Development and validation of an animal model of treatment resistant depression

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Do not go gentle into that good night,
Old age should burn and rave at close of day;
Rage, rage against the dying of the light.

Though wise men at their end know dark is right,
Because their words had forked no lightning they
Do not go gentle into that good night.

Good men, the last wave by, crying how bright
Their frail deeds might have danced in a green bay,
Rage, rage against the dying of the light.

Wild men who caught and sang the sun in flight,
And learn, too late, they grieved it on its way,
Do not go gentle into that good night.

Grave men, near death, who see with blinding sight
Blind eyes could blaze like meteors and be gay,
Rage, rage against the dying of the light.

And you, my father, there on the sad height,
Curse, bless, me now with your fierce tears, I pray.
Do not go gentle into that good night.
Rage, rage against the dying of the light.

~ Dylan Thomas

Abstract

The current investigation focused on the development and validation of an animal model of treatment resistant depression (TRD). In addition, an in-depth review of biomarkers of depression was included which aimed to identify relevant biomarkers that would support the construct validity of the model. In order to publish this work, however, the scope of the review was extended to include biomarkers of mood and psychotic disorders (i.e. depression, bipolar disorder and schizophrenia). Insights into psychotic disorders are therefor limited to the biomarker review (Manuscript A) while the study itself focuses on depression, more specifically TRD. Despite significant research efforts aimed at understanding the neurobiological underpinnings of mood and psychotic disorders, the diagnosis and evaluation of the treatment of these disorders are still based solely on relatively subjective assessment of symptoms which may be partly to blame for the incidence of poor treatment outcome and treatment resistance. Therefore, biological markers aimed at improving the current classification of mood and psychotic disorders, and that will enable clinicians to categorize their patients and diagnose these disorders on a biological basis into more homogeneous clinically distinct subgroups, are urgently needed. The attainment of this goal can be facilitated by identifying biomarkers that accurately quantify and reflect pathophysiologic processes in these disorders and developing animal models that accurately emulate the aberrancies identified in patients suffering from non-response to pharmacotherapy.

The high occurrence of non- or partial response to antidepressant treatment in the depressed population creates a major problem in effectively treating and managing the disorder. Up to half of patients fail to achieve a full response when treated with first-line antidepressant drugs and, even after applying several treatment strategies in this population, approximately 30% of these patients still do not respond to treatment. As with depression, TRD is believed to be heterogeneous in nature and, although most pathophysiological factors contributing to depression appear to be similar in TRD, many of these conditions are significantly exaggerated in the resistant form, resulting in more severe symptoms. However, a shortage of suitable and validated animal models of TRD is a major contributing factor to our current lack of understanding of the pathophysiology of TRD. Recent

studies have therefore set out to explore the processes that underlie treatment resistance in animal models.

In recent years it has become widely accepted that genetic susceptibility combined with adverse environmental situations are an important prodromal confluent for the development of depression. Thus, animal models that are based on this construct may contribute significantly to our knowledge of mood and anxiety disorders.

The Flinders sensitive line (FSL) rat is a well-studied genetic animal model of depression with robust construct, predictive and face validity. Considering the strong comorbidity between depression and post-traumatic stress disorder (PTSD), and that depression in patients with PTSD is more treatment resistant, we have developed an animal model of TRD based on the premise that exposing animals genetically predisposed to depressive-like behaviour to a PTSD-related paradigm would yield a model presenting with exacerbated and pronounced depressive-like behaviour that are resistant to traditional antidepressant treatment. To this end we have considered time-dependent sensitization (TDS; or stress re-stress) as a model of PTSD. TDS is based on a trauma plus contextual reminder principle of PTSD, and has previously shown good predictive, construct and face validity for PTSD.

In the first section of the study, subsequent to confirming the depressive-like phenotype of the FSL rat relative to that of the FRL rat, exposing FSL rats to TDS resulted in either bolstered or sustained reduction in coping response and increased depressive-like behaviours, combined with altered monoaminergic profiles in the hippocampal and frontocortical brain regions. Furthermore, the addition of TDS to FSL rats significantly abrogated the antidepressant-like effects of imipramine at most behavioural levels (climbing and immobility) and with respect to limbic serotonergic signalling.

Drug-centred approaches to manage TRD emphasize the use of agents with improved efficacy as well as the combination of drugs with different mechanisms of action. Hence, to extend the predictive validity of the model, we investigated sub-chronic treatment in TDS-exposed FSL rats with either a serotonin and noradrenaline reuptake inhibitor (SNRI), i.e. venlafaxine, or a N-methyl-D-aspartate (NMDA) receptor antagonist, i.e. ketamine, as monotherapy and in combination with imipramine in an augmentative approach.

In the second section of the study, we subsequently demonstrated that non-response is not only observed with the traditional antidepressant, imipramine, but also following treatment with either ketamine or venlafaxine as monotherapy. However, combining either venlafaxine or ketamine with imipramine led to enhanced antidepressant-like effects as measured in the FST, together with altered response in monoaminergic signalling in the animal model of TRD.

Taken together, an in-depth review of the literature revealed that mood and psychotic disorders are currently associated with a multitude of biomarkers that still require illumination regarding their exact etiological or diagnostic roles and that it is of the utmost importance that proposed biomarkers with confirmed involvement in the trait and state of mood and psychotic disorders be dissected to a point of absolute comprehension. We confirmed that monoamines remain a major biomarker for the pathophysiology of depression and, as the majority of clinically available and effective antidepressants still remain those that target monoaminergic signalling, correlates that are associated with said monoaminergic functioning was identified as strong markers of depression, forming the foundation of the neurochemical analyses applied in our investigation. The results from the current investigation confirm the hypothesis that exposure of FSL rats to a PTSD-like paradigm results in more severe depressive-like behaviour that is resistant to traditional antidepressant treatment, albeit responsive to treatment regimens that combine various mechanisms of antidepressant action. The model therefore provides an important example of a gene-x-environment approach to mimic TRD and provides a foundation for further investigation into the underlying pathophysiology responsible for treatment resistance in these animals.

Keywords: biomarkers, depression, FSL, gene-environment, imipramine, ketamine, noradrenalin, PTSD, serotonin, treatment resistance, venlafaxine

Opsomming

Die fokus van hierdie ondersoek was gesetel in die ontwikkeling en validering van 'n diermodel van behandelingsweerstandige depressie (BWD). Tydens 'n ondersoek na relevante biomerkers nom die konstrukgeldigheid van hierdie model te ondersteun, is 'n in-diepte literatuurstudie oor biomerkers voltooi en die bevindings daarvan hierby ingesluit. Vir publikasiedoeleindes is die reikwydte van die oorsig egter uitgebrei om biomerkers van gemoeds- én psigotiese toestande (d.i. depressie, bipolêre gemoedsteurnis en skisofrenie) in te sluit – hierdie dekking van psigotiese toestande word dus beperk tot die biomerkeroorsig (Manuskrip A) omdat die sentrale fokus van die studie wentel om depressie – meer spesifiek BWD. Ten spyte van die groot aantal pogings om die neurologiese onderbou van gemoeds- (depressie; bipolêre gemoedsversteuring) en –psigotiese afwykings beter te verstaan, berus die diagnose en evaluering van hierdie afwykings steeds slegs op die relatief subjektiewe assessering van simptome. Hierdie gebruik is deels verantwoordelik vir swak behandelingsresultate en behandelingsweerstandigheid – dus bestaan daar 'n dringende behoefte aan biologiese merkers wat gebruik kan word om die huidige klassifikasie van gemoeds- en psigotiese afwykings te verbeter en klinici in staat sal stel om hul pasiënte te kategoriseer en genoemde afwykings te diagnoseer in meer homogene, klinies-beduidende subgroepe. Die identifisering van biomerkers wat patofisiologiese prosesse in hierdie afwykings kwantifiseer en weerspieël, asook die ontwikkeling van diermodelle wat behandelingsweerstandigheid in pasiënte naboots, kan bydra ten einde hierdie doel te bereik.

Die hoë insidensie van gedeeltelike of selfs totale weerstandigheid teenoor behandeling in depressielyers skep 'n aansienlike struikelblok in die doeltreffende behandeling en hantering van die toestand. Ongeveer die helfte van depressielyers se toestand word nie effektief beheer na behandeling met eerste-linie antidepressiewe middels nie en tot en met 30% van pasiënte reageer glad nie op enige behandelingsstrategie nie. Net soos in die geval van depressie word daar vermoed dat BWD heterogeen van aard is en, alhoewel dit voorkom asof die meeste patofisiologiese faktore onderliggend aan depressie ooreenkom met dié in BWD, die meerderheid van hierdie faktore oordrewe is in BWD en daarom tot meer ernstige simptome aanleiding kan gee. 'n Tekort aan gepaste en gevalideerde diermodelle van BWD is 'n belangrike bydraende faktor tot ons huidige

gebrek aan kennis en begrip van die patofisiologie van die toestand en het gelei tot studies wat poog om die prosesse onderliggend aan behandelingsweerstandigheid in diermodelle te ondersoek.

Huidig word daar algemeen aanvaar dat genetiese vatbaarheid in kombinasie met ongunstige omgewingsomstandighede 'n belangrike voorloper is vir die ontwikkeling van depressie. Daarom kan diermodelle wat gebaseer is op hierdie konsep 'n belangrike bydrae lewer tot ons begrip van gemoeds- en angsversteurings.

Die Flinders-sensitiewelyn- (FSL) rot is 'n deeglik-bestudeerde genetiese diermodel van depressie met sterk konstruk-, sig- en voorspelbaarheidsgeldigheid. Deur die sterk ko-morbiditeit tussen depressie en posttraumatiese spanningsversteuring (PTSV), asook die feit dat depressie in pasiënte met PTSV meer behandelingsweerstandig is, in ag te neem, het ons 'n diermodel van BWD ontwikkel. Hierdie model is gebaseer op die uitgangspunt dat blootstelling van diere – geneties geneig tot depressie-agtige gedrag – aan 'n PTSV-verwante paradigma 'n model sal lewer met oordrewe en uitgesproke depressie-agtige gedrag met 'n gepaardgaande weerstandigheid teenoor tradisionele antidepressantbehandeling. Ten einde hierdie doelwit te bereik, het ons tydsafhanklike sensitisering (TAS) as 'n model van PTSV aangewend. TAS word gebaseer op 'n trauma-plus-samehangende-herinnering-beginsel en is bewys om goeie konstruk-, sig- en voorspelbaarheidsgeldigheid vir PTSV te openbaar.

In die eerste afdeling van die studie is die depressie-agtige fenotipe van die FSL-rot relatief tot dié van die Flinders-weerstandige-lyn (FWL)-rot bevestig en het blootstelling van FSL-rotte aan TAS gelei tot óf 'n ondersteunde óf volgehoue onderdrukking van uithougedrag en 'n toename in depressie-agtige gedrag en het dit ook gewysigde monoamienergiese profiele in die hippokampus en frontokortikale breinstreke veroorsaak. Boonop het die toevoeging van TAS in FSL-rotte die antidepressant-agtige effekte van imipramien op die meeste gedragsparameters (klim en beweeglosheid) in die geforseerde swemtoets asook limbiese serotonergiese aktiwiteit beduidend opgehef.

Geneesmiddelgesentreerde benaderings in die kliniese hantering van behandelingsweerstandigheid beklemtoon die gebruik van middels met verbeterde effektiwiteit sowel as die kombinasie van

middels met verskillende werkingsmeganismes. Om die voorspelbaarheidsgeldigheid van die model uit te brei, het ons subkroniese behandeling met óf 'n serotonien- en noradrenalienheropname-inhibeerder (SNHI), d.i. venlafaksien, óf 'n N-metiel-D-aspartaat (NMDA)-reseptorantagonis, d.i. ketamien, in TAS-blootgestelde FSL-rotte ondersoek.

Gevolglik het ons in die tweede afdeling van die studie bevind dat 'n gebrek aan reaksie nie nét met die tradisionele antidepressant, imipramien, waargeneem word nie, maar óók na behandeling met óf venlafaksien óf ketamien as monoterapie. Daarenteen het die kombinasie van óf venlafaksien óf ketamien met imipramien tot versterkte antidepressant-agtige effekte (soos bepaal in die geforseerde swemtoets), asook tot 'n gewysigde reaksie in monoamienergiese aktiwiteit in dié diermodel van BWD, gelei.

Samevattend het 'n deeglike oorsig van die literatuur daarop gedui dat gemoeds- en psigotiese afwykings tans geassosieer word met 'n menigte biomerkers waarvan die presiese etiologiese en diagnostiese rolle nog onduidelik is en dat dit van uiterste belang is dat biomerkers met bewese betrokkenheid in die kenmerke en simptome van gemoeds- en psigotiese afwykings ontleed word tot 'n punt waar dit ten volle verstaan word. Ons het bevestig dat monoamiene steeds 'n beduidende biomerker vir die patofisiologie van depressie is en, siende dat antidepressante wat monoamienergiese seingewing teiken, steeds effektief en in die meerderheid bly, het ons merkers wat geassosieer word met sodanige monoamienergiese seingewing geïdentifiseer as sterk biomerkers van depressie en het dit gevolglik die fondasie van die neurochemiese analises in ons huidige ondersoek, gevorm. Die resultate van die huidige ondersoek bevestig die hipotese dat blootstelling van die FSL-rot aan 'n PTSV-agtige paradigma aanleiding gee tot oordrewe depressie-agtige gedrag wat weerstandig is teenoor tradisionele antidepressantbehandeling, ofskoon sensitief is teenoor 'n behandelingsregimen wat verskeie meganismes van antidepressiewe werking kombineer. Die model bied daarom 'n belangrike toonbeeld van 'n geen-x-omgewing-benadering om BWD na te boots en bied 'n grondslag vir verdere ondersoeke na die onderliggende patofisiologie verantwoordelik vir behandelingsweerstandigheid in hierdie diere.

Sleutelwoorde: behandelingsweerstandigheid, biomerkers, depressie, FSL, geen-omgewing, imipramien, ketamien, PTSV, venlafaksien

Congress Proceedings

Results from the current investigation were presented as follows (presenting author underlined):

BRAND, S.J., WEGENER, G., HARVEY, B.H. Time dependent sensitisation exaggerates depressive-like symptoms in Flinders sensitive line (FSL) rats, a genetic animal model of depression. "28th Annual European College of Neuropharmacology Congress": 29 August – 1 September 2015, Amsterdam, The Netherlands

BRAND, S.J., HARVEY, B.H. Exploring stress re-stress as a mechanism to exacerbate depressive-like symptoms and induce antidepressant treatment resistance in Flinders Sensitive Line (FSL) rats. "2nd African College of Neuropsychopharmacology Congress": 30 - 31 July 2016, Cape Town, South Africa

Publications

Results from the current investigation have been published as follows:

BRAND, S.J., MOLLER-WOLMARANS, M., HARVEY, B.H. 2015. A review of biomarkers in mood and psychotic disorders: A dissection of clinical vs. preclinical correlates. *Current Neuropharmacology* (13)3: pp 324-368 (DOI: 10.2174/1570159X13666150307004545).

BRAND, S.J., WEGENER, G., HARVEY, B.H. 2015. P.1.h.014 Time dependent sensitisation exaggerates depressive-like symptoms in Flinders sensitive line (FSL) rats, a genetic animal model of depression. *European Neuropsychopharmacology* (25), pp. S288-S289.

BRAND, S.J., HARVEY, B.H. 2016. Exploring a post-traumatic stress disorder paradigm in Flinders sensitive line rats to model treatment resistant depression I: Bio-behavioural validation and response to imipramine. *Acta Neuropsychiatrica* pp. 1-14 (DOI: 10.1017/neu.2016.44).

BRAND, S.J., HARVEY, B.H. 2016. Exploring a post-traumatic stress disorder paradigm in Flinders sensitive line rats to model treatment resistant depression II: Response to antidepressant augmentation strategies. *Acta Neuropsychiatrica* pp. 1-15 (DOI: 10.1017/neu.2016.50).

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"Want ek loop in my pa se skoene,
ek loop in my pa se jas.
Ek weet ná soveel jare
'n pa het 'n hart van glas"
~ Coenie de Villiers

Dankie dat Mamma se hart altyd wawyd oop is en ons altyd eerste gekom het.

"want jy maak my groot
in jou krom klein handjies
jy beitel my met jou swart oë
en spits woorde
jy draai jou leiklipkop
jy lag en breek my tente op
maar jy offer my elke aand
vir jou Here God
jou moesie-oor my enigste telefoon
jou huis my enigste bybel
jou naam my breekwater teen die lewe"
~ Antjie Krog

***

"*Life was meant for good friends and great adventures*"

De Wet, jy is 'n kosbare vriend. Dankie dat jy in my glo en my soveel gun in die lewe. By jou hoef ek nooit my gedagtes te weeg of my woorde te meet nie – dankie dat jy luister selfs wanneer ek nie praat nie.

"*The glory of friendship is not the outstretched hand, nor the kindly smile nor the joy of companionship; it is the spiritual inspiration that comes to one when he discovers that someone else believes in him and is willing to trust him.*" ~ *Ralph Waldo Emerson*

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Table of Contents

CHAPTER 1

1 Introduction

1.1 Thesis layout

The current thesis is compiled in article format, as prescribed and approved by the North-West University. The main body of the thesis is presented as three manuscripts that have been published in international, peer reviewed neuroscience journals.

Chapter 1 provides a concise description of the project problem statement, study questions, aims, expected outcomes and a framework of the study layout, while Chapter 2 covers the literature background supporting the current project. The first manuscript (Manuscript A, Chapter 3) titled *'A Review of Biomarkers in Mood and Psychotic Disorders: A Dissection of Clinical vs. Preclinical Correlates'*, is a comprehensive review that has been first authored by the candidate and assisted by two co-authors. Chapters 4 and 5 contain the key findings of the current investigation in two separate manuscripts that have been published as companion papers by the same journal (Manuscripts B and C), first authored by the candidate and assisted by one co-author. These manuscripts have been prepared according to the 'Instructions to Authors' provided by each journal (indicated at the beginning of each chapter) and will be presented as such. Chapter 6 summarizes the key findings of the project and concludes the study as a whole. The addendums contain 'Instructions to Authors' from the different journals, letters of permission of co-authors for subjecting manuscripts A – C for examination purposes, and confirmations of article publications.

1.2 Candidate, study supervisor and co-supervisor contributions to the thesis:

- Sarel J. Brand conceptualized and executed all aspects of the experimental work contained in the thesis and interpreted the results of the experimental work. He wrote and compiled the initial versions of all the chapters as well as the final version of the thesis.
- Linda Brand was study co-supervisor and assisted in the planning of the project and translation of the abstract from English to Afrikaans. She also proofread the thesis in preparation for its final version.
- Brian H. Harvey was the study supervisor and assisted in the planning and funding of the project. He also assisted in the interpretation of results of the experimental work and proof read the thesis in preparation for its final version.

The contributions of each of the authors to the publications emanating from this investigation are provided on the title pages of each of the Manuscripts A – C.

1.3 Problem statement and hypothesis

The treatment of depression is based on theories developed over 60 years ago when the first generation of antidepressants was discovered by chance (Kuhn, 1958). These drugs were later shown to act on monoamines and ever since the treatment of depression has focused primarily on modulation of monoaminergic systems. A major problem with these classes of drugs is that they have a slow onset of action and are fairly inefficient, inducing a response rate of approximately 50% (Nestler *et al.*, 2002). Therefore, up to half of depressed patients are considered to be treatment resistant. While subsequent treatment steps have remained largely speculative in the past, the STAR*D study has put forward empirically based treatment steps for the later stages of TRD (Rush *et al.*, 2006). This study was mainly restricted to monoaminergic modulating antidepressants that were associated with extremely low response rates in the final stages of the study, imposing practical limitations on treatment options for patients demonstrating complete resistance to current therapies. As such, an important need for drugs acting via completely novel mechanisms was highlighted which resulted in an exponential growth in research during the last decade with respect to novel antidepressant agents demonstrating improved time-to-onset-of-action as well as improved efficacy (Duman *et al.*, 2012). Among the most exciting approaches in this regard has been the use of dual serotonin-noradrenalin reuptake inhibitors like venlafaxine, and more recently the use of glutamatergic modulators, particularly those acting as antagonists of the NMDA receptor (Machado-Vieira *et al.*, 2009).

Despite significant research efforts aimed at understanding the neurobiological underpinnings of mood and psychotic disorders, the diagnosis and evaluation of the treatment of these disorders are still based solely on relatively subjective assessment of symptoms as well as psychometric evaluations. We therefore set out to identify the most promising biomarkers that could potentially aid in the development of biomarker panels that may assist physicians to categorize their patients.

Venlafaxine has been found to be slightly more effective than several SSRIs in patients with severe MD (Bauer *et al.*, 2013, Smith *et al.*, 2002) and acts by increasing both serotonergic and noradrenergic activity (Smith *et al.*, 2002). This provides an advantage over drugs only acting on 5HTergic

mechanisms, seeing that drug-centred approaches to manage TRD emphasize the combination of drugs with different mechanisms of action (Culpepper *et al.*, 2015, Philip *et al.*, 2010) and thus makes it a popular treatment choice in patients resistant to SSRI treatment (Rush *et al.*, 2006). Also, despite similar actions to, for example, imipramine on NA and 5HT neuronal reuptake, venlafaxine boasts a "cleaner" receptor affinity profile (Muth *et al.*, 1986) than imipramine that has a high affinity for other neuronal receptors, such as the $5HT_{1A}$ receptors (Haddjeri *et al.*, 1998).

The glutamatergic system has been directly linked to processes of neuroplasticity, a process demonstrated to be essential to attain optimal antidepressant effect (Li *et al.*, 2010). The monoaminergic systems are far removed from these neuroplastic events, thereby possibly contributing to the delay in antidepressant effects of drugs that target these systems, ultimately culminating in their limited overall clinical efficacy (Berton *et al.*, 2006). However, the direct association of the glutamatergic system to neuroplasticity translates into rapid and effective antidepressant properties for modulators of these pathways (Autry *et al.*, 2011). Indeed, the non-selective NMDA antagonist, ketamine, displays a rapid *and* sustained antidepressant response in MD and high antidepressant efficacy rates in TRD patients following administration of a single dose (Berman *et al.*, 2000, Zarate Jr *et al.*, 2006). More recently, however, several studies have also applied repeated dosing strategies in TRD patients which achieved superior outcomes compared to single administration approaches (Aan het Rot *et al.*, 2010; Murrough *et al.*, 2013; Shiroma *et al.*, 2014). Likewise, in preclinical studies, chronic ketamine treatment has also been applied in rats using the FST compared to known antidepressants (Owolabi et al., 2014) and also in animals exposed to CMS (Garcia *et al.*, 2009, Zhang *et al.*, 2015, Parise *et al.*, 2013) where repeated ketamine treatment was associated with long-term anxiolytic- and antidepressant-like effects (Parise *et al.*, 2013). Unfortunately, the psychotomimetic properties and abuse potential of ketamine has prevented its clinical acceptance as a mainstay antidepressant (Berman *et al.*, 2000) and motivated the search for compounds resembling the antidepressant, *but not adverse*, properties of ketamine.

However, research into drugs effective for TRD is hindered by the lack of a suitable animal model. An ideal animal model for testing putative antidepressant compounds should closely resemble response rates observed for current antidepressants in TRD in order to elucidate the existence of

improved efficiencies of novel agents. To date, a putative animal model that most closely resembles clinical TRD statistics is based on the chronic mild stress (CMS) paradigm (Willner, 1997). However, this model has distinct limitations, i.e. being labour intensive and displaying poor reproducibility (Samuels *et al.*, 2011), and hence there is a great need for the development of a more robust animal model of TRD.

Therefore, the current study will consider the gene-x-environmental aetiology of depression in developing an animal model of TRD by combining a genetic model of depression, i.e. the Flinders Sensitive Line (FSL) rat (Overstreet *et al.*, 2013), with an environmental stress model of PTSD, i.e. time-dependent sensitization (TDS) (Oosthuizen *et al.*, 2005). Both models have robust face, construct and predictive validity for the respective human illnesses that they are modelling, *viz*. depression and PTSD (Overstreet *et al.*, 2013, Harvey *et al.*, 2003, Liberzon *et al.*, 1997, Harvey *et al.*, 2006). The development of the TRD model is based on the strong correlation between treatment resistance and comorbidity of depression and anxiety disorders (Papakostas *et al.*, 2008, Fava *et al.*, 2008), *especially* PTSD (Kessler *et al.*, 1995). We hypothesize that exposure of a genetically susceptible animal to a severely traumatic event should result in enhanced depressive-like behaviours that will be resistant to standard antidepressant treatments. Furthermore, development of a suitable TRD animal model would provide an ideal platform to test combination strategies that may have therapeutic capabilities to treat TRD, but also future studies investigating novel compounds.

We therefore propose that 1) based on our review of the current literature, we would be able to identify the most promising biomarkers of MD and other psychiatric disorders and, as such, be able to select appropriate biomarkers to apply as a measure of the underlying pathophysiology and treatment response in the current investigation. Moreover the combined FSL-TDS model will 2) demonstrate resistance to a traditional first line antidepressant treatment option, *viz*. imipramine, 3) show strong concordance with the molecular constructs suspected to underlie the neurobiology of TRD in humans, 4) demonstrate enhanced response to the STAR*D advocated level 2 drug, venlafaxine compared to imipramine, 5) demonstrate enhanced response to drugs that selectively target components of the glutamate-NMDA signalling cascade, *viz*. ketamine, and 6) demonstrate

an augmented response following combined treatment of either imipramine plus venlafaxine or imipramine plus ketamine.

1.4 Study questions

The present investigation was designed to address the following study questions pertaining to the validation and translational application of a putative animal model of TRD:

1) Manuscript A: Has the understanding of mood and psychotic disorders developed to such an extent that physicians may soon be able to stratify patient diagnoses and subsequent treatment according to results obtained from biomarker panels?
2) Manuscript A: Could this review of the current body of literature assist in the selection of specific biomarkers to analyse in the current investigation?
3) Manuscript B: Will FSL rats present with depressive-like behaviour and an altered monoaminergic profile relative to FRL rats?
4) Manuscript B: Will the combination of a genetic animal model of depression, i.e. the FSL-rat, with a PTSD stress paradigm, i.e. TDS, result in a model that resembles the behavioural and neurobiological deficits observed in clinical TRD, compared to TDS-naïve FSL animals?
5) Manuscript B: As in the case of clinical TRD, will such an animal model be non-responsive to sub-chronic treatment with a traditional drug indicated for the treatment of major depression, e.g. imipramine?
6) Manuscript C: Furthermore, if such deficits as described in (4) and (5) are demonstrated, will the bio-behavioural deficits observed in FSL+TDS rats respond to sub-chronic treatment with a drug advocated to have improved efficacy (SNRI; e.g. venlafaxine)?
7) Manuscript C: If such deficits as described in (4) and (5) are demonstrated, will the bio-behavioural deficits observed in FSL+TDS rats respond to sub-chronic treatment with a drug advocated to have improved efficacy (SNRI; e.g. venlafaxine)?
8) Manuscript C: Will augmentation therapy, i.e. treatment with combinations of imipramine with either venlafaxine or ketamine result in greater behavioural and neurobiological responses compared to either of the three drugs alone?

1.5 Project aims

To address the study questions of the current investigation we will aim to:

- develop a gene-x-environment animal model of TRD by exposing a genetic animal model of depression, *viz.* the FSL rat, to a stress paradigm related to PTSD, *viz.* TDS;
- identify relevant biomarkers (by means of an in-depth literature review on the subject) that may support the construct validity of such a model;
- elucidate the behavioural and neurobiological physiognomies of such a model and compare these to findings from TDS-naïve FSL control rats. This will be attained by:
    - measuring performance of individuals in the open field test (OFT) and forced swim test (FST) (Porsolt *et al.*, 1978), validated screening tests for measuring locomotor- and depressive-like behaviours, and
    - determining frontal-cortical and hippocampal concentrations of noradrenalin (NA) and 5-hydroxyindoleacetic acid (5HIAA), a reliable marker of serotonergic neurotransmission (Maes *et al.*, 1999)
- establish whether the behavioural and neurobiological aberrations observed in TDS-exposed, *but not* TDS-naïve FSL animals, demonstrate resistance to 7-day imipramine (10 mg/kg/day) administration, a traditional 1st line treatment for MD.
- determine whether TDS-exposed animals will demonstrate improved behavioural response as measured in the OFT and FST to 7-day treatment with venlafaxine (10 mg/kg/day) or ketamine (10 mg/kg/day) and therefore emulate the treatment response of clinical TRD;
- determine whether co-administration of imipramine with either venlafaxine or ketamine (all in concentrations of 10 mg/kg/day) will result in an augmented behavioural response compared to that achieved by either drug administered as monotherapy;
- associate any changes in the behavioural response measured with specific changes in monoaminergic functioning by comparing the frontal-cortical and hippocampal NA and 5HIAA concentrations measured in each of the TDS-exposed treatment groups to that of TDS- and treatment naïve FSL animals.

1.6 Project layout

Outline of TDS paradigm and treatment administration

To address the study questions as explained above, the current project has been divided into two main sections, employing male FRL and FSL animals (40 days of age) randomly divided between the separate groups. Subsequently animals were either subjected to a TDS protocol (as outlined in Figure 1-1) or left unstressed prior to bio-behavioural analysis (please refer to Tables 1-1 and 1-2).

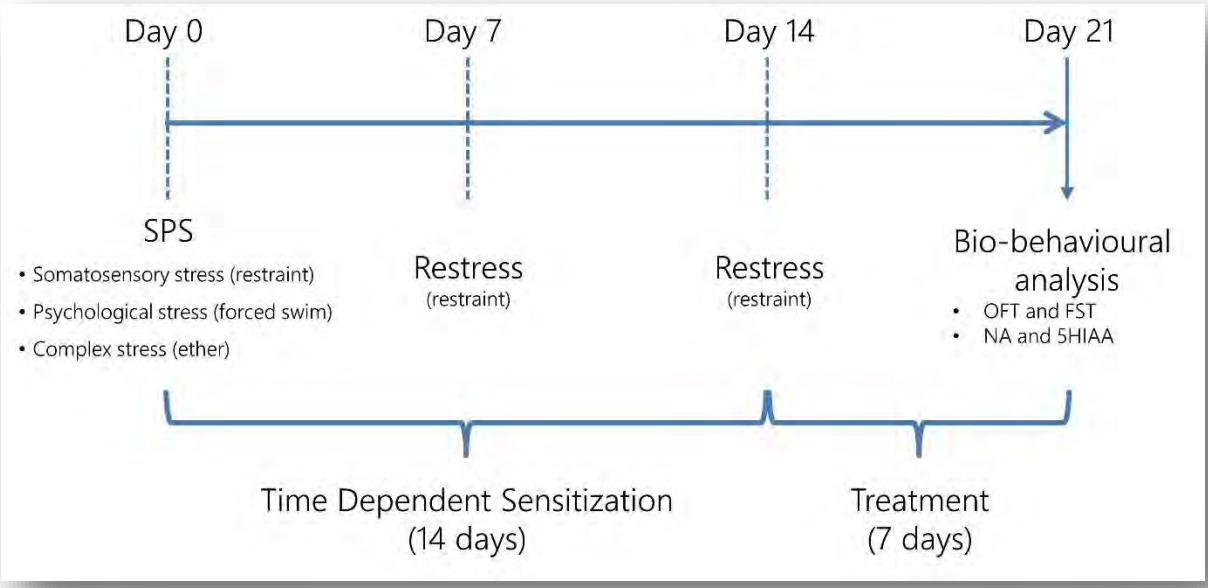

Figure 1-1: Schematic outline of the TDS procedure. At the start of the procedure (indicated as Day 0), rats are exposed to single prolonged stress (SPS) – a triple stressor sequence comprising a somatosensory stressor (restraint), a psychological stressor (forced swimming with brief submersion), and a complex stress-stimuli (exposure to ether vapours) followed by re-exposure to restraint stress, as a situational reminder of the initial SPS procedure, 7 and 14 days later. After the final restress, animals are left undisturbed for 7 days before performing behavioural assessments (OFT and FST) and monoaminergic analyses (NA and 5HIAA)

5HIAA: 5-hydroxyindoleacetic acid; FST: forced swim test; NA: noradrenalin; OFT: open field test; SPS: single prolonged stress; TDS: time-dependent sensitization

Section 1 (Manuscript B)

Behavioural, neurochemical and pharmacological validation of the TRD model.

In Section 1, experimental groups were structured as described in Table 1-1. Unstressed vehicle-treated FRL rats (the more resilient, stress-resistant counterpart of the FSL) served as a control to unstressed vehicle-treated FSL rat to confirm the depressive-like stereotype of the FSL rat. For further details, please refer to Manuscript B.

Table 1-1: Treatment Layout (Section 1)

	Group
Behavioural Analysis (n = 12 per group)	FRL (n/s; vehicle)
	FSL (n/s; vehicle)
	FSL (n/s; imipramine)
Neurochemical Analysis (n = 8 per group)	FSL (TDS; vehicle)
	FSL (TDS; imipramine)

Section 2 (Manuscript C)

Behavioural and neurochemical characterisation of TRD model following administration of novel drug treatment and augmentation strategies.

In Section 2, experimental groups were structured as described in Table 1-2. This section will only employ TDS exposed FSL animals. For further details, please refer to Manuscript C.

Table 1-2: Treatment Layout (Section 2)

	Group
Behavioural Analysis (n = 12 per group)	Vehicle
	Imipramine
	Venlafaxine
	Imipramine+ Venlafaxine
Neurochemical Analysis (n = 8 per group)	Ketamine
	Imipramine + Ketamine

1.7 Ethical considerations

The AnimCare animal research ethics committee (NHREC reg. number AREC-130913-015) of the North-West University approved all experiments. Animals were bred, supplied, and housed at the Vivarium (SAVC reg. number FR15/13458; SANAS GLP compliance number G0019) of the Pre-Clinical Drug Development Platform of the North-West University. All 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-00111-12-A5).

The investigation was evaluated by the supervisor, co-supervisor and investigator with respect to the so-called 3R guidelines: Replace, Refine and Reduce.

Replace: The aim of the study was to develop and validate an animal model of TRD, making the use of animals a necessity. However, careful consideration was given to the selection of the strains of animals (FSL and FSL) used in this investigation subsequent to a thorough review of the available literature, as expressed in this thesis. Also, although TRD has a prominent prevalence in female patients, the use of female rats in developing translational models of psychiatric disorders (especially MD) poses a very well-known complexity when considering both physiological and biological variances induced by the estrous cycle (Slattery and Cryan, 2014). These include discrepancies in drug metabolism (Kokras *et al.*, 2011), oxytocin receptor (OXT-R, which may influence stress response on both psychological and physiological levels (Marusak *et al.*, 2015)) expression (Bale *et al.*, 1995) and HPA-axis activity (Atkinson and Waddell, 1997). As a result, the majority of preclinical studies on MD employ male subjects (Slattery and Cryan, 2014). Hence, this approach was also followed in the current study.

Refine: Group layout and animal numbers were empirically based. Furthermore, the layout was structured in such a way as to prevent the duplication of data sets and to employ the smallest number of animals while ensuring that sufficient data points would be guaranteed for reliable statistical analysis.

Reduce: A reductionist approach was taken in the layout of the study, as discussed above in "Refine" to ensure that as few subjects as possible were employed during the investigation.

1.8 Expected results

Study Question	*Expected Result*
1 *(Manuscript A)*	We predict that recent advances in sampling and analysis techniques as well as an abundance of current literature on the preclinical and clinical correlates of major depressive disorder, will contribute to a better understanding of robust and reliable markers of depression that will aid the clinician to accurately discern between different sub-types of psychiatric disorder according to its specific biochemical construct.
2 *(Manuscript A)*	Following from the above, we hypothesize that both clinical and pre-clinical correlates of major depressive disorder would be separated into clusters of poor, weak and strong markers of depression based on the significance and consistency of their associations with clinical depression. We further hypothesize that, as the majority of clinically available and effective antidepressants still are those that target monoaminergic signalling, correlates that are associated with said monoaminergic functioning would be identified as strong markers of depression, forming the foundation of the neurochemical investigations of the current study.
3 *(Manuscript B)*	In line with literature demonstrating the depressive-like traits of the FSL rat, they will demonstrate depressive-like behaviours vs. FRL controls, as characterized by altered behaviour in the forced swim test (FST). Furthermore, we hypothesize that FSL animals will demonstrate alterations in markers of monoaminergic signalling compared to FRL control animals.
4 *(Manuscript B)*	The combination of a genetic animal model of depression, *viz.* the FSL rat, with a PTSD-like stress paradigm, *viz.* TDS, will result in an animal model that presents with enhanced depressive-like behaviour and monoaminergic deficits vs. TDS-naive FSL rats, thus resembling the behavioural and neurobiological aberrancies observed in clinical TRD.

5 *(Manuscript B)*	TDS-exposed, but not TDS naive FSL rats, will be non-, or at best only partially responsive to imipramine, a traditional antidepressant.
6 *(Manuscript C)*	TDS-exposed FSL rats will not respond, or at best show a partial behavioural response, to sub-chronic monotherapy treatment with new generation antidepressants, *viz.* venlafaxine and ketamine.
7 *(Manuscript C)*	TDS-exposed FSL rats will not respond, or at best show a partial neurochemical response, to sub-chronic monotherapy treatment with new generation antidepressants, *viz.* venlafaxine and ketamine.
8 *(Manuscript C)*	Augmentation of the traditional antidepressant, imipramine, with either venlafaxine or ketamine will counter resistance to treatment observed in monotherapeutic approaches, resulting in significant antidepressive-like behavioural effects in the FST and appropriate alterations in cortico-limbic monoaminergic response.

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CHAPTER 2

2 Literature Review

2.1 Introduction

Major depression, as an illness, has been described, defined and redefined during the 20th century, eventually resulting in a seemingly well-construed mood disorder. Despite this, major depression has historically taken on many different appearances and has been associated with various afflictions.

The ancient Greeks and Romans described depression as melancholia – meaning "black bile" and although the proposed biological origins of depression by the likes of Aristotle and Hippocrates (Akiskal *et al.*, 2007) was influenced by astronomy (Pagel, 1965) coupled with elementary knowledge of biochemistry, observations of patients suffering from the disorder closely relate to the well-structured symptomatology described by the Diagnostic and Statistical Manual (fifth edition; DSM-V) today (Akiskal *et al.*, 2007). These ancient physicians also observed a relationship between depression and anxiety (Nestler *et al.*, 2002) – a connection which has been well characterized in recent years. Since the introduction of standard criteria to be used in the diagnosis of mental disorders in the DSM, depression has been re-conceptualized as major depression (MD) with patients suffering from less severe symptoms being diagnosed with dysthymia (Nestler *et al.*, 2002).

Several decades have passed since the discovery of prototypic antidepressant drug classes, *viz.* the tricyclic antidepressants (TACs) and the monoamine oxidase inhibitors (MAOIs). Once these agents were added to the treatment strategy of MD, the management and treatment outcomes of the disorder were forever changed. However, despite the frequent use of these drugs, they are reckoned to be at best only 65% effective, are plagued by troublesome side effects, e.g. sleep abnormalities, GIT disturbances, sexual dysfunction and several anticholinergic effects (Bet *et al.*, 2013, Kikuchi *et al.*, 2012), and have a notoriously slow onset of action, ranging from several weeks to months (Fava, 2003, Holtzheimer *et al.*, 2006, Machado-Vieira *et al.*, 2009, Nestler *et al.*, 2002). Moreover, since then only modest advances have been made in developing novel drugs with improved efficacy, while the exact mechanisms by which these drugs mediate their mood elevating effects still remain controversial (Holtzheimer *et al.*, 2006). Shortfalls in understanding the genetic and neurobiological foundations of MD further add to the complexity of the disorder (Nestler *et al.*, 2002), compelling

one to define MD as a multifactorial illness, comprising not only genetic and environmental determinants but also consisting of mood, cognitive, endocrine and neuronal abnormalities (Krishnan *et al.*, 2008, Nestler *et al.*, 2002).

2.2 Aetiology and Pathophysiology

Pinpointing the exact causes of MD has proven to be an arduous task and, as a result, consensus about the exact causative elements of MD remains elusive. Instead, as noted above, it has become generally accepted that the aetiology of MD is multifactorial while development of the disorder strongly correlates with prior exposure to stressful life events, genetic risk (heritability ≈40%), and the presence of various genes which may predispose an individual to its development (Fava *et al.*, 2000, Kendler *et al.*, 2001) (refer to Manuscript A for a discussion on this subject). Furthermore, the development of MD may also be idiopathic, resulting from a drug induced side-effect (e.g. interferon-α or isotretinoin) or manifesting secondary to systemic illness (Drevets, 2001, Nestler *et al.*, 2002). To date the pathogenesis of MD has been ascribed to abnormal hypothalamic-pituitary-adrenal (HPA) axis activity (Belvederi Murri *et al.*, 2014) or altered monoaminergic signalling (Haase *et al.*, 2015), as well as abnormal neurotrophic signalling and abnormal hippocampal neurogenesis (Krishnan *et al.*, 2008).

The prevalence of MD has increased steadily despite economic development, improved health care and a thriving antidepressant industry (Lambert, 2006) – an increase which may be attributed to several factors, including industrialization, a higher incidence of stress-exposure at a tender age (Robinder, 1999), the progressive occurrence of physical inactivity, unhealthy diets (Beydoun *et al.*, 2010, Thomson *et al.*, 2010) and chronic lifestyle related illnesses such as type 2 diabetes mellitus, cardiovascular disorders, hypertension and obesity (Patten, 2005). When considering the vast biological and non-biological processes, environmental aspects and various risk factors that may contribute to the development of MD in any given individual, it seems unlikely that MD could be associated with any one specific cause (Hankin, 2006, Maja *et al.*, 2010). This has effectively prevented attempts to develop a unified theory of the disorder and as such, MD is now regarded as a heterogeneous disorder (Krishnan *et al.*, 2008).

As eluded to earlier, it is generally accepted that the onset of MD results when a genetically susceptible individual is exposed to adverse environmental situations such as stressful life events – an observation that has led to the gene-by-environment hypothesis of MD (Caspi *et al.*, 2003). This interaction is generally associated with the onset of lifestyle related illnesses involving the cardiovascular and neuroendocrine systems. Indeed, it is interesting that certain drugs used to treat the aforementioned disorders, such as certain hypoglycaemic agents (e.g. pioglitazone) (Sadaghiani *et al.*, 2011), antihypertensive agents (e.g. the ACE-inhibitor, captopril) (Giardina *et al.*, 1989), and anti-inflammatory agents (e.g. celecoxib) (Vital *et al.*, 2013) have also been found to have antidepressant-like effects in animals and to improve mood in humans (Chen *et al.*, 2010, Deicken, 1986, Kemp *et al.*, 2009). But beyond this, the complex neurobiology of MD is further illustrated by the different treatment methods currently available that act on different pathways. This was clearly demonstrated in serotonin (5-HT) 1A receptor knockout mice which were found to respond to TCAs but remained insensitive to selective serotonin reuptake inhibitor (SSRI) treatment, thereby indicating that independent molecular pathways may be involved during serotonergic and adrenergic changes associated with antidepressant response (Santarelli *et al.*, 2003). Paradoxical effects are even found within the *same* pathway, e.g. antidepressant effects of agents with opposite actions on the serotonergic system, *viz.* SSRIs vs. tianeptine that, respectively, increase and decrease extracellular levels of 5HT (Wagstaff *et al.*, 2001).

2.2.1 Genetic risk

It has become quite evident that MD frequently affects several individuals from the same family – observations which were confirmed by the results of a meta-analysis of the genetic epidemiology of MD performed by Sullivan *et al.* (2000), providing substantial supportive data to characterize MD as a familial disorder – this feature primarily being a genetic consequence.

As mentioned earlier, the development of MD is a result of a combination of gene and environment rather than either one alone (Sullivan *et al.*, 2000). Instead of only focussing on the discovery of susceptibility genes, more recent research has explored the influence of environmental risks on gene reactivity (Moffitt *et al.*, 2005). An initial report of patients expressing neurotic behaviour carrying a low-expressing short (S) allele of the 5-HT transporter linked polymorphic region (5-HTTLPR) (Lesch *et al.*, 1996) catapulted research into genetic variants in psychiatric disorders and the finding was

later underscored by the fact that patients carrying the S-allele presented with heightened fear responses (Hariri *et al.*, 2002) and depressive tendencies after exposure to stressful circumstances (Caspi *et al.*, 2003). Furthermore, the presence of two copies of the short allele is associated with an increase in stress sensitivity (Kendler *et al.*, 2005) and the absence thereof with a decreased incidence of MD and anxiety (Weissman *et al.*, 2005). Another popular marker of note in MD is the Met allele on the Val66Met polymorphism of brain-derived neurotrophic factor (BDNF) and has, interestingly, also been associated with individuals' responses to adverse events (Aguilera *et al.*, 2009).

However, despite the discovery of a substantial amount of potential genetic markers, genome-wide association studies (GWAS; including 9 000 subjects) have failed to isolate specific candidate genes, exemplifying the complexity of MD and the difficulty in pin-pointing relevant gene-gene and gene-environment interactions (Hek *et al.*, 2013, Major Depressive Disorder Working Group of the Psychiatric, 2013).

2.2.2 Exposure to stress

Glucocorticoids facilitate adaptation to stress and restore homeostasis under physiologic conditions (de Kloet *et al.*, 2008). However, under pathological conditions regulation of these hormones may become distorted resulting in detrimental effects. The physiological response to stress is regulated by the HPA axis and hyperactivity of this system is a consistent abnormality observed in MD, resulting in elevated levels of circulating glucocorticoids (Porter *et al.*, 2006). Considering previous mention of increased stress sensitivity in individuals predisposed to develop MD, this system may be viewed as a potential chink in the armour, protecting against stress-related pathology. Activity of the HPA axis is largely regulated by negative feedback mechanisms involving the hippocampus. The hippocampus expresses large numbers of glucocorticoid receptors which, upon activation, induce an increase in inhibitory neurotransmission within the HPA axis via activation of hippocampal neurons (de Kloet *et al.*, 2008). Therefore, a rise in glucocorticoids will invariably lead to reduced HPA axis activity. One of the major causes of elevated glucocorticoid levels is exposure to prolonged and severe stressors (Smith *et al.*, 1995). MD is characterised by hippocampal shrinkage that has been ascribed to hypercortisolemia and the aforementioned elevated presence of glucocorticoid receptors (Campbell *et al.*, 2004). Damage to the hippocampus compromises its ability to regulate

the stress response (Gilbertson *et al.*, 2002) and thus creates a vicious cycle of HPA axis activation resulting in further hippocampal damage.

2.2.3 Neural pathway abnormalities in major depression

Several biomarkers that reflect pathophysiologic processes evident in MD (and other mood and psychotic disorders) have been identified to date and are discussed here. For additional information, the reader is referred to Chapter 3 (Manuscript A; Brand *et al.*, 2015) in which these molecules and processes are reviewed extensively. Additionally, what this review has attempted to do is to consider putative biomarkers of mood and psychotic disorders and the correlation, if any, between animal models and the human disorder. It also discusses the relevance of individual markers as well as their potential application in clinical practice as utilities in the improved treatment and diagnosis of mood and psychotic disorders.

2.2.4 The monoamines

For a substantial part of the past decades, our understanding of the pathophysiology of MD was dominated by the amine hypothesis which postulates that MD is caused by a deficit in monoamine function in the brain, specifically noradrenalin (NA) and 5-HT (Berton *et al.*, 2006). Both are widely distributed throughout the brain, with notable prominence in the reward and cortico-limbic regions of the brain, including the ventral striatum, hippocampus, frontal cortex, hypothalamus, amygdala and olfactory bulb. The dilemma with this perspective, however, is that drugs which enhance monoaminergic function, including MOAIs, TCAs and serotonin reuptake inhibitors (SRIs), elicit acute molecular effects (Krishnan *et al.*, 2008) that do not translate into prompt behavioural effects. In fact, the mood-enhancing effects of currently prescribed antidepressant drugs may take several weeks or even months to achieve their full effect (Machado-Vieira *et al.*, 2009). Nevertheless, the fact that almost all currently used antidepressants act via monoamine receptors or related processes, reinforces the important construct validity of this hypothesis (Brand *et al.*, 2015).

2.2.5 Cholinergic-adrenergic regulation

MD has also been hypothesized to be a result of imbalances between central cholinergic and adrenergic neurotransmitter activity in areas of the brain tasked with regulating affect, with MD being

a disease of cholinergic dominance (Janowsky *et al.*, 1972). Contrary to this, mania may be the result of elevated noradrenergic, compared to cholinergic, transmission (Fritze *et al.*, 1995).

Cholinergic neurotransmission innervates both the hippocampus and frontal cortex (Mash *et al.*, 1986, Spencer Jr *et al.*, 1986) and influences attention, learning and memory (Everitt *et al.*, 1997, Sarter *et al.*, 1999) – indeed, MD is also associated with deficits in cognitive processes, that are largely influenced by cholinergic function (Deutsch, 1971, Jerusalinsky *et al.*, 1997).

In further attempts to clarify the role of the cholinergic system, it was also revealed that cholinomimetic agents induce depressive symptoms, such as anhedonia, in healthy volunteers (Risch *et al.*, 1981). Indeed a number of antidepressants, such as citalopram (Egashira *et al.*, 2006) and vortioxetine (David *et al.*, 2016) have been reported to improve cognitive deficits by an ability to *enhance* acetylcholine release. Furthermore, the cholinergic system has been suggested to exert mood modulating effects via its interaction with other signalling systems, such as the nitric oxide-cyclic guanosine monophosphate (NO-cGMP) pathway, where it has been proposed to play an important role in the antidepressant effects of phosphodiesterase-5 (PDE-5) inhibitors (Brink *et al.*, 2008, Liebenberg *et al.*, 2010). However, despite evidence of cholinergic involvement in MD and antidepressant action, there have also been a number of inconsistencies in the literature involving cholinergic-based drug therapies for the treatment of MD (Dagyte *et al.*, 2011, Ferguson *et al.*, 2000, Gatto *et al.*, 2004, Goldman *et al.*, 1983, Shytle *et al.*, 2002) – foremost among these being that anticholinergic agents have proved ineffective as antidepressants even though MD is said to underlie a hyper-cholinergic state (Fritze *et al.*, 1995, Gillin *et al.*, 1995, Goldman *et al.*, 1983). Keeping the heterogeneity of MD in mind, the diverse underlying mechanisms involved in the pathogenesis of the disorder may possibly explain the unpredictable response to anticholinergic therapy. Importantly, centrally acting anticholinergics may be rapidly effective in treatment resistant depression (TRD) (Drevets *et al.*, 2010, Furey *et al.*, 2006, Furey *et al.*, 2010) which underscores the involvement of the cholinergic system in MD. Moreover, a widely applied genetic animal model of MD, the Flinders sensitive line (FSL) rat, presents with increased activity of the cholinergic system in a number of limbic brain regions (Overstreet *et al.*, 2005). Therefore, despite evidence for an involvement in MD, the precise role of acetylcholine in this regard is unclear.

2.2.6 Structural abnormalities – the roles of stress and neurotrophic factors

The brain has the ability to undergo structural alterations in reaction to various stimuli (Duman, 2002) and where this process fails to function normally, several neuroplastic changes may result, e.g. loss of synaptic interactions, increased atrophy and apoptosis, suppressed neural cell proliferation and changes in receptor density (Duman, 2002). Various other molecules have also been implicated, including cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) and BDNF – that are also altered by stress (Chapter 3, Manuscript A, Figure 2) and antidepressant treatment (Duman, 2002).

The hippocampus, a prominent brain structure intimately involved in the neurocircuitry of MD, has been demonstrated to undergo small but significant volume reductions of 10-15% in depressed patients (Campbell *et al.*, 2004). The hippocampus, a central component of the limbic system, plays a definitive role in the regulation of mood and behaviour. Not surprisingly, structural alterations in the hippocampus have been linked to certain emotional aspects of MD, such as feelings of worthlessness, despair and guilt, and general cognitive deficits such as memory impairments (Krishnan *et al.*, 2008).

Stress-induced increases in glucocorticoid levels have been demonstrated to result in decreased synthesis of neurotrophic factors, particularly BDNF, which is an effective neuroprotective factor and protagonist of neurogenesis (Nestler *et al.*, 2002). Additionally, abnormal elevations of glucocorticoid levels induce atrophy in regions expressing large numbers of GRs, most notably the hippocampus (Sapolsky, 2000).

Moreover, the hippocampus is extensively innervated by the monoaminergic system and a reduced hippocampal volume subsequently results in waning monoamine levels, indicating that changes in monoamines occur downstream of the major events that drive the development of MD (Pittaluga *et al.*, 2007). Conversely, increases in monoamine levels lead to increased hippocampal neurogenesis (Santarelli *et al.*, 2003), thereby establishing a bi-directional relationship between hippocampal volume and monoaminergic neurotransmission.

Elevated levels of glucocorticoids also enhance glutamatergic transmission by increasing the expression of the glutamate ionotropic N-methyl-D-aspartic acid receptor (NMDAR) and by

increasing the synthesis and extracellular concentrations of glutamate (Lu *et al.*, 2003). Synaptic NMDARs consist of NR2A subunits that are associated with neuroprotective mechanisms and are activated under normal physiological conditions. In contrast, extrasynaptic NMDARs exclusively comprise NR2B subunits that are associated with atrophic and apoptotic processes and are only activated under extreme conditions, such as exceedingly high extracellular glutamate levels (Lu *et al.*, 2003). Interestingly, BDNF has been found to stimulate NR2A expression and suppress NR2B expression, whereas glucocorticoids have been found to stimulate NR2B expression and suppress NR2A expression (Glazner *et al.*, 2000), further demonstrating the opposing effects of neurotrophic factors and stress in MD.

As discussed in Chapter 3 (Manuscript A), increased glutamatergic transmission has also been reported as a feature of MD (Zarate Jr *et al.*, 2003) and directly linked to the neuroplastic events associated with the disorder (Musazzi *et al.*, 2011). Furthermore, chronic antidepressant treatment reverses these abnormalities through dampening glutamatergic neurotransmission, probably via increased activation of monoaminergic heteroreceptors (Pittaluga *et al.*, 2007), while inhibition of down-stream nitric oxide synthase (NOS) has also been suggested (Harvey, 2008). Importantly, support for this has ensued from pre-clinical work where increased stress sensitivity in a genetic animal model of MD has been associated with pronounced aberrations in glutamate-NMDA-NOS signalling (Wegener *et al.*, 2010).

2.2.7 The role of inflammation

In addition to its role in the immune system, cytokines may activate neural pathways within the central nervous system and have been found to mediate important mood-related processes (Dantzer *et al.*, 2008). Even though overproduction of cytokines may be the result of trauma and/or disease, it has been argued that these molecules should not merely be seen as inflammatory mediators involved in pathological processes but that they also serve as neuromodulators (Merrill, 1992, Vitkovic *et al.*, 2000). In fact, at lower concentrations they are likely involved in the normal development and functioning of the nervous system (Merrill, 1992, Vitkovic *et al.*, 2000). The CNS may be exposed to increased cytokine levels via several mechanisms. These include volume diffusion (Vitkovic *et al.*, 2000), access via the blood brain barrier subsequent to exaggerated peripheral production (Banks, 2006) and through upregulation of Toll-like receptors located on macrophages in the

circumventricular organs and choroid plexus in response to immune signals and intracellular signalling components (Schroder *et al.*, 2006) that ultimately leads to the production of pro-inflammatory cytokines (Dantzer *et al.*, 2008). Chronically elevated cytokine levels are implicated in neurotransmitter changes that may be interpreted by the brain as stressors and ultimately contribute to the development of MD (Anisman *et al.*, 2003). In this regard, depressed patients present with increased levels of pro-inflammatory cytokines (Anisman *et al.*, 2003) that strongly contribute toward various pathophysiological domains characterizing MD, including neurotransmitter metabolism, neuroendocrine function, synaptic plasticity and behaviour (Danner *et al.*, 2003, Ford *et al.*, 2004, Miller *et al.*, 1999, Musselman *et al.*, 2001, Raison *et al.*, 2006, Tiemeier *et al.*, 2003). Moreover, pro-inflammatory cytokines produced either peripherally or centrally may lead to sickness behaviour and the development of symptoms of MD in vulnerable individuals but the exact mechanisms by which cytokines bring about these behavioural effects remain elusive (Dantzer *et al.*, 2008). Pro-inflammatory cytokines also stimulate HPA-axis hormones, including corticotrophin releasing hormone (CRH), in both the hypothalamus and the amygdala, the latter having an important role in fear and anxiety (Raison *et al.*, 2006). Furthermore, administration of cytokines to human subjects results in altered brain functions in regions relevant to the development of depressive symptoms, while many patients treated with IFNα develop a behavioural syndrome closely related to MD (Capuron *et al.*, 2004), incidentally also being responsive to standard antidepressant therapy (Musselman *et al.*, 2001). Cytokine-induced MD is associated with alterations in 5HT metabolism through the enzyme indoleamine 2, 3, dioxygenase (IDO) (Capuron *et al.*, 2002) and alterations in CRH function, (Capuron *et al.*, 2003) while MD induced by IFNα involves the nitric oxide (NO) cascade (Hashioka *et al.*, 2007, Suzuki *et al.*, 2003). Clinical evidence for this is founded in the use of anti-inflammatory drugs to treat depression. Celecoxib has been demonstrated to decrease depressive symptoms (Akhondzadeh *et al.*, 2009, Chen *et al.*, 2010) and significantly enhance the effects of fluoxetine when administered as adjunctive treatment (Akhondzadeh *et al.*, 2009) while aspirin accelerates the onset of action of SSRIs (Mendlewicz *et al.*, 2006). Also in preclinical studies, aspirin has been associated with antidepressant-like behavioural and endocrine effects in rats comparable to that of imipramine and fluoxetine (Guan *et al.*, 2014) and has proved to be an effective augmentation agent in rats resistant to treatment with fluoxetine (Wang *et al.*, 2011). Moreover,

chronic inflammation is a major component of many lifestyle and other dreaded diseases that are often co-morbid with MD, such as cancer, diabetes mellitus, coronary artery disease and metabolic syndrome, thus highlighting how inflammation may contribute to an increased risk of developing MD.

2.2.8 Metabolic activity

Considering all the brain structures involved in mood and cognitive function and the stress response, it can be expected that several brain regions will be affected by mood disorders. As such, metabolic abnormalities in specific brain regions have been proposed to be central to the pathophysiology of MD with significant involvement of the prefrontal cortex and limbic region, most notably the hippocampus, amygdala and ventral striatum (Nestler *et al.*, 2002).

Furthermore, the hypothalamus is especially important for its role in the stress response and intermediary metabolism, both of which are strongly affected in MD, leading to, for example, altered biological rhythms, hypercortisolemia, sleep disturbances, altered immune function, as well as altered metabolic profile – the latter leading to altered glucose metabolism and obesity (Gardner *et al.*, 2011, Harvey, 2008).

Brain imaging studies have also utilised alterations in blood flow and glucose metabolism as measures to implicate certain brain regions affected in MD by comparing the resting brain state activity of depressed patients to that of healthy patients (Koenigs *et al.*, 2009). Positron emission tomography (PET) has been applied to measure regional brain glucose metabolism, indicating that the cortical and limbic circuitry is functionally compromised in patients suffering from MD (Kennedy *et al.*, 2001) and that depressed patients with elevated glucocorticoid levels present with increased activation of the amygdala (Drevets *et al.*, 2002). Moreover, such dysfunctions are reversed, at least to some extent, by antidepressant treatment (Drevets *et al.*, 2002, Kennedy *et al.*, 2001).

2.3 Incidence & Demographics

Citing its World Mental Health (WMH) surveys, the World Health Organization (WHO) suggested that half of the individuals in six countries (including South Africa and the United States) will suffer from a psychiatric disorder during their lifetime (Kessler *et al.*, 2007), while current statistics indicate

that the lifetime prevalence of MD is about ten percent in South Africa (Tomlinson *et al.*, 2009). The lifetime prevalence rate of MD is currently estimated at 8% to 15% (Rickards, 2005), making MD one of the most common neuropsychiatric disorders and one of the most significant causes of disability worldwide (Kessler *et al.*, 2005). In 2010, just shy of 300 million people suffered from MD (Ferrari *et al.*, 2013), while the Global Burden of Disease Study conducted by the WHO has determined that MD is now the second leading cause of disability worldwide. MD also contributes to the burden of disease of suicide and ischemic heart disease (Ferrari *et al.*, 2013). Of importance is that modern lifestyles and an increased occurrence of stressful events have a growing influence on the development of MD, thereby leaving many incapacitated with a poor quality of life, that ultimately place severe strain on society (Rickards, 2005). With regards to lifestyle-related determinants, co-morbidity between metabolic disorders and MD is evident (Skilton *et al.*, 2007) with obesity being implicated as the primary factor responsible for subsequent development of MD (Capuron *et al.*, 2004). Also, positive correlations between the prevalence of mood and anxiety disorders (including MD) and both screen time (time spent watching television, playing video games, etc.) and a lack of physical activity, have been found in adolescents (Goldfield *et al.*, 2016, Maras *et al.*, 2015) – both of which may also be predicative of adolescent obesity.

The International Consortium of Psychiatric Epidemiology (ICPE) has indicated that up to 50% of people with a lifetime history of MD also have a history of at least one anxiety disorder. In further support of this, patients with anxiety disorders in all countries have been observed to regularly present with co-morbid MD and that the presence of anxiety may serve as a reliable predictor of the first onset of secondary MD (Andrade *et al.*, 2003). Population studies have also shown that MD presents almost twice as often in female patients than in males, possibly related to differences in sex hormones, gender-related personality and social traits and other gender-related aspects such as endocrine stress reactions and neuropsychological processes (Kuehner, 2003). Locally, the South African Stress and Health (SASH) study found that mood and anxiety disorders have the highest incidence among common mental health disorders (Herman *et al.*, 2009), with MD having a lifetime prevalence of 9.7% and occurring more frequently in individuals with a low level of education (Tomlinson *et al.*, 2009).

2.4 Symptomology & Diagnosis

The most notable symptoms associated with MD are depressed mood, anhedonia (reduced ability to experience pleasure from natural rewards such as food, sex and social interaction), fatigue, irritability, difficulties in concentrating, and abnormalities in appetite and sleep (also referred to as 'neurovegetative symptoms') (Knol *et al.*, 2006, Krishnan *et al.*, 2008, Nestler *et al.*, 2002). Diagnosis of MD is, however, complicated when other psychiatric disorders co-occur, e.g. anxiety disorders (Brunello *et al.*, 2000) and Parkinson's disease (Friedman *et al.*, 2004) due to a degree of shared symptomatology amongst these disorders.

Despite the leaps made in the understanding of the disorder, diagnosis of MD remains highly subjective – a very important motivator to develop reliable methods by which clinicians would be able to measure biological markers associated with MD and, in this way, improve diagnosis and treatment of individual patients (please refer to Manuscript A for a comprehensive review of this subject). Currently, the diagnosis of MD is based on the presence of a combination of at least five of the symptoms described in Table 2-1 below, as stipulated by criteria specified in the DSM-V. The presenting symptoms must be evident for a period longer than two weeks (Nestler *et al.*, 2002) with at least one of the symptoms being either depressed mood or loss of interest or pleasure (American Psychiatric Association, 2000).

Table 2-1: Diagnostic criteria for major depression (adapted from Nestler *et al.* (2002) and Akechi *et al.* (2009)
Depressed mood
Irritability
Low self esteem
Feelings of self-reproach, worthlessness and guilt
Decreased ability to concentrate and think
Decreased or increased appetite
Weight loss or weight gain
Insomnia or hypersomnia
Low energy, fatigue or increased agitation
Decreased interest in pleasurable stimuli
Recurrent thoughts of death and suicide

2.5 Treatment

The prevailing treatment approach for MD is pharmacotherapy, however other treatment options are available which are also effective to varying extent, e.g. electroconvulsive therapy (ECT), magnetic stimulation, vagal nerve stimulation and deep brain stimulation (Berton *et al.*, 2006, Nestler *et al.*, 2002). Total sleep deprivation has also been demonstrated to rapidly improve depressive symptoms in ≈50% of patients (Giedke *et al.*, 2002) – an effect probably mediated by attuning circadian parameters (Wehr *et al.*, 1979). The effect, however, is short-lived and limited to only a few days (Giedke *et al.*, 2002). Exercise and physical activity also has proven benefits in alleviating depressive symptoms (Blumenthal *et al.*, 2007, Dunn *et al.*, 2005). Although the exact means by which these somatic approaches to treatment elicit their effects are not totally understood, they provide a valuable aid in treating depressed individuals by providing rapid effects. Considering pharmacotherapy, treatment has almost exclusively focused on an interaction with monoaminergic systems, particularly by applying drug therapies which are known to augment 5HT and/or NA mediated systems in the brain (Lenox *et al.*, 2002).

2.5.1 Traditional options

Antidepressant drugs are known to mediate monoaminergic interactions by various mechanisms including – but not limited to – inhibition of 5HT or NA reuptake by the monoamine reuptake inhibitors (e.g. TCAs and SSRIs) and by inhibition of monoamine oxidase, the major catabolic enzyme for monoamine neurotransmitters (e.g. (MAOIs)) (Nemeroff, 2008, Nestler *et al.*, 2002).

Among the first antidepressant compounds were the MAOIs (Lopez-Munoz *et al.*, 2009), with the antitubercular drug, iproniazid, being recognized as the first MAOI antidepressant (Shulman *et al.*, 2013). In 1952 it was discovered that iproniazid produced mood-elevating effects superior to those observed with isoniazid and inhibited monoamine oxidase (MAO) (Lopez-Munoz *et al.*, 2009) with a subsequent increase in central monoamines (Leusen, 1960). After the introduction of tranylcypromine to the treatment of MD in 1959, the MAO inhibitors became popular antidepressants (Atkinson *et al.*, 1965). Currently, MAOIs are not first choice antidepressants, and are usually reserved for use by specialists and in patients who exhibit poor outcomes or tolerance with other drug treatments (Shulman *et al.*, 2013).

The potential of the TCA, imipramine, as antidepressant was discovered in 1957 (Kuhn, 1958) and was once considered first-line treatment for MD (Pare, 1965). However, despite its potentiating effect on NA and 5HT, TCA's are plagued by various side-effects, including anticholinergic effects, e.g. constipation, dry mouth, dizziness and abnormal vision (Trindade *et al.*, 1998) which may negatively affect treatment outcomes resulting from patient non-compliance (Machado *et al.*, 2006). After reports of the ability of TCAs to inhibit 5HT reuptake (Carlsson *et al.*, 1969) researchers attempted to develop a drug exploiting this mechanism in a more selective manner, resulting in a landmark of rational psychiatric drug discovery, the SSRIs (Vaswani *et al.*, 2003).

The first SSRI, i.e. zimelidine, was introduced to the market in the 1980's (Vaswani *et al.*, 2003) but despite extensive use, it was withdrawn due to an associated risk of developing Guillain-Barre syndrome (Fagius *et al.*, 1985, Stanford, 1999). In 1987, fluoxetine was introduced to the market (Stanford, 1999), with a number of other SSRIs following in quick succession (Vaswani *et al.*, 2003). While the SSRIs are generally considered safe, mainly due to the absence of severe side-effects or death due to overdose, they do still have some common side-effects, e.g. nausea, anxiety and sexual

dysfunction (Vaswani *et al.*, 2003), Much of these side-effects, however, are temporary and dose-related (Vaswani *et al.*, 2003). Even though it may be argued that the more selective drugs, e.g. SSRIs, are potentially less or just as effective antidepressants as the TCAs, their use is still favoured due to their safer and improved tolerability profile (Anderson, 2000, Gallo, 1999, Vaswani *et al.*, 2003, Vetulani *et al.*, 2000). In addition, the non-TCA SNRIs (e.g. venlafaxine and duloxetine) are associated with a much more selective mechanism of action relative to TCAs (refer to Manuscript C) which also contributes to more favourable side-effect profiles while maintaining the benefit of its action on *both* 5HT and NA.

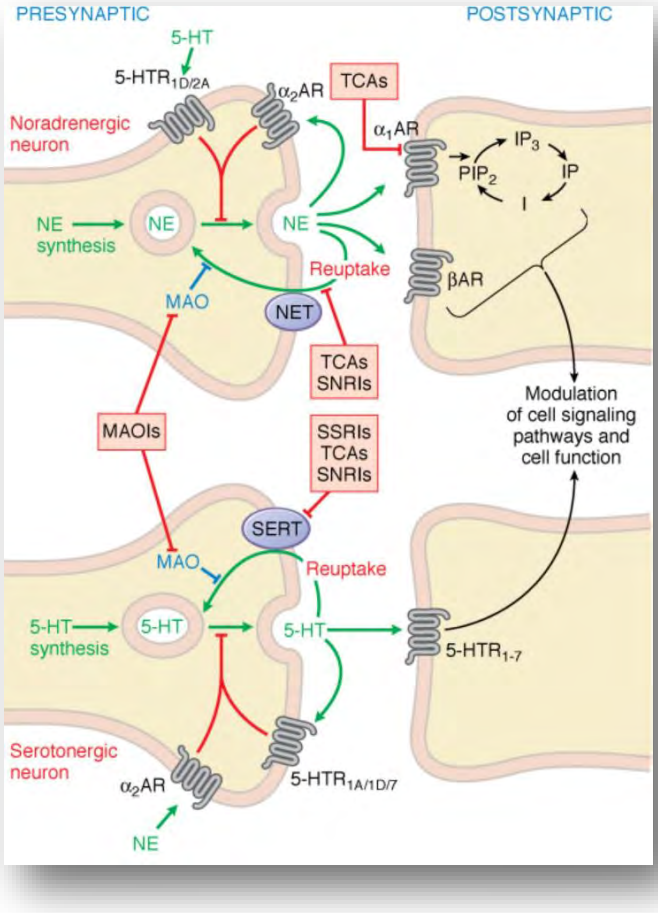

Figure 2-1: Sites of action of traditional antidepressants. Schematic representation of noradrenergic (top) and serotonergic (bottom) nerve terminals. SSRIs, SNRIs and TCAs increase noradrenergic or serotonergic neurotransmission by blocking the NA or 5HT transporter at presynaptic terminals (NET; SERT). MAOIs inhibit catabolism of NA and 5HT. Chronic treatment with a number of antidepressants desensitizes presynaptic autoreceptors and heteroreceptors, producing long-lasting changes in monoaminergic neurotransmission. Post-receptor effects of antidepressant treatment, including modulation of G-protein coupled receptor signalling and activation of protein kinases and ion channels, are involved in the mediation of the long-term effects of antidepressant drugs (Adapted from Brunton *et al.* (2011))

2.5.2 Novel options

Considering that available antidepressant treatments have limited efficacy, display a delay in the onset of clinical effects and that they cause a multitude of adverse effects (Fava, 2003, Holtzheimer *et al.*, 2006, Nestler *et al.*, 2002), there is an increasing need to explore novel targets for the treatment of MD which may produce rapid, robust and lasting antidepressant effects accompanied by a less daunting side-effect profile. Various atypical antidepressants have also been introduced that target monoaminergic or other systems in a way that is different to the traditional approaches described above. Such agents include for example mirtazepine and mianserin, multi-target antidepressants with antagonistic activity on the α_2-adrenergic receptor, trazodone, that mainly blocks $5HT_{2c}$ and α_1-adrenergic receptors, bupropion that inhibits NA and dopamine (DA) reuptake (Berton *et al.*, 2006, Holtzheimer *et al.*, 2006, Papakostas *et al.*, 2008), and more recently the melatonergic antidepressant, agomelatine (Howland, 2009). Tianeptine – in contrast with TCAs and SSRIs – increases the uptake of 5HT in the brain while inhibiting neuronal atrophy associated with stress (Wagstaff *et al.*, 2001) while vortioxetine induces 5HT receptor modulation combined with SERT inhibition, resulting in enhanced synaptic plasticity and cognitive function (Sanchez *et al.*, 2015).

The direct link between the glutamatergic system and neuroplasticity has prompted the development of many clinical trials that have aimed to test the antidepressant effects of glutamatergic modulators. Among these, the NMDAR antagonists have demonstrated the most promising results due to their rapid antidepressant effects and high response rates (Berman *et al.*, 2000, Maeng *et al.*, 2008, Zarate Jr *et al.*, 2006). Ketamine acts as a non-selective antagonist of NMDARs and has been demonstrated to induce rapid antidepressant action, often as quick as two hours after administration and sustained for up to two weeks following administration of a single dose (Maeng *et al.*, 2008). The rapid response to ketamine has recently been ascribed to the activation of mTOR signalling pathways that is functionally linked to synaptic protein synthesis (refer to Manuscript A, Figure 2), resulting in increased synaptic and dendritic spine formation (Li *et al.*, 2010). The antidepressant effects of ketamine are furthermore dependent on co-activation of the ionotropic glutamate α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor (Autry *et al.*, 2011, Li *et al.*, 2010). Ketamine also induces rapid increases in BDNF levels – a process

which occurs mainly via translational mechanisms rather than increased gene expression, as previously thought (Autry *et al.*, 2011, Garcia *et al.*, 2008).

Despite the presence of these differing antidepressant pathways (via manipulating monoaminergic, melatonergic, GABA-ergic and other pathways) existing evidence suggests that the efficacy of various antidepressant treatments ultimately converge by inducing an increase in neurogenesis, now regarded as an essential event for antidepressant effect (Santarelli *et al.*, 2003). This process, involving the reversal of stress-associated neuroplastic changes through the modulation of several signalling pathways, has been observed for all known classes of antidepressants (Nestler *et al.*, 2002). However, the neuroplastic changes associated with antidepressant action occur downstream of the elevated monoamine levels associated with treatment, and is therefore considered to be the rate limiting step of current therapeutic strategies, thereby contributing to the delayed effects observed for antidepressant treatment (Duman *et al.*, 2012). The common construct of antidepressant-induced neurogenesis resulted in wide acceptance that neuronal atrophy is a universal event of MD, although it is not clear whether it is a cause or consequence of the disorder (Pittenger *et al.*, 2008).

2.5.3 Treatment difficulties

Although pharmacotherapy, psychotherapy and electroconvulsive therapy may all be effective in treating MD, many patients do not respond sufficiently to the available forms of treatment. Current treatments remain sub-optimal, with only half of MD patients demonstrating complete remission (Nestler *et al.*, 2002), while up to 20% show minimal or no response to even the most aggressive interventions (Fava, 2003). In addition, intolerable side effects as well as a slow onset of action prompts many patients to prematurely discontinue their medication leading to a diverse range of complications, foremost among these being an increased risk of relapse and recurrence (Harvey *et al.*, 2003, Holtzheimer *et al.*, 2006). It is also generally accepted that long-term treatment is necessary to limit the number and intensity of subsequent depressive episodes (Holtzheimer *et al.*, 2006).

Ideally, an antidepressant drug should have a fast onset of action, a favourable side-effect profile and induce 100% remission rates, as well as an absence of relapse (Rosenzweig-Lipson *et al.*, 2007). Even the SSRIs, which are regarded as the mainstay of MD treatment, are associated with a host of side-effects, e.g. nausea, anorexia, diarrhoea, insomnia, nervousness, anxiety (Brambilla *et al.*, 2005,

Trindade *et al.*, 1998). Of these adverse effects, gastrointestinal disturbances, headache, nervousness and anxiety were observed to be even more problematic in patients treated with SSRIs relative to patients treated with TCAs (Brambilla *et al.*, 2005, Trindade *et al.*, 1998). Evidently, currently available antidepressants still have several shortcomings, as presented in Table 2-2.

Table 2-2: Unmet clinical needs for marketed antidepressants. (Adapted from Rosenzweig-Lipson et al., 2007)

Efficacy in refractory patients
Efficacy in treatment resistant depression
Faster onset of antidepressant action
Reduction of cognitive deficits
Treatment of symptomatic pain accompanying depression
Decreased side-effect profile

As has been highlighted earlier, the cause of MD is far from being a simple deficiency of central monoamines. Traditional antidepressants produce immediate increases in monoamine transmission (Krishnan *et al.*, 2008), whereas their mood-enhancing properties require weeks to reach antidepressant effect. In fact, many patients do not show adequate improvement, even after months of treatment (Machado-Vieira *et al.*, 2009). This indicates that enhanced serotonergic or noradrenergic neurotransmission per se is not immediately responsible for the clinical actions of these drugs (Nestler *et al.*, 2002). In fact, neurotrophins, neurogenesis and the concepts of neuroplasticity have now taken centre stage in our understanding of MD and the mechanisms of action of antidepressants (Krishnan *et al.*, 2008, Manji *et al.*, 2003), while a realization that genetic factors, an adverse environment and neuroendocrine and metabolic dysfunction may contribute to the eventual development of MD, is providing a new framework for understanding the neurobiology and treatment of MD.

2.5.3.1 Treatment resistance

One of the major concerns regarding treatment with typical antidepressants is the low rates of efficacy associated with these therapies. Drug resistance is therefore a major obstacle in the effective treatment of many cases of MD. Treatment resistant depression (TRD), first described by Heimann (1974), is defined as MD that does not respond to an adequate trial of at least one class of

antidepressant (Rush *et al.*, 2004). Clinical reports show that 50% of depressed patients fail to respond to an initial trial of antidepressant treatment, a value that was replicated by the Sequenced Treatment Alternatives to Relieve Depression (STAR*D) study (Rush *et al.*, 2004). The STAR*D study was designed to mimic clinical conditions by incorporating the most commonly used strategies in treating patients exhibiting drug resistance and thereby provided practitioners with empirical data regarding the most effective treatment options. Treatments were tiered according to four levels of non-response, viz. level 1 (citalopram), level 2 (venlafaxine), level 3 (nortryptiline) and level 4 (tranylcypromine) (Rush *et al.*, 2004).

Although most pathophysiological factors contributing to MD, e.g. adverse environmental factors and neuro-biologic deficits, appear to be similar in TRD, many of these conditions are significantly exaggerated in the resistant form, resulting in more severe symptoms (Wijeratne *et al.*, 2008). However, even though TRD may be seen as an aggravated manifestation of MD, a few unique features are believed to counteract treatment response. For instance, a major discrepancy between TRD and MD is specific structural brain changes. In this regard, TRD patients have been shown to present with greater right frontal-striatal atrophy as well as significant left hippocampal volume reductions (Shah *et al.*, 2002). Moreover, increased structural atrophy observed in TRD has been correlated with greater decreases in BDNF levels (Wolkowitz *et al.*, 2011), while depressed patients failing to respond to SSRI treatment present with lower BDNF levels prior to treatment when compared to treatment responsive patients (Wolkowitz *et al.*, 2011).

