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2-PHENYLETHYLAMINE: POSSIBLE ROLE IN ANTIDEPRESSANT DRUG ACTION

by

PAUL R. PAETSCH

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

IN

MEDICAL SCIENCES (PSYCHIATRY)

EDMONTON, ALBERTA

FALL, 1992

ISBN 0-315-77399-5

UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR: Paul R. Paetsch

TITLE OF THESIS: 2-Phenylethylamine: Possible role in antidepressant drug action.

DEGREE FOR WHICH THESIS WAS PRESENTED: Doctor of Philosophy

YEAR THIS DEGREE GRANTED: 1992

9935 83 Avenue, #101
Edmonton, Alberta
T6E 2B7

DATED July 14, 1992

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled 2-PHENYLETHYLAMINE: POSSIBLE ROLE IN ANTIDEPRESSANT DRUG ACTION by PAUL R. PAETSCH in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Medical Sciences (Psychiatry).

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ABSTRACT

Studies were undertaken to examine the possible role of 2-phenylethylamine (PEA) in antidepressant drug action. A gas chromatographic assay was developed for the analysis of phenylalanine, the precursor of PEA, in rat brain. Phenylalanine distribution in rat brain correlated poorly with PEA distribution. Treatments that alter accumulation of PEA in rat caudate nucleus, namely neurotoxic lesions of the substantia nigra or the drug reserpine, had no effect on phenylalanine levels in the caudate nucleus. These data indicate that changes in PEA are not simply related to changes in phenylalanine levels. Brain levels of phenylalanine and other large neutral amino acids (LNAAs) were increased by systemic administration of β_2-adrenoceptor agonists and a concurrent decrease in plasma LNAA levels was observed. Long-term antidepressant drug treatment had no effect on the β_2-adrenoceptor agonist-induced changes in LNAA availability. Brain PEA concentrations were increased 10-fold by long-term administration of a combination of exogenous PEA and (-)-deprenyl (DEP), an inhibitor of PEA catabolism. This combination also decreased the behavioral effects of β_2-adrenoceptor stimulation in a manner similar to the effect of the monoamine oxidase (MAO) inhibitor phenelzine, whereas treatment with PEA or DEP alone did not. The effects of long-term elevation of PEA by the DEP/PEA combination and of long-term administration of antidepressant drugs on catecholamine receptor binding were examined. β-Adrenoceptor density was decreased in the cortex, but not the cerebellum, of animals treated with antidepressants for 28 days. The decrease in β-adrenoceptor density was due to a selective decrease in the β_1 subtype population. Long-term treatment with MAO inhibitor antidepressant drugs reduced the density of both D_1 and D_2 dopamine-related binding sites in the striatum. The tricyclic antidepressant desipramine reduced the density of only the D_1-related binding site in this tissue. Long-term elevation of PEA had effects on these catecholamine receptors that were similar, but not identical, to long-term treatment with antidepressant drugs. These data indicate that elevation of PEA may play a role in the changes seen in brain catecholamine receptors after long-term administration of MAO inhibitors.

ACKNOWLEDGEMENTS

I would like to express my gratitude to Dr. Andrew J. Greenshaw and Dr. Glen B. Baker for their supervision, advice and encouragement during my studies at the Neurochemical Research Unit. I would also like to thank Dr. R. T. Coutts and Dr. M. Martin-Iverson for their help and advice on aspects of this work and Gail Rauw, Carolyn Kuefler and Jordyce van Muyden for their technical expertise and assistance in many aspects of this thesis.

Financial support from the Medical Research Council of Canada and the Alberta Heritage Foundation for Medical Research (stipend, research allowance and travel assistance) is greatly appreciated. Sincere thanks to the Province of Alberta Graduate Scholarship Fund, the University of Alberta Walter H. Johns Graduate Fellowship Fund (further stipend support) and to the University of Alberta Faculty of Graduate Studies and Research/Alma Mater Fund (travel assistance).

I would like to express my thanks to Sally Omura for her help with word-processing and her patience in this matter. I would like to wish my fellow graduate students, past and present, all the best in their endeavors and would like to thank them for making my experience an enjoyable one.

Finally, I am most grateful to my wife Sandra for her encouragement that helped me bring this project to completion.

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5-HIAA	5-hydroxyindole acetic acid
5-HT	5-hydroxytryptamine; serotonin
5-MeODMT	5-methoxy-N,N-dimethyltryptamine
6-OHDA	6-hydroxydopamine
8-OH-DPAT	8-hydroxy-2-(di-n-propylamino)tetraline
AAAD	aromatic *L*-amino acid decarboxylase
amu	atomic mass unit
ANOVA	analysis of variance
ATP	adenosine triphosphate
B_{max}	maximum density of binding sites (in fmol mg^{-1} protein)
cAMP	cyclic adenosine monophosphate
CNS	central nervous system
COMT	catechol-*O*-methyltransferase
CSF	cerebrospinal fluid
d	day
DA	dopamine
DEHPA	di(2-ethylhexyl)phosphoric acid
DEP	(-)-deprenyl
DHA	dihydroalprenolol
DMI	desmethylimipramine; desipramine
DOPAC	3,4-dihydroxyphenylacetic acid
ECD	electron capture detection(or)
ECS	electroconvulsice shocks
ECT	electroconvulsive shock therapy
eV	electron volt
fmol	femtomole (10^{-15})
g	gram
G protein	guanine nucleotide binding regulatory protein
GABA	γ-aminobutyric acid
GC	gas chromatography
h	hour
HP	Hewlett Packard
HPLC	high-pressure liquid chromatography
HVA	homovanillic acid
i.d.	internal diameter

i.p.	intraperitoneally
IMI	imipramine
K_d	dissociation constant (in nM)
kg	kilogram
K_i	inhibition constant (in nM)
KPa	kilopascals
l	litre
L-DOPA	3,4-dihydroxy-*L*-phenylalanine
LNAA	large neutral amino acid
M	molar
MAO	monoamine oxidase
mg	milligram
MHPG	3-methoxy-4-hydroxyphenylglycol
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	messenger ribonucleic acid
ms	millisecond
MS	mass spectrometry
NA	noradrenaline
ng	nanogram
nM	nanomolar
PAA	phenylacetic acid
PEA	2-phenylethylamine
PET	positron emission tomography
PLZ	phenelzine
s	second
s.c.	subcutaneous
TCA	tricyclic antidepressant
TCP	tranylcypromine
Tris	Tris buffer
VEH	vehicle
VMA	vanillylmandelic acid
µl	microliter
µM	micromolar
°C	degree Celsius

A. LITERATURE REVIEW

A.1 INTRODUCTION

The idea that depression may be due to an underlying biochemical imbalance can be traced back as far as the 5th Century B.C. Hippocrates suggested that mood depends upon the balance of four substances, namely blood, phlegm, yellow bile and black bile and that "melancholia" (i.e. depression) was a result of an excess of black bile (Sachar, 1985). Now, centuries later, scientists no longer believe that "black bile" is responsible for depression and several psychological theories of depression have been proposed but many are still looking for an underlying biochemical cause of the disease (Davison and Neale, 1986). The more recent serendipitous discovery that the drugs iproniazid and imipramine (IMI) are useful antidepressant agents (Selikoff *et al.*, 1952; Kuhn, 1957) and that these agents increase availability of brain monoamines (Brodie *et al.*, 1956; Glowinski and Axelrod, 1964) lead to the formation of the biogenic amine hypothesis of depression (Schildkraut, 1965; Coppen, 1967). Iproniazid increases brain levels of the monoamines noradrenaline (NA) and serotonin [5-hydroxytryptamine, 5-HT] (Brodie *et al.*, 1956) *via* the inhibition of monoamine oxidase [MAO] (Zeller *et al.*, 1952), an enzyme that participates in the catabolism of monoamines. IMI increases the synaptic availability of NA and 5-HT by the inhibition of their uptake into the nerve terminal (Axelrod *et al.*, 1961; Glowinski and Axelrod, 1964). In the simplest form, the monoamine hypothesis of depression states that a deficiency in the monoamine(s) NA and/or 5-HT may account for depressive illness.

The biogenic amine hypothesis of depressive illness was further substantiated by several findings. Reserpine, a drug that depletes monoamines (Pletscher *et al.*, 1956), can cause depressive symptoms in humans (Klein, 1968). Precursors

of NA (Goldberg, 1980; Gelenberg *et al.*, 1982; van Praag, 1983) or 5-HT (Wirz-Justice, 1977; Murphy *et al.*, 1978; van Praag 1984; Young, 1984) may be able to ameliorate depression, especially when combined with a MAO inhibitor (Coppen *et al.*, 1963; Glassman and Platman, 1969; Ayuso-Gutierrez and Lopez-Ibor, 1971; Pare, 1973; Birkmayer *et al.*, 1984; Young, 1991), although this continues to be an area of controversy. Amphetamine, which releases catecholamines and also blocks their uptake (Carlsson 1970; Scheel-Kruger, 1972), transiently elevates mood in people (Satel and Nelson, 1989). Some studies have also indicated that concentrations of metabolites of NA and/or 5-HT may be reduced in the urine, cerebrospinal fluid (CSF) or postmortem brain tissue in some groups of depressive patients, although this literature is full of discrepancies (for review: Davis, 1989).

The validity of the biogenic amine hypothesis in its original form has been questioned by many researchers. Problems with this hypothesis have been extensively documented (Dewhurst, 1969; Sulser, 1982; Johnstone, 1982; Sugrue, 1983; Maj *et al.*, 1984a). The two principal problems are: (a) biochemical changes occur within hours after antidepressant drug administration whereas the time-course for the clinically beneficial antidepressant effects is days to weeks (Oswald *et al.*, 1972; Quitkin *et al.*, 1986; Post *et al.*, 1987); and (b) newer antidepressants, often termed "novel" or "second generation" antidepressants (e.g. mianserin, trazodone and iprindole) include some drugs that are neither MAO inhibitors nor, in some cases, potent amine uptake blockers (Freeman and Sulser, 1972; Rosloff and Davis, 1974; Sanghvi and Gershon, 1975; Coppen *et al.*, 1976; Sulser *et al.*, 1978).

Twenty years ago, Ashcroft and coworkers proposed a modified amine hypothesis of depression (Ashcroft *et al.*, 1972). These workers suggested that interactions between various neuronal systems and also alterations of postsynaptic

receptor sensitivity should be considered in affective disorders. Indeed changes in the function and/or density of receptors for NA and/or 5-HT are observed after long-term treatment with the uptake-blocking tricyclic antidepressants (TCAs), the MAO inhibitor drugs and newer antidepressant drugs (for review: Maj *et al.*, 1984a; Blier and deMontigny, 1985a; Sulser, 1986; Baker and Greenshaw, 1989). Results from these preclinical studies have suggested that antidepressants exert their effects on neurotransmitter receptors with a time-course comparable to their antidepressant action in the clinic (Enna *et al.*, 1981; Coryell *et al.*, 1982; Sugrue, 1983; Heninger and Charney, 1987). The time-course of clinical effects, however, is problematic as there appears to be an heterogenous distribution of the response of individuals to antidepressant drugs. Katz *et al.* (1987) found that changes in affect, cognitive function and behavior occur during the first week of antidepressant treatment in some patients. These patients showed further improvement after two and a half weeks of antidepressant drug treatment. Other researchers have shown that at least six weeks or longer of antidepressant treatment is needed for some patients to show clinical improvement (Quitkin *et al.*, 1984; 1986; Georgotas *et al.*, 1989). Taken together these data indicate that for maximal antidepressant effects to occur in the greatest number of patients long-term treatment is needed.

The majority of work on the role of amine systems in affective disorder has focused on NA and/or 5-HT. There has also been some work suggesting a role for dopamine (DA) (Randrup *et al.*, 1975; Randrup and Braestrup, 1977; Willner 1983a,b,c). The brain also contains several other amines including the octopamines, tyramines, tryptamine and 2-phenylethylamine (PEA) which have received much less attention. These amines are present in small quantities in mammalian brain (<5 ng g^{-1} whole brain) and thus are often referred to as "trace" amines. These compounds are synthesized and metabolized very rapidly and are

suggested to be physiologically important (Dewhurst, 1968; Boulton, 1976; Boulton and Juorio, 1982). It has been proposed that these trace amines are neurotransmitters and/or neuromodulators in their own right, and recent neurochemical and electrophysiological data support this proposal (Boulton, 1979; Boulton and Juorio, 1982; Jones, 1983; van Nguyen and Juorio, 1989; Paterson *et al.*, 1990). The structural similarity between PEA and amphetamine has lead some researchers to propose that PEA may be involved in the etiology of depression (Dewhurst and Marley, 1965; Dewhurst 1968; Fischer *et al.*, 1968; Sabelli and Mosnaim, 1974). Studies have, however, indicated that PEA excretion in urine and PEA concentrations in plasma generally do not differ between depressive patients and controls (for review, Davis, 1989). Metabolites of PEA may be decreased in some groups of depressed patients, although there is considerable controversy in this area (Davis, 1989). The amino acid precursor of PEA, L-phenylalanine, has been reported to have mood-stimulating effects and may be effective in the alleviation of depression when combined with a MAO inhibitor (Fischer *et al.*, 1975; Beckman *et al.*, 1977; Birkmayer *et al.*, 1984). The administration of MAO inhibitors results in large increases in brain concentrations of PEA, the increases being much greater than the MAO inhibitor-induced increases in levels of catecholamines or 5-HT (Boulton *et al.*, 1973; Boulton, 1976; Philips and Boulton, 1979; McKim *et al.*, 1980; Philips *et al.*, 1980; Baker *et al.*, 1988; Hampson *et al.*, 1988). These studies suggest that increasing PEA availability may be linked to the alleviation of depression.

A.2 ANTIDEPRESSANT DRUGS

Prior to the 1950's, the pharmacotherapy of depression consisted of the use of stimulants such as amphetamine for psychomotor retardation and barbiturates for agitation (Baldessarini, 1985). Various physical treatments were also used for

more severe depression; these treatments included various "shock" treatments, namely hypoglycemia induced by large doses of insulin or convulsive treatments (Baldessarini, 1985). Convulsions were induced either through the inhalation of gases (e.g. camphor, flurothyl or Indoklon) or through electrical currents applied directly to the head. Of these treatments only the use of electrical currents, which is called electroconvulsive shock therapy (ECT), is still used for the treatment of depression. ECT is an effective tool in the treatment of depression (Brandon *et al.,* 1984), but now is usually used only after pharmacotherapy has failed. ECT produces changes in brain neurotransmitter receptors that are very similar to those produced with antidepressant drugs (Lerer, 1987; Baker and Greenshaw, 1989). This thesis will focus, however, on antidepressant drug treatment which is now the most common therapy for affective disorders.

A.2.1 MAO Inhibitor antidepressant drugs

The discovery that inhibitors of the enzyme MAO were effective antidepressants was serendipitous. It was noticed that tuberculosis patients treated with the drug iproniazid demonstrated hyperactive behavior and euphoria (Selikoff *et al.,* 1952). Iproniazid was subsequently found to be an inhibitor of MAO (Zeller *et al.,* 1952). MAO is an ubiquitous metabolic enzyme that carries out oxidative deamination of a variety of amines (Fowler and Ross, 1984). There are two forms of MAO defined by the preference of the enzyme for substrates and by the selectivity of certain inhibitor drugs (Johnson, 1968). 5-HT is the preferential substrate for MAO-A and this enzyme is selectively inhibited by the MAO inhibitor clorgyline (Johnson, 1968). PEA is the preferential substrate for MAO-B, and this enzyme is selectively inhibited by (-)-deprenyl [DEP] (Finberg and Youdim, 1983).

The most frequently prescribed MAO inhibitors for depression are phenelzine (PLZ) and tranylcypromine (TCP), nonselective irreversible inhibitors of

MAO (Murphy *et al.*, 1985; Tipton and Fowler, 1984). DEP is frequently prescribed for Parkinson's disease (Tetrud and Langston, 1989) and, along with PLZ and TCP, is used in the studies described in this thesis (Figure 1). These drugs are administered orally and are rapidly absorbed and metabolized (Lader, 1980). Possible metabolic routes for these drugs have been reviewed recently, and several of the metabolic products are active (Baker and Coutts, 1989).

The most common potentially serious side-effect of MAO inhibitors is orthostatic hypotension (Murphy *et al.*, 1985; Keck *et al.*, 1991). Other problems include some occurrence of anticholinergic effects including dry mouth, constipation, sexual dysfunction and hepatotoxicity (Baldessarini, 1985). PLZ and TCP, however, appear to be less hepatotoxic than other MAO inhibitors (Zisook, 1985). The most serious toxic effect of these drugs is their ability to provoke acute hypertensive crisis that can lead to headaches and stroke (Sjoqvist, 1965; Baldessarini, 1985). This effect is thought to be due to interactions with sympathomimetic amines including adrenaline, ephedrine, amphetamine and tyramine. Classically this reaction has been termed the "cheese reaction" as it is often associated with tyramine which is a natural by-product of bacterial fermentation and is found in great quantities in foods such as ripe cheeses, red wines, brewer's yeast, caviar and pickled herring (Baldessarini, 1985). The proposed mechanism of this "cheese reaction" is that tyramine, which usually is catabolized by MAO, acts to displace NA, which also is usually catabolized by MAO. The greatly exaggerated release of NA is thought to over-stimulate α-adrenergic receptors and cause a dramatic elevation in blood pressure (Marley and Blackwell, 1970; Sandler, 1981). This severe reaction can be prevented by giving patients a list of foodstuffs to avoid. When MAO inhibitors are combined with TCAs there is a potentially lethal interaction which may also be due to an overabundance of amines. MAO-inhibitors also interact with hepatic enzymes that

are involved in the metabolism of many drugs and, therefore, may potentiate the effects of barbituates, benzodiazepines, phenothiazines, antihistamines, opioid analgesics and alcohol (Baldessarini, 1985).

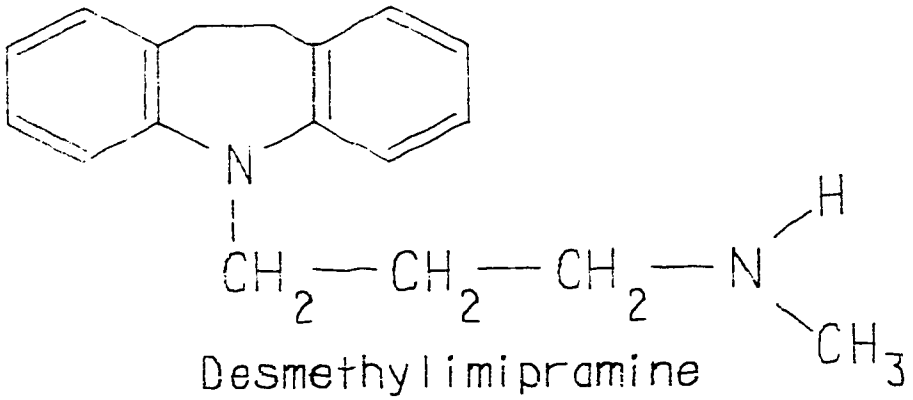
The MAO inhibitors increase levels of catecholamines and of 5-HT *via* inhibition of the main catabolic enzyme for these amines. It is interesting to note that the most widely prescribed MAO-inhibitors, PLZ and TCP, have a plethora of other effects. These drugs increase PEA and tryptamine in brain to a relatively greater extent than they increase catecholamines and 5-HT (Baker *et al.*, 1988). Both drugs also stimulate release and block uptake of monoamines (Burgen and Iversen, 1965; Hendley and Snyder, 1968; Baker *et al.*, 1978, 1980; Dyck, 1985) and inhibit a number of other enzymes in addition to MAO (Baker *et al.*, 1991; McManus *et al.*, 1992; Yu and Boulton, 1992). Clorgyline, a widely prescribed MAO-A inhibitor, also displays high affinity for opiate sigma receptors (Itzhak and Kassim, 1990).

A.2.2 Tricyclic antidepressant drugs (TCAs)

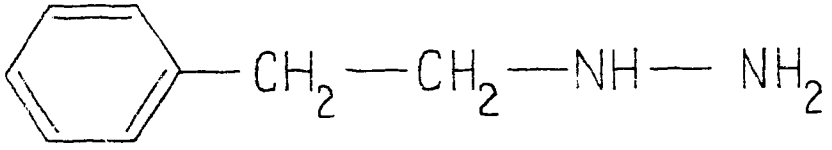
The discovery that drugs with a tricyclic structure (i.e. IMI) produced mood-altering effects and were clinically useful as antidepressant drugs was also serendipitous. Chlorpromazine, an antipsychotic agent, had first been developed as an antihistaminic sedative drug. It was given to a group of agitated psychotic patients and unexpectedly abolished some of their symptoms (for review: Caldwell, 1970). In the search for other possible antipsychotic compounds IMI, with a similar structure to that of chlorpromazine, was synthesized. IMI was tested as an antipsychotic but instead demonstrated mood-elevating and behavior-activating effects which resembled antidepressant actions (Kuhn, 1957 as cited in Caldwell, 1970).

TCAs are so named because of their characteristic structure of two benzene rings joined through a central seven-member ring (Figure 1). Other TCAs are similar to IMI in structure but have variations in either the side chain or central ring structure. The TCAs are well absorbed after oral administration and once absorbed are widely distributed. They are lipophilic and are strongly bound to tissue and plasma proteins (Baldessarini, 1985). Metabolism of these drugs is extensive, resulting in both inactive and active products. TCAs are initially dealkylated and/or oxidized by liver enzymes and then conjugated with glucuronic acid and excreted (Rudorfer and Potter, 1985). The major route of metabolism of tertiary amines such as IMI is by initial N-demethylation of the side chain. This demethylation leads to the formation of the secondary amine product (for example desmethylimipramine [desipramine, DMI] from IMI). Several of these secondary amines are also used clinically as antidepressants (Mindham, 1979).

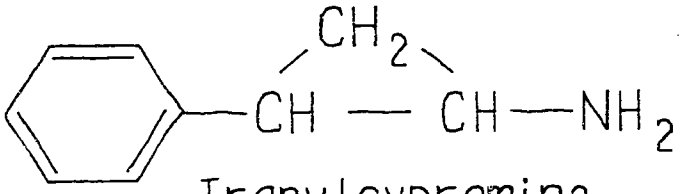
Side-effects of TCAs include anticholinergic effects such as blurred vision, dry mouth, constipation and excessive sweating. Other side-effects such as tachycardia, arrhythmias, orthostatic hypotension and sedative effects are thought to be due to the antagonism of α-adrenoceptors by these drugs (Abramowicz, 1980). Tolerance to some side-effects develops and occasionally a withdrawal syndrome, consisting of malaise, chills and muscle aches, occurs after abrupt discontinuation of treatment (Baldessarini, 1985). Interactions with other drugs may be problematic as many other drugs are competitors for the same liver enzymes; conversely other drugs may stimulate liver enzymes. Drugs such as phenothiazines and methylphenidate slow the degradation of TCAs by competing for catabolic enzymes (Dubovsky, 1987). Barbituates, alcohol and anticonvulsants stimulate the degradation of TCAs by inducing catabolic enzymes (Rudorfer and Potter, 1985). The anticholinergic effects of drugs such as TCAs, antihistamines



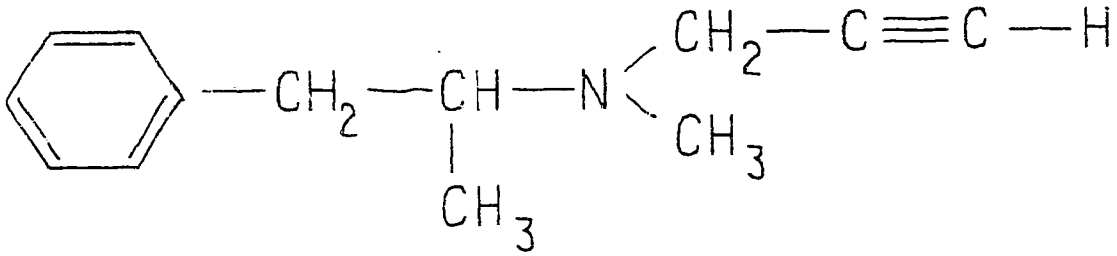
Desmethylimipramine



Phenelzine



Tranylcypromine



Deprenyl

Figure 1: Chemical structures of the TCA drug DMI and of the MAO inhibitors PLZ, TCP and DEP which are used in the studies in this thesis.

and phenothiazines are also additive (Sjoqvist, 1965). As mentioned previously, there is a potentially lethal interaction between TCAs and MAO inhibitors.

Within a short period of time after administration, TCAs block the uptake of amines back into the nerve terminal, thus increasing the synaptic availability of the amines (Glowinski and Axelrod, 1964; Iversen, 1965; Ross and Renyi, 1969; see reviews: Klein *et al.*, 1980; Richelson, 1984; Baker and Greenshaw, 1988). The TCAs that are tertiary amines, such as IMI, primarily block the uptake of 5-HT, whereas the TCAs that are secondary amines, such as DMI, primarily block the uptake of NA. These drugs usually have a much weaker effect on the uptake of DA. TCAs also block several neurotransmitter receptors, including muscarinic (Snyder and Yamamura, 1977), histaminergic (Diffley *et al.*, 1980; Green and Maayani, 1977), adrenergic (U'Prichard *et al.*, 1978) and serotonergic receptors (for reviews: Snyder and Peroutka, 1984; Richelson, 1984).

A.2.3 Novel antidepressant drugs

The high incidence of side effects seen with MAO inhibitors and TCAs and the delay of their therapeutic onset have lead to the search for novel antidepressants that lack these problems. Several antidepressants have been developed that are not MAO-inhibitors or, in some cases, are not potent blockers of uptake of amines (for review: Damlouji *et al.*, 1985; Rudorfer and Potter, 1989). Many of these drugs do appear to have a lower incidence of side effects (Cassidy and Henry, 1987; Brogden *et al.*, 1978; Conti *et al.*, 1979; McCormick *et al.*, 1985), but appear to still have the same time course of action as the older antidepressants (Rudorfer and Potter, 1989).

Fluoxetine has become one of the most widely used antidepressants in North America (Ciraulo and Shader, 1990). This drug is a 5-HT uptake inhibitor but differs from other uptake inhibitors, including the TCAs, in that both the drug

and its desmethylated metabolite are extremely potent and very selective blockers for 5-HT uptake (Benfield *et al.,* 1986). This drug appears to lack some side effects associated with TCA treatment (Schatzberg *et al.,* 1987), accounting for its great popularity. Nevertheless, fluoxetine does have some side effects including gastrointestinal system upset, and, in a small number of patients, a transient increase in nervousness, headaches and insomnia may be observed (Montgomery, 1988).

A.3 LONG-TERM ANTIDEPRESSANT TREATMENT

As stated earlier, it is widely believed that antidepressant drugs must usually be administered for 2 to 3 weeks before clinical improvement is seen. In this context the effects of long-term antidepressant treatment may be deemed as more interesting than their immediate effects. In this literature review, long-term antidepressant treatment refers to treatment administered for a minimum of 14 consecutive days. Vetulani and Sulser (1975) observed that 21-day administration of the antidepressant drugs DMI and iproniazid resulted in a reduction in the activity of NA-stimulated adenylate cyclase in rat brain tissue. This finding stimulated a large amount of work examining changes in receptor density, affinity and function following long-term antidepressant treatment. Much of this work has relied heavily on radioligand binding studies to measure the density and affinity of binding sites (review: Boulton *et al.,* 1986). Long-term treatment with antidepressant drugs induces changes in several different neurotransmitter receptor systems and their associated cellular components, but before this aspect is examined, a very brief overview of receptor-activated transduction pathways is provided.

Neurons interact with each other at synapses by the action of chemical mediators called neurotransmitters. The targets or receptors for these

neurotransmitters are proteins found on the extracellular surface of plasma membranes (Darnell *et al.*, 1986). Receptors can be located either on the same cell that released the neurotransmitter (i.e. autoreceptors) or on a different cell (i.e. heteroreceptors). Neurotransmitters may "bind" to (i.e. form a complex with) a specific protein receptor initiating a transduction pathway. This allows an external signal, carried by the neurotransmitter, to be changed into an internal signal, carried by an ion or second messenger, which regulates one or more cellular processes (Berridge, 1985). In the CNS the two most common receptor complexes are receptors coupled to ion channels and receptors coupled to guanine nucleotide binding regulatory proteins (G proteins). The majority of the receptors directly relevant to this thesis are linked through G proteins to the production of second messengers that regulate various cell processes (review: Dixon *et al.*, 1988). The most common second messenger systems are the adenylate cyclase system which is linked to the production of cyclic adenosine monophosphate (cAMP) and the phosphodiesterase system which is linked to the production of diacylglycerol, inositol triphosphate and an increase in free cytosolic Ca^{2+} ions.

A.3.1 Noradrenaline receptors

Noradrenaline receptors have been divided into subtypes based upon the pharmacological characteristics of the receptor. Initially the drug isoproterenol was used to subdivide these receptors into α-adrenoceptors and β-adrenoceptors (Lands *et al.*, 1967a,b). Receptors that responded more to the endogenous agonists, NA and adrenaline, than to isoproterenol were classified as α-adrenoceptors. Receptors that responded more to isoproterenol than to NA and adrenaline were classified as β-adrenoceptors. These receptors have subsequently been further divided into α_1-, α_2-, β_1-, and β_2-adrenoceptors based on their

function, the relative potency of NA and adrenaline, and by the selectivity of a variety of drugs (Cooper *et al.*, 1986). The linkage of NA receptors to their respective messengers is reviewed by Janowsky and Sulser (1987). In the brain α_1-adrenoceptors are coupled to the phosphodiesterase system probably *via* G proteins, and their stimulation leads to the production of diacylglycerol and inositol-1,4,5-triphosphate. In the periphery α_2-adrenoceptors are linked to inhibition of adenylate cyclase activity; this may also be the case in brain. Both β-adrenoceptor subtypes in brain are coupled *via* G proteins to adenylate cyclase and their activation leads to increased intracellular levels of cAMP.

A.3.1.1 β-Adrenoceptors

The most consistent and widely reported experimental finding with antidepressant drugs is that after long-term administration they decrease cortical β-adrenoceptor number and/or the activity of the receptor-linked adenylate cyclase. The first researchers to report this finding were Vetulani and Sulser (1975), but there have been numerous reports since demonstrating this effect for TCAs, MAO inhibitors, electroconvulsive shocks (ECS) and atypical agents (for review: Baker and Greenshaw, 1989). Initially the antidepressants mianserin, maprotiline and fluoxetine were reported not to reduce the density of β-adrenergic receptors (Mishra *et al.*, 1980; Charney *et al.*, 1981; Maggi *et al.*, 1980; Peroutka and Synder, 1980; Wong *et al.*, 1985; Barbaccia *et al.*, 1986; Baron *et al.*, 1988). This is an area of controversy, however, as more recent studies have found changes in density of β-adrenoceptors in some brain areas after chronic administration of these drugs (Asakura *et al.*, 1987; Byerley *et al.*, 1988; Eison *et al.*, 1991). Mood altering stimulants, such as amphetamine and cocaine, have also been reported to decrease the density of β-adrenoceptors (Banerjee *et al.*, 1979; Charney *et al.*, 1981) or decrease the activity of the β-adrenoceptor-linked

adenylate cyclase system (Baudry *et al.*, 1976). Other non-antidepressant drugs such as most antipsychotics and anxiolytics do not, however, alter β-adrenoceptor number in rat brain (Charney *et al.*, 1981).

