REGIONAL NEUROCHEMICAL CHARACTERIZATION OF THE FLINDERS SENSITIVE LINE RAT WITH REGARD TO GABA AND CHOLINERGIC SIGNALLING PATHWAYS

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Abstract

Despite their acknowledged efficacy, currently available antidepressants still demonstrate undesirable side effects, shortfalls in effectiveness and a delayed onset of action. All these agents act *via* monoaminergic mechanisms, although recent studies have begun to note the potential role of the cholinergic system as well as the amino acid pathways in affective disorders. It has been suggested that glutamate NMDA receptor activation may be involved in hippocampal degeneration seen in patients with depression, as well as contributing as a molecular target for the antidepressant action of known antidepressant drugs. Glutamate either separately or *via* the release of nitric oxide, regulates the release of various transmitters in the brain critical for affective state, e.g. monoamines (noradrenaline, dopamine), indoleamines (5HT), γ-aminobutyric acid (GABA) and acetylcholine. The aim of this study was to investigate N-methyl-D-aspartate (NMDA) and muscarinic M_1 receptor characteristics and also GABA and acetylcholine levels in a genetic animal model of depression, the Flinders Sensitive Line (FSL) rat, with respect to its control, viz. Flinders Resistant Line (FRL) rat, thereby establishing a possible role for the amino acid and cholinergic pathways in the hippocampus and frontal cortex, two brain areas implicated in depression. In addition, anxiety-like behaviours were assessed using the open field and social interaction tests. A sensitive liquid chromatography tandem mass spectrometer (LC/MS/MS) method was used in the quantification of acetylcholine as well as high performance liquid chromatography with electrochemical detection (HPLC-ECD) for the quantification of GABA in the above-mentioned brain areas of FSL and FRL rats. NMDA and muscarinic M_1 receptor characteristics were expressed in terms of receptor density (B_{max}) and affinity (K_d) values and were performed using [^{3}H]-MK801 (27.5 Ci/mmol) and quinuclidinyl benzilate (52.0 Ci/mmol) for NMDA and M_1 receptors, respectively. In addition, to provide evidence for face validity, behavioural

assessments were routinely performed using the open field test and social interaction test.

Significantly elevated levels of acetylcholine were found in the frontal cortex but with significantly reduced levels in the hippocampus of FSL rats. Cortical and hippocampal muscarinic receptor binding characteristics remained unchanged, while no differences with regard to GABA levels and NMDA receptor binding characteristics were noted in these brain areas. In concordance with studies from the literature, aversive and locomotor behaviour as measured in the open field test, provided evidence of anxiogenic behaviour in the FSL rat, evinced by significantly less social interaction than their FRL counterparts. In addition, evidence for a lack in general activity of the FSL rat in the open field was also noted. Our data therefore suggest the presence of a cholinergic dysfunction in both the frontal cortex and hippocampus of the FSL rat, although this is not accompanied by simultaneous changes in muscarinic M_1 receptor binding in key limbic brain regions. Although increased cholinergic drive is a recognised characteristic of FSL rats and is representative of the model's construct validity, we suggest that the depressive phenotype of these animals is not related to altered cholinergic activity in a single brain region, but instead involves various limbic brain regions, possibly being more dependent on opposing cholinergic deficits in the cortex and hippocampus.

Key words: Depression, Flinders Sensitive Line, glutamate, GABA, cholinergic pathway, frontal cortex, hippocampus

Opsomming

Ten spyte van hulle erkende effektiwiteit het huidige antidepressante steeds ongewensde newe-effekte, tekortkominge in hulle bruikbaarheid en 'n stadige aanvang van werking. Hierdie middels het almal monoaminergiese werkingsmeganismes, hoewel onlangse studies die moontlike rol van die cholinergiese stelsel asook aminosuurweë in affektiewe versteurings aangetoon het. Dit is voorgestel dat aktivering van die glutamaat-NMDA-reseptor betrokke kan wees by die degenerasie van die hippokampus soos waargeneem in pasiënte met depressie en ook as 'n molekulêre teiken tot die werking van antidepressante kan bydra. Óf alleen óf *via* die vrystelling van stikstofmonoksied reguleer glutamaat verskeie oordragstowwe wat in die brein krities vir affektiewe toestande is, waaronder monoamiene (noradrenalien, dopamien), indoolamiene (5HT), γ-aminobottersuur (GABA) en asetielcholien. Die doel van hierdie studie was om die eienskappe van N-metiel-D-aspartaat-(NMDA) en muskariniese M_1-reseptore asook die vlakke van GABA en asetielcholien te bestudeer in 'n genetiese diermodel van depressie, naamlik die sensitiewe lyn Flindersrot (FSL) teenoor die kontrole, nl. die weerstandige lyn Flindersrot (FRL) om sodoende 'n moontlike rol van die aminosuur- en cholinergiese weë in die hippokampus en frontale korteks, twee dele van die brein wat by depressie betrokke is, te bepaal. Daarby is angsagtige gedrag beoordeel deur die toetse van oop veld en sosiale interaksie te gebruik. 'n Sensitiewe metode van vloeistofchromatografie en tandemmassa-spektrometrie (VC/MS/MS) is vir die kwantifisering van asetielcholien gebruik terwyl hoëdoeltreffendheid-vloeistofchromatografie met elektrochemiese deteksie (HDVC-ECD) vir die kwantifisering van GABA in die bogenoemde breindele van FSL- en FRL-rotte gebruik is. Die eienskappe van NMDA- en M_1-reseptore is as reseptordigtheid (B_{maks}) en affiniteit (K_d) uitgedruk en dit is gemeet deur [^{3}H]-MK801 (27.5 Ci/mmol) en kinuklidinielbensilaat (52.0 Ci/mmol) vir NMDA- en M_1-reseptore onderskeidelik te gebruik. Daarby is

beoordelings van gedrag roetinegewys met toetse van oop veld en sosiale interaksie gedoen om getuienis van gesigsgeldigheid te verkry.

Beduidende hoër vlakke asetielcholien is in die frontale korteks van FSL-rotte gekry, maar met beduidende laer vlakke in die hippokampus. Die eienskappe van muskariniese reseptorbinding in die korteks en in die hippokampus het onveranderd gebly, terwyl geen veranderings in die GABA-vlakke en eienskappe van NMDA-reseptorbinding in hierdie breindele waargeneem is nie. In ooreenstemming met gegewens in die literatuur, het aversiewe en lokomotoriese gedrag, soos deur die oopveldtoets gemeet, getuienis vir angswekkende gedrag in FSL-rotte gelewer soos bevestig deur beduidend minder sosiale interaksie as hulle FRL-eweknieë. Daarby is 'n gebrek aan algemene aktiwiteit van die FSL-rotte in die oop veld ook opgemerk. Ons data toon dus cholinergiese disfunksie in sowel die frontale korteks as die hippokampus van die FSL-rot aan, hoewel dit nie terselfdertyd met veranderings in muskariniese M_1-reseptorbinding in belangrike limbiese dele van die brein gepaardgaan nie. Hoewel sterker cholinergiese dryfkrag 'n erkende eienskap van FSL-rotte is en die model se konstrukgeldigheid bepaal, reken ons dat die depressiewe fenotipe van hierdie diere nie met veranderde cholinergiese aktiwiteit in die brein verband hou nie, maar eerder verskeie limbiese breindele betrek en moontlik meer van opponerende cholinergiese tekorte in die korteks en hippokampus afhanklik is.

Sleutelwoorde: depressie, Flinders Sensitiewe Lyn, glutamaat, GABA, cholinergiese weg, frontale korteks, hippokampus

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List of Abbreviations

5-HT_{1A}	Serotonin-1A receptor
8-OH-DPAT	8-hydroxy-2-(di-n-propylamino)tetralin
% RSD	Percentage relative standard deviation
ACh	Acetylcholine
B_{max}	Receptor densitiy
BSA	Bovine serum albumin
CH_3COOK	Potassium acetate
DALYs	Disability in adjusted life years
DSM-IV-TR	Diagnostic and Statistical Manual of Mental Disorders (text revision)
EDTA	Ethylenediaminetetraaceticacid
ESI	Electron spray ionisation
FC	Frontal cortex
FRL	Flinders Resistant Line
FSL	Flinders Sensitive Line
FST	Forced swim test
GABA	γ-aminobutyric acid
HC	Hippocampus

$HClO_4$	Perchloric acid
HPLC/ ECD	High performance liquid chromatography coupled to electrochemical detector
"iso-ACh"	3-Carboxypropyl)trimethylammonium
K_d	Receptor affinity
LC/MS/MS	Liquid chromatography coupled to tandem mass spectrometer
LOD	Limit of detection
LOQ	Limit of quantification
M_1	Muscarinic-1 receptor
MDD	Major depressive disorder
MRM	Multiple reaction monitoring
NMDA	N-methyl-D-aspartate
NOS	Nitric oxide synthase
N.S	Non significance
OFT	Open field test
OPA	*orto*-phtaldialdehyde
PA	Peak area
QNB	quiniclidinyl benzilate
SD	Standard deviation

Chapter 1 Introduction

1.1 Problem statement

Major depression is a neuropsychiatric disorder that is usually expressed in terms of disability in adjusted life years and is currently globally the fourth most major cause of disability (Skolnick, 1999; Elhwuegi, 2004). The seriousness of depression is furthermore emphasized by the approximate 121 million people affected worldwide (Rosenzweig-Lipson *et al.*, 2007). A proper definition as well as classification for depression remains vague due to the heterogeneous nature (co-morbid symptoms) of the illness. Despite effective drug treatment being introduced as early as the 1950's with the availability of the tricyclic antidepressants (TCA's), followed some decades later by the selective serotonin reuptake inhibitors (SSRI's) and atypical antidepressants, patients still experience difficulties in response to treatment. Only 25 - 45% of people on different antidepressants reach remission (Thase *et al.*, 2001), while 30% of patients with depression do not respond to the current treatments available (Skolnick, 1999; Rosenzweig-Lipson *et al.*, 2007). In addition, patients with depression often display co-morbid conditions e.g. cardiovascular disease, whereas certain antidepressant drug treatments (TCAs, SSRI's) could also increase the risk of cardiac complications (Feenstra *et al.*, 1999). Indeed, it is predicted that depression, together with cardiovascular disease, will be the leading cause of disability in the year 2020 (Murray & Lopez, 1997). Drug discontinuation as a result of adverse effects (Fava, 2000), the period of $\geq$2 weeks before a therapeutic effect is reached (Stassen & Angst, 1998; Skolnick, 1999; Skolnick *et al.*, 2001) or even recurrent episodes of depression (Solomon *et al.*, 2000), could probably increase the risk of suicidal attempt. In turn, the clinical importance and need for novel treatment strategies is exemplified by the fact that depression may be a fatal illness if not addressed early and aggressively.

Apart from improving the efficacy of antidepressants for depression but including response of the heterogeneous symptoms of depression such as anxiety, cognitive deficits and somatic complaints, another important aspect that will promote an improvement in treatment outcome is to address the adverse effects of current antidepressants. In essence, the adverse effects of antidepressants as well as its efficacy in heterogeneous symptoms can be addressed by increasing the specificity for target receptors that are proven beyond doubt to be causally related to the symptoms of depression. Since the underlying neurobiology of depression remains diverse and debatable (Nestler *et al.*, 2002), this by implication means that multi-target agents may be the more successful approach in novel drug development (Millan, 2006). The obvious next step is to delineate the targets for multi-target agents, and for this to be achieved it is imperative that researchers identify novel therapies and mechanisms of action through which an improved treatment outcome can be achieved. One of the cornerstones of this quest is the development and application of well-validated animal models of depression (Rupniak, 2003).

A substantial body of evidence supports the involvement of acetylcholine (ACh) in depressive behaviours (Overstreet, 1986; Chau *et al.*, 2001; Rada *et al.*, 2006), as evinced by patients with depression exhibiting an increased sensitivity to challenge with cholinergic agonists (Janowsky *et al.*, 1980; Nurnberger *et al.*, 1989; Janowsky *et al.*, 1994). Cholinergic projection sites are distributed throughout the brain with high densities found in the cortex and hippocampus (Spencer *et al.*, 1986; Mash & Potter, 1986). The role of ACh in depression, together with the cholinergic transmission in the above mentioned brain areas, indicates the clinical importance of identifying not only suitable molecular targets for treating depression, but also identifying the neuroanatomical substrates of the illness. Indeed, the limbic system has received considerable attention with regard to the dense cholinergic (Spencer *et al.*, 1986), serotonergic (Bloom, 2001) and also the γ-aminobutyric acid (GABA)-glutamate pathways (Fritschy *et al.*, 1998) that are apparent in these regions. All are involved to varying degrees in limbic functions, such as memory, motivation and emotional behaviour (Afifi & Bergman, 1998), all of which are compromised in affective disorders. The hippocampus is also

interconnected with the frontal cortex and plays an important role in mood (emotion) as well as cognitive function (Bear *et al.*, 2001) while both regions are densely innervated by the cholinergic system. Recent evidence has suggested that depression can be linked to a neurodegenerative component, particularly in the hippocampus, and which seems to be a direct result of prolonged major depression (Sapolsky, 2000; Sheline *et al.*, 2003). This degenerative component of depression has recently been linked to imbalances in excitatory and inhibitory pathways, especially glutamate and GABA (McEwen, 1999). In turn, accumulating acetylcholine has been found to increase the activity of the glutamatergic system resulting in neural damage (McDonough & Shih, 1997; Weissman & Raveh, 2008)

The focus of research on depression has developed over the past years from the premise that depression involves the dysfunction of a single neurotransmitter, to hypotheses that describe a multifaceted disorder involving various central neurotransmitters in its pathophysiology. Glutamate and GABA are two of the key amino acid neurotransmitters that regulate euthemic state in depression through their mutual counterbalancing effects (Lydiard, 2003) as well as regulatory effects on other important monoamine transmitters (Prast & Philippu, 2001; Slattery *et al.*, 2005; Enna *et al.*, 2006). Consequently, despite the widely accepted notion that 5-HT is key to mood regulation, abnormalities in either or both the GABA'ergic and glutamatergic pathways could directly and indirectly result in mood disorders (Petty, 1995; Sanacora *et al.*, 1999; Sanacora *et al.*, 2004).

The importance of animal models in pre-clinical research into affective disorders cannot be underestimated. However, it is the validity of these models to accurately measure what they are expected to measure, and whether the said parameter being measured is closely analogous to the symptoms, biology and treatment of depression, requires rigorous testing. The absence of a universally accepted animal model of depression, as well as the relative paucity of truly valid animal models of depression, still remains a problem. Apart from the use of animal models exposed to a stress environment, it was soon realized that the genetic diathesis also plays a major role in the aetiology of major depression (Sullivan *et al.*, 2000). In 1982

Overstreet introduced a genetic animal model of depression, the Flinders Sensitive Line (FSL) rat (Overstreet *et al.*, 2005) that was bred for increased sensitivity to cholinergic challenge (Overstreet & Janowsky, 1991). This increased cholinergic sensitivity in the FSL rats was proposed to closely comply with the cholinergic hypothesis of depression, a theory that was built around evidence of increased cholinergic sensitivity in depressed patients (Janowsky *et al.*, 1980; Nurnberger *et al.*, 1989).

It is therefore evident that various neurotransmitters play a vital role in major depression and that disturbances in mood may involve multiple neuronal systems. The importance of determining the neurochemical abnormalities in genetic and other models of depression play an important role in validating the given model with respect to depression. Well validated models can then take their place as invaluable tools in the quest for a better understanding of the neurobiology and treatment of depression.

1.2 Project objectives

The main aim of the present study is the neurochemical characterization (construct validity) of the Flinders Sensitive Line (FSL) rat, a genetic animal model of depression, and their control, the Flinders Resistant Line (FRL) rat, with regard to differences in hippocampus (HC) and frontal cortex (FC) muscarinic M_1 and glutamate NMDA receptor binding, as well as total GABA and acetylcholine levels. To establish evidence for face validity, additional behavioural assessments will be performed on FSL and FRL rats.

Objectives:

- Acetylcholine and GABA analysis in the HC and FC will be analysed using liquid chromatography separation techniques coupled with mass spectrometry (development of technique) and electrochemical detection, respectively.

- Receptor binding characteristics of M_1 and NMDA receptors in the HC and FC will be analysed using [^{3}H] radio-labelled ligand assays.

- Behavioral assessments to establish differences in the levels of inherent anxiety between FSL and FRL rats will be studied using the open field and social interaction tests.

- $5HT_{1A}$-agonist induced hypothermia will be studied in order to establish suitable separation of the FSL/FRL with regard to construct validity.

1.3 Project design

Briefly, this project is divided into two experimental procedures, namely *neurochemical* and *behavioural* studies. The neurochemical assays will be performed on different groups of FSL and FRL rats (n = 10) to establish where and to what extent the FSL and FRL rats differ neurochemically with regard to the quantification of acetylcholine and GABA in two distinct limbic associated brain areas, the hippocampus and frontal cortex. M_1 muscarinic receptors and NMDA glutamatergic receptor characteristics will be analysed in the same brain areas. Pooled hippocampal tissue and pooled frontal cortical tissue of two rats will be used to perform radioligand binding studies. The behavioural studies (open field test and social interaction) will be randomly performed on different groups of FSL and FRL rats (n = 10/group) during three different occasions in order to establish face validity. 8-OH-DPAT – induced hyporthermia will also be evaluated in the FSL and FRL rats (n = 10/group) with the intent of construct validity.

1.4 Expected results

According to evidence underlining the FSL rat model of depression as well as theories based on the neurobiology of depression, the following results are expected:

- The Flinders Sensitive Line rat model was genetically developed with an increased cholinergic sensitivity (Overstreet & Russell, 1982; Russell & Overstreet, 1987; Overstreet, 2002). An expected enhanced cholinergic activity with regard to acetylcholine levels and muscarinic

M_1 receptor binding in frontal cortex and hippocampus brain areas of FSL and FRL rats would therefore explain the depressive phenotype, since previous studies reported an increased acetylcholine synthesis and endogenous acetylcholine levels in the cortex and striatum of FSL rats respectively while an increased concentration of muscarinic cholinergic receptors in striatal and hippocampal brain areas were found (Overstreet *et al.*, 1984; Overstreet *et al.*, 1988).

- The GABA hypothesis (Lloyd *et al.*, 1989) stated that depression is due to a deficiency in brain GABA levels and that effective treatment tends to achieve normalization in mood by restoring the GABA balance. It was found that patients with depression had significant lower levels of GABA in the cortex brain area compared with healthy controls (Sanacora *et al.*, 1999; Sanacora *et al.*, 2004). Since GABA neurons are widely distributed throughout the brain, low levels in the hippocampus and frontal cortex can be indicative of a dysfunctional GABAergic system evident in depression. GABA also regulates glutamate through counterbalancing effects (Yamada *et al.*, 1999; Lydiard, 2003), such that abnormalities in any one of these neurotransmitter pathways may be a precipitating factor for the development of a mood disorder (Gerner *et al.*, 1984; Petty, 1994; Petty, 1995; Sanacora *et al.*, 1999; Sanacora *et al.*, 2004). A dysfunctional GABA'ergic pathway precipitated in mood disorders could therefore influence acetylcholine regulation (Ikarashi *et al.*, 1999) as well as adaptations in glutamate receptors (NMDA) (Nowak *et al.*, 1995; Karolewicz *et al.*, 2005) as a result of glutamate dysfunction. It is therefore expected that the FSL rat would have increased NMDA receptor binding activity that enhance the release of neurotransmitters such as acetylcholine (Morari *et al.*, 1998; Knauber *et al.*, 1999), with the latter in part responsible for the depressive phenotype in these rats.

- Previous studies reported that the FSL rat exhibit a markedly greater hypothermic response to the $5HT_{1A}$-agonist, 8-OH-DPAT, than did FRL rats (Overstreet *et al.*, 1994; Overstreet *et al.*, 1998; Shayit *et al.*,

2003). The hypothermic challenge is therefore expecting to result into suitable separation of the two lines with regard to construct validity.

- Congruent with evidence indicating that patients with depression exhibit co-morbid anxiety disorders (Vaschetto *et al.*, 1996; Mineka *et al.*, 1998; Kaufman & Charney, 2000; Nemeroff, 2002) and psychomotor retardation (Parker *et al.*, 1993; American Psychiatric Association, 2000), are anxiety-like behaviours assessed in the social interaction test and deficits in general activity in the open field of the FSL rat. These behavioral differences could therefore reveal further evidence of face validity and support of depressogenic phenotype of these animals.

Chapter 2
Literature review

2.1 Major depression

Major depressive disorder (MDD) or unipolar depression is a serious heterogeneous mood disorder in terms of symptoms and underlying diseases. In addition to MDD, the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV-TR) (American Psychiatric Association, 2000) also described other classes of depressive disorders (dysthymia and "depressive disorders not otherwise specified"), where major depression is ranked the worse in terms of severity of symptoms. MDD is further characterized by the presence of one or more major depressive episodes for a duration of at least two weeks (American Psychiatric Association, 2000)

2.1.1 Epidemiology

Epidemiology is the study of populations to determine the frequency and distribution of diseases and to measure the risk factors. These studies focus on parameters like prevalence and incidence and are important for producing etiologic hypotheses and for administrative and planning purposes.

The DSM-IV (American Psychiatric Association, 2000) revealed a point prevalence for MDD between 10 - 25% for women and 5 - 12% for men. A national household survey conducted in South Africa (SA) between 2002 - 2004 using 4351 adults revealed a lifetime prevalence of 9.8% for mood disorders (Stein *et al.*, 2008). The results of prevalence in SA are therefore in line with the above prevalence range revealed by the DSM-IV. While data of various incidence studies are inconsistent, the data from eight reports indicated a range of 0.13 - 0.201% for men and 0.32 - 0.5% for women (Bland, 1997)

Risk factors are usually assessed by comparing certain demographic variables (e.g. gender, age and genetic related features). Incidence differences in males and females to develop MDD are well documented (Weissman *et al.*, 1996; Patten *et al.*, 2006) where MDD is twice as common in females as in males (American Psychiatric Association, 2000). Depressive disorders can strike virtually at any time of life but normally during the mid teens, 20's, or 30's (Beers, 2006). Disturbingly, the age at onset of major depression is beginning to decline (Weissman *et al.*, 1992) with the highest rates of first onset of depression in one epidemiological study found to be between age groups of 12-24 (Patten, 2000). Furthermore, various studies suggested that earlier onset of major depression can result in risk of higher rates of recurrent episodes (Lewinsohn *et al.*, 1994; Klein *et al.*, 1999). Thus, the presence of major depressive episodes in adolescents can result in difficulty to maintain remission in later stages of life. Major depression was ranked to become the second leading cause of disability-adjusted life years (DALYs) in the year 2020 (Murray & Lopez, 1997). Depressive illnesses have also been accompanied by alcohol abuse or dependence, panic disorder (Weissman *et al.*, 1996), and cardiovascular heart disease (McConnell *et al.*, 2005). The increased rate of DALYs can be a direct cause of the disability associated cardiovascular disease (CVD), since CVD will be the leading cause of DALYs in 2020 (Murray & Lopez, 1997).

2.1.2 Heterogeneous symptoms and conditions

As previously mentioned, the criteria for major depression is the presence of depressive episodes for at least two weeks that persist nearly every day (American Psychiatric Association, 2000). These episodes present itself with two core symptoms that is either the inability to experience pleasure (melancholy or anhedonia) or depressed mood. In addition, the patient also has to experience three or four additional symptoms (see figure 1). A major depressive episode is characterized by a symptom that is newly present or became worse when compared to a patient's previous symptom condition (American Psychiatric Association, 2000) and is usually the concern of the psychiatrist when various symptoms persist for weeks or months since a

lowered state of mood can be experienced by the majority of people during some stage of their live.

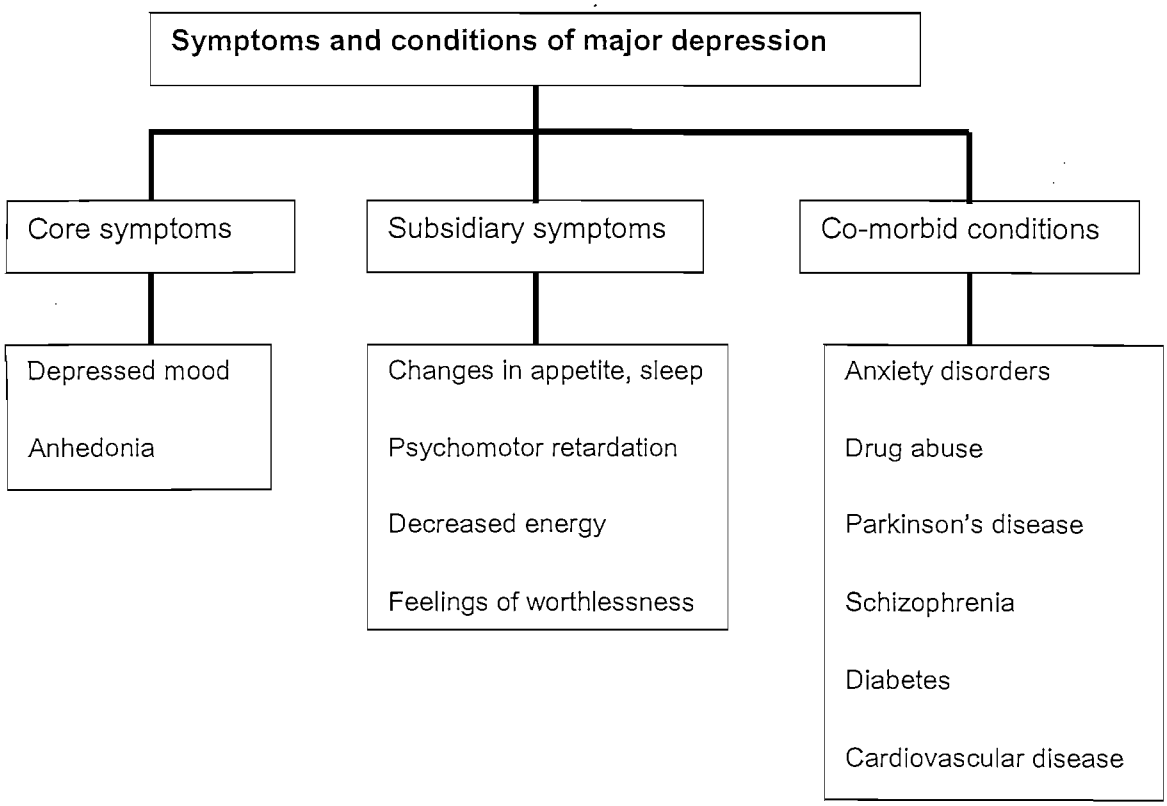

Figure 1: Adapted from (Millan, 2006) and (American Psychiatric Association, 2000) Major depression can be characterized by both (or one) core symptoms and any four (or three) subsidiary symptoms. The characteristic symptoms of major depressive disorder are almost never present on their own and in addition accompanied by various underlying co-morbid conditions

Major depression is also generally subdivided into four groups according to different subsets of symptoms experienced:

1 The psychotic subgroup: Patients may have delusions and auditory or visual hallucinations.

2 Catatonic subgroup: Characterized by severe psychomotor retardation or excessive activity (agitation).

3 Melancholic subgroup: The loss of pleasure in nearly all activities, unchanging emotional expression, excessive guilt, waking too early in the morning (terminal insomnia) and significant anorexia or weight loss.

