Evaluation of agomelatine treatment on neuroendocrine and behavioural markers in social isolation reared rats

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PREFACE

It is a rollercoaster ride and a heck of a fight, maar voor jy moed op gee, begin jou lewe te draai

Wals, Wals Willemien – Laurika Rauch

***

Life is no straight and easy corridor along

which we travel free and unhampered,

but a maze of passages

through which we must seek our way,

lost and confused, now and again

checked in a blind alley.

But always, if we have faith,

a door will open for us,

not perhaps one that we ourselves

would ever have thought of,

but one that will ultimately

prove good for us

A.J. Cronin

***

The best laid schemes o' mice and men often go astray

Robert Burns

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“By the grace of God there goes I. “

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ABSTRACT

Anxiety disorders are extremely prevalent and co-morbid in psychiatric disorders with a complex neurobiology and aetiology. Current treatment options are not sufficiently effective and there is therefore a demand for new and improved anxiolytic treatments. Agomelatine is a new generation antidepressant that acts as a serotonin 5-HT_{2C} antagonist and a melatonin MT_1/MT_2 agonist, where it acts to re-entrain circadian rhythms purported to be dysregulated in mood disorders. Although it has been shown to have anxiolytic effects in clinical and preclinical studies and may possibly be beneficial as an alternative anxiolytic compound, there remains a paucity of preclinical studies in this regard. Animal models make it possible to study and determine the mechanism underlying the neurobiology, aetiology and pharmacological interventions of anxiety disorders. Adverse life-events are believed to play a particular important role in increasing the vulnerability to develop an anxiety disorder. Social isolation rearing (SIR) is an animal model that resembles early-life adversity and results in behavioural alterations akin to that observed in schizophrenia, depression and anxiety. SIR involves isolating a rat from post-natal day (PND) 21 for 8 weeks, thereby inducing neurodevelopmental abnormalities in the rat culminating in late-life emergent pathological behaviour. These behavioural alterations can be measured by means of specific behavioural tests, such as those to determine anxiety-like behaviours and applied in the current study. This study therefore used the SIR animal model to evaluate the effect of early life social isolation on the development of anxiety-like behaviours later in life as well as on neurotransmitters and endocrine bio-markers important in anxiety. Clinical studies have demonstrated that females are twice as prone to developing an anxiety disorder, and subsequent preclinical studies have identified gender as an important susceptibility factor in the development of anxiety. Consequently, the current study has investigated gender-related differences concerning anxiety-like behaviours and response to agomelatine treatment in the SIR model.

Animals were bred and housed at the DST/NWU PCDDP Vivarium and all experiments were approved by the AnimCare animal research ethics committee of the NWU (Ethics approval number NWU-00347-15-S5). Male and female Sprague-Dawley rats were randomly allocated into 6 groups, consisting of 12 rats per group. Rats were randomly divided into either socially reared groups (3 animals per cage) or SIR groups (1 animal per cage). Socially reared animals only received vehicle treatment, whereas SIR animals were divided into groups receiving agomelatine treatment (40 mg/kg/day) or vehicle treatment, administered at 16:00 daily. Treatment commenced on PND 61 and continued for 16 days. Thereafter animals were subjected to the open field test (OFT) on day 13 of treatment to access general locomotor activity, social interaction (SIT) on day 14 of treatment to access interactive behaviour with peers, and the elevated plus maze (EPM) on day 15 of treatment to access avoidance behaviour. Together these

tests provide an overall impression of the animal's general state of awareness and level of anxiety. Rats were euthanized (via decapitation) 36 hours after the last behavioural test, followed by the collection of trunk blood and dissection of frontal cortices for neuroendocrine analysis. N-methyl-D-aspartate (NMDA) receptor density and γ-amino butyric acid (GABA) levels were measured in the frontal cortices of male rats by radioligand binding and high performance liquid chromatography with electrochemical detection (HPLC-EC), respectively. Corticosterone was measured in the plasma of both genders using HPLC with ultraviolet (UV) detection.

The results indicate that SIR tends to increase locomotor activity in both genders compared to socially reared animals in the OFT, with agomelatine significantly reducing SIR-induced locomotor hyperactivity in both genders. SIR, in both male and female rats, induces anxiety-like behavioural alterations in the EPM and SIT. In the SIT SIR significantly decreased social interaction in both genders compared to their social reared counterparts. Agomelatine significantly increased the time spent anogenital sniffing in male rats and a trend towards increased time spent together and times approaching each other in male rats. Agomelatine tended to increase the time spent together in female rats. Rearing behaviour (self-directed behaviour) in the SIT was also significantly decreased in SIR rats in both genders with agomelatine significantly increasing such behaviour in both genders. SIR significantly increased anxiogenic behaviour in the EPM compared to socially reared animals, with agomelatine showing a trend in both genders towards reversal of this behaviour.

SIR significantly decreased the plasma corticosterone in both genders vs. socially reared animals, although agomelatine did not correct this anomaly. The results indicated no differences between SIR and socially reared animals with regard to GABA levels and NMDA receptor density in the frontal cortex, although agomelatine did show a trend to increase GABA levels and NMDA receptor density in male rats.

This study indicates that SIR is a reliable neurodevelopmental animal model to resemble anxiety-like behaviours akin to symptoms observed in anxiety disorders, with anxiety being evident to a similar extent in both genders. Agomelatine decreased SIR-induced anxiety-like behaviours in both genders, although the treatment response in male rats was superior and more consistent. The study further suggests that the behavioural alteration observed in the SIR animals were arguably not associated with alterations regarding GABA, glutamate or corticosterone, but further research in this regard is warranted.

Keywords

Social isolation rearing, female, male, anxiety-like behaviours, neuroendocrine analysis, agomelatine

OPSOMMING

Angstoestande is besonder algemeen, kom dikwels gelyktydig met ander psigiatriese toestande voor en besit ŉ ingewikkelde neuropatologie en etiologie. Die huidige behandelingsopsies, toon onbevredigende effektiwiteit en daar bestaan dus ŉ groot behoefte aan nuwe en beter behandelingsopsies. Agomelatien is ŉ nuwe generasie antidepressant en sy werking behels die antagonering van die serotonien 5-HT_{2C} reseptore en die stimulasie van die melatonien MT_1/MT_2 reseptore. Agomelatien kan ook versteurde sirkadiese ritmes wat ŉ rol speel by gemoedsversteurings, korrigeer. Alhoewel beide kliniese en pre-kliniese studies al aangetoon het dat agomelatien oor angsiolitiese eienskappe beskik en moontlik gebruik kan word as ŉ alternatiewe angsiolitikum, is daar steeds 'n tekort aan pre-kliniese studies in hierdie verband. Diere modelle maak dit moontlik om die meganismes ter sprake by die neuropatologie, etiologie en farmakologiese ingrepe van angstoestande te bestudeer en op te klaar. Negatiewe gebeure in ŉ persoon se lewe kan ŉ belangrike predisponerende faktor wees by die ontwikkeling van ŉ angsversteuring. Sosiaal geïsoleerde huisvesting (SGH) is ŉ dieremodel wat sodanige negatiewe gebeure in ŉ vroeë lewensstadium naboots, en gedragsveranderinge soortgelyk aan dié van skisofrenie, depressie en angs tot gevolg het. Tydens SGH word ŉ rot na spening (dag 21 na geboorte) vir 8 weke geïsoleer, ŉ proses wat neuro-ontwikkelings-afwykings in die rot induseer en dan op ŉ later stadium as patologiese gedragsversteurings presenteer. Hierdie gedragsafwykings kan gemeet en gedefinieer word met die hulp van spesifieke gedragstoetse, soos in die huidige studie gebruik is om angs-agtige gedrag in rotte te bepaal. Hierdie studie het dus die SGH-model gebruik om die effek van sosiale isolasie, met betrekking tot die ontwikkeling van angs-agtige gedrag, te evalueer. Bykomend is die effek op sentraalsenuweestelsel oordragstowwe en endokriene bio-merkers wat ŉ belangrike rol speel in angstoestande, ook bepaal. Kliniese studies dui aan dat vroue twee keer meer geneig is tot die ontwikkeling van 'n angsversteuring en pre-kliniese studies het geslag geïdentifiseer as 'n belangrike vatbaarheidsfaktor in die ontwikkeling van angs. Om dié rede het die huidige studie geslagsverwante verskille met betrekking tot angs-agtige gedrag asook die respons op agomelatien behandeling in die SGH-model ondersoek.

Die diere is geteel en gehuisves by die DST/NWU PCDDP Vivarium en alle diere eksperimente is vooraf goedgekeur deur die AnimCare dierenavorsing etiekkomitee van die NWU (Etiek goedkeuringsnommer NWU-00347-15-S5). Manlike en vroulike Sprague-Dawley rotte is lukraak verdeel in 6 groepe, met 12 rotte per groep. Die rotte is verdeel in sosiaal-gehuisveste groepe (3 rotte per hok) of SGH groepe (1 rot per hok). Die sosiaal-gehuisveste rotte het net die draagstof ontvang as behandeling, terwyl die SGH rotte verdeel is in groepe wat of die draagstof of agomelatien (40 mg/kg/dag) behandeling ontvang het. Daaglikse behandeling om 16:00 elke

middag van die rotte is op nageboorte dag 61 begin en volgehou vir 16 dae. Die diere is op dag 13 van behandeling aan die oop veld toets (OVT) onderwerp om hulle lokomotoriese aktiwiteit te evalueer. Op dag 14 van behandeling is die diere aan die sosiale interaksie toets (SIT) onderwerp om interaktiewe gedrag teenoor hulle eweknieë te bestudeer. Laastens, op dag 15 van behandeling is die diere onderwerp aan 'n verhoogde plus-vorm doolhof (VPD) om vermydingsgedrag te evalueer. Hierdie toetse saam gee 'n geheelbeeld van die dier se algemene bewussyn- en angsvlakke. Die rotte is 36 uur na die laaste gedragtoets gedekapiteer, waarna die bloed uit die hoofslagaar opgevang is en die frontale korteks gedissekteer is vir neuro-endokriene analises. N-metiel-D-aspartaat (NMDA) reseptordigtheid en γ-aminobottersuur (GABS) vlakke is in die frontale korteks van manlike rotte met behulp van onderskeidelik radioligandbindingsanalise en hoëverrigting- vloeistofchromatografie met elektrochemiese deteksie, gemeet. Kortikosteroonvlakke is in die plasma van beide geslagte met behulp van hoëverrigting-vloeistofchromatografie met ultraviolet (UV) deteksie bepaal.

Die resultate dui daarop dat SGH 'n geneigdheid toon om die lokomotoriese gedrag van beide geslagte soos bepaal met behulp van die OVT, te verhoog in vergelyking met die sosiaal-gehuisveste rotte. Agomelatien het hierdie verhoging in SGH-geïnduseerde lokomotoriese gedrag statisties-beduidend verlaag in beide manlike en vroulike rotte. In beide geslagte het SGH angs-agtige gedrag soos bepaal in die VPD en SIT, geïnduseer. SGH het verder ook sosiale interaktiewe gedrag in die SIT statisties-beduidend verlaag in beide geslagte, in vergelyking met die sosiaal-gehuisveste rotte. Agomelatien het die tyd wat manlike rotte bestee het aan anogenitale snuiwing statisties-beduidend verhoog, maar slegs 'n neiging getoon om die tyd saam spandeer en die hoeveelheid kere wat die rotte mekaar genader het, te verhoog. In vroulike rotte het agomelatien geneig om die tyd wat die rotte saam spandeer te verhoog. Self-gerigte gedrag in die SIT, soos wanneer die rotte op hulle agterpote staan, is ook in beide geslagte statisties-beduidenddeur SGH verminder. Agomelatien het hierdie gedrag statisties-beduidend verhoog in beide geslagte, met ander woorde, die selfgerigte gedrag genormaliseer. SGH het angs-agtige gedrag in die VPD in vergelyking met die sosiaal gehuisveste rotte statisties-beduidend verhoog en agomelatien het 'n neiging in beide geslagte getoon om hierdie gedrag om te keer.

