best fit determined by linear regression analysis from which the Hill coefficients ($n_H = \text{slope}$) were derived.
Saturation experiments were performed to verify that the allosteric effects of GABA on benzodiazepine agonist binding were due to changes in receptor affinity. The apparent affinity or Kd for [3H]-flunitrazepam was decreased from 3.03 nM in the absence of GABA to 1.76 nM in the presence of GABA (100 μM), resulting in a GABA ratio of 1.86 (Figure 14). These results are representative of many similar experiments conducted in our laboratory (Abdel-Malek et al., unpublished observations) in which GABA produced an average GABA ratio of 1.57 ± 0.38 (mean ± SEM, n=18) and support the conclusion that the primary effect of GABA is to increase the receptor affinity for benzodiazepine agonists. It should be noted that the number of binding sites was also slightly increased from 1.11 nM to 1.34 nM by the addition of GABA (Figure 14). This small (approximately 20%) increase in the density of binding sites was consistently observed (Abdel-Malek et al., unpublished observations). Computer modeling indicates that [3H]-flunitrazepam binding both in the presence and absence of GABA is best fit to a single site. This result seems to rule out the possibility that in the absence of GABA a small portion of the receptor population exists in a lower affinity state which is then converted to a higher affinity state in the presence of GABA. If such a lower affinity state of the BZ receptor
Figure 13: Concentration-Effect Curve for GABA-Mediated Allosteric Modulation of Benzodiazepine Agonist and Inverse Agonist Binding. Rat cerebral cortical membranes were incubated with either 0.29 nM [3H]-flunitrazepam (●) or 1.2 nM [3H]-Ro 15-4513 (○) in Buffer A containing the indicated concentrations of GABA and specific binding determined as described in Methods. The data are plotted as the maximal stimulation or inhibition of specific binding, respectively. Specific binding of [3H]-flunitrazepam increased from 11.9 to 28.8 fmol/assay and [3H]-Ro 15-4513 specific binding decreased from 50.3 to 39.9 fmol/assay at maximally effective concentrations of GABA.
Figure 14: Scatchard Plot of [³H]-Flunitrazepam Binding to Rat Cerebral Cortical Membranes in the Presence and Absence of GABA. Membranes were incubated with increasing concentrations of [³H]-flunitrazepam (0.08 - 27.3 nM) in the absence (○) or the presence (●) of 100 μM GABA. The ratio of the specific bound to free drug is plotted on the ordinate versus the concentration of free ligand on the abscissa. The solid lines represent the best fit predicted values for a single site model.
does exist, it is not detectable by the current filtration assay method.

GABA-mediated Modulation of Benzodiazepane Inverse Agonist RO 15-4513 Binding to Rat Brain Membranes

A number of compounds, including β-carbolines, such as ethyl β-carboline carboxylate (β-CCE), DMCM, FG7142; the pyrazoloquinoline CGS 8216, and the benzodiazepine RO 15-4513 are known to interact at the GABA/Bz receptor complex producing a profile of behavioral and pharmacological activity opposite to that of benzodiazepine agonists. The effects of these compounds on GABA-mediated Cl⁻ flux measured by either biochemical or electrophysiological techniques are also opposite that of benzodiazepine agonists. Finally, the binding of these drugs to the GABA/Bz receptor complex is modulated by GABA in a manner opposite to that of agonists, i.e., GABA produces a decrease in receptor affinity or a negative "GABA shift" for these ligands. These compounds with behavioral, physiological, and biochemical properties opposite those of benzodiazepine agonists have been given the name inverse agonists.

We sought to characterize the effect of GABA on the binding properties of the partial inverse agonist, Ro 15-4513, in order to use this as a further test of the
functional capacity of the receptor complex. In whole rat brain membranes prepared by Method 2, Ro 15-4513 inhibited \[^3\text{H}\]-flunitrazepam binding in a concentration-dependent manner over the concentration range of \(10^{-9}\) to \(10^{-6}\) M (Figure 15) with a \(K_i\) value of approximately \(6\) nM. As shown previously, GABA (100 \(\mu\)M) increased \[^3\text{H}\]-flunitrazepam binding, but Ro 15-4513 was still capable of effectively inhibiting this increased binding (Figure 15). However, the potency of Ro 15-4513 for inhibiting \[^3\text{H}\]-flunitrazepam binding was significantly decreased in the presence of GABA, as shown by a rightward shift in the competition curve (Figure 16) or Hill plot (Figure 17). Typically, the \(IC_{50}\) for Ro 15-4513 was increased by approximately 40% to 200% in the presence of GABA, resulting in a GABA ratio less than 1 (approximately 0.4-0.7). Ro 15-4513 competition curves were better fit to single binding site model and Hill coefficients approached unity suggesting no heterogeneity in the binding sites. As described in detail in a subsequent section of this paper, we used direct binding of \[^3\text{H}\]-labelled Ro 15-4513 to demonstrate that the apparent decrease in the receptor affinity for Ro 15-4513 induced by GABA was dependent upon the concentration of GABA (Figure 13) and was due to a decrease in receptor affinity for the inverse agonist (Figure 27). Taken together, these data demonstrate that the binding of the partial inverse agonist, Ro 15-4513,
Figure 15: Effect of GABA on Competitive Inhibition of $^3$H-Flunitrazepam Binding by the Partial Inverse Agonist, Ro 15-4513. Specific binding was determined following incubation of rat cerebral cortical membranes (Method 2) with 0.3 nM $^3$H-flunitrazepam and the indicated concentrations of Ro 15-4513 in the absence (○) or the presence (●) of 100 μM GABA. GABA increased $^3$H-flunitrazepam specific binding by 52% (13.1 to 20.0 fmol/assay). Both competitive inhibition curves were significant better fit to a single component model (solid lines). Each point is the mean ± S.D. of triplicate determinations. These results are representative of >20 similar experiments.
Figure 16: Demonstration of the Negative "GABA Shift" with Normalized Ro15-4513 Competition Curves Measured in the Presence and Absence of GABA. The data from Figure 15 was normalized for each curve by converting the specific binding to a percentage of the maximal specific binding (13.1 fmol/assay for -GABA; 20.0 fmol/assay for +GABA). The IC50 value for Ro 15-4513 increased from 6.53 nM in the absence of GABA to 12.64 nM in the presence of GABA resulting in a GABA ratio of 0.52.
Figure 17: Indirect Hill Plots of Ro 15-4513 Inhibition of $[^3H]$-Flunitrazepam Binding in the Presence and Absence of GABA. The specific binding data from Figure 15 were converted to $\log \left[ \frac{B}{(B_0-B)} \right]$ where $B = \text{specific bound}$ and $B_0 = \text{maximal specific binding measured in the absence of competing drug}$. The solid lines are the best fits determined by linear regression analysis which gave Hill coefficients of 0.89 ($r^2 = 0.99$) and 0.94 ($r^2 = 0.99$) without and with GABA, respectively.
is modulated by GABA in a manner similar to the GABA-mediated modulation of agonist-receptor binding, except that the changes in receptor affinity are in opposite directions.

