

REGIONAL NEUROCHEMICAL CHARACTERIZATION
OF THE FLINDERS SENSITIVE LINE RAT WITH
REGARD TO GLUTAMATE - NITRIC OXIDE AND cGMP
SIGNALLING PATHWAYS

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Abstract

The serious nature of MDD has intensified the need to identify and elucidate new neurobiological targets for antidepressant drug action. Depression presents with evidence for degenerative pathology that relates to disturbances in excitatory glutamatergic pathways, particularly the N-methyl-D-aspartate (NMDA) receptor-mediated release of the pleiotropic molecule, nitric oxide (NO), and cyclic guanosine monophosphate (cGMP). The contribution of the glutamate-NO/cGMP pathway may realize great importance as a fundamental substrate underlying the pathophysiology of major depression. In the next generation of antidepressant drugs, the nitric oxide pathway could play a dynamic role in addressing urgent therapeutic needs. In this study, we have used a genetic model of depression, the Flinders Sensitive Line (FSL) rat, to investigate the surrogate markers of the NO/cGMP pathway.

The aim was to determine whether the depressive-like behaviour of the hypercholinergic FSL rat is accompanied by altered activation of the NO/cGMP pathway. To this end, the extent to which the FSL and Flinders Resistant Line (FRL) rats differ neurochemically with regard to basal hippocampal and frontal cortical NOS-activity, as well as nitric oxide (NO) and cGMP accumulation, were determined. Additionally, select behavioural assessments were performed to confirm the anxiogenic phenotype of the FSL strain.

For neurochemical determinations a sensitive fluorometric reversed phase high-performance liquid chromatographic (HPLC) assay was developed to analyze total nitrite and nitrate in brain tissue. Nitrate was enzymatically converted to nitrite before derivatization with 2,3-diaminonaphthalene (DAN). The stable and highly fluorescent product, 2,3-naphthotriazole (NAT), was quantified. Secondly, the quantity of the amino acid L-citrulline was measured by HPLC with electrochemical detection after *o*-phthalaldehyde (OPA) derivatization. L-citrulline formation was used as an index for nNOS activity. Finally, a direct, competitive enzyme immunoassay kit was used to determine the downstream activity of the NO-pathway in brain tissue.

FSL rats were compared to FRL rats with respect to sensitivity to serotonin 5-HT_{1A} receptor-mediated hypothermia under our lab-conditions. The Open Field Test (OFT) behavioural assessment was performed to compare FSL with FRL groups under baseline conditions according to their level of inherent anxiety. The parameters used to measure anxiety were number of line crosses (locomotor activity), time spent in middle blocks and social interaction time between pairs of rats. As an additional behavioural assessment, the Forced Swim Test (FST) was performed to assess behavioural restraint measured as time of immobility.

Basal cGMP levels in the frontal cortex were found to be significantly less in FSL than in FRL rats, whereas the levels in the hippocampus did not differ significantly. No other significant differences with respect to NO and nNOS activity were apparent in either of the brain areas. The hypothermia test confirmed a significantly greater decrease in temperature in the FSL rat than the FRL rat. The FST did not confirm any differences in immobility time between the two rat strains. In the OFT, FSL rat groups exhibited behaviour that indicated significantly more anxiety than FRL rats.

Under basal conditions, FSL rats do not present with significant changes in markers of the NO cascade in the hippocampus and frontal cortex compared to FRL controls, including NOS activity as well as NO accumulation. However, cGMP levels were found to be significantly lower in the frontal cortex of FSL rats versus FRL rats, although not in the hippocampus. Since the FSL rat is known to be hypercholinergic, these data support an interaction between the NO/cGMP pathway and the cholinergic system in the frontal cortex but not hippocampus of FSL animals. The mechanisms and implications of such a mutual involvement need further clarification. Further, this anatomical differentiation may have important implications for understanding the role of NO in the depressive-like behaviour of the FSL rat and, indeed, may reveal more on the neurobiology and treatment of depression. Through the performed behavioural assessments, the FSL and FRL rats were successfully separated with respect to their anxiety phenotype as well as their heightened response to serotonergic challenge, thus confirming a contribution of both the serotonergic and cholinergic systems to the depressogenic nature of these animals.

As concluding remark can be said that under normal basal conditions markers of the NO/cGMP signalling cascade are not altered in FSL vs FRL rats, although cGMP levels are reduced in the frontal cortex of FSL rats, supportive of an NO-independent mechanism of cGMP regulation, possibly involving ACh.

Keywords: *nitric oxide/ cGMP signal transduction pathway; neuronal nitric oxide synthase activity; Flinders Sensitive Line rat; animal model of depression; stress*

Die gevolgtrekking kan gemaak word dat merkers van die NO/cGMP seinkaskade in FSL-rotte onder normale basistoestande nie van dié van FRL-rotte verskil nie, hoewel cGMP-vlakke in die frontale korteks van FSL-rotte laer is, wat 'n NO-onafhanklike meganisme van cGMP-regulasie ondersteun waarby ACh moontlik betrokke is.

Sleutelwoorde: *stikstofoksied/cGMP-seintransduksieweg, aktiwiteit van neuronale stikstofoksiedsintase, sensitiewe lyn Flindersrot (FSL), diermodel van depressie, stres*

kompetitiewe immuunbepaling met ensieme gebruik om die aktiwiteit laer af in die NO-weg in breinweefsel te bepaal.

Die sensitiwiteit vir hipotermie, bewerkstellig deur die serotonien-5-HT_{1A}-reseptor in FSL-rotte, is onder plaaslike laboratoriumtoestande met dié van FRL-rotte vergelyk. Beoordeling van gedrag is met die oopveldtoets (OVT) gedoen om die vlak van inherente angs van FSL-groepe onder basislyntoestande met dié van FRL-groepe te vergelyk. Die parameters wat gebruik is om angs te meet, was die aantal kere wat lyne oorgesteek is (lokomotoriese aktiwiteit), tyd wat in die middelste blokke deurgebring is en sosiale interaksie tussen pare. As bykomende beoordeling van gedrag is die geforseerdeswemtoets (GST) gedoen deur die tyd van immobiliteit te meet.

Daar is gevind dat basale vlakke van cGMP in die frontale korteks van FSL-rotte beduidend laer is as in FRL-rotte terwyl die vlakke in die hippokampus nie beduidend verskil het nie. Geen ander beduidende verskille in die aktiwiteit van NO en nNOS is in enige van hierdie breinareas waargeneem nie. Die hipotermietoets het die beduidend groter afname in die temperatuur van die FSL-rotte teenoor dié van die FRL-rotte bevestig. Die GST het geen verskille in immobiliteit van die twee groepe aangetoon nie. In die OVT het die FSL-rotte gedrag vertoon wat beduidend meer angs as in die FRL-rotte aangetoon het.

Onder basistoestande het FSL-rotte nie beduidende verskille in merkers van die NO-kaskade in die hippokampus en frontale korteks, waaronder NOS-aktiwiteit en NO-opbou, met FRL-kontroles vertoon nie. Daar is egter gevind dat die vlakke van cGMP in die frontale korteks, maar nie in die hippokampus nie, van FSL-rotte beduidend laer is as dié van FRL-rotte. Omdat dit bekend is dat FSL-rotte hipercholinergies is, ondersteun hierdie data 'n interaksie tussen die NO/cGMP-weg en die cholinergiese stelsel in die frontale korteks van FSL-diere, maar nie in die hippokampus nie. Die meganismes en implikasies van sodanige onderlinge betrokkenheid moet verder opgeklar word. Verder kan hierdie anatomiese verskille belangrike implikasies hê vir die begrip van die rol van NO in die depressiewe gedrag van FSL-rotte en kan selfs meer oor die neurobiologie en die behandeling van depressie openbaar. Deur die beoordeling van gedrag is die FSL- en FRL-rotte suksesvol onderskei ten opsigte van hulle fenotipe teenoor angs asook hulle respons op blootstelling aan serotonien en bevestig dus so 'n bydrae van sowel die serotonergiese as cholinergiese stelsels in die depressiewe aard van hierdie diere.

Opsomming

Die ernstige aard van major depressie het die noodsaak versterk om nuwe neurobiologiese teikens vir die werking van antidepressante te identifiseer en te ontrafel. Depressie gaan gepaard met tekens van degeneratiewe patologie wat verband hou met versteurings in eksitatoriese glutaminergiese weë en veral die vrystelling van die pleiotropiese molekule, stikstofoksied (NO), en sikliese guanosienmonofosfaat (sGMP) wat bewerkstellig word deur die N-metiel-D-aspartaat (NMDA)-reseptor. Die glutamaat-NO/cGMP-weg kan 'n belangrike bydraende oorsaak in die onderliggende patofisiologie van major depressie wees. Die stikstofoksiedweg kan 'n dinamiese rol speel in die volgende generasie antidepressante wat dringend vir behandeling benodig word. In hierdie studie het ons 'n genetiese model van depressie, die sensitiewe lyn Flindersrot (FSL), gebruik om die surrogaatmerkers van die NO/cGMP-weg te ondersoek.

Die doel was om te bepaal of die depressiewe gedrag van die hipercholinergiese FSL-rot met verandering in die aktivering van die NO/cGMP-weg gepaardgaan. Ten einde hierdie doel te bereik, is die mate waarin FSL-rotte en weerstandige lyn Flindersrotte (FRL) neurochemies verskil ten opsigte van stikstofoksied sintase (NOS) -aktiwiteit in die hippokampus en frontale korteks asook opbou van stikstofoksied (NO) en cGMP bepaal. Daarmee saam is beoordelings van geselekteerde gedragpatrone gedoen om die angsagtige fenotipe van die FSL-stam te bevestig.

Vir neurochemiese bepaling is 'n sensitiewe fluorometriese omgekeerde fase hoëdoeltreffendheid-vloeistofchromatografiese (HDVC) metode ontwikkel om nitriet en nitraat in breinweefsel te bepaal. Nitraat is voor derivatisering met 2,3-diaminonaftaleen (DAN) ensiematies na nitriet omgeskakel. Die stabiele en hoogs fluoresserende produk, 2,3-naftotriasool (NAT), is gekwantifiseer. Tweedens is die hoeveelheid van die aminosuur, L-sitrullien, na derivatisering met o-ftaalaldehyd (OPA) met behulp van HDVC en elektrochemiese deteksie gemeet. Vorming van L-sitrullien is as maatstaf van nNOS-aktiwiteit gebruik. Laastens is 'n stel vir direkte

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God is the Creator of the human brain: 'the most complex and orderly arrangement of matter in the universe', Isaac Asimov.

Congress Proceeding

The work of the current study was presented at a congress as follows:

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List of Abbreviations

ACh	Acetylcholine
AChE	Acetylcholine esterase
ACPC	1-aminocyclopropanecarboxylic acid
ACTH	Adrenocorticotropic hormone
AMPA	α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
AP-7	2-amino-7-phosphonoheptanoic acid
AUC	Area under the curve
BH ₄	Tetrahydrobiopterin
CAPON	Carboxy-terminal PDZ ligand of nNOS
CaM	Calmodulin
cAMP	Cyclic adenosine monophosphate
cGMP	Guanosine 3',5'-cyclic monophosphate
CMS	Chronic mild stress
CNS	Central nervous system
CRH	Corticotropin-releasing hormone
CRF	Corticotropin releasing factor
DA	Dopamine
DFP	Diisopropylfluorophosphate
eNOS	Endothelial nitric oxide synthase
EDRF	Endothelial-derived relaxing factor
EPSP	Excitatory postsynaptic potential
FAD	Flavin adenine dinucleotide disodium salt

	hydrate
FMN	Riboflavin 5'-monophosphate sodium salt dihydrate
FRL	Flinders resistant line
FSL	Flinders sensitive line
FST	Forced swim test
GABA	γ -aminobutyric acid
GAD	Glutamic acid decarboxylase
HPA-axis	Hypothalamic-pituitary-adrenal-axis
HPLC	High performance liquid chromatography
5-HIAA	5-hydroxyindole acetic acid
5-HT	Serotonin or 5-hydroxytryptamine
iNOS	Inducible nitric oxide synthase
ISO/ IEC 17025	International organization for standardization and international electrotechnical commission.
IPSP	Inhibitory postsynaptic potential
LHPA-axis	Limbic hypothalamic-pituitary-adrenal-axis
LOC	Level of confidence
LOD	Limit of detection
LOQ	Limit of quantification
LTP	Long-term potentiation
M1-mAChR	M1 – muscarinic acetylcholine receptors
MAOI	Monoamine-oxidase inhibitor
MDD	Major Depressive Disorder
MK-801	Dizocilpine
nAChR	Nicotinic acetylcholine receptors
NA	Noradrenalin or -epinephrine
NADPH	β -Nicotinamide adenine dinucleotide phosphate
NARI	Noradrenaline reuptake inhibitor
NMDA	N-methyl-D-asparaginian acid
nNOS	Neuronal nitric oxide synthase

NO	Nitric oxide
NO_x	Total mono-nitrogen oxides (NO and NO₂)
OFT	Open field test
8-OH-DPAT	8-hydroxy-2-(di-<i>n</i>-propylamino)tetralin
OPA	o-phthalaldehyde
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PIN	Protein inhibitor of nNOS
PSD-95	Post-synaptic density protein 95
REM	Rapid eye movement
RSD	Relative standard deviation (in percentage)
ROS	Reactive oxygen species
sGC	Soluble guanylate cyclase
SANS 17025	South African national standard
SNRI	Selective noradrenaline reuptake inhibitor
SSRI	Selective serotonin reuptake inhibitor
SSRE	Selective serotonin reuptake enhancer
SOD	Superoxide dismutase
TCA	Tricyclic antidepressant
WHO	World health organization

List of Figures

Figure 1:	Classification of antidepressant drugs (Trevor <i>et al.</i> , 1998)	17
Figure 2:	Classification of neurotransmitters according to receptor function (Leonard, 2003).....	19
Figure 3:	Glutamate – NO/cGMP <i>signal transduction pathway</i> in the mammal brain (Contestabile <i>et al.</i> , 2003; Ledo <i>et al.</i> , 2004)	24
Figure 4:	Binding of glutamate to the postsynaptic NMDA receptor moiety	25
Figure 5:	Activated NMDA receptor	26
Figure 6:	Illustration of NOS: the involvement of cofactors and co-substrates in the NOS catalyzed enzyme reaction (Marletta, 1993).....	27
Figure 7:	NO autoxidation reaction sequence derived from NO: oxygen stoichiometry (4:1) (Mayer <i>et al.</i> , 1995; Contestabile <i>et al.</i> , 2003).....	32
Figure 8:	The hippocampus and the frontal cortex (part of limbic brain system) of the human and rodent brain.....	33
Figure 9:	Frontal cortex and hippocampal circuits and depression (Dobson, 2002)	35
Figure 10:	Stress, the HPA-axis and the the hippocampus (LHPA-axis) (Dubac, 2002).....	36
Figure 11:	Interactions between NO and other neurotransmitter systems in major depressive disorder	41
Figure 12:	Schematic presentation of reaction steps of NO _x determination and the reaction of nitrite with 2,3-diaminonaphthalene (DAN) to yield 2,3-	

naphthotriazole (NAT) under acidic conditions (Li <i>et al.</i> , 2000; Jobgen <i>et al.</i> , 2007)	99
Figure 13: Cycling of β -NADPH by enzyme mixture (Verdon <i>et al.</i> , 1995)	100
Figure 14: HPLC chromatogram representing a rat brain sample derivatized with 2,3-Diaminonaphthalene (DAN) to yield NAT (2,3-Naphthotriazole)	101
Figure 15: Reaction of nitrite with 2,3-diaminonaphthalene (DAN) to yield 2,3-Naphthotriazole (NAT) under acidic conditions	101
Figure 16: Standard concentration curve of NO _x without precautions taken against environmental NO _x contamination.....	108
Figure 17: Standard concentration curve of NO _x following precautions taken against environmental NO _x contamination.....	109
Figure 18: Schematic presentation of reaction steps of NOS activity assessment by measuring L-citrulline.....	110
Figure 19: HPLC chromatogram representing a rat brain sample derivatized with OPA.....	111
Figure 20: The reaction of OPA with TRIS (Shiue <i>et al.</i> , 2005)	118
Figure 21: Study outlay of the FST and OFT stress-naive behavioural assessments	121
Figure 22: Hypothermia challenge.....	124
Figure 23: The behavioural parameters distinguished in the forced swim test (Cryan <i>et al.</i> , 2002)	128
Figure 24: Hypothermia challenge. Basal temperature differences after 30 minutes and 60 minutes following administration of a serotonin agonist (8-OHDPAT) in the Flinders Resistant Line and Flinders Sensitive Line rats. Data were analyzed statistically using the Mann-Whitney U Test and statistical significance was indicated as *P<0.05 and ***P<0.001. (n=10; mean \pm SD)	129
Figure 25: All groups line graph, total number of line crosses of stress-naive rats. Data were analyzed statistically using the Two-way ANOVA.....	130

- Figure 26:** Number of line crosses in different groups of stress-naive Flinders Resistant Line and Flinders Sensitive Line rats on three separate occasions. Data were analyzed statistically using the Mann-Whitney U Test and statistical significance were indicated as * $P < 0.05$ and non-significance (N.S). as $p > 0.05$ ($n = 6-10$; mean \pm SD)..... 131
- Figure 27:** All groups line graph, time spent in middle blocks. Data were analyzed statistically using the Two-way ANOVA 132
- Figure 28:** Time spent in middle blocks between Flinders Resistant Line and Flinders Sensitive Line rats during three separate occasions. Data were analyzed statistically using the Mann-Whitney U Test and statistical significance was indicated as * $P < 0.05$ and non-significance (N.S) as $p > 0.05$ ($n = 6-10$; mean \pm SD) 133
- Figure 29:** All groups line graph, Social interaction. Data were analyzed statistically using the Two-way ANOVA 134
- Figure 30:** Total social interaction in stress-naive Flinders Resistant Line and Flinders Sensitive Line rats on occasion 1-3. Data were analyzed statistically using the Mann-Whitney U Test and statistical significance was indicated as * $P < 0.05$ and non-significance (N.S) as $p > 0.05$ ($n = 13$; mean \pm SD) 134
- Figure 31:** All groups line graph, Forced Swim Test. Data were analyzed statistically using the Two-way ANOVA 135
- Figure 32:** Forced Swim Test total immobility in stress-naive Flinders Resistant Line and Flinders Sensitive Line rats on occasion 1-3 Data were analyzed statistically using the Mann-Whitney U Test and statistical significance was indicated as ** $P < 0.01$ and non-significance (N.S) as $p > 0.05$ ($n = 10$; mean \pm SD) 135

List of Figures (Chapter 3: Article)

Figure 1 a	Figure 1 b	72
Figure 2 a	Figure 2 b	73
Figure 2 c	Figure 2 d	74
Figure 3 a	Figure 3 b	75

List of Figures (Addendum 1: Statistical Chromatographic Method Validation)

Figure 1:	NOx standard regression curve	155
Figure 2:	Comparison between 12.5 μ M NAT standards after 24 hours.....	158
Figure 3:	Comparison between 25 μ M NAT standards after 24 hours.....	159
Figure 4:	Amino acid standard regression curve.....	165
Figure 5:	Plotted L-citrulline AUCs.....	168
Figure 6:	Plotted L-arginine AUCs	168

List of Tables

Table 1:	Neurochemical analysis	6
Table 2:	Behavioural assessments	7
Table 3:	Classic hypothesis explaining the cause of depression (Leonard, 2003; Wells <i>et al.</i> , 2003; Belmaker & Agam, 2008).....	14
Table 4:	Novel hypotheses explaining the cause of depression	15
Table 5:	Symptoms of Major Depressive Disorder, and brain regions involved (American Psychiatric Association, 2000; Adell <i>et al.</i> , 2005; Cahill, 2007; Belmaker & Agam, 2008)	16
Table 6:	Timeline narrating NO research progression from science to neuroscience, reproduced from (Wells, 2003).....	22
Table 7:	Cross-talk mechanisms between neurotransmitters	43
Table 8:	Dependant validity criteria (Geyer & Markow, 2000; Willner & Mitchell, 2002)	48
Table 9:	Similarities among depressed individuals and FSL rats (Overstreet <i>et al.</i> , 1998, 2005).....	50
Table 10:	Neurochemical features of the FSL rat.....	51
Table 11:	Standard protein concentration series.....	98
Table 12:	List of reagents and chemicals used for quantification of NO _x	102
Table 13:	Calculation of 15 mU nitrate reductase	104
Table 14:	Simple dilutions, dilution factors and working concentration range of nitrite and nitrate	105
Table 15:	Components of NaNO ₂ and NaNO ₃	106
Table 16:	Concentrations and AUCs of standard solutions	107

Table 17:	The influence of environmental contamination on the NO _x standard concentration curve	107
Table 18:	NO _x standard concentration AUCs after precautions against contamination have been taken	108
Table 19:	List of reagents and chemicals used for quantification of L-citrulline .	111
Table 20:	Reaction cocktail component volumes and concentrations	113
Table 21:	Calculation of 25.2 mU nitrate reductase	114
Table 22:	Simple dilutions, dilution factors and working concentration range of amino acids	116
Table 23:	Concentrations and AUCs of standard solutions	117
Table 24:	Programmed HPLC sequence	118

List of Tables (Addendum 1: Statistical Chromatographic Method Validation)

Table 1:	Regression Statistics	154
Table 2:	NO _x regression curve values	155
Table 3:	Repeatability and reproducibly of standards	157
Table 4:	L-citrulline regression curve values	164
Table 5:	Regression Statistics	165
Table 6:	AUCs of standards.....	167
Table 7:	AUCs of Real Sample 1	168
Table 8:	Critical values of F for a one-tailed test ($p = 0.05$).....	171
Table 9:	DIXON	172
Table 10:	The t-distribution	172

Table of Contents

Abstract	I
Opsomming	IV
Acknowledgements	VII
Congress Proceeding	IX
List of Abbreviations	X
List of Figures	XIII
List of Tables	XVII
Table of Contents	XX
CHAPTER 1: INTRODUCTION	1
1.1 Project Motivation and Problem Statement	1
1.2 FSL rat as a genetic rodent model of depression	4
1.3 Project Aims	4
1.4 Project Layout	5
1.5 Expected Results	7
1.5.1 <i>Neurochemical analysis</i>	7
1.5.2 <i>Behavioural Assessments</i>	7
1.5.2.1 <i>Hypothermia</i>	7
1.5.2.2 <i>Open Field Test</i>	8
1.5.2.3 <i>Forced Swim Test</i>	9
1.6 Prologue	9
CHAPTER 2: LITERATURE REVIEW	10
2.1 Major Depressive Disorder	11
2.1.1 <i>Prevalence</i>	11
2.1.2 <i>Aetiology</i>	13
2.1.3 <i>Symptomatology</i>	15

2.1.4	<i>Current antidepressant drug therapy</i>	17
2.2	Classification of neurotransmitters involved in synaptic transmission.....	19
2.3	The role of the glutamatergic system and the cGMP/NO pathway in depression ...	21
2.3.1	<i>History of NO in the nervous system</i>	21
2.3.2	<i>Characterization of neuronal nitric oxide</i>	23
2.3.3	<i>Glutamate – NO/cGMP signal transduction pathway</i>	24
2.3.4	<i>Biochemistry of nitric oxide: nitric oxide synthase and co-factors</i>	26
2.3.5	<i>NO-derived formation of cGMP</i>	28
2.3.6	<i>Regulation of NO biosynthesis</i>	29
2.3.7	<i>Nitric oxide: protector or terminator of neuronal allostasis?</i>	30
2.4	Brain structures implicated in stress and depression	33
2.5	Stress system (LHPA-axis) and depression	36
2.6	Novel psychotropic drugs modulating the glutamatergic/ nitric oxide system in depression	38
2.7	Cross-talk mechanisms in depression	41
2.7.1	<i>Cholinergic-adrenergic hypothesis of depression*</i>	42
2.7.2	<i>Other cross-talk mechanisms</i>	43
2.8	Pathogenesis of depression.....	44
2.9	The Flinders Sensitive Line rat	45
2.9.1	The Flinders Sensitive Line rat as genetic animal model of depression	45
2.9.2	<i>NO in the Flinders Sensitive Line rat</i>	50
2.9.3	<i>Validity of the Flinders Sensitive Line rat as animal model of depression</i>	56
2.10	Closing remarks	57
CHAPTER 3: ARTICLE		58
	Abstract.....	60
1	Introduction.....	61
2	Materials and methods	63
3	Experimental animals	63
4	Neurochemical analysis	64
4.1	Tissue dissection and storage	64
4.2	Chemicals	64
4.3	Nitric oxide analysis	64
4.3.1	<i>NOx determination</i>	64
4.3.2	<i>NOS activity assessment</i>	67
4.4	cGMP analysis	71
4.5	Statistical analysis.....	71

5	Results	72
5.1	Nitric oxide analysis	72
5.1.1	<i>NOx determination</i>	72
5.1.2	<i>NOS activity assessment</i>	72
5.2	cGMP analysis	74
6	Discussion	75
7	Conclusion	81
8	Acknowledgements	81
9	References	81
10	Figure legends	95
CHAPTER 4: CHROMATOGRAPHIC METHODS		97
4	General information	97
4.1	NOx determination	98
4.2	NOS-activity determination	109
4.3	Conclusion	119
CHAPTER 5: BEHAVIOURAL ASSESSMENTS		120
5.1	Overview	120
5.2	Materials and Methods.....	121
5.2.1	<i>Experimental Animals</i>	121
5.2.2	<i>Apparatus</i>	122
5.2.3	<i>Drug and Chemicals</i>	122
5.2.4	<i>Experimental Protocols</i>	122
5.3	Statistical analysis of data.....	128
5.4	Results	129
5.4.1	<i>Hypothermia challenge</i>	129
5.4.2	<i>Open Field Test results</i>	130
5.4.3	<i>Forced Swim Test results</i>	134
5.5	Discussion.....	136
5.6	Conclusion	137
CHAPTER 6: CONCLUSION AND RECOMMENDATIONS		138
GLOSSARY		142
ADDENDUM 1: STATISTICAL CHROMATOGRAPHIC METHOD VALIDATION .		149
1	Validation of 2, 3-Naphtatriazole (NOx) detection method procedure	150

2	Validation of L-citrulline detection method procedure.....	160
	ADDENDUM 2: GUIDE FOR AUTHORS.....	173
1.1	Scope: Appropriate Section	173
1.2	Preparation of the electronic manuscript	173
1.3	Electronic submission of articles via the Web (http://ees.elsevier.com/bres).....	174
1.4	Organization of the article	174
1.5	Illustrations and Tables	176
1.6	Proofs, Offprints, Miscellaneous	178
1.7	Policy Issues	178
	BIBLIOGRAPHY	179

CHAPTER 1

Introduction

1.1 Project Motivation and Problem Statement

At the turn of the new millennium, major depressive disorder (MDD) globally claimed roughly one million lives (Bertelote, 2001), whilst in South Africa, a 2004 survey reported approximately 8000 fatal annual suicide attempts (Schlebusch L, 2004). Currently, the country's high HIV-AIDS prevalence bolsters suicide behaviour even more (Lönnquist, 2001). It is evident that MDD is an extremely serious mental disease. If left untreated or inappropriately treated - MDD has neuropathological complications and future psychological consequences, that are obvious and final (Freeman *et al.*, 2004). Even if treated, the disorder still presents with a strong tendency of relapse and recurrence (Ramana *et al.*, 1995).

Current antidepressant drug therapy is far from ideal, including a delayed onset of action, a variable range of side-effects, while a single treatment seldom is curative (Rosenzweig-Lipson *et al.*, 2007). Pre-treatment diagnostic guidelines require at least five symptoms of depression to be present during a two-week *major depressive episode*, before the disorder can be positively diagnosed in the patient (American Psychiatric Association, 2000). The biogenic amine deficiency *hypothesis*, also referred to as the monoamine hypothesis (Leonard, 2003) still forms the cornerstone of contemporary antidepressant drug treatment. The monoamine *hypothesis*, together with other acclaimed classic hypotheses, has laid the groundwork for the exploration of internal causes underlying the *pathophysiology* of major depression (Belmaker & Agam, 2008). These earlier models have helped in our understanding of antidepressant action, while more recent discoveries have built on these models to provide a better knowledge of neurotransmission and transmitter cross-talk, and as such to assist in identifying new antidepressant targets. One such novel messenger molecule is nitric oxide (NO) which, after first being discovered in the brain in 1988 (Garthwaite *et al.*, 1988), has in recent years been increasingly implicated in the

pathology and treatment of depression (Harvey *et al.*, 1996; Wegener *et al.*, 2003; Millan, 2006).

Apart from the important physiological functions of NO in the nervous system, such as regulation of synaptic plasticity, *long-term potentiation*, neuromodulation and neuroprotection, NO can also exert damaging effects on neurones when present at inappropriately high concentrations, as for example under pathological conditions (O'Dell *et al.*, 1991; Schuman & Madison, 1991; Arancio *et al.*, 1996; Christopherson & Bredt, 1997; McEwen, 1999; Sapolsky, 2001; Kim & Diamond, 2002; Contestabile *et al.*, 2003; Calabrese *et al.*, 2007). Indeed, NO is often regarded as a 'Janus-faced' molecule, having both beneficial and detrimental actions (Contestabile *et al.*, 2003; Calabrese *et al.*, 2007).

The role of NO in *affective* disorders has been investigated for more than a decade (Harvey *et al.*, 1994; Harvey 1996; Dawson & Dawson, 1996; McLeod *et al.*, 2001; Bal-Price & Brown, 2001; Suzuki *et al.*, 2001). Recent evidence regarding the possible role of degenerative phenomena in depression (MacQueen *et al.*, 2003), and considering the role of NO in neurodegeneration (Calabrese *et al.*, 2007), has flagged the NO pathway as an attractive option for novel drug development. Duport & Garthwaite (2005) reported that exposure of the hippocampus to three times higher than physiological concentrations of NO caused extensive neural damage that was not reversible with nitric oxide synthase (NOS) -inhibitors. NO overproduction occurs due to the persistent stimulation of neuronal NOS (Guix *et al.*, 2005), an event that may follow chronic stress and as such underlie the suppression of hippocampal *neurogenesis* observed in depression (Zhou *et al.*, 2007).

Although depression and NO have been extensively investigated, its role in a genetic rodent model of depression, specifically the Flinders Sensitive Line (FSL) rat, has not yet been undertaken. Preliminary findings from collaborators at the University of Aarhus, Denmark, have found that subacute stress induces an elevation in hippocampal expression of nNOS and associated proteins in the FSL rat compared to its normal healthy control, the Flinders Line resistant (FRL) rat (Wegener *et al.*, 2008). In this project, we have investigated evidence for differences in selected markers of the NO pathway in the hippocampus and frontal cortex of unstressed FSL versus -FRL rats. Critical to this study was the development of an in-house High performance liquid chromatography (HPLC)-method for the analysis of NO accumulation in rat brain.

Apart from the distinct behavioural attributes of the FSL rat that are akin to depressive symptoms in humans, at the neurochemical level FSL rats present with a hypercholinergic response (Overstreet *et al.*, 2005). Making use of the FSL rat model of depression has thus not only permitted us to use a behavioural animal model of depression to intercompare associated behavioural and neurochemical changes, but also to elaborate on current theories that underlie the neuropathological features of depression, like cross-talk mechanisms, and in particular the connection between the NO and cholinergic systems (Kendler, 1998; Harvey, 2008).

How does the stress-response involve NO and what connection is there between the nitrenergic and cholinergic systems in the FSL rat? Depression has been associated with an increase in cholinergic drive, the so-called cholinergic *hypothesis* of depression (Janowsky *et al.*, 1972), while the administration of a cholinergic agonist to depressed patients results in an increased adrenal response (increased cortisol levels) (Perlis *et al.*, 2002). Earlier studies have found that the Hypothalamic-pituitary-adrenal-axis (HPA-axis) is hyperactive in FSL rats, albeit only under stressful conditions (Owens *et al.*, 1991; Ayensu *et al.*, 1995). Acetylcholine (ACh) release in various brain regions is under NO regulation (Prast and Philippu, 2001). Recent data from our laboratory have described a unique antidepressant response in animals treated simultaneously with an antimuscarinic agent and sildenafil, an agent that bolsters down-stream NO signalling onto guanylyl cyclase-cyclic guanosine monophosphate (cGMP) synthesis, but with neither drug having any effect alone (Brink *et al.*, 2008). This described a co-operative relationship between the NO-cGMP and cholinergic systems with respect to mood regulation. Consequently, the increase in NOS activity in stressed hypercholinergic FSL rats vs. normocholinergic FRL rats (Wegener *et al.*, 2008) indicates that the HPA-axis as well as the nitrenergic and cholinergic systems are connected. The question now arises whether basal NO-cGMP and cholinergic activity are altered in the FSL rat, i.e. under non-stressed conditions, thereby confirming altered NO activity as either a state or trait dependent variable in these animals, and by implication in major depressive illness.

1.2 FSL rat as a genetic rodent model of depression

Genetic *animal models* are an extremely useful investigative tool to acquire valuable insights into the neuropathology of a clinical illness, such as depression. From these data, new strategies may be devised from which more effective antidepressant treatments can be developed. The analysis of tissue from animal models provide a useful means of investigating illness cause and effect as opposed to using the post-mortem brains of suicide victims. Moreover, a genetic model of depression means not having to 'create' a model in the conventional manner. Instead the effects of the genetic manipulation can be used to obtain more knowledge about the neuropathology of MDD. Nevertheless, all animal models, whether natural, induced, or genetic, need to comply with important validation criteria before their application in research related activities for a given human disorder. In the present study, the FSL/FRL rat, a selectively bred hypercholinergic model of depression together with its normocholinergic control, will be used. The model has demonstrated good face and some construct and predictive validity for major depression (Overstreet, 1993).

1.3 Project Aims

The current project aims to contribute to the elucidation of nitrenergic mechanisms in depressive illness by investigating the NO signalling pathway in the FSL rat and its healthy control, the FRL rat. These findings will expand on existing knowledge concerning the *pathophysiology* of depression and in this manner contribute towards further advancement in the pharmacotherapy of *affective* illnesses.

The project aspires to:

- Establish where and to what extent the FSL and FRL rat differs neurochemically, with regard to regional (hippocampal and frontal cortical) NOS-activity, as well as to determine the accumulation of nitrogen oxide- (NOx) and cGMP in the aforementioned brain areas.
- Determine if the standard depressive-like behaviour of the selectively bred hypercholinergic FSL rat line (Overstreet *et al.*, 2005) is accompanied by elevated levels of measured markers of the NO/cGMP pathway in aforementioned brain areas, as compared to levels in the FRL rat line.

- Develop and optimise two new analytical methods for the analysis of the NOS activity and NO_x accumulation in rat brain samples.
- Using the Open Field Test (OFT), to perform behavioural assessments on all study populations of FSL and FRL rats (randomly performed) to evaluate the prerequisite behavioural characteristics of FSL rats, such as level of inherent anxiety and locomotor activity. An additional assessment, namely the Forced Swim Test (FST) will be used to confirm learned helplessness in the FSL rat.
- Investigate the serotonergic cholinergic interaction in the FSL rat through determining the sensitivity of the FSL rat to the serotonin agonist, receptor subtype 1A (5-HT_{1A}-R agonist), 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT) in a hypothermia challenge.

1.4 Project Layout

The project can be split into a main component involving neurochemical analysis and a secondary component involving the behavioural assessments. This protocol is outlined in tables 1 and 2.

Neurochemical analysis will comprise NOS activity and NO_x accumulation analysis using HPLC, and cGMP analysis by radioimmunoassay (Table 1). Analysis of the NO pathway will be performed following the development of in-house methods for the assay of rat brain NOS activity by analyzing NOS-mediated conversion of L-arginine to L-citrulline, with the latter determined, by means of HPLC. NO_x accumulation will also be determined by HPLC, measuring total nitrite derived from endogenous nitrite and nitrate converted to nitrite. Determination of the down-stream messenger of the NO pathway, cGMP, will be determined using a commercially available radioimmunoassay kit.

Table 1: Neurochemical analysis

Neurochemical analysis	Substance measured	Method
1. Nitric Oxide pathway analysis		
1.1 NO _x determination	Total nitrite & nitrate	HPLC – fluorescence detection
1.2 NOS activity determination	L-citrulline	HPLC – electrochemical detection
2. cGMP direct competitive immunoassay analysis	cGMP	Direct competitive immunoassay (ELISA) kit

Behavioural assessments will be performed throughout the duration of the study as a means of validating the FSL rodent model of depression for prerequisite inbred behavioural characteristics as stipulated by Overstreet *et al.*, 2005. These will be corroborated with locomotor activity scores courtesy of the OFT. The FST, that will be performed additional as an additional assessment must not be considered as a precipitant of depressive like state ('stress' within the *diathesis*/stress framework), but is rather regarded as a psychometric instrument that measures 'the intensity of the pre-existing condition', without in itself being responsible for psychological changes (Willner & Mitchell, 2002).

Finally, FSL and FRL rats can be separated phenotypically with respect to a hypothermic response to the 5-HT_{1A}-R agonist, 8-OH-DPAT. This test will also be routinely performed in both FSL and FRL rats to further confirm the validity of the FSL/FRL rat model for the current study. In all cases, FRL rat group will serve as the normal healthy control.

The above three assessments will be performed at the preferred rat biorhythmic cycles. Firstly, the OFT and the FST will be performed nocturnally (OFT: ± 6:00 p.m. - 7:30 p.m.; FST: ± 9:30 p.m. – 10:00 p.m.), when rats are most active, while the hypothermia challenge will be performed from 8:00 a.m. – 1:00 p.m.

Table 2: Behavioural assessments

Time of day	Behavioural assessments	Parameters	
8:00 a.m. -1:00 p.m.	1. Hypothermia challenge	↓ core body temperature	
6:00 p.m. – 10:00 p.m.	2. Open Field Test (OFT)	a. Locomotor crosses	Number of line activity
		b. Seconds in four middle blocks	Anxiety
		c. Social Interaction in seconds	Sniffing/physical contact
	3. Forced Swim Test (FST)	Time of immobility seconds	

The Hypothermia challenge will be performed on a single occasion with a group of 10 FSL and 10 FRL rats.

The OFT and FST will be performed on three separate occasions, using three different groups of rats (FSL vs. FRL). These rats will not be handled in any way other than to be assessed for their behaviour as described in Table 2.

1.5 Expected Results

1.5.1 Neurochemical analysis

Taking into account that the FSL rat is an *animal model* of depression with extensive face validity (Overstreet 1993) with regard to the current theories underlying the neuropathology of depression, we anticipate that FSL rats will be characterised by an overall increase in NOx, NOS-activity and cGMP levels, as compared to the FRL rat.

1.5.2 Behavioural Assessments

1.5.2.1 Hypothermia

With evidence of serotonergic dysfunction in FSL rats (Wallis *et al.*, 1988), there is a consistency of reports confirming the increased response of these animals to the 5-HT_{1A}-R agonist, 8-OH-DPAT (Overstreet *et al.*, 1998). Indeed, FSL rats routinely display a 2-fold greater decrease in temperature compared to the FRL rat

subsequent to administration of 8-OH-DPAT (Overstreet *et al.*, 1994). We anticipate a similar result in the current study.

1.5.2.2 Open Field Test

The Open Field Test (OFT) is a measure of the inherent anxiety levels of the animal, as well as being a measure of the overall locomotor activity of the animals (Overstreet & Griebel, 2005). The FSL rats display a greater stress-susceptibility than FRL rats (Overstreet *et al.*, 2005), which explains their differential reaction in the OFT (Koene *et al.*, 2003).

a) *Number of line crosses*

Number of line crosses in the OFT serves as a measure of locomotor activity (Overstreet & Griebel, 2005). In the FST (see below), locomotor activity can be a confounding factor in interpreting behaviour in this test. Line crosses in the OFT ideally should not differ significantly between the two rat strains, so that altered swimming behaviour in the FST are more attributed to learned helplessness than to altered locomotor activity.

b) *Time spent in middle blocks in seconds*

The time the rats spend in the middle blocks serve as an indication of anxiety-like behaviour (Koene *et al.*, 2003). More anxious rats will spend less time in the centre of the open field arena. Due to the greater stress sensitivity of the FSL rats, and given the anxiogenic environment of the OFT, it is expected that FRL rats will spend a significantly greater amount of time in the middle blocks than FSL rats (Overstreet *et al.*, 2004).

c) *Time spent in social interaction in seconds*

Anxious rats spend less time in social interaction with their cage mates (File & Seth, 2003; Overstreet *et al.*, 2004; Overstreet & Griebel, 2005). Due to their greater anxious nature in a stressful environment, FSL rats are expected to spend less time in social interaction in the OFT than the FRL rat – with pairs of FSL rats tending to avoid each other rather than grooming, licking, sniffing or crawling over or under one another (Overstreet, 2002; Overstreet *et al.*, 2005).

1.5.2.3 Forced Swim Test

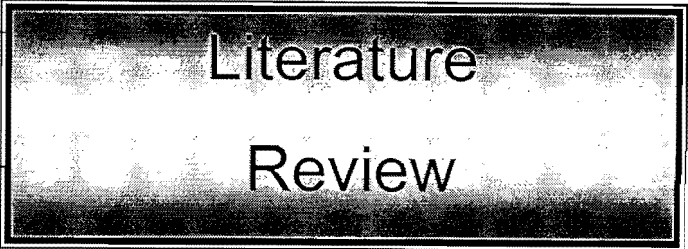
A natural response of an animal to an aversive environment is to attempt to escape. Although rats are good swimmers, they will avoid water, or if placed in water they will strive to escape the water by actively indulging in various swimming and diving behaviours (Porsolt *et al.*, 1978; Cryan *et al.*, 2002). These responses are futile due to the high smooth walls of the swim tank. On repeated exposure, animals realise the futility of the struggle and take on an immobile posture, choosing rather to float in the water and to await rescue by the investigator. More stress sensitive animals, however, will spend less time struggling to escape the tank than their stress-resilient counterparts (Overstreet *et al.*, 2005). Consequently, FSL rats should display greater immobility (in seconds) than FRL rats (Overstreet, 1986; Overstreet, 1993; Overstreet *et al.*, 1995; Zangen *et al.*, 1997; Overstreet *et al.*, 1998; Overstreet, 2002; Overstreet *et al.*, 2005). However, these behaviours need to be related to their locomotor performance in the OFT since swimming performance can be related to either altered locomotor activity or altered drive to escape the swim tank, i.e. learned helplessness. The latter, termed avolition, is a key symptom of MDD.

1.6 Prologue

This dissertation was written and submitted in the article format for thesis/dissertation submission, as approved by North-West University. This format includes an introductory chapter, a chapter covering the relevant literature overview, and a chapter containing a full length article for submission to a peer-reviewed, accredited neuroscience journal. Carefully selected, novel and high impact data from the study will be used for this submission. To this end, the article will be prepared according to the house style and author instructions of that particular journal. The guidelines for authors are provided in Appendix 1. Chapter 4 will present the behavioural assessments done in the current study, and Chapter 5 includes additional information concerning the method development of the two chromatographic methods.

Additional methods and results regarding the validation of neurochemical chromatographic methods are provided in Addendum 1.

CHAPTER 2



Literature
Review

This chapter consists of a concise literature overview on relevant topics concerning MDD from a neuropharmacological perspective.