TRD has been associated with hyperactivity of the HPA axis, which corresponds to higher glucocorticoid levels compared to depressed patients that respond to treatment (Juruena *et al.*, 2009). The HPA-axis dysfunction observed in TRD mainly arises from an impaired negative feedback response due to GR resistance (Bauer *et al.*, 2003) most probably resulting from chronically elevated glucocorticoid levels. Furthermore, alterations in glucocorticoid levels are accompanied by a flattening of the diurnal rhythm, which is proposed to prevent the ability of SSRI treatment to elevate 5HT transmission in the forebrain, thereby adding another dimension to treatment resistance (Gartside *et al.*, 2003).

Several abnormalities associated with specific receptors have also been highlighted in TRD. A dysfunction of 5-HT_{1A} receptor signalling is most commonly linked to TRD, where over-expression of this receptor, due to a polymorphism in the promoter region of the Htr1a (5-Hydroxytryptamine (5HT) Receptor 1A) gene, has been demonstrated to result in an increased resistance to SSRI treatment (Samuels *et al.*, 2011). A reduced density of tropomyosin receptor kinase B (TrkB), a receptor for which BDNF displays a high affinity, has also been correlated with MD (Dwivedi *et al.*, 2001) and is reversed by antidepressant treatment (Nibuya *et al.*, 1995). Due to the drastic reductions in BDNF levels in TRD, it is probable that TrkB may share a similar fate. However, the exact association between this receptor and TRD is unclear and still requires further elucidation.

Various clinical MD treatment guidelines provide approaches to manage treatment resistance after optimization of the dosage and treatment duration of an initial course of a first-line antidepressant. These recommendations generally include switching to another antidepressant or augmenting the initial approach with a second-generation antipsychotic with antidepressant properties, e.g. quetiapine or aripiprazole. Another popular approach is the combination of different traditional antidepressants – a strategy which lacks sufficient clinical research but may prove useful (Connolly *et al.*, 2011) due to synergistic activity and/or inclusion of additional mechanisms of action. In this regard, the use of the atypical traditional antidepressants bupropion and mirtazepine in combination therapy is supported by data from various clinical trials, including STAR*D (Al-Harbi, 2012). Other common literature-reported approaches include the combination of TCA + SSRI, SSRI + SSRI, SSRI + venlafaxine and TCA + venlafaxine (Al-Harbi, 2012).

2.5.3.2 Effects of comorbid anxiety on treatment response

The treatment of MD may be further complicated by the co-occurrence of other underlying psychological disorders. The prevalence rate of a coexisting anxiety disorder in patients receiving treatment for MD is reported at between 50%-60% (Rush *et al.*, 2006, Zarate Jr *et al.*, 2006) – a figure which significantly increases to 72% in MD patients who present with severe treatment resistance (Rush *et al.*, 2006). Of all anxiety disorders, PTSD (more details to follow) is more commonly diagnosed in patients suffering from MD and has a prevalence rate of 17.8% in this population – this number, however, significantly increases to 22.4% in the TRD population (Rush *et al.*, 2006). Conversely, according to the National Comorbidity Survey-Replication, 50% of patients seeking

treatment for PTSD are diagnosed with comorbid MD (Elhai *et al.*, 2011). The high rate of comorbidity between MD and PTSD stems largely from an overlap of several symptoms such as anhedonia, sleep difficulty, irritability and concentration difficulty (Elhai *et al.*, 2011). In addition, MD and PTSD also share certain aspects of their aetiology, often requiring exposure to stressful events for onset (although stressful events that precipitate PTSD are usually more traumatic in nature) (Elhai *et al.*, 2011). Importantly, both MD and PTSD exhibit reduced hippocampal volumes due to glucocorticoid-induced atrophy (Manji *et al.*, 2003). Patients with PTSD also exhibit reduced BDNF and TrkB levels, another feature shared with MD (Liberzon *et al.*, 2012). Furthermore, HPA-axis abnormalities, such as a flattened diurnal rhythm, are shared among patients suffering from PTSD and MD (Tucker *et al.*, 2004). Interestingly, depressed patients with comorbid anxiety disorders were shown to demonstrate even greater impairment of the HPA-axis feedback mechanism, a hallmark of TRD (Kara *et al.*, 2000). All these features culminate in greater symptom severity during MD-PTSD comorbidity which ultimately results in significant delays in treatment response as well as increased treatment resistance (Green *et al.*, 2006, Hegel *et al.*, 2005).

2.5.3.3 PTSD: A major contributor to TRD

PTSD, classified as an anxiety disorder, present with long-lasting anxiety coupled with fear-related symptomology (Vieweg *et al.*, 2006), including re-experiencing, hyperarousal and avoidance symptoms (APA, 2013). PTSD is a chronic and often persistent disorder that develops subsequent to stress (trauma) exposure (Neria *et al.*, 2000) and it has been suggested that the development of either MD or PTSD after exposure to trauma may also be determined by the type and/or severity of the trauma (Kessler *et al.*, 1995). Also, MD patients have an increased sensitivity to the PTSD-inducing effects of trauma (Bromet *et al.*, 1998) and pre-existing PTSD increases the risk of developing MD (Kessler *et al.*, 1995). Considering these observations, the high rate of comorbidity between these two disorders may suggest common vulnerabilities and/or pathophysiologic features. Even more, it is likely that inter-individual vulnerabilities play an important role in the development of either of these disorders when keeping in mind that not all individuals exposed to stressful events will suffer from MD or PTSD (Kendler *et al.*, 1998, Kessler, 1997).

Patients suffering from PTSD present with structural deficits – possibly due to neurotoxic effects of elevated glucocorticoids subsequent to stress (McEwen, 1999, Sapolsky, 2000). Such deficits are

mainly contained within the hippocampus (Bremner, 2002), a brain structure involved in emotional processing and memory (McEwen, 1999), while functional alterations in the medial prefrontal cortex (Bremner, 2002, Zubieta *et al.*, 1999), a structure involved in fear response and conditioning through interaction with the amygdala (Akirav *et al.*, 2001), have been demonstrated.

Various antidepressants have proven to be beneficial in the treatment of PTSD, including SRIs, MAOIs and TCAs (Hageman *et al.*, 2001). $5HT_{1A}$ and $5HT_{2A}$ receptor densities are upregulated in the hippocampus and prefrontal cortex of fear-sensitized rats (Kalynchuk *et al.*, 2001) and both the antidepressant and anxiolytic effects of SRIs are reliant on interaction with these receptors in limbic areas (Harvey, 1997).

NA also plays a central role in PTSD as it is also involved in fear signalling and functioning of the amygdala (Shin *et al.*, 2006), while it has been argued that the hyperarousal and re-experiencing symptoms observed in PTSD are associated with altered reactivity of the NAergic system. (Southwick *et al.*, 1997) – a feature that is characterized by increased α_2 receptor sensitivity coupled with exaggerated arousal subsequent to exposure to uncontrollable chronic stress (Nisenbaum *et al.*, 1992).

The current body of evidence on the pathophysiology of PTSD suggests prominent roles for alterations in the monoaminergic system and HPA-axis (Bailey *et al.*, 2013) as well as structural deficits and altered functioning of the hippocampus and prefrontal cortex – features which have also proven to be prominent in the pathophysiology of MD.

2.6 Modelling mood and anxiety disorders in animals

A number of studies have confirmed the importance of using a pathological animal model as opposed to a normal healthy animal, especially when determining the action and efficacy of pharmaceuticals. Animal models are designed to imitate disorders occurring in humans and thereby allow for more invasive examinations of the underlying mechanisms associated with the specific disorder (Hammack *et al.*, 2012). In order for an animal model to be validated, a series of stringent criteria must be met, allowing findings in the animal model to be carefully extrapolated to the human condition (Duman *et al.*, 2006). These criteria assess whether the animal model expresses behaviour that resemble that observed in the human disorder (face validity), whether such symptomology

correspond with physiological and biological changes that can be objectively measured (construct validity) and whether it can be reversed by various treatments effective in the human condition (predictive validity) (McKinney *et al.*, 1969). Animal models are, however, faced with many limitations, particularly when modelling a disorder as heterogeneous as MD. A major limiting factor in MD studies is that no single animal paradigm will sufficiently model all aspects of MD. Therefore, a series of tests used in conjunction provides a more accurate account of depressive phenotypes (Duman *et al.*, 2006, Moekoena *et al.*, 2015).

As outlined in the Introduction, this study will focus on the development of an animal model of TRD, based on the high comorbidity of MD with PTSD and the tendency of this combination to present with treatment resistance (see Chapters 4 and 5). However, for the sake of brevity we will only focus on the models selected for this study, viz. the FSL and TDS models of MD and PTSD, respectively. A brief overview of other translational animal models of MD and PTSD are also presented in Table 2-3 and 2-4, respectively.

Table 2-3: Brief overview of animal models of MD.
Owing to the heterogeneity of MD, animal models of the disorder usually aim to replicate a specific endophenotype of the disorder – features determined by the inducing conditions (Czéh *et al.*, 2016)

Basis of model	Key features
Stress-based (e.g. chronic mild stress)	Induces behavioural and neurochemical changes relevant to depression, especially anhedonia-like behaviour. However, difficulty to establish the model in a new laboratory, labour-intensiveness and being time consuming are potential drawbacks. (Willner, 1997)
Social (e.g. social defeat)	Induces decreased exploratory and sexual behaviour, increased submissive behaviour, immobility in the FST, anhedonia-like behaviour and HPA-axis dysregulation. Effects of long-term exposure are reversible by chronic, but not acute, antidepressant treatment. (Hollis *et al.*, 2014)
Circadian dysregulation (e.g. sleep deprivation)	Induces cognitive deficits (McEwen, 2006), anhedonia-like behaviour as well as HPA-axis (Becker *et al.*, 2010, Novati *et al.*, 2008) and $5HT_{1A}$ (Novati *et al.*, 2008) abnormalities. Neonatal exposure leads to reduced sexual activity and sleep abnormalities later in life (Feng *et al.*, 2003). Note: sleep deprivation may also be applied to induce antidepressant-like effects in animals with depressive-like symptoms (Lopez-Rodriguez *et al.*, 2004).
Genetic (e.g. inbred Wistar Kyoto (WKY) and FSL rats)	Hyper-reactivity to stress, dysregulation of HPA-axis, increased immobility in FST reversible by chronic, but not acute, antidepressant treatment (Lahmame *et al.*, 1997).
Olfactory bulbectomy	Alterations in circadian rhythm, increased ACTH and corticosterone and decreased 5-HT and 5-HIAA levels, decreased sexual activity and cognitive deficits. Proposed as a screening tool for novel antidepressants, however, inter-species differences may limit effectiveness. (Hendriksen *et al.*, 2015)

Table 2-4: Brief overview of animal models of PTSD.
Animal models of PTSD are usually based on stress-exposure that induce features of the PTSD phenotype – features which are characterized by measurements of molecular, endocrine and behavioural responses (Daskalakis *et al.*, 2013)

Exposure-type	Key features
Physical stress (e.g. single prolonged stress (SPS))	Enhanced HPA negative feedback (Liberzon *et al.*, 1997), hyperarousal, enhanced freezing-behaviour and fear memory and increased anxiety-like behaviour (Imanaka *et al.*, 2006).
Psychosocial stress (e.g. social defeat)	Increased anxiety-like behaviour, increased avoidance behaviour, blunted corticosterone levels (Krishnan *et al.*, 2007). Identified as a useful model to study neurobiological abnormalities in PTSD (Whitaker *et al.*, 2014).
Early-life stress (e.g. maternal separation	Long-lasting HPA-axis abnormalities and increased CRF levels (Pihoker *et al.*, 1993), blunted stress response (Daniels *et al.*, 2004). Results reported to be inconsistent (Whitaker *et al.*, 2014).
Psychogenic stress (e.g. predator stress)	Increased anxiety-like behaviour, heightened fear memory, reduced basal glucocorticoid levels, increased cardiovascular reactivity and exaggerated response to yohimbine (Zoladz *et al.*, 2008, Zoladz *et al.*, 2012).

2.6.1 Modelling MD: The Flinders Sensitive Line (FSL) rat

The FSL rat is a validated genetic model of MD with an increased susceptibility to stress (Overstreet, 1993) that was originally developed by selectively breeding Sprague-Dawley rats that display super-sensitivity to the organophosphate anticholinesterase agent, di-isopropyl fluorophosphate (Overstreet *et al.*, 2005). This selective breading pattern resulted in rats that were more sensitive to muscarinic receptor agonists due to an increase in muscarinic receptor densities in several brain regions (Overstreet *et al.*, 1984). This increased sensitivity to muscarinic receptor agonist parallels a number of human studies that have reported an increased sensitivity to cholinergic agonists among depressed patients (Janowsky *et al.*, 1972, Janowsky *et al.*, 1994, Jones *et al.*, 1985, Sokolski *et al.*, 1996), as well as that scopolamine displays rapid antidepressant effects in unipolar depressive patients (Furey *et al.*, 2010). Importantly, the FSL rat has also demonstrated high levels of compliance when subjected to the three major criteria required for animal model validation:

a) Face validity: Despite displaying normal hedonic responses and cognitive function, FSL rats commonly display traits reminiscent of those observed in depressed patients, including reduced appetite and psychomotor function and sleep and immune abnormalities (Overstreet, 1993). These overlapping depressive behaviours between FSL rats and human MD support the face validity of the model.

b) Construct validity: Although the similarity in cholinergic irregularities between FSL rats and depressed individuals at the biological and physiological levels (Overstreet *et al.*, 1982, Overstreet *et al.*, 1984) represents an important feature of the model, other prominent biological correlates between MD and the FSL rat include abnormalities in serotonergic, adrenergic and dopaminergic systems (Overstreet *et al.*, 2005), as well as aberrant glutamate-NOS signalling in response to an environmental stressor (Wegener *et al.*, 2010). Evidently, the FSL model therefore offers good construct validity.

c) Predictive validity: The FSL rat has been demonstrated to respond to most antidepressant drugs using the forced swim test (FST). Furthermore, elevated 5HT and 5HIAA levels (Zangen *et al.*, 1997) and increased immobility in the FST (Kanemaru *et al.*, 2009, Yadid *et al.*, 2000) are reversed following chronic, but not acute, treatment with antidepressants. The FSL model therefore has a high degree of predictive validity.

2.6.2 The forced swim test (FST): Measuring depressive-like behaviour

Porsolt and colleagues (1978) developed the FST for use in rodents (rats and mice) and it is extensively applied in preclinical assessment of antidepressant-like activity. The FST also boasts various advantages, including ease of use, reliability across laboratories and ability to detect activity of a variety of antidepressant agents (Borsini *et al.*, 1988). The FST is based on the observation that rats develop an immobile posture – subsequent to initial escape-directed movement – when placed in an inescapable cylinder filled with water. Development of this immobile posture has been proposed to reflect failure of persisting to attempt escape and has also been proposed as a model of behavioural despair (Lucki, 1997). When reintroducing animals to the FST after 24 hours (subsequent to a so-called 15 minute pre-swim to induce this behaviour), and following adequate treatment with an antidepressant, animals actively persist in escape-directed behaviour for longer periods relative to vehicle-treated control animals. The major drawback of the traditional FST, however, is that it fails

to detect the activity of SSRI-type antidepressants (Detke *et al.*, 1995). In attempts to enhance the sensitivity of the traditional FST to SSRIs, several modifications were made to the test's methodology (Cryan *et al.*, 2002, Lucki, 1997), including increased water depth and/or discriminating differential behavioural components, i.e., climbing, swimming and immobility. The modified FST has since demonstrated improved efficacy and reliability (Reneric *et al.*, 2001, Reneric *et al.*, 2002). An important advantage of the modified FST is its ability to distinguish between enhanced central 5HT and NA-mediated behaviours. As such, apart from decreasing immobility in the FST, agents that enhance NA levels are typically associated with increased climbing activity, while agents that enhance 5HT levels, increase swimming behaviour (Cryan *et al.*, 2000, Lucki, 1997). Application of these useful qualities of the modified FST and its use in corroborating the behavioural and neurochemical effects of putative antidepressant compounds is described in Chapters 4 and 5 (Manuscript B and C).

When applying the FST to antidepressant response in the FSL model, it has been found that an initial pre-swim is redundant. In fact, these animals spontaneously exhibit an immobile posture (Overstreet, 1993), likely as a result of an already increased stress-sensitivity, making the need for pre-swim unnecessary.

2.6.3 PTSD: Time-dependent Sensitization (TDS)

TDS is an animal model of PTSD based on the finding that animals exposed to a stressful situation, and followed by re-exposure to a situational reminder, exhibit significant physiological and behavioural alterations that show a time-dependent sustaining or worsening in the absence of the initiating stressor (Yehuda *et al.*, 1993). Not only are these changes reminiscent of human PTSD, but the model also meets criteria for face validity as it emulates the behavioural responses observed in PTSD, e.g. increased aversive behaviour and cognitive deficits (Harvey *et al.*, 2003, Harvey *et al.*, 2006), and evidence for inflammation and its reversal with a glucocorticoid synthesis inhibitor (Harvey *et al.*, 2004). TDS also demonstrates a construct similar to PTSD, as both the animal model and the clinical condition is characterized by reduced levels of glucocorticoids (Harvey *et al.*, 2003, Harvey *et al.*, 2006, Liberzon *et al.*, 1997), while alterations in DA, NA, 5HT and γ-aminobutyric acid (GABA) levels as well as immunological changes that are present in PTSD (Yehuda *et al.*, 1993) have also been demonstrated in animals subjected to TDS stress. The predictive validity of the TDS model is

established by its response to various drugs shown to induce a response in PTSD, particularly fluoxetine (Harvey *et al.*, 2004).

2.6.4 Modelling treatment resistance in animals

A major weakness in TRD research is the shortage of suitable and validated animal models thereof and contributes to the current shortfalls in our understanding of the pathophysiology involved in this form of MD. Chronic mild stress (CMS) is the only animal model to date that has successfully reproduced response rates resembling those observed in clinical studies (Jayatissa *et al.*, 2006) and was originally identified as a model of MD due to the procedure's ability to induce anhedonic-like behaviour (Katz, 1982). It has since been demonstrated to result in antidepressant response rates of ±50% in rats receiving chronic treatment with escitalopram (Jayatissa *et al.*, 2006) – the first line of MD treatment adopted in the STAR*D study. The CMS procedure is, however known to be very labour intensive and time consuming, while it has proven difficult to accurately replicate across different laboratories (Samuels *et al.*, 2011). As mentioned previously, the FSL rat is one of the more robust and widely used models of MD with congruence across many laboratories. Hence, a recent study subjected FSL rats to maternal separation in order to elucidate the involvement of certain biomarkers during treatment response in its response to various antidepressants (Carboni *et al.*, 2010). This study was based on the hypothesis that MD results from a complex interaction between genetic predisposition and environmental adversity (Caspi *et al.*, 2003); also, early life trauma has been identified as an important precursor of MD (Heim *et al.*, 2001). Although this group did not set out to develop a TRD model, their paradigm did exhibit certain biological correlates akin to that observed in human TRD. As such, certain aspects of this gene-environment paradigm do offer some valuable construct validity in modelling TRD and therefore emphasizes the potential of the approach to develop a suitable animal model of TRD.

Animal models have served as a major component in understanding the underlying pathophysiologic mechanisms involved in MD as well as the mechanisms by which antidepressant drugs mediate their antidepressant effects. As such, the increased incidence of treatment resistance among patients suffering from MD and the failure to develop effective drug-based strategies to manage this manifestation of the disorder has been a source of tremendous disappointment and frustration among scientists and clinicians alike (Willner *et al.*, 2015).

It is therefore clear that an increased need has developed for research which focuses on the development of new and improved models, especially those which might be able to reliably replicate symptoms and non-response observed in patients suffering from TRD. This would aid the search for novel therapeutic approaches and provide an additional platform to study the pathophysiological construct of the disorder. Ideally, preclinical models which attempt to replicate the features of TRD should be based on a model featuring a predisposition to the development of depressive-like behaviour and that these animals should be non-responsive to treatment with a traditional antidepressant (Willner *et al.*, 2015).

2.7 Synopsis

TRD, like MD, is a heterogeneous disorder with both stressful life events and genetic components contributing to its development. MD is one of the most common neuropsychiatric disorders, is a major cause of disability and poor quality of life and is a significant contributor to suicide and ischemic heart disease-related mortality. The pathophysiological foundations of TRD are multifactorial and may involve various abnormalities, including alterations in HPA-axis activity, monoaminergic regulation and structural deficits. Several genes, as well as exposure to stress have been implicated in predisposing individuals to the development of MD and treatment resistance. Hence, the incidence of TRD has increased in modern society due to an increased exposure to environmental stressors, which may include stressful and unhealthy lifestyles. Coincidentally, these factors also increase the incidence of stress- and lifestyle-associated chronic illnesses which are also involved in the development of comorbid MD. Several biomarkers of MD have been identified, including monoaminergic (5HT and NA), cholinergic, immune, metabolic and brain-structural aberrancies. Of these, monoaminergic alterations have been considered a key feature and also served as a basis for the development of most known antidepressant drug treatment strategies.

Patients suffering from MD may display a host of symptoms, including mood and cognitive deficits, sleep abnormalities, while the diagnosis of the disorders is primarily based on subjective evaluation of these and other symptoms according to criteria provided by diagnostic manuals. Subsequently, one or more treatment modalities, e.g. pharmacotherapy, psychotherapy and various somatic approaches, may be prescribed in attempts to, ideally, establish remission without subsequent

relapse. Unfortunately, despite the armamentarium of antidepressant drugs available, up to half of patients fail to reach complete remission. Furthermore, as many as 20% of MD patients fail to respond to several antidepressant treatment approaches, establishing a state of treatment resistance which is accompanied by the presence of more severe symptomatology. Furthermore, it is evident that a significant proportion of the MD population suffers from comorbid mood and anxiety disorders and this rate of comorbidity is even higher in TRD patients. In this regard, PTSD has a considerable presence among TRD patients, highlighting the impact of environmental adversity on the development of mood disorders in predisposed individuals. Although several novel treatment approaches have been proposed for improved treatment outcomes in TRD, research is hampered by the lack of preclinical models to clarify the biological construct underlying treatment non-response and to assess and investigate novel targets for treatment of the disorder.

In light of this, the present investigation will attempt to develop and validate a gene-x-environment animal model of TRD by exposing the FSL rat, genetically predisposed to develop depressive-like behavior, to TDS, a well-validated animal model of PTSD, and to validate the model with respect to the following criteria for TRD:

Face validity: behavioural responses of FSL-rats exposed to TDS will be measured in the FST, a well-validated and reliable tool to detect the presence of and measure depressive-like behavior, relative to that of unstressed FSL rats.

Construct validity: monoaminergic (5HT and NA) alterations – a cornerstone of the pathophysiological processes relevant to MD – will be measured in FSL-rats exposed to TDS and compared to that of unstressed animals. Additionally these alterations may be indicative of biological processes responsible for behavioural responses measured in the FST and will be applied as such.

Predictive validity: treatment response to a traditional antidepressant (imipramine) will be measured in TDS-exposed FSL rats versus unstressed animals by means of behavioural (FST) assessment and neurochemical (5HT and NA) analysis. Subsequent to this, response to drugs utilized in the management of TRD (e.g. venlafaxine and ketamine) will also be measured when administered in monotherapeutic and augmented regimens.

In the following chapters the current study will be presented based on the study questions noted in Chapter 1 – as such, manuscripts containing a literature review of biomarkers in mood and psychotic disorders (Chapter 3) and data, as well as the discussion thereof, gathered during the development and validation of and animal model of TRD (Chapters 4 and 5) are included.

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CHAPTER 3

3 Manuscript A

Article published in *Current Neuropharmacology* titled:

"A Review of Biomarkers in Mood and Psychotic Disorders: A Dissection of Clinical vs. Preclinical Correlates"

Author Contributions

- Sarel J Brand prepared the first draft as well as the final version of the manuscript
- Marisa Möller contributed to the initial and final versions of the manuscript.
- Brian H Harvey devised the concept and lay-out of the manuscript, as well as finalized the pre-submission version of the manuscript.

Important Information

- Instructions to the author are included in Addendum A.
- The published article is provided in PDF format in Addendum C.
- As per the instructions to the author, figures, tables, and legends are provided at the end of the manuscript.
- Both co-authors provided consent for the paper to be assessed as part of the Ph.D. thesis of *Sarel Jacobus Brand* (Addendum B).

A Review of Biomarkers in Mood and Psychotic Disorders: A Dissection of Clinical vs. Preclinical Correlates

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Abstract

Despite significant research efforts aimed at understanding the neurobiological underpinnings of mood (depression, bipolar disorder) and psychotic disorders, the diagnosis and evaluation of treatment of these disorders are still based solely on relatively subjective assessment of symptoms as well as psychometric evaluations. Therefore, biological markers aimed at improving the current classification of psychotic and mood-related disorders, and that will enable patients to be stratified on a biological basis into more homogeneous clinically distinct subgroups, are urgently needed. The attainment of this goal can be facilitated by identifying biomarkers that accurately reflect pathophysiologic processes in these disorders. This review postulates that the field of psychotic and mood disorder research has advanced sufficiently to develop biochemical hypotheses of the etiopathology of the particular illness and to target the same for more effective disease modifying therapy. This implies that a "one-size fits all" paradigm in the treatment of psychotic and mood disorders is not a viable approach, but that a customized regime based on individual biological abnormalities would pave the way forward to more effective treatment. In reviewing the clinical and pre-clinical literature, this paper discusses the most highly regarded pathophysiologic processes in mood and psychotic disorders, thereby providing a scaffold for the selection of suitable biomarkers for future studies in this field, to develop biomarker panels, as well as to improve diagnosis and to customize treatment regimens for better therapeutic outcomes.

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BH Harvey devised the concept and layout of the manuscript, as well as finalized the pre-submission version of the manuscript. S Brand prepared the first draft as well as the final version of the manuscript. M Möller contributed to the initial and final versions of the manuscript.

Conflicts of Interest

The authors declare that over the past three years Brian Harvey has participated in speakers/advisory boards and received honoraria from Servier, and has received research funding from Lundbeck and Servier. The authors declare that, except for income from the primary employer, research funding to BHH from the South African Medical Research Council as well as the honoraria described above, no other financial support or compensation has been received from any individual or corporate entity over the past three years for research or professional services, and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

Introduction

Major depression (MD), bipolar disorder (BPD) and psychotic disorders (e.g. schizophrenia) are often misdiagnosed, leading to inadequate treatment and devastating consequences [1, 2]. MD is among the most debilitating diseases worldwide, with a lifetime prevalence of up to 20% [3], and even though major advances have been made in developing new drugs, less than 50% of patients achieve remission after antidepressant treatment [4]. Bipolar disorder affects approximately 1.2% of the population worldwide [5], and differs from MD with a unique hallmark of mania (elevated mood or euphoria, hyper-activity with a lack of need for sleep, and an increased optimism) which frequently leads to a deficit in the patient's judgment [6]. On the other hand, schizophrenia is a debilitating neuropsychiatric disorder, typically emerging during adolescence or early adulthood and continuing to plague patients suffering from the disease to varying degrees throughout their lifetime [7]. Approximately 1% of the general population worldwide is affected by the disorder and the life expectancy of patients with schizophrenia has been demonstrated to be nearly 20% shorter than that of the general population [8].

Despite an abundance of research, the pathogenesis and aetiology of mood and psychotic disorders remain unclear, challenging the diagnosis and treatment of these disorders [9], mainly for the following reasons:

- Diagnoses of typical psychiatric disorders are primarily based on operationalized behavioural diagnostic systems as either self-reported symptoms by patients or observations by clinicians, being confirmed against diagnostic criteria set out in the Diagnostic and Statistical Manual of Mental Disorders $4^{th}/5^{th}$ ed. (DSM IV / V) and International Statistical Classification of Diseases, 10^{th} Revision [10].
- Laboratory diagnostic and screening tools, such as a non- invasive blood-based test, remain elusive [11], while mood and psychotic symptoms may overlap with other neurological and psychiatric problems [9].

Clinically useful biomarkers in these disorders could therefore significantly improve diagnosis and treatment and has been one of the holy grails of MD, BPD and schizophrenia research [12]. However, the likelihood of any single biomarker achieving a high enough degree of sensitivity and specificity

for mood and psychotic disorders is relatively low. Biomarker panels may represent an attainable alternate to a single-biomarker approach [13]. Common features of this method include correlates attributed to the individual that may determine the presence or absence of a state of sickness or that may even predict response to treatment [13]. Biomarkers may also indicate the presence of a pathophysiological process that can be addressed with a preventive treatment [14], as well as highlight "state" and/or "trait" markers. Therefore, the identification of biomarkers prior to onset of depressive and bipolar symptoms or psychosis has enormous potential importance for the design of future preventive strategies.

The Biomarkers Definitions Working Group of the National Institutes of Health Group [15] (2001) defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [15]. Biomarkers should also provide high levels of sensitivity and specificity (>80%) in the detection and classification of MD, BPD and psychotic disorders [16], in order to be clinically useful.

There are many potential biomarkers for mood and psychotic disorders and previous studies have tested specific biomarkers based on the hypotheses of monoamine dysfunction, altered immune-inflammatory processes, neuroendocrine dysfunction and disturbances in neuroplasticity [17]. However, considering the most recent neuroanatomical basis for these illnesses, as well as relevant hypotheses of their aetiology, this review will provide an overview of potential biomarkers that could contribute to an initial multi-analyte biomarker panel of mood and psychotic disorders. Such biomarkers include molecules and/or processes directly or indirectly connected with growth factors, neurotransmitters, oxidative stress, inflammation, neuro-imaging, genetic, proteomic and neuronal resilience markers. In order to do this, we have collated neuroimaging and neurobiological findings from clinical studies as well as data from validated translational animal models, to assist in developing a putative, uniform biomarker panel for MD, BPD and schizophrenia.

There can be no doubt as to the value of translational animal models in drug discovery and in identifying novel neurobiological targets. Considering mood and psychotic disorders, these models include for example social isolation rearing (SIR) and the glutamate N-methyl D-aspartate (NMDA)

antagonist models of schizophrenia [18, 19], and the Flinders Sensitive Line (FSL) or chronic mild stress (CMS) rat models of MD [20, 21]. Developing an appropriate animal model to mimic BPD has proven to be an arduous task, it being difficult to establish a model that combines symptoms of MD, mania and euthymia in an alternating manner as is observed in BPD. Instead, animal models have been developed to express features central to either MD or mania using pharmacologic (amphetamines and ouabain), environmental (e.g. behavioural despair; sleep deprivation), or genetic (e.g. FSL rat) models. Therefore, in parallel with our analysis of the clinical scenario, we will also closely scrutinize appropriate animal models for correlation with clinical findings. This review will cover MD, BPD and schizophrenia with respect to the noted biomarkers and across clinical and pre-clinical correlates.

The neuroanatomy and neurocircuitry of psychiatric illness

Clinical Correlates

Neuroimaging methods, such as structural magnetic resonance imaging (MRI), functional magnetic resonance imaging (fMRI), diffusion tensor imaging (DTI) and positron emission tomography (PET), provide important evidence for underlying biological factors of MD, BPD and psychotic disorders [22]. Generally, areas of the limbic system, the hippocampus and frontal cortical areas are under scrutiny with regard to structural and functional neuroimaging research in mood disorders [23]. Importantly, there is increasing awareness of the interplay between specific neurocircuitry of the brain and behavioural pathology.

Depression

Neuroimaging studies have been central in identifying the key structures involved in the pathophysiology of MD, showing decreases in hippocampal volume of up to 15% in depressed patients [24], as well as reductions in grey-matter volume and glial density in the prefrontal cortex and the hippocampus [25]. Other studies in MD indicated large volume reductions in the anterior cingulate cortex (ACC) and orbitofrontal cortex (OFC) accompanied by lesser reductions in the prefrontal cortex, along with moderate reductions in the hippocampus, the putamen, and the caudate nucleus [26]. Lorenzetti et al. [27] reported volume reductions of the hippocampus, basal ganglia, the OFC and subgenual prefrontal cortex in patients suffering from MD, while more

persistent forms of MD (which may include recurrent episodes or relapses and extended illness duration) are accompanied by an increased effect on regional brain volumes [28]. While reductions in hippocampal volume in MD may have a genetic component [29], it is also a function of illness duration [30] as well as poor compliance [28]. Moreover, PET studies have revealed consistently increased regional blood flow and glucose metabolism in the amygdala, orbital cortex, and medial thalamus but decreased blood flow in the dorsomedial/dorsal-anterolateral prefrontal cortex and anterior cingulate cortex in un-medicated MD patients [31]. N-acetyl-aspartate (NAA), an indicator of neuronal viability, was also reduced in frontal cortex and in subcortical regions of MD patients [32, 33].

Regarding antidepressant therapy, Frodl et al. [34] demonstrated increases in hippocampal volume in patients who were subjected to continual treatment with antidepressants for three years, while Mayberg et al. reported that patients who responded to antidepressant treatment presented with increased anterior cingulate metabolism at baseline when compared to non-responders and to healthy controls [35]. Importantly, Macqueen et al. noted a greater degree of hippocampal shrinkage in patients with a prior history of switching antidepressants, indicating the possible deleterious effect of non-compliance on hippocampal integrity [28]. Furthermore, it has been proposed by structural neuroimaging studies that the volume of regional structures, for example the ACC and hippocampus, may provide an estimate of response to treatment [36]. Clinical response has also been demonstrated to be predicted by activity in the rostral ACC region as measured by electroencephalography (EEG) studies which identified activity localised to the rostral ACC region as being predictive of a clinical response to antidepressant medication [37]. Finally, favourable treatment outcomes have repeatedly been demonstrated to be associated with integrity of perigenual anterior cingulate volume [38]. Moreover, direct electrical stimulation of the striatum has been found to elicit a positive response in patients suffering from resistant MD and is bolstered by reports proposing the striatum as an important relay system between limbic and cortical structures [39]. ACC activity has also been positively related to a variety of treatment responses, including antidepressant pharmacotherapy and sub-chronic and experimental treatment strategies, including sleep deprivation [40], which suggests that ACC response is generalised across different treatment types.

Bipolar Disorder

In patients with BPD, neuroimaging studies have found enlargement of the amygdala [41] and reductions in the dorsal and ventral prefrontal cortices [42] while PET studies have found decreases in cortical metabolism and increased normalized subcortical metabolism in depressed patients with BPD [43]. Two meta-analyses of neuroimaging studies concluded that patients with BPD suffered from hypo- activation and gray matter reductions in cortical-cognitive brain structures and increased activation of the para- hippocampal gyrus and amygdala [44].

Schizophrenia

In schizophrenia and other psychotic disorders, regions such as the ACC and dorsolateral prefrontal cortex (DLPC) have been emphasised [45]. Using cognitive paradigms, fMRI studies have demonstrated alterations in cerebellar activity in patients with schizophrenia, anxiety disorders and dementia (see review [46]). However, Farrow et al. [47] found that lateral and medial frontal regions and bilateral posterior temporal lobe regions feature structural losses in schizophrenia, whereas alterations in patients suffering from BPD were limited to bilateral inferior temporal gyri while deficits observed subsequently were limited to the ACC. Temporal lobe regions present with decreased activation in patients suffering from schizophrenia [48]. Additionally, EEG studies have demonstrated a reduction in the P300 wave amplitude, elicited in the process of decision making, in BPD and schizophrenia patients compared to control subjects [49]. Studies utilizing structural MRI have consistently observed temporal lobe abnormalities in schizophrenia, although results in BPD are less dependable [50]. Previous fMRI studies have also consistently reported anomalies in the prefrontal cortex in patients suffering either from a first episode or established schizophrenia [51, 52]. However, some of the evidence points to dorsolateral hyper-frontality, and especially for tasks which demand working memory, as well as increased activity in parietal regions [53]. Considering the progression from the prodromal phase to established chronic illness, patients with first episode and established schizophrenia show a gradual deterioration in frontal and striatal activation [54]. The most consistent findings in schizophrenia relating to cognition are detriments in executive tasks requiring prefrontal cortical function, e.g. a self-ordered working memory task [55] or anti-saccade eye movements [56], olfactory identification [57], and tasks that rely on rapid processing of information (e.g. story recall)

[58]. A recent 1H-MRS study in schizophrenia patients measuring NAA and N-acetyl-aspartylglutamate (NAAG) found a significant increase in NAAG/NAA ratio in the ACC but no difference in the left frontal lobe, although an inverse correlation between frontal lobe NAAG and negative symptoms was observed [59].

Pre-Clinical Correlates

Depression

Reductions in hippocampal volume have been observed in FSL rats, a genetic model of MD, when compared to Flinders Resistant Line (FRL) controls and is associated with a decrease in the number of neurons and synapses in the hippocampus – these alterations are reversed after chronic imipramine therapy [60].

Bipolar Disorder

In the ouabain-induced rat model of bipolar mania, PET imaging suggests reduced cerebral glucose metabolism, and is prevented by pre-treatment with lithium which concurs with similar decreases in cerebral metabolism noted in BPD patients [61]. Furthermore, lithium prevented stress-induced alterations in the amygdala by preventing increases in dendritic branching of pyramidal neurons in this structure [62]. Unfortunately, a paucity of MRI studies remains a shortcoming in animal models of BPD.

Schizophrenia

Previous studies indicated that the SIR model, a neurodevelopmental animal model of schizophrenia, presents with significantly reduced PFC volume, reduced accumbal dendritic length and spine density, cytoskeletal alterations and loss of parvalbumin (PV)-containing interneurons [18, 63, 64]. Among the most robust pathologies observed in schizophrenia is a decrease in gamma-aminobutyric acid (GABA) signaling (discussed in section 3.1.4), deficits of which are limited to the class of GABAergic interneurons containing the calcium binding protein PV [65]. These neurons synapse on the cell body or axon initial segment of glutamatergic neurons and thus are positioned to potently regulate pyramidal cell output. Furthermore a decrease in PV interneuron functionality

may lead to reduced inhibitory control over pyramidal cell activity and also reduce coordination in activity of large brain networks [66].

SIR rats without an enriched environment also present with a decrease in dendritic spine density in the dorsolateral striatum when compared to rats from an enriched environment [67, 68]. Moreover, a NMDA receptor antagonist model of schizophrenia, *viz*. the phencyclidine (PCP) model, presents with decreased synaptic spine density on frontal cortical neurones [64]. Interestingly, rats treated chronically with MK-801 (another NMDA receptor antagonist model) also show a reduction in the amount of PV-containing neurones in the dentate gyrus and CA1 region of the hippocampus, although this is not accompanied by alterations in the PFC [69]. Furthermore, chronic intermittent exposure to PCP decreases NAA and NAAG levels in the temporal cortex, while it raises hippocampal NAAG levels [70]. Similarly, SIR reduces NAA in the temporal cortex without changes observed in the hippocampus, striatum or frontal cortex [71]. These changes may indicate neuronal dysfunction that mirrors alterations observed in schizophrenia, as discussed in the clinical section [59].

In order to determine which regions exhibit the most disease-relevant information as well as the most potential for predictive capacity, these neuroanatomical correlates need to be linked to biological and genetic markers to more accurately predict both the pathology underlying the disease and the clinical outcomes. Predicting clinical response will assist in early identification and to further stratify patients who may benefit from more intensive, alternative, or combined therapies.

Biological, genetic and protein biomarkers

Neuroendocrine and Circadian Rhythms

Various hormones, especially the HPA-axis, thyroid hormones, insulin, as well as altered circadian rhythm, have a pronounced influence on neurodevelopment and the neurobiology of MD, BPD and schizophrenia [72]. They also influence other hormones such as sex steroids, orexin, arginine vasopressin etc. that are also implicated in these disorders but for reasons of space cannot be covered here.

Circadian Rhythms

Clinical Correlates

Altered circadian rhythms occupy a critical role in how the brain copes with stressful experiences and ultimately in regulating behavioural responses [73, 74]. The influence of circadian rhythm on mood and behaviour has received much attention in recent years and implicates not only hormonal dysregulation in these disorders, but also includes disturbances in neurochemical, redox and inflammatory cascades in its sphere of influence [75, 76]. Indeed, these processes will be discussed in subsequent sections of this review. Output from the suprachiasmatic nucleus (SCN) of the hypothalamus, the master biological clock, is under regulation by serotonergic (5-HT2c) and melatonergic (MT1/2) receptors, the expression of which are regulated by various clock genes. Indeed, melatonin-mediated regulation of hippocampal plasticity as well as clock gene expression in hippocampal neurons suggests a hitherto poorly recognized aspect in our understanding and treatment of these disorders [78]. Altered SCN output to other hypothalamic centres, but also monoaminergic cell bodies in the brain stem, will lead to wide-spread disturbances in neuroendocrine as well as monoaminergic function [78]. It is therefore not surprising that a significant amount of preclinical and clinical data has described the association between altered circadian rhythms with genetic, environmental and developmental abnormalities precedent to the development of MD, BPD (mania symptoms) and schizophrenia (see recent reviews by Wulff et al. [75] and Karatsoreos [76]). It is of relevance that agomelatine, a recently available antidepressant that acts via re-entrainment of circadian rhythms, may also have a therapeutic role in disorders other than MD, in particular BPD and schizophrenia [77].

Pre-Clinical Correlates

Biomarkers of circadian rhythms in animals remain a shortcoming, with such studies relying heavily on endocrine markers (as will be clear in the following section). Nevertheless, alterations in this system have on numerous occasions been shown to not only be of importance in humans suffering from MD, BPD and schizophrenia, but also in translational animal models for these disorders. Most animal models selectively bred to display characteristics of MD feature disturbed diurnal rhythms, e.g. the FSL rat [79], Wistar Kyoto rat [80] and mice bred for spontaneous helplessness [81]. Also,

depriving animals of REM sleep has been suggested to model mania [82]. In animal models of schizophrenia, blind-drunk (Bdr) mice demonstrate fragmented rest and activity rhythms under a light/dark cycle – which is reminiscent of altered sleeping patterns in schizophrenic patients [83].

Cortisol

Clinical Correlates

A dysfunctional hypothalamic-pituitary-adrenal (HPA) axis has been implicated in MD, BPD and also schizophrenia [84-86], affecting adrenocorticotrophic hormone (ACTH) release and cortisol secretion from the adrenal cortex [87]. Elevated salivary levels of cortisol after waking may represent a biomarker for depression in adolescence [88]. Importantly, an abnormal cortisol response, such as a flatter diurnal cortisol pattern, implies an abnormal stress reactivity that correlates with a greater severity of depression [73, 74], suggesting that altered circadian rhythms occupies a critical role in how the brain copes with stressful experiences and ultimately in regulating mood. Although the dexamethasone suppression test has attracted interest as a promising diagnostic test for MD, there has not been a consistent approach to evaluate its clinical usefulness [89].

Steen et al. [90] found no significant difference in cortisol release during a mental challenge in schizophrenia and BPD patients, although blunted cortisol release was observed in male patients compared to controls in both disorders [90]. A significant increase in systemic cortisol metabolism in both schizophrenia and BPD patients has been described, with results in patients with schizophrenia vs. controls being most consistent [87]. Interestingly, studies in children at risk for developing psychosis lend further support to the suggestion that illness onset is predated by a degree of HPA axis abnormalities, rather than being a subsequent epiphenomenon [91]. A blunted cortisol awakening response may embody an early marker of susceptibility to develop psychosis which may even be genetically mediated, whilst increases in diurnal cortisol levels may develop only proximate to disease onset [91]. These studies reaffirm the status of the HPA-axis, particularly cortisol levels and metabolism, as a putative biomarker in MD, BPD and schizophrenia, and warrants further study.

Pre-Clinical Correlates

FSL rats have been found to be hypocortisolemic, while Wistar Kyoto rats present with increased levels of corticotropin releasing hormone (CRH) and ACTH. Brain levels of dehydroepiandrosterone (DHEA), an adrenal androgen known to have antidepressant-like effects, has been demonstrated to be decreased in both FSL and Wistar Kyoto rats vs. healthy controls, Sprague Dawley and Wistar rats respectively [92]. Mice showing high reactivity to stress also present with symptoms resembling that in depressed patients and were demonstrated to have a flattened diurnal rhythm of glucocorticoid secretion [93]. Similarly, mice exposed to 6 weeks CMS presented with high plasma corticosterone levels and decreased hippocampal expression of glucocorticoid receptors [94]. Sleep deprivation, which has been used to induce an animal model of mania, leads to a marked increase in CRH [95]. Cortisol levels have also been found to be increased both in the frontal cortex and periphery of rats exposed to prenatal stress, which has relevance in that prenatal stress may predict the development of these disorders. In addition to increased corticosterone release, Ward et al. [96] found rats exposed to prenatal stress to also have adrenal hypertrophy with increased expression of CRF-1 receptors in the amygdala [97]. Furthermore, olanzapine treatment was able to reverse the increased cortisol observed in the prefrontal cortex following prenatal stress [98].

Thyroid Hormones

Clinical Correlates

The relation between thyroid dysfunction and mental disorders has long been recognized, ranging from depression [99], anxiety [100] and schizophrenia [101]. A recent study explored thyroid-stimulating hormone (TSH) in patients with acute schizophrenia, unipolar depression, bipolar depression and bipolar mania and, apart from measuring TSH disturbances in all the disorders, observed a definite higher prevalence of thyroid dysfunction in patients with both unipolar and bipolar mood disorders vs. controls [102]. Another study observed significant thyroid dysfunction (hypothyroidism and hyperthyroidism) in schizophrenia as well as BPD patients [88]. Interestingly, autoimmune thyroid disease was more common in schizophrenia [103], emphasising an immune-inflammatory basis for the illness. Santos et al. [104] reviewed research on thyroid function in schizophrenia, relating interrelations between the pituitary- thyroid axis and major neuro-signaling

systems involved in schizophrenia (including serotonin (5-HT), dopamine (DA), glutamate and GABA networks), as well as myelination and inflammatory processes. These processes are all convergent on the pathology of this disorder, as will be discussed. The authors conclude that thyroid hormone deregulation is a common feature in schizophrenia. Together, these studies emphasize the relevance of thyroid hormonal status as possible biomarkers in MD, BPD and schizophrenia, although further work in this regard is required to establish its putative role as a biomarker.

Pre-Clinical Correlates

Wistar Kyoto rats, an animal model of depression, have increased TSH levels that, together with ACTH, remains elevated after the diurnal peak [80]. To the best of our knowledge, the current body of literature on preclinical BPD and schizophrenia research does not contain significant data on thyroid hormones in animal models of mood and psychotic disorders.

Neurochemical markers

The majority of drugs used clinically to treat MD, BPD and psychotic disorders such as schizophrenia target monoamine (DA, 5-HT and noradrenaline (NA)) receptors, reuptake transporters and monoamine metabolism [105, 106]. DA-ergic, 5-HT-ergic and/or NA-ergic neurotransmission affects behaviour by regulating motivation, reward seeking, aggression, and activity level – all symptoms that play an important role in the pathophysiology of these disorders [107, 108]. However, the cause of mood disorders is far from being a simple dysregulation of central monoamines. For example, monoamine oxidase inhibitors and monoamine reuptake inhibitors produce immediate increases in monoamine transmission [109], whereas their mood-enhancing properties are only fully realised following 4-6 weeks of sustained treatment. In fact, some patients do not show adequate improvement even after many months of treatment [110]. This indicates that enhanced serotonergic or noradrenergic neurotransmission per se is not immediately responsible for the clinical actions of these drugs [111]. Indeed, neurotrophins, neurogenesis and the concepts of neuroplasticity has now taken centre stage in our understanding of mood and psychiatric disorders and the mechanism of action of antidepressants [109, 112, 112a]. Thus, and apart from some data based on NA (see later), selecting an antidepressant based on its monoamine selectivity remains to be substantiated. The same can be said for antipsychotic drugs. Furthermore, a realization that neuroendocrine and

metabolic dysfunction also contribute to the eventual development of these disorders, has provided a new framework for understanding their neurobiology and treatment. Nevertheless, their contribution towards the understanding and treatment of these disorders warrants closer scrutiny with respect to viable clinical biomarkers.

Dopamine

Clinical Correlates

Depression

Depressive symptoms (e.g. avolition, guilt, suicidality and social withdrawal) are ascribed to frontal cortical hypo-dopaminergia [109]. Striatal DA levels in MD are also reduced [113], being linked to symptoms such as anhedonia, reduced motivation and decreased energy levels. Patients presenting with MD episodes have been demonstrated to have significantly decreased dopamine transporter (DAT) binding potential, with binding potential correlating to receptor density and affinity [114]. Anhedonic MD patients exhibit significantly decreased levels of DAT in basal ganglia which are in accordance with the hypothesis linking impaired DA transmission to an impaired reward system [115]. It has also been suggested that decreased striatal D2 receptor density may underlie depressive symptoms, while increased striatal D2 receptor density/affinity has been observed in patients after successful SSRI treatment [116] which coincides with evidence of 5-HT modulating DA pathways [117].

Bipolar Disorder

Pharmacological evidence supports evidence that excessive DA neurotransmission mediates manic symptoms in BPD patients [118], while DA receptor D2 antagonists are robust anti-manic agents [119].

Schizophrenia

The DA hypothesis of schizophrenia proposes that a hyper-dopaminergic state in the striatum mediates positive symptom expression, while a hypo-dopaminergic state in the frontal cortex mediates cognitive and negative symptoms [120]. In line with this hypothesis, post-mortem studies in schizophrenia patients have described frontal cortical hypo-dopaminergia [121] and elevated DA levels in the striatum [120]. However, a previous study reviewing clinical evidence for DA involvement

in schizophrenia came to the conclusion that multiple "hits" (i.e. adverse environment, infection, chronic substance abuse etc.) interact to result in DA dysregulation, thereby producing the final common pathway to psychosis in schizophrenia [122]. In fact, MD [123, 124] and BPD [125, 126] are also correlated to early life trauma. It is pathways related to the latter that are deemed critical prodromal events in early life adversity, such as neurotoxicity, oxidative stress and inflammation that may hold the clue to identifying more appropriate biomarkers for these illnesses. Investigators have noted a strong correlation between D2 receptor binding and response to an antipsychotic, with a minimum 70% receptor occupancy necessary for antipsychotic action [127]. In fact, the success of treatment with antipsychotic agents depend on dopamine D2 receptor blockade, while Howes and Kapur [122] recently suggested various genetic and environmental factors to be implicated in compromising the brain and ultimately leading to dysregulation of DA.

Pre-Clinical Correlates

Depression

CMS decreases *in vivo* DA release [129] and leads to a decrease in D2 and D3 receptors in the limbic forebrain which is reversed by chronic treatment with imipramine [130]. A decrease in DA release in the nucleus accumbens has been observed as well as increased DA levels in limbic regions in FSL rats (together with elevated 5-HT and NA; see below) – possibly due to an increased synthesis and decreased release of DA [131].

Bipolar Disorder

Models of mania which incorporate dopaminergic agents, e.g. amphetamine, have been demonstrated to be superior to other similar models [132]. Alpha-methyl-para-tyrosine (AMPT) mediated catecholamine depletion mitigates some mania-related characteristics in DAT knockdown (KD) mice [133], while treatment with valproate reverses locomotor hyperactivity in these animals [134]. Also, treatment with lithium and valproate reverses increased extracellular DA and oxidative damage in a dextro-amphetamine-induced rat model of mania [135]. Furthermore, hyperactive rats exposed to CMS display significantly reduced HVA (homovanillic acid – DA metabolite) compared to DA in the nucleus accumbens, indicating decreased DA release in this brain region [136].

Schizophrenia

Previous evidence on the SIR model has indicated elevated striatal and decreased frontal cortical DA, dihidroxylphenylacetic acid (DOPAC) and homovanillic acid (HVA) levels [137]; increased or decreased frontal cortical DA and unchanged striatal DA [138]; as well as decreased frontal cortical and elevated nucleus accumbens DA, DOPAC and HVA [139, 140]. Another study in the SIR model observed reduced PFC D1 receptor density [141]. However, changes in mesolimbic dopamine D2 receptor expression are inconsistent, describing down-regulation in striatum [142], but no change in mesolimbic [143], hippocampal, PFC or amygdala areas [144]. SIR also induces a hyper-responsiveness in DA release in the PFC in response to systemic administration of the atypical antipsychotics clozapine and olanzapine, but not haloperidol [145]. Moreover, microdialysis data show that both basal and stress-induced PFC DA levels are reduced in rats chronically treated with PCP [146, 147].

Serotonin (5-HT)

Clinical Correlates

Depression

Although 5-HT2c antagonists are ineffective alone in the treatment of MD, they do show benefit when combined with other mood-regulating mechanisms, such as 5-HT reuptake inhibition (SRIs) or melatonin agonism (e.g., agomelatine) [78]. Since 5-HT2C receptor activation inhibits NA and DA release [78], the suppression of these monoamines by elevated 5-HT contradicts traditional views that antidepressant response typically involves an increase in brain 5-HT, as well as NA and DA. Indeed, elevated 5-HT-mediated suppression of DA and NA release will be counter-productive [148], such as causing emotional detachment and failure to address the anhedonic symptoms of MD [78]. Clearly there are valid reasons to doubt whether an elevation in brain 5-HT is in any way essential for antidepressant response. In fact, a sustained increase in 5-HT does not appear to be a requirement for anxiolytic/antidepressant effects of an SRI [149]. Further on this point, 5-HT agonists are ineffective as antidepressants while the 5-HT reuptake enhancer, tianeptine, is an effective antidepressant despite having the exact opposite effect on synaptic levels of 5-HT than SRIs [150].

This evidence contradicts the simplistic view that brain 5-HT needs to be elevated to improve mood, and has been instrumental in fuelling the search for new generation antidepressants.

Post-mortem studies have indicated that suicidal patients with MD present with low cerebrospinal fluid (CSF) levels of 5-hydroxyindole-acetic acid (5-HIAA), the metabolite of 5-HT [151, 152], along with increased 5-HT2A receptor binding sites in platelets [153] and prefrontal cortical sites [154] as well as increased limbic and decreased frontal cortical 5-HT1A receptors (reviewed in [155]). MD patients also present with reduced 5-HT2A receptor density in the frontal cortex [155a]. Interestingly, limbic density and activity of monoamine oxidase (MAO) is elevated in MD [156, 157] which in turn will influence a number of components of monoamine signalling.

Bipolar Disorder

The role of 5-HT in the pathogenesis of BPD is less studied, although a post-mortem study has indicated that subjects with DSM-III-R diagnoses of BPD who died while depressed had significantly reduced levels of 5-HIAA in frontal (−54%) and parietal cortex (−64%) [158]. A deficit in 5-HT uptake sites has also been observed in the brains of depressed BPD patients after death [159]. Furthermore, 5-HIAA levels were found to be decreased in the CSF of depressed BPD patients [160] and elevated in manic BPD patients [161].

Schizophrenia

Post-mortem studies in patients with schizophrenia [162,163] as well as psychotic patients [164] have observed reduced frontal cortex 5-HT2A and increased 5-HT1A receptor density. Another study also indicated increased striatal but diminished frontal cortical 5-HT uptake sites in schizophrenia patients [165]. In line with these findings, CSF, genetic and neuroimaging studies have demonstrated an increase in central 5-HT-ergic neurotransmission in schizophrenia [166, 167] and typified by the serotonergic psychedelics such as lysergide. A previous review suggested that the positive symptoms observed in schizophrenia (delusions, hallucinations etc.) could be associated with an excess of 5-HT in the striatum [168]. Despite the above evidence for 5-HT involvement in schizophrenia, clinical studies have found selective 5-HT2A/2C antagonists to be ineffective as antipsychotics (reviewed in [169]) and that alterations of brainstem 5-HT transporters are generally not associated with schizophrenia [170].

Preclinical Correlates

Depression

Excessive activation of the 5-HT2C receptor is anxiogenic [171] while 5-HT2C receptor antagonists are rapid acting with sustained anxiolytic actions [172]. 5-HT and 5HIAA levels were noted to be higher in limbic structures in the brains of FSL rats compared to normal Sprague Dawley rats [173], while 5-HT2/3 receptor density is compromised in the nucleus accumbens leading to a lack of DA-5-HT interaction [174]. Furthermore, CMS leads to an increase in 5-HT2a receptors in the cortex which is reversed by imipramine treatment [20]. However, contrary to human subjects, FSL rats present with decreased 5-HT synthesis [175] and SERT- expression [176].

Bipolar Disorder

5-HT-related data are limited in animal models of BPD and mania. However, a mutPOLG transgenic (Tg) mouse model of BPD has been demonstrated to have enhanced 5-HT turnover, accompanied by reduced 5-HT levels, in the amygdala and hippocampus when compared to non-Tg animals [177].

Schizophrenia

Studies on the SIR model has observed decreased cortical (or striatal) 5-HT/5-HIAA [138, 178], decreased frontal cortical and elevated nucleus accumbens and striatal 5-HT and 5-HIAA levels [140, 179]. Deficits in prefrontal 5-HT following SIR is also linked to the behavioural impairments associated with schizophrenia [180]. Evidence of altered 5-HT levels in the NMDA receptor antagonist model is limited with only one study indicating that 5-HT3 receptor antagonists can attenuate the behavioural hyperactivity caused by PCP [181].

Noradrenaline (NA)

Clinical Correlates

Depression

NA is of major importance in MD (reviewed in [182]). Previous studies have observed reduced levels of NA transporters in the locus coeruleus [183], altered density and sensitivity of frontal cortical α2A-adrenoceptors [184, 185], and a reduction of NA levels in non-compliant MD patients [186]. Further,

a positive relationship between urine NA levels and MD has been confirmed [187]. Symptoms of anxiety were also associated with increased NA excretion in the urine [187]. Moreover, studies have demonstrated that low urinary excretion of the NA metabolite, 3-methoxy-4-hydroxyphenylglycol (MHPG), predict a positive response to NA-selective drugs such as imipramine, nortriptyline, desipramine, or maprotiline [188, 189]. These studies illustrate the significance of urinary noradrenergic measurements as a biomarker in guiding treatment selection and predicting efficacy. Expression of adrenoreceptor density has also been investigated in individuals suffering from MD. However, even though dysregulation in alpha and beta-adrenoreceptor systems have been noted, it remains unclear whether alterations in the expression of these receptors are causative in the pathology of MD. Considering the heterogeneity of the disorder, the value of adrenoreceptor dysregulation as a biomarker is unclear [190].

Bipolar Disorder

NA studies in BPD are limited although an increased turnover of NA has been shown to be central to the pathology of the disorder [158]. Furthermore, post-mortem studies in schizophrenia associated with the positive symptoms of the illness describe elevated brain NA levels as mentioned above [191]. NA has also been shown to be one of the primary neurotransmitters targeted during carbamazepine therapy in BPD patients [192].

Schizophrenia

An earlier review found consistent evidence that the positive and negative symptoms observed in schizophrenia are associated with over-activity and under-activity of central NA, respectively [191]. Moreover, increased NA reactivity and/or tone have been linked to anxiety observed in schizophrenia [193].

Pre-Clinical Correlates

Depression

Data relating to NA as a biomarker in preclinical models of MD are limited, although increased catecholamines, including NA, has been reported in limbic regions in FSL rats [194].

Bipolar Disorder

Interestingly, very little data is currently available in animal models of BPD to support the role of NA as a biomarker, although a preclinical study has suggested a noradrenergic role for lamotrigine, producing an anti-immobility effect in the mouse forced swimming test (FST) while investigating the depressive facet of the disorder [195].

Schizophrenia

Similarly, evidence in support of NA in a schizophrenia animal model is extremely limited. However, a recent study on the SIR model in our laboratory has demonstrated elevated frontal cortical NA as well as striatal NA and MHPG, with decreased frontal cortical MHPG levels, in SIR rats [179]. Earlier SIR studies found an increase in NA turnover in the hippocampus, cerebellum and cortex of Wistar rats [196].

Glutamate and Gamma-Aminobutyric Acid (GABA)

Glutamate and aspartate, and GABA and glycine, are the preeminent excitatory and inhibitory amino acids respectively, in the brain. Their diffuse presence in interneurons (GABA) or as relay neurons and interneurons (glutamate) allows them to play a profound role in regulating the function of most neurotransmitter systems in the brain. As a result of their ubiquitous presence they are implicated in the neurobiology of probably all central nervous system disorders, in particular MD, BPD and schizophrenia. GABA-glutamate interactions have importance in kindling, a mechanism suggested to underlie the development of rapid cycling of mood or psychotic episodes, and how stressful life events adversely impact long-term outcome. GABA pathways exert a permissive role on the kindling action of glutamate, with excessive glutamatergic activity associated with synaptic remodeling and neurodegeneration.

Clinical Correlates

Depression

Abnormalities resulting in an increase in glutamate transmission have been reported in patients with MD [197]. Elevated levels of glutamate act on extrasynaptic NMDA receptors leading to an influx of

Ca2+ into the neurons, which results in the toxic accumulation of reactive oxygen species (ROS) [198], with increased nitric oxide (NO) production playing a key role in MD pathology and treatment response [199, 200]. We have earlier proposed that the NO pathway may play an important role in relapse and treatment resistance [201] as well as influencing the effect of non-compliance on treatment outcome [202]. In MD, glutamatergic hyper-function seems to be closely related to the lack of 5-HT-ergic and noradrenergic neurotransmission noted to underlie the core symptoms of MD. Indeed, studies examining peripheral blood of MD patients have demonstrated the glutamatergic system to be overly activated [203, 204]. Elevated glutamate levels have also been found in the occipital cortex of un-medicated subjects with MD [205]. Accordingly, reduced glycine binding (where it acts to abrogate NMDA receptor activity) has been described in the frontal and temporal cortex of suicide victims and MD patients [206, 207], leading to hyperglutamatergia. Glutamate, in combination with quinolinic acid (QA), a glutamate agonist derived from the kynureine pathway (see later), may contribute to excitotoxicity in the central nervous system [208]. While several factors may influence the levels of kynurenine and its metabolites (e.g. inflammation), a decrease in tryptophan (TRP) may generally be observed in patients suffering from MD resulting in reduced 5-HT levels. In general, depression is associated with lowered TRP, increased indoleamine 2,3 dioxygenase (IDO) activity as well as reduced levels of kynurenic acid [209, 210]. Furthermore, microglial levels of QA have been demonstrated to be upregulated in MD [211].

Bipolar Disorder

A recent review of magnetic resonance spectroscopy (MRS) studies in patients with BPD observed the cingulate and prefrontal cortices to contain higher glutamate levels, and possibly associated with illness state [212], while a decrease in NMDA receptor binding has also been noted in the CA3 region of the hippocampus [213]. In a post-mortem morphological study, an increase in QA positive microglia has been observed in the subgenual anterior cingulate cortex of BPD patients, commensurate with increased glutamatergic activity [211]. Drug therapy with the pyrimidine compound, cytidine, reduces glutamine/glutamate levels in BPD and possibly related to symptom severity, suggesting that the presence of glutamatergic dysfunction is an important factor in the underlying pathology of BPD [214]. Furthermore, the presence of genetic mutations affecting the glutamate pathway has also been suggested to be implicated in BPD [215].

Schizophrenia

Release of DA from cortical and limbic striatal structures are controlled by glutamate-GABA-glutamate feedback loops situated on pyramidal cells of the frontal cortex, the disturbances of which underlie the behavioral manifestations of schizophrenia [216, 217]. GABA'ergic interneurons in the brain stem monoaminergic nuclei, *viz.* raphe nuclei, locus coeruleus, ventral tegmentum, also modulate ascending serotonergic, noradrenergic and dopaminergic pathways, resulting in tonic inhibition of NA and DA release in the PFC, resulting in the emotional, mood and cognitive deficits associated with MD and schizophrenia [78].

The "glutamate" hypothesis of schizophrenia emerged from the observation that NMDA receptor inhibition induces schizophrenia-like behaviors in humans. Cortical hypoglutamatergia compromises DA release in the ventral tegmentum leading to meso-limbic hyperdopaminergic (positive symptoms) and meso-cortical hypodopaminergia (negative symptoms) [217]. Mitochondrial dysfunction, pro-inflammatory cytokines and increased IDO-mediated conversion of tryptophan to QA (supported by clinical evidence for elevated QA [218]), the latter an NMDA receptor agonist, may be directly or indirectly implicated in eliciting glutamate hyperactivity thereby increasing NMDA receptor activation, altered redox balance and oxidative stress [217]. Schizophrenia has also been likened to the kindling phenomenon, a process of increased excitatory glutamatergic activity coupled with a relative loss of inhibitory GABA'ergic tone [217]. In post-mortem schizophrenia studies, deficits of glutamate systems have been described in the temporal cortex, medial temporal lobe and striatal regions [219, 220], together with losses of glutamate uptake sites [221] and increases in NMDA receptors in the same brain regions [207]. Previous studies also emphasize the impact of NO metabolism via glutamate and GABA on NMDA receptor mediated neurotransmission in schizophrenia [222, 223]. NO is an important second messenger for the glutamate NMDA receptor pathway, and its overproduction is implicated in schizophrenia. Excessive NO release include impairment of NMDA-receptor mediated neurotransmission, disturbed DA metabolism, excessive ROS generation and mitochondrial dysfunction with cell-death (reviewed in [222, 223]). However, altered NO metabolism is not unique to, or indicative of, schizophrenia as disturbances in this signalling cascade has been noted in MD and BPD [222], as noted earlier. A recent clinical study also indicated elevated GABA and glutamate levels in the medial prefrontal cortex of un-medicated

patients, with no alterations in medicated schizophrenia patients, suggesting possible normalization of GABA and glutamate with antipsychotic treatment [224].

Pre-Clinical Correlates

Depression

Under conditions of chronic stress, elevated glucocorticoid levels enhance glutamatergic transmission by increasing the expression of the glutamate ionotropic NMDA receptors, as well as increasing the synthesis and extracellular concentrations of glutamate [225]. Abnormalities resulting in an increase in glutamate–NO transmission have been reported in FSL rats [226]. The possible mechanisms whereby NO can contribute to mood disorders is obscure, although persistent research has highlighted various possibilities including the actions of the NO/cyclic guanosine 3'5'-monophosphate (cGMP) pathway. Modulators of the NO- pathway have also gained relevance in MD research due to NO-inhibitors demonstrating antidepressant effects in models predictive of antidepressant activity [227, 228]. By activating soluble guanylate cyclase (sGC) which converts guanosine 5'-triphosphate (GTP) to the intracellular messenger cGMP [229, 230], NO is enabled to mediate many cellular processes, particularly the regulation of ion channels, activation and inhibition of cyclic nucleotide hydrolysis by phosphodiesterase, activation of G-kinase and modulation of neurotransmitter release [229, 231]. Interestingly, neuronal nitric oxide synthase (nNOS) inhibitors (e.g. methylene blue) [232] as well as PDE5 inhibitors (e.g. sildenafil) [233] have antidepressant and anxiolytic effects in animal models [234, 235] (Fig. 1), as do clinically relevant antidepressants [228]. These effects however are due to interference with constitutive nNOS-mediated effects and not inducible NOS (iNOS), which rather plays an important role in chronic stress associated with inflammation [231] (Fig. 1). Stressed vs. naive FSL/FRL rats show elevated hippocampal glutamate-NO signalling [224], indicating that a chronic environmental stressor is required in order to demonstrate altered glutamate-NO signalling in FSL rats [236]. This is congruent with the fact that MD involves a prior and/or ongoing chronic stressor [237, 238]. Considering these findings, it is not surprising that NMDA antagonists such as dizocilpine (MK-801) [239], ketamine [240], memantine [241] and others [242] exert antidepressant effects, while disinhibition of glutamate-NO signalling follows antidepressant discontinuation after chronic treatment [243, 244]. A possible explanation

could be that NMDA receptor antagonists increase 5-HT levels in the brain [245], while also having a modulatory effect on pathways involved in neuroplasticity and cellular resilience [202].

Preliminary evidence also supports the use of NMDA antagonists such as ketamine in treatment-resistant MD [246]. How this happens still needs illumination, although animal studies have begun to delve into the possible mechanisms involved [247]. The latter work has indicated a mutual cooperation with glutamate AMPA receptors [247], resulting in activation of mammalian target of rapamycin (mTOR) [248] and inhibition of glycogen synthase kinase-3β (GSK-3β) [249] signalling (Fig. 2). The mTOR pathway plays a pivotal role in protein synthesis by stimulating mRNA translation via interaction with its downstream targets [248], and leads to prolonged elevation of synapse-associated proteins in the prefrontal cortex [248]. Diminished activity of the mTOR pathway could underlie synaptic deficits in the PFC as previously reported in MD [250]. Furthermore, this evidence is supported by the behavioural responses to ketamine being blocked in mice which express constitutively active GSK-3β [251]. Considering the contributory role of oxidative stress in MD, inactivation of GSK-3β is linked to the regulation of redox homeostasis via stress responsive genes that protect cells against inflammation and oxidative stress [252, 253] (Fig. 2).

Bipolar Disorder

The standard treatment for BPD, lithium salts, target the glutamate-NO system [254, 255]. Unfortunately, current literature lacks sufficient data to elaborate on the role of glutamate/GABA as a biomarker in preclinical models of mania and BPD.

Schizophrenia

Decreased glutamate release has been observed in the frontal cortices of Homer1 mutant mice, a putative animal model of schizophrenia [256], while chronic phencyclidine (PCP) administration in rats is associated with a decreased expression of glutamate receptors in the prefrontal cortex [257] and a reduced number of cortical and hippocampal PV-immunoreactive neurones [64]. Confirming this, partial deletion of the NMDA receptor in mice is associated with behavioural alterations akin to that observed in PCP treated mice [258], while increased NMDA receptor binding has been described in the frontal cortex of SIR animals [141].

Neuronal Growth Factors

Growth factors are intricately involved in the survival, growth and differentiation of specific groups of neurons. Their relevance is gaining in importance in the light of increasing evidence that mood and psychotic disorders are associated with structural brain changes and that alterations in growth factors may precipitate or exacerbate depressive, BPD and psychotic episodes [13, 259, 260].

Brain-Derived Neurotrophic Factor (BDNF)

Clinical Correlates

Depression

Extensive studies have established that altered BDNF plays a pivotal role in MD. BDNF and the transcription factor, cyclic adenosine monophosphate (cAMP) response element binding protein (CREB), are intimately linked biochemically (see Fig. 2), playing a critical role in cellular resilience and neuroplasticity. Antidepressant treatment up- regulates CREB in the cortex and hippocampus of humans [261]. Both serum BDNF levels and CREB phosphorylation and protein levels are reduced in depressed individuals [262]. Moreover, an inverse relationship exists between serum levels of BDNF and the severity of MD [263], while antidepressant treatment is able to reverse the deficit in BDNF observed in MD [264, 265] and to increase phosphorylation and binding of CREB [266, 267]. BDNF is expressed throughout the body [268], but the exact origins of circulating BDNF remain elusive. BDNF has been shown to originate from several sources including brain neurons, vascular endothelial cells and platelets. It has also been shown to cross the blood-brain barrier [269] so that plasma BDNF levels may reflect central BDNF levels [270]. BDNF regulates synaptic plasticity in neuronal networks and appears to be a particularly relevant factor for mood disorders with associated cognitive dysfunction [271-273].

CREB is responsible for regulating BDNF expression [274]. Activation of CREB is associated with the regulation of synaptic plasticity as well as transcription of specific target genes involved in the production of proteins, BDNF being one example [275, 276]. Post-mortem studies have reported decreased hippocampal BDNF in MD patients who committed suicide, but elevated levels in patients who were being treated with antidepressant agents at the time of death [268, 277]. Considering the

growing evidence for an interaction between MD and metabolic and redox-related conditions [278-280], our group recently showed that altered serum BDNF may be linked to metabolic and redox factors, with BDNF levels indicating either a counter-regulatory action on the effects of glutathione oxidation or that BDNF may mediate the redox effects itself, leading to the development of a mood disorder [281].

Various factors associated with an increased risk of developing MD, e.g. smoking [282, 283] and type II diabetes mellitus [284], have been linked to BDNF deficits. Thus decreased levels of BDNF have been found in smoking individuals when compared to non-smokers [285, 286], while smoking cessation leads to improved BDNF levels [285, 286]. Likewise, serum levels of BDNF have been shown to be significantly lower in subjects with Type II diabetes when compared to healthy controls [287, 288], while cerebral output of BDNF is inhibited in the presence of high blood glucose levels [287]. These findings reiterate the causal link between metabolic diseases, altered BDNF and the development of MD noted earlier. On the other hand, for example, physical exercise has been shown to increase BDNF [289, 290], to be neuroprotective, to improve mood and to have antidepressant effects [291, 292].

Bipolar Disorder

Decreased peripheral BDNF levels have been observed in BPD patients, possibly associated with the pathophysiology and severity of manic symptoms [293]. Exercise has also been shown to decrease depressive symptoms in BPD patients and to even increase the frequency of mania [294]. The latter would indicate that elevated levels of BDNF may not always be beneficial, as has been proposed in a study in patients with MD [281]. The authors suggest that, by adversely affecting resilience, BDNF facilitates activity- dependent plasticity that may translate to a variable effect on mood and other plasticity-dependent functions. In fact, BDNF has been noted to induce paradoxical depressogenic effects [281]. As a mediator of synaptic plasticity, maladaptive secretion of BDNF (e.g. a response to environmental adversity) may set in motion counterregulatory responses that are counterproductive.

Schizophrenia

Decreased peripheral BDNF levels have been observed in schizophrenia [295]. Importantly, a recent study indicated that clinically stable schizophrenia patients present with significantly increased serum levels of BDNF after exposure to cognitive training targeted at improving neuroplasticity [296]. Furthermore, post-mortem studies reported a decreased concentration of BDNF-positive neurons [297] and BDNF concentrations in brain tissue of schizophrenic patients, which include the cortical areas and the hippocampi [298].

Pre-Clinical Correlates

Depression

Antidepressant treatment up-regulates CREB in the cortex and hippocampus of rats [299], while an overexpression of CREB in the dentate gyrus results in antidepressant effects in the FST and a learned helplessness paradigm – both animal models of MD [300]. In the latter, decreased hippocampal BDNF levels were described. Although contrary to that in human subjects, some studies have also noted increased serum levels of BDNF [301, 302]. In FSL rats, serum and whole blood BDNF levels have been found to be significantly increased compared to control but significantly decreased in the hippocampus, with no differences noted in the frontal cortex and CSF [303], suggesting that BDNF is differentially regulated in hippocampus, serum, and whole blood in these animals. The latter is not unlike similar paradoxical findings in humans, where BDNF has been suggested to play a counter-regulatory role [281]. Preclinical studies have indicated that BDNF administration produces antidepressant-like behaviour [304], while antidepressants and electroconvulsive therapy increase BDNF levels [305]. After animals were subjected to repeated stress, they constantly presented with decreased BDNF levels as measured in the hippocampus and serum, while corticosterone levels returned to normal levels, suggesting that changes in brain plasticity occur following a second stressful event [306]. The presence of decreased serum BDNF levels accompanied by normal serum cortisol levels may therefore represent a relevant biomarker for identifying individuals who are more likely to develop depressive symptoms in the subset of a population which may be predisposed to developing affective disorders. These alterations may even be expanded to other disorders provoked by stressful life events, for instance schizophrenia [307].

With smoking having been found to affect BDNF levels in humans, decreased levels of BDNF have also been found in rats repeatedly exposed to nicotine [308]. Similarly, physical exercise also increases BDNF in animals [289, 290].

Bipolar Disorder

BDNF levels are decreased in both the amygdala and hippocampus of rats in the ouabain model of mania, and reversed by lithium [309]. Moreover, in an amphetamine-induced model of mania, BDNF was also decreased in the hippocampus and increased by valproate and lithium [119].

Schizophrenia

Neonatal PCP administration produced a sustained elevation of BDNF in the hippocampus and the entorhinal cortex of 8-week-old rats [310]. However, studies in the SIR model observed significantly reduced medial PFC BDNF levels [311] as well as decreased hippocampal BDNF [312].

Insulin-Like Growth Factor

Insulin-like growth factor-1 (IGF-1) is involved in regulating peripheral cell growth and metabolism [313]; and plays a crucial role in the growth and differentiation of nerves and also in the synthesis and release of neurotransmitters [314].

Clinical Correlates

Depression

Unfortunately, there have not been sufficient clinical studies to determine whether peripheral IGF-1 is altered in MD patients or following antidepressant administration.

Bipolar Disorder

In BPD patients, a previous study observed altered IGF signalling in post-mortem brain tissue [315].

Schizophrenia

Antipsychotic-free schizophrenia patients have been found to present with a decrease in plasma IGF levels [316].

Pre-Clinical Correlates

Depression

Unfortunately, we are not aware of any extensive IGF-related data in established animal models of MD. Nevertheless, preclinical studies have indicated that peripheral IGF-1 administration reduces immobility in the FST [317], increases central BDNF mRNA [318] and produces antidepressant-like behavioural responses in mice exposed to CMS [317]. Moreover, after chronically treating rats with antidepressants, elevated IGF-1 expression was observed in the brains of these animals [319]. Finally, IGF-1 has been found to regulate adult hippocampal neurogenesis in rats [320].

Bipolar Disorder, Schizophrenia

To the best of our knowledge there is no pre-clinical data in established animal models of IGF-1 as a preclinical biomarker in BPD or schizophrenia.

Vascular Endothelial Growth Factor

Vascular endothelial growth factor (VEGF) acts as a neurotrophic factor, is a cytokine implicated in angiogenesis [321] and has been related to the vascular niche hypothesis of adult neurogenesis [322]. This hypothesis attributes increases in the proliferation of neurons in the adult hippocampus to VEGF-induced angiogenesis. VEGF is purported to play a role in several features associated with neuronal growth, including neuronal regeneration and differentiation as well as axonal outgrowth [323].

Clinical Correlates

Depression

MD patients present with higher plasma VEGF levels which can be reversed with antidepressant treatment [324], while earlier studies have confirmed said increase in MD [325]. Furthermore, remitted MD patients have significantly elevated VEGF levels, while MD patients with a family history of psychiatric disorders also have higher baseline levels of VEGF, compared to MD patients without a family history and healthy controls [326]. This may be indicative of a role for VEGF in the pathology of MD, possibly hinting of a neuroprotective role to counter reduced neurogenesis in MD.