SGH het die plasma kortikosteroonvlakke statisties-beduidend verlaag in beide geslagte in vergelyking met die sosiaal-gehuisveste rotte en agomelatien kon nie daarin slaag om hierdie abnormaliteit reg te stel nie. Geen verskille tussen die SGH rotte en die sosiaal-gehuisveste rotte met betrekking tot GABS- vlakke asook NMDA- reseptordigtheid in die frontale korteks is gevind nie. Agomelatien het wel 'n geneigdheid om die GABS- vlakke en NMDA- reseptordigtheid in die frontale korteks van manlike rotte te verhoog, getoon.

Hierdie studie dui daarop dat SGH ’n betroubare neuro-ontwikkelings dieremodel is om angs-agtige gedrag soortgelyk aan waargenome simptome in pasiënte met angsversteurings. Daar is ook waargeneem dat angs-agtige gedrag in beide geslagte tot ’n soortgelyke mate ontwikkel. Agomelatien het die SGH-geïnduseerde angs-agtige gedrag in beide geslagte verlaag, hoewel manlike rotte beter en meer konsekwent op die behandeling gereageer het. Die studie stel verder voor dat die gedragsveranderinge wat waargeneem is in die GSH rotte moontlik nie verband hou met veranderinge in GABS, glutamaat of kortikosteroon nie, maar verdere ondersoek in hierdie verband is nodig.

Sleutelterme

Sosiaal-geïsoleerde huisvesting, vroulik, manlik, angs-agtige gedrag, neuro-endokriene analises, agomelatien

CONGRESS PROCEEDINGS

Excerpts from this study were presented as follows:

Evaluation of agomelatine treatment on anxiety-like behaviours in social isolation reared rats, and its relation with gender

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The results were presented as a podium presentation for the Young Pharmacologist competition of the South African Society for Basic and Clinical Pharmacology 2016. The student, as first and presenting author, won the 1st prize in the “Basic Pharmacology” category.

The abstract submitted for the congress is presented in Addendum C.

LIST OF ABBREVIATIONS

A

ACTH	Adrenocorticotropic hormone
ADHD	Attention deficits hyperactivity disorder
ANOVA	Analysis of variance

B

BDNF	Brain-derived neurotrophic factor
BLA	Basolateral amygdala complex
BNST	Bed nucleus of the stria terminalis

C

CeA	Central nucleus of the amygdala
CNS	Central nervous system
CRH	Corticotrophin-releasing hormone
CRH_2	Corticotrophin-releasing hormone receptor 2
CT	Corticosterone
CV	Coefficient of variation

D

DA	Dopamine
DAD	Diode array detector

DHEA	Dehydroepiandrosterone
DSM-5	Diagnostic and Statistical Manual of Mental Disorders
DST	Department of Science and Technology
DX	Dexamethasone

E

EC	Electrochemical detections
% EOA	Percentage of entries onto the open arms
EPM	Elevated plus maze

F

fMRI	Functional magnetic resonance imaging
FRL	Flinders Resistant Line
FSL	Flinders Sensitive Line

G

GABA	γ-amino butyric acid
GABA-T	GABA-transaminase
$GABA_A$	γ-amino butyric acid receptor A
GAD	Generalized anxiety disorder

H

HEC	1% Hydroxyethylcellulose
HPA-axis	Hypothalamic-pituitary-adrenal axis
HPLC	High performance liquid chromatography

I

i.p.	Intraperitoneally

L

LLOD	Lower limit of detection
LLOQ	Lower limit of quantification
LTP	Long-term potentiation

M

Min	Minutes
MT_1	Melatonin receptor 1
MT_2	Melatonin receptor 2

N

NA	Noradrenaline
$Na_2HPO_4.2H_2O$	Sodium phosphate dibasic
Na_2EDTA	Ethylenediaminetetraacetic acid
NMDA	N-methyl-D-aspartate

NO	Nitric oxide
NRF	National Research Foundation

O

O_2	Oxygen
OCD	Obsessive compulsive disorder
OFT	Open field test

P

PCDDP	Pre-Clinical Drug Development Platform
PTSD	Posttraumatic Stress Disorder
PND	Post-natal day

S

SCN	Suprachiasmatic nucleus
SD	Sprague-Dawley
SEM	Standard error of the mean
SIR	Social isolation rearing
SIT	Social interaction test
SNRIs	Serotonin and noradrenaline reuptake inhibitors
SPE	Solid phase extraction
SS	Standard solution
SSRIs	Selective serotonin reuptake inhibitors

T

TAD	Tricyclic antidepressants
% TOA	Percentage of time spent on the open arms
TRD	Treatment resistant depression

U

UV	Ultraviolet

V

V_{1B}	Vasopressin receptor subtype 1B

Numbers

5-HT	Serotonin
$5\text{-}HT_{1A}$	Serotonin receptor subtype 1A
$5\text{-}HT_{1B}$	Serotonin receptor subtype 1B
$5\text{-}HT_{2A}$	Serotonin receptor subtype 2A
$5\text{-}HT_{2B}$	Serotonin receptor subtype 2B
$5\text{-}HT_{2C}$	Serotonin receptor subtype 2C
α_2	Alpha 2 receptor
α_{2C}	Alpha receptor subtype 2C
$\alpha_{2\text{-}\delta}$	Alpha 2 delta protein subunit

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1 Dissertation Approach and Layout

This dissertation is presented in the article format for submission as approved by the North-West University. The format includes an introductory chapter (Chapter 1), a chapter containing a relevant literature overview (Chapter 2), and a chapter whereby the key data is prepared as an article for submission to a peer-review scientific journal (Chapter 3). In the final chapter (Chapter 4) the conclusion of the study is described, as well as providing recommendations for future studies. Study data not included in the article are presented in various addenda, together with details on methods and method validation.

This introductory chapter serves as an orientation to the dissertation and study as a whole , describing (1) the article format (i.e. dissertation approach and layout), (2) the problem statement (concise literature overview, which is elaborated on in Chapter 2), (3) study objectives and (4) the study layout (experimental design/approach).

The following outline serves to assist the reader where to find key elements of the study in the dissertation:

- **Problem statement, study objectives and study layout:**

Chapter 1

- **Literature background**

Chapter 2 (Literature Review)

Chapter 3 (Article Introduction)

- **Materials and methods**

Chapter 3 (Materials and Methods for the generation of data presented in the Article)

Addendum A and Addendum B (Additional Materials and Methods)

- **Results and discussion**

Chapter 3 (Results and Discussion of studies presented in the Article)

Addendum A (Additional Results and Discussion)

- **Summary and conclusions**

Chapter 3 (Conclusion of findings presented in the Article)

Chapter 4 (For the study as a whole)

1.2 Problem statement

Anxiety is a natural response and a warning adaption that can be triggered by fearful/ stressful situations or a novel unfamiliar environment (Nuss, 2015). However, anxiety is deemed pathological when its manifestations become excessive and uncomfortable, caused by no specific threat (American Psychiatric Association, 2013). Moreover, anxiety disorders are persistent in that full symptomatic remission is uncommon (Garner *et al.*, 2009) and characterized by the manifestation of debilitating anxiety symptoms that vary in nature, severity, frequency and consequences (Dell'Osso *et al.*, 2010). Anxiety disorders are classified as one of the most prevalent of psychiatric disorders (Dell'Osso *et al.*, 2010), occurring in both developed and developing countries (Stein & Nesse, 2011; Stein *et al.*, 2008). They incur substantial social and economic impact on affected patients, their families and the general public (Dell'Osso *et al.*, 2010). Correspondingly, in South Africa anxiety disorders present with a lifetime prevalence of 15.8% (Stein *et al.*, 2008). Moreover, anxiety disorders are commonly comorbid with other psychiatric disorders, as approximately 75% of patients with an anxiety disorder will also meet the diagnostic criteria for at least one other psychiatric disorder (Dell'Osso *et al.*, 2010), such as depression (McEvoy *et al.*, 2011) and schizophrenia (Braga *et al.*, 2013).

Furthermore, available epidemiological data suggests that women are more vulnerable to develop an anxiety disorder, being diagnosed 2.25 times more often than men (Ter Horst *et al.*, 2012). This higher incidence rate in women is sustained across all the different types of anxiety disorders (Maeng & Milad, 2015). Despite this statistic, preclinical studies have predominantly focused on male rodent models (Ter Horst *et al.*, 2012). The main reason for not using female rodents is their fluctuating estrous cycle and the subsequent effect of circulating sex hormones on the animal behaviour (Ter Horst *et al.*, 2012). Given the higher prevalence of anxiety disorders in the female gender, this is clearly a contradiction where the arguments against the use of female animals are of a practical kind. At the same time the role of progesterone and oestrogen in psychiatric disorders are increasingly being acknowledged (Ter Horst *et al.*, 2012).

Although existing treatments benefit some patients with anxiety disorders, a great number of patients do not respond adequately to pharmacological treatment (Garner *et al.*, 2009). It is therefore important to identify novel treatments and/or biological targets for anxiety disorders. Recent evidence has highlighted the contributory role of circadian rhythms in the development of an anxiety disorder (McClung, 2013; Sipilä *et al.*, 2010; Verma *et al.*, 2010). However, more commonly recognized is the role of disordered circadian rhythm in mood disorders where agomelatine has developed into a new generation antidepressant acting to correct disrupted circadian rhythms (Lemoine *et al.*, 2007; McClung, 2013). Agomelatine is a melatonin MT_1/MT_2

receptor agonist and a serotonin 5-HT_{2C} antagonist (De Berardis *et al.*, 2015). Over and above its demonstrable antidepressant effects (Jhanjee *et al.*, 2010; San & Arranz, 2008), agomelatine is anxiolytic in animals (Millan *et al.*, 2005; Papp *et al.*, 2006) and has also shown anxiolytic properties in recent clinical studies as well (Stein et al., 2008). Its anxiolytic effects have been demonstrated in various anxiety-like behavioural tests in rodents, including the elevated plus maze (EPM), the Vogel conflict test and the social defeat test (Guardiola-Lemaitre *et al.*, 2014; San & Arranz, 2008). However, these actions have yet to be studied in a neurodevelopmental animal model of anxiety. The specific binding of agomelatine to melatonin and $5HT_{2C}$ receptors is derived from an extensive literature describing the central role of these receptors in the entrainment of biological rhythms (Racagni *et al.*, 2011) and especially the latter in the development of mood disorders such as depression and more recently in anxiety disorders (Bagdy *et al.*, 2001; De Berardis *et al.*, 2015; Millan *et al.*, 2005). The 5-HT_{2C} receptors are concentrated in the prefrontal cortex, hippocampus and amygdala (Millan *et al.*, 2003), brain regions that are strongly implicated in anxiety and the stress-response. Melatonin, on the other hand, has also shown to decrease anxiety in clinical studies (Caumo *et al.*, 2009) as well as anxiety-like behaviours in preclinical studies (Papp *et al.*, 2006). Thus, the anxiolytic effects of agomelatine may be mediated at least in part via antagonising the 5-HT_{2C} receptor, especially in the amygdala and in the hippocampus (De Berardis *et al.*, 2015), and agonism of the melatonin receptors (Racagni *et al.*, 2011).