In the opening section, contextual information such as the formal definition of MDD, its history and the global and local prevalence will be provided. The *aetiology* and typical *symptomatology* and diagnosis of the disease will be appraised, covering initial and more recently postulated central *hypotheses*. The serendipitous discovery of antidepressant actions of the antitubercular drug iproniazid (Monoamine-oxidase inhibitor - MAOI) and the antihistamine imipramine (Tricyclic antidepressant - TCA), led to the recognition of the important role of the monoaminergic system (postulated as the *biogenic amine -deficiency hypothesis*) in the *pathophysiology* and treatment of depression. To improve on these initial drugs concerning tolerability and safety profile, second generation drugs, such as serotonin reuptake inhibitors (SRIs) and other atypical antidepressants (eg. tianeptine) were engineered from the knowledge provided from more recent theories, such as the serotonin and *neuroplasticity* hypotheses. A brief classification of available antidepressant drugs will be provided. All the existing modes of action of antidepressants directly or indirectly affect neurotransmitter synthesis, degradation or receptor binding, and as such modify synaptic transmission. However, the efficacy of currently available antidepressants remains far from ideal, such that new targets underlying the neurobiology of depression, as well as for antidepressant action, are urgently needed.

It is now recognised that depression is not a single neurotransmitter disorder (Millan et al., 2000). This, plus that depression is associated with neurodegenerative changes in certain brain regions (Sapolsky, 2001) has prompted the suggestion that improvement in therapy may lie in addressing multiple transmitter systems, or alternatively targeting a central pathway involved in *neuromodulatory* actions on various physiological functions. It is especially neurodegenerative and neuroplasticity processes in depression that have taken center stage in recent years. In this regard,

the glutamatergic system has become increasingly important, and will form the main focus of this literature overview. The glutamate – NO/cGMP *signal transduction pathway* and the detrimental consequences of its activation will be discussed in detail. Finally the hypercholinergic FSL rat model of depression used in this study will be described and discussed.

2.1 Major Depressive Disorder

MDD is a serious, complex, and often chronic mental disease characterized by a long-lasting depressed mood and *anhedonia*. According to the *Oxford Dictionary*, depression is a state of “low spirits or vitality”. The disorder undeniably has a powerful and extending negative impact on lives, reducing patients’ overall competency and life quality, especially in the absence of effective medical intervention (Cahill, 2007).

An early medical term generally used to describe depression is ‘melancholia’, a state of mind presumably due to disturbances in the soul (Radden, 2003). Since these earlier times, a vast change has occurred regarding the insight and treatment of depression. In the 1950’s clinicians observed that reserpine and isoniazid, agents known at the time to alter monoamine levels but used to treat separate disorders (hypertension and tuberculosis, respectively), affected the symptoms of depression in patients with concurrent MDD. This observation symbolized the turning point in the history of the treatment of depression by inspiring a new theory, namely that clinical depression is a chemical imbalance in neurotransmitters in the brain (Schildkraut, 1965). Since then, many novel hypotheses have been postulated to try and explain the aethiology of depression.

2.1.1 Prevalence

The number of suicides due to depression has globally increased by nearly 60% between 1950 and 1995 (Bertelote, 2001). This drastic increase in global prevalence of depression accentuates the seriousness of the disease and need for new and improved treatment.

A national co-morbidity study ranked depression as the fourth medical condition with the worst disease burden in the USA. In the 1990’s the disorder accounted for 3.7% of total national disability adjusted life years (Murray & Lopez, 1997; Fava & Kendler,

2000). A later project, funded by the World health organization (WHO) reported that in the year 2000, global depression was still fourth, with 4.4% total national disability adjusted life years - causing the largest amount of non-fatal burden (almost 12%) of total years lived with disability worldwide (Mathers & Loncar, 2006). Roughly one million people worldwide committed suicide in the year 2000 (Bertelote, 2001), one death every 40 seconds worldwide and one attempt in every three seconds (Schlebusch L, 2005). The National Co-morbidity report by Murray and Lopez (1997) predicted that depression could become the condition with the second greatest disease burden worldwide by 2020 – with about 1.53 million people committing suicide annually (Bertelote, 2001).

At present 53% people that commit suicide are from the 5-44 years age group, most falling between the ages of 35 and 44 years. The global ratio of males to females is almost 4:1, except in rural China, where medial female rates are 1.3 times higher than in males (Bertelote, 2001; Schlebusch L, 2004).

In South Africa, a national treatment program for depression was compiled 25 years ago, illustrating that depression was then already regarded as a major problem in the country (Schlebusch, 1990). The total suicide rate in 1990 was higher than the global average (17.2 per 100 000 of the population in South Africa, against 16 per 100 000, worldwide). The current high HIV-AIDS prevalence, increases the chances of suicidal behaviour even more (Lönnquist, 2001). The average age for fatal suicides in South Africa (36) agrees with the global depression situation where a shift from the elderly towards younger people is occurring. Statistics revealed that more younger age groups are suffering from depression than 10 years ago. Patients as young as 6 years make attempts and some as young as 10 years old have died from suicide (Schlebusch L, 2005). In South Africa, suicide accounts for nearly 10% of all unnatural deaths, with up to five times more males than females committing suicide (Matzopoulos, 2002; Schlebusch L, 2004). Annually, approximately 8000 South African people die of suicide, with 20 to 40 suicide attempts every hour (Schlebusch L, 2004).

2.1.2 Aetiology

MDD is the product of a multitude of interconnected factors. These factors can be roughly categorized into internal (neurological or other anomalies – probably genetic) (Harvey *et al.*, 2007; Belmaker & Agam, 2008) and external (*psychosocial* or drug-induced) causes. Neuropsychopharmacology primarily focus on internal causes underlying the *pathophysiology* of MDD.

Several acknowledged neurotransmitter dysregulation hypotheses explaining the possible cause of depression have been postulated (see table 1). It is evident that some of these classic *hypotheses* overlap leading to other novel hypotheses – further underlining current thinking of depression as a multi-dysfunctional illness.

Table 3: Classic hypotheses explaining the cause of depression (Leonard, 2003; Wells *et al.*, 2003; Belmaker & Agam, 2008)

Hypothesis	Explanation	Confirmation
Biogenic amine - deficiency <i>hypothesis</i> (monoaminergic <i>hypothesis</i>) and Role of dopamine (DA)	Depression is due to a relative deficiency of <i>biogenic amine neurotransmitters</i> in the <i>synaptic cleft</i> . <i>Hypothesis</i> has a valuable ability to predict antidepressant effect of newly synthesized norepinephrine (NE) or 5-HT reuptake inhibiting-compounds.	<ul style="list-style-type: none"> • TCAs and MAOIs reversed this shortage by preventing the inactivation of these neurotransmitters by reuptake through transport receptor or metabolism by intracellular monoamine-oxidase (MAO) enzymes respectively. • ↓ 5-hydroxyindole acetic acid (5-HIAA) concentration in several brain regions & CSF. • ↓ HVA (main dopamine metabolite) in cerebrospinal fluid.
Joseph J. Schildkraut's catecholamine <i>hypothesis</i>	The etiology of depression revolves around NE (drive and motivation).	<ul style="list-style-type: none"> • Reserpine, the antihypertensive drug, which depletes both the central nervous system (CNS) and peripheral nervous system's vesicular stores of catecholamines like NE, often brings on depression in patients during remission.
Postsynaptic changes in receptor density (Receptor sensitivity <i>hypothesis</i>)	Receptor sensitivity changes may lead to depression.	<ul style="list-style-type: none"> • ↑ in density of 5-HT_{2A} receptors in limbic regions in depression. • Adaptive ↑ in density of β-adrenoceptors in cortical regions because of ↓ NE function in depressed patients. • ↑ in muscarinic ACh receptor density in limbic regions in depression.

Today, a number of novel theories/hypotheses on the *pathogenesis* of depression have been proposed (see table 4).

Table 4: Novel hypotheses explaining the cause of depression

Theory	Explanation
Cholinergic-adrenergic <i>hypothesis</i> (Janowsky <i>et al.</i> , 1972)	Disturbance in balance between norepinephrine (NE) and the cholinergic system accounts for depression. An increase in NE counters cholinergic activity.
HPA-axis (Swaab <i>et al.</i> , 2005)	Overactive HPA-axis exists in depression with neurotoxic consequences.
γ -aminobutyric acid dysregulation (GABAergic dysregulation) (Belsham, 2001; Hemrick-Luecke & Evans, 2002; Choudary <i>et al.</i> , 2005)	Disturbance in GABA-glutamate balance – excessive glutamate leads to neurotoxicity.
Disturbance in neuronal plasticity (Kempermann, 2002)	Decrease in <i>neurogenesis</i> leads to depression.
Inflammation due to circulatory cytokines (Raison <i>et al.</i> , 2006)	Higher levels of proinflammatory cytokines in depressed patients.
NO and depression	Significantly increased plasma nitrate levels in suicidal depressive patients (Kim <i>et al.</i> , 2006). Inappropriate formation of NO is neurotoxic/ causes neurodegeneration.
Interaction between NE-DA systems (Schildkraut, 1965; Mongeau <i>et al.</i> , 1997; Trevor <i>et al.</i> , 1998)	Dysregulation of NE accounts for depression.

In forthcoming sections of chapter 2, some of these theories will be discussed or reviewed according to their relevance to this project.

2.1.3 Symptomatology

MDD is diagnosed when a *major depressive episode* lasts for more than a week and interferes with a patient's life in a *psychosocial* and *psychophysiological* manner.

The American Psychiatric Association first published a manual for the diagnosis of mental disorders in 1952. Since then there have been five revisions, the last being

published in 1994 with a "text revision" in 2000. Diagnostic criteria are descriptions of the manifesting symptoms of the mental disorder. At least five of the listed symptoms have to be present, in the absence of mania or hypomania, during the same two-week period. The symptoms have to be in contrast with the individual's *psychophysiological* functioning prior to the *major depressive episode*. Symptoms of MDD (Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition) can be classified into four main categories:

Table 5: Symptoms of Major Depressive Disorder, and brain regions involved
(American Psychiatric Association, 2000; Adell *et al.*, 2005; Cahill, 2007; Belmaker & Agam, 2008)

<i>Affective (essential symptoms)</i>	<i>Psychomotor or behavioural</i>	<i>Psychopathologic or cognitive (prefrontal cortex)</i>	<i>Somatic (hypothalamus)</i>
<i>Anhedonia (nucleus accumbens),</i>	Change in observable behaviour	Distorted thinking patterns	Change in sleeping habits (insomnia/hypersomnia)
Strong feelings of guilt or worthlessness, helplessness, hopelessness, pessimism	A noticeable 'slowing down'	Thoughts of suicide	Significant weight change, increased or decreased appetite
Depressed mood (<i>limbic system</i>)	Restlessness or irritability (amygdaloid complex)	Waning of concentration	Headaches or Chronic pain
A deep feeling of sadness	Fatigue or loss of energy	Impaired decision making	Digestive problems
Persistent sad, anxious or "empty" moods(amygdaloid complex)	<i>Psychomotor agitation</i> or <i>Psychomotor retardation</i>	Constant self-criticism and pessimism	Malfunction of the Limbic - HPA-axis (hormonal changes)

The mistaken belief that suicide is a cry for help is usually untrue. It is more plausible that the person sees no salvation left – the situation feels hopeless and beyond help with no light at the end of a dark tunnel (Beck, Kovacs, & Weissman, 1975).

Prominent risk factors of depression in individuals with certain genetic predispositions include a personal/ family history of depressive disorder; substance abuse; lack of social support and stressful life events (Kendler, 1998; Schlebusch L, 2005). It is

important for clinicians to diagnose depression correctly, because as Dunitz put it: “Even if suicide is the result of an individual decision, it neither originates nor is committed in a vacuum” (Dunitz, 2001).

2.1.4 Current antidepressant drug therapy

The classic biogenic amine -deficiency hypotheses caused a surge in development of new antidepressant therapy (Figure 1), mostly NE and 5-HT reuptake inhibiting – compounds, but also MAO enzyme inhibitors and drugs that block Alpha₂ receptors. The drugs act to either inhibit reuptake of *biogenic amine neurotransmitters* out of the *synaptic cleft*, or inhibit the enzyme – MAO responsible for their metabolism. Alpha₂ receptor blockers potentiate the release of NE and 5-HT resulting in a mood elevation (Trevor *et al.*, 1998).

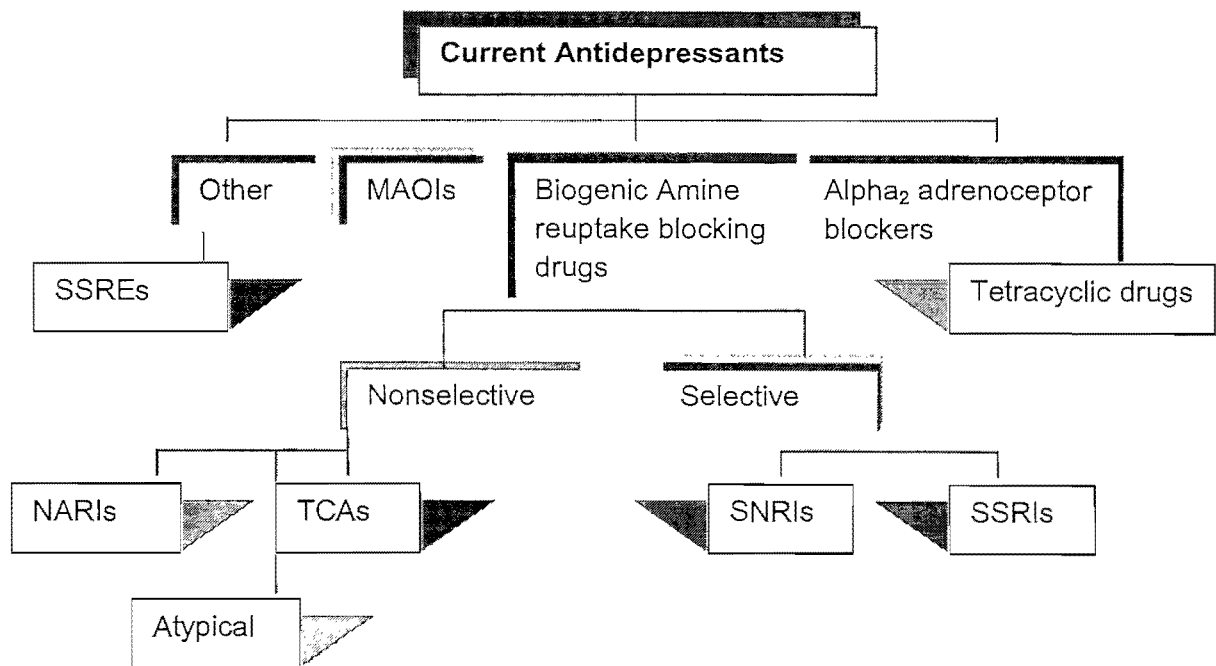


Figure 1: Classification of antidepressant drugs (Trevor *et al.*, 1998)

Abbreviations - MAOIs: Monoamine-oxidase inhibitors; SSREs: Selective serotonin reuptake enhancers; NARIs: Noradrenaline reuptake inhibitors; TCAs: Tricyclic antidepressants; SNRIs: Selective noradrenaline reuptake inhibitors; SSRIs: Selective serotonin reuptake inhibitors.

Despite the vast array of different agents available, current antidepressant drug-treatment does not satisfy all clinical needs. Deficits include: a delayed onset of action; ineffectiveness in refractory patients and those with treatment resistant depression; inadequate reduction of *cognitive* deficits caused by depression; weak

symptomatic pain treatment, as well as troublesome side effect profile (sexual dysfunction, gastrointestinal events, weight gain and cardiovascular side-effects) (Pacher & Kecskemeti, 2004; Rosenzweig-Lipson *et al.*, 2007).

Antidepressant drugs usually take two to three weeks to reach full clinical effect, even though they have acute presynaptic inhibiting actions on monoamine reuptake transporters or on the intracellular metabolizing enzyme MAO (Trevor *et al.*, 1998), (Trevor *et al.*, 1998; Freeman *et al.*, 2004). MDD patients from a study population that received antidepressant drug treatment, showed only 20% improvement in symptoms within 2 weeks (Stassen *et al.*, 1997). Suicidal ideation in individuals with MDD should disappear if the depression is effectively treated, but if the patient is critically suicidal, the medication may not work in time. The therapeutic effect of antidepressant drugs is presumably unassociated with the biochemical mode and site of action of the drugs within the monoaminergic system. A possible explanation for the lag in onset is that adaptive correctional changes in pre- and postsynaptic receptors (adrenoceptors, 5-HT, DA and GABA_B) first have to take place. These receptors regulate the physiological effect of neurotransmitters (Leonard, 2003).

Antidepressant drug therapy is usually ineffective in refractory patients and those with treatment resistant depression (Harvey, 1996). Approximately 80% of individuals will experience at least one more episode of MDD during their lifetime, after the initial episode (American Psychiatric Association, 2000). One in three patients will in all probability relapse within a year (Piccinelli & Wilkinson, 1994; Ramana *et al.*, 1995; Lin *et al.*, 2003). For some patients depressive episodes occur frequently followed by partial remission between episodes (Pintor *et al.*, 2003).

In addition to its more well known modes of action described above, chronic antidepressant therapy presumably also indirectly decreases excessive glucocorticoid and glutamate plasma concentrations in depressed patients (Nowak *et al.*, 1996; Maes *et al.*, 1998; Sapolsky, 2000). This activity at glutamatergic neurons, for instance is thought to be modified by TCAs (Bouron & Chatton, 1999) Excessive glutamate and glucocorticoids levels in the cortical-limbic system cause oxidative stress. Glutamate arbitrates regionally selective changes in the N-methyl-D-asparaginian acid (NMDA) receptor complex and regulates the NO/cGMP cascade (Nowak *et al.*, 1996) – see Figure 3. Given that glucocorticoid and glutamate are some of the main aggravators of the underlying *pathogeneses* of depression, and that they are only indirectly addressed by current antidepressants (Nowak *et al.*,

1996), an important question is *which main signal transduction pathway involved in mood regulation should be targeted by novel psychotropic drug activity?*

2.2 Classification of neurotransmitters involved in synaptic transmission

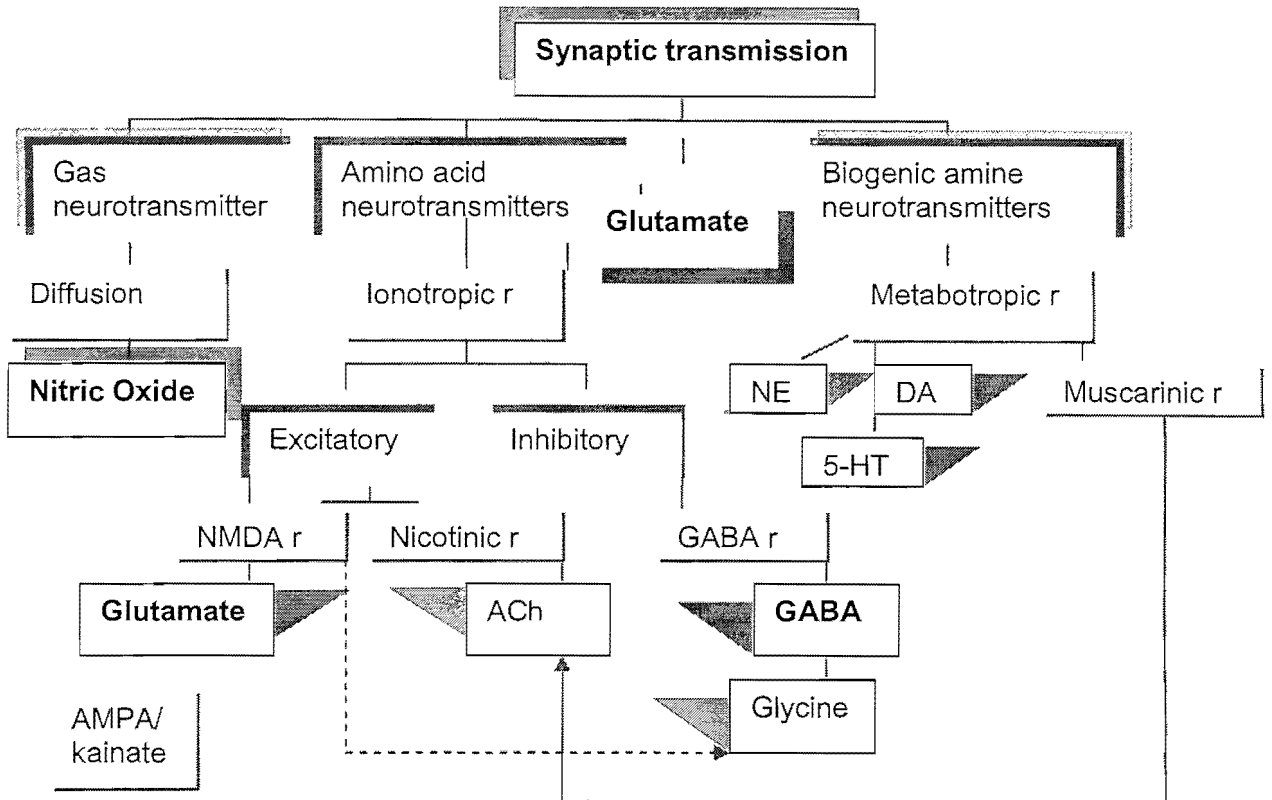


Figure 2: Classification of neurotransmitters according to receptor function (Leonard, 2003)

As can be seen in Figure 2, neurotransmitters can be divided into amino acid neurotransmitters, acting on *Ionotropic and metabotropic receptors*, *biogenic amine neurotransmitters* (Figure 2) acting on *metabotropic receptors* and the gas neurotransmitter, nitric oxide.

Without dismissing the role of the biogenic amine neurotransmitter pathways in the *aetiology* of MDD (section 2.2.1), ample recent evidence points to the involvement of amino acid neurotransmitters, particularly excitatory NMDA mediated glutamatergic and inhibitory GABA pathways in the *neuropathology* of MDD (Choudary *et al.*, 2005;

Harvey, 2006). Amino acid neurotransmitters either have *excitatory* or *inhibitory* actions on receptors. Repeated *excitatory postsynaptic potentials* (EPSP's) generate an *action potential* allowing nerve cells to carry a signal over a distance by releasing a neurotransmitter from the nerve terminal into the *synaptic cleft*. Glutamate is the main excitatory neurotransmitter, utilized by almost 60% of neurons in the brain (Nieuwenhuys, 1994; Squire, 2003; Javitt, 2004). It acts mainly on complex postsynaptic NMDA receptors to induce the down-stream release of NO and cGMP after α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors provide the primary depolarization needed to unblock NMDA receptors and to permit Ca^{2+} entry into the cell (Figure 3) (Javitt, 2004). Glutamate also acts on *metabotropic receptors* which assist to regulate glutamatergic neurotransmission both pre- and post-synaptically (Nieuwenhuys, 1994; Javitt, 2004).

ACh is a non-amino acid, excitatory neurotransmitter that acts on receptors of both the *ionotropic* nicotinic type and the *metabotropic* muscarinic type. Inhibitory *ionotropic* type amino acid neurotransmitters includes the primary transmitter GABA (GABA receptor action) and glycine (inhibitory, but acts on NMDA receptors). GABA inhibits signalling and subsequent neurotransmitter release. Presynaptic inhibition takes place via autoinhibitory Alpha_2 adrenoceptors (Figure 2) when there are high concentrations of neurotransmitters (GABA, NE, DA and 5-HT) in the *synaptic cleft* (Leonard, 2003). *Biogenic amine neurotransmitters* such as NE, DA and 5-HT (and the non-amine neurotransmitter ACh) normally act on *metabotropic* type receptors linked to intracellular second messenger systems.

Contrary to conventional neurotransmitters, gaseous transmitters such as NO are not stored in presynaptic vesicles or discharged by exocytosis. Rather NO is synthesized *de novo* and released immediately following up-stream glutamate signalling, whereafter it simply diffuses into adjacent nerve terminals (Vander *et al.*, 2001) to act as a *neuromodulator* and neurotransmitter. After synthesis, NO has an available half life of a few seconds before being rapidly inactivated by oxidation (Sun *et al.*, 2003; Jobgen *et al.*, 2007).

2.3 The role of the glutamatergic system and the cGMP/NO pathway in depression

2.3.1 History of NO in the nervous system

NO was always medically regarded as nothing more than a chemically reactive environmental pollutant. However, the delineation of its diverse physiological functions in the human body is one of the most intriguing tales of discovery in medicine (see table 6).

In 1803, John Dalton discovered that the reaction of nitric oxide with oxygen takes place, in the exact ratio and proportion: $\text{NO} + \text{O} \rightarrow \text{NO}_2$, while he was studying nitrogen oxides for Dr. Priestley's test for percentage of nitrogen in air (Moody & Bridges, 2007). This reaction ratio became very useful when; nearly two centuries later several researchers concluded that the so-called *endothelium-derived relaxing factor* (EDRF) was in fact NO (Ignarro *et al.*, 1987; Bredt & Snyder, 1989). This discovery by Ignarro, Furchgott and Murad in 1987 has revolutionized how this simple pleiotropic gas molecule is regarded in science and opened up a vast amount of possibilities for new research directions.

The neurotransmission properties of NO were discovered one year later, when John Garthwaite and colleagues recognized NO in the brain. They found that the excitatory messenger molecule glutamate acts on nerve cells to release a chemical similar to EDRF (Garthwaite *et al.*, 1988). This signal cascade with NO as intermediary product, in turn triggers nearby cells to release other classical neurotransmitters. NO is referred to as a *neuromodulator* (Harvey, 1996) by virtue of its ability to modify glutamate and other signal transduction systems (Prast and Philippu, 2001). Later research has shown that the NO producing enzyme, neuronal NOS is more abundant in the brain than in any other organ (Christopherson & Bredt, 1997; Wells, 2003), and particularly in brain regions involved in defensive behaviours (Krukoff and Khalilli, 1993), thereby suggesting an important role in anxiety and mood disorders. The role of NO in inflammation and immunity was also discovered during this time and represents another important area of intense research.

On December 10, 1998, Drs. L. Ignarro, F. Murad and R.F Furchgott won the Nobel Prize in medicine for their pioneering work on NO (Wright.P., 1996).

Table 6: Timeline narrating NO research progression from science to neuroscience, reproduced from (Wells, 2003)

Year	Discovery	Scientist
1628	It is established that blood circulates	William Harvey
1733	Blood pressure is measured	Stephen Hales
1803	Exact ration and proportion of NO reaction	John Dalton
1846	The explosive nitroglycerin is synthesized	Ascanio Sobrero
1854	First indirect blood pressure measurement is made	Karl von Vierordt
1879	Nitroglycerin as an angina treatment is established	William Murrell
1977	NO increases the activity of guanylyl cyclase and relaxes smooth muscles.	Ferid Murad
1979	NO is bubbled into a solution near an artery and gets a relaxation response	Louis Ignarro
1980	The endothelium releases a factor (EDRF) that relaxes blood vessels.	Robert Furchgott
1981	NO inhibits platelet aggregation and increases cGMP.	Louis Ignarro
1981	Mammals make nitrate	Steven Tannenbaum
1983	Blood vessel relaxation is associated with increased cGMP	Ferid Murad, and later others
1985	Inorganic nitrite and nitrate made by mouse macrophages is detected	Michael Marletta
1986	Speculation of Ignarro and Furchgott at a conference that EDRF is NO	Ignarro and Furchgott
1987	Independent publication of chemical evidence that EDRF is NO.	Salvador Moncada and Ignarro
1987	Arginine increases nitrite and nitrate formation in macrophages	John Hibbs and Michael Marletta
1988	NO is synthesized out of L-arginine	Salvador Moncada
1988	Detection of NO made by nerve cells	John Garthwaite
1991	bNOS and 1-NOS is cloned (purification of enzyme)	David Bredt and Solomon Snyder
1998	Receives Nobel Prize for Physiology or Medicine	Furchgott, Murad, and Ignarro

2.3.2 Characterization of neuronal NO

NO gas is a pleiotropic messenger molecule that acts as a regulatory neurotransmitter in the brain.

NO is a unique neurotransmitter in that its low molecular weight and hydrophobic properties allows it to effortlessly diffuse as much as 100-200 μm far from its place of origin to adjacent cells (Meulemans, 1994; Schuman & Madison, 1994; Schuman & Madison, 1994; Ledo *et al.*, 2004). This observation sparked off a theory that NO may be suitable for the mediation of *neuronal plasticity*, which underlies brain development and information storage processes like *long-term potentiation* (LTP) in the hippocampus (Christopherson & Brecht, 1997). In 1991, NO was labeled as a *retrograde messenger* after two independent research groups observed LTP after NO was synthesized at specific hippocampal subregion post-synaptic synapses (Schuman & Madison, 1991; O'Dell *et al.*, 1991; Arancio *et al.*, 1996; Ledo *et al.*, 2004). Evidence for NO's connection with LTP was also illustrated by the finding that NOS inhibitors prevent long-lasting changes in synaptic strength caused by repeated neuronal stimulation (Schuman & Madison, 1994; Boulton *et al.*, 1995; Christopherson & Brecht, 1997). The intracellular signalling pathways triggered by glutamate NMDA receptors manage development, gene expression, *senescence* and disease, in addition to LTP (Dawson & Dawson, 1996; Dawson & Dawson, 1998) and *neuromodulatory* actions (Dawson & Dawson, 1996), with NO playing a key role in these responses. NO also acts as the major activator of guanylyl cyclase leading to increased cGMP formation. cGMP in the brain plays a prominent role in modulating the release of certain neurotransmitters (Wegener *et al.*, 2003) critical for *affective* state, such as biogenic monoamines (NE, DA,) and glutamate, ACh (Garthwaite, 1991; Prast & Philippu, 1992; Hanbauer *et al.*, 1992; Hirsch *et al.*, 1993), GABA (Ohkuma *et al.*, 1995) and the presynaptic uptake of neurotransmitters like 5-HT (Miller & Hoffman, 1994).

NO is a very unstable free radical. It is rapidly oxidized to nitrite and/or nitrate in the presence of oxygenated haemoglobin (iron-containing protein), oxygen and superoxide (Helmke & Duncan, 2007). NO in fact, reacts swiftly with molecular oxygen, superoxide and iron-containing proteins, producing reactive intermediates that cause oxidative stress (Mayer *et al.*, 1995). The $t_{1/2}$ (half life) of NO in biological matrix ranges from less than one second in the presence of haemoglobin to approximately 30 seconds (Sun *et al.*, 2003; Jobgen *et al.*, 2007). This makes it

difficult to measure authentic NO as its short $t_{1/2}$ renders it inaccessible for accurate analytical measurement. The analysis of the oxidative metabolites (NOx) of nitric oxide, namely nitrite and nitrate represented a reliable method to assess NO levels.

2.3.3 Glutamate – NO/cGMP signal transduction pathway

This study revolves around the activation of the NO pathway which, in the CNS, represents an important down-stream messenger of the glutamate-NMDA receptor signalling cascade (Figure 3), and is of particular interest for the *pathogenesis* of MDD.

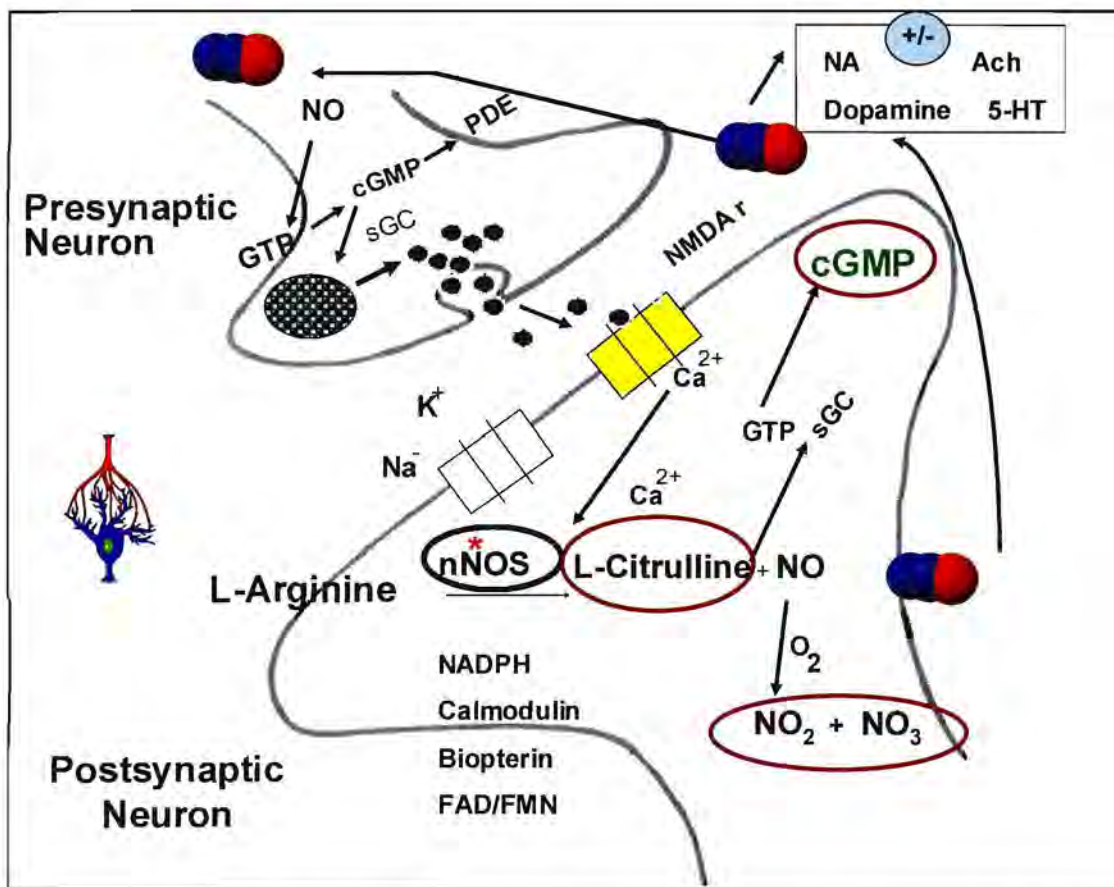


Figure 3: Glutamate – NO/cGMP signal transduction pathway in the mammal brain (Contestabile *et al.*, 2003; Ledo *et al.*, 2004)

a) *Glutamate induced signal transduction cascade*

The glutamate signaling cascade encompasses the following sequence of events through a transduction system that lasts as little as parts of a millisecond (illustrated in figure 3).

After its synthesis from glucose and glutamine in presynaptic neuron terminals, glutamate is released from its presynaptic effector vesicle (Belsham, 2001). Glutamate release occurs by the process of Ca^{2+} -triggered non-constitutive exocytosis. It docks at the presynaptic membrane, fuses with it - and glutamate is discharged into the *synaptic cleft* by a class II *metabotropic* autoreceptor (Squire, 2003). From there it diffuses across the cleft and a fraction acts on the binding moiety of postsynaptic NMDA receptors to culminate in an effect, Figure 4 (Vander *et al.*, 2001; Venter, 2003). The action of glutamate is terminated by reuptake into the presynaptic neuron or metabolism by glutamic acid decarboxylase (GAD) (Belsham, 2001).

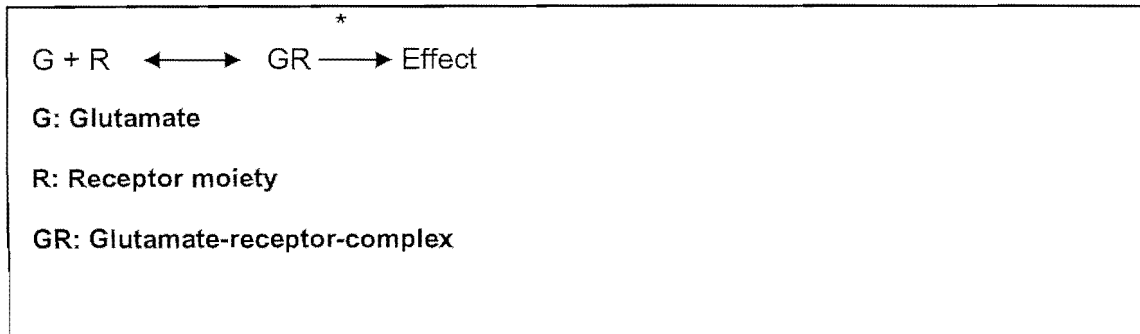


Figure 4: Binding of glutamate to the postsynaptic NMDA receptor moiety

b) NMDA receptor coupling and NOx, L-citrulline and cGMP formation

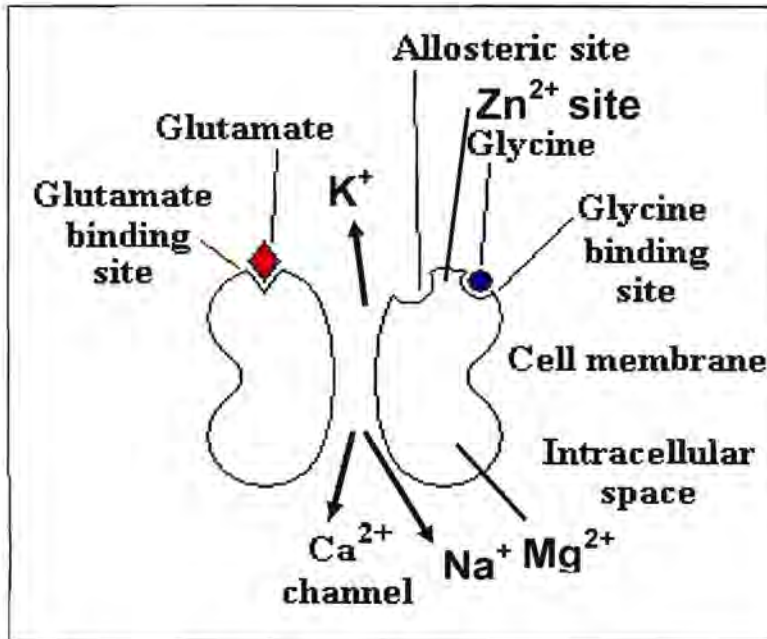


Figure 5: Activated NMDA receptor

The *voltage-gated ion channel* NMDA receptor is activated (Figure 5) following glutamate binding to the receptor moiety (receptor binding site), resulting in ion channel opening. This channel is nonselective for cations, this allowing the flow of Na^+ and small amounts of Ca^{2+} ions into the cell (effect of equation 1) and K^+ out of the cell (Ledo *et al.*, 2004). The Ca^{2+} influx into the postsynaptic neuron is the key regulator of nNOS activation. In the presence of calmodulin, cofactors and co-substrates, L-citrulline and NO are subsequently formed from the substrate, L-arginine (Bredt & Snyder, 1990). The released NO then activates soluble guanylate cyclase (sGC) to convert GTP into the second messenger, cyclic guanosine monophosphate (cGMP).

2.3.4 Biochemistry of nitric oxide: nitric oxide synthase and co-factors

In the brain, NO is formed from the guanidine nitrogen of L-arginine by the enzyme nNOS. This synthesis takes place through an oxidation process that consumes five electrons and results in the stoichiometric formation of L-citrulline together with previously mentioned NO metabolites (Figure 3) (Dawson & Snyder, 1994). For this reaction to optimally take place, several cofactors -tetrahydrobiopterin (BH_4), flavin adenine dinucleotide disodium salt hydrate (FAD) & riboflavin 5'-monophosphate sodium salt (FMN) must be present together with mandatory co-substrates

(molecular oxygen and β -nicotinamide adenine dinucleotide phosphate -NADPH), heme and calmodulin (CaM), which act as *prosthetic groups* (figure 6) (Gross & Levi, 1992; Pearce *et al.*, 1997; Ashwal *et al.*, 1999).

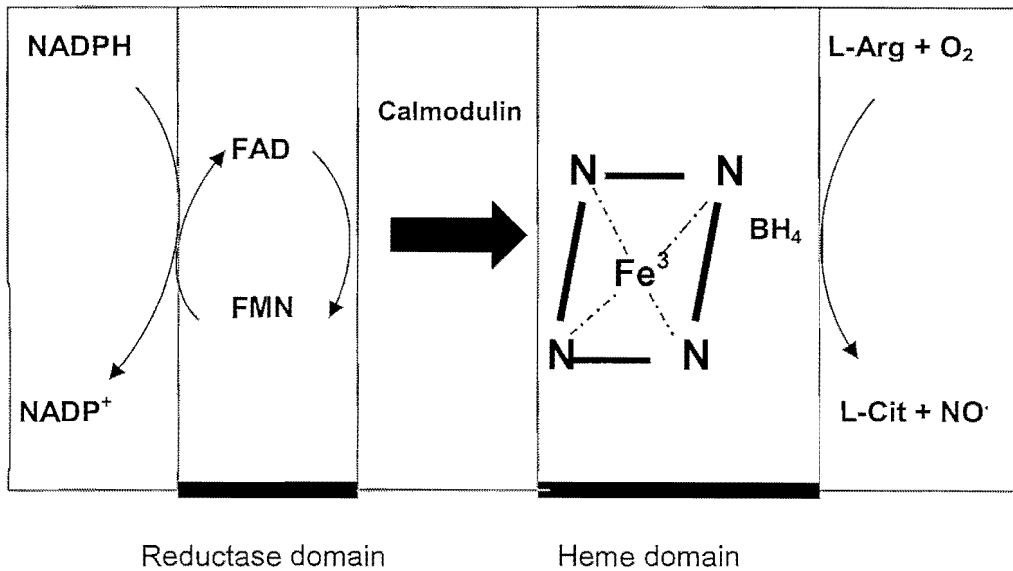


Figure 6: Illustration of NOS: the involvement of cofactors and co-substrates in the NOS catalyzed enzyme reaction (Marletta, 1993)

BH₄ is firmly regulated and can be rate-limiting for NOS activity if present in sub-effective quantities (Pearce *et al.*, 1997).

The function of BH₄ in the NOS reaction is still vague (Pfeiffer *et al.*, 1997). It may function as reactant (electron-transferring) cofactor (Dawson & Snyder, 1994). The allosteric effects of BH₄ binding results in great change in protein conformation. The mechanism of electron transfer probably occurs through a calmodulin-triggered (Klatt *et al.*, 1994) reduction of FAD by the co-substrate NADPH, which subsequently reduces FMN. FMN then transfers electrons to the ferric heme so that NOS can interact with molecular oxygen (Dawson & Snyder, 1994; Luzzi & Marletta, 2005)

Neuronal NOS also requires calcium for activity (Bredt & Snyder, 1989; Bredt & Snyder, 1990). An up-stream increase in intracellular calcium concentration through activation of the NMDA receptor is the key regulator of the whole reaction. Calmodulin mediates flavin to haem transfer as previously mentioned (Klatt *et al.*, 1994). Calmodulin also stimulates NOS in the absence of L-arginine, the substrate

for NOS (Stuehr *et al.*, 1995). Three isoforms of the enzyme nitric oxide synthase (NOS) have been identified (Bredt & Snyder, 1990). They are neuronal NOS (nNOS), endothelial NOS (eNOS) and immunologic NOS (iNOS), named after the tissue from which they were originally cloned (Dawson & Dawson, 1996). These three NOS isoenzymes are divided into two categories: 1) a constitutive Ca^{2+} - and calmodulin-dependent form (nNOS and eNOS) and 2) a cytokine-inducible form that does not require Ca^{2+} for its activation (iNOS) (Marletta, 1993; Knowles & Moncada, 1994; Contestabile *et al.*, 2003).

nNOS represents the majority of constitutively expressed NOS in rat brain (Dinerman *et al.*, 1994; Knowles & Moncada, 1994) and is the subject of investigation for NOS activity in this study.

