THE ROLE OF THE NO-cGMP PATHWAY AS A PUTATIVE TARGET IN ANTIDEPRESSANT ACTION

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ABSTRACT

Depressive disorders are among the most frequent psychiatric diseases in the Western world with prevalence between 9% and 18%. Poor compliance and inappropriate antidepressant discontinuation invokes long-term morbidity, and appear linked to hippocampal shrinkage.

Despite major advances in pharmacological treatment of the illness over the past 30-40 years, currently available agents have distinct shortfalls both in clinical efficacy and in maintenance of response. This implies a greater long-term morbidity with significant impact on the patient, the patient's family as well as economic implications to health care managers and providers. The major reason for this state of affairs is our poor understanding of the neurobiology of depression and hence, of antidepressant (AD) action. AD drugs are thus not addressing the crucial neurobiological target underlying the illness, and new strategies and treatments are urgently needed. In recent years, depression has been associated with disturbances in excitotoxic glutamatergic activity, yet this has not been systematically evaluated.

While the role of neurotransmitters such as serotonin, noradrenaline and dopamine has been extensively studied, new evidence suggests a role for the unique neurotransmitter nitric oxide (NO). Nitric oxide (NO), is activated by glutamatergic systems in various limbic and other regions of the brain, and has recently also been implicated in anxiety and affective disorders. Of special interest is the putative role of NO in cellular memory, synaptic plasticity and cell survival, all-important processes in the neuropathology and neurodevelopment of depression.

Recent clinical studies have provided evidence of the role of the NO-pathway in depression, while preclinical studies have demonstrated the anxiolytic and antidepressant actions of nitric oxide synthase (NOS)-inhibitors. Moreover, NO interacts with other classical transmitters that have a regulatory role on mood, particularly the monoamines, as well as glutamate and gamma-aminobutyric acid (GABA).

In the current study the role of the NO-cGMP pathway in AD action was investigated, after chronic imipramine (IMI) and after IMI withdrawal, using a learned helplessness paradigm. Behavioral changes, hippocampal NOS activity and cGMP accumulation was determined together with pharmacological manipulation of the NO-cGMP pathway.

Chronic IMI, 15 mg/kg/day intraperitoneal (ip) administration induced a pronounced reduction in swim immobility time in the forced swim test (FST), with no effect on horizontal or vertical locomotor activity. These behavioural changes were accompanied by a significant reduction in NOS enzyme activity and cGMP accumulation. In order to confirm the involvement of the NO-cGMP pathway in the AD action of IMI, chronic (3 weeks) IMI treatment was followed by an acute withdrawal of 7 days. Acute withdrawal, after chronic IMI treatment, resulted in a significant increase in swim immobility time and an increase in NOS enzyme activity and cGMP levels. In fact, NOS activity was raised above that of control, not just higher than the effect of chronic IMI.

In order to assess the possible role of the NMDA-NO-cGMP pathway in AD withdrawal, the NMDA receptor antagonist, memantine, and the NOS/guanylyl cyclase (GC) inhibitor, methylene blue (MB), were administered during the 7 day IMI withdrawal period.

Memantine (5 mg/kg/d ip), during the 7 day IMI withdrawal period, significantly reversed the increase in immobility time evoked after IMI withdrawal. This was accompanied by a significant reduction in NOS enzyme activity and a tendency to decrease cGMP levels. This data confirms that the antidepressant action of IMI, as well as IMI withdrawal, is associated with actions on the NMDA-Glu-NO-cGMP pathway. Particularly, IMI withdrawal evokes an increase in glutamate activity that is responsible for NOS activation.

During the 7 day IMI withdrawal period, MB (15 mg/kg/d ip) also significantly reversed the increased immobility time after IMI withdrawal and was accompanied by a tendency to decrease NOS enzyme activity and cGMP levels in the rat hippocampus, however statistical significance was not reached. Although not emphatic, this data implies a possible role of the NO-cGMP pathway in AD action and AD withdrawal.

In order to determine whether the observed IMI withdrawal effects on the NO-cGMP pathway may occur through an initial destabilisation in the serotonergic system, the 5-$HT_{2A/2C}$ receptor antagonist, ritanserin (4 mg/kg/d ip), was administered during the IMI withdrawal period.

These studies revealed that antidepressant withdrawal evokes an increase in 5-HT_2-mediated activity, and that antidepressant-induced NOS activation after withdrawal has its origin in serotonergic hyperactivity. Clearly, this is supportive of a distinct relationship between the NO and serotonergic system in antidepressant response. On its own, ritanserin was found to increase NOS and cGMP levels, yet during IMI withdrawal this response was lost, suggesting that IMI withdrawal alters the response to a 5-HT_2 receptor antagonist, which may have major clinical implications.

In conclusion, the AD action of IMI, as well as chronic IMI withdrawal, involves actions on the NO-cGMP pathway. Withdrawal of ADs is associated with a loss of AD efficacy together with an increase in release of NO and cGMP. The NMDA antagonist, memantine, and the NOS/GC inhibitor, MB, reversed these responses therefore suggesting that the NMDA-Glu-NO-cGMP pathway may be a new putative target in understanding the neurobiology of AD action. Finally, NOS activation following withdrawal suggest that inappropriate withdrawal during the treatment of depression may mediate neurodegenerative pathology observed in recurrent depression, possibly by severely increased hippocampal NOS activity which is toxic to neurons.

KEY WORDS: DEPRESSION; ANTIDEPRESSANT; WITHDRAWAL; IMIPRAMINE; NO-cGMP PATHWAY; METHYLENE BLUE; MEMANTINE; RITANSERIN

OPSOMMING

Depressie is een van die algemeenste psigiatriese siektetoestande in the Westerse wêreld met 'n voorkomsyfer van 9-18%. Swak pasiëntmeewerkendheid en ondeurdagte staking van antidepressante veroorsaak in die langtermyn morbiditeit wat waarskynlik met krimping van die hippokampus verband hou.

Ten spyte van geweldige vooruitgang in die farmakologiese behandeling van hierdie siektetoestand gedurende die afgelope 30-40 jaar het die huidige beskikbare middels besliste tekortkominge in sowel kliniese effektiwiteit en handhawing van die respons. Dit impliseer 'n groter mate van langtermyn neerslagtigheid met 'n betekenisvolle impak op die pasiënt, die pasiënt se familie, asook ekonomiese implikasies vir gesondheidsorgbestuurders en -verskaffers. Die hoofrede daarvoor is die gevolg van 'n gebrek aan insig in die neurobiologie van depressie en gevolglik antidepressantwerking. Antidepressante spreek dus nie die belangrike onderliggende neurobiologiese aspek van depressie aan nie en nuwe strategieë en behandelings word dringend benodig. Tans word depressie geassosieer met versteurings in eksitotoksiese glutamaataktiwiteit alhoewel dit nog nie sistematies geëvalueer is nie.

Die rol van senuweeoordragstowwe soos serotonien, noradrenalien en dopamien is reeds op groot skaal bestudeer maar nuwe bevindings dui ook op 'n moontlike rol vir die unieke oordragstof, stikstofoksied (NO). NO word deur die glutamaatsisteem in verskeie limbiese en ander breinareas geaktiveer, en word tans geïmpliseer in angs en affektiewe steurnisse. Die moontlike rol van NO in sellulêre geheue, sinaptiese plastisiteit en seloorlewing is van besondere belang - almal is belangrike prosesse in die neuropatologie en neuro-ontwikkeling van depressie.

Onlangse kliniese studies lewer bewys van die rol van die NO-baan in depressie terwyl die angsiolitiese en antidepressanteffek van NOS-inhibeerders deur pre-kliniese studies gedemonstreer is. Boonop interreageer NO met klassieke oordragstowwe wat 'n regulerende rol in gemoedstoestande het, veral monoamiene, maar ook glutamaat en aminobottersuur (GABA).

In hierdie studie word die rol van die NO-cGMP baan in antidepressantwerking na chroniese imipramienbehandeling en na imipramienontrekking met behulp van 'n "aangeleerde

hulpeloosheidsparadigma" ondersoek. Gedragsveranderinge, NO-sintetase(NOS)-aktiwiteit en akkumulasie van cGMP in die hippokampus is tydens farmakologiese manipulasie van die NO-cGMP baan bepaal.

Chroniese toediening van imipramien, 15 mg/kg/dag intraperitoneaal (ip), het 'n opvallende verlaging in die duur van swem-immobiliteit in die geforseerde swemtoets tot gevolg gehad, met geen effek op die horisontale of vertikale lokomotoraktiwiteit nie. Hierdie gedragsveranderinge het gepaard gegaan met 'n beduidende verlaging in NOS-aktiwiteit asook cGMP-akkumulasie. Ten einde die betrokkenheid van die NO-cGMP baan in die antidepressantwerking van imipramien (IMI) te bevestig, is chroniese (3 weke) IMI-behandeling opgevolg met n skielike ontrekkingsperiode van 7 dae. Akute onttrekking, na die chroniese IMI behandeling, het 'n beduidende verhoging in swem-immobiliteitsduur, NOS-ensiemaktiwiteit en cGMP-akkumulasie tot gevolg gehad. Inteendeel, NOS-ensiemaktiwiteit was selfs hoër as die kontrolegroep, en nie slegs hoër as die chroniese IMI behandelings groep nie.

Om die moontlike rol van die N-metiel-D-aspartaat(NMDA)-NO-cGMP-baan tydens ontrekking van die antidepressant vas te stel, is die NMDA-antagonis, memantien en die NOS/guaniliel siklase(GC)-inhibeerder metileenblou (MB) tydens die 7-dag-onttrekkingsfase toegedien.

Tydens die 7-dag IMI-onttrekkingsperiode het memantien (5 mg/kg/d i.p.) die verhoging in immobiliteitsduur, veroorsaak na IMI onttrekking, merkwaardig omgekeer. Hierdie waarneming het gepaard gegaan met beduidende verlaging in NOS-ensiemaktiwiteit en 'n neiging na verlaagde cGMP-vlakke. Hierdie data bevestig dat die antidepressantwerking van IMI, asook die onttrekking van IMI, geassosieer word met aksies op die NMDA-Glu-NO-cGMP-baan. Merkwaardig is dat IMI onttrekking 'n verhoging in glutamaataktiwiteit veroorsaak, wat verantwoordelik is vir NOS-aktivering.

Gedurende die 7-dag IMI-onttrekkingsperiode het MB (15 mg/kg/d i.p.) ook die verhoogde immobiliteitsduur na IMI onttrekking beduidend verlaag met onbeduidende neigings om NOS-ensiemaktiwiteit en cGMP-vlakke in die rot hippokampus te verlaag. Alhoewel hierdie data nie volstrek is nie, inisieer dit 'n moontlike rol vir die NO-cGMP baan in die antidepressantwerking sowel as in antidepressantonttrekking.

Om te bepaal of die waargeneemde IMI-onttrekkingseffekte op die NO-cGMP baan moontlik deur 'n aanvanklike destabilisering in die serotonergiese sisteem plaasvind, is die 5-$HT_{2A/2C}$-reseptorantagonis, ritanserien, gedurende die IMI onttrekkingstydperk toegedien.

Hierdie studie het getoon dat onttrekking van die antidepressant 'n verhoging in 5-HT_2-gedrewe aktiwiteit veroorsaak, en dat die antidepressant-geïnduseerde NOS-aktivering na onttrekking sy oorsprong in serotonergiese hiperaktiwiteit het. Dit ondersteun dus ongetwyfeld 'n verband tussen die NO- en serotonergiese sisteem in die antidepressantrespons. Tydens sub-akute behandeling met ritanserien is verhoogde NOS en cGMP vlakke gevind, maar gedurende die IMI onttrekkingsperiode het hierdie effek verlore geraak, wat suggereer dat IMI ontrekking die respons op 'n 5-HT_2 reseptor antagonis verander en dus belangrike kliniese implikasies mag hê.

Die gevolgtrekking van hierdie studie is dat die antidepressantwerking van IMI, asook die ontrekking na chroniese IMI-behandeling, aksies op die NO-cGMP-baan insluit. Onttrekking van antidepressante word geassosieer met 'n verlies in antidepressanteffektiwiteit en 'n verhoging in vrystelling van NO en cGMP. Die NMDA-reseptorantagonis, memantien, en die NOS/GC-inhibeerder, MB, het hierdie effekte omgekeer, wat impliseer dat die NMDA-Glu-NO-cGMP-baan 'n veronderstelde nuwe teiken is om die neurobiologie van antidepressantwerking te verstaan. Dus, NOS-aktivering na onttrekking impliseer dat ondeurdagte staking van antidepressantbehandeling kan lei tot neurodegeneratiewe patologie wat waarneembaar is in terugkerende depressie, moontlik as gevolg van erge verhoogde NOS-aktivering in die hippokampus en wat toksies is vir neurone.

SLEUTELWOORDE: DEPRESSIE; ANTIDEPRESSANT; ONTREKKING; IMIPRAMIEN; NO-cGMP BAAN; METILEENBLOU ; MEMANTIEN; RITANSERIEN

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"Go confidently in the direction of your dreams. Live the life you have imagined."

-Henry David Thoreay

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ABBREVIATIONS

AC	:	adenylyl cyclase
Ach	:	acetylcholine
ACTH	:	adreno-corticotrophic hormone
AD	:	antidepressant
AMPA	:	α-amino-3-hydroxy-5-methyl-4-isoxazoleprophionic acid
ANOVA	:	one-way analysis of variance
ATP	:	adenosin triphosphate
BDNF	:	brain-derived neurotrophic factor
BH_4	:	(6R)-5,6,7,8-tetrahydrobiopterin dihydrochloride
BSA	:	bovine serum albumin
CA	:	catecholamines
Ca^{2+}	:	calcium ions
CaM	:	calmodulin
c-AMP	:	cyclic adenosine 3', 5'- monophosphate
cGMP	:	cyclic guanosine 3', 5'- monophosphate
cNOS	:	constitutive nitric oxide synthase
CNS	:	central nervous system
CO	:	carbon monoxide
COMT	:	catechol-O-methyltransferase
CRE	:	cAMP response element
CREB	:	cAMP response element binding protein
CRF	:	corticotropin releasing factor
CRH	:	corticotrophin-releasing hormone

CSF	:	cerebrospinal fluid
DA	:	dopamine
DAAM	:	Digiscan Animal Activity Monitor
DAG	:	diacylglycerol
DNA	:	deoxynucleic acid
DOPA	:	dihydroxyphenylalanine
DTT	:	DL-dithiothreitol
DRL-72s	:	differential reinforcement of low-rate 72-s
DSM-IV	:	diagnostic and statistical manual of mental disorders, fourth edition
EAA	:	excitatory amino acids
EAE	:	experimental autoimmune encephalomyelitis
ECT	:	electroconvulsive shock treatment
EDRF	:	endothelium derived relaxing factor
EDTA	:	ethylenediaminetetraacetic acid
EGTA	:	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
eNOS	:	endothelial nitric oxide synthase
FAD	:	flavin adenine dinucleotide
FMN	:	flavin mononucleotide
FSL	:	Flinders Sensitive Line
FST	:	forced swim test
GABA	:	gamma-aminobutyric acid
GAD	:	glutamic acid decarboxylase
GC	:	guanylate cyclase
Glu	:	glutamate
Gly	:	glycine
GTP	:	guanosine 5'-triphosphate
5-HIAA	:	5-hydroxyindole acetic acid
H_2O	:	water
H_2O_2	:	hydrogen peroxide

HIV : Human Immunodeficiency Virus

HO : haeme oxygenase

HPA : hypothalamic pituitary-adrenal axis

HPT : hypothalamic pituitary-thyroid

5-HT : 5-hydroxy-tryptamine

5-HTP : 5-hydroxytryptophan

iGluR : ionotropic glutamate receptors

IMI : imipramine

iNOS : inducible nitric oxide synthase

IFN-γ : interferon-gamma

IP_3 : inositol triphosphate

IRS : inflammatory response system

KA : kainic acid

l-Arg : L-arginine

LDT : laterodorsal-tegmental

L-NAME : N^G-nitro-arginine methyl ester

L-NMMA : N^G-monomethyl-L-arginine

L-NNA : N^G-nitro-L-arginine

LOX : lipoxygenase

LPS : lipopolysaccharide

LTD : long term depression

LTP : long term potentiation

MAO : monoamine oxidase

MAOI : monoamine oxidase inhibitors

MB : methylene blue

Mg^{2+} ; magnesium ions

MGluR : metabotropic glutamate receptor

MnSOD : manganese superoxide dismutase

mRNA : messenger RNA

NA	:	noradrenaline
Na^{2+}	:	sodium ions
NADPH	:	reduced nicotinamide adenine dinucleotide phosphate
NANC	:	non-adrenergic-non-cholinergic
NARI	:	noradrenaline reuptake inhibitors
NASI	:	noradrenergic selective reuptake inhibitors
NaSSA	:	noradrenergic and specific serotonergic antidepressant
NDRI	:	noradrenaline and dopamine reuptake inhibitors
NF-kB	:	nuclear factor kappa B
7NI	:	7-nitroindazole
NMDA	:	N-methyl-D-aspartate
nNOS	:	neuronal nitric oxide synthase
NO	:	nitric oxide
NO_2^-	:	nitrite
NO_3^-	:	nitrate
NOS	:	nitric oxide synthase
O_2	:	molecular oxygen
$O_2^{\cdot-}$	:	superoxide anion
ODQ	:	1H-[1,2,4]oxadiazolo[4,3-a]-quinoxalin-2-one
$OH^{\cdot}$	:	hydroxyl radical
$ONOO^-$	:	peroxynitrite
PARS	:	poly (ADP-ribose) synthase
PC	:	phospholipase C
PCP	:	phencyclidine
PDE	:	cyclic nucleotide phosphodiesterase
PFC	:	prefrontal cortex
PIN	:	protein inhibitors of nNOS
PIP_2	:	phosphatidyl inositol 4,5-biphosphate
PKA	:	cyclic adenosine 3' 5' -monophospate (cAMP)-dependent protein kinase

PKC	:	protein kinase
PLC	:	phospholipase C
PTSD	:	post traumatic stress disorder
REM	:	rapid eye movement
ROS	:	reactive oxygen species
SGZ	:	subgranular zone
SNRI	:	serotonin and noradrenaline reuptake inhibitors
SOD	:	super oxide dismutase
SPD	:	Spraque – Dawley
SSRIs	:	selective serotonin reuptake inhibitors
SVZ	:	subventricular zone
SwLo	:	Swim Low-Active
TCA	:	tricyclic antidepressant
TD	:	tardive dyskinesia
TNF-α	:	tumor necrosis factor-alpha
TRH	:	thyrotropin-releasing hormone
TRIM	:	1-(2-trifluoromethylphenyl)imidazole
Zn^{2+}	:	zink ions

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INTRODUCTION

Depression is an affective disorder, characterized by pervasive disturbances in mood, sleep, energy, motivation, appetite and thinking (Leonard, 1997). It is a chronic and recurrent illness associated with disability, morbidity and mortality (Harvey *et al.*, 2003). Over 10% of a population will have depression within their lifetime and it is expected to be the second largest cause of burden of disease by 2020 (Danileviciute & Sveikata, 2002).

Previous work on depression has emphasized the role of monoamine systems in mediating disease pathogenesis (Stein & Stahl, 2000), treatment response (Bell & Nutt, 2001), and withdrawal effects (Schatzberg *et al.*, 1997). However, agents that selectively modify these systems, including the TCAs and SSRIs have shortfalls both in clinical efficacy (Anderson, 2000) and in maintenance of response (Nierenberg, 2002). This implies a greater long-term morbidity with significant impact on the patient, the patient's family as well as economic implications for health care managers and providers. The major reason for this state of affairs is our poor understanding of the neurobiology of depression and hence, of antidepressant action. Scientists and clinicians are becoming increasingly aware that current antidepressant drugs are not addressing the crucial neurobiological target underlying the illness, and that new strategies and treatments are urgently needed. This has prompted the shift to more diverse models to explain antidepressant action, particularly the role of the NO-cGMP pathway.

All classes of ADs, including electro-convulsive therapy, have been found to involve suppression of NMDA receptor activity (Skolnick, 1999; Stewart & Reid, 2002). Nitric oxide (NO), a second messenger system activated by glutamatergic systems in various limbic and other regions of the brain, has been implicated in anxiety and affective disorders (Harvey, 1996; Suzuki *et al.*, 2001; Bernstein *et al.*, 1998). Studies have demonstrated that NMDA antagonists (Skolnick, 1999) as well as inhibitors of downstream activation of NOS (Harkin *et al.*, 1999) and cGMP (Eroglu & Caglayan, 1997; Heiberg *et al.*, 2002), have all demonstrated distinct antidepressant-like effects in animal models of depression. Moreover, AD, over an above their more traditional actions on monoamine uptake, inhibit NOS in the hippocampus (Wegener *et al.*, 2003). Since the hippocampus represents an important brain area implicated in the neurobiology of depression, as well as in antidepressant action, these above studies are strongly supportive of a role for the NO-cGMP pathway in the pathology and pharmacology of depression.

The physiological function of glutamate and NO in the brain in synaptic plasticity (McCleod *et al.*, 2001) as well as cell death and neuro-protection (Garthwaite & Boulton, 1995) hold great promise in revealing more on the neurobiology of depression, and especially the dynamics of antidepressant action. Glutamate and NO-mediated regulation of synaptic plasticity and cellular aspects of memory may reveal more on why antidepressants take up to 4-6 weeks to work, while their immediate synaptic effects on NA or 5-HT occur almost immediately. It may also reveal more information as to why poor compliance is associated with greater incidences of relapse and treatment resistance, or why the hippocampi of patients suffering from depression display profound structural as well as functional changes. Of particular interest regarding the latter evidence of hippocampal shrinkage in depressives is that it appears to be correlated with the total lifetime of depression and not with the age of the patient (Sheline *et al.*, 1999), suggesting that recurrent depressive episodes may inflict cumulative hippocampal damage. Non-compliance is notorious among those taking AD's (Basco & Rush, 1995; Lin *et al.*, 1995). Apart from troublesome "withdrawal effects", inappropriate stopping and starting AD's may have a profound negative impact on the long-term outcome of depression (Harvey *et al.*, 2004), while recent evidence suggests that hippocampal shrinkage may be correlated to increased stopping, starting and switching of AD medication (MacQueen *et al.*, 2003).

Acute AD discontinuation has been found to evoke a marked change in glutamatergic activity in key limbic areas of the brain (Harvey et al., 2002), which may have significant effects on the underlying neurobiology of the illness that predict a relapse of dysphoric mood. Thus, it has been postulated that untoward effects on glutamate function will cause neuronal dysfunction, altered "wiring" of synapses and changes in neuronal sprouting that will compromise long-term outcome (Harvey *et al.*, 2003). The important fact that NO release induced by glutamate may have neuroprotective and neurotoxic actions, and in this way neuroplastic effects in the CNS, makes the NO-cGMP pathway a potentially valuable new neurobiological target in the treatment and understanding of depression.

Using an animal model of depression, this project will study the possible role of the NO-cGMP pathway in antidepressant action from the point of view of chronic treatment, as well as after AD withdrawal. Furthermore, the study will address the neurobiology of AD withdrawal more closely, using targeted, pharmacological probes aimed at the NMDA-NO-cGMP pathway.

The study will therefore determine the following:

- The effect of chronic IMI treatment on swim immobility time and locomotor activity, as well as NOS enzyme activity and cGMP levels in rat hippocampus (HC),
- The effect of withdrawal of IMI, after three weeks chronic exposure, on swim immobility time, locomotor activity as well as NOS enzyme activity and cGMP levels in the rat HC.
- Glu-NO-cGMP pathway involvement on the behavioral and neurochemical effects caused by IMI withdrawal by adding, during the withdrawal period, the NMDA antagonist, memantine, and the NOS/guanylyl cyclase inhibitor, methylene blue (MB).
- Using the 5-$HT_{2A/2C}$ receptor antagonist, ritanserin, administered during the 7-day withdrawal period, whether the observed IMI withdrawal effects on NO-cGMP may occur through an initial destabilisation in the serotonergic system.
- The effects of the drugs alone, used during IMI withdrawal on the various parameters analysed in the earlier studies.

The clinical importance of inappropriate AD discontinuation is not fully appreciated, and both clinicians and patients alike all too often view AD withdrawal effects as troublesome, yet transient, side effects that wain within a week or so, and do not have any lasting significance on illness progression. But is this, in fact, so? Recent studies has begun to provide evidence that this is not the case, and that poor compliance and spontaneous patient-initiated alteration of AD treatment is both counter-productive and possibly a protagonist of late relapse and treatment resistance. This study hopes to address the uncertainty in this area and to increase awareness of the perils of poor compliance and AD, particularly inappropriate discontinuation.

THE NEUROBIOLOGY OF DEPRESSION

Chapter 1

1.1 Introduction

Depression is a chronic and severe mental illness that affects a large number of individuals in all countries (Machado-Vieira *et al.*, 2004). It is one of the most pervasive and costly brain diseases (Tamminga *et* al., 2002), and it is expected to be the second largest cause of burden of disease by 2020 (Danilevičiūtė & Sveikata, 2002). The lifetime risk for major depressive disorder in community samples is 10% to 25% for women and 5% to 12% for men (Danilevičiūtė & Sveikata, 2002). Over 10% of a population will have depression within their lifetime. This multifactorial illness has a diversity of clinical symptoms that can vary greatly and represents a significant source of distress for patients suffering from it and their families, while it is also a great burden on the economy (Machado-Vieira *et al.*, 2004).

The illness has a 10% mortality rate due to suicide, while there is an increasing rate of serious accidents among persons with active mood disorders. It is associated with several comorbid psychiatric disorders and often goes undetected, especially in children and adolescents (Tamminga *et al.*, 2002), and is characterized by similar manifestations and symptoms irrespective of country, cultural group or socio-economic status. Major depression usually develops early in life and can last for a lifetime, during which time it will impair the overall function (with regard to occupation and social roles), and affect the quality of life of the affected individual (Weissman *et al.*, 1988).

Depression is often underdiagnosed and inadequately treated. A variety of methods, such as psychotherapy, pharmacological, electroconvulsive, and magnetic therapies, can be used to effectively treat depression but still with limited success (Nestler, 1998; Ressler & Nemeroff, 1999). Apart from the increased risk of suicide associated with depression, the illness is associated with an increased risk of cardiovascular illnesses, cerebrovascular disorders, and accidents (Angst *et al.,* 2002). Clearly depression must not only be recognized, but also aggressively treated.

1.2 Clinical Presentation and Diagnostic Criteria

Depression refers to the affective state of sadness that occurs in response to a variety of human situations such as loss of a loved one, failure to achieve goals, or disappointment in love (Leonard, 1997). Many factors probably contribute to the underdiagnosis and undertreatment of depression. It can have a misleading presentation that includes somatic symptoms such as sleep disturbances, headache, gastrointestinal upset, fatigue, or weight loss. Mental illness and its stigma is another factor that influences the diagnosis of depression.

Most depressed patients express feelings of hopelessness, worthlessness, sadness, guilt and desperation. Frequently patients exhibit loss of appetite, insomnia, crying, diminished sexual desire, loss of ambition, fatigue, and motor retardation or agitation. Physical symptoms may include localized pain, severe digestive disturbances, and difficult breathing. Loss of self-esteem is very common and is combined with a complete sense of hopelessness about the future, which may end in suicide (Leonard, 1997).

Given the high prevalence of depression, some recommend that routine screening be done in the outpatient setting, e.g. the "SAD A FACES" mnemonic and the Zung assessment scale are helpful tools in assessing the primary criteria for Major Depressive Episode. As defined in the *Diagnostic and Statistical manual of mental Disorders,* fourth edition (DSM-IV) these are outlined below.

1.2.1 Criteria for Major Depressive Episode

S = sleep – insomnia / hypersomnia
A = appetite or weight change
D = dysphoria – depressed mood, irritable or sad

A = anhedonia – lack of interest or pleasure, lack of sex drive

F = fatigue
A = agitation / retardation
C = concentration diminished
E = esteem (low) / guilt
S = suicide / thoughts of death

Patients should be diagnosed with major depression episode when they exhibit five or more of the above symptoms for at least 2 weeks and must represent a change from previous functioning. At least one of the symptoms must be either depressed mood or loss of interest or pleasure (American Psychiatric Association, 1994).

There are two listings for Major Depressive Disorder, namely Single Episode and Recurrent. Single Episode can be classified according to following criteria:

- presence of a single (two or more) Major Depressive Episode/s
- The Major Depressive Episode (Episodes) is not better accounted for by Schizoaffective Disorder and is not superimposed on Schizophrenia, Schizophreniform Disorder, Delusional Disorder, or Psychotic Disorder not otherwise specified
- There has never been a Manic Episode, a Mixed Episode, or a Hypomanic Episode

This exclusion does not apply if all of the manic-like, mixed-like, or hypomanic-like episodes are substance or treatment induced or due to the direct physiological effects of a general medical condition (American Psychiatric Association, 1994).

1.3 Neuroanatomy of Depression

Depressed patients exhibit distinct pathological changes in selective brain regions (Manji *et al.*, 2001). These changes are observed in limbic (hippocampus, basal ganglia and amygdala) and cortical brain regions implicated in the affective and cognitive impairments observed in depression (Figure 1.1) (Manji *et al.*, 2001).

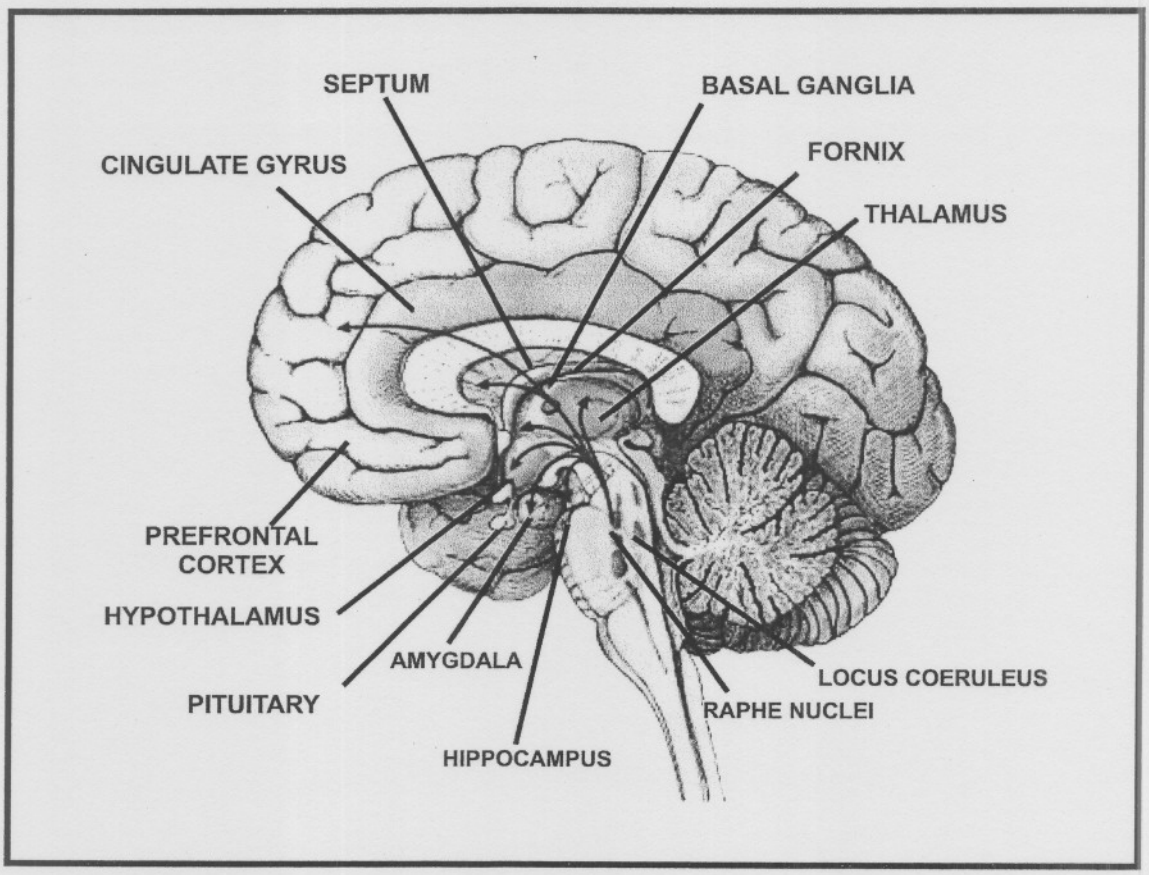


Figure 1.1: Limbic and Cortical Brain Regions (After Albany, 2004).

The two main neuroanatomical circuits believed to be involved in the pathophysiology of mood disorders are the limbic-thalamic-cortical circuit (LTCC), that includes the amygdala, the mediodorsal nucleus of thalamus, and medial and ventrolateral prefrontal cortex; and the limbic-striatal-pallidal-thalamic-cortical circuit (LSPTCC), connecting various parts of the basal ganglia, such as the striatum, the ventral pallidum, and the regions of the LTCC (Soares & Mann, 1997) (Figure 1.1 & 1.2).

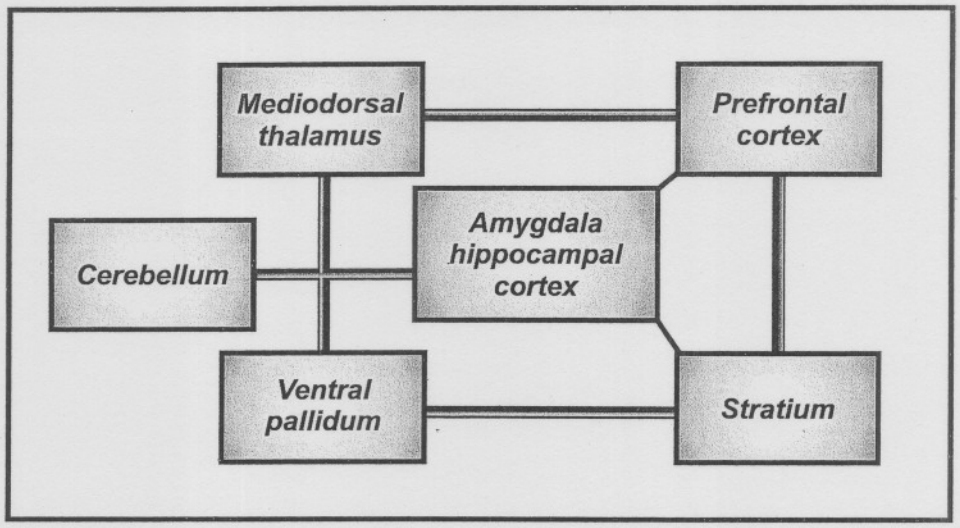


Figure 1.2: The key brain areas participating in mood regulation (After Soares & Mann, 1997).

A neuroanatomic model of depression, formulated by Krishnan (1992), provides a framework with which to interpret clinical depression. According to Krishnan depression can be divided into emotional expression, behavioral and emotional experience, and emotional evaluation, it is discussed more fully below.

Emotional expression:

The components of emotional expression can be divided into autonomic and vegetative, humoral, and skeletomotor. Autonomic expression consists of sympathetic and parasympathetic components that are mediated by projections originating in the hypothalamus. The amygdala and the medial orbital frontal cortex are also involved in the regulation of autonomic changes. The descending projections from the amygdala to the brainstem likely modulate monoamine systems, which in turn have effects on other areas of the brain involved in emotional expression, evaluation, and experience (Byrum *et al.*, 1999). Autonomic activation leads to release of a number of hormones. Sympathetic activation leads to the release of epinephrine from the adrenal medulla. The secretion of cortisol probably arises initially from the amygdala through its connection to the hypothalamic nuclei via the stria terminalis. This pathway stimulates the release of corticotropin releasing factor (CRF), which in turn stimulates the release of adrenocorticotropin from the anterior pituitary and leads to excessive cortisol production. The skeletomotor component of emotional expression is modulated by connections between the amygdala and the basal ganglia. The amygdala has direct projections to both the basal ganglia and the cortical motor areas and also provides an indirect pathway to the dopamine neurons of the brainstem (Graybiel, 1990). This latter pathway appears to be involved in motivation (Everitt & Robbins, 1992).

Behavioral and emotional experience

The brain structures that allow subjective awareness of feelings are obscure (Ledoux, 1986). However, stimultion of the amygdala can lead to emotional experience (Gloor *et al.*, 1982), while frontal and cingulated cortex, basal ganglia, amygdala, and hippocampus may also prove to be important.

Emotional evaluation

Emotional evaluation is the process by which the brain compares sensory input with knowledge and processes the emotional meaning of these stimuli. The mutual connections between the amygdala and cortex are thought to be involved in cognitive modulation necessary for emotional experience. The amygdala deals with information in an abstract form, such that emotional significance can be assigned to meaningful concepts. The medial orbital surface of the frontal cortex, and the cingulated gyrus are important for the processing and storage of emotionally laden information (Nauta, 1971). The amygdala also receives input from the hippocampus and from other cortical regions including other prefrontal areas. These areas probably provide the knowledge basis (memories) for the evaluation of information (Byrum *et al.*, 1999).

The above three pathways are in a state of dynamic flux, and collectively mediate emotional experience, emotional evaluation, and the expression of emotion (Byrum *et al.*, 1999). Pathways of emotional expression through the hypothalamic center can mediate common symptoms of depression such as decreased sexual drive and altered appetite. Other clinical symptoms that may arise through the mechanisms of emotional expression include altered sleep (mediated through the thalamus and the brain stem), fatigue (mediated through the basal ganglia circuits), and apathy (modulated by both the amygdala and basal ganglia circuits) (Byrum *et al.*, 1999).

Since the structures in the limbic-cortical-striatal-pallidal-thalamic circuit are all involved in emotional regulation, damage to any portion of this circuit could potentially produce depression, either directly by neuronal damage or indirectly by changes in neurotransmitter balance (Sheline, 2003). Abnormalities in these brain circuits may be manifested by structural changes, or restricted to a functional level. These abnormalities could trigger the onset of mood disorders, or confer a biological vulnerability, that in combination with environmental factors, results in affective disorders (Soares & Mann, 1997).

In support of this, positron emission tomography (PET) imaging studies have revealed multiple abnormalities of regional blood flow and glucose metabolism in limbic and prefrontal cortex (PFC) structures in mood disorders (Manji *et al.*, 2003). Structural imaging studies have also demonstrated reduced gray matter volumes in areas of the orbital and medial PFC, ventral striatum, and hippocampus, and enlargement of third ventricle of patients with mood-disorders relative to healthy control samples (Beyer & Krishnan, 2002). Complementary postmortem neuropathologic studies have shown abnormal reductions in cortex volume, glial cell counts, and/or neuron size in the subgenual PFC, orbital cortex, dorsal anterolateral PFC, amygdala, and in basal ganglia and dorsal raphe nuclei (Manji et al., 2003). The emerging picture thus is that cellular loss and volume decrease is causally associated with depressive disorders (D'Sa & Duman, 2002).

1.3.1 Hippocampus

The hippocampal formation is a brain region that is suggested to play a critical role in depressive disorder, and is particularly susceptible to the structural impairments induced by stress (McEwen, 2000). The function of the hippocampal formation is complex and poorly understood. However, three central properties of the hippocampal formation, which are all modified in depressed patients, may be identified.

The function of the hippocampus relates to memory and cognition (Dremencov *et al.*, 2003). Secondly, part of the hippocampus known as the Papez emotional circuit, controls emotional behavior (Chronister & Hardy, 1997). Thirdly, the hippocampus plays a central role in the regulation of neuroendocrine function related to stress. Hippocampal neurons express both glucocorticoid and mineralocorticoid receptors, with efferents projecting to the hypothalamus important for corticosterone-induced inhibition of ACTH release (Lathe, 2001). The function of the hippocampus may be described as cognitive recognition of the emotional meaning of environmental stimuli, by comparing between predicted results of the behavior and real output from the environment (Foster *et al.*, 2000). Chronic anxiety and stress may result in a pathological process within the hippocampus (Mongeau *et al.*, 1997), with human brain imaging studies finding that stress (Czeh *et al.*, 2001) and depression (Bremner *et al.*, 2000) are associated with hippocampal atrophy.

These hippocampal changes may arise from neuronal loss through chronic hypercortisolemia, glial cell loss, stress-induced reduction in neurotrophic factors, or stress-induced reduction in neurogenesis, but the precise mechanisms are not completely known (Sheline, 2000).

Because the hippocampus is involved in negative-feedback control of cortisol (Jacobson & Sapolsky, 1991), hippocampal dysfunction may result in reduction of the inhibitory regulation of the hypothalamic-pituitary-adrenal axis, which could then lead to hypercortisolemia (Davidson *et al.,* 2002b). Chronic hypercorticosolemia also results in hippocampal atrophy (Sapolsky, 2000a), dendritic reorganization (Wellmann, 2001), a reduction in synthesis of brain-derived neurotrophic factor (BDNF) and memory difficulties (Schaaf *et al.*, 2000). Structural imaging studies in people with recurrent major depression demonstrate that a smaller hippocampus is associated with greater lifetime duration of depression (Sheline *et al.,* 1999), suggesting that recurrent depressive episodes may inflict cumulative hippocampal atrophy and possibly permanent damage (Harvey *et al.*, 2003). Although the stress-induced changes in the hippocampus may not explain the affective symptoms of depression, they provide a cellular basis for understanding the structural impairments observed in this brain region as well as in other regions associated with depression (D'Sa & Duman, 2002).

1.3.2 Frontal Cortex

The prefrontal cortex is believed to have a major role in volition, working memory inhibition, motivation, and mood regulation (Weinberger, 1993). Dysfunction in this structure, indicated by decreased blood flow and metabolism, and perhaps atrophy, is consistent with a role in the pathophysiology of unipolar and bipolar depression (Soares & Mann, 1997). Volume reductions in frontal cortex ranging from 7% overall reduction in frontal lobe volume in major depression (Coffey *et al.*, 1992) to 48% in the subgenual prefrontal cortex (Drevets *et al.,* 1997) have been reported. The prefrontal cortex is particularly important as a target of monoamine projections while the orbitomedial prefrontal cortex has high concentrations of glucocorticoid receptors, potentially rendering it vulnerable to stress-mediated damage (Sheline, 2003).

1.3.3 Amygdala

Depressive illness is associated with a hyperactivation of the amygdala (Drevets *et al.*, 1992) and more recently with an enlargement of the amygdala in first episode major depression (Frodl *et al.,* 2002). In recurrent major depression of long duration, however, the amydala may undergo shrinkage (Sheline *et al.,* 1998). It is thus possible that initial hypertrophy gives way to atrophy in this important brain structure. Studies have shown that acute restraint stress increases anxiety and promotes a growth response of neurons within the medial and central amygdala of mice (Pawlak *et al.,* 2003) and that chronic stress causes increased anxiety and hypertrophy of neurons within the basolateral and central amygdala of rats (Conrad *et al.,* 1999). Because the amygdala is a central structure in directing the encoding of emotional

memories through projections to the hippocampus (Drevets, 2000), and sends outputs via the central nucleus for autonomic arousal and via the basal nucleus for more active aspects of coping, the elevation of amygdala activity may be a first step that leads to overactivation of systems involved in physiological and behavioral coping (McEwen, 2003). Noradrenaline release in the amygdala plays a critical role in some types of emotional learning, and the activation of noradrenaline release is facilitated by glucocorticoid secretion (Ferry *et al.*, 1999). Elevated noradrenaline and cortisol secretion are seen in some depressed patients (Schatzberg & Schildkraut, 1995), which in the presence of amygdala activation may increase the likelihood that ordinary social or sensory stimuli are perceived or remembered as being aversive or emotionally arousing (Drevets, 2001).

The Neurodegenerative hypothesis of depression will be discussed in Section 1.6.

1.4 Neurochemistry of Depression

Depression has been associated with a variety of neuroendocrine, neurochemical, neurophysiological, and neuromorphometric abnormalities (Manji *et al.*, 2001). Biological contributors to normal and pathological mood include not only the neurotransmitter systems supposedly involved in depression, including serotonin, noradrenaline, dopamine, and acetylcholine, but also the influence of two endocrine axes, the hypothalamic pituitary-adrenal (HPA) axes and hypothalamic pituitary-thyroid (HPT) axes, as well as alterations in immune function (Tamminga *et al.,* 2002). Abnormalities have also been demonstrated in a variety of neuropeptide, neuroendocrine, and other neurotransmitter systems in mood disorders. Elevated activity of the HPA axis is one of the most common findings in major depression, such that the illness appears to be associated with both a negative feedback disturbance and an increased drive by central processes (Drevets *et al.,* 2002). Several neurotransmitter systems and their metabolic pathways in the brain, and their role in depression, have been elucidated, including, glutamate, γ-aminobutyric acid (GABA), serotonin, noradrenaline, and dopamine, as have the membrane-bound signal transduction elements in the intracellular signaling systems, including gene transcription and protein synthesis (Tamminga *et al.,* 2002). The alterations in such a variety of neurotransmitter systems demonstrates the complexity of this illness. Furthermore, this may also explain the difficulty in understanding the neurobiology of depression and in finding new and effective treatments. It is likely that depression represents a common final pathway of multiple underlying pathophysiologies. Thus, the inability to find completely "effective" treatments may be hampered by the difficulty in defining specific depression subgroups because it is probable that groups with neurobiologic disturbances will respond differently to a given drug.

Although dysfunction within these neurotransmitter and neuroendocrine systems is likely to play a role in the pathophysiology of depression, there is a growing expectation that they may represent downstream effects of other, more primary abnormalities (Davidson *et al.*, 2002a). Figure 1.3 highlights the fact that one must address all the different physiological levels of depression for complete understanding of its pathophysiology (i.e., molecular, cellular, systems, and behavioral).

