A proteomic analysis of the ventral and dorsal hippocampal brain areas of serotonin knockout rats

Lorren R. Fairbairn

Thesis presented in partial fulfillment of the requirements for the degree of Masters of Neuroscience at the University of Stellenbosch.

Supervisors: Prof. William M.U. Daniels and Prof. Dan J. Stein

March 2008

Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature : …….

Date : …….

Abstract

For many centuries, scientists have engaged in a theoretical debate concerning the etiology of mood disorders, with very few ancient scholars speculating about the importance of genetic factors and affective temperaments as factors in the etiology of depression. Mood, emotion and cognition have been shown to be modulated by the serotonergic midbrain raphe system; implicated in the pathogenesis of psychiatric disorders like those of the affective spectrum. Evidence from neuroscience, genetics, and clinical investigation demonstrate that depression is a disorder of the brain. Brain imaging research is revealing that in depression, neural circuits responsible for moods, thinking, sleep, appetite, and behavior fail to function properly, and that the regulation of critical neurotransmitters is impaired. Genetics research, including studies of twins, indicates that genes play a role in depression. Vulnerability to depression appears to result from the influence of multiple genes acting together with environmental factors. Other research has shown that stressful life events, particularly in the form of loss such as the death of a close family member, may trigger major depression in susceptible individuals. Depression and anxiety have often been successfully treated by means of selective serotonin reuptake inhibitors. However, selective serotonin reuptake inhibitors do not solve all the problems inherent to the treatment of depression, for approximately 30 % of depressed patients do not respond to treatment and 20 % experience relapses whilst on treatment. Of consideration is the fact that the majority of drugs today are based on proteins, with 50 % of therapeutics on the market targeting cell membrane proteins. Up to this day the precise pathophysiology of mood disorders remains obscure, as does the neurobiology of normal mood regulation. Accordingly, there is a need for methods to identify the structural and/or signaling components which lead to changes in the brain, particularly the hippocampus, of subjects having mood disorders such as bipolar depressive disorder, chronic major depressive disorder and the like. Similarly, there is a need for the early detection, screening and diagnosis of individuals at risk for a mood disorder. As the serotonin tranpsorter is the primary target for therapeutic intervention in the treatment of numerous psychiatric disorders and considering the fact that at the structural level this protein's function as transporter in membranes remains incompletely understood, investigating its function in psychiatric disorders are of importance . The objective of this study was to determine the role of the serotonin transporter in wild type and serotonin knockout rats, with regards to the hippocampus. Rat hippocampi were fractionated into cytosolic and membrane components, which were run and further separated in two dimensions. Firstly separation occurred by isoelectrical focusing (pI), follwed by gel

electrophoresis (molecular weight). Gels were compared to see whether protein spots have changed between animals that have been differentially bred. Differentially expressed protein spots, as determined by PD Quest software, were excised, digested and analyzed by means of mass spectrometry. Our results indicated that metabolic, structural and cell signaling proteins were differentially expressed in both the ventral and dorsal hippocampus of the serotonin knockout rat. Futhermore, cellular stress proteins were found to be only differentially expressed in the ventral hippocampus. The majority of proteins identified in both hippocampal areas as well as both fractions, were assigned to energy metabolism. The cytosolic protein profile mirrored the pattern of the membrane protein profile. In conclusion, this proteomic study identified various protein groups that interacted with one another, thus establishing compensation for disrupted serotonin homeostasis.

Opsomming

Oor die eeue heen het wetenskaplikes in ‘n teoretiese debat betrokke geraak betreffende die etiologie van gemoedsversteurings. Bevindings van neurowetenskap, genetika en kliniese ondersoeke demonstreer dat depressie ‘n versteuring van die brein is. Verder het brein beeldingstudies onthul dat neurale netwerke, wat verantwoordelik is vir gemoed, denke, slaap, eetlus en gedrag, faal om korrek te funksioneer en dat die regulasie van kritieke neurotransmittore versteur is in stoornisse soos bv. depressie. Genetiese studies, insluitend studies op tweelinge, het sterk aangedui dat gene ‘n belangrike ‘n rol speel in depressie. Kwesbaarheid tot die ontwikkeling van depressie blyk die gevolg te wees van die invloed van verskeie gene wat saamwerk met omgewingsfaktore. Ander navorsing het getoon dat stresvolle lewenservaringe, byvoorbeeld in die vorm van verlies as gevolg van die dood van ‘n familielid, depressie mag ontlok in vatbare individue. Hierdie verskynsel is veral opmerklik in gevalle waar jong kinders ouers verloor, en gevolglik in die latere volwasse stadium gemoedstoornis ontwikkel. Tans is die neurobiologie van normale gemoedsregulering asook die presiese patofisiologie van gemoedsversteurings nog onduidelik. Een van die redes hiervoor is die tekort aan gepaste modelle om die verskillende siektetoestande te ondersoek. Byvoorbeeld daar heers ‘n groot behoefte aan sensitiewe metodes om die rol van strukturele proteïene en/of seintransduksie paaie, wat sentraal is tov neuronfunksie, te bestudeer. Daar word gespekuleer dat abnormaliteite op hierdie vlakke kan lei tot veranderinge in die funksie van sekere brein areas soos die hippokampus. Pasïente met gemoedsversteurings soos bipolêre depressiewe versteuring en kroniese uitgebreide depressiewe versteuring blyk om morfologies abnormale hippokampusse te hê. Soortelyk heers daar ‘n behoefte vir metodes om die vroeë waarneming, skandering en diagnosering van individue met ‘n risiko tot ‘n gemoedsversteuring te bewerkstellig. Gemoed, emosie en kognisie word betekenisvol deur die serotonien midbrein raphe sisteem gemoduleer en dus word hierdie neurotransmittorsisteem in die patogenese van ‘n aantal psigiatriese versteurings geïmpliseer. Bewyse hiervoor spruit uit die suksesvolle behandeling van depressie en angs met selektiewe serotonien heropname blokkeerders. Ongelukkig is hierdie sukses gedeeltelik, want ongeveer 30 % van depressie-lyers reageer nie op die behandeling nie en 20 % ervaar terugslae terwyl hulle op behandeling is. Interresant is die feit dat die oorgrootte meerderheid van huidige medikasie teiken proteïene, veral membraan proteïene. Die serotonien transporter is ‘n primêre teiken van ‘n hele aantal terapeutiese intervensies in die behandeling van sommige psigiatriese versteurings. Ten spyte hiervan is die bydraes van hierdie transporter tot gedragssiektetoestande nog nie goed bestudeer nie. Die doel van ons studie was dan juis om die rol van die serotonien transporter in die neurobiologie

van gedrag verder te ondersoek. Normale kontrole rotte en serotonien transporter knock-out diere is bestudeer. Die hippokampusse van hierdie diere was gefraksioneer in sitosoliese en membraan komponente wat verder geskei was in twee dimensies. Eerstens het skeiding plaasgevind op grond van isoelektriese fokusering, gevolg deur gelelektroforese wat proteïene volgens grootte skei. Hierdie gels was dan vergelyk om proteïene wat verander het tussen die verskillende rotgroepe. Differensieël uitgedrukte proteïene, soos uitgeken deur PD Quest sagteware, was uitgesny, verteer en dan onderwerp aan verdere analisering deur middel van massa spektrometrie. Ons resultate dui daarop dat metaboliese, strukturele en seintransduksie proteïene differensieël uitgedruk was in beide die ventrale en dorsale hippokampusse van die serotonien knock-out rot. Verder was sellulêre stres proteïene slegs differensieël uitgedruk in die ventrale hippokampus. Die meerderheid van proteïene wat geïdentifiseer is, in beide hippokampus areas sowel as beide fraksies, was toegeken aan energie metabolisme. Die sitosoliese proteïen profiel het die patroon van die membraan proteïen profiel weerspieël. Ten slotte het hierdie proteomiese studie die verskillende proteïengroepe wat op mekaar inwerk, geïdentifiseer, wat dus ‘n kompensasie daarstel vir die ontwrigte serotonien homeostase.

Acknowledgements

My sincerest gratitude to all family members, friends and neuroscience colleagues who traveled along this difficult, yet fulfilling, journey with me. Whose love, support, knowledge and technical assistance is valued.

A special word of thanks to my supervisors, Prof W. Daniels and Prof D. Stein, our collaborators at the Radboud University of Nijmegen The Netherlands for the opportunity afforded to partake in this novel study, as well as for their guidance.

Last but not least, the National Research Foundation and Medical Research Council for their financial support.

List of abbreviations

NCDs	= noncommunicable diseases
DALYs	= disability adjusted life years
WHO	= world health organization
ECSA	= Eastern, Central and Southern Africa
SPECT	= single-photon emission computed tomography
CNS	= central nervous system
CSF	= cerebrospinal fluid
HPA-axis	= hypthalamic-pituitary-adrenal axis
ACTH	= adrenocorticotropin hormone
CRF	= corticotropin releasing factor
TRH	= thyrotropin-releasing hormone
GHRF	= growth hormone releasing factor
GABA	= gama aminobutyric acid
NE	= norepinephrine
NET	= norepinephrine transporter
DAT	= dopamine transporter
VMAT	= vesicular monoamine transporter

SERT	= serotonin transporter
5-HTP	= 5-hydroxytryptophan
5-HIAA	= 5-hydroxyindole-3-acetic acid
5HT	= serotonin
5-HTTLPR	= serotonin transporter gene-linked polymorphic region
SSRIs	= selective serotonin reuptake inhibitors
L-AADC	= L-aromatic amino acid decarboxylase
MAOA	= monoamine oxidase A
[^{3}H]	= tritium
KO	= knockout
ENU	= N-ethyl-N-nitrosurea
DNA	= deoxyribonucleic acid
mRNA	= messenger ribonucleic acid
A	= adenosine
C	= cytosine
G	= guanine
T	= thymine
U	= uracil

bp	= base pair
Gb	= gigabase
PCR	= polymerase chain reaction
-/-	= homozygous
l	= long
s	= short
IEF	= isoelectric focusing
pI	= isoelectric point
IPG	= immobilized pH gradient
DTT	= dithiothreitol
CHAPS	= 3- [(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonate
2-D	= 2-dimensional
Mr	= molecular weight
Da	= Dalton
kDa	= kilo Dalton
SDS	= sodium dodecyl sulfate
SDS-PAGE	= sodium dodecyl sulfate polyacrylamide gel electrophoresis
V	= volt

Vh	= voltage hours
MS	= mass spectrometer
ESI	= electrospray ionization
ESI-QUAD-TOF-MS	= electrospray ionization-quadropole-time-of-flight-mass spectrometer
HPLC	= high performance liquid chromatography
MASCOT	= matrix science search engine
MOWSE	= molecular weight search
NCBI	= National Center for Biotechnology Information
Na^+	= sodium
K^+	= potassium
Cl^-	= chloride
SLC1/6	= sodium chloride coupled transporter 1/6
LSD	= lysergic acid diethylamide
ATP	= adenosine triphosphate
Hsp	= heat shock protein

List of Tables

		Page
Table 1 :	Disability Adjusted Life Years (DALYs) of psychiatric disorders by region	2
Table 2 :	Principles of a mass spectrometer	28
Table 3 :	Differentially expressed cytosolic proteins of ventral hippocampus as identified by SwissProt. The SSP number as assigned by PD Quest software.	33 - 35
Table 4 :	Differentially expressed cytosolic proteins of dorsal hippocampus as identified by SwissProt. The SSP number as assigned by PD Quest software.	36 - 37
Table 5 :	Functionally grouped cytosolic proteins of ventral hippocampus.	38 - 40
Table 6 :	Functionally grouped cytosolic proteins of dorsal hippocampus.	41 - 42
Table 7 :	Differentially expressed membrane proteins of ventral and dorsal hippocampus as identified by SwissProt. The SSP number as assigned by PD Quest software.	43 - 44
Table 8 :	Functionally grouped membrane proteins of ventral and dorsal hippocampi.	45 - 46

List of figures

		Page
Figure 1	**:** The synthetic and metabolic pathways of serotonin	10
Figure 2	**:** Schematic representation of the serotonin transporter and location of the ENU-induced mutation	16
Figure 3	**:** The standard curve of a Bradford protein determination	22
Figure 4	**:** The standard curve of a *RC DC* assay protein determination	23
Figure 5		
(a)	**:** A gel image of a test sample run on an IPG strip of pH 3 - 10	24
(b)	**:** A gel image of a test sample run on an IPG strip of pH 5 – 8	24
Figure 6	**:** A flow diagram of a proteomic experiment	25
Figure 7	**:** The basic components of a mass spectrometer	27
Figure 8	**:** An interface of the search engine MASCOT	30
Figure 9	**:** An example of a Gaussian curve generated by MASCOT	31
Figure 10		
(a)	**:** Gel image of a ventral hippocampal cytosolic fraction of the wild type	47
(b)	**:** Gel image of a ventral hippocampal cytosolic fraction of the knockout	47
Figure 11		
(a)	**:** Gel image of a dorsal hippocampal cytosolic fraction of the wild type	48
(b)	**:** Gel image of a dorsal hippocampal cytosolic fraction of the knockout	48

Figure 12

(a) : Gel image of a ventral hippocampal membrane fraction of the wild type 49

(b) : Gel image of a ventral hippocampal memrane fraction of the knockout 49

Figure 13

(a) : Gel image of a dorsal hippocampal membrane fraction of the wild type 50

(b) : Gel image of a dorsal hippocampal membrane fraction of the knockout 50

Figure 14

(a) : An example of a differentially expressed protein spot in the cytosolic fraction of the ventral hippocampus of the wild type 51

(b) : An example of a differentially expressed protein spot in the cytosolic fraction of the ventral hippocampus of the knockout 51

Figure 15 : A Venn diagram illustrating cytosolic proteins identified in the ventral and dorsal hippocampal regions 52

Figure 16 : A Venn diagram illustrating membrane proteins identified in the ventral and dorsal hippocampal regions 53

Figure 17 : A Venn diagram illustratring the distinct and common proteins between the cytosolic and membrane fraction. 54

Table of Contents

	Page
Declaration	i
Abstract	ii - iii
Opsomming	iv - v
Acknowledgements	vi
List of abbreviations	vii - x
List of tables	xi
List of figures	xii - xiii
Prologue	1
Chapter 1	
Introduction	2 - 19
1.1 Global burden of diseases	2
Table 1: Disease burden of psychiatric disorders by region	2
1.2 Noncommunicable disease in South Africa	3
1.3 Depression	3
1.3.1 Disease of malignant sadness	3 – 4
1.3.2 Types of depression	4
1.3.3 Epidemiology, genetics and etiology in brief	4 – 5
1.3.4 Hypotheses of depression	5
1.3.4.1 Psychodynamic theories	5 – 6
1.3.4.2 Biological theories	6

1.3.5 Contemporary models of depression 6
1.3.5.1 Dysregulation model 6 - 7
1.3.5.2 Neuroendocrine hypothesis 7
1.3.5.3 Gluccocorticoid cascade hypothesis 7
1.3.5.4 The biogenic/monoamine hypothesis 7
1.3.5.4.1 The monoamine neurotransmitters 7 – 8
1.3.5.4.2 Regulation of chemical transmission 8

1.3.6 The central serotonergic system 8- 9
1.3.6.1 5HT synthesis/metabolism 9
Figure 1: Serotonin synthesis and degradation 10

1.3.7 Genes 10

1.3.8 The neurotransmitter transporters 10 – 11
1.3.8.1 Serotonin transporter 12 – 13
1.3.8.2 5-HTTLPR variants 13 – 14

1.3.9 Knockout technology 14
1.3.9.1 Knockout mice 14 - 15
1.3.9.2 Knockout rats 15 – 16
Figure 2: Schematic representation of SERT gene and location of induced knockout mutation 16

1.3.10 Genome opposed to proteome 17

1.3.11 Proteomics 17 – 18
1.3.12 The current study 18 – 19

Chapter 2
Materials and Methods 20 - 31

2.1 Animals 20

2.2 Study design 20 - 21

2.3 Tissue collection 21

2.4 Sample preparation for 2-D gel electrophoresis 21
2.4.1 Protein extraction 21 – 22
2.4.2 Protein determination 22
Figure 3: Bradford standard curve 22
2.4.3 Cleanup 23
2.4.4 *RC DC* protein assay 23
Figure 4: *RC DC* standard curve 23
2.4.5 Global protein expression profile 24
Figure 5: Global protein expression profile 24

Figure 6: Proteomic experimental work flow 25

2.5 Proteomics 26
2.5.1 Isoelectric focusing 26
2.5.2 2-D gel electrophoresis 26 – 27
Figure 7: Components of a mass spectrometer 27
Table 2: Principle of mass spectrometry 28
2.5.3 Mass Spectrometer 27 – 29
2.5.4 Data analysis and bioinformatics 29 - 31
2.5.4.1 Protein sequence determination 29
Figure 8: Search engine (MASCOT) interface 30
Figure 9: Gaussian graph generated by MASCOT 31

2.6 Statistical analysis 31

Chapter 3
Results 32 – 54

3.1 Summary of results obtained 32

Table 3: Cytosolic proteins identified in ventral hippocampus 33 - 35

Table 4: Cytosolic proteins identified in dorsal hippocampus 36 - 37

Table 5: Functionally grouped cytosolic proteins of ventral hippocampus 38 - 40

Table 6: Functionally grouped cytosolic proteins of dorsal hippocampus 41 - 42

Table 7: Membrane proteins identified in ventral and dorsal hippocampi 43 - 44

Table 8: Functionally grouped proteins of ventral and dorsal hippocampi 45 - 46

Figure 10: Gel images of ventral hippocampus cytosolic fraction 47

Figure 11: Gel images of dorsal hippocampus cytosolic fraction 48

Figure 12: Gel images of ventral hippocampus membrane fraction 49

Figure 13: Gel images of dorsal hippocampus membrane fraction 50

Figure 14: Gel images of a specific annotated protein spot 51

Figure 15: Venn diagram of cytosolic proteins in hippocampal regions 52

Figure 16: Venn diagram of membrane proteins in hippocampal regions 53

Figure 17: Venn diagram of proteins identified in cytosolic and membrane fractions 54

Chapter 4

Discussion and Conclusion 55 – 66

4.1 Introduction 55

4.2 Aims of study 55

4.3 Characterization of the proteomic technique 55 - 57

4.4 Differentially expressed proteins in rat hippocampus 58 - 65

4.5 Conclusion 65

Epilogue 66

References

Prologue

Ten years before the fateful night, of 18 July 1965, when she walked into the sea at Three Anchor Bay, Afrikaans poet Ingrid Jonker wrote these prophetic words:
“My lyk lê uitgespoel in wier en gras
op al die plekke waar ons eenmaal was”/
“My corpse lies washed up in grass and wrack
wherever memory should call us back”

She was 31.

It is well known that Jonker had a long history of mental illness. Close friends suggesting that she suffered from major depression; others saying that if she were alive today, would now have been diagnosed with bipolar disorder. In the fifties and sixties, mental illness was still taboo and highly stigmatised, with little being done to educate the public in this area. Jonker was most probably treated with the old tricyclic antidepressants and records show she received electro-convulsive therapy (ECT). Unfortunately, plagued by silent suffering, fear of stigma and rejection, these therapies were not enough.

The modern day diagnostic system is immensely more refined than in Jonker’s time. Being developed around that time was the Diagnostic and Statistical Manual of Mental Disorders (DSM), which clinicians refer to when, making a diagnosis. A greater variety of psychiatric medication and more specific treatments are also available. However, even with the advent of newer and better antidepressants, relapses still occur.

Greater understanding of the interrelationship between hormonal, metabolic and molecular intracellular signaling pathways involved in psychiatric conditions may provide answers toward the inexplicable mechanisms of antidepressants.

CHAPTER 1
INTRODUCTION

1.1 Global burden of diseases

A single measure allowing comparison of the burden of disease across many different conditions, by including both death and disability, was developed for the Global Burden of Disease study. This measure was called Disability Adjusted Life Years (DALYs) (Table 1). Therefore, DALYs measure lost years of healthy life whether owing to premature death or disability with the disability component weighted for severity of the disability. As illustration, disability caused by major depression was found to be equivalent to blindness or paraplegia whereas active psychosis seen in schizophrenia produces disability equal to quadriplegia (Sanderson & Andrews, 2000). A Global Burden of Disease study conducted by the World Health Organization (WHO), the World Bank, and Harvard University, revealed that mental illness, including suicide, accounts for over 15 percent of the burden of disease in established market economies, such as the United States (WHO, 2002 b), being greater than the disease burden caused by all cancers. Schizophrenia, bipolar disorder, obsessive-compulsive disorder, panic disorder, and post-traumatic stress disorder also contributed significantly to the total burden of disease attributable to mental disorders. With the aging of the world population and the conquest of infectious diseases, psychiatric and neurological conditions are projected to increase their share of the total global disease burden by almost half, estimated from 10.5 percent of the total burden to almost 15 percent in 2020 (Murray & Lopez, 1996).

Table 1

Disease Burden of Selected Major Psychiatric Disorders, By Region, 2001

	DALYs Lost Annually per One Million Population			
Region	**Schizophrenia**	**Bipolar Disorder**	**Depression**	**Panic Disorder**
Sub-Saharan Africa	1,716	1,803	4,905	777
Latin America and the Caribbean	2,049	1,678	9,919	777
Middle East and North Africa	2,247	1,830	6,544	852
Europe and Central Asia	1,630	1,400	8,944	713
South Asia	2,087	1,612	10,507	779
East Asia and the Pacific	2,126	1,685	7,594	757
High-income countries	1,201	1,137	9,054	577
World	1,894	1,583	8,431	740

Table 1: Tabled here are the DALYs lost per year for a population of one million for selected psychiatric disorders: schizophrenia, bipolar disorder, depression, and panic disorder. While these disorders are not significant causes of mortality, they account for a substantial proportion of the global disease burden. *Source: Disease Control Priorities in Developing Countries, second edition, 2006, Table 31.1*

1.2 Noncommunicable disease in South Africa

Infectious diseases remain responsible for the largest burden of disease in sub-Saharan Africa, but noncommunicable diseases (NCDs) are becoming a significant burden as well. Examples of NCDs include depression, which in conjunction with cardiovascular diseases and diabetes, are responsible for a large proportion of death and disability in Eastern, Central and Southern Africa (ECSA).

Current figures, as released by the South African Society of Psychiatrists, on people suffering from a psychiatric disorder, estimates that between four and a half to five million in South Africans are affected (SADAG, 2007). Additionally, if alcohol and drug abuse are included, this figure rockets to an alarming 15 million people. Further estimates reveal that 20 % of South African children suffer from a mental illness due to the levels of violence and family problems experienced, eventually resulting in a quarter of the entire population suffering from a depressive disorder. Statistics also show that 50 % of visits to general practitioners are usually due to some type of mental problem.

Psychiatric illness is costing the country millions of Rands each year in lost working days and lowered productivity, in addition to the resultant immense human suffering. A significant contribution toward this detrimental fact is that about ten thousand young people commit suicide yearly, most of whom are economically active. A study done on a rural, so-called coloured, Western Cape community, from which 481 adults were randomly selected, found 27.1 % suffered from a mental illness, with depressive and anxiety disorders identified as the most common (MHIC, 2007). The results of a recent South African survey showed that as many as 42 % of people with mental illness did not disclose their status to family members. Furthermore, 22 % of the sample population had not told their partners. Stigma, surrounding mental illness, remains the single most significant reason why people do not seek help or do not receive appropriate care.

1.3 Depression

1.3.1 Disease of malignant sadness

Depression is perhaps the most common of all mental illnesses, currently felt to affect one in every four adults to some degree. This pathological mood state is characterized by persistent negative emotions and thoughts such as irritability, sadness, and apprehensiveness, which coexist with alterations in motivation, sleep, energy, appetite, and libido; possibly resultant from neurochemical disturbances (DSM IV, 2000).