Glutamate, γ-amino butyric acid (GABA) and corticosterone have all been implicated in stress and anxiety disorders (Bergink *et al.*, 2004; Harvey & Shahid, 2012; Heim & Nemeroff, 2001). Agomelatine's effects on GABA have not been studied extensively and one study indicated no significant effect of agomelatine on GABA transmission (Tardito *et al.*, 2010). Nevertheless, a preclinical study did show that melatonin may enhance GABA transmission (Cheng *et al.*, 2012). Agomelatine have shown to reduce glutamate transmission (Popoli, 2009; Tardito *et al.*, 2010). Thus, agomelatine may have beneficial effects to increase the inhibitory transmitter and reduce the excitatory transmitter, to establish a balance which is required for normal behaviour (Bergink *et al.*, 2004; Harvey & Shahid, 2012). The role of the hypothalamus–pituitary–adrenal (HPA) axis in anxiety and stress is well described (Steiger, 2002). Upon exposure to stress, corticotrophin-releasing hormone (CRH) is released, which leads to the secretion of adrenocorticotropic hormone (ACTH) from the pituitary gland which in turn stimulates the adrenal glands to secrete cortisol in humans or corticosterone in rodents (Heim & Nemeroff, 2001). Agomelatine have shown to normalize corticosterone elevations in rodent's urine after exposure to stress (Popoli, 2009; Schmelting *et al.*, 2014), whereas another study observed that agomelatine did not normalize the corticosterone in the plasma of mice (Barden *et al.*, 2005). Thus, there are still

discrepancies regarding agomelatine's effect on the HPA-axis, GABA as well as glutamate and this study may reveal the effects of agomelatine on these neurotransmitters and corticosterone.

Clinical and preclinical studies have indicated that early life stress increases the vulnerability to develop depression, anxiety disorders or both (Heim & Nemeroff, 2001; Lukkes *et al.*, 2012; McEwen *et al.*, 2012). Early life experiences may be a contributing factor to individual differences in susceptibility to developing an anxiety-related disorder, especially since such events are widely recognised to adversely affect the development of key brain structures (McEwen *et al.*, 2012). Social isolation rearing (SIR), whereby animals are separated from their littermates (one animal/cage) at weaning and kept apart for several weeks, is an animal model of early-life stress (Fone & Porkess, 2008; Lukkes *et al.*, 2009). The resulting neurodevelopmental changes that ensue is purported to result in several behavioural alterations later in life related to those observed in humans with depression, anxiety and/or schizophrenia (Fone & Porkess, 2008; Lukkes *et al.*, 2009; Yorgason *et al.*, 2013). In this study we will use the SIR animal model in order to emulate a neurodevelopmental abnormality that culminates in late-life bio-behavioural changes that resemble the pathophysiology of anxiety. Previous work in our laboratory has revealed this model to be a reliable and well validated model presenting with numerous bio-behavioural alterations of schizophrenia (Möller *et al.*, 2011; Möller *et al.*, 2012; Möller *et al.*, 2013) but also depression (Coutts, 2015). Other laboratories have also established its translational relevance with respect to the neurochemical and behavioural basis of anxiety (Bledsoe *et al.*, 2011; Lukkes *et al.*, 2009; Yorgason *et al.*, 2013). This model thus offers the opportunity to extend the validity of agomelatine in the treatment of anxiety disorders, while at the same time allow a better understanding of its mode of action at a behavioural and neuroendocrine level. However, much needs to be uncovered regarding the anxiolytic action of agomelatine, particularly the effects of agomelatine on anxiety-like behaviour, anxiety-related corticosterone changes, the role of GABA-glutamate signalling, and possible gender-specific differences with regards to treatment response. To this end, testing in a translational pathological animal model will have proven value in this endeavour.

1.3 Study hypothesis and objectives

Hypothesis:

We propose that the rats subjected to SIR will present with various behavioural, neurochemical and endocrine anomalies akin to that seen in patients with anxiety disorders. The alterations induced by SIR will show gender specific differences. Furthermore, we hypothesize that these bio-behavioural alterations will be reversed by sub-chronic agomelatine treatment in gender-dependent manner.

Primary objectives:

- To establish the severity of 8 weeks SIR in rats on various anxiety-like behavioural manifestations, as determined in the EPM, the social interaction test (SIT) and open field test (OFT), compared to socially reared control animals.
- To investigate any gender-specific differences in SIR-induced anxiety-like behaviour.
- To investigate whether SIR induces altered release of corticosterone (as determined in plasma), in comparison to socially reared controls and whether these alterations are gender-specific.
- To determine whether the observed behavioural and corticosterone alterations induced by SIR (if any) can be reversed by sub-chronic treatment with agomelatine.
- To investigate if there are any gender specific improvements in SIR-induced bio-behavioural alterations after agomelatine treatment.

Secondary objectives:

- To investigate whether SIR evokes changes in the male rats with regards to NMDA receptor binding and GABA levels in the frontal cortex.

1.4 Project layout

This study was designed to firstly establish whether SIR could be used as an appropriate model for anxiety under our laboratory conditions and to present with a relevant anxiety phenotype. Secondly, this model was then used to establish whether females and males react differently to early-life SIR stress with late-life behavioural anomalies. Thereafter the study will focus on the efficacy of agomelatine to reverse SIR-induced anxiety manifestations and an attempt to allocate a possible neuroendocrine mechanism for these responses. Post-weaning SIR will take place over 8 weeks as described previously (Möller *et al.*, 2011), with drug treatment administered during the last 16 days of the 8 week isolation period (PND 61 – PND 77). Groups will consist of both female and male cohorts. On days 13 - 15 of the treatment period, animals will be subjected to various behavioural tests, as indicated in Figure 1-1. The same animals will be used to evaluate behavioural as well as endocrine and neurochemical alterations. SIR rats will receive either agomelatine 40 mg/kg i.p. or vehicle at 16:00 (Coutts, 2015). Grouped-housed rats will only receive the vehicle treatment; based on previous findings that agomelatine had no significant effects in group-housed rats (Coutts, 2015). Animals will be sacrificed the day after their last treatment between 09:00 and 12:00. Trunk blood will be collected for plasma corticosterone analyses and the frontal cortex dissected for brain neurochemical assay.

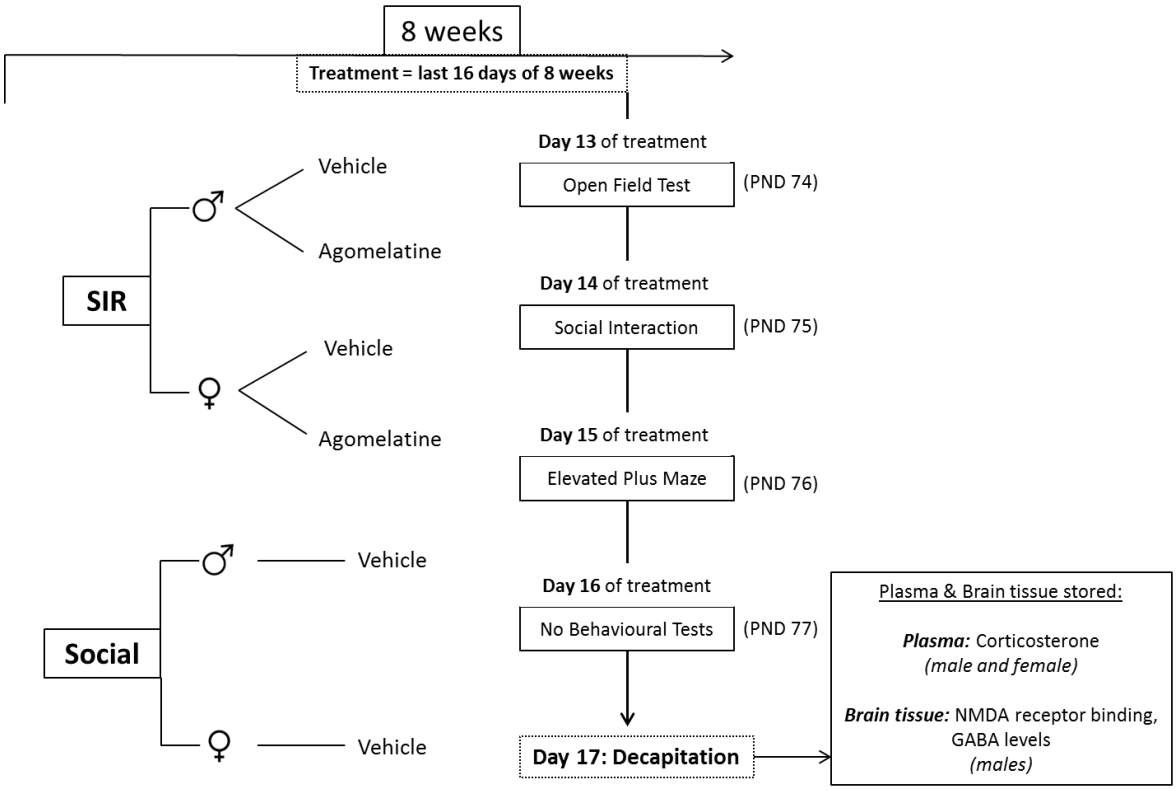

Figure 1-1: Experimental design of the study. Throughout the study SIR- or social groups will be reared accordingly for 8 weeks. Treatment consists of agomelatine 40 mg/kg i.p. or vehicle in the SIR groups, with social groups only receiving vehicle treatment in order to validate the model. Each group consists of both females and males, with 12 rats / individual treatment group. The study will therefore use a total of 72 rats. The same animals will be used for behavioural testing as well as endocrine and neurochemical analysis. Behavioural tests will focus on locomotor, social and anxiety-related behaviours and will be performed from day 13 to day 15 (PND 74 – PND 76); these tests will progress from least to most stressful, as shown. Animals will be sacrificed on day 17 (PND 78) and trunk blood collected for corticosterone analysis and the brain dissected for frontal cortical neurochemical analysis (NMDA receptor binding and GABA levels).

1.5 Expected Results

The working hypothesis for this study is that SIR will induce profound anxiety manifestations, with agomelatine demonstrating significant anxiolytic actions. Moreover, we propose that anxiety symptoms will be more prominent in female animals, while the anxiolytic action of agomelatine will not be gender-specific. We postulate that SIR will lead to increased release of peripheral corticosterone in comparison to socially reared control animals, with a more prominent response in females, together with alterations in frontal cortical NMDA receptor density and a decrease in GABA levels, the latter in male animals. The behavioural, corticosterone and neurochemical alterations induced by SIR will be reversed by sub-chronic treatment with agomelatine.

1.6 Ethical Approval

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 NWU. All experiments were approved by the AnimCare animal research ethics committee (NHREC reg. number AREC-130913-015) of the NWU. 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-00347-15-S5).

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CHAPTER 2

LITERATURE REVIEW

The current chapter will firstly review scientific literature on the aetiology, classification, manifestation and neurobiology of anxiety disorders, as well as the treatment thereof. Thereafter it will review our current understanding of the role of circadian rhythms in the neurobiology of these disorders, the mechanism on how agomelatine may correct circadian rhythm disruptions and possible exert an anxiolytic effect. Lastly, current animal models utilized to investigate anxiety disorders as well as the animal model used in this study will be discussed.

2.1 Anxiety disorders

2.1.1 Introduction

From an evolutionary point of view, in order to survive it is important that the animal or person is aware of their environment to enable them to respond appropriately to all kinds of stimuli, be it noxious, life-threatening or pleasant (Bouwknecht *et al.*, 2007). A set of interrelated limbic structures are responsible to evaluate the extent to which such a stimulus is threatening for the individual and to select appropriate responses in order to generate adequate patterns of defence (Bergink *et al.*, 2004). Therefore, anxiety may be seen as normal behaviour, as a defence mechanism in raising awareness in order to respond accordingly to a changing environment (Nuss, 2015). However, anxiety can also manifest in inappropriate situations and to varying degrees and should then be considered pathological. The Diagnostic and Statistical Manual of Mental Disorders-5 (DSM-5) states that: “Anxiety should be considered pathological when anxiety, worry or physical symptoms cause clinically significant distress or impairment in social, occupational or other important areas of functioning” (American Psychiatric Association, 2013). Although anxiety and fear are alike it should not be confused; fear is a state that can be directed to a specific threat, whereas anxiety is a nonspecific state with no definite threat (Nuss, 2015). Fear is the emotional response to a threat that is about to happen and anxiety on the other hand, is a state of excessive worry for an anticipated fear (American Psychiatric Association, 2013).