4 Atypical subgroup: Characterized by elevated mood in reaction to positive events and rejection sensitivity. These reactions lead to overreaction to criticism or rejection. Hypersomnia and increased appetite and consequently weight gain can also be present. (Beers, 2006)

The different classifications of above symptoms help to differentiate between various mood disorders that require different management (Remick, 2002). The diagnosis of major depression is considerably difficult in patients with co-morbid conditions (figure 1) and the management of both the underlying medical condition (co-morbid condition) and the major depressive disorder are adversely affected (e.g. longer episodes, reduced response to treatment of major depression) (American Psychiatric Association, 2000). The complicated diagnosis of depression is further exemplified by the co-existence of depression with other psychiatric disorders e.g. anxiety disorders (Brunello *et al.*, 2000), schizophrenia (see above subdivision of depression) and Parkinson's disease (Friedman & Chou, 2004), that share certain symptoms of major depression. The co-morbid conditions of depression are not only psychiatric-related as can be seen in figure 1. However, this detail is beyond the scope of this review.

2.1.3 Social and economical impact

Depression disability may affect the economic (Trivedi *et al.*, 2004; Andlin-Sobocki & Wittchen, 2005) and social (Donohue & Pincus, 2007) quality of life of the individual and has considerable impact on lost of productivity in the work environment. Data from two national surveys revealed a work disability cost almost similar to the direct cost of depression treatment. It could therefore be advisable to encourage treatment to increase productivity in the

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workplace that will promote economical improvement (Kessler *et al.*, 1999). Not only do the productivity and treatment costs contribute to the total healthcare cost of depression, but mortality costs as a consequence of suicide (Stoudemire *et al.*, 1986; Rice & Miller, 1995) are considerable, making up 7% of the total healthcare cost in the year 2000 (Greenberg *et al.*, 2003). Despite the distress of depressive disorders on a person's daily functioning, it is also evident that interpersonal adjustment is diminished. For example, it was found that infants or children of depressed patients showed an increased sensitivity for general problems (e.g. social and academic difficulties) as well as an increased risk for developing depression (Downey & Coyne, 1990).

2.2 Management drawbacks

For more than 40 years, the monoamine hypothesis has been recognized as the best supported theory explaining the mechanism of action of antidepressants and importantly, in providing a basis for our understanding of the neurobiology of depression. However, clinical features like difference in response by various patients, treatment resistance, adverse effects that lead to drug discontinuation and the delayed onset of action, emphasise the need for a better understanding of the pathophysiology of depression and for identifying and investigating novel neurochemical pathways beyond the biogenic monoamine hypothesis.

2.2.1 Monoamine hypothesis

The mechanisms of action of antidepressants currently available on the market all support the monoamine hypothesis of depression, which simply states that mental depression is due to a deficiency of brain monoaminergic activity and that depression is treated by drugs that increase this activity (Schildkraut, 1967). However, this hypothesis poses some limitations, e.g. there are drugs that can increase brain monoaminergic activity (e.g. cocaine and amphetamine) but which are not effective clinically as antidepressants. Second, not all depressed patients respond equally to the same antidepressant. Third, and most importantly, these changes in the

monoamine levels at the synapse take place within hours after the administration of the antidepressants, while the therapeutic response requires the continuous administration of these drugs for weeks (Baldessarini, 1989). These drawbacks have lead to the modified monoamine theory of depression which suggests that the acute increase in the levels of the monoamines at the synapse may be only an early step in a potentially complex cascade of events that ultimately results in antidepressant activity (Pineyro & Blier, 1999). It also suggests that the monoamine hypothesis is not the only hypothesis involved in depression. Some authors even suggest that the increased monoamine levels are in fact responsible for the delayed onset of action and adverse effects of antidepressants (Gittos, 2000).

2.2.2 Treatment failure

The ideal antidepressant is characterized by a profile of fast onset of action, no side effects, 100% remission rates and the absence of relapse. How novel antidepressants may be expected to contribute to the unmet needs of current therapeutic agents, as described above, are illustrated in table 1 (Rosenzweig-Lipson *et al.*, 2007)

Treatment resistant depression (TRD)
Fast onset of action of antidepressants
Fewer cognitive abnormalities in depressed patients
Treatment of symptomatic pain that is associated with depression
Reduction in side-effects
- Sexual dysfunction
- Gastrointestinal symptoms

Table 1: Unmet needs of novel antidepressants

Thus, it is clear that new antidepressants with an improved clinical profile are needed to treat the heterogeneous symptoms of depression.

2.3 Pathophysiology of depression

In the previous section, the classical monoamine hypothesis of depression and the role of monoamines in depression were described. Since the focus of the current study is on cholinergic and amino acid signalling pathways in a rat model of depression, the rest of this literature review will be devoted to the role of these neurotransmitters in depression.

2.3.1 Neuroanatomy of depression

It is evident that the limbic system of the brain plays a major role in the aetiology of depression. The limbic system is defined as the limbic lobe and all the cortical and subcortical structures related to it, comprising of:

- septal nuclei,
- amygdala,
- hypothalamus, thalamus, epithalamus,
- brain stem,
- neocortical areas in the frontotemporal region,
- olfactory cortex and
- ventral parts of the striatum.

However, the most closely related structures of the limbic system are the hippocampus, amygdala and hypothalamus (see figure 2).

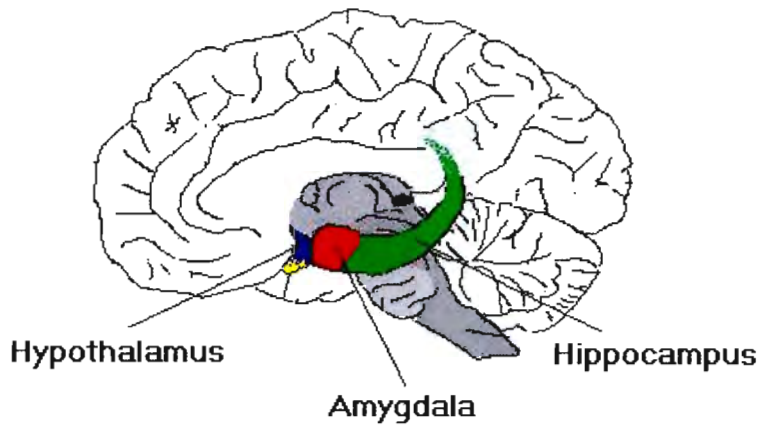

Figure 2: **Structures associated with the limbic system and intimately connected to the septum, basal ganglia, cingulate gyrus and prefrontal cortex. Figure adapted from (Boeree, 2002)**

All the above structures play a vital role in memory, motivation and emotional behaviour (Afifi & Bergman, 1998) all of which are involved in mood disorders. The hippocampus especially plays a key role in memory related functions. This brain region is part of the limbic system and forms part of the temporal lobe where it is interconnected with the entorhinal cortex, perirhinal cortex and parahippocampal cortex. All of these structures, including the hippocampus, are collectively referred to as the medial temporal lobe structures which are of great importance for declarative memory consolidation. Declarative memory based on the ability to store, remember and express memories are mostly associated with the hippocampus brain area (Eichenbaum *et al.*, 1996). The frontal cortex is also believed to be responsible for learning and memory since it is interconnected with the medial temporal lobe (see figure 3). Despite the memory related functions, the hippocampus is also involved in emotional behaviour where the cingulate cortex forms a circuit with the hippocampus (medial temporal lobe structures), the so-called "Papez circuit" (figure 3). All of the structures in the Papez circuit are responsible for different emotional functions, e.g. cingulate cortex for emotional experience and the hypothalamus for emotional expression.

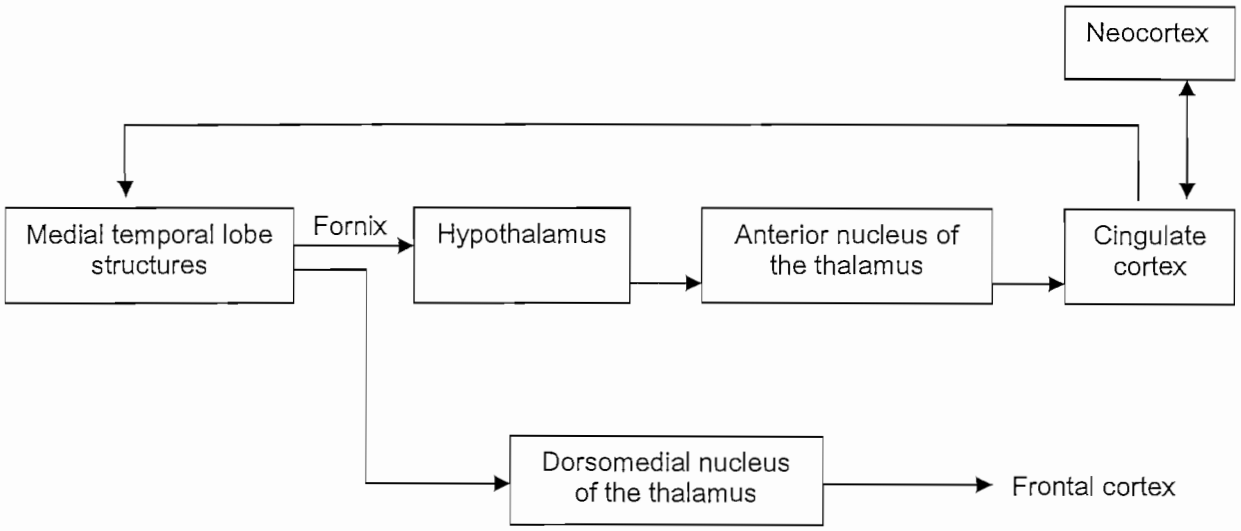

Figure 3: The "Papez circuit" and connection of the frontal cortex with this circuit in mediating different functions involved in depression. Figure adapted from Bear *et al.*, 2001

Both the hippocampus and frontal cortex are innervated by a dense cholinergic neurotransmission (Spencer *et al.*, 1986; Mash & Potter, 1986) that originates from two cholinergic nuclei of the basal forebrain, the medial septum and the nucleus basalis magnocellularis (NBM) (Zola-Morgan & Squire, 1993), as indicated in figure 4. The cholinergic neurotransmission that is implicated in attention, learning and memory (Everitt & Robbins, 1997; Sarter & Bruno, 1999) suggests a functional role in mood disorders. As can be seen in figure 4, the cholinergic projections innervate the brain areas that mediate certain functions affected in depression (e.g. emotion and cognitive functions).

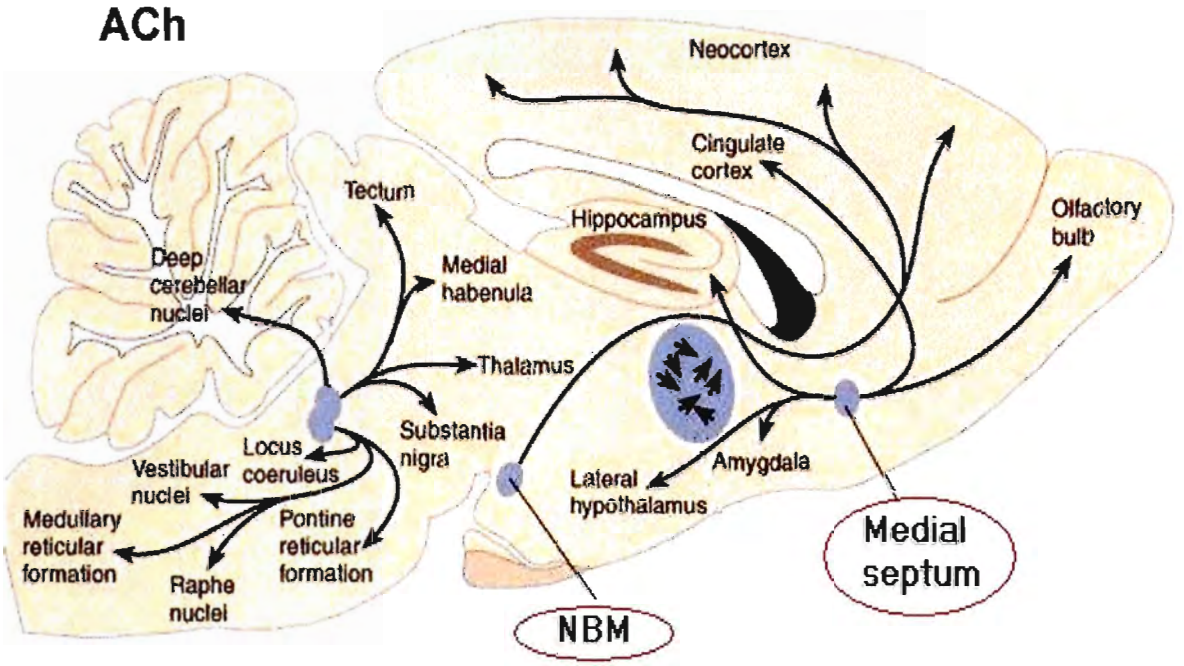

Figure 4: Acetylcholine (ACh) projections from different cholinergic nuclei. Figure adapted from Salinas, 2006

Serotonin transmission, with its acknowledged clinical role in major depression exemplified by the monoamine hypothesis and the clinical effectiveness of SRIs, is relative evenly distributed throughout the brain. Serotonergic neurons find their origin in nine nuclei near the raphe regions of the pons and upper brainstem (Steinbusch & Mulder, 1984). Two of these nuclei innervate the frontal cortex and hippocampus. The median raphe nucleus makes a major contribution to the limbic system whereas the dorsal raphe nucleus innervates the cortical regions (Bloom, 2001).

From the above, it is apparent that cholinergic and serotonergic transmission are both involved at some level in the regulation of mood, possibly with the former being influenced by the serotonergic system (Steinbusch & Mulder, 1984). Both these neurochemical pathways are involved in passive avoidance behaviour (Riekkinen, 1994) which is important to determine memory function commonly disrupted in depression (Overstreet, 1993). Furthermore, cholinergic plasticity (important underlying theory of depression)

(Duman *et al.*, 1999) is dependent on the integrity of the serotonergic system in the hippocampus and cortical brain area (Alonso & Soubrie, 1991). It has also been found that the hippocampal cholinergic system is regulated by the serotonergic system in modulation of anxiety, often a co-morbid condition with depression. For example, the anxiogenic effects of a nicotinic receptor antagonist, mecamylamine, can be blocked by co-administration of a 5-HT_{1A}-antagonist, suggesting that mecamylamine enhances serotonergic transmission. Indeed, mecamylamine enhances [^{3}H]-5-HT release from hippocampus (File *et al.*, 2000). Therefore, not only are the serotonergic and cholinergic systems anatomically related but they also co-regulate one another, such that dysfunction in either one of these signalling systems may underlie the development of depression (Overstreet *et al.*, 1998).

γ-aminobutyric acid (GABA) ergic and glutamatergic neurotransmission are also both involved in mood disorders (Petty, 1994; Sanacora *et al.*, 1999; Sanacora *et al.*, 2004; Luján, 2007). GABA is widely distributed throughout the brain and accounts for over 40% of all synapses in the cortex alone (see figure 5). However, the distribution of GABA is heterogeneous, with the highest concentrations present in the basal ganglia, followed by the hypothalamus, the peri-aqueductal grey matter and the hippocampus (Leonard, 2003b). Moreover, the glutamatergic cells represent 90% of hippocampal neurons (the dense distribution in limbic system can be seen in figure 5) whereas its counterbalancing amino acid GABA, represents the remaining 10% (Fritschy *et al.*, 1998).

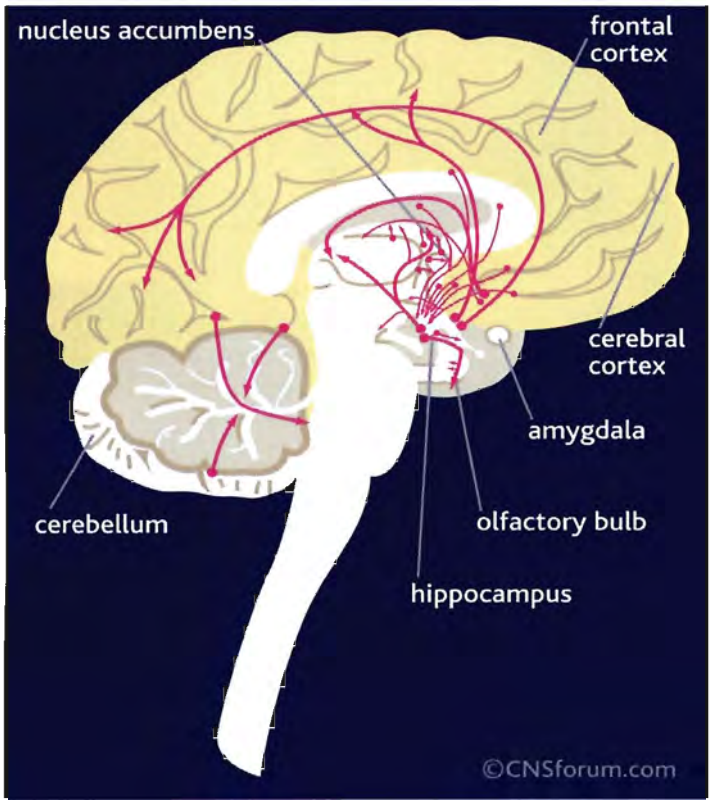

Figure 5: **Distribution of GABA neurotransmission in normal human brain**

Morphological changes in the hippocampus as a result of stress has been extensively studied. It is now well known that hippocampal changes associated with hippocampal shrinkage, are mediated by stress (Bremner *et al.*, 1995; Gurvits *et al.*, 1996) and which in turn is dependent on the nature and duration of the stressor. This too has been observed in patients with a history of recurrent major depression (Sheline *et al.*, 1996; Sheline *et al.*, 2003). Depression is causally linked to ongoing stress (Robbins, 2005; Bale, 2006), while the 20% shrinkage of the hippocampus observed in these patients has been related to atrophy of neurons (Woolley *et al.*, 1990; Sapolsky, 2000). A reduction in cortical volume has also been described in patients with mood disorders (Drevets *et al.*, 1997). It has been proposed that the process of neuronal atrophy that mediates these events is driven primarily by the neurotoxic action of glucocorticoids, and the subsequent release of glutamate and nitric oxide (Sapolsky, 2000). Important to note however, is that this process appears reversible following successful treatment (Sheline *et al.*, 2003) and remission of the illness. In addition, dysregulation of neuropeptides

may also contribute to above described hippocampal atrophy. Other hormone abnormalities involve growth hormone, thyroid-stimulating hormone and prolactin responses. Brain imaging studies have also found alterations in cerebral blood flow and metabolism in some individuals, including increased blood flow in limbic regions and decreased blood flow in the prefrontal cortex (American Psychiatric Association, 2000). While the involvement of the limbic system in mood disorders is non-debatable, the precise role and interaction of different substructures of the limbic system in depression remain vague.

2.3.2 Neurochemistry of depression

2.3.2.1 Acetylcholine

2.3.2.1.1 Introduction

Acetylcholine (ACh), a neurotransmitter present in the autonomic and central nervous system was one of the first neurotransmitters discovered (Hasey, 1996). Although a possible role for enhanced cholinergic transmission in the neurobiology of depression was originally proposed in 1972 (Janowsky et al, 1972), the biogenic amine hypothesis proved the most attractive for drug development, especially with early monoamine enhancing drugs being found to be powerful mood elevating agents, while anticholinergic agents proved to be of little value (Janowsky & Overstreet, 2002). Nevertheless, it's role in neuropsychiatric disorders has received significant attention only during the last few years, especially with the advent of improved methods for determining the presence of ACh (Cooper *et al.*, 2002b).

The synthesis of ACh in the cytoplasm of synaptic terminals is catalyzed by the enzyme choline acetyltransferase (ChAT) from the compounds choline and acetyl-CoA (synthesized in the mitochondria), as indicated by equation 1. The availability of choline is the rate limiting factor in the synthesis of ACh where 50% - 85% of the choline crosses the cell membranes by means of high-affinity transport (Cooper *et al.*, 2002b).

Acetyl CoA + choline $\rightleftharpoons$ ACh + CoA **Equation 1**

After the release of ACh by electrical stimuli and activation of postsynaptic receptors, ACh is metabolised by two enzymes viz. acetylcholinesterase and butyrylcholinesterase which are also known as "true" and "pseudo" cholinesterase, respectively (Leonard, 2003b). The enzyme released choline following the rapid breakdown of acetylcholine and is then transported back into neurons to be reused in the synthesis of ACh (Vander *et al.*, 1998). Physostigmine and neostigmine are examples of reversible cholinesterase inhibitors. Apart from their important clinical utility for improvement of memory (Blount *et al.*, 2002), these agents are also used for the purpose of increasing ACh levels in order to bolster assay sensitivity (Kehr *et al.*, 1998) (see method for analyzing ACh in addendum 1).

2.3.2.1.2 Acetylcholine hypothesis and role in depression

ACh has been associated with memory and learning (Hasselmo, 2006). Of considerable interest is that blockade of muscarinic receptors has been found to impair the formation of new memories, but not the recovery of previously gained memories (Atri *et al.*, 2004).

The cholinergic model of depression was first suggested by Janowsky (Janowsky *et al.*, 1972) who postulated the adrenergic-cholinergic hypothesis. It simply states that depression is due to an over activity of the cholinergic neurons relative to the adrenergic neurons whereas mania is the result of under-activity of the cholinergic neurons with respect to adrenergic neurons. The adrenergic and cholinergic balance in mood disorders was supported by the fact that physostigmine-induced depression could be antagonized by the adrenergic drug, methylphenidate and on the other hand, methylphenidate-induced depression was rapidly antagonized by physostigmine (Janowsky *et al.*, 1973a; Janowsky *et al.*, 1973b). The important role of the cholinergic system in mood disorders has been increasingly accepted over the years and

together with studies defining anatomical distribution, have led to the development of the cholinergic hypothesis of depression. Patients with affective disorders displayed an increased sensitivity towards cholinergic agents (Janowsky *et al.*, 1980; Nurnberger *et al.*, 1989; Furey & Drevets, 2006) whereas the blockade of cholinergic receptors has also been found to produce antidepressant like effects in rats (Chau *et al.*, 1999). However, the latter observation has not been found to be reproducible or definitive (Brink et al, 2008, Overstreet personal communication). Interestingly, relatives of patients with affective disorders exhibit an increased cholinergic response when compared to healthy relatives (Sitaram *et al.*, 1987), suggesting a possible genetic marker for cholinergic supersensitivity. The antidepressant effects of muscarinic blocking agents have also recently been assessed in a randomized clinical human trials where the antimuscarinic drug, scopolamine produced rapid and potent antidepressant and antianxiety effects in patients with major and bipolar depression (Furey & Drevets, 2006). A confounding factor in accepting the possible contribution of heightened cholinergic drive in depression is that the tricyclic antidepressants (TCA) such as imipramine, which have strong anticholinergic activity, as well as selective serotonin re-uptake inhibitors (SSRI's), which do not have affinity for muscarinic receptors, are all equally effective in treating depression (Howland *et al.*, 2005). Furthermore, several studies have indicated the shortfalls in clinical efficacy of antimuscarinic agents in treating depression (Fritze, 1993; Gillin, 1995).

The hypothalamic-pituitary-adrenal axis (HPA) that is involved in the stress response is also impaired in patients with depression. Studies have shown that elevated cortisol and adrenocorticotropic hormone (ACTH) release are present in depressed patients (Janowsky & Overstreet, 2002). These studies are further supported by evidence indicating that cholinergic drugs can increase CRF, ACTH and cortisol release in animals, healthy individuals and psychiatric patients (Risch *et al.*, 1983a; Risch *et al.*, 1983b; Janowksy & Craig Risch, 1984). The link between stress and ACh release has also been confirmed in studies where ACh release was determined in different brain areas following exposure of rats to different stressors. These studies found that stress causes a marked increase in ACh release in the hippocampus

followed by a compensatory down-regulation of muscarinic receptors (Gilad, 1987). By using microdialysis, a recent study described a long lasting increase in ACh levels in the nucleus accumbens following but not during the exposure to inescapable stress (Rada *et al.*, 2006). Similar findings were also reported in the rat hippocampus and frontal cortex although during the restraint stress itself (Mark *et al.*, 1996).

Thus, while a considerable amount of evidence supports the fact that the cholinergic system has a contributing role in stress and behavioural depression, it is not known if the changes in ACh levels are the direct cause or result of depression and how much involves its interaction with other transmitters and neuromodulators involved in mood regulation.

2.3.2.2 Muscarinic receptors

2.3.2.2.1 Introduction

Five different muscarinic receptors have been identified in the brain as well as cloned. Receptor classification has been based on the degree of affinity for the muscarinic antagonist, pirenzepine, with the M_1 muscarinic receptor having the highest affinity for pirenzepine. Current nomenclature includes the M_1 – M_5 muscarinic receptors with M_1 and M_3 found postsynaptically and M_2 and M_4 receptors located presynaptically. The function of the latter is to regulate the release of ACh (autoreceptors). These receptors are all found in the central nervous system (CNS) (Leonard, 2003b) with different distributions. High densities of M_1 receptors are found in the cerebral cortex, hippocampus, dentate gyrus, medial and basolateral amygdala, nucleus accumbens and caudate/putamen, while muscarinic M_2 receptors are distributed throughout the brain (Spencer *et al.*, 1986).

Muscarinic receptors are classified as metabotropic receptors in that they transmit ligand receptor binding into intracellular response through coupling to G-protein mediated signalling and the activation of phospholipase C (Leonard, 2003b). The cholinergic system is also able to adapt to changes in receptor regulation. Thus, chronic administration of muscarinic agonists results in

decreased M_1 receptor density while increased M_1 receptor density is evident following the administration of muscarinic antagonists (Leonard, 2003b).

2.3.2.2.2 Role of muscarinic receptors in depression

As discussed in section 2.3.2.1, patients with affective disorders display increased cholinergic sensitivity. Unfortunately, studies that have tried to explain cholinergic supersensitivity in terms of muscarinic receptor changes have been inconclusive (Overstreet & Janowsky, 1991). Nevertheless, elevated muscarinic receptor binding has been found in the frontal cortex of suicide victims compared to control patients (Meyerson *et al.*, 1982), while a higher density of muscarinic receptors has been described in fibroblasts of patients with affective disorders and a history of affective disorders (Nadi *et al.*, 1984). However, most other studies have not been able to replicate these findings (Kaufmann *et al.*, 1984; Lenox *et al.*, 1985; Katerina *et al.*, 2004). Contrary to the aforementioned studies, muscarinic cholinergic agonists and antagonists alter mood related behavior in animal models of depression (Chau *et al.*, 2001), while behavioral depression in rats induced by stress increases ACh levels together with a compensatory downregulation in muscarinic receptors (Rada *et al.*, 2006).

