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REGULATION OF ADRENERGIC AND IMIDAZOLE PREFERING RECEPTORS IN THE RABBIT

by

MOMOH A. YAKUBU
B. Sc. (Honours) Ibadan, Nigeria

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN THE FACULTY OF MEDICINE OF THE UNIVERSITY OF GLASGOW.

DEPARTMENT OF MATERIA MEDICA
STOBHILL GENERAL HOSPITAL
GLASGOW G21 3UW
SCOTLAND.
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DEDICATION

This piece of work is dedicated to the everlasting memory of my beloved father, the Late ALHAJI YAKUBU AUDU EJULE, who died tragically on the 10th February, 1981. His encouragement has been the driving force that has brought me this far. For this, I will forever be grateful.
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This work would not have been possible without the financial assistant from the University of Maiduguri - Nigeria which is gratefully acknowledged.
DECLARATION

I declare that this thesis has been composed by myself and is a record of research performed by myself. It has not been admitted previously for a higher degree.

This research was carried out in the Department of Materia Medica, Glasgow University between January, 1986 to December, 1988, under the supervision of Dr. C.A. Hamilton.

June, 1989

M. A. Yakubu
PRESENTATIONS AND PUBLICATIONS INCLUDING WORK UNDERTAKEN FOR THIS THESIS


SUMMARY
SUMMARY

The studies reported in this thesis were designed to investigate the effects of chronic adrenoceptor drug treatment on central and peripheral alpha\textsubscript{2}-adrenoceptor number in the rabbit. In addition, functional studies were carried out using the central depressor response to clonidine injection and vascular pressor response to bolus doses of alpha-methylnoradrenaline injection to examine central and peripheral responses, respectively. The effects of chronic adrenergic drug treatment on alpha- and beta-adrenoceptor number were examined in rabbit forebrain and hindbrain membranes and were compared with the effects in the periphery (kidney membranes) where appropriate. Also changes in receptor number were then compared with functional changes.

In preliminary experiments, it was observed that $[^3\text{H}]$yohimbine and $[^3\text{H}]$idazoxan which have been described as alpha\textsubscript{2}-adrenoceptor ligands bind to the tissues used in these studies with different characteristics. This observation led to detailed examination and characterization of these binding sites (chapter three). The displacement of these ligands from their binding sites by a range of adrenergic drugs was examined. It was found that $[^3\text{H}]$yohimbine bound to alpha\textsubscript{2}-receptors while $[^3\text{H}]$idazoxan in addition to binding at alpha\textsubscript{2}-receptors bound principally to a non-adrenergic site.

In chapter four, the effects of chronic treatment with the alpha\textsubscript{2}-adrenoceptor antagonists yohimbine and idazoxan
on alpha₂-adrenergic receptors were examined and compared with effects on the non-adrenergic site labelled by \[^3H\]idazoxan. Functional changes occurring as a result of these treatments were also examined. Chronic yohimbine and idazoxan treatments significantly attenuated both vascular pressor responses to alpha-methylnoradrenaline bolus doses and the central depressor response to intracisternal clonidine. Yohimbine treatment significantly elevated \[^3H\]yohimbine binding to both forebrain and hindbrain but reduced \[^3H\]idazoxan binding to kidney membranes with no change in the brain. Idazoxan treatment significantly increased \[^3H\]yohimbine binding to the forebrain and decreased \[^3H\]idazoxan binding to the kidney.

In chapter five, the effects of chronic amitriptyline treatment either alone or in combination with idazoxan or yohimbine on alpha₂-adrenergic and non-adrenergic binding sites along with effects on beta-adrenoceptor number were studied. Increasing catecholamine concentrations in the brain indirectly by chronic amitriptyline administration, significantly reduced \[^3H\]yohimbine binding to the hindbrain but not the forebrain. \[^3H\]Idazoxan binding sites were not significantly affected by this treatment. Neither treatment with amitriptyline alone nor when combined with alpha₂-adrenoceptor antagonists had any significant effects on the number of \[^3H\]dihydroalprenolol (\[^3H\]DHA) binding sites.

Chapter six examined the effects of direct infusion of catecholamines into the rabbit brain via
intracerebroventricular infusion. Neither adrenaline nor noradrenaline had any significant effect on $[^3H]DHA$ or $[^3H]yohimbine$ binding sites although chronic adrenaline but not noradrenaline infusion significantly attenuated the depressor response to clonidine injection. Adrenaline infusion significantly reduced $[^3H]idazoxan$ binding to the right cerebrum.

Chapter seven studied effects of chronic guanabenz infusion on both number and function of alpha$_2$-adrenoceptors. Chronic infusion with the alpha$_2$-adrenoceptor agonist guanabenz significantly reduced $[^3H]yohimbine$ binding to both forebrain and hindbrain although no changes in kidney membranes were observed, while the number of $[^3H]idazoxan$ binding site in the kidney but not the forebrain or hindbrain was significantly reduced. Both the depressor and pressor responses to clonidine and alpha-methylnoradrenaline respectively were significantly attenuated by this treatment.

Chapter eight was aimed at bringing all the results in this thesis together, making comparisons, drawing conclusions and making proposals for future studies.

In this thesis it was observed that agonists can cause down regulation and antagonists up-regulation of the $[^3H]yohimbine$ binding site. Differences in regulation between tissues were observed which may have been due to differences in the alpha$_2$-adrenoceptor subtype predominating in the tissues or differences in drug concentrations in different tissues. Down-regulation was in general
accompanied by an attenuation of functional responses. In contrast, the \[^{3}\text{H}]\text{idazoxan}\ binding site was not regulated as expected, as no consistent changes were observed in brain although \text{yohimbine, idazoxan and guanabenz all caused down regulation in the kidney. This differential regulation of the \[^{3}\text{H}]\text{yohimbine and \[^{3}\text{H}]}\text{idazoxan}\ binding sites further shows that they are different sites in the rabbit. The nature of the idazoxan binding site is yet to be understood and calls for studies looking at its function and the second messenger to which it is coupled. This will further the understanding of this binding site and help to unravel the complexity of alpha\(_2\)-adrenoceptors and related sites.}

Finally, no changes in beta-adrenoceptor number were observed in the rabbit brain during either catecholamine or amitriptyline treatments. This contrasts with observations in rat brain and reports of changes in beta-adrenoceptor number during catecholamine infusion into the periphery of rabbits. Small subtype changes may have occurred which were not detected, or alternatively, beta-adrenoceptors in rabbit brain are relatively resistant to down-regulation.
CHAPTER ONE

GENERAL INTRODUCTION
CHAPTER ONE
INTRODUCTION

1.1 HISTORY OF ADRENOCEPTOR CLASSIFICATION.

At the beginning of the century, a British physiologist Langley introduced the term "receptive substance" to describe the site at which chemical messengers might act. This also gave the key to the action of antagonists that might bind to the "receptive substance" and prevent the action of stimulant molecules (Langley, 1905).

Oliver and Shafer (1895) discovered that extracts from suprarenal gland induced a vasopressor effect. The active principle of this extract was identified as adrenaline and was later synthesized chemically (Stolz, 1904). The discovery of adrenaline led to the idea that sympathetic neurotransmission might be mediated by an adrenaline-like substance (Elliot, 1904). Early literature on this substance was greatly confused as a result of the diverse effects that could be produced by exogenously applied adrenaline or its derivatives and also by adrenergic nerve stimulation. For example, it was proposed that either an inhibitory or excitatory substance ("sympathin I" and "sympathin E") could be liberated from adrenergic nerve endings on stimulation (Cannon and Rosenbluth, 1937; Greer et al., 1938; Gaddum and Goodwin, 1947). Dale (1906) building on the concept of "receptive substance" introduced by Langley, proposed the existence of subtypes of adrenergic receptors at myoneural junctions, one of which he said mediated inhibitory effects.
while the other mediated excitatory effects. This proposal was consequent to the differential effect of interaction of adrenaline and ergot alkaloids on smooth muscles.

Despite subsequent changes in methodology and technology, the pharmacological tools that Dale and his colleagues (1906 and 1910) used as a means to differentiate receptors are still those that are used today; agonists, antagonists and structure-activity relationships.

Ahlquist (1948) introduced the first formal classification of adrenoceptors into alpha- and beta-adrenoceptors on the basis of the differing rank order of potency of a number of sympathomimetic amines in a variety of tissues. The function of these receptors were determined by effects of some catecholamine on some animal preparations. Where adrenaline appeared to be most potent and isoprenaline least potent, the receptor was classed as an alpha-adrenergic receptor. The alpha-adrenoceptor was shown to be linked with excitatory functions such as vasoconstriction and stimulation of smooth muscles (e.g. uterus and gastro-intestinal tract). At the beta-adrenergic receptor the order of potency was shown to be isoprenaline > adrenaline >> noradrenaline. Beta-adrenergic receptors were associated with inhibitory effects such as inhibition of uterine contraction, bronchial smooth muscles and vasodilation. It has also one excitatory function namely myocardial stimulation. The acceptance of the dual concept (alpha/beta) was slow, largely because of unavailability of subtype selective drugs. However, the discovery of
pharmacological agents and catecholamines (Moran and Perkins, 1958; Powell and Slater, 1958) altered this situation and in a short time a large number of tissue responses were designated alpha- or beta-adrenoceptor mediated effects on the basis of their selective blockade with alpha- or beta-adrenoceptor antagonists.

1.1.1 Beta-adrenoceptor subclassification.

By the mid 1960s Ahlquist's alpha/beta-adrenoceptor hypothesis was fairly well established. However careful quantitative studies and the introduction of further adrenoceptor agonists and antagonists suggested that a further subclassification of alpha- and beta-adrenoceptors was needed. Moran (1966) was the first to observe that beta-adrenoceptors were not of a homogeneous type in all tissues and proposed the subdivision of beta-adrenoceptors based on a differential tissue-dependent blockade of responses to isoprenaline by alpha-methylated derivatives of dichloroisoprenaline and related compounds. This proposal was taken further by Furchgott (1959; 1967) and Lands et al., (1967) who subclassified beta-adrenoceptors into beta_1 and beta_2 receptors. This subclassification was based on differences in the order of potencies of 12 agonists and antagonists in several isolated organ bath preparations.

The subdivision was validated and confirmed with the development and promotion of subtype selective antagonists
and agonists, practolol for beta₁-adrenoceptor (Dunlop and Shanks, 1968) and salbutamol for beta₂-adrenoceptors (Cullman et al., 1969). At the beta₁-adrenoceptor, isoprenaline was found to have an efficacy greater than adrenaline = noradrenaline. The beta₁-adrenoceptor controls fatty acid mobilization, cardiac stimulation and inhibition of the contraction of rabbit small intestine. At beta₂-adrenoceptors, the relative potency was isoprenaline > adrenaline > noradrenaline. The beta₂-adrenoceptor controls inhibition of uterine contraction. With the development of subtype selective agonists and antagonists, this classification has been further validated. It has been reported that the pharmacology of beta₁- and beta₂-adrenoceptors are identical in all mammalian species (Minneman et al., 1981).

1.1.2 Alpha-adrenoceptor subclassification.

Unlike the beta-adrenoceptor where subclassification was relatively straightforward, identification of alpha-adrenoceptor subtypes was much more difficult. This was due in part to the more complex physiological responses elicited via alpha-adrenergic receptors and the lack of subtype selective drugs.

The first attempt at subclassifying alpha-adrenoceptor was based on anatomical location of the receptors with the suggestion that alpha-adrenergic receptors could be subdivided into postsynaptic and presynaptic subtypes.
This subclassification was based on observed differences in the potency of phenoxybenzamine in inhibiting pre-synaptic transmitter release and postsynaptic contractility of the cat spleen. The presynaptic alpha-adrenoceptors were shown to be involved in the regulation of transmitter release (e.g. noradrenaline) through the negative feedback mechanism mediated via stimulation by the transmitter itself (Langer, 1974; Starke, 1977), while postsynaptic alpha-adrenoceptors (postjunctional receptors) led to production of an effect (e.g. contraction of smooth muscles).

The presynaptic alpha-adrenoceptors could be differentiated from the postsynaptic receptors using the relative activities of various agonists and antagonists (Langer, 1973; Starke, 1981). The postsynaptic receptor was classified as an alpha₁-adrenoceptor with a relatively high affinity for the antagonist prazosin and the agonist methoxamine. The presynaptic alpha-adrenoceptor was classified as an alpha₂-adrenoceptor and showed a high affinity for the agonists clonidine and alphamethylnoradrenaline and the antagonists yohimbine (Starke, 1977; Wikberg, 1979) and recently idazoxan (Doxey et al., 1983).

While it appears that all presynaptic alpha-adrenoceptor are of the alpha₂-subtype there is now evidence for alpha₂-adrenoceptors outside noradrenergic terminal axons (Timmermans and Zwieten, 1982). Typical "presynaptic"
adrenoceptors have been demonstrated to be present on human platelets (Hoffman an Lefkowitz, 1980), cultured cells (Sabol and Nirenberg, 1979), and frog skin, and postsynaptically, on smooth muscles (Pettinger, 1977; Digges and Summers, 1983). Hence, these observations impose limitations on the anatomical subclassification of alpha-adrenoceptor proposed by Langer, (1974).

As a result of the shortcomings of the anatomical classification of alpha-adrenoceptors, Berthelsen and Pettinger (1977) then proposed a functional basis for classification. According to their proposal, alpha_1- and alpha_2-adrenoceptors mediate excitatory and inhibitory responses, respectively.

The development and firm establishment of the concept of two distinct types of alpha-adrenoceptors has depended heavily on the availability of subtype selective drugs (Timmermans and van Zwieten, 1982). Currently, agonists as well as antagonists are available with a high degree of selectivity and specificity for either alpha-adrenoceptor subtype. The best definition for alpha_1- versus alpha_2- is based on the antagonists prazosin and yohimbine. Bylund (1981) and Bylund and U’Prichard (1983) have shown that at the alpha_1-adrenoceptor site prazosin is more potent than yohimbine, while at the alpha_2-adrenoceptor site yohimbine is more potent than prazosin. More recently idazoxan has also been shown to be more potent at alpha_2- than alpha_1-adrenoceptor sites (Dettmar et al., 1983; Doxey et al., 1983).
1.1.3 Alpha$_2$-adrenoceptor heterogeneity.

Evidence has been emerging in the past few years indicating that neither alpha$_1$-nor alpha$_2$-adrenoceptors have identical characteristics in all tissues and species. These observed differences are much more pronounced in the case of alpha$_2$-adrenoceptor (Cheung et al., 1982; Bylund, 1985).

With the introduction of the alpha$_2$-adrenoceptor antagonist yohimbine and its diastereoisomer rauwolscine as [$^3$H]ligands in the 1980s and [$^3$H]clonidine earlier on (Motulsky et al., 1980), evidence for the heterogeneity of alpha$_2$-adrenergic binding sites has been mounting (Motulsky et al., 1980; Cheung et al., 1982; Bylund, 1981; 1985). Hoffman and Lefkowitz (1980), using the platelet as a model system, developed a two state model for the alpha$_2$-adrenergic receptor. This model showed agonists bind with high affinity to the alpha$_2$-[H] but with low affinity to alpha$_2$-[L] state, while antagonists bind both states with equal affinity. Guanine nucleotides shift the equilibrium in favour of the alpha$_2$-[L] state whereas Mg$^{2+}$ favours the alpha$_2$-[H] state (Hoffman and Lefkowitz, 1980). On the basis of this model, one would expect that the number of [$^3$H]antagonists binding sites ($B_{max}$) would either be greater than, or equal to, the number of binding sites for [$^3$H]agonists. However, Bylund (1981) suggested the existence of alpha$_2$-adrenergic receptor subtypes after comparing the number of binding sites for [$^3$H]yohimbine and [$^3$H]clonidine.
in several tissues and species. He observed a considerable variation in both the Bmax values for $[^3\text{H}]$yohimbine and $[^3\text{H}]$clonidine as well as the ratio between them. The Bmax ratio of $[^3\text{H}]$yohimbine to $[^3\text{H}]$clonidine for the rodents (rat and guinea pig) was consistently lower than the ratio for the non-rodents (cat and human) in all brain regions observed. He also observed that $[^3\text{H}]$yohimbine has five to ten times higher affinity in the non-rodents than in rodents, that oxymetazoline is more potent in the non-rodent species and that prazosin is relatively more potent than yohimbine at alpha$_2$-adrenoceptors in rodents compared to non-rodents.

Using various adrenergic agents to displace $[^3\text{H}]$yohimbine and $[^3\text{H}]$rauwolscine from rat cerebral cortex and human platelet membranes, Cheung et al. (1982) proposed the existence of alpha$_2$-adrenoceptor heterogeneity. Alabaster et al. (1986) also made comparisons between alpha-adrenergic antagonist affinity for $[^3\text{H}]$rauwolscine binding sites and observed wide variations between species and effects of drugs.

Bylund (1985) and Petrash and Bylund (1986), demonstrated the existence of two population of alpha$_2$-adrenoceptor sites in some regions of the human and rat brain. Based on the differences in their affinity for prazosin, these sites have been designated alpha$_{2A}$ and alpha$_{2B}$ with the alpha$_{2A}$ having low affinity for prazosin (250 nM). The alpha$_{2A}$ is predominantly found on human platelets. The alpha$_{2B}$ subtype has a high affinity
for prazosin (5 nM), and neonatal rat lung is the prototype tissue for this subtype. Using differences in the radioligand binding between alpha₂-adrenoceptors in kidney and platelet of the rabbit, Nahorski et al. (1985) proposed a similar definition for these subtypes (alpha₂A and alpha₂B). In addition, characterization of alpha₂-adrenoceptors on OK cells, a cell line derived from an Opossum kidney, led Murphy and Bylund (1988) to propose the existent of a third alpha₂-adrenoceptor subtype. This receptor subtype was initially thought to be similar to the alpha₂B-subtype because of its high affinity for prazosin hence it was termed "alpha₂B-like" receptor. With more extensive characterization of the OK cell, it has been shown that this receptor is different from other alpha₂-adrenoceptor subtypes and has been termed alpha₂C (Bylund, 1988).

The successful cloning of the genes encoding alpha₂-adrenoceptors from human platelet and kidney by Kobilka et al. (1987) and Regan et al. (1988) have shown that the DNA sequences coding for the two receptors reside on different chromosomes. It was also reported that the two receptors have similar but unique primary amino acid sequences. The human platelet receptor was found to be derived from chromosome 10, while the kidney receptor has its encoding gene on chromosome 4. These receptors were referred to as alpha₂-C10 and alpha₂-C4, respectively. Based on pharmacological evidence and the correlation between data
from the receptor expressed in COS-7 cells, platelet and HT29 cell, the alpha$_2$-C10 receptor was identified as the alpha$_{2A}$-receptor. While the alpha$_2$-C4 was identified as the alpha$_{2B}$-receptor. An indication for the existence of a third subtype of alpha$_2$-adrenoceptor has been presented by Kobilka et al. (1987) when they found another receptor to reside on chromosome 2. This receptor has however not yet been cloned.

Boyajian and Leslie (1987) and Boyajian et al. (1987), using quantitative autoradiographic techniques have presented both anatomical and pharmacological evidence for differential binding and distribution of [$^3$H]rauwolscine and [$^3$H]idazoxan binding sites within rat brain. It was not clear, however, to which subtypes of alpha$_2$-adrenoceptor the two ligands were binding.

1.1.4 Imidazoline binding sites.

Several investigators have suggested that the recognition sites of some alpha$_2$-adrenoceptor agonists with an imidazoline moiety differ from those with a phenylethylamine-type structure (Ruffolo et al., 1977; Tayo, 1979; Mottram, 1983; Mottram and Thakar, 1984; Bond et al., 1986). Earlier Ruffolo et al. (1977) proposed that imidazolines interact at sites other than alpha$_2$-adrenoceptors when they failed to observe a cross-desensitization between the two compounds, imidazolines and phenylethylamines, on rat vas differentia preparations. Recently, several studies have shown [$^3$H]idazoxan and
[³H]para-aminoclonidine binding to non-adrenergic sites as well as alpha₂-adrenergic receptors in rabbit kidney and guinea pig brain tissues (Ernsberger et al., 1988; Lachaud et al., 1988; Wikberg, 1988). However, it is not known if these non-adrenergic sites which have been referred to as imidazol(in)e, idazoxan or clonidine binding sites (Yablonsky et al., 1988; Ernsberger et al., 1986; Wikberg, 1988) are a homogeneous receptor site.

Clonidine has recently been shown to bind preferentially to imidazoline binding sites as well as alpha₂-adrenoceptors in the ventrolateral medulla from bovine brain (Ernsberger et al., 1986) and a low molecular weight substance has been isolated from both bovine and rat brain membranes (Atlas and Burstein, 1984; Meeley et al., 1986). This substance has been shown to potently displace [³H]clonidine and its analogue [³H]para-aminoclonidine from their binding sites in bovine ventrolateral medulla membranes with a higher affinity for non-adrenergic sites (Atlas and Burstein, 1984; Meeley et al., 1986). The substance has been named clonidine displacing substance (CDS) (Meeley et al., 1986; Felsen et al., 1987). The isolated clonidine displacing substance has been shown to be biologically active. Bousquet et al. (1986) and Meeley et al. (1986) have shown that rostral ventrolateral medulla injection of CDS in rats or cats modifies arterial blood pressure. It has also been reported to potently contract rat gastric fundus (Felsen et al., 1987) and block electrically
stimulated contraction of rat vas deferens (Diamant and Atlas, 1986).

1.1.5 Alpha₁-adrenoceptor heterogeneity.

Variation has also been observed in the pharmacological properties of alpha₁-adrenoceptors in different tissues and species and this has led to speculation on the existence of more than one alpha₁-adrenoceptor subtype (Drew and Whiting, 1979; McGrath, 1982). Flavahan and Vanhoute (1986) have also suggested the existence of two subtypes of alpha₁-adrenoceptor after analysing the pA₂(affinity) values of prazosin and yohimbine on alpha₁-adrenoceptor contractile responses in blood vessels from rodents and rabbits. They observed that one of the subtypes of this receptor has a higher affinity for prazosin than yohimbine while the other has low affinity for these alpha-adrenoceptor antagonists. As a result of the differences observed in the affinities of clonidine and yohimbine for aortic vessels from six different species, Ruffolo and Waddel (1982) had earlier proposed the existence of alpha₁-adrenoceptor subtypes. Based on the actions of indanidine and phenoxybenzamine, Coats et al. (1982) also proposed the existent of a subtype (alpha₁S) which they said was a subset of alpha₁-adrenoceptors. In this experiment, the alpha₁S was shown to be activated by indanidine and to be particularly sensitive to phenoxybenzamine, while the classical alpha₁-adrenoceptor was defined as one where noradrenaline but not indanidine
was active. In order to explain the differential effects (potency) of imidazolines and phenylethylamines, McGrath (1982) proposed two subtypes (alpha₁a and alpha₁b). He noticed that imidazolines and phenylethylamines were highly potent in some tissues which he called alpha₁a, while imidazolines were not effective in the remaining tissues which he referred to as alpha₁b.