The finding, with few exceptions, that long-term administration of antidepressant drugs decreases β-adrenoceptor number has lead to the trial of β-adrenoceptor agonists for the therapy of depression. Clenbuterol and salbutamol have been reported to be effective antidepressant drugs (Lecrubier *et al.*, 1980; Simon *et al.*, 1978, 1984). Earlier studies are divided on the subject of decreased numbers of cortical β-adrenoceptor following clenbuterol administration, with most reporting a reduction (Hall *et al.*, 1980; Nimgaonkar *et al.*, 1985, 1986; Finnegan *et al.*, 1987), but others finding no change (Dooley and Hauser, 1983; Frazer *et al.*, 1986; Beer *et al.*, 1987).

Electrophysiological studies have also demonstrated a decrease in the β-adrenoceptor-mediated responses following repeated treatment with antidepressant drugs including TCAs, other uptake inhibitors, MAO inhibitors, iprindole and mianserin (for review: Aghajanian, 1981; Blier and deMontigny, 1985a; Baker and Greenshaw, 1989). This effect has been demonstrated with cortical neurons (Olpe and Schellenberg, 1980, 1981) and cerebellar Purkinje cells (Siggins and Schultz, 1979), but not with hippocampal cells (de Montigny and Aghajanian, 1978; Gallager and Bunney, 1979; de Montigny *et al.*, 1981; Blier *et al.*, 1984, 1986a,b). The reason for this discrepancy between results obtained from hippocampus and those obtained from cortex and cerebellum is unknown. Nevertheless, it is clear that the response of β-adrenoceptors to NA does vary with the brain region studied (Blier and de Montigny, 1985a).

Behavioral studies have also been used to assess the effects of repeated antidepressant treatment on β-adrenoceptor function *in vivo*. This approach assesses the function of the receptor system of interest by measuring changes in

the behavioral effects of a drug that is thought to act through the receptor of interest (Baker and Greenshaw, 1989). Salbutamol, a selective β_2-adrenoceptor agonist, induces behavioural hypoactivity in rats (Przegalinski *et al.*, 1980). It has been demonstrated that long-term administration of uptake-inhibiting antidepressants, MAO inhibitors, mianserin or ECS reduces salbutamol-induced hypoactivity in rats (Przegalinski *et al.*, 1983, 1984a,b; McManus and Greenshaw, 1991b; McManus *et al.*, 1991).

In the cortex both β_1- and β_2-adrenoceptors are present (Minneman *et al.*, 1979a; Dooley *et al.*, 1986). Initial studies, largely confined to examining the effects of DMI, suggest that it is the β_1-subtype that is regulated by antidepressant treatment (Minneman *et al.*, 1979b; Dooley and Bittiger, 1987). A recent study (Heal *et al.*, 1989) indicates that other monoamine uptake inhibitors, ECS and the MAO inhibitor TCP also decrease only β_1-adrenoceptor number in rat cortex. The procedure used in the more recent studies, introduced by Dooley *et al.* (1986), uses a selective β_1-adrenoceptor antagonist to displace ^{3}H-dihydroalprenolol (^{3}H-DHA) from β_1-adrenoceptors. The number of β_2-adrenoceptors was calculated indirectly by subtracting the number of β_1-adrenoceptors from the total number of β-adrenoceptors calculated from ^{3}H-DHA binding. Although binding studies indicate that brain β_1-adrenoceptors are decreased whereas the β_2-subtype may be unaltered, the previously described behavioral studies indicate that β_2-adrenoceptors are altered. Johnson *et al.* (1989) have also recently demonstrated that both β_1- and β_2-adrenoceptors are increased in number following central denervation with 6-hydroxydopamine (6-OHDA), in contrast to their earlier findings (Minneman *et al.*, 1979b). In light of these findings, the possible involvement of β_2-adrenoceptors in antidepressant action may warrant reappraisal.

Recent studies indicate that clenbuterol, unlike other antidepressant drugs, decreases β_2- but not β_1-adrenoceptors in cortex (Frazer *et al.*, 1986; Beer *et al.*,

α_1-adrenoceptor antagonist, ^{3}H-prazosin, it has been reported that chronic antidepressant treatments either increase or do not change α_1-adrenoceptor density (Campbell and McKernan, 1982; Maj *et al.*, 1983; Vetulani *et al.*, 1983; Menkes *et al.*, 1983; Palfreyman *et al.*, 1986; Stockmeier *et al.*, 1987). There is even more disagreement in the literature concerning effects of long-term antidepressant treatment on α_2-adrenoceptors (review: Baker and Greenshaw, 1989). These discrepancies may, however, be related to the length of treatment with antidepressant drugs, with increases at 4 to 7 days, no effect at 14-21 days and decreases at 21-28 days in the density of ^{3}H-clonidine binding being reported (Johnson *et al.*, 1980; Asakura *et al.*, 1982; Cohen *et al.*, 1982; Pilc and Vetulani, 1982a,b; Reisine *et al.*, 1982; Sethy *et al.*, 1983).

Electrophysiological studies indicate that the sensitivity of α_1-adrenoceptor is enhanced after long-term antidepressant treatment (review: Maj *et al.*, 1984a). Long-term treatment with TCAs and iprindole have been reported to enhance the response to iontophoretically applied NA in areas of rat brain where NA exerts its actions *via* an α_1-adrenoceptor, including the facial motor nucleus and the ventral lateral geniculate nucleus (Menkes *et al.*, 1980; Menkes and Aghajanian, 1981; Blier and de Montigny, 1985a). Electrophysiological data indicate that α_2-adrenoceptor sensitivity is decreased following long-term treatment with some antidepressants. The decrease in firing of locus coeruleus neurons after the administration of clonidine, an effect which may be mediated by α_2-adrenoceptors (Cedarbaum and Aghjanian, 1977), is not observed in rats chronically pretreated with TCAs or zimelidine (Svensson and Usdin, 1978; Scuvée-Moreau and Svensson, 1982), but is still observed after MAO inhibitors (Blier and de Montigny, 1985b).

Behavioral experiments indicate an enhanced response to α_1-adrenoceptor agonists after long-term treatment with antidepressant drugs. For example, prazosin-induced exploratory activity is enhanced after long-term administration of

DMI (Mogilnick *et al.*, 1987) and aggressive behaviour elicited in mice by a high dose of clonidine is increased after treatment with various antidepressant drugs (Maj *et al.*, 1980, 1982). At low doses clonidine is believed to be a selective presynaptic α_2-adrenoceptor agonist, but at high doses it is believed to induce aggressive behaviour *via* postsynaptic α_1-adrenoceptors (Morpurgo, 1968; Maj *et al.*, 1980). Low doses of clonidine induce both hypothermia and locomotor hypoactivity in rats, effects which are considered to be caused by α_2-adrenoceptor stimulation (Maj *et al.*, 1984a). A reduction in the sedative and hypothermic actions of clonidine has been observed after a variety of long-term antidepressant treatments, including TCAs and maprotiline (Delini-Stula, 1978; Von Voigtlander *et al.*, 1978; Spyraki and Fibiger, 1980; Gorka and Zacny, 1981; Passarelli and Scotti de Carolis, 1982; Pilc and Vetulani, 1982a;), MAO inhibitors (Greenshaw *et al.*, 1988a; McKenna *et al.*, 1991b), ECS (Heal *et al.*, 1981; Pilc and Vetulani, 1982b), and salbutamol (Mogilnicka, 1982).

A.3.2 Dopamine receptors

DA receptors were also first divided into subtypes based on their pharmacological profile (Cooper *et al.*, 1986). D_1 receptors are positively linked to adenylate cyclase and SKF 38393 and SCH 23390 were thought to be a selective agonist and antagonist, respectively. D_2 receptors are negatively coupled to adenylate cyclase and quinpirole was thought to be a selective agonist and (-)sulpiride a selective antagonist. Molecular biologists have cloned these two receptors and numerous other DA receptors that loosely fit into these two classifications. Other subtypes of DA receptors will be discussed in more detail in a subsequent section [A.4.5.2].

Studies examining the effects of long-term antidepressant treatments on DA receptor binding are limited. Initial studies reported a decrease in the number of

D_1-like DA receptors labelled by ^{3}H-SCH 23390 in certain brain regions (Klimek and Nielsen, 1987; De Montis *et al.*, 1990) but no change in the density of D_2-like DA receptors labelled by ^{3}H-spiperone (Martin-Iverson *et al.*, 1983; Klimek and Nielsen, 1987). In addition, the affinity of DA binding sites labelled by ^{3}H-spiperone for the DA agonist quinperole in the limbic system may be altered by long-term treatment with IMI and mianserin (Klimek and Maj, 1989) and differential effects of antidepressants on the turnover of ^{3}H-spiperone binding sites have been reported (Nowak and Zak, 1991). There is a paucity of data describing effects of MAO inhibitors on DA receptor binding characteristics. Only the reverisible MAO-A inhibitors moclobemide and brofaromine have been assessed and found not to alter ^{3}H-SCH 23390 or ^{3}H-spiperone binding (Klimek and Maj, 1990).

Behavioral studies have generally indicated that long-term antidepressant treatments may significantly alter DA systems. The behavioural responses to direct and indirect DA agonists are generally enhanced after long-term treatment with some antidepressants (Maj *et al.*, 1979; 1984b; 1987; 1989; Spyraki and Fibiger, 1981; Martin-Iverson *et al.*, 1983; Campbell *et al.*, 1985; De Ceballos *et al.*, 1985; Maj and Wedzony, 1985; Smialowsk and Maj, 1985; Plaznik and Kostowski, 1987; Klimek and Maj, 1989; Przegalinski and Jurkowska, 1990) In contrast, behavioural responses to low doses of apomorphine, an agonist believed to be selective for D_2 autoreceptors, are usually inhibited by long-term antidepressant treatment (Serra *et al.*, 1979, 1981; Antelman and Chiodo, 1981; Arnt *et al.*, 1984; Volosin *et al.*, 1991). These findings suggest that a decrease in DA autoreceptors, which are negatively coupled to DA synthesis, may be responsible for the behavioural sensitization of the DA system seen after repeated antidepressant treatment. Limited electrophysiological and neurochemical data also generally indicate that DA autoreceptor activity is reduced following long-term antidepressant treatment (Nielsen, 1986). However, chronic DMI administration has been reported to have

no effect on interstitial DA concentration in the nucleus accumbens and not to attenuate the decrease in interstitial DA induced by low dose apomorphine (Nomikos *et al.*, 1991). This latter finding has lead to the suggestion that the potentiation of behavioural effects of DA agonists may be due to a decrease in DA uptake sites, but to date there are no data on such changes in uptake site density (Nomikos *et al.*, 1991).

It is clear from the above data and the discovery that several other DA receptors exist in the CNS that the effects of long-term antidepressant treatment on the DA system in the brain must be further characterized. This is especially compelling in the context of the anhedonia or lack of pleasure seen in depression as there is a well characterized role of DA in maintaining rewarded behaviour (Fibiger and Phillips, 1987).

A.3.3 5-HT receptors

Like catecholamine receptors, 5-HT receptors were first divided into subtypes based upon their pharmacological characteristics. Two classes of receptors initially emerged, the 5-HT_1 receptor defined by nanomolar affinity for 5-HT and labelled by ^{3}H-5-HT and the 5-HT_2 receptor defined by nanomolar affinity for 5-HT antagonists such as ketanserin, mesulergine and d-lysergic acid diethylamide (LSD) and labelled by ^{3}H-LSD (Peroutka and Snyder, 1979). More recently radioligand binding methods have identified 5-HT_3 binding sites in rat brain (Kilpatrick *et al.*, 1987). These sites have moderate affinity for agonists such as 5-HT and 2-methyl-5-HT, have subnanomolar affinity for antagonists such as BRL 43694, zacopride and ICS 205930, and are labelled by ^{3}H-GR 65630. Molecular biological and pharmacological techniques have revealed further subtypes of 5-HT receptors. Many reviews on the pharmacology, biochemistry, molecular biology, function and clinical relevance of the subtypes of 5-HT

receptors have recently been published (Peroutka, 1988; Sanders-Bush, 1989; Glennon, 1990; Peroutka, 1990; Bonate, 1991; Goldfarb, 1991). In general, 5-HT_1 receptors include the 5-HT_{1a}, 5-HT_{1b} and 5-HT_{1d} subtypes and are linked negatively to adenylate cyclase. 5-HT_{1c}, 5-HT_{2a} and 5-HT_{2b} receptors are linked to phosphoinositide turnover. Up to three subtypes of 5-HT_3 receptors have been proposed and these receptors are believed to be linked to an ion channel. Other 5-HT sites are either controversial or have been discovered too recently to be included in any available reviews. These include the 5-HT_{1e} site (Titeler and Herrick-Davis, 1988) and the 5-HT_4 site which is believed to be positively coupled to adenylate cyclase (Dumuis *et al.*, 1989).

Radioligand binding studies have generally revealed that antidepressant drugs including TCAs, MAO inhibitors, mianserin and iprindole decrease 5-HT_2 receptor density in rat brain (Peroutka and Snyder, 1980; Kellar *et al.*, 1981; Blackshear and Sanders-Bush, 1982; Zsilla *et al.*, 1983; Kellar *et al.*, 1985; Scott and Crews, 1986). A decrease in the associated process of 5-HT-mediated inositol phosphate formation has also been reported (Kendall and Nahorski, 1985; Newman and Lerer, 1988a). Conversely ECS has been reported to increase 5-HT_2 receptor density (Kellar *et al.*, 1981; Green *et al.*, 1983; Kellar and Bergstrom, 1983) with no effect on the associated inositol phosphate formation (Godfrey *et al.*, 1987; Newman *et al.*, 1987). The effects of long-term administration of the antidepressant fluoxetine on 5-HT_2 receptors is at present controversial with either a slight reduction of 5-HT_2 density being reported (Eison *et al.*, 1991) or no change (Peroutka and Snyder, 1980; Baron *et al.*, 1988).

Chronic administration of MAO inhibitors has been reported to decrease the number of 5-HT_1 binding sites (Peroutka and Snyder, 1980; Keller *et al.*, 1981). Other reports indicate either a reduction or no change in 5-HT_1 binding sites following repeated administration of various other antidepressants (Peroutka and

Snyder, 1980; Charney *et al.*, 1981; Blier *et al.*, 1990). There are also reports that ECS or DMI reduce the inhibition of forskolin-stimulated adenylate cyclase by 5-HT, suggesting that 5-HT_{1a} receptors are altered after long-term antidepressant treatment (Newman and Lerer, 1988b). An autoradiographic binding study, however, indicates that the binding of ^{3}H-8-hydroxy-2-(di-n-propylamino)tetraline (8-OH-DPAT), a selective 5-HT_{1a} agonist, is not decreased by long-term TCA treatment but is decreased by long-term fluoxetine treatment (Welner *et al.*, 1989). Long-term treatment with MAO inhibitors generally do not alter ^{3}H-8-OH-DPAT binding as measured by autoradiography, although there was a slight increase in binding in the hypothalamus after PLZ and clorgyline treatment (Welner *et al.*, 1989). A more recent radioligand binding study found a reduction in the density and affinity of ^{3}H-8-OH-DPAT binding in the frontal cortex but not the hippocampus of rats after long-term DMI treatment (Lund *et al.*, 1992).

Using radioligand binding studies Schmidt and Peroutka (1989) reported that several antidepressant drugs, including 5-HT uptake inhibitors like fluoxetine, exhibit nanomolar affinity for 5-HT_3 binding sites in rat cortex. Hoyer *et al.* (1989) found, however, that antidepressant drugs did not have nanomolar affinity for 5-HT_3 binding sites in rat entorhinal cortex or the neuroblastoma cell line N1E-115.

There is now a large body of literature on the effects of long-term antidepressant treatments on electrophysiological measures of 5-HT function (for review: Blier and deMontigny, 1985a; Baker and Greenshaw, 1989; Blier *et al.*, 1990). Based on an electrophysiological paradigm, Blier *et al.* (1990) have suggested that all effective antidepressant treatments enhance 5-HT neurotransmission but may do so *via* different mechanisms. Long-term administration of TCAs increase the response of neurons in the rat hippocampus, amygdala, ventral lateral geniculate nucleus and the facial motor nucleus to microiontophoretically applied 5-HT (de Montigny and Aghajanian, 1978; Gallager

and Bunney, 1979; Menkes *et al.*, 1980; Wang and Aghajanian, 1980: de Montigny *et al.*, 1981; Gravel and de Montigny, 1983; Blier *et al.*, 1984). Repeated treatment with mianserin and ECT also induces a sensitization of these neurons to 5-HT (Blier *et al.*, 1984; de Montigny *et al.*, 1989). The 5-HT_{1a} agonist 8-OH-DPAT also stimulates hippocampal neurons when applied microiontophoretically and this stimulation is enhanced following long-term TCA treatment (de Montigny *et al.*, 1989). This finding has led to the suggestion that postsynaptic 5-HT_{1a} receptors have increased sensitivity to 5-HT after repeated TCA, mianserin and ECT treatment (de Montigny *et al.*, 1989; Blier *et al.*, 1987, 1990). Nevertheless, as stated earlier, initial binding studies using ^{3}H-8-OH-DPAT have reported no changes in affinity or density of hippocampal 5-HT_{1a} binding sites after these treatments (Welner *et al.*, 1989; Lund *et al.*, 1992).

Blier *et al.* (1990) have proposed that long-term administration of MAO-inhibitors enhances 5-HT transmission by increasing the availability of releasable 5-HT. Indeed, suppression of the firing of hippocampal pyramidal neurons induced by the stimulation of the ascending 5-HT pathway was increased by long-term treatment with the MAO inhibitors PLZ and clorgyline (Blier *et al.*, 1986b). In contrast, clorgyline administration decreases the response of these neurons to ionophoretically applied 5-HT (Blier *et al.*, 1986b). Taken together, these data have led to the suggestion that the presynaptic effect of MAO inhibition and resultant increase in the availability of releasable 5-HT overcomes the observed postsynaptic functional desensitization (Blier *et al.*, 1990). Long-term administration of β_2-adrenoceptor agonists also results in an enhancement of 5-HT transmission similar to that induced by MAO inhibitors (Bouthillier *et al.*, 1989).

Behavioral studies have also been used to assess 5-HT receptor function after long-term treatment with antidepressant drugs. A decrease in behaviors induced by the 5-HT_{1a} receptor agonist 8-OH-DPAT is seen following long-term

administration of several antidepressants (Goodwin *et al.*, 1985, 1987). Behavioral responses to 5-HT and the direct postsynaptic agonist 5-methoxy-N,N-dimethyl-tryptamine (5-MeODMT) are enhanced in rats treated chronically with TCAs and mianserin (Mogilnicka and Klimek, 1979; Friedman *et al.*, 1983; Stolz and Marsden, 1982). In contrast, others have reported a decrease in 5-MeODMT-induced behaviors in mice after chronic tricyclic antidepressant or mianserin treatment (Fuxe *et al.*, 1982; Blackshear and Sanders-Bush, 1982). MAO inhibitors are reported to prevent 5-MeODMT-induced behaviors (Lucki and Frazer, 1982).

It is evident from the discrepancies among results obtained from binding, electrophysiological and behavioral studies that further study on the effects of long-term antidepressant treatment on 5-HT receptors is needed. Development of drugs that are more selective for the numerous subtypes of 5-HT receptors may help in clarifying some of these discrepancies.

A.3.4 Other receptors

The effects of long-term antidepressant treatments on other receptor systems have also been examined. Binding studies have indicated that repeated treatment with TCAs or ECS either increases muscarinic acetylcholine receptors in several different areas of rat brain (Rehavi *et al.*, 1980; Koide and Matsushita, 1981; Gulati *et al.*, 1982; Goldman and Erickson, 1983) or does not change these receptors (Maggi *et al.*, 1980; Peroutka and Snyder, 1980; Deakin *et al.*, 1981). In contrast the few studies that have examined receptor-mediated responses to carbachol a cholinergic agonist after long-term antidepressant treatment have reported no change (Menkes and Aghajanian, 1981) or a decrease in response (Newman and Lerer, 1988b). It is now evident that several muscarinic receptor subtypes exist (Kerlavage *et al.*, 1987), complicating the interpretation of the above data.

Changes in γ-aminobutyric acid (GABA) binding sites have also been reported after long-term antidepressant treatment. The $GABA_A$ site is associated with a benzodiazepine binding site (Pritchett *et al.*, 1989; Haefely, 1989) and a chloride ion channel (Enna and Gallagher, 1983). Binding studies in rat brain tissue have indicated that the $GABA_A$ and benzodiazepine binding sites are decreased after long-term antidepressant treatment (Suranyi-Cadotte *et al.*, 1985; Suzdak and Gianutsos, 1985; Barbaccia *et al.*, 1986) although others have reported no change in benzodiazepine receptor density (Kimber *et al.*, 1987). A recent report also indicates that binding of ^{35}S-t-butylbicyclophosphorothionate directly to the chloride ion channel is decreased in the CA_3 pyramidal cell layer of the hippocampus after long-term antidepressant treatment (Suranyi-Cadotte *et al.*, 1990). Decreased function of this receptor complex after long-term antidepressant treatment has also been suggested by neurochemical, electrophysiological and behavioral experiments (Borsini *et al.*, 1986; Bouthillier and de Montigny, 1987; Fernandez-Teruel *et al.*, 1989).

It has been proposed that long-term treatment with antidepressants of all classes increases the density of $GABA_B$ receptors in the frontal cortex of rats and mice (Lloyd *et al.*, 1985, 1989; Suzdak and Gianutsos, 1986). Other researchers, however, have failed to find any significant changes in $GABA_B$ receptor density (Cross and Horton 1987, 1988; McManus and Greenshaw, 1991a). Yet still others have reported both an increase or no change in $GABA_B$ receptor density depending on the radiolabelled ligand used (Szekely *et al.*, 1987). Results from behavioural and neurochemical tests examining $GABA_B$ receptor-mediated also are varied after long-term antidepressant treatment with either no change or an increase in $GABA_B$ receptor-mediated responses being reported (Borsini *et al.*, 1986; Suzdak and Gianutsos, 1986; Gray *et al.*, 1987; Gray and Green, 1987; Szekely *et al.*, 1987; McManus and Greenshaw, 1991b).

A.3.5 Interactions of receptor systems

There has been considerable interest in the 5-HT/NA link in brain in the context of receptor changes observed after long-term administration of antidepressants (Manier *et al.*, 1987; Green, 1987; Asakura *et al.*, 1987). Evidence from lesion studies indicates that the antidepressant-induced decrease in ^{3}H-DHA binding is dependent on an intact 5-HT system (Stockmeier *et al.*, 1985; Manier *et al.*, 1987). Conversely, there is evidence that lesions of the NA system may also prevent the antidepressant-induced increase in 5-HT_2 sensitivity (Green and Deakin, 1980; Gravel and de Montigny, 1983). A recent study using a more selective β-adrenoceptor ligand, ^{125}I-iodopindolol, found that lesioning 5-HT neurons failed to prevent the down-regulation of β-adrenoceptors induced by chronic administration of DMI (Hensler *et al.*, 1991). Furthermore, Stockmeier and Kellar (1988) demonstrated that repeated ECS decreases β-adrenoceptor density despite lesions of 5-HT neurons. It is clear that the relationship between 5-HT and NA systems and their involvement in the long-term effects of antidepressant drugs on receptors needs to be further elucidated.

The possible interaction of DA with either NA or acetylcholine systems has also been examined in the context of long-term antidepressant-induced receptor changes. Initial reports indicated that the ECS-induced potentiation of apomorphine-induced behaviors was blocked by prior lesions of NA neurons (Green and Deakin, 1980). Martin-Iverson *et al.* (1983) failed, however, to observe any effect of NA lesions on DMI-induced increases in amphetamine-induced behaviour. These latter researchers found instead that anticholinergic activity of several antidepressant drugs correlates with the enhancement of DA-mediated behaviors after long-term antidepressant treatment.

A.3.6 Clinical context of receptor changes

As stated earlier, there is an apparent parallel between the time course of receptor changes in animals and that of clinical improvement in humans. The clinical relevance of this parallel, however, remains to be demonstrated definitively. Due to problems of species differences and the inadequacies of "animal models" of depression (Greenshaw *et al.,* 1988b), it is apparent that assessment of receptor function in humans is important. Human studies are obviously limited due to ethical constraints. Some postmortem receptor binding studies have used human tissue and these may provide limited information on the possible abnormalities in receptor systems in depressed patients. Indeed one such study has reported an increase in the number of β-adrenergic and 5-HT_2 receptors in the brains of suicide victims as opposed to controls (Mann *et al.,* 1986). Other approaches are also being applied including the measurement of peripheral receptor subtypes and the indirect assessment of receptor sensitivity using pharmacological probes. The more recent technique of *in vivo* imaging of receptor activity using positron emission tomography (PET) scanning may be particularly useful in the future (review: Frey, 1989). In general, results from studies in humans have paralleled the preclinical animal studies. These results have been recently reviewed (Heninger and Charney, 1987; Baker and Greenshaw, 1989).

Based on the data presented above, it is evident that long-term antidepressant treatments induce changes in the number and/or function of a large variety of neurotransmitter receptors. In the studies described in this thesis, the effects of long-term treatment with PEA and antidepressant drugs on catecholamine receptor systems have been examined. The next two sections provide a brief overview of the characteristics of DA, NA and PEA systems in the CNS.

A.4 DOPAMINE AND NORADRENALINE

The catecholamines DA and NA are neurotransmitters in the CNS. Although these compounds are now thought of as classical neurotransmitters it was not until the mid to late 1960's that it was widely accepted that they act as neurotransmitters in the CNS (for review: Carlsson, 1987). Since that time our knowledge of their action in the CNS has greatly expanded, and this section will try to briefly summarize this field of research.

A.4.1 Synthesis

The synthetic pathway for DA and NA is well characterized and shown schematically in Figure 2. The precursor amino acid L-tyrosine can be obtained from dietary protein or can be formed in the liver from L-phenylalanine, an essential dietary amino acid, *via* the enzyme phenylalanine hydroxylase. L-Tyrosine may also be formed in the brain by the hydroxylation of L-phenylalanine *via* the enzyme tyrosine hydroxylase (Murrin and Roth, 1976; Dyck *et al.,* 1983). Both of these enzymes have been purified and require the essential cofactors tetrahydrobiopterin and molecular oxygen (for review: Joh *et al.,* 1986).

It is widely accepted that catecholamine synthesis begins with L-tyrosine. The first step in the synthetic pathway is the hydroxylation of the m-position of L-tyrosine to form 3,4-dihydroxy-L-phenylalanine (L-DOPA). This synthetic step is believed to be rate-limiting for the synthesis of catecholamines. Since L-tyrosine appears to be at saturating amounts for tyrosine hydroxylase in the CNS, there is an abundance of research on the regulation of this enzyme. It appears that long-term regulation of tyrosine hydroxylase is related to changes in the quantity of the enzyme present. This may be accomplished at the transcriptional and/or translational level of enzyme synthesis and at the level of enzyme degradation. Short-term regulation is accomplished by the activation of the enzyme by

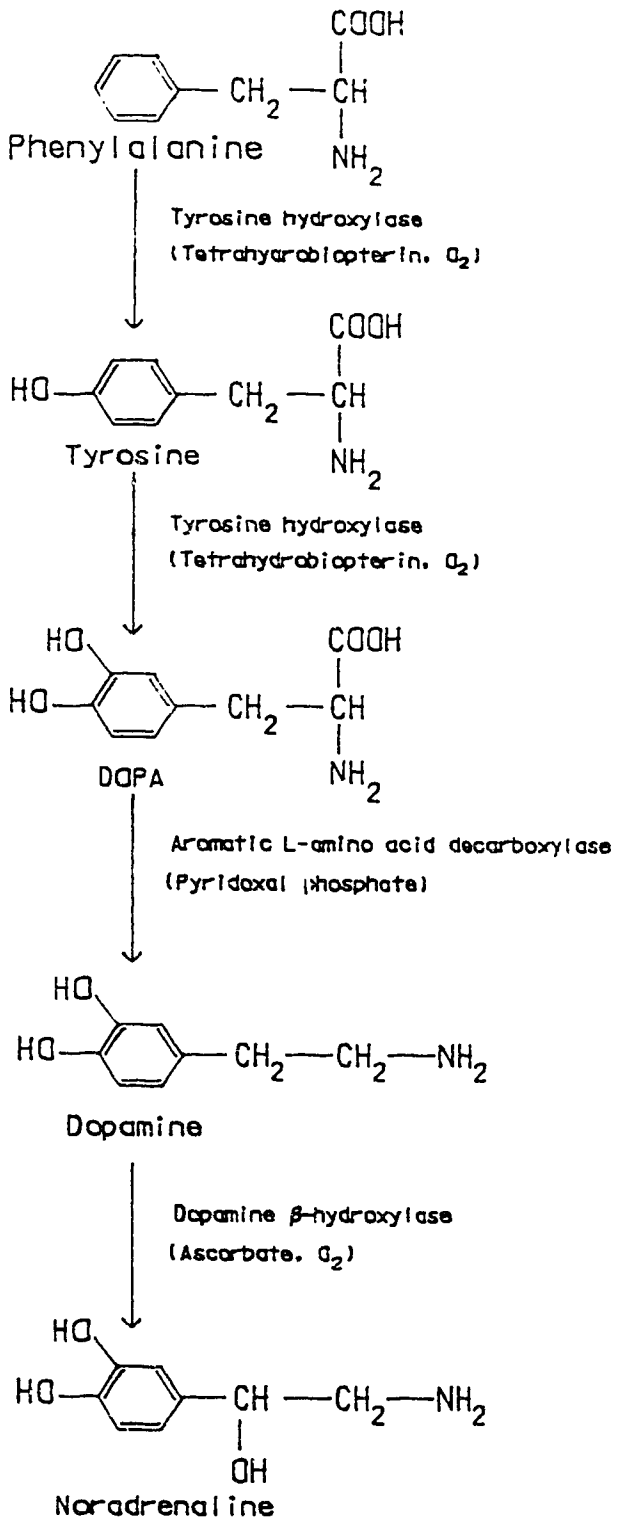


Figure 2: Synthetic pathway for DA and NA.

phosphorylation. For a brief review of these regulatory mechanisms the reader is referred to Joh *et al.* (1986). Another possible regulatory mechanism may be *via* the control of the levels of the essential cofactor tetrahydrobiopterin, which is at subsaturating concentrations in catecholamine neurons (Weirner and Molinoff, 1989). This enzyme hydroxylates L-tyrosine and to a much lesser extent L-phenylalanine. It will not act on D-tyrosine or L-tryptophan and is inhibited by analogues of L-tyrosine such as α-methyl-*p*-tyrosine (Cooper *et al.*, 1986; Weiner and Molinoff, 1989).