Thus, the correlation of cholinergic supersensitivity with muscarinic receptor alteration in depression is restricted to post mortem studies of tissue from suicide victims, as well as limited by the mixed findings from other studies as described above. Nevertheless, there is distinct direct and indirect evidence supporting a functional role for muscarinic receptors in mood disorders, as suggested by the regulation of acetylcholine by drugs targeting muscarinic receptors, as well as alteration in mood related behavior in animals. Given the role of muscarinic receptors in the pharmacological effects of certain antidepressant drugs, nicotinic cholinergic receptors have also been implicated in depression, especially with evidence that many antidepressants have actions on these receptors (Shytle *et al.*, 2002). However, review of this information is beyond the scope of this work.

2.3.2.3 γ-aminobutyric acid

2.3.2.3.1 Introduction

γ-aminobutyric acid (GABA) is an inhibitory amino acid that is present in the mammalian brain in very high concentrations in some regions and in approximately 200-1000 times higher concentrations than other neurotransmitters involved in depression, e.g. acetylcholine, noradrenaline, and serotonin. Since GABA does not penetrate the blood brain barrier even after systemic administration (Cooper *et al.*, 2002a), the brain forms the primary source of GABA.

GABA is synthesized in the nerve terminal from glutamine and glutamate in a reaction that is catalyzed by the glutaminase and glutamate decarboxylase enzymes, as reflected by figure 6. Glutamine is converted from glutamate, the latter being synthesized in the mitochondria of glial cell. After the release of GABA its action is terminated by transportation of GABA into the glial cell or by transportation back into the nerve terminal by active transportation (Leonard, 2003b).

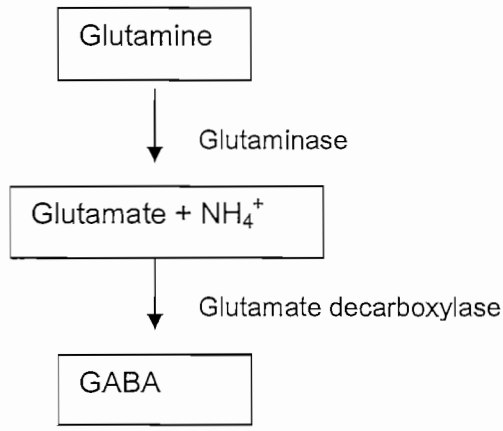

Figure 6: The synthesis pathway of GABA in the GABA terminal. Adapted from (Leonard, 2003b)

The functions of GABA are synonymous with its wide distribution in the brain and is involved in vigilance, consciousness, arousal, thermoregulation,

learning, food consumption, hormonal control, motor control and the control of pain (Leonard, 2003b).

2.3.2.3.2 GABA hypothesis and role in depression

Although GABA has been frequently related to anxiety (Lydiard, 2003; Nemeroff, 2003), various studies have also been conducted on its role in depression.

The GABA hypothesis of depression has already been proposed nearly 20 years ago (Lloyd *et al.*, 1989) based on collective findings. Those findings led to the hypothesis that depression is due to a deficiency in brain GABA levels and that effective treatment tends to achieve normalization in mood by restoring the GABA balance. Indeed, it was found that patients with depression had significant lower levels of GABA in the cortex brain area compared with healthy controls (Sanacora *et al.*, 1999; Sanacora *et al.*, 2004). A considerable amount of data also found lower levels of GABA in the CSF (Gerner & Hare, 1981; Kasa *et al.*, 1982; Gerner *et al.*, 1984) and plasma (Petty & Schlesser, 1981; Petty, 1994).

In a behavioral animal model of depression, stress-induced depression was found to reduce GABA release, whereas administration of a GABA antagonist (bicuculline) in the hippocampus resulted in identical depression behavior (Petty & Sherman, 1981). The role of the hippocampus in depression is therefore exemplified by the involvement of GABA in this brain area. The low levels of GABA are normalized by most effective antidepressant treatments (Sanacora *et al.*, 2002), while simultaneous alterations in GABA receptors have also been described (Lloyd *et al.*, 1985; Malatynska *et al.*, 1999; Sands *et al.*, 2004).

The GABA deficiency hypothesis is supported furthermore by the significant role that $GABA_B$ receptors play in depression. The $GABA_B$ receptors are widely distributed in GABA neurons as well as in other neurons where neurotransmitter release is modulated (Leonard, 2003b). Glutamate as well as GABA release is regulated through presynaptic $GABA_B$ autoreceptors

(Yamada *et al.*, 1999) further emphasizing the unique balance that exists between glutamate and GABA and the fact that an imbalance can probably give raise to affective disorders. $GABA_B$ receptors also regulate the release of biogenic amines, acetylcholine, neuropeptides and hormones via autoreceptors (Cooper *et al.*, 2002a). Various clinical used antidepressants increase $GABA_B$ receptor binding (Lloyd *et al.*, 1985; Sands *et al.*, 2004) in rat frontal cortex and hippocampus brain area. Since it is known that increased GABAergic transmission caused by blockade of presynaptic $GABA_B$ receptors may be an indication of antidepressant effects, it is interesting that $GABA_B$ receptor downregulation by antidepressants over time results in a compensatory upregulation/activity of the $GABA_B$ receptor (Sands *et al.*, 2004). The antidepressant effects of $GABA_B$ receptor blockade has also been demonstrated by various animal models of depression (Nowak *et al.*, 2006), providing further supportive evidence for the important contribution of GABA transmission to mood regulation.

Petty also proposed a GABAergic hypothesis (Petty, 1995), stressing its importance as a genetic marker of vulnerability to develop depression. Petty suggested that low GABA levels in certain patients indicate a vulnerability to develop depression and that symptoms of depression were the results of environmental factors (e.g. alcohol, menstrual cycle and gender) that increase extracellular GABA levels. Treatment restored the balance of GABA to its original levels along with remission of depression symptoms. Animal studies also found either decreased brain GABA levels in various depression models (Borsini *et al.*, 1988; Grønli *et al.*, 2007), or did not find any significant difference in GABA levels (Post *et al.*, 1980). Over recent years the important contribution of GABA towards the development of depression, and especially the possible degenerative nature of the illness, has been associated with its strong interaction with and regulation of excitatory transmission, especially that of glutamate.

Currently, the excitatory neurotransmitter, glutamate, is also increasingly being implicated in the aetiology and pathophysiology of depressive disorders. Several studies suggest the presence of an imbalance between glutamate and GABA, particularly a hyper-glutamatergic and a hypo-GABA'ergic state,

as being one of the major contributing factors in the development of depression (Sanacora *et al.*, 1999; Sanacora *et al.*, 2004).

2.3.2.4 NMDA receptor

2.3.2.4.1 Introduction

Receptors for glutamate can be divided into two groups of receptors, namely ionotropic receptors (NMDA, AMPA and kainate) and metabotropic receptors (Group I, II and III). The most important ionotropic receptor, NMDA, mediates relatively slow excitatory responses and also plays a role in neuroplasticity (Leonard, 2003b).

The NMDA receptor consists of various binding sites that are involved in the regulation of glutamate neurotransmission. The binding of glycine to the glycine binding site is necessary to exert functions, such as open time of channels and desensitization rate, and has been the focus of extensive research. However, binding of both glycine and glutamate is necessary to full functioning of the channel (Javitt, 2004). The enriched environment of NMDA modulatory sites that surrounds the channel has led to the development of ligands of varying potency that may be useful in regulating NMDA channel activity. The full antagonist, dizocilpine (MK801), is effective when the receptor is activated regardless of the level of activation (even under normal physiological conditions) (Javitt, 2004), while memantine, which is regarded as an open channel blocker, only blocks the channel when it is excessively activated (Chen *et al.*, 1992; Lipton, 2007).

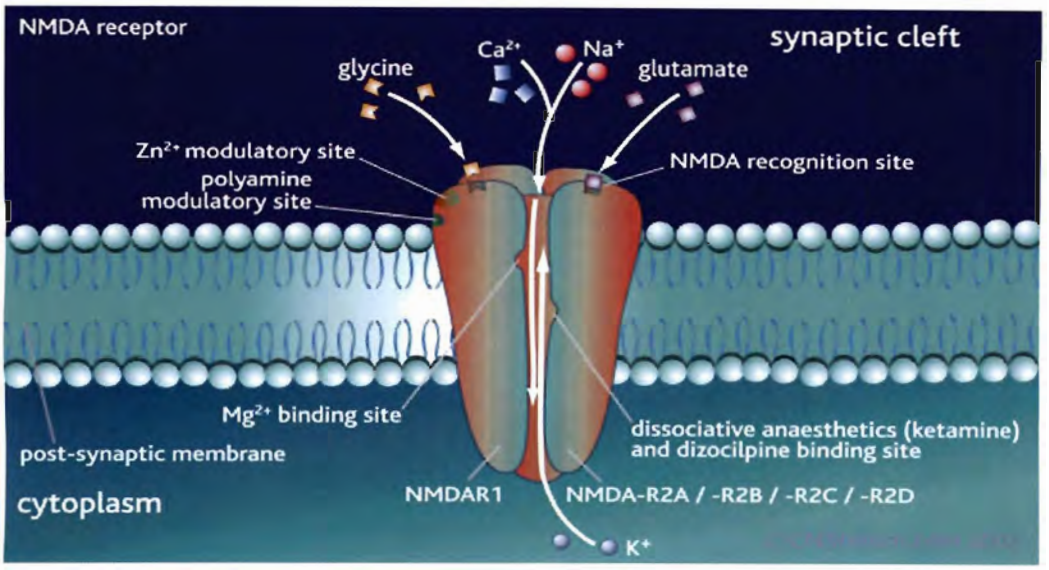

Figure 7: The ionotropic NMDA receptor with different regulatory binding sites

2.3.2.4.2 Role of NMDA receptor in depression

The importance of NMDA receptors in depression has been documented in both preclinical and clinical studies of depression, suggesting that NMDA receptor antagonists not only may modulate antidepressant activity (Layer *et al.*, 1995; Przegalinski *et al.*, 1997) but also have antidepressant actions of their own (Berman *et al.*, 2000; Boylan *et al.*, 2002), as will be discussed below.

Suppression of the functioning of the NMDA receptor channel has been shown to mimic effects of antidepressants in the forced swim test in rodents (Trullas & Skolnick, 1990; Layer *et al.*, 1995; Padovan & Guimarães, 2004), a test used to screen for antidepressants. A variety of chronically but not acutely administered antidepressants were also shown to produce adaptive changes in NMDA receptors (Skolnick *et al.*, 1996) by influencing the potency of glycine binding on the NMDA receptor binding site (Paul *et al.*, 1994; Nowak *et al.*, 1995). Therefore, adaptive changes to the NMDA receptor produced by monoaminergic antidepressants is akin to that evoked by NMDA antagonists (Skolnick, 1999), thus supportive for a definite role of the NMDA

receptors in depression and antidepressant action. Nevertheless, the exact mechanism of their involvement remains undefined. It has been suggested that NMDA receptors antagonists downregulate β-receptors (Layer *et al.*, 1995; Papp & Moryl, 1996); an effect also induced by chronic treatment with monoaminergic antidepressants (Heal *et al.*, 1989). What is important is that NMDA antagonists bolster the antidepressant actions of typical antidepressants in animals, while in concordance with this, the NMDA antagonist, ketamine, has been found not only to produce antidepressant effects in patients with major depression (Berman *et al.*, 2000), but also may have benefit in treatment resistant patients (Zarate *et al.*, 2006). Despite these promising results, NMDA antagonists present with undesirable side effects, such as cognitive impairments, that preclude their general clinical use as antidepressants (Malhotra *et al.*, 1996).

2.4 Relevant animal models of depression

2.4.1 Introduction

An animal model can be described as an "experimental preparation developed for the purpose of studying a condition in the same or different species" (Yadid *et al.*, 2000). Animal models can be used to study the neurobiology of a disease or the screening of potentially novel drugs in a pharmaceutical environment. Indeed, over the years a variety of models with different purposes have been developed (Seligman & Beagley, 1975; Porsolt *et al.*, 1978; File & Hyde, 1978) and improved (Vollmayr & Henn, 2001; Cryan *et al.*, 2002; File & Seth, 2003) to screen for antidepressants and model the behavioral effects of depression with regard to symptomatology and etiology. The ideal animal model thus would be a model that represents all the clinical features of depression (e.g. symptoms, antidepressant response and etiology). Since depression is a heterogeneous psychiatric disease with unknown causes and neurobiological disturbances, effective modeling is compromised. However, most of the animal models used today are capable of differentiating effectively between antidepressants and non-antidepressant drugs, while the forced swim test (FST) has gained broad acceptance in its

ability to screen antidepressant drugs (Cryan *et al.*, 2005). Despite the potential value of animal models in furthering our knowledge of illness pathology and treatment, one must be cautious not to make assumptions in depressed individuals based on findings in animal models (Newman, 1998).

With relevance to the current study, the subsequent sections will focus on genetic and environmental animal models of depression.

2.4.2 Validation criteria

The validity of a model is defined as the extent to which a model is useful for a given purpose (Geyer & Markou, 1995). An animal model is usually assessed according to three validity criteria: face validity, construct validity and predictive validity (Willner, 1984). Of the three levels of validation, construct validity is the most important. However, as will be discussed, construct validity is difficult to determine since the underlying neurobiology of most if not all psychiatric illnesses remains unclear. Therefore, a balanced approach is necessary in which all three criteria are considered to form a overall view of the validity of an animal model (Willner & Mitchell, 2002).

2.4.2.1 Face validity

Face validity refers to the similarity between the behavior exhibited by the animal model and the specific symptoms of the human conditions (Geyer & Markou, 1995). While face validity could be a starting point for the validity of an animal model, it is not useful for insight or scientific support for the model (Geyer & Markou, 1995) and therefore not a necessary criterion. As mentioned before, it can be difficult to model the entire depression syndrome since certain symptoms are difficult (e.g. anhedonia) or impossible (e.g. suicidal ideation) to model. Furthermore, depression is co-morbid with many other illnesses that not only complicate diagnosis but also defining its underlying neurobiology (Millan, 2006).

2.4.2.2 Construct validity

Construct validity is the evaluation of the theoretical rationale of an animal model and is achieved by bringing the theoretical rationale of both the disorder itself and the disorder of the animal into association (Willner & Mitchell, 2002). However, despite the importance of this criterion, construct validation is limited by the pathology of the disease and until the exact mechanisms involved in the neuropathology of depression are elucidated and confirmed beyond dispute, this level of validation will be largely speculative, diverse and incomplete. For example, the well known monoamine hypothesis of depression is still inconclusive in that both an under-activity and over-activity of monoamines has been reported (Muck-Seler *et al.*, 1991; Leonard, 2003a; Overstreet *et al.*, 2005; Sullivan *et al.*, 2006; Barton *et al.*, 2008). Furthermore, clinically useful antidepressants may increase monoamine reuptake, e.g. tianeptine, or inhibit reuptake, e.g. SRI's, yet both have equal clinical efficacy. Together with the neurobiology, studying the etiology of the disorder could also be used to evaluate construct validity and is usually well demonstrated in animal models of depression by means of responses to different stressors (Willner & Mitchell, 2002).

2.4.2.3 Predictive validity

The predictive validity is defined as the ability of a test to predict a criterion that is of interest to the investigator and usually refers to the model's ability to identify drugs with potential therapeutic value in humans (Geyer & Markou, 1995). The predictive validity should be both sensitive and specific by means of responding to effective antidepressant treatments ("true positives") and fail to respond to ineffective agents ("true negatives"). Further, a model should minimize identification of "false positives" and "false negatives" (Willner & Mitchell, 2002).

2.4.3 Types of animal models

Animal models of depression are usually classified based on different causes (etiology) of depression. These classifications rely on two basic contributors of depression viz. the diathesis and the stress concept (see table 2). The term diathesis refers to a predisposition for developing a disorder (in this case, depression) while stress refers to a precipitant (Willner & Mitchell, 2002). It is well known that stressful events can contribute to the development of depression (Kessler, 1997; Kendler *et al.*, 1999; Fava & Kendler, 2000). These stressful events together with other stressors e.g. menopause, parkinson's disease and thyroid insufficiency would be considered as precipitants (Willner & Mitchell, 2002). Indeed, the focus for the past 40 years was mainly on animal models where behavioral depression / despair was induced by stress (Seligman & Beagley, 1975; Porsolt *et al.*, 1978; Malatynska & Kostowski, 1984; Willner *et al.*, 1987). According to Fava and Kendler (Fava & Kendler, 2000), major depression is also the result of adverse childhood experiences, certain personality traits and genetic involvement (Sullivan *et al.*, 2000; Fava & Kendler, 2000; Nestler *et al.*, 2002). All these factors are associated with a predisposition to developing depression (diathesis) and have been more recently been identified as important components for the development of animal models (Owens & Nemeroff, 1991; Overstreet, 1993; Escorihuela *et al.*, 1999; Song & Leonard, 2005; Holmes *et al.*, 2005). In this study we used a diathesis animal model of depression, viz. the genetic FSL rat model, which will be discussed in more detail following table 2 that provides an outline of stress and diathesis models of depression.

Stress models		
Stress models	Acute stress	Learned helplessness Behavioral despair Tail suspension test
	Chronic stress	Chronic mild stress
	Social stress	Social hierarchy Social separation

Diathesis models

Developmental models	Early life stress	Maternal deprivation Prenatal / neonatal stress
Lesion models		Olfactory bulbectomy
Genetic models		Flinders Sensitive Line (FSL) Congenital learned helplessness Roman high-avoidance Fawn Hooded High DPAT sensitivity animals Swim high-active animals
Genomic		HPA transgenic 5-HT transporter knockout CRH receptor subtypes knockout

		Tachykinin receptor knockout

Table 2: Classification of animal models of depression based on etiology (stress and diathesis concept) (Willner & Mitchell, 2002; Fuchs & Flügge, 2006; El Yacoubi & Vaugeois, 2007)

2.5 The Flinders Sensitive Line (FSL) model of depression

2.5.1 Introduction

The original purpose of the Flinders Sensitive Line (FSL) rat was to develop a line that was more resistant to the effects of the organophosphate anticholinesterase agent, diisopropyl fluorophosphate (DFP) to compare the mechanisms of resistance to tolerance. However, a line was ultimately developed that was genetically more sensitive to the effects of DFP and not more resistant. The control, viz. the Flinders Resistant Line (FRL) rat was developed to be more resistant to DFP relative to the FSL and not to normal outbred control rats (Overstreet *et al.*, 2005). It was these outbred Sprague-Dawley rats from which the FSL was originally derived and data suggested that there were no differences between the FRL and randomly bred rats, therefore making them both less sensitive for DFP than the FSL (Overstreet, 1993; Overstreet *et al.*, 2005).

The following section will discuss important similarities between the FSL rat and depressed patients with regard to validation criteria, supportive of the usefulness of this animal model in studying the neurobiology of depression.

2.5.2 Validity of the FSL rat model of depression

As mentioned before, the symptoms of major depression is heterogeneous varying from psychomotor retarded to agitated, insomnia or hypersomnia, or decreases or increases in appetite. Such fluctuations in symptoms can make the simulation of symptoms through a model particularly difficult (Overstreet, 1993). However, a lot of behavioral and neurochemical evidence has been

collected for the FSL model to adequately comply to the three validation criteria, viz. face validity, construct validity and predictive validity.

2.5.2.1 Face validity

Various behavioral aspects of FSL rats resemble that of depression, as outlined in table 3.

Symptom/characteristic	FSL rats	Depressives
General activity	Decrease	Decrease
REM-sleep amount & latency	Increase	Increase
Appetite	Decrease	Decrease
Cognitive impairment	Yes/no	Yes
Anhedonia	Yes	Yes
Anxiety	Yes	Yes
Enhanced response to antidepressants	Yes	Yes

Table 3: Behavioral characteristics modeled in FSL rats that reflect symptoms of depressed patients. Table adapted from Yadid *et al.*, 2000

2.5.2.1.1 General activity

Patients with depression exhibit psychomotor retardation as well as psychomotor agitation (Parker *et al.*, 1993; American Psychiatric Association, 2000). It has been suggested that psychomotor retardation can be associated with both a reduction in locomotor activity as well as a reduction of certain activities that previously were enjoyed (anhedonia) (Overstreet, 1993). The

reduced body movements associated with depression have also been noted in FSL rats exposed to an open field arena (Overstreet & Russell, 1982; Overstreet *et al.*, 1986; Overstreet & Griebel, 2004; Overstreet *et al.*, 2004). However, others have not been able to reproduce this observation (Matthews *et al.*, 1996). However, it must be mentioned that these behavioral differences in immobility between the FSL and FRL control have been noted to be more apparent following simultaneous stress exposure, e.g. to a mild foot shock (Overstreet, 1986; Overstreet *et al.*, 1988; Overstreet *et al.*, 1989; Overstreet & Janowsky, 1991). Numerous studies have also described an increased stress-induced immobility in FSL rats compared to FRL rats exposed to the forced swim test (Overstreet, 1986; Zangen *et al.*, 1999; Overstreet *et al.*, 2004; Overstreet & Griebel, 2005). The forced swim test represents behavioral despair (Porsolt *et al.*, 1978), which is a core symptom in depression, but is not associated with deficits in general activity.

These data therefore suggest that the FSL rat is an animal model of genetic vulnerability for stress-induced depression, since the depressive behavior is best seen upon exposure to stressors (Overstreet, 1993).

2.5.2.1.2 Anhedonia

Anhedonia, the inability to experience pleasure, is one of the core symptoms experienced by patients with depression (American Psychiatric Association, 2000). A common method for assessing the hedonic state of depression is through the chronic mild stress model (CMS) model and by determining saccharin or sucrose consumption / preference in animals exposed to CMS (Willner, 1997). Rats normally exhibit very high preference for saccharin solution, which decrease when exposed to CMS (Willner *et al.*, 1987). However, various studies have not able to detect any significant baseline differences between the FSL and FRL rats (Overstreet, 1993; Pucilowski *et al.*, 1993; Overstreet *et al.*, 2005) with similar results noted following other methodologies, e.g. the electrical brain stimulation hedonic method (Matthews *et al.*, 1996). Identical to that observed following stress induced-immobility,

stress was found to induce a significant reduction in sucrose preference in the FSL rat (Overstreet, 1993; Pucilowski *et al.*, 1993).

The simulation of anhedonia through animal models is still debatable. Nevertheless, it has been suggested that anhedonia may be responsible for the psychomotor retardation observed in the open field and the immobility in the forced swim test in FSL rats (Overstreet, 1993).

2.5.2.1.3 Cognitive impairment

Depression is associated with cognitive dysfunction that is characterized by impaired memory, concentration or decision making (American Psychiatric Association, 2000). Depression can also precede severe memory disruptions e.g. dementia (Kasahara *et al.*, 2006) due to an organic cause (degeneration) (Overstreet, 1993). This severe form of memory disruption (degeneration) could possibly be the result of hippocampal shrinkage since cognitive disorders in depression have been linked to the hippocampus and prefrontal cortex that are susceptible to stress (Woolley *et al.*, 1990; Duman *et al.*, 1999; Sapolsky, 2000).

Passive and active avoidance tasks are generally used to assess the cognitive abilities of rats. The avoidance tasks consist of two-compartment chambers and require the animal either to move actively from one lighted section of the chamber to the dark section (active avoidance) or to remain in one compartment (light section) (passive avoidance) to avoid shock (Overstreet & Janowsky, 1991). By exposing the FSL rats to passive avoidance, they exhibited good memory by remaining in the light section significantly longer than the FRL rats (Overstreet, 1986; Overstreet, 1993). In contrast, FSL rats performed poorly in the active avoidance test (Overstreet *et al.*, 1990a). These data could therefore suggest that FSL rat remains immobile in the active and passive avoidance tasks due to stress-induced immobility as result of psychomotor retardation and anhedonia and not necessarily better memory function per se (Overstreet, 1993). The cognitive impairment is therefore still inconclusive and further studies need to be conducted.

2.5.2.1.4 Appetite

Patients with depression usually experience a reduced appetite that leads to lower body weight, with some patients that persist with sub-clinical features of major depression (melancholic features) usually display severe weight loss while others (atypical depression) experienced increased appetite (American Psychiatric Association, 2000).

Even for the relatively few detailed studies conducted with regard to a correlation between body weight and consumption in FSL rats, generally a lower body weight was found in FSL rats as compared to FRL rats.

2.5.2.1.5 Sleep disturbance

The most common sleep disturbance associated with depression is insomnia (American Psychiatric Association, 2000), with increased rapid eye movement (REM) sleep and shortened latency between REM cycles (Hartmann *et al.*, 1966; Gottesmann & Gottesman, 2007).

Sleep disturbances in the FSL rat also correlates with that seen in depression, where increased REM and decreased latency of REM sleep have been documented in these animals (Benca *et al.*, 1996; Overstreet *et al.*, 2005).

2.5.2.1.6 Anxiety

It is almost inevitable that patients with depression exhibit co-morbid anxiety disorders (Vaschetto *et al.*, 1996; Mineka *et al.*, 1998; Kaufman & Charney, 2000; Nemeroff, 2002) where it is estimated that between 33% - 85% of patients with depression also display anxiety disorders. Similarly, up to 90% of patients with anxiety disorders display co-morbid depression (Gorman, 1996).

Although depression and anxiety is difficult to differentiate in animal models exposed to stressors, a few important models have been validated for determining anxiety related behaviors (Fuchs & Flügge, 2006). Initially, it was found that FSL rats spent the same amount of time in the open arms of the elevated plus maze as their FRL controls (Schiller *et al.*, 1991; Overstreet *et*

al., 1995). This prompted investigators to propose that the FSL rat represents a model of depression without the anxiety component. However, anxiogenic effects in FSL rats have been observed in other anxiety tests. The social interaction test, which is used to screen between different anxiolytic drugs and is non-sensitive to antidepressants (File, 1985), is one such example. Anxiety is usually analyzed by placing pairs of rats in a brightly illuminated arena so that their social interaction (licking, grooming and sniffing) is reduced. The administration of anxiolytics increase time spent in social interaction. Using this test, anxiogenic effects have been observed in FSL versus FRL rats (Overstreet *et al.*, 2004). Concluding, it is therefore apparent that FSL rats display anxiety related behaviors in some, but not all applied anxiety tests (Overstreet *et al.*, 2005).

