Evaluating the antidepressant-like properties of *Sceletium tortuosum*, alone and as adjunctive treatment

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“Science without religion is lame.
Religion without science is blind."

Albert Einstein

ABSTRACT

BACKGROUND: Millions of people worldwide suffer from major depressive disorder (MDD) which can cause physical and emotional suffering for patients and their loved-ones. Unfortunately current treatment fails in approximately a third of MDD patients. This may be attributed to numerous side effects, delayed onset of action, and ineffective targeting of the many different, interrelated biological systems. This includes structural and functional brain alterations driven especially by decreased brain-derived neurotrophic factor (BDNF), and neurotransmitter abnormalities such as reduced monoamines (serotonin (5-HT), dopamine (DA) and norepinephrine (NE)). Evidently, new and more effective treatments are urgently needed. Fortunately, complementary medicines like plants and herbs with antidepressant effects offer untapped potential.

This study set out to evaluate the antidepressant potential of the South African plant, *Sceletium tortuosum* (L.) N.E.Br. (Zembrin®) (ST), which displays diverse pharmacological attributes that offer potential antidepressant activity, e.g. 5-HT transporter (SERT) inhibition, upregulation of vesicular monoamine transporter 2 (VMAT-2), mild MAO-A inhibition, and inhibition of phosphodiesterase 4 (PDE4). These mechanisms have almost all been studied *in vitro,* leaving an urgent need for *in vivo* studies. Furthermore, ST has not been tested in combination with known antidepressant compounds to evaluate its potential as a possible augmentative therapy for MDD.

AIM: To use acute and sub-chronic treatment paradigms with biobehavioural parameters to evaluate the antidepressant-like properties of ST, alone and in combination with the selective 5-HT reuptake inhibitor (SSRI), escitalopram (ESC), in the FSL rat.

METHODOLOGY: 1) A fingerprint analysis of the alkaloid profile of ST was done using an ultra-performance liquid chromatography - tandem mass spectrometer (UPLC-MS). 2) Behavioural confirmation of the FSL model of MDD. 12 saline-treated FSL and 6 Flinders Resistant Line (FRL) rats (reference control) were used to confirm the depressive phenotype of the FSL rat, using the open field test (OFT) and forced swim test (FST). 3) Acute dose response studies in FSLs, using a 3-tier dose response with escitalopram oxalate (ESC) (3 groups (n = 10); 5, 10 or 20 mg/kg), and a 5-tier dose response with ST (5 groups (n = 10); 5, 10, 25, 50 or 100 mg/kg). Treatment spanned over 24 hours, followed by the OFT and FST. The results were used to establish a low-dose of ESC, and a therapeutic dose of ST. 4) A sub-chronic treatment response study wherein four groups of FSL rats (n = 12) received a) saline, b) low dose of ESC, c) a therapeutic dose of ST or d) combination therapy of ESC + ST, over 15 days. After the OFT and FST on days 13 and

15, animals were decapitated, with hippocampus and frontal cortex samples harvested for analysis of monoamines and BDNF.

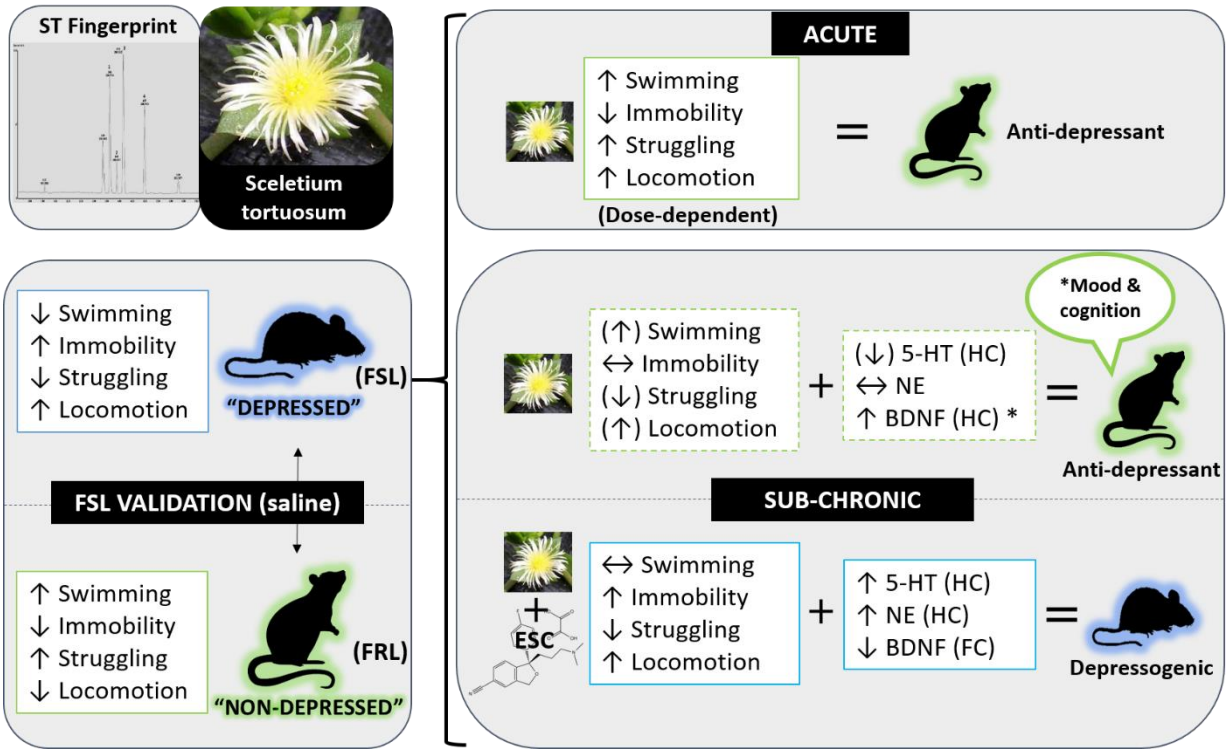
RESULTS: Four main alkaloids were identified and quantified in an UPLSC-MS chromatogram. FSL rats presented with significantly decreased swimming and struggling, and increased immobility versus FRL controls, thus reaffirming its face validity as a rodent model of MDD. ST and ESC showed dose-dependent antidepressant responses following acute treatment. ESC 5 mg/kg (ESC 5) was chosen as a low dose of ESC and ST 50 mg/kg (ST 50) was selected as the therapeutic dose of ST. Sub-chronically, ESC 5 and ST 50 alone displayed similar neurochemical changes with no significant antidepressant-like effects. ESC+ST significantly increased hippocampal 5-HT and NE, and locomotor activity, which is normally deemed positive in MDD treatment. However, increased immobility and decreased struggling are indicative of depressogenic effects. Furthermore, hippocampal 5-HT was significantly increased, possibly due to synergistic serotonergic effects of ST 50 and ESC 5. A hypersensitive inhibitory 5-HT_{1A} response, characteristic of FSL rats, could have prompted 5-HT_{1A} receptor-mediated "5-HT behavioural syndrome" presenting as passive coping in the FST and increased locomotion in the OFT. The apparent lack of antidepressant effects in the alone treatment groups may be due to delayed response attributed to delayed desensitization of inhibitory 5-HT_{1A} receptors, typical of SSRIs. ST 50 alone increased hippocampal BDNF indicating potential of ST in the treatment of impaired cognition, ESC + ST reduced frontal cortical BDNF levels.

CONCLUSION: ST and ESC induced dose-dependent antidepressant-like effects after acute treatment, however not after sub-chronic treatment. Combined sub-chronic treatment with ESC + ST appears to be depressogenic, displaying significant serotonergic activity at behavioural and neurochemical levels. ST 50 increasing hippocampal BDNF levels suggests promise in the treatment of mood and cognitive disorders.

Keywords

Major depressive disorder (MDD), Sceletium tortuosum, forced swim test, open field test, 5-HT_{1A}, 5-HT_{2A}, brain-derived neurotrophic factor (BDNF), neuroplasticity, serotonin behavioural syndrome.

Graphical abstract



CONGRESS PROCEEDINGS

Excerpts from this study were presented as a podium presentation at the 2019 SANS symposium as part of the Biological Psychiatry Congress in Cape Town, South Africa (20 – 23 September 2019) under the title:

Preliminary evidence of the antidepressant-like properties of *Sceletium tortuosum* (Zembrin™) in a genetic animal model of depression

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δ_2- opioid	Delta-opioid 2 receptor
5-HIAA	5-hydroxyindole acetic acid
5-HMT	5-hydroxy-Nω-methyptryptamine oxalate (internal standard)
5-HT	Serotonin
5-HT_{1A}	Serotonin 1A receptor
5-HT_{2A}	Serotonin 2A receptor
5-HT_{2C}	Serotonin 2C receptor
5-HTTLPR	Serotonin-transporter-linked polymorphic region
8-OH-DPAT	8-hydroxy-2-(di-n-propylamino) tetralin
95% CI	95% confidence interval
AC	Adenylate cyclase
ACh	Acetylcholine
AChE	Acetylcholinesterase
ACTH	Adrenocorticotropic hormone
Animcare	Animal Care, Ethics committee
ANOVA	Analysis of variance
AREC	Animal Research Ethics Committee
ARRIVE	Animal Research: Reporting of *In Vivo* Experiments
BDNF	Brain-derived neurotrophic factor
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CBT	Cognitive behavioural therapy
c-fos	Proto-oncogene
cGMP	Cyclic guanosine monophosphate
CNS	Central nervous system

List of Abbreviations

CPP	Conditioned Place Preference
CREB	cAMP response element-binding protein
CRF	corticotropin releasing factor
CSF	Cerebrospinal fluid
CYP2D6	Cytochrome P450 2D6
CYP3A4	Cytochrome P450 3A4
D_1	Dopamine 1 receptor
D_2	Dopamine 2 receeptor
DA	Dopamine
DAT	Dopamine transporter
DFP	Diisopropyl fluorophosphates
DOPA	Dihydroxyphenylalanine
DRN	Dorsal raphe nucleus
DSM-V	Diagnostic and Statistical Manual of Mental Disorders
e.g.	Example
ECT	electroconvulsive therapy
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EP4	Prostaglandin EP4 receptor
EPM	Elevated Plus Maze
EPSP	Excitatory postsynaptic potential
ESC 5	Escitalopram 5 mg/kg
ESC	Escitalopram
Fig.	Figure
fMRI	Functional magnetic resonance imaging
FRL	Flinders Resistant Line rat
FSL	Flinders Sensitive Line rat

List of Abbreviations

FST	Forced swim test
g	Gram
GABA	Gamma-aminobutyric acid
$G_{i/o}$	Inhibitory G-protein
GIRK	G-protein inwardly rectifying potassium
GLP	Good Laboratory Practice
GLU	Glutamate
HIV/AIDS	Human Immunodeficiency Virus, Acquired Immunodeficiency Syndrome
HPA axis	Hypothalamic–pituitary–adrenal axis
HPLC-EC	High-performance liquid chromatography with electrochemical detection
IDO	Indolamine-2,3-dioxygenase
IL	Interleukin
IPSP	Inhibitory postsynaptic potential
K^+	Potassium
kg	Kilogram
LC	Locus coeruleus
LPS	Lipopolysaccharides
mAChR	muscarinic acetylcholine receptor
MAO	Monoamine oxidase
MAOI	Monoamine oxidase inhibitors
MAPK/ERK	Mitogen-activated protein kinases, extracellular signal-regulated kinases
MDD	Major depressive disorder
ml	Millilitres
N/O	Novel object
NA'ergic	Noradrenergic
NE	Norepinephrine
NET	Norepinephrine transporter

NGF	Nerve growth factor
NHREC	National Health Research Ethics Council
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
nORT	Novel object recognition test
NPY	Neuropeptide Y
NRI	Norepinephrine reuptake inhibitor
NSAIDs	Non-steroidal anti-inflammatory drugs
NWU	North-West University
OFT	Open field test
PBS	Phosphate buffered solution
PCDDP	Pre-clinical Drug Development Platform
PDE4	Phosphodiesterase 4
PKA	Protein kinase A
PVC	Polyvinyl chloride
R^2	Coefficient of Determination
Rcf	Relative centrifugal force
SADAG	South African Depression and Anxiety Group
SANAS	South African National Accreditation System
SAVC	South African Veterinary Council
SD	Sprague Dawley rat
SEM	Standard error of the mean
SERT	Serotonin transporter
SJW	St John's wort
SLC6A4	Solute Carrier Family 6 Member 4
SNRI	Serotonin-norepinephrine reuptake Inhibitor
SOP	Standard operating procedures

List of Abbreviations

SSRI	Selective serotonin reuptake inhibitor
ST 50	*Sceletium tortuosum* 50 mg/kg
ST	*Sceletium tortuosum*
SwHi	Swim high-active
Sw-Lo	Swim low-active
TCA	tricyclic antidepressant
TH	Tyrosine hydroxylase
TRD	Treatment resistant depression
TrkB	Tropomyosin receptor kinase B
TUT	Tshwane University of Technology
UPLC-MS	Ultra performance liquid chromatography - tandem mass spectrometer
viz.	Namely
VMAT-2	Vesicular monoamine transporter 2
VTA	Ventral tegmental area
WHO	World Health Organization
α-AR	alpha adrenoceptors
β-AR	beta adrenoceptors
μ- opioid,	Mu-opioid receptor

GLOSSARY

Acupuncture	A system of complementary medicine during which fine needles are inserted in the skin at specific points along what are considered to be lines of energy (meridians), used in the treatment of various physical and mental conditions.
Anergia	Abnormal lack of energy
Anhedonia	Inability to feel pleasure in normally pleasurable activities
Anxiolytic	Medication used to reduce anxiety
Apathy	Lack of interest, enthusiasm, or concern
Augmentation	Combination of two or more drugs to achieve better treatment results
Autoreceptors	Type of receptor located in the membranes of presynaptic nerve cells. It serves as part of a negative feedback loop in signal transduction. It is only sensitive to the neurotransmitters or hormones released by the neuron on which the autoreceptor sits
Circadian rhythm	The natural cycle of physical, mental, and behavior changes that the body goes through in a 24-hour cycle.
Complementary medicine	Any of a range of medical therapies that fall beyond the scope of conventional medicine but may be used alongside it in the treatment of disease and ill health.
Construct validity	Neurochemical abnormalities resembling that seen in the human disorder

Cytokines	Any of a number of substances, such as interferon, interleukin, and growth factors, which are secreted by certain cells of the immune system and have an effect on other cells
Cytoprotection	Process by which chemical compounds provide protection to cells against harmful agents.
Depressogenic	Induced depression
Desensitization	Decreased responsiveness that occurs with repeated or chronic exposure to agonist
Diathesis	A tendency/predisposition to suffer from a particular medical condition
Discrimination index (DI)	DI = $\frac{\text{(time spent at novel object – time spent at familiar object)}}{\text{(time spent at the novel object + time spent at familiar object)}}$
Ethnopharmacology	The scientific study of ethnic groups and their use of drugs. It is distinctly linked to plant use, ethnobotany, as this is the main delivery of pharmaceuticals.
Face validity	Behaviour resembling symptoms of the human disorder
Fingerprint analysis	Methodology for the quality control of herbal samples. It is applied to identify closely related plant species, to detect adulterations, to control the extraction process or to study the quality of a finished product
Gene-X-environment	A disorder can be produced when a person with a genetic predisposition toward development of a disorder is exposed to environmental stressors
Heteroreceptors	A receptor regulating the synthesis and/or the release of mediators other than its own ligand.

Hyperreflexia	Overactive or overresponsive reflexes, e.g. twitching or spastic tendencies. Indicative of upper motor neuron disease as well as the lessening or loss of control ordinarily exerted by higher brain centers of lower neural pathways
Hyperthermia	Condition of having a body temperature greatly above normal.
Hypervigilance	Enhanced state of sensory sensitivity accompanied by an exaggerated intensity of behaviours whose purpose is to detect activity. Hypervigilance may bring about a state of increased anxiety which can cause exhaustion.
Iatrogenic	Relating to illness caused by medical examination or treatment
Immobility (FST)	Floating behaviour in the forced swim test (FST) during which a rodent only makes the necessary movements to stay afloat
In vitro	studies are performed with microorganisms, cells, or biological molecules outside their normal biological context
In vivo	The effects of various biological entities as tested on whole, living organisms or cells, usually animals, including humans, and plants
Interleukin	Glycoproteins produced by leucocytes for regulating immune responses.
Locomotor activity	General movement of an organism from one area to another
Mydriasis	Dilation of the pupil of the eye
Myoclonus	Quick, involuntary muscle jerk.

Neurogenesis	Neurogenesis refers to neurons that are newly born in certain parts of the brain which includes the hippocampus, olfactory bulb and possibly the striatum in adults
Neuroplasticity	Neuroplasticity is the ability of the brain to structurally adapt to internal and external changes, while synaptic plasticity is the ability to sense, access and store information, and to change synaptic transmission according to subsequent stimuli
Neurotransmitter	a chemical substance which is released at the end of a nerve fibre by the arrival of a nerve impulse and, by diffusing across the synapse or junction, effects the transfer of the impulse to another nerve fibre, a muscle fibre, or some other structure.
Neurotrophins	A family of proteins that induce the survival, development, and function of neurons. They belong to a class of growth factors, secreted proteins that are capable of signaling particular cells to survive, differentiate, or grow.
Oral gavage	Liquid compounds may be administered directly into the stomach of mice and rats via this technique. In this procedure a stainless steel bulb tipped gavage needle or a flexible cannula or tube is attached to a syringe and used to deliver the compound into the stomach.
Oxidative stress	An imbalance between free radicals and antioxidants in your body. Free radicals are oxygen-containing molecules with an uneven number of electrons. The uneven number allows them to easily react with other molecules.
Polymorphisms	Genetic variations
Postsynaptic	Occurring after synapsis Of, occurring in, or being a nerve cell by which a wave of excitation is conveyed away from a synapse a postsynaptic membrane.
Preclinical research	A study to test a drug, a procedure, or another medical treatment in animals. The aim of a preclinical study is to collect data in support of the safety and efficacy of the new treatment
Predictive validity	Model responds to treatments generally used for the disorder
Presynaptic	Relating to or denoting a nerve cell that releases a transmitter substance into a synapse during transmission of an impulse

Psychogenic	Having a psychological origin or cause rather than a physical one
Psychomotor retardation	Slowing-down of thought and a reduction of physical movements in an individual. Can cause a visible slowing of physical and emotional reactions, including speech and affect.
Serotonin syndrome	A group of symptoms that may occur with the use of certain serotonergic medications or drugs
Struggling (FST)	Climbing or struggling behaviour in an upward direction against the sides of the swim chamber during the forced swim test (FST)
Swimming (FST)	Movement across the water surface, crossing quadrants of the swim chamber in the forced swim test (FST)
Synapse	A junction between two nerve cells, consisting of a minute gap across which impulses pass by diffusion of a neurotransmitter.
Synergistic	Relating to the interaction or cooperation of two or more substances to produce a combined effect greater than the sum of their separate effects
Thigmotaxis	Tendency of rodents to explore mainly the peripheral zone of an open space to prevent feeling exposed to danger.
Vivarium	Facilities in which experimental animals like rodents are bred, kept, and cared for. Experimental procedures can also be conducted in these facilities.

CHAPTER 1

INTRODUCTION

1.1. Dissertation layout

This dissertation is compiled according to article format as prescribed and approved by the North-West University. The focus of this dissertation will thus be Manuscript A (Chapter 3), which will be prepared for submission to a peer-review journal in the field of ethnopharmacology or neuroscience. Any supplementary data will be presented in the addenda.

The dissertation format is as follows:

- Abstract
- Congress Proceedings
- Acknowledgements
- List of Tables and Figures
- List of Abbreviations
- Glossary
- Chapter 1: Introduction:
  Problem statement; aims and objectives,
  expected results; study design and animal groups; ethical considerations.
- Chapter 2: Literature review
- Chapter 3: Concept article (Manuscript A)
- Chapter 4: Unifying discussion, conclusion, limitations and future recommendations
- Addendum A: Validation of the FSL model of depression
- Addendum B: Dopamine results
- Addendum C: Novel Object Recognition Test
- Addendum D: FST and OFT Methodology
- Addendum E: High Performance Liquid Chromatography (HPLC): Methodology - Monoamines
- Addendum F: ELISA Kit Method
- Addendum G: Certificate of Analysis of Zembrin®
- Addendum H: Animal Monitoring: Monitoring Sheets and Weights of Animals
- Letters of consent from contributing authors

1.2. Problem statement

Everyday life often overwhelms us with challenges that can cause various anxiety and stress-related conditions, often with short-lived depressed mood and emotional responses. But for millions of people, the depressed mood is persistent and can be identified as the chronic condition known as Major Depressive Disorder (MDD) (World Health Organization, 2018a). MDD is a serious and potentially lethal disorder that affects about 300 million people worldwide, although these numbers may be even higher due to low reporting rates driven by stigma (Willner et al., 2013; World Health Organization, 2018a; Wu et al., 2017). Although there are limited statistics on MDD within the South African context, studies between 2008 and 2012 revealed a 41% increase in the incidence of mental disorders in general in SA, with a lifetime prevalence of about 30.3% (Kessler et al., 2003; Kessler et al., 2005; Kessler and Bromet, 2013; Osuch and Marais, 2017). Additionally, there is a higher prevalence of this disorder in women than in men (World Health Organization, 2018a). According to the World Health Organization (2018a), MDD is the leading cause of disability globally and is a major problem in the overall worldwide burden of disease. Another problem is that countless patients do not receive the effective care and treatment they need. This is ascribed to a lack of resources, insufficient amounts of qualified healthcare professionals, incorrect diagnoses and wrongfully prescribed medicines, low patient compliance, as well as reluctance to seek professional help due to the social stigma around mental disorders (Andersson et al., 2013; World Health Organization, 2018a).

MDD takes a heavy toll on different aspects of life, including relationships and work, this due to numerous debilitating symptoms viz. negativity, impaired cognition and memory, impaired coping, helplessness, increased/decreased appetite, anergia, anxiety, anhedonia, sleep disturbances, impaired emotional regulation, impaired motoric functions, and suicidal ideation amongst others (Dean and Keshavan, 2017; Jesulola et al., 2018; World Health Organization, 2018a; Willner et al., 2013). These symptoms are also included as diagnostic criteria in the DSM-V (American Psychiatric Association, 2013). In addition, MDD causes significant economic burden as it interferes with the daily life and responsibilities of patients, while increasing the risk of developing other chronic diseases such as cardiovascular and metabolic diseases and cancer (World Health Organization, 2018a; World Health Organization, 2018b). Importantly, MDD can be lethal as it is one of the leading causes of suicide. In fact, it is estimated that about 800 000 people lose their lives annually due to suicide, especially patients between the ages of 15 and 29 years (Dean and Keshavan, 2017; Michaud et al., 2006).

It is now accepted that MDD is a multifactorial illness with a number of pathological processes underlying its aetiology (Brand et al., 2015). Current treatments for MDD, which includes selective serotonin (5-HT) reuptake inhibitors (SSRIs), 5-HT and norepinephrine (NE) reuptake inhibitors (SNRIs), and NE reuptake inhibitors (NRIs), tricyclic antidepressants (TCAs), and monoamine

oxidase inhibitors (MAOIs), among others, have shown a measure of effectivity (Willner et al., 2013). However, these treatments mainly target one or two mechanisms of MDD which could prevent full recovery due to one or more systems implicated in the disorder not being targeted (Bennett and Smith, 2018; Bennett et al., 2018; Willner et al., 2013). Alarmingly, only about 30-40% of patients respond to treatment, thus calling for the exploration of novel drug targets and alternative treatments for the treatment of MDD. Such treatments should be effective as monotherapy or in combination with other treatments in order to gain synergistic effects (Trivedi et al., 2006; Willner et al., 2013). Therefore, new treatment strategies should target the copious pathophysiological changes in MDD. To this end, a typical multi-target antidepressant should not only address reduced monoamines in MDD (5-HT, NE and dopamine (DA)) levels, but also decreased neuroplasticity and neurogenesis, hypothalamic-pituitary-adrenal (HPA) axis dysfunction, disordered circadian rhythms and neuroinflammation (Dean and Keshavan, 2017; Jesulola et al., 2018). Evidence suggests that these changes can be caused by different factors, including chronic stress, genetic predisposition, and other environmental factors, or a combination thereof (also known as the diathesis/stress model) (Dean and Keshavan, 2017; Jesulola et al., 2018; Willner et al., 2013). If these treatments are successful, it could thus improve inter-personal relationships and job productivity, increase the quality of life, and so potentially save the lives of patients suffering from MDD.

With the current treatment strategies plagued by many drawbacks, especially delayed onset of action, high side effect profiles, decreased patient compliance, and overall treatment failure, many patients turn to complementary and alternative treatment strategies (Akil et al., 2018; Bennett et al., 2018; Carhart-Harris and Nutt, 2017; Willner et al., 2013). These alternative treatment strategies include cognitive behavioural therapy (CBT), dietary changes, increased physical activity, acupuncture, and the use of plants and herbs (Davis et al., 2008; Ritsner, 2013; Willner et al., 2013). The use of natural substances like plants and herbs has shown promise for the treatment of MDD, e.g. St. John's wort (SJW) with its multiple mechanisms of action (Sarris, 2018). However, some substances are more effective in combination with other drugs, e.g. lavender combined with imipramine has shown better efficacy with less side effects than either treatment alone (Akhondzadeh et al., 2003). Despite their many potential advantages, unsupervised use of these natural substances, and especially in combination with other substances like prescribed antidepressants, could lead to toxicity or drug interactions (Ravindran and da Silva, 2013). For instance, SJW in combination with serotonergic antidepressants can lead to a potentially life-threatening 5-HT syndrome (Sarris, 2018). Additionally, the many constituents of plant/herb extracts could also interact with each other. Based on this, there is a clear need for in-depth studies of the pharmacological and pharmacokinetic profiles of natural substances (Krstenansky, 2017; Sarris, 2018; Sarris et al., 2010; Yeung et al., 2018). In fact, in their traditional medicine strategy for 2014 – 2023 (World Health Organization, 2013), the WHO has recommended that evidence-based research should be used to evaluate the safety and

efficacy of these natural substances. Keeping this in mind, the present study will investigate the South African plant, *Sceletium tortuosum* (ST) in order to evaluate its antidepressant effects alone and in combination with the commonly prescribed SSRI, escitalopram (ESC).

The indigenous Khoi-San people of Southern Africa have used ST for many generations for a variety of purposes, including as a thirst and appetite suppressant during hunting trips, as a mood elevator, hypnotic, analgesic, and anxiolytic, and for its intoxicating/euphoric effects (Gericke and Viljoen, 2008; Harvey et al., 2011). Interestingly, ST shows no abuse liability despite its intoxicating effects (Gericke and Viljoen, 2008; Loria et al., 2014; Nell et al., 2013). Another exciting aspect of this particular plant is its low side effect profile (Schifano et al., 2015). ST contains a large number of bioactive alkaloids, such as mesembrine and mesembrenone, which have been associated with inhibition of the 5-HT transporter (SERT) and phosphodiesterase 4 (PDE4) (Gericke, 2001; Gericke and Viljoen, 2008; Harvey et al., 2011). These actions suggest monoaminergic actions that may be of benefit in treating mood-related disorders like MDD. PDE4 is associated with neuroplasticity and the release of neurotrophins like brain-derived neurotrophic factor (BDNF) via the cyclic adenosine monophosphate (cAMP)/cAMP response element binding protein (CREB) pathway. Neuroplasticity and immune-inflammatory cascades have been causally linked to MDD (Bennett and Smith, 2018; Duman et al., 2000; Kraus et al., 2017). Furthermore, its up-regulatory effects on vesicular monoamine transporter 2 (VMAT-2) can increase 5-HT release. Other putative neuropharmacological mechanisms include inhibition of the metabolic enzyme monoamine oxidase A (MAO-A), and inhibition of NE and DA transporters (NET, DAT) (Bennett and Smith, 2018; Coetzee et al., 2016; Duman et al., 2000; Gericke and Viljoen, 2008; Harvey et al., 2011; Krstenansky, 2017).

Needless to say, the favourable pharmacologic properties and low side effect profile of ST advocates its possible use as an antidepressant. Although some animal studies have hinted at its possible value as an antidepressant in MDD (Gericke and Viljoen, 2008; Harvey et al., 2011), most of the above-mentioned mechanisms have been established by *in vitro* means. To our knowledge, only one study has tested ST as a stand-alone treatment in a validated translational model of MDD, viz. in BALB/c mice. That study demonstrated that ST has subacute antidepressant effects in the forced swim test (FST) vs. paroxetine (Schell, 2014). However, no study to date has studied ST in a translational animal model to consider its chronic treatment response, nor has it been assessed as a possible adjunctive treatment. Furthermore, studies that correlate behavioural responses to ST regional brain neurochemistry have also not been done. There is thus a need for an in-depth study of the antidepressant properties of ST. Importantly, ST has shown dose-dependent effects with respect to *in vitro* receptor and transporter binding assays (Coetzee et al., 2016; Gericke and Viljoen, 2008; Smith, 2011), as well as *in vivo* models exploring anxiolytic and antidepressant effects in an *in vivo* chick model (Carpenter et al., 2016), as well as the previously mentioned rodent study by Schell (2014). However, the *in vivo* studies

mentioned here had its own limitations, e.g. lack of a comprehensive dose response study (Schell, 2014) and lack of antidepressant effects in the chosen model which could possibly be attributed to the type of formulation used or inconsistencies between this model used and previous models (including that seen in Schell (2014)). From this it is clear that a comprehensive dose-response study to identify an effective antidepressant dose of ST is lacking.

The Flinders Sensitive Line (FSL) rat is a genetic animal model of depression. It shows adequate construct validity, including serotonergic abnormalities and attenuated neuronal plasticity among many others; face validity due to its psychomotor retardation, cognitive impairment, increased passivity or behavioural despair; and predictive validity as it responds to chronic antidepressant treatments (Overstreet, 1993, 2012; Overstreet et al., 2005; Overstreet and Wegener, 2013). Considering the many overlaps between the bio-behavioural abnormalities present in FSL rats and human MDD and the pharmacological mechanisms of action of ST, this model provides a valuable research platform with which to investigate the antidepressant effects of ST. In fact, the model has been used to successfully characterize the antidepressant properties of other plant/herbal preparations (Oberholzer et al., 2018).

Taking into account that FSL rats show behavioural similarities to MDD patients in terms of psychomotor retardation and feelings of helplessness, the forced swim test (FST) and open field test (OFT) can be implemented to quantify these symptoms, respectively. The FST is a model of learned helplessness in which a rat is forced to swim in a water-filled cylinder, where immobility or floating behaviour is associated with despair and depressive-like behaviour (Brand and Harvey, 2017; Slattery and Cryan, 2012). That said, there are some authors insisting that immobility could be seen as a passive coping mechanism, possibly mediated by postsynaptic 5-HT_{1A} receptors (Commons et al., 2017; de Kloet et al., 1999; Puglisi-Allegra and Andolina, 2015). Nevertheless, most studies agree that swimming behaviour and struggling behaviour are related to 5-HT and NE activity, respectively (Brand and Harvey, 2017; Cryan et al., 2005), regarded as escape-directed movements. One can thus relate regional brain monoamines to swimming/climbing behaviour in order to more accurately interpret behavioural results at a neurochemical level.

The frontal cortex and hippocampus are especially implicated in the pathophysiology of MDD. The frontal cortex is involved in mood control, motility, cognition and attention (Millan et al., 2000), while the hippocampus is primarily involved in memory and learning (Dean and Keshavan, 2017). Consequently, dysfunction of these regions may contribute to MDD symptoms like psychomotor retardation, impaired mood regulation or helplessness, and cognitive impairments. The OFT can be used as a measure of locomotor activity, where a decrease in distance travelled in a 1 m x 1 m box can indicate psychomotor retardation (Brand and Harvey, 2017; Dean and Keshavan, 2017). Furthermore, neurotransmitters like NE can be valuable in the interpretation of OFT results due to its involvement in movement (Chen and Reith, 1995). The OFT can also aid in the interpretation of FST data in terms of drug-related effects on movement that could be a

confounder in interpreting the psychogenic basis for swimming/climbing behaviour (Lavi-Avnon et al., 2005).

The novel object recognition test (nORT) is a behavioural test used to evaluate memory and visual learning in rodents (Mokoena et al., 2015; Oberholzer et al., 2017). The concept of nORT lies in the natural exploratory behaviour of rats where they will explore novel objects more than familiar objects (Ennaceur and Delacour, 1988; Oberholzer et al., 2017). This test can give an indication of cognitive deficits as seen in human patients and can be linked to decreased neuroplasticity and altered states of BDNF in the frontal cortex and especially the hippocampus (Antunes and Biala, 2012; Sarkisyan and Hedlund, 2009).

In this study we first asserted the behavioural validation of the FSL rat versus its reference control, the Flinders Resistant Line (FRL) rat. Thereafter, we established the predictive validity of the model using a 3-tier acute dose challenge with the SSRI, escitalopram in FSL rats (5, 10 and 20 mg/kg). Here we set out to identify a low-dose and effective antidepressant dose of ESC. This was followed by an acute 5-tier dose response study with ST (5, 10, 25, 50, and 100 mg/kg) in order to identify an appropriate antidepressant dose for this extract. In all instances, we used the FST and OFT. We then used the above identified doses of ESC and ST in a sub-chronic 15 day treatment study to evaluated their antidepressant effects of ST alone and in combination with low-dose ESC using the FST and OFT. We also measured frontal cortical and hippocampal monoamines to further interpret these behaviours. Furthermore, we exposed the rats to the nORT and measured BDNF in the frontal cortex and hippocampus in order to interpret these results from a neuroplasticity point of view.

1.3. Aims and objectives

1.3.1. Aim of the study

The aim of the study was to assess the antidepressant-like effects of *Sceletium tortuosum* extract in the FSL rat alone, vs. an active comparator, escitalopram. Thereafter, to assess *Sceletium tortuosum* as a potential adjunctive therapy in combination with escitalopram.

1.3.2. Primary objectives

1. To do a fingerprint analysis of the Zembrin® used in this study in order to determine the amount of the different alkaloid constituents in this standardized extract as a measure of quality assurance. The chemotype of Zembrin® should be constant between all samples

Acute studies

2. In an acute treatment design, to confirm the presence of depressive behaviour in drug-naive male FSL vs. Sprague Dawley (SD) and Flinders Resistant Line (FRL) control rats using specific behavioural tests (OFT, FST)
3. To establish the most appropriate antidepressant dose of *Sceletium tortuosum* in FSL rats using an acute dose response study with 5 different doses, as assessed in the OFT and FST
4. To establish the most appropriate low dose of ESC by performing a dose response study consisting of 3 doses, as assessed in the FST and OFT.

Sub-chronic studies

5. To evaluate the bio-behavioural response to an optimal antidepressant dose of ST and low dose ESC vs. vehicle in FSL rats over 15 days, using behavioural tests (FST, OFT, and nORT) and hippocampal and prefrontal cortical biomarkers (monoamines and BDNF).
6. To assess whether the chosen antidepressant dose of ST can bolster the response to low dose escitalopram with respect to behaviour in the FST, OFT and nORT, as well as whether such behavioural changes are mirrored in neurobiomarker analysis vs. that compared to either drug alone.

1.3.3. Secondary objectives

1. Although used primarily for ethical obligations and health monitoring, to assess animals for possible signs of 5-HT syndrome using an adapted monitoring sheet. This was considered based on the theoretical possibility of inducing 5-HT syndrome when combining serotonergic actions of both ST and ESC.

1.4. Expected results

Zembrin Fingerprint results

It will be possible to identify and quantify the main alkaloid constituents as stipulated in the certificate of analysis (Addendum G) obtained from the manufacturing company, using the UPLC-MS method.

Acute studies

1. FSL rats will show face validity for MDD, viz. depressive-like behaviour as related to increased immobility and decreased swimming and struggling in the FST, as well as decreased locomotor activity in the OFT in an acute treatment design, when compared to control SD and FRL rats.
2. FSL rats will show predictive validity for MDD, by demonstrating a low-therapeutic and a full therapeutic dose of ESC from the 3 tested doses in an acute treatment design in FSL rats. This will be determined based on increased swimming and struggling and decreased immobility in the FST, as well as increased locomotor activity in the OFT.
3. We will be able to determine the most appropriate antidepressant dose of ST from the 5 tested doses in the acute treatment design in FSL rats. This will be determined based on increased swimming and struggling and decreased immobility in the FST, as well as increased locomotor activity in the OFT.

Sub-chronic study

4. In a sub-chronic treatment design vs. saline-treated FSL rats over 15 days, the chosen dose of ST alone will show similar or more effective reversal of depressive-like behaviour in the OFT (increased locomotor activity), FST (increased swimming and struggling; decreased immobility), and nORT (more time spent exploring novel objects) versus a low dose of ESC alone.
5. In a sub-chronic treatment design, FSL rats will present with anticipated neurobiomarker abnormalities associated with depression, viz. decreased regional brain (frontal cortex and hippocampus) monoamines and BDNF. These changes will be reversed by the chosen dose of ST alone to the same or even higher extent as the chosen low dose of ESC.
6. In a sub-chronic treatment design, the combination of the chosen doses of ST and ESC will show better antidepressant effects in bio-behavioural markers mentioned above. Thus, ST will bolster the antidepressant response of low dose ESC which will indicate good potential for its use as an augmentation strategy in the treatment of MDD.

Secondarily:

7. Since ST has actions on the 5-HT system, there is a theoretical possibility of inducing the serotonin syndrome in combination with an SSRI. However due to the combination of ST with a low dose of escitalopram this is not expected to happen.

1.5. Working hypothesis

The FSL rats will present with the typical depressive-like behaviour found in this strain compared to the FRL rat control group. ST will show acute dose-dependent antidepressant effects in terms of behaviour. ESC will show dose-dependent antidepressant effects. Sub-chronically, alone treatment with ST will show biobehavioural results indicating antidepressant effects compared to the control group, with better antidepressant efficacy than the sub-therapeutic dose of ESC. When combined with a low dose of ESC, there will be a bolstered biobehavioural response with even better or equal antidepressant effects than when used alone.

1.6. Study design and animal groups

1.6.1. Fingerprint analysis of Zembrin®

Firstly, before animal experiments, a sample of the Zembrin® used in this study will be analysed using the UPLC-MS method by Prof Alvaro Viljoen at Tshwane University of Technology (TUT) to determine the main constituents and quantities thereof in the standardised extract as a measure of quality control. Furthermore, this information may be useful in the interpretation of data based on literature comparing the binding profiles and pharmacological effects of these components on their own.

1.6.2. General information regarding animal experiments

This study is divided into 2 substudies: an acute treatment study (acute confirmation of the FSL model and an acute dose response with ST and ESC) and a sub-chronic treatment response study. In total, 158 male rats were used with 12 SDs, 6 FRLs and 140 FSLs

- All animals were randomly divided into their groups according to “weight classes” in order to simplify logistics as the animals were only used once they reached the target weight of 200 – 230 g. Weights differed within litters born on the same day, however if this was not the case, rats from the same litter were also allocated to separate home cages.
- The maximum volume that can be administered via oral gavage in a rat of 200 – 230 g is 3.5 ml.
- Both ST (Zembrin standardized extract) and ESC is highly soluble in saline and no pH adjustments were needed. Thus, all drugs were thoroughly dissolved in saline and administered via oral gavage.
- All animals were weighed daily in order to determine when treatment would commence, as well as to be able to calculate the dose to be administered based on body weight (mg/kg).

- After they were weighed, monitoring sheets (adapted with criteria for 5-HT syndrome for the combination treatment group) were completed for each rat. They were also monitored additionally directly after, as well as the morning after the FST.
- All behavioural tests commenced from 19:00 at night in order to allow the animals to completely wake up after the lights switched off at 18:00. However, the first experimental groups of the acute studies received their first dose from 18:00. All experiments were conducted under red or yellow light so as to prevent interruption of the day/night cycle of the nocturnal rats.
- **Due to the strong smell or taste of the *Sceletium tortuosum* extract (Zembrin) solution,** the gavage needle was rinsed in saline before carrying out the oral gavage procedure to prevent the rats from tasting it on the needle to avoid startling the rats.

1.6.3. Acute studies

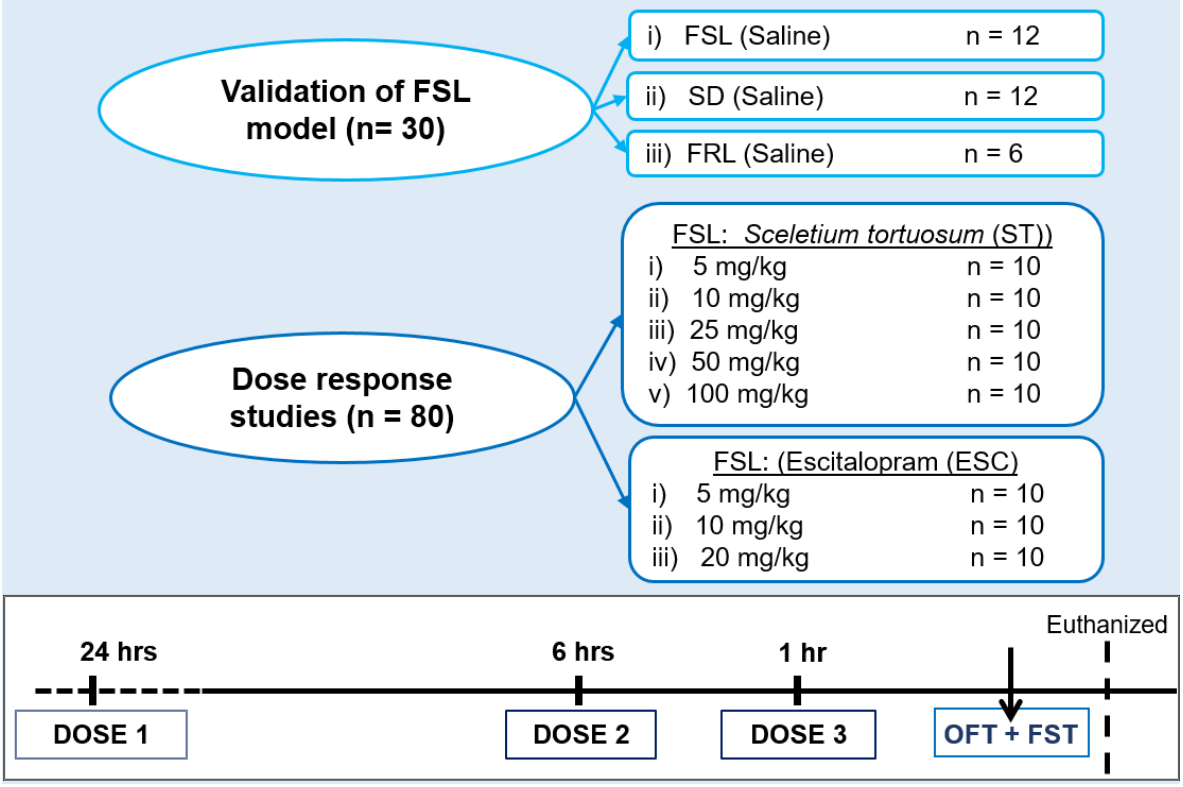

Fig. 1.1: Layout of acute study: Validation of the FSL model; Dose responses of ST and ESC. n – number of animals used, hrs – hours.

1.6.3.1. Validation of the FSL genetic model for depressive behaviour, vs. SD and FRL controls

The animals groups consisted of 12 FSL rats, 12 SD rats, and 6 FRL rats. All animals received three doses of 3 ml physiological saline via oral gavage at the same frequencies discussed under

the acute dose response study (Section 1.6.3.2.). 24 hours after the first administration, the FSL, FRL, and SD rats were subjected to the OFT, and FST and euthanized according to Vivarium protocols.

Since FRL rats are widely used as controls for FSL rats (Overstreet et al., 2005; Overstreet and Wegener, 2013), we opted to only use 6 animals of this strain in order to conserve animal numbers. Furthermore, considering our earlier work with FRL control rats (Brand and Harvey, 2017; Hamman et al., 2015; Steyn et al., 2018), 6 FRL rats would still be enough to produce robust data.

These groups were used to confirm that the FSL rats express depressive-like behaviour by comparing their behavioural data (OFT and FST) to that of the control SD and FRL rats (Fig 1.1). The FRL rats and FSL rats were derived/inbred from SD rats, thus SDs represents a pure strain, presenting with normal/healthy behaviour. Nevertheless, the FRL is widely used as controls for FSLs (Overstreet et al., 2005). This FSL group also served as the vehicle (saline) control groups for the acute dose response studies (Section 1.6.3.2).

1.6.3.2. Acute ST dose response vs. ESC dose response

With this acute dose response study, we set out to determine the most appropriate doses of ESC and ST, viz. a low and a therapeutic dose of ESC and a therapeutic dose of ST, to use in the sub-chronic treatment response study (section 1.5.2). 50 FSL rats were divided into five equal groups (n = 10) and treated orally with escalating doses of Zembrin® (5, 10, 25, 50, and 100 mg/kg) (Figure 1.1). The ESC dose response study (Figure 1.1) comprised of three groups (n = 10) treated with three escalating doses (5, 10 and 20 mg/kg) of escitalopram oxalate.

Each dose was administered at intervals of 24 hours, 6 hours and 1 hour before the animals were subjected to the OFT and FST. The rats were then euthanized according to Vivarium protocols. The rats used in the acute validation study (Section 1.6.3.1.) also served as the vehicle controls of these groups. The best antidepressant dose of ST and a low dose of ESC were used in the subchronic study, as motivated elsewhere, and as determined in the OFT and FST.