2.3.5 NO-derived formation of cGMP

The most important NO receptor in the brain is sGC (Prast & Philippu, 1992). Guanylate cyclase is an enzyme catalyzer line for the conversion of GTP to cGMP (see Figure 3). Guanylate cyclase consists of both membrane-bound and soluble isoforms that are expressed in nearly all cell types, with sGC occurring mostly in the CNS (Dawson & Dawson, 1995; Domek-lopacinska & Strosznajder, 2005).

NO plays a dynamic role in endogenously activated sGC (de Vente *et al.*, 1998) and the subsequent formation of cGMP (Fedele *et al.*, 2001). Some of the most important actions of NO in the CNS are through the parallel formation of cGMP (Southam & Garthwaite, 1991; de Vente *et al.*, 1998) activating *G-kinase* with consequential effects on cyclic adenosine monophosphate (cAMP), ion channels, G-proteins, and neurotransmitter release (Prast & Philippu, 1992; Harvey, 1996). cGMP is hydrolyzed by the phosphodiesterase (PDE) family (Harvey, 1996; Ledo *et al.*, 2004) among which PDE 3 and PDE 5 are considered specific for cGMP metabolism (Soderling & Beavo, 2000). A further degradation occurs through sGC desensitization (negative feedback mechanism), of which the level is determined by cGMP (Domek-lopacinska & Strosznajder, 2005). These regulation mechanisms of cGMP metabolism appear to be different in various brain regions and are influenced by altered physiological and pathological conditions (Domek-lopacinska & Strosznajder, 2005).

The importance of the NO/cGMP pathway in the pathology and treatment of depression is becoming more evident (Harvey & Nel, 2003; Millan, 2006), particularly with respect to neuronal and brain function (Oosthuizen, 2003; Harvey *et al.*, 2004).

According to Domek-Lopacinska and Strosznajder, 2005, NO and cGMP are the main messengers in the glutamatergic system. It seems that cGMP is also a very important messenger for the cholinergic system, where it could be involved in cross-talk between cholinergic and other neurotransmitter mediated pathways (de Vente, 2004). Although it is unlikely that a single neurochemical on its own will prove to be the neurobiological basis for major depression (Belsham, 2001), this project will participate in helping to uncover the future role of NO and cGMP in major depression, and possibly contribute to novel treatment strategies. Indeed, the exact role of cGMP in many biological processes has not yet been clearly established. Nevertheless, its well-established role in neuronal signal transduction (Wang & Robinson, 1997) hints of an important yet poorly understood role in the *aetiology* and treatment of *affective* disorders.

2.3.6 Regulation of NO biosynthesis

Validation experiments conducted by Pearce *et al.*, 1997, suggested that a NOS-independent mechanism of NO production contributed negligibly to their measurements of NOS-activity. For this project, the biosynthesis of NO was therefore accepted to occur through the earlier discussed glutamate – NO/cGMP *signal transduction pathway*.

Although NO is synthesized on demand (Dawson & Dawson, 1996), its biosynthesis by nNOS is tightly regulated (Ledo *et al.*, 2004). Unlike classic neurotransmitters that are metabolized by either reuptake mechanisms or enzymatic degradation, NO's membrane permeability prevents the cell from controlling local concentrations of the molecule in the conventional manner. Instead, NO simply diffuses from nerve terminals and are converted to other forms through autoxidation (Dawson & Snyder, 1994). For NO synthesis to occur optimally, cofactor concentrations should be sufficient. However, the conversion reaction will not occur at all in the absence of co-substrates, heme or calmodulin. NO presumably induces feedback inhibition of NOS through its breakdown products, as was seen in experiments conducted by Mayer *et al.*, 1995 where NOS-induced citrulline formation was inhibited by *superoxide dismutase* (SOD). This observation makes it wise to use both NO_x and L-citrulline formation as an indication of NOS-activity.

As alluded to earlier, the influence of cofactor availability on nNOS is an important issue. Cofactor availability has been found to be a major determinant of NOS activity in post-ischaemic infarct regions (Ashwal *et al.*, 1999). Other studies found that NO-

induced sGC formation was inhibited by high BH₄ concentrations (Mayer *et al.*, 1995) and nNOS was inhibited by NO through its interaction with the heme prosthetic group (Griscavage *et al.*, 1994). Since evidence for increased (Wegener *et al.*, 2003; Spiacci *et al.*, 2007; Zhou *et al.*, 2007 & Wegener *et al.*, 2008) and decreased (Bernstein *et al.*, 1998; Srivastava, 2001) NOS activity and NO release has been implicated in major depression, additional studies on cofactor availability will assist in clarifying the nature and cause of NOS dysregulation described in the disorder.

Abovementioned cofactors contribute to NO synthesis, but the key control of nNOS activity and subsequent NO synthesis is believed to be a specific stimulus allowing Ca²⁺-influx into the cell in the presence of calmodulin (Bredt & Snyder, 1989; Dawson & Dawson, 1996; Dawson & Dawson, 1998). This stimulus occurs through activation of the NMDA receptor by glutamate. The availability of glutamate is therefore a crucial regulator of NO biosynthesis.

Other important NOS regulatory systems include postsynaptic density protein 95 (PSD95), protein inhibitor of nNOS (PIN) and carboxy-terminal PDZ ligand of nNOS (CAPON). PSD95 is a scaffold protein in the postsynaptic density required for the efficient coupling of neuronal NOS to the glutamate NMDA receptor (Sattler *et al.*, 1999). CAPON is a cytoplasmic protein that competes with PSD95 for binding to nNOS and as such interferes with NMDA receptor-NOS coupling (Jaffrey *et al.*, 1998), while PIN is a cytoskeletal transport protein that inhibits nNOS (Jaffrey *et al.*, 1996). The close regulation by these proteins of glutamate-NOS coupling is of particular importance since glutamate NMDA receptor changes have been described in depression (Nowak *et al.*, 1995; Nudmamud-Thanoi *et al.*, 2004), while antidepressant treatments illicit changes in the NMDA receptor ion channel (Skolnick, 1999; Harvey *et al.*, 2002; Paul & Skolnick, 2001; Stewart and Reid, 2002).

2.3.7 Nitric oxide: protector or terminator of neuronal allostasis?

Elevated plasma and platelet levels of glutamate have been detected in depressed patients (Mauri *et al.*, 1998), while glutamate has been found to be an important contributor towards various neurodegenerative phenomena (Choudary *et al.*, 2005). Indeed, recent evidence strongly suggests that major depression, especially prolonged severe depression, is associated with structural brain changes that may have a neurodegenerative basis. Preclinical studies on depression reported atrophy, death and a decrease in *neurogenesis* of stress-vulnerable neurons in the hippocampus and prefrontal cortex, presumably due to exposure to stress or

activation of the HPA axis and consequential increase of glucocorticoids (Duman *et al.*, 1999). Brain imaging studies found depressive patients to have a significantly smaller left hippocampal total and gray matter volume than healthy subjects (Sapolsky, 1996; Sheline *et al.*, 1996; Bremner *et al.*, 1999; Frodl *et al.*, 2002).

Glutamate- induced excessive NO formation may therefore promote oxidative stress through reactive nitrogenous species, free radicals and nitrosative stress and in this manner contribute towards the *pathogenesis* of depression. (McLeod *et al.*, 2001; Contestabile *et al.*, 2003; Zhou *et al.*, 2007). In fact, evidence has confirmed the presence of increased oxidative stress in patients with depression (Mayer *et al.*, 1995), while anti-oxidants such as N-acetyl cysteine have anti-depressant-like properties in *animal models* (Ferreira *et al.*, 2008). Depression presents with a lack of hippocampal *neurogenesis*, possibly due to inhibition thereof by nNOS-derived NO (Zhou *et al.*, 2007).

Ongoing stress is a known contributor to the development of depression (McLeod *et al.*, 2001; Bao *et al.*, 2008), while active structural degenerative changes in the hippocampus are induced by stress, as has been demonstrated by both pre-clinical and clinical studies (Tsankova *et al.*, 2006). Interestingly, these structural changes or the inhibition of *neurogenesis* are reversed by antidepressant drugs and NOS-inhibitors (Malberg & Schechter, 2005).

NO-related species can also be responsible for mitochondrial damage (Brown, 1999), DNA damage (Salgo *et al.*, 1995), neuronal death and inflammation (Bal-Price & Brown, 2001), all of which are known to contribute towards the overall biochemical pathology of major depression (Harvey, 2008).

a) *Inappropriate formation of NO: neurodegeneration*

The various reactive forms of NO, account for its chemical diversity and the wide variety of biological effects that are evoked by NO. The free-radical nature of the molecule makes it highly reactive with molecular oxygen, superoxide and iron-containing proteins (Mayer *et al.*, 1995). The neurodegenerative effects mediated by NO probably evolve from its interaction with oxygen to form reactive intermediates, such as peroxynitrite, that cause oxidative damage to DNA (Mayer *et al.*, 1995). It also reacts with iron-containing proteins/ enzymes, the most well-recognized being the stimulation of sGC upon binding of NO to its regulatory prosthetic heme group (Mayer *et al.*, 1995).

The inappropriate and excessive formation of NO, via reaction sequences described in Figure 6, thus leads to neurotoxicity that may be associated with MDD and a variety of other CNS disorders (Dawson & Dawson, 1996).

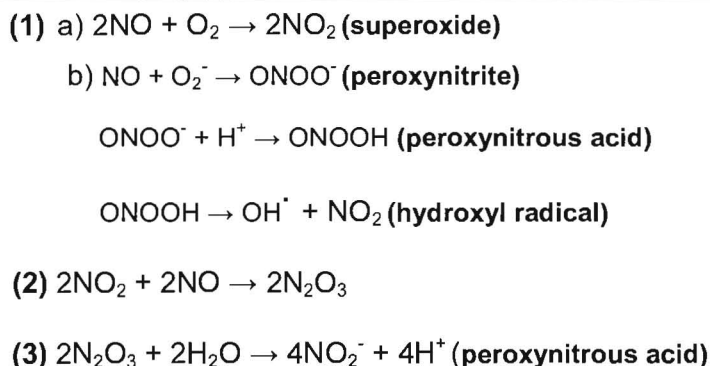


Figure 7: NO autoxidation reaction sequence derived from NO: oxygen stoichiometry (4:1) (Mayer *et al.*, 1995; Contestabile *et al.*, 2003)

Reaction with superoxide (1a) generates peroxynitrite (1b), the cause of oxidative damage to proteins, lipids, nucleic acids and key cellular organelles, such as mitochondria (Contestabile *et al.*, 2003). The enzyme *superoxide dismutase* (SOD) can also catalyze the dismutation of superoxide into oxygen and hydrogen peroxide (break-down products (Mayer *et al.*, 1995). NO also forms N_2O_3 in a two-step reaction (1 and 2). N_2O_3 and peroxynitrite is rapidly inactivated when reduced to the corresponding peroxynitrous acid (1b and 3), which produces a very neurotoxic and highly *reactive, oxygen species (ROS)*, the hydroxyl radical OH^\cdot . Finally, it is the decomposition of peroxynitrous acid through intermediate isomerization reactions that forms very neurotoxic and highly responsive *ROS* intermediates.

b) NO as neuroprotector

Controlled NO production on the other hand, mediates normal synaptic transmission because NO is a neuroregulator (Prast and Philippu, 2001; Wegener *et al.*, 2000; 2003). NO, ONOO^- and NO_2 contribute to oxidative damage, but may actually protect neurons by scavenging toxic free radicals in damaged brain (Farinelli *et al.*, 1996; Contestabile *et al.*, 2003). These neuroprotective effects of NO seem to depend on the stage of *neurogenesis* and the cell types producing NO.

Allostasis is defined as the ability to achieve stability through change (McEwen, 2003). The dual role of NO in the regulation of neuronal *allostasis* has been coined the 'NO-paradox' which allows it to function as either a protector or terminator of neuronal *allostasis*. However, the exact "borderline" between physiological and pathological actions of glutamate-derived NO still remains inconclusive.

2.4 Brain structures implicated in stress and depression

Two distinct brain areas, the hippocampus and frontal cortex will be investigated in this study (Figure 8).

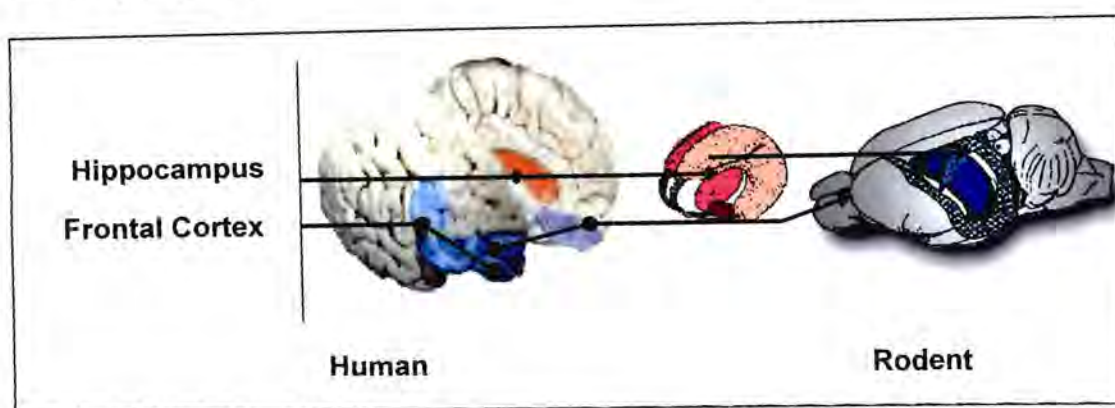


Figure 8: The hippocampus and the frontal cortex (part of limbic brain system) of the human and rodent brain

These two brain areas have been selected because of their prominent involvement in the symptoms and neuropathology of depression.

The frontal cortex is implicated in the *psychopathologic* and *cognitive* symptoms of depression, with the hippocampus more involved in the *somatic* symptoms of the illness (Table 5). Furthermore, the hippocampal and frontal cortical areas are believed to be a part of the LHPA-axis and are centrally located within the *limbic-cortical* network (McLeod *et al.*, 2001). Both of these structures are primary targets of stress hormones involved in the *pathogenesis* and *pathophysiology* of MDD (McLeod *et al.*, 2001; Frodl *et al.*, 2002; MacQueen *et al.*, 2003; Diamond *et al.*, 2004; Caetano *et al.*, 2004), making them appropriate anatomical areas for investigating the role NO in mood and antidepressant action. Moreover, NO plays a role in cortical perfusion, learning and memory as well as in *neuroplasticity*, which are all important

processes involved in depression (Contestabile *et al.*, 2003). The involvement of the LHPA-axis in depression will be discussed in the next section.

Certain other brain areas within the *limbic-cortical network* also relate to the symptoms of depression, including the amygdaloid complex and nucleus accumbens (Table 5). Although these areas will not be investigated in this study, they could be implicated in secondary changes triggered by the frontal cortex and hippocampus (Adell *et al.*, 2005).

The hippocampus is involved in long term learning and memory and neuroendocrine regulation of stress hormones (Sapolsky, 2001; Kim & Diamond, 2002). Recurrent depressive episodes cause cumulative hippocampal atrophy, which is normally reversible with treatment, but can be permanent in refractory depression (McEwen *et al.*, 1997; McEwen, 1999; Sapolsky, 2001; MacQueen *et al.*, 2003). To investigate whether hippocampal shrinkage precedes the first *major depressive episode*, rather than depression being the cause of this shrinkage, Frodl *et al.* (2002) conducted a study where the differences in hippocampal grey and white matter between healthy subjects and patients with a first depressive episode were compared. A significant decrease in hippocampal matter was found in depressed versus healthy subjects. The hippocampal shrinkage was also more pronounced in male subjects – relating to global information on the ratio of male to female suicide patients being nearly 4:1 (Bertelote, 2001). A study, comparing hippocampal volume and function from first *major depressive episode* patients to patients with several depressive episodes revealed a logarithmic relationship between disorder duration and hippocampal volume (MacQueen *et al.*, 2003). Persistent shrinkage of the hippocampus is also associated with reduced function, resulting not only in *cognitive* deficits, but also loss of neuroendocrine regulatory control and escalating cortisol-mediated hippocampal damage (Sapolsky, 1996). A vicious circle is thus imposed.

The frontal cortex is involved in *cognitive* functions (Duman *et al.*, 1999), such as decision making and experiencing *cognitive* emotions, particularly optimistic feelings (left frontal cortex) and pessimistic ones (right frontal cortex) (Dubac, 2002). The left frontal cortex possibly helps to inhibit negative emotions caused by hyperactive limbic structures present in depression. The latter is particularly prominent in depressed patients, so much so that the setting of goals and believing in oneself becomes very difficult (Dubac, 2002).

The top-down control of the hippocampus and other sub-cortical regions by the frontal cortex is important for mood regulation and the development of depression.

Three major circuits are involved in the top-down control over limbic responses, namely the fronto-amygdala, fronto-striatal, and fronto-hippocampal circuits. The *pathogenesis* of depression can be conceptualized as an “abnormality in emotional regulation due to a combination of pathogenic processes involving hyper responsive limbic structures to stress, and deficient fronto-limbic circuits that mediate the inhibitory control over limbic responses” (Dobson, 2008).

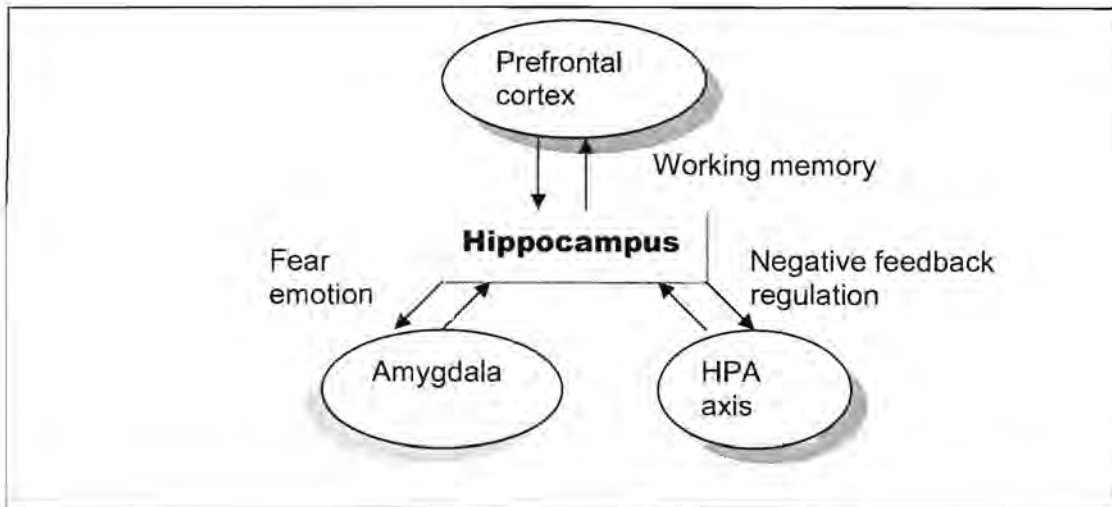


Figure 9: Frontal cortex and hippocampal circuits and depression (Dobson, 2002)

In the fronto-hippocampal circuit, the connection of the hippocampus with the prefrontal cortex and amygdala (Figure 9) are involved in context regulation of affect. Clinical studies have shown that hippocampal dysfunction may disrupt the negative feedback regulation of the HPA-axis (Jacobson & Sapolsky, 1991; Johnstone *et al.*, 2007), thus impairing the frontal-hippocampal circuit and leading to defective context coding of stress-responses, instigating generalized stress responses in inappropriate contexts (Dobson, 2002). Consequently, context inappropriate emotional responding may lead to a hyper responsive stress system – when sad affect persists beyond the relevant situation – and may eventually lead to depression (Davidson *et al.*, 2002). The persistence of deficits in executive functioning in remitted depressives have been related to abnormalities in hippocampal prefrontal circuits which may represent

'underlying *cognitive* vulnerability for depressive relapses' (Dobson, 2002; Clarke *et al.*, 2005).

2.5 Stress system (LHPA-axis) and depression

The limbic hypothalamic-pituitary-adrenal (LHPA) axis is a system of direct effects and feedback interactions between the hypothalamus, pituitary gland and adrenal glands. The hippocampal and prefrontal cortical areas are also believed to be part of this circuit and are consequently involved in both the stress response and depression (McLeod *et al.*, 2001), as was discussed in the previous section.

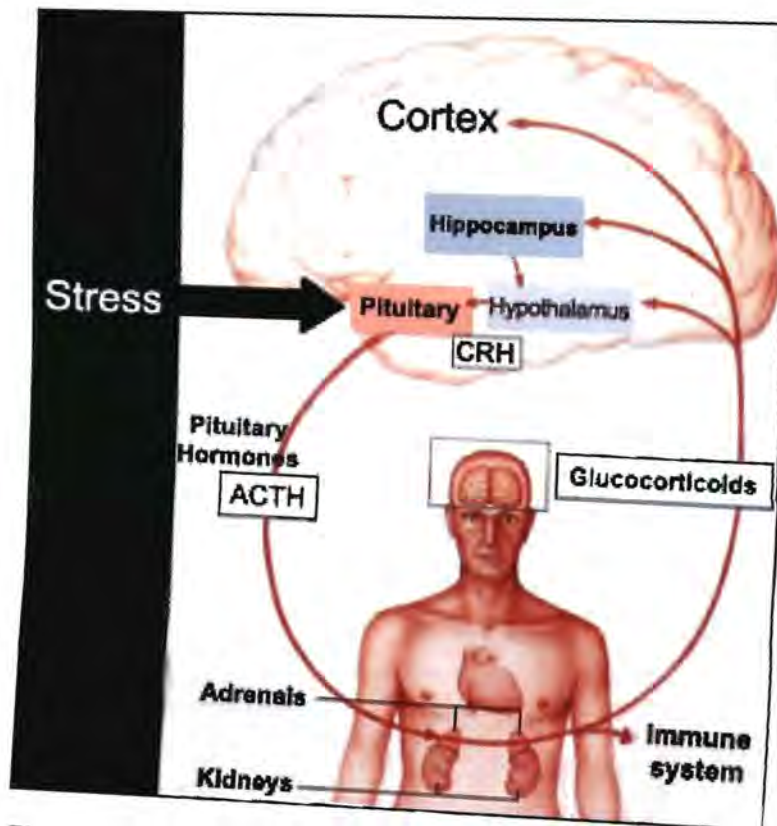


Figure 10: Stress, the HPA-axis and the the hippocampus (LHPA-axis) (Dubac, 2002)

Following exposure to a stressful stimulus, this negative experience is perceived by the cortex which then sends a signal to the hypothalamus to release corticotropin-releasing hormone (CRH). CRH then provokes the release of adrenocorticotrophic

hormone (ACTH) from the pituitary gland which, in turn, induces the release of corticosteroids from the adrenal glands. Released corticosteroids also exert a negative feedback on the hippocampus, pituitary and hypothalamus through mineralocorticoid and glucocorticoid receptors. The main glucocorticoid in humans is cortisol (Bear *et al.*, 2001; Swaab *et al.*, 2005; Bao *et al.*, 2008) while in the rodent, corticosterone is the primary glucocorticoid.

MDD presents with a hyperactive HPA-axis where CRH is excessively increased in MDD patients (Swaab *et al.*, 2005). Similar to NO, glucocorticoids also play a dual role in neuroprotection and neurodegeneration, particularly in the hippocampus (Sapolsky, 2000). Glucocorticoids are involved in restraining the neuronal responses initiated by the stressor, but in event of LHPA-axis dysfunction, excessive release of glucocorticoids as a result will target the hippocampus, with neurotoxic consequences (Sapolsky, 2000). Glucocorticoids are proposed to be the principle instigator of hippocampal volume loss following stress and in depressed patients, although the primary conveyor of these degenerative changes is corticosteroid-dependent glutamate release (Sapolsky, 2001).

Astrocytes constitute a major proportion of all brain cells and function to regulate the *homeostasis* of ions and amino acids in the extracellular milieu (Hansson & Ronnbock, 1995), particularly of glutamate and GABA (Hertz, 1979). Astroglial cells supply glycogen to the brain. Glycogen physiological functions as an energy metabolite during aglycaemia, but are also constantly utilized by the conscious brain (Brown *et al.*, 2004). The amount of glycogen is controlled by neurotransmitters having astrocyte receptors, as well as by hormones and local energy status (Brown *et al.*, 2004). Neurons and glia are also vitally important for the control of glutamate uptake following their release from neurons, although astroglia are the primary means of regulating this process (Hansson & Ronnbock, 1995). In the event of stress and depression, excessive release of glucocorticoids may impair glutamate uptake by affecting hippocampal astrocyte transport (Virgin *et al.*, 1991). An extracellular glutamate overload will activate NMDA receptors leading to a cascade of excitotoxic neurodamaging events, as discussed in section 2.4. Findings of Virgin and coworkers suggest that glucocorticoids compromise the ability of astrocytes to support hippocampal neurons in a stress situation by impairing their ability to clear the *synaptic cleft* of extracellular glutamate.

In addition to the multiple functions of NO in the CNS, glutamate-induced NO seems to also act as a modulator of the HPA-axis during stress. Indeed, the release of CRH, ACTH, oxytocin, luteinizing hormone and growth hormone are increased by NOS inhibitors (Virgin *et al.*, 1991; Boulton *et al.*, 1995; Giordano *et al.*, 1996; Dawson & Dawson, 1996; van Amsterdam & Opperhuizen, 1999).

2.6 Novel psychotropic drugs modulating the glutamatergic/ nitric oxide system in depression

A shift in treatment approach of MDD is expected to occur in the near future as researchers acquire more insight into the neurobiology of depression, and especially with regard to identifying new targets for antidepressant drug action. In this regard, the contribution of the glutamate – NO and cGMP pathways may realize great importance as a fundamental substrate underlying the *pathophysiology* of MDD.

However, any novel *psychotropic drug* must address the deficits that are evident in current antidepressant drug therapies. Some limited progress has been achieved, including novel drugs that achieve a possible faster onset of action, that are effective for resistant depression, that demonstrate improvement of cognition and that have a reduced side-effect profile (Rosenzweig-Lipson *et al.*, 2007). Examples of these advancements will be discussed briefly.

Faster onset

The combination of a NMDA antagonist and a SSRI improves drug efficacy (as demonstrated by *animal models* of depression) and accelerates increase in 5-HT levels (Layer *et al.*, 1995; Rogoz *et al.*, 2002; Owen & Whitton, 2005).

Augmentation in resistant depression

Consistent with evidence that the cholinergic system is hypersensitive in depression (Janowsky *et al.*, 1972) – clinical evidence has been shown that the antimuscarinic drug scopolamine, acts as an antidepressant and anxiolytic, producing fast and substantial effects in patients with resistant depression (Furey & Drevets, 2006). Furthermore scopolamine presents with a rapid onset of symptom relief (within 3 days after administration). Scopolamine has no serious adverse effects unlike treatments that have an effect within one week but have many undesired effects and sometimes needs to be taken over an extended time to remain beneficial

(electroconvulsive therapy, high-dose TCA drug administration, total sleep deprivation and the NMDAR-antagonist - ketamine use) (Gerner *et al.*, 1979; Segman *et al.*, 1995; Furrey & Drevets, 2006). Scopolamine's working-mechanism is still largely unknown, but it is known that, similar to other *somatic* antidepressant drug treatments, its action involves modulation of NMDAR function (Furrey & Drevets, 2006) through NMDAR gene expression regulation (by the muscarinic acetylcholine receptor). This modulation decreases glutamatergic transmission by reducing NMDAR function and thereby alleviates depression (Liu *et al.*, 2004).

Cognition

The formerly discussed brain structures investigated in this project (hippocampus and frontal cortex) are linked to *cognitive* disorders in MDD (Duman *et al.*, 1999; Sapolsky, 2001; Dubac, 2002; MacQueen *et al.*, 2003; Adell *et al.*, 2005). Antidepressant drug treatment increases hippocampal *neurogenesis* (Malberg & Schecter, 2005). If pathophysiological changes in these brain parts can be partly reversed by current antidepressant-induced *neurogenesis*, then antidepressant drugs that modulate these circuits possess so much more potential to enhance cognition (Rosenzweig-Lipson *et al.*, 2007). Tricyclic antidepressants impair cognition (Riedel & Van Praag, 1995), while SSRIs can impair vigilance (Schmitt *et al.*, 2002) but do not have direct effect on cognition except for allowing faster recovery of *cognitive* function in depressed patients on SSRIs relative to TCAs (Levkovitz *et al.*, 2002). Some novel drugs show potential effects on cognition, like memantine, a NMDAR antagonist, that has clinically been proven to have rapid symptomatological, *cognitive* effects and long-term neuroprotective effects (Parsons *et al.*, 2007).

Sexual dysfunction

Sexual dysfunction is not only a major symptom of depression (Kennedy *et al.*, 1999), but is also a complication of many antidepressant treatments (Nurnberg *et al.*, 2002). The cGMP-potentiating drug, sildenafil, has recently demonstrated efficacy in treating minor depression in people with combined erectile dysfunction (Nurnberg *et al.*, 2002). In such cases, sildenafil might be an alternative drug to conventional SSRI treatment. Sildenafil works to inhibit PDE5, which is responsible for degradation of cGMP release, thereby subsequently increasing levels of cGMP (Rosenzweig-Lipson *et al.*, 2007). A recent study in animals has also demonstrated that sildenafil does indeed have antidepressant-like properties, although only under conditions of

attenuated cholinergic tone (Brink et al, 2008). This study thus emphasises a mutual interaction between the cholinergic system and cGMP in the regulation of mood.

Various novel experimental drugs acting on glutamate signalling have been identified as having potential as therapeutic agents in the treatment of MDD (Trullas & Skolnick, 1990; Kelly *et al.*, 1997; Belsham, 2001; Linden & Schoepp, 2006). NMDA-antagonists, for example MK-801, are one such example (Mitani *et al.*, 2006). NMDA-agonists also have potential in that they reduce excessive glutamate, thereby restoring *allostasis*. Unfortunately their psychotogenic side-effects limit their clinical usefulness in humans (Javitt, 2004). Drugs acting as group-3 *metabotropic* glutamate receptor (mGluR) agonists also produce antidepressant effects in animals by decreasing glutamate release (Cryan *et al.*, 2003; Palucha *et al.*, 2004), although the mechanisms of action of these drugs are still unclear (Javitt, 2004).

Drugs that decrease NO availability should however, do so in a very controlled manner. Excessive NO production is neurotoxic and must be lowered. However, too rapid a drop is also detrimental, especially in lieu of the various isoforms of NOS that each contribute to the diverse functions of NO both in the CNS and in the periphery (see section 2.3.4). Ideal NOS inhibitors should therefore be selective for the nNOS isoform of NOS, so that side-effects in tissues associated with eNOS (blood pressure and organ blood perfusion) and iNOS (produced in macrophages –fights inflammation and autoimmune diseases) can be prevented (Contestabile *et al.*, 2003; Wells, 2003).

A number of pharmacological interventions in the NO/cGMP pathway could theoretically achieve a decrease in NO availability. However, L-arginine antagonists or the interference with vital cofactors/ co-substrates limit the potential to achieve selective NOS isoform inhibition (Contestabile *et al.*, 2003). If inhibitor drug molecules could be designed to interact only with catalytic sites that differ between isoforms, a more specific effect may be achieved. Remarkable success was achieved in the late 1990's when the dual highly selective inhibitor of nNOS and lipid peroxidation: *BN-80933* was designed (Marin *et al.*, 2000). This drug featured in the journal: "Drugs of the future" in 2006 (Van der Schyf *et al.*, 2006) as a neuroprotective drug that also remarkably reduces neuronal damage. BN 80933 might prove to be a potentially useful therapeutic agent for the treatment of stroke, trauma (Marin *et al.*, 2000) and possibly neurodegenerative disorders like MDD that involve both NO and ROS.

Unfortunately the greater amount of experimental drugs that have been tested up to date show low selectivity between NOS isoforms. Prospective NO-related antidepressant drug therapy will therefore likely be focused on achieving selective inhibition of NOS isoforms, the augmentation of drugs with agents like antioxidants to enhance cell proliferation (*neurogenesis*), or the direct or indirect targeting of neurogenic factors (Contestabile *et al.*, 2003; Malberg & Schecter, 2005).

2.7 Cross-talk mechanisms in depression

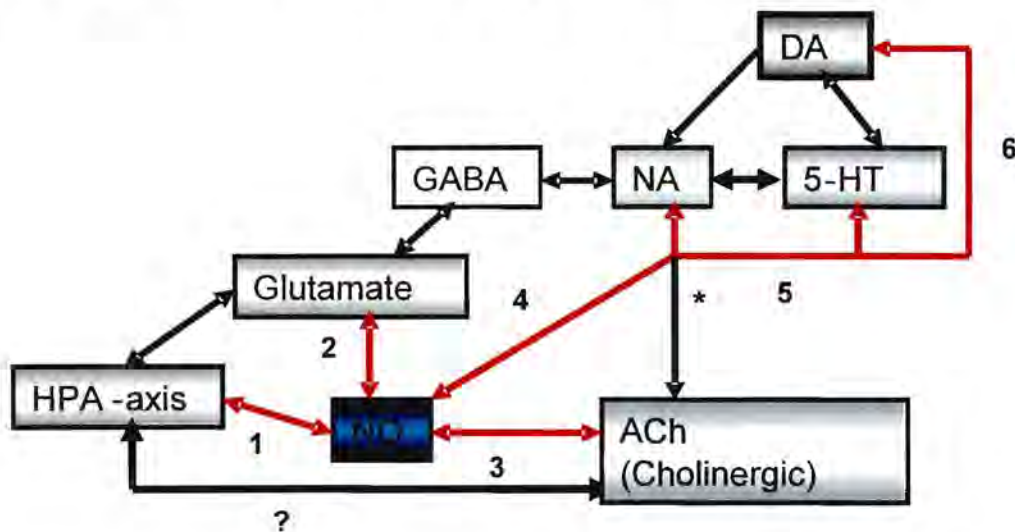


Figure 11: Interactions between NO and other neurotransmitter systems in major depressive disorder (*: see section 2.7.1)

NO has direct interactions with the HPA-axis (1), glutamate (2), the cholinergic system (3) as well as with conventional neurotransmitters (4, 5, 6: NA, 5-HT and DA), making it a very promising candidate for future drug development. In order to develop novel, more efficient antidepressant drugs, scientists must intervene at brain regions that are vulnerable to the *pathogenesis* of MDD, such as the hippocampus and frontal cortex (figure 1). Because of multi-transmitter involvement in depression, it is logical to search for a *neuromodulator*, such as NO, that plays a central role in cross-talk mechanisms between the various signalling systems purported to underly the *aetiology* and pathology of depression.

As illustrated in figure 11, NO directly interacts with various neurotransmitters and signalling systems that are well recognized to play a role in depression (figure 11, interactions 1-6). However, other meaningful interactions, such as the cholinergic-adrenergic interaction (2.7.1) also exist. Table 4 in section 2.7.2 gives a concise summary of other interactions - some of which have already been discussed in detail in previous sections. The interaction between the cholinergic system and the HPA-axis and the interaction between NO and the cholinergic system will be discussed in section 2.8 and particularly section 2.9. The latter will be considered in the light of investigating markers of the NO/cGMP pathway in the Flinders Sensitive and Flinders Resistant Line (FSL/FRL) rat, a genetic *animal model* of depression used in the current study that presents with hypercholinergic activity (Overstreet *et al.*, 2005). Research on the connection between NO and the cholinergic system in this particular rat strain will add important new information on the subject, contributing significantly towards new ideas for novel antidepressant drug development.

2.7.1 Cholinergic-adrenergic hypothesis of depression*

Janowsky and coworkers articulated the cholinergic-adrenergic *hypothesis* of depression in the early 1970's (Janowsky *et al.*, 1972). These early findings indicated that central cholinergic factors (like those seen in the depression-inducing effects of cholinomimetic drugs like physostigmine, and antidepressant effects of anticholinergic TCAs), can provide a framework contributing to the explanation of *affective* disorders (Janowsky *et al.*, 1972; Janowsky *et al.*, 1994). According to the cholinergic-adrenergic *hypothesis*, the *aetiology* of depression is partly derived from the "overactivity of cholinergic neurons relative to adrenergic neurons in specific brain regions" (Hasey, 1996). Depressed patients may therefore present with supersensitive muscarinic acetylcholine receptors (Leonard, 2003). Interestingly, it is the anticholinergic effects of the TCAs that made them unpopular (because of peripheral side-effects) and which led to the later development of second generation antidepressants that lack anticholinergic side-effects, yet still have a high efficacy. Nevertheless, many investigators are of the opinion that TCAs could have superior effectivity above newer agents in cases of severe depression or resistant depression (Leonard, 2003), arguing that their additional anticholinergic properties allow for a greater efficacy in more severe intractable cases of depression.

Since an imbalance between central noradrenergic and cholinergic systems could account for depression (Leonard, 2003), aside from the direct anticholinergic effects

of the TCAs, the anticholinergic effect of antidepressants is also possibly an indirect effect arising from an elevation in central noradrenergic activity (Hasey, 1996).

2.7.2 Other cross-talk mechanisms

Table 7 lists other neuronal cross-talk mechanisms, besides those discussed in section 2.7.1.

Table 7: Cross-talk mechanisms between neurotransmitters

Neuronal cross-talk mechanisms	References
Glutamate and GABA	
GABA pathways curb excessive glutamatergic release via presynaptic GABA _B heteroreceptors.	(Yamada <i>et al.</i> , 1999; Belsham, 2001; Hemrick-Luecke & Evans, 2002; Choudary <i>et al.</i> , 2005; Linden & Schoepp, 2006; Harvey, 2008)
HPA-axis and glutamate	
Excessive release of glucocorticoids may impair glutamate uptake.	(Virgin <i>et al.</i> , 1991; Hauger <i>et al.</i> , 2006)
GABA and NA	
Involvement of GABA _B receptors in ↑ NA release in the cortex. ↓ in GABA _B receptor activity can thus add to the ↓ CNS NA levels in depressed patients.	(Leonard, 2003; Braga <i>et al.</i> , 2004).
DA, NA + 5-HT and NA	
DA is hydroxylated to NA inside the synaptic vesicle. Contradicting results regarding NA levels in MDD changed the early catecholamine <i>hypothesis</i> (proposing a deficiency of NA in depression) – to simply propose that a dysregulation of NA exists in depression. The catecholamine <i>hypothesis</i> similarly stated an increase in 5-HT neurotransmitters. Both NA and the 5-HT systems are involved in the <i>aetiology</i> of depression and the actions of antidepressant drugs. Combinations of antidepressant drugs that affect both, are more effective and faster acting than monotherapy. This suggests a combined, side-by-side dysfunction of the NA-5-HT system in MDD. Various possible types of interactions between these two systems have been confirmed by extensive behavioral and neurochemical evidence.	(Schildkraut, 1965; Mongeau <i>et al.</i> , 1997; Trevor <i>et al.</i> , 1998)

2.8 Pathogenesis of depression

Cumulative anatomical and functional anomalies occur through stepwise neurodegenerative changes that take place throughout the development and manifestation of MDD (Frodl *et al.*, 2002; MacQueen *et al.*, 2003). Recurrent depressive episodes increasingly aggravate hippocampal atrophy, especially in refractory depression (McEwen *et al.*, 1997; McEwen, 1999; Sapolsky, 2001; MacQueen *et al.*, 2003). Patients suddenly stopping therapy experience increased hippocampal shrinkage and are more difficult to treat with commencement of therapy (Fava *et al.*, 2006). Hippocampal shrinkage generally occurs due to various underlying factors responsible for the neuropathology of MDD, particularly relating to disturbances in the glutamate NMDA signalling cascade and NO release (McEwen, 1999; Harvey *et al.*, 2003).

Allostasis is achieved through normal adaptation of the body to cope with potentially adverse events and to improve the response to a later similar stressor. Mediators of *allostasis* have short-term protective effects, but if the body is unable to restore baseline levels, these chronic disturbances increase *allostatic load* and lead to the development of pathologies characteristic of MDD (MacQueen *et al.*, 2003; McEwen, 2008). Mediators of *allostasis* include glucocorticoids, excitatory amino acids, 5-HT and certain endogenous factors (McEwen *et al.*, 1997; McEwen, 1999; McEwen, 2008). Plausible mechanisms whereby underlying factors cause allostatic disturbances revolve around the fact that major depression is ultimately a stress-related disorder (Sapolsky, 2001). Ongoing and unresolved stress inhibits *neurogenesis*, decreases cell proliferation (Kempermann, 2002), causes shrinkage of neuronal networks and induces neurotoxicity by the up-regulation of excitatory pathways that mediate *synaptic plasticity* (Sapolsky, 2001). Glucocorticoids are the most likely instigators of the degenerative processes that affect the hippocampus (Sapolsky, 2001).

Despite their destructive role in depressive illness, glucocorticoids in the hippocampus help to contain neuronal responses initiated by the stressor, through glucocorticoid-mediated negative feedback that inhibits the HPA-axis (McEwen & Sapolsky, 1995). While the excitatory amino acid, glutamate, mediates many of the effects of glucocorticoid actions in the brain, such as inducing the release of NO and cGMP (Javitt, 2004), inhibitory GABA activity is responsible for protecting allostatic processes by presynaptically inhibiting excessive glutamatergic transmission during

stress (Hemrick-Luecke & Evans, 2002; Harvey, 2006) via GABA_B heteroreceptors (Yamada *et al.*, 1999). NO, a fast-acting *neuromodulator* (Dawson & Dawson, 1996) is involved in the mediation of neuronal plasticity through inducing cGMP release (Schuman & Madison, 1991; O'Dell *et al.*, 1991; Arancio *et al.*, 1996; Christopherson & Bredt, 1997; Ledo *et al.*, 2004), which modulates the release of various conventional neurotransmitters involved in mood regulation, such as 5HT, NA, ACh, GABA and glutamate (Garthwaite, 1991; Prast & Philippu, 1992; Hanbauer *et al.*, 1992; Ohkuma *et al.*, 1995; Wegener *et al.*, 2003).

During MDD, a dysfunctional LHPA-axis leads to an increase in glucocorticoid release that targets the hippocampus, eventually inflicting damage (Sapolsky, 2000; see section 2.5). Stress also compromises the GABAergic system (Harvey *et al.*, 2004), while reduced GABA levels are often reported in depressed patients (Petty, 1995; Choudary *et al.*, 2005) resulting in an imbalance between inhibitory GABA and excitatory glutamate pathways (Linden & Schoepp, 2006). Presumably most forms of glutamate neurotoxicity are initiated by the influx of intracellular Ca²⁺ after NMDA receptor activation. Elevated plasma and platelet levels of excitatory neurotransmitters, particularly glutamate have been detected in depressed patients and are possibly causally related to the shrinkage of the hippocampus (Dawson & Dawson, 1996; Mauri *et al.*, 1998; Maes *et al.*, 1998). Analysis of cerebral tissue from deceased depressed patients have demonstrated a down-regulation of glutamate/neuronal amino acid transporter protein as well as a reduction in expression of the enzyme that converts glutamate to its non-toxic form glutamine (Choudary *et al.*, 2005). Increased glutamate leads to an inappropriate excessive formation of NO, causing neurodegeneration due to oxidative stress from the formation of ROS.