The Effects of Ethanol Intake on Allosteric Binding Interactions

Having established an in vitro assay capable of measuring the allosteric binding interactions between GABA and benzodiazepine agonists and inverse agonists, we next used these assays to test the hypothesis that protracted administration of ethanol would reduce the functional capacity of the GABA receptor-coupled chloride channel and that this reduced functional capacity might be reflected in a decreased ability of GABA to modulate the binding of benzodiazepine agonists and inverse agonists. In collaboration with Drs. P. Prather, M. Rezazadeh, and H. Lal, a study was undertaken to measure the capacity of GABA to modulate flunitrazepam and Ro 15-4513 binding to GABA receptors in distinct brain regions both before and during selected periods of withdrawal from ethanol.

Rats were fed a nutritionally complete liquid ethanol (4.5% w/v) diet for 4 days and at various times after the last dose of ethanol (0, 12, 24, and 72 hours), rats were sacrificed and extensively washed brain membrane fractions
(Method 2) were prepared from different brain regions. Using cortical membrane suspensions, competitive inhibition of $[^3\text{H}]$-flunitrazepam binding by either flunitrazepam or Ro 15-4513 was performed in the absence and presence of GABA ($10^{-4}$ M) (See Figures 10-12 and 15-17). The IC$_{50}$ value, the concentration of flunitrazepam and RO 15-4513 required to inhibit $[^3\text{H}]$-flunitrazepam binding by 50%, and the GABA ratio [IC$_{50}$ (-GABA)/IC$_{50}$ (+GABA)] were used to quantitate the effects of GABA on receptor binding. IC$_{50}$ values were determined by linear regression analysis of indirect Hill plots of the competitive inhibition data. Neither the IC$_{50}$ value for flunitrazepam (Figure 18, left panel) nor Ro 15-4513 (Figure 18, right panel) was significantly different from control at 12 or 72 hours after the last dose of ethanol (Table 1 & 2). The capacity of GABA to modulate the receptor affinity for both agonist and inverse agonist was determined by calculation of the GABA ratio value. This analysis revealed that addition of GABA resulted in a positive GABA shift of approximately 1.7 fold for flunitrazepam and a negative GABA shift of 0.57 fold for Ro 15-4513 across all the treatments (Table 2). Statistical analysis revealed no significant change in the GABA ratio values at 12, 24, or 72 hours following chronic ethanol administration when compared with paired-fed controls for
Table 1. The capacity of GABA to modulate receptor affinity for benzodiazepine agonist flunitrazepam is not altered during withdrawal from chronic ethanol.

<table>
<thead>
<tr>
<th>Hours post chronic EtOH</th>
<th>IC50 -GABA</th>
<th>IC50 +GABA</th>
<th>GABA Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.43 +/-0.05</td>
<td>2.07 +/-0.23</td>
<td>1.67 +/-0.22</td>
</tr>
<tr>
<td>12</td>
<td>4.05 +/-0.26</td>
<td>2.37 +/-0.51</td>
<td>1.75 +/-0.24</td>
</tr>
<tr>
<td>72</td>
<td>3.39 +/-0.30</td>
<td>2.09 +/-0.21</td>
<td>1.74 +/-0.23</td>
</tr>
</tbody>
</table>

Rats were fed a liquid ethanol (4.5%) or control diet for 4 days (protocol 1). Animals were sacrificed 12 or 72 hrs after discontinuation of ethanol and cerebral cortical membrane suspensions were prepared (see method 2). Competitive inhibition of ³H-flunitrazepam binding by flunitrazepam (10⁻¹⁰M to 10⁻⁷M) was performed in the absence or presence of GABA (10⁻⁵M) (see Fig. 10). IC50 values were then derived from linear regression analysis of indirect Hill Plots (see Fig. 12), and GABA ratio values were subsequently calculated by the following formula: [IC50(-GABA)/IC50(+GABA)]. Values shown are the mean +/- standard deviation of assays performed using four different membrane preparations for each treatment group.
Table 2. The capacity of GABA to modulate receptor affinity for the benzodiazepine inverse agonist RO15-4513 is not altered during withdrawal from chronic ethanol.

RO 15-4513

<table>
<thead>
<tr>
<th>Hours post chronic EtOH</th>
<th>IC50</th>
<th>IC50</th>
<th>GABA Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-GABA</td>
<td>+GABA</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6.35+/-0.45</td>
<td>11.29+/-1.83</td>
<td>0.57+/-0.04</td>
</tr>
<tr>
<td>12</td>
<td>7.06+/-2.47</td>
<td>12.22+/-3.98</td>
<td>0.57+/-0.02</td>
</tr>
<tr>
<td>72</td>
<td>7.11+/-0.81</td>
<td>13.68+/-1.87</td>
<td>0.52+/-0.02</td>
</tr>
</tbody>
</table>

Rats were fed a liquid ethanol (4.5%) or control diet for 4 days. Animals were sacrificed 12 or 72 hrs after discontinuation of ethanol and cerebral cortical membrane suspensions were prepared (see method 2). Competitive inhibition of $^3$H-flunitrazepam binding by RO 15-4513 ($10^{-10}$M to $10^{-7}$M) was performed in the absence or presence of GABA ($10^{-5}$M) (see Fig. 15). IC50 values were then derived from linear regression analysis of indirect Hill Plots (see Fig. 17), and GABA ratio values were subsequently calculated by the following formula: \([\text{IC50(-GABA)/IC50(+GABA)}]\). Values shown are the mean +/- standard deviation of assays performed using four different membrane preparations for each treatment group.
Figure 18. Representative Indirect-Hill Plots derived from specific $^3$H-flunitrazepam data. a, b, and c (left panels) represent competition of $^3$H-flunitrazepam by flunitrazepam, while d, e, and f (right panels) depict competition by RO15-4513. Assays were performed using cerebral cortical membrane preparations from animals sacrificed either 0 (a & d), 12 (b & e), or 72 (c & f) hr after the last dose of ethanol. The open circles represent competition in the absence of GABA, while the filled circles represent competition in the presence of GABA. Log Z is defined by the following: $\log \frac{[B]}{[B_{max}-B]}$, where $B$=specific $^3$H flunitrazepam bound at each ligand concentration, and $B_{max}$=Maximal amount bound in the absence of competing drug.
either agonist flunitrazepam [$F(2,11) = 0.236, p<0.796$] or inverse agonist RO 15-4513 [$F(2,11) = 3.435, p<0.101$].