1.6.4. **Sub-chronic study**

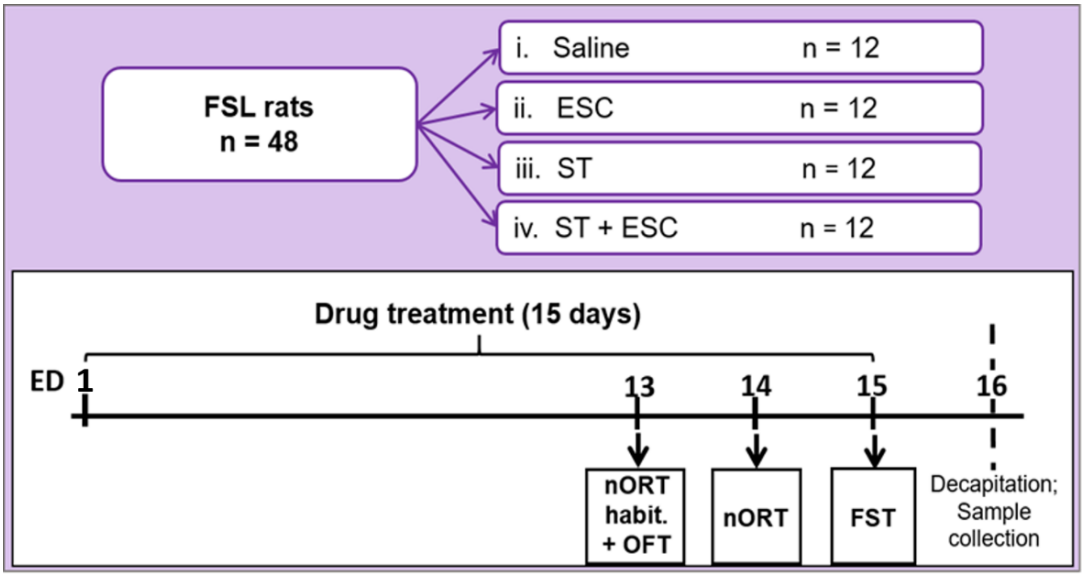

Fig. 1.2. Study layout and timeline of the various sub-chronic treatment response studies. ED – Experimental day, n – number of animals used, habit. – habituation.

The purpose of this study was to use the designated doses of ST and ESC described in Section 1.5.3.2. above to determine the efficacy of ST in reversing the bio-behavioural changes akin to MDD, as sub-chronic monotherapy (compared to low-dose ESC monotherapy), and as augmentation therapy in combination with low dose ESC.

FSL rats (n=48) were randomly divided into four groups containing 12 rats each (Figure 1.2). Group (i) received saline and served as the control group. Group (ii) received the chosen dose of escitalopram and group (iii) the chosen dose of ST (both as determined by the acute dose response study – Section 1.6.3.2). Group (iv) received a combination of the chosen dose of ST and the low dose of ESC. The combination of ST plus low dose ESC was done in order to better demonstrate an augmenting effect, as well as to prevent the risk of inducing serotonin syndrome in combination with a relatively unknown entity purported to have serotonergic properties, viz. ST (Boyer and Shannon, 2005). All rats were treated between 07:00 and 09:00 for 15 days. On day 13, each rat was subjected to the nORT habituation procedures which also served as the OFT, and on day 14, the nORT familiarization and test/acquisition trials. The animals were then subjected to the FST on day 15. After these behavioural tests, the rats were decapitated on day 16, 24 hours after the last dose of drug was administered. The hippocampus and frontal cortex were dissected out for monoamine and BDNF analyses, and blood samples collected for future analysis.

1.7. Ethical considerations

- All procedures were approved by the North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC) (NHREC reg. number AREC-130913-015) of the North-West University.
- All animals in this study were bred, supplied and housed at the Vivarium (AAALAC accreditation international file #1717"; SAVC reg. number FR15/13458; SANAS GLP compliance number G0019) of the Pre-Clinical Drug Development Platform (PCDDP) associated with the national Department of Science and Technology (DST) and the North-West University (NWU). A constant ambient temperature of 22 ± 2°C and relative humidity of 40 – 60% was maintained, with a full spectrum of light in a 12:12h light/dark cycle (switched on at 06:00 and off at 18:00). The animals were housed in groups of 3 to 4 rats per cage with corncob bedding in polypropylene cages (380×380×230 mm) and environmental enrichment in the form of PVC pipes and standard Vivarium nesting material, with positive air pressure and an air exchange rate of 18/hr. Food (standard rat chow) and water was supplied ad libitum. Animals were maintained and procedures performed in accordance with the code of ethics in research, training and testing of drugs in South Africa and complied with national legislation.
- This study was classified as a category 4 (very severe) study due to the FST.
- Study-specific (modified for 5-HT syndrome – See Addendum H) NWU Vivarium monitoring sheets were completed daily to monitor animal welfare, as well as facilitate early detection of any deterioration of health or when humane endpoints would be reached. Thereby the sheets enabled early intervention to avoid any unnecessary suffering
- The animals were prior handled (during daily weighing and health monitoring) from 21 days old (weaning age) to familiarise them to human handling and thereby minimize stress during administration of medication and other procedures.
- The animals were weighed daily to determine the test date and normal growth and development of the animals.
- This study used the ARRIVE guidelines in order to ensure that the study design required the least amount of animals for robust data, and to ensure that animals were handled and research conducted in a humane way.

1.8. Declaration of contributions

- Johané Gericke (MSc student):
    - Played a major role in the planning, study design, and write-up of the proposal for this study
    - Obtained ethical approval from Animcare.
    - Carried out all experiments
    - Collected and contributed to the analysis of biological samples
    - Gathered, processed and interpreted data
    - Wrote all first drafts of the concept article, chapters and addenda found in this dissertation
    - Made all changes and improvements based on the comments from the study leaders
- Prof Brian Harvey (Study leader):
    - Played a major part in the planning, design, and the write-up of the proposal for this study
    - Reviewed and edited all drafts by the student
    - Assisted in the interpretation and write-up of the results in the concept article, chapters and addenda found in this dissertation
    - Obtained funding for this study
- Dr. Makhotso Lekhooa (Assistant study leader)
    - Played a major part in the planning, design, and the write-up of the proposal for this study
    - Reviewed and edited all drafts by the student
    - Assisted in the interpretation and write-up of the results in the concept article, chapters and addenda found in this dissertation
- Prof Alvaro Viljoen (collaborator from TUT)
    - Involved in the planning of the study
    - Provided fingerprint analyses of the ST (Zembrin®) used in this study
    - Provided funding for this study
- Dr Stephan Steyn
    - Gave inputs and assistance in the practical application of experiments
    - Assisted with scoring and processing of results of the behavioural tests
    - Assisted with statistical analyses of all results
- Dr Francois Viljoen
    - Assisted with the HPLC analyses of the brain monoamines

- Mr Walter Dreyer
    - Assisted with the ELISA analyses of the brain BDNF
- Ms Antoinette Fick and Mr Kobus Venter (Vivarium personnel)
    - Husbandry of animals
    - Training of the student with regards to animal handling and procedures like oral gavage
    - Euthanisation and decapitation of animals for sample collection
- HG&H
    - Provided the Zembrin® used in this study
    - Provided information regarding ST (Zembrin®)
    - Provided partial funding for this study
- Lundbeck
    - Provided the escitalopram oxalate through a donation to Dr De Wet Wolmarans

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CHAPTER 2

LITERATURE REVIEW

2.1. Epidemiology and diagnosis of MDD

Clinical depression, also known as major depressive disorder (MDD), is a severe psychiatric disorder that affects about 350 million people of all ages globally, making it the most common psychiatric disorder. MDD is more prevalent in women than in men, possibly due to the higher likelihood of women seeking help compared to men (Mayoclinic, 2017). A high prevalence is also noted among menopausal women and mothers who have suffered from post-partum depression (10 to 20%) (Akil et al., 2018; Jesulola et al., 2018; Organization, 2017; World Health Organization, 2018a). About 10 to 15% of the global population will be diagnosed with MDD in their lifetime, with symptoms generally starting during adolescence or early adulthood (Freudenberg et al., 2015; Jesulola et al., 2018; World Health Organization, 2018a; World Health Organization, 2018b). MDD can also be seen as a "silent killer" due to its impact on physical, psychological and social wellbeing of sufferers, especially when considering that MDD is the leading cause of suicide attempts, especially in patients between the ages of 15 and 29 years (World Health Organization, 2018b; Porche, 2005). Almost 50% of all patients who have attempted suicide showed definite symptoms of MDD (Cummins et al., 2015).

According to the South African Depression and Anxiety Group (SADAG), MDD is a major national health concern. In 2017, statistics showed that the lifetime prevalence of MDD in South Africa is 9.7% of the population – meaning 4.5 million people in South Africa alone suffers from this devastating illness. Statistics also show that roughly 8 000 South Africans commit suicide every year (South African Depression and Anxiety Group, 2017). MDD prevalence in South Africa, a developing country, is also higher in low-income populations due to increased financial stress and limited access to treatment (Hamad et al., 2008). Other factors also contribute to the high prevalence of MDD are, for example, comorbidity with diseases such as HIV/AIDS, obesity, type-2 diabetes, cardiovascular disease, and neurodegenerative diseases like Parkinson's disease (Du and Pang, 2015; Kurhe et al., 2014; Pappin et al., 2012).

2.2. Symptoms and diagnosis of MDD

Clinical MDD is a mood disorder characterised by continuous feelings of sadness, apathy and anhedonia (Mayoclinic, 2017). It influences the patient's feelings, thoughts and behaviour and

can lead to many emotional and physical problems that interfere with day-to-day tasks (Mayoclinic, 2017).

MDD is characterized by symptoms such as decreased motor functions, anhedonia or loss of pleasure, decreased motivation, weight loss or weight gain, fatigue, cognitive impairments such as decreased concentration and memory, impaired emotional regulation (feelings of guilt, hopelessness, sadness), anxiety, sleep disturbances, lowered self-esteem, negative worldview, negative view of the future, and in serious cases suicidal thoughts or suicide attempts (Dean and Keshavan, 2017; Jesulola et al., 2018; Willner et al., 2013). MDD is diagnosed using criteria from the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) (American Psychiatric Organization, 2013). MDD also precipitates other complications such as strained relationships, substance abuse, self-mutilation, anxiety and social isolation, amongst others (Mayoclinic, 2017; Organization, 2017).

2.3. Treatment of MDD

2.3.1. Current treatment of depression: Benefits, risks and pitfalls

Currently, antidepressants are targeting the most commonly researched mechanisms involved in the pathophysiology of MDD. These drugs include classes such as the selective 5-HT reuptake inhibitors (SSRI), 5-HT and NE reuptake inhibitors (SNRIs), monoamine oxidase inhibitors (MAOIs), NE reuptake inhibitors (NRIs) and atypical antidepressants like buspirone, agomelatine, vortioxetine and mirtazepine (Cipriani et al., 2018; Willner et al., 2013). However, for the most part these drugs are associated with decreased patient compliance and high side effect profiles (apart from agomelatine (Cipriani et al., 2018)), and delayed onset of action (Akil et al., 2018; Bet et al., 2013; Carhart-Harris and Nutt, 2017). Moreover, only about a third of patients receiving these treatments actually respond after about 14 weeks of treatment, thus contributing to the prevalence of treatment resistant depression (TRD) (Willner et al., 2013).

In the pharmacotherapy of MDD, treatment could fail for many different reasons. None-response to one class of antidepressants could be indicative that the wrong pathophysiological mechanism is being targeted and that a different class of antidepressant with a different or additional mechanism of action could elicit a positive response (Bennett et al., 2018; Willner et al., 2013). Antidepressant combinations could also be required to maximize response when more than one system is causally involved in MDD (Dodd et al., 2005; Sarris, 2018; Sarris et al., 2010). Initial treatment that elicited a positive response could become ineffective over time and which underlies the complexity of TRD (Shelton, 2007). Importantly, in appropriate discontinuation, e.g. repeated stopping and starting and random switching of antidepressants, as well as general non-compliance, is recognised as a forerunner of TRD (Harvey and Slabbert, 2014).

Treatment options for MDD and TRD entail many different strategies according to different staging models (Kay and Atiq, 2006; McIntyre et al., 2014; Ruhé et al., 2012). Basic strategies also entail optimizing treatment by adjusting doses and duration of therapy, switching to more appropriate antidepressants with more favourable side effect profiles and associated with better patient compliance, combinations of different antidepressants with synergistic effects, psychotherapy and electroconvulsive therapy (ECT) and other neurostimulation strategies like deep brain stimulation (DBS) (Kay and Atiq, 2006). Augmentation strategies are also used. Evidence show improved response when using lithium (Heit and Nemeroff, 1998; Nelson et al., 2014), thyroid hormone therapy (Aronson et al., 1996), some atypical antipsychotics (Moukaddam and Shah, 2016) and buspirone (Joffe and Schuller, 1993; Wang et al., 2014) as augmentation therapies. Other promising treatments for TRD include NMDA antagonists like ketamine, S-ketamine and rapastinel that modulates glutamate, or drugs that positively influence the kynurenine pathway (Allen et al., 2018; McIntyre et al., 2014; Thase, 2017). Augmentation strategies using anti-inflammatory agents like celecoxib and some other non-steroidal anti-inflammatory drugs (NSAIDs) have also shown positive responses in TRD treatment (Abbasi et al., 2012). Despite TRD being a serious condition, very little research is conducted to identify novel biological pathways involved in TRD and finding more effective treatment strategies.

Additional factors such as high adverse effect profiles including sexual dysfunction, gastrointestinal disturbances, weight gain, anxiety, and restlessness, et cetera can also call for discontinuation of treatment of commonly prescribed antidepressants (Bet et al., 2013; Shrestha et al., 2014). Furthermore, dangers of concomitant use of more than one serotonergic agent (SSRIs, SNRIs, MAOIs, tramadol etc.) could induce the potentially life-threatening serotonin syndrome (Pilgrim et al., 2011; Suchowersky and Devries, 1990), which is characterized by a triad of mental-status changes, autonomic overactivity and neuromuscular abnormalities (Boyer and Shannon, 2005). Combining drugs with multiple metabolic pathways, narrow therapeutic indices, and competition for metabolism with two drugs with the same metabolic pathway, also increase the risk (Pilgrim et al., 2011). Manifestations of serotonin syndrome in humans include tremor, mydriasis, excessive sweating, tachycardia, hypertension, hyperreflexia and myoclonus, agitation or hypervigilance, pressured speech, head turning behaviour (repetitive rotation of head with the neck held in moderate extension), seizures, etc. (Boyer and Shannon, 2005; Sternbach, 1991). Life-threatening toxicity can present with severe hypertension and tachycardia which can rapidly deteriorate into shock, usually accompanied by agitated delirium, muscular rigidity and hypertonicity (especially in the lower limbs). In life-threatening cases, muscle hyperactivity can cause hyperthermia with core temperatures rising to more than 41.1°C (Boyer and Shannon, 2005). Combined antidepressant treatments should thus be implemented carefully.

For the purpose of this dissertation, it is important to pay special attention to the SSRI escitalopram (ESC) (Cipriani et al., 2009; Kennedy et al., 2006), that works by increasing the

activity of 5-HT in the central nervous system, due to inhibition of the 5-HT transporter (SERT) and thereby inhibition of 5HT reuptake from the synapse (Cipriani et al., 2009; Owens et al., 2001). SSRIs have been claimed to also act as indirect agonists of 5-HT receptors such as the $5HT_{1A}$ receptor (Samuels et al., 2011). It is the S-enantiomer of citalopram, and is the most potent and selective of all SSRIs with a quicker onset of action (Zhong et al., 2012), as well as showing a more prolonged 5-HT reuptake inhibition compared to other SSRIs like citalopram (El Mansari et al., 2005; Montgomery et al., 2001; Owens et al., 2001; Zhong et al., 2012). ESC has no direct effect on norepinephrine and dopamine reuptake (Lin et al., 2016). However, in addition to its inhibitory effect on SERT, SSRIs has been shown to improve cAMP signalling in the brain (Fujita et al., 2017), and increase neurogenesis and cell proliferation in the hippocampus (Bjørnebekk et al., 2010; Jayatissa et al., 2006). However, whereas increased BDNF levels are typically associated with antidepressant activity, Jacobsen and Mørk (2004) found that chronic ESC treatment induced a decrease in BDNF levels in the hippocampus and frontal cortex, although this effect could be dose-dependent and could also be related to the treatment period; in the mentioned study 10 mg/kg/day was used for 21 days. ESC (10 mg/kg/day) is associated with significantly quicker desensitization of the $5\text{-}HT_{1A}$ receptor after two weeks of treatment compared to 3 weeks of citalopram (20 mg/kg/day) treatment (El Mansari et al., 2005), thus further illustrating its high potency.

2.3.2. Complementary medicines as alternative treatment of MDD

Problems like impaired patient compliance, delayed onset of action and side effects profiles as previously mentioned have necessitated the exploration of alternative and complementary medicines, including the use of herbs and plants (Fajemiroye et al., 2016; Sarris, 2018; Sarris et al., 2010).

According to reviews by Fajemiroye et al. (2016) and Sarris (2018), many complementary treatments including herbal/plant substances have shown potential antidepressant-like effects in preclinical and clinical studies. These include *Hypericum perforatum* (MAO inhibition; DA, 5-HT and NE reuptake inhibition; NMDA receptor antagonism; reduced release of pro-inflammatory interleukin 6(IL-6)), *Piper methysticum* (MAO-B inhibition, blocked reuptake of NE), and *Lavandula angustifolia* (effects on $5\text{-}HT_{1A}$ receptors), *Garcinia mangostana Linn* inhibits cAMP-PDE and blocks $5\text{-}HT_{2A}$ receptors (Oberholzer et al., 2017), among others. Whereas these mechanisms are evidently similar to the mechanisms of action of clinically used antidepressants, many of the substances possess multiple mechanisms of action. Most of these substances show potential as stand-alone treatment, whereas others show better results when combined with a known antidepressant, e.g. the concomitant use of lavender (*Lavandula angustifolia*) and

imipramine has shown better efficacy with less side effects than either treatment alone (Akhondzadeh et al., 2003).

These natural/herbal substances have proven to be potentially helpful as antidepressants. However, the commercial availability thereof and the resultant unsupervised use these compounds might be problematic due to the possibilities of drug interactions and toxicity, especially if combined with prescribed medicines (Ravindran and da Silva, 2013). One example includes the use of St. John's wort (a plant with serotonergic properties) in conjunction with other serotonergic antidepressants like SSRIs, which has been shown to induce life-threatening serotonin syndrome (Sarris, 2018). Furthermore, different effects of plant constituents, especially when using raw extracts instead of isolated components, call for further research of the pharmacokinetic and pharmacological profiles of herbal medicines (Krstenansky, 2017; Sarris, 2018; Sarris et al., 2010; Yeung et al., 2018).

Based on the abovementioned, the World Health Organization has postulated a traditional medicine strategy for 2014 - 2023 (World Health Organization, 2018a), stating that the safety and efficacy of complementary medicine should be evaluated using evidence-based research as a tool.

2.3.3. *Sceletium tortuosum* as a putative antidepressant

Sceletium tortuosum (ST) is an indigenous South African plant that has been used by the Khoi-San for generations. It is also known as *Canna* or *Kougoed* (Afrikaans for "chew stuff") as it was chewed to obtain its pharmacological effects (Gericke and Viljoen, 2008)

The herbal monograph of ST (as provided by HG&H) contains information regarding the physical characteristics and geographical distribution of ST. These succulent plants are mainly found in the Cape provinces of South Africa, especially in the Karoo and Namaqualand. Khoisan people used the whole plant to concoct teas, tinctures, snuff, and it can also be smoked (Gericke and Viljoen, 2008; Smith, 1966; Waterhouse et al., 1979). The leaves of these plants are about 4 cm in length, while flowers measure at 2-3 cm in diameter and can be seen in spring and summer (Fig. 2.1.A). Flower colours can range from white to different shades of pink and yellow. Derived from the Latin word "sceletus" meaning skeleton, the genus name (*Sceletium*) refers to the clear vein-like lines resembling a skeleton which can be seen in old, withered and dry leaves (Fig. 2.1.B).

Currently, the plant is not regarded as an endangered species according to the Red List of South African plants (redlist.sanbi.org).

Fig. 2.1. Pictures of the *Sceletium tortuosum* plant showing A) the full blooming plant, and B) the skeleton-like veins on dry, withered leaves as described in-text.

Traditional uses: ST is used by the indigenous population for many different purposes, such as a mood-elevator, sedative, hypnotic, thirst and appetite suppressant, analgesic, to combat fatigue, and for its intoxicating effects in the form of euphoria (Gericke and Viljoen, 2008).

Chemical composition: ST contains many different alkaloids which are variably responsible for its pharmacological effects (Krstenansky, 2017). The most prominent alkaloids include mesembrine, mesembrenone, mesembrenol, and mesembrenol (**Fig. 2.2)** with mesembrine being the most abundant constituent (Krstenansky, 2017; Murbach et al., 2014; Schifano et al., 2015; Terburg et al., 2013).

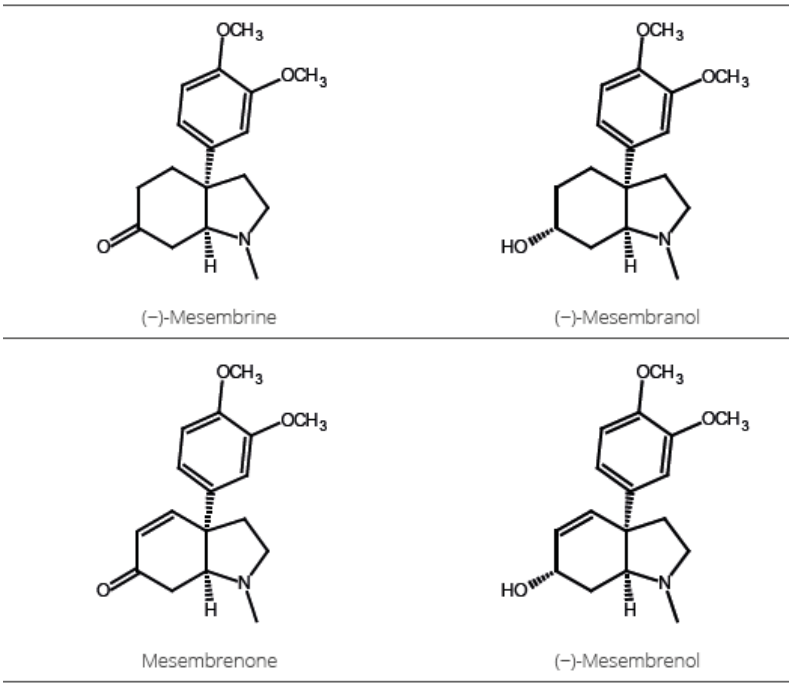

Fig. 2.2. Chemical structures of four of the most abundant alkaloids found in ST

Pharmacological mechanism of action: Furthermore, studies have found that each alkaloid constituent possesses specific mechanisms of action, with some having more pronounced pharmacological effects and/or potencies than others (Gericke and Viljoen, 2008; Loria et al., 2014). Pharmacological effects of mesembrine, mesembrenone; mesembrenol and alkaloid enriched fractions (Carpenter et al., 2016; Loria et al., 2014) are summarized below in Table 2.1. The latter studies included behavioural results only.

Furthermore, previous *in vitro* and *in vivo* studies (summarized in Table 2.2) showed that ST has antidepressant, anxiolytic and immunomodulatory effects, thus suggesting promise in the treatment of psychiatric disorders like depression and anxiety disorders.

In vitro cell culture studies: Studies have shown that ST inhibits 5-HT reuptake by decreasing the expression of SERT (Coetzee et al., 2016; Harvey et al., 2011). ST also increases monoamines by up-regulating vesicular monoamine transporter (VMAT-2), which plays a role in monoamine release and storage (Coetzee et al., 2016), while also showing limited inhibition of NE and DA reuptake at higher concentrations (Harvey et al., 2011; Schell, 2014). Furthermore, ST is a potent inhibitor of PDE4, thus enabling cAMP-mediated signal transduction (Duman et al., 2000; Gericke and Viljoen, 2008; Halene and Siegel, 2007; Harvey et al., 2011; Harvey et al., 2018; Krstenansky, 2017). Mild inhibition of AChE and MAO-A is also evident *in vitro* (Coetzee et al., 2016; Khatib and Yuliana, 2010). At high doses of ST *in vitro*, there is a concentration-dependent inhibitory activity on GABA, δ_2- and μ- opioid, EP4 prostaglandin, melatonin-1, and cholecystokinin-1 (or –A) receptors (Harvey et al., 2011; Loria et al., 2014). ST has immunomodulatory effects (Bennett and Smith, 2018). The latter affords it with significant cytoprotective, anti-inflammatory and antioxidant effects, although these have only been studied in vitro. In this regard, higher doses of ST increase levels of the anti-inflammatory cytokine IL-10 while decreasing that of the pro-inflammatory cytokine IL-6 in human monocytes (Bennett and Smith, 2018).

In vivo: Very little neurochemical data is currently available on ST, with most data based *on in vitro* findings. The majority of *in vivo* studies investigated behaviour without neurochemistry markers. A preclinical study in chicks by Carpenter et al. (2016) found evidence of anxiolytic-like effects in different behavioural tests, while healthy SD rats treated with ST was used to evaluate side effects of ST (Loria et al., 2014) (see Table 2.2 for more detail). That said, five clinical studies (Chiu et al., 2014; Dimpfel et al., 2016; Dimpfel et al., 2017; Nell et al., 2013; Terburg et al., 2013) gave been done in healthy human subjects (see Table 2.2). In fact, the majority of *in vivo* ST studies focused mainly on healthy subjects. To our knowledge, only one *in vivo* study revealed antidepressant effects in the OFT and FST at a low compared to a high dose of ST in a mouse (BALB-c) model of MDD (Schell, 2014) (see Table 2.2. for more detail). Evidently, there exists a need for *in vivo* ST studies in depressed patients and animal models of MDD, with the inclusion of neurochemical data to expand our knowledge of ST as a potential antidepressant.

Table 2.1. Summary of currently available data on specific pharmacological effects of the most abundant alkaloids found in ST

Alkaloid	Pharmacological mechanisms	References
Mesembrine	SERT inhibition;	(Carpenter et al., 2016; Coetzee et al., 2016; Gericke and Viljoen, 2008; Harvey et al., 2011; Krstenansky, 2017)
	PDE-4 inhibition	(Carpenter et al., 2016; Gericke and Viljoen, 2008)
	Anti-inflammatory; cytoprotective	(Bennett and Smith, 2018)
	upregulates VMAT-2	(Coetzee et al., 2016; Krstenansky, 2017)
	mild inhibition of AChE	(Coetzee et al., 2016)
	Mild inhibition of MAO-A	(Coetzee et al., 2016)
	limited reuptake of NE and DA at high concentrations	(Gericke and Viljoen, 2008)
Mesembrenone	SERT inhibition	(Carpenter et al., 2016; Harvey et al., 2011)
	PDE-4 inhibition	(Harvey et al., 2011)
Mesembrenol	SERT inhibition	(Carpenter et al., 2016; Harvey et al., 2011)
	PDE-4 inhibition	(Carpenter et al., 2016)
Mesembranol	No data found	
Alkaloid enriched fraction (in vivo; behavioural)	Anxiolytic	(Carpenter et al., 2016)
	Antidepressant in FST	(Loria et al., 2014)

Clinical application and indications: The psychological effects of ST, as noted in its traditional use (Gericke and Viljoen, 2008; Harvey et al., 2011), suggests potential to alleviate symptoms in anxiety disorders and depression (Smith, 2011). In fact, a functional magnetic resonance imaging (fMRI) study in healthy patients found that ST reduces amygdala reactivity to unattended facial expressions of others that convey fear, and can also decouple amygdala-hypothalamus connectivity, thus inducing anxiolytic effects (Silvert et al., 2007; Terburg et al., 2013). Due to its ability to target mechanisms associated with antidepressant effects, namely SERT inhibition and inhibition of MAO and PDE4, ST may be a valuable treatment for depression. Moreover, its anti-inflammatory and cytoprotective properties suggest therapeutic potential in MDD. Despite its possible benefits as an add-on treatment or augmentation therapy, to our knowledge, this approach has not been studied either clinically or pre-clinically. It is in the area of MDD where it may offer significant potential, which is the focus of this study.

Side effects: ST has a relatively low side effect profile (Schifano et al., 2015), its most common side effects being headache, hypertension, nausea, insomnia, irritability and anxiety (Schifano et al., 2015). Although no studies have been performed to-date that have evaluated its monoaminergic properties, given its inhibitory actions on MAO, SERT and VMAT-2 suggests caution when combining ST with other serotonergic agents for fear of precipitating serotonin syndrome (Krstenansky, 2017; Schifano et al., 2015). To our knowledge, no cases have been reported to date. ST has little to no abuse liability (Gericke and Viljoen, 2008; Harvey et al., 2011; Loria et al., 2014; Nell et al., 2013).

Previous studies: Previous *in vitro* (Bennett and Smith, 2018; Coetzee et al., 2016; Harvey et al., 2011) and *in vivo* studies (Carpenter et al., 2016; Loria et al., 2014; Terburg et al., 2013), in addition to anecdotal claims (Gericke and Viljoen, 2008), showed that ST have antidepressant, anxiolytic and immunomodulatory effects, thus suggesting promise in the treatment of MDD and anxiety-related disorders. Table 2.2. provides a summary of the abovementioned *in vitro* and *in vivo* studies performed on ST.

Table 2.2: Summary of previous studies on ST

SOURCE	TYPE OF STUDY	SCELETIUM PREPARATION	DOSE AND ROUTE OF ADMINISTRATION	FINDINGS	CONCLUSION
Gericke and Viljoen (2008)	**Clinical case reports:** 1) Depressed woman	Not specifically stated. Tablets	50 mg daily oral (tablet) Withdrawn after 4 weeks	Improved mood Decreased general anxiety Insomnia improved (at onset) No symptoms of withdrawal after discontinuation	Effective anxiolytic and mood elevator
	2) Dysthymic woman with personality disorder	Not specifically stated. Tablets	50 mg daily oral (tablets) Doubled dose to 100 mg daily for exams (month later)	Mood lifted within 10 days More focused More engaged and less socially distant Decreased anxiety Less inclined to over-indulge in alcohol	Mood elevator, anxiolytic and more contained feeling
	3) Woman with MDD Failed treatment with St. John's Wort	Not specifically stated. Tablets	100 mg daily oral (tablets)	Mood lifted within first day Hypersomnia improved Increase in energy 6 weeks treatment: fully recovered and maintained	Mood elevator
Harvey et al. (2011)	***In vitro* studies on mammalian cells** Receptors, enzymes and other targets	Zembrin® and Purified alkaloid extracts: Mesembrine Mesembrenone mesembrenol	N/A	Serotonin reuptake inhibition (inhibits serotonin transporter (SERT)) Potent inhibition of phosphodiesterase 4 (PDE4) and to lesser extent PDE3 At high doses, inhibition of GABA, δ_2-opioid, μ-opioid, cholecystokinin-1, melatonin-1, and EP4 prostaglandin receptors	Potent 5-HT reuptake inhibitor Potent PDE4 inhibitor

Loria et al. (2014)	***In vivo* animal study** Healthy Sprague Dawley rats Behavioural tests: Conditioned Place Preference (CPP)* Hotplate test Forced Swim Test (FST) Elevated Plus Maze (EPM) Rotarod	Alkaloid enriched fraction Mesembrine Alkaloid extract	5, 10, 20 mg/kg* 20 mg/kg 5, 10, 20 mg/kg* 20 mg/kg 25, 50, 100 mg/kg* 100 mg/kg Intraperitoneally	Reduced float time in FST Ataxia in rotarod Altered nociceptive responses approximate to morphine No psychoactive properties in EPM or CPP	Antidepressant with ataxia Analgesic without abuse liability or ataxia No anxiolytic effects, no abuse liability
Schell (2014)	***In vivo* animal study** BALB/c mice (MDD model)	Direct acid extraction of mesembrine	10 mg/kg/day or 80 mg/kg/day	Decreased immobility in FST at low dose	Antidepressant
Carpenter et al. (2016)	***In vivo* animal study** **Chick anxiety-depression model** (distress vocalizations after social separation)	Alkaloid enriched fraction	10, 20, or 30 mg/kg (Experiment 1) 50, 75, or 100 mg/kg (Experiment 2)	No antidepressant effects (contradictory of literature in rodents) – could be due to altering effects of constituents on each other, narrower dose range for antidepressant effects, comorbid anxiety symptom overlaps, translational problems from rodents to avian model. Good anxiolytic effects	Anxiolytic
Bennett and Smith (2018)	***In vitro*: human monocytes** Immunomodulatory effects – cytokine release and mitochondrial viability	Lyophilised extract (Trimesamine®)	0.01 mg/ml or 1.0 mg/ml ± *E.coli* lipopolysaccharides (LPS)	Increased mitochondrial viability Upregulated anti-inflammatory cytokine IL-10 Prevented decreased mitochondrial viability (LPS-induced) Acute inflammatory response to LPS not negatively affected Best dose: 0.01 mg/ml	Cytoprotective against oxidative stress Mild anti-inflammatory properties Beneficial for cytokine-induced depression and systemic low-grade inflammation

Terburg et al. (2013)	**Acute human studies:** pharmaco-fMRI on anxiety-related activity in amygdala. Healthy subjects. Perceptual-task and emotion-matching task	Zembrin®	Single 25 mg dose	Attenuated amygdala reactivity to fearful faces under low perceptual load conditions Decreases amygdala-hypothalamus coupling	Anxiolytic potential by decreasing amygdala reactivity to unattended facial fear
Coetzee et al. (2015)	**In vitro studies** Human astrocytes and mouse hippocampal cells: SERT; VMAT-2 MAO-A; AChE	Trimesamine™ High in Mesembrine	1, 0.1, 0.01, 0.001, 0.0001 mg/ml	Down-regulated SERT expression similar to citalopram VMAT-2 upregulated significantly Relatively mild inhibition of AChE and MAO-A	SERT inhibition activity is a secondary function to monoamine-releasing activity of high-mesembrine extract
Dimpfel et al. (2016)	Acute clinical study in healthy subjects. Psychophysiological characterization of ST. EnkephaloVision method (EEG analysis during cognitive and emotional challenges)	Zembrin®	25 and 50 mg vs placeo	Significant increase in delta and theta spectral power in the frontal brain indicating positive effects on electrical activity of the brain during cognitive processing. Increased calmness and decreased depressive symptoms; increased memory and attention	Positive action on cognitive and emotional processes in the brain
Chiu et al. (2014)	Clinical study in healthy subjects. Double-blind placebo-controlled croos-over design. CNS Vital Signs, Hamilton depression rating scale (HAM-D). Monitored side effects with treatment emergence adverse events scale	Zembrin®	25 mg capsule Once daily for 3 weeks vs placebo	Significantly improved cognitive set flexibility and executive function. Positive changes in mood and sleep. Well tolerated	Promising cognitive enhancing effects. Possible treatment of early Alzheimer's dementia.
Dimpfel et al. (2016b)	Clinical study in healthy subjects. EEG 1 hour before and after administration of Zembrin.	Zembrin®	25 or 50 mg Daily for 6 weeks vs placebo	Increased delta and theta activity (50 mg). Increased alpha 1 spectral power. Improvement during performance of the	Improves some aspects of cognitive function, decreases anxiety, enhances mood

	6 Cognitive tests: d2-test, memory test, calculation performance test, reaction time test, number identifying test, number connection test. 3 Questionnaires (HAM-D, sleep questionnaire, Profile of Mood States.			arithmetic calculation test and number connection test. Decreased anxiety after 6 weeks. Significant activity on questionnaires, psychometry and quantative EEG.	
Nell et al. (2013)	Clinical study in healthy subjects. Assessed safety and tolerability with full vital signs, ECG, physical examination, laboratory assessments (hematology, biochemistry, urinalysis), Recorded adverse events	Zembrin®	8 mg and 25 mg once daily for 3 months vs placebo	Well tolerated. No significant differences in any of the tested parameters after 3 months. Most common adverse events: headache, abdominal pain, upper respiratory tract infections (all greater incidence in placebo groups. Improved wellbeing. Improved coping with stress and sleep	Both doses well tolerated in healthy humans for 3 months

2.4. MDD Pathophysiology

MDD is a complicated disorder which is not fully understood, although several hypotheses and possible mechanisms of its underlying pathophysiology have been identified (Dean and Keshavan, 2017). Some of these mechanisms and hypotheses include, among others, the monoamine deficit hypothesis (Dean and Keshavan, 2017; Haase and Brown, 2015; Jesulola et al., 2018), the glutamate hypothesis (Brand et al., 2015), hypothalamic-pituitary-adrenal axis (HPA axis) dysregulation (Dean and Keshavan, 2017; Du and Pang, 2015), stress-related responses (Binder and Nemeroff, 2010; Dean and Keshavan, 2017; Jesulola et al., 2018), inflammatory conditions and increased cytokines (Dean and Keshavan, 2017; Haase and Brown, 2015; Jeon and Kim, 2017; Shariq et al., 2018), dysfunctional neurogenesis and neuroplasticity (Belzung et al., 2015; Brown et al., 2017; Dean and Keshavan, 2017; Jesulola et al., 2018; Kraus et al., 2017; Miller and Hen, 2015), environmental and genetic influences (Belzung et al., 2015; Jesulola et al., 2018), circadian rhythm misalignment and changes in melatonin (Brown et al., 2017), endocrine factors (Jesulola et al., 2018), faulty neurocircuitry (Dean and Keshavan, 2017), increased acetylcholine (Janowsky et al., 1974; Mineur and Picciotto, 2010; Shytle et al., 2002), and abnormalities in second messenger systems (Deutschenbaur et al., 2016; Funk et al., 2012; Ghosal et al., 2017) such as the adenylate cyclase-cyclic adenosine monophosphate (cAMP)-phosphodiesterase 4 (PDE4) cascade (Halene and Siegel, 2007; O'Donnell and Zhang, 2004).

Furthermore, dysregulation of brain regions like the frontal cortex, implicated in mood control, cognition, attention and motility (Millan et al., 2000), and the hippocampus (part of the limbic system), which facilitates memory, learning and cognition (Dean and Keshavan, 2017), are causally implicated in the pathophysiology of MDD. Previous studies have found reduced volumes of the frontal cortex (including prefrontal cortex (PFC) and medial prefrontal cortex (mPFC)) and hippocampus in patients diagnosed with MDD (Belleau et al., 2018; Brand et al., 2015). These brain areas are susceptible to stress which ultimately leads to brain structural damage via stress-induced hypothalamic-pituitary-adrenal (HPA) axis dysregulation, inflammation, hyperglutamatergic activity, oxidative stress, and abnormalities in neurotransmitters (Belleau et al., 2018). These abnormalities will be discussed in more detail later in this chapter. The hippocampus is especially susceptible to these factors (Willner et al., 2013), eventually presenting with severe impairments in neuroplasticity and neurogenesis, the latter due to reduced brain-derived neurotrophic factor (BDNF) (Belleau et al., 2018; Duman et al., 2000; Saaltink and Vreugdenhil, 2014).

For the purpose of this dissertation, the monoamine hypothesis and the role of PDE4 in the regulation of the inflammation and neurogenesis in MDD will be elaborated on, with special attention to these changes in the frontal cortex and hippocampus. Furthermore, the use of the FSL rat as a genetic model of MDD prompts the need to touch on the genetic influences in MDD.

2.4.1. Monoamine reduction

Changes in the levels of monoamines like serotonin (5-HT), dopamine (DA) and norepinephrine (NE), are implicated in the aetiology of MDD (Dean and Keshavan, 2017). Decreased levels of monoamines can cause impaired neurotransmission and cognitive performance which are expressed as symptoms such as low mood, tiredness, decreased motivation, psychomotor agitation and retardation, and cognitive and memory disturbances (Jesulola et al., 2018). One suggestion for decreased monoamines is over-activity of the enzyme monoamine oxidase (MAO) (Meyer et al., 2006), which is responsible for the degradation of monoamines in the synaptic cleft, thus less monoamines are available for binding to their receptors and subsequent neurotransmission (Jesulola et al., 2018; Osuch and Marais, 2017; Willner et al., 2013). However, other key processes involved in monoaminergic transmission and that contribute to decreased monoamine levels could be dysregulated protein transporter function at pre-synaptic monoamine reuptake sites (Jesulola et al., 2018), especially the 5-HT reuptake transporter (SERT), NE transporter (NET) and the DA transporter (DAT). These transporters facilitate reuptake of intact monoamines from the synaptic cleft into the neuron where they are stored in vesicles for later transmission, or degraded by the enzyme monoamine oxidase (Andrews, 2015; Olivier, 2015).

Receptor dysfunction could also contribute to reduced transmission as the number and affinity of receptors are altered in such a way that neurotransmitters cannot couple with their receptors, leading to faulty and reduced transmission (Osuch and Marais, 2017).

Factors such as inflammation can also cause a decrease in neurotransmitters (Maes et al., 2011; Sahin et al., 2016). For example, reductions in 5-HT can be caused by pro-inflammatory cytokine-mediated activation of indolamine 2,3-dioxygenase (IDO). IDO is responsible for the conversion of tryptophan (the precursor of serotonin) to kynurenine and the activation thereof causes a shift in the metabolism of tryptophan away from 5-HT, favouring the production of kynurenine, resulting in decreased 5-HT production and bioavailability (Maes et al., 2011; Sahin et al., 2016).

2.4.2. Dopamine

DA is a neurotransmitter that is synthesized from tyrosine by the rate-limiting enzyme tyrosine hydroxylase (TH) (Salvatore et al., 2016). DA itself is also the precursor for NE synthesis via DA β-hydroxylase (El Mansari et al., 2010). Distinct pathways are involved in DA neurotransmission which can be seen in Fig. 2.3. These include the nigrostratial pathway originating from the substantia nigra (controls movement and sensory stimuli), the mesolimbic pathway originating from the ventral tegmental area (VTA) (also known as the reward pathway, controlling pleasure, emotional regulation, addiction and perception), and the mesocortical pathway originating from the VTA (controls memory, learning, cognition, attention, and emotional behaviour) (Ayano, 2016). Levels of DA can be regulated via various mechanisms, e.g. the D_2 autoreceptor provides

inhibition of further neuronal firing, release of DA, and synthesis via decreased TH phosphorylation (Salvatore et al., 2016). This receptor also upregulates DAT, consequently promoting reuptake of DA into presynaptic neurons with a nett effect of decreased DA and DA signalling (Dickinson et al., 1999).

DA is involved in motoric behaviour, cognition and emotion (Grace, 2016). Furthermore, it regulates reward and motivation, attention, working memory and aggression (Grace, 2016; Jesulola et al., 2018). Attenuated DA activity is usually associated with MDD, with mesolimbic DA and mesocortical pathways (also known as the reward pathway) believed to be decreased in MDD, accompanied by hypoactive D_1 receptors (Ayano, 2016). This leads to the despair, decreased motivation, anhedonia, impaired cognition and memory, and dysregulated emotions most commonly associated with MDD (Ayano, 2016; Dean and Keshavan, 2017; Panksepp and Watt, 2011).

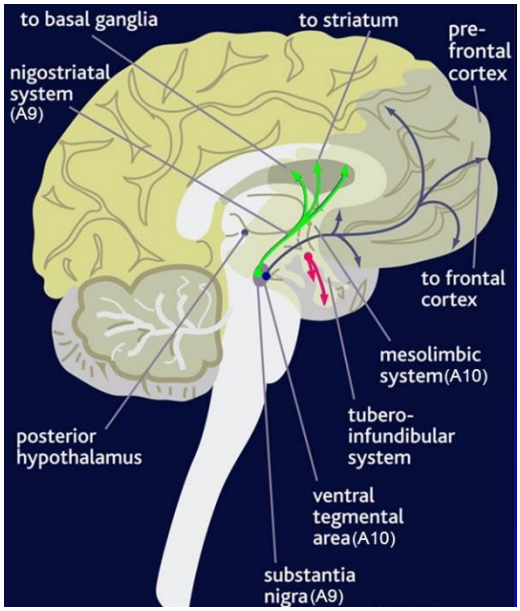

Fig. 2.3. Dopaminergic pathways of the brain (Hamman et al., 2015)

Stress also has a major effect on the regulation of DA levels, which can play an important role in anhedonia attributed to acute stress (Lemos et al., 2012). While CRF normally increases the release of DA in the nucleus accumbens to induce a rewarding effect, this is converted to an aversive response when exposed to stress (Lemos et al., 2012; Willner et al., 2013). Stress can also cause neuroadaptive changes in the brain, which can alter the expression of BDNF and hence neuroplasticity (Nestler and Carlezon, 2006). Moreover, the hippocampus is greatly susceptible to stress, which can give rise to dysfunctional release of DA (Willner et al., 2013). Therefore, impaired hippocampal activity as seen in MDD can decrease excitatory DA cell firing via its indirect connection to the VTA of the midbrain (Cooper et al., 2006).

Other neurotransmitters can also influence DA levels. Studies have shown that 5-HT receptors can have inhibitory or excitatory effects on DA systems in different areas of the brain, such as

reducing DA release in the striatum to cause Parkinsonism (Dean and Keshavan, 2017; Shimizu and Ohno, 2013). NE increases DA release by stimulating α_1 adrenoreceptors (α_1-ARs) and decreases its release by inhibiting α_2 receptors (α_2-ARs) (Dean and Keshavan, 2017). DA can have different effects in different regions of the brain involved in MDD, for instance inducing hyperactivity in the prefrontal cortical area and hyper-responsivity of the amygdala to emotionally charged stimuli, especially to negative affective components, thereby precipitating depressed mood (Grace, 2016).

2.4.3. Serotonin

Serotonin (5-hydroxytryptamine or 5-HT) is a chemical messenger which is found in abundance throughout the body and is involved in the regulation of numerous bodily functions. This includes, among many others, cardiovascular functioning, bowel motility and bladder control, ejaculation, and even platelet aggregation (Berger et al., 2009). Moreover, 5-HT is a very important neurotransmitter in the central nervous system and is instrumental in the regulation of mood and behavioural and somatic functions such as appetite, sleep patterns, libido, response to pain, regulation of body temperature, circadian rhythm, impulse control, cognition, and memory and learning (Akimova et al., 2009; Alenina and Klempin, 2015). It is also crucial in the modulation of anxiety and fear, and impulsivity associated with violence and suicide (Akimova et al., 2009). Due to all of these effects, 5-HT is also implicated in the pathophysiology of MDD and other mood and anxiety disorders (Berger et al., 2009; Berumen et al., 2012; Dean and Keshavan, 2017).

Studies have found that decreased 5-HT neurotransmission can be a cause of depression, although the opposite has been found in some subtypes of depression like melancholic depression (Andrews et al., 2015). Many overall mechanisms causing decreased monoamines (as described in Section 2.4.1) that could cause decreased 5-HT neurotransmission include inhibition of 5-HT release due to presynaptic autoreceptors, reduced synthesis of 5-HT due to depleted tryptophan (a precursor of 5-HT), increased pro-inflammatory cytokines that directly decrease 5-HT synthesis via the kynurenine pathway, genetic abnormalities in 5-HT transmission causing altered receptor expression, and depression-induced stress that causes interactions between norepinephrine, glutamate and histamine to decrease the production of 5-HT (Dean and Keshavan, 2017; Jesulola et al., 2018; Osuch and Marais, 2017; Strawbridge et al., 2017).