When this diagnosis is present, the individual will exhibit at least five of the following symptoms during the depressive periods:

- Depressed mood, most of the day or every day
- Markedly diminished interest in all or almost all activities
- Significant weight loss or gain or appetite disturbance
- Insomnia or excessive sleeping
- Psychomotor agitation or retardation (restlessness)
- Low energy level or chronic tiredness
- Feelings of inadequacy, loss of self-esteem, and/or self-deprecation
- Decreased attention, concentration, or ability to think clearly
- Recurrent thoughts of death or suicide, an expressed desire to be dead

1.3.2 Types of depression

There are three main types of depressive disorders: major depressive disorder, dysthymic disorder, and bipolar disorder (manic-depressive illness). Major depression is the leading cause of disability (measured by the number of years lived with a disabling condition) in the U.S. and worldwide among persons age five and older (Murray & Lopez, 1996). In epidemiological surveys depression has been, consistently, found to be highly comorbid with other mental disorders; generally strongest with anxiety disorders e.g. generalized anxiety disorder and panic disorder (Kessler et al., 1996).

1.3.3 Epidemiology, genetics and etiology in brief

Epidemiological and pathological studies indicate a role for both genetic and environmental factors in the etiology and pathogenesis of these disorders, but specific disease-associated genes or pathogenic agents have not as yet been identified. There are, at present, no biological or pathological markers which are highly associated with specific psychiatric disorders.

Lifetime prevalence for major depressive disorder is estimated at 10 % for men, and 20 % for women. This contrasts with only a 1 % prevalence for bipolar disorder (Frude, 1998). Evidence exists that both major depressive and bipolar disorders are genetically linked. Twin, adoption, and family studies indicate that genetic factors contribute substantially to the liability for developing both acquired factors in disease expression (Drevets, 2003). For example in monozygotic, or identical, twins, there was a concordance rate of 54 – 65 % for major depressive disorder, with the concordance rate even higher in bipolar disorder, at

79 %. A concordance rate of only 14 – 19 % percent in dizygotic or fraternal twins were found in both cases. This clearly showed a genetic link for both major depressive and bipolar disorders (Frude, 1998; Gershon, 1990). Additionally, there is evidence of a cross-link between bipolar and major depressive disorders, as of the 32 monozygotic twins who were positively concordant for an affective illness, 11 were major depressive, 14 were bipolar, and 7 had one twin major depressive and the other bipolar (Gershon, 1990). Gershon (1990) further gives evidence against a purely "nurture" hypothesis for affective disorders through a review of literature regarding adoptees. Of adoptees with affective disorders, their adoptive parents had an affective disorder only 12 percent of the time, while the biological parents manifested an affective disorder 29 %. This study therefore showed that environmental factors may also have an influence on the development of affective disorders.

The etiology of major depressive disorder remains unclear. The function of the hypothalamic-pituitary-adrenal axis (HPA) is abnormal in a substantial proportion of major depressive disorder cases. Severe depression has been associated with increased cerobrospinal fluid (CSF) levels of corticotropin releasing factor (CRF), a blunted adrenocorticotropin hormone (ACTH) response to CRF administration, hypersecretion of ACTH and cortisol, pituitary and adrenal gland enlargement, and reduced sensitivity to negative feedback inhibition of cortisol secretion (Plotsky et al, 1998; Wong et al, 2000). In addition, it has been shown that post-mortem suicide victims have decreased CRF receptor density in the frontal cortex along with reduced pituitary messenger ribonucleic acid (mRNA) levels indicative of chronic HPA axis activation (Heim et al., 1997). Major depressive disorder has also been associated with abnormalities in serotonergic, dopaminergic, noradrenergic, cholinergic, gamma-aminobutyric acid (GABA), and peptidergic function (Sachar & Baron, 1978). The serotonin system has received particular interest, as selective serotonin reuptake inhibitors (SSRIs) exert antidepressant effects, and other antidepressant drugs also increase serotonin transmission (Guelfi et al., 1995; Benkert et al., 1997).

1.3.4 Hypotheses of depression

1.3.4.1 Psychodynamic hypotheses

Rudimentary, supernatural explanations for mental illness prevailed until the early part of the 20th century. However, with the emergence of the psychoanalytic theory of Sigmund Freud, an emphasis on early life trauma in the development of adult psychopathology was born (Freud, 1895). Furthermore, the term “psychobiology” as utilized by Adolf Meyer, emphasized the importance of the interaction between genetic factors and life events in the causation of mental illness (Jackson, 1986). Approximately 50 years ago, scientists

developed successful psychopharmacologic medications to treat severe depression, ultimately paving the way for the development of biological theories of mood disorders.

1.3.4.2 Biological hypothesis

The biogenic amine hypothesis of depression was posited, virtually simultaneously, in the 1960s, by researchers from the United States and Europe. This concept held that depression was caused by a deficiency in the catecholeamine, norepinephrine (NE), and/or the indoleamine, serotonin (5HT). Implying that the biochemical alterations of these monoamine systems are genetically determined (Akiskal, 1995), thus minimizing any possible environmental influence. This model provides important associations with the clinical phenomena of, and the pharmacologic treatments employed in, mood disorders. However, it is now known from subsequent research, that mere deficiency of the biogenic amines is insufficient for the development of depression (Delgado et al., 1999). Further evidence against the biogenic amine hypothesis, includes the fact that traditional antidepressant medications, which primarily target norepinephrine and/or serotonin neurons, are ineffective in approximately 40 % of patients with major depression (Schatzberg, 2000). In addition to the abovementioned, the following observations reinforce the argument against this hypothesis: tianeptine, an antidepressant, enhances serotonergic uptake, while still retaining antidepressant efficacy (Wagstaff et al., 2001), and a clear discrepancy exists between the synaptic monoamine levels and therapeutic effect (Yamada & Yamada, 2007).

1.3.5 Contemporary models of depression

Other non-monoamine neurochemical systems have been demonstrated to likely play a role in the etiology and treatment of depression. Patients diagnosed with certain mood and anxiety disorders show symptoms of a dysregulated HPA axis in conjunction with dysregulated neurotransmitter systems. Various immune system components, such as cytokines, and the neuropeptide neurotransmitters, such as corticotropin-releasing factor (CRF), thyrotropin-releasing hormone (TRH), somatostatin, and growth hormone releasing factor (GHRF) have been implicated. These latter discoveries have served as a force for researchers to seek alternative rational treatment modalities based on these non-monoamine neurotransmitter systems; holding promise for improved therapeutic response with more favourable side-effect profiles.

1.3.5.1 Dysregulation Model

The dysregulation model of depression, as proposed by Siever and Davis (1985), suggests that depression is due to inappropriate (i.e. less selective) environmental responsiveness, and defective habituation (i.e. a slower return to baseline functioning following a

perturbation). These authors believe that this is due to chronic abnormality within the pattern and degree of responsiveness of a neurotransmitter to a stressful situation.

1.3.5.2 Neuroendocrine Hypothesis

The neuroendocrine hypothesis is seen as secondary to other pathology - that occurring within the neurotransmitters. The hormones within the neuroendocrine system are largely secreted in response to neurotransmitter function, and thus, while they may exact a primary effect in the affective disorders, they must be viewed as being "downstream" from the root cause of the problem.

1.3.5.3 Glucocorticoid Cascade Hypothesis

According to abovementioned hypothesis, glucocorticoid overproduction is driven indefinitely by chronic stressors. Overproduction of glucocorticoids has been suggested to cause HPA axis hyperactivity and subsequent brain abnormalities that include hippocampal volume reductions (Sapolsky et al, 1986a). However, insufficient signaling of glucocorticoids has also been implicated in the pathology of stress-related disorders (Riason & Miller, 2003).

1.3.5.4 The Biogenic/Monoamine Hypothesis

The general tenet of the monoamine hypothesis is, that a functional deficit of monoamines, particularly serotonin and norepinephrine, in the neurotransmitter synapses, cause depression. Suggesting that dysfunctional, deficient neurotransmission of these monoamines underlie the symptoms of depression. The biogenic amine hypothesis has been the cornerstone of research on depression for more than 30 years.

1.3.5.4.1 The monoamine neurotransmitters

Neurotransmitters are endogenous substances that are released from neurons, act on receptor sites that are typically present on membranes of postsynaptic cells, and produce a functional change in the properties of the target cell. Before a substance is designated a neurotransmitter, the following criteria should be met:

- A neurotransmitter must be synthesized by and released from neurons.
- The substance should be released from nerve terminals in a chemically or pharmacologically identifiable form.
- A neurotransmitter should reproduce at the postsynaptic cell the specific events that are seen after stimulation of the presynaptic neuron.
- The effects of a putative neurotransmitter should be blocked by competitive antagonists of the transmitter in a dose-dependent manner.

- In addition treatments that inhibit synthesis of the transmitter candidate should block the effects of presynaptic stimulation.
- There should be active mechanisms to terminate the action of the putative neurotransmitter i.e. reuptake of the substance into the presynaptic neuron or glial cells through specific transporter molecules, or enzymatic inactivation.

1.3.5.4.2 Regulation of chemical transmission

In the brain messages are passed between two nerve cells via a synapse. The presynaptic cell sends information, via neurotransmitter release, into the synapse. After they have been released at the synapse, neurotransmitters activate pre- and/or postsynaptic receptors. Once inside the neuronal cell, neurotransmitters can be further transported into synaptic vesicles by vesicular carriers; these processes being responsible for the homeostasis of neurotransmitter pools within nerve endings. The neurotransmitter is then recognized by receptors on the surface of the postsynaptic cell, which upon this stimulation relays the signal. About 10 % of neurotransmitters are lost during this process, the remaining 90 % are released from the receptor and taken up again by monoamine transporters into the presynaptic cell i.e. reuptake. Chemically mediated transmission, by the activity-dependent secretion of neurotransmitters at synapses, is the major mode of neuronal communication. Neurotransmitter actions may be terminated actively or passively. Among the active mechanisms are reuptake of the neurotransmitter through specific transporter proteins on the presynaptic neuron and/or glial cells and enzymatic degradation to an inactive substance (Iversen, 1975). Consequently, the plasma membrane transporters are an important determinant of extracellular neurotransmission. In the central nervous system (CNS), monoaminergic neurotransmission supports major physiological and behavioural functions, including locomotor activity, reward, attention and mood. Therefore, those factors which alter or modulate the activity of transporters at the cell surface are critical factors in regulating monoaminergic neurotransmission.

1.3.6 The central serotonergic system

Serotonin was first isolated in 1933; a rather slow-acting neurotransmitter that is associated with sleep, appetite, energy, alertness, and mood. It's an intermediate product of tryptophan metabolism located primarily in the enterochromaffin cells of the intestine, serotonergic neurons of the brain, and blood platelets. Interestingly, the brain accounts for only about 1 % of body stores of 5HT. The major site of serotonin cell bodies is found in the upper pons and midbrain, specifically the median and dorsal raphe nuclei; these neurons project to the basal ganglia, limbic system and cerebral cortex (Sadock & Sadock, 2007); with the midbrain raphe nuclei to limbic hippocampal/amygdala path being the most important (Janicak et al,

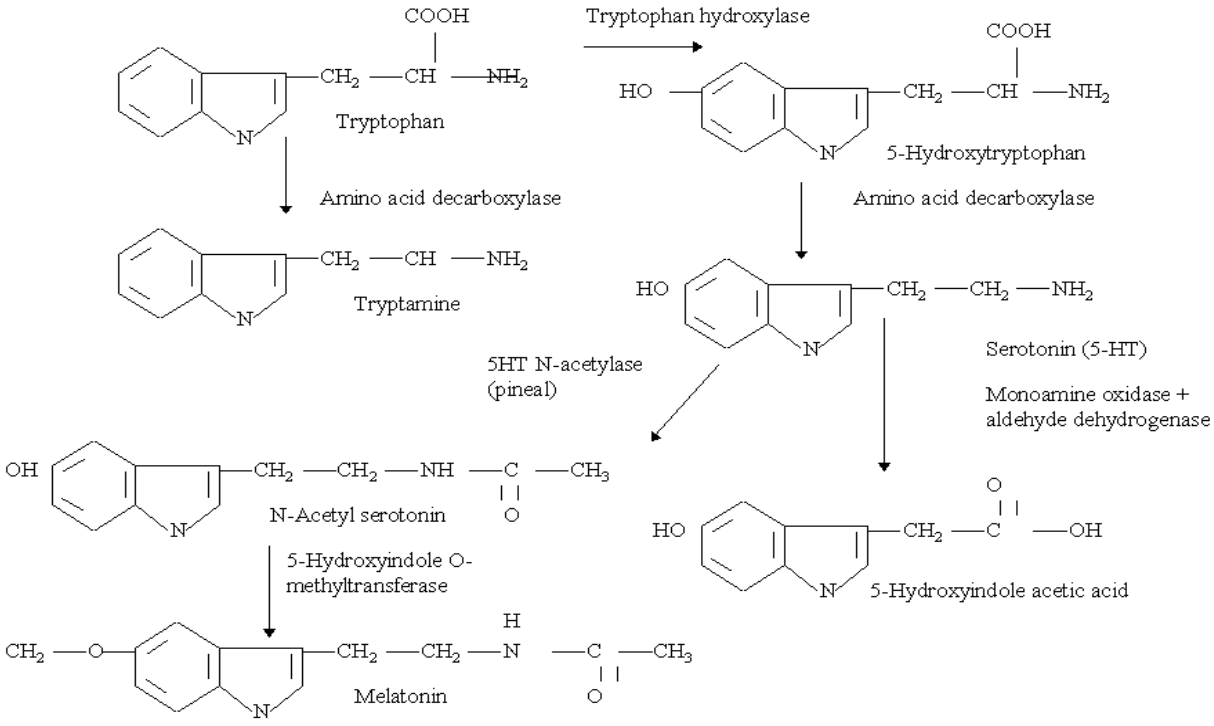
1993). The dorsal and ventral hippocampus receive serotonin projections predominantly from the median raphe nucleus and dorsal raphe nucleus, respectively. The finding that the chemical structure of 5HT is similar to that of Lysergic Acid Diethylamide (LSD) led theories that associated abnormalities in 5HT function to schizophrenia and depression. The physiological actions of 5HT are mediated by different types of receptors, but terminated by a single 5HT transporter (SERT) (Blakely et al., 1991; Hoffman et al, 1991; Lesch et al, 1993; Ramamoorthy et al, 1993).

1.3.6.1 5HT synthesis/metabolism

The basic process of 5HT biosynthesis involves the peripheral amino acid, tryptophan, gaining entry into the brain and its metabolism in serotonergic neurons via a series of enzymatic steps. This amino acid normally circulates in the blood at low concentrations and is converted to serotonin in the brain, specifically in the raphe nuclei (Siegel et al., 1999). Tryptophan in the neuron is hydroxylated/metabolized sequentially by tryptophan hydroxylase. Tryptophan hydroxylase is the rate-limiting step in 5HT synthesis, giving rise to the 5HT precursor 5-hydroxytryptophan (5-HTP), which is then decarboxylated by L-aromatic amino acid decarboxylase (L-AADC) to yield serotonin (Cooper et al, 1996). 5HT is accumulated by the vesicular monoamine transporter (VMAT). When released, 5HT can interact with both postsynaptic receptors and presynaptic autoreceptors. 5HT is taken up by the high-affinity 5HT transporter (SERT), and once inside the neuron it can be reaccumulated by vesicular transporter or inactivated metabolically/oxidized by monoamine oxidase A (MAOA) and other enzymes. Metabolic degradation of 5HT proceeds first through monoamine oxidase, which is covalently modified with a flavin cofactor, and then through aldehyde dehydrogenase, which uses a solution-phase nicotinamide cofactor (Wise & Shear, 2006). The intermediate in this catabolism, 5-hydroxyindole-3-acetaldehyde, typically is degraded rapidly in vivo to the primary metabolite 5-hydroxyindole-3-acetic acid (5-HIAA) (Wise & Shear, 2006).

Figure 1

Serotonin synthesis and degradation



The synthetic and metabolic pathways of serotonin. The amino acid tryptophan is hydrolyzed by the enzyme tryptophan hydroxylase, resulting in 5-hydroxytryptophan which is further metabolized by L-amino acid decarboxylase into serotonin. *Source: Cooper J.R., Bloom F.E., Roth R.H. The biochemical basis of neuropharmacology, (1996) p.355, 7th Edition New York, Oxford University Press.*

1.3.7 Genes

Risk for mood and anxiety disorders has been found to be partly genetic (Kendler, 2001). Gene mutations and polymorphisms in proteins that regulate the brain monoamine systems, such as transporters and catabolic enzymes, are attractive candidate genes for emotional disorders given the weight of evidence implicating monoamines in these conditions.

1.3.8 The neurotransmitter transporters

The transmembrane transport of neurotransmitters is of fundamental significance for proper signaling between neurons. Transport processes are mediated by distinct classes of membrane transport proteins that have key roles in controlling the neurotransmitter concentrations in the synaptic cleft. These neurotransmitter transporters are plasma membrane proteins that take up extracellular neurotransmitters after release and thereby

terminate the transmitters' action at extracellular receptor sites. They represent the first step in the process of transmitter recycling. Transporters can be classed as intracellular vesicular transporters that are responsible for sequestering transmitters from the cytoplasm into synaptic vesicles, and plasma membrane transporters that are responsible for sequestering released transmitters from extracellular space. The two major subclasses of transmembrane/plasma membrane transporters are; the high-affinity glutamate-transporters (SLC1 gene family) (Kanai & Hediger, 2004) and the sodium-chloride (Na^+-Cl^-)-coupled transporters (SLC6 gene family) (Chen et al 2004). The latter subclass is the largest and includes the dopamine transporter (DAT), serotonin transporter (SERT) (Saier, 1999), norepinephrine transporter (NET), Glycine and gamma-aminobutyric acid (GABA) transporters (Chen et al, 2004). All neurotransmitter transporters are polytopic membrane proteins that mediate transmembrane ion-coupled secondary active transport and an internal negative membrane potential for transport of their substrate neurotransmitters (Rudnick & Clark, 1993). Hence SLC1/6 transporters operate as Na^+-dependent co-transporters that use the transmembrane Na^+ gradient to couple 'downhill' transport of Na^+ with 'uphill' transport (i.e. against a concentration gradient) of their substrate from the extracellular to the intracellular environment (Chen et al, 2004; Kanai & Hediger, 2004).

Thus the primary mechanism for termination of monoaminergic neurotransmission, is mediated through rapid reuptake of the released neurotransmitter back into the presynaptic terminals by high-affinity transporters; with reuptake transporters usually being located on the presynaptic nerve terminals or surrounding glia (Vialou et al, 2006). The importance of the SLC6 transporter family is highlighted by the fact that they are major targets for psychostimulants, antidepressants and gates for certain neurotoxins (Giros & Caron 1993; Torres et al, 2003; Chen et al., 2004). These psychoactive drugs act to slow the transport activity of the neurotransmitters, hence slowing the removal of released neurotransmitters from the synapse.

Furthermore, it has been shown that naturally occurring mutations in the transporter genes cause, or increase, the risk of developing certain diseases. Mutations in the gene encoding SERT are associated with symptoms of obsessive-compulsive disorder, Ausperger's syndrome, anorexia and autism (Kilic et al, 2003; Sutcliffe et al, 2005). Therefore comprehensive knowledge of these proteins can promote therapeutic intervention in the treatment of numerous psychiatric disorders, such as depression, anxiety and perhaps even schizophrenia as well as drug abuse.

1.3.8.1 Serotonin transporter

The plasma membrane SERT belongs to a family of proteins with 12 putative transmembrane domains (Amara & Kuhar 1993). SERT selectively transports 5-hydroxytryptamine (5HT) into nerve cells together with Na^+ and Cl^- and, in the same reaction, transports a potassium (K^+) ion out of the cell. Thus the sodium gradient drives active reuptake of released 5HT. These proteins have been shown, in the adult human brain, to be localized/distributed not only on synaptic terminals but to a great extent on axon terminals as well as on cell bodies and dendrites (Zhou et al, 2000; Blakely et al 1994, 2006; Vialou et al, 2006).

SERT catalyzes an intricate reaction incorporating both symport and antiport transport mechanisms. In 5HT transport, SERT binds Na^+, Cl^- and 5HT in a 1:1:1 stoichiometry, after which it undergoes a conformational change that occludes the binding site from the extracellular medium and exposes it to the cytoplasm (Nelson & Rudnick, 1979). However, only after binding a cytoplasmic K^+ ion and releasing it to the extracellular medium, can dissociation of Na^+, Cl^- and 5HT allow the transporter to return to its original conformation. Therefore the overall stoichiometry of this process is a 1:1:1:1 electroneutral exchange of K^+ with Na^+, Cl^- and 5HT (Rudnick & Nelson, 1978; Talvenheimo et al, 1983; Rudnick, 1998). The studies that provided the original evidence for this mechanism utilized platelet plasma membrane vesicles (Rudnick, 1977).

The SERT is the primary target for therapeutic intervention in the treatment of numerous psychiatric disorders, such as depression, anxiety and even schizophrenia; specifically being the target of antidepressant drugs such as tricyclics and the selective serotonin reuptake inhibitors (SSRIs) (Thomas et al, 1987; Dechant & Clissold, 1991), which enhance 5HT neurotransmission by blocking reuptake and thereby increasing extracellular 5HT levels (Invernizzi et al, 1995). Amongst the variety of compounds that inhibit SERT are: fluoxetine (Prozac), sertraline (Zoloft), paroxetine (Paxil) and citalopram (Celexa). However, at the structural level this protein's function as a transporter in membranes remains incompletely understood.

The human SERT gene contains a genetic polymorphism with allelic variation in transcriptional activity and protein expression. Although this genetic variant does not directly affect amino acid sequence, it can alter SERT expression (Bradley et al, 2005; Lesch et al, 1996). The common polymorphism in SERT gene 5'-regulatory promoter region (SERT gene-linked polymorphic region – 5-HTTLPR) comprises a tandemly repeated sequence of guanine cytosine (GC)-rich, 20 – 23 base pairs (bp) long repeat elements. 5-HTTLPR

genetic variants have been associated with a variety of emotion-related behaviors and conditions, including shyness, aggression, anxiety, bipolar disorder, posttraumatic stress disorder, autism, and attention-deficit/hyperactivity disorder (Arbelle et al, 2005; Cho et al, 2005; Curran et al, 2005; Gerra et al, 2005; Lee et al, 2005; Yirmiya et al, 2001; You et al, 2005). Interestingly, population association studies demonstrated that the SERT gene promoter polymorphism accounts for 4 - 5 % of population variation of anxiety-related behavioural traits (Heils, 1997).Therefore the SERT gene is a promising candidate for assessing the heritability of interindividual variation in personality and the genetic susceptibility for various psychiatric diseases (Michaelovsky et al, 1999).

1.3.8.2 5-HTTLPR variants

The effect of 5-HTTLPR length variability on SERT function was determined by studying the relationship between 5-HTTLPR genotype, SERT gene transcription and 5HT uptake activity in human lymphoblastoid cell lines. A deltion/insertion in the 5-HTTLPR was first reported to create a short (s) allele and a long (l) allele (14- and 16- repeats, respectively), representing the composition of the majority of alleles (Nakamura et al, 2000). When fused to a luciferase reporter gene and transfected into human SERT expressing cell lines, the short (s) and long (l) 5-HTTLPR variants differentially modulate transcriptional activity of the SERT gene promoter (Lesch et al, 1996). Specifically, allele s, generated by a deletion of 44 bp involving repeat six to eight, reduced transcriptional efficiency, gene expression, and 5-hydroxytryptamine uptake and was associated with increased neurotiscism scores (Lesch et al, 1996, 1997; Delbrück et al, 1997). Further evidence from studies of SERT promoter activity in other cell lines (Mortensen et al, 1999), messenger ribonucleur acid (mRNA) concentrations in the raphe complex of human postmortem brain (Little et al, 1998), platelet 5HT uptake and content (Hanna et al 1998; Greenberg et al 1999; Nobile et al, 1999) and in vivo single-photon emission computed tomography (SPECT) imaging of human brain SERT (Heinz et al, 1999) confirmed that the s form is associated with lower SERT expression and function. The 5-HTTLPR s allele-associated impaired ability of the SERT for rapid 5HT clearance following release into the synaptic cleft may elicit acute increases of 5HT in the vicinity of serotonergic cell bodies and dendrites in the raphe complex and may exert a somatodendritic 5-HT1A receptor-mediated negative feedback that leads to an overall decrease of 5HT neurotransmission in patients with the 5-HTTLPR s allele. Furthermore, inheritance of the SERT-s allele has been associated with a heightened response of the limbic system to emotional stimuli (Hariri et al, 2005; Pezawas et al, 2005), elevated levels of subclinical depressive symptoms (Gonda et al, 2005), and an increased incidence of major depression and suicidal behavior (Hoefgen et al, 2005; Lin & Tsai, 2004).