Bipolar Disorder

BPD patients present with higher plasma VEGF levels during acute episodes vs. healthy controls [327], emphasizing that a depressive and manic episode in mood disorders may be associated with the neuroprotective role of VEGF. Interestingly, a recent study indicated that VEGF mRNA levels were significantly decreased in BPD patients treated with lithium vs. healthy controls [328], suggesting that VEGF may be a useful marker in BPD and as an indicator of lithium response.

Schizophrenia

VEGF data in schizophrenia patients are limited, although previous studies have not observed any differences in serum VEGF in schizophrenia vs. healthy individuals [285]. However, significantly reduced levels of VEGF mRNA have been observed in the DLPFC of patients with schizophrenia [329].

Pre-Clinical Correlates

Depression

The relationship between central and peripheral levels of VEGF still needs clarification. In a genetic rat model of MD, Elfving and colleagues found decreased levels of VEGF in the brain but no variations in serum VEGF levels [301].

Bipolar Disorder

To the best of our knowledge, there is no data available on VEGF as a biomarker in preclinical models of BPD.

Schizophrenia

Similarly, no pre-clinical data is currently available on VEGF in the SIR or NMDA antagonist models of schizophrenia. However, a pre-clinical study did observe that VEGF levels are increased in rat hippocampi following 14 days haloperidol or olanzapine treatment [303]. Interestingly, in the case of haloperidol treatment this increase was lost 45 days later, while olanzapine treatment bolstered the initial increase in VEGF [330], reaffirming that first and second generation antipsychotics are not therapeutically equivalent. This underlines VEGF as a possible marker in schizophrenia treatment but not diagnosis per se.

Neuronal Resilience Markers

Several neurochemical markers have been associated with neuroprotective effects and positive antidepressant treatment response. With the increased evidence for a neurodegenerative profile for MD, BPD and schizophrenia and the progressive nature of these illnesses, identifying neuroresilience markers is gaining in relevance. In this regard, resilience markers linked to the BDNF pathway are especially attractive.

Stress and environmental adversity is a common thread throughout all three illnesses under review [252, 331, 332]. Stress-induced increases in glucocorticoid levels have been shown to decrease the synthesis of neurotrophic factors, particularly BDNF, which is an effective neuroprotective factor and protagonist of neurogenesis [333]. These neurotrophic effects are mainly mediated through inhibition of cell death pathways and activation of mitogen-activated protein kinases (originally extracellular signal-regulated kinases or MAPK/ERK) signalling pathways and phosphotidylinositol-3 kinase (PI-3K)/Akt (protein kinase B) pathways (see Fig. 2) [334]. As noted earlier, BDNF expression is decreased during MD, BPD and schizophrenia, a response that is reversed by effective pharmacological treatment [295, 335-337]. Furthermore, increased structural atrophy observed in treatment resistant MD has been correlated with greater decreases in BDNF levels [338] in patients failing to respond to SRI treatment compared to treatment responsive patients.

The cAMP cascade is activated following increased serotonergic and adrenergic receptor activity which results in downstream activation of CREB [339]. The ensuing elevation in cAMP ultimately leads to increased BDNF expression which subsequently activates the MAPK/ERK pathway, a major pathway involved in cell growth and proliferation [340, 341] (see Fig. 2). Monoaminergic neurons experience an increase in growth following MAPK/ERK pathway activation thereby accounting for how BDNF modulates the monoaminergic system [342].

Activation of the PI-3K cascade by BDNF leads to phosphorylation of Akt, a molecule at the crossroad of cell survival and cell death [343] (see Fig. 2). Activation of Akt following phosphorylation leads to enhanced activity of mTOR which is responsible for regulating the expression of several genes involved in cell growth, particularly a group of synapse-associated genes that have been directly linked to neuroplastic events [248]. Conversely, inactivation of Akt by dephosphorylation leads to a

decrease in phosphorylation and subsequent activation of Bcl-xL/Bcl-2-associated death promoter (Bad), a pro-apoptotic molecule [343]. It is therefore evident that in combination, the MAPK/ERK and PI-3K pathways are largely accountable for the neuroplastic events occurring during antidepressant response and, furthermore, directly links the actions of BDNF to these processes.

Clinical Correlates

Depression

Decreased cAMP levels and lower MAPK/ERK pathway activity has been associated with MD, which has been shown to be reversed by increasing BDNF levels [344].

Bipolar Disorder

GSK-3β has been demonstrated to be an important role-player in BPD with lithium, an inhibitor of GSK-3β, having served as a mainstay in the treatment of BPD. It also regulates various proteins and is involved in neuroplasticity and neurotransmission [345]. Therefore, agents involved in the modulation of GSK-3β and its downstream pathways may serve as valuable biomarkers in the diagnosis and treatment of BPD – e.g., several molecules involved in both cell survival and apoptosis, such as CREB [346] and p53 [347], respectively. GSK-3β also plays an important role in the regulation of the Wnt [347] and PI-3K [348] signalling pathways linked to cellular resilience [348, 349] (Fig. 2). It has also been suggested that progranulin (PGRN) may serve as a neurotrophic factor-modulating neurite outgrowth as well as neuronal differentiation and survival [350]. Furthermore, plasma levels of PGRN are decreased in BPD patients [351, 352] and GSK-3β has been implicated in mediating PGRN activity [351]. GSK-3β protein expression is decreased in the platelets of BPD patients [349]. Even though previous studies could not find alterations in brain expression of GSK-3β, decreased protein expression in platelets can be reversed by mood stabilizers – but not antidepressants – thereby emphasizing a valuable role for GSK-3β as a peripheral biomarker and even a state – rather than trait – marker of BPD [353].

Schizophrenia

A recent post-mortem study in patients with schizophrenia found increased levels of various proteins involved in the MAPK- and cAMP-associated pathways, as expressed in frontal cortical structures [354]. In line with these observations are studies indicating alterations in several proteins in the MAPK-associated pathway: extracellular signal-regulated kinase (ERK)-2, immediate early genes c-fos and c-Jun levels were elevated in the thalamus on both protein and transcription level, whereas c-Jun protein and Elk-1, CREB, and ATF-2 protein levels were elevated in the cerebellar vermis [355, 356]. Moreover, other proteins involved in the MAPK pathway, including MEK1, MEK2, RSK1, B-Raf, and CREB were found to be reduced in the frontal cortex of schizophrenia patients [357]. With regards to the cAMP pathways, decreased DA- and cAMPregulated phosphoprotein Mr 32 kDa (DARPP-32) was observed in the frontal cortex and thalamus of schizophrenia patients [358, 359]. Furthermore, a recent review highlights numerous evidence and theories in support of a novel mTOR based hypothesis of the neuropathology of schizophrenia [360]. Control of protein synthesis is the primary role of this signalling cascade while it is also regulated by known extracellular and environmental factors implicated in the pathology of schizophrenia [360].

Pre-Clinical correlates

Depression

Blocking MAPK signalling leads to depressive-like behaviour in the FST in rats and inhibits the antidepressant effects of ketamine [340]. These findings provide some insight on how glutamate-NMDA signalling interacts with monoaminergic-cAMP pathways to mitigate a faster onset of action or to treat refractory MD. Furthermore, SIR in rats, a putative neurodevelopmental animal model of MD and schizophrenia [18], leads to an enhanced expression of mitogen-activated kinase phosphatase and apoptosis-related genes in the prefrontal cortices of Sprague-Dawley rats [361].

Bipolar Disorder

Transgenic mice that overexpress GSK-3 present with decreased habituation and an increase in activity that has been related to hyperactivity in mania [362]. β-catenin, a downstream molecule of GSK-3 (Fig. 2), was found to be decreased in the hippocampi of black Swiss mice, a putative model

of mania [363], while the behaviour of transgenic mice overexpressing β -catenin was found to have a behavioural phenotype similar to that of lithium-treated animals [364]. Of significance is that lithium stabilizes β - catenin by inhibiting GSK-3β thus reducing neuronal vulnerability to apoptosis [365].

Schizophrenia

Gururajan and Van den Buuse [360] have explained the involvement of mTOR in schizophrenia by referring to numerous animal models of schizophrenia. However, direct measurement of neuronal resilience markers in the SIR model and the NMDA antagonist model is limited, with only one study indicating that MK-801 administration elevates phosphorylation of MAPK in the frontal cortex of rats [19].

Oxidative Stress Markers

Normal oxidative metabolism in cells results in the production of various ROS. Oxidative stress occurs when cellular antioxidant defence mechanisms, such as SOD, catalase, glutathione peroxidase, fail to counterbalance and control endogenous production of ROS such as O_2^- and H_2O_2. This leads to a free radical attack on proteins, DNA and lipids [366, 367]. SOD is the primary defence against oxidative stress by converting O_2^- to H_2O_2 [368]. Hydrogen peroxide in turn is converted to water and glutathione (GSSG) by catalase and glutathione peroxidase [369], with GSSG rapidly being converted to reduced glutathione (GSH) by glutathione reductase [370]. The brain has relatively low levels of antioxidant defences, as well as a high lipid content that is highly susceptible to attack by ROS [371]. Thus, a reduction in GSH, and an increase in GSSG, is regarded as being indicative of increased oxidative stress.

Many of the changes in oxidative status may be directly related to increased inflammatory response due to the presence of other systemic illnesses, such as endocrine and metabolic disorders and cardiovascular disorders [281]. Furthermore, changes in certain neurotransmitter systems in the brain, especially glutamate and GABA, increase the risk of oxidative stress in the brain and subsequent neuronal oxidation and cell death [279]. Moreover, oxidative stress in its own right may mediate altered monoaminergic activity [372] that underlies the pathology of many neuropsychiatric illnesses associated with oxidative stress [373]. One of the more prominent redox active molecules

released by changes in glutamate activity in the brain is NO, and which is well described as being a contributing factor towards the development of MD [374], schizophrenia [223] and possibly BPD [375]. In this regard, both constitutive NOS-, such as nNOS, and iNOS-mediated NO synthesis needs to be considered, with nNOS being more involved in neurotransmission and iNOS in inflammation. Fig. 1 provides an outline of how glutamate, NO and redox systems interact to produce oxidative stress.

Clinical Correlates

Depression

In recent years MD has been associated with several changes in redox status, presenting as either an increase in oxidative stress and/or diminished oxidative defence systems [279]. Elevated plasma malondialdehyde (an indication of lipid peroxidation) levels and susceptibility of red blood cells to oxidation, as well as an increase in serum SOD activity, has been observed in MD patients [376, 377]. However, Srivastava et al. [378] found no alterations in the activities of SOD and glutathione peroxidation in polymorphonuclear leukocytes from patients with MD. In their clinical study, Berk and colleagues noted only limited support for the role of antioxidant and glutathione precursor, N-acetyl cysteine (NAC), as an adjunctive therapy for MD, although further such clinical studies are required [379]. A high incidence of co-morbid metabolic syndrome and MD have been observed [380] with inflammation a major mediator in the development of both MD and metabolic syndrome [381]. In support of this, substantial evidence exists linking insulin- and NO-mediated pathways in the brain. In fact, insulin upregulates expression of nNOS [382] while a role for increased NO and insulin/peroxisome proliferator-activated receptor (PPAR) signalling has been noted following stress, thus presenting as a susceptibility factor in the subsequent development of MD [383].

Bipolar Disorder

BPD patients present with changes in antioxidant enzymes, for example Andreazza et al. [384] reported manic and depressive phases to be associated with increased SOD activity, but unaltered activity in euthymia. This is corroborated in part by Machado-Vieira [260] who found untreated manic bipolar patients to present with increased activity of SOD. Furthermore, patients who were euthymic presented with decreased catalase activity [384], while activity was increased in manic

patients who did not receive treatment [260]. Increases in lipid peroxidation due to oxidative stress unrelated to the phase of illness have also been reported [260, 384]. In addition, BPD patients were found to express increased lipid peroxidation in the cingulate cortex [385], while clinical studies have indicated that the antioxidant, NAC, is effective as adjunctive treatment in BPD [386-388].

Schizophrenia

Evidence has accumulated in recent years that antioxidant systems are impaired in schizophrenia [389]. Gawryluk et al. [390] reported reduced levels of GSH in post-mortem prefrontal cortices of patients with schizophrenia. Do and colleagues [391] found a 52% decrease in GSH levels in the prefrontal cortex of patients with schizophrenia. Interestingly, a significant deficit in total antioxidant status was inversely associated with some domains of cognitive deficits in schizophrenia patients, such as attention and immediate memory [392]. Moreover, plasma SOD activity was negatively correlated with positive symptoms in first- episode schizophrenia patients [393]. Lower levels of total antioxidant status, catalase and glutathione peroxidation has been described in first episode schizophrenia patients, with GSH levels positively associated with executive function [394]. Furthermore, clinical studies have described the clinical utility of NAC as an adjunctive treatment in schizophrenia [387, 395], as well as the combination of ω-3 fatty acids with vitamins E and C [396]. However, we have been unable to demonstrate efficacy for ω-3 polyunsaturated fatty acids (PUFA) plus alpha-lipoic acid in preventing relapse in patients who had responded well to antipsychotic treatment after a single episode of psychosis [397]. Further studies in this regard are nevertheless warranted.

Pre-Clinical Correlates

Depression

With regard to significant animal studies, SOD and catalase activities have been found to be decreased in rats exposed to a chronic stress model of MD, and could be reversed by tianeptine [398]. Similarly, animal studies have found NAC to be as effective an antidepressant as imipramine [399]. Of particular interest is that exposure to ozone worsens anxiety, cognitive and depressive-like behaviour in the FSL rat model of MD, suggesting that genetically susceptible individuals exposed to high levels of oxidative stress are at higher risk of developing mood and/or anxiety disorders [400].

Moreover, exacerbating levels of oxidative stress (e.g. with ozone) can attenuate antidepressant action [400]. In fact, stress-related activation of the NMDA- NOS cascade has been proposed to be a vulnerability factor in stress-sensitive FSL rats [236].

Considering the connection between inflammation, NO and insulin/PPAR signalling in MD, it is not surprising that PPARγ has been associated with suppression in immune response through its ability to inhibit the expression of inflammatory cytokines [401] and to have actions on pathways involved in apoptosis, cellular proliferation and cellular resilience [402]. Moreover, it has been demonstrated that metabolites of 5-HT act as PPARγ-agonists in the periphery [403], which further indicates the possibility of an underlying link between biochemical pathways of mood disorders and metabolic syndrome. The recently discovered prostaglandin and endogenous PPARγ ligand, 15d-PGJ2, presents with anti-inflammatory properties [404], increases the neuronal metabolism of glucose, prevents stress-induced suppression of glutamate uptake [405] and has been suggested to be a possible marker for psychiatric diseases [406]. Indeed, animal studies have also described the antidepressant activity of PPARγ agonists [407].

Bipolar Disorder

SOD was found to be increased and catalase (CAT) decreased in an ouabain-induced rat model of BPD [408], while lithium and valproate protect against amphetamine-induced oxidative stress in the same model, thus further supporting a role for oxidative stress in BPD [119]. When considering the proposed role of oxidative stress and inflammatory process in BPD it is important to note that some mood stabilizers, e.g. lithium, valproate (VPA), carbamazepine, lamotrigine, suppress (brain) pro-inflammatory mediators such as cyclooxygenase-2 (COX-2) and prostaglandins [409-411], indicating possible anti- inflammatory properties. Moreover, NAC reverses and protects against oxidative protein damage induced by d- amphetamine in a rat model of mania [412].

Schizophrenia

Animal studies, too, have confirmed that schizophrenia involves redox imbalance and oxidative stress. Using the ketamine challenge model, researchers have noted a decreased expression of PV-interneurons (relating to GABA; see section 2 and section 3.1.4) in the hippocampus [413] – a recent review by Bitanihirwe and Woo, 2011 [366] explain that GABAergic pathways innervate primary

neurons and could increase intracellular calcium levels and subsequently trigger oxidative damage. Elevations in nicotinamide adenosine dinucleotide phosphate (NADPH) oxidase 2 (Nox2) are observed in the prefrontal cortex of rats exposed to the SIR model of schizophrenia [414]. Nox2 is a major source of ROS and controls glutamate release in the prefrontal cortex ([415]; reviewed in [416]). We have also observed increased SOD activity, a decrease in the ratio of oxidized vs. reduced glutathione and an increase in lipid peroxidation in both the striatum and frontal cortex of SIR rats [373]. Importantly, all the latter alterations could be reversed with clozapine treatment [373]. The latter not only emphasizes the validity of these findings but also highlights the ability of contemporary treatments like clozapine to address disturbances in redox balance. Preclinical studies in the SIR model of schizophrenia also confirmed NAC's utility as adjunctive treatment to an anti-psychotic [137].

Inflammatory Markers

Increasing evidence indicates that inflammation may have a critical role in the pathophysiology of MD, BPD and schizophrenia [386, 417]. Inflammation is also a closely associated phenomenon with oxidative stress, discussed above. Cytokines play a crucial role as signalling molecules in the immune system and have the ability to cross the blood–brain barrier (BBB), granting it both central and peripheral activity [418]. Cytokines have been demonstrated to exert activity in almost every area relevant to the pathophysiology of MD, BPD and psychotic disorders including neurotransmitter metabolism, neuroendocrine function, and neural plasticity [386, 419-421]. The pro-inflammatory cytokines, such as interleukin (IL)-1, tumor necrosis factor (TNF)-α, and IL-6, can inhibit neurogenesis *in vivo* [422, 423], induce apoptosis [424, 425] and negatively affect synaptogenesis, synaptic plasticity and connectivity, and also the structure of synaptic membranes [426, 427]. On the other hand, anti-inflammatory cytokines such as IL-10 and IL-4 dampen the immune and inflammatory response [421] so that an inflammatory state is generally determined by an imbalance between pro- and anti- inflammatory mediators. Fig. 3 provides an outline of how inflammatory mediators and oxidative stress are related to regulate immune response and redox status.

Clinical Correlates

Depression

A strong relationship has been demonstrated between MD and the presence of inflammation and its associated inflammatory mediators [428, 429]. These mediators include the pro-inflammatory cytokines, IL-1, -2, -6 and -8, interferon (IFN)-γ and TNF-α [430] that, when administered to a healthy individual, may induce sickness behaviour [431, 432]. Sickness behaviour describes a state in which many of the symptoms coincide with that of MD [420]. Furthermore, it has been proposed that constant elevation of cytokine levels may lead to neurotransmitter changes which are interpreted by the brain as stressors that further allow these molecules to contribute to the development of MD [429]. Not only do pro-inflammatory cytokines contribute towards altered neurotransmitter metabolism, neuroendocrine function, synaptic plasticity and behaviour characteristic of MD [419, 433-435], but also stimulate hypothalamic-pituitary- adrenal (HPA)-axis hormones as well as CRH in both the hypothalamus and the amygdala. The latter play an important role in fear responses and anxiety-related behaviour [419].

Cytokine-induced MD is associated with alterations in 5-HT metabolism through the activation of IDO [436], as well as alterations in CRH function [430, 436]. Importantly, MD induced by IFN-γ also involves the activation of iNOS [437], the latter known to play an important role in stress- related inflammation [438]. Peripheral levels of IL-1, IL-6 and TNF-α is increased in patients with MD [299, 439], and these effects are normalized following antidepressant treatment [440, 441]. Increased levels of IL-1 and IL-6 have also been measured in the CSF of patients suffering from MD [442]. Furthermore, an elevation in IL-6 levels has been proposed as an early marker for cognitive symptoms and has been found to correspond to the severity of MD as well as increased activity of the HPA axis [430, 443, 444]. Interestingly, IL-6 levels have been demonstrated to be increased in patients with treatment-resistant depression compared to treatment responders [445], which indicates that altered IL-6 levels in the blood of depressed individuals may serve as a marker of possible response to antidepressant treatment [208]. In line with these findings, a recent clinical study found that administration of the non-specific COX inhibitor acetylsalicylic acid leads to an

improved onset of action during fluoxetine treatment and also increased the response rate to the drug when compared to patients receiving only fluoxetine [446].

Bipolar Disorder

BPD may be associated with moderately increased plasma levels of pro-inflammatory cytokines, such as IL-6 and TNF-α, along with increased IL-1, and IL-1 receptor antagonist protein, while elevated mRNA levels have been observed in post-mortem frontal cortex of BPD patients [447-449].

Schizophrenia

Schizophrenia patients and their first-degree relatives have been found to present with significantly elevated pro- vs. anti-inflammatory cytokines [450], as well as a significant elevation in pro-inflammatory cytokines in first episode psychosis patients, with a positive correlation between IL-6 and duration of illness [418]. IL-6 has been found to be significantly increased in early and late stage schizophrenia, with IL-10 reported to decrease in the late stages [451].

The presence of elevated levels of IL-1 in the CSF has been suggested as a marker of acute psychotic relapse, while immune-inflammatory dysfunctions may be implicated in the underlying processes mediating relapse, considering that the relationship between cytokine abnormalities and acute exacerbations of schizophrenia appear not to be related to treatment with antipsychotics. On this point, certain cytokines have been suggested to represent state markers for acute exacerbations of psychosis (see [217] for review). However, a number of studies have described inconsistent effects on plasma/serum cytokines, namely IL-4 [452], [453], IL-6 (reviewed in [454]), IFN-γ [452, 455]; reviewed in [456], and TNF-α (reviewed in [456]). Furthermore, a positive correlation between the severity of cognitive deficits and levels of IL-1β, IL-6 and TNF-α have been described in schizophrenia [252].

Pre-Clinical Correlates

Depression

Acetylsalicylic acid has proved to be an effective augmentation agent to fluoxetine in rats resistant to standard fluoxetine treatment using the CMS animal model of MD [457]. Furthermore, it has been

demonstrated in gene-environment animal models of MD that peripheral levels of inflammatory markers and regulators of metabolic pathways, including glucocorticoids, MAPK's and cytokines are altered in these animals when compared to healthy controls [458].

Bipolar Disorder

Using the ouabain model of BPD, investigators have noted an increased activation of signalling pathways linked to inflammation in the brain [459].

Schizophrenia

Elevated pro-inflammatory cytokines (TNF-α, IFN-γ) and decreased anti-inflammatory (IL-4) and dual-action (IL-6) cytokines has been described in the SIR model, and which are for the most part reversed with clozapine treatment [137]. More interesting is that NAC is able to augment select behavioural, neurochemical and anti-inflammatory responses to clozapine in SIR animals [137]. Other pre-clinical studies have also indicated that IL-6 and TNF-α have direct inhibitory effects on adult hippocampal neurogenesis [422, 460], which may attenuate antidepressant and antipsychotic efficacy by decreasing hippocampal neurogenesis or interfering with the neurogenic properties of these drugs.

Kynurenine Pathway Markers

An important link with the inflammatory cascade is the kynurenine pathway whereby TRP is catabolized to kynurenine via hepatic TDO and via IDO in the central nervous system, lungs and placenta [461] (Fig. 3). Kynurenine is then metabolised to either kynurenic acid (KYNA) or 3-hydroxykynurine (3-OHK) and then to anthranilic acid, 3-hydroxyanthranilic acid (3-OHAA) and QA [462]. Conversion along this highly regulated pathway accounts for the metabolism of approximately 80% of non-protein bound TRP, an essential amino acid necessary for the synthesis of 5-HT (Fig. 3; [463]). QA, a NMDA receptor agonist and excitotoxin, along with 3OHK, a mediator of neuronal apoptosis, and 3-OHAA, a free radical, are all capable of inducing neurodegenerative changes in the brain [462, 464, 465]. KYNA, on the other hand, acts as an antagonist at the facilitatory glycine site on the NMDA receptor ion channel, thus having potential neuroprotective properties [466]. The activation of IDO by pro-inflammatory cytokines (e.g. INF-γ and TNF-α) in the CNS also leads to

increased TRP degradation into kynurenine and QA, thereby reducing the bioavailability of TRP for 5-HT synthesis (Fig. 3) (reviewed in [467]). Hence, increased pro-inflammatory and decreased anti-inflammatory actions in the CNS can contribute to central 5-HT deficiency, which plays an important role in the pathogenesis of MD and BPD but also the negative symptoms of schizophrenia [386, 468, 469].

Clinical Correlates

Depression

Previous studies have observed decreased TRP and 5-HT along with increased kynurenine in the peripheral blood of depressed patients receiving the pro-inflammatory cytokine, IFN-γ [470, 471]. Significant decreases in the concentration of KYNA have also been observed in the plasma of depressed individuals [465]. Alterations in the symptoms of depressed patients were significantly positively correlated with kynurenine and negatively correlated with levels of 5- HT [471]. A previous study also indicated the correlation of increased IL-6 production in vitro with decreased TRP levels in depressed patients, emphasizing the influence of IL-6 on 5-HT metabolism via TRP in these patients [444]. A post- mortem study also indicated elevated QA levels in the brain of patients with MD [472]. This supports the hypothesis that the development of depressive symptoms may be mediated by increased TRP metabolism via IDO along the KYN pathway [436, 473].

Bipolar Disorder

Levels of TRP and kynurenine-dependent TRP index have been demonstrated to be diminished in bipolar mania [467]. Furthermore, increased kynurenine was found post-mortem in the ACC in patients with BPD, which corresponded with increased density and intensity of TDO positive glial cells [474].

Schizophrenia

In patients with schizophrenia clinical post-mortem studies found elevated levels of TRP, 3-OHAA, kynurenine and QA in various brain regions [475, 476]. Moreover, individuals suffering from schizophrenia present with increased levels of TRP in the CSF and plasma whether they received treatment or not [477, 478]. Although increases in KYNA levels have been observed in the post-

mortem brain tissue of schizophrenia patients who received treatment [218], Myint and colleagues [479] have described a significant reduction in plasma levels of KYNA accompanied by a decrease in the neuroprotective ratio (a measure of the relationship between KYNA and kynurenine levels) in treated and non-treated patients suffering from schizophrenia.

Pre-Clinical Correlates

Depression

In a stress-induced rat model of MD the kynurenine pathway was observed to be activated, leading to increased expression and activity of hepatic TDO as well as its expression in the cortex [480]. This increased TDO activity was associated with elevated circulating kynurenine concentration and a reduction in circulating TRP concentration [480]. Furthermore, induction of depressive and anxiety-like symptoms via the administration of a viral mimetic led to reductions in BDNF signalling and activation of the kynurenine pathway [481]. Recently, the potential for using the kynurenine 3-monooxygenase inhibitor, Ro 61-8048 (to elevate kynurenine levels), as an antidepressant has been suggested ([482], while the TDO inhibitor, allopurinol, has been noted for its antidepressant-like effects in rats [480].

Bipolar Disorder

Current animal models of BPD have not produced data related to the role of the kynurenine pathway, and data is this regard is eagerly awaited.

Schizophrenia

Recent pre-clinical studies in the SIR model of schizophrenia have described elevated plasma TRP, kynurenine, anthranilic acid, 3-OHAA and QA with reduced KYNA and neuroprotective ratio, with all alterations reversed with the antipsychotic, clozapine [137, 483].

Genetic Markers

Full remission of a psychiatric illness is often impeded by variability in an individual's response to psychoactive drugs, so that being able to predict responses to psychotropic drugs holds great promise in improving treatment outcome. Genotyping, where proteins involved in

neurotransmission and cellular signalling specific for illness pathology and drug response are identified and targeted, will allow the selection of an appropriate psychopharmacologic agent to best suit an individual's pathologic and metabolic characteristics.

Clinical Correlates

Depression

The development of MD has been demonstrated to be critically influenced by genetic factors which provide the opportunity to investigate mechanisms underlying the disorder [13]. Polymorphisms on genes encoding the 5-HT transporter, 5-HT2A-receptor, BDNF, and tryptophan hydroxylase have been identified as candidate genes implicated in the pathology of MD [484], while several studies have suggested the Met/Met genotype (of the gene coding for COMT) to be predictive of antidepressant response rates [485, 486]. The Val/Met polymorphism in MD has not been consistent and has not been linked to any single effect [487]. Nevertheless, although contradictory findings have been presented, research into polymorphisms on the COMT variant may still hold promise [488].

Bipolar Disorder

Efforts to identify possible genetic markers and isolation of genes implicated in the pathogenesis of BPD have been challenging and at times contradictory [489]. Nevertheless, a recent study by Mahon et al. [490] suggested that abnormalities in lower white matter in the temporal lobe might be a marker for genetic risk of BPD. It has been suggested that the presence of an unknown gene on chromosome 12q22-q23.2 may predispose especially men to develop MD and perhaps even BPD [491]. Various studies have explored a possible link between variations in tryptophan hydroxylase II (TPH2) [492, 493] and the development of BPD, although its link with schizophrenia has been dismissed [494]. As in MD, the Val/Met polymorphism in BPD has not been consistent and has not been linked to any single effect [487].

Schizophrenia

Although the heritability for schizophrenia is estimated to be as high as 70%, the illness clearly does not have a pattern of inheritance in any population or even in single families that is consistent with the effect of a single gene [14]. Moreover, according to Riley and Kendler [495], genetics is not a determinant of schizophrenia but rather a means by which the disease is mediated. It is therefore necessary for several genes to interact and be influenced by environmental factors to lead to a patient presenting with the array of symptoms associated with schizophrenia. Like in MD and BPD, the Val/Met polymorphism is involved in predisposition to the development of schizophrenia [487] but further work is needed. The introduction of the SchizophreniaGene (SzGene) database by Allen et al. has made a substantial contribution to schizophrenia genomics [496]. Using the SzGene database, Sun et al. [497] described a collection of highly ranked genes that may be utilized as a working blueprint in the future. These genes, amongst others, include:

i. Disrupted in Schizophrenia 1 (DISC1). A gene that encodes a protein that has been implicated in neurite outgrowth and cortical development by interacting with other proteins.
ii. Dystrobrevin Binding Protein 1 (DTNBP1). A gene that encodes a protein purported to influence organelle biogenesis associated with melanosomes, platelet dense granules, and lysosomes.
iii. Catechol-O-Methyl Transferase (COMT). A catabolic enzyme involved in the degeneration of various molecules that are biologically active, including DA.
iv. D-Amino Acid Oxidase (DAO). This is a NMDA- receptor-mediated signalling gene.
v. Regulator of G Protein Signalling 4 (RGS4). This is a regulatory molecule that acts as a GTPase activating protein.
vi. Neuregulin 1 (NRG1). This is a protein essential for normal development and function of the nervous system (reviewed in [498]).
vii. Metabotropic Glutamate Receptor 3 (GRM3) gene. This gene, coding for the GRM3 glutamate receptor, is linked to inhibition of the cAMP cascade and has been associated with susceptibility to develop mood disorders.

Another possibly important genetic marker, and that links with oxidative stress, is NOS as discussed earlier. Weber et al. [499] indicated that a genetic variance in nitric oxide synthase NOS1 results in a reduction in the expression of the gene in the prefrontal brain region which adds to schizophrenia burden, and that NOS1 interacts with NOS1 adapter protein (NOS1AP) in doing so. The interaction observed in NOS1, NOS1AP and the PDZ binding domain may therefore establish a novel drug target for treating schizophrenia [499].

Pre-Clinical Correlates

Depression

mRNA expression of neuropeptide Y have been demonstrated to be decreased in various brain regions of FSL rats [500, 501] while basal mRNA levels of several genes involved in the synthesis of neurotransmitters are similarly altered. These include tyrosine hydroxylase (TH), DA β- hydroxylase, phenylethanolamine N-methyltransferase (PNMT) and GTP cyclohydrolase I – all of which were elevated in FSL rats [502]. Also, protein expression of BDNF, CREB and Bcl-2 were reduced in rats after exposure to CMS [503].

Bipolar Disorder

The lack of viable genetic animal models of BPD has left a void in the search for genetic markers at pre-clinical level. However, black Swiss mice (a genetic model of mania) present with increased mRNA expression of β-catenin in the hippocampus, as opposed to other brain regions [363], while increased CSF levels of S100B (a neuronal trophic factor released by astrocytes) has been observed in the ouabain-induced model of mania [504]. Furthermore, transgenic mice expressing increased S100B have been demonstrated to be hyperactive [505].

Schizophrenia

The SIR model has shown increased expression of metabotropic glutamate receptor (mGluR) 6 and ionotropic AMPA3 receptor subunit genes in the PFC [361], as well as reduced mGluR1 and mGluR5 expression [506]. These findings are consistent with the proposal that dysregulation of glutamatergic activity may contribute to the behavioural/ cognitive deficits associated with SIR [64]. Furthermore, the PCP model produces up regulation of genes coding for frontal cortical NMDA receptors and

produces differential expression of frontal cortical genes coding for BDNF [252]. Another clear indication of the involvement of genetic markers in schizophrenia is the numerous genetic animal models with validity for schizophrenia, such as: the DISC1 knockout (KO) model, the DA transporter KO model, the Homer1a KO model, insulin receptor KO model and the mGluR 1 and 5 KO models (reviewed in [507]).

Proteomic Markers

The proteome is the entire collection of "proteins encoded by the genome of an organism at a specific point in time, incorporating the set of isoforms, posttranslational modifications, covalent structures and complex protein- protein interactions present therein" [508]. Proteomics provides an insight into the character and interactions of proteins and thus of signalling pathways – understanding a proteome allows for development of effective predictive biomarkers [509].

Clinical Correlates

Depression

Ditzen et al. [510] observed several aspects in the CSF which were significantly altered in depressed patients when compared to controls, including 11 proteins and 144 peptide features. A recent large proteomic study in MD and schizophrenia patients observed that insulin was the marker with the highest statistical significance in MD patients compared to controls [2]. Their findings are consistent with the observation that MD is frequently linked with insulin resistance [511]. The increased comorbidity between type 2 diabetes and MD [512] and the strong link between MD and metabolic syndrome [513] further supports this hypothesis. Moreover, increased levels of chromogranin A, a secretory protein and precursor for functional peptides, are linked to dysregulated insulin levels in patients with schizophrenia [514].

Bipolar Disorder

Novikova et al. [515] identified several protein biomarkers which may possibly be involved in the pathology of BPD, e.g. MB-18.5, CBF2, DECR2, BYSL, ANKARD12, ALDOC and DKK2 (part of the Wnt signalling cascade) [516]. The Wnt cascade is a group of signal transduction pathways comprising proteins that pass signals from outside to inside of a cell through cell surface receptors. These

proteins appear to be compromised in patients with BPD [517]. Importantly, these proteins interact with GSK3-B, BDNF, oxidative stress mediators and cytokines relevant to BPD [349].

Schizophrenia

Cyclophilin A is a protein biomarker that plays an important role in cerebral cortical plasticity [518]. Differing levels of this protein has been noted postmortem in patients with schizophrenia and BPD, suggesting contradictory influences on plasticity specifically in the DLPFC in these diseases [515]. We have earlier noted the importance of neurotrophins like BDNF and VEGF in the neurobiology of psychiatric disorders. Proteomic studies [2] have also observed significant differences in various growth factors and neurotrophins in patients with schizophrenia, influencing somatic and dendritic growth in the hippocampus and prefrontal cortex, such as BDNF, VEGF or stem cell factor. To a lesser extent, this may include members of the chemokine/cytokine family. In line with these findings, previous studies have indeed observed decreased VEGF in serum of patients with schizophrenia [259, 519], although this has not always been reproducible [520]. The current body of evidence relating to BDNF in schizophrenia provides little congruence, with increased, decreased, or no change in serum or plasma BDNF levels noted [259, 521].

Another study in schizophrenia patients identified distinctive profiles of peptides and proteins in the CSF [522] that are potentially specific for schizophrenia, including a VEGF-derived peptide sequence consisting of 40 amino acids, a transthyretin protein cluster (a serum and CSF carrier of the thyroid hormone thyroxin), and another smaller protein cluster also associated with transthyretin, with 95% specificity and 80 to 88% sensitivity [522]. Recent studies also observed that serum concentrations of insulin and chromogranin A were increased in schizophrenia patients [514, 523].

Pre-Clinical Correlates

Depression

Yang et al. [524] recently published proteomic data from the CMS rat model of MD, noting decreases in glyoxalase-1 and dihydropyrimidinase-related protein-2 (DRP-2) in the prefrontal cortex, which translates to alterations in energy and glutathione pathways. Carboni and colleagues [458] detected significantly increased levels of leptin, IL-1α and BDNF in FSL compared to FRL rats.

Bipolar Disorder

To the best of our knowledge there are no current proteomic data on animal models of BPD – this may be due to the lack of comprehensive animal models presenting with cyclic behaviour ranging from depressive to manic.

Schizophrenia

Proteomic studies in preclinical models of schizophrenia are limited. One study, however, describes the prohibitin protein, a potential marker of synaptic pathology, to be up- regulated in chronic schizophrenia patients as well as in the ketamine animal model of schizophrenia [525]. Another proteomics study describes the neuroprotective action of abrogated COX-II expression in insulin receptor KO mice as a validated animal model of schizophrenia [526]. Using the PCP animal model to access frontal cortical levels of chromogranin A, B and secretoneurin (large protein molecules of the chromogranin family), acute PCP treatment caused a decrease in secretoneurin, while chronic PCP treatment elevated this protein [527].

Micro-RNAs

Clinical Correlates

MicroRNAs (miRNAs), a large family of small non- coding RNAs, are potent regulators of gene expression with proposed roles in brain development and function [528]. These miRNAs may play a substantial role in the pathophysiology of mood and psychotic disorders and may even have an influence on the effect of drugs used to treat these disorders [529].

Depression

Bocchio-Chiavetto et al. [529] found 28 miRNAs to be up-regulated and 2 miRNAs to be down-regulated following antidepressant treatment – they further demonstrated that these differentiations could be associated with alteration of neuronal pathways and may be involved in the underlying mechanisms of MD.

Schizophrenia and BPD

Perkins et al. [530] observed that from 264 human miRNAs, 16 were differentially expressed, 15 were expressed at lower levels and 1 at a higher level in post mortem prefrontal cortex tissue of schizophrenia patients vs. controls. Similarly, post-mortem studies in schizophrenia or BPD found under-expression of several miRNAs of the adult prefrontal cortex [528], while in a study evaluating 667 miRNAs in post-mortem prefrontal cortex tissue of schizophrenia and BPD patients, 22 miRNAs were found differentially expressed between cases and controls, 7 deregulated in schizophrenia and 15 in BPD [531]. Furthermore, these 22 miRNAs were found to target brain specific genes involved in neurodevelopment, behaviour as well as the development of schizophrenia and BPD [531]. It has also been observed that a considerable modification to post-transcriptational regulation may be defined by an overall increase in miRNA expression in two regions of the cerebral cortex in post-mortem brain tissue from patients suffering from schizophrenia [532].

These studies highlight miRNAs as possible biomarkers for mood and psychotic disorders, although it is clear that further research is needed to evaluate the relationship between miRNA alterations and disease development and progression. The fact that altered expression of miRNAs is reflected also in the blood of patients suffering from mood disorders potentially give rise to the idea that peripheral miRNAs may be screened as an aid in the diagnosis and treatment of these disorders. However, as with most peripheral biomarkers, the correlation between central and peripheral expression of miRNAs remains a topic of debate [533, 534].

Pre-Clinical Correlates

By referring to selected animal models of depression, recent reviews have evaluated the molecular biology of miRNAs in relation to the pathophysiology of clinical depression as well as the utility of targeting miRNAs for antidepressant treatment [533, 535]. They confirm the dysregulation of a large number of miRNAs in these depression models. Moreover, miRNAs to some extent may be associated with treatment and onset of BPD and schizophrenia. The reader is advised to consult these papers for further detail.

Developing a biomarker panel

To date the diagnosis and treatment of patients suffering from mood and psychotic disorders have almost exclusively been based on behavioural symptomatology observed in these individuals, with laboratory testing and confirmation of diagnosis being absent due to the diverse aetiologies and underlying neurochemical abnormalities associated with these disorders. However, in recent years several biological markers have been linked to neuronal changes associated with the pathological processes and/or treatment response in these disorders. Being able to identify discrepancies in these markers in humans and using them in the diagnosis and treatment of mood and psychotic disorders will surely improve treatment efficacy and potentially even allow for provisional measures to be taken to counter neuronal deficits and prevent the onset and/or progression of symptoms and structural brain changes associated with these disorders. Quantifying the underlying abnormalities in these disorders may also be helpful in understanding the pathogenesis of these disorders and mechanisms causing the delayed response to drugs observed in their treatment. But then again, as mentioned in the introduction the likelihood of identifying any single biomarker with sensitivity and specificity for MD, BPD and schizophrenia is relatively low so that a feasible alternative to the single-biomarker approach could be the development of biomarker panels.

From the data discussed in this review, we have attempted to distinguish between biomarkers that provide mostly substantial evidence in support of them being a strong or moderate biomarker of either MD, BPD of schizophrenia, as opposed to markers where there is less of an evidence base to link it to the neuroanatomy or pathophysiology of these disorders. Furthermore, we distinguish between markers observed in clinical vs preclinical studies in order to highlight affirmation as well as the possibility/importance of further investigation. Table 1 describes a putative biomarker panel based on the clinical and preclinical data described in this article, where strong, moderate and weak candidate biomarkers are color coded for easy identification and interpretation with the text.

Altered endocrine responses, indicated in the 2nd row of Table 1, are typical of a number of these illnesses, and can in many instances be related to altered circadian rhythm. Moreover, such changes, for example cortisol, can be instrumental in driving many of the behavioural and pathological changes evident in these disorders, e.g. hyper-glutamatergia, structural brain changes, etc.

Furthermore, the recent introduction of the 5-HT2c antagonist/M1/2 agonist, agomelatine, as the first antidepressant acting to re-establish altered circadian rhythms provides robust validation of the importance of these processes in the development and treatment of depression [78]. Moreover, its actions on frontal cortical DA function hints of possible value in the treatment of schizophrenia.

Regarding the neuroanatomy and neurocircuitry of MD, BPD and schizophrenia, it is evident (and quite expected) that there are distinct alterations in the volume of certain brain structures in mood and psychotic disorders, highlighting the importance of altered neuroplasticity and structural brain changes in these illnesses, indicated in the 1st row of Table 1. Decreased volumes have been recorded for various limbic structures in both MD and schizophrenia, specifically the prefrontal cortex. Although several observations have been made in BPD, current data is limited or of little clinical value and further research into the neuroanatomy of this disorder is necessary. Overall, there remains speculation as to whether the above-described neuroplastic changes are cause or effect.

Neuroimaging studies, indicated in the 1st row of Table 1, have also given insight into altered neurocircuitry, blood flow and metabolic rate in affected brain structures in these disorders and largely parallel the neurocircuitry described above. Of importance is the decreased activity in cerebellar, prefrontal, frontal and cortical structures in clinical studies of schizophrenia as well as decreases in cerebral glucose metabolism in both preclinical and clinical studies of BPD and alterations in NAA in MD and schizophrenia.

Current pharmacotherapy of MD, BPD and schizophrenia in many ways provides robust construct and predictive validity for the importance of monoamines as biomarkers for these illnesses. At the neurochemical level, indicated in the 3rd row of Table 1, patients with MD present with a decrease in DA, 5-HIAA, NA and MHPG; BPD patients present with an increase in DA, associated with manic symptoms, and a decrease in 5-HIAA; schizophrenia patients present with an increase and decrease in DA in the striatum and prefrontal cortex respectively, along with increased 5-HT transmission and increased NA levels. Decreased levels of DA have been correlated with anhedonic behaviour in MD in both clinical and preclinical studies. Increases in DA as well as NA have, however, been linked to manic symptoms of BPD and models of mania as well as clinical and preclinical studies of schizophrenia. Glutamate on the other hand is increased peripherally and in the cortex of MD and

BPD patients, is reversed with ketamine treatment, while in un-medicated schizophrenia patients the literature indicates an increase of glutamate and GABA in post-mortem prefrontal cortex and striatum tissue. However, as noted earlier, schizophrenia is proposed to involve cortical hypoglutamatergia that in turn drives meso-limbic hyperdopaminergia and meso-cortical hypodopaminergia. Decreases in the level of 5HIAA, a 5-HT metabolite, have also been noted in both MD and BPD patients suffering from depressive symptoms, while 5HT transmission is increased in patients suffering from schizophrenia.

With regards to the growth factors, indicated in the 4th row of Table 1, the current literature supports a general decrease in BDNF in MD, BPD and schizophrenia; none to limited data of IGF-1 in MD, no changes of IGF-1 in BPD and decreased IGF-1 in schizophrenia; VEGF was increased in MD and BPD, but mRNA of VEGF was decreased in schizophrenia. While VEGF studies in humans report conflicting results and with preclinical data still lacking, it may still hold promise as a biomarker of psychiatric/mood disorders – especially considering the divergent results seen in MD vs schizophrenia patients. Recently published meta-analyses on BDNF are emphatic that altered serum BDNF is a state marker in MD, BPD and schizophrenia [536], although peripheral BDNF levels are not a sufficient measure of disease severity in MD [337] neither does it adequately discriminate between MD, BPD and schizophrenia. Animal studies tend to echo these sentiments, although serum BDNF levels may act as marker of predisposition to develop symptoms in rats used to model these disorders [306, 307]. Interesting enough, it does differentiate between mood states in BPD [336] and between acute and remitted states in MD [536]. In schizophrenia, a significant positive correlation between BDNF levels and positive and negative syndrome scale (PANSS) positive subscore has been described, as well as higher BDNF levels in the paranoid subtype of schizophrenia [537]. Low BDNF levels at the onset of psychosis may therefore contribute to the pathogenesis of schizophrenia and could perhaps be a candidate biological marker for positive symptoms. It may also play an important role as a marker of disease progression in BPD due to an observed association between peripheral BDNF levels and age and duration of illness [336, 538].

Markers of neuronal resilience, indicated in the 5th row of Table 1, have aroused considerable interest and play an important role in programmed cell death and plasticity. GSK-3 has surfaced as a possible marker in BPD via its regulation of several biochemical pathways, including the Wnt pathway. It has

also been connected to the effects of lithium treatment in BPD. Decreased cAMP and activity of the MAPK/ERK pathway has been demonstrated in both humans and animal models of MD and may serve as a valuable biomarker in the disorder. However, data in BPD and schizophrenia is less clear. Increases in MAPK have been noted in animal models of schizophrenia but need further investigation in a clinical setting as well.

Numerous findings in MD, BPD and schizophrenia patients indicate the presence of a prooxidative state, indicated in the 6th row of Table 1. However, elevations in SOD and lipid peroxidation are relatively non-specific, although BPD patients present with an increase and decrease in catalase in manic and euthymic symptoms respectively, making catalase a possible specific marker in the latter disorder. Furthermore, schizophrenia and BPD patients present with decreased levels of GSH [539]. Importantly, studies in translational animal models have provided evidence that the associated oxidative stress occurring with MD-related behaviors can be reversed by antidepressant treatment [400] and that the antioxidant and glutathione precursor, NAC, is antidepressant in rats [399]. Moreover, exacerbated levels of oxidative stress can attenuate antidepressant action [400]. However, preliminary clinical studies have only been able to provide limited support for the use of NAC as an adjunctive therapy for MD [379]. Similar studies in humans and animals have described reversal of redox changes by lithium and/or antipsychotics in BPD [119, 135] and schizophrenia [137, 373, 387, 395], respectively. This review is adamant that both oxidant and antioxidant systems and redox balance play a pathophysiological role in MD, BPD and psychotic disorders such as schizophrenia. This realisation has opened the door to the possible clinical utility of antioxidant drugs (e.g. NAC) in the treatment of these illnesses alone and as an adjunctive treatment [12, 118, 540].

As has been noted, inflammation and oxidative stress are closely linked. Significant increases in the levels of proinflammatory cytokines have been reported in humans and animal models of MD, BPD and schizophrenia, indicated in the 7th row of Table 1. Along with the above-mentioned oxidative stress observations is the evidence that MD, BPD and schizophrenia patients present with an elevation in proinflammatory cytokines (IL-1, 6, IFN-γ and TNF-α), that is reversed with antidepressant treatment in MD. A recent animal study in the SIR model of schizophrenia indicates that clozapine and NAC can also reverse this proinflammatory state [137]. This review suggests that the presence of a pro-inflammatory state is a non-specific pathological marker for MD, BPD and schizophrenia yet

underscores the pathological role of inflammation in these disorders, especially give their reversal by typical drug treatment. Furthermore, anti-inflammatory cytokines have been found to be decreased in both humans and animal models of schizophrenia. However, it remains unclear whether activation of inflammatory pathways in the CNS during MD, BPD and psychotic disorders is rooted in the periphery (e.g., as a function of overt or nascent medical illness or psychological stress) and/or whether stress or other yet to be identified processes (e.g., vascular insults in late life MD/psychosis) induce inflammatory responses directly within the brain. Strong evidence supports a prenatal inflammatory event as a prodromal event to the development of schizophrenia [541]. Prenatal immune challenge with either a systemic endotoxin or viral mimic vs. an inducer of local inflammation suggests that neurodevelopment of the fetus may rather be affected by circulating cytokines and/or fever as opposed to direct effects evoked on the fetus by the agent responsible for maternal infection [542]. What is evident from work in animals is that these pathological processes seem to have their origin in a disturbance in the mitochondria [179], and explains why redox dysfunction is such a central feature of these illnesses [373]. Cytokine activity may elicit several effects on the brain, affecting the synthesis, release and reuptake of several neurotransmitters, including monamines, which have the ability to influence mood [417].

An interesting observation is that MD and BPD patients present with a decrease in TRP along with an increase in kynurenine and QA levels, while schizophrenia patients (corroborated by animal studies using the SIR model [137]), indicates an increase in TRP, 3OHAA, kynurenine and QA along with a decrease in KYNA [475, 476, 479]. These findings are indicated in the 8th row of Table 1. The decrease in TRP could therefore possibly be specific markers for MD and BPD, with the increase in TRP along with the decrease in KYNA specific to schizophrenia. The relative induction of KYNA versus QA may determine the effects of cytokines on the CNS and remains an important area for future investigation, including the therapeutic targeting of IDO and kynurenine enzymatic pathways in MD, BPD and schizophrenia [465, 467, 475].

Even though these disorders are known to be hereditary to some extent, the exact genetic basis still needs further elucidation, indicated in the 9th row of Table 1. Val/Met polymorphisms have been studied in MD, BPD and schizophrenia and even though various reports have been made, data remains inconclusive. Polymorphisms in 5-HT transporters and receptors, as well as BDNF and

tryptophan hydroxylase hold promise in MD and warrants further research to pinpointing exact genetic markers involved in the development of MD [484]. DISC1 is a well-researched candidate gene for schizophrenia and affective disorders with a range of functions relating to neurodevelopment, although studies into its role in these disorders remains promising albeit conflicting [543]. Gene variations in the SzGene database [496] also hold promise in schizophrenia research and also needs further research to clarify predisposition in developing schizophrenia. Tracking genetic variants in patient blood may therefore serve to compliment biomarker panels by providing more information relating genotypes to MD, BPD and psychotic disorders and their respective treatment responses.

With regards to proteomic markers, indicated in the 10th row of Table 1, it is clear that utilizing modern proteomic techniques, especially mass spectrometric approaches, may support attempts to understand the biochemical processes that accompany psychiatric disorders and may in turn lead to the development of diagnostics and better therapeutics. In MD, abnormalities in insulin secretion has been observed [511] and the disorder is also accompanied by decreased levels of glyoxalase-1 and dihydropyrimidinase-related protein-2 and increased leptin, IL-1 and BDNF protein levels in animal models [458, 524]. This supports the hypothesis for a shared etiopathology with an inflammatory underbuild in patients with co-morbid MD and metabolic syndrome and/or type II diabetes mellitus, which highlights the proposed utility of the PPARγ-pathway in the treatment of MD [544]. Increased insulin levels have also been reported in patients suffering from schizophrenia and may be accompanied by increases in cyclophillin A [518], suggesting increased support for the role of inflammation in the disorder with cyclophillin A already being linked to a variety of disorders with an inflammatory component – amongs others, type II diabetes [545]. Moreover, these findings may relate to the confounding observation of weight gain and metabolic syndrome in this disorder, and that may be worsened by certain antipsychotic drugs [546]. Various proteins involved in the WNT cascade may possibly serve as proteomic biomarkers of BPD which may lead to these markers aiding in the diagnosis and treatment of the disorder and therefore warrants further investigation. Thus, screening peripheral compartments, such as serum and CSF, in patients and controls for altered expression of proteins and metabolites known to be involved in the pathophysiology of the disease or associated with comorbid states could serve in developing a "fingerprint" for identifying persons

at risk of developing MD, BPD or schizophrenia. However, it is critical that we bring together knowledge on the biology of these illnesses, co-morbid states, illness severity and treatment resistance to enable proteomic markers to realize this potential.

Finally, studies suggest that micro-mRNAs, indicated in the 11th row of Table 1, may play a valuable role as a biomarker in the diagnosis and treatment of mood and psychotic disorders, however further research is warranted and the relation between central and peripheral expression still needs elucidation.

This review has focussed primarily on suitable disease-specific biomarkers with especially predictive validity. However, we have on occasion made reference in the aforegoing sections to physiological markers. It is maybe incumbent to mention that these markers are gaining in interest, with some recently been found to have value in predicting treatment response. Thus for example, we earlier described that clinical response to antidepressants can be predicted by assessing activity in the rostral ACC region via electroencephalography (EEG) [37]. Similarly, we noted that by studying P304 wave amplitude, EEG can be used to assess decision making in BPD and schizophrenia patients [49]. In a study based on the "disconnection hypothesis" of schizophrenia [547], and with accumulating evidence of abnormal functional connectivity in schizophrenia, Takahashi and colleagues argued that neurophysiologic signals may provide a retrospective window with which to view disordered neural dynamics in schizophrenia [548]. Using a novel entropy-based approach for measuring dynamical complexity in physiological systems, they observed abnormal dynamical EEG signal complexity in anterior brain areas in schizophrenia that normalized selectively in fronto-central areas following antipsychotic treatment. This approach has also been proven successful in MD [488]. Another promising neurophysiological marker is electroretinography (ERG), a specialized measure of retinal function, which has been studied in schizophrenia and BPD [549]. ERG abnormalities may reflect altered phospholipid metabolism and/or impaired dopaminergic transmission. With all patients receiving stable psychotropic medications at least for 2 weeks before the first assessment, the authors found that retinal dysfunctions are specific for schizophrenia, as compared with BPD, and are confined to the acute stage of the illness. Another potential physiological marker is event-related-potentials (ERPs) (voltage fluctuations in an EEG depicting neural activity), which are specific for cognitive dysfunction in schizophrenia [550] and also studied in MD [551]. Although physiology

markers have not been as extensively studied as biological markers, there is a literature describing their use in animals, such as employing EEG and related markers in translational animal models of MD [552] and schizophrenia [553]. For example, blind-drunk (Bdr) mice demonstrate fragmented rest and activity rhythms under a light/dark cycle, reminiscent of altered sleeping patterns in schizophrenic patients [83], while depriving animals of REM sleep, which can be studied as an EEG-related marker, has been suggested to model mania [82].

Discussion and conclusion

The search for blood biomarkers can essentially be divided into screening putative markers inferred by our current knowledge of the given illness, e.g. BDNF, CREB etc., or through exploring candidate pathways through the use of "omic" procedures, such as proteomic or transciptomic profiling that offer an unbiased view of these pathways. Either approach has its own set of advantages and disadvantages, yet studies deploying either still lack the critical requirement of reproducibility and selectivity [13]. Another important challenge in identifying possible biomarkers is the predictive efficacy of a specific biomarker in the treatment of MD, BPD and schizophrenia. Very few biomarkers of these illnesses have shown utility in regards to predictive efficacy following drug treatment. Having this in hand offers the possibility of introducing tailored pharmacotherapy. Furthermore, demonstrating a dose-dependent response of these markers under the conditions of treatment may aid in more accurately establishing an appropriate dose selection during clinical trials, thereby optimizing drug discovery and development. Knowing this brings the focus of future research to a more optimized translational approach [554]. Ideally, questions from a clinical situation should be translated into a valid animal model, where such animal data can be integrated with patients' data in order to identify predictive biomarkers. Thereafter extensive validation should be performed on these biomarkers before diagnostic kits with predictive value can be developed and marketed [554]. In the end, the final requirement is that the aforegoing process should allow clinicians to make evidence-based decisions that will reinforce the decision to treat, with what agent/s, and with a higher likelihood of success than that provided by current approaches. However, there are a number of obstacles to overcome before realising a biomarker panel that is sensitive and specific enough to be implemented as a reliable tool for diagnosis and treatment, which include:

- difficulties in translating findings observed in animal models to clinical studies and correlating markers measured in animal subjects with those measured in human patients [13, 555];
- employing clinical studies in a larger population in order to validate specific findings [13, 555, 556];
- attributing measured biomarkers to one specific pathway [2] as measured in a specific disorder, thus ensuring biomarker specificity [13] and the possible presence of underlying comorbid disorders [13, 547];
- the complexity of underlying pathophysiologic and ethiologic origins of these disorders combined with demographics [556], and interpatient variables, e.g. smoking [2];
- correlating data from observations made in different locations and utilizing different sampling techniques [2];
- accounting for disease state [13, 556] and previous/current drug therapy [2, 557];
- correlating levels of markers measured in different tissue samples, e.g. plasma vs CSF [2, 13];
- the influence of the time at which biomarkers are measured, e.g. the influence of circadian rhythm and disease state/progression [2, 558];
- and the inability to measure biological markers in brain tissue in live patients due to the invasive nature thereof [559].

Nevertheless, recent studies using "omics" approaches have demonstrated that careful selection of appropriate biomarker panels can provide good separation between diseased and healthy states, as well as predict response to treatment. Two recent studies by Pajer [555] and Redei [556] set out to investigate the validity of potential biomarkers of MD and found that several of these markers may have possible use in discriminating between depressed and non- depressed individuals and may even predict response to therapy. Furthermore, Redei et al. found several blood markers identified in animal models of depression to correlate with levels measured in depressed human individuals. Importantly, these studies affirm the approach taken in this review that clinical data and that obtained from validated translational animal models are supportive and should be used together when developing a biomarker panel [555]. Indeed, the latter studies corroborate that genes expressing transcripts belonging to processes related to transcription, neurodevelopment, neurodegeneration and redox are causally related to at least MD [555], which concurs with molecular mechanisms linked

to these processes and highlighted in this review. Although metabolomics per se has not been covered in this review as it addresses much the same processes as do genomic and proteomic methods, studying the metabolome has great potential to map potential biomarkers in neuropsychiatric disorders. Indeed, as has been noted, mood and psychotic disorders are linked to a range of disturbances in metabolic pathways, e.g. neurotransmitter systems, TRP-kynurenine metabolism, oxidative stress, etc., so that generating a metabolic signature for a specific disorder will aid in metabolic phenotyping and contribute to discovering disease-specific biomarkers as well as predicting treatment response [560].

Based on this review, the ever-increasing availability of new pre-clinical and clinical studies is beginning to forge a way through the neuropathologic complexity of illnesses like MD, schizophrenia and BPD, so much so that we are in a position to portray how altered neuroendocrine, anatomical, neurochemical and other pathologies can be linked to a specific disorder. The role of the endocrine system has long been linked to mood and psychotic disorders with MD patients presenting with increased saliva cortisol as well as HPA-axis activation. Similarly, patients suffering from BPD and schizophrenia have been found to have increased systemic cortisol metabolism. Dysfunction of the hypothalamic-thyroid axis has been demonstrated in all three disorders.

In MD, decreased hippocampal volume as well as reductions in the size of the prefrontal cortex and basal ganglia is accompanied by reduced levels of monoamine neurotransmitters (NA, 5-HT and DA), decreased levels of the 5-HT metabolite, 5-HIAA, and an increase in the levels of glutamate. However, DA has been noted to be increased in BPD and increased in the striatum and decreased in the frontal cortex of both schizophrenic patients and most animal model studies of the disorder. In contrast with MD and BPD, increased 5-HT transmission and NA levels characterize neurotransmission in schizophrenia and is accompanied by decreased NAAG levels in the temporal cortex while increased in the hippocampus as well as reduced activity in various brain regions, including the cerebellar and temporal lobes, prefrontal cortex, cortex and striatum.

Although a variety of observations have been made regarding BDNF, VEGF and IGF, data currently available report conflicting findings. However, hypotheses and data surrounding these markers make a strong case for their involvement in these disorders – whether as a cause or result of

underlying pathology. Continued investigation will, more than likely, eventually lead to pinpointing the exact roles of these markers in the pathophysiology and/or progression of mood and psychotic disorders and establish them as valid biomarkers in the diagnosis and/or treatment of MD, BPD and schizophrenia.

Inflammation has emerged as a central role player in the pathophysiology of all three disorders discussed in this review with levels of pro-inflammatory cytokines being observed to be markedly increased in MD, BPD and schizophrenia and a decrease in anti-inflammatory cytokines also contributing to the inflammatory component of schizophrenia. Inflammation is thus not illness specific but a residual marker of ongoing pathology. Increased levels of kynurenine add to the immune response in these disorders with TRP levels being decreased in MD and BPD but increased in schizophrenia. Closely associated with inflammation, nitrosative and oxidative stress deepens the extent of neuronal stress in these disorders – increased lipid peroxidation accompanied by raised levels of SOD are a feature in all three disorders, accompanied by decreased GSH in schizophrenia. The individual inflammatory components that characterize said inflammation in these disorders may in the end prove to be more illness-specific markers. Thus for example, we noted earlier that a decrease in TRP may be specific markers for MD and BPD, while the increase in TRP and decrease in KYNA is more specific to schizophrenia. Similarly, certain components of the inflammatory response such as NO may be pro- or anti-inflammatory depending on the cellular mileux and/or pathways activated (eg see nNOS vs iNOS-mediated pathways in Fig. 1).

The current body of literature features data on a wide variety of possible biomarkers linked to mood and psychotic disorders. To improve diagnostic techniques and treatment strategies, it is of great importance that possible trait and state markers of these disorders are scrutinized to a point where they can be incorporated into an appropriate panel of biomarkers (as presented in Table 1) which may serve as adjunct to current diagnostic criteria. Furthermore, such a panel may assist in treatment strategies being tailored to the unique context in which mood and psychotic disorders present in each individual. In this manner we may move forward from the current "one-size-fits-all" approach to treating an individual to one that addresses the biological processes underlying the disorder and specific for that particular patient.

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Figure 1

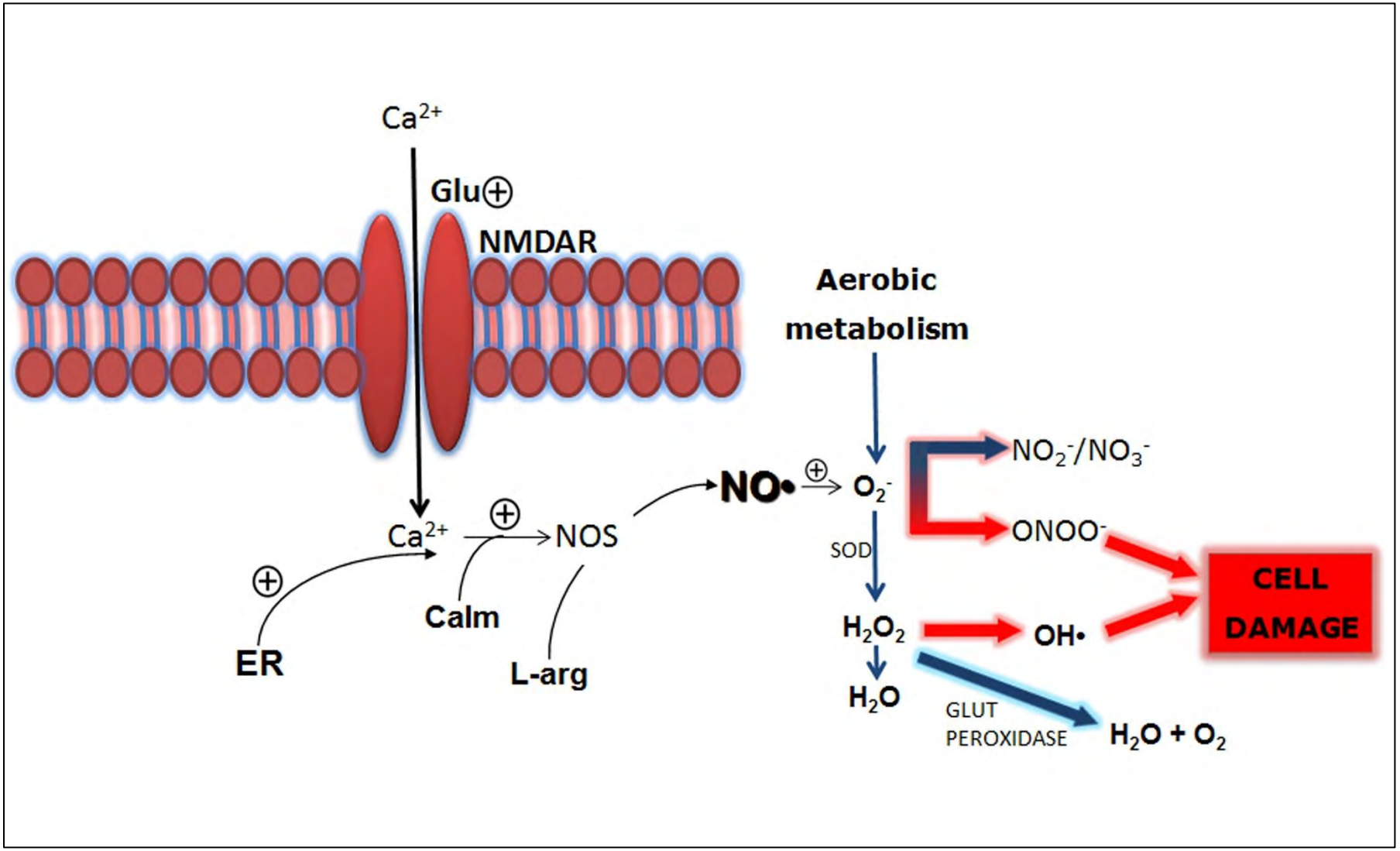
Ca2+
Glu⊕
NMDAR
Aerobic
metabolism
NO•
⊕
O2-
NO2-/NO3-
ONOO-
SOD
CELL
DAMAGE
H2O2
OH•
H2O
GLUT
PEROXIDASE
H2O + O2
Ca2+
⊕
NOS
⊕
Calm
ER
L-arg

Figure 2

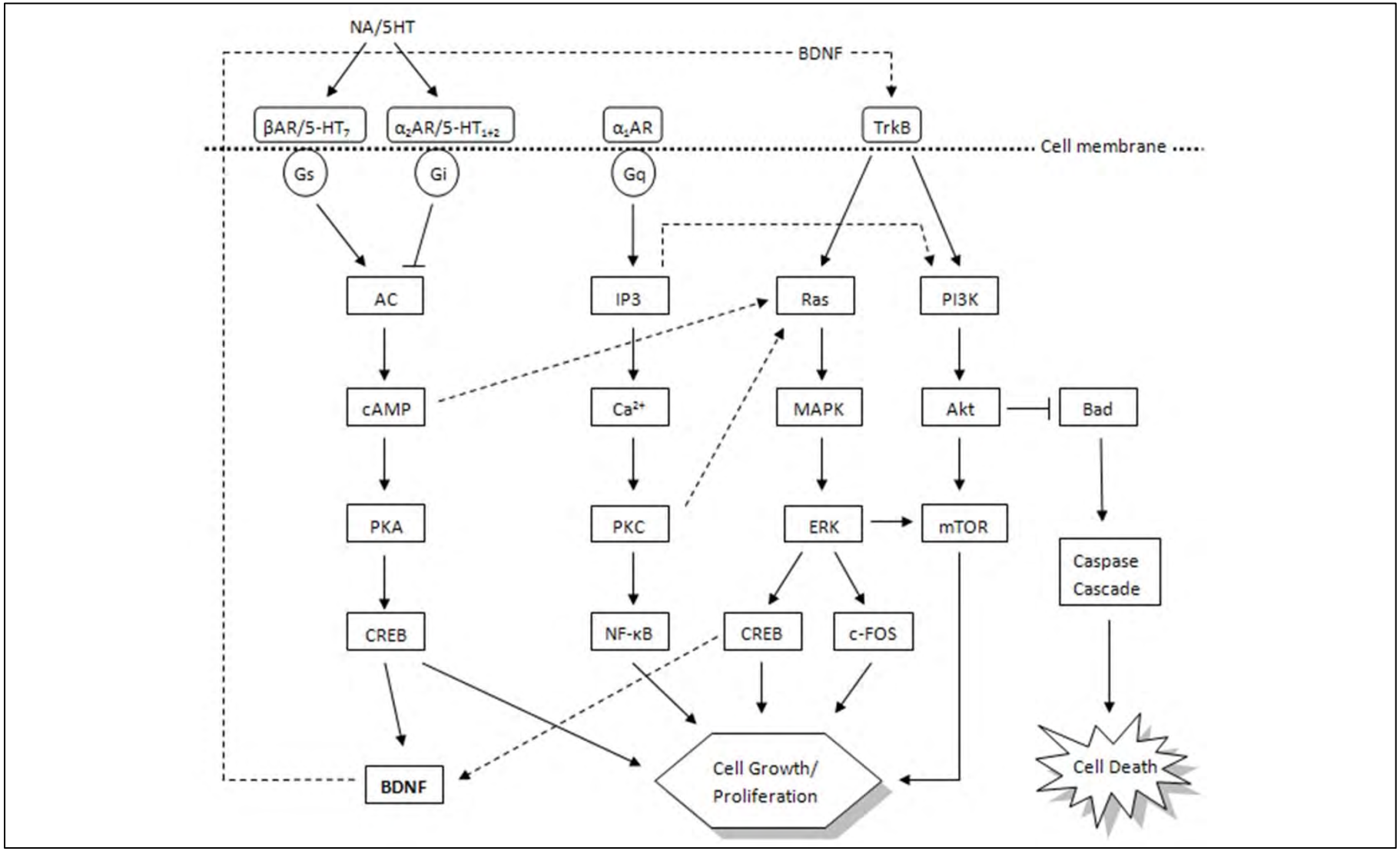
NA/5HT
BDNF
βAR/5-HT7
α2AR/5-HT1+2
α1AR
TrkB
Cell membrane
Gs
Gi
Gq
AC
IP3
Ras
PI3K
cAMP
Ca2+
MAPK
Akt
Bad
PKA
PKC
ERK
mTOR
Caspase Cascade
CREB
NF-κB
CREB
c-FOS
BDNF
Cell Growth/ Proliferation
Cell Death

Figure 3

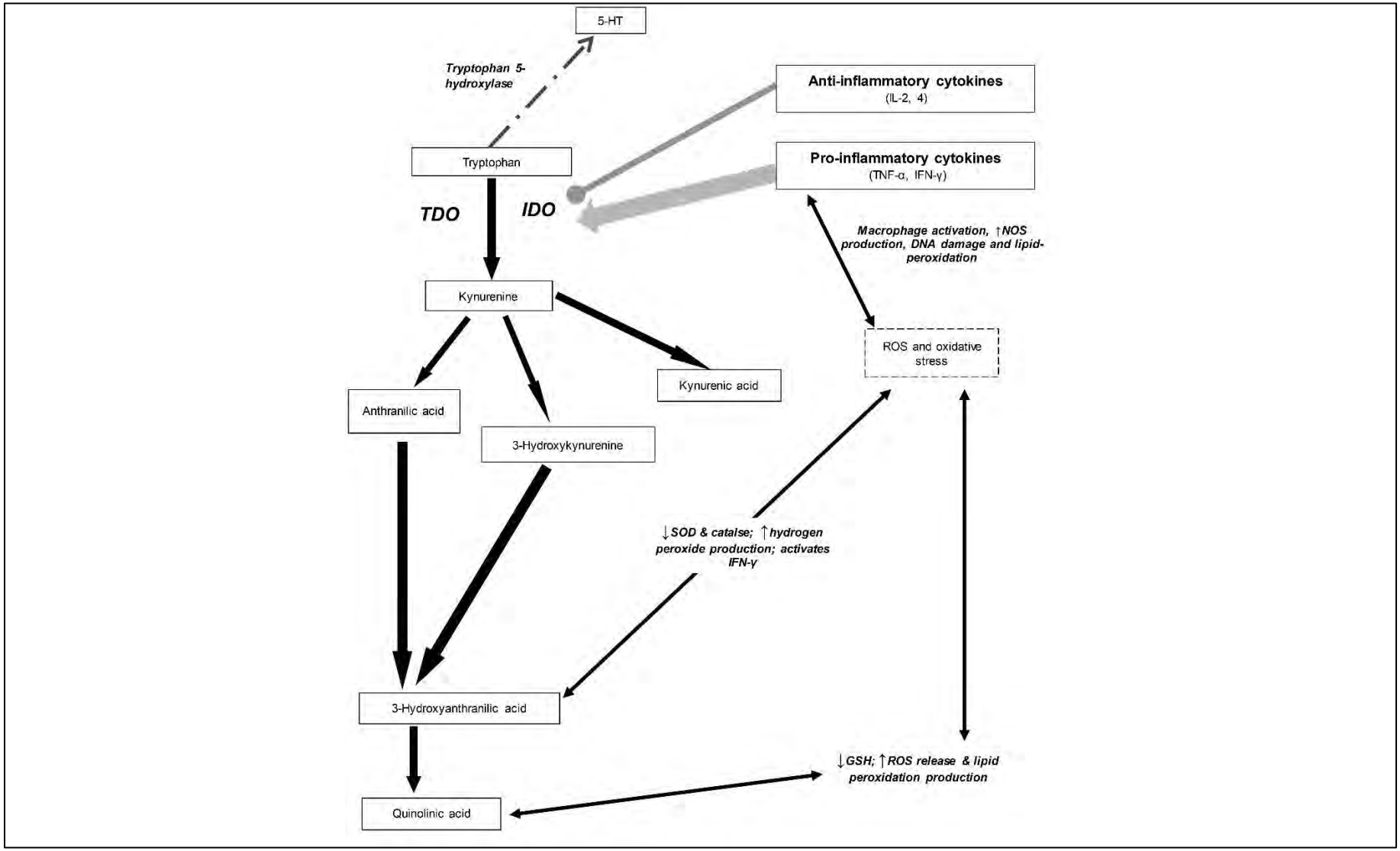
5-HT
Tryptophan 5-hydroxylase
Anti-inflammatory cytokines
(IL-2, 4)
Tryptophan
Pro-inflammatory cytokines
(TNF-α, IFN-γ)
TDO
IDO
Macrophage activation, ↑NOS production, DNA damage and lipid-peroxidation
Kynurenine
ROS and oxidative stress
Kynurenic acid
Anthranilic acid
3-Hydroxykynurenine
↓SOD & catalse; ↑hydrogen peroxide production; activates IFN-γ
3-Hydroxyanthranilic acid
↓GSH; ↑ROS release & lipid peroxidation production
Quinolinic acid

Table 1: A putative biomarker panel, based on clinical and pre-clinical data described in this article, for depression, bipolar disorder and schizophrenia.