However, it should be appreciated that all the abovementioned factors form an interconnected series of pathological events that together lead to the disruption of normal allostatic neuronal processes. Clearly, treating depression involves a multi-targeted approach for optimal results.

2.9 The Flinders Sensitive Line rat

2.9.1 The Flinders Sensitive Line rat as genetic *animal model* of depression

Animal models of depression are regularly used as screening tests for the discovery and testing of novel antidepressant drugs regarding their neuropharmacological

mechanisms and effects, and also as simulations for investigating the *pathogenesis* of depression (Willner & Mitchell, 2002). Rats have been the principal subject for behavioural pharmacology studies as well as for the investigation of brain chemistry, anatomy and physiology in depressive illness (Whishaw & Kolb, 2005). Studying rat brain also has some advantages above studying post-mortem brain tissue from suicide victims (Leonard, 2003), as outlined below:

In suicide victims:

- a) depression could be falsely diagnosed,
- b) autopsy usually takes place several hours post-mortem and the time between different autopsies varies,
- c) the manner of suicide can affect central monoamine neurotransmitter function, for example in case of death by an overdose with a combination of alcohol and drugs.

These variables have to be carefully controlled for valid results, which is unnecessary when brain from a well-validated *animal model* is used for analysis.

Genetic *animal models* are a means to acquire valuable insights into the neuropathology of depression so that new treatment strategies may be devised. Using a genetic rodent model of depression usually focus on monitoring symptoms, neurochemistry and behaviour of rats that were genetically altered to partially resemble depressed individuals. However, when considering depression, two symptoms of depression in humans, namely recurrent thoughts of death and prevailing feelings of guilt, are impossible to model in phylo-genetically lower species. The unassailable question: "can we ever know whether a laboratory animal is depressed?"(Cryan *et al.*, 2002), repeatedly resurfaces. The other popular question is: to what extent can studies done on a rat model of depression be extrapolated to depressed human individuals? Conventional animal studies of most mental diseases, including depression are based solely on the direct relationship between the clinical efficacy of known antidepressants and their behavioural effect on a pharmacologically-, or stress-induced test model of depression. Correlation of these pharmacological tests of antidepressants with the existing neuropathology of major depression is not possible (Porsolt *et al.*, 1978). Tests can do nothing to reveal new insight into the disorder that should ideally lead to new drug discovery (Porsolt *et al.*,

1978). For that, a genetic *animal model* of depression, like the Flinders Sensitive Line (FSL) rat and its control, the Flinders Resistant Line (FRL) rat could prove to be extremely valuable (Zangen *et al.*, 1999).

The Sensitive strain was selectively bred to be genetically hypercholinergic by being more sensitive to an organophosphate: diisopropyl fluorophosphate (DFP), in contrast with its normocholinergic control, the FRL rat (Overstreet *et al.*, 2005). The FSL rat model is based on the *hypothesis* that central cholinergic systems are involved in depression. The possibility that the FSL rat might be an *animal model* of depression was raised when investigators reported that depressed individuals were more sensitive to cholinergic agonists (Davidson, 1972). However, the association between the FSL rat and depression goes further than just its increased cholinergic activity, as will be highlighted below.

FSL rats are more susceptible to stress-induced behavioural disturbances (Overstreet, 2002; Overstreet *et al.*, 2005). A more accurate description of the FSL rat is therefore probably that it is an *animal model* with a 'predisposition to contract depression' or depressive *diathesis*. Its depressive-like behaviour is often revealed only when it is subjected to a precipitating stressor, like subacute chronic stress or chronic mild stress (CMS) (Overstreet, 2002; Willner & Mitchell, 2002).

Validity of the *animal model* refers to the degree of use it has for the purpose for which it has been selected (Geyer & Markow, 2000). Validity criteria for an *animal model* can be divided into an independent variable and a dependent measure. The independent variable is the manipulation used to induce the abnormality. Not having to 'create' a model makes genetic animal studies superior to and simpler than conventional *animal models*. Only the second measure, the dependent measure, i.e. the measure used to assess the behavioural and neurochemical effects of genetic manipulation, has been used to evaluate this genetic *animal model*. The following dimensions are usually addressed when evaluating the validity of *animal models* of depression (Table 8):

Table 8: Dependant validity criteria (Geyer & Markow, 2000; Willner & Mitchell, 2002)

Dependent measure	Description
Construct validity	For theoretical rationale evaluation, theories about biopsychological mechanisms of MDD itself must correlate with the biopsychological theory of behavioural of the FSL rat (<i>animal model</i>). The construct validity is however limited by the undeveloped state of theories of the pathology of depression as displayed by controversial findings of monoamine levels in depressive patients.
Face validity	How well the behaviour of the <i>animal model</i> complies with the symptoms of MDD.
Predictive validity	The predictive validity of <i>animal models</i> is usually determined by their response to antidepressant drugs. Also, capability of predicting what the application of behavioural parameter outcomes or differences in neurotransmitters between the test (FSL) and control (FRL) rats, on humans will be.
Reliability	Consistency and stability of observations: <ul style="list-style-type: none"> a. objective measurements, b. reasonably small inter- and intra-sample variations (sometimes acceptable if large variation occur – forming part of the study), c. reproducibility of the behavioural and neurochemical effect of the genetic engineering.

Regarding specific depressive *symptomatology*, the FSL rat partially resembles depressed individuals because it exhibits reduced appetite and psychomotor function but displays normal hedonic responses and *cognitive* function. Certain symptoms/ observations of depressed individuals compare to behavioural measures in FSL rats. These include reduced appetite, altered sleep patterns and associated variables, like immune abnormalities, asthma and anxiety (Overstreet *et al.*, 2005). These symptoms or variables will be outlined in Table 9. The two most prominent similarities between the FSL rat and depressed individuals are altered sleep patterns and

immune abnormalities. The FSL rat displayed a reduced dormancy to and greater amount of REM sleep than the FRL rat (similar to depressed individuals) (Benca *et al.*, 1996). Concerning immunological deficits in FSL rats, they are similar to those in depressed humans, although it may be limited to type I responses (Friedman *et al.*, submitted for publication). The behavioural despair exhibited by the rats can be effectively countered by antidepressants, thereby conferring predictive validity on the model. In fact, the FSL rat model has been very useful as a screen for antidepressants because known antidepressants reduced swim test immobility when given chronically and psychomotor stimulants did not (Overstreet, 2002).

Neurochemically, the FSL rat presents with a hyper-responsive cholinergic system which is consistent with the cholinergic model of depression. Despite the increased cholinergic drive in the FSL rat and its hypersensitivity to cholinergic agonists (Overstreet, 2002) – which is akin to the hypersensitivity of depressed individuals to cholinergic agonists (Janowsky *et al.*, 1994), anticholinergic drugs exhibit no antidepressant effects in the FSL rat or depressed human (Schiller *et al.*, 1992). It remains to be determined which, if any, of the neurochemical changes in the FSL rat are related to the behavioral alterations that they exhibit.

Table 9: Similarities among depressed individuals and FSL rats (Overstreet *et al.*, 1998, 2005)

Characteristic	Depressed individuals	FSL rats
A. Neurochemical		
<i>Increased cholinergic sensitivity</i>	Yes	Yes
<i>Increased 5-HT_{1A}-R sensitivity</i>	No	Yes
B. Core symptoms		
<i>Decreased locomotor activity</i>	Yes	Yes
<i>Suicidal ideation/ attempt</i>	Yes	No
Increased REM sleep	Yes	Yes
High sweet intake craving	Yes	Yes
Reduced appetite/ loss of weight	Yes	Yes
<i>Immobility after stress</i>	Yes	Yes
Antidepressant response	Yes	Yes
C. Associated variables		
Immune abnormalities	Yes	Yes
Higher incidence of asthma/ greater airway sensitivity to antigen	Yes	Yes
Anxiety in some types/ tasks	Yes	Yes

2.9.2 NO in the Flinders Sensitive Line rat

As can be deduced from the foregoing sections, the FSL rat is a useful model to test selected novel *hypotheses* of depression. Indeed, various theoretical models and associated hypotheses of depression have been confirmed in these rats (Table 10). As was illustrated in Table 4, the role for ACh and GABA in the *aetiology* of depression, as well as a possible interaction between NO and the cholinergic system in depression, have received much less attention than neurotransmitters involved in the classical hypotheses (Table 1).

Table 10: Neurochemical features of the FSL rat

Theoretical model	Associated hypothesis	Confirmation in FSL rat	Depression in humans
Cholinergic model	-	Hypersensitive to cholinergic agonists (Overstreet, 2002) Increases in muscarinic binding in the hippocampus and other brain parts (Daws & Overstreet, 1999).	Hypersensitive to cholinergic agonists (Janowsky <i>et al.</i> , 1994), although there was no difference in muscarinic acetylcholine receptors (Meyerson <i>et al.</i> , 1982; Kaufmann <i>et al.</i> , 1984) Centrally active cholinomimetic drugs induce depressed moods in patients (Nurnberger <i>et al.</i> , 1983; Janowsky <i>et al.</i> , 1974; 1995).
		Anticholinergic agonist exhibits no antidepressant effects (Schiller <i>et al.</i> , 1992).	
Serotonergic model	Biogenic amine hypothesis	↑ 5-HT _{1A} -R sensitivity (Wallis <i>et al.</i> , 1988; Janowsky <i>et al.</i> , 1994; Zangen <i>et al.</i> , 1999). More sensitive to the hypothermic effects of 8-OH-DPAT, a 5-HT _{1A} -R agonist (Overstreet, 2002)	Inconsistent results ↑5-HT _{1A} -R sensitivity ↓ 5-HT-R sensitivity Most antidepressants affect 5-HT-Rs/ NE-Rs. Less sensitive to hypothermic effects of similar agents (Lesch, 1991)
Noradrenergic model	Catecholamine hypothesis	No change in sensitivity—some research report ↑ DA and NE levels – normalized with antidepressant therapy (Serova <i>et al.</i> , 1998; Zangen <i>et al.</i> , 1999; Overstreet <i>et al.</i> , 2005)	Altered sensitivity (Leonard, 2003)
Dopaminergic model	Catecholamine hypothesis	Reduced DA transporter (Overstreet <i>et al.</i> , 2005)	Reduced DA transporter (Leonard, 2003)
GABAergic model	-	↑ GABA _A receptor concentration and sensitivity (Pepe, 1998)	↓ levels GABA in the cortex, cerebrospinal fluid (Gerner <i>et al.</i> , 1984) and plasma (Petty, 1994; Sanacora <i>et al.</i> , 1999; 2004)

Theoretical model	Associated hypothesis	Confirmation in FSL rat	Depression in humans
HPA-axis	-	<ul style="list-style-type: none"> • HPA-axis not hyperactive when no stress - <p>Normal to diminished corticosterone (Owens <i>et al.</i>, 1991; Ayensu <i>et al.</i>, 1995)</p> <ul style="list-style-type: none"> • Administration of arecoline → ↑ corticosterone release (FRL rats also presents with an ↑)(Overstreet <i>et al.</i>, 1986) 	<ul style="list-style-type: none"> • HPA axis hyperactive <p>↑ cortisol (Swaab <i>et al.</i>, 2005; Bao <i>et al.</i>, 2008a)</p> <ul style="list-style-type: none"> • Administration of cholinergic agonist → ↑ in cortisol release (Perlis <i>et al.</i>, 2002)

a) Cholinergic model

The FSL rat strain (Overstreet *et al.*, 1986) and patients suffering from MDD (Perlis *et al.*, 2002) are hypersensitive to cholinergic agonists (Ayensu *et al.*, 1995). However, the FSL rat does not comply with the cholinergic-adrenergic *hypothesis* proposed by Janowsky *et al.* (1972) in that the rat line is only a cholinergic model. As earlier mentioned in the “cholinergic-adrenergic *hypothesis* of depression” (Section 2.7.1), the anticholinergic effect of antidepressants is possibly an indirect effect arising because most antidepressant drugs elevate central noradrenergic activity (Leonard, 2003). In the FSL rat, NE levels do not deviate from FRL rats’ levels.

Moreover, increased cholinergic drive doesn’t seem to correlate with the key behavioural characteristics of the FSL rat since treatment that alter cholinergic receptor function do not affect swim time in these animals (Schiller *et al.*, 1992). Even though the FSL rat line was selectively bred to be hypercholinergic, other neurochemical mechanisms possibly underlie the depressed-like behaviour of these rats. (Overstreet *et al.*, 2005). The model may however possess some predictive validity, at least on this level, if the fact that anticholinergic drugs also do not act as antidepressants in humans is taken into account. This shows some degree of overlap with regard to cholinergic disturbances in depression and the FSL rat.

A possible co-operative relationship between the NO/cGMP and cholinergic system in depression was suggested by recent studies in our laboratory (Brink et al, 2004, 2008).

The nitric oxide-cGMP pathway is implicated in the neurobiology of depression, as evidenced by the antidepressant-like response of the phosphodiesterase type 5 (PDE5) inhibitor sildenafil. Sildenafil was found to bolster the mAChR signalling capacity of neuroblastoma cells – implicating an antidepressive effect (Brink *et al.*, 2008) that is only observed, after attenuation of central muscarinic cholinergic activity (Brink *et al.*, 2008) – achieved during the combination of sildenafil with the administration of the anticholinergic drug atropine in the FSL rat. The sildenafil bolsters neurotransmission (through increase in cGMP), while its elevating effect on the cholinergic system is likely cancelled out by atropine – yielding an antidepressive netto-effect (Liebenberg *et al.*, Submitted for publication).

b) The FSL rat, NO and the HPA – axis model

The FSL rat is an *animal model* with a “predisposition to depression” for its depressive-like behaviour is often revealed only when it is subjected to a stressor (Overstreet, 2002). In contrast with depressed humans though, the HPA axis is not hyperactive. In fact, the FSL rat displayed reduced CRF levels in the median eminence and reduced serum levels of ACTH (Overstreet *et al.*, 2005), but serum corticosterone (cortisol in humans) showed no difference between FSL and FRL rats in studies conducted under nonstressful conditions (Ayensu *et al.*, 1995).

According to Ayensu and coworkers, these findings suggest that cholinergic mechanisms do not play a vital role in adrenal response to chronic unpredictable stress in the FSL rat and that the FSL rat – HPA-axis is even diminished under nonstressful conditions, although the axis has a similar response to the administration of acute cholinergic agonists as in depressed patients (Owens *et al.*, 1991; Ayensu *et al.*, 1995).

These animals present with an increase in corticosterone levels (Overstreet *et al.*, 1986) following the administration of acute cholinergic agonist, arecoline, and that is similar in response to that observed in depressed patients following the administration of cholinergic agonists, i.e. an increase in cortisol levels (Perlis *et al.*, 2002). Furthermore, hypercholinergic FSL and normocholinergic FRL rats display no significant difference in hormonal response to chronic mild stress. On the whole,

however, a significant elevation in corticosterone levels were noted in stressed FSL and in stressed FRL groups compared to stress-naive controls (Ayensu *et al.*, 1995). These findings show that the HPA-axis is activated in response to chronic mild stress in both rat lines, in spite of the cholinergic status of the rats, suggesting that the cholinergic system is not the decisive factor in the adrenal response to stress in these animals. It has, however, been suggested that FSL rats present with a possible diminished HPA-axis activity under non-stressful conditions, but the axis can react to acute cholinergic stimulation, in the same manner than in many depressed patients (Overstreet *et al.*, 1986; Ayensu *et al.*, 1995). Since a fully functional HPA-axis is necessary for stress coping, diminished HPA-axis function in FSL rats may predispose them to a maladaptive response to stressful challenges, resulting in the more pronounced development of learned helplessness behaviour. According to Ayensu *et al.*, 1995, the hypersensitive state of the FSL rat to some effects of dopaminergic agonists (hypothermia, aggression, locomotion) (Overstreet, 1993), might account for the lack of difference in HPA-axis activity between the two rat strains, since DA agonists abrogate stress-induced cholinergic transmission.

c) *NO and the cholinergic system*

A study conducted by Hartlage-Rübsamen & Schliebs (2001) approached the relationship between NO and the cholinergic system in depression by relating cortical cholinergic transmission with the cortical actions of NO in the rat brain. They put forward that, since the dysfunction of cortical cholinergic transmission is associated with similar functions to that mediated by cortical NO mechanisms (eg. cortical perfusion, learning and memory as well as *neuroplasticity*), a link between the basal forebrain cholinergic system and cortical NO-mediated mechanisms exist (Hartlage-Rübsamen & Schliebs, 2001). By lesioning rat basal forebrain cholinergic neurones, they found a significant decrease in nNOS activity, although total cortical and hippocampal nNOS protein did not change. They conclude that a decrease in nNOS activity presumably occurred due to cortical ACh not being able to stimulate M1-mACh receptors and/or NMDA-like neuronal ACh receptors to trigger Ca²⁺-induced intracellular signalling cascades (Colquhoun & Patrick, 1997; Hartlage-Rübsamen & Schliebs, 2001).

NO on the other hand also acts as a modulator for ACh release (Kraus & Prast, 2001). Furthermore other researchers have concluded that NO could have an

anterograde messenger role in cholinergic neurons, meaning that ACh release could be directly influenced by NO-mediated cGMP synthesis (de Vente *et al.*, 2000).

Despite the evidence supporting the connection between NO and the cholinergic system, there has been no concrete evidence for increased cortical mACh density in tissue derived from post mortem patients who committed suicide (Stanley, 1984; Daws and Overstreet, 1998).

Furthermore, uncertainty about the role of the cholinergic system in depression still prevails, predominantly due to the formerly mentioned failure of anticholinergic agents to alleviate human and rodent symptoms of depression. Nevertheless, the addition of an anticholinergic agent to existing treatment can be beneficial in treatment resistant depressed patients (Furey and Drevets, 2006). Moreover, recent work in our laboratory has found behavioural and neuroreceptor evidence in support of a mutual interaction between the cholinergic and cGMP signalling pathways in antidepressant response (Brink *et al.*, 2008).

Subacute stress has been found to induce an increase in hippocampal expression of nNOS and PSD95 proteins (coexpressed with nNOS in several neuronal populations (Brenman *et al.*, 1996) in the hypercholinergic FSL rat versus its healthy control, while the expression of PIN was decreased (Wegener *et al.*, 2008). The authors conclude that the more stress sensitive FSL rats shows a greater sensitivity of the NMDA-NOS cascade over the FRL rat, and which may underlie the pro-depressive nature of these animals (Wegener *et al.*, 2008). These results may also indicate that not only the nitric and cholinergic systems but also the HPA-axis are connected when depression is concerned.

Apart from the aforementioned study performed in cooperation with collaborators in Denmark, the connection between NO and the cholinergic system, and the expected influence of the FSL hypercholinergic system on nitric oxide metabolite (NO_x or total nitrite and nitrate), nNOS activity and cGMP concentration, have not been studied. This connection will be studied in the current project, albeit under baseline conditions. Further details on the current study and of the possible association between NO and acetylcholine in the FSL rat will follow in the coming chapters.

2.9.3 Validity of the Flinders Sensitive Line rat as *animal model* of depression

Evaluation of the FSL rat regarding validity, after taking behavioural and neurochemical characteristics into account amounts to the following:

Table 12: Evaluation of FSL rat according to dependant validity criteria (Willner & Mitchell, 2002)

Hypercholinergic Flinders Sensitive Line Rat		
Validity	Predictive	+
	Face	++
	Construct	0
Evaluation	Depression	+
	<i>Dysthymia</i>	+

- 0 no positive evidence, or no excess of positive evidence over negative;
- + small amount of positive evidence over negative;
- ++ Moderate amount of positive evidence over negative.

In summary, as *animal model* of depression the FSL rat has minimal, if any construct validity and limited predictive validity for major depression. However the model remains one of the most promising *animal models* of depression due to its moderate amount of face validity (Overstreet, 1993; Willner & Mitchell, 2002).

2.10 Closing remarks

Research into the processes underlying the *pathogenesis* of MDD; have failed to answer a fundamental question: *what is the unknown-factor (possibly of a genetic nature) that prevents the body from restoring normal allostatic mechanisms after a chronic stress response? Further, what are the targets upon which drugs must act to keep this maladaptive response from re-occurring after completion of a course of antidepressant medication?*

Further research should compare conditions of normal allostatic physiological regulation to pathophysiological conditions present during disorders like major depression in an attempt to determine why rebound regulation after stress is not achieved in certain patients with a predisposition to depression. Due to the undeniable and still unclear role that predisposition (genetic factors) plays in the *aetiology* of MDD, future drugs need not only be of a curative nature, but should also have prophylactic value to prevent relapse, or protect high risk patients from developing depression.

Current literature discusses the nature and mechanisms of imbalance that occur during depression in the hope of obtaining more insight into the mental illness. This project will contribute to this endeavour by investigating the role of the NO/cGMP pathway, in a genetically predisposed *animal model* of depression, in an attempt to further elucidate the “biochemical basis” underlying MDD (Harvey, 1996).

“Research on nitric oxide, its physiological and pathological roles and clinical potential is currently one of the most exciting sources of knowledge in biology (Wright et al., 1996)”.

One fact remains clear, whatever the face of antidepressant drugs of the future, nitric oxide could play a dynamic role in addressing urgent therapeutic needs in the next generation of antidepressant drugs.

Assessment of the nitric oxide-cGMP cascade in the frontal cortex and hippocampus of Flinders Sensitive Line rats, a rodent model of depression

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Abstract.

Depression presents with evidence of degenerative pathology as well as disturbances in the excitatory glutamatergic pathway, particularly of the N-methyl-D-aspartate (NMDA)-nitric oxide (NO)-cyclic guanosine monophosphate (cGMP) signalling pathway. Hippocampal atrophy in depression may have its origins in excessive NO formation which is neurotoxic and disruptive of normal allostatic neuronal processes. Elucidating the possible mechanisms of glutamatergic-NO involvement in depression may improve our understanding of its pathology and treatment. The aim of this study was to determine if the depressive-like behaviour of the Flinders Sensitive Line (FSL) rat, a genetic model of depression, is accompanied by altered levels of markers of the NO/cGMP pathway. We investigated basal hippocampal and frontal cortical differences between FSL rats and their healthy controls (Flinders Resistant Line, FRL rats) with respect to accumulation of nitrogen oxides (NO_x) and cGMP, as well as neuronal nitric oxide synthase (nNOS) activity. Total neuronal nitrite and nitrate was determined by fluorometric High-Performance Liquid Chromatography (HPLC) assay, L-citrulline by HPLC-electrochemical detection, and cGMP by competitive enzyme-linked immunoassay. Basal cGMP levels in the frontal cortex were found to be significantly less in the FSL rats versus FRL rats, with no differences evident in the hippocampus of these animals. No significant differences with respect to NO_x and nNOS activity were apparent in either of the brain areas studied. Concluding, under normal basal conditions markers of the NO/cGMP signalling cascade are not altered in FSL vs FRL rats, although cGMP levels are reduced in the frontal cortex of FSL rats, supportive of an NO-dependent mechanism of cGMP regulation, possibly involving ACh.

Keywords: *nitric oxide; cGMP; nitric oxide synthase; Flinders Sensitive Line rat; animal model; depression; stress*

1 Introduction

Major depressive disorder (MDD) is a serious affective disorder with an increasingly high global prevalence and mortality rate (Murray & Lopez, 1997; Fava & Kendler 2000; Bertelote, 2001; Mathers & Loncar, 2006). A shift in treatment approach of MDD is expected to occur in the near future as researchers acquire more insight into the neurobiology of depression, and especially with regard to identifying new targets for antidepressant drug action. In this regard, recent evidence supporting a causal role for the nitric oxide/ cyclic guanosine monophosphate (NO/cGMP) pathway (McLeod *et al.*, 2001; Heiberg *et al.*, 2002; Contestabile *et al.*, 2003; Spiacci *et al.*, 2007) may realize great importance as a fundamental substrate of the disorder.

The neuroanatomical substrates of MDD have been related to two specific brain areas, namely the frontal cortex and hippocampus. The frontal cortex is implicated in the psychopathologic and cognitive symptoms of depression, while the hippocampus is more implicated in somatic manifestations, particularly the malfunction of the limbic hypothalamic-pituitary-adrenal-axis (LHPA-axis), with consequential hormonal changes characteristic of major depression (American Psychiatric Association, 2000; Adell *et al.*, 2005; Cahill, 2007; Belmaker & Agam, 2008). The hippocampus therefore is involved in long term learning and memory as well as neuroendocrine regulation of the hypothalamic-pituitary-adrenal stress axis (Sapolsky, 2001; Kim & Diamond, 2002). The hippocampal as well as the frontal cortical areas are in fact part of the LHPA-axis and are centrally located within the limbic-cortical network (McLeod *et al.*, 2001). This makes both structures primary targets of stress hormones released following stressful conditions, latter being an integral component of MDD (McLeod *et al.*, 2001; Frodl *et al.*, 2002; MacQueen *et al.*, 2003; Diamond *et al.*, 2004; Caetano *et al.*, 2004). NO plays a central role in many of these functions, including cortical perfusion, learning and memory as well as in neuroplasticity (Contestabile *et al.*, 2003).

In the brain, NO formation takes place following N-methyl-D-aspartate (NMDA) receptor activation and the subsequent increase in intracellular calcium which stimulates the enzyme nitric oxide synthase (NOS) to convert L-arginine to NO and the amino acid L-citrulline. Immediately after its formation, NO rapidly distributes within the neuron or diffuses to adjacent neurons where it activates guanylyl cyclase to increase synthesis of the second messenger, cGMP (de Vente *et al.*, 1998). NO is

also rapidly oxidised to its inactive metabolites, nitrate and nitrite (Dawson & Snyder, 1994).

In addition to mediating neuronal plasticity that underlies long term potentiation (Schuman & Madison, 1991; O'Dell *et al.*, 1991; Arancio *et al.*, 1996; Ledo *et al.*, 2004), NO also acts as a regulatory neurotransmitter (Wegener *et al.*, 2003; Leonard, 2003). NO and cGMP are the main messengers in the neuronal glutamatergic system (Domek-Lopacinska and Strosznajder, 2005). However, cGMP is also a very important messenger for the neuronal cholinergic system (Prast & Philippu, 1992), where it is involved in cross-talk between cholinergic and other neurotransmitter mediated pathways, such as the cholinergic-adrenergic pathway (de Vente, 2004). According to the cholinergic-adrenergic hypothesis, (Janowsky *et al.*, 1972) the aetiology of depression is partly derived from the "overactivity of cholinergic neurons relative to adrenergic neurons in specific brain regions" (Hasey, 1996). cGMP activates G-kinase, and also activates or inhibits phosphodiesterase (PDE) with subsequent effects on cellular levels of cyclic adenosine monophosphate (cAMP), activation of ion channels and G-proteins (de Vente, 2004). cGMP also modulates the release of certain neurotransmitters (Harvey 1996; Prast and Philippu 2001; Wegener *et al.*, 2003) critical for affective state, such as biogenic monoamines, norepinephrine (NE), dopamine (DA), glutamate, acetylcholine (ACh) (Garthwaite, 1991; Prast & Philippu, 1992; Hanbauer *et al.*, 1992; Hirsch *et al.*, 1993) and γ -aminobutyric acid (GABA) (Ohkuma *et al.*, 1995). Cyclic GMP also regulates presynaptic uptake of neurotransmitters like serotonin (5-HT) (Miller & Hoffman, 1994). NO may also be a protector of neuronal allostasis, with NO, peroxynitrite (ONOO⁻) and nitrogen dioxide (NO₂) contributing to oxidative damage, but also protecting neurons by scavenging toxic free radicals (Farinelli *et al.*, 1996; Contestabile *et al.*, 2003). Under pathological conditions such as disease and senescence (Dawson & Dawson, 1996; Dawson & Dawson 1998), NO is inappropriately and excessively synthesized leading to neurotoxicity that may be associated with degenerative phenomena (Dawson & Dawson, 1996). Drawing on the above knowledge, it is clear that the NO/cGMP pathway may play a diverse and unpredictable role in affective disorders (Harvey *et al.* 2003; Millan, 2006).

The Flinders line sensitive (FSL) rat presents with extensive face and predictive validity for depression (Overstreet 1993; Willner & Mitchell, 2002), although has to date not been studied with respect to changes in the NO/cGMP pathway. The FSL

rat also displays a hypercholinergic response (Overstreet *et al.*, 2005) which, due to the cholinergic hypothesis of depression (Janowsky *et al.*, 1972), has further raised its putative construct validity for depression. Since NO is a modulator (Kraus & Prast, 2001) as well as an anterograde messenger (de Vente *et al.*, 2000) for ACh release, the FSL rat represents a useful model with which to explore the contributory role of NO, and also NO-ACh interactions, in the depressive phenotype of these animals.

In order to determine whether depressive-like behavior of the selectively bred hypercholinergic FSL rat line (Overstreet *et al.*, 2005) is accompanied by altered markers of the NO/cGMP pathway, cortico-hippocampal neuronal NOS activity, nitrogen oxides, total nitrite and nitrate (NO_x) and cGMP accumulation were studied in FSL rats under basal conditions and compared to that of the Flinders Resistant (FRL) rat Line.

2 Materials and methods

The rapid metabolism and diffusion of NO between adjacent cells (Meulemans, 1994; Schuman & Madison, 1994; Ledo *et al.*, 2004) makes it inaccessible for accurate analytical measurement. The best index of NO production is therefore the sum of its oxidative metabolites, total nitrate and nitrite (NO_x) (Casey & Hilderman, 2000), while L-citrulline concentration can act as a surrogate marker of neuronal NOS activity.

Nitric oxide production, as indicated by the concentration of the accumulated oxidated metabolites (NO_x) and basal NOS activity as well as cGMP, respectively, were analysed in both the hippocampus and frontal cortex of different groups of stress-naive FSL and FRL rats. Separate groups of FSL/FRL rats (n=10/group) were used for each analysis.

3 Experimental animals

Approval of the study protocol was granted by the Animal Ethics Committee of the North-West University (Ethics approval number NWU0003207S2). All animals were treated according to the code of ethics in research as laid down by this Animal Ethics Committee.

Breeding pairs of the FSL and FRL rats were originally purchased from Dr. David Overstreet at the University of South Carolina, USA. For this study, young, adult male rats, weighing 200 ± 20 g were provided by the Animal Centre of the North West University Potchefstroom campus, after being reared in corncob cages. Controlled temperatures ($21^{\circ}\text{C} \pm 1^{\circ}\text{C}$) with relative humidity $55 \pm 5\%$, positive air pressure in animal rooms and airtight doors (maintaining positive air-pressure barriers between departments) were maintained during the duration of the study. Animal Centre air was exchanged 16 – 18 times the volume (fresh uncirculated air) per hour, with Air quality controlled with HEPA - high efficiency filters. Alternating fluorescent “daylight” tubes that supply full spectrum white light (350 lux, 1m off the ground) was provided over a nycthemeral cycle (12h/12h). Food and water were supplied *ad libitum*.

4 Neurochemical analysis

4.1 Tissue dissection and storage

Animals were sacrificed by decapitation, after which the brain was swiftly removed and the hippocampus and frontal cortex dissected out on an ice-cooled stainless steel slab. The dissected tissue was individually placed in eppendorf tubes and immediately snap-frozen in liquid nitrogen to be stored at -86°C until the day of analysis.

4.2 Chemicals

Chemicals were of analytical grade or higher purity and stored at specified conditions. All aqueous solutions were prepared using High-Performance liquid chromatography (HPLC) -grade water.

4.3 Nitric oxide analysis

4.3.1 NO_x determination

Total neuronal nitrite and nitrate was measured with HPLC coupled to fluorescence detection. Statistical method validation met the general requirements of ISO 17025, 2005 and SANS 17025 (Anon, 2005).

The three-step method used for NO_x (total nitrite and nitrate) determination is based on the derivatization of nitrite with the highly fluorescent compound 2, 3-diaminonaphthalene (DAN) (Li *et al.*, 2000; Jobgen *et al.*, 2007). After sample

preparation, nitrate was converted into nitrite and the resultant nitrite was derivatized so that it could fluorescently be detected after HPLC separation of DAN and 2,3-naphthotriazole (NAT) at excitation and emission wavelengths of 363 nM and 425 nM respectively, and a flow rate of 1 ml/min under chromatographic conditions as stipulated in section 4.3.1 b.

a) Standards and reagents

A 100 µg/ml stock solution containing 13.7 mg sodium nitrate (NaNO₃) and 15.0 mg sodium nitrite (NaNO₂), dissolved in 100 ml Tris(hydroxymethyl)-aminomethan (TRIS) buffer, pH 7.6 was freshly prepared daily.

A standard series of working concentrations in the range of 10 - 300 ng/ml or 0.22 – 10.75 µM was prepared from stock solutions by appropriate dilution before use. Standard regression displayed a positive, significant, almost perfect linearity ($r^2 = 0.9988$). All standards were made up in and samples homogenized with TRIS buffer. All buffers were made up with HPLC-grade water and stored at -20 °C.

β-nicotinamide adenine dinucleotidephosphate (β-NADPH) was freshly prepared daily, by dissolving 0,3 mg in 275 ml of HPLC-grade water (1.3 µM, final concentration: 0.22 µM). This lower concentration was used in combination with an enzyme solution (176 mg D-glucose-6-phosphate monosodium salt (G-6-P) with 100 IU/ 0.05 mg glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (GD) in 26, 04 ml of a 170 mM sodium phosphate buffer, pH 7.4) to cycle the NADP⁺ to NADPH. The enzyme solution was stable for at least a month at -80°C.

Immediately before use, 50 µl of the enzyme mixture was added to freshly prepared nitrate reductase (NR, Sigma-Aldrich) to yield a final NR concentration of 15 mU (Misko *et al.*, 1993; Li *et al.*, 2000; Woitzik *et al.*, 2001; Sigma-Aldrich, 2008). DAN was freshly prepared daily by dissolving an excess of 3,2 mg of DAN powder in 5 ml of 0.76 M hydrochloric acid (HCl) (4 mM, final concentration: 0.57 mM). The solution was protected from light and stored on ice, but allowed to reach room temperature before addition to sample.

b) Chromatographic conditions

HPLC was performed using an Agilent 1100 series HPLC system, equipped with an isocratic pump, autosampler, Shimadzu RF-551 fluorescence detector with excitation 363 nM and emission 425 nM, and Chemstation Rev. A.06.02 data acquisition and

analysis software for calculating peak areas and sample concentrations. An Eclipse XDB-C18 column, 4.6 x 150 mm, 5 μ M (Agilent) was used and protected by a guard column SecurityGuard™, HPLC Guard Cartridge System, with SecurityGuard Cartridges, C18 - 4.0x3.0 mm (Phenomenex, Torrance, CA). The isocratic elution mobile phase comprised of 47.8 % of 15 mM sodium phosphate buffer (pH=7.5) and 52.2 % HPLC-grade methanol (Merck) adjusted with orthophosphoric acid (85%, ACE) and was delivered at a flow rate of 1 ml/min at a temperature of 26 °C.

c) *NO_x assay procedure*

Tissue extraction and preparation:

On the day of analysis, samples were removed from – 86 °C storage and allowed to thaw. The tissue was then weighed and immediately homogenized in approximately 750 μ l of ice-cold TRIS buffer (1mM Ethylene glycol-bis (β -aminoethyl ether), N-N-N' tetraacetic acid (EGTA), Sigma-aldrich; 1 mM Ethylenediamino-etetraacetic acid (EDTA), PAL chemicals and 25 Mm TRIS, Saarchem, pH=7.4, adjusted with 10% HCl, from 32% uniVar), respective to the weight, with a Heidolph-Elektro KG glass/Teflon homogenizer (15 strokes at \pm 22500 U/min) at \pm 2 - 6 °C. A sufficient amount of tissue homogenate was stored at – 86 °C for determination of sample protein concentration.

The sample's soluble fraction was obtained by centrifugation for 1 hour at 5 400 xg, 4 °C (Pearce *et al.*, 1997). 200 μ l Supernatant was directly transferred into an amber glass HPLC-vial and kept on ice until use.

Conversion of nitrate into nitrite:

50 μ l of β -NADPH, Sigma-Aldrich Chemicals, St. Louis, MO (1.3 μ M) was added to the sample/ standard, immediately followed by a separate addition of 50 μ l nitrate reductase (NR, Sigma-Aldrich) (15 mU) in enzyme mixture, to avoid inhibition of NR by pre-incubation with NADPH (Woitzik *et al.*, 2001). The nitrate in the brain sample/ standard was optimally converted into nitrite by incubating the reaction mixture for 45 minutes at 20 °C (Misko *et al.*, 1993).

Derivatization of resultant nitrite:

The conversion reaction was terminated; and a new reaction initiated with addition of 50 μ l DAN (Sigma-Aldrich) in HCl (Woitzik *et al.*, 2001). This reaction is optimal at 24

°C. After 10 minutes, the pH was adjusted with 25 μ l of 1.71 M sodium hydroxide (NaOH) (Saarchem) to stop the derivatization reaction, stabilize the product (NAT) and adjust the reaction mixture's pH so that fluorescence detection of NAT is possible.

A final centrifugation of vials at 60 rpm, for 5 minutes was necessary to form a pellet of remaining protein. The HPLC sample injection needle was set at a level above the protein pellet in the HPLC-vial, before sample injection.

HPLC-fluorescence detection was used to perform separation of DAN and NAT. The runtime per sample was 9 minutes.

NO_x quantity was expressed as μ M per 2 - 5 mg/ml protein.

*Volumetric glass apparatus was used throughout the analysis to make up all reagents and standards. All pipette tips, eppendorf- and HPLC-vials were pre-rinsed with TRIS-buffer for at least 3 times before use to remove trace amounts of nitrite that interfered at low standard concentrations (1 - 10 ng/ml).

4.3.2 NOS activity assessment

Levels of the amino acid, L-citrulline, an indication of nNOS activity (Dawson & Snyder, 1994), was determined in the frontal cortex and hippocampus with a customized simple, sensitive and reproducible isocratic reversed-phase liquid chromatography method with amperometric electrochemical detection (Harvey, 2002; Harvey & Nel, 2003). We modified the method which was originally developed for the determination of NOS activity through NO_x by means of HPLC fluorescence detection (Fernández-cancio *et al.*, 2001) to the above-mentioned HPLC, electrochemical detection-method. This method was previously validated for the quantification of L-citrulline, L-arginine, L-glutamate and GABA in rat brain tissue. The method involves the *in vitro* activation of the basal nNOS enzyme in the brain tissue sample by addition of a cofactor, co-substrate reaction cocktail and an enzyme mixture (i).

Additional to this standard measurement of basal nNOS activity in rat brain samples, the enzyme was further investigated by introducing an L-arginine challenge into the assay. L-arginine was found to be the only substrate for all the NOS isoforms (Wiesinger, 2000).

An amount of the reaction substrate, L-arginine was added to the reaction cocktail to compare the L-citrulline levels that were induced by the L-arginine challenge (Test sample levels) between rat strains (ii). This comparison was also made to investigate the amount of increase in NOS activity, following L-arginine challenge (iii) (also see section 4.3.2 d). Control samples, measuring basal NOS activity contained all of the above components, except L-arginine, which was replaced by an equal volume of borate buffer. The assay was carried out at an enzyme concentration of 30 – 50 µg protein, adequate to convert the added substrate to product, but prior to reaching steady state (Harvey *et al.*, 2004).

a) *Standards and reagents*

A 100 µg/ml amino acid containing stock solution (all obtained from Sigma-Aldrich) was freshly prepared daily by dissolving 1 mg L-citrulline, 1.21 mg L-arginine, 1 mg GABA and 1 mg glutamate in 10 ml borate buffer, pH 7.5. A standard series of working concentrations in the range of 0.1 - 5 µg/ml (0.57 – 28.54 µM L-citrulline) was prepared from stock solutions by appropriate dilution before use. Standard regression displayed a positive, significant linearity ($r^2 = 0.9970$).

The enzyme mixture used for cycling β -NADPH, and nitrate reductase (NR) was prepared according to the same procedure as in the NO_x assay. β -NADPH was freshly prepared daily by dissolving 0.5 mg in 3 ml HPLC-grade water (0.2 mM). The NO_x assay TRIS buffer, was also replaced with a borate buffer to avoid the competition of the amino group (-NH₂) in TRIS, with *o*-phthalaldehyde (OPA, Pierce) (Shiue *et al.*, 2005).

The final concentration of each component in the reaction cocktail was β -NADPH, 27.36 µM; calmodulin (CaM) 13.7 µg/ml; flavin adenine dinucleotide disodium salt hydrate (FAD) 1 µM; riboflavin 5'-monophosphate sodium salt dihydrate (FMN) 1 µM; tetrahydrobiopterin (BH₄) 4 µM and L-arginine, 18.3 µM.

b) *Chromatographic conditions*

HPLC was performed using an Agilent 1100 series HPLC system, equipped with an isocratic pump, autosampler, GBC LC 1260, electrochemical detector, and Chemstation Rev. A.06.02 data acquisition and analysis software for calculating peak areas and sample concentrations. The glassy carbon electrode was used at a potential of +0.600 V, range: 5 nA - 500 pA, polarity: positive, filter: 64 point, filter

(backside): 0.5 Hz. A Luna C18-2 column, 75 x 4.6 mm, 5 μm , 100 \AA pores, 17.8% carbon load, endcapped (Phenomenex, Torrance, CA - Column L1, USP 24, 2000, p 1925), was used and protected by a guard column SecurityGuard™, HPLC Guard Cartridge System, with SecurityGuard Cartridges, C18 - 4.0x3.0 mm (Phenomenex, Torrance, CA). The mobile phase comprised of 0.1 M sodium phosphate dibasic, 0.13 mM ethylenediaminetetraacetic acid (EDTA disodium salt Na_2EDTA) and 28% (to 35%) methanol, pH \pm 6.4 – adjusted with orthophosphoric acid (85%) and was delivered at a flow rate of 0.8 ml/min to 1.5 ml/min at a temperature of 26 °C.

c) *L-citrulline assay procedure*

Tissue extraction and preparation:

On the day of analysis, samples were weighed and homogenized in approximately 1 ml of ice-cold borate buffer (15 mM, pH 7.6 – adjusted with HCl, sodium tetraborate decahydrate, Riedel – de Haën and boric acid powder, Merck) with a Heidolph-Elektro KG glass/Teflon homogenizer (15 strokes at \pm 22500 U/min) at 2 - 6 °C. Samples were centrifuged for 1 hour at 5 400 xg, 4 °C (Pearce *et al.*, 1997) and the supernatant decanted, separated from the tissue pellet. The standard amount of supernatant extraction for a particular brain part that contains approximately 30 - 50 μg protein (Harvey *et al.*, 2004) was calculated after spectrophotometric determination of protein concentration (Bradford, 1976). Supernatant aliquots were transferred to an amber glass HPLC-vial and kept on ice until use.

nNOS enzyme activation:

To simulate the *in vitro* enzyme reaction, a reaction cocktail containing 15 μl BH_4 (100 μM), 5 μl FAD (100 μM) and FMN (100 μM), 50 μl β -NADPH (0.2 mM) and 12, 5 μl CaM; (400 $\mu\text{g/ml}$) (all from Sigma-Aldrich) was added to the brain supernatant aliquot after the reaction cocktail has been incubated for 2 minutes at 37 °C (Venturini *et al.*, 1999). Test samples were spiked with 134 μl L-arginine (50 μM , Sigma-Aldrich) while control samples received 134 μl of borate buffer. Immediately thereafter, 84 μl (25.2 mU) NR dissolved in the cycling enzyme mixture (G-6-P and GD, glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (GD) and D-glucose-6-phosphate monosodium salt, Fluka Biochemika in a phosphate buffer, pH 7.6, potassium dihydrogen orthophosphate (KH_2PO_4) and Sodium Chloride (NaCl), Acros) were added to the vial and the nNOS enzyme reaction initiated with 10 μl calcium chloride (CaCl_2 ; 130 mM).

