AN INVESTIGATION INTO THE POSSIBLE NEUROPROTECTIVE ROLE OF ANTIDEPRESSANT DRUGS

Thesis

Submitted in Fulfilment of
the requirements for the Degree of

MASTER OF SCIENCE (PHARMACY)

OF

RHODES UNIVERSITY

BY

CLAIRE STEINER
NOVEMBER 2001

Contents

Acknowledgments i

Abbreviations ii

List of Figures vi

List of Tables ix

Abstract x

Chapter One

Literature Review

1.1 Depression 1

1.1.1 Introduction 1

1.1.2 Theories of depression 2

1.1.2.1 Biogenic Amine Hypothesis 2

1.1.2.2 Dopamine Hypothesis 3

1.1.2.3 Permissive Hypothesis 3

1.1.2.4 Cholinergic-Adrenergic Hypothesis 4

1.1.2.5 Additional Theories of Depression 5

1.1.3 Antidepressant Therapy 5

1.1.3.1 Tricyclic Antidepressants 5

1.1.3.1.1 Nortriptyline 8

1.1.3.1.2 Trimipramine 9

1.1.3.2 Selective Serotonin Reuptake Inhibitors 10

1.1.3.2.1 Fluoxetine 10

1.1.4 Tryptophan and Depression 11

1.1.4.1 Tryptophan 2.3-Dioxygenase 12

1.1.4.2 Tryptophan 2,3-dioxygenase and the Kynurenine Hypothesis 13

1.1.4.3 Tryptophan 2,3-dioxygenase and Melatonin 13

1.1.5 Depression and the Medically Ill 14
1.1.5.1 Depression and Parkinson's Disease 14
1.1.5.2 Depression and Dementia 15

1.2 Mechanisms of Neurodegeneration 16
1.2.1 Oxidant Stress Hypothesis 16
1.2.1.1 Superoxide Radical 17
1.2.1.2 Nitric Oxide 17
1.2.1.3 Hydrogen Peroxide 18
1.2.1.4 Hydroxyl Radical 19

1.2.2 Metals and Free Radicals 21

1.2.3 Excitotoxicity 23
1.2.3.1 Glutamate 23
1.2.3.1.1 Functional Role of Glutamate 23
1.2.3.1.2 Glutamate Excitotoxicity 24
1.2.3.1.2.1 Kainate 25
1.2.3.1.2.2 Quinolinic Acid 26
1.2.3.1.3 Glutamate Receptors 28
1.2.3.1.3.1 NMDA Receptors 28
1.2.3.1.3.2 AMPA Receptors 29
1.2.3.1.3.3 Kainate receptors 29
1.2.3.1.3.4 Metabotropic Receptors 30
1.2.3.2 Calcium Mediated Excitotoxicity 30
1.2.3.3 Excitotoxicity and Oxidant Stress 31

1.3 Mechanisms of Defence 32
1.3.1 Antioxidants 32
1.3.2 Melatonin 33
1.3.2.1 Introduction 33
1.3.2.2 Biosynthesis and Distribution 34
1.3.2.3 Photoperiodic Control of Melatonin Synthesis 34
1.3.2.4 Melatonin and Aging 35

1.3.2.5 Melatonin and the Sleep/Wake Cycle 36
1.3.2.6 Melatonin as an Antioxidant 36
1.3.2.6.1 Antioxidant Actions of Melatonin *In Vitro* 37
1.3.2.6.2 Antioxidant Actions of Melatonin *In Vivo* 38
1.3.2.7 Melatonin and Other Antioxidants 38

1.4 Neurodegenerative Diseases 39
1.4.1 Parkinson's Disease 39
1.4.1.1 Introduction 39
1.4.1.2 Oxidant Stress in Parkinson's Disease 40
1.4.1.3 Animal Model for Parkinson's Disease 41

1.4.2 Alzheimer's Disease 42
1.4.2.1 Introduction 42
1.4.2.2 Metals and Alzheimer's Disease 43
1.4.2.3 Excitotoxicity and Alzheimer's Disease 43

1.4.3 Huntington's Disease 44
1.4.3.1 Introduction 44
1.4.3.2 Metals and Huntington's Disease 44
1.4.3.3 Excitotoxicity and Huntington's Disease 45
1.4.3.4 Animal Model for Huntington's Disease 45

1.5 Research Objectives 46

Chapter Two

Lipid Peroxidation of rat forebrain brain homogenate

2. Introduction 47

2.1 Experiment 1: *In vitro* effect of Nortriptyline hydrochloride, Trimipramine maleate and Fluoxetine hydrochloride on quinolinic acid induced lipid peroxidation 48

2.1.1 Introduction 48
2.1.2 Materials and Methods
2.1.2.1. Animals 48
2.1.2.2 Chemicals and reagents 49
2.1.2.3 Methods
2.1.2.3.1 Preparation of tissue homogenate 49
2.1.2.3.2 Lipid peroxidation assay 49
2.1.2.3.3 Statistical analysis 50
2.1.3 Results 50
2.1.4 Discussion 55

2.2 Experiment 2: *In vivo* effect of Nortriptyline hydrochloride, Trimipramine maleate and Fluoxetine hydrochloride on rat forebrain homogenate after quinolinate intrastriatal injection 56

2.2.1 Materials and Methods
2.2.1.1 Animals 56
2.2.1.2 Surgical procedures 56
2.2.1.3 Treatment Regimes 56
2.2.1.4 Chemicals and Reagents 57
2.2.1.5 Methods
2.2.1.5.1 Preparation of tissue homogenate 57
2.2.1.5.2 Lipid peroxidation assay 57
2.2.1.5.3 Statistical Analysis 58
2.2.2 Results 58
2.2.3 Discussion 60

2.3 Experiment 3: *In vitro* effect of Nortriptyline hydrochloride, Trimipramine maleate and Fluoxetine hydrochloride on iron induced lipid peroxidation

2.3.1 Introduction 61
2.3.2 Materials and methods
2.3.2.1. Animals 61

2.3.2.2 Chemicals and reagents 62
2.3.2.3 Instrumentation 62
2.3.2.4 Chromatographic conditions 62
2.3.2.5 Methods
2.3.2.5.1 Preparation of tissue homogenate 63
2.3.2.5.2 Lipid peroxidation assay 63
2.3.2.5.3 Statistical analysis 64
2.3.3 Results 65
2.3.4 Discussion 68

Chapter Three

Histological investigation

3.1 Introduction 70
3.2 Materials and methods
3.2.1 Chemicals and reagents 70
3.2.2 Animals 71
3.2.3 Surgical procedures 71
3.2.4 Treatment Regimes 71
3.2.5 Brain Removal 71
3.2.6 Histological Technique
i. Fixing the Brain 71
ii. Specimen Preparation and Embedding 72
iii. Blocking Out 72
iv. Sectioning 73
v. Transferring Sections to Slides 73
vi. Staining 74
vii. Mounting of the Slides 74
3.2.7 Photo-microscopy 75
3.3 Results 75
3.4 Discussion 78

Chapter 4

Superoxide Anion Lipid Peroxidation

4.1 Introduction 80

4.2 Materials and Methods

4.2.1 Animals 80

4.2.2 Chemicals and Reagents 80

4.2.3 Methods

4.2.3.1 Preparation of tissue homogenate 81

4.2.3.2 Nitroblue Tetrazolium assay 81

4.2.3.3. Protein assay 81

4.2.3.4 Statistical analysis 82

4.3 Results 82

4.4 Discussion 85

Chapter 5

Liver Tryptophan-2,3-dioxygenase Activity

5.1 Introduction 86

5.1.1 Experiment 1: *In vitro* effect of Nortriptyline hydrochloride, Trimipramine maleate and Fluoxetine hydrochloride on liver tryptophan-2,3-dioxygenase activity 88

5.1.2 Materials and methods

5.1.2.1 Animals 88

5.1.2.2 Chemicals and reagents 88

5.1.2.3. Methods

5.1.2.3.1 Preparation of liver homogenate 89

5.1.2.3.2 Determination of TDO activity 89

5.1.2.3.3 Protein assay 90

5.1.2.3.4 Statistical analysis 90

5.1.3 Results 91
5.1.4 Discussion 95

5.2 Experiment 2: *In vivo* effect of Nortriptyline hydrochloride, Trimipramine maleate and Fluoxetine hydrochloride on liver tryptophan-2,3-dioxygenase activity 96

5.2.1 Materials and methods
5.2.1.1 Animals 96
5.2.1.2. Chemicals and reagents 96
5.2.1.3 Methods
5.2.1.3.1 Preparation of liver homogenate 96
5.2.1.3.2 Determination of TDO activity 96
5.2.1.3.3 Protein assay 97
5.2.1.3.4 Statistical analysis 97
5.2.2 Results 97
5.2.3 Discussion 99

Chapter 6

Electrochemical investigation and UV/Visible *in vitro* investigation

6.1 Introduction 100
6.2 Electrochemistry
6.2.1 Introduction 101
6.2.2 Materials and Methods
6.2.2.1 Chemicals and reagents 103
6.2.2.2 Cyclic voltametry 103
6.2.3 Results 103
6.2.4 Discussion 109

6.3 UV/Visible Studies
6.3.1 Introduction 111
6.3.2 Materials and methods

6.3.2.1 Chemicals and reagents 111
6.3.2.2 UV/Visible analysis 111
6.3.3 Results 112
6.3.4 Discussion 114

Chapter 7

Conclusions and Recommendations for Future Work

7.1 Summary of Results
7.1.1 Chapter 2 115
7.1.2 Chapter 3 115
7.1.3 Chapter 4 116
7.1.4 Chapter 5 116
7.1.5 Chapter 6 116

7.2 Conclusions 117

7.3 Recommendations for future study 118

Appendices 120
References 125

Acknowledgements

First and foremost, I would like to thank my family, especially my parents Sue and Ian Pollock for their endless support, encouragement and patience over the last two years. Without them, this would not have been possible. Furthermore, my heartfelt thanks go to Chris Gray for his advice, enthusiasm and loving support, which has played no small part in this undertaking and words, no matter how extensive, cannot express my gratitude. I am also grateful for the devoted support of the Leiper family.

I would then like to express my sincere gratitude to Professor Santy Daya, my supervisor for his guidance and faith during this research experience. I am also grateful to the academic staff in the Faculty of Pharmacy at Rhodes University, Grahamstown.

Thank-you to my laboratory colleagues, especially Paula Heron, Janice Limson, Linda Heidemann and Shai Anoopkumar-Dukie for their ever willing hands on help and moral support throughout the time we have spent together. I am grateful to Dave and Sally Morley for guaranteeing that I had all the materials necessary for this study and their unfailing encouragement.

I appreciate the help from members of staff from the Department of Microbiology and Biochemisty and for the use of equipment in the Electorn Miccroscopy Unit, Rhodes University, Grahamstown.

Finally, I would like to thank the NRF and Rhodes University for financial assistance towards this study.

Abbreviations

$^{\cdot}OH$:	Hydroxyl radical
5-HIAA:	5-Hydroxyindole acetic acid
5-HT:	Serotonin
Ach:	Acetylcholine
AD:	Alzheimer's disease
AMPA:	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
Ag:	Silver
Ag/Cl:	Silver chloride
aMT:	Melatonin
ANOVA:	One way analysis of variance
AUC:	Area under curve
AUFS:	Absorbance units full scale
Aβ:	β-amyloid
BHT:	Butylated Hydroxytoluene
BSA:	Bovine serum albumen
Ca^{2+}:	Calcium (II)
cAMP:	Cyclic adenosine monophosphate
Cl:	Chlorine
CNS:	Central nervous system
CSF:	Cerebrospinal fluid
Cu^{2+}:	Copper II
CV:	Cyclic voltammogram
DA:	Dopamine
DNA:	Deoxyribonucleic acid
$E^{0'}$:	Formal reduction potential
E_{pa}:	Anodic peak potential
E_{pc}:	Cathodic peak potential
EAA:	Excitatory amino acid
ECT:	Electroconvulsive therapy
EDTA:	Ethylenediaminetetra-acetic acid
Fe^{2+}:	Iron II

Fe^{3+}:	Iron III
FL:	Fluoxetine
g/l:	Grams per litre
g:	Gram
GABA:	γ-aminobutyric acid
GCE:	Glassy carbon electrode
GTP:	Guanidine triphosphate
H:	Hydrogen
H_1:	Histamine 1 receptor
H_2O:	Water
H_2O_2:	Hydrogen peroxide
HCl:	Hydrochloric acid
HD:	Huntington's disease
HPLC:	High performance liquid chromatography
HSAB:	Hard soft acid base
HSD:	Honest significant difference
i_{pa}:	Anodic peak current
i_{pc}:	Cathodic peak current
i.p.:	Intra peritoneal
K^+:	Potassium
KCl:	Potassium chloride
KCN:	Potassium cyanide
kg:	Kilogram
LPS:	Lipopolysaccaride
M:	Molar
MAO:	Monoamine oxidase
MAOI:	Monoamine oxidase inhibitor
MDA:	Malondialdehyde
mg:	Milligram
Mg^{2+}:	Magnesium
ml:	Millilitre
mM:	Millimolar
MPP^+:	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MPTP:	1-Methyl-1,2,3,6-tetrahydropyridine

n:	Sample size
NA:	Noradrenaline
Na^+:	Sodium
NaCl:	Sodium chloride
NAD(P)H:	Nicotinamide adenine dinucleotide (phosphate)
NAD:	Nicotinamide adenine dinucleotide
NaOH:	Sodium hydroxide
NBD:	Nitroblue diformazan
NBT:	Nitroblue tetrazolium
NE:	Norepinephrine
nm:	nanometre
NMDA:	N-methyl-D-aspartate
$NO^{\cdot}$:	Nitric oxide
NT:	Nortriptyline
$O_2^{\cdot -}$:	Superoxide radical
O_2:	Oxygen
^oC:	Degrees Celsius
PBS:	Phosphate buffered saline
PD:	Parkinson’s disease
QA:	Quinolinic acid
ROS:	Reactive oxygen species
rpm:	Revolutions per minute
s.c:	Subcutaneous
SCN:	Suprachiasmatic nuclei
SD:	Standard deviation
SEM:	Standard error of mean
SOD:	Superoxide dismutase
SPE:	Solid phase extraction
SSRI:	Selective serotonin reuptake inhibitor
TBA:	2-Thiobarbituric acid
TBA-MDA:	Thiobarbituric acid- malondialdehyde
TCA:	Trichloroacetic acid
TCA:	Tricyclic antidepressant

TDO:	Tryptophan-2,3-dioxygenase
TM:	Trimipramine
Tris:	Tris(hydroxymethyl)-aminomethane
TRP:	Tryptophan
UK:	United Kingdom
USA:	United Sates of America
UV:	Ultraviolet
w/v:	Weight per volume
V:	Volts
μA:	Microampres
μg:	Microgram
μl:	Microlitre
μM:	Micromolar

List of Figures

FIG. 1.1	Chemical structures of some tricyclic antidepressants	7
FIG. 1.2	Chemical structure of nortriptyline hydrochloride	8
FIG. 1.3	Chemical structure of trimipramine maleate	9
FIG. 1.4	Chemical structure of fluoxetine hydrochloride	10
FIG. 1.5	One-electron transfer reactions demonstrating the relationship Between H_2O_2 and the oxyradicals	18
FIG. 1.6	The Fenton reaction	19
FIG. 1.7	A representation of free radical chain reactions affecting polyunsaturated fatty acids in membranes	20
FIG. 1.8.	Kynurenine pathway in the brain	27
FIG. 1.9	Antioxidant interaction with peroxyl radicals	33
FIG. 1.10	Chemical structure of melatonin	34
FIG. 1.11	Presumed mechanism of direct free radical trapping by aMT and its indolyl cation radical	37
FIG. 2.1	QA induced lipid peroxidation in rat forebrain homogenate	51
FIG. 2.2	The effect of various concentrations of NT hydrochloride on QA-induced lipid peroxidation	52
FIG. 2.3	The effect of various concentrations of TM maleate on QA-induced lipid peroxidation	53
FIG. 2.4	The effect of various concentrations of FL hydrochloride on QA-induced lipid peroxidation	54
FIG. 2.5	The effect of FL hydrochloride, NT hydrochloride and TM maleate on *in vivo* QA intrastriatal injection	59
FIG. 2.6	The effect of NT hydrochloride on iron induced lipid peroxidation	65
FIG. 2.7	The effect of FL hydrochloride on iron induced lipid peroxidation	66
FIG. 2.8	The effect of TM maleate on iron induced lipid peroxidation	67

FIG. 3.1	Schematic representation of a block with brain embedded in the centre	73
FIG. 3.2	Micrograph of cells in the CA1 region of the hippocampus from an animal treated with 0.9% saline prior and post QA intrastriatal injection	76
FIG. 3.3	Micrograph of cells in the CA1 region of the hippocampus from an animal treated with antidepressant prior and post QA intrastriatal injection	76
FIG. 3.4	Micrograph of cells in the CA3 region of the hippocampus from an animal treated with 0.9% saline prior and post QA intrastriatal injection	77
FIG. 3.5	Micrograph of cells in the CA3 region of the hippocampus from an animal treated with antidepressant (TCA) prior and post QA intrastriatal injection	77
FIG. 3.6	Micrograph of cells in the CA3 region of the hippocampus from an animal treated with antidepressant (SSRI) prior and post QA intrastriatal injection	78
FIG. 4.1	The lipid peroxidative effect of increasing concentrations of KCN on rat brain homogenate	83
FIG. 4.2	The effect of increasing concentrations of NT hydrochloride, TM maleate and FL hydrochloride on superoxide anion lipid peroxidation as induced by 2mM KCN	84
FIG. 5.1	Indole metabolism pathway	87
FIG. 5.2	The *in vitro* effect of NT hydrochloride on rat live TDO activity	92
FIG. 5.3	The *in vitro* effect of TM maleate on rat live TDO activity	93
FIG. 5.4	The *in vitro* effect of FL hydrochloride on rat live TDO activity	94
FIG. 5.5	The in vivo effect of various antidepressants on rat liver TDO activity	98

FIG. 6.1	A typical cyclic voltammogram (for illustrative purposes only)	102
FIG. 6.2	Successive cyclic voltammograms of TM maleate (4×10^{-5}M)	104
FIG. 6.3	Successive cyclic voltammograms of aMT (2×10^{-6}M)	105
FIG. 6.4	Successive cyclic voltammograms of TM maleate (4×10^{-5}M) in the presence of increasing concentrations of Fe^{2+}	106
FIG. 6.5	successive cyclic voltammograms of aMT (2×10^{-6}M) in the presence of increasing concentrations of Fe^{2+}	107
FIG. 6.6	Successive cyclic voltammograms of TM maleate (4×10^{-5}M) in the presence of increasing concentrations of Fe^{3+}	108
FIG. 6.7	Successive cyclic voltammograms of TM maleate (4×10^{-5}M) in the presence of increasing concentrations of Cu^{2+}	109

FIG. 8.1	Lipid peroxidation standard curve	122
FIG. 8.2	NBT assay standard curve	123
FIG. 8.3	Protein standard curve	124

List of Tables

Table 1.1	Role of metal ions converting less to more reactive species	21
Table 1.2	Examples of pathways utilizing glutamate as the neurotransmitter	24
Table 1.3	Excitotoxicity	31
Table 2.1	Treatment regimes for each group of animals	57
Table 3.1	Procedure for embedding brains in paraffin was	72
Table 3.2	Procedure for dewaxing and rehydrating	74
Table 3.3	Procedure for dehydrating brain sections after staining	74
Table 5.1	Scheme for the TDO enzyme assay	90
Table 6.1	λ-Max values as determined for NT hydrochloride, TM maleate, FL hydrochloride and aMT	112
Table 6.2	The effect of TM maleate and aMT on Fe^{2+} absorbance values	113
Table 6.3	The effect of Fe3+ on TM maleate and aMT absorbance values	113

Abstract

Antidepressants are widely used in the treatment of depressive illnesses associated with neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and Huntington's disease. Neuroprotection in such disorders is of vital importance in order to delay the progression of the primary disorder.

The pathology of neurodegeneration is not fully understood. It is however widely accepted that oxidative stress and excitotoxicity play a major role. Brain tissue is rich in phospholipids, which are especially prone to oxidation due to the high level of oxygen utilization in the brain. In addition, the brain lacks defence mechanisms to protect it against the wrath of free radicals.

Presently, there is a wide variety of antidepressant drugs available. These range from the original tricyclic antidepressants to the newer selective serotonin reuptake inhibitors. It is not known whether antidepressant drugs, old or new, offer neuroprotection or how the existing state and/or the progression of neurodegeneration, is influenced by these agents. The present study was undertaken to determine how nortriptyline, trimipramine and fluoxetine affect neurodegeneration.

Initial *in vitro* and *in vivo* studies show that all three of the antidepressants (0-1mM) studied provide neuroprotection from quinolinic acid induced lipid peroxidation. A histological investigation supported these findings by showing that a marginal degree of neuroprotection is apparent when treating animals with antidepressants (10mg/kg) before and following quinolinic acid intrastriatal injection.

Further studies were undertaken in an attempt to determine the mode of neuroprotective action of the agents studied. An *in vitro* study of superoxide anion induced lipid peroxidation indicates that these agents do not act as antioxidants.

The influence of the antidepressants on tryptophan 2,3-dioxygenase activity was assessed, based on the understanding that inhibition of this enzyme results in increased levels of the known antioxidant indoleamine, melatonin. Nortriptyline

hydrochloride is seen to inhibit tryptophan 2,3-dioxygenase activity and as such it is possible that this antidepressant can indirectly provide neuroprotection by increasing available melatonin.

Electrochemical and UV/visible studies show that trimipramine maleate interacts with free iron (II) and iron (III) ions. Free metal ions can catalyse the formation of damaging free radicals. Through interaction with trimipramine maleate, these ions will be unavailable to the system and thus cannot contribute to oxidative stress.

The findings of this study indicate that antidepressants may be able to provide neuroprotection to neuronal cells. The mode of such neuroprotective actions need to be further examined so that patients suffering from depression coexisting with neurodegenerative diseases can be safely and effectively treated.

Chapter 1

Literature Review

1.1 Depression

1.1.1 Introduction

Depression is a common disease of adulthood. During their lifetime, approximately 30% of adults will have had depression. The risk of depression is 2 to 3 times higher among women than men, and 2 to 3 times higher among first degree relatives of depressed persons than among other persons (Richelson, 1994b). Clinically recognisable depression is present in approximately 10% of elderly patients (65 years and older), however, the rate of depression may be increasing in association with a decrease in age at onset (Klerman and Weissman, 1989).

This debilitating disease impacts not only on the individual sufferer, but also on the economy and society as a whole. Broadhead *et al*, (1990) showed that there is a substantial negative economic effect in relation to work days lost (disability days) as a result of depression affecting members of the working population. As such, the medical profession has undertaken extensive research to understand and classify this disease, so as to be able to provide effective and safe medication.

Cooper and Magnus, (1984) broadly categorized depressive reactions into three types: reactive (e.g. neurotic depression, grief and depression associated with a personality disorder), symptomatic (e.g. depression following the use of drugs such as reserpine, or secondary to organic or other psychiatric disorders) and endogenous (e.g. the depression of unipolar and bipolar disorders). Depression in the elderly may present in a variety of ways, often uncharacteristic of depressive symptoms seen in younger patients and coexisting with symptoms of other illnesses (e.g. dementia, paranoid states and schizophrenia) (Shillcutt and Easterday, 1984).

Core symptoms of depression as described by Richelson (1994b) are:

- Depressed mood
- Diminished pleasure or interest in activities
- Major change in appetite or weight

- Alterations in sleep (insomnia or hypersomnia)
- Psychomotor agitation or retardation
- Fatigue or loss of energy
- Inability to concentrate
- Indecisiveness
- Thoughts of death, dying or suicide

1.1.2 Theories of depression

1.1.2.1 Biogenic Amine Hypothesis

This theory of depression predicts that a fundamental disturbance in biogenic amines, specifically the catecholamines, noradrenaline (NA), dopamine (DA) and the indoleamine, serotonin (5-HT), underlies the pathology of affective disorders (Harvey, 1997). Mania is said to be associated with excess NA and DA release, whereas depression is due to a synaptic deficiency of these neurotransmitters. Ashcroft *et al* (1972) suggested that in some patients the problem might be one of a low rate of synthesis of amines while in others it might be one of reduced sensitivity of the amine receptors to normal levels of transmitters.

The theory however fails to fully explain the aetiology of mood disorders for two main reasons:

1) No consistent pattern of abnormality in cerebrospinal fluid (CSF), urinary and serum transmitter metabolites was found to exist in depressed patients

Studies undertaken to determine whether any biochemical abnormalities exist in CSF, blood or urine of depressed or manic patients have problems in relating changes in concentrations of various metabolites to changes in transmitter function because (a) many secondary factors can affect their concentration such as diet, transport between CSF, blood and urine, or release of monoamines from non-cerebral sites and (b) many patients receive drug treatment that markedly affects the metabolite concentrations (Rang *et al,* 1995).

2) Immediate synaptic action of various antidepressant drugs do not correlate with the onset of their therapeutic efficacy which is usually 2-4 weeks (Schildkraut, 1965)

It has been suggested that due to the delayed onset of therapeutic effectiveness of antidepressant drugs, despite their immediate pharmacological action, that it may be the secondary, adaptive changes in the brain, rather than the primary drug effect, that are important in producing clinical improvement (Rang *et al*, 1995). Charney (cited in Nelson, 1991) supports this idea by suggesting that changes in different receptor systems occur during chronic antidepressant treatment, and it is as a result of these changes that depression is relieved.

Extensive studies have been undertaken to elucidate the changes in monoamine systems following chronic antidepressant therapy. Banerjee, (1977) showed a decrease in density of central β-adrenoceptors after repeated administration of a number of antidepressants. Another finding by Blier *et al*, (1987) involved serotonergic firing in the raphe nuclei. Shortly after antidepressant administration, a decrease in the firing rate is noted. This then returns to predrug levels after about 2-3 weeks, despite continued therapy.

1.1.2.2 Dopamine Hypothesis

The DA hypothesis implies that DA plays a major role in depression. This theory is based on evidence that the levels of the DA metabolite, homovanillic acid are lower than those of the NA metabolite, 4-methoxy-3-hydroxyphenyl glycol in comparison to normal controls in the central nervous system (CNS) (Syvälahti, 1994). The involvement of DA in depression is further supported by the fact that some effective antidepressants are potent inhibitors of DA uptake in rat brain synaptosomes (Randrup and Braestrup, 1977).

1.1.2.3 Permissive Hypothesis

The permissive hypothesis (Prange *et al*, 1974) emphasised the importance of 5-HT as a neuromodulator and led to it being identified as a neurological target for select

antidepressant action (Harvey, 1997). This hypothesis states that 5-HT deficiency along with reduced catecholamine levels will lead to depression and increased catecholamine levels will cause mania.

Critical involvement of serotonin in mood regulation is widely supported for a number of reasons (as reviewed by Harvey, 1997):

- 5-HT dependent prolactin release is reduced in depressed patients
- Depletion of the 5-HT precursor, L-tryptophan, in the diet of remitting depressives results in relapse of symptoms
- Reduced platelet 5-HT uptake is observed in depressives
- CSF of suicide victims reveals significant reduction in the 5-HT metabolite, 5-hydroxyindole acetate (5-HIAA)

1.1.2.4 Cholinergic-Adrenergic Hypothesis

This theory implicates monoamines and other biological amines, such as acetylcholine (Ach) in affective disorders (Janowsky *et al*, 1972a, 1972b). High monoamine and low Ach levels are said to give rise to mania, and low monoamine with high Ach concentrations result in depression.

Physostigmine is a cholinomimetic agent in humans. Administration of this agent to depressed patients promoted depression symptomology (Janowsky *et al*, 1972b; Modestein *et al*, 1973). Additionally, an acute dose of physostigmine had a sedative-like effect in a person suffering from mania that eventually resulted in a state of psychomotor retard depression (Janowsky *et al*, 1972a). These findings are in strong support of the cholinergic-adrenergic hypothesis. Further evidence in favour of this theory is that reserpine, an indirectly acting cholinomimetic compound, induces depression when used for the treatment of hypertensive patients (Janowsky *et al*, 1972b; Sulser, 1964).

1.1.2.5 Additional Theories of Depression

Further theories for depression include the *modified* biogenic amine hypothesis, the 5-HT/NA and the γ-aminobutyric acid (GABA)/glutamate balance theory (Rang *et al*, 1995). These theories emphasised that the pathological basis of affective disorders rested on multiple pathway involvement.

1.1.3 Antidepressant Therapy

It is in actual fact the mechanism of action of antidepressants that have provided many clues to help understand the pathology of depression.

The major classes of drugs used to treat depressive illnesses are:

- Tricyclic antidepressants (TCA) (e.g. imipramine, nortriptyline)
- Monoamine oxidase inhibitors (MAOI) (e.g. phenelzine, tranylcypromine)
- Selective 5-HT reuptake inhibitors (SSRI) (e.g. fluoxetine, fluvoxamine)
- 'Atypical' antidepressants – those that act similarly to TCAs, but with a different chemical structure (e.g. nomifensine, maprotiline)
- Lithium

Electroconvulsive therapy (ECT) is also effectively used for the treatment of depression, and usually acts more rapidly than antidepressant drugs (Rang *et al*, 1995). Use of ECT however is increasingly reserved for patients who fail to respond to antidepressant medication, or who are unable to tolerate such medications (Frankel *et al*, 1978 cited in Sackheim *et al*, 1990).

TCAs and SSRIs are the most commonly prescribed antidepressants in clinical use at present. This review will be limited to these two classes of drugs.

1.1.3.1 Tricyclic antidepressants

These agents form the most important group of antidepressants in current clinical use. Their antidepressant action was discovered serendipitously when imipramine, with a

structure closely related to the phenothiazines, was first produced as a potential neuroleptic drug. Though ineffective as an antischizophrenic, it was found to possess antidepressant properties. Thus TCAs were born.

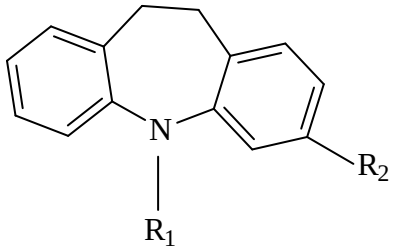
The first TCAs produced were the tertiary amines e.g. amitriptyline. These agents are rapidly demethylated *in vivo*, to secondary amines, which are in their own right antidepressants (figure 1.1). Hence the development of secondary amine TCAs; e.g. nortriptyline (NT), desipramine.

The main effect of TCAs is to block the uptake of amines by nerve terminals, by competition for the carrier that forms part of this membrane transport system. The conventional TCAs show little selectivity between NA and 5-HT uptake. Furthermore, the major metabolites of TCAs have considerable pharmacological activity, that may differ from that of the parent drug in respect of their NA/5-HT selectivity (Rang *et al*, 1995).

NA is believed to be as important a neurotransmitter in depression as 5-HT and consequently, greater selectivity for 5-HT over NA does not offer any significant benefits since we are comparing two desirable properties of an antidepressant. It is interesting to note that antidepressants, regardless of selectivity for 5-HT or NA, produce similar changes in biogenic amine metabolites (Potter *et al*, 1989 cited in Harvey, 1997; Frazer 1997) and there is little evidence to distinguish between the efficacy of the two types of antidepressants, or to differentiate among the patients that these agents help (Frazer, 1997; Rickels and Schweizer, 1990). Moreover, chronic use of an antidepressant, regardless of differing selectivity for the various transmitters, induces similar changes to cell function over time (Harvey, 1997)

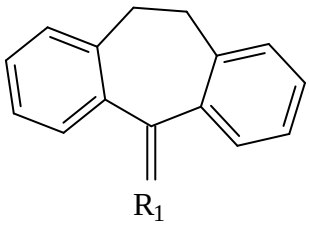
TCAs may affect one or more types of neurotransmitter receptor, including muscarinic Ach receptors, histamine receptors and 5-HT receptors (Rang *et al*, 1995). The clinical use of TCAs is limited by their side effect profile, potential cardiac toxicities and lethality in overdose (Ninan, 1999).

Dibenzazepines:



Drug	R_1	R_2
Imipramine	$CH_2CH_2CH_2N(CH_3)_2$	H
Desipramine	$CH_2CH_2CH_2NHCH_3$	H
Clomipramine	$CH_2CH_2CH_2N(CH_3)_2$	Cl

Dibenzcycloheptenes:



Drug	R_1
Amitriptyline	$CHCH_2CH_2N(CH_3)_2$
Nortriptyline	$CHCH_2CH_2NHCH_3$

Figure 1.1 Chemical structures of some tricyclic antidepressants

The two TCAs studied for the purpose of this thesis are NT and trimipramine (TM).

1.1.3.1.1 Nortriptyline

NT is a secondary amine TCA, and is the first metabolite of amitriptyline (Dollery, 1991). NT inhibits the uptake of NA and 5-HT at nerve terminals, displaying a greater effect on NA reuptake (Horn, 1980 and Waldmeier *et al*, 1976 sited in Dollery *et al*, 1991).

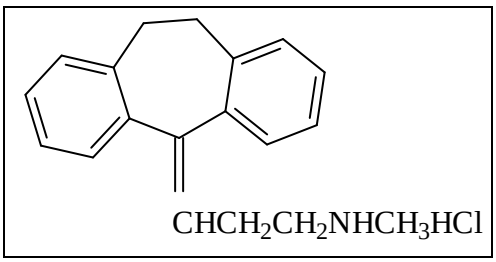


Figure 1.2: Chemical structure of NT Hydrochloride

NT is an effective antidepressant drug in clinical practice. Blockade of amine uptake is established rapidly and steady state concentrations in plasma are achieved about 10 days after initiation of therapy, but full antidepressant activity is only apparent after approximately 2-3 weeks. The initial recommended dose is 10mg three times daily, gradually increasing to a maximum of 100mg daily (Dollery *et al*, 1991).