	Depression	Bipolar disorder	Schizophrenia
Neuroanatomy	↓Hippocampal, prefrontal cortex, orbitofrontal cortex and basal ganglia volume *[24; 25; 26; 27]* [60]#*	Enlarged amygdala *[41]** ↓Dorsal and ventral prefrontal cortical volume *[42]** ↔Temporal lobe volume *[50]#*	↓Prefrontal cortex and temporal lobe volume *[50; 51; 52]* [322]#* Loss of PV-interneurons in hippocampus and ↓frontal cortical spine density *[64]#*
Neurocircuitry	↑Blood flow and glucose metabolism in amygdala, orbital cortex, and medial thalamus and ↓Blood flow in the prefrontal cortex and anterior cingulate cortex *[31]** ↓NAA in frontal cortex and subcortical regions *[32; 33]**	↓Activation and gray matter in cortical-cognitive brain and ↑ activation of the para-hippocampal gyrus and amygdala *[44]**. ↓Cerebral glucose metabolism *[61]*#* ↓P300 wave amplitude *[49]**	↓NAAG and NAA in temporal cortex and ↑NAAG in hippocampus *[59]* [68; 69]#* ↓Cerebellar and temporal lobe activity [46; 48]* ↓P300 wave amplitude *[49]** ↓Frontal cortical and striatal activation *[54]** ↓Prefrontal cortical function *[55; 56; 58]**
Neuroendocrine and circadian rhythms			
- *Cortisol*	↑Saliva cortisol *[76]** ↑Peripheral argenine-vasopressin *[77]** ↑HPA-axis activation *[78]**	↔Saliva cortisol *[79]** ↑Systemic cortisol metabolism *[75]**	↔Saliva cortisol *[79]** *↑Systemic cortisol metabolism [75]**
- *Circadian rhythms*	Reviewed in *[81]*#* and *[82]*#*	Reviewed in *[81]*#* and *[82]*#*	Reviewed in *[81]*#* and *[82]*#*
- *Thyroid hormones*	Thyroid and TSH dysfunction *[83]* [86]**	Thyroid and TSH dysfunction *[84]* [86]* [87]**	Thyroid and TSH dysfunction *[85]* [86]* [87]** *↑Autoimmune thyroid disease [87]** Thyroid regulation involved in crucial brain networks *[88]**

Neurochemical markers - *Monoamines*	↓DA *[74]* [20; 96]#* ↓5-HIAA *[117]** ↑5-HT & 5-HIAA in limbic structures *[139]#* ↓NA *[152; 153]** ↓MHPG *[154]**	↑ DA (manic symptoms) *[83]* [100]#* ↓5-HIAA (depressed) *[124]** ↑ 5-HIAA (manic symptoms) *[127]** ↓5-HT in amygdala and hippocampus *[143]#* ↑ NA *[124]* [158]#*	↑DA (striatum) & ↓DA (frontal cortex) *[85; 86]* [102; 103; 104]#* ↑5-HT transmission *[132; 133]** ↓5-HT/5-HIAA in frontal cortex and ↑5-HT/5-HIAA in nucleus accumbens and striatum [102; 104]# ↑NA *[156; 157]* [498]#*
- *Glutamate and GABA*	↑Glutamate *[168; 169]* [188]#*	↑Glutamate *[177]**	↑Glutamate & ↓GABA *[179]** ↓Glutamate *[181; 182]* [217; 218; 106]#*
Neuronal growth factors - *BDNF*	↓BDNF *[223; 224]* [264; 266]#* ↑BDNF *[242]* [262; 263]#*	↓BDNF *[221]* [84]#*	↓BDNF *[256; 259]* [271; 272]#* ↑BDNF in paranoid schizophrenia *[492]**
- *IGF -1*	↓IGF-1 *[277; 279]#*	↔IGF-1 *[275]**	↓IGF-1 *[276]**
- *VEGF*	↑VEGF *[284; 285]** ↓ or ↔ VEGF *[262]#*	↑VEGF *[287; 288]**	↓ or ↔ VEGF *[246; 289]**
Neuronal resilience markers	↓cAMP and MAPK/ERK pathway activity *[304]** ↑Expression of MKP *[322]#*	↓PGRN plasma levels *[311; 312]** ↔ GSK-3 brain expression *[313]* [324;325]#*	↑Expression of MAPK *[322; 19]#* ↑MAPK-and cAMP proteins *[314]** ↓DARPP-32 *[319]**
Oxidative stress markers	↑SOD, lipid peroxidation *[338; 339]**. ↓SOD and catalase *[359]#*	↑SOD *(manic and depressed symptoms)* ↑catalase *(manic symptoms)* & ↓catalase *(euthymic symptoms)* ↑ lipid peroxidation *[345; 221]* [369]#*	↑SOD, lipid peroxidation *[354]* [335]#* ↓GSH *[351; 352]** ↓PV-IR interneurons *[374]#* ↑Nox2 *[375]#*
Inflammatory markers	↑Pro-inflammatory cytokines (IL-1,6 ; IFN-γ; TNF-α) *[260; 285; 405]* [420]#*	↑Pro-inflammatory cytokines (IL-1,6 ; TNF-α) *[409; 410; 411]**	↑Pro-inflammatory cytokines (IL-1,6 ; IFN-γ; TNF-α) *[412; 380]* [102]#* ↓Anti-inflammatory cytokines (IL-10; IL-4) *[413]* [102]#*

Kynurenine pathway markers	↓Tryptophan & ↑Kynurenine *[432; 433]* [465]#* ↑QA *[434]** ↓KYNA *[427]**	↓Tryptophan & ↑Kynurenine *[429; 436]**	↑Tryptophan & ↑Kynurenine & ↑QA & ↑3-OHAA *[437; 436]* [443; 102]#* ↓ KYNA *[440]* [443; 102]#* or ↑KYNA *[180]**
Genetic markers	Polymorphisms in: 5-HT transporter, 5-HT receptor-2A, BDNF, and tryptophan hydroxylase *[444]* [462]#* Val/Met polymorphism *[447; 448]** ↓Neuropeptide Y expression *[460; 461]#*	Val/Met polymorphism *[447]** Tryptophan hydroxylase II gene variations *[452; 453]** ↑β-catenin expression *(manic model) [325]#*	Val/Met polymorphism *[445; 446]** NOS1 gene variance *[459]** Possible SzGene database (DISC1, DTNBP1, COMT, DAO, RGS4, NRG1, GRM3 gene variations) *[456]** ↑mGluR6 and AMPA3 gene expression *[323]#* ↓mGluR1 and mGluR5 expression *[466]#*
Proteomic markers	Abnormal insulin secretion *[471; 472; 473; 474]** ↓Glyoxalase-1 and dihydropyrimidinase-related protein-2 *[486]#* ↑Leptin, IL-1, BDNF proteins *[487]#*	Proteins involved in the WNT cascade (MB-18.5, CBF2, DECR2, BYSL, ANKARD12, ALDOC and DKK2) *[326]#*	↑Insulin and Cyclophilin A *[475; 485]** VEGF-derived peptide sequence, a transthyretin protein cluster, and another smaller cluster related to transthyretin *[484]** ↔Secretoneurin (Chromogranin) *[490]#* ↑Prohibitin protein *[488]*#*
Micro-RNAs (miRNAs)	28 miRNAs up-regulated and 2 miRNAs down-regulated in treatment *[516]**	Under expression of several miRNAs *[515]** 15 miRNAs (involved in neurodevelopment and behaviour regulation) deregulated *[518]**	16 miRNAs differentially expressed, 15 miRNAs down-regulated and 1 miRNA up-regulated *[517]* [515]** 22 miRNAs (involved in neurodevelopment and behaviour regulation) deregulated *[518]** ↑miRNA expression in cerebral cortex *[519]**

**Clinical data; #Pre-clinical data however see text for additional discussion and bibliography; Strong marker; Moderate marker; Not a strong marker/limited data*

Abbreviations: ↓, Decrease; ↑, Increase; ↔, Unchanged or differing results; PV, parvalbumin; NAA, N-acetyl aspartate; HPA, Hypothalamic-pituitary-adrenal; TSH, Thyroid stimulating hormone; DA, Dopamine; 5-HIAA, 5-Hydroxyindole-acetic acid; NA, Noradrenaline; MHPG, 3-Methoxy-4-hydroxyphenylglycol; GABA, Gamma-aminobutyric acid; BDNF, Brain-derived neurotrophic factor; IGF-1, Insulin growth factor-1; VEGF, Vascular endothelial growth factor; cAMP, Cyclic adenosine monophosphate; MAPK/ERK, Mitogen-activated protein kinases / extracellular signal-regulated kinases; MKP, Mitogen-activated kinase phosphatase; PGRN, Progranulin; GSK-3, Glycogen synthase kinase; DARPP-32, Dopamine-and cAMP-regulated neuronal phosphoprotein-32; SOD, Superoxide dismutase; GSH, Glutathione; PV-IR, Parvalbumin-interneurons; Nox2, Nicotinamide adenosine dinucleotide phosphate (NADPH) oxidase 2; QA, Quinolinic acid; KYNA, Kynurenic acid; 3-OHAA, 3-Hydroxyanthranilic acid; NOS-1, Nitric oxide synthase-1.

Figure Captions

Figure 1: Glutamate-mediated effects on the cGMP-NO system leading to monoamine release that in turn can be targeted by pharmacological means, e.g. PDE5 and NOS inhibitors, as well as known antidepressants.

This pathway can also lead to oxidative stress if excessive glutamate-mediated NO synthesis combines with O_2^- from aerobic metabolism. Also depicted is the effect of inflammatory mediators that promote iNOS-mediated NO synthesis thereby promoting the formation of cell-damaging reactive oxygen and nitrogen species. These pro-oxidative mechanisms can be abrogated by endogenous antioxidant systems such as superoxide dismutase (SOD) and glutathione that act as a sink to quench excessive NO and/or O_2^-.

Abbreviations: calmodulin (Calm); cyclic guanosine monophosphate (cGMP); endoplasmic reticulum (ER); glutamate (Glu); glutathione peroxidase (glut peroxidase); guanosine triphosphate (GTP); inducible nitric oxide synthase (iNOS); l-arginine (L-arg); neuronal nitric oxide synthase (nNOS); nitric oxide (NO); NMDA receptor (NMDAR); phosphodiesterase (PDE); superoxide dismutase (SOD); superoxide (O_2-); hydrogen peroxide (H_2O_2).

Figure 2: An overview of key neuroprotective and neurotoxic molecules involved in drug-induced neuroplasticity.

The monoaminergic system mainly exerts its effect on BDNF expression via the cAMP cascade, while BDNF in turn exerts its effect on monoaminergic neurons through the TrkB-receptor via the MAPK/ERK cascade and the phospholipase C (PLC) signalling system. Adapted from [342]. Abbreviations: beta adrenoceptor (βAR); alpha-2 adrenoceptor (α2AR); BDNF receptor (TrkB), see text for further details/ abbreviations not noted here.

Figure 3: A simplified diagram of the kynurenine pathway, indicating the principle enzymes, IDO and tryptophan-2,3-dioxygenase (TDO), and the subsequent formation of kynurenine and its metabolites from TRP.

The diagram indicates the inter-relationship of kynurenine metabolites, particularly QA, kynurenic acid and 3-hydroxyanthranilic acid. This diagram also depicts the activation and inhibition of IDO via pro- and anti-inflammatory cytokines respectively, as well as the influence of oxidative stress processes that will eventually determine cellular resilience or susceptibility to neurotoxic insults. Increased activity of the tryptophan-kynurenine synthesis (by TDO/IDO) will also diminish the availability of tryptophan for serotonin synthesis via tryptophan-5-hydroxylase, with resulting effects on mood and behavior.

Abbreviations: glutathione (GSH); interferon (IFN)-γ; interleukin (IL)-2 and -4; nitric oxide synthase (NOS); reactive oxygen species (ROS); serotonin (5-HT); tumour necrosis factor (TNF)-α. Adapted from [452, 453].

CHAPTER 4

4 Manuscript B

Article accepted for publication in *Acta Neuropsychiatrica* titled:

"Exploring a post-traumatic stress disorder paradigm in Flinders sensitive line rats to model treatment resistant depression I: Bio-behavioural validation and response to imipramine"

Author Contributions

- *Sarel J Brand* designed the investigation in consultation with *Brian H Harvey*, performed all behavioural, pharmacological, and statistical analyses, wrote the first version of the manuscript, and edited the manuscript following input from the co-authors.
- *Brian H Harvey* was study supervisor, devised the concept of the study, assisted in the interpretation of results, served as corresponding author for submission of the final manuscript to *Acta Neuropsychiatrica* and finalized the pre-submission version of the manuscript.

Important Information

- Instructions to the author are included in Addendum A.
- As per the instructions to the author, figures, tables, and legends are provided at the end of the manuscript.
- The co-author provided consent for the paper to be assessed as part of the Ph.D thesis of *Sarel J Brand* (Addendum B).
- The published article is provided in PDF format in Addendum D.

Exploring a Post-Traumatic Stress Disorder Paradigm in Flinders Sensitive Line Rats to Model Treatment Resistant Depression I: Bio-Behavioural Validation and Response to Imipramine

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Keywords: Treatment resistant depression; animal model; PTSD; antidepressant; gene-environment model

Abstract

Objective: In developing a preclinical model of treatment resistant depression (TRD), we combined animal models of depression with posttraumatic stress disorder (PTSD) to produce an animal with more severe as well as treatment resistant depressive-like behaviours.

Methods: Male Flinders sensitive line (FSL) rats, a genetic animal model of depression, were exposed to a stress re-stress model of PTSD (time dependent sensitisation; TDS) and compared to stress-naive controls. Seven days after stress, depressive-like and coping behaviours as well as hippocampal and cortical NA and 5HIAA levels were analysed. Response to sub-chronic imipramine treatment (IMI; 10mg.kg^{-1} s.c. x 7 days) was subsequently studied.

Results: FSL rats demonstrated bio-behavioural characteristics of depression. In FSL rats, exposure to TDS stress correlated negatively with weight gain, reduced swimming behaviour and increased immobility vs. unstressed FSL rats. IMI significantly reversed depressive-like (immobility) behaviour and enhanced active coping behaviour (swimming and climbing) in FSL rats. The latter was significantly attenuated in FSL rats exposed to TDS vs. unstressed FSL rats. IMI reversed reduced 5HIAA levels in unstressed FSL rats while exposure to TDS negated this effect. Lowered NA levels in FSL rats were sustained after TDS with IMI significantly reversing this in the hippocampus.

Conclusion: Combining FSL rats with a PTSD paradigm produces exaggerated depressive-like symptoms that display an attenuated response to antidepressant treatment. This work confirms combining FSL rats with TDS exposure as a putative animal model of TRD.

Abbreviations: 5-hydroxytryptamine (5HT); chronic mild stress (CMS); chronic unpredictable mild stress (CUMS); Diagnostic and Statistical Manual 4th Edition (DSM-IV); dopamine (DA); Flinders Resistant Line (FRL); Flinders Sensitive Line (FSL); forced swim test (FST); hypothalamic-pituitary-adrenal (HPA); imipramine (IMI); noradrenaline (NA); open field test (OFT); post-traumatic stress disorder (PTSD); Pre-clinical Drug Development Platform (PCDDP); Sequenced Treatment Alternatives to Relieve Depression (STAR*D); serotonin transporter (SERT); single prolonged stress (SPS); time dependent sensitization (TDS); treatment resistant depression (TRD); vehicle (VEH)

Significant outcomes

- Exposure of Flinders sensitive line (FSL) rats to time-dependent sensitisation (TDS) stress reduces active coping, amplifies depressive-like behaviour and attenuates the antidepressant effects of imipramine (IMI) in FSL rats.
- The beneficial effects of IMI on limbic monoamine levels in FSL rats are compromised in combined FSL+TDS exposed rats, especially its effects on the serotonergic system.
- Posttraumatic stress disorder (PTSD) is highly co-morbid with depression and contributes to the development of treatment resistant depression (TRD). Combining a genetic animal model of depression with a PTSD paradigm may represent a putative animal model of TRD.

Limitations

- The initial (severe) stress sequence and subsequent re-stresses may promote several adaptive responses in the animals that complicate understanding of monoaminergic responses. Limiting the procedure to a single re-stress could be considered.
- Assessment of corticosterone levels immediately post severe stress as well as before and after re-stress may provide a more comprehensive picture of the bio-behavioural responses observed and their relevance to TRD.
- Behavioural assessment of anhedonia (sucrose preference test), which has been demonstrated to be an important symptom of TRD, would be a valuable addition.
- Challenging FSL+TDS animals with first-line antidepressants (SSRIs, NSRI's) and/or ketamine would expand predictive validity, and is presented in a companion paper to this manuscript.
- Would this model present with altered biomarkers of TRD that contribute to construct validity?

1. Introduction

The occurrence of non- or partial response to antidepressant treatment in the depressed population creates a major problem in effectively treating and managing the disorder. Less than two thirds of patients respond to drug-centred therapy (1) and up to half of patients fail to achieve a full response when treated with first-line antidepressant drugs (2). These initial observations were confirmed by the Sequenced Treatment Alternatives to Relieve Depression (STAR*D) study, designed to mimic clinical conditions by incorporating the most commonly used strategies in treating patients exhibiting drug resistance (3). Even after applying several treatment strategies in this population, approximately 30% of these patients still did not respond to treatment (4).

On-going work has described the underlying biology of depression as being driven by the presence of chronic psychosocial stress and associated disturbances in monoaminergic, GABA-glutamate, neuroendocrine (5) and cardio-metabolic and immune-inflammatory disturbances (6). However, the exact cause of treatment resistant depression (TRD) remains obscure. As with depression, TRD is believed to be heterogeneous in nature (7) and, although most pathophysiological factors contributing to depression appear to be similar in TRD, many of these conditions are significantly exaggerated in the resistant form, resulting in more severe symptoms (8).

The treatment of depression may be further complicated by the co-occurrence of other underlying psychological disorders. The prevalence rate of a co-existing anxiety disorder is 50%-60% (9, 10) – a figure that increases to 72% in TRD (9). With a prevalence rate of 17.8%, post-traumatic stress disorder (PTSD) is one of the more commonly co-occurring anxiety disorders in patients with depression, and increases to 22.4% in TRD (9). Conversely, more than half of patients seeking treatment for PTSD are diagnosed with comorbid depression (11). This high comorbidity stems largely from overlapping symptoms of anhedonia, sleep difficulty, irritability and poor concentration (DSM-IV criteria) (12). Both depression and PTSD require exposure to stressful events for onset (12), while both illnesses exhibit hippocampal atrophy related to hypothalamic-pituitary-adrenal (HPA) axis abnormalities (13).

In recent years it has become widely accepted that genetic susceptibility plus adverse environmental situations are an important prodromal event to the development of depression (14-16). Animal

models that are based on this construct have contributed significantly to our knowledge of mood and anxiety disorders (5, 17). However, a shortage of suitable and validated animal models of TRD is a major contributing factor to our current lack of understanding of the pathophysiology of TRD. Recent studies have therefore set out to explore the processes that underlie treatment resistance in animal models (18). In their review, Willner and Belzung (19) emphasize models that incorporate predisposing factors leading to heightened stress responsiveness. Chronic mild stress (CMS), a paradigm primarily identified as a depression model (20), has been demonstrated to successfully reproduce antidepressant treatment response rates resembling those observed in clinical studies, with chronic escitalopram treatment found to induce response rates of only 50% (21). However, it being labour intensive and exhibiting poor cross-laboratory reproducibility is a concern (22, 23).

The Flinders Sensitive Line (FSL) rat, a genetic animal model of depression, is a robust and well-studied preclinical model of depression with good construct, predictive and face validity (24-27). Furthermore, FSL rats only display anhedonic responses after exposure to CMS (28, 29), thus tagging the strain as a good candidate for gene-X-environment studies. Indeed, FSL and FRL rats display differential sensitivity to rearing conditions (early-life stress) and rat strain (genes) that in turn modify treatment response by altering serotonin transporter (SERT) receptors (30). This is a valuable quality, seeing that abnormal SERT function has been implicated in the pathology of depression (31, 32). Interestingly, by exposing FSL rats to maternal separation, Carboni et al (33) demonstrated the induction of biological correlates reminiscent of those observed in human TRD, prompting them to propose that the gene-environment paradigm offers important construct validity in modelling TRD. However, the model lacked predictive validity due to the inability of antidepressant treatment to alter immobility time in maternally separated FSL rats either before or after treatment when compared to control animals (33).

Considering the strong comorbidity between depression and PTSD, and that depression in patients with PTSD is more treatment resistant (34, 35), we have developed an animal model of TRD based on the premise that exposing animals genetically predisposed to depressive-like behaviour to a PTSD-related paradigm would yield animals displaying more pronounced depressive-like behaviour. Moreover, such behaviour would be resistant to antidepressant treatment. To this end we have considered the time-dependent sensitization (TDS) or stress re-stress model of PTSD. TDS is based

on a trauma plus contextual reminder principle of PTSD (36), and has shown good predictive, construct and face validity for PTSD (37-40). Face, construct and predictive validity were assessed in the forced swim test (FST) using a behavioural sampling method to study serotonergic and noradrenergic driven behaviours, assessment of limbic NA and 5HIAA levels, and response to chronic treatment with the tricyclic antidepressant, imipramine (IMI).

2. Materials and Methods

2.1 Subjects

Animals were bred, supplied, and housed at the Vivarium (SAVC reg. number FR15/13458; SANAS GLP compliance number G0019) of the Pre-Clinical Drug Development Platform of the North-West University. Ambient temperature was maintained at 22 ± 2°C with a relative humidity of 40 – 60% and full spectrum of light in a 12-hour light/dark cycle, with lights switched on at 06:00 AM and off at 06:00 PM. Food and water were provided ad libitum. All experiments were approved by the AnimCare animal research ethics committee (NHREC reg. number AREC-130913-015) of the North-West University. All 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-00111-12-A5).

Subjects were male adult FSL (n = 48 for behavioural assessment and n = 32 for monoamine analysis each) and FRL (n = 12 for behavioural assessment and n = 8 for monoamine analysis) rats. Table 1 describes the layout of the experimental groups. Half of the FSL animals in each of the above groups were subjected to TDS (see below) at the start of the protocol with behaviour in the open field test (OFT) and FST assessed at the end of the protocol (2 weeks later). Monoamine analysis was performed in animals naive to behavioural assessment. The animals were housed four males per cage, with the TDS paradigm initiated at an age of 40 (± 1) days in order to conclude the experiments while the rats were still of an appropriate weight for the behavioural assessments. Handling of the animals was initiated one week before starting the experimental procedure by taking bodyweight measurements daily until the last day of the study to monitor weight gain and calculate drug dosages.

2.2 Time dependent sensitization (TDS)

TDS is an animal model of PTSD. Animals exposed to a severely traumatic situation, and followed by subsequent but less stressful contextual reminders, exhibit significant physiological and behavioural alterations that show a time-dependent sustaining or worsening in the absence of the initiating stressor (41, 42).

The TDS paradigm used in this study incorporated an acute single prolonged stress (SPS) sequence comprising a somatosensory stressor (restraint), a psychological stressor (forced swimming with brief submersion), and a complex stress-stimuli (exposure to ether vapours) followed by re-exposure to restraint stress 7 and 14 days later (42).

2.2.1. Restraint stress: Rats were placed in Perspex® restrainers for 2 hours with the tail-gates adjusted to keep each animal well contained without impairing circulation to the limbs. The same procedure was followed on days 7 and 14 during the re-stress phase of the TDS protocol.

2.2.2. Forced swim stress: Rats were placed individually in cylindrical Perspex® swim tanks containing 40 cm of ambient water (25 °C) and allowed to swim for 15 min while being forcefully submerged for the last 20 seconds. Thereafter animals were removed from the cylinders, dried and returned to their home cages to recover for 15 minutes. Forced swimming was performed 21 days *before* behavioural testing (only as part of the SPS procedure and not during re-restress) in the FST so that any possible conditioned response to swim stress in the FST would be unlikely.

2.2.3. Exposure to ether vapours: Fifteen minutes after swim stress, rats were exposed to 5 ml of 100% ether vapours in a 5 L sealed plastic container until loss of consciousness (±2 min). Ether was poured onto a paper towel at the bottom of the container with the animal placed on a raised metal platform to avoid direct contact with the substance. After loss of consciousness, the animals were immediately removed from the container, returned to their home cage for observation until regaining full consciousness and then returned to their holding room. Animals were left mostly undisturbed, only subjecting them to routine handling until re-exposure to restraint-stress during the re-stress phase of the TDS protocol.

2.3 Open Field Test (OFT)

This test is generally performed prior to the FST to control for locomotor activity. The OFT was performed half an hour before subjecting animals to the FST. Rats were individually placed in a square arena (100 x 100 x 50 cm) facing the centre and their behaviour recorded for 5 minutes using a ceiling-mounted digital camera. The video files were subsequently analysed using EthoVision® XT software (Noldus® Information Technology, Wageningen, The Netherlands). Total distance moved was used as a measure of locomotor activity.

2.4 Forced swim test (FST)

The FST can reliably predict antidepressant-like effects after drug treatment and is considered a model of behavioural despair that is typically manifest in human depression, and expressed in rodents as a decrease in escape-driven behaviour, i.e. increased immobility (43). During behavioural analysis, rats were placed individually in cylindrical Perspex® swim tanks containing 30 cm of ambient water (25 °C) for 7 minutes and their behaviour recorded. The first and last minute of the video files were discarded and the remaining five minutes of swimming behaviour scored for characteristic escape-directed behaviours, including swimming, climbing (struggling), and immobility. These sub-scores of the FST provide useful information relating to serotonergic (swimming) and noradrenergic (climbing) directed behaviours that may extend whole brain monoamine analyses (44).

2.5 Drug administration

After weighing all animals daily (between 09:00 AM and 11:00 AM), IMI (Sigma-Aldrich) was dissolved in physiological saline (0.9 % NaCl) and administered subcutaneously at a dose of 10 mg/kg (45, 46) to unstressed animals (FSL+IMI) and animals exposed to TDS (TDS+IMI). Treatment started on day 15 (after completing the TDS protocol on day 14) and persisted for 7 days before behavioural testing commenced on the evening of day 21. This duration of treatment is adequate for establishing an antidepressant response in rats (44, 47, 48). Stressed and unstressed control animals (FSL+FRL) were injected with saline vehicle according to the same procedure as in IMI-treated animals.

2.6 Quantitative analysis of brain NA and 5HIAA

Several valid indices of 5HT central activity may be applied, including 5HT and 5HIAA levels and the 5HIAA/5HT ratio (49). In this regard, *in vivo* microdialysis is a more reliable method to directly measure extracellular levels of 5HT, whereas whole- or regional brain monoamine analysis provides total levels of 5HT – both extracellular and unreleased from nerve terminals (50). 5HT is metabolized primarily to 5HIAA, hence 5HIAA has been demonstrated to reflect reliable insights into time-dependent alterations in serotonin response (51). 5-HIAA has previously been correlated with 5HT function (49) and was therefore applied as an indicator of 5HT-ergic function in the current study. Following sacrifice of the rats by decapitation, total hippocampus and frontal cortices were dissected out on an ice-cooled dissection slab, weighed, snap frozen in liquid nitrogen and stored at -80 °C until the day of analysis, as described previously (41). Quantification of NA and 5HIAA was performed by high performance liquid chromatography (HPLC) coupled with electrochemical detection (HPLC-EC), as previously described (42). An Agilent 1200 series HPLC, equipped with an isocratic pump, auto sampler and coupled to an ESA Coulochem Electrochemical detector and Chromeleon® Chromatography Management System software (version 6.8), was used. NA and 5HIAA concentrations in the tissue samples were determined by comparing the area under the peak of each monoamine to that of the internal standard, isoprenaline (range 5 – 50 ng/ml). Linear standard curves (regression coefficient greater than 0.99) were found in this particular range. Monoamine concentrations were expressed as ng/g wet weight of tissue (mean ± S.E.M.).

2.7 Body weight analysis

Decreased bodyweight and loss of appetite have been observed in both depressed individuals and FSL rats (*Introduction*). Sustained decreases in weight gain have been reported in rats following chronic stress (52, 53) which may be initiated by increased energy metabolism during stress coupled with acute increases in stress-related peptides (53). In order to establish the impact of the applied stressors on the well-being of the animals, body weight was measured daily from seven days consecutive to SPS and continued until the final day of the experiment.

2.8 Statistical analysis

Statistical analyses were performed using Graphpad Prism® 6 and IBM® SPSS® 22 software under the guidance of the Statistical Consultation Service of the North-West University. In pairwise comparisons of the behaviour and neurochemistry between unstressed FRL and FSL animals, unpaired student's t-tests with Welch's correction (normally distributed data as indicated by Shapiro-Wilk test for normality $p > 0.05$) or Mann-Whitney U-tests (data not distributed normally) were performed. Two-way repeated measures analysis of variance (RM-ANOVA) followed by Bonferroni post-hoc analysis was applied to comparisons of the treatment naïve cumulative weight gain of FRL and stressed and unstressed FSL animals. Time and cohort was set as within-subject factors, while weight was set as between-subject factor. Ordinary two-way ANOVA was applied in between-group comparisons of behaviour and neurochemistry in treatment-naïve and IMI treated unstressed and stressed FSL animals. In this case, exposure to TDS and treatment was set as within-subject factors, while the respective behavioural and neurochemical parameters were set as between-subject factors. Significance was set at $p < 0.05$ for all comparisons. Where Cohen's d effect sizes were calculated, large effect sizes are indicated by $d > 0.8$ and very large effect sizes by $d > 1.3$.

3. Results

3.1 Bodyweight

Data are represented in Figure 1. Two-way RM-ANOVA revealed a significant interaction between time and cohort ($F(81, 1188) = 61.98$) with respect to the mean cumulative weight gain of animals, while both time ($F(27, 1188) = 13940$, $p < 0.0001$) and cohort ($F(3, 44) = 102.0$, $p < 0.0001$) respectively also had significant main effects on weight gain. Although the mean cumulative daily weight gain of rats between the respective cohorts demonstrated no significant differences before SPS (day 0), significant age and stress related differences between FSL and FRL animals, both within (FSL) and between strain, became apparent post-SPS. From day 3 post-SPS, unstressed FSL animals lagged behind the FRL controls (day 3, 45.6±3.2 vs. 51.4±3.9g, $p = 0.004$). Moreover the weight gain in stressed FSL rats soon lagged behind that of unstressed control animals (Table 2). On day 8 post-SPS the difference in weight gain between stressed and unstressed FSL rats began to reveal significance (69.8±4.2 vs 75.6±3.3; $p = 0.005$). The observed disparities in the rates of weight gain

in the various groups persisted until the last day of observation. In addition to the curbed weight gain of TDS-exposed FSL rats, they were also observed to present with a general decrease in fur quality and porphyrin staining around the eyes (visual inspection; data not shown).

3.2 Behaviour

In order to establish the translational relevance of the FSL rat for depression, data and statistics relating to the behavioural comparisons made between stress and treatment-naive FRL and FSL animals are provided in Table 3. The behavioural differences between FSL vs. FRL rats are henceforth described separately under the OFT and FST sections below.

3.2.1. OFT

3.2.1.1 Locomotor activity

FSL and FRL rats were similar with respect to distance travelled in the OFT (2119±505.4 vs. 2273±307 cm, Table 3).

Data describing the effects of stress and IMI treatment in FSL animals are presented in Figure 2A. Considering the locomotor activity of FSL animals, two-way ANOVA did not reveal a significant interaction between TDS-exposure and treatment ($F(1, 11) = 0.95$, $p = 0.35$). However, a main effect of treatment was observed ($F(1, 11) = 5.67$, $p = 0.04$) in the locomotor activity of the TDS exposed group with post hoc analysis revealing a trend towards decreased locomotor activity in IMI treated animals that narrowly missed statistical significance (1640±422.8 vs. 2296±971.7cm, $p = 0.07$, $d = 0.94$).

3.2.2. FST

3.2.2.1. Swimming

FSL rats presented with significantly reduced swimming behaviour compared to FRL rats (59.9±15.2 vs 70.4±14.8s; $p < 0.05$, Table 3).

Data describing the effects of stress and IMI treatment in FSL animals are presented in Figure 3A. While no significant interaction between TDS and treatment was displayed in the behaviour of FSL animals ($F(1,44) = 1.54$, $p = 0.2$), both factors had statistically significant main effects on swimming

behaviour (TDS, $F(1, 44) = 11.8$, $p = 0.001$; treatment, $F(1, 44) = 5.32$, $p = 0.03$). As such, post-hoc analysis demonstrated that treatment-naive FSL animals exposed to TDS showed an even greater reduction in the average swimming time compared to unstressed treatment-naïve rats (52.9±15.2 vs. 24.4±9.8s, $p = 0.004$). Finally, IMI treatment significantly reversed the reduced swimming time in TDS-exposed FSL animals (46.0±36.7 vs. 24.4±9.8s, $p = 0.03$), while failing to affect the behaviour of animals in the unstressed group.

3.2.2.2. Climbing

FSL rats presented with significantly reduced climbing behaviour compared to FRL rats (35.0±9.2 vs 117.9±38.0s; $p < 0.0001$, Table 3).

Data describing the effects of stress and IMI treatment in FSL animals are presented in Figure 3B. A significant interaction was displayed between TDS and treatment ($F(1, 44) = 28.5$, $p < 0.0001$) while both treatment ($F(1, 44) = 49.6$, $p < 0.0001$) and stress ($F(1, 44) = 57.1$, $p < 0.0001$) had significant main effects on climbing behaviour. Although no difference between the climbing behaviour of treatment naïve stressed and unstressed FSL animals was demonstrated, post-hoc analysis revealed that IMI treatment significantly increased climbing in unstressed FSL animals (96.8±29.2 vs. 35.0±9.3s, $p < 0.0001$), but was without effect in FSL+TDS animals (32.4±13.9 vs. 24.0±7.9s, n/s). In fact, in comparing FSL+TDS to non-stressed FSL rats receiving IMI, exposure to TDS significantly negated the response to IMI in FSL animals (32.4±13.9 vs. 96.8±29.2s, $p < 0.0001$).

3.2.2.3. Immobility

FSL rats presented with significantly increased immobility compared to FRL rats (212.1±18.8 vs 111.7±33.70s; $p < 0.0001$, Table 3).

Data describing the effects of stress and IMI treatment in FSL animals are presented in Figure 3C. As a significant two-way interaction was revealed between TDS and treatment with respect to the immobility scores of FSL animals ($F(1, 44) = 6.8$, $p = 0.01$), simple main effects of both factors were run. Exposure to TDS ($F(1, 44) = 64.4$, $p < 0.0001$) significantly increased the average immobility score of treatment naïve FSL animals (251.7±14.7 vs. 212.1±18.8s, $p = 0.0008$) and although IMI treatment ($F(1, 44) = 45.4$, $p < 0.0001$) resulted in significant reductions in the immobility scores of

both unstressed (143.8±27.1 vs. 212.1±18.8, $p < 0001$) and stressed (221.5±35.7 vs. 251.7±14.2, $p = 0.01$) FSL rats. However, immobility in IMI-treated FSL+TDS animals *remained* significantly greater than that in unstressed FSL animals receiving IMI (221.5±35.7 vs. 143.8±27.1, $p < 0.0001$).

3.3 Monoamine analysis

In order to establish the translational relevance of the FSL rat for depression, data and statistics relating to the neurochemical comparisons made between stress and treatment-naive FRL and FSL animals are provided in Table 3. The neurochemical differences between FSL vs. FRL rats are henceforth described separately below.

3.3.1. 5HIAA

FSL rats presented with significantly increased 5HIAA levels in the frontal cortex (268.4±51.3 vs. 170.4±22.8 ng.mg^{-1}; $p < 0.005$) and hippocampus (244.1±40.3 vs. 177.2±37.2 ng.mg^{-1}; $p < 0.05$) vs. FRL rats (Table 3).

Data describing the effects of stress and IMI treatment in FSL animals are presented in Figure 4A. Two-way ANOVA revealed significant interactions between TDS and treatment in both brain areas (frontal cortex, $F(1, 27) = 7.6$, $p = 0.01$; hippocampus, $F(1, 27) = 4.45$, $p = 0.04$) and simple main effects were run. As such, TDS *and* treatment significantly influenced the concentrations of 5HIAA measured in both the frontal cortex (TDS, $F(1, 27) = 30.8$, $p < 0.0001$; treatment, $F(1, 27) = 6.1$, $p = 0.02$) and hippocampus (TDS, $F(1, 27) = 15.72$, $p = 0.0005$; treatment, $F(1, 27) = 11.1$, $p = 0.002$). As such, post-hoc analyses revealed that 5HIAA levels in treatment naïve stressed FSL animals tended to be lower compared to the unstressed FSL animals (4Ai, 216.4±45.6 vs. 268.4±51.3 ng.mg^{-1}, d=1.07; 4Aii, 201.5±59.1 vs. 244.1±40.4 ng.mg^{-1}, $d = 0.85$). While IMI treatment significantly increased both frontocortical (4Ai, 366.0±69.0 vs. 268.4±51.3 ng.mg^{-1}, p=0.007) and hippocampal (4Aii, 369.1±87.3 vs. 244.1±40.4 ng.mg^{-1}, p=0.004) 5HIAA levels in unstressed FSL rats, exposure to TDS negated this effect (4Ai, frontal cortex, 210.8±32.4 vs. 216.4±45.6 ng.mg^{-1}, n/s; 4Aii, hippocampus, 229.7±58.7 vs. 201.5±59.0 ng.mg^{-1}, n/s) and resulted in significantly lower levels of 5HIAA levels measured in IMI-treated animals after TDS-exposure relative to stress-naive animals (4Ai, frontal cortex, 210.8±32.4 vs. 366.0±69.0 ng.mg^{-1}, $p < 0.0001$; 4Aii, hippocampus, 229.7±58.7 vs. 369.1±87.3 ng.mg^{-1}, $p = 0.0005$).

3.3.2. Noradrenaline

FSL rats presented with significantly reduced NA levels in the frontal cortex (188.7±77.5 vs. 412.1±27.7 ng.mg^{-1}; $p < 0.0001$) and hippocampus (202.9±78.4 vs. 451.9±95.3 ng.mg^{-1}; $p < 0.0001$) vs. FRL rats (Table 3).

Data describing the effects of stress and IMI treatment in FSL animals are presented in Figure 4B. No significant two-way interactions between TDS and treatment were observed in either brain area (frontal cortex, $F(1, 27) = 0.01$, $p = 0.9$; hippocampus, $F(1, 26) = 1.3$, $p = 0.3$). However, treatment demonstrated a main effect on NA concentrations in the frontal cortex ($F(1, 27) = 8.4$, $p = 0.007$) and hippocampus ($F(1, 26) = 11.29$, $p = 0.002$). Although IMI resulted in trends toward increased NA in both brain areas of unstressed FSL animals (frontal cortex, 285.2±119.1 vs. 188.7±77.5 ng.mg^{-1}, $d = 0.98$; hippocampus, 308.5±126.1 vs. 202.9±78.4 ng.mg^{-1}, $d = 1.03$), it significantly increased the hippocampal NA levels in stressed FSL animals to levels comparable to that observed in unstressed animals (4Bii, 364.9±212.8 vs. 151.9±41.4 ng.mg^{-1}, $p=0.04$). Furthermore, although narrowly missing statistical significance, IMI also tended to increase NA in the frontal cortex of stressed FSL animals (4Bi, 243.8±96.2 vs. 154.0±51.1 ng.mg^{-1}, $p=0.054$, $d=1.2$).

4. Discussion

As expected, FSL rats presented with significant depressive-like manifestations versus their FRL controls at both the behavioural and neurochemical level (Table 3), with IMI for the most part reversing these changes (Figures 3 and 4). Exposure of FSL rats to TDS profoundly inhibited growth (Figure 1), with behavioural and neurochemical sequelae (Figures 3 and 4). TDS further reduced active coping (swimming) behaviour and amplified depressive-like behaviour (immobility) in FSL rats (Figure 3A, C). Importantly, the above-noted antidepressant-like effects of IMI in FSL rats was significantly attenuated after TDS exposure. While IMI altered brain monoamine levels in unstressed FSL rats, it failed to do so in combined FSL+TDS rats – especially effects on 5HIAA (Figure 4). As such, combining FSL+TDS stress may represent a novel animal model of TRD, a schematic outline of which is depicted in Figure 5.

Depression is a multifactorial disorder (7) with both genetics and environmental stress contributing to its development (14, 15). The FSL rat is a well validated genetic animal model of depression (27).

Considering the high co-morbidity of PTSD in depression as a contributing factor in treatment resistance (34, 35), introducing these animals to conditions conducive to PTSD may serve as a suitable gene-X-environment model of TRD. The aim of this study was therefore to explore this notion by studying behaviour and neurochemistry in such a model and, in so doing, to aid preclinical research into TRD and developing novel drug options for the disorder. By using FRL rats as a control, we demonstrated the depressive phenotype of the FSL rat, thereafter subjecting this stress-sensitive animal to a TDS paradigm and assessing its response to standard antidepressant treatment. The negative impact of TDS on physical development, as illustrated by its detrimental effects on growth during a four week period (Figure 1), is indicative of the degree to which the physical and, no doubt, psychological wellbeing of these animals were affected by these interventions. The results of the comparison between cumulative weight gain in FRL rats and stressed and unstressed FSL rats provide an accurate portrayal of the character and resilience of the two strains. At baseline, FSL rats already displayed decreased ability to gain weight even before exposure to environmental stressors. Bearing this in mind, TDS expectedly proved to further worsen the overall well-being of these animals.

Rats exposed to CMS have previously been observed to exhibit impaired locomotor activity (22), although TDS did not negatively affect locomotor activity in the current study (Figure 2). Although IMI treatment resulted in a trend toward decreased locomotor activity in TDS exposed animals, this finding failed to reach statistical significance. As such, this finding provides a robust departure point for interpreting treatment effects in the FST without having to consider any confounding effects on locomotor activity.

Immobility time is a characteristic behaviour measured in the FST, while the assessment of swimming and climbing behaviour allows for generating a more holistic account of animal behaviour and also aids in understanding the behavioural effects of drug treatment (54). Results obtained from the FST showed that FSL rats displayed significantly less active coping (swimming and climbing) behaviour as well as being significantly more immobile than their FRL counterparts (Table 3). Important to note is that both decreased swimming behaviour and increased immobility observed in FSL control animals were augmented to a significant degree following exposure to TDS (Figure 3A and C). Of even greater importance is that the antidepressant-like effect exhibited by IMI treatment in unstressed FSL animals was negated in TDS-exposed FSL rats in respect to climbing and immobility

(Figure 3B and C) although not swimming (Figure 3A). Further, the anti-immobility effects of IMI in FSL animals were also significantly compromised by TDS compared to that in unstressed FSL animals (Figure 3C). Thus TDS stress exaggerates depressive-like (immobility) behaviour evident in a genetic animal model of depression and abrogates the antidepressant-like effects of IMI in these animals. This not only supports the validity of combining FSL rats with a PTSD paradigm as a gene-X-environment model of TRD, but reinforces the clinical presentation of TRD in depressed patients with a history of severe psychological trauma.

In spite of diffuse distribution of 5HT throughout the central nervous system (CNS), uncertainty still surrounds the exact function of 5HT and its relationship to other neurochemicals (55, 56). TDS represents a severely traumatic series of events which prompts the activation of various bio-behavioural responses geared to maximize the animal's survival. Serotonin is crucial in survival behaviour and has been suggested to play a critical role in adapting to aversive events (57, 58). In fact, stress re-stress has been demonstrated to alter 5HT receptors in limbic structures that in turn adversely affect memory and other cognitive processes (37). Further, the changes in 5HT concentration in response to stress vary between brain regions and also according to the duration of stress applied (59). FSL rats have previously been characterised by increased levels of 5HT and 5HIAA in limbic regions that are altered in response to antidepressant treatment (60). Increased cortical and hippocampal 5HIAA levels in FSL rats compared to FRL controls in the current study concur with this observation (Table 3) and would suggest a compromised 5HT-ergic system. 5HIAA levels were decreased in FSL rats one week after TDS (Figure 4A) – this decrease correlating with significantly reduced swimming activity measured in the FST and concurs with decreased 5HIAA levels measured in the frontal cortex of Sprague Dawley rats subjected to CUMS (61). Although TDS may be viewed as a series of aversive and traumatic events (36), it should be kept in mind that the current data reflects NA and 5HIAA changes one week subsequent to completion of the TDS procedure, and thus represents a late emerging event that may be pathological. Previous data demonstrated that an initial increase in 5HT levels after SPS was followed by decreased levels after restress (42) which may be suggestive of an adaptive response to stress.

The above-mentioned coping strategies employed in the FST have been found to present with significant correlations with altered monoamines and to be of relevance for the neurochemical basis

of depression (62). Thus, noradrenergic processes have been demonstrated to be altered in depression, but also in anxiety and PTSD, such as adrenal receptor dysregulation in depression (63), increased NA precursors accompanied by a decrease in adrenergic receptor affinity in patients suffering from PTSD with comorbid depression (64), the association between catechol-O-methyl transferase (COMT) single nucleotide polymorphisms and suicide risk in TRD patients (65), and increased 3-methoxy-4-hydroxyphenylglycol (MHPG) levels measured in patients suffering from anxiety disorders (66). Furthermore, uncontrollable stress in animal models is associated with decreased central levels of NA (67, 68) and may be the result of insufficient synthesis of the neurotransmitter relative to its utilization (67). A general decrease in NA levels were measured in the frontal cortex and hippocampus of treatment-naive FSL animals (Table 3). Owing to the premise that increased climbing and swimming behaviour in the FST may be a result of enhanced noradrenergic and serotonergic neurotransmission, respectively (62), the decreased frontal-hippocampal NA levels in both stressed and unstressed FSL rats (Figure 4Ai and ii; Table 3) as well as the trend to raise NA levels as well as significantly elevate NA levels in the cortex and hippocampus respectively by IMI in TDS+FSL rats (Figure 4B) were expected and congruent with the current thinking on the role of NA in depression (5). However, it is apparent that TDS-exposure abrogated the climbing-enhancing effect of IMI in FSL animals (Figure 3B) as well as sustained lowered NA in the cortex and hippocampus of untreated animals (Figure 4Bi and ii). Indeed, TDS has been found to significantly increase NA after SPS, eventually falling to levels significantly lower than baseline one week after restress (42). The inability of IMI to increase climbing behaviour in stressed FSL rats (Figure 3B), despite its tendency to elevate NA in the cortex as well as significantly increase NA in the hippocampus, is of interest but may be a result of a decrease in adrenergic receptor density and/or affinity as previously reported in both humans (64, 69) and animals (70, 71) exposed to stress.

Given that limbic brain structures are involved in the stress response, changes in 5HT-related responses may be linked to changes in hippocampal and cortical 5HT neurotransmission (37, 39). A general decrease in 5HIAA levels were measured in the frontal cortex and hippocampus of treatment-naive FSL animals (Table 3). TDS worsened swimming deficits as well as duration of immobility (Figure 3A-C) and sustained reduced cortical and hippocampal 5HIAA levels (Figure 4A). While IMI significantly reduced immobility in unstressed and stressed FSL rats, immobility in the latter

group remained significantly higher than that of unstressed IMI-treated rats (Figures 3B and C) and *failed* to reverse lowered 5HIAA in FSL+TDS animals (Figure 4A).

Tricyclic antidepressants such as IMI act by increasing the extracellular levels of noradrenaline *and* serotonin (72). IMI significantly reversed deficits in swimming in FSL+TDS animals (Figure 3A) but failed to reverse lowered limbic 5HIAA levels in these animals (Figure 4A). On the other hand, IMI failed to reverse suppressed climbing in FSL+TDS animals (Figure 3B) despite provoking a tendency (in the FC) and to significantly (in the hippocampus) reverse lowered NA in these animals (Figure 4B). This paradox with respect to limbic monoamine levels and coping strategies may indicate other adaptive changes that underlie coping responses following sustained exposure to stress. Furthermore, it cannot be assumed that the effects of antidepressant drugs are simply to reverse and/or normalise dysfunctions in the brain (73), including those of animals. This has been exemplified by CMS-induced behavioural effects on mice, demonstrating that, while being reversed by fluoxetine, the drug failed to alter most of the underlying stress-induced biological effects (74).

In conclusion, exposing FSL rats to TDS resulted in either bolstered or sustained reduction in coping and increase in depressive-like behaviours, combined with altered monoaminergic profiles in hippocampal and frontocortical brain regions. Furthermore, the addition of TDS to FSL rats significantly abrogated the antidepressant-like effects of IMI at most behavioural levels (climbing and immobility) and with respect to limbic serotonin. Data presented here therefore supports the proposed hypothesis that exposure of a genetic animal model of depression to a PTSD-like paradigm results in a more severe depressive-like profile that is resistant to traditionally effective antidepressant treatment. The results of the current study have potential value in the search for a suitable animal model of TRD and warrants further investigation. Challenging FSL+TDS animals with first-line antidepressants (SSRIs, NSRI's) and/or ketamine would expand predictive validity, and is presented in a companion paper to this manuscript.

Author disclosures

Author contributions: SJ Brand performed all behavioural procedures, including treatment of the animals, performed all neurochemical analyses, undertook the statistical analysis, and prepared the first draft as well as the final version of the manuscript. BH Harvey devised the concept of the study as well as the layout of the manuscript, and finalized the pre-submission version of the manuscript.

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Figure 1

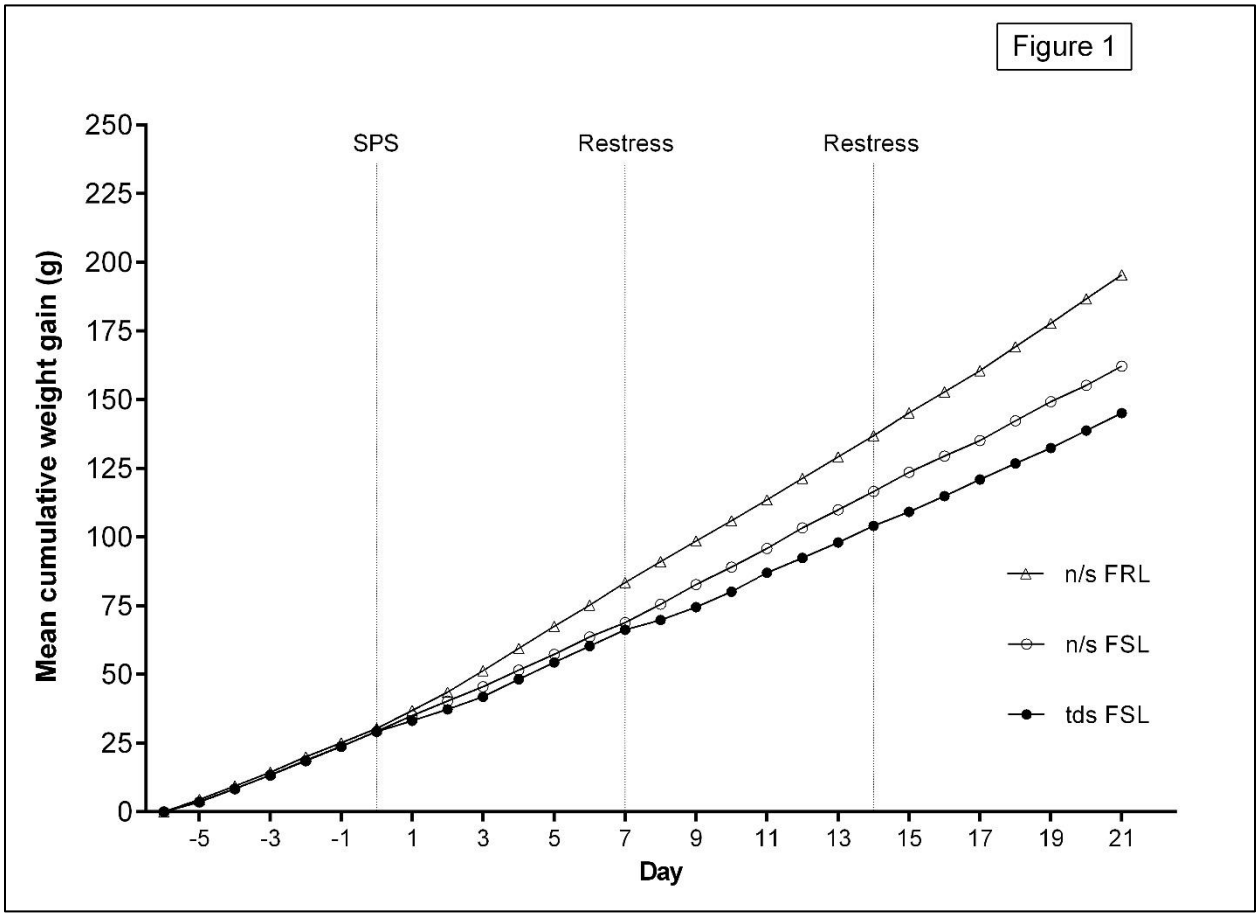
Figure 1
SPS
Restress
Restress
Mean cumulative weight gain (g)
250
225
200
175
150
125
100
75
50
25
0
-5
-3
-1
1
3
5
7
9
11
13
15
17
19
21
Day
n/s FRL
n/s FSL
tds FSL

Figure 2

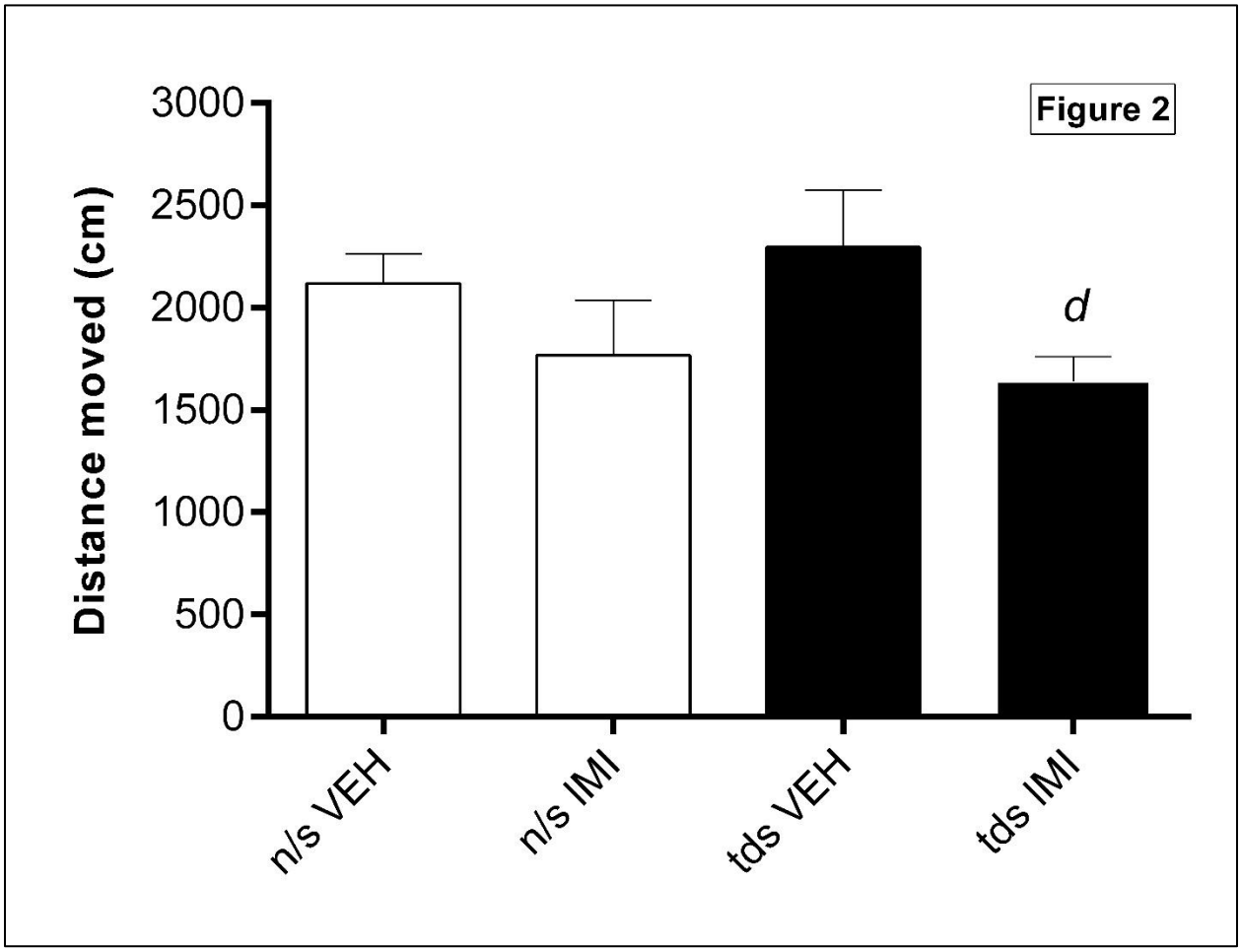
Figure 2
Distance moved (cm)
3000
2500
2000
1500
1000
500
0
d
n/s VEH
n/s IMI
tds VEH
tds IMI

Figure 3

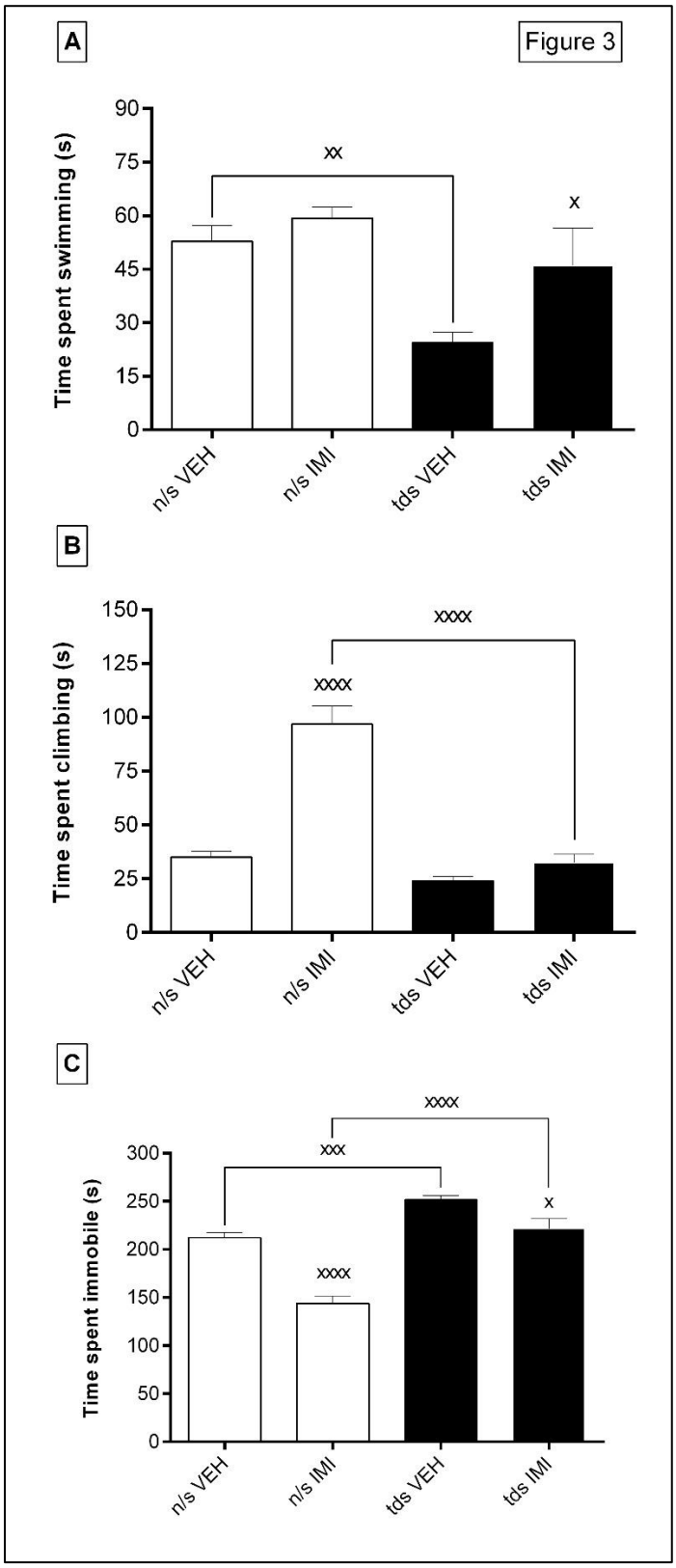
A
Figure 3
Time spent swimming (s)
90
75
60
45
30
15
0
xx
x
n/s VEH
n/s IMI
tds VEH
tds IMI
B
Time spent climbing (s)
150
125
100
75
50
25
0
xxxx
xxxx
n/s VEH
n/s IMI
tds VEH
tds IMI
C
Time spent immobile (s)
300
250
200
150
100
50
0
xxxx
xxx
x
xxxx
n/s VEH
n/s IMI
tds VEH
tds IMI

Figure 4

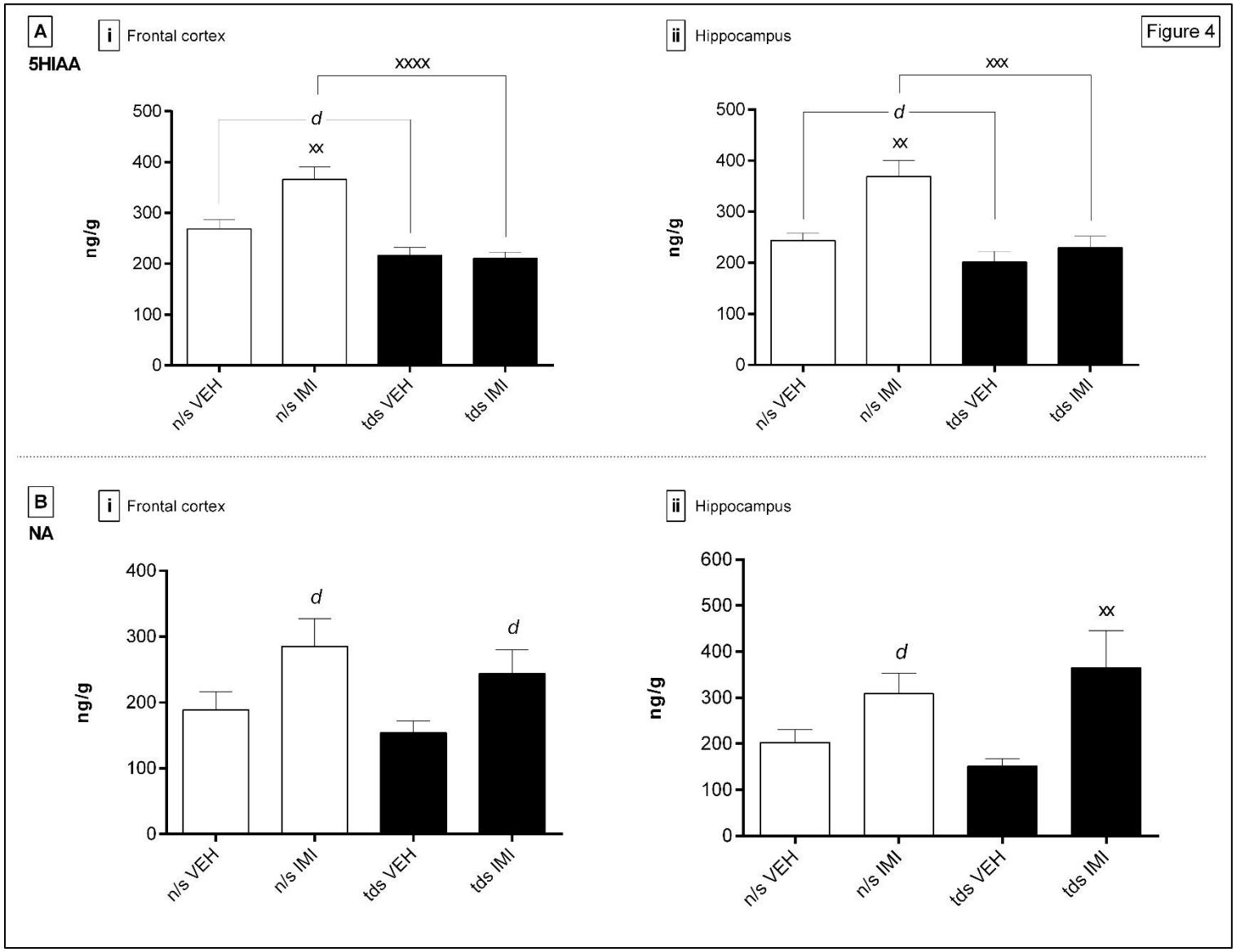
A
5HIAA
i Frontal cortex
ii Hippocampus
Figure 4
ng/g
500
400
300
200
100
0
d
xx
xxx
xxxx
n/s VEH
n/s IMI
tds VEH
tds IMI
B
NA
i Frontal cortex
ii Hippocampus
600

Figure 5

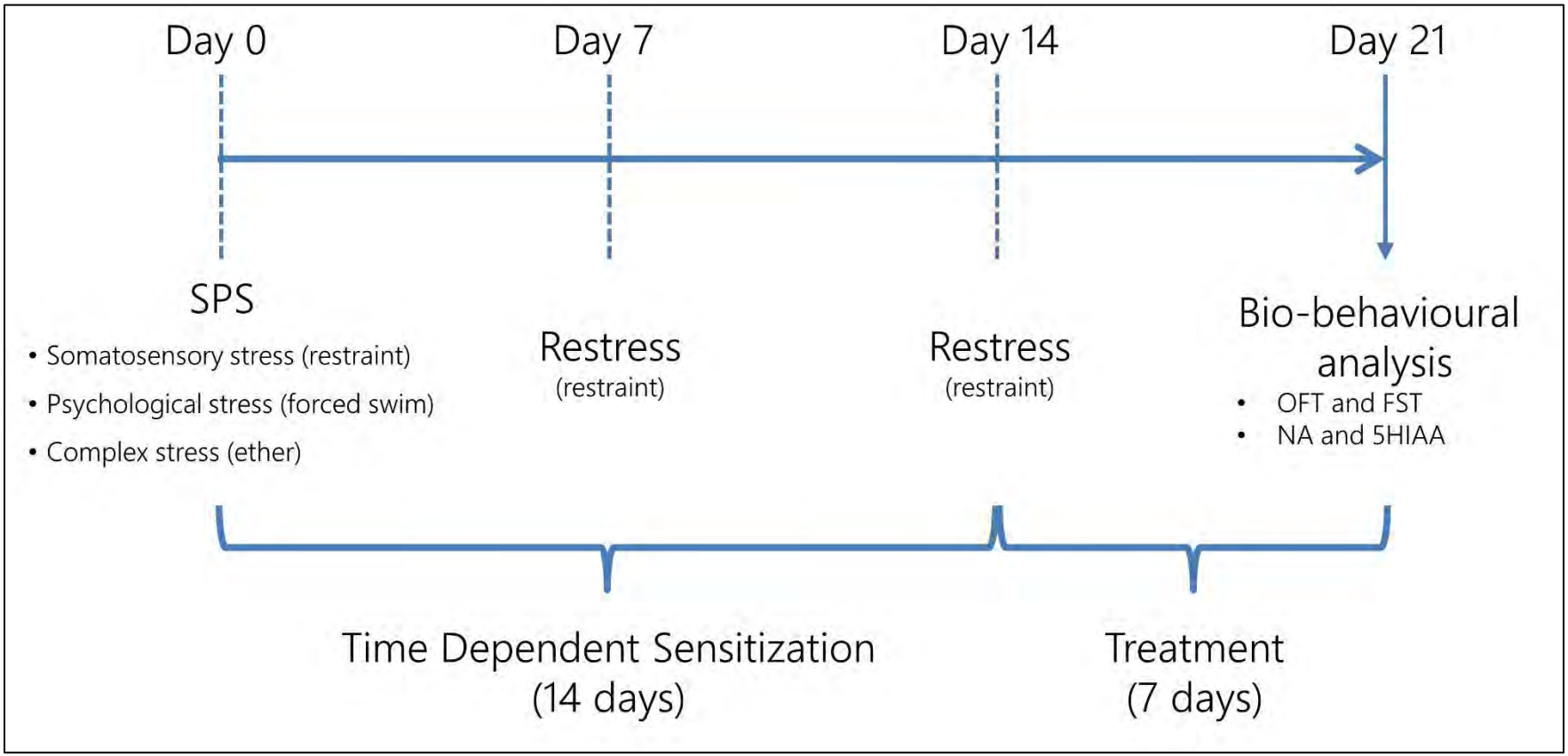
Day 0
Day 7
Day 14
Day 21
SPS
• Somatosensory stress (restraint)
• Psychological stress (forced swim)
• Complex stress (ether)
Restress
(restraint)
Restress
(restraint)
Bio-behavioural analysis
• OFT and FST
• NA and 5HIAA
Time Dependent Sensitization
(14 days)
Treatment
(7 days)

Table 1: Group Layout

Group Name *(text)*	Group Name *(figures)*	Group Description	Dosage	*n*	
				Behaviour	*Bio-Molecular*
FRL	*FRL n/s VEH*	Treatment naive unstressed FRL	1ml.kg^{-1}	12	8
FSL-TDS+VEH	*n/s VEH*	Treatment naive unstressed FSL	1ml.kg^{-1}	12	8
FSL-TDS+IMI	*n/s IMI*	Imipramine treated unstressed FSL	1mg.ml^{-1}.kg^{-1}	12	8
FSL+TDS+VEH	*tds VEH*	Treatment naive stressed FSL	1ml.kg^{-1}	12	8
FSL+TDS+IMI	*tds IMI*	Imipramine treated stressed FSL	1mg.ml^{-1}.kg^{-1}	12	8

Table 2: Cumulative weight gain over time

Mean cumulative weight gain of rats measured daily from one week prior to commencement of the TDS protocol until the final day of behavioural testing. Data is provided as mean of matched daily values in each group.

Day	Mean cumulative weight gained ± SD (g)			Significance of comparison (*p*-value)	
	FRL	*FSL-TDS*	*FSL+TDS*	*FRL vs FSL-TDS*	*FSL-TDS vs FSL+TDS*
0 (ASS)	30.4±2.5	29.3±3.8	29.3±1.7	n/s	n/s
2	43.6±2.7	40.3±3.5	37.3±2.9	n/s	n/s
3	51.4±3.9	45.6±3.2	41.9±3.2	0.0006	n/s
5	67.5±3.2	57.3±2.6	54.4±3.3	0.0001	n/s
7 (restress)	83.5±3.8	68.9±3.3	66.3±3.4	<0.0001	n/s
8	91.1±4.1	75.6±3.3	69.8±4.2	<0.0001	0.0007
14 (restress)	136.9±5.2	116.7±4.5	104.1±3.5	<0.0001	<0.0001
21 (final day)	195.5±6.8	162.3±6.2	145.2±6.4	<0.0001	<0.0001

Two-Way Repeated Measures ANOVA with Bonferroni post-hoc

Table 3: – Comparisons of data relating to open field and forced swim test behavior and frontal-cortical and hippocampal markers of monoamine function in unstressed FRL and FSL animals.

	FRL	*FSL*	*Significance*
Open Field Test			
Total distance traveled (cm)	2273±307.2	2119±505.4	-
Forced Swim Test			
Swimming (s)	70.4±14.8	52.9±15.2	** (p = 0.009)
Climbing (s)	117.9±38.0	35.0±9.2	^{xxxx} (p < 0.0001; U = 2)
Immobility (s)	111.7±33.7	212.1±18.8	**** (p < 0.0001)
Neurochemistry			
5HIAA (ng.mg^{-1})			
Frontal cortex	170.4±22.8	268.4±51.3	*** (p = 0.0007)
Hippocampus	177.2±37.2	244.1±40.3	^{x} (p = 0.021; U = 10)
Noradrenalin (ng.mg^{-1})			
Frontal cortex	412.1±27.7	188.7±77.5	****(p < 0.0001)
Hippocampus	451.9±95.3	202.9±78.4	**** (p < 0.0001)

**Unpaired student t-test; *Mann-Whitney U-test*

Figure Captions

Figure 1: Mean cumulative weight gain in FRL and TDS naïve and TDS exposed FSL animals

TDS, time-dependent sensitization; n/s, non-stressed; SPS, single prolonged stress; FRL, Flinders resistant line; FSL, Flinders sensitive line. Data are represented as the mean of 12 animals. Descriptive statistics are provided in Table 2.

Figure 2: Comparison between locomotor activity of unstressed and TDS exposed FSL rats before (white) and after (black) sub-chronic IMI treatment

TDS VEH vs. TDS IMI; d = 0.94

All data analysed by two-way analysis of variance (ANOVA) followed by Bonferroni post-hoc tests and Cohen's d analysis. Data are represented as mean ± SEM. TDS, time-dependent sensitization; n/s, non-stressed; VEH, vehicle; IMI, imipramine.

Figure 3: Comparisons between behavioral parameters measured in the forced swim test (panel A = time swimming, panel B = time climbing, panel C = immobility time) in unstressed and TDS exposed FSL rats before and after sub-chronic IMI treatment

Panel A – Time spent swimming (s). n/s VEH vs. TDS VEH, $^{xx}p < 0.01$; TDS VEH vs. TDS IMI, $^{x}p < 0.05$

Panel B – Time spent climbing (s). n/s VEH vs n/s IMI, $^{xxxx}p < 0.0001$; n/s IMI vs TDS IMI, $^{xxxx}p < 0.0001$

Panel C – Time spent immobile (s). n/s VEH vs. n/s IMI, $^{xxxx}p < 0.0001$; n/s IMI vs. TDS IMI, $^{xxxx}p < 0.0001$; n/s VEH vs TDS VEH, $^{xxx}p < 0.001$; TDS VEH vs. TDS IMI, $^{x}p < 0.05$

All data analysed by two-way analysis of variance (ANOVA) followed by Bonferroni post-hoc tests and Cohen's d analysis. Data are represented as mean ± SEM. TDS, time-dependent sensitization; n/s, non-stressed; VEH, vehicle; IMI, imipramine.

Figure 4: Comparisons between frontocortical and hippocampal 5HIAA (panel A) and NA (panel B) in unstressed and TDS exposed FSL rats before and after sub-chronic IMI treatment

Panel Ai – Frontal-cortical 5HIAA concentrations. n/s VEH vs n/s IMI, $^{xx}p < 0.01$; n/s IMI vs. TDS IMI, $^{xxxx}p < 0.0001$; n/s VEH vs. TDS VEH, $d = 1.07$

Panel Aii – Hippocampal 5HIAA concentrations. n/s VEH vs. n/s IMI, $^{xx}p < 0.01$; n/s IMI vs. TDS IMI, $^{xxx}p < 0.001$, n/s VEH vs. TDS VEH, $d = 0.87$

Panel Bi – Frontal cortical NA concentrations. n/s VEH vs n/s IMI, d = 0.98; TDS VEH vs. TDS IMI, d = 1.22

Panel Bii – Hippocampal NA concentrations. TDS VEH vs. TDS IMI, $^{xx}p < 0.01$; n/s VEH vs. n/s IMI, d = 1.03

All data analysed by two-way analysis of variance (ANOVA) followed by Bonferroni post-hoc tests and Cohen's d analysis. Data are represented as mean ± SEM. 5HIAA, 5-hydroxyindoleacetic acid; NA, noradrenalin; FC, frontal cortex; HC, hippocampus; TDS, time-dependent sensitization; n/s, non-stressed; VEH, vehicle; IMI, imipramine.

Figure 5: Schematic outline of the TDS procedure

At the start of the procedure (indicated as Day 0), rats are exposed to single prolonged stress (SPS) – a triple stressor sequence comprising a somatosensory stressor (restraint), a psychological stressor (forced swimming with brief submersion), and a complex stress-stimuli (exposure to ether vapours) followed by re-exposure to restraint stress, as a situational reminder of the initial SPS procedure, 7 and 14 days later. After the final restress, animals are left undisturbed for 7 days before performing behavioural assessments (OFT) and monoaminergic analyses (NA and 5HIAA)

5HIAA: 5-hydroxyindoleacetic acid; FST: forced swim test; NA: noradrenalin; OFT: open field test; SPS: single prolonged stress; TDS: time-dependent sensitization

CHAPTER 5

5 Manuscript C

Article submitted for publication in *Acta Neuropsychiatrica* titled:

"Exploring a post-traumatic stress disorder paradigm in Flinders sensitive line rats to model treatment resistant depression II: Response to antidepressant augmentation strategies"

Author Contributions

- *Sarel J Brand* designed the investigation in consultation with *Brian H Harvey*, performed all behavioural, pharmacological, and statistical analyses, wrote the first version of the manuscript, and edited the manuscript following input from the co-authors.
- *Brian H Harvey* was study supervisor, devised the concept of the study, assisted in the interpretation of results, served as corresponding author for submission of the final manuscript to *Acta Neuropsychiatrica* and finalized the pre-submission version of the manuscript.

Important Information

- Instructions to the author are included in Addendum A.
- As per the instructions to the author, figures, tables, and legends are provided at the end of the manuscript.
- The co-author provided consent for the paper to be assessed as part of the Ph.D thesis of *Sarel J Brand* (Addendum B).
- The published article is provided in PDF format in Addendum E.

Exploring a post-traumatic stress disorder paradigm in Flinders sensitive line rats to model treatment resistant depression II: Response to antidepressant augmentation strategies

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Keywords: Serotonin reuptake inhibitor; noradrenaline reuptake inhibitor; animal model; stress re-stress; NMDA antagonist; antidepressant resistance

Abstract

Objective: Posttraumatic stress disorder (PTSD) shows high comorbidity with major depression and treatment resistance. We investigated the predictive validity of a gene-x-environment model of treatment resistant depression (TRD) by testing behavioural and monoaminergic responses to venlafaxine and ketamine in FSL rats. This model has displayed exaggerated depressive-like symptoms and an abrogated response to imipramine.

Methods: Male FSL rats, a genetic model of depression, were exposed to a time dependent sensitisation (TDS) model of PTSD and compared to stress-naive controls. Seven days after re-stress, immobility and coping (swimming and climbing) behaviours in the forced swim test (FST) as well as hippocampal and cortical 5HIAA and NA levels were analysed. Response to imipramine, venlafaxine and ketamine treatment (all 10mg.kg^{-1}x 7 days) alone and in combination was subsequently studied.

Results: TDS exacerbated depressive-like behaviour of FSL rats in the FST. Imipramine, venlafaxine and ketamine were ineffective as monotherapy in TDS-exposed FSL rats. However, combining imipramine with either venlafaxine or ketamine resulted in significant anti-immobility effects and enhanced coping behaviours. Only ketamine + imipramine (frontal-cortical 5HIAA and NA), ketamine alone (frontal-cortical and hippocampal NA) and venlafaxine + imipramine (frontal-cortical NA) altered monoamine responses vs. untreated TDS-exposed FSL rats.

Conclusion: Exposure of FSL rats to TDS inhibits antidepressant response at behavioural and neurochemical levels. Congruent with TRD, imipramine + venlafaxine or ketamine overcame treatment resistance in these animals. These data further support the hypothesis that exposure of FSL rats to a PTSD-like paradigm produces a valid animal model of TRD and warrants further investigation.

Abbreviations: 5-hydroxytryptamine (5HT); chronic mild stress (CMS); chronic unpredictable mild stress (CUMS); Flinders sensitive line (FSL); forced swim test (FST); major depression (MD); N-methyl-D-aspartate (NMDA); noradrenaline (NA); open field test (OFT); post-traumatic stress disorder (PTSD); Sequenced Treatment Alternatives to Relieve Depression (STAR*D); single prolonged stress (SPS); Time dependent sensitization (TDS); treatment resistant depression (TRD).

Significant outcomes

- Sub-chronic treatment with imipramine, venlafaxine and ketamine as monotherapy *failed* to evoke antidepressant-like effects in the FST in FSL rats exposed to TDS-stress, suggesting treatment resistance to multiple classes of antidepressants.
- Combining imipramine with either venlafaxine or ketamine produced a significant reversal of treatment resistance in all behavioural parameters in the FST.
- Only ketamine + imipramine (frontal-cortical 5HIAA and NA), ketamine alone (frontal-cortical and hippocampal NA) and venlafaxine + imipramine (frontal- cortical NA) increased NA and 5HIAA responses vs. untreated TDS-exposed FSL rats, supporting evidence of a more robust response following combination treatment.

Limitations

- Behavioural assessment of anhedonia (sucrose preference test), which has been demonstrated to be an important symptom of TRD, would be a valuable addition.
- This study is limited to observations made after sub-chronic antidepressant (7 days) treatment. Extending treatment duration (inadequate treatment duration is often a reason for antidepressant non-response) and increasing dosages may provide additional support for current findings.
- Applying additional biochemical measures, e.g. monoamine responses via *in vivo* microdialysis and/or determination of putative molecular biomarkers of TRD such as $5HT_{1A}$-receptor expression, would bolster construct validity.

1. Introduction

Major depression (MD) is a commonly occurring disorder with a lifetime prevalence rate of approximately 16% (1). Despite several classes of antidepressants being available to clinicians (2), pharmacological management remains suboptimal. High rates of recurrence are a constant challenge, with symptom severity serving as the greatest predictor of a poor outcome (3) and more than 50% of patients still experiencing persistent symptoms of MD after treatment with a first choice antidepressant drug (4). In fact, the Sequenced Treatment Alternatives to Relieve Depression (STAR*D) study found that up to 30% of patients did not achieve remission despite being subjected to multiple antidepressant drug treatments (5). STAR*D was designed to replicate clinical settings, and highlighted the low remission rates associated with treatment resistant depression (TRD) (6).

Relative to MD, TRD is associated with more severe symptomatology (7) as well as increased morbidity and mortality (8). In addition, an increased presence of somatic symptoms, e.g. pain and fatigue (9), may predict increased treatment resistance (8). The impact of TRD on healthcare expenditure is proportional to the degree of resistance (10), requiring more frequent hospitalisation (11), increased use of pharmacotherapy (11) together with an increased disease burden (8). Despite important strides in our understanding of the neurobiology and treatment of MD, as well as increased use of antidepressants (12-14), TRD remains an undeniable concern. Nevertheless, various strategies have been employed to alleviate the non- or partial response to antidepressant treatment (15).