Recently, Han et al. (1987) have provided evidence for the existence of alpha₁a and alpha₁b-subtypes in which functional responses at the second messenger level were incorporated in their definition. In their experiments, they noticed that rat vas deferens contains two subpopulations of alpha₁-adrenoceptors (alpha₁a and alpha₁b), each subtype having a different function in coupling Ca²⁺ release by neurotransmitters and hormones.

Table 1.1 shows the proposed adrenoceptor classification.

1.1.6 Radioligand binding studies of adrenergic receptors.

The introduction of direct radioligand binding studies has changed the entire complexion of the experimental approach to the study and understanding of adrenergic receptors and the events they mediate. Radioligands have made much more accurate quantification of adrenergic receptors possible and have increased our understanding of
Table 1.1

Proposed classification of adrenoceptors.

(Adapted from Bylund, 1988).

Gs = Stimulatory guanine nucleotide binding protein,
Gi = Inhibitory guanine nucleotide binding protein,
Gx = Guanine nucleotide binding protein not yet characterized.
drug-receptor interactions.

The first ligands employed in radioligand binding experiments were the \(^3\text{H}\)catecholamines, but this pioneering effort met with limited success because of the low specific activity and the high non-specific binding of these ligands (U'Prichard and Snyder, 1977; Davies and Lefkowitz, 1981). Successful radioligand binding was first reported for beta-adrenergic receptors using \(^3\text{H}\)alprenolol and \(^3\text{H}\)dihydroalprenolol, while \(^3\text{H}\)dihydroergocryptine and \(^3\text{H}\)clonidine were used for alpha-adrenoceptors (Aurbach et al., 1974; Levitzki et al., 1974; Lefkowitz et al., 1974; Williams and Lefkowitz, 1976; Greenberg and Snyder, 1977).

Several radioligands with high affinity have been developed and used to study beta- and alpha-adrenergic receptors in a variety of tissue systems. These include \(^3\text{H}\)dihydroalprenolol, \(^{125}\text{I}\)iodohydroxybenzylpindolol, \(^3\text{H}\)hydroxybenzylisoproterenol (Hoffman and Lefkowitz, 1980) and recently \(^{125}\text{I}\)iodocyanopindolol (ICYP) (Brodde et al., 1981) for beta-adrenoceptors. While ligands such as \(^3\text{H}\)dihydroergocryptine (\(^3\text{H}\)DHE), \(^3\text{H}\)WB4101, \(^3\text{H}\)clonidine, \(^3\text{H}\)epinephrine (Hoffman and Lefkowitz, 1980) and recently \(^3\text{H}\)yohimbine and \(^3\text{H}\)idazoxan (Motulsky et al., 1980; Covents et al., 1987) have been used to study alpha-adrenergic receptors. This has led to a variety of different binding patterns. These different patterns may relate to heterogeneity of alpha-adrenergic receptors and the varying regulatory properties of these receptors.
In addition to binding to specific receptors radioligands may bind at other sites. For radioligand binding sites to be recognised as functional receptors, criteria were set first by Cuatracesas (1974) and recently by Motulsky and Insel (1984). These conditions have to be satisfied to demonstrate that radioligand binding sites may be equated with receptor sites. These conditions as defined by Motulsky and Insel (1984) are as follows:

i) Stereospecific criteria must be satisfied. Where stereoisomerism has biological relevance as in the catecholamines the receptor must be stereospecific i.e. -isomers more potent than +isomers.

ii) The affinity of the binding site and kinetic rate constants of binding should correlate with the biological properties of the ligand used in binding studies.

iii) Specific binding should be saturable and proportional to tissue concentration.

iv) Binding should be as rapid and reversible as are the physiological responses to the ligand.

v) Unlabelled ligands should compete for radioligand binding sites with similar relative potencies as those obtained from pharmacological experiments.
vi) Radioligands should remain unaltered throughout the course of the experiment.

vii) Alternative methods should give approximately identical values for the parameters (equilibrium dissociation ($K_D$) constant and maximum number of binding sites ($B_{max}$)).

These criteria are necessary but may not be sufficient for identifying a receptor since transport systems for a hormone may satisfy all these criteria and yet may not be physiologically relevant receptor sites.

However, the basic principles of this technique entails incubation of a tissue homogenate with a tritiated or iodinated form of an adrenergic agonist or antagonist in the presence or absence of a non-labelled displacing drug. Commonly used displacing agents include prazosin for $\alpha_1$-, yohimbine for $\alpha_2$-, practolol for $\beta_1$- and metoprolol for $\beta_2$-receptors. Specific binding is defined as the difference between radioactivity observed in the absence of unlabelled ligand (total binding) and that in the presence (non-specific binding). The specific binding should approach a maximum ($B_{max}$) in a saturable manner. The concentration of ligand that binds to half of these receptors is the dissociation constant ($K_D$). These parameters are obtained from the analysis of the Scatchard or other suitable plots (Scatchard 1947).

In contrast to specific binding sites which are finite
and saturable, it is assumed that non-specific binding sites are effectively infinite.

1.2 BIOCHEMICAL MECHANISMS OF SIGNAL TRANSDUCTION.

Many extracellular signals (first messengers), such as neurotransmitters, hormones and growth factors, do not permeate the plasma membrane. Instead, they interact with the recognition sites (receptors) at the cell surface, which via a series of events finally leads to a physiological response in the form of contraction, relaxation, secretion, inhibition, or growth, etc. The processes linking receptor activation to intracellular events is known as transduction and was first used to describe the transfer of information between the receptors for a variety of hormones and adenylate cyclase, the enzyme responsible for the production of cyclic adenosine monophosphate (cAMP) (Rodbell et al., 1969). Adenylate cyclase and other systems that relay messages from the exterior are collectively referred to as "second messengers".

As a model for information transfer, a tripartite model has been suggested (Rodbell, 1980). This model is composed of recognition, transduction, and effector or amplifying components (fig. 1.1). Recognition is represented by the surface receptor, transduction involves members of a family of guanine nucleotide-binding regulatory proteins (G-proteins) and effector or amplifying component such as cyclic
Components of transmembrane signalling and processes involved in adenylate cyclase activation.

(Adapted from Rodbell, 1980). Receptor (R), Guanine nucleotide protein (Gs), Adenylate cyclase (AC), Guanosine triphosphate (GTP), Adenosine triphosphate (ATP), Guanosine diphosphate (GDP), Cyclic adenosine monophosphate (cAMP).
AMP. Decades of research have led to the understanding that these steps are carried out by distinct macromolecular components.

1.2.1 **Beta-adrenoceptor effector coupling.**

Sutherland and Rall, (1960) discovered that catecholamines and sympathomimetic agents acting via beta-adrenoceptors transmit their messages to the cell interior by stimulating the synthesis of cyclic adenosine monophosphate (cAMP) an intracellular nucleotide "second messenger" that is present at micromolar concentrations. Cyclic AMP is one of the most intensively investigated second messengers (Ross and Gilman, 1980).

Binding of the agonist to the receptor (R) leads to a conformational change in the receptor molecule (R'), the complex (agonist-R') formed then binds to the guanine nucleotide (Gs) protein to form a transient agonist-R'-Gs ternary complex. The formation of this complex facilitates the conversion of GTP to GDP by ATPase, and this causes the dissociation of the Gs-protein from the agonist-R' complex. The G-protein then binds to and activates adenylate cyclase (AC) (figure 1.1). The activation of adenylate cyclase leads to the conversion of adenosine triphosphate (ATP) to adenosine-3-5-monophosphate (cAMP) which serves as "second messenger" and activates intracellular protein kinases. These in turn cause phosphorylation of enzymes that lead to the cellular response such as smooth muscle relaxation.
glycolysis, lipolysis, etc.

In radioligand binding studies, agonist and antagonist binding characteristics differ (Lefkowitz et al., 1983). The inhibition of radioligand antagonist binding by unlabelled antagonist follows simple Mass Action kinetics (Kent et al., 1980). The slope of antagonist competition curves are steep with Hill coefficients that are approximately equal to one. Analysis of the curves indicate that antagonists bind to a single receptor affinity state (Kent et al., 1980). In contrast, antagonist radioligand binding inhibition by agonists results in competition curves with "shallow" slopes and Hill coefficients of less than one (De Lean et al., 1980), which is consistent with the agonist binding to more than one affinity state of the receptor (Kent et al., 1980). These "shallow" curves are characteristic of agonists competing against labelled antagonist ligands. The ability of agonists to promote the formation of a receptor-G-protein complex corresponds to the ability of the agonist to form a high affinity state of receptor. The binding of guanosine triphosphate (GTP) to the ternary complex initiates two events, one is to destabilise the ternary complex so that the receptor returns to its low affinity state (HR). This explains the GTP induced decrease in apparent affinity for agonists and the steepening of the agonist competition displacement curve so that agonists in the presence of GTP appear to interact with a single state of the receptor (lower affinity state) (Nahorski and
Barnett, 1982; Motulsky and Insel, 1982). A second simultaneous consequence of GTP binding to agonist-receptor-G-protein complex is activation of the G-protein (probably by a conformational change) (Bottari et al., 1979). The activated G-protein-GTP complex then activates the catalytic moiety of adenylate cyclase (Stadel et al., 1981).

So far, at least three G-proteins have been suggested to exist. Of these, two have been widely studied, Gs- and Gi- which have been shown to have stimulatory and inhibitory actions respectively (Rodbell, 1980). Gs and Gi can be distinguished by cholera and pertusis toxins. Gs is inhibited by cholera toxin and Gi by pertusis toxin. The third type which has not been extensively studied has been postulated to regulate membrane processes other than adenylate cyclase and is referred to as the Gx-protein (Rodbell, 1980). It is not inhibited by either cholera toxin or pertusis toxin. The G-proteins consist of 3 subunits (alpha, beta and gamma). The beta and gamma subunits are conserved in all types of G-proteins while the alpha subunit differs.

1.2.2 **Alpha2-adrenoceptor effector-coupling.**

Like the beta-adrenoceptors, alpha2-adrenoceptors are coupled to adenylate cyclase but unlike the beta-adrenoceptors, activation of alpha2-receptors leads to inhibition of cAMP production, since they are negatively coupled to adenylate cyclase (Fain and Garcia-Sainz, 1980).
However, little information is available in the literature on hormonal activation of alpha\textsubscript{2}-adrenoceptors and its coupling to the second messenger. As for beta-adrenoceptors, agonists bind to two affinity states (high and low) at the alpha\textsubscript{2}-adrenoceptor, while antagonists recognise a single affinity state (Bylund and U'Prichard, 1983; Nahorski et al., 1985). Adenylate cyclase inhibition occurs through activation of a different guanine nucleotide binding protein (Gi) which is distinct from the one responsible for stimulation (Gs) (Jakobs et al., 1984; Jakobs, 1985). Jakobs and Schultz (1983), have reported a decrease in cAMP levels in platelets, hepatocytes, cerebral microblastoma cells on alpha\textsubscript{2}-adrenoceptor stimulation. However, opinions are divided on the mechanisms by which this guanine nucleotide protein causes a decrease in adenylate cyclase activity. Both direct mechanisms via the beta, gamma subunits and indirect mechanisms via phospholipase A or Na\textsuperscript{+}/H\textsuperscript{+} exchange have been postulated.

1.2.3 \textbf{Alpha\textsubscript{1}-adrenoceptor effector coupling.}

In contrast to alpha\textsubscript{2}- and beta-adrenoceptors, alpha\textsubscript{1}-adrenoceptors do not transmit their signal to the cell interior via cAMP.

Agonist binding to alpha\textsubscript{1}-adrenoceptor activates membrane bound phospholipase C an enzyme that catalyses the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP\textsubscript{2})
to myoinositol-1,4,5-trisphosphate - (IP$_3$) and diacylglycerol (DG) (Homcy and Graham, 1975; Michell 1983; 1985; Berridge 1984). The inositol-1,4,5-trisphosphate produced then activates the release of Ca$^{2+}$ from intracellular stores into the cytosol (Streb et al., 1983). This can in turn activate cellular responses such as actin-myosin coupling and hence tissue/muscle contraction (Michell, 1985) (figure 1.2). The location of the inositol-1,4,5-trisphosphate sensitive Ca$^{2+}$ pool is uncertain, but it may be in some element of the endoplasmic reticulum membrane system.

1,2-Diacylglycerol (1,2-DG), the other product of PtdIns-4,5-bisphosphate (PIP$_2$) breakdown is the first conclusively identified membrane associated "second messenger" molecule; it activates a novel phospholipid- and Ca$^{2+}$ dependent protein kinase known as protein kinase C (Nishizuka, 1983). Among other actions, protein kinase C may influence the influx of extracellular calcium. Thus the mobilization of Ca$^{2+}$ from intracellular vesicles and the influx of extracellular Ca$^{2+}$ appears to be linked to the activation of the alpha$_1$-adrenoceptor.

Figure 1.2 shows a diagramatic representation of some of the mechanisms of transmembrane signalling used by cell surface receptors.
Figure 1.2
Mechanisms of transmembrane signalling used by cell surface adrenergic receptors. (Adapted from Michell, 1987).
1.3 ADRENERGIC RECEPTOR REGULATION.

The phenomenon of denervation supersensitivity and drug tachyphylaxis have intrigued pharmacologists for many years. It is now possible to investigate the molecular mechanisms involved in this regulatory process using radioligand binding techniques. Receptor regulation refers to the processes that modify an agent's ability to bind to a receptor and induce an effect. After a cell or tissue has been exposed for a period of time to an agonist or hormone such as a catecholamine, that tissue often becomes less responsive to further stimulation by that particular agent. A variety of terms have been employed to describe such processes including tolerance, desensitization, refractoriness and tachyphylaxis. This phenomenon has clearly been demonstrated for at least some beta-adrenergic and alpha-adrenergic responses (Davies and Lefkowitz, 1981; 1984). The regulation of adrenergic effects may involve changes in receptor number or affinity, altered coupling of the receptor to adenylate cyclase or other effector mechanisms, and may be demonstrable in a variety of experimental conditions e.g. intact animals, whole cell suspensions or isolated membrane preparations (Brodde et al., 1982; Harden, 1983; Insel and Motulsky, 1987).

It should be noted that down regulation and desensitization are not synonymous; down regulation refers to a fall in receptor number while desensitization refers to a reduction in biological responses to a hormone or agonist.
stimulus. A receptor system may be down regulated or desensitized but not necessarily both. Similarly, up-regulation and supersensitization are not synonymous.

Desensitization phenomenon have been classified into two broad categories, agonist-specific or "homologous" and agonist non-specific or "heterologous". Homologous desensitization refers to the form of regulation where, after exposure to an agonist for some time, the cell becomes refractory to a further stimulation specifically by that agonist, while actions of other agents or hormones remain unaffected. Conversely, heterologous, or agonist non-specific desensitization indicates that exposure of a receptor system to an agonist leads to attenuation of the response to other agonists operating through distinct receptors (Hertel and Perkins, 1984; Davies and Lefkowitz, 1981; Sibley et al., 1987). It has been proposed that these two processes occur by distinct mechanisms. The detailed processes responsible for these receptor alterations are however not known at present.

1.3.1 Desensitization and down regulation of beta-adrenergic receptors.

Desensitization has been extensively studied for the beta-adrenoceptor coupled adenylate-cyclase system (Harden, 1983; Hertel and Perkins, 1984; Sibley and Lefkowitz, 1985). Long term exposure of responsive cells to a beta-adrenergic agonist leads to a rapid loss in agonist-stimulated
adenylate cyclase activity.

The mode of loss of receptor sensitivity after chronic stimulation by agonists or hormones occurs in stages. The first stage involves the uncoupling of receptors from adenylate cyclase within the plasma membrane without any loss of beta-adrenergic receptors as measured by binding of hydrophobic antagonists such as $[^3]H$-dihydroalprenolol or $[^{125}]I$-labelled pindolol. It is believed that there are alterations in the conformational state of the receptor so that it is unable to activate adenylate cyclase. The process is rapid and involves conversion of the receptor from a high to a low affinity state which occurs within 1-2 minutes (Harden et al., 1979; Su et al., 1979; 1980; Homburger et al., 1980).

The process of beta-adrenoceptor uncoupling also involves the Gs-protein. The uncoupling process is rapidly followed by sequestration of the uncoupled receptor into vesicles away from the cell surface (Staehelin and Simonis, 1982; Stadel et al., 1983). The receptors are therefore said to be internalized and become less accessible to hydrophobic agonist and antagonist binding ligands such as $[^3]H$CGP-12177. Further evidence for redistribution of receptors was observed from differential centrifugation. When cell lysates or homogenates were analysed by sucrose density gradient centrifugation, it was noted that some of the receptors were shifted from plasma membrane to a light vesicle fraction which sediments at higher centrifugal forces (Fishman and
Perkins, 1988). With the loss of antagonist binding sites at the cell surface, there was at the same time an appearance of $[^3H]$dihydroalprenolol binding sites in the soluble fraction of the disrupted cells. This observation led to the hypothesis that agonist modified receptors are redistributed in the cytosol (Davies and Lefkowitz, 1981; Staehelin et al., 1983; Mahan et al., 1985). This might well be compatible with an internalization mechanism. Redistribution only appears to involve physical separation of beta-adrenergic receptor from Gs-protein. The redistributed receptors can still be quantified by conventional antagonist radioligand binding techniques which label total receptor populations.

Homologous desensitization of beta-adrenergic receptors appears to involve phosphorylation of the receptor by a novel protein kinase which is independent of cAMP. The enzyme termed beta-adrenergic receptor kinase (BARK) is common in mammalian tissues and appears to be cytosolic (Benovic et al., 1986; Strasser et al., 1986). The BARK is specific for beta-adrenergic receptors and possibly other receptors which are coupled to adenylate-cyclase. It has a unique property of phosphorylating only the agonist-occupied form of the receptor, the antagonist occupied or unoccupied receptor is not a substrate (Strasser et al., 1986; Sibley et al., 1987; Lefkowitz and Caron, 1988). This type of phosphorylation reaction can therefore be described as substrate-activated rather than kinase-activated. Agonist occupation of the receptor leads to translocation of beta-
adrenergic receptor kinase from the cytosol to the plasma membrane where it phosphorylates the agonist occupied receptor (Strasser et al., 1986; Sibley et al., 1987). This is followed by functional uncoupling of the receptors from adenylate-cyclase. The receptors then become sequestered or internalized into low density membrane particles or vesicles. The nature of this internalized compartment and the mechanisms by which receptor translocation occur are not yet understood. The internalized receptors may be dephosphorylated and functionally redistributed to the cell surface. A reduction in agonist concentration or its removal may lead to receptor redistribution to basal levels. However prolonged agonist stimulation will result in more receptors being internalized for longer and could lead to receptor degradation and loss which can cause the reduction in total receptor number, usually referred to as down-regulation.

It has been speculated that all adenylate-cyclase coupled receptors share a common domain where phosphorylation by BARK takes place and that this domain is exposed on occupation of the receptor. Agonist-occupied receptors are substrates for the enzyme while uncoupled receptors will escape desensitization. Figure 1.3 presents molecular events which may be involved in homologous desensitization of beta-adrenergic receptors.

Mechanisms of heterologous desensitization of beta-adrenergic receptors are said to involve phosphorylation of the receptors by cAMP-dependent protein kinases (protein
kinases A and C). Activation of adenylate cyclase by any of several hormones leads to increased concentrations of cAMP, protein kinases become activated and the beta-adrenergic receptor is phosphorylated. Evidence for this mechanism of regulation of beta-adrenoceptors comes from studies with avian erythrocytes, which present a close correlation between catecholamine-induced heterologous desensitization and beta-adrenergic phosphorylation (Stadel et al., 1983; Nambi et al., 1985). Phosphorylation of beta-adrenergic receptors in vitro by both protein kinase A and C has also been reported (Benovic et al., 1986; Bouvier et al., 1987). The rate but not the extent of the reaction is enhanced by agonist occupation of the receptor. Phosphorylation reduces the ability of the agonist-occupied receptor to stimulate the GTPase activity of this coupled Gs-protein, hence it appears to be a functionally significant modification. Since any hormone or drug that increases cAMP levels will presumably lead to phosphorylation, this type of regulatory mechanism is referred to as heterologous. Receptor phosphorylation and desensitization has been reported after treatment of intact cells with phorbol esters, which can directly activate protein kinase C (Kelleher et al., 1984; Sibley et al., 1987; Lefkowitz and Caron, 1988). Activation of protein kinase C by phosphatidylinositol (PI) hydrolysis may also promote heterologous desensitization.
Figure 1.3
Molecular mechanisms involved in homologous desensitization of beta-adrenoceptors. (Adapted from Lefkowitz and Caron, 1988).

BAR = Beta-adrenoceptor
Ns = Nucleotide stimulatory protein
C = Adenylate cyclase
BARK = Beta-adrenoceptor kinase
PO$_4$ = Phosphate
1.3.2 Desensitization and down-regulation of alpha-adrenergic receptors.

The mechanisms involved in the desensitization processes described have been entirely derived from studies of beta-adrenergic receptor responses. Unlike beta-adrenoceptors, the regulation of alpha-adrenergic receptor mediated responses have been much less explored. The lack of understanding of the mechanisms of action of alpha-adrenoceptors coupled with the absence of suitable hydrophilic radioligands, might partly be responsible for the lack of information on the regulation of the alpha$_2$-adrenergic receptor system which is negatively coupled to adenylate cyclase.

1.3.2.1 Mechanisms of alpha$_2$-adrenoceptor desensitization.

Alpha$_2$-adrenoceptors are negatively coupled to adenylate cyclase via the inhibitory guanine nucleotide binding protein (Gi). The mechanism of desensitization of alpha$_2$-adrenoceptors has been much less studied. Much information on alpha$_2$-adrenoceptor coupling and post-receptor mechanisms has come from studies using platelets (Motulsky et al., 1986; Insel and Motulsky, 1987). Despite their usefulness for such studies, platelets, unlike most cells, lack nuclei and are deficient in protein synthesis. Hence, for more understanding of the metabolism and cellular
dynamics of alpha\textsubscript{2}-adrenoceptors, such as regulation, cell types other than platelets, will be more appropriate for such studies. To overcome this problem, McKernan et al. (1987) described a cell type, human erythroleukemia (HEL) cells, which possess surface markers for platelets/megakaryocytes as well as monocytes. This HEL cell model system derived from peripheral blood cells, grown in suspension, is related to human platelets. It has made it possible to study alpha\textsubscript{2}-adrenergic receptors on intact cells. The HEL cells system has been demonstrated to have alpha\textsubscript{2}-adrenoceptors like any other system (McKernan et al., 1987; 1988). Using radioligand binding to study the mechanisms of desensitization of alpha\textsubscript{2}-adrenoceptors in HEL cell systems, McKernan et al. (1987) demonstrated that the alpha\textsubscript{2}-adrenoceptor on this system can be detected in three different states:

i) A high affinity state of the receptor which has been shown to represent a ternary complex (agonist-receptor-G\textsubscript{i} protein). This state is only identified in the cell membranes in the absence of guanine nucleotides which dissociate the complex (Bylund and U’Prichard, 1983; Limbird, 1983; Neubig et al., 1985).

ii) A low affinity state of the receptor for the agonist. This represents binding of agonist to the receptor only (Bylund and U’Prichard, 1983; Limbird, 1983; Neubig et al., 1985).
iii) A sequestered state. This state represents receptors which cannot be detected by epinephrine in an intact cell in the presence of GTP. All sites are however detected by UK 14,304, the difference between the sites detected by UK 14,304 but not by epinephrine is that they are sequestered. Although receptor redistribution or sequestration has been reported for other receptors, this is the first for alpha$_2$-adrenoceptors.