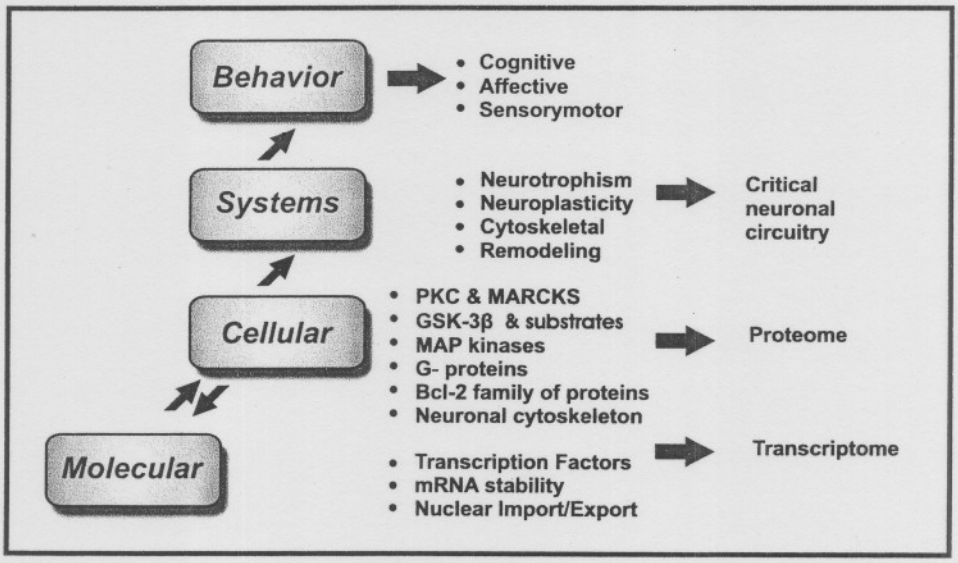


Figure 1.3: The different physiological levels in the neurobiology of depression. For a complete understanding of the pathophysiology of depression one must address its neurobiology at different physiological levels. (PKC, protein kinase C; MARCKS, myristoylated alanine-rich C kinase substrate; GSK-3, glycogen synthase kinase-3; MAP kinase, mitogen-activated protein kinase; Bcl-2, B-cell leukemia/lymphoma; proteome, the population of cellular protein species and their expression level; transcriptome, the population of cellular messenger messenger RNA species and their expression level) (After Manji & Lenox, 2000).

1.5 Neurotransmitters in Depression

1.5.1 Monoamines

Monoaminergic pathways are higly responsive to aversive stimuli and play a crucial role in the control of affect, cognition, endocrine secretion, chronobiotic rhythms, appetite, and motor function, all of which are profoundly disrupted in depressive states (Milan, 2004). Accordingly, all antidepressant drugs in clinical use increase the availability of these monoamines at the synapse either by inhibiting their neuronal reuptake, inhibiting their intraneuronal metabolism, or

increasing their release by blocking α_2 auto-and heteroreceptors receptors on the monoaminergic neuron (Elhwuegi, 2004).

1.5.1.1 Synthesis, release and metabolism of monoamines.

The catecholamines (CA), noradrenaline (NA), adrenaline and dopamine (DA), are neurotransmitters and/or hormones in the periphery and in the central nervous system (CNS). Catecholamines are formed in brain, chromaffin cells, sympathetic nerves and sympathetic ganglia (Cooper *et al.*, 1996). These three monoamines share a common pathway in their synthesis, where they are synthesized from tyrosine which is converted inside the nerve terminal by tyrosine hydroxylase (TH) to 3, 4 dihydroxyphenylalanine (DOPA). DOPA is then converted to DA, which is converted to NA by dopamine-β-hydroxylase (Iverson, 1991).

The indoleamine, serotonin, is a transmitter that is synthezised within the nerve ending from the amino acid L-tryptophan obtained from dietary and endogenous sources (Rang *et al.*, 1999). After being transported into the brain and nerve terminal by an active transport system, L-tryptophan is hydroxylated inside the nerve terminal to 5-hydroxytryptophan (5-HTP) by tryptophan hydroxylase to 5-hydroxy-tryptamine (5-HT). In the pineal gland, 5-HT is converted enzymatically to melatonin (Rang *et al.*, 1999).

When the action potential reaches the monoamine nerve terminals, it causes the opening of voltage-activated calcium channels. Calcium entry promotes vesicle fusion with the presynaptic membrane and the release of the monoamine into the synaptic cleft in a process known as exocytosis (Elhwuegi, 2004) (Figure 1.4).

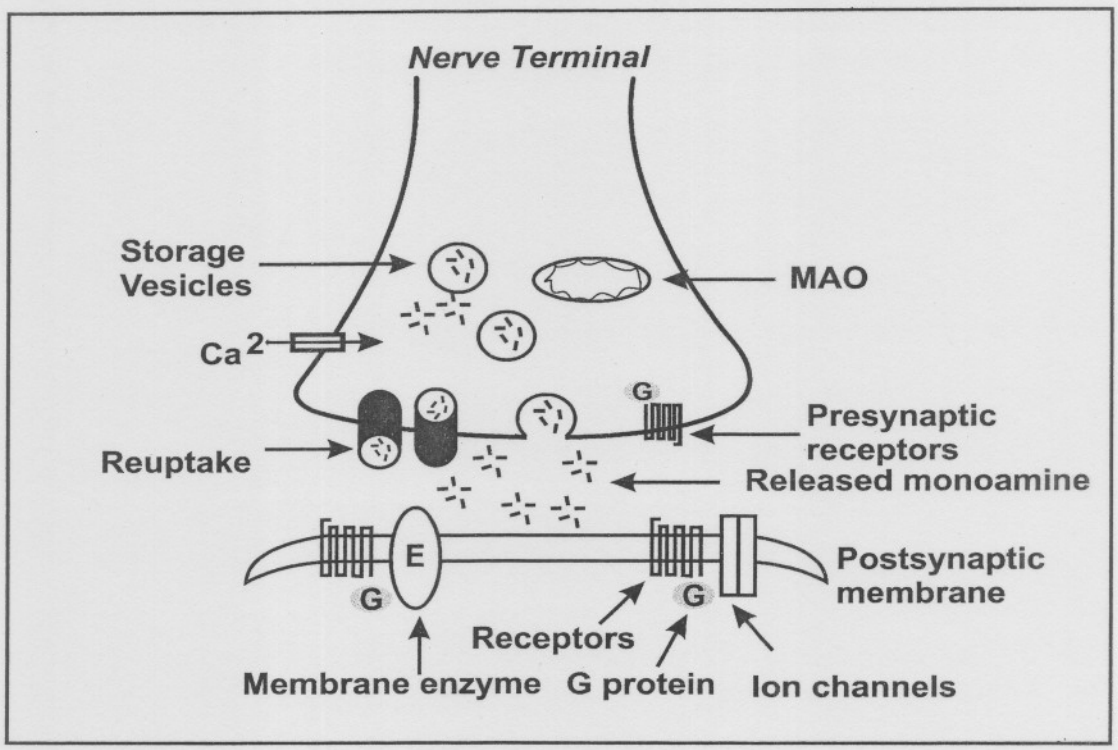


Figure 1.4: The monoamine neuron (After Elhwuegi, 2004).

The released monoamine will act on specific receptors located either on postsynaptic or presynaptic membranes (Figure 1. 4). Stimulation of the postsynaptic receptors results in changes in the properties of the postsynaptic membrane with either a shift in membrane potential when the receptors are coupled to ion channels (ionotropic), or biochemical changes in intracellular cyclic nucleotides, protein kinase activity, and related substrate proteins when the receptors are coupled to G-proteins (metabotropic receptors) (Elhwuegi, 2004). On the other hand, stimulation of the presynaptic receptors located on the nerve terminal will regulate vesicular release, thereby providing a feedback mechanism that controls the concentration of the transmitter in the synaptic cleft (Boehm & Kubista, 2002). If the pre-synaptic regulatory receptors are present on the same neuron releasing the neurotransmitter, they are called autoreceptors, but if they are present on another neuron releasing different neurotransmitters, they are called heteroreceptors. These regulatory receptors may be either ionotropic or metabotropic (Boehm & Kubista, 2002) and are an important mechanism whereby neurons can communicate, a process described as cross-talk (Harvey, 1997).

Non-synaptic transmission describes the functional interaction between neurons without morphological "synaptic" contacts. In this case, the transmitter released from the axon terminal, without a synaptic contact with another neuron, will diffuse away from the release site and activates remote receptors of high affinity on another axon terminal (Vizi, 2000). A typical

example is the gaseous transmitter, nitric oxide (NO), which act as a modulator of monoamine release (Prast & Phillipu, 2001). The actions of all monoamines are terminated by active reuptake of the monoamines either into the presynaptic neuron (known as uptake 1) and/or into glial cells. The uptake 1 mechanism utilize Na^+ /Cl^- dependant transporters and is voltage-dependant (Sonders, *et al.,* 1997).

After release of the monoamine into the synapse, and activation of its specific receptors, it undergoes rapid biotransformation by monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT). Monoamine oxidase exists in two different forms, a type A and type B, and occurs in virtually all tissues, where it appears to be bound to the outer mitochondrial membrane (Rang *et al.*, 1999).

The efficiency of monoaminergic signaling can be enhanced by molecular changes at single synapses such as up-regulation of post-synaptic receptors and/or down-regulation or desensitization of presynaptic autoreceptors that are negatively coupled to exocytotic neurotransmitter release (Schloss & Henn, 2004). Additional possibilities include enhanced neurotransmitter synthesis/release or downregulation/desensitization of transmitter transporters. A different possibility takes into account that the efficiency of monoaminergic signaling is not only modulated at single synapses but also by the density of synaptic contact sites (Schloss & Henn, 2004).

1.5.1.2 Noradrenergic neurotransmission : A CNS Overview

Noradrenaline is found in most brain regions. The most noradrenergic neurons arise either in the locus ceruleus (LC) of the pons, or in neurons of the lateral tegmental portion of the reticular formation (Moore & Bloom, 1979) and project to (1) frontal cortex to regulate mood, (2) limbic areas to regulate emotions and anxiety, and, (3) hypothalamus for regulation of eating, appetite, weight, sex drive, and pleasure (Figure 1.5). In addition one unique noradrenaline projection to the frontal cortex regulates cognition and attention, and another to cerebellum may modulate motor movements (Stahl, 2002).

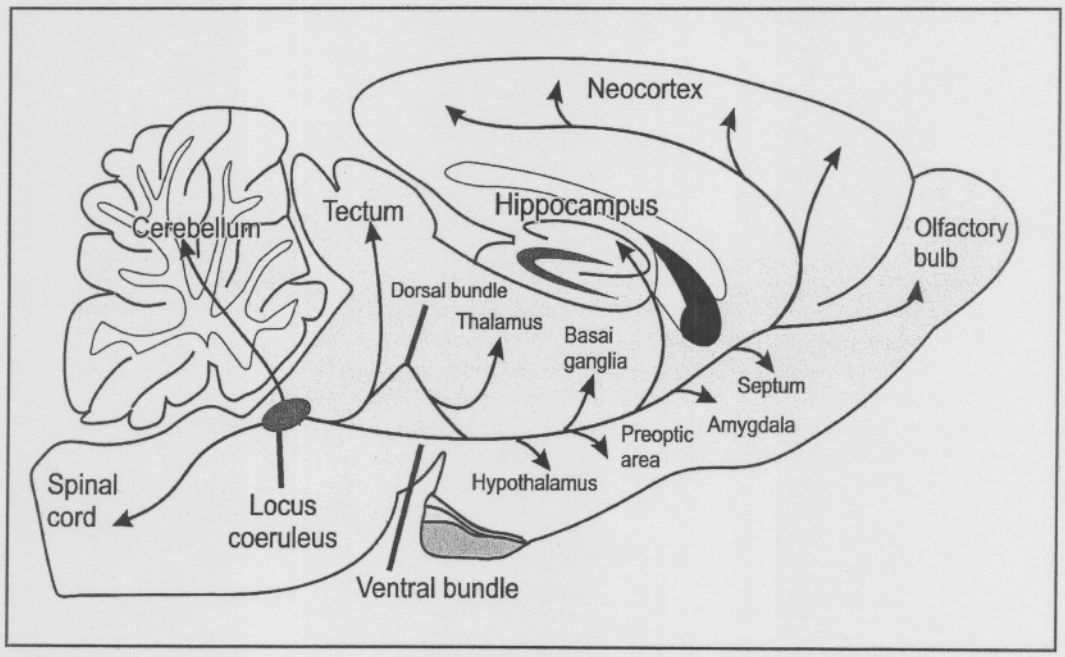


Figure 1.5: Anatomical distribution of the central noradrenergic system as mapped in the rat brain (Anon, 2001).

Adrenergic responses and receptors are classified into two broad categories, alpha and beta of which there are three families, viz alpha1 (α_1), alpha2 (α_2) and beta (β) (Bylund *et al.,* 1994). All have been described in the CNS. The β-adrenoceptors are currently divided into β_1, β_2 and β_3, the latter occurring exclusively in adipose tissue (Lefkowitz *et al.,* 1992). These three subtypes of adrenergic receptors are all coupled to stimulation of membrane adenylyl cyclase (AC) activity through G_s protein leading to the formation of the second messenger cyclic adenosine monophosphate (c-AMP) (Taussig & Gilman, 1995). c-AMP will activate cellular c-AMP-dependant protein kinase A (PKA) that causes phosphorylation of various cellular proteins, which produces the specific β –adrenergic receptor response (Elhwuegi, 2004).

α_1 adrenoceptors are excitatory in nature and are coupled to stimulation of membrane phospholipase C (PLC), leading to the formation of the second messengers' inositol triphosphate (IP_3) and diacylglycerol (DAG) through G_q proteins. Activation of the α_1 receptors on some adrenergic target neurons produces depolarization due to an increase in K^+ conductance, thus making the neuron more excitable (Elhwuegi, 2004).

Molecular cloning has revealed four different subtypes of α_2 receptor designated as α_{2A} , α_{2B}, α_{2C}, and α_2 (Bylund *et al.,* 1994). The α_2 receptors are all inhibitory in nature, mediating hyperpolarization of the neuronal membrane by increasing K^+ conductance via activation of G protein-gated K^+ channels, making the neuron less excitable. α_2 adrenoceptors are also coupled to inhibition of AC activity through the inhibitory G_i family (Liggett, 1996). The main function of the α_2 adrenoceptor is the presynaptic regulation of transmitter release, where these receptors have been implicated in the inhibitory control of adrenergic and serotonergic pathways innervating the frontal cortex (Dennis *et al.*, 1987). The activation of α_2 adrenoceptors decreases NA output and also suppresses the firing activity of 5-HT neurons (Clement *et al.,* 1992).

1.5.1.2.1 Alterations in the noradrenergic system in depression

Apart from the antidepressant efficacy of agents whose biochemical effects includes increasing NA, diverse evidence has accumulated over the decades implicating the NA system in depression (Manji *et al.*, 2001). These include reduced cerebrospinal fluid and urinary 3-methoxy 4-hydroxyphenylglycol (MHPG) (major NAmetabolite) and elevated plasma NAin depression, as well as a blunted neuroendocrine responses to the α_2 agonist, clonidine. Altered α_2AR and β-AR density and responsivity in peripheral cells, altered densities of α_2ARs and β-ARs in areas of postmortem brain in depressives, and reduced internal jugular venoarterial NA metabolite concentration gradients have been observed in patients with depression (Manji *et al.*, 2001). Figure 1.6 illustrates antidepressant drugs that appear to act, among others, on noradrenergic neurotransmission (Gareri *et al.*, 2000).

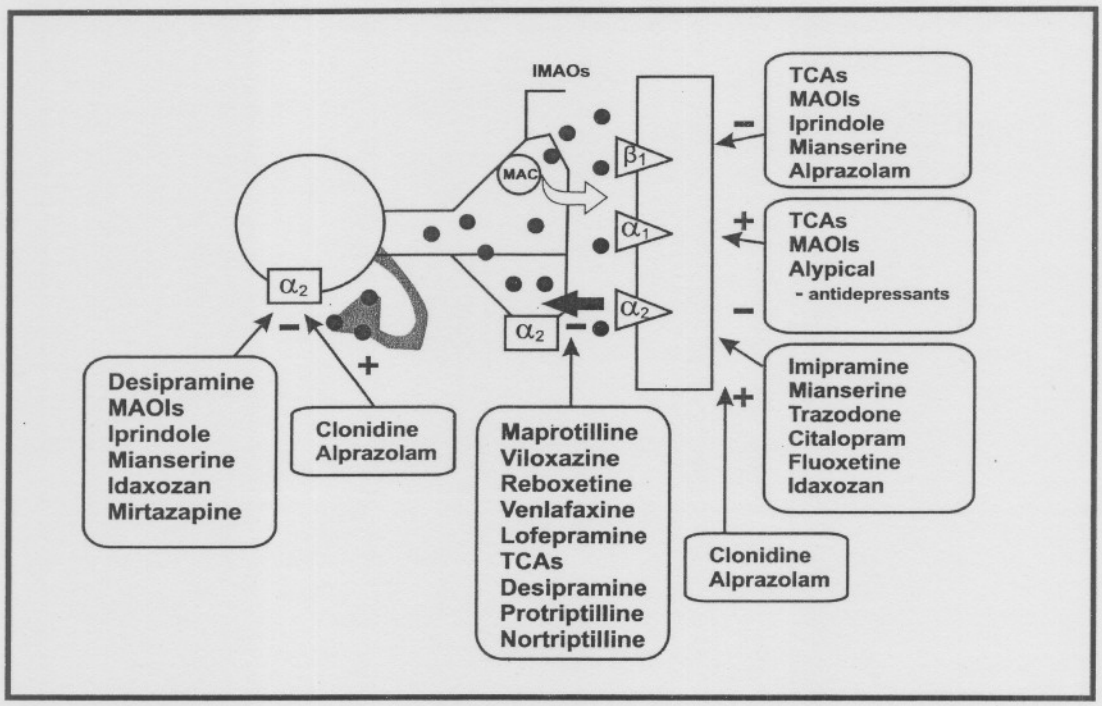


Figure 1.6: Antidepressant drugs acting on noradrenergic neurotransmission (Gareri *et al.*, 2000).

1.5.1.3 Dopaminergic neurotransmission : A CNS Overview

Due to its prominent role in the reward system (Koob, 1996), dopamine is a natural candidate for a neurotransmitter involved in the etiology of depression and the action of antidepressant drugs. DA is also involved in the control of movement, behavior and some endocrine functions (Elhwuegi, 2004). Specially, enhanced dopaminergic activity may play a role in the pathogenesis of psychotic depression. Several studies suggest that increased DA activity may occur as a result of increased corticosteroid activity, which may induce tyrosine hydroxylase activity (Nathan *et al.,* 1995). The first hypothesis implicating a role for dopamine in depression and mania was proposed over 25 years ago (Randrup *et al.*, 1975). Several studies since have indicated that behavioral responses to dopaminergic stimulation are increased by antidepressant treatments (Maj *et al.,* 1989) and in particular by electroconvulsive shock treatment (ECT) (Antkiewicz-Michaluk *et al.*, 1994). A common characteristic of antidepressants is an enhancement in extracellular levels of dopamine in the frontal cortex by either blockade of cortical NA transporters, which are responsible for taking up DA, or blockade of α_2-adrenoceptors and 5-HT_{2C} receptors, which inhibit mesocortical dopaminergic projections (Devoto *et al.*, 2004). DA is a particularly widespread neurotransmitter in the brain with more than half the CNS content of catecholamines being DA (Elhwuegi, 2004). Dopaminergic pathways in the brain arise from groups of cells in the midbrain and hypothalamus (Roth & Elsworth, 1995). In the midbrain, cell groups designated A8, A9, and A10 form the DA neurons

of the ventral mesencephalon, also collectively referred to as the ventral mesotelencephalic DA system (Roth & Elsworth, 1995). The A8 and A9 neurons contain approximately 70% of brain dopamine and are involved in the modulation of motor behavior (Kapur & Mann, 1992). The A10 DA neurons arise from the ventral tegmental area and innervate both mesolimbic regions (nucleus accumbens, olfactory tubercle, septum) and mesocortical sites (cingulated, entorhinal, prefrontal, and pyriform cortices) (Kapur & Mann, 1992).

In the hypothalamus, there are four discrete groups of DA cell bodies which project to the median eminence and neurohypophysis and which modulate neuroendocrine regulation of prolactin secretion (Kapur & Mann, 1992). It is particularly the long DA systems, or the nigrostriatal, mesolimbic, and mesocortical pathways that play a prominent role in psychiatric illness (Pickar, 1986). The other DA system of significance in the CNS is the intermediate-length tuberoin-fundibular DA pathway.

Dopaminergic pathways are interconnected with the limbic system, especially the nucleus accumbens (Schultz *et al.*, 1997) and the dopaminergic neurons of the ventral tegmental area (Blackburn *et al.,* 1992). Dopaminergic activity in the nucleus accumbens has been associated with hedonia (pleasure), incentive motivation, and reward-related behavior. Motor hyperactivity, as a manic-like symptom, has been associated with the activation of the mesolimbic dopaminergic system, originating from the ventral tegmental area to the nucleus accumbens (D'Aquila *et al.,* 2000).

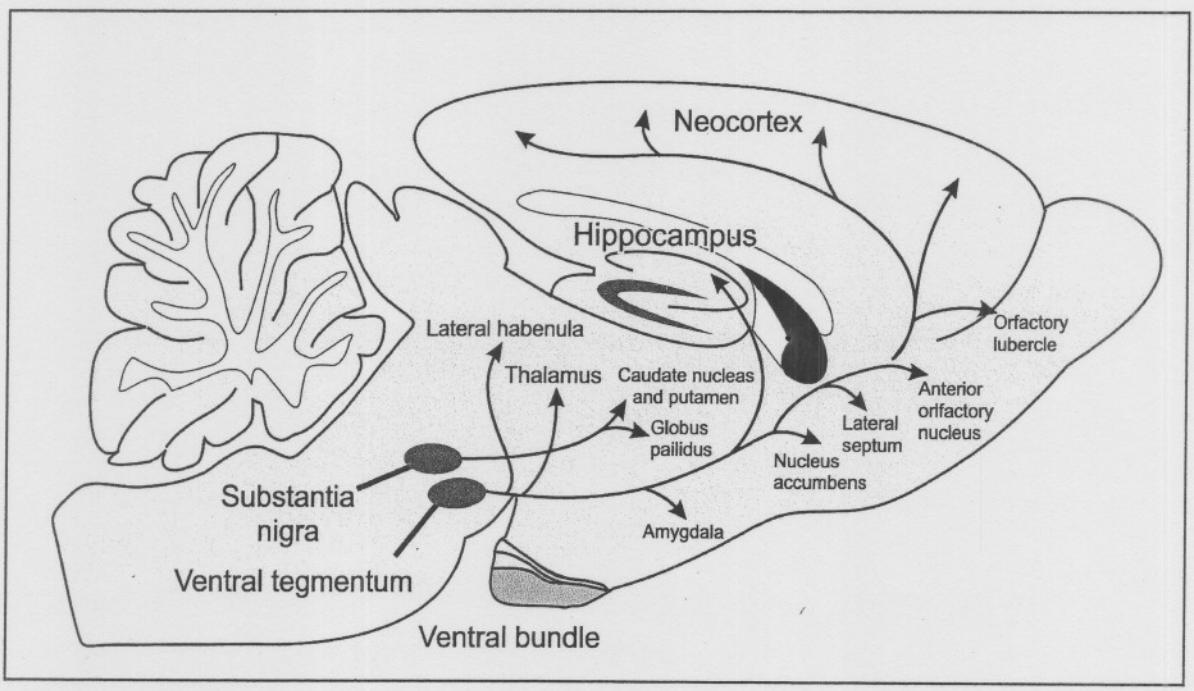


Figure1.7: Anatomical distribution of the central dopaminergic system as mapped in the rat brain (Anon, 2001).

Five DA receptor types (D_1-D_5) have been identified, and can be divided into D_1 and D_2 receptor families, forming part of the superfamily of seven transmembrane, G protein-coupled receptors. The D_2 family consists of D_2, D_3, and D_4 receptors (Van Tol *et al.*, 1991), whereas the D_1 family consists of the D_1 and D_5 receptors (Dearry *et al.*, 1990). The D_1 receptor are the most widespread dopamine receptors and are found in areas known to be under dopaminergic control such as the striatum, nucleus accumbens, and olfactory tubercle. It is also found in the limbic system, hypothalamus and thalamus (Gareri *et al.*, 2000). The D_2 receptor is found at both pre-and post-synaptic levels in the brain and occurs mainly in the striatum, olfactory tubercle and nucleus accumbens (Le Moine & Bloch, 1995). D_3 receptors are present in a high density in the limbic areas and have low expression in the striatum (Gareri *et al.*, 2000). D_4 receptors are located in the frontal cortex and amygdala, with relatively low density in the basal ganglia and limbic areas (Leonard, 1997). The D_1 receptors activate adenylyl cyclase (AC), and increasing intracellular c-AMP. The D_2 receptors couple to multiple effector systems, including the inhibition of AC activity, suppression of Ca^{2+} currents, and activation of K^+ currents (Vallar & Meldolesi, 1989).

1.5.1.3.1 Alterations in the dopaminergic system in depression

Apart from the prominent role for DA in reward and motivation circuits, evidence for an involvement for DA in depression includes reduced CSF homovanillic acid (major DA metabolite), and blunted neuroendocrine and temperature responses to DA agonists in patients with depression (Manji *et al.*, 2001). Agents whose biochemical effects includes increasing DA, have antidepressant properties, while a-methyl-para-tyrosine, and inhibitor of catecholamine biosynthesis, and reserpine, are depressogenic in susceptible individuals (Manji *et al.*, 2001). Reduced internal jugular venoarterial DA metabolite concentration gradients, is evident in depression, while the fact that depression often occurs in Parkinson disease, is indirect circumstantial evidence for low DA as a cause of depression (Manji *et al.*, 2001). Figure 1.8 illustrates antidepressant drugs that appear to act, among others, on dopaminergic neurotransmission (Gareri *et al.*, 2000).

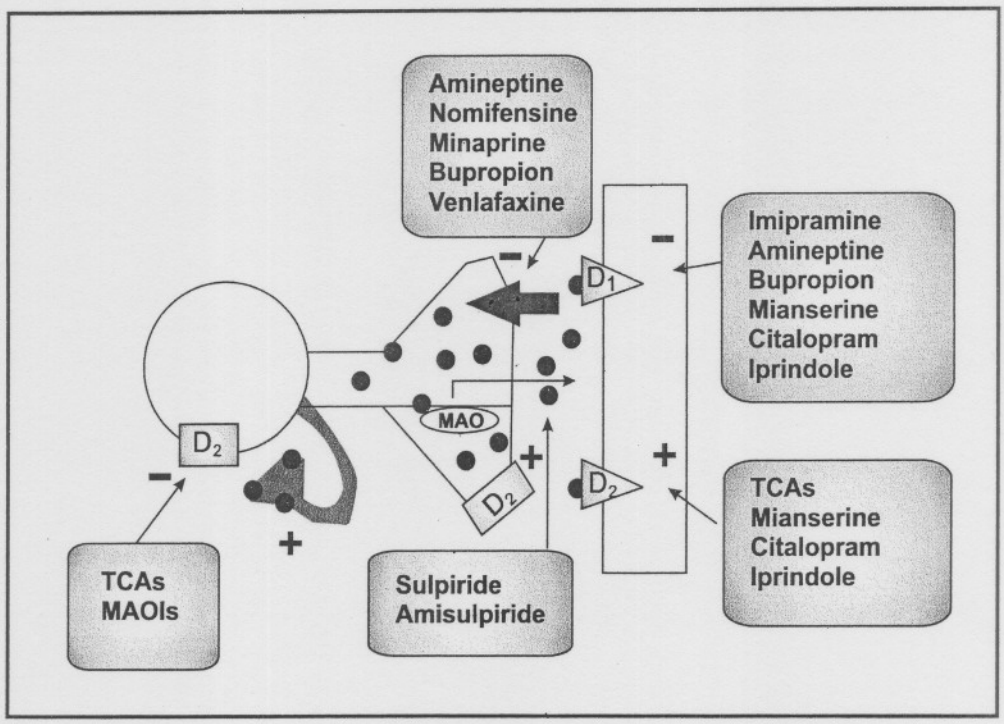


Figure 1.8: Antidepressant drugs acting on dopaminergic neurotransmission (After Gareri *et al.*, 2000).

1.5.1.4 Serotonergic neurotransmission : A CNS Overview

Serotonergic neurons are found in nine nuclei lying in or adjacent to the midline (raphe) regions of the pons and upper brainstem. The dorsal raphe nucleus (DRN) is the largest of the brainstem serotonergic nuclei containing about 50-60% of 5-HT neurons in the human CNS. The cell bodies of the neurons are located in the midbrain raphe with axons projecting to the frontal cortex where they may have important regulatory functions for mood, to the basal ganglia where they may regulate movements, and to limbic areas where they may modulate emotions, particularly anxiety (Figure 1.9). Serotonergic projections also impinge on the hypothalamus where they regulate eating, appetite, and weight as well as sex drive and pleasure, while projections to the sleep centers regulate the sleep-wake cycle (Stahl, 2002).

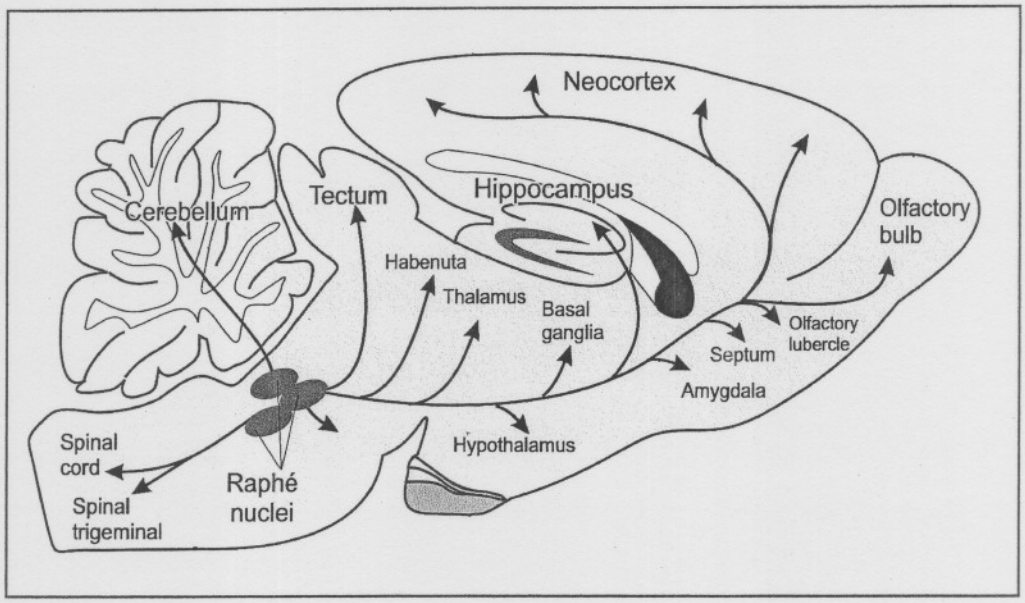


Figure 1.9: Anatomical distribution of the central serotonergic system as mapped in the rat brain (Anon, 2001).

Pharmacological and biochemical data indicate the presence of at least four major 5-HT receptor families- 5-HT_1, 5-HT_2, 5-HT_3, and 5-HT_4 - with some families containing multiple subtypes (Frazer & Hensler, 1999). To date, at least 14 distinct mammalial 5-HT receptor subtypes have been identified. This includes the 5-HT_1 and 5-HT_2 receptors, which are both G protein-coupled receptors. The 5-HT_1 receptors are subdivided into at least five receptor subtypes, viz 5-HT_{1A} , 5-HT_{1B} , 5-HT_{1D}, 5-HT_{1E}, 5-HT_{1F}). These receptors are linked to inhibition of AC activity through G_i protein, thus inhibiting formation of the second messenger c-AMP, or to regulate the K^+ or Ca^{2+} channels. Activation of 5-HT_{1A} receptors leads to the opening of an inwardly rectifying K^+ conductance, which leads to hyperpolarization and neuronal inhibition (Frazer & Hensler, 1999). 5-HT_{1A} receptors are abundantly expressed on the serotonergic cell bodies and dendrites of the DRN (somatodendritic receptors) and on the nerve terminal of the forebrain projection areas of the frontal cortex, hippocampus and the amygdala (Hensler *et al.*, 1991).

Somatodendritic 5-HT_{1A} autoreceptors in the DRN causes inhibition of the firing rate of 5-HT neurons and therefore inhibit 5-HT release at the nerve terminals (Piñeyro & Blier, 1999). 5-HT release from 5-HT neurons is also under the control of terminal 5-$HT_{1B/1D}$ autoreceptors (Starkey & Skingle, 1994). These receptors, unlike the somatodendritic ones, can modify 5-HT release without altering 5-HT neuron firing activity (Crespi *et al.*, 1990).

There are three subtypes of 5-HT_2 receptors, of which the most important are the 5-HT_{2A} receptors that show high densities in prefrontal cortex, but are also evident in the HC.

The 5-HT_3 receptor is a ligand-gated ion channel with a high density found in the nucleus tractus solitarri and in the area postrema where they participate in the emetic response. 5-HT_3 receptors have no role in the regulation of the firing activity of 5-HT neurons (Piñeyro & Blier, 1999). The 5-HT_4, 5-HT_5, 5-HT_6 and 5-HT_7 subgroups have been identified by molecular cloning and characterized biochemically, but their exact role remains poorly defined.

1.5.1.4.1 Alterations in the serotonergic system in depression

Apart from the well-known antidepressant efficacy of agents that increase intrasynaptic 5-HT, there is also supportive evidence from studies indicating, for example reduced CSF 5-hydroxyindole acetic acid (5-HIAA) (major serotonin metabolite) in depression, as well as blunted neuroendocrine and temperature responses to 5-HT agonists (Manji *et al.*, 2001). In addition reduced [^{3}H]IMI binding in platelets and postmortem brain of depressives, as well as reduced 5-HT_{1A} receptor binding in living brain and postmortem brain tissue, have been observed (Manji *et al.*, 2001). Finally, tryptophan depletion in antidepressant-treated patients is depressogenic. Figure 1.10 illustrates antidepressant drugs that appear to act, among others, on serotonergic neurotransmission (Gareri *et al.*, 2000). One novel and distict AD mechanism related to the SSRIs is exemplified by tianeptine. This agent acts by enhancing serotonin reuptake in contrasts with most clinical AD that inhibit serotonin reuptake.

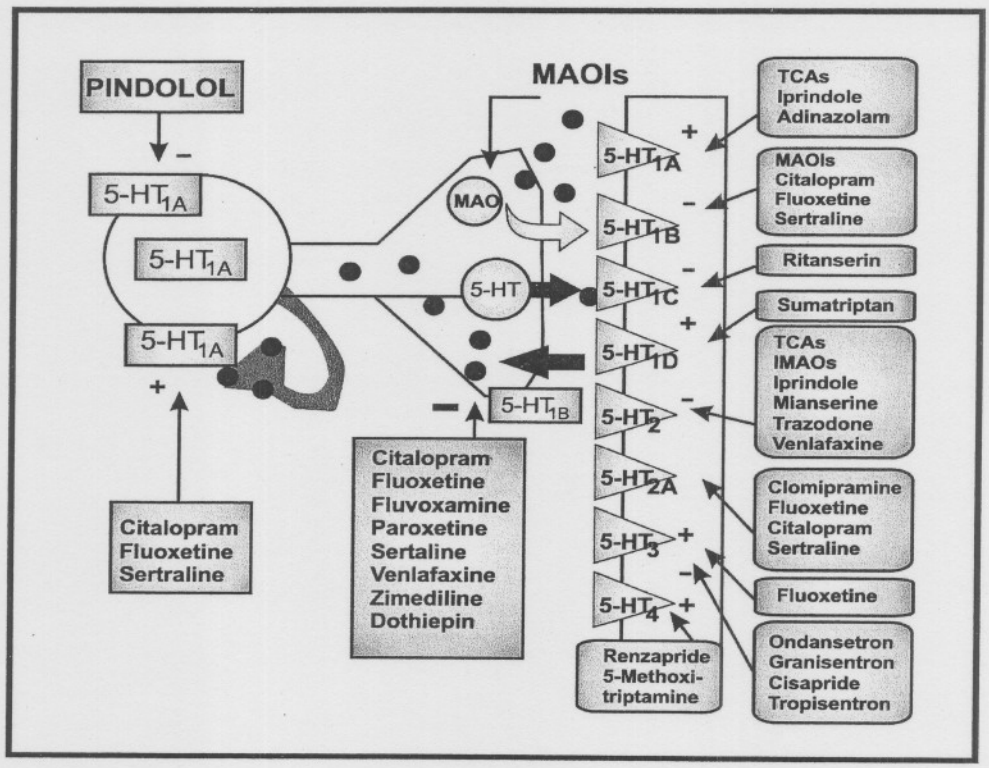


Figure 1.10: Antidepressant drugs acting on serotonergic neurotransmission (After Gareri *et al.*, 2000).

1.5.1.5 Monoamine hypothesis of depression

The idea that biogenic monoamines are involved in the aetiology of depression came initially from three main lines of evidence (Blier, 2003). Firstly, the antihypertensive drug, reserpine, (later found to act as a depleter of central nervous system stores of NA, DA and 5-HT), could induce symptoms of depression in susceptible patients (Goodwin & Bunney, 1971). Secondly, depressed patients may have reduced levels of monoaminergic metabolites in especially the cerebrospinal fluid. Finally, drugs that relieve depression seem to attenuate the mechanisms by which serotonin and noradrenaline are inactivated (Blier, 2003). Thus, this theory simply states that mental depression is due to the deficiency of brain monoaminergic activity and that depression is treated by drugs that increase this activity (Elhwuegi, 2004).

However, this theory suffered several drawbacks and failed to explain several facts. First, there are drugs that can increase brain monoaminergic activity (e.g. cocaine and amphetamine) but are not effective clinically as antidepressants. Second, not all depressed patients respond equally to the same antidepressant. Third, and most importantly, these changes in the monoamine levels at the synapse take place within hours after administration of the antidepressants, but the therapeutic response requires the continuous administration of these drugs for a number of weeks (Baldessarini, 1989)

The modified amine theory suggest that the acute increase in the levels of the monoamines at the synapse may be only an early step in a potentially complex cascade of events that ultimately results in antidepressant activity (Piñeyro & Blier, 1999). This acute increase in the amount of the monoamine at the synapse has been found to induce desensitization of the inhibitory auto- and heteroreceptors located in certain brain regions. The desensitization of these receptors would result in higher central monoaminergic activity that coincides with the appearance of the therapeutic response. These adaptive changes responsible for the therapeutic effect depend on the availability of the specific monoamine at the synapse, as depletion of this monoamine will either reverse the antidepressant effect or cause a relapse in the state of drug-free depressed patient previously treated with antidepressant drugs. Furthermore, blocking the somatodendritic and nerve terminal autoreceptors increases the response rate in the treatment of major and treatment resistant depression, providing further support to the assumption that antidepressant effect results from the long-term adaptive changes in the monoamine auto- and heteroregulatory receptors (Elhwuegi, 2004).

Up-regulation of β-adrenergic receptors is a consistent finding in patients with depression and their down-regulation is regarded as a marker for antidepressant activity (Leonard, 1997).

Although the functional significance of the decrease of β-adrenergic activity induced by most antidepressant treatments (Elhwuegi, 2004), is plausible in some respect, more recent evidence suggests that interactions between NA and 5-HT (Blier, 2003) may be important for mediating the down regulation of β-adrenoceptors, in conjunction with the 5-HT system, which argues against an exclusive central role for a single neurotransmitter system in the mechanism of action of antidepressants.

Central α_2 autoreceptors and heteroreceptors are hypersensitive in depression, which results in low aminergic activity in the prefrontal cortex. Chronic antidepressant treatments desensitize these α_2 receptors and may increase the sensitivity of the stimulatory α_1 receptors, thus increasing the release of 5-HT and NA (Elhwuegi, 2004).

However, the NA and 5-HT hypothesis are unable to explain the delayed therapeutic action of TADs and SSRIs, the failure of L-DOPA and tryptophan as affective antidepressants, why some antidepressants decrease biogenic amines and their metabolites (Song & Leonard, 2000), and why some antidepressants increase synaptic reuptake, e.g. tianeptine, and others decrease reuptake, e.g. SSRIs. More recently, there has been increasing awareness of the impotant role played by excitatory and inhibitory neurotransmitters in the regulation of mood. Indeed, it has been suggested that glutmate and GABA pathways may be the over-arching neuroregulatory pathway controlling and mediating the CNS actions of the biogenic amines (Harvey, 1996). These two pathways are also integral in explaining the evidence that depression may be a degenerative disorder.

1.5.2 Amino acids

The excitatory N-methyl-D-aspartate, (NMDA)-glutamate (Skolnick, 1999) and inhibitory GABAergic pathways (Petty, 1995) are now undoubtedly implicated in the development of affective disorders, although the depth and mechanism of their involvement remains uncertain. Glutamate- and GABA systems have major implications for biological psychiatry. First, the number of synapses using these transmitters is much greater than those using all other types of monoamines and peptides combined. Second, both glutamate- and GABA-containing neurons are widely distributed within the brain, in contrast to the more restricted distribution of other neurotransmitter neurons. Third, both glutamate and GABA have other biochemical and metabolic actions in the body in addition to their transmitter functions (Wilcox & Gonzales, 1995).

1.5.2.1 Glutamate : A CNS Overview

Glutamate-containing neurons are found in the cortex, subcortical structures, such as hippocampus, caudate nucleus, thalamic nuclei, and the cerebellum (Rajkowska, 2000a). While glutamate is best known for its involvement in intermediary metabolism and neurotransmission within the CNS, glutamate is also present in many tissues of the body including blood and cerebral spinal fluid (Sanacora *et al.*, 2003).

1.5.2.1.1 Synthesis, release, and metabolism of glutamate

In the brain, glutamate is synthesized in the nerve terminals from glucose via the Krebs cycle and transamination of α-oxoglutarate, and from glutamine (synthesized in glial cells). The latter is transported into the nerve terminals and converted by glutaminase to glutamate (Cooper *et al.*, 1996). The majority of glutamate in the brain, however, is involved in intermediary metabolism in both neurons and glia and only a fraction of the total glutamate pool participates in neuronal signaling (McDonald & Johnston, 1990). Glutamate, stored in synaptic vesicles, is released from presynaptic terminals by membrane depolarization in a Ca^{2+}-dependant fashion (Dingledine & McBain, 1999).

Once released into the synaptic cleft, glutamate can depolarize the post-synaptic neuronal membrane by binding to either ionotropic or metabotropic receptors (McDonald & Johnston, 1990). The excitatory actions of glutamate are limited by neuronal presynaptic and glial high-affinity uptake systems.

1.5.2.1.2 Glutamatergic receptors

Glutamate is primarily an excitatory neurotransmitter and nearly all neurons are depolarized by glutamate through the activation of the various glutamate receptors (Bisaga & Popik, 2000). These receptors have been pharmacologically classified into either metabotropic- and ionotropic receptors. Metabotropic receptors are coupled to G proteins and linked to the activation of phospholipase C (PLC) or the inhibition or activation of adenylyl cyclases (AC) (Michaelis, 1998). Ionotropic receptors are ligand-gated ion channels that mediate rapid changes in sodium, calcium, and potassium permeability. The ionotropic receptors are further subdivided into *N*–methyl-D-aspartate (NMDA), kainic acid (KA), and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Bisaga & Popik, 2000). The metabotropic receptors are also divided into three sub groups (Bordi & Ugolini, 1999). Both ionotropic and metabotropic

receptors initiate multiple signaling cascades that determine neuronal growth, differentiation and survival (Michaelis, 1998).

1.5.2.1.2.1 Ionotropic glutamate receptors

Glutamatergic NMDA receptors are permeable to Ca^{2+}, and blocked by Mg^{2+} at or close to resting membrane potential. This block is quickly relieved by membrane depolarization. The resulting influx of the second messenger Ca^{2+} then stimulates a variety of calcium-dependant enzymes, including NOS, protein kinase C, Ca^{2+}, calmodulin-dependant kinases, calcium-dependant proteases, phosphatases, etc. They respond slowly to glutamate and their primary contribution is to the slow component of excitatory postsynaptic currents (Michaelis, 1998). The NMDA receptor also possesses a binding site for the inhibitory transmitter glycine (Macdonald *et al.,* 1996) responsible for greatly potentiating peak NMDA currents, and speeding the rate of recovery from desensitization (McBain & Mayer, 1994). The channel also contains recognition sites for divalent cations, polyamines, NO, Zn^{2+} and H^+ (Bisaga & Popik, 2000). Figure 1.11 illustrates the NMDA-receptor complex and its modulatory sites.

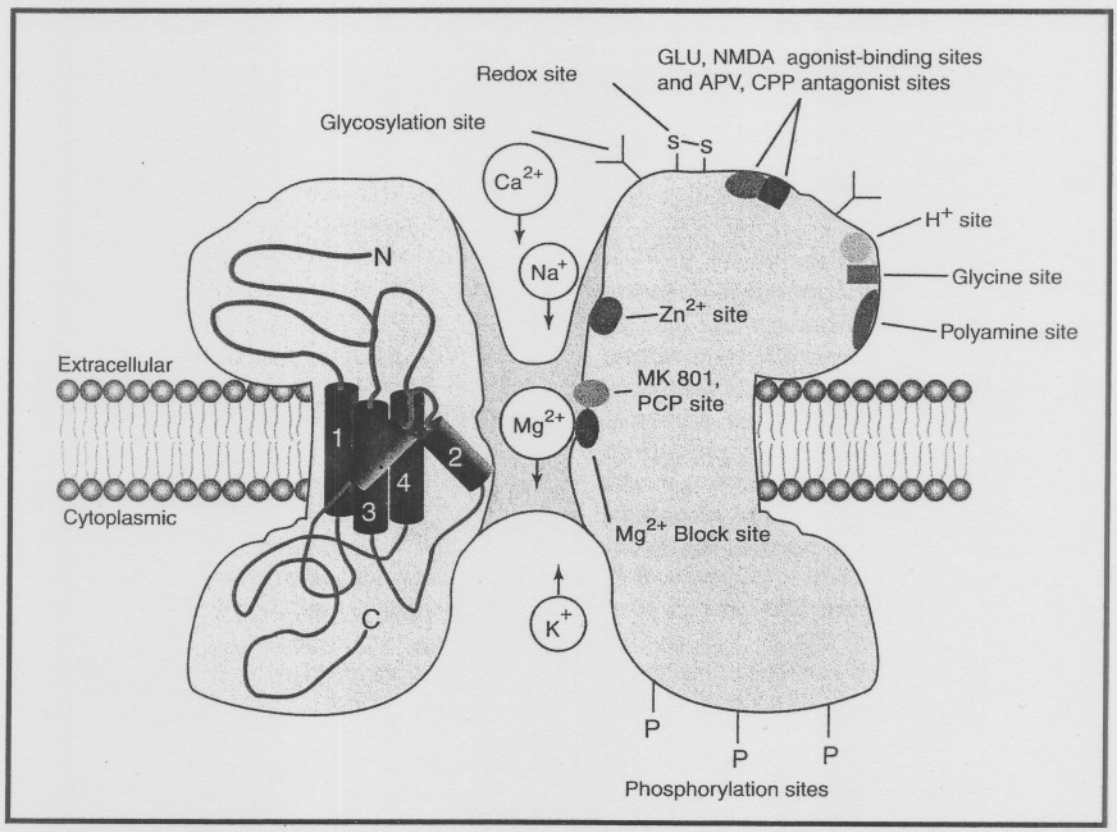


Figure 1.11: The NMDA ion-channel receptor complex and its modulatory sites. (PCP: phencyclidine) (After Waxham, 2004).