GABA Modulation of $^3$H-Flunitrazepam Binding in Discrete Rat Brain Regions at Various Intervals after Ethanol Cessation

The ability of GABA to enhance specific $^3$H-flunitrazepam binding in various regions of rat brain at various intervals after ethanol withdrawal was compared based on the idea that ethanol might have a selective effect on GABA receptors in different brain areas. The increase of specific $^3$H-flunitrazepam binding stimulated by GABA (rather than complete competition curves and IC50 values) was used as a measure of the capacity of GABA to modulate receptor function during various periods of withdrawal from ethanol (Figure 19). Although a greater percent change in specific $^3$H-flunitrazepam binding in the presence of GABA was observed in the hippocampus and cerebellum than in cortex, no specific alteration in the capacity of GABA to increase $^3$H-flunitrazepam binding was noted in any brain area, across all withdrawal time periods, when compared to pair-fed controls. Taken together these data suggest that four days of ethanol administration does not reduce the functional capacity of the GABA$_A$ receptor as assessed by measuring allosteric binding interactions.
Figure 19. The ability of GABA to enhance specific $^{3}$H-flunitrazepam (0.3nM) binding in discrete brain areas at various interval after ethanol cessation. Rats were fed a nutritionally complete liquid ethanol (4.5% w/v) diet for 4 days. At various times after the last dose of ethanol (0, 12, 24, & 72 hrs), rats were sacrificed and washed membrane fractions from the brain areas shown were prepared. Values represent mean +/- standard deviations of assays performed using four different membrane preparations for each brain area. Percent change refers to the increase in the specific $^{3}$H-flunitrazepam binding in the presence of GABA (10μM).
Two Classes of $[^3]H$-RO 15-4513 Binding Sites in Rat Cerebellum

It has been demonstrated by photoaffinity labelling, ligand binding, and autoradiographic studies that the benzodiazepine receptors expressed in the cerebellum have a unique pharmacological profile compared to receptor populations in other brain areas. Recent studies have shown that the $\alpha_6$ and $\gamma$ subunits of the $\text{GABA}_A$ receptor are expressed almost exclusively by cerebellar granule cells and that cultured mammalian cells engineered to coexpress recombinant $\alpha_6$, $\beta_2$, and $\gamma_2$ subunits possess a GABA/BZ receptor with ligand binding properties corresponding to the cerebellar receptor. A key feature of this putative $\alpha_6$-$\text{GABA}_A$ receptor is that it displays high affinity for the benzodiazepine inverse agonist and ethanol antagonist, Ro 15-4513, and very low affinity for the classical benzodiazepine agonists like diazepam. Based on radioligand binding studies (Taylor et al., 1990), this putative receptor has been designated as the diazepam-insensitive benzodiazepine receptor (DZ-IS receptor). The ability of Ro 15-4513 to antagonize some of the neurochemical, electrophysiological, and behavioral effects of ethanol, coupled with the recent report that an alcohol non-tolerant
line of rats has a higher density of a diazepam-insensitive benzodiazepine receptor (DZ-IS receptor) than a corresponding tolerant line of rats (Uusi-Oukari and Korpi, 1990) has led to the proposal that the antagonism of alcohol-induced motor impairment by Ro 15-4513 is effected through action at the DZ-IS receptor (Lueddens et al., 1990; Harris, 1990). This proposal prompted us to investigate the ligand binding properties of this putative cerebellar GABA/BZ receptor and whether the properties of these receptors are changed by chronic exposure of animals to ethanol.

Initial experiments to characterize [³H]-Ro 15-4513 binding were performed using membranes prepared from whole rat brain without cerebellum, since we had already accumulated a considerable amount of data on the GABA/benzodiazepine binding properties of this tissue preparation. [³H]-Ro 15-4513 bound to whole brain membranes with high affinity, and, using excess nonradioactive Ro 15-4513 to define nonspecific binding, we found that greater than 90-95% of the binding was specific (data not shown). Flunitrazepam competitively inhibited [³H]-Ro 15-4513 binding to these membranes with an IC₅₀ value of 7 nM (Figure 20). The Hill coefficient for this data was 0.93, i.e., approaching unity (r²=0.99). In addition, the data were significantly better fit to a model of mass action
Figure 20: Competitive Inhibition of [3H]-Ro 15-4513 Binding to Brain Membranes by the Benzodiazepine Agonist, Flunitrazepam. Membranes were prepared (Method 2) from whole rat brain dissected free of the cerebellum, incubated with 1.36 nM [3H]-Ro 15-4513 in the presence of the indicated concentrations of flunitrazepam, and specific binding determined as described in Methods. The inset shows the indirect Hill plot of the same data.
Figure 21: Demonstration of Two [$^3$H]-Ro 15-4513 Binding Sites with Different Affinity for the Benzodiazepine Agonist Diazepam in the Rat Cerebellum. Rat cerebellar membranes (Method 3) were incubated with 1.12 nM [$^3$H]-Ro 15-4513 in the presence of the indicated concentrations of unlabelled diazepam and specific binding was determined as previously described. The data were fit to a model for either one or two independent binding sites with differing affinities for diazepam (see text for values). The inset shows the indirect Hill plot of the same data.
binding at a single rather than multiple binding sites (Ki=6.6 nM; p<0.05). Thus, this data by itself provides no evidence for heterogeneous binding sites for Ro 15-4513/flunitrazepam in whole brain areas other than the cerebellum.

A similar experiment, this time using diazepam to displace \(^3\)H-Ro 15-4513 binding, was applied to membranes prepared from cerebellum (Method 3) with strikingly different results. In contrast to the steep inhibition curve for flunitrazepam observed in whole brain membranes (Figure 20), the displacement curve for diazepam in cerebellum was very shallow (Figure 21). Low concentrations (3x10^{-9}M to 1x10^{-7}M) of diazepam effectively displaced approximately 50\% of the \(^3\)H-Ro 15-4513 specific binding. However, diazepam was much less effective in displacing the remaining radioactivity, i.e., almost a plateau in filter-bound radioactivity was observed with no further displacement of radiolabelled ligand binding occurring until concentrations greater than 1x10^{-5} M diazepam were used. As shown in the inset to Figure 21, the Hill plot for this data was clearly nonlinear with a hill coefficient of 0.39 indicative of a heterogenous population of binding sites for diazepam. Further analysis by nonlinear regression indicated the data was significantly better fit to a two binding site model compare to a single binding site model.
(Figure 21). The high affinity binding site had a Ki for diazepam of 33 nM and represented 54% of the total binding at this concentration of \(^3\)H-Ro 15-4513, while the low affinity binding site had a Ki = 65 mM and accounted for 46% of the binding. This second site with very low affinity for the benzodiazepine agonist diazepam is the so called "diazepam-insensitive" (DZ-IS), Ro 15-4513 binding site.

The analysis of two binding sites with different affinity for ligands is only possible if the radiolabelled ligand in nonselective for the sites. In other words, in order to define two sites with different affinities for diazepam or other ligands, the \(^3\)H-Ro 15-4513 must bind to both sites with equal or nearly equal affinity. We tested for selectivity of \(^3\)H-Ro 15-4513 by both competitive inhibition studies and by analysis of saturation data. Figure 22 shows the results of incubating cerebellar membranes with \(^3\)H-Ro 15-4513 in the presence of increasing concentrations of unlabelled Ro 15-4513. The steep slope of the curve, Hill coefficient 0.94 (Figure 22, inset), suggests that the binding sites are apparently homogeneous in their affinity for Ro 15-4513. Furthermore, the data are best fit to a single site binding model (solid line in Figure 22). Thus, these data suggest that Ro 15-4513 is nonselective for the cerebellar binding sites.
The nonselective nature of $[^3H]$-Ro 15-4513 binding to the cerebellar membranes was also suggested from results obtained from saturation experiments. Cerebellar membranes were incubated with increasing concentrations of $[^3H]$-Ro 15-4513 (0.3 - 40 nM) in the absence of competing ligand to measure total binding, in the presence of $10^{-5}$ M diazepam to define the diazepam-insensitive component of the binding (see Figure 21), and in the presence of $10^{-5}$ M unlabelled Ro 15-4513 to define nonspecific binding (see Figure 22). The results of this experiment, shown in Figure 23, indicate that $[^3H]$-Ro 15-4513 binding to cerebellar membranes is of relatively high affinity and is saturable. Nonspecific binding is linear with increasing concentrations of radioligand and accounts for very little of the total binding at low ligand concentrations and increases to only 13% of the total binding at the highest ligand concentration (data not shown).