Following incubation for 10 minutes at 37 °C (Venturini *et al.*, 1999; Harvey & Nel, 2003), the samples were removed from the oven (Horo Stuttgart-Hedelf) and the reaction was stopped by dilution with 50 µl ice-cold stop buffer (50 mM 4-(2-hydroxymethyl) piperazine-1-ethanesulfonic acid (HEPES), 5 mM EDTA, pH: 5.5 – NaOH). The pH of the sample was adjusted to pH 9 with the addition of 25 µl of potassium acetate (CH₃COOK, 10 M).

As a pre-final step, the HPLC-vials were centrifuged for 2.5 minutes at 60 rpm for removal of any remaining protein. 50 µl of the resultant supernatant was then finally pipetted into a HPLC vial insert and placed into the HPLC autosampler after programming the software's injector program for precolumn *ortho*-phthaldialdehyde derivatization and subsequent injection of 50 µl into the HPLC for separation of amino acids.

For the shortest possible runtime with good peak separation the flow rate was increased during latent times so that the last peak's (GABA) retention time was within 30 minutes.

d) Calculation of NOS activity

NOS – activity in the two brain areas was determined by

- i. Comparing the differences between FRL rat endogenous (control) L-citrulline concentrations and FSL rat endogenous (control) L-citrulline concentrations (basal NOS activity),
- ii. comparing the differences between FRL rat induced (test) L-citrulline concentrations and FSL rat induced (test) L-citrulline concentrations (as illustrated in Figure 2a & b).
- iii. comparing the L-citrulline concentration of test samples (L-arginine-challenged) with that of control samples (endogenous),

All differences in NOS activity resulted from differences in active enzyme concentration. All results were expressed in µM per 30 – 50 µg protein.

4.4 cGMP analysis

Since NO_x determination is a surrogate marker of authentic NO, and L-citrulline for NOS activity, a third assay method of NO pathway activity, namely the measurement of cGMP levels, would complement nitric oxide data, as there exists a parallel relationship between the enzyme nNOS and the NO-mediated accumulation of cGMP in the rat brain (Southam & Garthwaite, 1991; de Vente *et al.*, 1998). For this purpose, determining down-stream activity of the NO pathway is very useful. The second messenger cGMP was measured in the aforementioned brain areas using a direct competitive immunoassay (ELISA) kit (Sigma-Aldrich) (Uzbay *et al.*, 2004). Differences in neuronal cGMP concentration between FSL and FRL rats were measured following the kit procedure as described in the Sigma-Aldrich product information technical bulletin (Catalog number CG200).

* Polyclonal antibody to cGMP binds the cGMP in the sample in a competitive manner.

1. Samples or standards, alkaline phosphatase conjugate and antibody were simultaneously incubated at room temperature in a secondary antibody coated microwell plate to increase the sensitivity of the assay. All samples were acetylated.
2. The excess reagents were then washed away and substrate was added.
3. After a short incubation time the enzyme reaction was stopped and the yellow color generated could be read on a microplate reader at 405 nm.
4. The intensity of the bound yellow color was inversely proportional to the cGMP concentration in the standards and samples.

* The measured optical density was used to calculate the cGMP concentration, expressed in pmol/mg/ml protein.

4.5 Statistical analysis

NO_x, NOS activity and cGMP data were analyzed with Statistica[®] and graphically presented with Graphpad Prism[®] (Statsoft Inc., 2007, Statistica Data Analysis Software System, version 8; Graphpad software, version 5.0 for Windows[®], San Diego, USA).

All data were non-parametrically analyzed in view of the irregular distribution of normal probability study plots. The Mann-Whitney U Test or the Wilcoxon Matched Pairs Test was used, as will be indicated under section 4, Results.

The representation of data was expressed as means \pm SD and statistical significance of marked tests were defined as $p < 0.05$ in all instances.

5 Results

5.1 Nitric oxide analysis

5.1.1 NO_x determination

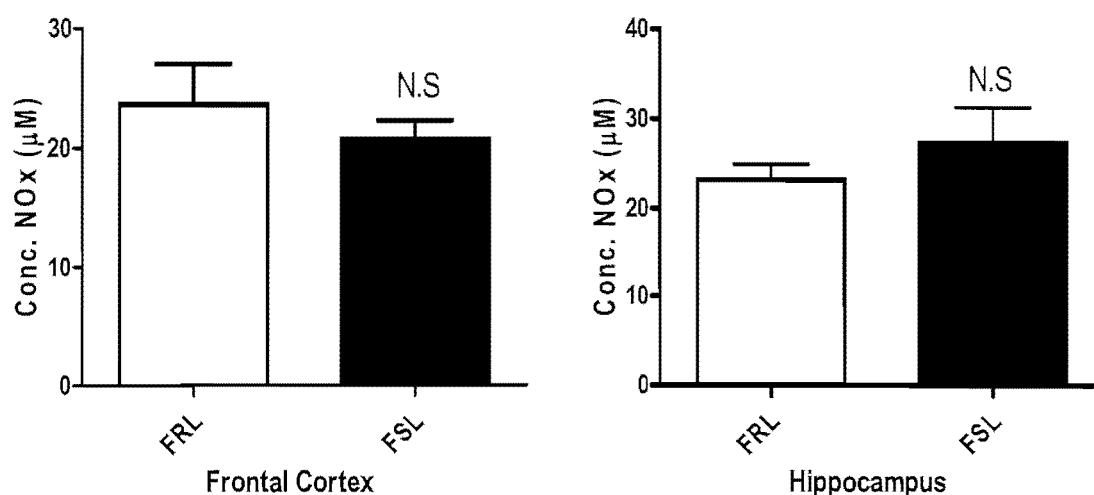


Figure 1 a

b

The Mann-Whitney U Test showed no significant differences in endogenous NO_x concentration between the FRL and FSL rat groups in either the frontal cortex (Figure 1a: $p = 0.94$), or the hippocampus (Figure 1b: $p = 0.33$).

5.1.2 NOS activity assessment

5.1.2.1 *L-citrulline concentration between FSL and FRL rats*

- i. Comparison of differences between FRL rat endogenous (control) L-citrulline concentrations and FSL rat endogenous (control) L-citrulline concentrations with the Mann-Whitney U Test, showed no significant differences in the frontal cortex (Figure 2a,i: $p = 0.545$) or the hippocampus (Figure 2b,i: $p = 0.9397$).

- ii. Comparison of differences between FRL rat induced (test) L-citrulline concentrations and FSL rat induced (test) L-citrulline concentrations with the Mann-Whitney U Test, also did not differ significantly in the frontal cortex (Figure 2a, ii: $p=0.1736$) or the hippocampus (Figure 2b, ii: $p=0.5453$)
- iii. Respective L-citrulline comparison between frontal cortical FRL rat control vs. test sample concentrations (Figure 2a, iii: $p=0.5$) and FSL rat control vs. test sample concentrations (Figure 2a, iii: $p=0.07$) as analyzed with the Wilcoxon signed-rank test, showed no significant differences.

Hippocampal FRL rat control vs. test L-citrulline sample concentrations (Figure 2b: $p=0.12$) and FSL rat control vs. test sample concentrations (Figure 2b: $p=0.07$) also showed no differences.

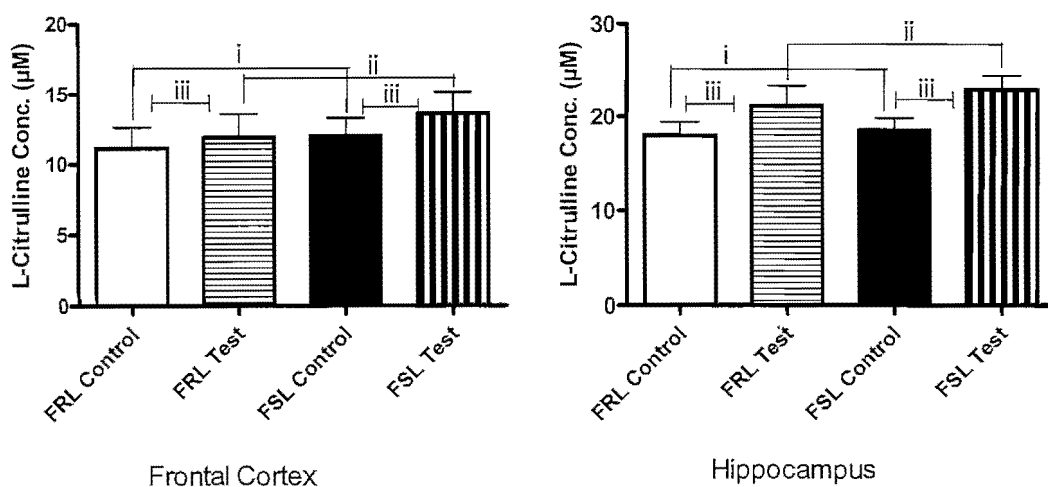


Figure 2 a

b

Statistical analysis:

i: Mann-Whitney U Test, NS

ii: Mann-Whitney U Test, NS

iii: Wilcoxon signed-rank test, NS

5.1.2.2 *n*NOS activity

L-arginine challenged NOS activity was also calculated by subtracting test (induced) L-citrulline sample concentrations from control (endogenous) L-citrulline sample concentrations. The difference in L-citrulline represents the increase in L-arginine - activated *n*NOS when the conversion reaction was initialized.

Results, analysed with the Mann-Whitney U Test showed no significant differences between FRL and FSL rat L-citrulline levels/ active *n*NOS in neither the frontal cortex (Figure 2c: $p=0.5$), nor the hippocampus (Figure 2d: $p=0.4$).

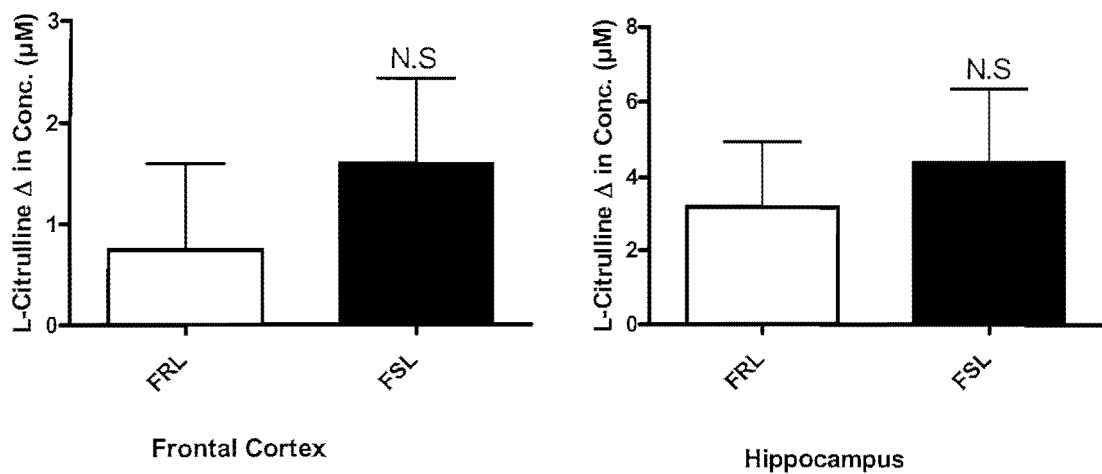


Figure 2 c

d

Statistical analysis: Mann-Whitney U Test

5.2 cGMP analysis

The Mann-Whitney U Test showed a significant difference in frontal cortical FSL and FRL endogenous cGMP concentrations (Figure 3a: $p=0.008$), with a decrease in FSL cGMP concentration relative to FRL concentration. There were no significant differences between hippocampal FRL and FSL cGMP concentrations (Figure 3b: $p=0.33$).

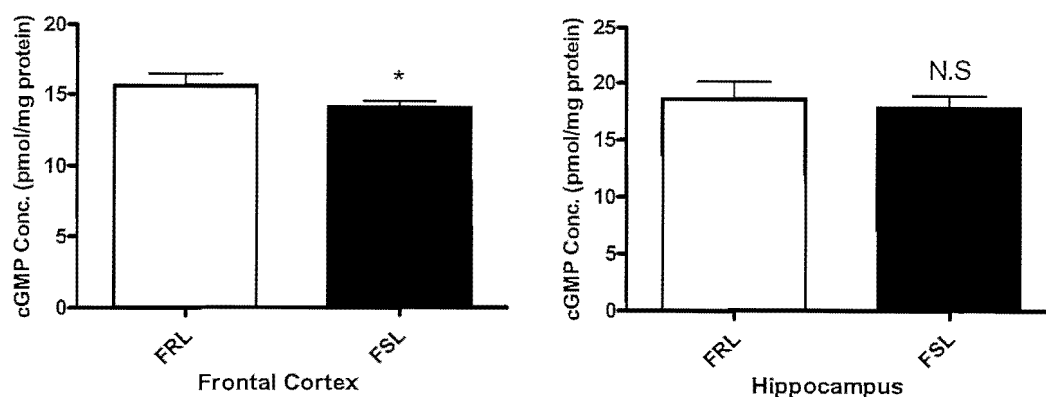


Figure 3 a

b

Statistical analysis: Mann-Whitney U Test

6 Discussion

In the present study, we have investigated basal hippocampal and frontal cortical differences between FSL rats and their healthy controls (FRL rats) with regard to accumulation of nitrogen oxides (NO_x) and cGMP, and neuronal nitric oxide synthase (nNOS) activity. The neurological complications of MDD particularly manifest in the frontal cortex and hippocampus (American Psychiatric Association, 2000; Adell *et al.*, 2005; Cahill, 2007; Belmaker & Agam, 2008). Although we were unable to detect significant differences in NO_x or NOS activity in FSL rats versus FRL rats in either of these brain areas, we found that basal frontal cortical cGMP levels were significantly lower in FSL rats compared to FRL rats, while levels in the hippocampus remained unaffected.

The FSL rat is an inbred animal characterized by increased cholinergic activity and heightened response to cholinergic challenge as compared to its normocholinergic control, the FRL rat (Overstreet, 2002). Due to well established face and some predictive validity (Overstreet, 1993; Willner & Mitchell, 2002), the FSL rat is a promising animal model of depression, particularly for testing selected novel hypotheses of depression. In this regard, it has been used to investigate various theoretical models, including the cholinergic (Daws & Overstreet, 1999; Overstreet, 2002), serotonergic (Wallis *et al.*, 1988; Janowsky *et al.*, 1994; Zangen *et al.*, 1999; Overstreet, 2002), noradrenergic (Serova *et al.*, 1998; Zangen *et al.*, 1999; Overstreet *et al.*, 2005), dopaminergic (Overstreet *et al.*, 2005), GABAergic (Pepe,

1998), HPA-axis (Ayensu *et al.*, 1995; Owens *et al.*, 1991) and associated hypotheses of depression.

Particularly with regard to the regulation of temperature (Bert *et al.* 2006; Daws & Overstreet 1999; Overstreet *et al.* 1998; Overstreet 2002), there is distinct evidence for the co-involvement of serotonergic and cholinergic systems in the expression of certain behaviours in FSL rats. Thus, hypercholinergic FSL rats demonstrate a marked hypothermic response to the 5-HT_{1A} receptor agonist, 8-OH-DPAT (Overstreet *et al.*, 1998), displaying a 2-fold greater decrease in temperature compared to the FRL rat (Overstreet *et al.*, 1994). Various *in vitro* studies (eg. Brink *et al.* 2004) have provided further evidence for the mutual interaction between 5HT_{1A}ergic and cholinergic signalling. Biochemical alterations to the serotonergic and cholinergic system suggested that the regulation of muscarinic acetylcholine receptor plasticity is dependent on the integrity of the serotonergic system (Alonso & Soubrie, 1991) and vice versa (Overstreet *et al.*, 1997). Accordingly, functional 5-HT release in the serotonergic system may be regulated by muscarinic acetylcholine receptors (Hery *et al.*, 1977). In affective disorders, an imbalance between the serotonergic and cholinergic neurotransmitter systems exists- presenting with cholinergic overactivity (Nurnberger *et al.*, 1983; Janowsky *et al.*, 1974; 1995) that may stimulate subsequent alterations in serotonergic function (Leonard, 2003), leading to decreased 5HT and eventually to depressive episodes (Overstreet *et al.*, 1997). The FSL rat model of depression is based on the hypothesis that the central cholinergic system is over-active in depression (cholinergic model of depression; Janowsky *et al.*, 1972). In line with this, *in vivo* animal studies by Overstreet (Overstreet, 1993; Overstreet *et al.*, 1997) have found FSL rats to be hypersensitive to cholinergic agonists (Ayensu *et al.*, 1995) as well as to serotonergic drugs (Overstreet, 2002), thus suggestive of a combined dysfunction of both serotonergic and cholinergic signalling in depression.

Despite the increased cholinergic drive in the FSL rat and its hypersensitivity to cholinergic agonists (Overstreet, 2002) – which is akin to the hypersensitivity of depressed individuals to cholinergic agonists (Janowsky *et al.*, 1994), anticholinergic drugs exhibit no antidepressant effects in the FSL rat or in depressed humans (Schiller *et al.*, 1992). This observation remains the cause for uncertainty about the role of the cholinergic system in depression. The lack of an antidepressant effect by anticholinergic agents could possibly be due to the inconsistent distribution of muscarinic acetylcholine receptors in the brain between different types of neurons,

reducing the chances of selective muscarinic receptor stimulation and overall antidepressant effect of anticholinergic drugs (de Vente, 2004).

Recent work in our laboratory has found behavioural and neuroreceptor evidence in support of a mutual interaction between the cholinergic and cGMP signalling pathways in antidepressant response (Brink et al, 2008). The importance of the NO/cGMP pathway in the pathology and treatment of depression and other stress-related illnesses is becoming more evident (see Harvey, 2006 for review; Millan, 2006), particularly with respect to neuronal and brain function (Harvey *et al.*, 2004). However, the interplay between ACh and NO, and its interaction with regard to the aetiology of depression in the FSL and other models of depression has received relatively little attention, especially when compared to studies on neurotransmitters involved in the classical biogenic amine/ monoamine hypotheses. According to Domek-Lopacinska and Strosznajder (2005), nitric oxide and cGMP are the main messengers in the glutamatergic system. It seems that cGMP is also a very important messenger for the cholinergic system, where it could be involved in cross-talk between cholinergic and other neurotransmitter-mediated pathways (de Vente, 2004). Indeed, certain psychotropic drugs, such as sildenafil (Brink et al, 2008) as well as lithium (Harvey et al, 1990a; 1990b) have demonstrated an interaction between the NO-cGMP and cholinergic systems

When considering the data from the current study in FSL vs. FRL rats, total nitrite and nitrate (NO_x) concentrations in the frontal cortex and hippocampus (Figure 1) of FSL and FRL rats did not differ significantly, demonstrating that under ambient (basal) conditions, activity of the NO cascade appears to be unchanged. Similarly, when considering NOS-activity (of which L-citrulline is an index), there was no change in basal limbic NOS activity in FSL rats as well as their counterparts.

The additional further investigation of the nNOS enzyme, by introducing an L-arginine challenge into the assay, should act as a type of external reaction stimulus in a similar context as that of the generally expected increase in stress-activation-response, of the NOS enzyme in FSL rats, in comparison to that of FRL rats. The L-arginine-induced challenge would be expected to prime a significant increase in enzyme activity, as the amino-acid. The challenge was however designed to involve the addition of such an amount of substrate into the reaction, relative to the composition of the reaction cocktail that the induced reaction product L-citrulline would still fall into the same instrumental detection range as that of the basally

induced L-citrulline, for the sake of distinctive comparison between the two. Keeping this in mind, the induced-NOS activity, should not be regarded as the optimal enzyme activity. The concentration of added substrate in this modified assay was not meant for the complete stimulation of substrate –product conversion. Rather, a reaction-challenge with approximately half (50 μ M; final concentration: 18 μ M) of the concentration L-arginine that is commonly used under similar reaction conditions (Fernández-cancio *et al.*, 2001), yielded a good L-citrulline response, without being above the instrumental threshold.

In retrospect, the results from the L-arginine challenge showed no difference in induced L-citrulline concentration between limbic regions in the two rat strains. Similarly, results did not confirm a significant stimulated increase in product formation in FSL rats, in comparison with basal levels of L-citrulline. Thus there was no significant increase in NOS activity in either of the rat lines, compared to basal NOS activity. A trend of the FSL rat to have higher induced NOS activity is apparent, but prospective studies are needed to investigate in-range product formation in the presence of higher L-arginine concentrations (between 50 and 100 μ M), in these rats, accordingly with adapted reaction cocktail concentrations to facilitate complete conversion of substrate by an increased amount of enzyme – where differences in available NOS activity might become apparent.

Despite the lack of change with regard to regional NOS activity described above, a significant decrease in frontal cortical cGMP was observed in FSL compared to FRL: rats. Considering the hypercholinergic status of the FSL rat, these data are congruent with earlier studies that have reported an association between cholinergic neurotransmission and cGMP synthesis (de Vente, 2004; Brink *et al.*, 2008). The current study describing changes in regional brain cGMP levels in a hypercholinergic rat supports the existence of a cholinergic-cGMP interaction.

Early results in the rat brain have confirmed the hippocampal, but not the frontal cortical increase of cGMP levels in response to the administration of a cholinergic agonist (Lenox *et al.*, 1980). The observed decrease in cGMP in the FC in the present study, interestingly, occurred within the presence of a high ACh concentration (in FSL rats), an observation that has been shown by others (Overstreet *et al.*, 2005) as well as in a parallel study (van Zyl, 2009). These observations agree with various early reports from researchers who found decreased cGMP levels in the cortex (Palmer and Duszynski, 1975; Palmer *et al.*, 1980) after

intrastratial injection of ACh. However, the earlier mentioned increase in cGMP levels in the hippocampus after the administration of a cholinergic agonist could not be reversed after the injection of a cholinergic antagonist (Puri *et al.*, 1978), while the administration of a cholinergic antagonist also increased cGMP (de Vente, 2004), suggesting a different role for cGMP-ACh interactions in these two brain regions. Indeed, the frontal cortex and hippocampus play separate yet interacting roles within the stress circuit of the brain. The frontal cortex plays an important role in the extinction of conditioned fear responses by modulating fear responding through inhibitory connections with the amygdala. These actions in turn play a crucial role in fear conditioning by directing the encoding of emotional memories through projections to the hippocampus (Harvey *et al.*, 2003). This decrease in cGMP observed selectively in the frontal cortex not only suggests neuroanatomical specific differences between the animals, but may also imply differences in cortical function in these animals, such as cognition and goal-directed behaviour, which all contribute to the symptoms of depression (Duman *et al.*, 1999). This anatomical diversity with respect to the cGMP response described in this study is further complicated by studies that show that cGMP-analogs both stimulate (Guevara-Guzman *et al.*, 1994; Prast *et al.*, 1998) and inhibit ACh release (Nordström and Bartfai, 1981; Nordström *et al.*, 1983; Suzuki *et al.*, 1993). NO also is an anterograde messenger in cholinergic neurons (de Vente *et al.*, 2000), meaning that it always facilitates a reaction in the frontward direction ACh release however, is not always directly influenced by NO-mediated cGMP synthesis (de Vente, 2004).

Glutamate receptors regulate cGMP efflux in the hippocampus (Fedele *et al.*, 1996; 1997; 1998; 2001). This, or that ACh levels were not increased in the hippocampus of FSL rats and as such could not stimulate cGMP synthesis (Lenox *et al.* 1980), could explain the unchanged baseline levels of cGMP in the hippocampus of FSL rats observed in the current study.

In cultured cortical and frontoparietal cortex and other neurons, NO donors enhance acetylcholine release (Prast *et al.*, 1995; Kopf *et al.*, 2001; Kraus and Prast, 2001; Buchholzer and Klein, 2002), yet NO levels were not enhanced in the frontal cortex or the hippocampus of the FSL rat in the current study. However, increased ACh in the frontal cortex of FSL rats might reflect a lack of anterograde suppression of ACh by the frontward influence of NO. The various contradictions in results from this and other studies emphasises that the effect of ACh on NO and ACh/ cGMP relationship

are still unclear. Further studies, however, are also needed to probe other possible explanations. For example earlier studies that describe an ability of sildenafil, a selective cGMP-PDE 5 inhibitor, to bolster cholinergic signalling (Devan et al, 2004; Brink et al, 2008) prompts further investigation into the regulation of cGMP levels in the FSL rat by phosphodiesterases, which might further elucidate the mechanisms underlying acetylcholine-NO-cGMP signalling in the FSL rat.

Many of the results described thus far with respect to ACh-cGMP may be related to the unchanged nitric oxide levels in FSL rats, which are otherwise normally driven by elevated NO levels (Bredt & Snyder, 1990). However, it is well known that stress activates NOS (Harvey et al, 2004; 2006). A recent study in our group has confirmed that FSL rats can only be separated from their FRL controls at the level of NO synthesis by exposing the animals to stress (Wegener et al, 2009; manuscript submitted for publication). These authors studied various molecular markers of the glutamate NMDA-NOS cascade in FSL and FRL rat in the presence and absence of a subacute stressor. In the presence of stress, but not under basal conditions, FSL rats displayed increased hippocampal expression of nNOS and post-synaptic density protein 95 (PSD95), a protein that is coexpressed with nNOS in several neuronal populations (Brenman *et al.*, 1996). In addition, the expression of the protein inhibitor of nNOS (PIN) was decreased, thereby confirming that stress mediates an exaggerated NO response in stress vulnerable FSL animals (Wegener *et al.*, 2009). Indeed, it is well recognized that the depressive phenotype of FSL rat is dependent on the animal's greater sensitivity to stress than FRL rats (Overstreet, 2002), thus suggesting that the increased sensitivity of these animals to stress, as well as their depressiogenic phenotype, may be causally related to increased activation of the NO-cascade during times of stress (Wegener et al, 2009; manuscript submitted for publication). Plausible mechanisms whereby underlying factors cause allostatic disturbances revolve around the fact that major depression is ultimately a stress-related disorder (Sapolsky, 2001). Ongoing and unresolved stress inhibits neurogenesis, decreases cell proliferation (Kempermann, 2002), causes shrinkage of neuronal networks and induces neurotoxicity by the up-regulation of excitatory pathways that mediate synaptic plasticity (Sapolsky, 2001).

The current study therefore confirms an earlier observation from our group that stress-naive FSL rats do not present with changes in limbic NO/cGMP signalling. However, that a significant decrease in frontal cortical cGMP was observed in FSL

rats is particularly noteworthy, possibly suggesting that ACh is an alternative NO-independent mechanism for activation of the NO cascade, thereby regulating cGMP, in this case suppressing its synthesis. One possible mechanism may involve the activation or inhibition of various cGMP-specific phosphodiesterases (Beavo, 1995; Soderling and Beavo, 2000).

7 Conclusion

Under basal conditions, FSL rats do not present with significant changes in markers of the NO cascade in the hippocampus and frontal cortex compared to FRL controls, including NOS activity as well as NO_x accumulation. However, cGMP levels are significantly lower in the frontal cortex of the stress-naive FSL rat, although not in the hippocampus. These data support an interaction between the NO/cGMP pathway and the cholinergic system in the frontal cortex but not hippocampus of FSL animals, although the mechanisms and implications of such involvement need further clarification. Further, this anatomical differentiation may have important implications for understanding the role of NO in the depressive-like behaviour of the FSL rat and, indeed, may reveal more on the neurobiology and treatment of depression.

8 Acknowledgements

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10 Figure legends

Figure 1a:

Concentration NO_x in μM per 2-5 mg/ml protein in the frontal cortex of Flinders Resistant Line and Flinders Sensitive Line rats. Data were analyzed using the Mann-Whitney U Test and non-significance indicated as $p > 0.05$ (N.S) ($n=10$; mean \pm SD).

Figure 1b:

Concentration NO_x in μM per 2-5 mg/ml protein in the hippocampus of Flinders Resistant Line and Flinders Sensitive Line rats. Data were analyzed using the Mann-Whitney U Test and non-significance indicated as $p > 0.05$ (N.S) ($n=10$; mean \pm SD).

Figure 2a:

Concentration L-citrulline in μM per 30-50 μg protein in the frontal cortex of Flinders Resistant Line and Flinders Sensitive Line rats. Data were analyzed using the Mann-Whitney U Test ($n=10$; mean \pm SD).

Figure 2b:

Concentration L-citrulline in μM per 30-50 μg protein in the hippocampus of Flinders Resistant Line and Flinders Sensitive Line rats. Data were analyzed using the Mann-Whitney U Test (N.S) ($n=10$; mean \pm SD).

Figure 2c:

Difference in L-citrulline concentration between Figure 2a test and control samples in μM per 50 μg protein in the frontal cortex of Flinders Resistant Line and Flinders Sensitive Line rats. Data were analyzed using the Mann-Whitney U Test and non-significance (N.S) indicated as $p > 0.05$ ($n=10$; mean \pm SD)

Figure 2d:

Difference in L-citrulline concentration between Figure 2b test and control samples in μM per 30-50 μg protein in the hippocampus of Flinders Resistant Line and Flinders Sensitive Line rats. Data were analyzed using the Mann-Whitney U Test and non-significance (N.S) indicated as $p > 0.05$ ($n=10$; mean \pm SD)

Figure 3a:

cGMP concentration in pmol/mg/ml protein in the frontal cortex of Flinders Resistant Line and Flinders Sensitive Line rats. Data were analyzed using the Mann-Whitney U Test and significance indicated as * $p < 0.05$ (n=10; mean \pm SD)

Figure 3b:

cGMP concentration in pmol/mg/ml protein in the hippocampus of Flinders Resistant Line and Flinders Sensitive Line rats. Data were analyzed using the Mann-Whitney U Test and non-significance (N.S) indicated as * $p > 0.05$ (n=10; mean \pm SD).

CHAPTER 4

Chromatographic Methods

This chapter will deal with developmental aspects of the High-Performance Liquid Chromatography (HPLC) methods for the analytical analysis of nitric oxide. Methods were developed in the Analytical Technology Laboratory (ATL) at the Pharmacology and Unit for Drug Research and Development, North-West University, Potchefstroom Campus. The complete reaction assay of each method was included into the article, Chapter 3.

4 General information

a) Chromatographic instrumentation

All chromatographic instrumentation and mobile phase compositions were listed in the article, Chapter 3.

b) Experimental instrumentation

Consort P901 electrochemical analyzer (pH meter), Sartorius BP211D balance, Sartorius BP211D balance, 96 well plate reader and 560 nm filter (Labsystems multiskan RC), Polytron[®] Homogenizer (Kinematica, Switzerland), Pierce, Reacti-Therm Heatingmodule, Horo Stuttgart-Hedelf oven, Beckman 61 electrochemical analyzer (pH meter), Sartorius BP211D balance, Eppendorf micropipettes.

c) Spectrophotometric determination of sample protein concentration

The protein concentration of all samples was determined with a Bradford Protein Assay Method for 96-Well Plates (Bradford, 1976) in the Laboratory for Applied Molecular Pharmacology (LAMB) at the North-West University, Potchefstroom campus.

The goal of this experiment was to quantify samples accurately, according to the amount of protein present so that results are reproducible.

Sample homogenate was obtained, by centrifugation of samples for 1 hour at 5 400 g, which is the lowest speed that yields a reproducible clear supernatant and preserves neuronal nitric oxide synthase (nNOS) (Pearce *et al.*, 1997).

A fixed amount of homogenate was diluted to give a protein concentration within the standard solution concentration range of 0.1 – 1.4 mg/ml.

BSA – bovine serum albumin (Sigma-Aldrich) was used for the standard series. The absorbance in each well was determined with a 96-well plate reader (Labsystems multiskan RC) at absorbance in the 595 nm region (shift in absorbance max from 465 – 595 nm). Protein concentration of the brain homogenate could be calculated using the mathematical equation obtained when the net absorbance is plotted against the protein concentration of the standards.

Table 11: Standard protein concentration series

Protein concentration (mg/ml)	Dilution procedure	
	Volume of 2 mg/ml BSA (µl)	Volume of Homogenization buffer (µl)
0.0	0	100
0.1	5	95
0.4	20	80
0.7	35	65
1.0	50	50
1.4	70	30

4.1 NO_x determination

Most compounds including nitrite and nitrate do not fluoresce, but compounds containing aromatic rings give the most intense and most useful molecular fluorescence emission (Skoog *et al.*, 1996).

Total brain NO_x (nitrite and nitrate) concentration was determined in the frontal cortex and hippocampus with a fluorometric, isocratic reversed-phase liquid chromatography method, which involves the pre-column derivatization of nitrite with 2,3-diaminonaphthalene (DAN) after the enzymatic conversion of nitrate into nitrite.

The interaction of nitrite with the relatively nonfluorescent compound DAN adds a fluorophore to the nitrite molecule, resulting in the highly fluorescent and stable

aromatic reaction product, 2,3 naphthotriazole (NAT). This simple and rapid derivatization enables the fluorometric detection of NAT, following HPLC separation of DAN and NAT. Statistical method validation met the general requirements of ISO 17025, 2005 and SANS 17025 (Addendum 1) (SABS, 2005; see also South African Bureau of Standards).

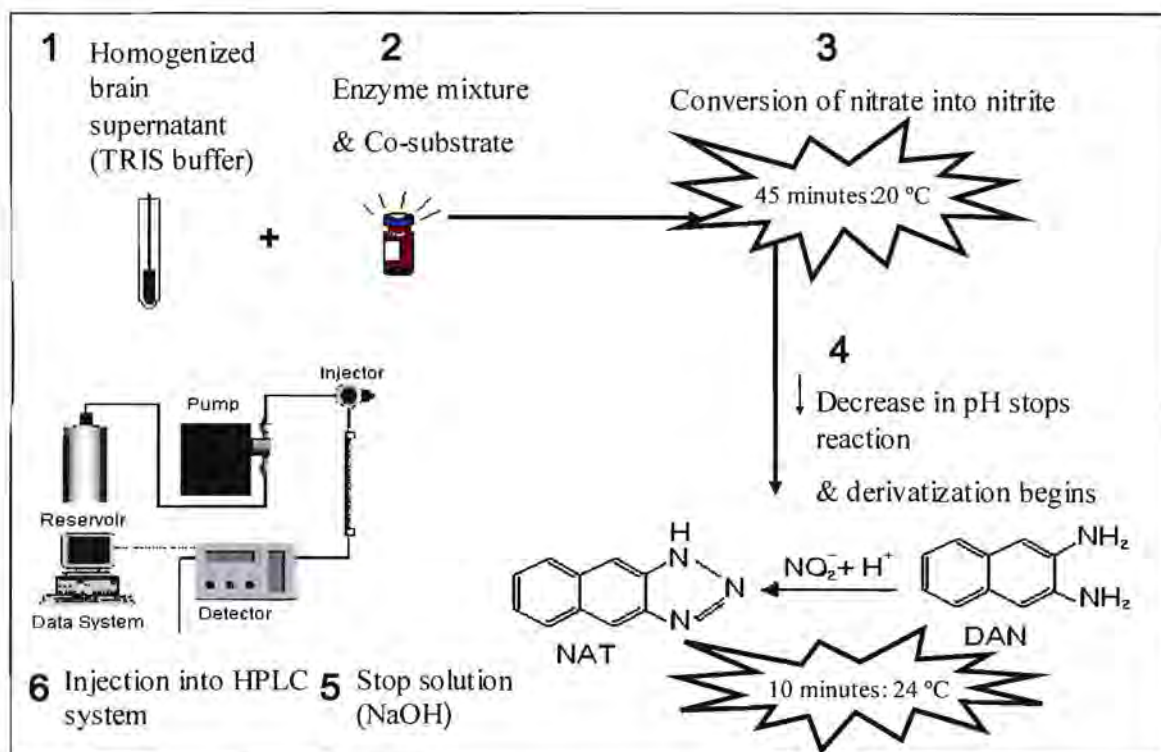


Figure 12: Schematic presentation of reaction steps of NO_x determination and the reaction of nitrite with 2,3-diaminonaphthalene (DAN) to yield 2,3-naphthotriazole (NAT) under acidic conditions (Li *et al.*, 2000; Jobgen *et al.*, 2007)

NAT acts as direct indication of NO_x concentration, when fully derivatized, because of its stoichiometric (1:1) relationship with total nitrite concentration (NO_x / converted nitrite + other nitrite).

The HPLC-fluorescence method is a popular and accurate way to analyze biological samples (Jobgen *et al.*, 2007), provided that important precautions be taken throughout the entire assay to prevent sample contamination (Fernández-Cancio *et al.*, 2001). The main assay interferences are nitrite contamination (as illustrated in Section 2.4) and quenching of fluorescence by β-nicotinamide adenine dinucleotidephosphate (β-NADPH). Environmental nitrite contamination affects nitrite

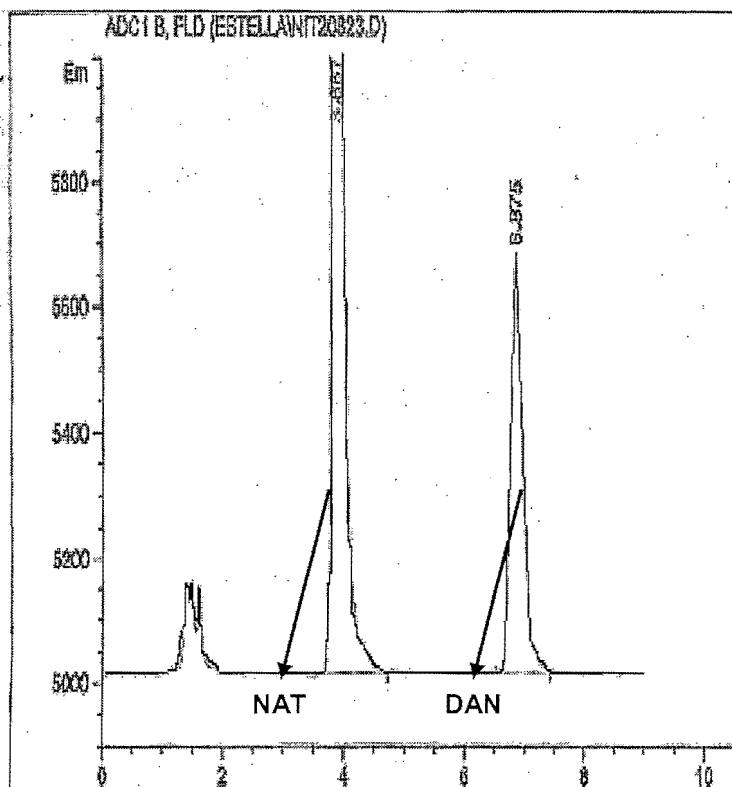


Figure 14: HPLC chromatogram representing a rat brain sample derivatized with 2,3-Diaminonaphthalene (DAN) to yield NAT (2,3-Naphthotriazole)

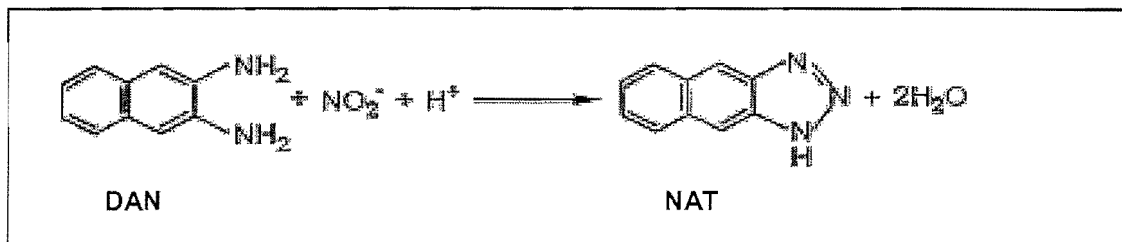


Figure 15: Reaction of nitrite with 2,3-diaminonaphthalene (DAN) to yield 2,3-Naphthotriazole (NAT) under acidic conditions

4.1.2 Chemicals and Reagents

Chemicals were of analytical grade or higher purity and stored at specified conditions. All aqueous solutions were prepared using HPLC-grade water. All chemicals employed are listed in table 12.

Table 12: List of reagents and chemicals used for quantification of NO_x

Category	Chemical name	Abbreviation	Supplier
Co-substrate	β -Nicotinamide adenine dinucleotide phosphate	β -NADPH	Sigma-Aldrich
Derivatization agent and pH adjustment	2,3-Diaminonaphtalene	DAN	
	Hydrochloric acid 32% uniVar	HCl	Saarchem
End or sub reaction products and Standards	Sodium nitrite	NaNO ₂	Sigma- Aldrich
	Sodium nitrate	NaNO ₃	
Enzyme mixture	Nitrate Reductase lyophilized powder, > 300 units/g solid from <i>Aspergillus sp.</i>	NR	
	D-glucose-6-phosphate monosodium salt	G-6-P	Fluka Biochemika
	glucose-6-phosphate Dehydrogenase from <i>Leuconostoc mesenteroides</i>	GD	
Phosphate buffer (enzyme mixture)	Potassium dihydrogen orthophosphate	KH ₂ PO ₄	Saarchem
Homogenization buffer	Tris (hydroxymethyl) aminomethane	TRIS	Saarchem
	Ethylenediamino-tetraacetic acid	EDTA	PAL chemicals
	Ethylene glycol-bis (β -aminoethyl eter), N-N-N' tetraacetic acid	EGTA	Sigma-Aldrich

Category	Chemical name	Abbreviation	Supplier
Mobile phase	Potassium Dihydrogen Orthophosphate	KH ₂ PO ₄	Saarchem
	Sodium hydroxide pearls	NaOH	Saarchem
	Methanol – HPLC Grade	CH ₃ OH	Merck
pH adjustment of Mobile Phase	Orthophosphoric Acid 85% AR	H ₃ O ₄ P	ACE
Stop Solution & Phosphate buffer pH adjustment	Sodium hydroxide pearls	NaOH	Saarchem

The preparation of reagents displayed in Table 12, was described in the article, Chapter 3, under NO_x determination, Section 4.3.1 a).

Additionally, in both the NO_x and NOS assays, it was important to add the required quantity of β -NADPH separately to the sample, during the experiment, after the master mix (containing NR in the enzyme mixture and buffer) was added to the sample. If NADPH is added to the master mix (and a few minutes allowed to elapse) before addition of sample, the reduction reaction does not proceed (Verdon *et al.*, 1995).

Furthermore, the solubility of DAN in HCl (0.76 M) was unsatisfactory, even after 5 minutes of sonication. A few drops of methanol solution, added to the DAN/ HCl mixture, enhanced solubility during sonication by breaking surface tension between grains of 2,3-diaminonaphtalene powder.

Concerning calculations for the Nitrate Reductase concentration:

The concentration of NR (15 mU) was calculated according to Sigma-Aldrich's product information leaflet which states that for 1 U/ml nitrate reductase: "one unit will reduce 1, 0 μ mole of nitrate per minute in the presence of β -NADPH at pH 7,5."

Table 13: Calculation of 15 mU nitrate reductase

NO_x assay calculation of nitrate reductase concentration

e.g. 41 samples x 50 μ l (15 mU)

= 2050 μ l

\approx 2100 μ l

15 mU = 50 μ l

630 mU = 2100 μ l

10 000 mU = 11.96 mg

630 mU = 0.75348 mg

.□

4.1.3 Simple dilution of Stock Solution to obtain standards

Two series of simple dilutions must be performed in order to obtain a final concentration range of 1 ng/ml-5 μ g/ml. Simple dilutions was chosen above serial dilutions to cancel out the possible carry-over of mistakes.