NMDA receptors are expressed in the forebrain, striatum, medial thalamus, and cortex (Buller *et al.*, 1994), the hippocampus, granule cells of the dentate gyrus, and Purkinje neurons of the cerebellum (Brose *et al.*, 1994). In some neuronal circuits there may be presynaptic NMDA "autoreceptors" that are involved in the regulation of transmitter release.

AMPA and kainic acid receptors subserve primarily rapid excitatory neurotransmission in the CNS and mediate the influx of Na^+ and K^+ (Michaelis, 1998). Like the NMDA receptor, the AMPA receptor is widespread in the CNS with a parallel distribution (Cooper *et al.*, 1996). High-affinity binding sites for kainate exists in the CNS, and exerts powerful excitatory actions on hippocampal neurons. Kainate channels thus contribute to the rapid phase of at least some excitatory postsynaptic potential in the hippocampus (Patneau *et al.*, 1993).

1.5.2.1.2.2 Metabotropic receptors

Metabotropic glutamate receptors (mGluR) act either directly through receptor-coupled G proteins or indirectly through stimulation of protein kinase C (PKC) that inhibit voltage gated L and N Ca^{2+} and voltage-dependant and Ca^{2+} -activated K^+ channels (Chavis *et al.*, 1994). They also act indirectly through depolarization and Ca^{2+} influx, to induce the release of arachidonic acid. Metabotropic receptors produce the most widespread physiological and biochemical effects on neurons of any of the excitatory amino acids (EAA) receptors (Michaelis, 1998). Eight mGluRs families exist and are divided into three groups. Group I consists of the $mGluR_1$ and $mGluR_5$ receptors, which are positively coupled to PLC. Group II receptors include the $mGluR_2$ and $mGluR_3$, which are negatively coupled to adenylate cyclase and cAMP formation (Michaelis, 1998). The receptors of group III are $mGluR_4$ and $mGluR_{6\text{-}8}$, which are also negatively coupled to adenylate cyclase (Conn & Pin, 1997). Metabotropic glutamate receptors are important for long-term potentiation (LTP) and also presynaptic inhibition of excitatory synaptic neurotransmission.

1.5.2.1.3 Alterations of the glutamatergic system in depression

In recent years, a prolific amount of research has brought to light the evidence for glutamate involvement in depression (Manji *et al.*, 2001). Stress increases Glu signaling, while drugs used in bipolar depression, such as Li+ and lamotrigine, facilitate Glu reuptake, or decreases Glu release, thereby decreasing synaptic Glu levels. In addition the NMDA antagonist, ketamine, has antidepressant effects (Berman *et al.,* 2000), while various ADs, including ECT, reduce NMDA receptor subunit expression and can modulate the release and/or reuptake of glutamate (Golembiowska & Zylewska, 1999). NMDA receptor antagonists are as effective as tricyclic

antidepressants in preclinical antidepressant screening procedures in rats (Wedzony *et al.*, 1995) and produce antidepressant-like properties in acute, preclinical screening procedures for AD efficacy (Li *et al.*, 2001). Finally, the evidence for neuronal atrophy and dendritic reshaping in depression correlates with the evidence for increased glutamate activity in depression and the neurotoxic nature of this amino acid.

As with traditional ADs, chronic NMDA antagonist treatment results in a reduction of the β-adrenoreceptor density and 5-HT_2 receptors in the forebrain (Wedzony *et al.*, 1995). These data suggest a cross-talk mechanism between adrenergic and glutamatergic pathways (Harvey *et al.*, 2003). Equally important is that a functional noradrenergic system is essential for antidepressants to reduce the activity of the glycine site on NMDA receptor (Harkin *et al.*, 2000). Interactions between glutamate and DA pathways also have relevance for a glutamatergic influence in affective disorders. Stress induced activation of mesocortical and mesolimbic dopaminergic systems are blocked by glycine and NMDA receptor antagonists (Toru *et al.*, 1994).

Since the NMDA receptor promotes Ca^{2+} influx, changes in intracellular Ca^{2+} and activation of NOS may mediate many effects of NMDA receptor activation. Thus, NO synthase (NOS) inhibitors and gc/cGMP inhibitors produce antidepressant-like effects in the forced swim test (FST) (Harkin *et al.*, 1999; Eroglu & Caglayan, 1997; Heiberg *et al*, 2002). Skolnick (1999) suggests that major depression is accompanied by changes in glutamate and subcellular NOS signaling, and that antidepressants may act by inducing alterations of glutamate-dependant calcium-calmodulin-NOS-guanylyl cyclase signaling pathway (Skolnick, 1999). Chronic antidepressant administration reduces glutamatergic transmission by decreasing the high activity glycine sites on NMDA receptor (Skolnick *et al.*, 1996).
Considering the importance of monoamine-glutamate cross talk described earlier, these antidepressant-induced adaptations may be secondary to an initial action at monoaminergic terminals. Indeed, noradrenaline and serotonin can influence the activity of glutamate neurons, both directly and via regulation of GABA-mediated synaptic inhibition (Bijak & Misgeld, 1997) and can also regulate glutamate release via presynaptic heteroreceptors. Because the above-mentioned monoaminergic receptors are downregulated after chronic antidepressant therapy, including ECT, these adaptive changes in their receptors may influence the release of glutamate and result in changes in post- synaptic glutamate receptors (Zahorodna & Bijak, 1999).

NMDA receptor abnormalities are observed in human suicide victims (Nowak *et al.*, 1995) while a reduction in the expression of $NMDAR_1$ mRNA in the hippocampus has been described in depressed subjects (Law & Deakin, 2001). Moreover, platelet glutamate receptor super-

sensitivity (Berk *et al.,* 2001) and elevated levels of nitrogen oxides (Suzuki *et al.,* 2001) are some of the abnormalities observed in depressed patients that are indicative of altered NMDA-NO-activity.

The role of glutamate in depression is not restricted to the NMDA receptors, and recent evidence highlights the role of metabotropic glutamate receptors in AD action (Pilc & Legutko, 1995; Zahorodna & Bijak, 1999; Smialowska, 2002; Marek *et al.* 2000), while AMPA receptors have also been implicated (Li *et al.*, 2001; Svenningsson, 2002; Legutko *et al.*, 2001).

It is thus clear, that diminishing or interfering with EAA signaling pathways will produce antidepressant-like effects, with chronic antidepressant treatments resulting in an adaptation of EAA signaling at multiple loci within the prefrontal cortex, hippocampus and brainstem, particularly monoaminergic nuclei at these sites. By direct regulation of Ca^{2+} influx and NO free radical production, antidepressant-induced regulation of EAA signaling stimulates neurogenesis and neurite outgrowth, which result in improvement of depressive symptomatology, as well as degenerative pathology evident in the disorder.

1.5.2.2 GABA

Gamma-aminobutryric acid (GABA) is the most important inhibitory neurotransmitter in the CNS and is found in all areas of the brain (Davies, 1996). GABA is a major regulator of many CNS functions such as seizure threshold, and has inhibitory input into other neurotransmitter systems such as Glu, NA and DA. Strong clinical and pharmacological evidence suggests that GABA metabolism is altered in affective disorders (Nathan *et al.,* 1995).

GABA is synthesized in GABAergic terminals by the removel of a carboxyl group from glutamate by glutamic acid decarboxylase (GAD). The principle supply of glutamate comes from glucose via the tricarboxylic acid (TCA) cycle. GAD is a cytosolic enzyme that requires the cofactor pyridoxal phosphate (vitamin B_6) to be active (Davies, 1996). Following synaptic release, GABA interacts with receptors located on adjacent neurons or on the GABAergic terminal itself (Davies, 1996). The effect of GABA is terminated by reuptake into nerve terminals or into glial cells by high-affinity uptake systems. Once taken up into the nerve terminal or glial cells, GABA is metabolized to succinic semialdehyde by GABA-transaminase (GABA-T) (Davies, 1996). A feedback inhibitory mechanism is also present which triggers the GABA autoreceptor on the presynaptic terminal, leading to a reduction in the transmitter release (Leonard, 1997).

GABA has a heterogeneous distribution, with the highest concentrations in the basal ganglia, followed by the hypothalamus, the periaqueductal grey matter and the hippocampus. Approximately equal concentrations are found in the cortex, amygdala and thalamus (Leonard, 1997). Specific GABA-containing interneurons are present in the striatum, in the nigrostriatal pathway and in the pallidonigral pathway (Leonard, 1997).

There are two major classes of GABA receptors, termed $GABA_A$ and $GABA_B$, and each is a member of a different receptor superfamily. The $GABA_C$ class of receptors is structurally related to the $GABA_A$ receptor (Olsen & DeLorey, 1999) but is unique in that they are activated by very low concentrations of GABA and are unlikely to be desensitized by prolonged exposure to GABA (Leonard, 1997)

The $GABA_A$ receptor, a member of the ligand-activated ion channel family, is closely associated with the chloride ion channel. Activation of this complex by GABA causes Cl^- ions to flow through the channel, resulting in hyperpolarization of the neuron. Binding sites on the $GABA_A$/Cl ion channel complex that facilitates GABA action, such as benzodiazepines, barbiturates, neurosteroids, and possible anesthetic agents, have sedative, hypnotic and anticonvulsive properties (Davies, 1996).

$GABA_B$ receptors are members of the large G protein-coupled receptor superfamily (Davies, 1996), and are widely distributed throughout the CNS and are also present on peripheral organs. On neurons, $GABA_B$ receptors have postsynaptic actions and act to modulate neurotransmitter release (Bowery, 1993). Selective $GABA_B$ antagonists may be useful therapeutic agents for the treatment of epilepsy (Davies, 1996), while their cognitive-enhancing, anxiolytic, and neuroprotective properties may have promise (Bowery, 1993)

1.5.2.2.1 Alterations in the GABAergic system in depression

Preclinical and clinical data suggests that GABA may play an important role both in the pathophysiology of mood disorders, particularly depression, and in the mechanisms of action of mood stabilizers such as lithium, carbamazepine, and divalproex, and various antidepressant treatments, including ECT (Shiah & Yatham, 1998). GABA concentrations are reduced in the brain, CSF and plasma of depressed patients (Shiah & Yatham, 1998), while GABA enhancing agents have been shown to have antidepressant-like actions in several rodent models of antidepressant activity (Lloyd *et al.*, 1987). This supports a GABA deficit in mood disorders (Petty, 1995).

In the prefrontal cortex and hippocampus, GABA facilitates noradrenaline, both presynaptically via $GABA_A$ receptor (Bonanno & Raiteri, 1987) and postsynaptically via the $GABA_B$ receptor (Suzdak & Gianutsos, 1985). GABA also inhibits glutamatergic transmission via presynaptically $GABA_B$ heteroreceptors (Yamada *et al.*, 1999). Moreover, $GABA_A$ and $GABA_B$ receptor agonists attenuate stress-induced release of NO (Ishizuka *et al.,* 2000), while $GABA_A$ receptor expression is regulated by NO (Kim & Oh, 2002). Both of these agonists also reduce hippocampal cGMP levels (Fedele *et al.,* 1997).

Classical antidepressants have significant effects on GABA function. For example, elevated GABA levels, enhanced GABA release in rat brains, and enhanced $GABA_B$ receptor binding have been reported after chronic administration of TCAs, SSRIs and MAOIs (Lloyd *et al.*, 1987). Moreover, progabide and fengabine (SL79229), both GABA-mimetic compounds, possess antidepressant properties in preliminary studies (Lloyd *et al.,* 1987). It has been suggested that low total brain GABA, reflected in low plasma GABA, is a genetic marker that leads to development, or recurrence, of depression in some individuals (Petty, 1995). Thus, regulation of both the GABAergic and glutamatergic neurotransmitter systems are altered during depressive episodes, and both systems contribute to both the pathophysiology and treatment of major depressive disorder. One particular molecule that has appeared as a target in both GABA and glutamate systems is the gaseous neurotransmitter, NO. NO has attracted great interest in recent years as a possible contributing factor in affective illnesses well as a possible new drug target and the treatment of depression.

It has been proposed that NO may be an important protagonist in affective disorders, particularly in setting the course, outcome and response to treatment of the illness (Harvey, 1996). In agreement with this, depressed patients have increased levels of nitrate, the end product of NO metabolism (Suzuki *et al.*, 2001), while various authors have described changes in the NO-pathway during depression (Harkin *et al.,* 1999; Heiberg *et al.*, 2002; Wegener *et al.*, 2003). In the CNS, NO is synthesized in neurons via NMDA-mediated activation of nNOS and from iNOS localized in astocytes, while its release can be strongly inhibited by GABA (Yamada *et al.,* 1999). In the CNS nitric oxide, derived from nNOS, acts as inter- and intracellular messenger playing an active role in a number of processes, including neurotransmitter release neurotrophic release, blood flow, neurogenesis and synaptic plasticity, learning and memory formation and neuronal cell death (Jurado *et al.*, 2004). NO-mediated activation of guanylyl cyclase and increased cGMP can alter cell responses by inhibition or activation of phosphodiesterase (PDE)-mediated cAMP metabolism, protein phosphorylation via G-kinase and effects on ion-channel conductance (Goy, 1991). These actions strongly suggest a possible role for the NO/cGMP pathway in the action of antidepressants. Given the central role of the

NO-cGMP pathway in the current study, this pathway is discussed in detail in Chapter 4 "The Nitric Oxide pathway".

While this chapter has focused primarily on the biogenic theory of depression, as well as the role of glutamate and GABA, the reader should not lose sight of the fact that depression is a heterogenous illness and very likely is a multi-transmitter disorder. For example, the cholinergic theory of mood disorder was proposed by Janowsky *et al.*, 1972, who suggested that the cholinergic-adrenergic hypothesis of mood disorder as an extension of the earlier biogenic amine hypothesis. Evidence for a role for Ach in depression is the depressogenic effect of cholinomimetics, and enhanced cholinergic sensitivity seen in depressives, its role in sleep EEG abnormalities, and the antimanic effects of cholinomimetics (Manji *et al.*, 2001).

Furthermore, recent evidence has revealed a potential role of neuroendocrine and neuropeptides important in depression, but discussion of these falls beyond the scope of the current study.

1.6 Neurodegenerative Hypothesis of Depression

Conventional definitions of neurodegeneration imply that it is a process of idiopathic progressive neuronal death, generally occurring in a selective distribution that often involves functionally related pathways (Calne *et al.*, 1992). Analyses of the hippocampus in depressed patients have revealed structural changes (Sheline, 2000) that go beyond volume loss and include gray matter alterations. The biochemical changes associated with depression affect general hippocampal morphology, hippocampal neurogenesis (Kempermann & Kronenberg, 2003) and also functioning. Studies on other brain regions have also reported reduced volume of the prefrontal cortex (Bremner *et al.*, 2000) and reduction in the size of neurons and number of glia, which could underlie the reduction in cortical volume (Ongur *et al.*, 1998).

Because stress regulates the rate of new cell formation most strongly in the hippocampus (Henn & Vollmayr, 2004), the HC has become the focus for this hypothesis and, indeed, in the current study.

Major depression is a recurrent illness; the interval between episodes tends to shorten while with each new episode there is an increasing risk for the occurrence of another (Kasper, 1993). This process resembles the process of neuronal kindling that is characterized by a diminishing cycle length as a function of prior episodes, the spontaneous occurrence of episodes, and the presence of a preceding symbolic stressor (Post & Weiss, 1998). While alerations in serotonergic tone enhance or delay the rate of kindling progression and have an impact on the

intensity or duration of seizures, the primary neural substrate of kindling involves glutamate and NMDA receptor activaton, with GABA pathways exercising a permissive role on the kindling action of glutamate (Post & Weiss, 1998). The fact that excessive glutamatergic and nitregic activity, particularly after stress-related release of glucocorticoids (McEwen, 1999), can lead to the neurodegenerative pathology in the hippocampus, emphasize the importance of the balance between GABA-glutamate pathways in the limbic areas of the brain. Stress-mediated glucocorticoid release will promote neuronal death by overt release of glutamate, disturbed Ca^{2+} homeostasis via excessive activation of NMDA receptors, inhibition of glucose transport, and an increase in reactive oxygen radicals ($O_2^{\cdot}$) and reactive nitrogen radicals ($NO^{\cdot}$) (Sapolsky, 2000b). Both stress and glucocorticoids increases glutamate concentrations in the hippocampal synapse (Moghaddam *et al.*, 1994). Figure 1.12 illustrates these glutamate receptor-mediated processes.

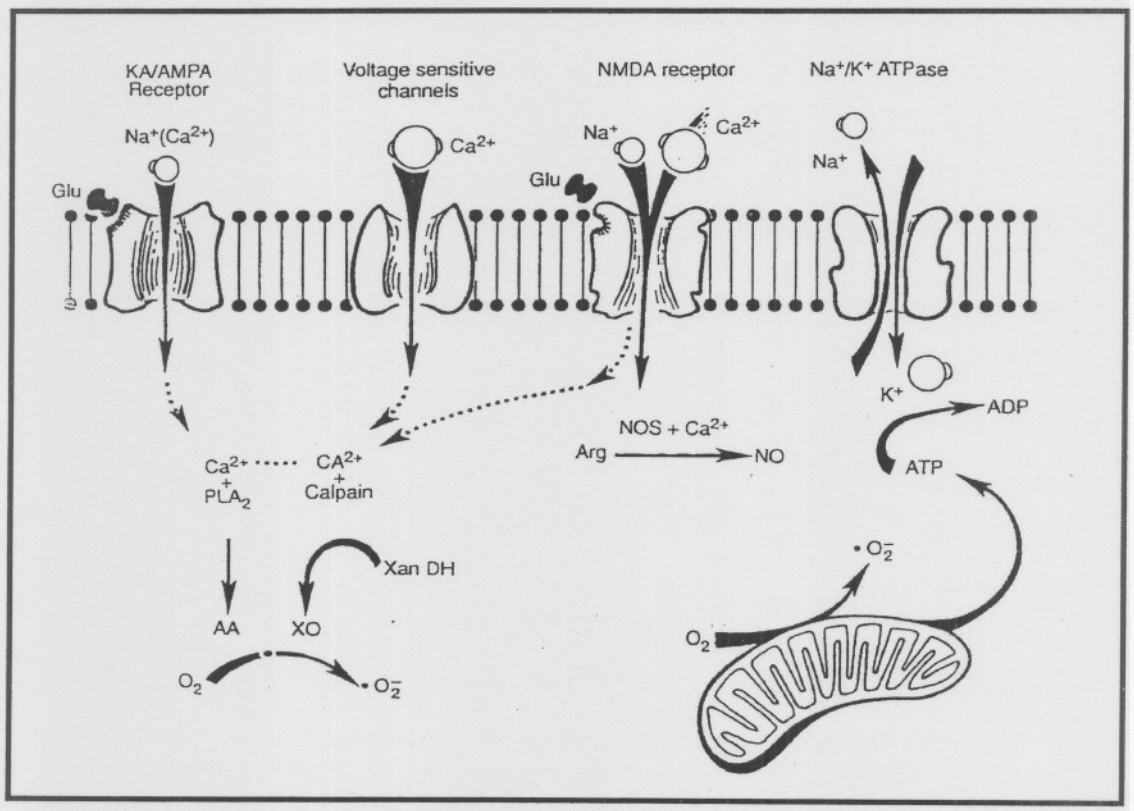


Figure 1.12: Activation of KA/AMPA and NMDA receptors by Glu permits Na^+ and Ca^{2+} to flow through its channels (Coyle & Puttfarcken, 1993). Calcium enters the neuron through channels or via the release from intracellular organelles, resulting in activation of calcium-dependant enzymes that are cell damaging, for example, phospholipase A_2 (PLA_2) that initiated the arachidonic acid metabolic cascade, proteases such as calpain that convert xanthine dehydrogenase (Xan DH) to xanthine oxidase (XO), and NO synthase. Depolarization also increases ATP consumption by the Na^+ / K^+ ATPase, which increases oxidative phosphorylation, yielding O_2^- as a by-product (After Coyle & Puttfarcken, 1993).

Hippocampal volume loss appears to be related to greater lifetime of depression (Sheline *et al.*, 1999) and tends to escalate with later episodes of depression (MacQueen *et al.,* 2003), suggesting that recurrent depressive episodes inflict increasing hippocampal atrophy and permanent damage. Since increased glucocorticoid concentrations in the hippocampus provides negative feedback regulation of the HPA axis, McEwen (1999) suggested that atrophy in depressed patients could lead to a recurrent and damaging cycle of HPA overactivation and sustained hippocampal atrophy (McEwen, 1999). In animal studies, hippocampal structural changes are mediated by elevated glucocorticoid secretion that produces its effects by enhancing neuronal glutamate signaling (McEwen, 1999). The fact that elevated cortisol levels in Cushing disease are associated with reduced hippocampal volume, as well as depression (Starkman *et al.,* 2001), is supportive of the role of the HPA axis in the apparent neurodegenerative pathology of depression. Moreover, stress decreases the expression of brain-derived neurotrophic factor (BDNF), and reduced levels could contribute to the atrophy and compromised function of stress-vulnerable hippocampal neurons (Figure 1.13). A number of pre-clinical studies have indicated that altered neurogenesis plays an important role in the etiology and treatment of depression (Duman *et al.,* 2001; Kempermann & Gage, 2002) and that the formation of new neurons might be a final pathway in the etiology of depression (Duman *et al.,* 2000; Jacobs *et al.*, 2000). Indeed, antidepressants tend to increase the expression of BDNF, and thereby reverse atrophy and protect neurons from further damage (Figure 1.13).

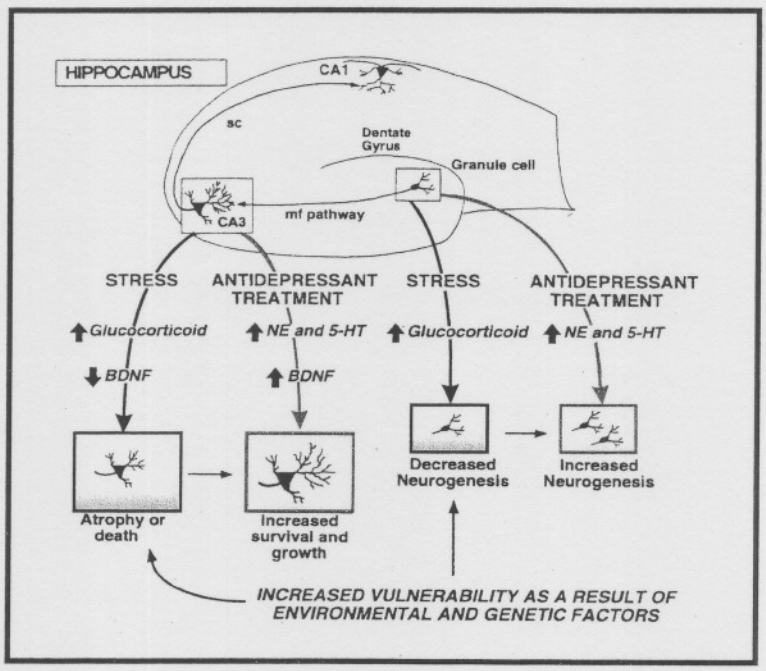


Figure 1.13: Diagrammatic representation of the actions of stress and antidepressant treatment on hippocampal neurons (After Duman *et al.*, 1999).

The time lag for therapeutic response of antidepressants has led to the hypothesis that adaptations are necessary for the clinical actions of antidepressants (Duman, 2004). These cellular adaptations can lead to alterations in cell morphology and even the birth of new neurons. Alterations in neuronal morphology and cell number represent a mechanism of neuronal plasticity that allow adaptation to environmental, behavioral, and pharmacological stimuli and thereby illicite long-term responses (Duman, 2004). Manifestations of neuroplasticity in the adult CNS have been characterized as changes in dendritic function, synaptic remodeling, long-term potentiation, axonal sprouting, neurite extension, synaptogenesis and neurogenesis (Manji *et al.*, 2003). Thus, another explanation for the cellular mechanism of antidepressants might involve the effect of these drugs on the rate of new neuron formation in the adult brain (Duman *et al.*, 2001). Different classes of antidepressants increase the proliferation as well as number of newly formed neurons in the hippocampus (Figure 1.13). However this increase is not seen with non-antidepressant psychotropic drugs, suggesting that neurogenesis may be a mechanism-specific action of AD treatment to reverse the stress-induced damage to hippocampal neurons (Malberg *et al.,* 2000)

Although there is no direct link between adult hippocampal neurogenesis and mood and emotion, the key role of the hippocampus in regulating diverse physiologic and neurovegetative functions suggests that impairments of neurogenesis may contribute to other facets of depression (D'Sa & Duman, 2002; Jacobs *et al.,* 2000). Furthermore, the putative role of GABA and Glu, as well as NO, in the underlying pathology of depression offers further evidence to the importance of this hypothesis.

Chapter 2
TREATMENT OF DEPRESSION

2.1 Introduction

Despite the growing awareness, acceptance and availability of effective antidepressants, the diagnosis and treatment of depression remain inadequate (Anderson, 2000). Depression is an illness that is more prevalent among primary care patients than hypertension (Jacobsen, 1996) and recently it has been found to be a risk factor in diseases such as diabetes and cardiovascular diseases (Ciechanowski *et al.*, 2000). This illness can be potentially fatal since most patients think about suicide, about 50% try to commit suicide and up to 15% of patients with severe depression die from suicide (Strakowski *et al.*, 1996). Depression is sometimes not treated aggressively and often goes underdiagnosed, it is missed in approximately 50% of patients in the primary care setting and it was found that only 34% of depressed patients is treated effectively (Montano, 1994). In view of the efficacy of drugs (about 60%) and ECT (80%-90%) (Nobler & Sackheim, 2001; Shergill & Katona, 2001) it is clear that we still have a need to understand the pathophysiology of the illness in order to develop effective medications.

Depression has been proposed to represent an inability to make the appropriate adaptive responses to stress or other aversive stimuli. As discussed in chapter 1, this could be attributed to dysfunction of the normal mechanisms underlying neural plasticity. Antidepressants may act by correcting this dysfunction by themselves or by inducing the appropriate adaptive responses (Duman *et al.*, 1999).

The treatment of depression was revolutionized about a half-century ago with the introduction of two classes of agents that were found to be effective antidepressants: the tricyclic antidepressants and the monoamine oxidase inhibitors. The original tricyclic agents (e.g., imipramine) arose from antihistamine research, whereas the early monoamine oxidase inhibitors (e.g., iproniazid) were derived from work on antitubercular drugs. Both classes of agents were demonstrated to dramatically improve the symptoms of depression (Nestler *et al.,* 2002b) and they are still the standard of clinical efficacy, despite multiple adverse effects (Yadid *et al.,* 2000). The only prior established somatic treatment for depression was electroconvulsive seizures or ECT (Nestler *et al.,* 2002b). Over the last 40 years, six other classes of antidepressants have become available: selective serotonin reuptake inhibitors (SSRIs; such as

fluvoxamine, fluoxetine citalopram, paroxetine and sertraline), 5-HT/NA reuptake inhibitor (SNRI; such as venlafaxine and milnacipram), serotonin reuptake inhibitor and 5-HT blocker (SARI; such as nefazodone), NA and specific 5-HT antidepressant (NaSSA; such as mitrazapine), NA selective reuptake inhibitors (NASI; such as reboxetine), and NA/DA reuptake inhibitors (NDRI; such as bupropion) (Yadid *et al.,* 2000).

The common basis of pharmacotherapy rests on the enhancement of serotonergic and/or noradrenergic neurotransmission by either inhibiting the intracellular degradation of the monoamines with monoamine oxidase inhibitors or blocking their reuptake back into the synaptic cleft by SSRI, SNRI, or TCA (Nemeroff, 1998). The shortfall in clinical efficacy of the above agents has emphasized, however, that 5-HT and NA are not the only mechanisms involved in depression. Moreover, the delay in onset of action indicates that several distinct receptor mechanisms' trigger different but converging intracellular signal cascades that activate transcription factors, which, in turn, promote the expression of genes encoding for proteins, that play a crucial role in restoring of neuronal functions involved in mood regulation (Vetulani & Nalepa, 2000).

The available antidepressant treatments attempt to alleviate the symptoms of the disorder, to decrease the functional disability of the affected individual, and to increase their subjective well being, quality of life and overall function (Yadid *et al.,* 2000). Treatment strategy is strongly influenced by factors such as the patient's previous response to treatment, drug-related mood instability, drug interactions, safety and tolerability profile, compliance, lethality in overdose, comorbid psychiatric disorders and type of depression (Zornberg & Pope, 1991). Alleviation of personal suffering is an important reason for developing more effective therapies, since the long period (2-6 weeks) that is required before significant improvement is seen, probably contributes to the high percentage (15%) of depressed patients that commit suicide (Akiskal, 1995). Furthermore, prolonged intractable depression, as well as poor compliance is both linked to poor long-term outcome as well as neurodegenerative pathology in limbic regions of the brain (Harvey *et al.*, 2003). Clearly, new treatment strategies are urgently needed if the impact of this illness on society is to be addressed more successfully.

A functional classification system classifies currently available antidepressant drugs into the following classes (Danilevičiūtė & Sveikata, 2002): Non-selective antidepressants, that inhibit the reuptake of serotonin and noradrenalin but with effects on multiple receptor systems and sodium conductance, (e.g. TCAs), or that block monoamine oxidase, (e.g. MAOI). Selective reuptake inhibitors include the selective serotonin reuptake inhibitors (SSRI), such as citalopram, fluoxetine and others, the selective noradrenaline reuptake inhibitors (NARI), such as reboxetine, the serotonin and noradrenaline reuptake inhibitors (SNRI), such as venlafaxine

and milnacipran, and the inhibitors of noradrenaline and dopamine reuptake inhibition (NDRI), such as bupropion.

Receptor blockers, on the other hand, include those agents that block serotonin (5-$HT_{2A \text{ and } 2C}$, 5-HT_3) receptors together with noradrenalin (α_2) receptor blockade, such as mirtazapine, and agents such as nefazodone, that block both serotonin (5-HT_{2A}) receptors as well as block serotonin reuptake.

Although it is usefull to discuss all the classes of antidepressants in detail, for the purpose of the current study, only the TCA class of drugs and the SSRI's will be described in more detail.

2.2 The Tricyclic antidepressants (TCA)

The TCAs were the first line medication therapy for depression for nearly 30 years, until the introduction of the SSRIs (Tamminga *et al.*, 2002). Because of their three benzene ring structure they are called tricyclics and are the most commonly used drugs in depression therapy. The TCAs have well-documented efficacy in the treatment of major depression, their pharmacological effects are essentially the same and differ primarily in their relative potency. In terms of the therapeutic actions of TCAs, they essentially work as negative allosteric modulators of the neurotransmitter reuptake process (Gareri *et al.*, 2000). TCAs thus inhibit NA and 5-HT reuptake, but while biochemical block is already evident on the first administration, the antidepressant effect is shown only after two or three weeks. However, the chronic administration of an antidepressant shows β-adrenergic postsynaptic receptor desensitization and a decrease in receptor density for 5-HT; suggesting that depression may be linked to a receptor supersensitivity (Garattini & Samanin, 1988). Additional neuropharmacological changes that may contribute to the clinical effects of TCAs include indirect facilitation of serotonin and perhaps dopamine neurotransmission through excitatory α_1 receptors on other monoaminergic neurons, or via desensitized, inhibitory α_2 receptors, as well as D_2 dopamine autoreceptors. Activated release of serotonin and dopamine may, in turn, lead to secondary down-regulation of serotonin 5-HT_1 autoreceptors, postsynaptic 5-HT_2 receptors, and perhaps dopamine D_2 autoreceptors and postsynaptic D_2 receptors (Leonard & Richelson, 2000). Other adaptive changes have also been observed in response to long-term treatment with TCAs, and include altered sensitivity of muscarinic acetylcholine receptors as well as decrease in gamma-aminobutyric acid receptors ($GABA_B$) (Leonard & Richelson, 2000). Altered cytoskeletal and other structural proteins may alter neuronal growth and sprouting (Wang *et al.*, 1991) via changes in nuclear genetic-regulatory factors, including the cyclic AMP-response-element binding protein (CREB) and brain-derived neurotrophic factor (BDNF) (Duman *et al.*, 1997)

TCAs can be divided into two main classes (Figure 2.1) (Potter *et al.*, 1998). The tertiary amines are generally dual (serotonin and noradrenaline) reuptake inhibitors, although are metabolized to secondary amines, and have a high burden of anticholinergic side effects (e.g., amitriptyline and imipramine) (Tamminga *et al.*, 2002). The secondary amines are generally more selective at blocking NE reuptake with somewhat reduced anticholinergic side effects (e.g., nortriptyline and desipramine) (Tamminga *et al.*, 2002).

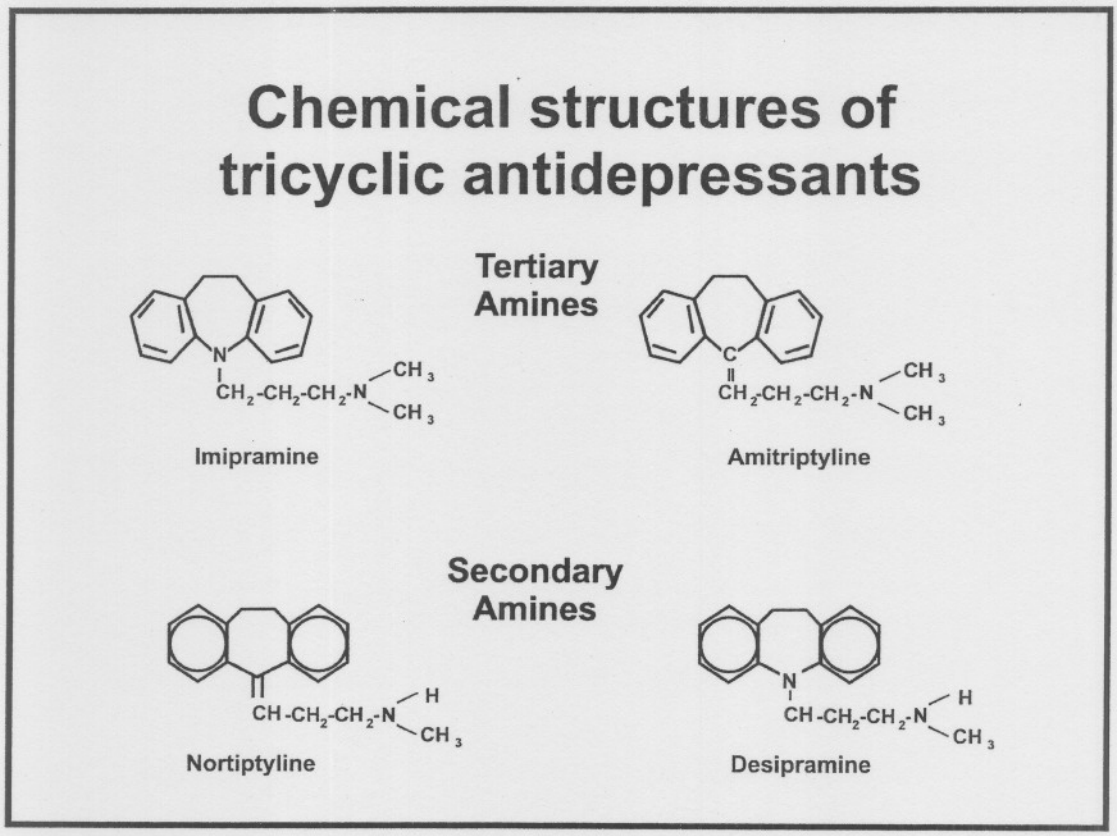


Figure 2.1: The chemical structures of some tricyclic antidepressant drugs.

In addition to blockade of neurotransmitter uptake, these compounds have affinities for several different heterogeneous receptors, including the histamine$_1$, α_1-adrenergic and muscarinic cholinergic receptors (Kamil, 1996). Consequently, these agents are associated with numerous adverse events, such as cardiac dysfunction, sedation, orthostatic hypotension, dry mouth, urine retention, constipation, cognitive impairment, tremor, sexual dysfunction, and weight gain. Associated anticholinergic effects prevent the use of TCAs in patients with comorbid conditions such as dementia, glaucoma, and coronary artery disease (Baldessarini, 2001). Cardiovascular adverse events may occur even at therapeutic doses (Gumnick & Nemeroff, 2000). TCAs inhibit α_1-adrenergic receptors, resulting in peripheral vasodilatation and orthostatic hypotension. Noradrenaline reuptake is blocked in the central and peripheral nervous systems, initially causing hypertension and tachycardia and eventually leading to refractory hypotension and

bradycardia. Cardiac conduction times are prolonged and contractility is impaired, leading to the potential arrythmias, especially with overdose (Baldessarini, 2001).

The toxic effects of TCAs may also result from pharmacokinetic and pharmacodynamic interactions (Masand, 2003). TCAs are metabolized primarily by the cytochrome P (CYP) 2D6 isozyme, and when a TCA is administrated concomitantly with inhibitors of this isozyme, serum levels of the TCA may increase by as much as 4 times the level reached in the absence of the CYP2D6 inhibitor (Gumnick & Nemeroff, 2000).

The treatment discontinuation rate with TCAs is 20%, and concerns about adverse events and toxicity, often lead to subtherapeutic dosing (Masand, 2003). Abrupt withdrawal of the TCAs may be characterized by a range of gastro-intestinal symptoms, sometimes with cardiac arrhythmias, insomnia followed by excessive, vivid dreams, extrapyramidal symptoms and acute psychiatric disturbances. These side-effects are likely caused by the cholinergic rebound after rapid discontinuation of TCAs (Kelsey & Nemeroff, 1998). Although TCAs are a relatively inexpensive choice of therapy, these dose-limiting adverse events restrict the use of TCAs as first-line agents (Masand, 2003).

2.3 The Selective serotonin reuptake inhibitors (SSRI)

SSRIs are a rationally designed class of psychotropic medication and have launched a new era in psychotropic drug development (Carlsson, 1999). The introduction of the SSRIs in the late 1980s has radically changed the treatment of depressive disorder worldwide and they have emerged as the first line of treatment for depressive disorders (Vaswani *et al.,* 2003). The therapeutic mechanism of action of SSRIs involves alteration in the 5-HT system. The explanation for therapeutic effects characteristic of SSRIs may be found in delayed neurochemical adaptations. A leading hypothesis for this action is desensitization of somatodendritic 5-HT_{1A} autoreceptors in the midbrain raphe nucleus, which increases 5-HT in critical brain regions and at key 5-HT receptor subtype(s), which may mediate the pathophysiology of depression (Goodwin, 1996). 5-HT is a neuromodulator that modulates homeostatic balance between dopamine, noradrenaline and GABA, responsible for mediating anxiety and mood, respectively (Davis *et al.*, 1999).

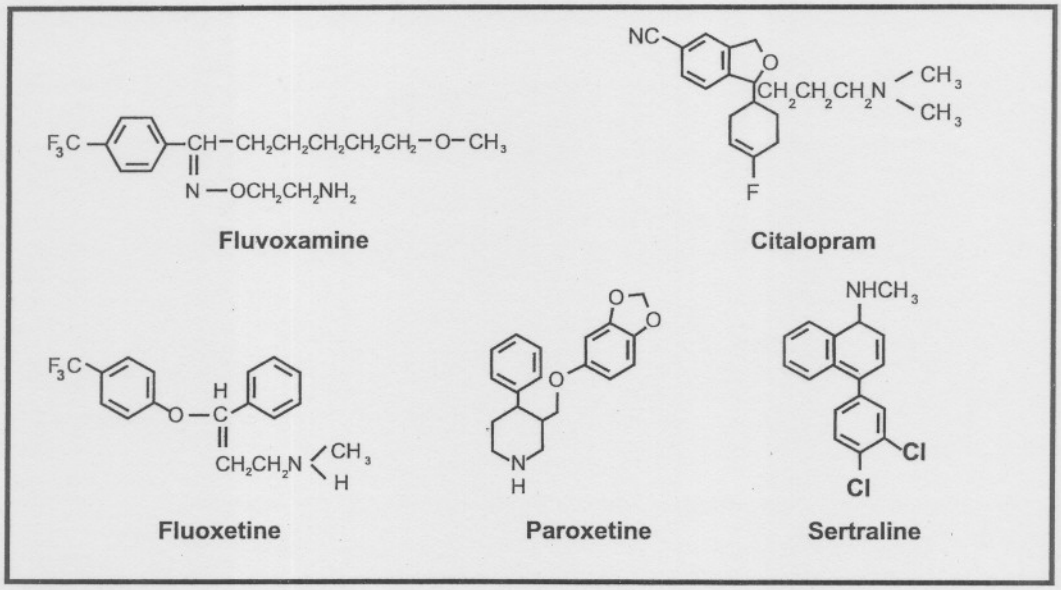


Figure 2.2: Selective 5-HT reuptake inhibitors (After Kelsey & Nemeroff, 1998).

The SSRIs are a heterogeneous group of compounds that are all structurally distinct entities (Harvey, 1997), they lack affinity for alpha-adrenergic, muscarinic and histaminergic receptors, and thus do not cause pronounced anticholinergic, cardiac or sedative effects (Cusack *et al.*, 1994).

The most important advantages of the SSRIs over TCAs are the absence of severe adverse effects and death from overdose (Vaswani *et al.,* 2003). The diversity of biological substrates, receptors and pathways acted upon by 5-HT are candidates to not only mediate the therapeutic actions of SSRIs, but also their side effects. Specifically, the immediate actions of SSRIs are mostly side effects mediated by the acute negative allosteric modulation of the 5-HT transporter (Vaswani *et al.*, 2003). These common side effects include anxiety, nervousness and headache, movement disorders, sexual dysfunction, gastrointestinal disturbances and 5-HT syndrome. SSRIs can inhibit the uptake of serotonin by platelets and under physiological circumstances this action can cause platelet aggregation that may be responsible for bruising or bleeding disorders (Kelsey & Nemeroff, 2000).

The "serotonin syndrome" is a rare but often lethal syndrome that often occurs as a result of the interaction of two or more drugs acting via different mechanisms to potentiate the central effects of 5-HT in the brain stem and spinal cord. The mechanism of this potentially lethal interaction appears to involve 5-HT mediated suppression of DA neurons within the basal ganglia (Harvey, 1997). The most frequent clinical features of this syndrome are changes in mental state, restlessness, myoclonus, hyper-reflexia, diaphoresis, shivering and tremors (Sternbach, 1991).

Abrupt interruption of SSRI treatment can result in a syndrome characterized by specific physical and psychological symptoms, which develop within 1 to 7 days of discontinuation or reduction in dosage of the SSRI (Black *et al.,* 2000). The relative risk of withdrawal symptoms is more likely to occur with SSRIs with short elimination $t^1/_2$, e.g. paroxetine, sertraline and fluvoxamine but less with fluoxetine (Price *et al.,* 1996). The common withdrawal symptoms are a sense of disequilibrium, gastrointestinal symptoms, flu like symptoms, sensory and sleep disturbances, extra pyramidal symptoms, anxiety, crying spells, irritability, confusion and aggression (Vaswani *et al.,* 2003). It has been postulated that discontinuation events result from a sudden decrease in the availability of synaptic 5-HT (Vaswani *et al.,* 2003). It has also been hypothesized that the symptoms may be caused by cholinergic rebound (Schatzberg *et al.,* 1997) especially with paroxetine, which has significant anticholinergic properties (Harvey, 1997) and also a faster elimination rate resulting in a relatively acute state of cholinergic and serotonergic dysregulation. Patients, by nature, may wean themselves off their antidepressant once they feel well, possibly by alternate-day dosing or by random skipping of doses. This inappropriate discontinuation may significantly increase the risk for relapse, recurrence and treatment refractoriness that has significant clinical implications (Harvey *et al.*, 2003). The best way to prevent the possible somatic and neurochemical consequences of AD withdrawal is to taper the dosage of the SSRI gradually (Vaswani *et al.,* 2003).

2.4 Onset of Antidepressant action and Subcellular Mechanisms

Most clinically effective antidepressants initially result in increases in synaptic concentrations of one or more monoamines (noradrenaline, serotonin, or dopamine). The onset of therapeutic action, however, appears 3-5 weeks after continuous administration (Manji *et al.*, 2001), even though stable plasma and brain concentrations are achieved within a week of treatment, suggesting that a cascade of downstream effects are ultimately responsible for their therapeutic effects (Manji *et al.,* 2003).

The lag in onset of therapeutic activity is ubiquitous amongst the different chemical classes of AD, but is not a characteristic of the disease, since some treatments, such as sleep deprivation and ECT, appear to work more quickly (Daly *et al.,* 2001). The existence of antidepressants that do not interact directly with monoaminergic neurons, non-pharmacological treatments (such as ECT and REM sleep deprivation), and the relatively modest success rate of the current drugs (estimated 65-70% in most studies) have led many investigators to conclude that actions at monoaminergic synapses alone cannot completely account for the actions of antidepressants

(Skolnick, 1999), but rather long-term neuroadaptive changes that may underlie the therapeutic effect (Schloss & Henn, 2004).

2.4.1 Molecular effects of antidepressant treatment

Onset of therapeutic activity is dependant on both extracellular and intracellular dynamics (Harvey, 1997). The presence of anatomical as well as functional receptor interactions epitomizes the way in which 5-HT may act as a 'permissive' modulator of neurotransmitter function. Serotonergic pathways make synaptic connections, via heteroreceptors, with dopaminergic (Devane, 1995), cholinergic (Hirano *et al.*, 1995) as well as noradrenergic (Leonard, 1993) pathways and thus modulate their function. The release of NA and 5-HT are both regulated by α_2 noradrenergic receptors on autoreceptors and heteroreceptors, conversely resulting in attenuation of their release (Leonard, 1995). Inhibition of these receptors will result in enhanced release of both NA and 5-HT (Harvey, 1997) (Figure 2.3).