2.5.2.2 Construct validity

Construct validity of an animal model is wholly dependent on how much is known regarding the underlying neurobiology of the particular illness it is attempting to model. When considering depression, table 4 outlines the diverse etiological basis of the disorder, and explains why establishing construct validity for an animal model of depression is in most cases speculative at best. Nevertheless, if the hypotheses suggested to be involved in depression can be established in an animal model, it does elevate its application and usefulness considerably, even though predictive and face validity remain the cornerstones of validation.

Theoretical model	FSL rats	Depressives
Cholinergic model	**Increased cholinergic sensitivity**	**Increased cholinergic sensitivity**
GABAergic model	Not determined	Decreased GABA levels
Serotonergic model	Increased 5-HT_{1A} sensitivity	Reduced 5-HT_{1A} sensitivity

Noradrenergic model	No change in sensitivity	Altered sensitivity
Dopaminergic model	**Reduced DA transporter**	**Reduced DA transporter**
HPA axis model	No change	Increased levels of CRF, ACTH and cortisol
Circadian rhythm model	**Phase advance** Normal amplitude	**Phase advance in some** Reduced amplitude
Neurotrophin model	Normal levels **Increased with AD treament**	Reduced levels **Increased with AD treatment**
Neuropeptide Y model	**Reduced NPY levels**	**Reduced NPY levels**

Table 4: Key findings in neurochemical models implicated in the neurobiology of depression. The findings indicated in bold suggest positive correlation between depression and FSL model and therefore agreement or partial agreement of neurochemical model. Table adapted from Overstreet *et al.*, 2005

2.5.2.2.1 Cholinergic model

As has been eluded to earlier, patients with depression have a higher sensitivity towards different cholinergic agents (Janowsky *et al.*, 1980; Nurnberger *et al.*, 1989; Furey & Drevets, 2006), and which is said to accompany an elevated number of muscarinic receptors in the frontal cortex (Meyerson *et al.*, 1982). Further studies, however, are needed to confirm these muscarinic receptor alterations in other brain areas.

In order to simulate the above, the FSL rat was developed as an animal model with increased cholinergic behavioral sensitivity to DFP and other cholinergic agents (e.g. physostigmine) (Overstreet & Russell, 1982; Russell & Overstreet, 1987; Overstreet *et al.*, 1988; Overstreet & Janowsky, 1991; Overstreet, 2002). The possibility that the above observations may be due to

changes in the sensitivity of acetylcholinesterase (AChE), the ACh degradation enzyme, has however been ruled out by Overstreet and colleges (Overstreet *et al.*, 1984), although this raises further questions, namely (1) are elevated ACh levels or its synthesis and (2) are differences in muscarinic ACh receptors responsible for the differences in cholinergic sensitivity between the two lines (FSL and FRL rats). In order to clarify these questions, an earlier study found an increase in ACh synthesis in the cerebral cortex of FSL rats while significant elevated levels of endogenous ACh have been found in the striatum (Overstreet *et al.*, 1984). In addition, this and other studies (Pepe *et al.*, 1988) also noted a significantly increased muscarinic cholinergic receptor density in the striatum and hippocampus of FSL rats.

Therefore, although increased muscarinic receptor density has been found in the hippocampus and striatum of FSL rats, these results are contradictory with clinical studies that have revealed no differences in muscarinic receptor binding (Kaufmann *et al.*, 1984; Lenox *et al.*, 1985; Katerina *et al.*, 2004). However, most of the latter studies have determined muscarinic binding activity in the cortex of suicide victims (Kaufmann *et al.*, 1984; Katerina *et al.*, 2004) and not in the hippocampus and striatum, as has been determined in the FSL studies. The latter could be regarded as an inappropriate region for studying muscarinic receptor binding (Overstreet & Janowsky, 1991). Thus, until more muscarinic binding studies are conducted in other brain areas of suicide victims and FSL rats, the construct validity of the FSL rat model of depression will remain largely incomplete.

2.5.2.2.2 GABAergic model

A substantial amount of evidence has accumulated over the years in support of a role for GABA in mood disorders. Most studies have indicated that depression is associated with a GABA deficiency, with low GABA levels reported in brain tissue (Sanacora *et al.*, 1999; Sanacora *et al.*, 2004), CSF (Gerner & Hare, 1981; Gerner *et al.*, 1984) and plasma (Petty & Schlesser, 1981; Petty, 1994). Moreover, this deficiency in GABA is corrected with antidepressants (Sanacora *et al.*, 2002).

Previous studies on the FSL rat have provided support for the proposed GABA deficiency model of depression (Petty, 1995). It has thus been found that FSL rats are more sensitive to the behavioral suppressant effects (locomotor activity) of a $GABA_A$ receptor agonist and a benzodiazepine (BD) agonist with an increase in benzodiazepine receptor concentration noted in the striatum and hippocampus compared to FRL rats (Pepe *et al.*, 1988). The FSL rat also displays a significantly greater degree of ethanol-induced hypothermia (ethanol is regarded as a $GABA_A$ agonist) (Overstreet *et al.*, 1990b). Since the BD receptor is linked to the $GABA_A$ receptor (Overstreet, 1993; Trevor *et al.*, 2002), an increase in BD receptors also reflects a $GABA_A$ receptor abnormality, while antidepressants tend to decrease $GABA_A$ receptor binding, and therefore increase GABA activity (Petty, 1995).

There is thus evidence for GABAergic dysfunction in the FSL rat, although more studies are necessary to convincingly correlate abnormalities seen in the FSL rats with depression. In this regard, studies to determine GABA turnover in different brain areas in the FSL rat are still lacking.

2.5.2.2.3 Serotonergic model

Before reflecting any serotonin activity in the FSL rat, some general issues with regard to serotonin activity in humans must be made clear. Regardless of the historical development of a serotonergic deficiency in the monoamine hypothesis, over-activity or no changes in serotonergic transmission have been reported (Cheetham *et al.*, 1989; Muck-Seler *et al.*, 1991; Reddy *et al.*, 1992; Sullivan *et al.*, 2006; Barton *et al.*, 2008). In addition to these conflicting results, $5\text{-}HT_2$ receptors have been found to be increased in blood platelets (Arora & Meltzer, 1989) and frontal cortex (Yates *et al.*, 1990) of depressed patients, while other studies have failed to reproduce these findings (Cheetham *et al.*, 1988). The $5\text{-}HT_{1A}$ receptor has been associated with anxiety and depression, with $5\text{-}HT_{1A}$ agonists having both anxiolytic and antidepressant effects (Blier & Ward, 2003). Moreover, $5\text{-}HT_{1A}$ receptor levels in depressed suicides have also revealed contradictory results, with high (Matsubara *et al.*, 1991), low (Cheetham *et al.*, 1990) or even no differences

(Lowther *et al.*, 1997) noted. It has been suggested that these contradictory results could be the cause of differences between studies with regard to psychiatric status of suicide victims at time of death, the diagnostic criteria used to establish the psychiatric diagnoses and brain region examined (Stockmeier *et al.*, 1997). However, despite these variations in results, the monoamine hypothesis has been derived from the proposed under-activity in serotonergic transmission (Yadid *et al.*, 2000), while the historical development of clinically effective antidepressants have all been based on their ability to increase monoaminergic activity (Overstreet *et al.*, 2005).

The FSL rat is more sensitive to the hypo/hyper-thermic effects of a variety serotonergic agonists that act on 5-HT_{1A} and 5-HT_2 receptors (Wallis *et al.*, 1988; Overstreet *et al.*, 1992; Overstreet *et al.*, 1998). On the other hand, a greater reduction in behaviors, such as locomotor activity and bar pressing for water reward, have been noted in the FSL rat in response to a serotonin agonist (Wallis *et al.*, 1988). Evidence would therefore suggest that serotonin supersensitivity in the FSL rat mediates the depressive phenotype seen in these animals subsequent to being subjected to the forced swim test. Rats randomly bred for increased hypothermic responses to selective 5-HT_{1A} receptor stimulation (Overstreet *et al.*, 2003) display immobility behavior in the forced swim test (Overstreet *et al.*, 1996) that is similar to the exaggerated immobility displayed by the FSL rat in the FST (Overstreet *et al.*, 1998). In contrast with the suggested serotonergic hypo-function in depression, previous studies have found an elevated level of serotonin and its metabolite in the FSL rat, and which could be corrected following chronic treatment with an antidepressant (Zangen *et al.*, 1997).

Thus, there is ample evidence in the FSL rat model of depression to indicate serotonergic abnormalities and to suggest the usefulness of such a model for further studies.

2.5.2.3 Predictive validity

Predictive validity reflects the ability of the animal model to respond to specific pharmacological treatments that are effective in the human disorder. Predictive validity is also used to extend and confirm construct validity.

Drug or drug class	Response in depression	Effect on swim test in FSL rats
Tricyclics Imipramine Desipramine	**Relief**	**Increased swimming**
SSRIs Fluoxetine Citalopram Paroxetine Sertraline	**Relief**	**Increased swimming**
Nefazodone	**Relief**	**Increased swimming**
Lithium	Relief	No change
Bright light exposure	**No effect**	**No change**
Nemifitide	**Relief**	**Increased swimming**
Amphetamine	**No effect**	**No change**
Scopolamine	**No effect**	**No change**
Nicotine	Limited data	Increased swimming

DFP	**No effect**	**No change**
Melatonin agonist	**Relief**	**Increased swimming**
CRF antagonist	**Relief**	**Increased swimming**
NGF	**Relief**	**Increased swimming**
Inositol	**Relief**	**Increased swimming**
Rolipram	**Relief**	**Reduced shock-induced depression**

Table 5: Effect of drugs in depression and FSL rats exposed to the forced swim test adapted from Overstreet *et al.*, 2005

As can be seen from table 5, the FSL rat responds positively to most clinically effective antidepressants, with no response to non-antidepressants e.g. amphetamine and scopolamine. The model therefore presents with good predictive validity. Given the positive response to various clinical antidepressants, it would be commendable to test a wider variety of non-antidepressants on this animal model since there have been only limited studies with drugs that do not possess antidepressant activity (Overstreet *et al.*, 2005).

2.5.2.4 Validity overview

Various animal models of depression have been developed, including genetic models, genomic models, developmental models and lesion models. All of these models fall into the diathesis framework (as described in above section 2.4.3) as a long-term predisposition to depression and are therefore not based on the traditional stress precipitant per se. Table 6 outlines the comparison between these models with that of the FSL rat model.

	Validity				Evaluation	
	Face	Construct	Predictive	Total	Depression	Dysthymia
Genetic models						
Flinders sensitive line (FSL)	**+ +**	**0**	**+**	**3**	**+**	**+**
Congenital learned helplessness	+	+	+	3	+	0
Roman high avoidance	0	0	0	0	0	0
Fawn Hooded	+	0	+	2	+	+
Genomic models						
HPA transgenic	0	+	+	2	+	+
5-HT transporter knockout	0	0	0	0	0	0
CRH receptor subtypes knockouts	0	0	0	0	0	0
Tachykinin receptor knockout	+	0	0	1	+	+
Developmental models						
Prenatal/neonatal stress	+	0	0	1	0	0
Lesion model						
Olfactory bulbectomy	+	+	+ +	4	+ +	+

Table 6: Validity scoring for Flinders Sensitive Line (FSL) rats compared to other models of predisposition to develop depression. Adapted from Willner & Mitchell, 2002

Referring to table 6, the validity of each criterion (face, construct and predictive) in the first three columns has been evaluated on a scale of 3 where (0) reflects no positive evidence, (+) small amount of evidence, (++) moderate amount of evidence and (+++) large amount of evidence. Column four reflects the sum of the first three columns with a total score of nine. An overall score of between 0-3 is given in column five for depression. In addition, the right column also evaluates the extent to which the criteria ratings comply with dysthymia. Dysthymia is another subcategory of depressive disorders and is characterized by at least two years of depressed mood and accompanied by additional symptoms that do not meet criteria for a major depressive episode (American Psychiatric Association, 2000).

Table 6 therefore shows that the olfactory bulbectomy model exhibits the best validity with a moderate total score of four. Therefore all the predisposition animal models, including the FSL model, demonstrate a relatively low overall validity. Willner and colleagues (2002) suggest that this low validity could be the cause of an absence of positive results rather than the presence of negative results (Willner & Mitchell, 2002). Indeed, environmental stress models have been implicated and validated thoroughly for a longer time. For example, the most widely studied animal model of depression, the learned helplessness model, was already developed in the 1960's (Overmier & Seligman, 1967) and ever since has been improved with regard to validity and reliability (Vollmayr & Henn, 2001). It could therefore be necessary to conduct more studies on these genetic and other diathesis animal models of depression before validity could be significantly improved. Clearly, this also applies to the FSL rat model, and indeed is the subject of the current study.

Despite the low construct validity of the FSL rat model of depression, the face and predictive validity criteria provide relatively good simulation of depression and reflect the importance of studying this animal model. Further, the conflicting results of neurochemical models in human depression restrict the improvement of such construct validity.

2.6 Conclusion

Major depression is a serious multifaceted psychiatric disorder that affects approximately 121 million people worldwide (Rosenzweig-Lipson *et al.*, 2007). The life time prevalence for major depression in South Africa is estimated at 9.8% (Stein *et al.*, 2008) and is twice as common in females than males (Nestler *et al.*, 2002). Major depression is characterized by depressive episodes for a period of at least two weeks during which there is either depressed mood or the loss of pleasure (American Psychiatric Association, 2000). These two core symptoms are accompanied by additional diagnostic symptoms and co-morbid non-diagnostic symptoms (Millan, 2006) that further complicate diagnoses of major depression. The cause of depression is still unclear but is believed to be a combination of genetic and environmental factors (Nestler *et al.*, 2002). It has been suggested that 40% - 50% of the risk to developed depression is genetic (Nestler *et al.*, 2002). Non-genetic factors are diverse and range from stress, emotional trauma, and adverse childhood experiences (Fava & Kendler, 2000).

It seems that depression does not affect specific brain areas but rather affects various brain structures that regulate certain functions, e.g. emotional and cognitive functions (Bear *et al.*, 2001) involved in mood disorders. Of considerable importance is the limbic system that contains complex circuits in regulating these functions (Bear *et al.*, 2001). Despite the recognizable role of monoaminergic neurotransmission in the above brain areas in depressive disorders, other neurochemical pathways have recently become an important focus point in developing novel targets for improving current therapies and understanding the neurobiology of depression. The cholinergic system which is involved in memory and learning (Hasselmo, 2006) has also been found to play a significant role in depression. Patients with depression exhibit an increased sensitivity to cholinergic agents (Janowsky *et al.*, 1980; Nurnberger *et al.*, 1989), while muscarinic antagonists display antidepressant effects in human and rat studies (Chau *et al.*, 1999; Furey & Drevets, 2006). The involvement of GABA in depression has been supported with mounting evidence. Low GABA levels in brain tissue (Sanacora *et al.*, 1999; Sanacora *et al.*, 2004) have been found in depressed patients that can be corrected with

effective antidepressants (Sanacora *et al.*, 2002). The presynaptic $GABA_B$ receptor is also responsible for regulating GABA and glutamate release (Yamada *et al.*, 1999) as well as euthymia through actions on these two pathways (Harvey, 1996). Therefore, antidepressant efficacy can be achieved by NMDA receptor alterations. Indeed, various antidepressants have been found to reduce glutamate neurotransmission through NMDA receptor alteration (Paul *et al.*, 1994; Nowak *et al.*, 1995).

Animal models of depression are widely utilized for screening of potential novel antidepressants (Overstreet & Griebel, 2004; Overstreet *et al.*, 2004; Overstreet & Griebel, 2005) and are also important for studying the neurobiology of depression (Yadid *et al.*, 2000). The use of animal models with good validity for depressive disorder have been extensively studied for a number of years (Willner & Mitchell, 2002). The genetic bred FSL rat shows great potential for elucidating the neurobiology of depression, although it needs substantially more validation efforts with respect to construct and predictive validity. It is the aim of this study to make significant in-roads in this regard.

Chapter 3

Article

For submission in the European Journal of Pharmacology (EJP)

This chapter complies with the instructions for authors that is outlined in Appendix 1 and found on the website:

http://www.elsevier.com/wps/find/journaldescription.cws_home/506087/description#description under "guide for authors"

This paper will discuss the neurochemical and behavioural differences in an animal model of depression as contributing factors in depression. The manuscript will contain a title page where after the article will be divided into the following section order: Abstract, Introduction, Materials and Methods, Results, Discussion, Conclusion, Acknowledgements, References and figure legends

TITLE PAGE

Cortical-hippocampal neurochemical characterization of the Flinders Sensitive Line rat with regard to amino acid and cholinergic signalling pathways

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Abstract

There is increasing evidence supporting a role for the cholinergic system as well as glutamate and γ-aminobutyric acid (GABA) pathways in affective disorders. We have investigated frontal cortical and hippocampal N-methyl-D-aspartate (NMDA) and muscarinic M_1 receptor binding characteristics, as well as GABA and acetylcholine levels, in the Flinders Sensitive Line (FSL) rat, a genetic animal model of depression, compared to its healthy control, the Flinders Resistant Line (FRL) rat. A sensitive liquid chromatography tandem mass spectrometry (LC/MS/MS) method and high performance liquid chromatography electrochemical detection (HPLC-ECD) method was used for the quantification of acetylcholine and GABA, respectively. NMDA and muscarinic M_1 radioligand binding studies were performed using [^{3}H]-MK801 and quinuclidinyl benzilate, respectively. In addition, anxiety-like behaviours were assessed using the open field and social interaction tests. Significantly elevated levels of acetylcholine were found in the frontal cortex of Flinders Sensitive Line rats, but with significantly reduced levels observed in the hippocampus. In both cases, muscarinic receptor binding was unaffected. Furthermore, no differences with regard to GABA levels and NMDA receptor binding characteristics were noted in either of the brain areas studied. Increased anxiety, evinced by significantly less social interaction, together with a trend towards reduced activity in the open field, was observed in Flinders Sensitive Line versus Flinders Resistant Line rats. Flinders Sensitive Line rats thus display broad cholinergic dysfunction in the frontal cortex and hippocampus, although without noteworthy changes in either muscarinic M_1 receptor density or affinity, which together may contribute to the depressive phenotype of these animals.

Key words: Depression, Flinders Sensitive Line, amino acid pathway, cholinergic pathway, frontal cortex, hippocampus

1 Introduction

Major depression is a heterogeneous neuropsychiatric disorder that is the fourth major cause of disability (Skolnick, 1999; Elhwuegi, 2004). Globally approximate 121 million people suffer from depression (Rosenzweig-Lipson *et al.*, 2007), with 10 - 25% women and 5 - 12% men afflicted by the illness (American Psychiatric Association, 2000). An important contributing factor for the development of depression is long-standing/chronic stressful life events (Fava & Kendler, 2000), while a genetic component to the development of the illness is also well recognised (Sullivan *et al.*, 2000). Consequently, depression presents with a significant gene X environment interaction.

The heterogeneous nature of depression suggests the involvement of diverse brain areas that interact as part of a complex system (Nestler *et al.*, 2002). Indeed, the limbic system has received considerable attention with regard to the dense serotonergic (Bloom, 2001), cholinergic (Spencer *et al.*, 1986) and γ-aminobutyric acid (GABA)-glutamate pathways (Fritschy *et al.*, 1998) that are apparent in these regions. All are involved to varying degrees in limbic functions, such as memory, motivation and emotional behaviour (Afifi & Bergman, 1998), all of which are compromised in affective disorders. The hippocampus and frontal cortex and their interconnection in particular, plays an important role in mood (emotion) as well as cognitive function (Bear *et al.*, 2001). Both regions are densely innervated by the cholinergic system. The hippocampus is also prone to neurodegenerative changes evoked by prolonged depression (Sapolsky, 2000; Sheline *et al.*, 2003) and which is causally related to the observed cognitive deficits seen in depressed patients.

Although the monoamine hypothesis has played a major role in explaining the pathophysiology of depression as well as in the development of antidepressant drugs, a substantial body of evidence now implicates the involvement of dysfunctional cholinergic transmission in depression (Overstreet, 1986; Chau *et al.*, 1999; Chau *et al.*, 2001; Atri *et al.*, 2004; Rada *et al.*, 2006; Furey & Drevets, 2006). Indeed, patients with depression display increased cholinergic sensitivity in response to cholinergic agents (Janowsky *et al.*, 1980; Nurnberger *et al.*, 1989; Janowsky *et al.*, 1994), while muscarinic

blocking agents have been found to produce rapid and potent antidepressant and anti-anxiety effects in patients with major and bipolar depression (Furey & Drevets, 2006). Despite this evidence, earlier studies have failed to confirm antidepressant activity for anticholinergic agents (Fritze, 1993; Gillin, 1995). Thus, although there is evidence to implicate altered cholinergic transmission in depression, its exact role in the aetiology of depression remains an enigma, although it is possibly more supplementary to the actions of GABA, glutamate and the monoamines. In fact, the focus of research on depression has in recent years begun to focus more on the premise that depression is a multifaceted disorder involving various central neurotransmitters and as such requiring a multi-targeted approach to treatment (Millan, 2006). Glutamate and GABA regulate mood through counterbalancing effects (Lydiard, 2003), such that abnormalities in any one of these neurotransmitter pathways may be a precipitating factor for the development of a mood disorder (Gerner *et al.*, 1984; Petty, 1994; Petty, 1995; Sanacora *et al.*, 1999; Sanacora *et al.*, 2004), probably by allowing inappropriate changes in monoamines and/ or acetylcholine through its various cross-talk mechanisms with these pathways. Indeed, it was found that NMDA receptor activation in the cortex may reduce acetylcholine release through negative feedback mechanisms and acetylcholine conversely depresses synaptic potentials mediated by glutamate and GABA (Metherate & Ashe, 1995). It was further found that $GABA_A$ receptors located on the cholinergic neurons in the striatum can directly modulate acetylcholine release.

The Flinders Sensitive Line (FSL) rat (Overstreet *et al.*, 2005) was bred for increased cholinergic sensitivity (Overstreet & Janowsky, 1991), which is in concordance with that found in depressive patients (Janowsky *et al.*, 1980; Nurnberger *et al.*, 1989; Janowsky *et al.*, 1994). In addition, the animals also display behavioural characteristics akin to that of depression, including psychomotor retardation, anhedonia, loss of appetite/weight, sleep disturbances and anxiety (Overstreet *et al.*, 2005). The FSL rat also displays increased responsiveness to environmental stressors (Overstreet, 1986; Pucilowski *et al.*, 1993). At the neurochemical level, FSL rats display disturbances in monoamine activity (Zangen et al, 1997) as well as

serotonergic function, as evinced by a marked hypothermic response to 5-HT_{1A} stimulation (Overstreet *et al.*, 1994; Shayit *et al.*, 2003) and elevated levels of serotonin in limbic brain areas (Zangen *et al.*, 1997). FSL rats have also been found to be more sensitive to the suppressant effects of GABA agonists with increased benzodiazepine binding activity, particularly in the striatum and hippocampus (Pepe *et al.*, 1988). As with glutamate in the Flinders Sensitive Line rat, the only proof of disturbances in this neurotransmitter pathway is underlined by a recently submitted article where NMDA-NOS signalling in the Flinders Sensitive Line and Flinders Resistant Line were investigated. It was found that the Flinders Sensitive Line and Flinders Resistant Line rats are similar with respect to hippocampal nNOS activity and protein expression under basal conditions, although subjecting the animals to stress induced pronounced changes in various molecular markers of the NMDA-NO signalling cascade in the genetically vulnerable Flinders Sensitive Line rats, thereby affirming its importance as a vulnerability factor in the depressive phenotype of the Flinders Sensitive Line rat (Wegener *et al.,* unpublished results)

In the current study we have studied the neurochemical differences between drug treatment- and stress-naive Flinders Sensitive Line and Flinders Resistant Line rat with regards to acetylcholine and GABA levels, and N-methyl-D-aspartate (NMDA) and muscarinic M_1 receptor binding activity in the frontal cortex and hippocampus. In addition, inherent anxiety-like behaviours were also assessed in Flinders Sensitive Line/ Flinders Resistant Line rats to provide evidence for face validity for depression.

2 Materials and methods

2.1 Animals

The use of Flinders Sensitive Line and Flinders Resistant Line rats were approved by the North-West University Ethics Committee (Ethics approval number 3207S2). Rats were bred and raised in the Experimental Animal

Centre of the North-West University, Potchefstroom campus where all the behavioural studies were conducted. Male rats (weighing 200 ± 20 g) were housed in pairs of 5/6 in a controlled environment with temperature of 22 ± 2°C and humidity of 40 – 60% under a 12:12 hour light/dark cycle, provided by a full spectrum of cold white light (350-400 lux). Water and food were freely available. Different groups of rats were used for the behavioural and neurochemical assays. All behavioural studies (open field test and social interaction) were conducted between the active 18:00 and 06:00 dark cycle.

2.2 Behavioural testing

In order to reaffirm the validity of the Flinders Sensitive Line rat model, and to establish the anxiogenic characteristics of Flinders Sensitive Line vs. Flinders Resistant Line rats in keeping with the former's reported heightened stress-sensitivity and depressogenic phenotype, various behavioural studies were performed in the animals in order to differentiate between the two lines. These behavioural tests included the open field and social interaction tests. In addition, according to Overstreet (Overstreet *et al.*, 1994; Overstreet *et al.*, 1998), Flinders Sensitive Line and Flinders Resistant Line animals were also tested with respect to their hypothermic response to the $5HT_{1A}$ agonist, 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT).

2.2.1 Open Field and Social interaction

The Two-way ANOVA with group and study as factor revealed a significant interaction between group and study and therefore study one, two and three are represented separately in figure 1 and 2 of the open field test (time spent in middle blocks and line crossings (section 3.1.1)

Flinders Sensitive Line and Flinders Resistant Line rats (in pairs of two) were placed in the middle blocks of a square test arena (100 × 100 cm; marked with sixteen 20 × 20 cm square blocks) from which escape was prevented by surrounding walls. The amount of social interaction (grooming, licking and sniffing) and the time spent in middle blocks were recorded during a 5 minute session. The social interaction test gives an indication of "anxiety-like"

behaviour where decreased social interaction indicates an increased anxiogenic effect while the time spent in middle blocks of arena gave an additional measurement of "anxiety-like" behaviour where increased time spent in middle blocks of arena indicates an increased anxiogenic response (File & Seth, 2003; Overstreet *et al.*, 2004). Since it was suggested that the pair of rats influences each other's behaviour (File & Seth, 2003), the behaviour of the rats was scored as a unit by using the total score for the pair of rats during each 5 minute session. In addition, the number of lines crossed were measured as motor activity in the open field and gave an indication of the general activity.

2.3 Hypothermic response

The hypothermic response is a necessary test to establish construct validity (Overstreet, 1993; Overstreet *et al.*, 2005) although most of the serotonergic changes (including serotonergic sensitivity) in the Flinders sensitive Line are not absolutely in accordance with that found in depression because of inconclusive results in the literature.