Current approaches to treating TRD includes both pharmacological and non-pharmacological (e.g. electroconvulsive therapy, psychotherapy and deep brain stimulation) approaches. Drug-centred approaches are based on switching between antidepressants either in the same or across drug classes or employing augmentative drug therapies (adding a drug from a different class or with a different mechanism of action) (2, 16). However, it would seem that switching within or between drug classes offers limited therapeutic benefit (17). Interestingly the latter study suggests that adjunctive treatment may accelerate symptom improvement and improve remission rates, although the authors hasten to note that the success of such a strategy requires the initial drug treatment to have at least some degree of efficacy (18) and that the adjunct treatment enhances these

improvements (17). Potential augmentation agents include selective serotonin reuptake inhibitors (SSRIs) and selective noradrenaline reuptake inhibitors (SNRIs) (e.g. venlafaxine), atypical antipsychotics and glutamatergic drugs (e.g. ketamine) (19). Venlafaxine has been found to be slightly more effective than several SSRIs in patients with severe MD (20, 21) and acts by increasing both serotonergic and noradrenergic activity (21). Ketamine, on the other hand, acts as an NMDA receptor antagonist (22) and is associated with a proven rapid onset of action (23) and high response rate (22).

Psychiatric co-morbidity is a common problem in patients with MD (24, 25), with co-occurrence of anxiety disorders ranging between 50 and 60% (5, 26). Importantly, such co-occurrence is increasingly being associated with antidepressant treatment failure (24, 27, 28). Posttraumatic stress disorder (PTSD) is one of the more commonly co-occurring anxiety disorders in MD and is especially prevalent in TRD (5). Furthermore, half of patients with PTSD have comorbid MD (29) with the high comorbidity attributed to an overlap in symptoms, e.g. anhedonia, sleep difficulty, irritability and poor concentration (DSM-IV criteria) (30, 31), while both MD and PTSD are precipitated by a chronic or severe traumatic event, respectively (30). Such co-occurrence is also positively associated with symptom severity (31) and treatment resistance (32, 33).

The complexity and heterogeneity of MD makes it unlikely that any one animal model will fully embody the behavioural and biological characteristics of the disorder. However, modifying existing models to represent specific phenotypes of the disorder may hold promise. The gene-x-environment hypothesis of MD has enabled the conceptualising of genetic susceptibility combined with environmental adversity as prodromal events to the subsequent development of MD (34-36). Moreover, Willner and Belzung (37) emphasize that the search for treatments for TRD may require models that incorporate predisposing factors leading to heightened stress responsiveness. The co-morbidity of PTSD and MD and its association with treatment resistance is thus noteworthy. Consequently, we have recently developed an animal model of TRD by superimposing a PTSD-related paradigm (time-dependent sensitisation; TDS) on the Flinders sensitive line (FSL) rat (38). FSL rats are a well-studied genetic animal model of MD (39) while TDS is based on a stress re-stress procedure (40) with proven predictive, construct and face validity for PTSD (41-44). In a companion paper (38) we describe how exposing FSL rats to TDS evokes more pronounced depressive-like

behaviour together with altered limbic monoamine levels vs. unstressed FSL rats, as well as engendering resistance to sub-chronic imipramine treatment. To extend the predictive validity of the model, we investigated sub-chronic imipramine treatment in TDS-exposed FSL rats compared to that of venlafaxine and ketamine monotherapy as well as versus imipramine plus venlafaxine or ketamine to simulate a TRD regime. Post-treatment cortico-limbic monoamines were analysed after behavioural analysis.

2. Materials and Methods

2.1 Subjects

Animals were bred, supplied, and housed at the Vivarium (SAVC reg. number FR15/13458; SANAS GLP compliance number G0019) of the Pre-Clinical Drug Development Platform of the North-West University. Ambient temperature was maintained at 22 ± 2°C with a relative humidity of 40 – 60% and full spectrum of light in a 12-hour light/dark cycle, with lights switched on at 06:00 AM and off at 06:00 PM. Food and water were provided ad libitum. All experiments were approved by the AnimCare animal research ethics committee (NHREC reg. number AREC-130913-015) of the North-West University. All 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-00111-12-A5).

Subjects were male adult FSL rats (n = 84 for behavioural assessment and n = 56 for monoamine analysis). Table 1 describes the layout of the experimental groups. Animals in all experimental groups were either subjected to the PTSD paradigm, *viz*. TDS, or left undisturbed (unstressed) in their home cages, after which behaviour of all animals was analysed in the open field test (OFT) and FST, with subsequent monoamine analyses performed in animals naive to behavioural assessment. Animals were housed four males per cage, with the TDS paradigm initiated at an age of 40 (± 1) days in order to conclude the experiments while the rats were still of an appropriate weight for the behavioural assessments. Handling of the animals was initiated one week before starting the experimental procedure by taking bodyweight measurements daily until the last day of the study to monitor weight gain and calculate drug dosages.

2.2 Time dependent sensitisation (TDS)

TDS is an animal model of PTSD. Animals exposed to a severely traumatic situation followed by subsequent, but less stressful, contextual reminders exhibit significant physiological and behavioural alterations that show a time-dependent sustaining or worsening in the absence of the initiating stressor (45, 46). The TDS paradigm used in this study incorporated an acute single prolonged stress (SPS) sequence comprising a somatosensory stressor (restraint), a psychological stressor (forced swimming with brief submersion), and a complex stress-stimuli (exposure to ether vapours) followed by re-exposure to restraint stress 7 and 14 days later (45).

2.2.1. Restraint stress: Rats were placed in Perspex® restrainers for 2 hours with the tail-gates adjusted to keep each animal well contained without impairing circulation to the limbs. The same procedure was followed on days 7 and 14 during the re-stress phase of the TDS protocol.

2.2.2. Forced swim stress: Rats were placed individually in cylindrical Perspex® swim tanks containing 40 cm of ambient water (25 °C) and allowed to swim for 15 min while being forcefully submerged for the last 20 seconds. Thereafter animals were removed from the cylinders, dried and returned to their home cages for 15 minutes to recover. Forced swimming as part of TDS was performed 21 days *before* behavioural testing in the FST so that any possible conditioned response to swim stress in the FST would be unlikely.

2.2.3. Exposure to ether vapours: Fifteen minutes after swim stress, rats were exposed to 5 ml of 100% ether vapours in a 5 L sealed plastic container until loss of consciousness (±2 min). Ether was poured onto a paper towel at the bottom of the container with the animal placed on a raised metal platform to avoid direct contact with the substance. After loss of consciousness, the animals were immediately removed from the plastic container, returned to their home cage for observation until regaining full consciousness and then returned to their holding room. Animals were left undisturbed, only subjecting them to routine handling until re-exposure to restraint-stress during the re-stress phase of the TDS protocol.

2.3 Open Field Test (OFT)

This test is generally performed prior to the FST to control for locomotor activity possibly contributing to altered swimming performance in the FST and thereby confounding interpretation of the results. The OFT was performed half an hour before subjecting animals to the FST. Rats were individually placed in a square arena (100 x 100 x 50 cm) facing the centre and their behaviour recorded for 5 minutes using a ceiling-mounted digital camera. The video files were subsequently analysed using EthoVision® XT software (Noldus® Information Technology, Wageningen, The Netherlands) with total distance moved applied as a measure of locomotor activity.

2.4 Forced swim test (FST)

The FST can reliably predict antidepressant-like effects after drug treatment and is considered a model of behavioural despair that is typically manifest in human MD, and expressed in rodents as a decrease in escape-driven behaviour, i.e. increased immobility (47). During behavioural analysis, rats were placed individually in cylindrical Perspex® swim tanks containing 30 cm of ambient water (25 °C) for 7 minutes and their behaviour recorded. The first and last minute of the video files were discarded and the remaining five minutes of swimming behaviour scored for characteristic escape-directed behaviours, including swimming, climbing (struggling), and immobility. The former two swimming parameters of the FST provide useful information relating to serotonergic (swimming) and noradrenergic (climbing) directed behaviours that may inform on the mode of antidepressant action, allowing possible correlation with whole-brain monoamine levels (48).

2.5 Drug administration

After weighing all animals daily (between 09:00 AM and 11:00 AM), imipramine (Sigma-Aldrich) (49, 50), venlafaxine (51) (Adcock Ingram, South Africa) and ketamine (52) (Fresenius-Kabi, South Africa) was dissolved in physiological saline (0.9 % NaCl) and administered subcutaneously at a dose of 10 mg/kg to FSL animals exposed to TDS (see Table 1). Treatment started on day 15 (after completing the TDS protocol on day 14) and persisted for 7 days before behavioural testing commenced on the evening of day 21. This duration of treatment is regarded adequate to establish an antidepressant response in rats (53-55). Stressed and unstressed control animals were injected with saline vehicle in the same manner as drug-treated animals.

2.6 Quantitative analysis of brain 5HIAA and NA

Several valid indices of 5HT central activity may be applied, including 5HT and 5HIAA levels and the 5HIAA/5HT ratio (56). In this regard, *in vivo* microdialysis has proven to be a reliable method to directly measure extracellular levels of 5HT. However whole- and regional brain monoamine analysis provides total levels of 5HT – both extracellular and unreleased from nerve terminals (57). 5HT is metabolized primarily to 5HIAA and has been demonstrated to reflect reliable insights into time-dependent alterations in serotonin response (58). Moreover, 5-HIAA levels have previously been correlated with 5HT function (56) and was therefore applied as an indicator of 5HT-ergic function in the current study. Quantification of NA and 5HIAA in the hippocampus and frontal cortex of animals was performed using a high performance liquid chromatography (HPLC) system with electrochemical detection (HPLC-EC), as previously described (45). An Agilent 1200 series HPLC, equipped with an isocratic pump, auto sampler and coupled to an ESA Coulochem Electrochemical detector with Chromeleon® Chromatography Management System software (version 6.8), was used. NA and 5HIAA concentrations in the tissue samples were determined by comparing the area under the peak of each marker to that of the internal standard, isoprenaline (range 5 – 50 ng/ml). Linear standard curves (regression coefficient greater than 0.99) were found in this particular range. 5HIAA and NA concentrations were expressed as ng/g wet weight of tissue (mean ± S.E.M.).

2.7 Statistical analysis

Statistical analyses were performed using Graphpad Prism® 6 and IBM® SPSS® 22 software under the guidance of the Statistical Consultation Service of the North-West University. In pairwise comparisons of the behaviour (n = 12 per group) and neurochemistry (n = 8 per group) between treatment naïve unstressed and stressed FSL animals, unpaired student's t-tests with Welch's correction (normally distributed data as indicated by Shapiro-Wilk test for normality $p > 0.05$) or Mann-Whitney *U*-tests (data not distributed normally) were performed. One-way analysis of variance followed by Tukey's post-hoc analysis (normally distributed data) or Kruskal-Wallis analysis of variance followed by Dunn's multiple comparisons was applied to comparisons of the behaviour (n = 12 per group) and neurochemistry (n = 8 per group) in treatment-naïve and treated stressed FSL animals. Treatment was set as within-subject factor, while the respective behavioural and

neurochemical parameters were set as between-subject factors. Significance was set at $p < 0.05$ for all comparisons. Where Cohen's d effect sizes were calculated, large effect sizes are indicated by $d > 0.8$ and very large effect sizes by $d > 1.3$.

3. Results

3.1 Behaviour

In order to confirm the translational relevance of the FSL rat for MD, data and statistics relating to the behavioural comparisons made between stress and treatment-naive FRL and FSL animals have been presented in the companion manuscript (38) but are reproduced here for the sake of completion (see Table 2). For the remainder of this study all data described were undertaken in FSL animals with/without concomitant exposure to TDS stress.

Comparison of treatment naive unstressed and TDS-exposed FSL animals is reported in Table 3, and described separately under the relevant sections below.

3.1.1. Locomotor activity (Figure 1)

Locomotor data from the pairwise comparison between the behaviour of treatment naïve unstressed and stressed FSL animals demonstrated no significant differences in overall activity (Table 3).

Considering the various drug treatments on TDS exposed FSL rats (Fig 1), one-way ANOVA revealed a significant effect of treatment on the mean locomotor activity scores ($F(5, 64) = 2.65$, $p = 0.03$). However, post-hoc Tukey's analysis failed to demonstrate statistically significant differences between the means of any of the respective treatments.

3.1.2. FST

3.1.2.1. Swimming (Figure 2A)

Data from the pairwise comparison between the swimming behaviour of treatment naïve unstressed and stressed FSL animals are provided in Table 3. Here we demonstrate that exposure to TDS significantly reduced the time spent swimming ($p < 0.0001$, $U = 6.0$).

Considering the various drug treatments on TDS exposed FSL rats (Fig 2A), Kruskal-Wallis analysis of variance revealed significant differences between the median swimming scores of animals in the respective treatment groups ($H(5) = 17.67$, $p = 0.003$). As such, pairwise comparisons were performed using Dunn's procedure with a Bonferroni correction for multiple comparisons and adjusted p values are presented (Fig 2A). Although a trend with a large effect size towards increased swimming behaviour was noted in animals treated with both imipramine ($d = 0.93$) and venlafaxine ($d = 1.07$) alone compared to vehicle treated animals, this increase was significant in the combined venlafaxine + imipramine ($p = 0.005$) and ketamine + imipramine ($p = 0.04$) groups, respectively. Moreover, venlafaxine and ketamine administered as monotherapy had no effect on swimming behaviour.

3.1.2.2. Climbing (Figure 2B)

Data from the pairwise comparison between the climbing behaviour of treatment naïve unstressed and stressed FSL animals (Table 3) revealed a significant decrease in the climbing behaviour of stressed FSL animals compared to the unstressed controls ($p < 0.001$, $U = 24.5$).

One-way ANOVA revealed significant differences between the climbing behaviour of TDS-exposed rats in the various treatment groups (Fig 2B, $F(5, 66) = 6.7$, $p < 0.0001$). Subsequently, Tukey post-hoc analysis revealed significant differences in climbing behaviour between treatment naïve control FSL animals and those treated with venlafaxine + imipramine ($p = 0.01$) and ketamine + imipramine ($p = 0.002$), respectively (Fig 2B). Furthermore, although a trend towards increased climbing behaviour was demonstrated in animals treated with imipramine alone compared to the vehicle treated controls ($d = 0.8$), no such trends were demonstrated in groups treated with either venlafaxine or ketamine as monotherapies. Rather, combining both venlafaxine and ketamine with imipramine resulted in bolstered effects on climbing behaviour compared to either venlafaxine ($p = 0.006$) and ketamine ($p = 0.007$) administered alone, indicating an augmenting effect (Figure 2B).

3.1.2.3. Immobility (Figure 2C)

FSL rats exposed to TDS demonstrated a significant increase in the time spent immobile compared to unstressed FSL controls (Table 3; $p < 0.0001$)

Kruskal-Wallis analysis of variance revealed significant differences between the median immobility scores of animals in the respective treatment groups ($H(5) = 33.61$, $p < 0.0001$). Subsequently, pairwise comparisons were performed using Dunn's procedure with a Bonferroni correction for multiple comparisons of which the adjusted p values are presented (Fig 2C). Although a trend with a large effect size ($d = 1.21$) towards a decrease in the time spent immobile was noted in animals treated with imipramine alone compared to vehicle treated animals, this decrease was strengthened by the concomitant administration of imipramine with either venlafaxine ($p < 0.0001$) or ketamine ($p = 0.0007$), respectively. Again, while neither venlafaxine or ketamine had significant effects on immobility scores when administered as monotherapy, combining both with imipramine resulted in bolstered effects on climbing behaviour compared to either venlafaxine ($p = 0.01$) and ketamine ($p = 0.03$) administered alone, indicating an augmenting effect.

3.2 5HIAA and NA analysis

3.2.1. 5HIAA (Figure 3A)

Data from the pairwise comparisons of frontal-cortical and hippocampal 5HIAA concentrations between the treatment naïve unstressed and stressed FSL animals are provided in Table 3. No significant differences were observed between the either the frontal-cortical or hippocampal 5HIAA concentrations measured. However, 5HIAA levels measured in TDS-exposed animals strongly trended toward a decrease in both the frontal cortex (d = 1.07) and the hippocampus (d = 0.84).

With respect to 5HIAA measurements in FSL animals, one-way ANOVA revealed significant differences between the mean frontal-cortical 5HIAA concentrations measured in animals of the different treatment groups (3Ai: $F(5, 41) = 4.97$, $p = 0.001$). As such, Tukey post-hoc analysis revealed a significant increase in frontal-cortical 5HIAA levels in ketamine + imipramine treated animals vs. vehicle-treated animals ($p = 0.006$), with none of the other treatments effective compared to the control group. In addition, frontal-cortical 5HIAA levels in rats treated with ketamine + imipramine

were significantly higher than that of animals treated with imipramine alone (p = 0.004) and compared to the combination of imipramine and venlafaxine (p = 0.003).

Kruskal-Wallis analysis was applied in comparisons between the hippocampal 5HIAA concentrations measured in the different treatment groups (Fig 3Aii). However, no significant differences could be displayed between the median 5HIAA concentrations of any of the groups compared (H(5) = 4.19, p = 0.52).

3.2.2. Noradrenaline (Figure 3B)

Data comparing the frontal-cortical and hippocampal NA concentrations of treatment naïve unstressed and stressed FSL animals (Table 3) failed to reveal significant differences in both the frontal cortex and hippocampus. However, NA levels measured in TDS-exposed animals trended toward a decrease in the hippocampus (d = 0.90).

Considering the various drug treatments on TDS exposed FSL rats (Fig 3B), one-way ANOVA revealed significant differences between the frontal-cortical NA concentrations in the different treatment groups (3Bi: F(5, 41) = 7.6, p < 0.0001). Tukey post-hoc analysis showed that venlafaxine + imipramine (p = 0.004), ketamine alone (p < 0.0001) and ketamine + imipramine (p = 0.0004) induced significantly elevated NA levels compared to vehicle-treated controls (Fig 3Bi).

Considering hippocampal NA measurements, Kruskal-Wallis analysis revealed significant differences in the median levels measured in animals across the different treatment groups (3Bii: H(5) = 15.3, p = 0.009). Pairwise comparisons were performed using Dunn's procedure with a Bonferroni correction for multiple comparisons of which the adjusted p values are presented (Fig 3Bii). Although trends toward increased NA was measured in imipramine (d = 0.83), venlaxine + imipramine (d = 0.92) and ketamine + imipramine (d = 1.2) treated animals compared to the vehicle treated controls, only ketamine monotherapy resulted in significantly elevated NA levels vs. vehicle treated controls (p = 0.01). A large effect size was also measured in venlafaxine + imipramine vs venlafaxine alone treated animals (d = 0.82).

4. Discussion

Several noteworthy observations have been made in this study. Exposing FSL rats to TDS exacerbates depressive-like behaviour which is depicted by reduced active coping behaviour (swimming and climbing) and increased immobility in the FST (Table 3). Where we noted a reversal of depressive-like behaviour in stress naive FSL rats with sub-chronic imipramine treatment in the companion paper (38), here we found that sub-chronic treatment with imipramine, venlafaxine and ketamine as monotherapy *failed* to evoke a similar response in TDS-exposed FSL rats, indicating treatment resistance to multiple classes of antidepressant (Fig 2A-C). However, combining imipramine with either venlafaxine or with ketamine produced a significant reversal of treatment resistance in all behavioural parameters (Fig 2A-C). Considering monoaminergic responses, TDS-exposed FSL rats displayed a trend towards lowered 5HIAA levels in both the hippocampus and frontal cortex and lowered NA in the hippocampus (Table 3). Where we had previously noted a reversal of limbic 5HIAA and NA changes in stress naive FSL rats with sub-chronic imipramine treatment (38), only ketamine + imipramine (frontal-cortical 5HIAA and NA), ketamine alone (frontal-cortical and hippocampal NA) and venlafaxine + imipramine (frontal- cortical NA) increased monoamine responses vs. untreated TDS-exposed FSL rats (Fig. 3Ai and Bi), indicating a more robust response following these combination treatments. Additionally, both venlafaxine + imipramine (d = 0.87) and ketamine + imipramine (d = 1.12) tended to increase NA compared to vehicle-treated animals.

In the clinical setting, acute dosing with ketamine has been proven to induce rapid and robust antidepressant effects in TRD (59, 60). More recently, however, several studies have also applied repeated dosing strategies in TRD patients which achieved superior outcomes compared to single administration approaches (61-63). Likewise, in preclinical studies, chronic ketamine treatment has also been applied in rats using the FST compared to known antidepressants (64) and also in animals exposed to CMS (52, 65, 66) where repeated ketamine treatment was associated with long-term anxiolytic- and antidepressant-like effects (66). Taken together, these results suggest that combining ketamine with classic antidepressants would improve antidepressant onset time with lasting and predictable effects (52). Similarly, preclinical (67, 68) and clinical (20, 21, 69) data have demonstrated venlafaxine to be equally if not more effective than SSRIs making it a popular treatment choice in patients resistant to SSRI treatment (69).

Compared to stress-naive FSL rats, TDS-exposed animals presented with severely exaggerated depressive-like behaviour in the FST, characterized by significant increases in immobility and decreased coping behaviour (swimming and climbing; Table 3). TDS on its own did not adversely affect locomotor activity. In the companion paper (38) we noted that sub-chronic imipramine treatment was an effective antidepressant in these animals. However, together with an enhanced depressive-like phenotype in TDS-exposed FSL rats we also observed a very modest (see Cohens d-effect sizes) albeit insignificant behavioural response to imipramine in the FST (Figure 2A-C). Interestingly, the response to monotherapy with either venlafaxine or ketamine *also* proved unsuccessful. Neither of the drug treatments had a significant impact on locomotor activity, although it tended to be lower in imipramine-treated animals. Thus any observed treatment effects in the FST can be assumed to be unrelated to an indirect effect on locomotor activity. Based on these findings, and that clinically comorbid MD and PTSD often present with TRD (32, 33), the presence of a PTSD-like paradigm in genetically predisposed animals significantly attenuates antidepressant-like response to imipramine but also to venlafaxine and ketamine. The latter two findings with ketamine and venlafaxine are especially interesting since both agents are generally considered effective antidepressants when applied as monotherapy and also offer improved efficacy in treatment resistance (61, 70). Although dose may be a reason for this observation, venlafaxine has demonstrated effectiveness in the FST after 10 days of treatment (51). On the other hand, it should be mentioned that sub-chronic venlafaxine treatment may be associated with non-response in the FST while still inducing monoaminergic alterations (71). Previous studies with ketamine applied doses of up to 20mg/kg twice daily for two weeks (66) while 10mg/kg for seven days (as applied here) have also proven to be sufficient to induce antidepressant-like effects (52). Interestingly, the latter study (52) was performed in rats exposed to a chronic unpredictable mild stress (CUMS) protocol – a model which has been described as presenting with the attributes of TRD (72). Therefore the doses of venlafaxine and ketamine used in the current work can be regarded as effective, with ketamine demonstrating efficacy under at least some TRD-related conditions (i.e. CUMS).

Exposing FSL rats to TDS stress may therefore represent a more profound state of treatment resistance that warrants a more robust treatment regimen. To test this supposition, we investigated the response to combined imipramine plus ketamine or venlafaxine, considering not only superiority

vs. imipramine alone but also vs. ketamine and venlafaxine monotherapy. This is also a typical approach taken for a failed monotherapy treatment response in human patients. Where all drugs administered as monotherapy failed to induce adequate anti-immobility effects in the FST, we found that venlafaxine + imipramine and ketamine + imipramine achieved successful attenuation of depressive-like manifestations in TDS-exposed FSL rats without notable effects on locomotor activity. This conclusion is supported by a significantly reduced immobility time (Figure 2C) as well as bolstered coping behaviour exhibited by significant increases in swimming and climbing behaviour (Figure 2 A,B) following combination treatments.

The mechanism whereby the combined use of a TCA and a SNRI or an NMDA receptor antagonist may engender a bolstered response in the current model of TRD is of particular interest. Despite a plethora of up-stream signalling pathways purported to be involved in the neurobiology of MD (see (73) for review), it is ultimately a resultant effect on NA and 5HT that may hold sway in the behavioural presentation of the illness and how antidepressants produce their desired effect. Considering 5HT, FSL rats present with deficits in serotonergic transmission (39), while TDS in its own right adversely affects this monoamine and its behavioural sequelae (41, 43), implying that TDS-exposed FSL rats may have a profoundly compromised serotonergic system. Indeed, 5HIAA was reduced in the frontal cortex (d = 1.07) and hippocampus (d = 0.84) of TDS-exposed FSL rats, although narrowly missed significance (Table 3). It is interesting that clinical studies have demonstrated that relapse of MD induced by a tryptophan depleting diet occurs primarily in remitted patients taking an SSRI and not another pharmacological or behavioural treatment (74, 75), indicating that loss of serotonergic function during treatment with serotonergic drugs mediate the relapse. Since both venlafaxine and imipramine act to increase extracellular levels of 5HT (and NA) (76), a synergistic action on 5HT may underlie the improved response observed in combination treatment. Drug-centred approaches for treating TRD also emphasizes adding a drug with a different mechanism of action) (2, 16). Thus, despite similar actions on NA and 5HT neuronal reuptake, imipramine has a high affinity for other neuronal receptors, such as the $5HT_{1A}$ receptors (77) versus the "cleaner" profile of venlafaxine (78), which may explain the increased swimming behaviour observed in imipramine alone and venlafaxine + imipramine combinations versus venlafaxine alone. Also worth considering is that venlafaxine only inhibits NA reuptake at higher therapeutic doses compared to its SSRI effects across the dose range

(76). This may explain the absence of climbing-enhancing effects in venlafaxine alone compared to imipramine + venlafaxine, which would have provided synergistic SNRI effects.

Regarding ketamine, mechanisms involving mammalian target of rapamycin (79) and glycogen synthase kinase-3 (80) may underlie its improved antidepressant response. However, ketamine is known to act via various mechanisms that may target 5HT indirectly (81), while at least acute ketamine administration produces a rapid increase in the activity of locus coeruleus NA neurons through an amplification of AMPA transmission (82). Ketamine has also been demonstrated to induce significant increases in NA release in the prefrontal cortex (83). These actions may underlie the observed additive response with imipramine in the FST. Therefore, combining imipramine with either venlafaxine or ketamine delivers an effective antidepressant response even in apparently treatment resistant animals. These data are important because, not only do they correlate to clinical data such as that presented in STAR*D, but reaffirms our earlier observation (38) that TDS-exposed FSL rats constitute a novel and useful animal model of TRD.

Coping behaviour in the FST is thought to be mediated by the same underlying mechanisms that determine effectiveness of chronic antidepressant therapy in humans (84), highlighting that in this case both combination treatments with imipramine improved serotonergic (swimming) and noradrenergic (climbing) activities. Additionally, discriminating between these coping behaviours may provide further insight into the role of monoaminergic neurotransmitter systems involved in mediating these effects (48). We have already demonstrated that FSL rats show significantly raised frontal cortical and hippocampal 5HIAA levels as well as significantly reduced NA levels in these brain regions vs. their FRL control (Table 2). In this study, monoamine data (Figure 3) reveals no alterations in 5HIAA or NA in the frontal cortex following treatment with either imipramine, venlafaxine or ketamine, although ketamine increased frontal-cortical NA vs vehicle treated animals, while also not markedly affecting swimming or climbing. However, ketamine + imipramine (frontal-cortical 5HIAA and NA), ketamine alone (frontal-cortical NA) and venlafaxine + imipramine (frontal-cortical NA) significantly increased 5HIAA and NA responses vs. untreated TDS-exposed FSL rats (Fig. 3Ai and Bi), indicating a more robust response following these combination treatments. Also, these combinations increased swimming and climbing (Figure 2). Considering that TCAs such as imipramine act by increasing the extracellular levels of NA and 5HT (85), TDS tends to prevent these

effects (Table 3) with only ketamine alone, ketamine + imipramine and venlafaxine + imipramine able to reverse the reduction in NA, while only ketamine + imipramine reverses TDS-associated reductions in 5HIAA (Figure 3Ai; 3Bi).

Significant increases in NA levels in the frontal cortex was measured in animals treated with venlafaxine + imipramine and ketamine + imipramine which corresponded with increases in climbing behaviour measured in the FST. This is especially interesting considering that neither imipramine nor venlafaxine, when administered alone, were able to achieve this. However while ketamine alone increased NA levels, this effect did not translate to climbing behaviour. Also, increased swimming behaviour was observed in rats treated with venlafaxine + imipramine while neither imipramine- nor venlafaxine-treated animals attained significance in this regard, despite a large effect size (d = 0.93 and 1.07, respectively). Contradictions between monoamine and FST data have been reported in several animal studies in response to stress (86, 87). In fact the paradox with respect to limbic monoamine levels and coping strategies may be indicative of adaptive changes that influence coping responses following repeated exposure to stress. However, independent of interplay between monoaminergic and behavioural responses, only augmentative treatments (venlafaxine + imipramine and ketamine + imipramine) induced significant alterations in both behavioural parameters in the FST and 5HIAA and NA responses, signifying the improved efficacy of combination vs mono-therapeutic antidepressant therapy in this model which further lends support to its validity as an animal model of TRD.

In conclusion, combining stress sensitive FSL rats with TDS results in a treatment resistant rat model of MD. Non-response is not only observed with the traditional antidepressant, imipramine, but also following treatment with either ketamine or venlafaxine. Exposure to TDS thus inhibits antidepressant response in FSL rats at both behavioural and neurochemical levels. However, combining venlafaxine or ketamine with imipramine leads to enhanced antidepressant-like effects, together with associated effects on neurochemistry. These data confirm the hypothesis that exposure of FSL rats to a PTSD-like paradigm results in more severe depressive-like behaviour which is resistant to traditional antidepressant treatment, albeit responsive to treatment regimens which combine various mechanisms of antidepressant action. Combining FSL+TDS therefore holds promise for future development as a suitable animal model of TRD.

Author disclosures

Author contributions: SJ Brand performed all behavioural procedures, including treatment of the animals, performed all neurochemical analyses, undertook the statistical analysis, and prepared the first draft as well as the final version of the manuscript. BH Harvey devised the concept of the study as well as the layout of the manuscript, and finalized the pre-submission version of the manuscript.

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Figure 1

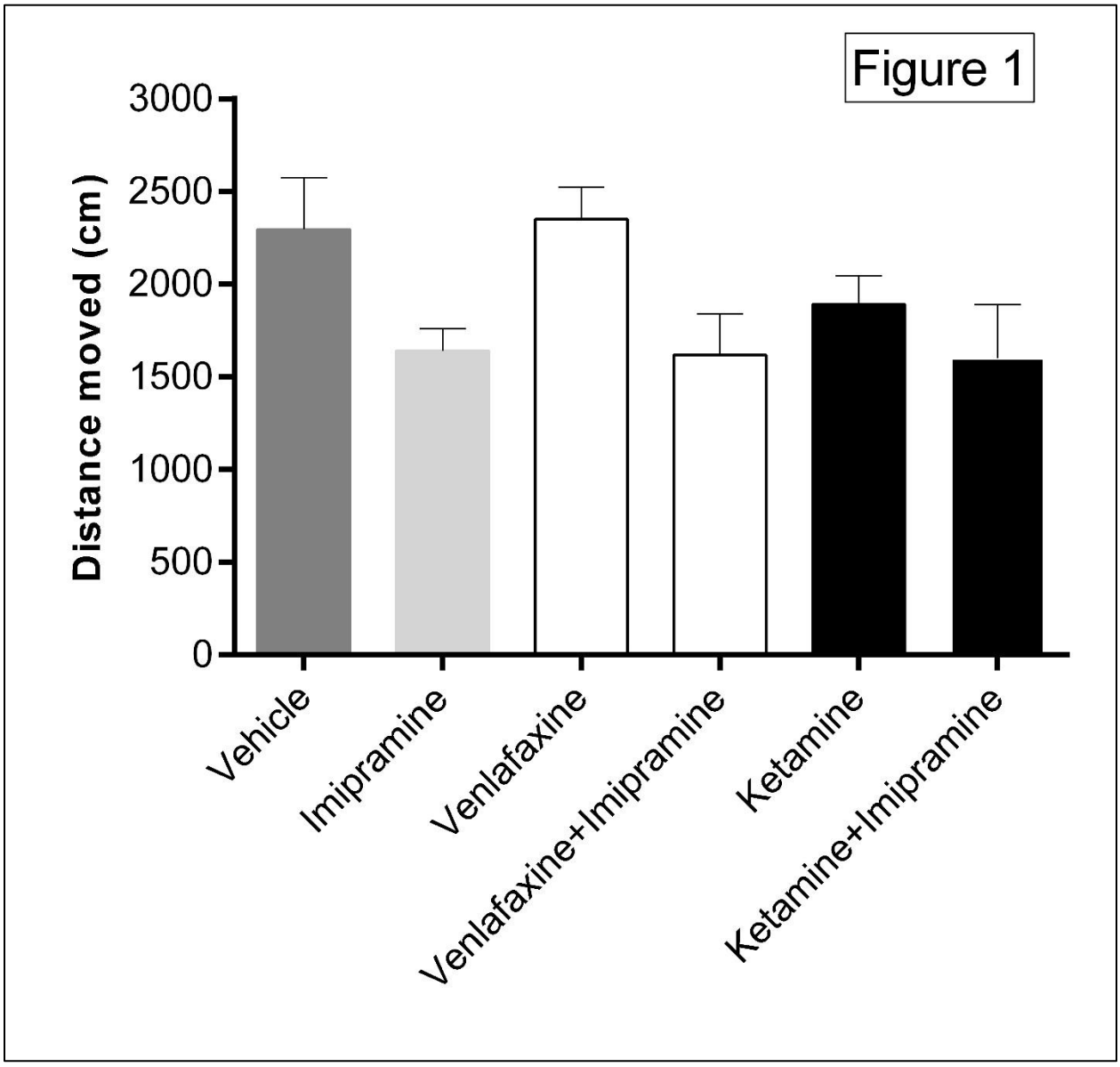
Figure 1
Distance moved (cm)
3000
2500
2000
1500
1000
500
0
Vehicle
Imipramine
Venlafaxine
Venlafaxine+Imipramine
Ketamine
Ketamine+Imipramine

Figure 2

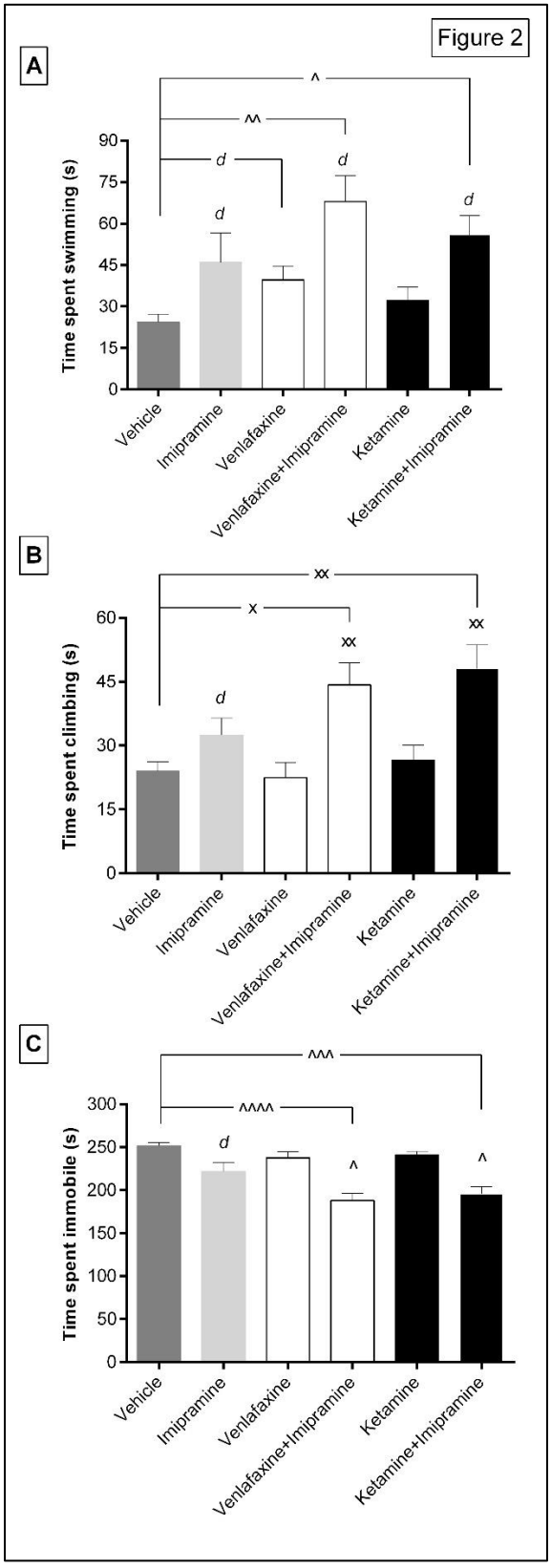
Figure 2
A
Time spent swimming (s)
90
75
60
45
30
15
0
^
^^
d
d
d
d
Vehicle
Imipramine
Venlafaxine
Venlafaxine+Imipramine
Ketamine
Ketamine+Imipramine
B
Time spent climbing (s)
60
45
30
15
0
xx
x
xx
xx
d
Vehicle
Imipramine
Venlafaxine
Venlafaxine+Imipramine
Ketamine
Ketamine+Imipramine
C
Time spent immobile (s)
300
250
200
150
100
50
0
^^^
^^^^
d
^
^
Vehicle
Imipramine
Venlafaxine
Venlafaxine+Imipramine
Ketamine
Ketamine+Imipramine

Figure 3

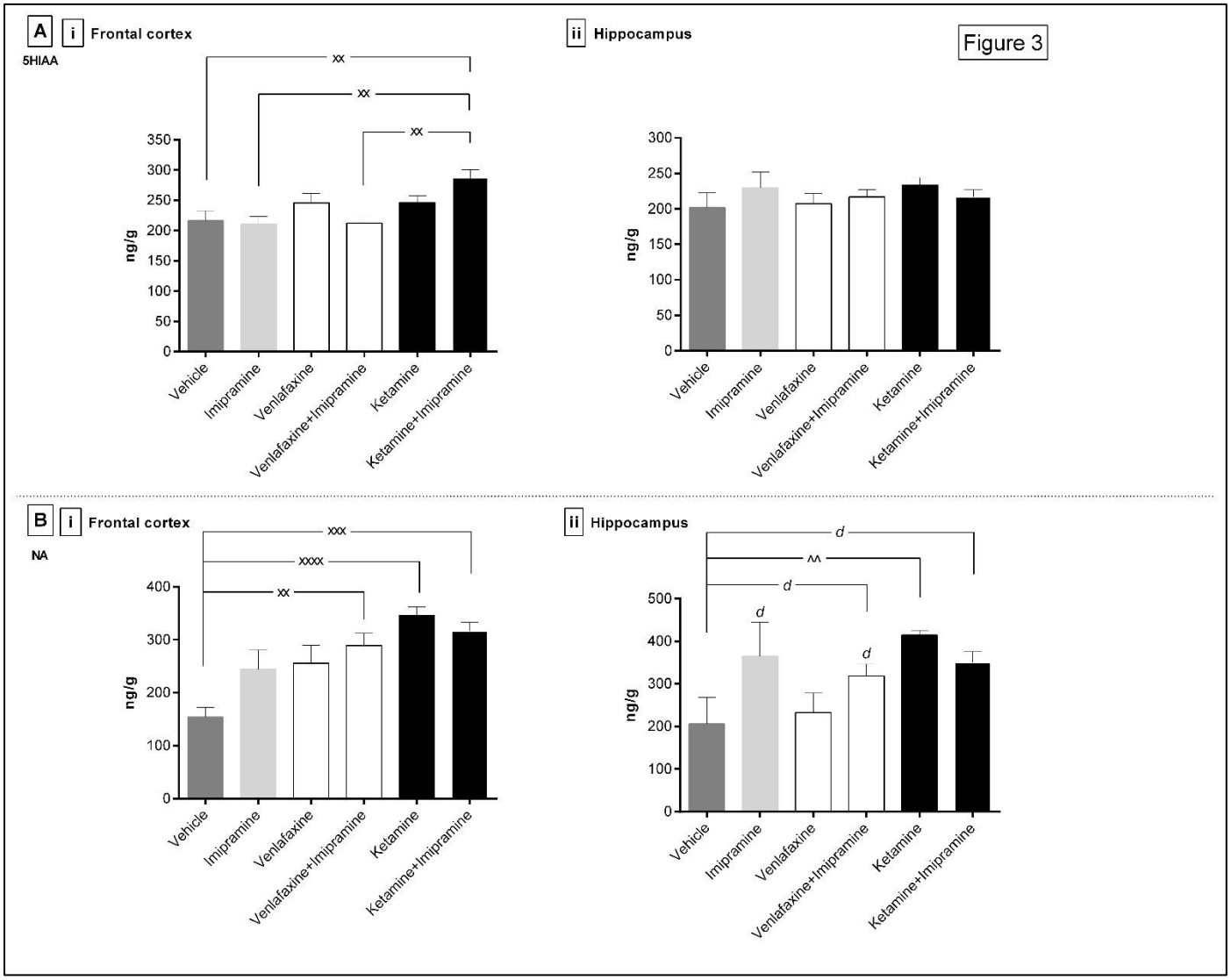
A
i
Frontal cortex
5HIAA
ii
Hippocampus
Figure 3
ng/g
Vehicle
Imipramine
Venlafaxine
Venlafaxine+Imipramine
Ketamine
Ketamine+Imipramine
B
i
Frontal cortex
NA
ii
Hippocampus

Table 1: Group Layout

Group name	*Group description*	*Dosage*	*n-value*	
			Behavioural	*Bio-molecular*
FSL-TDS+VEH	Antidepressant naive unstressed FSL rats	1ml.kg^{-1}	12	8
FSL+TDS+VEH	Antidepressant naive stressed FSL rats	1ml.kg^{-1}	12	8
FSL+TDS+IMI	Imipramine treated stressed FSL rats	10mg.1ml^{-1}.kg^{-1}	12	8
FSL+TDS+VEN	Venlafaxine treated stressed FSL rats	10mg.1ml^{-1}.kg^{-1}	12	8
FSL+TDS+KET	Ketmaine treated stressed FSL rats	10mg.1ml^{-1}.kg^{-1}	12	8
FSL+TDS+IMI+VEN	Imipramine/venlafaxine treated stressed FSL rats	10mg.1ml^{-1}.kg^{-1}	12	8
FSL+TDS+IMI+KET	Imipramine/ketamine treated stressed FSL rats	10mg.1ml^{-1}.kg^{-1}	12	8

Table 2: Comparisons of data relating to open field and forced swim test behavior and frontal-cortical and hippocampal markers of monoamine function in unstressed FRL and FSL animals.

	FRL	*FSL*	*Significance*
Open Field Test			
Total distance traveled (cm)	2273±307.2	2119±505.4	-
Forced Swim Test			
Swimming (s)	70.4±14.8	52.9±15.2	** (p = 0.009)
Climbing (s)	117.9±38.0	35.0±9.2	xxxx (p < 0.0001; U = 2)
Immobility (s)	111.7±33.7	212.1±18.8	**** (p < 0.0001)
Neurochemistry			
5HIAA (ng.mg^{-1})			
Frontal cortex	170.4±22.8	268.4±51.3	*** (p = 0.0007)
Hippocampus	177.2±37.2	244.1±40.3	x (p = 0.021; U = 10)
Noradrenalin (ng.mg^{-1})			
Frontal cortex	412.1±27.7	188.7±77.5	****(p < 0.0001)
Hippocampus	451.9±95.3	202.9±78.4	**** (p < 0.0001)

**Unpaired student t-test; xMann-Whitney U-test*

Table 3: Comparisons of data relating to open field and forced swim test behaviour and frontal-cortical and hippocampal markers of monoamine function in treatment naïve unstressed and TDS-exposed FSL animals.

	TDS-naïve FSL	*TDS-exposed FSL*	*Significance / effect size*
Open Field Test			
Total distance traveled (cm)	2119±505.4	2296±971.7	-
Forced Swim Test			
Swimming (s)	52.9±15.2	24.4±9.8	^{xxxx} ($p < 0.0001$; $U = 6.0$)
Climbing (s)	35.02±9.2	24.0±7.9	^{xx} ($p = 0.005$; $U = 24.5$)
Immobility (s)	212.1±18.8	251.7±14.2	**** ($p < 0.001$)
Neurochemistry			
5HIAA (ng.mg^{-1})			
Frontal cortex	268.4±51.3	216.4±45.6	$d = 1.07$
Hippocampus	244.1±40.4	201.5±59.0	$d = 0.84$
Noradrenalin (ng.mg^{-1})			
Frontal cortex	188.7±77.5	154.0±51.0	-
Hippocampus	202.9±78.4	205.5±167.0	$d = 0.9$

**Unpaired student t-test; ˣMann-Whitney U-test*

Figure Captions

Figure 1: Comparisons between locomotor activity of treatment-naive and treated TDS exposed FSL rats

Panel B – vehicle vs venlafaxine (d = 0.94); vehicle vs ketamine (d = 1.02); vehicle vs combinations of venlafaxine and imipramine (d = 1.02) and ketamine and imipramine (d = 0.9)

Data are represented as mean ± SEM.

Figure 2: Comparisons between behavioral parameters measured in the forced swim test of treatment-naive and treated TDS exposed FSL rats

Panel A – Time spent swimming (s). vehicle vs venlafaxine + imipramine, $^{\wedge\wedge}p < 0.001$; vehicle vs ketamine + imipramine $^{\wedge}p < 0.05$; vehicle vs imipramine, d = 0.93; vehicle vs venlafaxine, d = 1.07; venlafaxine + imipramine vs venlafaxine, d = 1.08;

Panel B – Time spent climbing (s). vehicle vs venlafaxine + imipramine, $^{x}p < 0.05$; vehicle vs ketamine + imipramine, $^{xx}p < 0.001$; venlafaxine vs venlafaxine + imipramine, $^{xx}p < 0.001$; ketamine vs ketamine + imipramine, $^{xx}p < 0.001$; vehicle vs imipramine, d = 0.8

Panel C – Time spent immobile (s). vehicle vs venlafaxine + imipramine, $^{\wedge\wedge\wedge\wedge}p < 0.0001$; vehicle vs ketamine + imipramine, $^{\wedge\wedge\wedge}p < 0.0001$; venlafaxine vs venlafaxine + imipramine, $^{\wedge}p < 0.05$; ketamine vs ketamine + imipramine, $^{\wedge}p < 0.05$; vehicle vs imipramine, d = 1.21

^{x}Two-way analysis of variance (ANOVA) followed by Bonferroni post-hoc tests; $^{\wedge}$Kruskal-Wallis Analysis of Variance followed by Dunn's multiple comparisons test. Data are represented as mean ± SEM.

Figure 3: Comparisons between frontal-cortical and hippocampal neurochemical markers in treatment-naive and treated TDS exposed FSL rats

Panel Ai – frontocortical 5HIAA concentrations: vehicle vs ketamine + imipramine, $^{xx}p < 0.001$; imipramine vs ketamine + imipramine, $^{xx}p < 0.001$; venlafaxine + imipramine vs ketamine + imipramine, $^{xx}p < 0.001$

Panel Aii – Hippocampal 5HIAA concentrations.

Panel Bi – Frontocortical NA concentrations. vehicle vs venlafaxine + imipramine, $^{xx}p < 0.001$; vehicle vs ketamine, $p < 0.0001$; vehicle vs ketamine + imipramine, $p < 0.0001$

Panel Bii – Hippocampal NA concentrations. vehicle vs ketamine, $^{\wedge\wedge}p < 0.001$; vehicle vs imipramine, $d = 0.83$; vehicle vs venlafaxine + imipramine, $d = 0.92$; vehicle vs ketamine + imipramine, $d = 1.2$; venlafaxine vs venlafaxine + imipramine , $d = 0.82$

[x] Two-way analysis of variance (ANOVA) followed by Bonferroni post-hoc tests; [^] Kruskal-Wallis Analysis of Variance followed by Dunn's multiple comparisons test. Data are represented as mean ± SEM. 5HIAA: 5-hydroxyindolacetic acid; NA: noradrenalin; FC: frontal cortex; HC: hippocampus

CHAPTER 6

6 Conclusion

The current investigation has made a significant contribution to the understanding of mood and psychotic disorders by 1) documenting and evaluating the most prominent biomarkers associated with these disorders and providing a framework for improved selection of biomarkers that will aid the development of biomarker panels for improved therapeutic outcomes, 2) conceptualizing a gene-x-environment based animal model of TRD that centres around bench to bed observations relating to MD and PTSD comorbidity, and 3) the subsequent development and validation of said model at behavioural, pharmacological and biological levels.

Mood and psychotic disorders, aside from their well-characterized symptomatology, also present with a wide range of biochemical and structural anomalies. Despite the presence of such potentially quantifiable biomarkers in these disorders, the diagnosis and treatment of psychiatric disease rely solely on the predominantly subjective assessment of patient-reported somatic and behavioural symptoms according to a predefined set of diagnostic criteria. Although this is the clinically accepted and only available approach, the absence of quantifiable physiologic parameters contribute to the poor treatment outcomes and non-response that are often experienced in severe manifestations of psychiatric disease, e.g. TRD. As such, Manuscript A reviewed the current state of biochemical and structural abnormalities associated with mood and psychotic disorders, providing the first published account of how these putative biomarkers are correlated at clinical *and* preclinical levels, thereby providing a more robust means of establishing their value as true biomarkers for a specific illness. We aimed at identifying the most important and relevant biomarkers in three major psychiatric disorders responsible for disability as identified by The Global Burden of Disease Study (2015), *viz*. MD, bipolar disorder and schizophrenia, through a systematic synthesis of the main findings from available clinical and pre-clinical literature on the array of potential biomarkers associated with these disorders. This review attempted to direct future research on neuropsychiatric markers and to provide a theoretical foundation for the eventual development of biomarker panels which may assist clinicians to more accurately diagnose their patients and even tailor treatment approaches to the unique pathophysiologic features that may underlie psychiatric illnesses. Ideally, this would result in improved diagnostic accuracy and prevention of relapse and treatment non-response. Drawing from

the current body of knowledge, it is clear that mood and psychotic disorders are currently associated with a multitude of biomarkers that still require illumination regarding their exact etiological or diagnostic roles. However, investigations into biomarkers remain resolute and, owing to ongoing attempts to clarify the pathophysiology of these disorders, the complexities thereof are gradually being unravelled. Hence, we are now increasingly able to explain how altered neuroendocrine, anatomical and neurochemical abnormalities may contribute to the development, progression and persistence of psychiatric disorders. To resolve the existing inconsistencies observed in biomarker measurements, specificity of these markers should be clarified, translation of preclinical findings to the clinical level be fine-tuned, potential interpatient- and interspecies variables established and studies of biomarkers be employed in larger populations. In order to realize the vision of biomarker panels and utilizing its potential benefits, it is thus of the utmost importance that proposed biomarkers with confirmed involvement in the trait and state of mood and psychotic disorders be dissected to a point of absolute comprehension. Another outcome to the writing of this review was to establish an array of likely biomarkers for the current animal study. Based on our synthesis of the available data, we established that a variety of potential biomarkers exist in MD and TRD, including markers of genetic, inflammatory, circadian and neuroendocrine processes. However, considering that monoaminergic processes still remain a very prominent pathological feature of the disorder and that the majority of pharmacological treatment options depend on interaction with this system, monoaminergic markers (i.e. 5HIAA and NA) were selected as a platform on which to base the construct validity of the TRD model developed in the current investigation. As such, we were also able to extrapolate behavioural data accumulated in the FST to the most relevant biomarkers underlying observations made regarding depressive-like and coping behaviours (swimming and climbing).

The treatment of MD is plagued by a high incidence of treatment resistance, while the development of improved therapeutic approaches is hindered by a lack of appropriate animal models (Willner *et al.*, 2015). The investigation into and development of such models are thus imperative. This realization prompted the need to develop and validate a gene-environment model of TRD in the current investigation. This work was prepared and submitted as companion papers to the same peer review journal (manuscripts B and C).

Manuscript B provides a proof of concept and offers a detailed report of the initial bio-behavioural and pharmacological findings made during the development of this model. To confirm the depressive-like phenotype of the FSL rat, a head-to-head comparison between these animals and the control FRL strain upheld previous observations made in FSL rats, i.e. increased immobility and decreased coping (swimming and climbing) behaviour in the FST as well as increased 5HIAA and decreased NA levels in limbic brain structures. Subsequently, we superimposed TDS, a putative model of PTSD, on this genetic animal model of MD in an attempt to exacerbate the already present depressive-like behaviour and in this way induce resistance to traditional antidepressant treatment. As hypothesized, TDS-exposed FSL rats displayed exaggerated immobility and poor coping behaviour in the FST and, *importantly*, demonstrated significant reductions in behavioural and limbic 5HIAA responses following imipramine-treatment compared to TDS naive FSL animals. Thus, these promising findings provided preliminary face, construct and predictive validity for the model and supported the notion that the concept of combining a genetic animal model of MD with a PTSD paradigm holds promise as a suitable animal model of TRD.

Elaborating on the above and attempting to emulate clinical findings presented by the STAR*D study, any putative animal model TRD would require more explorative predictive validity studies, in particular with respect to its response to established treatment regimens for TRD. For this purpose, the response of TDS-exposed FSL animals was evaluated with respect to its response to venlafaxine and ketamine, the findings of which are presented in Manuscript C. We established that not only sub-chronic monotherapy with imipramine, but also venlafaxine and ketamine proved to be ineffective in attenuating depressive-like symptomology in TDS-exposed FSL rats. However, the key finding of this investigation was that combinations of imipramine with either venlafaxine or ketamine not only produced significant reductions in immobility and resulted in enhanced swimming and climbing behaviour after sub-chronic treatment, but also altered the frontal-cortical monoamine response compared to TDS naive animals. We therefore demonstrated that while exposure to TDS inhibits the bio-behavioural responses to sub-chronic imipramine, venlafaxine and ketamine treatment, such resistance to treatment can be countered by co-administering a traditional TCA, *viz.* imipramine with antidepressants of a different class and/or mode of action. Since both venlafaxine and imipramine act to increase extracellular levels of 5HT (and NA) (Gillman, 2007), a synergistic

action on 5HT may underlie the improved response observed in combination treatment while ketamine may contribute via its actions on mammalian target of rapamycin (Li *et al.*, 2010), glycogen synthase kinase-3 (Beurel *et al.*, 2011) and its indirect actions on 5HT (du Jardin *et al.*, 2016) and NA (El Iskandrani *et al.*, 2015, Kubota *et al.*, 1999) – effects that, if combined with that of imipramine, may produce more pronounced antidepressant-like effects. These findings are congruent with clinical TRD, in that the current antidepressants available to our disposal are not sufficient as is evident when considering the rates of poor or non-response, as well as that non-response to traditional first line antidepressants can be countered by a sequential addition of so-called augmentation agents, e.g. venlafaxine (Epstein *et al.*, 2014). Moreover, ketamine was used as it too is realizing strong clinical acceptability as an effective treatment for TRD, either alone or in combination with a more traditional agent (Epstein *et al.*, 2014). Before preclinical research can have a meaningful impact on the development of novel approaches to manage treatment refractive MD, reliable and specific animal models of the disorder as well as potential biomarkers for non-response are needed. Also, by integrating appropriate biomarkers and animal models of TRD, novel avenues may be created to properly classify and treat patients suffering from MD and prevent poor treatment outcomes.

In conclusion, the data presented in Manuscripts A, B and C provide sufficient answers to the study questions proposed during the current investigation and confirm the proposed hypothesis that exposing FSL rats, a genetic model of depression, to a PTSD-like paradigm will result in a gene-x-environment model of TRD characterized by more severe depressive-like behaviour that is resistant to mono-therapeutic antidepressant treatment approaches, however being responsive to combination treatment modalities utilizing both traditional and novel antidepressants. These findings are strongly reminiscent of the STAR*D findings that has outlined a typical TRD approach in the clinic, and is provocative for future work that will reveal more of the underlying neurobiology of TRD as well as provide a platform for the testing of novel treatments.

6.1 Shortcomings and Suggestions for Future Studies

This study has validated a novel TRD model with respect to behavioural, biological and treatment response and found to be a reasonable analogue of the human disorder. As a result, the model may now be further studied as well as used to investigate the biological underpinnings responsible for treatment resistance, and to test novel compounds. In retrospect, however, the following suggestions may be made to strengthen the model as well as for future investigations using this model:

Owing to the recovery period between the (initial) single prolonged stress (SPS) sequence and the initial and second re-stress, several adaptations may occur regarding the neurochemical profile of these animals and, importantly, the corticosterone response. Indeed, as may be gathered from the literature review, glucocorticoids play a considerable role, not only in MD and PTSD, but also in TRD. It would therefore be a valuable addition if monoamines and corticosterone levels are measured not only after completion of the TDS procedure, but also before and after SPS and also during the initial and second re-stress, in order to get a more comprehensive picture of monoaminergic and glucocorticoid fluctuations during the whole procedure. In this regard, the inclusion of *in vivo* microdialysis may prove to be of great value to illuminate monoaminergic alterations induced by TDS in FSL rats. Furthermore, additional behavioural assessments may also illuminate the response of these animals to TDS, e.g. anxiety-like behaviour in, for example, the elevated plus maze (EPM), as well as anhedonic responses. In fact, anhedonia is an important symptom of TRD and has been demonstrated to be highly prevalent in rats subsequent to stress-exposure (Henningsen et al., 2009), while it is reversible by the administration of ketamine (a novel antidepressant with proven efficacy in TRD) (Garcia et al., 2009). Considering the importance of anhedonic responses in TRD and the prevalence thereof in animals subsequent to stress-exposure, the sucrose preference test (SPT) should be considered as an alternative to the FST to measure depressive-like symptoms in TDS-exposed animals. Although the experimental design of the current study allowed 21 days between forced swim stress (during SPS) and the FST to render any effects that previous swimming exposure may have on results gathered in the FST unlikely, employing the SPT as alternative behavioural measure may eliminate this possible limitation entirely. Also, measuring $5HT_{1A}$ receptor expression in the limbic regions of TRD animals (e.g. Samuels et al., 2011) could bolster the construct validity of

the model significantly, especially since we have found in our review of the literature that these receptors play an important role in treatment resistance. Gene polymorphisms affecting this receptor have been connected to antidepressant response (Lemonde, Du, Bakish, Hrdina, & Albert, 2004) and the effects of the interplay between gene and environment have also been demonstrated to have a significant impact on the way that this receptor is influenced by antidepressant treatment (Shrestha et al., 2014). Importantly, the antidepressant effects of ketamine have also been reported to be hinged on activation of $5HT_{1A}$-receptors (du Jardin et al., 2016).

Findings made during this investigation was limited to behavioural and neurochemical alterations after sub-chronic antidepressant (7 days) treatment with relatively conservative dosages. Considering that inadequate treatment duration and dosage are often important causes for treatment resistance (Philip, Carpenter, Tyrka, & Price, 2010), extending treatment duration and increasing dosages may provide additional support for our observations.

Furthermore, it may also be of value to reconsider the restress process and perhaps limit the procedure to a single restress. The frequency of exposure to situational reminders adds to maintaining disturbances induced by TDS over time (Oosthuizen, Wegener, & Harvey, 2005) and it may therefore be possible to limit the procedure to a single restress and still measure relevant appropriate behavioural and biomarker alterations. This would also allow extension of the treatment period without delaying the age at which behavioural analyses are performed. Animal weight and age are important factors of consideration to ensure reproducibility of results in behavioural and neurochemical assessments.

TRD has a prominent prevalence in female patients (Slattery and Cryan, 2014) and further studies examining gender bias in the current model may be valuable to reinforce its relevance. However, the use of female rats in developing translational models of psychiatric disorders (especially MD) poses a very well-known complexity when considering both physiological and biological variances induced by the estrous cycle (Slattery and Cryan, 2014) (refer to section 1.7).

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Addendum A –
Instructions to the Authors

Manuscript A – Current Neuropharmacology

Instructions for online manuscript submissions are available on the journal's website at the following URL:

http://benthamscience.com/journals/current-neuropharmacology/author-guidelines/

Manuscript B and C – Acta Neuropsychiatrica

Instructions for contributors are available in a downloadable PDF file on the journal's website at the following URL:

http://journals.cambridge.org/action/displayMoreInfo?jid=NEU&type=ifc

Addendum B –
Co-Authors Letters of Consent

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To whom it may concern,

I, M Möller, second author of ***A Review of Biomarkers in Mood and Psychotic Disorders: A Dissection of Clinical vs. Preclinical Correlate; Current Neuropharmacology, 13(3): 324-368*** hereby gives permission that S. Brand may use this article as part of his PhD thesis.

Yours sincerely,

.....

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24 June 2016

To whom it may concern

RE PHD THESIS – S.J. BRAND
PERMISSION TO INCLUDE MANUSCRIPTS FOR EXAMINATION PURPOSES

As study leader and senior corresponding author on three manuscripts first-authored by Mr. Sarel Brand, I hereby approve that these manuscripts (as listed below) be included as part of the requirements for fulfilment of the PhD degree, and that these manuscripts be submitted for examination of Mr. Brand's thesis.

The three articles are as follows:

Manuscript A
A Review of Biomarkers in Mood and Psychotic Disorders: A Dissection of Clinical vs. Preclinical Correlates
Published in Current Neuropharmacology, 2015, 13, 324-368

Manuscript B
Exploring a post-traumatic stress disorder paradigm in Flinders sensitive line rats to model treatment resistant depression I: Bio-behavioural validation and response to imipramine.
In submission to Acta Neuropsychiatrica

Manuscript C
Exploring a post-traumatic stress disorder paradigm in Flinders sensitive line rats to model treatment resistant depression II: Response to antidepressant augmentation strategies
In submission to Acta Neuropsychiatrica

The published version of Manuscript A (*Current Neuropharmacology*) and cover pages for Manuscripts B and C provided by *Acta Neuropsychiatrica* after submission to this journal are included in the Addendums section of the thesis.

I trust you will find this in order.

Yours sincerely,

Brian H. Harvey, PhD
Co-director, MRC Unit for Anxiety and Stress Disorders
Sub-programme leader, Translational Neuroscience and Neurotherapeutics

Addendum C –
Published PDF of Manuscript A (Chapter 3)

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324 *Current Neuropharmacology, 2015, 13,* 324-368

A Review of Biomarkers in Mood and Psychotic Disorders: A Dissection of Clinical vs. Preclinical Correlates

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B.H. Harvey

Abstract: Despite significant research efforts aimed at understanding the neurobiological underpinnings of mood (depression, bipolar disorder) and psychotic disorders, the diagnosis and evaluation of treatment of these disorders are still based solely on relatively subjective assessment of symptoms as well as psychometric evaluations. Therefore, biological markers aimed at improving the current classification of psychotic and mood-related disorders, and that will enable patients to be stratified on a biological basis into more homogeneous clinically distinct subgroups, are urgently needed. The attainment of this goal can be facilitated by identifying biomarkers that accurately reflect pathophysiologic processes in these disorders. This review postulates that the field of psychotic and mood disorder research has advanced sufficiently to develop biochemical hypotheses of the etiopathology of the particular illness and to target the same for more effective disease modifying therapy. This implies that a "one-size fits all" paradigm in the treatment of psychotic and mood disorders is not a viable approach, but that a customized regime based on individual biological abnormalities would pave the way forward to more effective treatment. In reviewing the clinical and pre-clinical literature, this paper discusses the most highly regarded pathophysiologic processes in mood and psychotic disorders, thereby providing a scaffold for the selection of suitable biomarkers for future studies in this field, to develope biomarker panels, as well as to improve diagnosis and to customize treatment regimens for better therapeutic outcomes.

Keywords: Antidepressant, biomarker panel, GABA-glutamate, genomics-proteomics, immune-inflammation-redox, kynurenine-cytokine, neurotransmitters, nitric oxide, schizophrenia.

INTRODUCTION

Major depression (MD), bipolar disorder (BPD) and psychotic disorders (e.g. schizophrenia) are often misdiagnosed, leading to inadequate treatment and devastating consequences [1, 2]. MD is among the most debilitating diseases worldwide, with a life-time prevalence of up to 20% [3], and even though major advances have been made in developing new drugs, less than 50% of patients achieve remission after antidepressant treatment [4]. Bipolar disorder affects approximately 1.2% of the population worldwide [5], and differs from MD with a unique hallmark of mania (elevated mood or euphoria, hyper-activity with a lack of need for sleep, and an increased optimism) which frequently leads to a deficit in the patient's judgment [6]. On the other hand, schizophrenia is a debilitating neuropsychiatric disorder, typically emerging during adolescence or early adulthood and continuing to plague patients suffering from the disease to varying degrees throughout their lifetime [7]. Approximately 1% of the general population worldwide is affected by the disorder and the life expectancy of patients with schizophrenia has been demonstrated to be nearly 20% shorter than that of the general population [8].

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Despite an abundance of research, the pathogenesis and aetiology of mood and psychotic disorders remain unclear, challenging the diagnosis and treatment of these disorders [9], mainly for the following reasons:

- Diagnoses of typical psychiatric disorders are primarily based on operationalized behavioural diagnostic systems either as self-reported symptoms by patients or observations by clinicians, being confirmed against diagnostic criteria set out in the *Diagnostic and Statistical Manual of Mental Disorders* 4^{th}/ 5^{th} *ed. (DSMIV/V) and International Statistical Classification of Diseases, 10th Revision* [10].
- Laboratory diagnostic and screening tools, such as a non-invasive blood-based test, remain elusive [11], while mood and psychotic symptoms may overlap with other neurological and psychiatric problems [9].

Clinically useful biomarkers in these disorders could therefore significantly improve diagnosis and treatment and has been one of the holy grails of MD, BPD and schizophrenia research [12]. However, the likelihood of any single biomarker achieving a high enough degree of sensitivity and specificity for mood and psychotic disorders is relatively low. Biomarker panels may represent an attainable alternate to a single-biomarker approach [13]. Common features of this method include correlates attributed to the individual which may determine the

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presence or absence of a state of sickness or that may even predict response to treatment [13]. Biomarkers may also indicate the presence of a pathophysiological process that can be addressed with a preventive treatment [14], as well as highlight "state" and/or "trait" markers. Therefore, the identification of biomarkers prior to onset of depressive and bipolar symptoms or psychosis has enormous potential importance for the design of future preventive strategies.

The Biomarkers Definitions Working Group of the National Institutes of Health Group [15] (2001) defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [15]. Biomarkers should also provide high levels of sensitivity and specificity (>80%) in the detection and classification of MD, BPD and psychotic disorders [16], in order to be clinically useful.

There are many potential biomarkers for mood and psychotic disorders and previous studies have tested specific biomarkers based on the hypotheses of monoamine dysfunction, altered immune-inflammatory processes, neuroendocrine dysfunction and disturbances in neuroplasticity [17]. However, considering the most recent neuroanatomical basis for these illnesses, as well as relevant hypotheses of their aetiology, this review will provide an overview of potential biomarkers that could contribute to an initial multi-analyte biomarker panel of mood and psychotic disorders. Such biomarkers include molecules and/or processes directly or indirectly connected with growth factors, neurotransmitters, oxidative stress, inflammation, neuro-imaging, genetic, proteomic and neuronal resilience markers. In order to do this, we have collated neuroimaging and neurobiological findings from clinical studies as well as data from validated translational animal models, to assist in developing a putative, uniform biomarker panel for MD, BPD and schizophrenia.

There can be no doubt as to the value of translational animal models in drug discovery and in identifying novel neurobiological targets. Considering mood and psychotic disorders, these models include for example social isolation rearing (SIR) and the glutamate N-methyl D-aspartate (NMDA) antagonist models of schizophrenia [18, 19], and the Flinders Sensitive Line (FSL) or chronic mild stress (CMS) rat models of MD [20, 21]. Developing an appropriate animal model to mimic BPD has proven to be an arduous task, it being difficult to establish a model that combines symptoms of MD, mania and euthymia in an alternating manner as is observed in BPD. Instead, animal models have been developed to express features central to either MD or mania using pharmacologic (amphetamines and ouabain), environmental (e.g. behavioral despair; sleep deprivation), or genetic (e.g. FSL rat) models. Therefore in parallel with our analysis of the clinical scenario, we will also closely scrutinize appropriate animal models for correlation with clinical findings. This review will cover MD, BPD and schizophrenia with respect to the noted biomarkers and across clinical and pre-clinical correlates.

THE NEUROANATOMY AND NEUROCIRCUITRY OF PSYCHIATRIC ILLNESS

Clinical Correlates

Neuroimaging methods, such as structural magnetic resonance imaging (MRI), functional magnetic resonance imaging (fMRI), diffusion tensor imaging (DTI) and positron emission tomography (PET), provide important evidence for underlying biological factors of MD, BPD and psychotic disorders [22]. Generally, areas of the limbic system, the hippocampus and frontal cortical areas are under scrutiny with regard to structural and functional neuroimaging research in mood disorders [23]. Importantly, there is increasing awareness of the interplay between specific neurocircuitry of the brain and behavioural pathology.

Depression

Neuroimaging studies have been central in identifying the key structures involved in the pathophysiology of MD, showing decreases in hippocampal volume of up to 15% in depressed patients [24], as well as reductions in grey-matter volume and glial density in the prefrontal cortex and the hippocampus [25]. Other studies in MD indicated large volume reductions in the anterior cingulate cortex (ACC) and orbitofrontal cortex (OFC) accompanied by lesser reductions in the prefrontal cortex, along with moderate reductions in the hippocampus, the putamen, and the caudate nucleus [26]. Lorenzetti *et al.* [27] reported volume reductions of the hippocampus, basal ganglia, the OFC and subgenual prefrontal cortex in patients suffering from MD, while more persistent forms of MD (which may include recurrent episodes or relapses and extended illness duration) are accompanied by an increased effect on regional brain volumes [28]. While reductions in hippocampal volume in MD may have a genetic component [29], it is also a function of illness duration [30] as well as poor compliance [28]. Moreover, PET studies have revealed consistently increased regional blood flow and glucose metabolism in the amygdala, orbital cortex, and medial thalamus but decreased blood flow in the dorsomedial/dorsal-anterolateral prefrontal cortex and anterior cingulate cortex in un-medicated MD patients [31]. N-acetyl-aspartate (NAA), an indicator of neuronal viability, was also reduced in frontal cortex and in subcortical regions of MD patients [32, 33].

Regarding antidepressant therapy, Frodl *et al.* [34] demonstrated increases in hippocampal volume in patients who were subjected to continual treatment with antidepressants for three years, while Mayberg *et al.* reported that patients who responded to antidepressant treatment presented with increased anterior cingulate metabolism at baseline when compared to non-responders and to healthy controls [35]. Importantly, Macqueen *et al.* noted a greater degree of hippocampal shrinkage in patients with a prior history of switching antidepressants, indicating the possible deleterious effect of non-compliance on hippocampal integrity [28]. Furthermore, it has been proposed by structural neuroimaging studies that the volume of regional structures, for example the ACC and hippocampus, may provide an estimate of response to treatment [36]. Clinical response has also been demonstrated

to be predicted by activity in the rostral ACC region as measured by electroencephalography (EEG) studies which identified activity localised to the rostral ACC region as being predictive of a clinical response to antidepressant medication [37]. Finally, favourable treatment outcomes have repeatedly been demonstrated to be associated with integrity of perigenual anterior cingulate volume [38]. Moreover, direct electrical stimulation of the striatum has been found to elicit a positive response in patients suffering from resistant MD and is bolstered by reports proposing the striatum as an important relay system between limbic and cortical structures [39]. ACC activity has also been positively related to a variety of treatment responses, including antidepressant pharmacotherapy and sub-chronic and experimental treatment strategies, including sleep deprivation [40], which suggests that ACC response is generalised across different treatment types.

Bipolar Disorder

In patients with BPD, neuroimaging studies have found enlargement of the amygdala [41] and reductions in the dorsal and ventral prefrontal cortices [42] while PET studies have found decreases in cortical metabolism and increased normalized subcortical metabolism in depressed patients with BPD [43]. Two meta-analyses of neuroimaging studies concluded that patients with BPD suffered from hypo-activation and gray matter reductions in cortical-cognitive brain structures and increased activation of the para-hippocampal gyrus and amygdala [44].

Schizophrenia

In schizophrenia and other psychotic disorders, regions such as the ACC and dorsolateral prefrontal cortex (DLPC) have been emphasised [45]. Using cognitive paradigms, fMRI studies have demonstrated alterations in cerebellar activity in patients with schizophrenia, anxiety disorders and dementia (see review [46]). However, Farrow *et al.* [47] found that lateral and medial frontal regions and bilateral posterior temporal lobe regions feature structural losses in schizophrenia, whereas alterations in patients suffering from BPD were limited to bilateral inferior temporal gyri while deficits observed subsequently were limited to the ACC. Temporal lobe regions present with decreased activation in patients suffering from schizophrenia [48]. Additionally, EEG studies have demonstrated a reduction in the P300 wave amplitude, elicited in the process of decision making, in BPD and schizophrenia patients compared to control subjects [49]. Studies utilizing structural MRI have consistently observed temporal lobe abnormalities in schizophrenia, although results in BPD are less dependable [50]. Previous fMRI studies have also consistently reported anomalies in the prefrontal cortex in patients suffering either from a first episode or established schizophrenia [51, 52]. However, some of the evidence points to dorsolateral hyper-frontality, and especially for tasks which demand working memory, as well as increased activity in parietal regions [53]. Considering the progression from the prodromal phase to established chronic illness, patients with first episode and established schizophrenia show a gradual deterioration in frontal and striatal activation [54]. The most consistent findings in schizophrenia relating to cognition are detriments in executive tasks requiring prefrontal cortical function, eg. a self-ordered working memory task [55] or anti-saccade eye movements [56], olfactory identification [57], and tasks that rely on rapid processing of information (eg. story recall) [58]. A recent ^{1}H-MRS study in schizophrenia patients measuring NAA and N-acetylaspartylglutamate (NAAG) found a significant increase in NAAG/NAA ratio in the ACC but no difference in the left frontal lobe, although an inverse correlation between frontal lobe NAAG and negative symptoms was observed [59].

Pre-Clinical Correlates

Depression

Reductions in hippocampal volume have been observed in FSL rats, a genetic model of MD, when compared to Flinders Resistant Line (FRL) controls and is associated with a decrease in the number of neurons and synapses in the hippocampus – these alterations are reversed after chronic imipramine therapy [60].

Bipolar Disorder

In the ouabain-induced rat model of bipolar mania, PET imaging suggests reduced cerebral glucose metabolism, and is prevented by pre-treatment with lithium which concurs with similar decreases in cerebral metabolism noted in BPD patients [61]. Furthermore, lithium prevented stress-induced alterations in the amygdala by preventing increases in dendritic branching of pyramidal neurons in this structure [62]. Unfortunately, a paucity of MRI studies remains a shortcoming in animal models of BPD.

Schizophrenia

Previous studies indicated that the SIR model, a neurodevelopmental animal model of schizophrenia, presents with significantly reduced PFC volume, reduced accumbal dendritic length and spine density, cytoskeletal alterations and loss of parvalbumin (PV)-containing interneurons [18, 63, 64]. Among the most robust pathologies observed in schizophrenia is a decrease in gamma-aminobutyric acid (GABA) signaling (discussed in section 3.1.4), deficits of which are limited to the class of GABAergic interneurons containing the calcium binding protein PV [65]. These neurons synapse on the cell body or axon initial segment of glutamatergic neurons and thus are positioned to potently regulate pyramidal cell output. Furthermore a decrease in PV interneuron functionality may lead to reduced inhibitory control over pyramidal cell activity and also reduce coordination in activity of large brain networks [66].

SIR rats without an enriched environment also present with a decrease in dendritic spine density in the dorsolateral striatum when compared to rats from an enriched environment [67, 68]. Moreover, a NMDA receptor antagonist model of schizophrenia, viz. the phencyclidine (PCP) model, presents with decreased synaptic spine density on frontal cortical neurones [64]. Interestingly, rats treated chronically with MK-801 (another NMDA receptor antagonist model) also show a reduction in the amount of PV-containing neurones in the dentate gyrus and CA1 region of the hippocampus, although this is not accompanied by alterations in the PFC [69]. Furthermore, chronic intermittent exposure to PCP

decreases NAA and NAAG levels in the temporal cortex, while it raises hippocampal NAAG levels [70]. Similarly, SIR reduces NAA in the temporal cortex without changes observed in the hippocampus, striatum or frontal cortex [71]. These changes may indicate neuronal dysfunction that mirrors alterations observed in schizophrenia, as discussed in the clinical section [59].

In order to determine which regions exhibit the most disease-relevant information as well as the most potential for predictive capacity, these neuroanatomical correlates need to be linked to biological and genetic markers to more accurately predict both the pathology underlying the disease and the clinical outcomes. Predicting clinical response will assist in early identification and to further stratify patients who may benefit from more intensive, alternative, or combined therapies.

BIOLOGICAL, GENETIC AND PROTEIN BIOMARKERS

Neuroendocrine and Circadian Rhythms

Various hormones, especially the HPA-axis, thyroid hormones, insulin, as well as altered circadian rhythm, have a pronounced influence on neurodevelopment and the neurobiology of MD, BPD and schizophrenia [72]. They also influence other hormones such as sex steroids, orexin, arginine vasopressin etc. that are also implicated in these disorders but for reasons of space cannot be covered here.

Circadian Rhythms

Clinical Correlates

Altered circadian rhythms occupy a critical role in how the brain copes with stressful experiences and ultimately in regulating behavioural responses [73, 74]. The influence of circadian rhythm on mood and behaviour has received much attention in recent years and implicates not only hormonal dysregulation in these disorders, but also includes disturbances in neurochemical, redox and inflammatory cascades in its sphere of influence [75, 76]. Indeed, these processes will be discussed in subsequent sections of this review. Output from the suprachiasmatic nucleus (SCN) of the hypothalamus, the master biological clock, is under regulation by serotonergic ($5\text{-}HT_{2c}$) and melatonergic ($MT_{1/2}$) receptors, the expression of which are regulated by various clock genes. Indeed, melatonin-mediated regulation of hippocampal plasticity as well as clock gene expression in hippocampal neurons suggests a hitherto poorly recognized aspect in our understanding and treatment of these disorders [78]. Altered SCN output to other hypothalamic centres, but also monoaminergic cell bodies in the brain stem, will lead to wide-spread disturbances in neuroendocrine as well as monoaminergic function [78]. It is therefore not surprising that a significant amount of preclinical and clinical data has described the association between altered circadian rhythms with genetic, environmental and developmental abnormalities precedent to the development of MD, BPD (mania symptoms) and schizophrenia (see recent reviews by Wulff *et al.* [75] and Karatsoreos [76]). It is of relevance that agomelatine, a recently available antidepressant that acts *via* re-entrainment of circadian rhythms, may also have a therapeutic role in disorders other than MD, in particular BPD and schizophrenia [77].