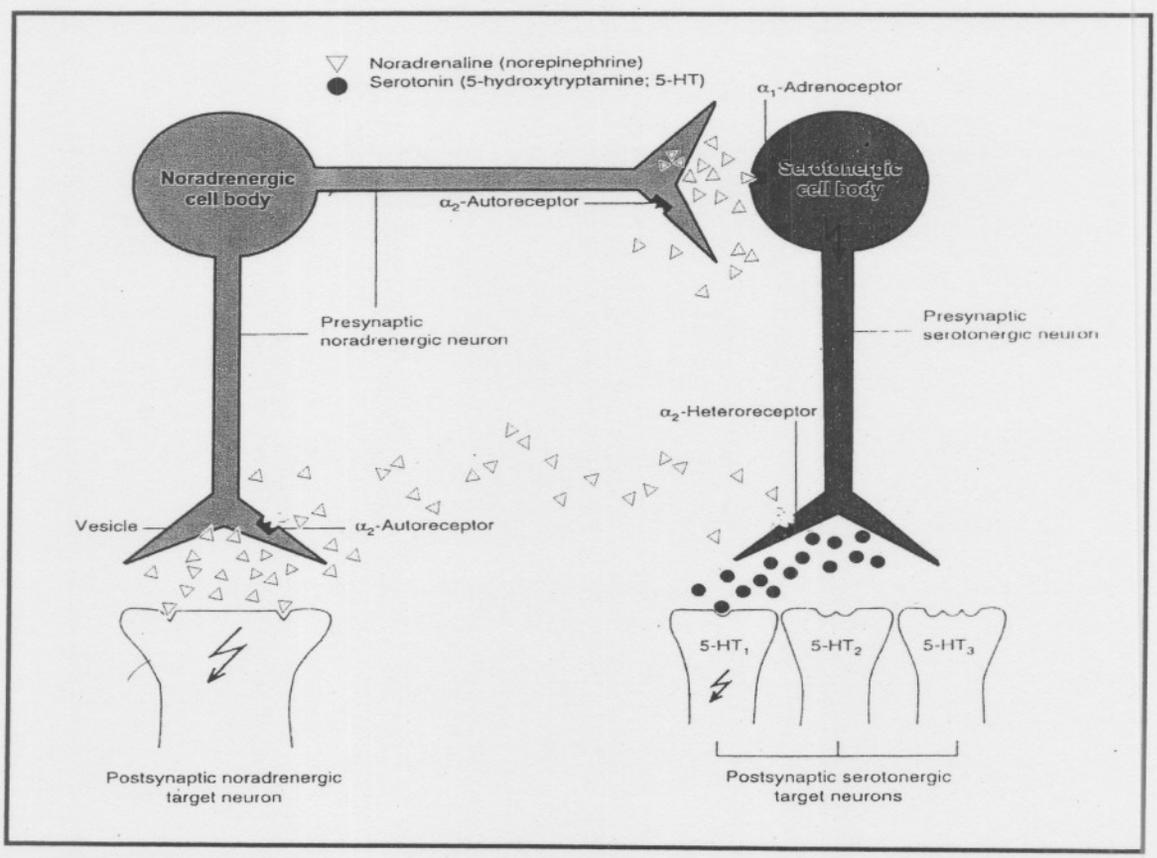


Figure 2.3: Auto- and heteroreceptors. When the presynaptic receptors are present on the same nerve terminal releasing the neurotransmitter, they are known as autoreceptors (e.g. 5-$HT_{1B/D}$). When the presynaptic receptors are present in nerve terminals releasing different neurotransmitter, they are known as heteroreceptors (e.g. α_2 adrenoreceptors) (De Boer *et al.*, 1995).

Functional cross-talk describes a subcellular interaction between different receptors within single cells/neurons via second messenger release acting as mutual co-regulators of cell function (Harvey, 1997). These subcellular interactions between different receptors allow a particular underactive/overactive pathway to be modulated, despite a selective action of the drug on another extracellular receptor, e.g. SSRIs modify dopaminergic transmission.

The acute increase in the levels of monoamines at the synapse is an early event in a complex cascade of events that ultimately results in antidepressant activity (Piñeyro & Blier, 1999). This acute increase in the amount of the monoamine at the synapse results in activation of somatodendritic and synaptosomal 5-HT_{1A} auto-receptors that inhibits 5-HT release, which delays the onset of AD action (Goodwin, 1996). Only once these receptors are down regulated via changes in sub cellular elements, can the full AD action be realized (Harvey, 1997).

Once a neurotransmitter binds to its receptor, the receptor must pass the signal encoded by the neurotransmitter to the interior of the receiving cell. Neurotransmitter receptors transmit the extracellular signal through the cell membrane into the cytoplasm and the nucleus where cell function is controlled. For the metabotropic receptors, the transmembrane signal is dependant on the functional interaction between the receptor and a transducer G-protein (e.g., 5-HT_1, 5-HT_2, mAch, alpha-1 and beta-adrenoreceptors) (Harvey, 1997). This complex is ultimately responsible for enhancing or reducing the activity of a transducing enzyme, e.g. adenylate cyclase or phospholipase C that is responsible for the synthesis/release of the second messenger, e.g. cAMP, IP_3, Ca^{2+} (Harvey, 1997). Ionotropic receptors such as the 5-HT_3 and NMDA receptors (Harvey, 1997) activate Ca^{2+} channels to increase influx of this ion into the cell. The second messengers can be stimulated by the same or different receptors and with different functions in different cells. Sub-cellular messengers, including cGMP, cAMP, inositol phosphates, Ca^{2+} and NO, phosphorylate critical regions within nuclear DNA, leading to the encoding of transcription factors which, in turn, modulate the transcription and translation of qualitatively modified proteins, e.g. receptors, neuropeptides, growth factors, neurotransmitters and enzymes (Harvey, 1997). This process and its relationship to time from immediate synaptic neurotransmitter changes to functional changes related to response are depicted in Figure 2.4.

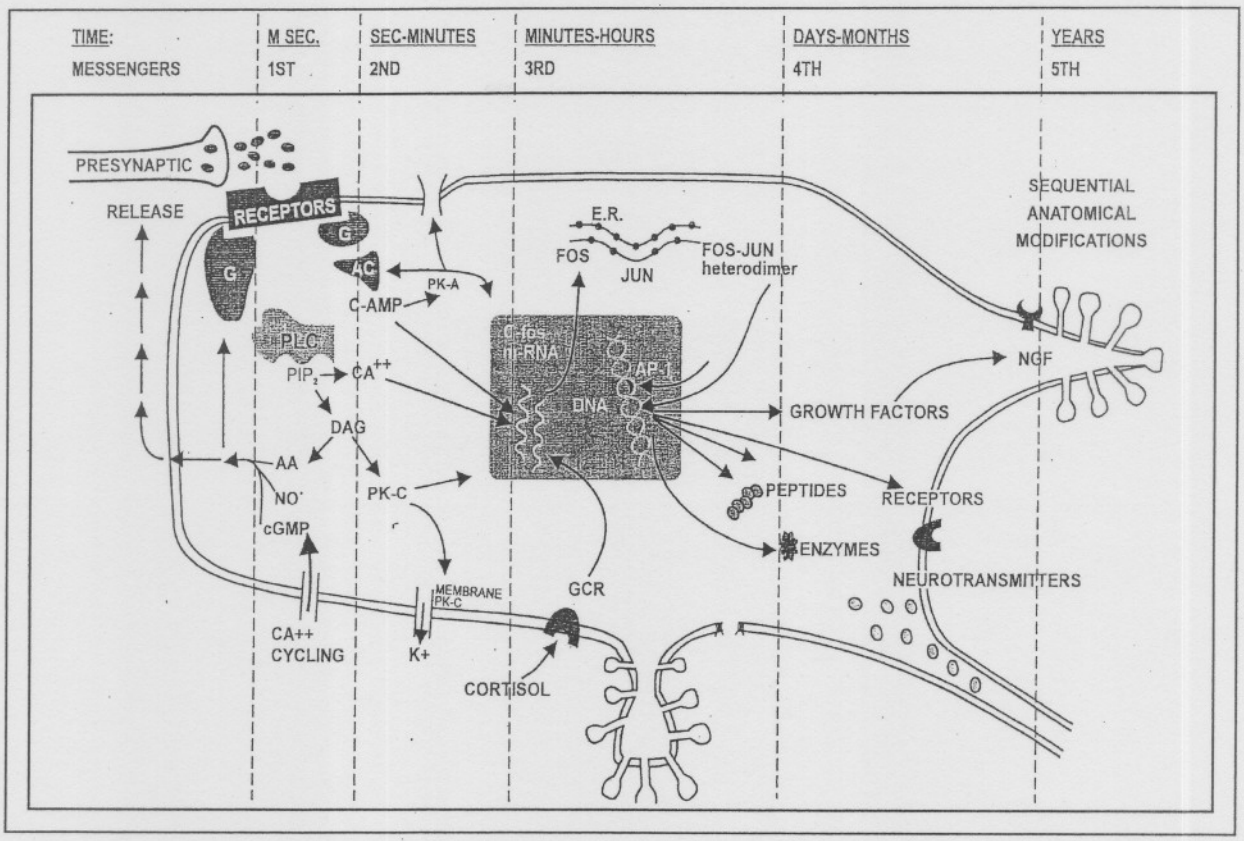


Figure 2.4: This schematic illustrates how synaptic events induced by external stimuli can exert longer-lasting effects on neuronal excitability and on the structure of the brain. Neurotransmitters activate receptors and second-messenger systems, which then induce immediate early genes (IEGs), such as c-fos and c-jun. Fos and Jun binds to DNA to further alter the transcription of late effector genes (LEGs) and other regulatory factors, the effects of which could last for months or years. (PLC, phospholipase; PIP2, phosphatidyl inositol 4,5-biphosphare; AA, arachidonic acid; DAG, diacylglycerol; PK-C, protein kinase C; AP-1, activator protein 1; E.R., endoplasmic reticulum; PK-A, protein kinase A) (After Post, 1992).

Due to prolonged 'starvation' of adequate stimulation, various critical receptors in the CNS of a depressed individual become upregulated in an attempt to maintain normal neurotransmission, e.g. β-adrenergic, muscarinic cholinergic (mAch), alpha-2 adrenergic, GABA-B, DA autoreceptors, 5-HT_{1A} and 5-HT_2 receptors (Leonard, 1992). Most, but not all, clinically effective AD drugs, including ECT, dowregulate these receptors after chronic use. This phenomenon parallels the delay in the onset of AD action (Leonard, 1993).

The efficiency of monoaminergic signaling can be enhanced by molecular changes at single synapses such as up-regulation of post-synaptic receptors and/or down-regulation or desensitization of presynaptic autoreceptors that are negatively coupled to exocytotic neurotransmitter release (Schloss & Henn, 2004). Additional possibilities include enhanced

neurotransmitter synthesis/release or downregulation / desensitization of transmitter transporters. The efficiency of monoaminergic signaling is not only modulated at single synapses but also by the density of synaptic contact sites (Schloss & Henn, 2004).

Thus, after a depressed individual starts to take an AD, the pathological 'upregulated' receptor proteins present in cerebral membranes have to be sequestrated and disposed of, while the newly induced receptor protein needs to be synthesized and then incorporated into the membrane. The complete process, from initial receptor stimulaton (0-6 hours) to resetting of cellular response (2-3 weeks), may account for the observed delay in AD action (Harvey, 1997) (Figure 2.4).

Subcellular mechanisms that have attracted the most attention with regards to AD action are the adenylate cyclase and phosphoinositide (PI) signaling pathways, these are discussed in more detail below.

2.4.1.1 Adenylate cyclase signaling pathway

Adenylate cyclase, of which there are several distinct forms, converts ATP to the second messenger, cyclic AMP. Most receptors that regulate cAMP action do so via their effect on G proteins. A recognized effect of cAMP is activation of protein kinase A (PKA), an enzyme that phoshorylates and regulates many proteins including ion channels, cytoskeletal elements, transcription factors and other enzymes. One of the transcription factors phosphorylated and thereby modulated by PKA is the cAMP response element binding protein (CREB). This protein binds to the cAMP response element (CRE), a gene sequence found in the promoter of certain genes (Gould & Manji, 2002) (Figure 2.5).

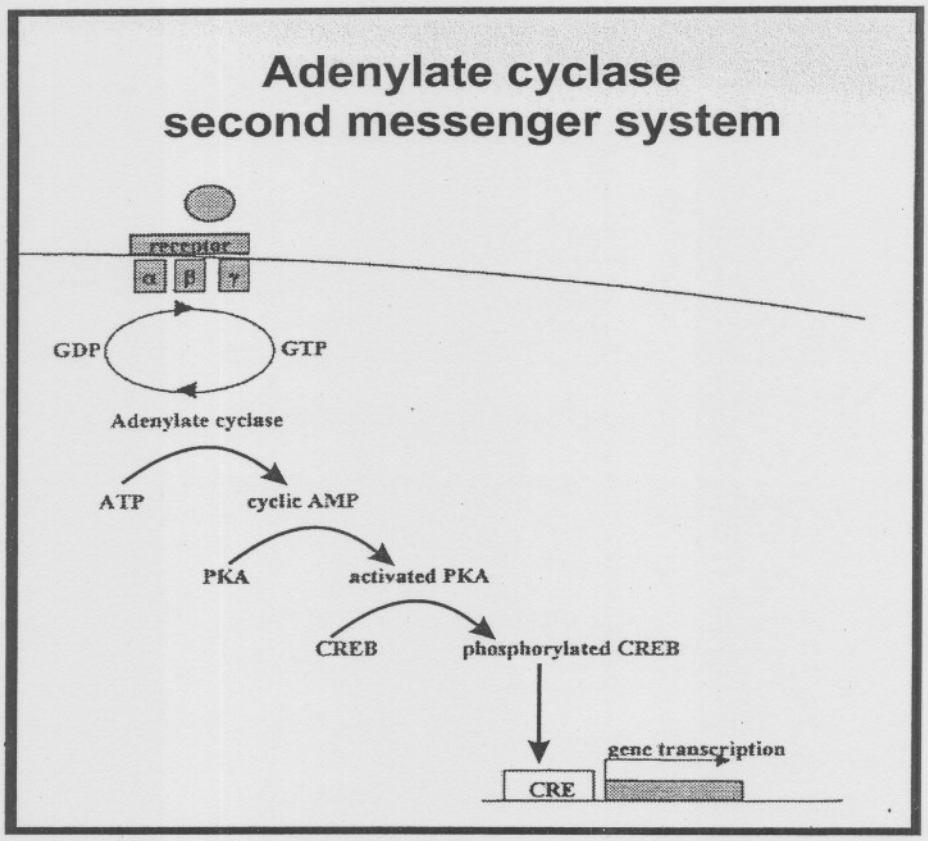


Figure 2.5: Adenylate cyclase second messenger system (After Gould & Manji, 2002).

CREB regulates the genes responsible for the expression of neurotransmitter synthetic enzymes (e.g., tyrosine hydroxylase), proteins involved in programmed cell death/neurogenesis (e.g., B-cell lymphoma protein-2; bcl-2) and for numerous neuropeptides (e.g., endogenous opioid peptides) (Manji *et al.*, 2000; Gould & Manji, 2002; Duman *et al.,* 2000). Enhanced transcriptional activity of phosphorylated CREB then induces the synthesis and release of BDNF, responsible for neurogenesis and increased synaptic connectivity (Mamounas *et al.,* 2000; Nestler *et al.,* 2002a). This action will regulate neuronal cell growth and suggests a neurotrophic effect for this pathway and possibly exploited by AD treatment (Nibuya *et al.,* 1996).

2.4.1.2 Phosphoinositide signaling pathway

A second well-characterized G protein-linked signal transduction pathway is the phosphoinositide-signaling pathway (Figure 2.6), which involves the breakdown of a cell membrane component, PIP_2, and its metabolism to various intracellular second messengers (Majerus, 1992).

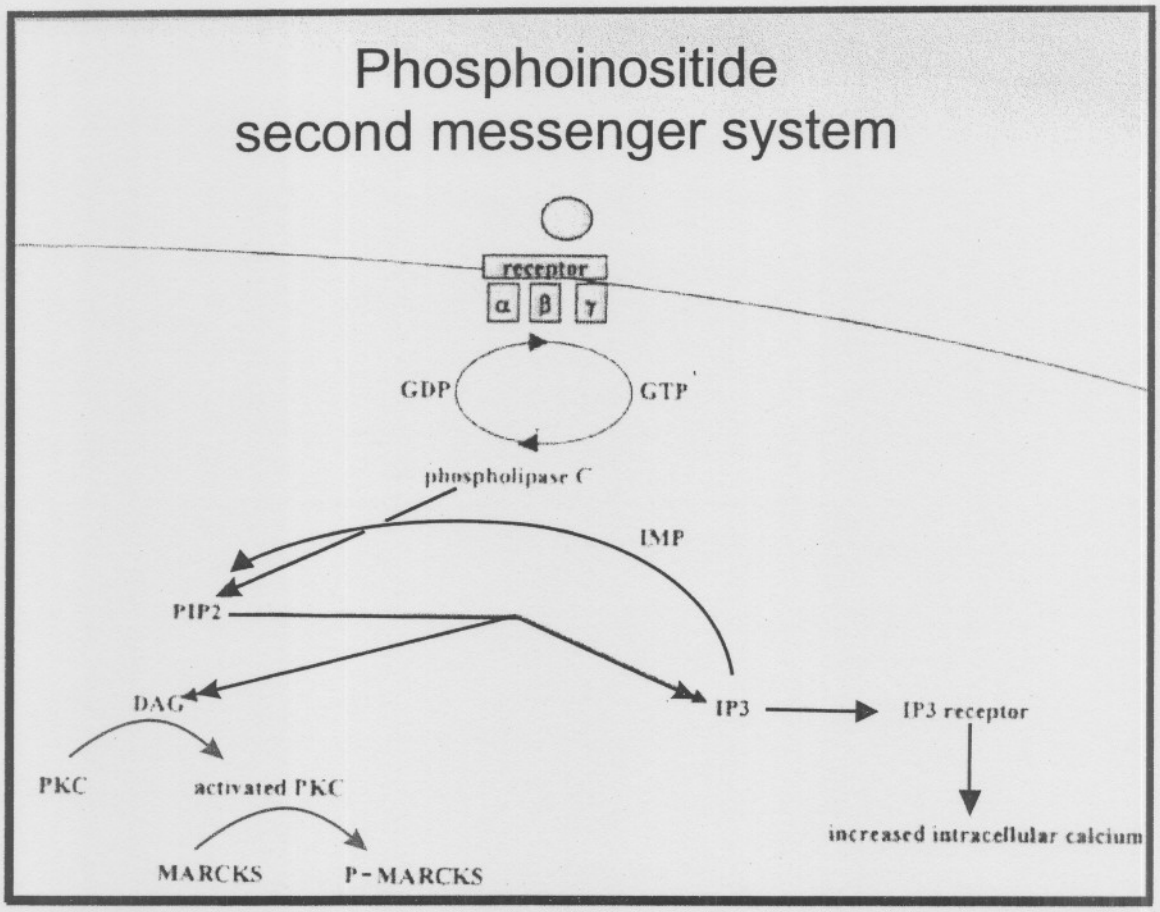


Figure 2.6: Phosphoinositide second messenger system (see text below) (After Gould & Manji, 2002).

Following binding of a ligand to its extracellular receptor, GTP binding induces hydrolysis of PIP_2 to form diacylglycerol (DAG) and inositol-1, 4, 5-triphosphate (IP_3). The hydrolysis of PIP_2 to DAG and IP_3 is under the control of phospholipase C. These products act as second messengers within two separate second messenger cascades. DAG activates a phosphorylating enzyme, protein kinase C (PKC), an enzyme that has both direct effects on cellular systems and is involved in phosphorylating other proteins in the cell. PKC has many growth regulating properties (Kanashiro & Khalil, 1998) as well as modulates many other cellular processes including secretion, exocytosis, gene expression, modulation of ion conductance, cell proliferation and downregulation of extracellular receptors (Nishizuka, 1992).

The second product of PIP_2 breakdown, IP_3, binds to the IP_3 receptor that functions to regulate calcium release in the cell. Increased IP_3 levels, concomitant with an increase of IP_3 binding to the IP_3 receptor, cause a release of intracellular calcium from the endoplasmic reticulum and the activation of calmodulin (Ca^{2+} -receptor protein calmodulin or CaM). Calmodulin then activates calmodulin-dependent protein kinases (CaM-Ks), leading to activation of ion channels, signaling

molecules, proteins regulating apoptosis, scaffolding proteins and transcription factors (Sodering, 2000).

Because of a convergence of these two signaling pathways, drugs affecting different signaling pathways may eventually produce the same final effect

2.5 Addressing Compliance, remittance and relapse of depression

The natural history of depression indicates that this is a life-long illness that is likely to relapse within several months of an index episode, especially if untreated, under-treated or if antidepressants are discontinued, and is prone to multiple recurrences that are possibly preventable by long-term antidepressant treatment (Stahl, 2000). Recurrence and relapse rates may be as high as 80% (Kupfer, 1991), treatment failures are common (40% to 60%), and as many as 20% of patients remain inadequately treated (Thase, 2002). Appropriate discontinuation would be that which is based on an informed decision by a clinician following thorough examination of the patient's mental state, response to the medication, and duration of treatment (Keller *et al.*, 2002). Patients with a first episode of depression should be treated for 6 to 12 months after which the antidepressant treatment is slowly tapered. Patients with more than 3 episodes of major depression, or those with two severe episodes, are candidates for ongoing maintenance therapy (Kelsey & Nemeroff, 1998). Factors such as advancing age, early age of onset, increased number of prior episodes, emergent rapid cycling, severity of prior episodes, and history of poor response to treatment should also be considered before a decision is made to begin discontinuation (Greden, 1993).

Because the profound effects that inappropriate discontinuation have on the long-term outcome of depression, and that poor compliance begets more episodes it is correlated to hippocampal shrinkage (MacQueen *et al.*, 2003), and it is imperative that this topic be addressed. Moreover, AD discontinuation and the neurobiology thereof, is the subject of this dissertation.

Among patients taking any kind of antidepressant, 28% have been found to discontinue treatment within the first month and 44% discontinued within 3 months of initiation of therapy. Among the understandable reasons for premature AD discontinuation is the poor tolerability of certain agents, although the introduction of novel antidepressants has diminished the relevance of this issue (Anderson, 2000).

More important, premature discontinuation may be a product of a lack of knowledge of the neurobiological mechanisms mediating untreated depression and antidepressant withdrawal and of their implications for the course and prognosis of depression (Harvey *et al.*, 2003). Studies in clinical settings (Masand, 2003), suggested that the reasons for nonadherence vary, but may be grouped into 3 categories as shown in Table 2.1:

Physician-Specific Issues	**Patient-Specific Issues**	**Medication-Specific Issues**
Poor communication with patient	Poor motivation; dislike taking medication; stigma associated with medication	Adverse events
Prescribed treatment does not meet needs of patient	No perceived symptom relief	Delayed onset of action
Suboptimal dosage	Premature conclusion that symptoms have fully resolved	Complicated dosing or titration schedule
Inadequate duration of therapy	Weak social support network	No effect on comorbid symptoms
	Cost of medication	

Table 2.1: Factors contributing to treatment discontinuation (After Masand, 2003)

The goals of AD treatment are full remission of all symptoms (defined as resolution of symptoms for at least 4 to 6 months) and prevention of recurrence (Masand, 2003). This will lead to a symptom-free state and a return to premorbid levels of functioning (Bakish, 2001). Response or partial remission refers to a clinically significant improvement; full remission occurs when symptoms have decreased to the extent that a reduction in intensity of treatment might be attempted; and recovery takes place when remission has lasted for a predefined period and discontinuation of treatment is being considered (Figure 2.7). This distinction between relapse and recurrence assumes that symptomatic improvement occurs before resolution of the underlying pathophysiology and that the risk of relapse decreases as the pathophysiology underlying the disorder resolves (Frank *et al.*, 1991).

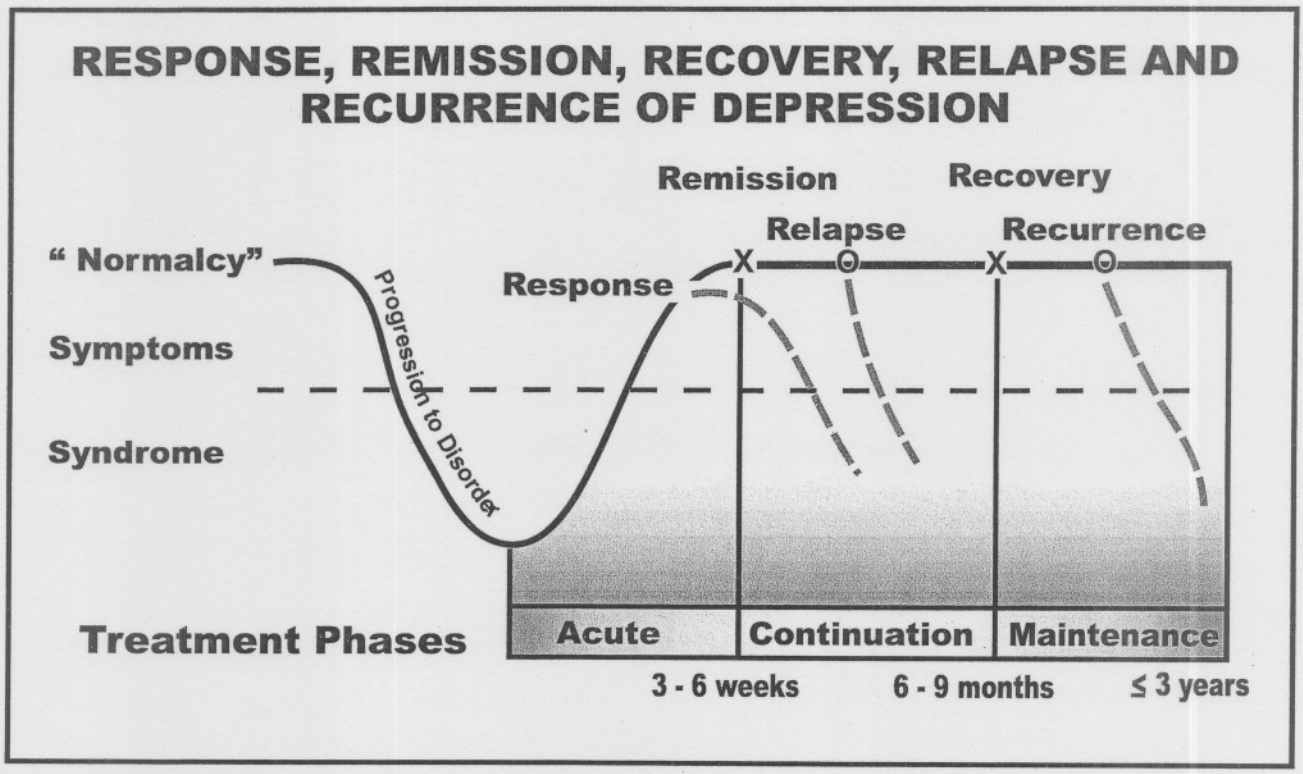


Figure 2.7: Response, remission, recovery, relapse and recurrence of depression. When depression returns before there is full remission of symptoms or within the first several months following remission of symptoms, it is called relapse. When depression returns after a patient has recovered, it is called recurrence (After Thase, 1990).

Compliance is the extent to which a person's behavior in terms of taking medication, following diets, or executing lifestyle changes coincides with medical or health advice (Haynes, 1979). Faulty compliance with medication may include errors of purpose, timing of dosage as well as total or partial omission, or use of inadvertent combinations. The outcome of compliance can be defined in categorical statements such as 'good' (75% to 100% intake), 'fair' (25% to 75% intake) or 'poor' (less than 25% intake). Hyper-compliance is defined as a more than 100% intake, often in the belief that this will accelerate the onset of action or will enhance the efficacy of the drug (Demyttenaere, 1997). In depressive patients, compliance was shown to be 68% after 3 weeks of treatment, but this percentage decreased after 6, 9 and 12 weeks to 63%, 50% and 40% respectively (Myers & Branthwaite, 1992). A cognitive deficit, helplessness, poor motivation and withdrawal all lead to forgetfulness and passive non-compliance (Gitlin *et al.*; 1989). Moreover, it is not always clear whether non-compliance precedes recurrence or whether non-compliance is a first 'symptom' of recurrence. Indeed, although maintenance treatment with antidepressants is an effective way of preventing recurrence, it does not provide a 100% guarantee of non-recurrence (Demyttenaere, 1997). Communication, education, planning of

dosing strategies, and visit scheduling are the principal strategies aimed at improving compliance to antidepressant therapy (Cramer, 1991).

2.6 The Neurobiology of antidepressant discontinuation and its clinical relevance

There are increasing awareness of the possibility that certain mood and anxiety disorders are associated with neuronal atrophy and cell death. Postmortem studies on depressed patients show atrophy and cell loss in cerebral cortical areas (Rajkowska, 2000b). Hippocampal volume reduction is not present in first episode patients but becomes increasingly evident in those with multiple episodes (MacQueen *et al,* 2003). It is also of interest that hippocampal shrinkage may be correlated with the total lifetime of depression but not with the age of the patient (Sheline *et al.*, 1999), suggesting that stress associated with recurrent depressive episodes may inflict cumulative hippocampal injury. Repeat episodes of depression are often of longer duration, more severe and less treatment-responsive, and similarly, greater number and longer duration of episodes of depression are associated with a higher risk of relapse and recurrence (Judd *et al.,* 1998).

The kindling theory of recurrent affective illness (Post & Weiss, 1998) has been viewed as having particular relevance for how synapses react to environmental and neurochemical stressors and how these can influence clinical response and outcome (Harvey *et al.*, 2003). Increased susceptibility to depression with subsequent episodes, or a 'kindled state', is distinctly linked to the number of prior episodes, the presence of stressful life events, and high genetic risk (Kendler et al, 2001). The primary neural substrate of kindling involves NMDA receptor activation (Post & Weiss, 1998), while GABA pathways exert essentially a permissive role on the kindling action of glutamate.

Using a learned helplessness paradigm, Harvey & collogues (2002) showed convincingly that sudden AD discontinuation after chronic treatment is associated with a profound increase in stress responsivity, as well as significant perturbations in NMDA receptors and GABA levels in the rat hippocampus. These behavioral and neurochemical changes were also blocked by the NMDA antagonist, dizocilpine (MK801). This involvement of GABA, glutamate, and increasing stress reactivity, have particular relevance in the neurobiology underlying the treatment and prognosis of depressive illness (Table 2.2) (Harvey *et al.*, 2003). Table 2.2 depicts that withdrawal of IMI causes an increase in stress responsiveness with an increase in GABA and NMDA receptor density, these changes were reversed with the NMDA antagonist MK801.

	Swim stress	GABA	NMDA B_{max}
Chronic IMI (no withdrawal)	↓a	↓*	↓a
Chronic IMI + withdrawal (saline)	↑#	↑#	↑b
Chronic IMI + withdrawal (MK801)	↓*	↓a	↓ac

Table 2.2: Changes observed after abrupt antidepressant withdrawal in rodents. [a]$p<0.05$ vs control; [b]$p<0.05$ vs chronic IMI; [c]$p<0.05$ vs chronic IMI + withdrawal; all other arrows indicate numerical changes compared to saline control* or chronic IMI (Harvey *et al.*, 2002).

Inappropriate antidepressant withdrawal-associated stress has been demonstrated in humans (Michelson *et al.*, 2000). Stress and its action on glutamate can have profound effects on synaptic plasticity and may change neuronal and synaptic structure in such a way that prior antidepressant response is altered (Figure 2.8). This response could lay the foundation for relapse, requirement for higher dose, and possibly treatment resistance (Harvey *et al*, 2003). Figure 2.8 shows the effect of antidepressant discontinuation and overt glutamate on synaptic plasticity as well as the neurodegenerative consequences.

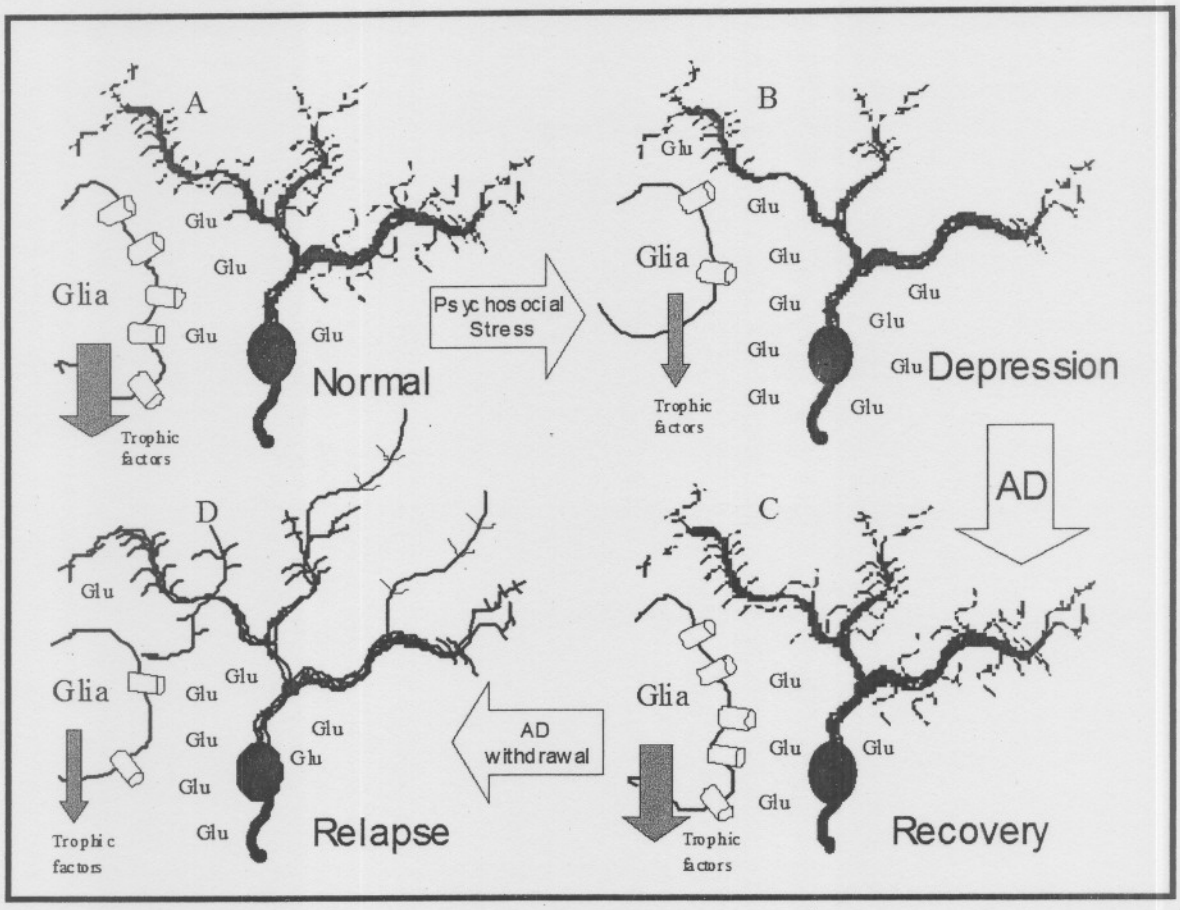


Figure 2.8: Psychosocial stressors provoke depressive illness by impacting negatively on neurogenesis and cellular resilience through attenuated expression of essential neurotrophins leading to the gradual loss of glial cells and reduced number of glial glutamate transporters (A-B). This leads to buildup of synaptic glutamate, the development of depression, and neurodegenerative consequences (B). AD treatment will reverse this pathology (C). Inappropriate AD withdrawal will result in conditions of overt glutamate activity (D). An increase in neuronal glutamate NMDA receptor density and the loss of neurotrophic cover, will underlie synaptic and neuronal remodeling that may be different to that of the previous episode (D). The result is a recurrent episode of depression that presents differently to the previous episode that may require higher dosage or alternative treatment (After Harvey *et al.*, 2003).

Tolerance and refractoriness is well documented with the antimanic drug, lithium (Harvey, 1996), and it is interesting to note that its propensity to induce treatment refractoriness is paralleled by its ability to potentiate excitatory glutamatergic response (Harvey *et al.*, 1994), and to augment glutamatergic-mediated NO synthesis in rat cerebral cortex (Harvey *et al.*, 1994). It is also noteworthy that depression is associated with altered dynamics of NO synthesis and NOS activity (Suzuki *et al.*, 2001) while ADs of different classes all inhibit NOS (Wegener *et al.*, 2003). It has been proposed that this ability of lithium to potentiate glutamatergic-NO-cGMP responses may underlie the cellular mechanisms leading to recurrence and refractoriness

(Harvey, 1996). This is noteworthy, especially since NO-mediated increases in cGMP can induce the expression of select genes that regulate neuronal plasticity (Arancio *et al.,* 1995), a process that is an essential component of neuronal adaptability (Leonard, 1997).

Many patients report drug-related side effects within the first days of AD treatment, including anxiety, irritability, nausea, headache, and other symptoms that are unrelated to clinical response (Harvey, 1997), while patients that abruptly withdraw their chronic antidepressant treatment also report an array of "withdrawal effects" that are distinct from relapse of clinical depression (see section 2.5). The symptoms that follow treatment discontinuation may be attributed to specific mechanisms evoked by withdrawal of the drug, since they are rapidly resolved when treatment is reinstated (Harvey, 1997; Schatzberg *et al.,* 1997). Some of the characteristic physical and psychological symptoms include dizziness, nausea, gastro-intestinal distress, headache, anxiety, and gait instability (Schatzberg *et al.,* 1997). Just as treatment initiation-related side effects may be precursors of subcellular neuronal changes involved in the clinical response that occur some time later, one may ask if there are also prominent long-term effects of AD withdrawal on neuronal function (Harvey *et al.*, 2003). Moreover, can these events represent neuronal events that are in some ways opposite to those seen in association with clinical response and so negatively influence the long-term treatment outcome of depression?

Although the severity of withdrawal symptoms may vary with the type of antidepressant and between patients, not enough emphasis is placed on the biochemical processes that may cause further neuronal dysfunction resulting in the phenomena of relapse, recurrence and treatment refractoriness (Harvey *et al*, 2003). Clinicians need to emphasize that premature antidepressant discontinuation is associated with relapse, that subsequent episodes may be more severe in a number of aspects, and that increased number and duration of depression episodes in turn becomes a poor prognostic indicator (Keller, 2001). They should also encourage patients to withdraw antidepressants, particularly those with short half-lives, only gradually and only after consultation with a psychiatrist. It is however to be emphasized that the decision to discontinue antidepressants should be made judiciously and on an individualized basis (Harvey *et al.*, 2003).

The aim of this study is to reveal more of the neurobiological underpinnings of AD discontinuation, and to relate said changes to the reported positive association between poor compliance and treatment resistance and relapse.

Chapter 3
ANIMAL MODELS OF DEPRESSION

3.1 Introduction

Animal models of depression can be used to dynamically study biological parameters that have been implicated in the expression of depression in humans. A model is defined as an experimental preparation (in this case an animal) developed for the purpose of studying a condition in the same or different species (Yadid, 1998). A depressive-like syndrome in animals is characterized by anhedonia, anorexia, body weight loss, decreased locomotor activity, and reduced exploratory and social behavior (Machado-Vieira *et al.*, 2004). Thus, animal models of depression are essential, both for screening new antidepressants and for studying the mechanism of action underlying the neurobiological effects of antidepressant treatments (Gambarana *et al.,* 2001).

3.2 Criteria for Evaluating Animal Models of Depression

Although an animal model of depression must respond appropriately to antidepressant drugs, the converse does not apply: the fact that behavior is sensitive to antidepressants is not in itself sufficient to define the procedure as an animal model of depression. Antidepressant drugs have side effects unrelated to their clinical action that may have irrelevant behavioral consequences (Willner, 1995). Antidepressants also have clinical actions outside depression, including a spectrum of anxiolytic effects in at least some animal models of anxiety, following chronic treatment. For these and other reasons, it is important at the outset to consider the criteria that a valid animal model of depression should meet (Willner, 1995).

Animal models of psychiatric conditions can be assessed on the basis of how well they fulfill three major criteria: face validity (how well the model resembles the psychiatric condition), construct validity (how well the model is consistent with theoretical rationale) and predictive validity (how well the model responds favorable to the same drugs as humans with the condition) (Yadid *et al.,* 2000). Thus, the animal model must be produced by factors similar to those factors that result in the human disorder, it must resemble human disorder in symptoms and manifestations, it must have an underlying pathophysiological basis similar to that of the

human disorder and it must respond to therapeutic treatments in the same way or similar, as the human disorder (Weiss & Kilts, 1995).

A fundamental aspect associated with the validity of an animal model of depression is its ability to mimic major depression in humans or to be responsive to pharmacologic treatments. Some of these models can mimic symptoms (despair), evaluate behavioral and development theories (cognitive theory of learned helplessness), or allow the study of neuropathological mechanisms (cerebrospinal fluid metabolites, genetic, pharmacological, and neurotransmitter), mostly to evaluate the effectiveness of new drugs in preclinical pharmacological trails.

The following models have been proposed to fulfill in part or totally all validity criteria (Machado-Vieira *et al.,* 2004) of which some will be discussed in more detail in this chapter:

- olfactory bulbectomy,
- predatory behavior (Muricide),
- kindling,
- isolation-induced hyperactivity,
- 5-hydroxytryptophan (5-HTP)-induced behavioral depression,
- chronic stress,
- forced-swimming test,
- learned helplessness,
- separation models,
- swim low-active (SwLo) line in rats,
- Flinders sensitive line (FSL) in rats,
- intracranial self-stimulation,
- DOPA potentiation, and
- behavioral despair.

3.3 Types of Animal Models

Animal models can be divided into two categories, namely animal assay models and homologous models (Weiss & Klits, 1995).

3.3.1 Animal Assay models

Models in this category use behavioral and/or physiological responses of animals to assess processes, usually physiological processes, that already-existing evidence indicates are important in a disorder. These models help to determine how various compounds affect physiological processes that are important in pathology; animal assay models make it possible to rapidly assess the potential usefulness of drug compounds and, in some cases, to discover new drugs that affect an important physiological process. Thus, the major use of assay animal models' is in drug screening and the development of new drugs (Weiss & Klits, 1995). In evaluating animal assays, it should be kept in mind that such assays use responses of animals, whether behavioral or physiological, in a manner that is not different from an in vitro assay; that is, the response of the animal even if it is a behavioral one, is no more than a "read-out" of drug action (Weiss & Klits, 1995). Examples of animal assay models are discussed briefly below.

3.3.1.1 Muricide

Some rats will spontaneously kill mice when the mice are presented to them. The acute administration of antidepressants (Weiss & Klits, 1995) such as TCAs, MAOIs and amphetamines block the tendency of rats to kill mice. It has been proposed that 5-HT acts as an inhibitory modulator of predatory aggression. This model can be assessed only for predictive validity as an animal model of depression due to the fact that the mouse-killing behavior in rats can also be selectively blocked by administration of antidepressants. However, this model cannot be assessed for face validity because muricidal behavior in rats does not present homology when compared with human depression symptoms (Machado-Vieira *et al.,* 2004).

3.3.1.2 Yohimbine lethality

The drug yohimbine can be administered to mice in dosages that are lethal. However, administration of sublethal doses is lethal when accompanied by the co-administration of TCAs and MAOs. Although atypical antidepressants also produce this effect, whereas antipsychotics,

tranquilizers and ECT do not (Weiss & Klits, 1995), the fact that anticholinergic and antihistaminics also act similarly is essentially indicative of poor predictive validity.

3.3.1.3 Amphetamine potentiation

Amphetamine produces a number of effects in rats, including hyperthermia, increased locomotor activity, and improved shuttle box avoidance performance. Acute administration of antidepressants like TCAs and MAOIs has been found to potentiate these responses. The ability of a drug to potentiate the effects of amphetamine primarily detects an ability to potentiate catecholaminergic neurotransmission (Weiss & Klits, 1995), and thus have some construct validity.

3.3.1.4 Kindling

Kindling is the progressive induction of seizure activity by repeated and appropriately spaced, sub threshold stimuli, up to a stage of spontaneous seizures. Electrical stimulation of certain brain regions apparently sensitizes the brain region to initiate activity so that, following such stimulation, considerably less electrical stimulation delivered to the brain region is required to initiate seizure activity than is the case when electrical stimulation of that brain region has not been given previously (Weiss & Klits, 1995). TCAs and ECT reduce the likelihood of development of seizures in "kindled" animals, but not MAOIs. Kindling models provide a framework that may contribute to the understanding of important aspects of the longitudinal course of recurrent affective disorders. These include illness progression and cyclicity, sensitization to environmental stressors, differential pharmacological responsivity at various stages of illness evolution, and the issues of tolerance and treatment-discontinuation-induced refractoriness (Kupka, 2001).

3.3.1.5 Circadian rhythm readjustment

Rats are normally active during the dark period of the day and are less active during the light period. The rapidity with which animals will shift their activity when their light-dark periods are switched is facilitated by imipramine or pargyline (Weiss & Klits, 1995). A disturbance in sleep is a typical symptom of depression and is possibly related to changes in circadian rhythm. SAD also suggests a disturbance in circadian rhythm in depression, thus indicating some predictive and face validity for this model.

3.3.1.6 Olfactory bulbectomy

The olfactory bulbectomized rat presents partial face, construct, and predictive validities as an animal model of depression. This model has been proposed to reproduce several biochemical and functional changes associated with human depression. Bilateral olfactory bulbectomy may alter body weight, sucrose preference levels, and open-field activity levels. This model induces behavioral changes like hyperactivity in the open-field, enhanced nocturnal hyperactivity, deficits in memory as passive avoidance behavior, and changes in food-motivated and conditioned taste aversion behavior (Machado-Vieira *et al.,* 2004). Changes in the serotonergic, cholinergic, GABAergic, noradrenergic, and glutamatergic systems, as well as an enhanced nocturnal secretion of corticosterone suppressed by dexamethasone are associated with olfactory bulbectomy (Kelly *et al.,* 1997). Specifically, it has been proposed as a model of hyposerotonergic depression (Connor *et al.,* 1999). Behavioral indicators of antidepressant activity in this model include attenuation of hyperactivity in the open field (Machado-Vieira *et al.,* 2004). Deficits in the avoidance behavior, particularly passive avoidance, are reversed by antidepressants such as TCAs (Weiss & Klits, 1995).

3.3.1.7 Differential operant responding for low reinforcement

The differential reinforcement of low-rate 72-s (DRL-72s) schedule, in which rats must withhold a response for at least 72 seconds to obtain a reward, is an attractive procedure for the characterization of potential antidepressant agents (Dekeyne *et al.*, 2002). Animals are taught to press a lever to receive food as a reward. After the response, the animal is placed on a "differential reinforcement of low rate 72 second" schedule, which requires the animal to wait 72 seconds between responses to obtain food as reward. The timing sequence is reset, when the animal makes a response before the 72 second period is expired, so that the animal must wait an additional 72 seconds to receive the reward (Weiss & Klits, 1995). Several antidepressants have been shown to improve efficiency in this model, either by decreasing response rates and/or by increasing reinforcement rates (Dekeyne *et al.,* 2002). ECT, TCAs, MAOIs, SSRIs and atypical antidepressants all improve the ability of animals to inhibit responding and then obtain reward (Weiss & Klits, 1995).

3.3.1.8 Isolation-induced hyperactivity

When rats are housed individually as soon as they are weaned, they are found to be hyperactive as adults when tested in a novel environment (Weiss & Klits, 1995). Isolation results in formation of some behavioral patterns usually absent in normally reared rats (Machado-Vieira

et al., 2004). Social deprivation in animals has been associated with changes in the intensity of locomotor activity, which has been shown to induce hyperactive behavior. Whereas effective antidepressant drugs limit hyperactivity in this model, other psychoactive drugs do not present the same effect, providing reliable predictive validity.