NT is a potent antagonist at the muscarinic Ach receptors and has weak antagonist effects at histamine (H_1) receptors and at α_1-adrenoceptors. The antimuscarinic action is most likely to be partially responsible for the cardiac effects, such as tachycardia and arrhythmias, experienced in clinical practice. NT and desipramine appear to be less sedating than many of the other TCAs and this is believed to be related to strong noradrenergic effects (Frazer, 1997).

1.1.3.1.2 Trimipramine

TM is a TCA that has been in use since 1960.

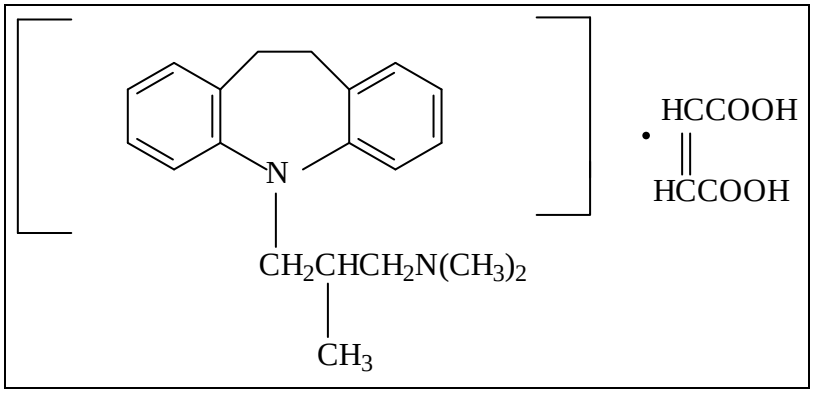


Figure 1.3: Chemical structure of TM Maleate

In contrast to the other TCAs, TM is a relatively weak NA and 5-HT reuptake blocker. It is however, a potent inhibitor of DA uptake and increases DA turnover. TM antagonises the effect of reserpine-induced depression in animals and the antidepressant effect takes about 2 weeks to become manifest (Dollery *et al*, 1991).

TM's DA receptor blocking properties seem to produce no side effects and this has been attributed to TM's relatively potent effects on muscarinic receptors (Richelson, 1994b). TM has potent antihistaminic activity and some anticholinergic activity and is thus particularly useful in depression associated with marked sleep disturbance or agitation and may obviate the need for concurrent hypnotic or major tranquilliser use (Settle and Ayd, 1980).

Acute administration of TM to rats caused a modest dose dependent increase in the spontaneous firing rate of noradrenergic locus coeruleus neurons. Chronic treatment of rats with TM caused a supersensitive response of cortical neurons to iontophoretically applied NA (Hauser *et al*, 1985). From such data it appears that TM may assist noradrenergic transmission, although the underlying mechanisms by which it does so are unknown.

1.1.3.2 Selective Serotonin Reuptake Inhibitors

As the name suggests, the SSRIs show selectivity for 5-HT over NA, but antidepressant actions are similar in efficacy and time-course to those of TCAs (Range *et al*, 1995). The benefit of SSRIs compared with MAOIs and TCAs are:

- Lack of anticholinergic and cardiovascular effects
- No problem with weight gain
- Low acute toxicity (low risk of overdose)
- No food reactions

The reduced frequency of side effects displayed by SSRIs in relation to TCAs is a result of less interaction with various receptor systems, such as the anticholinergic system and histamine receptors. These agents were developed once the pharmacologic actions of older antidepressants, that contributed to side effects but not efficacy, were identified (Richelson, 1994a). These drugs do have their own side effect profiles, but general tolerability by patients for these agents is better (Frazer, 1997).

The most commonly prescribed antidepressant at present is fluoxetine (an SSRI), and thus fluoxetine (FL) was an appropriate choice in our study to compare the above-mentioned TCAs.

1.1.3.2.1 Fluoxetine

FL is a potent and highly selective inhibitor of 5-HT uptake (Stark *et al*, 1985).

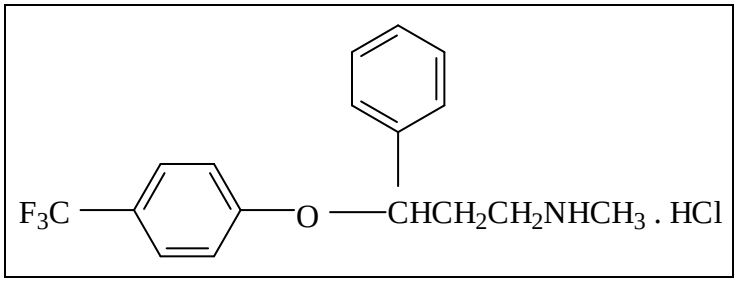


Figure 1.4: Structure of FL Hydrochloride

The preferred dose for initiation and maintenance therapy for depression is 20mg daily. Larger doses of 40-60mg per day have no greater efficacy (Wernicke *et al*, cited in Dollery *et al*, 1991). Fluoxetine exhibits little affinity for adrenoceptors, histamine, GABA 'B', muscarinic or 5-HT receptors.

There is data showing that the presence of 5-HT is necessary for the maintenance of an acute antidepressant effect of SSRIs (Frazer, 1997). Delgado *et al* (1990,1991) discovered that when patients that had responded to and were being maintained on antidepressants, followed a diet that was low in tryptophan (TRP), but high in other amino acids, 70% of those taking SSRIs or MAOIs relapsed. Only 20% of those patients treated with NT, desipramine or bupropion relapsed. TRP is the dietary precursor of 5-HT and the presumed effect of this diet is to lower brain content of 5-HT in patients. Selective inhibitors of NA uptake are much less dependent on the availability of 5-HT for their beneficial effects. Further studies showed that it was likely that antidepressants that inhibit NA and/or 5-HT reuptake, or inhibit MAO, need the availability of these respective transmitters for efficacy (Delgado *et* al, 1990, 1991).

1.1.4 Tryptophan and Depression

TRP is an essential amino acid obtained from the diet in human beings. TRP is the precursor for a number of important molecules including 5-HT and melatonin (aMT) (Walsh *et al*, 1994).

Brain 5-HT is synthesised from TRP that is taken up from the peripheral circulation (Daya and Anoopkumar-Dukie, 2000). The cerebral concentration of TRP is thus an important factor in brain 5-HT synthesis and concentration (Fernstrom and Wurtman, 1971). A number of studies have shown an apparent decrease in the availability of TRP for uptake into the brain in depression (Bender, 1982), with lower than normal concentrations of total or freely diffusible TRP in plasma, in relation to the concentrations of amino acids that compete with TRP for transport.

Unge *et al*, (1977 cited in Bender, 1982)) showed that the fall in plasma TRP following prolonged haemodialysis correlates with depression. Cyclical variation in plasma TRP is seen in bipolar manic-depressive patients with a short cycle, where high plasma TRP concentrations correlate with manic behaviour, and low plasma TRP with depressive phases (Rees *et al*, 1974). Coppen *et al* (1973) showed that diffusible plasma TRP was lower than normal during depression in unipolar recurrent depressive patients, and that these levels rose to normal on recovery. Furthermore, as previously mentioned, the finding that remitted depressed patients relapse very quickly after acute TRP withdrawal, implicates TRP and its derivatives specifically in the pathophysiology of depression (Delgado *et al*, 1990).

1.1.4.1 Tryptophan 2,3-Dioxygenase

One of the hypotheses that account for depression proposes that tryptophan 2,3-dioxygenase (TDO) is the major peripheral determinant of brain TRP and ultimately 5-HT levels. Curtailed serum and cerebral TRP levels and brain 5-HT turnover, as a consequence of an enhanced TDO activity, are causally linked to the pathogenesis of the clinically characterised depressive episodes in humans (Curzon and Green, 1969).

TDO is the haem-dependent cytosol enzyme that catalyses the conversion of TRP to formylkynurenine (Badawy and Evans, 1975). TDO exists in two forms – the active holoenzyme and the apoenzyme that requires the addition of exogenous haematin for demonstration of its activity.

TDO activity is regulated by two mechanisms. Firstly, glucocorticoids cause a hormonal-type induction involving the synthesis of new apoenzyme, and secondly TRP produces a substrate-type enhancement involving decreased degradation of pre-existing apoenzyme in the presence of the normal rate of its synthesis. Badawy and Evans (1975) showed that administration of haematin, or of its precursor 5-amino-laevulinate enhances the activity of rat liver TDO. It is thus suggested that there exists another mechanism - a cofactor (haem) mechanism - regulating the activity of rat liver TDO.

1.1.4.2 Tryptophan 2,3-dioxygenase and the Kynurenine Hypothesis

This theory states that there is an increase in plasma cortisol which stimulates TDO synthesis, thereby increasing TRP catabolism and thus diverting free plasma TRP away from 5-HT formation in the brain in depression (Curzon and Green, 1969; Curzon and Bridges, 1970). The basis for this theory is the observation that hepatic concentrations of TDO are increased by hydrocorticosteroids (Knox and Auerbach, 1955).

1.1.4.3 Tryptophan 2,3-dioxygenase and Melatonin

A relationship exists between the levels of aMT and TDO activity. Walsh *et al* (1994) showed that aMT causes a dose dependent inhibition of the enzyme and the effect of aMT on the enzyme appears to be ultimately dependent on TRP concentration.

A fraction of free circulating TRP is required for indoleamine synthesis and a large portion of the remainder is catabolised by TDO to ultimately yield nicotinate and related compounds (Walsh *et al*, 1994). Nocturnally, the need for free serum TRP increases because a) requirements with respect to the TRP-nicotinate pathway need to be met, and b) TRP is needed for increased indoleamine synthesis by both the brain and pineal. aMT could enable this relatively high serum TRP concentration by displacing TRP from serum albumin (Walsh *et al,* 1991). Moreover, through the modulation of TDO activity, aMT could allow sufficient TRP to escape catabolism for uptake across the blood brain barrier.

Antidepressant drugs have been shown to have the ability to increase the conversion of TRP to aMT, and increased levels of aMT have been found in the pineal and plasma of rats after acute and chronic treatment with antidepressants (Wirz-Justice *et al,* 1980; Heydorn *et al,* 1982). Efficacious antidepressants such as the TCA desipramine and SSRIs (FL) are said to have as part of their mechanism of action the ability to inhibit TDO, and thus increase plasma TRP concentrations and consequently 5-HT and aMT levels (Hardeland and Rensing, 1968; Walsh and Daya, 1998).

1.1.5 Depression and the Medically Ill

Rates of depression concurrent with medical disorders vary from illness to illness, but are generally in excess of the rates for the general population (Cunningham, 1994). Depression has been especially associated with, among others, cardiovascular disease, stroke, cancer, Parkinson's disease (PD), diabetes mellitus and dementia. Furthermore, concurrent depression may be associated with increased morbidity and mortality from the coexisting medical condition.

It is obvious that choosing an antidepressant for the medically ill requires careful consideration. For example, an antidepressant with DA receptor blocking activity may worsen the movement disorder of PD. An anticholinergic drug may aggravate the central cholinergic defects implicated in Alzheimers disease (AD). As such, clinicians need to carefully consider the pathology of the illness coexisting with depression, in order to effectively treat patients.

1.1.5.1 Depression and Parkinson's Disease

Depression and anxiety are the most common psychiatric conditions that accompany PD (Allain *et al*, 2000), and depression is believed to affect 40-50% of PD patients (Zesiewicz *et al* , 1999; Cummings and Masterman, 1999). Diagnosis of depression in PD is difficult as depressive symptomology may overlap with that of PD (Allain *et al*, 2000) and furthermore, coexisting depression is often qualitatively different from primary major depression (Taylor *et al*, 1986).

The severity of depression is believed to contribute to the cognitive disorders in PD. A study of patients with PD who did not have dementia showed that depression was associated with a significantly increased risk of developing dementia (Marder *et al*, 1995).

As such, it is clear that depression associated with PD must be treated. NT has been shown to be significantly superior to placebo in relieving depressive symptoms in PD,

and has little effect on the movement disorder (Anderson *et al* 1980 cited in Cunningham, 1994). Most of the experience reviewed by Cunningham (1994) indicates that bupropion or TCAs, especially NT, are the most useful antidepressants in parkinsonian patients. SSRIs may be safely used in PD, however use in combination with selegiline must be avoided, as the interaction between these agents may lead to the development of serotonin syndrome (Toyama and Iacono, 1994).

1.1.5.2 Depression and Dementia

The prevalence of depression in dementia is reported to be as high as 30% (Lazarus *et al*, 1987). Fischer *et al* (1990) found that there is an inverse relationship between the prevalence of depression and the severity of the dementia in a sample of patients with AD. The effective treatment of depression in patients with dementia has many advantages. It can reduce suffering of the patient and the caregiver, improve overall functioning by alleviating the 'excess disability' and can help the clinician discern the stage of dementia by reducing the cognitive disturbance contributed by depression (Alexopoulos, 1996).

A problem in diagnosing depression in patients with dementia is that depressive symptomatology appears transient and recurrent (Roth, 1955). Other factors hindering the diagnosis of depression in demented patients include cultural biases of patients and physicians, unreliable reporting of symptoms and overlap of symptoms of depression with those of dementia (Alexopoulos, 1996).

Secondary amines such as NT and desipramine are preferred over tertiary amines in treating geriatric depression because these have less complex metabolism and milder side effects (Alexopoulos, 1992). Anticholinergic drugs however may worsen cognitive defects. SSRIs and bupropion have less adverse effects on cognition than TCAs and are not as sedative, making them preferred agents (Cunningham, 1994), though agitation may occur, requiring the need for caution when making use of these agents.

1.2 Mechanisms of Neurodegeneration

As previously mentioned, there is a need to carefully consider the pathology of diseases concomitant with depression when treating patients. As depression is often seen to coexist with neurodegenerative diseases, analysis to determine how specific antidepressants impact on the existing state and/or the progression of neurodegeneration, is crucial.

Neuronal cells of the CNS, with few exceptions, cannot divide or regenerate, and as such, pathological processes causing neuronal loss generally have irreversible consequences (Rang *et al*, 1995). At present the oxidative stress hypothesis and excitotoxicity are the main areas of focus attempting to explain or improve our understanding of neurodegenerative disease. These are attractive theories in that explanations for acute and delayed onset damage that progresses over time are provided (Dawson and Dawson, 1996).

1.2.1 Oxidant Stress Hypothesis

A free radical is defined as any species capable of independent existence, that contains one or more unpaired electrons (Halliwell, 1992). Much interest has been placed on the role of free radicals in aging. Additionally, it is widely accepted that an altered redox balance exists in neurodegenerative disease (Sayre *et al*, 1999).

Oxidant stress is due to the formation of reactive oxygen species (ROS). The reactive species derived from oxygen (O_2) include hydrogen peroxide (H_2O_2), the superoxide anion radical ($O_2^{\cdot}$-) and the hydroxyl radical ($^{\cdot}OH$) (Fahn and Cohen, 1992). Oxygen radicals are continuously generated by chemical reactions and metabolic processes *in vivo* (Dawson and Dawson, 1996), as a consequence of aerobic metabolism. Oxidative stress can thus be described as an imbalance between biochemical processes leading to production of ROS and the cellular antioxidant cascade.

1.2.1.1 Superoxide Radical

Superoxide is formed *in vivo* in a variety of ways. Some of the electrons passing through the electron transport chains in the mitochondria and endoplasmic reticulum "leak" from intermediate electron carriers onto O_2 (Halliwell, 1992). As O_2 accepts electrons one at a time, $O_2^{\cdot-}$ is produced. At physiological concentrations of O_2, the rate of leakage is probably less than 5% of total electron flow and rises as O_2 concentration increases (Fridovich, 1978, 1989). As such, the toxicity of excess O_2 may be due to increased formation of $O_2^{\cdot-}$

Superoxide anions are generated enzymatically by a number of oxidases, other than cytochrome oxidase of the mitochondria. These include xanthine oxidase and the oxidase that is found in the plasmalemma of phagocytic cells (Reiter *et al*, 1995b).

The number of targets sensitive to $O_2^{\cdot-}$ within mammalian cells is believed to be small (Halliwell, 1992), and controlled production is a necessary process. Superoxide anions produced from activated phagocytic cells are used to destroy engulfed bacteria (Curnutte and Babior, 1987) and tumour cells (Fogler and Fidler, 1989). Such phagocytes include monocytes, neutrophils, eosinophils and various macrophages, including microglial cells of the brain (Colton and Gilbert, 1987). Logically however, excessive activation of phagocytic cells (as in chronic inflammation) can lead to oxidative stress.

1.2.1.2 Nitric Oxide

Nitric oxide ($NO^{\cdot}$) is mainly released by vascular endothelial cells and phagocytes (Moncada *et al* 1991). At physiological pH, $NO^{\cdot}$ reacts with $O_2^{\cdot-}$ to yield peroxynitrite, a nonradical (Saran *et al*, 1990 cited in Halliwell, 1992). Peroxynitrite may be directly cytotoxic by oxidising thiol groups, and it may also decompose to form $^{\cdot}OH$ (Beckman, 1991; Radi *et al*, 1991).

It appears that the interaction of $NO^{\cdot}$ with $O_2^{\cdot-}$ is extremely relevant to normal brain metabolism and to neurodegenerative diseases (Beckman, 1991). $NO^{\cdot}$ has been

suggested to be involved in both normal functioning of excitatory amino acids (EAA) such as glutamate and in the damaging effects produced by their generation in excess (Dawson *et al*, 1991). $NO^{\cdot}$ or peroxynitrite could cause the release of glutamate by activation of astrocytes (Pauwels and Leysen,1992; Meffert *et al*, 1994; Montague, 1994) and/or inhibit glutamate uptake (Pogun *et al*, 1994) and thereby raise the extracellular glutamate concentration and promote damage through possible nitric oxide-independent mechanisms.

Additionally, it has been suggested that $O_2^{\cdot -}$ and $NO^{\cdot}$ are produced as 'antagonistic agents' in vascular endothelial cells to allow for fine control over vascular tone (Halliwell, 1989).

1.2.1.3 Hydrogen Peroxide

Superoxide dismutases (SODs) convert $O_2^{\cdot -}$ to H_2O_2. Other enzymes that produce H_2O_2 include L-amino acid oxidase, glycollate oxidase, and monoamine oxidase (MAO). H_2O_2 is not a radical, as it does not contain an unpaired electron, and can be removed from within human cells by catalases and selenium-dependent glutathione peroxidases (Halliwell, 1992).

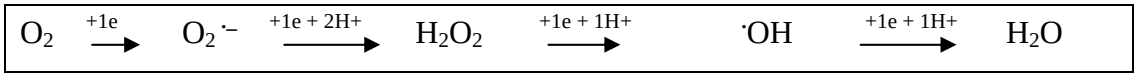


Figure 1.5: One-electron transfer reactions demonstrating the relationship between H_2O_2 and the oxyradicals (Fahn and Cohen, 1992)

Toxicity of this molecule is not usually mediated by a direct effect, but rather due to interaction with Fe^{2+}, to form $^{\cdot}OH$.

1.2.1.4 Hydroxyl radical

The hydroxyl radical is produced via the Fenton reaction as represented in figure 1.6.

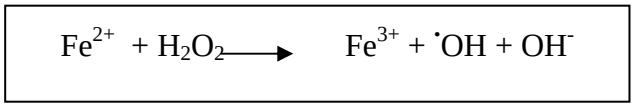


Figure 1.6: The Fenton reaction

$OH^{\bullet}$ react rapidly with almost all molecules in living cells including deoxyribose nucleic acid (DNA), carbohydrates and membrane lipids. Action on DNA results in stand breakage and chemical alterations of the deoxyribose and of the purine and pyrimidine bases.

A main physiological target of free radicals is the polyunsaturated fatty acids of cell membranes and the resultant degradation causes alterations in membrane structure and function (Viani *et al*, 1991). Hydroxyl radicals are capable of initiating lipid peroxidation by abstracting a hydrogen atom from the polyunsaturated fatty acid side chain (represented as 'lipid-H' in figure 1.7), resulting in a carbon-centered radical in the membrane (lipid$^{\bullet}$). These radicals react with O_2 *in vivo* to form peroxyl radicals that can attack membrane proteins and also abstract hydrogen atoms from adjacent fatty acid side chains (Halliwell, 1992). It is thus evident that the abstraction of a single hydrogen can set off a chain reaction to produce damaging lipid hydroperoxides.

Decomposition products from reactions include hydrocarbon gases and a wide range of toxic carbonyl compounds, including aldehydes (e.g. malondialdehyde). These aldehydes can continue to damage adjacent cells. They also have the ability to inactivate membrane-bound enzymes and receptors. It can thus be seen that protein essential for membrane function can be damaged not only by attack of peroxyl radicals, but also due to covalent modification by aldehyde end products of peroxidation (Halliwell, 1992).

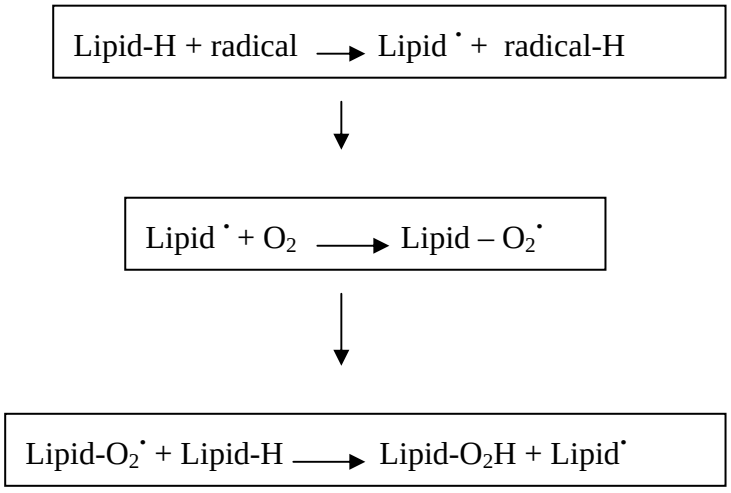


Figure 1.7: A representation of free radical chain reactions affecting polyunsaturated fatty acids in membranes (Halliwell, 1992)

Neuronal cell membranes are rich in unsaturated fatty acids, and consequently, are especially vulnerable to oxidative damage. Vulnerability is further intensified by the fact that the brain consumes about 20% of total body oxygen and is relatively deficient in protective mechanisms (Dawson and Dawson, 1996).

1.2.2 Metals and Free Radicals

There are a number of metal ions present in nervous tissue that are capable of converting less reactive to more reactive species as depicted in table 1.1

Table 1.1 Role of metal ions converting less to more reactive species (Halliwell, 1992)

$O_2^{\cdot-}$, H_2O_2	Fe/Cu[a]	$^{\cdot}OH$
Lipid Peroxides (ROOH)	Fe/Cu[b]	$RO^{\cdot}$ (alkoxyl), $RO_2^{\cdot}$ (peroxyl), cytotoxic aldehydes
Thiols (RSH)	Fe/Cu plus O_2[c]	$O2^{\cdot-}$, H_2O_2, thiyl ($RS^{\cdot}$), $^{\cdot}OH$
NAD(P)H	Fe/Cu plus O_2[c]	$NAD(P)^{\cdot}$, $O_2^{\cdot}$-, H_2O_2, $^{\cdot}OH$
Ascorbic acid	Cu/Fe[d]	Semidehydroascorbate radical, $^{\cdot}OH$, H_2O_2, Degradation products of ascorbate
Catecholamines, related autoxidizable molecules	Fe/Cu/Mn plus O2[c]	$O_2^{\cdot}$-, H_2O_2, $^{\cdot}OH$, semiquinones (or other radicals derived from the oxidising compounds)

[a] The iron- or copper-catalyzed Haber-Weiss reaction
[b] Lipid peroxide decomposition is metal iron dependent
[c] Most 'autoxidations' are stimulated by traces of transition metal ions, and proceed by free radical mechanisms
[d] Copper ions are especially effective in decomposing ascorbic acid, and ascorbate/copper or ascorbate/iron mixtures are cytotoxic

Several areas of the human brain are rich in iron, such as the globus pallidus and the substantia nigra (Youdim, 1988 cited in Halliwell 1992). Brain tissue has the ability to readily accumulate iron (Dawson and Dawson, 1996) and this observation is

important as iron (and copper) ions can contribute to lipid peroxidation in two ways. Firstly, they catalyze formation of free radical species and secondly, decompose hydroperoxides to peroxyl and alkoxyl radicals (Halliwell and Gutteridge,1990), which are able to abstract hydrogen and lead to further peroxidation.

Under normal physiological conditions, iron is stored in specific proteins that minimise its reaction with reduced oxygen metabolites. One of the most important storage proteins for iron is ferritin (Linert and Jameson, 1999). Iron for involvement in free radical reactions however appears to be available within cells, both as a mobile pool and as iron releasable from ferritin.

Transferrin, an iron transporter molecule, binds iron in the ratio of 1:2. In normal human plasma, there is an excess of iron binding capacity, thus the content of free iron in the plasma should effectively be nil (Weinberg, 1992 cited in Halliwell, 1992). As the content of transferrin in the CSF is very low, it has no significant iron-binding capacity (Gruener *et al*, 1991). Transferrin content of CSF has been reported to be ~ 0.24μM from normal subjects, and total iron values in the CSF are in the range of 0.2-1.1μM. As such, data suggests that CSF transferrin is often at, or close to, iron saturation (reviewed by Gutteridge, 1992 cited in Halliwell, 1992).

There is increasing evidence that suggests that reactive iron becomes available during disease states (Gutteridge *et al*, 1984). Antioxidants therefore need to scavenge $O_2^{\bullet -}$ and H_2O_2 before coming into contact with the available free iron (Halliwell and Gutteridge, 1989). It has been suggested that the nature of damage done to cells by excess formation of H_2O_2 and $O_2^{\bullet -}$ will be affected by the location of metal ion catalysts within the cells. If these ions are unavailable then $O_2^{\bullet -}$ and H_2O_2 will have limited, if any, damaging effects (Halliwell, 1992).

1.2.3 Excitotoxicity

Excitotoxicity is a concept that shows that EAAs (e.g. glutamate and aspartate) could cause acute neuronal degeneration (Leigh And Meldrum, 1996). The term "excitotoxins" refers to EAAs, which share neurotoxic properties, since their neurodegenerative action is thought to occur as a direct result of an ability to depolarise neurons (Olney, 1974 cited in Foster *et al*, 1983). Injury appears to be predominantly mediated by excessive influx of calcium (Ca^{2+}) into neurons through ion channels that are triggered by activation of glutamate receptors (Choi, 1985).

1.2.3.1 Glutamate

1.2.3.1.1 Functional Role of Glutamate

Glutamate is the principle excitatory amino acid in the brain and fulfils the criteria for a neurotransmitter (Cotman *et al*, 1987):

- Ca^{2+} dependent release upon stimulation
- High affinity uptake into nerve terminals
- Presence of the amino acid and synthetic enzymes in nerve terminals
- Blockade of synaptic transmission by glutamate receptor antagonists
- Cellular and molecular effects of receptor activation

Glutamate interacts with specific membrane receptors and is required for many neurologic functions, including cognition, memory, movement and sensation (Gasic and Hollmann, 1992). A number of biological functions including intermediary metabolism in neuronal tissues and detoxification of ammonia in the brain, involve glutamate (Tilson and Mundy, 1995). Excessive activation of glutamate receptors however, may mediate neuronal injury and death (Lipton and Rosenberg, 1994).

Table 1.2 Examples of pathways utilizing glutamate as the neurotransmitter (Tilson and Mundy, 1995)

Allocortical – CA3 hippocampal to lateral septim
Auditory nerve
Cerebellum – granule cells, climbing fibers
Corticostiratal pathway – caudate, substantia nigra
Hippocampus – entorhinal cortex to hippocampus, Schaffer collaterals within Hippocampus
Retina – photoreceptor cell, bipolar cells, rods
Thalamus – ventral lateral thalamic nuclei from cortex
Vagus nerve – afferents of arterial barorecptors
Visual cortex – ipsilateral to lateral geniculate

Under normal physiological conditions, when the presynaptic terminal is depolarised, glutamate is released in a Ca^{2+}-dependent process. It diffuses across the synaptic cleft where it can activate its specific receptors (Rothstein, 1996). Glutamate is removed from the synaptic cleft and the extracellular space by sodium (Na^{+})-dependent, high affinity monocarboxylic acid carriers located in both neurons and glia (Obrenovitch and Urenjak, 1997).The glia convert glutamate into glutamine for transport into neurons, where it is subsequently reconverted into glutamate for release into the synapses when required (Lipton and Rosenberg, 1994).

Intraneuronal concentrations of glutamate are in the region of 10mmol, whereas extracellular glutamate levels are approximately 1μmol (Lipton and Rosenberg, 1994). Glutamate concentrations in the region of 0.2 to 1mmol are sufficient to elicit excitotoxicity (Leigh and Meldrum, 1996). There is evidence that suggest that inefficient glutamate transport leads to the accumulation of excessive neurotransmitter in the synapse, with subsequent neurotoxicity (Rothstein, 1996).

1.2.3.1.2 Glutamate Excitotoxicity

Glutamate has been implicated in various neurodegenerative diseases such as AD, PD and Huntington's disease (HD) (Choi, 1988). Subsequent to the activation of glutamate receptors, a series of intracellular cascades can be activated, which are believed to account for its neurotoxic properties: excess calcium influx, activation of

oxidant-generating enzymatic pathways, including increased formation of $NO^{\cdot}$, peroxynitrite, $^{\cdot}OH$, and $O_2^{\cdot -}$ (Choi, 1988).

Reynolds and Hastings (1995) demonstrated that excitotoxic concentrations of glutamate rapidly produce ROS in cultured forebrain neurons after N-methyl-D-aspartate (NMDA) receptor activation. Gunassekar *et al* (1995) have shown that glutamate induced concurrent generation of $NO^{\cdot}$ and ROS by activation of NMDA receptors through a Ca^{2+}-mediated process. Oxidative stress has been shown to increase the release of glutamate (Pellegrini-Giampietro *et al*, 1990), which in turn increases ROS formation and thus triggers a feedback loop, that produces progressive neuronal damage.

Glutamate induces necrotic and apoptotic cell death (Cebers *et al*, 1997). Necrotic neuronal death is typically characterised by rapid cell and organelle swelling and lysis, whereas apoptotic is characterised by cell shrinkage, relocalization of organelles, condensation of intracellular chromatin, and DNA fragmentation (Bredesen, 1995). Ankarcrona *et al* (1995) showed that glutamate induced time- and agonist concentration-dependent patterns of necrotic and apoptotic cell death in cerebellar granule cells. Higher concentrations of glutamate destroyed neurons rapidly through necrosis-like cell death, whereas lower concentrations induce delayed, apoptosis-like cell death.

1.2.3.1.2.1 Kainate

Kainate is a non-NMDA glutamate receptor agonist that binds to ionotropic glutamate receptors and opens cation channels generating Na^+ influx, occasionally Ca^{2+} influx, and potassium (K^+) efflux (von Blankenfeld and Kettenmann, 1991).

Dykens *et al* (1987) have shown that, in primary culture of cerebellar neurons, the neurotoxicity of kainate can be prevented by inhibiting the enzyme xanthine oxidase, a cellular source of cytotoxic superoxide radicals. Further evidence that the generation of free radicals is central to the mechanism of kainate-elicited neuronal death is provided by Chow *et al* (1994). It was found that in primary neuronal cultures, free

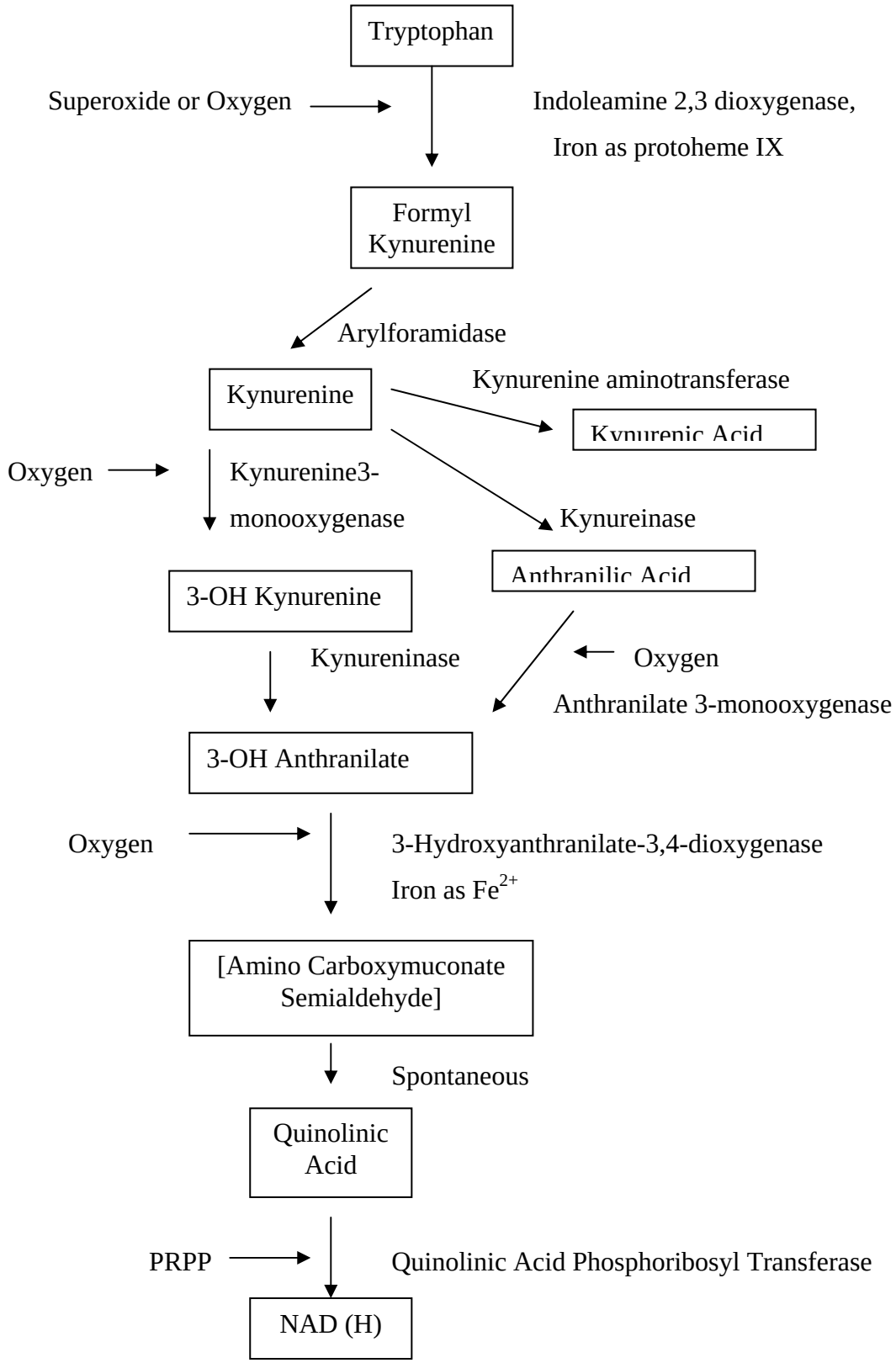
radical scavengers such as trolox (a vitamin E analogue) protect from excitotoxicity triggered by agonists for non-NMDA glutamate receptors, but not from the toxicity of NMDA receptor activation.