A concentration of diazepam, $10^{-5}$ M, was chosen that inhibits essentially all of the $[^3H]$-Ro 15-4513 binding to the high affinity component, but inhibits very little of the $[^3H]$-Ro 15-4513 binding to the low affinity component (see Figure 21) to define the diazepam-sensitive (DZ-S) and the diazepam-insensitive (DZ-IS) components of the $[^3H]$-Ro 15-4513 binding. In the presence of $10^{-5}$ M diazepam the diazepam-sensitive component of $[^3H]$-Ro 15-4513 binding was
Figure 22: Ro 15-4513 Is Nonselective for Cerebellar Binding Sites. Competitive inhibition of $[^3H]$-Ro 15-4513 by unlabelled Ro 15-4513 was performed using rat cerebellar membrane suspensions. Each point is the mean ± S.D. of specific binding of triplicate determinations. The solid line is the values predicted from a single site model. The singular affinity for ligand is also suggested by the linear indirect Hill plot (inset; $n_H=0.94$).
Cerebellar membrane suspensions (Method 3) were incubated with the indicated free concentrations of $[^3]H$ Ro15-4513 (abscissa) in the absence (O; Total Binding) or the presence (●; Diazepam-Insensitive) of $10^{-5}$ M diazepam and specific binding determined as described in Methods. Diazepam-sensitive binding was calculated by subtracting diazepam-insensitive specific binding from total specific binding. Nonspecific binding determined with parallel incubations containing $10^{-5}$M Ro 15-4513 (data not shown) has been subtracted and for each condition only specific binding is shown (ordinate).
Figure 24: Scatchard Plots of Total and Diazepam-Insensitive $[^3H]$ Ro15-4513 Binding to Rat Cerebellar Membranes. The data from Figure 23 are replotted as Scatchard plots. The amount of $[^3H]$ Ro15-4513 specifically bound under each condition is plotted on the abscissa versus the ratio of specific bound to free ligand on the ordinate.
inhibited and saturable $[^3]H$-Ro 15-4513 binding was achieved at the remaining binding sites (Figure 23). Figure 24 shows the Scatchard transformations of this data. There was a high density of $[^3]H$-Ro 15-4513 binding sites on cerebellar membranes ($B_{\text{max}} = 2.09 \text{ pmol/mg protein}$) with relatively high affinity for the ligand ($K_d = 9.9 \text{ nM}$). Total specific $[^3]H$-Ro 15-4513 binding gave a linear scatchard plot and was best fit by nonlinear regression to a population of binding sites with a single affinity for the ligand, again suggesting that $[^3]H$-Ro 15-4513 is nonselective for the two sites. Diazepam-insensitive binding measured in the presence of $10^{-5} \text{ M}$ diazepam also gave a linear scatchard plot with apparent affinity very similar to that observed for total specific binding ($K_d = 9.7 \text{ nM}$). Diazepam-insensitive sites accounted for approximately 30% of the total binding sites ($B_{\text{max}} = 0.67 \text{ pmol/mg protein}$). An estimate of the diazepam-sensitive component of the binding was calculated by subtracting the diazepam-insensitive binding from the total specific binding (Figure 24). These DS binding sites had a $K_d = 10.29 \text{ nM}$ and a $B_{\text{max}} = 1.43 \text{ nM}$ . Thus, $[^3]H$-Ro 15-4513 nonselectively binds to two different sites on cerebellar membranes. One site represents 60-70% of the total receptors and has high affinity for diazepam, flunitrazepam, and other benzodiazepine full agonists. The
second site, accounting for 30-40% of the receptors, has very low affinity for these agonists.

Is the Diazepam-Insensitive Ro15-4513 Binding Site Linked to a GABA Receptor?

Since the diazepam-insensitive Ro 15-4513 binding site has low affinity for classical benzodiazepine agonists, it could be a novel receptor not associated with the GABAA receptor complex. To examine this question, we tested for GABA-mediated modulation of \( ^{3}\text{H}\)-Ro 15-4513 binding to the two sites in cerebellum, reasoning that if the binding properties of both sites were modified by GABA then this would suggest that both of these sites are probably coupled in some way to the GABAA receptor.

Competitive inhibition of \( ^{3}\text{H}\)-Ro 15-4513 binding to cerebellar membranes by unlabelled Ro 15-4513 was performed in the absence and the presence of maximally effective concentrations of GABA (100 μM) (Figure 25). In the absence of GABA, Ro 15-4513 bound to population of sites with an apparent homogenous affinity for the ligand (IC\(_{50}\) = 4.9 nM). \( ^{3}\text{H}\)-Ro 15-4513 binding was significantly inhibited by the addition of GABA and as predicted this effect could be attributed to a decrease in the apparent affinity for Ro 15-4513, as shown by the shift to right in the indirect Hill
The IC₅₀ value for Ro 15-4513 was increased from 4.9 nM to 7.1 nM in the presence of GABA, resulting in a GABA ratio of 0.69. However, the +GABA curve was also best fit to a single site model, and so, even though GABA significantly modified inverse agonist binding, experiments with this nonselective ligand provide no evidence as to which of the two binding sites is being effected by GABA.

In an attempt to demonstrate a differential effect of GABA on diazepam-sensitive vs diazepam-insensitive receptors, competitive inhibition of the nonselective ligand, [³H]-Ro 15-4513 binding to cerebellar membranes by the selective ligand, diazepam, was performed in the absence and the presence of maximally effective concentrations of GABA (100 μM) (Figure 26). Specific [³H]-Ro 15-4513 binding measured in the absence of competing ligand was inhibited by 37% in the presence of GABA, indicating that at least one of the receptor sites was sensitive to modulation by GABA. Consistent with earlier results (Figure 21), the inhibition curves for diazepam reached a plateau at concentrations between 10⁻⁷ M and 10⁻⁵ M, both in the presence and absence of GABA (Figure 26). Within this concentration range it was difficult to see any differences in the binding with and without GABA. Curve fitting analysis suggested that both curves were readily fit to a
Figure 25: Effect of GABA on Total $[^3H]$ Ro15-4513 Binding and Competitive Inhibition by Unlabelled Ro15-4513 in Cerebellar Membranes. Rat cerebellar membranes were incubated with 1.42 nM $[^3H]$ Ro15-4513 and the indicated concentrations of unlabelled Ro15-4513 in absence (○) or presence (●) of 100 μM GABA and specific binding was determined as previously described. The solid lines are the predicted one site model values. The inset is an indirect Hill plot of the data (nH=0.91 for -GABA; nH=0.86 for +GABA).
In the presence of GABA, the Ki values for both the high and the low affinity sites were increased (Kih \(-\text{GABA} = 42 \text{ nM}; \ Ki_h +\text{GABA} = 52 \text{ nM}; \ Ki_L -\text{GABA} = 3.9 \mu M; \ Ki_L +\text{GABA} = 7.9 \mu M\)). The change in apparent receptor number was almost entirely reflected in the high affinity component (BH \(-\text{GABA} = 95 \text{ fmol}; \ BH +\text{GABA} = 44 \text{ fmol}; \ BL -\text{GABA} = 65 \text{ fmol}; \ BL +\text{GABA} = 64 \text{ fmol}\)). This data suggests that the high affinity binding component is clearly effected by the presence of GABA. However, the results on the lower affinity component are harder to interpret since there appears to be some shift in the apparent affinity for diazepam in the presence of GABA but there is little change in the number of binding sites.