Anxiety disorders are not only associated with great personal distress, a reduction in quality of life and an increase in morbidity and mortality, but also carries an immense economic burden (Wittchen & Jacobi, 2005). Anxiety disorders account for one-third of the costs of mental disorders, with indirect costs (e.g. impact on work) playing a particularly important role (Stein *et al.*, 2008b). Furthermore, generalized anxiety disorder (GAD) is responsible for 110 million disability days per annum in the United States (American Psychiatric Association, 2013). This

negative impact on the overall wellbeing of affected individuals has a vast impact on the global economy. Moreover, pathological anxiety or anxiety disorders are chronic or recurring disorders in which full symptomatic remission is uncommon (Garner *et al.*, 2009). Although existing treatments benefit some patients with anxiety disorders (or anxiety symptoms), a great number of patients do not respond adequately to pharmacological treatment (Garner *et al.*, 2009). It is therefore important to identify novel treatments or biological targets for anxiety disorders as well as anxiety symptoms.

Anxiety may be comorbid or a symptom of numerous other neuropsychiatric disorders, such as depression and schizophrenia (Hamilton, 1960; Huppert *et al.*, 2001), which complicates diagnosis as well as effective management of the illness. A recent study observed that anxiety or symptoms of anxiety are easily overlooked in schizophrenia patients, even though it is a significant source of morbidity in these patients (Braga *et al.*, 2013). Moreover, meta-analyses indicated that 38.3% of schizophrenia patients suffer from at least one comorbid anxiety disorder (Braga *et al.*, 2013). Other studies also observed an estimated prevalence of 67% of GAD in patients with major depression (Judd *et al.*, 1998; McEvoy *et al.*, 2011), while many others have anxiety symptoms without meeting criteria for a specific disorder (Young *et al.*, 2004). Another study observed that 75% of patients with depression had a lifetime comorbid anxiety disorder, whereas 81% of patients with an anxiety disorder had a lifetime prevalence of comorbid depression (Lamers *et al.*, 2011). In most cases, anxiety precedes depression and interestingly, comorbidity with preceding depression is associated with a shorter duration of depressive symptoms and/or anxiety symptoms when compared to preceding anxiety (Lamers *et al.*, 2011). With a prevalence rate of 17.8%, post-traumatic stress disorder (PTSD) is one of the more commonly co-occurring disorders with features of anxiety, in patients with depression, and which escalates to 22.4% in treatment resistant depression (TRD) (Rush *et al.*, 2006), emphasizing that co-presenting of severe anxiety can worsen treatment outcome of the co-presenting illness. This highlights how pervasive anxiety disorders can be, and that it shouldn't be overlooked or seen as the secondary problem. It is therefore important to assess all psychiatric disorders routinely regardless of the primary diagnosis (Lamers *et al.*, 2011). This is especially important since patients presenting with comorbid anxiety show a specific vulnerability pattern, with increased childhood trauma, neuroticism, and higher severity and duration of anxiety and/or depressive symptoms (Lamers *et al.*, 2011). Furthermore, the same trend is observed in patients with bipolar disorder, where lifetime comorbid anxiety disorders are common and decreases the possibility of recovery (Simon *et al.*, 2004). These patients also experience more severe symptoms and impairment, less time in a euthymic state and have an increased risk of suicide (Simon *et al.*, 2004). This emphasises the need for enhanced clinical attention to anxiety in this population so

that treating the anxiety symptoms may possibly improve bipolar disorder severity and response to treatment (Simon *et al.*, 2004).

An association between anxiety disorders and physical disorders such as Parkinson's disease, cardiovascular diseases, an increase in gastrointestinal illnesses and metabolic disorders, is also evident (Menza *et al.*, 1993; Sareen *et al.*, 2005). Indeed, a recent clinical study indicated that GAD is associated with gastrointestinal illnesses (such as gastrointestinal ulcers), while panic attacks and agoraphobia are associated with cardiovascular diseases (such as hypertension) and interestingly, phobias are associated with metabolic/autoimmune disorders (Sareen *et al.*, 2005). The incidence of a physical disorder together with an anxiety disorder may lead to a more disabling condition and optimal treatment of both the anxiety disorder and the physical disorder concurrently may lead to positive outcomes (Sareen *et al.*, 2005).

Important to consider is that males and females respond differently to psychological stressors, possibly relating to hormonal differences (Ter Horst *et al.*, 2012). Sex differences are prominent in mood and anxiety disorders and may provide a window into revealing more on the mechanisms of onset and maintenance of affective disturbances in men and women (Altemus *et al.*, 2014). Epidemiology studies indicate that women are twice as likely to develop mood disorders as men (Maeng & Milad, 2015); while women are diagnosed 2.25 times more often than men with anxiety disorders (Ter Horst *et al.*, 2012). Epidemiology studies in South Africa also concluded that mood and anxiety disorders are significantly higher in women than men (Stein *et al.*, 2008b). This higher incidence rate in women is sustained across all the different types of anxiety disorders e.g. GAD and social anxiety (Maeng & Milad, 2015). The exceptions are obsessive compulsive disorder (OCD) and bipolar disorder, which have similar prevalence in men and women (Altemus *et al.*, 2014). However, even in these disorders, men and women have differences in disease presentation and course (Altemus *et al.*, 2014). Previous studies also indicate that women experience anxiety symptoms to a greater degree (Altemus *et al.*, 2014; McLean *et al.*, 2011). Furthermore, anxiety disorders in women are associated with more missed work days and greater comorbidity together with anxiety disorders (McLean *et al.*, 2011). Moreover, boys are more susceptible to developing a psychiatric disorder than girls *before* puberty (Palanza, 2001); these differences suggest the important role of gonadal hormones in the onset and prevalence of anxiety disorders (Maeng & Milad, 2015; Palanza, 2001). For example, a preclinical study showed that female rats with high levels of progesterone and oestrogen present with less anxiety-like behaviours as seen in the open field test (OFT) and elevated plus maze (EPM) (Hrubá *et al.*, 2012). Furthermore, high levels of these hormones increase exploration on the open arms in the EPM (Hrubá *et al.*, 2012).

2.1.2 Diagnosis and classification of anxiety disorders

The American Psychiatric Association DSM-5 describes the following diagnostic criteria for an anxiety disorder (American Psychiatric Association, 2013):

A) Excessive anxiety and worry (apprehensive expectation), occurring more days than not for at least 6 months, about a number of events or activities (such as work or school performance).

B) The person finds it difficult to control the worry.

C) The anxiety and worry are associated with three (or more) of the following six symptoms (with at least some symptoms present for more days than not for the past 6 months). Note: Only one item is required in children. *(1)* Restlessness or feeling keyed up or on edge; *(2)* Being easily fatigued; *(3)* Difficulty concentrating or mind going blank; *(4)* Irritability; *(5)* Muscle tension and *(6)* Sleep disturbance.

D) The anxiety, worry, or physical symptoms cause clinically significant distress or impairment in social, occupational, or other important areas of functioning.

E) The disturbance is not due to the direct physiological effects of a substance (e.g. a drug of abuse, a medication) or a general medical condition (e.g. hyperthyroidism)

F) The disturbance is not better explained by another mental disorder.

Insomnia, a symptom not a diagnosis, is part of a widespread set of psychiatric disorders such as depression and anxiety (Berk, 2009). Furthermore, sleep disturbances, such as difficulty falling or staying asleep, or restless unsatisfying sleep, are important symptoms and diagnosing criteria for anxiety disorders (American Psychiatric Association, 2013). Clinical and preclinical studies have also shown the connection between circadian rhythm disruptions and anxiety disorders as well as anxiety-like behaviours, as discussed in section 2.2 (Sipilä *et al.*, 2010; Tapia-Osorio *et al.*, 2013). Moreover, most patients who suffer from a mood disorder have disruptions in circadian rhythms and the sleep-wake cycle, seen as altered sleep patterns, and used as important diagnostic criteria for mood disorders (McClung, 2013).

The DSM-5 distinguishes between five types of anxiety disorders: GAD, panic disorder, agoraphobia, phobias and social anxiety disorders. The different types of anxiety disorders vary from one another based on the object or situation that induces fear, anxiety, avoidance behaviour and associated thoughts (American Psychiatric Association, 2013).

2.1.2.1 Generalized anxiety disorders (GAD)

GAD was originally conceptualized as a remaining category for patients whose anxiety symptoms did not meet the criteria for other anxiety disorders (Uys *et al.*, 2003). However, GAD can nowadays be specifically described, e.g. GAD is characterized by excessive and inappropriate worrying that is persistent (six months or longer) (Baldwin *et al.*, 2005). Moreover, the symptomatology of GAD is strongly associated with repetitive negative thinking, described as cognitive perseveration on negative themes, with worry and rumination (McEvoy *et al.*, 2015). The focus of the anxiety and worry is not narrowed to a specific and definite feature like in any of the other anxiety disorders, e.g. being embarrassed in public as observed in social phobia (American Psychiatric Association, 2013). Furthermore, patients have physical anxiety symptoms and key psychological symptoms such as restlessness, fatigue, difficulty concentrating, irritability, muscle tension and disturbed sleep (Baldwin *et al.*, 2005). GAD is often comorbid with major depression, panic disorder, phobic anxiety disorders and OCD (Baldwin *et al.*, 2005).

2.1.2.2 Panic disorder

Panic disorder is characterized by recurrent unexpected surges of severe anxiety with varying degrees and a constant concern of having additional panic attacks (American Psychiatric Association, 2013; Mineka & Oehlberg, 2008). Panic attacks are distinct periods of intense fear or discomfort, accompanied by at least four of the following physical or psychological symptoms: palpitations, sweating, trembling, sensation of shortness of breath, chest pain, nausea, feeling dizzy, fear of losing control, fear of dying, chills and hot flashes (Mineka & Oehlberg, 2008). Panic attacks usually reach their peak within ten minutes from onset and last around 30 – 45 minutes (Baldwin *et al.*, 2005). Around two-thirds of patients with panic disorder develop agoraphobia (Baldwin *et al.*, 2005).

2.1.2.3 Agoraphobia

Defined as fear in places or situations from which escape might be difficult or in which help might not be available (American Psychiatric Association, 2013). These situations include being in a crowd, being outside the home or using public transport (Baldwin *et al.*, 2005). These situations are either avoided or endured with significant personal distress, avoidance is sometimes necessary for diagnosis (Baldwin *et al.*, 2005). When agoraphobias lead to persistent avoidance of such a situation it may impair an individual's ability to travel to work or to perform day-to-day tasks (American Psychiatric Association, 2013).

2.1.2.4 Specific phobia

Specific phobias are characterized by excessive or unreasonable fear for, and restricted to, people, animals, objects, or situations (e.g. flying, dentists, and seeing blood) which are either avoided or are endured with significant personal distress (Baldwin *et al.*, 2005).

2.1.2.5 Social anxiety disorder

Social anxiety disorder is characterized by a marked, persistent and unreasonable fear of social performances or situations, being evaluated negatively by other people or being embarrassed (Baldwin *et al.*, 2005). Exposure to a social performance almost always triggers an immediate anxiety response (American Psychiatric Association, 2013).