Dilution steps are illustrated in table 14. The first step was to make up a Stock Solution. For Stock Solution (SS), 15.0 mg NaNO₂ and 13.7 mg NaNO₃ was weighed out and made up to 100 ml with TRIS buffer; the final concentration of each component was 100 μ g/ml. From the SS, solution 11 was made up by diluting 100 μ l of the SS with 9,900 μ l of TRIS buffer to a final volume of 10 ml.

The rest of the solutions were obtained in a similar manner, by diluting specific aliquots of either solution 11 or the SS (as indicated in table 14) with TRIS buffer.

Table 14: Simple dilutions, dilution factors and working concentration range of nitrite and nitrate

	Molarity (μM -nM)		Concentration of NO_2 and NO_3 respectively (ng/ml & $\mu\text{g}/\text{ml}$)	Dilution	+	TRIS Buffer	=	Tot. Vol.	Dilution Factor
	NO2	NO3							
	Make up to 10 ml								1:x
1	21.74 nM	16.13 nM	1 ng/ml	10 μl (11)	+	9990 μl	=	10 ml	1000
2	108.7 nM	80.66 nM	5 ng/ml	50 μl (11)	+	9950 μl	=	10 ml	200
3	217 nM	161 nM	10 ng/ml	100 μl (11)	+	9900 μl	=	10 ml	100
4	543 nM	403 nM	25 ng/ml	250 μl (11)	+	9750 μl	=	10 ml	40
5	1.08 μM	807 nM	50 ng/ml	500 μl (11)	+	9500 μl	=	10 ml	20
6	1.63 μM	1210 μM	75 ng/ml	750 μl (11)	+	9250 μl	=	10 ml	13.3
7	2.17 μM	1610 μM	100 ng/ml	10 μl (SS) or 1000 μl (11)	+	9990 μl	=	10 ml	10000 (SS) or 400
8	5.43 μM	4030 μM	250 ng/ml	25 μl (SS)	+	9975 μl	=	10 ml	400
9	10.87 μM	8.07 μM	500 ng/ml	50 μl (SS)	+	9950 μl	=	10 ml	200
10	16.3 μM	12.10 μM	750 ng/ml	75 μl (SS)	+	9925 μl	=	10 ml	133.3
11	21.74 μM	16.13 μM	1 ng/ml	100 μl (SS)	+	9900 μl	=	10 ml	100
12	108.7 μM	80.66 μM	5 $\mu\text{g}/\text{ml}$	500 μl (SS)	+	9500 μl	=	10 ml	20

4.1.4 Working concentration range of standard solutions

The working range of standard solutions contained 5–150 ng/ml NO₂ and NO₃. The percentage of sodium NaNO₂ and NaNO₃ compounds were compensated for to ensure a final SS containing 100% pure NO₂ and NO₃ respectively.

Table 15: Components of NaNO₂ and NaNO₃

Reagent	Molecular weight (MW)
NaNO ₂	69.00 (100%)
Na	23.0 (33.33%)
NO ₂	46.0 (66.67%)
NaNO ₃	84.99 (100%)
Na	23.0 (27.07%)
NO ₃	61.99 (72.93%)

In each standard solution, the NO₃ (nitrate) was converted to NO₂ (nitrite) with nitrate reductase before derivatization with DAN, as previously explained. The reputed recovery rate after this nitrate conversion is approximately 98% (Li *et al.*, 2000). For conformation, standard solutions containing only nitrite were analyzed. Nitrate solutions amounted to approximately half of the AUC – and therefore half of the concentration, of standard solutions that contained both unconverted nitrite and converted nitrite.

The total concentration of a standard solution, therefore comprises of approximately 50% unconverted NO₂ and 49% converted NO₂, thus instead of the SS being 200 ng/ml nitrite, the estimated concentration was 198%.

Table 16, represents standard solutions in ng/ml or nM concentrations used to quantify samples after the regression curve of NO_x, from table 16, displayed a positive, almost perfect linearity of 0.9988 (Addendum 1).

Table 16: Concentrations and AUCs of standard solutions

ng/ml	nM	AUC
9.90	215.23	3491.30
19.80	429.66	3670.10
49.50	1075.14	5534.70
99.00	2138.40	9270.20
148.5	3227.40	12559.4
198.0	4296.60	15477.9
295.0	10751.4	37499.7

4.1.5 Experimental precautions

Effect of environmental contamination on NOx standard curve

Figure 16 represents a standard curve after standards were prepared in glass apparatus.

Pipette tips were not pre-flushed during the dilution procedure or before each addition in the assay. Glass apparatus and HPLC-vials were also rinsed with HPLC-grade water and dried before use.

Table 17: The influence of environmental contamination on the NOx standard concentration curve

Conc. (ng/ml)	AUC
0.99	11409.2
4.95	6257.9
9.9	5537.5
24.75	7922.7
49.5	13854.7
74.25	18562.8
99	25522.7

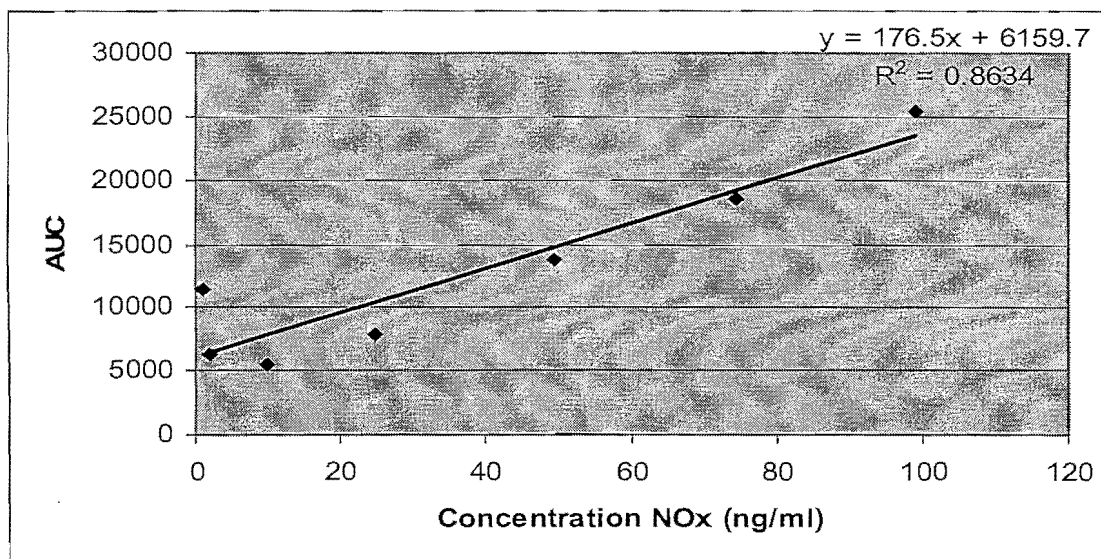


Figure 16: Standard concentration curve of NOx without precautions taken against environmental NOx contamination

In Figure 16, the effect of environmental contamination can be observed at especially the lower NOx standard concentrations (1 and 5 ng/ml or approximately between 20 and 200 nM).

Table 18: NOx standard concentration AUCs after precautions against contamination have been taken

Std conc. μM	Std conc. nM	Std conc. (ng/ml)	AUC
0.02	20.000	0.990	2964.10
0.22	215.25	9.900	3491.30
0.43	429.66	19.80	3670.10
1.08	1075.14	49.50	5534.70
2.14	2138.4	99.00	9270.20
3.23	3227.4	148.5	12559.4

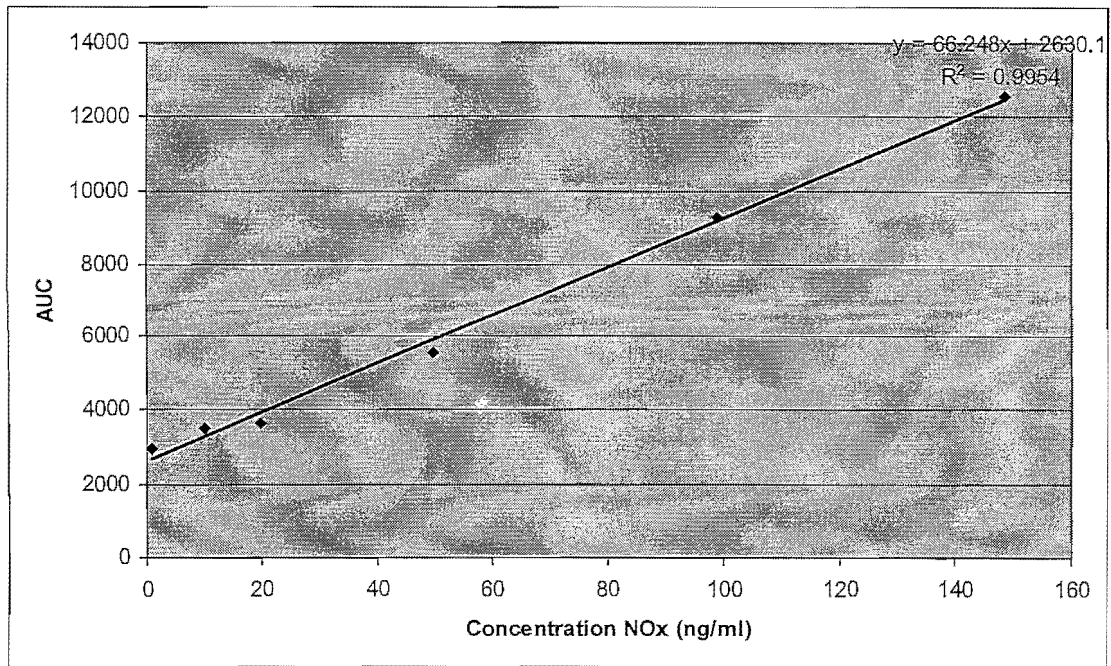


Figure 17: Standard concentration curve of NOx following precautions taken against environmental NOx contamination

As can be observed in figure 17, contamination could be drastically reduced with the necessary precautions being taken throughout sample preparation. These precautions included that all pipette tips were flushed a minimum of three times with TRIS buffer and then again with the particular reagent to be pipetted. All eppendorf vials were also rinsed with TRIS buffer and left to dry before use.

4.2 NOS-activity determination

The amino acid, L-citrulline, which serves as an indication of nNOS – activity, (see Chapter 2, section 2.3.4; chapter 3, section 4.1.3.2), were determined in the frontal cortex and hippocampus with a customized simple, sensitive and reproducible isocratic reversed-phase liquid chromatography method with amperometric electrochemical detection (Harvey *et al.*, 2002). This method can also be used for the quantification of L-citrulline, L-arginine, L-glutamate and γ -aminobutyric acid (GABA) in rat brain tissue.

The method principle was based on a fluorescence detection, HPLC method, developed for the determination of NOS activity through NOx (Fernández-cancio *et*

al., 2001), but on our HPLC, with electrochemical determination – the method can be used for the quantification of L-citrulline, L-arginine, L-glutamate and GABA in rat brain tissue. The method involves the *in vitro* activation of the nNOS enzyme in the brain tissue sample by addition of a cofactor, co-substrate reaction cocktail and an enzyme mixture as well as the NOS-substrate L-arginine as illustrated in Figure. 18. Control samples contained all of the above components, except L-arginine. The assay was carried out at an enzyme concentration of 30 – 50 μg protein, adequate enough to convert the added substrate to product, but prior to reaching steady state (Harvey *et al.*, 2004).

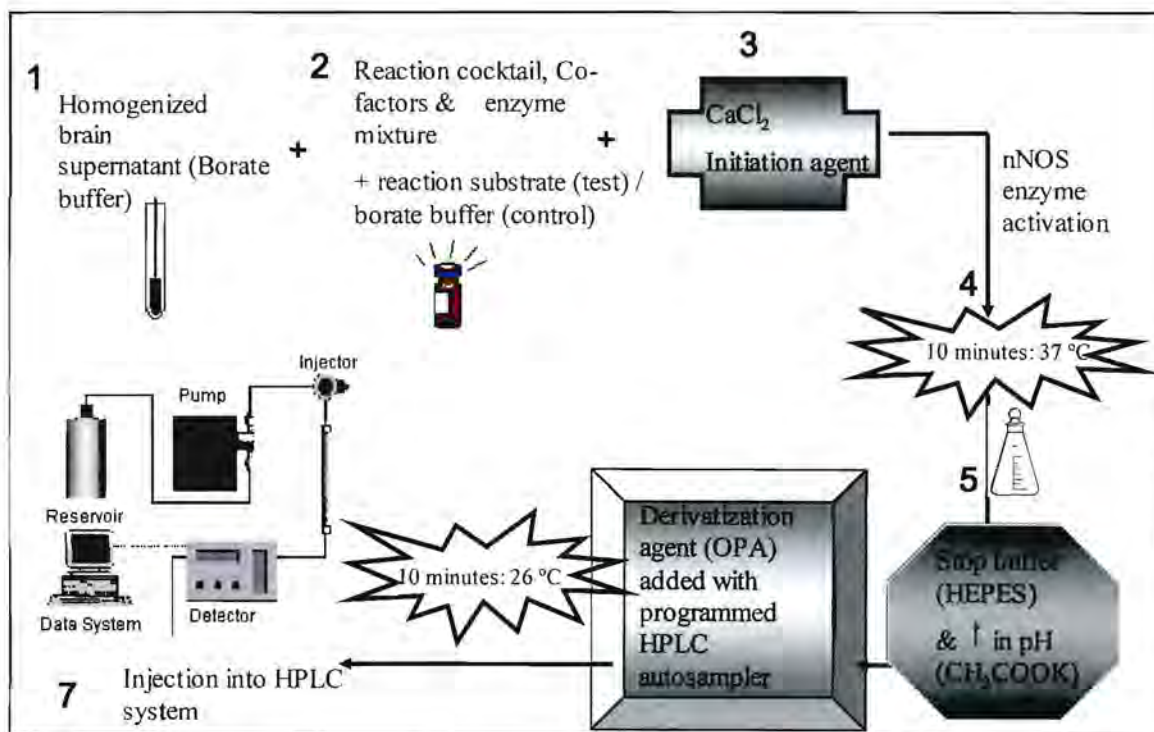


Figure 18: Schematic presentation of reaction steps of NOS activity assessment by measuring L-citrulline

4.2.1 NOS Chromatogram

Figure 19 displays an example of the resultant chromatogram after L-citrulline, L-arginine, Glutamate and GABA in a rat brain sample was derivatized with OPA.

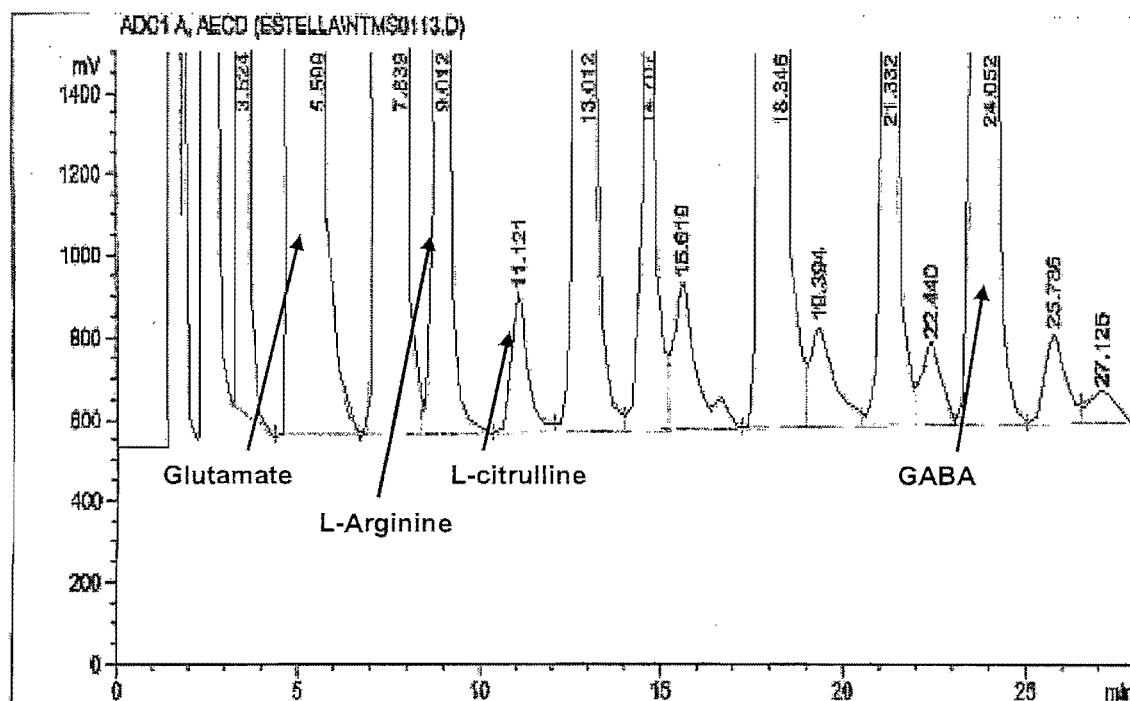


Figure 19: HPLC chromatogram representing a rat brain sample derivatized with OPA

4.2.2 Chemicals and Reagents

Chemicals were of analytical grade or higher purity and stored at specified conditions. All aqueous solutions were prepared using HPLC-grade water. All chemicals employed are listed in table 19.

Table 19: List of reagents and chemicals used for quantification of L-citrulline

Category	Chemical name	Abbreviation	Supplier
Co-factors	Riboflavin 5'-monophosphate sodium salt dihydrate	FMN	Sigma-Aldrich
	Flavin adenine dinucleotide disodium salt hydrate	FAD	
	(6R)-5,6,7,8-Tetrahydrobiopterin dihydrochloride	BH ₄	
Co-substrates	β -Nicotinamide	β -NADPH	

Category	Chemical name	Abbreviation	Supplier
	adenine dinucleotide phosphate		
	Calmodulin from bovine brain	CaM	
Derivatization agent	o-phthalaldehyde	OPA	Pierce
Enzyme mixture	Nitrate Reductase lyophilized powder, > 300 units/g solid from <i>Aspergillus sp.</i>	NR	Sigma-Aldrich
	D-glucose-6-phosphate monosodium salt	G-6-P	Fluka Biochemika
	glucose-6-phosphate Dehydrogenase from <i>Leuconostoc mesenteroides</i>	GD	
End-product and Standard	L-citrulline	$C_6H_{13}N_3O_3$	Sigma-Aldrich
Homogenization buffer (Borate buffer)	sodium tetraborate decahydrate	-	Riedel – de Haën
	Sodium chloride	$NaCl_2$	Acros
	Boric acid powder	H_3BO_3	Merck
	Hydrochloric acid 32% uniVar	HCl	Saarchem
Initiation agent	Calcium chloride	$CaCl_2$	Saarchem
Mobile Phase	di-sodium Hydrogen Phosphate	-	Merck
	Ethylenediamine-tetra-acetic acid	EDTA dinatriumsalt Na_2EDTA	Sigma-Aldrich
	Methanol (HPLC-grade)	CH_3OH	Merck
	orthophosphoric acid	H_3O_4P	ACE
pH adjustment	Potassium acetate	CH_3COOK	BDH
Reaction substrate	L-arginine hydrochloride	$C_4H_9NO_2$	Sigma-Aldrich
Stop buffer	HEPES	-	Sigma-Aldrich
	Ethylenediamine-	EDTA	

Category	Chemical name	Abbreviation	Supplier
	tetra-acetic acid		
	Sodium hydroxide pearls	NaOH	Saarchem

The initial and final concentrations of components of the reaction cocktail are listed in table 20. Final concentrations were adequate for the reaction to optimally occur (Brenda *et al.*, 2009).

Table 20: Reaction cocktail component volumes and concentrations

Substance	Mw	Volume added in assay (µl)	Amount weighed (mg)	Volume of SS (ml)	Initial (SS) concentration	Final concentration in assay	Final volume of assay for reaction
BH₄	314.2	15	0.25	8	100 µM	4 µM	365.5 µl
FAD	829.51	5	0.66	8	100 µM	1. µM	
FMN	478.3	5	0.38	8	100 µM	1 µM	
CaCl₂	110	10	57.2	4	130 mM	3.5 mM	
CaM		12.5	0.8	2	400 µg/ml	13.7 µg/ml	
NADPH	833.4	50	0.5	3	0.2 mM	27.36 µM	
L-Arg	174	134	0.87	100	50 µM	18.3 µM	

A nitrate reductase concentration of 25, 2 mU was calculated for each assay, with the specifications for NR by Sigma-Aldrich (see Section 4.1.2) as in the following example:

Table 21: Calculation of 25.2 mU nitrate reductase

**NOS activity assessment calculation
of nitrate reductase concentration**

e.g. 80 samples x 84 μ l (25.2 mU)

= 6720 μ l.

\approx 6.75 ml.

25.2 mU in 84 μ l

2025 mU in 6750 μ l

2025 mU = 2.4219 mg

The preparation procedure of reagents displayed in Table 19, was described in the article, Chapter 3, under NOS-activity assessment, Section 4.1.3.2 a).

4.2.3 Simple dilution of Stock Solution to obtain standards

Two series of simple dilutions must be performed in order to obtain a final concentration range of 0.1 μ g/ml-5 μ g/ml. Simple dilutions was chosen above serial dilutions to cancel out the possible carry-over of mistakes.

Dilution steps are illustrated in table 22. The first step was to make up a Stock Solution (SS) by weighing 1 mg GABA, 1 mg L-Glutamic acid, 1 mg L-citrulline and 1.21 mg L-arginine and making it up to 10 ml with borate buffer; the final concentration of each component in the SS was 100 μ g/ml.

From the SS, solution A was made up by diluting 2000 μl of the SS with 8000 μl of Borate buffer to a final volume of 10 ml.

The rest of the solutions were obtained in a similar manner, by diluting specific aliquots of solution A (as indicated in table 22) with borate buffer.

Table 22: Simple dilutions, dilution factors and working concentration range of amino acids

	GABA	Glutamate	L-citrulline	L-arginine	Concentration ($\mu\text{g/ml}$)	Dilution	+	Borate Buffer	=	Total Volume
	Molarity									
	(μM)	(μM)	(μM)	(μM)						
1	0.97	0.68	0.57	0.57	0.1	50 μl (A)	+	9950 μl	=	10ml
2	2.43	1.70	1.43	1.44	0.25	125 μl (A)	+	9875 μl	=	10ml
3	4.85	3.39	2.85	2.87	0.5	250 μl (A)	+	9750 μl	=	10ml
4	7.27	5.10	4.28	4.30	0.75	375 μl (A)	+	9625 μl	=	10ml
5	9.70	6.80	5.71	5.74	1	500 μl (A)	+	9500 μl	=	10ml
6	24.25	17.0	14.27	14.35	2.5	1250 μl (A)	+	8750 μl	=	10ml
7	48.50	34.0	28.54	28.70	5	2500 μl (A)	+	7500 μl	=	10ml
C	72.75	50.98	42.81	43.04	7.5	3750 μl (A)	+	6250 μl	=	10ml
B	97.0	67.98	57.08	57.39	10	5000 μl (A)	+	5000 μl	=	10ml
A	193.9864	135.9619	114.1618	114.7842	20	2000 μl (SS)		8000 μl	=	10ml

4.2.4 Working concentration range of standard solutions

The working range of standard solutions contained 0.1 µg/ml–5 µg/ml amino acids.

In table 23, standard solutions are represented in the ng/ml or µM concentrations that were used to quantify samples after the regression curve of the amino acids, from table 9, displayed a positive, significant linearity of 0.9970 (Addendum 1).

Table 23: Concentrations and AUCs of standard solutions

ng/ml	µM	GABA	µM	Glutamate	µM	L-citrulline	µM	L-arginine
100	0.97	2718.8	0.68	485.83	0.57	456.54	0.57	1394.0
250	2.43	3075.7	1.70	849.18	1.43	706.17	1.44	1955.7
500	4.85	4262.2	3.39	1572.6	2.85	1262.6	2.87	2634.1
750	7.27	3060.3	5.10	2095.3	4.28	1685.8	4.30	3095.9
1000	9.70	5868.5	6.80	3027.6	5.71	2447.5	5.74	4045.5
2500	24.25	9729.3	17.0	3.05E+04	14.27	6054.7	14.35	8028.3
5000	48.50	2.04E+04	34.0	1.02E+05	28.54	1.33E+04	28.70	1.49E+04

4.2.5 Experimental precautions and conditions

a) TRIS buffer and OPA interaction

Initially TRIS buffer (25 mM) without EGTA and EDTA, as not to sequester added CaCl₂ (essential for initiation of the reaction), was used. TRIS is the conventional buffer used for enzymatic reactions (Sambrook & Russel, 2000). However, literature does not recommend the use of TRIS buffer with OPA because of the preferable binding of the primary amine group to OPA, as illustrated in Figure 20 (Shiue *et al.*, 2005). The buffer was consequentially adapted to a borate buffer, as was also used in a similar assay than ours (Tcherkas *et al.*, 2001; for the analysis of aminoacids with HPLC-coupled electrochemical detection and OPA derivatisation).

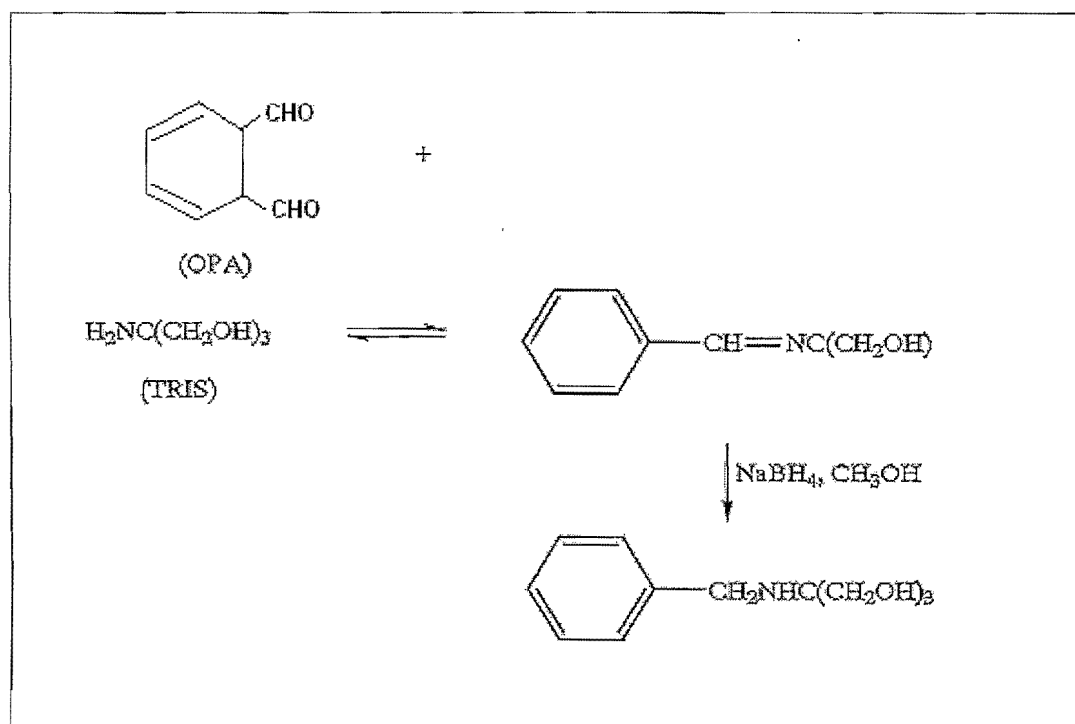


Figure 20: The reaction of OPA with TRIS (Shiue *et al.*, 2005)

b) HPLC programming in OPA derivatization step

The HPLC software's injector program was programmed for precolumn *ortho*-phthalaldehyde derivatization and subsequent injection of 50 μl sample into the HPLC for separation of amino acids.

For the shortest possible runtime with good peak separation the flow rate was increased during latent times so that the last peak's (GABA) retention time was within 30 minutes.

Table 24: Programmed HPLC sequence

Row	Action
1	Draw 5.0 μl from air.
2	Draw 10.0 μl from vial 81.
3	Eject 15.0 μl into sample.
4	Draw 60.0 μl from sample.
5	Eject 60.0 μl into sample.
6	Wait 5.00 min.
7	Draw 50.0 μl from sample.
8	Inject.

Table 11: Flowrate adjustment

Time	Flowrate
0.00	1.00 ml/min
10.00	1.00 ml/min
11.00	1.50 ml/min
29.00	1.50 ml/min
29.90	1.00 ml/min

4.3 Conclusion

Reliable and reproducible NO_x and L-citrulline results were obtained with these in-house developed methods. Both methods displayed an acceptable reproducibility and repeatability. These two HPLC-methods produce results under the described chromatographic and experimental conditions that are repeatable and reproducible with the necessary precautions taken - as was discussed in this chapter. Both methods are suitable for the analysis of rodent brain samples. The HPLC-method for NO_x determination with fluorescence detection offers the advantages of easy sample preparation and derivatization, a stable and highly fluorescent derivative, a short retention time, acceptable sensitivity and specificity and minimized assay interferences.

The HPLC-method for L-citrulline determination with electrochemical detection, offers a reproducible, simple, more sensitive and safer method than the conventionally used radiometric L-citrulline assay, based on the use of radiolabeled arginine and the radiochemical determination of citrulline (Bredt & Snyder, 1990). Radiometric methods have the inconveniences of handling radioactive materials and radioactive Arginine. It usually also involves an added step of arginine separation from citrulline.

Our HPLC-method method can also be used for the simultaneous quantification of L-citrulline, L-arginine, L-glutamate and GABA in rat brain tissue.

CHAPTER 5

Behavioural Assessments

This chapter reports the results and conclusions obtained through behavioural assessments in the current study. Other main topics presented also include an overview of the study layout; experimental animals; apparatus; chemicals; drug dosage and protocols.

The behavioural assessments were performed in the Animal Research Centre at the Potchefstroom Campus of the North-West University that offered the necessary facilities and trained personnel to assist with the study.

5.1 Overview

The behavioural part of this study is divided into the main study, comprising of three different types of behavioural assessments, namely the Hypothermia Challenge, Open Field Test (OFT) and a supplemental assessment, the Forced Swim Test (FST) – as outlined in Chapter 1, Section 1.4, Project Layout.

As illustrated in Figure 21, all behavioural assessments, except for the hypothermia test, were conducted on three separate occasions distributed over the course of the project. The rats were not handled in any way other than to be assessed for their behaviour. Separate groups of Flinders Sensitive Line (FSL) and Flinders Resistant Line (FRL) rats (groups 1, 2 and 3) were used to conduct the OFT. On all three occasions, the OFT was conducted in the second phase of the day/night noctymeral Animal Centre cycle – approximately 6:00 p.m. – 7:30 p.m. After a two hour rest period, the same group of rats (FSL vs. FRL) was used in the FST from approximately 9:30 p.m. – 11:00 p.m.

After completion the assessment on each occasion, the animals were sacrificed. Different groups of rats were therefore used for the neurochemical studies to ensure that the behavioural studies would not influence neurotransmission and thereby bias the neurochemical results.

Schematic study outlay

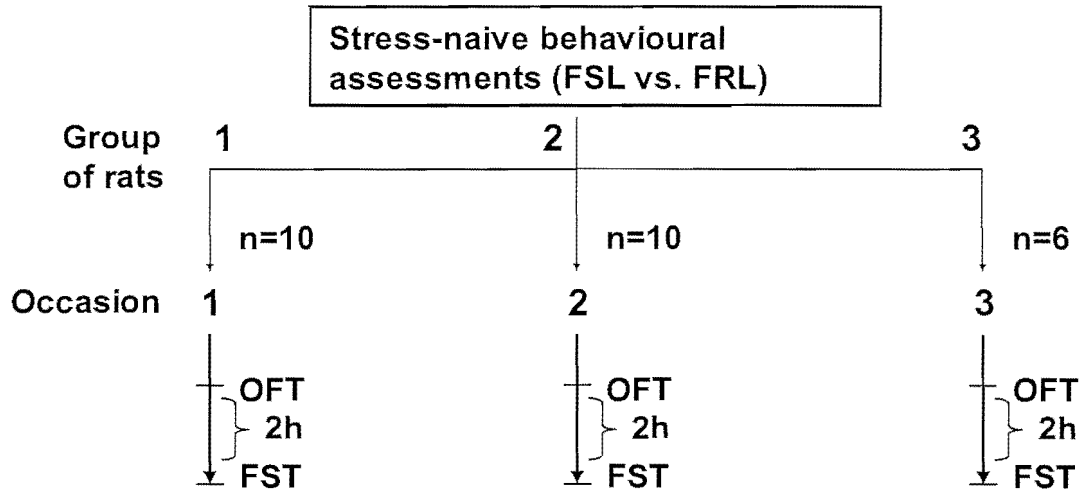


Figure 21: Study outlay of the FST and OFT stress-naïve behavioural assessments

Study aims:

- Behavioural assessments: The aim of these behavioural assessments was to verify key prerequisite congenital behavioural differences (Overstreet *et al.*, 2005) between the stress-naïve FSL rat and its control, the FRL rat. These assessments were thus necessary for validation of the FSL rat model used in the current project.

5.2 Materials and Methods

5.2.1 Experimental Animals

Young adult male FSL and FRL rats weighing 200 ± 20 g were provided by the Animal Centre of the North-West University Potchefstroom campus, after being reared in corn cob cages under conditions described in Chapter 3, Section 3.

Approval of the study protocol was granted by the Animal Ethics Committee of the North-West University (Ethics approval number NWU0003207S2). All animals were treated according to the code of ethics in research as laid down by this Animal Ethics Committee.

All the behavioural assessments as well as the hypothermia test were previously validated under the conditions in our laboratory

5.2.2 Apparatus

Perspex cylinders (diameter 180 mm, height 400 mm); Sony Digital Video Camera Recorder (model: DCR-TRV330E); Open field arena (1x1 m, divided into 16 evenly spaced squares); Digital thermometer; disposable syringes and needles for hypothermia challenge –procedure; Eppendorf micropipettes; Sartorius BP211D balance and Spidy Stop Watch.

5.2.3 Drug and Chemicals

Sodium chloride 0.9% solution (w/v), Isotonic saline, 8-hydroxy-2-(di-*n*-propylamino)tetralin (5-OH-DPAT, Sigma-Aldrich), Petroleum jelly as lubricant.

5.2.4 Experimental Protocols

5.2.4.1. Hypothermia Challenge

The FSL adult rat is supersensitive to the hypothermic effects of serotonin agonists (5HT_{1A}-mediated) with high levels of serotonin being described in the hippocampus (Zangen *et al.*, 1997) compared to the FRL line. Upon challenge of each rat with a serotonergic agonist such as 8-OH-DPAT (Shayit *et al.* 2003), a 2-fold greater decrease in temperature in FSL rats, compared to the FRL rat, is routinely elicited (Overstreet *et al.*, 1994).

Moreover, while FSL rats are bred to be hypercholinergic (as discussed in Chapter 2), it is also important to note that there is distinct evidence for an interaction between serotonergic and cholinergic systems in the FSL rat, and hence in the regulation of temperature (Bert *et al.* 2006; Daws & Overstreet 1999; Overstreet *et al.* 1998; Overstreet 2002). Both systems are also implicated in the neurobiology of depression.

In the hypothermia challenge, FSL and FRL rat groups were injected with a serotonergic agonist, whereafter the degree of hypothermic response was measured

after a time period of 30 and 60 minutes respectively following the injection. The aim of this test was to validate the FSL rat by verifying for prerequisite phenotypical difference in 5-HT_{1A}-R sensitivity (Overstreet *et al.*, 1998) between the FSL and its control, the FRL rat. FRL rats are not hypercholinergic, their core body temperatures should correlate with the expected normal body temperature in rats. The normal body temperature of rats varies from a daytime average of about 37,3°C to a night time mean of approximately 38,1°C (Whishaw & Kolb 2005).

Because of its high affinity for 5HT_{1A} receptors (Middlemiss & Fozard 1983), 8-OH-DPAT was used as the serotonergic agonist.

Accepted dosage and preparation of 8-OH-DPAT

1.5 mg 8-OH-DPAT powder was weighed and made up to 15 ml with isotonic saline solution in a volumetric flask. The flask was sonicated to ensure that the drug was completely dissolved in the vehicle. For a 200 g rat, the dosage of the solution was 0.05 mg/ 0.5 ml (0.25 mg/kg), as described in pilot studies previously conducted at the NWU Pharmacology department. The volume of each injection was adapted for each individual rat, according to its body weight to meet the specified dosage. Higher concentrations of 2 - 4 mg/kg may result in serotonin syndrome (Scott *et al.* 1994), leading to characteristic movements such as hindlimb abduction, forepaw treading, lateral headweaving, resting tremor, hyperactivity, random circling, rigidity or hypertonicity, straight tail and salivation.

Experimental Procedure

The hypothermia challenge was performed between 8:00 a.m. and 1:00 p.m. After transfer to the experimental room, the rats were allowed a habituation period of 30 minutes (White *et al.*, 2004) before initiation of the hypothermia challenge procedure.

As illustrated by figure 22, the core body temperature of each marked and weighed rat was recorded by inserting a lubricated thermometer into the rectum. Temperature was recorded after 15 seconds to the nearest 0.1°C. After performing a duplicate baseline recording, 0.25 mg/kg of the 5-HT_{1A}-R agonist, 8-OH-DPAT in a saline vehicle was subcutaneously (Fuller & Snoddy 1987) injected into each rat. Rat core body temperature was again recorded, 30 minutes and 60 minutes after the injection.

After the completion of the challenge, all used needles and syringes were disposed of correctly in a biological waste container for sharps.

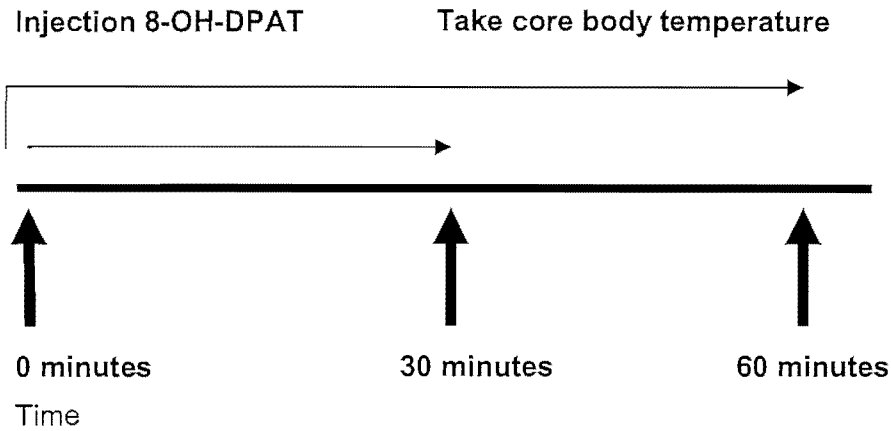


Figure 22: Hypothermia challenge

The data were expressed as mean \pm SEM temperature change from baseline values.

5.2.4.2. Open Field Test

The open field test was used to compare the level of anxiety and the locomotor activity of the FSL rat to the FRL rat. The behavioural parameters utilized in the OFT, were number of line crosses and time spent in middle blocks of each rat, that were placed in the open field arena in pairs.

Rats usually avoid novel, open spaces (Koene *et al.*, 2003), so the OFT environment induces anxiety and allows for measurement of anxiety-stimulated locomotor activity and exploratory behaviors (Overstreet & Griebel, 2005). The OFT challenge displays the conflict between the innate fear that rodents have of the central area of a novel or open field versus their desire to explore new environments (Koene *et al.*, 2003). When anxious, a rodent's nature is to stay close to the walls in the open field arena (thigmotaxis). In this context, anxiety-related behavior and locomotor activity is measured by the degree to which the rodent avoids the center of the OFT arena (time spent in middle blocks), as well as the number of line crosses – representing locomotor activity of the rats, which relates to the exploratory behaviour of the rats. Lastly anxious rats spend less time in social interaction with their cage mates (File & Seth, 2003; Overstreet *et al.*, 2004; Overstreet & Griebel, 2005). A pair of rat normally interact with one another by displaying grooming, licking, sniffing or crawling over or under one another (Overstreet, 2002; Overstreet *et al.*, 2005). In the social interaction test, these parameters were compared between the two rat strains.

Aim

The aim of performing the Open Field Test (OFT) was two-fold. First and foremost, the test served to detect the anxiety/ stress levels of the rats, with the FSL rat being predisposed to depression – in which stress acts as a precipitant (time spent in middle blocks as well as social interaction). Secondly, the difference in locomotor activity between the two rat lines, acted as a further validation of the rat line, as the FSL rat is expected to be more immobile than their FRL counterparts (Overstreet & Griebel, 2005).

Experimental Procedure

The OFT was performed in the evening, during the dark 12 hour cycle of the Animal Centre, during the peak activity period of the rodents (Sharp & La Regina 1998).

For the Open Field Test, rats were always placed in pairs into the centre of a square 'open-field' black test arena (1 x 1 m, divided into 16 symmetrical, squares with white tape).

Recording of behaviour

The total behaviour of the rats during the challenge in the open field arena was recorded with an overhead Digital Video Camera Recorder (model: DCR-TRV330E), over the first 5 minute time period that the rats spent in the OFT.

Scoring technique

- (a) The total number of line crossings for each individual animal during the session (with all four paws), was manually counted while viewing the recorded 5 - minute time period locomotion activity.
- (b) Total time, in seconds spent in the four middle blocks of individual animals was also assessed.
- (a) For social interaction, pairs of rats, placed in the centre of the arena, was scored for the amount of time, in seconds, engaged in behaviour such as grooming, licking, sniffing or crawling over or under each other. This sampling procedure was used for the pair of rats as a unit.

After each assessment session, the test arena was cleaned.

The data were expressed as mean \pm SEM of (a), (b) or (c) respectively.

5.2.4.3. Forced Swim Test

This test was originally described by Porsolt *et al.*, 1978. and is founded by the observation that rats exhibit lethargic behaviour (immobility, except for the minimum movement necessary to keep the rat's head above the water) when they are forced to swim in a limited, inescapable space.

A natural response of an animal to an aversive environment is to attempt to escape, so although rats are good swimmers, they will avoid water, or if placed in water they will strive to escape the water by actively indulging in various swimming and diving behaviours (Porsolt *et al.*, 1978; Cryan *et al.*, 2002). These responses are futile due to the high smooth walls of the swim tank. On repeated exposure, animals realise the futility of the struggle and take on an immobile posture, choosing rather to float in the water and to await rescue by the investigator. More stress sensitive animals, however, will spend less time struggling to escape the tank than their stress-resilient counterparts (Overstreet *et al.*, 2005). This immobility indicates a state of despair in the rat that can be reversed by antidepressant treatment (Porsolt *et al.*, 1978).

The FST was validated in our laboratory in a previous project (Liebenberg, 2006, O3D09). The greater immobility of FSL in comparison with FRL rats (Overstreet 1986; 1993; 2002), makes the pre-test - performed in the original FST (Porsolt *et al.*, 1978), unnecessary (Overstreet *et al.* 2005). In Sprague-Dawley rats, a stressor/ pre-swim of 15 minutes is usually necessary, because the rats are not predisposed to stress. With a pre-swim, differences between the test and control groups are obtained after the rats are placed again into the cylinders, to swim for the normal duration of 5 minutes. The FSL rats however are already sensitive to stress deeming a pre-swim unnecessary (Overstreet *et al.*, 2005). Further stress will only enhance the immobility of FSL rats in the FST.