3.3.2 Homologous Animal Models

These models endeavor to create the human disorder in animals. In these models, treatments are administered to animals in the hope of causing the animal to resemble individuals who are afflicted with the disorder (Weiss & Klits, 1995). It is possible to study the model as a means to discover the underlying cause in the disorder (i.e., the physiological defect). Because a perfected homologous model reproduces the disorder without making any assumptions regarding its physiological basis, researchers can test any potential treatment on the animal model to see if it can reverse the disorder (Weiss & Klits, 1995).

3.3.2.1 Reserpine-induced reduction of motor activity

Reserpine and reserpine-like compounds inactivate the ability of synaptic vesicles to retain monoamines. As a consequence of this action, release of monoamines follows administration of these drugs, and there is then a long-term reduction in monoamine stores, resulting in depletion of dopamine, noradrenaline, adrenaline, and serotonin in the brain and periphery (Weiss & Klits, 1995). Vernier and colleagues (1962) reported that imipramine would antagonize reserpine-induced sedation, which was quantified largely by decreased motor activity. In addition to imipramine, other TCAs also antagonize reserpine induced depression of motor activity. MAOIs have a similar effect. The "reserpinized rodent" constitutes one of the earliest animal models of depression. The observation that reserpine suppresses motor activity was apparently a key factor in suggesting the catecholamine hypothesis of depression (Weiss & Klits, 1995). Thus, this model has reliable face, construct and predictive validity

3.3.2.2 5-HTP-induced behavioral depression

5-HTP-induced depression has been proposed to be due to an excess of free 5-HT acting at postsynaptic receptors, which is called the hypersensitive postsynaptic 5-HT receptor theory of depression (Nagayama *et al.,* 1981). The reduction in active behavior seen following increased 5-HT release due to administration of 5-HTP is blocked by TCAs as well as non-TCAs (i.e., mianserin, iprindole) (Nagayama *et al.,* 1981). However, two features of the model that do not

reproduce that seen in depression include the fact that acute administration of antidepressants is effective in blocking the behavioral depression, and that drugs that block 5-HT reuptake increased 5-HTP-induced behavioral depression markedly (Nagayama et al., 1980), so that this class of antidepressants exacerbates rather than prevents depression in this model, indicating poor face and predictive validity.

3.3.2.3 Forced Swim Test (FST)

Because this study has made extensive use of this model, it is discussed in more detail.

The forced swim test, also known as the Porsolt test, is the most widely used animal model in depression research, more specifically as a screen for antidepressant activity (Porsolt, 2000). The introduction of "stress" during forced swimming has been described as the means whereby the FST is sensitive to the effects of antidepressant drugs. The test is based on a "learned helplessness" model. In clinical depression, this trait is evident in symptoms such as decreased volition (drive), apathy, lack of self-worth and esteem and locomotor retardation. Initial exposure of the animal to the procedure produces consequences similar to these, but is measured as a lack of responsiveness to the stressors which, in the current model, is a gradual increased immobility in response to an extended period of swim stress. Effective antidepressants counter the "stress-induced" immobility response in the test. The method has good face, construct and predictive validity. However, a possible drawn back is that both acute and chronic AD administration is effective, contrary to that seen clinically. Nevertheless, it is widely used in pharmacological research to access the antidepressant potential of a given drug.

A typical procedure that has been used is to place the animals individually in inescapable Perspex cylinders containing water. During a preconditioning period, the animals are placed into the cylinders and observed for 15 minutes. After an initial phase of vigorous activity, the animal adopts a characteristic immobile posture (Redrobe & Bourin, 1999). The animal is judged to be immobile when the animal remains afloat making only necessary movements to keep its head above the water (Redrobe & Bourin, 1999). A rat in the characteristic immobility posture during a forced swim test is illustrated in Figure 3.1(right). The following day, the animal is returned to the water tank for 5 minutes where immobility is timed. Effective antidepressants cause the rats to show less immobility in the second exposure than is shown by the vehicle treated animals (Porsolt *et al.*, 1978).

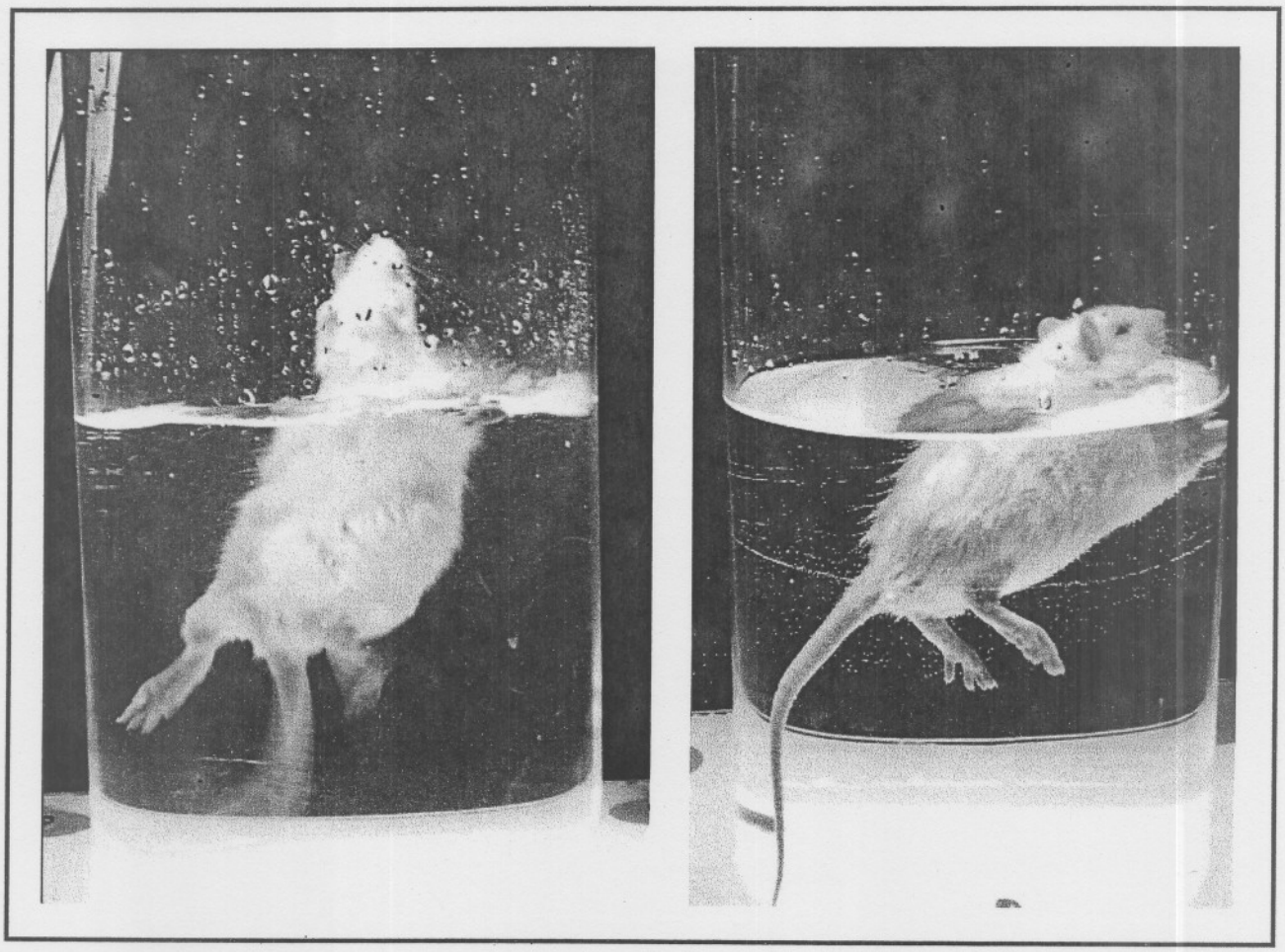


Figure 3.1: Rat showing the struggling behavior (left) and the characteristic immobility posture, making only necessary movements to keep its head above (right), during the forced swimming test

A variant of the forced swim test, used in mice, is the *tail suspension test*. Here mice are suspended by their tails and the time it takes for the animal to become immobile (to hang passively upside down) is measured (Nestler *et al.,* 2002). The major advantage of the forced swim test (or tail suspension test) is its relatively high throughput and ease of use. The test also provides a useful model in which to study the neurobiological and genetic mechanisms underlying stress and antidepressant responses (Lucki, 2001; Porsolt, 2000). It detects a wide range of antidepressant treatments, including al the TCA's, MAOIs, atypical antidepressants and ECT (Redrobe & Bourin, 1999).

Because antidepressants may increase locomotor activity as part of their overall CNS effects, a decrease in immobility time as measured in the FST in response to the drug may reflect an increase in motor activity. Thus, an improved response to the FST may not necessarily reflect an improvement in psychological response to environmental adversity, such as increase in drive

(volition) and diminished "helplessness", the main determinants measured in the FST. To avoid this confounding variable, total horizontal and vertical activity is measured to ensure that changes in swim motivation are based only on an antidepressant response, and not due to an indirect effect of the drug on locomotor activity.

3.3.2.4 Clonidine withdrawal

Acute administration of clonidine causes rats to be inactive in the swim test, but it is also possible to produce long-lasting inactivity in this test in drug-free animals (Weiss & Klits, 1995). When clonidine is administrated to rats for 2 weeks and then abruptly withdrawn, animals that are drug-free, show a depression of activity in the swim test that lasts for several weeks. The long-lasting nature of this reduction in motor activity permits examination of the effects of long-term treatment regimens. Only DMI, however, has been tested to date and further studies of this model have, to date, not been conducted (Weiss & Klits, 1995).

3.3.2.5 Neonatal clomipramine

In this model, rat pups are injected with clomipramine twice daily on postnatal days 8 through 21. When tested at 90+ days of age, these animals show decreased sexual activity, intracranial self-stimulation, aggressive behavior, and motor hyperactivity in a novel situation (Hartley *et al.*, 1990). Clomipramine-injected rats also show changes in sleep behavior that have been associated with depression, including reduced latency to enter REM sleep following sleep onset and frequent onset of REM periods. One drawback of this model appears to be the hyperactivity evidenced by the animals in that patients manifesting "endogenous" depression are likely to be the most seriously affected who will show psychomotor retardation rather than hyperactivity (Weiss & Klits, 1995). Testing of treatment efficacy on this model has been very limited. According to Vogel, imipramine treatment for 4 days reduced locomotor hyperactivity of the animals treated neonatally with clomipramine (Vogel *et al.*, 1990)

3.3.2.6 Lesioning of the dorsomedial amygdala in dogs

When the dorsomedial amygdala is lesioned eletrolytically in dogs, one of the most dramatic syndromes resembling severe depression is produced. Fonberg has reported that these animals display lethargy, negativism, reluctance to eat, and, to the extent that this can be judged in a dog, saddened facial expression (Fonberg, 1972). The effect of antidepressant medication on this syndrome has not yet been reported. Despite its dramatic appearance, the

model has been little studied, and perhaps is likely to remain so given the problems of using dogs as subjects (Weiss & Klits, 1995).

3.3.2.7 Isolation / separation-induced depression in monkeys

Behavioral responses that appear similar to those seen in severe depression have been elicted by separating young monkeys from their mothers and normal social setting or from their juvenile peers. Following separation, animals initially display high activity accompanied by much vocalization; this is labeled the stage of "agitation" or "protest" and usually lasts 24-36 hours. Following this, the behavior pattern changes markedly, with the animal then showing decreased activity, huddling and self-clasping, dejected and saddened facial expression, and generally decreased activity and exploration. Depression-related behavior are diminished by long-term administration of imipramine, thus paralleling what is seen with antidepressant administration in humans, and symptoms reappear if the drug is withheld (Suomi *et al.*, 1978).

3.3.2.8 Separation of Siberian hamsters

Siberian dwarf hamsters form stable male-female mating pairs that remain together after pups are born, with the male participating in rearing activities (Weiss & Klits, 1995). Crawley developed a model, in which the pairs were separated, 3-4 weeks after they had form mating pairs and were housed in individual cages (Crawley, 1984). During the period after separation, the male hamsters showed decreased daily running-wheel activity, decreased activity when tested in an open field, increased body weight, and decreased social interaction when an unfamiliar animal of the opposite sex was introduced into the cage. Separated females showed a smaller decrease in running-wheel activity but no change in the other measures. All of these changes could be reversed by reuniting the separated pair (Weiss & Klits, 1995). Administration of imipramine for 2 weeks following separation eliminated a number of activity-related changes shown by male hamsters in the open field, but did not eliminate the increase in body weight or the decrease in social interaction with an unfamiliar animal of the other sex (Weiss & Klits, 1995).

3.3.2.9 Exhaustion stress

Female rats are exposed to three sessions of forced running, separated with 24-hour rest period, in an activity wheel until the animals are exhausted. Spontaneous motor activity in the rats is markedly depressed for several weeks. Motor activity returns to normal by daily injections of imipramine; a therapeutic response is seen only after the drug was administered for longer

than 10 days (Weiss & Klits, 1995). The other characteristic described for this model is that a marked increase in intensity of histochemical fluorescence of noradrenergic cell bodies in the brain accompanies the exhaustion-induced decrease in motor activity, thereby indicating that activation of noradrenergic neurons in the brain appears to be increased by the exhaustion-stress procedure (Weiss & Klits, 1995).

3.3.2.10 Chronic mild stress-induced anhedonia

The chronic mild stress model was developed to mimic anhedonia, a fundamental symptom of depressive disorder (Willner, 1997). In rats, anhedonia is assessed by measuring the intake of a sweet solution (Yadid *et al.,* 2000). According to this model, chronically stressed rats' present behavioral and neuroendocrine abnormalities that resemble some of the most prominent features of clinical depression. In contrast to acute stress exposure, rats submitted to a regimen of chronic, mild and nontraumatic stressors, such as deprivation of food and water, overnight illumination, cage tilt, or change of cage-mate develop a decreased sensitivity to reward characterized as anhedonia (Machado-Vieira *et al.,* 2004). The exposure to stressful situations results in a decrease in open-field activity. That is reduced by TCAs, MAOIs, atypical antidepressants and ETC (Weiss & Kilts, 1995). The neurochemical effects in rats in the serotonergic, dopaminergic, and noradrenergic systems induced by the CMS paradigm and reversed by antidepressant administration strongly support the construct validity of this model (Machado-Vieira *et al.,* 2004).

3.3.2.11 Learned helplessness

Learned helplessness is a behavioral phenomenon in which animals are exposed to aversive stimuli under circumstances in which they cannot control or predict these stimuli (Seligman *et al.,* 1975). Exposure to such stimuli results in a long-lasting deficit in escape performance, which has been termed learned helplessness. It has been argued that the model has good face validity because there is similarity between the behavioral characteristics of learned helplessness of animals and signs of depression in humans (Willner, 1986). Animals with learned helplessness exhibit loss of appetite and weight, exhibit decreased locomotor activity (Wagner *et al.,* 1977), and perform poorly in both appetitively- and aversively-motivated tasks (Zacharko *et al.,* 1982).

These behavioral characteristics are considered to correspond to the loss of appetite and weight, psychomotor retardation, and anhedonia, demonstrated by depressed humans (D$M-IIR). The learned helplessness model appears to have good predictive validity for identifying potentially useful pharmacotherapies for depression in humans. The construct validity of the

learned helplessness model of depression rests on the following assumptions: that animals exposed to uncontrollable aversive events do become helpless, that a similar state is induced in people by uncontrollable aversive events, and that helplessness in people is the central symptom of depression (Yadid *et al.*, 2000).

3.3.3 Models Using Selective Breeding

The use of selective breeding procedures has attempted to correct the shortcomings of existing animal models and has set out to produce populations of animals that either have a higher likelihood of showing the appropriate characteristics than in a normal population of animals, or display characteristics of the disorder to a more pronounced extent than is seen in normal populations (Weiss & Kilts, 1995).

3.3.3.1 Swim low-active line rat

The swim low-active (SwLo) model integrates genetic components associated with a classical behavioral model of depression called "forced-swim." In the forced-swim model, observations have suggested that the amount of immobility during the forced- swimming test is directly associated with a depressive rat (see section 3.3.2.3). Sprague-Dawley (SPD) albino rats were selectively bred for low motor activity (little struggling time/much floating time) as a genetic model of depression (Weiss *et al.*, 1998). Dopaminergic function has shown to be reduced in SwLo rats. According to its pharmacological response, this model has been proposed to represent an animal model of atypical depression (West & Weiss, 1998).

3.3.3.2 Flinders sensitive line of rats

FSL rats resemble depression in humans, presenting both reduced locomotor activity and body weight, increased REMS, and learning difficulties (Yadid *et al.*, 2000). The FSL rats, which are selectively bred for increased responses and sensitivity to behavioral and hormonal effects of cholinergic (muscarinic) agonists, also present changes in circadian rhythms and thus, represent a cholinergic and circadian rhythm model of depression. This model also presents altered expression on neurotransmitter functions that have been implicated in depression and many behavioral similarities in response to antidepressants (Overstreet, 1993). Both FSL rats and depressed individuals have similar changes in serotonergic and dopaminergic systems as a result of chronic treatment with antidepressants. Thus, this model appears to have both face, construct and predictive validities (Yadid *et al.*, 2000).

Of all the above-described animal models of depression, the forced swim test is probably one of the most frequently used models. In the current study, this particular model of depression was chosen since it is simple to perform, quick and reliable. Since the FST is associated with the induction of a stress-response, it further appears to be the ideal model to evaluate the "anti-stress" effects of the applied treatment regimens, especially since stress is one of the most important premorbid events in depressive illness. Moreover, it also has relevance in mediating hippocampal histological and anatomical changes during depression and with antidepressant withdrawal (see section 2.6).

Chapter 4
THE NITRIC OXIDE PATHWAY

4.1 Introduction

The identification of nitric oxide (NO) as a gaseous, nonconventional neurotransmitter in the central nervous system has led to an explosion of studies aimed at the learning about the roles of NO, not only at a cellular level, but also in regulating the activity of specific physiological systems that are coordinated by the brain.

Although NO was first identified as the "endothelium derived relaxing factor" (EDRF) (Haung & Lo, 1998), it is now clear that this molecule serves as a major intercellular/intracellular messenger within and among the neurons of the peripheral and central nervous systems. NO is involved in various physiological processes including, among others, smooth muscle relaxation, neurotransmission, platelet aggregation, and host defense mechanisms (Krumenacker *et al.*, 2004). It is implicated in the regulation of various behavioral, cognitive and emotional processes, e.g. learning, aggression, locomotion, anxiety, and depression (Heiberg *et al.*, 2002). In addition, NO is also implicated in the pathology of several inflammatory disease states such as arthritis, myocarditis, colitis, nephritis, and other pathological conditions such as cancer, diabetes, and neurodegenerative diseases (Krumenacker *et al.*, 2004).

Many of the cellular actions of NO involve guanylate cyclase and production of the intracellular messenger cGMP. Because the NO/cGMP pathway is an important system for signal transduction in intra and intercellular regulation, and owing to the extensive distribution of this pathway, it is clear that inappropriate functioning of this pathway can lead to pathological states. The aim of this chapter is to present the physiological and pathological roles of NO, especially in the CNS, and in so doing consolidate its possible value as a pharmacological target in neuropsychiatric illness.

4.2 Synthesis and Metabolism of NO

Nitric oxide is synthesized in several types of cells, such as neurons, endothelial cells and macrophages by a family of three isoenzymes termed nitric oxide synthases (NOS) (Prast & Philippu, 2001). NOS are present in the brain, retina, adrenal medulla, in the myenteric plexus neurons of the intestine, in platelets, neutrophils and in macrophages (Rang *et al.*, 1999). NOS catalyses NO biosynthesis via a reaction involving the conversion of L -arginine to L -citrulline (Mayer & Hemmens, 1997). (Fig 4.1)

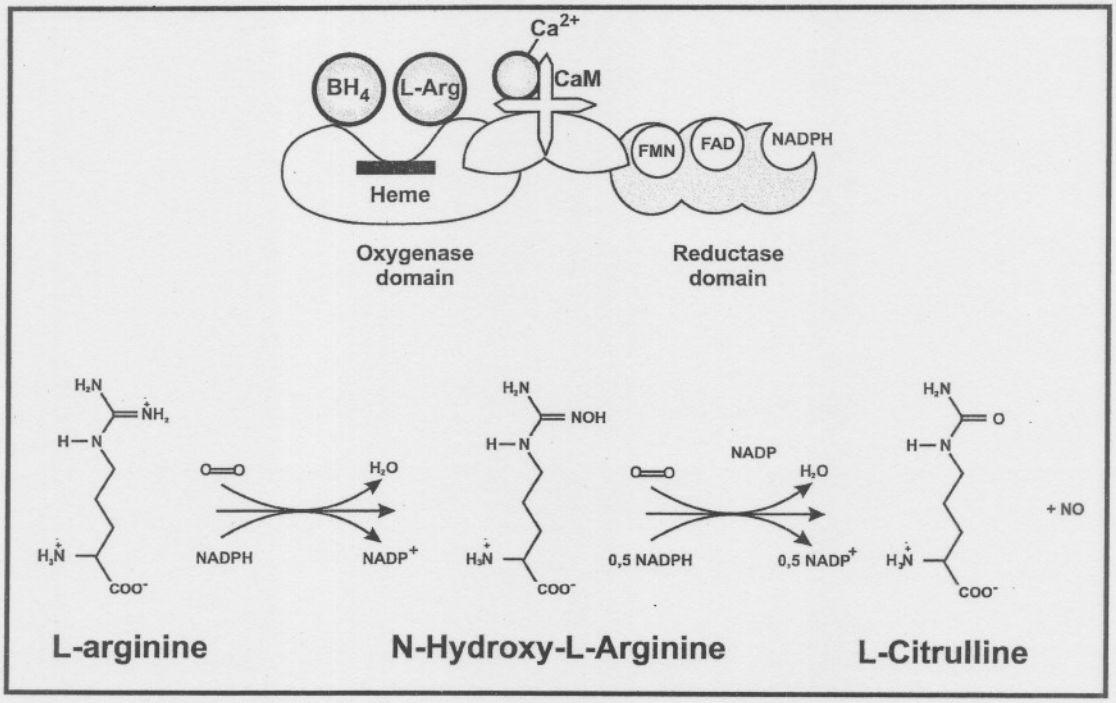


Figure 4.1: Biosynthesis of NO involves a two-step oxidation of L-arginine to L-citrulline, with concomitant production of NO (Cirino *et al.,* 2002)

The three subtypes of NOS described to date include NOS-1, also referred to as neuronal NOS (nNOS), found in high levels in neuronal cells, NOS-2, or inducible NOS (iNOS), found in macrophages, hepatocytes, smooth muscle cells and others, and NOS-3, or endothelial NOS (eNOS), the enzyme originally found in the vascular endothelium. nNOS and eNOS are activated by calmodulin during conditions of elevated calcium, and produce NO that acts as a neurotransmitter (Bredt & Snyder, 1992). iNOS is the only isoform that is activated independent of calcium. NOS-1 and NOS-3 are commonly referred to as constitutive NOS enzymes due to their constitutive expression and regulation by calcium/calmodulin (Krumenacker *et al.*, 2004). NOS-2 activity is normally absent, unless the enzyme is induced by cytokines, and is thus especially important in inflammatory states.

The enzyme functions as a dimer consisting of two domains, a reductase domain and an oxygenase domain (Figure 4.1). Within the reductase domain, five electrons are transferred from the nicotinamide adenine dinucleotide phosphate reduced (NADPH) to the L-arginine through flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). In the oxygenase domain, the enzymes have domains that bind heme, tetrahydrobiopterin (BH_4), and calmodulin (CaM), which are required for NOS activity. In the presence of O_2, the guanidine nitrogen of L-arginine acts as a final acceptor of the electrons, and then produces L-citrulline and NO (McLeod *et al.*, 2001).

In neurons NO synthesis is stimulated by Ca^{2+} influx, which is induced by activation of glutamate receptors, preferentially NMDA receptors (Prast & Philippu, 2001). Unlike conventional neurotransmitters, NO is not stored in lipid-lined vesicles or metabolized by hydrolytic enzymes, but is synthesized de novo. Once synthesized, NO diffuses freely across cell membranes, obviating the need for a transport system. Therefore, NO can diffuse from a cell in which it is produced to neighboring cells (Denninger & Marletta, 1999), making it an especially good candidate as a retrograde and inter-cellular messenger. NO is extraordinary labile, with a half-life of only about 3 to 5 seconds (Bredt, 1999), such that the extent of NO diffusion in tissue depends on the balance between its rate of production, its high diffusion constant, its half-life and its chemical reactivity, with locally produced chemicals and proteins, e.g. O_2^+, $O_2^{\bullet}$ (superoxide) (Contestabile, 2000).

NO reacts with O_2 and undergoes spontaneous oxidation to the inactive metabolites nitrite and nitrate (NO_2^- and then NO_3^-), and also with $O_2^{-\bullet}$ to form an unstable intermediate peroxynitrite ($ONOO^-$), therefore no separate mechanism is required for its illumination (Denninger & Marletta, 1999). In the mitochondria, ONOO- acts on the respiratory chain (I-IV) complex and manganese superoxide dismutase (MnSOD), to generate superoxide anions and hydrogen peroxide (H_2O_2) respectively (Figure 4.2).

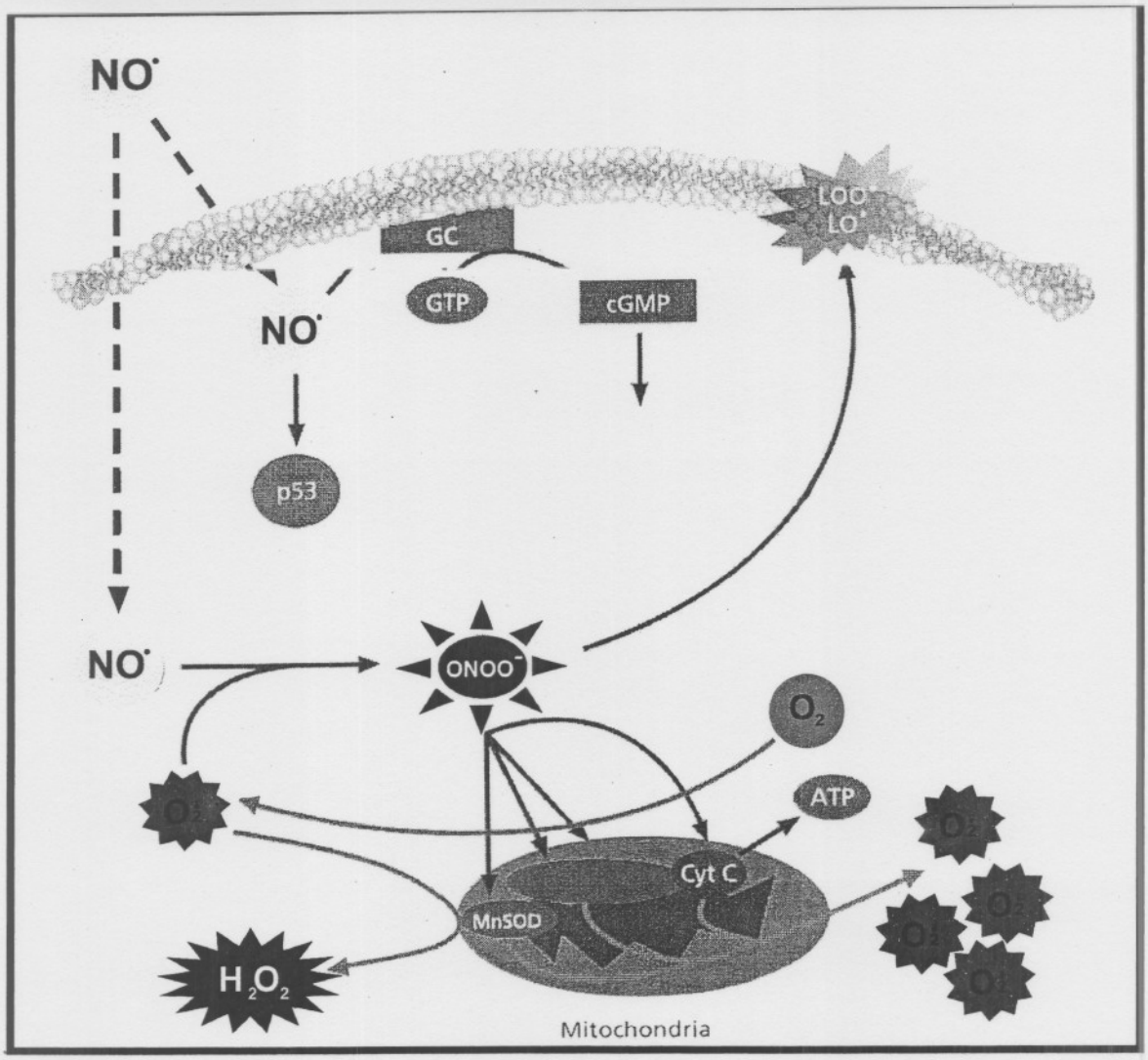


Figure 4.2: The interaction of NO with $O_2^{-\cdot}$ to form an unstable intermediate peroxynitrite ($ONOO^-$). In the mitochondria, ONOO- acts on the respiratory chain (I-IV) complex and manganese superoxide dismutase (MnSOD), to generate superoxide anions and hydrogen peroxide (H_2O_2) respectively (After Sigma-Aldrich, 2002).

4.3 Role of NO in Signal Transduction

Due to its gaseous nature NO can easily diffuse from the producing cells into adjacent neurons where it can induce changes in neuronal and signaling-related functions by several ways. The chemical reactivity of NO is critical to its action as an antioxidant, a cytotoxin and as an inter and intracellular messenger (Mayer & Hemmens, 1997). One interaction of note is that NO interacts with heme complexes in proteins, such as sGC, hemoglobin, cytocrome P450, or protoporphyrin IX. However, NO can also interact with non-heme iron proteins, other transition metals such as zinc, protein radicals and various enzymes (Krumenacker *et al.*, 2004).

The majority of NO effects on neuronal excitability depends on increasing cGMP synthesis via activation of sCG (Prast & Philippu, 2001). NO binds to the haem-iron of sGC resulting in the formation of a penta-coordinate nitrosyl-haem complex, exposing the catalytic site to cyclic guanosine 5'-triphosphate (GTP) and thereby constitutes enzyme activation resulting in the conversion of GTP to cGMP (Hobbs, 1997). The resulting increase of cGMP then may directly affect ion channels, activate protein kinases, activate or inhibit phosphodiesterase and activation of ADP ribosyl cyclase (Contestabile, 2000) (Figure 4.3). These actions result in various changes in neuronal function, e.g. membrane ion flux, changes in presynaptic release of neurotransmitters, such as Ach and NA, as well as uptake of 5-HT, promotion of neutrite outgrowth and changes to various glial cell-directed events (Harvey, 1996).

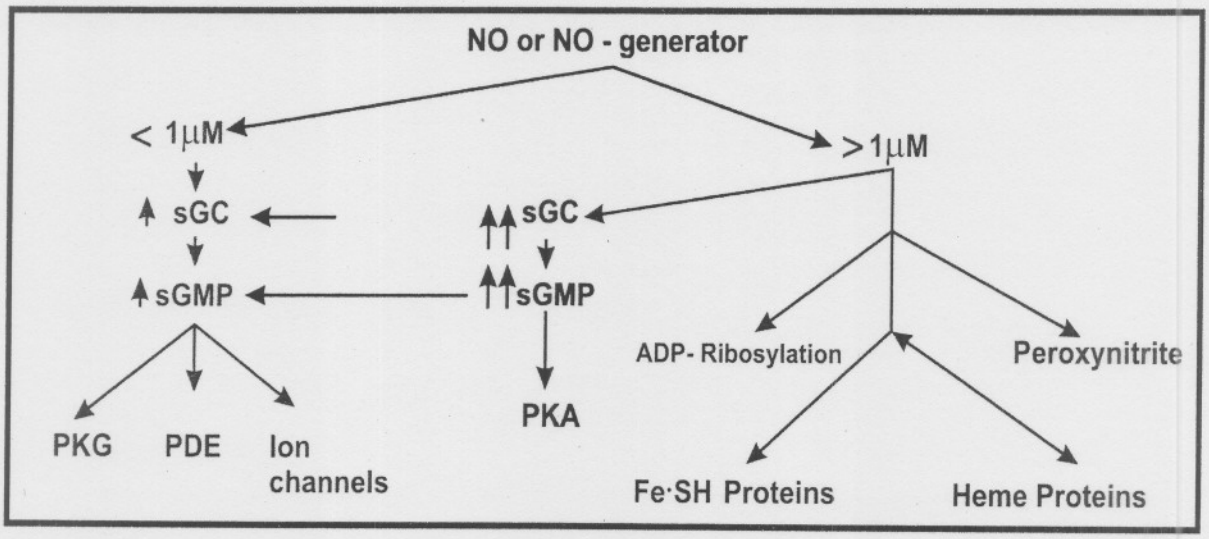


Figure 4.3: NO may have effects on cells related to its concentration. At micromolar concentrations (<1μM), NO selectively activates soluble guanylate cyclase (sGC). At higher concentrations, NO produce a variety of effects. Robust stimulation of sGC by supramicromolar (>1μM) concentrations of NO may produce inordinately high concentrations of soluble guanylate cyclase (cGMP) that may "cross over" and activate cAMP-dependant protein kinase (PKA). PKG, cGMP-dependant protein kinase; PDE, cyclic nucleotide phosphodiesterases; Fe-SH proteins, iron-sulpher cluster proteins. (After Lincoln *et al.*, 1996).

However, NO can also modulate neuronal function in a cGMP-independent way. Thus, it has been demonstrated that NO, induced by NMDA receptor stimulation, activates the p21 (ras) signal transduction pathway via a cascade involving extra-cellular signal-regulated kinases and phosphoinositide 3-kinase (Dawson *et al.*, 1998). Glutamate binding to NMDA receptors leads to Ca^{2+} influx and the activation of nNOS. NO is released postsynaptically and, because of its lipophylic character, diffuses to the presynaptic neurons where it induces an increase in glutamate release and an increase in synaptic transmission (Moncada & Higgs, 1993) (Figure

4.4). Other enzymes that constitute cellular targets for NO are cyclooxygenases, ribonucleotide reductase, some mitochondrial enzymes while a mechanism of feedback control negatively regulates NOS itself (Contestabile, 2000).

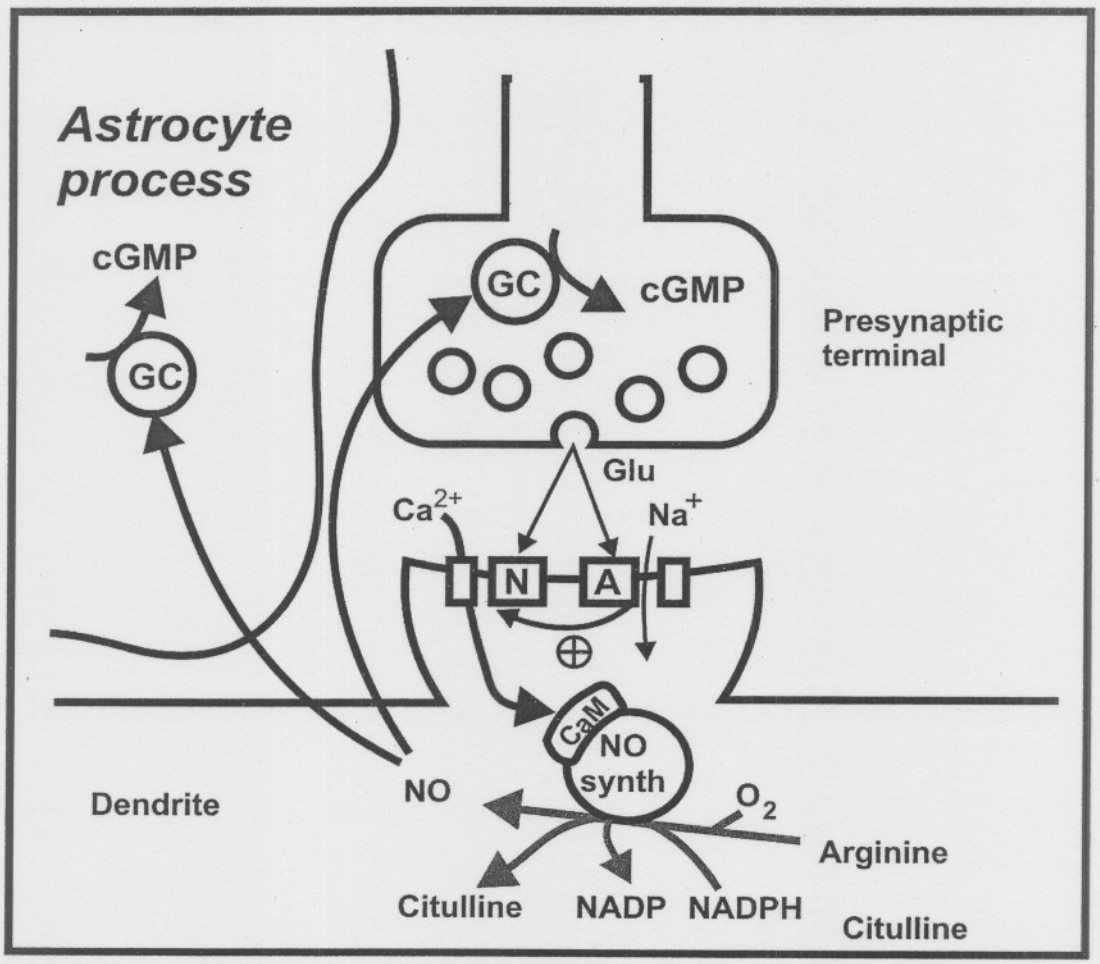


Figure 4.4: The nitric oxide transduction mechanism in the central nervous system. Glutamate (Glu) released from the presynaptic terminal acts upon NMDA (N) and AMPA (A) receptors. When the postsynaptic membrane is depolarized the magnesium (Mg^{2+}) block of the NMDA channel is reduced, Ca^{2+} enters and, via camodulin (CaM), activates NO synthase. The NO formed diffuses out to neighbouring astrocyte fibres and/or to the presynaptic terminals, where it activates guanylate cyclase (GC) to form cGMP (After Garthwaite, 1991).

4.4 Physiological roles of NOS isoforms

As mentioned earlier, NO is generated in many mammalian tissue by three NOS isoforms. These have a wide variety of physiological and pathophysiological roles in the body. Table 1 summarizes some of the postulated roles for NO synthesized by the three NOS isoforms (Knowles & Moncada, 1998), some of which will be discussed in more detail in section 4.5.

nNOS	iNOS	eNOS
CNS :	**Non-specific immunity :**	**Cardiovascular**
Responses to Glu	**Resistance to infection by**	**Regulation of**
Synaptic plasticity	Protozoa	Tissue conductance
Peripheral :	Fungi	Blood flow
GI tract	Bacteria	Blood pressure
Urogenital system	Viruses	**Inhibition of**
Blood flow	**Pathological roles :**	platelet aggregation
Skeletal muscle	**Shock states**	**Protect**
Pathological roles :	Septic, endotoxic	vascular endothelium
Ischaemic brain damage	Cytokine-induced	from toxic mediators
Huntington's disease	**Inflammatory/autoimmune**	and atherosclerosis
Parkinson's disease	Acute inflammation	
Tardive Dyskinesia	Ulcerative colitis	
Depression	Asthma	
Schizophrenia	Transplant rejection	
Anxiety	Arthritis	
Epilepsy	Multiple sclerosis	
Menstrual Migraine	Dementias (Alzheimer's)	
Nociception	**Tumours**	

Table 4.1 Postulated roles for NO synthesized by the three NOS isoforms

Although NO has been implicated in non-adrenergic-non-cholinergic (NANC) transmission in the periphery, with purported roles in the gastrointestinal and genitourinary system, and in the cardiovascular and muskuloskeletal system (Esplugues, 2002), given the focus of this particular study, this chapter will only address NO in the brain and in neuropsychiatric illness.

4.4.1 Neuronal NOS

The brain contains the highest activity of nNOS in any tissue, and is predominantly present in neurons, but also in some cerebral vessels and in glial cells (Esplugues, 2002). The role of NO acting as a diffusible messenger, upon NMDA receptor activation, has been demonstrated in the cortex, the striatum, the hippocampus, the cerebellum, the spinal cord as well as in cell cultures and synaptosomal preparations (Contestabile, 2000). Although nNOS neurons represent only 1% of cell bodies in the cerebral cortex, virtually every neuron in the cortex is exposed to nNOS nerve terminals (Esplugues, 2002).

4.4.1.1 nNOS in the central nervous system

4.4.1.1.1 Synaptic plasticity

Because of its short half-life and lipophilic character, NO readily diffuses across cellular membranes and so contributes to both anterograde and retrograde signaling at the synapse (Haung & Lo, 1998). Thus, NO is uniquely suited to mediate induction of two forms of synaptic plasticity; namely long-term potentiation (LTP), a process that requires information transfer from the postsynaptic to the presynaptic terminal (Brenman & Bredt, 1997) and that contributes to learning (Hawkins *et al.*, 1998), as well as long-term depression (LTD) (Esplugues, 2002). LTP and LTD are activity-dependent forms of modified transmission and occur in various brain regions (Prast & Philippu, 2001).

LTP, a property of many central excitatory synapses, is characterized by a prolonged enhancement of synaptic transmission, or an activity-dependant increase in synaptic strength, lasting from hours to weeks. The process involves glutamate acting at AMPA and/or NMDA receptors, activating a series of events in which Ca^{2+}/calmodulin-dependant protein kinase II, NOS and protein tyrosine kinase are implicated (Esplugues, 2002). Guanylate cyclase is the main effector of NO in induction of LTP (Esplugues, 2002).

NO produced in the postsynaptic neuron via the action of glutamate on NMDA/AMPA receptors, travels through the extracellular space, to diffuse into the presynaptic neuron to evoke further pre-synaptic activation, glutamate release and thus, produce long-lasting potentiation. Pre-existing NOS in the presynaptic neuron can also be activated by receptor-mediated calcium influx that occurs on stimulation. Inhibitors of NOS and scavengers of NO block LTP, and application of exogenous NO produces activity-dependant enhancement of synaptic transmission (Haung & Lo, 1998), supporting the hypothesis that NO acts as a retrograde

messenger during LTP (Hawkins *et al.*, 1998). NO as the retrograde messenger, goes back to the presynaptic neurons to indicate that the signal had been received and to strengthen or weaken the synapse in question (Figure 4.5). The process of LTP forms the physiological basis of how memories are laid down, be it emotional or motor-related memory, in brain regions such as the hippocampus and the striatum, respectively.

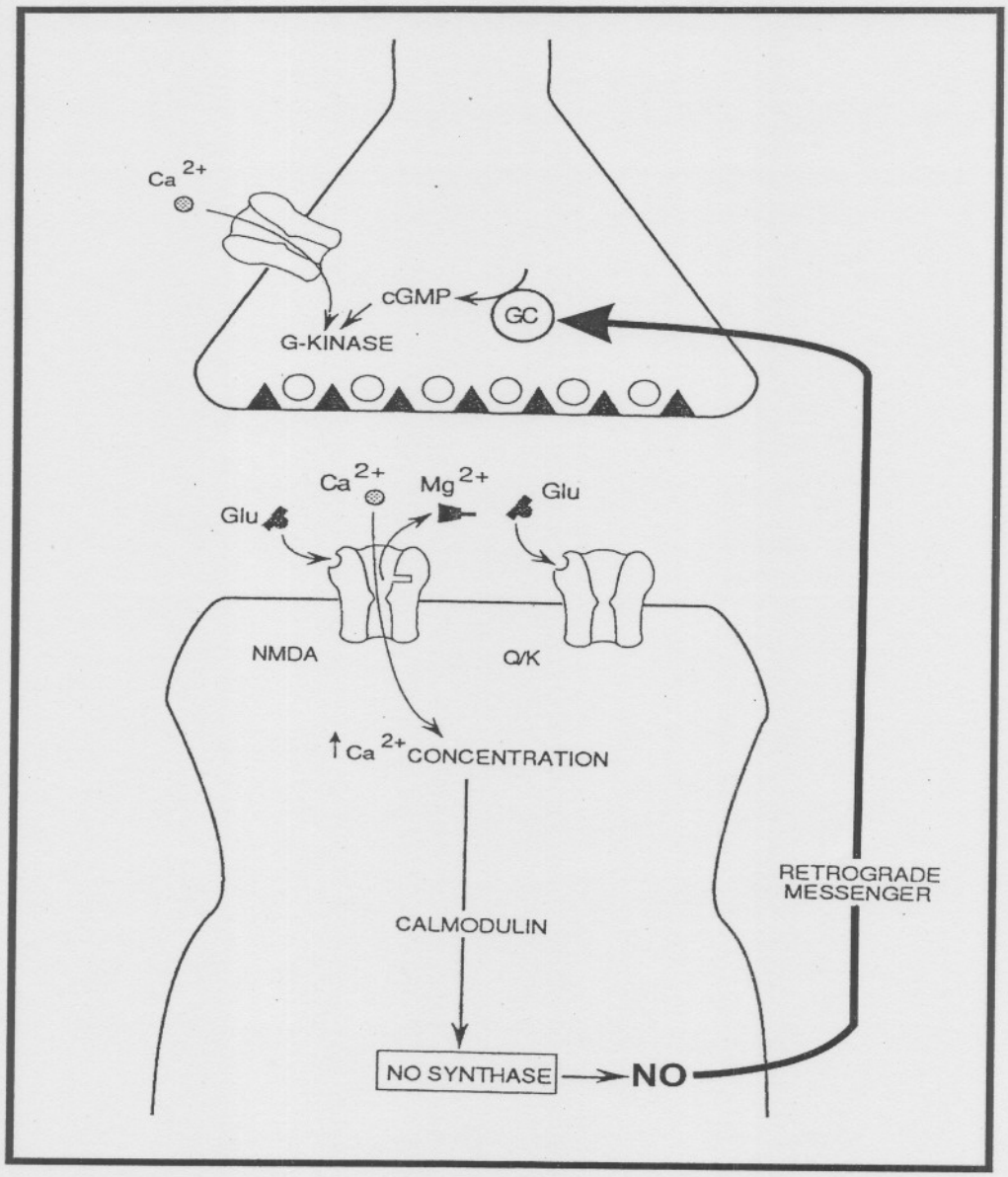


Figure 4.5: Molecular mechanism contributing to LTP. NO acts as a retrograde messenger, causing long-lasting increase in transmitter release from the presynaptic terminals by stimulating guanylyl cyclase (GC) and cGMP-dependant protein kinase. The molecular cascade is amplified or enabled by concurrent spike activity in the presynaptic neurons, perhaps due to a synergistic effect of Ca^{2+} that enters the cell during activity (After Hawkins *et al.*, 1998).