2.1.3 Epidemiology and Aetiology

Anxiety disorders are the most prevalent psychiatric disorder in developed and developing countries (Stein & Nesse, 2011; Stein *et al.*, 2008b). Correspondingly, in South Africa it has also been identified as the most prevalent psychiatric disorder, with a lifetime prevalence of 15.8% and an age of onset of 32 years (Stein *et al.*, 2008b). In Europe, Australia and America (Dell'Osso *et al.*, 2010; Kessler *et al.*, 2005; McEvoy *et al.*, 2011), epidemiological studies indicate a lifetime prevalence of approximately 13.6% – 21% in Europe (Dell'Osso *et al.*, 2010), a 12-month prevalence of 11.8% and a lifetime prevalence of 20% in Australia (McEvoy *et al.*, 2011) and a lifetime prevalence of 28.8% in the USA (Kessler *et al.*, 2005). Interestingly, these epidemiology studies indicated that the median age of onset of anxiety disorders in the USA is 11 years, which is earlier than any other psychiatric disorder (Kessler *et al.*, 2005). Similarly, in Australia the median age of onset is 19 years, also earlier than any other psychiatric disorder (McEvoy *et al.*, 2011). Thus, since the first onset is usually in childhood or adolescence, interventions or treatment should focus on the youth (Kessler *et al.*, 2005; McEvoy *et al.*, 2011).

Genetic and environmental factors have both been implicated to increase the vulnerability in developing an anxiety disorder (Garner *et al.*, 2009). Numerous studies have shown that genetic alterations, such as serotonin (5-HT) transporter gene variations, affect anxiety-related behaviours, cognitive bias and neural mechanisms involved in threat processing (Garner *et al.*, 2009). In addition to these studies, twin studies confirm that GAD has genetic risk factors; these data also suggest that GAD and the genetic risk factors are associated with volumetric and spectroscopic changes in limbic structures involved in fear processing (Hettema *et al.*, 2012). Novel evidence also indicates that the development of anxiety could be as a result of environmental stressors, as well as the effect of environmental challenges on gene expression (Garner *et al.*, 2009). Moreover, studies reveal an association between environmental factors

such as adverse parenting (e.g. overprotection) and a higher risk to develop an anxiety disorder (Heider *et al.*, 2008). Meta-analyses studies have shown that panic disorder, GAD and phobias all have a substantial familial aggregation, explained by genetic factors rather than shared familial environmental factors (Garner *et al.*, 2009). Thus, it could be hypothesized that environmental and genetic factors interact, leading to improper operation of regulatory mechanisms, within diverse localizations in the brain that could lead to pathological anxiety symptoms (Saavedra *et al.*, 2011).

Epigenetics, the study of modifications in gene expressions without altering the DNA sequence, may explain how life events, like stress, can cause persistent changes in the brain and in behaviour (McEwen *et al.*, 2012). Epigenetics is of particular interest in disorders such as anxiety and depression where epigenetic changes or adverse life events increase susceptibility to developing an anxiety disorder (Lukkes *et al.*, 2009; McEwen *et al.*, 2012). Both clinical and preclinical studies have indicated that early life stress increases the vulnerability to develop depression, anxiety disorders or both (Heim & Nemeroff, 2001; Lukkes *et al.*, 2009; McEwen *et al.*, 2012). Early life experiences may be a contributing factor to individual differences regarding anxiety-related behaviours, as it may affect the development of key brain structures (McEwen *et al.*, 2012). Furthermore, the quantity, quality as well as the consistency of maternal care determines the anxiety profile via epigenetic mechanisms and improves cognitive and social development (McEwen *et al.*, 2012). Although adverse events during development may predispose these individuals to develop a wide array of physiological and physical disorders, it is not the only significant factor (Figure 2-1) (Heim & Nemeroff, 2001). Thus, the manifestation of anxiety disorders is a function of genetic disposition, early trauma, and recent life stress (Heim & Nemeroff, 2001). As illustrated in Figure 2-1, exposure to adverse experiences in early life may shape a preceding genetic susceptibility towards mood, anxiety and stress-related disorders, which may result in a stable phenotype with a certain risk to develop a psychiatric disorder, such as anxiety, in response to further stress exposure (Heim & Nemeroff, 2001).

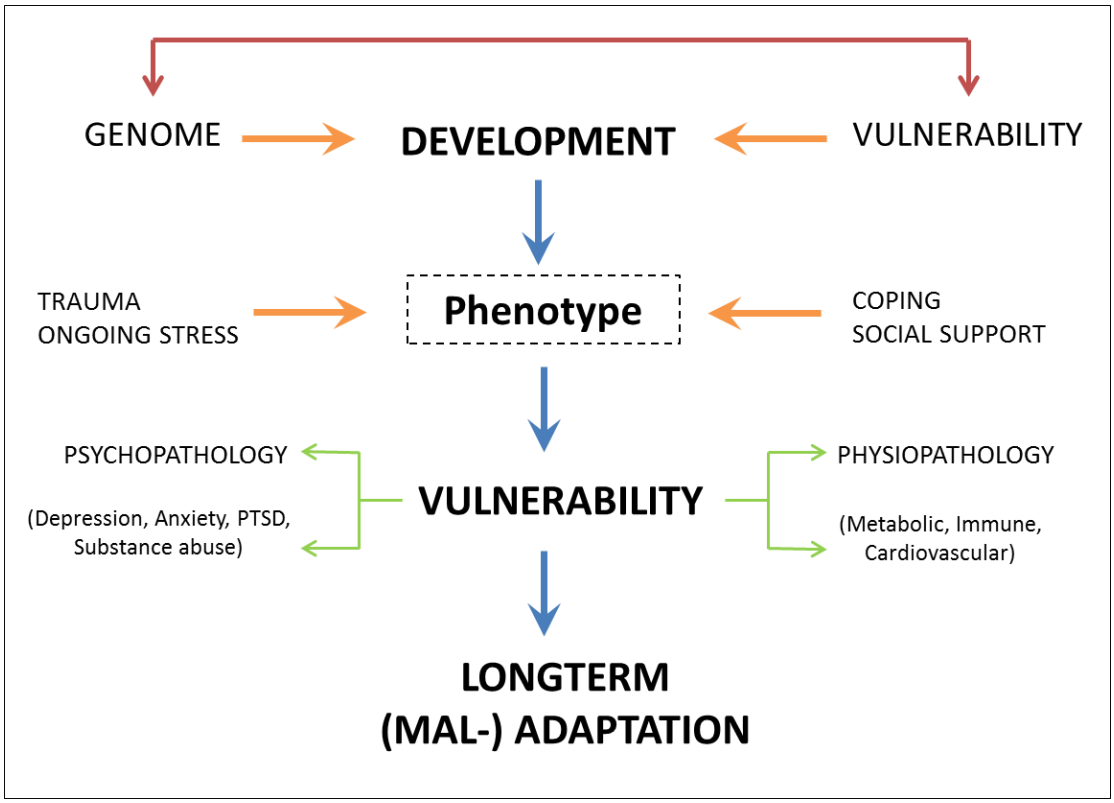

Figure 2-1: A schematic proposed model of the possible interaction between genetic disposition and early environment leading to a vulnerable phenotype. Subsequently, exposure to stress or trauma throughout the life span may exacerbate the underlying vulnerability. Social support or coping styles may decrease the effects of early life stress on vulnerability. Illustration adapted from Heim & Nemeroff (2001).

Other prominent hypotheses regarding anxiety disorders involve inflammatory responses (e.g. cytokines), dysregulation of neurotransmitters (e.g. monoamines and 5-HT) or neuropeptides and the endocrine system (e.g. cortisol secretion) (Petrik *et al.*, 2012). Although immune responses are necessary for survival, the excessive formation of inflammatory mediators and oxidative radicals in the brain may lead to cellular injury and death. It is well established that this excessive brain inflammation play a critical role in the pathophysiology of anxiety disorders (Saavedra *et al.*, 2011).

2.1.4 Pathophysiology

2.1.4.1 Neuroanatomy of anxiety disorders

The brain is the central organ for providing adaptation to stress, by perceiving and determining what may be threatening, as well as setting up the behavioural and physiological responses to the stressor. Structures that are especially important in this regard include the hippocampus, the amygdala and areas of the hypothalamus, as well as the periaqueductal grey matter of the midbrain (Bergink *et al.*, 2004).

2.1.4.1.1 The Amygdala

The amygdala, in particular, seems to play a vital role in these responses (Rauch *et al.*, 2003). The correlation between the activation of the amygdala and anxiety is evident in multiple clinical studies (Nuss, 2015). Fear-conditioning studies in rats as well as functional magnetic resonance imaging (fMRI) studies in humans have also confirmed a state of amygdala hyperactivity in anxiety (Bishop *et al.*, 2004; Garner *et al.*, 2009). Thus, normalizing the hyperactivity of the amygdala is deemed important for successfully treating anxiety disorders with cognitive behavioural therapy as well as pharmacological treatments (Rauch *et al.*, 2003; Straube *et al.*, 2006).

While the amygdala consists of a number of nuclei, the basolateral amygdala complex and the centromedial amygdala complex, mainly the central nucleus, are particularly important in anxiety disorders (Nuss, 2015). The basolateral amygdala complex receives information of potential threatening stimuli from the thalamus and the sensory association cortex (Figure 2-2). Subsequently the basolateral amygdala complex activates the central nucleus, in the centromedial amygdala complex, directly through an excitatory glutamatergic pathway as well as activating a relay of inhibitory γ-amino butyric acid (GABA) interneurons (Figure 2-2) (Nuss, 2015). The GABAergic interneurons (intercalated neurons) are situated between the basolateral amygdala complex and the central nucleus, and exert an inhibitory influence upon the latter (Figure 2-2) (Nuss, 2015). The central nucleus is the main output pathway from the amygdala. Inhibitory GABAergic neurons project from the central nucleus to the hypothalamus and brainstem, the activation of these neurons leads to the somatic manifestations of anxiety (Figure 2-2) (Nuss, 2015). Furthermore, projections to other basal forebrain nuclei such as the ventrotegmental area and the locus coeruleus may be involved in the dysphoria associated with anxiety (Nuss, 2015). In addition to activation of the central nucleus, neurons from the basolateral amygdala complex also activate cells in the adjacent bed nucleus of the stria terminalis, which project to the same areas as the central nucleus and apparently play a similar role (Nuss, 2015). This circuitry has been established from research on experimental animals and, although fMRI results are consistent with this model, it should be noted that these pathways have not all been demonstrated conclusively in the human brain (Nuss, 2015).

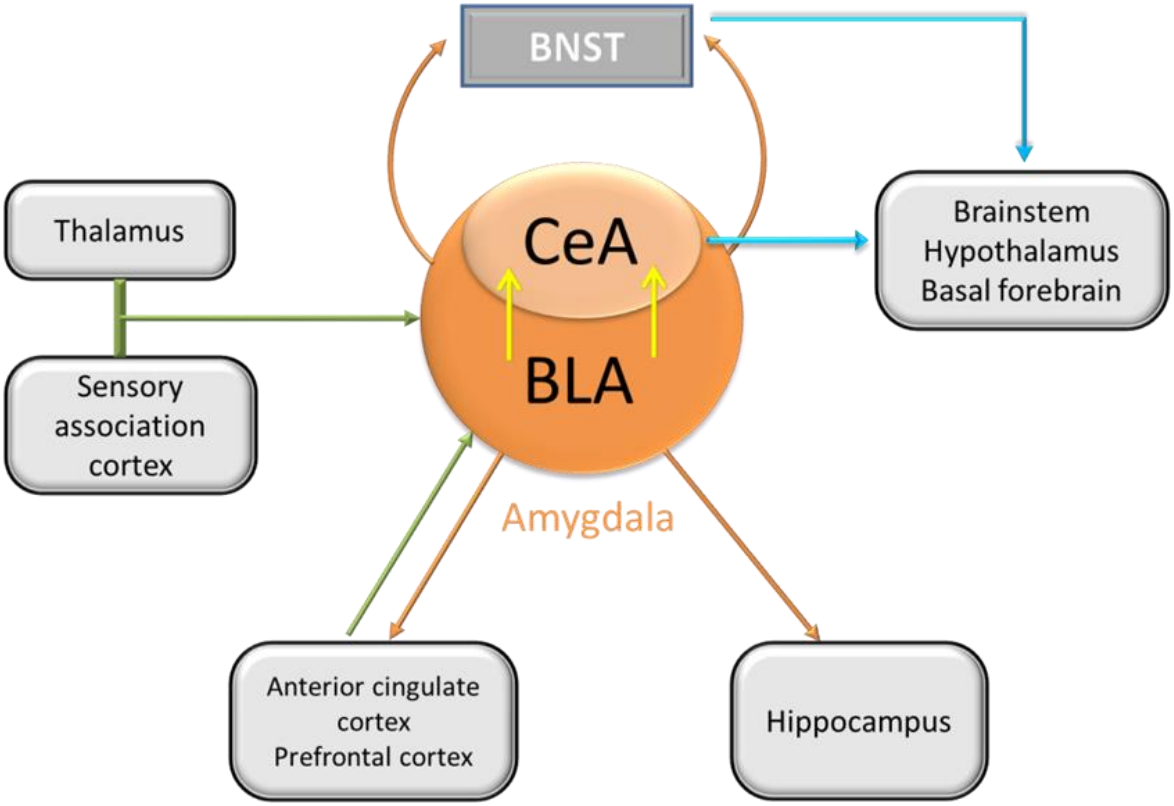

Figure 2-2: The neural circuitry implicated in anxiety disorders. Illustration adapted from (Nuss, 2015). Abbreviations: **CeA,** central nucleus of the amygdala; **BLA,** basolateral amygdala complex; **BNST,** bed nucleus of the stria terminalis. Green arrows: main inputs to the BLA; orange and yellow arrows: main outputs of the BLA; blue arrows: main outputs of the CeA and BNST.