The high affinity $5HT_{1A}$ agonist, 8-OH-DPAT, was purchased from Sigma-Aldrich, Germany and dissolved in saline. A dosage of 2-4 mg/kg produces a serotonin syndrome (Scott *et al.*, 1994) and hypothermic effect in rats, which is more pronounced in Flinders Sensitive Line rats (Overstreet *et al.*, 1994). For the current study, a dose of 0.25mg/kg that gave the required hypothermia effect according to a previous pilot study in our laboratory, was injected subcutaneously (s.c.) in a volume of approximately 0.5 ml.

Rats were transferred to the experimental room in the Animal centre and allowed to acclimatize to the conditions of the testing room. The hypothermia test was performed during the non-active 06:00 – 18:00 light cycle on a different group of rats (10 Flinders Sensitive Line and 10 Flinders Resistant Line control rats) than those used for the other behavioural tests. The experiment was performed according to the results of a previous pilot study to establish dose and response (unpublished results) and conducted in our animal centre. The study was initiated by weighing and measuring core body

temperature via the insertion of a lubricated thermocouple probe 1-8 cm (based on size of rat) into the rectum. 8-OH-DPAT (0,25 mg/kg/0,5 ml) was administered subcutaneously followed by a second baseline measurement. After a 30 minute and 60 minute period following 8-OH-DPAT administration, body temperature was measured again and the difference from baseline notated in both the Flinders Sensitive Line and Flinders Resistant Line rats.

2.4 Neurochemical assays

2.4.1 Extraction of hippocampus and frontal cortex tissue for GABA and acetylcholine analysis

Flinders Sensitive Line rats and Flinders Resistant Line control rats were decapitated and the brains were rapidly removed. The hippocampus and frontal cortex brain areas were dissected on ice and immediately placed in separate poly vials, fixed in liquid nitrogen (-196°C) and stored at -80°C until later analysis. At the day of analysis, brain tissue were weighed (± 100 mg hippocampal and frontal cortical tissue for acetylcholine and ± 30 mg hippocampal and frontal cortical tissue for GABA analysis) and a solution of 1ml of 0.1M $HClO_4$ (and 4 μM Eserine® for acetylcholine) were added to each vial of brain tissue. The vials were then sonicated for 2 × 10 seconds at 21 μ after which it was centrifuged at 20 000 xg for 15 minutes (4°C) in a Sigma 3K15 bench top centrifuge. The preparation of the supernatant was performed on ice on the day of GABA / acetylcholine analysis.

2.4.2 High performance liquid chromatography (HPLC) determination of GABA levels

The quantification of GABA was performed by a high performance liquid chromatography method according to previous studies in our laboratory (Harvey *et al.*, 2002; Harvey *et al.*, 2004) with minor modifications.

The chromatographic system consists of a Agilent 1100 series HPLC, equipped with an isocratic pump, autosampler and a GBC LC 1260 electrochemical detector. A Luna C18-2 column (75 × 3 mm) was used to

separate the GABA from internal standard and other interfering compounds. The column was connected to a Phenomenex C18 guard column (4.0 × 3.0 mm). Detection of compounds was made possible by a glassy carbon electrode with a positive polarity (potential of 0.600 V; range of 5 nA). The mobile phase consisted of 0.1 M sodium phosphate dibasic, 0.13 mM Ethylenediaminetetraaceticacid (EDTA disodiumsalt Na_2EDTA) and 28% (to 35%) methanol. The solution was prepared by dissolving 14.2 g Na_2HPO_4 acid and 0.05 g Na_2EDTA in 720 ml purified water (Milli Q). The pH was adjusted to 6.4 with 85% phosphoric acid where after 280 ml of 35% methanol was added and thoroughly mixed. The solution was degassed and filtered under vacuum through a 0.45 µm membrane filter. The software's injector program was programmed for precolumn *orto*-phtaldialdehyde derivatization of GABA. An amount of 200 µl supernatant was added to 100 µl of 5 µg/ml homoserine internal standard. The pH was adjusted to approximately 6 with 50 µl of 10M potassium acetate (CH_3COOK) where after the solution was vortexed. Finally, an amount of 55 µl was added to an insert vial and 50 µl withdrawn from the vial and injected on the column for high-performance liquid chromatography (HPLC) analysis.

The data was acquired using Chemstation Rev. A.06.02 data acquisition and analysis software. A linear graph was drawn with a ratio of peak area of GABA standards plotted against respective GABA concentrations that yielded a regression coefficient of 0.9992. The concentrations of peak areas of GABA measured in samples were ultimately determined with linear equation of GABA standards.

2.4.3 Liquid chromatography/ mass spectrometry (LC/MS/MS) determination of acetylcholine levels

The quantification of acetylcholine was performed by a liquid chromatography method with electron spray ionization tandem mass spectrometer detection (Hows *et al.*, 2002) with minor modifications

The chromatographic system consisted of a Agilent G1312A binary pump, a G1379B micro vacuum degasser and a thermostat autosampler fitted with a

six port injection valve with a 100 µl loop capillary. The analytes acetylcholine, choline and internal standard, neostigmine, were separated on a cation-exchange column (Hamilton PRP-X200; 150 × 4.1 mm internal diameter). The temperature of the column was maintained at 25 °C. Compounds were detected with a Agilent 6410 liquid chromatography/ triple-quadrupole mass spectrometer (LC/MS/MS). The most abundant fragment ion was selected for each compound by performing a product ion scan. Multiple reaction monitoring (MRM) transitions of 146.2→87.1 for acetylcholine; 104→60 for choline and 223.2→72.1 for neostigmine were chosen according to the most abundant fragment ion. Since acetylcholine, choline and neostigmine are positively ionized in an environment with low pH, multiple reaction monitoring was performed in positive electron spray ionisation (ESI) mode. Compound analysis was optimized prior to sample analysis by direct infusion with a liquid chromatography flow rate of 0.3 ml/min. The collision energy voltage, fragmentation voltage and capillary voltage were adjusted to give the highest sensitivity with the injection program and set at voltages of 20, 80 and 5 000 respectively. Nitrogen was used as nebulizer gas and desolvation gas. The gas temperature (°C), gas flow (l/min) and nebulizer pressure (psi) were set at 300, 10 and 45 respectively.

The mobile phase consisted of 5mM ammonium acetate and 100% acetonitrile. The solution was prepared by dissolving 115.6 mg ammonium acetate in 300 ml of purified water (Milli Q). The pH was adjusted to 4.0 with glacial acetic acid where after 700 ml of acetonitrile was added and thoroughly mixed. The solution was filtered under vacuum through a 0.45 µm membrane filter. The high performance liquid chromatography (HPLC) system was purged with increased eluent flow before adjusting the flow to 0.3 µl/min for sample analysis with isocratic elution.

The supernatant (250 µl) was added to 20 µl of 0.1 mg/ml neostigmine internal standard. An amount of 100 µl was added to an insert vial and 10 µl withdrawn from the vial and injected on the column.

2.4.4 Extraction of hippocampus and frontal cortex for NMDA and muscarinic binding assays

Rats were decapitated and brains rapidly removed. The hippocampus and frontal cortex brain areas were dissected on ice and immediately "snap frozen" in liquid nitrogen. The frozen brain areas were stored at -80°C in separate "poly" vials until the day of analysis.

At the day of analysis, three frontal cortices and three hippocampi were pooled respectively to obtain a sufficient amount of protein. The pooled brain tissue was "washed" by suspending the tissue in 25 ml of Tris (for muscarinic M_1 assay) / HTS (HEPES & Tris buffer) buffer (for NMDA assay) by using a Polytron PT 10 homogenizer (setting 6, 10 seconds). The suspension was then centrifuged with a Sorvall discovery 90 SE ultracentrifuge at 48 000 ×g for 15 minutes at 4°C where after it was homogenized by a Teflon homogenizer. The centrifugation step was repeated and the pellet finally re-suspended in 60 volumes of buffer prior to a final polytron homogenizing step. The homogenate was kept on ice and an aliquot of the homogenate was used for protein determination.

2.4.5 NMDA receptor binding assay

The NMDA receptor binding assay was performed according to a previous radioligand binding method in our laboratory (Harvey *et al.*, 2002).

A series of eight [^{3}H-MK801] concentrations (specific activity 27.5 Ci/mmol) were prepared through dilution with HTS buffer (5 mM HEPES and 4.5 mM TRIS) where after increasing concentrations (0.7 - 25 nM) of radioligand were added to a 300 μl tissue homogenate, 50 μl 300 μM glycine, 50 μl 100 μM *l*-glutamate, 50 μl buffer or MK801 and 50 μl [^{3}H]-MK801 mixture and incubated for 90 minutes at 25°C. The cold ligand, (+) MK801 (25 µM), was added to define non-specific binding and the replacement of the cold ligand with buffer was added to define total binding. The binding reaction was initiated as soon as the radioligand was added to each tube and for that reason added last to the incubation mixture. Drug-receptor binding reaction was terminated by

rapid vacuum filtration through Whatman GF/B filters pre-soaked with ice-cold buffer on a Hoeffler apparatus. The whole procedure was performed as quickly as possible by rinsing the filters twice with 4 ml ice-cold buffer after adding the contents to the filter.

Finally, the filters were transferred to polypropylene scintillation vials and 3 ml scintillation fluid (Filter Count®) was added to each vial for determination of radioactivity by liquid scintillation spectrometry (Packard Tri-Carb 4660). Specific binding was defined as the total binding minus binding in the presence of 25 μM MK801. Receptor binding data were analyzed by non-linear regression analysis using Prism from GraphPad Software, Inc. (www.graphpad.com) to give affinity (K_d) and receptor density (B_{max}) values. NMDA receptor density was expressed in fmol/mg protein and affinity in nM, with protein routinely assayed using the Bradford assay.

2.4.6 Muscarinic M_1 receptor binding assay

The muscarinic M_1 receptor binding assay was performed according to a previous radioligand binding method in our laboratory (Brand *et al.*, 2008).

A concentration range of seven [^{3}H]-quinuclidinyl benzilate (QNB) concentrations (0.2055 – 10.96 nM) was prepared by dilution with TRIS buffer. The incubation mixture (increasing concentrations of radioligand, homogenate and buffer / cold ligand) was incubated for 15 minutes at 25°C. The cold ligand, atropine sulphate (15 mM), was added to define non-specific binding. Drug-receptor binding reaction was terminated by rapid vacuum filtration through Whatman GF/B filters pre-soaked with ice-cold buffer on a Hoeffler apparatus. Filters were rinsed twice with 4 ml ice-cold buffer after adding the contents to the filter.

The filters were ultimately transferred to polypropylene scintillation vials and 3 ml scintillation fluid (Filter Count®) added for counting of radioactivity with a liquid scintillation spectrometer (Packard Tri-Carb 4660). Specific binding was defined by subtracting the non-specific binding, in the presence of 15 mM atropine sulphate, from the total binding and GraphPad software was used to

obtain the K_d and B_{max} values through non-linear regression. As for the NMDA receptor binding, the muscarinic receptor density was expressed in fmol/mg protein and affinity values in nM.

2.5 Statistical analysis

GraphPad Prism® version 5.0 (GraphPad Software, San Diego, CA, USA) was used for the data representation while Statistica® version 8.0 (StatSoft, Inc., 2007) was used for statistical analysis. All behavioural and neurochemical data were analyzed using the non-parametric, Mann-Whitney U Test and Wilcoxon Matched Pairs Test respectively. Two-way ANOVA (analysis of variance) was performed on all behavioural studies to determine the effect of factors group and study. The representation of data was expressed as mean ± SD and statistical significance was defined as $p<0.05$ in all instances.

3 Results

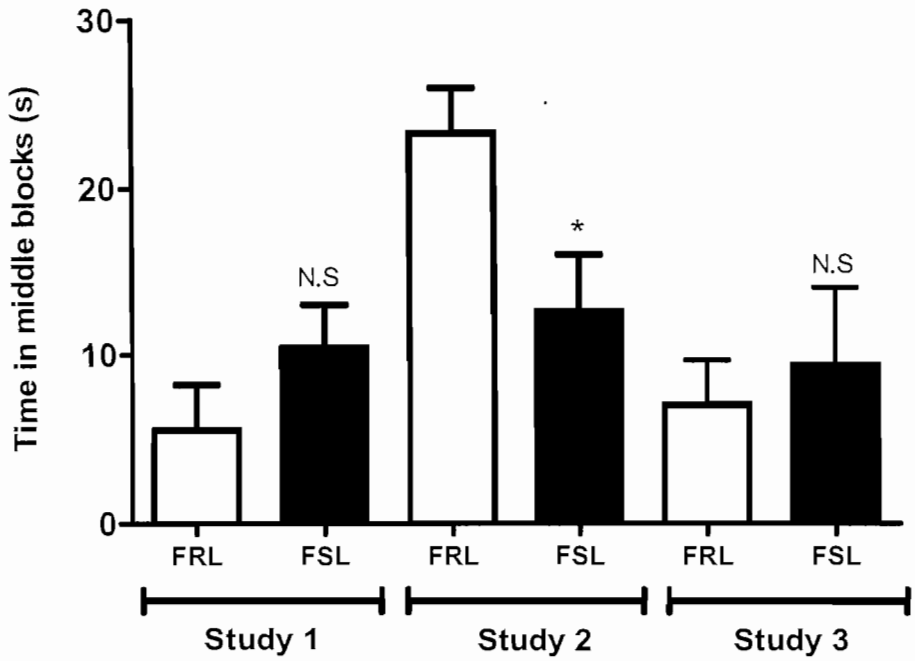
3.1 Behavioural testing

3.1.1 Open field by measuring time spent in middle blocks and line crossings

The time the Flinders Sensitive Line rats spent in middle blocks was calculated for study 1 (10.4 s ± 5.899; n=10), study 2 (12.6 s ± 7.7; n=10), study 3 (9.333 s ± 8.144; n=6) and compared with the time the control Flinders Resistant Line rats spent in middle blocks for study 1 (5.526 s ± 6.014; n=10), study 2 (23.2 s ± 5.975; n=10) and study 3 (7.0 s ± 4.582; n=6). The Two-way ANOVA with group and study as factor revealed a significant interaction between group and study ($p=0.036$) and therefore study one, two and three are represented separately in figure 1.

The Mann-Whitney U Test in study 1 and study 3 revealed no significant differences between the Flinders Sensitive Line and Flinders Resistant Line rats in the time they spent in middle blocks but differed significantly ($p < 0.05$;

figure 1) in study 2 with the Flinders Sensitive Line rat that made fewer entries into the middle blocks.



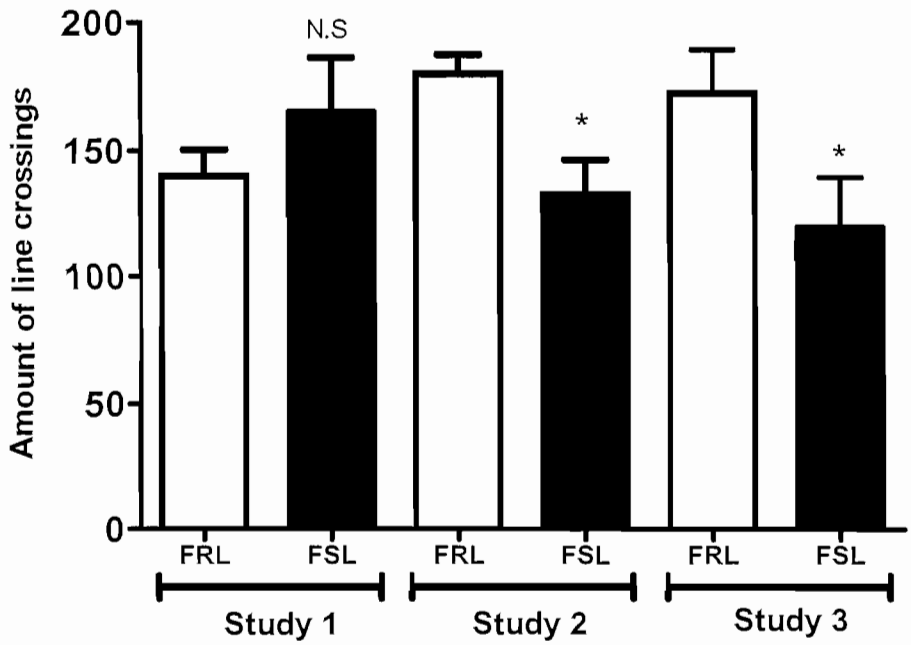
Statistics: *p < 0.05 vs Flinders Resistant line rats (Mann-Whitney U Test)
(n=6-10; mean ± SD)

Figure 1

The amount of line crossings for Flinders Sensitive Line rats in study 1 (164.8 ± 47.531; n=10), study 2 (132.0 ± 31.329; n=10), study 3 (118.667 ± 32.275; n=6) were compared to those of the Flinders Resistant Line rats in study 1 (139.8 ± 23.467; n=10), study 2 (179.6 ± 16.742; n=10), study 3 (172.0 ± 29.138; n=6) using the Mann-Whitney U Test. The Two-way ANOVA with group and study as factor revealed a significant interaction between group and study (p=0.029) and therefore study one, two and three are represented separately in figure 2.

Figure 2 revealed a significant reduction in line crossings for Flinders Sensitive Line rats in both study 2 (p=0.0163; Mann-Whitney U Test) and study 3 (0.0495; Mann-Whitney U Test) compared to Flinders Resistant Line

rats in which the Flinders Sensitive Line demonstrated a lack in general activity, but study 1 ($p>0.05$; Mann-Whitney U Test) failed to reveal any statistical significance.



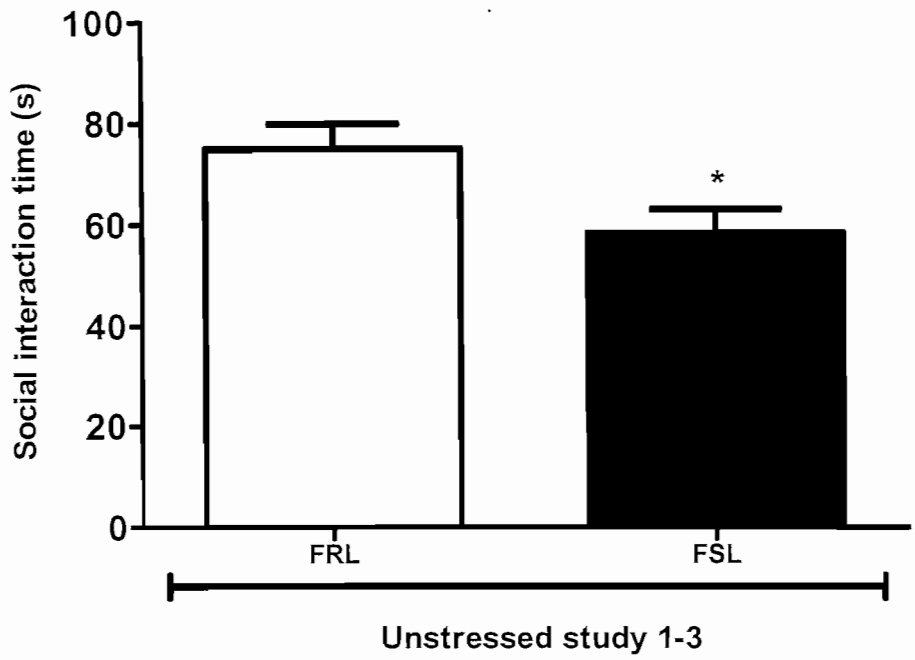
Statistics: *p < 0.05 vs Flinders Resistant Line rats (Mann-Whitney U Test).
(n=6-10; mean ± SD)

Figure 2

3.1.2 Social interaction

Social interaction in the Flinders Sensitive Line rats in study group 1-3 (58 s ± 17.18; n=26) was compared with social interaction in Flinders Resistant Line rats in study group 1-3 (74.85 s ± 17.61; n=26) using the Mann-Whitney U Test (figure 3). Two-way ANOVA with group and study as factor revealed no significant interaction between group and study ($p>0.05$) and therefore study one, two and three are grouped as represented in figure 3.

The Flinders Sensitive Line rats displayed significantly less social behaviour ($p=0.0273$; figure 3; Mann-Whitney U Test) than their control, the Flinders Resistant Line rats, in study one to three.



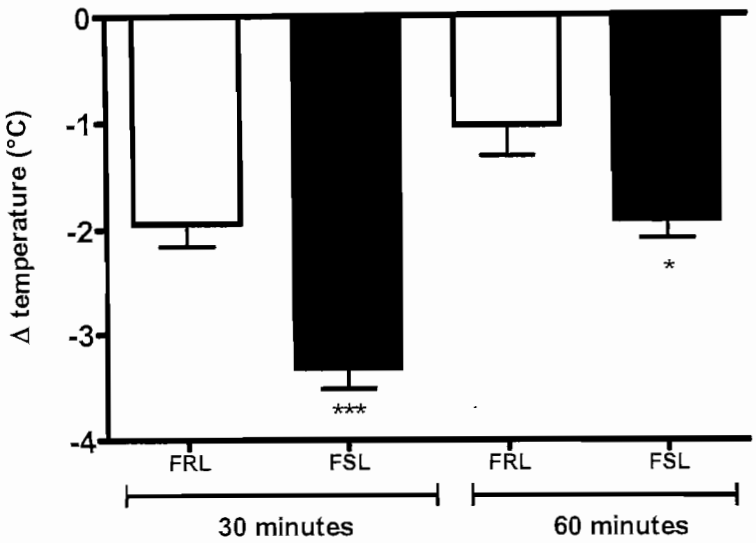
Statistics: *P < 0.05 vs Flinders Resistant Line rats (Mann-Whitney U Test)
(n=6-26; mean ± SD)

Figure 3

3.1.3 Hypothermia test

The hypothermia test was performed after 30 minutes following administration of 8-OH-DPAT to Flinders Sensitive Line (-3.320°C ± 0.6161; n=10) and Flinders Resistant Line (-1.940°C ± 0.6753; n=10) rats and 60 minutes following 8-OH-DPAT treatment in Flinders Sensitive Line (-1.910°C ± 0.5547; n=10) and Flinders Resistant Line rats (-1.030°C ± 0.8982; n=10).

Thirty minutes after treatment, the serotonin agonist, 8-OH-DPAT provoked a significant reduction from baseline body temperature in the Flinders Sensitive Line rats compared to Flinders Resistant Line rats (p=0.000861; figure 4; Mann-Whitney U Test). 60 minutes after treatment with 8-OH-DPAT, body temperature of Flinders Sensitive Line rats were still significantly reduced in comparison with those of Flinders Resistant Line rats (p=0.0253; figure 4; Mann-Whitney U Test).



Statistics: *p < 0.05 vs Flinders Resistant Line rats (Mann-Whitney U Test)
***p < 0.001 vs Flinders Resistant Line rats (Mann-Whitney U Test)
(n=10; mean ± SD)

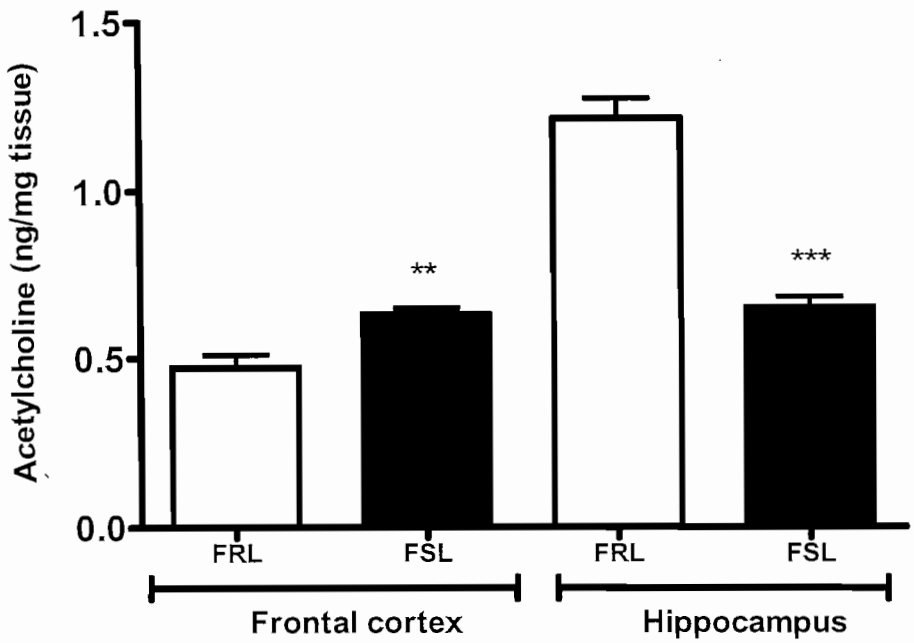
Figure 4:

3.2 Neurochemical assays

3.2.1 Acetylcholine levels in frontal cortex and hippocampus

Endogenous acetylcholine levels were determined in frontal cortex of Flinders Sensitive Line (0.627 ng/mg ± 0.064; n=10) and Flinders Resistant Line (0.472 ng/mg ± 0.119; n=10) rats and in hippocampus of Flinders Sensitive Line (0.641 ng/mg ± 0.11; n=10) and Flinders Resistant Line (1.208 ng/mg ± 0.185; n=10) rats (Figure 5).

Statistical comparison between endogenous acetylcholine in Flinders Sensitive Line and Flinders Resistant Line rats, revealed significant differences between the Flinders Sensitive Line and Flinders Resistant Line rats with the Flinders Sensitive Line rats having elevated acetylcholine levels in frontal cortex (p=0.00407; figure 5; Wilcoxon Matched Pairs Test) but significant lower levels in hippocampus (p=0.000157; figure 5; Wilcoxon Matched Pairs Test).



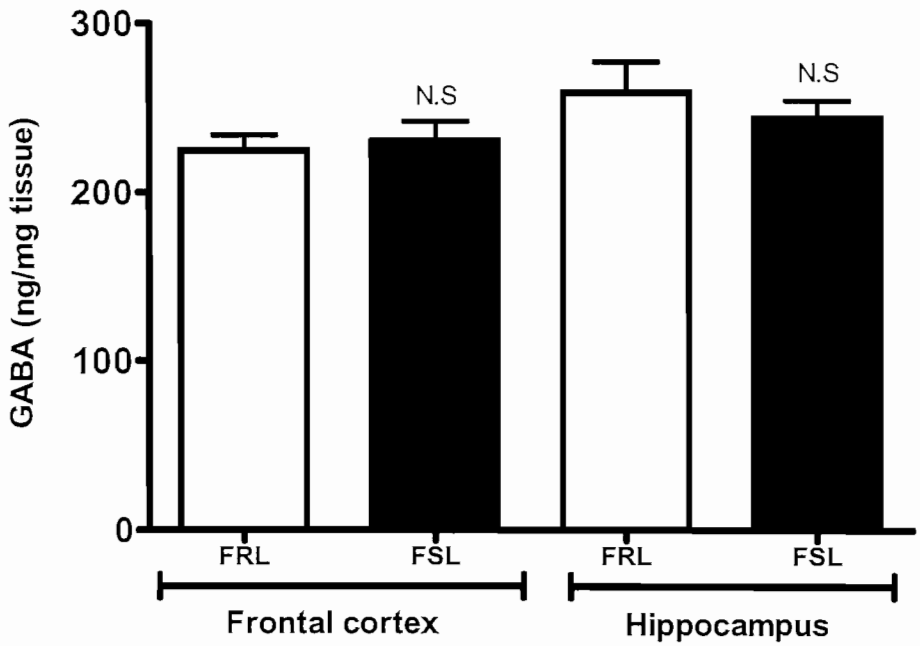
Statistics: **p < 0.01 (Wilcoxon Matched Pairs Test)
***p < 0.001 (Wilcoxon Matched Pairs Test)
(n = 10; mean ± SD)

Figure 5

3.2.2 GABA levels in frontal cortex and hippocampus brain area

Endogenous GABA levels were determined in the frontal cortex of Flinders Sensitive Line (230.8 ng/mg ± 35.73; n=10) and Flinders Resistant Line (225.1 ng/mg ± 28.94; n=10) rats and hippocampus brain area of Flinders Sensitive Line (243.8 ng/mg ± 32.82; n=10) and Flinders Resistant Line (259.2 ng/mg ± 56.3; n=10) rats (figure 6).