Alpha$_2$-adrenergic receptor translocation from the cell surface to the sequestered compartment has been demonstrated after agonist treatment, but a consistent decrease in the function of the receptors in inhibiting cAMP accumulation was not observed (McKernan et al., 1987). This observation gave rise to the suggestion that this system has a great deal of spare receptors such that receptors can be desensitized or removed from the cell surface without changes in function. Acute desensitization is not a common feature of alpha$_2$-adrenergic receptors (Insel and Motulsky, 1987).

Beta-adrenergic receptor kinase has also been shown to regulate or phosphorylate receptors that are negatively coupled to adenylate cyclase (Benovic et al., 1986; Mayor et al., 1987; Lefkowitz and Caron, 1989).
1.3.2.2 **Mechanism of alpha₁-adrenoceptor desensitization.**

Alpha₁-adrenoceptors have been reported to undergo desensitization. Changes in adrenergic neuronal activity have been reported to alter alpha₁-adrenoceptor density in brain (Reader and Briere, 1982; Vetulani et al., 1984; Johnson et al., 1987). Rapid loss of alpha₁-adrenergic receptor binding sites have been reported to occur within 6 hours after intrathecal infusion of phenylephrine (Astrachan et al., 1983). Incubation of hamster vas deferens smooth muscle with phorbol esters has been reported to cause attenuation of alpha₁-receptor mediated release of inositol phospholipids (Leeb-Lundberg et al., 1985), although chronic intracerebroventricular infusion of norepinephrine had no significant effect on the density of $[^{125}\text{I}]$BE 2254 binding sites or on the magnitude of any of alpha₁-adrenergic receptor-mediated responses (Johnson et al., 1987).

Recently, Leeb-Lundberg et al. (1987) have attempted to explain the molecular mechanisms involved in alpha₁-adrenoceptor desensitization as a result of agonist treatment. They have reported that occupation of alpha₁-adrenoceptor by agonists such as noradrenaline leads to desensitization and phosphorylation of the receptor within minutes of occupany. Activation of the receptor causes hydrolysis of phosphatidylinositol-4,5-bisphosphate which leads to the generation of diacylglycerol. The liberated
diacylglycerol activates cAMP formation which then phosphorylates \( \alpha_1 \)-adrenoceptors as a process of feedback regulation thus leading to functional desensitization of the receptor. In addition to phosphorylation, agonist activation of the receptor causes a reduction in the accessibility of the receptor to ligands by promoting sequestration away from the plasma membrane cell surface. Hence receptor uncoupling appears to require only phosphorylation while receptor sequestration requires both phosphorylation and agonist occupancy. \( \alpha_1 \)-adrenergic agonists as well as other agonists which use diacylglycerol as a second messenger can undergo functional desensitization of their receptors in a like manner. Phorbol esters which act directly on protein kinase C have also been reported to cause functional desensitization of the \( \alpha_1 \)-adrenoceptor via the mechanisms described (Kelleher et al., 1984; Lefkowitz and Caron, 1987; Mayor et al., 1987; Sibley et al., 1987).

1.3.3 Hypersensitivity and Up-regulation.

The mechanism of hypersensitivity or up-regulation is not well understood. However, this phenomenon may be a consequence of blocking tonic down regulation induced by physiologic fluctuations in the levels of agonist (circulating catecholamines). Increases in receptor density have been observed concomitant with a fall in the levels of circulating catecholamines. Destruction of noradrenergic neurons or adrenergic antagonist treatment may lead to
tissue hypersensitivity to catecholamines and an up-regulation of receptor number (Davies and Lefkowitz, 1981). Surgical interruption of innervation has also been reported to cause an increase in beta-adrenoceptor density in rat skeletal muscle (Bannerjee et al., 1977). Similarly, destruction of noradrenergic neurons by administration of 6-hydroxydopamine leads to an increase in both alpha- and beta-adrenoceptor density (Hamilton and Reid, 1982; U'Prichard et al., 1979). Increases in beta-adrenoceptor number as a result of these treatments has been reported to be accompanied by parallel increases in isoprenaline stimulated cAMP production (Sporn et al., 1976).

Pharmacological denervation such as treatment with receptor antagonists can also lead to receptor up-regulation (Davies and Lefkowitz, 1981). Chronic treatment with propranolol has been reported to cause an increased beta-adrenoceptor density in human lymphocytes (Aarons et al., 1980) and doubling of beta-adrenoceptor number in rat heart (Glaubiger and Lefkowitz, 1977). Bylund and Martinez (1978) have reported increased numbers of alpha- and beta-adrenoceptors in rats after treatment with the catecholamine depleting agent reserpine. Chronic guanethidine has also been reported to increase beta-adrenoceptor density in rat hearts (Glaubiger et al., 1978). Treatment with alpha-adrenoceptor antagonists have also been reported to increase receptor number (Johnson et al., 1980; Vetulani et al., 1984).
Beta-adrenergic hypersensitivity has been implicated in the "propranolol withdrawal syndrome" observed after cessation of treatment with propranolol. It has been attributed to the persistent up-regulation of beta-adrenoceptors (Alderman et al., 1974). The excessive number of receptors left exposed after propranolol withdrawal may account for the increased sensitivity observed (Davies and Lefkowitz, 1981). Patients with orthostatic hypotension have been reported to have low circulating catecholamine levels and increased alpha_2- and beta-adrenoceptor density on platelets and lymphocytes. Hence, administration of catecholamines leads to enhanced responses in these subjects (Hui and Connolly, 1981; Davies et al., 1981).

1.4 EFFECTS OF PATHOPHYSIOLOGICAL CONDITIONS ON ADRENERGIC RECEPTOR REGULATION.

1.4.1 Phaeochromocytoma.

The effects of phaeochromocytoma on alpha-adrenoceptors has not been well defined with some investigators finding no change while others observed a reduction in platelet alpha_2-adrenoceptors (Davies et al., 1981; Davies and Lefkowitz, 1981; 1984; Snavely et al., 1982; Brodde et al., 1982; Brodde and Bock, 1984). Lymphocyte beta-adrenoceptors have been reported to be down regulated in phaeochromocytoma (Snavely et al., 1982).

A rat model of phaeochromocytoma has been developed producing a distinct clinical syndrome which is due to the excessive production of catecholamines (40-50 fold greater
than normal) by the tumour (Snavely et al., 1983; Tsujimoto et al., 1984). In this model, the beta<sub>1</sub>-adrenoceptor was observed to be down regulated in the heart and adipocytes with no significant changes in beta<sub>2</sub>-adrenoceptors (Tsujimoto et al., 1984). The down-regulation of beta<sub>1</sub>-adrenoceptors was proposed to be due to the excessive noradrenaline produced which has higher affinity for postjunctonal beta<sub>1</sub>-adrenoceptors which are particularly sensitive to noradrenaline released from nerve endings. Similarly, Snavely et al. (1983) reported selective down-regulation of adrenergic receptor subtypes in tissues of rat models of phaeochromocytoma, with decreases in beta<sub>1</sub>-adrenoceptors in renal cortical and pulmonary membranes without decrease in beta<sub>2</sub>-adrenergic receptor number. In addition, a 70% decrease in the number of alpha<sub>1</sub>- but not alpha<sub>2</sub>-adrenergic receptors in membranes from renal cortex and lung was reported. This subtype- and tissue selective-down regulation of alpha<sub>1</sub>- and beta<sub>1</sub>-adrenergic receptors may reflect a response to the excess noradrenaline in circulation as a result of this pathological condition.

1.4.2 Hypertension.

Increased sympathetic activity has been implicated in the pathogenesis of essential hypertension (Abboud, 1982; Folkow, 1982; Weber and Drayer, 1982). In animal models of hypertension, physiological and biochemical evidence has
been presented for reduced beta-adrenergic responsiveness and parallel alterations in beta-adrenergic mediated adenylate cyclase activity (Feldman et al., 1984). Altered adrenergic responsiveness may be an important factor in the pathogenesis and maintenance of the hypertensive state (Vlachakis et al., 1977). However, in man, the physiological evidence of reduced beta-adrenergic responsiveness in hypertension is much less convincing than in animal models (Feldman et al., 1984). In contrast to beta-adrenoceptors, alpha-adrenergic hypersensitivity has been demonstrated (Davies and Lefkowitz, 1981). Studies on vascular adrenoceptors have provided evidence for an imbalance between vasoconstriction mediated by alpha-adrenoceptors and vasodilation mediated by beta-adrenoceptors. Decreased beta-adrenoceptor numbers from aorta of spontaneously hypertensive rats have been reported (Limas and Limas, 1978; Yamada et al., 1980), while increased alpha-adrenoceptor number has been reported from cerebral vessels (Koybayashi et al., 1985). These changes however were not universal, since there was no change in alpha-adrenoceptor density in other tissues (kidney, spleen and ventricles) from the same model (Hick et al., 1983).

1.4.3 Heart failure.

Increased sympathetic activity has been reported as a compensatory response to heart failure followed by an increase in catecholamine levels at a later stage of the
The increased catecholamine has been reported to be predominantly noradrenaline (Thomas and Marks, 1978; Brodde et al., 1986a). This increased circulation of agonist could lead to changes in responsiveness and receptor density (Fowler et al., 1986). Reduced beta-adrenoceptor number in lymphocytes of patients with heart failure has been observed (Colucci et al., 1981). Also Bristow et al. (1982) have demonstrated changes in beta-adrenoceptor density in left ventricles. A decrease of 50% in binding sites was accompanied by an almost equal reduction in isoprenaline-stimulated adenylate cyclase activity in patients with heart failure. Furthermore, a reduced contractile response of the failing heart to isoprenaline stimulation has been reported (Brodde et al., 1986a). Brodde et al. (1986a) also reported a selective down regulation of beta_1-adrenoceptor density in cardiac tissues of patients with congestive heart failure. Under these conditions, beta_2-adrenoceptors are not affected which may explain the beneficial effects of beta_2-agonists in severe congestive heart failure.

1.4.4 Depression.

Much of what is known about the biological basis of depressive disorders results from an understanding of the biochemical pharmacology of antidepressant drugs. The tricyclic antidepressants and the monoamine oxidase
inhibitors are thought to act by increasing the synaptic concentration of biogenic amines, while clinical depression can be produced by the administration of the monoamine depleting agent reserpine. These observations gave rise to the "biogenic amine hypothesis of depression". This hypothesis proposed that depression is caused by a deficiency in biogenic amine concentration at the synapses (Schildkraut, 1965; Charney et al., 1981; Stahl, 1984). However, studies have failed to discover an abnormality in biogenic amine metabolism in depressed patients (Charney et al., 1981; Sugrue, 1983; Stahl, 1984).

The importance of an increased concentration of intrasynaptic biogenic amine as a necessary and sufficient mechanism for antidepressant effect has been questioned on several grounds (Sulser et al., 1979):

i) Clinically effective "atypical" antidepressants (mianserin, trazodone, and iprindole) fail to significantly inhibit MAO's or re-uptake transport of monoamines (Roslof and Davies, 1974; Gluckman and Baum, 1969; Goodlet et al., 1977).

ii) Effective amine uptake blockers such as amphetamine, cocaine, femoxetine appear not to be useful in the treatment of depression (Overall et al., 1962; Post et al., 1974; Ghose et al., 1977).

iii) Inconsistencies are apparent between the time course of drug effects on amine availability and
the clinical amelioration of depression. Re-uptake blockade and inhibition of amine catabolism occurs minutes after the first dose, whereas clinical improvement takes 2-3 weeks before it manifest (Oswald et al., 1972; Sulser, 1979; Charney et al., 1981).

These observations indicated that antidepressant effects on amine availability can not satisfactorily account for their therapeutic effect. Hence, emphasis on research into the mechanism of action of antidepressant treatments has changed from acute presynaptic effects to changes in noradrenergic and serotonin receptors caused by chronic antidepressant treatment.

Changes in presynaptic receptors have been reported after chronic antidepressant therapy (Charney et al., 1981; Campbell et al., 1986) in both animals and depressed patients. Down-regulation of beta-adrenoceptors in parallel with reduced sensitivity of adenylate cyclase to isoproterenol and noradrenaline stimulation has been reported after chronic but not acute treatment (Banerjee et al., 1977; Campbell et al., 1979; Charney et al., 1981). Alongside changes in beta-adrenoceptors, 5-HT$_2$ receptors are also down-regulated (Peroutka and Snyder, 1980 a,b; Enna et al., 1981). These observations have led to a new hypothesis: "Neurotransmitter receptor hypothesis of depression", which proposes that depressive illness is caused by an abnormality in the regulation of neurotransmitter receptors which can be corrected by
 Evidence that led to this hypothesis was predominantly from observations in normal rat brain and very little is known about CNS receptor changes in depressed patients or animal model of depression. However, abnormalities in neuroendocrine (ACTH, TSH and growth hormone) secreting cells of anterior pituitary have been reported and are said to be reversed by drug therapy (Caroll et al., 1981; Heninger et al., 1984; Stahl and Kravitz, 1986). In addition, preliminary evidence for abnormalities in beta- and 5-HT receptors exist in depression with the observation of night time decreased secretion of melatonin (beta-mediated) and prolactin (5-HT mediated) after tryptophan challenge (Heninger et al., 1984; Thompson et al., 1985).

It has been reported that platelet 5-HT reuptake and $[\text{H}]imipramine binding sites are significantly reduced in patients with depression (Stahl, 1983; Stahl and Kravitz, 1986) and $[\text{H}]imipramine binding sites in patients who commit suicide are reduced (Stanley et al., 1982). Platelet alpha$_2$-adrenoceptor binding sites are reportedly increased in depressed patients when agonist sites (i.e. $[\text{H}]clonidine$) are labelled and not when antagonist sites (e.g $[\text{H}]yohimbine$) are labelled (Stahl et al., 1983; Kafka and Paul, 1986) but antagonist sites are said to be reduced after antidepressants therapy (Stahl et al., 1983; Finberg, 1987). A reduction in noradrenaline-stimulated adenylate
cycase of lymphocyte beta$_2$-adrenoceptors has been reported in depressed patient but total numbers of receptors are not reduced (Mann et al., 1985).
CHAPTER TWO

GENERAL MATERIALS AND METHODS.

New Zealand white rabbits weighing 2.00 - 3.00 kg from Cheshire Rabbit Farm Ltd were used for all experiments. The animals on arrival were housed individually in cages and allowed free access to food and water ad libitum. They were allowed to acclimatize for about three to four days before being used for any experiment. Except where indicated, groups of six or more animals were used for all the experiments.

2.1 Measurement of blood pressure and heart rate.

Arterial catheters were inserted into the central ear artery of the rabbit under local anaesthesia (2% lignocaine). For direct monitoring of mean arterial blood pressure (MAP) (mmHg), the arterial catheter was connected to a Grass polygraph recorder model 7B via a statham P231D transducer. The heart rate (HR) (beats/min.) was counted directly from the blood pressure tracing. Animals were allowed 30-60 minutes to rest before any readings or experiment was carried out on them.

2.2. Drug administration.

2.2.1 Acute infusions.

Acute drug infusions were carried out through a catheter which had been inserted into the marginal ear vein under local anaesthesia. The drug was infused via a Braun
2.2.2 Chronic infusions.

Long term chronic drug infusions were carried out using osmotic minipumps (figure 2.1) model 2ml1 or 2002 depending on the duration of the drug infusion and drug solubility. This method of drug administration involves the implantation of the minipump filled with the drug which is infused via a catheter as described below.

2.2.2.1 Intravenous infusion.

Intravenous drug infusion was carried out via the femoral vein. Cannulation and implantation of the pump was performed under a general anaesthestic. The rabbits were anaesthetized with 1-2 ml of pentobarbitone sodium (60 mg/kg) then shaved in the upper region of the thigh. The skin was cut open, the femoral vein located and isolated from the nerve and artery. The vein was then cannulated and the pump with the catheter firmly attached was embedded in the thigh muscle. After the operation the wound was stitched back and chloramphenicol sodium succinate (120 mg) was given intramuscularly and the rabbits were allowed to recover from the surgical procedure for an hour before being housed individually without restraint.
2.2.2.2 Principle of osmotic minipump.

These devices sustain a continuous infusion of a drug solution when implanted subcutaneously or intramuscularly into the animal. The ALZET® osmotic minipump (Alza Corp., Palo Alto, Cal., U.S.A.) comes in various models; models 2ml1 and 2ml2 hold 2.3mls of drug and infuse for one and two weeks, respectively while models 2001 and 2002 hold 230ul and infuse for one and two weeks, respectively.

The osmotic minipump consists of a water permeable capsule, a flexible reservoir, an osmotic material sandwiched between the reservoir and the capsule and a flow modulator. Figure 2.1 shows a diagram in which different components of the pump are shown.

The device acts via the following mechanism: After filling the reservoir with a drug solution it is implanted subcutaneously or intramuscularly. The osmotic material will then absorb water from the biological environment. The volume of the osmotic material increases and this gradually compresses the central compartment (reservoir) causing a highly constant outflow of the drug’s solution. The infusion rates vary from 0.5 to 50 ul/hr depending on the model.
Figure 2.1

Diagramatic representation of osmotic minipump showing different components.
2.2.2.3 Intracerebroventricular infusions (head plate operations).

This method of drug administration was employed in infusing adrenaline and noradrenaline into the cerebral ventricle of rabbits since these drugs do not cross the blood brain barrier when administered peripherally.

For insertion of a cannula into the cerebral ventricle, a head plate was used as a guide to hold the indwelling cannula in position. After the rabbit had been anaesthetised and the head shaved, the skull was exposed by cutting the skin. The skull was then cleared of connective tissue to make the sutures on the skull visible (figure 2.2). The reference point for mounting the head plate is the meeting point of coronal and sagittal sutures (bregma) on which the reference point on the head plate (figure 2.3) was mounted. With the reference point of the head plate on the bregma, the plate was held in place by stainless steel self-tapping screws after a hole had been drilled on the skull with a hand drill. When the head plate was in position, 1.5 mm behind the bregma (coronal suture) and 2 mm lateral, a hole was bored for the insertion of the cannula. The cannula was 15 mm long to enable it to be well within the cerebral ventricle when inserted (figure 2.3). (Figure 2.4 shows cerebral ventricle from different brain sections). The cannula was then screwed on to the head plate. P.E 10 tubing attached to an osmotic minipump (model 2002) which had been filled with the drug was then connected to the exposed end
Figure 2.2
Rabbit skull showing reference points.
Figure 2.3
Diagramatic representation of head plate and cannula.
Figure 2.4

Sections of rabbit brain showing position of ventricles.
of the cannula and the minipump was embedded in the shoulder muscle. At the end of the operation the rabbit was given 120 mg of chloramphenicol sodium succinate intramuscularly and allowed to recover from the anaesthesia before being returned to its cage.

In pilot studies dye was injected to check that the position of injection and of the cannula was right. The method was mastered before being used for the study.

2.3 Intracisternal injection.

For intracisternal injection animals were anaesthetized lightly using pentobarbitone sodium and shaved over the occiput and back of the neck. Clonidine was injected into the cisterna magna as appropriate by a free hand transcutaneous injection in a total volume of 100 μl (Chalmers and Wurtman, 1971) and blood pressure and heart rate monitored as described in each section.

2.4 Radioligand binding assays.

2.4.1 Tissue preparations.

At the end of the experiments, rabbits were killed with an overdose of intravenously injected pentobarbitone sodium. The brain and kidney were removed, placed on ice and cleansed of excess blood. The brain was dissected into forebrain and hindbrain, at the level of colliculi. The
tissues were chopped finely in ice cold 0.32 M sucrose (40 ml), then homogenised using a Brinkman Polytron at a setting of 5 and centrifuged for 15 minutes at 1500 rpm. The supernatant was collected discarding the pellet consisting of fibrous tissues, red cells and other high density debris. The supernatant was recentrifuged twice at 30,000 rpm for 15 minutes at 4°C. The resulting membranes were washed in ice cold Tris HCl buffer 50 mM pH 7.5 and recentrifuged under the same conditions. The pellet was finally resuspended in 50 mM Tris HCl buffer and assayed at a wet weight concentration of 20-25 mg/ml or stored at -70°C until required for assay.

2.4.2 **Alpha\textsubscript{2}- and Beta-adrenocetor binding assays.**
Receptor binding studies were carried out on a final volume of 1 ml consisting 0.8ml of tissue homogenate, 0.1ml radioligand and 0.1ml of buffer or phentolamine $10^{-5}$M for alpha\textsubscript{2}-adrenoceptor assays or propranolol $4 \times 10^{-7}$M for beta-adrenoceptor assays. The tubes in duplicate were incubated at 25°C for 25 minutes in a shaking water bath. Incubation was terminated by rapid vacuum filtration through glass fibre filters (Whatman GF/B): The filters were rinsed twice with 5 ml of Tris HCl buffer, placed in glass vials and left to dry overnight. The vials plus filters were then filled with 10 ml of Optiphase Triton X scintillation liquid. The radioactivity was counted in a Packard Tri-cab model 3255 liquid scintillation counter at an efficiency of 36%.
The following ligands were used: $[^3\text{H}]$yohimbine (1.25-37.5 nM), $[^3\text{H}]$idazoxan (0.5-20.0 nM or $[^3\text{H}]$dihydroalprenolol (0.15-7.2 nM).

2.4.3 Data analysis.

Specific binding was defined as the difference in radioactivity bound between samples containing buffer and those containing displacing agent. The maximum number of binding sites (Bmax) and dissociation constant (K_D) were obtained from modified Scatchard plot (1949) analysis of free/bound vs free this being preferred to the more usual bound/free vs bound plot. Bmax was then corrected for protein which was assayed by the method of Lowry et al. (1951) and expressed as fmoles/mg protein.

2.5 Statistical analysis.

The data generated in this thesis from radioligand binding assays were subjected to unpaired t-test analysis. Other analyses carried out are as indicated in the appropriate section.

2.6 Tissue catecholamines.

Sucrose homogenates of tissues were prepared by homogenising tissues in 40 ml of 0.32 M sucrose. This
sucrose homogenate was taken and an equal volume of 0.2% perchloric acid added. The mixture centrifuged at 2,000 rpm for 15 minutes at 4°C. The supernatant was harvested into tubes and stored at -70°C until assayed using high performance liquid chromatography with electrochemical detection (HPLC-ECD) (Hashimoto and Maruyama, 1977; Howes et al., 1983).