In addition, 5-HTTLPR genetic variation is emerging as an important factor contributing to structural changes in the brain. A recent study done by Pezawas and colleagues (2005) found that normal, nonpsychiatric SERT-s carriers have a 25 % reduction in the volume of the anterior cingulate cortex and a 15 % reduction in the volume of the amygdala. Additionally the study identified functional alterations in limbic circuits in SERT-s carriers. Identification of anatomical changes in these limbic circuits raises the possibility that SERT-s carriers possess a unique brain structural phenotype predisposing them to depression and related disorders.

1.3.9 Knockout Technology

The search for a single, defective gene responsible for each mental illness has given way to the current understanding that multiple gene variants, acting together with yet unknown environmental risk factors or developmental events, account for the expression of psychiatric disorders. However, identifying these genes has proven extremely difficult. One of the most recent and effective means of determining gene function is by a targeted knockout (KO) of the gene.

Target-selected mutagenesis, also known as gene-driven mutagenesis or targeting induced local lesions in genomes (TILLING), is a powerful approach to manipulate a genome and its coding capacity permanently. A specific gene is rendered nonfunctional, for example if this technique is applied to the SERT, it results in a reduced capacity to remove 5HT from the extracellular space following release. In some respects SERT KO, models native genetic variation in SERT expression recently discovered to occur in the normal human population. In these, reduced SERT expression is linked to an increase in anxiety-related personality traits (Li, 2006).

1.3.9.1 Knockout mice

Elucidation of the murine SERT gene sequence allowed the generation of an animal model with targeted disruption of this gene by homologous recombination (Bengel et al, 1998). This generation of stable knockouts, by homologous recombination in embryonic stem cells, as is common for the mouse (Smits & Cuppen, 2006), is not available for most higher organisms, including the rat, because of the lack of pluripotent embryonic stem cells.

The relation between the plasma 5HT level and the uptake ability of SERT has been demonstrated using SERT knockout mice (Bengel et al, 1998), polymorphisms in the SERT-linked promoter region, and SERT inhibitors (Heils et al, 1996; Lesch et al, 1996; Johnson et al, 2003). Mice lacking the SERT exhibit highly elevated extracellular concentrations of 5HT

(Bengel et al, 1998) and induces several behavioural phenotypes such as reduced aggression (Holmes et al, 2002a), and altered responses to drugs of abuse (Bengel et al, 1998; Sora et al, 1998). Of interest is the fact that SERT knockout mice develop anxiety and depression-like adult behavioral phenotypes despite the presence of high levels of 5HT throughout development and into adulthood (Holmes et al, 2002b, 2003; Lira et al, 2003; Adamec et al, 2006). Deletion of exon 2 results in an inactive gene and complete absence of 5HT reuptake activity in homozygous (-/-) SERT mice.

1.3.9.2 Knockout rats

The rat is one of the most important model organisms for biomedical and pharmacological research. A three-way comparison of the rat sequence with the human and mouse genomes has revealed immense new information about the mammalian genome evolution. The rat genome of 2.75 gigabases (Gb) is smaller than the human genome (2.9 Gb) but larger than that of the mouse (2.6 Gb). Global comparison of the three genomes reveals large chromosomal regions, referred to as orthologous chromosomal segments, which have been inherited with negligible rearrangement of gene order from the primate to rodent ancestor (Mullins & Mullins, 2004). Therefore studies done on knockout rodents can be extrapolated to humans, because of the conservation of certain genes.

In the rat, the knockout approach starts with random mutagenesis of the male germline by intraperitoneal injection of the small chemical and mutagenic agent N-ethyl-N-nitrosurea (ENU), which introduces random deoxyribonucleic acid (DNA) damage that becomes fixed as point mutations in the DNA during point spermatogenesis (Zan et al, 2003). ENU is a powerful alkylating agent; preferentially alkylating adenosine tymine base pairs. After a period encompassing a full round of spermatogenesis (~50 – 70 days) mutagenised males are mated with untreated females to generate a large population of F1 progeny that harbour many random heterozygous point mutations in their genomes. Interestingly mutants are outcrossed and bred to homozygosity (Smits & Cuppen, 2006). Next, DNA is extracted from each F1 individual, which is then subsequently screened for induced mutations in the coding or regulatory regions of genes of interest. By assessing mutation rates at a number of visible marker loci, it has been estimated that ENU can induce functional mutations at gene loci at the rate of approximately one per 1 000 genomes. Although this assumption has been upheld in some cases, actual per locus mutation rates can vary from one in 300 genomes to almost one in 5 000 genomes screened. These exonic mutations could be silent, change an amino acid (misense), or induce a premature stop codon (nonsense).

ENU-driven target-selected mutagens is therefore the systematic generation of knockout and protein function altering alleles in the rat; with the resulting induced rat models being powerful tools for studying many aspects of a wide variety of human diseases (Smits et al, 2006). SERT knockout rats (SLC6A4^{1Hubr}) are generated by target-selected ENU-induced mutagenesis (Smits et al, 2006); this model results from a single point mutation which introduces a premature stopcodon. The stop codon is any of 3 mRNA nucleotide sequences thymine/uracil guanine adenosine, thymine/uracil adenosine guanine, thymine/uracil adenosine adenosine (T/UGA, T/UAG, T/UAA) that do not code for an amino acid and thus signal the end of translation, ultimately the end of protein synthesis. With the introduction of a characterization study on the SERT knockout rat in 2007 by Homberg et al., it was demonstrated that the homozygous SERT animal had a severely affected 5HT homeostasis, their SERT mRNA and functional protein was completely absent, 5HT tissue levels and depolarization-induced 5HT release was reduced, basal extracellular hippocampal 5HT levels were increased by 9-fold, behaviour appeared normal when compared to wild type controls and no major adaptations were seen in the non-serotonergic systems. Overall these findings signify a decreased 5HT uptake and increased extracellular 5HT levels.

Figure 2

Schematic of serotonin transporter gene and location of ENU-induced mutation

Figure 2: Schematic representation of the serotonin transporter gene and induced knock out mutation obtained by target-selected mutagenesis. The arrows indicate the location of ENU-induced cytosine to adenosine nucleotide base transversion that results in the change of amino acid 209 from a cysteine to a stop codon. *Source: Homberg JR et al., Characterization of the serotonin transporter knockout rat: a selective change in the functioning of the serotonergic system, Neuroscience 146; 1662 - 1676 (2007).*

1.3.10 Genome opposed to proteome

The genome of an organism is generally static, whereas the proteome is very dynamic i.e an organism will have a fixed number of genes, a certain number of which will be expressed at a given time. Most cells contain the same genome regardless of the cell type, developmental stage or environmental conditions. The proteome, however, varies considerably in these differing conditions due to different patterns of gene expression and different patterns of protein modification. Using proteomics the products of these genes can be studied, focusing on the proteins that are present in that organism at the time of sampling. In this way the response to different stimuli or comparisons between a control and disease condition can be investigated. Furthermore, studying the proteome is important because proteins represent the actual functional molecules in the cell. When mutations occur in the DNA, the proteins are ultimately affected. In addition, the beneficial effects of drugs are due to their interaction with proteins. Consequently, measuring gene expression at the protein level is potentially more informative than the corresponding measurement at the mRNA level.

1.3.11 Proteomics

Proteomics is the simultaneous study of all proteins produced by a cell type and organism, potentially including the full complement of proteins encoded by the genome at any given time i.e. the proteome. By definition, the proteome refers to specific subsets of proteins found in a specific region of cell type under specific conditions (Pandey & Mann, 2000). Proteins as major catalysts of biological function contain several dimensions of information that collectively indicate the actual, rather than the potential, functional state as indicated by mRNA analysis, therefore representing a higher complexity than the transcriptome. These dimensions include the abundance, state of posttranslational modification, subcellular localization, and association with each other (Griffin & Aebersold, 2001). The human genome has an estimated 20 000 – 25 000 genes, of which there are probably more than 500 000 different proteins, due to alternative splicing and posttranslational modifications (Alam et al, 1999). Thus proteins display a higher degree of dynamics due to posttranslational modifications.

The majority of proteomic techniques involves some form of separation procedure in order to isolate individual proteins, such as 2-dimensional (2-D) gel electrophoresis and high performance liquid chromatography (HPLC), ionization of peptides, followed by detection and identification by mass spectrometry (MS) (Steen & Mann, 2004). 2-D gel electrophoresis is the oldest method for large scale protein separation since 1975 and still remains the most popular method for protein display and quantification. It permits

simultaneous detection, display, purification, identification and quantification. 2-D gel electrophoresis is followed by in-gel digestion where a specific spot is removed from the gel and then incubated with trypsin. Trypsin is the most widely used protease for proteomic studies and cleaves proteins at the carboxyl side of lysine or arginine residues, unless followed by proline.

These days, proteomics techniques find a wide application in neuroscience research (Engidawork & Lubec, 2001; Lubec, 2003; Fountoulakis 2001; Fountoulakis, 2004). Three kinds of proteomics exist, namely expressional, functional and structural proteomics. Expressional proteomics is concerned with the display, measurement and analysis of global changes in protein expression. It monitors changes arising from developmental, environmental or disease perturbations. It has been used mainly for protein screening in brain tissue in healthy and diseased states, for the detection of drug targets and diagnostic markers (Fountoulakis et al., 1999; Freidl, 2001; Kim et al., 2001; Korolainen, 2002; Weitzdoerfer, 2001). Proteomics is the ideal tool for studying protein-protein interactions and post-translational modifications. Previously, neuroproteomics has mainly been applied to the analysis of total brain tissue (Fountoulakis et al., 1999; Langen et al., 1999). Such an analysis provides us with a general pattern of the proteins expressed in all brain regions, the preparation of subfractions and the isolation of organelles, each containing a lesser number of total proteins (Fountoulakis & Tak'acs, 2002; Fountoulakis, 2004).

1.3.12 The current study

Previous results of the SERT knockout rat have shown a lack in the binding of a radio actively labeled SSRI [^{3}H] 5HT citalopram to brain slices of SERT homozygous (-/-) knockout rats and a 40 % decreased binding in SERT heterozygous (+/-) knockout rats, with hypothermia being completely absent and reduced in SERT (-/-) and (+/-) knockout rats respectively, the animals also displayed normal behavioural responses when compared to wild type animals (Homberg et al, 2007). These data suggests that there was no properly folded and functionally active SERT protein in the SERT
(-/-) knockout rats, which resulted in a general decrease in 5HT uptake and increase in extracellular 5HT levels.

The aim of this study was to further characterize the serotonin transporter knockout model, by getting a more comprehensive profile of the proteins, in the hippocampus, affected, controlled, and/or regulated by the serotonin transporter. The hippocampus was chosen, as this brain structure has been extensively studied in patients with mood disorders and is known to be strongly innervated by serotonergic neurons from the raphe nucleus. We

decided to investigate the dorsal and ventral horns, of the hippocampus separately as these areas are implicated in learning, memory and anxiety-related behaviours respectively. In addition, we distinguished between cytosolic and membrane fractions of the specific horns, to elucidate the role of proteins in processes such as metabolism, signaling and receptor binding as well as generating information about the structural integrity of neurons.

CHAPTER 2
MATERIALS AND METHODS

All chemicals used were of purest grade, highest standard and commercially available. These were purchased mainly from Bio-Rad Laboratories Inc., CA, USA and Sigma-Aldrich.

2.1 Animals

Wildtype and SERT knockout male Wistar rats, age 11 – 15 weeks, were used for experiments. Animals were housed under standard conditions in groups of two to four per Macrolon type III cage per gender under controlled experimental conditions (12-h light/dark cycle, 21± 1 °C, 60 % relative humidity, food and water *ad libitum*). All procedures were approved by the Animal Care Committee of the Royal Dutch Academy of Science and the Radboud University Nijmegen, according to ethical guidelines.

The generation of knockout rats was performed at Radboud University Nijmegen. The SERT knockout rat ($Slc6a4^{1Hubr}$) was obtained by applying target-selected ENU-induced mutagenesis techniques, which introduces a premature stop-codon in the second extracellular loop of the transporter protein. This technique results in a complete nonfunctional serotonin transporter (Homberg et al., 2007).

2.2 Study design

The primary focus of this study was to do a proteomic analysis of rat hippocampus on animals subjected to knockout technology in order to identify protein changes in response to the lack of serotonin transporters. A top-down proteomic approach was chosen, which incorporated protein mixture separation, protein digestion into peptides, peptide separation, and finally peptide analysis. A mass spectrometer was used to determine peptide sequence coverage and ultimately identification of proteins.

Rats were genetically bred; hippocampi were dissected and further divided into dorsal and ventral hippocampal areas. The respective hippocampi were then fractionated into cytosolic, membrane, nuclear and cytoskeletal components. The cytosolic and membrane proteins, so isolated, were run and separated in two further dimensions, firstly by isoelectrical focusing (IEF), followed by polyacrylamide gel electrophoresis. Subsequently gels were compared for protein expression, i.e. to see whether the density of protein spots have changed between animals that have been differentially bred. More than two-fold differentially expressed protein spots, as is generally accepted in the proteomics field, were considered as being of biological significance. Expression differences were subsequently confirmed by visual inspection of each original gel image. The differentially expressed protein spots, as

determined by PD Quest software (Bio-Rad Laboratories Inc., CA, USA), were excised, digested and peptides were analyzed by means of mass spectrometry. Each sample was prepared from a single animal and analyzed on three replicate gels. Samples belonging to control (wild type) or SERT knockout (homozygous) animals, were analyzed at the same time under the same conditions.

2.3 Tissue collection

Frozen whole brains were obtained from the Department of Psychoneuropharmacology, Radboud University Nijmegen, The Netherlands to the Division of Medical Physiology, Department of Biomedical Sciences (University of Stellenbosch, Tygerberg, South Africa). The dorsal and ventral hippocampi were dissected on ice and snap frozen in liquid nitrogen until further analysis.

2.4 Sample preparation for 2-D gel electrophoresis

2.4.1 Protein extraction

A major challenge in functional proteomics is the separation of complex protein mixtures to allow detection of low abundance proteins and provide for reliable quantitative and qualitative analysis of proteins. Prerequisites for the success of such analysis are standardized and reproducible procedures for sample preparation prior to 1- or 2-D gel electrophoresis and/or liquid chromatography (LC). In order to maximize the coverage of the proteome and to increase the chance to visualize low-abundance proteins, it is required to prepare standardized partial proteomes of a given organism, due to the complexity of total proteomes and divergence of protein properties. Therefore sample preparation is key to successful proteomic techniques. Consideration must be given to break all non-covalent protein-protein, protein-DNA, protein-lipid interactions, as well as disrupt disulfide bonds. Simultaneously proteolysis, accidental phosphorylation, oxidation, cleavage and deamination of proteins must be prevented. In addition sample preparation procedures must remove substances that might interfere with the separation buffers such as salts, polar detergents e.g. sodium dodecyl sulfate (SDS), lipids, polysaccharides and nucleic acids and finally proteins must be kept soluble during both phases of the electrophoresis process.

The commercially available, ProteoExtract® Subcellular Proteome Extraction Kit (Catatlog # 539790, EMD Biosciences, Inc., an Affiliate of Merck KGaA, Darmstadt, Germany) enables the differential extraction of proteins according to their subcellular localization and is therefore a useful tool to produce a comprehensive proteome. Subsequently proteomes generated, in this way, has been fractionated into four sub proteomes of decreased complexity. The kit contains four extraction buffers prepared with ultra-pure chemicals to ensure high reproducibility, protease inhibitor cocktail to prevent protein degradation and

Benzonase® nuclease to remove contaminating nucleic acids. The procedure starts with hippocampal tissue being resuspended in the first extraction buffer and homogenized with a glass homogenizer. With extraction buffer I cytosolic proteins were released (fraction 1). Subsequently, membranes and membrane organelles were solubilized with extraction buffer II, without impairing the integrity of nucleus and cytoskeleton (fraction 2). Next, nucleic proteins were enriched with extraction buffer III (fraction 3), whereafter components of the cytoskeleton were finally solubilized with extraction buffer IV (fraction 4).

2.4.2 Protein determination

The Quick Start Bradford (Catalog #500-0205, Bio-Rad Laboratories, Inc., CA, USA) protein assay is a simple and accurate procedure for determining the concentration of protein in solution. The Bradford assay is a protein determination method that involves the binding of Coomassie Brilliant Blue G-250 dye to proteins. The dye exists in three forms: cationic (red), neutral (green), and anionic (blue). Under acidic conditions, the dye is predominantly in the doubly protonated red cationic form (Amax = 470 nm). However, when the dye binds to protein, it is converted to a stable unprotonated blue form (Amax = 595 nm). It is this blue protein-dye form that is detected at 595 nm in the assay using a spectrophotometer. The protein standard used was, 2 mg/ml bovine serum albumin (BSA). Therefore a relative standard and not an absolute reference standard was generated (Figure 3), as would normally be the practice. The determination of protein content in the various sub fractions was important to ensure equal loading of sample onto the IEF apparatus.

Figure 3

Protein determination

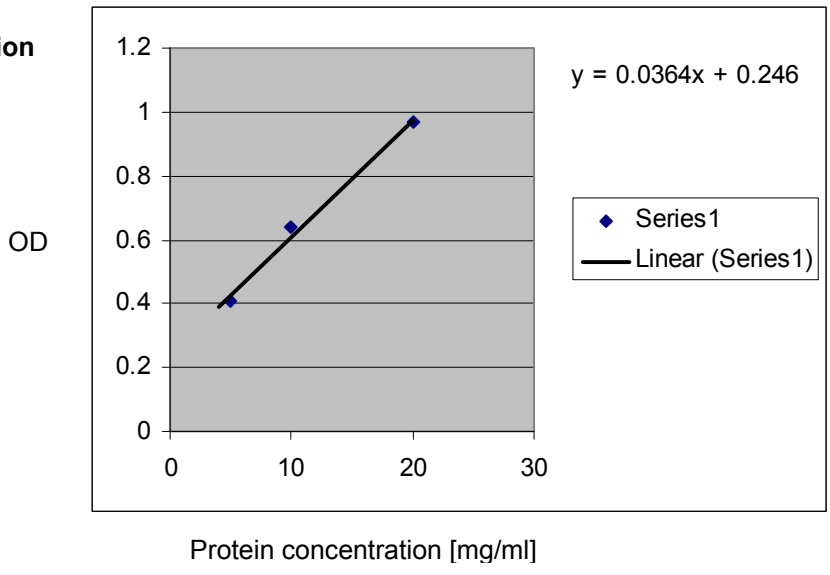


Figure 3: Graph representing protein determination as performed with Bradford assay. Protein optical density is plotted on the y-axis, with protein concentration [mg/ml] plotted on the x-axis.

2.4.3 Cleanup

The ReadyPrep™ 2-D Cleanup Kit (Catalog # 163-2130, Bio-Rad Laboratories, Inc., CA, USA) was used on fractionated samples. This kit facilitates the preparation of low conductivity samples suitable for isoelectric focusing (IEF) and 2-D gel electrophoresis. Additionally, the kit concentrates proteins from samples that are too dilute, allowing for higher protein loads that can improve subsequent spot detection on the gel. Application of the Ready-Prep™ 2-D Cleanup Kit therefore generates improved 2-D results by reducing streaking, background staining, and other gel artifacts associated with substances contaminating 2D/IEF samples. The procedure works by quantitatively precipitating and concentrating proteins in a sample while leaving behind and washing away substances like ionic detergents, salts, nucleic acids, lipids and plant-derived phenolic compounds, all of which are known to interfere with IEF. After precipitation, the proteins are washed and then resuspended in the IEF/2-D-compatible rehydration/sample buffer (Catalog #163-2106, Bio-Rad Laboratories, Inc., CA, USA), ready for isoelectric focusing.

2.4.4 *RC DC* Protein Assay

It has been found that before IEF can be performed, a more sophisticated protein determination is necessary for very low protein concentrations. The commercially available *RC DC* Protein Assay (Catalog # 500-0119, Bio-Rad Laboratories, Inc., CA, USA), which is based on the Lowry assay, was modified to be reducing agent compatible (*RC*) as well as detergent compatible (*DC*). This assay also incorporated a standard curve (Figure 4) and absorbances were read at 750 nm with a spectrophotometer.

Figure 4

Protein determination

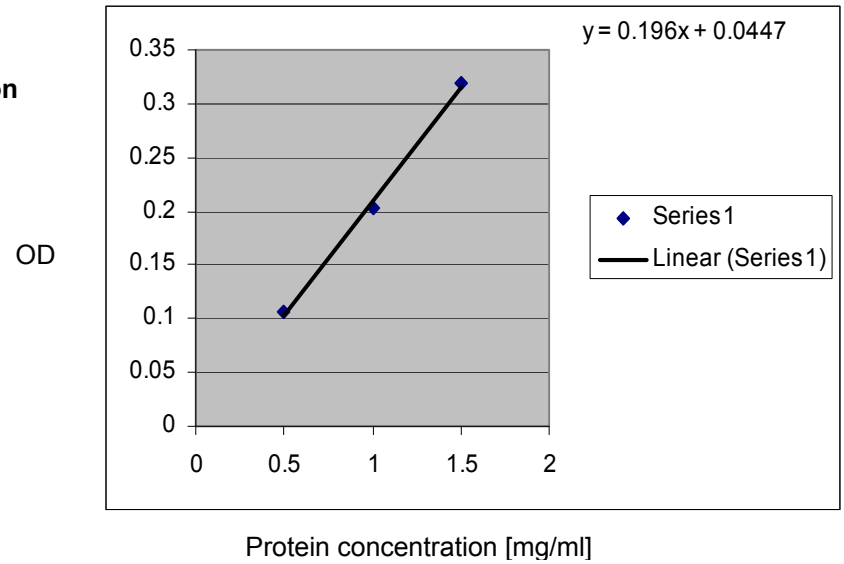


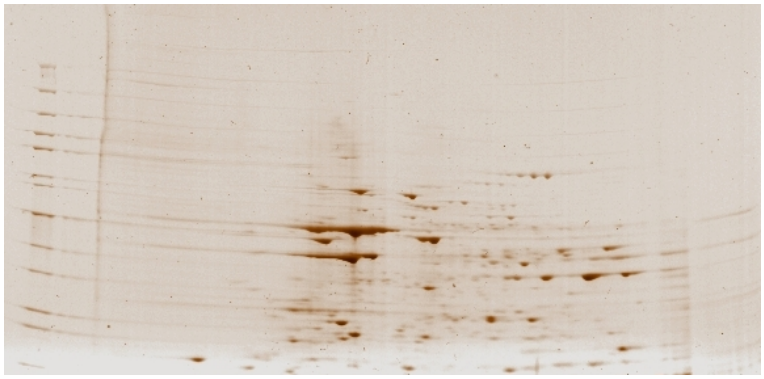
Figure 4: Graph representing protein determination as performed with *RC DC* assay and based on the Lowry assay. Protein optical density is plotted on the y-axis, with protein concentration [mg/ml] plotted on the x-axis.

2.4.5 Global protein expression profile

To acquire general information on protein distribution in the presence of serotonin transporter absenteeism, 150 micrograms (µg) of total protein lysate was loaded on immobilized pH gradient (IPG) strips with a pH range 3-10 (Figure 5 a). Approximately 90% of the proteins were observed within the pH range 5-8 (Figure 5 b). Therefore, subsequent experiments such as differential protein expression analysis and subsequent identification of differentially expressed proteins by electrospray ionization-quadrupole-time-of-flight-mass spectrometer (ESI-QUAD-TOF-MS) were subjected to IEF with pH range 5-8 with 150 µg total protein per strip. The experiments were run in triplicate i.e. repeated three times, resulting in a total of 6 gels per experiment (wild type versus knockout).