The next step in catecholamine synthesis is the decarboxylation of L-DOPA to form DA (Figure 2). This is accomplished by the relatively non-specific enzyme aromatic L-amino acid decarboxylase (AAAD) which requires pyridoxal phosphate as a cofactor (for review: Bowsher and Henry, 1986). Kinetic experiments have indicated that L-DOPA is efficiently converted to DA, resulting in very low amounts of L-DOPA being present in catecholamine-containing neurons (Christenson *et al.*, 1970). This is the final step in neurons that contain DA as a neurotransmitter. AAAD plays a role in the synthesis of other neuroactive monoamines, including PEA. This role will be discussed further in a subsequent section [A.5.2].

In NA neurons the next and final step in the synthetic pathway is the hydroxylation of the side chain of DA at the β-position to form NA (Figure 2). The cofactors for this reaction are ascorbate and molecular oxygen (for review: Nagatsu, 1986). This enzyme contains Cu^{2+}, which is essential for the chemical reaction. Consequently, copper chelators are potent inhibitors of this enzyme.

A.4.2 Storage and release

Conversion of tyrosine to L-DOPA and L-DOPA to DA takes place in the cytoplasm. DA is then taken up into storage vesicles and in NA neurons is converted to NA in these vesicles (Weiner and Molinoff, 1989). Catecholamines

are concentrated in vesicles by an ATP-dependent proton pump (Holz, 1978). These vesicles protect the catecholamines from catabolism and contain ATP, proteins called chromogranins and in NA neurons dopamine-β-hydroxylase. The drug reserpine is an inhibitor of the vesicular amine pump, and treatment with this drug causes depletion of endogenous catecholamines in neurons (Weiner and Molinoff, 1989). Vesicles also help to mediate the release of catecholamines from neurons. Vesicular release is initiated by the influx of Ca^{2+} ions into the terminal of the axon (for review: Augustine *et al.*, 1987; Smith and Augustine, 1988; Zimmermann, 1990). This Ca^{2+} influx is generated by the arrival of an action potential. Vesicles are thought to fuse with the cell membrane and release their contents in an exocytotic manner. In addition to vesicular release there is a Ca^{2+}-independent continuous release of catecholamines from nerve terminals (Zimmermann, 1990).

A.4.3 Uptake and catabolism

To terminate the action of catecholamines at the synapse, the presynaptic terminal is equipped with an uptake process. Although this process was hypothesized much earlier it was originally observed by Axelrod in 1971. There are NA- and DA-specific uptake carriers but a variety of phenylethylamines can act as competitors for these carriers (Coyle and Snyder, 1969). The uptake carrier is linked to a NA^+/K^+ ATPase and uptake is an energy-dependent process (Weiner and Molinoff, 1989). As mentioned earlier, TCAs are inhibitors of these amine uptake systems.

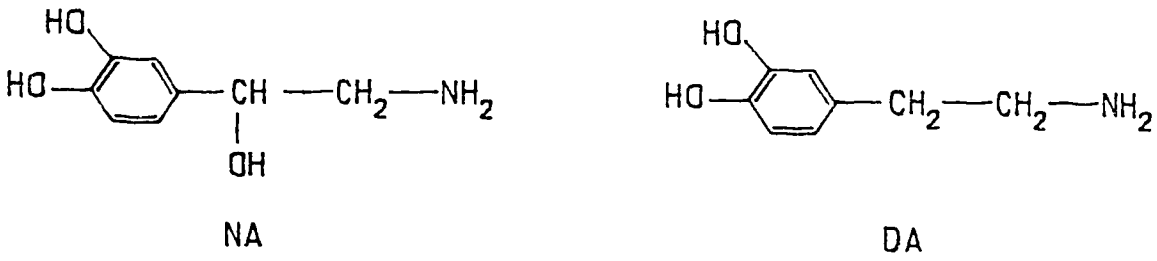
There are two enzymes that are primarily responsible for the catabolism of catecholamines. MAO oxidatively deaminates the amine function of catecholamines to the corresponding aldehyde. This aldehyde is then converted by aldehyde reductase or aldehyde dehydrogenase to form a glycol or acids. The

second catabolic enzyme is catechol-O-methyltransferase (COMT) which methylates the 3-hydroxy position on the catechol ring. The reactions involved in the catabolism of NA and DA are shown in Figure 3.

MAO is a flavin-containing enzyme located on the outer membrane of mitochondria and therefore plays a role in the inactivating of catecholamines that are free in the cytosol of neurons (for reviews: Glover and Sandler, 1986; Yu, 1986; Hsu *et al.*, 1989; Weyler *et al.*, 1990). As previously mentioned there are two forms of MAO based upon the pharmacology of the enzymes. Molecular biologists have now confirmed that MAO-A and MAO-B arise from separate genes (Bach *et al.*, 1988). These two enzymes are differentially and heterogeneously distributed throughout the CNS (Konradi *et al.*, 1987, 1988; Thorpe *et al.*, 1987). As will be discussed in a subsequent section [A.5.2], MAO is involved in the catabolism of other neuroactive amines, including PEA.

COMT is found in nearly all cells and is believed to act on extraneuronal catecholamines (Cooper *et al.*, 1986; Weiner and Molinoff, 1989). This enzyme requires Mg^{2+} and S-adenosylmethionine from which it transfers a methyl group to the catechol (for review: Park, 1986).

In the CNS, the principal metabolite of NA is 3-methoxy-4-hydroxyphenylglycol (MHPG) whereas in the periphery the principal metabolite is vanillylmandelic acid [VMA] (Figure 4). The principal metabolite of DA in the human CNS is homovanillic acid (HVA) but in the rat CNS the principal metabolite is 3,4-dihydroxyphenylacetic acid [DOPAC] (Figure 4). A smaller quantity of other metabolites are formed from the catabolism of catecholamines including 3,4-dihydroxymandelic acid, 3,4-dihydroxyphenylglycol, 3,4-dihydroxyphenylethanol, 3-methoxy-4-hydroxyphenylethanol, normetanephrine and 3-methoxytyramine (Cooper *et al.*, 1986).



ENZYMES AND PRIMARY PRODUCTS

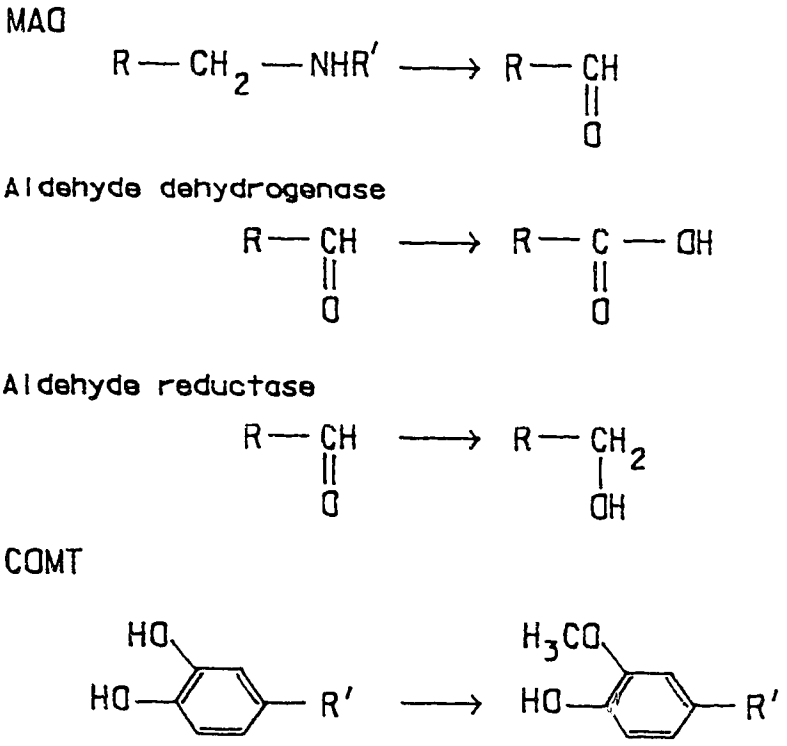
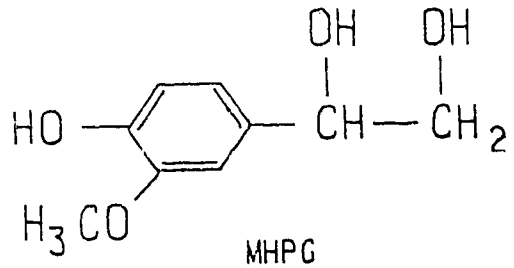
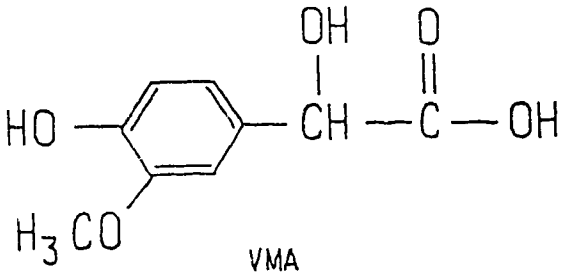


Figure 3: Catabolic reactions involved in the degradation of NA and DA.

Principal metabolites of NA:

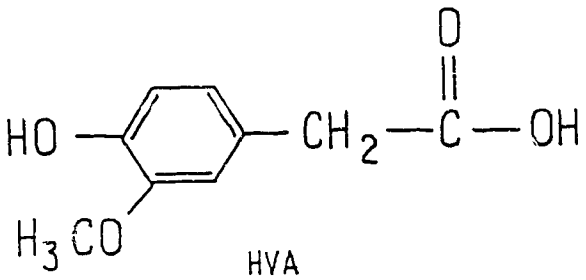


MHPG

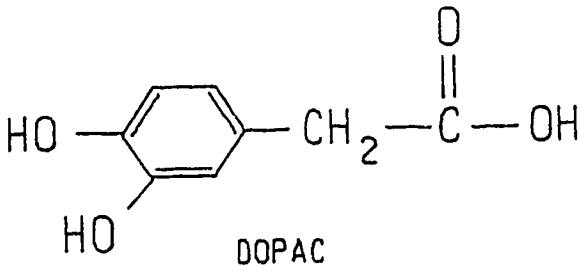


VMA

Principal metabolites of DA:



HVA



DOPAC

Figure 4: Principal products formed as metabolites of NA and DA.

A.4.4 CNS pathways

The development of a formaldehyde histofluorescence method was the initial impetus for the mapping of CNS NA-containing and DA-containing neurons (Falck *et al.*, 1962). Since this time other sensitive techniques have been used alone and in combination to map the catecholamine systems (Moore and Card, 1984; Bjorkland and Lindvall, 1984). There are two major NA pathways and an additional minor pathway (Moore and Card, 1984). Several DA pathways exist in the CNS (Bjorkland and Lindvall, 1984). Although catecholamine pathways were initially mapped in the rat, the pathways appear to be very similiar higher up the phylogenetic scale including primates (Bradford, 1986).

The locus coeruleus, located in the upper pons region of the brainstem, is a group of NA-containing cell bodies. The majority of these neurons project rostrally in bundles but some also project caudally to the spinal cord and brainstem and others project to the cerebellum. The second major pathway arises in a diffuse set of cells that are caudal to the locus coeruleus. These cells project forward jointly and give rise to the central tegmental bundle. Projections from the locus coeruleus join this bundle and the two systems travel forward as the ventral NA bundle. After a short distance the projections from the locus coeruleus branch of2f to form the dorsal NA bundle, but the majority of these fibres later join up with the ventral bundle to form the medial forebrain bundle. Along these pathways neurons branch off and innervate various structures, including the hypothalamus, amygdala, hippocampus, septum, various cortical areas, and finally the projection terminates in the olfactory
bulbs. The minor NA pathway projects from groups of cell bodies in the lateral tegmentum to the spinal cord, brainstem, hypothalamus and basal forebrain.

One of the major DA-containing ascending pathways projects from the substantia nigra pars compacta to the striatum as the nigrostriatal tract. Other

cells of the substantia nigra project to the cingulum. The other major forward projecting DA-containing system is the mesolimbic system, sometimes called the meso-limbo-cortical system. This system arises in the ventral tegmental area, which is medial to the substantia nigra and projects, as part of the medial forebrain bundle, to the amygdala, septum, nucleus accumbens, olfactory tubercle and frontal cortex. There are several minor DA-containing systems, including the incertohypothalamic and the tuberoinfundibular systems. These systems innervate the hypothalamus from groups of cell bodies known as A12 located just dorsal to the hypothalamus. The olfactory bulb also has a group of DA-containing neurons although these cells do not appear to exhibit axons. The inner plexiform layer of the retina also contain DA neurons.

A.4.5 Receptors

A brief overview of neurotransmitter receptors and their coupling to a transduction pathway has been provided in an earlier section. This section will briefly review what is known about brain β-adrenoceptors and DA receptors.

A.4.5.1 β-Adrenoceptors

As stated earlier, NA receptors have been classified into α- and β-adrenoceptors based upon their pharmacology. β-Adrenoceptors have been further characterized by radioligand binding studies and subdivided into two subtypes (Stadel and Lefkowitz, 1991). The β_2-adrenoceptor, which was solubilized as early as 1976 (Caron and Lefkowitz, 1976), has been purified by a variety of methods (Caron *et al.*, 1979; Rashidbaigi and Ruohjo, 1981; Shorr *et al.*, 1982a; Benovic *et al.*, 1984). Subsequently the β_2-adrenoceptor was cloned and its primary sequence published (Dixon *et al.*, 1986). The β_1-adrenoceptor has since been cloned and its primary sequence published (Frielle *et al.*, 1987). The

β_2-adrenoceptor is the prototype of a variety of other G protein-coupled receptors and is very similar in structure to these receptors which include the α_1-, α_2-, β_1-adrenergic receptors, as well as the rhodopsin, acetylcholine muscarinic and DA receptors (Caron and Lefkowitz, 1991).

The purification and cloning of the β_2-adrenoceptors has been the impetus for the dramatic increase in understanding of the structure and function of G protein-coupled receptors (for review: Caron and Lefkowitz, 1991). These receptors appear to have seven membrane spanning domains with the amino acid terminus on the extracellular side and the carboxy terminal tail on the intracellular side. These receptors are glycosylated near the amino terminus but the functional significance of this is unclear (Benovic *et al.*, 1987; Dixon *et al.*, 1987). The sites on the receptor involved in ligand binding and G protein coupling are currently being explored (for review: Caron and Lefkowitz, 1991; Strosberg, 1991; Tota *et al.*, 1991). There are also ongoing studies into the structural basis of receptor regulation and the involvement of phosphorylation in this process (for reviews: Perkins *et al.*, 1991; Stiles, 1991; Strosberg, 1991).

In addition to the β_1- and β_2-adrenoceptors, a third β-adrenergic receptor subtype has been cloned (Emorine *et al.*, 1989). This receptor also belongs to the G-protein-coupled receptor family. Expression of this receptor in eukaryotic cells leads to expression of a receptor that has different binding properties than other β-adrenoceptors (Emorine *et al.*, 1989). It appears that this receptor is involved in the lipolytic effect of catecholamines in adipose tissue, although this has not been demonstrated clearly (Emorine *et al.*, 1989). To date it is unknown if the β_3-adrenoceptor is present in the CNS.

Using radioligand binding and autoradiography techniques, the distribution of β-adrenoceptors has been determined in mammalian brain. Initial studies indicated that β_1-adrenoceptors are heterogeneously distributed throughout the

rat forebrain, whereas β_2-adrenoceptors are distributed much more homogeneously (Palacios and Kuhar, 1982). A later study with much more selective compounds found that both β_1- and β_2-adrenoceptors were heterogeneously distributed (Rainbow *et al.*, 1984). Areas high in β-adrenoceptor density include the cortex, caudate-putamen, nucleus accumbens, olfactory tubercles, substantia nigra, nucleus interpeduncularis, subiculum and pia mater (Palacios and Kuhar, 1982). Areas that appear to be specifically enriched with β_1-adrenoceptors include area CA_1 of the hippocampus, the dentate gyrus, the Islands of Calleja, certain thalamic nuclei (gelatinosus, ventroposterior and dorsal lateral geniculate) and all layers of the cerebral cortex except layer IV. Areas specifically enriched with β_2-adrenoceptors include the molecular layer of the cerebellum, the olfactory tubercle, certain thalamic areas (lateral posterior, paraventricular and reticular nuclei) and layer IV of the cortex (Wolfe, 1991). Interestingly β-adrenoceptors appear not to be restricted to areas of high NA innervation as the caudate-putamen, olfactory tubercles and substantia nigra all have a high density of β-adrenoceptors but are innervated with a low density of NA terminals (Swanson and Harman, 1975; Moore and Bloom, 1979; Palacios and Kuhar, 1982). The location of β-adrenoceptors on individual cells has also been studied. In humans it appears that β_1-adrenergic receptors are localized on neuronal cells, whereas β_2-adrenergic receptors are predominantly associated with glial cells (Cash *et al.*, 1986).

A.4.5.2 DA receptors

As stated earlier, DA receptors have been classified into D_1 and D_2 receptors based upon their pharmacological profile. Presently several DA receptor subtypes have been characterized using molecular biological techniques, and other DA receptors may still remain to be isolated (Anderson *et al.*, 1990). The rat

D_2 receptor was the first DA receptor cloned (Bunzow *et al.*, 1988). The cloning of this receptor was accomplished by taking advantage of its similarity to the previously cloned β_2-adrenoceptor (Bunzow *et al.*, 1988). To date, all DA receptors cloned belong to the G protein-linked receptor family and are similar in structure to the β_2-adrenoceptor. The D_2 receptor exists in two isoforms that differ by the inclusion or exclusion of a 29-amino acid sequence between the 5th and 6th membrane-spanning regions (Giros *et al.*, 1989; Monsma *et al.*, 1990). Other receptors that pharmacologically resemble the D_2 receptor have been cloned and characterized, namely the rat and human D_3 receptor (Sokoloff *et al.*, 1990; Giros *et al.*, 1990) and the human D_4 receptor (Van Tol *et al.*, 1991). Three variants of the D_3 receptor have now been described (Giros *et al.*, 1991). The radioligand binding characteristics of the D_3 and D_4 receptors are similar to, but not identical to, those of the D_2 receptor.

Subsequent to the cloning of the D_2 receptor the D_1 receptor was cloned and characterized from human and rat brain (Zhou *et al.*, 1990; Monsma *et al.*, 1990). An additional D_1-like receptor, namely the human D_5 receptor, has been isolated and may only differ from the D_1 receptor in that it binds DA with approximately 10-fold higher affinity (Sunahara *et al.*, 1991).

Radioligand binding and autoradiographical techniques have been used to map the distribution of D_1 and D_2 receptors in the brain, although the existence of the various other subtypes of these receptors now complicates the interpretation of these data. In general D_1 and D_2 receptors are found in areas of high DA input such as the caudate nucleus, nucleus accumbens, olfactory tubercle, substantia nigra and globus pallidus (for review: Waddington and O'Boyle, 1988). Most areas appear to have a higher density of D_1 than D_2 receptors. In the striatum the ratio is about 3:1 in favor of D_1 receptors (Boyson *et al.*, 1986; Richfield *et al.*, 1987). Similar to the NA system, there are areas of mismatch such as regions of the

cortex and cerebellum that are relatively high in D_2 receptors despite lacking any notable DA innervation (Bouthenet *et al.*, 1987; Camus *et al.*, 1986). The cerebellum also unexpectedly contains D_1 receptors (Boyson *et al.*, 1986; Savasta *et al.*, 1986).

Using in situ hybridization and northern blotting the regional distribution of the messenger ribonucleic acid (mRNA) for a variety of subtypes of DA receptors has been studied. D_1 and D_2 mRNAs are most abundant in the caudate nucleus, nucleus accumbens and olfactory tubercles with D_2 mRNA also present in the substantia nigra (Mansour *et al.*, 1990). D_3 mRNA is distributed similarly to D_2 mRNA with the exception that the dorsolateral striatum is devoid of mRNA for D_3 receptors but possesses a large amount of mRNA for D_2 receptors (Sokoloff *et al.*, 1990). D_3 receptor mRNA is most abundant in the nucleus accumbens, Islands of Calleja and cingulate cortex, the last two areas having more RNA for D_3 receptors than D_2 (Sokoloff *et al.*, 1990). The locations of D_4 and D_5 receptor mRNAs are less well characterized but appear to be very similar to those of D_2 and D_1 receptor mRNA respectively (Van Tol *et al.*, 1991; Sunahara *et al.*, 1991). These last data should be interpreted cautiously as the probes used to locate D_4 and D_5 mRNA may have some affinity for D_2 and D_1 mRNA respectively. In additional, one must remember that mRNA is usually found in the cell body, whereas many receptors are located on nerve terminals. Immunocytohistochemistry may be a useful technique to allow mapping of different DA receptors, but, due to the great similarity in primary structure of this group of receptors there may be problems of cross-reactivity. Detailed mapping of brain DA receptor subtypes probably awaits development of selective radioligands for these receptors.

A.5 2-PHENYLETHYLAMINE (PEA)

PEA is an endogenous amine found in the mammalian CNS and is structurally similar to amphetamine. This similarity has lead some researchers to propose that PEA may be involved in the etiology of depression (Dewhurst and Marley, 1965; Dewhurst 1968, 1969; Fischer *et al.*, 1968; Sabelli and Mosnaim, 1974). Interest in PEA has increased following the introduction of highly specific and sensitive techniques for the quantification of this amine, as well as the characterization of its metabolic pathways in the CNS (for review: Boulton and Jourio, 1982; Paterson *et al.*, 1990).

A.5.1 Distribution

PEA is distributed heterogeneously in the mammalian CNS, with the highest concentrations in the hypothalamus and mesolimbic structures such as the nucleus accumbens, olfactory tubercles and olfactory bulbs (Paterson *et al.*, 1990). Other DA-containing areas such as the caudate nucleus also have relatively high concentrations of PEA. The synthesis of PEA appears to be specific to neurons since astrocytes do not contain AAAD, the synthetic enzyme for PEA (Jourio *et al.*, 1987). 6-OHDA-induced lesions of the substantia nigra result in a decrease in the MAO inhibitor-induced accumulation of PEA in the caudate nucleus, indicating that PEA could possibly be co-localized in DA-containing neurons (Greenshaw *et al.*, 1986). Another possibility is that PEA is localized in a set of non-dopaminergic neurons that are sensitive to 6-OHDA. Indeed certain neurons in the area of the substantia nigra contain AAAD but lack tyrosine hydroxylase (Jaeger *et al.*, 1984) and could possibly contain PEA. MAO inhibitor-induced PEA accumulation in the caudate nucleus is not affected by 5-HT-depleting lesions of the dorsal and median raphé nuclei (Greenshaw *et al.*, 1986).

A.5.2 Metabolism

The synthesis and catabolism of PEA are depicted in Figure 5. The essential amino acid L-phenylalanine is decarboxylated by the enzyme AAAD to form PEA. PEA is synthesized in rat brain at a rate similar to the synthesis of DA (Brodie *et al.*, 1966; Durden and Philips, 1980). AAAD has 100 times less affinity for L-phenylalanine than for L-DOPA so although AAAD is not a rate-limiting step for catecholamines it could be for the production of PEA (Bowsher and Henry, 1986). This suggests that the rate of synthesis of PEA will be dependent on the concentration of L-phenylalanine. L-Phenylalanine is a substrate for several other enzymes including tyrosine hydroxylase (Murrin and Roth, 1976; Dyck *et al.*, 1983). The activity of these other enzymes could therefore affect the synthesis of PEA. Indeed electrical stimulation of the substantia nigra in rats increases the turnover of DA and decreases the MAO inhibitor-induced accumulation of PEA (Jourio and Paterson, 1988). The inhibition of tyrosine hydroxylase blocks both the increased turnover of DA and the decrease in PEA accumulation, indicating that modulating the activity of tyrosine hydroxylase may alter PEA synthesis (Juorio and Paterson, 1988); however, this is inconsistent with other pharmacological data. Treatment with reserpine which depletes DA, presumably resulting in an increase of DA synthesis, increases the rate of PEA accumulation in the mouse striatum (Juorio *et al.*, 1988). Clearly the relationship between PEA and L-phenylalanine availability remains to be elucidated.

PEA is oxidatively deaminated by MAO-B, an enzyme with a high affinity for PEA (Yang and Neff, 1973; Yu, 1986). The resultant product, phenylacetic acid (PAA), is the major metabolite of PEA and has been found in the mammalian brain (Durden and Boulton, 1982; Mosnaim *et al.*, 1984). It has been proposed that PAA can also be formed from phenylalanine *via* a shunt that bypasses PEA (Karoum *et al.*, 1984). Despite PEA being synthesized as rapidly as DA, it is catabolized

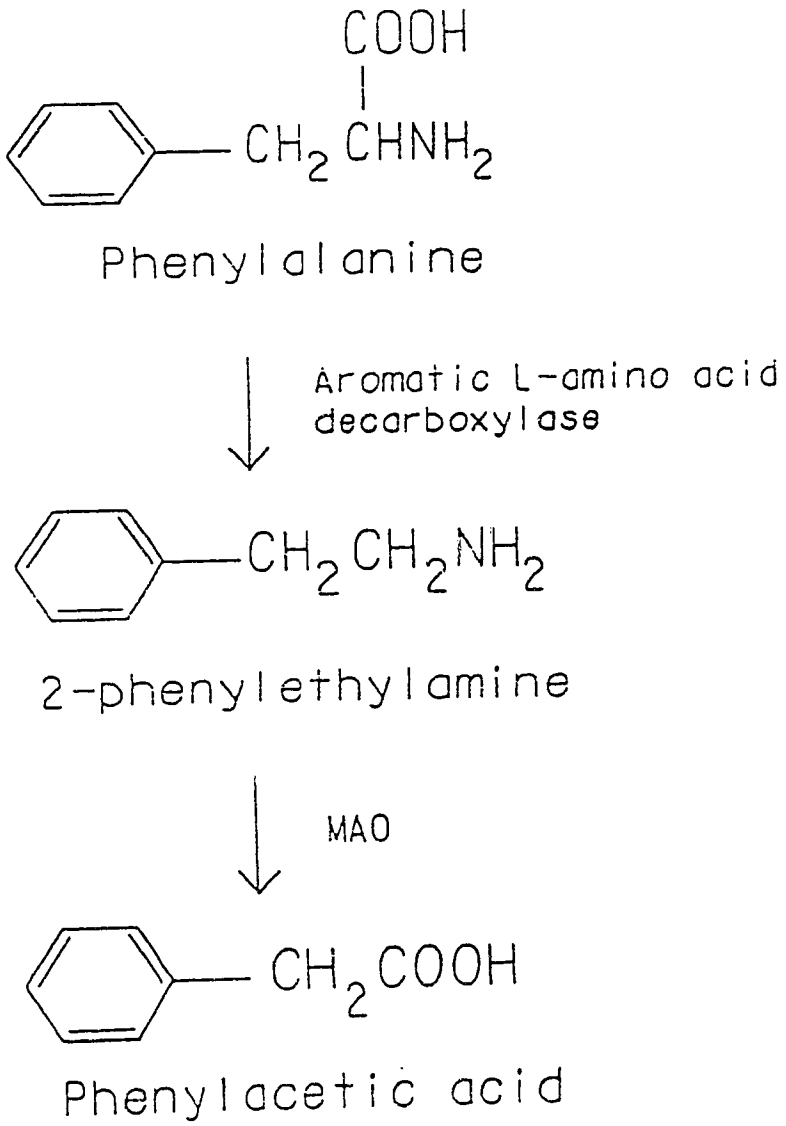


Figure 5: Principal route of formation and degradation of PEA.

much more rapidly, resulting in its presence in low quantities (<10 ng/g whole brain) in the CNS (Durden and Philips, 1980).

A.5.3 Possible neuromodulatory role of PEA

PEA, unlike the catecholamines, does not appear to be stored in vesicles. As stated earlier, reserpine, which disrupts vesicular stores of catecholamines, actually increases the accumulation of PEA (Juorio *et al.*, 1988). PEA appears to be released in a concentration-dependent manner and stimulation of neurons does not seem to cause PEA release (Henry *et al.*, 1988, Dyck, 1989). ^{3}H-PEA has been found to bind to tissue homogenates in a specific and saturable manner (Hauger *et al.*, 1982), but this binding may be to the enzyme MAO (Nguyen and Juorio, 1989). In fact, ^{3}H-PEA fails to bind specifically to brain homogenates in the presence of a MAO inhibitor (Li *et al.*, 1992). Based on these results it can be concluded that PEA may not act like a conventional transmitter. Nevertheless, recent neurochemical, electrophysiological and behavioral evidence points to a neuromodulatory role of PEA in the CNS.

PEA has a relatively potent effect on the uptake and release of the catecholamines, and a weaker effect in this regard on 5-HT. PEA inhibits the uptake of these amines by synaptosomal preparations (Horn and Snyder, 1973; Raiteri *et al.*, 1977). PEA also stimulates release of these amines from synaptosomes (Raiteri *et al.*, 1977), brain slices (Dyck, 1983) and directly from the caudate nucleus as measured using an *in vivo* push-pull cannula (Philips and Robson, 1983; Philips, 1986; Bailey *et al.*, 1987). A large dose of peripherally administered PEA (100 mg/kg) depletes central catecholamines and 5-HT (Fuxe *et al.*, 1967) and moderate to large doses also result in an increase in the central concentration of metabolites of 5-HT (Antelman *et al.*, 1977; McQuade and Juorio, 1982). These results are consistent with the releasing and uptake blocking

properties of PEA. The release and blocking of uptake of catecholamines generally occurs at high nM to low μM concentrations of PEA which are much greater than the endogenous concentration of PEA of approximately 2nM (Paterson *et al.,* 1990). These presynaptic actions of PEA, nevertheless, may be important in some clinical settings, such as after the administration of a MAO inhibitor.