2.7 Materials.

New Zealand white rabbits were obtained from Cheshire Rabbits Farm, Tarpoley, Cheshire. [^{3}H]Yohimbine, [^{3}H]idazoxan and [^{3}H]dihydroalprenolol were supplied by Amersham International U.K. Clonidine HCl, (-)adrenaline bitartrate, (-)noradrenaline HCl, phentolamine mersylate, yohimbine HCl, rauwolscine HCl, propranolol, oxymetazoline, haloperidol, metanephrine and normetanephrine were bought from Sigma U.K. Metoprolol and ICI 118551 were obtained from ICI U.K and amitriptyline HCl from M.S.D U.K. Idazoxan (Reckitt and Coleman, Kent), guanabenz (Wyeth, U.K.) and prazosin (Pfizer, U.K.) were gifts. Alzet osmotic minipumps were obtained from Scientific Marketing, London.
CHAPTER THREE

CHARACTERISTICS OF $[^3H]$YOHIMBINE AND $[^3H]$IDAZOXAN BINDING SITES
CHAPTER THREE

CHARACTERISTICS OF $[^{3}H]^\text{YOHIMBINE}$ AND $[^{3}H]^\text{IDAZOXAN}$ BINDING SITES.

3.1.1 INTRODUCTION.

Alpha$_2$-adrenoceptor ligands have proved to be valuable tools in elucidating receptor mechanisms and classification. Early ligands used in classifying alpha$_2$-adrenoceptors included $[^{3}H]$ adrenaline, $[^{3}H]$ noradrenaline and $[^{3}H]$ dihydroergocryptine (U'Prichard and Snyder, 1977; Greenberg and Snyder, 1977), but these ligands could not distinguish between alpha$_1$- and alpha$_2$-receptors and even sometimes labelled other adrenergic receptors (Lefkowitz and Williams, 1977; Tittler et al., 1977). The agonist ligands $[^{3}H]$ clonidine and its analogue $[^{3}H]$ para-aminoclonidine together with the antagonist ligands $[^{3}H]$ yohimbine and $[^{3}H]$ rauwolscine were the first selective alpha$_2$-ligands developed (Perry and U'Prichard, 1981; Nomura et al., 1984). Anomalies in binding characteristics between the two ligands soon became apparent. These differences were initially thought to be due to agonists binding preferentially to the high affinity state of the receptor while the antagonist ligands bind both high and low affinity states equally (Hoffman and Lefkowitz, 1980). This however would not explain all the differences observed and based on a comparison of $[^{3}H]$ clonidine and $[^{3}H]$ yohimbine binding in a series of tissues, the possibility of alpha$_2$-adrenoceptor subtypes was suggested (Bylund, 1981). Differences in the
displacement of \([^3\text{H}]\)rauwolscine and \([^3\text{H}]\)yohimbine from their binding sites have been observed (Cheung et al., 1982; Neylon and Summers, 1985; Alabaster et al., 1986). These observations also led the proposal of heterogeneity of the alpha\(_2\)-receptor. These ligands appear to bind to two distinct sites which are most readily distinguished by their affinities for prazosin and oxymetazoline. Variations between the relative densities of the binding sites of the ligands have also been observed between species and tissues (Bylund, 1985; Dickinson et al., 1986).

Recently, another alpha\(_2\)-adrenoceptor ligand \([^3\text{H}]\)idazoxan has become available. Idazoxan is an imidazoline derivative and has been shown to be a selective and potent alpha\(_2\)-adrenoceptor antagonist (Dettmar et al., 1983; Hannah et al., 1983; Convents et al., 1987). \([^3\text{H}]\)Idazoxan has also been shown to bind to alpha\(_2\)-adrenoceptor sites in rat tissues (Doxey et al., 1983). However, Boyajian et al. (1987) have recently presented autoradiographic and pharmacological evidence suggesting that \([^3\text{H}]\)idazoxan labels two distinct alpha\(_2\)-adrenoceptor populations in rat brain with high affinity, only one of which is labelled by \([^3\text{H}]\)rauwolscine. To confuse the picture further, \([^3\text{H}]\)clonidine and its analogue \([^3\text{H}]\)para- amino-clonidine have been reported to bind to non-adrenergic sites in addition to binding at adrenergic sites (Ernsberger et al., 1987). Recently, evidence has been presented showing that idazoxan may also label sites distinct from the alpha\(_2\)-adrenoceptor site. These sites have been referred to as
imidazoline or idazoxan binding sites (Hamilton et al., 1988; Lachaud et al., 1988; Wikberg, 1988; Yablonsky et al., 1988; Vigne et al., 1989).

This chapter (3), examines the characteristics of $[^3\text{H}]$yohimbine and $[^3\text{H}]$idazoxan binding sites using various adrenergic drugs to displace them from their binding to forebrain and kidney membranes from rabbit and rat brain tissues.

3.1.2 METHODS.

Rabbit forebrain and kidney membranes were prepared as described in methods 2.4 ($n = 6$). Rat forebrain membranes were prepared in a similar manner. Saturation binding assays were carried out on the tissue homogenates as described in methods section 2.5. In addition, the inhibition of specific binding of $[^3\text{H}]$yohimbine (6.25 nM) and $[^3\text{H}]$idazoxan (2.5 nM) were studied in the presence and absence of increasing concentrations of a range of alpha-adrenergic agonists and antagonists. The tissue homogenates plus the ligand and competing drug in a total volume of 1ml were incubated as described in section 2.5 of the methods. The monoamine oxidase inhibitor pargyline (10 nM) was added to assays containing adrenaline and noradrenaline along with ascorbate to prevent oxidation. Agonist displacement assays were incubated at 0°C for 16 hours to maintain the receptors in the high affinity state. The inhibition curves were plotted
and the concentration of displacing agent capable of reducing the specific binding of the ligands by 50% (IC\textsubscript{50}) was obtained from the inhibition curves and used as a measure of the relative affinity of the displacing agents for the binding site. The displacing agents examined were as follows: adrenaline, noradrenaline, clonidine, guanabenz, oxymetazoline, yohimbine, rauwolscine, idazoxan, prazosin, phentolamine, haloperidol, methysergide and propranolol.

3.1.3 RESULTS.

3.1.3.1 Saturation binding experiments.

Significant differences were observed in the number of binding sites of both ligands in both forebrain and kidney membranes. \[^{3}H\]Yohimbine labelled 126 ± 55 fmoles/mg protein in the forebrain which was significantly different (p < 0.05) from the number of sites labelled by \[^{3}H\]idazoxan 58 ± 19 fmoles/mg protein. In the kidney, \[^{3}H\]idazoxan labelled 209 ± 64 fmoles/mg protein which was significantly (p < 0.01) greater than the number of sites labelled by \[^{3}H\]yohimbine 70 ± 8 fmoles/mg protein. There was no significant difference between the affinities of the ligands in either tissue studied (Table 3.1).

3.1.3.2 Inhibition experiments.

The ability of various adrenergic drugs to displace the specific binding of \[^{3}H\]yohimbine (6.25 nM) and \[^{3}H\]idazoxan (2.5 nM) from rabbit brain and kidney membranes and rat
brain tissues were examined. The potency of alpha₂-adrenoceptor agonists in displacing [³H]yohimbine and [³H]idazoxan from their binding to rabbit brain membranes are illustrated in figures 3.1 and 3.2, respectively. At the [³H]yohimbine binding site, the order of potency of oxymetazoline > guanabenz > clonidine was observed while at the [³H]idazoxan binding site the order was completely different, being guanabenz >> clonidine > oxymetazoline. In addition, the endogenous adrenoceptor agonists adrenaline and noradrenaline were unable to displace [³H]idazoxan from its binding site in the rabbit brain but displaced [³H]yohimbine with an equal potency with IC₅₀ of 450 nM (Table 3.2). However, [³H]idazoxan binding to the rat forebrain was displaced by adrenaline and noradrenaline with an IC₅₀ of 180 and 400 nM, respectively (Table 3.2).

In the antagonist inhibition experiments, the order of potency of yohimbine > rauwolscine > idazoxan > phentolamine >> prazosin was observed at [³H]yohimbine binding sites (figure 3.3) while at [³H]idazoxan binding sites the order of potency observed was idazoxan > phentolamine > yohimbine = rauwolscine > prazosin (figure 3.4). The IC₅₀'s for propranolol, methysergide, haloperidol at both ligands binding sites were high, in the micromolar (µm) region. In the kidney, the displacement of [³H]idazoxan by adrenergic drugs is consistent with the order obtained in the forebrain and the same is true for inhibition of [³H]yohimbine. The IC₅₀ values are summarised in Table 3.2.
Since the Hill coefficient was significantly less than 1 in some cases for agonist displacement, relative drug potencies are expressed as IC$_{50}$ instead of Ki. For individual points on the curves the standard deviation varied between 5 - 20%, mean values only shown on the figures for clarity.
<table>
<thead>
<tr>
<th>Ligands:</th>
<th>Forebrain</th>
<th></th>
<th>Kidney</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bmax</td>
<td>K&lt;sub&gt;D&lt;/sub&gt;</td>
<td>Bmax</td>
<td>K&lt;sub&gt;D&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td>(fmoles/mg protein)</td>
<td>(nM)</td>
<td>(fmoles/mg protein)</td>
<td>(nM)</td>
</tr>
<tr>
<td>[{superscript}3H{superscript}]Yohimbine</td>
<td>126 ± 55</td>
<td>9 ± 3</td>
<td>58 ± 19</td>
<td>9 ± 4</td>
</tr>
<tr>
<td>[{superscript}3H{superscript}]Idazoxan</td>
<td>70 ± 6*</td>
<td>4 ± 2</td>
<td>209 ± 64**</td>
<td>6 ± 2</td>
</tr>
</tbody>
</table>

Result: - mean ± S.D  * P < 0.05, ** P < 0.01, using unpaired t-test.
Figure 3.1
Displacement of specific [³H]yohimbine binding from rabbit brain membranes by alpha₂-adrenoceptor agonists: O guanabenz, △ oxymetazoline, • clonidine. n = 4 - 6.
Figure 3.2
Displacement of specific \[^3^H\]idazoxan binding from rabbit brain membranes by alpha\(_2\)-adrenoceptor agonists: O guanabenz, • clonidine, △ oxymetazoline, \(n = 4 - 6\).
<table>
<thead>
<tr>
<th></th>
<th>[(^3)H]Yohimbine</th>
<th>[(^3)H]Idazoxan</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rabbit</td>
<td>Rabbit</td>
</tr>
<tr>
<td>FB</td>
<td>kidney</td>
<td>FB</td>
</tr>
</tbody>
</table>

### Alpha\(_2\)-agonists

<table>
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<tr>
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<th>Rabbit kidney</th>
<th>Rat FB</th>
<th>Rat FB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenaline</td>
<td>356</td>
<td>278</td>
<td>1200</td>
<td>&gt;5000</td>
</tr>
<tr>
<td>Noradrenaline</td>
<td>341</td>
<td>208</td>
<td>1600</td>
<td>&gt;5000</td>
</tr>
<tr>
<td>Guanabenz</td>
<td>1999</td>
<td>-</td>
<td>-</td>
<td>2.8</td>
</tr>
<tr>
<td>Oxymetazoline</td>
<td>600</td>
<td>-</td>
<td>-</td>
<td>&gt;5000</td>
</tr>
<tr>
<td>Clonidine</td>
<td>&gt;5000</td>
<td>-</td>
<td>-</td>
<td>800</td>
</tr>
<tr>
<td>Metanephrine</td>
<td>&gt;5000</td>
<td>-</td>
<td>-</td>
<td>&gt;5000</td>
</tr>
<tr>
<td>Normetanephrine</td>
<td>&gt;5000</td>
<td>-</td>
<td>-</td>
<td>&gt;5000</td>
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</table>

### Alpha\(_2\)-antagonists

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<th>Rabbit kidney</th>
<th>Rat FB</th>
<th>Rat FB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phentolamine</td>
<td>107</td>
<td>180</td>
<td>40</td>
<td>487</td>
</tr>
<tr>
<td>Yohimbine</td>
<td>22</td>
<td>22</td>
<td>24</td>
<td>&gt;5000</td>
</tr>
<tr>
<td>Rauwolscine</td>
<td>10</td>
<td>7</td>
<td>-</td>
<td>&gt;5000</td>
</tr>
<tr>
<td>Idazoxan</td>
<td>127</td>
<td>76</td>
<td>50</td>
<td>1.7</td>
</tr>
</tbody>
</table>

### Others

<table>
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<tr>
<th>Drug</th>
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<th>Rabbit kidney</th>
<th>Rat FB</th>
<th>Rat FB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prazosin</td>
<td>&gt;5000</td>
<td>&gt;5000</td>
<td>&gt;5000</td>
<td>&gt;5000</td>
</tr>
<tr>
<td>Propranolol</td>
<td>&gt;5000</td>
<td>&gt;5000</td>
<td>&gt;5000</td>
<td>&gt;5000</td>
</tr>
<tr>
<td>Methysergide</td>
<td>&gt;5000</td>
<td>&gt;5000</td>
<td>-</td>
<td>&gt;5000</td>
</tr>
<tr>
<td>Haloperidol</td>
<td>&gt;5000</td>
<td>&gt;5000</td>
<td>-</td>
<td>&gt;5000</td>
</tr>
</tbody>
</table>

FB = forebrain

n = 4 – 6

**TABLE 3.2**

RELATIVE AFFINITIES OF ALPHA\(_2\)-ADRENOCEPTOR DRUGS ON [\(^3\)H]YOHIMBINE AND [\(^3\)H]IDAZOXAN BINDING SITES. (IC\(_{50}\) nM)
Figure 3.3
Displacement of specific $[^3\text{H}]$yohimbine binding from rabbit brain membranes by alpha-adrenoceptor antagonists: △ yohimbine, ▲ rauwolscine, ◦ idazoxan, ○ phentolamine, □ prazosin, n = 4 - 6.
Figure 3.4
Displacement of specific $[^3H]idazoxan$ binding from rabbit brain membranes by alpha-adrenoceptor antagonists:
- idazoxan, O phentolamine, □ prazosin, △ yohimbine,
△ rauwolscine, n = 4 – 6.
Figure 3.5
Compounds with imidazol(in)e or closely related structures.
3.1.4 DISCUSSION.

The data presented indicate that the ligands $[^3\text{H}]$yohimbine and $[^3\text{H}]$idazoxan bind to receptor populations which are significantly different in both rabbit forebrain and kidney membranes. The number of binding sites for the two ligands differed significantly. In addition, the inhibition experiments indicated the difference between the binding properties of these ligands in the rabbit, while differences between rat and rabbit provide further evidence for adrenoceptor heterogeneity between rodents and other species. The high IC$_{50}$ (uM) obtained for non alpha$_2$-adrenergic drugs namely propranolol, prazosin, methysergide and haloperidol suggest that the differences observed between the two ligands were not as a result of either ligand binding to beta-, alpha$_1$-, serotonin or dopamine sites. The IC$_{50}$ values obtained with $[^3\text{H}]$yohimbine were similar to those reported in rabbit tissues by others and are consistent with binding at alpha$_2$-adrenoceptor sites (Cheung et al., 1982, Bylund, 1985, Neylon and Summers, 1985, Dickinson et al., 1986). $[^3\text{H}]$Idazoxan binding has not been extensively studied in rabbit membranes, but the IC$_{50}$ values obtained in rat forebrain were consistent with those reported by others (Doxey et al., 1983; 1984) and consistent with the ligand binding to an alpha$_2$-adrenoceptor. However, the high values of IC$_{50}$ obtained for displacement of $[^3\text{H}]$idazoxan by catecholamines and several of the alpha$_2$-adrenoceptor antagonists in the rabbit membranes are hard to reconcile with the ligand binding to alpha$_2$-adrenoceptors.
Idazoxan has been shown to be a potent and selective alpha$_2$-adrenoceptor antagonist in vivo in the rabbit and rat (Dettmar et al., 1983; Doxey et al., 1983; Hannah et al., 1983). It is possible that the failure to observe specific binding to alpha$_2$-adrenceptors with $[^3H]$idazoxan in the rabbit membranes was related to the procedures used resulting in degradation of either the ligand or the alpha$_2$-adrenoceptor binding site. However, this is unlikely since using identical methodology, the binding of $[^3H]$idazoxan to rat membranes and $[^3H]$yohimbine to rabbit membranes was entirely consistent with binding to alpha$_2$-adrenoceptors.

The compounds most potent at displacing $[^3H]$idazoxan binding all had imidazoline or closely related structures (figure 3.5). An imidazoline binding site has been proposed and compounds with imidazoline/imidazole structures have been shown to bind at this site (Coupry et al., 1987; Ernsberger et al., 1987; Bricca et al., 1988; Wikberg, 1988; Yablonsky et al., 1988; Vigne et al., 1989). It has also been reported that imidazoline/imidazole drugs including clonidine, ST 589, cirazoline produce dose-dependent hypotension when micro-injected into the rostral ventrolateral medulla and nucleus reticularis of rat (Meeley et al., 1986; Bousquest et al., 1984; Tibirica et al., 1988). In contrast, injection of catecholamines such as alpha-methylnoradrenaline and noradrenaline which have high selectivity for alpha$_2$-receptors did not have any effect on blood pressure (Bousquet et al., 1984). These hypotensive effects have
been shown to be independent of a mechanism involving alpha-adrenoceptors (Meeley et al., 1986). These observations are similar to the report that on the peripheral alpha-adrenoceptors structural requirements of catecholamine sensitive sites are different from those of imidazoline sensitive sites in smooth muscle (Ruffolo et al., 1977; 1982; Ruffolo and Waddell, 1982). In addition, a low molecular weight substance has been isolated from bovine brain which has been shown to potently displace \(^{3}\text{H}\)clonidine and \(^{3}\text{H}\)para-aminoclonidine binding to alpha\(^{2}\) adrenoceptors in rat brain (Atlas and Burstein., 1984; Diamant and Atlas, 1986; Meeley et al., 1986). This substance has been named clonidine displacing substance and has been reported to be biologically active (Meeley et al., 1986; Bousquet et al., 1986; Felsen et al., 1987). It has been proposed that this substance may be producing its effect via the imidazoline binding site (Meeley et al., 1986). It is also possible that this site can be equated with the idazoxan binding site. However, one potential problem that might arise as a result of using antagonists ligands is that they may not identify the exact site at which the endogenous neurotransmitter acts, agonists tend to be small molecules with hydrophobic centre (Ariens and Simonis, 1983), while the characteristics of antagonists tend to favour binding to lipophilic sites which are close to the polar sites acted upon by agonists. Idazoxan could bind to an allosteric site at or adjacent to the alpha\(^{2}\)-adrenoceptor. In support of the latter hypothesis, parallels
may be drawn with the the methysergide / 5-HT binding site. Methysergide readily displaces \[ ^3\text{H}\]ketanserin from brain membranes but it only displaces a small portion of specifically bound ketanserin from platelets of several species. It has been suggested that this may be due to methysergide binding to an allosteric site which is distinct from the 5-HT\(_2\) receptor (Fenken and Kaumann, 1987). However, such explanations are tentative and further investigations including functional studies need to be undertaken to explain the characteristics of \[^3\text{H}\]idazoxan binding to rabbit tissue membranes observed in this study.

3.2 SPECIFICITY OF \[^3\text{H}\]DIHYDROALPRENOLOL AS A BETA-ADRENOCEPTOR LIGAND.

3.2.1 INTRODUCTION.

Early ligands employed in the characterization of beta-adrenoceptors included \[^3\text{H}\]adrenaline, \[^3\text{H}\]alprenolol and \[^3\text{H}\]dihydroalprenolol (U'Prichard and Snyder, 1977; Greenberg and Snyder, 1977). These ligands were found to have low specific activity. The development of high specific activity ligands such as \[^{125}\text{I}\]iodocyanopindolol and \[^{125}\text{I}\]dihydrobenzylpindolol followed, however both ligands have been shown to bind to other receptor sites in the brain, serotonin and dopamine for example (Engel et al., 1981; Hoffman and Lefkowitz, 1980). Thus despite the low specific activity of \[^3\text{H}\]DHA, it has the advantages of not binding to
other receptor sites and has been increasingly employed in characterizing beta-adrenoceptors in various tissues and species (Bylund and Snyder, 1976; Davies and Lefkowitz, 1981; Stone and U'Prichard, 1981; Chang and Lotti, 1983).

This section is intended to substantiate the binding characteristics of $[^{3}\text{H}]$DHA by displacement from its binding sites in the rabbit brain by the beta-adrenoceptor antagonists propranolol, ICI 118551 and metoprolol.

3.2.2 METHODS.

Rabbit tissue homogenates were prepared as described in methods (2.4.1) (n = 4). The non selective beta-adrenoceptor ligand $[^{3}\text{H}]$dihydroalprenolol ($[^{3}\text{H}]$DHA) was used to measure the specificity of its binding to rabbit brain homogenates in the presence or absence of increasing concentrations of beta-adrenoceptor drugs. Propranolol, ICI 118551 and metoprolol were incubated with $[^{3}\text{H}]$DHA and tissue homogenate as described in section 2.4.2 of the methods. Displacement curves were plotted as percentage inhibition against the negative logarithum of the drug concentration. The concentration of drugs required to displace 50% ($IC_{50}$) of the specifically bound radioactivity was calculated from the displacement curves and used as measure of drug affinity.
3.2.3 RESULTS.

The displacement of specific $[^3H]DHA$ binding rabbit brain tissue homogenates was studied and the $IC_{50}$ (nM) obtained from displacement curves. The order of potency at displacing $[^3H]DHA$ from its binding site was propranolol (70 nM) $>$ ICI 118551 (80 nM) $>$ metoprolol (200 nM). However, the Hill slopes for metoprolol and ICI 118551 were shallow. A summary of the results is presented in table 3.3.
**Table 3.3**

Relative potencies of beta-adrenoceptor antagonists on [³H]dihydroalprenolol

<table>
<thead>
<tr>
<th>Drugs</th>
<th>IC₅₀ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>propranolol</td>
<td>70</td>
</tr>
<tr>
<td>ICI 118551</td>
<td>80</td>
</tr>
<tr>
<td>metoprolol</td>
<td>200</td>
</tr>
</tbody>
</table>
3.2.4 DISCUSSION.

The beta-adrenergic receptor antagonist \[^3\text{H}]\text{DHA}\) has been widely used to label total receptor numbers in membranes from various organs and species (Bylund and Snyder, 1976; Stone and U'Prichard, 1981; Brodde et al., 1981). The site labelled by this ligand has been shown to have characteristics which are very similar to the beta-adrenoceptor (Lefkowitz, 1978). Correlations have also been observed in the ability of various beta-adrenoceptor drugs to compete for \([^3\text{H}]\text{DHA}\) binding sites and the ability of the same agents to stimulate or antagonise beta-adrenoceptor coupled adenylate cyclase (Murkerjee et al., 1976). This observation is consistent with the \([^3\text{H}]\text{DHA}\) binding site representing the physiological beta-adrenergic receptors.