2.1.4.1.2 The Frontal Cortex

In addition to the amygdala, the medial prefrontal cortex and anterior cingulate cortex also play important roles in the pathophysiology of anxiety (Figure 2-3) (Nuss, 2015). The roles of these cortical areas are to send and receive excitatory glutamatergic projections to and from the basolateral amygdala complex, and are activated along with the amygdala during the presentation of emotional stimuli (Figure 2-2) (Kober *et al.*, 2008; Nuss, 2015).

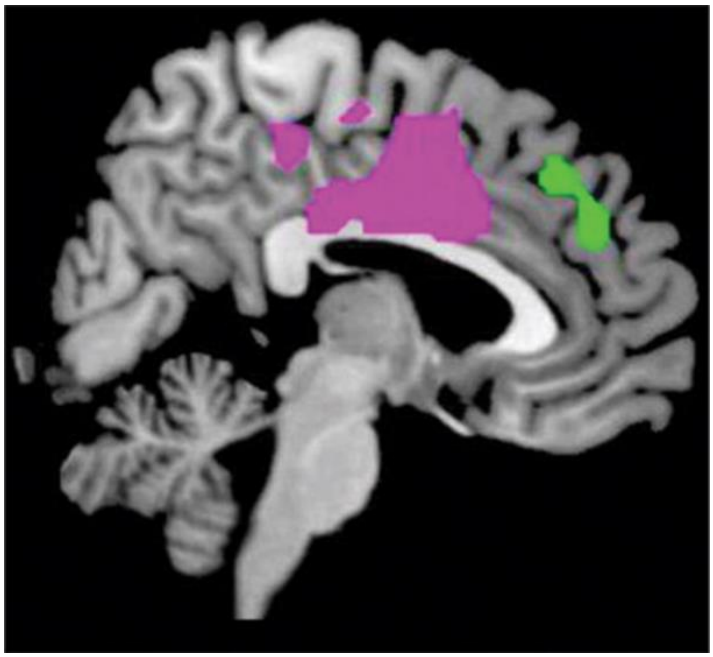

Figure 2-3: The dorsal anterior cingulate and medial prefrontal cortex (highlighted in pink) and the dorsal medial prefrontal cortex (highlighted in green) show increased positive connectivity with the amygdala during the processing of fearful faces under stress using fMRI (Robinson *et al.*, 2014).

Indeed, dysfunctional prefrontal cortex activity has been observed in patients with anxiety disorders (Brooks & Stein, 2015). In GAD, fMRI studies observed a hypofunction of the prefrontal cortex; this is in contrast to other anxiety disorders (e.g. social anxiety disorder) where a hyperfunction of the prefrontal cortex is observed (Brooks & Stein, 2015). In an important finding, Monk *et al.* (2006) established using fMRI that adolescents with GAD show greater right ventrolateral prefrontal cortex activation (Figure 2-4) but that as the ventrolateral prefrontal cortex activation increased, the severity of the anxiety symptoms in these patients reduced (Monk *et al.*, 2006). Thus, the activation of the prefrontal cortex may serve as a compensatory response (Monk *et al.*, 2006).

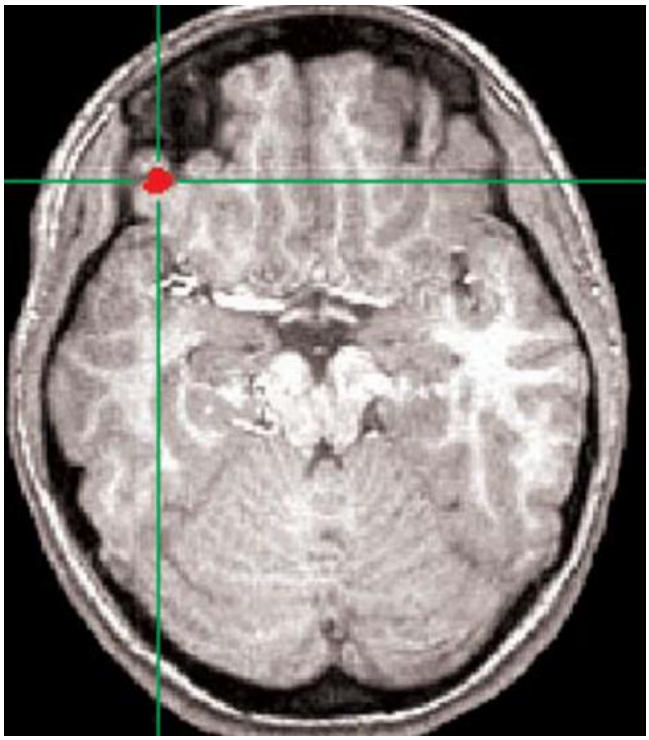

Figure 2-4: The site in the right ventrolateral prefrontal cortex where activation was greater in adolescents with GAD as a compensatory response, in comparison to healthy subjects (Monk *et al.*, 2006).

Acute and chronic stress causes an imbalance of the neural circuitry important for cognition, decision making, anxiety as well as mood, which alters the expression of behaviour in these situations (McEwen *et al.*, 2012). During situations involving acute stress or a threatening environment, the brain will increase fearful vigilance and anxiety, which can be regarded as adaptive changes (McEwen *et al.*, 2012). When the danger passes but the behavioural state persists, along with the changes in neural circuitry, intervention is needed (McEwen *et al.*, 2012). Exposure to chronic stress also leads to extensive architectural changes in various brain regions (Arnsten, 2009; Grillo *et al.*, 2015; McEwen *et al.*, 2012), with the prefrontal cortex being particularly sensitive to these changes (Arnsten, 2009). In rats, prefrontal cortical neurons lose dendritic material following chronic stress, e.g. decrease in dendrite length, branching and spine density (Arnsten, 2009). These changes in the prefrontal cortex are seen after only one week of stress or even a single exposure, whereas structural changes in the hippocampus are seen after several weeks of stress (Arnsten, 2009). Furthermore, chronic stress impairs extinction of a fear conditioning task, which is a function of the prefrontal cortex (McEwen *et al.*, 2012). Moreover, chronic stress may affect hippocampal-dependant behaviours, such as spatial memory and an increase in sensitivity to glucocorticoids, which appears to be involved and mediate some of the behavioural changes (McEwen *et al.*, 2012). Interestingly, dendrites in the amygdala expand in response to chronic stress; thus chronic stress weakens the structures that provide negative feedback on the stress response and strengthens the structures that promote the stress response

(Arnsten, 2009). Chronic stress and hyperactivity of the amygdala also impairs amygdala-dependent unlearned fear and fear conditioning and increases aggression between animals living in the same cage (McEwen *et al.*, 2012). Moreover, chronic corticosterone treatment produces an anxiogenic effect that could be due to the glucocorticoid enhancement of corticotrophin-releasing hormone (CRH) activity in the amygdala (McEwen *et al.*, 2012).

2.1.4.1.3 The Hippocampus

The ability to learn and remember spatial locations, and to associate them with stimuli, is a vital adaptive behaviour required for survival (Bannerman *et al.*, 2014). Spatial navigation and spatial memory are primarily associated with the hippocampus in rodents and humans (Bannerman *et al.*, 2014). Furthermore, apart from the hippocampus' cognitive functions, the hippocampus has a role in anxiety responses towards environmental signals (Bergink *et al.*, 2004; Engin & Treit, 2007). The hippocampus seems to code contextual cues associated with fearful environments and not fear itself (LeDoux, 2003). Preclinical studies suggest that the hippocampus has a distinct role in anxiety, independent of its roles in learning and memory (Engin & Treit, 2007). The dorsal hippocampus (posterior hippocampus in primates) is involved in spatial memory functions of the hippocampus e.g. in the water maze and radial maze (illustrated in Figure 2-5) (Bannerman *et al.*, 2014). The ventral hippocampus (anterior hippocampus in primates), on the other hand, mediates the anxiolytic effects of the hippocampus e.g. exposure to the EPM (illustrated in Figure 2-5) (Bannerman *et al.*, 2014).

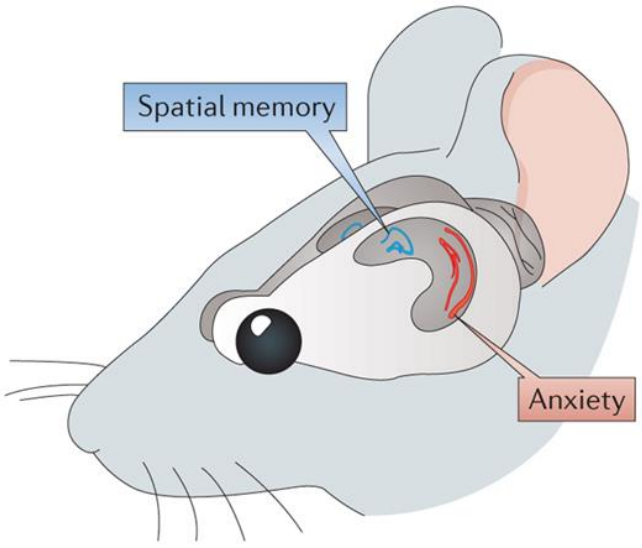

Figure 2-5: This picture illustrates the distinct contributions of the dorsal and ventral hippocampus with regards to memory and anxiety behaviour in rodents (Bannerman *et al.*, 2014).

The ventral hippocampus is much more directly associated with subcortical structures such as the amygdala and the hypothalamus–pituitary–adrenal (HPA) axis (Bannerman *et al.*, 2014). The hippocampus has regulatory functions on the HPA axis, for example, lesions to the hippocampus impair control of the hormonal stress response (Fanselow & Dong, 2010). Furthermore, elevations of stress hormones lead to hippocampal dysfunction in both humans and rodents (Fanselow & Dong, 2010). In humans, decreased hippocampal volumes and hippocampal dysfunction are associated with psychological disorders with strong affective components such as PTSD (Fanselow & Dong, 2010).

Another important aspect regarding anxiety and the hippocampus includes the role of neuroplasticity in anxiety disorders.