1.2.3.1.2.2 Quinolinic Acid

Quinolinic acid (QA) is an endogenous glutamate agonist with a relative selectivity for the NMDA receptor (Tsuzuki *et al*, 1989). It is a neurotoxic metabolite present in the brain in low nanomolar concentrations and is derived from L-TRP via the kynurenine pathway (Heyes *et al*, 1996) as represented in figure 1.8. The mechanism by which QA exerts it neurotoxic effects has been ascribed to its ability to induce excessive NMDA receptor activation and consequent massive Ca^{2+} entry into the cells (Stone, 1993).

Only certain cell types have been shown to produce QA. Macrophages, which can infiltrate the brain in many conditions of brain inflammation, have particularly high capacity for synthesizing QA and might be important sources of QA within the CNS. Microglia are the “resident” macrophages within the brain and are activated in many conditions of brain immune response, including trauma and microbial infection (Heyes *et al*, 1996).

Figure 1.8 Kynurenine pathway in the brain. The rate-limiting enzyme in the pathway (indoleamine 2,3-dioxygenase) is replaced by tryptophan 2,3-dioxygenase in the liver (Dale *et al*, 2000).



Generally accepted estimates of the prevalence of microglia in the normal brain are approximately 3-4% (Adams and Duchen, 1992 cited in Heyes *et al,* 1996). Once activated under neuropathological conditions (e.g. autoimmune disease, physical trauma and infection of the brain with micro-organisms), this network of cells can represent a prominent volume of the brain tissue, and QA levels in the CSF and brain tissue are increased (Heyes *et al,* 1992; 1993). QA concentrations in brain tissue exceed those in CSF, and the levels in both brain and CSF can exceed those in the blood (Heyes *et al,* 1991; Heyes *et al,* 1993). This observation suggests that brain QA derives from intracerebral synthesis rather than being derived from blood or meninges.

1.2.3.1.3 Glutamate Receptors

Glutamate receptors have been the major focus of research into the excitotoxic basis of neurodegenerative disease (Chen *et al,* 1995). Ionotropic glutamate receptors can be divided into three major types based on their selective agonists: NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and kainate (Lipton and Rosenberg, 1994). These receptors are termed ionotropic because they open cation-specific channels upon their activation by an agonist (Mori and Mishina, 1995; Bettler and Mulle, 1995). Metabotropic glutamate receptors belong to a family of receptors coupled to GTP-binding proteins.

1.2.3.1.3.1 NMDA Receptors

NMDA receptors are distributed throughout the brain but occur in greatest density in the hippocampus (Maragos *et al,* 1988). The NMDA-receptor channels have a number of distinctive properties (Obrenovitch and Urenjak, 1997):

1. Ligand and voltage gating (gating requires occupation of the NMDA/glutamate binding site by an agonist and relief by depolarisation of the voltage-dependent magnesium (Mg^{2+}) block within the ion channel
2. High permeability to Ca^{2+}
3. Activation at the low glutamate concentration
4. Prolonged opening time

5. multiple modulatory mechanisms

These receptors are activated by transmitters in a voltage-dependent manner (Farooqui and Horrocks, 1991). Activation of NMDA receptors opens a cation channel, which conducts monovalent and divalent cations non-specifically (MacDermott and Dale, 1987). This increase in ionic permeability generates depolarising responses (Ascher and Nowak, 1987).

Ankarcrona *et al,* (1995) suggest that glutamate induced cell death is mediated through NMDA receptors. This is based on the finding that the neurotoxic effects of glutamate are fully blocked by the administration of a specific NMDA receptor antagonist. Manganese and copper accumulation in the striatum following QA intrastriatal injection appears to be exclusively dependent on NMDA receptor activation (Santamaria *et al,* 1996 cited in Santamaria *et al* 1996), as dizocilpine (an NMDA antagonist) completely prevents this increase.

1.2.3.1.3.2 AMPA Receptors

AMPA appears to be involved in fast excitatory synaptic transmission (Young *et al,* 1991 cited in Tilson and Mundy, 1995). These receptors are ionotropic and voltage independent, permitting exchange of Na^+ and K^+ when activated. AMPA receptors are found on glial cells, as well as on neurons.

Cebers *et al (*1997) reported that AMPA receptors play a significant role in glutamate-induced cell death in rat cerebellar granule cells. The neurotoxicity is under certain conditions characterised by morphological changes that are largely recognised as typical for both necrotic and apoptotic cell death.

1.2.3.1.3.3 Kainate Receptors

These are ionotropic receptors (Tilson and Mundy, 1995). Amano *et al* (1994) found that marked cell death of striatal cultures was induced by kainate and not by NMDA nor AMPA. These findings strongly suggest that glutamate-induced cytotoxicity in

the striatal cultures is mediated by the kainate receptors among the glutamate receptors.

1.2.3.1.3.4 Metabotropic Receptors

Evidence from molecular biology indicates that there are at least five subtypes of metabotropic glutamate receptors, some of which are coupled negatively to cyclic adenosine monophosphate (cAMP) and some to stimulation of phosphoinositide metabolism (Farooqui and Horrocks, 1991). The agonist binding site is not coupled to an ion channel, but rather to a second messenger system. These receptors, like AMPA receptors, are found on glial cells, as well as on neurons.

Metabotropic receptors have the potential to modulate a variety of intracellular signal transduction events (Obrenovitch and Urenjak, 1997). So far, multiple metabotropic receptors have been subdivided into three subgroups, according to their amino acid sequence identities (Pin and Duvoisin, 1995). Depending on the cell type and receptor subtype, these receptors might mediate inositol phosphate metabolism, release of arachidonic acid, or cAMP levels, with either excitatory or inhibitory effects, exerted post- or pre-synaptically.

1.2.3.2 Calcium Mediated Excitotoxicity

Excessive amounts of calcium can contribute to the overstimulation of normal cellular processes causing the neuron to digest itself by protein breakdown, free radical formation and lipid peroxidation (Lipton and Rosenberg,1994). The complex cascade of events is briefly described in table 1.3. The paradox of excitotoxicity is that the mammalian brain is especially vulnerable to its own excitatory neurotransmitters.

Table 1.3: Excitotoxicity (Leigh and Meldrum, 1996)

Entry of Na^+, Cl^-, H2O	→ cell swelling
	→ altered Na^+/Ca^{2+} exchange
Entry of Ca^{2+} (early phase)	→ activation of enzymes:
▪ Proteases (calpains, xanthine oxidase)	
▪ Protein kinases (PKA; calcium-calmodulin-dependent kinase)	
▪ Phosphatases	
▪ Phospholipases (A_2, C)	
▪ Ornithine decarboxylase	
▪ Nitric oxide synthase	
▪ Endonucleases	
Substantial increases in intracellular Ca^{2+}, cell fate depends on:	
▪ Excitotoxin involved	
▪ Cell type	
▪ Interactions with energy metabolism	
▪ Interacting neuroprotective mechanisms	

The neurotoxicity due to Ca^{2+} is a relatively slow process; after stimulation of the glutamate receptor, at least 12 hours is required for the complete death of neurons (Choi, 1987).

Na^+ may also play a critical role in excitotoxic neuronal damage (Taylor and Meldrum, 1995). Transport of Ca^{2+} out of neurons is dependent on a Na^+/Ca^{2+} exchange mechanism that can act in reverse, resulting in net Ca^{2+} influx. Glutamate release during ischemia may be mediated mainly by reversed Na^+-dependent glutamate transport, so blockade of Na^+ channels in this situation may have a significant neuroprotective effect (Leigh and Meldrum, 1996).

1.2.3.3 Excitotoxicity and Oxidant Stress

Several observations have suggested that the mechanisms of oxygen free radical and excitatory amino acid toxicity may be at least partially interdependent (summary as provided by Volterra *et al*, 1994):

1. Kainate-induced degeneration of cerebellar neurons in culture can be prevented by free radical scavengers
2. Glutamate administration in a neuronal cell line lead to glutathione depletion with peroxide formation
3. 21-amino-steroid compounds, inhibitors of lipid peroxidation, partially reduce excitotoxic neuronal death of cortical cultures
4. Massive release of EAA in hippocampal slices subjected to "*in vitro* ischemia" is blocked by free radical scavengers and reproduced by free radical-generating systems
5. Oxidative inhibition of glutamine synthetase in parallel to free radical production is observed during early postischemic reperfusion *in vivo*

1.3 Mechanisms of Defence:

1.3.1 Antioxidants

Defence mechanisms have evolved in organisms to limit levels of reactive oxidants. Almost all of these defences appear to be inducible, as concentrations of antioxidants increase in response to increased levels of damage (Ames *et al*, 1993).

Defensive enzymes include SODs, catalase and glutathione peroxidase. Glutathione peroxidase is believed to be the major H_2O_2 detoxifying enzyme in the brain. Behl *et al*, (1997) proposed that EAA neurotoxicity may be caused by the induction of an imbalance in antioxidant enzyme systems and a reduction in intracellular glutathione levels. The drop in glutathione levels has been shown to arise from glutamate competing with the glutamate/cystine antiporter, leading to an imbalance in the homeostasis of cystine, the precursor of glutathione formation (Murphy *et al*, 1989).

Structural defences involve sequestration of H_2O_2-generating enzymes and chelation of any free iron or copper salts (Ames *et al*, 1993).

Additionally, antioxidants are obtained from dietary sources, such as ascorbic acid (vitamin C), α-tocopherol (vitamin E) and carotenoids, can scavenge damaging free

radicals. Antioxidants inhibit the chain reaction of lipid peroxidation by scavenging intermediate peroxyl radicals as depicted in figure1.9

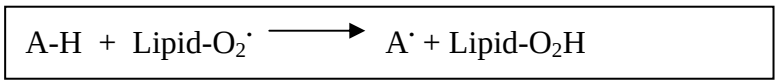


Figure 1.9: Antioxidant interaction with peroxyl radicals

Vitamin E is believed to be the most important chain breaking antioxidant inhibitor of lipid peroxidation in humans (Burton and Ingold, 1989). The fact that vitamin E, and high extracellular cystine levels protect hippocampal cells from glutamate toxicity indicate that the glutamate induced cell death occurs via an oxidative pathway (Davis and Maher, 1994).

There is a high concentration of vitamin C in the gray and white matter of the CNS. Vitamin C in the absence of transition metals has well-established antioxidant properties (Frei *et al*, 1989). Ascorbate/iron and ascorbate/copper mixtures however have the ability to generate free radicals (Halliwell and Gutteridge, 1990). If iron was to become available in the CNS as a result of injury, ascorbic acid present may stimulate ·OH generation within the brain and CSF.

The role of aMT as an endogenous free radical scavenger has been extensively studied and it is widely believed that this neurohormone may be of therapeutic use for protection against neurodegeneration.

1.3.2 Melatonin

1.3.2.1 Introduction

aMT was first defined as the hormone that mediates the annual fluctuations in seasonally breeding animals, as well as influencing numerous aspects of circadian biology (Reiter *et al*, 1996). Subsequently, aMT's functions have been shown to significantly exceed those related to circannual and circadian biology.

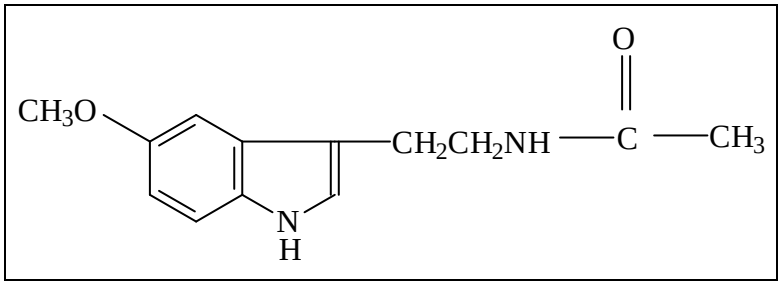


Figure 1.10: Chemical structure of Melatonin

1.3.2.2 Biosynthesis and distribution

aMT, an indoleamine, is the principle secretory product of the mammalian pineal gland (Limson *et al*, 1998). aMT is also synthesised in the retina and gastrointestinal tract (Yu, 1994; Huether, 1993 cited in Reiter1995a), however blood levels of aMT are essentially derived from the pineal gland.

Pineal TRP is converted to 5-hydroxytryptophan, which is in turn converted to 5-HT. Serotonin is subsequently changed to *N*-acetylserotonin by the nocturnal increase in *N*-acetyltransferase activity. Hydroxyindole *O*-methyltransferase converts N-acetylserotonin to aMT (Limson *et al*, 1998). aMT is secreted directly from the pineal gland into the blood stream.

About 60-70% of circulating aMT is bound to plasma proteins (mainly albumin) and in the CSF, aMT is present in its free form (Cardinali *et al*, 1972). The turnover rate of aMT is fairly short and inactivation occurs by rapid hepatic hydroxylation (Freuer, 1990).

aMT is highly lipophilic and relatively small, allowing it to enter cells and gain access to all subcellular compartments (Acnũa-Castroviejo *et al*, 1997). It is thus widely distributed in cells and has actions in the membrane, cytosol and nucleus (Tan *et al*, 1993).

1.3.2.3 Photoperiodic Control of Melatonin Synthesis

The production and discharge of aMT from the pineal gland is under the control of the photoperiod, acting by way of the suprachiasmatic nuclei (SCN) of the hypothalamus

(Reiter, 1991). During the night, the SCN sends a neuronal signal to the pineal gland through the peripheral autonomic nervous system, where postganglionic sympathetic neurons release norepinephrine (NE) onto the pinealocytes, resulting in the increased production of aMT. Melatonin concentrations found in human plasma at night are in the nanomolar range (Olney *et al*, 1971). Conversely, during the day, retinal messages quell the activity of the SCN and shut down aMT synthesis.

As such, the organism is aware of the photoperiodic environment to which it is exposed by virtue of the daily nocturnal rise in blood aMT levels. Thus the signal provides important information that adjusts the organism's physiology on a seasonal and possibly daily basis. Biological timekeeping has been shown to be affected by administration of exogenous aMT. When aMT is administered in the morning, it delays circadian rhythms (Lewy *et al*, 1992), whilst in the afternoon or early evening aMT advances circadian rhythms (Sack *et al*, 1991).

1.3.2.4 Melatonin and Aging

As vertebrates age, the pineal gland gradually loses its ability to synthesise aMT (Reiter, 1992; 1995b). In humans, a substantial reduction in the nocturnal levels of aMT is reported to occur during sexual maturation, and the drop is believed to initiate and sustain pubertal development (Waldhauser *et al*, 1991).

Studies indicate a persistent steady reduction in the synthetic capability of the pineal gland with advancing age and the amplitude of the aMT rhythm is only a fraction of that of young animals (Reiter *et al*, 1980; 1981). The rate of aging and the time of onset of age-related diseases in rodents can be retarded by the administration of aMT, in a manner which preserves the endogenous rhythm of aMT formation (Sandyk, 1990; Poeggeler *et al*, 1993; Oaknin *et al*, 1995 cited in Exposito *et al*, 1995).

Aging associated with an impaired function of the pineal gland, and insufficient aMT secretion has been proposed as one of the contributors to age-linked pathologies (Reiter, 1992). Interestingly, preliminary evidence suggests that individuals with Alzheimers dementia have a virtual absence of a nocturnal aMT rise (Skene *et al*, 1990).

1.3.2.5 Melatonin and the Sleep/Wake Cycle

aMT is the main synchronizer of the sleep/wake cycle (Bersani and Garavini, 2000). The indoleamine alters the timing of sleep in part by its effect on the central circadian pacemaker in the SCN in order to modify the phase of the internal clock (Dawson and Encel, 1993). Melatonin exerts a therapeutic effect by normalising the sleep/wake cycle in several sleep phase disorders (e.g. subject suffering from jet-lag, advanced sleep onset and delayed sleep phase syndrome) (Zisapel, 1999).

The evening, oral administration of aMT to manic patients, has been shown by Bersani and Garavini, (2000) to produce an overall reduction in manic symptomology. This indicates a beneficial effect exerted by aMT upon the manic state itself by the normalisation of sleep/wake cycle. This could be in accordance with the hypothesis of a pathogenic role of sleep disturbances on maintaining the manic state.

1.3.2.6 Melatonin as an Antioxidant

There is extensive evidence that convincingly demonstrates aMT's *in vitro* and *in vivo* antioxidant abilities.

The most important part of the molecule is the pyrrole ring, given that ring opening results in the formation of a series of potent biogenic amines (Leone and Silman, 1984; Beck and Jonsson, 1981). Ring opening through interactions with free radicals could explain the antioxidant properties displayed by this compound (figure 1.11).

Once oxidised, aMT cannot be reduced or involved in regenerating processes that may cause auto-oxidative radical formation or toxic redox recycling, unlike other low molecular weight antioxidants (Hardeland *et al*, 1993 cited in Melchiorri *et al*, 1995). This unique scavenging mechanism of aMT makes it less toxic to DNA and more suitable for protection of DNA damage than many other free radical scavengers (Shaikh *et al*, 1997) and the tendency of aMT to be present in the nucleus provides an effective means of on-site protection against DNA damage (Tan *et al*, 1994).

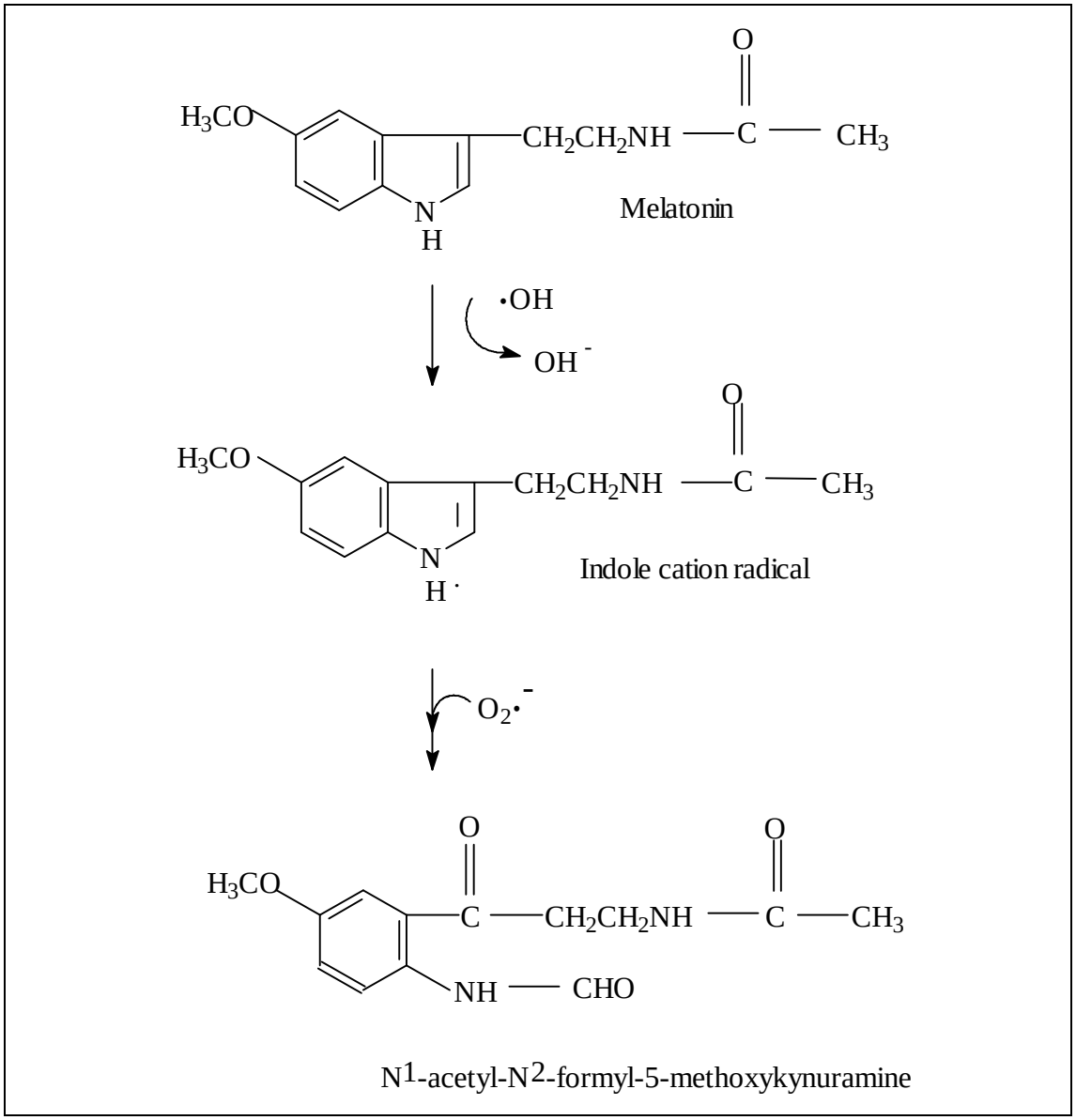


Figure 1.11: Presumed mechanism of direct free radical trapping by aMT and its indolyl cation radical (Hardeland *et al*, 1993 cited in Melchiorri *et al*, 1995)

1.3.2.6.1 Antioxidant Actions of Melatonin *In Vitro*

Sewerynek *et al* (1996) used pharmacological levels of aMT to overcome lipid peroxidation (due to oxygen based radicals) in brain homogenates. All brain regions investigated were protected including the cerebral cortex, striatum, hypothalamus, hippocampus, and the cerebellum.

Giusti *et al,* (1995) showed that the neurotoxic action of kainate in primary neuronal cultures can be counteracted by aMT. Kainate can 1) induce a direct primary axon sparing lesion; 2) excite certain neuronal pathways, which in turn may cause secondary excitotoxic damage; and 3) trigger seizures that could induce hypoxia and edema, thus leading to tertiary brain damage (Sperk, 1994). The neuroprotection was not due to aMT action on kainate-sensitive glutamate receptors or GABA receptors, and the concentration of aMT required for neuroprotection surpassed the concentrations needed for the action of this hormone on specific aMT receptors. It is thus postulated that aMT –provided neuroprotection could originate in the antioxidative properties of the aMT molecule.
Additionally, Melchiorri *et al* (1995), showed that aMT prevented kainate-induced lipid peroxidation.

1.3.2.6.2 Antioxidant Actions of Melatonin *In Vivo*

Tan *et al,* (1993) conducted a series of experiments using safrole, a highly toxic carcinogen that generates free radicals which damage nuclear DNA. The DNA adducts quantified in the liver of rats that were treated with aMT and safrole showed significantly reduced damage (up to 95%) in comparison to those animals treated with safrole alone. It was further shown that when safrole was administered to animals at night, when endogenous aMT levels are known to be high, the amount of damage to hepatic DNA was significantly less compared to daytime exposure (Tan *et al*, 1994).

Lipopolysaccharide (LPS) induces extensive tissue damage and multiple organ failure in humans (Ghezzi *et al*, 1986). LPS is believed to damage polyunsaturated fatty acids in membranes through the generation of toxic oxygen radicals. Sewerynek *et al* (1995) treated animals with LPS, and the resulting damage to lipids was readily counteracted by systemic aMT administration.

1.3.2.7 Melatonin and Other Antioxidants

Melatonin has been shown to possess antioxidant capabilities greater than that of other known antioxidants in humans. Pieri *et al,* (1994) provide evidence that aMT is twice as potent as vitamin E in scavenging the peroxyl radical. *In vitro* experiments

carried out by Tan *et al*, (1993) show that aMT is 5-and 14-fold more potent than glutathione and mannitol respectively, in scavenging ˙OH.

It is however the synergistic action of aMT and other antioxidants that is believed to be important to the mammalian organism. Reiter (1995a) observed that when aMT is co-incubated with other chain-breaking antioxidants (e.g. ascorbate or glutathione), the synergistic antioxidant action is much greater than the sum of the actions of each compound individually. Barlow-Walden *et al* (1995) demonstrated that aMT is able to induce an increase in the activity of glutathione peroxidase in the brain of rats. Thus it was postulated that even low concentrations of aMT may greatly enhance the antioxidative action of other scavengers.

1.4 Neurodegenerative diseases

1.4.1 Parkinson's Disease

1.4.1.1 Introduction

PD is a progressive disorder of movement, occurring most commonly in the elderly. It is believed to affect approximately 1% of the population over 55years in age (Linert and Jameson, 2000).

The main symptoms of PD are:

- Tremor at rest
- Muscle rigidity
- Decrease in the frequency of voluntary movements (hypokinesia)

Dementia is commonly associated with PD, and is a result of the same degenerative process affecting other parts of the brain (Rang *et al*, 1995).

Parkinsonian symptoms can be related to an extreme deficiency of the neurotransmitter DA in the striatum (Linert and Jameson, 2000) as a result of neuronal degeneration in the substantia nigra pars compacta (Lange *et al*, 1997).

1.4.1.2 Oxidant Stress in Parkinson's Disease

As previously discussed, free iron has been implicated in undergoing redox transitions *in vivo* with consequential generation of free radicals. Abnormally high levels of iron and oxidative stress have been demonstrated in a number of neurodegenerative disorders (Sayre *et al*, 1999) including PD. It has been shown that the iron concentration is significantly increased in the substantia nigra of patients who die from PD, where it appears to be accumulated in the melanin particles in the remaining cells (Fahn and Cohen, 1992; Linert and Jameson, 2000). As yet however, there is insufficient data as to whether iron accumulation in parkinsonian brains is an early or late event in the disease process.

Fahn and Cohen (1992) have compiled convincing evidence to favour the oxidant stress hypothesis in PD:

1. Neurotoxins with the ability to selectively destroy dopaminergic neurons in the substantia nigra, such as 6-hydroxydopamine and 1-methyl-1,2,3,6-tetrahydropyridine (MPTP), appear to act via oxidant stress
2. Findings of evidence of increased lipid peroxidation and decreased reduced glutathione in substantia nigra of patients with PD
3. Total iron is increased and ferritin is reduced in the substantia nigra pars compacta in patients with PD
4. Neuromelanin, a product of DA auto-oxidation, can serve as a reservoir for iron, promoting the generation of oxyradicals
5. Findings of decreased activities of glutathione peroxidase and catalase in the parkinsonian substantia nigra indicates reduced antioxidant defence mechanisms

A further suggestion supporting the involvement of oxidative stress as a cause of PD, was made by Cohen (1988 cited in Halliwell, 1992), by proposing that the main

catabolic pathway for DA within DA nerve terminals involves oxidative deamination by MAO. An accelerated turnover of DA in patients with PD leads to increased H_2O_2 formation, provoking oxidant stress within surviving DA terminals, so accelerating their destruction. Such a sequence of events can partly explain the progressive nature of the disease.

Possible neuroprotective strategies in PD are largely based on the belief that oxidant stress is an important pathogenic factor (Riederer and Lange, 1992). These include free radical scavengers, MAO-B inhibitors, iron chelators and DA agonists.

1.4.1.3 Animal Model for Parkinson's Disease

A deficiency in complex I of the respiratory chain in the mitochondria is demonstrated in patients suffering from PD (Mizuno *et al*, 1989). This deficiency was found to involve a selective reduction of NADH-CoQ reductase activity – an enzyme specific to complex I (Schapira *et al*, 1990a, 1990b cited in Fahn and Cohen, 1992). Enzyme loss is found to be limited to the substantia nigra of patients with PD.

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPP^+) is widely used to reproduce PD symptoms in animals, since this drug depletes DA from the striatum (Sundstorm *et al*, 1987) and the active uptake of MPP^+ is believed to be responsible for the selective vulnerability of nigrostriatal neurons (Ludolph, 1995).

When MPTP is administered exogenously to the brain, the lipophilic compound readily crosses the blood-brain barrier (Ludolph, 1995). It is immediately oxidised to MPP^+ by MAO-B in astrocytes (Castagnoli *et al*, 1985; Heikkila *et al*, 1985). MPP^+ is actively transferred to dopaminergic neurons from the astrocytes, where it is accumulated into the mitochondria and provokes energy failure by inhibition of the respiratory chain at the site of complex I (Singer *et al*, 1987; Vyas *et al*, 1986).

There is evidence that EAAs are involved in the neurotoxic effects of MPTP (Lange *et al*, 1993). It has been shown that MPP^+ causes a release of glutamate and aspartate in the rat brain (Carboni *et al*, 1990). Glutamate antagonists which block the NMDA receptor subtype (competitively or non-competitively) protect against destruction of

dopaminergic nigral neurons following MPP^+ injected directly into the substantia nigra pars compacta of rats (Turski *et al*, 1991), thereby supporting the hypothesis that NMDA receptor-mediated events are involved in the neurotoxicity of MPP^+.

Neuronal energy deprivation could alter normal functioning of cell membranes and cause a partial depolarisation leading to a release of the voltage-dependent Mg^{2+} block of the NMDA receptor ion channels (Nowak *et al*, 1984). Removal of the Mg^{2+} block enables EAAs to persistently excite their receptors, to open the ion channels and to become neurotoxic (Novelli *et al*, 1988).

1.4.2 Alzheimer's Disease

1.4.2.1 Introduction

AD is a progressive neurological disorder characterised by neuronal degeneration accompanied by cognitive deterioration (Daya, 1999). Behavioural disturbances are frequently present, and delirium, delusions and depressed mood can be superimposed on the dementia (Pepeu, 2000).

It is accepted that AD has a genetic component, but not all cases of AD can be traced back to a genetic origin. Other factors impacting on the onset and progression of AD include advanced age (recent reports claim about 30% of the population over 85 years of age succumb to AD), dietary and nutritive factors (Cutler and Sramek, 2001).

The main pathological features of dementia of the Alzheimer's type comprise of β-amyloid (Aβ) plaques, neurofibrillary tangles and a loss of neurons, especially cholinergic neurons of the basal forebrain (Rang *et al*, 1995). Cellular losses in the locus ceruleus, dorsal raphe nucleus, and serototonergic central superior nucleus (Zubenko and Moossy, 1988) are linked to symptoms of depression in AD.

Amyloid β-protein associated with plaques in the brains of patients with AD are believed to be cytotoxic to neurons (Behl *et al*, 1994). Beal (1992) proposes that the role of amyloid in AD is to make neurons more susceptible to glutamate neurotoxicity, which could then contribute to neuronal degeneration. Recently it was

reported that Aβ plaques are redox active, and reduce Cu^{2+} or Fe^{3+} which then produce H_2O_2 by electron transfer to O_2 (Huang *et al*, 1999 sited in Bush, 2000).

1.4.2. Metals and Alzheimer's Disease

Several recent studies have indicated that there are imbalances of trace elements including aluminium, silicone, lead, mercury, zinc, copper and iron in AD (Sayre *et al*, 1999).

Aluminum ions are known to be neurotoxic. Aluminosilicates have been identified at the cores of senile plaques and aluminium has been found within neurons bearing neurofibrillary tangles (Birchall and Chappell, 1988; Good *et al*, 1992). The accumulation of aluminium in the brain could be due to a disrupted blood-brain barrier in AD (Halliwell, 1992). Aluminium ions alone have been shown incapable of promoting lipid peroxidation (Gutteridge *et al* 1985), but under certain circumstances, are able to accelerate Fe^{2+}-dependent peroxidation. Aluminium may also interfere with cellular iron metabolism, allowing for iron to be more available to catalyze free radical reactions (Good *et al*, 1992; Youdin, 1988b cited in Halliwell 1992). Superoxide radicals have been reported to form a complex with aluminium ions that is a stronger oxidising agent than $O_2^{\cdot-}$ itself (Kong *et al*, 1992). From this evidence, it is likely that co-accumulation of iron and aluminium may facilitate oxidative damage, but this needs to be proven relevant in AD.

Zinc, copper and iron binding proteins have also been shown to be significantly altered in the AD brain (Bush, 2000). Sayre *et al* (1999) undertook studies that indicate that the accumulation of redox active iron and probably also copper in AD pathology are major producers of ROS. These metabolites are responsible not only for the numerous oxidative stress markers that appear on neurofibrillary tangles and amyloid plaques, but also for the general oxidative stress observed in AD.