In these experiments, it has been observed that specific \([^3\text{H}]\text{DHA}\) binding was inhibited with high potencies by propranolol a non-selective beta-adrenoceptor antagonist and ICI 118551 a beta\(_2\)-selective antagonist and metoprolol a beta\(_1\)-selective antagonist. Displacement curves for metoprolol and ICI 118551 were shallow with Hill slopes less than one. This would be consistent with the presence of both beta\(_1\)- and beta\(_2\)-adrenoceptors in the rabbit brain. The relatively low potency exhibited by metoprolol could be due to the fact that both beta\(_1\)- and beta\(_2\)-receptors exist in the brain with beta\(_2\)-receptor being present in greater numbers than beta\(_1\)-adrenoceptors. The results obtained in these experiments are consistent with previous studies (Lefkowitz, 1978; Brodde et al., 1981). Thus, results
indicate that $^{3}\text{H}]$DHA labels beta-adrenoceptor in the rabbit brain in these studies.
CHAPTER FOUR

EFFECTS OF CHRONIC IDAZOXAN AND YOHIMBINE TREATMENTS ON ADRENOCEPTORS IN THE RABBIT.
CHAPTER FOUR

EFFECTS OF CHRONIC IDAZOXAN AND YOHIMBINE TREATMENTS ON ADRENOCEPTORS IN THE RABBIT.

4.1 INTRODUCTION.

Previous studies on central and peripheral adrenergic receptors have shown that long term receptor stimulation in vivo or chronic agonist administration desensitizes (down-regulates) receptors (Hoffman and Lefkowitz, 1980; Davies and Lefkowitz, 1981), while noradrenergic nerve depletion or chronic receptor blockade supersensitizes (up-regulates) both alpha- and beta-adrenoceptors (U’Prichard and Snyder, 1978; U’Prichard et al., 1979; Lefkowitz et al., 1983; Nahorski and Barnett, 1982).

Up-regulation of alpha$_2$-adrenoceptor number has been reported to occur in rat brain after treatment with the alpha$_2$-adrenoceptor antagonist yohimbine (Johnson et al., 1980), while treatment with reserpine a catecholamine store depleting agent has been reported to cause an increase in $[^3]$H]catecholamine binding sites in the rat brain (U’Prichard and Snyder, 1978). Bylund and Martinez (1980) also reported an increase in rat salivary gland alpha$_2$-adrenoceptor number after reserpine treatment. In addition, surgical and pharmacological denervation by administration of 6-hydroxydopamine have been said to cause up-regulation of both alpha- and beta-adrenoceptor density in the brain (U’Prichard et al., 1979; Sporn et al., 1979). Increases in beta-adrenoceptors in rat heart and skeletal muscle following guanethidine treatment and denervation have been
demonstrated (Banerjee et al., 1977; Glaubiger et al., 1978). Chronic administration of propranolol to healthy volunteers has been reported to lead to a significant increase in beta2-adrenoceptor density in circulating lymphocytes (Aarons et al., 1980; Aarons and Molinoff, 1982). Such an enhanced adrenoceptor density after chronic antagonist treatment has been implicated in tachyphylaxis and withdrawal phenomena and could also account for side effects observed after some treatments, enhanced beta-adrenoceptor density after chronic propranolol treatment has been proposed to be responsible for "propranolol withdrawal syndrome" (Alderman et al., 1974; Davies and Lefkowitz, 1981). However, increases in receptor density after antagonist treatment are not an ubiquitous finding. Administration of beta-adrenoceptor antagonists with partial agonist activity like pindolol have been reported to cause a significant decrease in lymphocyte beta2-adrenoceptor density (Aarons and Molinoff, 1982; Giudicelli et al., 1976; Brodde et al., 1986b). Neve and Molinoff (1986) also reported decrease in beta-adrenoceptor density after treatment with celiprolol and pindolol beta-adrenoceptor antagonists with intrinsic sympathomimetic activities.

Yohimbine and idazoxan have been shown to be potent and selective alpha2-adrenoceptor antagonists (Perry and U'Prichard, 1981; Doxey et al., 1983; Hannah et al., 1983; Convents et al., 1987; Ruffolo et al., 1988). However, recently idazoxan has been reported to bind principally to a
non-adrenergic site in addition to binding to alpha$_2$-adrenergic receptors (Coupry et al, 1987; Lachaud et al., 1988; Yablonsky et al, 1988; Wikberg 1988; Vigne et al., 1989; chapter 3).

This chapter [4], is therefore intended to compare effects of chronic yohimbine and idazoxan treatment on $[^3H]$yohimbine and $[^3H]$idazoxan binding sites in tissue membranes from rabbits. In addition effects on alpha$_2$-adrenoceptor mediated pressor responses to bolus doses of alpha-methylnoradrenaline injection in conscious rabbits and the depressor response induced by intracisternal injection of clonidine to anaesthetised rabbits were examined.

4.2 METHODS.

The experiments were performed using male New Zealand white rabbits (2.5-3.0 kg) from Cheshire Rabbit Farms. The animals were housed individually and allowed access to food and water ad libitum.

4.2.1 In vivo drug infusion.

Groups of rabbits ($n = 8 - 12$) received either acute (30 minutes) or chronic (5 days) idazoxan infusions, while yohimbine was administered intraperitoneally for 5 days. Since idazoxan was a relatively new drug and few chronic studies have been carried out using this animal model, it was necessary to carry out a series of preliminary studies.
with short term infusions to select the appropriate effective dose for chronic study. In contrast, the dose used for the yohimbine study was selected from previous publications (Johnson et al., 1980; Scott and Crews, 1983). Yohimbine had to be given intraperitoneally as solubility problems limited its administration via osmotic minipump. Control groups received 0.9% saline.

4.2.1.1 Idazoxan infusions.

Idazoxan dissolved in 0.1% ascorbate was acutely infused via a marginal ear vein catheter inserted under local anaesthesia (2% lignocane). The infusion was carried out (for 30 minutes) at the rate of 0.56 or 1.1 mg/hr using a Braun perfusor pump.

Chronic (5 days) infusions were carried out using Alzet® osmotic minipumps (model 2ml1) (figure 2.1), implanted as described in section 2.2.2. The concentration of idazoxan in the pump was adjusted so that the infusion rate (1.1 mg/kg) was the same as in the acute experiments. Control groups were implanted with pumps delivering vehicle ascorbate.

4.2.1.2 Yohimbine treatment.

Because of problems with solubility, yohimbine 15 mg/kg/day in divided doses was administered intraperitoneally for 5 days and was not given continuously
via osmotic minipumps. Control groups received vehicle ascorbate by a similar route.

4.2.2 **Pressor dose-response curves to alpha-methylnoradrenaline.**

Dose-response curves to alpha-methylnoradrenaline (5-100 ug) were constructed in groups of rabbits before and after pretreatment with yohimbine or idazoxan. Intravenous and arterial lines were inserted for infusion of drugs and measurement of blood pressure as described in section 2.1 and 2.2.1.2 of methods.

4.2.3. **Intracisternal injections of clonidine.**

To observe effects of treatment on depressor responses to clonidine, clonidine 1 ug/kg was injected intracisternally to groups of rabbits treated chronically with saline, idazoxan or yohimbine. Clonidine was administered by a free hand transcutaneous injection (Chalmers and Wurtman, 1971) in a total volume of 100 ul while the rabbits were under light anaesthesia (sodium pentobarbitone 30-60 mg/kg). Changes in mean arterial pressure (MAP) and heart rate (HR) were monitored before and continuously for 5 minutes after the injection then at 15 minutes interval thereafter until 60 minutes after the injection.
4.2.4 **Tissue preparation.**

At the end of the experiment rabbits were killed with an overdose of sodium pentobarbitone (I.V). The brain and kidney removed, placed on ice and cleansed of connective tissue and blood. The brain was dissected at the point of colliculi into fore and hindbrain. The tissues were prepared as described in section 2.4.1 of the methods.

4.2.5. **Radioligand binding assays.**

Receptor binding studies were performed on tissue homogenates using $[^3H]$idazoxan (0.5-20 nm) for labeling idazoxan / imidazoline binding sites and $[^3H]$yohimbine (1.25-37.5 nm) for alpha$_2$-adrenoceptors and $[^3H]$DHA for beta-adrenoceptors and incubated as described in section 2.4.2. The radioactivity was counted using a Packard Tri-Cab Model 3255 liquid scintillation counter at an efficiency of 36%.
4.2.6. Data analysis.

To analyse the alpha-methylnoradrenaline dose response curves, curves were constructed for each individual and the dose required to increase MAP by 50 mmHg for yohimbine treated groups and 30 mmHg for idazoxan treated groups and their respective controls were obtained from the dose-response curves. The difference between the treated and control groups were analysed by unpaired t-test. In addition the shift in the dose response curves in the treated groups relative to the controls were calculated as dose ratios. The binding parameters Bmax and KD were obtained from the modified Scatchard analysis. Statistical analysis of the differences between groups of data generated by Scatchard plots were analysed using unpaired t-test. Data from the depressor response induced by intracisternal injection of clonidine were analysed by analysis of covariance using the package Rummage (Bryce, 1980) run on an ICL 2988 mainframe computer. The preinjection blood pressure was used as the covariate. All results are expressed as mean ± S.D.

4.3 RESULTS.

4.3.1 Blood pressure and heart rate.

There was no significant change in the resting blood pressor or heart rate of the rabbits during either idazoxan or yohimbine treatment, neither were there any obvious behavioural changes during the course of the experiments.
4.3.2 Effects of antagonist treatments on pressor responses to alpha-methylnoradrenaline.

In preliminary experiments, idazoxan infusion for 30 minutes attenuated the response to bolus doses of alpha-methylnoradrenaline, the dose required to raise the MAP by 30 mmHg was increased from 17.4 ± 3.2 for control to 22.4 ± 4 ug for 0.56 mg/kg/hr infused and the curve was shifted to the right with a dose ratio of 1.3, while for 1.1 mg/kg/hr infusion, the dose was increased from 17.4 ± 2 for control to 27.5 ± 5 ug for treated and the curve was shifted to the right with a dose ratio of 1.6 (figure 4.1). On the basis of these results the higher dose was selected for detailed chronic study. In the chronic study with 1.1 mg/kg/hr, the dose required to increase the MAP by 30 mmHg was increased from 19.1 ± 5 for control to 35.5 ± 4.3 ug for treated group and the curve was shifted to the right with a dose ratio of 2.0 (figure 4.2).

Treatment with yohimbine (15 mg/kg/day) caused attenuation of the alpha-methylnoradrenaline induced pressor response with the dose required to increase the MAP by 50 mmHg increased from 16 ± 4 to 42 ± 15 ug for control and treated groups, respectively. The dose response curve was shifted to the right with a dose ratio of 2.6 (figure 4.3).
4.3.3 Effects of antagonists treatment on intracisternal clonidine injection.

Intracisternal administration of clonidine 1 μg/kg caused a prolonged fall in blood pressure and heart rate in control ascorbate treated rabbits with the maximum fall occurring at between 5 and 15 minutes after injection.

Chronic treatment with idazoxan (1.1 mg/kg/hr) attenuated the fall in blood pressure induced by clonidine injection. This was significantly (p < 0.05) different when compared to the control group at 15, 30 and 45 minutes (figure 4.4). Treatment with yohimbine 15 mg/kg/day also attenuated the depressor response and was significantly (p < 0.05) different from controls at 5, 15, 30, and 45 minutes (figure 4.5). There were no significant treatment effects of the antagonists on the heart rate effects of clonidine.

4.3.4 Effects of treatments on in vitro \[^{3}\text{H}]\text{Yohimbine} and \[^{3}\text{H}]\text{Idazoxan} binding.

The treatment with yohimbine 15 mg/kg/day caused 63% and 103% increases in \[^{3}\text{H}]\text{yohimbine} binding to the forebrain and kidney membranes, respectively. These increases in Bmax were significantly different (p < 0.02 and p < 0.002) when compared to the respective control groups. There was no significant treatment effect on the hindbrain (figure 4.6) nor was the K_D significantly altered by yohimbine treatment (Table 4.1). \[^{3}\text{H}]\text{Idazoxan} binding to kidney membrane was
Figure 4.1
Effects of acute 0.56 and 1.1 mg/kg/hr idazoxan infusions on pressor responses to bolus doses of alpha-methylnoradrenaline.

- ○○○ control
- ●●● idazoxan treated

n = 8 - 12.
Figure 4.2
Effects of chronic 1.1 mg/kg/hr idazoxan infusion on pressor responses to bolus doses of alpha-methylnoradrenaline.

- ○ ○ control
- ● ● idazoxan treated

n = 8 - 12.
Figure 4.3

Effects of chronic yohimbine 15 mg/kg/day treatment on pressor responses to bolus doses of alpha-methylnoradrenaline.

- control
- Yohimbine treated

n = 8 - 12.
significantly ($p < 0.001$) decreased by 52%. This decrease in $B_{\text{max}}$ was accompanied by a significant ($p < 0.04$) reduction in the $K_D$. There were no significant changes in $[^3\text{H}]$idazoxan binding to the forebrain or hindbrain membranes and no significant changes in $K_D$ were observed as a result of yohimbine treatment (figure 4.7 and Table 4.1).

Chronic idazoxan infusion 1.1 mg/kg/hr caused a significant ($p < 0.001$) increase in the number of $[^3\text{H}]$yohimbine binding sites ($B_{\text{max}}$) by 83% in the forebrain with no significant treatment effect on the hindbrain or kidney (figure 4.8). There was no significant treatment effect on the $K_D$ for $[^3\text{H}]$yohimbine binding in any tissue examined (Table 4.1). $[^3\text{H}]$Idazoxan binding to kidney membranes was significantly ($p < 0.001$) decreased by 89% after chronic 1.1 mg/kg/hr idazoxan infusion (figure 4.9). This was accompanied by a significant decrease in $K_D$. No change in the number of binding sites was observed in the forebrain or hindbrain. There was a significant increase ($p < 0.04$) in the $K_D$ of $[^3\text{H}]$idazoxan binding to the forebrain in the group treated with idazoxan (Table 4.1).
Figure 4.4

Effects of chronic idazoxan 1.1 mg/kg/hr infusion on the depressor response to intracisternal clonidine injection.

\[ n = 8 - 12, \quad * \ p < 0.05. \]
Figure 4.5

Effects of yohimbine 15 mg/kg/day treatment on the depressor response to intracisternal clonidine injection.

- control
- yohimbine treated

n = 8 - 12, * p < 0.05.
Figure 4.6

Effects of yohimbine treatment on the number of $[^3H]$yohimbine binding sites.

- control
- yohimbine treated

$n = 8 - 12$, * $p < 0.05$. 
Figure 4.7

Effects of yohimbine treatment on the number of $[^{3}\text{H}]$idazoxan binding sites.

- $n = 8 - 12$, $* p < 0.05$. 
### TABLE 4.1

Effects of yohimbine and idazoxan treatments on the dissociation constant (K_d) nM for [³H]yohimbine and [³H]idazoxan binding.

<table>
<thead>
<tr>
<th></th>
<th>[³H]Yohimbine</th>
<th></th>
<th>[³H]Idazoxan</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>forebrain</td>
<td>hindbrain</td>
<td>kidney</td>
<td>forebrain</td>
</tr>
<tr>
<td>Control</td>
<td>8 ± 3</td>
<td>12 ± 3</td>
<td>15 ± 8</td>
<td>4 ± 3</td>
</tr>
<tr>
<td>Idazoxan</td>
<td>15 ± 6</td>
<td>10 ± 4</td>
<td>12 ± 4</td>
<td>7 ± 4*</td>
</tr>
<tr>
<td>Control</td>
<td>8 ± 2</td>
<td>11 ± 5</td>
<td>8 ± 4</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>Yohimbine</td>
<td>13 ± 7</td>
<td>11 ± 6</td>
<td>16 ± 7</td>
<td>4 ± 2</td>
</tr>
</tbody>
</table>

**Result:** mean ± S.D, *p < 0.05, **p < 0.01, n = 8 - 12
Figure 4.8

Effects of idazoxan infusion on the number of $[^3H]$yohimbine binding sites.

- □ control
- ■ idazoxan treated

$n = 8 - 12$, * $p < 0.05$. 
Figure 4.9

Effects of idazoxan infusion on the number of $[^3H]$idazoxan binding sites.

- □ control
- ■ idazoxan treated

$n = 8 - 12$, $* p < 0.05$. 
4.4 DISCUSSION.

Studies on the pharmacological effects of alpha-adrenoceptor antagonists have been limited by the relatively low alpha_2/alpha_1-adrenoceptor selectivity and specificity of the antagonists available until yohimbine and rauwolscine were introduced (Tanaka et al., 1978; Weitzel et al., 1979; Motulsky et al., 1980; Yamada et al., 1980). These drugs were shown to be more selective and specific for alpha_2-adrenoceptors compared to other alpha_2-antagonists available then (Bylund, 1981; Bylund and U'Prichard, 1983). Recently, idazoxan has been proposed to be a potent and highly selective antagonist at alpha_2-adrenoceptors (Dettmar et al., 1983; Doxey et al., 1983). However, idazoxan has been shown to bind to non-adrenergic as well as adrenergic sites (Coupry et al., 1987; Lachaud et al., 1988; Yablonsky et al., 1988). In this Chapter (4), studies to compare effects of acute and chronic infusions of idazoxan and yohimbine on alpha_2-adrenoceptor mediated effects and on [^3^H]yohimbine and [^3^H]idazoxan binding sites were reported.

Idazoxan infusion attenuated the pressor responses induced by intravenous injection of alpha-methylnoradrenaline and caused rightward shifts of dose response curves with dose ratios of 1.3 and 1.6 for acute dosing with idazoxan 0.56 and 1.1 mg/kg/hr while chronic treatment with 1.1 mg/kg/hr shifted the curves with a dose ratio of 2.0. Pretreatment with yohimbine also attenuated the hemodynamic effects of the alpha_2-adrenoceptor agonist.
alpha-methylnoradrenaline with a rightward shift in the dose response curves and a dose ratio of 2.6 which is comparable to that observed with chronic idazoxan 1.1 mg/kg/hr infusion. These results are consistent with yohimbine and idazoxan acting as alpha$_2$-receptor blockers (Tanaka et al., 1978; Weitzell et al., 1979; Dettmar et al., 1983).

Intracisternal injection of clonidine induces a depressor response (Reynoldson et al., 1979; Hannah et al., 1983), which has been shown to be mediated via the stimulation of central alpha$_2$-adrenoceptors (Schmitt et al., 1967; Dollery and Reid, 1973; Haeusler, 1974; Kobinger, 1978). These receptors are believed to be postsynaptic (Haeusler, 1974; Bousquet and Schwartz, 1983) and are located principally in the medulla oblongata (Schmitt and Schmitt, 1969). The depressor response induced by clonidine injection can be attenuated or abolished by treatment with alpha$_2$-adrenoceptor antagonists (Bolme et al., 1974; Timmermans et al., 1981; Hannah et al., 1983). Pretreatment with yohimbine and the higher dose of idazoxan (1.1 mg/kg/hr), significantly antagonised the hypotension induced by intracisternal injection of clonidine, consistent with the drugs roles as alpha$_2$-adrenoceptor blockers.

In the radioligand binding experiments, the increase in $[^3]$H]yohimbine binding in the forebrain and kidney after chronic yohimbine treatment plus the increase in binding to the forebrain after chronic 1.1 mg/kg/hr idazoxan infusion is also consistent with the alpha$_2$-adrenoceptor antagonist properties of these drugs (Doxey et al., 1984; Weitzell et
al., 1979; Doxey et al., 1983; Dettmar et al., 1983; Hannah et al., 1983). Up-regulation in both alpha- and beta-adrenoceptors has been reported after chronic administration of adrenergic receptor antagonists and noradrenergic terminal destruction or depletion in animals (U'Prichard and Snyder, 1978; U'Prichard et al., 1979; Johnson et al., 1980; Davies and Lefkowitz, 1981). The reason for the localized up-regulation in [3H]yohimbine binding sites as a result of treatment is not known, and the effect of chronic treatment on receptor number appeared to depend on the brain region examined. The pre- and postsynaptic location of the receptor, the density of innervation and the alpha2-adrenoceptor subtype predominating in that region may all be contributory factors. In addition selective accumulation of drug in different brain regions could modify the receptors in that area specifically. Tissue and drug specific changes in adrenoceptor binding have been observed centrally and in the periphery by others (Sporn et al., 1976; Winter et al., 1986; Minneman et al., 1981). In contrast to the expected effects of antagonists on receptor binding sites, [3H]idazoxan binding to kidney membranes was significantly reduced by pretreatment with yohimbine and idazoxan. The reductions were accompanied by significant decreases in KD. These observations are very hard to reconcile with the action of these drugs as alpha2-adrenoceptor antagonists. However, idazoxan has been reported to have partial agonist properties (Hannah et al., 1983). Consistent with this
observation, chronic treatment with beta-adrenoceptor antagonists with partial agonist activity have been shown to cause down-regulation of beta-receptors (Giudicelli et al., 1984; Brodde et al., 1986b; Neve and Molinoff, 1986).

Idazoxan has been reported to bind to a non-adrenergic receptor site (Bricca et al., 1988; Wikberg 1988; Yablonsky et al., 1988), it is therefore possible that both yohimbine and idazoxan may be acting as agonists on this site in the periphery thus causing down-regulation. However, most drugs showing a higher affinity for the idazoxan binding site are imidazol(in)es or with related structures. Yohimbine does not have an imidazole structure and showed a low affinity for the \[^3\text{H}\]idazoxan site in \textit{in vitro} displacement studies (chapter 3). It is possible that yohimbine is acting indirectly to cause down-regulation of the idazoxan site. Both yohimbine and idazoxan treatment resulted in a reduction in $K_D$ for \[^3\text{H}\]idazoxan binding suggesting that these compounds are not simply down-regulating the binding site. A small but significant increase in $K_D$ of \[^3\text{H}\]idazoxan binding was observed in the forebrain after idazoxan treatment. The reason for this is unknown. However the level of significance was low and it may be unwise to attribute biological significance to this single result.

In conclusion, in the \textit{in vivo} functional studies both idazoxan and yohimbine behaved as expected for alpha$_2$-adrenoceptor antagonists. In contrast anomalies were observed in the radioligand binding studies, no changes in the number of idazoxan binding sites were observed in either
forebrain or hindbrain during yohimbine or idazoxan treatment, while $[^3H]$yohimbine binding sites in the forebrain but not in the hindbrain were significantly increased by both idazoxan and yohimbine treatments. In the kidney, $[^3H]$yohimbine binding sites were significantly increased by yohimbine but not idazoxan treatment but $[^3H]$idazoxan binding was significantly decreased by both yohimbine and idazoxan treatments. Thus idazoxan and yohimbine binding sites can be differentially regulated, which further supports the hypothesis that the site labelled by idazoxan is distinct from the alpha$_2$-adrenoceptor site labelled by $[^3H]$yohimbine.
CHAPTER FIVE

EFFECTS OF CHRONIC AMITRIPTYLINE ALONE OR IN COMBINATION WITH IDAZOXAN OR YOHIMBINE ON CENTRAL ADRENOCEPTORS IN THE RABBIT.
CHAPTER FIVE

EFFECTS OF CHRONIC AMITRIPTYLINE ALONE OR IN COMBINATION WITH IDAZOXAN OR YOHIMBINE ON CENTRAL ADRENOCEPTORS IN THE RABBIT.