No statistical significant difference in GABA levels was found in either the frontal cortex or hippocampus of Flinders Sensitive Line and Flinders Resistant Line rats ($p>0.05$; figure 6; Wilcoxon Matched Pairs Test).



Statistics: $p > 0.05$ (N.S) vs Flinders Resistant Line rats (Wilcoxon Matched Pairs Test)
(n = 10; mean ± SD)

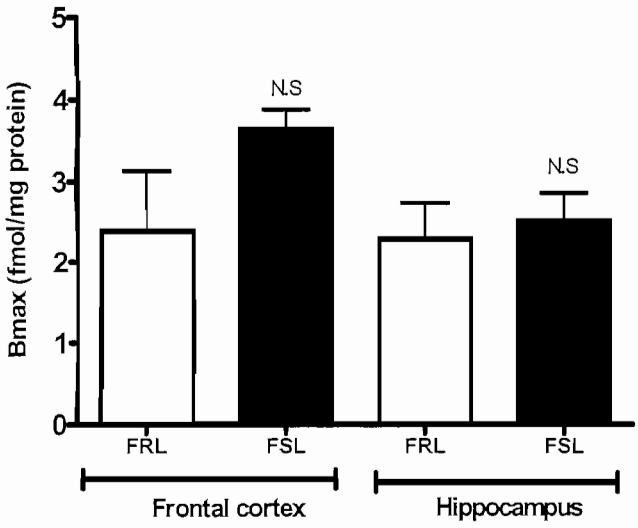
Figure 6

3.2.3 Muscarinic M_1 receptor binding in frontal cortex and hippocampus

Muscarinic M_1 receptor density was determined in the frontal cortex of Flinders Sensitive Line (3.634 fmol/mg ± 0.42; n=3) and Flinders Resistant Line (2.393 fmol/mg ± 1.469; n=4) rats and in hippocampus of Flinders Sensitive Line (2.517 fmol/mg ± 0.587; n=3) and Flinders Resistant Line (2.292 fmol/mg ± 0.886; n=4) rats (figure 7). The affinity values of the muscarinic M_1 receptors were also determined in the frontal cortex of Flinders Sensitive Line (1.336 nM ± 0.497; n=4) and Flinders Resistant Line (3.11 nM ± 1.775; n=3) and in hippocampus of Flinders Sensitive Line (6.102 nM ± 6.426; n=4) and Flinders Resistant Line (3.463 ± 0.638; n=3) rats (figure 8).

Both brain areas (frontal cortex and hippocampus) in Flinders Sensitive Line and Flinders Resistant Line rats revealed no statistical significant difference in muscarinic receptor density (B_{max}) ($p>0.05$; figure 7; Wilcoxon Matched Pairs

Test). No statistical significant differences were found in the affinity values of muscarinic receptors between Flinders Sensitive Line and Flinders Resistant Line rats ($p > 0.05$; figure 8; Wilcoxon Matched Pairs Test)



Statistics: $p > 0.05$ (N.S) vs Flinders Resistant Line rats (Wilcoxon Matched Pairs Test) (n=3-4; mean ± SD)

Figure 7:

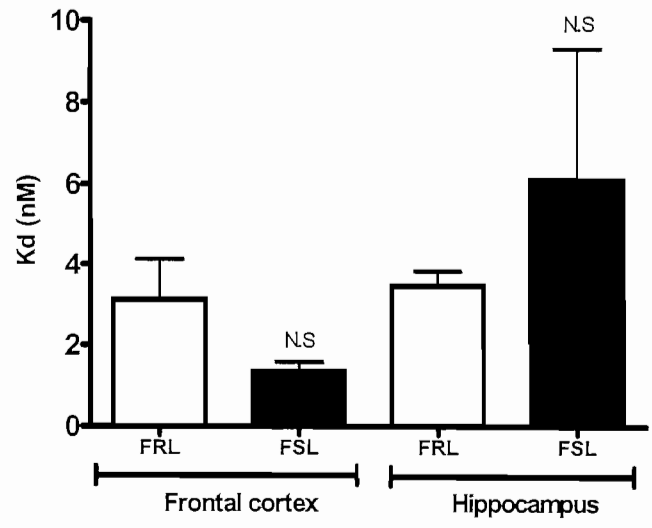


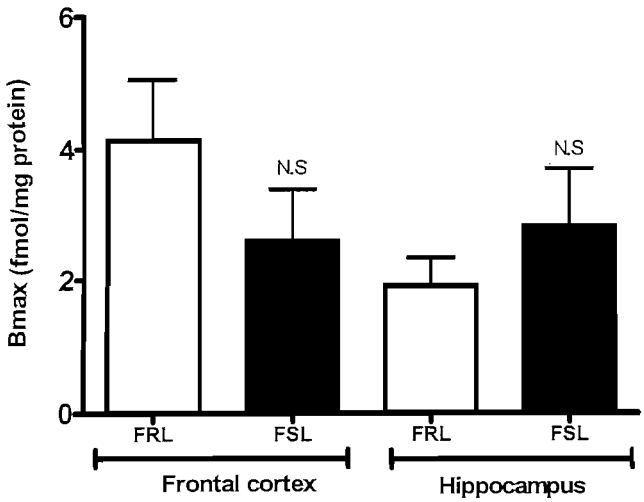
Figure 8: Statistics: $p > 0.05$ (N.S) vs Flinders Resistant Line rats (Wilcoxon Matched Pairs Test) (n=3-4; mean ± SD)

Figure 8

3.2.4 NMDA receptor binding in frontal cortex and hippocampus

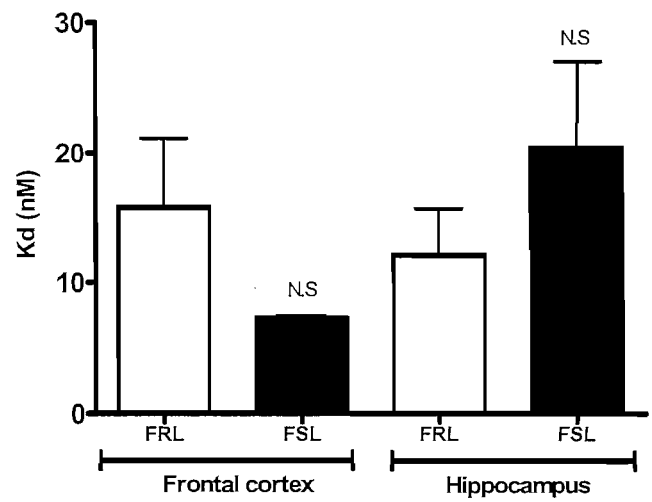
Glutamate NMDA receptor density (B_{max} values) was determined in frontal cortex of Flinders Sensitive Line (2.583 fmol/mg ± 1.371; n=3) and Flinders Resistant Line (4.138 fmol/mg ± 1.614; n=3) rats and in hippocampus of Flinders Sensitive Line (2.81 fmol/mg ± 1.97; n=5) and Flinders Resistant Line (1.911 fmol/mg ± 0.954; n=10) rats (figure 9). The affinity values of the NMDA receptors were also determined in the frontal cortex of Flinders Sensitive Line (7.289 nM ± 0.242; n=3) and Flinders Resistant Line (15.72 nM ± 9.44; n=3) and in hippocampus of Flinders Sensitive Line (20.44 nM ± 14.85; n=5) and Flinders Resistant Line (12.12 ± 7.957; n=5) rats (figure 10).

No statistical significant differences in B_{max} values were revealed in any of the brain areas (frontal cortex and hippocampus) of Flinders Sensitive Line and Flinders Resistant Line rats ($p>0.05$; figure 9; Wilcoxon Matched Pairs Test). There were also no significant differences between the K_d values in frontal cortex and hippocampus brain areas of Flinders Sensitive Line and Flinders Resistant Line rats ($p>0.05$; figure 10; Wilcoxon Matched Pairs Test).



Statistics: $p > 0.05$ (N.S) vs Flinders Resistant Line rats (Wilcoxon Matched Pairs Test) (n=3-5; mean ± SD)

Figure 9



Statistics: $p > 0.05$ (N.S) vs Flinders Resistant Line rats (Wilcoxon Matched Pairs Test) (n=3-5; mean ± SD)

Figure 10

4 Discussion

In the present study we determined acetylcholine levels and muscarinic M_1 receptor binding activity, as well as GABA'ergic levels and NMDA receptor binding activity in the frontal cortex and hippocampus of Flinders Sensitive Line versus Flinders Resistant Line rats, a genetic animal model of depression. In addition, inherent anxiety-like behaviours in the Flinders Sensitive Line and Flinders Resistant Line rats were assessed using the open field and social interaction tasks. Differences between Flinders Sensitive Line and Flinders Resistant Line rats with respect to serotonergic challenge with 8-OH-DPAT were also performed. As previously described, we found that Flinders Sensitive Line rats displayed a markedly greater hypothermic response to 8-OH-DPAT than do Flinders Resistant Line rats, thereby confirming suitable separation of the two lines (Overstreet *et al.*, 1994; Overstreet *et al.*, 1998; Shayit *et al.*, 2003). This observation also predicts separation of the animals with respect to stress response (Overstreet, 1986; Pucilowski *et al.*, 1993; Zangen *et al.*, 1997; Overstreet *et al.*, 2004).

Acetylcholine levels were significantly elevated in the frontal cortex ($p=0.004$; figure 5), but significantly reduced in the hippocampus of Flinders Sensitive Line rats vs. Flinders Resistant Line rats ($p=0.0002$; figure 5). However, cholinergic muscarinic M_1 receptor binding in Flinders Sensitive Line rats vs. Flinders Resistant Line rats remained unchanged in both the frontal cortex and hippocampus ($p>0.05$; figure 7). The Flinders Sensitive Line rat model was genetically developed with an increased behavioural sensitivity to cholinergic agonists (Overstreet & Russell, 1982; Russell & Overstreet, 1987; Overstreet, 2002). The cholinergic supersensitivity model of depression (Janowsky *et al.*, 1980; Nurnberger *et al.*, 1989; Furey & Drevets, 2006) has been postulated to underlie the mechanisms of increased stress sensitivity of Flinders Sensitive Line rats (Wegener *et al.*, unpublished results), while this increase has been associated with increased acetylcholine synthesis in the cortex and an increased concentration of muscarinic cholinergic receptors in striatal and hippocampal brain areas (Overstreet *et al.*, 1984; Overstreet *et al.*, 1988). Significantly elevated levels of endogenous acetylcholine have also

been found in the striatum (Overstreet *et al.*, 1984). Our data are in agreement with these earlier studies, as well as supporting the cholinergic hypothesis of depression.

The frontal cortex plays an important role in motivational planning as well as in other important cognitive processes involving dopamine, acetylcholine and glutamate. Indeed, the cholinergic system may influence frontal cortical dopaminergic function, which will in turn affect cognitive processing such as memory and motivation (Nieoullon, 2002; Tanda *et al.*, 2007) which is grossly affected in depression. Moreover, the NMDA/ NO pathway is involved in regulating acetylcholine levels. Blockade of NMDA receptors in the cortex increase acetylcholine levels whereas stimulation of the nitric oxide pathway also enhance acetylcholine release (Prast & Philippu, 1992; Del Arco *et al.*, 2008). The cholinergic neurotransmission that is implicated in attention, learning and memory (Everitt & Robbins, 1997; Sarter & Bruno, 1999) also significantly innervate the cortex (Spencer *et al.*, 1986) which in turn is also involved in above depression-related functions (Everitt & Robbins, 1997; Sarter & Bruno, 1999). Interestingly, our study also revealed significantly lower levels of acetylcholine in the hippocampus of Flinders Sensitive Line rats. The hippocampus is well recognized for playing an important role in mood, cognition and neuroendocrine function. However, the cholinergic neurons of the central nervous system such as the limbic system, of which the hippocampus is central, are under multiple regulatory mechanisms (Mesulam, 1995) including the GABAergic neurons and nitric oxide (NMDA/ NO pathway) (Suzuki *et al.*, 1997) that regulate hippocampal acetylcholine turnover rate and concentration (Wood *et al.*, 1979; Sethy & Francis, 1988). The cholinergic system also innervates the hippocampus that is in turn also responsible for memory and emotion (Bear *et al.*, 2001) just as the cholinergic system. However, it is the disruption of the interactive network between the hippocampus and the cortex that is critical for the regulation of mood and associated cognitive behaviour, and which forms the basis of the limbic-cortical model of depression (Mayberg, 1997). Indeed, it has been reported that depression is associated with decreased activation of cortical regions and increased activation of limbic regions as result of imbalances in connectivity in

this circuit (Anand *et al.*, 2005). In fact, cortical-hippocampal dysfunction has been noted in models of acute and chronic stress and which has been linked to increased aversive behaviour and cognitive disturbance (Harvey *et al.*, 2003; Harvey *et al.*, 2006). These regulatory functions between brain areas important in depression could therefore involve various neurotransmitter pathways that make synaptic contact with cholinergic neurons.

The hippocampus is particularly rich in GABA'ergic neurons (Banks *et al.*, 2000; Kalueff & Nutt, 2007) negatively regulating for example the activity of septohippocampal cholinergic neurons (Wood *et al.*, 1979; Sethy & Francis, 1988). Inhibitory GABA tone innervating the hippocampus could therefore be responsible for lowering levels of acetylcholine in this brain area, although no changes in hippocampal GABA were observed in the current study. Muscarinic receptors in the hippocampus and cortex are important in regulating acetylcholine levels through different presynaptic muscarinic receptors which proved to enhance acetylcholine levels through selectively blocking its receptors (Vannucchi & Pepeu, 1995). Muscarinic receptors from Flinders Sensitive Line rats have been found to be increased in the striatum and hippocampus. (Overstreet *et al.*, 1984; Pepe *et al.*, 1988). However, that acetylcholine levels were raised in the frontal cortex, yet decreased in the hippocampus of Flinders Sensitive Line versus Flinders Resistant Line rats, yet not associated with changes in M_1 receptor binding, suggests that M_1 receptor-mediated actions on acetylcholine synthesis are less involved in this instance. However, until comprehensive cell signalling studies are performed, this cannot be regarded as definitive. Another possible explanation could involve an influence from NMDA receptor function. Counterbalancing glutamatergic activity is involved in the neurobiology of depression, particularly the NMDA glutamate receptor (Paul *et al.*, 1994; Nowak *et al.*, 1995). NMDA receptors are also the primary target for regulating acetylcholine and nitric oxide release in hippocampus (Kraus & Prast, 2001). Neither NMDA receptor density nor GABA levels were altered in Flinders Sensitive Line vs. Flinders Resistant Line rats, making it difficult to associate changes in the cholinergic system to altered GABA function or NMDA receptor activity. The role of GABA-glutamate pathway in the Flinders Sensitive Line

rat however still remains an unexplored area of research, although a recent study found pronounced changes in various molecular markers of the NMDA-NO signalling cascade by subjecting the Flinders Sensitive Line rats to stressful events (Wegener *et al.,* unpublished results).

Behavioral assessments to confirm previously reported parameters of face validity were performed to differentiate between aversive and locomotor behaviors in the Flinders Sensitive Line/ Flinders Resistant Line rats. Social interaction is a valuable behavioral paradigm for testing anxiolytic drugs (Fuchs & Flügge, 2006) and for screening of novel potential antidepressants that may have anxiolytic activity (Overstreet & Griebel, 2004; Overstreet & Griebel, 2005). Previous studies have reported that the Flinders Sensitive Line rat spends the same amount of time in the open arms of the elevated plus maze as does its Flinders Resistant Line control (Schiller *et al.*, 1991; Overstreet, 1993), and that it primarily represents a model of depression without the anxiety component. However, using the social interaction task we have demonstrated that Flinders Sensitive Line rats spend significantly less time interacting with each other (licking, sniffing, grooming) than do their Flinders Resistant Line controls ($p=0.0273$; figure 3), which is congruent with anxious behavior and in concordance with earlier studies (Overstreet *et al.*, 2004; Overstreet *et al.*, 2005). The time spent in the middle blocks of the open field also provides an index of anxiety behavior (Ramos *et al.*, 1997; Overstreet *et al.*, 2004), and it has been reported that Flinders Sensitive Line rats make less centre entries than Flinders Resistant Line counterparts (Overstreet *et al.*, 2004). The time spent in middle blocks of the open field in the current study are likened to the aforementioned data. However, our studies revealed conflicting results, with only study two supporting an anxiety-like profile in Flinders Sensitive Line rats (results; figure 1; $p > 0.05$). We were therefore unable to consistently confirm the anxiety behavior in the Flinders Sensitive Line rat with regard to time spent in middle blocks of the open field test.

Patients with depression normally exhibit psychomotor retardation (American Psychiatric Association, 2000). The reduced body movements associated with depression have also been noted in Flinders Sensitive Line rats exposed

to an open field arena (Overstreet & Russell, 1982; Overstreet *et al.*, 1986), which are even more apparent after stress exposure (Overstreet *et al.*, 1988; Overstreet *et al.*, 1989). In the current study, locomotor activity (line crossings) were reflected in three separate studies in which study two ($p=0.016$; figure 2) and three ($p=0.0495$; figure 2) revealed significantly less general activity in Flinders Sensitive Line rats compared to the Flinders Resistant Line rats, although no significant difference with regard to general activity was obtained in study one. Therefore, the current study indicated some evidence for deficits in general activity in the Flinders Sensitive Line rat that supports previous studies.

5 Conclusion

In conclusion our data confirm and expand on the hypercholinergic hypothesis of depression in the genetic Flinders Sensitive Line rat model of depression. Increased acetylcholine levels are evident in the frontal cortex of the Flinders Sensitive Line rat, although lower acetylcholine levels are observed in the hippocampus of these animals vs. Flinders Resistant Line rats. This suggests that the depressive phenotype of these animals is dependent on a much broader scale of cholinergic dysfunction in the cortex and hippocampus, and which may underlie the depressive phenotype of these animals. However, changes in frontal cortical and hippocampal acetylcholine were not associated with any changes in muscarinic receptor binding characteristics in these regions, neither with any alterations in GABA levels or NMDA receptor binding. Finally, Flinders Sensitive Line rats displayed evidence for increased anxiety-like behaviour and significantly reduced social interaction compared to Flinders Resistant Line rats, which is in agreement with the general depressogenic phenotype of these animals.

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Figure legends

Figure 1:

The measured time spent in middle blocks in the open field between Flinders Sensitive Line and Flinders Resistant Line rats during three separate studies.

Figure 2:

The measured line crossings in the open field between Flinders Sensitive Line and Flinders Resistant Line rats during three separate studies.

Figure 3:

The measured social interaction between Flinders Sensitive Line and Flinders Resistant Line rats in combined study 1-3.

Figure 4:

Basal temperature differences after 30 minutes and 60 minutes after administration of serotonin agonist (8-OH-DPAT) in the Flinders Sensitive Line and Flinders Resistant Line rats.

Figure 5:

Endogenous acetylcholine levels in frontal cortex and hippocampus of Flinders Sensitive Line and Flinders Resistant Line rats.

Figure 6:

GABA levels in frontal cortex and hippocampus brain area of Flinders Sensitive Line and Flinders Resistant Line rats.

Figure 7:

B_{max} values of muscarinic M_1 receptors in frontal cortex and hippocampus of Flinders Sensitive Line and Flinders Resistant Line rats.

Figure 8:

K_d values of muscarinic M_1 receptors in frontal cortex and hippocampus of Flinders Sensitive Line and Flinders Resistant Line rats.

Figure 9:

B_{max} values of NMDA receptors in frontal cortex and hippocampus of Flinders Sensitive Line and Flinders Resistant Line rats.

Figure 10:

K_d values of NMDA receptors in frontal cortex and hippocampus of Flinders Sensitive Line and Flinders Resistant Line rats.

Chapter 4 Conclusion

Major depression is a common neuropsychiatric disorder affecting approximately 121 million people worldwide while it is suggested that the illness will be the second leading cause of death by the year 2020 arising from suicide and cardiovascular complications (Rosenzweig-Lipson *et al.*, 2007). Treatment is further restricted by antidepressants presenting with undesirable side effects, shortfalls in effectiveness and a delayed onset of action. It is therefore of considerable importance to understand the neurophysiology of depression, especially the neurotransmitters involved in the illness as well as those targeted by antidepressants before novel antidepressants can be developed.

Animals (rats) subjected to environmental stress, such as the forced swim test and open field, present with behavioural changes that resemble depressive behaviours in humans (Porsolt *et al.*, 1978; File & Hyde, 1978). Apart from monoaminergic changes that are believed to underlie many of the symptoms of depression, the study of animal models related to stress exposure has given rise to the suggestion that other neurotransmitters and their receptors are also involved in depression (e.g. acetylcholine/ muscarinic receptor, γ-aminobutyric acid (GABA) and glutamate/ NMDA receptor) (Borsini *et al.*, 1988; Layer *et al.*, 1995; Mark *et al.*, 1996; Rada *et al.*, 2003; Rada *et al.*, 2006; Belozertseva *et al.*, 2007; de Groote & Linthorst, 2007). Considering the involvement of stress, the potential role of genetic animal models has realised new importance with the realisation that the risk of developing depression can largely be accounted for by an interaction between environmental and genetic factors (Sullivan *et al.*, 2000; Fava & Kendler, 2000; Nestler *et al.*, 2002). In the current study, a genetic animal model of depression, the Flinders Sensitive Line (FSL) rat, originally bred for its ability to demonstrate increased sensitivity to cholinergic agonists (also evident in

humans with depression), and its healthy control, the Flinders Resistant Line (FRL) rat, were studied with respect to the following neurochemical (construct validity) and behavioural (face validity) outcomes:

- Cholinergic activity, namely acetylcholine levels and M_1 receptor binding, in frontal cortex and hippocampus
    - As part of determining the acetylcholine levels in brain tissue, the development of a sensitive LC/MS method was essential (addendum 1)
- Amino acid transmitter characteristics, namely GABA levels and glutamate NMDA receptor binding, in above brain areas
- Sensitivity to 5-HT_{1A}-agonist induced hypothermia
- Behavioural assessment of anxiety, as measured in the open field and social interaction tests

The following conclusions were drawn from the study:

- Significantly elevated acetylcholine levels were noted in the frontal cortex of FSL compared to FRL rats, together with significantly lower levels of acetylcholine in the hippocampus. This latter observation was of considerable interest considering that previous studies in FSL rats have found increased cholinergic activity in both the cortex and striatum with regard to acetylcholine synthesis and acetylcholine levels respectively. Thus, an increase in acetylcholine was expected. This over-activity in the frontal cortex and hypo-activity in the hippocampus is somewhat paradoxical. This observation could not be explained according to cholinergic muscarinic receptor changes as no changes in muscarinic receptor binding in both frontal cortex and hippocampus were observed. Since depression cannot be related to neurochemical changes in a single brain region, we suggest that the depressive phenotype of these animals involves bidirectional dysfunction in

cholinergic activity in the limbic brain regions, in this case the frontal cortex and hippocampus. Thus, elevated cholinergic activity in the cortex, and attenuated cholinergic function in the hippocampus may explain why anticholinergic agents are not effective as antidepressants, since such an approach would possibly benefit certain hypercholinergic pathways active in depression, but be counterproductive in other brain regions where increased cholinergic tone may be desired. Indeed, the cholinergic hypo-activity in the hippocampus may also underlie the depressive phenotype of the FSL rat, and not just the more recognised hypercholinergic profile.

- A LC/MS method with good sensitivity and selectivity was developed to analyse the ACh levels in brain tissue

- GABA levels as well as NMDA receptor binding activity were unchanged in the FSL rat in both the frontal cortex and hippocampus. Since GABA may modulate acetylcholine levels, these results suggest that GABA could not be a protagonist of the acetylcholine differences found in these brain regions. Nevertheless, further studies on the amino acid pathways in FSL rats need to be done since the role of GABA and glutamate in the depressiogenic phenotype of these animals remains speculative.

- We found that FSL rats displayed a markedly greater hypothermic response to the 5-HT_{1A}-agonist, 8-OH-DPAT, than did FRL rats, thereby confirming suitable separation of the two lines with regard to construct validity (Overstreet *et al.*, 1994; Overstreet *et al.*, 1998; Shayit *et al.*, 2003).

- FSL rats were found to present with some evidence of anxiety-like behavior as well as deficits in general activity, and thus supportive of the depressogenic phenotype of these animals.

- Our behavioral data revealed inconsistent behavioral differences with regard to line crossings (general activity) and anxiety as measured in "time spent" in middle blocks. However, we found that the anxiety-

related behaviors were sufficiently modulated in the social interaction test which is in agreement with previous studies (Overstreet *et al.*, 2004; Overstreet *et al.*, 2005). The line crossings, although not consistent, are reasonably congruent with psychomotor retardation and therefore in concordance with earlier studies (Overstreet & Russell, 1982; Overstreet *et al.*, 1986).

4.1 Recommendations and prospective studies

The hippocampus and frontal cortex play an important role in regulating cognitive and emotional function. However, other brain areas associated with the limbic system, e.g. striatum and nucleus accumbens (part of ventral striatum), also seems to be involved in depression (Overstreet *et al.*, 1984; Daws & Overstreet, 1999; Chau *et al.*, 2001; Rada *et al.*, 2003; Rada *et al.*, 2006), although this may be mediated by the interplay of cholinergic and amino acid signaling pathways. It could therefore be meaningful for future studies to investigate cholinergic and amino acid characteristics as well as their role in a wider variety of brain areas.