Pre-Clinical Correlates

Biomarkers of circadian rhythms in animals remain a shortcoming, with such studies relying heavily on endocrine markers (as will be clear in the following section). Nevertheless, alterations in this system have on numerous occasions been shown to not only be of importance in humans suffering from MD, BPD and schizophrenia, but also in translational animal models for these disorders. Most animal models selectively bred to display characteristics of MD feature disturbed diurnal rhythms, eg. the FSL rat [79], Wistar Kyoto rat [80] and mice bred for spontaneous helplessness [81]. Also, depriving animals of REM sleep has been suggested to model mania [82]. In animal models of schizophrenia, blind-drunk (Bdr) mice demonstrate fragmented rest and activity rhythms under a light/dark cycle – which is reminiscent of altered sleeping patterns in schizophrenic patients [83].

Cortisol

Clinical Correlates

A dysfunctional hypothalamic-pituitary-adrenal (HPA) axis has been implicated in MD, BPD and also schizophrenia [84-86], affecting adrenocorticotrophic hormone (ACTH) release and cortisol secretion from the adrenal cortex [87]. Elevated salivary levels of cortisol after waking may represent a biomarker for depression in adolescence [88]. Importantly, an abnormal cortisol response, such as a flatter diurnal cortisol pattern, implies an abnormal stress reactivity that correlates with a greater severity of depression [73, 74], suggesting that altered circadian rhythms occupies a critical role in how the brain copes with stressful experiences and ultimately in regulating mood. Although the dexamethasone suppression test has attracted interest as a promising diagnostic test for MD, there has not been a consistent approach to evaluate its clinical usefulness [89].

Steen *et al.* [90] found no significant difference in cortisol release during a mental challenge in schizophrenia and BPD patients, although blunted cortisol release was observed in male patients compared to controls in both disorders [90]. A significant increase in systemic cortisol metabolism in both schizophrenia and BPD patients has been described, with results in patients with schizophrenia vs. controls being most consistent [87]. Interestingly, studies in children at risk for developing psychosis lend further support to the suggestion that illness onset is predated by a degree of HPA axis abnormalities, rather than being a subsequent epiphenomenon [91]. A blunted cortisol awakening response may embody an early marker of susceptibility to develop psychosis which may even be genetically mediated, whilst increases in diurnal cortisol levels may develop only proximate to disease onset [91]. These studies reaffirm the status of the HPA-axis, particularly cortisol levels and metabolism, as a putative biomarker in MD, BPD and schizophrenia, and warrants further study.

Pre-Clinical Correlates

FSL rats have been found to be hypocortisolemic, while Wistar Kyoto rats present with increased levels of corticotropin releasing hormone (CRH) and ACTH. Brain levels of dehydroepiandrosterone (DHEA), an adrenal androgen

known to have antidepressant-like effects, has been demonstrated to be decreased in both FSL and Wistar Kyoto rats vs. healthy controls, Sprague Dawley and Wistar rats respectively [92]. Mice showing high reactivity to stress also present with symptoms resembling that in depressed patients and were demonstrated to have a flattened diurnal rhythm of glucocorticoid secretion [93]. Similarly, mice exposed to 6 weeks CMS presented with high plasma corticosterone levels and decreased hippocampal expression of glucocorticoid receptors [94]. Sleep deprivation, which has been used to induce an animal model of mania, leads to a marked increase in CRH [95]. Cortisol levels have also been found to be increased both in the frontal cortex and periphery of rats exposed to prenatal stress, which has relevance in that prenatal stress may predict the development of these disorders. In addition to increased corticosterone release, Ward *et al*. [96] found rats exposed to prenatal stress to also have adrenal hypertrophy with increased expression of CRF-1 receptors in the amygdala [97]. Furthermore, olanzapine treatment was able to reverse the increased cortisol observed in the prefrontal cortex following prenatal stress [98].

Thyroid Hormones

Clinical Correlates

The relation between thyroid dysfunction and mental disorders has long been recognized, ranging from depression [99], anxiety [100] and schizophrenia [101]. A recent study explored thyroid-stimulating hormone (TSH) in patients with acute schizophrenia, unipolar depression, bipolar depression and bipolar mania and, apart from measuring TSH disturbances in all the disorders, observed a definite higher prevalence of thyroid dysfunction in patients with both unipolar and bipolar mood disorders vs. controls [102]. Another study observed significant thyroid dysfunction (hypothyroidism and hyperthyroidism) in schizophrenia as well as BPD patients [88]. Interestingly, autoimmune thyroid disease was more common in schizophrenia [103], emphasising an immune-inflammatory basis for the illness. Santos *et al*. [104] reviewed research on thyroid function in schizophrenia, relating interrelations between the pituitary-thyroid axis and major neuro-signaling systems involved in schizophrenia (including serotonin (5-HT), dopamine (DA), glutamate and GABA networks), as well as myelination and inflammatory processes. These processes are all convergent on the pathology of this disorder, as will be discussed. The authors conclude that thyroid hormone deregulation is a common feature in schizophrenia. Together, these studies emphasize the relevance of thyroid hormonal status as possible biomarkers in MD, BPD and schizophrenia, although further work in this regard is required to establish its putative role as a biomarker.

Pre-Clinical Correlates

Wistar Kyoto rats, an animal model of depression, have increased TSH levels that, together with ACTH, remains elevated after the diurnal peak [80]. To the best of our knowledge, the current body of literature on preclinical BPD and schizophrenia research does not contain significant data on thyroid hormones in animal models of mood and psychotic disorders.

Neurochemical Markers

The majority of drugs used clinically to treat MD, BPD and psychotic disorders such as schizophrenia target monoamine (DA, 5-HT and noradrenaline (NA)) receptors, reuptake transporters and monoamine metabolism [105, 106]. DA-ergic, 5-HT-ergic and/or NA-ergic neurotransmission affects behaviour by regulating motivation, reward seeking, aggression, and activity level – all symptoms that play an important role in the pathophysiology of these disorders [107, 108]. However, the cause of mood disorders is far from being a simple dysregulation of central monoamines. For example, monoamine oxidase inhibitors and monoamine reuptake inhibitors produce immediate increases in monoamine transmission [109], whereas their mood-enhancing properties are only fully realised following 4-6 weeks of sustained treatment. In fact, some patients do not show adequate improvement even after many months of treatment [110]. This indicates that enhanced serotonergic or noradrenergic neurotransmission *per se* is not immediately responsible for the clinical actions of these drugs [111]. Indeed, neurotrophins, neurogenesis and the concepts of neuroplasticity has now taken centre stage in our understanding of mood and psychiatric disorders and the mechanism of action of antidepressants [109, 112, 112a]. Thus, and apart from some data based on NA (see later), selecting an antidepressant based on its monoamine selectivity remains to be substantiated. The same can be said for antipsychotic drugs. Furthermore, a realization that neuroendocrine and metabolic dysfunction also contribute to the eventual development of these disorders, has provided a new framework for understanding their neurobiology and treatment. Nevertheless, their contribution towards the understanding and treatment of these disorders warrants closer scrutiny with respect to viable clinical biomarkers.

Dopamine

Clinical Correlates

Depression

Depressive symptoms (e.g. avolition, guilt, suicidality and social withdrawal) are ascribed to frontal cortical hypo-dopaminergia [109]. Striatal DA levels in MD are also reduced [113], being linked to symptoms such as anhedonia, reduced motivation and decreased energy levels. Patients presenting with MD episodes have been demonstrated to have significantly decreased dopamine transporter (DAT) binding potential, with binding potential correlating to receptor density and affinity [114]. Anhedonic MD patients exhibit significantly decreased levels of DAT in basal ganglia which are in accordance with the hypothesis linking impaired DA transmission to an impaired reward system [115]. It has also been suggested that decreased striatal D_2 receptor density may underlie depressive symptoms, while increased striatal D_2 receptor density/affinity has been observed in patients after successful SSRI treatment [116] which coincides with evidence of 5-HT modulating DA pathways [117].

Bipolar Disorder

Pharmacological evidence supports evidence that excessive DA neurotransmission mediates manic symptoms in BPD patients [118], while DA receptor D_2 antagonists are robust anti-manic agents [119].

Schizophrenia

The DA hypothesis of schizophrenia proposes that a hyper-dopaminergic state in the striatum mediates positive symptom expression, while a hypo-dopaminergic state in the frontal cortex mediates cognitive and negative symptoms [120]. In line with this hypothesis, post-mortem studies in schizophrenia patients have described frontal cortical hypo-dopaminergia [121] and elevated DA levels in the striatum [120]. However, a previous study reviewing clinical evidence for DA involvement in schizophrenia came to the conclusion that multiple ''hits'' (i.e. adverse environment, infection, chronic substance abuse etc.) interact to result in DA dysregulation, thereby producing the final common pathway to psychosis in schizophrenia [122]. In fact, MD [123, 124] and BPD [125, 126] are also correlated to early life trauma. It is pathways related to the latter that are deemed critical prodromal events in early life adversity, such as neurotoxicity, oxidative stress and inflammation that may hold the clue to identifying more appropriate biomarkers for these illnesses. Investigators have noted a strong correlation between D_2 receptor binding and response to an antipsychotic, with a minimum 70% receptor occupancy necessary for antipsychotic action [127]. In fact, the success of treatment with antipsychotic agents depend on dopamine D_2 receptor blockade, while Howes and Kapur [122] recently suggested various genetic and environmental factors to be implicated in compromising the brain and ultimately leading to dysregulation of DA.

Preclinical Correlates

Depression

CMS decreases *in vivo* DA release [129] and leads to a decrease in D_2 and D_3 receptors in the limbic forebrain which is reversed by chronic treatment with imipramine [130]. A decrease in DA release in the nucleus accumbens has been observed as well as increased DA levels in limbic regions in FSL rats (together with elevated 5-HT and NA; see below) – possibly due to an increased synthesis and decreased release of DA [131].

Bipolar Disorder

Models of mania which incorporate dopaminergic agents, eg. amphetamine, have been demonstrated to be superior to other similar models [132]. Alpha-methyl-para-tyrosine (AMPT) mediated catecholamine depletion mitigates some mania-related characteristics in DAT knockdown (KD) mice [133], while treatment with valproate reverses locomotor hyperactivity in these animals [134]. Also, treatment with lithium and valproate reverses increased extracellular DA and oxidative damage in a dextro-amphetamine-induced rat model of mania [135]. Furthermore, hyperactive rats exposed to CMS display significantly reduced HVA (homovanillic acid – DA metabolite) compared to DA in the nucleus accumbens, indicating decreased DA release in this brain region [136].

Schizophrenia

Previous evidence on the SIR model has indicated elevated striatal and decreased frontal cortical DA, dihidroxylphenylacetic acid (Dopac) and homovanillic acid (HVA) levels [137]; increased or decreased frontal cortical DA and unchanged striatal DA [138]; as well as decreased frontal cortical and elevated nucleus accumbens DA, Dopac and HVA [139, 140]. Another study in the SIR model observed reduced PFC D_1 receptor density [141]. However, changes in mesolimbic dopamine D_2 receptor expression are inconsistent, describing down-regulation in striatum [142], but no change in mesolimbic [143], hippocampal, PFC or amygdala areas [144]. SIR also induces a hyper-responsiveness in DA release in the PFC in response to systemic administration of the atypical antipsychotics clozapine and olanzapine, but not haloperidol [145]. Moreover, microdialysis data show that both basal and stress-induced PFC DA levels are reduced in rats chronically treated with PCP [146, 147].

Serotonin (5-HT)

Clinical Correlates

Depression

Although $5HT_{2c}$ antagonists are ineffective alone in the treatment of MD, they do show benefit when combined with other mood-regulating mechanisms, such as 5-HT reuptake inhibition (SRIs) or melatonin agonism (e.g., agomelatine) [78]. Since $5\text{-}HT_{2C}$ receptor activation inhibits NA and DA release [78], the suppression of these monoamines by *elevated* 5-HT contradicts traditional views that antidepressant response typically involves an increase in brain 5-HT, as well as NA and DA. Indeed elevated 5-HT-mediated suppression of DA and NA release will be counter-productive [148], such as causing emotional detachment and failure to address the anhedonic symptoms of MD [78]. Clearly there are valid reasons to doubt whether an elevation in brain 5-HT is in any way essential for antidepressant response. In fact, a sustained increase in 5-HT does not appear to be a requirement for anxiolytic/antidepressant effects of an SRI [149]. Further on this point, 5-HT agonists are ineffective as antidepressants while the 5-HT reuptake enhancer, tianeptine, is an effective antidepressant despite having the exact *opposite* effect on synaptic levels of 5-HT than SRIs [150]. This evidence contradicts the simplistic view that brain 5-HT needs to be elevated to improve mood, and has been instrumental in fueling the search for new generation antidepressants.

Post-mortem studies have indicated that suicidal patients with MD present with low cerebrospinal fluid (CSF) levels of 5-hydroxyindole-acetic acid (5-HIAA), the metabolite of 5-HT [151, 152], along with increased $5\text{-}HT_{2A}$ receptor binding sites in platelets [153] and prefrontal cortical sites [154] as well as increased limbic and decreased frontal cortical $5\text{-}HT_{1A}$ receptors (reviewed in [155]). MD patients also present with reduced $5\text{-}HT_{2A}$ receptor density in the frontal cortex [155a]. Interestingly, limbic density and activity of monoamine oxidase (MAO) is elevated in MD [156, 157] which in turn will influence a number of components of monoamine signalling.

Bipolar Disorder

The role of 5-HT in the pathogenesis of BPD is less studied, although a post-mortem study has indicated that

subjects with DSM-III-R diagnoses of BPD who died while depressed had significantly reduced levels of 5-HIAA in frontal (−54%) and parietal cortex (−64%) [158]. A deficit in 5-HT uptake sites has also been observed in the brains of depressed BPD patients after death [159]. Furthermore, 5-HIAA levels were found to be decreased in the CSF of depressed BPD patients [160] and elevated in manic BPD patients [161].

Schizophrenia

Post-mortem studies in patients with schizophrenia [162, 163] as well as psychotic patients [164] have observed reduced frontal cortex 5-HT_{2A} and increased 5-HT_{1A} receptor density. Another study also indicated increased striatal but diminished frontal cortical 5-HT uptake sites in schizophrenia patients [165]. In line with these findings, CSF, genetic and neuroimaging studies have demonstrated an increase in central 5-HT-ergic neurotransmission in schizophrenia [166, 167] and typified by the serotonergic psychedelics such as lysergide. A previous review suggested that the positive symptoms observed in schizophrenia (delusions, hallucinations etc.) could be associated with an excess of 5-HT in the striatum [168]. Despite the above evidence for 5-HT involvement in schizophrenia, clinical studies have found selective $5HT_{2A/2C}$ antagonists to be ineffective as antipsychotics (reviewed in [169]) and that alterations of brainstem 5-HT transporters are generally not associated with schizophrenia [170].

Preclinical Correlates

Depression

Excessive activation of the 5-HT_{2C} receptor is anxiogenic [171] while 5-HT_{2C} receptor antagonists are rapid acting with sustained anxiolytic actions [172]. 5HT and 5HIAA levels were noted to be higher in limbic structures in the brains of FSL rats compared to normal Sprague Dawley rats [173], while 5-$HT_{2/3}$ receptor density is compromised in the nucleus accumbens leading to a lack of DA-5-HT interaction [174]. Furthermore, CMS leads to an increase in $5HT_{2a}$ receptors in the cortex which is reversed by imipramine treatment [20]. However, contrary to human subjects, FSL rats present with decreased 5-HT synthesis [175] and SERT-expression [176].

Bipolar Disorder

5-HT-related data are limited in animal models of BPD and mania. However, a mutPOLG transgenic (Tg) mouse model of BPD has been demonstrated to have enhanced 5-HT turnover, accompanied by reduced 5-HT levels, in the amygdala and hippocampus when compared to non-Tg animals [177].

Schizophrenia

Studies on the SIR model has observed decreased cortical (or striatal) 5-HT/5-HIAA [138, 178], decreased frontal cortical and elevated nucleus accumbens and striatal 5-HT and 5-HIAA levels [140, 179]. Deficits in prefrontal 5-HT following SIR is also linked to the behavioural impairments associated with schizophrenia [180]. Evidence of altered 5-HT levels in the NMDA receptor antagonist model is limited with only one study indicating that 5-HT_3 receptor antagonists can attenuate the behavioural hyperactivity caused by PCP [181].

Noradrenaline (NA)

Clinical Correlates

Depression

NA is of major importance in MD (reviewed in [182]). Previous studies have observed reduced levels of NA transporters in the locus coeruleus [183], altered density and sensitivity of frontal cortical α_{2A}-adrenoceptors [184, 185], and a reduction of NA levels in non-compliant MD patients [186]. Further, a positive relationship between urine NA levels and MD has been confirmed [187]. Symptoms of anxiety were also associated with increased NA excretion in the urine [187]. Moreover, studies have demonstrated that low urinary excretion of the NA metabolite, 3-methoxy-4-hydroxyphenylglycol (MHPG), predict a positive response to NA-selective drugs such as imipramine, nortriptyline, desipramine, or maprotiline [188, 189]. These studies illustrate the significance of urinary noradrenergic measurements as a biomarker in guiding treatment selection and predicting efficacy. Expression of adrenoreceptor density has also been investigated in individuals suffering from MD. However, even though dysregulation in alpha and beta-adrenoreceptor systems have been noted, it remains unclear whether alterations in the expression of these receptors are causative in the pathology of MD. Considering the heterogeneity of the disorder, the value of adrenoreceptor dysregulation as a biomarker is unclear [190].

Bipolar Disorder

NA studies in BPD are limited although an increased turnover of NA has been shown to be central to the pathology of the disorder [158]. Furthermore, post-mortem studies in schizophrenia associated with the positive symptoms of the illness describe elevated brain NA levels as mentioned above [191]. NA has also been shown to be one of the primary neurotransmitters targeted during carbamazepine therapy in BPD patients [192].

Schizophrenia

An earlier review found consistent evidence that the positive and negative symptoms observed in schizophrenia are associated with over-activity and under-activity of central NA, respectively [191]. Moreover, increased NA reactivity and/or tone have been linked to anxiety observed in schizophrenia [193].

Preclinical Correlates

Depression

Data relating to NA as a biomarker in preclinical models of MD are limited, although increased catecholamines, including NA, has been reported in limbic regions in FSL rats [194].

Bipolar Disorder

Interestingly, very little data is currently available in animal models of BPD to support the role of NA as a

biomarker, although a preclinical study has suggested a noradrenergic role for lamotrigine, producing an anti-immobility effect in the mouse forced swimming test (FST) while investigating the depressive facet of the disorder [195].

Schizophrenia

Similarly, evidence in support of NA in a schizophrenia animal model is extremely limited. However, a recent study on the SIR model in our laboratory has demonstrated elevated frontal cortical NA as well as striatal NA and MHPG, with decreased frontal cortical MHPG levels, in SIR rats [179]. Earlier SIR studies found an increase in NA turnover in the hippocampus, cerebellum and cortex of Wistar rats [196].

Glutamate and Gamma-Aminobutyric Acid (GABA)

Glutamate and aspartate, and GABA and glycine, are the preeminent excitatory and inhibitory amino acids respectively, in the brain. Their diffuse presence in interneurons (GABA) or as relay neurons and interneurons (glutamate) allows them to play a profound role in regulating the function of most neurotransmitter systems in the brain. As a result of their ubiquitous presence they are implicated in the neurobiology of probably all central nervous system disorders, in particular MD, BPD and schizophrenia. GABA-glutamate interactions have importance in kindling, a mechanism suggested to underlie the development of rapid cycling of mood or psychotic episodes, and how stressful life events adversely impact long-term outcome. GABA pathways exert a permissive role on the kindling action of glutamate, with excessive glutamatergic activity associated with synaptic remodeling and neurodegeneration.

Clinical Correlates

Depression

Abnormalities resulting in an increase in glutamate transmission have been reported in patients with MD [197]. Elevated levels of glutamate act on extrasynaptic NMDA receptors leading to an influx of Ca^{2+} into the neurons, which results in the toxic accumulation of reactive oxygen species (ROS) [198], with increased nitric oxide (NO) production playing a key role in MD pathology and treatment response [199, 200]. We have earlier proposed that the NO pathway may play an important role in relapse and treatment resistance [201] as well as influencing the effect of non-compliance on treatment outcome [202]. In MD, glutamatergic hyper-function seems to be closely related to the lack of 5-HT-ergic and noradrenergic neurotransmission noted to underlie the core symptoms of MD. Indeed, studies examining peripheral blood of MD patients have demonstrated the glutamatergic system to be overly activated [203, 204]. Elevated glutamate levels have also been found in the occipital cortex of un-medicated subjects with MD [205]. Accordingly, reduced glycine binding (where it acts to abrogate NMDA receptor activity) has been described in the frontal and temporal cortex of suicide victims and MD patients [206, 207], leading to hyperglutamatergia. Glutamate, in combination with quinolinic acid (QA), a glutamate agonist derived from the kynureine pathway (see later), may contribute to excitotoxicity in the central nervous system [208]. While several factors may influence the levels of kynurenine and its metabolites (eg. inflammation), a decrease in tryptophan (TRP) may generally be observed in patients suffering from MD resulting in reduced 5-HT levels. In general, depression is associated with lowered TRP, increased indoleamine 2,3 dioxygenase (IDO) activity as well as reduced levels of kynurenic acid [209, 210]. Furthermore, microglial levels of QA have been demonstrated to be upregulated in MD [211].

Bipolar Disorder

A recent review of magnetic resonance spectroscopy (MRS) studies in patients with BPD observed the cingulate and prefrontal cortices to contain higher glutamate levels, and possibly associated with illness state [212], while a decrease in NMDA receptor binding has also been noted in the CA3 region of the hippocampus [213]. In a post-mortem morphological study, an increase in QA positive microglia has been observed in the subgenual anterior cingulate cortex of BPD patients, commensurate with increased glutamatergic activity [211]. Drug therapy with the pyrimidine compound, cytidine, reduces glutamine/glutamate levels in BPD and possibly related to symptom severity, suggesting that the presence of glutamatergic dysfunction is an important factor in the underlying pathology of BPD [214]. Furthermore, the presence of genetic mutations affecting the glutamate pathway has also been suggested to be implicated in BPD [215].

Schizophrenia

Release of DA from cortical and limbic striatal structures are controlled by glutamate-GABA-glutamate feedback loops situated on pyramidal cells of the frontal cortex, the disturbances of which underlie the behavioral manifestations of schizophrenia [216, 217]. GABA'ergic interneurons in the brain stem monoaminergic nuclei, viz. raphe nuclei, locus coeruleus, ventral tegmentum, also modulate ascending serotonergic, noradrenergic and dopaminergic pathways, resulting in tonic *inhibition* of NA and DA release in the PFC, resulting in the emotional, mood and cognitive deficits associated with MD and schizophrenia [78].

The "glutamate" hypothesis of schizophrenia emerged from the observation that NMDA receptor inhibition induces schizophrenia-like behaviors in humans. Cortical hypoglutamatergia compromises DA release in the ventral tegmentum leading to meso-limbic hyperdopaminergic (positive symptoms) and meso-cortical hypodopaminergia (negative symptoms) [217]. Mitochondrial dysfunction, pro-inflammatory cytokines and increased IDO-mediated conversion of tryptophan to QA (supported by clinical evidence for elevated QA [218]), the latter an NMDA receptor agonist, may be directly or indirectly implicated in eliciting glutamate hyperactivity thereby increasing NMDA receptor activation, altered redox balance and oxidative stress [217]. Schizophrenia has also been likened to the kindling phenomenon, a process of increased excitatory glutamatergic activity coupled with a relative loss of inhibitory GABA'ergic tone [217].

In post-mortem schizophrenia studies, deficits of glutamate systems have been described in the temporal cortex, medial temporal lobe and striatal regions [219, 220], together with losses of glutamate uptake sites [221] and increases in NMDA receptors in the same brain regions [207]. Previous studies also emphasize the impact of NO metabolism *via* glutamate and GABA on NMDA receptor mediated neurotransmission in schizophrenia [222, 223]. NO is an important second messenger for the glutamate NMDA receptor pathway, and its overproduction is implicated in schizophrenia. Excessive NO release include impairment of NMDA-receptor mediated neurotransmission, disturbed DA metabolism, excessive ROS generation and mitochondrial dysfunction with cell-death (reviewed in [222, 223]). However, altered NO metabolism is not unique to, or indicative of, schizophrenia as disturbances in this signalling cascade has been noted in MD and BPD [222], as noted earlier. A recent clinical study also indicated elevated GABA and glutamate levels in the medial prefrontal cortex of un-medicated patients, with no alterations in medicated schizophrenia patients, suggesting possible normalization of GABA and glutamate with antipsychotic treatment [224].

Preclinical Correlates

Depression

Under conditions of chronic stress, elevated glucocorticoid levels enhance glutamatergic transmission by increasing the expression of the glutamate ionotropic NMDA receptors, as well as increasing the synthesis and extracellular concentrations of glutamate [225]. Abnormalities resulting in an increase in glutamate–NO transmission have been reported in FSL rats [226]. The possible mechanisms whereby NO can contribute to mood disorders is obscure, although persistent research has highlighted various possibilities including the actions of the NO/cyclic guanosine 3'5'-monophosphate (cGMP) pathway. Modulators of the NO-pathway have also gained relevance in MD research due to NO-inhibitors demonstrating antidepressant effects in models predictive of antidepressant activity [227, 228]. By activating soluble guanylate cyclase (sGC) which converts guanosine 5'-triphosphate (GTP) to the intracellular messenger cGMP [229, 230], NO is enabled to mediate many cellular processes, particularly the regulation of ion channels, activation and inhibition of cyclic nucleotide hydrolysis by phosphodiesterase, activation of G-kinase and modulation of neurotransmitter release [229, 231]. Interestingly, neuronal nitric oxide synthase (nNOS) inhibitors (eg. methylene blue) [232] as well as PDE_5 inhibitors (e.g. sildenafil) [233] have antidepressant and anxiolytic effects in animal models [234, 235] (Fig. **1**), as do clinically relevant antidepressants [228]. These effects however are due to interference with constitutive nNOS-mediated effects and *not* inducible NOS (iNOS), which rather plays an important role in chronic stress associated with inflammation [231] (Fig. **1**). Stressed vs. naive FSL/FRL rats show elevated hippocampal glutamate-NO signalling [224], indicating that a chronic environmental stressor is required in order to demonstrate altered glutamate-NO signalling in FSL rats [236]. This is congruent with the fact that MD involves a prior and/or ongoing chronic stressor [237, 238]. Considering these findings, it is not surprising that NMDA antagonists such as dizocilpine (MK-801) [239], ketamine [240], memantine [241] and others [242] exert antidepressant effects, while disinhibition of glutamate-NO signalling follows antidepressant discontinuation after chronic treatment [243, 244]. A possible explanation could be that NMDA receptor antagonists increase 5-HT levels in the brain [245], while also having a modulatory effect on pathways involved in neuroplasticity and cellular resilience [202].

Preliminary evidence also supports the use of NMDA antagonists such as ketamine in treatment-resistant MD [246]. How this happens still needs illumination, although animal studies have begun to delve into the possible mechanisms involved [247]. The latter work has indicated a mutual cooperation with glutamate AMPA receptors [247], resulting in activation of mammalian target of rapamycin (mTOR) [248] and inhibition of glycogen synthase kinase-3β (GSK-3β) [249] signalling (Fig. **2**). The mTOR pathway plays a pivotal role in protein synthesis by stimulating mRNA translation *via* interaction with its downstream targets [248], and leads to prolonged elevation of synapse-associated proteins in the prefrontal cortex [248]. Diminished activity of the mTOR pathway could underlie synaptic deficits in the PFC as previously reported in MD [250]. Furthermore, this evidence is supported by the behavioural responses to ketamine being blocked in mice which express constitutively active GSK-3β [251]. Considering the contributory role of oxidative stress in MD, inactivation of GSK-3β is linked to the regulation of redox homeostasis *via* stress responsive genes that protect cells against inflammation and oxidative stress [252, 253] (Fig. **2**).

Bipolar Disorder

The standard treatment for BPD, lithium salts, target the glutamate-NO system [254, 255]. Unfortuantely, current literature lacks sufficient data to elaborate on the role of glutamate/GABA as a biomarker in preclinical models of mania and BPD.

Schizophrenia

Decreased glutamate release has been observed in the frontal cortices of Homer1 mutant mice, a putative animal model of schizophrenia [256], while chronic phencyclidine (PCP) administration in rats is associated with a decreased expression of glutamate receptors in the prefrontal cortex [257] and a reduced number of cortical and hippocampal PV-immunoreactive neurones [64]. Confirming this, partial deletion of the NMDA receptor in mice is associated with behavioural alterations akin to that observed in PCP treated mice [258], while increased NMDA receptor binding has been described in the frontal cortex of SIR animals [141].

Neuronal Growth Factors

Growth factors are intricately involved in the survival, growth and differentiation of specific groups of neurons. Their relevance is gaining in importance in the light of

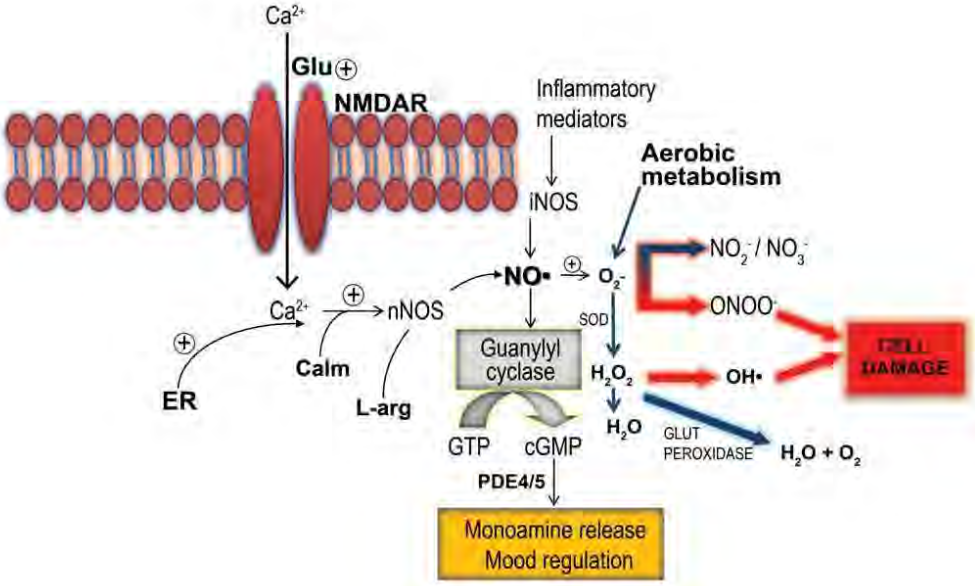

Fig. (1). Glutamate-mediated effects on the cGMP-NO system leading to monoamine release that in turn can be targeted by pharmacological means, eg. PDE5 and NOS inhibitors, as well as known antidepressants. However this pathway can also lead to oxidative stress if excessive glutamate-mediated NO synthesis combines with O_2^- from aerobic metabolism. Also depicted is the effect of inflammatory mediators that promote iNOS-mediated NO synthesis thereby promoting the formation of cell-damaging reactive oxygen and nitrogen species. These pro-oxidative mechanisms can be abrogated by endogenous antioxidant systems such as superoxide dismutase (SOD) and glutathione that act as a sink to quench excessive NO and/or O_2^-.
Abbreviations: calmodulin (Calm); cyclic guanosine monophosphate (cGMP); endoplasmic reticulum (ER); glutamate (Glu); glutathione peroxidase (glut peroxidase); guanosine triphosphate (GTP); inducible nitric oxide synthase (iNOS); l-arginine (L-arg); neuronal nitric oxide synthase (nNOS); nitric oxide (NO); NMDA receptor (NMDAR); phosphodiesterase (PDE); superoxide dismutase (SOD); superoxide (O_2^-); hydrogen peroxide (H_2O_2).

increasing evidence that mood and psychotic disorders are associated with structural brain changes and that alterations in growth factors may precipitate or exacerbate depressive, BPD and psychotic episodes [13, 259, 260].

Brain-Derived Neurotrophic Factor (BDNF)

Clinical Correlates

Depression

Extensive studies have established that altered BDNF plays a pivotal role in MD. BDNF and the transcription factor, cyclic adenosine monophosphate (cAMP) response element binding protein (CREB), are intimately linked biochemically (see Fig. **2**), playing a critical role in cellular resilience and neuroplasticity. Antidepressant treatment up-regulates CREB in the cortex and hippocampus of humans [261]. Both serum BDNF levels and CREB phosphorylation and protein levels are reduced in depressed individuals [262]. Moreover, an inverse relationship exists between serum levels of BDNF and the severity of MD [263], while antidepressant treatment is able to reverse the deficit in BDNF observed in MD [264, 265] and to increase phosphorylation and binding of CREB [266, 267]. BDNF is expressed throughout the body [268], but the exact origins of circulating BDNF remain elusive. BDNF has been shown to originate from several sources including brain neurons, vascular endothelial cells and platelets. It has also been shown to cross the blood-brain barrier [269] so that plasma BDNF levels may reflect central BDNF levels [270]. BDNF regulates synaptic plasticity in neuronal networks and appears to be a particularly relevant factor for mood disorders with associated cognitive dysfunction [271-273].

CREB is responsible for regulating BDNF expression [274]. Activation of CREB is associated with the regulation of synaptic plasticity as well as transcription of specific target genes involved in the production of proteins, BDNF being one example [275, 276]. Post-mortem studies have reported decreased hippocampal BDNF in MD patients who committed suicide, but elevated levels in patients who were being treated with antidepressant agents at the time of death

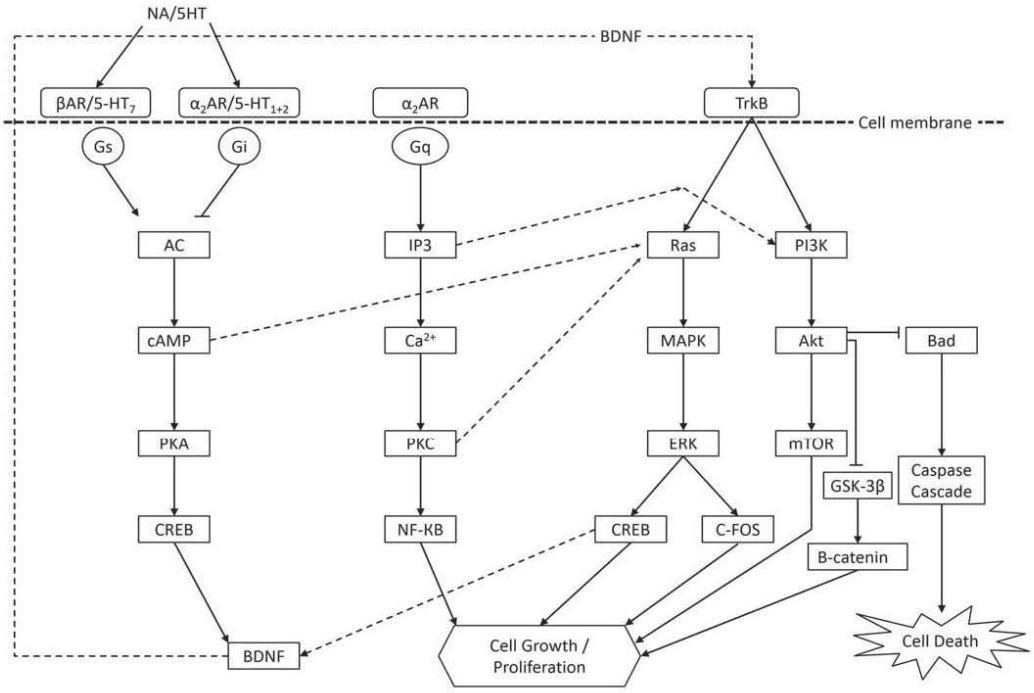

Fig. (2). An overview of key neuroprotective and neurotoxic molecules involved in drug (antidepressant, lithium etc.)-induced neuroplasticity. The monoaminergic system mainly exerts its effect on BDNF expression *via* the cAMP cascade, while BDNF in turn exerts its effect on monoaminergic neurons through the TrkB-receptor *via* the MAPK/ERK cascade and the phospholipase C (PLC) signalling system. Adapted from [342]. Abbreviations: beta adrenoceptor (βAR); alpha-2 adrenoceptor (α2AR); BDNF receptor (TrkB), see text for further details/ abbreviations not noted here.

[268, 277]. Considering the growing evidence for an interaction between MD and metabolic and redox-related conditions [278-280], our group recently showed that altered serum BDNF may be linked to metabolic and redox factors, with BDNF levels indicating either a counter-regulatory action on the effects of glutathione oxidation or that BDNF may mediate the redox effects itself, leading to the development of a mood disorder [281].

Various factors associated with an increased risk of developing MD, e.g. smoking [282, 283] and type II diabetes mellitus [284], have been linked to BDNF deficits. Thus decreased levels of BDNF have been found in smoking individuals when compared to non-smokers [285, 286], while smoking cessation leads to improved BDNF levels [285, 286]. Likewise, serum levels of BDNF have been shown to be significantly lower in subjects with Type II diabetes when compared to healthy controls [287, 288], while cerebral output of BDNF is inhibited in the presence of high blood glucose levels [287]. These findings reiterate the causal link between metabolic diseases, altered BDNF and the development of MD noted earlier. On the other hand, for example, physical exercise has been shown to increase BDNF [289, 290], to be neuroprotective, to improve mood and to have antidepressant effects [291, 292].

Bipolar Disorder

Decreased peripheral BDNF levels have been observed in BPD patients, possibly associated with the pathophysiology and severity of manic symptoms [293]. Exercise has also been shown to decrease depressive symptoms in BPD patients and to even increase the frequency of mania [294]. The latter would indicate that elevated levels of BDNF may not always be beneficial, as has been proposed in a study in patients with MD [281]. The authors suggest that, by adversely affecting resilience, BDNF facilitates activity-dependent plasticity that may translate to a variable effect on mood and other plasticity-dependent functions. In fact, BDNF has been noted to induce paradoxical depressogenic effects [281]. As a mediator of synaptic plasticity, maladaptive

secretion of BDNF (eg. a response to environmental adversity) may set in motion counterregulatory responses that are counterproductive.

Schizophrenia

Decreased peripheral BDNF levels have been observed in schizophrenia [295]. Importantly, a recent study indicated that clinically stable schizophrenia patients present with significantly increased serum levels of BDNF after exposure to cognitive training targeted at improving neuroplasticity [296]. Furthermore, post-mortem studies reported a decreased concentration of BDNF-positive neurons [297] and BDNF concentrations in brain tissue of schizophrenic patients, which include the cortical areas and the hippocampi [298].

Preclinical Correlates

Depression

Antidepressant treatment up-regulates CREB in the cortex and hippocampus of rats [299], while an overexpression of CREB in the dentate gyrus results in antidepressant effects in the FST and a learned helplessness paradigm – both animal models of MD [300]. In the latter, decreased hippocampal BDNF levels were described. Although contrary to that in human subjects, some studies have also noted increased serum levels of BDNF [301, 302]. In FSL rats, serum and whole blood BDNF levels have been found to be significantly increased compared to control but significantly decreased in the hippocampus, with no differences noted in the frontal cortex and CSF [303], suggesting that BDNF is differentially regulated in hippocampus, serum, and whole blood in these animals. The latter is not unlike similar paradoxical findings in humans, where BDNF has been suggested to play a counter-regulatory role [281]. Preclinical studies have indicated that BDNF administration produces antidepressant-like behaviour [304], while antidepressants and electroconvulsive therapy increase BDNF levels [305]. After animals were subjected to repeated stress, they constantly presented with decreased BDNF levels as measured in the hippocampus and serum, while corticosterone levels returned to normal levels, suggesting that changes in brain plasticity occur following a second stressful event [306]. The presence of decreased serum BDNF levels accompanied by normal serum cortisol levels may therefore represent a relevant biomarker for identifying individuals who are more likely to develop depressive symptoms in the subset of a population which may be predisposed to developing affective disorders. These alterations may even be expanded to other disorders provoked by stressful life events, for instance schizophrenia [307].

With smoking having been found to affect BDNF levels in humans, decreased levels of BDNF have also been found in rats repeatedly exposed to nicotine [308]. Similarly, physical exercise also increases BDNF in animals [289, 290].

Bipolar Disorder

BDNF levels are decreased in both the amygdala and hippocampus of rats in the ouabain model of mania, and reversed by lithium [309]. Moreover, in an amphetamine-induced model of mania, BDNF was also decreased in the hippocampus and increased by valproate and lithium [119].

Schizophrenia

Neonatal PCP administration produced a sustained elevation of BDNF in the hippocampus and the entorhinal cortex of 8-week-old rats [310]. However, studies in the SIR model observed significantly reduced medial PFC BDNF levels [311] as well as decreased hippocampal BDNF [312].

Insulin-Like Growth Factor

Insulin-like growth factor-1 (IGF-1) is involved in regulating peripheral cell growth and metabolism [313]; and plays a crucial role in the growth and differentiation of nerves and also in the synthesis and release of neurotransmitters [314].

Clinical Correlates

Depression

Unfortunately, there have not been sufficient clinical studies to determine whether peripheral IGF-1 is altered in MD patients or following antidepressant administration.

Bipolar Disorder

In BPD patients, a previous study observed altered IGF signalling in post-mortem brain tissue [315].

Schizophrenia

Antipsychotic-free schizophrenia patients have been found to present with a decrease in plasma IGF levels [316].

Preclinical Correlates

Depression

Unfortunately, we are not aware of any extensive IGF-related data in established animal models of MD. Nevertheless, preclinical studies have indicated that peripheral IGF-1 administration reduces immobility in the FST [317], increases central BDNF mRNA [318] and produces antidepressant-like behavioural responses in mice exposed to CMS [317]. Moreover, after chronically treating rats with antidepressants, elevated IGF-1 expression was observed in the brains of these animals [319]. Finally, IGF-1 has been found to regulate adult hippocampal neurogenesis in rats [320].

Bipolar Disorder, Schizophrenia

To the best of our knowledge there is no pre-clinical data in established animal models of IGF-1 as a preclinical biomarker in BPD or schizophrenia.

Vascular Endothelial Growth Factor

Vascular endothelial growth factor (VEGF) acts as a neurotrophic factor, is a cytokine implicated in angiogenesis [321] and has been related to the vascular niche hypothesis of adult neurogenesis [322]. This hypothesis attributes increases in the proliferation of neurons in the adult hippocampus to VEGF-induced angiogenesis. VEGF is purported to play a role in several features associated with neuronal growth, including neuronal regeneration and differentiation as well as axonal outgrowth [323].

Clinical Correlates

Depression

MD patients present with higher plasma VEGF levels which can be reversed with antidepressant treatment [324], while earlier studies have confirmed said increase in MD [325]. Furthermore, remitted MD patients have significantly elevated VEGF levels, while MD patients with a family history of psychiatric disorders also have higher baseline levels of VEGF, compared to MD patients without a family history and healthy controls [326]. This may be indicative of a role for VEGF in the *pathology* of MD, possibly hinting of a neuroprotective role to counter reduced neurogenesis in MD.

Bipolar Disorder

BPD patients present with higher plasma VEGF levels during acute episodes vs. healthy controls [327], emphasizing that a depressive and manic episode in mood disorders may be associated with the neuroprotective role of VEGF. Interestingly, a recent study indicated that VEGF mRNA levels were significantly decreased in BPD patients treated with lithium vs. healthy controls [328], suggesting that VEGF may be a useful marker in BPD and as an indicator of lithium response.

Schizophrenia

VEGF data in schizophrenia patients are limited, although previous studies have not observed any differences in serum VEGF in schizophrenia vs. healthy individuals [285]. However, significantly reduced levels of VEGF mRNA have been observed in the DLPFC of patients with schizophrenia [329].

Preclinical Correlates

Depression

The relationship between central and peripheral levels of VEGF still needs clarification. In a genetic rat model of MD, Elfving and colleagues found decreased levels of VEGF in the brain but no variations in serum VEGF levels [301].

Bipolar Disorder

To the best of our knowledge, there is no data available on VEGF as a biomarker in preclinical models of BPD.

Schizophrenia

Similarly, no pre-clinical data is currently available on VEGF in the SIR or NMDA antagonist models of schizophrenia. However, a pre-clinical study did observe that VEGF levels are increased in rat hippocampi following 14 days haloperidol or olanzapine treatment [303]. Interestingly, in the case of haloperidol treatment this increase was lost 45 days later, while olanzapine treatment bolstered the initial increase in VEGF [330], reaffirming that first and second generation antipsychotics are not therapeutically equivalent. This underlines VEGF as a possible marker in schizophrenia treatment but not diagnosis *per se*.

Neuronal Resilience Markers

Several neurochemical markers have been associated with neuroprotective effects and positive antidepressant treatment response. With the increased evidence for a neurodegenerative profile for MD, BPD and schizophrenia and the progressive nature of these illnesses, identifying neuroresilience markers is gaining in relevance. In this regard, resilience markers linked to the BDNF pathway are especially attractive.

Stress and environmental adversity is a common thread throughout all three illnesses under review [252, 331, 332]. Stress-induced increases in glucocorticoid levels have been shown to decrease the synthesis of neurotrophic factors, particularly BDNF, which is an effective neuroprotective factor and protagonist of neurogenesis [333]. These neurotrophic effects are mainly mediated through inhibition of cell death pathways and activation of mitogen-activated protein kinases (originally extracellular signal-regulated kinases or MAPK/ERK) signalling pathways and phosphotidylinositol-3 kinase (PI-3K)/Akt (protein kinase B) pathways (see Fig. **2**) [334]. As noted earlier, BDNF expression is decreased during MD, BPD and schizophrenia, a response that is reversed by effective pharmacological treatment [295, 335-337]. Furthermore, increased structural atrophy observed in treatment resistant MD has been correlated with greater decreases in BDNF levels [338] in patients failing to respond to SRI treatment compared to treatment responsive patients.

The cAMP cascade is activated following increased serotonergic and adrenergic receptor activity which results in downstream activation of CREB [339]. The ensuing elevation in cAMP ultimately leads to increased BDNF expression which subsequently activates the MAPK/ERK pathway, a major pathway involved in cell growth and proliferation [340, 341] (see Fig. **2**). Monoaminergic neurons experience an increase in growth following MAPK/ERK pathway activation thereby accounting for how BDNF modulates the monoaminergic system [342].

Activation of the PI-3K cascade by BDNF leads to phosphorylation of Akt, a molecule at the crossroad of cell survival and cell death [343] (see Fig. **2**). Activation of Akt following phosphorylation leads to enhanced activity of mTOR which is responsible for regulating the expression of several genes involved in cell growth, particularly a group of synapse-associated genes that have been directly linked to neuroplastic events [248]. Conversely, inactivation of Akt by dephosphorylation leads to a decrease in phosphorylation and subsequent activation of Bcl-xL/Bcl-2-associated death promoter (Bad), a pro-apoptotic molecule [343]. It is therefore evident that in combination, the MAPK/ERK and PI-3K pathways are largely accountable for the neuroplastic events occurring during antidepressant response and, furthermore, directly links the actions of BDNF to these processes.

Clinical Correlates

Depression

Decreased cAMP levels and lower MAPK/ERK pathway activity has been associated with MD, which has been shown to be reversed by increasing BDNF levels [344].

Bipolar Disorder

GSK-3β has been demonstrated to be an important role-player in BPD with lithium, an inhibitor of GSK-3β, having served as a mainstay in the treatment of BPD. It also regulates various proteins and is involved in neuroplasticity and neurotransmission [345]. Therefore, agents involved in the modulation of GSK-3β and its downstream pathways may serve as valuable biomarkers in the diagnosis and treatment of BPD – e.g., several molecules involved in both cell survival and apoptosis, such as CREB [346] and p53 [347], respectively. GSK-3β also plays an important role in the regulation of the Wnt [347] and PI-3K [348] signalling pathways linked to cellular resilience [348, 349] (Fig. **2**). It has also been suggested that progranulin (PGRN) may serve as a neurotrophic factor- modulating neurite outgrowth as well as neuronal differentiation and survival [350]. Furthermore, plasma levels of PGRN are decreased in BPD patients [351, 352] and GSK-3β has been implicated in mediating PGRN activity [351]. GSK-3β protein expression is decreased in the platelets of BPD patients [349]. Even though previous studies could not find alterations in brain expression of GSK-3β, decreased protein expression in platelets can be reversed by mood stabilizers – but not antidepressants – thereby emphasizing a valuable role for GSK-3β as a peripheral biomarker and even a state – rather than trait – marker of BPD [353].

Schizophrenia

A recent post-mortem study in patients with schizophrenia found increased levels of various proteins involved in the MAPK- and cAMP-associated pathways, as expressed in frontal cortical structures [354]. In line with these observations are studies indicating alterations in several proteins in the MAPK-associated pathway: extracellular signal-regulated kinase (ERK)-2, immediate early genes c-fos and c-Jun levels were elevated in the thalamus on both protein and transcription level, whereas c-Jun protein and Elk-1, CREB, and ATF-2 protein levels were elevated in the cerebellar vermis [355, 356]. Moreover, other proteins involved in the MAPK pathway, including MEK1, MEK2, RSK1, B-Raf, and CREB were found to be reduced in the frontal cortex of schizophrenia patients [357]. With regards to the cAMP pathways, decreased DA- and cAMP-regulated phosphoprotein Mr 32 kDa (DARPP-32) was observed in the frontal cortex and thalamus of schizophrenia patients [358, 359]. Furthermore, a recent review highlights numerous evidence and theories in support of a novel mTOR based hypothesis of the neuropathology of schizophrenia [360]. Control of protein synthesis is the primary role of this signalling cascade while it is also regulated by known extracellular and environmental factors implicated in the pathology of schizophrenia [360].

Preclinical Correlates

Depression

Blocking MAPK signalling leads to depressive-like behaviour in the FST in rats and inhibits the antidepressant effects of ketamine [340]. These findings provide some insight on how glutamate-NMDA signalling interacts with monoaminergic-cAMP pathways to mitigate a faster onset of action or to treat refractory MD. Furthermore, SIR in rats, a putative neurodevelopmental animal model of MD and schizophrenia [18], leads to an enhanced expression of mitogen-activated kinase phosphatase and apoptosis-related genes in the prefrontal cortices of Sprague-Dawley rats [361].

Bipolar Disorder

Transgenic mice that overexpress GSK-3 present with decreased habituation and an increase in activity that has been related to hyperactivity in mania [362]. β-catenin, a downstream molecule of GSK-3 (Fig. **2**), was found to be decreased in the hippocampi of black Swiss mice, a putative model of mania [363], while the behaviour of transgenic mice overexpressing β -catenin was found to have a behavioural phenotype similar to that of lithium-treated animals [364]. Of significance is that lithium stabilizes β-catenin by inhibiting GSK-3β thus reducing neuronal vulnerability to apoptosis [365].

Schizophrenia

Gururajan and Van den Buuse [360] have explained the involvement of mTOR in schizophrenia by referring to numerous animal models of schizophrenia. However, direct measurement of neuronal resilience markers in the SIR model and the NMDA antagonist model is limited, with only one study indicating that MK-801 administration elevates phosphorylation of MAPK in the frontal cortex of rats [19].

Oxidative Stress Markers

Normal oxidative metabolism in cells results in the production of various ROS. Oxidative stress occurs when cellular antioxidant defence mechanisms, such as SOD, catalase, glutathione peroxidase, fail to counterbalance and control endogenous production of ROS such as $O_2^{\cdot}$ and H_2O_2. This leads to a free radical attack on proteins, DNA and lipids [366, 367]. SOD is the primary defense against oxidative stress by converting $O_2^{\cdot}$ to H_2O_2 [368]. Hydrogen peroxide in turn is converted to water and glutathione (GSSG) by catalase and glutathione peroxidase [369], with GSSG rapidly being converted to reduced glutathione (GSH) by glutathione reductase [370]. The brain has relatively low levels of antioxidant defences, as well as a high lipid content that is highly susceptible to attack by ROS [371]. Thus, a reduction in GSH, and an increase in GSSG, is regarded as being indicative of increased oxidative stress.

Many of the changes in oxidative status may be directly related to increased inflammatory response due to the presence of other systemic illnesses, such as endocrine and metabolic disorders and cardiovascular disorders [281]. Furthermore, changes in certain neurotransmitter systems in the brain, especially glutamate and GABA, increase the risk of oxidative stress in the brain and subsequent neuronal oxidation and cell death [279]. Moreover, oxidative stress in its own right may mediate altered monoaminergic activity [372] that underlies the pathology of many neuropsychiatric illnesses associated with oxidative stress [373]. One of the more prominent redox active molecules released by changes in glutamate activity in the brain is NO, and which is well

described as being a contributing factor towards the development of MD [374], schizophrenia [223] and possibly BPD [375]. In this regard, both constitutive NOS-, such as nNOS, and iNOS-mediated NO synthesis needs to be considered, with nNOS being more involved in neurotransmission and iNOS in inflammation. Fig. **1** provides an outline of how glutamate, NO and redox systems interact to produce oxidative stress.

Clinical Correlates

Depression

In recent years MD has been associated with several changes in redox status, presenting as either an increase in oxidative stress and/or diminished oxidative defence systems [279]. Elevated plasma malondialdehyde (an indication of lipid peroxidation) levels and susceptibility of red blood cells to oxidation, as well as an increase in serum SOD activity, has been observed in MD patients [376, 377]. However, Srivastava *et al.* [378] found no alterations in the activities of SOD and glutathione peroxidation in polymorphonuclear leukocytes from patients with MD. In their clinical study, Berk and colleagues noted only limited support for the role of antioxidant and glutathione precursor, N-acetyl cysteine (NAC), as an adjunctive therapy for MD, although further such clinical studies are required [379]. A high incidence of co-morbid metabolic syndrome and MD have been observed [380] with inflammation a major mediator in the development of both MD and metabolic syndrome [381]. In support of this, substantial evidence exists linking insulin- and NO-mediated pathways in the brain. In fact, insulin upregulates expression of nNOS [382] while a role for increased NO and insulin/peroxisome proliferator-activated receptor (PPAR) signalling has been noted following stress, thus presenting as a susceptibility factor in the subsequent development of MD [383].

Bipolar Disorder

BPD patients present with changes in antioxidant enzymes, for example Andreazza *et al.* [384] reported manic and depressive phases to be associated with increased SOD activity, but unaltered activity in euthymia. This is corroborated in part by Machado-Vieira [260] who found untreated manic bipolar patients to present with increased activity of SOD. Furthermore, patients who were euthymic presented with decreased catalase activity [384], while activity was increased in manic patients who did not receive treatment [260]. Increases in lipid peroxidation due to oxidative stress unrelated to the phase of illness have also been reported [260, 384]. In addition, BPD patients were found to express increased lipid peroxidation in the cingulate cortex [385], while clinical studies have indicated that the antioxidant, NAC, is effective as adjunctive treatment in BPD [386-388].

Schizophrenia

Evidence has accumulated in recent years that antioxidant systems are impaired in schizophrenia [389]. Gawryluk *et al.* [390] reported reduced levels of GSH in post-mortem prefrontal cortices of patients with schizophrenia. Do and colleagues [391] found a 52% decrease in GSH levels in the prefrontal cortex of patients with schizophrenia. Interestingly, a significant deficit in total antioxidant status was inversely associated with some domains of cognitive deficits in schizophrenia patients, such as attention and immediate memory [392]. Moreover, plasma SOD activity was negatively correlated with positive symptoms in first-episode schizophrenia patients [393]. Lower levels of total antioxidant status, catalase and glutathione peroxidation has been described in first episode schizophrenia patients, with GSH levels positively associated with executive function [394]. Furthermore, clinical studies have described the clinical utility of NAC as an adjunctive treatment in schizophrenia [387, 395], as well as the combination of ω-3 fatty acids with vitamins E and C [396]. However, we have been unable to demonstrate efficacy for ω-3 polyunsaturated fatty acids (PUFA) plus alpha-lipoic acid in preventing relapse in patients who had responded well to antipsychotic treatment after a single episode of psychosis [397]. Further studies in this regard are nevertheless warranted.

Preclinical Correlates

Depression

With regard to significant animal studies, SOD and catalase activities have been found to be decreased in rats exposed to a chronic stress model of MD, and could be reversed by tianeptine [398]. Similarly, animal studies have found NAC to be as effective an antidepressant as imipramine [399]. Of particular interest is that exposure to ozone worsens anxiety, cognitive and depressive-like behaviour in the FSL rat model of MD, suggesting that genetically susceptible individuals exposed to high levels of oxidative stress are at higher risk of developing mood and/or anxiety disorders [400]. Moreover, exacerbating levels of oxidative stress (eg. with ozone) can attenuate antidepressant action [400]. In fact, stress-related activation of the NMDA-NOS cascade has been proposed to be a vulnerability factor in stress-sensitive FSL rats [236].

Considering the connection between inflammation, NO and insulin/PPAR signalling in MD, it is not surprising that PPARγ has been associated with suppression in immune response through its ability to inhibit the expression of inflammatory cytokines [401] and to have actions on pathways involved in apoptosis, cellular proliferation and cellular resilience [402]. Moreover, it has been demonstrated that metabolites of 5-HT act as PPARγ-agonists in the periphery [403], which further indicates the possibility of an underlying link between biochemical pathways of mood disorders and metabolic syndrome. The recently discovered prostaglandin and endogenous PPARγ ligand, 15d-PGJ2, presents with anti-inflammatory properties [404], increases the neuronal metabolism of glucose, prevents stress-induced suppression of glutamate uptake [405] and has been suggested to be a possible marker for psychiatric diseases [406]. Indeed, animal studies have also decribed the antidepressant activity of PPARγ agonists [407].

Bipolar Disorder

SOD was found to be increased and catalase (CAT) decreased in an ouabain-induced rat model of BPD [408], while lithium and valproate protect against amphetamine-

induced oxidative stress in the same model, thus further supporting a role for oxidative stress in BPD [119]. When considering the proposed role of oxidative stress and inflammatory process in BPD it is important to note that some mood stabilizers, eg. lithium, valproate (VPA), carbamazepine, lamotrigine, suppress (brain) pro-inflammatory mediators such as cyclooxygenase-2 (COX-2) and prostaglandins [409-411], indicating possible anti-inflammatory properties. Moreover, NAC reverses and protects against oxidative protein damage induced by d-amphetamine in a rat model of mania [412].

Schizophrenia

Animal studies, too, have confirmed that schizophrenia involves redox imbalance and oxidative stress. Using the ketamine challenge model, researchers have noted a decreased expression of PV-interneurons (relating to GABA; see section 2 and section 3.1.4) in the hippocampus [413] – a recent review by Bitanihirwe and Woo, 2011 [366] explain that GABAergic pathways innervate primary neurons and could increase intracellular calcium levels and subsequently trigger oxidative damage. Elevations in nicotinamide adenosine dinucleotide phosphate (NADPH) oxidase 2 (Nox2) are observed in the prefrontal cortex of rats exposed to the SIR model of schizophrenia [414]. Nox2 is a major source of ROS and controls glutamate release in the prefrontal cortex ([415]; reviewed in [416]). We have also observed increased SOD activity, a decrease in the ratio of oxidized vs. reduced glutathione and an increase in lipid peroxidation in both the striatum and frontal cortex of SIR rats [373]. Importantly, all the latter alterations could be reversed with clozapine treatment [373]. The latter not only emphasizes the validity of these findings but also highlights the ability of contemporary treatments like clozapine to address disturbances in redox balance. Preclinical studies in the SIR model of schizophrenia also confirmed NAC's utility as adjunctive treatment to an anti-psychotic [137].

Inflammatory Markers

Increasing evidence indicates that inflammation may have a critical role in the pathophysiology of MD, BPD and schizophrenia [386, 417]. Inflammation is also a closely associated phenomenon with oxidative stress, discussed above. Cytokines play a crucial role as signalling molecules in the immune system and have the ability to cross the blood–brain barrier (BBB), granting it both central and peripheral activity [418]. Cytokines have been demonstrated to exert activity in almost every area relevant to the pathophysiology of MD, BPD and psychotic disorders including neurotransmitter metabolism, neuroendocrine function, and neural plasticity [386, 419-421]. The pro-inflammatory cytokines, such as interleukin (IL)-1, tumor necrosis factor (TNF)-α, and IL-6, can inhibit neurogenesis *in vivo* [422, 423], induce apoptosis [424, 425] and negatively affect synaptogenesis, synaptic plasticity and connectivity, and also the structure of synaptic membranes [426, 427]. On the other hand, anti-inflammatory cytokines such as IL-10 and IL-4 dampen the immune and inflammatory response [421] so that an inflammatory state is generally determined by an imbalance between pro- and anti-inflammatory mediators. Fig. **3** provides an outline of how inflammatory mediators and oxidative stress are related to regulate immune response and redox status.

Clinical Correlates

Depression

A strong relationship has been demonstrated between MD and the presence of inflammation and its associated inflammatory mediators [428, 429]. These mediators include the pro-inflammatory cytokines, IL-1, -2, -6 and -8, interferon (IFN)-γ and TNF-α [430] that, when administered to a healthy individual, may induce sickness behaviour [431, 432]. Sickness behaviour describes a state in which many of the symptoms coincide with that of MD [420]. Furthermore, it has been proposed that constant elevation of cytokine levels may lead to neurotransmitter changes which are interpreted by the brain as stressors that further allow these molecules to contribute to the development of MD [429]. Not only do pro-inflammatory cytokines contribute towards altered neurotransmitter metabolism, neuroendocrine function, synaptic plasticity and behaviour characteristic of MD [419, 433-435], but also stimulate hypothalamic-pituitary-adrenal (HPA)-axis hormones as well as CRH in both the hypothalamus and the amygdala. The latter play an important role in fear responses and anxiety-related behaviour [419].

Cytokine-induced MD is associated with alterations in 5-HT metabolism through the activation of IDO [436], as well as alterations in CRH function [430, 436]. Importantly, MD induced by IFN-γ also involves the activation of iNOS [437], the latter known to play an important role in stress-related inflammation [438]. Peripheral levels of IL-1, IL-6 and TNF-α is increased in patients with MD [299, 439], and these effects are normalized following antidepressant treatment [440, 441]. Increased levels of IL-1 and IL-6 have also been measured in the CSF of patients suffering from MD [442]. Furthermore, an elevation in IL-6 levels has been proposed as an early marker for cognitive symptoms and has been found to correspond to the severity of MD as well as increased activity of the HPA axis [430, 443, 444]. Interestingly, IL-6 levels have been demonstrated to be increased in patients with treatment-resistant depression compared to treatment responders [445], which indicates that altered IL-6 levels in the blood of depressed individuals may serve as a marker of possible response to antidepressant treatment [208]. In line with these findings, a recent clinical study found that administration of the non-specific COX inhibitor acetylsalicylic acid leads to an improved onset of action during fluoxetine treatment and also increased the response rate to the drug when compared to patients receiving only fluoxetine [446].

Bipolar Disorder

BPD may be associated with moderately increased plasma levels of pro-inflammatory cytokines, such as IL-6 and TNF-α, along with increased IL-1, and IL-1 receptor antagonist protein, while elevated mRNA levels have been observed in post-mortem frontal cortex of BPD patients [447-449].

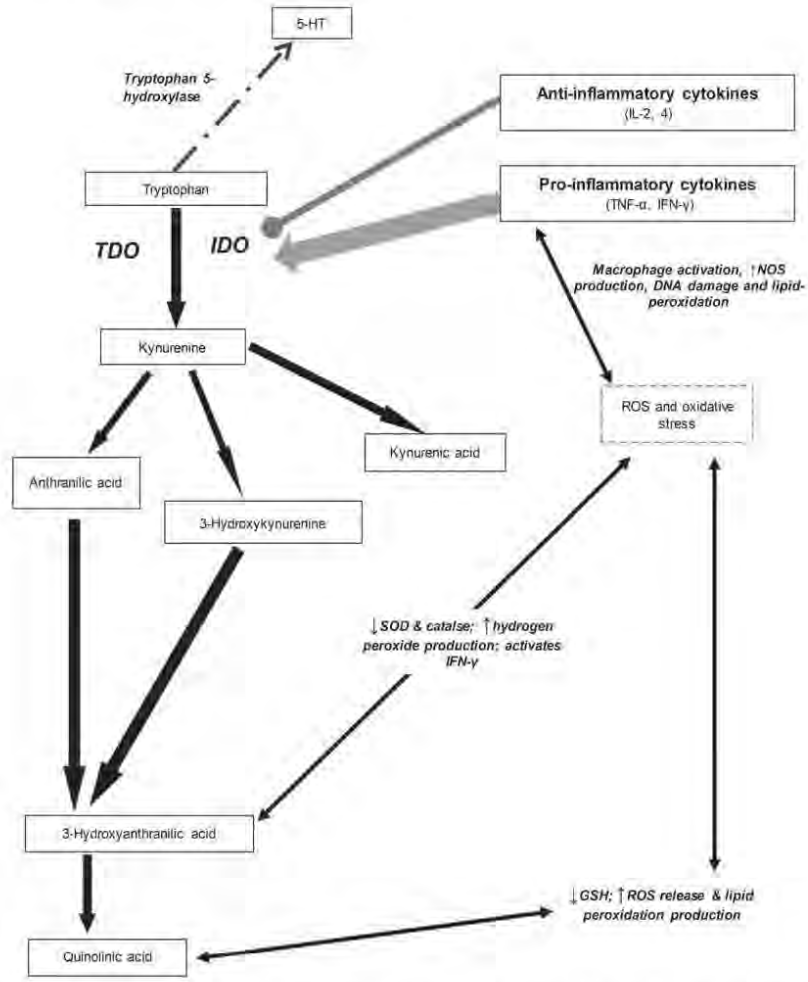

Fig. (3). A simplified diagram of the kynurenine pathway, indicating the principle enzymes, IDO and tryptophan-2,3-dioxygenase (TDO), and the subsequent formation of kynurenine and its metabolites from TRP. The diagram indicates the inter-relationship of kynurenine metabolites, particularly QA, kynurenic acid and 3-hydroxyanthranilic acid. This diagram also depicts the activation and inhibition of IDO *via* pro- and anti-inflammatory cytokines respectively, as well as the influence of oxidative stress processes that will eventually determine cellular resilience or susceptibility to neurotoxic insults. Increased activity of the tryptophan-kynurenine synthesis (by TDO/IDO) will also diminish the availability of tryptophan for serotonin synthesis *via* tryptophan-5-hydroxylase, with resulting effects on mood and behavior.
Abbreviations: glutathione (GSH); interferon (IFN)-γ; interleukin (IL)-2 and -4; nitric oxide synthase (NOS); reactive oxygen species (ROS); serotonin (5-HT); tumour necrosis factor (TNF)-α. Adapted from [452, 453].

Schizophrenia

Schizophrenia patients and their first-degree relatives have been found to present with significantly elevated pro- vs. anti-inflammatory cytokines [450], as well as a significant elevation in pro-inflammatory cytokines in first episode psychosis patients, with a positive correlation between IL-6 and duration of illness [418]. IL-6 has been found to be significantly increased in early and late stage schizophrenia, with IL-10 reported to decrease in the late stages [451].

The presence of elevated levels of IL-1 in the CSF has been suggested as a marker of acute psychotic relapse, while

immune-inflammatory dysfunctions may be implicated in the underlying processes mediating relapse, considering that the relationship between cytokine abnormalities and acute exacerbations of schizophrenia appear not to be related to treatment with antipsychotics. On this point, certain cytokines have been suggested to represent state markers for acute exacerbations of psychosis (see [217] for review). However, a number of studies have described inconsistent effects on plasma/serum cytokines, namely IL-4 [452], [453], IL-6 (reviewed in [454]), IFN-γ [452, 455]; reviewed in [456], and TNF-α (reviewed in [456]). Furthermore, a positive correlation between the severity of cognitive deficits and levels of IL-1β, IL-6 and TNF-α have been described in schizophrenia [252].

Preclinical Correlates

Depression

Acetylsalicylic acid has proved to be an effective augmentation agent to fluoxetine in rats resistant to standard fluoxetine treatment using the CMS animal model of MD [457]. Furthermore, it has been demonstrated in gene-environment animal models of MD that peripheral levels of inflammatory markers and regulators of metabolic pathways, including glucocorticoids, MAPK's and cytokines are altered in these animals when compared to healthy controls [458].

Bipolar Disorder

Using the ouabain model of BPD, investigators have noted an increased activation of signalling pathways linked to inflammation in the brain [459].

Schizophrenia

Elevated pro-inflammatory cytokines (TNF-α, IFN-γ) and decreased anti-inflammatory (IL-4) and dual-action (IL-6) cytokines has been described in the SIR model, and which are for the most part reversed with clozapine treatment [137]. More interesting is that NAC is able to augment select behavioural, neurochemical and anti-inflammatory responses to clozapine in SIR animals [137]. Other pre-clinical studies have also indicated that IL-6 and TNF-α have direct inhibitory effects on adult hippocampal neurogenesis [422, 460], which may attenuate antidepressant and antipsychotic efficacy by decreasing hippocampal neurogenesis or interfering with the neurogenic properties of these drugs.

Kynurenine Pathway Markers

An important link with the inflammatory cascade is the kynurenine pathway whereby TRP is catabolized to kynurenine *via* hepatic TDO and *via* IDO in the central nervous system, lungs and placenta [461] (Fig. **3**). Kynurenine is then metabolised to either kynurenic acid (KYNA) or 3-hydroxykynurine (3-OHK) and then to anthranilic acid, 3-hydroxyanthranilic acid (3-OHAA) and QA [462]. Conversion along this highly regulated pathway accounts for the metabolism of approximately 80% of non-protein bound TRP, an essential amino acid necessary for the synthesis of 5-HT (Fig. **3**; [463]). QA, a NMDA receptor agonist and excitotoxin, along with 3OHK, a mediator of neuronal apoptosis, and 3-OHAA, a free radical, are all capable of inducing neurodegenerative changes in the brain [462, 464, 465]. KYNA, on the other hand, acts as an antagonist at the facilitatory glycine site on the NMDA receptor ion channel, thus having potential neuroprotective properties [466]. The activation of IDO by pro-inflammatory cytokines (e.g. INF-γ and TNF-α) in the CNS also leads to increased TRP degradation into kynurenine and QA, thereby reducing the bioavailability of TRP for 5-HT synthesis (Fig. **3**) (reviewed in [467]). Hence, increased pro-inflammatory and decreased anti-inflammatory actions in the CNS can contribute to central 5-HT deficiency, which plays an important role in the pathogenesis of MD and BPD but also the negative symptoms of schizophrenia [386, 468, 469].

Clinical Correlates

Depression

Previous studies have observed decreased TRP and 5-HT along with increased kynurenine in the peripheral blood of depressed patients receiving the pro-inflammatory cytokine, IFN-γ [470, 471]. Significant decreases in the concentration of KYNA have also been observed in the plasma of depressed individuals [465]. Alterations in the symptoms of depressed patients were significantly positively correlated with kynurenine and negatively correlated with levels of 5-HT [471]. A previous study also indicated the correlation of increased IL-6 production *in vitro* with decreased TRP levels in depressed patients, emphasizing the influence of IL-6 on 5-HT metabolism *via* TRP in these patients [444]. A post-mortem study also indicated elevated QA levels in the brain of patients with MD [472]. This supports the hypothesis that the development of depressive symptoms may be mediated by increased TRP metabolism *via* IDO along the KYN pathway [436, 473].

Bipolar Disorder

Levels of TRP and kynurenine-dependent TRP index have been demonstrated to be diminished in bipolar mania [467]. Furthermore, increased kynurenine was found post-mortem in the ACC in patients with BPD, which corresponded with increased density and intensity of TDO positive glial cells [474].

Schizophrenia

In patients with schizophrenia clinical post-mortem studies found elevated levels of TRP, 3-OHAA, kynurenine and QA in various brain regions [475, 476]. Moreover, individuals suffering from schizophrenia present with increased levels of TRP in the CSF and plasma whether they received treatment or not [477, 478]. Although increases in KYNA levels have been observed in the post-mortem brain tissue of schizophrenia patients who received treatment [218], Myint and colleagues [479] have described a significant reduction in plasma levels of KYNA accompanied by a decrease in the neuroprotective ratio (a measure of the relationship between KYNA and kynurenine levels) in treated and non-treated patients suffering from schizophrenia.

Preclinical Correlates

Depression

In a stress-induced rat model of MD the kynurenine pathway was observed to be activated, leading to increased expression and activity of hepatic TDO as well as its expression in the cortex [480]. This increased TDO activity was associated with elevated circulating kynurenine concentration and a reduction in circulating TRP concentration [480]. Furthermore, induction of depressive and anxiety-like symptoms *via* the administration of a viral mimetic led to reductions in BDNF signalling and activation of the kynurenine pathway [481]. Recently, the potential for using the kynurenine 3-monooxygenase inhibitor, Ro 61-8048 (to elevate kynurenine levels), as an antidepressant has been suggested ([482], while the TDO inhibitor, allopurinol, has been noted for its antidepressant-like effects in rats [480].

Bipolar Disorder

Current animal models of BPD have not produced data related to the role of the kynurenine pathway, and data is this regard is eagerly awaited.

Schizophrenia

Recent pre-clinical studies in the SIR model of schizophrenia have described elevated plasma TRP, kynurenine, anthranilic acid, 3-OHAA and QA with reduced KYNA and neuroprotective ratio, with all alterations reversed with the antipsychotic, clozapine [137, 483].

GENETIC MARKERS

Full remission of a psychiatric illness is often impeded by variability in an individual's response to psychoactive drugs, so that being able to predict responses to psychotropic drugs holds great promise in improving treatment outcome. Genotyping, where proteins involved in neurotransmission and cellular signalling specific for illness pathology and drug response are identified and targeted, will allow the selection of an appropriate psychopharmacologic agent to best suit an individual's pathologic and metabolic characteristics.

Clinical Correlates

Depression

The development of MD has been demonstrated to be critically influenced by genetic factors which provide the opportunity to investigate mechanisms underlying the disorder [13]. Polymorphisms on genes encoding the 5-HT transporter, 5-HT_{2A}-receptor, BDNF, and tryptophan hydroxylase have been identified as candidate genes implicated in the pathology of MD [484], while several studies have suggested the Met/Met genotype (of the gene coding for COMT) to be predictive of antidepressant response rates [485, 486]. The Val/Met polymorphism in MD has not been consistent and has not been linked to any single effect [487]. Nevertheless, although contradictory findings have been presented, research into polymorphisms on the COMT variant may still hold promise [488].

Bipolar Disorder

Efforts to identify possible genetic markers and isolation of genes implicated in the pathogenesis of BPD have been challenging and at times contradictory [489]. Nevertheless, a recent study by Mahon *et al.* [490] suggested that abnormalities in lower white matter in the temporal lobe might be a marker for genetic risk of BPD. It has been suggested that the presence of an unknown gene on chromosome 12q22-q23.2 may predispose especially men to develop MD and perhaps even BPD [491]. Various studies have explored a possible link between variations in tryptophan hydroxylase II (TPH2) [492, 493] and the development of BPD, although its link with schizophrenia has been dismissed [494]. As in MD, the Val/Met polymorphism in BPD has not been consistent and has not been linked to any single effect [487].

Schizophrenia

Although the heritability for schizophrenia is estimated to be as high as 70%, the illness clearly does not have a pattern of inheritance in any population or even in single families that is consistent with the effect of a single gene [14]. Moreover, according to Riley and Kendler [495], genetics is not a determinant of schizophrenia but rather a means by which the disease is mediated. It is therefore necessary for several genes to interact and be influenced by environmental factors to lead to a patient presenting with the array of symptoms associated with schizophrenia. Like in MD and BPD, the Val/Met polymorphism is involved in predisposition to the development of schizophrenia [487] but further work is needed. The introduction of the SchizophreniaGene (SzGene) database by Allen *et al.* has made a substantial contribution to schizophrenia genomics [496]. Using the SzGene database, Sun *et al.* [497] described a collection of highly ranked genes that may be utilized as a working blueprint in the future. These genes, amongst others, include:

i Disrupted in Schizophrenia 1(DISC1). A gene that encodes a protein that has been implicated in neurite outgrowth and cortical development by interacting with other proteins.

ii Dystrobrevin Binding Protein 1 (DTNBP1). A gene that encodes a protein purported to influence organelle biogenesis associated with melanosomes, platelet dense granules, and lysosomes.

iii Catechol-O-Methyl Transferase (COMT). A catabolic enzyme involved in the degeneration of various molecules that are biologically active, including DA.

iv D-Amino acid Oxidase (DAO). This is a NMDA-receptor-mediated signalling gene.

v Regulator of G Protein Signalling 4 (RGS4). This is a regulatory molecule that acts as a GTPase activating protein.

vi Neuregulin 1 (NRG1). This is a protein essential for normal development and function of the nervous system (reviewed in [498]).

vii Metabotropic Glutamate Receptor 3 (GRM3) gene. This gene, coding for the GRM3 glutamate receptor, is linked to inhibition of the cAMP cascade and has been associated with susceptibility to develop mood disorders.

Another possibly important genetic marker, and that links with oxidative stress, is NOS as discussed earlier. Weber *et al.* [499] indicated that a genetic variance in nitric oxide synthase NOS1 results in a reduction in the expression of the gene in the prefrontal brain region which adds to schizophrenia burden, and that NOS1 interacts with NOS1 adapter protein (NOS1AP) in doing so. The interaction observed in NOS1, NOS1AP and the PDZ binding domain may therefore establish a novel drug target for treating schizophrenia [499].

Preclinical Correlates

Depression

mRNA expression of neuropeptide Y have been demonstrated to be decreased in various brain regions of FSL rats [500, 501] while basal mRNA levels of several genes involved in the synthesis of neurotransmitters are similarly altered. These include tyrosine hydroxylase (TH), DA β-hydroxylase, phenylethanolamine *N*-methyltransferase (PNMT) and GTP cyclohydrolase I – all of which were elevated in FSL rats [502]. Also, protein expression of BDNF, CREB and Bcl-2 were reduced in rats after exposure to CMS [503].