5.1 INTRODUCTION.

Research into the mechanisms of action of psychotropic drugs has largely been responsible for the development of the hypotheses implicating monoamine deficiency in depression. These hypotheses are based on the observations that monoamine depleting agents such as reserpine can produce clinical depressive states in humans while clinically effective antidepressants of the tricyclic and monoamine oxidase inhibitors (MAOI) classes act rapidly to enhance the synaptic concentration of amines and serotonin in brain (Schildkraut, 1965; Bunney, 1965; Charney et al., 1981; Stahl, 1984). Many investigators measured monoamine metabolites such as 3-methoxy-4-hydroxyphenethyleneglycol (MHPG), a noradrenaline metabolite, and 5-hydroxyindoleacetic acid (5-HIAA), a 5-HT metabolite in the urine, plasma and cerebro-spinal fluid (CSF) of depressed patients before and after administration of antidepressant drugs (Dekirmenjian and Maas, 1974; Maas et al., 1976; Ursillo et al., 1980). These studies failed to identify metabolic disorders of biogenic amine metabolites in the untreated depressed patients as a group (Stahl, 1984; Charney et al., 1981). However, one finding was that some patients with low CSF 5-HIAA concentrations may be prone to
suicidal attempts (Stanley et al., 1982). Hence, biogenic amine metabolites have not been useful in identifying a biochemical abnormality in depressed patients. The importance of increased availability of synaptic monoamines as a necessary and sufficient mechanism for antidepressant effects has been questioned increasingly for several reasons (Sulser et al., 1978). The tricyclic antidepressants blockade of noradrenaline and/or serotonin uptake is rapid yet a lag phase of one to three weeks exists before the onset of a clinical amelioration: furthermore atypical antidepressants such as iprindole (Gluckman and Baum, 1969; Lahti and Maichel, 1971) and mianserin (Leonard, 1974; Goodlet et al., 1977) are essentially devoid of effects on monoamine uptake in vivo yet are effective antidepressants (Zis and Goodwin, 1979). Lastly, effective amine uptake inhibitors such as amphetamine, femoxetine and cocaine appear not to be useful in the treatment of depression (Overall et al., 1962; Post and Goodwin, 1974; Ghose et al., 1977). The tricyclic antidepressants are however widely employed in the treatment of depression although it has not been possible to identify a common mode of action for this class of drugs (Charney et al., 1981).

Recently, neurochemical events which occur with approximately the same time course as antidepressant effects have been described (Enna et al., 1981). Chronic administration of tricyclic antidepressants (2-3 weeks) has been associated with changes in beta-adrenoceptor number in rat brain (Banerjee et al., 1977; Bergstrom and Kellar,
1979; Campbell et al., 1979; 1984; Keith et al., 1988). The
down-regulation is accompanied by a loss in sensitivity of
adenylate cyclase to stimulation by noradrenaline or
isoproterenol (Sulser, 1978; Enna et al., 1981; Richardson
and Hertz, 1983; Campbell et al., 1984). These observations
on the pharmacological effects of chronic antidepressant
treatments have led to the suggestion that the therapeutic
effect of antidepressants is due to a progressive
desensitization of central beta-adrenoceptors (Sulser, 1979)
and evolved into the "neurotransmitter receptor" hypothesis
of antidepressant action which postulates that adrenergic
and/or serotonergic receptors are responsible for
mediating the clinical effects of antidepressant drugs
(Charney et al., 1981; Stahl, 1984).

The down-regulation of beta-adrenoceptors observed
after chronic antidepressant treatment may be due to an
increased synaptic concentration of biogenic amines (Wolfe
et al., 1978; Sugrue, 1983). An increase in the amount of
noradrenaline release per nerve impulse during chronic but
not acute antidepressant treatment has been observed in the
peripheral nerves (Crews and Smith, 1978). The increase in
release may be due to adaptive changes which must occur
before subsensitivity of postsynaptic beta-adrenoceptor
manifests itself. The alpha_2-adrenergic receptors located
presynaptically on noradrenergic neurons and on
noradrenergic cell bodies of the locus coeruleus have been
proposed to become desensitized upon chronic antidepressant
treatment (Svensson and Usdin, 1978). The desensitization would lead to increased catecholamine release (Hughes, 1978). Thus, down-regulation may result from initially increased neurotransmitter (agonist) levels leading to desensitization in which the receptor becomes uncoupled from adenylate cyclase system followed by a second irreversible stage in which the receptors become internalized. However, while down-regulation of beta-adrenoceptors is a consistent observation with antidepressant drug treatment, not all of these drugs have been shown to increase synaptic noradrenaline levels. Down-regulation is therefore seen as a compensatory reaction to increased agonists levels. Alternatively, down-regulation may take place via a process independent of increased agonist (catecholamine) levels. This implies that the action of antidepressant drugs is to reduce the activity of beta-adrenoceptor mediated neuronal pathways directly (Susler, 1979; Charney et al., 1981; Sugrue, 1983; Maj et al., 1984).

Although, most reports suggested that the beta-adrenoceptors down-regulate, the effects of antidepressants treatment on alpha-adrenoceptor numbers have been diverse with reports of up-regulation and no changes in receptor number (Charney et al., 1981; Asakura et al., 1982; Vetulani et al., 1984; Stahl and Palazidou, 1986; Finberg, 1987). However, desensitization of alpha₂-presynaptic receptors has been demonstrated following chronic treatment of rats with imipramine or desipramine. A reduction in alpha₂-adrenoceptor sensitivity has been invoked to explain the
enhanced release of catecholamines by desipramine (Crews and Smith, 1978; Svensson and Usdin, 1978) and reduced binding of alpha\textsubscript{2}-adrenoceptor ligands to cerebral cortical membranes from rats treated chronically with MAOI and tricyclic antidepressant has been reported (Smith et al., 1981; Cohen et al., 1982). The antidepressant effects were proposed to correct a pre-existing defect where alpha\textsubscript{2}-presynaptic receptor number is enhanced in depression and a reduction is observed after antidepressant treatment. These reports have therefore indicated that alpha\textsubscript{2}-adrenergic receptors may also be involved in therapeutic effects of antidepressants.

Since increases in catecholamine concentrations at the synapses has been indicated as an important aspect of antidepressant therapy and also as a prerequisite to reduction in beta-adrenoceptor function, desensitization of alpha\textsubscript{2}-adrenoceptor (presynaptic) could be a rate limiting step in the efficacy of antidepressant therapy. It was then proposed that coadministration of alpha\textsubscript{2}-adrenoceptor antagonists would result in a more rapid antidepressant response and the down-regulation of central beta-adrenoceptors would be enhanced and accelerated (Crew et al., 1978; Johnson et al., 1980; Wiech and Ursillo, 1980). Consistent with this hypothesis are several studies which have demonstrated that beta-adrenoceptors down-regulation is indeed enhanced and accelerated after coadministration of antidepressants with alpha\textsubscript{2}-receptor antagonist in rat brain (Scott and Crews, 1983; Salama et al., 1983; Campbell et al., 1983).
Most of the experiments described above report changes in rat brain alpha2- and beta-adrenoceptor number. However, marked differences in alpha2-receptors between rodents and other species have been reported. In this chapter (5) effects of chronic amitriptyline either alone or in combination with yohimbine or idazoxan on a non-rodent species, rabbit, were studied. Idazoxan in addition to its reported alpha2-adrenoceptor antagonist property (Doxey et al., 1983; Dettmar et al., 1983), has been reported to possess some antidepressant effect (Stahl and Palazidou, 1986). Effects of these treatments on idazoxan and yohimbine binding sites in addition to beta-adrenoceptor numbers in rabbit brain were examined.

5.2 METHODS.

5.2.1 Drug administrations.

Male New Zealand white rabbits (2.5-3.00 kg) from Cheshire Rabbit Farm Ltd were used for all the studies. Six treatment groups of animals (n = 8 - 12) were studied: one group of rabbits was treated with amitriptyline 30 mg/kg/day intraperitoneally for 6 or 21 days (10 mg/kg tid), one group received combined amitriptyline (30 mg/kg/day) and yohimbine (15 mg/kg/day) for 6 days (I.P). While a third group received idazoxan (0.56 mg/kg/hr) intravenously via osmotic minipumps and amitriptyline (30 mg/kg/day) (I.P). Control groups received 0.1% ascorbate for either 6 or 21 days as
appropriate. The effects of yohimbine treatment alone was studied in the previous chapter (4). However the dose of idazoxan use for this study was lower than the dose studied in chapter four.

5.2.2 Tissue preparation.

At the end of the experimental period rabbits were killed with an overdose of pentobarbitone sodium and the brain tissue removed and prepared as described in methods section 2.4.1.

5.2.3 Receptor binding assays.

Radioligand binding assays were performed using \([^3]H\)idazoxan (0.5-20.0 nM), \([^3]H\)yohimbine (1.25-37.5 nM) and \([^3]H\)dihydroalprenolol (0.15-7.2 nM) for beta-adrenoceptors. Incubations were carried out as described in section 2.4.2 of the Methods.

5.2.4 Data analysis.

Differences between groups were examined using unpaired t-test for the two groups treated for 21 days while analysis of variance was used for the three groups treated for 6 days.
5.3 RESULTS.

5.3.1 Effects of 21 days amitriptyline treatment.

Twenty one days amitriptyline treatment significantly reduced the number of $[^3\text{H}]$yohimbine binding sites ($B_{\text{max}}$) in the hindbrain by 54% ($p < 0.01$) but not in the forebrain (figure 5.1). The reduction observed in the hindbrain was as a result of changes in $B_{\text{max}}$ rather than changes in the affinity since no significant change in the $K_D$ was observed (Table 5.1). There was no significant treatment effect on either the $B_{\text{max}}$ or $K_D$ of the $[^3\text{H}]$idazoxan binding site in forebrain or hindbrain (figure 5.2); neither was $[^3\text{H}]$dihydroalprenolol binding to either fore or hindbrain significantly affected by treatment (figure 5.3). Although $[^3\text{H}]$yohimbine binding to hindbrain was significantly reduced by 21 days treatment with amitriptyline, 6 days treatment had no significant effect ($B_{\text{max}}$ were 100 $\pm$ 15 and 90 $\pm$ 11 fmoles/mg protein for control and treated groups respectively).

5.3.2 Effects of 6 days coadministration of amitriptyline with yohimbine or idazoxan.

Coadministration of amitriptyline with either yohimbine or idazoxan had no significant treatment effects on either $[^3\text{H}]$idazoxan or $[^3\text{H}]$yohimbine binding in the forebrain or hindbrain (figures 5.4 and 5.5). There was no significant changes in $K_D$ of $[^3\text{H}]$idazoxan binding to forebrain or
hindbrain also there was no significant treatment effect on the $K_D$ for $[^3H]$yohimbine binding in either brain region (Table 5.1). Coadministration of amitriptyline with either yohimbine or idazoxan did not significantly affect $[^3H]$dihydroalprenolol binding sites in either brain regions (figure 5.6) and the $K_D$ was unchanged (table 5.1). However, there was a slight decrease in the forebrain but this did not reach statistical significance.
Figure 5.1

Effects of 21 days of amitriptyline treatment on the number of \([^3]H\)yohimbine binding sites.

- □ control
- ■■ amitriptyline

\(n = 8 - 12, * p < 0.05\).
Figure 5.2
Effects of 21 days amitriptyline treatment on the number of $[^3$H]idazoxan binding sites.

- control
- amitriptyline

$n = 8 - 12.$
Figure 5.3

Effects of 21 days amitriptyline on the number of $[^3H]$dihydroalprenolol binding sites.

- Control
- Amitriptyline

$n = 8 - 12.$
TABLE 5.1

Effects of 21 days amitriptyline either alone or in combination with yohimbine or idazoxan on the dissociation constant $K_D$ (nM).

<table>
<thead>
<tr>
<th></th>
<th>[${}^3$H]Yohimbine</th>
<th>[${}^3$H]Idazoxan</th>
<th>[${}^3$H]DHA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FB</td>
<td>HB</td>
<td>FB</td>
</tr>
<tr>
<td>Control</td>
<td>$8 \pm 3$</td>
<td>$8 \pm 4$</td>
<td>$3 \pm 1$</td>
</tr>
<tr>
<td>Amitriptyline</td>
<td>$11 \pm 3$</td>
<td>$7 \pm 4$</td>
<td>$4 \pm 2$</td>
</tr>
<tr>
<td>Control</td>
<td>$8 \pm 2$</td>
<td>$13 \pm 5$</td>
<td>$4 \pm 1$</td>
</tr>
<tr>
<td>Amt + Yoh</td>
<td>$10 \pm 3$</td>
<td>$7 \pm 4$</td>
<td>$3 \pm 2$</td>
</tr>
<tr>
<td>Amt + Idaz</td>
<td>$8 \pm 4$</td>
<td>$10 \pm 6$</td>
<td>$5 \pm 2$</td>
</tr>
</tbody>
</table>

Results: mean $\pm$ S.D, n = 8 - 12.

FB = forebrain, HB = Hindbrain, Amt = amitriptyline, Yoh = yohimbine, Idaz = idazoxan.
Figure 5.4

Effects of 6 days amitriptyline combined treatments with yohimbine or idazoxan on the number of $[^3H]$idazoxan binding sites.

- □ control,
- ■ amitriptyline and yohimbine,
- ▬ amitriptyline and idazoxan

n = 8.
Figure 5.5

Effects of 6 days amitriptyline combined treatments with yohimbine or idazoxan on the number of $[^3H]$yohimbine binding sites.

- □ control,
- ■ amitriptyline with yohimbine,
- ● amitriptyline with idazoxan

$n = 8$. 
Figure 5.6
Effects of 6 days amitriptyline combined treatments with yohimbine or idazoxan on the number of [3H]dihydroalprenolol binding sites.

- control,
- amitriptyline with yohimbine,
- amitriptyline with idazoxan

n = 8.
5.4 DISCUSSION.

Chronic drug treatment can lead to modifications in receptor sensitivity which appears to be one of the means by which synaptic transmission is regulated. Long term receptor activation or blockade in vivo has been reported to lead to receptor desensitization or supersensitization (Creese and Sibley, 1981; Davies and Lefkowitz, 1981; Lefkowitz et al., 1983). Investigations into the comparative effects of acute and chronic antidepressant treatment on the turnover of brain monoamines in rats has led to the suggestion that the chronic administration of antidepressants initiates an adaptive process which could result in enhanced transmitter release (Shildkraut et al., 1970; 1971; Campbell et al., 1979) and a reduction in the number of beta-adrenoceptor, these changes may be responsible for the mechanisms of action of the antidepressants. This hypothesis has been supported to a certain degree by the observation that chronic and not acute antidepressant treatment leads to clinical alleviation of depression (Denber, 1975; Charney et al., 1981; Sugrue, 1983).

In the experiments described here, 21 days of amitriptyline treatment significantly reduced the number of $[^3H]$yohimbine binding sites in the hindbrain but not in the forebrain. The reduction was as a result of a reduction in $B_{\text{max}}$ and not as a result of a reduction in affinity since the $K_D$ was not significantly affected by the treatment. The reduction observed in the $[^3H]$yohimbine binding is
consistent with the postulate that alpha$_2$-adrenoceptor sensitivity and possibly number will be reduced as a consequence of increased catecholamine concentrations in the central nervous system caused by the actions of antidepressants (Crews and Smith, 1978; Svensson and Usdin, 1978). Consistent with this observation is the reduction in the binding of alpha$_2$-adrenoceptor ligands to cerebral membranes from rats treated chronically with both tricyclic and monoamine oxidase inhibitor antidepressants (Smith et al., 1981; Cohen et al., 1982). However, the reason for the localized reduction in $[^3\text{H}]$yohimbine binding after amitriptyline treatment is not known, but penetration of the drug into the CNS and concentration in localized areas, pharmacokinetic interactions, receptor susceptibility to changes, and the receptor population predominating in this region could partially explain this observation. In support of this localized decrease in $[^3\text{H}]$yohimbine binding is the finding that chronic amitriptyline lowers $[^3\text{H}]$clonidine binding in rat limbic structures (Crews and Smith, 1978) with no changes in binding to cortical slices (Peroutka and Snyder, 1980; Tang et al., 1981; Sugrue, 1982; 1983). In addition Scott and Crews, (1983) have reported localized changes in beta-adrenoceptors in rat brain after treatment with desipramine. In other studies, the number of alpha$_2$-receptors in the CNS has been shown to be increased or decreased after antidepressant treatment (Creese and Sibley, 1981; Finberg, 1987), and the direction of the change in alpha$_2$-adrenoceptor number appears to depend on the
duration, and dose of drug administration and the brain region examined (Smith and Hollingsworth, 1984) plus concentration of noradrenaline in that region. The type of ligands, agonist or antagonist used in the studies has also been reported to affect the direction of change in alpha-receptor numbers (Salama et al., 1983). However, a reduction in \(^3\text{H}\)clonidine and \(^3\text{H}\)yohimbine binding to cortical membranes after chronic clorgyline treatment has been reported (Cohen et al., 1982). In contrast to the changes in \(^3\text{H}\)yohimbine binding there was however no significant treatment effect on \(^3\text{H}\)idazoxan binding sites and \(K_D\) in either forebrain or hindbrain after 21 days amitriptyline treatment. Differences between yohimbine and idazoxan could relate to their binding to different sites (chapter 3).

The coadministration of amitriptyline with either yohimbine or idazoxan did not have any significant effect on \(^3\text{H}\)yohimbine or \(^3\text{H}\)idazoxan binding sites in forebrain or hindbrain. Also there were no significant changes observed in the \(K_D\) of both \(^3\text{H}\)idazoxan and \(^3\text{H}\)yohimbine binding in either brain region examined. This observation is not consistent with the postulated reduced alpha\(_2\)-adrenoceptor sensitivity after tricyclic antidepressant treatment (Finberg, 1987). In addition, there was a trend towards a reduction in \(^3\text{H}\)yohimbine binding in the group treated with combined yohimbine and amitriptyline. It is possible that a longer duration of treatment or higher doses of both yohimbine and amitriptyline could lead to reduction in the
number of binding sites. Changes in alpha$_2$-adrenoceptor number have been observed by some groups after coadministration of antidepressants with alpha$_2$-adrenoceptor antagonists. Ursillo et al. (1980) reported an increase in $[^3$H]para-aminoclonidine binding sites in cerebral cortex after four days desipramine and yohimbine treatment.

In the experiments involving treatment with either yohimbine or idazoxan alone it was observed that both treatments significantly increased the number of $[^3$H]yohimbine binding sites in the rabbit brain (chapter 3). Hence, in these studies, it seems that the coadministration of antagonists with amitriptyline may be having opposing effects with amitriptyline increasing the biogenic amines (agonists) concentration leading to down-regulation while the antagonists up-regulates. This could be responsible for the lack of change in receptor number in this experiment.

In these studies, $[^3$H]dihydroalprenolol binding was not significantly affected by treatment with amitriptyline either alone or in combination with yohimbine or idazoxan. Although there was a slight reduction in groups treated with amitriptyline in combination with yohimbine or idazoxan, this did not reach significance. In contrast to these observations in rabbit, numerous groups have reported decreases in $[^3$H]DHA binding in rats after tricyclic antidepressant treatment with an increase in the extent and rate of decrease when the antidepressant was given in combination with an alpha$_2$-adrenoceptor antagonist. Wiech and Ursillo (1980) and Charney et al. (1986) reported a
significant reduction in [$^3$H]DHA binding to cerebral cortex after 4 days and 6 days treatment with desipramine, respectively. Also a significant decrease in beta-adrenoceptor number has been reported in the cortex by Minneman et al. (1982) after 10 days desipramine treatment. In contrast, Ursillo et al. (1980) were only able to observe a significant reduction in the number of [$^3$H]DHA binding sites after 42 days when desipramine alone was used. The administration of imipramine alone was reported by Keith et al. (1988) to cause progressive reduction in the number of [$^3$H]DHA binding reaching maximum after four weeks of treatment in rat cerebral cortex. While coadministration of imipramine with alpha$_2$-adrenoceptor antagonists resulted in accelerated down-regulation of beta-adrenoceptor with maximum reduction occurring only after one week of treatment. It is however, possible, that changes in beta-adrenoceptor number in the rabbit brain may occur in localized areas and were not detected in this study. In addition, longer treatment or higher doses of drugs may result in a significant reduction in receptor number. [$^3$H]DHA has a similar affinity for both beta$_1$- and beta$_2$-adrenoceptors and subtype selective down-regulation may have occurred which was not detected in this experiment. Minneman et al. (1978; 1982) have reported selective regulation of beta$_1$- and beta$_2$-adrenoceptors while Beer et al., (1987) also reported independent regulation of beta$_1$- and beta$_2$-adrenoceptors in rats after treatment with desipramine. In
addition, amitriptyline treatment may have greater effects on muscarinic cholinergic receptors (Rehavi et al., 1980) and serotonin transport / uptake (Koe, 1976) than noradrenaline uptake and beta-adrenoceptors. However, these results emphasise the danger of generalising from findings with receptor binding experiments done across tissues and species and suggest that beta-adrenoceptor down-regulation may not be a universal response to tricyclic antidepressant treatment.
CHAPTER SIX

CHRONIC INTRACEREBROVENTRICULAR INFUSION OF ADRENALINE AND NORADRENALINE: EFFECTS ON CENTRAL ADRENOCEPTORS IN THE RABBIT.
CHAPTER SIX

EFFECTS OF CHRONIC INTRACEREBROVENTRICULAR INFUSION OF ADRENALINE AND NORADRENALINE ON CENTRAL ADRENOCEPTORS IN THE RABBIT.