1.4.2.3 Excitotoxicity and AD

Maragos *et al* (1987) propose that glutaminergic overstimulation due to excessive synthesis or release of glutamate, decreased inhibition of glutamatergic neurons, or

impaired reuptake or catabolism of glutamate can produce an excitotoxic effect leading to neuronal degeneration and eventual formation of neurofibrillary tangles.

The above proposal and the obvious involvement of trace metal elements in AD provide compelling evidence for excitotoxicity and oxidative stress playing an important role in this progressive neurodegenerative disease. Consequently, current therapeutic approaches include drugs such as estrogen and antioxidants, with the intent of providing neuroprotection to slow neurodegeneration (Cutler and Sramek, 2001).

1.4.3 Huntington's Disease

1.4.3.1 Introduction

HD is an autosomal dominant neurological disorder resulting in brain degeneration, which usually becomes apparent in midlife. It leads to progressive dementia and choreiform movements, which makes speech and feeding increasingly more difficult (Rang *et al,* 1995). Behavioural disturbances are often evident including paranoia, both auditory and visual delusions, increased aggression and depression (Bird, 1980).

The major basis of the clinical symptoms of HD is the death of striatal neurons in the basal ganglia (Figueredo-Cardenas e*t al,* 1994). This is observed in the early stages of the pathologic process, with selective degeneration of medium sized spiny neurons, but later the disease becomes generalised, involving neuronal loss in most parts of the brain (Taylor-Robinson *et al*, 1996).

1.4.3.2 Metals and Huntington's Disease

Dexter *et al,* (1991 cited in Perez, 1996) produced evidence of increased concentrations of copper and iron in caudate nucleus, putamen, substantia nigra and cerebral cortex of post-mortem HD brains. This pattern of increased copper in HD patients is not shared by other neurodegenerative diseases of the basal ganglia, such as PD, suggesting that changes in transition metals are linked to the different neuronal

populations affected (Santamaria *et al*, 1996). Once again, trace metals are seen to be available to induce oxidant stress.

1.4.3.3 Excitotoxicity in Huntington's Disease

Glutamate-mediated excitotoxicity involving striatal and cortical neurons has been postulated (Rang *et al*, 1995). Bird and Iversen (1974) discovered on examination of post-mortem brains from patients with HD a number of abnormalities including a 75% reduction in the activity of glutamic acid decarboxylase and a variable reduction in the activity of choline acetyltransferase. It is further believed that the loss of GABA-mediated inhibition in the striatum produces a hyperactivity of dopaminergic synapses, partly accounting for the choreiform movements of HD. Perry and Hansen, (1990) found that postmortem study of patients dying with HD shows reduced glutamate and normal glutamine levels in the caudate nucleus. It was thus suggested that the failure of glutamate reuptake mechanisms from the extracellular space could lead to reduced levels of intracellular striatal glutamate, with increased toxic levels of glutamate in the intrasynaptic cleft.

Although the nature of the primary biochemical defect remains undetermined, overall evidence available suggests that an excitotoxic process may be involved in HD (Figueredo-Cardenas *et al*, 1994).

1.4.3.4 Animal Model for Huntington's Disease

QA has been shown to be able to reproduce some neurochemical and histopathological features of HD, such as GABA depletion and striatal spiny cell loss, if administered topically into the rat corpus striatum (Beal *et al*, 1986). Shoham *et al*, (1992) have shown that after a single unilateral injection of QA and kainate into the rat ventral-striatal region, iron is accumulated in high concentrations in the basal ganglia areas such as globus pallidus and substantia nigra pars reticulata. Reynolds *et al* (1988) however found that QA concentrations were not significantly elevated in the striatum of individuals with HD. Although QA toxicity is the most useful animal model available to help our understanding of possible pathological pathways in HD, it does not necessarily reproduce the actual events that cause the disease in man.

1.5 Objectives

The objectives of this study were to determine whether NT, TM and FL could act as neuroprotective agents and if so, what the possible mechanism for neuroprotective action could be.

Potential neuroprotective properties would be investigated by:

a. Determination of the effect of antidepressants on lipid peroxidation induced by QA (*in vitro* and *in vivo*) and Fe^{2+} (*in vitro*)
b. Histological investigations following QA administration alone or in combination with antidepressants
c. Determination of the effect of antidepressants on damage induced by superoxide anion free radicals
d. Determination of the effect of antidepressants on TDO activity (*in vitro* and *in vivo*)
e. Determination of interaction between antidepressants and trace metals using electrochemical- and UV/Visible-studies

Antidepressants are often used to treat depression that coexists with a number of neurodegenerative diseases such as PD, AD and HD. Unfortunately neither depression nor neurodegenerative disease are fully understood. Medical professionals thus have to make use of available understanding and hypotheses in order to treat patients. Elucidating the role that certain commonly used antidepressants may play in the progression of neurodegeneration could help towards the goal of safe and effective therapy for patients.

Chapter Two

Lipid Peroxidation

2. Introduction

The utilization of oxygen for biochemical reactions by aerobically functioning cells results in the formation of highly reactive free radical species (Ottino and Duncan, 1997). These free radicals are able to induce damage in many macromolecular targets (Ottino and Duncan, 1977; Volterra *et al*, 1994) such as DNA, proteins and cellular membranes. Peroxidation of polyunsaturated fatty acids results in the formation of lipid peroxides and aldehydes which are cause extensive damage to membrane structure and integrity (Halliwell, 1992). Oxidative stress is believed to mediate damage following trauma and may play a role in disorders including AD, HD and PD (Dawson and Dawson, 1996).

The thiobarbituric acid (TBA) test is the most widely used assay for measuring lipid peroxidation (Gutteridge and Halliwell, 1990) and involves the reaction between one molecule of malondialdehyde (a lipid peroxidation end product) and two molecules of TBA to yield a pink chromogen which is measured colorimetrically at 532nm using a spectrophotometer (Southgate and Daya, 1999). Acid hydrolysis with TCA is required for the formation of the complex and the release of protein-bound MDA (Gutteridge and Halliwell, 1990). Recently, this method has been modified (Anoopkumar-Dukie *et al*, 2001) to reduce the impact of various limitations. The main limitation cited is that of TBA- reactive substances which absorb light in the same region as the TBA-MDA complex (Draper and Hadley, 1990).

Both methods are used in this chapter in order to determine whether the various antidepressants studied have any *in vitro* or *in vivo* effect on lipid peroxidation caused by oxidative stress.

2.1 Experiment 1: *In vitro* Effect of Nortriptyline Hydrochloride, Trimipramine Maleate and Fluoxetine Hydrochloride on Quinolinic Acid Induced Lipid Peroxidation

2.1.1 Introduction

QA is a neurotoxic metabolite of TRP (Heyes *et al*, 1996) and acts as an endogenous agonist of the NMDA glutamate receptor subtype (Štípek, *et al*, 1997). It is able to reproduce the features of HD (Popoli *et al*, 1994) and plays an important role in neurodegenerative, inflammatory and infectious diseases.

QA induces a chain of events that culminate in the generation of free radicals (Stone, 1993), to which neuronal cells are especially susceptible, as their surface membranes are largely composed of vulnerable unsaturated fatty acids (Southgate and Daya, 1999). In addition, the brain is relatively deficient in defense mechanisms to protect it against the wrath of free radical attack (Southgate and Daya, 1999; Viani *et al*, 1991).

The fact that brain tissue is especially vulnerable to oxidative stress becomes especially important when using drugs for neurological disorders, in cases where the brain is already compromised. Such a situation could arise when using antidepressant therapy in patients with pre-existing neurodegenerative disorders such as PD, AD and HD. Whether antidepressant drugs exert a protective or deleterious effect on the state of brain lipids is not known and thus the following investigation was undertaken.

2.1.2 Materials and Methods

2.1.2.1. Animals

The animals used were male Wistar rats weighing 250-300g, and were maintained and sacrificed as described in appendix 1 and 2. Brains were removed and either used immediately or rapidly frozen in liquid nitrogen and then stored at –70^{o}C until needed.

2.1.2.2 Chemicals and reagents

2-Thiobarbituric acid (TBA), 1,1,3,3-tetramethoxypropane (98%), butylated hydroxytoluene (BHT), NT hydrochloride, TM maleate and QA were purchased from Sigma Chemical Corporation (USA). Trichloroacetic acid (TCA) was obtained from Saarchem (Pty) Ltd (South Africa). Butanol was purchased from Holpro Chemical Company (South Africa). FL hydrochloride was supplied by Union Quimico Farmaceurica (South Africa). All other reagents used were obtained locally and were of the highest purity available.

2.1.2.3 Methods

2.1.2.3.1 Preparation of tissue homogenate

Frozen tissue was allowed to thaw and was then weighed. A 10% w/v homogenate was prepared using 50mM Tris-HCL buffer, pH 7.4.

2.1.2.3.2 Lipid peroxidation assay

Lipid peroxidation was determined using a modified method of the TBA assay, as described by Ottino and Duncan, 1997.
1ml aliquots of rat brain homogenate containing QA (0 - 1mM) alone or in combination with varying concentrations of the antidepressants (0 – 1mM) were incubated at 37^{o}C for 1 hour in an oscillating water bath. After the incubation period, 0.5ml BHT (0.5g/l in absolute ethanol) and 1ml TCA (25% w/v in aqua) were added to the mixture. The samples were centrifuged at 2000xg for 20 minutes at 4^{o}C to avoid adsorption of malondialdehyde onto insoluble protein, after which 2ml of protein free supernatant was removed. 0.5ml TBA (33% w/v in aqua) was added to this fraction and the mixture incubated at 95^{o}C for 1 hour. Following incubation 2ml butanol was added to the samples in order to extract the TBA-MDA complexes. Absorbance readings were taken at 532nm

using a Shimadzu UV-160A UV visible recording spectrophotometer and the MDA levels were determined from the standard curve generated from 1,1,3,3-tetramethoxypropane (Appendix 3). The standard curve was obtained using the method described above, except that the standards were made up with distilled water and absorbance values plotted against the molar equivalent weight of MDA in the complex assayed.

2.1.2.3.3 Statistical analysis

Results were tested to determine whether they were normally distributed prior to using a one-way analysis of variance (ANOVA). The differences between individual means were analyzed using the Tukey honest significant difference (HSD) test, and the level of significance was accepted at $p<0.05$.

2.1.3 Results

Results were expressed as nmoles MDA. Optimum incubation time and temperature for lipid peroxidation was determined (results not shown) and taken to be 60 minutes and 37^{o}C respectively.

A dose dependent increase in lipid peroxidation was observed on the exposure of rat brain homogenate to increasing concentrations of QA (0-1mM). Co-treatment of brain homogenate with QA (0.75mM) and increasing concentrations (0-1mM) of NT hydrochloride, TM maleate and FL hydrochloride respectively resulted in an overall statistically significant decline in MDA production.

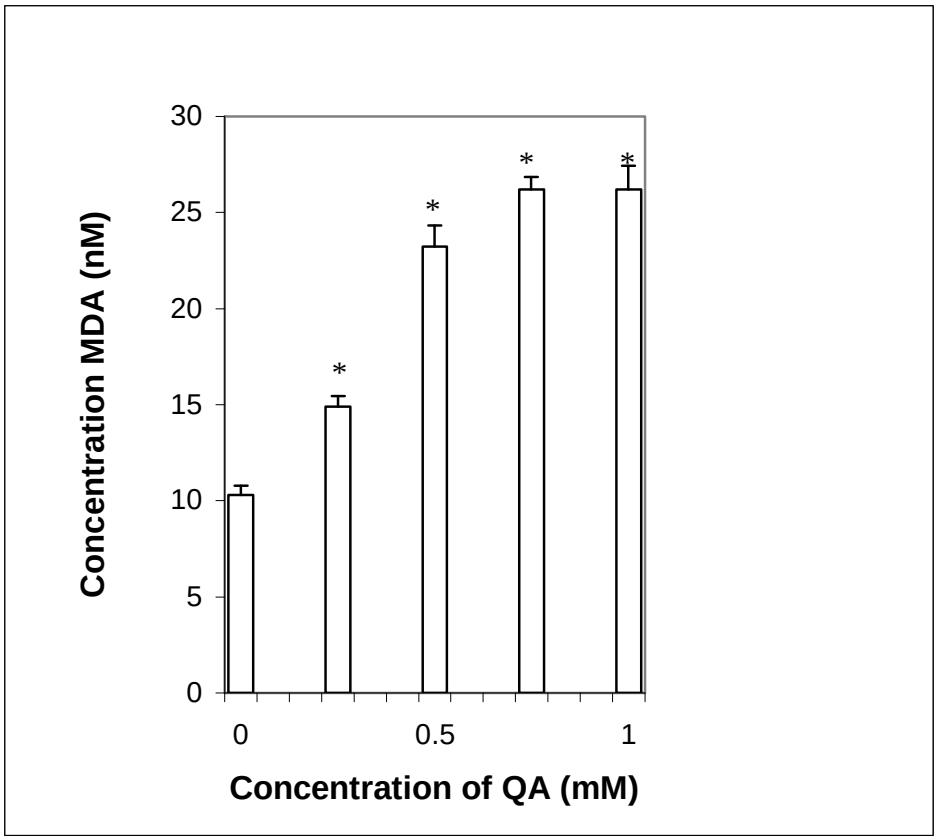


Figure 2.1: QA induced lipid peroxidation in rat forebrain homogenate. Each bar represents mean ± SEM. n=3 (0mM QA vs increasing concentrations QA are all statistically significant [*p<0.001]). Tukey HSD test

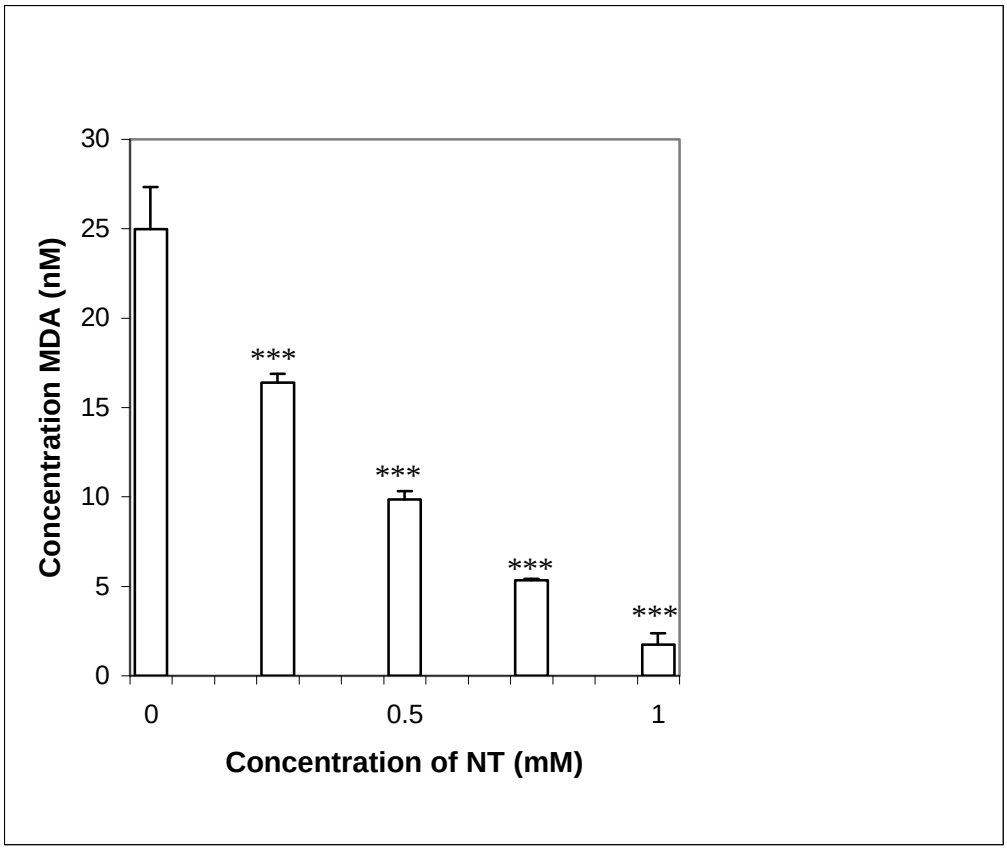


Figure 2.2: The effect of various concentrations of NT hydrochloride on QA (0.75mM) induced lipid peroxidation. Each bar represents mean ± SEM. n=3. (***p<0.001 *vs* 0mM antidepressant). Tukey HSD test

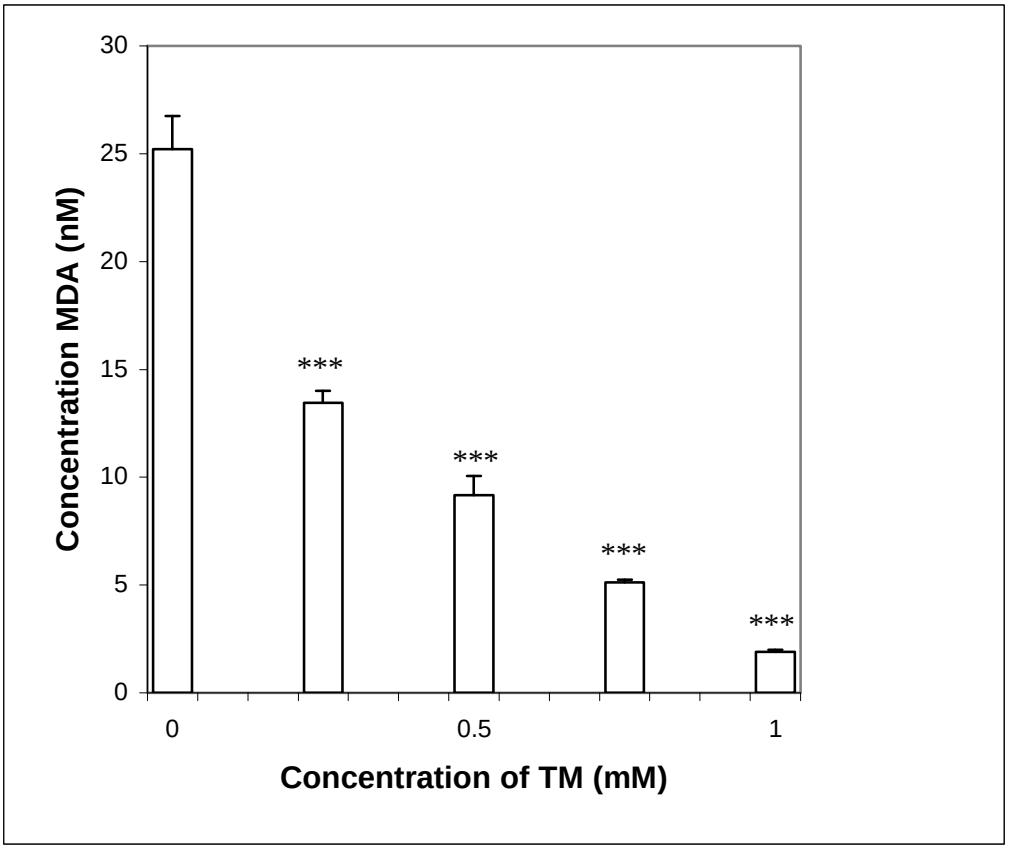


Figure 2.3: The effect of various concentrations of TM maleate on QA (0.75mM) induced lipid peroxidation. Each bar represents mean ± SEM. n=3. (***p<0.001 *vs* 0mM antidepressant). Tukey HSD test

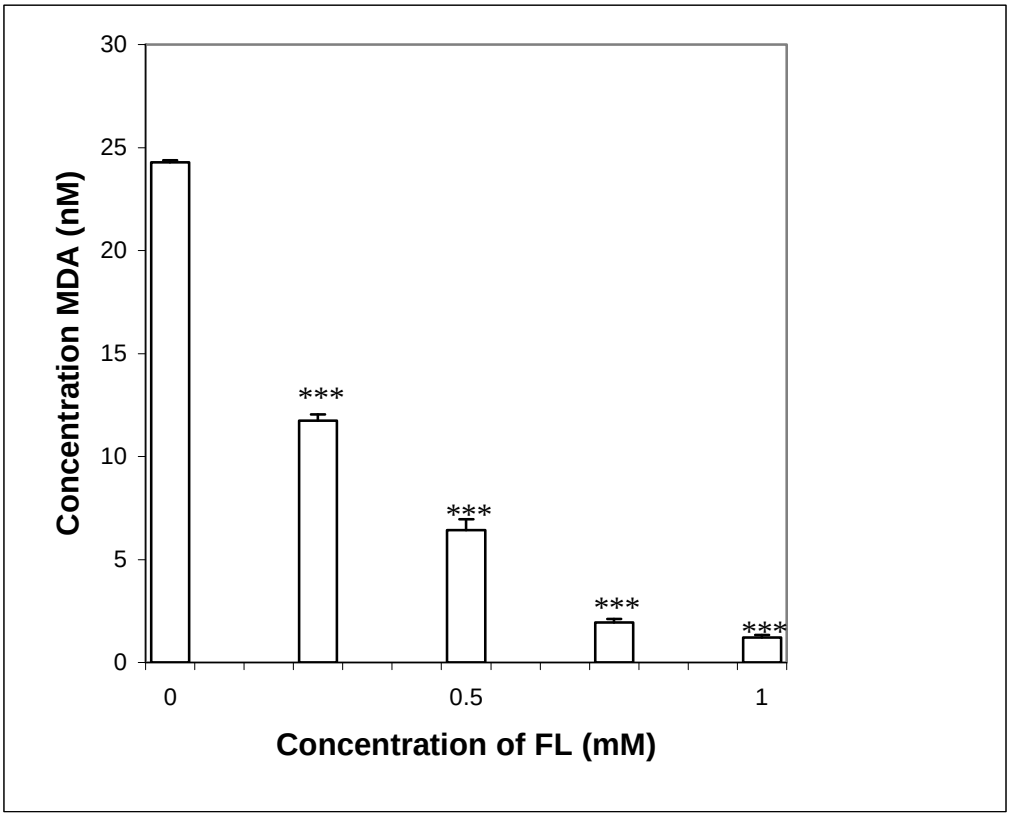


Figure 2.4: The effect of various concentrations of FL hydrochloride on QA (0.75mM) induced lipid peroxidation. Each bar represents mean ± SEM. n=3. (***p<0.001 *vs* 0mM antidepressant). Tukey HSD test

2.1.4 Discussion

The results obtained are the first to show that the antidepressants, NT, TM and FL, even though separated into two very different classes of antidepressants, are able to reduce the extent of QA-induced lipid peroxidation in rat brain homogenates. Whilst pharmacological concentrations of these agents were used, this can be justified by the high concentrations of QA required to induce lipid peroxidation *in vitro*.

The most obvious mechanism by which these antidepressants reduce the QA-induced lipid peroxidation would be due to blockade of NMDA receptors thereby minimizing Ca-induced damage. The scavenging free radicals induced by QA, despite the differences in structure between the TCAs and FL, can be an alternative or additional mechanism of neuroprotection. . Thus, it appears that these antidepressants do have the potential to offer neuroprotection and could be used for the treatment of depression associated with neurodegenerative disorders.

2.2 Experiment 2: *In vivo* effect of Nortriptyline Hydrochloride, Trimipramine Maleate and Fluoxetine Hydrochloride on Rat Forebrain Homogenate of Rat Brains which received Quinolinate by Intrastriatal Injection

2.2.1 Materials and methods

2.2.1.1 Animals

Adult, male Wistar rats weighing 250-300g were used in the experiment. The rats were randomly assembled into groups of four, and maintained as described in appendix 1.

2.2.1.2 Surgical Procedures

i. Bilateral Intrahippocampal Injections of Quinolinic acid

Male Wistar rats were anaesthetized with ether, and secured in a surgical apparatus. The skull was orientated according to the König and Klippel (1963) stereotaxic atlas. The bregma suture was located after a sagittal cut in the skin of the skull, and holes were drilled with an electrical drill at co-ordinates derived from lamda; 2.58mm anterior, 3.5mm lateral and 4mm ventral from the dura. Care was taken toprevent lesion to the meninges. A Hamilton syringe (cannula diameter of 0.3mm) was used to inject the various solutions used. The injection was administered at a rate of 1μl per minute, and the cannula left in place for a further 3 minutes following injection to allow for passive diffusion away from the canula tip. The cannula was then slowly removed and the skin sutured.

2.2.1.3 Treatment Regimes

Animals were divided into groups and treated as shown in table 2.1

Table 2.1: Treatment regimes for each group of animals

Group	**Received daily doses for 3 days prior to operation (s.c)**	**Intrahippocampal injection consisted of:**	**Received daily doses for 5 days post operation**
Control A	0.9% Saline	0.9% Saline	0.9% Saline
Control B	0.9% Saline	1µmol QA in H_2O	0.9% Saline
Drug - NT	NT in 0.9% saline (10g/kg)	1µmol QA in H_2O	NT in 0.9% saline (10g/kg)
Drug - TM	TM in 0.9% saline (10g/kg)	1µmol QA in H_2O	TM in 0.9% saline (10g/kg)
Drug - FL	FL in 0.9% saline (10g/kg)	1µmol QA in H_2O	FL in 0.9% saline (10g/kg)

Animals were treated with the various antidepressants at 10h00 daily for 3 days prior to and 5 days following surgery. These were then sacrificed and their brains obtained as described in appendix 2.

2.2.1.4 Chemicals and reagents

Chemicals and reagents were obtained from sources described in section 2.2.1.2

2.2.1.5 Methods

2.2.1.5.1 Preparation of tissue homogenate

Rat brain homogenate was prepared as explained in section 2.1.2.3.1

2.2.1.5.2 Lipid peroxidation assay

Lipid peroxidation was determined using a modified method of the TBA assay as described by Ottino and Duncan,1997.
1ml aliquots of rat brain homogenate were incubated at 95^{o}C for 1 hour in an oscillating water bath. After the incubation period the samples were cooled, 0.5ml BHT (0.5g/l in absolute ethanol) and 1ml TCA (25% w/v in aqua) were added to the mixture. The assay continued as described in section 2.1.2.3.2.

2.2.1.5.3 Statistical analysis

Results were analyzed as described in section 2.1.2.3.3

2.2.2 Results

Results were expressed as nmol MDA/mg protein. MDA values were divided by the protein content of the rat brain homogenate in order to standardize the values determined, thus enabling comparisons to be made between the treated animals. Optimum incubation time and temperature were taken to be 60 minutes and 37^{o}C respectively.

No significant statistical difference in MDA levels was seen between those animals that received saline intrastriatal injections (control A) and those receiving QA intrastriatal injections (control B) (results not shown). There was however a statistically significant reduction in the level of lipid peroxidative damage determined in those animals that had antidepressant treatment prior and post intrastriatal QA injection compared to those animals receiving injections of 0.9% saline.

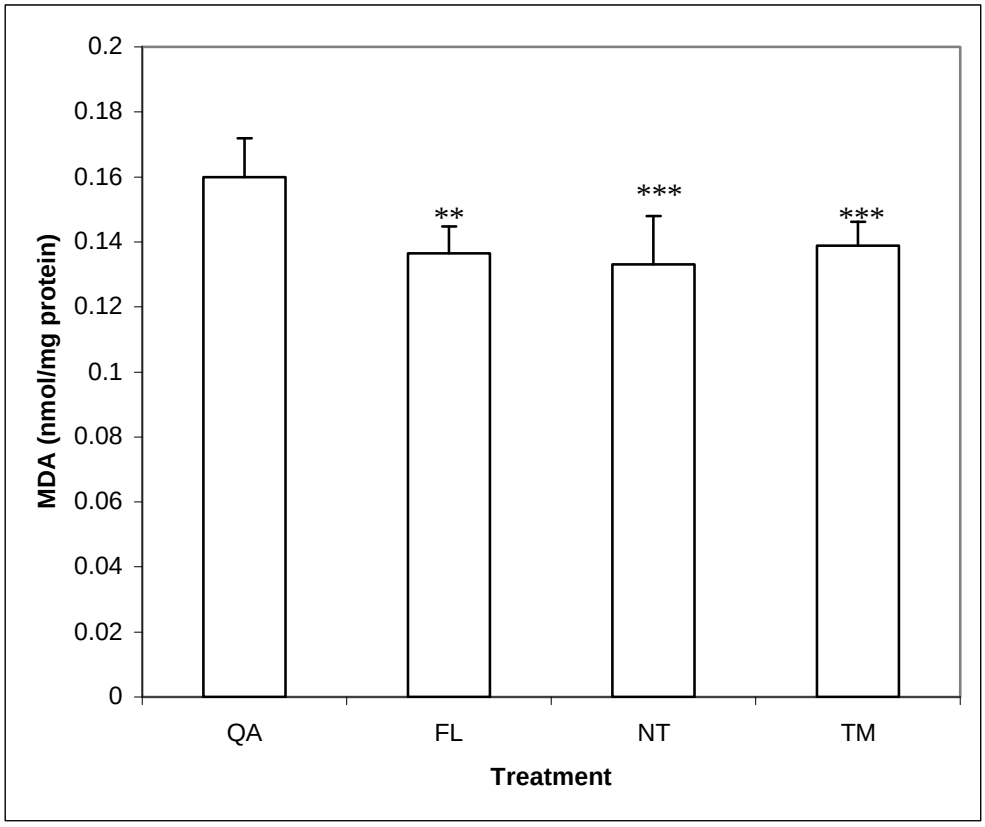


Figure 2.5 The effect of FL hydrochloride, NT hydrochloride and TM maleate (10mg/kg) on *in vivo* QA intrastriatal administration

Each bar represents mean ± SEM, n=5 (**p<0.01; ***p<0.001 in comparison with QA). Tukey HSD test.

2.2.3 Discussion

There was a significant reduction in the degree of lipid peroxidation in rat brain tissue of those animals that were treated with the various antidepressants prior and post intrastriatal QA injection in comparison to the controls. These results concur with the findings in section 2.1.4 where the antidepressants showed protective properties against QA-induced lipid peroxidation *in vitro*. Such findings once again point to the possibility that NT, TM and FL have the potential to provide neuroprotection.

The finding that lipid peroxidation induced by saline and QA intrahippocampal injections was shown to be statistically similar may be attributed to the fact that the stereotaxic procedure performed on the animals was an invasive procedure. Brain tissue would naturally be damaged upon mechanical trauma and injection of an exogenous solution, with a resultant increase in free radical production and therefore increased lipid peroxidation. An isotonic saline solution was used for the intrahippocampal injection in the control (A) group of rats in preference to a buffer such as PBS, so as to minimize the level of impurities being injected into the brain. However, the fact that a significant difference between control groups A and B (both yielding a high level of neuronal damage) and antidepressant treated groups was still evident, these results can be taken as a positive indicator for neuroprotection.

2.3 Experiment 3: *In vitro* effect of Nortriptyline Hydrochloride, Trimipramine Maleate and Fluoxetine Hydrochloride on Iron II Induced Lipid Peroxidation, as analyzed by HPLC

2.3.1 Introduction

Iron, required by the Fenton reaction, assists free radical formation in the brain (Halliwell, 1992). Its involvement in free radical and lipid peroxidation reactions has been extensively studied (Stipek *et al*, 1996; Viani *et al*, 1991). Free iron or chelated forms thereof are involved in radical reactions at many different levels (Braughler and Hall, 1989), for example, Fe^{2+} is oxidized in the presence of H_2O_2 to form $^{\cdot}OH$ and a ferryl ion (Fe^{3+} -OH):

$$H_2O_2 + Fe^{2+} \rightarrow HO^{\cdot} + {}^{-}OH + Fe^{3+}$$

This is known as the Fenton reaction, and the effective oxidizing agent in the system is the $^{\cdot}OH$ (Halliwell, 1992).
These are potent oxidants and may react with a wide range of biological substrates (Ottino and Duncan, 1997; Dawson and Dawson, 1996). Furthermore, it has been shown that lipid peroxidation in rat brain homogenates increases substantially after addition of ferrous ions with ascorbate, and the observed increase is inhibited by iron chelators, such as desferoxamine (Zaleska and Floyd, 1985).

2.3.2 Materials and methods

2.3.2.1 Animals

Adult male Wistar rats were used in this experiment and were maintained and sacrificed as described in appendix 1 and 2.

2.3.2.2 Chemicals and reagents

Methanol (HPLC grade) and ferric sulphate was purchased from BDH Laboratory Supplies (UK). IsoluteJ C_{18} solid-phase extraction (SPE) columns were obtained from International Sorbent Technology (UK). All other chemicals and reagents were obtained as described in section 2.1.2.2.

2.3.2.3 Instrumentation

Samples were analyzed on a modular, isocratic high-performance liquid chromatographic (HPLC) system. The system consisted of a spectraphysics iso chrom LC pump, a linear spectra 100 variable wavelength detector and a spectraphysics analytical data jet integrator. Samples were introduced into the system using a rheodyne fixed loop injector, fitted with a 20-ul loop. Eluted solvents were evaporated using an N-EVAP analytical evaporator.

2.3.2.4 Chromatographic conditions

C_{18} (Waters Spherisorb, 5um, 250 x 4.6 mm i.d.) solid-phase extraction (SPE) columns were used for analytical separation. A 2-µm pre-column filter (Upchurch Scientific) filtered the samples following injection.
A 14% methanol in Milli-Q water solution was used as the mobile phase and was degassed before use with a 0.45-µm membrane filter. The flow rate of the mobile phase was set at 1.2ml/min and the chart speed recorder was 5mm/min. Detector sensitivity was set at 0.1 aufs (absorbance units full scale) and the TBA-MDA complex was detected at 532nm. Resorcinol was used as an internal standard.

2.3.2.5 Methods

2.3.2.5.1 Preparation of tissue homogenate

Rat brain homogenate was prepared as explained in section 2.2.1.3.1

2.3.2.5.2 Lipid peroxidation assay

Lipid peroxidation was determined using the method described by Anoopkumar-Dukie *et al*, 2000.