Figure 5

(a) IPG strip: pH 3 – 10



(b) IPG strip: pH 5 – 8

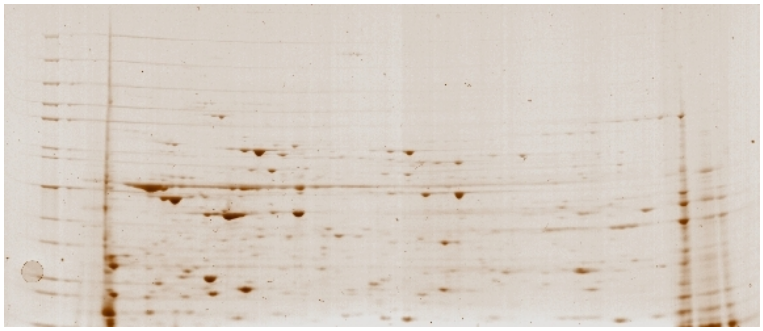


Figure 5: Gel images of a test sample run, in order to elucidate the distribution of protein spots. The molecular weight marker and protein spots were visualized. Figure 5 (a) demonstrates the distribution of proteins on an IPG strip with a pH of 3 – 10. Figure 5 (b) demonstrates the distribution of proteins on an IPG strip with a pH of 5 – 8, resulting in higher resolution.

Figure 6

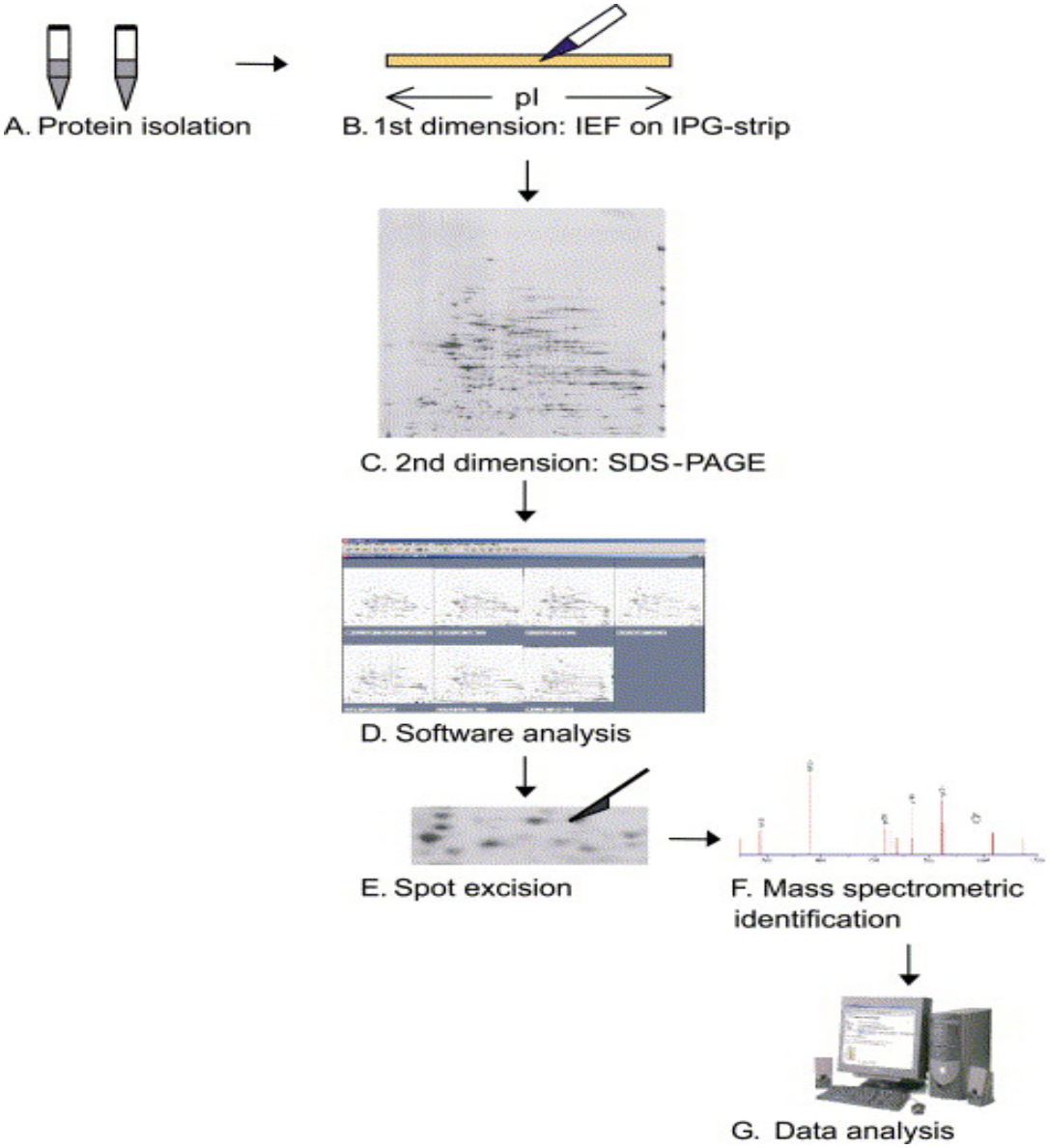


Figure 6: Proteomic experiment based on two-dimensional electrophoresis to identify differential protein expression in different protein extracts. A typical setup of this experiment consists of seven stages. In stage A, proteins are extracted from cells or tissues, after which these proteins are separated by means of isoelectric focusing (IEF) on immobilized pH gradient (IPG) strips (B). In the second dimension of 2D-PAGE, proteins with similar isoelectric points are further separated according to their molecular weight on a sodium disulphate polyacrylamide (SDS-PAGE) gel (C), and proteomic patterns are visualized by means of, e.g. silver staining, Coomassie staining or fluorescent staining. In stage D, the proteomic patterns of the samples are compared by means of software, to identify differential spots on the gels. Differential spots are excised (E) and proteins in these spots are enzymatically degraded, usually by means of trypsin, and subsequently identified by means of mass spectrometry (F) and database searching (G).

2.5 Proteomics

2.5.1 Isoelectric focusing

IEF was carried out on 11 cm immobilized pH gradient strips (5-8 pH gradient, Bio-Rad Laborotories Inc., CA, USA). Proteins migrate through the IPG strip until their net charge is neutral (pI), thus IEF is a first dimension separation technique. The strips were rehydrated for 12 h with 150 µg protein lysate in 200 microliter (µl) of solubilizing /2-D sample buffer (urea, CHAPS, Dithiothreitol:DTT,IPG buffer, Bio-Rad Laborotories Inc., CA, USA). Focusing was carried out at 20^0C for 40 000 Voltage hours (Vh) at a maximum of 8 000 Volts (V) in a Protean IEF Cell (Bio-Rad Laborotories Inc., CA, USA). Immobilized pH gradient strips (IPG) were then incubated with gentle shaking in buffer I, an SDS equilibration solution/buffer (6mMurea, 2 % DTT, Tris pH 8.8, 30 % glycerol,2 % w/v SDS), for 15 minutes followed by equilibration with buffer II (DTT was replaced by % iodoacetamide) for 15 minutes. The IPG strip was then placed on top of 4 – 12% pre-cast sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), prepared in running buffer (XT-MOPS, Catalog # 161-0788, Bio-Rad Laboratories Inc., CA, USA) and an electric current applied, subjecting it to second dimension separation.

2.5.2 2-D gel electrophoresis

The 2-D method separates proteins in two dimensions according to the pI (isoelectric point) and molecular weight (Mr). Following separation in the first dimension, proteins are separated in the orthogonal direction using electrophoresis in acrylamide gels containing SDS. SDS imparts a net negative charge allowing protein separation by mass. Two-dimensional gel electrophoresis is an analytic method that can quantify the levels of individual protein species in complex biological samples and allows comparison between diagnostic groups. 2-D gel electrophoresis is robust, increasingly reproducible, simple, cost effective, scalable and parallelizable; providing pI, Mr, and quantity.

Samples were analyzed in triplicate to reduce experimental variation, thus a total of 6 gels were run per experiment, i.e. 3 gels run for the control sample and 3 gels run for the experimental sample. We incorporated a protein standard on every gel used to reduce gel-to-gel variation and to standardize 2-D gel results. A protein mass marker, PeqGold (Optima Scientific) is loaded onto the gel, after which the IPG strip is transferred, an agarose overlay (Catalog # 163-2111, Bio-Rad Laboratories Inc., CA, USA) applied and running buffer added to the system. Second dimension gels were run at 200 V/ mA for 55 minutes. The gel slabs were fixed in 40 % methanol and 7 % acetic acid for1 hour, whereafter the fixed solution was removed and 50 ml colloidal Coomassie blue (Sigma) was added to each gel and incubated on gently continuous rocker at room temperature overnight. The following day, gels were

destained for 1 minute in 40 % methanol and 10 % acetic acid, followed by a second destaining step for 1 hour in 25 % methanol. Images were captured on a GS-800 calibrated densitometer (Bio-Rad Laboratories Inc., CA, USA) and analyzed with PD Quest 8 Advanced software (Bio-Rad Laboratories Inc., CA, USA). Significantly ($p < 0.05$) differentially expressed spots, as assessed by PD Quest, were excised and sent for protein identification by ESI-QUAD-TOF-mass spectrometer analysis.

2.5.3 Mass Spectrometer

Mass spectrometer (MS) analysis is a technique used for determination of molecular mass under high vacuum, by measuring the mass-to-charge ratio (m/z) of an ion (Figure 7). Basically the molecule of interest is ionized, i.e. a charge is put on the analyte (Table 2). Measurements on how the trajectories of the resulting charged ions respond, in vacuum, to various combinations of electric and magnetic fields are then taken. Ions are generated by inducing either the loss or gain of a charge from a neutral species. Once formed, ions are electrostatically directed into a mass analyzer where they are separated according to *m/z* and finally detected (Table 2). The result of molecular ionization, ion separation, and ion detection is a spectrum that can provide molecular mass and even structural information.

Figure 7

Components of mass spectrometer

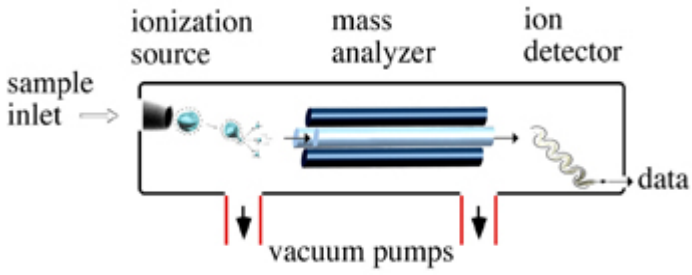


Figure 7: Above are the basic components, that are common to many mass spectrometers: a sample inlet, an ionization source, a mass analyzer and an ion detector. Some instruments combine the sample inlet and the ionization source, while others combine the mass analyzer and the detector. Sample molecules are introduced into the instrument through a sample inlet. Once inside the instrument, the sample molecules are converted to ions in the ionization source, before being electrostatically propelled into the mass analyzer. Ions are then separated according to their *m/z* within the mass analyzer. The detector converts the ion energy into electrical signals, which are then transmitted to a computer.

Table 2

Principle of mass spectrometry

Ion Source	Mass analyzer	Detector
Ion production i.e. ionization and vaporization of sample molecules	Ion separation according to their mass/charge ratio (m/z)	Ion detection i.e multiply and detect signal from ionized molecules

In the present study the proteins were identified by electrospray ionization-quadropole-time-of-flight-mass spectrometer (ESI-QUAD-TOF-MS) on the basis of peptide mass matching, following in-gel digestion with trypsin. The goal of protein digestion is to isolate the protein digest from the gel matrix and generate peptides of 6-20 amino acid lengths, which are best suited for MS analysis and database comparisons. The peptide masses were matched with the theoretical peptide masses of all proteins from all databases.

John B. Fenn, the originator and 2002 Nobel Laureate in Chemistry for advancing electrospray ionization (ESI) based this technique on an ion evaporation model. In brief, samples/peptides are injected, in conjunction with an aerosole, through a high positive voltage needle/tip into the mass spectrometer. The needle voltage produces an electrical gradient, maintained at a potential of 700 V to 5 000 V, on the fluid which separates the charges at the surface; forcing the liquid to emerge from the needle as a cone. The tip of the cone protrudes as a filament until the liquid reaches the limit where the surface tension and electrostatic repulsion are equal and the highly charged droplets leave the filament. Thus the needle disperses the solution into a fine spray of charged droplets. Droplets that break away from the filament are attracted to the entrance of the mass spectrometer due to the high opposite voltage at the mass analyzer's entrance. As the droplet moves towards the analyzers, the repulsion on the surface exceeds the surface tension, and the droplet explodes into smaller droplets ultimately releasing ions. Thus ESI produces gaseous ionized molecules directly from a liquid solution; by creating a fine spray of highly charged droplets in the presence of an electric field. Either dry gas, heat, or both are applied to the droplets at atmospheric pressure, thus causing the solvent to evaporate from each droplet. ESI is conducive to the formation of singly charged small molecules, but also produces multiple charged ions of larger molecules. Multiple charging makes it possible to observe very large molecules with an instrument having a relatively small mass range, such as a mass spectrometer which measures the mass-to-charge ratio (*m/z*). ESI is commonly used on quadrupole mass spectrometers (MS), which basically are four mass analyzers.

ESI-MS is a powerful technique, achieving higher sensitivity due to higher surface-to-volume ratios. This technique enables identification of proteins, after digestion, and peptide mass mapping with a mass range of 200 000 Daltons (Da).

ESI protein's molecular weight is calculated from the protein's charge distribution:

n_1 = charge on peak 1

m_H = mass of proton

n = number of

Mr = molecular weight

$$n_1 = \frac{m/z_2 - m_H}{m/z_1 - m/z_2} \quad ; \quad Mr = n_1 (m/z_1 - m_H)$$

Mass spectral data were applied to the search engine Matrix Science (MASCOT; Figure 8), whereafter results were generated from protein databases which store protein sequence information.

2.5.4 Data analysis and Bioinformatics

2.5.4.1 Protein sequence determination

Trypsin digested proteins of interest, resulted in peptides that were eluted and subjected to electrospray mass spectrometry.

Measured peptide masses were analyzed by MASCOT software tools (http://www.matrixscience.com) (Matrix Science, London, UK) (Perkins et al, 1999), which incorporates a probability-based scoring (Figure 9). MASCOT was used to search SwissProt and NCBI nonredundant databases with Mammalia (mammals) as taxonomic category. Database interrogation was carried out using monoisotopic peptide masses, 0.2 Da peptide mass tolerance and 0.5 Da fragment mass tolerance, and 1 as the maximum number of missed tryptic cleavages (Figure 8). The molecular masses of the intact proteins and associated isoelectric (pI) points were taken from the 2-D maps. The interrogation also included possible modifications such as oxidation of methionine and the alkylation of cysteine residues by iodoacetamide, and carboxyamidomethylation of cysteines as fixed modification (Figure 8). MASCOT provides rank, score, threshold and expectation value per identification; important properties which have to be tabled (Table 3, 4 & 7) for reference purposes. The criteria for positive identification of proteins were set as follow: protein scores were considered significant when the probability of random events was less than 36 ($p < 0.05$).

Figure 8

MASCOT interface

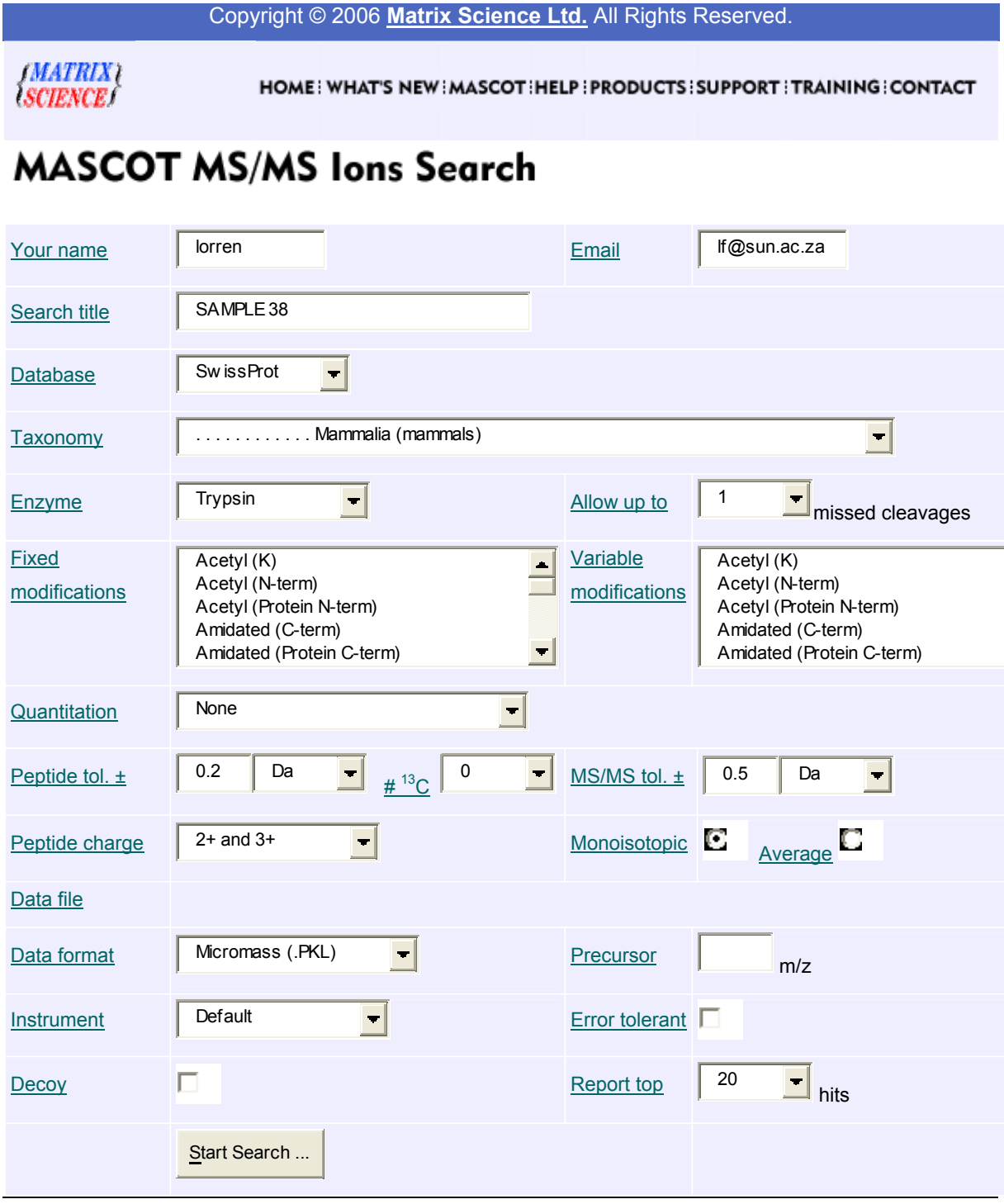


Figure 8: The interface of the search engine used to identify proteins generated from mass spectrometry data. Various parameters must be set and kept constant throughout analyses.

For MASCOT analyses, proteins with a probability-based MOWSE (Molecular Weight Search: scoring system based on peptide frequency distribution from the OWL non redundant protein Database) score greater than the threshold calculated by the tool algorithm were considered significant identifications (Figure 9). Probability-based scoring offers a simple numerical and graphical assessment of whether a result is significant. In addition this scoring system is more reliable than simple mass or number of peptide match techniques. The deduced peptide sequence(s) were compared against the sequence database, SwissProt, for known matches. All protein identifications were verified using the expected size range based on their position in the gel.

Figure 9

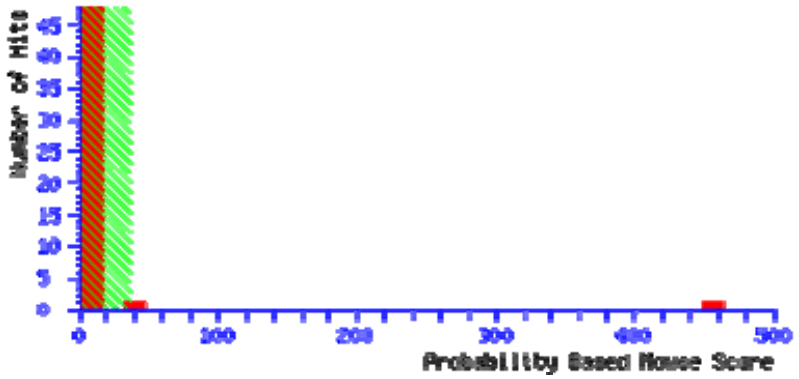


Figure 9: A Gaussian graph generated by MASCOT, which incorporates the probability based MOWSE score. A score greater than the threshold calculated by the tool algorithm is considered to be a significant identification.

2.6 Statistical analysis

Protein levels were evaluated as volumes (spot area X optical density) for the protein spots matched among gels. Spot volume was normalized for each gel on total density in valid spots. Data were log transformed and analyzed with Student's *t* test with the statistics tools included in the version 8 PD Quest software. Spots which gave significant results ($p < 0.05$) were verified visually to exclude artifacts. Volume values of satisfactory spots were re-checked by Student's *t* test on the 4th version of Prism software (GraphPad Software Inc., San Diego, CA, USA).

CHAPTER 3
RESULTS

3.1 Summary of results obtained

The proteomic analyses were carried out on two parallel, independent groups of rats (wild type and SERT knockout). Figures 10 to 13 are representative gel images of rat hippocampus proteins, extracted and separated on an immobilized pH 5 – 8 gradient strip followed by separation on a 4 – 12 % gradient polyacrylamide gel. The gel was stained with Coomassie blue. Maps were obtained in which ± 300 spots were detectable. For proteins that were unambiguously matched among the gels, levels were evaluated as volumes (spot area X optical density) with the image analysis software. The comparison between groups with image analysis followed by statistical testing revealed that the ventral hippocampal area had a 2 fold and greater expression of proteins as compared with the dorsal hippocampal area. In addition the SERT knockout group demonstrated significantly altered protein profiles as compared to the wild type (control) group.

The spots representing the identified proteins are indicated in Table 3, 4 & 7 and are designated with their accession numbers of SwissProt or NCBI database. The number of matching peptides, probability of assignment (MASCOT score), theoretical molecular weight (Mr) and pI values are given. Important brain proteins including cell signaling, structural, metabolic and cell stress proteins were identified (Table 5, 6 & 8) by ESI-QUAD-TOF MS, representing many individual protein cascades and pathways. The majority of these proteins have been previously identified, as part of the brain proteome, by others (Gauss et al., 1999; Fountoulakis et al., 2002, 2005; Yang et al., 2004, 2005). After clustering and functional categorization of the identified proteins, 47 cytosolic and 30 membrane proteins were identified (Tables 5, 6 and 8 respectively). Among these proteins, 5.64 % were present in only the cytosolic fraction, and 1.08 % were present in only the membrane fraction.

Figure 14 is a 2-D gel representation of a specific differentially expressed protein, namely glia maturation factor beta, as annotated by PD Quest software. The Venn diagrams in Figures 15 to 17, illustrate the number of differentially expressed proteins in specific hippocampal brain areas (ventral and dorsal) and fractions (cytosolic and membrane).

Table 3

Data represents the differentially expressed cytosolic proteins of the ventral hippocampus between SERT knockout and wild type animals. The SSP number is assigned by PD Quest software. The molecular weight (Mr), isoelectric point (pI), MASCOT score, number of peptides, protein name, SwissProt accession number (#) and percentage (%) sequence coverage is assigned by the database SwissProt using the search engine MASCOT.