The effects of GABA on the \([3^H]-\text{Ro 15-4513}\) binding properties of the two sites in the cerebellum were tested further by generating saturation isotherms in the presence and absence of GABA (Figure 27). As expected, at lower ligand concentrations \([3^H]-\text{Ro 15-4513}\) binding was decreased in the presence of GABA. Scatchard analysis showed that this decrease in binding was due to a decrease in the apparent affinity, i.e., the Kd increased from 7.14 nM in the absence of GABA to 13.1 nM in the presence of GABA, rather than to a change in the number of binding sites (Bmax-\text{GABA}=0.23 \text{ nM}; Bmax+\text{GABA}=0.22 \text{ nM}). Once again these data were better fit to a homogeneous population of sites.
Figure 26: Effects of GABA on Competitive Inhibition of Total [³H] Ro15-4513 Binding to Cerebellar Membranes.
Competitive inhibition of [³H] Ro15-4513 binding was performed in the absence (○) and the presence (●) of 100 µM GABA. The solid lines represent the computer-generated best fit to two site models (see text for values). Each point is the mean of triplicate determinations of specific binding.
indicating that Ro15-4513 was nonselectively for cerebellar
binding. In addition, the fact that GABA decreased the
affinity for Ro15-4513 for at least a portion of these sites
was confirmed. However, we still had no evidence as to
whether one or both sites were being modulated by GABA's
action.

To overcome some of these problems, another approach
was taken for determining the binding properties of the
diazepam-insensitive binding site. Binding parameters were
determined by performing assays in the presence of $10^{-5}$ M
diazepam to inhibit essentially all of the diazepam-
sensitive component. In this way, any contributions from
the diazepam-sensitive site were eliminated.

Characteristics of the Diazepam-Insensitive Sites

Several benzodiazepine receptor ligands were tested for
inhibition of RO 15-4513 binding to DZ-IS sites. A
concentration of diazepam ($10^{-5}$ M) was used to saturate the
diazepam sensitive sites, therefore only the DZ-IS sites
were available for ligand binding. Figure 25 shows the
competition curve of Ro 15-4513 vs. $[^3H]$-Ro 15-4513 for the
cerebellum binding sites. DZ-IS sites binds RO 15-4513 with
the same affinity as the DZ-sensitive sites. In
comparision, DMCM, a full inverse agonist, has much lower
Figure 27: The effects of GABA on the $[^3H]$RO 15-4513 binding properties of the two sites in rat cerebellum saturation isotherms in the presence and absence of GABA. At lower ligand concentrations, binding was decreased in the presence of GABA (upper panel). Scatchard analysis showed that the apparent affinity was decreased in the presence of GABA, the Kd increased from 7.14 nM in the absence of GABA to 13.1 nM in the presence of GABA (Lower panel), but the number of binding sites was not changed.
affinity to DZ-IS sites compared to RO 15-4513 (Fig. 28). Several compounds whose binding to the receptor are known to be allosterically modulated by GABA were tested to determine if the DZ-IS sites were GABA receptor linked. Displacement of \[^3H\] RO 15-4513 binding (in the presence of diazepam, DZ-IS sites) by either the partial inverse agonist RO 15-4513 (data not shown) or full inverse agonist DMCM (Figure 29) were carried out in the presence or absence of GABA (100 μM). \[^3H\]- Ro15-4513 binding to the DZ-IS sites was not affected by the presence of GABA (Data not shown). In contrast, the DMCM competition curve was shifted to the right in the presence of 100 μM of GABA (Figure 29). Undoubtedly, diazepam-sensitive sites were modulated by GABA, suggesting that these sites are linked to the GABA receptor. Although the evidence above for DZ-IS sites is not conclusive, it also indicated that they may be linked to GABA receptors.

The Effects of Chronic Ethanol Intake on
\[^3H\]RO 15-4513 Binding Properties
of Cerebellar DZ-S and DZ-IS GABA Receptors.

Chronic ethanol treatment produces intoxication, eventual tolerance to the effects of ethanol, and physical
Figure 28. Competitive inhibition of $^3$H RO15-4513 binding by DMCM in rat cerebellum in the presence of 10μM diazepam. DMCM competitively inhibits $^3$H RO15-4513 binding to cerebellum diazepam insensitive sites. But the apparent affinity is much lower compared to RO 15-4513 and the apparent IC50 value for DMCM is approximately 0.3 μM.
Figure 29: Effect of GABA on DMCM binding. Specific \(^3\)H RO 15-4513 binding was competitively inhibited by increasing concentrations of DMCM +/- GABA. The data were converted to percent of maximal binding. DMCM, a full inverse agonist of benzodiazepine receptor, binds to the cerebellum diazepam insensitive sites with a IC50 value of approximately 0.3 \(\mu\)M. In the presence of 100 \(\mu\)M GABA, the curve was shifted slightly to the right indicating that there is a small decrease in the apparent affinity for DMCM.
dependence. The cellular basis for both the acute and chronic effects of ethanol is incompletely understood. However, there have been some studies that have demonstrated changes in receptor number or in the properties of benzodiazepine or GABAA receptors after chronic ethanol treatment. For example, chronic administration of ethanol was reported to produce an increase in the specific binding of the putative ethanol antagonist $^3$H-RO 15-4513 in rat brain cerebral cortex and cerebellum. This increase suggested that RO 15-4513 binding sites on the GABAA receptor complex were altered by chronic ethanol administration, and supported the notion of a unique role of RO 15-4513 as an ethanol antagonist. The experiments above demonstrate the existence of benzodiazepine receptor subtypes in the rat cerebellum; that is two binding sites for RO 15-4513 with different pharmacological profiles. Taken together, it appears that either DZ-IS sites or both of the sites might play a role in the ethanol antidote effects of RO 15-4513. We tested the hypothesis that chronic ethanol administration and withdrawal induces changes in the binding properties of one or both of these sites of the putative GABAA receptor.