LTD, on the other hand, is characterized by a long lasting depression of parallel fibre synapses in the hippocampus, which follows repeated excitation of the climbing fibres of Purkinje cells. This reduction in synaptic strength is a result of diminished sensitization of postsynaptic AMPA receptors that is mediated by activation of protein kinases C and G and the NO-cGMP signaling pathway (Esplugues, 2002).

Apart from its established role in memory, LTP, and the role of glutamate and NO in this process, has also been suggested as a means whereby psychiatric illnesses, such as depression, induce lasting changes to neuronal function and in so doing, determines the response to treatment and the long-term outcome of the illness (Harvey, 1996; Harvey *et al.*, 2003)

4.4.1.1.2 Neurotransmitter release

The NO-cGMP pathway displays the ability to modulate neurotransmitter release, but the precise mechanism by which this occurs is not well understood. However direct S-nitrosylation of receptors, activation of cyclic GMP-dependant protein phosphorylation cascades, regulation of neuronal energy dynamics and a modulating effect on transporters are potentially involved (Esplugues, 2002).

NO has been linked to the release of various neurotransmitters and the effects which they produce in the CNS, e.g. 5-HT, DA, GABA, glutamate (Wegener *et al.*, 2000), Ach, NA, adenosin triphosphate (ATP), bombesin, carbon monoxide, opioids and endothelin (Esplugues, 2002).

NO, via cGMP, modulate presynaptic 5-HT uptake (Zhu *et al.*, 2004). It has been reported that low concentrations of NO donors' decreases 5-HT outflow, whereas higher concentrations of these donors enhance the release of this monoamine, thus regulating 5-HT in a biphasic way (Prast & Philippu, 2001; Inan *et al.*, 2004).

A low concentration of NO inhibits the activity of excitatory and inhibitory amino acid neurons, while elevated NO levels lead to their stimulation (Prast & Philippu, 2001). NO indirectly modulates Ach release via excitatory and inhibitory amino acids (Prast & Philippu, 2001). As mentioned above, depending on the concentration of NO, glutamate release is either enhanced or decreased. Glutamate stimulates NMDA and AMPA/kainite receptors located on cholinergic neurons, thus enhancing the release of acetylcholine, while inhibition of glutamate release exerts the opposite effect on cholinergic transmission. $GABA_A$ receptors on cholinergic neurons

inhibits Ach release, while inhibition of GABA release, enhances Ach output (Prast & Philippu, 2001).

NO influences the function of dopaminergic terminals and plays an inhibitory role in NMDA-induced DA release in the striatum. Endogenously produced NO may influence the activity of the DA transporter, which might have special importance in the regulation of extracellular transmitter concentration in the striatum (Kiss *et al.*, 1999). It has been suggested that NO does not influence DA release directly via cGMP, but indirectly possibly via a stimulatory action on cholinergic and GABAergic neurons or, by a mechanism not involving cGMP (Kendrick *et al.*, 1998).

4.4.1.2 Pathological roles of nNOS in the CNS

4.4.1.2.1 Ischaemic brain damage

The neuronal damage that accompanies cerebral ischeamia involves an excessive release of glutamate and a subsequent activation of NMDA receptors that, if maintained for a sufficient period of time, induces a massive influx of Ca^{2+} into the postsynaptic neurone, which in turn, triggers the activation of nNOS and overproduction of NO (Esplugues, 2002). The neurotoxicity of NO in ischemic brain appears to result from the simultaneous production of superoxide and NO that leads to formation of peroxynitrite. However, increases in endothelial NOS activity will protect against ischaemia (Zhang *et al.*, 2001), by maintaining regional cerebral blood flow. It is also pertinent to know that inflammatory conditions and transient ischaemic periods can also induce the expression of iNOS in various populations of cerebral cells, although iNOS expression occurs later than that of nNOS and eNOS, and its expression is dependant on the nature of injury (Esplugues, 2002).

It thus seems that NO can either be neuroprotective or neurotoxic depending on the timing of exposure to NO (Zhang *et al.*, 2001).

4.4.1.2.2 Neurodegenerative Diseases

The excessive release of both glutamate and NO (Esplugues, 2002), coupled with mitochondrial electron transport chain damage (Stewart & Heales, 2002) has been implicated in a number of neurodegenerative disorders. NO and its toxic metabolite peroxynitrite ($ONOO^-$) can inhibit the mitochondrial respiratory chain, leading to energy failure and ultimately cell death. Evidence suggests that $NO/ONOO^-$ causes release of neuronal glutamate, leading to glutamate-induced

activation of neuronal NOS and generation of further damaging species (Stewart & Heales, 2002). Nitrotyrosine immunoreactivity (a marker for peroxynitrite and NO-derived oxidants) has been found in Alzheimer's disease (Smith *et al.*, 1997), multiple sclerosis (Bagasra, 1995) and in animal models of Parkinson's disease (Hantraye *et al.*, 1996) and Huntington's disease (Schultz *et al.,* 1996).

Evidence for hippocampal degenerative pathology in post traumatic stress disorder (PTSD) has been found at both anatomical (volume reduction) and behavioral (cognitive deficit) levels (Bremner, 1999). Pre-clinical work suggests that structural changes of the hippocampus are mediated by the neurotoxic effect of elevated glucocorticoids following stress and the release of neurotoxic molecules, particular glutamate and NO (Sapolsky, 2000a). The NMDA-nNOS pathway plays an important role in anxiety-related behaviors, while mice lacking a fully functional glutamate NMDA receptor are less sensitive to stress induced by the elevated plus-maze, light-dark box, and FST (Miyamoto *et al.,* 2002).

A general hypotheses in the neuropathology of schizophrenia is that an over activity of the DA system exists. Excessive NO production might result in elevated DA release, and the ability of NO to modulate the release of other neurotransmitters might increase the possible role of NO in schizophrehnia (Meltzer & Deutch, 1999). NOS expression is altered in psychiatric patients, and it has been reported that the density of NADPH-d containing neurons (which express NOS) was significantly lower in the gray matter and higher in the white matter of the prefrontal cortex and the lateral temporal lobe of patients with schizophrenia compared with controls (Xing *et al.*, 2002)

The pathophysiological basis of tardive dyskinesia (TD) remains speculative. Harvey and Nel (2003), however, suggest that the risk of TD may be linked directly to overt suppression of striatal NO modulation, while the ability of the atypical antipsychotic olanzapine, to treat TD may be linked to an indirect action to evoke recovery of striatal NOS.

4.4.1.2.3 Depression

The diverse effects of NO and cGMP on subcellular events, the unique physico-chemical properties of NO and its involvement in cellular memory processes and synaptic plasticity makes it an ideal regulator of short-and long-term adaptive changes associated with mood regulation (Harvey, 1996). This statement is thus of particular importance for the present study, so that this topic is addressed more comprehensively.

Inhibitors of the down-stream activation of NOS and cGMP formation, including NOS-inhibitors (Harkin *et al.*, 1999) and guanylyl cyclase-cGMP inhibitors (Eroglu & Caglayan, 1997; Heiberg *et al.*, 2002) have all demonstrated distinct antidepressant-like effects in animal models of depression. Down-regulation of cortical β-adrenoreceptors, which is observed in animals chronically treated with many clinically effective antidepressants, is also observed following chronic NOS inhibitor administration (Karolewicz *et al.*, 1999). Moreover, depressed patients have increased levels of nitrate, the end product of NO metabolism (Suzuki *et al.*, 2001).

Dysfunction of monoamine neurotransmitters is involved in the pathogenesis of depression (Harvey, 1997). NO also modulates the extracellular levels of various neurotransmitters in the CNS, e.g. serotonin, DA, GABA and glutamate (Prast & Phillipu, 2001). Wegener et al. (2003) suggest that the 5-HT-glutamate-NOS-NO interactions may play a critical role in maintaining psychic well being, as well as having a role in the neurobiology and treatment of depression.

NO is a regulator of both short-and long-term neuronal adaptive changes (Paul & Skolnick, 2003) and consequently, may play a role in neuronal adaptation to AD drugs. NO mediated cGMP synthesis also mediates induction of immediate early gene expression that is implicated in long-term synaptic changes (Harvey, 1996) and more recently has been implicated in the mechanism of action of AD drugs (Harkin *et al.*, 1999). Antidepressants, over an above their more traditional actions on monoamine uptake, inhibit NOS in the hippocampus (Wegener *et al.*, 2003), a important brain area involved in the neurobiology of depression.

Considering that NMDA receptors represent the principle mechanism of NOS activation in the limbic brain regions, including the hippocampus and cortex, it is of particular interest that NMDA antagonists exert synergistic antidepressant-like effects with classical antidepressants (Rogóż *et al.*, 2002), that behavioral effects of IMI withdrawal can be reversed by NMDA antagonists (Harvey *et al.*, 2002) and that chronic AD treatment, in turn have significant impact on limbic NMDA receptors (Skolnick, 1999; Harvey *et al.*, 2002). Rosa et al (2003) demonstrated that the antidepressant-like effect of zinc is mediated, at least in part, by the inhibition of NMDA receptors and an interaction with the L-arginine-nitric oxide pathway. More recently, the role of NO in the apparent neurodegenerative symptoms of depression have attracted attention (Harvey *et al.*, 2003), especially regarding the increase in NOS containing neurons in the hippocampus and the neurotoxic nature of excessive NO and glutamate. Clearly, the physiological function of glutamate and NO in the brain with regards to synaptic plasticity (McLeod *et al.*, 2001) as well as cell death and neuro-protection (Garthwaite & Boulton, 1995) hold great promise in revealing more on the neurobiology of depression, and especially the dynamics of antidepressant action.

4.4.2 Inducible NOS

The effects of iNOS are a result of its unique features of Ca^{2+}-independence and inducible, high level expression following exposure to lipopolysaccharides (LPS) and cytokines such as interferon-gamma (IFN-y) and tumor necrosis factor-alpha (TNF-α) (Dawson & Dawson, 1998). iNOS expression can be induced by inflammatory mediators in most types of vascular cells, including endothelial cells, cardiac myocytes, and smooth muscle cells, as well as macrophages, which, as a result of the high NO output, can have damaging consequences (Andrew & Mayer, 1999). Following ischemic insult, trauma, viral or bacterial infections or other immunologic challenges, iNOS can be expressed in the brain, predominantly in microglia and astrocytes.

NO synthesized by iNOS in activated macrophages is an important host defense mechanism, killing pathogens such as leishmania, Mycobacterium tuberculosis, malaria parasites, and certain fungi. It also mediates "non-specific" immunity and is toxic to tumour cells (Vallance & Collier, 1994). Expression of iNOS in response to cytokines or endotoxin is part of the inflammatory response and contributes to vasodilatation, vascular leakage, and tissue damage in some inflammatory conditions (Vallance & Collier, 1994).

4.4.2.1 Pathological roles in the CNS

Overproduction of NO by iNOS is implicated in the pathology of various inflammatory disorders of the CNS. It plays a role in viral and bacterial infections, disruption of the blood-brain barrier during inflammatory conditions, and is a potential toxic mediator in inflammatory encephalitic diseases (Esplugues, 2002).

4.4.2.1.1 Demyelinating diseases

Multiple sclerosis (MS) and animal models such as experimental autoimmune encephalomyelitis (EAE) are inflammatory diseases characterized by early damage to the blood-brain barrier (Ye & Sontheimer, 1998). NO has been implicated as a potential mediator of microglia-dependant primary demyelination, a characteristic of multiple sclerosis, and iNOS induction has been noted in the brains of patients with this autoimmune disease (Esplugues, 2002). Cross et al. (1996) showed that iNOS transcript and enzyme activity correlate with active EAE (Ye & Sontheimer, 1998).

4.4.2.1.2 Alzheimer's diseases

Increasing evidence suggest NO metabolism is affected in Alzheimer's disease. Studies indicate that both NOS activity and the number of NADPH diaphorese-positive neurons are increased in Alzheimer's disease (Stewart & Heales, 2002). Nitrotyrosine residues have been detected in postmortem Alzheimer's tissue but not in age-matched controls, indicating the presence of $ONOO^-$. The pathophysiology of Alzheimer's disease suggest that β-amyloid induces a cascade of cellular mechanisms including the activation of astrocytes and the subsequent production of pro-inflammatory cytokines and NO, which may lead to neuronal damage (Minagar *et al.*, 2002).

4.4.2.1.3 HIV Dementia

It is estimated that 15% of Human Immunodeficiency Virus (HIV) infected patients with AIDS are affected with varying severity of HIV dementia. Localization of the virus in the CNS is nearly exclusively in blood-derived macrophages, micro-glia and multi-nucleated giant cells (Dawson & Dawson, 1998). Neurotoxicity induced by certain HIV coat proteins is mediated by activation of NOS, whereas HIV patients who develop dementia exhibit a substantial increase in cortical iNOS (Esplugues, 2002). It is likely that the HIV coat protein, gp41 on HIV infected cells, stimulates local cells to express iNOS and NO for sustained periods of time which can lead to neuronal damage and ultimately the severity of HIV dementia (Dawson & Dawson, 1998).

4.4.2.1.4 Stress

An increase in the expression of iNOS has been noted to occur in the limbic regions following restraint stress in rats (Madrigal *et al.,* 2001). iNOS involves a gradual induction over time, resulting in the prolonged release of large amounts of NO, that may have direct implications for neuronal degenerative and atrophy evident in patients with PTSD. Supportive data for a prominent role for NOS, particular iNOS, in the behavioral and neurochemical response to repeated stress has recently been reported (Harvey *et al.*, 2004).

4.5 NO: Neurotoxicity vs Neuroprotection

As a reactive free radical, NO, can give rise to both nitrosative and oxidative stress through nitrosation, nitration or nitrosylation of various organic molecules, lipid peroxidation, DNA damage, impairmant of mitochondrial function and eventually neuronal death (Dawson & Dawson, 1998). Dysregulation of NO production and release, has been therefore proposed as an important primary or aggravating factor in several neurodegenerative pathologies. However, increasing evidence suggests that NO can be cell protective and in particular neuroprotective (Contestabile & Ciani, 2004).

Many of the neurotoxic actions of $NO^{\bullet}$ are mediated by derived nitrogen species, including NO_2 radical and peroxynitrite (ONOO-) (Ledo *et al.*, 2004). Peroxynitrite formation is initiated via NMDA receptor activation, intracellular Ca^{2+} increase, and augmented NO synthesis (Figure 4.6). It is formed by reaction of $NO^{\bullet}$ with $O_2^{\bullet -}$ and this complex rapidly decomposes into $NO_2^{\bullet}$ and hydroxyl radical ($OH^{\bullet}$), or a reactive intermediate with $OH^{\bullet}$-like activity (Dalkara *et al.*, 1998). Peroxynitrite is a potent oxidizing agent that can initiate lipid peroxidation in biological membranes, hydroxylation and nitration of aromatic amino acid residues, and sulphydryl oxidation of proteins (Schultz *et al.*, 1995) (Figure 4.6).

Various mechanisms have been proposed whereby $NO/ONOO^-$ can cause neuronal damage. These include the modulation of neurotransmitter release, disruption of cellular metabolism and DNA synthesis, DNA damage and poly(ADP-ribose) (PARS) activation, activation of lipoxygenase (LOX) and cyclooxygenase (COX), mediation of apoptosis, inihibtion of glial Glu uptake, inhibition of superoxide dismutase and inactivation of reduced glutathione, as well as the formation of nitrotyrosine (Liew, 1998).

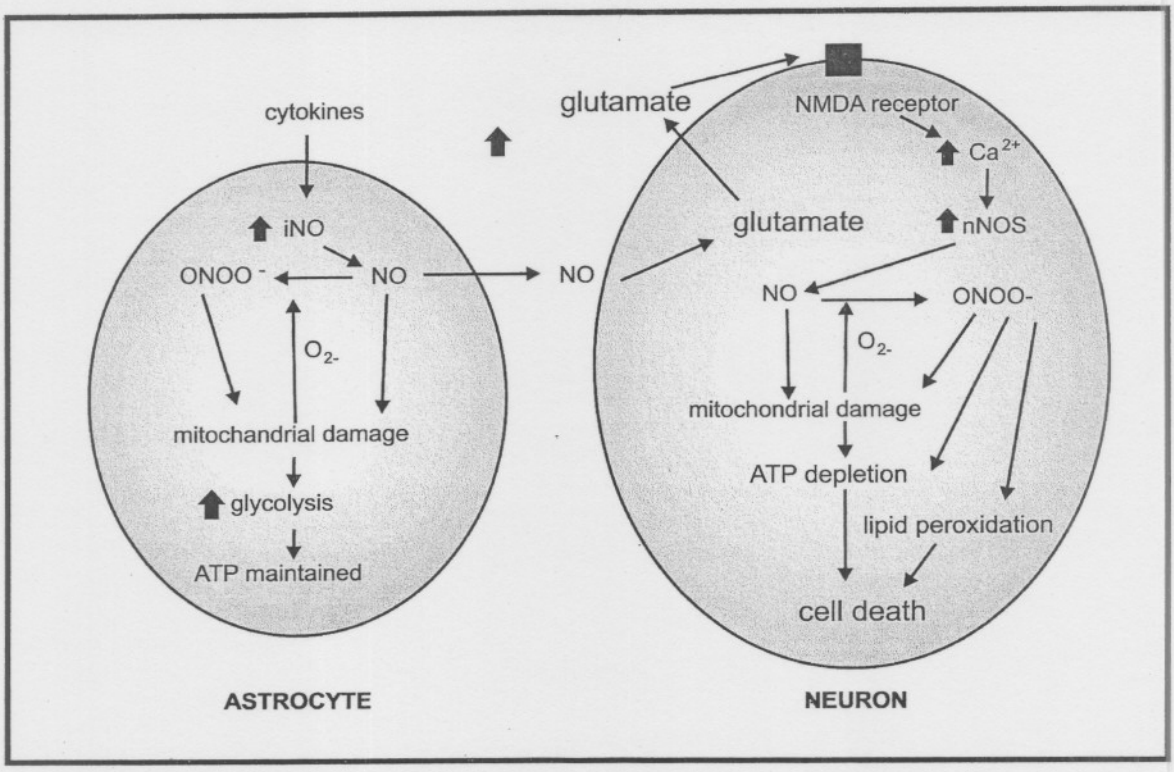


Figure 4.6: Mechanisms of NO-mediated neurotoxicity. Exposure of astrocytes to cytokines leads to the induction of iNOS, generation of NO, and mitochondrial respiratory chain damage. Superoxide radicals react with NO, forming $ONOO^-$ and causing more mitochondrial damage. NO causes neuronal glutamate release, which then activates NMDA receptors, triggers Ca^{2+} influx and activation of nNOS. $NO/ONOO^-$ initiates neuronal mitochondrial damage, lipid peroxidation, and ATP depletion (After Stewart & Heales, 2002).

However, NO itself, is a relatively nontoxic molecule, which in the absence of superoxide anion will not kill cells even at extremely high concentrations (Dawson & Dawson, 1998). Moreover, NO can act as both a reducing as well as an oxidizing agent. Consequently NO can reduce the generation of reactive oxygen species (ROS), such as hydrogen peroxide and superoxide, and prevent lipid peroxidation (Colasanti & Suzuki, 2000). Thus, despite the formation of reactive nitrogen species by NO, NO can also protect against oxidative stress mediated by ROS and exercise a positive effect on oxidative tissue damage, for example in an animal model of TD (Harvey & Bester, 2000).

Excessive activation of glutamate NMDA receptors, termed "excitotoxicity", represents a general mechanism underlying many neurological disorders, ranging from Alzheimer's disease, Parkinson's disease, multiple sclerosis to AIDS dementia. It is $NO^{\bullet}$ that mediates much of glutamate-mediated toxicity (Dawson & Dawson, 1998). However, there is evidence that the NMDA receptor is also downregulated by NO in a negative feedback loop by downregulating the

NMDA receptor by an S-nitrosylation at the redox-sensitive site on the receptor, thus exerting a neuroprotective function (McCaslin & Oh, 1995).

Thus, the redox versality of NO$^{\cdot}$ permits its conversion from a neuroprotective molecule to a neurotoxin and visa versa depending on surrounding conditions, such as the neuronal redox milieu, the balance between reactive species (such as superoxide anion and H_2O_2) and the presence of neuroprotective compounds such as ascorbate and glutathione (Ledo *et al.*, 2004).

Because the excessive release of both glutamate and NO, coupled with mitochondrial dysfunction and oxidative stress, has been implicated in a number of neurodegenerative diseases, it highlights the potential therapeutic role for specific NOS inhibitors as pharmacological tools in the treatment of these disorders (Esplugues, 2002).

4.6 Regulation of NO

4.6.1 Physiological

The most important regulator of constitutive NOS (eNOS and nNOS) activity is the elevation in intracellular Ca^{2+}, which stimulates NOS through interaction with calmodulin. Ca^{2+} promotes the binding of CaM to the enzyme's CaM-binding site, which triggers electron flux from FMN to haem, thereby coupling the oxygenase and reductase domains and activating the enzyme, producing small amounts of NO (Lowenstein *et al.*, 1994). Therefore, any factor blocking calmodulin binding may inhibit Ca^{2+}-induced NO formation (Schmidt *et al.*, 1993). When Ca^{2+} concentration falls, it dissociates from the calmodulin, which in turn dissociates from NOS, thus acting as a switch that turns the enzyme on and off (Esplugues, 2002). The activity of NOS may also be affected by the availability of L-arginine, molecular oxygen and BH_4. Drugs capable of sequestering calmodulin have been shown to inhibit NOS activity and decrease NO production (Dawson *et al.*, 1995).

Studies show that phosphorylation of eNOS by protein kinase Akt results in an increase in electron flux through the reductase domain, and subsequently an increase in NO production (McCabe *et al.*, 2000). The catalytic activity of nNOS is decreased following phosphorylation by cyclic AMP-dependant kinase (PKA), protein kinase C or Ca^{2+}/calmodulin-dependant protein kinase II (Esplugues, 2002). Frequent increases in the intracellular Ca^{2+} concentration and phosphorylation of NOS by Ca^{2+}/calmodulin-dependant protein kinase II might cause a negative feedback regulation of NOS activity.

In the CNS, NO synthesis is predominantly regulated by the influx of Ca^{2+} following postsynaptic stimulation of NMDA receptors by glutamate. The amino acid terminal of nNOS possesses a PDZ domain that interacts with the postsynaptic density protein PSD-95, whereas the NMDA receptor contains a Ser/Thr-X-Val motif (tSXV) that also binds with PSD-95. PSD-95 directly exposes nNOS to the flux of Ca^{2+} entering the ion channel of activated NMDA receptors, whereas transient Ca^{2+} fluxes from other receptors is too diluted to have a similar effect by the time they reach the vicinity of nNOS (Esplugues, 2002). NO regulates NMDA receptor activity in a biphasic manner playing a positive (via activation of GC) and negative (via feedback effects on the NMDA receptor resulting in decreased NMDA receptor and NOS activity) modulatory role in NMDA receptor mediated events. Thus, NO can either facilitate or inhibit NMDA receptor mediated events such as LTP, seizures (see Section 4.4.1.1.1), nociception, neurotoxicity and anxiolysis (Harkin *et al.*, 1999). PIN, a protein inhibitor of nNOS, binds specifically to the nNOS isoform and inhibit its action by destabilizing the active NOS dimer (Bredt, 1999).

Neurotrophins, despite their general role in attenuating excitotoxic neuronal injury, increase nNOS neurons, nNOS protein and nNOS catalytic activity in cortical cultures (Dawson *et al.*, 1998). nNOS is also dynamically regulated in response to a variety of stimuli, including nerve and brain injury, neuronal damage in the cerebral cortex induced by the middle cerebral artery occlusion (Dawson *et al.*, 1998), aging, lactation, hypoxia, stress, gonadectomy, light exposure, pharmacological treatment and exercise (Esplugues, 2002). The existence of presynaptic automodulation in nitrergic neurons has also been proposed; this action may result from the combination of NO with the haem group of NOS, which inhibits the enzyme.

eNOS activation can be induced by hormones such as catecholamines, autacoids such as bradykinin and histamine, platelet-derived mediators such as serotonin, and mechanical forces such as shear stress (Andrew & Mayer, 1999).

Glucocorticoids may either increase or inhibit NO production. Suppression of glucocorticoid production leads to an increase in iNOS expression, while the basal levels of nNOS expression is restored after glucocorticoid replacement (Lopez-Figueroa *et al.*, 1998). Excess glucocorticoids can stimulate glutamate, and subsequently NO, playing a critical role in neuronal plasticity and the response of neuronal cells to external stressors (Sapolsky, 2000a). Proinflammatory cytokines, endotoxin, HIV envelope proteins (gp120), hypoxia, β-amyloid and pathological processes such as infection, autoimmune, ischaemia etc. can induce iNOS expression, while anti-inflammatory cytokines, dexamethosone, chemokines, heat shock protein 70 and neurotransmitters such as NA and Glu can suppress the expression of iNOS (Loihl & Murphy, 1998).

The expression of iNOS requires the activation of nuclear factor κB (NF-κB) and interferon-responsive element (Liew, 1998). Physiological concentrations of NO suppress iNOS activity and gene expression by downregulating NF-κB (Taylor *et al.*, 1997), thus limiting processes such as immune response and inflammation (Colasanti & Suzuki, 2000). When a pathological process occurs, immune cytokines or lipopolysaccharide shift the equilibrium towards phosphorylated nNOS or eNOS, resulting in the abrupt fall in intracellular NO levels. The NO-induced inhibitory effect is removed and NF-κB is activated, followed by the expression of the gene encoding iNOS and the production of high levels of NO. Inhibitors of nNOS or eNOS might mimic this situation (Colasanti & Suzuki, 2000).

4.6.2 Pharmacological

NOS can be exogenously evoked with compounds that release NO such as:

- sodium nitroprusside,
- molsidomine,
- S-nitro-*N*-acetylpenicillamine (SNAP),
- and peripherally administered L-arginine.

Conversely, NO can be inhibited with:

- L-arginine analogous, such as N^G-nitro-L-arginine (L-NNA), N^G-nitro-arginine methyl ester (L-NAME), N^G-monomethyl-L-arginine (L-NMMA), and 7-nitroindazole (7NI) (Paul & Skolnick, 2003),
- methylene blue that acts as a direct inhibitor of NOS as well as of sGC. It also inactivates NO extracellularly through generation of superoxide anions (Eroglu & Caglayan, 1997),
- aminoguanidine, a selective inhibitor of iNOS (Harvey et al., 2004) and,
- 1-(2-trifluoromethylphenyl)imidazole (TRIM), a selective inhibitor of nNOS (Volke *et al.*, 2003).

Activation of the NMDA receptor complex stimulates the enzymatic production of NO. A decrease in NMDA receptor density will result in a subsequent decrease in nNOS-activation and thus a decrease in NO levels (McCaslin & Oh, 1995). Therefore, NMDA receptor antagonists can indirectly reduce the activity of NOS (Garthwaite, 1991). Antidepressants including paroxetine, citalopram, tianeptine and imipramine decrease NOS activity as shown *in vivo* using microdialysis (Wegener *et al.*, 2003), although earlier data do not support this (Jopek *et al.*, 1999). Wegener et al (2003) suggest that the effects of these AD occur secondary to their inhibitory effects of NMDA receptors, after increased glutamatergic activity that may occur in

depression. Zinc is another endogenous inhibitor of the NMDA receptor complex and has also been shown to inhibit brain constitutive NOS (Rosa *et al.*, 2003).

Mayer et al (1993) showed that methylene blue (MB) acts *in vitro* as a direct inhibitor of purified cerebellar NOS although it is a much less specific and potent inhibitor of guanylate cyclase than previously assumed (Mayer *et al.*, 1993). Nevertheless, Volke et al., (1999) found that MB inhibits brain NOS activity in vivo using microdialysis.

The quinoxalin derivate 1H-[1,2,4]oxadiazolo[4,3-a]-quinoxalin-1-one (ODQ) binds in an NO-competitive manner to inhibit NO-stimulated activity leaving basal enzymatic activity unchanged. Oxidation of the heme iron has been identified as the underlying mechanism (Koesling *et al.*, 2004).

The anti-inflammatory and cytotoxic drug, methotrexate acts by inhibiting the synthesis of tetrahydrobiopterin, an essential cofactor for the induction and activity of NO (Vallance & Collier, 1994). Angiotensin converting enzyme inhibitors block the breakdown of bradykinin, which stimulates release of NO from the endothelium (Vallance & Collier, 1994).

The antipsychotic drug, haloperidol, has been found to directly inhibit nNOS in vitro (Hu *et al.*, 1994), while chronic haloperidol treatment in rats has also been found to decrease NO activity in the striatum (Nel & Harvey, 2004).

Clearly, a number of agents may modulate NOS activity, some selectively, other indirectly. Whether the action of drugs such as antidepressants and antipsychotics on NOS are therapeutically relevant remains an important question. Thus, in light of the diverse functions of NO and its interactions with neurotransmitter as well as the fact that antidepressants have a modulating effect on the NO-cGMP pathway, it is important to determine the role of NO in affective disorders and whether actions on NOS represent an important mechanism of action of AD in depression.

Chapter 5
MATERIAL AND METHODS

5.1 Objectives of the study

Abrupt antidepressant discontinuation is associated with a stress response (Michelson *et al.*, 2000) that may be instrumental in perpetuating the illness and also negatively impacting on the neuroanatomy and function of the hippocampus. The neurobiology of AD withdrawal, however, remains unexplored despite the important relevance of this phenomenon. The current study includes a behavioral and neurochemical analysis of the tricyclic antidepressant, imipramine, after chronic treatment and withdrawal and its effect on the nitric oxide-cGMP pathway.

It has been proposed that NO may be critical in affective disorders, particularly in setting the course, outcome and response to treatment of the illness (Harvey, 1996). In agreement with this, depressed patients have increased levels of nitrate, the end product of NO metabolism (Suzuki *et al.*, 2001), while various animal studies have described the involvement of the NO-pathway in antidepressant action (Harkin *et al.,* 1999; Heiberg *et al.*, 2002; Volke *et al.*, 2003; Wegener *et al.*, 2003). Pre-clinical studies have demonstrated the antidepressant-like properties of NMDA antagonists (Skolnick, 1999), while inhibitors of the downstream activation of NOS and cGMP formation (Harkin *et al.*, 1999; Heiberg *et al.,* 2002), have demonstrated distinct antidepressant-like effects in animal models of depression and anxiety.

These pathways may hold the key to the neurodegenerative pathology noted in severe depression and why poor compliance is associated with greater incidences of relapse and treatment resistance. The regulation of the NMDA-NO pathway could be an important mechanism in antidepressant action; therefore this study will investigate the role of the NMDA-NO-cGMP pathway in AD action and particularly during AD withdrawal, while a pharmacological challenge study during IMI withdrawal, using the NOS/guanylyl cyclase (GC) inhibitor, methylene blue and the NMDA receptor antagonist, memantine will address the neurobiology of withdrawal specifically. Finally, the 5-HT_{2A}/5-HT_{2C} receptor antagonist, ritanserin, will be used to determine the role of serotonin in the NO-cGMP response to AD withdrawal.

The study was divided into three parts:

Firstly, two groups of 12 rats each received daily intra-peritoneal injections of the tricyclic antidepressant (TCA) imipramine (15 mg/kg/day; Harvey *et al.*, 2002) or saline, for three weeks (21 days). After chronic treatment, the animals were studied in the forced swim test (FST) (day 20/21) as well as subsequent evaluation of locomotor activity in a Digiscan animal activity monitor (day 20) to ensure that changes in swim motivation are based on an antidepressant response, and not due to an indirect effect of the drug on locomotor activity. These two groups formed the chronic imipramine vs. control group.

The second part of the study was the AD withdrawal paradigm:

Four groups of rats (12 animals/group) were treated with imipramine 15 mg/kg/day intraperitoneally (ip) for three weeks. From week 4 (day 21 to 28), the animals were withdrawn from imipramine. During this time, the four groups were allocated a withdrawal drug, being one of the following:

- the NMDA receptor antagonist, memantine (5 mg/kg/day; Rogoz *et al.*, 2002),
- the NOS/guanylyl cyclase (GC) inhibitor, methylene blue (MB; 15 mg/kg/day; Eroğlu & Cağlayan, 1997),
- the 5-HT_{2A} / 5-HT_{2C} receptor antagonist, ritanserin (4 mg/kg/day; Einat *et al.*, 2001), or
- saline control

After the withdrawal period, the animals were studied in the FST, and locomotor activity was tested. These four groups formed the imipramine-withdrawal groups and the effect of memantine, methylene blue and ritanserin on imipramine withdrawal.

The third part of the study was the sub-acute treatment paradigm:

In order to access the effects of the withdrawal drugs alone on the various biochemical and behavioral parameters, four groups (12 animals/group) received saline (ip) for three weeks followed by either memantine, methylene blue, ritanserin or saline for seven days, at dosages described earlier. After this treatment period, the animals were subjected to the FST- and assessment of locomotor activity, as described later in Section 5.4.

Immediately after behavioral testing (FST and LB), the animals were sacrificed by decapitation, the hippocampus was identified and dissected out, fixed in liquid nitrogen and stored for later neurochemical and enzyme assays, which included the following:

- NOS activity assay (Harvey & Nel, 2003)
- cGMP assay (Harvey & Bester, 2000)

5.2 Animals

The study protocol was approved by the Ethics Committee (Medical) (Evaluation Sub-committee for Experimental Animals) of the North West University (Potchefstroom campus). Ethical approval number : 03D10.

Male Sprague-Dawley rats, initially weighing 200–250 g, were used throughout the study and were provided by the Animal Research Center of the North West University. At the end of the chronic treatment period, the animals weighed between 300-350 g. The rats were kept on a natural 12 hour light / dark cycle with free access to food and water. The rats were housed in cages (4 rats per cage) with a width of 28 cm, a length of 44,5 cm and a height of 12,5 cm. The conditions in the animal center were controlled at 21 ± 0, 5ºC and 50 ± 5% relative humidity. Full spectrum cold white light, with a light intensity of 350 – 400 lux was provided over a 12 hours light – 12 hours dark cycle. A positive air pressure was maintained in the laboratory with air filtration 99,7% effective for a particle size of 2 micron and 99,9% for a particle size of 5 micron. All behavioral studies were performed in the Animal Research Centre.

Rats received standard rat pellets containing: Protein (180 g/kg), Fat (25 g/kg), Fibre (60 g/kg), Calcium (18 g/kg), Phosphor (7 g/kg) and Moisture (120 g/kg).

5.3. Drugs and Chemicals

Drugs and reagents used were of the highest and purist available commercially, and were purchased from the suppliers indicated in Table 5.1.

Suppliers	Chemicals
Sigma, England	• imipramine, • memantine, • methylene blue, • ritanserin
Alpha Pharm, South Africa	• Saline
Merck	• TRIS(hydroxymethyl)-aminomethan, • $CaCl_2$, • NaOH, • Na_2CO_3, • K^+/Na^+-tartrate, • $CuSO_4$
PAL chemicals	• Ethylenediaminetetraacetic acid (EDTA)
Sigma Chemicals, St Louis, USA	• Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N' –tetraacetic acid (EGTA) • B-nicotinamide adenine dinucleotide phosphate reduced form tetrasodium (β-NADPH), • calmodulin, • (6R)-5,6,7,8-tetrahydrobiopterin dihydrochloride (BH_4), • L-Arginine, • L-[^{3}H]arginine, • flavin mononucleotide sodium (FMN), • flavin adenine dinucleotide disodium (FAD), • DL-dithiothreitol (DTT), crystalline bovine serum albumin (BSA), • Dowex AG 50W × 8, • Folin & Ciocalteus

Fluka biochemika	• [4-(2-Hydroxymethyl)piperazine-1-ethanesulonic acid] (HEPES)
Sigma-Aldrich, Germany	• Bradford Reagent (protein dye reagent)
Amersham Ltd, Buckinghamshire, UK	• Cyclic GMP radio-immunoassay kit

Table 5.1: Suppliers and chemicals used in the study.

5.4 Forced swim test studies

As has been described earlier (section 3.3.2.3), the introduction of "stress" during forced swimming has been described as the means whereby the FST is sensitive to the effects of antidepressant drugs. The test is based on a "learned helplessness" model that bears a strickly similarly to decreased volition (drive), apathy, lack of self-worth and esteem and locomotor retardation seen in depression. Effective antidepressants counter the "stress-induced" immobility response in the test.

Animals were placed individually in inescapable Perspex cylinders (diameter 18cm; height 40cm) containing 18cm of water and maintained at a constant temperature of 25°C by immersion in a surrounding water bath (157cm x 26cm x 25cm). During a preconditioning period, the animals were placed into the cylinders and observed for 15 minutes. The risk of drowning is zero since the depth of the water is pre-determined to allow the animal to keep its nose above water by using its tail for support. Furthermore, the animals are also under constant surveillance. Thereafter, the animals were transferred to a Tempedair drier maintained at 35°C. Immediately after drying, each animal received the last dose of test drug and was returned to its cage and housed overnight. The following day, each rat was placed into the separate cages of the Digiscan animal motor activity monitor for assessment of locomotor activity (see below). Thereafter, they were returned to the water tank for 5 minutes where immobility was timed. The total immobility time was recorded using video cameras and in-house observers. The animal was judged to be immobile when it remained afloat making only the necessary movements to keep its head above the water (Redrobe & Bourin, 1999).

5.4.1 Assessment of locomotor behaviour

Selected parameters for locomotor behaviour were identified, including horizontal- and vertical activity, and were monitored using a Digiscan Animal Activity Monitor (DAAM, AccuScan Instruments, Columbus, OH, USA) (Fig. 5.1).

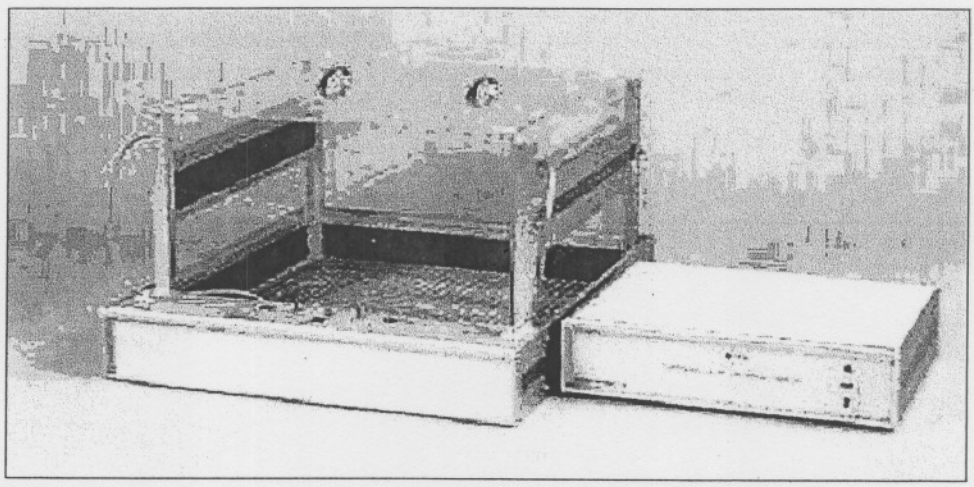


Figure 5.1: The Digiscan Animal Activity Monitor (DAAM, AccuScan Instruments, Columbus, OH, USA).

This automated method provides continual computerized monitoring of the animal that is more sensitive than simple observation and, above all, is without risks of investigator bias (Sanberg *et al.,* 1983; 1987). The cages employed in these observations are surrounded by a series of horizontal infra-red light beams (16 beams spaced 2.5 cm apart), with one set of beams at ground level and a second set 10cm above the first. This array of infra-red beams enables the computerized collection of all locomotor activity by a digital analyser that effectively determines the position of the animal 100 times per second. This high-speed analysis provides a dynamic picture of all aspects of the animal's activity throughout the observation period (AccuScan Instruments Incorporated, 2000). The interruption of any beam is recorded as an activity score while interruptions of two or more consecutive beams is a movement score.

On the last day of study for each group (day 21 for the chronic IMI and day 28 for the withdrawal group), motor activity for each rat was recorded over a period of 10 minutes. Thereafter the animals were subjected to the FST for 5 minutes.

All behavioral studies were performed between 08h00 and 12h00 to reduce the possible influence of the animals' diurnal cycles.

5.5 Pharmacological Studies

Ten groups of rats (12 animals / group) were involved in this study. The various drug treatments, dosages and duration of treatment for each group are shown in Table 5.1. All drugs were administered at 8h30.

Group Number	Drug Treatment and Dose	Duration of Treatment	Withdrawal Treatment & Dose	Duration of Withdrawal Treatment
Group 1	Imipramine 15 mg/kg/d	3 weeks	-----	-----
Group 2	Saline	3 weeks	-----	-----
Group 3	Imipramine 15 mg/kg/d	3 weeks	Saline	1 week
Group 4	Imipramine 15 mg/kg/d	3 weeks	Memantine 15 mg/kg/d	1 week
Group 5	Imipramine 15 mg/kg/d	3 weeks	Methylene Blue 15 mg/kg/d	1 week
Group 6	Imipramine 15 mg/kg/d	3 weeks	Ritanserin 4 mg/kg/d	1 week
Group 7	Saline	3 weeks	Memantine 15 mg/kg/d	1 week
Group 8	Saline	3 weeks	Methylene Blue 15 mg/kg/d	1 week
Group 9	Saline	3 weeks	Ritanserin 4 mg/kg/d	1 week
Group 10	Saline	3 weeks	Saline	1 week

Table 5.2: Drug treatment, dosage and duration of treatment of the various treatment groups as indicated.

5.5.1 The chronic imipramine vs control saline group

Groups 1 and 2 were treated for three weeks with 15 mg/kg/day imipramine or saline intraperitoneally (ip). Drug or vehicle were administered at 8h30 each morning. On the next last day of the study (day 20) the rats were allowed to swim for 15 minutes where after the final dosages of imipramine or saline were administered. The rats were then removed from the water and placed in a Tempedair drier before they were returned to their cages. On the second day (day 21) each rat was placed into separate cages in the Digiscan animal motor activity monitor for assessment of locomotor activity. Thereafter each rat was allowed to swim for 5 minutes while its activity was videotaped. The videotaped behaviour and vertical and horizontal activity of rats was subsequently analysed. The duration of immobility was defined as the absence of active, escape-orientated behaviour such as swimming, jumping and diving, was recorded. After the 5-minute swim stress, the rats were decapitated. The hippocampus of each rat brain was quickly removed and frozen in liquid nitrogen (-196ºC). The tissue was subsequently stored at -80ºC until used for NOS and cGMP assays.

5.5.2 The withdrawal treatment groups

Group 3, 4, 5, 6 were treated for three weeks with 15 mg/kg/day (ip) imipramine followed by one week of "withdrawal". Group 3 received saline intraperitoneal during this time, Group 4 received memantine (5 mg/kg/day ip), Group 5 received methylene blue (15 mg/kg/day) and Group 6 received ritanserin (4 mg/kg/day). The drugs were administered at 8h30 each morning. Similar to groups 1 and 2, the rats were exposed to the FST on the second last day for 15 minutes (day 27), followed by the final dosage of the day. On the next day (day 28) each rat was placed into a separate cage in the Digiscan animal motor activity monitor for assessment of locomotor activity. Thereafter each rat was allowed to swim in the FST for 5 minutes while its activity was videotaped. After the 5-minute re-stress, the rats were decapitated. The hippocampus and prefrontal cortex of each rat brain was quickly removed and frozen in liquid nitrogen (-196ºC). The tissue was subsequently stored at -80ºC until used for NOS and cGMP assay. The videotaped behavior and vertical and horizontal activity of the rats was subsequently analyzed. Immobility was defined as the absence of active, escape-orientated behaviour such as swimming, jumping and diving.

5.5.3 The sub-acute treatment groups

Group 7, 8, 9 and 10 received saline intraperitoneally (ip) for three weeks followed by one-week treatment during which time the withdrawal drugs was administered. This was done in order to access the effects of the respective withdrawal drugs alone on the various biochemical and behavioral parameters. Group 7 received memantine (5 mg/kg/day ip), Group 8 received methylene blue (15 mg/kg/day ip), Group 9 received ritanserin (4 mg/kg/day ip) and Group 10 received saline.

The drugs were administered at 8h30 each morning. Similar to groups 1,2,3,4,5,6 the rats were exposed to the FST on the second last day for 15 minutes (day 27), followed by final dosage of the day. On the next day (day 28) each rat was placed into separate cages in the Digiscan animal motor activity monitor for assessment of locomotor activity. Thereafter each rat was allowed to swim for 5 minutes in the FST while its activity was videotaped. The videotaped behaviour and vertical and horizontal activity of rats was subsequently analysed as described earlier. After the 5-minute re-stress, the rats were decapitated. The hippocampus and prefrontal cortex of each rat brain was quickly removed and frozen in liquid nitrogen (-196ºC). The tissue was subsequently stored at -80ºC until used for NOS and cGMP assay.

5.6 Tissue dissection and extraction for NOS and cGMP assays

Male Spraque-Dawley rats were sacrificed by decapitation, after which the brains were rapidly dissected on an ice-cooled slab. The hippocampi were removed, and immediately fixed in liquid nitrogen (-196ºC). The hippocampus of each rat was then stored at -80ºC until the day of NOS – and cGMP assay.

5.6.1 NOS assay

On the day of the NOS-assay, the hippocampus of each rat was removed from -80ºC storage and allowed to thaw. The tissue was then homogenized with a Teflon homogeniser (15 strokes on ice) in 1 ml homogenizing buffer containing 25mM TRIS, 1mM EDTA, and 1mM EGTA (pH = 7.2). The homogenate was then centrifuged at 10 000 g for 30 minutes at 4ºC using a Sigma 3K15 Centrifuge (Wirsam Scientific). The supernatant was then separated from the tissue pellet, and kept on ice until used for NOS assay (see section 5.8 and 5.9). The endogenous L-arginine were removed by applying the supernatant to a 0.3 ml Dowex AG-50W-×8 column, which was

pre-equilibrated with 1 ml homogenizing buffer. Dowex (Sigma) is an anion-exchange column used to separate l-citrulline, which is neutral, from l-arginine, which is positively charged. It was then eluted with 0.5 ml homogenizing buffer.

5.6.2 cGMP assay

On the day of the cGMP-assay, the hippocampus of each rat were removed from -80°C storage and allowed to thaw. The tissue was then homogenized with a Teflon homogeniser (for 1 minute on ice) at a concentration of 100 mg tissue per 1 ml 50 µM HCl Tris-EDTA buffer pH 7.5 containing EDTA (4mM). The homogenate was heated at 100°C for 3 minutes, cooled on ice and centrifuged at 2000 g for 45 minutes in a Sigma 3K15 Centrifuge (Wirsam Scientific). These conditions, i.e. Ca^{2+} chelation and heat, prevent post–mortem changes in cyclic nucleotide levels by inhibiting the action of synthesizing (NOS, sGC) and hydrolytic (phosphodiesterase) enzymes through restriction of co-factor and pH requirements (Harvey *et al.,* 1994). The supernatant was used for the determination of cGMP (see section 5.9).