SSP	Mr (Da)	pI	MASCOT score	No of peptides	Protein Name	SwissProt Accession #	% Sequence Coverage
2004	20902	5.48	153	9	Phosphatidylethanolamine-binding protein 1	P31044	59
1004	21941	5.34	93	7	Peroxiredoxin-2	P35704	27
1106	25165	5.14	233	10	Ubiquitin carboxyl-terminal hydrolase isozyme	Q00981	43
2002	16897	5.32	206	6	Glia maturation factor beta	Q63228	49
5004	16073	5.88	153	3	Superoxide dismutase (Cu-Zn)	P07632	23
5003	22539	5.96	147	4	Nucleoside diphosphate kinase A	Q05982	31
6501	47440	6.16	507	23	Alpha-enolase	P04764	55
5505	47440	6.16	593	17	Alpha-enolase	P04764	41
6102	28928	6.67	60	4	Phosphoglycerate mutase 1	P25113	22
6104	27345	6.89	192	9	Triosephosphate isomerase	P48500	21
7101	28928	6.67	60	4	Phosphoglycerate mutase 1	P25113	22
8101	27345	6.89	149	10	Triosephosphate isomerase	P48500	44
8101	28928	6.67	80	10	Phosphoglycerate mutase 1	P25113	48
9001	17386	6.92	122	6	Nucleoside diphosphate kinase B	P19804	46
7301	36090	8.14	153	8	Glyceraldehyde-3-phosphate dehydrogenase	P04797	27
5203	36631	6.16	259	11	Malate dehydrogenase,cytoplasmic	O88989	28
5102	20190	6.32	277	8	Protein DJ-1	O88767	32
6201	36631	6.16	199	6	Malate dehydrogenase,cytoplasmic	O88989	20
5101	24860	5.64	518	15	Peroxiredoxin-6	O35244	53
4203	30952	5.69	340	12	F-actin-capping protein subunit beta	Q5XI32	31
4202	33118	5.57	221	6	F-actin-capping protein subunit alpha-2	Q3T1K5	30
4304	40044	6.47	413	10	Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial precursor	Q99NA5	24
4204	36874	5.7	435	13	L-lactate dehydrogenase B chain	P42123	34

Table 3 continued

Data represents the differentially expressed cytosolic proteins of the ventral hippocampus between SERT knockout and wild type animals. The SSP number is assigned by PD Quest software. The molecular weight (Mr), isoelectric point (pI), MASCOT score, number of peptides, protein name, SwissProt accession number (#) and percentage (%) sequence coverage is assigned by the database SwissProt using the search engine MASCOT.

SSP	Mr (Da)	pI	ASCOT sco	No of peptides	Protein Name	SwissProt Accession #	% Sequence Coverage
4204	36712	8.45	100	3	L-lactate dehydrogenase A chain	P04642	11
5201	36874	5.7	180	7	L-lactate dehydrogenase B chain	P42123	21
5201	31805	5.75	75	5	N (G), N (G)-dimethylarginine dimethylaminohydrolase 1	O08557	18
5301	40044	6.47	422	8	Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial precursor	Q99NA5	21
4301	42983	5.39	453	15	Creatine kinase B-type	P07335	36
4501	51554	5.56	588	17	Guanine deaminase	Q9WTT6	40
4501	42983	5.39	84	3	Creatine kinase B-type	P07335	11
404	47510	5.03	468	19	Gamma-enolase	P07323	51
404	47440	6.16	133	5	Alpha-enolase	P04764	11
1302	42108	5.31	180	14	Actin,cytoplasmic 2	P63259	45
1302	47510	5.03	65	3	Gamma-enolase	P07323	12
1101	25165	5.14	134	4	Ubiquitin carboxyl-terminal hydrolase isozyme L 1	Q00981	19
1101	20977	5.12	76	4	Lactoylglutathione lyase	Q6P7Q4	14
102	20977	5.12	195	7	Lactoylglutathione lyase	Q6P7Q4	26
806	72473	5.07	605	22	78 kDa glucose-regulated protein precursor	P06761	31
806	71055	5.37	77	3	Heat shock cognate 71 kDa protein	P63018	5
1906	89977	5.14	205	22	Transitional endoplasmic reticulum ATP-ase	P46462	36
1904	93997	5.13	415	13	Heat shock 70 kDa protein 4	P83581	17
1903	94795	5.13	456	18	Heat shock 70 kDa protein 4	P83582	23
3803	71055	5.37	335	22	Heat shock cognate 71 kDa protein	P63018	33
4802	74097	5.97	522	15	Stress-70 protein,mitochondrial precursor	P48721	27
3705	61088	5.91	332	23	60 kDa heat shock protein,mitochondrial precursor	P63039	34
3205	37156	5.51	95	3	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-3	P52287	8

Table 3 continued

Data represents the differentially expressed cytosolic proteins of the ventral hippocampus between SERT knockout and wild type animals. The SSP number is assigned by PD Quest software. The molecular weight (Mr), isoelectric point (pI), MASCOT score, number of peptides, protein name, SwissProt accession number (#) and percentage (%) sequence coverage is assigned by the database SwissProt using the search engine MASCOT.

SSP	Mr (Da)	pI	ASCOT sco	No of peptides	Protein Name	SwissProt Accession #	% Sequence Coverage
3205	32947	5.57	54	1	F-actin-capping protein subunit alpha-2	Q3T1K5	3
3201	39299	6.2	186	5	Pyruvate dehydrogenase E 1 component subunit beta, mitochondrial precurso	ODPB_RAT	17
3201	33493	5.44	179	5	Pyridoxal phosphate phosphatase	Q8VD52	14
5802	70682	6.09	195	15	Serum albumin precursor	P02770	29
6701	62638	5.95	197	15	Dihydropyrimidinase-related protein 2	P47942	37
6606	57256	6.28	426	12	D-3 phosphoglycerate dehydrogenase	O08651	26
7601	57256	6.28	94	6	D-3 phosphoglycerate dehydrogenase	O08651	12
7601	56514	6.77	61	4	Cytosol aminopeptidase	Q68FS4	10
7801	63158	6.4	303	13	Stress-induced phosphoprotein-1	O35814	21
9901	86121	7.87	410	14	Aconitate hydratase,mitochondrial precursor	Q9ER34	20
9703	58294	6.63	222	15	Pyruvate kinse isozymes M1/M2	P11980	29
9702	61719	8.05	237	9	Glutamate dehydrogenase 1,mitochondrial precurso	P10860	18
9702	58294	6.63	101	4	Pyruvate kinse isozymes M1/M2	P11980	10

Table 4

Data represents the differentially expressed cytosolic proteins of the dorsal hippocampus between SERT knockout and wild type animals. The SSP number is assigned by PD Quest software. The molecular weight (Mr), isoelectric point (pI), MASCOT score, number of peptides, protein name, SwissProt accession number (#) and percentage (%) sequence coverage is assigned by the database SwissProt using the search engine MASCOT.

SSP	Mr (Da)	pI	MASCOT score	No of peptides	Protein Name	SwissProt Accession #	% Sequence Coverage
4901	71055	5.37	502	10	Heat shock cognate 71 kDa protein	P63018	16
5801	71055	5.37	1000	19	Heat shock cognate 71 kDa protein	P63018	33
6801	68625	5.42	1083	21	Vacuolar ATP synthase catalytic subunit A		37
6901	74097	5.97	1017	18	Stress-70 protein,mitochondrial precursor	P48721	31
5701	61088	5.91	1314	24	60 kDa heat shock protein,mitochondrial precursor	P63039	48
1502	47510	5.03	948	17	Gamma-enolase	P07323	47
1401	47510	5.03	1052	15	Gamma-enolase	P07323	53
1401	47326	7.08	259	5	Beta-enolase	P15429	17
1401	47440	6.16	194	4	Alpha-enolase	P04764	14
1401	50788	4.94	74	1	Tubulin alpha-1 A chain	P68370	1
1401	50804	4.94	74	1	Tubulin alpha-1 B chain	Q6P9V9	1
1401	50590	4.94	74	1	Tubulin alpha-1 C chain	Q6AYZ1	2
1401	50612	4.97	74	1	Tubulin alpha-3 chain	Q68FR8	2
1401	50634	4.97	74	1	Tubulin alpha-4 A chain	Q5XIF6	2
1401	50690	4.97	74	1	Tubulin alpha-8 chain	Q6AY56	2
2302	42052	5.29	267	5	Actin,cytoplasmic 1	P60711	17
2302	42108	5.31	267	5	Actin,cytoplasmic 2	P63259	17
1001	20902	5.48	153	9	Phosphatidylethanolamine-binding protein 1	P31044	59
3001	25165	6.23	548	8	Ubiquitin carboxyl-terminal hydrolase isozyme L1	Q00981	5
3001	27703	6.23	58	1	NADH dehydrogenase flavoprotein 2	P19234	5
4001	20902	5.48	571	10	Phosphatidylethanolamine-binding protein 1	P31044	59
6101	38151	5.6	361	6	Guanine nucleotide-binding protein G(I)/G(S) subunit beta-1	P54311	26
6101	38048	5.6	179	3	Guanine nucleotide-binding protein G(I)/G(S) subunit beta-2	P54313	10
6301	42983	5.39	789	5	Creatine kinase B-type	P07335	47
6301	42052	5.29	90	2	Actin,cytoplasmic 1	P60711	7

Table 4 continued

Data represents the differentially expressed cytosolic proteins of the dorsal hippocampus between SERT knockout and wild type animals. The SSP number is assigned by PD Quest software. The molecular weight (Mr), isoelectric point (pI), MASCOT score, number of peptides, protein name, SwissProt accession number (#) and percentage (%) sequence coverage is assigned by the database SwissProt using the search engine MASCOT.

SSP	Mr (Da)	pI	MASCOT score	No of peptides	Protein Name	SwissProt Accession #	% Sequence Coverage
6301	42108	5.31	90	2	Actin,cytoplasmic 2	P63259	7
6102	36874	5.7	510	10	L-lactate dehydrogenase B-chain	P42123	30
6102	42983	5.39	268	3	Creatine kinase B-type	P07335	12
6102	36712	8.45	69	8	L-lactate dehydrogenase A-chain	P04642	7
6502	51554	5.56	470	17	Guanine deaminase	Q9WTT6	15
6502	50788	4.94	137	2	Tubulin alpha-1 A chain	P68370	5
6502	50804	4.94	137	1	Tubulin alpha-1 B chain	Q6P9V9	5
6502	50590	4.94	137	1	Tubulin alpha-1 C chain	Q6AYZ1	5
6602	57044	5.88	186	10	Protein disulfide-isomerase A3 precursor	P11598	8
6602	56857	5.57	181	19	Vacuolar ATP synthase subunit B,brain isoform	P62815	8
6602	62638	5.95	159	8	Dihydropyrimidinase-related protein-2	P47942	8

Differentially expressed cytosolic proteins of dorsal hippocampus

Table 5

Data represents functionally grouped cytosolic proteins differentially expressed in the ventral hippocampus between SERT knockout and wild type animals. The pathway indicates the function of a protein, with change in protein levels of SERT knockout animals in comparison to wild type animals.

Protein Name	Pathway	Change
Metabolism		
Energy Metabolism		
Phosphatidylethanolamine-binding protein 1	Energy metabolism; Presynaptic cholinegic neurons	↑
Nucleoside diphosphate kinase A	Energy metabolism	↑
Nucleoside diphosphate kinase B	Energy metabolism	↑
Alpha-enolase	Glycolytic Pathway	↑
Pyruvate kinse isozymes M1/M2	Glycolysis; Energy metabolism	↑
Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial precursor	Tricarboxylic Acid Cycle (Energy)	↑
L-lactate dehydrogenase B chain	Lactate metabolism; Anaerobic Glycolysis	↑
L-lactate dehydrogenase A chain	Lactate metabolism; Anaerobic Glycolysis	↑
Phosphoglycerate mutase 1	Glycolysis; Acetylation	↓
Pyridoxal phosphate phosphatase	Energy metabolism	↓
Malate dehydrogenase,cytoplasmic	Malate metabolism (Energy)	↓
Pyruvate dehydrogenase E 1 component subunit beta, mitochondrial precursor	Energy metabolism	↓
Transitional endoplasmic reticulum ATP-ase	Energy metabolism; Membrane transport	↔
Creatine kinase B-type	Energy metabolism	↔
Protein Metabolism		
D-3 phosphoglycerate dehydrogenase	Amino acid biosynthesis	↑
Cytosol aminopeptidase	Processing/Turnover of intracellular proteins	↑
Ubiquitin carboxyl-terminal hydrolase isozyme L 1	Protein degradation	↓
Ubiquitin carboxyl-terminal hydrolase isozyme	Protein degradation	↑

Table 5 continued

Data represents functionally grouped cytosolic proteins differentially expressed in the ventral hippocampus between SERT knockout and wild type animals. The pathway indicates the function of a protein, with change in protein levels of SERT knockout animals in comparison to wild type animals.

Protein Name	Pathway	Change
Metabolism		
Carbohydrate Metabolism		
Triosephosphate isomerase	Carbohydrate biosynthesis; Gluconeogenesis	↑
Aconitate hydratase,mitochondrial precursor	Carbohydrate metabolism	↓
Triosephosphate isomerase	Carbohydrate biosynthesis; Gluconeogenesis	↔
Signal Transduction		
78 kDa glucose-regulated protein precursor	Chaperone	↑
Heat shock 70 kDa protein 4	Chaperone	↑
Stress-70 protein,mitochondrial precursor	Chaperone	↑
60 kDa heat shock protein,mitochondrial precursor	Chaperone	↑
Stress-induced phosphoprotein-1	Chaperone mediator	↑
Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-3	Signaling; Transmembrane modulator	↑
Heat shock cognate 71 kDa protein	Chaperone	↔
Structure		
F-actin-capping protein subunit alpha-2	Actin-binding; Structure	↑
F-actin-capping protein subunit beta	Actin-binding; Structure	↑
Actin,cytoplasmic 2	Structure	↓

Table 5 continued

Data represents functionally grouped cytosolic proteins differentially expressed in the ventral hippocampus between SERT knockout and wild type animals. The pathway indicates the function of a protein, with change in protein levels of SERT knockout animals in comparison to wild type animals.

Protein Name	Pathway	Change
Cellular Stress		
Peroxiredoxin-6	Redox regulation; Antioxidant	↑
Superoxide dismutase (Cu-Zn)	Antioxidant	↑
Protein DJ-1	Redox sensitive chaperone	↑
N (G), N (G)-dimethylarginine dimethylaminohydrolase 1	Nitric oxide generation	↓
Peroxiredoxin-2	Redox regulation; Antioxidant	↔
Lactoylglutathione lyase	Glyoxal Pathway; Antioxidant	↓
Lactoylglutathione lyase	Glyoxal Pathway; Antioxidant	↔
Miscellaneous		
Gamma-enolase	Neuroprotection	↑
Guanine deaminase	Neuroplasticity	↔
Dihydropyrimidinase-related protein 2	Neuroplasticity	↓
Glyceraldehyde-3-phosphate dehydrogenase	Neuronal transcription; Apoptosis (overexpressed)	↑
Serum albumin precursor	Transporter protein	↓
Glutamate dehydrogenase 1, mitochondrial precursor	Memory and Learning	↑
Glia maturation factor beta	Brain cell differentiation & regeneration	↑

Table 6

Data represents functionally grouped cytosolic proteins differentially expressed in the dorsal hippocampus between SERT knockout and wild type animals. The pathway indicates the function of a protein, with change in protein levels of SERT knockout animals in comparison to wild type animals.

Protein Name	Pathway	Change
Metabolism		
Energy Metabolism		
Phosphatidylethanolamine-binding protein 1	Energy; Presynaptic cholinegic neurons	↑
Alpha-enolase	Glycolytic Pathway	↑
Vacuolar ATP synthase subunit B,brain isoform	Energy metabolism	↑
Creatine kinase B-type	Energy metabolism	↑
L-lactate dehydrogenase A-chain	Lactate metabolism; Anaerobic Glycolysis	↑
L-lactate dehydrogenase B-chain	Lactate metabolism; Anaerobic Glycolysis	↑
NADH dehydrogenase flavoprotein 2	Energy metabolism; Electron Transfer	↓
Phosphatidylethanolamine-binding protein 1	Energy metabolism; Presynaptic cholinegic neurons	↔
Vacuolar ATP synthase catalytic subunit A	Energy metabolism	↔
Protein Metabolism		
Ubiquitin carboxyl-terminal hydrolase isozyme L1	Protein degradation	↓
Signal Transduction		
Guanine nucleotide-binding protein G(I)/G(S) subunit beta-1	Signaling; Transmembrane modulator	↑
Guanine nucleotide-binding protein G(I)/G(S) subunit beta-2	Signaling; Transmembrane modulator	↑
Stress-70 protein,mitochondrial precursor	Chaperone	↑
Heat shock cognate 71 kDa protein	Chaperone	↓
60 kDa heat shock protein,mitochondrial precursor	Chaperone	↔

Table 6 continued

Data represents functionally grouped cytosolic proteins differentially expressed in the dorsal hippocampus between SERT knockout and wild type animals. The pathway indicates the function of a protein, with change in protein levels of SERT knockout animals in comparison to wild type animals.

Protein Name	Pathway	Change
Structure		
Tubulin alpha-1 A chain	Structure; Microtubules	↑
Tubulin alpha-1 B chain	Structure; Microtubules	↑
Tubulin alpha-1 C chain	Structure; Microtubules	↑
Tubulin alpha-3 chain	Structure; Microtubules	↑
Tubulin alpha-4 A chain	Structure; Microtubules	↑
Tubulin alpha-8 chain	Structure; Microtubules	↑
Actin,cytoplasmic 1	Structure;Cell motility	↑
Actin,cytoplasmic 2	Structure;Cell motility	↑
Beta-enolase	Structure; Muscle Development	↑
Protein disulfide-isomerase A3 precursor	Structure; Disulfide bond rearrangement	↑
Actin,cytoplasmic 1	Structure;Cell motility	↓
Actin,cytoplasmic 2	Structure;Cell motility	↓
Miscellaneous		
Gamma-enolase	Neuroprotection	↔
Guanine deaminase	Neuroplasticity	↑
Dihydropyrimidinase-related protein-2	Neuroplasticity	↑

Table 7

Data represents the differentially expressed membrane proteins of the ventral and dorsal hippocampi between SERT knockout and wild type animals. The SSP number is assigned by PD Quest software. The molecular weight (Mr), isoelectric point (pI), number of peptides, protein name, SwissProt accession number (#) and percentage (%) sequence coverage is assigned by the database SwissProt using the search engine MASCOT. The symbol^{VHC} indicates proteins uniquely expressed in the ventral hippocampus, with the symbol$^{+\ VHC}$ indicating proteins expressed in both the ventral and dorsal hippocampi.Protein names without a symbol represent those identified in the dorsal hippocampus.

SSP	Mr (Da)	pI	No of peptides	Protein Name	SwissProt Accession #	% Sequence Coverage
1204	47110	4.8	8	Gamma Enolase	P07323	27
1304	50417	4.9	3	Pacsin 1 Protein kinase C	Q9Z0W5	6
303	50119	4.7	7	Tubulin alpha 1 B	Q6P9V9	20
303	24255	4.7	2	Tubulin 5 isoform 2	P69897	9
303	50027	4.5	2	Tubulin 6 beta	Q4QQV0	4
7201	46984	7.2	3	Beta Enolase	P15429	11
6202	47086	6.1	7	Alpha Enolase	P04764	19
6202	46984	7.2	2	Beta Enolase	P15429	8
6001	18751	6.1	4	ATP synthase D chain	P31399	35
7	14506	4.5	6	Synuclein alpha	P37377	54
7	14495	4.2	3	Synuclein beta	Q63754	23
7	22412	4.6	3	HPCA neuron specific	P84076	16
7	17584	4.9	13	ATP synthase delta	P35434	14
9	23300	4.4	2	SNAP 25	P60881	12
1403	72302	4.8	11	Heat shock protein 78kDa	P06761	21
2101	41709	5.1	5	Actin cytoplasmic	P60711	17
2202	52815	5.4	3	Ubiquinol cytochrome	Q68FY0	5
2204	52815	5.4	5	Ubiquinol cytochrome	Q68FY0	10
3102	39588	6.4	3	Isocitrate dehydrogenase	Q99NA5	8
3106	38957	6.2	9	Pyruvate dehydrogenase$^{+\ VHC}$	P49432	28
1102	24822	4.9	6	Ubiquitin carboxyl-terminal hydrolase isozyme L1$^{+\ VHC}$	Q00981	33
3105	37155	5.4	1	Guanine nucleotide binding protein	P52287	3
3001	29801	5.4	7	Prohibitin	P67779	28
4102	30609	5.5	7	F-actin capping protein$^{+\ VHC}$	Q5XI32	26

Table 7 continued

Data represents the differentially expressed membrane proteins of the ventral and dorsal hippocampi between SERT knockout and wild type animals. The SSP number is assigned by PD Quest software. The molecular weight (Mr), isoelectric point (pI), number of peptides, protein name, SwissProt accession number (#) and percentage (%) sequence coverage is assigned by the database SwissProt using the search engine MASCOT. The symbol^{VHC} indicates proteins uniquely expressed in the ventral hippocampus, with the symbol$^{+ VHC}$ indicating proteins expressed in both the ventral and dorsal hippocampi. Protein names without a symbol represent those identified in the dorsal hippocampus.

SSP	Mr (Da)	pI	No of peptides	Protein Name	SwissProt Accession #	% Sequence Coverage
5101	39588	6.4	6	Isocitrate dehydrogenase	Q99NA5	16
6101	39588	6.4	5	Isocitrate dehydrogenase	Q99NA5	14
6401	68686	6	8	Serum albumin precursor	PO2770	15
5303	57043	5.8	6	Protein disulfide isoform a 3	P11598	13
5302	67123	8.6	6	Dihydrolipoyllysine	P08461	12
5301	57043	5.8	10	Protein disulfide isoform a 3	P11598	23
5301	67123	8.6	2	Dihydrolipoyllysine	P08461	4
3302	60917	5.8	16	Heat shock protein 60kDa$^{+ VHC}$	P63039	34
2302	60917	5.8	9	Heat shock protein 60kDa$^{+ VHC}$	P63039	19
3404	73811	5.8	11	Heat shock protein 9 a	P48721	20
3401	69598	5.3	6	Heat shock protein a 2	P14659	11
2405	69598	5.3	6	Heat shock protein a 2	P14659	9
4102	20788	5.3	2	Phosphatidylethanol-binding protein^{VHC}	P31044	14
3103	24822	4.9	5	Ubiquitin carboxyl-terminal hydrolase isozyme L1$^{+ VHC}$	Q00981	29
3104	21770	5.2	2	Peroxiredoxin 2^{VHC}	P35704	10
4209	38957	6.2	5	Pyruvate dehydrogenase$^{+ VHC}$	P49432	17
4209	32946	5.4	2	F-actin capping protein$^{+ VHC}$	Q3T1K5	9
4606	60917	5.8	10	Heat shock protein 60kDa$^{+ VHC}$	P63039	21
4602	60917	5.8	12	Heat shock protein 60kDa$^{+ VHC}$	P63039	28
3608	60917	5.8	10	Heat shock protein 60kDa$^{+ VHC}$	P63039	22

Table 8

Data represents functionally grouped membrane proteins differentially expressed in the ventral and dorsal hippocampus between and SERT knockout and wild type animals. The pathway indicates the function of a protein, with change in protein levels of SERT knockout animals in comparison to wild type animals. The symbol^{VHC} indicates proteins uniquely expressed in the ventral hippocampus, with the symbol$^{+ VHC}$ indicating proteins expressed in both the ventral and dorsal hippocampi. Protein names without a symbol represent those identified in the dorsal hippocampus.

Protein Name	Pathway	Change
Metabolism		
Energy Metabolism		
Pacsin 1 Protein kinase C	Metabolism	↑
Phosphatidylethanol-binding protein^{VHC}	Energy metabolism; Presynaptic cholinegic neurons	↑
ATP synthase delta	Energy metabolism	↑
Pyruvate dehydrogenase$^{+ VHC}$	Energy metabolism	↑
Isocitrate dehydrogenase	Tricarboxylic Acid Cycle (Energy)	↑
Alpha Enolase	Glycolytic Pathway	↑
ATP synthase D chain	Energy metabolism	↑
Dihydrolipoyllysine	Energy metabolism	↑
Protein Metabolism		
Ubiquitin carboxyl-terminal hydrolase isozyme L1$^{+ VHC}$	Protein degradation	↑
Ubiquinol cytochrome	Mitochondrial respiratory chain	↑
Signal Transduction		
Heat shock protein 60kDa$^{+ VHC}$	Chaperone	↑
Heat shock protein 9 a	Chaperone	↔
Heat shock protein a 2	Chaperone	↔
Heat shock protein 78kDa	Chaperone	↑
Guanine nucleotide binding protein	Signaling; Transmembrane modulator	↔

Table 8 continued

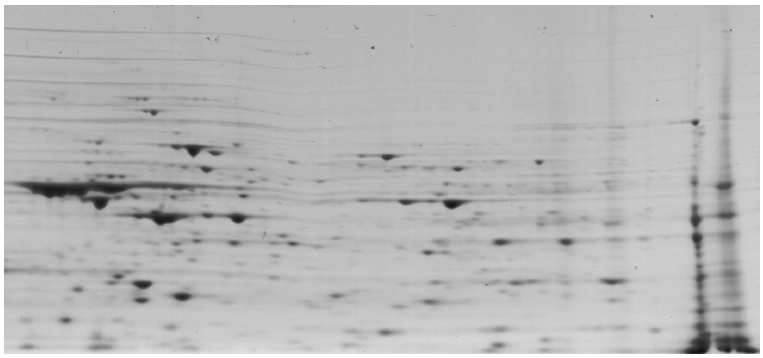
Data represents functionally grouped membrane proteins differentially expressed in the ventral and dorsal hippocampus between SEF knockout and wild type animals. The pathway indicates the function of a protein, with change in protein levels of SERT knockout animals in comparison to wild type animals. The symbol ^{VHC} indicates proteins uniquely expressed in the ventral hippocampus, with the symbol $^{+ VHC}$ indicating proteins expressed in both the ventral and dorsal hippocampi.