1ml Aliquots of rat brain homogenate containing 1mM ascorbate, 100µM EDTA, 5mM iron sulphate and H_2O_2 alone or in combination with varying concentrations of the antidepressants (0 – 1mM) were incubated at 37^oC for 1 hour in an oscillating water bath. After the incubation period, 0.5ml BHT (0.5g/l in methanol) and 1ml TCA (15% w/v in aqua) were added to the mixture. The tubes were then heated in a boiling water bath for 15 minutes to release the protein-bound MDA, and then cooled and centrifuged at 2000xg for 15 minutes. 2ml of the protein free supernatant obtained was removed and 0.5ml TBA (0.33% w/v in aqua) was added to this fraction. Tubes were sealed and incubated at 95^oC for 30 minutes.

After the samples were cooled, TBA-MDA was separated from other possible TBA reactive substances using an IsoluteJ C_{18} SPE column. The columns were washed with 2ml methanol and then 2ml distilled water prior to the loading of 1ml of sample into the column, which was subsequently washed with 2ml distilled water. The TBA-MDA complex was eluted with 1ml methanol, which was then evaporated using an N-EVAP analytical evaporator at 60^oC under a gentle stream of nitrogen. The pink residue was dissolved in 0.5ml distilled water containing 0.1mg/ml resorcinol and these samples were analyzed using HPLC as described above. The MDA levels were obtained from a calibration curve generated using the compound1,1,3,3-tetramethoxypropane as described above.

2.3.2.5.3 Statistical analysis

Results were analyzed as described in section 2.1.2.3.3

2.3.3 Results

The ratio of the area under the curve (AUC) of the TBA-MDA peak and the AUC of the resorcinol (internal standard) peak was plotted against the concentration of MDA in the complex injected. Final results were expressed as nmol MDA.
The retention time for the TBA-MDA complex and the resorcinol was approximately 2.9 and 6.4 minutes respectively.

As shown in figures 2.6, 2.7 and 2.8 the three antidepressants used in increasing concentrations had no significant effect on iron induced lipid peroxidation.

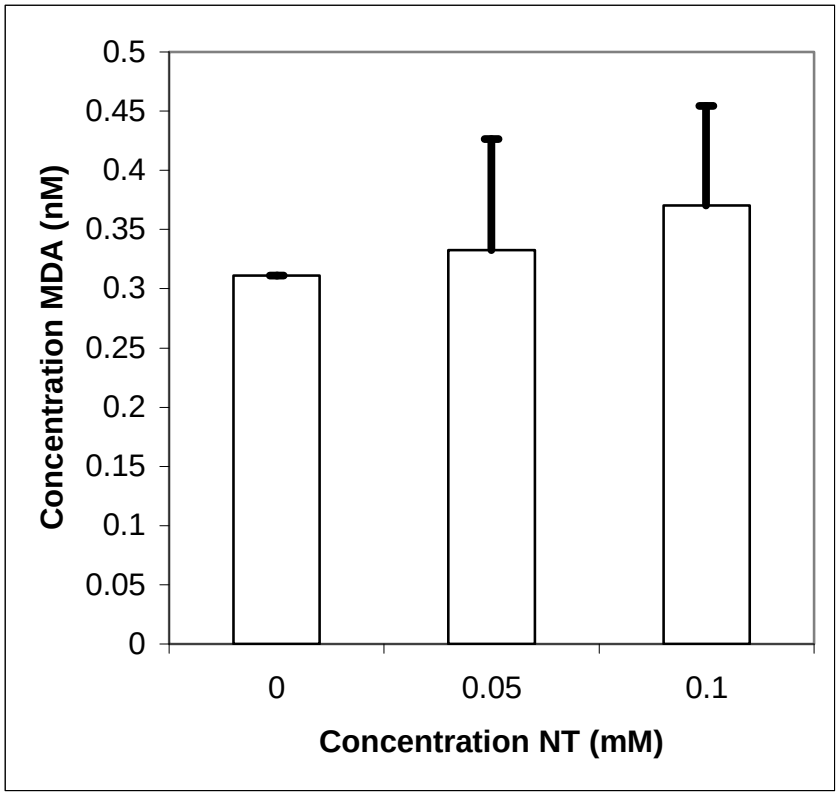


Figure 2.6: The effect of NT hydrochloride on iron induced lipid peroxidation in rat brain homogenate. Each bar represents mean ± SEM. n=3. Tukey HSD test.

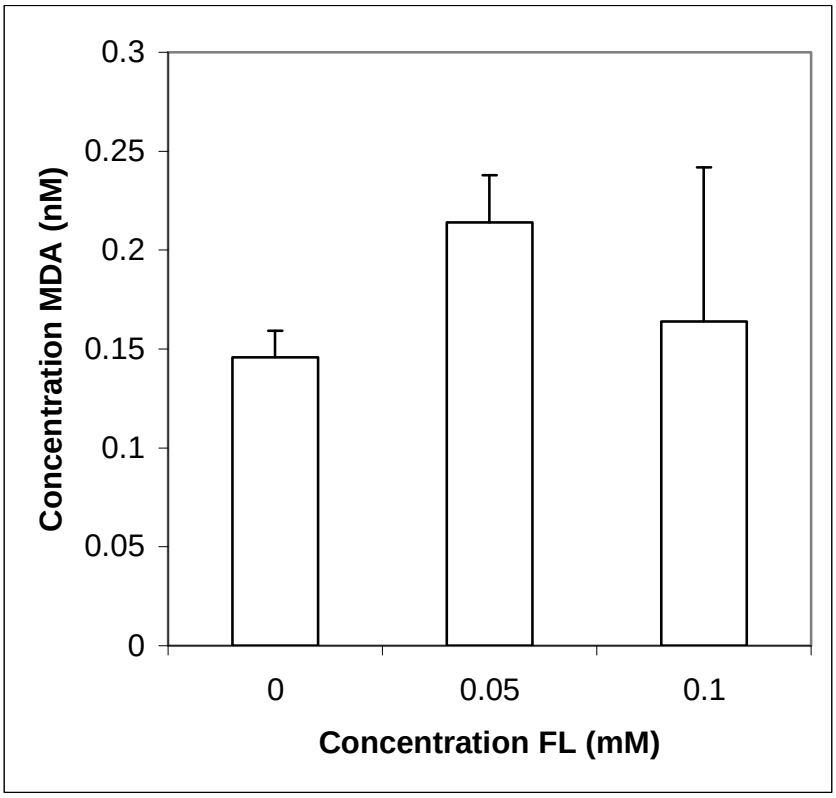


Figure 2.7: The effect of FL hydrochloride on iron induced lipid peroxidation in rat brain homogenate. Each bar represents mean ± SEM. n=3. Tukey HSD test

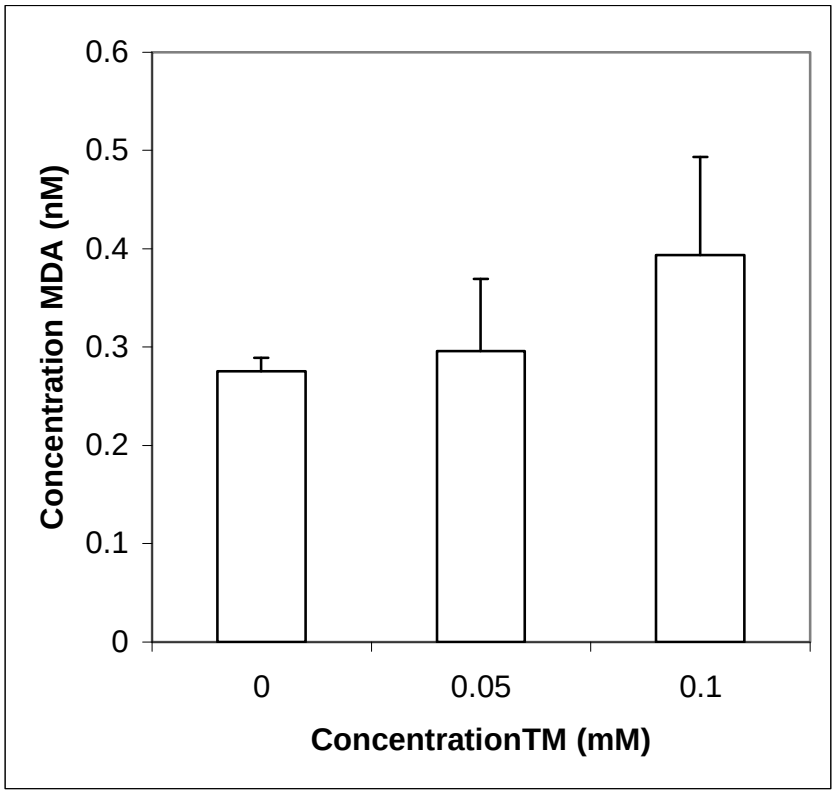


Figure 2.8: The effect of TM maleate on iron induced lipid peroxidation in rat brain homogenate. Each bar represents mean ±SEM. n=3. Tukey HSD test

2.3.4 Discussion

The TBA test for lipid peroxidation has limitations (Gutteridge and Halliwell, 1990). A number of other TBA reactants and pigments that are present in the reaction mixture may interfere with the MDA content of many materials, so it has been suggested that isolation of the TBA-MDA complex should be undertaken (Draper and Hadley, 1990). The method designed by Anoopkumar-Dukie *et al* (2001) to eliminate these variables was used for this experiment.

Numerous repetitions of the assay continually yielded data with unacceptably high standard errors and consequently results were poorly reproducible. Moreover, due to the consistently large standard errors obtained, the antidepressant drugs used in their varying concentrations failed to show any significant effect on iron induced lipid peroxidation.

Two possible reasons for such results could be: (1) Fe^{2+} used to induce lipid peroxidation was used in the form of iron II sulphate dissolved in degassed Milli-Q water. This is an unstable compound in solution and easily oxidizes to Fe^{3+} hence one cannot be certain that exactly 5mM Fe^{2+} is consistently available to the 1ml homogenate in order to induce lipid peroxidation. (2) It is uncertain as to whether the TBA-MDA complexes that were extracted with methanol and dried under nitrogen were completely dissolved when reconstituted in water containing 0.1mg/ml resorcinol, hence available for detection.

A further consideration is that the lipid peroxidation induced by Fe^{2+} is as result of a different chain of events compared to the lipid peroxidation induced by QA. QA induced lipid peroxidation is as a result of activation of NMDA receptors and a consequent massive influx of Ca^{2+} into the cell. Calcium influx is followed by a complex cascade of events, culminating in free radical generation, lipid peroxidation and protein breakdown (Lipton and Rosenberg, 1994).

Fe^{2+} contributes to lipid peroxidation by catalyzing the formation of free radical species and decomposing hydroperoxides to peroxyl and alkoxyl radicals (Halliwell and

Gutteridge, 1990). Obviously, the concentration of free Fe^{2+} available and the levels of oxygen in the tissue determine the amount of reactive oxygen species that are generated and able to induce lipid peroxidation.

Taking these factors into consideration, it might be that the results obtained in this experiment cannot be taken to be a true reflection of how the antidepressants tested influence lipid peroxidation induced by Fe^{2+}. However, it is clear that different mechanisms are involved in the induction of lipid peroxidation by Fe^{2+} and QA respectively, and that the antidepressants do not have the same affect on these events.

Chapter Three

Histological investigation

3.1 Introduction

Histology, a word derived from the Greeks meaning web or tissue, is a means by which we are able to examine preserved, sectioned and stained tissue.

In this experiment the possibility that NT hydrochloride, TM maleate and FL hydrochloride could offer neuroprotection against QA-induced intrahippocampal lesions in the rat brain was further investigated. This was done by microscopically examining sections of rat hippocampal cells after QA intrastriatal injection with prior and post antidepressant treatment, to determine if there was any evidence of morphological changes in the tissue.

Glutamate induces necrotic and apoptotic cell death (Cebers *et al*, 1997). The first sign of glutamate toxicity develops within minutes after exposure to glutamate (Choi *et al*, 1987). Necrotic neuronal death is typically characterized by rapid cell and organelle swelling and lysis. QA, an endogenous glutamate analogue, is expected to induce the same cellular response of necrotic neuronal damage, through activating NMDA receptors.

3.2 Materials and methods

3.2.1 Chemicals and reagents

QA, NT hydrochloride and TM maleate were purchased from Sigma Chemical Company (USA). FL hydrochloride was supplied by Union Quimico Farmaceutica (South Africa). Paraffin was obtained from Lasec (South Africa). Cresyl violet stain was purchased from BDH Chemicals Ltd (England) and DPX was purchased from Philip Harris Ltd (England). Haupt's adhesive was made up of: 1g gelatine, 100ml water, 2g phenol and

15ml glycerol. All other chemicals were obtained locally and were of the highest purity available.

3.2.2 Animals

Adult, male Wistar rats were used in this experiment and were maintained as described in appendix 1.

3.2.3 Surgical procedures

Surgical procedures undertaken were the same as those described in section 2.2.1.2

3.2.4 Treatment Regimes

Treatment regimes were the same as those outlined in section 2.2.1.3

3.2.5 Brain Removal

Rats were sacrificed and their brains removed as described in appendix 2.

3.2.6 Histological Technique

i. Fixing the Brain

Immediately following death, animal tissues begin to break down as a result of autolysis (self-digestion) and bacterial attack. The aim of preservation and fixation is to prevent this breakdown in order to observe tissue and cell structure.

Brains were removed and immediately placed in a mixture of formol (30%), glacial acetic acid and ethanol (2:1:7v/v) for 2 hours. It is essential that the brain be removed from the

fixative and stored in 70% ethanol after the recommended 2 hours of fixation, as longer periods result in excessive tissue hardening, and sectioning becomes extremely difficult.

ii. Specimen Preparation and Embedding

The brain tissue was embedded in a supporting medium (in this case, paraffin) in order to be cut.
Firstly the tissue was dehydrated using increasing concentrations of ethanol and then the ethanol was removed using xylene. Molten paraffin wax was then used to infiltrate the tissue and remove the xylene in the absence of water.

Table 3.1: Procedure for embedding brains in paraffin wax

Step	Embedding Agent	Time
1	70% Ethanol	1 hour
2	90% Ethanol	1 hour
3	Absolute Ethanol I	1 hour
4	Absolute Ethanol II	1 hour
5	Xylene I	1 hour
6	Xylene II	1 hour
7	Melted Paraffin wax I ($57^{o}C$)	1 hour
8	Melted Paraffin wax II ($57^{o}C$)	1 hour

iii. Blocking Out

Brain tissue was fixed in a block that could be cut using a microtome. Moulds were made from sellotape wrapped around the base of a suitable sized “polytop” tube. The moulds were filled with fresh molten wax and warmed forceps were used to transfer the tissue from the wax in the oven to the moulds as quickly as possible. The surface of the wax was gently blown with air until the surface solidified. The entire mould was then immersed in a dish of cold water to allow for rapid cooling to prevent the formation of crystals that might disrupt the tissue, and left overnight.

iv. Sectioning

The sellotape was removed from the wax disk, which was then trimmed into a block with two sides parallel and the other two converging (see figure 3.1).

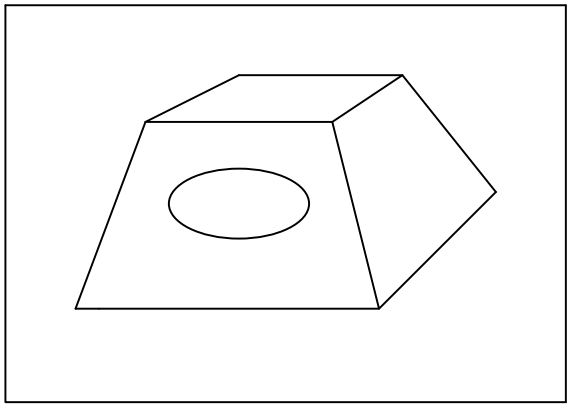


Figure 3.1: Schematic diagram of a wax block with brain embedded in the center

Molten wax was used to attach the wax block to a small wooden base. This was secured to the microtome chuck, ensuring that the parallel sides of the block were parallel to the knife edge. Sectioning was done using a rotary microtome that was set to cut at 5µm. Sections cut stuck together to form ribbons, which were removed and placed on a clean sheet of paper. Those sections containing the hippocampus were selected and placed in a water bath (40^{o}C) in order to "iron out" the wax wrinkles.

v. Transferring Sections to Slides

A thin layer of Haupt's adhesive was spread onto the slides, which were then carefully dipped under the ribbons of wax floating on the water bath, and lifted out. The slides were left overnight in an oven set at 40^{o}C.

vi. Staining

Cresyl violet was used to stain the sections. Sections needed to be dewaxed and rehydrated as the stain is water soluble. This was done as per table 3.2.

Table 3.2: Procedure for dewaxing and rehydrating slides

Step	Processing Agent	Time
1	Xylene I	5 minutes
2	Xylene II	5 minutes
3	Xylene/Absolute Ethanol (1:1)	3 minutes
4	Absolute Ethanol I	5 minutes
5	Absolute Ethanol II	5 Hours at 30^{o}C

Sections were placed in a 0.1% cresyl violet solution for 2 hours in order to be stained. Slides were then rinsed in a flat dish with 95% ethanol until the background was clear and thereafter were dehydrated as per table 3.3.

Table 3.3: Procedure for dehydrating brain sections after staining

Step	Processing Agent	Time
1	Absolute Ethanol I	5 minutes
2	Absolute Ethanol II	5 minutes
3	Xylene I	5 minutes
4	Xylene II	5 minutes

vii. Mounting of the Slides

Sufficient DPX was added to just cover the tissue sections, which were kept moist with xylene. A cover slip was carefully placed over the tissue and the slides allowed to dry on a flat surface for 48 hours.

3.2.7 Photo-microscopy

The slides were photographed using a combination Olympus camera and light microscope.

3.3 Results

The CA1 and CA3 regions of the hippocampus were investigated. Evidence of damage in the neurons of all the animals treated with QA was apparent due to roundness and swelling of neuronal cells, with signs of debris.

Micrographs of the cells presented in figures 3.2 – 3.6 are representative of the histological investigations undertaken in this experiment.

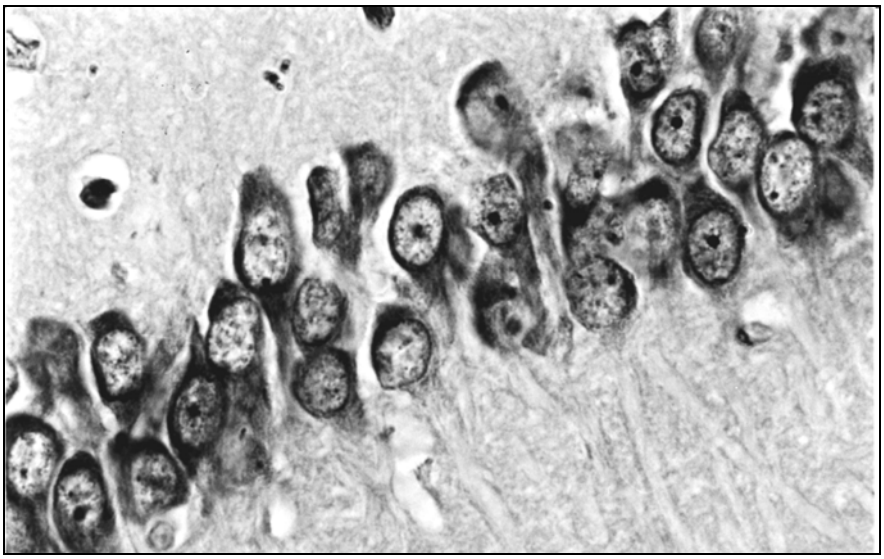


Figure 3.2: Micrograph of cells in the CA1 region of the hippocampus from an animal treated with 0.9% saline prior and post QA intrastriatal injection.
Magnification x 1320

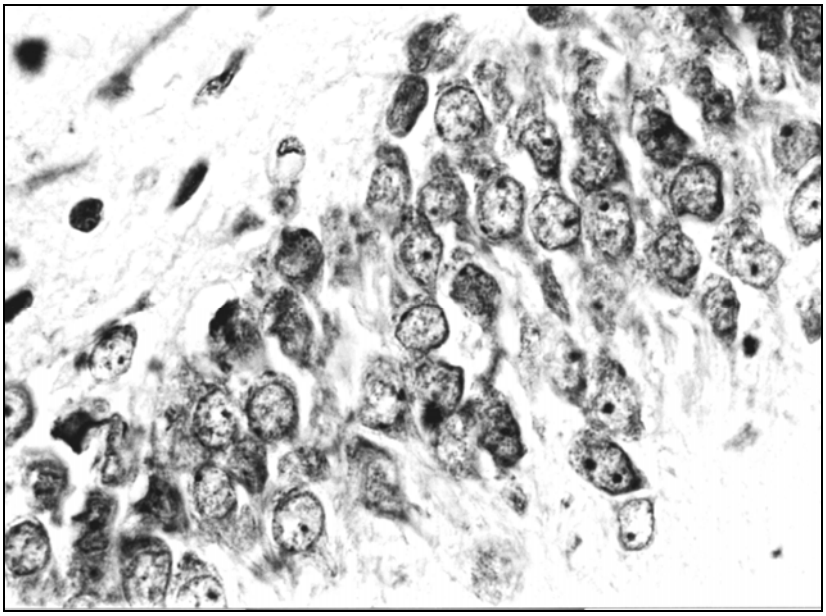


Figure 3.3: Micrograph of cells in the CA1 region of the hippocampus from an animal treated with antidepressant prior and post QA intrastriatal injection.
Magnification x 1320

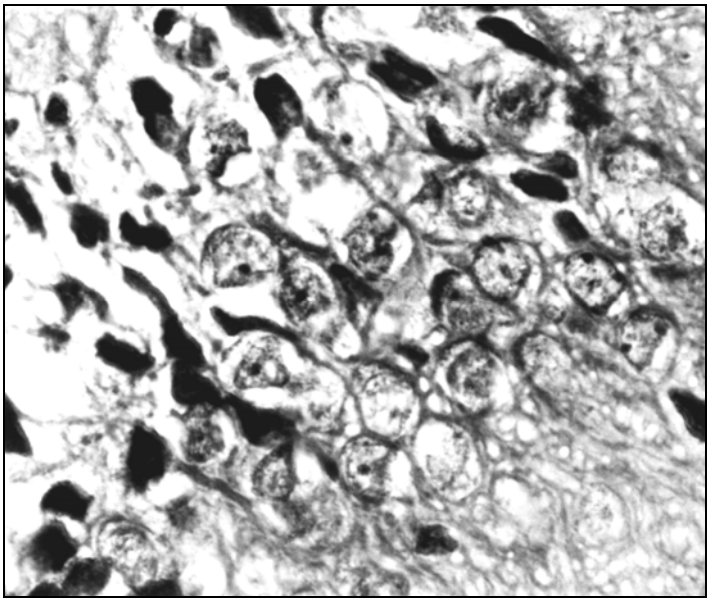


Figure 3.4: Micrograph of cells in the CA3 region of the hippocampus from an animal treated with 0.9% saline prior and post QA intrastriatal injection.
Magnification x 1320

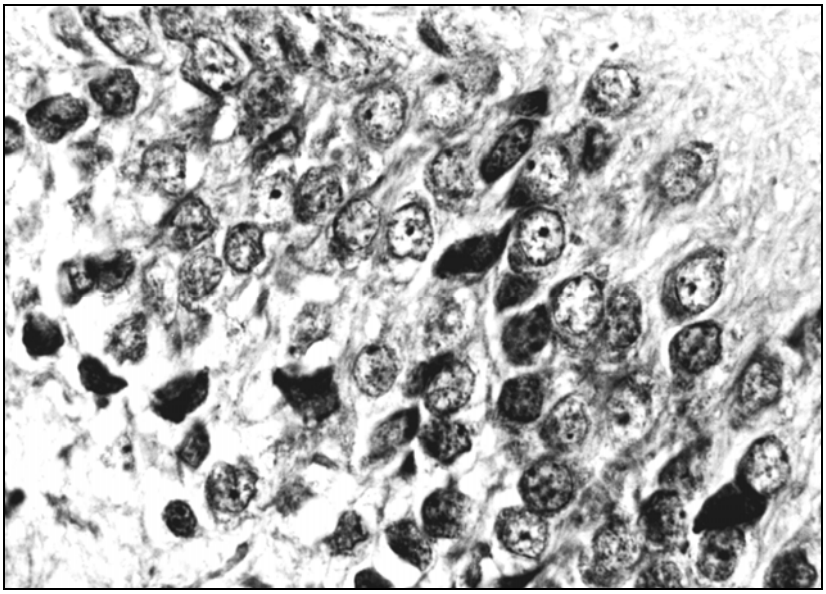


Figure 3.5: Micrograph of cells in the CA3 region of the hippocampus from an animal treated with antidepressant (TCA) prior and post QA intrastriatal injection.
Magnification x 1320

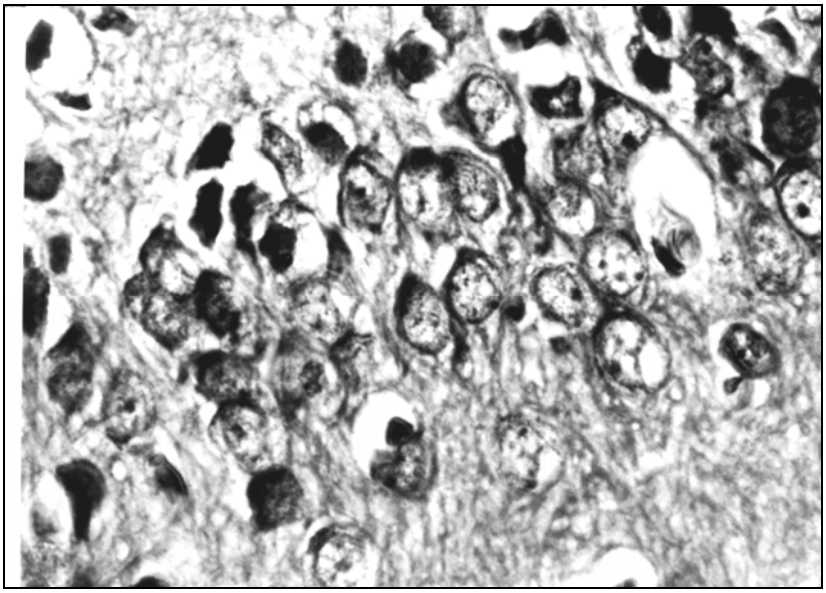


Figure 3.6: Micrograph of cells in the CA3 region of the hippocampus from an animal treated with antidepressant (SSRI) prior and post QA intrastriatal injection. Magnification x 1320

3.4 Discussion

It could be argued that the neuronal swelling evident in the micrographs of those animals that received antidepressant treatment is not as severe as those that received saline injections. However, as this is not overtly the case, it is believed that this histological investigation shows that the antidepressants used, provide only a marginal degree of neuroprotection. Consideration must be given to the fact that *in vivo* concentrations of the antidepressants in the CNS may be insufficient to afford significant neuroprotection against the unnaturally high concentrations of QA necessary to induce damage.

In chapter 2, it was seen that NT, TM and FL have the ability to significantly reduce the lipid peroxidative damage induced by QA. Determination of lipid peroxidation is quantifiable, by measuring the concentration of MDA. In this case however, damage induced by QA, is being accessed by observing the degree of neuronal swelling. Obviously, these two aspects of neuronal damage, and hence degree of protection

afforded by the antidepressants, need to be viewed in combination. As such, evidence suggests that NT, TM and FL do have neuroprotective abilities. The extent and significance of these abilities have to be further elucidated in order to determine to what degree they would be of benefit to patients suffering from neurodegenerative diseases.

Chapter Four

Superoxide Anion Induced Lipid Peroxidation

4.1. Introduction

Cyanide neurotoxicity has been attributed to production of cellular anoxia in the brain (Yamato and Tang, 1996). As a number of antioxidant enzymes are inhibited by cyanide, it is believed that oxidative stress also plays an important role in cyanide induced neurotoxicity (Ardelt *et al*, 1989). Furthermore, it has been proposed that influx of intracellular calcium following cyanide treatment generates ROS culminating in lipid peroxidation and neuronal damage (Johnson *et al*, 1987).

The nitro-blue tetrazolium method is generally accepted as a simple and reliable method of assaying the superoxide and possibly other free radicals (Ottino and Duncan, 1997). Thus this assay was used in an investigation to determine whether NT hydrochloride, TM maleate and FL hydrochloride are able to scavenge free radicals generated by cyanide in rat brain homogenate *in vitro*.

4.2. Materials and methods

4.2.1 Animals

Adult, male Wistar rats, weighing 250 – 300g were used in this experiment and were maintained as described in appendix 1.

4.2.2. Chemicals and reagents

NT hydrochloride, TM maleate, nitroblue diformazan (NBD) and nitroblue tetrazolium (NBT) were purchased from the Sigma Chemical Corporation (USA). FL hydrochloride was supplied by Union Quimico Farmaceurica (South Africa). Glacial acetic acid was obtained from Saarchem (Pty) Ltd (South Africa). Potassium cyanide (KCN) was

supplied by BDH Laboratory Supplies (UK). All other chemicals and reagents were purchased from local sources and were of the highest chemical purity available.

4.2.3 Methods

4.2.3.1 Preparation of tissue homogenate

Rat brain tissue was obtained as described in appendix 2 and immediately homogenized (10% w/v) with 0.1M PBS, pH 7.4. This was necessary in order to prevent lysosomal damage of the tissue.

4.2.3.2 Nitroblue Tetrazolium assay

A modification of the method described by Ottino and Duncan, 1997, was used for this set of experiments.
1ml aliquots of homogenate containing KCN (0 – 4mM) alone or in combination with varying concentrations of the antidepressants (0 – 4mM) were incubated with 0.4ml of NBT (0.1% w/v in aqua) in an oscillating water bath at 37^{o}C for 1 hour. After the incubation period, samples were centrifuged at 2000g and the resultant pellet was resuspended with 2ml glacial acetic acid in order to extract the reduced NBT. Absorbance of this fraction was measured at 560nm using a Shimadzu UV-160A UV visible recording spectrophotometer and converted to µmoles diformazan using a standard curve generated from NBD (appendix 4).

4.2.3.3 Protein assay

Protein estimation was determined using the method described by Lowry *et al* (1951). 5ml of alkaline copper reagent (1ml 1% copper sulphate, 1ml 2% sodium tartrate and 98ml of 2% sodium carbonate in 0.1M sodium hydroxide) was added to 1ml homogenate in a set of clean test tubes. The tubes were mixed and allowed to stand for 10 minutes. 0.3ml Folin-Ciocalteu reagent was added to the tubes, which were left to stand in the dark

for 30 minutes, at room temperature. Absorbance was then read at 500nm using a Shiamdzu UV-160 A UV-Visible spectrophotometer. A standard curve (0-300µg/ml) was generated in the same manner as above, using 1ml BSA in Milli-Q water rather than homogenate (appendix 5).

4.2.3.4 Statistical analysis

Results were analyzed as described in section 2.1.2.3.3

4.3 Results

Results were expressed as µmoles diformazan/mg protein.
A dose dependant increase in diformazan concentration was observed on exposure of rat brain homogenate to increasing concentrations of KCN (0 - 4mM). Co-treatment of brain homogenate with KCN (2mM) and increasing concentrations (0 - 4mM) of NT hydrochloride resulted in a significant increase in diformazan concentration, whereas FL hydrochloride and TM maleate displayed no significant effect on superoxide anion peroxidation.

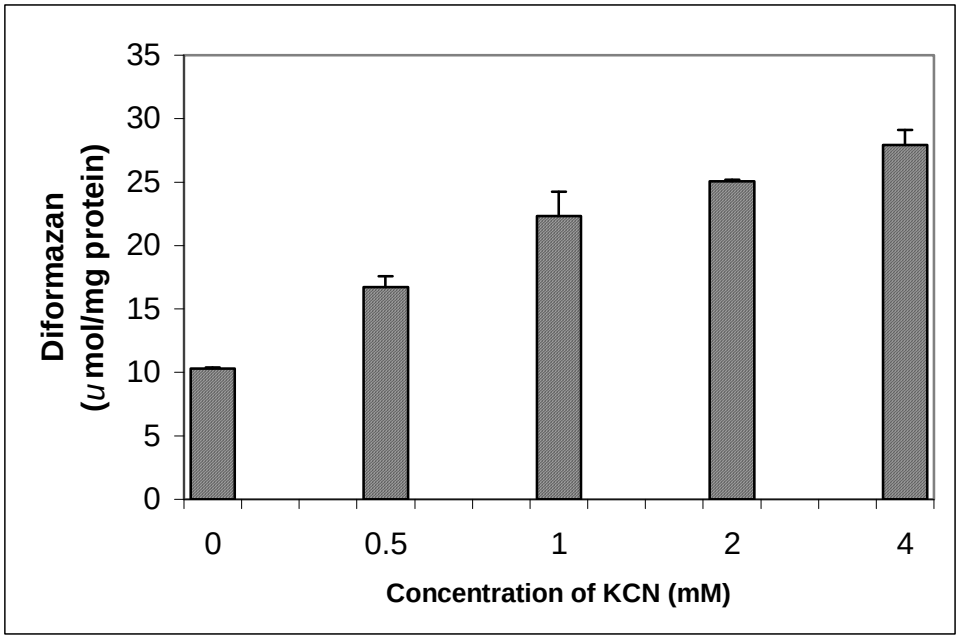


Figure 4.1: The lipid peroxidative effect of increasing concentrations of KCN on rat brain homogenate. Each bar represents mean ± SEM. n=3. (0mM vs increasing concentrations KCN are all statistically significant $p<0.001$). Tukey HSD test.