The midbrain raphe nuclei are the central serotonergic neurons in the brain (Celada et al., 2004; Samuels et al., 2011). These cell bodies innervate almost every brain region (Fig. 2.4), including the hippocampus and frontal cortex which play major roles in depression (Berumen et al., 2012). Serotonergic effects are then mediated by the binding of 5-HT to various 5-HT post-synaptic (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, 5-HT_3, 5-HT_4, 5-HT_5, 5-HT_6, 5-HT_7) and pre-synaptic

(5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}) receptors to increase or decrease firing of serotonergic neurons, respectively (Blier and El Mansari, 2013; Samuels et al., 2011).

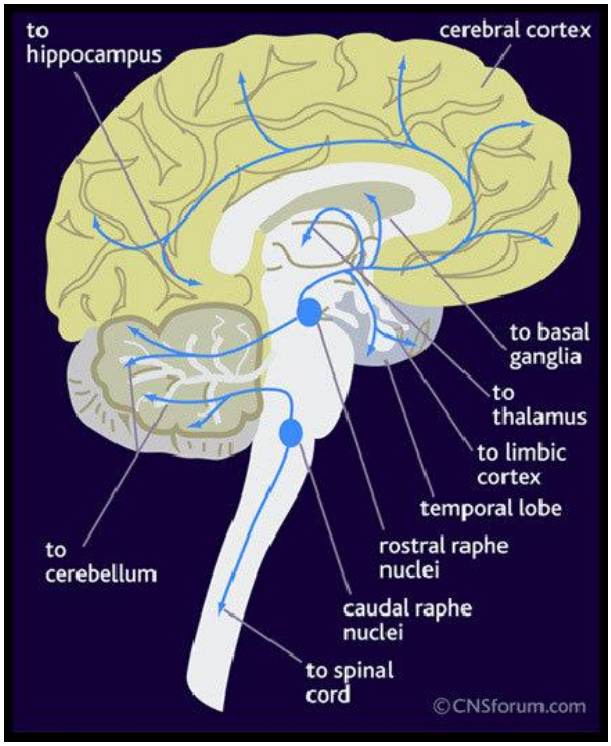

Fig. 2.4. Diagram depicting the serotonergic pathways in the brain (Hamman et al., 2015)

Increased 5-HT levels are typically regulated through negative feedback mechanisms that eventually decrease synaptic 5-HT levels. This can be due to the reuptake of the 5-HT into the presynaptic serotonergic neurons via SERT or the vesicular monoamine transporter (VMAT-2) where it is consequently either stored in vesicles to be released again when needed, or degraded by MAO-A to form the metabolite 5-hydroxyindole acetic acid (5-HIAA) (Olivier, 2015). In addition, increased 5-HT activates 5-HT_{1B} (in mice) or 5-HT_{1A} (in humans) autoreceptors, which in turn attenuates further release of 5-HT (Barnes and Sharp, 1999; Olivier, 2015). The activation of 5-HT_{1A} autoreceptors also leads to inhibition of neuronal firing, thereby reducing its activity and preparing the neuron for new discharge (Artigas, 2013). Importantly, post-synaptic 5-HT_{1A} heteroreceptors modulate the release of other neurotransmitters implicated in mood, anxiety and stress, e.g. the release of acetylcholine (ACh), NE, DA, glutamate and GABA from their respective neurons (Albert and Vahid-Ansari, 2018; Barnes and Sharp, 1999; Shimizu and Ohno, 2013).

2.4.3.1. The 5-HT transporter

The 5-HT transporter (SERT) plays an important role in the pathophysiology and treatment of MDD (Canli and Lesch, 2007). SERT is a sodium chloride-dependent transporter located on glia cells and 5-HT nerve terminals, which regulates the uptake of amino acids and 5-HT from the

extracellular space (Kovačević et al., 2010). Transport proteins increases availability of neurotransmitters needed for sustained neurotransmission by increasing and facilitating their presynaptic reuptake (Bondy, 2002; Jesulola et al., 2018). This in turn limits the amount of neurotransmitter available for degradation by MAO enzymes in the extracellular space, so that dysregulation of these transporters could cause decreased monoamines as seen in MDD (Bondy, 2002; Jesulola et al., 2018; Owens et al., 2011). However, there are no extracellular enzymes responsible for 5-HT breakdown and thus 5-HT needs to be transported by SERT into the presynaptic neuron where it is metabolized by MAO-A to 5-HIAA. Excessive synaptic reuptake will cause a decrease in extracellular 5-HT, presumably leading to depressive symptoms (Andrews et al., 2015). MAO-A levels in the limbic areas have also been found to be increased in MDD (Barton et al., 2008; Brand et al., 2015). Depressed suicide victims have shown decreased and increased 5-HIAA in the cerebrospinal fluid (CSF) and plasma, respectively (Mitani et al., 2006), although low plasma levels were found in a study by (Spreux-Varoquaux et al., 2001), and a review by Brand et al. (2015) found that decreased 5-HIAA is a strong marker of MDD.

Furthermore, stress-induced release of inflammatory cytokines can upregulate SERT activity via cytokine-dependent pathways, which ultimately leads to decreased synaptic 5-HT levels, decreased BDNF levels and associated decreased neuroplasticity (see Section 2.4.7), and thus precipitating MDD symptoms like impaired memory and cognition (Haase and Brown, 2015). SSRI-induced inhibition of SERT causes an elevation of extracellular 5-HT, which enhances signalling through post- and presynaptic 5-HT receptors, thus potentiating antidepressant affects (Haase and Brown, 2015).

SERT expression and function can differ in depressed patients due to genetic polymorphisms in the expression of the polymorphic region 5-HTTLPR on the SLC6A4 gene which only codes for SERT (Canli and Lesch, 2007; Willner et al., 2013). People with a specific short variant of 5-HTTLPR are twice as likely to develop MDD when exposed to severe stressors such as loss of a loved one, illness, job loss, or romantic disasters (Canli and Lesch, 2007). These short alleles create reduced SERT expression, thus resulting in a dysfunctional regulation of 5-HT (Belsky et al., 2009). Individuals with the short allele have been shown to have increased HPA stress reactivity, thus presenting with a higher risk of developing MDD when exposed to stress (Gotlib et al., 2008). This also supports the idea that a person with a genetic predisposition towards MDD is more likely to develop MDD once exposed to severe environmental stressors. This is known as the Diathesis-Stress Model of MDD (Dean and Keshavan, 2017; Jesulola et al., 2018).

2.4.3.2. The 5-HT_{1A} receptor

The pre- and postsynaptic 5-HT_{1A} receptors are important in MDD and its treatment (Akimova et al., 2009). Post-mortem studies have found increased density of 5-HT_{1A} receptors in the limbic regions, including the hippocampus (pre- and postsynaptically), as well as lower densities (postsynaptically) in the frontal cortex of suicide victims diagnosed with MDD (Blier and Ward, 2003; Pasqualetti et al., 1996; Polter and Li, 2010). These receptors contribute to dynamic modulation of serotonergic activity, impacting functions like cognition and emotion, and also playing a crucial role in neuronal migration, neurite growth and synapse formation as part of neurodevelopment (Savitz et al., 2009; Whitaker-Azmitia et al., 1995).

5-HT_{1A} receptors are expressed on neurons in many areas of the brain, such as serotonergic neurons in the raphe nuclei, the pyramidal neurons in the hippocampus and cortex, and cholinergic neurons in the septum (Barnes and Sharp, 1999). 5-HT_{1A} receptors have a high affinity for 5-HT (Hoyer et al., 1994), with its primary function to dominate 5-HT transmission under normal circumstances (Carhart-Harris and Nutt, 2017).

Presynaptic 5-HT_{1A} receptors act as somatodendritic autoreceptors and are situated on serotonergic neurons in the raphe nuclei (Akimova et al., 2009). Activation of these receptors reduce the firing rate of serotonergic neurons through hyperpolarization, thereby attenuating 5-HT synthesis and turnover, and decreases further release of 5-HT (Akimova et al., 2009). Postsynaptic 5-HT_{1A} receptors are mainly situated on glutamatergic and GABAergic pyramidal neurons in the frontal and entorhinal cortices and limbic regions (Akimova et al., 2009; Pasqualetti et al., 1996). Studies have shown that limbic areas, especially the hippocampus, contain the highest density of 5HT_{1A} receptors compared to other areas such as the frontal cortex, which has an intermediate level of expression (Blier and Ward, 2003; Pasqualetti et al., 1996). Studies also suggest that 5-HT_{1A} might be pre- and postsynaptic on the pyramidal cells in the hippocampus, correlating with previous data from rat studies (Berumen et al., 2012; Pasqualetti et al., 1996).

The following summary of the signalling pathways of 5-HT_{1A} receptors was derived from reviews by (Huang et al., 2017; Newman-Tancredi et al., 2017; Samuels et al., 2011; Shimizu and Ohno, 2013) and are visually illustrated in Fig. 2.5. below (taken from Shimizu and Ohno (2013)). Binding of 5-HT to 5-HT_{1A} receptors (1) causes coupling with an inhibitory $G_{i/o}$ protein (2). Specific G-protein subunits, like the $G_{\alpha o}$ subunit in the hippocampus, inhibits adenylate cyclase (AC) (3) which consequently decreases cyclic adenosine monophosphate (cAMP) formation (4) and as a result inhibits protein kinase A (PKA)-mediated protein phosphorylation (5), ultimately causing a decrease in neuronal activity (6). At the same time, activation of GIRK) (7) causes hyperpolarization (9) due to the efflux of intracellular K+ (8), ultimately inhibiting neuronal activity (10).

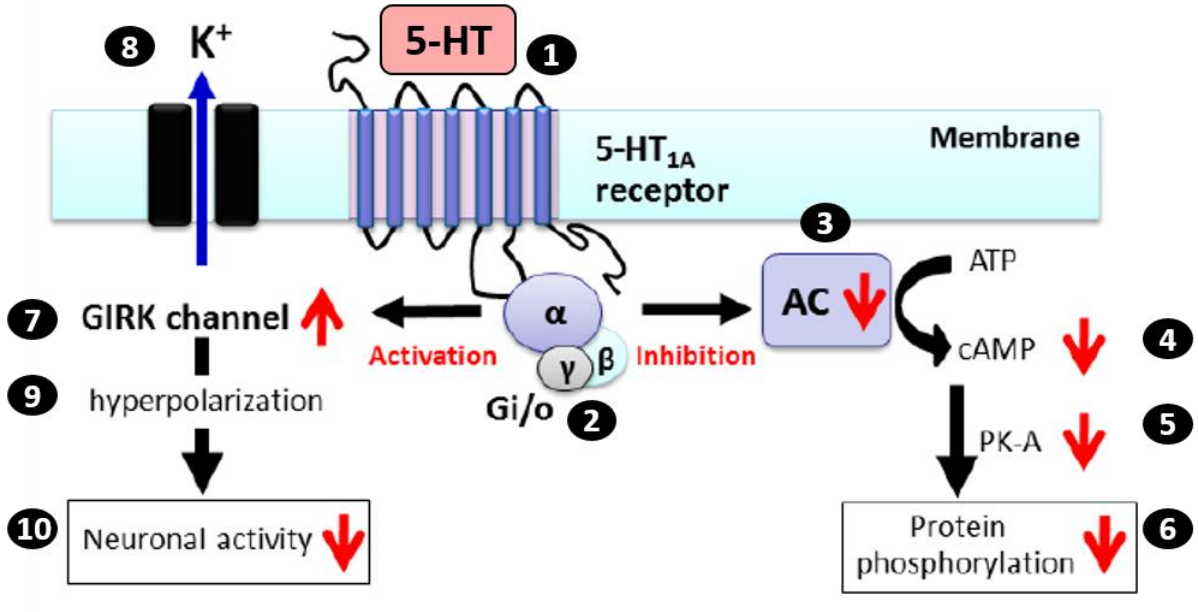

Fig. 2.5: Signal transduction pathways of 5-HT$_{1A}$ receptors. Refer to text for description. Adapted from Shimizu and Ohno (2013).

In times of increased 5-HT, for example when antidepressants are administered or during normal regulation of increased 5-HT levels, 5-HT$_{1A}$ receptors play a step-wise inhibitory role on 5-HT synthesis and release via the mechanisms described graphically in Fig. 2.6. (Albert and Vahid-Ansari, 2018; Blier and Ward, 2003; Carhart-Harris and Nutt, 2017). Increased extracellular 5-HT levels (1), causes 5-HT to bind to postsynaptic 5-HT$_{1A}$ and other postsynaptic receptors (2) resulting in increased firing of postsynaptic neurons (3). When 5-HT activates presynaptic 5 HT$_{1A}$ receptors (4), hyperpolarization and decreased 5-HT synthesis and release form the neuron takes place (5), thus decreasing extracellular 5-HT (6). Less 5-HT is available to bind to postsynaptic receptors and neuronal firing is decreased (7).

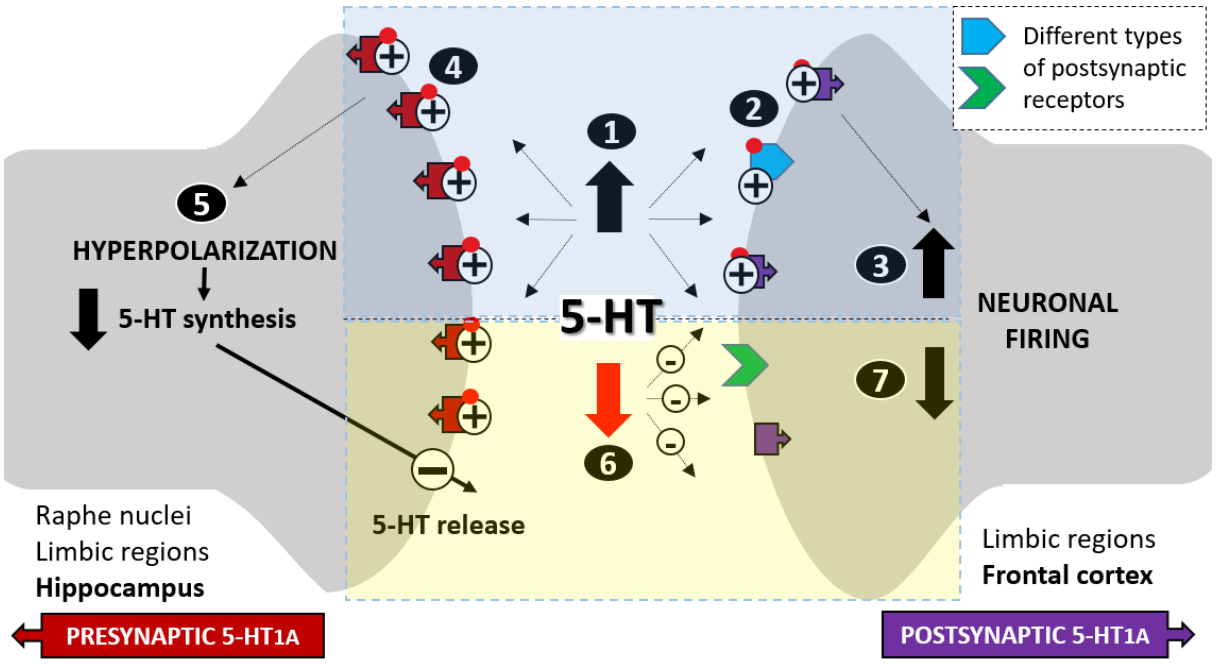

Fig. 1.6. Representation of the effects of increased 5-HT on pre- and postsynaptic 5-HT_{1A} receptors. Refer to text for a step-wise description.

Acute treatment with antidepressants like SSRIs could theoretically cause an initial increase in 5-HT due to its direct pharmacological effects on SERT inhibition. Nevertheless, acute treatment with SSRIs have shown that extracellular levels of 5HT in frontal cortex did not increase much, possibly due to the concomitant activation of the 5-HT_{1A} autoreceptors (Ceglia et al., 2004) by mechanisms described above in Fig. 2.6.

Chronic treatment can cause a compensatory decrease in 5-HT in attempt to restore homeostasis after acute SSRI treatment-induced disruption in energy homeostasis (Andrews et al., 2015). When antidepressants like SSRIs or 5-HT_{1A} agonists like 8-OH-DPAT are deployed as chronic treatment, it can induce the recovery of normal firing rate of serotonergic neurons through desensitization of the 5HT_{1A} autoreceptors which ultimately leads to increased synaptic 5-HT levels (Blier and Ward, 2003; Celada et al., 2004).

Receptor desensitization is a complicated process that could be mediated by different mechanisms, as reviewed in detail by Albert and Vahid-Ansari (2018). In short, some of these mechanisms include acute protein kinase mediated mechanisms, or downregulation of 5-HT_{1A} receptors by changes in G protein-coupling with the receptors or differential transcriptional regulation of the 5-HT_{1A} promoter in pre- and postsynaptic neurons. These mechanisms differ depending on the type of receptor (auto- or heteroreceptor), brain area, specific cell types, and developmental stage. They also concluded that 5-HT_{1A} autoreceptors appear to be more sensitive to 5-HT-induced desensitization than heteroreceptors (Mehta, 2008).

One mechanism of desensitization can include the decrease in levels of inhibitory G proteins like $G_{\alpha o}$ in the hippocampus, thus reversing the inhibitory effects of 5-HT_{1A} (Samuels et al., 2011). This increase in 5-HT levels leads to an antidepressant effect (Shrestha et al., 2012; Shrestha et al., 2014). According to Le Poul et al. (1995), desensitization after treatment with SSRIs can probably also result from continuously increased stimulation of 5-HT_{1A} receptors by the accumulated 5-HT in the extracellular spaces following inhibition of 5-HT reuptake. This desensitization process of the 5-HT_{1A} receptor is time dependent; this is suggested to underlie the delayed onset of therapeutic action (about 2 to 3 weeks) of serotonergic antidepressants (Shrestha et al., 2012; Shrestha et al., 2014). It has been suggested that the use of a 5-HT_{1A} receptor antagonist, for example pindolol, could accelerate the onset of action of an SSRI by suppressing the time-dependent desensitization of the 5-HT_{1A} receptor (Celada et al., 2004).

5-HT_{1A} receptor agonists like 8-OH-DPAT have also been shown to increase the release of acetylcholine (ACh) in the hippocampus and cortex of rats through mechanisms involving postsynaptic 5-HT_{1A} receptors (Barnes and Sharp, 1999; Izumi et al., 1994). This spontaneous release of ACh has been associated with increased locomotor activity by disinhibiting 5-HT-mediated suppression of the cholinergic system (Barnes and Sharp, 1999; Day et al., 1991; Izumi et al., 1994; Jordan et al., 2014). Moreover, 5-HT_{1A} agonists also increase the release of NE in many brain regions including the hippocampus, hypothalamus and frontal cortex through postsynaptic activation (Barnes and Sharp, 1999), as well as increase the expression of the intermediate-early gene, *c-fos*, in the locus coeruleus (the main source of NE projections). This suggests that the increase in NE could be attributed to an action on locus coeruleus afferents via postsynaptic 5-HT_{1A} mediated process (Barnes and Sharp, 1999; Chen and Reith, 1995; Hajós-Korcsok and Sharp, 1996). From this, it is evident that 5-HT_{1A} receptors play an important role in mediating the influences of 5-HT on cholinergic and noradrenergic pathways.

2.4.3.3. The 5-HT_{2A} receptor

$5HT_{2A}$ is an excitatory G protein-coupled receptor expressed in many brain areas, including the hippocampus, cortex, DRN and locus coeruleus, but is considerably more abundant in cortical than subcortical regions (Andrade, 2011; Carhart-Harris and Nutt, 2017; Hall et al., 2000; Willner et al., 2013). It is also highly abundant on excitatory glutamatergic neurons in the prefrontal cortex, while only about 30% of GABAergic interneurons in these same areas also contain 5-HT_{2A} receptors (De Almeida and Mengod, 2007). 5-HT_{2A} evoke excitatory postsynaptic potentials (EPSPs) in the frontal cortex, while inducing inhibitory postsynaptic potentials (IPSPs) via GABAergic interneurons in the hippocampus (Aghajanian and Marek, 1999; Jaggar and Vaidya, 2018; Narla et al., 2015; Puig et al., 2003). 5-HT_{2A} also has effects on neuroplasticity as described in Section 2.4.8.

Carhart-Harris and Nutt (2017) and Jennings (2013) suggested that, under normal circumstances, 5-HT_{1A} and its functions dominate 5-HT transmission, while 5-HT_{2A} receptors play an increasingly important role in states where 5-HT release is highly elevated (Carhart-Harris and Nutt, 2017; Quesseveur et al., 2012; Quesseveur et al., 2013). Due to their sensitivity to basal 5-HT concentrations, 5-HT_{2A} receptors can upregulate when 5-HT is low and downregulate when 5-HT is high (Benekareddy et al., 2010; Carhart-Harris and Nutt, 2017; López et al., 1999), the latter initiating a negative feedback mechanism to decrease 5-HT release from the raphe nuclei (Carhart-Harris and Nutt, 2017; Quesseveur et al., 2012; Quesseveur et al., 2013). However, the receptor can exist in a high (G protein-coupled) or low (G protein-uncoupled) affinity state (Carhart-Harris and Nutt, 2017; Sleight et al., 1996). During extreme conditions high affinity 5-HT_{2A} receptors can become upregulated and 5-HT_{1A} receptors can become down-regulated.

According to Arango et al. (1997), post-mortem studies of depressed patients showed an increase in cortical 5-HT_{2A}, which is associated with decreased 5-HT levels (Carhart-Harris and Nutt, 2017). In fact, untreated patients recovering from MDD have also shown increased 5-HT_{2A} binding in the PFC (Bhagwagar et al., 2006), thus confirming reduced 5-HT in MDD. Additionally, blocking 5-HT_{2A} (Celada et al., 2004) and 5-HT_{2C} (Barnes and Sharp, 1999; Loo et al., 2002) receptors have been shown to be valuable in the treatment of depression, and in fact is a key design feature of a number of clinically used antidepressants such as nefazodone, trazadone, mirtazapine and agomelatine (Bennett and Smith, 2018; Blier and El Mansari, 2013; Blier and Ward, 2003; De Boer et al., 1995; Horst and Preskorn, 1998; Loo et al., 2002; Millan, 2005). Stimulation of the 5-HT_{2C} receptors under both basal and activated conditions can inhibit DA release and activity (Berg et al., 2008), and can cause anxiogenic effects after acute SSRI treatment, however chronic treatment with the aforementioned antagonists can stimulate dopaminergic and adrenergic pathways while progressively downregulating 5-HT_{2C} with resultant antidepressant and anxiolytic effects (Millan, 2005).

2.4.4. Norepinephrine

Norepinephrine (NE) is a monoamine neurotransmitter that is mainly synthesized in the locus coeruleus (LC), from where projections are sent to many different brain areas, including the cortex, hippocampus, amygdala, cerebellum, thalamus and hypothalamus, and basal ganglia (Atzori et al., 2016; El Mansari et al., 2010; Maletic et al., 2017) (Fig. 2.7).

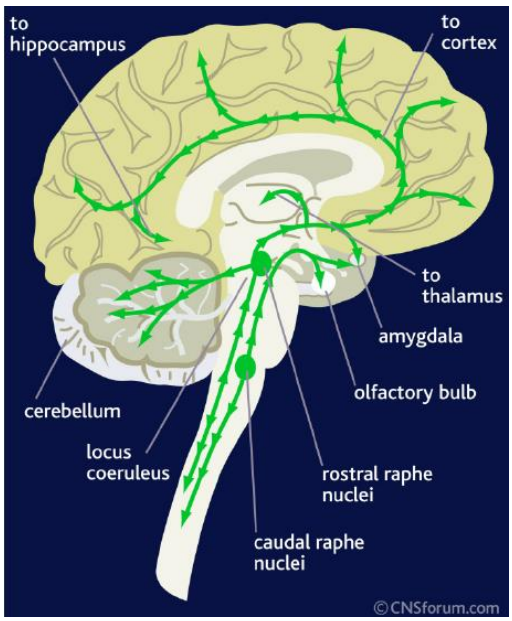

Fig. 2.7. Norepinephrine pathways in the brain (Hamman et al., 2015)

Importantly, noradrenergic receptors are located on NE neurons that originate from the LC (Maletic et al., 2017). NE receptors, including α- and β- adrenoceptors (α-AR, β-AR) are found in a variety of locations, including on gamma-aminobutyric (GABA), glutamate (GLU), DA, 5-HT, and histamine neurons, and on glial and immune cells (Maletic et al., 2017), suggesting noradrenergic regulation of these pathways. Noradrenergic receptors like α_1 and β_1 can be stimulatory whereas others like α_2- and β_2-receptors can be inhibitory (Drago et al., 2011). Stimulatory receptors increase signalling by increasing intracellular cAMP and phospholipase A, while inhibitory receptors inhibit these processes (Drago et al., 2011; Maletic et al., 2017). Stimulation of the cAMP pathway can increase CREB, consequently increasing BDNF and neuroplasticity (see Section 2.4.7.). NE signalling in turn is modulated by GLU (excitatory) and GABA (inhibitory) (Chandley et al., 2013; Jin et al., 2016). Regulation of NE is controlled by, amongst others, α_2-AR and high-affinity NE transporters (NET), the latter being found presynaptically on NE neurons where they facilitate reuptake of extracellular NE into the neuron (Blier, 2001).

Dysregulation of NE (usually decreased levels) can be implicated in many symptoms of MDD-like depressed mood, exhaustion, reduced motor activity, cognitive impairment like decreased concentration, disrupted sleep patterns, as well as altered circadian rhythms, and immune responses (Chen and Reith, 1995; Maletic et al., 2017). Many factors can cause dysregulated NE in MDD, but which are beyond the scope of this dissertation. Two central players, viz. the NE transporter (NET) and the α_2-AR are discussed presently.

2.4.4.1. The NET and α_2-adrenoceptor

Characteristic features of MDD include decreased density of the NE transporter (NET), which leads to lowered NE neurotransmission (Maletic et al., 2017), and increased expression of α_2-ARs which leads to increased signaling of these receptors (Cottingham and Wang, 2012; Pytka et al., 2015).

α_2-autoreceptors can be found presynaptically on NE cell bodies where they are involved in negative feedback mechanisms that inhibit further firing of NE neurons in the LC (Invernizzi and Garattini, 2004). Previous work has found that a_2-ARs have a higher affinity for NE than α_1-ARs, meaning that low concentrations of NE can inhibit neural activity while stimulatory effects via α_1-ARs and β-ARs happen at high concentrations (Maletic et al., 2017; Ramos and Arnsten, 2007). Receptor abnormalities, such as desensitized α_1-autoreceptors and increased affinity and density of inhibitory α_2-autoreceptors in the LC and prefrontal cortex, affects NE levels in such a way that neurotransmission is impaired (García-Sevilla et al., 1999; Maletic et al., 2017). Other systems, like the serotonergic system can also influence NE activity, e.g. 5-HT_{2A} receptors (see Section 2.4.3.3) on GABAergic interneurons are also linked to a decrease in NE levels (Dremencov et al., 2007; Guiard and Giovanni, 2015; Quesseveur et al., 2012; Quesseveur et al., 2013). Activation of these receptors potentiates the release of GABA in the synaptic cleft, which in turn inhibits the release of NE form the LC (Dremencov et al., 2007; Guiard and Giovanni, 2015; Quesseveur et al., 2012; Quesseveur et al., 2013).

Antidepressants, like NE reuptake inhibitors (NRIs) e.g. desipramine and reboxetine, block the NET resulting in decreased reuptake of NE into the neuron thus increasing extracellular levels of NE (Invernizzi and Garattini, 2004). Chronic treatments can thus increase extracellular NE in order to activate postsynaptic adrenoceptors in order to induce noradrenergic effects (Blier, 2001; Linnér et al., 1999). Additionally, long-term exposure to these types of antidepressants can cause desensitization of presynaptic α_2-ARs, thus attenuating the inhibitory effects of these receptors on NE neuron firing, which ultimately leads to antidepressant effects (Invernizzi and Garattini, 2004; Mongeau et al., 1994). The use of α_2-AR antagonists like risperidone in combination with other antidepressants like escitalopram (SSRI) can augment the response by risperidone's ability to inhibit escitalopram-induced reductions in NE via the 5-HT_{2A} receptor (Dremencov et al., 2007). Blocking α_2-ARs have been shown to be valuable in the treatment of MDD, e.g. mianserin and mirtazapine. Importantly though, various subclasses of α_2-ARs, viz. α_{2A}-AR and α_{2C}-AR, have opened up new avenues for targeting NE mechanisms in treating MDD (Uys et al., 2017).

2.4.4.2. NE and the stress response

The presence of the enzyme tyrosine hydroxylase (TH), an enzyme needed for the synthesis of DA and NE from tyrosine, is increased in MDD (Maletic et al., 2017; Zhu et al., 1999). The higher

level of the TH is indicatory of a compensatory effect where low levels of NE causes up-regulation of TH to increase the synthesis of NE in the LC (Zhu et al., 1999). In response to stress, corticotrophin-releasing factor (CRF) is released, which stimulates TH to quicken NE turnover (Leonard, 2001; Melia and Duman, 1991). However, suicide victims in one study (Nemeroff et al., 1988) showed reduced CRF binding sites in the frontal cortices, indicating that chronic stress can downregulate CRF function. The LC of these victims also contained 23% less neurons, which could thus lead to the conclusion that MDD might be worsened by decreased NE neurons (Chan-Palay and Asan, 1989; Leonard, 2001).

The central NE system is also linked to the immune system, as discussed by (Leonard, 2001): Many organs forming part of the immune system is innervated by the NE system, including the spleen, thymus, lymph organs, and bone marrow. Interestingly, immune system cells including lymphocytes and mast cells contain adrenoceptors which, when stimulated by NE, release important inflammatory cytokines including interleukin (IL-1, IL-2, and IL-6). NE is further involved in a process that increases release of glucocorticoids, and as a result of glucocorticoid receptor desensitization in MDD, potentiates the pro-inflammatory reactions in MDD as described in Section 2.4.8. Interestingly, inflammatory cytokines like IL-1β can also increase amine (NE) release via the nitric oxide, cyclic guanosine monophosphate pathway (Leonard, 2001; Licinio and Wong, 1999; Vitkovic et al., 2001).

It is thus clear that dysregulation of the NE system can contribute to the dysregulation of mood, and that this may involve a pro-inflammatory state (Leonard, 2001; Maletic et al., 2017).

2.4.5. Functional connectivity of the monoamines

Monoamines have many interacting effects in different parts of the brain, which emphasizes the complexity that underlies the pathophysiology in MDD. For instance, as explained by Dean and Keshavan (2017) and El Mansari et al. (2010), NE release is inhibited by DA in the LC. DA release in the ventral tegmental area is inhibited by the stimulation of α_2 ARs and excited by stimulation of α_1 ARs by NE. 5-HT release from the dorsal raphe nucleus is mediated by dopamine through D_2 receptors and NE through α_1 ARs. And NE activity in the LC is non-specifically modulated by glutamate, meaning fluctuations in glutamate levels can influence NE levels (Maletic et al., 2017). This suggests that even supposedly selective drugs, like SSRIs and SNRIs, eventually have non-selective actions at the neuronal and sub-cellular level that have far-reaching implications in understanding their efficacy as antidepressants as well as their side effects (Harvey, 1997). These are but a few examples of the interrelated actions of the monoamines.

2.4.6. Summary of the monoamine hypothesis

To conclude, Table 2.3. contains a summary of the changes in monoamines which constitutes the monoamine hypothesis which was described in full in Section 2.4.1 – 2.4.4

Table 2.3. Summary of the major abnormalities in the monoamine hypothesis

PARAMETER	CONCENTRATION /R-BINDING	REFERENCE
5-HT	↓ (or ↑)	(Andrews et al., 2015; Brand et al., 2015; Dean and Keshavan, 2017; Jesulola et al., 2018; Willner et al., 2013)
SERT	↑	(Brand et al., 2015)
	↓	(Arango et al., 1997; Willner et al., 2013)
5-HT_{1A}	↑ limbic ↓ frontal cortex	(Blier and Ward, 2003; Brand and Harvey, 2017; Brand et al., 2015; Pasqualetti et al., 1996; Polter and Li, 2010)
5-HT_{2A}	↑ frontal cortex ↓ limbic	(Andrade, 2011; Carhart-Harris and Nutt, 2017; Hall et al., 2000; Willner et al., 2013)
5-HIAA	↓CSF ↑plasma	(Brand et al., 2015; Mitani et al., 2006)
	↑ limbic	(Brand and Harvey, 2017)
5-HT turnover	↑	(Barton et al., 2008; Mitani et al., 2006)
NE	↓	(Dean and Keshavan, 2017; Jesulola et al., 2018)
NET	↓	(Cottingham and Wang, 2012; Pytka et al., 2015)
α_2-AR	↑	(Cottingham and Wang, 2012; Pytka et al., 2015)
DA	↓	(Brand et al., 2015; Dunlop and Nemeroff, 2007)

2.4.7. The role of phosphodiesterase 4 (PDE4) in MDD

PDE4 is an important role-player in the pathophysiology of MDD. PDE4 is an enzyme that regulates the cAMP/CREB (cyclic adenosine monophosphate (c-AMP); c-AMP response element-binding protein (CREB)) cascade, being responsible for mediating the hydrolysis of mainly cAMP (Figure 2.3) (Bellamy and Garthwaite, 2001; Halene and Siegel, 2007). This cascade is critical for intracellular signal transduction. Some mechanisms set in motion by enhanced breakdown of c-AMP by PDE4 include modifications in cellular functions mediating apoptosis, lipogenesis, differentiation, glycogenolysis, gluconeogenesis, neurogenesis, and muscular contractions (Halene and Siegel, 2007). Levels of PDE4 are controlled via a cAMP-PKA-PDE4 negative feedback loop, where PKA phosphorylation of PDE4 increases its activity (Chay et al., 2016).

PDE4 is especially responsible for increased neuroinflammation via activates microglia which causes the release of pro-inflammatory cytokines while decreasing anti-inflammatory cytokines as summarized in Fig. 2.8., while also decreasing neuroplasticity and neurogenesis via its down-regulatory effects on the cAMP/CREB/BDNF pathway (Fig. 2.8) (Wang et al., 2017). These cascades are correlated with negative effects on cognition and memory attributed to a reduction in BDNF, which is further elaborated on in Section 2.4.7) (Duman et al., 2000; Halene and Siegel, 2007).

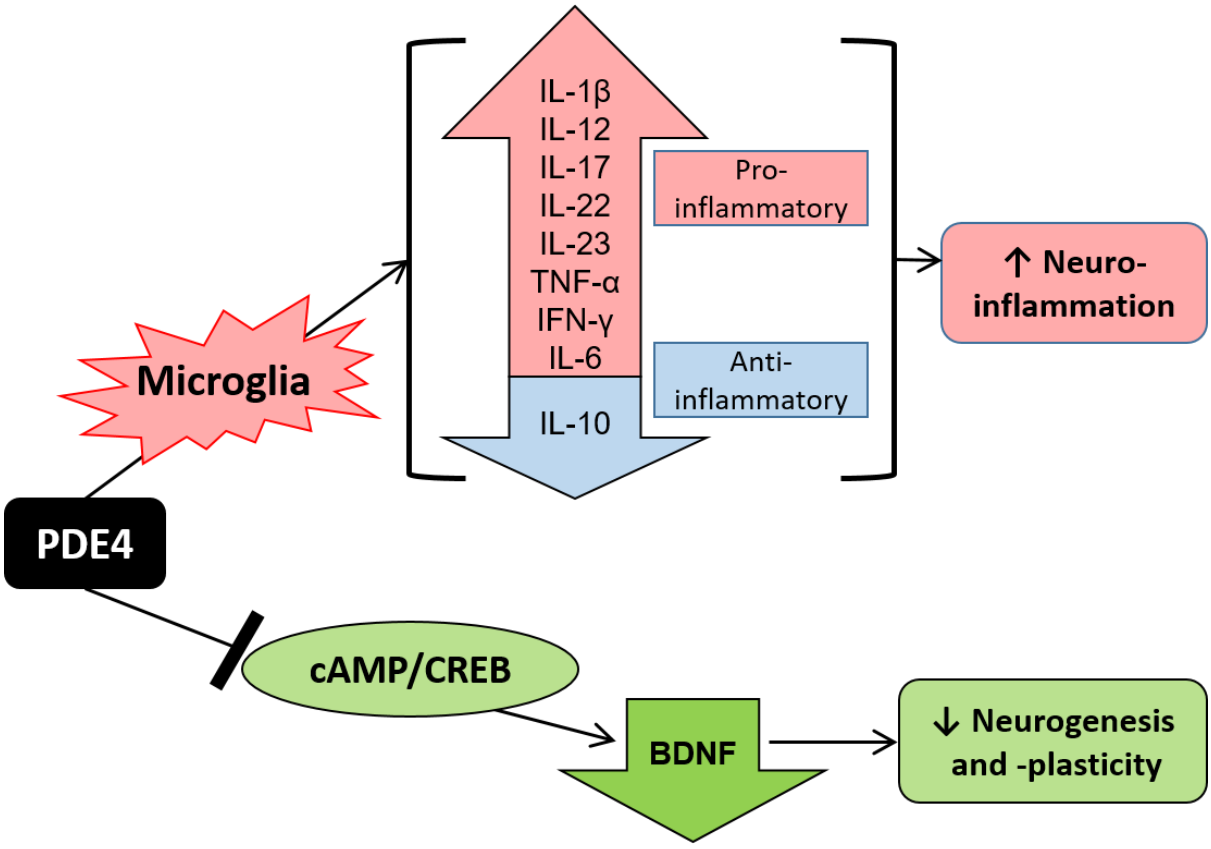

Fig. 2.8. Diagram of the main actions of PDE4, namely increasing neuroinflammation and decreasing neuroplasticity (Adapted from Wang et al. (2017)).

Itoh et al. (2004) found that the PDE4 inhibitor rolipram has antidepressant effects concomitant with increased levels of BDNF in the hippocampus and frontal cortex, alone and in combination with the tricyclic antidepressant imipramine. N-methyl-D-aspartate (NMDA) receptors in the hippocampal CA1 subregions possess an important regulatory effect on synaptic plasticity, memory and learning by means of increasing cAMP (Zhang et al., 2000). Blocking these receptors would thus decrease hippocampal cAMP, resulting in impaired memory and learning (Zhang et al., 2000). Moreover, PDE4 might increase cognitive deficits due to decreasing effects on cAMP (O'Donnell and Zhang, 2004; Zhang et al., 2000). According to Zhang et al. (2000), administration of PDE4 inhibitors like rolipram has shown to increase NMDA receptor-mediated increases in cAMP. These increases could be induced by mechanisms facilitated by NMDA receptor-mediated Ca^{2+} influx (Suvarna and O'Donnell, 2002). Exploring the use of PDE4 inhibitors as treatment for impaired memory and cognition could thus be very beneficial for MDD patients. NMDA antagonists like ketamine exerts its effects by blocking NMDA receptors on inhibitory GABAergic neurons causing a decrease in GABA, which disinhibits glutamate release and transmission, and ultimately increases BNDF release (Deutschenbaur et al., 2016). However, stimulation of NMDA receptors can also increase cAMP levels in the hippocampus (Chay et al., 2016; Zhang et al., 2000). This increase could be induced by mechanisms facilitated by NMDA receptor-mediated Ca^{2+} influx (Suvarna and O'Donnell, 2002).

Neuroinflammation is also an important factor in the pathophysiology of MDD (Jesulola et al., 2018; Sahin et al., 2016). Studies have shown that PDE4 activates microglia which causes the release of pro-inflammatory cytokines while decreasing anti-inflammatory cytokines (see summary in Fig. 2.8). PDE4 inhibitors displays anti-inflammatory effects which contributes to its potential use as an antidepressant (Zhu et al., 2001). Pro-inflammatory mechanisms of PDE4 and the effects of inflammation on neuroplasticity is discussed in more detail in Section 2.4.7. Inhibition of PDE4 can improve symptoms of MDD such as memory impairment through enhanced neuroplasticity, as well as learned helplessness in rat models (Halene and Siegel, 2007). There are many sub-families of PDE, like PDE4A, PDE4B and PDE4D, each having its own functions in different parts of the brain. Studies suggest that PDE4D is especially involved in the pathology of MDD (Halene and Siegel, 2007; Houslay et al., 2005). Further evidence for this stems from a study by Zhang et al. (2002) where PDE4D knockout mice presented with behaviours similar to antidepressant-like effects such as decreased immobility in the forced swim and tail suspension tests. Importantly, PDE4 inhibitors have shown antidepressant effects in humans (Fujita et al., 2012; Gericke and Viljoen, 2008; Zeller et al., 1984) and animals (Itoh et al., 2004; Loria et al., 2014; O'Donnell and Zhang, 2004). Prominent PDE4 inhibitors include rolipram, ibudilast, roflumilast, and cilomilast (Beardsley et al., 2010; Brown, 2007; Fujita et al., 2012; Gericke and Viljoen, 2008; Terburg et al., 2013). Although PDE4 inhibitors like rolipram have shown potential as antidepressants, these drugs have not advanced past clinical trials due to side effects such as severe nausea (Teixeira et al., 1997; Zhu et al., 2001).

Additionally, rolipram has also been shown to stimulate tyrosine hydroxylase (TH), an enzyme responsible for the conversion of tyrosine to DOPA, which consequently is converted to DA and then to NE (Bräutigam et al., 1999). This is most likely due to local cAMP-mediated activation of TH via allosteric interactions (Kehr et al., 1985). This could increase the synthesis and release of NE, consequently enhancing central noradrenergic transmission (Stewart, 2014; Zhu et al., 2001). There is thus an indication that cAMP signalling regulates PDE4, mediated by β-ARs, and that inhibition of PDE4 might cause antidepressant effects in part by modifying NE-mediated neurotransmission (O'Donnell and Zhang, 2004).

2.4.8. Brain-derived neurotrophic factor (BDNF) in MDD

BDNF is an important neurotrophic factor in the brain which, when dysregulated, can cause symptoms of MDD like impaired memory and learning due to the involvement of brain areas like the hippocampus and frontal cortex (Antunes and Biala, 2012; Duman et al., 2000; Sarkisyan and Hedlund, 2009). BDNF is also an important factor in the cAMP-CREB cascade which promotes neuronal survival, synaptogenesis, neuroplasticity and neurogenesis (Dean and Keshavan, 2017; Willner et al., 2013). Neurotrophins like BDNF are substances in the developing and adult nervous system which facilitates neuroplasticity, dendritic spine morphology, as well as neuronal differentiation, survival and proliferation (Duman et al., 2007; Willner et al., 2013).

Several brain structural changes have been identified in MDD. Reduced volumes has been shown in brain areas such as the hippocampus, prefrontal cortex, anterior cingulated cortex, caudate nucleus, putamen and limbic regions (Brown et al., 2017; Deutschenbaur et al., 2016). Structural brain changes underlie cognitive dysfunction that is responsible for many symptoms of MDD, such as anhedonia, psychomotor retardation, suicidality, negativity, impaired memory, and decreased concentration (Brown et al., 2017; McIntyre et al., 2013). It can therefore be said that in MDD, pathways that mediate neuroplasticity and survival are impaired. These pathways include the cAMP/CREB and neurotrophic factor-mitogen-activated protein (MAP) kinase cascades. cAMP is a second messenger for many hormones like NE, epinephrine, corticotrophin, glucagon, parathyroid hormone, thyroid stimulating hormone, calcitonin, and vasopressin, where it mediates cellular responses to these hormones and neurotransmitters (Scolnick, 2006).

The mechanism of the cAMP-CREB cascade derived from review articles by is illustrated below in Fig. 2.9, (Duman et al., 2000; O'Donnell and Zhang, 2004; Willner et al., 2013; Zhang et al., 2000). In summary, BDNF production is increased when NE and 5-HT bind to β-adrenergic and serotonergic receptors, activating the cAMP-CREB pathway which increased BDNF production, leading to neurogenesis and neuroplasticity in neural progenitor cells. While postsynaptic 5-HT_{1A} receptors can decrease phosphorylation of CREB by inhibition of PKA, binding of 5-HT to this receptor can also increase BDNF production via Ca^{2+}-dependent kinase or mitogen-activated

protein kinase (MAPK) pathways. Alternatively, neurogenesis can also be achieved by direct activation of the 5-HT$_{1A}$ receptor on the neural progenitor cell.