Protein Name	Pathway	Change
Structure		
Tubulin alpha 1 B	Structure; Microtubules	↑
Tubulin 5 isoform 2	Structure; Microtubules	↑
Tubulin 6 beta	Structure; Microtubules	↑
Beta Enolase	Structure; Muscle Development	↑
Actin cytoplasmic	Structure;Cell motility	↑
F-actin capping protein $^{+ VHC}$	Actin-binding; Structure	↑
Cellular Stress		
Peroxiredoxin 2 ^{VHC}	Redox regulation; Antioxidant	↑
Miscellaneous		
Serum albumin precursor	Transporter protein	↑
Gamma Enolase	Neuroprotection	↑
Prohibitin	DNA synthesis	↑
Synuclein alpha	Monoamine release & transport	↑
Synuclein beta	Monoamine release & transport	↑
HPCA neuron specific	Rhodopsin phosphorylation	↓
SNAP 25	Neurotransmitter regulation	↑
Protein disulfide isoform a 3	Disulfide bonds	↔

Figure 10

Representative 2-D gel image of significantly differentially expressed ventral hippocampal cytosolic proteins of wild type and SERT knockout animals.

(a) Ventral hippocampal cytosolic fraction wild type



(b) Ventral hippocampal cytosolic fraction knockout

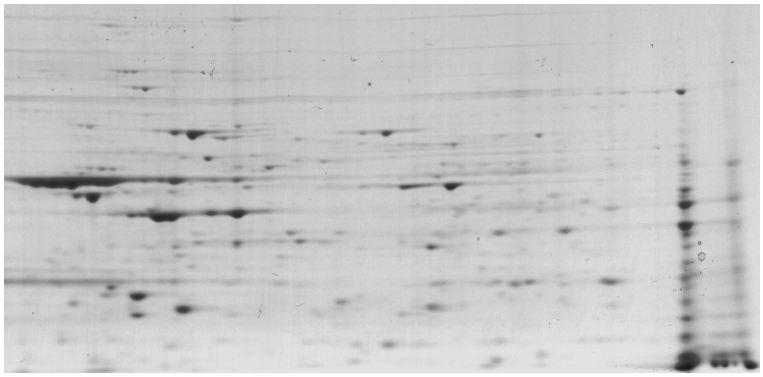
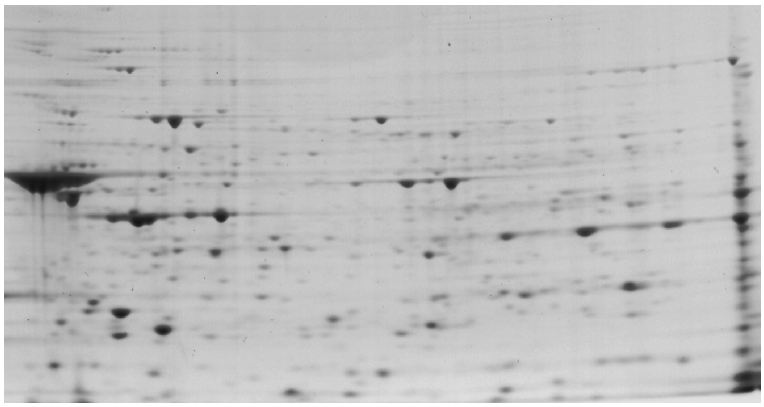


Figure 10: Representative 2-D SDS PAGE image of significantly differentially expressed ventral hippocampal cytosolic proteins. The pH range of 5 – 8. The acrylamide gradient is 4 - 12. The gel was stained with Coommasie Brilliant Blue stain. Proteins that showed differential expression after knockout of the serotonin transporter are reported in Table 3. All gels were run together in the same apparatus at the same time to reduce gel to gel variation.

Figure 11

Representative 2-D gel image of significantly differentially expressed dorsal hippocampal cytosolic proteins of wild type and SERT knockout animals.

(a) Dorsal hippocampal cytosolic fraction wild type



(b) Dorsal hippocampal cytosolic fraction knockout

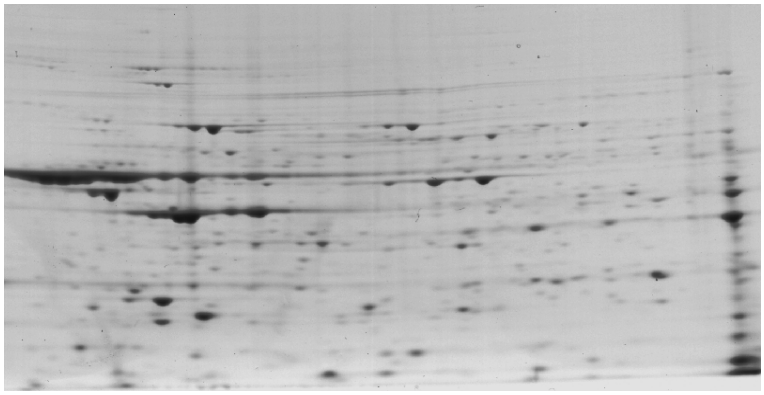
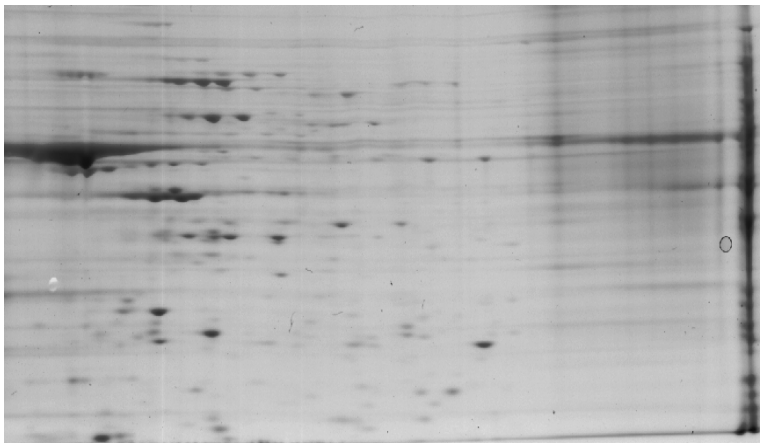


Figure 11: Representative 2-D SDS PAGE image of significantly differentially expressed dorsal hippocampal cytosolic proteins. The pH range of 5 – 8. The acrylamide gradient is 4 - 12. The gel was stained with Coommasie Brilliant Blue stain. Proteins that showed differential expression after knockout of the serotonin transporter are reported in Table 4. All gels were run together in the same apparatus at the same time to reduce gel to gel variation.

Figure 12

Representative 2-D gel image of significantly differentially expressed ventral hippocampal membrane proteins of wild type and SERT knockout animals.

(a) Ventral hippocampal membrane fraction wild type



(b) Ventral hippocampal membrane fraction knockout

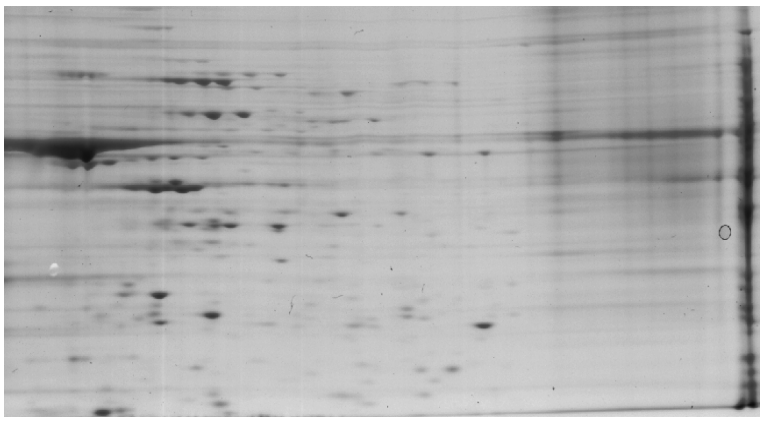


Figure 12: Representative 2-D SDS PAGE image of significantly differentially expressed ventral hippocampal membrane proteins. The pH range of 5 – 8. The acrylamide gradient is 4 - 12. The gel was stained with Coommasie Brilliant Blue stain. Proteins that showed differential expression after knockout of the serotonin transporter are reported in Table 7. All gels were run together in the same apparatus at the same time to reduce gel to gel variation.

Figure 13

Representative 2-D gel image of significantly differentially expressed dorsal hippocampal membrane proteins of wild type and SERT knockout animals.

(a) Dorsal hippocampal membrane fraction wild type

(b) Dorsal hippocampal membrane fraction knockout

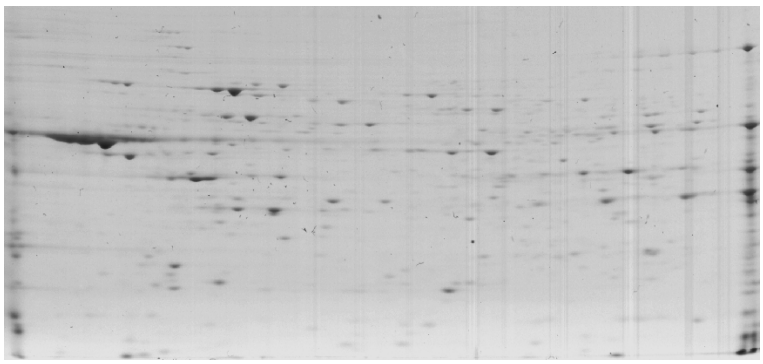
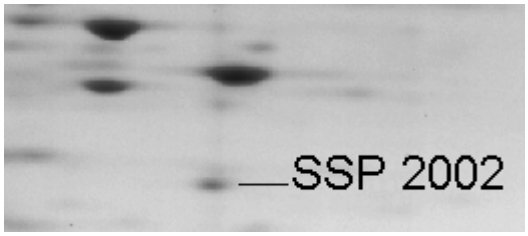


Figure 13: Representative 2-D SDS PAGE image of significantly differentially expressed dorsal hippocampal membrane proteins. The pH range of 5 – 8. The acrylamide gradient is 4 - 12. The gel was stained with Coommasie Brilliant Blue stain. Proteins that showed differential expression after knockout of the serotonin transporter are reported in Table 7. All gels were run together in the same apparatus at the same time to reduce gel to gel variation.

Figure 14

Representative 2-D gel image of a differentially expressed protein, glia maturation factor beta.

(a) Cytoslic fraction of wild type ventral hippocampus



(b) Cytosolic fraction of SERT knockout ventral hippocampus

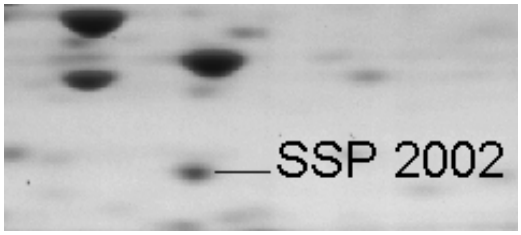


Figure 14: Representative image of differential expressed protein, glia maturation factor beta (SSp 2002, Table 3) between (a) wild type and (b) SERT knockout animals. Glia maturation factor beta shows a 2-fold increase in SERT knockout animals relative to wild type (Table 5).

Figure 15

Cytosolic proteins identified in the ventral and dorsal hippocampal regions

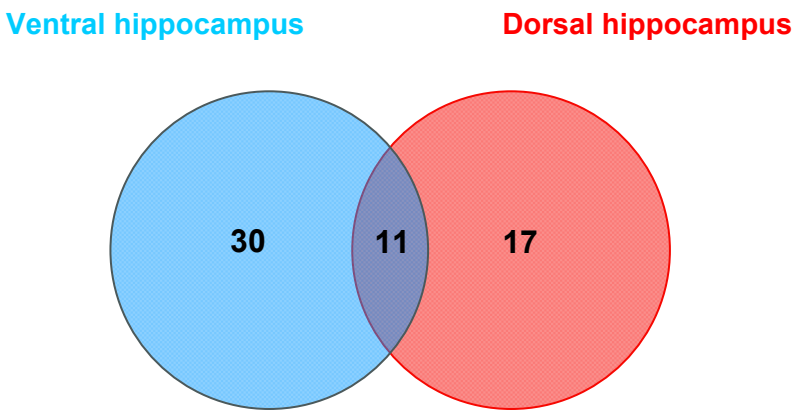


Figure 15: Distinct and common cytosolic proteins identified in the hippocampus. The Venn diagram illustrates the number of distinct proteins identified in the ventral (blue) and dorsal hippocampal regions (red). The overlapping region of the diagram (grey) illustrates the group of proteins present in both ventral and dorsal regions of the hippocampus.

Figure 16

Membrane proteins identified in the ventral and dorsal hippocampal regions

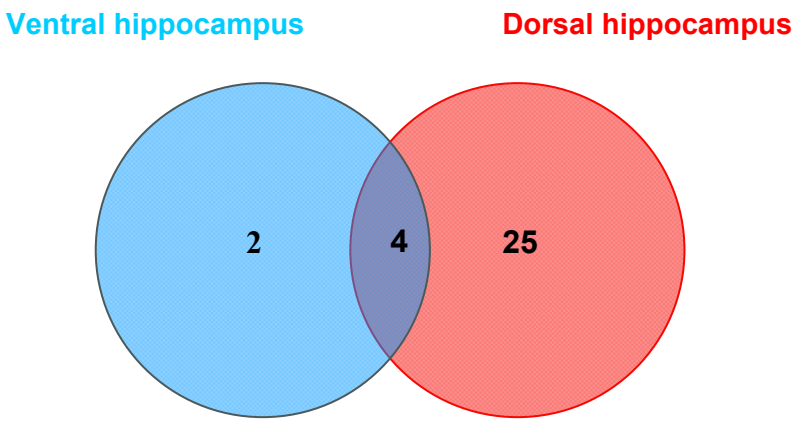


Figure 16: Distinct and common membrane proteins identified in the hippocampus. The Venn diagram illustrates the number of distinct proteins identified in the ventral (blue) and dorsal hippocampal regions (red). The overlapping region of the diagram (grey) illustrates the group of proteins present in both ventral and dorsal regions of the hippocampus.

Figure 17

Proteins identified in the cytosol and membrane fractions

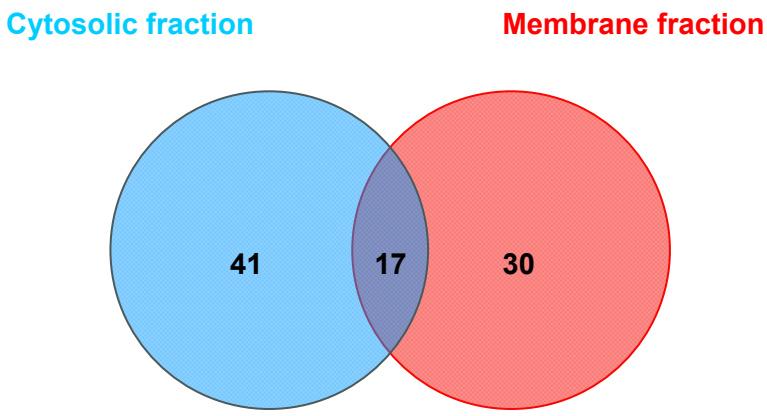


Figure 17: Distinct and common proteins in the cytosolic and membrane fractions. The Venn diagram illustrates the number of distinct proteins identified in the cytosolic (blue) and membrane fractions (red). The overlapping region of the diagram (grey) illustrates the group of proteins present in both cytosolic and membrane fractions.

CHAPTER 4
DISCUSSION AND CONCLUSION

4.1 Introduction

Clinical abnormalities in serotonin metabolism have been implicated in the pathophysiology of various psychiatric disorders. Decreased serotonergic signaling, resulting from low levels of 5HT in the synaptic cleft, 5HT receptor desensitization, decreased 5HT receptor expression, or changes in monoaminergic transporter expression can lead to the development of mood disorders such as depression (Owens & Nemeroff, 1994; Malison et al., 1998; Bianchi et al., 2003). Equally, increasing the 5HT concentration in the synaptic cleft can lead to the development of affective disorders such as schizophrenia (Powchik et al., 1998). Thus changing the bioavailability of 5HT in the synaptic cleft is a major mechanism of regulating 5HT signaling, which occurs principally via reuptake by the monoaminergic, sodium and chloride dependent, serotonin transporter (Blakely et al., 1991). Any modulation in the expression or action of SERT would therefore be expected to have consequences on behaviour and mood. Studies have demonstrated that specific polymorphic variants in the human SERT gene correlate with predisposition to a number of neurological and psychiatric disorders, suggesting that individuals with a particular combination of polymorphisms may respond differently to the same medications e.g. SSRIs. More specifically, modulation of SERT expression has been strongly associated with depression (Owens & Nemeroff, 1994; 1998).

4.2 Aims of study

In complex systems such as the brain, animal models represent useful tools to study molecular processes, occurring in health and disease states. Such studies provide necessary platforms to unravel signal transduction pathways and intercellular interaction networks between proteins and other molecules that influence cellular function, which prove unfeasible with humans. In the present study proteomic techniques were used to determine protein profiles expressed in the ventral and dorsal hippocampal regions of normal Wistar rats and this was compared to the protein expression profiles of SERT knockout animals. Therefore, the aim of this study was to further characterize the serotonin transporter knockout model, by getting a more comprehensive profile of the proteins, in the hippocampus, affected, controlled, and/or regulated by the serotonin transporter.

4.3 Characterization of the proteomic technique

Proteomics has a number of key advantages over other techniques, such as microarrays. The most important being a look at the proteins present in the cell, not the mRNA from which

it is translated. Another advantage is the possibility to identify proteins that have been posttranslationally modified, as well as the simultaneous measurement and quantification of protein expression levels and protein–protein interactions.

Protein modifications play a key role in the function of many proteins; therefore researchers' ability to look at and identify changes in these proteins can be of key importance. It should be noted that posttranslational modifications of gene products as well as protein translocation and activity are not intrinsically encoded in gene sequences and therefore cannot be derived from mRNA expression. Previously a lack of correlation between transcriptional profiles and actual protein levels in cells (Anderson & Seilhamer, 1997) have been shown; with abundant mRNA resulting from strong gene expression, not necessarily corresponding to abundant or indeed active proteins in the cell. Proteomic analysis allows for these modifications, which produce changes in the size, the pI or both to be identified on 2-D gels by the anomalous migration of the protein under investigation. This technique therefore offers a unique means of identifying and characterizing proteins that are expressed in a cell or tissue at any given time-point and advances understanding of any modifications that they may undergo. In the current study, proteomic techniques identified a vast pool of proteins expressed within the ventral hippocampus (42 cytosolic proteins and 6 membrane proteins, Figures 15 & 16 respectively) and the dorsal hippocampus (29 cytosolic proteins and 29 membrane proteins, Figures 15 & 16 respectively) of both SERT knockout and normal animals.

However, a drawback of this method is that low abundance, highly charged and very hydrophobic proteins often go undetected (Mann et al., 2001). This phenomenon has been suggested to probably be due to protein/gel interactions during IEF (Adessi et al., 1997). Furthermore, in many cases only the most abundant proteins such as metabolic proteins dominate and are therefore identified. Some larger proteins may also be lost and it has been suggested to be due to size exclusion when the proteins are loaded onto the gel. Other disadvantages include possible contamination with regards to subfractionation i.e. nuclear constituents might be present in the cytosolic fraction, the necessity to distinguish between two proteins per spot i.e. one excised spot identified as two different proteins, a lack in reproducibility between labs and finally it is a time-consuming technique. These aforementioned points are to be considered when undertaking proteomic work, however, troubleshooting is always possible. For example (refer to Table 3 SSP 3205), to determine which of the two proteins are to be attributed to the excised spot, one must consider the confidence of the identification as being indicated by the number of matching and total peptides as well as the MASCOT score; the higher the score the greater the confidence in

the result. In the present study a peptide number of greater than 1 was chosen and a MASCOT score above 30 was selected. In view of SSP 3205, the likely protein would therefore be Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-3 and not F-actin-capping protein subunit alpha-2 (Table 3).

A series of cytosolic and membrane hippocampal proteins were identified by ESI-QUAD-TOF MS and representative gels are shown in Figures 10 - 13. All gels were run together in the same apparatus at the same time to reduce gel to gel variation. Figure 14 is a representative image of a specific differential expressed protein, glia maturation factor beta (SSp 2002, Table 3) between (a) wild type and (b) SERT knockout animals. Glia maturation factor beta shows a 2-fold increase in SERT knockout animals relative to wild type (Table 5). The Venn diagrams in Figures 15 & 16, respectively illustrate the number of cytosolic and membrane proteins, distinct and common to the ventral and dorsal hippocampal brain areas. Figure 17 is a Venn diagram illustrating the distinct and common proteins between the two fractions i.e. the cytosolic and membrane fractions. Data in Tables 3 & 4 represents the differentially expressed cytosolic proteins of the ventral and dorsal hippocampi, respectively, between SERT knockout and wild type animals. The SSP number is assigned by PD Quest software. The molecular weight (Mr), isoelectric point (pI), number of peptides, protein name, SwissProt accession number (#) and percentage (%) sequence coverage is assigned by the database SwissProt using the search engine MASCOT. Data in Table 5 & 6 represents functionally grouped cytosolic proteins differentially expressed in the ventral and dorsal hippocampi, respectively, between SERT knockout and wild type animals. The pathway indicates the function of a protein, with change in protein levels of SERT knockout animals in comparison to wild type animals. Data in Table 7 represents the differentially expressed membrane proteins of the ventral and dorsal hippocampi between SERT knockout and wild type animals. The SSP number is assigned by PD Quest software. The molecular weight (Mr), isoelectric point (pI), number of peptides, protein name, SwissProt accession number (#) and percentage (%) sequence coverage is assigned by the database SwissProt using the search engine MASCOT. The symbol VHC indicates proteins uniquely expressed in the ventral hippocampus, with the symbol + VHC indicating proteins expressed in both the ventral and dorsal hippocampi. Data in Table 8 represents functionally grouped membrane proteins differentially expressed in the ventral and dorsal hippocampi between SERT knockout and wild type animals. The pathway indicates the function of a protein, with change in protein levels of SERT knockout animals in comparison to wild type animals. The symbol VHC indicates proteins uniquely expressed in the ventral hippocampus, with the symbol + VHC indicating proteins expressed in both the ventral and dorsal hippocampi.

4.4 Differentially expressed proteins in rat hippocampus

Notwithstanding these considerations, a 2-D map of proteins in the SERT knockout rat hippocampus was constructed and we identified 106 individual protein spots and grouped them according to previously attributed functions (Table 5, 6 & 8). Furthermore, it was observed, from the current study, that the majority of the identified proteins serve in "housekeeping" such as in metabolic pathways and cellular structure. Intermediate metabolic enzymes are considered housekeeping gene products and therefore can be used for normalisation of protein data. They mainly consist of carbohydrate handling enzymes, those involved in energy metabolism, the respiratory chain and citric acid cycle (Bajo et al., 2002; Brookes et al., 2002; Rabilloud et al., 2002a). When protein levels are determined in neuronal cells or brain tissue, it is of high importance that the data be normalised against a housekeeping protein and a protein marker for neuronal density to correct for neuronal loss. The use of neuron-specific enolase represents such a marker and could be recommended for this purpose.