5.7 Protein Determination

5.7.1 NOS assay

Protein determination of tissue homogenate carried out on the day of the NOS assay was based on the method described by Lowry (1951). The phenolyic hydroxyl groups of the amino acid-side chains present in the proteins were estimated by the Folin-Ciocalteus colour reaction. Crystalline bovine serum albumin (BSA) was used as a standard (1 mg/ml).

For the construction of a standard curve, six test tubes were each filled with 50 µl homogenizing buffer. In the first test tube, 950µl water (distilled and deionised) was added with no BSA (blank). The second received 940µl water and 10µl of BSA (equal to 10µg of protein), the third 930µl water and 20µl of BSA (equal to 20µg of protein) etc. until the sixth, which received 900µl water and 50µg of BSA (equal to 50µg of protein). For the determination of protein concentration of the homogenates, 900µl water plus 50µl of the homogenate was added.

To each tube was added 1 ml freshly prepared tartrate solution (a solution of 10% Na_2CO_3 in 0.5M NaOH containing 1% potassium tartrate and 5% $CuSO_4$ in a ratio of 100:10:1). The test tubes were incubated at room temperature for ten minutes. Thereafter, 3ml of Folin-Ciocalteus phenol reagent (diluted 1:10 with distilled, deionised water) was added to each test tube, followed by a 10 minute incubation at 50°C. The tubes were then allowed to cool to room

temperature and absorbance (A_{650}) read at a wavelength of 650 nm using a MultiScan RC spectrophotometer (Amersham). Homogenate protein concentrations were then extrapolated from a standard curve of A_{650} against protein concentration.

5.7.2 cGMP assay

cGMP levels were expressed as *f*mol/µg protein. Consequently the Bradford protein assay was used to determine the protein levels in the hippocampus of each rat. Protein blanks, standards and unknowns were prepared as follows. 2 mg BSA was dissolved in 1 ml distilled water. 100 µl of each of the following dilutions of BSA were prepared:

Protein concentration	Volume of 2mg/ml BSA	Volume of Buffer
0 mg/ml (blanco)	0 µl	100 µl
0.1 mg/ml	5 µl	95 µl
0.4 mg/ml	20 µl	80 µl
0.7 mg/ml	35 µl	65 µl
1.0 mg/ml	50 µl	50 µl
1.4 mg/ml	70 µl	30 µl

5 µl of each unknown, in triplicate, were placed into separate wells of a 96-well plate, 250 µl of the Bradford reagent were add to each well and mixed for 30 sec, and thereafter incubated at room temperature for 15 min. Absorbance (A_{650}) were read at a wavelength of 650 nm using a MultiScan RC spectrophotometer (Amersham). Homogenate protein concentrations were then extrapolated from a standard curve of A_{650} against protein concentration.

5. 8 Determination of Nitric Oxide Synthase activity

Determination of NOS activity was based on the method described by Bredt and Schmidt (1996) as recently modified for our laboratory conditions (Harvey & Nel, 2003). Radioactive arginine, the substrate for NOS, was added to brain homogenate derived from the hippocampi and the mixture was incubated. During the incubation NOS converts the arginine precursor to NO and citrulline. Adding an EDTA containing buffer terminated the reaction. The arginine-precursor was separated from the citrulline product, by using a Dowex clean-up column. Dowex (Sigma) is an anion-exchange column used to separate l-citrulline, which is neutral, from l-arginine, which is positively charged. NO and l-citrulline are formed in equal quantities. Therefore, by measuring the concentration of radioactive citrulline with a scintillation spectrometer, the amount of NO as well as the activity of NOS was determined.

A reaction cocktail was prepared containing the following:

1M TRIS (pH = 7.4)
1mM BH_4
4mM DTT
0.1mM FAD
0.1mM FMN
10mM NADPH
10 µg/µl Calmodulin
5 µM L-[^{3}H]arginine
125mM $CaCl_2$
Distilled water (so that final volume is 500 µl)

All of above were incubated for 2 minutes at 37ºC. The reaction was initiated by adding tissue homogenates containing approximately 50 µg protein to the reaction cocktail and incubated for 10 minutes at 37ºC. After 10 minutes, the samples were removed from incubation and the reaction stopped by dilution with 500 µl of ice-cold stop-buffer (50 mM HEPES, 5mM EDTA, pH = 5.5).

Reaction mixtures were then applied to 1 ml Dowex AG-50W-× 8 columns that were pre-equilibrated with 3×1 ml stopbuffer. Thereafter, 2×0.5 ml stopbuffer was added to columns and eluted into scintillation vials containing 3 ml scintillation liquid. The eludated L- [^{3}H]citrulline activity was measured by scintillation counting (TriCarb 2100TR, Packard United Technologies). Blanks, containing all the reagents except the tissue homogenates, were used to determine the extent of L-[^{3}H]citrulline formation in the absence of NOS activity, and 5 µM L-[^{3}H]arginine alone was used to determine total counts per picomole.

NOS activity was expressed as picomoles citrulline formed per mg protein present in the homogenate per minute (pmol/mg/min).

5.9 Determination of Cyclic GMP

Cyclic GMP levels were determined using the radioimmunoassay method described by Amersham Ltd, Buckinghamshire, U.K.

Cyclic GMP functions as an important second messenger for NO, and include mediating the effect of NO on smooth muscle relaxation, platelet aggregation and adhesion,

neurotransmission and hormonal secretion (Chapter 4). Measuring both NO and cGMP levels can be of potential value to detect and follow alterations in the NO-cGMP pathway after pharmacological or pathological changes during depression. The use of a radio-immunoassay method has enabled the determination of hippocampal cGMP levels and, in combination with the NOS Enzyme activity assay, the action of the NO-cGMP pathway in depression can be reflected.

The competition between unlabelled cGMP and a fixed quantity of the tritium labelled compound for binding to an antiserum form the basis of this assay. The antiserum has a high affinity and specificity for cGMP. The amount of labelled cGMP bound to the antiserum is inversely related to the amount of cGMP present in the sample. Ammonium sulphate is used in the assay to separate the antibody-bound cGMP from the unbound nucleotide, followed by centrifugation. The precipitate containing the antibody-bound complex is dissolved in water and its activity determined by liquid beta-scintillation counting. The concentration of unlabelled cGMP in the sample is then determined from a linear standard curve.

120 assay tubes in duplicate were set up plus enough tubes to cover the unknown samples. 50 µl tritiated cGMP was added to all the tubes. Tubes one and two with 100 µl Tris-EDTA buffer reflect the zero-dose binding. In six pairs of tubes respectively, 100 µl of each cGMP serial dilution (0,06–1pmol) was added to provide a standard calibration curve. Tube fifteen and sixteen contained 100 µl of blank reagent (blank counts per minute). The remainder of the allotted tubes received 100 µl of unknown in duplicate. Antiserum (50 µl) was added to all the tubes, which were then mixed and placed in a refrigerator at 2-8°C for 1.5 hours.
After 1.5 hours, 60% saturated, ice-cold, ammonium sulphate was added to all the tubes, mixed and allowed to stand for 5 min before centrifugation at 12000 rpm for 2 minutes. The resulting supernatant was decanted and discarded and 1.1 ml distilled water was used to dissolve the sediment. From each tube, 1 ml aliquots were removed, placed into a counting vial containing a water-soluble scintillant and radioactivity was determined using a TriCarb 2100TR Scintillation Counter (Packard United Technologies). Data were expressed as *f*mol cGMP /µg protein.

5.10 Statistical analysis

Statistical analysis was done in collaboration with the Statistical Consultation Service of the North West University (Potchefstroom campus).

All of the chronic, withdrawal and sub-acute study's data were analyzed using a one-way analysis of variance (ANOVA). If statistical significant differences were observed across the

groups using the ANOVA test, post hoc tests were performed to compare means of different treatment groups using the Tukey or Dunnett's test.

All data are expressed as the mean ± SEM with statistical significance defined at the 95% ($p<0.05$) level.

Chapter 6 RESULTS

6.1 Introduction

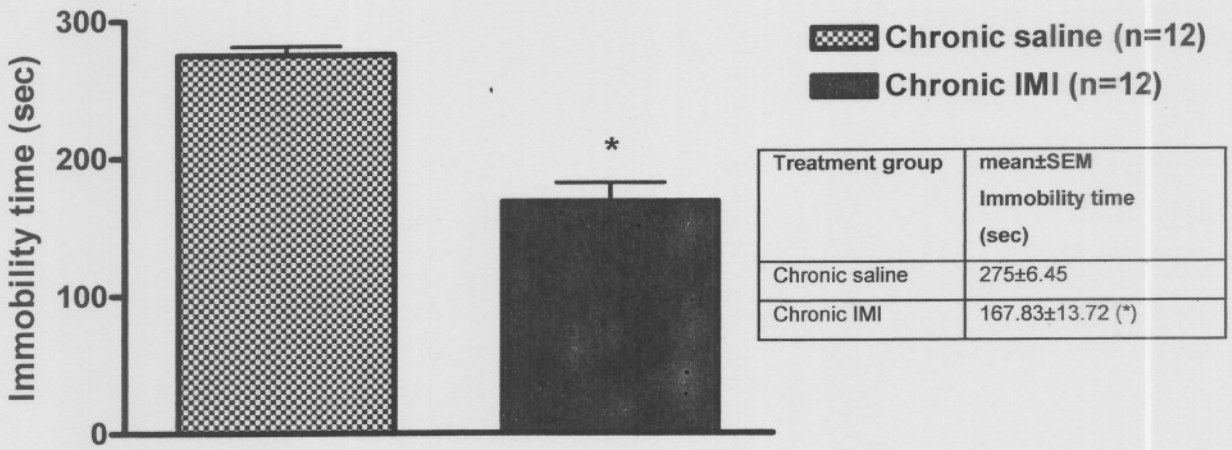
The current study consists of three parts, 1) a chronic antidepressant study, 2) an antidepressant withdrawal study, and 3) a sub-acute antidepressant study. In this chapter the results of all the behavioral evaluations and neurochemical analyses (as described in chapter 5), will be presented.

6.2 Chronic imipramine vs control group

Rats received chronic imipramine (15 g/kg/day ip) or chronic saline (control group) for 21 days. After treatment, the animals were studied in the FST as well as subsequent evaluation of locomotor activity. The hippocampi of each rat was used for the NOS enzyme activity assay as well as for the assay of cGMP (as described in chapter 5). Data were analysed using a one-way analysis of variance (ANOVA) across all the groups, and were subsequently subjected to the Tukey post-hoc test. Data were expressed as the mean ± SEM with statistical significance defined at the 95% ($p<0.05$) level. Although all the groups were analysed together, since they were treated over the same period, individual one-on-one comparisons were necessary and are presented graphically for greater emphasis.

6.2.1 Forced swimming test (FST)

Data obtained from the ANOVA revealed a significant difference between the treatment groups [F(2;30)=37.73;p=0.00001]. Data presented in Figure 6.1 show the outcome of chronic IMI treatment for 21 days on swim immobility time compared to chronic saline treatment. Chronic IMI induced a significant decrease in immobility time compared to chronic saline treatment. (p=0.0001;Tukey's test;Figure 6.1).



Treatment group	mean±SEM Immobility time (sec)
Chronic saline	275±6.45
Chronic IMI	167.83±13.72 (*)

*p=0.0001 vs chronic saline (Tukey's test)

Figure 6.1: The effect of chronic IMI (15 mg/kg/day ip) treatment on the duration of swim immobility time in the FST (mean ± SEM). Descriptive statistics are provided in the table insert.

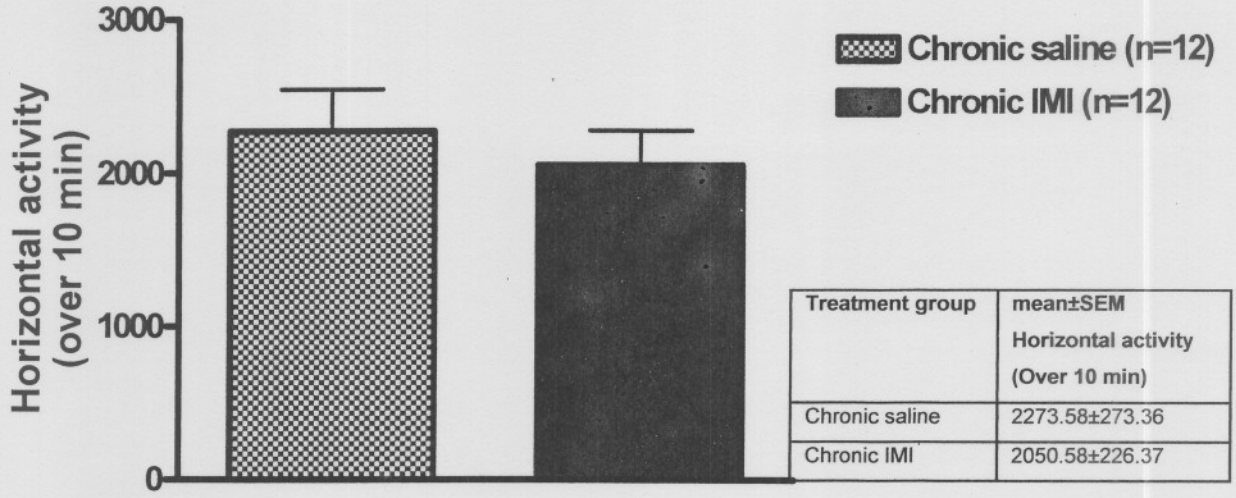
6.2.2 Locomotor Activity

Locomotor activity was evaluated to ensure that changes in swim motivation are based on an antidepressant response, and not due to an indirect effect of the drug on locomotor activity (section 5.4).

Data obtained from the ANOVA revealed no difference across the groups with respect to horizontal activity [F(2;30)=0.83;p=0.447], and vertical activity [F(2;30)=1.26;p=0.298). Data, obtained from Tukey's test, presented in Figure 6.2, show the effect of chronic IMI (21 days) on horizontal locomotor activity (a) and vertical locomotor activity (b) compared to the control saline treated group (21 days). With regards to horizontal activity, chronic IMI showed no difference to chronic saline.

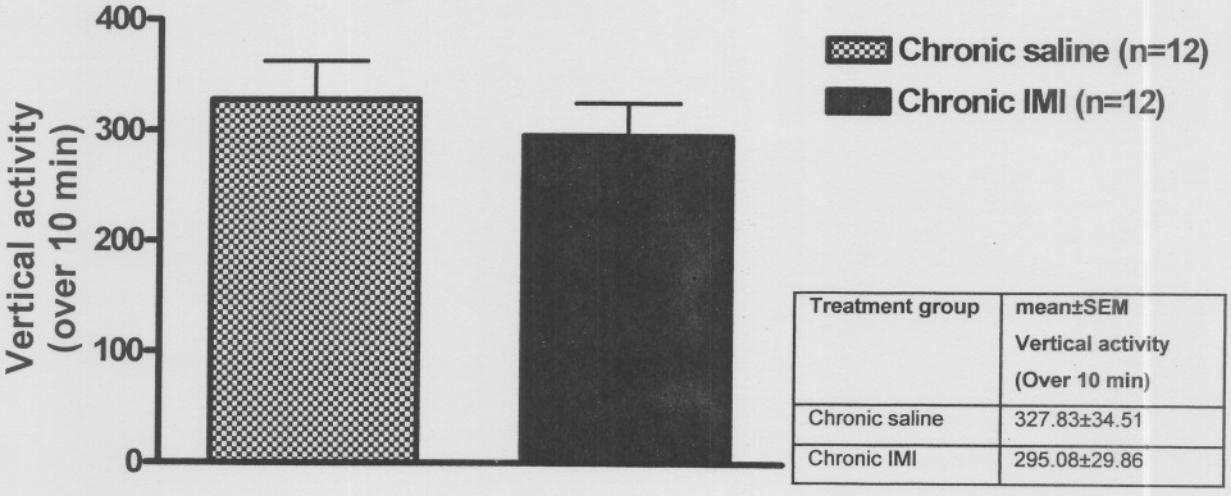
Vertical activity after chronic IMI treatment similarly showed no difference to chronic saline treatment. These data confirm that the anti-immobility effects of IMI depicted in Fig 6.1 are indicative of an antidepressant effect, and that these actions are not due to an indirect effect of the drug on locomotor activity (section 5.4).

(a)



Treatment group	mean±SEM Horizontal activity (Over 10 min)
Chronic saline	2273.58±273.36
Chronic IMI	2050.58±226.37

(b)



Treatment group	mean±SEM Vertical activity (Over 10 min)
Chronic saline	327.83±34.51
Chronic IMI	295.08±29.86

Figure 6.2: Behavioral analysis (over 10 minutes) of chronic IMI treatment (15 mg/kg/day ip) on horizontal (a) and vertical (b) locomotor activity (mean ± SEM). Descriptive statistics are provided in the table inserts.

6.2.3 NOS Enzyme Activity

As for any quantitative enzyme assay, it is important to verify that reaction conditions are such that the assay is linear with respect to time and tissue concentration (Bredt & Smith, 1996). Figure 6.3 is a representative standard curve, which indicates that concentration citrulline formation is linear with respect to time under the current laboratory conditions.

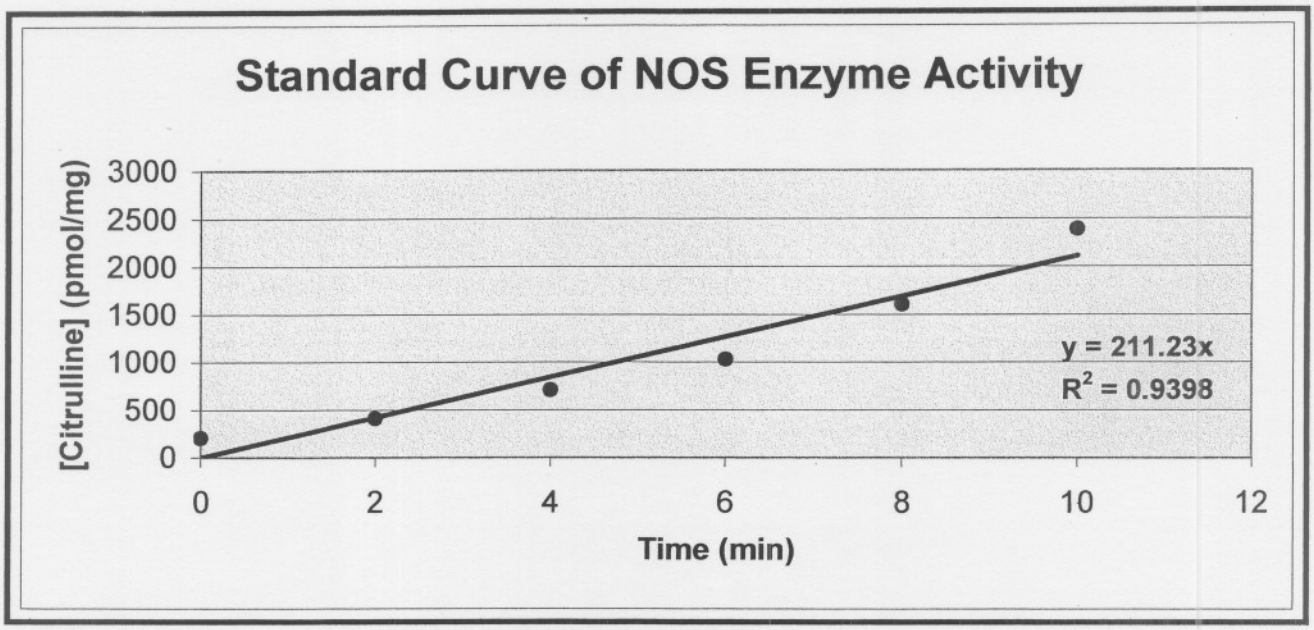
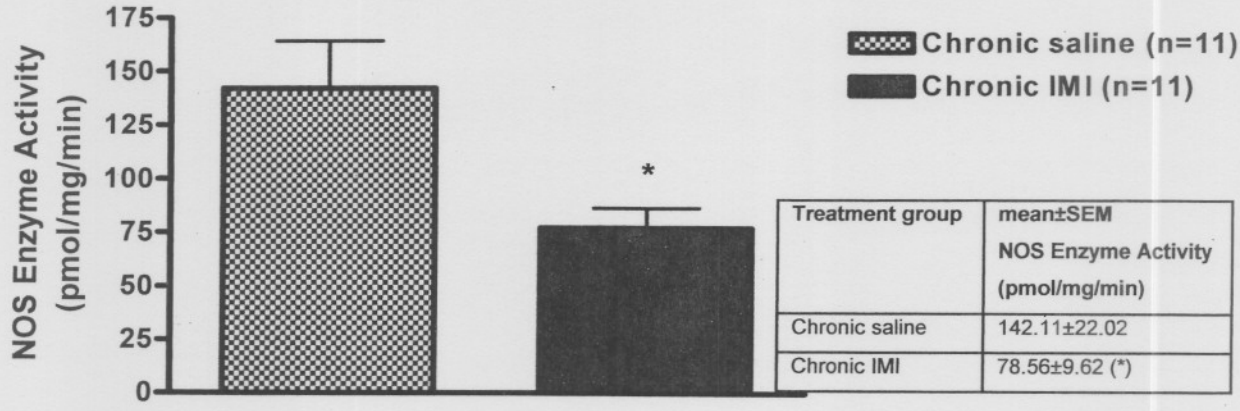


Figure 6.3: A representative standard curve of hippocampal NOS enzyme activity at various times over 10 min.

Data obtained from the one-way ANOVA revealed a significant difference in NOS enzyme activity across the treatment groups [F(2;30)=29.07;p=0.00001). Data presented in figure 6.4 shows that NOS activity was significantly decreased after chronic IMI treatment compared to the control group (p=0.019;Tukey's test;Figure 6.4).



Treatment group	mean±SEM NOS Enzyme Activity (pmol/mg/min)
Chronic saline	142.11±22.02
Chronic IMI	78.56±9.62 (*)

*p=0.019 vs chronic saline (Tukey's test)

Figure 6.4: The effect of chronic IMI (15 mg/kg/day ip) treatment (21 days) on NOS enzyme activity (mean ± SEM). Descriptive statistics are provided in the table insert.

6.2.4 Cyclic GMP Levels

Rat hippocampal extracts were prepared as described in section 5.6.2, and assayed according to the procedure described in section 5.9. Amersham reports the cGMP data as pmol/incubation tube or pmol/200µl. A representative standard curve performed under current laboratory conditions is shown in Figure 6.5. However, because the protein concentration of each rat hippocampus differs, it was decided to express all experimental data in the study as *f*mol cGMP/µg protein. Protein determination of each rat hippocampus was done as described in section 5.7.2.

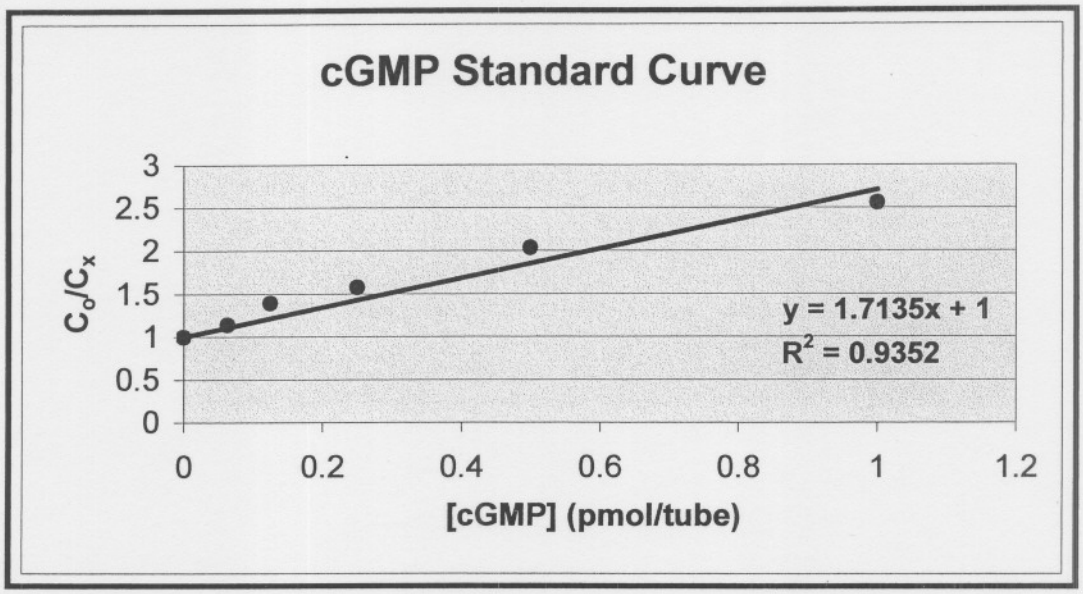
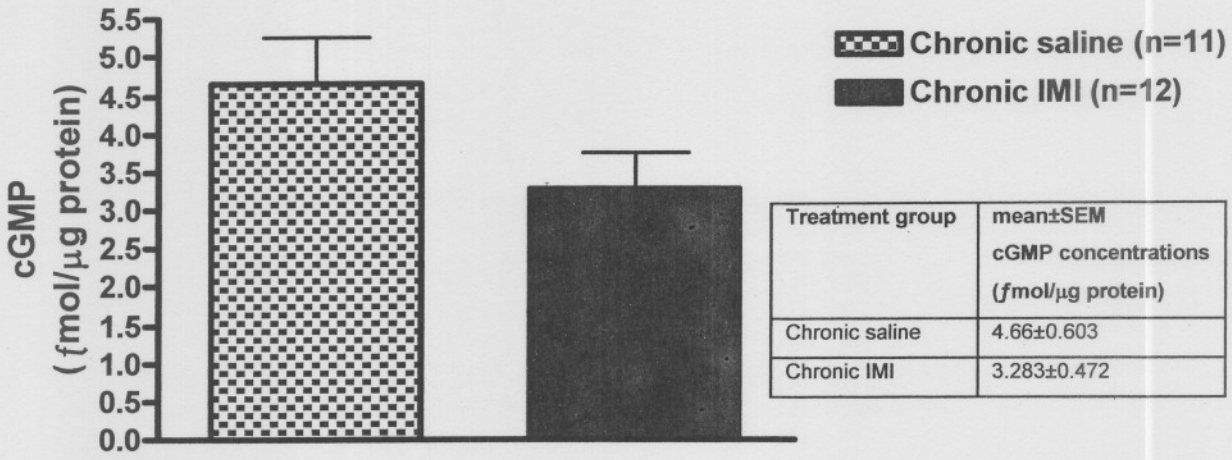


Figure 6.5: Representative standard curve for the cGMP assay from which the concentration in the sample is determined by extrapolation. Co/Cx = Counts per minute in the absence of unlabelled cGMP/ Counts per minute in the absence of standard or unknown cGMP.

Data obtained from the one-way ANOVA revealed no differences in hippocampal cGMP levels after chronic treatment with imipramine [F(2;30)=1.148;p=0.331). Subsequently, the Tukey test also revealed no differences when comparing chronic IMI treatment with control group (Figure 6.6), although there was a distinct trend towards reduced levels in the IMI group.



Treatment group	mean±SEM cGMP concentrations (fmol/µg protein)
Chronic saline	4.66±0.603
Chronic IMI	3.283±0.472

Figure 6.6: The effect of chronic IMI (15 mg/kg/day ip) treatment (21 days) on rat hippocampal cGMP levels (mean ± SEM). Descriptive statistics are provided in the table insert.

6.3 Antidepressant withdrawal study

Rats received imipramine for 21 days, whereafter they were withdrawn from the drug for 7 days, receiving saline ip during this time. This group formed the IMI withdrawal group. Comparing this group with the chronic imipramine and chronic saline treated groups demonstrated the effect of imipramine's withdrawal on the behavioral models and neurochemical parameters described earlier. The aim of this study is to confirm the behavioral and neurochemical effects of IMI, since these actions should be qualitatively reversed when the drug is withdrawn. Furthermore, this study hopes to shed more light on the neurobiological consequences and possible sequelae of inappropriate AD discontinuation after chronic administration.

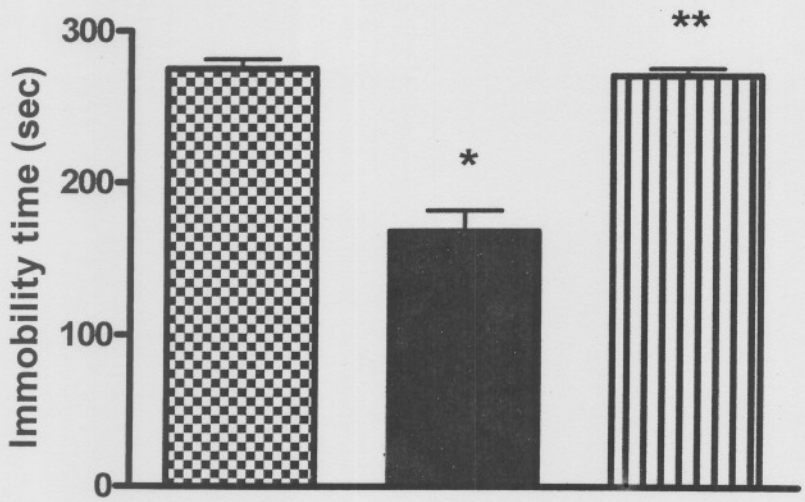
In the second part of the withdrawal study, three groups of animals received IMI for 21 days and again withdrawn from IMI for 7 days. However, instead of receiving saline during withdrawal, they received either memantine, methylene blue (MB), or ritanserin at dosages described in Chapter 5. Considering the profound effects of IMI treatment on NOS-cGMP after chronic treatment described in section 6.2, comparing these groups with the imipramine withdrawal group, would determine whether the effects of IMI withdrawal on NOS and cGMP could be

reversed with drugs that act selectively on specific targets on the NO-pathway. Data are expressed as the mean ± SEM with statistical significance defined at the 95% ($p<0.05$) level.

6.3.1 Effects of imipramine withdrawal

6.3.1.1 Forced swimming test (FST)

Data obtained from ANOVA revealed a significant difference between the treatment groups [$F(2;30)=37.73;p=0.00001$]. Data presented in Figure 6.7 show the effect of IMI withdrawal on swim immobility time compared to the control saline and chronic IMI treatment groups (21 days). IMI withdrawal induced a significant increase in immobility time compared to chronic IMI treatment ($p=0.0001$;Tukey's test;Figure 6.7) approaching levels obtained with the saline treated group. The effect of chronic IMI treatment compared to the saline treated group is also presented on this figure for visual comparison of the effect of IMI withdrawal on behaviour (Section 6.2).



Treatment group	mean±SEM Immobility time (sec)
Chronic saline	275±6.45
Chronic IMI	167.83±13.72 (*)
IMI + saline	270.17±4.79 (**)

*p=0.0001 vs chronic saline (Tukey's test)

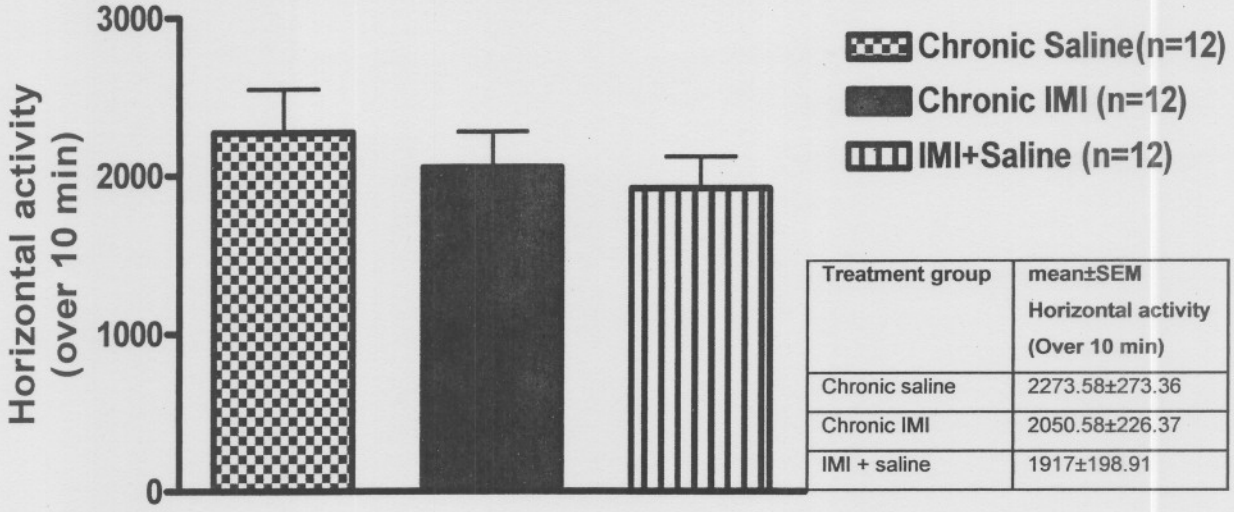
**p=0.0001 vs chronic IMI (Tukey's test)

Figure 6.7: The effect of chronic IMI treatment and IMI withdrawal on the duration of swim immobility time in the FST (mean ± SEM). Descriptive statistics are provided in the table insert.

6.3.1.2 Locomotor Activity

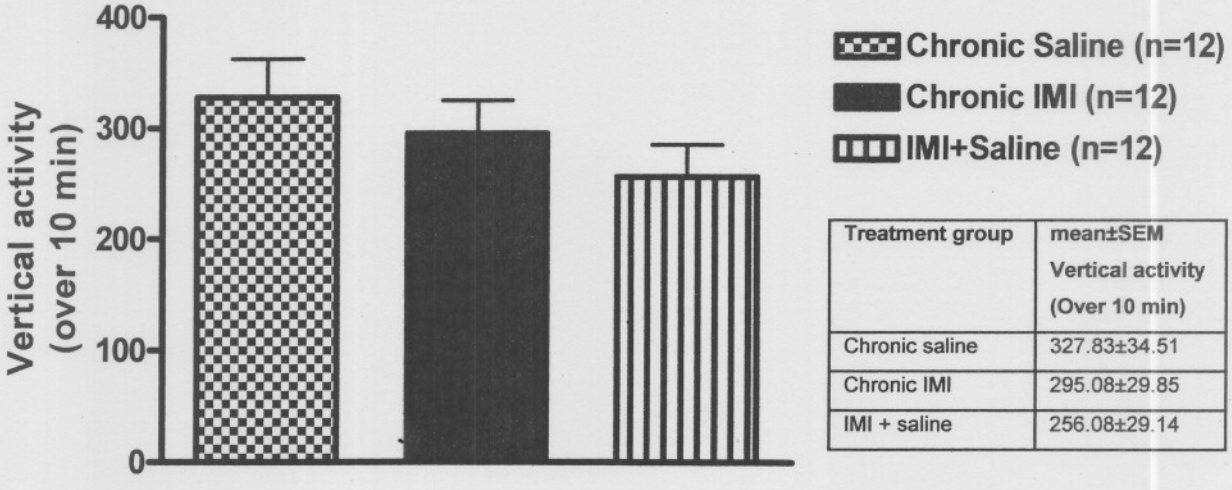
Data obtained from ANOVA revealed no differences between the treatment groups, with respect to horizontal activity [F(2;30)=0.83;p=0.447), or vertical activity [F(2;30)=1.26;p=0.298). Data, obtained from Tukey's test, presented in Figure 6.8 (a) + (b) reveal little difference in horizontal activity (a) or vertical activity (b) between the groups. Thus, IMI and IMI withdrawal had little effect on locomotor activity of the animals.

(a)



Treatment group	mean±SEM Horizontal activity (Over 10 min)
Chronic saline	2273.58±273.36
Chronic IMI	2050.58±226.37
IMI + saline	1917±198.91

(b)

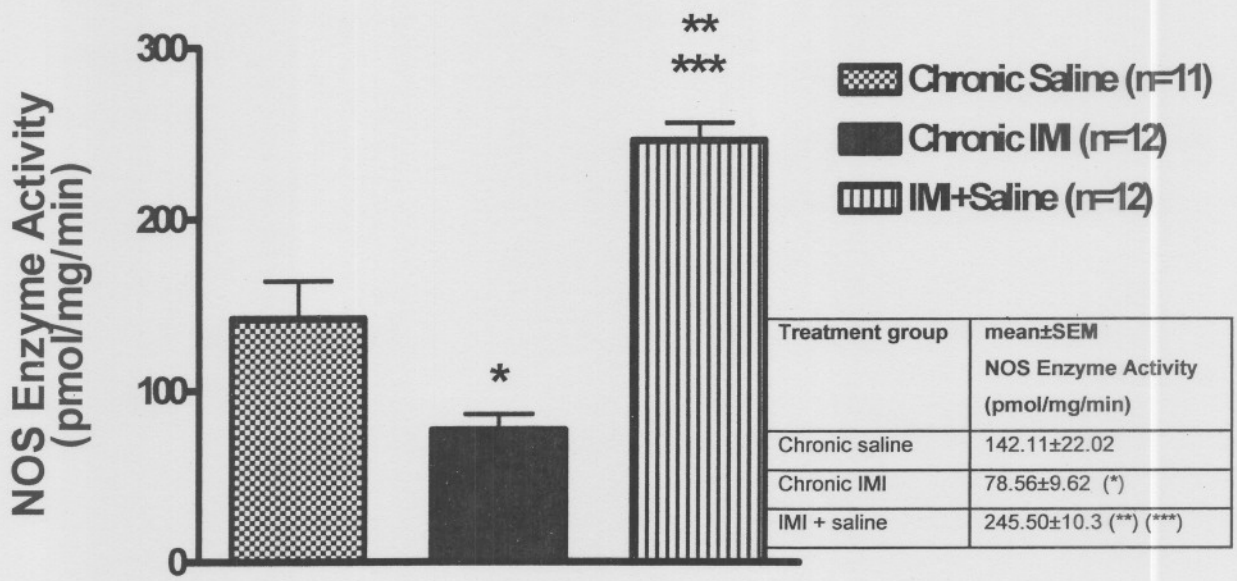


Treatment group	mean±SEM Vertical activity (Over 10 min)
Chronic saline	327.83±34.51
Chronic IMI	295.08±29.85
IMI + saline	256.08±29.14

Figure 6.8: Behavioral analysis (over 10 minutes) of saline, chronic IMI (15 mg/kg/day ip) and IMI withdrawal on horizontal locomotor activity (a) and vertical locomotor activity (b) (mean ± SEM). Descriptive statistics are provided in the table inserts.

6.3.1.3 NOS Enzyme Activity

Data obtained from the one-way ANOVA revealed a significant difference in the NOS activity across the various groups [F(2;30)=29.07;p=0.00001). Data presented in figure 6.9 show that NOS activity significantly decreased after chronic IMI treatment compared to the control saline treated group (p=0.019;Tukey's test;Figure 6.9) while NOS activity significantly increased after IMI withdrawal compared with chronic IMI treatment (p=0.0001;Tukey's test;Figure 6.9). Of interest is that NOS activity was also significantly increased after IMI withdrawal, compared to the control saline treated group as well (p=0.0003;Tukey's test;Figure 6.9).



Treatment group	mean±SEM NOS Enzyme Activity (pmol/mg/min)
Chronic saline	142.11±22.02
Chronic IMI	78.56±9.62 (*)
IMI + saline	245.50±10.3 (**) (***)

*p=0.019 vs chronic saline (Tukey's test)

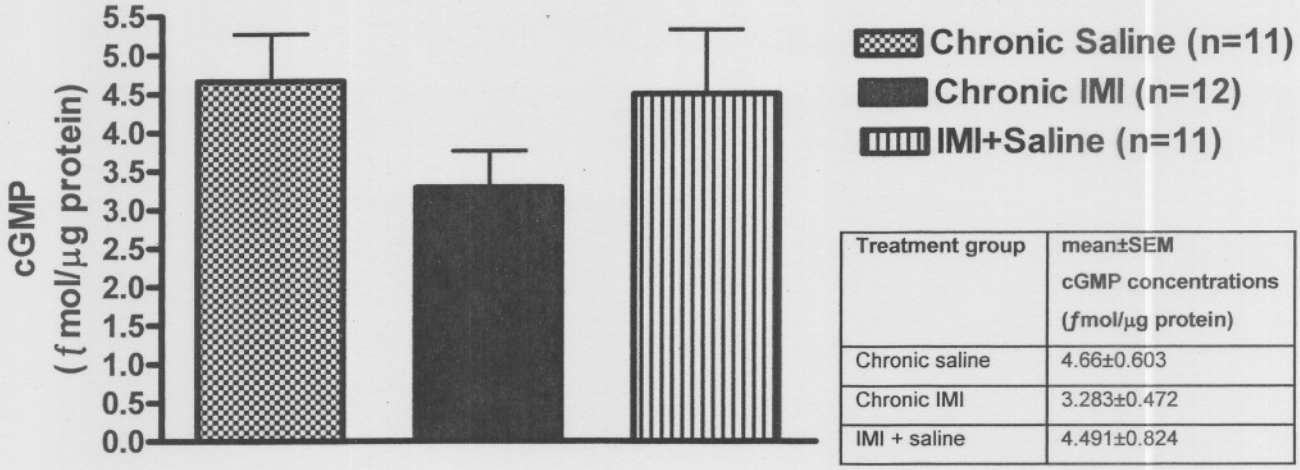
**p=0.0001 vs chronic IMI (Tukey's test)

***p=0.0003 vs chronic saline (Tukey's test)

Figure 6.9: The effect of chronic imipramine treatment and imipramine withdrawal on NOS enzyme activity (mean ± SEM). Descriptive statistics are provided in the table insert.

6.3.1.4 Cyclic GMP Levels

One-way ANOVA showed no differences between the different treatment regimens on cGMP levels in the hippocampus [F(2;30)=1.148;p=0.331]. Tukey's test similarly revealed no difference on cGMP levels in the hippocampus after chronic IMI treatment or after withdrawal (Figure 6.10), although cGMP levels tended to increase back to chronic saline treatment levels after IMI withdrawal.



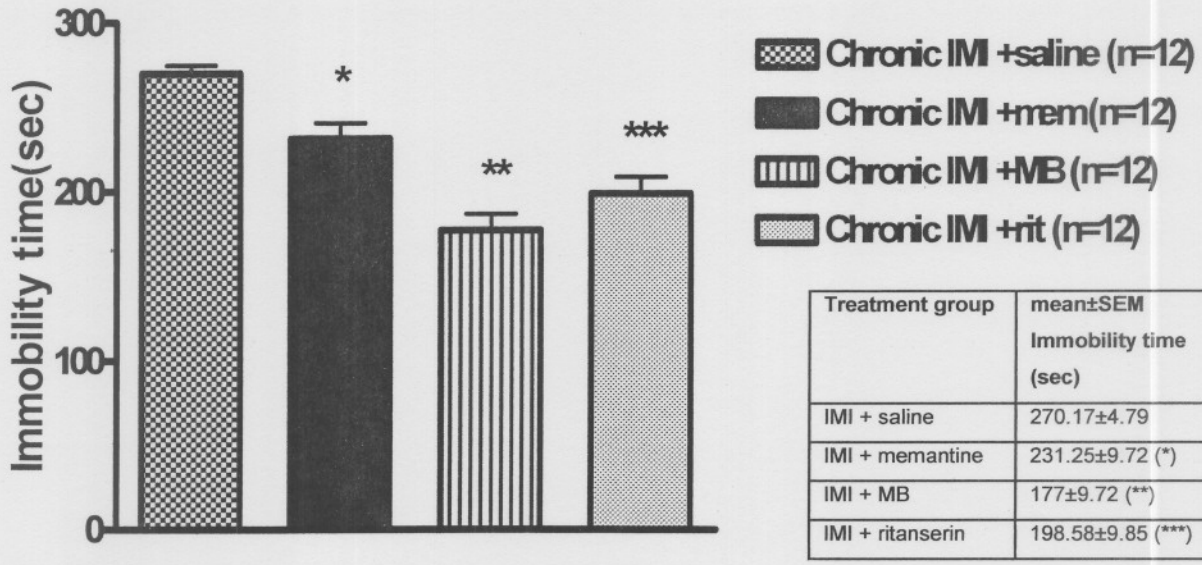
Treatment group	mean±SEM cGMP concentrations (fmol/µg protein)
Chronic saline	4.66±0.603
Chronic IMI	3.283±0.472
IMI + saline	4.491±0.824

Figure 6.10: The effect of chronic IMI treatment and IMI withdrawal on hippocampal cGMP levels (mean ± SEM). Descriptive statistics are provided in the table insert.

6.3.2 Effects of drug treatments on imipramine withdrawal

6.3.2.1 Forced swimming test (FST)

Data obtained from the one-way ANOVA for the FST showed significant differences across the groups [F(3;42)=22.02;p=0.0001). The withdrawal effects of IMI on swim immobility time were significantly reversed by memantine, methylene blue and ritanserin administered during the 7-day withdrawal period (p<0.001;Dunnett's test;Figure 6.11).



Treatment group	mean±SEM Immobility time (sec)
IMI + saline	270.17±4.79
IMI + memantine	231.25±9.72 (*)
IMI + MB	177±9.72 (**)
IMI + ritanserin	198.58±9.85 (***)

*p=0.002 vs chronic IMI + saline (Dunnett's test)

**p=0.000009 vs chronic IMI + saline (Dunnett's test)

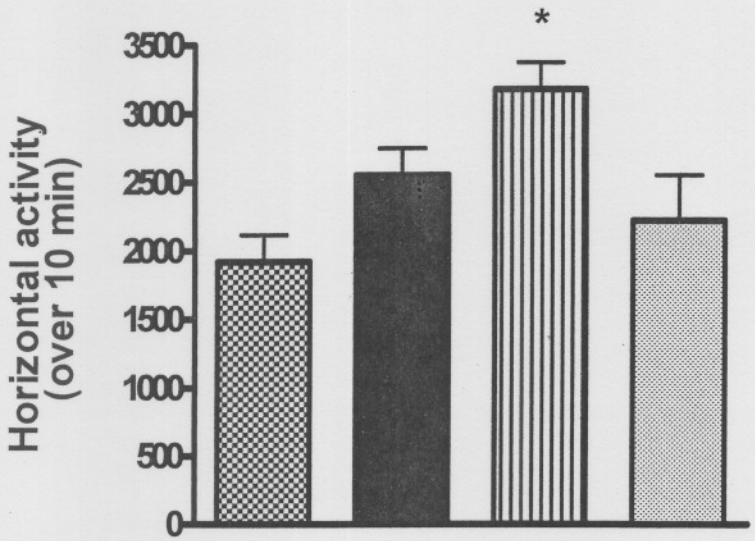
***p=0.00001 vs chronic IMI + saline (Dunnett's test)

Figure 6.11: Effects of memantine, methylene blue and ritanserin on IMI withdrawal during the FST (mean ± SEM). Descriptive statistics are provided in the table insert.