Electrophysiological studies have revealed that large doses of PEA, like NA and DA, when applied iontophoretically to caudate nucleus and cortical neurons depressed their firing rates (Henwood *et al.,* 1979). It was later found that intravenous administration of PEA inhibited the firing of NA neurons in the locus coeruleus and that this action was potentiated by MAO inhibitors and α_2-adrenoceptor antagonists (Lundberg *et al.,* 1985). In cortical neurons it was found that iontophoretically applied PEA induced responses similar to those observed following iontophoretic application of NA. The PEA-induced response was prevented by ipsilateral lesions of the locus coeruleus and by pretreatment with either reserpine, a NA depleting agent, or DMI, a NA uptake blocker (Paterson, 1988a,b). These data indicated that these responses to PEA are indirect effects dependent on endogenous NA and possibly due to PEA-induced NA release and/or PEA blockade of NA uptake. A separate effect of PEA observed in electrophysiological studies is that iontophoretic application of PEA with low currents potentiates subsequent cortical neuron responses to NA (Paterson and Boulton, 1988). Metabolites of PEA do not have this effect, nor is the effect abolished by electrolytic lesions of the locus coeruleus, or pretreatment with reserpine, DMI or α-methyl-*p*-tyrosine (an inhibitor of catecholamine synthesis) indicating that endogenous NA or NA presynaptic terminals are probably not involved (Paterson, 1988a,b). Extraneuronal uptake of NA and the subsequent release of this pool of NA by PEA has also been ruled out as PEA fails to inhibit

the sodium-independent uptake of NA into astrocytes (Paterson and Hertz, 1989). Electrophysiological studies have indicated that PEA also potentiates neuronal responses to iontophoretically applied DA (Jones and Boulton, 1980; Paterson *et al.*, 1990). The ionophoretic application of PEA has no effect on the spontaneous firing rate or neuronal responses to glutamate, GABA, acetylcholine or 5-HT. The above data have lead to a proposed model of PEA as a neuromodulator of catecholamine transmission (Paterson *et al.*, 1990). This model suggests that PEA may act as an allosteric activator of postsynaptic catecholamine receptors. At present there is no direct evidence to support this model.

Administration of PEA to animals results in behavioral effects that are similar, but not identical, to those caused by amphetamine administration. It has been well documented that PEA administration can cause decreases in feeding behavior, increases in locomotor behavior and stereotypical behavior patterns (Jackson, 1975, 1978, 1988; Danielson *et al.*, 1976; Moja *et al.*, 1976; Borison *et al.*, 1977; Dourish and Boulton, 1981; Dourish, 1982, 1985; Dourish *et al.*, 1983; Ortmann *et al.*, 1984; Popplewell *et al.*, 1985). These effects could be mediated by presynaptic release of DA as they are only evident after high doses of PEA or after administration of PEA in combination with a MAO inhibitor. Chronic administration of PEA, like chronic administration of amphetamine, also results in behavioral supersensitivity to the drug (Borison and Diamond, 1978; Dourish, 1981; Kuruki *et al.*, 1990). PEA has a reinforcing effect in animals as observed in place-preference, self-administration and self-stimulation studies and it has been suggested that endogenous PEA may play a role in brain reinforcement processes (Yokel and Pickens, 1973; Risner and Jones, 1977; Gilbert and Cooper, 1983; Greenshaw, 1984, 1988; Greenshaw *et al.*, 1985). PEA may differ from amphetamine in that it lacks the aversive properties seen with amphetamine in the conditioned taste aversion paradigm using rats (Greenshaw and Dourish, 1984),

although another study using different conditions found a transient taste aversion to PEA with mice (Kutscher, 1986).

A.6 RATIONALE OF EXPERIMENTS

In this section a brief description of the purpose and form of each experiment in this thesis is given.

A.6.1 Quantification of phenylalanine

A novel gas chromatographic (GC) procedure was developed for the analysis of phenylalanine in brain tissue. This method was initially applied to whole brain samples from control rats and rats treated with phenylalanine methyl ester HCl. Subsequently the regional distribution of phenylalanine was determined in drug-naive rats. The procedure was also used in subsequent experiments in this thesis and details of the assay method are given in section B.4.

A.6.2 Effects of treatments that alter PEA availability on phenylalanine concentrations

Various pharmacological manipulations alter the accumulation of PEA after inhibition of MAO with DEP. Reserpine, which depletes DA stores,, increases the DEP-induced accumulation of PEA in the caudate nucleus (Juorio *et al.*, 1988). By contrast, lesions of the DA-containing neurons of the nigrostriatal pathway decrease the accumulation of PEA in rat caudate nucleus after DEP (Greenshaw *et al.*, 1986). To address the possibility that these changes in PEA accumulation may be secondary to altered concentrations of phenylalanine, levels of phenylalanine were measured in the rat caudate nucleus after reserpine treatment or 6-OHDA lesions of the substantia nigra. Both experiments were done in the absence or presence of MAO inhibition. In addition to phenylalanine, levels of DA,

DOPAC, HVA, 5-HT and 5-HIAA were measured to verify the effectiveness of reserpine treatment or of the 6-OHDA-induced lesions.

A.6.3 Effects of long-term (28 day) administration of antidepressants and PEA on rat brain levels of PEA

One objective of this thesis was to assess receptor changes after long-term treatment with antidepressant drugs and after long-term elevation of brain PEA levels. Brain PEA levels were, therefore, determined after each antidepressant treatment used and after treatment with either DEP, PEA or the combination of these two treatments. PEA levels were quantitated in whole brain tissue less the cortex and caudate nuclei which were used for receptor binding analysis.

A.6.4 Efficacy of long-term (28 day) drug administration

MAO activity was determined after chronic treatment with various MAO inhibitors to confirm the efficacy of drug treatment. After long-term treatment with DMI, brain levels of the drug were quantitated. MAO activity and DMI levels were determined in brain samples equivalent to those used for determining PEA levels.

A.6.5 Functional analysis of β-adrenoceptors

A.6.5.1 Effects of long-term (21 day) antidepressant and PEA treatment on the behavioral response to salbutamol

A consequence of chronic treatment with most antidepressants is the emergence of a decrease in the density of cortical β-adrenoceptors (for review, Baker and Greenshaw, 1989). In addition to directly measuring changes in β-adrenoceptor density, this phenomenon has also been assessed in behavioural tests with an agonist such as salbutamol. It has recently been reported that chronic treatment with the MAO inhibitor PLZ reduces behavioral response to

salbutamol in rats (McManus and Greenshaw, 1991b; McManus *et al.,* 1991). It has been proposed that PEA, a substrate for MAO (Yang and Neff, 1973) and also a metabolite of PLZ (Baker *et al.,* 1982, Dyck *et al.,* 1985), may be one mediator of the effects of chronic PLZ on β-adrenoceptor function (McManus *et al.,* 1991). This hypothesis was based on the following observations by McManus *et al.* (1991). Chronic doses of 5 and 10 mg kg^{-1} per day of PLZ sulphate resulted in equipotent inhibition of MAO but only the higher dose reduced the behavioral response to salbutamol. Additionally, differential effects of these PLZ doses on liver levels of PEA have been observed, with only the higher dose resulting in a significant increase in PEA levels. The present study was conducted to assess the proposed role of increased brain PEA concentrations in functional down-regulation of β-adrenoceptors. The behavioral response to salbutamol was examined after chronic elevation of PEA concentrations in rats. PEA was administered chronically (21 days) to rats alone and in combination with a low dose of the MAO inhibitor DEP. Other groups of animals were treated chronically (21 days) with DEP, PLZ or vehicle treatment.

A.6.5.2 Effects of long-term (28 day) antidepressant treatment on the salbutamol-induced changes in amino acid concentrations in brain and plasma

Recent studies have indicated that the relative concentrations of large neutral amino acids (LNAAs) in brain and plasma may be regulated by an adrenoceptor-mediated mechanism (Eriksson *et al.,* 1984; Eriksson and Carlsson, 1988). The LNAAs, which include phenylalanine, tyrosine, tryptophan, leucine, isoleucine and valine, are subject to active transport across the blood-brain barrier by a common carrier (Pardridge and Oldendorf, 1975; Pardridge, 1977). Altered availability of phenylalanine, tyrosine and tryptophan, which are precursors of

various neuroactive amines, may be relevant to neural function under both normal and pathological conditions. It is evident that altered availability of these amino acids may be important in the context of normal cases such as prolonged exercise (Conlay *et al.*, 1989) and pathological states such as affective disorders (Birkmayer *et al.*, 1984; van Praag and Lemus, 1986). Furthermore, manipulation of L-tryptophan or L-phenylalanine levels in humans has been reported to alter mood (Birkmayer *et al.*, 1984; Young *et al.*, 1985; van Praag and Lemus, 1986; Delgado *et al.*, 1990).

Previous studies have demonstrated that administration of β-adrenoceptor agonists isoproterenol, salbutamol and clenbuterol reduced rat plasma levels of LNAAs while concurrently increasing their brain concentrations (Eriksson and Carlsson, 1988; Edwards and Sorisio, 1988; Edwards *et al.*, 1989). In the study by Eriksson and Carlsson (1988) the response of all LNAAs to the non-selective β-adrenoceptor agonist isoproterenol was measured. The studies concerning selective β_2-adrenoceptor agonists (Edwards and Sorisio, 1988; Edwards *et al.*, 1989), however, focussed only on tyrosine and tryptophan in brain, leaving it unclear whether the salbutamol-induced effects were related to a global alteration in LNAA content in brain and plasma.

The behavioral effects of salbutamol are altered by chronic antidepressant treatment [Section A.3.1.1]. In view of this, it is possible that chronic treatment with antidepressants may affect brain LNAA availability. The present study was undertaken to examine the actions of the nonselective β-adrenoceptor agonist isoproterenol and of the β_2-adrenoceptor agonist salbutamol on whole brain and plasma concentrations of each of the six LNAAs described above and to determine whether the changes induced by salbutamol are altered by long-term administration (28 days) of antidepressant drugs. For this purpose PLZ and DMI

were chosen as representatives of MAO inhibitors and tricyclic antidepressant drugs, respectively.

A.6.6 Radioligand binding to β-adrenoceptors

A.6.6.1 Effects of long-term (28 day) antidepressant drug treatment

Most studies examining cortical β-adrenoceptors after long-term treatment with antidepressant drugs have used ^{3}H-DHA to label the receptors and recent work has questioned the specificity of this ligand [Section A.3.1.1]. The effects of long-term administration of PLZ, DMI and tranylcypromine (TCP) on total cortical β-adrenoceptors were assessed using the new more selective β-adrenoceptor ligand ^{3}H-CGP 12177.

Early studies indicated that the β_1-adrenoceptor subtype is decreased in cortex after long-term antidepressant treatment. Nevertheless, some evidence points toward β_2-adrenoceptor-mediated changes after long-term antidepressant treatment [Section A.3.1.1]. In view of this the effects of PLZ, DMI and TCP on cortical β_1 and β_2 subtypes of adrenoceptors were also studied. Competition studies for ^{3}H-CGP 12177 binding with the selective β_1 antagonist ICI 89406 were conducted.

Rat cortex predominantly contains β_1-adrenoceptors, whereas rat cerebellum predominantly contains β_2-adrenoceptors. Binding to cerebellar β-adrenoceptors was therefore carried out after long-term treatment (28 days) with PLZ, DMI and TCP. Total β-, β_1- and β_2-adrenoceptors were assessed.

A.6.6.2 Effects of long-term (28 day) administration of PEA and/or DEP

Brain concentrations of PEA are greatly increased by MAO inhibitors and PEA has been proposed to be a neuromodulator of the NA system [Section A.5.3]. The effects of long-term treatment (28 days) with PEA alone or in combination with

DEP on cortical β-adrenoceptors were examined. The total population of β-adrenoceptors was initially examined and subsequently the effects of the drug treatments on subtypes of β-adrenoceptors were examined. This was done in parallel to the studies on the effects of long-term antidepressant treatment [Section A.6.6.1] and β-adrenoceptors were studied in cortex and cerebellum.

A.6.7 Radioligand binding to DA receptors

A.6.7.1 Effects of long-term (28 day) antidepressant drug treatment

At present there are only relatively selective radioligands for D_1 and D_2 receptors as initially defined by pharmacological studies. The ligand ^{3}H-SCH 23390 has been used to label D_1-like receptors, whereas ^{3}H-spiperone has been used to label D_2-like receptors. Few studies have examined the effects of long-term administration of antidepressant drugs on DA receptor binding [Section A.3.2], and MAO inhibitors have largely been ignored. The effects of long-term administration (28 days) of PLZ, DMI and TCP on ^{3}H-SCH 23390 and ^{3}H-spiperone binding have therefore been examined in the studies described in this thesis. Rat striatum was chosen as the tissue of choice as it has a high density of DA receptors of the D_1 and D_2 subtypes.

A.6.7.2 Effects of long-term (28 day) administration of PEA and/or DEP

Concentrations of PEA are greatly increased by MAO inhibitors and this amine has been proposed to be a neuromodulator of the DA system [Section A.5.3]. Therefore an attempt was made to examine the effects of long-term treatment (28 days) with PEA alone or in combination with DEP on striatal DA receptors. This was done in parallel to the studies on the effects of long-term antidepressant treatment on DA receptors in striatum [Section A.6.7.1].

B. MATERIALS AND METHODS

B.1 CHEMICALS

Table 1: Chemicals used in the studies described in this thesis.

Chemicals	Suppliers
acetic acid - glacial	British Drug Houses [BDH] (Toronto, ON)
acetic anhydride	Caledon Laboratories (Georgetown, ON)
acetonitrile, HPLC grade distilled-in-glass	BDH
L-alanine	Aldrich (Milwaukee, WI)
alprenolol HCl	Sigma (St. Louis, MO)
γ-aminobutyric acid	Aldrich
ascorbic acid	Fisher Scientific (Edmonton, AB)
benzoyl chloride	Aldrich
benzylamine HCl	Sigma
bovine serum albumin	Sigma
(+)-butaclamol HCl	Research Biochemicals Inc. [RBI] (Natick, MA)
[^{3}H]-CGP 12177	Amersham Canada Ltd. (Oakville, ON)
calcium [illegible]ride	Fisher Scientific
chlor[illegible], reagent grade	Fisher Scientific
cupric sulfate	Fisher Scientific

deoxycholate	Fisher Scientific
(-)-deprenyl HCl	RBI
desmethylimipramine HCl	Sigma
dicyclohexylcarbodiimide	Aldrich
diethyl ether	BDH Chemicals
di(2-ethylhexyl) phosphoric acid	Sigma
3,4-dihydroxyphenylacetic acid	Sigma
dopamine HCl	Sigma
epinine HCl	Sigma
ethyl acetate	BDH
ethylenediamine tetraacetate, disodium salt	Fisher Scientific
o-fluorophenylalanine	Sigma
folin - phenol reagent	Sigma
glycine	Sigma
homovanillic acid	Sigma
hydrochloric acid, 37-38%	Fisher Scientific
5-[2-^{14}C]-hydroxytryptamine binoxalate	New England Nuclear [NEN] Products, Dupont (Boston, MA)
5-hydroxyindole-3-acetic acid	Sigma
5-hydroxytryptamine creatinine sulfate	Sigma
6-hydroxydopamine HCl	Sigma

ICI 89406	Imperial Chemical Industries (Mississauga, ON)
isobutyl chloroformate	Aldrich
L-isoleucine	Sigma
isopropanol	BDH
isoproterenol HCl	Sigma
ketanserin (+)-tartrate	RBI
L-leucine	Sigma
magnesium chloride	Fisher Scientific
maprotiline	Ciba-Geigy (Summit, NJ)
methoxyflurane	MTC Pharmaceuticals (Mississauga, ON)
D,L-norleucine	Aldrich
pentafluorobenzyl alcohol	Aldrich
pentofluorophenol	Aldrich
perchloric acid, 60%	Fisher Scientific
phenelzine sulfate	Sigma
L-phenylalanine	Sigma
L-phenylalanine methyl ester HCl	Sigma
2-[ethyl-1-^{14}C]-phenylethylamine HCl	NEN
2-phenylethylamine HCl	Sigma
phosphoric acid, 85%	Fisher Scientific

potassium biphosphate	J.T. Baker Chemicals Co. (Phillipsberg, NJ)
potassium carbonate anhydrous	Fisher Scientific
potassium chloride	Fisher Scientific
prazosin HCl	Sigma
reserpine	Sigma
salbutamol hemisulfate	Sigma
SCH 23390 (N-methyl-[^{3}H])	NEN Products
scintillation fluid (Ready Safe)	Beckman Instruments Inc. (Edmonton, AB)
sodium bicarbonate	Fisher Scientific
sodium carbonate anhydrous	Fisher Scientific
sodium chloride	Fisher Scientific
sodium hydroxide	Fisher Scientific
sodium phosphate, dibasic, anhydrous	Fisher Scientific
sodium phosphate, monobasic	Fisher Scientific
sodium potassium tartrate	Allen & Hanbury's (Toronto, ON)
spiperone (benzene ring-[^{3}H]-)	NEN
toluene, glass-distilled	BDH
toluene, reagent grade	BDH
(±)-tranylcypromine HCl	Sigma
tri-n-octylamine	Sigma

Tris (hydroxymethyl) aminomethane	Fisher Scientific
L-tryptophan	Raylo Chemicals Ltd. (Edmonton, AB)
p-tyrosine	Fisher Scientific
L-valine	Sigma

B.2 INSTRUMENTATION AND APPARATUS

B.2.1 Gas Chromatography (GC)

For phenylalanine level determinations a Hewlett-Packard (HP, Palo Alto, CA, U.S.A.) 5880 gas chromatograph equipped with a fused silica column, an electron-capture detector (ECD) with a radioactive source of 15 mCi ^{63}Ni and an HP 5880A integrator were used. The carrier gas, helium, was set at a flow rate of 2 ml/min. Argon-methane (95%-5%), flow rate 35 ml/min, was the make-up gas used in the detector. The injection port temperature was 200°C and the detector temperature was 325°C.

For determinations of valine, leucine and isoleucine amino acid levels and PEA levels a HP 5890 gas chromatograph equipped with a fused silica column, an ECD with a radioactive source of 15 mCi ^{63}Ni, a HP 7673A automatic sampler and an HP 3392A integrator was used. Gases, injection port temperature and detector temperature were as described in the previous paragraph.

For tricyclic antidepressant level determinations a HP Model 5890 gas chromatograph equipped with a fused silica column and a nitrogen phosphorus detector linked to a HP 3392A integrator was used. The carrier gas was pure helium at a flow rate of 3.0 ml/min. The detector was purged with pure hydrogen

(3.5 ml/min) mixed with dry air at 80 ml/min. The injection port temperature was 200°C and the detector temperature was 325°C.

B.2.2 Mass spectrometry (MS)

Chemical structures of the derivative of phenylalanine were confirmed by combined GC-MS. The mass spectrometer was a HP 5985A. The system also consisted of a HP 5840A gas chromatograph as the inlet, a HP 2648A graphics terminal, a HP 9876A printer, a HP 7920 disc drive and a HP 21 MX Series E computer. Operating conditions were as follows: ion source temperature, 200°C; interface temperature, 275°C; column pressure, 34.5 kPa; accelerating voltage, 2200 V; ionization energy, 70 eV; scan speed, 100 amu s^{-1}; dwell time, 200 ms. Methane was used as the reactant gas in the chemical ionization analysis. The capillary column was the same as that used in the gas chromatographic procedure.

B.2.3 High pressure liquid chromatography (HPLC)

For tryptophan, tyrosine, DA, DOPAC, HVA, 5-HT and 5-hydroxyindole acetic acid (5-HIAA) level determinations, a HPLC system consisting of a solvent delivery system (Model 510, Waters Associates, Milford, MA, U.S.A.) coupled to an automatic injector (WISP, Waters model 710B) was used. Compounds of interest were separated on an Econosphere C_{18} column (4.6 mm i.d. x 250 mm, 5 μm particle size, Applied Science Labs, Avondale, PA). A precolumn (4.6 mm i.d. x 30 mm) with the same packing material as that in the analytical column was used. Eluants from the column were detected by an electrochemical detector (model 460, Waters) with the applied potential set at 0.90 volt. Chromatographic peaks were recorded and integrated using a model 740 integrator (Waters). The mobile phase, pumped at a flow rate of 0.7 ml/min, consisted of 55 mM sodium

phosphate monobasic, 0.73 mM sodium octyl sulfate, 0.37 mM disodium EDTA and 6.5% v/v acetonitrile: the pH was adjusted to a value of 2.75 with phosphoric acid.

B.2.4 Stereotaxic equipment

The rat stereotaxic instrument was obtained from David Kopf Instruments (Tujunga, CA).

B.2.5 Locomotor activity monitoring system

The activity monitoring system (Acadia Insts. Ltd., Saskatoon, SK) consisted of six 17" x 17" x 12" arcrylic test cages each placed in a 12 x 12 beam infra-red grid system (Acadia Infra-red Grid Model 17-12 with vertical sensors). The test cages' sensors were interfaced with a microcomputer system (Acadia 6502 Data Gatherer) for data-logging and temporal analysis of activity counts.

B.2.6 Centrifuges

A Sorvall GLC-2B or Sorvall GLC-1 General Laboratory Centrifuge (Dupont Instruments, Wilmington, DE) was used for low-speed, small volume centrifugations. Higher speed and/or larger volume centrifugations were carried out in a Damon-IEC B-20 (Needham Heights, MA) refrigerated high-speed centrifuge or a Beckman L755 (Palo Alto, CA) vacuum refrigerated ultracentrifuge.

B.2.7 Filtration

A Brandel Cell Harvester (Gathersberg, MD) equipped with Whatman GF/C filters was used for the filtration step in receptor binding assays.

B.2.8 Liquid scintillation spectrometry

A Beckman LS 7500 liquid scintillation spectrometer coupled to a Datamex 43 printer was used for counting radioactivity in all *ex vivo* receptor binding and MAO inhibition studies.

B.2.9 Ultraviolet spectrophotometer

A Pye Unicam SP 1700 (Cambridge, UK) ultraviolet spectrophotometer was used for determination of protein concentrations in receptor binding homogeneities.

B.2.10 Tissue homogenizer

A combination of a TRI-R S63C (TRI-R Instruments, Rockville Center, NY) variable speed laboratory motor with a Teflon® glass pestle and a glass grinding tube was used for homogenizing tissue samples.

B.2.11 Shaker-Mixer

Two types of vortex-shakers were used: Ika-Vibrax VXR2 Shaker (Janke and Kunkel GMBH and Co., Germany) and a thermolyne Maxi Mix vortex mixer (Thermolyne Corp., Dubuque, IO).

B.2.12 Weighing balances

A Mettler AE 160 (Zurich, Switzerland) electronic balance was used for weighing chemicals and biological samples.

B.2.13 Glassware cleaning

All glassware was rinsed with tap water and washed out with biodegradable Sparkleen (Fisher Scientific Co.) solution. Further washing was accomplished with

a dishwasher (Miele Electronic 6715, Germany). For test tubes, an additional cleaning step was added; test tubes were sonicated (Ultra-sonic cleaner, Mettler Electronics) in a solution of Decon 75 concentrate (BDH Chemicals) before the dishwasher wash. All glassware was then air-dried in a mechanical convection oven (Model 28, Precision Scientific Group, Chicago, IL).

B.3 ANIMALS

Male Sprague-Dawley rats (200-250 g) were obtained from Bio-Science Animal Services, Ellerslie, Alberta. The animals were housed in plastic cages on cedar chip bedding in a room with an alternate 12 h light/dark schedule (lights on 7:30 am); the temperature of the room was maintained at 21 ± 1°C. Food and water were freely available. The animal feed (Lab-Blox feed, Wayne Feed Division, Continental Grain Company, Chicago, IL, U.S.A.) was 4.0% (min) crude fat, 4.5% (max) crude fibre and 24% (min) crude protein. Procedures involving the use of rats were approved by the University of Alberta Health Sciences Animal Welfare Committee and were conducted according to the guidelines established by the Canadian Council on Animal Care.

B.3.1 Drug administration

For long-term drug administration animals were randomly allocated to drug or vehicle treatment conditions. Each animal was deeply anesthetized with methoxyflurane, as an inhalant, and under aseptic conditions Alzet osmotic minipumps (2ML4) were implanted subcutaneously in the interscapular region. Each pump was filled with a drug solution individually adjusted in concentration (Greenshaw, 1986) or distilled water vehicle according to each animal group allocation to provide constant infusion. The incisions were closed with wound clips and after recovery the animals were placed in normal housing conditions and

handled approximately every second day. All long-term drug treatment in this study was for 28 days. Doses of these drugs reported in this study are in mg kg^{-1} per d of the respective salt of the drug [PLZ sulphate, DMI HCl, DEP HCl, TCP HCl, PEA HCl].

For acute drug treatment with phenylalanine methyl ester HCl, the compound was dissolved in 0.9% saline. All injections were performed intraperitoneally (i.p.) and in a volume of 2 ml kg^{-1}. For acute drug treatment with DEP the drug was dissolved in 0.9% saline and administered subcutaneously (s.c.) in a volume of 1 ml kg^{-1}. Reserpine was dissolved with 50-100 μl of glacial acetic acid and diluted with isotonic glucose solution. Reserpine was injected s.c. and in a volume of 1 ml kg^{-1}. Salbutamol hemisulphate and isoproterenol HCl were dissolved in 0.9% saline, administered i.p. and in a volume of 1 ml kg^{-1}. In each study the control animals were injected with the corresponding vehicle solution.

B.3.2 Sample collection and storage

Following drug administration, animals were killed by guillotine decapitation, approximately 5 ml of blood collected if needed and the brains rapidly removed. If no further dissection was necessary the brain was immediately frozen in isopentane over solid carbon dioxide. All brain dissection was done as quickly as possible over ice and the tissue frozen over solid carbon dioxide. Blood was collected in a Corex tube containing 0.5 ml of 1 % EDTA solution. The blood was then centrifuged at 1500 x g for 10 min and the plasma removed. Plasma and brain samples were stored at -80°C until the time of analysis.

B.4 ANALYSIS OF PHENYLALANINE IN RAT BRAIN AND PLASMA

A novel GC procedure was developed for the analysis of phenylalanine. Brain tissues were homogenized in 5 volumes of ice-cold 0.1 M perchloric acid

containing 10 mg% EDTA and 0.05 mM ascorbic acid. The homogenates were centrifuged at 8000 x g for 10 min at 4°C to remove the protein precipitate. To 1 ml of each plasma sample, 2 ml of 0.4 M perchloric acid containing 40 mg% EDTA and 0.20 mM ascorbic acid were added. Samples were then vortexed and centrifuged as described above. A 700 μl aliquot of the supernatant from brain or plasma was used for the analysis. o-Fluorophenylalanine (5.0 μg) was added as an internal standard to all samples. Each sample was made basic with 75 μl 25% potassium carbonate, and 700 μl benzoyl chloride solution (5 μl/ml toluene/acetonitrile, 9:1, v/v) were added to each. The samples were shaken for 10 min, transferred to 1.5 ml conical polypropylene microcentrifuge tubes with snap-on caps (Bio Plas, San Francisco, CA, U.S.A.) and centrifuged to separate the aqueous and organic phases. After separation, the organic layers were removed by aspiration. The remaining aqueous layers were transferred into a set of glass test tubes and 1.5 ml 2 M sodium phosphate buffer (pH 5.2) followed by 50 μl of 6 M HCl was added to each tube. After brief mixing, 1 μl dicyclohexylcarbodiimide, a coupling agent, and 1 μl pentafluorobenzyl alcohol dissolved in 3 ml of chloroform were added to each tube. The samples were shaken for 15 min and then centrifuged, after which the aqueous layers were aspirated off and the remaining organic layers transferred to a clean set of tubes and each sample was taken to dryness under a steady stream of nitrogen. The residues were redissolved in 300 μl toluene and washed briefly with 500 μl distilled water. Aliquots of the toluene layers were used for GC analysis. Chromatographic separation was accomplished using the following automatic oven temperature program: initial temperature of 140°C for 0.5 min, increasing at a rate of 8°C/min to 220°C where it was held for 8.5 min. The column used was a fused silica capillary column (SP-2100, 0.25 mm i.d. x 25 m, 0.25 μm film thickness, Supelco,

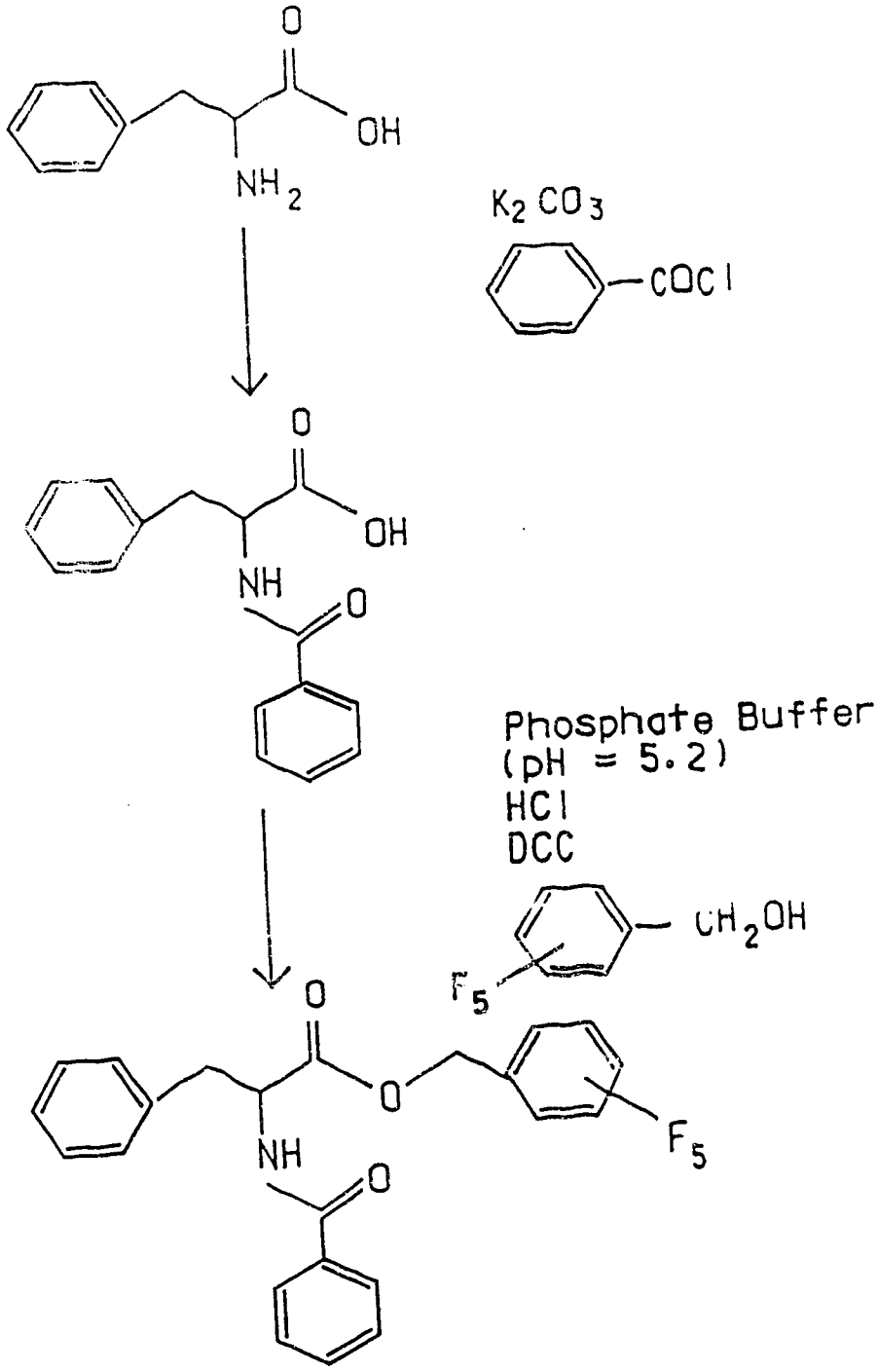


Figure 6: Flowchart of derivatizing procedure for analyzing phenylalanine in rat brain and plasma. The resultant derivative was detected by GC-ECD.