Since previous data (Overstreet, 1986; Pucilowski *et al.*, 1993; Zangen *et al.*, 1997; Overstreet *et al.*, 2004) as well as data from our laboratory have demonstrated differences in behavioral response in the FSL/FRL rats with regard to stressors, further explorative work on the role of stress in depression, especially how resilience and susceptibility are determined. Stress alone is not sufficient to cause depression and some people become depressed after stresses that are quite mild while other people do not become depressed even after serious stressful experiences. It is therefore evident that depression is caused by an interaction between a genetic predisposition of the individual and environmental factors (Nestler *et al.*, 2002). Further investigation into the interaction of genetic diathesis and environmental precipitants as well as the effect of different stressors on behavioral differences in FSL and FRL rats and how these stressors effect the neurochemistry in brain areas relevant to depression, would contribute substantially to our understanding of the etiology of depression.

The current study formed part of two phases. The first phase (current study), or non-pharmacological phase, was carried out by establishing the neurochemical differences with regard to amino acid (GABA levels and NMDA receptor) and cholinergic pathways (acetylcholine levels and muscarinic M_1 receptor) between the FSL rat and its control, the FRL rat, as well as prerequisite behavioral differences. These baseline behavioral and neurochemical differences will have distinct value in interpreting data in the future for phase two studies in FSL and FRL rats.

The second phase, or pharmacological phase, will employ the technique of *in vivo* microdialysis. In this work, the *in vivo* release of endogenous ACh evoked under basal conditions as well as under KCl evoked potential, in the presence and absence of acute antidepressant administration through reverse microdialysis, or preceded by chronic treatment with these drugs, will be carried out in different limbic brain regions in live FSL and FRL rats. Antidepressants will include members of different classes, e.g. selective serotonin uptake inhibitors (e.g. fluoxetine), triclyclic antidepressants (e.g. imipramine) and atypical agents (e.g. tianeptine and mirtazepine). This will provide definitive proof of the putative involvement of the cholinergic system in antidepressant action.

LC/MS/MS analysis of acetylcholine

Addendum 1

1.1 Introduction

The reliable quantification of basal levels of acetylcholine (ACh) in the peripheral and central nervous system (CNS) still remains a challenge for today's analyst. The most commonly approach to detect ACh levels was by using high performance liquid chromatography (LC) with electrochemical detection (ECD). However, the most sensitive and direct method used to detect ACh was performed by using LC coupled with mass spectrometry (MS) (Zhu *et al.*, 2000). The LC/MS technique of detection of ACh in biological tissue has not been done previously in our laboratories and was therefore developed in the current study. One of the major advantages of using the LC/MS is that compounds can be identified by both their retention times and molecular weight and therefore represents a method with good sensitivity and specificity.

1.2 Materials and methods

1.2.1 Chemicals

Acetylcholine chloride	Sigma Aldrich; Germany
(3-Carboxypropyl)trimethylammonium ("*iso-Acetylcholine*")	Sigma Aldrich; Germany
Choline Chloride	Sigma Aldrich; Germany
Eserine® (physostigmine)	Sigma Aldrich; Germany

Neostigmine Bromide	Sigma Aldrich; Germany
Ammonium Acetate	SAARChem, Krugersdorp
Acetonitrile	Acros organics; USA
Perchloric acid (60%)	SAARChem; Krugersdorp
Acetic acid (glacial)	SAARChem; Gauteng

1.2.2 Instrumentation and conditions for ACh analysis

The chromatographic system consisted of an Agilent G1312A binary pump, a G1379B micro vacuum degasser and a thermostat autosampler fitted with a six port injection valve with a 100 µl loop capillary. The analytes ACh, choline and internal standard, neostigmine, were separated on a cation-exchange column (Hamilton PRP-X200; 150 × 4.1 mm internal diameter). The temperature of the column was maintained at 20 °C.

Compounds were detected with a Agilent 6410 LC/MS/MS. The most abundant fragment ion was selected for each compound by performing a product ion scan. Multiple reaction monitoring (MRM) transitions of 146.2→87.1 for ACh; 104→60 for choline and 223.2→72.1 for neostigmine were chosen according to the most abundant fragment ion. Since ACh, choline and neostigmine are positively ionized in an environment with low pH, MRM was performed in positive electron spray ionisation (ESI) mode. Compound analysis was optimized prior to sample analysis by direct infusion with a LC flow rate of 0.3 ml/min. The collision energy voltage, fragmentation voltage and capillary voltage were adjusted to give the highest sensitivity with the injection program and set at voltages of 20, 80 and 5000 respectively. Nitrogen was used as nebulizer gas and desolvation gas. The gas temperature (°C), gas flow (l/min) and nebulizer pressure (psi) were set at 300, 10 and 45 respectively.

The mobile phase consisted of 5 mM ammonium acetate and 100% acetonitrile. The solution was prepared by dissolving 115.6 mg ammonium acetate in 300 ml of purified water (Milli Q). The pH was adjusted to 4.0 with glacial acetic acid where after 700 ml of acetonitrile was added and thoroughly mixed. The solution was filtered under vacuum through a 0.45 µm membrane filter. The HPLC system was purged with increased eluent flow before adjusting the flow to 0.3 µl/min for sample analysis with isocratic elution.

1.2.3 Standard solutions

Stock solutions were prepared by dissolving 3 mg of ACh and choline in 50 ml and 1 mg neostigmine (internal standard) in 10 ml solution of 0.1M $HClO_4$ and 4 µM Eserine® (physostigmine). The solution was stored at 4°C and used in less than one week. Standard solutions were prepared on the day of analysis by diluting the stock solution with a mixture of $HClO_4$ and Eserine® to yield a series of standard concentrations.

1.2.4 Tissue dissection and extraction

Male Flinders Sensitive Line (FSL) rats and its control group, the Flinders Resistant Line (FRL) rats were decapitated and brains rapidly removed. The hippocampus and frontal cortex brain areas were dissected and immediately placed in separate poly vials and "snap frozen" in liquid nitrogen (-196°C). The frozen brain sections were then stored at -80°C.

On the day of analysis, brain areas were weighed (± 100 mg) and a solution of 1 ml of 0.1M $HClO_4$ and 4 µM Eserine® was added to each vial of brain tissue. The vials were then sonicated for 2 × 10 seconds at 21 µ after which it was centrifuged at 20 000 ×g for 15 minutes (4°C). The preparation of the supernatant was performed on ice.

The supernatant (250 µl) was added to 20 µl of 0.1 mg/ml neostigmine internal standard. An amount of 100 µl was added to an insert vial and 10 µl withdrawn from the vial and injected on the column.

1.3 Validation

1.3.1 Linearity

Linearity is the ability of an analytical method to obtain a response that is directly proportional to known concentrations of analytes in the sample within a given range (USP, 2008). The USP recommends that, for the establishment of linearity, a minimum of five concentrations have to be used and the range has to extend from 80% to 120% of the test concentration. USP further recommends that the correlation coefficient (r^2), used to approximate the linearity, should not have a r^2 value less than 0.95 (USP, 2008)

1.3.1.1 Method

A concentration range was chosen that included the endogenous concentrations of ACh. For the purpose of the study, only endogenous ACh levels were quantified and its standard solutions entirely validated while endogenous concentrations of choline were found to be outside the concentration range and the endogenous "iso-ACh" compound was found to be an interference peak (see section 1.3.3). The peak area (PA) ratios (equation 1) for ACh and choline were plotted against seven standard concentrations to determine linearity through Equation 2.

$$\text{PA-ratio} = \frac{\text{PA of standard}}{\text{PA of internal standard}} \qquad \textbf{(Equation 1)}$$

$$y = mx + c \qquad \textbf{(Equation 2)}$$

where

y = PA-ratio

m = gradient

x = concentration (ng/ml)

c = y-intercept

1.3.1.2 Results

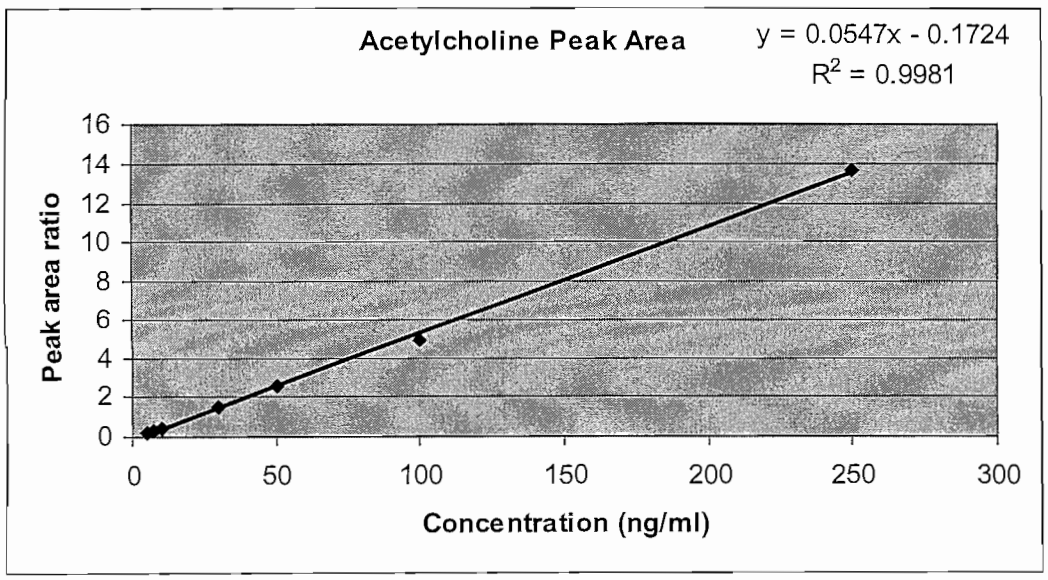


Figure 1: Linearity validation: Acetylcholine PA ratio vs. concentrations

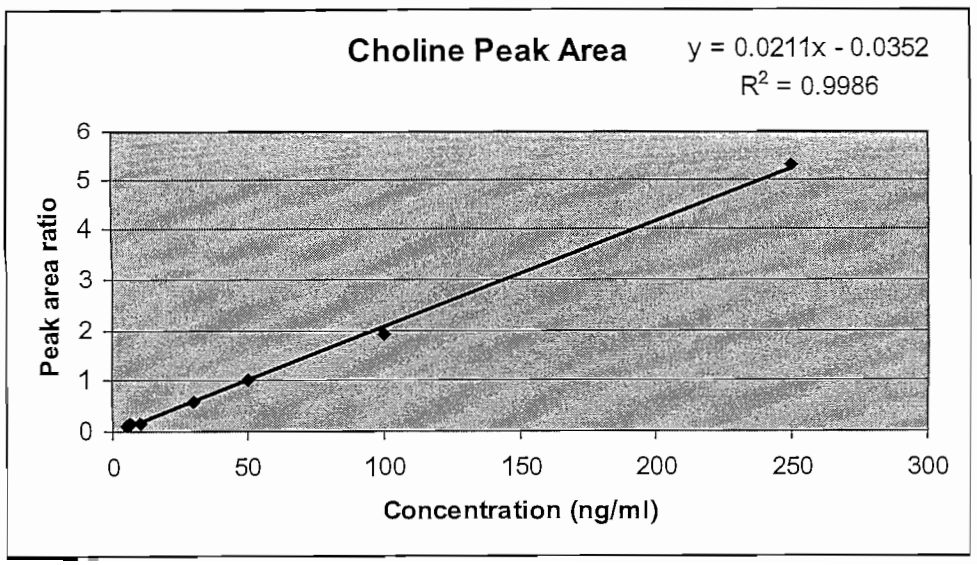


Figure 2: Linearity validation: Choline PA ratio vs. concentrations

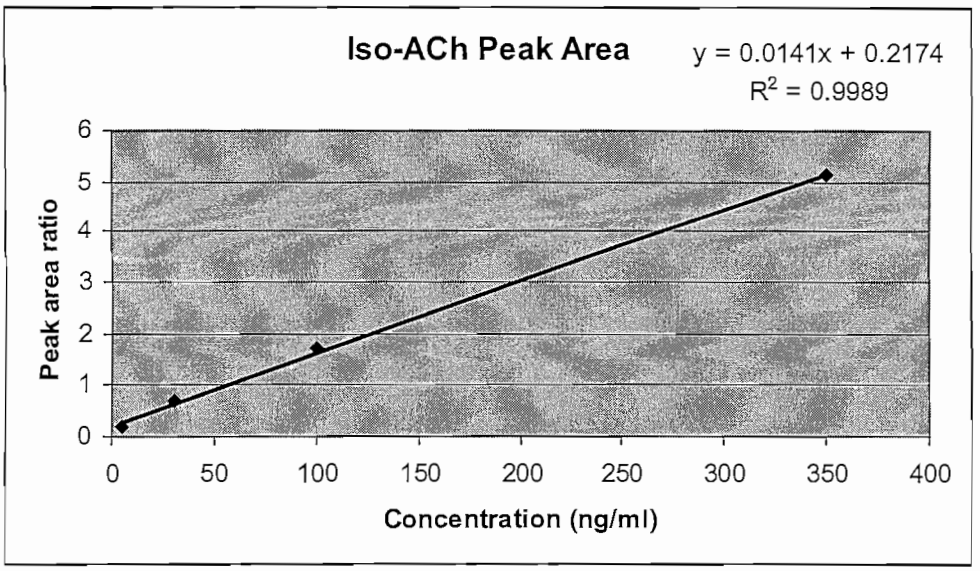


Figure 3: Linearity validation: "iso-Acetylcholine" PA ratio vs. concentrations

Analyte	Gradient (m)	Y-intercept (c)	Regression (R)
PA Curve			
Acetylcholine	0.0547	-0.1724	0.9981
Choline	0.0211	-0.0352	0.9986
"iso-Acetylcholine"	0.0141	0.2174	0.9989

Table 1: Linearity validation: Gradient, y-intercept and regression coefficient for Acetylcholine, Choline and "iso-Acetylcholine"

1.3.1.3 Conclusion

A regression coefficient of one is described as a straight line and as indicated in table 1, the results for acetylcholine, choline and "iso-Acetylcholine" demonstrated linearity with the regression coefficients in the acceptable range above 0.95. Although the four points used for "iso-acetylcholine" are not according to the minimum criteria in the USP, it is still acceptable for drawing a calibration line (Jonker, 2001)

1.3.2 Precision

The precision of an analytical method is the closeness among different test results after repeated measurements (USP, 2008) and can be divided into intra-assay precision and intermediate precision. The USP recommends that repeatability should be assessed using a minimum of three concentrations with three replicates of each concentration.

1.3.2.1 Intra-assay precision

Precision that is expressed under the same operating conditions over a short period of time.

1.3.2.1.1 Method

The precision is expressed as the percentage relative standard deviation (% RSD) of a series of eight measurements for ACh and four measurements for choline over the calibration range of three concentrations (Equation 3).

$$\%\ RSD = \frac{\text{SD of mean concentration}}{\text{Mean concentration}} \times 100 \quad \textbf{(Equation 3)}$$

The mean concentration was obtained from the PA ratio (Equation 1) and respective regression line (Equation 2).

1.3.2.1.2 Results

Acetylcholine		
Concentration	**Mean % RSD**	**Number of injections (n)**
5 ng/ml	5.59	8
50 ng/ml	4.672	8
350 ng/ml	1.777	8
Choline		
5 ng/ml	2.962	4
50 ng/ml	3.348	4
350 ng/ml	1.959	4

Table 2: Intra-precision validation: % RSD values for Acetylcholine and choline

1.3.2.1.3 Conclusion

A precision value lower than 15% for bioanalytical applications are referred to as good precision (Lindholm, 2004). The % RSD values for both acetylcholine and choline in Table 2 were found to be less than 6% and unknown brain concentrations could therefore be accurately determined.

1.3.2.2 Intermediate precision

Precision is expressed through within-laboratory variation in terms of different days or different analysts or equipment (USP, 2008).

1.3.2.2.1 Method

The same calculation for expressing precision values in terms of % RSD was used for intermediate precision (see Equation 3). Three different concentrations of ACh and choline were injected on three different days. Other variations e.g. different analysts and equipment were kept constant throughout the period.

1.3.2.2.2 Results

Acetylcholine		
Concentration	**Mean % RSD**	**Number of days of injections (n)**
50 ng/ml	5.419	3
100 ng/ml	1.995	3
350 ng/ml	1.895	3
Choline		
50 ng/ml	8.853	3
100 ng/ml	4.572	3
350 ng/ml	5.204	3

Table 3: Intermediate precision validation: % RSD values for Acetylcholine and choline

1.3.2.2.3 Conclusion

Despite the variation of preparation of standard solutions and equipment response, an additional variant was included in intermediate precision viz. injection at different days. Good precision values under 15% were obtained at higher concentrations with fewer repeated injections as for intra-assay precision. Results are reflected in table 3.

1.3.3 Specificity and selectivity

Selectivity is the ability of an analytical method to differentiate and quantify an analyte in the presence of other components (United States Department of Health and Human Services, 2001) whereas specificity refers to a method that produces a response for only one analyte. Since most methods respond to a number of analytes, the term selectivity is usually used (Lindholm, 2004).

By using an LC/MS/MS method in the current study, the selectivity issue could be managed in most circumstances, because of relatively clean chromatogram spectra and for identification by both retention time and molar mass. However, isomers of the compound of interest can result in selectivity problems.

1.3.3.1 Method

In evaluating the chromatogram spectra for interferences, which could result from contamination, two blank standards were injected with each calibration run. A "iso-ACh" standard was also injected to identify the presence of the possible endogenous form of this compound that was reported in previous studies (Zhu *et al.*, 2000; Zhang *et al.*, 2007).

1.3.3.2 Results

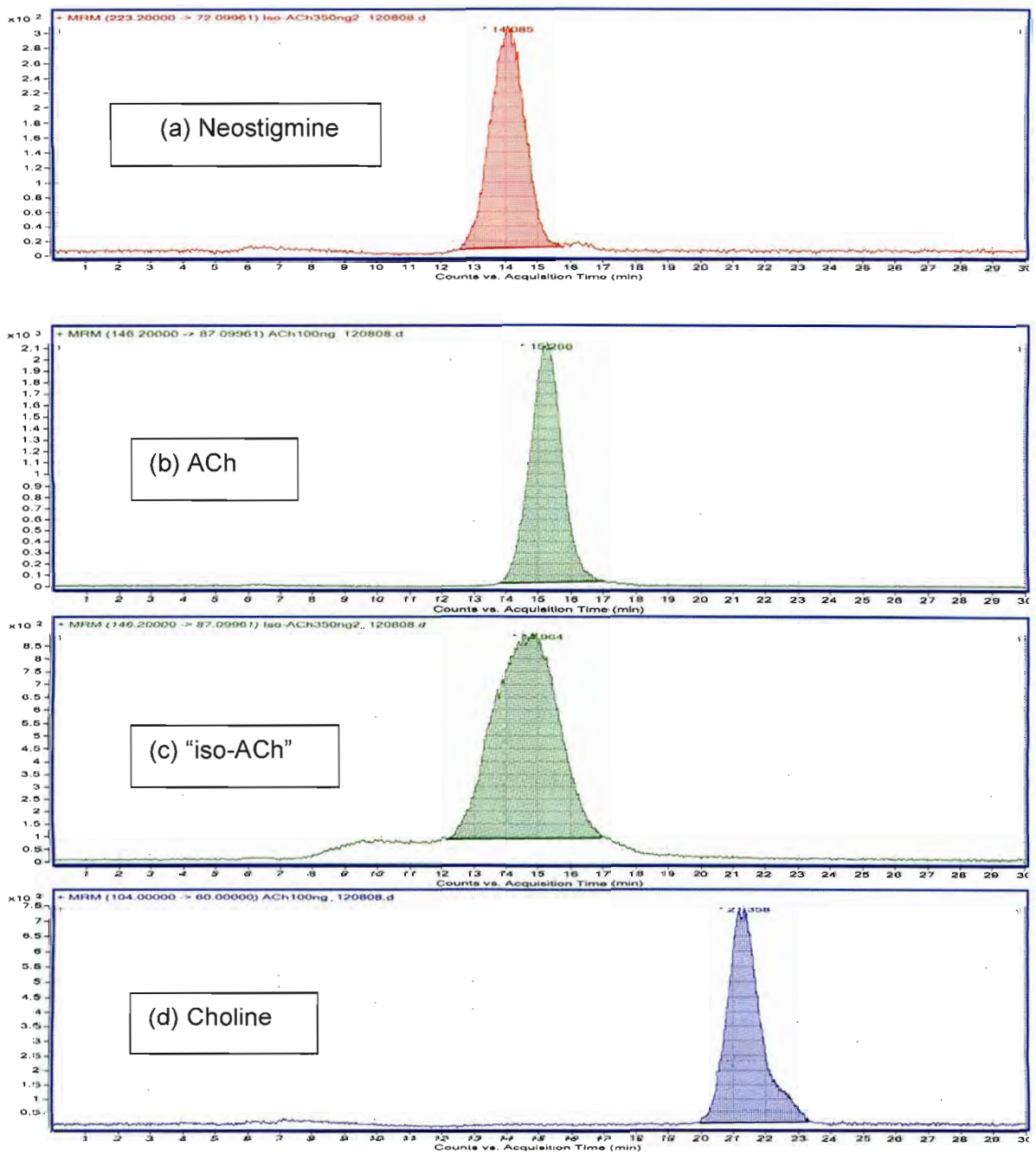


Figure 4: Selectivity validation: Chromatograms of neostigmine ACh, "iso-ACh" and choline standards

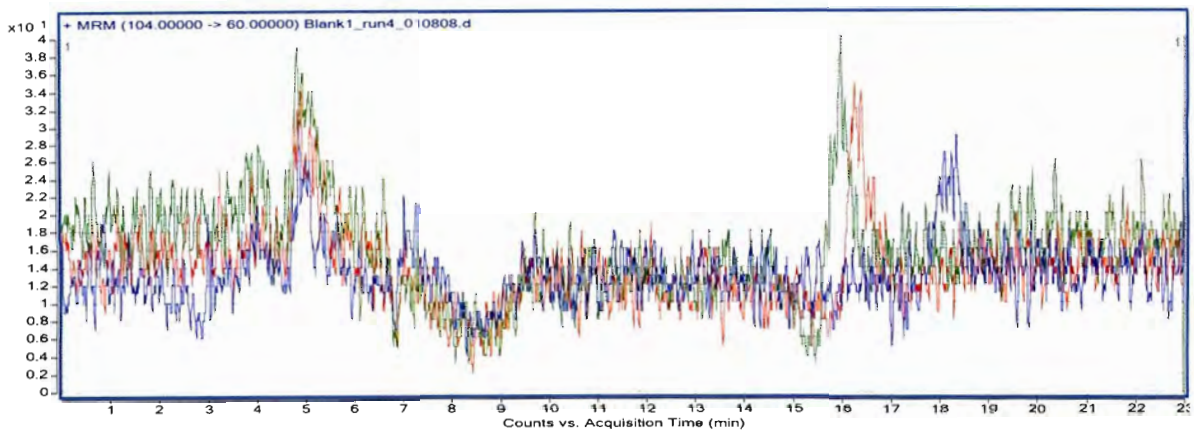


Figure 5: Selectivity validation: Chromatogram of blank solution

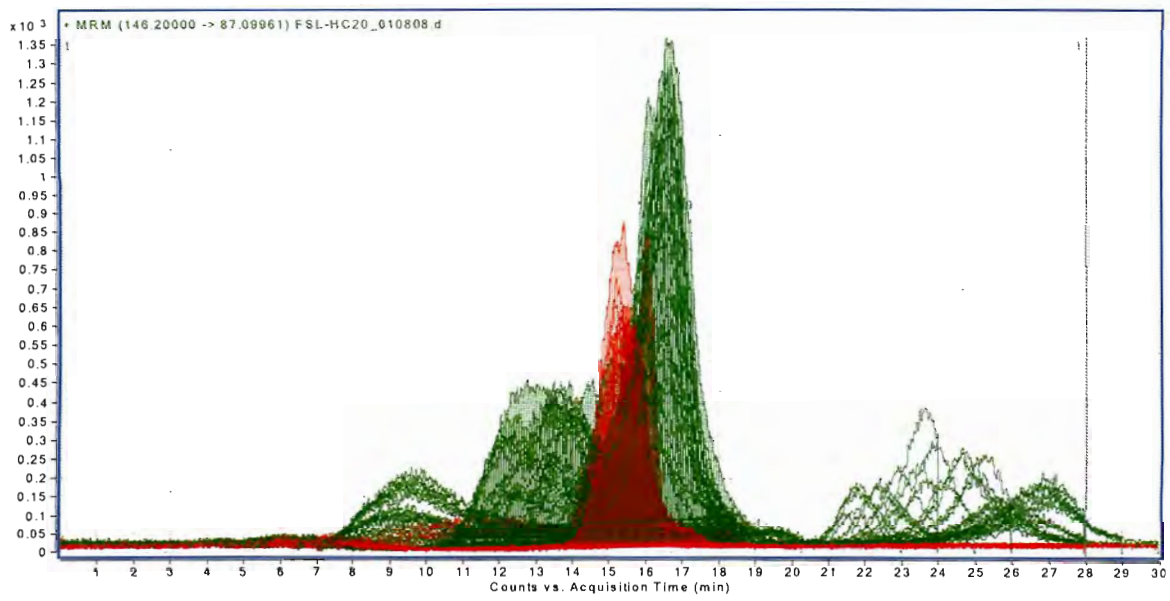


Figure 6: Selectivity validation: Acetylcholine (green peaks; time interval 12-19) and neostigmine (red peaks) in overlay view of multiple samples

1.3.3.3 Conclusion

The blank solution in figure 5 reveals the chromatogram with negligible contamination peaks at time intervals of 16-17 and 18-19 minutes and therefore did not interfere with the quantification of compounds of interest. Another interference compound, "iso-Ach" identified through its standard

solution (figure 4c; "iso-ACh), was found to elute in the presence of acetylcholine (shifted in time interval 12-19; figure 6) and therefore led to the integration of both these compounds as one compound, viz. acetylcholine. Other compound with the same mass transition as acetylcholine and its isomere ("iso-ACh") was found in the samples but successfully separated from ACh peaks (time interval 8-11 and 22-28; figure 6).

1.3.4 Limit of Quantification & Limit of Detection

The limit of quantification (LOQ) is the lowest standard on the calibration curve which can be quantitatively determined with suitable precision and accuracy (United States Department of Health and Human Services, 2001) whereas the limit of detection (LOD) is the lowest amount of analyte that can be detected but not necessarily quantified.