Bipolar Disorder

The lack of viable genetic animal models of BPD has left a void in the search for genetic markers at pre-clinical level. However, black Swiss mice (a genetic model of mania) present with increased mRNA expression of β-catenin in the hippocampus, as opposed to other brain regions [363], while increased CSF levels of S100B (a neuronal trophic factor released by astrocytes) has been observed in the ouabain-induced model of mania [504]. Furthermore, transgenic mice expressing increased S100B have been demonstrated to be hyperactive [505].

Schizophrenia

The SIR model has shown increased expression of metabotropic glutamate receptor (mGluR)6 and ionotropic AMPA3 receptor subunit genes in the PFC [361], as well as reduced mGluR1 and mGluR5 expression [506]. These findings are consistent with the proposal that dysregulation of glutamatergic activity may contribute to the behavioural/cognitive deficits associated with SIR [64]. Furthermore, the PCP model produces up regulation of genes coding for frontal cortical NMDA receptors and produces differential expression of frontal cortical genes coding for BDNF [252]. Another clear indication of the involvement of genetic markers in schizophrenia is the numerous genetic animal models with validity for schizophrenia, such as: the DISC1 knockout (KO) model, the DA transporter KO model, the Homer1a KO model, insulin receptor KO model and the mGluR 1 and 5 KO models (reviewed in [507]).

PROTEOMIC MARKERS

The proteome is the entire collection of "proteins encoded by the genome of an organism at a specific point in time, incorporating the set of isoforms, posttranslational modifications, covalent structures and complex protein-protein interactions present therein" [508]. Proteomics provides an insight into the character and interactions of proteins and thus of signalling pathways – understanding a proteome allows for development of effective predictive biomarkers [509].

Clinical Correlates

Depression

Ditzen *et al.* [510] observed several aspects in the CSF which were significantly altered in depressed patients when compared to controls, including 11 proteins and 144 peptide features. A recent large proteomic study in MD and schizophrenia patients observed that insulin was the marker with the highest statistical significance in MD patients compared to controls [2]. Their findings are consistent with the observation that MD is frequently linked with insulin resistance [511]. The increased comorbidity between type 2 diabetes and MD [512] and the strong link between MD and metabolic syndrome [513] further supports this hypothesis. Moreover, increased levels of chromogranin A, a secretory protein and precursor for functional peptides, are linked to dysregulated insulin levels in patients with schizophrenia [514].

Bipolar Disorder

Novikova *et al.* [515] identified several protein biomarkers which may possibly be involved in the pathology of BPD, e.g. MB-18.5, CBF2, DECR2, BYSL, ANKARD12, ALDOC and DKK2 (part of the Wnt signalling cascade) [516]. The Wnt cascade is a group of signal transduction pathways comprising proteins that pass signals from outside to inside of a cell through cell surface receptors. These proteins appear to be compromised in patients with BPD [517]. Importantly, these proteins interact with GSK3-B, BDNF, oxidative stress mediators and cytokines relevant to BPD [349].

Schizophrenia

Cyclophilin A is a protein biomarker that plays an important role in cerebral cortical plasticity [518]. Differing levels of this protein has been noted postmortem in patients with schizophrenia and BPD, suggesting contradictory influences on plasticity specifically in the DLPFC in these diseases [515]. We have earlier noted the importance of neurotrophins like BDNF and VEGF in the neurobiology of psychiatric disorders. Proteomic studies [2] have also observed significant differences in various growth factors and neurotrophins in patients with schizophrenia, influencing somatic and dendritic growth in the hippocampus and prefrontal cortex, such as BDNF, VEGF or stem cell factor. To a lesser extent, this may include members of the chemokine/cytokine family. In line with these findings, previous studies have indeed observed decreased VEGF in serum of patients with schizophrenia [259, 519], although this has not always been reproducible [520]. The current body of evidence relating to BDNF in schizophrenia provides little congruence, with increased, decreased, or no change in serum or plasma BDNF levels noted [259, 521].

Another study in schizophrenia patients identified distinctive profiles of peptides and proteins in the CSF [522] that are potentially specific for schizophrenia, including a VEGF-derived peptide sequence consisting of 40 amino acids, a transthyretin protein cluster (a serum and CSF carrier of the thyroid hormone thyroxin), and another smaller protein cluster also associated with transthyretin, with 95% specificity and 80 to 88% sensitivity [522]. Recent studies also observed that serum concentrations of insulin and chromogranin A were increased in schizophrenia patients [514, 523].

Preclinical Correlates

Depression

Yang *et al*. [524] recently published proteomic data from the CMS rat model of MD, noting decreases in glyoxalase-1 and dihydropyrimidinase-related protein-2 (DRP-2) in the prefrontal cortex, which translates to alterations in energy and glutathione pathways. Carboni and colleagues [458] detected significantly increased levels of leptin, IL-1α and BDNF in FSL compared to FRL rats.

Bipolar Disorder

To the best of our knowledge there are no current proteomic data on animal models of BPD – this may be due to the lack of comprehensive animal models presenting with cyclic behaviour ranging from depressive to manic.

Schizophrenia

Proteomic studies in preclinical models of schizophrenia are limited. One study, however, describes the prohibitin protein, a potential marker of synaptic pathology, to be up-regulated in chronic schizophrenia patients as well as in the ketamine animal model of schizophrenia [525]. Another proteomics study describes the neuroprotective action of abrogated COX-II expression in insulin receptor KO mice as a validated animal model of schizophrenia [526]. Using the PCP animal model to access frontal cortical levels of chromogranin A, B and secretoneurin (large protein molecules of the chromogranin family), acute PCP treatment caused a decrease in secretoneurin, while chronic PCP treatment elevated this protein [527].

Micro-RNAs

Clinical Correlates

MicroRNAs (miRNAs), a large family of small non-coding RNAs, are potent regulators of gene expression with proposed roles in brain development and function [528]. These miRNAs may play a substantial role in the pathophysiology of mood and psychotic disorders and may even have an influence on the effect of drugs used to treat these disorders [529].

Depression

Bocchio-Chiavetto *et al*. [529] found 28 miRNAs to be up-regulated and 2 miRNAs to be down-regulated following antidepressant treatment – they further demonstrated that these differentiations could be associated with alteration of neuronal pathways and may be involved in the underlying mechanisms of MD.

Schizophrenia and BPD

Perkins *et al*. [530] observed that from 264 human miRNAs, 16 were differentially expressed, 15 were expressed at lower levels and 1 at a higher level in post mortem prefrontal cortex tissue of schizophrenia patients vs. controls. Similarly, post-mortem studies in schizophrenia or BPD found underexpression of several miRNAs of the adult prefrontal cortex [528], while in a study evaluating 667 miRNAs in postmortem prefrontal cortex tissue of schizophrenia and BPD patients, 22 miRNAs were found differentially expressed between cases and controls, 7 deregulated in schizophrenia and 15 in BPD [531]. Furthermore, these 22 miRNAs were found to target brain specific genes involved in neurodevelopment, behaviour as well as the development of schizophrenia and BPD [531]. It has also been observed that a considerable modification to post-transcriptational regulation may be defined by an overall increase in miRNA expression in two regions of the cerebral cortex in post-mortem brain tissue from patients suffering from schizophrenia [532].

These studies highlight miRNAs as possible biomarkers for mood and psychotic disorders, although it is clear that further research is needed to evaluate the relationship between miRNA alterations and disease development and progression. The fact that altered expression of miRNAs is reflected also in the blood of patients suffering from mood disorders potentially give rise to the idea that peripheral miRNAs may be screened as an aid in the diagnosis and treatment of these disorders. However, as with most peripheral biomarkers, the correlation between central and peripheral expression of miRNAs remains a topic of debate [533, 534].

Pre-Clinical Correlates

By referring to selected animal models of depression, recent reviews have evaluated the molecular biology of miRNAs in relation to the pathophysiology of clinical depression as well as the utility of targeting miRNAs for antidepressant treatment [533, 535]. They confirm the dysregulation of a large number of miRNAs in these depression models. Moreover, miRNAs to some extent may be associated with treatment and onset of BPD and schizophrenia. The reader is advised to consult these papers for further detail.

DEVELOPING A BIOMARKER PANEL

To date the diagnosis and treatment of patients suffering from mood and psychotic disorders have almost exclusively been based on behavioural symptomatology observed in these individuals, with laboratory testing and confirmation of diagnosis being absent due to the diverse aetiologies and underlying neurochemical abnormalities associated with these disorders. However, in recent years several biological markers have been linked to neuronal changes associated with the pathological processes and/or treatment response in these disorders. Being able to identify discrepancies in these markers in humans and using them in the diagnosis and treatment of mood and psychotic disorders will surely improve treatment efficacy and potentially even allow for provisional measures to be taken to counter neuronal deficits and prevent the onset and/or progression of symptoms and

structural brain changes associated with these disorders. Quantifying the underlying abnormalities in these disorders may also be helpful in understanding the pathogenesis of these disorders and mechanisms causing the delayed response to drugs observed in their treatment. But then again, as mentioned in the introduction the likelihood of identifying any single biomarker with sensitivity and specificity for MD, BPD and schizophrenia is relatively low so that a feasible alternative to the single-biomarker approach could be the development of biomarker panels.

From the data discussed in this review, we have attempted to distinguish between biomarkers that provide mostly substantial evidence in support of them being a strong or moderate biomarker of either MD, BPD of schizophrenia, as opposed to markers where there is less of an evidence base to link it to the neuroanatomy or pathophysiology of these disorders. Furthermore, we distinguish between markers observed in clinical vs preclinical studies in order to highlight affirmation as well as the possibility/importance of further investigation. Table **1** describes a putative biomarker panel based on the clinical and preclinical data described in this article, where strong, moderate and weak candidate biomarkers are color coded for easy identification and interpetation with the text.

Altered endocrine responses, indicated in the 2nd row of Table **1**, are typical of a number of these illnesses, and can in many instances be related to altered circadian rhythm. Moreover, such changes, for example cortisol, can be instrumental in driving many of the behavioural and pathological changes evident in these disorders, eg. hyper-glutamatergia, structural brain changes, etc. Furthermore, the recent introduction of the 5-HT_{2c} antagonist/$M_{1/2}$ agonist, agomelatine, as the first antidepressant acting to re-establish altered circadian rhythms provides robust validation of the importance of these processes in the development and treatment of depression [78]. Moreover, its actions on frontal cortical DA function hints of possible value in the treatment of schizophrenia.

Regarding the neuroanatomy and neurocircuitry of MD, BPD and schizophrenia, it is evident (and quite expected) that there are distinct alterations in the volume of certain brain structures in mood and psychotic disorders, highlighting the importance of altered neuroplasticity and structural brain changes in these illnesses, indicated in the 1st row of Table **1**. Decreased volumes have been recorded for various limbic structures in both MD and schizophrenia, specifically the prefrontal cortex. Although several observations have been made in BPD, current data is limited or of little clinical value and further research into the neuroanatomy of this disorder is necessary. Overall, there remains speculation as to whether the above-described neuroplastic changes are cause or effect.

Neuroimaging studies, indicated in the 1st row of Table **1**, have also given insight into altered neurocircuitry, blood flow and metabolic rate in affected brain structures in these disorders and largely parallel the neurocircuitry described above. Of importance is the decreased activity in cerebellar, prefrontal, frontal and cortical structures in clinical studies of schizophrenia as well as decreases in cerebral glucose metabolism in both preclinical and clinical studies of BPD and alterations in NAA in MD and schizophrenia.

Current pharmacotherapy of MD, BPD and schizophrenia in many ways provides robust construct and predictive validity for the importance of monoamines as biomarkers for these illnesses. At the neurochemical level, indicated in the 3rd row of Table **1**, patients with MD present with a decrease in DA, 5-HIAA, NA and MHPG; BPD patients present with an increase in DA, associated with manic symptoms, and a decrease in 5-HIAA; schizophrenia patients present with an increase and decrease in DA in the striatum and prefrontal cortex respectively, along with increased 5-HT transmission and increased NA levels. Decreased levels of DA have been correlated with anhedonic behaviour in MD in both clinical and preclinical studies. Increases in DA as well as NA have, however, been linked to manic symptoms of BPD and models of mania as well as clinical and preclinical studies of schizophrenia. Glutamate on the other hand is increased peripherally and in the cortex of MD and BPD patients, is reversed with ketamine treatment, while in un-medicated schizophrenia patients the literature indicates an increase of glutamate and GABA in post-mortem prefrontal cortex and striatum tissue. However, as noted earlier, schizophrenia is proposed to involve cortical hypoglutamatergia that in turn drives meso-limbic hyperdopaminergia and meso-cortical hypodopaminergia. Decreases in the level of 5HIAA, a 5-HT metabolite, have also been noted in both MD and BPD patients suffering from depressive symptoms, while 5HT transmission is increased in patients suffering from schizophrenia.

With regards to the growth factors, indicated in the 4th row of Table **1**, the current literature supports a general decrease in BDNF in MD, BPD and schizophrenia; none to limited data of IGF-1 in MD, no changes of IGF-1 in BPD and decreased IGF-1 in schizophrenia; VEGF was increased in MD and BPD, but mRNA of VEGF was decreased in schizophrenia. While VEGF studies in humans report conflicting results and with preclinical data still lacking, it may still hold promise as a biomarker of psychiatric/mood disorders – especially considering the divergent results seen in MD vs schizophrenia patients. Recently published meta-analyses on BDNF are emphatic that altered serum BDNF is a state marker in MD, BPD and schizophrenia [536], although peripheral BDNF levels are not a sufficient measure of disease severity in MD [337] neither does it adequately discriminate between MD, BPD and schizophrenia. Animal studies tend to echo these sentiments, although serum BDNF levels may act as marker of predisposition to develop symptoms in rats used to model these disorders [306, 307]. Interesting enough, it does differentiate between mood states in BPD [336] and between acute and remitted states in MD [536]. In schizophrenia, a significant positive correlation between BDNF levels and positive and negative syndrome scale (PANSS) positive subscore has been described, as well as higher BDNF levels in the paranoid subtype of schizophrenia [537]. Low BDNF levels at the onset of psychosis may therefore contribute to the pathogenesis of schizophrenia and could perhaps be a candidate biological marker for positive symptoms. It may also play an important role as a marker of disease progression in BPD due to

Table 1. A putative biomarker panel, based on clinical and pre-clinical data for depression, bipolar disorder and schizophrenia.

	Depression	Bipolar disorder	Schizophrenia
Neuroanatomy **Neurocircuitry**	↓Hippocampal, prefrontal cortex, orbitofrontal cortex and basal ganglia volume [24; 25; 26; 27]* [60]#	Enlarged amygdala [41]* ↓Dorsal and ventral prefrontal cortical volume [42]*	↓Prefrontal cortex and temporal lobe volume [50; 51; 52]* [18]# Loss of PV-interneurons in hippocampus and ↓frontal cortical spine density [64]#
	↑Blood flow and glucose metabolism in amygdala, orbital cortex, and medial thalamus and ↓Blood flow in the prefrontal cortex and anterior cingulate cortex [31]* ↓NAA in frontal cortex and subcortical regions [32; 33]*	↔Temporal lobe volume [50]# ↓ Cortical activation, ↓ cortical gray matter and ↑ activation of the para-hippocampal gyrus and amygdala [44]*. ↓Cerebral glucose metabolism [61]*# ↓P300 wave amplitude [49]*	↓NAAG and NAA in temporal cortex and ↑NAAG in hippocampus [59]* [70; 71]# ↓Cerebellar and temporal lobe activity [46; 48]* ↓P300 wave amplitude [49]* ↓Frontal cortical and striatal activation [54]* ↓Prefrontal cortical function [55; 56; 58]*
Neuroendocrine and circadian rhythms **-Circadian rhythms** **-Cortisol** **-Thyroid hormones**	Circadian misalignment and changes in sleep-wake cycles [75, 76]*# ↑Saliva cortisol [88]* ↑HPA-axis activation [86]* Thyroid and TSH dysfunction [99, 102]* ↑TSH [80]*	Altered sleep patterns, mutated core-clock-gene, Clock, leads to manic symptoms [75, 76]*# ↔Saliva cortisol [90]* ↑Systemic cortisol metabolism [87]* Thyroid and TSH dysfunction [98, 102, 103]*	Sleep onset and sleep maintenace insomnia and altered melatonin rhythm [75, 76]*# ↔Saliva cortisol [90]* ↑Systemic cortisol metabolism [87]* Thyroid and TSH dysfunction [101, 102, 103]* ↑Autoimmune thyroid disease [103]* Thyroid regulation involved in monoamine, myelination and inflammation networks [104]*
Neurochemical markers **-Monoamines** **-Glutamate and GABA**	↓DA [109]* [130; 131]# ↓5-HIAA [139]* ↑5-HT & 5-HIAA in limbic structures [151]# ↓NA [186; 187]* ↓MHPG [188]* ↑Glutamate [204; 205]* [226]#	↑ DA (manic symptoms) [118]* [135]# ↓5-HIAA (depressed) [158]* ↑ 5-HIAA (manic symptoms) [161]* ↓5-HT in amygdala and hippocampus [177]# ↑ NA [158]* [161]# ↑Glutamate [215]*	↑DA (striatum) & ↓DA (frontal cortex) [120; 121]* [137; 139; 146; 147]# ↑↓DA (frontal cortex) & ↔DA (striatum) [138]# ↑5-HT transmission at 5-HT_{2A} receptors [166; 167]* ↓5-HT/5-HIAA in frontal cortex and ↑5-HT/5-HIAA in nucleus accumbens and striatum [140; 179]# ↑NA [191; 193]* [179]# ↑Glutamate & ↓GABA [217]* ↓Glutamate [219; 220]* [256; 257; 141]#
Neuronal growth factors **-BDNF** **-IGF-1** **-VEGF**	↓BDNF [262; 263]* [304; 306]# ↑BDNF [281]* [301; 302]# ↓IGF-1 [317; 319]# ↑VEGF [324; 325]* ↓ or ↔ VEGF [301]#	↓BDNF [504]* [119]# ↔IGF-1 [315]* ↑VEGF [327; 328]*	↓BDNF [295; 298]* [311; 312]# ↑BDNF in paranoid schizophrenia [537]* ↓IGF-1 [316]* ↓ or ↔ VEGF [285; 329]*
Neuronal resilience markers	↓ cAMP and MAPK/ERK pathway activity [344; 349]* ↑Expression of MKP [18]#	↓PGRN plasma levels [351; 352]* ↔ GSK-3 brain expression [353]* [363; 364]#	↑Expression of MAPK [18; 19]# ↑MAPK-and cAMP proteins [354]* ↓DARPP-32 [359]*

Table 1, contd....

	Depression	Bipolar disorder	Schizophrenia
Oxidative stress markers	↑SOD, lipid peroxidation [376; 377]*. ↓SOD and catalase [398]#	↑SOD (manic and depressed symptoms) ↑catalase (manic symptoms) & ↓catalase (euthymic symptoms) ↑ lipid peroxidation [384; 260]* [408]#	↑SOD, lipid peroxidation [393]* [373]# ↓GSH [390; 391]* ↓PV-IR interneurons [413]# ↑Nox2 [414]#
Inflammatory markers	↑Pro-inflammatory cytokines (IL-1,6 ; IFN-γ; TNF-α) [299; 439; 443]* [458]#	↑Pro-inflammatory cytokines (IL-1,6 ; TNF-α) [447; 448; 449]*	↑Pro-inflammatory cytokines (IL-1,6 ; IFN-γ; TNF-α) [450; 418]* [104]# ↓Anti-inflammatory cytokines (IL-10; IL-4) [442]* [137]#
Kynurenine pathway markers	↓Tryptophan & ↑Kynurenine [436; 471]* [480]# ↑QA [472]* ↓KYNA [465]*	↓Tryptophan & ↑Kynurenine [467; 474]*	↑Tryptophan & ↑Kynurenine & ↑QA & ↑3-OHAA [476; 475]* [137; 483]# ↓ KYNA [479]* [137; 483]# or ↑KYNA [218]*
Genetic markers	Polymorphisms in: 5-HT transporter, 5-HT receptor-2A, BDNF, and tryptophan hydroxylase [484]* [502]# Val/Met polymorphism [487; 488]* ↓Neuropeptide Y expression [500; 501]#	Val/Met polymorphism [487]* Tryptophan hydroxylase II gene variations [492; 493]* ↑β-catenin expression (manic model) [363]#	Val/Met polymorphism [485; 486]* NOS1 gene variance [499]* Possible SzGene database (DISC1, DTNBP1, COMT, DAO, RGS4, NRG1, GRM3 gene variations) [496]* ↑mGluR6 and AMPA3 gene expression [361]# ↓mGluR1 and mGluR5 expression [506]#
Proteomic markers	Abnormal insulin secretion [2; 511; 512; 513]* ↓Glyoxalase-1 and dihydropyrimidinase-related protein-2 [524]# ↑Leptin, IL-1, BDNF proteins [458]#	Proteins involved in the WNT cascade (MB-18.5, CBF2, DECR2, BYSL, ANKARD12, ALDOC and DKK2) [364]#	↑Insulin and Cyclophilin A [514; 523]* VEGF-derived peptide sequence, a transthyretin protein cluster, and another smaller cluster related to transthyretin [522]* ↔Secretoneurin (Chromogranin) [527]# ↑Prohibitin protein [525]*#
Micro-RNAs (miRNAs)	28 miRNAs up-regulated and 2 miRNAs down-regulated in treatment [529]*	Under expression of several miRNAs (prefrontal cortex) [530]* Deregulation of 15 miRNAs (involved in neurodevelopment and behaviour regulation) [531]*	↓6 miRNAs differentially expressed, 15 miRNAs down-regulated and 1 miRNA up-regulated [530; 532]* 22 miRNAs (involved in neurodevelopment and behaviour regulation) deregulated [533]* ↑miRNA expression in cerebral cortex [534]*

Strong marker Moderate marker Not a strong marker/limited data

*Clinical data; #Pre-clinical data (Please refer to text for additional discussion)

Abbreviations: ↓, decrease; ↑, increase; ↔ , Unchanged or differing results; PV, parvalbumin; NAA, N-acetyl aspartate; HPA, Hypothalamic-pituitary-adrenal; TSH, Thyroid stimulating hormone; DA, Dopamine; 5-HIAA, 5-Hydroxyindole acetic acid; NA, Noradrenaline; MHPG, 3-Methoxy-4-hydroxyphenylglycol; GABA, Gamma-aminobutyric acid; BDNF, Brain-derived neurotrophic factor; IGF-1, Insulin growth factor-1; VEGF, Vascular endothelial growth factor; cAMP, Cyclic adenosine monophosphate; MAPK/ERK, Mitogen-activated protein kinases / extracellular signal-regulated kinases; MKP, Mitogen-activated kinase phosphatase; PGRN, Progranulin; GSK-3, Glycogen synthase kinase; DARPP-32, Dopamine and cAMP-regulated neuronal phosphoprotein 32; SOD, Superoxide dismutase; GSH, Glutathione; PV-IR, Parvalbumin-interneurons; Nox2, Nicotinamide adenosine dinucleotide phosphate (NADPH) oxidase 2; QA, Quinolinic acid; KYNA, Kynurenic acid; 3-OHAA, 3-Hydroxyanthranilic acid; NOS-1, Nitric oxide synthase-1.

an observed association between peripheral BDNF levels and age and duration of illness [336, 538].

Markers of neuronal resilience, indicated in the 5th row of Table 1, have aroused considerable interest and play an important role in programmed cell death and plasticity. GSK-3 has surfaced as a possible marker in BPD *via* its regulation of several biochemical pathways, including the Wnt pathway. It has also been connected to the effects of lithium treatment in BPD. Decreased cAMP and activity of the MAPK/ERK pathway has been demonstrated in both humans and animal models of MD and may serve as a valuable biomarker in the disorder. However, data in BPD and schizophrenia is less clear. Increases in MAPK have been noted in animal models of schizophrenia but need further investigation in a clinical setting as well.

Numerous findings in MD, BPD and schizophrenia patients indicate the presence of a prooxidative state, indicated in the 6th row of Table **1**. However, elevations in SOD and lipid peroxidation are relatively non-specific, although BPD patients present with an increase and decrease in catalase in manic and euthymic symptoms respectively, making catalase a possible specific marker in the latter disorder. Furthermore, schizophrenia and BPD patients present with decreased levels of GSH [539]. Importantly, studies in translational animal models have provided evidence that the associated oxidative stress occurring with MD-related behaviors can be reversed by antidepressant treatment [400] and that the antioxidant and glutathione precursor, NAC, is antidepressant in rats [399]. Moreover, exacerbated levels of oxidative stress can attenuate antidepressant action [400]. However, preliminary clinical studies have only been able to provide limited support for the use of NAC as an adjunctive therapy for MD [379]. Similar studies in humans and animals have described reversal of redox changes by lithium and/or antipsychotics in BPD [119, 135] and schizophrenia [137, 373, 387, 395], respectively. This review is adamant that both oxidant and antioxidant systems and redox balance play a pathophysiological role in MD, BPD and psychotic disorders such as schizophrenia. This realisation has opened the door to the possible clinical utility of antioxidant drugs (e.g. NAC) in the treatment of these illnesses alone and as an adjunctive treatment [12, 118, 540].

As has been noted, inflammation and oxidative stress are closely linked. Significant increases in the levels of pro-inflammatory cytokines have been reported in humans and animal models of MD, BPD and schizophrenia, indicated in the 7th row of Table **1**. Along with the above-mentioned oxidative stress observations is the evidence that MD, BPD and schizophrenia patients present with an elevation in pro-inflammatory cytokines (IL-1, 6, IFN-γ and TNF-α), that is reversed with antidepressant treatment in MD. A recent animal study in the SIR model of schizophrenia indicates that clozapine and NAC can also reverse this pro-inflammatory state [137]. This review suggests that the presence of a pro-inflammatory state is a non-specific pathological marker for MD, BPD and schizophrenia yet underscores the pathological role of inflammation in these disorders, especially give their reversal by typical drug treatment. Furthermore, anti-inflammatory cytokines have been found to be decreased in both humans and animal models of schizophrenia. However it remains unclear whether activation of inflammatory pathways in the CNS during MD, BPD and psychotic disorders is rooted in the periphery (e.g., as a function of overt or nascent medical illness or psychological stress) and/or whether stress or other yet to be identified processes (e.g., vascular insults in late life MD/psychosis) induce inflammatory responses directly within the brain. Strong evidence supports a prenatal inflammatory event as a prodromal event to the development of schizophrenia [541]. Prenatal immune challenge with either a systemic endotoxin or viral mimic vs. an inducer of local inflammation suggests that neurodevelopment of the fetus may rather be affected by circulating cytokines and/or fever as opposed to direct effects evoked on the fetus by the agent responsible for maternal infection [542]. What is evident from work in animals is that these pathological processes seem to have their origin in a disturbance in the mitochondria [179], and explains why redox dysfunction is such a central feature of these illnesses [373]. Cytokine activity may elicit several effects on the brain, affecting the synthesis, release and reuptake of several neurotransmitters, including monamines, which have the ability to influence mood [417].

An interesting observation is that MD and BPD patients present with a decrease in TRP along with an increase in kynurenine and QA levels, while schizophrenia patients (corroborated by animal studies using the SIR model [137]), indicates an increase in TRP, 3OHAA, kynurenine and QA along with a decrease in KYNA [475, 476, 479]. These findings are indicated in the 8th row of Table **1**. The decrease in TRP could therefore possibly be specific markers for MD and BPD, with the increase in TRP along with the decrease in KYNA specific to schizophrenia. The relative induction of KYNA versus QA may determine the effects of cytokines on the CNS and remains an important area for future investigation, including the therapeutic targeting of IDO and kynurenine enzymatic pathways in MD, BPD and schizophrenia [465, 467, 475].

Even though these disorders are known to be hereditary to some extent, the exact genetic basis still needs further illucidation, indicated in the 9th row of Table **1**. Val/Met polymorphisms have been studied in MD, BPD and schizophrenia and even though various reports have been made, data remains inconclusive. Polymorphisms in 5-HT transporters and receptors, as well as BDNF and tryptophan hydroxylase hold promise in MD and warrants further research to pinpointing exact genetic markers involved in the development of MD [484]. DISC1 is a well researched candidate gene for schizophrenia and affective disorders with a range of functions relating to neurodevelopment, although studies into its role in these disorders remains promising albeit conflicting [543]. Gene variations in the SzGene database [496] also hold promise in schizophrenia research and also needs further research to clarify predisposition in developing schizophrenia. Tracking genetic variants in patient blood may therefore serve to compliment biomarker panels by providing more information relating genotypes to MD, BPD and psychotic disorders and their respective treatment responses.

With regards to proteomic markers, indicated in the 10th row of Table **1**, it is clear that utilizing modern proteomic techniques, especially mass spectrometric approaches, may support attempts to understand the biochemical processes that accompany psychiatric disorders and may in turn lead to the development of diagnostics and better therapeutics. In MD, abnormalities in insulin secretion has been observed [511] and the disorder is also accompanied by decreased levels of glyoxalase-1 and dihydropyrimidinase-related protein-2 and increased leptin, IL-1 and BDNF protein levels in animal models [458, 524]. This supports the hypothesis for a shared etiopathology with an inflammatory underbuild in patients with co-morbid MD and metabolic syndrome and/or type II diabetes mellitus, which highlights the proposed utility of the PPARγ-pathway in the treatment of MD [544]. Increased insulin levels have also been reported in patients suffering from schizophrenia and

may be accompanied by increases in cyclophillin A [518], suggesting increased support for the role of inflammation in the disorder with cyclophillin A already being linked to a variety of disorders with an inflammatory component – amongs others, type II diabetes [545]. Morever, these findings may relate to the confounding observation of weight gain and metabolic syndrome in this disorder, and that may be worsened by certain antipsychotic drugs [546]. Various proteins involved in the WNT cascade may possibly serve as proteomic biomarkers of BPD which may lead to these markers aiding in the diagnosis and treatment of the disorder and therefore warrants further investigation. Thus, screening peripheral compartments, such as serum and CSF, in patients and controls for altered expression of proteins and metabolites known to be involved in the pathophysiology of the disease or associated with comorbid states could serve in developing a "fingerprint" for identifying persons at risk of developing MD, BPD or schizophrenia. However, it is critical that we bring together knowledge on the biology of these illnesses, co-morbid states, illness severity and treatment resistance to enable proteomic markers to realize this potential.

Finally, studies suggest that micro-mRNAs, indicated in the 11th row of Table **1**, may play a valuable role as a biomarker in the diagnosis and treatment of mood and psychotic disorders, however further reseach is warranted and the relation between central and peripheral expression still needs elucidation.

This review has focussed primarily on suitable disease-specific biomarkers with especially predictive validity. However, we have on occasion made reference in the aforegoing sections to *physiological markers*. It is maybe encumbent to mention that these markers are gaining in interest, with some recently been found to have value in predicting treatment response. Thus for example, we earlier described that clinical response to antidepressants can be predicted by assessing activity in the rostral ACC region *via* electroencephalography (EEG) [37]. Similarly, we noted that by studying P304 wave amplitude, EEG can be used to assess decision making in BPD and schizophrenia patients [49]. In a study based on the "disconnection hypothesis" of schizophrenia [547], and with accumulating evidence of abnormal functional connectivity in schizophrenia, Takahashi and colleagues argued that neurophysiologic signals may provide a retrospective window with which to view disordered neural dynamics in schizophrenia [548]. Using a novel entropy-based approach for measuring dynamical complexity in physiological systems, they observed abnormal dynamical EEG signal complexity in anterior brain areas in schizophrenia that normalized selectively in fronto-central areas following antipsychotic treatment. This approach has also been proven successful in MD [488]. Another promising neurophysiological marker is electroretinography (ERG), a specialized measure of retinal function, which has been studied in schizophrenia and BPD [549]. ERG abnormalities may reflect altered phospholipid metabolism and/or impaired dopaminergic transmission. With all patients receiving stable psychotropic medications at least for 2 weeks before the first assessment, the authors found that retinal dysfunctions are specific for schizophrenia, as compared with BPD, and are confined to the acute stage of the illness. Another potential physiological marker is event-related-potentials (ERPs) (voltage fluctuations in an EEG depicting neural activity), which are specific for cognitive dysfunction in schizophrenia [550] and also studied in MD [551]. Although physiology markers have not been as extensively studied as biological markers, there is a literature describing their use in animals, such as employing EEG and related markers in translational animal models of MD [552] and schizophrenia [553]. For example, blind-drunk (Bdr) mice demonstrate fragmented rest and activity rhythms under a light/dark cycle, reminiscent of altered sleeping patterns in schizophrenic patients [83], while depriving animals of REM sleep, which can be studied as an EEG-related marker, has been suggested to model mania [82].

DISCUSSION AND CONCLUSION

The search for blood biomarkers can essentially be divided into screening putative markers inferred by our current knowledge of the given illness, e.g. BDNF, CREB etc, or through exploring candidate pathways through the use of "omic" procedures, such as proteomic or transciptomic profiling that offer an unbiased view of these pathways. Either approach has its own set of advantages and disadvantages, yet studies deploying either still lack the critical requirement of reproducibility and selectivity [13]. Another important challenge in identifying possible biomarkers is the predictive efficacy of a specific biomarker in the treatment of MD, BPD and schizophrenia. Very few biomarkers of these illnesses have shown utility in regards to predictive efficacy following drug treatment. Having this in hand offers the possibility of introducing tailored pharmacotherapy. Furthermore, demonstrating a dose-dependent response of these markers under the conditions of treatment may aid in more accurately establishing an appropriate dose selection during clinical trials, thereby optimizing drug discovery and development. Knowing this brings the focus of future research to a more optimized translational approach [554]. Ideally, questions from a clinical situation should be translated into a valid animal model, where such animal data can be integrated with patients' data in order to identify predictive biomarkers. Thereafter extensive validation should be performed on these biomarkers before diagnostic kits with predictive value can be developed and marketed [554]. In the end, the final requirement is that the aforegoing process should allow clinicians to make evidence-based decisions that will reinforce the decision to treat, with what agent/s, and with a higher likelihood of success than that provided by current approaches. However, there are a number of obstacles to overcome before realising a biomarker panel that is sensitive and specific enough to be implemented as a reliable tool for diagnosis and treatment, which include:

- difficulties in translating findings observed in animal models to clinical studies and correlating markers measured in animal subjects with those measured in human patients [13, 555];
- employing clinical studies in a larger population in order to validate specific findings [13, 555, 556];

- attributing measured biomarkers to one specific pathway [2] as measured in a specific disorder, thus ensuring biomarker specificity [13] and the possible presence of underlying comorbid disorders [13, 547];
- the complexity of underlying pathophysiologic and ethiologic origins of these disorders combined with demographics [556], and interpatient variables, eg. smoking [2];
- correlating data from observations made in different locations and utilizing different sampling techniques [2];
- accounting for disease state [13, 556] and previous/ current drug therapy [2, 557];
- correlating levels of markers measured in different tissue samples, eg. plasma vs CSF [2, 13];
- the influence of the time at which biomarkers are measured, eg. the influence of circadian rhythm and disease state/progression [2, 558];
- and the inability to measure biological markers in brain tissue in live patients due to the invasive nature thereof [559].

Nevertheless recent studies using "omics" approaches have demonstrated that careful selection of approporiate biomarker panels can provide good separation between diseased and healthy states, as well as predict response to treatment. Two recent studies by Pajer [555] and Redei [556] set out to investigate the validity of potential biomarkers of MD and found that several of these markers may have possible use in discriminating between depressed and non-depressed individuals and may even predict response to therapy. Furthermore Redei *et al.* found several blood markers identified in animal models of depression to correlate with levels measured in depressed human individuals. Importantly, these studies affirm the approach taken in this review that clinical data and that obtained from validated translational animal models are supportive and should be used together when developing a biomarker panel [555]. Indeed, the latter studies corroborate that genes expressing transcipts belonging to processes related to transcription, neurodevelopment, neurodegeneration and redox are causally related to at least MD [555], which concurs with molecular mechanisms linked to these processes and highlighted in this review. Although metabolomics per se has not been covered in this review as it addresses much the same processes as do genomic and proteomic methods, studying the metabolome has great potential to map potential biomarkers in neuropsychiatric disorders. Indeed, as has been noted, mood and psychotic disorders are linked to a range of disturbances in metabolic pathways, eg. neurotransmitter systems, TRP-kynurenine metabolism, oxidative stress, etc, so that generating a metabolic signature for a specific disorder will aid in metabolic phenotyping and contribute to discovering disease-specific biomarkers as well as predicting treatment response [560].

Based on this review, the ever-increasing availability of new pre-clinical and clinical studies is beginning to forge a way through the neuropathologic compexity of illnesses like MD, schizophrenia and BPD, so much so that we are in a position to portray how altered neuroendocrine, anatomical, neurochemical and other pathologies can be linked to a specific disorder. The role of the endocrine system has long been linked to mood and psychotic disorders with MD patients presenting with increased saliva cortisol as well as HPA-axis activation. Similarly, patients suffering from BPD and schizophrenia have been found to have increased systemic cortisol metabolism. Dysfunction of the hypothalamic-thyroid axis has been demonstrated in all three disorders.

In MD, decreased hippocampal volume as well as reductions in the size of the prefrontal cortex and basal ganglia is accompanied by reduced levels of monoamine neurotransmitters (NA, 5-HT and DA), decreased levels of the 5-HT metabolite, 5-HIAA, and an increase in the levels of glutamate. However, DA has been noted to be increased in BPD and increased in the striatum and decreased in the frontal cortex of both schizophrenic patients and most animal model studies of the disorder. In contrast with MD and BPD, increased 5-HT transmission and NA levels characterize neurotransmission in schizophrenia and is accompanied by decreased NAAG levels in the temporal cortex while increased in the hippocampus as well as reduced activity in various brain regions, including the cerebellar and temporal lobes, prefrontal cortex, cortex and striatum.

Although a variety of observations have been made regarding BDNF, VEGF and IGF, data currently available report conflicting findings. However, hypotheses and data surrounding these markers make a strong case for their involvement in these disorders – whether as a cause or result of underlying pathology. Continued investigation will, more than likely, eventually lead to pinpointing the exact roles of these markers in the pathophysiology and/or progression of mood and psychotic disorders and establish them as valid biomarkers in the diagnosis and/or treatment of MD, BPD and schizophrenia.

Inflammation has emerged as a central role player in the pathophysiology of all three disorders discussed in this review with levels of pro-inflammatory cytokines being observed to be markedly increased in MD, BPD and schizophrenia and a decrease in anti-inflammatory cytokines also contributing to the inflammatory component of schizophrenia. Inflammation is thus not illness specific but a residual marker of ongoing pathology. Increased levels of kynurenine add to the immune response in these disorders with TRP levels being decreased in MD and BPD but increased in schizophrenia. Closely associated with inflammation, nitrosative and oxidative stress deepens the extent of neuronal stress in these disorders – increased lipid peroxidation accompanied by raised levels of SOD are a feature in all three disorders, accompanied by decreased GSH in schizophrenia. The individual inflammatory components that characterize said inflammation in these disorders may in the end prove to be more illness-specific markers. Thus for example, we noted earlier that a decrease in TRP may be specific markers for MD and BPD, while the increase in TRP and decrease in KYNA is more specific to schizophrenia. Similarly, certain components of the inflammatory response such as NO may be pro- or anti-

inflammatory depending on the cellular mileux and/or pathways activated (eg see nNOS vs iNOS-mediated pathways in Fig. **1**).

The current body of literature features data on a wide variety of possible biomarkers linked to mood and psychotic disorders. To improve diagnostic techniques and treatment strategies, it is of great importance that possible trait and state markers of these disorders are scrutinized to a point where they can be incorporated into an appropriate panel of biomarkers (as presented in Table **1**) which may serve as adjunct to current diagnostic criteria. Furthermore, such a panel may assist in treatment strategies being tailored to the unique context in which mood and psychotic disorders present in each individual. In this manner we may move forward from the current "one-size-fits-all" approach to treating an individual to one that addresses the biological processes underlying the disorder and specific for that particular patient.

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AUTHOR CONTRIBUTIONS

BH Harvey devised the concept and lay-out of the manuscript, as well as finalized the pre-submission version of the manuscript. S Brand prepared the first draft as well as the final version of the manuscript; M Möller contributed to the initial and final versions of the manuscript.

CONFLICTS OF INTEREST

The authors declare that over the past three years Brian Harvey has participated in speakers/advisory boards and received honoraria from Servier, and has received research funding from Lundbeck and Servier. The authors declare that, except for income from the primary employer, research funding to BHH from the South African Medical Research Council as well as the honoraria described above, no other financial support or compensation has been received from any individual or corporate entity over the past three years for research or professional services, and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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Addendum D –
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Exploring a post-traumatic stress disorder paradigm in Flinders sensitive line rats to model treatment-resistant depression I: bio-behavioural validation and response to imipramine

Brand SJ, Harvey BH. Exploring a post-traumatic stress disorder paradigm in Flinders sensitive line rats to model treatment-resistant depression I: bio-behavioural validation and response to imipramine.

Objective: Co-morbid depression with post-traumatic stress disorder (PTSD) is often treatment resistant. In developing a preclinical model of treatment-resistant depression (TRD), we combined animal models of depression and PTSD to produce an animal with more severe as well as treatment-resistant depressive-like behaviours.
Methods: Male Flinders sensitive line (FSL) rats, a genetic animal model of depression, were exposed to a stress re-stress model of PTSD [time-dependent sensitisation (TDS)] and compared with stress-naive controls. Seven days after TDS stress, depressive-like and coping behaviours as well as hippocampal and cortical noradrenaline (NA) and 5-hydroxyindoleacetic acid (5HIAA) levels were analysed. Response to sub-chronic imipramine treatment (IMI; 10 mg/kg s.c. × 7 days) was subsequently studied.
Results: FSL rats demonstrated bio-behavioural characteristics of depression. Exposure to TDS stress in FSL rats correlated negatively with weight gain, while demonstrating reduced swimming behaviour and increased immobility versus unstressed FSL rats. IMI significantly reversed depressive-like (immobility) behaviour and enhanced active coping behaviour (swimming and climbing) in FSL rats. The latter was significantly attenuated in FSL rats exposed to TDS versus unstressed FSL rats. IMI reversed reduced 5HIAA levels in unstressed FSL rats, whereas exposure to TDS negated this effect. Lowered NA levels in FSL rats were sustained after TDS with IMI significantly reversing this in the hippocampus.
Conclusion: Combining a gene-X-environment model of depression with a PTSD paradigm produces exaggerated depressive-like symptoms that display an attenuated response to antidepressant treatment. This work confirms combining FSL rats with TDS exposure as a putative animal model of TRD.

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Keywords: animal model; antidepressant; gene-environment model; post-traumatic stress disorder; treatment-resistant depression

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Significant outcomes

- Exposure of Flinders sensitive line (FSL) rats to time-dependent sensitisation (TDS) stress reduces active coping, amplifies depressive-like behaviour and attenuates the antidepressant effects of imipramine (IMI) in FSL rats.
- The beneficial effects of IMI on limbic monoamine levels in FSL rats are compromised in combined FSL + TDS-exposed rats, especially its effects on the serotonergic system.
- Post-traumatic stress disorder (PTSD) is highly comorbid with depression and contributes to the development of treatment-resistant depression (TRD). Combining a genetic animal model of depression with a PTSD paradigm may represent a putative animal model of TRD.

Limitations

- The initial (severe) stress sequence and subsequent re-stresses may promote several adaptive changes in the animals that complicate interpretation of monoaminergic responses. Limiting the procedure to a single re-stress could be considered.
- Assessment of corticosterone levels immediately post severe stress as well as before and after re-stress may provide a more comprehensive picture of the bio-behavioural responses observed and their relevance to TRD.
- Behavioural assessment of anhedonia (sucrose preference test), which has been demonstrated to be an important symptom of TRD, would be a valuable addition.
- Challenging FSL+TDS animals with first-line antidepressants (SSRIs, NSRI's) and/or ketamine would expand predictive validity, and is presented in a companion paper to this manuscript.
- Would this model present with altered biomarkers of TRD that contribute to construct validity?

Introduction

The occurrence of non- or partial response to antidepressant treatment in the depressed population creates a major problem in effectively treating and managing the disorder. Less than two-thirds of patients respond to drug-centred therapy (1) and up to half of patients fail to achieve a full response when treated with first-line antidepressant drugs (2). These initial observations were confirmed by the Sequenced Treatment Alternatives to Relieve Depression (STAR D) study, designed to mimic clinical conditions by incorporating the most commonly used strategies in treating patients exhibiting drug resistance (3). Even after applying several treatment strategies in this population, approximately 30% of these patients still did not respond to treatment (4).

On-going work has described the underlying biology of depression as being driven by the presence of chronic psychosocial stress and associated disturbances in monoaminergic, γ-amino-butyric acid (GABA)-glutamate, neuroendocrine (5) and cardio-metabolic and immune-inflammatory disturbances (6). However, the exact cause of TRD remains obscure. As with depression, TRD is believed to be heterogeneous in nature (7) and, although most pathophysiological factors contributing to depression appear to be similar in TRD, many of these conditions are significantly exaggerated in the resistant form, resulting in more severe symptoms (8).

The treatment of depression may be further complicated by the co-occurrence of other underlying psychiatrc disorders. The prevalence rate of a co-existing anxiety disorder is 50–60% (9,10) – a figure that increases to 72% in TRD (9). With a prevalence rate of 17.8%, PTSD is one of the more commonly co-occurring anxiety disorders in patients with depression, and increases to 22.4% in TRD (9). Conversely, more than half of patients seeking treatment for PTSD are diagnosed with comorbid depression (11). This high comorbidity stems largely from overlapping symptoms of anhedonia, sleep difficulty, irritability and poor concentration (Diagnostic and Statistical Manual of Mental Disorders, 4th Edition criteria) (12). Both depression and PTSD require exposure to stressful events for onset (12), whereas both illnesses exhibit hippocampal atrophy related to hypothalamic–pituitary–adrenal (HPA) axis abnormalities (13).

In recent years, it has become widely accepted that genetic susceptibility plus adverse environmental situations are an important prodromal event to the development of depression (14–16). Animal models that are based on this construct have contributed significantly to our knowledge of mood and anxiety disorders (5,17). However, a shortage of suitable and validated animal models of TRD is a major contributing factor to our current lack of understanding of the pathophysiology of TRD. Recent studies have therefore set out to explore the processes that underlie treatment resistance in animal models (18). In their review, Willner and Belzung (19) emphasise models that incorporate predisposing factors leading to heightened stress responsiveness. Chronic mild stress (CMS), a paradigm primarily identified as a depression model (20), has been demonstrated to successfully reproduce antidepressant treatment response rates resembling those observed in clinical studies, with chronic escitalopram treatment found to induce response rates of only 50% (21). However, it being labour intensive and exhibiting poor cross-laboratory reproducibility is a concern (22,23).

The FSL rat, a genetic animal model of depression, is a robust and well-studied preclinical model of depression with good construct, predictive and face validity (24–27). Furthermore, FSL rats only display anhedonic responses after exposure to CMS (28,29), thus tagging the strain as a good candidate for gene-X-environment studies. Indeed, FSL and Flinders Resistant Line (FRL) rats display differential sensitivity to rearing conditions (early-life stress) and rat strain (genes) that in turn modify treatment response by altering serotonin transporters (SERT) (30). This is a valuable quality, seeing that abnormal SERT function has been implicated in the pathology of depression (31,32). Interestingly, by exposing FSL

rats to maternal separation, Carboni et al. (33) demonstrated the induction of biological correlates reminiscent of those observed in human TRD, prompting them to propose that the gene-environment paradigm offers important construct validity in modelling TRD. However, the model lacked predictive validity due to the inability of antidepressant treatment to alter immobility time in maternally separated FSL rats either before or after treatment when compared with control animals (33).

Considering the strong comorbidity between depression and PTSD, and that depression in patients with PTSD is more treatment resistant (34,35), we have developed an animal model of TRD based on the premise that exposing animals genetically predisposed to depressive-like behaviour to a PTSD-related paradigm would yield animals displaying more pronounced depressive-like behaviour. Moreover, such behaviour would be resistant to antidepressant treatment. To this end we have considered the TDS or stress re-stress model of PTSD. TDS is based on a trauma plus contextual reminder principle of PTSD (36), and has shown good predictive, construct and face validity for PTSD (37–40). In this study, face, construct and predictive validity were assessed in the forced swim test (FST) using a behavioural sampling method to study serotonergic and noradrenergic-driven behaviours, assessment of limbic noradrenaline (NA) and 5-hydroxyindoleacetic acid (5HIAA) levels, and response to chronic treatment with the tricyclic antidepressant, IMI.

Materials and methods

Subjects

Animals were bred and housed at the Vivarium (SAVC reg. number FR15/13458; SANAS GLP compliance number G0019) of the Pre-Clinical Drug Development Platform of the North-West University. Ambient temperature was maintained at 22 ± 2°C with a relative humidity of 40–60% and full spectrum of light in a 12-hour light/dark cycle, with lights switched on at 06:00 a.m. and off at 06:00 p.m. Food and water were provided ad libitum. All experiments were approved by the AnimCare animal research ethics committee (NHREC reg. number AREC-130913-015) of the North-West University. 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-00111-12-A5).

The original colonies of FSL and FRL rats were obtained from Dr. David H Overstreet, University of North Carolina, USA. Subjects were male adult FSL ($n = 48$ for behavioural assessment and $n = 32$ for monoamine analysis each) and FRL ($n = 12$ for behavioural assessment and $n = 8$ for monoamine analysis) rats. Table 1 describes the layout of the experimental groups. Half of the FSL animals in each of the above groups were subjected to TDS (see below) at the start of the protocol with behaviour in the open field test (OFT) and FST assessed at the end of the protocol (3 weeks following single prolonged stress (SPS)). Monoamine analysis was performed in animals naive to behavioural assessment. The animals were housed four per cage, with the TDS paradigm initiated at an age of 40 (±1) days in order to conclude the experiments while the rats were still of an appropriate weight for the behavioural assessments. Handling of the animals was initiated 1 week before starting the experimental procedure by taking bodyweight measurements daily until the last day of the study to monitor weight gain and calculate drug dosages.

Time-dependent sensitisation (TDS)

TDS is an animal model of PTSD. Animals exposed to a severely traumatic situation, and followed by subsequent but less stressful contextual reminders, exhibit significant physiological and behavioural alterations that show a time-dependent sustaining or worsening in the absence of the initiating stressor (41,42).

The TDS paradigm used in this study incorporated an acute SPS sequence comprising a somatosensory stressor (restraint), a psychological stressor (forced swimming with brief submersion) and a complex stress-stimuli (exposure to ether vapours) followed by re-exposure to restraint stress 7 and 14 days later (42).

Restraint stress. Rats were placed in Perspex® restrainers for 2 h with the tail-gates adjusted to keep each animal well-contained without impairing circulation to the limbs. The same procedure was followed on days 7 and 14 during the re-stress phase of the TDS protocol.

Forced swim stress. Rats were placed individually in cylindrical Perspex® swim tanks containing 40 cm of ambient water (25°C) and allowed to swim for 15 min while being forcefully submerged for the last 20 s. Thereafter animals were removed from the cylinders, dried and returned to their home cages to recover for 15 min. Forced swimming was performed 21 days *before* behavioural testing (only as part of the SPS procedure and not during re-stress) in the FST so that any possible conditioned response to swim stress in the FST is unlikely.

Table 1. Layout of experimental groups

Group name (text)	Group name (figures)	Group description	Dosage	*n* Behaviour	*n* Bio-molecular
FRL	FRL n/s VEH	Treatment-naive unstressed FRL	1 ml/kg	12	8
FSL – TDS + VEH	n/s VEH	Treatment-naive unstressed FSL	1 ml/kg	12	8
FSL – TDS + IMI	n/s IMI	IMI-treated unstressed FSL	10 mg/ml/kg	12	8
FSL + TDS + VEH	TDS VEH	Treatment-naive stressed FSL	1 ml/kg	12	8
FSL + TDS + IMI	TDS IMI	IMI-treated stressed FSL	10 mg/ml/kg	12	8

FRL, Flinders resistant line; FSL, Flinders sensitive line; IMI, imipramine; n/s, non-stressed; TDS, time-dependent sensitisation; VEH, vehicle.

Exposure to ether vapours. 15 min after swim stress, rats were exposed to 5 ml of 100% ether vapours in a 5 l sealed plastic container until loss of consciousness (±2 min). Ether was poured onto a paper towel at the bottom of the container with the animal placed on a raised metal platform to avoid direct contact with the substance. After loss of consciousness, the animals were immediately removed from the container, returned to their home cage for observation until regaining full consciousness and then returned to their holding room. Animals were left mostly undisturbed, only subjecting them to routine handling until re-exposure to restraint stress during the re-stress phase of the TDS protocol.

Open field test (OFT)

This test is generally performed before the FST to control for locomotor activity. The OFT was performed half an hour before subjecting animals to the FST. Rats were individually placed in a square arena (100 × 100 × 50 cm) facing the centre of the arena. Behaviour was recorded for 5 min using a ceiling-mounted digital camera. The video files were subsequently analysed using EthoVision® XT software (Noldus® Information Technology, Wageningen, The Netherlands). Total distance moved was used as a measure of locomotor activity.

Forced swim test (FST)

The FST can reliably predict antidepressant-like effects after drug treatment and is considered a model of behavioural despair that is typically manifest in human depression, and expressed in rodents as a decrease in escape-driven behaviour, i.e. increased immobility (43). During behavioural analysis, rats were placed individually in cylindrical Perspex® swim tanks containing 30 cm of ambient water (25°C) for 7 min and their behaviour recorded. The first and last minute of the video files were discarded and the remaining 5 minutes scored for characteristic escape-directed behaviours, including swimming, climbing (struggling) and immobility. These sub-scores of the FST provide useful information relating to serotonergic (swimming) and noradrenergic (climbing) directed behaviours that may extend whole brain monoamine analyses (44).

Drug administration

After weighing all animals daily (between 09:00 a.m. and 11:00 a.m.), IMI (Sigma-Aldrich, Kempton Park, South Africa) was dissolved in physiological saline (0.9% NaCl) and administered subcutaneously at a dose of 10 mg/kg (45,46) to unstressed animals (FSL – TDS + IMI) and animals exposed to TDS (FSL + TDS + IMI) (Table 1). Treatment started on day 15 (after completing the TDS protocol on day 14) and persisted for 7 days before behavioural testing commenced on the evening of day 21. This duration of treatment is adequate for establishing an antidepressant response in rats (44,47,48). Stressed and unstressed control animals (FSL and FRL) were injected with saline vehicle according to the same procedure as in IMI-treated animals.

Quantitative analysis of brain NA and 5HIAA

Several valid indices of central serotonergic activity may be applied, including serotonin (5-hydroxytryptamine; 5HT) and 5HIAA levels and the 5HIAA/5HT ratio (49). In this regard, *in vivo* microdialysis is a more reliable method to directly measure extracellular levels of 5HT, whereas whole- or regional brain monoamine analysis provides total levels of 5HT – both extracellular and unreleased from nerve terminals (50). 5HT is metabolised primarily to 5HIAA, hence 5HIAA has been demonstrated to reflect reliable insights into time-dependent alterations in 5HT response (51). 5HIAA has previously been correlated with 5HT function (49) and was therefore applied as an indicator of serotonergic function in the current

study. Following sacrifice of the rats by decapitation, total hippocampus and frontal cortices were dissected out on an ice-cooled dissection slab, weighed, snap frozen in liquid nitrogen and stored at –80°C until the day of analysis, as described previously (41). Quantification of NA and 5HIAA was performed by high-performance liquid chromatography (HPLC) coupled with electrochemical detection (HPLC-EC), as previously described (42). An Agilent 1200 series HPLC (Agilent Technologies, California, USA), equipped with an isocratic pump, auto sampler and coupled to an ESA Coulochem Electrochemical detector (Dionex, California, USA), and Chromeleon® Chromatography Management System software (version 6.8), was used. NA and 5HIAA concentrations in the tissue samples were determined by comparing the area under the peak of each monoamine with that of the internal standard, isoprenaline (range 5–50 ng/ml). Linear standard curves (regression coefficient >0.99) were found in this particular range. Monoamine concentrations were expressed as ng/g wet weight of tissue (mean ± SEM).

Bodyweight analysis

Decreased bodyweight and loss of appetite have been observed in both depressed individuals (20) and FSL rats (26). Sustained decreases in weight gain have been reported in rats following chronic stress (52,53) which may be initiated by increased energy metabolism during stress coupled with acute increases in stress-related peptides (53). In order to establish the impact of the applied stressors on the well-being of the animals, bodyweight was measured daily from 7 days prior to SPS and continued until the final day of the experiment.

Statistical analysis

Statistical analyses were performed using Graphpad Prism® 6 and IBM® SPSS® 22 software under the guidance of the Statistical Consultation Service of the North-West University. In pairwise comparisons of the behaviour and neurochemistry between unstressed FRL and FSL animals, unpaired student's *t*-tests with Welch's correction (normally distributed data as indicated by Shapiro–Wilk test for normality $p > 0.05$) or Mann-Whitney *U*-tests (data not distributed normally) were performed. Two-way repeated measures analysis of variance (RM-ANOVA) followed by Bonferroni *post-hoc* analysis was applied to comparisons of the treatment naive cumulative weight gain of FRL and stressed and unstressed FSL animals. Time and cohort was set as within-subject factors, whereas weight was set as between-subject factor. Ordinary two-way ANOVA was applied in between-group comparisons of behaviour and neurochemistry in treatment-naive and IMI-treated unstressed and stressed FSL animals. In this case, exposure to TDS and treatment was set as within-subject factors, whereas the respective behavioural and neurochemical parameters were set as between-subject factors. Significance was set at $p < 0.05$ for all comparisons. Where Cohen's *d* effect sizes were calculated, large effect sizes are indicated by $d > 0.8$ and very large effect sizes by $d > 1.3$.

Results

Bodyweight

Data are represented in Fig 1. Two-way RM-ANOVA revealed a significant interaction between time and cohort [$F(81,1188) = 61.98$] with respect to the mean cumulative weight gain of animals, whereas both time [$F(27,1188) = 13\,940$, $p < 0.0001$] and cohort [$F(3,44) = 102.0$, $p < 0.0001$], respectively, also had significant main effects on weight gain. Although the mean cumulative daily weight gain of rats between the respective cohorts demonstrated no significant differences before SPS (day 0), significant age and stress-related differences between FSL and FRL animals, both within (FSL) and between strain, became apparent post-SPS. From day 3 post-SPS, unstressed FSL animals lagged behind the FRL controls (day 3, 45.6 ± 3.2 vs. 51.4 ± 3.9 g, $p = 0.004$). Moreover weight gain in stressed FSL rats soon lagged behind that of unstressed control animals (Table 2). On day 8 post-SPS the difference in weight gain between stressed and unstressed FSL rats began to reveal significance (69.8 ± 4.2 vs. 75.6 ± 3.3; $p = 0.005$). The observed disparities in the rates of weight gain in the various groups persisted until the last day of observation. In addition to the curbed weight gain of TDS-exposed FSL rats, they were also observed to present with a general decrease in fur quality and porphyrin staining around the eyes (visual inspection; data not shown).

Behaviour

In order to establish the translational relevance of the FSL rat for depression, data and statistics relating to the behavioural comparisons made between stress and treatment-naive FRL and FSL animals are provided in Table 3. The behavioural differences between FSL versus FRL rats are henceforth described separately under the OFT and FST sections below.

Open field test (OFT)

Locomotor activity. FSL and FRL rats were similar with respect to distance travelled in the OFT (2119 ± 505.4 vs. 2273 ± 307 cm, Table 3).

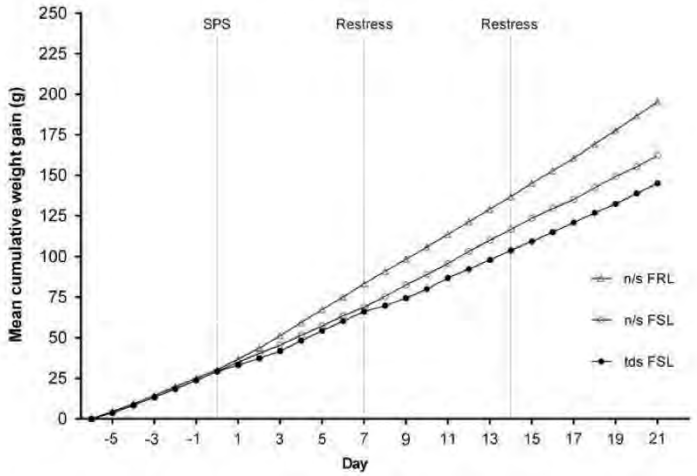

Fig. 1. Mean cumulative weight gain in FRL and TDS naive and TDS exposed FSL animals. Data are represented as the mean of 12 animals. Descriptive statistics are provided in Table 2. FRL, Flinders resistant line; FSL, Flinders sensitive line; n/s, non-stressed; SPS, single prolonged stress; TDS, time-dependent sensitisation.

Table 2. Cumulative weight gain over time

	Mean cumulative weight gained ± SD (g)			Significance of comparison (*p*-value)	
Days	FRL	FSL – TDS	FSL + TDS	FRL vs. FSL – TDS	FSL – TDS vs. FSL + TDS
0 (SPS)	30.4 ± 2.5	29.3 ± 3.8	29.3 ± 1.7	n/s	n/s
2	43.6 ± 2.7	40.3 ± 3.3	37.3 ± 2.9	n/s	n/s
3	51.4 ± 3.9	45.6 ± 3.2	41.9 ± 3.2	0.0006	n/s
5	67.5 ± 3.2	57.3 ± 2.6	54.4 ± 3.3	0.0001	n/s
7 (restress)	83.5 ± 3.8	68.9 ± 3.3	66.3 ± 3.4	<0.0001	n/s
8	91.1 ± 4.1	75.6 ± 3.3	69.8 ± 4.2	<0.0001	0.0007
14 (restress)	136.9 ± 5.2	116.7 ± 4.3	104.1 ± 3.5	<0.0001	<0.0001
21 (final day)	195.5 ± 6.8	162.3 ± 6.2	145.2 ± 6.4	<0.0001	<0.0001

FRL, Flinders resistant line; FSL, Flinders sensitive line; IMI, Imipramine; n/s, non-stressed; SPS, single prolonged stress; TDS, time-dependent sensitisation.
Mean cumulative weight gain of rats measured daily from 1 week before commencement of the TDS protocol until the final day of behavioural testing.
Data are provided as mean of matched daily values in each group.
Two-way repeated measures ANOVA with Bonferroni *post-hoc*.

Data describing the effects of stress and IMI treatment in FSL animals are presented in Fig 2. Considering the locomotor activity of FSL animals, two-way ANOVA did not reveal a significant interaction between TDS-exposure and treatment [$F(1,11) = 0.95$, $p = 0.35$]. However, a main effect of treatment was observed [$F(1,11) = 5.67$, $p = 0.04$] in the locomotor activity of the TDS-exposed group with *post-hoc* analysis revealing a trend towards decreased locomotor activity in IMI-treated animals that narrowly missed statistical significance (1640 ± 422.8 vs. 2296 ± 971.7 cm, $p = 0.07$, $d = 0.94$).

Forced swim test (FST)

Swimming. FSL rats presented with significantly reduced swimming behaviour compared with FRL rats (59.9 ± 15.2 vs. 70.4 ± 14.8 s; $p < 0.05$, Table 3).

Data describing the effects of stress and IMI treatment in FSL animals are presented in Fig 3a. Although no significant interaction between TDS and treatment was displayed in the behaviour of FSL animals [$F(1,44) = 1.54$, $p = 0.2$], both factors had statistically significant main effects on swimming behaviour [TDS, $F(1,44) = 11.8$, $p = 0.001$; treatment, $F(1,44) = 5.32$, $p = 0.03$]. As such, *post-hoc* analysis demonstrated that treatment-naive FSL animals exposed to TDS showed an even greater reduction in the average swimming time compared with unstressed treatment-naive rats (52.9 ± 15.2 vs. 24.4 ± 9.8 s, $p = 0.004$). Finally, IMI treatment significantly reversed the reduced swimming time

Table 3. Open field test, forced swim test and frontal-hippocampal monoamine data in unstressed Flinders resistant line (FRL) vs. Flinders sensitive line (FSL) animals

	FRL	FSL	Significance
Open field test			
Total distance travelled (cm)	2273 ± 307.2	2119 ± 505.4	–
Forced swim test			
Swimming (s)	70.4 ± 14.8	52.9 ± 15.2	$p = 0.009$**
Climbing (s)	117.9 ± 38.0	35.0 ± 9.2	$p < 0.0001$; $U = 2^{xxxx}$
Immobility (s)	111.7 ± 33.7	212.1 ± 18.8	$p < 0.0001$****
Neurochemistry			
5HIAA (ng/mg)			
Frontal cortex	170.4 ± 22.8	268.4 ± 51.3	$p = 0.0007$***
Hippocampus	177.2 ± 37.2	244.1 ± 40.3	$p = 0.021$; $U = 10^{x}$
Noradrenalin (ng/mg)			
Frontal cortex	412.1 ± 27.7	188.7 ± 77.5	$p < 0.0001$****
Hippocampus	451.9 ± 95.3	202.9 ± 78.4	$p < 0.0001$****

*Unpaired Student's *t*-test; ^xMann–Whitney *U*-test.

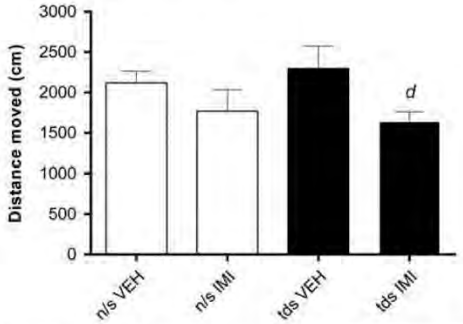

Fig. 2. Comparison between locomotor activity of unstressed and TDS exposed FSL rats before (white) and after (black) sub-chronic IMI treatment. TDS VEH versus TDS IMI: $d = 0.94$. All data analysed by two-way analysis of variance followed by Bonferroni's *post-hoc* tests and Cohen's *d* analysis. Data are represented as mean ± SEM. IMI, imipramine; n/s, non-stressed; TDS, time-dependent sensitisation; VEH, vehicle.

in TDS-exposed FSL animals (46.0 ± 36.7 vs. 24.4 ± 9.8 s, $p = 0.03$), whereas failing to affect the behaviour of animals in the unstressed group.

Climbing. FSL rats presented with significantly reduced climbing behaviour compared with FRL rats (35.0 ± 9.2 vs. 117.9 ± 38.0 s; $p < 0.0001$, Table 3).

Data describing the effects of stress and IMI treatment in FSL animals are presented in Fig 3b. A significant interaction was displayed between TDS and treatment [$F(1,44) = 28.5$, $p < 0.0001$], whereas both treatment [$F(1,44) = 49.6$, $p < 0.0001$] and stress [$F(1,44) = 57.1$, $p < 0.0001$] had significant main effects on climbing behaviour. Although no difference

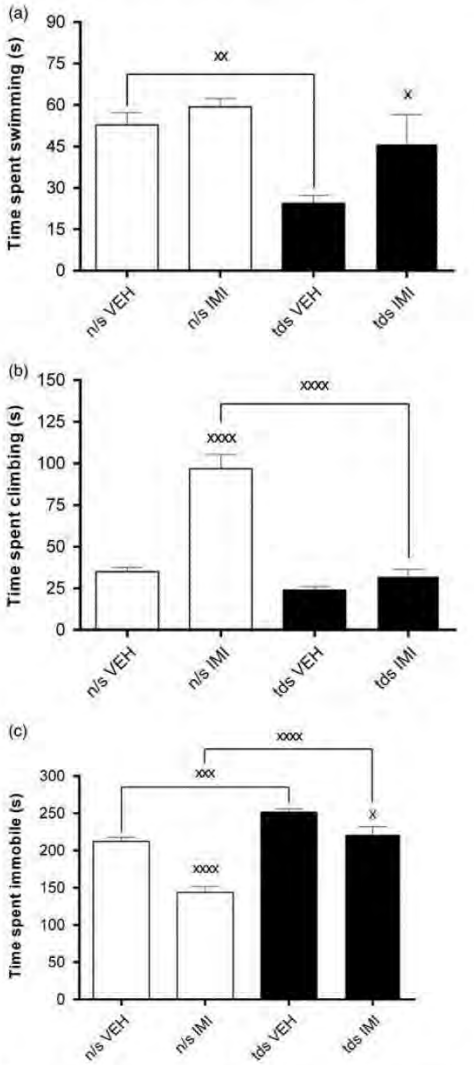

Fig. 3. Comparisons between behavioural parameters measured in the forced swim test [panel (a) = time swimming, panel (b) = time climbing, panel (c) = immobility time] in unstressed and TDS-exposed FSL rats before and after sub-chronic IMI treatment. (a) Time spent swimming (s). n/s VEH versus TDS VEH, $^{xx}p < 0.01$; TDS VEH versus TDS IMI, $^{x}p < 0.05$. (b) Time spent climbing (s). n/s VEH versus n/s IMI, $^{xxxx}p < 0.0001$; n/s IMI versus TDS IMI, $^{xxxx}p < 0.0001$. (c) Time spent immobile (s). n/s VEH versus n/s IMI, $^{xxxx}p < 0.0001$; n/s IMI versus TDS IMI, $^{xxxx}p < 0.0001$; n/s VEH versus TDS VEH, $^{xxx}p < 0.001$; TDS VEH versus TDS IMI, $^{x}p < 0.05$. All the data were analysed by two-way analysis of variance followed by Bonferroni's *post-hoc* tests and Cohen's *d* analysis. Data are represented as mean ± SEM. IMI, imipramine; n/s, non-stressed; TDS, time-dependent sensitisation; VEH, vehicle.

between the climbing behaviour of treatment naive stressed and unstressed FSL animals was demonstrated, *post-hoc* analysis revealed that IMI treatment significantly increased climbing in unstressed FSL animals (96.8 ± 29.2 vs. 35.0 ± 9.3 s, $p < 0.0001$), but was without effect in FSL+TDS animals (32.4 ± 13.9 vs. 24.0 ± 7.9 s, n/s). In fact, in comparing FSL+TDS with non-stressed FSL rats receiving IMI, exposure to TDS significantly negated the response to IMI in FSL animals (32.4 ± 13.9 vs. 96.8 ± 29.2 s, $p < 0.0001$).

Immobility. FSL rats presented with significantly increased immobility compared to FRL rats (212.1 ± 18.8 vs. 111.7 ± 33.70 s; $p < 0.0001$, Table 3).

Data describing the effects of stress and IMI treatment in FSL animals are presented in Fig 3c. As a significant two-way interaction was revealed between TDS and treatment with respect to the immobility scores of FSL animals [$F(1,44) = 6.8$, $p = 0.01$], simple main effects of both factors were run. Exposure to TDS [$F(1,44) = 64.4$, $p < 0.0001$] significantly increased the average immobility score of treatment naive FSL animals (251.7 ± 14.7 vs. 212.1 ± 18.8 s, $p = 0.0008$). Although IMI treatment [$F(1,44) = 45.4$, $p < 0.0001$] resulted in significant reductions in the immobility scores of both unstressed (143.8 ± 27.1 vs. 212.1 ± 18.8, $p < 0001$) and stressed (221.5 ± 35.7 vs. 251.7 ± 14.2, $p = 0.01$) FSL rats, immobility in IMI-treated FSL+TDS animals *remained* significantly greater than that in unstressed FSL animals receiving IMI (221.5 ± 35.7 vs. 143.8 ± 27.1, $p < 0.0001$).

Monoamine analysis

In order to establish the translational relevance of the FSL rat for depression, data and statistics relating to the neurochemical comparisons made between stress and treatment-naive FRL and FSL animals are provided in Table 3. The neurochemical differences between FSL versus FRL rats are henceforth described separately below.

5HIAA. FSL rats presented with significantly increased 5HIAA levels in the frontal cortex (268.4 ± 51.3 vs. 170.4 ± 22.8 ng/mg; $p < 0.005$) and hippocampus (244.1 ± 40.3 vs. 177.2 ± 37.2 ng/mg; $p < 0.05$) versus FRL rats (Table 3).

Data describing the effects of stress and IMI treatment in FSL animals are presented in Fig 4a. Two-way ANOVA revealed significant interactions between TDS and treatment in both brain areas [frontal cortex, $F(1,27) = 7.6$, $p = 0.01$; hippocampus, $F(1,27) = 4.45$, $p = 0.04$] and simple main effects were run. As such, TDS *and* treatment significantly influenced the concentrations of 5HIAA measured in both the frontal cortex [TDS, $F(1,27) = 30.8$, $p < 0.0001$; treatment, $F(1,27) = 6.1$, $p = 0.02$] and hippocampus [TDS, $F(1,27) = 15.72$, $p = 0.0005$; treatment, $F(1,27) = 11.1$, $p = 0.002$]. As such, *post-hoc* analyses revealed that 5HIAA levels in treatment naive stressed FSL animals tended to be lower compared with the unstressed FSL animals (Fig 4ai, 216.4 ± 45.6 vs. 268.4 ± 51.3 ng/mg, $d = 1.07$; Fig 4aii, 201.5 ± 59.1 vs. 244.1 ± 40.4 ng/mg, $d = 0.85$). Although IMI treatment significantly increased both frontocortical (Fig 4ai, 366.0 ± 69.0 vs. 268.4 ± 51.3 ng/mg, $p = 0.007$) and hippocampal (Fig 4aii, 369.1 ± 87.3 vs. 244.1 ± 40.4 ng/mg, $p = 0.004$) 5HIAA levels in unstressed FSL rats, exposure to TDS negated this effect (Fig 4ai, frontal cortex, 210.8 ± 32.4 vs. 216.4 ± 45.6 ng/mg, n/s; Fig 4aii, hippocampus, 229.7 ± 58.7 vs. 201.5 ± 59.0 ng/mg, n/s) and resulted in significantly lower levels of 5HIAA levels measured in IMI-treated animals after TDS-exposure relative to stress-naive animals (Fig 4ai, frontal cortex, 210.8 ± 32.4 vs. 366.0 ± 69.0 ng/mg, $p < 0.0001$; Fig 4aii, hippocampus, 229.7 ± 58.7 vs. 369.1 ± 87.3 ng/mg, $p = 0.0005$).

NA. FSL rats presented with significantly reduced NA levels in the frontal cortex (188.7 ± 77.5 vs. 412.1 ± 27.7 ng/mg; $p < 0.0001$) and hippocampus (202.9 ± 78.4 vs. 451.9 ± 95.3 ng/mg; $p < 0.0001$) versus FRL rats (Table 3).

Data describing the effects of stress and IMI treatment in FSL animals are presented in Fig 4b. No significant two-way interactions between TDS and treatment were observed in either brain area [frontal cortex, $F(1,27) = 0.01$, $p = 0.9$; hippocampus, $F(1,26) = 1.3$, $p = 0.3$]. However, treatment demonstrated a main effect on NA concentrations in the frontal cortex [$F(1,27) = 8.4$, $p = 0.007$] and hippocampus [$F(1,26) = 11.29$, $p = 0.002$]. Although IMI resulted in trends toward increased NA in both brain areas of unstressed FSL animals (frontal cortex, 285.2 ± 119.1 vs. 188.7 ± 77.5 ng/mg, $d = 0.98$; hippocampus, 308.5 ± 126.1 vs. 202.9 ± 78.4 ng/mg, $d = 1.03$), it significantly increased the hippocampal NA levels in stressed FSL animals to levels comparable with that observed in unstressed animals (Fig 4bii, 364.9 ± 212.8 vs. 151.9 ± 41.4 ng/mg, $p = 0.04$). Furthermore, although narrowly missing statistical significance, IMI also tended to increase NA in the frontal cortex of stressed FSL animals (Fig 4bi, 243.8 ± 96.2 vs. 154.0 ± 51.1 ng/mg, $p = 0.054$, $d = 1.2$).

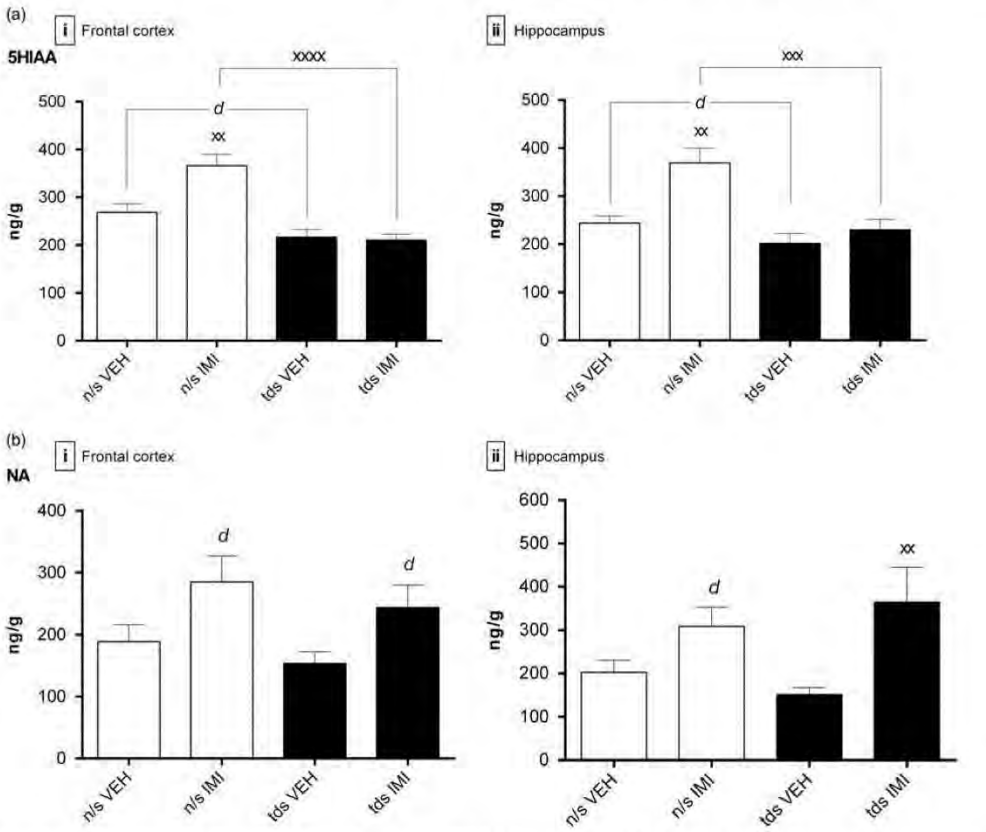

Fig. 4. Comparisons between frontocortical and hippocampal 5HIAA [panel (a)] and NA [panel (b)] in unstressed and TDS-exposed FSL rats before and after sub-chronic IMI treatment. Panel (ai) Frontal-cortical 5HIAA concentrations. n/s VEH versus n/s IMI, $^{xx}p < 0.01$; n/s IMI versus TDS IMI, $^{xxxx}p < 0.0001$; n/s VEH versus TDS VEH, $d = 1.07$. Panel (aii) Hippocampal 5HIAA concentrations. n/s VEH versus n/s IMI, $^{xx}p < 0.01$; n/s IMI versus TDS IMI, $^{xxx}p < 0.001$, n/s VEH versus TDS VEH, $d = 0.87$. Panel (bi) Frontal-cortical NA concentrations. n/s VEH versus n/s IMI, $d = 0.98$; TDS VEH versus TDS IMI, $d = 1.22$. Panel (bii) Hippocampal NA concentrations. TDS VEH versus TDS IMI, $^{xx}p < 0.01$; n/s VEH versus n/s IMI, $d = 1.03$. All data analysed by two-way analysis of variance followed by Bonferroni's *post-hoc* tests and Cohen's *d* analysis. Data are represented as mean ± SEM. FC, frontal cortex; HC, hippocampus; 5HIAA, 5-hydroxyindoleacetic acid; IMI, imipramine; NA, noradrenaline; n/s, non-stressed; TDS, time-dependent sensitisation; VEH, vehicle.