Some of the proteins presented here (e.g. malate dehydrogenase, heat shock cognate 71 kDa, synuclein alpha; Table addendum) have been published in previous reports on human and rodent brain proteomes (Langen et al., 1999; Krapfenbauer et al., 2003; Stevens et al., 2003; Yang et al., 2004). Of greater significance is the fact that the majority of proteins identified in the current study, coincide with the profile of protein expression variations in the rat hippocampus as established by Fountoulakis et al (1999). Amongst these, proteins were found to assist in energy metabolism, cytoskeletal regulation, signal transduction, antioxidant reactions, molecular chaperone proteins and some miscellaneous products (Tables 3 - 8). Fountoulakis et al (1999) also showed that a greater number (2.4 fold) of proteins are localized in the cytosol compared to the mitochondrial membranes. The present study reported a similar pattern of protein localization between the cytosolic and membrane fractions; i.e. cytosol 2 fold greater than the membrane. The predominant of cytosolic proteins were identified as being uniquely expresssed in either the ventral (30 proteins) or dorsal (17 proteins) hippocampal regions, with 12 proteins found to be commonly expressed between the two areas (Figure 15). With regards to the membrane proteins 4 were commonly expressed in the ventral and dorsal hippocampal regions, with 2 and 25 found to be unique in ventral and dorsal hippocampal regions respectively (Figure 16).

Proteomic techniques identified 42 cytosolic proteins in the ventral hippocampus common to both SERT knockout and normal rats (Table 5 & Figure 15). Levels of these expressed proteins were either up- or down regulated, with some interestingly remaining unchanged between the two groups (Table 5). Ventral hippocampal cytosolic proteins were shown to be

involved in metabolism, neuronal structure, signal transduction, cell stress and other processes such as neuroplasticity and neuroprotection to name but a few (Table 5).

The ventral hippocampus has been suggested to have a preferential role in brain processes associated with anxiety-related behaviours (Bannerman et al., 2004); however, it is interesting to note that the present study did not identify any proteins that may play a role in the behavioural pathways of anxiety or depression. This finding was not the first to reveal no association between the ventral hippocampus and its putative role in anxious manifestations. The absence of 'anxious' proteins in this knockout model corroborates the behavioural findings of Homberg et al (2007), where no difference in behaviour between SERT knockout and wild type (normal) animals was found. It has been suggested that protein interactions govern behaviour; therefore the lack of anxious-like behavioural manifestation could be due to the lack of alteration in the expression and/or structure of anxious proteins.

However, the expressed protein profile could also be indicative of a reactive ventral hippocampus. Since the deficiency in serotonin was present throughout the lifespan of the knockout animals, compensatory molecular mechanisms could have been established, resulting in normal behavioural functionality. Characterizing the SERT knockout rat, Homberg et al (2007) found homozygous animals to have reduced serotonin tissue levels, hence disrupted neurotransmission. Considering that many cellular functions rely on adenosine triphosphate (ATP) consumption, especially in those cellular compartments where a high rate of ATP is fundamental to maintain signaling pathways, such as the synapse (Wang et al., 2004). Possible compensatory mechanisms in the current study may therefore include the up-regulation of energy proteins in the SERT knockout rat as compared to the normal animal (Table 5 & 8). By increasing energy metabolism, neuronal cell firing is increased; ultimately resulting in increased neurotransmission; an energy-dependent process.

A deficiency in SERT has also been shown to result in increased levels of 5HT within the synaptic cleft which not only activates the postsynaptic receptors, but also flood presynaptic autoreceptors, which serve as a feedback sensor for the cell. Activation of autoreceptors triggers the throttling of serotonin production. Seeing that serotonin is synthesized from the amino acid L-tryptophan and implicated in regulation of carbohydrate metabolism (Goldberg et al., 2004), it was not surprising to note that amino acid biosynthesis and carbohydrate metabolism protein expression levels of for example D-3 phosphoglycerate dehydrogenase, cytosol amino peptidase and triosephosphate isomerase (Table 5) were increased in response to elevated extracellular 5HT concentration.

Another plausible reason for the increase in energy metabolism seen in SERT knockout rats is based on previous studies which showed that a dysregulation in serotonin metabolism results in hypervigilant behaviour (Sugden et al., 2006). The dynamic processes of constantly being alert and seeking for escape routes, requires a general increase in energy production i.e. brain for alert state and body for locomotion. However, a more detailed behavioural analysis is required from the Homberg et al. (2007) study to confirm whether SERT knockout rats displayed such behaviour when compared to normal animals.

At the structural level, cytoskeletal proteins specifically tubulin, were also expressed (Table 6 & 8). The significance of this result is implicated in these proteins' fundamental known function i.e. to serve as scaffolding structures and their role in transport and signaling of various organelles and vesicular components (dos Remedios & Thomas, 2001). In response to firing of 5HT neurons, vesicular movement occurs along microtubules, present in the presynaptic neuron, resulting in the release of 5HT into synaptic clefts. Microtubules, constituents of the cytoskeleton, are synthesized from the protein tubulin. Matus et al (1981) demonstrated the distribution of tubulin and high molecular weight proteins in the brain, as determined by immunoperoixdase histochemistry with specific antisera, and found tubulin in the microtubules of both neurons and glial cells. Rat glial cells have also been shown to contain tubulin via polymerase chain reaction (PCR). Since the present proteomic study investigated the hippocampus as a whole, and did not distinguish between different cell types, the increase in tubulin could have been due to changes within glial cells present in tissue samples. Glial cells are known to support neuronal function by acting as structural foundations to provide a framework that allow the networks of neurons to remain connected. They also remove debris after neuronal waste.

Motor proteins are implicated in vesicular transport, and are known to utilize chemical energy (ATP) to orchestrate movements. ATP hydrolysis drives and controls protein conformational changes that result in sliding/walking movements of one molecule relative to another. Therefore, microtubules mediating intracellular motion of organelles/vesicles rely on these cytosolic motor proteins known as dyneins and kinesins. These proteins possess microtubule-activated ATPase activity which can bind microtubules, in the presence of ATP, and move them through a solution. It has been shown in neuronal axons that dyneins move vesicles from synaptic termini toward the cell body. Hence, it may be possible that the expression of cytoskeletal (e.g. tubulin alpha-3; Table 6) and metabolic proteins (e.g. vacuolar ATP synthase subunit B; Table 5) were increased in the SERT knockout rats to improve vesicular movement of 5HT along the microtubule toward the synapse for release,

with structural proteins such as actin cytoplasmic-2 (Table 5, 6 & 8) merely being an indication of the presence of glial cells.

Stressful conditions have also been shown to affect neuronal plasticity and 5HT neurotransmission in the hippocampus (Bianchi et al., 2003). Neuronal plasticity which is typically referred to as a structural adaptation of neurons to functional needs, require more dynamic forms of microtubules (cytoskeletal component). The alpha-tubulin, which is a major component of microtubules, can be posttranslationally modified. Neurotransmitters capture chemical energy in the form of ATP synthesis. The sodium pump (Na^+, K^+-ATPase) is an integral protein of the plasma membrane. Potassium is required within the cell to activate a variety of processes, whereas high intracellular sodium concentrations are inhibitory. The transmembrane gradients of Na^+ and K^+ and the attendant gradient of Cl^- and other ions provide the means by which neurons communicate. They also serve to regulate cellular volume and shape. Animal cells also depend upon these gradients to drive transport processes involving amino acids, sugars, nucleotides and other substances. Maintenance of these gradients consumes large amounts of energy in animal cells: 20 – 40 % of total metabolic energy in many cases and up to 70 % in neural tissue. ATP hydrolysis occurs on the cytoplasmic side of the membrane and the net movement of 1 + charge outward per cycle makes the sodium pump electrogenic in nature. Biosynthesis involves the formation of new covalent bonds, and an input of chemical energy is necessary to drive such endergonic processes. ATP generated by catabolism provides energy for biosynthesis of complex biomolecules (proteins, nucleic acids, lipids and polysaccharides) from simpler precursors, aka anabolism. NADPH is a source of high-energy electrons for reductive reactions of anabolism.

Therefore SERT knockout-mediated increased synaptic 5HT concentrations, which heighten autoreceptor activation resulting in increased intracellular 5HT production, require structural and metabolic regulation.

The bulk of proteins identified in this proteomic study were key metabolic enzymes (e.g. trioesphosphate isomerase, alpha enolase, ubiquinol cytochrome; Table 5, 6 & 8 respectively); capable of sensing the momentary metabolic needs of cells and adjusting their catalytic rates accordingly. These adjustments can be viewed as reactive compensatory mechanisms; ensuring that the living state is promoted and preserved, by means of integrating the diverse and divergent metabolic activities of the cell.

In homozygous SERT knockout rats a hypothermic response was completely absent following *d*-fenfluramine administration (Homberg et al., 2007), suggesting serotonin homeostasis is severely affected. The multi-gene family of stress-inducible heat shock proteins (Hsp) have been shown to be enriched in the mammalian nervous system compared with non-neural tissue and is present at high levels in neuronal cell bodies (Manzerra et al, 1993, 1997). Hsp levels were up-regulated (Table 5, 6 & 8) in SERT knockout, as opposed to normal, animals. Additionally, previous studies have identified the presence of a host of these stress signaling related proteins in the brain (Langen et al., 1999; Simpson and Dorow, 2001). It is suggested that under control conditions, Hsp 70 could be involved in postsynaptic mechanisms of local protein synthesis related to synaptic plasticity; evident in the current study by the presence of expressed neuroplastic proteins. These proteins act as molecular chaperones, assisting in protein synthesis and protein translocation across membranes, shepherding proteins to their ultimate cellular destinations i.e. from synapse to nucleus (Suzuki et al., 1999). With increased amino acid synthesis (Table 5, 6 & 8), thus increased protein synthesis being evident in the present study, a recognized role for Hsp in the SERT knockout model can be ascribed. Previously perinuclear accumulations have been described and been shown to be associated with cytoplasmic ribosomes (Welch and Suhan, 1986). The Hsp70-assisted folding proteins bind to nascent polypeptide chains while they are still on ribosomes, recognizing exposed, extended regions of polypeptides that prevent nonproductive associations and thus keeps the polypeptide in an unfolded state until productive folding interactions can occur. Completion of folding requires energy-dependent release of the protein from Hsp70 and is therefore driven by ATP hydrolysis. Again the metabolic proteins, expressed in the present study, are implicated (Table 5, 6 & 8).

Serotonin is known to regulate the thermal response, hence a disruption in serotonin homeostasis resulting from SERT knockout, would lead to disruption in temperature homeostasis. Thus the present finding contributes to that of Homberg et al. (2007); suggesting an acquired thermotolerance, via a heat shock response. The suggested thermotolerance mechanism is based upon findings of Morimoto et al. (1997). These researchers showed that cells induce the classic heat shock response, in reaction to stressful stimuli such as exposure to lethal temperatures; resulting in cellular repair and protective mechanisms.

Since synapses are critical points of information transfer, their functionality must be preserved during stressful conditions to prevent communication breakdown. The presence of Hsp, specifically in synapses, suggests a reparative and/or protective effect related to

synaptic proteins as has been postulated under stress conditions (Bechtold et al., 2000). The increased extracellular 5HT and/or a lack of 5HT neurotransmission can be perceived as a stressful condition, as is observed with the 5HT syndrome. Consequently Hsp proteins were expressed in this knockout model as participants in neuroprotective mechanisms; preserving synaptic function. Heat shock proteins have also been shown to protect tissues and organisms from other forms of stress and cell death (Marimoto et al., 1997). The presence and increase in expression level of ubiquitin carboxyl-terminal hydrolase isozyme (Table 5, 6 & 8) indicates protein degradation. Thus Hsp could also have been increased to protect against this threatening event.

Hsp synthesis as observed in the present study, could not only be induced after hyperthermia (Homberg et al., 2007), but also following alterations in the intracellular redox environment. Previously studies in patients with major depression (Khanzode et al., 2003) have found serum levels of lipid peroxidation products and superoxide dismutase to be increased. These and present observations of cellular stress in this SERT knockout animal model, where numerous proteins involved in apoptosis (e.g. glyceraldehyde-3-phosphate dehydrogenase, Table 5) and redox regulation (e.g. peroxiredoxin-6, Table 5) are expressed; indicate a role for impaired CNS serotonergic signaling in oxidative stress. It is of interest to note that the oxidative stress proteins were only expressed in the ventral and not in the dorsal hippocampus (Table 5 versus Table 6), which might suggest a further differentiation between these two brain areas.

Membrane proteins constitute a 1/4th to a 1/3rd of the mammalian proteome; however upon comparing the 6 ventral hippocampal membrane proteins (Table 8 & Figure 16) to that of the 42 ventral hippocampal cytosolic proteins (Table 5 & Figure 15), this figure drops substantially. Nevertheless it should be noted that more proteins could have been expressed in this fraction, but due to the size/amount of tissue samples, the yield may have been too small to detect a greater protein profile. The ventral hippocampal membrane protein profile was analogous to that of the cytosolic protein profile, where functional groups consisted of metabolism, cellular stress, cell signaling and structure (Table 5 & 8). Once more the metabolic proteins, specifically involved in energy metabolism, dominated.

The current proteomics study also identified 29 cytosolic proteins in the dorsal hippocampus common to both SERT knockout and normal rats (Table 6 & Figure 15). Interestingly, the majority of these expressed proteins were those with a role in energy metabolism (Table 6). The remainder of the proteins was shown to be involved in neuronal structure, cell signaling and neuroplasticity. While the dorsal hippocampus is suggested to have a preferential role

in certain forms of learning and memory, notably spatial learning (Bannerman et al., 2004), it is interesting to note that the neuroplastic proteins guanine deaminase and dihydropyrimidinase-related protein 2 (Table 5 & 6) were expressed in the minority. However, these proteins were significantly expressed in SERT knockout rats as compared to their controls. A previous characterization study on the SERT knockout rat (Homberg et al., 2007) lacked behavioural tests on memory and learning, such as the Water Morris maze. The present proteomic study is unable to further elucidate the role of dorsal hippocampal proteins in this regard.

The present study identified 29 dorsal hippocampal membrane proteins (Figure 16). The dorsal hippocampal protein profile again mirrored the cytosolic equivalent, where proteins were functionally divided into metabolic, structural, cell signalling, neuroprotection and miscellaneous clusters (Table 8).

In the cytosolic fraction 11 proteins were found to be commonly expressed i.e. both the ventral and dorsal hippocampi contained these proteins (Figure 15). These commonly expressed proteins included alpha enolase, gamma enolase, L-lactate dehydrogenase, dihydropirimidinase-related protein 2, phosphatidylethanolamine-binding protein 1, creatine kinase, actin cytoplasmic, guanine nucleotide-binding protein, ubiquitin carboxyl-terminal hydrolase isozyme L1, heat shock cognate 71 kDa and heat shock protein 60 kDa (Table 5 & 6). However, the majority of cytosolic proteins expressed, were distinct i.e. only found in either the ventral or dorsal hippocampus (Figure 15). Region-specific proteins included cytosol aminopeptidase, superoxide dismutase and malate dehydrogenase in the ventral hippocampus (Table 5), with vacuolar ATP synthase, protein disulfide isomerase and tubulin being expressed in the dorsal hippocampus (Table 6). The pattern of the membrane fraction protein profile was similar to that of the cytosolic fraction protein profile, i.e. the majority of membrane proteins expressed was distinct with only 4 membrane proteins being commonly expressed between the ventral and dorsal hippocampi (Figure 16). These commonly shared proteins included pyruvate dehydrogenase, heat shock protein 60 kDa, F-actin binding capping protein and ubiquitin carboxyl-terminal hydrolase isozyme L1 (Table 8). Unique membrane proteins expressed in the ventral hippocampus were peroxiredoxin-2 and phosphatidylethanolamine-binding protein (Table 8), with synuclein, synaptosoma-associated protein and prohibitin being expressed only in the dorsal hippocampus (Table 8). Interestingly, cellular stress proteins (e.g. peroxiredoxin-2) were once again only expressed in the ventral hippocampus as opposed to the dorsal hippocampus (Table 8).

Considering the findings of this study (Table 3 – 8), various protein groups were expressed that interacted systematically with one another. This observation emphasized that cellular processes, in response to environmental changes, should always be considered as a whole and not in isolation i.e. structure cannot be segregated from metabolism or cell signaling. This interplay of different cellular components can be of importance when considering the current limitations in neuropharmacology.

4.5 Conclusion

The many identified enzymes from intermediary metabolism including those from citric acid cycle, respiratory chain, energy metabolism *etc.* make the results a useful reference for the research of inborn errors of metabolism or other metabolic disorders related to 5HT dysregulation. A series of heat shock proteins could be characterized and this may serve in the investigation of neurodegenerative diseases where aberrant heat shock proteins (Hsp) have been indicated. This also holds for the apoptosis related proteins, signaling proteins, and cytoskeletal elements. Neuronal, synaptosomal and glial proteins listed may serve as corresponding markers and basis for the normalization of brain proteins, in particular when neuronal loss or glial proliferation can be expected. Selective spatially and temporally controlled expression and turn-over of proteins reflects area-specific differentiation and response to environmental stimuli. Individual regions of the brain, in particular across its various developmental stages and pathological disorders, are likely to have distinct protein composition.

In conclusion this work showed that changes in protein levels could be detected in the hippocampus of rats subjected to ENU-mutagenesis resulting in a total non functional serotonin transporter system in the brain. However this dramatic change in central 5HT transmission did not result in marked behavioural changes. A likely reason for this apparent unaffected behavioural state may result from the ability of the animal to adapt and develop new homeostatic set points. Evidence of these adaptations resides within the differential expression of proteins within the SERT KO animal (Table 5, 6 & 8). This encompassed alterations in structural, metabolic and signaling systems.

Finally the current study highlights the importance of a systematic approach in order to generate a more holistic and integrative hypothesis of underlying biological mechanisms driving disease states.

Epilogue

Journal exerpts from one of South Africa's most celebrated, unfortunately posthumously, poetesses Ingrid Jonker:

"If i rest, I think inward, I go mad..."

"...outcast on a cold star, unable to feel anything but an awful helpless numbness."

These are more than autobiographical accounts of a person's mental breakdown, more than a confessional...it is a painful call to the awareness of psychiatric disorders and their treatment...

Through her words,
Through her lines,
Through her thoughts,
Through her phrases…

She opened her soul,
She opened her heart,
She cried out!
All on deaf ear…

Decades later Society Read
Society See
Decades later Society Feel
Society Hear

Today Research abounds…
Hope survives!
Through Her Past
We Can Save Lives…

Recalling the limited existent knowledge of SERT - the major target of affective disorder therapeutics such as SSRI's – further studies are warranted to ultimately unravel the complexities surrounding psychiatric disorders.

REFERENCES

Adamec R., Burton P., Blundell J., Murphy D.L. and Holmes A. Vulnerability to mild predator stress in serotonin transporter knockout mice. Behavioural Brain Research (2006), 170:126 – 140.

Adessi C., Miege C. and Rabilloud T. Two-dimensional electrophoresis of membrane proteins: a current challenge for immobilized pH gradients. Electrophoresis (1997), 18:127 – 135.

Akiskal HS. Mood disorders: introduction and overview. In: Kaplan HI, Sadock BJ, eds. Comprehensive Textbook of Psychiatry. 6th ed. Baltimore, Md: Lippincott, Williams & Wilkins; 1995:1067-1079.

Alam M.N., Szymusiak R., Gong H., King J. and McGinty D. Adenosinergic modulation of rat basal forebrain neurons during sleep and waking: neuronal recording with microdialysis. The Journal of Physiology (1999), 521:679 - 690.

Amara S.G. and Kuhar M.J. Neurotransmitter transporters: recent progress. Annu Rev Neurosci (1993), 16:73 – 93.

Anderson L. and Seilhamer J. A comparison of selected mRNA and protein abundances in human liver. Electrophoresis (1997), 18:533 – 537.

Arbelle S., Benjamin J., Golin M., Kremer I., Belmaker R.H. and Ebstein R.P. Relation of shyness in grade school children to the genotype for the long form of the serotonin transporter promoter region polymorphism. Am J Psychiatry (2005), 160:671 – 676.

Bajo M., Fruehauf J., Kim S.H., Fountoulakis M. and Lubec G. Proteomic evaluation of intermediary metabolism enzyme proteins in fetal Down's syndrome cerebral cortex. Proteomics (2002), 2(11):1539 - 46.

Bannerman D.M., Rawlins J.N., Mchugh S.B., Deacon R.M., Yee B.K., Bast T., Zhang W.N., Othuizen H.H. and Feldon J. Regional dissociations within the hippocampus – memory and anxiety. Neurosci Biobehav Rev (2004), 28 (3):273 – 283.

Bechtold D.A., Rush S.J. and Brown I.R. Localization of the heat-shock protein Hsp70 to the synapse following hyperthermic stress in the brain. J Neurochem. (2000), (2):641 - 6.

Bengel D., Murphy D.L., Andrews A.M. Wichems C.H., Feltner D., Heils A., Mossner R., Westphal H. and Lesch K-P. Altered brain serotonin homeostasis and locomotor insensitivity to 3,4-methylenedioxy-methamphetamine ("ecstacy") in serotonin tranpsorter-deficient mice. Molecular Pharmacology (1998), 53:649 – 655.

Benkert O., Szegedi A., Wetzel H., Staab H.J., Meister W. and Philipp M. Dose escalation vs. continued doses of paroxetine and maprotiline: a prospective study in depressed out-patients with inadequate treatment response. Acta Psychiatr Scand. (1997), 95(4):288 - 96.

Bianchi P. Seguelas M.M, Parini A. and Cambon. Activation of pro-apoptotic cascade by dopamine in renal epithelial cells is fully dependent on hydrogen peroxide generation by monoamine oxidase. Journal of Am. Soc. Nephrol. (2003), 14:855 – 862.

Blakely R.D., Berson H.E., Fremau R.T., Caron M.G., Peek M.M., Prince H.K. and Bradley C.C. Cloning and expression of a functional serotonin transporter from rat brain. Nature (1991), 354:66–70.

Blakely R.D., Felice L. and Hartzell H. Molecular physiology of norepinephrine and serotonin transporters. J Exp Biol (1994), 196:263 – 281.

Bradley S.L., Dodelzon K., Sandhu H.K. and Philibert R.A. Relationship of serotonin transporter gene polymorphisms and haplotypes to mRNA transcription. Am J Med Genet B Neuropsychiatr Genet (2005), 136:58 – 61.

Brookes P.S., Pinner A., Ramachandran A., Coward L., Barnes S., Kim H., and Darley-Usmar V.M. High throughput two-dimensional blue-native electrophoresis: a tool for functional proteomics of mitochondria and signaling complexes. Proteomics. (2002), 2(8):969 - 77.

Charney D.S. Monoamine dysfunction and the pathophysiology and treatment of depression. Journal of Clinical Psychiatry (1998), 59:11.

Cho H.J., Meira-Lima I., Cordeiro Q., Michelon L., Sham P. and Vallada H. Population-based and family-based studies on the serotonin transporter gene polymorphisms and bipolar disorder: a systematic review and meta-analysis. Molecular Psychiatry (2005), 10:771 – 781.

Cooper J.R., Bloom F.E. and Roth R.H. Serotonin (5-Hydroxytryptamine) and Histamine. The Biochemical basis of neuropharmacology, 7th ed., New York: Oxford University Press, (1996), p. 352-409.

Curran S., Purcell S., Craig I., Asherson P. and Sham P. The serotonin transporter gene as a QTL for ADHD, Am J Med Genet B Neuropsychiatr Genet (2005), 134:42 – 47.

Dechant K.L. and Clissold S.P. Paroxetine. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic potential in depressive illness. Drugs (1991), 41(2):225 – 53.

Delbrück S.J., Wendel B., Grunewald I., Sander T., Morris-Rosendahl D., Crocq M.A., Berrettinin W.H. and Hoche M.R. A novel allelic variant of the human serotonin transporter gene regulatory polymorphism. Cytogenetic Cell Genetics (1997), 79 (3 - 4):214 – 220.