Inc., Bellafonte, PA, U.S.A.). A flow diagram of the procedure is shown in Figure 6.

In this and the other GC and HPLC procedures used in this thesis, a standard (calibration) curve was prepared with each assay run to permit quantification of the drug or neurochemical of interest in the brain or plasma homogenate supernatants. This curve was constructed by adding known, varying amounts of authentic standard and a fixed amount of internal standard (same amount as added to the supernatants) to a series of tubes and carrying these tubes through the assay procedure in parallel with the sample tubes. The peak height ratios of each neurochemical to the internal standard was determined at several concentrations and plotted. The corresponding peak height ratios from the homogenate supernatants were then plotted on this curve to determine the quantity of neurochemical present.

B.5 ANALYSIS OF VALINE, LEUCINE AND ISOLEUCINE IN RAT BRAIN AND PLASMA

A GC assay developed by Wong *et al.* (1990) was used for the analysis of valine, leucine and isoleucine in rat brain and plasma. A 25 μl aliquot of the supernatant from brain or plasma from section B.4 was used for the analysis. Norleucine (0.25 μg) was added as an internal standard to all samples. To each sample 1 ml 2.5% potassium carbonate and 1 ml isobutyl chloroformate solution (5 μl/ml toluene/acetonitrile, 9:1, v/v) were added. The samples were shaken for 10 min and centrifuged to separate the aqueous and organic phases. After separation, the organic layers were removed by aspiration. The remaining aqueous layers were transferred into a set of glass test tubes and 1.5 ml 2 M sodium phosphate buffer (pH 5.2) followed by 50 μl of 6 M HCl were added to each tube. After brief mixing, 1 μl dicyclohexylcarbodiimide and 1 μl

pentafluorophenol dissolved in 3 ml of chloroform were added to each tube. The samples were shaken for 15 min and then centrifuged, after which the aqueous layers were removed and the remaining organic layers transferred to a clean set of tubes and each was taken to dryness under a steady stream of nitrogen. Each residue was redissolved in 300 μl toluene and washed briefly with 500 μl distilled water. Aliquots of the toluene layers were used for GC analysis. Chromatographic separation was accomplished using the following automatic oven temperature program: initial temperature of 100°C for 0.5 min, increasing at a rate of 25°C/min to 200°C where it was held for 0.5 min, increasing at a rate of 3°C/min to 230°C where it was held for 5 min. The chromatograph column used was a fused silica capillary column, crosslinked 5 % phenylmethylsilicone phase (0.31 mm i.d. x 25 m, 1.03 μm film thickness, HP).

B.6 ANALYSIS OF TYROSINE, TRYPTOPHAN, DA, 5-HT AND ACID METABOLITES IN RAT BRAIN AND PLASMA

A modification of an existing HPLC method (Baker *et al.*, 1987) was used to analyze brain levels of tryptophan and tyrosine. This involved altering the concentrations of the ion-pairing reagent and the organic modifier in the mobile phase and increasing the applied voltage on the detector so that tyrosine and tryptophan can be included in the analysis (see section B.3 for details). T aliquots of the clear supernatant obtained in section B.4 200 ng of the internal standard epinine were added. The aliquots were then each injected into the HPLC system described in section B.3. This procedure was also used for the analysis of DA, DOPAC, HVA, 5-HT and 5-HIAA.

B.7 6-OHDA-INDUCED LESIONS IN RATS

Rats were deeply anesthetized with pentobarbital (initial dose 50 mg kg^{-1} i.p.) and placed on a stereotaxic frame with the incisor bar set at 2.4 mm below the interaural line. The right substantia nigra was targeted with the coordinates based on the rat brain atlas of König and Klippel (1963): 3.0 mm anterior, 2.0 mm lateral to the interaural zero and 8.0 mm ventral to the skull surface. After a small hole was made in the skull, the rats received an infusion of 6-OHDA (8 μg free base in 0.9% saline containing 0.1 % ascorbic acid) by a 10 μl Hamilton syringe connected through polyethylene tubing to a 28 gauge internal cannula with the tip at the above coordinates. The total infusion volume was 4 μl which was delivered over a period of 4 min with the cannula left in place for a further 2 min to allow diffusion of the neurotoxin. Another group of animals went through the same procedure but received sham lesions where only 0.9% saline containing 0.1% ascorbic acid was infused into the substantia nigra. The incisions were then cleaned and sutured and the animals were then kept warm under a heat lamp until they recovered, after which they were returned to their home cages.

B.8 ANALYSIS OF PEA IN RAT BRAIN

A modification of the GC assay developed by Baker *et al.* (1986) was used for the analysis of PEA in rat brain. A 3 ml aliquot of the supernatant from brain (section B.4) was used for the analysis and 125 ng of benzylamine was added as an internal standard to all samples. The samples were basified by the addition of solid potassium bicarbonate and the precipitate removed by a brief centrifugation. The resultant samples were transferred to another set of tubes, one-tenth the volume of sodium phosphate buffer (pH=7.8) was added, and the samples were extracted by shaking with 5 ml of chloroform containing the liquid ion-pairing reagent di(2-ethylhexyl)phophoric acid (DEHPA, 2.5%, v/v). Following a brief

centrifugation, the top aqueous layers were removed by aspiration and the chloroform layers were each shaken vigorously with 4 ml of 0.5 M HCl for 5 min. After centrifuging the acidic aqueous layer was transferred to another set of tubes and basified with solid sodium bicarbonate. A solution (4 ml) of ethyl acetate-acetonitrile-pentafluorobenzenesulfonyl chloride (9:1:0.01) was added to each tube. The samples were shaken vigorously for 15 min and centrifuged; the top organic layers were transferred to a set of clean tubes and taken to dryness under a stream of nitrogen. Each residue was taken up in 300 μl of toluene and an aliquot (1 μl) was used for GC analysis. Chromatographic separation was accomplished using the following automatic oven temperature program: initial temperature 105°C for 0.5 min, increasing at a rate of 15°C/min to 270°C which was maintained for 10 min. The chromatographic column used was a fused silica capillary column, crosslinked 5% phenylmethylsilicone phase (0.31 mm i.d. x 25 m, 1.03 μm film thickness, HP).

B.9 DETERMINATION OF MAO ACTIVITY

Monoamine oxidase (MAO) activity was determined using a modification of the procedure of Wurtman and Axelrod (1963). Rat brain tissues were homogenized in ice-cold isotonic KCl to give a 4% homogenate; 25 μl of this homogenate were added to each test tube and in the case of blank controls, 25 μl of isotonic KCl were added instead. All tubes were placed on ice and to each 250 μl 0.5 M sodium phosphate buffer (pH = 7.4) were added. Aliquots (25 μl) of solutions of ^{14}C-5-HT and ^{14}C-PEA (substrates for MAO type A and type B, respectively), appropriately diluted with respective unlabeled compounds, were added to each tube to give a final concentration of 100 μM of substrate in each. Tubes were then incubated at 37°C for 20 min and after cooling to room temperature, 200 μl of 2M HCl was added to each tube to terminate the reaction. Toluene (6 ml) was added

to all tubes and the mixtures were shaken for 5 min. After brief centrifugation, the tubes were stored at -80°C until the aqueous layers were frozen (> 1 h). The toluene layers were then decanted into scintillation vials into which 9 ml of scintillation fluid was added. Vials were thoroughly mixed and radioactivity was monitored in a liquid scintillation spectrometer. The amount of radioactivity in blank tubes was subtracted from all samples. Values from sample controls were averaged. The radioactivity (in dpm) in the sample was divided by that in controls and the value multiplied by 100 to give % activity. Percent inhibition was determined by subtracting % activities from 100.

B.10 ANALYSIS OF DMI IN RAT BRAIN

A modification of the procedure of Drebit *et al.* (1988) was used. Rat brain tissue was homogenized in 6 volumes of distilled water and a portion (2 ml) of this homogenate was used for analysis. The internal standard, maprotiline (1 μg), was added to the homogenate which was centrifuged 1000 x g for 10 min. The clear supernatant was collected and basified with solid sodium bicarbonate. Acetylation was then carried out using the procedure of Martin and Baker (1977). The acetylated DMI and maprotiline were extracted by shaking with ethyl acetate (5 ml) for 10 min. After a brief centrifugation, the organic phase was transferred to another set of tubes and evaporated to dryness under a stream of nitrogen. The samples were redissolved in toluene (200 μl). Aliquots of these solutions were injected onto a gas chromatograph equipped with a fused silica capillary column [see section B.2.1] and a nitrogen-phosphorus detector. Chromatographic separation was accomplished using the following automatic oven temperature program: initial temperature of 105°C for 0.5 min, increasing at a rate of 25°C/min to 295°C where it was held for 5 min.

B.11 BEHAVIORAL ANALYSIS

The effects on spontaneous locomotor activity of salbutamol, a β-adrenoceptor agonist were assessed in animals that received long-term treatment with vehicle, PLZ, PEA, (-)-deprenyl or a combination of PEA and (-)-deprenyl. A randomly selected half of each long-term treatment group received salbutamol on day 21 and saline on day 22. The other half of each treatment group received saline on day 21 and salbutamol on day 22. 15 minutes following the administration of salbutamol (3 mg kg^{-1} i.p.) the locomotor activity of the animals was measured for 30 min using the apparatus described in section B.2.5. The activity of the rats was measured under conditions of low-intensity illumination and in a quiet environment. At the end of the test period the rats were returned to their respective housing-cages.

B.12 RECEPTOR BINDING ANALYSIS

B.12.1 Membrane preparation

Cerebral cortices, cerebellae or striata were homogenized in 9 ml of the appropriate ice-cold buffer (see Table 2). The homogenate was decanted into centrifuge tubes and buffer was added to yield a total volume of approximately 50 ml. This homogenate was centrifuged at 25000 x g at 4°C for 20 min. The supernatant was discarded and the pellet resuspended in 9 ml of buffer, subsequently made up to 50 ml. The resultant suspension was recentrifuged and washed once again as described above. After the second centrifugation, the final pellet was resuspended in 9 ml of buffer and this suspension used in the binding experiments.

B.12.2 ^{3}H-CGP 12177 saturation binding analysis

For the quantification of total β-adrenoceptor population the ligand ^{3}H-CGP 12177 was used. Saturation binding analyses were performed in triplicate in a

total volume per tube of 1 ml of 50 mM Tris including 100 μl of tissue suspension and 50 to 200 μl of ^{3}H-CGP 12177 (final concentration 0.05 to 2.0 nM). To measure non-specific binding 100 μl of alprenolol (final concentration 10 μM) was used. Tubes were allowed to incubate for 120 min at 25°C, binding was terminated by rapid filtration and the filters were washed with a large excess of ice-cold buffer. Filters were counted for radioactivity in 5 ml of Ready Safe liquid scintilation cocktail at an efficiency of approximately 50%. An outline of ^{3}H-CGP 12177 binding parameters is shown in Table 2.

Data obtained from saturation curves were analyzed using the equilibrium binding data analysis (EBDA) program (McPherson, 1985) to give initial estimates of the K_d and B_{max}. These estimates were then used in further analysis with the non-linear regression analysis program LIGAND (Munson and Rodbard, 1980). The final B_{max} estimate was expressed in fmol mg^{-1} protein.

B.12.3 ^{3}H-SCH 23390 saturation binding analysis

For the quantification of dopamine D_1-like receptor population the ligand ^{3}H-SCH 23390 was used and the buffer consisted of 50 mM Tris (pH=7.4) thatcontained 120 mM NaCl and 5 mM KCl. Saturation binding analyses were performed in triplicate in a total volume per tube of 1 ml of buffer including 100 μl of tissue suspension and 50 to 200 μl of ^{3}H-SCH 23390 (final concentration 0.05 to 2.0 nM). To preclude binding to 5-HT$_2$ sites 100 μl of ketanserin (final concentration 40 nM) were added to all tubes. To measure non-specific binding 100 μl of (+)butaclamol (final concentration 1 μM) were used. Samples were allowed to incubate for 60 min at 25°C were then filtered, washed, and counted. The data were analyzed as described above [Section B.10.2]. An outline of ^{3}H-SCH 23390 binding parameters is shown in Table 2.

Table 2: Conditions for radioligand binding saturation analysis.

BINDING SITE	β-ADRENERGIC	β-ADRENERGIC	DOPAMINE D_1	DOPAMINE D_2
[^{3}H]LIGAND (spec. act.) [Conc.]	CGP 12177 (30 Ci mmol^{-1}) [0.05 - 2.0 nM]	CGP 12177 (36 Ci mmol^{-1}) [0.05 - 2.0 nM]	SCH 23390 (87 Ci mmol^{-1}) [0.05 - 2.0 nM]	Spiperone (23 Ci mmol^{-1}) [0.01 - 0.5 nM]
BUFFER	50 mM Tris (pH=7.4)	50 mM Tris (pH=7.4)	50 mM Tris (pH=7.4) 120 mM NaCl 5 mM KCl 40 nM Ketanserin	50 mM Tris (pH=7.4) 120 mM NaCl 5 mM KCl 10 mM $MgCl_2$ 2 mM $CaCl_2$ 40 nM Ketanserin 40 nM Prazosin
NON-SPECIFIC BINDING DEFINED BY	10 μM *Alprenolol*	10 μM *Alprenolol*	1 μM *(+)Butaclamol*	1 μM *(+)Butaclamol*
TISSUE	cortex	cerebellum	striatum	striatum
PROTEIN/TUBE	500-700 μg ml^{-1}	300-400 μg ml^{-1}	80-120 μg ml^{-1}	80-120 μg ml^{-1}

B.12.4. ^{3}H-Spiperone saturation binding analysis

For the quantification of dopamine D_2-like receptor population the ligand ^{3}H-spiperone was used and the buffer consisted of 50 mM Tris (pH=7.4) that contained 120 mM NaCl, 5 mM KCl, 10 mM $MgCl_2$ and 2mM $CaCl_2$. Saturation binding analyses were performed in triplicate in a total volume per tube of 1 ml of buffer including 100 μl of tissue suspension and 50 to 200 μl of ^{3}H-spiperone (final concentration 0.01 to 0.5 nM). To preclude binding to 5-HT_2 and α_1-adrenergic binding sites, respectively, 100 μl of ketanserin (final concentration 40 nM) and 100 μl of prazosin (final concentration 40 nM) were added to all tubes. To measure non-specific binding 100 μl of (+)butaclamol (final concentration 1 μM) were added. Samples were incubated for 60 min at 25°C and then filtered, washed and counted. The data were analyzed as described above (Section B.10.2). An outline of ^{3}H-spiperone binding parameters is shown in Table 2.

B.12.5 ICI 89406 competition binding analysis

Competition experiments with the drug ICI 89406 competing for ^{3}H-CGP 12177 binding sites were used to determine the β_1 and β_2 adrenoceptor population in cortex and cerebellum. Competition binding analyses were performed in triplicate in a total volume per tube of 1 ml of 50 mM Tris including 100 μl of tissue suspension, 25 to 100 μl of ICI 89406 (final concentration 2.5 to 1000 nM) and 200 μl of ^{3}H-CGP 12177 (final concentration 0.2 nM). Samples were incubated for 120 min at 25°C and then filtered, washed and counted. The data were analyzed as described above [Section B.10.2].

B.12.6 Analysis of protein concentrations

Protein concentrations in rat brain homogenates used for binding analysis were determined according to the procedure of Lowry *et al.* (1951). To an aliquot

(50 μl) of brain homogenate, 750 μl distilled water and 200 μl of membrane digestor (1:1, v/v, 1 M sodium hydroxide:1% sodium deoxycholate) were added. The mixture was vortexed and incubated at room temperature for 10 min. 5 ml of reagent A (1/0.01/0.01, v/v/v, 2% sodium carbonate/1% cupric sulfate/2% sodium potassium tartrate) were added and the samples were mixed by vortexing and incubated for 10 min. Folin reagent (500 μl, 1M) was added, the samples mixed and incubated for a minimum of 30 min. A standard curve was run in parallel with the tissue samples, using bovine serum albumin as protein standard. All samples were run through a spectrophotometer (wave length = 660 nm) to determine protein concentrations.

B.13 STATISTICAL ANALYSIS

Behavioral data were analyzed using the Kruskal-Wallis analysis of variance (ANOVA), followed by the Mann-Whitney U-test where appropriate. The critical 2-tailed probability was $p \leq 0.05$ and the Bonferroni procedure was used to maintain this level with multiple comparisons (Holm, 1979; Krauth, 1988). Non-parametric statistics were used for the behavioral data as these data violated the ANOVA assumption of a homogeneity of variance ($F_{max}=33.11$). All other analysis used ANOVA as described by Winer (1971) and the critical 2-tailed probability was $p \leq 0.05$. Binding experiments examining the effects of antidepressants and PEA on receptors were run in parallel and for financial and ethical reasons the same control group is used in the separate statistical analysis of these experiments.

C. RESULTS

C.1 QUANTIFICATION OF PHENYLALANINE IN RAT BRAIN

The novel GC procedure developed for analysis of brain phenylalanine provided a derivative with good chromatographic properties (typical gas chromatograms are shown in Figure 7). The stability of the derivative also permitted unattended injection with an automatic sampler. The derivative of phenylalanine was shown to be highly sensitive to electron-capture detection, with the minimum detectable quantity in a sample being < 70 pg "on column" (< 0.02 ng/g tissue). Calibration curves constructed using a range of concentrations of phenylalanine were linear (correlation coefficients > 0.99 obtained routinely). A molecular ion was absent in the electron-impact and chemical ionization mass spectra of the derivative, but the significant fragment ions are supportive of the structure shown in Figure 8.

C.1.1 Whole rat brain phenylalanine concentrations

The whole brain phenylalanine concentration from 9 saline-treated rats was 57 ± 2 nmol g^{-1} tissue. In rats treated with phenylalanine methyl ester HCl [(400 mg kg^{-1} (n=5) or 800 mg kg^{-1} (n=6), i.p., 90 min)], whole brain levels of phenylalanine increased to 150 ± 8 nmol g^{-1} and 342 ± 53 nmol g^{-1}, respectively [$F(2,17)=29.71$].

C.1.2 Phenylalanine concentrations in rat brain regions

Regional levels of phenylalanine were determined in untreated rats (n=5) and the values obtained are shown in Table 3. Concentrations of phenylalanine varied between regions [$F(8,35)=4.83$)]. The cerebellum had a significantly higher concentration of phenylalanine than all other areas. The hypothalamus and nucleus

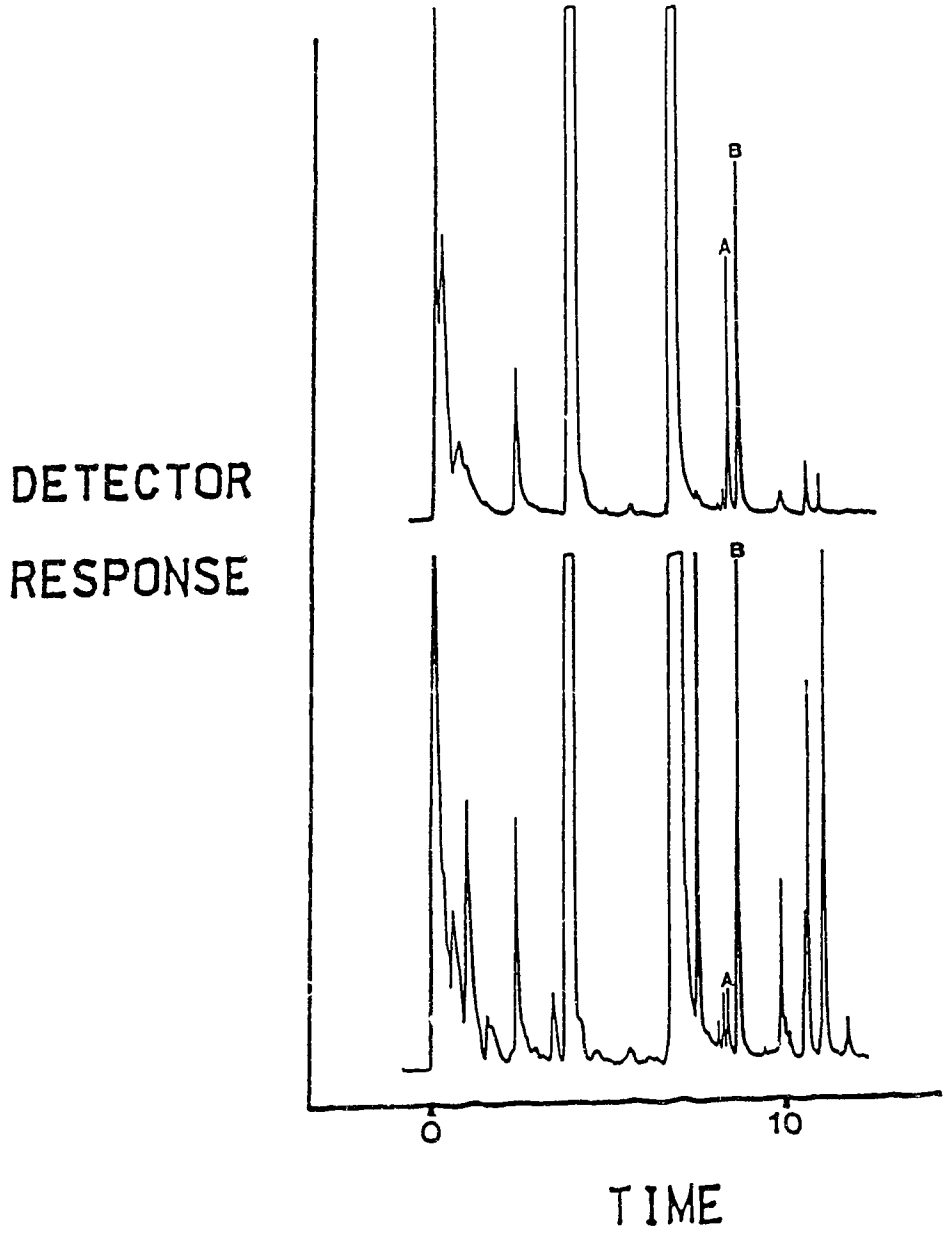


Figure 7: Typical GC traces for a standard sample (top) and an extracted brain sample (bottom). Retention times for derivatized phenylalanine (A) and internal standard (B) are 8.05 and 8.31 min, respectively.

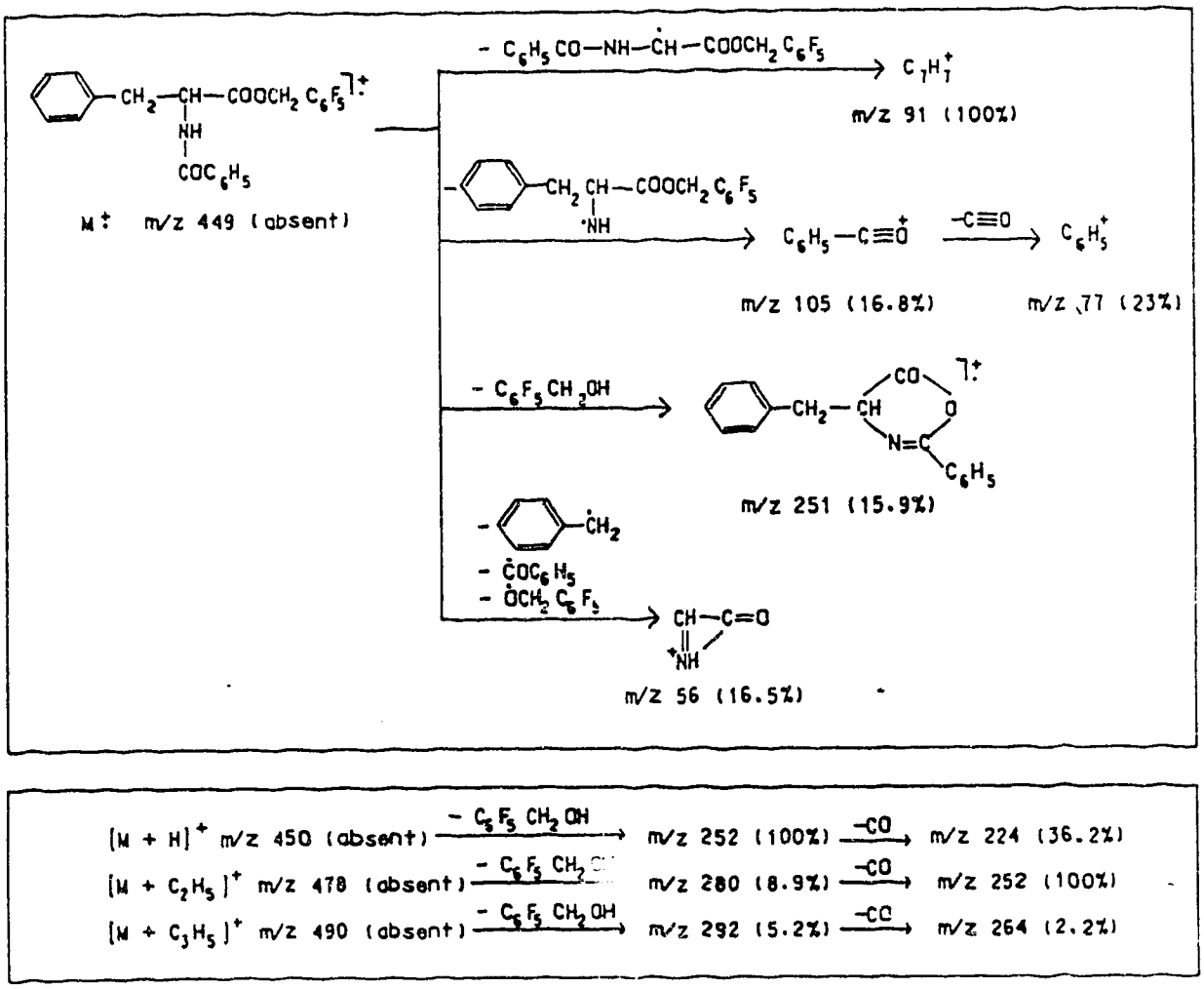


Figure 8: Proposed mass fragmentation pattern (top - electron-impact mode: bottom - chemical ionization mode) of the derivatives of phenylalanine. Values in parentheses represent relative abundance.

Table 3: Regional distribution of phenylalanine in rat brain.

Brain Region	phenylalanine (nmol g^{-1} tissue)
Olfactory Tubercles	54 ± 5
Hippocampus	56 ± 4
Rest of Cortex	56 ± 7
Frontal Cortex	59 ± 3
Brainstem	66 ± 3
Caudate Nucleus	67 ± 2
Hypothalamus	75 ± 3*
Nucleus Accumbens	76 ± 10*
Cerebellum	94 ± 11**

Note: Values are expressed as nmol g^{-1} tissue. **The cerebellum contained significantly more phenylalanine than any other brain region ($p \leq 0.05$). *The hypothalamus and nucleus accumbens had significantly more phenylalanine than the olfactory tubercles, hippocampus and cortex ($p \leq 0.05$).

accumbens also had significantly higher concentrations of phenylalanine than the olfactory tubercles, hippocampus and cortex.

C.2 EFFECTS OF TREATMENTS THAT ALTER PEA AVAILABILITY ON PHENYLALANINE CONCENTRATIONS IN RAT BRAIN

C.2.1 Effects of reserpine

The effects of reserpine (1 or 10 mg kg^{-1}, s.c. 2 h) on caudate nucleus concentrations of amines, their metabolites and phenylalanine are displayed in Table 4. The effects of reserpine after DEP (2 mg kg^{-1}, s.c., 4h) pretreatment on caudate nucleus concentrations of the same neurochemicals are displayed in Table 5. Reserpine is known to increase the DEP-induced accumulation of PEA in the caudate nucleus and this change is also reported in the tables (from Juorio *et al.*, 1988). DA was significantly decreased in animals treated with either 1 or 10 mg kg^{-1} reserpine and this effect was seen in the presence [$F(2,18)=27.20$] or absence [$F(2,18)=38.96$] of DEP pretreatment. Corresponding significant increases in DOPAC and HVA were seen only following administration of 10 mg kg^{-1} reserpine in animals treated with DEP [DOPAC $F(2,18)=23.70$, HVA $F(2,18)=23.20$] and those treated with vehicle [DOPAC $F(2,18)=16.80$, HVA $F(2,18)=12.17$]. 5-HT was significantly reduced and 5-HIAA significantly increased by both doses of reserpine in animals pretreated with deprenyl [5-HT $F(2,18)=7.89$, 5-HIAA $F(2,18=9.96$]. 5-HT was also significantly reduced and 5-HIAA significantly increased in animals pretreated with vehicle followed by 10 mg kg^{-1} reserpine [5-HT $F(2,18)=4.49$, 5-HIAA $F(2,18)=8.32$]. Phenylalanine was unaltered by reserpine treatment in animals pretreated with DEP [$F(2,18)=0.95$] or vehicle [$F(2,18)=0.35$].

Table 4: The effect of reserpine on caudate nucleus levels of amines, their metabolites and phenylalanine in rats.

	Vehicle (n=8)	1 mg kg^{-1} Reserpine (n=6)	10 mg kg^{-1} Reserpine (n=7)
DA	10260 ± 770	8010 ± 420*	3320 ± 300*
DOPAC	1680 ± 60	1600 ± 60	2160 ± 90*
HVA	870 ± 70	990 ± 60	1610 ± 180*
5-HT	450 ± 50	408 ± 20*	290 ± 30*
5-HIAA	550 ± 30	690 ± 40*	750 ± 50*
Phenylalanine	9480 ± 850	8850 ± 860	9770 ± 640
PEA[a]	1.5 ± 0.7	1.9 ± 0.7	
PEA[a]	2.5 ± 0.6		2.0 ± 0.7

Note: Values are expressed in ng g^{-1} tissue, mean ± S.E.M. *Denotes different from to vehicle ($p \leq 0.05$). [a]Values obtained from Juorio et al. (1988). Rats were administered saline (s.c., 4 h) and then reserpine or the appropriate vehicle (s.c., 2 h).