Discussion

As expected, FSL rats presented with significant depressive-like manifestations versus their FRL controls at both the behavioural and neurochemical level (Table 3), with IMI for the most part reversing these changes (Figs 3 and 4). Exposure of FSL rats to TDS profoundly inhibited growth (Fig 1), with behavioural and neurochemical sequelae (Figs 3 and 4). TDS further reduced active coping (swimming) behaviour and amplified depressive-like behaviour (immobility) in FSL rats (Fig 3a, c). Importantly, the above-noted antidepressant-like effects of IMI in FSL rats were significantly attenuated after TDS exposure. Although IMI altered brain monoamine levels in unstressed FSL rats, it failed to do so in combined FSL+TDS rats – especially effects on 5HIAA (Fig 4). As such, combining FSL+TDS stress may represent a novel animal model of TRD, a schematic outline of which is depicted in Fig 5.

Depression is a multifactorial disorder (7) with both genetics and environmental stress contributing to its development (14,15). The FSL rat is a well-validated genetic animal model of depression (27). Considering the high comorbidity of depression in PTSD and as a

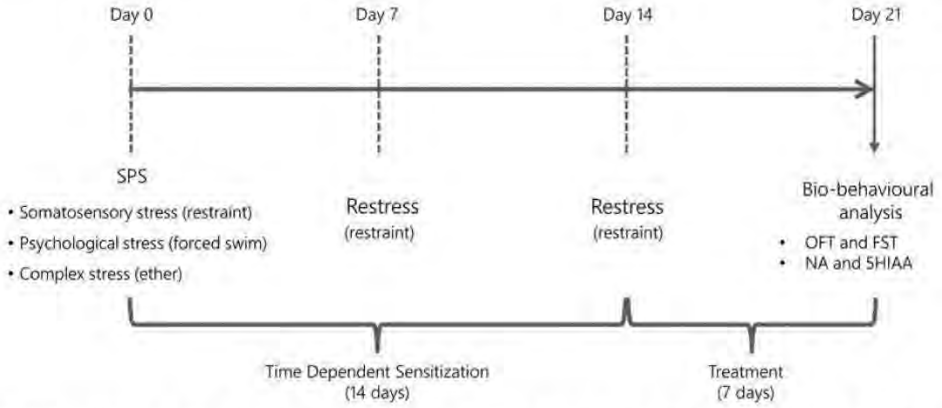

Fig. 5. Procedural outline of the treatment-resistant depression (TRD) model. The TRD model is a coming together of two translational animal models, namely exposing a genetic animal model of depression (FSL rats) with the time-dependent sensitisation (TDS) model of post-traumatic stress disorder (PTSD). Rats are exposed to single prolonged stress (SPS; day 0) – a triple stressor sequence comprising a somatosensory stressor (restraint), a psychological stressor (forced swimming with brief submersion), and a complex stress-stimuli (exposure to ether vapours), followed by a less stressful but situational reminder of the original stressor (restraint stress on days 7 and 14). The latter is to enable consolidation of contextual fear memory to promote the progression from an acute stress disorder to PTSD. Thereafter, the animals are left undisturbed for another 7 days before being subjected to behavioural and neurochemical analysis (day 21; two separate cohorts of animals). Drug treatment takes place during the latter 7-day period immediately prior to bio-behavioural testing. FST, forced swim test; 5HIAA, 5-hydroxyindoleacetic acid; FSL, Flinders sensitive line; NA, noradrenalin; OFT, open field test.

contributing factor in treatment resistance (34,35), introducing these animals to conditions conducive to PTSD may serve as a suitable gene-X-environment model of TRD. The aim of this study was therefore to explore this notion by studying behaviour and neurochemistry in such a model and, in so doing, to aid preclinical research into TRD and developing novel drug options for the disorder. By using FRL rats as a control, we demonstrated the depressive phenotype of the FSL rat, thereafter subjecting this stress-sensitive animal to a TDS paradigm and assessing its response to standard antidepressant treatment. The negative impact of TDS on physical development, as illustrated by its detrimental effects on growth during a 4-week period (Fig 1), is indicative of the degree to which the physical and, no doubt, "psychological" well-being of these animals were affected by these interventions. The results of the comparison between cumulative weight gain in FRL rats and stressed and unstressed FSL rats provide an accurate portrayal of the character and resilience of the two strains. At baseline, FSL rats already displayed decreased ability to gain weight even before exposure to environmental stressors. Bearing this in mind, TDS expectedly proved to further worsen the overall well-being of these animals.

Rats exposed to CMS have previously been observed to exhibit impaired locomotor activity (22), although TDS did not negatively affect locomotor activity in the current study (Fig 2). Although IMI treatment resulted in a trend toward decreased locomotor activity in TDS exposed animals, this failed to reach statistical significance. As such, this finding provides a robust departure point for interpreting treatment effects in the FST without having to consider any confounding effects on locomotor activity.

Immobility time is a characteristic depressive-like behaviour measured in the FST, whereas the assessment of swimming and climbing behaviour allows for generating a more holistic account of coping behaviour and also aids in understanding the behavioural effects of drug treatment (54). Results obtained from the FST showed that FSL rats displayed significantly less active coping (swimming and climbing) behaviour as well as being significantly more immobile than their FRL counterparts (Table 3). Important to note is that both decreased swimming behaviour and increased immobility observed in FSL control animals were augmented to a significant degree following exposure to TDS (Fig 3a and c). Of even greater importance is that the antidepressant-like effect exhibited by IMI treatment in unstressed FSL animals was negated in TDS-exposed FSL rats in respect to climbing and immobility (Fig 3b and c) although not swimming (Fig 3a). Further, the anti-immobility effects of IMI in FSL animals were also significantly compromised by TDS compared with

that in unstressed FSL animals (Fig 3c). Thus TDS stress exaggerates depressive-like (immobility) behaviour evident in a genetic animal model of depression and abrogates the antidepressant-like effects of IMI in these animals. This not only supports the validity of combining FSL rats with a PTSD paradigm as a gene-X-environment model of TRD, but reinforces the clinical presentation of TRD in depressed patients with a history of severe psychological trauma.

In spite of diffuse distribution of 5HT throughout the central nervous system, uncertainty still surrounds the exact function of 5HT and its relationship to other neurochemicals (55,56). TDS represents a severely traumatic series of events which prompts the activation of various bio-behavioural responses geared to maximise the animal's survival. Serotonin is crucial in survival behaviour and has been suggested to play a critical role in adapting to aversive events (57,58). In fact, stress re-stress has been demonstrated to alter 5HT receptors in limbic structures that in turn adversely affect memory and other cognitive processes (37). Further, the changes in 5HT concentration in response to stress vary between brain regions and also according to the duration of stress applied (59). FSL rats have previously been characterised by increased levels of 5HT and 5HIAA in limbic regions that are altered in response to antidepressant treatment (60). Increased cortical and hippocampal 5HIAA levels in FSL rats compared with FRL controls in the current study concur with this observation (Table 3) and would suggest a compromised serotonergic system. 5HIAA levels were decreased in FSL rats 1 week after TDS (Fig 4a) – this decrease correlating with significantly reduced swimming activity measured in the FST and concurs with decreased 5HIAA levels measured in the frontal cortex of Sprague–Dawley rats subjected to CMS (61). Although TDS may be viewed as a series of aversive and traumatic events (36), it should be kept in mind that the current data reflects NA and 5HIAA changes 1 week subsequent to completion of the TDS procedure, and thus represents a late emerging event that may be pathological. Previous data demonstrated that an initial increase in 5HT levels after SPS was followed by decreased levels after re-stress (42) which may be suggestive of an adaptive response to stress.

The above-mentioned coping strategies employed in the FST have been found to present with significant correlations with altered monoamines and to be of relevance for the neurochemical basis of depression (62). Thus, noradrenergic processes have been demonstrated to be altered in depression, but also in anxiety and PTSD, such as adrenergic receptor dysregulation in depression (63), increased NA precursors accompanied by a decrease in adrenergic receptor affinity in patients suffering from PTSD with comorbid depression (64), the association between catechol-O-methyl transferase single nucleotide polymorphisms and suicide risk in TRD patients (65), and increased 3-methoxy-4-hydroxyphenylglycol levels measured in patients suffering from anxiety disorders (66). Furthermore, uncontrollable stress in animal models is associated with decreased central levels of NA (67,68) and may be the result of insufficient synthesis of the neurotransmitter relative to its utilisation (67). A general decrease in NA levels were measured in the frontal cortex and hippocampus of treatment-naive FSL animals (Table 3). Owing to the premise that increased climbing and swimming behaviour in the FST may be a result of enhanced noradrenergic and serotonergic neurotransmission, respectively (62), the decreased frontal–hippocampal NA levels in both stressed and unstressed FSL rats (Fig 4bi and 4bii; Table 3) as well as the trend to raise NA levels and significantly elevate NA levels in the cortex and hippocampus, respectively, by IMI in TDS + FSL rats (Fig 4b) were expected and congruent with the current thinking on the role of NA in depression (5). However, it is apparent that TDS-exposure abrogated the climbing-enhancing effect of IMI in FSL animals (Fig 3b) as well as sustained lowered NA in the cortex and hippocampus of untreated animals (Fig 4bi and bii). Indeed, TDS has been found to significantly increase NA after SPS, eventually falling to levels significantly lower than baseline 1 week after re-stress (42). The inability of IMI to increase climbing behaviour in stressed FSL rats (Fig 3b), despite its tendency to elevate NA in the cortex as well as significantly increase NA in the hippocampus, is of interest but may be a result of a decrease in adrenergic receptor density and/or affinity as previously reported in both humans (64,69) and animals (70,71) exposed to stress.

Given that limbic brain structures are involved in the stress response, changes in 5HT-related responses may be linked to changes in hippocampal and cortical 5HT neurotransmission (37,39). A general decrease in 5HIAA levels were measured in the frontal cortex and hippocampus of treatment-naive FSL animals (Table 3). TDS worsened swimming deficits as well as duration of immobility (Fig 3a and c) and sustained reduced cortical and hippocampal 5HIAA levels (Fig 4a). Although IMI significantly reduced immobility in unstressed and stressed FSL rats, immobility in the latter group remained significantly higher than that of unstressed IMI-treated rats (Fig 3c) and *failed* to reverse lowered 5HIAA in FSL + TDS animals (Fig 4a).

Tricyclic antidepressants such as IMI act by increasing the extracellular levels of NA *and* 5HT (72). IMI significantly reversed deficits in swimming in FSL+TDS animals (Fig 3a), but failed to reverse lowered limbic 5HIAA levels in these animals (Fig 4a). On the other hand, IMI failed to reverse suppressed climbing in FSL+TDS animals (Fig 3b) despite provoking a tendency (in the FC) and to significantly (in the hippocampus) reverse lowered NA in these animals (Fig 4b). This paradox with respect to limbic monoamine levels and coping strategies may indicate other adaptive changes that underlie coping responses following sustained exposure to stress. Furthermore, it cannot be assumed that the effects of antidepressant drugs are simply to reverse and/or normalise dysfunctions in the brain (73), including those of animals. This has been exemplified by CMS-induced behavioural effects in mice, demonstrating that while aberrant behavior was reversed by fluoxetine, the drug failed to alter most of the underlying stress-induced biological effects (74).

In conclusion, exposing FSL rats to TDS resulted in either bolstered or sustained reduction in coping and an increase in depressive-like behaviours, combined with altered monoaminergic profiles in hippocampal and frontocortical brain regions. Furthermore, the addition of TDS to FSL rats significantly abrogated the antidepressant-like effects of IMI at most behavioural levels (climbing and immobility) and with respect to limbic 5HT. Data presented here therefore supports the proposed hypothesis that exposure of a genetic animal model of depression to a PTSD-like paradigm results in a more severe depressive-like profile that is resistant to traditionally effective antidepressant treatment. The results of the current study have potential value in the search for a suitable animal model of TRD and warrants further investigation. Challenging FSL+TDS animals with first-line antidepressants (serotonin selective reuptake inhibitor or SSRI, or noradrenaline serotonin reuptake inhibitor or NSRI) and/or ketamine would expand predictive validity, and is presented in a companion paper to this manuscript (75).

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Conflicts of Interest

The authors declare that over the past 3 years, Brian Harvey has participated in advisory boards and received honoraria from Servier®, and has received research funding from Servier® and Lundbeck®. The authors declare that, except for income from the primary employer and research funding to BHH from the MRC, NRF, or the above-mentioned exceptions, no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional services, and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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Addendum E –
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Exploring a post-traumatic stress disorder paradigm in Flinders sensitive line rats to model treatment-resistant depression II: response to antidepressant augmentation strategies

Brand SJ, Harvey BH. Exploring a post-traumatic stress disorder paradigm in Flinders sensitive line rats to model treatment-resistant depression II: response to antidepressant augmentation strategies.

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Objective: Post-traumatic stress disorder (PTSD) displays high co-morbidity with major depression and treatment-resistant depression (TRD). Earlier work demonstrated exaggerated depressive-like symptoms in a gene × environment model of TRD and an abrogated response to imipramine. We extended the investigation by studying the behavioural and monoaminergic response to multiple antidepressants, viz. venlafaxine and ketamine with/without imipramine.
Methods: Male Flinders sensitive line (FSL) rats, a genetic model of depression, were exposed to a time-dependent sensitisation (TDS) model of PTSD and compared with stress naive controls. 7 days after the TDS procedures, immobility and coping (swimming and climbing), behaviours in the forced swim test (FST) as well as hippocampal and cortical 5-hydroxyindoleacetic acid (5HIAA) and noradrenaline (NA) levels were analysed. Response to imipramine, venlafaxine and ketamine treatment (all 10 mg/kg × 7 days) alone and in combination were subsequently studied.
Results: TDS exacerbated depressive-like behaviour of FSL rats in the FST. Imipramine, venlafaxine and ketamine were ineffective as monotherapy in TDS-exposed FSL rats. However, combining imipramine with either venlafaxine or ketamine resulted in significant anti-immobility effects and enhanced coping behaviours. Only ketamine + imipramine (frontal-cortical 5HIAA and NA), ketamine alone (frontal-cortical and hippocampal NA) and venlafaxine + imipramine (frontal-cortical NA) altered monoamine responses versus untreated TDS-exposed FSL rats.
Conclusion: Exposure of FSL rats to TDS inhibits antidepressant response at behavioural and neurochemical levels. Congruent with TRD, imipramine plus venlafaxine or ketamine overcame treatment resistance in these animals. These data further support the hypothesis that exposure of FSL rats to a PTSD-like paradigm produces a valid animal model of TRD and warrants further investigation.

Keywords: animal model; NMDA antagonist; noradrenaline reuptake inhibitor; serotonin reuptake inhibitor; stress re-stress

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Significant outcomes

- Sub-chronic treatment with imipramine, venlafaxine and ketamine as monotherapy *failed* to evoke antidepressant-like effects in the forced swim test (FST) in Flinders sensitive line (FSL) rats exposed to time-dependent sensitisation (TDS)-stress, suggesting treatment resistance to multiple classes of antidepressants.
- Combining imipramine with either venlafaxine or ketamine produced a significant reversal of treatment resistance in all behavioural parameters in the FST.
- Only ketamine + imipramine [frontal-cortical 5-hydroxyindoleacetic acid (5HIAA) and noradrenaline (NA)], ketamine alone (frontal-cortical and hippocampal NA) and venlafaxine + imipramine (frontal-cortical NA) increased NA and 5HIAA responses versus untreated TDS-exposed FSL rats, supporting evidence of a more robust response following combination treatment.

Limitations

- Behavioural assessment of anhedonia (sucrose preference test), which has been demonstrated to be an important symptom of treatment-resistant depression (TRD), would be a valuable addition.
- This study is limited to observations made after sub-chronic antidepressant (7 days) treatment. Extending treatment duration (inadequate treatment duration is often a reason for antidepressant non-response), and increasing dosages may provide additional support for current findings.
- Applying additional biochemical measures, for example monoamine responses via *in vivo* micro-dialysis and/or determination of putative molecular biomarkers of TRD such as $5HT_{1A}$-receptor expression, would bolster construct validity.

Introduction

Major depression (MD) is a commonly occurring disorder with a lifetime prevalence rate of ~16% (1). Despite several classes of antidepressants being available to clinicians (2), pharmacological management remains suboptimal. High rates of recurrence is a constant challenge, with symptom severity serving as the greatest predictor of a poor outcome (3). In fact, >50% of patients still experience persistent symptoms of MD after treatment with a first line antidepressant (4). The Sequenced Treatment Alternatives to Relieve Depression (STAR*D) study found that up to 30% of patients did not achieve remission despite being subjected to multiple antidepressant drug treatments (5). STAR*D was designed to replicate clinical settings, and highlighted the low remission rates associated with TRD (6).

Relative to MD, TRD is associated with more severe symptomatology (7) as well as increased morbidity and mortality (8). In addition, an increased presence of somatic symptoms, for example pain and fatigue (9), may predict increased treatment resistance (8). The impact of TRD on healthcare expenditure is proportional to the degree of resistance (10), requiring more frequent hospitalisation (11), increased use of pharmacotherapy (11) together with an increased disease burden (8). Despite important strides in our understanding of the neurobiology and treatment of MD, as well as increased use of antidepressants (12–14), TRD remains an undeniable concern. Nevertheless, various strategies have been employed to alleviate the non- or partial response to antidepressant treatment (15).

Current approaches to treating TRD include both pharmacological and non-pharmacological (e.g. electroconvulsive therapy, psychotherapy and deep brain stimulation) approaches. Drug-centred approaches are based on switching between antidepressants either in the same or across drug classes or employing augmentative drug therapies (adding a drug from a different class or with a different mechanism of action) (2,16). However, it would seem that switching within or between drug classes offers limited therapeutic benefit (17). Interestingly, the latter study suggests that adjunctive treatment may accelerate symptom improvement and improve remission rates, although the authors hasten to note that the success of such a strategy requires the initial drug treatment to have at least some degree of efficacy (18), and that the adjunct treatment enhances these improvements (17). Potential augmentation agents include selective serotonin reuptake inhibitors (SSRIs) and selective NA reuptake inhibitors (SNRIs) (e.g. venlafaxine), atypical antipsychotics and glutamatergic drugs (e.g. ketamine) (19). Venlafaxine has been found to be slightly more effective than several SSRIs in patients with severe MD (20,21), and acts by increasing both serotonergic and noradrenergic activity (21). Ketamine, on the other hand, acts as an N-methyl-D-aspartate (NMDA) receptor antagonist (22) and is associated with a proven rapid onset of action (23)

and high response rate (22), with benefits demonstrated following acute and chronic treatment (22).

Psychiatric co-morbidity is a common problem in patients with MD (24,25), with co-occurrence of anxiety disorders ranging between 50% and 60% (5,26). Importantly, such co-occurrence is increasingly being associated with antidepressant treatment failure (24,27,28). Post-traumatic stress disorder (PTSD) is one of the more commonly co-occurring anxiety disorders in MD, and is especially prevalent in TRD (5). Furthermore, half of patients with PTSD have co-morbid MD (29) with the high co-morbidity attributed to an overlap in symptoms, for example anhedonia, sleep difficulty, irritability and poor concentration (Diagnostic and Statistical Manual of Mental Disorders criteria) (30,31), whereas both MD and PTSD are precipitated by a chronic or severe traumatic event, respectively (30). Such co-occurrence is also positively associated with symptom severity (31) and treatment resistance (32,33).

The complexity and heterogeneity of MD makes it unlikely that any one animal model will fully embody the behavioural and biological characteristics of the disorder. However, modifying existing models to represent specific phenotypes of the disorder may hold promise. The gene × environment hypothesis of MD has enabled the conceptualising of genetic susceptibility combined with environmental adversity as prodromal events to the subsequent development of MD (34–36). Moreover, Willner and Belzung (37) emphasise that the search for treatments for TRD may require models that incorporate predisposing factors leading to heightened stress responsiveness. The co-morbidity of PTSD and MD and its association with treatment resistance is thus noteworthy. Consequently, we have recently developed an animal model of TRD by superimposing a PTSD-related paradigm, viz. time-dependent sensitization (TDS), on the Flinders Sensitive Line (FSL) rat (38). FSL rats are a well-studied genetic animal model of MD (39), whereas TDS is based on a stress re-stress procedure (40) with proven predictive, construct and face validity for PTSD (41–44). In a companion paper (38), we describe how exposing FSL rats to TDS evokes more pronounced depressive-like behaviour together with altered limbic monoamine levels versus unstressed FSL rats, as well as engendering resistance to sub-chronic imipramine treatment. To extend the predictive validity of the model, we investigated sub-chronic imipramine treatment in TDS-exposed FSL rats compared with that of venlafaxine and ketamine monotherapy as well as versus imipramine plus venlafaxine or ketamine to simulate a typical TRD regime. Post-treatment cortico-limbic monoamines were analysed after behavioural analysis.

Materials and methods

Subjects

Animals were bred and housed at the Vivarium (SAVC reg. number FR15/13458; SANAS GLP compliance number G0019) of the Pre-Clinical Drug Development Platform of the North-West University (NWU). Ambient temperature was maintained at 22 ± 2°C with a relative humidity of 40–60% and full spectrum of light in a 12 h light/dark cycle, with lights switched on at 06:00 a.m. and off at 06:00 p.m. Food and water were provided *ad libitum*. All experiments were approved by the AnimCare animal research ethics committee (NHREC reg. number AREC-130913-015) of the NWU. All 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-00111-12-A5).

The original colonies of FSL rats and their control Flinders resistant line (FRL) rats were obtained from Dr. David Overstreet, University of North Carolina, USA. Subjects were male adult FSL rats ($n = 84$ for behavioural assessment and $n = 56$ for monoamine analysis). Table 1 describes the layout of the experimental groups. Animals in all experimental groups were either subjected to the PTSD paradigm, namely TDS, or left undisturbed (unstressed) in their home cages, after which behaviour of all animals was analysed in the open field test (OFT) and FST, with subsequent monoamine analyses performed in animals naive to behavioural assessment. Animals were housed four per cage, with the TDS paradigm initiated at an age of 40 (±1) days in order to conclude the experiments while the rats were still of an appropriate weight for the behavioural assessments. Handling of the animals was initiated 1 week before starting the experimental procedure by taking bodyweight measurements daily until the last day of the study to monitor weight gain and to calculate drug dosages.

Time dependent sensitization (TDS)

TDS is an animal model of PTSD. Animals exposed to a severely traumatic situation followed by subsequent, but less stressful, contextual reminders exhibit significant physiological and behavioural alterations that show a time-dependent sustaining or worsening in the absence of the initiating stressor (45,46). The TDS paradigm used in this study (see Fig. 1) incorporated an acute single prolonged stress (SPS) sequence comprising a somato-sensory stressor (restraint), a psychological stressor (forced swimming with brief submersion), and a complex stress-stimuli (exposure to ether vapours) followed by re-exposure to restraint stress 7 and 14 days later (45).

Table 1. Layout of experimental groups

Group name	Group description	Dosage	*n*-value: Behavioural	*n*-value: Bio-molecular
FSL – TDS + VEH	Antidepressant naïve unstressed FSL rats	1 ml/kg	12	8
FSL + TDS + VEH	Antidepressant naïve stressed FSL rats	1 ml/kg	12	8
FSL + TDS + IMI	Imipramine-treated stressed FSL rats	10 mg/ml/kg	12	8
FSL + TDS + VEN	Venlafaxine-treated stressed FSL rats	10 mg/ml/kg	12	8
FSL + TDS + KET	Ketamine-treated stressed FSL rats	10 mg/ml/kg	12	8
FSL + TDS + IMI + VEN	Imipramine/venlafaxine-treated stressed FSL rats	10 mg/ml/kg	12	8
FSL + TDS + IMI + KET	Imipramine/ketamine-treated stressed FSL rats	10 mg/ml/kg	12	8

FSL, Flinders sensitive line; TDS, time-dependent sensitisation; VEH, vehicle; IMI, imipramine; KET, ketamine; VEN, velafaxine.

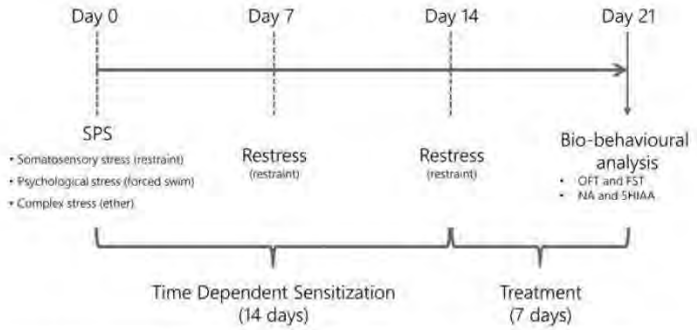

Fig. 1. Schematic outline of the treatment-resistant depression (TRD) procedure. On day 0, rats are exposed to single prolonged stress (SPS) followed by re-exposure to restraint stress on days 7 and 14. Subsequently, behavioural assessments [open field test (OFT) and forced swim test (FST)] and monoaminergic analyses [noradrenaline (NA) and 5-hydroxyindoleacetic acid (5HIAA)] are performed on day 21.

Restraint stress. Rats were placed in Perspex® restrainers (Instrument Makers, NWU) for 2 h with the tail-gates adjusted to keep each animal well-contained without impairing circulation to the limbs. The same procedure was followed on days 7 and 14 during the re-stress phase of the TDS protocol.

Forced swim stress. Rats were placed individually in cylindrical Perspex® swim tanks (Instrument Makers, NWU) containing 40 cm of ambient water (25°C) and allowed to swim for 15 min while being forcefully submerged for the last 20 s. Thereafter, animals were removed from the cylinders, dried and returned to their home cages for 15 min to recover. Forced swimming was performed 21 days *before* behavioural testing (only as part of the SPS procedure and not during re-stress) in the FST so that any possible conditioned response to swim stress in the FST is unlikely.

Exposure to ether vapours. After 15 min of swim stress, rats were exposed to 5 ml of 100% ether vapours in a 5 l sealed plastic container until loss of consciousness (±2 min). Ether was poured onto a paper towel at the bottom of the container with the animal placed on a raised metal platform to avoid direct contact with the substance. After loss of consciousness, the animals were immediately removed from the plastic container, returned to their home cage for observation until regaining full consciousness and then returned to their holding room. Animals were left undisturbed, thereafter only subjecting them to routine handling until re-exposure to restraint stress during the re-stress phase of the TDS protocol.

Open field test (OFT)

This test is generally performed before the FST to control for locomotor activity possibly contributing to altered swimming performance in the FST and thereby confounding interpretation of the results. The OFT was performed half an hour before subjecting animals to the FST. Rats were individually placed in a square arena (100 × 100 × 50 cm) facing the centre and their behaviour recorded for 5 min using a ceiling-mounted digital camera. The video files were subsequently analysed using EthoVision® XT software (Noldus® Information Technology,

Wageningen, The Netherlands) with total distance moved applied as a measure of locomotor activity.

Forced swim test (FST)

The FST can reliably predict antidepressant-like effects after drug treatment and is considered a model of behavioural despair that is typically manifest in human MD, and expressed in rodents as a decrease in escape-driven behaviour (i.e. increased immobility) (47). During behavioural analysis, rats were placed individually in cylindrical Perspex® swim tanks containing 30 cm of ambient water (25°C) for 7 min and their behaviour recorded. The first and last minute of the video files were discarded and the remaining 5 min of swimming behaviour scored for characteristic escape-directed behaviours, including swimming, climbing (struggling) and immobility. The former two swimming parameters of the FST provide useful information relating to serotonergic (swimming) and noradrenergic (climbing) directed behaviours that may inform on the mode of antidepressant action, allowing possible correlation with whole-brain monoamine levels (48).

Drug administration

After weighing all animals daily (between 09:00 a.m. and 11:00 a.m.), imipramine (Sigma-Aldrich, Kempton Park, South Africa) (49,50), venlafaxine (51) (Adcock Ingram, Midrand, South Africa) and racemic ketamine (52) (Fresenius-Kabi, Midrand, South Africa) was dissolved in physiological saline (0.9% NaCl) and administered subcutaneously at a dose of 10 mg/kg to FSL animals exposed to TDS (see Table 1). Treatment started on day 15 (after completing the TDS protocol on day 14) and persisted for 7 days before behavioural testing commenced on the evening of day 21 (Figure 1). This duration of treatment is regarded adequate to establish an antidepressant response in rats (53–55). Stressed and unstressed control animals were injected with saline vehicle in the same manner as drug-treated animals.

Quantitative analysis of brain 5HIAA and NA

Several valid brain indices of 5-hydroxytraptamine (serotonin; 5HT) activity may be applied, including 5HT and 5HIAA levels and the 5HIAA/5HT ratio (56). In this regard, *in vivo* micro-dialysis has proven to be a reliable method to directly measure extracellular levels of 5HT. However, whole and regional brain monoamine analysis provides total levels of 5HT – both extracellular and unreleased from nerve terminals (57). 5HT is metabolised primarily to 5HIAA and has been demonstrated to reflect reliable insights into time-dependent alterations in 5HT response (58). Moreover, 5HIAA levels have previously been correlated with 5HT function (56), and was therefore applied as an indicator of 5HT-ergic function in the current study. Quantification of NA and 5HIAA in the hippocampus and frontal cortex of animals was performed using a high performance liquid chromatography (HPLC) system with electrochemical detection (HPLC-EC), as previously described (45). An Agilent 1200 series HPLC (Agilent Technologies, Santa Clara, CA, USA), equipped with an isocratic pump, auto sampler and coupled to an ESA Coulochem Electrochemical detector (Dionex, Sunnyvale, CA, USA) with Chromeleon® Chromatography Management System software (version 6.8), was used. NA and 5HIAA concentrations in the tissue samples were determined by comparing the area under the peak of each marker with that of the internal standard, isoprenaline (range 5–50 ng/ml). Linear standard curves (regression coefficient >0.99) were found in this particular range. 5HIAA and NA concentrations were expressed as ng/g wet weight of tissue (mean ± SEM).

Statistical analysis

Statistical analyses were performed using Graphpad Prism® 6 and IBM® SPSS® 22 software under the guidance of the Statistical Consultation Service of the North-West University. In pairwise comparisons of the behaviour ($n = 12$ per group) and neurochemistry ($n = 8$ per group) between treatment naive unstressed and stressed FSL animals, unpaired Student's *t*-tests with Welch's correction (normally distributed data as indicated by Shapiro–Wilk's test for normality $p > 0.05$) or Mann–Whitney *U*-tests (data not distributed normally) were performed. One-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* analysis (normally distributed data) or Kruskal–Wallis ANOVA followed by Dunn's multiple comparisons was applied to comparisons of the behaviour ($n = 12$ per group) and neurochemistry ($n = 8$ per group) in treatment naive and treated stressed FSL animals. Treatment was set as within-subject factor, whereas the respective behavioural and neurochemical parameters were set as between-subject factors. Significance was set at $p < 0.05$ for all comparisons. Where Cohen's *d*-effect sizes were calculated, large effect sizes are indicated by $d > 0.8$ and very large effect sizes by $d > 1.3$.

Results

Behaviour

In order to confirm the translational relevance of the FSL rat for MD, data and statistics relating to the

Table 2. Open field test (OFT), forced swim test (FST) and frontal–hippocampal monoamine data in unstressed Flinders Resistant Line (FRL) vs. Flinders sensitive line (FSL) animals

	FRL	FSL	Significance
OFT			
Total distance travelled (cm)	2273 ± 307.2	2119 ± 505.4	–
FST			
Swimming (s)	70.4 ± 14.8	52.9 ± 15.2	$p = 0.009$**
Climbing (s)	117.9 ± 38.0	35.0 ± 9.2	$p < 0.0001$****; $U = 2$
Immobility (s)	111.7 ± 33.7	212.1 ± 18.8	$p < 0.0001$****
Neurochemistry			
5HIAA (ng/mg)			
Frontal cortex	170.4 ± 22.8	268.4 ± 51.3	$p = 0.0007$***
Hippocampus	177.2 ± 37.2	244.1 ± 40.3	$p = 0.021$*; $U = 10$
Noradrenaline (ng/mg)			
Frontal cortex	412.1 ± 27.7	188.7 ± 77.5	$p < 0.0001$****
Hippocampus	451.9 ± 95.3	202.9 ± 78.4	$p < 0.0001$****

5HIAA, 5-hydroxyindoleacetic acid; TDS, time dependent sensitization; FSL, Flinders Sensitive Line.
*Unpaired Student's *t*-test; *Mann–Whitney *U*-test.

Table 3. Comparisons of data relating to open field and forced swim test behaviour and frontal-cortical and hippocampal markers of monoamine function in treatment naïve unstressed and time-dependent sensitisation (TDS)-exposed Flinders sensitive line (FSL) animals

	TDS-naïve FSL	TDS-exposed FSL	Significance/effect size
Open field test			
Total distance travelled (cm)	2119 ± 505.4	2296 ± 971.7	–
Forced swim test			
Swimming (s)	52.9 ± 15.2	24.4 ± 9.8	$p < 0.0001$****; $U = 6.0$
Climbing (s)	35.02 ± 9.2	24.0 ± 7.9	$p = 0.005$*; $U = 24.5$
Immobility (s)	212.1 ± 18.8	251.7 ± 14.2	$p < 0.001$****
Neurochemistry			
5HIAA (ng/mg)			
Frontal cortex	268.4 ± 51.3	216.4 ± 45.6	$d = 1.07$
Hippocampus	244.1 ± 40.4	201.5 ± 59.0	$d = 0.84$
Noradrenaline (ng/mg)			
Frontal cortex	188.7 ± 77.5	154.0 ± 51.0	–
Hippocampus	202.9 ± 78.4	205.5 ± 167.0	$d = 0.9$

5HIAA, 5-hydroxyindoleacetic acid; TDS, time dependent sensitization; FSL, Flinders Sensitive Line.
*Unpaired Student's *t*-test; *Mann–Whitney *U*-test.

behavioural comparisons made between stress and treatment naive FRL and FSL animals have been presented in the companion manuscript (38), but are reproduced here for the sake of completion (see Table 2). For the remainder of this study, all data described were undertaken in FSL animals with/without concomitant exposure to TDS stress.

Comparison of treatment naive unstressed and TDS-exposed FSL animals is reported in Table 3, and described separately under the relevant sections below.

OFT (Table 3, Fig. 2). Locomotor data from the pairwise comparison between the behaviour of treatment naive unstressed and stressed FSL animals demonstrated no significant differences in overall activity (Table 3).

Considering the various drug treatments on TDS-exposed FSL rats (Fig. 2), one-way ANOVA revealed a significant effect of treatment on the mean locomotor activity scores [$F(5, 64) = 2.65$, $p = 0.03$]. However, *post-hoc* Tukey's analysis failed to demonstrate statistically significant differences between the means of any of the respective treatments.

FST-Swimming (Table 3, Fig. 3a). Data from the pairwise comparison between the swimming behaviour of treatment naive unstressed and stressed FSL animals are provided in Table 3. Here we demonstrate that exposure to TDS significantly reduced the time spent swimming ($p < 0.0001$, $U = 6.0$).

Considering the various drug treatments on TDS-exposed FSL rats (Fig. 3a), Kruskal–Wallis ANOVA

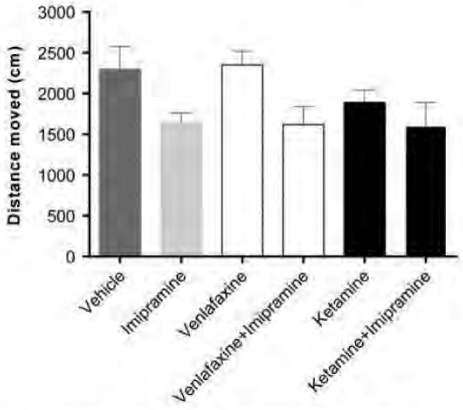

Fig. 2. Comparison between locomotor activity of treatment naive and treated time-dependent sensitisation (TDS)-exposed Flinders sensitive line (FSL) rats ($n = 12$ per group). Vehicle vs. venlafaxine ($d = 0.94$); vehicle vs. ketamine ($d = 1.02$); vehicle vs. combinations of venlafaxine and imipramine ($d = 1.02$) and ketamine and imipramine ($d = 0.9$). Data are represented as mean ± SEM.

revealed significant differences between the median swimming scores of animals in the respective treatment groups [$H(5) = 17.67$, $p = 0.003$]. As such, pairwise comparisons performed using Dunn's procedure with a Bonferroni's correction for multiple comparisons and adjusted p values are presented (Fig. 3a). Although a trend with a large effect size towards increased swimming behaviour was noted in animals treated with both imipramine ($d = 0.93$) and venlafaxine ($d = 1.07$) alone compared with vehicle-treated animals, this increase was significant in the combined venlafaxine + imipramine ($p = 0.005$) and ketamine + imipramine ($p = 0.04$) groups, respectively. Moreover, venlafaxine and ketamine administered as monotherapy had no effect on swimming behaviour.

FST-Climbing (Table 3, Fig. 3b). Data from the pairwise comparison between the climbing behaviour of treatment naive unstressed and stressed FSL animals (Table 3) revealed a significant decrease in the climbing behaviour of stressed FSL animals compared with the unstressed controls ($p < 0.001$, $U = 24.5$).

One-way ANOVA revealed significant differences between the climbing behaviour of TDS-exposed rats in the various treatment groups [Fig. 3b, $F(5, 66) = 6.7$, $p < 0.0001$]. Subsequently, Tukey's *post-hoc* analysis revealed significant differences in climbing behaviour between treatment naive control FSL animals and those treated with venlafaxine + imipramine ($p = 0.01$) and ketamine + imipramine ($p = 0.002$), respectively (Fig. 3b). Furthermore, although a trend towards increased climbing behaviour was demonstrated in animals treated with imipramine alone compared with the vehicle-treated controls ($d = 0.8$), no such trends were demonstrated in groups treated with either venlafaxine or ketamine as monotherapies. Rather, combining both venlafaxine and ketamine with imipramine resulted in bolstered effects on climbing behaviour compared with either venlafaxine ($p = 0.006$) and ketamine ($p = 0.007$) administered alone, indicating an augmenting effect (Fig. 3b).

FST-Immobility (Table 3, Fig. 3c). FSL rats exposed to TDS demonstrated a significant increase in the time spent immobile compared with unstressed FSL controls (Table 3; $p < 0.0001$).

Kruskal–Wallis ANOVA revealed significant differences between the median immobility scores of animals in the respective treatment groups [$H(5) = 33.61$, $p < 0.0001$]. Subsequently, pairwise comparisons were performed using Dunn's procedure with a Bonferroni correction for multiple comparisons of which the adjusted p values are presented (Fig. 3c). Although a trend with a large effect size ($d = 1.21$) towards a decrease in the time spent immobile was noted in animals treated with imipramine alone compared with vehicle-treated animals, this decrease was strengthened by the concomitant administration of imipramine with either venlafaxine ($p < 0.0001$) or ketamine ($p = 0.0007$), respectively. Again, although neither venlafaxine or ketamine had significant effects on immobility scores when administered as monotherapy, combining both with imipramine resulted in bolstered effects on climbing behaviour compared with either venlafaxine ($p = 0.01$) and ketamine ($p = 0.03$) administered alone, indicating an augmenting effect.

5HIAA and NA analysis

5HIAA (Table 3, Fig. 4a). Data from the pairwise comparisons of frontal-cortical and hippocampal 5HIAA concentrations between the treatment naive unstressed and stressed FSL animals are provided in Table 3. No significant differences were observed between the either the frontal-cortical or hippocampal 5HIAA concentrations measured. However, 5HIAA levels measured in TDS-exposed animals strongly tended towards a decrease in both the frontal cortex ($d = 1.07$) and the hippocampus ($d = 0.84$).

With respect to 5HIAA measurements in drug-treated FSL animals, one-way ANOVA revealed significant differences between the mean frontal-cortical 5HIAA concentrations measured in animals

of the different treatment groups [Fig. 4ai: $F(5, 41) = 4.97$, $p = 0.001$]. As such, Tukey's *post-hoc* analysis revealed a significant increase in frontal-cortical 5HIAA levels in ketamine + imipramine treated animals versus vehicle-treated animals ($p = 0.006$), with none of the other treatments effective compared with the control group. In addition, frontal-cortical 5HIAA levels in rats treated with ketamine + imipramine were significantly higher than that of animals treated with imipramine alone ($p = 0.004$), and compared with the combination of imipramine and venlafaxine ($p = 0.003$).

Kruskal–Wallis analysis was applied in comparisons between the hippocampal 5HIAA concentrations measured in the different treatment groups (Fig. 4aii). However, no significant differences could be displayed between the median 5HIAA concentrations of any of the groups compared [$H(5) = 4.19$, $p = 0.52$].

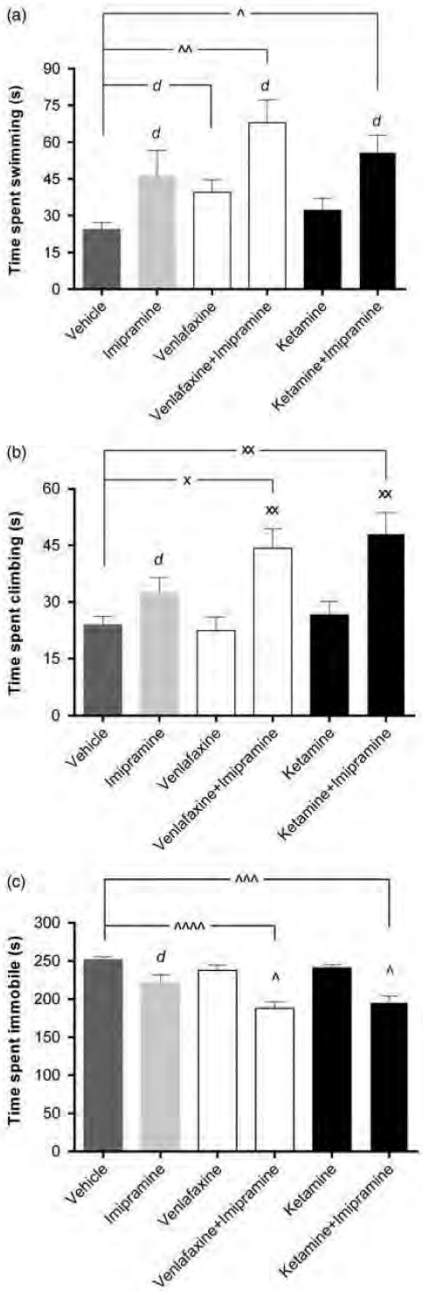

Fig. 3. Comparison between behavioural parameters measured in the forced swim test of treatment naive and treated time-dependent sensitisation exposed Flinders sensitive line rats ($n = 12$ per group). (a) Time spent swimming (s). Vehicle vs. venlafaxine + imipramine, $^{\wedge\wedge}p < 0.001$; vehicle vs. ketamine + imipramine $^{\wedge}p < 0.05$; vehicle vs. imipramine, $d = 0.93$; vehicle vs. venlafaxine, $d = 1.07$; venlafaxine + imipramine vs. venlafaxine, $d = 1.08$. (b) Time spent climbing (s). Vehicle vs. venlafaxine + imipramine, $^{x}p < 0.05$; vehicle vs. ketamine + imipramine, $^{xx}p < 0.001$; venlafaxine vs. venlafaxine + imipramine, $^{xx}p < 0.001$; ketamine vs. ketamine + imipramine, $^{xx}p < 0.001$; vehicle vs. imipramine, $d = 0.8$. (c) Time spent immobile (s). Vehicle vs. venlafaxine + imipramine, $^{\wedge\wedge\wedge\wedge}p < 0.0001$; vehicle vs. ketamine + imipramine, $^{\wedge\wedge\wedge}p < 0.0001$; venlafaxine vs. venlafaxine + imipramine, $^{\wedge}p < 0.05$; ketamine vs. ketamine + imipramine, $^{\wedge}p < 0.05$; vehicle vs. imipramine, $d = 1.21$. ^{x}Two-way analysis of variance (ANOVA) followed by Bonferroni *post-hoc* tests; $^{\wedge}$Kruskal–Wallis ANOVA followed by Dunn's multiple comparisons test. Data are represented as mean ± SEM.

NA (Table 3, Fig. 4b). Data comparing the frontal-cortical and hippocampal NA concentrations of treatment naive unstressed and stressed FSL animals (Table 3) failed to reveal significant differences in both the frontal cortex and hippocampus. However, NA levels measured in TDS-exposed animals trended towards a decrease in the hippocampus ($d = 0.90$).

Considering the various drug treatments on TDS-exposed FSL rats, one-way ANOVA revealed significant differences between the frontal-cortical NA concentrations in the different treatment groups [Fig. 4bi: $F(5, 41) = 7.6$, $p < 0.0001$]. Tukey's *post-hoc* analysis showed that venlafaxine + imipramine ($p = 0.004$), ketamine alone ($p < 0.0001$) and ketamine + imipramine ($p = 0.0004$) induced significantly elevated NA levels compared with vehicle-treated controls (Fig. 4bi).

Considering hippocampal NA measurements, Kruskal–Wallis analysis revealed significant differences

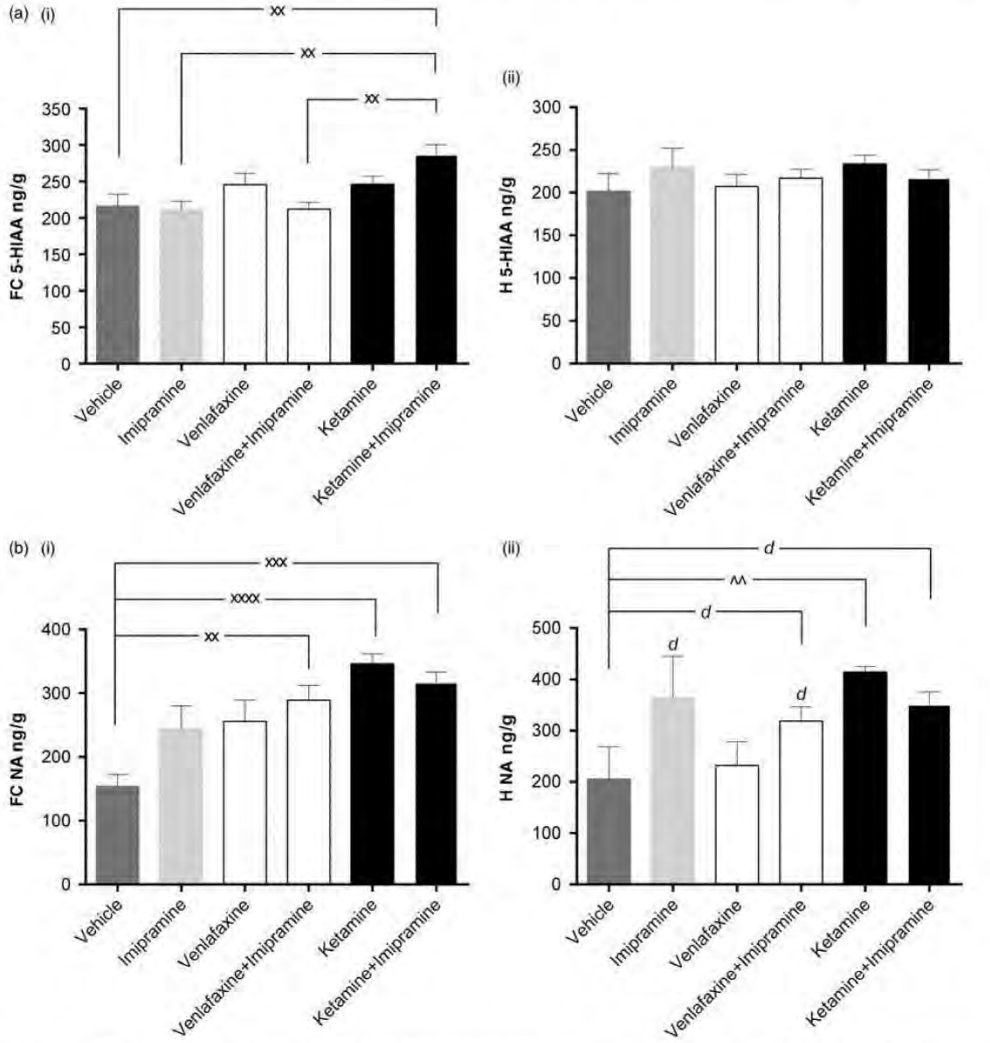

Fig. 4. Comparisons between frontal-cortical (FC) and hippocampal (H) neurochemical markers in treatment naive and treated time-dependent sensitisation exposed Flinders sensitive line rats ($n = 8$ per group). (ai) Frontocortical 5-hydroxyindoleacetic acid (5HIAA) concentrations: vehicle vs. ketamine + imipramine, $^{xx}p < 0.001$; imipramine vs. ketamine + imipramine, $^{xx}p < 0.001$; venlafaxine + imipramine vs. ketamine + imipramine, $^{xx}p < 0.001$. (aii) Hippocampal 5HIAA concentrations. (bi) Frontocortical noradrenaline (NA) concentrations. Vehicle vs. venlafaxine + imipramine, $^{xx}p < 0.001$; vehicle vs. ketamine, $p < 0.0001$; vehicle vs. ketamine + imipramine, $p < 0.0001$. (bii) Hippocampal NA concentrations. vehicle vs. ketamine, $^{\wedge\wedge}p < 0.001$; vehicle vs. imipramine, $d = 0.83$; vehicle vs. venlafaxine + imipramine, $d = 0.92$; vehicle vs. ketamine + imipramine, $d = 1.2$; venlafaxine vs. venlafaxine + imipramine, $d = 0.82$. ^{x}Two-way analysis of variance (ANOVA) followed by Bonferroni *post-hoc* tests; $^{\wedge}$Kruskal–Wallis ANOVA followed by Dunn's multiple comparisons test. Data are represented as mean ± SEM.

in the median levels measured in animals across the different treatment groups [Fig. 4bii: $H(5) = 15.3$, $p = 0.009$]. Pairwise comparisons were performed using Dunn's procedure with a Bonferroni correction for multiple comparisons of which the adjusted p values are presented (Fig. 4bii). Although trends towards increased NA was measured in imipramine ($d = 0.83$), venlaxine + imipramine ($d = 0.92$) and

ketamine + imipramine ($d = 1.2$) treated animals compared with the vehicle-treated controls, only ketamine monotherapy resulted in significantly elevated NA levels versus vehicle-treated controls ($p = 0.01$). A large effect size was also measured in venlafaxine + imipramine versus venlafaxine alone treated animals ($d = 0.82$).

Discussion

Several noteworthy observations have been made in this study. Exposing FSL rats to TDS exacerbates depressive-like behaviour which is depicted by reduced active coping behaviour (swimming and climbing) and increased immobility in the FST (Table 3). Where we noted a reversal of depressive-like behaviour in stress naive FSL rats with sub-chronic imipramine treatment in the companion paper (38), here we found that sub-chronic treatment with imipramine, venlafaxine and ketamine as monotherapy *failed* to evoke a similar response in TDS-exposed FSL rats, indicating treatment resistance to multiple classes of antidepressant (Figs 3a–c). However, combining imipramine with either venlafaxine or with ketamine produced a significant reversal of treatment resistance in all behavioural parameters (Figs 3a–c). Considering monoaminergic responses, TDS-exposed FSL rats displayed a trend towards lowered 5HIAA levels in both the hippocampus and frontal cortex and lowered NA in the hippocampus (Table 3). Where we had previously noted a reversal of limbic 5HIAA and NA changes in stress naive FSL rats with sub-chronic imipramine treatment (38), only ketamine + imipramine (frontal-cortical 5HIAA and NA), ketamine alone (frontal-cortical and hippocampal NA) and venlafaxine + imipramine (frontal-cortical NA) increased monoamine responses versus untreated TDS-exposed FSL rats (Figs 4ai and bi), indicating a more robust response following these combination treatments. In addition, both venlafaxine + imipramine ($d = 0.87$) and ketamine + imipramine ($d = 1.12$) tended to increase NA compared with vehicle-treated animals.

In the clinical setting, acute dosing with ketamine has been proven to induce rapid and robust antidepressant effects in TRD (59,60). More recently, however, several studies have also applied repeated dosing strategies in TRD patients which achieved superior outcomes compared with single administration approaches (61–63). Likewise, in pre-clinical studies, chronic ketamine treatment has also been applied in rats using the FST compared with known antidepressants (64) and also in animals exposed to chronic mild stress (CMS) (52,65,66) where repeated ketamine treatment was associated with long-term anxiolytic- and antidepressant-like effects (66). Taken together, these results suggest that combining ketamine with classic antidepressants would improve antidepressant onset time with lasting and predictable effects (52). Similarly, pre-clinical (67,68) and clinical (20,21,69) data have demonstrated venlafaxine to be equally if not more effective than SSRIs making it a popular treatment choice in patients resistant to SSRI treatment (69).

Compared with stress naive FSL rats, TDS-exposed animals presented with severely exaggerated depressive-like behaviour in the FST, characterised by significant increases in immobility and decreased coping behaviour (swimming and climbing; Table 3). TDS on its own did not adversely affect locomotor activity. In the companion paper (38), we noted that sub-chronic imipramine treatment was an effective antidepressant in FSL rats. However, together with an enhanced depressive-like phenotype in TDS-exposed FSL rats, we also observed a very modest (see Cohen's *d*-effect sizes) albeit insignificant behavioural response to imipramine in the FST (Figs 3a–c). Interestingly, the response to monotherapy with either venlafaxine or ketamine *also* proved unsuccessful. Neither of the drug treatments had a significant impact on locomotor activity, although it tended to be lower in imipramine-treated animals. Thus any observed treatment effects in the FST can be assumed to be unrelated to an indirect effect on locomotor activity. Based on these findings, and that clinically co-morbid MD and PTSD often present with TRD (32,33), the presence of a PTSD-like paradigm in genetically predisposed animals significantly attenuates antidepressant-like response to imipramine, but also to venlafaxine and ketamine. The latter two findings with ketamine and venlafaxine are especially interesting as both agents are generally considered effective antidepressants when applied as monotherapy, and also offer improved efficacy in treatment resistance (61,70). Although dose may be a reason for this observation, venlafaxine has demonstrated effectiveness in the FST after 10 days of treatment (51). On the other hand, it should be mentioned that sub-chronic venlafaxine treatment may be associated with non-response in the FST while still inducing monoaminergic alterations (71). Previous studies with ketamine applied doses of up to 20 mg/kg twice daily for 2 weeks (66), whereas 10 mg/kg for 7 days (as applied here) have also proven to be sufficient to induce antidepressant-like effects (52). Interestingly, the latter study (52) was performed in rats exposed to a CMS protocol – a model which has been described as presenting with the attributes of TRD (72). Therefore, the doses of venlafaxine and ketamine used in the current work can be regarded as effective, with

ketamine demonstrating efficacy under at least some TRD-related conditions (i.e. CMS).

Exposing FSL rats to TDS stress may therefore represent a more profound state of treatment resistance that warrants a more robust treatment regimen. To test this supposition, we investigated the response to combined imipramine + ketamine or venlafaxine, considering not only superiority versus imipramine alone, but also versus ketamine and venlafaxine monotherapy. This is also a typical approach taken for a failed monotherapy treatment response in human patients (as evident in STAR*D). Where all drugs administered as monotherapy failed to induce adequate anti-immobility effects in the FST, we found that venlafaxine + imipramine and ketamine + imipramine achieved successful attenuation of depressive-like manifestations in TDS-exposed FSL rats without notable effects on locomotor activity. This conclusion is supported by a significantly reduced immobility time (Fig. 3c) as well as bolstered coping behaviour exhibited by significant increases in swimming and climbing behaviour (Figs 3a and b) following combination treatments.

The mechanism whereby the combined use of a TCA and a SNRI or an NMDA receptor antagonist may engender a bolstered response in the current model of TRD is of particular interest. Despite a plethora of up-stream signalling pathways purported to be involved in the neurobiology of MD [see (73) for review], it is ultimately a resultant effect on NA and 5HT that may hold sway in the behavioural presentation of the illness and how antidepressants produce their desired effect. Considering 5HT, FSL rats present with deficits in serotonergic transmission (39), whereas TDS in its own right adversely affects this monoamine and its behavioural sequelae (41,43), implying that TDS-exposed FSL rats may present with a profoundly compromised serotonergic system. Indeed, 5HIAA was reduced in the frontal cortex ($d = 1.07$) and hippocampus ($d = 0.84$) of TDS-exposed FSL rats, although narrowly missed significance (Table 3). It is interesting that clinical studies have demonstrated that relapse of MD induced by a tryptophan depleting diet occurs primarily in remitted patients taking an SSRI and not another pharmacological or behavioural treatment (74,75), indicating that loss of serotonergic function during treatment with serotonergic drugs mediate the relapse. As both venlafaxine and imipramine act to increase extracellular levels of 5HT (and NA) (76), a synergistic action on 5HT may underlie the improved response observed in combination treatment. Drug-centred approaches for treating TRD also emphasise adding a drug with a different mechanism of action (2,16). Thus, despite similar actions on NA and 5HT neuronal reuptake, imipramine has a high affinity for other neuronal receptors, such as the $5HT_{1A}$ receptors (77) versus the 'cleaner' profile of venlafaxine (78), which may explain the increased swimming behaviour observed in imipramine alone and venlafaxine + imipramine combinations versus venlafaxine alone. Also worth considering is that venlafaxine only inhibits NA reuptake at higher therapeutic doses compared with its SSRI effects across the dose range (76). This may explain the absence of climbing-enhancing effects in venlafaxine alone compared with imipramine + venlafaxine, which would have provided synergistic SNRI effects.

Regarding ketamine, mechanisms involving mammalian target of rapamycin (79) and glycogen synthase kinase-3 (80) may underlie its improved antidepressant response. However, ketamine is known to act via various mechanisms that may target 5HT indirectly (81), whereas at least acute ketamine administration produces a rapid increase in the activity of locus coeruleus NA neurons through an amplification of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) transmission (82). Ketamine has also been demonstrated to induce significant increases in NA release in the prefrontal cortex (83). These actions may underlie the observed additive response with imipramine in the FST. Therefore, combining imipramine with either venlafaxine or ketamine delivers an effective antidepressant response even in apparently treatment-resistant animals through broad actions on serotonergic and noradrenergic signaling. These data are important because, not only do they correlate to clinical data such as that presented in STAR*D, but reaffirms our earlier observation (38) that TDS-exposed FSL rats constitute a novel and useful animal model of TRD.

Coping behaviour in the FST is thought to be mediated by the same underlying mechanisms that determine effectiveness of chronic antidepressant therapy in humans (84), highlighting that in this case both combination treatments with imipramine improved serotonergic (swimming) and noradrenergic (climbing) activities. In addition, discriminating between these coping behaviours may provide further insight into the role of monoaminergic neurotransmitter systems involved in mediating these effects (48). We have already demonstrated that FSL rats show significantly raised frontal-cortical and hippocampal 5HIAA levels as well as significantly reduced NA levels in these brain regions versus their FRL control (Table 2) (38). In this study, monoamine data (Fig. 4) reveals no alterations in 5HIAA or NA in the frontal cortex following treatment with either imipramine, venlafaxine or ketamine, although ketamine increased frontal-cortical NA versus vehicle-treated animals, whereas also not markedly affecting swimming or climbing. However, ketamine + imipramine

(frontal-cortical 5HIAA and NA), ketamine alone (frontal-cortical NA) and venlafaxine + imipramine (frontal-cortical NA) significantly increased 5HIAA and NA responses versus untreated TDS-exposed FSL rats (Figs 4ai and bi), indicating a more robust response following these combination treatments. Also, these combinations increased swimming and climbing (Fig. 3a,b). Considering that TCAs such as imipramine act by increasing the extracellular levels of NA and 5HT (85), TDS tends to prevent these effects (Table 3) with only ketamine alone, ketamine + imipramine and venlafaxine + imipramine able to reverse the reduction in NA, whereas only ketamine + imipramine reverses TDS-associated reductions in 5HIAA (Figs 4ai and bi).

Significant increases in NA levels in the frontal cortex was measured in animals treated with venlafaxine + imipramine and ketamine + imipramine, which corresponded with increases in climbing behaviour measured in the FST. This is especially interesting considering that neither imipramine nor venlafaxine, when administered alone, were able to achieve this. However, although ketamine alone increased NA levels, this effect did not translate to climbing behaviour. Also, increased swimming behaviour was observed in rats treated with venlafaxine + imipramine, whereas neither imipramine nor venlafaxine-treated animals attained significance in this regard, despite a large effect size ($d = 0.93$ and 1.07, respectively). Contradictions between monoamine and FST data have been reported in several animal studies in response to stress (86,87). In fact, the paradox with respect to limbic monoamine levels and coping strategies may be indicative of adaptive changes that influence coping responses following repeated exposure to stress. However, independent of interplay between monoaminergic and behavioural responses, only augmentative treatments (venlafaxine + imipramine and ketamine + imipramine) induced significant alterations in both behavioural parameters in the FST and 5HIAA and NA responses, signifying the improved efficacy of combination versus mono-therapeutic antidepressant therapy in this model, which further lends support to its validity as an animal model of TRD.

In conclusion, combining stress sensitive FSL rats with TDS results in a treatment-resistant rat model of MD. Non-response is not only observed with the traditional antidepressant, imipramine, but also following treatment with either ketamine or venlafaxine. Exposure to TDS inhibits antidepressant response in FSL rats at both behavioural and neurochemical levels. However, combining venlafaxine or ketamine with imipramine leads to enhanced antidepressant-like effects, together with associated effects on neurochemistry. These data confirm the hypothesis that exposure of a gene-environment model of depression with a PTSD-like paradigm results in more severe depressive-like behaviour which is resistant to traditional antidepressant treatment, albeit responsive to treatment regimens which combine various mechanisms of antidepressant action. Combining FSL rats + TDS therefore holds promise for future development as a suitable animal model of TRD.

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Conflicts of Interest

The authors declare that over the past 3 years, B.H.H. has participated in advisory boards and received honoraria from Servier®, and has received research funding from Servier® and Lundbeck®. The authors declare that, except for income from the primary employer and research funding to B.H.H. from the MRC, NRF and the above-mentioned exceptions, no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional services, and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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Addendum F –
Determination of tissue monoamine levels VIA HPLC-ECD

Introduction

This section describes the full validated method used to determine regional rat brain tissue levels of noradrenaline (NA) and its main metabolite, 3-methoxy-4-hydroxyphenylglycol (MHPG), serotonin (5-HT) and its main metabolite, 5-hydroxyindole-3-acetic acid (5-HIAA) and dopamine (DA) and its main metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) using a HPLC-ECD system. The method forms part of the standard methods of the Analytical Technology Laboratory (ATL) of the Centre of Excellence for Pharmaceutical Sciences (Pharmacen) of the North-West University, Potchefstroom. The method is maintained and validated by the ATL and has been described in the literature (Harvey et al., 2006; Möller et al., 2011; Möller et al., 2013)

Chromatographic conditions

Analytical Instrument:	Agilent 1200 series HPLC, equipped with an isocratic pump, autosampler, coupled to an ESA Coulochem III Electrochemical detector (with Coulometric flow cell) and Chromeleon® Chromatography Management System version 6.8.
Column/Stationary phase:	Kinetix C18, 4.6 x 150mm, 2.6µm, 100Å (Phenomenex, Torrance, CA, USA).
Guard column:	4.0 x 3.0 mm C18 SecurityGuard™, HPLC Guard Cartridge System (Phenomenex, Torrance, CA, USA).
Mobile Phase:	0.1 M (6.801g/l) Sodium formate buffer, 5 mM (1.01125g/l) sodium heptane sulphonic acid, 0.17 mM (20 mg/l) ethylene-diamine-tetra-acetic acid (EDTA disodium salt Na_2EDTA), 60 ml methanol (6%) and 40ml (4%) acetonitrile. The pH of the mobile phase was set at ± pH 3.5-4.1 with orthophosphoric acid (85%).
Flow rate:	0.50 ml/min.
Injection volume:	20 µl
EC Detector settings:	ESA 5011A Analytical Cell Potential settings Coulometric Electrochemical Detection Volts: E1: -150mV, E2: +750mV Gain range: 20nA Polarity: Positive Reaction: Oxidation Guardcell Potential setting: +350mV

Chemicals and reagents

The reagents for the mobile phase and the homogenization solution were purchased from Merck (Pty) Ltd., Modderfontein, Gauteng, South Africa.

The monoamine salts and the internal standard, isoprenaline, were purchased from Sigma-Aldrich Pty. Ltd., Johannesburg, Gauteng, South Africa.

Preparation of the Homogenization solution

All standards and samples are prepared with this solution. The following are required:

0.5 mM sodium metabisulphite

0.3 mM Na2EDTA

0.1 M perchloric acid (60% strong solution).

Preparation: 0.09505 g sodium metabisulphite and 0.111672 g Na2EDTA is weighed off and dissolved in 800 ml distilled water. 10.87 ml perchloric acid is then added to the above solution and made up to 1000 ml.

Preparation of Monoamine Standards

The monoamine standards were each prepared in 10ml of the homogenization solution and made up to a stock solution with a concentration of 100µg/ml for each analyte. Table 1 reports the substances and amounts used to prepare the stock solution for each analyte. The stock solution was subsequently used to prepare a series of dilutions to produce a standard concentration range between 1.25 - 50ng/ml.

Table 1. Preparation of the various monoamine standard stock solutions

Analyte	Molar Mass (g/mol)	Substance	Molar Mass (g/mol)	Amount dissolved in 10 ml homogenization solution (mg)	Final concentration of analyte
MHPG	184.09	3-methoxy-4-hydroxyphenylglycol hemipiperazinium salt	454.5	2.47	100µg/ml
NA	169.18	/-noradrenaline hydrochloride	205.6407	1.22	
DOPAC	168.15	3,4-dihydroxyphenylacetic acid	168.15	1	
DA	153.18	3-hydroxytyramine hydrochloride	189.64	1.24	
HVA	182.18	homovanillic acid	182.18	1	
5-HT	176.2	5-hydroxytryptamine creatinine sulphate	405.43	2.3	
5-HIAA	191.19	5-Hydroxyindole-3-acetic acid	191.19	1	

Preparation of Internal Standard

The internal standard is a known concentration of a substance possessing physico-chemical characteristics as close as possible to that of the analyte of interest (United Nations Office on Drugs and Crime, 2009). Internal standards are used to correct for variability in the sample preparation process and are usually employed as a measure to improve the precision of a quantitative analysis. The internal standard should ideally provide a response that is similar to that of the analyte, but provide a signal that is distinct from the signal of the analyte. The rationale is that factors that could affect the signal of the analyte will similarly affect the signal of the internal standard. The ratio of the two signals should therefore exhibit a lower degree of variability than the signal of the analyte alone (Center for Drug Evaluation and Research; 1994; Magee & Herd, 1999; United Nations Office on Drugs and Crime, 2009).

1mg of the internal standard, isoprenaline (N-isopropyl-DL-noradrenaline hydrochloride), was dissolved in 10 ml homogenization solution to make up a stock solution with a concentration of 100µg/ml. To prepare a working internal standard solution of 1500 ng/ml, 30µl of this stock solution was added to 1970 µl of the homogenization solution.

Sample preparation of brain tissue and determination of monoamine content

1. Following dissection, brain tissue (hippocampus or striatum, dissected as described in Addendum D and E) of each animal is placed individually into polypropylene tubes, marked and snap frozen with liquid nitrogen. The samples are stored at -70°C until the day of analyses.
2. On the day of analysis, samples are weighed, thawed and 1 ml of homogenization solution is added to each tube. The tissue in each tube is then ruptured by sonication (2 x 12 seconds, at amplitude of 14 µ) (Keller et.al. 1976).
3. The tubes are left to stand on ice for a period of 20 minutes to complete perchlorate precipitation of protein and extraction of monoamines.
4. Following this period, samples were centrifuged at 4°C in an ultra-centrifuge for 20 minutes at 16 000 revolutions per minute (rpm) (24 000 g).
5. The supernatant of the tissue extract (± 1ml) is pipetted into a 2 ml amber polypropylene tube.
6. The pH of the sample is adjusted to pH 5 with the addition of 1 drop/ml of 10 M potassium acetate.
7. 200 µl of the tissue extract sample or standard is pipetted into another 1.5 ml polypropylene tube.
8. 20 µl of the internal standard working solution is added to the sample.
9. This final sample is vortexed and then centrifuged again for 5 minutes.
10. The whole sample is pipetted into a 300 µl glass insert, which fits into an amber HPLC glass vial. The vial is then placed into the sample tray of the Agilent 1200 series autosampler.
11. The instrument's software is programmed to inject 20 µl of the sample onto the HPLC column.

12. The peak area data of each sample is collected, divided by the peak area of the internal standard, and converted to a concentration value in ng/ml by making use of the relevant analyte's standard linear calibration curves (regression >0.98) (see Table 3).

13. The concentration value of the monoamine is expressed in terms of ng/g wet tissue weight.

HPLC Chromatographic Information

The retention times for the respective analytes are reported in Table 2, with Figure 1 representing a typical chromatogram generated by the solution containing all the monoamine standards at a monoamine standard concentration of 10 ng/ml.

Table 2: Retention times of the various analytes

Analyte	Retention time (minutes)
MHPG	± 5.97
NA	± 6.15
DOPAC	± 10.61
DA	± 13.95
5-HIAA	± 20.18
Internal Standard	± 22.90
HVA	± 24.93
5-HT	± 39.45

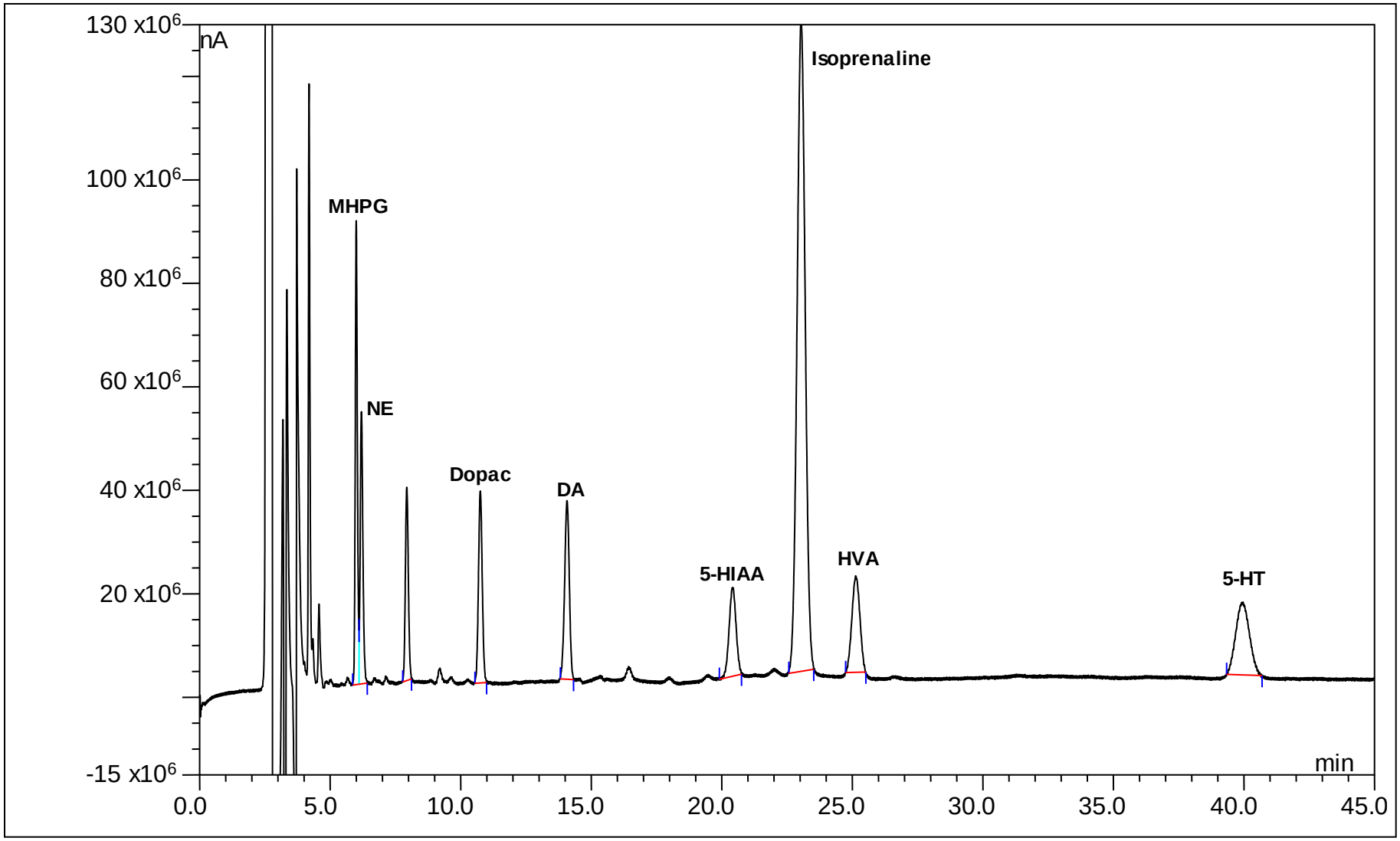

Figure 1: Representative chromatogram of monoamine standard, isoprenaline (10 ng/ml), as well as all other analyte standards (10 ng/ml) in a typical brain tissue sample.

Mini method validation

A mini method validation, otherwise referred to as analytical method verification, is performed before starting routine analyses of samples with the objective of demonstrating that the method and analytical instrument used will still provide the same accurate and valid results as generated with the original validation of the analytical method (Geetha et.al. 2012). This method has been validated in the Analytical Technology Laboratory (ATL) of the Centre of Excellence for Pharmaceutical Sciences (Pharmacen) of the North-West University, Potchefstroom (Basson et al., 1988).

Mini method validation parameters

The analytical parameters used in this mini method validation are as follows: a) linearity (calibration curve), b) repeatability, c) lower limit of quantification (LLOQ) and d) lower limit of detection (LLOD).

Results for system suitability

a) Linearity / Calibration curve

The acceptable criteria for regression (r^2), the coefficient of determination for biomolecules, must be at least 0.95 or greater (Shabir, 2006). The linearity/calibration curve used in this validation process comprised of the following 6 standard concentrations for each analyte: 1.25, 2.5, 5.0, 10, 20 and 50.0 ng/ml.

The internal standard method was used to set up the calibration curve as well as to calculate the test sample results according to the following response ratio:

$$Response\ factor\ ratio\ (x) = \frac{Peak\ Area\ of\ Standard}{Peak\ Area\ of\ Internal\ Standard}$$

The calibration curve was set up with the 6 standard concentrations of the specific analyte on the x-axis of the graph with the corresponding response factor ratio on the y-axis. These linear regression results for the 7 analytes are reported in Table 3 on the next page.

b) Repeatability

The repeatability measures the precision of the method as reported by the % coefficient of variation (CV) and is determined at 3 concentrations (lowest, middle and highest concentrations) and using 3 repetitions each (Huber, 2010). The % CV is determined by dividing the standard deviation of the 3 repetitions by their mean and multiplying this number by 100. The repeatability determined at each of the 3 concentration levels should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV.

The 3 concentrations used to measure repeatability were 1.25, 5.0 and 50 ng/ml. See the repeatability results in the table below.

Table 3: Linear regression and repeatability (%CV) for each analyte

Analyte	Calibration curve Linear Regression (r^2)	Concentration (ng/ml)	Average CV (%)
NA	0.996	1.25	4.48
		10.0	4.01
		50.0	2.63
MHPG	0.994	1.25	4.82
		10.0	2.09
		50.0	0.28
DA	0.992	1.25	2.50
		10.0	4.85
		50.0	2.44
DOPAC	0.994	1.25	7.94
		10.0	3.96
		50.0	4.14
HVA	0.984	1.25	3.58
		10.0	7.68
		50.0	4.51
5-HT	0.985	1.25	5.22
		10.0	4.91
		50.0	4.19
5-HIAA	0.987	1.25	6.40
		10.0	1.99
		50.0	4.34

c) Lower limit of quantification (LLOQ)

The lower limit of quantification was found to be 1.25 ng/ml, which corresponds with the lowest concentration on the calibration curve.

d) Lower limit of detection (LLOD)

The lower limit of detection was found to be 1.0 ng/ml.

Limitations

Since this analytical procedure analyses biological samples, a few unknown peaks are produced on the chromatogram. Furthermore, the monoamine molecules are structurally (physico-chemically) very similar to each other, resulting specifically in the retention times of MHPG and of NA to lie very close to each other in this HPLC method.

The validation of analytical methods for endogenous molecules/biomarkers (such as the monoamines) has been complicated by the absence of official guidelines (Van de Merbel, 2008). Most researchers apply "The method-validation principles for the analysis of drugs" issued by the US Food and Drug Administration (FDA; 2001) to their methods when measuring endogenous molecules/biomarkers, to ensure that their results have an acceptable and comparable level of quality (Van de Merbel, 2008). The FDA principles were however meant for drug analysis and not for endogenous molecules/biomarkers and therefore direct application of these principles are not possible, requiring analyte-specific modifications (Van de Merbel, 2008).

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