The water level was adapted to 18 cm, instead of the originally used 30 cm, because of the smaller size of rats, 200 ± 20 g, instead of the traditionally used 300 - 350 g rats.

Experimental Procedure

On the evening of the challenge, two hours after the completion of the OFT, the same two groups of rats (FSL vs. FRL) were exposed to the FST.

Rats were individually placed into upright plexiglass cylinders (diameter 180 mm, height 400 mm height: 40 cm; diameter: 20 cm) separated with opaque screens and filled with ambient water (25°C) to a depth of 18 cm. Behaviour was recorded with a video camera for 5 minutes to score on a later occasion. Rats were placed in their home cages with paper towels to dry for 15 minutes whereafter they were taken back to their usual quarters.

Recording of behaviour

The total active and passive behaviour of the rats during the challenge was separately assessed for each rat during a 5 minute period with a Digital Video Camera Recorder (model: DCR-TRV330E), whereafter different behavioural components were distinguished – as explained in “Scoring technique”. Total immobility time (seconds) that serves as an indication of passive behaviour and represents despair/ depression experienced by the rat, was documented.

Scoring technique

Forced Swim Test Parameters

Active behaviour includes climbing (“upward directed movements of forepaws usually along the side of the swim chamber”) and swimming (“horizontal movement throughout the swim chamber which includes crossing across quadrants of the cylinder”) (see fig 23) in order to escape.

Passive behaviour include immobility which is defined as the absence of active escape oriented behaviour (such as swimming, diving and climbing) or the minimum movement necessary to keep the rat’s head above the water. The rats “disengage active escape and conserves energy while waiting for events to happen that might enhance the chances of escape” (Cryan *et al.*, 2005).

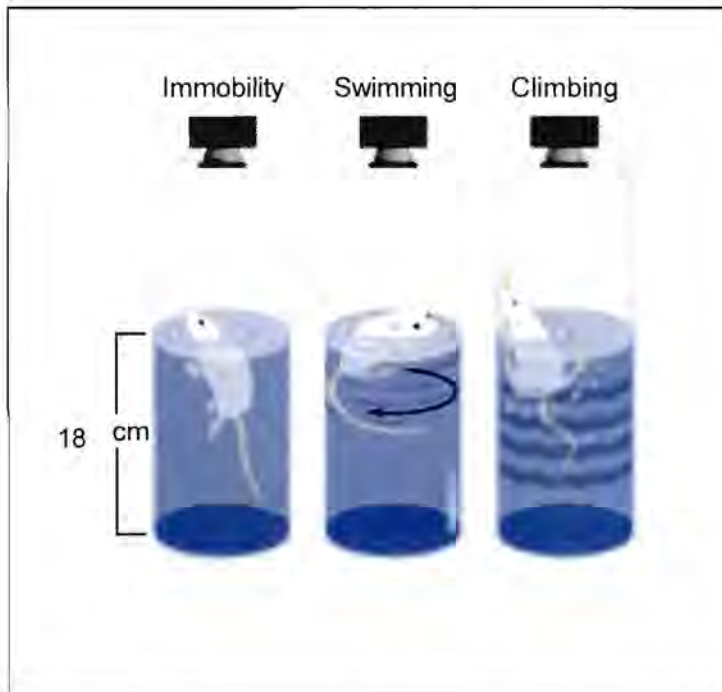


Figure 23: The behavioural parameters distinguished in the forced swim test (Cryan *et al.*, 2002)

Each rat was separately scored for time of immobility in seconds. The FSL group's time of immobility was compared to that of the FRL group and the data were expressed as mean \pm SEM time of immobility.

5.3 Statistical analysis of data

Behavioural data were analyzed with Statistica[®] and graphically presented with Graphpad Prism[®] (Statsoft Inc., 2007, Statistica Data Analysis Software System, version 8; Graphpad software, version 5.0 for Windows[®] San Diego, USA). All data were non-parametrically analyzed, because of the irregular distribution of normal probability study plots, using the Mann-Whitney U Test as will be described shortly.

The representation of data was expressed as means \pm SD and statistical significance of marked tests were defined as * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$ in all instances.

A two-way ANOVA analysis with group and study as factors was performed to determine the interaction between different studies.

5.4 Results

5.4.1 Hypothermia challenge

In the hypothermia challenge the difference in basal temperatures at 30 – and 60 minute time intervals, following injection of 8-OH-DPAT was compared between the FSL and FRL rat groups.

At 30 minutes, the FSL group illustrated an extremely significant decrease in basal temperature (Figure 24: $p=0.000881$) compared to the FRL group. At 60 minutes the FSL group still exhibited a significant basal temperature decrease compared to the FRL group (Figure 24: $p=0.0253$).

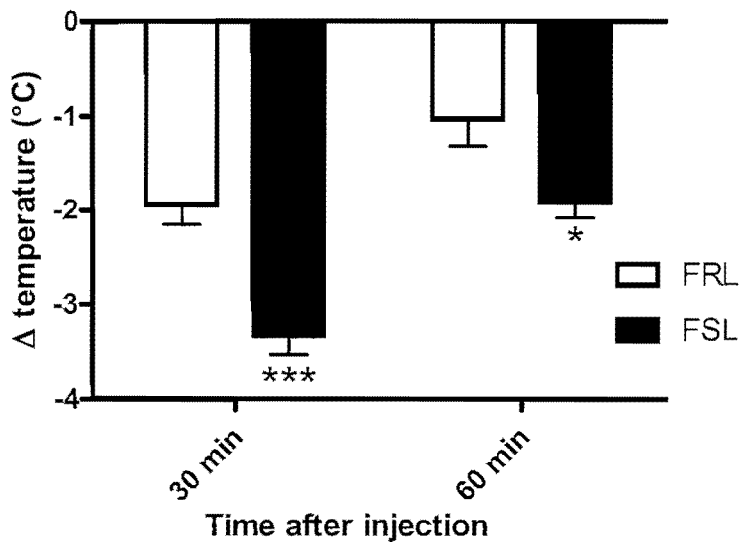


Figure 24: Hypothermia challenge. Basal temperature differences after 30 minutes and 60 minutes following administration of a serotonin agonist (8-OHDPAT) in the Flinders Resistant Line and Flinders Sensitive Line rats. Data were analyzed statistically using the Mann-Whitney U Test and statistical significance was indicated as * $P<0.05$ and *** $P<0.001$. ($n=10$; mean \pm SD)

5.4.2 Open Field Test results

In the OFT all parameters, namely the total number of line crossings, time spent in middle blocks and social interaction were compared between the FSL and FRL rat groups, on all three different occasions. A group study compared all three occasions, to illustrate the effect of the studies on each other (Figure 25, 27 and 29).

a) Total number of line crossings

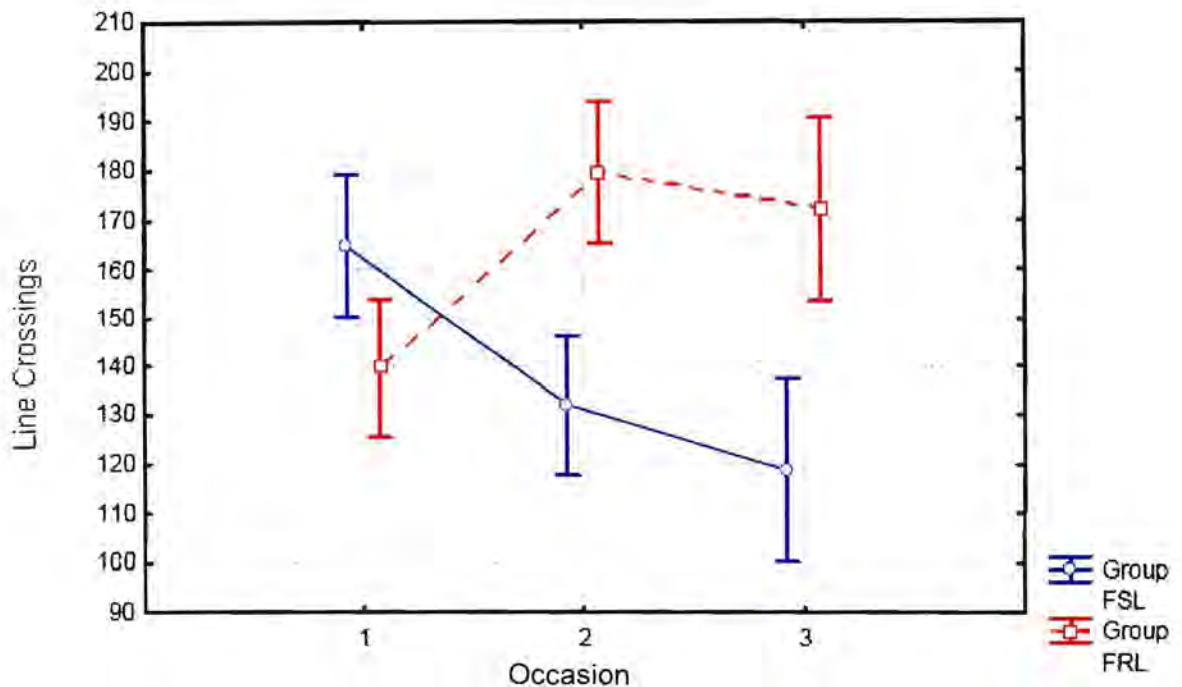


Figure 25: All groups line graph, total number of line crosses of stress-naive rats. Data were analyzed statistically using the Two-way ANOVA

As can be seen in Figure 25, there was a significant interaction between the results of study occasion 1, 2 and 3 ($p=0.029710$). Results were therefore not pooled, but kept separate to avoid the masking of trends in the results, by other occasion's results.

As evident in Figure 26 the number of line crossings on occasion 1, was not significantly different between FSL and FRL rat groups ($p=0.117186$).

On occasion 2, the line crossings of the FSL rat group were significantly less than that of the FRL rat group ($p=0.016294$).

Occasion 3 also showed a significant decrease in line crossings between FSL and FRL rat groups ($p=0.049535$).

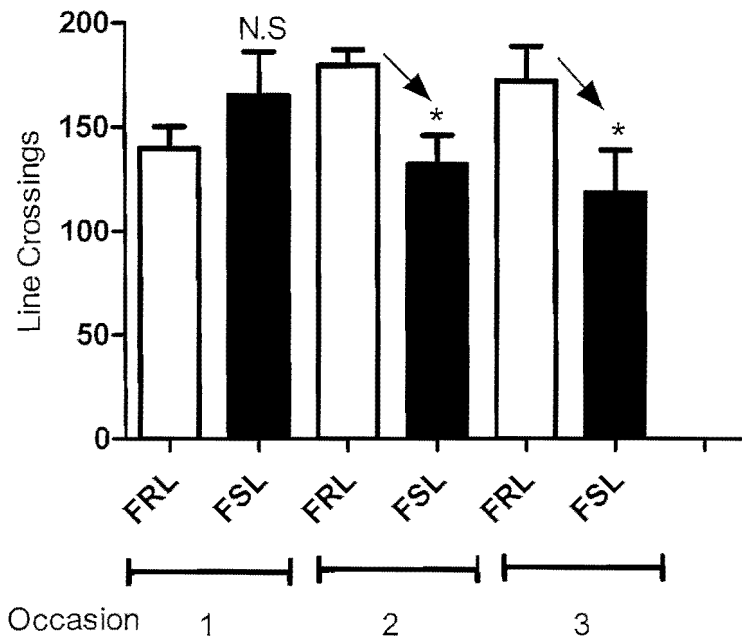


Figure 26: Number of line crosses in different groups of stress-naive Flinders Resistant Line and Flinders Sensitive Line rats on three separate occasions. Data were analyzed statistically using the Mann-Whitney U Test and statistical significance were indicated as $*P<0.05$ and non-significance (N.S.) as $p>0.05$ ($n=6-10$; mean \pm SD)

b) Time spent in middle blocks

As illustrated in Figure 27, there was a significant interaction between the results of study occasion 1, 2 and 3 ($p=0.036070$). Results were therefore also not pooled, but kept separate to avoid the masking of trends in the results, by other occasion's results.

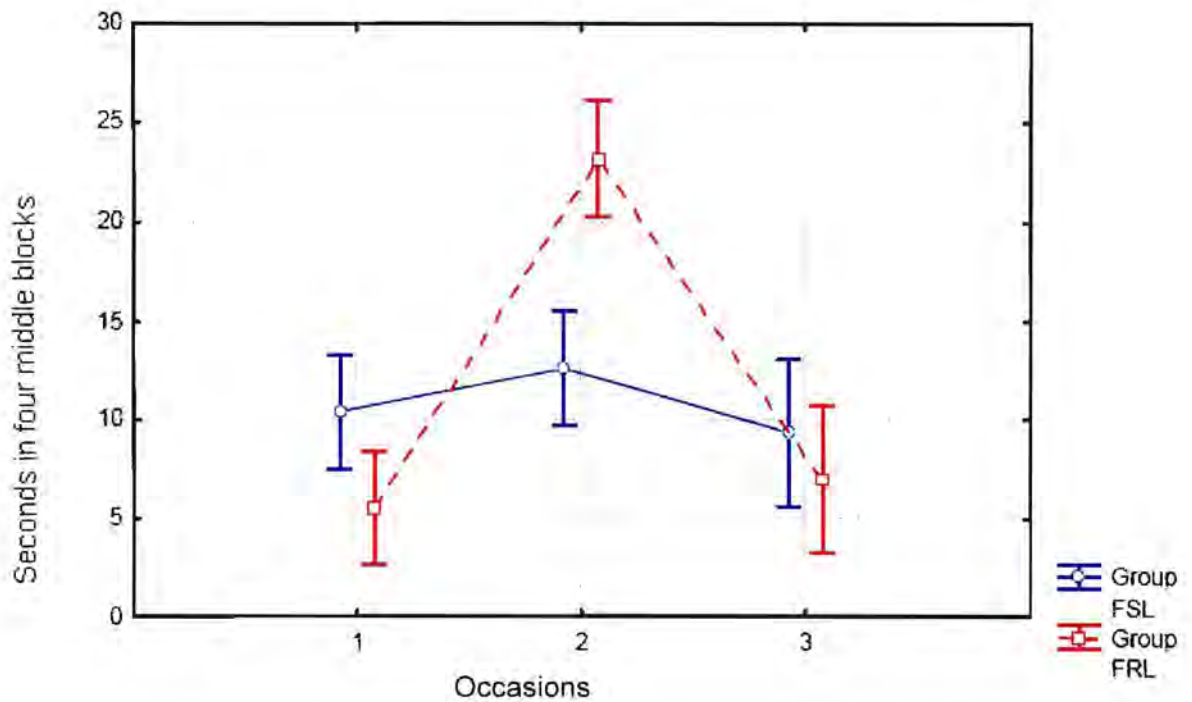


Figure 27: All groups line graph, time spent in middle blocks. Data were analyzed statistically using the Two-way ANOVA

In Figure 28 it can be seen that the time spent in middle blocks on occasion 1, was not significantly different between FSL and FRL rat groups ($p=0.141239$).

On occasion 2, the time spent in middle blocks of the FSL rat group were significantly less than that of the FRL rat group ($p=0.047203$).

Occasion 3 did not show a significant difference in time spent in middle blocks between FSL and FRL rat groups ($p=0.512691$).

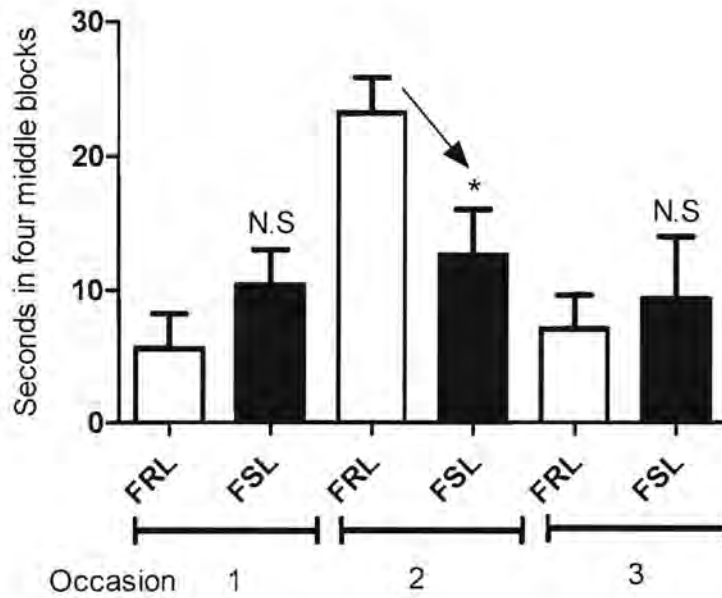


Figure 28: Time spent in middle blocks between Flinders Resistant Line and Flinders Sensitive Line rats during three separate occasions. Data were analyzed statistically using the Mann-Whitney U Test and statistical significance was indicated as * $P < 0.05$ and non-significance (N.S) as $p > 0.05$ ($n = 6-10$; mean \pm SD)

c) *Social interaction*

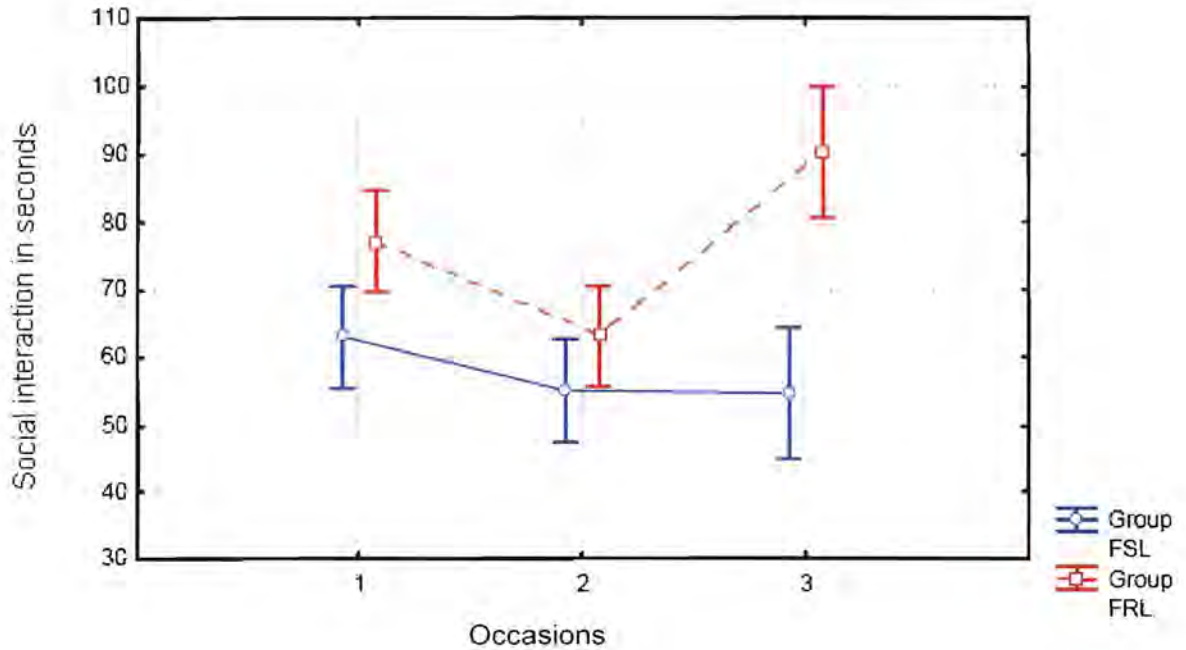


Figure 29: All groups line graph, Social interaction. Data were analyzed statistically using the Two-way ANOVA

From Figure 29 it is evident that there was no significant interaction in the results of the Social Interaction among study occasion 1, 2 and 3 ($p=0.293125$) which implies that the results may be pooled.

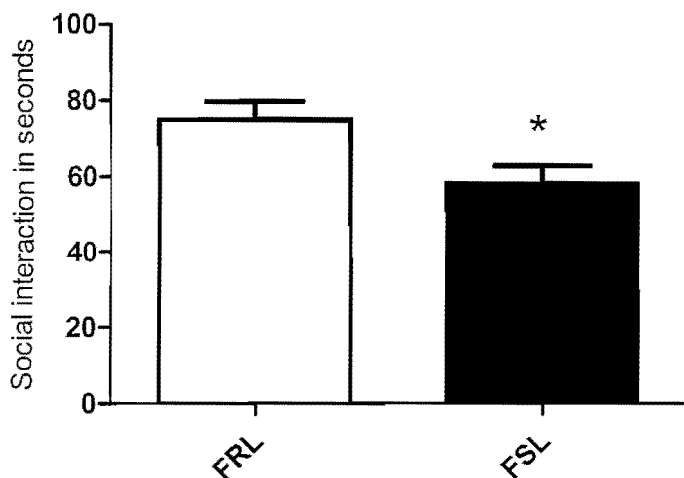


Figure 30: Total social interaction in stress-naive Flinders Resistant Line and Flinders Sensitive Line rats on occasion 1-3. Data were analyzed statistically using the Mann-Whitney U Test and statistical significance was indicated as * $P<0.05$ and non-significance (N.S) as $p>0.05$ ($n=13$; mean \pm SD)

As evident from Figure 30 the total social interaction time during occasions 1 - 3, was significantly lower in the FSL rat groups than in the FRL groups ($p=0.027261$).

5.4.3 Forced Swim Test results

Figure 32 showed no significant difference in immobility time among study occasion 1, 2 and 3 ($p=0.993401$). The group studies (Figure 31) illustrated that the different studies will not have a masking effect on each other. The time of immobility was respectively compared between the FSL and FRL rat groups after the three different occasion's results were pooled.

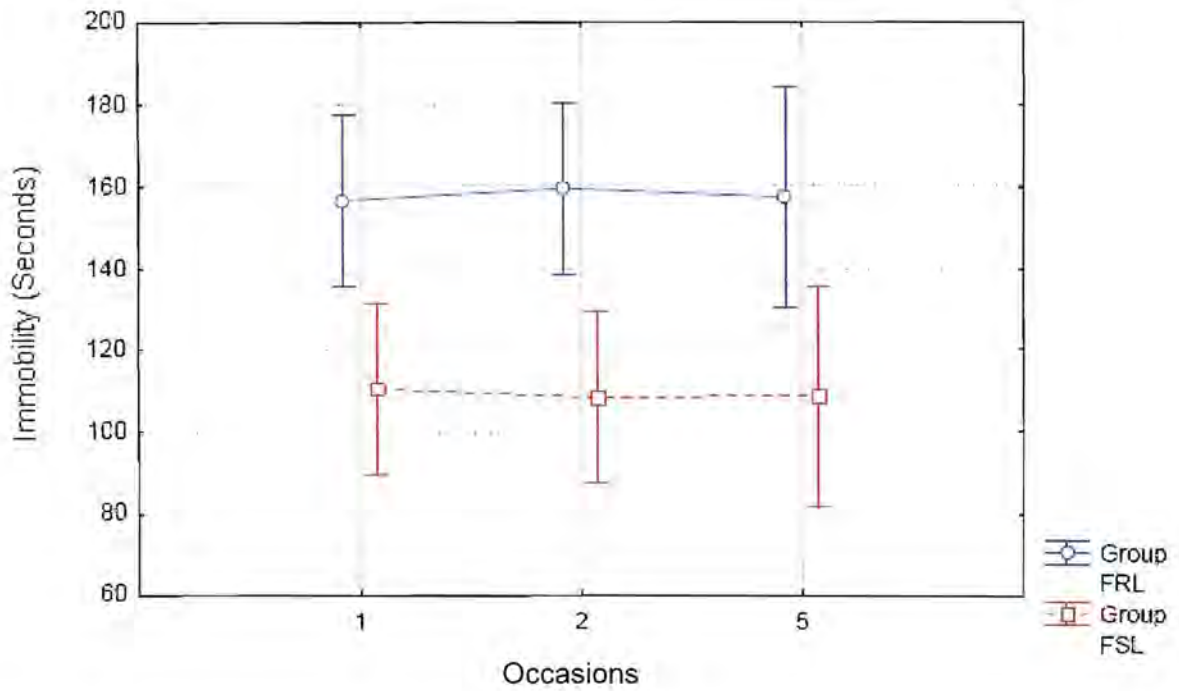


Figure 31: All groups line graph, Forced Swim Test. Data were analyzed statistically using the Two-way ANOVA

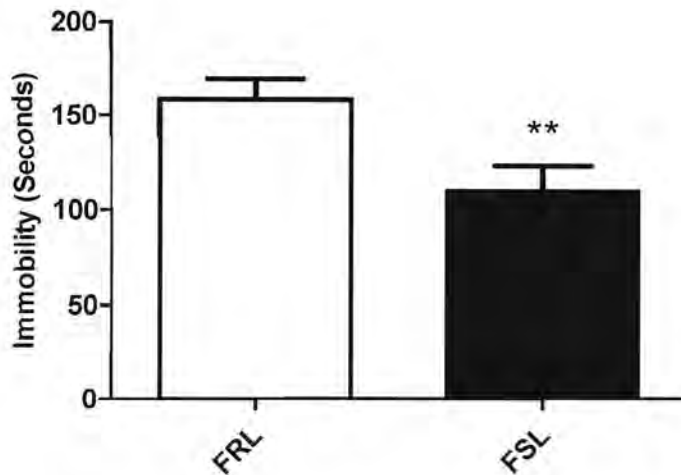


Figure 32: Forced Swim Test total immobility in stress-naive Flinders Resistant Line and Flinders Sensitive Line rats on occasion 1-3 Data were analyzed statistically using the Mann-Whitney U Test and statistical significance was indicated as $**P < 0.01$ and non-significance (N.S) as $p > 0.05$ ($n=10$; mean \pm SD)

5.5 Discussion

Regarding the hypothermia challenge, the FSL rats were two-fold more sensitive to the hypothermic effect of the 5-HT_{1A} receptor agonist 8-OH-DPAT relative to the FRL rats, at 30 minutes after the injection. At 60 minutes after the injection they were still significantly more sensitive to 8-OH-DPAT than the FRL rats. This is in consistency with numerous reports in the literature (Overstreet et al, 1994, 1998). This result is therefore evident of a serotonergic dysfunction in FSL rats (Wallis *et al.*, 1988).

In the OFT, the total number of line crosses served as a measure of locomotor activity (Overstreet & Griebel, 2005). Locomotor activity on occasion 1 did not differ significantly between FSL and FRL rats; however, on occasions 2 and 3 there was a significant decrease in locomotor activity in the FSL rat. FSL rats were found to have lower locomotor activity than FRL rats when exposed to an open-field apparatus for short periods of time under baseline conditions (Overstreet & Russel, 1982, Overstreet, 1986). The OFT is also a measure of the inherent anxiety levels of the animal (Koene *et al.*, 2003). Significant inactivity of the FSL rats therefore implicated that the FSL rats were more anxious than the FRL rats. Psychomotor retardation (or locomotor activity) was found to be even more pronounced in FSL rats after exposure to stressors (Overstreet *et al.*, 1996), due to the FSL rats' greater stress-susceptibility compared to FRL rats (Overstreet *et al.*, 2005).

The time the rats spend in the middle blocks also served as an indication of anxiety-like behaviour (Koene *et al.*, 2003). More anxious rats spent less time in the centre of the open field arena. Due to the greater stress sensitivity of the FSL rats, and given the anxiogenic environment of the OFT, it was expected that FRL rats would have spend a significantly greater amount of time in the middle blocks than FSL rats (Overstreet *et al.*, 2004). Our results did not show any significant difference in time spent in middle blocks between FSL and FRL groups on occasions 1 and 3, but on occasion 2, the FSL rat spent significantly less time in the middle blocks, indicative of anxiety.

Results of the pooled Social Interaction assessments, confirmed that the FSL rats were more anxious than the FRL rats supportive of literature that those rats which are anxious, spent less time in social interaction with their cage mates (File & Seth, 2003; Overstreet *et al.*, 2004; Overstreet & Griebel, 2005).

Results from the FST under basal conditions were disappointing as one would expect that FSL rats should display more immobility than FRL rats in the Forced Swim Test (Overstreet, 1986; Overstreet, 1993; Overstreet *et al.*, 1995; Zangen *et al.*, 1997; Overstreet *et al.*, 1998; Overstreet, 2002; Overstreet *et al.*, 2005), a result that was also reproduced in our laboratory, but after subacute mild stress, comprising daily injection stress plus daily handling for two weeks (Lieberberg *et al.*, submitted for publication).

5.6 Conclusion

- Hypothermia test results obtained under our laboratory-conditions were supportive with literature reporting a prerequisite phenotypical difference between FSL and FRL rat lines, in sensitivity to the 5-HT_{1A} receptor agonist, 8-OH-DPAT (Overstreet *et al.*, 1998) FSL rats routinely displays a 2-fold greater decrease in temperature compared to the FRL rat (Overstreet *et al.*, 1994), results that was reproduced in the current study.
- Open Field Test results are in support of more anxious behaviour of the FSL rats compared to FRL rats (Overstreet & Russel, 1982; Overstreet *et al.*, 1986, File & Seth, 2003; Overstreet *et al.*, 2004; Overstreet & Griebel, 2005).
- Concerning the Forced Swim Test, Figure 12, illustrates that the rats' immobility behaviour followed the same trend in all three stress-naïve studies. On occasion 1, 2 and 3 stress-naïve FSL rats were not more immobile than FRL rats, in contrast to previous studies in our laboratory that were conducted after subacute mild stress and showed a significant immobility of FSL rats compared to FRL rats (Lieberberg *et al.*, submitted for publication).

Through the hypothermia challenge and OFT behavioural assessments, the FSL and FRL rat was successfully validated regarding phenotypical and behavioural differences – acting as confirmation of the reported face validity of the FSL rat and thereby ultimately providing fundamental support for the study's neurochemical results.

CHAPTER 6

Conclusion and
Recommendations

The current study has expanded our existing knowledge concerning the NO/cGMP pathway in depression, thereby helping to elucidate the biochemical basis of Major depressive disorder with regard to nitrergic mechanisms. In this manner the study could contribute towards further advancement in the pharmacotherapy of affective illnesses.

The study has confirmed and achieved the following:

- **Confirmation of interaction between the NO/cGMP pathway and cholinergic system**

Concerning the neurochemical characterization of the FSL rat, the anticipation was that the hypercholinergic state of the rodent model of depression, under baseline conditions, would be accompanied by altered markers of the NO/cGMP pathway, as compared to the FRL rat. Our data did concur with this premise to a degree, as evidenced by the decreased cGMP levels noted in the frontal cortex (but not hippocampus) of stress-naive FSL rats, although no other differences were found with respect to up-stream markers of cGMP synthesis, viz. activation of the NO cascade, including accumulation of NO_x and activation of NOS. This decrease in cGMP observed selectively in the frontal cortex not only suggests neuroanatomical specific differences between the animals which may imply differences in cortical function in these animals, such as cognition and goal-directed behaviour, but differences at the neurochemical level with respect to cGMP synthesis suggest that cGMP may be modulated in an NO-independent manner. Given the hypercholinergic state of FSL rats (Daws & Overstreet, 1998; Van Zyl, 2009), this regulation may be taking place through the cholinergic system. On the other hand, given that all tests were conducted in stress-naive animals, it is possible that actions on the NO cascade are dependent on the presence of stress in the animals, as has recently been

described (Wegener et al, 2009). While these data are exciting and provocative, further explorative studies are needed to unravel the mechanism of nitrenergic-cholinergic interactions in the FSL/FRL rat

• **Successful analytical method development**

Reliable and reproducible NO_x and L-citrulline results were obtained with the analytical methods developed in-house. Both methods displayed an acceptable reproducibility and repeatability and are suitable for the analysis of rodent brain samples.

The HPLC-method for NO_x determination with fluorescence detection offers the advantages of easy sample preparation and derivatization, a stable and highly fluorescent derivative, a short retention time, acceptable sensitivity and specificity and minimized assay interferences.

The HPLC-method for L-citrulline determination with electrochemical detection offers a reproducible, simple and safe method of analysis. The method can also be used for the quantification of L-citrulline, L-arginine, L-glutamate and GABA in rat brain tissue, which, if simultaneously analyzed provides a more holistic perspective of the biochemical landscape of the FSL rat model of depression.

• **Validation of Flinders Sensitive Line rat as a genetic animal model of depression**

Through the behavioural assessments, the FSL and FRL rat was successfully separated with respect to levels of inherent anxiety, and as such confirming the reported face validity of the FSL rat. These observations provide fundamental support for the neurochemical data found in the study and their possible involvement in anxiety-like behaviours in these animals. The latter of course represent a core symptom of depression.

- Data from the Open Field Test demonstrated that the FSL rat groups exhibited behaviour that was significantly more anxious than that in the FRL rats.
- Hypothermia test results obtained under our laboratory-conditions reported increased sensitivity to the 5-HT_{1A} receptor agonist, 8-OH-DPAT, in evoking

hypothermia in FSL rats, thereby confirming firstly a fundamental disturbance in serotonergic transmission in these animals, as well as providing evidence of an interaction between the serotonergic and cholinergic systems in FSL rat.

Recommendations and Prospective studies

• Post-stress neurochemical studies

There are as yet, no published studies reporting levels of cGMP, ACh and GABA or receptor binding studies in the FSL rat after acute stress. .Since FSL rats are more stress sensitive than their FRL controls, evidence from the current study, as well as other studies in our laboratory, suggest that investigation of the above neurochemical markers of depression in FSL rats after exposure to a stress paradigm, such as acute stress, may contribute to new knowledge of the aetiology of major depression.

• Post-stress behavioural studies

Results from a pilot FST study performed by another group in our laboratory (Liebenberg, 2008) showed that following exposure of handled rats to the forced swim test, the immobility difference between the two rat lines showed a significant difference, with the FSL rats demonstrating increased immobility in the test, thus confirming their increased stress sensitivity as well as their depressiogenic nature. A further investigation of the influence of mild subacute chronic stress on the immobility of FSL rats, through the comparison of results to the current study's results from the stress-naive FSL rats, might further confirm the contribution of stress sensitivity in these rats to their depressiogenic nature.

• Further investigation of cGMP involvement, through the investigation of cyclic GMP specific phosphodiesterases (PDE).

Limited reports describing the distribution of cGMP isozymes in the brain exist. The investigation of the homeostatic regulation of cGMP through characterizing the involved phosphodiesterases, may further unlock the acetylcholine-NO-cGMP signal transduction pathway (de Vente, 2004).

- **Combination of current results with collaborating studies**

Current results should be viewed in combination with data from a simultaneously run parallel study conducted under the same experimental conditions (Van Zyl, 2009). This latter study measured muscarinic - and NMDA-receptor characteristics as well as total ACh and GABA levels in the hippocampus and frontal cortex of stress-naive FSL/FRL rats. Combining these data will provide a more holistic impression of cholinergic interactions with the excitatory NMDA-NOS cascade and the GABA inhibitory pathway in FSL rats.

- **Prospective microdialysis studies**

This project will act as forerunner to projects to be initiated in the near future where NO-cholinergic signalling in the FSL/FRL animal model of depression will be investigated using a newly established *in vivo* microdialysis procedure

NO-cholinergic signalling in the FSL/FRL animal model of depression should be investigated using this *in vivo* microdialysis procedure in order to look at basal and stimulated release of ACh, GABA, glutamate and NO in the presence and absence of antidepressant treatments in FSL and FRL rats. This will provide irrevocable proof of cholinergic-NMDA-NO interactions in these animals.

Glossary

Action potential: in neurophysiology, the action potential is a self-regenerating wave of electrochemical activity that allows nerve cells to carry a signal over a distance.

Aetiology: the study of causation.

Affective: influenced by or resulting from the emotions.

Allostasis: is the process of Achieving stability, or homeostasis, through physiological or behavioral change. This can be carried out by means of alteration in HPA axis hormones, the autonomic nervous system, cytokines, or a number of other systems, and is generally adaptive in the short term.

Allostatic load: refers to the physiological costs of chronic exposure to the neural or neuroendocrine stress response. Allostatic load specifically refers to a composite index of indicators of cumulative strain on multiple organs and tissues which accumulates via the wear and tear associated with acute shifts in physiologic activity in response to negative stimuli.

Anhedonia: loss of interest and pleasure in activities normally enjoyed.

Animal model: an animal that has a disease or injury that is similar to a human condition, like depression – hence “animal model of depression”.

Biogenic amine neurotransmitters: includes histamine, 5-HT, catecholaminergic neurotransmitter's – NA, Epinephrinee and DA – and tryptamine.

Bulbectomized rat: The olfactory bulb of the rat is removed by a surgical procedure and results in similarities to brain chemistry seen in depressed humans such as altered DA and 5-HT brain levels. Olfactory bulb ablation also leads to an enlarged lateral and 3rd ventricle, as well as decreased hippocampal volume, as in depression.

The *bulbectomized rat* is one of the best available models to predict antidepressant activity.

Cognitive: of, relating to, being, or involving conscious intellectual activity (as thinking, reasoning, or remembering).

Deafferentation: the elimination or interruption of sensory nerve impulses by destroying or injuring the sensory nerve fibers.

Diathesis: an elegant term for a predisposition or tendency to depression.

Dysthymia: a mood disorder that falls within the depression spectrum. It is considered a chronic depression, but with less severity than major depression.

Endothelium-derived relaxing factor: nitric oxide produced and released by the endothelium that results in smooth muscle relaxation. It is released in response to a variety of chemical and physical stimuli. It causes the smooth muscle in the vessel wall to relax by activating the soluble guanylate cyclases (sGC), increasing the cyclic guanosine monophosphate (cGMP) concentration and activating the protein kinase G, resulting in vasodilation.

Excitatory synapse: a synapse in which an action potential in the presynaptic cell increases the probability of an action potential occurring in the postsynaptic cell (see also: EPSP)).

At an *excitatory synapse*, the neurotransmitter opens sodium (Na^+) channels, a slight depolarization of the receiving neuron occurs because of the movement of the positively charged sodium ions into the cell.

Excitatory postsynaptic potential (EPSP): is a temporary depolarization of postsynaptic membrane potential caused by the flow of positively charged ions into the postsynaptic cell as a result of opening of ligand-sensitive channels.

Genotype: an organism's full hereditary information, even if not expressed. Exact genetic make-up or particular set of genes it possesses.

G-kinase: cGMP dependent kinase – an enzyme that catalyze the transfer of a phosphate group from a donor, such as ADP or ATP, to an acceptor.

Gross morphology: prominent aspects of an organism's outward appearance (shape, structure, colour, pattern).

Homeostasis: is the property of a living organism that regulates its internal environment so as to maintain a healthy, stable, constant and condition.

Hypothesis: in contex, the hypotheses of depression are the suggested explanations for the observed behaviour in depressed patients. These postulted hypotheses can be explained by or are often based on previous observations or on extentions of scientic theories and are then tested for conformation according to the scientific method.

Inhibitory synapse: a synapse in which an action potential in the presynaptic cell decreases the probability of an action potential occurring in the postsynaptic cell (see also: IPSP).

Inhibitory postsynaptic potential (IPSP): the change in membrane voltage of a postsynaptic neuron which results from synaptic activation of inhibitory neurotransmitter receptors. The most common inhibitory neurotransmitters in the nervous system are GABA and glycine.

Ionotropic receptors / Channel-linked receptors: a group of transmembrane ion channels that are opened or closed in response to the binding of a chemical messenger, such as a neurotransmitter.

Limbic system: a set of brain structures including the hippocampus, amygdala, anterior thalamic nuclei, and limbic cortex, which support a variety of functions including emotion, behavior, long term memory, and olfaction. The limbic system operates by influencing the endocrine system and the autonomic nervous system. It is highly interconnected with the nucleus accumbens, the brain's pleasure center.

Limbic-cortical network: the limbic system is tightly connected to the prefrontal cortex. Some scientists contend that this connection is related to the pleasure obtained from solving problems.

Long-term potentiation: in neuroscience, long-term potentiation (LTP) is the long-lasting improvement in communication between two neurons that results from stimulating them simultaneously. LTP and its opposing process, long-term depression, are widely considered the major cellular mechanisms that underlie learning and memory.

Major depressive episode: A major depressive episode is when a person experiences 5 or more symptoms during most parts of the day for a two week period.

Metabotropic receptors: are indirectly linked with ion-channels on the plasma membrane of the cell through signal transduction mechanisms, often G proteins. Hence, they are a type of G Protein Coupled Receptor.

Neurochemical: A neurochemical is an organic molecule that participates in neural activity. This term is often used to refer to neurotransmitters and other molecules such as neuro-active drugs that influence neuron function. *Neurochemistry is a branch of science that studies neurochemicals.

Neurogenesis: (birth of neurons) is the process by which neurons are created. Most active during pre-natal development, neurogenesis is responsible for populating the growing brain.

Neuromodulator: those substances that may be released with a transmitter, but which do not produce a direct effect on a receptor; a neuromodulator seems to work by modifying the responsiveness of the receptor to the action of the transmitter.

Neuroplasticity or Synaptic plasticity: refers to the changes that occur in the organization of the brain as a result of experience. According to the theory of neuroplasticity, thinking, learning, and acting change both the brain's physical structure, or anatomy, and functional organization.

Neuropsychopharmacology: More precisely, neuropsychopharmacology is an interdisciplinary science related to psychopharmacology (how drugs affect the mind) and fundamental neuroscience. It entails research of mechanisms of neuropathology, pharmacodynamics (drug action), psychiatric illness, and states of consciousness. These studies are instigated at the detailed level involving neurotransmission/receptor activity, bio-chemical processes, and neural circuitry. Neuropsychopharmacology supersedes psychopharmacology in the areas of "how" and "why", and additionally addresses other issues of brain function.

Pathogenesis: is the mechanism by which an aetiological factor causes the disease, it also means the step by step development of a disease due to a series of changes in the structure and /or function of a tissue being caused by a chemical agent (in depression).

Pathophysiology: the underlying physiological (mechanical, physical, and biochemical functions) manifestations of a disease or disorder.

Prosthetic groups: non-protein (non-amino acid) component of a conjugated protein that is important in the protein's biological activity.

Psychoactive drug or Psychotropic substance: a chemical substance that acts primarily upon the central nervous system where it alters brain function, resulting in temporary changes in perception, mood, consciousness and behaviour.

Psychotropic drug: a drug having an altering effect on perception, emotion, or behavior.

Psychomotor agitation: is a series of unintentional and purposeless motions that stem from mental tension of an individual. This includes pacing around a room, wringing one's hands, pulling off clothing and putting it back on and other similar actions. In more severe cases, the motions may become harmful to the individual, such as ripping, tearing or chewing at the skin around one's fingernails or lips to the point of bleeding.

Psychomotor retardation: comprises a slowing down of thought and a reduction of physical movements in a person. This is most commonly seen in people with major depression where it indicates a degree of severity.

Psychopathologic: the manifestation of a mental or behavioral disorder.

Psychophysiological: the branch of psychology that is concerned with the physiological bases of psychological processes. Psychophysiology looks at the way psychological activities produce physiological responses. It is the perspective of studying the interface of mind and body that makes psychophysicologists most distinct.

Psychosocial: an individual's psychological development in a social environment as well as the interaction of the person with the social environment.

Phenotype: The observable physical or behavioural characteristics of an organism, as determined by both genetic makeup and environmental influences. Also actual observed properties, such as morphology (outward appearance – shape, structure, colour, and pattern), development or behaviour.