Rats were fed a liquid diet of 4.5% ethanol for 7 days, gavaged with a 3g/kg dose of ethanol (protocol 2 in
methods), and then rats were sacrificed after 2 hours, 12 hours (acute withdrawal), or 4.5 days (protracted withdrawal) from the last dose. Saturation binding of $[^{3}\text{H}]$-RO 15-4513 to cerebellar membranes was performed in the absence (total binding) or presence of 10 μM diazepam (DZ-IS binding). Diazepam-sensitive binding was calculated as the difference between total binding and diazepam-sensitive binding. Nonlinear regression analysis showed that each class of binding sites fit a model of mass action binding to a single, noninteractive population of sites (Figure 24). The apparent affinity (Kd) and apparent number of receptors (Bmax) for $[^{3}\text{H}]$-Ro15-4513 of each class of binding sites for control and ethanol treated animals are showed in Table 3 and 4. No significant differences were observed between any of the treatment groups in the apparent affinity (Kd) for $[^{3}\text{H}]$-Ro 15-4513 binding at total, DZ-IS, or DZ-S sites following chronic ethanol treatment or withdrawal. In addition, no significant differences were observed in the apparent number of DZ-S or DZ-IS binding sites or the ratio of DZ-S to DZ-IS sites. These data suggest that seven days of chronic ethanol intake does not induce changes in the number or affinity of cerebellar $[^{3}\text{H}]$ Ro15-4513/GABAA receptor binding sites.
Table 3. $^3$H-Ro15-4513 Binding Properties During Ethanol Withdrawal: Apparent Receptor Affinity Is unchanged during ethanol withdrawal

<table>
<thead>
<tr>
<th>Condition</th>
<th>Total Binding</th>
<th>Diazepam Insensitive</th>
<th>Diazepam Sensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.99±1.26</td>
<td>6.94±1.33</td>
<td>9.12±1.83</td>
</tr>
<tr>
<td>Acute Withdrawal</td>
<td>7.68±1.41</td>
<td>7.83±0.67</td>
<td>6.87±1.92</td>
</tr>
<tr>
<td>Protracted Withdrawal</td>
<td>10.17±1.35</td>
<td>5.88±1.52</td>
<td>12.84±0.2</td>
</tr>
</tbody>
</table>

Rats were fed a liquid ethanol (4.5%) or control diet for 7 days. On day seven of treatment, rats were gavaged with a 3g/Kg dose of ethanol in liquid diet of ethanol. 12 hr (acute withdrawal) or 4 days (protracted withdrawal) after the last dose of ethanol the animals were sacrificed and cerebellar membrane suspensions were prepared (see method 3). Saturation curves for $^3$H-RO 15-4513 (0.2-40nM) were generated in the absence (total) or presence of 10$^{-5}$M diazepam (diazepam insensitive). Diazepam sensitive binding was calculated by substracting diazepam insensitive from total binding. Kd values (mean+/−S.E.M.) (Control n=10;
Acute n=7; Protracted n=4) were determined by computer-assisted nonlinear regression analysis.

Table 4. The Number of $^3$H-RO 15-4513 Binding Sites (Bmax)
Is Not Changed During Ethanol Withdrawal

<table>
<thead>
<tr>
<th>Condition</th>
<th>Total</th>
<th>Diazepam Insensitive</th>
<th>Diazepam Sensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.76±0.14</td>
<td>0.52±0.14</td>
<td>1.13±0.18</td>
</tr>
<tr>
<td>Acute Withdrawal</td>
<td>1.46±0.23</td>
<td>0.53±0.17</td>
<td>0.91±0.26</td>
</tr>
<tr>
<td>Protracted Withdrawal</td>
<td>1.70±0.15</td>
<td>0.49±0.12</td>
<td>1.14±0.18</td>
</tr>
</tbody>
</table>

See legend of Table 3 for details.

DISCUSSION

Data obtained from neurochemical, electrophysiological and behavioral studies suggest that acute ethanol treatment enhances GABA-mediated neurotransmission. For example, acute ethanol administration results in enhancement of GABA stimulated Cl⁻ flux both by a direct action at pharmacologically relevent doses (Mehta and Ticku, 1988;
Suzdak et al, 1986), and/or indirectly by potentiation of the effect of GABA (Allan and Harris, 1987; Ticku et al, 1986). These actions of ethanol can be prevented or reversed by benzodiazepine inverse agonists, which inhibit GABA stimulated chloride flux (Suzdak et al, 1986; Mehta and Ticku, 1988). In addition, several behavioral studies have also demonstrated that drugs which reduce GABAergic neurotransmission diminish or reverse acute ethanol induced motor-impairment (Hakkinen et al, 1976; Hellevuo et al, 1989), loss of righting-reflex (Mendelson et al, 1985), as well as the effect of ethanol on sleep, locomotion and thermal regulation (Liljequist and Engel, 1982). The motor impairing and anticonvulsant effects of ethanol have been shown to be potentiated when administered in combination with drugs which elevate GABA levels (Frye et al, 1983; Rastogi and Ticku, 1986).

In contrast, chronic ethanol treatment results in tolerance and this tolerance to ethanol may be due to a compensatory decrease in GABA-mediated inhibition in the brain (Buck and Harris, 1990; Morrow et al, 1988). During acute withdrawal from ethanol, there are reduced behavioral responses to intranigral administration of the GABA-agonist muscimol (Gonzalez and Czachura, 1989).

Many symptoms such as the occurrence of audiogenic (Frye et al, 1983) and spontaneous seizures (Nordberg et al,
have been correlated with decreases in GABA neurotransmission following chronic ethanol administration. Finally, in clinical situations, GABA-mimetic drugs suppress (Gallimberti et al, 1989), while GABA antagonists potentiate ethanol withdrawal signs and symptoms (Lister and Karanian, 1987). This evidence suggests that withdrawal symptoms occurring after abrupt discontinuation of chronic alcohol might result from an ethanol induced GABAergic deficit (Idemudia et al, 1989; Littleton, 1989).

Anxiety experienced during ethanol withdrawal is one of the first symptoms to appear and one of the last to abate (Roelofs, 1985). Recent findings indicate that withdrawal induced anxiety may be related to a GABAergic deficit. This is evidenced by both the development of tolerance to the anxiolytic effect of drugs in the direct relation to their ability to enhance GABA function (Lal et al, 1988), as well as an enhancement in the anxiogenic properties of drugs which diminish GABAergic neurotransmission (Idemudia et al, 1989). These alterations in GABA function occur up to three days following the last dose of ethanol, when the overt signs and symptoms have subsided. However, the results from the present study indicate that the capacity of GABA to modulate receptor affinity for benzodiazepine agonists and inverse agonists in rat cortex, cerebellum and hippocampus is not altered during withdrawal from ethanol.
These results are consistent with Rostogi et al (1986) who found no changes in GABA-modulation of agonist binding immediately after or 24 hours following ethanol cessation. A more recent study observed no change in GABA modulation of agonist or inverse agonist binding immediately following chronic ethanol exposure (Buck and Harris, 1990). Our findings extend previous findings by others to show that 12 hours following the cessation of chronic ethanol administration, during the time period when maximal withdrawal induced anxiety occurs, there is no alteration in the capacity of GABA to modulate benzodiazepine agonist and inverse agonist binding affinity. Further, no alteration in GABA modulation appears for up to three days after the last dose of ethanol, when behavioral experiments show that a GABAergic deficiency is still evident.