6.1 INTRODUCTION.

The effects of acute and chronic catecholamine exposure on peripheral alpha- and beta-adrenoceptors have been extensively studied (Mickey et al., 1975; Perkins, 1981; Maura et al., 1985; Jones et al., 1986; Neve and Molinoff, 1986; Deighton et al., 1986; 1988), but for technical reasons very few studies have looked directly at the effects of chronic changes in central catecholamine levels on central adrenoceptors. Investigators have, however, employed various methods of altering central catecholamine levels indirectly by administration of drugs which influence the levels of adrenaline and noradrenaline in the brain. Such drugs include tricyclic antidepressants and monoamine oxidase inhibitors which increase the synaptic concentration of the biogenic amines via inhibition of uptake mechanisms and degradation of catecholamines by monoamine oxidase, respectively (Gluckman and Baum, 1969; Cohen et al., 1980; Charney et al., 1981; Asakura et al., 1982; Sugrue, 1983; Campbell et al., 1979). Other methods include the destruction of central catecholaminergic pathways by the administration of 6-hydroxydopamine or reserpine which deplete neuronal catecholamine storage (U'Prichard and Snyder, 1978; U'Prichard et al., 1979; Bylund and Martinez, 1980; Hamilton and Reid, 1982). In addition to these
treatments, treatment with alpha₂-adrenoceptor agonists and antagonists which readily cross the blood brain barrier have been used to study regulation of central alpha₂-adrenoceptors. These include the imidazoline and guanidine groups such as clonidine, idazoxan, guanfacine, phentolamine, guanethidine, guanabenz and B-HT 933 (Oates et al., 1979; Timmermans and van Zwieten, 1980; Thoolen et al., 1982; Maura et al., 1985; Finberg and Kopin, 1987). However, the effects of such treatments on central adrenoceptor function and density have been conflicting (Charney et al., 1981; Armstrong et al., 1983; Finberg, 1987). In addition, all the drugs used in these studies have imidazole or related structures. The effects of direct injection of phenylethylamine and imidazole type of drugs into rostral ventrolateral medulla have been shown to differ and this has led to the suggestion that the two classes of compound may be acting at different sites (Bousquet et al., 1984). Lack of cross-desensitization between these classes of drugs in the periphery has given support to this hypothesis (Ruffolo et al., 1977). Furthermore, it has recently been shown that clonidine and its analogue para-aminoclonidine, bind to sites distinct from alpha₂-adrenoceptor sites and a low molecular weight substance which can displace [³H]clonidine and [³H]para-aminoclonidine from their binding sites has been isolated from bovine brain membrane (Atlas and Burstein, 1984; Ernsberger et al., 1986; Meeley et al., 1986; Felsen et al., 1987). More recently,
idazoxan, an imidazoline derivative, has also been reported to bind with high affinity to a site distinct from the alpha$_2$-adrenoceptor site (Coupry et al., 1987; Lachaud et al., 1988; Wikberg, 1988; Yablonsky et al., 1988). Thus the effects of chronic treatment with imidazoline type of agonists may not reflect changes occurring during chronic elevation of the endogenous catecholamines adrenaline and noradrenaline.

Most of the studies reporting changes in alpha$_2$-adrenoceptor function and number on chronic treatment with catecholamines have been carried out in the periphery while central regulation of alpha$_2$-adrenoceptors has not been widely studied particularly in response to stimulation by catecholamines. This has been due to the fact that peripherally administered catecholamines do not cross the blood-brain barrier (Hardebo and Owman, 1980; Oldendorf, 1971; Minneman, 1983). However, intracerebroventricular infusion has been described recently (Jefferies and Orzechowski 1985; Wu and Wei 1984; Correa et al., 1982; Johnson et al., 1987). This method has made it possible for direct drug infusion into the brain via the cerebral ventricle and it has been used to study the effects of some centrally acting drugs such as carbachol and saralasin (Brunner et al., 1983; Wu and Wei, 1984). This method has therefore been used to study effects of chronic catecholamine infusion directly into the brain on functions mediated by alpha$_2$-adrenoceptors and on densities of central adrenergic receptors in the rabbit.
6.2 METHODS.

6.2.1 Doses and drug infusions.

Ten day infusions of adrenaline (100 μg/kg/hr) or noradrenaline (150 μg/kg/hr) were carried out in groups of rabbits (n = 6 - 8). The dose of adrenaline infusion was reduced from 150 μg/kg/hr to 100 μg/kg/hr because in pilot studies, at the higher dose, some rabbits (3) died within 24-36 hours of commencing infusion. Control groups studied in parallel received 0.1% ascorbate. Alzet osmotic minipumps (figure 2.1) model 2002 with a nominal flow rate of 0.5 ul/hr were loaded with either the drug or vehicle. Drugs were infused continuously into the left cerebral ventricle via intracerebral cannulae implanted and held in place by a head plate as described in the methods section 2.2.2.4.

6.2.2 Effects on intracisternal injection of clonidine.

To monitor effects of chronic catecholamine infusion on alpha₂-adrenoceptor mediated function, the depressor response to intracisternal injection of clonidine (1 μg/kg) was examined in anaesthetized treated and control groups. Changes in mean arterial blood pressure (MAP) mmHg were monitored at intervals for 60 minutes (methods 2.3).
6.2.3 **Tissue preparation.**

At the end of the blood pressure monitoring, rabbits were killed by an overdose of pentobarbitone sodium (60 mg/ml). The brain was removed and dissected into forebrain and hindbrain at the point of colliculi. The forebrain was then dissected into left and right cerebrum at the mid brain. The brain tissues were prepared by homogenization and centrifugation as described in methods (2.4.1) for radioligand binding assays (2.4.2) and for tissue catecholamine assay (methods 2.6).

6.2.4 **Radioligand binding assays.**

In order to study effects of chronic catecholamine infusion on central adrenoceptors, radioligand binding experiments were performed using tissue homogenates and the ligands [*H*]yohimbine (1.25-37.0 nM), [*H*]idazoxan (0.5-20.0 nM) and [*H*]dihydroalprenolol (0.15-7.2 nM) as described in the methods section 2.4.2.

6.2.5 **Data and statistical analysis.**

Radioligand binding parameters and $K_D$ were obtained from modified Scatchard plot analysis (section 2.4.3). Bmax and $K_D$ for control, adrenaline and noradrenaline treated animals were then compared using one way analysis of variance. The differences between the depressor response to clonidine injection in catecholamine and vehicle treated
6.3 RESULTS.

No significant changes in the mean arterial blood pressure and heart rate of the animals used in this experiment were observed throughout the period of drug infusion. Also, there were no obvious behavioural changes during the infusion.

6.3.1 Effects of catecholamine infusion on tissue catecholamine levels.

The effect of chronic catecholamine infusion on tissue catecholamine levels as assayed by HPLC-ECD is presented in table 6.1. There was variation within the groups in the levels of catecholamine assayed but the highest levels were consistently observed in the left cerebrum, the area into which the drugs were infused, with lower levels in the right cerebrum and hindbrain. In the assay, adrenaline concentrations in saline/ascorbate and noradrenaline infused rabbits, were below the level of detection, while groups that received either adrenaline or noradrenaline, showed higher levels of catecholamines with increases of 50 fold or greater in the left cerebrum and around 10 fold in the hindbrain during noradrenaline infusion. Although, the
tissues were washed thoroughly, it is possible that not all perfusate was removed and this could have contributed to the high catecholamine levels observed in the left cerebrum.

6.3.2 Effects of clonidine injection (I.C).

Intracisternal clonidine injection (1 ug/kg) produced a prolonged depressor response with a maximum fall of $-26 \pm 11$ mmHg at 15 minutes after the injection in the control group. Chronic adrenaline infusion 100 ug/kg/hr significantly attenuated the depressor response induced by intracisternal clonidine injection. The maximum fall was significantly reduced to $-10 \pm 4$ mmHg and was significantly attenuated when compared to controls at 5, 15 and 30 minutes after the injection (figure 6.1). However, noradrenaline infusion did not have any significant effect in attenuating the fall in blood pressure induced by the clonidine injection (figure 6.2). Neither of these treatments had significant effects on the heart rate changes induced by clonidine injection.
Table 6.1
Effects of chronic intracerebroventricular infusion of catecholamines adrenaline and noradrenaline concentration (pm/mg protein) in the rabbit brain tissues.

<table>
<thead>
<tr>
<th>Treatments:</th>
<th>Control</th>
<th>Adrenaline</th>
<th>Noradrenaline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NA</td>
<td>ADR</td>
<td>NA</td>
</tr>
<tr>
<td>Lt cerebrum</td>
<td>5</td>
<td>4</td>
<td>ND</td>
</tr>
<tr>
<td>Rt cerebrum</td>
<td>5</td>
<td>4</td>
<td>ND</td>
</tr>
<tr>
<td>Hindbrain</td>
<td>4</td>
<td>4</td>
<td>ND</td>
</tr>
</tbody>
</table>

Result: - mean ± S.D, n = 6 - 8.

ND = Not detectable, Rt = right, Lt = left.
NA = Noradrenaline, ADR = Adrenaline.
6.3.3 Effects of chronic catecholamine infusion on:-

6.3.3.1 \(^3\text{H}\)Idazoxan binding sites.

Chronic adrenaline (100 ug/kg/hr) infusion significantly reduced the maximum number (Bmax) of \(^3\text{H}\)idazoxan binding sites from 55 + 9 to 33 + 9 fmoles/mg protein in the right cerebrum (figure 6.3). There was no significant treatment effect on the binding sites in either left cerebrum or hindbrain. Chronic noradrenaline infusion did not have any significant effect on \(^3\text{H}\)idazoxan binding sites in any brain region examined (figure 6.4). There was no significant changes in the \(K_D\) as a result of these treatments (Table 6.2).

6.3.3.2 \(^3\text{H}\)Yohimbine binding sites.

Neither chronic adrenaline (100 ug/kg/hr) nor noradrenaline (150 ug/kg/hr) had any significant effect on the maximum number (Bmax) of \(^3\text{H}\)yohimbine binding sites in any of the brain areas studied (figures 6.5 and 6.6). Although Bmax from the group that received adrenaline tended to be reduced (from 137 + 33 for control to 109 + 15 fmoles/mg protein for adrenaline treated) this did not reach significant level. However, a significant increase in the dissociation constant (\(K_D\)) was observed in both left and right cerebrum but not in the hindbrain with both adrenaline and noradrenaline infusions (Table 6.2).
6.3.3.3  \(^{[3\text{H}]}\text{Dihydroalprenolol binding sites.}\)

Neither chronic adrenaline nor noradrenaline infusion had any significant treatment effects on either the binding sites or the affinity state of \(^{[3\text{H}]}\)dihydroalprenolol binding at any of the brain regions studied (figures 6.7 and 6.8) (Table 6.2).
Figure 6.1
Effects of chronic adrenaline (100 ug/kg) infusion (icv) on the depressor response to intracisternal clonidine (1ug/kg) injection.

Control

Adrenaline

\[ n = 6 - 8, \, * \, P < 0.05. \]
Figure 6.2
Effects of chronic noradrenaline (150 ug/kg) infusion (icv) on the depressor response intracisternal clonidine (1ug/kg) injection.

- Control
- Noradrenaline.

n = 6 - 8.
Effects of chronic adrenaline (100 ug/kg) infusion on the number of $[^3H]$idazoxan binding sites.

- Control
- Adrenaline

$n = 6 - 8$, $* P < 0.05.$
Figure 6.4
Effects of chronic noradrenaline (150 ug/kg) on the number of $[^3\text{H}]$idazoxan binding sites.

- Control
- Noradrenaline

$n = 6 - 8.$
Figure 6.5
Effects of chronic adrenaline (100 μg/kg) infusion on the number of $[^3H]$yohimbine binding sites.

- Control
- Adrenaline

$n = 6 - 8$. 
Table 6.2

Effects of chronic catecholamine infusion on the dissociation constant (K_D) nM.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Control</th>
<th>Adrenaline</th>
<th>Noradrenaline</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>[^3H]Idazoxan</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rt cerebrum</td>
<td>2 ± 1</td>
<td>3 ± 1</td>
<td>3 ± 2</td>
</tr>
<tr>
<td>Lt cerebrum</td>
<td>2 ± 1</td>
<td>2 ± 1</td>
<td>3 ± 2</td>
</tr>
<tr>
<td>Hindbrain</td>
<td>2 ± 1</td>
<td>3 ± 1</td>
<td>3 ± 1</td>
</tr>
<tr>
<td><strong>[^3H]Yohimbine</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rt cerebrum</td>
<td>8 ± 3</td>
<td>13 ± 3*</td>
<td>11 ± 5*</td>
</tr>
<tr>
<td>Lt cerebrum</td>
<td>6 ± 3</td>
<td>13 ± 7*</td>
<td>13 ± 5*</td>
</tr>
<tr>
<td>Hindbrain</td>
<td>8 ± 5</td>
<td>8 ± 5</td>
<td>10 ± 4</td>
</tr>
<tr>
<td><strong>[^3H]Dihydroalprenolol</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rt cerebrum</td>
<td>0.9 ± 0.6</td>
<td>0.9 ± 0.2</td>
<td>1.6 ± 0.7</td>
</tr>
<tr>
<td>Lt cerebrum</td>
<td>1.0 ± 0.6</td>
<td>1.0 ± 0.2</td>
<td>1.5 ± 0.5</td>
</tr>
<tr>
<td>Hindbrain</td>
<td>0.8 ± 0.6</td>
<td>0.5 ± 0.1</td>
<td>0.7 ± 0.5</td>
</tr>
</tbody>
</table>

Results: mean ± S.D, *p < 0.05, n = 6 – 8.
Effects of chronic noradrenaline (150 ug/kg) infusion on the number of $[^3H]$yohimbine binding sites.

- Control
- Noradrenaline

$n = 6 - 8$. 
Figure 6.7
Effects of chronic adrenaline (100 ug/kg) infusion on the number of [^3]Hdihydroalprenolol binding sites.

- Control
- Adrenaline

n = 6 – 8.
Right cerebrum  Left cerebrum  Hindbrain

Figure 6.8
Effects of chronic noradrenaline (150 ug/kg) infusion on the number of [^H]dihydroalprenolol binding sites.

□  Control

Noradrenaline

n = 6 - 8.
6.4 DISCUSSION.

Intracerebroventricular infusion of catecholamines causes increased adrenaline and noradrenaline levels in the rabbit brain as assayed using HPLC-ECD methods (Hashimoto and Maruyama, 1977; Howes et al., 1983). The increases varied from 2 to more than 50 fold in the different brain regions when compared to the controls as shown in table 6.1. The highest concentrations of catecholamine being observed in the left cerebrum into which the drug was infused. These results show that the method of infusion used was effective at delivering drug into the brain (Wu and Wei, 1984; Jefferies and Orzechowski, 1985; Johnson et al., 1987). However, contamination from the infusate could contribute to the high levels of catecholamines observed.

Chronic intracerebroventricular infusion of adrenaline but not noradrenaline significantly attenuated the depressor response induced by intracisternal injection of clonidine. In addition, adrenaline was more toxic as it caused death at 150 ug/kg/hr before it was reduced to 100 ug/kg/hr while the same dose of noradrenaline had no obvious adverse effects. In the periphery, adrenaline but not noradrenaline has been reported to attenuate platelet aggregation and decrease receptor number while noradrenaline but not adrenaline decreased receptor number in the kidney (Brodde et al., 1982; Deighton et al., 1986; Hamilton et al., 1987). These effects could be related to the subtype of alpha_{2}-adrenoceptor found in platelets and kidney and the greater responses to adrenaline observed in these experiments could
be related to the alpha$_2$-subtype predominating within the brain. The existence of alpha$_2$-adrenoceptor subtypes between and within species has been widely reported (Neylon and Summers 1985; Bylund 1981; 1985; 1988). Bylund (1988) reported that alpha$_2$-adrenoceptors on human platelets are exclusively of the alpha$_2A$-subtype. Alpha$_2A$-subtypes also predominate in rat cerebral cortex and submandibular gland and in human caudate nucleus, while neonatal rat lung has a high proportion of alpha$_2B$ subtype. In human cortex and cerebrum, the ratio of the alpha$_2A$ to alpha$_2B$ is 100:0. The subtype of receptor predominating in a region or tissue may determine the effects of drugs in that tissue. Adrenaline has a higher affinity for the alpha$_2A$-subtype which occurs exclusively in platelets and predominates in many brain regions. Moreover, although both catecholamines have been implicated in the regulation of blood pressure (Kobinger, 1978), it has been reported that adrenaline may be responsible for mediating sympathetic cardiovascular and baroreflex vasodepressor responses (Fuller et al., 1980; Granata et al., 1985; Ruggiero and Reis, 1988) and this is consistent with the greater effect of chronic adrenaline than noradrenaline in attenuating the depressor response to clonidine in these experiments.

No reduction in the number of [$^3$H]yohimbine binding sites in the hindbrain was observed. However attenuation of response without loss in number may precede a down-regulation in number. In previous studies, chronic
amitriptyline treatment in rabbits also caused attenuation of the clonidine depressor response without a change in number, but the attenuation was related to an alteration in affinity state of the receptor (Hamilton et al., 1986). In the forebrain there was a tendency towards a decrease in $^3$H]yohimbine binding and a longer infusion time or higher dose of infusion might lead to a significant decrease in the number of binding sites.

Alterations in the dissociation constant of $^3$H]yohimbine binding to both the left and right cerebrum were observed after adrenaline and noradrenaline infusion. This could be due to agonist retention. Consistent with this observation was the reported increase in apparent dissociation constant for $^3$H]yohimbine with no change in Bmax after incubation of platelets with epinephrine (Karliner et al., 1982).

Changes in $^3$H]yohimbine binding were less marked than has been reported for guanabenz (chapter 7) after 5 days infusion. Pharmacokinetic and pharmacodynamic properties plus metabolism of guanabenz may be different from that of catecholamines. In addition guanabenz could also have different affinities for subtypes of alpha$_2$-receptor.

The decrease in the number of $^3$H]idazoxan binding sites in the right cerebrum after adrenaline infusion was surprising as throughout this thesis no other treatment caused changes in the number of $^3$H]idazoxan binding sites in the brain, in addition, although adrenaline was infused via the left side of the brain no significant change was
observed on this side. This result is therefore difficult to explain on physiological or pharmacological grounds. Taking a significant level of $p < 0.05$, one in 20 t-tests would give a false positive result, thus considering the number of comparisons made in this thesis, it would not be surprising if one outlying result is obtained which may not have any significant biological importance.

No changes in the number of $[^3H]$dihydroalprenolol binding sites was observed in either the brain regions examined which is consistent with lack of change in density after amitriptyline treatment (Hamilton et al., 1986; Chapter 5). In the rat, changes in the number of beta-adrenoceptors have been reported (Minneman et al., 1978; Ursillo et al., 1980; Beer et al., 1986; Charney et al., 1986). Rabbit beta-adrenoceptors may be more resistant to changes although Minneman (1983) also reported rat beta-adrenoceptors to be resistant to regulation.

In this study, the depressor response to clonidine injection (i.c) was attenuated by chronic adrenaline but not noradrenaline infusion although no changes in $[^3H]$yohimbine binding were obtained, $[^3H]$idazoxan binding to the right cerebrum was reduced by adrenaline infusion but this may not be of any biological significance. Thus this study has demonstrated that receptor mediated functions may be regulated by chronic putative agonist treatment without meaningful changes in the density of receptor binding sites.
CHAPTER SEVEN

EFFECTS OF CHRONIC GUANABENZ INFUSION ON RABBIT $\alpha_2$- AND IMIDAZOLE TYPE RECEPTORS.
7.1 INTRODUCTION.

Guanabenz, a guanidine derivative has been shown to have antihypertensive properties. Its mechanism of action is by stimulation of central alpha\textsubscript{2}-adrenoceptors, thereby reducing sympathetic outflow (Baum and Shropshire, 1976; Ohata et al., 1982). Guanabenz lowers blood pressor and heart rate in both normotensive and hypertensive rats (Baum et al., 1970; Baum and Shropshire, 1976; Misu and Kubo, 1982). It has been reported to reduce noradrenaline turnover in the brain (Braestrup and Nielsen, 1976) and to possess a diuretic property (Strandhoy et al., 1980). It has a pharmacological profile similar to that of clonidine (Baum et al., 1970; Baum and Shropshire, 1976). However, differences between guanabenz and clonidine's mechanisms of action have been reported. Gutkin and Enero (1983) have shown that the cardiovascular effects of clonidine but not guanabenz were antagonised by uptake blockers. In the periphery, guanabenz induced pressor effects in pithed rats via postsynaptic alpha\textsubscript{2}-adrenoceptors while clonidine activated both vascular alpha\textsubscript{1}- and alpha\textsubscript{2}-adrenoceptors (Gutkin et al., 1986). Gutkin and Enero, (1987) further showed differences in the pharmacological interaction of clonidine and guanabenz with antidepressant drugs and postulated that these drugs might be acting on different receptors. It has been suggested that idazoxan may label a
novel imidazole-like idazoxan receptor (Yablonsky et al., 1988; Bricca et al., 1988; Wikberg, 1988; Vigne et al., 1989). In radioligand displacement experiments (chapter 3) guanabenz showed a high affinity (2.8 nM) for \[^{3}H\text{idazoxan}\] binding sites while clonidine showed a relatively low affinity (800 nM) for the same binding site. Both drugs (clonidine and guanabenz) showed a similar but fairly low affinity for the \[^{3}H\text{yohimbine}\] binding site.

Chronic agonist treatment may lead to receptor down regulation and desensitization. The effects of acute and chronic clonidine treatment on receptor number and function have been a subject of extensive study (Reid et al., 1977; Brodde et al., 1982; Maura et al., 1985; Finberg and Kopin, 1987), but the effects of chronic guanabenz treatment on alpha\(_{2}\)-adrenoceptor number and function have not been as widely studied and may differ from those of clonidine. In previous studies, treatment of hypertensive patients with guanabenz did not lead to changes in platelet alpha\(_{2}\)-adrenoceptor (Motulsky et al., 1983) but Brodde et al., (1982) reported reduced platelet alpha\(_{2}\)-adrenoceptor number in patients treated with clonidine. This chapter (7) is therefore aimed at examining effects of chronic guanabenz treatment on alpha\(_{2}\)-adrenoceptor mediated function and the effects of treatment on the \[^{3}H\text{yohimbine}\] and \[^{3}H\text{idazoxan}\] binding sites in rabbit brain and kidney membranes.
7.2 METHODS.

The experiments were performed using groups (n = 6 - 9) of rabbits (2.5-3.0 kg) from Cheshire Rabbit Farms Ltd. The animals were housed individually and allowed free access to water and food ad libitum.

7.2.1 In vivo infusion.

Groups of rabbits received guanabenz acetate dissolved in 40% alcohol and 60% 0.1% ascorbate at a concentration of 50 mg/ml. The drug was infused via a minipump implanted as described in the methods section 2.2.2.2 for 5 days at the rate of 237 ug/kg/hr. Control groups received vehicle. In acute studies the drug was infused into a peripheral ear vein at the same rate for 5 hours.

7.2.2 Pressor responses to alpha-methylnoradrenaline.

Dose-response curves were constructed for bolus injection of alpha-methylnoradrenaline 5 - 100 ug in both control and guanabenz (acute and chronic) treated groups. The dose required to increase the mean arterial pressure (MAP) by 50 mmHg in individual animals was obtained from the curves. The intravenous line for injection of drugs and the arterial lines for measurement of blood pressure were inserted under local anaesthesia as described in the methods section 2.1. In the acute studies pressor dose response curves were constructed before and after 5 hours infusion of
guanabenz, thus in these experiments the animals acted as their own controls.