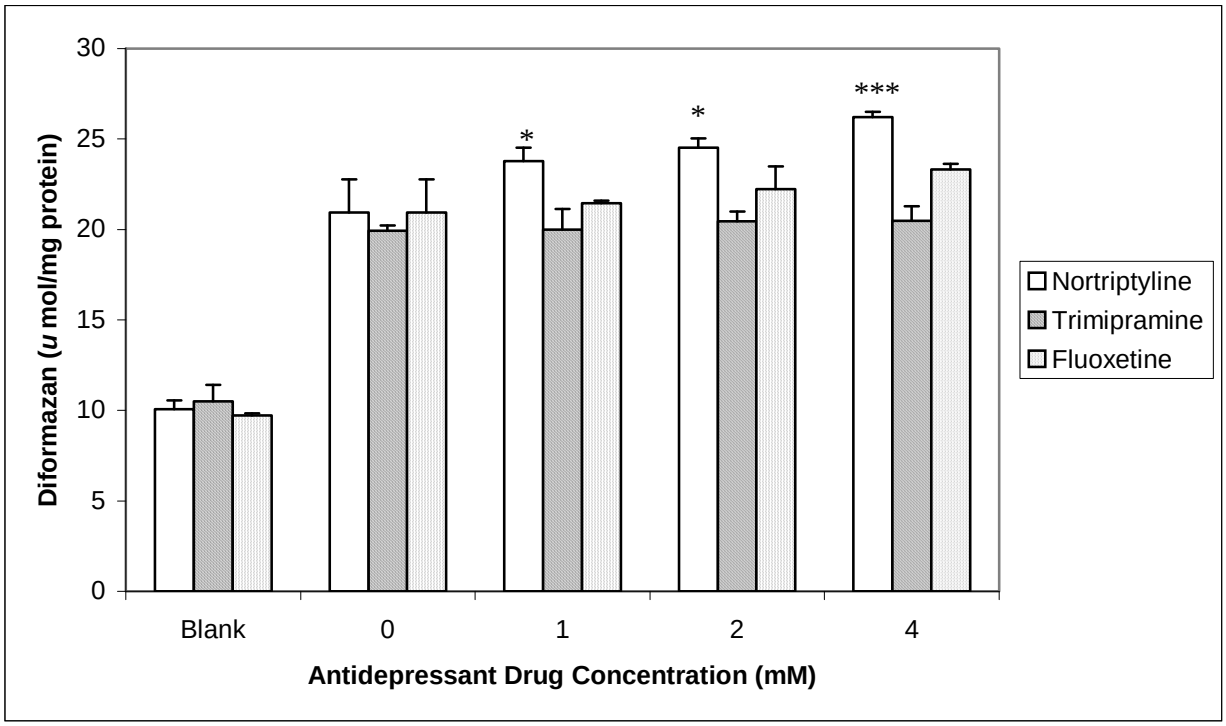


Figure 4.2: The effect of increasing concentrations of NT hydrochloride, TM maleate and FL hydrochloride on superoxide anion lipid peroxidation as induced by 2mM KCN (blank contains no KCN or antidepressants). Each bar represents mean ± SEM. n=3. (***p<0.005, *p<0.05 vs 0mM antidepressant). Tukey HSD test.

4.4 Discussion

KCN was used to generate the superoxide free radicals known to induce neuronal damage in rat brain homogenate. Considering the finding that the antidepressants studied displayed significant neuroprotective properties against QA-induced lipid peroxidation *in vitro* and *in vivo* (chapter 2), this investigation was undertaken to determine whether their mode of protection was due to superoxide anion scavenging abilities.

The results obtained in this experiment indicate that the antidepressants do not provide protection from cyanide induced lipid peroxidation. NT hydrochloride was shown to significantly increase damage induced by cyanide alone, in a dose dependent manner. Such results suggest that the neuroprotective properties displayed by the antidepressants against QA induced damage is due to a mechanism other than the scavenging of superoxide anion radicals.

Such mechanisms could include interaction with various trace metals involved in the production of free radicals or possible action involving NMDA receptors to prevent over stimulation and thereby inhibit resultant excessive Ca^{2+} influx into cells.

In summary, it appears that the antidepressants tested lack the ability to scavenge free radicals once they are produced, and as such, any possible protective properties may be as a result of actions prior to the generation of ROS.

Chapter 5

Liver Tryptophan-2,3-dioxygenase Activity

5.1 Introduction

TDO is a haem-dependent liver cytosolic metalloenzyme (Badawy and Evans,1975) that functions as the most important peripheral determinant of plasma tryptophan levels (Walsh and Daya, 1998). It catalyses the irreversible insertion of molecular oxygen into the pyrrole moiety of L-TRP to produce N-formylkynurenine (Walsh *et al*, 1994). TDO exists in two forms: the active holoenzyme (in the reduced form) and the inactive apoenzyme that requires addition of exogenous haematin for demonstration of its activity.

TRP is an essential amino acid that serves as the precursor for various biogenic amines in the brain, including 5-HT and aMT (Walsh *et al*, 1994). The availability of TRP for 5-HT synthesis appears to be dependent on the rate of TDO activity, thus suggesting an inverse relationship between 5-HT and aMT synthesis and TDO activity as represented in figure 5.1 (Badawy and Evans, 1981). Furthermore, a metabolite of TRP as described by the kynurenine pathway (Martin and Beal, 1992) is QA which has been shown to be an excitatory neurotoxin (Rios and Santamaria, 1991).

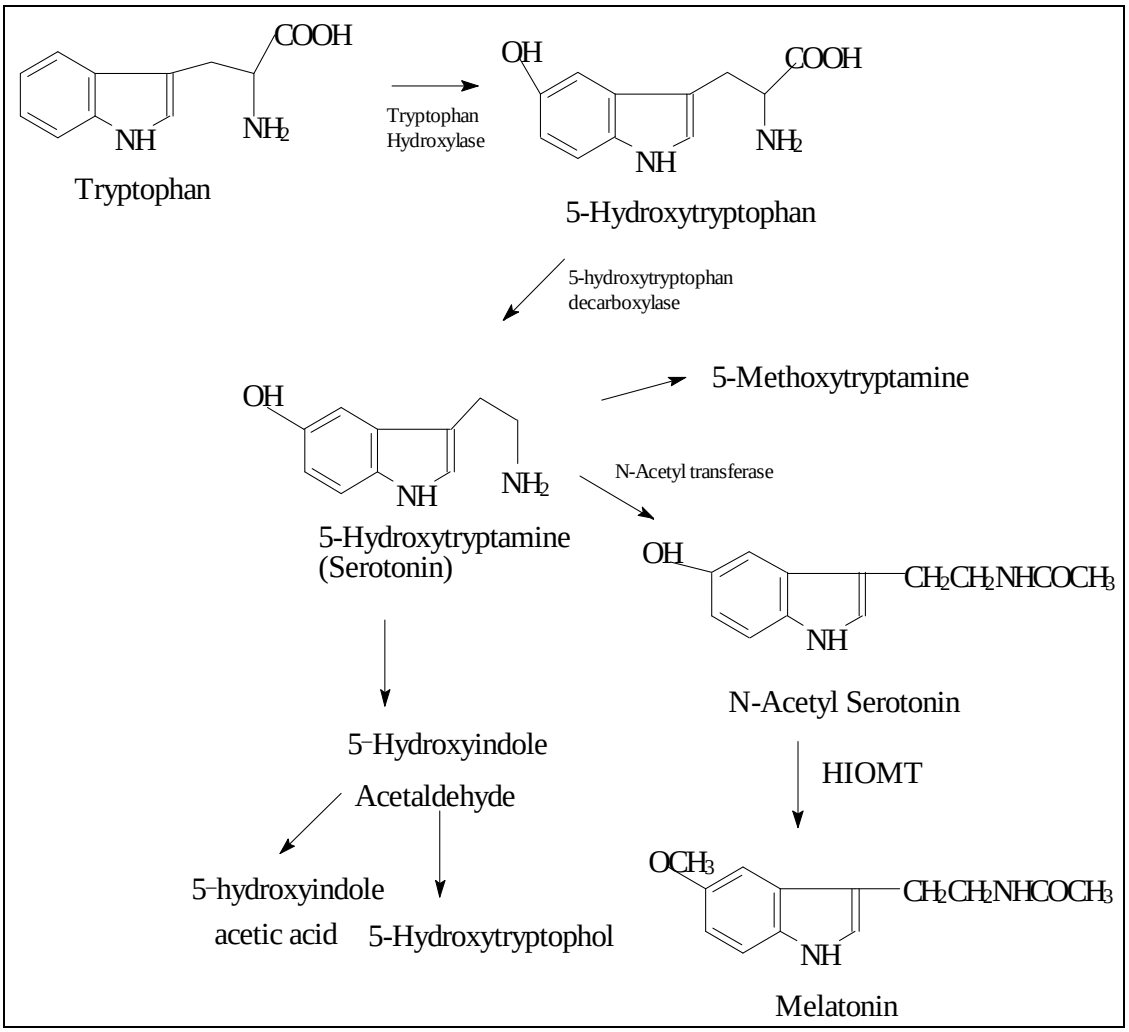


Figure 5.1: Indole metabolism pathway (modified from Young and Silman, 1982)

Results from previous studies suggest that many antidepressants inhibit TDO activity and hence increase brain TRP and consequently, 5-HT concentrations (Badawy and Evans, 1981). This is important when one considers the modified amine hypothesis for depression, which cites a functional deficit of neurotransmitter amines as the cause for depression (Harvey, 1997). There is considerable evidence that TRP metabolism is disturbed during depression and inhibition of TDO could in part explain the mode of action of these drugs alleviating depression.

The aim of this experiment is to determine whether the antidepressants being studied are able to inhibit TDO and thus ultimately increase the amount of aMT in the brain and reduce the levels of the neurotoxic metabolite, QA being formed. aMT has been shown to be neuroprotective through its antioxidant properties (Reiter *et al*, 1996) and is able to protect against QA-induced lipid peroxidation in rat brain homogenate (Southgate and Daya, 1999). A neuroprotective side effect could thus be achieved if the antidepressants were able to inhibit TDO.

The activities of both the holoenzyme and the apoenzyme should be measured in order to determine the site of action of the drugs that are able to alter TDO activity. A modification of the method described by Badawy and Evans (1975) was used, and the activity of the enzyme determined by measuring the formation of kynurenine from L-TRP.

5.1.1 Experiment 1: *In vitro* effect of Nortriptyline Hydrochloride, Trimipramine Maleate and Fluoxetine Hydrochloride on Liver Tryptophan-2,3-Dioxygenase Activity

5.1.2 Materials and methods

5.1.2.1 Animals

Adult, male Wistar rats were used for these experiments and were maintained as described in appendix 1.

5.1.2.2 Chemicals and reagents

NT hydrochloride, TM maleate and haematin were purchased from Sigma Chemical Company (USA). Fluoxetine hydrochloride was supplied by Union Quimico Farmaceutica (South Africa). L-TRP was supplied by Merck (South Africa). All other chemicals and reagents were obtained locally and were of the highest purity available.

5.1.2.3 Methods

5.1.2.3.1 Preparation of liver homogenate

Rats were sacrificed and livers were carefully removed as described in appendix 2. A 10% w/v homogenate was prepared using equal volumes of 140mM KCL/2.5mM NaOH buffer (pH 7) and 0.2M PBS (pH 7). The liver tissue was homogenized and then sonicated.

5.1.2.3.2 Determination of TDO activity

TDO activity was determined using a modification of the assay described by Badawy and Evans (1975). A scheme of the assay is outlined in table 5.1.
A 15ml aliquot of homogenate is added to 12.5ml distilled water and 2.5ml 0.03M L-TRP, to determine holo-enzyme activity. To the same components in a second reaction beaker, 0.1ml of 1.2M haematin was added in order to determine total-enzyme activity. 3ml aliquots of the reaction mixtures were transferred to clean boiling tubes kept on ice. NT hydrochloride, TM maleate and FL hydrochloride were then added to their respective tubes in increasing concentration (0 – 1mM) The tubes were saturated with carbogen (95% O_2: 5% CO_2), sealed and incubated at 37^{o}C in an oscillating water bath for 1 hour. The reaction was terminated by the addition of 2ml of 0.9M TCA, and shaken for a further 2 minutes before being filtered through Whatman No.1 filter paper. 2.5ml of the filtrate was transferred to another set of clean tubes containing 1.5ml 0.6m NaOH. The absorbance of this fraction was measured at 365nm using a Shimadzu UV-160A UV-visible spectrophotometer with 2ml TCA and 1.5ml NaOH as the blank.

Table 5.1: Scheme for the TDO enzyme assay

	Holo Enzyme	**Total Enzyme**
Homogenate (10% w/v)	15ml	15ml
Distilled H_2O	12.5ml	12.5ml
L-Tryptophan (0.03M)	2.5ml	2.5ml
Haematin (1.2M)	-	0.1ml
	Continue in Triplicate	
Transfer to clean tubes	3ml	3ml
Incubate in carbogen saturated atmosphere for 1 hour at 37^oC		
TCA (0.9M)	2ml	2ml
Shake for 2 min and filter through Whatman No. 1 Filter paper		
Transfer to clean tubes	2.5ml	2.5ml
NaOH (0.6M)	1.5ml	1.5ml
	Read absorbance at 365nm	

5.1.2.3.3 Protein assay

Protein estimation was determined using the method described by Lowry *et al* (1951), as previously detailed in section 4.2.3.3.

5.1.2.3.4 Statistical analysis

The data were analysed using a one way analysis of variance (ANOVA) following determination of Cochran C to ensure variables were normally distributed. Statistical

variance among specific means was determined using the Tukey –HSD test. A $p<0.05$ between groups was accepted as being statistically significant.

5.1.3 Results

The activity of the enzyme is determined by measuring the formation of kynurenine from L-TRP. The concentration of kynurenine was calculated by applying the Beer-Lambert Law. The molar extinction coefficient of kynurenine is 4540 l/mole.cm (Badawy and Evans, 1975). Results were expressed as moles of kynurenine formed/minute/mg protein. Holoenzyme activity was taken to be the activity present without exogenous haematin added, and total enzyme activity as that in the presence of added haematin. Apoenzyme activity was taken as the difference between the two.

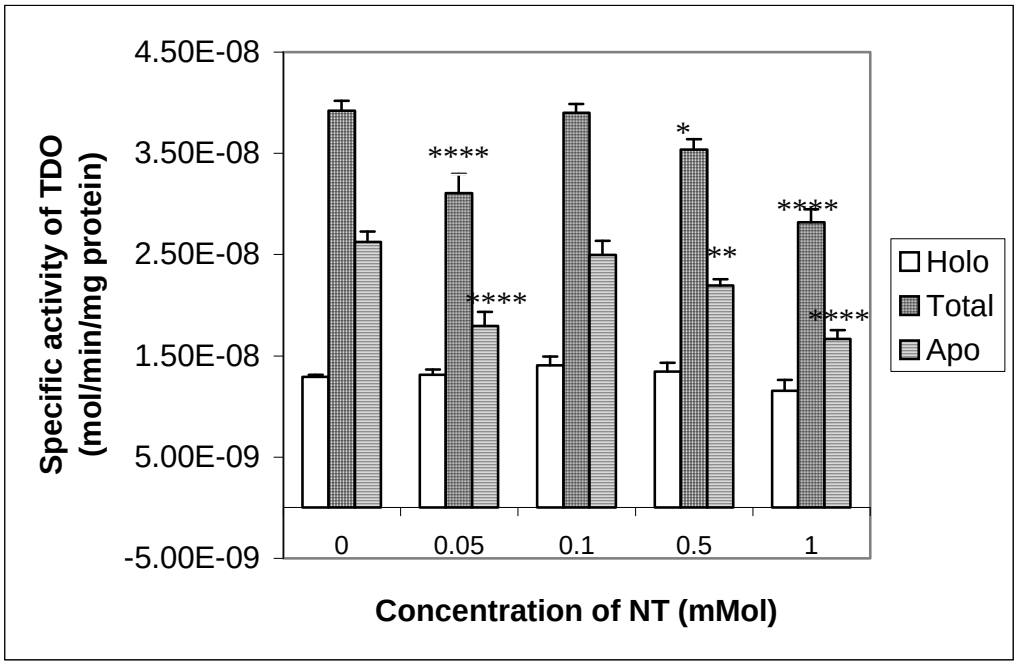


Figure 5.2: The *in vitro* effect of NT hydrochloride on rat liver TDO activity. Each bar represents mean ± SEM; n=3. (* $p<0.05$; ** $p<0.01$, ***$p<0.005$; ****$p<0.001$ in comparison with 0mM NT) Tukey HSD test.

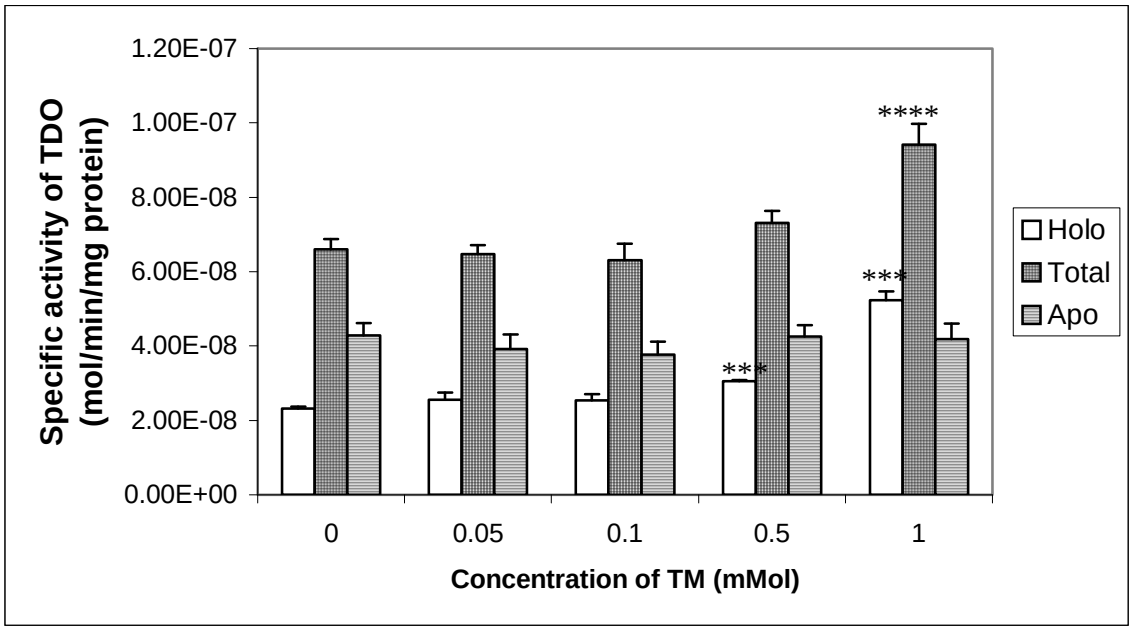


Figure 5.3: The *in vitro* effect of TM maleate on rat liver TDO activity. Each bar represents mean ± SEM; n=3. (* $p<0.05$; ** $p<0.01$, ***$p<0.005$; ****$p<0.001$ in comparison with 0mM TM) Tukey HSD test.

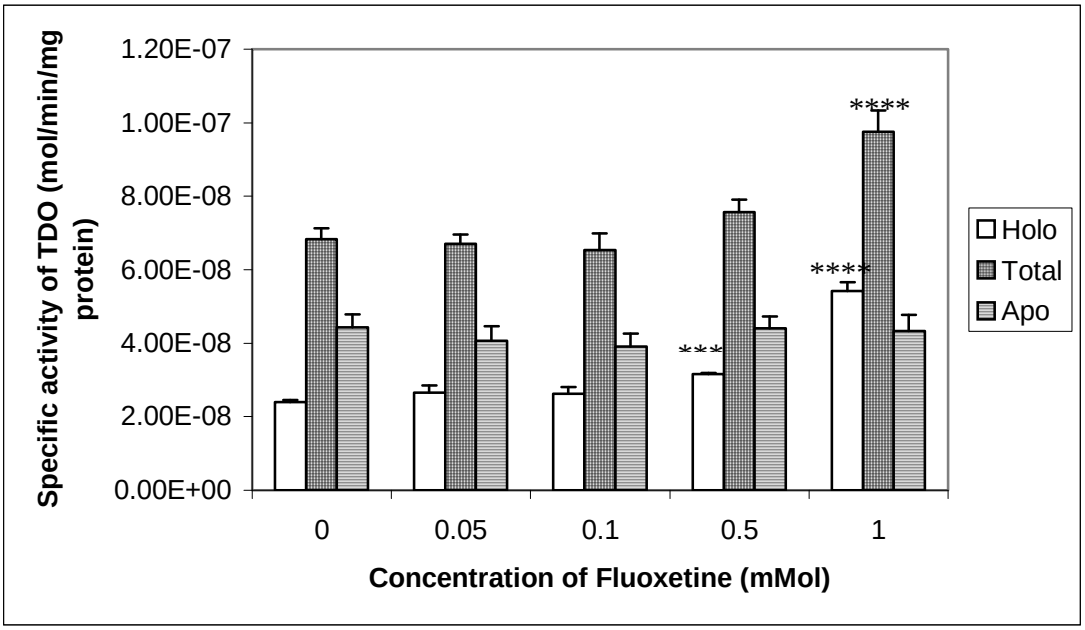


Figure 5.4: The *in vitro* effect of FL hydrochloride on rat liver TDO activity. Each bar represents mean ± SEM; n=3. (* $p<0.05$; ** $p<0.01$, ***$p<0.005$; ****$p<0.001$ in comparison with 0mM FL) Tukey HSD test.

5.1.4 Discussion

From figure 5.2 it can be observed that higher concentrations of NT hydrochloride resulted in a statistically significant inhibition of TDO total and apoenzymes. This is consistent with the findings of Badawy and Evans (1980). It could therefore be postulated that due to this inhibition, neurological tissue may be protected from excess QA formation and furthermore, a resultant elevated level of aMT production in the brain tissue could provide neuroprotection.

The results presented involving higher concentrations of TM maleate and FL hydrochloride however showed a significant stimulation of TDO holo and total enzyme activity. The assay was repeated a number of times to ensure that the increase noted was reproducible and not as a result of experimental error.

Badawy and Evans (1980) suggest that the apoenzyme activity that is inhibited by the various antidepressants tested, is due to a prevention of the conjugation of the apoenzyme with its cofactor haem. Considering that only holo and total enzyme activity is affected by these two antidepressants suggests that they are influencing levels of TDO through a mechanism different to that of NT. The finding that TDO activity is actually increased is interesting considering the monoamine hypothesis of depression and the consequent speculation that inhibition of TDO may be, in part, the mode of action of antidepressants in treating depression.

5.2 Experiment 2: *In vivo* effect of Nortriptyline Hydrochloride, Trimipramine Maleate and Fluoxetine Hydrochloride on Rat Liver Tryptophan-2,3-Dioxygenase Activity

5.2.1 Materials and methods

5.2.1.1 Animals

Adult, male Wistar rats weighing 250-300g were used for this experiment. Rats were randomly assembled into groups of five, and maintained as described in appendix 1. The control group (n = 5) received 0.25ml 0.9% saline subcutaneously. The three remaining groups (n = 5 for each group) received 0.25ml of 10mg/kg of their respective antidepressants dissolved in 0.9% saline subcutaneously. Animals were injected at 10h00 daily for 5 days.

5.2.1.2. Chemicals and reagents

Chemicals and reagents used were obtained from the sources described in section 5.2.1.2

5.2.1.3 Methods

5.2.1.3.1 Preparation of liver homogenate

Livers were obtained from rats sacrificed as described in appendix 2. The homogenate was prepared as described in section 5.2.1.3.1.

5.2.1.3.2 Determination of TDO activity

TDO activity was determined in the same way as described in section 5.2.1.3.2, though it was unnecessary to add the antidepressant drugs to the 3ml reaction mixtures.

5.2.1.3.3 Protein assay

Protein concentration was determined as described in section 4.2.3.3

5.2.1.3.4 Statistical analysis

Results were analyzed as described in section 5.1.2.3.4

5.2.2 Results

Results were obtained as described in section 5.2.2

.

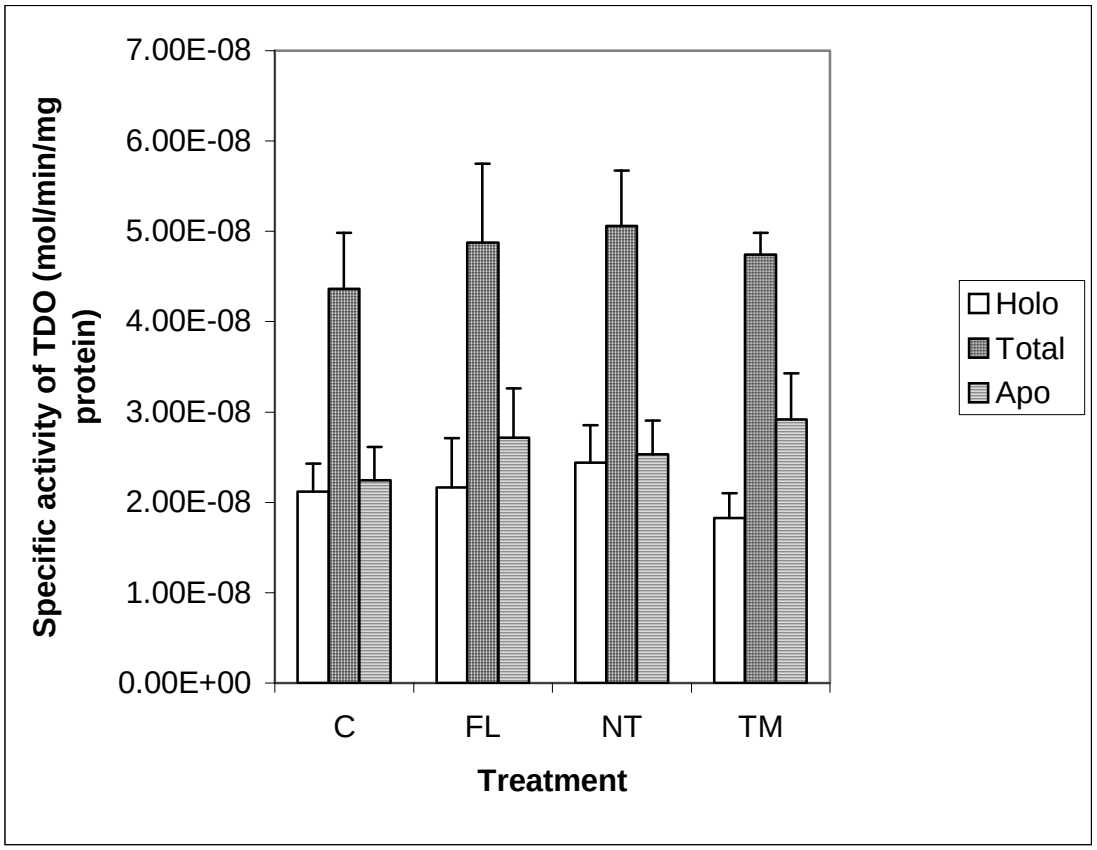


Figure 5.5: The *in vivo* effect of various antidepressants on rat liver TDO activity. Each bar represents mean ± SEM; n=5.C= control animals, FL= animals treated with FL hydrochloride, NT= animals treated with NT hydrochloride, TM= animals treated with TM maleate. Tukey HSD test.

5.2.3 Discussion

The results show that none of the antidepressants used in the experiment had a significant effect on TDO *in vivo*. It has been suggested that the ability of compounds to inhibit TDO may depend on the extent of the haem saturation of the apoenzyme and therefore on the status of haem *in vivo* (Badawy, 1980). Anoopkumar-Dukie (2000) determined that saturation of TDO with haem (via haematin or 5-ALA) increases activity of TDO, resulting in increased catabolism of TRP. It is thus possible that the concentration of haem in the livers of the animals, and furthermore, the concentration of haem added to determine total enzyme activity, was sufficiently high to overcome any possible inhibitory influence of the added antidepressants.

From the *in vitro* study is was apparent that TM maleate and FL hydrochloride did not affect apoenzyme conjugation with haem, and stimulated the activity of the TDO holo enzyme. There are many contributing factors that influence *in vivo* experimental procedures compared to the controlled environment created in *in vitro* testing and, as such it is often difficult to extrapolate results determined *in vitro* to living systems. This is an example of a situation where the living system remains unaffected by drug administration, probably due to homeostatic mechanisms in place to protect fluctuations from the healthy norm. It is also important to consider that the final concentration of the parent compound in the livers of the animals cannot be equivalent to those concentrations used *in vitro*, nor will the concentration of the antidepressants in the liver be consistent from one animal to the next.

From the results obtained it is apparent that the antidepressants used are able to influence the activity of TDO. The form of interaction and whether pharmacological concentrations used are able to influence TDO activity, need to be further elucidated.

Chapter 6

Electrochemical and UV/Visible *in vitro* investigation

6.1 Introduction

Data is rapidly accumulating to show that metallochemical reactions might be a common denominator underlying neurodegenerative disease, including: AD, amyotrophic lateral sclerosis, prion disease, cataracts and PD. Highly reactive and biologically damaging $^{\bullet}OH$ can be formed in biological material when suitable transition metal ion catalysts are available (Halliwell and Gutteridge, 1985; Aust *et al*, 1985). The most abundant metal ion likely to catalyse this reaction is iron (Gutteridge, 1986). Both copper and iron are redox-active metal ions that are able to promote the formation of reactive oxygen species within humans (Bush, 2000).

It is widely accepted that oxidant stress is one of the primary causes leading to neurodegeneration. This arises from an imbalance between free radical generation and various enzymatic and non-enzymatic antioxidant defence systems (Ames *et al*, 1993). It is important to realise that total concentrations of copper, iron, zinc and manganese in brain tissue are sufficient to damage or deregulate many proteins and metabolic systems, and efficient homeostatic mechanisms and buffers must be in place to prevent abnormal decompartmentalisation of these ions. There is increasing evidence that reactive iron becomes available during disease states (Gutteridge and Halliwell, 1984), and that this may be the case concerning other metal ions too.

With this understanding, we undertook to determine whether NT, TM and FL have the ability to interact with copper and iron *in vitro.* Interaction with these trace metal ions could prevent them from catalysing the formation of reactive oxygen species and as such, these antidepressants could provide neuroprotection *in vivo*.

aMT is a neurohormone secreted from the pineal gland. There is extensive evidence that aMT has the ability to act as an antioxidant and free radical scavenger *in vivo* and *in vitro* (Tan *et al,* 1993, Reiter *et al,* 1995; Lezoualc'h *et al,* 1996; Cho *et al,* 1997).

Melatonin is able to quench especially ˙OH (Daya, 1999). Furthermore, it is known to be highly lipophilic, allowing it to enter cells and gain access to all subcellular compartments (Daya, 1999). At present there is much research being undertaken involving the possible therapeutic use of aMT as a drug for neuroprotection. For example. aMT has been shown to be neuroprotective in the MPTP model for PD (Acun a-Castroviejo *et al*, 1996) and its role as a neuroprotectant in AD has been investigated (Daya, 1999).

aMT was included in our electrochemical and UV/visible studies so that comparisons could be made between the actions of a known neuroprotectant and the antidepressants that are commonly used by patients suffering from coexisting depression and neurodegenerative illnesses.

6.2 Electrochemistry

6.2.1 Introduction

Cyclic voltammetry is an electrochemical technique used to characterise species in solution. Species that are electroactive in solution yield characteristic redox patterns when a potential window is scanned. Electrons generated at the electrode when scanning, produce a current. This current is then plotted as a peak vs. potential. A typical cyclic voltammogram (CV) provides information on reversibility, kinetics and formal reduction potential of reactions (figure 6.1).

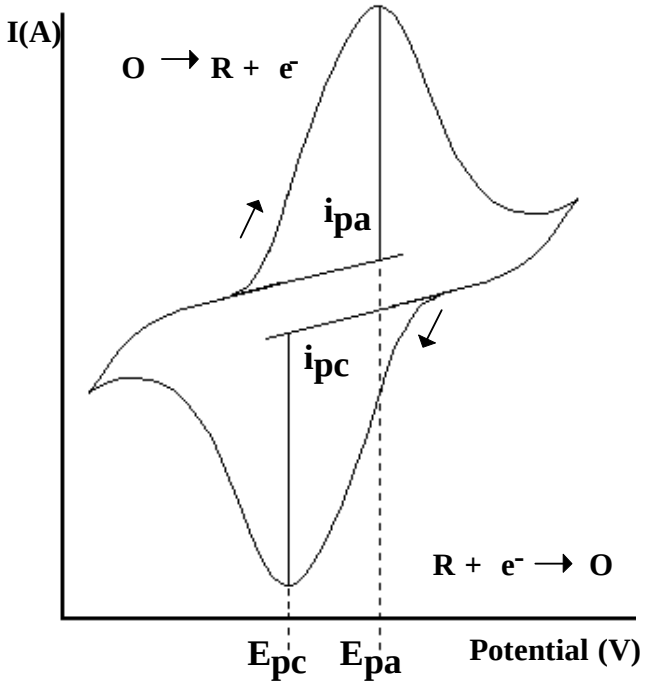


Figure 6.1 A typical cyclic voltammogram (for illustrative purposes only).

i_{pa} is the anodic peak current produced by scanning positively until the electrode becomes an adequately strong oxidant to oxidise the analyte. The current peak decays when all the species at the electrode are oxidised. The reverse scan allows for the reduction of the species, yielding i_{pc}, the cathodic peak current. The magnitude of the current is obtained from extrapolating the baselines of these peaks. The cathodic peak potential, E_{pc}, and the anodic peak potential, E_{pa} can be read from the voltammograms and the formal reduction potential, $E^{0'}$ of a redox couple is determined according to the equation below (Wang, 1994; Heinemann and Kissinger, 1996):

$$\mathbf{E^{0'} = (E_{pa} + E_{pc})/2}$$

E0' = formal reduction potential

E_{pa} = anodic peak potential

E_{pc} = cathodic peak potential

Changes in the potentials or intensity of current response when various compounds are added to a solution, in comparison to scans produced by the analytes alone, are strong indications of new species in solution.