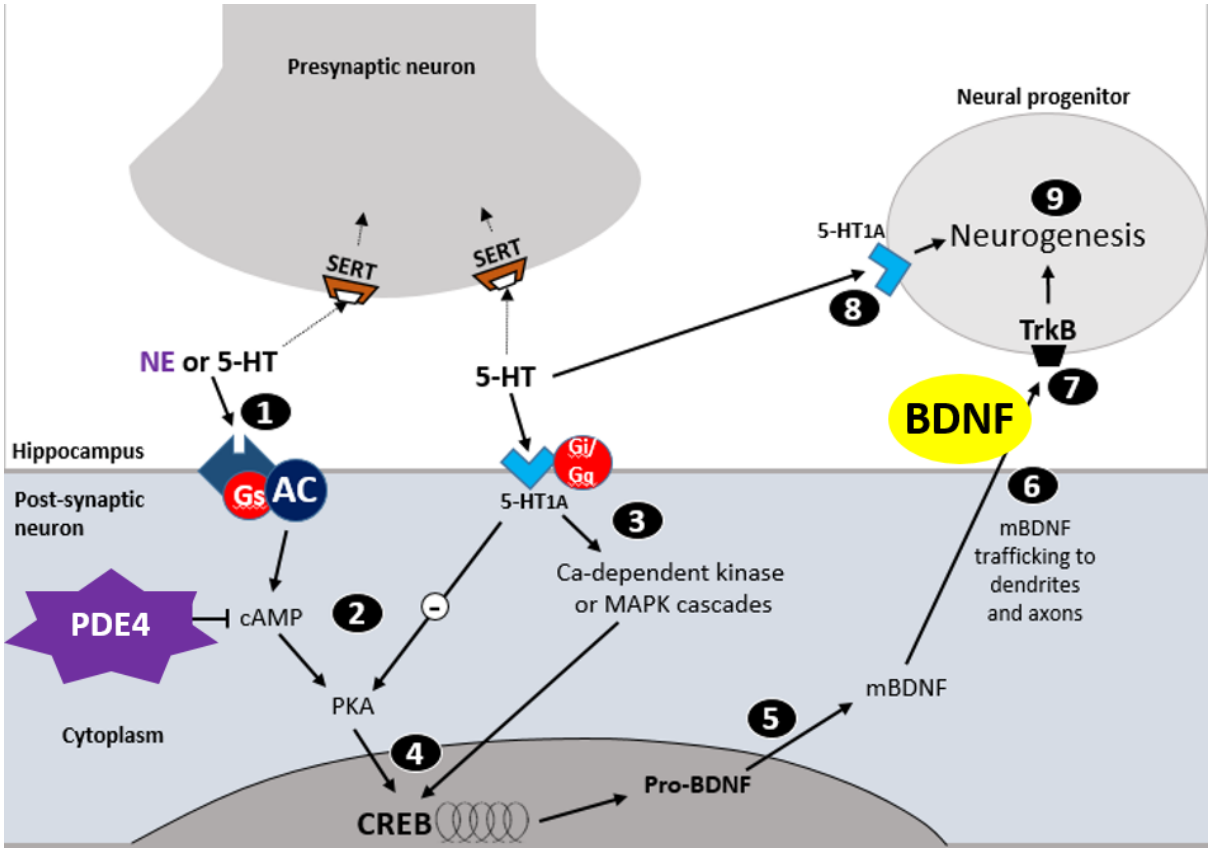

Figure 2.9: Normal postsynaptic mechanisms following an increase in synaptic monoamines. In cases where BDNF levels are low, upregulatory effects takes place via various second messenger systems. Firstly, 5-HT and NE binds to postsynaptic G_s-coupling receptors (β-noradrenergic/5-HT receptors like 5-HT$_4$ and 5-HT$_7$) in the hippocampus (1). This then activates adenyl cyclase (AC), which potentiates the change of ATP into cAMP, consequently activating and increasing protein kinase A (PKA) (2). Impairment of this pathway, for example by the increase of the enzyme phosphodiesterase 4 (PDE-4), which decreases cAMP in the pathway, inactivates the remainder of the cascade and decreases neuroplasticity – ultimately contributing to MDD symptoms. Moreover, Gi- or Gq-coupled 5-HT$_{1A}$ receptors activates Ca^{2+}-dependent cascades and MAPK (3). These pathways phosphorylate cAMP response element-binding protein (CREB) in the nucleus (4), causing an increase in BDNF gene transcription of the BDNF gene into pro-BDNF (5), whereafter it matures into mBDNF and is trafficked to dendrites and axons (6). From here, BDNF binds to tropomyosin receptor kinase B (TrkB) receptors on neural progenitor cells (7) leading to increased neurogenesis and maturation and differentiation of new hippocampal neurons (9). Alternatively, the last mentioned can also be increased the activation of 5-HT$_{1A}$ receptors on progenitor cells (8).

Although 5-HT$_{1A}$ receptors (Section 2.4.3.2.) are inhibitory on the cAMP-CREB cascade via inhibition of PKA, neuroplasticity is increased following the stimulatory effects of 5-HT$_{1A}$ on mitogen-activated protein kinase (MAPK) as illustrated in Figure 2.9. (Polter and Li, 2010; Willner et al., 2013). MAPK is also an important regulator of neuronal development and neuroplasticity through increased activity of the extracellular signal-regulated kinases 1 and 2 (ERK1/2) pathway, which eventually leads to increased CREB expression, ion channel activation, gene expression,

and increased neuroplasticity. This pathway is discussed in detail elsewhere (Polter and Li, 2010; Willner et al., 2013).

As explained in detail by Haase and Brown (2015), SERT and BDNF regulates each other through feedback mechanisms. Under circumstances where BDNF is low, normal compensatory mechanisms increase BDNF (Kraus et al., 2017; Polter and Li, 2010; Willner et al., 2013). Low levels of BDNF causes an attenuation of SERT activity and thus reduced clearance of 5-HT, resulting in an increase in extracellular 5-HT. This causes increased postsynaptic receptor activity and ultimately results in increased activation of the cAMP/CREB pathway, resulting in increased BDNF expression and neurogenesis. However, when there is a dysregulation in the pathways responsible for this as typically seen in MDD, these compensatory mechanisms are impaired. Consequently, antidepressant intervention can be applied in order to restore BDNF levels. However, while chronic treatment with SSRIs have shown upregulated BDNF, this effect is not seen in acute treatment (Nibuya et al., 1996).

5-HT_{2A} also plays an important role in neuroplasticity in conditions where 5-HT levels are high (Carhart-Harris and Nutt, 2017; Quesseveur et al., 2012; Quesseveur et al., 2013; Vaidya et al., 1999). Previous rodent studies have shown that activation of 5-HT_{2A} receptors may decrease BDNF mRNA in the hippocampus which bares similarities to that observed post-stress (Vaidya et al., 1999). In times of excessively high 5-HT levels, 5-HT can activate 5-HT_{2A} receptors on GABAergic interneurons to increase GABA release, which ultimately decreases BDNF in the hippocampus (as mentioned below) (Carhart-Harris and Nutt, 2017; Jaggar and Vaidya, 2018; Narla et al., 2015; Piguet and Galvan, 1994; Quesseveur et al., 2012; Quesseveur et al., 2013; Vaidya et al., 1999). Vaidya et al. (1999) found that downregulation of hippocampal BDNF mRNA in these circumstances are predominantly influenced by $5HT_{2A}$ rather than 5-HT_{1A} receptors. $5HT_{2A}$ has also shown to induce EPSPs in the neocortex, and IPSPs in the hippocampus (Section 2.4.3.3), thus regulating BDNF expression in the neocortex and downregulating BDNF in the hippocampus (Jaggar and Vaidya, 2018).

Glutamate and GABA (gamma aminobutyric acid) also have important effects on neuroplasticity, especially in the hippocampus CA1 subregion (Zhang et al., 2000). The activation of Ca^{2+}-dependant kinases via ionotropic glutamate receptors can mediate the phosphorylation of CREB, thus leading to increased neuroplasticity and NMDA-receptor-mediated cAMP signalling (Duman et al., 2000). This has been suggested to mediate positive effects on cognition and memory processes (Zhang et al., 2000). Moreover, GABA overactivity causes an overall inhibitory effect on the cAMP-CREB cascade, causing decreased BDNF and impaired neuroplasticity, while glutamate's excitatory effects increase synaptogenesis by increasing BDNF and subsequent neuroplasticity and neurogenesis. Blocking the NMDA receptor thus decreases GABA and increases glutamate which boost neuroplastic events and so exerts an antidepressant effect. (Deutschenbaur et al., 2016; Duman et al., 2000; Maeng and Zarate, 2007; Vetencourt et

al., 2008). However, excessive amounts of glutamate can be neurotoxic, worsening MDD pathophysiology (Brand et al., 2015; Drago et al., 2011). Abnormal increases in glutamate, as seen in MDD patients, can act on extrasynaptic NMDA receptors which causes a flood of Ca^{2+} into the neurons, thereby causing toxic accumulation of reactive oxygen species and increased production of nitric oxide (Brand et al., 2015).

2.4.9. The role of inflammation in MDD

MDD is known to represent a pro-inflammatory state (Brand et al., 2015). Chronic inflammation due to many mechanisms, such as chronic stress, can lead to prolonged sickness behaviour and subsequent development of MDD (Dean and Keshavan, 2017; Haase and Brown, 2015; Jesulola et al., 2018; Rosenblat et al., 2014). This causes symptoms similar to MDD such as lethargy, anhedonia, social withdrawal (Rosenblat et al., 2014), loss of appetite and sleep disturbances (Dantzer et al., 2008).

During inflammatory conditions, pro-inflammatory cytokines such as interleukin 1 (IL-1) and IL-6 and tumour necrosis factor (TNF), as well as anti-inflammatory cytokines such as IL-4, IL-8, IL-10 and IL-13 (Dinarello, 1997), are released. Pro-inflammatory cytokines increase inflammation, subsequently causing psychological stress (which in itself is pro-inflammatory), and depression. Thus, there is a positive feedback loop between depression and inflammation (Dean and Keshavan, 2017). Chronic inflammation can cause depression due to its effects on many different pathways such as excessive glutamatergic neurotransmission, overactive HPA axis, oxidative stress, activation of microglia and the IDO (indolamine 2,3-dioxygenase) pathway, neurotransmitter metabolism, decreased neurogenesis, amongst others (Brand et al., 2015; Sahin et al., 2016).

An important effect of inflammation is the activation of IDO by cytokines. IDO is an enzyme that converts the precursor of 5-HT, tryptophan, into kynurenine. Increased activation of IDO thus causes a shift in the metabolism of tryptophan, favouring kynurenine above 5-HT, thus causing a reduction in 5-HT which contributes to the depressive symptoms mentioned earlier (Haase and Brown, 2015; Jeon and Kim, 2017; Maes et al., 2011). Kynurenine metabolism also leads to the production of quinolinic acid which acts as an NMDA receptor agonist, leading to overly active glutamatergic neurotransmission and consequently glutamate excitotoxicity as seen in MDD as previously mentioned (Brand et al., 2015; Maes et al., 2011; Mathews et al., 2012).

The glial inflammatory pathway is an important factor in depression pathophysiology, especially when considering its role in impaired neuroplasticity (See Section 2.4.8) (Sahin et al., 2016). The majority of cells in the CNS are glial cells, including astrocytes and microglia, which play an important role in the release of neurotransmitters, neuroinflammatory and neurotrophic factors,

and neuroplasticity (Beardsley et al., 2010). Microglia can be activated by inflammatory mediators to release neurotoxic substances (like pro-inflammatory cytokines and glutamate) as well as neuroprotective substances (like BDNF and anti-inflammatory cytokines IL-4 and IL-10) (Smith et al., 2012). An imbalance in favour of neurotoxic factors can lead to loss of neuroplasticity and neurotoxicity, as well as neurotransmitter imbalances, ultimately leading to MDD (McNally et al., 2008). Moreover, astrocytes responsible for releasing neurotrophic factors and recycling neurotoxic excitatory amino acids such as glutamate, is decreased in MDD (McNally et al., 2008). This action is dependent on the cAMP-PDE4 cascade which acts to modulate the activity of astrocytes and microglia (Beardsley et al., 2010). PDE4 inhibition and a resulting increase in cAMP signalling will decrease pro-inflammatory cytokines and other neurotoxic factors in addition to the increase in production and release of neurotrophic factors (Beardsley et al., 2010).

Interestingly, increased PDE4 activity and/or chronic stress-induced upregulation of microglia can increase levels of pro-inflammatory cytokines, which can then upregulate SERT activity via cytokine-dependent pathways (p38 MAPK), ultimately leading to decreased 5-HT, decreased binding of 5-HT on receptors, and inactivation of the cAMP-CREB pathway. This results in a decrease in BDNF and neuroplasticity (Haase and Brown, 2015; Li et al., 2018). See Fig. 2.10. for a visual illustration hereof.

Moreover, as explained in Fig. 2.10., cytokines can reduce 5-HT levels by upregulating SERT via the p38 MAPK pathway and increasing reuptake and metabolism of 5-HT (Haase and Brown, 2015; Raison et al., 2006). That said, cytokines like IL-1β can increase nitric oxide (NO) production, resulting in stimulation of CRF and the guanylate cyclase-cyclic guanosine monophosphate (cGMP) pathway, resulting in the release of amines like NE (Leonard, 2000; Leonard, 2001). NO, in turn, can decrease the release of monoamines via the production of prostaglandins, which is elevated in MDD patients (Leonard, 2000; Leonard, 2001; Licinio and Wong, 1999; Vitkovic et al., 2001). It is not surprising then that NOS inhibitors are antidepressant in animal models (Wegener and Volke, 2010). Another mechanism includes the overstimulation of the HPA axis by pro-inflammatory cytokines to activate CRF, adrenocorticotropic hormone (ACTH) and cortisol which disrupts negative feedback via glucocorticoid receptor inhibition (Raison et al., 2006).

White blood cells (WBC) including neutrophils, eosinophils, basophils, monocytes, and lymphocytes, are involved in the immune response (Davis et al., 2008). Neutrophils are first-line responders in the immune response and secrete many inflammatory cytokines like IL-6 and TNF-α which can worsen the inflammation seen in MDD (Sunbul et al., 2016). Lymphocytes, however, are specific inflammatory mediators and are normally a reflection of general health due to its regulatory and protective properties (Mazza et al., 2018).

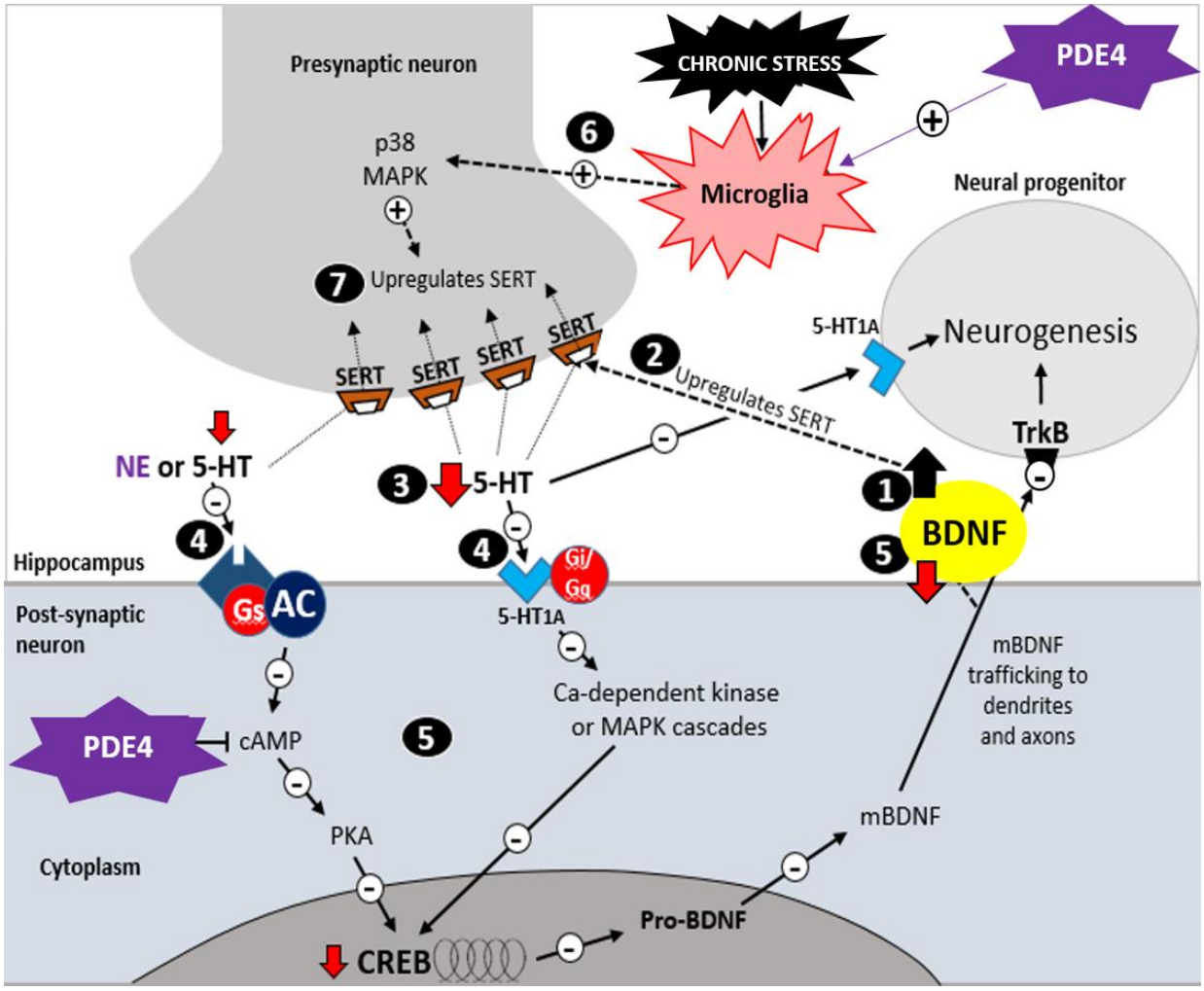

Figure 2.10: Feedback mechanism between SERT and BDNF, when BDNF levels are high, or during stress-induced pro-inflammatory responses. Under inflammatory conditions, microglia are activated (6) and SERT activity is upregulated through cytokine-dependent pathways like the p38 MAPK pathway (7). Upregulated SERT attenuates extracellular 5-HT levels (3), resulting in impaired 5-HT receptor-dependent BDNF expression (5). Alternatively, when BDNF levels are high (1), it causes an upregulation in SERT activity (2). Increased SERT causes increased clearance of 5-HT and thus decreased extracellular 5-HT levels (3). From here, the decrease in 5-HT leads to decreased receptor binding (4) and downregulated activity of the cAMP/CREB pathway resulting in decreased expression of BDNF (5). Minus signs in circles represents inhibition or inactivation; plus signs in circles represent activation or stimulation. Adapted from Polter and Li (2010) and Willner et al. (2013).

By quantifying the neutrophils and lymphocytes in a blood smear sample, a neutrophil to lymphocyte ratio (NLR) can be calculated which can be used as an indicator of the inflammatory response of patients suffering from MDD, with studies showing a higher NLR in depressed patients compared to healthy controls (Mazza et al., 2018; Sunbul et al., 2016).

2.5. Genetic influences on the pathophysiology of MDD

MDD is strongly influenced by genetic predisposition which, combined with stress, could prompt the manifestation of MDD (O'Donnell and Zhang, 2004). Severe stressors such as death of a loved one, abuse, war, social isolation, divorce, retrenchment et cetera are main contributing environmental factors leading to MDD (Brown et al., 1994; Lopez-Leon et al., 2008).

Interestingly, MDD is more hereditary in women than in men (Kendler et al., 2001). In short, genetic predisposition could include a wide variety of genetic polymorphisms in various systems that can have marked relationships with the pathophysiology of MDD (Dean and Keshavan, 2017). This includes, but is not limited to, altered protein transporters, e.g. SERT (see Section 2.4.3.1.) which can indirectly affect other systems including receptor function (Jennings et al., 2008), as well as directly altered expression of receptor genes which can cause dysregulation of receptor expression and function, e.g. increased 5-HT_{2A} receptors in the cortex, amongst many others (Arango et al., 1997; Brouwer et al., 2006; Lopez-Leon et al., 2008; Willner et al., 2013).

Additionally, genetic abnormalities may lead to poor response to antidepressant treatments (Ampong, 2018). For example, altered expression of genes coding for metabolizing enzymes of antidepressants, like CYP2D6 and CYP3A4, can increase risks for failed antidepressant treatment as ultra-rapid metabolism may prevent pharmacological effects due to premature breakdown, while poor metabolism may cause accumulation of antidepressants which could lead to serious side effects and toxicity (Ampong, 2018).

2.6. Flinder's Sensitive Line (FSL) rats

The FSL rat is a genetic model of depression which traces its origin to Australia after being bred from the Sprague-Dawley strain (Brand and Harvey, 2017; Nuno et al., 1997; Overstreet and Wegener, 2013). Originally, the rats were genetically modified to be resistant to the effects of an anticholinesterase agent called diisopropyl fluorophosphanate (DFP) in an attempt to elucidate the mechanisms of tolerance to DFP. However, the opposite was produced, viz. rats were *more* sensitive to DFP and hypersensitive to cholinergic agonists (Overstreet et al., 1994; Overstreet et al., 2005; Overstreet and Wegener, 2013; Russell and Overstreet, 1987). In addition to hypercholinergic responses of FSL rats, the muscarinic acetylcholine receptor (mAChR) is increased in the hippocampus, striatum and hypothalamus of these animals (Daws and Overstreet, 1999). Another strain resembling the original Sprague Dawley (SD) rat strain, the Flinder's Resistant Line (FRL) rat, was found to be relatively more resistant to DFP, however not to the same extent as the original Sprague Dawley rat (Overstreet et al., 1979; Overstreet and Wegener, 2013), and has since been regarded as the in-bred control for the FST rat. During this

period of development by the team of,David Overstreet, David Janowsky first marked the similarities between the FSL and human MDD patients in 1984 in a meeting with Overstreet (Overstreet and Wegener, 2013). This after observations were made that FSL rats, but not FRL rats, displayed hypersensitivity towards cholinergic agonists with an increased tendency to display a stress-sensitive, depressive-like phenotype which will be further discussed below (Overstreet et al., 2005; Overstreet and Wegener, 2013). The FSL rat is thus referred to as a "genetic model" because of its origin through selective breeding, leading to the bio-behavioural studies (Overstreet et al., 2005).

The FSL genetic model of depression has since been proven to have good construct, face, and predictive validity for depression (Du Jardin et al., 2017; Overstreet and Wegener, 2013). These characteristics are described below, and summarized in Table 2.4.

Face validity described as behaviour resembling the symptoms of the human disorder, is evident in FSL rats displaying reduced locomotor activity, reduced body weight, increased REM sleep and circadian rhythm abnormalities, cognitive impairment (Overstreet, 1993; Overstreet et al., 2005), increased passivity or inhibitory behaviour (as shown by exaggerated immobility during the forced swim test (FST) in rats) (Overstreet et al., 2005; Overstreet and Wegener, 2013), and increased response to environmental stressors or factors (Pucilowski et al., 1993; Wegener et al., 2010).

Construct validity: FSLs also share many similar neurochemical abnormalities to those found in depressed humans which indicate good construct validity. The cholinergic supersensitivity that is seen in FSL is in agreement with depressed humans (Perlis et al., 2002), and abnormalities in the serotonergic system (that are normalised by antidepressants), as well as reduced 5-HT synthesis (Hasegawa et al., 2006; Zangen et al., 1997) are evident in FSL rats and humans. Other similarities also include decreased neuropeptide Y (NPY)(Wu et al., 2011); circadian rhythm abnormalities (Overstreet et al., 2005); decreased activity of the dopaminergic system (Overstreet et al., 2005); immune system abnormalities like decreased natural killer cells and increased plasma concentrations of some cytokines (Friedman et al., 1996); changes in corticolimbic basal ACh and NO/c-GMP signalling (Brand et al., 2012); impaired neuroplasticity due to decreased BDNF and vascular endothelial growth factor (VEGF) (Elfving et al., 2010a; Elfving et al., 2010b) and thus hippocampal atrophy and decreased neuronal and synapse numbers (Brand et al., 2012; Chen et al., 2010); increased oxidative stress (Mokoena et al., 2015), and increased hippocampal lipid peroxidation (Oberholzer et al., 2017). Coinciding with humans (Pytka et al., 2015), FSL rats present with and higher SERT densities and increased α2-AR compared to SD rats (Kovačević et al., 2010; Lillethorup et al., 2015). Interestingly, FRL rats show an even higher increase in the latter receptors compared to both the FSL and SD rats (Lillethorup et al., 2015). Similar comorbidities as those seen in human patients, like hyperinsulinemia or diabetes and cardiovascular diseases like myocardial infarction, has also been found to present in FSL rats

(Solskov et al., 2010). The FSL model thus resembles depression in humans in many different ways.

Predictive validity: The improvement of depressive-like symptoms in FSL rats by clinically relevant antidepressants, such as increased immobility during the forced swim test, indicates good predictive validity of the FSL model (Overstreet and Wegener, 2013). Indeed, response to treatment with SSRIs, tricyclic antidepressants (TCAs) and melatonin agonists shows predictive validity (Overstreet et al., 2005). Indeed, the model has also shown response to complimentary and herbal medicines (eg. Oberholzer et al. (2017). Other treatments that also have a positive effect on increased immobility during FST in FSL rats include corticotrophin releasing factor 1 (CRF1) antagonists (Overstreet et al., 2004), NGF and BDNF, rapidly acting nemefitide, and NPY agonists (Overstreet et al., 2005).

Other genetic rat models include the Wistar-Kyoto rat, Fawn-hooded rat, SwHi (swim high-active) and SwLo (swim low-active) rats, and high-reactivity and low-activity rat breeds. These models are not immediately relevant to this dissertation, and are described elsewhere (see (Overstreet and Wegener, 2013).

Although the FSL model can be seen as a 'pure' depression model due to the apparent absence of anxious behaviour in the elevated plus maze (EPM), these rats do nonetheless display anxiogenic properties in social interaction tasks (Overstreet and Wegener, 2013). Anhedonia, another characteristic of depression, is absent in FSL rats during baseline. However, exposure to chronic mild stress can induce anhedonic behaviour in the sucrose preference test (Brand and Harvey, 2017; Overstreet et al., 2005).

2.6.1. FSL hypersensitivity to 5-HT_{1A} agonists like 8-OH-DPAT

Interestingly, FSL rats show hypersensitive hypothermic reactions to a 5-HT_{1A} receptor (see Section 2.4.3.2.) agonist like 8-OH-DPAT (Overstreet et al., 1979; Overstreet et al., 2005; Yadid et al., 2000). Interestingly, 5-HT syndrome (see Addendum H for more information) can be induced when combining 5-HT_{1A} agonists with other 5-HT enhancing drugs (Overstreet et al., 2005). This hypersensitive reaction can be attributed to an approximate 20% higher density of 5-HT_{1A} receptors in FSL rats compared to control strains like FRL or Sprague Dawley rats. This attribute has been used to confirm the validity of the FSL rat as a model of depression so to ensure reproducible results (Hasegawa et al., 2006; Overstreet et al., 2005).

A study by Hajós-Korcsok and Sharp (1996) also found that administration of 5-HT_{1A} agonists can increase hippocampal NE which is most likely due to stimulation of postsynaptic rather than somatodentritic 5-HT_{1A}. They further explained that 5-HT_{1A} receptors mediating extracellular NE increase might be more sensitive than those mediating the 5-HT behavioural syndrome. The authors then postulated that the former has a high receptor reserve despite being located

postsynaptically. Furthermore, they found evidence of possible dopamine 1 (D_1) receptor involvement in the 5-HT_{1A}-mediated release of NE, while low dose 8-OH-DPAT caused

Table 2.4: Table summarizing the validity of the FSL rat as a genetic model of MDD

FACE VALIDITY	CONSTRUCT VALIDITY	PREDICTIVE VALIDITY
Reduced locomotor activity (reduced activity in OFT)	Cholinergic supersensitivity	Nicotine
Reduced body weight and appetite	Abnormal serotonergic system and reduced 5-HT synthesis	Chronic antidepressants: SSRIs TCAs
Increased rem sleep	Decreased neuropeptide Y (NPY)	NPY agonists
Circadian rhythm abnormalities	Circadian rhythm abnormalities	Corticotrophin releasing factor 1 (CRF1) antagonists
Cognitive impairment	Decreased dopaminergic activity	5-HT3 antagonist (ondansetron)
Increased passivity or inhibitory behaviour (immobility in FST)	Immune system abnormalities (decreased natural killer cells, increased plasma cytokines	Rapidly acting nemefitide
Increased response to environmental stressors or factors	Changes in corticolimbic basal ach and NO/c-GMP signalling	Melatonin agonists
Psychomotor retardation (less activity in OFT)	Impaired neuroplasticity due to decreased BDNF and (VEGF)	NGF and BDNF
	Hippocampal atrophy and decreased neuronal and synapse numbers	-
-	Increased oxidative stress	-
	Aberrant glutamate- Nos signalling in response to an environmental stressor	
-	Similar comorbidities (hyperinsulinemia, diabetes, cardiovascular diseases, etc.)	-
(Brand, 2017; Neumann et al., 2011; Overstreet, 1993, 2012; Overstreet et al., 2005; Overstreet and Wegener, 2013)		

significantly increased DA by increasing DA cell-firing in the prefrontal cortex (PFC) of rats (Arborelius et al., 1993; Hajós-Korcsok and Sharp, 1996). However, it's important to point out that this hypersensitivity to 5-HT_{1A} agonists is not expressed in human depressed patients (Overstreet et al., 2005). Even so, other animal experiments suggest that over-activation of the 5-HT_{1A} receptor can also increase the risk of developing 5-HT syndrome in humans (Haberzettl et al., 2013). Human studies also found an increased risk of developing 5-HT syndrome when combining 5-HT_{1A} agonists with other drugs that enhance levels of 5-HT (Haberzettl et al., 2013; Parks et al., 2012).

Administration of 5-HT_{1A} receptor agonists and cholinergic agonists instigates hypothermic reactions in the FSL rat due to its supersensitivity towards these agents. The agonist-induced hypothermic reaction can increase immobility when these animals are subjected to the FST, with a positive correlation between the degree of hypothermic response and the time spent immobile (Overstreet et al., 1994; Yadid et al., 2000). However, Overstreet et al. (1994) found that the increase in immobility is more closely related to its 5-HT_{1A} supersensitivity, rather than its cholinergic supersensitivity (Yadid et al., 2000). The immobility response may suggest that FSLs tend to adopt a passive coping strategy (Overstreet et al., 1994; Puglisi-Allegra and Andolina, 2015).

2.6.2. Monoaminergic abnormalities in the FSL rat

FSL rats have been shown to possess specific serotonergic abnormalities compared to control SD and FRL rats (Yadid et al., 2000). These abnormalities include significantly increased levels of 5-HT and 5-HIAA in the limbic regions and, to a lesser extent, the prefrontal cortex compared to controls (Yadid et al., 2000; Zangen et al., 1997). These increases may be due to increased synthesis of 5-HT as a compensatory response after decreased postsynaptic 5-HT neurotransmission in limbic areas (Zangen et al., 1997). Moreover, the increased 5-HT might also suggest that there is reduced elimination of 5-HT and 5-HIAA from neuron terminals (Zangen et al., 1997). Despite this, studies have found lower 5-HT synthesis in FSLs than FRLs and SDs (Hasegawa et al., 2006). Zangen et al. (1997) et al. found that increased 5-HIAA and 5-HT levels in limbic areas of FSL rats was normalized by desipramine, thus suggesting that an increase in these transmitters could cause behavioural deficits, i.e. increased immobility in the FST. However, none of these effects were observed in other non-limbic areas, thus indicating site-specific action of desipramine: an antidepressant that is mainly a norepinephrine reuptake inhibitor (NRI). Chronic SSRI treatment reduces densities of SERT to larger extent in FSLs compared to SDs (Kovačević et al., 2010).

Moreover, FRL rats present with higher densities of 5-HT_{1A} compared to FSL and SD rats, with no significant differences between the latter two strains (Nishi et al., 2009). Humans also show decreased 5-HT_{1A} in cortex and HC (Albert and Lemonde, 2004; Albert and Vahid-Ansari, 2018). Furthermore, Lillethorup et al. (2015) found upregulated α_2-AR in FSL compared to SD rats. When the α_2-autoreceptors in the LC are activated, it causes a decrease in tonic NE release in the hippocampus which in turn increases 5-HT cell firing and release (Mongeau et al., 1994; Zangen et al., 1997). Chronic treatment with desipramine, which increases synaptic NE desensitizes α_2 autoreceptors, results in reduced inhibitory adrenergic tone followed by an increase in 5-HT release in certain brain areas. Thus using similar antidepressants can down-regulate α_2-autoreceptors and normalize 5-HT levels in FSL rats (Mongeau et al., 1994; Zangen et al., 1997).

2.7. Behavioural assessments relevant to MDD in rodents

Behavioural assessments in animal models like the FSL rat are valuable to determine the face validity of models (Nelson et al., 2014; Overstreet et al., 2005; Overstreet and Wegener, 2013) and to gain a clearer understanding of results relating to neurochemistry. Many different behavioural assessments can be done in MDD, including the tail suspension test as a measure of learned helplessness, sucrose preference test as a measure of anhedonia, and social interaction test as a measure of social anxiety among others (Neumann et al., 2011). However, for the purposes of this study, we will only focus on the following tests:

2.7.1. The Forced Swim Test (FST)

The FST is a validated behavioural model that screens for manifestations of behavioural despair in response to a forced swimming challenge, and is related to that typically seen in depression (Porsolt, 2000; Porsolt et al., 1978). In this test, animals are forced to swim in water-filled cylinders for a set amount of time, whereafter behaviour is scored based on swimming, struggling/climbing, and immobility (Brand and Harvey, 2017; Oberholzer et al., 2017). Faced with the inescapable swimming stressor, animals exhibit impaired escape-driven behaviour leading to behavioural despair or learned helplessness (Bogdanova et al., 2013). This manifests in rodents as decreased swimming and struggling/climbing, and increased immobility (Bogdanova et al., 2013; Brand and Harvey, 2017; Slattery and Cryan, 2012). Importantly, the FST on its own can also be used as a stressor (Flemmer et al., 1990; Paredes et al., 2005).

Antidepressant treatment increases escape-directed behaviour, seen as bolstered coping strategies, viz. swimming and climbing behaviour. Importantly, swimming and climbing is directly linked to serotonergic and noradrenergic-mediated activities, respectively (Cryan et al., 2005; Oberholzer et al., 2017). These behaviours can assist in categorizing the antidepressant as either

SSRI, NRI or SNRI, especially if linked to concomitant regional brain monoamine analyses (Brand and Harvey, 2017; Oberholzer et al., 2017).

Although this model has good construct (serotonergic and noradrenergic effects) (Cryan et al., 2005), face (immobility indicating helplessness) (Bogdanova et al., 2013) and predictive validity (response to SSRIs, SNRIs and NRIs, etc.) (Bogdanova et al., 2013; Cryan et al., 2005; Slattery and Cryan, 2012), there are some problems with this test as a test of depression.

One problem includes the risk of obtaining false positives or negatives when testing different kinds of drugs (Slattery and Cryan, 2012). For instance, use of non-antidepressant drugs viz. psychostimulants, tranquilizers, GABAergic and anticholinergic drugs can produce false positive results (Bogdanova et al., 2013; Bourin et al., 2001; Detke et al., 1995). False negatives are apparent when there is a decrease in immobility which contradicts the specific mechanism of action of the drug (Bogdanova et al., 2013). Furthermore, antidepressants like SSRIs have delivered false negatives, with a lack of change in immobility contradicting its known antidepressant action (Bogdanova et al., 2013; Cryan et al., 2002; Lucki, 1997). That said, modifications of the FST have helped to address some of these issues. This includes increasing water depth from 15-18 cm to 30 cm in order to bolster swimming behaviour correlated with increased serotonergic activity of SSRIs (Cryan et al., 2002). This also helped simplify scoring criteria due to inability of the rats to adapt by touching the bottom with their tails, among others. Additionally, modifications in scoring as explained by Cryan et al. (2005), can significantly affect results. Even the facing of the camera (from above or from the side) can alter scoring due to exclusion of the hind limbs for buoyancy. Some facilities record swims for 7 minutes, where the first and last minutes are excluded and the remaining video scored. By doing this, factors like trapped air pockets in the fur of animals which could increase buoyancy in the first minute. The last minute is excluded as some animals reach “full” immobility by that time, thus preventing dilution of treatment effects (Oberholzer et al., 2017). Furthermore, inclusion of additional tests for locomotor activity (e.g. open field test) has been implemented prior to the FST in order to determine if treatment-induced changes in general activity levels may confound interpretation of swimming activity in the FST, thus serving as an additional indicator of false positive or false negative results (Bogdanova et al., 2013; Molendijk and de Kloet, 2015; Slattery and Cryan, 2012).

Although most sources use immobility as a measure of increased depressive-like behaviour Bogdanova et al. (2013), some authors like Commons et al. (2017) and Molendijk and de Kloet (2015) strongly disagree on the basis that coping behaviour can either be passive (increased floating and reduced struggling) or active (increased swimming and struggling) (de Kloet et al., 1999; Puglisi-Allegra and Andolina, 2015). Moreover, De Kloet and Molendijk (2016) reviewed the possible adaptive mechanism of rodents in the FST (please see review for detail). In his review, De Kloet quoted the hypotheses involving adaptive coping as follows*: ““immobility is the*

result of extinction-like inhibitory learning involving all available escape responses due to the inescapable/unavoidable nature of the FST", and that "immobility is beneficial in preventing the rats from sinking": rodents that float longer probably live longer" (Campus et al., 2015; Colelli et al., 2014; De Kloet and Molendijk, 2016; Nishimura et al., 1988).

Moreover, active and passive coping has been shown to be mediated by different receptors, with 5-HT_{1A} receptors being associated with increased immobility, indicative of passive coping behaviour in the FST, and 5-HT_{2A} receptors being associated with decreased immobility indicative of active coping (Carhart-Harris and Nutt, 2017; Owens and Nemeroff, 1994; Puglisi-Allegra and Andolina, 2015; Yadid et al., 2000).

2.7.2 Open Field Test (OFT)

The OFT is a test used to measure locomotor activity in rodents (Essman, 1968). Animals are placed in a square arena where the total horizontal distance moved is recorded and analysed. Depressed rodents are less active in the OFT, which correlates with psychomotor retardation seen in human patients with depression (Neumann et al., 2011). Locomotor retardation may also indicate anxiety-like behaviour, and is related to fear-induced freezing (Brenes Sáenz et al., 2006; Tejani-Butt et al., 2003). As a result, more anxious animals will explore less and move close to the walls of the arena to avoid exposure in the middle of the arena, which is known as thigmotaxis (Prut and Belzung, 2003). Apart from the above behavioural read-outs, this test is done before the FST to determine if altered locomotor activity is psychogenic and not due to general alterations in locomotor activity (Lavi-Avnon et al., 2005) and if anomalies in locomotion may have contributed to or abrogated swimming behaviour in the FST, as mentioned in Section 2.7.1. (Lavi-Avnon et al., 2005).

Locomotor activity has been attributed to different biological factors, for instance, studies have shown that increased locomotor activity (albeit in serotonin behavioural syndrome) is mediated by postsynaptic 5-HT_{1A} receptors (Chen and Reith, 1995; Haberzettl et al., 2013; Mignon and Wolf, 2002). Also, Chen and Reith (1995) found that an increase in NE can also increase locomotor activity, most likely via indirect effects of postsynaptic 5-HT_{1A} receptors on noradrenergic neurons. Another important neurotransmitter for locomotion is acetylcholine (ACh), which has been positively correlated with increased locomotor activity (Day et al., 1991; Flicker and Geyer, 1982). Region-specific increases in DA can also increase locomotor activity (Ikemoto and Panksepp, 1999; Tye et al., 2013).

2.7.3. Novel Object Recognition Test (nORT)

The nORT is a validated behavioural model used to assess visual learning and declarative memory in rodents (Mokoena et al., 2015; Oberholzer et al., 2017). Depression is associated with these cognitive deficits which makes the nORT very useful in depression studies (Bremner et al., 1997; Mokoena et al., 2015). The model is based on the observation that rats prefer exploring novel objects above familiar ones and that a shorter period of novel object exploration may indicate impaired cognition (Ennaceur and Delacour, 1988; Oberholzer et al., 2017).

The procedure involves placing a rat into a box where they are first habituated in order to decrease stress in response to a new environment (Antunes and Biala, 2012). Habituation is an important step as this attenuates provoked stress responses (Antunes and Biala, 2012). The animals are then exposed to two trials, namely the acquisition trial during which the rat is exposed to two identical objects for 5 minutes, followed by the retention trial 90 minutes later during which the rats are exposed to a familiar and an unfamiliar (novel) object (Mokoena et al., 2015; Oberholzer et al., 2017). Rats that explore the novel objects for longer can remember the familiar object from the acquisition trial, and will be less likely to explore the familiar object, while rats that spend shorter periods exploring the novel objects shows impaired memory and cognitive deficits (Antunes and Biala, 2012; Ennaceur and Delacour, 1988). Since there are no positive or negative reinforcements, this test gives a measure of the natural preference for novel objects (Antunes and Biala, 2012).

This test is commonly modified to fit the objectives of a specific study, for example, increasing the retention interval (period between acquisition and retention trials) can aid in testing long-term memory (Taglialatela et al., 2009). Other modifications are also frequently made with regards to the types of objects used, nORT boxes and its physical properties, habituation periods, placements of the objects, and even environmental factors like lighting and cleaning agents. These modifications are reviewed in detail by (Antunes and Biala, 2012). Despite all the different modifications, nORT always consists of three phases: habituation, familiarization, and test phase (Antunes and Biala, 2012).

Specific brain areas influence the behaviour and different type of memory assessed in the nORT, for example the involvement of the perirhinal cortex in object recognition memory (Aggleton et al., 2010). Many different types of memory are consolidated with different brain structures, including the hippocampus and cortical regions, especially the perirhinal cortex (see Antunes and Biala (2012) for more detail. The rate of hippocampal neurogenesis has also been associated with spatial memory consolidation (Antunes and Biala, 2012; Sarkisyan and Hedlund, 2009), thus there is also a correlation between the nORT and hippocampal BDNF levels (described in Section 2.4.8). Other neurotransmitters like NE and 5-HT, and receptors like 5-HT_{1A} are also involved in memory and cognitive impairment (Meltzer and Sumiyoshi, 2008; Ramos and Arnsten, 2007).

nORT has many different applications and advantages. However, it is limited to memory of an object, its placement, and its context, and unfortunately cannot be used as a measure of strength of memory of an event (Antunes and Biala, 2012).

2.8. Synopsis

Overall, MDD is a major mental health concern that affects millions of people from different backgrounds, ages, and genders (Mayoclinic, 2017; World Health Organization, 2018a; World Health Organization, 2018b). Due to its debilitating effects – physically, psychologically, socially and economically – this disease impairs the quality of life of patients to such an extent that in extreme cases, patients take their own lives. The root of this problem lies in the complicated pathophysiology of the illness, which includes a wide variety of dysregulated systems that in turn produce a wide range of symptoms, especially anhedonia, intense feelings of sadness, cognitive impairments, fatigue, and emotional dysregulation (Dean and Keshavan, 2017; Jesulola et al., 2018; Willner et al., 2013). These pathophysiological mechanisms include, but is not limited to, HPA axis dysregulation, neuroinflammation, decreased neuroplasticity and neurogenesis, and decreased monoamines (Dean and Keshavan, 2017; Jesulola et al., 2018; Willner et al., 2013).

Although there currently are a variety of treatments for MDD, many patients fail to respond, while TRD is a growing clinical concern. Based on the pathophysiology, it is difficult to treat MDD as there are so many possible biological processes that could be involved, but that are not targeted by current treatments. Other contributing factors, including delayed onset of action and high side effect profiles, further complicates current treatments. Patients are therefore increasingly resorting to the exploration of alternative or complementary treatment strategies like natural medicines (Akil et al., 2018; Carhart-Harris and Nutt, 2017; Willner et al., 2013), this with or without the approval of the prescribing physician. The use of these medicines can be potentially beneficial as an antidepressant, as many may have favourable therapeutic and side effect profiles. However, unsupervised use of these medications could increase risks of inducing toxicity or medicine interactions when combined with other medications (Ravindran and da Silva, 2013). Furthermore, many of these medicines have not been thoroughly tested to establish possible clinical efficacy or side effect risk. For this reason, the WHO has urged in their traditional medicine strategy for 2014 – 2023 (World Health Organization, 2013), that evidence-based research of these substances be undertaken in order to evaluate their safety and efficacy as mono- or augmentative therapies.

In this study, we have explored the antidepressant effects of the South African plant, *Sceletium tortuosum* (ST), alone and in combination with an SSRI (ESC). ST was chosen based on its diverse mechanisms of action that can prove useful in the treatment of neuropsychiatric disorders like MDD. The anti-inflammatory actions of ST, as well as SERT inhibition, VMAT-2 up-regulatory effects, and ability to increase BDNF via PDE4 inhibition (Coetzee et al., 2016; Gericke and

Viljoen, 2008; Harvey et al., 2011; Krstenansky, 2017) dovetails well with the pathophysiology of MDD. This makes ST an ideal multi-target antidepressant. However, these mechanisms have mainly been investigated *in vitro* with only a few studies performed in mainly healthy humans and rodents (Loria et al., 2014; Terburg et al., 2013). Indeed, only one in vivo study (to our knowledge) has been conducted in a mouse model of depression (Schell, 2014).

Therefore, there is a need for further investigation into ST as an antidepressant in a validated model of depression that encompasses key pathological features of MDD, such as its genetic underpinnings, stress sensitivity and broad range of bio-behavioural correlates. Due to its good face, construct and predictable validity (Overstreet et al., 2005; Overstreet and Wegener, 2013), the FSL rat is a theoretically suitable model to with which to study the antidepressant abilities of ST. Due to its face validity, we opted to subject the FSLs to the FST as a measure of behavioural despair, the OFT as a measure of locomotor activity, and the nORT as an evaluation of cognitive deficits or impaired memory. Additionally, monoamines and BDNF were measured to correlate neurobiology with expressed behaviours in the OFT, FST and nORT. Initially, behavioural validation of the FSL rat was undertaken using the FRL rat as control, followed by a dose ranging study with ESC and ST using an acute treatment FST design to establish pharmacologically active doses and to investigate any obvious untoward effects of ST in the animals. Thereafter, the most appropriate dose of ST and ESC were selected for testing in a sub-chronic treatment study design. A low dose of the ESC and therapeutic dose of ST. Due to a lack of definitive dose response studies, especially considering all the differences in ST preparations used in different studies, it was necessary to do a dose response with ST to determine which dose presented with antidepressant effects with the specific preparation (standardized extract Zembrin) in this study. Here a maximally effective dose of ST was compared to that of a low dose of ESC and a saline-treated group, all performed in FSL rats. A low dose of ESC was used in order to establish whether ST can bolster the effects of a low dose SSRI as this could potentially deliver better pharmacological effects with a better side effect profile. Furthermore, given the novelty of this study, we were concerned with the possible risks of inducing a potentially life-threatening 5-HT syndrome (Haberzettl et al., 2013) when combining ST with an SSRI. This is especially relevant given the *in vitro* evidence for the serotonergic actions of ST.

2.9. References

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CHAPTER 3: MANUSCRIPT A

CONCEPT ARTICLE

This chapter contains the concept article titled:

"**Evaluating the antidepressant properties of *Sceletium tortuosum* (L.) N.E. Br., alone and in combination with escitalopram, in the Flinders Sensitive Line rat."**

The article reports on a pre-clinical study of the antidepressant-like effects of *Sceletium tortuosum* (ST) in a rodent animal model of depression, as evaluated in acute and sub-chronic study designs. We report the acute dose-dependent antidepressant-like effects of ST, as well as its ability to bolster serotonergic effects in combination with the SSRI escitalopram (ESC) in a sub-chronic treatment response over 15 days. However, behavioural results indicate that the combination treatment has depressogenic effects in the forced swim test. This may have been due to an induced effect resembling serotonin behavioural syndrome which could be attributed to 5-HT_{1A} receptor supersensitivity of the Flinders Sensitive Line rat (FSL) used in this study. Treatments with the ESC or ST alone showed no significant antidepressant-like effects in the sub-chronic treatment period. Nevertheless, this article indicated that ST has definite serotonergic and noradrenergic effects in an *in vivo* model of depression which could prove useful in the treatment of depression. Furthermore, ST alone treatment in the sub-chronic study showed increased hippocampal BDNF. The combined serotonergic and noradrenergic activity, as well as the positive effects on BDNF, indicates promise of ST in the treatment of mood and cognitive disorders.