7.2.3 **Intracisternal injection of clonidine.**

The effects of chronic guanabenz infusion on the depressor response to clonidine injected intracisternally, was studied at the end of 5 days infusion. Rabbits were anaesthesized with pentobarbitone sodium (30-60 mg/kg) and clonidine 1 ug/kg was injected into the cisterna magna by a free hand transcutaneous injection in a total volume of 100 ul (Chalmers and Wurtman, 1971). Blood pressure and heart rate changes were monitored continuously for 5 minutes and then every 15 minutes thereafter for 60 minutes.

7.2.4 **Tissue preparation.**

At the end of the experiments, rabbits were killed by an overdose of pentobarbitone sodium (i.v). The brain and kidney were removed, placed on ice and cleansed of blood and connective tissue. The brain tissue was then dissected into forebrain and hindbrain at the point of colliculi. The tissues were prepared by homogenization and centrifugation as described in the methods section 2.4.1.

7.2.5 **Radioligand binding assays.**

Radioligand binding assays were performed on tissue homogenates using [³H]yohimbine (1.25 - 37.5 nM) and
[3H]idazoxan (0.5 – 20.0 nM). Incubations were carried out as described in section 2.4.2. The radioactivity was counted using a Packard Tri-Cab Model 3255 liquid scintillation counter at an efficiency of 36%.

7.2.6 Data analysis.

The binding parameters were obtained from modified Scatchard plot analysis as described in section 2.4.3. Data generated from analysis of Scatchard plots (Bmax and KD) were subjected to unpaired t-tests. To compare responses to alpha-methylnoradrenaline in treated and control animals, pressor dose-response curves were constructed in individual animals and the dose required to raise mean arterial pressure by 50 mmHg calculated. The differences between the groups was tested using unpaired t-test. The depressor response after clonidine injection was analysed by analysis of covariance using the package Rummage (Bryce, 1980) run on an ICL 2988 mainframe computer. The preinjection blood pressure was used as covariant.

7.3 RESULTS.

There were no significant changes in blood pressure or heart rate throughout the period of drug infusion. The blood pressure was 90 ± 9 for control and 80 ± 10 mmHg for guanabenz treated animals. No obvious behavioural changes were observed.
7.3.1 Effects of bolus doses of alpha-methylnoradrenaline.

The effect of treatment with guanabenz on pressor responses induced by bolus doses of alpha-methylnoradrenaline was examined by comparing the dose required to increase the mean arterial blood pressure by 50 mmHg. The dose required to increase the blood pressure by 50 mmHg was shown to be significantly (p < 0.01) increased to $63 \pm 10$ ug for chronically treated animals from $20 \pm 5$ ug in control animals when responses in individual animals were compared. The treatment with guanabenz shifted the dose-response curve rightwards with a dose ratio of 3 (figure 7.1) when pooled data from guanabenz treated and control rabbits were compared. Acute infusions of guanabenz (5 hours) had no effect on pressor responses to alpha-methylnoradrenaline bolus doses.

7.3.2 Effects of intracisternal clonidine injection.

The response to intracisternal clonidine (1 ug/kg) injection was attenuated by 5 days of guanabenz infusion. The maximum fall in blood pressure was observed at 5 minutes after injection for the guanabenz treated group ($-14 \pm 10$ mmHg) and at 15 minutes ($-26 \pm 15$ mmHg) for the control group. The fall in blood pressure was significantly (p < 0.05) attenuated at 5, 15, 30, and 45 minutes after
injection in the guanabenz treated group (figure 7.2). There was no significant treatment effect on the heart rate.

7.3.3 \[^{3}H\]Yohimbine and \[^{3}H\]Idazoxan binding sites.

Five days guanabenz infusion significantly reduced the number of \[^{3}H\]yohimbine binding sites (Bmax) in both the forebrain and hindbrain by 47% and 45%, respectively (p < 0.003 and p < 0.02). Yohimbine binding sites in the kidney were not significantly affected by this treatment (figure 7.3). The dissociation constant (K_D) was not affected by five days guanabenz treatment in any tissue studied (Table 7.1). In contrast, 5 days of guanabenz treatment significantly (p < 0.002) decreased \[^{3}H\]idazoxan binding sites in the kidney by 41%, but no significant treatment effect on Bmax was observed in the forebrain or hindbrain (figure 7.4). However, the K_D of \[^{3}H\]idazoxan for the binding site was significantly (p < 0.03) increased (K_D: 3 ± 2 and 6 ± 3 nM) in the hindbrain as a result of this treatment. No change in affinity constants were observed in either the forebrain or the kidney (table 7.1).
Figure 7.1
Effects of chronic guanabenz (237 ug/kg/hr) infusion on pressor responses to bolus doses of alpha-methylnoradrenaline.

control 

guanabenz 

n = 6 - 9.
Figure 7.2

Effects of chronic guanabenz (237 ug/kg/hr) infusion on the depressor response to clonidine (1 ug/kg) injection (i.c).

- Control
- Guanabenz

n = 6 - 9, * p < 0.05.
Figure 7.3

Effects of chronic guanabenz (237 ug/kg/hr) infusion on the number of [^3H]yohimbine binding sites.

- Control
- Guanabenz

n = 6 - 9, * p < 0.05.
Figure 7.4
Effects of chronic guanabenz (237 μg/kg/hr) infusion on the number of [³H]idazoxan binding sites.

□ Control
■ Guanabenz

n = 6 - 9, * p < 0.01.
TABLE 7.1

Effects of chronic guanabenz infusion on dissociation constants ($K_D$ nM).

<table>
<thead>
<tr>
<th></th>
<th>[³H]Yohimbine</th>
<th>[³H]Idazoxan</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>forebrain</td>
<td>hindbrain</td>
</tr>
<tr>
<td>Control</td>
<td>$8 \pm 2$</td>
<td>$11 \pm 6$</td>
</tr>
<tr>
<td>Guanabenz</td>
<td>$10 \pm 4$</td>
<td>$13 \pm 5$</td>
</tr>
</tbody>
</table>

Results: mean $\pm$ S.D, $n = 6 - 9$, * $p < 0.05$. 
Many excitable cells exhibit homeostatic mechanisms that change their responsiveness to stimulation when neural inputs are altered. Prolonged exposure to drugs or hormones may result in an attenuation of the physiological response (Trendeleburg, 1963; Harden, 1983; Hayes et al., 1984). Although this has been most extensively studied for beta-adrenoceptors (Lefkowitz et al., 1983; Toews and Perkins, 1984; Strasser et al., 1985), it has also been examined for alpha-adrenoceptors (Majewski and Story, 1976; Brodde et al., 1982; Maura et al., 1985; Jones et al., 1986). Prolonged exposure of receptors to agonists may lead to loss of receptor number in addition to desensitization. This has also been described in many tissues (Catt et al., 1979; Davies and Lefkowitz, 1981; Finberg, 1987) and cell preparations (Remold-O'Donnell, 1974; Sabol and Nirenberg, 1979; Brodde et al., 1982; Hamilton et al., 1987) for alpha-adrenoceptors.

Clonidine and guanabenz have been reported to act by activating central and peripheral alpha2-adrenoceptors which leads to reductions in catecholamine turnover and hence reduced sympathetic outflow (Haeusler, 1974; van Zwieten, 1975; Kobinger, 1978; Meachen et al., 1980; Misu and Kubo, 1982). In addition, prolonged activation of this receptor may result in desensitization (Brodde et al., 1982; Engberg et al., 1982; Maura et al., 1985; Finberg and Kopin, 1987). It has been postulated that the withdrawal syndrome observed after cessation of clonidine and guanabenz treatment could
be related to desensitization and down-regulation of central alpha$_2$-adrenoceptors (Paker and Atkinson, 1982; Conway and Jarrott, 1982; Finberg and Kopin, 1987). However, down-regulation on chronic clonidine treatment has only been convincingly shown in hypertensive rats (Armstrong et al., 1983).

In these experiments, chronic infusion with guanabenz attenuated the pressor response to bolus doses of alpha-methylnoradrenaline. The observed desensitization of the pressor response to the alpha$_2$-adrenoceptor selective agonist alpha-methylnoradrenaline after chronic guanabenz is consistent with the peripheral alpha$_2$-adrenoceptor agonist properties of this drug (Gutkin et al., 1986) and with the observations of reduced sensitivity of alpha$_2$-adrenoceptors after chronic treatment with other alpha$_2$-adrenoceptor agonists (Takeyaku et al., 1982; Ishii and Koto, 1984; Deighton et al., 1986; Jones et al., 1986; Hamilton et al., 1987). The depressor response induced by intracisternal injection of clonidine was also significantly attenuated by chronic guanabenz infusion. Consistent with this finding is the observed desensitization of central alpha$_2$-adrenoceptor mediated responses after chronic treatment with other alpha$_2$-agonists (Maura et al., 1985; Raiteria et al., 1986; Finberg and Kopin, 1987).

Decreases in [$^3$H]yohimbine binding sites in the hindbrain were observed and are consistent with the attenuation of the depressor response induced by
intracisternal clonidine injection. Decreases in receptor number were also observed in the forebrain but with no change in the kidney. The lack of change in $[^3\text{H}]$yohimbine binding to the kidney may possibly be due to differences in guanabenz concentration at this site and in the brain. In addition, subtypes of $\alpha_2$-adrenoceptor predominating in different tissues may be a contributory factor to the localized changes in receptor density observed in this study. Localized changes in receptor number have also been reported after chronic treatment with other drugs (Minneman 1983; Winter et al., 1986).

A different pattern of down regulation for $[^3\text{H}]$idazoxan binding sites with no change in brain but down regulation in the kidney was observed after chronic guanabenz infusion. Differential regulation of idazoxan and yohimbine binding sites supports the hypothesis that the two ligands bind to distincts sites. Guanabenz appears to have an affinity for both binding sites (yohimine and idazoxan) and to regulate them independently.

In conclusion, chronic guanabenz infusion significantly attenuated both peripheral and central $\alpha_2$-adrenoceptor mediated functions. The density of $[^3\text{H}]$yohimbine and $[^3\text{H}]$idazoxan binding sites in brain and kidney membranes were differentially regulated which further shows that both ligands bind to different entities in the brain and kidney membranes. In addition, the changes in $[^3\text{H}]$yohimbine observed in the brain could help explain the mechanism behind withdrawal reactions reported after sudden
withdrawal of centrally acting drugs.
CHAPTER EIGHT

GENERAL SUMMARY, DISCUSSIONS AND CONCLUSION.
CHAPTER EIGHT

GENERAL SUMMARY, DISCUSSIONS AND CONCLUSION.

This chapter is intended to bring all the results in this thesis together, discuss them and suggest directions for future studies.

Activation of peripheral blood vessel alpha$_2$-adrenoceptors has been shown to cause vasoconstriction leading to increased blood pressure while central adrenoceptor stimulation causes reduced sympathetic tone and a reduction in blood pressure. These functions have been used to study effects of adrenoceptor drugs on alpha$_2$-receptor sensitivity (Hannah et al., 1983; Hamilton and Reid, 1985; Hamilton et al., 1986; Deighton et al., 1988). Bolus doses of alpha-methylnoradrenaline and intracisternal injection of clonidine were used to study effects of acute and chronic treatments with adrenoceptor drugs on peripheral and central alpha$_2$-receptor responsiveness respectively. Chronic treatment with the antagonists idazoxan and yohimbine attenuated both pressor and depressor responses to alpha-methylnoradrenaline and intracisternal clonidine injection. Acute and chronic treatment with idazoxan caused a similar attenuation of responses. Thus these studies did not show any changes in responses during chronic drug treatment with antagonists. In contrast, differences were observed between acute and chronic treatment with agonists: chronic but not acute treatment with guanabenz attenuated both depressor and pressor responses induced by clonidine and alpha-methylnoradrenaline injections, while the
depressor response to clonidine injection was significantly attenuated by intracerebroventricular adrenaline but not noradrenaline infusion (Table 8.1). Attenuation of responses during agonist treatment is consistent with receptor desensitization and down-regulation. These observations are also consistent with previous studies in which desensitization of peripheral alpha$_2$-adrenoceptors were observed during agonist infusion (Hamilton et al., 1987; Deighton et al., 1988). The inability of noradrenaline to influence the central alpha$_2$-adrenoceptors mediated function may have been related to the low levels of noradrenaline achieved in hindbrain during infusion. It may also be related to noradrenaline's selectivity and potency at the alpha$_2$-adrenoceptor. Adrenaline has been shown to be a more potent agonist at alpha$_2$-adrenoceptors than noradrenaline. In addition, the subtype of alpha$_2$-receptor predominating in the region of the brain examined may account for this discrepancy (Petrash and Bylund 1986; Bylund 1988; Reid et al., 1988). In the periphery, different effects of adrenaline and noradrenaline on platelet and kidney alpha$_2$-adrenoceptors have been reported (Deighton et al., 1988a,b) which are consistent with the differences in subtypes of alpha$_2$-receptors identified in these tissues. Table 8.1 presents a summary of functional changes observed in these studies during chronic drug treatment.
### TABLE 8.1

**SUMMARY OF FUNCTIONAL CHANGES AS A RESULT OF CHRONIC DRUG TREATMENTS.**

<table>
<thead>
<tr>
<th></th>
<th>Alpha-MeNA injection (iv) (pressor effect)</th>
<th>Clonidine injection (ic) (depressor effect)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Treatments:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Antagonists:</strong></td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>Idazoxan:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>acute</td>
<td>attenuated</td>
<td></td>
</tr>
<tr>
<td>0.56 &amp; 1.1 mg/kg/hr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chronic</td>
<td>attenuated</td>
<td>attenuated</td>
</tr>
<tr>
<td>1.1 mg/kg/hr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yohimbine</td>
<td>attenuated</td>
<td>attenuated</td>
</tr>
<tr>
<td>15 mg/kg/day</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Agonists:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adrenaline</td>
<td></td>
<td>attenuated</td>
</tr>
<tr>
<td>100 ug/kg/hr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Noradrenaline</td>
<td></td>
<td>No significant effect</td>
</tr>
<tr>
<td>150 ug/kg/hr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Guanabenz</td>
<td>attenuated</td>
<td>attenuated</td>
</tr>
<tr>
<td>237 ug/kg/hr</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
In radioligand binding studies $[^3\text{H}]$yohimbine has been shown to label alpha$_2$-adrenoceptors with high affinity (Motulsky et al., 1980; Bylund, 1981; 1985). Chronic treatment with yohimbine and idazoxan significantly increased $[^3\text{H}]$yohimbine binding sites in the forebrain but not in the hindbrain; an increase in $[^3\text{H}]$yohimbine binding in the kidney was also observed with yohimbine but not with idazoxan treatment. Agonist treatment with guanabenz significantly reduced $[^3\text{H}]$yohimbine binding to both forebrain and hindbrain while amitriptyline which may cause increases in catecholamine concentrations in the brain significantly reduced the number of binding sites in the hindbrain. These changes in binding sites are consistent with changes occurring at receptor sites after chronic agonist or antagonist treatment, with antagonists causing up-regulation and agonists down-regulation. The changes in receptor number observed in these studies in the hindbrain are consistent with the changes observed in response to intracisternally administered clonidine. Amitriptyline which down-regulated $[^3\text{H}]$yohimbine binding has previously been shown to cause attenuation of the depressor response to clonidine (Hamilton et al., 1986). Similarly guanabenz caused down-regulation in both $[^3\text{H}]$yohimbine binding in the hindbrain and in the response to intracisternally administered clonidine injection. In contrast, treatment with the antagonists idazoxan and yohimbine caused an up-regulation of receptor number in forebrain but no changes in the hindbrain and no
evidence for altered depressor responses to clonidine were observed during chronic treatment with these drugs. Chronic adrenaline and noradrenaline had no significant treatment effects on \([^3H]yohimbine\) binding sites, although adrenaline infusion attenuated responses to intracisternal clonidine injection. However, desensitization of functional responses frequently precedes down-regulation in receptor number and it has been shown that treatment with low doses of amitriptyline may cause attenuation of responses to clonidine without any concomitant decrease in \([^3H]yohimbine\) binding.

Differences in regulation in different brain regions have been observed. This may be due to differences in drug concentrations in different brain areas. Adrenaline and noradrenaline were infused into the brain and much higher concentrations of catecholamine were observed in the forebrain than in the hindbrain; amitriptyline is assumed to cause increases in biogenic amines including adrenaline and noradrenaline concentrations in the brain synapses via uptake blockade and may cause higher concentrations of these amines in the hindbrain. In addition, the density of innervation of different brain regions may account for these localized changes. Furthermore, drug concentrations could be selectively elevated in the regions with high receptor density or innervation. Finally, subtypes of \(\alpha_2\)-adrenoceptor predominating in different brain regions (Petrash and Bylund, 1986; Bylund, 1988) could contribute to the differential regulation of receptors in different areas and tissues. Table 8.2 presents summary of receptor.
regulation observed with adrenoceptor drug treatments in these studies.

In contrast, to alpha$_2$-adrenoceptor binding, beta-adrenoceptor binding sites in the brain as identified by [$^3$H]dihydroalprenolol were not significantly affected by the drug treatments examined in this thesis (Table 8.2). However changes in the periphery have been observed (Tsujimoto and Hoffman, 1985; Deighton et al., 1988; Elfellah et al., 1988). The lack of change in beta-adrenoceptor number in rabbit brain contrasts with changes observed in rat brain after chronic treatment (Bergstrom and Kellar, 1979; Minneman et al., 1982; Sugrue, 1983; Toews and Perkins, 1984; Beer et al., 1986). It is however possible that subtype selective changes might have occurred. Dihydroalprenolol is a non-selective drug and small changes in beta$_1$- or beta$_2$- subtypes might not have been detected. Alternatively, beta-adrenoceptors in rabbit brain may be more resistant to agonist regulation.

During preliminary studies, it was observed that [$^3$H]idazoxan and [$^3$H]yohimbine which have been defined as typical alpha$_2$-adrenoceptor ligands (Cheung et al., 1982; Motulsky et al., 1980; Convents et al, 1987; Ruffolo et al., 1988) did not bind to rabbit brain or kidney membranes with similar characteristics. This observation led to the displacement studies described in chapter three using various adrenoceptor drugs to displace these ligands from their binding sites. It was observed in these experiments
<table>
<thead>
<tr>
<th></th>
<th>[³H]Yohimbine</th>
<th>[³H]Idazoxan</th>
<th>[³H]DHA</th>
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<tbody>
<tr>
<td></td>
<td>FB</td>
<td>HB</td>
<td>KID</td>
</tr>
<tr>
<td>Idaz</td>
<td>UP</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>Yoh</td>
<td>UP</td>
<td>NC</td>
<td>UP</td>
</tr>
<tr>
<td>Amit</td>
<td>NC</td>
<td>DOWN</td>
<td>_</td>
</tr>
<tr>
<td>Amit + Yoh</td>
<td>NC</td>
<td>NC</td>
<td>_</td>
</tr>
<tr>
<td>Amit + Idaz</td>
<td>NC</td>
<td>NC</td>
<td>_</td>
</tr>
<tr>
<td>Adr</td>
<td>NC</td>
<td>NC</td>
<td>_</td>
</tr>
<tr>
<td>Norad</td>
<td>NC</td>
<td>NC</td>
<td>_</td>
</tr>
<tr>
<td>Gbz</td>
<td>DOWN</td>
<td>DOWN</td>
<td>NC</td>
</tr>
</tbody>
</table>

FB = forebrain, HB = hindbrain, KID = kidney, Amit = amitriptyline, Yoh = yohimbine, Idaz = idazoxan, Adr = adrenaline, Norad = noradrenaline, Gbz = guanabenz, DOWN = reduction in receptor number, UP = increase in receptor number, NC = No change, ? = Reduction in the right cerebrum, - = Not studied.
that \[^{3}\text{H}]\text{yohimbine}\] bound principally to \(\alpha_2\)-adrenoceptors and was displaced from this site with high affinity by \(\alpha_2\)-adrenoceptor drugs, while \[^{3}\text{H}]\text{idazoxan}\] was not displaced by typical adrenergic drugs such as adrenaline and noradrenaline but was displaced by drugs with imidazoline or related structures with high affinity. These observations have been confirmed by others (Boyajian et al., 1987; Lachaud et al., 1987; Bricca et al., 1988; Wikberg 1988; Yablonsky et al., 1988; Vigne et al., 1989).

In addition, these ligands labelled different populations of binding sites in both the brain and the kidney, with more \[^{3}\text{H}]\text{yohimbine}\] binding in the brain and more \[^{3}\text{H}]\text{idazoxan}\] binding in the kidney. These observations were followed up by looking at the effects of chronic drug treatments on the \[^{3}\text{H}]\text{idazoxan}\] binding site and comparing with effects on the \[^{3}\text{H}]\text{yohimbine}\] binding site. In the \textit{in vivo} studies in which animals were treated chronically with \(\alpha_2\)-adrenoceptor drugs, the idazoxan binding site was regulated independently of the yohimbine binding site. The idazoxan binding sites in the brain were not significantly affected by any of the chronic drug treatments except adrenaline which significantly reduced binding to the right cerebrum. However, in the kidney, chronic guanabenz infusion significantly reduced the number of idazoxan binding sites, while chronic treatment with the \(\alpha_2\)-adrenoceptor antagonists yohimbine and idazoxan also significantly down-regulated this site. These effects on the number of idazoxan
binding sites did not conform with the antagonist properties of these drugs. The changes observed with yohimbine were surprising since yohimbine only displaced $[^3H]$idazoxan in the in vitro displacement experiments with low affinity (in micromolar range). Thus this effect may be an indirect effect, and a decrease in the $K_D$ was observed along with the reduction in number. It is possible that yohimbine and idazoxan might be acting as agonists on this binding site. Alternatively the reduction in binding sites may be related to the Na$^+/K^+$ antiporters, since the Na$^+/K^+$ blocker amiloride has been reported to displace $[^3H]$idazoxan from its binding sites in rabbit adipocyte membranes with high affinity (Langin and Lafontan, 1989). However, this might not be the case since amiloride has also been reported to be a promiscuous drug that binds with micromolar affinities to many other receptors including adrenergic receptors (Howard et al., 1987).

In conclusion, regulation of alpha$_2$-adrenoceptors in the central nervous system by agonists and antagonists is consistent with current concepts of agonist down-regulation and antagonist up-regulation of receptors. These changes were accompanied by functional regulation. Differences between the regulation of alpha$_2$-receptors in the brain and the periphery have been observed. In addition, differences between brain regions were also observed and this may be due to the predominance of different subtypes of receptors in different regions. The newly labelled idazoxan binding site has been shown to be distinct from the alpha$_2$-adrenoceptor binding
site. The nature and integrity of this binding site is not yet fully known, but it appears to be resistant to drug regulation in the brain. For further insights into the properties of this binding site an extensive study is required looking at the nature of the second messenger to which this site is coupled. In addition, the function of this site requires to be elucidated. It is possible that the idazoxan site may have functions which have previously been attributed to alpha_2-adrenoceptors. Such studies will further the understanding of the nature of the central nervous system and peripheral receptors and help to unravel the complex pharmacology of alpha-adrenergic receptors.
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denervation and [3H]prazosine binding sites in rat
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