In contrast to the experiments which examine the ability of GABA to modulate benzodiazepine affinity, Buck and Harris (Buck and Harris, 1990) did show that chronic exposure to ethanol attenuated the activity of a benzodiazepine agonist to augment muscimol-stimulated uptake of chloride, and enhanced the actions of benzodiazepine inverse agonists to inhibit this response. The attenuation of increased agonist activity by muscimol was only evident shortly after ethanol discontinuation, while the change in inverse agonist function remained evident for up to eight
days. They suggested that following chronic exposure, changes in coupling of benzodiazepine agonist and inverse agonist receptor sites with the chloride channel might be affected. All these evidence suggested that ethanol may interact with GABAergic transmission and might have a binding site in the GABA/BZ chloride channel complex as showed in figure 30. It is generally believed that ethanol acting at this receptor complex modulate the stimulatory effects of GABA on chloride flux and at higher concentration ethanol might have a direct effect on Cl⁻ channel. Further studies need to be performed to characterize GABA sensitivity following chronic ethanol exposure during periods when maximal withdrawal induced behavioral alterations such as anxiety occur.

On the other hand, the imidazobenzodiazepine RO 15-4513 has been found to bind to benzodiazepine receptor sites on the GABAa receptor complex. In electrophysiological and behavioral studies, RO 15-4513 had the pharmacological properties of a partial inverse agonist (Bonetti et al, 1989). Sieghart et al (1987) demonstrated that a portion of the irreversible binding of RO 15-4513 in the brain was not displaceable by diazepam. This diazepam insensitive binding was localized to the olfactory granule cell layer and cerebellar granule cells and corresponded to a peptide of Mr 57000 daltons on gel electrophoresis in sodium dodecyl
Figure 30: The GABA/BZ chloride channel complex indicating the possible binding site for ethanol. This figure schematically illustrates the different receptor sites within the complex and give examples of their various ligands. Drugs acting at the complex modulate the effects of GABA on chloride influx. Ethanol may interact with this receptor modulating stimulatory effects of GABA on the chloride channel and at higher concentrations ethanol might have a direct action on Cl⁻ flux.
sulfate. However, because the diazepam-insensitive binding appeared not to be displaceable by diazepam, clonazepam, flunitrazepam, 2-oxoquazepam, CL 218-872 and triazolam, and only displaceable by Ro 15-1788, the parent compound of RO 15-4513, at an inhibition constant Ki of more than 100 times those concentrations that compete effectively at the diazepam sensitive sites, Sieghart et al (1987) believed that these sites were largely irrelevant to benzodiazepine-GABA pharmacology. We have explored the characteristics of reversible binding of RO 15-4513 to these sites and believe that they represent a subpopulation of GABAA receptors. RO 15-4513 binding to the classical benzodiazepine receptors is designated as binding to the diazepam sensitive sites, because the sites are sensitive to diazepam; and RO15-4513 binding to the unique sites are designated as binding to diazepam insensitive sites because they are insensitive to diazepam.

As reported by Turner et al. (1991) from autoradiography and Sieghert et al. (1987) for irreversible binding, we observed a high density of DZ-IS binding sites in rat cerebellum. It can also be seen there is more than one binding site for RO 15-4513 in rat cerebellum. In rat cerebellum, a 2 fold abundance diazepam sensitive sites exists relative to the DZ-IS sites.
DZ-IS sites have some pharmacological similarities to the DZ-S sites but display a unique ligand specificity. The most obvious difference is the inability of the classical ligands of the DZ-S site, the benzodiazepines, to bind to the DZ-IS sites. Diazepam did not inhibit \(^3\)H-RO 15-4513 binding to diazepam insensitive sites at physiologically relevant concentrations. It was reported (Turner et al. 1991) that neither diazepam, RO 5-4864, nor flurazepam inhibited \(^3\)H-RO 15-4513 DZ-IS binding; clonazepam was only able to inhibit the binding partially. Other classes of benzodiazepine receptor ligands bound to the DZ-IS site with lower affinities than to the DZ-S sites. In decreasing the order of affinity for the DZ-IS sites these included: the pyrazoloquinoline CGS 8216 (IC\(_{50}=50\)nM), the imidazobenzodiazepinone RO15-1788 (IC\(_{50}=80\)nM) and \(\beta\)-carbolines DMCM, ZK95962, ZK 94326, ZK 91296, ZK94323, \(\beta\)CCE and \(\beta\)CCM (active at 0.1-2\(\mu\)M). Most of these compounds are antagonists or inverse agonists at the classical benzodiazepine receptors. However, the possibility that the DZ-IS sites have a unique conformational requirement for compounds which are benzodiazepine receptor antagonists or inverse agonists is not absolute, because two of the modulately active \(\beta\)-carbolines (ZK91296 and ZK 94323) are actually agonists at the classical benzodiazepine receptors.
The DZ-IS sites do not bind CL 218872 and clearly do not correspond to the previously defined benzodiazepine-binding site subtypes. However, that these sites do bind many benzodiazepine receptor ligands (Turner et al. 1987) supports the possibility that DZ-IS sites represent a novel type of GABA receptor.

The present studies explored this possibility by testing whether the DZ-IS sites could be modulated by GABA. Clearly GABA was able to modulate DZ-S binding as expected (Fig.26). GABA modulation of DMCM binding to DZ-IS sites (Fig.29) was demonstrated, but no effect of GABA on RO 15-4513 binding to these sites could be demonstrated. However, if Ro 15 4513 binds to the DZ-IS sites as an antagonist, modulation by GABA would not be expected since, typically, GABA increases the affinity for agonists, decreases affinity for inverse agonists, and has no effects on antagonist binding. Further experiments revealed that GABA decreased the capacity of the full inverse agonist DMCM to displace the binding of RO 15-4513 to DZ-IS sites, although it is only weakly modulated. If these sites behave like other benzodiazepine receptors with respect to the GABA shift, RO 15-4513 appears to bind as an antagonist to the DZ-IS sites and DMCM bind as inverse agonist.

GABA modulation of inverse agonist binding to DZ-IS sites is consistent with other observations. Turner et al.
(1991) observed allosteric modulation by GABA of $^3$H-RO 15-4513 binding to DZ-IS sites in bovine cerebellum. Halminiem et al. (1989) found GABA enhancement of $^3$H-RO 15-4513 binding to DZ-IS sites in primary cultured cerebellar cells, as well as in actual cerebellar membranes from neonatal rats. Uusi-Oukari et al. (1990) demonstrated differential diazepam displacement of RO 15-4513 binding in two rat strains showing high and low alcohol sensitivity. Diazepam displaced all RO 15-4513 binding in the cerebellum in alcohol sensitive rats whereas displaced only 75% of the binding in the alcohol insensitive rats, i. e., only rats with low sensitivity to ethanol had significant DZ-IS sites. However, DZ-IS sites are present in normal alcohol sensitive Sprague-Dawley rats (Turner et al. 1991; Sieghart et al. 1987), cows and humans. It is important to note that this novel GABAA receptor with sensitivity to RO 15-4513 but not benzodiazepines agonists is present in human brain (Turner et al. 1991).