Delgado PL, Miller HL, Salomon RM, et al. Tryptophan-depletion challenge in depressed patients treated with desipramine or fluoxetine: implications for the role of serotonin in the mechanism of antidepressant action. Biol Psychiatry. (1999), 46:212-220.

Diagnostic and Statistical Manual of Mental Disorders IV (2000), American Psychiatric Association, Washington D.C.

dos Remedios C.G. and Thomas D.D. An overview of actin structure and actin-binding proteins. Results Probl Cell Differ. (2001), 32:1 – 7.

Drevets W.C. Neuroimaging abnormalities in the amygdala in mood disorders. Ann N Y Acad Sci (2003), 985:420 – 444.

Engidawork E. and Lubec G. Protein expression in Down syndrome brain. Amino Acids (2001), 21:331 - 361.

Frazer A., Gerhardt G. and Daws L. New views of biogenic amine transporter function:implications for neuropsychopharmacology. (1999).

Freidl M., Gulesserian T., Lubec G., Fountoulakis M. and Lubec B. Journal of Neural Transmission (2001), 61:47.

Freud S. General theory on neuroses. In: Freud A, ed. The standard edition of the complete psychological works of Sigmund Freud. London: Hogarth Press (1895).

Fountoulakis M., Schuller El, Hardmeier R., Berndt P. andLubec G. Rat brain proteins: Two-dimensional protein database and variations in the expression level. Electrophoresis (1999), 20:3572 – 3579.

Fountoulakis M. Proteomics: Current technologies and applications in neurological disorders and toxicology. Amino Acids (2001), 21:363 - 381.

Fountoulakis M. and Tak'acs B. Enrichment and proteomic analysis of low-abundance bacterial proteins. Methods Enzymol. (2002), 358:288 - 306.

Fountoulakis M. Application of proteomics technologies in the investigation of the brain. Mass Spectrom Rev (2004), 23:231 -258.

Fountoulakis M., Tsangaris G.T., Maris A. and Lubec G. The rat hippocampus proteome. Journal of Chromatography (2005), 819:115 – 129.

Frude N. Understanding Abnormal Psychology (1998), Malden, MA: Blackwell, Publishers.

Gauss C., Kalkum M., Lowe M., Lehran H. and Klose J. Analysis of the mouse proteome: brain proteins separation by two-dimensional electrophoresis and identification by mass spectrometry and genetic variation. Electrophoresis (1999), 20:575 – 600.

Gerra G., Garofano L., Castaldini L., Rovetto F., Zaimovic A. and Moi G. Serotonin transporter promoter polymorphism genotype is associated with temperament, personality traits and illegal drugs use among adolescents. J Neural Transm (2005), 112:1397 –1410.

Gershon E. S. Genetics. In F. K. Goodwin & K. R. Jamison (Eds.), Manic-depressive illness (1990), 369-401, New York, NY: Oxford University Press.

Giros B. and Caron M.G. Molecular characterization of the dopamine transporter. TRENDS Pharmacol Sci (1993), 14:43 – 49.

Goldberg F., Burdick K.E., Endick C.J. Preliminary randomised, double-blind placebo-controlled trial of pramipexole added to mood stabilisers for treatment-resistant bipolar depression. American Journal of Psychiatry (2004), 16:564 – 566.

Gonda X., Juhasz G., Laszik A., Rihmer Z. and Bagdy G. Subthreshold depression is linked to the functional polymorphism of the 5HT transporter gene. Journal of Affective Disorders (2005), 87:291 – 297.

Greenberg B.D., Tolliver T.J., Huang S.J., Li Q., Bengel D. and Murphy D.L. Genetic variation in the serotonin transporter promoter region affects serotonin uptake in human blood platelets. American Journal of Medical Geneteics (1999), 88:83 -87.

Griffin T.J. and Aebersold R. Advances in proteome analysis by mass spectrometry. J Biol Chem (2001), 276:45497 – 45500.

Guelfi J.D., White C. and Hackett D. Effectiveness of venlafaxine in patients hospitalized for major depression and melancholia. J Clin Psychiatry (1995), 56:450 – 458.

Hanna G.L., Himle J.A., Curtis G.C., Koram D.Q., Weele J.V., Leventhal B.L. and Cook E.H. Serotonin transporter and seasonal variation in blood serotonin in families with obsessive–compulsive disorder. Neuropsychopharmacology (1998), 18:102 – 111.

Hariri A.R., Drabant E.M., Munoz K.E., Kolachana B.S., Mattay V.S., Egan M.F. and Weinberger D.R. A susceptibility gene for affective disorders and the response of the human amygdale. Arch Gen Psychiatry (2005), 62:146 –152.

Heim C., Owens M.J., Plotsky P.M. and Nemeroff C.B. The role of early adverse life events in the etiology of depression and posttraumatic stress disorder. Focus on corticotropin-releasing factor. Psychobiology of Posttraumatic Stress Disorder. Ann. N.Y. Acad. Sci. (1997), 821:194 – 207.

Heils A., Teufel Al, Petri S., Stober G., Riederer P., Bengel D. and Lesch K-P. Allelic variation of human serotonin transporter gene expression. Journal of Neurochemistry (1996), 66 (6):2621 – 2624.

Heils A., Lesch K-P. And Mossner R. The human serotonin transporter gene polymorphism: basic research and clinical implications. Journal of Neural Transmission (1997), 104 (10):1005 – 1014.

Heinz A., Jones DW., Mazzanti C., Goldman D. A relationship between serotonin transporter genotype and in vivo protein expression and alcohol neurotoxity. Biol Psychiatric (1999) 47:643 – 649.

Hoefgen B., Schulze T.G., Ohlraun S., von Widdern O., Hofels S. and Gross M. The power of sample size and homogenous sampling: association between the 5-HTTLPR serotonin transporter polymorphism and major depressive disorder. Biological Psychiatry (2005), 57**:**247 – 251.

Hoffman B.J., Mezey E. and Brownstein M.J. Cloning of a serotonin transporter affected by antidepressants. Science (1991), 254:579 – 580.

Holmes A., Murphy D.L. and Crawley J.N. Reduced aggression in mice lacking the serotonin transporter. Psychopharmacology (2002 a), 161:160 – 167.

Holmes A., Yang J.L., Murphy D.L. and Crawley J.N. Evalution of antidepressant-related behavioural responses in mice lacking the serotonin transporter. Neuropsychopharmacology (2002 b), 27:914 – 923.

Holmes A., Li Q., Murphy D.L., Gold E. and Crawley J.N. Abnormal anxiety-related behaviour in serotonin transporter null mutant mice: the influence of genetic background. Genes Brain Behaviour (2003), 2:365 – 380.

Homberg J.R., Pattij T., Janssen M.C., Ronken E., De Boer S.F., Schoffelmeer A.N. and Cuppen E, Serotonin transporter deficiency in rats improves inhibitory control but not behavioural flexibility. European Journal of Neuroscience (2007), 7:2066 – 2073.

Invernizzi R., Bramante M. and Samanin R. Extracellular concentrations of serotonin in the dorsal hippocampus after acute and chronic treatment with citalopram. Brain Res (1995), 696:62 – 66.

Iversen L.L. Dopamine receptors in the brain. Science. (1975), 188(4193):1084 - 9.

Jackson S.W. Melancholia and depression: from Hippocratic times to modern times. New Haven Conn: Yale University Press (1986).

Janicak P.G., Davis J.M., Preskorn S.H. and Ayd F.J. Principles and practice of psychopharmacotherapy (1993), Baltimore, MD: Williams & Wilkins.

Johnson C., Willeit M., Levitan R., Partonen T., Smedh C. and Del Favero J. The serotonin transporter promoter repeat length plymorphism, seasonal affective disorder and seasonality. Psychological Medicine (2003), 33:785 – 792.

Kanai Y. and Hediger M.A. The glutamate neutral amino acid transporter family SLC1. Pfluger Archives (2004), 477:469 – 479.

Kendler K.S. Twin studies of psychiatric illness : an update. Arch Gen Psychiatry.(2001),58 : 1005 – 1014.

Kessler R.C., Nelson C.B., McGonagle K.A., Liu J., Swartz M. and Blazer D.G. Comorbidity of DSM-III-R major depressive disorder in the general population: results from the U.S. National Comorbidity Survey. British Journal of Psychiatry (1996), 168:7 – 30.

Khanzode S.D., Dakhale G.N., Khanzode S.S., Saoji A. and Palasodkar R. Oxidative damage and major depression: the potential antioxidant action of selective serotonin reuptake inhibitors. Redox Rep. (2003), 8(6):365 - 70.

Kilic F., Murphy D.L. and Rudnick G. A human serotonin transporter mutation causes constitutive activation of transport activity. Molecular Pharmacology (2003), 64:440 -446.

Kim S.H., Dierssen M., Ferreres J.C., Fountoulakis M. and Lubec G. Journal of Neural Transmission (2001), 61:272.

Korolainen M.A., Yoo B.C., Fountoulakis M., Mitrova E. and Lubec G. Electrophoresis (2002), 23:1245.

Krapfenbauer K., Engidawork E., Cairns N., Fountoulakis M. and Lubec G. Aberrant expression of peroxiredoxin subtypes in neurodegenerative disorders. Brain Research (2003), 967:152 – 160.

Langen H., Gray C., Röder D., Juranville J-F., Takács B., Fountoulakis M. Electrophoresis (1997), 18:1184 – 1192.

Langen H., Berndt P., Röder D., Cairns N., Lubec G. and Fountoulakis M. Electrophoresis (1999), 20:907.

Lee H.J., Lee M.S., Kang R.H., Kim H., Kim S.D. and Kee B.S. Influence of the serotonin transporter promoter gene polymorphism on susceptibility to posttraumatic stress disorder. Depress Anxiety (2005), 21:135–139.

Lesch K.P., Wolozin B.L., Murphy D.L. and Reiderer P. Primary structure of the human platelet serotonin uptake site: identity with the brain serotonin transporter. Journal of Neurochemistry (1993), 60:2319 -2322.

Lesch K.P., Bengel D., Heils A., Sabol S.Z., Greenberg B., Petri S., Benjamin J., Muller C.R., Hamer D.H. and Murphy D.L. Association of anxiety-related traits with a polymorphism in the serotonin transporter gene regulatory region. Science (1996), 274:1527 – 1531.

Lesch K.P., Meyer J., Glatz K., Flugge G., Hinney A., Hebebrand J., Klauck S.M., Poutska A, Poutska F., Bengel D., Mossner R., Riederer P. and Heils A. The 5-HT transporter gene-linked polymorphic region in evolutionary perspective: Alternative biallelic variation in rhesus monkeys. Journal of Neural Transmission (1997), 104:1259 – 1266.

Li Q. Cellular and molecular alterations in mice with deficient and reduced serotonin transporters. Molecular Neurobiology (2006), 34(1):51 – 65.

Lin P.Y. and Tsai G.. Association between serotonin transporter gene promoter polymorphism and suicide: Results of a meta-analysis. Biol Psychiatry (2004), 55:1023 – 1030.

Lira A., Zhou M., Castanon N., Ansorge M.S., Gordon J.A., Francis J.H. Bradley-Moore M., Lira J., Underwood M.D. Arango V., Kung H.F., Hofer M.A., Hen R. and Gingrich J.A. Altered depression-related behaviours and functional changes in the dorsal raphe nucleus of serotonin transporter-deficient mice. Biological Psychiatry (2003), 54:960 – 971.

Little K.Y., McLaughlin D.P., Zhang L., Livermore C.S., Dalack G.W.and McFinton P.R. Cocaine, ethanol, and genotype effects on human midbrain serotonin transporter binding sites and mRNA levels. American Journal of Psychiatry (1998), 155:207 – 213.

Lubec G., Krapfenbauer K. and Fountoulakis M. Proteomics in brain research: potentials and limitations. Prog. Neurobiol (2003), 69:193 - 211.

Mann M., Hendrickson R.C. and Pandey A. Analysis of proteins and proteomes by mass spectrometry. Annu Rev Biochem (2001), 70:437 – 473.

Manzerra P., Rush S.J. and Brown I.R. Temporal and spatial distribution of heat shock mRNA and protein (hsp70) in the rabbit cerebellum in response to hyperthermia. J Neurosci Res. (1993), 36:480 – 490.

Manzerra P., Rush S.J. and Brown I.R. Tissue-specific differences in heat shock protein hsc 70 and hsp 20 in the control and hyperthermic rabbit. J. Cell Physiol. (1997), 170 : 130-137.

Malison R.T., Price L.H., Berman R., van Dyck C.H., Pelton G.H. and Carpenter L. Reduced brain serotonin transporter availability in major depression as measured by [123I]-2 beta-carbomethoxy-3 beta-(4-iodophenyl)-tropane and single photon emission computed tomography. Biological Psychiatry (1998), 44:1090 – 1098.

Matus A., Bernhardt R., Hugh – Jones T. High molecular weight microtubule – associated proteins are preferentially associated with dendritic microtubules in brain. Proc Natl Acad Sci USA (1981 May); 78 (5) : 3010 – 3014.

MHIC: **www.mentalhealthsa.co.za**

Michaelovsky E., Frisch A., Rockah R., Peleg L., Magal N., Shohat M. and Weizman R. A novel allele in the promoter region of the human serotonin transporter gene, Molecular Psychiatry (1999), 4 (1):97 – 99.

Morimoto R.I., Kline M.P., Bimston D.N. and Cotto J.J.The heat- shock response : regulation and function of heat shock proteins and molecular chaperones. Essays Biochem. (1997) 32 : 17 – 29.

Mortensen O.V., Thomassen M., Larsen M.B., Whittemore S.R. and Wibory O. Functional analysis of a novelhuman serotonin transporter gene promoter in immortalized raphe cells. Brain Res Mol Brain Res (1999), 68:141 – 148.

Mullins L.J. and Mullins J.J. Insight from the rat genome. Genome Biology (2004), 5:221.

Murray C.J.L. and Lopez A.D. Evidence-based health policy--lessons from the Global Burden of Disease Study. Science (1996), 274 (5288):740 -743.

Nakamura M., Ueno S., Sano A. and Tanabe H. The human serotonin transporter gene linked polymorphism (5-HTTLPR) shows ten novel allelic variants. Mol Psychiatry (2000), 5:32 – 38.

Nelson P.J. and Rudnick G. Journal of Biological chemistry (1979), 254:10084 – 10089.

Nelson P.J. and Rudnick G. Coupling between platelet 5 – hydroxtryptamine and potassium transport. J. Biol. Chem. (1979) 254: 10084 – 10089.

Nobile M., Begni B., Giorda R., Frigerio A., Marino C. and Molteni M. Effects of serotonin transporter promoter genotype on platelet serotnin transporter functionality in depressed children and adolescents, Journal of America Acad Child Adolescent Psychiatry (1999), 38:1396 – 1402.

Owens M.J. and Nemeroff C.B. Role of serotonin in the pathophysiology of depression: focus on the serotonin transporter. Clinical Chemistry (1994), 40:288 – 295.

Owens M.J. and Nemeroff C.B. The serotonin transporter and depression. Depress Anxiety. (1998), 8 (1):5-12.

Pandey A. and Mann M. Proteomics to study genes and genomes. Nature (2000), 405, 837 –846.

Perkins D.N., Papin D.J., Creasy D.M. and Cottrell J.S. Probability based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis (1999), 20:3551 – 3567.

Pezawas L., Meyer-Lindenberg A., Drabant E.M. 5-HTTLPR polymorphism impacts human cingulate-amygdala interactions: a genetic susceptibility mechanism for depression. Nat Neurosci. 2005, 8(6):828 - 34.

Plotsky P.M., Owens M.J. and Nemeroff C.B. Psychoneuroendocrinology of depression. Hypothalamic-pituitary-adrenal axis. Psychiatr Clin North Am (1998), 21:293 – 307.

Powchik P., Davidson M., Haroutunian V., Gabriel S.M., Purohit D.P., Perl D.P., Harvey P.D. and Davis K.L. Postmortem studies in schizophrenia. Schizophr Bull. (1998), 24(3):325 - 41.

Rabilloud T., Strub J.M, Carte N., Luche S., Van Dorsselaer A., Lunardi J., Giegé R. and Florentz C. Comparative proteomics as a new tool for exploring human mitochondrial tRNA disorders. Biochemistry. (2002), 41(1):144 - 50.

Ramamoorthy S., Bauman A.L., Moore K.R., Han H., Yang-Feng T. and Chang A.S. Antidepressant- and cocaine-sensitive human serotonin transporter: molecular cloning, expression, and chromosomal localization. Proc Natl Acad Sci (1993), 90:2542 – 2546.

Riason C.L. and Miller A.H. When not enough is too much: The role of insufficient glucocorticoid signaling in the pathophysiology of stress-related disorders. Am J Psychiatry (2003), 160:1554 – 1565.

Rudnick G. and Nelson P.J. Biochemistry (1978), 17:4739 - 4742.

Rudnick G. and Clark J. From synapse to vesicle: the reuptake and storage of biogenic amine neurotransmitters. Biochim Biophys Acta (1993), 1144:249 – 263.

Rudnick G. Active transport of 5-hydroxytryptamine by plasma membrane vesicles isolated from human blood platelets. J Biol Chem (1977), 252:2170 -2174.

Rudnick G. Journal of Bioenergy Biomemb (1998), 30:173 – 185.

Rudnick G. Neurotransmitter transporters, structure, function and regulation, 2nd edition (2000), pp. 25 – 52, Humana Press, Totowa.

Sachar E.J. and Baron M. The biology of affective disorders. Ann Rev Neurosci (1978), 2:505 – 518.

SADAG: **www.sadag.co.za**

Saier M.H. Genome archeology leading to the characterization and classification of transport proteins. Curr Opin Microbiol (1999), 2:555 – 561.

Sanderson K and Andrews G. Why does the burden of disease persist? Relating the burden of anxiety and depression to effectiveness of treatment. Bull World Health Org. (2000); 78: 446 – 454.

Sapolsky R.M. and Meaney M.J. Maturation of the adrenocortical stress response: neuronendocrine control mechanisms and the sttress hyporesponsive period. Brain Research (1986), 396:64 -76.

Schatzberg AF. Current status of managing depression: is there a need for new treatment strategies? Program and abstracts of the 153rd Annual Meeting of the American Psychiatric Association; May 13-18, 2000; Chicago, Ill: Abstract 13A.

Siegel G.J., Agranoff B.W., Fisher S.K., Albers R.W. and Uhler M.D. Understanding the neuroanatomical organization of serotonergic cells in the brain provides insight into the functions of this neurotransmitter**.** Basic Neurochemistry**,** sixth edition (1991), Lippincott Williams and Wilkins.

Siever L. and Davis K. Toward a dysregulation Hypothesis of depression. American Journal of Psychiatry (1985), 142:1017 - 1031.

Simpson R.J. and Dorow D.S. Cancer proteomics: from signaling networks to tumor markers. TRENDS Biotechnol. (2001), 19:S40 – 8.

Smits B.M. and Cuppen E. Rat genetics: the next episode. TRENDS in Genetics (2006), 22: 232 - 240.

Smits B.M., Mudde J.B., van de Belt J., Verheul M., Olivier J., Homberg J., Guryev V., Cools A.R., Ellenbroek B.A., Plasterk R.H. and Cuppen E. Generation of gene knockouts and mutant models in the laboratory rat by ENU-driven target-selected mutagenesis. Pharmacogenetic Genomics (2006), 16:159 – 169.

Sora I., Wichems C., Takahashi N., Li X.P., Zeng Z., Revay R., Lesch K.P., Murphy D.L. and Uhl G.R. Cocaine reward models: conditioned place preference can be established in dopamine and in serotonin tranpsorter knockout mice. Proc Natl Acad Sci (1998), 13:7699 – 7704.

Steen H. and Mann M. The ABC's (and XYZ's) of peptide sequencing. Nat Rev Mol Cell Biol (2004), 5:699 – 711.

Stevens S.M., Zharikova A.D. and Prokai L. Proteomic analysis of the synaptic plasma membrane fraction isolated from rat forebrain. Molecular Brain Research (2003), 117:116 – 128.

Sugden S.G., Kile S.J. and Hendren R.L. Neurodevelopmental pathways to aggression: a model to understand and target treatment in youth. Journal of Neuropsychiatry and Clinical Neuroscience (2006), 18:302 – 317.

Sutcliffe J.S., Delahanty R.J., Prasad H.C., McCauley J.L., Han Q., Jiang L., et al. Allelic heterogeneity at the serotonin transporter locus (SLC6A4) confers susceptibility to autism and rigid-compulsive behaviours. American Journal of Human Genetics (2005), 77:265 – 279.

Suzuki M., Hirata H., Yamamura I., Yasuda K. Separate cis – acting DNA elements control cell type – and tissue specific expresion of collagen binding molecular chaperone HSP 47. J. Biol. Chem. (1999), 274(50):35703 – 35710

Talvenheimo J.,Fishkes H., Nelson P.J., Rudnick G. The serotonin transporter-imipramine reseptor : Different sodium requirements for imipramine binding and serotonin translocation.J. Biol. Chem. (1983), 258 : 6115 – 6119.

Thomas D.R., Nelson D.R. and Johnson A.M. Biochemical effects of the antidepressant paroxetine, a specific 5-hydroxytryptamine uptake inhibitor. Psychopharmacology (1987), 93:193 – 200.

Torres G.E. and Caron M.G. Center stage for the serotonin transporter: a gain-of-function polymorphism in persons with obsessive-compulsive disorder. Mol Pharmacol (2003), 64:196 - 198.

Vailou V., Balasse L., Dumas S., Giros B. and Gautron S. Neurochemical characterization of pathways expressing plasma membrane transporter in the brain. Neuroscience (2006), 14:17110048.

Wagstaff A.J., Omrod D. and Spencer C.M. Tianeptine: a review of its use in depressive disorders. CNS Drugs (2001), 15 (3):231 – 59.

Wang X., Wu H. and Miller A.H. Interleukin-1 alpha-induced activation of p38 mitogen-activated kinase inhibits glucocorticoid receptor function. Mol Psychiatry (2004), 9:65 –75.

Weitzdoerfer R., Dierssen M. Fountoulakis M. and Lubec G. Journal of Neural Transmission (2001), 61:59.

Welch W.J. and Suhan J.P. Cellular and biochemical events in mammalian cells during and after recovery from physiological stress. J Cell Biol. (1986), 103(5):2035 - 52.

World Health Organization Burden of disease project: mortality and DALYs. 2002 b. **www.who.int/whosis**

Wong D.T., Bymaster F.P. and Dreshfield-Ahmad L. Effectiveness of duloxetine and venafaxine in producing increases in extracellular levels of serotonin and norepinephrine in brain. Presented at the American College of Neuropsychopharmacology (2000).

Yamada M. and Yamada M. Identification of molecular systems responsible for the therapeutic effect of antidepressants. Breathing, Feeding and Neuroprotection (2007).

Yang J.W., Czech T. and Lubec G. Proteomic profiling of human hippocampus. Electrophoresis (2004), 25:1169 – 1174.

Yang J.W., Juranville J.F., Höger H., Fountoulakis M. and Lubec G. Molecular diversity of rat brain proteins as revealed by proteomic analysis. Molecular Diversity (2005), 9:385 – 396.

Yirmiya N., Pilowsky T., Nemanov L., Arbelle S., Feinsilver T., Fried I. and Ebstein R.P. Evidence for an association with the serotonin transporter promoter region polymorphism and autism. Am J Med Genet (2001), 105:381 – 386.

You J.S., Hu S.Y., Chen B. and Zhang H.G. Serotonin transporter and tryptophan hydroxylase gene polymorphisms in Chinese patients with generalized anxiety disorder. Psychiatr Genet (2005), 15:7–11.

Zan Y., Haag J.D., Chen K.S. Shepel L.A., Wigington D., Wang Y.R., Hu R., Lopez-Guajardo C.C, Brose H.L., Porter K.L., Leonard R.A. Hitt A.A., Schommer S.L., Elegbede A.F. and Gould M.N. Production of knockout rats using ENU mutagenesis and a yeast-based screening assay. Nat. Biotech (2003), 21:645 – 651.

Zhou F.C., Sari Y. and Zhang J.K. Expression of serotonin transporter protein in developing rat brain. Brain Res Dev Brain Res (2000), 119:33 – 45.