Table 5: The effect of reserpine on caudate nucleus levels of amines, their metabolites and phenylalanine in DEP pretreated rats.

	Vehicle (n=7)	1 mg kg^{-1} Reserpine (n=7)	10 mg kg^{-1} Reserpine (n=7)
DA	11350 ± 460	7730 ± 760*	4630 ± 680*
DOPAC	1200 ± 70	1060 ± 40	1570 ± 50*
HVA	680 ± 40	700 ± 60	1260 ± 90*
5-HT	510 ± 30	390 ± 40*	350 ± 20*
5-HIAA	590 ± 30	570 ± 40	750 ± 20*
Phenylalanine	9620 ± 840	9570 ± 650	10900 ± 820
PEA[a]	11.9 ± 0.5	15.5 ± 1.0*	
PEA[a]	8.8 ± 0.5		17.3 ± 0.4*

Note: Values are expressed in ng g^{-1} tissue, mean ± std. error. *Denotes different from to vehicle ($p \leq 0.05$). [a]Values obtained from Juorio et al. (1988). Rats were administered DEP (s.c., 4 h) and then reserpine or the appropriate vehicle (s.c., 2 h).

C.2.2 Effects of lesions

The effects of 6-OHDA on caudate nucleus concentrations of amines, their metabolites and phenylalanine are displayed in Table 6. The effects of 6-OHDA followed by DEP (2 mg kg^{-1}, s.c., 2h) on the same neurochemicals are displayed in Table 7. 6-OHDA is known to decrease the DEP-induced accumulation of PEA in the caudate nucleus and the degree of this change is also reported in the tables (from Greenshaw *et al.*, 1986). For ease of comparison each value is expressed as a percentage of the concentration of the neurochemical level on the ipsilateral side compared to the contralateral side. Control levels of neurochemicals were similar to those obtained in the reserpine study. 6-OHDA induced a significant depletion of DA [$F(1,10)=127.89$], DOPAC [$F(1,10)=71.37$] and HVA [$F(1,10)=49.13$] and an increase in 5-HIAA [$F(1,10)=5.49$] in vehicle pretreated animals. 6-OHDA also induced a significant depletion of DA [$F(1,10)=91.44$], DOPAC [$F(1,10)=70.75$] and HVA [$F(1,10)=41.36$] in DEP-pretreated animals. 6-OHDA had no significant effect on phenylalanine [$F(1,10)=1.44$] or 5-HT [$F(1,10)=0.14$] in vehicle treated animals; nor did it have any effect on phenylalanine [$F(1,10)=0.14$], 5-HT [$F(1,10)=0.60$] or 5-HIAA [$F(1,10)=0.71$] in DEP-treated animals.

C.3 EFFECTS OF LONG-TERM (28 DAY) ADMINISTRATION OF ANTIDEPRESSANTS AND PEA ON RAT BRAIN LEVELS OF PEA

The effects of chronic drug treatment on brain levels of PEA are displayed in Figure 9. Control levels of PEA were 3.5 ± 0.7 ng g^{-1} tissue. The combination of PEA/DEP and the higher dose of PLZ respectively increased brain levels of PEA to a significant extent [$F(7,76)=21.23$]. The increases in PEA levels following either TCP, DEP or the lower dose of PLZ did not reach statistical significance. Treatment

Table 6: The effect of unilateral 6-OHDA lesions of the substantia nigra on caudate nucleus levels of amines, their metabolites and phenylalanine in rats.

Treatment	DA	DOPAC	HVA	5-HT	5-HIAA	PHE	PEA[a]
Shams (n=5)	94 ± 4	99 ± 2	92 ± 3	98 ± 6	99 ± 3	90 ± 6	75 ± 25
Lesions (n=7)	32 ± 4*	46 ± 5*	46 ± 5*	101 ± 6	128 ± 10*	101 ± 7	64 ± 20

Note: Values are expressed for the ipsilateral side as a percentage of the contralateral side, mean ± S.E.M. *Denotes $p \leq 0.05$ compared to shams. [a]Values obtained from Greenshaw et al. (1986).

Table 7: The effect of unilateral 6-OHDA lesions of the substantia nigra on caudate nucleus levels of amines, their metabolites and phenylalanine in DEP-treated rats.

Treatment	DA	DOPAC	HVA	5-HT	5-HIAA	PHE	PEA[a]
Shams (n=6)	100 ± 6	104 ± 6	102 ± 8	101 ± 6	102 ± 6	121 ± 16	97 ± 3
Lesions (n=6)	32 ± 4*	39 ± 5*	44 ± 4*	107 ± 4	109 ± 5	114 ± 9	55 ± 6*

Note: Values are expressed for the ipsilateral side as a percentage of the contralateral side, mean ± S.E.M. *Denotes $p \leq 0.05$ compared to shams. [a]Values obtained from Greenshaw et al. (1986).

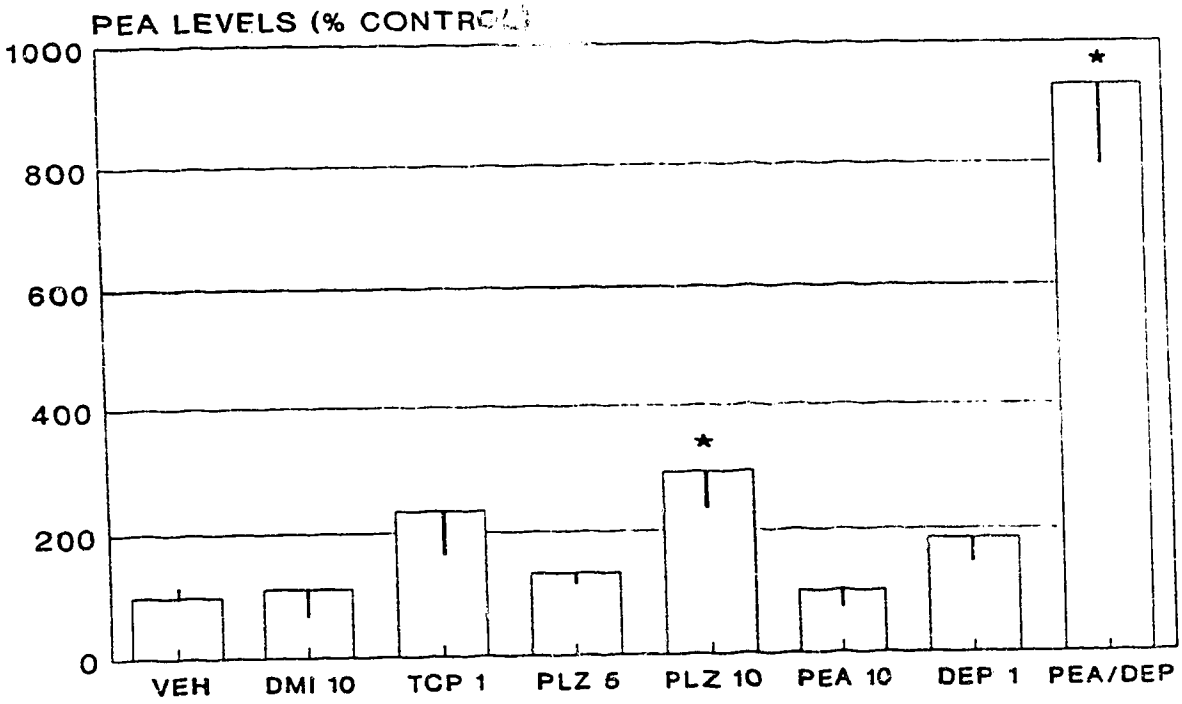


Figure 9: Effects of long-term (28 day) drug treatment on brain levels of PEA.

*Indicates significantly different from vehicle (ANOVA, $p<0.05$).

with PEA did not result in any increase in brain levels of this amine in the absence of a MAO inhibitor.

C.4 EFFICACY OF LONG-TERM (28 DAY) DRUG ADMINISTRATION

C.4.1 Brain MAO inhibition

The degree of MAO inhibition following long-term treatment with antidepressant drugs and PEA is shown in Table 8. As expected, MAO inhibitor drugs significantly inhibited both types of MAO [MAO-A $F(7,79)=208.80$, MAO-B $F(7,79)=156.99$]. Both doses of PLZ had equipotent effects on MAO activity. TCP inhibited MAO to the same extent as PLZ. DEP inhibited MAO-A to a lesser degree than the other MAO inhibitors but was equipotent in its inhibition of MAO-B. Neither PEA nor DMI signficantly inhibited brain MAO activity.

C.4.2 Brain DMI levels

Brain levels of DMI were determined to be 2765 ± 246 ng g^{-1} of tissue in DMI-treated animals ($n=16$). DMI was not detectable in the brains of vehicle-treated animals.

C.5 FUNCTIONAL ANALYSIS OF β-ADRENOCEPTORS

C.5.1 Effects of long-term (21 day) antidepressant and PEA treatment on the behavioral response to salbutamol

Chronic drug treatment did not result in any significant changes in spontaneous locomotor activity after saline injections ($H=7.01$, $p > 0.20$). The effects of salbutamol on spontaneous locomotor activity for each treatment group are displayed in Figure 10. In control animals salbutamol reduced activity to less than 40% of that observed after saline injections. A significant reduction in the

Table 8: The effects of long-term (28 day) treatment with antidepressant drugs and PEA on the activity of MAO in rat brain.

Drug	Dose (mg kg^{-1} per d)	n	**MAO-A** (% inhibition)	**MAO-B** (% inhibition)
VEH	0	19	0 ± 3.9	0 ± 4.5
DMI	10	10	-6.7 ± 4.7	-5.6 ± 5.2
TCP	1	10	90.5 ± 0.6*	90.0 ± 0.9*
PLZ	5	10	93.5 ± 0.7*	88.1 ± 1.2*
PLZ	10	10	94.4 ± 1.1*	87.1 ± 2.5*
PEA	10	9	9.0 ± 3.1	-7.6 ± 5.9
DEP	1	9	68.1 ± 1.7**	83.5 ± 1.1*
PEA/DEP	10/1	10	69.0 ± 1.7**	85.8 ± 0.5*

Note: Values are means ± S.E.M. *Denotes significantly different from vehicle ($p \leq 0.05$). **Denotes significantly different from vehicle and also different from other MAO inhibitors.

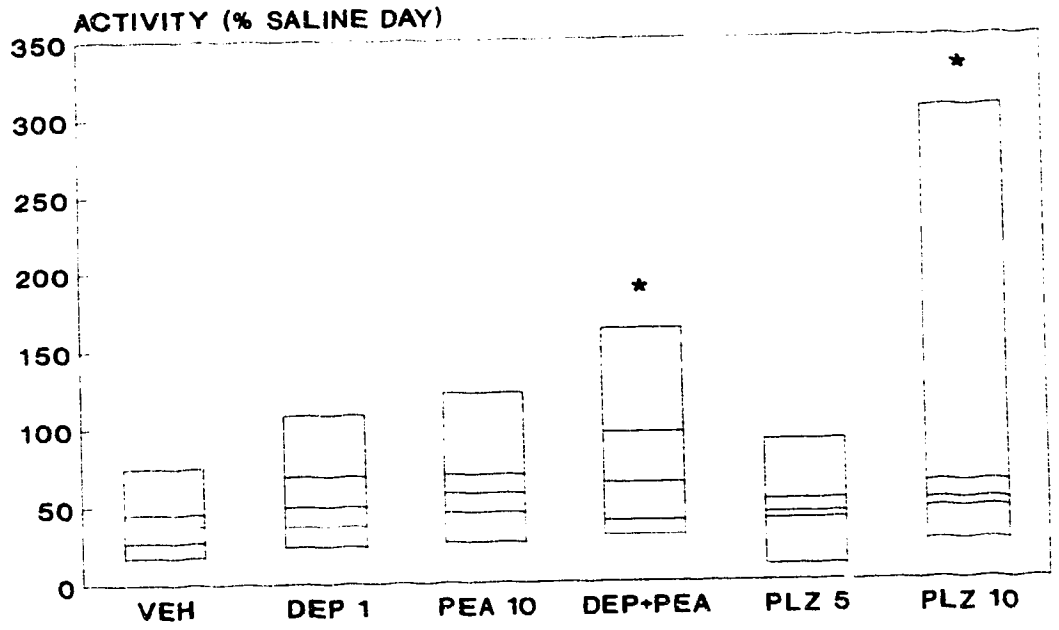


Figure 10: Effects of long-term (21 day) drug treatment on the degree of salbutamol-induced suppression of locomotor activity expressed as a percentage of activity exhibited on the equivalent saline control day. The values obtained are plotted as quartile ranges. The responsiveness to the motor suppressant effects of salbutamol hemisulphate was reduced by chronic treatment with either PLZ (10 mg kg^{-1}) or the combination of DEP (1 mg kg^{-1}) and PEA (10 mg kg^{-1}). All other drug treatments were ineffective in altering responsiveness to salbutamol (Kruskal-Wallis ANOVA, $p<0.05$).

response to salbutamol ($H=13.45$, $p < 0.05$) was evident with animals receiving 10 mg kg^{-1} per d of PLZ sulphate whereas those receiving 5 mg kg^{-1} per d of PLZ sulphate did not exhibit any significant change in this measure. PEA and DEP when administered alone failed to significantly alter the response to salbutamol. The combination of PEA and DEP, however, induced a significant reduction in the response to salbutamol.

C.5.2 Lack of effects of long-term (28 day) antidepressant treatment on the salbutamol-induced changes in amino acid concentrations in brain and plasma

C.5.2.1 Acute effects of agonists on brain and plasma concentrations of LNAAs

The effects of administration of isoproterenol (3 mg kg^{-1}, i.p., 60 min) and salbutamol (3mg kg^{-1} or 10 mg kg^{-1}, i.p., 60 min) on rat brain and plasma levels of LNAAs are shown in Tables 9 and 10. Brain levels of tryptophan [$F(3,18)=18.06$], tyrosine [$F(3,18)=9.87$], phenylalanine [$F(3,18)=5.22$] and valine [$F(3,18)=6.57$] were significantly increased by each drug treatment. The increases seen were approximately the same for each drug treatment, as none of the LNAA levels within groups treated with β-adrenergic agonists significantly differed from each other. The largest increases were seen in tryptophan (165-190% of controls) followed by tyrosine (133-155% of controls). Phenylalanine and valine both increased by approximately the same amount (130-140% of controls). Neither leucine [$F(3,18)=0.17$] nor isoleucine [$F(3,18)=0.47$] was significantly altered by treatment with isoproterenol or salbutamol.

Concurrent with changes in brain levels of LNAAs, isoproterenol and salbutamol induced a significant decrease in plasma concentrations of tryptophan

Table 9: Effects of isoproterenol and salbutamol on brain levels of LNAAs.

	Vehicle	*Isoproterenol (3 mg kg^{-1})*	*Salbutamol (3 mg kg^{-1})*	*Salbutamol (10 mg kg^{-1})*
Tryptophan	21 ± 1(6)	40 ± 2(4)*	36 ± 1(6)*	35 ± 3(6)*
Tyrosine	43 ± 3(6)	58 ± 5(4)*	67 ± 2(6)*	62 ± 4(6)*
Phenylalanine	63 ± 6(6)	88 ± 6(4)*	81 ± 4(6)*	83 ± 3(6)*
Valine	61 ± 4(6)	79 ± 4(4)*	85 ± 5(6)*	82 ± 4(6)*
Leucine	70 ± 3(6)	70 ± 4(4)	67 ± 4(6)	67 ± 4(6)
Isoleucine	33 ± 2(6)	32 ± 2(4)	32 ± 3(6)	43 ± 14(6)

Note: Data are means ± SEM (n) for each amino acid in nmol g^{-1} wet tissue. * Denotes significantly different from vehicle $p<0.05$, ANOVA. Vehicle and drug treatments were administered i.p. (1h).

Table 10: Effects of isoproterenol and salbutamol on plasma levels of LNAAs.

	Vehicle	*Isoproterenol (3 mg kg^{-1})*	*Salbutamol (3 mg kg^{-1})*	*Salbutamol (10 mg kg^{-1})*
Tryptophan	63 ± 2(6)	34 ± 4(4)*	55 ± 2(6)*	43 ± 1(6)*
Tyrosine	113 ± 6(6)	69 ± 6(4)*	89 ± 6(6)*	91 ± 9(6)*
Phenylalanine	57 ± 3(6)	48 ± 3(4)	52 ± 4(6)	48 ± 2(6)
Valine	144 ± 3(6)	55 ± 9(4)*	75 ± 9(6)*	82 ± 11(6)*
Leucine	122 ± 2(6)	46 ± 12(4)*	54 ± 6(6)*	63 ± 10(6)*
Isoleucine	80 ± 3(6)	26 ± 8(4)*	36 ± 3(6)*	39 ± 7(6)*

Note: Data are means ± SEM (n) for each amino acid in nmol g^{-1} wet tissue. * Denotes significantly different from vehicle $p<0.05$, ANOVA. Vehicle and drug treatments were administered i.p. (1h).

[F(3,18)=32.75], tyrosine [F(3,18)=9.87], valine [F(3,18)=19.92], leucine [F(3,18)=20.44] and isoleucine [F(3,18)=20.47]. The largest decreases were seen in the aliphatic amino acids leucine (38-50% of controls), isoleucine (33-49% of controls) and valine (38-57% of controls). Tryptophan (55-87% of controls) and tyrosine (61-81% of controls) decreased to a lesser extent. Phenylalanine (85-93% of controls) also showed a tendency to decrease in plasma but this was not a statistically significant effect [F(3,18)=1.89].

C.5.2.2 Effects of salbutamol on brain and plasma LNAA concentrations following long-term (28 day) antidepressant drug treatment

Effects of salbutamol and chronic antidepressant treatment on brain LNAA concentrations are presented in Tables 11 and 12. DMI or PLZ administered continuously for 28 days did not significantly alter brain concentrations of LNAAs nor did they alter the salbutamol-induced increases in tryptophan, tyrosine, phenylalanine, or valine. The salbutamol-induced increase in tyrosine levels appears to be augmented by DMI: however, a two-way ANOVA revealed that this was not a statistically significant interaction.

Effects of salbutamol and chronic antidepressant treatment on plasma LNAA concentrations are presented in Tables 11 and 12. DMI or PLZ administered continuously for 28 days did not significantly alter plasma concentrations of LNAAs nor did they alter the salbutamol-induced decreases in tryptophan, tyrosine, valine, leucine, or isoleucine. The details of the statistical analysis of the above data are available in Tables 13 and 14.

Table 11: Effects of salbutamol and chronic (28 day) antidepressant treatment on brain LNAA concentrations.

		Vehicle	*Desipramine 10 mg kg^{-1} per d*	*Phenelzine 10 mg kg^{-1} per d*
Tryptophan	Vehicle	20 ± 1(8)	20 ± 1(8)	21 ± 1(8)
	Salbutamol*	30 ± 2(7)	33 ± 3(8)	31 ± 2(8)
Tyrosine	Vehicle	54 ± 3(7)	55 ± 6(5)	48 ± 6(8)
	Salbutamol*	63 ± 4(7)	91 ± 5(8)	74 ± 8(8)
Phenylalanine	Vehicle	45 ± 4(8)	50 ± 5(8)	48 ± 3(8)
	Salbutamol*	62 ± 4(7)	72 ± 4(8)	62 ±6(8)
Valine	Vehicle	125 ± 6(8)	109 ± 7(8)	135 ± 10(8)
	Salbutamol*	151 ± 12(7)	143 ± 10(8)	154 ± 5(8)
Leucine	Vehicle	78 ± 2(8)	77 ± 5(8)	73 ± 6(8)
	Salbutamol	75 ± 3(7)	79 ± 5(8)	70 ± 2(8)
Isoleucine	Vehicle	35 ± 1(8)	31 ± 2(8)	31 ± 2(8)
	Salbutamol	35 ± 2(7)	35 ± 2(8)	31 ± 1(8)

Note: Data are means ± SEM (n) for each amino acid in nmol g^{-1} wet tissue. * Denotes that the effect of salbutamol was significantly different from vehicle $p<0.05$, ANOVA. There were no effects of antidepressant treatments (28 d s.c. via osmotic minipumps, Alzet 2ML4). Salbutamol dose was 3 mg kg^{-1} (i.p., 1h).

Table 12: Effects of salbutamol and chronic (28 day) antidepressant treatment on plasma LNAA concentrations.

		Vehicle	*Desipramine 10 mg kg^{-1} per d*	*Phenelzine 10 mg kg^{-1} per d*
Tryptophan	Vehicle	72 ± 3(8)	61 ± 5(8)	79 ± 4(8)
	Salbutamol*	60 ± 4(8)	52 ± 4(8)	59 ± 3(8)
Tyrosine	Vehicle	52 ± 4(8)	50 ± 2(8)	66 ± 3(8)
	Salbutamol*	42 ± 2(8)	45 ± 5(8)	47 ± 4(8)
Phenylalanine	Vehicle	52 ± 5(7)	48 ± 4(8)	53 ± 3(8)
	Salbutamol	45 ± 3(7)	48 ± 3(8)	48 ± 3(8)
Valine	Vehicle	150 ± 7(8)	132 ± 5(8)	146 ± 7(8)
	Salbutamol*	84 ± 5(8)	82 ± 9(8)	74 ± 6(8)
Leucine	Vehicle	133 ± 6(8)	122 ± 6(8)	128 ± 6(8)
	Salbutamol*	64 ± 5(8)	61 ± 7(8)	56 ± 6(8)
Isoleucine	Vehicle	64 ± 5(8)	68 ± 3(8)	72 ± 4(8)
	Salbutamol*	36 ± 3(8)	36 ± 4(8)	33 ± 4(8)

Note: Data are means ± SEM (*n*) for each amino acid in nmol g^{-1} wet tissue. * Denotes that the effect of salbutamol was significantly different from vehicle $p<0.05$, ANOVA. There were no effects of antidepressant treatments (28 d s.c. via osmotic minipumps, Alzet 2ML4). Salbutamol dose was 3 mg kg^{-1} (i.p., 1h).

Table 13: F values obtained from 2 way ANOVA of data in Table 11.

	Factor A (Salbutamol)	Factor B (Antidepressant)	Interaction
Tryptophan	$F(1,41)=54.65$	$F(2,41)=0.35$	$F(2,41)=0.45$
Tyrosine	$F(1,37)=24.71$	$F(2,37)=3.53$	$F(2,37)=2.74$
Phenylalanine	$F(1,41)=23.49$	$F(2,41)=1.58$	$F(2,41)=0.41$
Valine	$F(1,41)=14.24$	$F(2,41)=2.41$	$F(2,41)=0.39$
Leucine	$F(1,41)= 0.15$	$F(2,41)=1.32$	$F(2,41)=0.24$
Isoleucine	$F(1,41)= 0.90$	$F(2,41)=2.69$	$F(2,41)=0.90$

Note: Post-hoc analysis reveals that there is a significant effect of DMI on brain tyrosine (1 way ANOVA of pooled means $F(2,44)=3.36$]). This is not apparent among the animals treated with acute saline and there is no interaction between acute and chronic treatments.

Table 14: F values obtained from 2 way ANOVA of data in Table 12.

	Factor A (Salbutamol)	Factor B (Antidepressant)	Interaction
Tryptophan	F(1,42)=18.47	F(2,42)=5.62	F(2,42)=1.07
Tyrosine	F(1,42)=15.62	F(2,42)=4.64	F(2,42)=2.04
Phenylalanine	F(1,40)= 1.91	F(2,40)=0.28	F(2,40)=0.52
Valine	F(1,42)=133.3	F(2,42)=1.19	F(2,42)=1.46
Leucine	F(1,42)=187.1	F(2,42)=0.84	F(2,42)=0.44
Isoleucine	F(1,42)=107.7	F(2,42)=0.23	F(2,42)=1.02

Note: Post-hoc analysis reveals that the significant effect of antidepressant treatment on plasma tryptophan is due to a difference between DMI and PLZ treated animals and that neither group differs from vehicle (1 way ANOVA of pooled means [F(2,45)=3.44]). Post-hoc analysis of plasma tyrosine reveals that there is no significant effect of antidepressants (1 way ANOVA of pooled means [F(2,45)=2.70]).

C.6 RADIOLIGAND BINDING TO β-ADRENOCEPTORS

C.6.1 Effects of long-term (28 day) antidepressant drug treatment on total cortical β-adrenoceptors

A typical binding isotherm and saturation curve for ^{3}H-CGP 12177 binding to cortical β-adrenoceptors is shown in Figures 11 and 12. The K_d and B_{max} values of cortical β-adrenoceptors after long-term treatment with antidepressant drugs are shown in Table 15. Antidepressants did not alter the affinity (K_d) of β-adrenoceptors as compared to vehicle-treated animals [$F(4,49)=2.60$]. The affinity after TCP, however, was different from the affinity after the higher dose of PLZ. All antidepressants caused a decrease in the density (B_{max}) of cortical β-adrenoceptors [$F(4,49)=19.34$]. The doses of MAO inhibitors used decreased β-adrenoceptor density by approximately 20%. The two doses of PLZ did not differ in their ability to alter the density of cortical β-adrenoceptors. The dose of DMI used in this study decreased cortical β-adrenoceptor density by approximately 33%.

C.6.2 Effects of long-term (28 day) antidepressant drug treatment on subtypes of cortical β-adrenoceptors

A typical Hofstee plot of the selective β_1 antagonist ICI 89406 competing for ^{3}H-CGP 12177 binding is shown in Figure 13. Ligand analysis revealed that a two-site model was a significantly better fit for these competition curves than a one-site model ($p<0.05$). This model revealed one site with a K_i in the range of 0.5 nM whereas the second site had a K_i in the range of 50 nM. The high affinity site is beleived to be the β_1 site and the lower affinity site the β_2 site. The effects of long-term treatment with antidepressant drugs on cortical β-adrenoceptor subtypes are shown in Tables 16 and 17. Long-term treatment with antidepressant

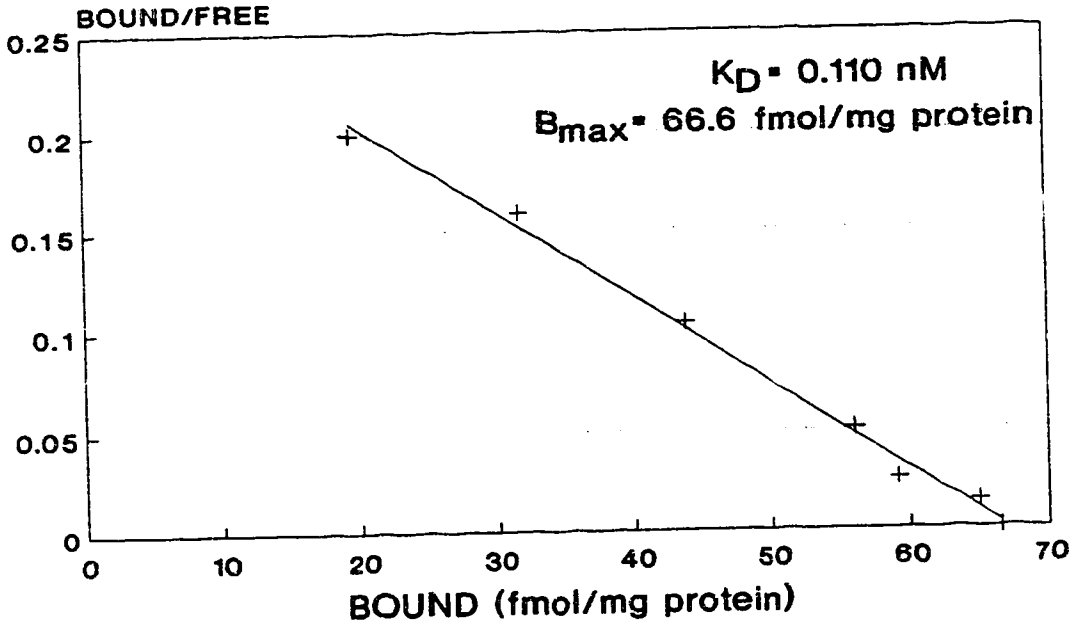


Figure 11: Typical saturation curve for ^{3}H-CGP 12177 binding to rat brain cortical β-adrenoceptors.

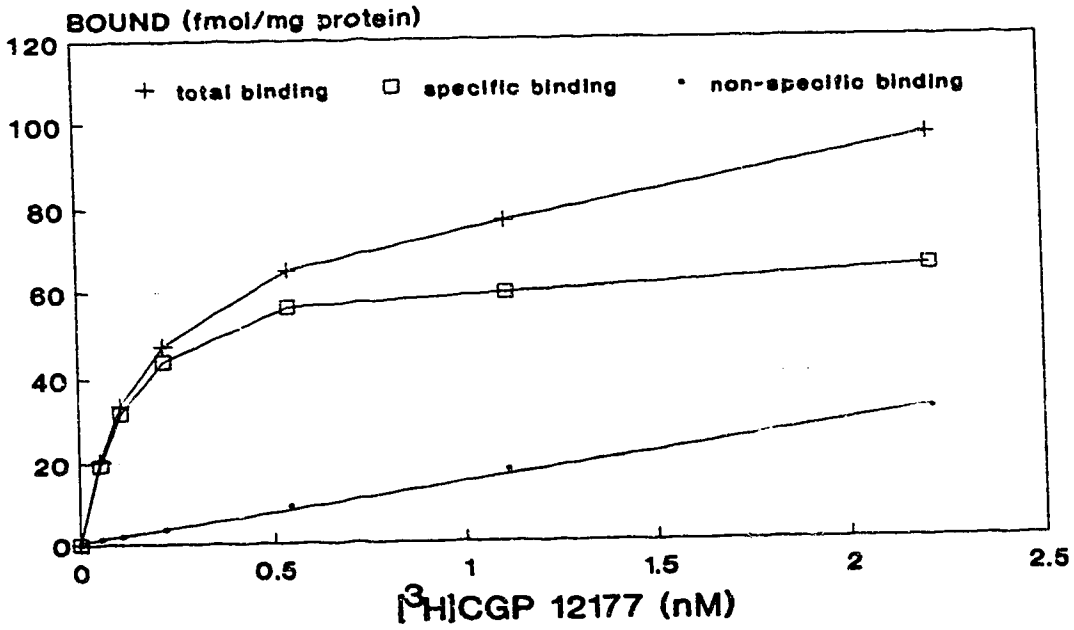


Figure 12: Typical binding isotherm for ^{3}H-CGP 12177 binding to rat brain cortical β-adrenoceptors.

Table 15: Effects of long-term (28 day) administration of antidepressant drugs on the K_d and B_{max} of total cortical β-adrenoceptors.