An additional suggestion of an alcohol connection for RO 15-4513 binding sites comes from the discovery that chronic ethanol administration to rats leads to an increase in the binding of $^3$H-RO 15-4513 but not $^3$H-flunitrazepam in several brain regions (Mhatre et al. 1988). Although the elevated RO 15-4513 binding was not described as being DZ-IS, it must have a novel low affinity for flunitrazepam
because high affinity binding of that ligand did not increase. These novel sites could figure in alcohol pharmacology and relatively low sensitivity to benzodiazepine. Further work clearly is needed to evaluate why the binding of RO 15-4513 varies with genetic susceptibility to alcohol action and chronic exposure to ethanol.

Seihgart et al. (1987) found a unique polypeptide of Mr 57000 daltons in cerebellum but not cortex that was photoaffinity labeled by $^3$H-RO 15-4513 in a DZ-IS manner. This is an Mr similar to that expected for numerous GABA\textsubscript{A} receptor subunits (Luddens et al. 1990). The possibility that at least some DZ-IS sites may be the result of GABA\textsubscript{A} receptor oligomers containing a novel polypeptide subunit composition was deduced from molecular cloning results: DZ-IS like binding was expressed in animal cells transfected with the $\alpha_6$, $\beta_2$ and $\gamma_2$ cDNAs (Luddens et al. 1990) suggesting that $\alpha_6$ gene product plays some role in receptors that have DZ-IS properties. The $\alpha_6$ mRNA is expressed almost exclusively in cerebellum (Luddens et al. 1990). Cerebellum has long been known to possess some unusual GABA\textsubscript{A} binding sites including some muscimol binding sites in granule cells that are apparently not coupled to benzodiazepine binding sites. Cerebellum also has an unusual assortment of GABA\textsubscript{A} subunit candidate mRNAs. It is
found that DZ-IS sites are not totally absent in other brain regions, but present at low level in hippocampus, cortex and elsewhere.

The novel efficacy of RO 15-4513, especially in regard to ethanol, and the existence of benzodiazepine receptor subtypes with unique pharmacological profiles, plus the animal strain differences in receptor subtypes and alterations by chronic ethanol administration suggest the possibility that the DZ-IS sites might mediate at least part of the alcohol antidote effects of RO 15-4513. If these sites were involved in alcohol antagonism, then other drugs active at DZ-IS sites could also be able to uniquely modulate alcohol effects. One can use the pharmacological differences between DZ-S and DZ-IS sites to determine which are more relevant. For example, FG 7142, another inverse agonist thought to possibly modulate ethanol effects through its inverse agonist properties (Nutt et al. 1989; Harris et al. 1988), does not bind to the DZ-IS sites. If the DZ-IS sites were to play some role in the blockade of ethanol action by RO 15-4513, this selectivity would be consistent with the idea that this drug specifically blocks GABA or pentobarbital more efficaciously than other partial inverse agonists, i.e., a selective efficacy against ethanol for RO 15-4513 (Sudzdak et al. 1988; Kulkarni et al. 1989). One possible explanation for its unique efficacy is that drugs
acting on benzodiazepine receptors differ in their affinity for different receptor subtypes (Olsen et al. 1990). Another possibility is that ethanol administration in some way increases the efficacy of partial inverse agonists. Evaluation of these hypotheses and understanding of the mechanism of the action of this novel drug will require more information.

Whereas DZ-IS sites appear to exist throughout the brain, the highest concentration in localized to the cerebellum. Thus, any effects of ethanol at this site, which could be reversed by RO 15-4513, may be expected to initially alter cerebellar functions, such as the incoordination seen early in alcohol intoxication. This leads to the postulation that the unique DZ-IS GABA receptor could be the first enhanced by ethanol, and additional differential ethanol effects could be expressed through the classical GABA/BZ receptors. Thus RO 15-4513 acting at DZ-IS sites might block early ethanol effects especially in the cerebellum, whereas other effects might be reversed by inverse agonist action at the DZ-S site. Further speculation is that the azido group of RO 15-4513, the only structural difference between the alcohol antagonist and the nonalcohol antagonist 15-1788, might physically block the chloride channel in receptors altered by ethanol.

Although the present investigation shows that chronic
ethanol intake produced no changes either in the apparent affinity (Kd) or the number of DZ-Iz and DZ-sensitive binding sites in rat cerebellum, it is still possible that ethanol induce some changes in these GABA receptor. The changes might be in other subunits or in the coupling of BZ agonist and inverse agonist receptor sites with the chloride channel. Thus, further studies, perhaps utilizing other techniques such as measuring uptake of Cl⁻ are needed to further characterize the effect of ethanol on GABA receptors and the functional Cl⁻-channel response.

In summary, DZ-IS sites represent a pharmacologically distinct site bearing certain similarities to but unique from the classical benzodiazepine receptor, possibly located on a unique GABA receptor. This GABA receptor subtype is most dense in the cerebellum, but not limited in this region. Although, significant differences in the apparent affinity or apparent receptor number for RO 15-4513 binding at total, DZ-S, and DZ-IS sites following 7 days of ethanol intake and subsequent withdrawal were not found in the present study, it is still possible that this site is involved in the modulation of the postulated ethanol effect on the GABAergic neurotransmission. More studies are required to answer the questions raised above.
CONCLUSIONS

1. Demonstration of allosteric interaction between BZ agonists, BZ inverse agonists and GABA requires special procedures for removal of endogenous GABA.

2. In the presence of GABA, the apparent affinity for flunitrazepam was increased approximately 1.7 fold and the apparent affinity for RO 15-4513 was decreased 1.7 fold.

3. No alteration in the capacity of GABA to modulate flunitrazepam or RO 15-4513 binding was observed in cerebral cortex membrane preparations either 12 or 72 hours following ethanol cessation. No changes in the GABA-modulation of flunitrazepam binding was evident at various times after the last dose of ethanol in membranes prepared from cortex, hippocampus or cerebellum.

4. Rat cerebellum expresses at least two different binding sites for $^3$H-RO 15-4513. These two putative GABA receptors bind RO 15-4513 with equal affinity, but are differentiated by their affinities for benzodiazepine agonists, such as diazepam.

5. Approximately 60-70% of the $^3$H-Ro 15-4513 binding sites in cerebellar membranes have relatively high affinity for diazepam, i.e., diazepam sensitive sites. Agonist and inverse agonist binding to this receptor is modulated by GABA. The second population of binding sites has very low
affinity for diazepam; i.e., this site is diazepam insensitive. The binding of Full inverse agonists like DMCM at this site is only weakly modulated by GABA.

6. No significant difference was observed between any of the treatment groups in the apparent affinity (Kd) for $^3$H-RO 15-4513 binding at total, DZ-S and DZ-IS sites following 7 days of ethanol intake and subsequent withdrawal. In addition, no significant difference in the density of diazepam-sensitive and insensitive binding sites was observed.
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