2.1.4.2 Neuroplasticity in anxiety disorders

Neuroplasticity includes dynamic processes such as adult neurogenesis, the development of dendritic spines and synaptic adaptions (Wainwright & Galea, 2013). These processes are vital, since humans' ability to process information, ultimately resulting in behaviour, is reliant on neural plasticity (Wainwright & Galea, 2013). Thus, neuroplasticity is an essential mechanism of neural adaption and studies have indicated that this mechanism is disrupted in disorders such as depression and anxiety (Gatt *et al.*, 2009; Pittenger & Duman, 2008). This is supported by post mortem studies in depressed and suicidal patients; altered synaptic and structural plasticity have been observed, especially in the hippocampus (Pittenger & Duman, 2008). Additionally, overwhelming evidence of altered synaptic and structural plasticity has been found in healthy rodents exposed to chronic stress (Pittenger & Duman, 2008). As described earlier, the frontal cortex, hippocampus and amygdala are sensitive to the effects of chronic stress and these structures involvement in anxiety disorders are evident (Arnsten, 2009; Grillo *et al.*, 2015; McEwen *et al.*, 2012). Early life stress such as post-natal social isolation for 8 weeks led to a decrease in neuroplasticity in the frontal cortex of rats (Pittenger & Duman, 2008). Thus, chronic stress leads to architectural changes in these structures, which in turn leads to behavioural alterations, indicating the important role of neural plasticity in anxiety disorders (Pittenger & Duman, 2008).

The survival and plasticity of neurons are affected by the availability of neurotrophins (Jiang *et al.*, 2005). Neurotrophins consists of signaling factors that have a critical role in the development, maintenance and function of the CNS (Jiang *et al.*, 2005). Brain-derived neurotrophic factor (BDNF) is one of the most prominent neurotrophins and is important in pre- and postsynaptic long-term potentiation (LTP) (Jiang *et al.*, 2005). Furthermore, BDNF modulates the early as well as the late phases of synaptic plasticity in the pre- and postsynaptic cells (Pittenger & Duman, 2008). Acute and chronic stress causes a reduction in hippocampal BDNF, indicating that stress

causes impairments in neuroplasticity (Pittenger & Duman, 2008) (Figure 2-6). Glucocorticoids decrease BDNF, possibly explaining how stress causes a decrease in BDNF expression (Pittenger & Duman, 2008). Preclinical and clinical studies have shown that antidepressant treatment increases neuroplasticity (i.e. neurogenesis and neural maturation) by increasing BDNF as illustrated in Figure 2-6 (Pittenger & Duman, 2008).

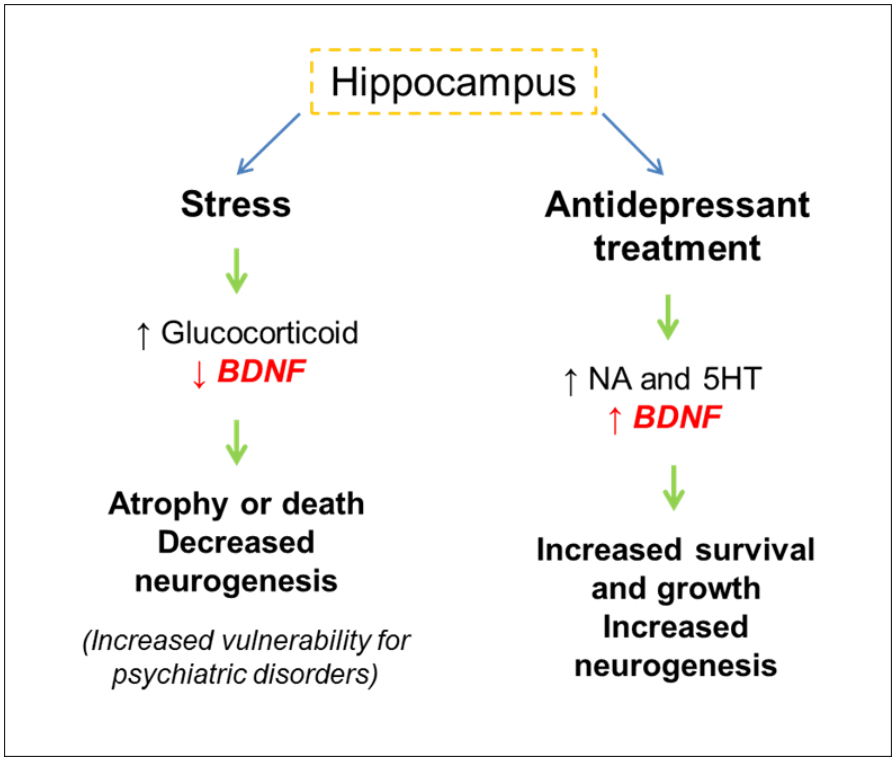

Figure 2-6: The effects of stress and glucocorticoids on BDNF expression and how antidepressants may oppose these effects. Illustration adapted from (Duman *et al.*, 1999).

2.1.4.3 The role of oxidative stress in anxiety disorders

Even though oxygen (O_2) is critical for aerobic life, excessive amounts of its metabolic by-products are highly unstable molecules and noxious to cellular signalling, physiological immunological responses and mitosis (Ng *et al.*, 2008). The main classes of free radicals are reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are collective terms for oxygen- and nitrogen derived radicals (Ng *et al.*, 2008).

Oxidative stress is when the amount of ROS exceeds the antioxidant ability of an organism and consequently oxidative damage is caused by this excessive amount of ROS to cellular components (Bouayed *et al.*, 2009). The cellular damage, as a result, may vary from damages to cellular structures, a decrease in mitosis, or apoptosis to cell necrosis, depending on the severity of oxidative stress (Ng *et al.*, 2008). The brain is exceedingly susceptible for oxidative stress, since it has high O_2 consumption, modest antioxidant defence and a lipid-rich constitution

(Bouayed *et al.*, 2009). The lipid-rich constitution of the brain aids lipid peroxidation, which results in a decrease in membrane plasticity, damaging membrane proteins, inactivating receptors, enzymes and ion channels (Bouayed *et al.*, 2009). Consequently, oxidative stress may alter neurotransmission, neural function and general brain activity (Bouayed *et al.*, 2009). Thus, oxidative stress has been implicated in numerous psychiatric disorders such as depression, schizophrenia and anxiety disorders (Bouayed *et al.*, 2009; Ng *et al.*, 2008). Although clinical studies regarding the role of oxidative stress in anxiety disorders are limited, a few studies have indeed linked anxiety and oxidative stress (Ng *et al.*, 2008). In social phobia and panic disorder an increase in lipid peroxidation were observed, and in social phobia this was corrected by 8 week treatment with citalopram (Ng *et al.*, 2008). A study in anxious women demonstrated that these women have a reduced total antioxidant capacity when compared to non-anxious controls (Ng *et al.*, 2008).

Preclinical studies have also made the link between anxiety-like behaviours in rodents and oxidative stress (De Oliveira *et al.*, 2007; Salim, 2014; Vollert *et al.*, 2011). Vitamin A has been shown to increase oxidative stress (i.e. increased lipid peroxidation, carbonylation, protein thiol oxidation as well as altered superoxide dismutase and catalase activity) in the rat hippocampus, together with anxiety-like behaviour (De Oliveira *et al.*, 2007). Another study observed an increase in anxiety-like behaviour after administration of l-buthionine-(S, R)-sulfoximine, a compound that increases oxidative stress (Salim, 2014), highlighting the relation between oxidative stress and anxiety. The latter behavioural deficits were corrected by the antioxidant, tempol (Salim, 2014).

2.1.4.4 Neurochemistry of anxiety disorders

With regards to neurotransmission dysfunction in anxiety, it is clear that the following neurotransmitters appear to have the most direct effect on anxiety, namely glutamate, GABA, 5-HT, dopamine (DA) and noradrenaline (NA) (Leonard, 2004). Clinical studies have indeed shown that patients with GAD exhibit a significant dysregulation of these neurotransmitters (Ballenger, 2001).

2.1.4.4.1 The GABAergic and Glutamatergic system

An increase in GABAergic activity results in a decrease in anxiety-like behaviour as shown in various animal models of anxiety (Leonard, 2004; Nuss, 2015), while the blockade of the $GABA_A$ receptor induces anxiety (Leonard, 2004). Benzodiazepines, as treatment for anxiety, increase GABA neurotransmission thereby decreasing excitatory output of the amygdala and resulting in anxiolytic effects (Bergink *et al.*, 2004). The inhibitory actions of GABA are opposed by the effects of glutamate, which is the major excitatory neurotransmitter in mammals (Bergink *et al.*, 2004). Normal brain function and behaviour require a balance between inhibitory (GABA) and excitatory

(glutamate) neurotransmission in the central nervous system (CNS) (Harvey & Shahid, 2012). Various studies have highlighted the importance of glutamate in anxiety and anxious behaviour (Bergink *et al.*, 2004; Bermudo-Soriano *et al.*, 2012; Cortese & Phan, 2005; Harvey & Shahid, 2012). Glutamate activity is also responsible for the regulation of neuroendocrine responses to stress (Harvey & Shahid, 2012) and increase the release of CRH in the amygdala (Bermudo-Soriano *et al.*, 2012). A preclinical study also indicated that acute stress may stimulate glutamate release in the rat prefrontal cortex whilst repeated exposure to stress can attenuate glutamate release as an adaptive response (Bermudo-Soriano *et al.*, 2012; Harvey & Shahid, 2012). N-methyl-D-aspartate (NMDA) receptors and glutamate are also involved in stress-induced shortening of dendrites in the medial prefrontal cortex (McEwen *et al.*, 2012). Moreover, chronic stress and the increase in cortisol may lead to glutamate-mediated neurotoxicity in the hippocampus which may result in hippocampal neuronal atrophy and death, reduced regeneration and reduced dendritic branching; this will cause impairment in hippocampal-dependent spatial learning tasks (Bermudo-Soriano *et al.*, 2012). Ionotropic and metabotropic glutamate receptors are widely distributed in the hippocampus, amygdala, anterior cingulate cortex and the medial prefrontal cortex, all structures which have been extensively linked to anxiety disorders (Bermudo-Soriano *et al.*, 2012) and are abundantly innervated with glutaminergic pyramidal cells (Bermudo-Soriano *et al.*, 2012). Moreover, administration of NMDA receptor antagonists into the basolateral amygdala complex has anxiolytic effects in animal models, such as disruption of acquisition in fear conditioning (Bergink *et al.*, 2004). Glutamate receptors in the basolateral amygdala also seem to be involved in eliciting anxiety responses, since the anxiety-like effects observed due to the administration of a $GABA_A$ receptor antagonist, can be reversed by the co-administration of a NMDA antagonist (Sajdyk & Shekhar, 1997). These findings suggest the need for a balance between GABA and glutamate in the basolateral amygdala to regulate behavioural and physiological responses associated with anxiety (Sajdyk & Shekhar, 1997). Moreover, the inhibition of GABA-transaminase (GABA-T), the catabolizing enzyme of GABA, leads to an increase in brain GABA levels and induces behavioural changes such as increased exploratory behaviour as measured in the EPM and OFT i.e. a decrease in anxiety-like behaviour (Sherif & Oreland, 1995).

2.1.4.4.2 The Noradrenergic and Dopaminergic system

The noradrenergic and dopaminergic systems seem to increase arousal in response to threat (Bergink *et al.*, 2004). For example, elevated DA levels in the nucleus accumbens noted in socially isolated rats show an increased reactivity and sensitivity for possible threatening stimuli (Yorgason *et al.*, 2013). Moreover, increased DA levels in the nucleus accumbens are associated with increased anxiety-like behaviour (a decrease in time spent on open arms in the EPM in rats) (Yorgason *et al.*, 2013), and the administration of a α_2 receptor antagonist (e.g. yohimbine)

increases NA and is anxiogenic in humans and animals (Garner *et al.*, 2009; Leonard, 2004). However, administration of clonidine, an α_2 receptor agonist that decreases NA release, is anxiolytic in patients with panic disorder but not in patients with GAD (Garner *et al.*, 2009), indicating a possible distinct difference between panic disorders and GAD in noradrenergic neuro-circuitry. However, targeting the α_2 receptor can be unpredictable, while the specific subtype of α_2 receptor, e.g. α_{2C} subtype, plays a deciding role in how the adrenergic system mediates or regulates anxious or fear-related behaviour (Erasmus, 2011).