1.3.4.1 Method

The LOQ was determined by the lowest value in the calibration range that gave good precision (see section 1.3.2). The LOD was theoretically calculated by using equation 4 which was substituted in equation 5. Linearity statistics from table 4 was obtained for calculating the LOD.

$$Y_{LOD} = a + 3S_a(S_{y/x}) \quad \textbf{(Equation 4)}$$

$$Y_{LOD} = bx_{LOD} + a \quad \textbf{(Equation 5)}$$

1.3.4.2 Results

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.999051126
R Square	0.998103152
Adjusted R Square	0.997723782
Standard Error	0.230952667
Observations	7

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	140.3326935	140.3326935	2630.951833	5.3242E-08
Residual	5	0.266695672	0.053339134		
Total	6	140.5993892			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	-0.172437115	0.111189188	-1.550844272	0.181634603	-0.458257554	0.113383324
X Variable 1	0.054707882	0.00106658	51.29280488	5.3242E-08	0.051966155	0.057449609

Table 4: Linearity statistics from sample calibration standards

Acetylcholine	
LOQ	**LOD**
5 ng/ml	1.408 ng/ml

Table 5: LOQ and LOD values for ACh

1.3.4.3 Conclusion

The method that was developed, yielded a LOD lower than most studies (Hows *et al.*, 2002; Zhang *et al.*, 2007) but not more than reported by a study with high sensitivity (Zhu *et al.*, 2000). Both LOQ and LOD values are reflected in table 5.

1.4 Conclusion

A sensitive high performance liquid chromatography-tandem mass spectrometer (LC/MS/MS) method was developed for the measurement of acetylcholine in brain tissue. The method yielded a successful linear regression above 0.95 which implies an accurate detection and quantification of ACh concentration in brain tissue. Intra-assay and intermediate precision revealed that the current method is reproducible in quantification of ACh in brain tissue after short periods of time and even between different days. The major advantages of using the LC/MS is that compounds can be selectively and sensitively detected and identified by both their retention times as well as molecular weight. However, a chromatogram of the current method was obtained with ACh eluting in the presence of interfering compounds which revealed the same mass transition as ACh. Although successful separation techniques were employed to separate ACh from these compounds, a compound previously identified as "iso-ACh" (Zhu *et al.*, 2000; Zhang *et al.*, 2007), could not be separated in the current study. Since the sensitivity of the current method (LOD) in the presence of a AChE inhibitor, physostigmine was found to be higher than other microdialysis studies (Hows *et al.*, 2002; Zhang *et al.*, 2007), the current method can be implicated for future microdialysis studies with small sample size.

HPLC analysis of GABA

Addendum 2

2.1 Introduction

The inhibitory amino acid, γ-aminobutyric acid (GABA), with its role in neuropsychiatric disorders has received significant attention with regard to analytical detection. A thoroughly validated method (Jonker, 2001) was followed with minor modifications and therefore only the basic validation criteria were performed (linearity and precision).

2.2 Materials and methods

2.2.1 Chemicals

γ-Amino-n-Butyric Acid (GABA)	Sigma Aldrich; USA
DL-Homoserine	Sigma Aldrich; USA
orto-phtaldialdehyde (OPA)	Pierce, USA
Perchloric acid - $HClO_4$ (60%).	SAARChem, Krugersdorp
Sodium phosphate dibasic	Merck, Germany
Ethylenediaminetetraaceticacid (EDTA) disodiumsalt (Na_2EDTA)	SAARChem, Kugersdorp
Methanol 28% (to 35%)	VWR International Ltd, England
orthophosphoric acid (85%).	SAARChem, Krugersdorp
potassium acetate (CH_3COOK)	BDH Chemical Ltd., Poole

	England

2.2.2 Instrumentation and conditions for GABA analysis

The chromatographic system consists of a Agilent 1100 series HPLC, equipped with an isocratic pump, autosampler and a GBC LC 1260 Electrochemical detector. The analyte, GABA, was separated from the internal standard and other compounds by a Luna C18-2 column (75 x 4.6 mm) that was protected by a Phenomenex C18 guard column (4.0 x 3.0mm).

The electrochemical detector comprises a Glassy Carbon Electrode with positive polarity. An electrode potential of 0.600 V with a range of 5 nA was used to detect GABA in the samples.

The mobile phase consisted of 0.1 M Sodium phosphate dibasic, 0.13 mM Ethylenediaminetetraaceticacid (EDTA) disodiumsalt (Na_2EDTA) and 28% - 35% methanol. The solution was prepared by dissolving 14.2 g Na_2HPO_4 acid and 0.05 g Na_2EDTA in 720 ml purified water (Milli Q). The pH was adjusted to 6.4 with 85% phosphoric acid where after 280 ml of 35% methanol was added and thoroughly mixed. The solution was degassed and filtered under vacuum through a 0.45 µm membrane filter.

The mobile phase was recycled for two weeks before it was replaced by fresh mobile phase solution. The HPLC system was purged with increased eluent flow before adjusting the flow to the desired flow rate for sample analysis. The flow rate was adjusted during the analysis as shown in the table below for the GABA peak to elude earlier.

Time	**Flow rate**
0.00	1.00 ml/min
15.00	1.00 ml/min

16.00	1.50 ml/min
27.00	1.50 ml/min
27.90	1.50 ml/min

The data were acquired using Chemstation Rev. A.06.02 data acquisition and analysis software.

2.2.3 Standard solutions

The stock solution was prepared by dissolving 1 mg γ-Amino-n-Butyric Acid (GABA) in 10 ml of a 0.1M perchloric acid ($HClO_4$) solution to yield a final concentration of 100 µg/ml GABA. Internal standard, homoserine, was prepared in a separate stock solution by dissolving 1 mg in 10 ml $HClO_4$. Standard solutions were prepared on the day of analysis by diluting the stock solution with $HClO_4$ solution.

2.2.4 Tissue dissection and extraction

The dissection procedure for the FSL and FRL rats was done according to the procedure for the acetylcholine assay (see addendum 1; section 1.2.4) where the hippocampus and frontal cortex brain areas were dissected and immediately placed in separate poly vials and "snap frozen" in liquid nitrogen (-196°C). The frozen brain sections were than stored at -80°C.

On the day of analysis, brains were weighed (± 30 mg) and a solution of 1 ml 0.1 M $HClO_4$ was added to each vial of brain tissue. The vials were then sonicated for 2 × 10 seconds at 21 µ after which it was centrifuged at 20 000 ×g for 15 minutes (4°C) in a Sigma 3K15 bench top centrifuge. The preparation of the supernatant was performed on ice.

The supernatant (200 µl) was added to 100 µl of 5 µg/ml homoserine internal standard. The pH was adjusted to approximately 6 with 50 µl of 10 M

CH_3COOK where after the solution was vortexed. Finally, an amount of 55 µl was added to an insert vial and 50 µl withdrawn from the vial and injected on the column.

2.2.5 Derivatization procedure

The derivatization procedure is necessary for electrochemical activation of amino acids. The software's injector program was programmed as follow for precolumn *orto*-phtaldialdehyde (OPA) derivatization:

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.

2.3 Validation

2.3.1 Linearity

See addendum 1; section 1.3.1 for a description of the criteria and method for the determination of linearity.

2.3.1.1 Method

The linearity of the method was determined with a series of standard concentrations that include the range of endogenous GABA concentrations usually found in the brain. The peak area (PA) ratios (Section 1.3.1.1; equation 1) for GABA were plotted against seven standard concentrations to determine linearity through Equation 2; section 1.3.1.1 of addendum 1

2.3.1.2 Results

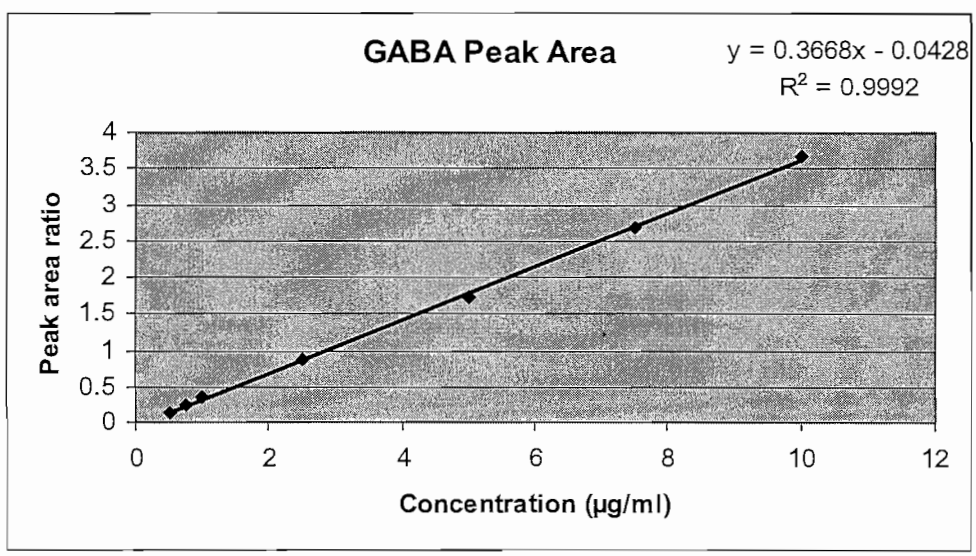


Figure 7: Linearity validation: GABA PA ratio vs. concentrations

Analyte	Gradient (m)	Y-intercept (c)	Regression (R)
Peak area			
GABA	0.3668	-0.0428	0.9992

Table 6: Linearity validation: Gradient, y-intercept and regression coefficient for GABA

2.3.1.3 Conclusion

The HPLC method for the detection and quantification of GABA was found to be linear in a range between 0.5-10 μg/ml. The regression coefficient is reflected in table 6.

2.3.2 Precision

See addendum 1; section 1.3.2 for a description of the criteria and method to establish / measure precision.

2.3.2.1 Method

The intra-assay precision was determined with six measurements of two concentrations in the range of expected concentrations. The precision is expressed in term of % RSD (see Equation 3; section 1.3.2.1.1 of addendum 1)

2.3.2.2 Results

GABA		
Concentration	**Mean % RSD**	**Number of injections (n)**
2.5 μg/ml	1.833	6
7.5 μg/ml	2.639	6

Table 7: Precision validation: % RSD values for GABA

2.3.2.3 Conclusion

The % RSD values for GABA in Table 8 were found to be less than 3% and unknown brain concentrations could therefore be accurately determined.

NMDA / M_1 radioligand binding assay

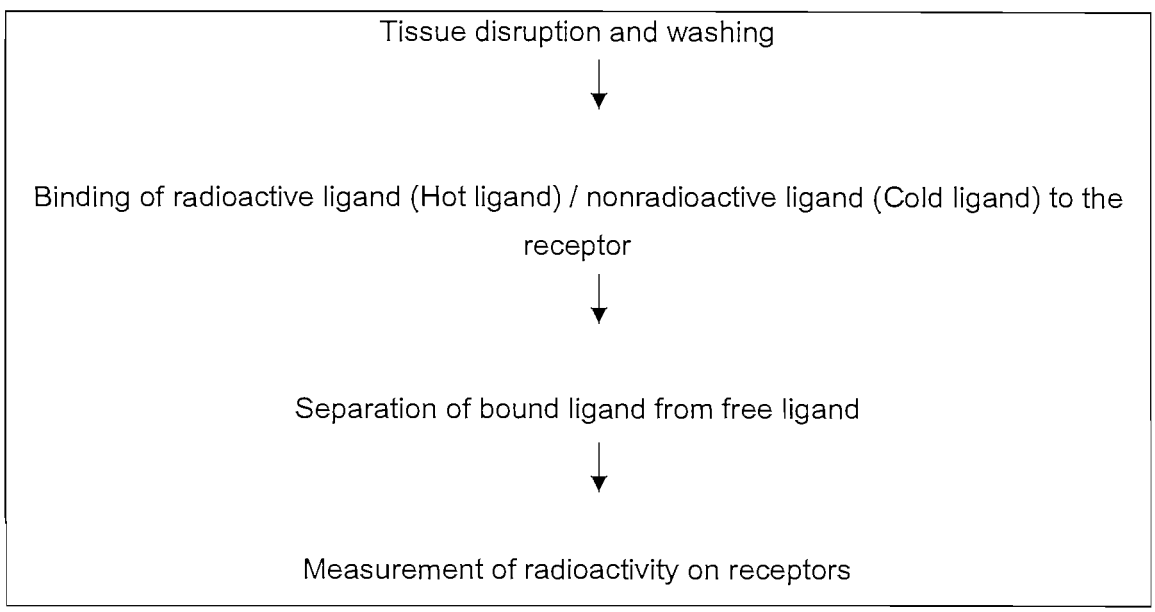
Addendum 3

3.1 Introduction

Proteins form the most important class of drug receptors where by hormones, growth factors and neurotransmitters modulate their intrinsic physiological functions (Goodman & Gilman, 2001). Little was known about the identity of neurotransmitter receptors, until studies from different laboratories in the early 1970's gave valuable insight in receptor characterization and laid the basis for ligand receptor binding studies (Leonard, 2003b).

3.2 Basic principles

Receptor binding studies consist of the following basic steps adjusted from (Deupree & Bylund, 2002)



Tissue disruption and washing

The standard procedure is to homogenize the tissue or cells of interest in a buffer by using a Polytron, glass dounce or a sonicater. The tissue sample is then centrifuged by a series of washing steps to remove any endogenous ligand.

Binding of ligand to receptor

The ligand receptor binding study consists of a reaction between the radioactive ligand / non radioactive ligand and tissue sample that contains the receptors. The binding of a radioligand to a receptor is therefore analogous to a bimolecular reaction according to the Law of Mass Action

$$L^* + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} L^*R \qquad \textbf{(Equation 6)}$$

Where

L^* = ligand

R = receptor

L^*R = ligand-receptor complex

Like a bimolecular reaction, the rate of the forward reaction (k_1) and the reverse reaction (k_2) can reach equilibrium that is also referred to as the steady state. The steady state of the reaction is where receptor binding does not further increase and therefore the rate of $k_1 = k_2$ (see Equation 6). For receptor saturation experiments, the steady state conditions are determined by measuring the smallest concentration of ligand at various times following incubation.

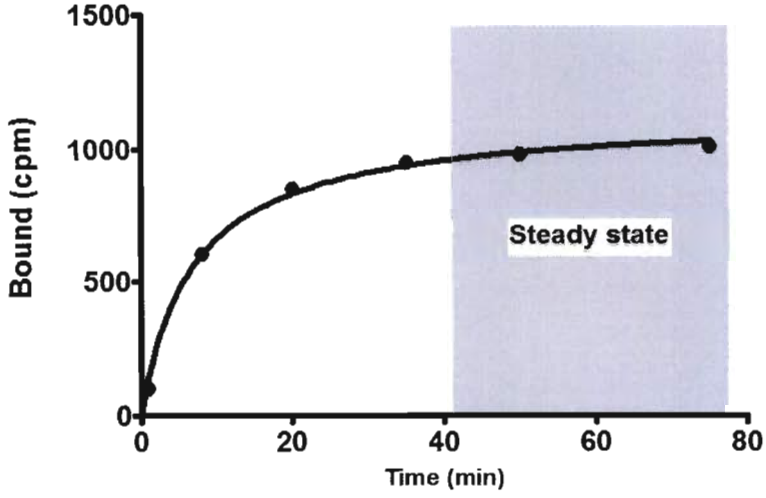


Figure 8: Measurement of steady state

Radioligand binding studies are performed to determine the B_{max} (receptor density) and the K_d value (affinity) of the ligand for the receptor. In saturation binding assays, the B_{max} value is determined by the amount of radioactivity measured at the concentration of radioligand that occupies 100% of the receptors of interest and the K_d value is the concentration of radioligand that occupies 50% of the total amount of specific receptors.

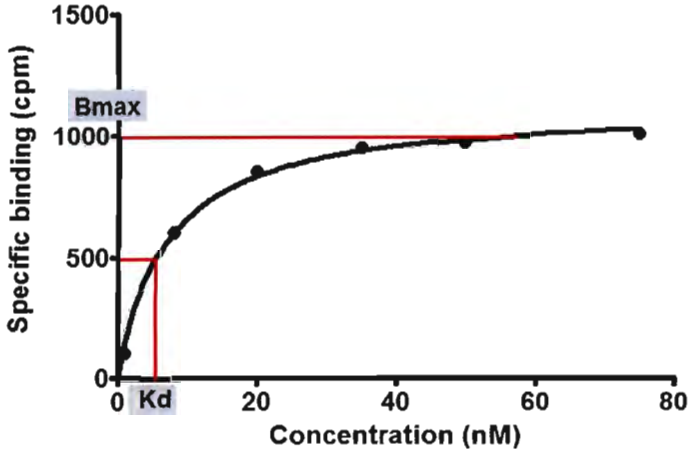


Figure 9: Measurement of K_d- and B_{max}- values by incubating increased concentrations of radioligand with tissue samples

Since the radioactive ligand does not only bind to the receptor of interest, the specific binding (Figure 9) is calculated by subtracting the non-specific binding from the total binding. The non-specific binding is determined by blocking the receptor of interest by a competitive antagonist (cold ligand).

Total bound – Non-Specific Bound = Specific Bound

Separation of bound ligand from free ligand

Following steady state most of the radioligand remains unbound and is therefore separated from bound ligand, usually by means of vacuum filtration. The unbound radioligand passes through the filters by vacuum whereas the radioligand-receptor complex is retained by the filter.

Measurement of radioactivity on receptors

The amount of radioactivity trapped on the filters can be determined using a scintillation counter. The scintillation counting is achieved by placing the filters (trapped with bound ligand) in a counting vial where after scintillation fluid is added.

3.3 Material and methods

3.3.1 Chemicals

NMDA receptor binding assay	
[^{3}H]-MK801	Perkin Elmer, USA
Tris (hydroxymethyl) aminomethane	SAARChem, Gauteng
HEPES	Sigma-Aldrich, USA
MK801	Sigma-Aldrich, USA
l-glutamate	Sigma-Aldrich, USA
Glycine	Sigma-Aldrich, USA
Scintillation fluid, Filter count®	Perkin Elmer, USA
Muscarinic receptor binding assay	
[^{3}H]-Quinuclidinyl benzilate	Perkin Elmer, USA
Tris	SAARChem, Gauteng
Atropine sulphate	Merck, Germany
Scintillation fluid, Filter count®	Perkin Elmer, USA

Table 8: Chemical substances used in the NMDA- and muscarinic receptor binding assays

3.3.2 Tissue dissection and extraction

Rats were decapitated and brains rapidly removed. The hippocampus and frontal cortex brain areas were dissected on ice and immediately "snap

frozen". The frozen brain areas were stored at -80°C in separate "poly" vials until the day of analysis.

On the day of analysis, three frontal cortices and three hippocampi were pooled to obtain a sufficient amount of protein. The pooled brain tissue was "washed" by suspending the tissue in 25 ml of Tris / HTS (HEPES and TRIS) buffer by using a Polytron homogenizer. The suspension was then centrifuged with a Sorvall discovery 90 SE ultracentrifuge at 48 000 ×g for 15 minutes at 4°C where after it was homogenized by a Teflon homogenizer. The centrifugation step was repeated and the pellet finally re-suspended in 60 volumes of buffer prior to a final polytron homogenizing step.

The homogenate was kept on ice and an aliquot of the homogenate was used for protein determination.

3.3.3 Protein determination

3.3.3.1 Chemicals

Bradford reagent	Sigma-Aldrich, USA
Bovine serum albumin (BSA)	Sigma-Aldrich, USA
HTS (HEPES+Tris)/ Tris (hydroxymethyl) aminomethane buffer	SAARChem, Gauteng; Sigma Aldrich, USA

Table 9: Chemicals used for the Bradford assay

3.3.3.2 Bradford assay

A 2 mg/ml BSA solution was prepared and diluted with the appropriate amount of buffer to obtain the following protein concentrations:

Protein concentration	Volume of 2 mg/ml BSA	Volume of HTS/Tris buffer
0 mg/ml	0 µl	100 µl
0.1 mg/ml	5 µl	95 µl
0.4 mg/ml	20 µl	80 µl
0.7 mg/ml	35 µl	65 µl
1.0 mg/ml	50 µl	50 µl
1.4 mg/ml	70 µl	30 µl

Table 10: Preparation of the BSA standards

An amount of 5 µl of membrane suspension and standard protein concentration was added in duplicate to each of the wells of a 96-well plate. An amount of 250 µl of Bradford reagent was added to each well and allowed to interact with the protein by the mixing feature of the plate reader and left to react for 15 minutes.

The absorbance was determined in the 560 nm region and the protein concentration of the membrane suspension was determined using the mathematical equation obtained from the linear graph of standard protein concentrations plotted against the netto absorbance values.

3.3.4 Assay for receptor density and affinity

3.3.4.1 NMDA receptor assay

3.3.4.1.1 Stock solutions

The HTS buffer used for the binding assay comprises of 5 mM HEPES and 4.5 mM TRIS and all the solutions in table 11 was prepared the previous day, stored at 4°C and kept on ice throughout the binding assay

Substance	Molar mass	Mole concentration	Mass concentration
HEPES	238.31 g/mol	5 mM	1.192 g/L
TRIS	121.14 g/mol	4.5 mM	0.545 g/L
Glycine	75.07 g/mol	300 μM	0.023 g/L
l-glutamate	169.1 g/mol	100 μM	0.017 g/L
MK801	337.4 g/mol	300 μM	0.101 g/L

Table 11: The molar mass, mole concentration and mass concentration of non-radioactive chemicals used in NMDA assay

The following series of [^{3}H]-MK801 concentrations were prepared:

Final assay concentration (nM)	Volume from previous concentration (μl)	Volume Buffer (μl)	Total volume (μl)
25.21	5 *	717	722
15.14	436	290	726

9.09	436	290	726
5.46	436	290	726
3.28	436	290	726
1.97	436	290	726
1.18	436	290	726
0.71	436	290	726

Table 12: Radioligand stock concentrations for NMDA binding assay

* An amount of 5 μl from [^{3}H]-MK801 container was added to 717 μl buffer.

3.3.4.1.2 Binding assay

The homogenate/ buffer/ cold ligand mixture was incubated for 90 minutes at 25°C with increasing concentrations of radioligand (see series of concentrations in table 12). The binding reaction was initiated as soon as the radioligand was added to each tube and for that reason added last to the incubation mixture. Binding reactions for all concentrations in table 12 were performed in duplicate.

Total binding (hot)	Non-specific binding (cold)
300 µl Tissue homogenate	300 µl Tissue homogenate
50 µl Glycine	50 µl Glycine
50 µl l-Glutamate	50 µl l-Glutamate
50 µl HTS buffer	50 µl MK801
50 µl radioligand	50 µl radioligand

Table 13: Incubation mixture for [^{3}H]-MK801 receptor assay

To separate the free ligand from the bound ligand, the contents of each tube were transferred to a Whatman GF/B filter pre-soaked with ice-cold buffer on a Hoeffler apparatus. To prevent dissociation of ligand from receptor, the whole procedure was performed as quickly as possible by rinsing the filters twice with 4 ml ice-cold buffer after adding the contents to the filter.

Finally, the filters were transferred to scintillation vials and 3 ml scintillation fluid was added to each vial for extraction of radioactivity.

In addition, the total amount of radioactivity was determined through adding 50 µl of each radioligand stock concentration (table 12) to a pre-soaked filter in a scintillation vial. Scintillation fluid was added where after it was counted in the scintillation counter. The measured radioactivity for each stock concentration was then used to draw a standard curve that was used in converting the cpm values for specific binding to a final concentration unit (see paragraph 3.3.6)

3.3.4.2 Muscarinic M_1 receptor assay

3.3.4.2.1 Stock solutions

The storage conditions of stock solutions for M_1 receptor binding assays were the same as described for NMDA binding assays (paragraph 3.3.4.1.1)

Substance	Molar mass	Mole concentration	Mass concentration
TRIS	121.14 g/mol	50 mM	6.057 g/L
Atropine sulphate	694.85 g/mol	15 mM	0.104 g/L

Table 14: The molar mass, mole concentration and mass concentration of non-radioactive chemicals used in muscarinic receptor assays

The following series of [^{3}H]-Quinuclidinyl benzilate (QNB) concentrations were prepared:

Final assay concentration (nM)	Volume from previous concentration (μl)	Volume Buffer (μl)	Total volume (μl)
10.96	4 *	698	702
6.576	450	300	750
3.288	300	300	600
1.644	300	300	600
0.822	300	300	600
0.411	300	300	600

0.2055	300	300	600

Table 15: Radioligand stock concentrations for the muscarinic M_1 binding assay

* An amount of 4 μl from [^{3}H]-QNB container was added to 698 μl buffer.

3.3.4.2.2 Binding assay

The incubation mixture to determine the total binding and non-specific binding was incubated for 15 minutes at 25°C. The assay for both total binding and non-specific binding was carried out in duplicate.

Total binding (hot)	Non-specific binding (cold)
400 μl Tissue homogenate	400 μl Tissue homogenate
50 μl TRIS buffer	50 μl Atropine sulphate
50 μl of respective radioligand	50 μl of respective radioligand

Table 16: Incubation mixture for [^{3}H]-QNB receptor assay

The separation of free radioligand from bound radioligand, and the preparation of scintillation vials were kept the same as for the NMDA receptor binding assay (see section 3.3.4.1.2)

3.3.5 Determination of radioactivity

The radioactivity on the filters (total bound and non-specific bound) was counted with a Packard Tri-Carb 2100TR liquid scintillation analyzer. The radioactivity was expressed in cpm and dpm values

3.3.6 Data analysis

The specific binding was calculated by subtracting the average cpm values of non-specific binding from average total binding. The cpm values (y-axis) for specific binding were then plotted against the nM concentration values (x-axis) to generate a saturation curve in the form of a hyperbola. The B_{max} value was determined from the y-axis (cpm) where 100% of the specific receptors were occupied whereas the K_d value was determined from the x-axis (nM) where 50% of the specific receptors were occupied. Both the B_{max}- and K_d -values of the respective binding assays were analyzed through non linear regression by Prism version 5 from GraphPad Software, Inc. The cpm / nM slope of the standard curve for the respective binding assays as discussed in paragraph 3.3.4.1.2 was used to convert the B_{max} values to concentration (nM) values and finally to fmol/mg protein values.

Instructions to Authors

Appendix 1

1 Instructions for Authors

European Journal of Pharmacology

1.1 BEFORE YOU BEGIN

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GDP, IDP	cytidine, guanosine, inosine
UDP	uridine
AMP etc.	adenosine 5'-monophosphate etc.
ADP etc.	adenosine 5'-diphosphate etc.
ATP etc.	adenosine 5'-triphosphate etc.
CM-cellulose	carboxymethylcellulose
CoA and acetyl-CoA	coenzyme A and its acyl derivatives
DEAE-cellulose	*O*-(diethylaminoethyl)-cellulose
DNA	deoxyribonucleic acid
EGTA	ethylene glycol-bis(*β*-aminoethyl ether)*N,N,N',N'*-tetraacetic acid
FAD	flavin-adenine dinucleotide
FMN	flavin mononucleotide
GSH, GSSG	glutathione, reduced and oxidized
Hepes	4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid
NAD	nicotinamide-adenine dinucleotide
NADP	nicotinamide-adenine dinucleotide phosphate
NMN	nicotinamide mononucleotide
P_i, PP_i	orthophosphate, pyrophosphate
RNA	ribonucleic acid
Tris	2-amino-2-hydroxymethylpropane-1,3-diol

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