This electrochemical technique was used to determine if any of the antidepressant drugs being studied showed interactions with selected trace metal ions in solution. If interaction of antidepressants with copper and iron ions is apparent, it could be extrapolated that these drugs have the ability to remove specific free ions from the system, and hence reduce their potential to form damaging free radicals.

6.2.2 Materials and methods

6.2.2.1 Chemicals and reagents

NT hydrochloride and TM maleate were purchased from Sigma Chemical Company (USA). FL hydrochloride was supplied by Union Quimico Farmaceutica (South Africa). Iron (II) suphate, copper (II) sulphate and iron (III) chloride were supplied by BDH laboratory supplies (UK). All other chemicals and reagents were obtained locally and were of the highest chemical purity available.

6.2.2.2 Cyclic Voltammetry

CVs were recorded in a C2 cell stand using a glassy carbon electrode (GCE). Appropriate concentrations of the various antidepressants, alone or in combination with selected trace metals, in Tris-HCl buffer (pH 7.4) were introduced into the glass cell and degassed with nitrogen for five minutes prior to scanning a potential window. Fe^{2+} was dissolved in a citric acid buffer (pH 3.5) to prevent oxidation to Fe^{3+} in solution.

6.2.3 Results

All three antidepressants in this study were dissolved and an appropriate potential window scanned. Only TM maleate yielded a reproducible scan, with peaks that corresponded to the redox patterns of the drug in solution. This appears to be the first

electrochemical characterisation of TM maleate. We proceeded to produce CVs for aMT and TM maleate and combinations of these analytes with various trace metals at appropriate concentrations.

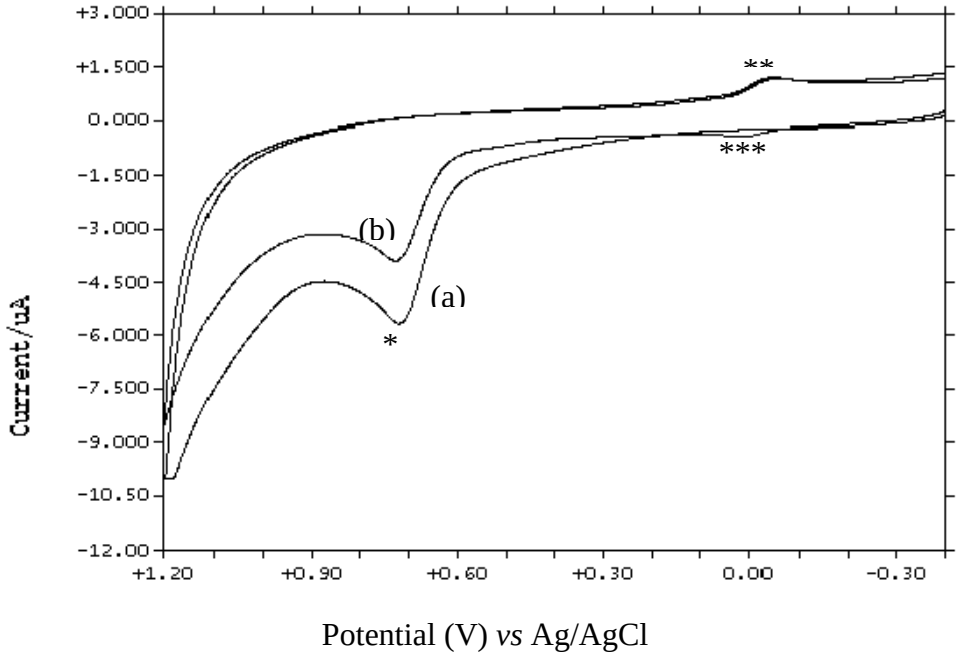


Figure 6.2: Successive cyclic voltammograms of TM maleate (4 x 10^{-5}M).
a = first scan, b = second scan

Figure 6.2 represents the CV of TM maleate. A peak due to oxidation of TM maleate is observed at 0.74 V *vs.* Ag/AgCl (*). At 0.0V weaker peak is observed on the return scan (**), corresponding to the adsorption of TM maleate at the electrode. Upon the successive scan a new peak was observed at 0.02V *vs.* Ag/AgCl (***) which is reversible with the peak at 0.0V. As this peak was not observed during the first scan, it may suggest the 'dimerization' of TM maleate, as observed by other authors looking at the anodic oxidation pathways of phenolic compounds (Papouchado *et al*, 1975). The intensity of the peak at 0.7V was reduced on the second scan, which may be related to the adsorption of TM maleate at the electrode surface.

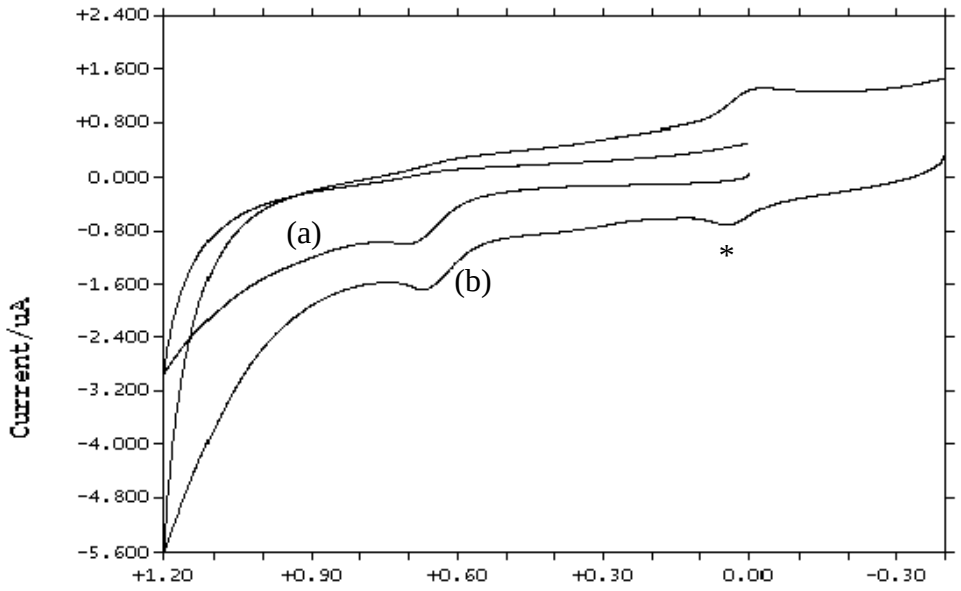


Figure 6.3: Successive cyclic voltammograms of aMT (2 x 10^{-6}M)

a = first scan, b = second scan

Figure 6.3 represents the CV of aMT. The scans are very similar to those produced by TM with an oxidation peak observed at 0.67 V vs. Ag/AgCl and a new peak formed on successive scanning at 0.043 V vs. Ag/AgCl (*). This also suggests dimerization of aMT. The formal reduction potential of the redox couple is:

$E^{0'} = (0.043 + [-0.018])/2$

$= 0.0125$ V

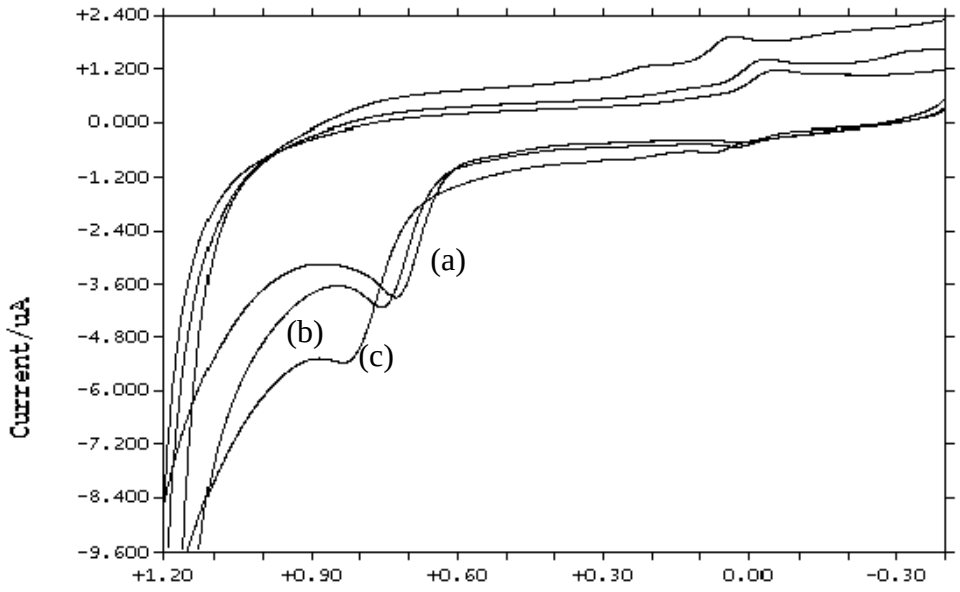


Figure 6.4: Successive cyclic voltammograms of TM maleate (4 x 10^{-5}M) in the presence of increasing concentrations of Fe^{2+} (a = 0M Fe^{2+}; b = 6 x 10^{-6}M Fe^{2+}; c = 8 x 10^{-6}M Fe^{2+}).

Figure 6.4 represents the CV of the interaction between TM maleate and increasing concentrations of Fe^{2+}. As one can see, Fe^{2+} shifts the TM maleate oxidation peak to more positive values (scan a = 0.724 V vs Ag/AgCl scan; b = 0.0753 V vs Ag/AgCl and scan c = 0.828 V vs Ag/AgCl). This is accompanied by a slight increase in peak intensity of TM maleate oxidation. It is unlikely that a new compound is being formed between TM maleate and Fe^{2+}. It is likely that these results show that a weak bond develops between Fe^{2+} and TM maleate in solution.

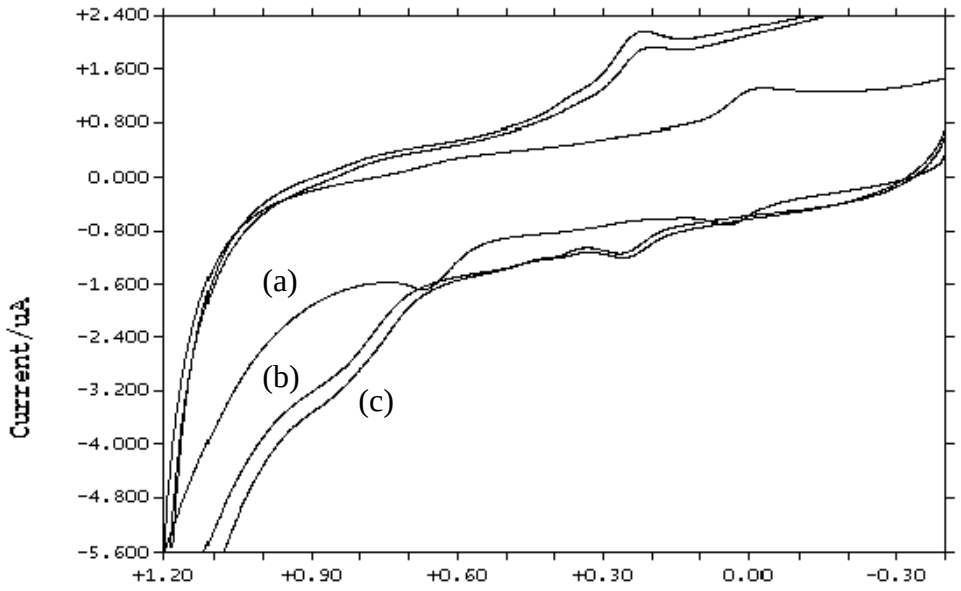


Figure 6.5: Successive cyclic voltammograms of aMT (2 x 10^{-6}M) in the presence of increasing concentrations of Fe^{2+} (a = 0M Fe^{2+}; b = 1 x 10^{-6}M Fe^{2+}; c = 2 x 10^{-6}M Fe^{2+}).

Figure 6.5 represents the CV of the interaction between aMT and increasing concentrations of Fe^{2+}. As with TM maleate, it is evident that Fe^{2+} shifts the aMT oxidation peak to more positive values (scan a = 0.724 V vs Ag/AgCl scan; b = 0.0753 V vs Ag/AgCl and scan c = 0.828 V vs Ag/AgCl). A significant decrease in the peak intensity for the oxidation of aMT was observed. It is thus apparent that there is an interaction between aMT and Fe^{2+} similar (though more evident) to that seen between TM maleate and Fe^{2+} in figure 6.3.

It is thus suggested that aMT and TM maleate may be forming a weak bond with Fe^{2+}.

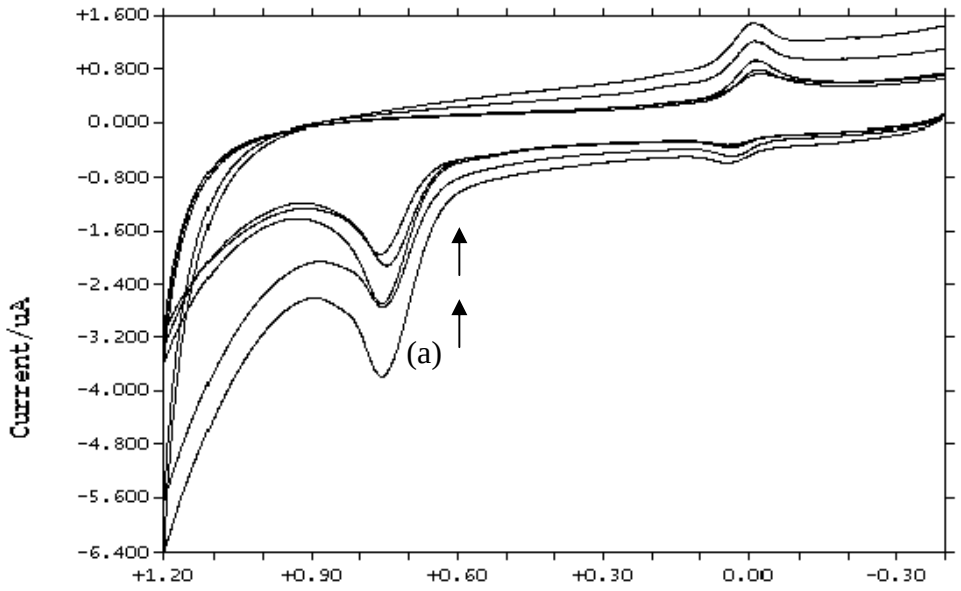


Figure 6.6: Successive cyclic voltammograms of TM maleate (4 x 10^{-6}M) in the presence of increasing concentrations of Fe^{3+} (a = 0M Fe^{3+}; 2 x 10^{-6}M; 6 x 10-6M; 8 x 10^{-6}M; 1 x 10^{-5}M).

Figure 6.6 represents the cyclic voltammograms determined from TM maleate and increasing concentrations of Fe^{3+}. The oxidation peak of TM maleate at 0.743 V vs. Ag/AgCl remains unchanged but successive scans of increasing concentrations of Fe^{3+} reduce the intensity of the peak in a concentration dependent manner.

The change in current intensity for TM maleate with increased Fe^{3+} points towards an interaction. The decrease in TM maleate may indicate that some TM maleate is interacting with Fe^{3+}, and therefore is not available for oxidation at the electrode. Increasing concentrations of Fe^{3+} results in even further reduction of the peak, as even less TM maleate is available. However, as there is no peak shift, it is suggested that the interaction is weak and intermediate.

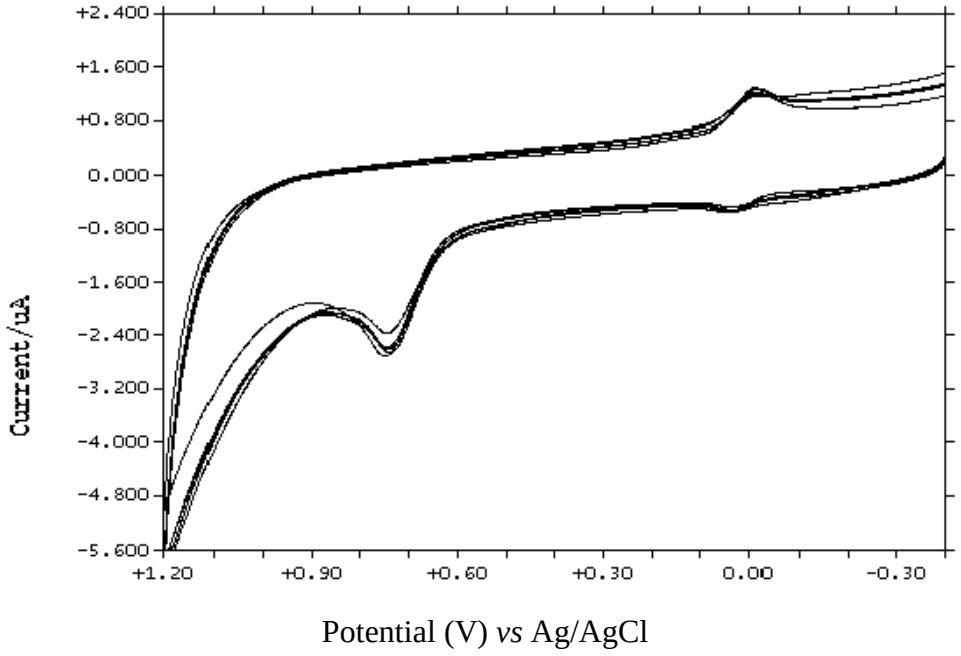


Figure 6.7: Successive cyclic voltammograms of TM maleate (4 x 10^{-6}M) in the presence of increasing concentrations of Cu^{2+}

Figure 6.7 represents the cyclic voltammograms determined on the mixing of TM maleate and solutions of increasing concentrations of Cu^{2+}. No significant changes were noted in the CVs obtained.

6.2.4 Discussion

Despite numerous attempts, using a wide range of buffers at various pH values, only TM maleate yielded a reproducible scan, with peaks that corresponded to the redox potentials of the drug in solution. It was thus clear that FL hydrochloride and NT hydrochloride are poorly electroactive, and hence this method of analyses was inappropriate for characterisation of those species in solution.

The author was however able to analyse TM maleate both alone, and in combination, with Fe^{2+}, Fe^{3+} and $Cu^{2+}$and make comparisons with aMT derived CVs.

As mentioned previously, aMT is known to have neuroprotective properties. Based on the results of our electrochemical analysis, the possibility exists that aMT has the

ability to interact with free Fe^{2+}. Interaction was seen to occur with increased concentrations of Fe^{2+}. Thus, aMT may act by 'mopping up' excess Fe^{2+} or by binding Fe^{2+} in a form that renders it unavailable for interaction with H_2O_2 to produce damaging free radicals. In an absorptive voltammetric study, Limson *et al* (1998) showed that aMT formed a bond with Fe^{3+} in solution.

As TM maleate in combination with Fe^{2+} was seen to behave in a similar electrochemical manner as aMT, it may be extrapolated that it too can provide neuroprotection by interacting with Fe^{2+}. It is believed that these results are cause for further work to be undertaken to examine the nature of the observed interactions. TM maleate was also shown to interact with Fe^{3+}. This interaction appears to be weaker than the interaction with Fe^{2+}, and the significance thereof is unclear.

The Hard Soft Acid Base (HSAB) Rule (Pearson, 1968; Huheey, 1978) could explain the differing levels interactions seen to occur in these analyses. Metal cations are classified as hard, soft and borderline acids that show preference for binding with respective hard, soft and borderline ligand bases. The hard and borderline acid metals used (Fe^{3+} and Fe^{2+}, Cu^{2+}respectively) preferably bond with various hard and borderline base functional groups on the antidepressants. However, further analyses is required in order to determine exact sites of interaction before conclusive statements can be made.

6.3 UV/Visible Studies

6.3.1 Introduction

A UV/visible study was undertaken in order to elucidate if an electronic interaction between NT, FL and TM and selected trace metals exists. As FL hydrochloride and NT hydrochloride proved to be poorly electroactive and thus could not be analysed using electrochemical techniques, UV/visible spectra were obtained in order to give an indication as to how these compounds behave in the presence of free metal ions *in vitro*.

6.3.2 Materials and methods

6.3.2.1 Chemicals and reagents

Chemicals and reagents were obtained from sources as outlined in section 6.2.2.1.

6.3.2.2 UV/Visible analysis

The individual antidepressants and metal compounds were dissolved in distilled deionised water and made up to 100ml to produce 1 x 10^{-4}M solutions. UV/Visible spectra were recoded using a Cary 500 UV/VIS/NIR over a wavelength range of 200 – 800nm. Firstly, spectra were recorded for all of the solutions alone at concentrations of 1 x 10^{-4}M, 5 x 10^{-5}M and 6.67 x 10^{-5}M. Spectra were then run for each of the antidepressants (5 x 10^{-5}M and 6 x 10^{-5}M) in combination with Fe^{2+}, Fe^{3+} or Cu^{2+} (5 x 10^{-5}M and 3 x 10^{-5}M) immediately after mixing and after 1 hour of incubation at room temperature.

6.3.3 Results

Each of the antidepressants studied and aMT produced spectra with characteristic λ-max absorbance values (see table 6.1)

Table 6.1: λ-max values as determined for NT hydrochloride, TM maleate, FL hydrochloride and aMT

Compound	Absorbance wavelength	Intensity
NT (5 x 10-5M)	202.4nm	1.835
NT (1 x 10-4M)	202.72nm	2.676
TM (5 x 10-5M)	248.48nm	0.605
TM (1 x 10-4M)	247.84nm	1.078
FL (5 x 10-5M)	262.88nm	0.069
FL (1 x 10-4M)	263.2nm	0.093
aMT (5 x 10-5M)	277.6nm	0.202
aMT (1 x 10-4M)	277.6nm	0.388

Fe^{2+}at both concentrations used, masked the characteristic absorbance peaks of all three antidepressants and melatonin. A peak corresponding to Fe^{2+} alone, at 300.64nm was therefore used to determine if any interaction was apparent.
Neither FL hydrochloride nor NT hydrochloride appeared to have an affect on the Fe^{2+} maximum absorbance value. Both TM maleate and aMT however appeared to increase the absorbance recorded at approximately 300nm for Fe` and there was no change in these elevated values noted after 1 hour incubation as shown in table 6.2

Table 6.2: The effect of TM maleate and aMT on Fe^{2+} absorbance values

Compound	Absorbance wavelength	Intensity
Fe^{2+} (5 x 10^{-5}M)	300.64nm	0.52
Fe^{2+} (3 x 10^{-5}M)	300.64nm	0.351
TM (5 x 10-^{5}M) + Fe^{2+} (5 x 10^{-5}M)	296.48nm	0.659
TM (5 x 10^{-5}M) + Fe^{2+} (5 x 10^{-5}M)-1h	295.52nm	0.66
TM (6.67x 10^{-5}M) + Fe^{2+} (3x 10^{-5}M)	295.52nm	0.601
aMT (5 x 10^{-5}M) + Fe^{2+} (5 x 10^{-5}M)	298.4nm	0.697
aMT (5 x 10^{-5}M) + Fe^{2+} (5 x 10^{-5}M)-1h	297.44nm	0.695
aMT (6.67 x10^{-5}M) + Fe^{2+} (3 x 10^{-5}M)	295.52nm	0.603

No significant changes in the absorbance spectra were seen with NT hydrochloride and FL hydrochloride in combination with varying concentrations of Fe^{3+} and Cu^{2+}, as with TM maleate and aMT in combination with Cu^{2+}.

An increase in TM maleate absorbance at 248.48nm on addition of 5 x 10^{-5}M Fe^{3+} was noted, but after 1 hour incubation, values returned to that which were expected for 5 x 10^{-5}M TM alone. aMT absorbance at 277.6nm also appeared to increase on the addition of Fe^{3+}, and remained elevated after the incubation period (refer to table 6.3).

Table 6.3: The effect of Fe^{3+} on TM and aMT absorbance values

Compound	Absorbance wavelength	Intensity
TM (5 x 10^{-5}M)	248.48nm	0.605
TM (5 x 10^{-5}M) + Fe^{3+} (5 x 10^{-5}M)	247.52nm	0.755
TM (5 x 10^{-5}M) + Fe^{3+} (5 x 10^{-5}M)-1h	248.48nm	0.623
aMT (5 x 10^{-5}M)	277.6nm	0.232
aMT (5 x 10^{-5}M) + Fe^{3+} (5 x 10^{-5}M)	276.32nm	0.359
aMT (5 x 10^{-5}M) + Fe^{3+} (5 x 10^{-5}M)-1h	275.36nm	0.35

6.3.4 Discussion

Analysis of the absorbance patterns of NT hydrochloride, TM maleate, FL hydrochloride and aMT was undertaken in order to ascertain whether there was a chemical interaction with selected trace metal ions in solution. Additionally, the experiment was done to compliment and possibly further clarify electrochemical findings as previously described in this chapter.

Characteristic spectra were produced for all three antidepressants being studied. We were thus able to show that neither FL hydrochloride nor NT hydrochloride interacted with any of the trace metal ions to a significant degree. The increases seen in the absorbance intensity values relating to TM maleate and aMT respectively, indicate that there is some form of interaction between these species and Fe^{2+} and Fe^{3+}. This compliments electrochemical findings in section 6.3.3 that showed that there is a weak reversible interaction with Fe^{3+} vs. a stronger interaction with Fe^{2+} and TM maleate.

It is possible that TM maleate and aMT form a bond with these ions, and such an interaction is contributing to the increased absorbance values noted. The apparent TM-Fe^{3+} bond is transient, as absorbance values decrease to original expected intensity over time, in contrast to TM maleate and aMT with added Fe^{2+} and aMT and Fe^{3+}. Such results suggest that TM and aMT are able to provide neuroprotection to nervous tissue by interacting with free ions of Fe^{2+} and Fe^{3+} in the CNS, before they are able to partake in free radical forming reactions.

Once more, the HSAB Rule may provide the basis for the apparent interactions. The being and nature of these interactions can only be verified by other methods, and these preliminary studies indicate that there is cause for further study to provide clarification and understanding.

Chapter 7

Summary of Results: Conclusions and Recommendations for Future Work

7.1 Summary of Results

7.1.1 Chapter 2 - Lipid Peroxidation of rat forebrain homogenate

NT hydrochloride, TM maleate and FL hydrochloride all significantly reduce lipid peroxidation induced by 0.75mM QA, in a dose dependent fashion (0-1mM) *in vitro*.

In vivo experimentation showed that the three antidepressants studied have the ability to reduce damage induced by QA intrastriatal injection. If administered at a dose of 10mg/kg prior to and post QA intrastriatal injection, NT hydrochloride, TM maleate and FL hydrochloride are seen to significantly reduce levels of lipid peroxidation.

Results obtained for *in vitro* Fe^{2+} induced lipid peroxidation as analysed by HPLC were poorly reproducible. As such it was unclear as to how these antidepressants may influence neurodegeneration as a result of metal ion availability. It is however apparent that the neuroprotection provided against QA induced lipid peroxidation involves a mechanism of action that is not evident in Fe^{2+} induced lipid peroxidation.

7.1.2 Chapter 3 – Histological investigation

NT hydrochloride, TM maleate and FL hydrochloride were administered to animals prior to and post QA intrastriatal injection, and micrographs of the resultant necrotic damage to hippocampal cells assessed. The antidepressants appeared to provide only a marginal degree of neuroprotection in comparison to control animals treated with 0.9% saline.

7.1.3 Chapter 4 – Superoxide anion induced lipid peroxidation

The antidepressants studied did not provide protection from cyanide induced lipid peroxidation. NT hydrochloride was shown to significantly increase damage induced by KCN (2mM) in a dose dependent manner (0-4mM) *in vitro*. TM maleate and FL hydrochloride had no significant effect on the damage induced by KCN.

7.1.4 Chapter 5 – Liver TDO activity

NT hydrochloride significantly inhibited TDO total and apoenzyme activity *in vitro*, where as TM maleate and FL hydrochloride appeared to significantly stimulate TDO holo and total enzyme activity.

In vivo experimentation showed that there is no significant influence of the antidepressants (administered at a dose of 10mg/kg for 5 days) on TDO total- or holo-enzyme activity.

7.1.5 Chapter 6 – Electrochemical and UV/Visible *in vitro* investigation

There was no interaction between FL hydrochloride and NT hydrochloride and the metal ions used (Fe^{2+}, Fe^{3+} and Cu^{2+}). Both these antidepressants are poorly electroactive, and as such could not be analysed electrochemically. UV/Visible analysis showed that there were no apparent interactions between these antidepressants and Fe^{2+}, Fe^{3+} or Cu^{2+}.

TM maleate, when studied electrochemically was seen to interact with both Fe^{2+} and Fe^{3+}. It is possible that TM maleate can form weak bonds with these metals, and the bond with Fe^{3+} appears to be less significant than that with Fe^{2+}. The electrochemical interactions observed are similar to those interactions seen between aMT and the metal ions used.

UV/Visible analysis confirms that TM maleate and aMT interact with Fe^{2+} and Fe^{3+}. TM maleate-Fe^{3+} bond is transient in contrast to the TM maleate-Fe^{2+} bond and the aMT-Fe^{2+}/Fe^{3+} bonds.

7.2 Conclusions

The objectives of this study were to determine whether NT, FL and TM could act as neuroprotective agents, and if so, what the possible mechanism for neuroprotective action could be.

All three of the antidepressants provided significant protection from QA induced lipid peroxidation *in vitro* and *in vivo*, and as such, it is believed that these agents have a strong potential to provide neuroprotection. As results from Fe^{2+} induced lipid peroxidation as analysed by HPLC proved to be unclear, no firm conclusions can be made from these observations.

Based on the histological investigation it is suggested that NT, TM and FL do have the ability to provide a low level of neuroprotection *in vivo* at pharmacological concentrations. Damage induced is as a result of NMDA receptor activation by QA. It is thus suggested that a possible site for neuroprotective action of the antidepressants is at the level of glutamate receptors.

The superoxide anion induced lipid peroxidation assay was undertaken to determine whether NT, TM and FL have any significant free radical scavenging abilities and are therefore able to provide neuroprotection. This proved not to be the case, and as such, it is clear these antidepressants have no antioxidant actions. Possible neuroprotective action must therefore occur prior to the generation of damaging free radicals or be as a consequence of activation of other defence systems in the organism.

With this understanding, influence of NT, TM and FL on TDO activity was determined. Inhibition of TDO activity is known to result in the increased synthesis of aMT and thus the antidepressants could provide neuroprotection indirectly by increasing the concentration of this antioxidant. This hypothesis could hold true for NT, as this antidepressant inhibits TDO *in vitro*. As the same inhibition was not apparent on *in vivo* testing, and neither TM nor FL inhibited TDO activity, it seems unlikely that these agents could provide neuroprotection as a result of increased aMT synthesis.

Finally, electrochemical and UV/visible analyses were undertaken in order to determine whether NT, TM, and FL could interact with trace metal ions. Through binding available ions the antidepressants could render metals unavailable to become involved in free radical generating reactions. This hypothesis may hold true for TM maleate which was the only antidepressant seen to interact with Fe^{2+} and Fe^{3+} ions.

All three of the antidepressants studied were shown to provide neuroprotection against QA induced damage. It is unlikely that the neuroprotection seen is as a consequence of antioxidant actions, or in the case of TM and FL, as a result of TDO activity inhibition. The latter may be a way in which NT can indirectly provide neuroprotection. TM maleate could possibly be neuroprotective through interaction with available free ions.

All things considered, it seems likely that these antidepressants that are commonly used to treat depression that coexists in patient with neurodegenerative diseases, may be of marginal benefit to sufferers. However, as NT is seen to exacerbate superoxide induced lipid peroxidation (chapter 4), higher therapeutic concentrations of the drug must be used cautiously. This is important as patients may be treated for depression with confidence that these agents are not worsening the primary neurodegenerative state.

7.3 Recommendations for Future Study

At present, possible neuroprotective actions appear to be as a result of differing modes of action/s of each of the antidepressants. It is clear that further research is required in order to elucidate how these agents influence the state and/or progression of neurodegenerative disease and the significance thereof.

Neuroprotection is provided against QA acid induced damage, and therefore action may be at the level of the glutamate receptors. Glutamate receptor binding studies would clarify this. Such studies were initially undertaken, however it was found that the experimental protocols used did not yield conclusive results. It is believed that modification and refinement of existing techniques is required in order to accurately determine how NT, TM and FL affect glutamate receptor binding.

The NT inhibition of TDO activity could lead to neuroprotection by inducing the formation of aMT. Further investigation is required to determine the level at which NT is able to inhibit TDO total- and apo-enzyme activity, and the significance thereof. Such an understanding would be interesting as TM maleate, despite falling into the TCA group of antidepressants, did not inhibit TDO enzyme activity.

Another area for future study that could prove to be of interest involves the nature of the interaction between TM maleate and trace metal ions. The site of interaction and the strength thereof needs to be elucidated in order to determine the significance of the discovered interaction.

Appendix 1

Animals

The work involving the use of animals was approved by the Rhodes University animal ethics committee. The animals used were male rats of the Wistar strain, weighing 250-300g. The housing provided comprised of opaque cages with metal grid floors and covers, under a diurnal lighting cycle 12 light:12 dark with food and water *ad libitum*. The light intensity during the 12 hour light phase was approximately 300μWatts/cm^2. The temperature in the animal room was maintained between 20^{o}C and 25^{o}C, and the cages were cleaned on a daily basis.