Drug	Dose (mg kg^{-1} per d)	n	K_d (nM)	B_{max} (fmol mg^{-1} protein)
VEH	0	15	0.108 ± 0.004	66.5 ± 2.0
DMI	10	12	0.122 ± 0.007	44.1 ± 1.9*
TCP	1	11	0.126 ± 0.014	53.1 ± 1.9*
PLZ	5	6	0.099 ± 0.011	53.1 ± 2.8*
PLZ	10	10	0.094 ± 0.005	53.3 ± 1.8*

Note: Values are means ± S.E.M. *Denotes significantly different from vehicle ($p \leq 0.05$).

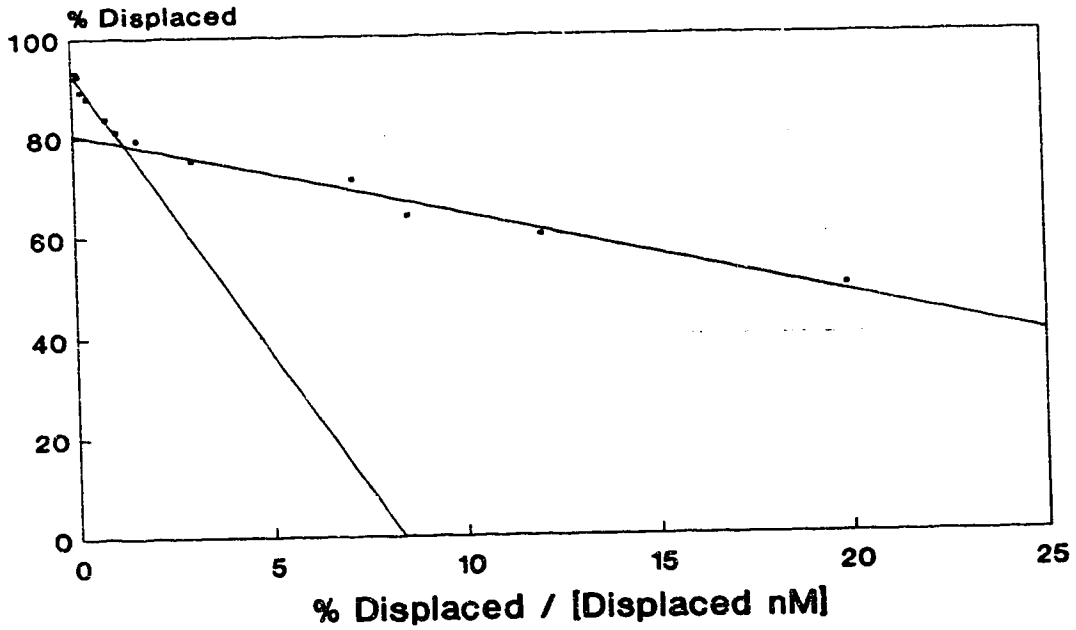


Figure 13: Typical Hostee plot of competition data for β-adrenoceptors.

Table 16: Effects of long-term (28 day) administration of antidepressant drugs on the K_i and B_{max} of cortical β_1-adrenoceptors.

Drug	Dose (mg kg^{-1} per d)	n	K_i (nM)	B_{max} (fmol mg^{-1} protein)
VEH	0	13	0.461 ± 0.025	53.0 ± 1.4
DMI	10	10	0.472 ± 0.062	28.3 ± 1.4*
TCP	1	11	0.545 ± 0.055	40.9 ± 2.5*
PLZ	5	6	0.434 ± 0.043	46.2 ± 2.5*
PLZ	10	9	0.438 ± 0.041	45.2 ± 1.7*

Note: Values are means ± S.E.M. *Denotes significantly different from vehicle ($p \leq 0.05$, ANOVA).

Table 17: Effects of long-term (28 day) administration of antidepressant drugs on the K_i and B_{max} of cortical β_2-adrenoceptors

Drug	Dose (mg kg^{-1} per d)	n	K_i (nM)	B_{max} (fmol mg^{-1} protein)
VEH	0	13	58.5 ± 4.4	12.5 ± 0.6
DMI	10	10	61.3 ± 5.3	12.0 ± 0.5
TCP	1	11	67.8 ± 9.2	10.6 ± 0.8
PLZ	5	6	77.5 ± 9.1	10.5 ± 0.7
PLZ	10	9	59.7 ± 7.8	11.2 ± 0.7

Note: Values are means ± S.E.M.

drugs did not alter the affinity of either of these sites (β_1 [F(4,44)=0.92], β_2 [F(4,44)=1.00]). All antidepressant drugs induced a decrease in the density of β_1-adrenoceptors [F(4,44)=25.42] but did not alter the density of β_2-adrenoceptors [F(4,44)=1.79]. The two doses of PLZ did not differ in their effects on the subtypes of cortical β-adrenoceptors.

C.6.3 Effects of long-term (28 day) antidepressant drug treatment on total cerebellar β-adrenoceptors

A typical saturation curve and binding isotherm for ^{3}H-CGP 12177 binding to cerebellar β-adrenoceptors is shown in Figures 14 and 15. The K_d and B_{max} of cerebellar β-adrenoceptors after long-term treatment with antidepressant drugs are shown in Table 18. Antidepressants did not alter the affinity (K_d) of β-adrenoceptors as compared to values in vehicle-treated animals [F(4,25)=0.24]. Antidepressant treatment also did not significantly change the density of β-adrenoceptors in the cerebellum [F(4,25)=1.43].

C.6.4 Effects of long-term (28 day) antidepressant drug treatment on subtypes of cerebellar β-adrenoceptors

Ligand analysis revealed that a two-site model was a significantly better fit for only four of the vehicle-treated animals. These animals had a K_i of 0.500 ± 0.043 nM. For further analysis the K_i of the β_1 site was set at 0.5 when using ligand analysis of the other competition curves. The K_i and B_{max} of the subtypes of cerebellar β-adrenoceptors are displayed in Tables 19 and 20. Long-term treatment with antidepressant drugs did not alter the affinity of the β_2 site [F(4,21)=0.32] nor did it alter the B_{max} of the β_1 [F(4,21)=1.37] or β_2 sites [F(4,21)=0.87].

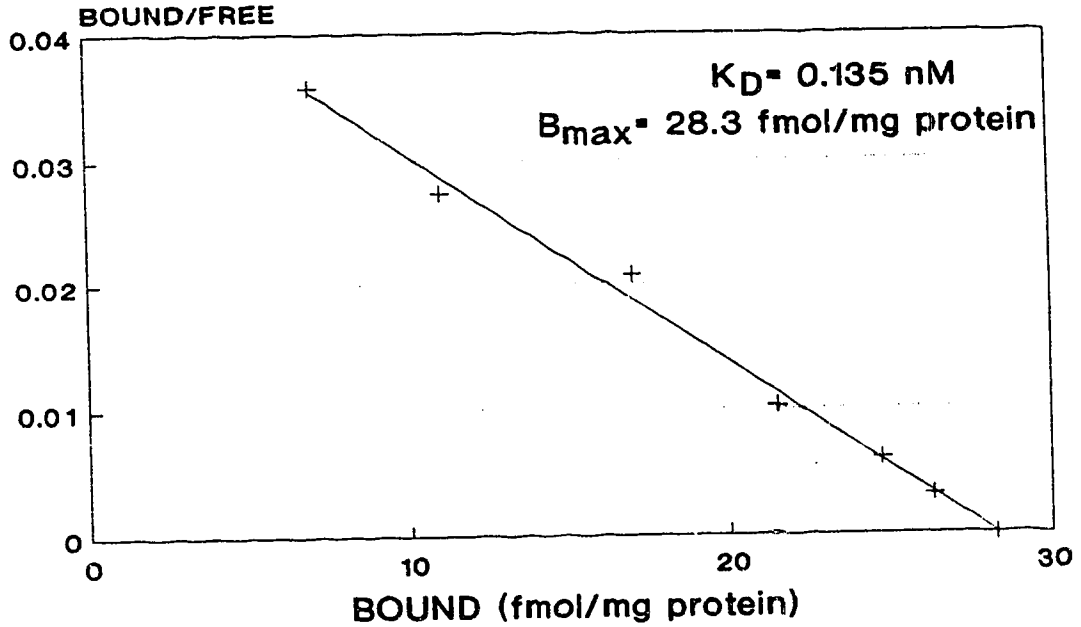


Figure 14: Typical saturation curve for ^{3}H-CGP 12177 binding to rat cerebellar β-adrenoceptors.

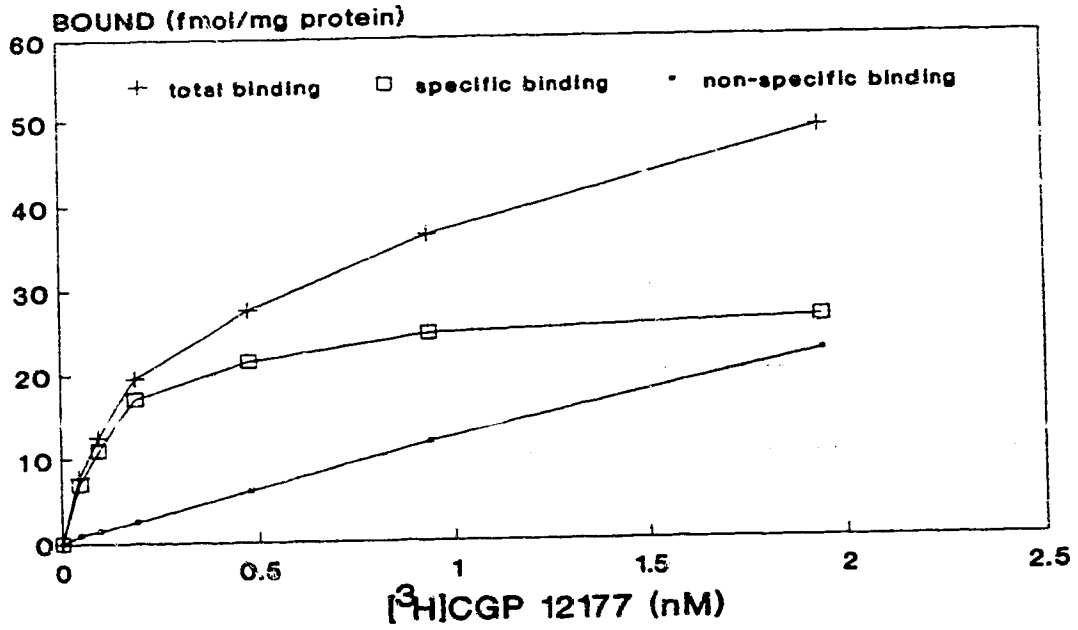


Figure 15: Typical binding isotherm for ^{3}H-CGP 12177 binding to rat cerebellar β-adrenoceptors.

Table 18: Effects of long-term (28 day) administration of antidepressant drugs on the K_d and B_{max} of total cerebellar β-adrenoceptors.

Drug	Dose (mg kg^{-1} per d)	n	K_d (nM)	B_{max} (fmol mg^{-1} protein)
VEH	0	6	0.146 ± 0.004	28.6 ± 1.0
DMI	10	6	0.144 ± 0.013	24.3 ± 1.7
TCP	1	6	0.139 ± 0.009	26.7 ± 1.7
PLZ	5	6	0.138 ± 0.003	27.8 ± 0.4
PLZ	10	6	0.137 ± 0.007	26.9 ± 1.5

Note: Values are means ± S.E.M.

Table 19: Effects of long-term (28 day) administration of antidepressant drugs on the K_i and B_{max} of cerebellar β_1-adrenoceptors.

Drug	Dose (mg kg^{-1} per d)	n	K_i (nM)	B_{max} (fmol mg^{-1} protein)
VEH	0	5	0.500 ± 0.043	3.9 ± 0.6
DMI	10	5	set 0.5	1.9 ± 0.6
TCP	1	5	set 0.5	1.8 ± 0.2
PLZ	5	6	set 0.5	2.4 ± 0.8
PLZ	10	5	set 0.5	2.6 ± 1.0

Note: Values are means ± S.E.M.

Table 20: Effects of long-term (28 day) administration of antidepressant drugs on the K_i and B_{max} of cerebellar β_2-adrenoceptors.

Drug	Dose (mg kg^{-1} per d)	n	K_i (nM)	B_{max} (fmol mg^{-1} protein)
VEH	0	5	65.4 ± 5.7	25.8 ± 2.1
DMI	10	5	67.0 ± 6.1	22.8 ± 1.9
TCP	1	5	66.6 ± 1.2	25.2 ± 0.7
PLZ	5	6	66.8 ± 7.1	26.8 ± 1.2
PLZ	10	5	59.7 ± 3.1	24.7 ± 1.9

Note: Values are means ± S.E.M.

C.6.5 Effects of long-term (28 day) administration of PEA and/or DEP on total cortical β-adrenoceptors

The K_d and B_{max} of cortical β-adrenoceptors after long-term treatment with PEA and/or DEP are shown in Table 21. The affinity (K_d) of β-adrenoceptors was not altered by drug treatment [DEP $F(1,31)=1.24$, PEA $F(1,31)=2.05$, DEP x PEA $F(2,31)=0.03$]. Two way ANOVA revealed a significant effect of DEP [$F(1,31)=42.82$] and PEA [$F(1,31)=14.40$] on the density of β-adrenoceptors and that there was a significant interaction between these two treatments [$F(2,31)=5.52$]. *Posthoc* analysis revealed that the DEP-treated group was significantly different from vehicle and that PEA alone did not alter the density of β-adrenoceptors. The combination of DEP and PEA also produced a significantly lower density of β-adrenoceptors than either of the treatments alone. DEP reduced β-adrenoceptors by 15% whereas the combination of DEP and PEA reduced β-adrenoceptors by 30%.

C.6.6 Effects of long-term (28 day) administration of PEA and/or DEP on subtypes of cortical β-adrenoceptors

The effects of long-term treatment with PEA and/or DEP on cortical β-adrenoceptor subtypes are shown in Tables 22 and 23. Long-term treatment with these drugs did not alter the affinity of either the β_1 site [DEP $F(1,29)=2.02$], PEA $F(1,29)=0.45$, DEP x PEA $F(1,29)=0.79$] or the β_2 site [DEP $F(1,29)=1.04$, PEA $F(1,28)=0.01$, DEP x PEA $F(1,29)=0.84$]. Two way ANOVA revealed that there was a significant effect of DEP [$F(1,29)=23.68$ and PEA [$F(1,29)=5.92$] on the density of β_1-adrenoceptors and that there was a significant interaction between these two treatments [$F(1,29)=4.26$]. *Posthoc* analysis revealed that DEP resulted in a significant decrease of 14% compared to controls and that PEA alone did not alter β_1-adrenoceptor density.

Table 21: Effects of long-term (28 day) administration of PEA and/or DEP on the K_d and B_{max} of total cortical β-adrenoceptors.

Drug	Dose (mg kg^{-1} per d)	n	K_d (nM)	B_{max} (fmol mg^{-1} protein)
VEH/VEH	0/0	15	0.108 ± 0.004	66.5 ± 2.0
PEA/VEH	10/0	5	0.120 ± 0.013	62.6 ± 2.8
VEH/DEP	0/1	8	0.117 ± 0.011	56.9 ± 2.3*
PEA/DEP	10/1	7	0.132 ± 0.013	46.6 ± 1.6**

Note: Values are means ± S.E.M. *Denotes significantly different from vehicle, **denotes significantly different from all other groups ($p \leq 0.05$).

Table 22: Effects of long-term (28 day) administration of PEA and/or DEP on the K_i and B_{max} of cortical β_1-adrenoceptors.

Drug	Dose (mg kg^{-1} per d)	n	K_i (nM)	B_{max} (fmol mg^{-1} protein)
VEH/VEH	0/0	13	0.461 ± 0.025	53.0 ± 1.4
PEA/VEH	10/0	5	0.405 ± 0.014	52.3 ± 3.3
VEH/DEP	0/1	8	0.480 ± 0.030	45.7 ± 3.4*
PEA/DEP	10/1	7	0.488 ± 0.054	35.5 ± 2.1**

Note: Values are means ± S.E.M. *Denotes significantly different from vehicle, **denotes significantly different from all other groups ($p \leq 0.05$).

Table 23: Effects of long-term (28 day) administration of PEA and/or DEP on the K_i and B_{max} of cortical β_2-adrenoceptors

Drug	Dose (mg kg^{-1} per d)	n	K_i (nM)	B_{max} (fmol mg^{-1} protein)
VEH/VEH	0/0	13	58.5 ± 4.4	12.5 ± 0.6
PEA/VEH	10/0	5	53.1 ± 4.3	10.7 ± 0.7
VEH/DEP	0/1	8	59.2 ± 6.4	10.7 ± 0.9
PEA/DEP	10/1	7	66.2 ± 10.6	8.8 ± 0.5*

Note: Values are means ± S.E.M. *Denotes significantly different from vehicle ($p \leq 0.05$).

The combination of DEP and PEA resulted in a 33% decrease in the density of β_1-adrenoceptors and this was significantly different from either DEP or PEA alone. Two way ANOVA revealed that there was a significant effect of DEP [$F(1,29)=7.29$] and PEA [$F(1,29)=4.21$] on the density of β-adrenoceptors and that this was additive as there was no interaction between these two treatments [$F(1,29)=0.13$]. The density of cortical β_2-adrenoceptors was reduced 14% by DEP, 15% by PEA and 30% by the combination.

C.6.7 Effects of long-term (28 day) administration of PEA and DEP on total cerebellar β-adrenoceptors

The K_d and B_{max} of cortical β-adrenoceptors after long-term treatment with PEA and/or DEP are shown in Table 24. The affinity (K_d) of β-adrenoceptors was not altered by drug treatment [DEP $F(1,20)=0.46$, PEA $F(1,20)=1.19$, DEP x PEA $F(1,20)=0.30$]. Two way ANOVA revealed a significant effect of DEP [$F(1,20)=6.12$] on the density of β-adrenoceptors and that PEA had no effect [$F(1,20)=0.88$]; there was no interaction between these two treatments [$F(1,20)=2.10$].

C.6.8 Effects of long-term (28 day) administration of PEA and/or DEP on subtypes of cerebellar β-adrenoceptors

The effects of long-term treatment with PEA and/or DEP on cerebellar β-adrenoceptor subtypes are shown in Tables 25 and 26. Long-term treatment with these drugs did not alter the affinity of the β_2 site [DEP $F(1,18)=0.17$, PEA $F(1,18)=4.00$, DEP x PEA $F(1,18)=0.61$]. Two-way ANOVA revealed that there was a significant effect of PEA [$F(1,18)=7.61$] on the density of β_1-adrenoceptors whereas the effect of DEP did not reach significance [$F(1,18)=2.24$] nor did the interaction between the two drugs [$F(1,18)=2.74$]. There was no significant effect of DEP [$F(1,18)=0.29$] or PEA [$F(1,18)=0.35$] on the density of β_2-adrenoceptors,

Table 24: Effects of long-term (28 day) administration of PEA and/or DEP on the K_d and B_{max} of total cerebellar β-adrenoceptors.

Drug	Dose (mg kg^{-1} per d)	n	K_d (nM)	B_{max} (fmol mg^{-1} protein)
VEH/VEH	0/0	6	0.146 ± 0.004	28.6 ± 1.0
PEA/VEH	10/0	6	0.158 ± 0.008	31.4 ± 1.6
VEH/DEP	0/1	6	0.145 ± 0.006	27.4 ± 0.5*
PEA/DEP	10/1	6	0.149 ± 0.010	26.8 ± 1.3

Note: Values are means ± S.E.M. *Denotes significant effect of DEP ($p<0.05$).

Table 25: Effects of long-term (28 day) administration of PEA and DEP on the K_i and B_{max} of cerebellar β_1-adrenoceptors.

Drug	Dose (mg kg^{-1} per d)	n	K_i (nM)	B_{max} (fmol mg^{-1} protein)
VEH/VEH	0/0	5	0.500 ± 0.043	3.9 ± 0.6
PEA/VEH	10/0	6	set 0.5	1.1 ± 0.4*
VEH/DEP	0/1	5	set 0.5	1.9 ± 0.8
PEA/DEP	10/1	6	set 0.5	1.2 ± 0.7

Note: Values are means ± S.E.M. *Denotes significant effect of PEA ($p<0.05$).

Table 26: Effects of long-term (28 day) administration of PEA and/or DEP on the K_i and B_{max} of cerebellar β_2-adrenoceptors

Drug	Dose (mg kg^{-1} per d)	n	K_i (nM)	B_{max} (fmol mg^{-1} protein)
VEH/VEH	0/0	5	65.4 ± 5.7	25.8 ± 2.1
PEA/VEH	10/0	6	54.7 ± 2.7	29.1 ± 1.7
VEH/DEP	0/1	5	64.0 ± 4.3	27.1 ± 1.3
PEA/DEP	10/1	6	59.3 ± 2.7	25.9 ± 1.8

Note: Values are means ± S.E.M.

nor was there any significant interaction between these two drugs [F(1,18)=1.62] on this measure.

C.7 RADIOLIGAND BINDING TO STRIATAL DA RECEPTORS

C.7.1 Effects of long-term (28 day) antidepressant drug treatment on striatal D_1-like DA receptors

A Scatchard plot and binding isotherm of ^{3}H-SCH 23390 binding to striatal DA D_1-like receptors are shown in Figures 16 and 17. The effects of long-term antidepressant treatment on the affinity (K_d) and density (B_{max}) of D_1-like receptors are shown in Table 27. None of the antidepressant treatments altered the affinity of the site for ^{3}H-SCH 23390 [F(F(4,23)=0.13]. The antidepressant drugs used induced a significant decrease of 9 to 12% in the density of D_1-like receptors [F(4,23)=2.84). Both doses of PLZ decreased D_1-like binding to the same extent.

C.7.2 Effects of long-term (28 day) antidepressant drug treatment on striatal D_2-like DA receptors

A Scatchard plot and binding isotherm of ^{3}H-spiperone binding to striatal DA D_2-like receptors is shown in Figures 18 and 19. The effects of long-term antidepressant treatment on the affinity (K_d) and density (B_{max}) of D_2-like receptors are shown in Table 28. None of the antidepressant treatments altered the affinity of the site for ^{3}H-spiperone [F(4,21)=0.21]. The MAO inhibitor antidepressant drugs used in this study induced a decrease of 15 to 20% in the density of D_2-like receptors [F(4,21)=3.22]. Both doses of PLZ decreased D_2-like binding to the same extent (15%). DMI had no effect on the density of these D_2-like receptors.

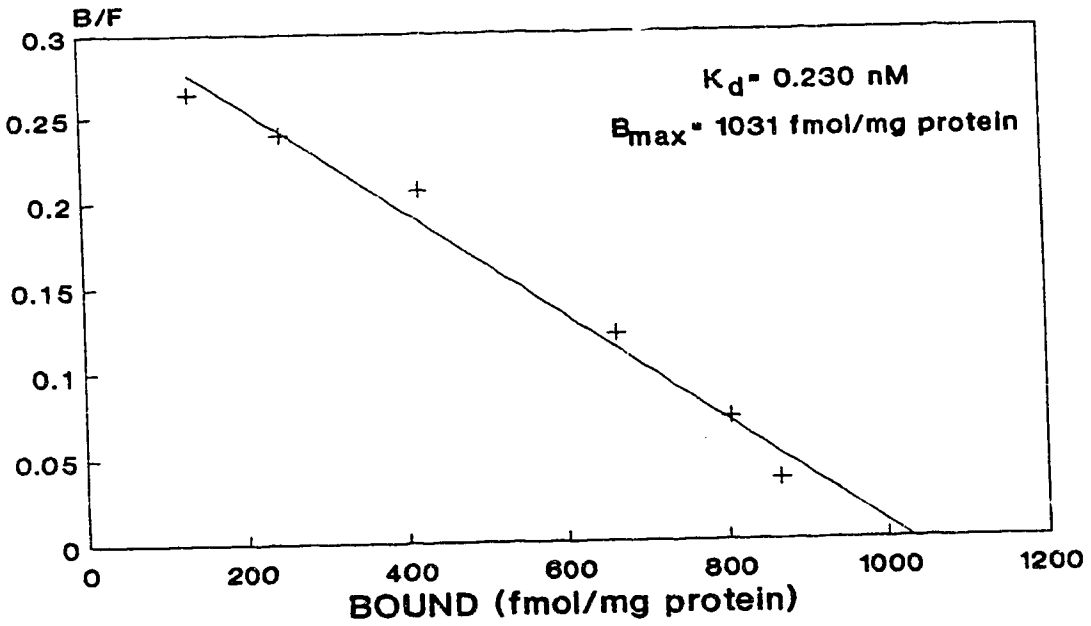


Figure 16: Typical saturation curve for ^{3}H-SCH 23390 binding to rat brain striatal D1 DA receptors.

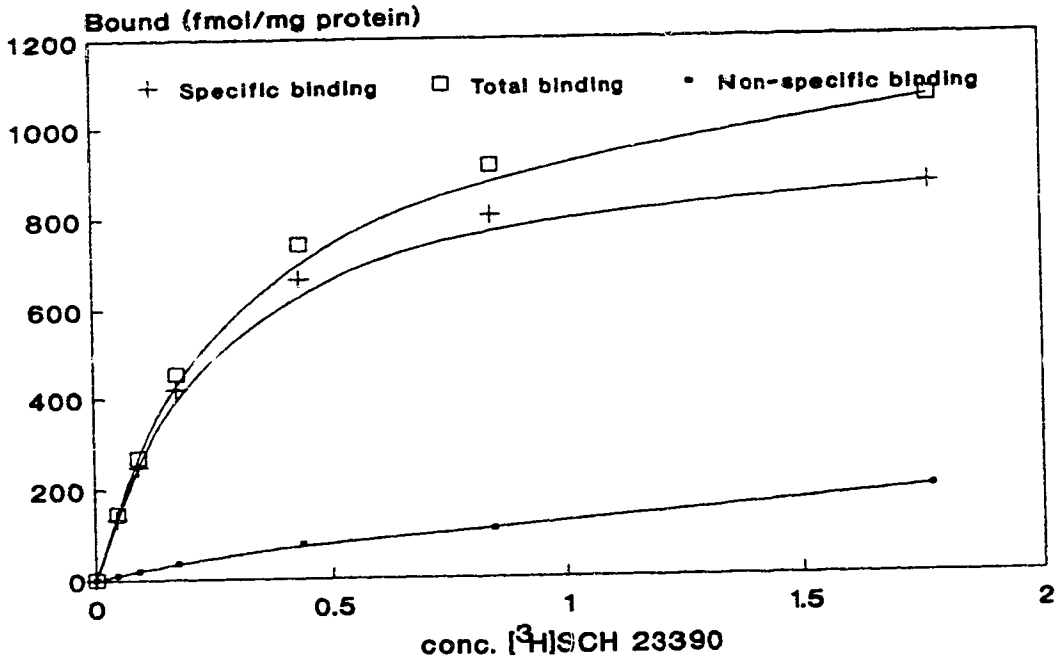


Figure 17: Typical binding isotherm for ^{3}H-SCH 23390 binding to rat brain striatal D1 DA receptors.

Table 27: Effects of long-term (28 day) administration of antidepressant drugs on K_d and B_{max} of striatal D_1 DA receptors.

Drug	Dose (mg kg^{-1} per d)	n	K_d (nM)	B_{max} (fmol mg^{-1} protein)
VEH	0	5	0.251 ± 0.023	995 ± 30
DMI	10	6	0.257 ± 0.017	872 ± 32*
TCP	1	6	0.247 ± 0.012	868 ± 33*
PLZ	5	6	0.241 ± 0.012	873 ± 38*
PLZ	10	5	0.247 ± 0.020	906 ± 8*

Note: Values are means ± S.E.M. *Denotes significantly different from vehicle ($p \leq 0.05$).

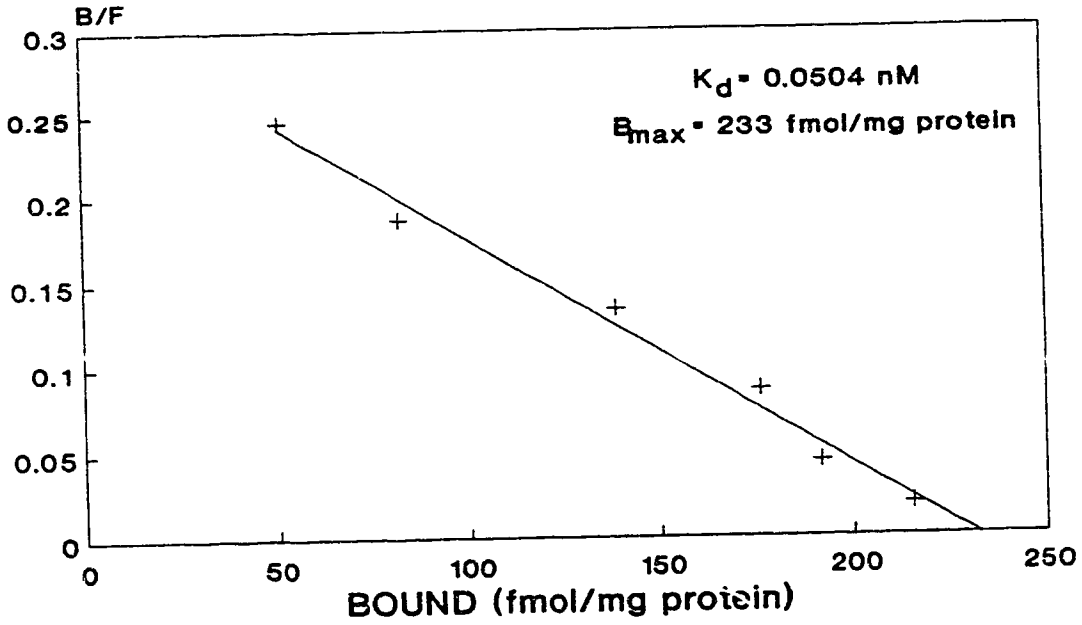


Figure 18: Typical saturation curve for ^{3}H-spiperone binding to rat brain striatal D2 DA receptors.