Receptor agonist: In pharmacology an agonist is a substance that binds to a specific receptor and triggers a response. It mimics the action of an endogenous ligand, like a hormone or neurotransmitter that binds to the same receptor.

Receptor antagonist: is a drug that blocks agonist-mediated responses without provoking a biological response itself upon binding to a receptor.

Reactive oxygen species (ROS): are ions or very small molecules that include oxygen ions, free radicals, and peroxides, both inorganic and organic. They are highly reactive due to the presence of unpaired valence shell electrons. ROS form as a natural byproduct of the normal metabolism of oxygen and have important roles in cell signalling. However, during times of environmental stress (such as for example, UV or heat exposure) ROS levels can increase dramatically, which can result in significant damage to cell structures. This cumulates into a situation known as oxidative stress.

Senescence: refers to the biological processes of a living organism approaching an advanced age.

Signal transduction pathway: in biology transduction refers to any process by which a cell converts one kind of signal or stimulus into another. Most processes of signal transduction involve ordered sequences of biochemical reactions inside the cell, which are carried out by enzymes, activated by second messengers, resulting in a signal transduction pathway. Such processes are usually rapid, lasting on the order of milliseconds in the case of ion flux. The number of proteins and other molecules participating in the events involving signal transduction increases as the process emanates from the initial stimulus, resulting in a "**signal cascade**," beginning with a relatively small stimulus that elicits a large response. This is referred to as amplification of the signal.

Somatic: of the body; bodily; physical.

Suicide ideation: is a common medical term for thoughts about suicide, which may be as detailed as a formulated plan, without the suicidal act itself.

Superoxide dismutase: The enzyme superoxide dismutase (SOD), catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. As such, it is an important antioxidant defense in nearly all cells exposed to oxygen.

Symptomatology: the branch of medical science that studies the symptoms of diseases.

Synaptic cleft: the site where neurons meet is called the synapse and consists of the axon terminal (transmitting end) of one cell and the dendrite (receiving end) of the next. A microscopic gap, about 20 nm wide exists between the neurons and is called a synaptic cleft.

Voltage-gated ion channels: a class of transmembrane ion channels that are activated by changes in electrical potential difference near the channel; these types of ion channels are especially critical in neurons.

ADDENDUM 1

**Statistical
Chromatographic Method
Validation**

Chromatographic methods were validated to meet the general requirements of ISO 17025 (2005) and SANS 17025 (Anon, 2005).

Linearity was demonstrated, calibration uncertainties determined, LOD and LOQ validated and precision (repeatability, reproducibility and stability) and specificity/sensitivity established after minimum validation criteria (VC 1-9) were established.

Statistical Data Tables 8-10 (de Beer, 2006), as listed as section 4 at the end of Appendix 2, were used to obtain all t_{crit} and F_{calc} values, as well as for the determination of outliers with DIXON's test for outliers (LOC values).

1 Validation of 2, 3- Naphtatriazole (NOx) detection method procedure

Validation of determination of 2, 3-naphtatriazole (NOx) in brain tissue samples by HPLC - **Fluorescence detection**.

1. Linearity
2. Calibration uncertainties
3. LOD
4. LOQ
5. Precision – repeatability, reproducibility and stability
6. Specificity/Sensitivity

This document was drawn up to comply with sections 4.3, 5.2, 5.3, 5.4 & 5.9 of ISO 17025 (2005).

METHOD

Brief description of the method is: 15,0 mg of sodium nitrite and 13,7mg of sodium nitrate were weighed out and then made up to 100 ml with Tris buffer as solvent. The stock solution was diluted and the standard series analyzed by HPLC-Fluorescence.

IMPORTANCE OF THE METHOD

For organization/student:

2,3 naphtatriazole will give an indication of total nitrite and nitrate (NOx) concentration in the Flinders Sensitive Line rat, a genetic rodent model of depression.

STUDENT and ORGANIZATION

Estella Minnaar, M.Sc student at the North-West University, Pharmacology department.

CHROMATOGRAPHIC INSTRUMENTATION

Analytical Instrument: Agilent 1100 series HPLC, equipped with an isocratic pump, autosampler, Shimadzu RF - 551 Fluorescence Detector and Chemstation Rev. A.06.02 data acquisition and analysis software.

Column: Agilent Eclipse XDB C18 column, 4.6 x 150 mm, 5 μ m,

Guard column: SecurityGuard™, HPLC Guard Cartridge System, with SecurityGuard Cartridges, C18, 4.0x3.0mm, Phenomenex, Torrance, CA.

Mobile Phase: 15 mM Sodium phosphate buffer. 52.2 % Methanol. The pH of the mobile phase was adjusted to \pm pH 7.5 with orthophosphoric acid (85%).

INSTRUMENT DOCUMENTATION: the following documentation is available from Analytical Technology Laboratory

1. Procurement: Necessary specification
2. Maintenance: Service contracts, Service periods, Availability of spare parts.
3. Service providers & instrument providers.
4. Manuals, software, calibration tables, calibration charts, calibration certificates.
5. Validation documentation of instrument.
6. Material safety data sheet (MSDS).

ANALYST: Estella Minnaar

LABORATORY ENVIRONMENT

Controlled environment:

1. Air conditioning: 20 ± 2 °C.
2. Extraction fan.
3. Laboratory sample preparation area has an analytical balance and HPLC-grade water supply.

VALIDATION CRITERIA (VC)

VC 1: $r^2 \geq 0.95$

VC 2: Working range of standards: 1-100 ng/ml.

VC 3: Number of standards: $n = 7$

VC 4: Significant linearity must be proven.

VC 5: LOD ≥ 0.2139 μM

VC 6: LOQ ≤ 0.713 μM

VC 7: Calibration sensitivity = $b \neq 0$

VC 8: m at least 3 of 4.

VC 9: RSD (Repeatability) $\leq 15\%$

CALIBRATION

1. Range of standards: 1-100 ng/ml (VC 2)
2. Number of standards: 7 (VC 3)

The standards were prepared from sodium nitrite and sodium nitrate bought from Sigma-Aldrich.

Table 1: Regression Statistics

SUMMARY OUTPUT

<i>Regression Statistics</i>	
Multiple R	0.9994
R Square	0.9988
Adjusted R Square	0.9986
Standard Error	448.68
Observations	7

ANOVA

	<i>df</i>	<i>SS</i>	<i>MS</i>	<i>F</i>	<i>Significance F</i>
Regression	1	850920740.6	850920740.6	4227.1	1.62962E-08
Residual	5	1006496.529	201299.3058		
Total	6	851927237.1			

	<i>Coefficients</i>	<i>Standard Error</i>	<i>t Stat</i>	<i>P-value</i>	<i>Lower 95%</i>	<i>Upper 95%</i>	<i>Lower 95.0%</i>	<i>Upper 95.0%</i>
Intercept	2216.5	231.9	9.558	0.0002	1620.4	2812.6	1620.4	2812.6
X Variable 1	3.2524	0.050	65.02	1.63E-08	3.1238	3.381	3.1238	3.381

Key (Table 2): r = Multiple R; r² = R Square; Sy/x = Standard Error; number of standards = Observations; Fcalc = F; Fcrit = Significance F;

a = Intercept; b = X Variable 1.

Table 2: NOx regression curve values

ng/ml	nM	AUC
9.90	215.23	3491.30
19.80	429.66	3670.10
49.50	1075.14	5534.70
99.00	2138.40	9270.20
148.5	3227.40	12559.4
198.0	4296.60	15477.9
295.0	10751.4	37499.7

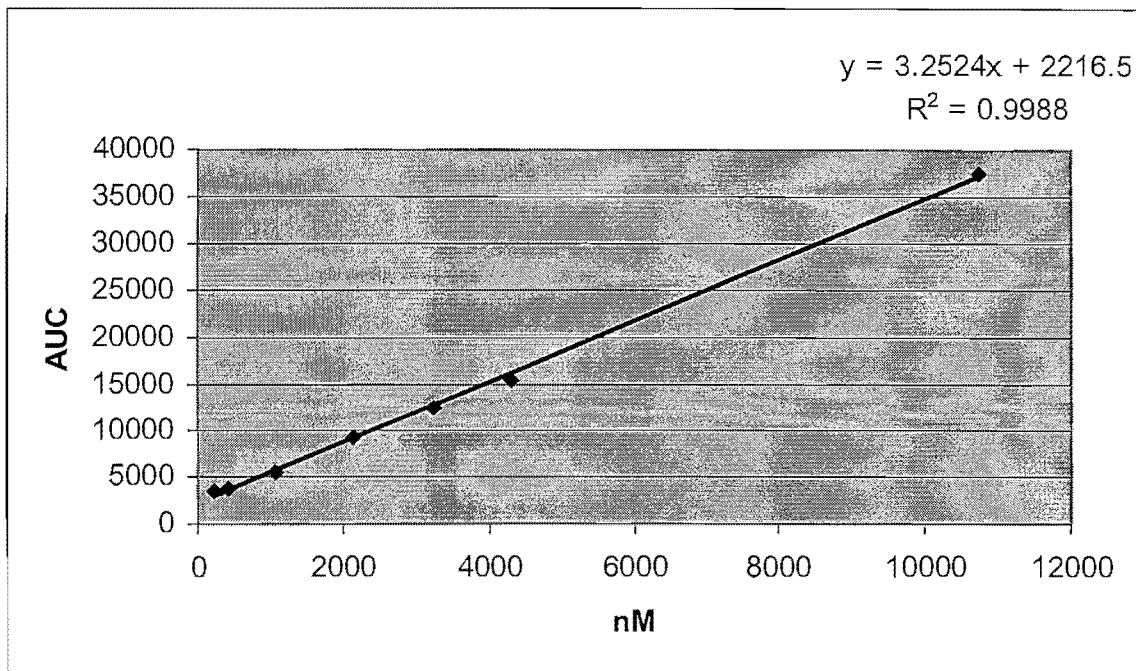


Figure 1: NOx standard regression curve

1.1 NOx LINEARITY

t-test: $r = 0.999$ $r^2 = 0.9988$, positive, almost perfect linearity.

$$t\text{-calc} = \frac{|r| \sqrt{n-2}}{\sqrt{1-r^2}}$$

$$= 64.485$$

$$t\text{-crit} = 2.57 \quad DF=n-2.$$

If $t\text{-calc} > t\text{-crit}$, then reject H_0 and linearity is significant.

$$\text{ANOVA: } F_{\text{calc}} = 4227.142$$

$$F_{\text{crit}} (1,5;0.05) = 6.608$$

Since $F_{\text{calc}} > F_{\text{crit}}$, Linearity is significant.

REGRESSION PARAMETERS

$$\text{Slope} = b = 3.252384$$

$$\text{Intercept} = a = 2216.514$$

$$\text{Regression line: } y = bx + a: y = 3.25x + 2216.514$$

1.2 CALIBRATION UNCERTAINTIES

$$\text{Random calibration uncertainty} = S_y/S_x = 448.6639$$

$$\text{Uncertainty in slope} = S_b = 0.05$$

$$\text{Uncertainty in intercept} = S_a = 231.9$$

Evaluation: $S_b < S_a$, working range is wide enough.

1. Since both S_b and $S_a < S_y/x$ – good general selection of standards.
2. $S_a / S_b = 4638$

The value can be lowered more if required, by excluding the highest concentration or by using more standards close to the blank.

- Sb < Sa – the range is wide enough.

1.3 VALIDATION OF THE LOD

$x \text{ LOD} = 3 S_a / b = 213.9 \text{ nM}$. 213.9 nM or 0.2139 μM is the lowest concentration the method can detect.

1.4 VALIDATION OF THE LOQ

$x \text{ LOQ} = 10 S_a / b = 713.02 \text{ nM}$ or 0.713 μM is the lowest concentration the method can quantify.

1.5 VALIDATION OF PRECISION: REPEATABILITY, REPRODUCIBILITY AND STABILITY

Table 3: Repeatability and reproducibly of standards

DAY	10 ng/ml AUC	50 ng/ml AUC	100 ng/ml AUC
1	3987.6	8197.3	16968.6
2	2905.5	8305.2	14994.5
3	3670.1	9270.2	15477.9
X bar	3521.1	8590.9	15813.7
S	556.23	590.76	1028.9
%RSD	15.797%	6.8766%	6.50697%

The % RSD of standards > 10 ng/ml (0.215 μM) \leq 100 ng/ml lies within RSD < 15% (VC 9). The method is thus repeatable as well as reproducible in the abovementioned concentration range.

Sample validity

The determination is valid, sample concentrations (20-30 μM) lies within LOD and LOQ.

Stability of NAT in standards

Two different concentrations of standards were injected into the HPLC and after it was left for 24 hours, the two vials were again injected to test for stability.

Standard number 131.D (12.5 μM) was injected 24 hours after 125.D (12.5 μM).

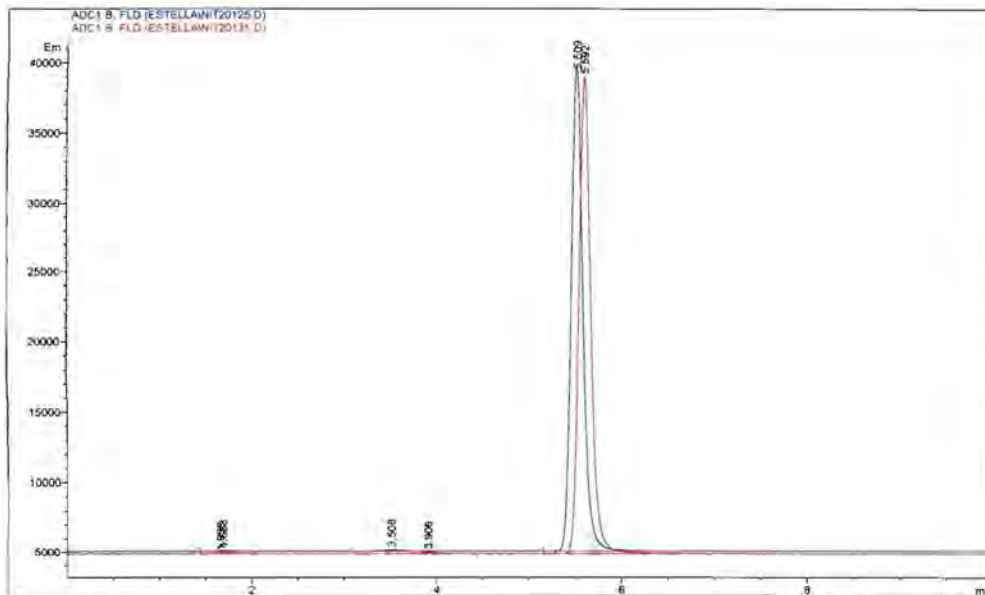


Figure 2: Comparison between 12.5 μM NAT standards after 24 hours

Standard number 132.D (25 μM) was injected 24 hours after 126.D (25 μM).

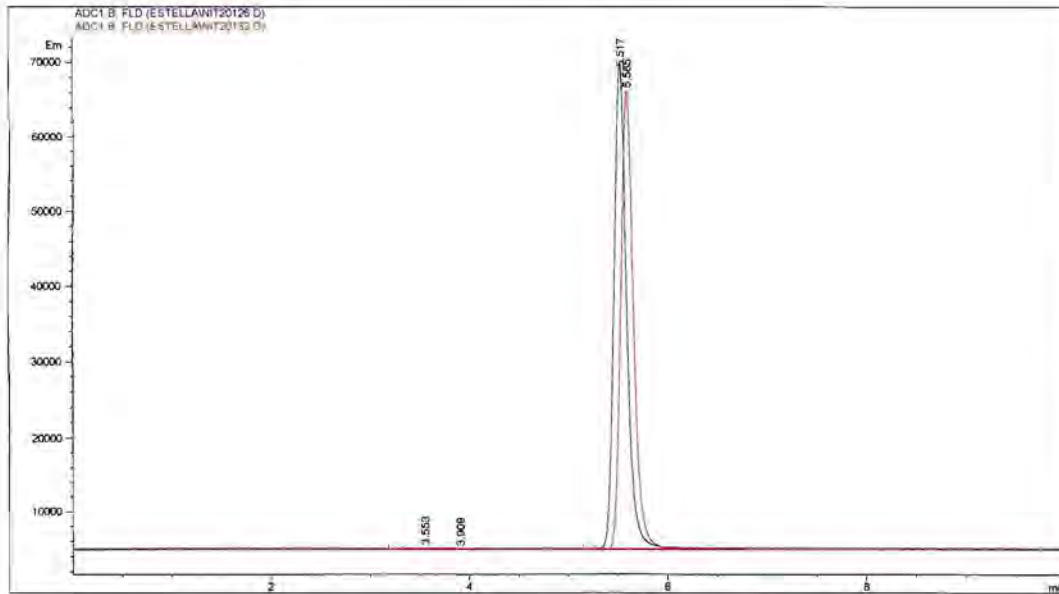


Figure 3: Comparison between 25 µM NAT standards after 24 hours

The % RSD of the two 12.5 µM standards complies with VC 9 ($S_x = 141.42$, $\bar{X} = 6900$, %RSD = 2%), so does the % of the two 25 µM standards. ($S_x = 325.98$, $\bar{X} = 307734.5$, %RSD = 0.1%).

The stability of standards correlates with literature that states that NAT in standards and samples stays stable for at least 24 hours at room temperature (20 – 25°C) (Li & Wu, 2000; Jobgen *et al.*, 2007). Brain samples were subsequently done in batches of 10 per assay to ensure adequate stability throughout the experiment.

Suggestion for further improvement of experiment:

Injection of higher standard series (10-40 µM).

1.6 VALIDATION OF SPECIFICITY/ SENSITIVITY

Calibration sensitivity = $b = 3.252384 \neq 0$ – the calibration sensitivity is acceptable.

2 Validation of L-citrulline detection method procedure

Validation of determination of **glutamate, Arginine, Citrulline and GABA** in brain tissue samples with HPLC by **Electrochemical Detection**.

2.1 Linearity

2.2 Calibration uncertainties

2.3 LOD

2.4 LOQ

2.5 Precision – repeatability, reproducibility and stability

2.6 Specificity/Sensitivity

This document was drawn up to comply with sections 4.3, 5.2, 5.3, 5.4 & 5.9 of ISO 17025 (2005).

METHOD

Brief description of the method is: 1 mg of GABA, L-Glutamic acid, L-citrulline and 1,21 mg L-arginine were weighed out and then made up to 10 ml with borate buffer as solvent. The stock solution was diluted and the standard series analyzed by HPLC-ECD.

IMPORTANCE OF THE METHOD

For organization/student:

L-citrulline will give an indication of NOS-activity in the Flinders Sensitive Line rat, a genetic rodent model of depression.

STUDENT and ORGANIZATION

Estella Minnaar, M.Sc student at the North-West University, Pharmacology department.

CHROMATOGRAPHIC INSTRUMENTATION

Analytical Instrument: Agilent 1100 series HPLC, equipped with an isocratic pump, autosampler, GBC LC 1260 Electrochemical detector, Shimadzu Fluorescence Detector and Chemstation Rev. A.06.02 data acquisition and analysis software.

Column: Luna C18-2 column, 75 x 4.6 mm, 5 μ m, 100 Å pores, 17.8% carbon load, endcapped, Phenomenex, Torrance, CA (Column L1, USP 24, 2000, p 1925).

Guard column: SecurityGuard™, HPLC Guard Cartridge System, with SecurityGuard Cartridges, C18, 4.0x3.0mm, Phenomenex, Torrance, CA.

Mobile Phase: 0.1 M Sodium phosphate dibasic, 0.13 mM Ethylenediaminetetraaceticacid (EDTA dinatriumsalt Na₂EDTA), 28% (to 35%) Methanol. The pH of the mobile phase was adjusted to \pm pH 6.4 with orthophosphoric acid (85%).

INSTRUMENT DOCUMENTATION:

the following documentation is available from Analytical Technology Laboratory

1. Procurement: Necessary specification
2. Maintenance: Service contracts, Service periods, Availability of spare parts.

3. Service providers & instrument providers.
4. Manuals, software, calibration tables, calibration charts, calibration certificates.
5. Validation documentation of instrument.
6. Medical safety data sheets (MSDS).

ANALYST: Estella Minnaar

LABORATORY ENVIRONMENT

Controlled environment:

1. Air conditioning: 20 ± 2 °C.
2. Extraction fan.
3. Laboratory sample preparation area has an analytical balance and HPLC-grade water supply.

VALIDATION CRITERIA

VC 1: $r^2 \geq 0.95$

VC 2: Working range of standards: 100 -5000 ng/ml.

VC 3: Number of standards: $n = 7$

VC 4: Significant linearity must be proven.

VC 5: LOD ≥ 0.92 μM

VC 6: LOQ ≤ 3.068 μM

VC 7: Calibration sensitivity = $b \neq 0$

VC 8: m at least 3 of 4.

VC 9: RSD (Repeatability) $\leq 5\%$

CALIBRATION

1. Range of standards: 100 – 5000 ng/ml (VC2)
2. Number of standards: 7 (VC 3)

The standards were prepared from amino-acids, GABA, L-Glutamic acid, L-citrulline and L-arginine bought from Sigma-Aldrich.

Calibration data:

Raw data was interpreted using MS-Excel, Data analysis: regression.

Table 4: L-citrulline regression curve values

ng/ml	µM	Glutamate	µM	L-arginine	µM	L-citrulline	µM	GABA
100	0.68	485.83	0.57	1394.0	0.57	456.54	0.97	2718.8
250	2.43	849.18	1.44	1955.7	1.43	706.17	2.43	3075.7
500	4.85	1572.6	2.87	2634.1	2.85	1262.6	4.85	4262.2
750	7.27	2095.3	4.30	3095.9	4.28	1685.8	7.27	3060.3
1000	9.70	3027.6	5.74	4045.5	5.71	2447.5	9.70	5868.5
2500	24.25	3.05E+04	14.35	8028.3	14.27	6054.7	24.25	9729.3
5000	48.50	1.02E+05	28.70	1.49E+04	28.54	1.33E+04	48.50	2.04E+04

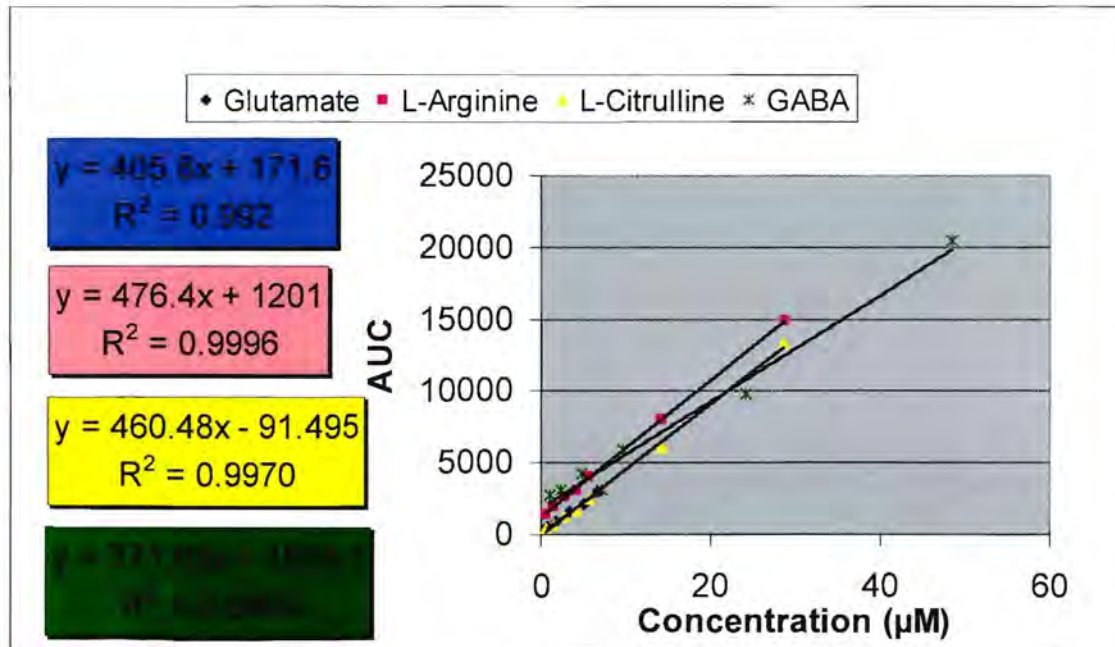


Figure 4: Amino acid standard regression curve

Table 5: Regression Statistics

SUMMARY OUTPUT

<i>Regression Statistics</i>	
Multiple R	0.997
R Square	0.995
Adjusted R Square	0.994
Standard Error	92.62
Observations	7

ANOVA

	<i>df</i>	<i>SS</i>	<i>MS</i>	<i>F</i>	<i>Significance F</i>
Regression	1	7930202.8	7930202.8	924.4	7.22082E-07
Residual	5	42891.912	8578.3824		
Total	6	7973094.9			

	<i>Coefficients</i>	<i>Standard Error</i>	<i>t Stat</i>	<i>P-value</i>	<i>Lower 95%</i>	<i>Upper 95%</i>	<i>Lower 95.0%</i>	<i>Upper 95.0%</i>
Intercept	-34.55	46.77	-0.739	0.493	-154.78	85.68	-154.8	85.68
X Variable 1	0.655	0.021	30.40	7.22E-07	0.5984	0.709	0.598	0.709

Key: r = Multiple R; r² = R Square; Sy/x = Standard Error; number of standards = Observations; Fcalc = F;

Fcrit = Significance F; a = Intercept; b = X Variable 1.

CITRULLINE LINEARITY

t-test: $r = 0.998$ $r^2 = 0.997$, positive linearity.

$$t\text{-calc} = |r| \sqrt{n-2}$$

$$\begin{aligned} & \frac{\text{-----}}{\sqrt{1-r^2}} \\ & = 2.232/0.055 \\ & = 40.743 \end{aligned}$$

t-crit = 2.57 DF=n-2.

If t-calc > t-crit, then reject H_0 and linearity is significant.

ANOVA: Fcalc = 1641.612

$$F_{\text{crit}}(1,5;0.05) = 6.608$$

Since Fcalc > Fcrit, Linearity is significant.

REGRESSION PARAMETERS

Slope=b= 460.7

Intercept = a= -92.37

Regression line: $y = bx + a$: $y = 460.7x - 92.37$.

CALIBRATION UNCERTAINTIES

Random calibration uncertainty = $S_y/S_x = 279.6361$

Uncertainty in slope = $S_b = 11.37$

Uncertainty in intercept = $S_a = 141.2126152$

Evaluation: $S_b < S_a$, working range is wide enough.

1. Since both S_b and $S_a < S_y/x$ – good general selection of standards.
2. $S_a/S_b = 12.42$.

The value can be lowered more if required, by using more standards close to the blank.

3. $S_b < S_a$ – the range is wide enough.

VALIDATION OF THE LOD

$x \text{ LOD} = 3 S_a / b = 0.92 \mu\text{M}$. 0.92 μM is the lowest concentration the method can detect.

CALIDATION OF THE LOQ

$x \text{ LOQ} = 10 S_a / b = 3.065 \mu\text{M}$ is the lowest concentration the method can quantify.

VALIDATION OF PRECISION: REPEATABILITY, REPRODUCIBILITY AND STABILITY

Repeatability of standards:

Table 6: AUCs of standards

Vial	0.5 $\mu\text{g/ml}$ AUC	2.5 $\mu\text{g/ml}$ AUC	5 $\mu\text{g/ml}$ AUC
1	2476.5	4407.2	9294.8
2	2487.5	4268.4	9246.7
3	1606.0	4349.9	9297.5
X bar	2190.0	4341.8	9279.7
S	505.79	69.75	28.60
%RSD	23.10%	1.61%	0.31%

The % RSD of standards $> 0.5 \mu\text{g/ml}$ (2.854 μM) $\leq 5 \mu\text{g/ml}$ (28.54 μM) lies within RSD =15% (VC 9). The method is thus repeatable as well as reproducible in the abovementioned concentration range.

Stability of Real Sample 1:

A single sample was injected four consecutive times to test for stability of endogenous arginine and citrulline. Four samples were injected at a time during the experiments.

Table 7: AUCs of Real Sample 1

Vial	Time (minutes)	Arginine AUC	Citrulline AUC
1	00:00	12724.80	324.16
2	01:26	16749.80	243.26
3	02:58	15761.90	372.67
4	03:44	13923.50	272.79

% RSD between Vial 1 and 3: 9.85%

% RSD between Vial 1 and 4: 12%, compliant with VC 9.

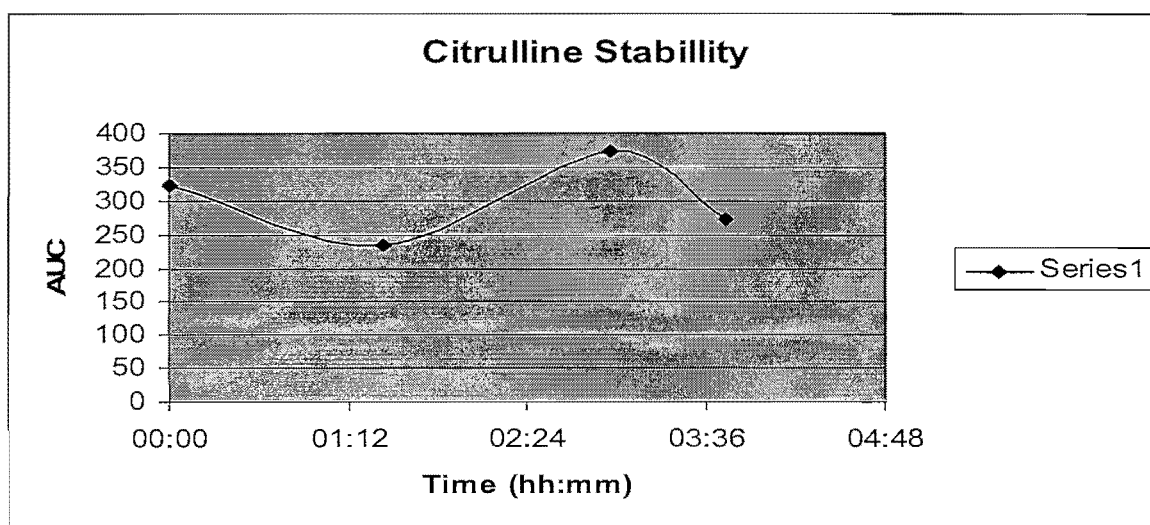


Figure 5: Plotted L-citrulline AUCs

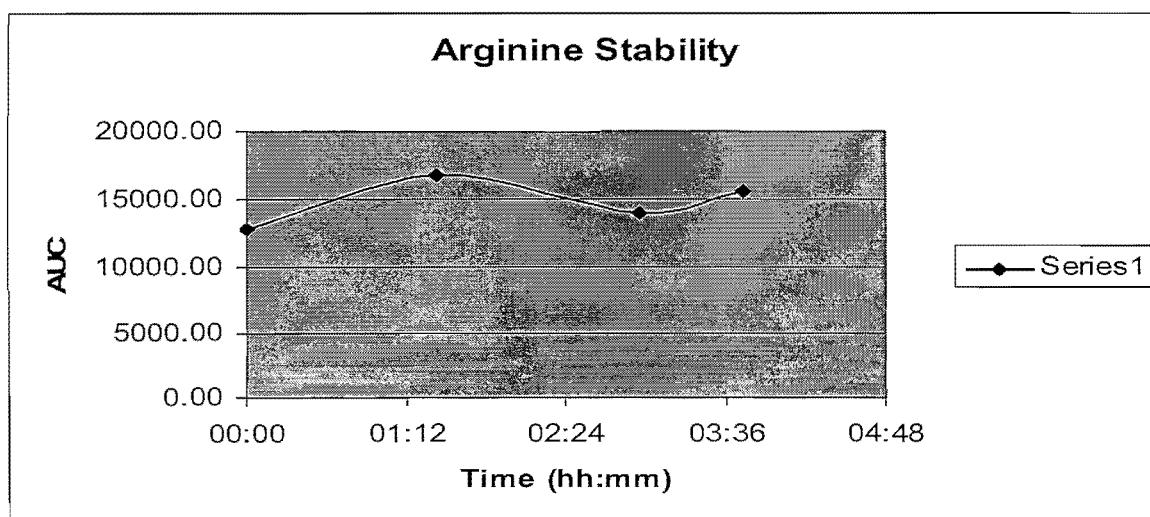


Figure 6: Plotted L-arginine AUCs

Suggestion to increase validity of determination

Sample concentrations (2-8 μM) lie within LOD but not within LOQ. This is because of the large b value. To improve the validity of the determination, a larger range of standards (current range = 0.6 – 29 μM / 100 – 5000 ng/ml) must be injected.

VALIDATION OF SPECIFICITY/ SENSITIVITY

Calibration sensitivity = $b = 460.7 \neq 0$ – the calibration sensitivity is acceptable.

3. Conclusion

Quality dependable NO_x and L-citrulline results were verified by the following findings:

3.1 NO_x determination

1. The standard regression curve, displayed a positive almost perfect linearity that was demonstrated to be significant.
2. The calibration uncertainties can be lowered by using more standards close to the blank. Since the working range was wide enough, it implies a good general selection of standards.
3. The lowest calculated concentration the method could detect was 0.2139 μM .
4. The lowest calculated concentration the method could quantify was 0.713 μM .
5. The method had an acceptable intra- and inter-repeatability and -reproducibility in the specified concentration range. Furthermore, sample determinations were valid and standards remained stable at room temperature for at least 24 hours.
6. The calibration sensitivity was acceptable.

3.2 L-citrulline detection

- 1 The standard regression curve, displayed a positive linearity that was demonstrated to be significant.

- 2 The calibration uncertainties can be lowered by using more standards close to the blank. Since the working range was wide enough, it implies a good general selection of standards.
- 3 The lowest calculated concentration the method could detect was 0.92 μM .
- 4 The lowest calculated concentration the method could quantify was 3.065 μM .
- 5 The method had an acceptable repeatability and reproducibility in the specified concentration range. Real sample 1 was found to be stable for the time of injection of four samples. Four samples were subsequently injected per assay to ensure sustained stability.

The calibration sensitivity was acceptable.

4. Statistical data tables

The following three statistical tables (de Beer, 2006) were used to obtain all t_{crit} and F_{calc} values, as well as for the determination of outliers with DIXON's test for outliers (LOC values).

Table 8: Critical values of F for a one-tailed test ($p = 0.05$)

V_1	1	2	3	4	5	6	7	8	9	10	12	15	20
V_2													
1	161.4	199.5	215.7	224.6	230.2	234.0	236.8	238.9	240.5	241.9	243.9	245.9	248.0
2	18.51	19.00	19.16	19.25	19.30	19.33	19.35	19.37	19.38	19.40	19.41	19.43	19.45
3	10.13	9.552	9.277	9.117	9.013	8.941	8.887	8.845	8.812	8.786	8.745	8.703	8.660
4	7.709	6.944	6.591	6.388	6.256	6.163	6.094	6.041	5.999	5.964	5.912	5.858	5.803
5	6.608	5.786	5.409	5.192	5.050	4.950	4.818	4.818	4.772	4.735	4.678	4.619	4.558
6	5.987	5.143	4.757	4.534	4.387	4.284	4.207	4.147	4.099	4.060	4.000	3.938	3.874
7	5.591	4.737	4.347	4.120	3.972	3.866	3.787	3.787	3.677	3.637	3.575	3.511	3.445
8	5.318	4.459	4.066	3.838	3.687	3.581	3.500	3.500	3.388	3.347	3.284	3.218	3.150
9	5.117	4.256	3.863	3.633	3.482	3.374	3.293	3.293	3.179	3.137	3.073	3.006	2.936
10	4.965	4.103	3.708	3.478	3.326	3.217	3.135	3.135	3.020	2.978	2.913	2.845	2.774
11	4.844	3.982	3.587	3.357	3.204	3.095	3.012	2.948	2.896	2.854	2.788	2.719	2.646
12	4.747	3.885	3.490	3.259	3.106	2.996	2.913	2.849	2.796	2.753	2.687	2.617	2.544
13	4.667	3.806	3.411	3.179	3.025	2.915	2.832	2.767	2.714	2.671	2.604	2.533	2.459
14	4.600	3.739	3.344	3.112	2.958	2.848	2.764	2.699	2.646	2.602	2.534	2.463	2.388
15	4.543	3.682	3.287	3.056	2.901	2.790	2.707	2.641	2.588	2.544	2.475	2.403	2.328
16	4.494	3.634	3.239	3.007	2.852	2.741	2.657	2.591	2.538	2.494	2.425	2.352	2.276
17	4.451	3.592	3.197	2.965	2.810	2.699	2.614	2.548	2.494	2.450	2.381	2.308	2.230
18	4.414	3.555	3.160	2.928	2.773	2.661	2.577	2.510	2.456	2.412	2.342	2.269	2.191
19	4.381	3.522	3.127	2.895	2.740	2.628	2.544	2.477	2.423	2.378	2.308	2.234	2.155
20	4.351	3.493	3.098	2.866	2.711	2.599	2.514	2.477	2.393	2.348	2.278	2.203	2.124

Table 9: DIXON

Number of observations n	LOC		
	(90%) Q0.10	(95%) Q0.05	(99%) Q0.01
3	0.941	0.970	0.994
4	0.765	0.829	0.926
5	0.642	0.710	0.821
6	0.560	0.625	0.740
7	0.507	0.568	0.680
8	0.468	0.526	0.634
9	0.437	0.493	0.598
10	0.412	0.466	0.568
15	0.338	0.384	0.475
20	0.300	0.342	0.425
25	0.277	0.317	0.393
30	0.260	0.298	0.372

Table 10: the t-distribution

Value of t for a confidence interval of	90%	95%	98%	99%
Critical value of t for p values of	0.10	0.05	0.02	0.01
Number of degrees of freedom (DF)				
1	6.31	12.71	31.82	63.66
2	2.92	4.30	6.96	9.92
3	2.35	3.18	4.54	5.84
4	2.13	2.78	3.75	4.60
5	2.02	2.57	3.36	4.03
6	1.94	2.45	3.14	3.71
7	1.89	2.36	3.00	3.50
8	1.86	2.31	2.90	3.36
9	1.83	2.26	2.82	3.25
10	1.81	2.23	2.76	3.17
12	1.78	2.18	2.68	3.05
14	1.76	2.14	2.62	2.98
16	1.75	2.12	2.58	2.92
18	1.73	2.10	2.55	2.88
20	1.72	2.09	2.53	2.85
30	1.70	2.04	2.46	2.75
50	1.68	2.01	2.40	2.68
∞	1.64	1.96	2.33	2.58

ADDENDUM 2

Guide for Authors

Brain Research

1.1 Scope: Appropriate Section

This article could appear under Sections:

1: Cellular and Molecular Biology of Nervous Systems. The study investigates the molecular dissection of intracellular and extracellular signal transduction pathways or

6: Regulatory Systems. Deals with the internal regulatory systems of the central and peripheral nervous systems, including: stress and the brain.

1.2 Preparation of the electronic manuscript

Text, tables and graphics (and any other items) was kept as separate files - *figures or tables were not imported into the text file*. Files were named using the correct extension, e.g. text.doc, fig1a.eps, fig1.tif, Fig1.jpg, tbl1-6.xls, etc.

Text files should be supplied in one of the following formats: Microsoft Word or WordPerfect, Windows or Macintosh formatted. Ensure that the letter "l" and the digit "1" (also letter "O" and digit "0") have been used properly, and format your article (tabs, indents, etc.) consistently. Characters not available on your wordprocessor (Greek letters, mathematical symbols, etc.) should not be left open, but indicated by a unique code (e.g. galpha, @, #, etc. for the Greek letter alpha). Such codes should be used consistently throughout the entire text. Please make a list of such codes and provide a key.

When accepted articles are processed, most formatting codes will be removed or replaced so there is no need to use excessive layout styling. In addition, do not use options such as automatic word breaking, justified layout, double columns or automatic paragraph numbering (especially for numbered references). However, do use bold face, italic, subscripts, superscripts etc. for scientific nomenclature. When

preparing tables, if you are using a table grid, please use only one grid for each separate table and not a grid for each row. If no grid is being used, use tabs to align columns, not spaces.

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Title page. The title page should contain the following items: (i) complete title (preferably no chemical formulas or arbitrary abbreviations); (ii) full names of all authors; (iii) complete affiliations of all authors; (iv) the number of text pages of the whole manuscript (including figures and tables) and the number of figures and tables; (v) the name and complete address of the corresponding Author (as well as telephone number, facsimile number and E-mail address, and if available URL address) to whom correspondence and proofs should be sent.

Abstract. This should provide a concise description of the purpose of the report or summary of the review and should not exceed 250 words. References should therefore be avoided, but if essential, they must be cited in full, without reference to the reference list.

Classification terms (to be placed under the Abstract) **Section:** One term should be selected from the 9 (sub) Section headings listed above under "Scope".

Keywords. Provide a maximum of six keywords, using American spelling and avoiding general and plural terms and multiple concepts (avoid, for example, "and", "of"). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Abbreviations. Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements. Collate acknowledgements in a separate section at the end of the article and do not, therefore, include them on the title page, as a footnote to the title or otherwise.

Research Reports and *Reviews* should be divided into **numbered sections** headed by a caption (e.g. Abstract, 1. Introduction, 2. Results, 3. Discussion, 4. Experimental Procedure, Acknowledgements, References).

Introduction. In this section, the objectives of the research should be clearly stated. Relevant background information and published studies should be described concisely, and be cited appropriately.

Results. The results should be described clearly and concisely, and in logical order without extended discussion of their significance. Results should usually be presented descriptively and be supplemented by photographs or diagrams.

Discussion. The results of the research should be discussed in the context of other relevant published work, and as concisely as possible. Extensive citations and discussion of published literature should be avoided.

Experimental Procedure. This section should contain all the details necessary to reproduce the experiments. Avoid re-describing methods already published; only relevant modifications should be included in the text.

Literature references. All references cited in the text should be listed at the end of the manuscript on a separate page, arranged in alphabetical order of first author then year of publication. The references should include only articles that are published or in press. Unpublished data, submitted manuscripts, or personal communications should be cited within the text only. Personal communications should be documented by a letter of permission. Abstracts of work presented at meetings may not be cited. All items in the list of references should be cited in the text and, conversely, all references cited in the text must be presented in the list. The abbreviations of journal titles should conform to those adopted by the *List of Serial Title Word Abbreviations*, CIEPS/ISDS, Paris, 1985 (ISBN 2-904938-02-8).

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Allan, K., Doyle, M.C., Rugg, M.D., 1996. An event related potential study of word-stem cued recall. *Cognitive Brain Res.* 4, 251-262.

Chapter in a book (within a series):

Mize, R.R., 1994. Conservation of basic synaptic circuits that mediate GABA inhibition in the subcortical visual system. In *Neuroscience: From the Molecular to the Cognitive. Progress in Brain Research*, Vol. 100, F.E. Bloom, ed. Elsevier, Amsterdam, pp. 123-132.

An entire book:

Swanson, L.W., Bjorklund, A., Hokfelt, T., 1966, *Integrated Systems of the CNS, Part III: Cerebellum, Basal Ganglia, Olfactory System. Handbook of Chemical Neuroanatomy*, Vol. 12, Elsevier, Amsterdam.

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For **other policy issues**, authors are referred to the policy guidelines of the Society for Neuroscience (see their website <http://www.jneurosci.org/misc/itoa.shtml>).

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