This manuscript will be submitted to the peer-reviewed Journal of Ethnopharmacology for publication. The Manuscript was thus prepared according to the author guidelines of this journal as found in

https://www.elsevier.com/journals/journal-of-ethnopharmacology/0378-8741/guide-for-authors

Data that does not appear in this article have been included in addenda (nORT and DA).

Author contributions

- *Johané Gericke* contributed towards the planning and design of the study, and undertook all bio-behavioural and statistical analyses. She wrote all the first drafts of the proposal for this study, as well as for the manuscript itself.
- *Prof Brian Harvey* was greatly involved in the design of the study. Furthermore, he proof-read and contributed towards the preparation of the manuscript.
- *Dr Makhotso Lekhooa* was involved in the design of the study. She contributed toward the write-up of the proposal for this study and the Manuscript.
- *Prof Alvaro Viljoen* performed the fingerprint analysis of the Zembrin used in this study and acted as a consultant with regards to *Sceletium tortuosum*

Evaluating the antidepressant properties of *Sceletium tortuosum* (L.) N.E. Br., alone and in combination with escitalopram, in the Flinders Sensitive Line rat.

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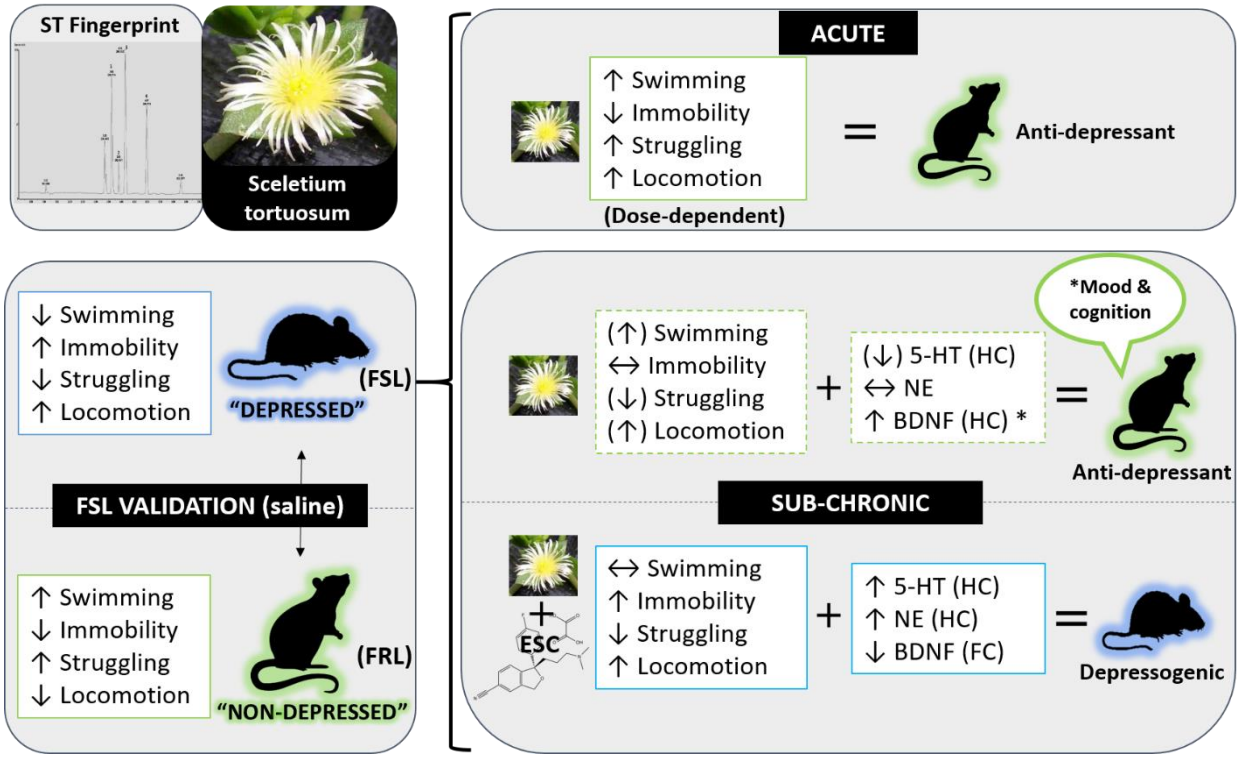
Graphical abstract



1. Abstract

Ethnopharmacological relevance: Based on its pharmacological mechanisms of action the South African plant, *Sceletium tortuosum* (L.) N.E.Br. (Zembrin®), has distinct potential as an antidepressant, and has been used as a mood elevator by indigenous communities.

Aim of the study: To evaluate the antidepressant-like activity of a standardized extract of *S. tortuosum* (Zembrin®) (ST) in the Flinder's Sensitive Line (FSL) rat, following acute and sub-chronic treatment as a single-entity or augmentative agent in combination with escitalopram.

Materials and methods: ST extract was analysed by an UPLCS-MS method. 12 saline-treated FSL and 6 Flinders Resistant Line (FRL) rats were used to face validate the FSL model in the forced swim test (FST). Thereafter 5 groups of FSL rats (n = 10) received 5, 10, 25, 50 or 100 mg/kg ST, with 3 groups (n = 10) receiving 5, 10 or 20 mg/kg escitalopram oxalate (ESC), and subjected to the open field test (OFT) and FST. This established a low dose of ESC and a therapeutic dose of ST. Four groups of FSL rats received 1) saline, 2) the selected dose of ESC, 3) the selected dose of ST, 4) combined ESC + ST, for 15 days, followed by the OFT and FST on days 13 and 15. Animals were decapitated on day 16, with hippocampus and frontal cortex harvested for analysis of serotonin (5-HT), norepinephrine (NE) and brain-derived neurotrophic factor (BDNF).

Results: Four main alkaloids were identified and quantified in an ST UPLSC_MS chromatogram. FSL rats showed significantly increased swimming and decreased immobility versus FRL controls. ST and ESC showed dose-dependent antidepressant responses following acute treatment, with ESC 5 mg/kg and ST 50 mg/kg selected for the sub-chronic study. These doses demonstrated trends to increase swimming and locomotor activity, decreased struggling, and unchanged immobility. Both treatments tended to decrease hippocampal 5HT and unchanged NE. Combined ESC+ST significantly increased hippocampal 5-HT and NE, increased immobility and locomotor activity, but decreased struggling, and had no effect on swimming. While ST 50 alone increased hippocampal BDNF, combined ESC 5 and ST 50 reduced frontal cortical BDNF levels. No treatment significantly affected frontal cortical 5-HT and NE.

Conclusions: ST and ESC show dose-dependent antidepressant effects after acute but not sub-chronic treatment, perhaps due to the model used. Combined sub-chronic ESC 5 and ST 50 is depressogenic, while displaying significant serotonergic activity. The latter, plus ST increasing frontal cortical BDNF levels, suggest promise in mood and cognitive disorders.

Keywords

Antidepressant, sceletium tortuosum, escitalopram, adjunctive treatment, monoamines, 5-HT$_{1A}$, 5-HT$_{2A}$, BDNF

2. Introduction

Major depressive disorder (MDD) affects about 300 million people worldwide (World Health Organization, 2018a; Willner et al., 2013). With only 30-40% of patients responding to current antidepressants, there is an urgent need for novel and alternative treatments (Trivedi et al., 2006; Willner et al., 2013). Multiple mechanisms are implicated in the pathophysiology of MDD (Dean and Keshavan (2017); Jesulola et al. (2018), including deficits in central brain monoamines, dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis, neuroinflammation, impaired neuroplasticity, genetic predisposition and environmental influences, among others (Brand et al., 2015). Given this complexity it is not surprising that current antidepressants that primarily target monoamines, are failing (Akil et al., 2018; World Health Organization, 2018a). Moreover, their high side effect profile and delayed onset of action (Bet et al., 2013; Carhart-Harris and Nutt, 2017) highlights a need not only for better monotherapies but also augmentative agents with synergistic actions when combined with current treatments (Dodd et al., 2005; Sarris, 2018). Interest in adjunctive treatments is therefore growing, especially complementary natural medicines like plants and herbs (Fajemiroye et al., 2016; Sarris, 2018). On the other hand, the commercial availability of complementary medicines often sees herbal preparations being used without medical supervision, even in combination with prescribed treatments (Ravindran and da Silva, 2013). This poses a serious risk for possible drug interactions and toxicity, e.g. serotonin (5-HT) syndrome when selective 5-HT reuptake inhibitors (SSRIs) are co-administered with ST John's wort (Sarris, 2018). These real-world issues, plus that plant constituents may have interacting effects with one another, warrants dedicated research into the pharmacokinetic and pharmacological profiles of herbal medicines (Krstenansky, 2017; Sarris, 2018; Sarris et al., 2010; Yeung et al., 2018).

According to Sarris (2018) and Fajemiroye et al. (2016), many herbal substances have antidepressant effects in preclinical and clinical studies, e.g. *Hypericum perforatum*, *Piper methysticum* and *Lavandula angustifolia*, often with mechanisms similar to that of traditional antidepressants. In this regard, these preparations variably demonstrate 5-HT_{1A} receptor modulatory actions, monoamine oxidase (MAO) inhibition, monoamine reuptake inhibition, antiglutamatergic and anti-inflammatory actions, thus emphasizing multi-target mechanisms of action. However, while most of these substances show potential as stand-alone treatment, others show better results when combined with antidepressants, e.g. concomitant use of lavender (*Lavandula angustifolia*) and imipramine has shown better efficacy with less side effects than either treatment alone (Akhondzadeh et al., 2003). Considering the above, the World Health Organization (WHO) has put forward a traditional medicine strategy for 2014-2023 (World Health Organization, 2013) recommending that evidence-based research be used as a tool to evaluate the safety and efficacy of complementary medicines. This study focusses on the indigenous South African plant, *Sceletium tortuosum (ST)*.

ST has for many years been used by the indigenous population for diverse purposes, including as a mood-elevator, hypnotic, analgesic, anxiolytic, thirst and hunger suppressant, and for its intoxicating/euphoric effects (Gericke and Viljoen, 2008; Harvey et al., 2011). Despite its intoxicating effects, it shows little to no abuse liability (Gericke and Viljoen, 2008; Loria et al., 2014; Nell et al., 2013). ST has a rich alkaloid profile, including mesembrine, mesembrenone, mesembrenol, and mesembranol (Krstenansky, 2017), each presenting with subtly different pharmacological properties (Gericke and Viljoen, 2008; Loria et al., 2014). Anecdotal evidence suggest ST to possess a relatively low side effect profile with the most commonly reported effects being nausea, headache, insomnia, hypertension, irritability and anxiety (Schifano et al., 2015). Its diverse neuropsychopharmacological actions have been noted to include increased 5-HT release by up-regulating vesicular monoamine transporter 2 (VMAT-2) and/or serotonin transporter (SERT) inhibition, and inhibition of phosphodiesterase 4 (PDE4) with upregulation of the cyclic adenosine monophosphate (cAMP)/cAMP response element binding protein (CREB) pathway, the latter bolstering neurotrophin levels (e.g. brain derived neurotrophic factor (BDNF). Other putative mechanisms include inhibition of MAO-A, inhibition of the noradrenaline (NE) and dopamine (DA) transporter (NET, DAT), and anti-inflammatory properties (Bennett and Smith, 2018; Coetzee et al., 2016; Duman et al., 2000; Gericke and Viljoen, 2008; Harvey et al., 2011; Krstenansky, 2017). With these actions dove-tailing well with current neuropathological theories of MDD (Brand et al., 2015), together with a low side effect profile (Schifano et al., 2015), prompts further exploration of the potential antidepressant actions of ST, either alone or as an adjunctive treatment. Only one study to-date has tested ST as stand-alone antidepressant treatment in a validated rodent model of depression, viz. BALC-c mice (Schell, 2014). Here, ST was found to induce antidepressant effects in the forced swim test (FST) after sub-acute treatment. While these findings are promising, they need to be extended to other translational models. Furthermore, ST is still to be tested for efficacy as a chronic treatment and as an adjunctive treatment, both being clinically important parameters to establish predictive validity.

The FSL rat is a genetic animal model of depression that shows good validity for MDD, including construct validity (e.g. serotonergic abnormalities and decreased neuronal plasticity), face validity (e.g. psychomotor retardation, cognitive impairment, increased passivity or behavioural despair), and predictive validity (proclivity to respond to chronic antidepressant treatments) (Overstreet, 1993, 2012; Overstreet et al., 2005; Overstreet and Wegener, 2013). Since certain actions of ST coincide with that of known antidepressants (e.g. SERT inhibition, PDE4 inhibition, increased BDNF) and that correlate with the bio-behavioural abnormalities evident in FSL rats and MDD, this model provides a valuable platform to establish the antidepressant effects of ST.

The first aim of this study was to define an effective antidepressant dose for ST in FSL rats, this to limit possible toxicity prior to testing in a chronic treatment paradigm. An acute dose response study was conducted with 5 doses of ST in the FST versus a reference control, the selective

serotonin reuptake inhibitor (SSRI) escitalopram. A 3-tier dose response of escitalopram was conducted in order to establish the predictive validity of the FSL model and to consider therapeutic and low-dose options of this agent. Since effective antidepressant treatments require chronic dosing to attain therapeutic efficacy (Carhart-Harris and Nutt, 2017), the above-noted most appropriate doses of each drug will be tested in a sub-chronic study, alone or in combination, and evaluated in the open field test (OFT) and FST. Finally, in order to relate to current constructs of MDD, regional brain monoamine and BDNF analysis was performed after all treatments. Lastly, quality control of ST was done before treatment commenced. This study mainly focuses on ST's effects on monoamines and BDNF. However, it should be considered that various other mechanisms of action of ST can also play important roles in the efficacy of ST as an antidepressant, alone and as augmentative therapy.

3. Materials and methods

3.1. Plant materials and quality control

A high-quality standardized extract of *Sceletium tortuosum* (L.) N.E. Br. (www.theplantlist.org, accessed October 2019), known as Zembrin® (Zembrin Lot: SCE0416-1407), was provided by HG&H Pharmaceuticals (Johannesburg, South Africa). ST is not presently and endangered species, and a legal benefit-sharing agreement has been signed between HG&H Pharmaceuticals and the South African San Council, who in turn signed an agreement with the Nama communities of Kamiesberg in Namaqualand to share financial benefits resulting from commercialization of the preparation.

3.1.1 Chromatographic fingerprinting

A methanolic extract of ST was analysed using a validated Ultra Performance Liquid chromatography - tandem mass spectrometer (UPLC-MS) (Shikanga et al., 2012) for quality control purposes and quantification of alkaloids.

3.2. Animals

Male adult Flinder Sensitive Line (FSL) rats (n=140), serving as the experimental model and male Flinder's Resistant Line (FRL) rats (n=7) serving as control animals, were bred, supplied and housed at the Vivarium (SAVC reg. number FR15/13458; SANAS GLP compliance number G0019; AAALAC accreditation international file #1717) of the Pre-Clinical Drug Development Platform of the North-West University (NWU). The original colonies were obtained from Dr David H Overstreet, University of North Carolina, Chapel Hill, North Carolina, USA. A constant ambient

temperature of 22 ± 2°C and relative humidity of 40 – 60% was maintained, with a full spectrum of light in a 12:12h light/dark cycle (switched on at 06:00 and off at 18:00). All animals weighed between 200g and 230g at the start of experiments. The animals were housed in groups of 3 to 4 rats per cage with corncob bedding in polypropylene cages ($380 \times 380 \times 230$ mm) environmentally enriched with PVC pipes and standard Vivarium nesting material. Positive air pressure was maintained throughout at an air exchange rate of 18/h. Food (standard rat chow) and water was supplied ad libitum. All animals were weighed and monitored daily. All experiments were approved by the NWU-AnimCareREC animal research ethics committee (NHREC reg. number AREC-130913-015) of the North-West University, and all steps of animal handling conformed to the South African National Standard (SANS) for the Care and Use of Animals for Scientific Purposes (SANS 10386:2008). Animals were maintained and procedures performed in accordance with the code of ethics in research, training and testing of drugs in South Africa and complied with national legislation (ethics approval number NWU-00168-18-A5).

3.3. Drug treatment

ESC oxalate, provided as a kind gift by Lundbeck AG (Copenhagen, Denmark), and ST (Zembrin®) sponsored by HG&H, were prepared by dissolving it in 3 ml physiological saline according to guidelines of the manufacturer (escitalopram: (Wolmarans et al., 2016; (Brand et al., 2013); and supplier (ST*:* HG&H). These compounds were dissolved in (control treatment for all experiments). All drugs were calculated and administered via oral gavage after each rat was weighed. An acute dose response study with ST was performed across 5 doses (5, 10, 25, 50, 100 mg/kg; denoted ST 5, ST 10, ST 25, ST 50 and ST 100, respectively) based on previous ST studies (Carpenter et al., 2016; Gericke and Viljoen, 2008; Loria et al., 2014; Schell, 2014) and 3 doses of ESC (5, 10, 20 mg/kg, denoted ESC 5, ESC10 and ESC 20, respectively). Although previous studies in our laboratory have demonstrated the efficacy of SSRIs like escitalopram and fluoxetine in the FST and in FSL rats (Mokoena et al., 2015; Schoeman et al., 2017), it was incumbent on us to verify the effective dose of ESC via oral gavage. Using 10 mg/kg ESC as a reference dose (Eren et al., 2007; Hui et al., 2016; Hui et al., 2010; Wang et al., 2014; Yilmaz et al., 2011), we also assessed a higher (20 mg/kg) and a lower (5 mg/kg) dose. A therapeutic dose of ST and a sub- and full therapeutic dose of ESC were explored in the OFT and FST. Thereafter an appropriate dose of ESC and ST was used in a 15-day sub-chronic treatment study. While the nominal doses would validate the FST in this study, the low dose of ESC (5 mg/kg/day) would be explored in the sub-chronic study to fully explore the adjunctive potential of ST, and to minimize the risk of inducing 5-HT syndrome during adjunctive treatment with a full therapeutic dose of ST.

3.4. Study Layout

Acute validation of the FSL model: In order to confirm the phenotype of the FSL rat as an animal model of depression for application in this study, 12 FSL and 6 FRL rats were randomly treated with saline following the same methods and study design as the acute dose response study (see below). Behaviour was assessed in the OFT and FST where FSL rats were compared to that of control FRL rats. The latter animals also served as the vehicle control groups for the acute dose response study.

Acute dose response study: In order to ensure predictive validity for the FST in this study, and to define an optimal dose for both agents (see earlier note), the 3-tier dose response for ESC, and a 5-tier dose response with ST was performed. Upon reaching the desired weight (200 – 230 g), animals from each group (n=10) were treated at intervals of 24 hours, 6 hours and 1 hour before being subjected to the OFT and to the FST 30 min later. Thereafter, the animals were euthanized according to standard protocols.

Sub-chronic dose response study: A low dose of ESC and a full antidepressant dose of ST, as determined in the acute dose response study above, were used in this study. At the start of the study, 48 FSL rats (200 -230 g) were randomly divided into 4 equal groups through sorting them according to weight groups (weights differed between litters of the same age), the first receiving saline (control group) with the second and third groups treated with low dose ESC and full dose ST, respectively. Group 4 was treated with a combination of ESC 5 and ST 50. These groups were treated once daily between 7:00 and 9:00 (Oberholzer et al., 2017) over a period of 15 days. Thereafter animals were subjected to the OFT and FST on day 13 and 15 respectively, as described below. On day 16 the animals were decapitated and brain samples and trunk blood collected for bio-analysis.

3.5. Behavioural tests

3.5.1. Open field test (OFT)

The OFT test is a measure of reduced locomotor activity (Brand and Harvey, 2017) which is a typical symptom manifestation of MDD according to the DSM-5 (Psychiatric, 2013). Furthermore, the OFT is used to exclude any confounding drug treatment effects on locomotor activity that could otherwise complicate interpretation of the FST data (Lavi-Avnon et al., 2005).

The OFT, performed in a black open field box (100 x 100 x 50 cm), was done according to previous studies (Brand and Harvey, 2017; Oberholzer et al., 2017). For the acute dose response phase, each rat was subjected to the OFT for 5 minutes, 30 minutes before the FST. In the sub-chronic study, the OFT was performed for 10 minutes as part of a habituation period for another

behavioural test not discussed in this paper in an attempt to simplify logistics. Behavioural tests (starting with OFT) were conducted from 19:00 according to the sleep/wake cycle of the animals using a ceiling-mounted digital camera. Rat movements were recorded under a 40 lux red light with the video footage analysed using EthoVision® XT software (Noldus® Information Technology, Wageningen, The Netherlands). Locomotor activity, assessed at total distance moved (cm), was scored by an investigator blind to treatment conditions.

3.5.2. Forced swim test (FST)

The FST is a validated model of behavioural despair used to measure depressive-like behaviour in rodents, where the test scores decreased escape-driven behaviour (Brand and Harvey, 2017; Slattery and Cryan, 2012). This test was performed according to a previously published method (Brand and Harvey, 2017). In brief, animals were placed in a Perspex® cylinder (diameter 18 cm, height 60 cm) containing 30 cm of 25°C water for a total of 7 minutes. Since air might still be trapped in the animal's fur which could increase buoyancy and decrease swimming, the first minute was excluded from scoring. The last minute was excluded as some animals might have reached full immobility at this point, thus preventing dilution of treatment effects (Oberholzer et al., 2018). The middle 5 minutes was analysed and manually scored (using randomly assigned video codes by a colleague to ensure unbiased scoring) with respect to swimming, climbing/struggling and immobility. Swimming, climbing/struggling inform on escape-directed coping behaviours with immobility an indicator of behavioural despair. Immobility (floating) is where the rat only makes the necessary movements to keep its head above the water. On the other hand, swimming is a horizontal movement on the water surface with quadrant crossing, associated with serotonergic activity, while struggling is an upward- directed movement against the sides of the cylinder, associated with noradrenergic activity (Cryan et al., 2005). These coping strategies can be related to regional brain monoamine levels to enable construct validation (Brand and Harvey, 2017). After removing the rats from the cylinders, they were gently dried and returned to their home cage (Brand, 2017; Oberholzer et al., 2017; Rygula et al., 2005). No animals (FSL, SD or FRL) were subjected to an FST pre-swim 24 hours before the test swim (Cryan et al., 2002; Slattery and Cryan, 2012), in order to establish and compare baseline swimming behaviour of these strains with the intent to confirm depressive-like behaviour in the FSL rat.

3.6. Neurochemical analyses

3.6.1. Brain tissue and trunk blood collection

After the rats are decapitated on the last day of the study (24 hrs after the final treatment dose), the frontal cortex and hippocampus were dissected on an ice-cooled glass slab as previously

described (Chiu et al., 2007; Möller et al., 2013). After removing the olfactory bulb, the frontal cortex and hippocampus were dissected and transferred to 1.5 ml Eppendorf tubes, snap frozen at -80°C in liquid nitrogen, and stored until the day of analysis. To avoid freeze-thaw-freeze changes and possible deactivation of components, prior to freezing brain tissue were pre-split into aliquots according to left or right hippocampus/frontal cortex for use in the different assays. Immediately following decapitation, trunk blood was collected in purple K_2 vacutainers containing EDTA. Thereafter the vacutainer was closed and tilted slowly to prevent coagulation. It was then kept on ice and centrifuged, after which the plasma supernatant was pipetted into Eppendorf tubes and stored in the -80°C freezer for future analyses of neurobiomarkers not included in this study.

3.6.2. Monoamines

Quantitative analysis of frontal cortical and hippocampal monoamines (NE and 5-HT) was performed using a high performance liquid chromatography with electrochemical detection (HPLC-EC) method previously validated in our laboratories (Viljoen et al. (2018). Monoaminergic concentrations were expressed as ng/mg wet weight brain tissue (Viljoen et al. (2018).

3.6.3. Brain-derived neurotrophic factor (BDNF)

The BDNF concentration in the hippocampus and frontal cortex measured using an enzyme-linked immunosorbent assay (ELISA (Elabscience®; Catalog No: E-EL-R1235, 96T) according to the protocol provided by the manufacturer's instructions, with the results derived from a standard curve and expressed as pg/ml.

3.7. Statistical analyses

GraphPad Prism 7 for Windows (GraphPad software, San Diego, USA) software was used for all statistical analysis and graphical presentations. Statistical analysis was performed under consultation with the Statistical Consultation Service of the NWU.

Unpaired student's t-tests with Welch's correction (Shapiro-Wilk test for normality $p < 0.05$ for normally distributed data) or Mann-Whitney U-tests (data not distributed normally) were used to compare the behaviour between the FSL and FRL groups. To analyse behaviour and neurochemistry between treated and untreated FSL animals, an ordinary one-way analysis of variance (ANOVA), followed by Tukey's post-hoc analysis (for normally distributed data) or Kruskal-Wallis ANOVA followed by Dunn' multiple comparisons (for data not distributed normally) was deployed. Significance for all comparisons was set at $p < 0.05$. Cohen's *d* values were

calculated to determine effect sizes, with only large effect size differences (d > 0.8) accepted as significant and reported.

4. Theory/calculation

Given the pharmacological potential of ST as an antidepressant, it would be valuable to evaluate its effectivity as a single-entity or augmentative agent in an *in vivo* translational model of depression (FSL rat) using bio-behavioural analysis.

5. Results

5.1. *Quality control of ST of Zembrin®*

Fig. 1 is a ST extract UPLC-MS chromatogram with four main peaks at 3.35, 3.62, 4.14, and 4.97 minutes confirmed as mesembrenol, mesembranol, mesembrenone and mesembrine respectively, which contributed to 3.84 µg/mg of total plant material. The composition of the four main alkaloids in descending order were mesembrenone (1.84 µg/mg), mesembrenol (1.23 µg/mg), mesembrine (0.51 µg/mg) and mesembranol (0.26 µg/mg).

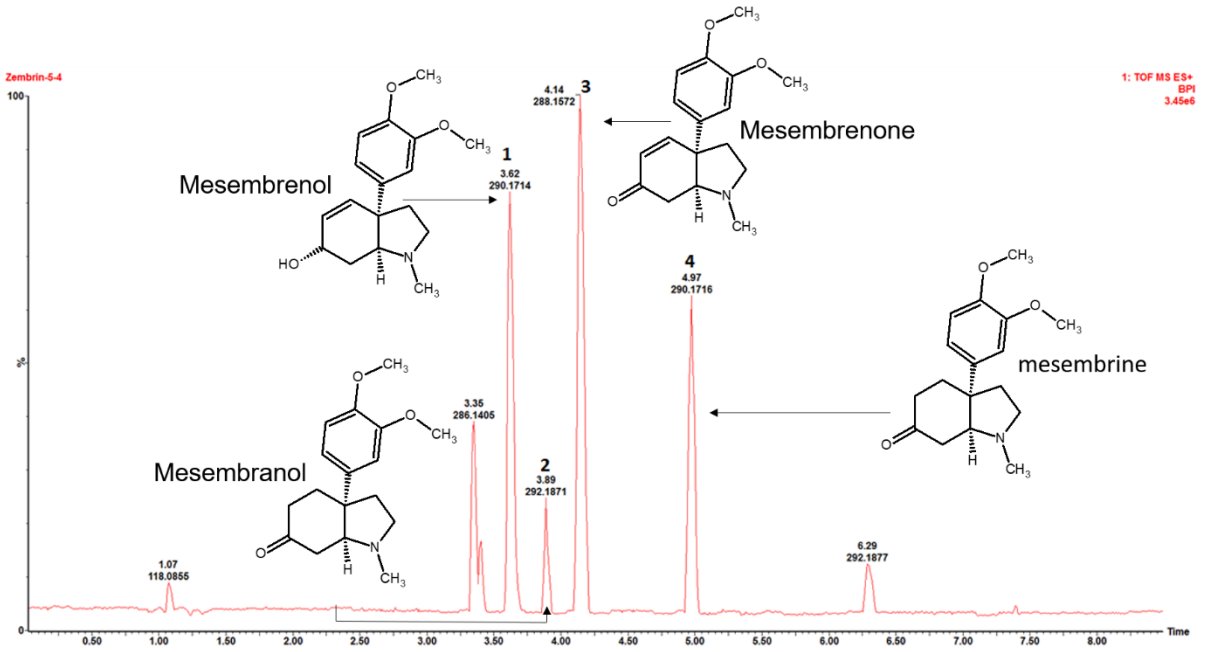


Fig. 1. Chromatographic fingerprint of the standardised extract of ST

5.2. *Verification of the FSL model*

Table 1: Swimming data was found not to be normally distributed (Shapiro-Wilke test). Subsequently the Mann-Whitney test indicated significant differences in swimming behaviour between the two groups ($U = 5$, $p = 0.002$), with FRL rats showing significantly increased swimming behaviour compared to the FSL rats. With regard to struggling behaviour, an unpaired t-test indicated significant differences between groups ($t(16) = 4.892$, $p = 0.0002$), with FRL rats showing a significantly higher duration of struggling behaviour compared to FSL rats. Unpaired t-test also indicated that FSL rats spent a significantly longer time immobile in the FST than FRL rats ($t(16) = 7.046$, $p < 0.0001$). Finally, an unpaired t-test showed that FRL rats moved significantly less in the OFT than FSL rats ($t(16) = 3.416$, $p = 0.003$)

Table 1: Verification of the FSL model. Immobility, swimming, and climbing behaviour as measured in the FST, and distance moved as a measure of locomotor activity in the OFT Data represent saline-treated FSL (n = 12) vs. FRL (n = 6) rats, expressed as (mean [95% CI]), $p < 0.05$ deemed significant.

	FRL	**FSL**	***p*-value**
Swimming	43.82 s [30.88 – 56.76]	26.6 s [22.15 – 31.4]	0.0020
Struggling	94.68 s [68.66 – 120.7]	48.51 s [38.69 – 58.33]	0.0002
Immobility	160 s [134.8 – 186.2]	220.1 s [212.5 – 227.7]	<0.0001
Distance moved (OFT)	2284 cm [1611 – 2958]	3247 cm [2921 – 3573]	0.0033

5.3. Acute dose response studies

5.3.1. Acute ESC dose response in FST

Immobility (Fig.2.A): All data sets showed normal distribution. The one-way ANOVA indicated significant differences between groups ($F(3.38) = 5.62$, $p = 0.003$), where multiple comparisons indicated that ESC 5 (195 s [176 – 208]) significantly decreased time spent immobile compared to the FSL-SAL group (220 s [213 – 228], $p = 0.002$). There was no statistically significant difference between FSL-SAL and ESC 10 (202 s [191 – 214]; $p = 0.073$).

Swimming (Fig.2.B.): Not all data sets represented normal distribution. The Kruskal-Wallis test indicated significant differences between groups ($\chi^2(3) = 13.1$, $p = 0.004$). According to multiple comparisons, ESC 10 (42.9 s [32.2 to 53.5 s]) significantly increased swimming behaviour relative to FSL-SAL (26.6 s [22.2 to 31.0 s], $p = 0.026$) and ESC 5 (28.0 s [19.1 to 37.0 s], $p = 0.036$).

Struggling (Fig.2.C): All data sets showed normal distribution. The one-way ANOVA indicated significant differences between groups ($F(3,38) = 3.1$, $p = 0.038$). Multiple comparisons test

showed that only ESC 20 (44.5 s [33.8 to 55.1 s]) displayed significantly decreased struggling behaviour when compared to ESC 5 (65.4 s [51.6 to 79.2 s], $p = 0.041$).

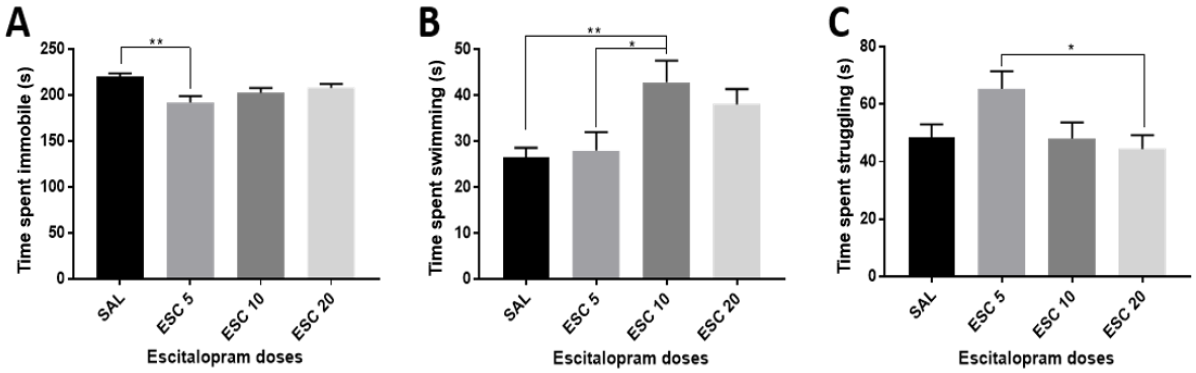


Fig.2. *Acute ESC dose response:* Acute antidepressant-like effects of various doses of ESC, as shown (n = 10 per group) in the FST, compared to saline-treated control FSL animals (n = 12). Parameters include time (s) spent A) immobile, B) swimming, and C) struggling. Data are expressed as (means [95% CI]). $p < 0.05$ deemed significant. A) $^{**}p < 0.015$; B) $^{*}p < 0.036$, $^{**}p < 0.026$; C) $^{*}p < 0.041$.

5.3.2. *Acute ESC dose response in OFT*

All data sets (Fig. 3) showed normal distribution. The one-way ANOVA indicated significant differences between groups ($F(3.39) = 8.18$, $p = 0.0002$). Multiple comparisons indicated that all three treatment groups showed significant differences compared to FSL-SAL (3247 cm [2921 – 3573]), where ESC 5 (4370 cm [3857 – 4884], $p = 0.001$), ESC 10 (4240 cm [3748 – 4733], $p = 0.005$), and ESC 20 (4342 cm [3849 – 4835], $p = 0.002$) significantly increased the distance moved.

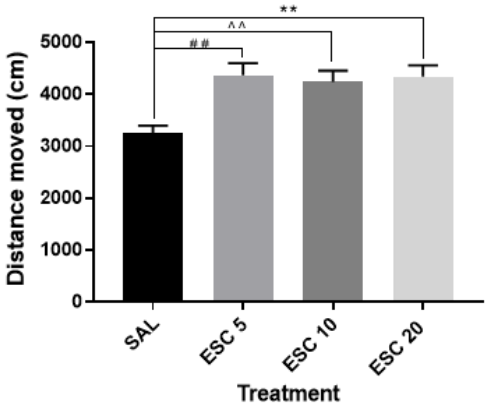


Fig. 3. *Acute ESC dose response:* Acute effects of the various drug treatments on locomotor activity, as shown (n = 10 per group) in the OFT, compared to saline-treated control FSL animals (n = 12). Parameter measured is distance travelled (cm) over 5 minutes. Data expressed as (means [95% CI]). $p < 0.05$ deemed significant. $^{**}p < 0.002$, $^{\wedge\wedge}p < 0.005$, $^{\#\#}p < 0.001$.

5.3.3. Acute ST dose response in FST

Immobility (Fig.4.A): All data sets showed normal distribution. The one-way ANOVA indicated significant differences between groups ($F(5.56) = 4.73$, $p = 0.001$). Multiple comparisons showed significant differences between FSL-SAL (220 s [213 – 228]), ST 25 (193 s [178 – 207] $p = 0.022$), and ST 50 (189 s [173 – 205], $p = 0.006$), where both treatment groups significantly decreased immobility. Additionally, immobility was decreased by ST 25 ($p = 0.041$) and ST 50 ($p = 0.013$) compared to ST 5 (219 s [201 – 238]).

Swimming (Fig.4.B): Not all data sets displayed normal distribution. The Kruskal-Wallis test indicated significant differences between groups ($\chi^2(5) = 11.8$, $p = 0.038$), however post-hoc tests showed no significant intergroup differences. However, Cohen's d-value showed trends towards increased swimming with ST 50 (38.1 s [24.9 – 51.3], $p = 0.436$, $d = 0.822$ [-0.032 – 1.727]) compared to FSL-SAL (26.6 s [22.2 – 31]).

Struggling (Fig.4.C): All data sets represented normal distribution. The one-way ANOVA indicated significant differences between groups ($F(5.56) = 3$, $p = 0.018$). Significant increases in struggling was observed with ST 25 (68.6 s [51 – 86.2], $p = 0.027$) as well as ST 50 (67.6 [48.4 – 86.7], $p = 0.036$) compared with ST 5 (38.3 s [25 – 51.6]).

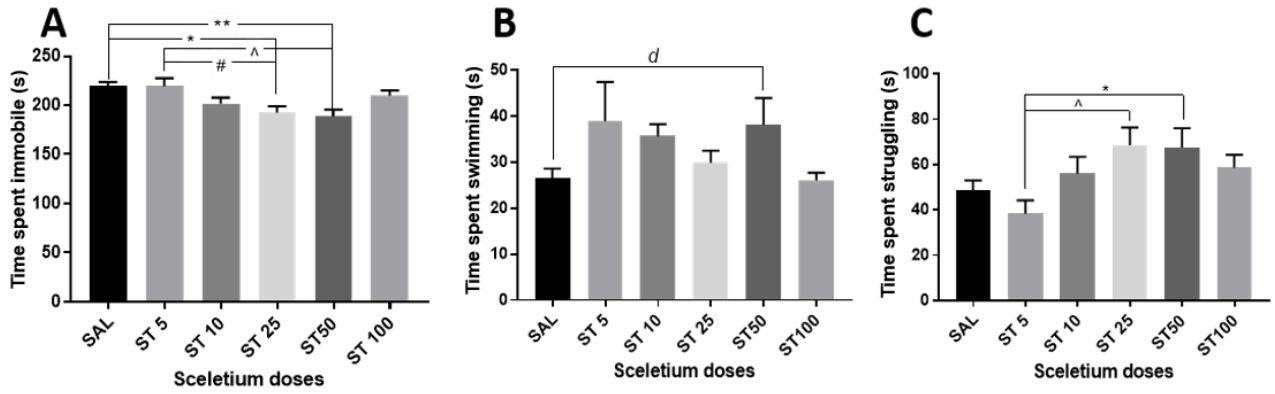


Fig.4. *Acute ST dose response:* Acute effects of various doses of ST as shown (n = 10 per group) in the FST, compared to saline-treated control FSL animals (n = 12). Parameters measured include time (s) spent A) immobile B) swimming, and C) struggling. Data expressed as (means [95% CI]). $p < 0.05$ deemed significant. A) $^{**}p < 0.006$, $^{*}p < 0.022$, $^{\wedge}p < 0.013$, $^{\#}p < 0.041$; B) $d = 0.822$; C) $^{*}p < 0.036$, $^{\wedge}p < 0.027$.

5.3.4. *Acute ST dose response in OFT*

All data sets (Fig. 5) displayed normal distribution. The one-way ANOVA showed significant differences between groups ($F(5.57) = 2.28$, $p = 0.059$). Multiple comparisons showed a significant increase in distance moved between ST 5 (2665 cm [2037 – 3294]) and ST 25 (3624 cm [2856 – 4391], $p = 0.045$). No other differences were evident.

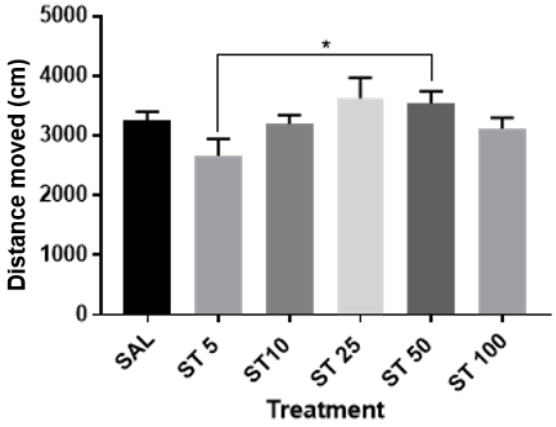


Fig. 5. *Acute ST dose response:* Acute effects of the various doses of ST on locomotor activity as shown (n = 10 per group) in the OFT, compared to saline-treated control FSL animals (n = 12). Parameter measured is distance travelled (cm) over 5 minutes. Data expressed as (means [95% CI]). $p < 0.05$ deemed significant. *$p < 0.045$

5.4. Sub-chronic study

The dose of ST (50 mg/kg) was chosen based on its statistically significant antidepressant-like effects in the FST and OFT, while the dose of ESC (5 mg/kg) was chosen as a sub-therapeutic dose. This is discussed more extensively under Section 6.1.

5.4.1. Sub-chronic study in FST

Immobility (Fig. 6.A): All data sets showed normal distribution. The one-way ANOVA indicates significant differences between groups *(*$F(3.44) = 7.29$, $p = 0.0005$). According to multiple comparisons, the combination group (211 s [196 – 226]) significantly increased immobility compared to FSL-SAL (177 s [156 – 198], $p = 0.014$), ESC 5 (163 s [151 – 175], $p = 0.0002$), and ST 50 (182 s [165 – 198], $p = 0.039$).

Swimming (Fig. 6.B): All data sets displayed normal distribution. The one-way ANOVA indicated significant differences between groups ($F(3.44) = 3.61$, $p = 0.021$). Multiple comparisons indicated a significant increase in swimming behaviour with ESC 5 (62.2 s [53.1 – 71.3]) compared to the combination treatment group (46.3 s [38.5 – 54.1], $p = 0.041$).

Struggling (Fig. 6.C): All data sets were not normally distributed. The Kruskal-Wallis test indicated significant differences between groups ($\chi^2(3) = 9.7$, $p = 0.021$). Multiple comparisons indicated that the combination treatment (37.6 s [27.4 – 47.8]) significantly decreased struggling behaviour compared to ESC 5 (60.4 s [49.9 – 71], $p = 0.031$).

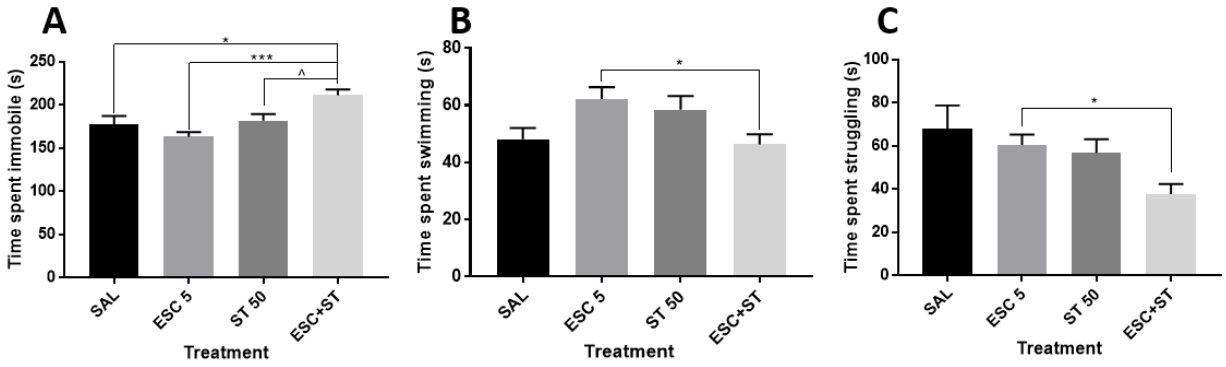


Fig. 6. *Sub-chronic study:* Effects of 5 mg/kg/day ESC, 50 mg /kg/day ST, and a combination of ESC 5 and ST 50, vs. saline-treated control animals in the FST (n = 12 per group). Parameters measured include A) immobility, B) swimming, and C) struggling behaviour. Data expressed as (means [95% CI]). $p < 0.05$ deemed significant. A) *$p < 0.014$, ***$p < 0.0002$, ^$p < 0.039$; B) *$p < 0.041$; C) *$p < 0.031$.

5.4.2. *Sub-chronic study in OFT*

All data sets (Fig. 7) presented with normal distribution. The one-way ANOVA showed significant differences between groups ($F(3.44) = 3.22$, $p = 0.032$). Multiple comparisons indicated that the combination treatment (5865 cm [5137 – 6594]) showed a significant increase in distance moved compared to FSL-SAL (4724 cm [4280 – 5168], $p = 0.019$).

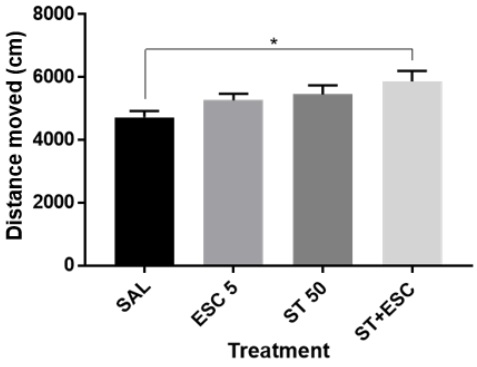


Fig. 7. *Sub-chronic study*. Effects of 5 mg/kg/day ESC, 50 mg/kg/day ST, and a combination of ESC 5 and ST 50, vs. saline-treated control animals on locomotor activity in the OFT. Parameter measured is distance travelled over 10 minutes. Data expressed as (means [95% CI]). $p < 0.05$ deemed significant. *$p < 0.019$

5.4.3. 5-HT levels in sub-chronic study

Hippocampal 5-HT (Fig. 8.A): All data sets were normally distributed. The one-way ANOVA indicated significant differences between groups ($F(3.44) = 11$, $p < 0.0001$). Multiple comparisons showed that the combination treatment (144 ng/g [118 – 171]) showed a statistically

significant increase in hippocampal 5-HT compared FSL-SAL (91.4 ng/g [58.5 – 124], $p = 0.009$), ESC 5 (60.6 ng/g [43.7 – 77.6], $p < 0.0001$), and ST 50 (70.1 ng/g [50.7 – 89.5], $p = 0.0002$).

Frontal cortex 5-HT (Fig. 8.B): Not all data sets are normally distributed. The Kruskal-Wallis test indicated significant differences between groups ($\chi^2(3) = 8.44$, $p = 0.038$). However, multiple comparisons did not show any statistically significant intergroup differences in 5-HT levels.