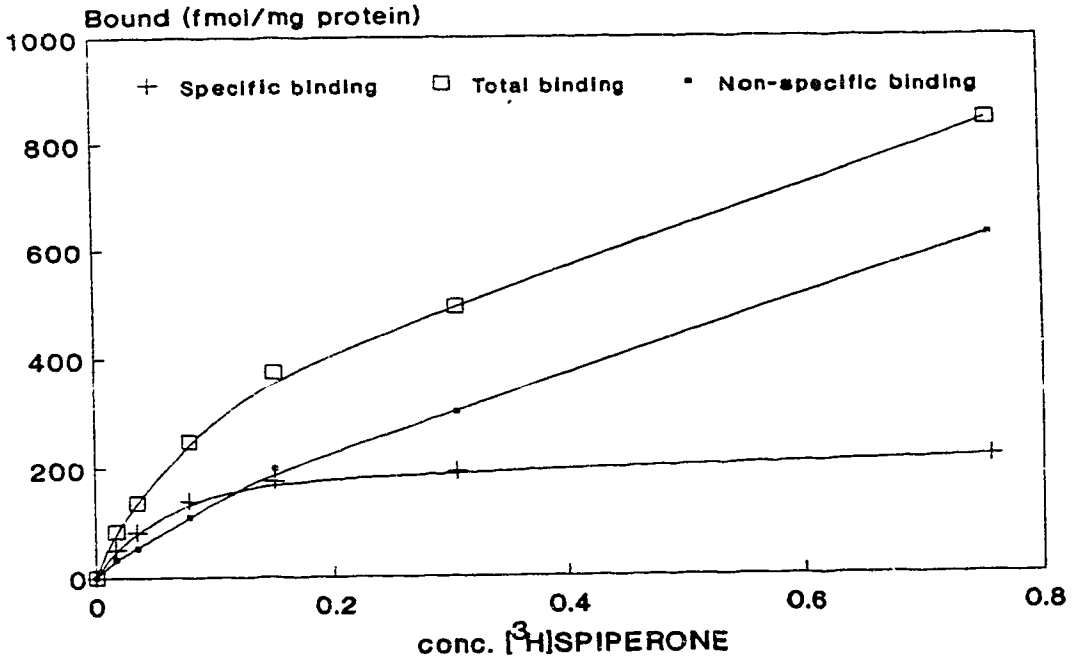


Figure 19: Typical binding isotherm for ^{3}H-spiperone binding to rat brain striatal D2 DA receptors.

Table 28: Effects of long-term (28 day) administration of antidepressant drugs on K_d and B_{max} of striatal D_2 DA receptors.

Drug	Dose (mg kg^{-1} per d)	n	K_d (nM)	B_{max} (fmol mg^{-1} protein)
VEH	0	5	0.0490 ± 0.0039	253 ± 12
DMI	10	6	0.0475 ± 0.0010	244 ± 9
TCP	1	6	0.0481 ± 0.0026	203 ± 11*
PLZ	5	6	0.0457 ± 0.0020	212 ± 18*
PLZ	10	5	0.0484 ± 0.0030	219 ± 8*

Note: Values are means ± S.E.M. *Denotes significantly different from vehicle ($p \leq 0.05$).

C.7.3 Effects of long-term (28 day) administration of PEA and/or DEP on striatal D_1-like DA receptors

The effects of long-term administration of PEA and/or DEP on the affinity (K_d) and density (B_{max}) of D_1-like receptors are shown in Table 29. These treatments did not alter the affinity of these receptors for ^{3}H-SCH 23390 [DEP $F(1,18)=0.05$, PEA $F(1,18)=0.01$, DEP x PEA $F(1,18)=0.10$]. Two way ANOVA indicated that DEP [$F(1,18)=8.23$] and PEA [$F(1,18)=8.56$] each induced a small but significant (9%) decrease in the density of D_1-like receptors. When administered together there was an additive effect of these treatments as no interaction was indicated [$F(1,18)=0.19$]. The two drugs administered in combination resulted in a 22% decrease in the density of D_2-like receptors.

C.7.4 Effects of long-term administration (28 day) of PEA and/or DEP on striatal D_2-like DA receptors

The effects of long-term administration of PEA and/or DEP on the affinity (K_d) and density (B_{max}) of D_2-like receptors are shown in Table 30. These treatments did not alter the affinity of these receptors for ^{3}H-spiperone [DEP $F(1,18)=0.25$, PEA $F(1,18)=0.23$, DEP x PEA $F(1,18)=0.01$]. Two way ANOVA indicated that there was no significant effect of either DEP [$F(1,18)=3.65$] or PEA [$F(1,18)=0.54$] on the density of D_2-like receptors, nor was there any interaction between these two drugs [$F(1,18)=0.12$] on this measure. Although DEP and PEA did not have a significant effect the mean decrease in D_2-like receptors was 13% in this study after the combined administration of these two drugs.

Table 29: Effects of long-term (28 day) administration of PEA and/or DEP on the K_d and B_{max} of striatal D_1 DA receptors.

Drug	Dose (mg kg^{-1} per d)	n	K_d (nM)	B_{max} (fmol mg^{-1} protein)
VEH/VEH	0/0	5	0.251 ± 0.023	995 ± 30
PEA/VEH	10/0	6	0.240 ± 0.021	908 ± 15*
VEH/DEP	0/1	6	0.246 ± 0.020	906 ± 56*
PEA/DEP	10/1	5	0.248 ± 0.017	788 ± 18*

Note: Values are means ± S.E.M. *Denotes significantly different from vehicle ($p \leq 0.05$).

Table 30: Effects of long-term (28 day) administration of PEA and/or DEP on the K_d and B_{max} of striatal D_2 DA receptors.

Drug	Dose (mg kg^{-1} per d)	n	K_d (nM)	B_{max} (fmol mg^{-1} protein)
VEH/VEH	0/0	5	0.0490 ± 0.0039	253 ± 12
PEA/VEH	10/0	6	0.0475 ± 0.0055	221 ± 18
VEH/DEP	0/1	6	0.0476 ± 0.0023	234 ± 12
PEA/DEP	10/1	5	0.0454 ± 0.0019	213 ± 15

Note: Values are means ± S.E.M.

D. DISCUSSION

D.1 QUANTIFICATION OF PHENYLALANINE

This procedure developed for measurement of phenylalanine proved to be simple, very rapid, convenient and relatively inexpensive. The method should be readily applicable to the quantification of phenylalanine in other body tissues and fluids. The whole brain total phenylalanine concentration obtained from this method is in good agreement with previously reported values (Perry, 1982; Eriksson and Carlsson, 1988).

It has been suggested that PEA may merely be a metabolic byproduct formed when the enzyme AAAD acts on phenylalanine. Regional values of phenylalanine do not, however, appear to parallel regional concentrations of PEA. PEA is lowest in cerebellum and highest in olfactory tubercles (Paterson *et al.*, 1990) whereas levels of phenylalanine are highest in cerebellum and lowest in olfactory tubercles. Other areas that are high in PEA, including the nucleus accumbens and hypothalmus, do have relatively high concentrations of phenylalanine. These data clearly indicate that phenylalanine levels are not the sole determinant of PEA levels in all brain areas.

Phenylalanine is an essential amino acid and must be supplied in the diet (Alberts *et al.*, 1983). Like other amino acids, it is used in the biosynthesis of proteins and can be catabolized to generate molecular energy (Alberts *et al.*, 1983). Phenylalanine is also the precursor for the amino acid tyrosine and also is involved in a variety of other biochemical pathways (Lehninger, 1978). Less than 2% of available tyrosine is used in the synthesis of catecholamines (Cooper *et al.*, 1986) and it would be expected that very little phenylalanine is used for the synthesis of PEA. The discrepancy between levels of phenylalanine and PEA in different brain regions may be attributed to this factor. Currently it is not possible

to specifically measure the pool of phenylalanine that may be available for PEA and catecholamine synthesis.

D.2 EFFECTS OF TREATMENTS THAT ALTER PEA AVAILABILITY ON PHENYLALANINE CONCENTRATIONS

Both reserpine pretreatment and 6-OHDA lesions had the expected effects on amines and their metabolites, confirming the efficacy of these treatments. Nevertheless, these treatments, which are known to alter PEA levels, do not appear to alter levels of the precursor amino acid phenylalanine. These data further support the proposal that no simple relationship between phenylalanine levels and PEA availability exists. Lesions of DA neurons may also destroy PEA-containing neurons or, alternatively, a group of DA neurons that regulate PEA availability.

Recent results from studies using dopaminergic neurons of the retina and striatum indicate that AAAD, the synthetic enzyme for PEA, may be regulated (Hadjiconstantinou *et al.*, 1988; 1989; Rosetti *et al.*, 1990). In the retina, stimulation of DA D_1 receptors results in a decrease in the activity of AAAD, whereas a decrease in DA D_1 receptor stimulation leads to an increase in the activity of AAAD. In the striatum of mice treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) antagonism of either D_1 and D_2 receptors resulted in an increase of AAAD activity, but these effects were not observed in the absence of MPTP pretreatment (Hadjiconstantinou *et al.*, 1989). MPTP is a highly selective toxin for DA cells in the substantia nigra pars compacta of mice and therefore depletes striatal DA content (Heikkila *et al.*, 1984). The above results indicate that under conditions of DA depletion, AAAD activity may be changed by DA receptor stimulation. Indeed, it is possible that reserpine treatment, which depletes DA *via* releasing the vesicular stores of DA, may lead to a decrease in DA stimulation and

thus an activation of AAAD leading to an increase in the synthesis of PEA. Other studies fit loosely into the framework of this hypothesis. Treatment with amphetamine, which results in an increase in the stimulation of DA receptors, decreases the rate of accumulation of PEA in the striatum (Juorio *et al.*, 1991). Administration of neuroleptics, which results in a decrease in the stimulation of DA receptors, increases the accumulation of striatal PEA (Jourio *et al.*, 1991). These data have led to the hypothesis that PEA synthesis changes in response to changes in the activity of AAAD which is negatively coupled to a presynaptic DA receptor (Paterson *et al.*, 1990). This hypothesis suggests the DA D_2 autoreceptor may be involved in regulating AAAD activity and PEA levels. Alternatively, it is possible that PEA is synthesized in non-DA containing neurons as previously mentioned [Section A.5.1]. These neurons may have DA D_1 receptors on them that are negatively coupled to AAAD. This second hypothesis may be more likely as studies in the retina indicate that under normal (i.e. non-DA depleted conditions) the D_1 and not D_2 receptor may be linked to AAAD. It is clear that further testing of these hypotheses is needed.

D.3 FUNCTIONAL ANALYSIS OF β-ADRENOCEPTORS

D.3.1 Effects of long-term antidepressant and PEA treatment on the behavioral response to salbutamol

The dose-dependent effect of PLZ on the behavioral response to salbutamol observed in this study replicated a previous finding (McManus *et al.*, 1991). In addition, only the higher dose of PLZ resulted in a significant increase in brain concentrations of PEA as determined with a GC-ECD assay. Chronic administration of PEA in combination with DEP resulted in an attenuation of the salbutamol response and a marked increase in brain concentrations of PEA. Treatment with either PEA or DEP alone failed to alter the salbutamol response or

to significantly increase brain levels of PEA. These observations indicate that PEA can induce a functional down-regulation of β-adrenoceptors. Additionally, the observation that only the high dose of PLZ altered the salbutamol response and increased brain levels of PEA indicates that PEA may, at least in part, mediate the effects of chronic administration of PLZ on β-adrenoceptor function.

Although salbutamol has previously been used to characterize altered β-adrenoceptor function, the fact that it exhibits poor penetrance of the blood-brain barrier may be viewed as problematic (Caccia and Fong, 1984). Clenbuterol has been used as an alternative but, although more lipophilic that salbutamol, this β_2-adrenoceptor agonist is also an antagonist at β_1-adrenoceptors (Ordway *et al.*, 1987; Vos *et al.*, 1987). There is, however, evidence that the effects of peripherally administered salbutamol are centrally mediated. The decrease in locomotor activity induced by salbutamol is evident following either peripheral or central injection (Frances *et al.*, 1979). These effects are not blocked by β-adrenoceptor antagonists which act peripherally and the effects of centrally active β-adrenoceptor antagonists on this salbutamol response are stereoselective (Mogilnicka, 1982; Przegalinski *et al.*, 1984a,b). The effects of peripherally administered salbutamol on food intake are also blocked by central administration of racemic propranolol, giving further support to a central component of the actions of salbutamol (Borsini *et al.*, 1982). These data taken together with the results of the present experiments imply that central β_2-adrenoceptors are altered after long-term treatment with PEA. These effects of PEA may represent a significant component of the actions of MAO inhibitor antidepressants, particularly PLZ. These findings are discussed further, in the context of changes in receptor binding studies, in a subsequent section [D.4.2].

D.3.2 Lack of effects of long-term antidepressant treatment on the salbutamol-induced changes in amino acid concentrations in brain and plasma

Previous studies have demonstrated that brain and plasma concentrations of LNAAs are altered by administration of β-adrenoceptor agonists (Eriksson *et al.*, 1984; Eriksson and Carlsson, 1988; Edwards and Sorisio, 1988; Edwards *et al.*, 1989). The present results confirm and extend this finding. The control values obtained for LNAA levels in this experiment are in good agreement with levels found in other studies (Perry, 1982; Eriksson and Carlsson, 1988; Edwards *et al.*, 1989). In addition to confirming the effects of isoproterenol on brain and plasma levels of LNAAs, this experiment revealed that salbutamol-induced changes in LNAA concentrations parallel those induced by both isoproterenol and clenbuterol. As in the study with clenbuterol, it is evident that salbutamol-induced changes in tryptophan were greater than those observed with tyrosine and phenylalanine. In fact tryptophan increased to a greater extent than any other LNAA in brain. No changes in brain leucine or isoleucine concentrations were observed in this or a previous study (Eriksson and Carlsson, 1988) although plasma levels of leucine and isoleucine were decreased to a greater extent than those of the other LNAAs. A possible explanation for this is that β-adrenoceptors may play a role in the regulation of whole body leucine and isoleucine metabolism through a mechansim different from that controlling LNAA transport between brain and periphery. In humans, adrenaline infusion causes a decrease in leucine flux (ie. between muscle tissue and blood) and an increase in the metabolic clearance of leucine, causing a decrease in plasma leucine concentrations (Kraenzlin *et al.*, 1989). The lack of β-agonist-induced increases in brain leucine concentrations may be due to increased oxidative metabolism, thus offsetting any effect of increased transport into brain.

Isoproterenol has two hydroxyl groups on the benzene ring and thus penetrates the blood-brain barrier very poorly (Oldendorf, 1973). It therefore appears that transport of LNAAs across the blood-brain barrier is under the control of peripheral β_2-adrenoceptors. It is possible that these receptors may play a role in the response to acute stress in rats. It is known that in the rat acute immobilization stress leads to increases in plasma adrenaline and NA (Kvetnansky *et al.*, 1978). Recently it was found that acute immobilization stress caused an increase in brain levels of several LNAAs, including tryptophan, phenylalanine, valine, leucine and isoleucine. There was also a corresponding decrease in plasma levels of phenylalanine, tyrosine, isoleucine and total tryptophan (Kennett *et al.*, 1986). It is possible that stress may affect LNAA transport *via* increase stimulation of β_2-adrenoceptors by plasma adrenaline and NA. In view of the purported relationship between chronic stress and depression (Anisman and Zacharko, 1982), this hypothesis should be assessed further.

In the present study there were no effects of DMI or PLZ on brain or plasma levels of LNAAs. Acute treatment with TCAs and MAO inhibitors has been shown to elevate brain tryptophan concentrations (Badawy and Evans, 1981, 1982; Edwards and Sorisio, 1988) but this is not apparent following chronic administration of the present compounds. Although chronic administration of each of these drugs with the present dose regimen results in an attenuation of the behavioral effects of salbutamol (McManus and Greenshaw, 1991b; McManus *et al.*, 1991), no interaction between antidepressant treatment and salbutamol was observed in the present study. Thus, it appears that functional changes in β-adrenoceptors following chronic antidepressant treatments do not extend to their effect on LNAA transport into the brain. These results imply that peripheral β_2-adrenoceptors are unaltered by long-term antidepressant treatment.

As indicated earlier, the β_2 agonists salbutamol and clenbuterol may be effective in the treatment of depression [Section A.3.1.1]. Recent studies using clenbuterol appear, however, to indicate that long-term treatment reduces the density of cortical β_2-adrenoceptors and not β_1-adrenoceptors, an effect that is opposite that of other antidepressant treatments [Section A.3.1.1]. This observation indicates that the mechanism of action of β_2-adrenoceptor agonists may differ from that of other antidepressant drugs. It is possible that part of the antidepressant efficacy of clenbuterol and salbutamol is due to these drugs increasing the levels of LNAAs including L-tryptophan and L-phenylalanine. As stated earlier, the manipulation of L-tryptophan levels and L-phenylalanine levels in humans alters mood [Section A.6.5.2].

D.4 RADIOLIGAND BINDING TO β-ADRENOCEPTORS

D.4.1 Effects of long-term antidepressant drug treatment on radioligand binding to β-adrenoceptors

The density of β-adrenoceptors measured in cortex using ^{3}H-CGP 12177 binding is in general much lower than the density observed in previous studies which used ^{3}H-DHA as a ligand (Baker and Greenshaw, 1989 for references). The density of 66.5 fmol mg^{-1} protein and K_d of 0.11 nM are, however, in good agreement with the work of Riva and Creese (1989a,b) who found that ^{3}H-CGP 12177 is a more selective ligand for β-adrenoceptors. The density of β-adrenoceptors also is in good agreement with the earlier work by Minneman *et al.*, (1979b) who used ^{125}I-iodohydroxybenzylpindolol as a ligand for β-adrenoceptors. In the present study, cortical β-adrenoceptors were found to consist of 80% β_1-adrenoceptors and 20% β_2-adrenoceptors. This ratio of β_1/β_2-adrenoceptors is in close agreement with previously reported data (Minneman *et al.*, 1979a; Riva and Creese, 1989b) but is higher than the figure reported by several other groups

who used ^{3}H-DHA as a ligand (Dooley and Bittiger, 1987; Beer *et al.*, 1987; Heal *et al.*, 1989).

Using the new more selective β-adrenoceptor ligand, ^{3}H-CGP 12177, it was found that both DMI and MAO inhibitor drugs decreased the density of rat cortical β-adrenoceptors but did not affect the affinity of these sites. The effect of DMI on β-adrenoceptors was shown to be due to a reduction in only the β_1 subtype of this receptor as has been previously reported (Minneman *et al.*, 1979b; Dooley and Bittiger, 1987; Heal *et al.*, 1989). To date the effects of long-term administration of MAO inhibitors on the subtypes of β-adrenoceptors in rat cortex have largely been ignored except for one existing study on the effects of TCP (Heal *et al.*, 1989). The present study indicates that the MAO inhibitors TCP and PLZ both induced changes in β-adrenoceptors by acting solely on the β_1-adrenoceptor subtype in cortex.

The density of cerebellar β-adrenoceptors measured in the present study also is in close agreement with a previous study (Minneman *et al.*, 1979b). In the cerebellum β-adrenoceptors consisted of 13% β_1- and 87% β_2-adrenoceptors, also in good agreement with the study cited above. The density and affinity of cerebellar β-adrenoceptors were unaltered by long-term treatment with DMI, TCP or PLZ. Due to the very low density of β_1-adrenoceptors in cerebellar tissue the results examining the effects of drugs on this population of receptors should be interpreted cautiously. There was a tendency for the β_1-site to be decreased but this did not reach statistical significance.

Overall the data in these experiments indicate that long term administration of either TCA or MAO inhibitor drugs induces a reduction in the density of β_1-adrenoceptors in rat brain but does not affect the density of β_2-adrenoceptors. β_2-Adrenoceptors appear to be unaltered after long-term antidepressant treatment in radioligand binding studies and in the functional studies examining the

salbutamol-induced changes in brain and plasma LNAA concentrations. The behavioral response to salbutamol was, however, altered after long-term antidepressant treatment. This lack of concordance between binding data and functional data is further examined in the next section.

D.4.2 Effects of long-term administration of PEA and/or DEP on radioligand binding to β-adrenoceptors

The combination of PEA and DEP raised brain PEA levels approximately 10 fold. This drug combination also resulted in a decrease in the density of cortical β-adrenoceptors that was significantly larger than the decrease seen after DEP alone. These data indicate that increasing PEA levels for a long-term period does cause a decrease in cortical β-adrenoceptor density. Administering PEA alone did not induce a change in brain levels of PEA using the present GC-ECD assay and also failed to alter cortical β-adrenoceptor density. In the cortex the decrease in β-adrenoceptor density observed after the MAO inhibitor DEP appeared to be due solely to a change in β_1-adrenoceptor density. This result parallels the findings with other MAO inhibitors. Statistical analysis indicated that both β_1- and β_2-adrenoceptors were decreased in the cortex following the combination of PEA and DEP. This contradicts the finding that cerebellar β_2-adrenoceptors were unaltered by this treatment. It may be that there is differential regulation of β_2-adrenoceptors in these two areas. Alternatively, it is conceivable that the effect of PEA and DEP on cortical β_2-adrenoceptors is a result of a Type 1 statistical error. Statistical analysis also indicated that the total number of cerebellar β-adrenoceptors is reduced by DEP. This may be due to a reduction in the number of β_1-adrenoceptors. Statistical analysis indicated that only PEA had a significant effect on cerebellum β_1-adrenoceptors. Nevertheless, as stated earlier, only the vehicle group had a significantly better two-site than a one-site fit in the competition

experiments examining cerebellar β-adrenoceptors. This indicates that the drug treatments used in this thesis, including DEP, may have nearly eliminated the β_1-adrenoceptor population in cerebellum.

The PEA-induced decrease in β-adrenoceptor density does parallel the previous observation that PEA can induce a functional down-regulation of β-adrenoceptors. Nevertheless, binding data indicate that PEA, like antidepressant drugs, predominately acts on β_1-adrenoceptors. As the behavioral response to salbutamol is altered by both PEA and antidepressant treatment, this is not easily attributed to changes in brain β_2-adrenoceptors as antidepressants failed to alter β_2-adrenoceptor density in brain. It is therefore probable that the changes in the behavioral response to salbutamol are not due solely to changes in brain β_2-adrenoceptors. Antidepressant drugs may alter post-receptor events and/or other receptor types that mediate the behavioral response to salbutamol. Nevertheless, it is clear that long-term elevation of PEA can alter β-adrenoceptors in binding studies. This may be the result of increased NA availability at the synapse as PEA is known to both block the uptake and stimulate the release of NA (Horn and Snyder, 1973; Raiteri *et al.*, 1977). Alternative explanations for this effect are also possible as it has been hypothesized that PEA may be an allosteric modulator at catecholamine receptors (Paterson and Boulton, 1988; Paterson *et al.*, 1990).

D.5 RADIOLIGAND BINDING TO DA RECEPTORS

D.5.1 Effects of long-term antidepressant treatment on radioligand binding to DA receptors

The density and affinity profile of D_1- and D_2-like receptors in this study was similar to those of previous studies using similar binding methodologies (Briere *et al.*, 1987; Urwyler and Coward, 1987; Gottberg *et al.*, 1989). In the present study

DMI induced a decrease in the density of D_1-like receptors but not D_2-like receptors, with no changes in K_d values for either receptor. These findings are in agreement with previous studies examining the effects of long-term administration of TCAs (Klimek and Nielsen, 1987; De Montis *et al.*, 1990). Long-term administration of MAO inhibitors in the present study induced significant changes in both D_1- and D_2-like receptor density but not in their affinities. This finding is in contrast to that of Klimek and Maj (1990) who found that long-term administration of two MAO-A inhibitors, brofaromine and moclobemide, did not alter D_1- or D_2-like receptor binding using the same ligands employed in the present study. This may be due to the fact that these reversible MAO-A inhibitors were assessed following 14 days of oral administraiton. In the present experiments the effects of irreversible MAO inhibitors were assessed following 28 days of constant drug infusion *via* minipumps. It is likely that the regimen of drug administration and reversibility of drug action may account for the previous negative report (Klimek and Maj, 1990). Klimek and Maj (1990) have suggested that D_1 and D_2 receptors may be differentially sensitive to increased DA concentrations as this would explain the ability of TCAs, which are often weak DA uptake inhibitors (Randrup and Braestrup, 1977), to only decrease the density of D_1 DA receptors. The MAO inhibitors may have a larger effect on DA concentrations and therefore may alter both D_1 and D_2 receptors. In the present experiments the effects of MAO inhibitors on D_1 and D_2 receptors were of a similar magnitude. If D_1 receptors are more sensitive to increased DA concentrations, it may be expected that there would be a considerably larger effect on D_1 than D_2 receptors. It appears, therefore, that differential sensitivity of these receptors to DA availability is not sufficient to explain the present findings. Additionally, long-term administration of antidepressant drugs decreased the density of DA receptors whereas equivalent treatments increased the functional responsiveness of DA

receptors [Section A.3.2]. Clearly more work on the effects of antidepressant drugs on the DA system is needed to help resolve the above discrepancies.

D.5.2 Effects of long-term administration of PEA and/or DEP on radioligand binding to DA receptors

Long-term elevation of brain PEA levels induced by the combined administration of DEP and PEA resulted in a decrease in the density of DA D_1 receptors in rat striatum. Statistical analysis of this effect surprisingly revealed that PEA administered alone was partially responsible for this result although neurochemical analysis did not reveal any significant effects on PEA levels. Although the combination of DEP and PEA resulted in a mean decrease of 17% in D_2 receptor density this effect was not statistically significant. In general it appears that chronic elevation of brain PEA levels has effects that are similar to long-term administration of antidepressants on striatal D_1 receptors. As with NA this could be due to either the DA releasing and uptake blocking properties of PEA or due to a neuromodulatory action of PEA at DA receptors. An increase in the dose of PEA administered may lead to a significant change in D_2 DA receptors. Nevertheless, the present results indicate that D_1 receptors and not D_2 receptors are altered by long-term PEA treatment. The selective D_1 receptor change is interesting as it is the DA D_1 receptor that is negatively coupled to AAAD, the enzyme involved in the synthesis of PEA [Section D.2]. This significance of this relationship remains to be elucidated.

D.6 SUMMARY

PEA is found in small quantities in the mammalian CNS and the structural similarity between this amine and amphetamine was the initial impetus for researchers to investigate its possible role in the pharmacotherapy of depression.

In the context of antidepressant drug action brain concentrations of this amine are greatly increased by administration of MAO inhibitor antidepressant drugs. The studies in this thesis provided some interesting insights into both the control of the availability of endogenous PEA and also into its possible role in the pharmacotherapeutic action of MAO inhbitors.

Several studies in this thesis indicate that brain levels of PEA are not simply related to the availability of its precursor phenylalanine, suggesting that PEA is not simply a metabolic by-product formed when AAAD acts on phenylalanine. Recent evidence from other laboratories indicates that AAAD may be negatively coupled to DA and in this thesis it has been shown that D_1 DA receptor density is decreased by increasing PEA availability is of interest. This relationship should be further explored.

Other studies in this thesis investigated the possible role that increased PEA availability may play in the action of MAO inhibitor antidepressant drugs. Long-term MAO inhibitor treatment decreased the density of brain β_1-adrenergic and DA D_1 and D_2 catecholamine receptors. Increasing the availability of PEA resulted in changes in catecholamine receptor density that were similiar to, but not identical to, the changes induced by MAO inhibitors. It was also demonstrated that both MAO inhibitor treatment and increasing the availability of PEA could alter the behavioral response to the β_2-adrenoceptor agonist salbutamol. Transport of LNAAs, which is probably regulated by β_2-adrenoceptors located outside the brain, was not altered by long-term treatment with antidepressant drugs including MAO inhibitors. Furthermore, brain β_2-adrenoceptor density was not altered by either long-term MAO inhibitor treatment or by increasing PEA availability. This lack of concordance between behavioral studies and receptor binding studies has not previously been reported. The parallels between the effects of increased PEA availability and of MAO inhibitor treatment on the catecholamine systems indicate

that increased availability of PEA may mediate some of the effects of MAO inhibitor treatment. The mechanism by which PEA induces changes in catecholamine receptor number and/or function remains unknown and should be further investigated. Nevertheless, this thesis demonstrates clear effects of PEA on catecholamine receptors and it is plausible that elevation of PEA during treatment with MAO inhibitors may play a role in the pharmacotherapy of depression.

E. CONCLUSIONS

1. A novel GC method for the quantification of brain phenylalanine has been developed. The method is rapid and convenient and the resultant derivative has good chromatographic properties and is stable, permitting unattended injection with an autosampler. The structure of the derivative was confirmed by GC-MS.

2. Brain levels of PEA were not simply related to brain levels of the precursor amino acid phenylalanine. 6-OHDA-induced lesions of the substantia nigra which depleted striatal DA and are known to decrease the MAO inhibitor-induced accumulation of PEA in the striatum had no effect on striatal levels of phenylalanine. Reserpine treatment which depleted striatal DA and 5-HT and is known to increase the MAO inhibitor-induced accumulation of PEA in the striatum also had no effect on striatal levels of phenylalanine. The lack of effect of these lesions and of reserpine on striatal phenylalanine levels has not been previously reported.

3. Long-term treatments that increase brain PEA levels in rats resulted in a decreased functional (behavioral) response to the administration of the β_2-adrenoceptor agonist salbutamol. This novel finding is similar to the results of long-term treatment with antidepressant drugs.

4. Brain and plasma levels of LNAAs were regulated by β_2-adrenoceptors that are probably located outside the brain. Stimulation of these receptors resulted in increases in brain levels of LNAAs at the expense of peripheral levels of these LNAAs. The β_2-adrenoceptors that regulated brain and plasma LNAA content were not altered by long-term treatment with either the TCA or MAO inhibitor antidepressant drugs used in this study. This last finding has not been previously reported.

5. Binding studies, using the selective β-adrenoceptor ligand ^{3}H-CGP 12177, indicated that the density of β-adrenoceptors in rat cortex was lower than earlier studies using ^{3}H-DHA indicated. Antidepressant drugs decreased the density of cortical β-adrenoceptors while not altering the affinity of these receptors. In the cerebellum, where the majority of β-adrencoeptors are of the β_2 subtype, antidepressant drugs did not alter the density of these receptors. This novel finding in combination with studies examining binding to β-adrenoceptors subtypes in cortex and cerebellum indicate that long-term antidepressant drug treatment decreases brain β_1- but not β_2-adrenoceptor density.

6. Increasing PEA levels resulted in a decrease in the density of brain β-adrenoceptors in rats. Similar to antidepressants, long-term elevation of PEA resulted in a decrease in cortical β_1-adrenoceptors but not in cerebellar β_2-adrenoceptors. These novel findings indicate that it is possible that the MAO inhibitor-induced down-regulation of β-adrenoceptors is partially mediated by increased brain levels of PEA.

7. Long-term administration of MAO inhibitor antidepressant drugs, as with TCAs, resulted in a decrease in the density of D_1-like DA receptors. MAO inhibitor antidepressant drugs also decreased the density of D_2-like DA receptors whereas DMI did not. The effects of MAO inhibitors on DA receptors have not been previously reported.

8. Similar to long-term antidepressant treatment, long-term elevation of PEA levels resulted in a decreased density of D_1-like DA receptors. Increasing PEA levels also had a tendency to decrease the density of D_2-like DA receptors although this did not reach statistical significance. This is the first report of the effects of PEA on DA receptors.

9. Overall these data indicate that PEA does not appear to be merely a metabolic by-product in the mammalian CNS. PEA has effects on catecholamine receptors that are similar to the effects of MAO inhibitors and it is plausible that elevation of PEA during treatment with MAO inhibitors may play a role in the pharmacotherapy of depression.

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