Appendix 2

Sacrificing and dissection of animals

The rats were killed as swiftly as possible by cervical dislocation followed by decapitation. Incisions were made on either side of the skull from the foramen magnum down towards the orbit, and forceps used to lift the skull to expose the brain. Brains were removed and either used immediately or rapidly frozen in liquid nitrogen and stored at –70^{o}C until needed.

In order to obtain liver tissue a mid-vertical incision was made through the abdominal musculature from the pelvic region to the posterior edge of the sternum. A transverse cut was made anteriorly to expose the liver, which was removed. The liver was then perfused with 0.9% saline solution in order to remove blood before clotting occurred, and used immediately or rapidly frozen and stored at -70^{o}C until needed.

Appendix 3

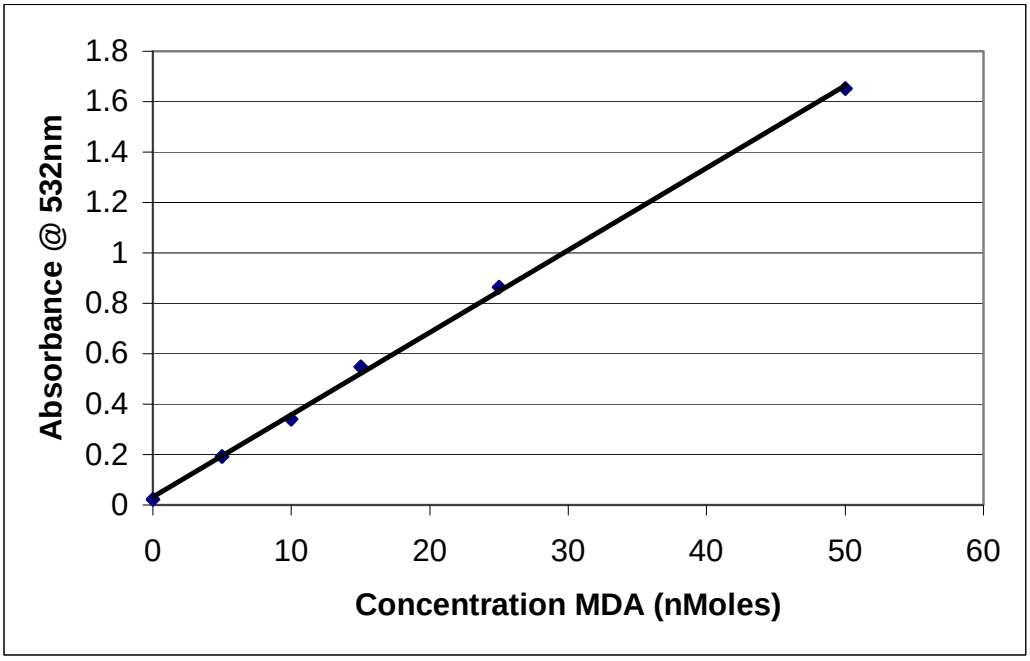


Figure 8.1: Lipid peroxidation standard curve (r^2=0.999)

Appendix 4

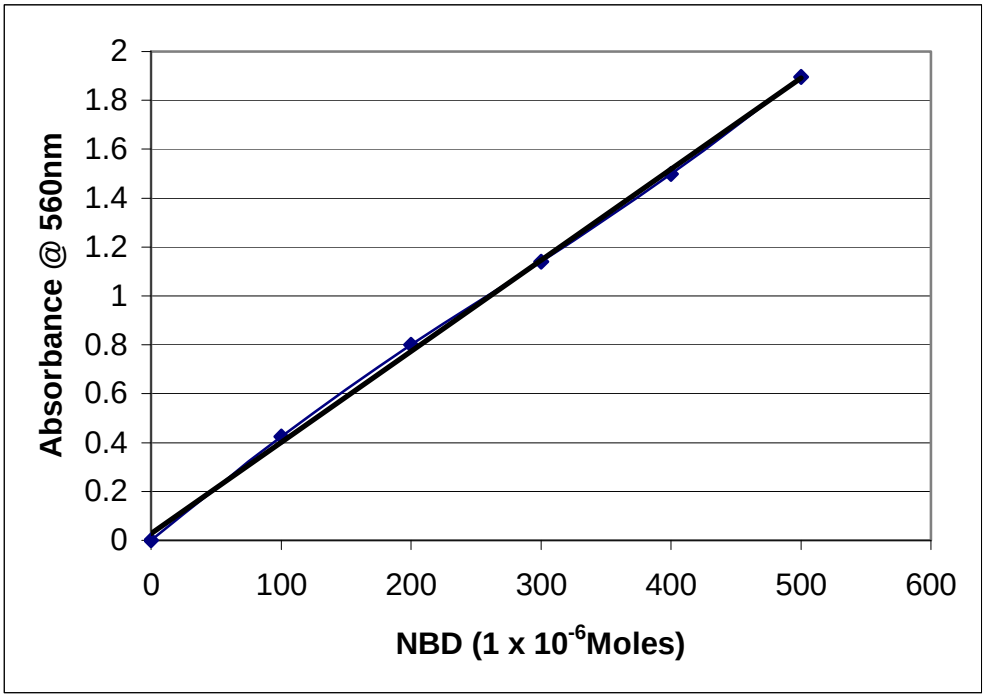


Figure 8.2: NBT assay standard curve (r^2=0.999)

Appendix 5

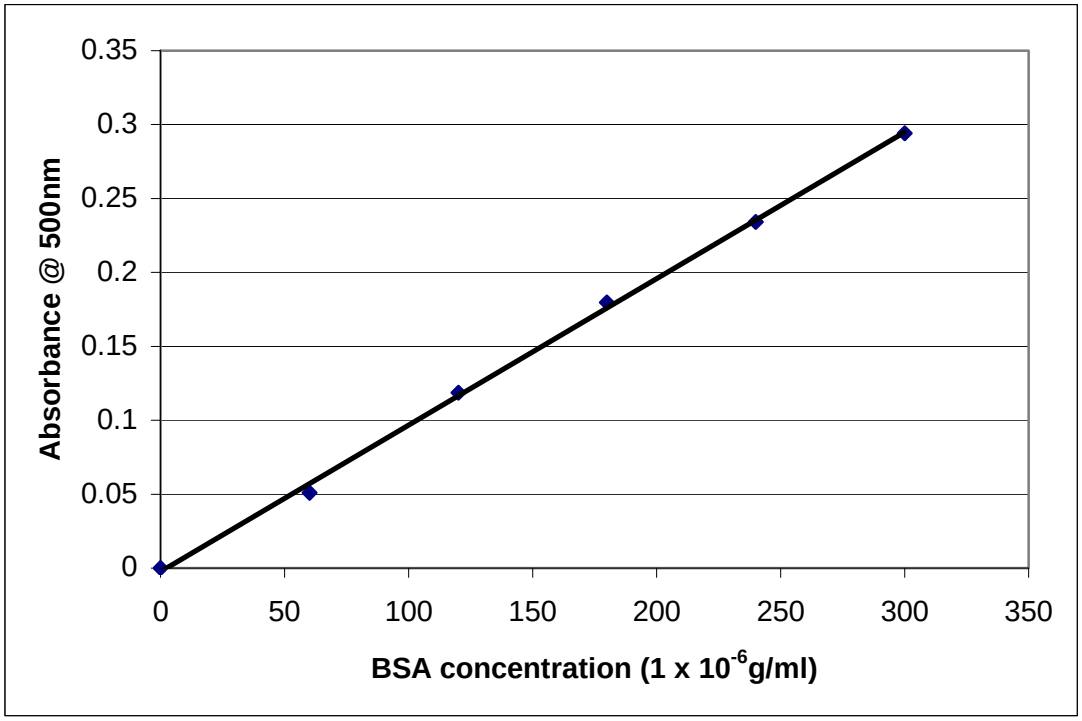


Figure 8.3: Protein standard curve (r^2=0.999)

References

Acun a-Castroviejo, D., Coto-Montes, A., Monti, M. G., Ortiz, G. G., Reiter, R. J., (1997) *Pharmacol. Lett.* **60**: 23

Alexopoulos, G. S., (1992) *Psychopharmacol., 2nd ed* :137

Alexopoulos, G. S., (1996) *J Clin Psychiatry* **57** (Suppl 14): 14

Allain, H., Schuck, S., Mauduit, N., (2000) *BMJ* **320**:1287

Amano, T., Ujihara, H., Matsubayashi, H., Sassa, M., Yakeharu, T., Tamura, Y., Akaike, A., (1994) *Brain Res.* **655**:61

Ames, B. N., Shigenaga, M. K., Hagen, T. M., (1993) *Proc. Natl. Acad. Sci. USA* **90**: 7915

Ankarcrona, M., Dypbukt, J. M., Bonfoco, E., Zhivotovsky, B., Orrenius, S., Lipton, S. A., Nicotera, P., (1995) *Neuron* **15**:961

Anoopkumar-Dukie, S., Walker, R. B., Daya, S., (2001) *Pharmacy and Pharmacol.* **53**: 263

Ardelt, B. K., Borowitz, J. L., Isom, G. E., (1989) *Toxicol.* **56**:147

Ascher, P., Nowak, L., (1987) *Trends Neurosci*. **10**:284

Ashcroft, G. W., Eccleston, D., Murry, L. G., Glen, A. I. M.,Crawford, T. B. B., Pullar, I. A., Shields, P. J., Walter, D. S., Blackburn, I. M., Connechan, J., Lonergan, M., (1972) *Lancet* **ii**:573

Aust, S. D., Morehouse, L. A., Thomas, C. E., (1985*) J. Free Radicals Biol. Med*. **1**:3

Badawy, A. A., Evans, M., (1975) *Biochem. J*. **150:** 511

Badawy, A. A., Evans, M., (1981) *Practical Pharmacol.* **30**: 1211

Banerjee, S. P., Kung, L. S., Chanda, S. K., (1977) *Nature* **268**:455

Barlow-Walden, L. R., Reiter, R. J., Abe, M., Pablos, M., Mene ndez-Pela ez, A., Chen, L. D., Poeggeler, B., (1995) *Neurochem. Int*. **26**:497

Beal, M. F., (1992) *Ann. Neurol.* **31**: 119

Beal, M. F., Kowall, N. W., Ellison, D. W., Mazurek, M. F., Swartz, K. J., Martin, J. B., (1986) *Nature* **321**: 186

Beck, O., Jonsson, G., (1981) *J. Neurochem*. **36**:2013

Beckman, J. S., (1991) *J. Dev. Physiol*. **15**:53

Behl, C., Davis, J. B., Lesley, R., Schubert, D., (1994) *Cell* **77**:817

Behl, C., Lezoualc'h, F., Trapp, T., Widmann, M., Skutella, T., Holsboer, F., (1997) *Endochrinology* **138**:11

Bender, D., (1982) *Molec. Aspects Med*. **6**: 101

Bersani, G., Garavini, A., (2000) *Prog. Neuro-Psychopharmacol. & Biol. Psychiat.* **24**:185

Bettler, B., Mulle, C., (1995) *Neuropharmacol.* **34**:123

Birchall, J. D., Chappell, J. S., (1988) *Lancett* **ii**:1008

Bird, E. D., (1980) *Ann. Rev. Pharmacol. Toxicol*. **20**: 33

Bird, E. D., Iversen, L. L., (1974) *Brain* **97**: 457

Blier, P., De Montigny, C., Chaput, Y., (1987) *J. Clin. Psychopharmacol*. **7**(Suppl 1): 24S

Boadhead, W. E., Blazer, D. G., George, L. K., Tse, C. K., (1990) *JAMA* **264**:2524

Braughler, J., Hall, E., (1989) *Free Rad. Biol. Med*. **6**:289

Bredesen, D. E., (1995) *Ann. Neurol*. **38**:839

Burton, G. W., Ingold, K. U., (1989) *Ann. NY Acad. Sci*. **570**:7

Bush, A., (2000) *Current Opinion in Chemical Biol.* **4**: 184

Carboni, S., Melis, F., Pani, L., Hadjiconstantinou, M., Rossetti, Z., (1990) *Neurosci. Lett.* **117**:129

Cardinali, D. P., Lynch, H. J., Wurtman, R. J., (1972) *Endochrinology* **91**:1213

Castagnoli, N., Chiba, K., Trevor, A. J., (1985) *Life Sci*. **36**:225

Cebers, G., Zhivotovsky, B., Ankarcrona, M., Liljequist, S., (1997) *Brain Res. Bull.* 43:393

Chen, Q., Harris, C., Brown, C. S., Howe, A., Surmeier J., (1995) *Experimental Neurology* **136**:212

Cho, S., Joh, T. H., Baik, H. H., Dibinis, C., Volpe, B. T., (1997) *Brain Res.* **755**: 335

Choi D. W., (1985) *Neurosci. Lett*. **58**:293

Choi, D. W., (1987) *J. Neurosci*. **7**:369

Choi, D. W., (1988) *Neuron* **1**:623

Chow, H. S., Lynch, J. J., Rose, K., Choi, D. W., (1994) *Brain Res*. **639**:102

Colton, C. A., Gilbert, D. L., (1987) *FEBS Lett*. **223**:284

Cooper, A. J., Magnus, R. V., (1984) *Can Med Assoc J*. **130**:383

Coppen, A., Eccleston, E. G., Peet, M., (1973) *Lancet* **ii**:60

Cotman, C. W., Monaghan, D. T., Otterson, O. P., Storm-Mathisen, J., (1987) *Trends Neurosci*. **10**:273

Cummings, J. L., Masterman, D. L., (1999) *Int. J. Geriatr. Psychiatry* **14**:711

Cunningham, L. A., (1994) *J Cin Pschiatry* **55**(Suppl A): 90

Curnutte, J. T., Babior, B. M., (1987) *Adv. Hum. Genet*. **16**:229

Curzon, G., Green, A. R., (1969) *Biochem. J*. **111**:15

Curzon. G., Bridges, P. K., (1970) *J. Neurol. Neurosurg. Psychiat*. **33**:698

Cutler, N. R., Sramek, J. J., (2001) *Prog. Neuro-Psychopharmacol. & Biol. Psychiat.* **25**:27

Dale, W. E., Dang, Y., Brown, O. R., (2000) *Free Radical Biology & Med.* **29**:191

Davis, J. B., Maher, P., (1994) *Brain Res*. **652**:169

Dawson, D., Encel, N., (1993) *J. Pineal Res*. **15**:1

Dawson, V. L., Dawson T. M., (1996) *Cell death and Differentiation* **3**: 71

Dawson, V. L., Dawson, T. M., London, E. D., Bredt, D. S., Synder, S. M., (1991) *Proc. Natl. Acad. Sci. USA* **88**:6368

Daya, S., (1999) *Specialist Medicine* 529

Daya, S., Anoopkumar-Dukie, S., (2000) *Life Sci*. **67**: 235

Degado, P. L., Price, L. H., Miller, H. L., (1991) *Psychopharmacol. Bull*. **27**:321

Delgado, P. L., Charney, D. S., Price, L. H., Aghajanian, G. K., Landis, H., Heninger, G. R., (1990) *Arch. Gen. Psychiatry* **47**:411

Dollery, C., (1991) *Therapeutic Drugs Vol 1&2*, Churchill Livingston Inc, New York

Draper, H. H., Hadley, M., (1990) *Methods in Enzymology*. 186, Academic Press, San Diego, pp 421

Dykens, J. A., Stern, A., Trenker, E., (1987) *J. Neurochem*. **49**:1222

Exposito, I., Mora, F., Zisapel, N., Oaknin, S., (1995) *NeuroReport* **6**:2399

Fahn, S., Cohen, G., (1992) *Ann Neurol* **32**: 804

Farooqui, A. A., Horrocks, L. A., (1991) *Brain Res. Rev*. **16**:171

Fernstorm, J. D., Wurtmann, R. J., (1971) *Science* **173**:149

Feuer, G., (1990) *Drug Metabolism and Drug Interactions* **8**: 203

Figueredo-Cardenas, G., Anderson, K. D., Chen, Q., Veeman, C. L., Reiner, A., (1994) *Experimental Neurology* **129**: 37

Fischer, P., Simanyi, M., Danielezyk, W., (1990) *Am. J. Psychiatry* **147**:1484-1487

Fogler, W. E., Fidler, I. J., (1989) *J. Immunol*. **136**:2311

Frazer, A., (1997*) J. Cin. Psychopharmacol.* **17**(Suppl 1): 2S

Frei B., England, L., Ames, B. N., (1989) *Proc. Natl. Acad. Sci. USA* **86**:6377

Fridovich, I., (1978) *Science* **209**:875

Fridovich, I., (1989) *J. Biol. Chem*. **264**:7761

Gasic, G. P., Hollmann, M., (1992) *Annu. Rev. Physiol* **54**:507

Ghezzi, P., Saccardo, B., Bianchi, M., (1986) *Immunopharmacology* **12**:241

Giusti, P., Gusella, M., Lipartiti, M., Milani, D., Zhu, W., Vicini, S., Manev, H., (1995) *Exp. Neurol*. **131**:39

Giusti, P., Lipartiti, M., Franceschini, D., Schiavo, N., Floreani, M., Manev, H., (1996) *The FASEB Journal* **10**: 891-896

Good, P. F., Perl, D. P., Bierer, L. M., Schmeidler, J., (1992) *Ann. Neurol*. **31**:286

Gruener, N., Gozlan, O., Goldstein, T., Davis, J., Besner, I., Iancu, T. C., (1991) *Clin. Chem.* **37**:263

Gunasekar, P. G., Kanthasamy, A. G., Borowitz, J. L., Isom, G. E., (1995) *J. Neurochem*. **65**:2016

Gutteridge J. M. C., Quinlan, G. J., Clark, I. A., Halliwell, B., (1985*) Biochem. Biophys. Acta* **835**:441

Gutteridge, J. M. C., (1986) *FEBS Letters* **201**:291

Gutteridge, J. M. C., Halliwell, B., (1990) *Trends Biochem. Sci*. **15**:129

Gutteridge, J. M. C., Halliwell, B., Rowely, D. A., (1984) *Life Chem. Rep*. **2**:15

Halliwell, B., (1989) *Free Radic. Res. Commun.* **5**:315

Halliwell, B., (1992) *J.of Neurochem.* **59**: 1609

Halliwell, B., Gutteridge, J. M. C., (1985) *Aspects Med.* **8**: 89

Halliwell, B., Gutteridge, J. M. C., (1989) *Free radicals in Biology and Medicine*, 2nd ed. Clarendon Press, Oxford

Halliwell, B., Gutteridge, J. M. C., (1990) *Methods Enzymol*. **186**:1

Hardeland, R., Rensing, L., (1968) *Nature* **219**:619

Harvey, B. H., (1997) *SAMJ* **87**: 540

Hauser, K., Olpe, H. R., Jones, R. S. G., (1985) *Eur. J. Pharmacol* **111**:23

Heikkila, R. E., Manzino, L., Cabbat, F. S., Duvoisin, R. C., (1985) *J. Neurochem*. **45**:1049

Heinemann, W. R., Kissinger, P. T., (1996) *Lab. Techniques in Electroanalytical Chem*, 2nd Ed. Marcel Dekker Inc. New York

Heydorn, W. E., Brunswick, D. J., Frazer, A., (1982*) J. Pharmac. Exp. Ther***. 222**:534

Heyes, M. P., Achim, C., Wiley, C. A., Major, E. O., Saito, K., Markey, S. P., (1996) *Biochem J* **320**: 595

Heyes, M. P., Brew, B. J., Martin, A., Price, R. W., Salazar, A. M., Sidtis, J. J., Yergey, J. A., Mouradian, M. M., Sadler, A. E., Keilp, J., Rubinow, D., Markey, S. P., (1991) *Ann. Neurol.* **29**:202

Heyes, M. P., Saito, K., Crowley, J., Davis, L. E., Demitrak, M. A., Der, M., Dilling, L., Kruesi, M. J. P., Lackner, A., Larsen, S. A., (1992) *Brain* **115**:1294

Heyes, M. P., Saito, K., Major, E. O., Milstein, S., Markey, S. P., Vickers, J. H., (1993) *Brain* **116**:1425

Huheey, J. E., (1978) *Inorganic Chemistry-Principles of Reactivity and Sturcture*, 2nd Ed., Harper and Row, New York

Janowsky, S., El-Yousuf, M. K., Davis, J. M., Sekerke, H. J., (1972a) *Lancett* **ii**(778):632

Janowsky, S., El-Yousuf, M. K., Davis, J. M., Sekerke, H. J., (1972b) *Psychopahrmacologia*. **27**(4):295

Johnson, J. D., Conroy, W. G., Burris, K. D., Isom, G. E., (1987) *Toxicology* **46**:21-28

Klerman, G. L., Weissman, M. M., (1989) *JAMA* **261**:2229

Knox, W. E., Auerbach (1955) *J. Biol. Chem*. **214**:307

Kong, S., Liochev, S., Fridovich, I., (1992) *Free Radic. Biol. Med*. **13**:79

König, J. F. R., Klippel, R. A., (1963) *The Rat Brain*, Williams and Wilkins, Baltimore

Lange, K. W., Kornhuber, J., Riederer, P., (1997) *Neurosci. and Biobehavioral Rev.* **21**: 393

Lange, K. W., Youdim, M. B. H., Riederer, P., (1993) *J. Neural Transm*. **38**(Suppl.)27

Lazarus, L. W., Newton, N., Cohler, B., (1987) *Am. J. Psychiatry* **144**:763

Leigh, P., Meldrum, B. S., (1996) *Neurology* **47** (Suppl 4): S221

Leone , R. M., Silman, R. E., (1984) *Endocrinology* **114**:1825

Lewy, A. J., Ahmed, S., Jackson J. M., Sack, R. L., (1992) *Chronobiol. Int*. **9**:380

Lezoualc´h, F., Skutella, T., Widmann, M., Behl, C., (1996) *Neuro Report* **7**: 2071

Limson, J., Nyokong, T., Daya, S., (1998) *J. of Pineal Res.* **24**: 15

Linert, W., Jameson, G. N. L., (2000) *J Inorganic Biochem*. **79**: 319

Lipton, S. A., Rosenberg, P. A., (1994) *New Eng. J. of Med.* **330:** 613

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., (1951) *J. Biol. Chem* **193**:265

Ludolph, A. C., (1995) *Neurotoxicology: Approaches and Methods* :671

MacDermott, A. B., Dale, N., (1987) *Trends Neurosci*. **10**:280

Maragos, W. F., Greenmyre, J. T., Penney, J. B., Young, A. B., (1987) *Trends Neurosci*. **10**:65

Maragos, W. F., Penney, J. B., Young, A. B., (1988) *J. Neurosci* **8**:493

Marder, K., Tang, M. X., Cote, L., Stern, Y., Mayeuf, R., (1995) *Arch. Neurol*. **52**:695

Meffert, M. K., Premack, B. A., Schulman, H., (1994) *Neuron* **12**:1235

Melchorri, D., Reiter, R. J., Attia, A. M., Hara, M., Rurgos, A., Nistico , G.,(1995) *Life Sci.* **56**:83

Mizuno, Y., Ohta, S., Tanaka, M., Takamiya, S., Suzuki, K., Sato, T., Oya, H., Ozawa, T., Kagawa, Y., (1989) *Biochem. Biophys. Res. Commun*. **169**:1450

Modestein, J., Hunger, J., Schwartz, R. B., (1973) *Arch. Psychiatr. Nervenkr*. **218**(1):67

Moncada, S., Palmer, R. M. P., Higgs, E. A., (1991) *Pharmacol. Rev*. **43**:109

Montague, P. R., Gancayco, C. D., Winn, M. J., Marchase, R. B., Friedlander, M. J., (1994) *Science* **263**:973

Mori, H., Mishina, M., (1995) *Neuropharmacol.* **34**:1219

Murphy, T. H., Miyamoto, M., Sastre, A., Schnaar, R. L., Coyle, J. T., (1989) *Neuron* **2**:1547

Nelson, J. C., (1991) *J. Clin. Psychiat.* **52**: 193

Ninan, P. T., (1999) *J. Clin. Psychiat*. **60**: 12

Novelli, A., reilly, J. A., Lysko, P. G., Henneberry, R. C., (1988) *Brain Res*. **451**:205

Nowak, L., Bregestovski, P., Ascher, P., Herbet, H., Prochiantz, A., (1984) *Nature* **307:**462

Obrenovitch, T. P., Urenjak, J., (1997) *Progress in Neurobiology* **51**:39

Olney, J. W., Ho, C., Van Ree (1971) *Exp. Brain Res*. **14**:61

Ottino, P., Duncan, R., (1997) *Free Rad. Biol. and Med.* **22**: 1145

Pauwels, P. J., Leysen, J. E., (1992) *Neurosci. Lett*. **143**:27

Pearson, R. G., (1968) *J. Chem. Ed*. **45**:643

Pellegrini-Giampietro, D. E., Cherici, G., Alesiani, M., Caria, V., Moroni, F., (1990) *J. Neurosci*. **10**:1035

Pepeu, G., (2000) *Prog. Neuro-Psychopharmacol. & Biol. Psychiat*. **25**:193

Perez, P., Flores, A., Santamaria, A., Rios, C., Galvan-Arzate, S., (1996) *Archives o*f *Med. Res.* **27**: 449-

Perry, T. L., Hansen, S., (1990) *Neurology* **40**:20

Pieri, C., Marra, M., Moroni, F., Recchioni, R., Marcheselli, F., (1994) *Life Sci*. **15**:271

Pin, J. P., Duvoisin, R., (1995) *Neuropharmacol.* **34**:1

Pogun, S., Dawson, V., Kuhar, M. J., (1994) *Synapse* **18**:21

Popoli, P., Pe zzola, A., Domenici, M. R., Ssagratella, S., Diana, G., Caporali, M. G., Bronzetti, E., Vega, J., Scotti De Carolis, A., (1994) *Brain Res. Bull.* **35**: 329

Prange, A., Wilson, I. C., Lynn, C. W., Alltop, L. B., Stikeleather, R. A., (1974) *Arch. Gen. Psychiat.* **30** (1):56

Radi, R., Beckman, J. S., Bush, K. M., Freeman, B. A., (1991) *J. Biol. Chem*. **266**:4244

Randrup, A., Braestrup, C., (1977) *Psychopharmacol.* **53**:309

Rang, H. P., Dale, M. M., Ritter, J. M., (1995) *Pharmacology*, 3rd Ed., Churchill Livingstone Inc. New York

Rees, J. R., Allsopp, M. N. E., Hullin, R. P., (1974) *Psychol. Med*. **4**:334

Reiter R. J., (1995b) *Exp. Gerontol*. **30**:199

Reiter R. J., Johnson, L. Y., Steger, R. W., Richardson, B. A., Peterborg, L. J., (1980) *Peptides* **1** (Suppl. 1):69

Reiter R. J.,Craft, C. M., Johnson, J. E., King, T. S., Richardson, B. A., Vaughan, G. M., Vaughan, M. K., (1981) *Endocrinology* **109**:1295

Reiter, R. J., (1991) *Trends in Endocrinol. Metabol.* **2**:13

Reiter, R. J., (1992) *BioEssays* **14**:169

Reiter, R. J., (1995a) *Aging Clin. Exp. Res*. **7**:340

Reiter, R. J., Melchiorri, D., Sewerynek, E., Poeggeler, B., Barlow-Walden, L., Chuang, J., Oritz, G. G., Acuna-Castroviejo, D., (1995) *J. Pineal. Res*. **18**:1

Reiter, R. J., Oh, C., Fujimori, O., (1996) *TEM* **7**:22

Reynolds, G. P., Pearson, S. J., Halket, J., Sandler, M., (1988) *J. Neurochem*. **50**:1959

Richelson, E., (1994a) *J. Clin Psychiatry* **55**(Suppl A): 34

Richelson, E., (1994b) *Mayo Clin. Proc*. **69**: 1069

Rickels, K., Schweizer, E., (1990) *J. Cin. Psychiatry* **51**:9

Riederer, P., Lange, K. W., (1992) *Curr. Opinion Neurol. Neurosurg*., **5**:295

Rios, C., Santamaria, A., (1991) *Neurochem. Res*. **16**: 1139

Roth, M., (1955) *J. Mental Sci*. **1**:281

Rothstein, J. D., (1996) *Neurology* **47**(Suppl 2):S19

Sack, R. L., Lewy, A. J., Blood, M. L., Stevenson, J. Keith, L. D., (1991) *J. Biol. Rhythms* **6**:249

Sackeim, H. A., Prudic, J., Devanand, D. P., Decina, P., Kerr, B., Malitz, S., (1990) *J. Clin. Psychophar,acol.* **10**:96

Santamaria, A., Ríos, C., Pérez, P., Flores, A., Galván-Arzate, Osorio-Rico, L., Solís, F., (1996) *Toxicol. Lett.* **87**: 113

Sayre, L. M., Perry, G., Smith, M. A., (1999) *Current Opinion in Chemical Biology* **3**: 220

Schildkraut, J. J., (1965) *Am. J. Psychiatry* **122**:509

Settle, E. C., Ayd, F. J., (1980) *J. Clin. Psychiatry* **41**:266

Sewerynek, E., Reiter, R. J., Melchiorri, D., Ortiz, G. G., Lewinski, A., (1996) *Hepato-Gastroenterology* **43**:898

Sewerynek, E., Abe, M., Reiter, R. J., Barlow-Walden, L. R., Chen, L. D., McCabe, T. J., Roman, L. J., Diaz-Lopez, B., (1995) *J. Cell. Biochem*. **58**:436

Shaikh, A. Y., Xu, J., Wu, Y., He, L., Hsu, C, Y., (1997) *Neurosci. Lett*. **229**:193

Shillcutt, S. D., Easterday, J. L., (1984) *Hosp Formul* **19**: 941

Shoham, S., Wertman, E., Ebstein, P., (1992) *Exp. Neurol*. **118**:227

Singer, T. P., Castagnoli, N., Ramsay, R. R., Trevor, A. J., (1987) *J. Neurochem*. **49**:1

Skene, D. J., Viven-Roels, B., Sparks, D. L., Hunsaker, J. C., Pevet, O., David, D., Swaab, D. F., (1990) *Brain Res*. **528**:170

Southgate, G., Daya, S., (1999) *Metab. Brain. Disease* **14**:165

Sperk, G., (1994) *Progress Neurobiol*. **42**:1

Stark, P., Fuller, R. W., Wong, D. T., (1985) *J. Clin. Psychiatry* **46**:7

Štípek, S., Štastny F., Pláteník, J., Crkovská J., Zima, (1997) *Neurochem. Int*. **30**: 233

Stone, T. W., (1993) *Pharmacol Rev*. **45**:309

Sulser, F., Bickel, M. H., Brodie, B. B., (1964) *J. Pharmac. Exp. Ther*. **144**:321

Sundstorm, E., Strommerg, I., Tsutsumi, T., Olson, L., Jonsson, G., (1987) *Brain Res*. **405**:26

Syvalahti, E. K. G., (1994) *Acta Psychiatr. Scand*. **89**:11

Tan, D. X., Poeggeler, B., Reiter, R. J., Chen, L. D., Chen, S., Manchester, L. C., Barlow-Walden, L. R., (1993) *Cancer lett*. **70**:65

Tan, D. X., Reiter, R. J., Chen, L. D., Poeggeler, B., Manchester, L. C., Barlow-Walden, L. C., (1994) *Carcinogenesis* **15**:215

Taylor, A., Saint-Cyr, J. A., Lang, A. E., Kenny, F. T., (1986) *Brain* **109**:279

Taylor, C. P., Meldrum, B. S., (1995) *Trends Pharmacol Sci*. **16**:309

Taylor-Robinson, S. D., Weeks, R. A., Bryant, D. J., Sargentoni, J., Marcus, A. D., Harding, A. E., Brooks, D. J., (1996) *Movement Disorders* **11**:167

Tilson, H. A., Mundy, W. R., (1995) *Neurotoxicology: Approaches and Methods* :359

Toyama, S. C., Iacono, R. P., (1994) *Ann. Pharmacother*. **28**:405

Tsuzuki, K., Iino, M., Ozawa, S., (1989) *Neurosci. Lett*. **105**:269

Turski, L., Bressler, K., Rettig, K. J., Löschmann, P. A., Wachtel, H., (1991) *Nature* **349**:414

Viani, P., Cervato, G., Fiorilli, A., Cestaro, B., (1991) *J Neurochem* **56**: 253

Volterra, A., Trotti, D., Tromba, C., Floridi, S., Racagni, G., (1994) *J. Neurosci*. **14**:2924

von Blankenfeld, G., Kettenmann, H., (1991) *Mol. Neurobiol*. **5**:31

Vyas, I., Heikkila, R. E., Nicklas, W. J., Studies, W. J., (1986) *J. Neurochem*. **46**:1501

Waldhauser, F., Boepple, P. A., Crowley, W. F., (1991) *Adv. Pineal Res*. **5**:329

Walsh, H. A., Daya, S., Whiteley, C. G., (1991) *Med. Sci. Res*. **19**:849

Walsh, H.A., Daya, S. and Whiteley, C.G., (1994) *J. Pineal Res*. **16:** 188

Walsh, H.A., Daya, S.,(1998) *Life Sciences* **62**: 2417

Wang, J., (1994) *Analytical Chem. 1st Ed*, VCH publishers, New York

Wirz-Justice, A., Arendt, J., Harston, A., (1980) *Experimentia* **63**:442

Yamoto, H., Tang, H., (1996) *Neuroscience Letters* **207**: 89

Zaleska, M., Floyd, R., (1985) *Neurochem. Res*. **10**:397

Zesiewicz, T. A., Gold, M., Chari, G., Hauser, R. A., (1999) *Am. J. Geriatr. Psychiatry* **7**:110

Zisapel, N., (1999) *Biol. Signals Recept.* **8**:84

Zubenko, G. S., Moosey, J., (1988) *Arch. Neurol*. **45**:1182