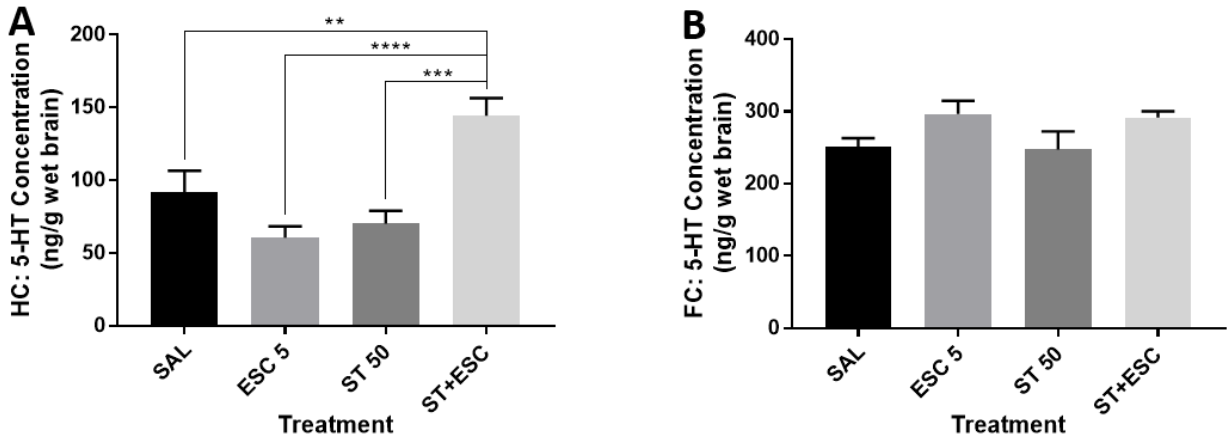


Fig. 8. *Sub-chronic study.* Tissue 5-HT levels (ng/g) in the various treatment groups as indicated vs. saline-treated FSL control animals (n = 12 per group) in A) the hippocampus, and B) the frontal cortex. Data expressed as (means [95% CI]). $p < 0.05$ deemed significant. A) $^{**}p < 0.009$, $^{****}p < 0.0001$, $^{***}p < 0.0002$

5.4.4. *NE levels in sub-chronic study*

Hippocampal NE (Fig. 9.A): All data sets did not show normal distribution. The Kruskal-Wallis test indicated significant differences between groups ($\chi^2(3) = 9.07$, $p = 0.028$). Multiple comparisons revealed that the combination treatment (243 ng/g [199 – 287]) significantly increased hippocampal NE levels compared to FSL-SAL (174 ng/g [156 – 193], $p = 0.038$).

Frontal cortex NE (Fig.9.B): All data sets were not normally distributed. The Kruskal-Wallis test revealed no statistically significant differences between groups ($\chi^2(3) = 6.6$, $p = 0.086$).

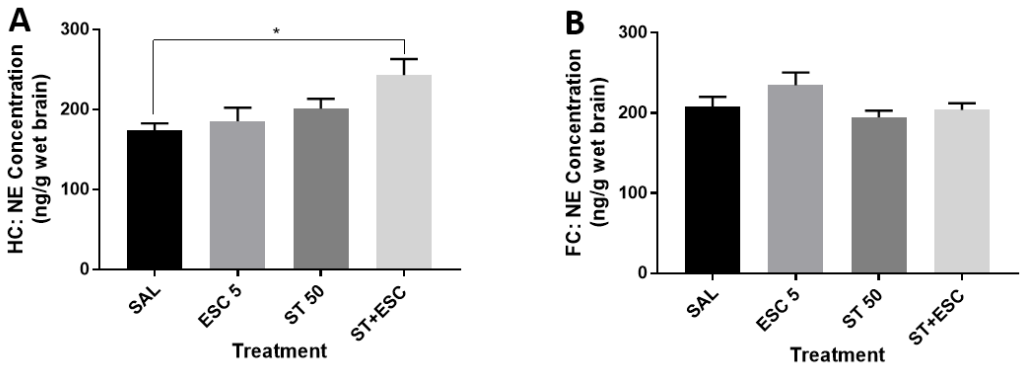


Fig. 9. *Sub-chronic study.* Tissue NE levels (ng/g) in the various treatment groups as indicated vs. saline-treated control animals (n = 12 per group) in A) the hippocampus, and B) the frontal cortex. Data expressed as (means [95% CI]). $p < 0.05$ deemed significant. A) *$p < 0.038$

5.4.5. *BDNF levels in sub-chronic study*

Hippocampal BDNF (Fig. 10.A): Not all data sets were normally distributed. The Kruskal-Wallis test indicated significant differences between groups ($\chi^2(3) = 9.58$, $p = 0.023$). Multiple comparisons showed that ST 50 (239 pg/ml [207 – 271]) significantly increased hippocampal BDNF levels compared to FSL-SAL (188 pg/ml [163 – 213], $p = 0.038$).

Frontal cortex BDNF (Fig. 10.B): All data sets showed normal distribution. The one-way ANOVA indicated statistically significant differences between groups ($F(3.44) = 7.71$, $p = 0.0003$). Multiple comparisons showed that both ESC 5 (80.1 pg/ml [74.5 – 85.7], p = 0.002) and the combination treatment (81.2 pg/ml [76.8 – 85.7], p = 0.006) significantly decreased BDNF levels compared to FSL-SAL (93.7 pg/ml [86.8 – 101]). Additionally, BDNF levels were significantly decreased by ESC 5 (p = 0.011) and the combination treatment (p = 0.026) compared to ST 50 (91.8 pg/ml [86.7 – 96.8]).

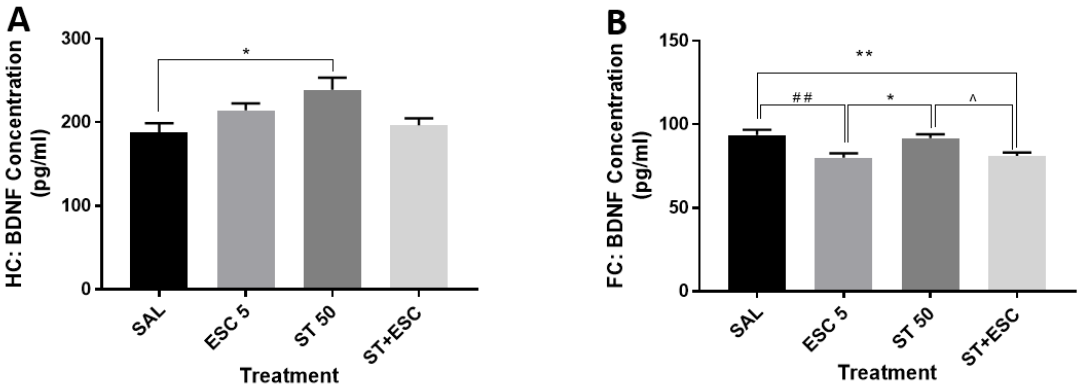


Fig. 10. *Sub-chronic study.* Tissue BDNF levels (pg/ml) in the various treatment groups as indicated vs. saline-treated control animals (n = 12 per group) in A) the hippocampus, and B) the frontal cortex. Data expressed as (means [95% CI]). $p < 0.05$ deemed significant. A) *$p < 0.038$; B) **$p < 0.006$, $^{\#\#}p < 0.002$, *$p < 0.011$, ^$p < 0.026$.

6. Discussion

Fingerprinting ensures authenticity and quantifies the plant extract composition being evaluated (Liu et al., 2019). In this study the ST extract comprised of, in order of decreasing mass of plant material, mesembrenone (1.84 µg/mg), mesembrenol (1.23 µg/mg), mesembrine (0.51 µg/mg) and mesembranol (0.26 µg/mg) (Fig. 1). These ratios should always be constant between batches of Zembrin® due to its chemotype. Interestingly, ST plants used for the production of Zembrin® are cultivated to constantly contain the same ratio of alkaloids in each individual plant. Previous ST studies (Krstenansky, 2017; Patnala and Kanfer, 2015; Shikanga et al., 2012) have identified similar alkaloids, though in varying concentration, which is normal owing to various factors (i.e. species, climatic conditions, analytic methods). The most important findings of this study are that ST and ESC show dose-dependent antidepressant-like effects after acute treatment. However, sub-chronic ESC 5 and ST 50 treatment did not demonstrate antidepressants effects separately, while the ST 50 and ESC 5 combination significantly increased immobility, decreased swimming behaviour and increased locomotor activity, indicating possible depressogenic effects. The latter behaviours occurred concurrent with significantly elevated hippocampal 5-HT levels and reduced frontal cortical BDNF levels.

Deficits in monoaminergic neurotransmission underlie the expression of diverse symptoms of MDD, such as low mood, decreased motivation, psychomotor agitation and retardation, and deficits in cognitive performance (Jesulola et al., 2018). Consequently, increasing these neurotransmitters is an important construct for antidepressant treatment (Dean and Keshavan, 2017; Jesulola et al., 2018). NE is more linked to locomotor and psychomotor activation, while 5-HT is especially implicated in behavioural and somatic systems (Chen and Reith, 1995; Dean and Keshavan, 2017; Jesulola et al., 2018). 5-HT and NE may be causally linked to behavioural despair in the FST and impaired locomotor activity in the OFT (Brand, 2017; Chen and Reith, 1995). New generation antidepressants like SSRIs are broadly effective in improving or normalizing aberrant behaviours observed in these tests (Brand, 2017; Chen and Reith, 1995; Dean and Keshavan, 2017) and provides the necessary predictive validity to explore novel compounds such as ST.

Prolonged monoaminergic deficits in MDD prompt up-regulation of 5-HT_{1A} and α_{2A}-adrenergic receptors (Albert and Lemonde, 2004; García-Sevilla et al., 1999). Acute SSRI treatment induces an initial increase in 5-HT synaptic levels through SERT inhibition, which in turn activates postsynaptic 5-HT_{1A} receptors in the hippocampus and frontal cortex, and presynaptic 5-HT_{1A} receptors in the raphe nuclei and hippocampus, ultimately leading to a post-acute depletion of 5-HT through hyperpolarization of the neuron (Blier and Ward, 2003; Ceglia et al., 2004). However, chronic treatment with SSRIs or 5-HT_{1A} agonists desensitizes the 5-HT_{1A} receptor, restoring 5-HT levels and ultimately producing antidepressant effects. This process underlies the delay in therapeutic action with these antidepressants (Blier and Ward, 2003; Ceglia et al., 2004).

We confirmed here that FSL rats present with a depressive phenotype versus FRL control rats, viz. decreased swimming and struggling, and increased immobility in the FST (Table 1). However, while FSL rats normally express decreased locomotor activity (Overstreet (1993), we observed a significant increase (Table 1). This anomaly will be discussed later on. Importantly though, FSL rats present with altered expression of 5-HT_{1A} and α_{2A}-ARs (Lillethorup et al., 2015; Nishi et al., 2009), in line with that observed in MDD (Albert and Vahid-Ansari, 2018; Invernizzi and Garattini, 2004; Lillethorup et al., 2015), and of importance when considering the action of ST.

6.1. Response to acute ESC and ST:

ST possesses high affinity for the SERT *in vitro* (Harvey et al., 2011; Zhong et al., 2012), suggesting concordance with an SSRI. By increasing VMAT-2, ST has monoamine releasing properties and is also a mild MAO-A inhibitor (Coetzee et al., 2016; Krstenansky, 2017). Thus ESC and ST can be regarded as serotonergic drugs, indirectly activating 5-HT_{1A} receptors via SERT inhibition. ST may have some NE actions too.

FST: Swimming and immobility

Swimming behaviours in the FST are linked to 5-HT'ergic activity (Brand, 2017; Cryan et al., 2005). Most studies agree that 10 mg/kg ESC represents a typical antidepressant dose in rodents (Bech et al., 2006; Montgomery et al., 2001). Indeed, acute ESC 10 mg/kg narrowly missed statistical significance to reverse immobility ($p = 0.073$) (Fig. 2A), but significantly increased swimming behaviour (Fig. 2B), which correlates with previous studies (El Mansari et al., 2005; Murdoch and Keam, 2005). The 20 mg/kg ESC dose induced a similar albeit lesser response, probably due to over-activation of pre-synaptic 5-HT_{1A} autoreceptors, lowering 5-HT levels and abrogating the behavioural response. On the other hand, ESC 5 mg/kg did not show a significant increase in swimming (Fig. 2B) yet was significantly antidepressant (decreased immobility; Fig. 2A). This response could be related to the dose-dependent effects of ESC on 5-HT_{1A} and 5-HT_{2A} receptors in the dorsal raphe nuclei (DRN), where a lower dose may limit overt 5-HT_{1A} autoreceptor mediated inhibition of 5-HT release.

ST exerted dose-dependent antidepressant effects (Fig. 4A), with 25 mg/kg (ST 25) and 50 mg/kg (ST 50) significantly reducing immobility; ST 50 being slightly more effective than ST 25 ($d = 0.187$). ST 50 also showed a large effect size increase in swimming (Fig.4B), correlating with an antidepressant action with at least *some* serotonergic action. These findings confirm the earlier acute treatment study of Schell in BALC-c mice (Schell (2014).

Struggling (FST) and locomotor activity (OFT):

Struggling in the FST is purported to reflect more noradrenergic mechanisms of action (Cryan et al., 2005). Both ESC 5 mg/kg/day (Fig. 2C) and ST 50 (Fig 4C) increased struggling behaviour.

Despite a selective action on SERT, SSRI's like ESC also modulate NA'ergic pathways, albeit indirectly (see Harvey (1997). Thus, studies have shown that 5-HT increases hippocampal NE via 5-HT_{1A} receptors in subacute but not chronic treatment (Chen and Reith, 1995; Szabo and Blier, 2001). Similarly, the penchant of ST to increase the activity of VMAT-2, inhibit MAO-A, and inhibit NET (see Introduction) will arguably also increase NE levels. Increased struggling with ESC and ST 50 therefore confirms the above-mentioned mechanisms to bolster NE activity, at least after acute dosing.

The increased locomotor activity following all doses of ESC (Fig 3), but not with any dose of ST (Fig 5), is confirmatory of a NA'ergic action at least for ESC. On the other hand, increased locomotor activity may imply a false positive response in the FST (de Kloet et al., 1999; Slattery and Cryan, 2012). However, this would have meant a general increase in swimming and struggling, as well as reduced immobility, across all doses, which did not happen. This is not the case for ST, which did not affect locomotor activity at any dose. Interestingly, FSL rats already presented with increased locomotor activity vs FRL control rats (Table 1). FSL rats have been found to present with increased (Du Jardin et al., 2016), decreased (Overstreet, 1993) or no change (Uys, 2016) in locomotor activity. Auto-inhibitory α_2-ARs suppress NE release (Invernizzi and Garattini (2004). Lillethorup et al. (2015) found increased α_2-adrenoreceptor densities in FSL vs Sprague Dawley rats, which suggests reduced NE (increased negative feedback), but reduced density versus FRL rats which suggests increased NE levels (decreased negative feedback). The latter may explain increased locomotor activity in FSL rats described here.

On the strength of the above findings, we selected the maximally effective dose of 50 mg/kg ST for the sub-chronic study, congruent with the 5 to 100 mg/kg dose range noted in other *in vivo* studies as previously mentioned. ESC 5 mg/kg/day was chosen as a suitable low dose in combination with ST 50 for the sub-chronic adjunctive treatment study. Low-dose ESC was deemed less likely to induce a detrimental increase in 5-HT levels when combined with ST, while the adjunctive potential of ST would be more amenable to testing when the dose of the primary treatment is lowered (Sarris et al., 2010).

6.2. Response to sub-chronic ESC and ST:

Although we did not measure basal 5-HT and NA levels in FRL rats in this study, previous literature indicates that hippocampal 5-HT and NE levels are increased in FSL versus FRL rats, although 5-HT synthesis is decreased (Overstreet et al., 2005; Overstreet and Wegener, 2013). However, earlier work in our facilities indicated that FSL rats present with significantly *lower* hippocampal 5-HT and NE levels versus FRL rats (Uys, 2016), thus congruent with the monoamine hypothesis of MDD. Nevertheless, these data concur that definite serotonergic abnormalities are evident in FSL rats (Overstreet et al., 2005). Thus, 5-HT_{1A} and SERT are

decreased and α_2-ARs are upregulated in FSL rats (Lillethorup et al., 2015; Nishi et al., 2009; Overstreet and Wegener, 2013; Owens et al., 2011), which will drive diverse brain monoamine changes in these animals. The central role of 5-HT_{1A} receptors in MDD and response to treatment is widely recognized, with up or down-regulation playing a key role in suppressing or increasing 5-HT release following acute or chronic antidepressant exposure, respectively (Blier and Ward, 2003). Similarly, α_2-ARs play a decisive role in NE regulation and antidepressant action (Uys et al., 2017a; 2017b). This would explain why FSL rats respond positively to various monoamine-selective antidepressants (Overstreet and Wegener, 2013).

While both ESC 5 mg/kg and ST 50 mg/kg separately tended to lower hippocampal 5-HT levels (Fig.8) or have no effect on NE (Fig. 9), their combination significantly increased the levels of both transmitters versus saline and either drug alone (5-HT; Fig. 8) or just versus saline (NE; Fig 9). Although we did not measure receptor expression and affinity, the state of SERT, NET, 5-HT_{1A} and α_2-ARs probably underlies these responses. These will be discussed separately.

Role of Serotonin (Fig. 8):

A down-regulated state of 5-HT_{1A} receptors would explain significantly increased hippocampal 5-HT levels (Fig. 8A) with combination therapy, suggesting that the effect of ST 50 and ESC 5 *separately* did not sufficiently desensitize the 5-HT_{1A} receptor during the 15-day treatment, but that the combination presumably did. ESC is associated with a quicker desensitization of the 5-HT_{1A} receptor due to its higher potency compared to other SSRIs (Ceglia et al., 2004; El Mansari et al., 2005). Assuming that 10 mg/kg/day delivers a therapeutic response within 2 weeks (El Mansari et al. (2005), it is not unreasonable that a lower dose of ESC could show delayed desensitization of 5-HT_{1A}, which would explain lower 5-HT levels in the hippocampus with low dose ESC monotherapy as well as a reduced, albeit not significantly so, antidepressant response (decrease in immobility; Fig. 6A). ST 50 alone showed similar effects to ESC 5 with regard to hippocampal 5-HT levels and immobility, although its exact mechanism of action is speculative. Nevertheless, its bolstering of hippocampal 5-HT in combination with ESC is strongly supportive of a serotonergic action. Indeed, the increased swimming in the ST alone group, and similar to ESC (Fig. 6B), support this argument. Interestingly, swimming was markedly *reduced* in the ESC+ST group, together with *increased immobility*, this concurrent with significantly elevated hippocampal 5-HT levels. The behavioural observations are indicative of a paradoxical depressogenic response, in spite of elevated 5-HT levels. Can this be so, or is there another explanation?

Interestingly, none of the treatments significantly altered frontal cortex 5-HT levels (Fig. 8B), possibly linked to low densities of 5-HT_{1A} and high densities of 5-HT_{2A} receptors in the frontal cortex versus the hippocampus (Carhart-Harris and Nutt, 2017). Also, the frontal cortex occupies a top-down regulatory role on the hippocampus (Miyashita, 2004), which may explain why drug-

induced changes are more prominent down-steam of the frontal cortex, i.e. the hippocampus. Clearly, further study into the mutual interaction between 5-HT and NE, and the role of 5-HT_{1A} and 5-HT_{2A} receptors in this regard are needed (e.g. (Quesseveur et al., 2013)).

Given their common SERT inhibitory properties, the increased hippocampal 5-HT levels in the combined treatment group (Fig.8) very likely represents a synergistic serotonergic response to ESC 5 plus ST 50. This increase in 5-HT had indirect 5-HT_{1A} agonistic effects and was sufficient to induce more rapid desensitization of 5-HT_{1A} receptors than either drug alone. Importantly, the FST data seems to suggest "depressogenic" effects for the combination treatment. This is paradoxical to the low serotonin hypothesis of depression, as well as our earlier work that showed *reduced* baseline 5-HT and NE levels in FSL vs. FRL rats (Uys, 2016). However, reduced swimming activity and increased immobility in the FST may suggest an attempt to conserve energy (viz. increased hang time, reduced swimming/climbing), and hence support a putative antidepressant response. This idea is well-supported by earlier work (Andrews et al., 2015). On the other hand, this response may also be attributed to 5-HT_{1A} receptor supersensitivity, which has been described in FSL rats (Overstreet et al., 1998; Shayit et al., 2003). Interestingly, while 5-HT_{1A} agonists are antidepressant in the FST (Detke et al., 1995), they induce "5-HT behavioural syndrome" in FSL rats (Haberzettl et al., 2013; Shayit et al., 2003). This is accompanied by hypothermia and increased immobility (El Yacoubi and Vaugeois, 2007; Overstreet, 2002; Shayit et al., 2003), the latter evident in the combined treatment group (Fig. 6A). Moreover, locomotor hyperactivity in the OFT (Fig. 7) is also associated with 5-HT behavioural syndrome and mediated by postsynaptic 5-HT_{1A} receptors (Haberzettl et al., 2013; Mignon and Wolf, 2002). 5-HT behavioural syndrome that may follow 5-HT_{1A} supersensitivity described in FSL rats (Overstreet and Wegener, 2013), may also underlie the paradoxical depressive-like state observed following ESC+ST treatment, described above (Yadid et al., 2000). Here, 5-HT_{1A} receptor agonism has been associated with passive coping in the FST, presenting as increased immobility (Puglisi-Allegra and Andolina, 2015). This evidence reinforces the notion that ESC+ST facilitates 5-HT mediated activity.

Role of norepinephrine (Fig. 9):

Both ESC and ST may harness direct and indirect actions on the noradrenergic systems of the brain (see earlier). Furthermore, FSL rats present with marked upregulation of α_2-AR binding (Lillethorup et al., 2015). Hyperlocomotion (Fig. 7) can also be correlated to the significantly increased levels of NE in the hippocampus (Fig. 9B)) (Chen and Reith, 1995). NE levels in the frontal cortex (Fig.9B) follows the same pattern as for 5-HT (Fig. 8B). Indeed, ESC+ST-induced struggling behaviour in the FST (Fig. 6C) was significantly reduced vs. ESC with a trend towards same vs saline control (p = 0.073). Presynaptic α_2-AR inhibit NE neuron firing and decrease extracellular levels of NE (Blier and Ward, 2003; Invernizzi and Garattini, 2004), which can be attenuated by desensitizing α_2-AR with chronic desipramine treatment, a noradrenergic

antidepressant (Blier and Ward, 2003; Invernizzi and Garattini, 2004). Therefore, increased NE in the ESC+ST combination reflects α_2-AR desensitization within the 15-day treatment period. Furthermore, α_{2A} heteroreceptors can also inhibit 5-HT release in the frontal cortex and hippocampus (Harvey and Slabbert, 2014), thus indirectly modulating the actions of 5-HT. However, 5-HT may also modulate the actions of NE. Indeed, Hajós-Korcsok and Sharp (1996) found increased hippocampal NE following treatment with the full 5-HT_{1A} receptor agonist, i.e. 8-OH-DPAT, highlighting how altered 5-HT_{1A} receptor sensitivity can affect NA'ergic signalling.

Role of BDNF (Fig. 10):

BDNF is important for neuronal growth and neuroplasticity, especially in the hippocampus and frontal cortex, where its dysregulation is associated with learning and memory deficits in MDD (Cattaneo et al., 2016; Duman et al., 2000). BDNF is invariably reduced in MDD (Brand et al., 2015). Although basal BDNF levels in FRL rats were not determined, we have earlier described a significant decrease in hippocampal BDNF in FSL versus FRL rats (Uys (2016). Similarly, Elfving et al. (2010) reported significantly decreased hippocampal BDNF and unchanged levels in the frontal cortex of FSL vs FRL rats.

ESC 5 tended to increase hippocampal BDNF (Fig. 10A), while combination treatment had no effect. However, ST 50 alone significantly increased hippocampal BDNF, although this increase was not associated with an antidepressant effect (Fig. 6A). Paradoxically though, ESC 5 and combined ESC 5 + ST 50 significantly *decreased* frontal cortical BDNF (Fig. 10B), the latter correlating with a depressogenic effect (Fig. 6A). That ST 50 alone increased hippocampal BDNF but reduced such levels in the cortex when combined with ESC are intriguing. In support of these findings, some animal (Castrén et al., 2007) and human (Harvey et al., 2013) studies have found elevated BDNF to be associated with depressive symptoms. In fact, our data also shows an inverse relation between 5HT and swimming activity, and between NA and struggle activity, which supports a counter-regulatory role for BDNF.

Alternatively, the BDNF findings could involve $5HT_{2A}$ receptor activation, which down-regulates hippocampal BDNF expression (Vaidya et al., 1999). This response may follow ESC 5+ST 50-induced 5-HT_{1A} down-regulation leading to increased 5-HT release from the DRN (Carhart-Harris and Nutt, 2017; Jaggar and Vaidya, 2018; Narla et al., 2015; Piguet and Galvan, 1994; Quesseveur et al., 2013; Vaidya et al., 1999). Alternatively, disturbances in α_2-AR sensitivity may also partly or wholly underlie the observed changes in neurotrophin release (Yanpallewar et al., 2010). Indeed, the ST-associated increase in NE via increased VMAT-2 and NET inhibition may underlie its ability to increase hippocampal BDNF (Fig. 10A).

7. Conclusion

Acute ESC and ST administration display dose-dependent antidepressant effects in FSL rats, with 25 and 50 mg/kg ST comparable in efficacy to effective doses of ESC (viz. 5 and 10 mg/kg). This suggests promise in the treatment of MDD. While low dose ESC and a full dose of ST were ineffective antidepressants following a 15-day sub-chronic treatment and did not alter hippocampal 5-HT levels, combined ESC+ST worsened depressive-like behaviour, significantly bolstered hippocampal 5-HT levels, and suppressed frontal cortical BDNF levels. These paradoxical actions may be a splinter symptom of 5-HT behavioural syndrome or an anomaly of the FSL rat. Alternatively, one could argue that the unexpected findings indicate that ST simply should not be used in combination therapy – at least not in combination with other serotonergic drugs. That ST alone increased hippocampal BDNF levels prompts consideration in mood and cognitive disorders. Adjunctive treatment with ESC confirms that ST has prominent serotonergic actions. However, further investigation in sub-chronic study designs, and perhaps in another depressed rat model free of factors like 5-HT_{1A} receptor hypersensitivity, are needed to gain a better and more accurate understanding of ST's therapeutic potential in MDD. One must also keep in mind that there are many different mechanisms of action of ST, as mentioned in the introduction, that have not been considered in this study which could have influenced the antidepressant activity of ST as monotherapy and augmentative treatment.

8. Limitations and future recommendations

5-HT_{1A} and α_2-AR binding assays would confirm arguments emphasizing 5-HT_{1A} receptors (and perhaps α_2-ARs) in the action of ST. More sub-chronic doses of ST and ESC and over longer treatment periods to validate the delayed 5-HT_{1A} receptor desensitization theory, could be considered. The supersensitive 5-HT_{1A} receptor state of the FSL rat prompts pharmacological interrogation in another MDD animal model. Studies testing the comparative and interactive effects of different components of ST, from both a pharmacodynamic and pharmacokinetic point of view, should be considered. Future studies should also focus on other mechanisms of action of ST that has not been investigated in this study

9. Conflicts of interest

HG&H (manufacturers of Zembrin®, provided an unrestricted educational grant towards this study and provided the Zembrin®. HG&H had no further role in this study. Prof Alvaro Viljoen (Tshwane University of Technology) provided funding for this study.

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CHAPTER 4

SUPPLEMANTARY DISCUSSION, CONCLUSION, LIMITATIONS AND RECOMMENDATIONS

4.1. Introduction

This section only comprises of a more detailed explanation of concepts mentioned in Manuscript A (Chapter 3) and the various addenda, or explanations that are not in Manuscript A, or the Addenda. Please refer to the data sets and statistical analyses under Results of Chapter 3, and addenda where noted.

4.2. Supplementary discussion of study findings

4.2.1. Acute studies

Confirmation of FSL rats vs FRL rats vs SD rats

Before implementing the acute dose response studies, the validity of the FSL rats used in this study was confirmed by comparing their behaviour in the OFT and FST to that of the control FRL group and SD group respectively. These data are presented in Addendum A. (*Fig. A1, Addendum A*). After the SD group surprisingly presented with no significant differences in immobility (*Fig. A1.A, Addendum A*) and struggling (*Fig. A1.C, Addendum A*) compared to the FSL rats, FRL rats were tested and selected as the more appropriate control animal for FSL rats and used thereafter throughout the study due to its significantly increased swimming and struggling, and decreased struggling vs the FSL group (*Fig. A1, Addendum A*).

Acute dose response study of ESC

The following is a discussion of the dose-dependent effects of ESC on 5-HT_{1A} and 5-HT_{2A} as mentioned in Manuscript A (6.1.; *FST – Swimming and immobility):*

The acute FST data (*Fig. 2, concept article, chapter 3*) showed that ESC 10 significantly increased swimming behaviour in the FST (*Fig. 2B, concept article, Chapter 3*) compared to the saline group, indicating that ESC 10 is a therapeutic dose due to potent SERT inhibition at higher doses, correlating with previous studies (El Mansari et al., 2005; Murdoch and Keam, 2005). 20 mg/kg (ESC 20) also induced increased swimming and decreased immobility (*Fig. 2A, concept article,*

Chapter 3) like ESC 10, yet to a lesser extent. This further corroborates that ESC 10 appears to give the best antidepressant effects. Higher doses of ESC could induce a faster increase in 5-HT and also activate the 5-HT_{1A} autoreceptor more rapidly, producing lower 5-HT levels which is linked to less emphatic swimming behaviour, compared to ESC 10. This also explains the slight trend towards decreased immobility compared to the saline group, and a slight trend towards increased immobility compared to ESC 10. As expected, ESC 5 served as a low dose of ESC and did not show a significant increase in swimming, probably due to lower levels of extracellular 5-HT after less pronounced SERT inhibition. At this low dose, 5-HT might not have been enough to exert effects on swimming, or the small amount of available synaptic 5-HT could have activated presynaptic 5-HT_{1A} receptors, thus resulting in reduced availability of 5-HT to induce swimming behaviour.

Elaboration on the mechanisms behind the increased NE pertaining to struggling, mentioned in Manuscript A (6.1. *Struggling (FST) and locomotor activity (OFT)*:

In spite of the low swimming behaviour with ESC 5, immobility decreased significantly (*Fig. 2A, concept article, Chapter 3*) which can be attributed to the increased struggling (Fig. 2C, *concept article, Chapter 3*) associated with increased NE. This could be related to the dose-dependent effects of ESC on 5-HT_{1A} and 5-HT_{2A} receptors in the dorsal raphe nuclei (DRN). According to Quesseveur et al. (2013), low doses of ESC preferentially trigger 5-HT_{1A} autoreceptors in the DRN, while higher doses could involve indirect pathways via the 5-HT_{2A} receptor in addition to 5-HT_{1A} in the DRN. 5-HT_{2A} activation is linked to a decrease in NE levels due to activation of 5-HT_{2A} on GABAergic interneurons, thus increasing GABA in the synaptic cleft which in turn inhibits the release of NE form the LC (Guiard and Giovanni, 2015; Quesseveur et al., 2012; Quesseveur et al., 2013). Considering this dose-dependent mechanism of ESC at low doses, the increased struggling at 5 mg/kg (*Fig. 2C, Concept article, Chapter 3*) could suggest that the indirect decrease of NE due to 5-HT_{2A} receptor activation has not yet taken place. Thus, higher levels of NE could increase struggling behaviour as seen with ESC 5. In turn, decreased struggling at the higher doses of 10 and 20 mg/kg ESC could suggest the involvement of the 5-HT_{2A} receptor due to an increase in synaptic 5-HT after potent SERT inhibition, resulting in an indirect decrease in NE and the subsequent decreased struggling compared to ESC 5.

Acute dose response study of ST

A possible indirect effect on monoamine levels which could influence FST and OFT results (not mentioned in the article). 5-HT could be increased indirectly via ST's anti-inflammatory properties by downregulating SERT via inhibition of the p38/MAPK pathway (Haase and Brown, 2015) – *See Chapter 2, Section 2.4.9*, However, this is only a theory and has not been proven yet).

4.4 Sub-chronic study

The importance of the 5-HT$_{1A}$ receptor in the sub-chronic study:

For background detail on the 5-HT$_{1A}$ receptor, please refer to *Chapter 2, Section 2.4.3.2*

The decreased swimming (*Fig. 5A, Concept article, Chapter 3*) behaviour despite the significant increase in 5-HT in the hippocampus during combined ST 50 and ESC 5 treatment could be due to hippocampal 5-HT$_{1A}$ receptor supersensitivity evident in adult FSL rats (Overstreet et al., 1998; Shayit et al., 2003). 5-HT$_{1A}$ receptor agonists are useful antidepressants, while increasing swimming behaviour in the FST following chronic treatment (Detke et al., 1995). In FSL rats, these agonists could cause "5-HT behavioural syndrome" (*Section 2.6.1, Addendum H*) (Haberzettl et al., 2013; Shayit et al., 2003), which includes a hypothermic response. All animals in this study were monitored daily (*see Addendum H for adapted monitoring sheet*) for signs of 5-HT syndrome, however no life-threatening symptoms and signs were evident here. Although not measured in this study, hypothermia usually accompanies an increase in immobility, as seen in the combination group (*Fig. 6A, Concept article, Chapter 3*) and is facilitated by postsynaptic 5-HT$_{1A}$ stimulation (Carhart-Harris and Nutt, 2017; Overstreet et al., 1994; Yadid et al., 2000). These effects are typically seen when a full 5-HT$_{1A}$ agonist, e.g. 8-OH-DPAT, is used to validate the FSL rat based on its supersensitivity reaction (El Yacoubi and Vaugeois, 2007; Overstreet, 2002; Shayit et al., 2003).

Studies reviewed by Borsini (1995) and Ceglia et al. (2004) suggest that SSRIs show very limited increases in 5-HT in the frontal cortex, probably due to the 5-HT-mediated activation of somatodendritic 5-HT$_{1A}$ receptors. This would decrease 5-HT levels and serotonergic effects in the frontal cortex (Ceglia et al., 2004), coinciding with findings in this study (*Fig. 8B, Concept article, Chapter 3*).

Previous studies have found that the hippocampus contains pre- and postsynaptic 5-HT$_{1A}$ receptors rather than just postsynaptic receptors as is the case in the frontal cortex (Berumen et al., 2012; Pasqualetti et al., 1996). There is generally a higher density of 5-HT$_{1A}$ in the hippocampus compared to the frontal cortex. This may account for significantly elevated 5-HT in the hippocampus but not in the frontal cortex described in the current study (*Fig. 8, Concept article, Chapter 3*). The stimulatory effect of the combination treatment could thus have taken place in both the DRN and hippocampus, subsequently increasing 5-HT levels faster and to a greater extent in the hippocampus than in the frontal cortex. The frontal cortex also has a higher density of 5-HT$_{2A}$ receptors than the hippocampus (Carhart-Harris and Nutt, 2017). The NE levels in the frontal cortex (*Fig.9B, Concept article, Chapter 3*) follows the same pattern as the 5-HT which further suggests that these effects are mediated indirectly by 5-HT$_{1A}$, 5-HT$_{2A}$ and α_2-ARs with less effects in the frontal cortex compared to the hippocampus.

Additionally, increased locomotor activity as seen in the OFT (*Fig. 7, Concept article, Chapter 3*) in the combination treatment has also been associated with 5-HT behavioural syndrome, and mediated by postsynaptic 5-HT_{1A} receptors (Haberzettl et al., 2013; Mignon and Wolf, 2002).

Effect of sub-chronic treatment on BDNF: The role of 5-H_{1A}, 5-HT_{2A}, and PDE4

Decreases in monoamines like 5-HT as well as enzymes like PDE4 can cause impaired expression of BDNF (Duman et al., 2000), although ongoing insurmountable stress is most often associated with reductions in BDNF (Belleau et al., 2018; Duman et al., 2000; Saaltink and Vreugdenhil, 2014; Willner et al., 2013). However, antidepressants are known to reverse such changes (Nibuya et al., 1995; Nibuya et al., 1996). In the current study we observed a trend towards increased hippocampal BDNF with ESC 5 treatment, and a significant increase in BDNF in the ST 50 group compared to the saline groups (*Fig. 10A, Concept article, Chapter 3*). However, the combination group showed no such increase.

As previously discussed, low levels of hippocampal 5-HT (*Fig. 8A, Concept article, Chapter 3*) due to delayed 5-HT_{1A} desensitization might not have been sufficient enough to activate 5-HT_{2A} receptors in the alone treatment groups as these receptors have been shown to have a lower affinity for 5-HT than that of 5-HT_{1A} (Barnes and Sharp, 1999). Consequently, 5-HT_{2A} plays a more pronounced role during states characterized by elevated 5-HT, as found in the hippocampus with combination treatment (*Fig. 8A, Concept article, Chapter 3*) (Carhart-Harris and Nutt, 2017). Despite 5-HT_{2A} being considered the major metabotropic 5-HT receptor in the hippocampus, cortex, DRN and LC (Jaggar and Vaidya, 2018) where it evokes mainly excitatory postsynaptic potentials (EPSPs), it is capable of inducing inhibitory postsynaptic potentials (IPSPs) via GABAergic interneurons in the hippocampus in the presence of increased 5-HT (Jaggar and Vaidya, 2018). In the combination group, the aforementioned increase in hippocampal 5-HT (Fig. 8A *Concept article, Chapter 3*) could, due to the hypersensitive reaction to 5-HT_{1A} receptors, have caused increased activation of the IPSP of 5-HT_{2A} in the hippocampus leading to an increase in GABAergic-mediated suppression of BDNF expression (*Fig. 10A, Concept article, Chapter 3*). Considering the earlier mentioned delayed desensitization of 5-HT_{1A} in the alone treatment groups, the significant increase in BDNF in the alone ST 50 group and the trend towards increased BDNF in the alone ESC 5 group, may follow increased BDNF-induced neuroplasticity and neurogenesis (Polter and Li, 2010; Willner et al., 2013). This action may be attributed to the stimulatory effects of 5-HT_{1A} on the MAPK/ERK pathway (Polter and Li, 2010; Willner et al., 2013). These effects are most probably supressed by 5-HT_{2A} in the combination treatment group, which was associated with high 5-HT levels thus inhibiting the release of BDNF.

As ST 50 showed similar effects to ESC 5 throughout this study, it was expected to have the same effects on hippocampal BDNF as ESC 5 due to overlapping serotonergic mechanisms of

action. However this is not the case as ST 50 alone significantly increased hippocampal BDNF (*Fig 10, Concept article, Chapter 3*). PDE4 hydrolyses and therefore decreases cAMP, consequently impairing the cAMP-CREB pathway and decreasing BDNF expression (Duman et al., 2000) (*also refer to Chapter 2; Section 2.4.8*.). In addition to its serotonergic effects, ST also inhibits PDE4 (Harvey et al., 2011), and action that may underlie the observed increments in BDNF following ST 50. Since memory processes are dependent on neurogenesis and neuroplasticity, the PDE4 inhibitor actions of ST would be expected to benefit certain cognitive processes, such as that assessed in the novel object recognition test (nORT) (see Addendum C). However, this study failed to find any significant differences between groups in the nORT, thus rendering these results inconclusive. This was most probably due to problems with the methodology as described in Addendum C. This was thus omitted from the Concept article (Manuscript A, Chapter 3).

Confusing is why the combination treatment did not show increased BDNF, considering the bolstering actions of ST 50 alone. ESC alone tended to do the same. Perhaps the opposing action for ST 50 can be attributed to more dominant effects of the 5-HT_{2A} receptor that overshadowed any effects of PDE4 inhibition. Alternatively, simultaneous positive effects of PDE4 inhibition and negative effects of 5-HT_{2A} on BDNF could drive a nett effect similar to that of ESC 5 alone, viz. unchanged hippocampal BDNF levels. Clearly further investigation is warranted.

On the other hand, BDNF levels in the frontal cortex (*Fig. 10B, Concept article, Chapter 3*) showed a reduction with the ESC 5 alone group *and* the combination group. This might be attributed to similar reasons as for the hippocampal BDNF in the combination treatment, and the lack of PDE4 inhibition by ESC. Furthermore it may be attributed to the dominant effect of 5-HT_{2A} in the frontal cortex which is inhibitory on BDNF under circumstances of high 5-HT activity. More likely is that the low levels of 5-HT in the frontal cortex might be directly correlated to decreased BDNF due to less activation of the cAMP/CREB pathway. This idea also warrants further investigation. All things considered, the increase in BDNF by ST 50 could be attributed to its PDE4 inhibition. Thus, ST shows promise to improve the deficits in learning and memory in MDD and other neuropsychiatric disorders characterized by reduced BDNF (Cattaneo et al., 2016; Duman et al., 2000).

Finally, it is worth noting that Yanpallewar et al. (2010) found that inhibition or desensitization of α_2-ARs after prolonged treatment with noradrenergic drugs like desipramine (Invernizzi and Garattini, 2004) can increase BDNF. This may take place through indirect mechanisms such as increased NE binding to postsynaptic adrenergic receptors and subsequent activation of the cAMP/CREB pathway (Willner et al., 2013). Alternatively, it may do so directly via inhibiting G protein-mediated inhibition of adenylate cyclase which inactivates of the cAMP/CREB pathway, thus increasing activity of this pathway. Indeed, the ST-associated trend towards increased NE

via increased VMAT-2 and NET inhibition may underlie its ability to increase hippocampal BDNF (*Fig. 10A, Concept article, Chapter 3*).

4.5. Summary of observations

Zembrin fingerprint profile

In summary, the findings of the Zembrin® fingerprint profile compared to the expected results as provided in the certificate of analysis (COA) were conclusive (Addendum G).
The following summary (Table 4.1) consists of the expected results regarding alkaloid content according to the COA (written fist) compared to found in the fingerprint analysis (written in *italics*)

Table 4.1. Summary of the expected results (COA) vs fingerprint analysis results for the alkaloid content of the Zembrin used in this study

Expected results (COA)	**Fingerprint results**
Mesembrenone > mesembrenol > mesembrine > mesembrenol.	Concurs
Total alkaloid content of sample: 0.42% Total alkaloid content specifications of Zembrin®: ≥ 0.35% - ≤ 0.45%	0.38 (not the same), however still within range

In conclusion, the fingerprint analysis confirmed the information provided in the COA.

Total alkaloid content of 0.38% was quantified in the fingerprint analysis. Although the latter was quantified as 0.42%, the quantities obtained in the fingerprint profile still falls between the specifications of ≥ 0.35% and ≤ 0.45%.

Animal experiments

Table 4.2. and 4.3. provides a summary of the objectives, expected outcomes, findings, and conclusions of this study

Acute studies ***(Table 4.2)***

- FSL rats showed depressive-like behaviour in the FST, evinced as decreased swimming and struggling, and increased immobility compared to control FRL rats. However, in the OFT, FSL rats showed higher levels of locomotion than FRL rats. Furthermore, the FRL rat rather than the SD rat proved to be a better control for FSL rat studies based on the lack of significant differences in immobility between the FSL and SD groups.
- In the acute dose response study, ST showed dose-dependent antidepressant-like effects, with ST 50 mg/kg prevailing as the most effective dose. Evidence for this was its decreased

immobility, increased struggling and trends toward increased swimming in the FST, in addition to increased locomotion in the OFT.

- In the acute dose response study, ESC 5 mg/kg presented with the best profile as a low dose of ESC. Evidence for this was its increased struggling and decreased immobility in the FST indicative of antidepressant-like behaviour, this despite low swimming activity. The other tested doses were also conclusive an important as these indicated dose-dependent antidepressant effects, while simultaneously confirming the predictive validity of the FSL rat.
- Sub-chronically, the ST 50 mg/kg group showed a similar behavioural response to that of ESC 5 mg/kg in the FST and OFT, although neither treatment was effective in any of these behavioural parameters vs the saline control group.