6.3.2.2 Locomotor Activity

Data obtained from one-way ANOVA showed a significant difference between the groups with regards to horizontal locomotor activity [F(3;42)=4.666;p=0.0067] but not vertical locomotor activity [F(3;42)=1.928;p=0.1397]. Data obtained from Dunnett's test reveal that only methylene blue significantly altered horizontal locomotor behavior (Figure 6.12a;p=0.003;Dunnett's test), while no drug altered vertical activity (Figure 6.12b).

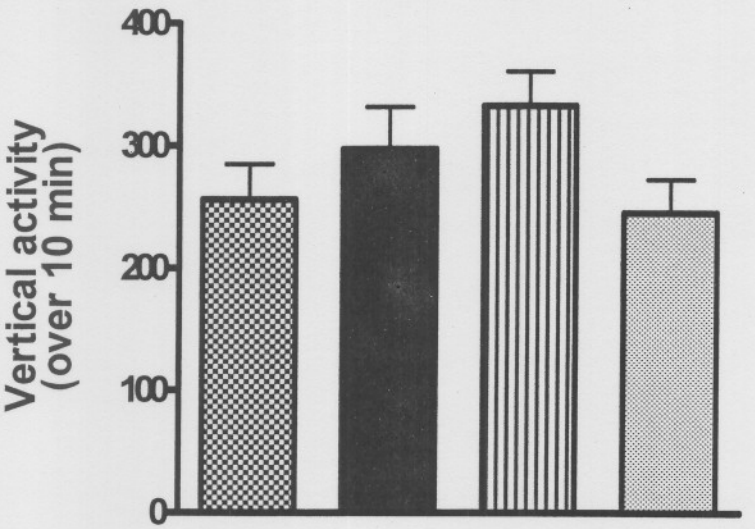
(a)



Treatment group	mean±SEM Horizontal activity (Over 10 min)
IMI + saline	1917±198.91
IMI + memantine	2549.92±196.94
IMI + MB	3171.58±196.43 (*)
IMI + ritanserin	2219.25±327.72

*p=0.003 vs chronic IMI + saline (Dunnett's test)

(b)

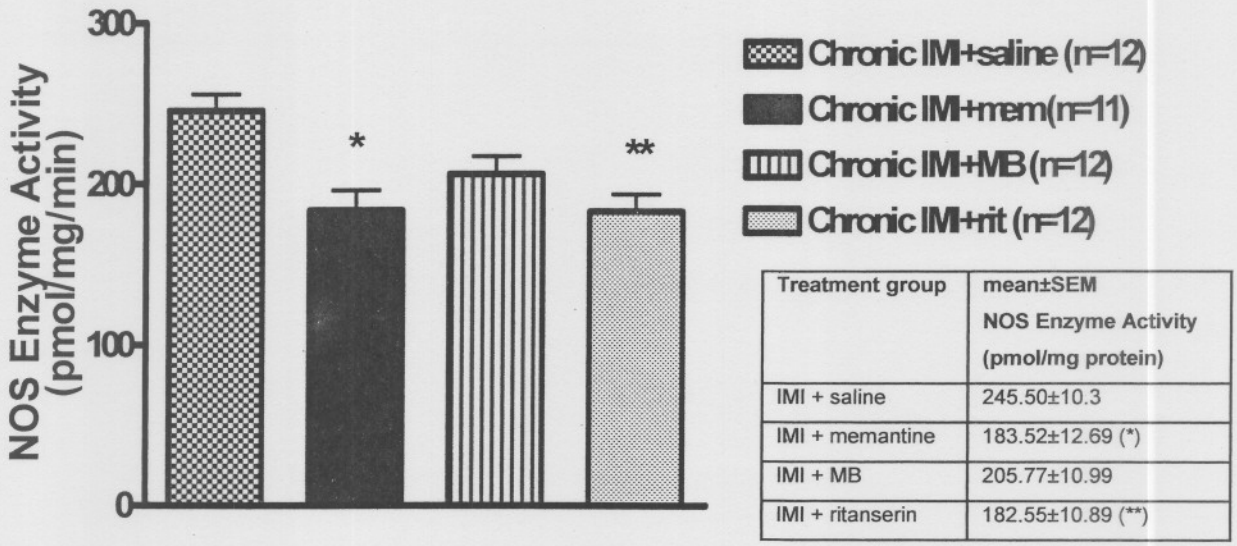


Treatment group	mean±SEM Horizontal activity (Over 10 min)
IMI + saline	256.08±29.14
IMI + memantine	297.42±34.10
IMI + MB	332.58±27.85
IMI + ritanserin	245±27.62

Figure 6.12: The effects of memantine, methylene blue and ritanserin, administered during the 7-day withdrawal period, on horizontal locomotor activity (a) and vertical locomotor activity (b) (mean ± SEM). Descriptive statistics are provided in the table inserts.

6.3.2.3 NOS Enzyme Activity

Data obtained from the one-way ANOVA for NOS activity showed significant differences across the groups [$F(3;42)=5.869;p=0.0019$]. Memantine and ritanserin, administered during the 7-day withdrawal period, significantly reversed the effect of IMI withdrawal on NOS activity (Figure 6.13;$p<0.01$;Dunnett's test), while methylene blue evoked a similar response, albeit not significantly.



Treatment group	mean±SEM NOS Enzyme Activity (pmol/mg protein)
IMI + saline	245.50±10.3
IMI + memantine	183.52±12.69 (*)
IMI + MB	205.77±10.99
IMI + ritanserin	182.55±10.89 (**)

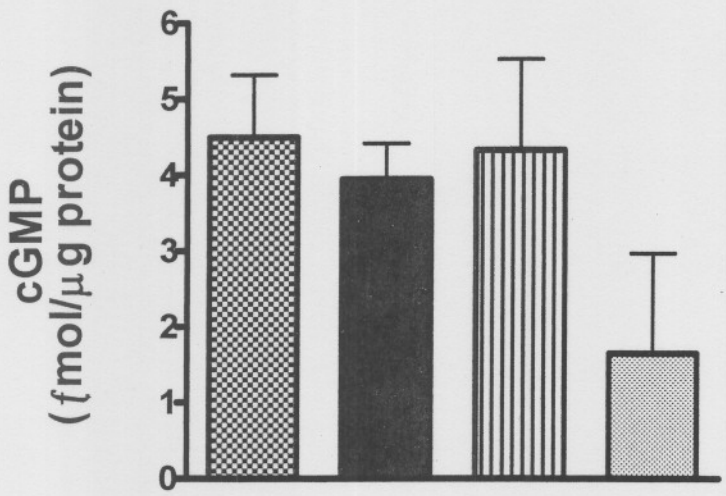
*p=0.003 vs chronic IMI+saline (Dunnett's test)

**p=0.002 vs chronic IMI+saline (Dunnett's test)

Figure 6.13: Effects of memantine, methylene blue and ritanserin on NOS activity when administered during the 7 day IMI withdrawal period (mean ± SEM). Descriptive statistics are provided in the table insert.

6.3.2.4 Cyclic GMP Levels

Data obtained from the one-way ANOVA revealed no changes in cGMP levels in the hippocampus across the various groups [F(3;42)=1.488;p=0.231]. Memantine and methylene blue were unable to change increased cGMP levels after IMI withdrawal, although ritanserin demonstrated a distinct suppresive action on this response, but did not reach significance (Figure 6.14).



Treatment group	mean±SEM cGMP concentrations (fmol/µg protein)
IMI + saline	4.491±0.824
IMI + memantine	3.942±0.472
IMI + MB	3.8±1.19
IMI + ritanserin	1.65±1.312

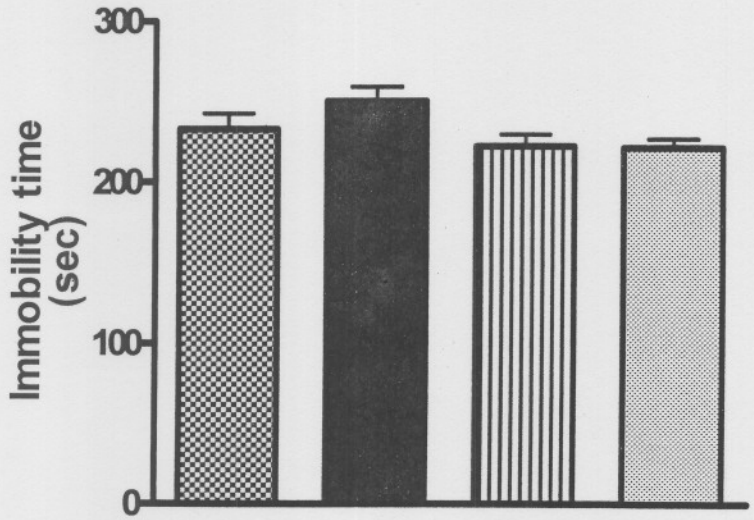
Figure 6.14: Effects of memantine, methylene blue and ritanserin on cGMP levels in hippocampus when administered during the 7 day IMI withdrawal period (mean ± SEM). Descriptive statistics are provided in the table insert.

6.4 Sub-acute study

In order to determine the effects of saline, memantine, methylene blue and ritanserin alone under similar treatment conditions, a sub-acute study was performed. Four groups of rats received saline for 21 days, whereafter one of the above mentioned drugs or saline was administered for 7 days for a total treatment period of 28 days (as described in chapter 5).

6.4.1 Forced swimming test (FST)

Data obtained from the one-way ANOVA for swim immobility time revealed significant differences across the groups [F(3;43)=3.154;p=0.034]. However, according to Dunnett's test, no significant differences were found between the groups (Figure 6.15).



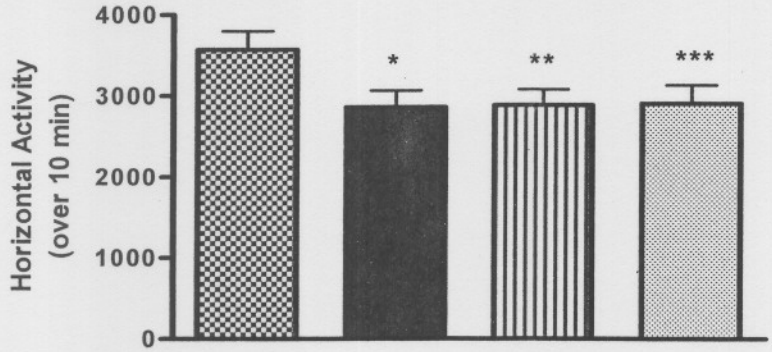
Treatment group	mean±SEM Immobility time (sec)
Saline (28 days)	233±9.95
Saline + memantine	250.33±9.04
Saline + MB	222.33±7.29
Saline + ritanserin	221.25±5.61

Figure 6.15: Effects of sub-acute memantine, methylene blue and ritanserin treatment on swim immobility time compared to the chronic + sub-acute saline treated group (28 days) (mean ± SEM). Descriptive statistics are provided in the table insert.

6.4.2 Locomotor Activity

Data obtained from the one-way ANOVA revealed significant differences among the various groups with regards to horizontal locomotor activity [F(3;43)=3.904;p=0.015] and vertical locomotor activity [F(3;43)=4.167;p=0.011] of the groups. Memantine, methylene blue and ritanserin all significantly decreased horizontal activity compared to saline (p<0.05;Figure 6.16a). However, only methylene blue significantly decreased vertical activity compared to saline (p=0.004;Figure 6.16b).

(a)



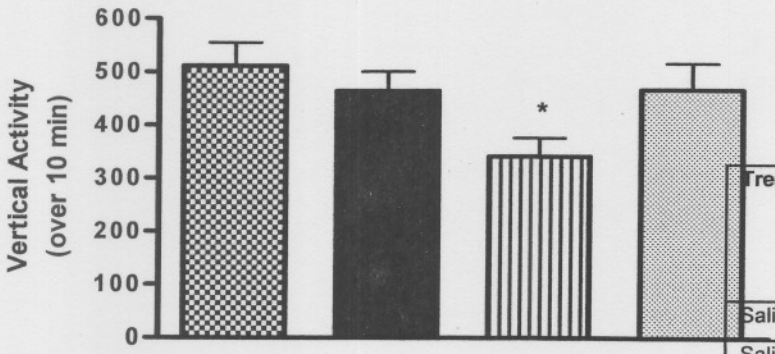
Treatment group	mean±SEM Horizontal activity (Over 10 min)
Saline (28 days)	3570.33±232.82
Saline + memantine	2861.33±204.02 (*)
Saline + MB	2886.25±189.27 (**)
Saline + ritanserin	2901.75±224.36 (***)

*p=0.016 vs saline (Dunnett's test)

**p=0.020 vs saline (Dunnett's test)

***p=0.023 vs saline (Dunnett's test)

(b)



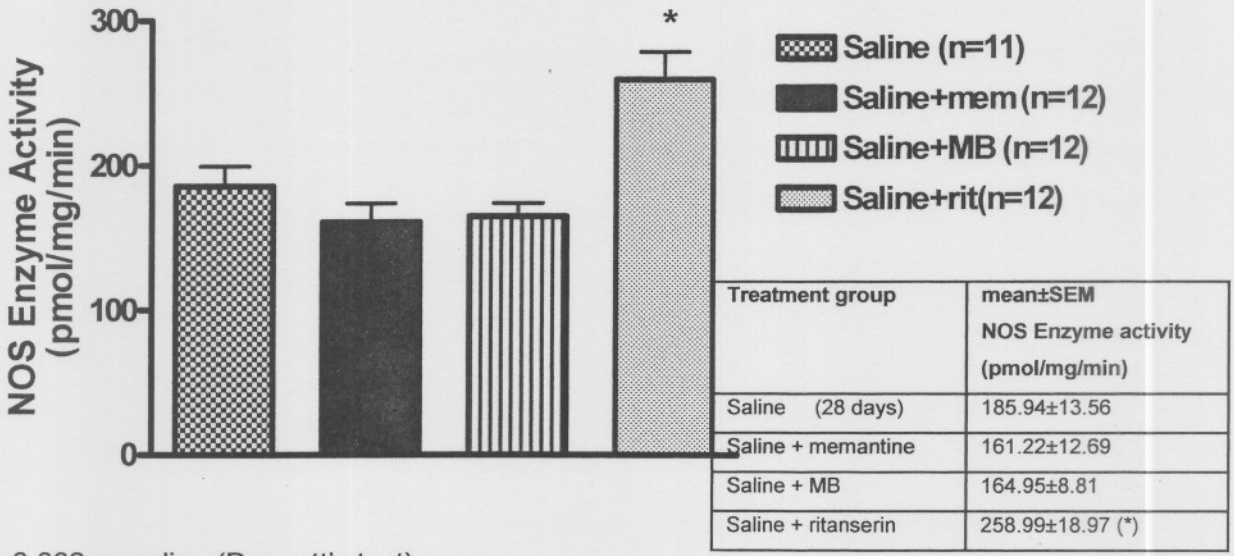
Treatment group	mean±SEM Vertical activity (Over 10 min)
Saline (28 days)	512.08±43.34
Saline + memantine	464.83±35.82
Saline + MB	341.42±34.33 (*)
Saline + ritanserin	466.83±49.40

*p=0.004 vs saline (Dunnett's test)

Figure 6.16: The effects of sub-acute memantine, methylene blue and ritanserin, on horizontal locomotor activity (a) and vertical locomotor activity (b) compared to the chronic + sub-acute saline treated group (28 days) (mean ± SEM). Descriptive statistics are provided in the table insert.

6.4.3 NOS Enzyme Activity

Data obtained from the one-way ANOVA for NOS activity revealed significant differences across the various groups [F(3;43)=10.741;p=0.00002]. Memantine and methylene did not significantly alter NOS enzyme activity, while ritanserin significantly increased NOS activity (p=0.002;Dunnett's test; Figure 6.17).



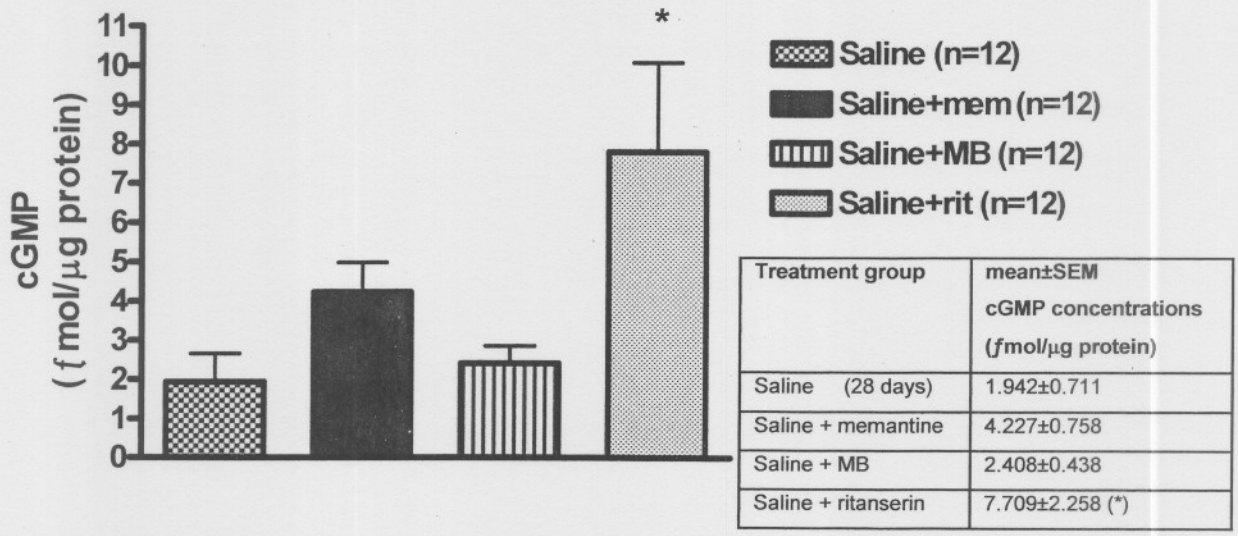
Treatment group	mean±SEM NOS Enzyme activity (pmol/mg/min)
Saline (28 days)	185.94±13.56
Saline + memantine	161.22±12.69
Saline + MB	164.95±8.81
Saline + ritanserin	258.99±18.97 (*)

*p=0.002 vs saline (Dunnett's test)

Figure 6.17: Effects of sub-acute memantine, methylene blue and ritanserin on NOS activity compared to the chronic + sub-acute saline treated group (28 days) (mean ± SEM). Descriptive statistics are provided in the table insert.

6.4.4 Cyclic GMP Levels

Data obtained from the one-way ANOVA for cGMP showed significant differences across the various groups [F(3;43)=4.232;p=0.010]. However, treatment with memantine and methylene did not significantly change cGMP levels in rat hippocampus. Ritanserin, however, significantly increased cGMP levels in rat hippocampus compared to the chronic + sub-acute saline treated group (28 days) (p=0.008;Dunnett's test;Figure 6.18).



Treatment group	mean±SEM cGMP concentrations (*f*mol/µg protein)
Saline (28 days)	1.942±0.711
Saline + memantine	4.227±0.758
Saline + MB	2.408±0.438
Saline + ritanserin	7.709±2.258 (*)

*p=0.008 vs saline (Dunnett's test)

Figure 6.18: Effects of sub-acute memantine, methylene blue and ritanserin on cGMP levels in rat hippocampus compared to the chronic + sub-acute saline treated group (28 days) (mean ± SEM). Descriptive statistics are provided in the table insert.

Chapter 7 DISCUSSION

Changes in central monoaminergic transmission have been considered to be involved in the mechanism of action of currently used antidepressants, since these drugs all exert effects on monoamine receptors or inhibit the uptake of amines. However, a relationship between these acute effects of ADs and their therapeutic action is questioned, especially since 2-3 weeks of administration is usually required to achieve a therapeutic effect, while the above actions on monoaminergic transmission is immediate (Harvey, 1997). This has prompted the shift to more diverse models to explain AD action. One particularly attractive alternative is the involvement of glutamate, but especially the role of the NO-cGMP pathway.

Neuronal 5-HT uptake can be modified by NO and cGMP (Miller & Hoffman, 1994; Zhu *et al.*, 2004), suggesting that 5-HT-NOS-NO interactions could play a critical role in the neurobiology and pharmacology of depression. Considering the recent evidence of neurodegenerative pathology in depression (Sapolsky, 2000a), potential neurotoxins, such as NO have attracted attention (Harvey et al., 2003), especially considering the high concentration of NOS containing neurons in the hippocampus, the brain region that is particularly vulnerable in depression. The neurotoxic nature of excessive NO and glutamate and their physiological function in the brain with regards to synaptic plasticity (McLeod *et al.*, 2001) as well as cell death and neuro-protection (Garthwaite & Boulton, 1995) holds great promise in revealing more on the neurobiology of depression, and especially the dynamics of antidepressant action

The aim of the present project was to study this hypothesis more closely. More specifically, the study sought to determine :

- The effect of chronic IMI treatment, a well known AD drug, on swim immobility time and locomotor activity, as well as NOS enzyme activity and cGMP levels in rat hippocampus (HC),
- The effect of withdrawal of IMI, after three weeks chronic exposure, on swim immobility time, locomotor activity as well as NOS enzyme activity and cGMP levels in the rat HC.
- Glu-NO-cGMP pathway involvement on the behavioral and neurochemical effects caused by IMI withdrawal by including, during the withdrawal period, the NMDA antagonist, memantine, or the NOS/guanylyl cyclase inhibitor, methylene blue (MB).

- Using the 5-$HT_{2A/2C}$ receptor antagonist, ritanserin administered during the 7 day withdrawal period, whether the observed IMI withdrawal effects on NO-cGMP pathway may occur through an initial destabilisation of the serotonergic system.
- The effects of the drugs alone used during IMI withdrawal on the various parameters analysed in the earlier studies.

7.1 Chronic IMI Studies

The prototypic tricyclic AD, IMI, is a potent inhibitor of neuronal noradrenaline and serotonin reuptake. Whether the activity of IMI in the FST is associated with increased NA and 5-HT activity alone, or whether a more deep-seated mechanism is involved, will have significant impact on understanding the neurobiological mechanism of AD action, and may assist in novel drug design that will address the current deficits in efficacy evident in ADs.

The aim of the chronic IMI study was to confirm the AD action of IMI, and to investigate relate said efficacy to the regulation of hippocampal NOS and cGMP levels.

Chronic treatment with IMI resulted in a significant reduction in swim immobility time, with no changes in horizontal or vertical locomotor activity observed. These behavioral changes in the presence of a situational stressor is indicative of an AD effect (Porsolt *et al.*, 1978), and that the increased psychological drive evoked by IMI in the FST is not due to an indirect effect of the drug on locomotor activity. Moreover, this AD action was accompanied by a significant reduction in hippocampal NOS enzyme activity and a corresponding tendency of decreased cGMP levels.

IMI may therefore provoke its antidepressant action through a mechanism that involves modulation of the NOS-cGMP pathway. The possible involvement of NO in AD action was proposed earlier by Harvey (1996), but it was Harkin et al (1999) who first demonstrated that L-Arg treatment could antagonize the immobility reducing effects of IMI in the FST, and that NOS inhibitors have AD actions in the FST, thus establishing a possible role for NO in AD action. More recently, Wegener et al (2003), using microdialysis, found that citalopram, paroxetine and IMI dose dependently decreased hippocampal NOS activity *in vitro* and *in vivo*. The current study is the first to describe the direct association between the AD action of an established AD, like IMI, and NOS inhibition in an animal model *in vivo*.

Chronic AD administration can modulate hippocampal NMDA receptor function (Harvey *et al.*, 2002). Indeed, ADs have been found to inhibit glutamatergic transmission by reducing the proportion of high affinity glycine sites on the NMDA receptor (Skolnick *et al.*, 1996) and by

reducing NMDA subunit messenger RNA in various limbic and subcortical structures (Boyer *et al.*, 1998). The NMDA receptor is a ligand-dependant Ca^{2+} channel that regulates Ca^{2+} influx, and consequently, the synthesis of NO.

AD response thus involves suppression of overt activity of glutamate-NMDA activity, and by implication its subcellular transduction mechanism, such as the activation of NOS. Chronic IMI is also known to suppress Ca^{2+} mobilisation and calcium-related events, such as calmodulin-regulated protein kinase and protein kinase C (Segovia *et al.*, 1994). This may further explain the reduced NOS enzyme activity evoked by IMI in this study.

A role for the glutamatergic-NOS system in the AD effect of TCA's and other ADs appears particularly relevant, given the qualitative similarities between NMDA receptor antagonists (Skolnick, 1999), NOS and cGMP inhibitors (Harkin *et al.*, 1999; Eroglu & Caglayan, 1997; Heiberg *et al.*, 2002) and TCA's in animal models and other biochemical test predictive of AD activity. Moreover, elevated NO_3 levels have been noted in depressed patients (Suzuki *et al.*, 2001). Increased NOS activity is associated with animal models of serotonin depletion (Tagliaferro *et al.*, 2001), while serotonin depletion is also associated with long-lasting increases in glutamatergic activity (Di Cara *et al.*, 2001), suggesting that decreased serotonin, that may be a causal factor in depression, is associated with increased glutamate and NOS activity. These elevations of glutamatergic and nitrergic activity may underlie the neurodegenerative pathology documented in the hippocampus of depressed patients (Sapolsky, 2000b), and may occur secondary to decreased serotonin evoked by conditions of chronic stress, as engendered by the FST (Tagliaferro *et al.*, 2001).

7.2 Antidepressant Withdrawal Studies

In order to confirm the above conclusions, an IMI withdrawal paradigm was investigated, with IMI being withdrawn after 21 days chronic treatment and the resultant effects on FST, locomotor behavior, NOS and cGMP investigated.

IMI withdrawal induced a significant increase in immobility time, approaching levels obtained with the saline treated group. This pronounced effect on swim immobility had no concomitant effects on the locomotor activity of the rats. Abrupt discontinuation of chronic IMI treatment thus reversed the AD action induced by chronic IMI treatment. Moreover, loss of the anti-immobility effects of IMI after withdrawal were accompanied by a significant increase in NOS enzyme activity compared to the chronic IMI groups as well as being significantly higher than control groups. An increase in cGMP levels in the hippocampus was also noted, but again it was disappointing that significance was not reached.

These behavioral data concur that acute withdrawal of IMI after chronic exposure is associated with a behavioral stress response. Earlier studies have found that AD withdrawal and increased stress responsivity is causally linked to an increase in HC glutamate activity and an increase in NMDA receptor density with reactive changes in GABA levels (Harvey *et al.,* 2002). Since NO is one of the intracellular messengers activated by NMDA receptor activation, the increase in NOS enzyme activity and cGMP levels observed after IMI withdrawal may be due to the release of glutamate, activation of NOS and subsequent release of NO and stimulation of cGMP. Chronic IMI withdrawal therefore reversed the decreased immobility time as well as the reduction in NOS and cGMP levels in rat HC noted after chronic IMI treatment, and it is irrefutable proof of the role of this pathway in the AD action of IMI. Clearly, AD effects and inhibitory effects on NOS during IMI treatment confirm a commonality of effect in AD action, and provide an important clue for novel drug development in the treatment of depression. However, of great significance was that decreased NOS activity was not only reversed by IMI withdrawal, but was significantly raised over control values. This has distinct pathological implications. Thus, AD response involves suppression of glutamate-NMDA-NOS activity, but abrupt discontinuation may severely disinhibit NMDA-NOS activity that may have important implications for the treatment and long-term outcome of the illness.

Considering this possible role of glutamate, NO and cGMP in the behavioral and neurochemical aftermath of AD withdrawal, it was decided to investigate whether administration of inhibitors of the NMDA-NO-cGMP pathway may modify these withdrawal responses. To do this, the NOS/guanylyl cyclase inhibitor, MB, and the NMDA receptor antagonist, memantine, were administered during the 7-day withdrawal period, and their effects on behavior and on the NO-cGMP pathway were observed.

7.3 Memantine Challenge

Memantine is a low-affinity non-competitive voltage dependant NMDA receptor antagonist (Sanacora *et al.*, 2003) that modulates glutamatergic neurotransmission. In states of reduced glutamate release, memantine results in an improvement in signal transmission and activation of neurons. However, during conditions of massive glutamate release, memantine blocks NMDA receptors, thereby blocking the excitotoxic action of glutamate on neurons (Manji *et al.*, 2003). Memantine treatment during the IMI withdrawal phase significantly reversed the increase in immobility time associated with IMI-withdrawal. There were no effects on either horizontal or vertical locomotor activity of the animals, indicating that it did not induce improvement by increasing locomotor behavior, which would constitute a false positive response.

Memantine reversed the increase in NOS enzyme activity significantly, with a similar, but insignificant trend in cGMP levels, observed after IMI withdrawal. This would suggest that IMI withdrawal is associated with increased excitotoxic glutamate and NMDA receptor driven activity, in agreement with earlier studies where the NMDA receptor antagonist, MK801, was found to re-establish an anti-stress response after withdrawal of IMI, following long-term administration (Harvey *et al.,* 2002). By blocking excessively active NMDA receptors, memantine decreases Ca^{2+} transmission and thus inhibits the downstream activation of NOS and subsequent activation of cGMP.

Considering that NMDA receptors represent the principle mechanism of NOS activation in the limbic brain regions, including the hippocampus and cortex, it is of particular interest that NMDA antagonists exert synergistic antidepressant-like effects with classical antidepressants (Rogoz *et al.*, 2002), that behavioral effects of IMI withdrawal can be reversed by NMDA antagonist (Harvey *et al.*, 2002) and that chronic AD treatment, in turn have significant impact on limbic NMDA receptors (Skolnick, 1999; Harvey *et al.*, 2002). Although these data have not been studied in the clinic, recent case reports have confirmed the AD efficacy of NMDA antagonists, especially in patients resistant to traditional treatment options (Sanacora *et al.,* 2004; Stryjer *et al.,* 2003).

These data confirm that the antidepressant action of IMI as well as IMI withdrawal, is associated with actions on the NMDA-glutamate-NO-cGMP pathway. That memantine reversed both the behavioral and neurochemical disturbances evoked by IMI withdrawal highlights this fact, but also emphasises the possible deleterious effects evoked by inappropriate and/or abrupt AD discontinuation. Since NO may be neurotoxic (Ledo *et al.*, 2004; Stewart & Heales, 2002), AD discontinuation may lay the foundation for neurodegenerative sequalae, which can be blocked by inhibiting NO release via NMDA receptor activation (Chen *et al.*, 1998).

Memantine on its own had no effect on swim immobility time. However, it decreased horizontal locomotor activity with no effect on vertical locomotor activity. A decrease in horizontal activity may imply that memantine would decrease performance in the FST, yet no effect was observed. However, NMDA antagonists are known to decrease locomotor behavior (Moryl *et al.*, 1993), which is clearly separate to its AD like actions. Decreased locomotor activity has been observed after amantadine treatment under certain conditions (Messiha, 1988), while it is interesting that Rogoz et al. (2002) found that co-administration of memantine with IMI and fluoxetine decreased exploratory activity. However, they found no changes in exploratory activity when memantine was given alone.

The fact that memantine had no effect on swim immobility time in this study is disappointing. As alluded to earlier, previous studies have shown that uncompetitive NMDA antagonists exert AD-like effects in animal models of depression (Moryl *et al.*, 1993; Skolnick, 1999; Rogoz *et al.*, 2002), although this appears more prominent in acute challenge studies. It is possible that the FST is not sensitive to chronic administration of memantine, although similar dosing of MK801 revealed profound anti-stress effects (Harvey *et al.*, 2002). Memantine also tended to decrease NOS enzyme activity and to decrease cGMP levels, but statistical significance was not reached. However, the fact that memantine reversed the increased immobility time and hyperfunction of NOS enzyme activity and raised cGMP levels in rat HC after IMI-withdrawal, is a significant finding, and is perfectly in keeping with the neuropharmacological profile of this drug. Memantine predominantly acts as an NMDA open-channel blocker, in a voltage-dependant manner, in the presence of prolonged elevation of glutamate concentration (on the scale of tens of minutes or longer). However, it is relatively inactive when glutamate is elevated for only milliseconds, as in normal synaptic transmission. The current study, as well as earlier studies (Harvey *et al.,* 2002), indicates that during IMI withdrawal, glutamate levels appear to be elevated. Thus memantine may act more predominantly on NMDA receptors during IMI withdrawal, than under normal conditions. In other words, memantine only acts under pathological raised glutamatergic function, clearly the case in AD withdrawal.

7.4 Methylene Blue Challenge

MB, a NOS/guanylyl cyclase inhibitor, significantly reversed the increase in immobility time observed after IMI withdrawal. Although an increase in horizontal locomotor activity was observed, indicating that the effect of MB may be due to an increase in locomotor activity, no changes were observed in vertical locomotor activity, suggesting that this may be an artifact. In agreement with this, previous studies by Eroglu & Caglayan (1997) have demonstrated that MB decreases swim immobility time with no concomitant effect on locomotor activity.

This reversal of increased immobility time after IMI withdrawal by MB was accompanied by a tendency to decrease NOS enzyme activity and cGMP levels in rat HC. MB, therefore, at least in part, reversed IMI withdrawal effects on behavioral and neurochemical parameters, supposedly through its inhibitory effect on NOS and guanylyl cyclase. Lack of effect on NOS-GC may be dose related since earlier studies have found that MB has a inverted U-shaped curve with respect to NOS inhibition and AD effects (Eroglu & Caglayan, 1997; Wegener, 2004 personal communication). The dose selected for this study, however, was found to be within the range of its most pronounced AD and anxiolytic actions (Eroglu & Caglayan, 1997). The fact that MB significantly reversed IMI withdrawal effects on swim immobility confirms this

conclusion, while its correlation with lowered NO and cGMP level emphasizes the probable role of the NO-cGMP pathway in AD action and AD withdrawal. Clearly, further studies are needed to confirm this, but the current data nevertheless allows the tentative suggestion that this pathway may play an important role in AD treatment and response.

Methylene blue treatment alone did not alter immobility time, despite a suppressive action on both measures of locomotor behavior. Nevertheless, dose-dependant reductions in the duration of immobility in the FST after acute MB administration has previously been reported (Eroglu & Caglayan, 1997), while MB has been found to decrease NOS in the brain using microdialysis (Volke *et al.*, 1999). However, no significant effects were observed on NOS or cGMP levels. Alone MB significantly decreased both vertical and horizontal locomotor activity in animals. This discrepancy between the stress-related and stress-unrelated effects of MB is disappointing and requires further investigation.

Mayer et al (1993) suggests that MB acts as a direct inhibitor of NOS and is a much less specific and a less potent inhibitor of guanylyl cyclase than previously assumed. Indeed, NOS seems to be more sensitive than sGC to the inhibitory effect of MB, since NOS is more completely inactivated at a concentration of MB that exerts no great effect on direct activation of sGC (Mayer *et al.,* 1993). Moreover, MB can inactivate NO extracellularly through generation of superoxide anions during auto-oxidation of a reduced form of MB (Wolin *et al.*, 1990), hinting of a more potent effect in the presence of raised NOS activity and increased NO release.

Previously studies on the locomotor behaviour on rats after MB treatment showed that MB had no effect on the locomotor activity (Eroglu & Caglayan, 1997). Although not a direct indication of anxiolytic properties, the decrease in locomotor activity may be indicative of sedative and anxiolytic effects of MB. Eroglu & Caglayan (1997) demonstrated that MB at 15 and 30mg/kg displayed both anxiolytic and AD effect in the elevated plus maze test, yet found no effect on locomotor activity over a 15 min observation period. Locomotor activity in this study was monitored over 10 min, thus variance in time may have an influence on the different data obtained. Nevertheless, the data with MB are less than convincing and further studies are needed to confirm these findings.

7.5 Ritanserin Challenge

AD discontinuation-related side effects has been proposed to be due to increased serotonergic activity due to decreased functioning of presynaptic 5-HT_{1A} receptors (Coupland *et al.,* 1996). It is also of interest that AD withdrawal phenomena include typical symptoms of a hyper-

serotonergic state, e.g. headache, anxiety, nausea (Section 2.6). Pre-clinical studies have also highlighted that excess serotonin not only is anxiogenic (Iversen, 1984), but may also be a protagonist for neurodegenerative phenomena (Joseph *et al.*, 1992). In particular, 5-HT-mediated neurotoxicity has been proposed to be mediated by 5-HT_{2A} receptors (Vaidya *et al.*, 1999), and to involve the release of glutamate (McEwen, 1996). In lieu of the current data describing increased NOS activity following IMI withdrawal and its reversal after administration of memantine, it was decided to evaluate to what extent these withdrawal-related changes are dependant on excess serotonergic activity following IMI withdrawal. In order to study this, the 5-$HT_{2A/2C}$ receptors antagonist, ritanserin, was administered during the withdrawal phase.

Ritanserin alone had no effect on swim immobility time, and reduced horizontal locomotor activity with no significant effect on vertical locomotor activity. Such a reduction in locomotor activity was also seen in a study done by Redrobe & Bourin (1997). However, previous studies have also shown that the blockade of 5-HT_{2A} and 5-HT_{2C} by ritanserin on its own has no effect on swim immobility time (Borsini, 1994; Redrobe & Bourin, 1997). These authors describe that whereas the stimulation of the 5-HT system may reduce the immobility time, the reduction in 5-HT transmission fails to alter this parameter.
IMI is a 5-HT and NA reuptake inhibitor and has been found to alter 5-HT_{2A} and 5-HT_{2C} receptor mediated functions in a genetic animal model of depression (Aulakh *et al.*, 1995). Moreover, Redrobe and Bourin (1997) found that ritanserin potentiates the anti-immobility effects of IMI, suggesting that the AD effects of IMI can be bolstered by antagonism of 5-HT_{2A} and 5-HT_{2C} receptors. Furthermore, 5-HT_{2C} receptor inactivation can potentiate the consequences of 5-HT re-uptake blockade (Cremers *et al.*, 2004).

The current study showed that ritanserin significantly reversed the increase in immobility time seen with IMI withdrawal, and is in keeping with the above thesis. Furthermore, this data also indicates that after chronic IMI withdrawal, 5-HT_{2A} and 5-HT_{2C} receptors are excessively active and that by blocking them after withdrawal, an AD response can be maintained, in spite of the withdrawal of IMI.

In agreement with these studies, 5-$HT_{2A/2C}$ receptor antagonism has a significant role in the mechanism underlying the antidepressant-like effect of conventional ADs in the FST (Redrobe & Bourin, 1997; Van Oekelen *et al.*, 2003), while the down regulation of 5-HT_{2A} receptors is proposed to mediate the long-term actions of AD use. Chronic blockade of 5-HT_{2A} receptors leads to a down regulation of these receptors, similarl to the down-regulation caused by agonists at these receptors (Smith & Whitton, 2000). While it may therefore be postulated that ritanserin reversed the increased immobility time after IMI withdrawal via a direct inhibitory action of the 5-$HT_{2A/2C}$ receptors, another mechanism may be evident.

The effects of ritanserin on the FST during withdrawal were accompanied by a significant reduction in NOS enzyme activity as well as a reduction in cGMP, although not significantly. Thus, the rescue effect of ritanserin on IMI withdrawal involves the suppression of the NOS pathway. This is of great interest since it directly links the aforementioned role of NOS in AD action to the serotonergic system, a well-recognised and important player in the design and efficacy of most currently used AD. Moreover, the association between AD withdrawal, increased NOS activity and increased 5-HT_2 activity has important implications for the possible role of neurotoxcicity associated with 5-HT_{2A} receptor activation described earlier.

Although cholinergic overdrive may be a contributory factor in especially tricyclic antidepressant withdrawal (Dilsaver, 1989), chronic antidepressant treatment results in desensitisation of post-synaptic and pre-synaptic 5-HT_{1A} receptors (Harvey, 1997). The hyper-serotonergic symptoms experienced after acute discontinuation has been ascribed to abrupt cessation of 5-HT reuptake inhibition following withdrawal, resulting in a temporary deficit of available 5-HT in the face of these down-regulated receptors (Coupland *et al.*, 1996). The result is a neurochemical and behavioral syndrome caused by loss of inhibitory 5-HT_{1A} mediated synaptic control and an increase in 5-HT. Increased hippocampal serotonergic activity, therefore, is a noteworthy sequelae to IMI withdrawal. This process may be viewed as negatively influencing the outcome of depression, especially since 5-$HT_{2A/2C}$ receptor antagonists have been described as having antidepressant properties in animal models (Sibille *et al.*, 1997; Patel *et al.*, 2004) and may even bolster the efficacy of more traditional antidepressant drugs (Yamada & Sugimoto, 2001). It is also disturbing that chronic stress-induced neuronal remodelling is driven by 5-HT_{2A} receptors and prevented by antagonist at this receptor (Vaidya *et al.*, 1999). Furthermore, since serotonergic-driven neurotoxicity appears to be mediated by glutamate, the current data suggests that AD withdrawal will, by increasing Glu-NOS activity, lay the foundation for neuronal loss that may occur after prolonged depression (Sapolsky, 2000a).

The ritanserin data can also be considered in another perspective. Of major significance is that ritanserin alone induced a profound increase in both NOS activity and cGMP accumulation in the rat hippocampus, contrary to its actions during IMI withdrawal. Decreased, or inhibited serotonergic function has been associated with increased NOS activity (Tagliaferro *et al.*, 2001), while serotonergic depletion also is a protagonist of glutamate release (Di Cara *et al.*, 2001). Thus, ritanserin alone and in the absence of any pathological conditions induced a prodigious increase in NOS activity and cGMP accumulation in the hippocampus. This can be construed as being a typical response to the drug under the current investigative conditions. This would indicate that under conditions of IMI withdrawal, ritanserin does not behave as it did under normal control conditions. In other words, AD withdrawal effectively altered the response to a 5-HT_2 receptor antagonist. The available data from this study implicate disturbed Glu-NOS-cGMP

activity as a strong causal factor in this altered response, although more in-depth study is required. Nevertheless, that serotonergic receptor responsiveness was markedly altered during IMI withdrawal has important ramifications, especially in the clinical treatment of depression. Increasing evidence suggests that repeated initiating and stopping AD treatment over a prolonged period can introduce treatment resistance, where the patient no longer respond to an AD that originally was effective (Harvey *et al.*, 2003; Keller, 2001). The altered response to a 5-HT_2 active drug in this study emphasises that drug response, notably on the serotonergic system, may be altered by AD withdrawal.

In the rat cortex and cerebellum, 5-HT_{2C} receptor activation inhibits the NMDA-NO-cGMP pathway (Maura *et al.*, 2000). If the role of the 5-$HT_{2A/2C}$ receptor in the hippocampus is to attenuate this pathway as well, it would be expected that a selective 5-$HT_{2A/2C}$ receptor antagonist such as ritanserin would further increase cGMP accumulation during IMI withdrawal and either worsen, or at best have no effect, on the behavioral changes induced by IMI withdrawal. This is not the case. Under conditions of IMI withdrawal where an increase in NOS activity and cGMP was found, ritanserin caused a paradoxical attenuation of this response. If increased NOS-cGMP activity is, indeed, a predictor of worsening prognosis as evinced in the FST, then the concomitant improvement in swim stress immobility and reduction of NOS-cGMP by 5-$HT_{2A/2C}$ inhibition confirms that these neurochemical changes induced by IMI withdrawal are pathological and are driven by excessive serotonergic activity in the hippocampus. These data are also provocative in their suggestion that IMI withdrawal may change the pharmacological response to a serotonergic-active drug, in this case from exerting a stimulatory action on basal 5-$HT_{2A/2C}$ -mediated NO-cGMP synthesis, to a reversed effect during conditions of IMI withdrawal. Since the 5-HT_{2A} receptor family are particularly important in drug response in depression (Sibille *et al.*, 1997;Patel *et al.*, 2004), this may have importance for the neurobiology of relapse and treatment refractoriness.

The current study has therefore provided behavioral and neurochemical evidence that AD action involves distinct pharmacological actions on the Glu-NOS pathway. This includes suppression of NOS after chronic treatment with IMI, and its reversal after withdrawal. Furthermore, this study has confirmed that this increased NOS activity after treatment withdrawal is mediated by serotonergic 5-HT_2 receptor activation, as well as a disinhibition of the glutamate pathway resulting in excessive activation of the NMDA receptor. Considering the important role that the Glu-NMDA-NO pathway has in the mechanisms related to cell death and cellular memory and how these processes are involved in the neurobiology of depression, one is left with the question whether current ADs, which are not 100% effective, are in fact addressing the correct target. The role of glutamate and NO in both neuromodulating, and neurotransmitter roles, as well as in the regulation of neuronal survival, makes a strong

statement as to where current drug development should be aiming and why current drugs are less that adequately effective. This study also raises the important question whether non-compliance and inappropriate AD discontinuation is, in ignorance of the potential long-term dangers, not being treated with enough concern and respect.

Chapter 8 CONCLUSION

Despite the growing awareness, acceptance and availability of effective antidepressants, the diagnosis and treatment of depression remain inadequate (Anderson, 2000). Moreover, there is a lack of knowledge about the underlying neurobiological mechanisms involved in mediating untreated depression and antidepressant withdrawal, as well as its implications for the course and prognosis of the disorder.

Using IMI as the archetypal AD, the current study has focussed on the role of the NO-cGMP pathway in antidepressant action and antidepressant withdrawal, and has provided the following evidence:

- The antidepressant action of chronic IMI treatment is strongly correlated to its ability to inhibit NOS enzyme activity and cGMP accumulation.
- Withdrawal of IMI results in a loss of AD efficacy that is associated with a profound increase in NOS enzyme activity.
- The NO-cGMP pathway is intimately involved in the AD action of IMI and in the neurobiology of depression.
- AD withdrawal and its associated increase in NO and cGMP levels may modify neuronal response to drug treatment, particularly of serotonergic drugs and thereby negatively impacting long-term outcome of depression.
- AD withdrawal induces an activation of the serotonergic system, particularly of the 5-$HT_{2A/2C}$ receptor.
- AD withdrawal activates Glu-NMDA activity directly, but most likely via a 5-HT_{2A}-dependant mechanism.

The current study has therefore shown that the NO-cGMP pathway plays an important role in the antidepressant action of IMI and that it may be a putative novel target in understanding the neurobiology of depression. Considering the potentially neurotoxic nature of glutamate and NO, the study has revealed that AD withdrawal may disinhibit NMDA-NOS driven events in the brain, with a host of untold possibilities that this may hold, but especially with regards to long-term treatment outcome of depression. Finally, clinicians need to seriously consider the possible consequences of inappropriate AD withdrawal and ensure that patients view compliance as a vitally important component of their treatment. Furthermore, AD medication should never be

stopped and re-initiated at the discretion of the patient. The time and duration of antidepressant discontinuation is a clinically informed decision that lies in the hands of a psychiatrist or suitable qualified person.

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