Table 4.2. Summary of each objective, its expected outcomes, findings and conclusion based on observations during the acute studies. *↓ decrease; ↑ increase; (↑) (↓) trend towards increase or decrease; ↔ no differences; * statistical significance; E = Expected outcomes; F = Findings; AD = antidepressant.*

#	OBJECTIVE	EXPECTED OUTCOME	SWIMMING		STRUGGLING		IMMOBILITY		LOCOMOTION		CONCLUSION
			E	F	E	F	E	F	E	F	
1	Validation of FSL model	FSL will show depressive-like behaviour (vs. FRL)	↓	↓ **	↓	↓ ***	↑	↑ ****	↓	↑ **	FSL showed depressive-like behaviour in the FST vs. FRLs, but not vs. SD SDs should preferably not be used as FSL controls based on lack of differences in immobility and struggling
		FRL will not show depressive-like behaviour (vs FSL)	↑	↑ **	↑	↑ ***	↓	↓ ****	↑	↓ **	
		SD will not show depressive-like behaviour (vs FSL)	↑	↑ **	↑	↔	↓	↔	↑	↔	
2	Determine AD dose of ST	Will be able to established an AD dose of ST (vs. SAL)	↑	↔ ST 50	↑	↑ * ST 50	↓	↓ ** ST 50	↑	↔ ST 50	**ST 50** showed the best antidepressant-like effects of all tested doses
3	Determine low dose ESC	Will be able to establish low dose of ESC (vs SAL)	**(↑) Or ↔**	↔ ESC 5	**(↑) Or ↔**	↔ ESC 5	**(↓) Or ↔**	↓ ** ESC 5	**(↑) Or ↔**	↑ ** ESC 5	**ESC 5** was chosen as low dose based on antidepressant-like effects on immobility and locomotion. No effects on swimming (linked with 5-HT), thus indicates safe dose for combination with ST without inducing 5-HT syndrome

*Sub-chronic study (**Table 4.3**)*

- ST 50 showed similar neurochemical effects to that of ESC 5 mg/kg. ST 50 mg/kg groups showed no significant differences in hippocampal and frontal cortical 5-HT or NE levels compared to the ESC 5 mg/kg group. Both groups also showed no significant differences compared to the saline control group. This further indicates that ST, at this dose, either shows no antidepressant-like effects in a sub-chronic setting, or that the treatment period was not long enough.
- ST 50 mg/kg trended to increase hippocampal BDNF vs ESC 5 mg/kg, but was significantly increased compared to saline controls. ST 50 mg/kg may have value in the treatment of disorders presenting with cognitive deficits, such as MDD. However, ST 50 failed to alter frontal cortical BDNF vs saline treated groups. Both ESC 5 mg/kg and combination group significantly reduced BDNF compared to the saline and ST 50 groups.
- ST 50 and ESC 5 combination significantly increased hippocampal 5-HT and NE vs. alone treatment groups and saline-treated groups, indicating that ST 50 mg/kg does present with 5-HT'ergic and NA'ergic properties, and that it is able to bolster the serotonergic and noradrenergic effects of ESC 5 mg/kg. The latter suggests promise as augmentative treatment for MDD.
- Combined ST 50 plus ESC 5 significantly decreased swimming and struggling and increased immobility in the FST, indicating possible depressogenic effects. However, increased locomotion in the FST indicates antidepressant effects.
- The bio-behavioural data of the combination group suggests evidence of induced 5-HT behavioural syndrome despite using a low dose of ESC in the combination. However, no frank toxicity or fatalities were evident, this supported by our routine vivarium monitoring sheets (see Addendum H).
- DA data further indicates interactions between the receptors focused on in this study (5-HT_{1A}, 5-HT_{2A} and α2-ARs) as discussed in Addendum DA, with alone treatment groups indicating medium trends toward increased DA in the frontal cortex indicating slight antidepressant effects, while levels were low in the combination treatment group. Hippocampal DA levels were below level of detection and intergroup comparisons were thus not possible.
- All nORT data showed no significant differences and was thus inconclusive. Data was omitted for the purpose of Manuscript A, but can be seen in Addendum B

Table 4.3. ***Summary of each objective, its expected outcomes, findings, and conclusion based on observations during sub-chronic study (as discussed in the Concept article (Manuscript A, Chapter 3)***. *↓ decrease; ↑ increase; ↔ no differences; * statistical significance; E = Expected outcomes; F = Findings; AD = antidepressant; FC = frontal cortex; HC = hippocampus*

#	OBJECTIVE	EXPECTED OUTCOME	SWIM.		STRUG.		IMMOB.		LOCO-MOTION		5-HT			NE			BDNF			CONCLUSION
			E	F	E	F	E	F	E	F	E	F HC	F FC	E	F HC	F FC	E	F HC	F FC	
5	AD-like effects of ST 50 vs ESC 5 alone (OFT + FST)	ST 50 will show similar or even more effective AD-like effects vs ESC 5 **ST 50 vs SAL**	↑	↔	↑	↔	↓	↔	↑	↔	-	-	-	-	-	-	-	-	-	Sub-chronically, ST 50 does not appear to be antidepressant in the FST despite its trend toward increased swimming and increased mobility in the OFT ST 50 shows similar effects to ESC 5 (low dose) sub-chronically
		ST 50 vs ESC 5	↑ Or ↔	↔	↑ Or ↔	↔	↓ Or ↔	↔	↑ Or ↔	↔	-	-	-	-	-	-	-	-	-	
6	AD-like effects of ST 50 vs ESC 5 alone (5-HT + NE + BDNF)	ST 50 will show similar or even more effective AD-like effects vs ESC 5 **ST 50 vs SAL**	-	-	-	-	-	-	-	-	↑	↔	↔	↑	↔	↔	↑	↑ *	↔	Sub-chronically, ST 50 does not appear to be antidepressant due to no significant differences in 5-HT and NE ST 50 shows similar effects to ESC 5 (low dose) sub-chronically with no significant differences in 5-HT or NE
		ST 50 vs ESC 5	-	-	-	-	-	-	-	-	↑ Or ↔	↔	↔	↑ Or ↔	↔	↔	↑ Or ↔	↔	↑ *	
7	AD-like effects of combination vs alone treatments	Combination will show better AD-like effects than ST and ESC alone **ESC+ST vs SAL**	↑	↔	↑	↔	↓	↑ *	↑	↑ *	↑	↑ **	↔	↑	↑ *	↔	↑	↔	↓ **	ESC+ST appears to be depressogenic in the FST (compared to SAL and alone treatments). However, it also appears to be antidepressant in the OFT and neurochemistry, with significantly increased 5-HT and NE in the HC. Here, ESC+ST shows superior AD-like effects vs alone treatments.
		ESC+ST vs ESC 5	↑	↓ *	↑	↓ *	↓	↑ ***	↑	↔	↑	↑ ****	↔	↑	↔	↔	↑	↔	↔	
		ESC+ST vs ST 50	↑	↔	↑	↔	↓	↑ *	↑	↔	↑	↑ ***	↔	↑	↔	↔	↑	↔	↓ *	
8	ESC+ST: 5-HT syndrome?	Low dose ESC will prevent SS	↑	↔	↑	↔	↓	↑ *	↑	↑ *	↑	↑ **	↔	↑	↑ *	↔	↑	↔	↓ *	Indications of possible 5-HT behavioural syndrome. However, daily monitoring using adapted monitoring sheets showed no signs of toxicity based on the included criteria

4.6. Conclusion

The behavioural test results from the acute treatment study indicate that ST has definite dose-dependent antidepressant-like effects. However, the sub-chronic bio-behavioural results demonstrated often opposite and/or paradoxical findings. This disparity very likely has its origin in the complex pharmacology presented by the ST preparation, and possibly the conditions of study including the unique neurobiology of the FSL rat. Although ST has not been proven to have agonist effects on 5-HT_{1A} receptors, the current data suggests that ST does indeed bolster the serotonergic and noradrenergic effects of low dose ESC, at least in this model. This provides potential for its use as augmentative treatment. However, due to the novelty of ST and limited information on its pharmacological and pharmacokinetic profile, we are unable to pinpoint which of the many mechanisms of action of ST are ultimately responsible for the apparent antidepressant effects. On top of that, *in vitro* profiling of ST might not accurately represent the effects seen *in vivo* which could be affected by many different biological systems. It would thus be valuable to do more neurochemical analyses, additional behavioural tests, and multiple chronic dose response studies in the future. We postulate that the bolstered serotonergic response is possibly produced by 5-HT_{1A} agonist-like effects similar to that induced by full 5-HT_{1A} agonists in the FSL rat, which resembles a 5-HT behavioural syndrome. Despite this, the alone treatment with ST 50 suggests comparable bio-behavioural effects versus low dose ESC, but with better potential in the treatment of cognitive dysfunction due to its positive effects on BDNF. Although the NORT proved unequivocal, ST's effects on BDNF may be associated with its PDE4 inhibitory effects. Nevertheless, this study has demonstrated definite antidepressant (acute treatment paradigm), serotonergic, noradrenergic and pro-BDNF effects for ST which could hold promise for the treatment of MDD. However, the seemingly depressogenic effects of the combination treatment could simply indicate that ST should not be used as augmentative therapy – at least not in combination with other serotonergic agents.

4.7. Limitations and future recommendations

Here, the limitations are listed first, with the recommendation following in italics:

- The dose response of ST was determined using acute treatment only; a similar design was <u>not</u> employed in the sub-chronic treatment study (for reasons of cost and time)
    - *A sub-chronic dose response study of ST would possibly have been more informative from a dose exploration point of view, especially regarding antidepressant effects in the FST*

- Response to a full therapeutic dose of ESC was not employed in the sub-chronic study for reasons of cost and time.
    - *This would be useful in order to compare ST a full therapeutic dose of ESC following sub-chronic treatment.*
- No FRL control groups was included in the sub-chronic treatment study
    - *An FRL control should be included in the sub-chronic treatment study to establish basal reference FRL values for all biological and behavioural data, and for comparison with treatment-naïve and drug treated FSL rats.*
- No receptor binding assays were included in the current study (for reasons of cost and time)
    - *Cortical-hippocampal 5-HT$_{1A}$, 5-HT$_{2A}$ and α2-AR receptor binding studies would be invaluable in the interpretation of both behavioural and neurochemical results and should be considered in future studies.*
- The nORT did not deliver any meaningful data
    - *These data highlight an urgent need to standardise and validate nORT methodology in our laboratory.*
- Treatment interventions were conducted over 15 days only
    - *Although 15 day treatment is a widely accepted treatment duration in animal studies relating to antidepressant-like activity, chronic treatment periods of 30 days or more could have allowed enough drug exposure time to evaluate the role of delayed 5-HT$_{1A}$ desensitization.*
- This study employed the FSL rat model of MDD. Although translational relevance to human MDD is good, the results obtained may not be immediately transferrable to another animal model of MDD. Our theory is based on this model's unique neurochemical profile. Consequently, the ST 50+ESC 5 combination treatment that induced 5-HT behavioural syndrome in FSL rats may be due to the presence of 5-HT$_{1A}$ hypersensitivity in this model.
    - *Use alternative rodent models of MDD, e.g. chronic mild stress in SD rats, in order to determine if these effects are model-dependent.*
    - *Measure body temperature of FSLs could help indicate if the results were indeed due to 5-HT$_{1A}$ super sensitivity of the FSL rat.*
    - *The above-noted 5-HT$_{1A}$ and 5-HT$_{2A}$ receptor binding studies may also provide clarity on this*
- Rats in the ST groups showed more struggling and aversive behaviour during dosing which could be attributed to the pungent taste and/or smell of the ST solution.
    - *For future reference, consider rinsing the oral gavage needle in saline (as done in this study) or sucrose solution right before administration to mask the taste. Also*

do not leave the solution open for long periods in order to limit permeation of the odour.

- The evaluation of only one extract of ST was done in the current study. Previous ST studies (Gericke et al., 2015; Gericke and Viljoen, 2008; Harvey et al., 2011; Loria et al., 2014) show that variance in types of preparations used, e.g. standardized extracts vs isolated components, can produce varying results due to different pharmacological effects of the individual components in these preparations. Extracts can vary according to geographical and climatological factors, extraction, purification as well as harvesting techniques, which need to be factored into the analysis.
    - *Repeat this study with different isolated alkaloid components in order to compare their antidepressant-like effects to that of the Zembrin extract. This could also identify any opposing effects of components that could mask the antidepressant effects of other components, or induce toxic interactions in combination with other drugs.*
- The availability of pharmacokinetic profiles of ST is currently lacking
    - *Pharmacokinetic studies will be valuable to predict the risk of drug interactions and to establish dosing schedules.*
- No inflammatory markers or PDE4 were measured
    - *Considering the pro-inflammatory effects of PDE4 (**Section 2.4.7),** and the putative inhibitory actions of ST on this enzyme, it would be valuable to measure markers like cytokines or lymphocytes, as well as PDE4 expression and activity, in brain and blood to further evaluate the PDE4 inhibitory and anti-inflammatory properties of ST.*
- Many other mechanisms of ST could have influenced the results in ways we have not considered in this study
    - *Explore other mechanisms of action, e.g. inflammation, in future studies to gain a better holistic perspective of ST as an antidepressant when given as monotherapy or augmentative therapy.*
- No monoamine or BDNF data were collected in a non-depressive control group, as the model was validated using behavioural data only. This complicated the interpretation of the magnitude of deviation from normal in the FSL rats, and would have enabled more context for the therapeutic changes reported.
    - *Include a non-depressive control group (FRL) which can be used to determine a baseline of neurochemical markers like monoamines and BDNF*

- Combination treatment of ST with ESC (an SSRI) indicated the possibility of induced serotonin syndrome.
    - *Future studies should include a combination of ST and a non-serotonergic agent*

4.8. Final concluding statement

Despite the identified limitations, the study objectives were achieved and the study has contributed significant knowledge on the neuropharmacology of ST in an animal model of MDD, thus laying the foundation for future studies exploring this plant and its pharmacological properties further in additional *in vivo* models of MDD.

4.9. References

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ADDENDUM A
CONFIRMATION OF THE FSL MODEL OF DEPRESSION

A.1. Introduction

As discussed in Chapter 2 (Section 2.6.), the Flinders Sensitive Line (FSL) rat has been established as a valid animal model of major depressive disorder (MDD) (Overstreet et al., 2005; Overstreet and Wegener, 2013). FSLs are genetically inbred from SD rats, originally in an attempt to produce a rat strain resistant to diisopropyl fluorophosphate (DFP), an anticholinesterase agent. However, these animals were found to be *sensitive* to DFP while the Flinders Resistant Line (FRL) rat proved resistant to cholinergic agonists (Overstreet et al., 2005) (see Section 2.6 for further discussion). FSL rats were later found to display behavioural manifestations akin to MDD while the FRL strain did not (Overstreet et al., 2005; Overstreet and Wegener, 2013).

In order to confirm that FSL rats used in this study did indeed show depressive-like behavioural manifestations, we compared the behaviour of FSL rats in the open field test (OFT) and forced swim test (FST) to control groups (Neumann et al., 2011). Firstly, Sprague Dawley (SD) rats were used as control as they are deemed the original "healthy" rat strain from which FSLs were developed. In addition, we also compared FSL rats to their in-bred control, the FRL rat. As we will present here, FSL rats did not separate adequately from SD rats with respect to their depressive phenotype, which were unexpected results. However, comparison to FRL rats confirmed their depressive phenotype. Here we present these findings.

In this study 12 FSL and 12 SD rats were used. Since FRL rats are widely used as controls for FSL rats (Overstreet et al., 2005; Overstreet and Wegener, 2013), we opted to only use 6 animals of this strain in order to conserve animal numbers. Furthermore, considering our earlier work with FRL control rats (Brand and Harvey, 2017; Hamman et al., 2015; Steyn et al., 2018), 6 FRL rats would still be enough to produce robust data.

As discussed in Chapter 2 (Section 2.7) the FST is a test of behavioural despair and learned helplessness (Bogdanova et al., 2013). This test is widely used as a measure of depressive-like behaviour which is characterized by increased floating/immobility and decreased swimming and struggling behaviour (Bogdanova et al., 2013; Cryan et al., 2005). The OFT is used to measure locomotor activity and can also be used to measure anxiety (Brenes Sáenz et al., 2006; Essman, 1968; Tejani-Butt et al., 2003) (See section 2.7.2; Chapter 2). It can also be used to determine false positive or false negative results in the FST. In this study, we expected to see decreased swimming and struggling, and increased immobility in the FST in

FSL rats versus FRL or SD control rats (Neumann et al., 2011). Furthermore we expected to see decreased locomotor activity (decreased distance travelled) in the OFT (Neumann et al., 2011). Both SD and FRL rat were expected to display the opposite behaviours in the FST and OFT, indicating no depressive-like behaviour.

All FST and OFT procedures were done according to the methods as explained in Addendum OFT FST, Manuscript A (concept article, Chapter 3), and Chapter 1.

A.2. Results

A.2.1. Forced swim test (FST)

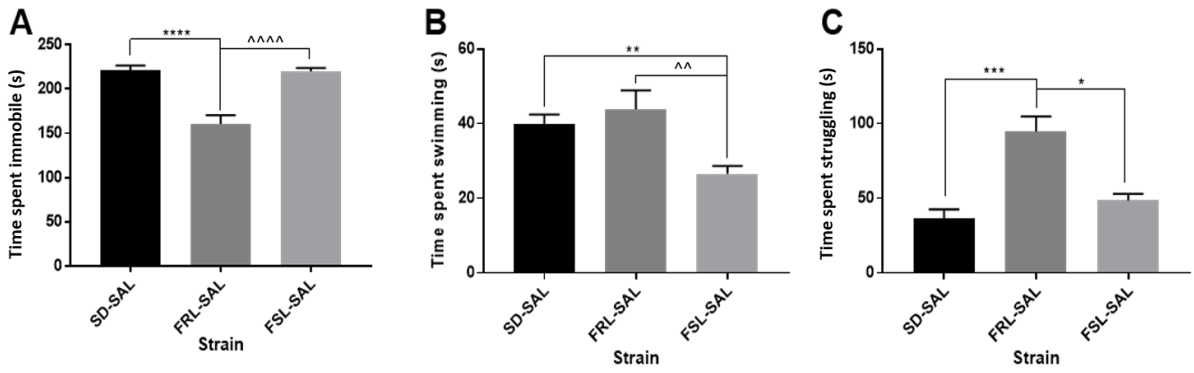

Fig. A.1. Comparison of behaviour in the FST between the control SD (n = 12) and FRL (n = 6) groups, and the FSL (n = 12) group. Parameters measured include time (s) spent A) immobile, B) swimming, and C) struggling. All animals were treated with saline according to the acute study design as explained in **Chapter 1.** Data are expressed as (means [95% CI]). $p < 0.05$ deemed significant. A) ****$p < 0.001$, ^^^^$p < 0.005$; B) **$p < 0.008$, ^^$p < 0.007$; C) ***$p < 0.001$, *$p < 0.044$

*Immobility (**Fig. A.1.A):*** Not all data sets were normally distributed. The Kruskal-Wallis test indicated significant differences between groups ($x^2(2) = 13.4$, $p = 0.001$). The FSL group (220 s [213 – 228]) unexpectedly showed no significant differences in immobility compared to the SD group (221 s [208 – 233]), but showed significantly increased immobility compared to the FRL group (161 s [135 - 186]; $p = 0.005$). FRL rats presented with significantly decreased immobility compared to SD rats ($p = 0.001$)

*Swimming (**Fig. A.1.B**):* Not all data sets were normally distributed. The Kruskal-Wallis test indicated significant differences between groups ($x^2(2) = 13$, $p = 0.002$). The FSL group (26.6 s [22.2 - 31]) showed significantly decreased swimming vs the SD group (39.7 s [33.7 – 45.7]; $p = 0.008$) and the FRL group (43.8 s [30.9 – 56.8]; $p = 0.007$).

*Struggling (**Fig. A.1.C**):* Not all data sets were normally distributed. The Kruskal-Wallis test indicated significant differences between groups ($x^2(2) = 13.1$, $p = 0.001$). The FSL group (48.5 s [38.7 – 58.3]) unexpectedly showed the same degree of struggling vs the SD group (36.4 s [22.8 – 50]), however the FSL group presented with significantly decreased struggling compared to the FRL group (94.7 s [68.7 – 121]; $p = 0.044$). Furthermore, FRL rats unexpectedly showed significantly increased struggling vs the SD group ($p = 0.001$).

A.2.2. Open field test (OFT)

All data sets were normally distributed. An one-way ANOVA indicated significant differences between groups ($F(2.3) = 15.9$; $p < 0.0001$). The FSL group (3247 s [2921-3573]) unexpectedly showed significantly increased distance travelled compared to the FRL group (2283 s [1741-2824]; p = 0.002), however FSL rats unexpectedly showed the same degree of locomotor activity as the SD group (3714 s [3399-4029]). Surprisingly, the FRL group revealed significantly decreased distance travelled compared to the SD group ($p < 0.0001$).

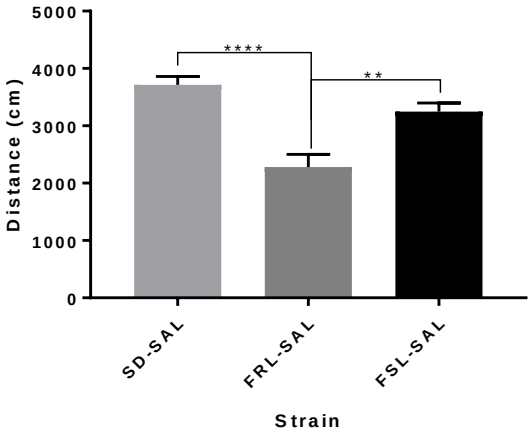

Fig. A.2. Comparison of the total distance travelled (cm) in the OFT between the control SD (n = 12) and FRL (n = 6) groups, and the FSL (n = 12) group. All animals were treated with saline according to the acute study design as explained in **Chapter 1.** Data are expressed as (means [95% CI]). $p < 0.05$ deemed significant. ****$p < 0.0001$, **$p < 0.002$

A.3. Discussion

The data obtained from the FST was interesting. While the FRL data fitted the profile of a "non-depressed" animal, i.e. increased swimming and struggling and decreased immobility, SD rats unexpectedly presented with decreased struggling (Fig. A.1.C) and increased

immobility (Fig. A.1.A), while simultaneously showing an expected increase in swimming behaviour (Fig. A.1.B).

As decreased struggling and increased immobility are indicators of depressive-like behaviour (Bogdanova et al., 2013), the current data suggests that the SD rats showed depressive-like behaviour comparable to that seen in the FSL rats. Surprisingly, despite the high immobility in the SD rats, increased swimming versus FSL rats does fit the profile of a "non-depressed" rat. Furthermore, SD rats showed no significant difference in locomotor activity in the OFT (Fig. A.2) compared to the FSLs, which further indicates depressive-like behaviour in the SD rats. The reasons for this are unknown and should be further investigated. Based on the inconsistencies in the data, it was not possible to discern whether the SD rats were the most appropriate control group to use for FSL rats, despite it being the original "healthy" version of an FSL (Overstreet et al., 2005; Overstreet and Wegener, 2013).

Importantly, since FSL rats did not separate from SD rats with respect to their depressive phenotype, either the FSL is no longer displaying depressive like characteristics, or the SD rat cannot be deemed a suitable control. In order to gain clarity on these critical issues, we found that FSL rats did indeed show significantly decreased struggling (Fig. A.1.C) and increased immobility (Fig. A.1.A) compared to FRL rats, while FSL and SD groups were similar in this regard. Also FSL rats showed significantly decreased swimming (Fig. A.1.B) vs the FRL group, with no significant differences compared to the SD group. This confirms the depressive phenotype of FSL rats versus FRL rats, and also indicates that SD rats are a less effective control group for FSLs, at least in the current study.

However, FRL rats unexpectedly showed decreased locomotor activity in the OFT compared to both the FSL and FRL groups, a trait that is normally associated with depressive-like behaviour as it resembles psychomotor retardation as seen in MDD patients (Dean and Keshavan, 2017; Jesulola et al., 2018). This could however be explained by significantly upregulated of α_2-ARs in FRL rats compared to both SDs and FSLs (Lillethorup et al., 2015). These autoreceptors inhibits the release of NE which could underlie the decreased levels of locomotor activity as seen in this data, as increased hippocampal NE can increase locomotion (Chen and Reith, 1995)

A.4. Conclusion and future recommendation

Based on the current data, we were unable to use SD rats as control for FSL rats in this study, and conclude that the FRL rat is a more appropriate control animal for FSL rats, at least under the current conditions of study. This matter should be further investigated in the future.

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ADDENDUM B
DOPAMINE RESULTS

B.1. Introduction

Dopamine (DA) is another monoamine that plays an important role in major depressive disorder (MDD) via its role in cognition, emotion regulation and motoric behaviour (Grace, 2016). Decreased DA can give rise to symptoms like anhedonia, decreased motivation, impaired cognition and memory, and despair among others (Ayano, 2016; Dean and Keshavan, 2017; Jesulola et al., 2018; Panksepp and Watt, 2011). Please see Chapter 2 (Section 2.4.2)

For reasons noted above, DA was quantified along with 5-HT and NE in this study via the HPLC method described in Addendum E.

B.2. Results

Although DA levels were measured in both the hippocampus and frontal cortex via HPLC, the majority of the hippocampal DA levels of the treatment groups were below limit of detection. These samples cannot be compared to each other accurately and was thus excluded from Manuscript A and this addendum. However, hippocampal levels will be regarded as low.

*Frontal cortex DA (**Fig. B.1.**)*: All data sets did not show normal distribution. The Kruskal-Wallis test did not show significant differences between groups ($x^2(3) = 1.27$; $p = 0.7368$). However, medium effects sizes were found between the saline group (105 ng/g [49.5 – 161]) and the ESC 5 group (243 ng/g [50.8 – 435]; $p > 0.9999$; $d = 0.6204$), and the ST 50 group (347 ng/g [12.6 – 682]; $p > 0.9999$; $d = 0.6417$).

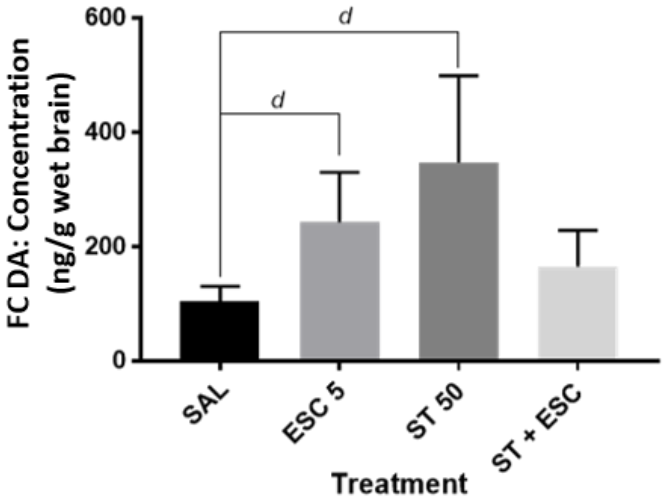

Fig. B.1. Tissue DA levels (ng/g) in the various treatment groups as indicated vs. saline-treated control animals (n = 12 per group) in the frontal cortex. Data expressed as (means [95% CI]). $p < 0.05$ deemed significant.

B.3. Discussion

It is well known that DA levels are low in the hippocampus (Borgkvist et al., 2012). Indeed, the majority of hippocampal DA levels for the alone and combination treatment groups were below limit of detection and thus conducting inter-group comparisons were impossible. Levels in the frontal cortex were above the limit of detection and thus these groups could be compared.

The hippocampus is impaired in MDD, probably as result of ongoing psychosocial stress (Willner et al., 2013). Attenuated DA levels, as seen in MDD, could be due to this hippocampal dysfunction. The hippocampus normally exerts indirect excitatory effects on the ventral tegmental area (VTA) of the midbrain to increase DA (Cooper et al., 2006). This mechanism can be impaired in MDD and could thus lead to decreased DA levels (Cooper et al., 2006; Willner et al., 2013).

Additionally, 5-HT has inhibitory effects on the release of DA from the nucleus accumbens and VTA (Daw et al., 2002; Millan et al., 2000). The significant increase in hippocampal 5-HT following combined ESC+ST treatment (see Fig. 8. Manuscript A, Chapter 3) could have had an inhibitory effect on the release of DA through stimulation of 5-HT_{2C} located in the VTA (Millan et al., 2000). Whether ESC or ST alone had less significant effects on DA in the hippocampus at the doses tested should be further investigated.

There is an increased density of 5-HT_{2A} receptors in the frontal cortex compared to the hippocampus (Carhart-Harris and Nutt, 2017; Willner et al., 2013). This receptor is excitatory and results in an increase in DA levels in the frontal cortex, as is apparent in this study after treatment with ESC or ST alone (medium effect size responses), but not after combined treatment (Fig.B.1.B). This also links with our argument in Manuscript A where we suggest that lower levels of 5-HT in the frontal cortex were further attenuated by increased 5-HT_{2A} activity, probably linked to the SSRI actions of these two drugs separately. Also, the medium effect size increase in DA with ST 50 alone may also be attributed to its ability to upregulate VMAT-2 and mild inhibition of MAO-A (Coetzee et al., 2016). However, the lack of a similar response following combined ESC+ST treatment is intriguing and warrants further investigation.

Overtly reduced hippocampal DA levels in FSL rats may follow the presence of hypersensitive 5-HT_{1A} receptors (Overstreet et al., 2005; Yadid et al., 2000) and upregulated α_2-ARs (Lillethorup et al., 2015). According to Millan et al. (2000), increased α_2-AR activity can decrease DA, 5-HT, NE and glutamate in corticolimbic structures, including the frontal cortex and hippocampus. Increased release of NE via 5-HT_{1A} stimulation (Hajós-Korcsok and Sharp, 1996) (the proposed dominant effect during the sub-chronic alone treatment before desensitization of 5-HT_{1A} (Manuscript A)) could allow for increased stimulation of inhibitory α_2-ARs, consequently causing the decreased levels of DA (Millan et al., 2000). This effect could be dose-dependent. The inhibitory effects of α_2-ARs are dominant when NE levels are lower, while stimulatory α_1-ARs play a more pronounced role when NE levels are higher (Maletic et al., 2017; Ramos and Arnsten, 2007). Thus, even small amounts of NE can bind to α_2-AR and cause diminished DA in the hippocampus and frontal cortex. As discussed in Manuscript A, the bio-behavioural data suggests that ST 50 (chosen as a therapeutic dose in the acute dose response study) showed similar effects to that of a low dose of ESC in a sub-chronic setting. The lower doses of these drugs may allow for smaller increases in 5-HT which can increase NE via the aforementioned mechanisms. Thus, due to the low doses of ST 50 and ESC 5, stimulation of α_2-ARs could result in decreased DA.

In conclusion, the data throughout this study could mostly be attributed to specific effects of 5-HT_{1A}, 5-HT_{2A} and α_2-AR receptors on monoamine levels, with further regulatory feedback mechanisms playing a role in various systems. The same is most probably true for the DA levels. However these mechanisms must be further investigated in future studies in order to more accurately link these results to the mechanisms of action of ST.

Considering the lack of significant differences or large effect size changes in DA levels in the frontal cortex, as well as the lack of measurable levels in the hippocampus, the DA data were

excluded from Manuscript A. However, since the behavioural changes measured in the FST are more immediately connected to NA'ergic and 5-HT'ergic effects, adding DA into the discussion was deemed superfluous and counterproductive. Indeed, a more appropriate behavioural correlate for DA activity would be the sucrose preference test, which is a measure of reward and where DA is known to play a more dominant role in MDD (Mathews et al., 2012).

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ADDENDUM C

NOVEL OBJECT RECOGNITION TEST

The novel object recognition test (nORT) showed no useful results and was thus not included in the concept article (Manuscript A).

C.1. Introduction

As previously discussed in detail in Chapter 2 (Section 2.7.3), the novel object recognition test (nORT) is a behavioural test used to measure visual learning and declarative memory in rodents (Mokoena et al., 2015; Oberholzer et al., 2018). According to the DSM-V, these are some of the cognitive processes that are impaired in MDD. Thus exploring drugs that can restore these cognitive functions could be invaluable in the treatment of MDD.

Memories of facts, places or events are termed declarative memory and is dependent on proper functioning of the hippocampus. This is due to its positive effects on organization and flexible application of stored information (Hammond et al., 2004). As the hippocampus and cortical regions are implicated in the pathophysiology of MDD (Hammond et al., 2004), we opted to measure behaviours dependent on these brain areas in order to evaluate the potential antidepressant effects of ST. This is specifically relevant considering that one of the putative biological actions of ST is PDE4 inhibition (Harvey et al., 2011). As discussed extensively in Section 2.4.7. PDE4 inhibits the formation of cAMP, thus inactivating the pathway responsible for BDNF production, with a resulting negative effect on neuroplasticity and neurogenesis (Duman et al., 2000), and learning and memory (Dean and Keshavan, 2017). Thus, the inhibitory effects of ST on PDE4 would increase BDNF production, consequently improving impaired cognition and memory, and should thus be able to show increased exploration of novel objects in the nORT.

Furthermore, other systems like NE, 5-HT, and receptors like 5-HT_{1A} also have pro-cognitive effects (Meltzer and Sumiyoshi, 2008; Ramos and Arnsten, 2007) which should reflect in the nORT. Also, due to its effects on these systems as discussed in detail in Manuscript A and Chapter 4, ST as well as ESC should also be expected to show positive effects in the nORT.

C.2. Methodology

As mentioned in **Chapter 2 (Section 2.7.3),** nORT consist of three phases: habituation, familiarization, and the test or acquisition phase. The following is a full description of the methods used in the current study.

These methods were based on those described by Abildgaard et al. (2011).

General

- All phases were carried out in a black open field box (100 x 100 x 50 cm) under a 40 lux red light.
- Rat movements were recorded using a ceiling-mounted digital camera. Thereafter, the footage was analysed using EthoVision® XT software (Noldus® Information Technology, Wageningen, The Netherlands).
- Boxes were cleaned with a 10% alcohol solution between every trial in order to remove scents that could alter behaviour.
- Exploration of an object was defined as movement within 4 cm of the objects, turning toward and sniffing the object, or physically exploring it.
- Results were calculated using the Discrimination Index (DI):

$$DI = \frac{\text{(time spent at novel object – time spent at familiar object)}}{\text{(time spent at the novel object + time spent at familiar object)}}$$

- Object preference varied between -1 and +1, where positive scores indicates more time spent with the novel object and negative scores indicates more time spent with the familiar object. Zero indicates no preference (Oberholzer et al., 2018). Thus, improvement in memory is associated with positive values.
- Three parameters were scored, namely:
    - *Nose point frequency:* Number of entries into the novel object zone (4cm area around novel object)
    - *Nose point cumulative duration*: Total amount of time (s) spent in the novel object zones of each treatment group
    - *Total distance moved* of each treatment group (cm)

- Objects used are depicted in Figure A.1.:

Fig. C.1. Objects that were used in the nORT were A) yellow rubber ducks (7.5 x 9.5 cm) and B) purple ceramic owls (5 x 5.5 cm).

Phase 1: Habituation

Animals were habituated once on day 13. This was done by placing a rat in an empty box and letting it explore for 10 minutes. The rat was then returned to its home cage.

Phase 2: Familiarization

Phase 2 was carried out 24 hours after phase one (day 14). In this phase, the animals were placed in the same box as *Phase 1* with two identical objects (either an owl or a duck) and left to explore for 5 minutes. They were then removed and returned to their home cages.

As seen in Fig. C.2., placement of the objects was done by dividing the boxes into quadrants, with the objects placed in the center of the quadrants. Objects were placed in diagonally opposing corners (Fig. C.2.). The diagonal directions were also alternated between rats to exclude location bias (Fig. C.4.)

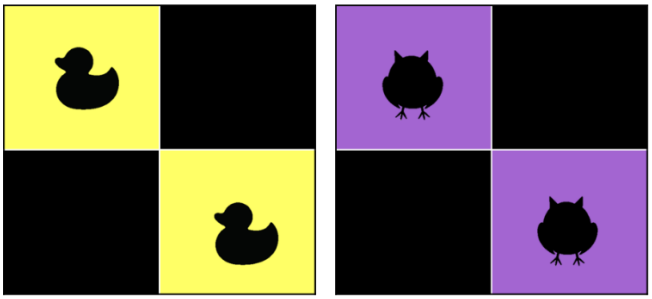

Fig. C.2. Placement of two identical objects (owls or ducks) in the familiarization phase

Phase 3: Test/Acquisition phase

This phase was carried out 90 minutes after *Phase 2* (Day 14). Herein, the rats were placed back in the box with one familiar and one novel object and recorded for 5 minutes. Animals in each group were alternatively exposed to either the owl or the duck as novel objects to exclude object bias (Fig. C.3). Diagonal directions of objects were also alternated to exclude location bias (Fig.C.4.)

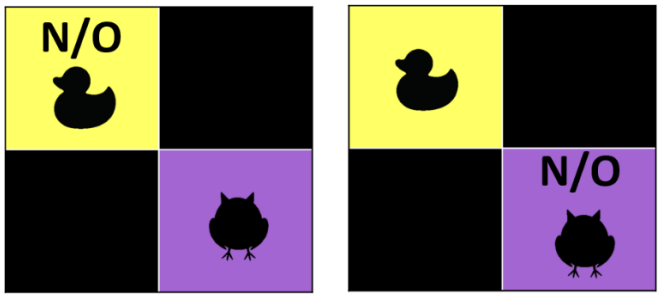

Fig. C.3. Novel objects were alternated between rats.

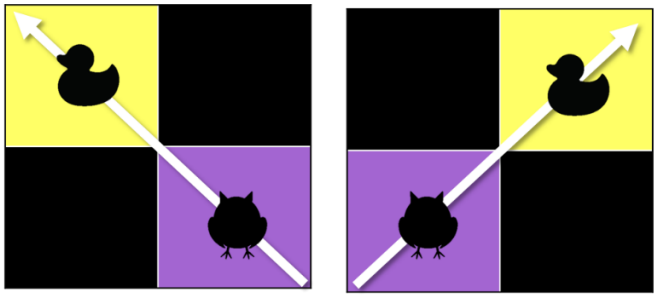

Fig. C.4. Diagonal directions of object placement were alternated between rats.

C.3. Results

Nose point frequencies: *Number of entries into the novel object zone*

All data sets were normally distributed. A one-way ANOVA indicated no significant differences between groups ($F(3.44) = 0.171$; $p = 0.916$).

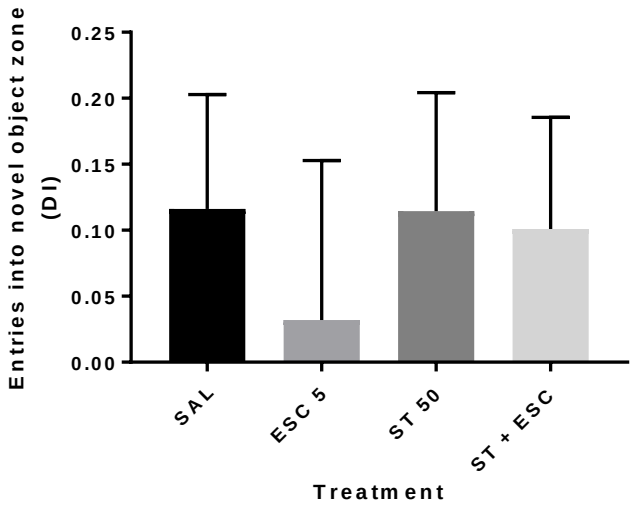

Fig. C.5. *Nose point frequency:* Number of entries into the novel object zone in the nORT of each treatment group (n = 12). Data expressed as (means [95% CI]). $p < 0.05$ deemed significant.

All data sets showed normal distribution. A one-way ANOVA indicated no significant differences between groups ($F(3.44) = 0.963$; $p = 0.419$).

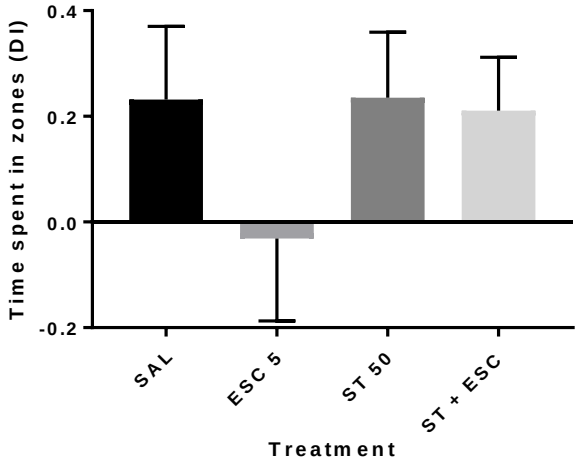

Fig. C.6. Nose point cumulative duration: Time spent in the zones of each treatment group (n = 12). Positive values represent the novel object zone and negative values represent the familiar object zone. Data expressed as (means [95% CI]). $p < 0.05$ deemed significant.

Total distance moved during Phase 3

All data sets were normally distributed. A one-way ANOVA indicated no statistically significant differences between groups ($F(3.44) = 0.941$; $p = 0.429$).

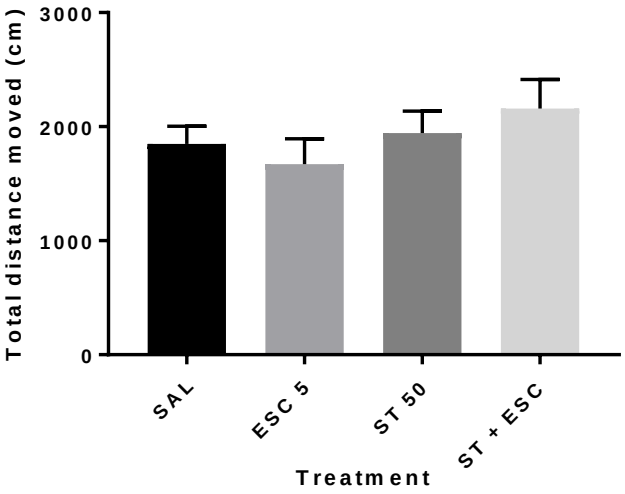

Fig. C.7. Total distance moved of each treatment group (n = 12) during the acquisition trial in the nORT. Data expressed as (means [95% CI]). $p < 0.05$ deemed significant.

C.4. Discussion

Due to the lack of significance between all treatment naive and drug treated FSL rats, these results were deemed inconclusive and were excluded from Manuscript A. This is contradictive of all other data obtained in this study, which showed differences between FRL and FSL rats, and between saline and drug treated FSL rats. This indicates that the most likely explanation for these unexpected results lie with the method itself. Many different modifications of this test have been used in previous studies, with each method tailored to fit the objectives of the studies. A comprehensive review regarding the nORT and its modifications was written by Antunes and Biala (2012). All NOR tests must comprise of the same basic three phases as described above (habituation, familiarization, test/acquisition phase), however, one can modify details of these phases, e.g. including longer times between the familiarization and test phases to test long-term memory, or by changing object locations to test spatial memory (Antunes and Biala, 2012). Table C.1. is a summary, based on the Antunes and Biala (2012) which contains the general modifications that can be made to tailor the test according to a specific study outcome.

Table C.1.: Summary of general modifications in nORT as reviewed by Antunes and Biala (2012), with a comparison of the methods followed in this study

TYPE	CHANGED FACTORS	REASON/OBJECTIVE AND EXTRA INFORMATION	MODIFICATIONS IN THIS STUDY
Basic procedure	Number of objects	Object identities an spatial memory	2 objects used testing object identification
	Novel objects divided 50/50 between animals	Prevent object and place preference	Was implemented
	Contextual change	Role of hippocampus in modulation of N/O preference	N/A to this study
	Test phase with 2 familiar objects, one displaced 90° from original placement	Spatial memory	N/A in this study as spatial memory was not tested
Animals	Special modifications; gender; age; strain	Differences in preferences	Male FSL animals were used between 200 g and 230 g
Exploration concept	Included: Nose orientated to object; within ≥2 cm (1-4 cm); touching and sniffing with nose; head orientation within 45°. Ambulation, Stationary, rearing	Excluded: running around or sitting/climbing on object	Were implemented
Habituation (H), familiarization (F), test days	Study specific H + F during 1 day with different duration and number of sessions; during 2-5 consecutive days	Study-specific	1 habituation 24 hours before the familiarization phase
Apparatus	Shapes	Rectangular; quadrangular; circular	Square
	Material	Plywood; acrylic; plastic; Plexiglas; wood; PVC; ABS	Perspex box
	Colour	Black; opaque; gray; white; transparent	Black
	Sizes	Study-specific	**100 x 100 x 50 cm** (keeping in mind the size of the rats)
Objects	Material	(metal, glass, glazed ceramic, rubber, durable non-toxic plastic, aluminium, wood), Not easily gnawed on; can be easily cleaned	Ceramic owl **Rubber duck (can be gnawed on)**** Easily cleanable with alcohol
	Shape and texture	Smooth (regular, cylindrical); complex (sharp angles, curves, extending features	Owl – simple, smooth shape Duck – complex, curved

	Appearance and colours	E.g. cans, bottles, tins, glasses, pots, pyramids, tower, cylinder, Playmobil toys, Lego toys, balls, mugs, pet toys, shuttlecock, candlestick, etc. (Preferably) not resembling living stimuli	Ceramic owl – purple Rubber duck – yellow **Both resembles living stimuli****
	Size	Small (<12 cm); large (>18 cm) Unable to climb and rest on it. Weight heavy enough not to move. Age-appropriate. No taller than twice the animal's size	Ducks (7.5 x 9.5 cm) Owls (5 x 5.5 cm). = Small objects **Could be climbed on**. Was heavy enough not to move and was fastened to the box base with "Prestik". No taller than twice the animal's size
Object positions	Extremes of apparatus. Exchange positions. N/O positions changed 50/50. Symmetrical (distance from sides etc.) Animals placed in center, nose pointed away from objects	Prevent object/side bias	Only changed diagonal direction as described above *between animals* to eliminate effects of spatial bias in the specific boxes. N/O positions were changed 50/50 Were placed symmetrically Animals were placed in the center with their noses pointed away from the objects
Cleaning between sessions	10% or 95% ethanol solution; 5% acetic acid; 70% isopropanol, diluted chlorine bleach	Not guided by odour cues	10% alcohol
Light and sound	Sound-isolated; low-level background noise; masking white noise (70 dB). <10 lux to 30-40 lux. Fluorescent bulbs 25- and 60-W bulbs suspended over box	Constant conditions	No background sounds (done at night to prevent other environmental sounds) 40 lux red light

Based on the Methodology (Section C.2.), the methods used in this study followed that of previous published papers (Abildgaard et al., 2011; Mokoena et al., 2015) and is in concordance with the above modifications and the reason/motivation behind it (Table C.1), except that the objects used (ducks and owls) resembled living beings which could have

affected behaviour according to (Antunes and Biala, 2012) (these objects have been used in our facilities in the past and has shown results). Although the ducks were made from rubber that could be gnawed on, it is unlikely that this could have had a significant effect on the results as the rats did not show object bias and this was especially prevented by alternating novel objects between rats. However the size of these objects were small and it was possible for the animals to climb on it. Based on these factors, it seems that the objects used might not have been ideal.

Previous studies in our facilities used smaller NOR boxes (70 x 70 x 40cm) (Möller et al., 2013; Uys, 2016) than those used in this study. This could have impacted behaviour by increasing the space being explored, thus attenuating their interest in the objects. It is possible that these boxes are too small for adult rats, "forcing" them to move near the objects which could affect automated scoring by the computer program, i.e. incidental touching of the objects. It was also argued that smaller boxes should be considered more for mice, thus prompting the decision to use the larger open field boxes in this study.

In conclusion, it is impossible to pinpoint reasons why the test did not deliver conclusive results based on the current data, especially considering the more emphatic bio-behavioural data presented in Manuscript A. Drug treatments should therefore have produced intergroup differences at least, especially considering the increase in hippocampal BDNF in the ST 50 alone treatment group as well as the overall treatment effects on regional brain monoamines (Manuscript A, Chapter 3).

Future recommendations would thus include that a nORT method should be validated and standardized in our facility (especially with regards to objects used) to prevent inter-study differences and deliver accurate, reliable and reproducible results.

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ADDENDUM D
FST AND OFT METHODOLOGY

1. Open field test (OFT)

This behavioural test is discussed in Chapter 2 (Section 2.7.2). and Manuscript A In short, this test can be used as a measure of locomotor activity (Brand and Harvey, 2017) which can be correlated with psychomotor retardation as seen in human MDD patients (Dean and Keshavan, 2017). Moreover, the OFT can be used to interpret FST data with regards to effects of drug treatments on locomotor activity which could affect swimming behaviour (Lavi-Avnon et al., 2005).

The methodology of the acute OFT test as implemented in this study is as follows:

- The researcher started camera recording boxes, and the EthoVision® XT software (Noldus® Information Technology, Wageningen, The Netherlands)
- Immediately after recording started, a rat was removed from its home cage and individually placed in an empty black open field box (100 x 100 x 50 cm).
- The rats were then left to explore the box for 6 minutes under red light (40 lux), whereof 5 minutes were scored according to distance travelled in cm. A minute extra was included for leeway to place the experimental groups (n = 4) in their separate boxes.
- After the 6 minutes elapsed, they were returned to their home cages.
- They were then left undisturbed for 30 minutes, after which they were exposed to the FST
- All boxes were thoroughly washed with soapy water between sessions

For the sub-chronic study, the same principals applied as mentioned above, the only exception being that the rats were recorded for 10 minutes as part of the nORT habituation (see Addendum C) on day 13 of the study. The FST was done on day 15.

Increased distance travelled (cm) was regarded as less psychomotor retardation and thus indicated absence of MDD-like behaviour.

2. Forced swim test (FST)

As described in full in Chapter 2, (Section 2.7.1), and Manuscript A. In this test, animals were forced to swim in water for a period of time, during which behaviour such as swimming, struggling, and immobility was scored as a measure of depressive-like behaviour.

The methods for the FST in the acute and sub-chronic study were implemented as follows:

- The water tank of the FST apparatus was filled with water and heated to 25°C
- Four individual cylinders Perspex® cylinder (diameter 18cm, height 60 cm) were filled with 30 cm of water while the rats were not in the room to prevent exposure to loud noises which could influence anxiety levels and swimming behaviour (See figure xxx for FST cylinder setup).

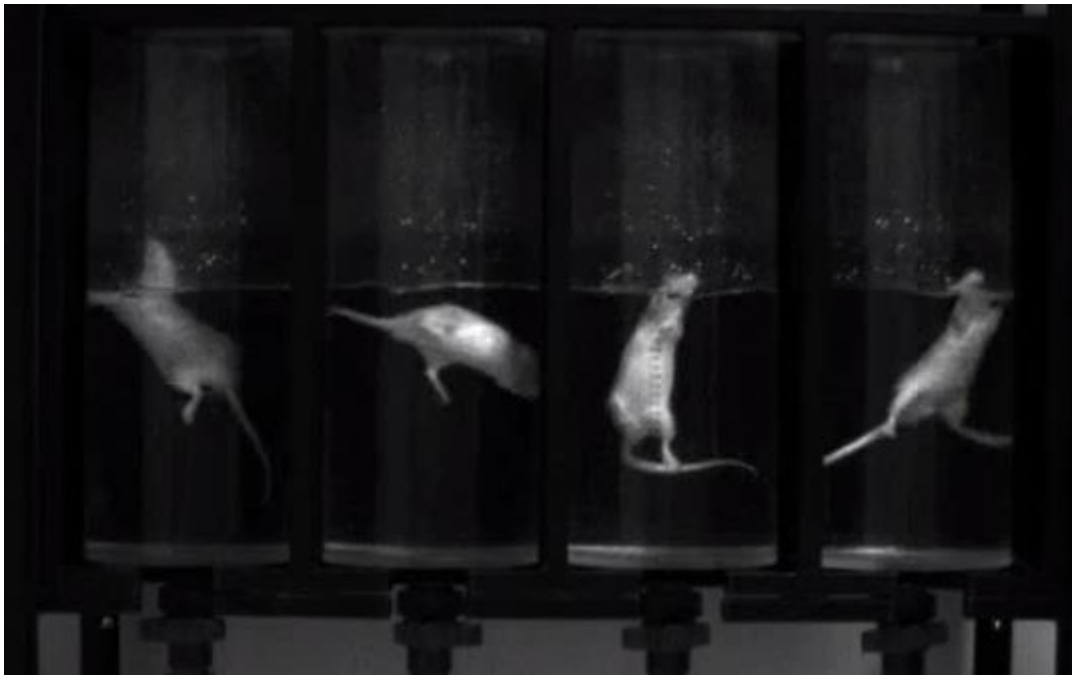

Fig. D.1. Experimental setup of rats swimming in the water-filled cylinders as explained in the text above

- Animals were taken to the FST room, where the camera and software program was prepared to record swimming behaviour (±10 minutes)
- The researcher started video recording
- Rats (maximum four per trial) were immediately removed from their home cages and individually placed in the water, where they were left to swim for 7 minutes under red light (40 lux). No animals received a 15 minute pre-swim as is usually done with control SD rats.
- The researcher left the room, but closely monitored the rats through a glass window, ready to intervene in at any sign of distress which could prove life-threatening
- After the time elapsed, animals were removed from the water and kept in holding cages while they were towel dried one at a time.
- They were then returned to their home cages and monitored according to the monitoring sheet (Addendum H) approximately one hour after the swim as well as the next morning.
- All cylinders were thoroughly rinsed out with water between sessions. Each rat swam in their own clean water.

Behaviours in the FST were manually scored using the FST Scoreboard program, based on the following criteria (Bogdanova et al., 2013; Cryan et al., 2005; Oberholzer et al., 2018):

- *Immobility*: floating on the water surface with only the necessary movements to keep its nose above water
- *Swimming*: horizontal movements across the water surface, while crossing quadrants of the cylinder.

- *Struggling/climbing*: deliberate upward-directed movements (resembling climbing behaviour) against the side of the cylinder
- *Diving*: Escape-driven submergence under water

However, despite the test's simplicity and sensitivity, Bogdanova et al. (2013) have identified and reviewed numerous factors which could influence rodent behaviour during the test. These factors are summarized in Table 2.5. and was considered during the planning and implementation of this test.

Table D.1: Table summarizing factors that could influence rodent behaviour the FST

BIOLOGICAL	**PRECONDITIONING AND TREATMENTS**	**TEST DESIGN**	**ENVIRONMENTAL CONDITIONS**
Animal strain	Handling	Time effects	Light
Body weight	Housing	Scoring	Noise
Individual differences	Endocrine manipulations	Combinations with other tests	Odour
Age	Diet	Statistical analysis	
Gender	Surgery and other manipulations	Equipment and settings	
	Stressors		
	Dosing schedule		
	Drug type and dose		

For full review, see Bogdanova et al. (2013)

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ADDENDUM E
HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC): METHODOLOGY - MONOAMINES

Monoamines (5-HT, NE, DA) play a major role in the pathophysiology of major depressive disorder (MDD) (Dean and Keshavan, 2017). Furthermore, the majority of clinically used antidepressants target monoamines (Dean and Keshavan, 2017; Jesulola et al., 2018; Willner et al., 2013). The drug in question in this study, *Sceletium tortuosum* (ST), also shows potential, at least based on data from in vitro studies, to increase monoamine levels (Harvey et al., 2011) and thus engender an antidepressant effect. Thus, by measuring frontal cortical and hippocampal monoamine levels in the control and drug treatment groups from the sub-acute study, we hope to gain valuable insight into the mechanisms and efficacy of ST as an antidepressant modality. For this study, we focused on NE and 5-HT concentrations in the abovementioned regions (as these two brain regions are especially implicated in MDD (Dean and Keshavan, 2017; Millan et al., 2000; Willner et al., 2013)) for the purposes of Manuscript A (Concept article, Chapter 3). However DA was also measured in these regions and is only included in Addendum B as the data did not contribute to the Concept article.

E.1. Methodology

Regional brain monoamine analyses were performed by high pressure liquid chromatography (HPLC) with electrochemical detection (ED) using the validated method of (Viljoen et al., 2018).

E.2. Sample preparation

After decapitation of animals coming from the sub-chronic study, the brains were collected and the hippocampus and frontal cortex were dissected and transferred to 1.5 ml Eppendorf tubes whereafter they were snap frozen in liquid nitrogen and stored in a - 80° freezer until analysis. On the day of analysis, the following steps were followed:

- Hippocampal and frontal cortical brain tissue samples were weighed and 1 ml of solution A was added to the Eppendorf tubes
- The samples were sonicated (2 x 12 s, at an amplitude of 14µ) to rupture cells

- In order to allow for completion of the perchlorate precipitation of proteins as well as extraction of the monoamines, the samples were placed on ice for 20 minutes
- The samples were centrifuged at 4 °C, for 25 minutes at 20 817 rcf (relative centrifugal force)
- Supernatant from the samples were pipetted into 2 ml amber Eppendorf tubes
- pH adjustment was done by adding one drop of 10 M potassium acetate to each sample to obtain a pH of 5.0
- 200 µl tissue extract or standard was pipetted into 1.5 ml Eppendorf tubes
- 20 µl of 5-HMT (internal standard) was added to each sample
- Each final sample was vortexed
- Final samples were centrifuged for 5 minutes at 20 817 rcf
- Samples were then transferred to an HPLC vial insert
- All excess tissue samples were stored in the -80°C

E.3. Internal standard solution preparation

The working internal standard solution containing 5-hydroxy-Nω-methyptryptamine oxalate (5-HMT) at a final concentration of 1500 ng/ml, was prepared via appropriate dilution from the standard 5-HMT stock solution (100 µg/ml) and the solvent, Solution A (0.1 M perchloric acid; 0.5 mM sodium metabisulphite; 0.3 Mm ethylenediaminetetraacetic acid disodium salt).

E.4. HPLC conditions

E.4.1. Instrumentation

An Agilent 1200 series HPLC (Agilent Technologies Inc., Santa Clara, CA USA), equipped with an isocratic pump and autosampler, coupled to an ESA Coulochem III Electrochemical detector with a coulometric flow cell (Model 5011A High Analytical Cell and Guard cell 5020) and Chromeleon® Chromatography Management System® version 6.8 (obtained from Thermo Fisher Scientific, Waltham, MA USA).

E.4.2. Mobile phase preparation

The mobile phase consisted of the following:

- 0.1 M sodium formate buffer
- 5 mM sodium 1-heptane-sulfonate
- 0.17 mM ethylenediaminetetraacetic acid (EDTA) disodium salt

- 5% v/v acetonitrile

The pH was adjusted to ± 4.0 using ortho-phosphoric acid.

Before usage, the mobile phase was filtered through a 0.22 µm nylon filter (Agela Technologies).

E.4.3. Instrumentation settings

HPLC instrument settings:

- 1.0 ml/min flow rate
- 20 µl Injection volume
- 50 minutes run time

Electrochemical detection settings:

- Cell potential settings:
    - Test electrode 1 (E1):-150 mV (to eliminate background noise)
    - Test electrode 2 (E2): +650 mV (to analyse the monoamines)
    - Guard Cell (EGC): +350 mV
    - Detection range: 500 nA
    - Filter: 0.5 seconds
    - Offset: 0%
    - Signal output: 0.1 V
- 20 Hz data collection rate.
-

E.5. Calibration curve

Table E.1. Coefficient of Determination and linear regression line equations of the calibration curve

Monoamine	Coefficient of Determination (R^2)	y = mx + c
5-HT	**R^2 = 0.999**	**y = 0.007x + 0.031**
5-HIAA	R^2 = 0.999	y = 0.006x + 0.027
DA	**R^2 = 0.999**	**y = 0.005x + 0.029**
DOPAC	R^2 = 0.999	y = 0.004x + 0.023
HVA	R^2 = 0.999	y = 0.005x + 0.035
3-MT	R^2 = 0.999	y = 0.005x + 0.025
NE	**R^2 = 0.999**	**y = 0.004x + 0.021**

In this study, we only used the 5-HT, DA and NE data highlighted above.

E.6. Chromatograms

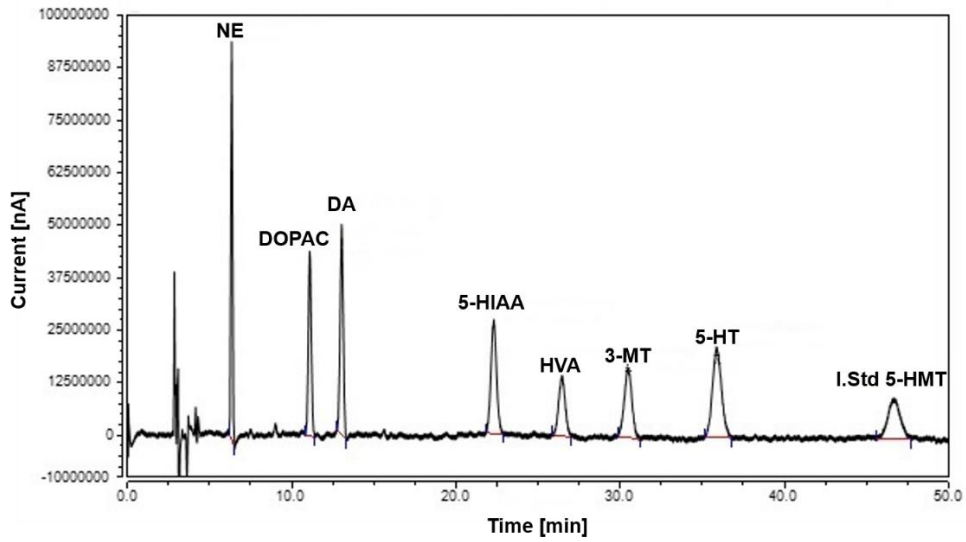

Fig. E.1. Chromatogram showing monoamine peaks of the internal standard

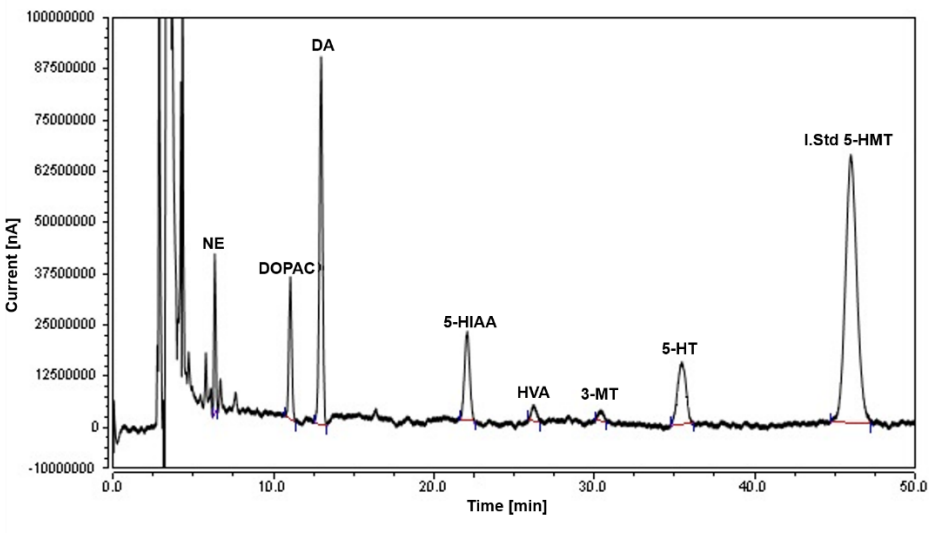

Fig. E.2. Chromatogram showing monoamine peaks of a frontal cortex sample

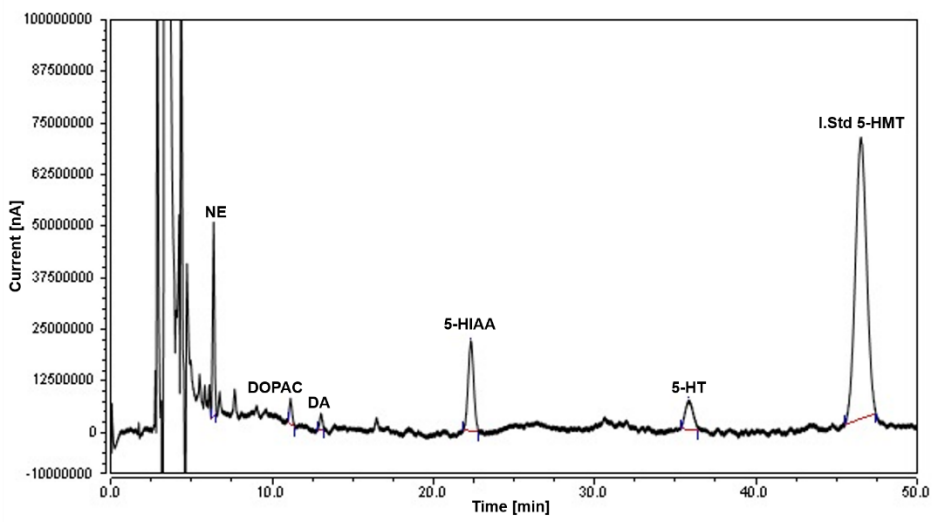

Fig. E.3. Chromatogram showing the monoamine peaks in a hippocampus sample

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ADDENDUM F
ELISA KIT METHOD: BDNF

F.1. Introduction

The phosphodiesterase (PDE) 4 enzyme hydrolyses cyclic adenosine monophosphate (cAMP), thus decreasing the activity of the cAMP/cAMP response element binding protein (CREB) pathway which contributes significantly to the production of brain-derived neurotrophic factor (BDNF (Duman et al., 2000). Based on the ability of ST to inhibit PDE4 (Harvey et al., 2011), we opted to measure regional brain levels of BNDF. Plasma BDNF is known to be reduced in MDD, while effective antidepressants are known to reverse these changes (Willner et al., 2013). As BDNF increases neuroplasticity and neurogenesis in brain regions such as the hippocampus, which is especially responsible for memory and learning, altered levels of BDNF should correlate with scores in the nORT (**Addendum C),** be linked to regional brain monoamines levels (Chapter 3), and respond to sub-chronic treatment with either ESC or ST or both, that is if the latter proves to be an effective antidepressant (Chapter 3). The BDNF data are presented in Manuscript A (Concept article, Chapter 3).

F.2. The ELISA kit

In vitro quantitative determination of rat brain BDNF (frontal cortex and hippocampus) was analysed using the Rat BDNF enzyme linked immunosorbent assay (ELISA) kit catalog No: E-EL-R1235, 96T from Elabscience (Houston, USA)).

Certificate of analysis

- Production No.: E-EL-R1235
- Lot No.: ZGZ5G46LZ5
- Date: 2019-06-28

Specification

- Sensitivity: 18.75pg/ml
- Detection Range: 31.25-2000pg/ml
- Specificity: BDBF in given sample. No significant interference or cross-reactivity between rat BDNF and analogues was found.
- Repeatability: Coefficient of variation is <10%.

Principle of test

Elabscience's ELISA kit uses the Sandwich-ELISA principle. The micro ELISA plate provided in the kit has been pre-coated with an antibody specific to Rat BDNF. Standards or samples were added to the micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for Rat BDNF and Avidin-Horseradish Peroxidase (HRP) conjugate were added successively to each micro plate well and incubated. Free components were washed away. The substrate solution was added to each well. Only those wells that contained Rat BDNF, biotinylated detection antibody and Avidin-HRP conjugate appear blue in colour. The enzyme-substrate reaction was terminated by the addiction of stop solution which turned yellow in colour. The optical density (OD) was measured spectrophotometrically at a wavelength of 450 nm. The OD value was proportional to the concentration of Rat BDNF. To calculate Rat BDNF concentration in the samples, the OD of the samples were compared to the rat BDNF standard curve provided in the kit and prepared and analysed according to the manufacturer's instructions.

F.3. Materials

Provided materials

- Manual
- Certificate of Analysis
- Micro ELISA plate (8 wells x 12 strips)
- Plate sealer (5 pieces)
- Reference Standard (2 vials)
- Concentrated Biotinylated Detection Ab (100x – 1 vial 120µl)
- Concentrated HRP Conjugate (100x – 1 vial 120µl)
- Reference standard & Sample diluent (1 vial 20ml)
- Biotinylated Detection Ab Diluent (1 vial 14ml)
- HRP Conjugate Diluent (1 vial 14ml)
- Concentrated Wash buffer (25x – 1 vial 30ml)
- Substrate reagent (1 vial 10ml)
- Stop solution (1 vial 10ml)

Other materials required

• Microplate reader with 450nm wavelength filter	BioTek FL600 Microplate Fluorescence reader (BioTek, Instruments, Inc., 381 Highland Park, Winooski, VT, USA)
• Incubator (37ºC)	SHAKER DTS-4
• High-precision transfer pipette	
• Eppendorf (EP) tubes and disposable pipette tips	
• Distilled water	
• Absorbent paper	
• Loading slot for wash buffer	BioTek

F.4. Sample preparation

1. The day before analysis, frontal cortical and hippocampal tissue were thawed on ice, rinsed with ice-cold phosphate buffered solution (PBS) (0.01M, pH 7.4), weighed and homogenised in PBS (tissue weight (g): PBS (ml) volume=1:9) by sonication.
2. The homogenates were centrifuged (5000xg, 5 minutes), thereafter the supernatant was collected and stored at -80ºC until the next day of analysis.

F.5. Reagent preparation

1. On the day of the analysis, all reagents were left to reach room temperature (18-25ºC).
2. The microplate reader was preheated 15 minutes before measuring the OD.
3. The wash buffer was prepared
   (For 750ml wash buffer – 30ml of the concentrated wash buffer was diluted with 720ml distilled water).
4. The standard working solution was prepared
   The standard was centrifuged – 10000xg (60 seconds). 1ml of reference standard and sample diluent was added and left to stand for 10 minutes. It was inverted very gently a few times. Once it was fully dissolved, it was carefully mixed with a pipette. This produced a 2000pg/ml working solution. Serial dilutions were prepared as needed (2000, 1000, 500, 250, 125, 62.5, 31.25, 0pg/ml were recommended). A visual demonstration of the dilution is presented in figure C.1. First, 7 EP tubes were prepared and 500µl of reference standard and sample diluent were added to each one. 500µl of the 2000pg/ml working solution were pipetted to the first EP tube and mix thoroughly to produce a 1000pg/ml working solution. This process was proceeded by pipetting 500µl from the former EP tube to the latter EP tube. The last EP tube served as a blank.

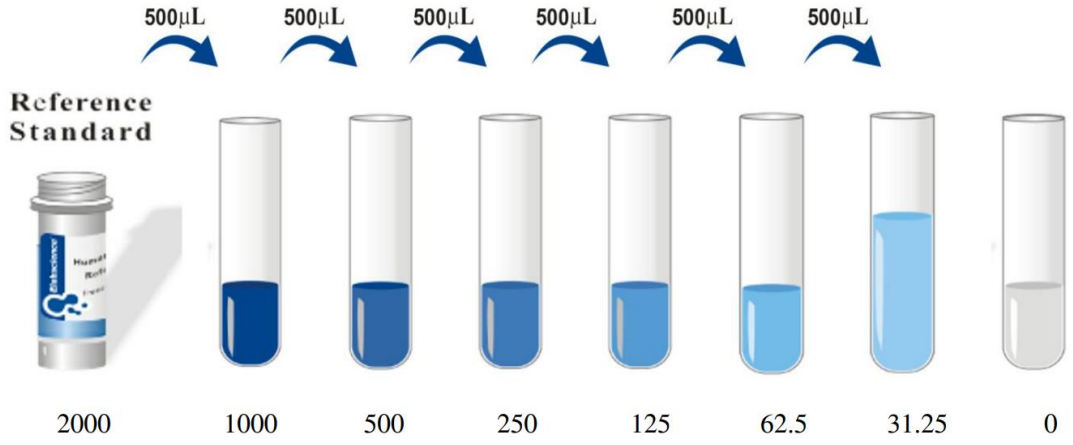

Fig. F.1: Dilution method for working solution. Figure taken from the provided ELISA kit manual

5. The Biotinylated Detection Ab working solution was prepared
   The exact amount needed was calculated before the experiment (100µl/well). All stock tubes were centrifuged before use. 1x working solution with Biotinylated Detection Ab Diluent was diluted with 100x Concentrated Biotinylated Detection Ab.
6. Concentrated HRP Conjugate working solution was prepared

The exact amount needed was calculated before the experiment (100µl/well). 1x working solution with Concentrated HRP Conjugate Diluent was diluted with 100x Concentrated HRP Conjugate.

F.6. Assay procedure

1. The Standard working solution (100µl) was added in the first two columns. All concentrations of the solution were added in duplicate.
2. A sample (100µl) was added to each individual well. With this assay all 48 samples from the frontal cortex were added in duplicate to the first set of 96 wells, thereafter 48 samples of the hippocampus were added in duplicate to the second set of 96 wells.
3. The plates were covered with a sealer and incubated (90 minutes 37ºC).
4. The liquid from each well was removed.
5. The Biotinylated Detection Ab (100µl) was immediately added to each individual well.
6. The plates were covered with one of the provided sealers and incubated (60 minutes - 37ºC). A SHAKER DTS-4 were used as an incubator.
7. The plates were aspirated and washed three times. This was done by adding wash buffer (350µl) to each well and letting the wells soak for 2 minutes before decanting the wash buffer. After this step, the plates were patted dry against absorbent paper. The plates were washed automatically by a microplate washer (BioTek) in the laboratory.
8. HRP Conjugate (100µl) was added to each individual well.
9. The plates were covered with one of the provided sealers and incubated (30 minutes - 37ºC).
10. The plates were aspirated and washed five times, as described in step 7.
11. Substrate reagent (90µl) was added to each individual well.
12. The plates were covered with one of the provided sealers and incubated (15 minutes - 37ºC). It was of utmost importance to protect the plates from light at this stage due to colour changes that may occur.
13. Stop solution (50µl) was added to each individual well.
14. The OD value (450nm) was determined immediately using a microplate reader.
15. The results were calculated.

F.7 Calculation of results

A computer with software capable of plotting a four-parameter logistic curve was used. The X-axis represented the standard concentration and the y-axis the OD values. Figure 2 depicts the logistic curve produced by the BDNF concentration in this experiment for three plates. Three plates were needed as 192 samples had to be analysed. Please see figure 3 for a detailed plate layout for all three plated used.

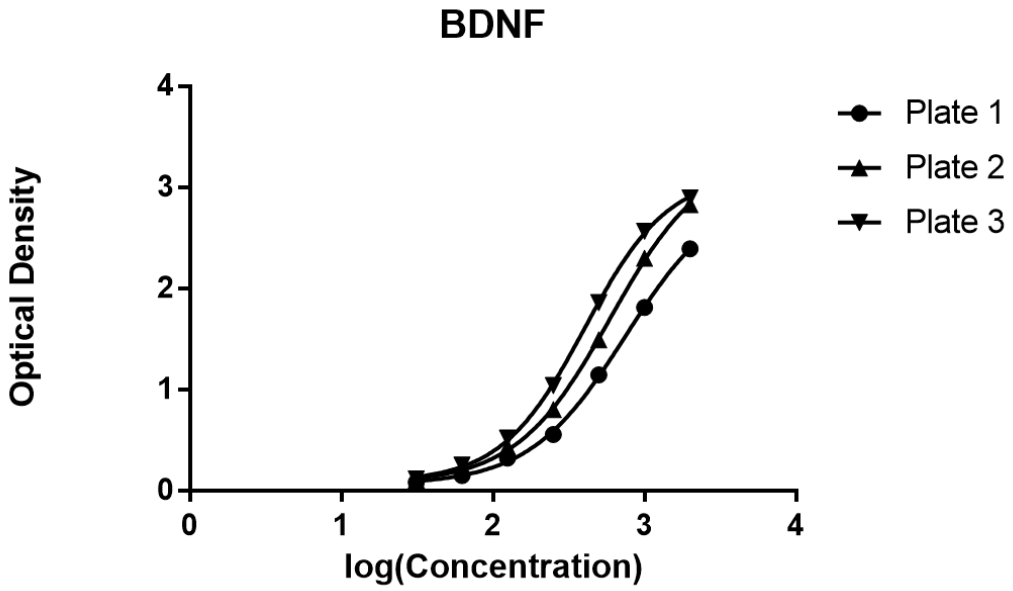

Fig. F.2: Standard logistic curve for rat BDNF measured in the frontal cortex and hippocampus.

Rat sample allocation (Fig. F.3)

- 1 – 12 = Saline group
- 13 – 24 = ST 50 group
- 25 – 36 = ESC 5
- 37 – 48 = ESC 5 + ST 50

PLATE 1												
	1 (Std.)	2 (Std.)	3	4	5	6	7	8	9	10	11	12
A	0	0	1	1	9	9	17	17	25	25	33	33
B	31,25	31,25	2	2	10	10	18	18	26	26	34	34
C	62,5	62,5	3	3	11	11	19	19	27	27	35	35
D	125	125	4	4	12	12	20	20	28	28	36	36
E	250	250	5	5	13	13	21	21	29	29	37	37
F	500	500	6	6	14	14	22	22	30	30	38	38
G	1000	1000	7	7	15	15	23	23	31	31	39	39
H	2000	2000	8	8	16	16	24	24	32	32	40	40
			Hippocampus samples									

PLATE 2												
	1 (Std.)	2 (Std.)	3	4	5	6	7	8	9	10	11	12
A	0	0	41	41	49	49	57	57	65	65	73	73
B	31,25	31,25	42	42	50	50	58	58	66	66	74	74
C	62,5	62,5	43	43	51	51	59	59	67	67	75	75
D	125	125	44	44	52	52	60	60	68	68	76	76
E	250	250	45	45	53	53	61	61	69	69	77	77
F	500	500	46	46	54	54	62	62	70	70	78	78
G	1000	1000	47	47	55	55	63	63	71	71	79	79
H	2000	2000	48	48	56	56	64	64	72	72	80	80
			Hippocampus samples		Frontal cortex samples							

PLATE 3												
	1 (Std.)	2 (Std.)	3	4	5	6	7	8	9	10	11	12
A	0	0	81	81	89	89	-	-	-	-	-	-
B	31,25	31,25	82	82	90	90	-	-	-	-	-	-
C	62,5	62,5	83	83	91	91	-	-	-	-	-	-
D	125	125	84	84	92	92	-	-	-	-	-	-
E	250	250	85	85	93	93	-	-	-	-	-	-
F	500	500	86	86	94	94	-	-	-	-	-	-
G	1000	1000	87	87	95	95	-	-	-	-	-	-
H	2000	2000	88	88	96	96	-	-	-	-	-	-
			Frontal cortex samples									

Fig. F.3: Plate 1- 3 sample layout for rat BDNF measured in the frontal cortex and hippocampus. Sample numbers indicated in the corresponding wells in which they were analysed.

References

Duman, R.S., Malberg, J., Nakagawa, S., D'Sa, C., 2000. Neuronal plasticity and survival in mood disorders. Biological Psychiatry 48(8), 732-739.

Harvey, A.L., Young, L.C., Viljoen, A.M., Gericke, N.P., 2011. Pharmacological actions of the South African medicinal and functional food plant Sceletium tortuosum and its principal alkaloids. Journal of ethnopharmacology 137(3), 1124-1129.

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ADDENDUM G

CERTIFICATE OF ANALYSIS OF ZEMBRIN®

PoliNat

CERTIFICATE OF ANALYSIS

Polifenoles Naturales

GENERAL INFORMATION

Product Name:	**ZEMBRIN®**	Manufacture Date :	14-07-2016
Latin Name:	*Sceletium tortuosum (L.) N.E. Br.*	Testing Date:	14-07-2016
Batch Number:	SCE0416-1407	Expiration Date	14-07-2019
Code:	SCE04	Shelf Life:	3 Years

ANALYSES

ITEM DESCRIPTION:	SPECIFICATION	TEST METHOD	RESULT
PRODUCT CHARACTERISTICS			
PLANT PART USED	AERIAL PARTS	ORGANOLEPTIC + UPLC	COMPLIES
CARRIER USED	MALTODEXTRIN	BY WEIGHT (≤ 40%)	COMPLIES
	SILICON DIOXIDE	BY WEIGHT (≤ 2%)	COMPLIES
PLANT:EXTRACT RATIO	2:1	INTERNAL VALID METHOD	COMPLIES
ID TESTING OF RAW MATERIAL USED TRACEABILITY CODE: RM-SCE	REFERENCE DESCRIPTION	MACROSCOPIC	COMPLIES
	Sceletium tortuosum	DNA BARCODING	COMPLIES
	PHYTOCHEMICAL FINGERPRINT	UHPLC FINGERPRINT	COMPLIES
PHYSICAL TESTS			
APPEARANCE	MILLED POWDER	VISUAL	COMPLIES
COLOR	LIGHT BROWN	VISUAL	COMPLIES
AROMA	CHARACTERISTIC SMELL	ORGANOLEPTIC	COMPLIES
FLAVOR	CHARACTERISTIC	ORGANOLEPTIC	COMPLIES
PARTICLE SIZE	100% THROUGH 80Mesh	US STANDARD SIEVE	COMPLIES
SOLUBILITY IN WATER	≥ 99 %	Column Elution/NF T20-045 AFNOR	99%
BULK DENSITY	0,5-0,7(g/cm^3)	USP<616> / EU Pharm. 2.9.34	0.52 (g/cm^3)
CHEMICAL TESTS			
TOTAL ALKALOIDS	≥ 0.35% - ≤ 0.45%	UPLC–DAD	0,42 %
MESEMBRENONE + MESEMBRENOL	≥ 60% contribution to total alkaloids	UPLC-DAD	78,6 %
MESEMBRINE	≤ 20% contribution to total alkaloids	UPLC-DAD	13,5 %
MESEMBRANOL	≥ 5% contribution to total alkaloids	UPLC-DAD	7,8 %
MOISTURE	≤ 7.0%	LOST OF DRYING / USP 921	4.96%
TOTAL HEAVY METAL	≤ 10.0 ppm	EU Pharmacopeia 2.4.8	*In progress*
ARSENIC	≤ 0.5 ppm	ICP-MS /EC 629/2008	*In progress*
LEAD	≤ 2 ppm	ICP-MS /EC 629/2008	*In progress*
CADMIUM	≤ 0.5 ppm	ICP-MS /EC 629/2008	*In progress*
MERCURY	≤ 0.5 ppm	ICP-MS /EC 629/2008	*In progress*
RESIDUAL SOLVENTS	≤ 0.05%	Ethanol - GC-FID/ USP-467 Class3 and European Directive 2009/32/CE	≤ 0.05%
PESTICIDES	Material complies with USP 565 and European Regulation 396/2005/CE		
CONTAMINANTS	Material complies with European regulation 1881/2006 and amendments setting maximum levels for contaminants in foodstuffs		
MICROBIOLOGICAL TEST			
TOTAL PLATE COUNT	≤ 10000 (cfu/g)	ISO 4833	200 (cfu/g)
YEAST & MOLD	≤ 1000 (cfu/g)	ISO 7954	< 10 (cfu/g)
TOTAL COLIFORMS (*)	< 10 (ufc/10g)	M0042-Part V. VRB (PCM)	< 10 (cfu/g)
E.COLI	NEGATIVE (cfu/10g)	EU Pharm. 2.6.13.	NEGATIVE (cfu/10g)
SALMONELLA	ABSENT (cfu/25g)	ISO 6579	ABSENT (cfu/25g)
STAPHYLOCOCCUS AUREUS	NEGATIVE (cfu/10g)	EU Pharm. 2.6.13.	NEGATIVE (cfu/10g)
(*) Quantitative testing method			

EXTRACTION METHOD	Water:ethanol extraction and spray dried.
PACKAGING and STORAGE	Original container in a cool, dry place.
PACKING	1kg / 2kg/ 5kg / 10kg sealed aluminium foil bags.
COUNTRY OF ORIGIN /MANUFACTURED	Spain
Non-ALLERGENS/GLUTEN STATEMENT	This product does not contain any of the food allergens cited in the Directive 2000/13/EC, amended by 2003/89/EC, 2006/142/EC, 2007/68/EC
Non-BSE/TSE STATEMENT	All ingredients in the product are of vegetable origin
Non-IRRADIATION STATEMENT	This material has not been subjected to irradiation
Non-GMO STATUS STATEMENT	This product is considered NOT genetically modified or NOT derived from a genetically modified organism as defined by the EC regulations 1830/2003/EC, 1829/2003/EC and any amending legislation
Total heavy metal, microbiological, residual solvents, pesticides and contaminants results are based on historical data review and routine testing	

Q.C. Beatriz Ercilla PoliNat Polifenoles Naturales, S.L. rev.18 created date: 04.07.2016

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ADDENDUM H

ANIMAL MONITORING: MONITORING SHEETS AND WEIGHTS OF ANIMALS

Serotonin syndrome is life-threatening condition which can be induced by combined use of serotonergic drugs (see Boyer and Shannon (2005) for review). At various points in the main study we expressed possible evidence for a "serotonin behavioural syndrome" as a means to explaining our findings. ST is a relatively unknown entity when tested *in vivo* in animals, with this study representing the first to explore its use as an adjunctive agent in combination with a known antidepressant in an animal model. The FSL rat is known to present with hypersensitive 5-HT_{1A} receptors (Overstreet et al., 1998; Shayit et al., 2003). Due to the known serotonergic effects of both ST and ESC (El Mansari et al., 2005; Harvey et al., 2011), a low dose of ESC was identified for use in combination with a therapeutic dose of ST, this in order to prevent dangerous increases in 5-HT which could lead to 5-HT syndrome and prove possibly fatal to the animals. The low dose of ESC would also aid in establishing the adjunctive potential of ST.

In order to address the ethical standards set out for this work (see Chapter 1, Section 1.6) a monitoring sheet was specifically adapted from standard Vivarium monitoring sheets to screen for 5-HT syndrome. Criteria was added (in bold) in the form of specific behaviours known to be associated with 5-HT syndrome in rodents, as described by Haberzettl et al. (2013).

Any abnormal behaviours or concerns were documented. Health of animals were determined using a scoring system, where scores indicate if the rats show normal behaviour, should be monitored carefully, if one should seek advice, or if one should intervene immediately. The scores were interpreted as follows:

- 00 - 04 = Normal
- 05 - 09 = monitor carefully, consider intervention
- 10 - 13 = Suffering, provide relief, observe regularly. Seek opinion from technologist as per callout sheet. Consider humane euthanasia.
- 14 - 15 = Sever pain; intervene immediately per humane endpoint, reconsider experimental protocol.

The table provided below (Table H.1) was used to observe and note abnormal behaviour. Furthermore, this table also contains criteria to monitor during drug administration via oral

gavage and for behavioural tests. In addition to routine daily monitoring, all animals were monitored approximately an hour after the FST, as well as the next morning.

As has been alluded to in Chapters 3 and 4, careful perusal of the monitoring sheets highlighted the presence of a so called “serotonin behavioural syndrome”, specifically the presence of hyperactivity during handling, dosing, during the OFT, and after the FST despite increased immobility during the FST. Further discussion of these behaviours as quantified in the behavioural tests can be found in the appropriate sections of the dissertation, viz. Manuscript A (Concept article, Chapter 3) and Chapter 4.

The adapted monitoring sheet was approved by the Animcare Ethics Committee of the NWU.

Table H.1. Adapted monitoring sheet with the bold, highlighted criteria added as signs and symptoms of serotonin syndrome (Haberzettl et al., 2013), with a table providing space for notes and observations during daily monitoring, after oral gavage, and during behavioural tests.

AnimCare 06-01a v4.10 — **General Health and Serotonin Syndrome Monitoring Sheet for Animal Studies**

Study title:	Evaluating the antidepressant-like properties of Sceletium tortuosum, alone and as adjunctive treatment																		**Year:**	2018/2019		
Ethics no.:	NWU-00168-18-S5		**Project head:**	Prof BH Harvey						**Observer / student:**	Johané Gericke								**Animal ID:**			
Parameter		**Score**	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
			Date	Date	Date	Date	Date	Date	Date	Date	Date	Date	Date	Date	Date	Date	Date	Date	Date	Date	Date	Date	Date
			Time	Time	Time	Time	Time	Time	Time	Time	Time	Time	Time	Time	Time	Time	Time	Time	Time	Time	Time	Time	Time
Appearance	Normal	0																					
	Lack of grooming	1																					
	Dull coat, ocular/nasal discharge	2																					
	Piloerection, hunched up, hind limb abduction, flat body posture	3																					
Weight	Normal	0																					
	< 5% weight loss	1																					
	5-15% weight loss	2																					
	>15% weight loss	3																					
Clinical signs	Normal	0																					
	Slight changes	1																					
	Respiratory increase ↑ 30%, **tremors**	2																					
	Respiratory increase ↑ 50%, convulsions, **increased body temp**	3																					
Natural behaviour	Normal	0																					
	Minor change	1																					
	Less mobile/alert, isolated, **decreased rearing, increased head weaving, hyperactivity**	2																					
	Vocalisation, restless or still	3																					
Provoked behaviour	Normal	0																					
	Minor depression	1																					
	Moderate change	2																					
	Reacts violently/weakly, precomatose	3																					
	TOTAL SCORE	0-15																					
Project-specific	Criterion 1																						
	Criterion 2																						
	Criterion 3																						
	Criterion 4																						
Other	Observation and/or comment (tick box ☐ if written on reverse side)		1☐ reverse	2☐ reverse	3☐ reverse	4☐ reverse	5☐ reverse	6☐ reverse	7☐ reverse	8☐ reverse	9☐ reverse	10☐ reverse	11☐ reverse	12☐ reverse	13☐ reverse	14☐ reverse	15☐ reverse	16☐ reverse	17☐ reverse	18☐ reverse	19☐ reverse	20☐ reverse	21☐ reverse
Decision	**✓ = normal / ? = monitor carefully / ! = seek advice / × = intervene immediately**																						
Signature *(please sign/initialise with each observation per column)*																							
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21

00 - 04 = Normal
05 - 09 = monitor carefully, consider intervention
10 - 13 = Suffering, provide relief, observe regularly. Seek opinion from technologist as per callout sheet. Consider humane euthanasia.
14 - 15 = Sever pain; intervene immediately per humane endpoint, reconsider experimental protocol.

<u>Observations and/or comments</u> – General health; Oral gavage; Behavioural tests (OFT/nORT habituation, nORT, FST); Serotonin Syndrome signs.

General health: Table, page 1

Oral gavage: Difficulty breathing, coughing, general signs of decline in health (Table, page 1)

Behavioural tests: Anxiety, distress (criteria in Table, page 1); drowning or prolonged submergence (FST)

Serotonin syndrome: Signs and symptoms in **bold** (Table, page 1)

1.	
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Furthermore, all rats were weighed (see Table H.2 for average weights of each treatment group for the duration of the sub-chronic study) daily as part of health monitoring according to the requirements of Animcare. These weights were also used to calculate the doses of drugs to be administered (doses calculated as mg/kg). Acute and sub-chronic treatment started as soon as the animals reached 200 – 230 g.

Table H.2.. Mean rat weights (gram) and standard error of means (SEM) of all rats in each treatment group on each experimental day of the 15-day sub-chronic study. Weights were used as part of health monitoring as required by the Animcare Ethics Committee, and to calculate doses each day before drug administration.

	SALINE		ESC 5		ST 50		ESC 5 + ST 50	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Day 1	218	1,89	212	2,38	217	2,18	220	2,02
Day 2	223	1,89	218	2,38	222	2,17	224	2,18
Day 3	230	1,64	224	2,41	230	1,9	230	2,12
Day 4	235	2,04	231	2,33	236	2,09	236	1,99
Day 5	244	2,27	238	2,44	242	1,68	244	2,25
Day 6	248	2,36	242	2,31	247	2,33	250	2,5
Day 7	252	2,3	248	2,57	248	2,57	252	2,3
Day 8	258	2,41	253	2,53	258	2,77	260	2,25
Day 9	264	2,51	257	2,96	260	2,75	262	2,49
Day 10	270	2,76	263	2,99	264	3,03	268	2,54
Day 11	274	2,12	268	3,13	267	3,33	271	3,1
Day 12	278	2,78	272	3,21	273	3,46	277	2,68
Day 13	283	2,78	280	3,26	275	3,64	282	3,03
Day 14	288	3,01	284	3,56	277	3,56	282	3,53
Day 15	290	3,27	284	3,51	281	3,84	285	2,72

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Overstreet, D.H., Daws, L.C., Schiller, G.D., Orbach, J., Janowsky, D.S., 1998. Cholinergic/serotonergic interactions in hypothermia: implications for rat models of depression. Pharmacology Biochemistry and Behavior 59(4), 777-785.

Shayit, M., Yadid, G., Overstreet, D.H., Weller, A., 2003. 5-HT1A receptor subsensitivity in infancy and supersensitivity in adulthood in an animal model of depression. Brain research 980(1), 100-108.

LETTERS OF CONSENT

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13 November 2019

Dear examiner

MSc DISSERTATION – J. GERICKE
PERMISSION TO INCLUDE MANUSCRIPTS FOR EXAMINATION PURPOSES

As study leader and senior corresponding author on Manuscript A, first authored by Miss Johané Gericke, I hereby approve that the concept manuscript listed below be included as part of the requirements for fulfilment of the MSc. degree, and that this manuscript may be submitted for examination purposes by the candidate.

The article is as follows:

Chapter 3, Manuscript A

Evaluating the antidepressant properties of *Sceletium tortuosum* (L.) N.E. Br., alone and in combination with escitalopram, in the Flinders Sensitive Line rat.

Sincerely,

Prof B.H. Harvey

Study leader
North-West University, Potchefstroom Campus, South Africa

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14 November 2019

Dear examiner

MSc DISSERTATION – J. GERICKE
PERMISSION TO INCLUDE MANUSCRIPTS FOR EXAMINATION PURPOSES

As co-supervisor and co-author on Manuscript A, first authored by Miss Johané Gericke, I hereby approve that the concept manuscript listed below be included as part of the requirements for fulfilment of the MSc. degree, and that this manuscript may be submitted for examination purposes by the candidate.

The article is as follows:

Chapter 3. Manuscript A

Evaluating the antidepressant properties of *Sceletium fortuosum* (L.) N.E. Br., alone and in combination with escitalopram, in the Flinders Sensitive Line rat.

Sincerely,

Dr M. Lekhooa

Co-supervisor
North-West University, Potchefstroom Campus, South Africa

Tshwane University of Technology
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13 November 2019

Dear Sir/Madam

MSc DISSERTATION – J. GERICKE
PERMISSION TO INCLUDE MANUSCRIPTS FOR EXAMINATION PURPOSES

I hereby approve that the concept manuscript listed below, first authored by Miss Johané Gericke with myself listed as co-author, be included as part of the requirements for fulfilment of the MSc. degree, and that this manuscript may be submitted for examination purposes by the candidate.

The article is as follows:

Chapter 3, Manuscript A

Evaluating the antidepressant properties of *Sceletium tortuosum* (L.) N.E. Br., alone and in combination with escitalopram, in the Flinders Sensitive Line rat.

Sincerely,

Dr A.M. Viljoen

Co-author
Tshwane University of Technology, South Africa

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13 November 2019

Dear Sir/Madam

MSc DISSERTATION – J. GERICKE

PERMISSION TO INCLUDE MANUSCRIPTS FOR EXAMINATION PURPOSES

We as collaborators and funders hereby approve that the dissertation and the concept manuscript listed below, first authored by Miss Johané Gericke, may be submitted as part of the requirements for fulfilment of the MSc. degree, and that these documents may be submitted for examination purposes by the candidate.

The dissertation is as follows:

Evaluating the antidepressant-like properties of Sceletium tortuosum, alone and as adjunctive treatment

The article is as follows:

Chapter 3. Manuscript A

Evaluating the antidepressant properties of Sceletium tortuosum (L.) N.E. Br., alone and in combination with escitalopram, in the Flinders Sensitive Line rat.

Sincerely,

Dr Ralph Tetteh-Amlalo
Managing Director
HG & H Pharmaceuticals (Pty) Ltd, South Africa