2.1.4.4.3 Serotonin

5-HT plays a definite role in anxiety, with several studies suggesting that a reduction in the activity of 5-HT in the limbic structures decreases anxiety-like behaviour (Leonard, 2004). Selective-serotonin receptor inhibitors (SSRIs) are anxiogenic following acute treatment (Garner *et al.,* 2009) but are anxiolytic after chronic treatment by down-regulating postsynaptic 5-HT receptors and reducing central 5-HTergic neurotransmission (Deakin, 2013; Deakin & Graeff, 1991). These studies imply that anxiety disorders are associated with increased 5-HT activity. Indeed, increased levels and activity of 5-HT in the limbic systems are associated with increased anxiety-like behaviour in rats when tested on an EPM (Rex *et al.*, 2004). However, as mentioned above, neurobiological studies have established that other neurotransmitter systems such as glutamate, GABA, DA and NA are also involved in the regulation of anxiety (Harvey & Shahid, 2012) and indeed are interrelated with 5-HT (Ballenger, 2001). For example, approximately 20% of serotonergic terminals from the dorsal raphe nucleus to the nucleus accumbens are in contact with the pre-synaptic DA terminals (Lukkes *et al.*, 2009). Thus, 5-HT may increase DA release via pre-synaptic mechanisms in the nucleus accumbens (Lukkes *et al.*, 2009). Furthermore, studies have revealed the existence of α_2 heteroreceptors on 5-HT nerve terminals in various brain regions of rats and humans (Harvey & Slabbert, 2014; Saito *et al.*, 1996) and administration of a α_2 adrenoreceptor agonist decreases 5-HT levels in the ventral hippocampus of the rat (Saito *et al.*, 1996). This would imply that drugs affecting 5-HT transmission may also, directly or indirectly, affect the neurotransmission of glutamate, GABA, DA and NA (Ballenger, 2001). Furthermore, numerous animal studies suggest that 5-HT aids to maintain certain sex differences in behaviour (Carrillo *et al.*, 2009; Guptarak *et al.*, 2010; Näslund *et al.*, 2013).

2.1.4.4.4 Histamine

Pharmacological as well as genetic studies in rodents indicate that histamine plays a role in anxiety, since an increase in histamine facilitates in heightening anxiety (Haas *et al.*, 2008). Anxiety-like behaviours was evaluated in mice using histamine agonists and antagonists and clearly implicates the role of histamine and its receptors in anxiety states (Yuzurihara *et al.*, 2000).

Thioperamide, a histamine H_3 receptor antagonist, increased histamine which in turn increased anxiety-like behaviour in mice (Yuzurihara *et al.*, 2000). Anxiety-like behaviour was also decreased by a H_1 receptor antagonist, mepyramine (Yuzurihara *et al.*, 2000). Cimetidine, a H_2 antagonist, when administered alone didn't have an effect on anxiety-like behaviour, although when administered together with thioperamide, was found to decrease anxiety-like behaviour (Yuzurihara *et al.*, 2000). The results from this study indicate that anxiety may be mediated by the stimulation of H_1 receptors, while H_2 receptors may inhibit the anxiety-like behaviours produced by the stimulation of H_1 receptors (Yuzurihara *et al.*, 2000). Correspondingly, mice genetically lacking the H_1 receptor showed less anxious behaviour compared to wild type mice (Haas *et al.*, 2008).

2.1.4.5 Neuroendocrine anomalies in anxiety disorders

2.1.4.5.1 The HPA-axis

Apart from neurotransmitter dysregulation, another important mediator of anxiety may be an acute physical or psychological stress reaction (Steiger, 2002) mediated by the hypothalamic-pituitary-adrenal (HPA) axis (Steiger, 2002). The cell bodies in the medial parvocellular region of the hypothalamic paraventricular nucleus contain CRH, which forms the central component of the HPA axis and constitutes the major neuroendocrine stress response system (Heim & Nemeroff, 2001). This stress reaction starts with the release of CRH, resulting in the secretion of adrenocorticotropic hormone (ACTH) from the pituitary gland which in turn stimulates the adrenal glands to secrete cortisol in humans or corticosterone in rats (see Figure 2-7) (Steiger, 2002).

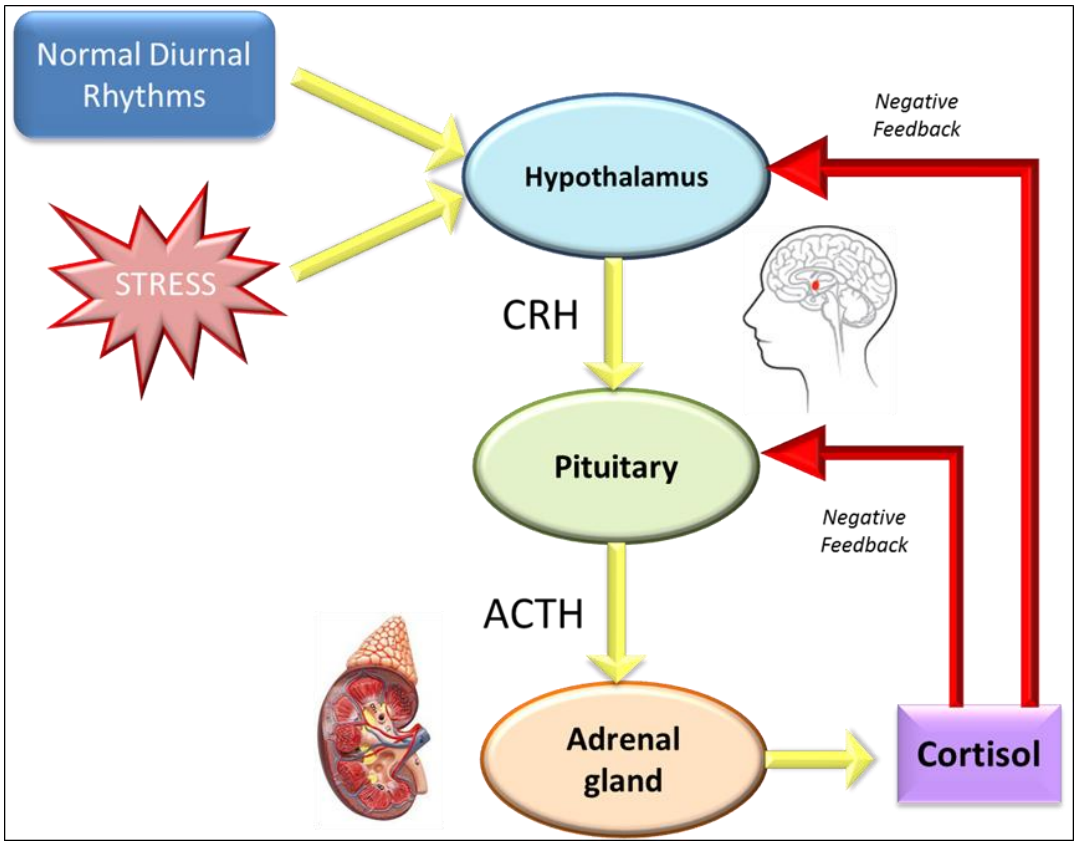

Figure 2-7: Systemic regulation of circulating cortisol levels by the HPA axis. Normal circadian rhythm or stress increases the release of CRH from the hypothalamus. This acts on the pituitary to increase the release of ACTH, which subsequently increase cortisol synthesis and secretion from the adrenal gland. Cortisol suppresses both CRH and ACTH at the pituitary and hypothalamus via a negative feedback loop. Illustration adapted from Hardy *et al.* (2012).

The relationship between early life stress and the development of major depression and anxiety disorders may be mediated by persistent changes in CRH neurotransmission (Heim & Nemeroff, 2001). CRH has been identified as a prominent neuropeptide responsible for initiating a wide variety of endocrine and behavioural responses to stress (Shekhar *et al.*, 2005). Apart from the role of CRH in the HPA axis, it also serves as a neurotransmitter within the CNS, mediating autonomic and behavioural stress responses (Heim & Nemeroff, 2001). Preclinical studies observed that administration of CRH directly into the CNS produces effects that are similar to stress, depression and anxiety such as increased heart rate, disruption of sleep, suppression of exploratory behaviour in a novel environment, facilitation of fear conditioning enhancement of shock-induced freezing and fighting behaviour (Heim & Nemeroff, 2001). Furthermore, the amygdala expresses a high density of CRH receptors, while CRH is released into the amygdala during stress which causes stress-induced alterations in affective behaviour (Shekhar *et al.*, 2005). Increased CRH activates glutamate receptors in the amygdala resulting in induction of synaptic plasticity and increased excitability of BLA neurons (Shekhar *et al.*, 2005). Moreover,

GABAergic neurons in the hippocampus and amygdala exert an inhibitory effect on central CRH neurons as well as stress responsiveness (Heim & Nemeroff, 2001).

A recent study indicates that 5-HT activates CRH in the periventricular nucleus and increases the release of ACTH, possibly indicating a connection between 5-HT, stress and cortisol or corticosterone release in humans or animals, respectively (Kumar *et al.*, 2014). Moreover, studies of major depression have clearly demonstrated HPA axis dysregulation, revealing increased cortisol and ACTH levels at baseline and defects in negative feedback (Young *et al.*, 2004). Another study indicated that patients with depression and a comorbid anxiety disorder had an exaggerated endocrine response, with increased ACTH responses and although the cortisol data did not show a significantly greater response, the results indicated a same direction of effect (Young *et al.*, 2004). The impact of comorbidity on the ACTH response seemed to be a function of having an anxiety disorder and not merely being anxious (Young *et al.*, 2004). Concluding, comorbid anxiety disorders might play a role in the increased activation of the HPA axis observed in patients with major depression (Young *et al.*, 2004).

2.1.4.5.2 DHEA

Another hormone that has been linked to the stress response as well as the genesis of anxiety, as proven in preclinical and clinical studies, is dehydroepiandrosterone (DHEA) (Boudarene *et al.*, 2002; Lang *et al.*, 2014; Prasad *et al.*, 1997; Strous *et al.*, 2003). DHEA is a steroid hormone (illustrated in Figure 2-8) of which the exact physiological role is not yet fully understood (Boudarene *et al.*, 2002; Lang *et al.*, 2014; Prasad *et al.*, 1997; Strous *et al.*, 2003). A clinical study indicated that an increase in cortisol is associated with a high state of anxiety, while a low state of anxiety is related to an increase in DHEA levels (Boudarene *et al.*, 2002). Increased levels of DHEA are associated with a decrease in repetitive negative thinking, an established characteristic of GAD (Boudarene *et al.*, 2002). Administration of DHEA has also been shown to decrease anxiety symptoms in schizophrenia patients (Strous *et al.*, 2003) as well as anxiety-like behaviour in animal models (Prasad *et al.*, 1997). However, intermediate anxiety is observed in subjects who present with increased cortisol and DHEA levels (Boudarene *et al.*, 2002). Furthermore, a preclinical study observed that DHEA is neuroprotective since it increased cell proliferation in the dentate gyrus of the hippocampus in male rats (Karishma & Herbert, 2002). DHEA also has the ability to antagonize the neurodegenerative and suppressive action on cell proliferation of corticosterone (Karishma & Herbert, 2002). Another important interaction between DHEA and cortisol is that DHEA may inhibit the action of cortisol, possibly due to the competition for synthesis and release by the adrenal gland (see Figure 2-8) (Boudarene *et al.*, 2002).

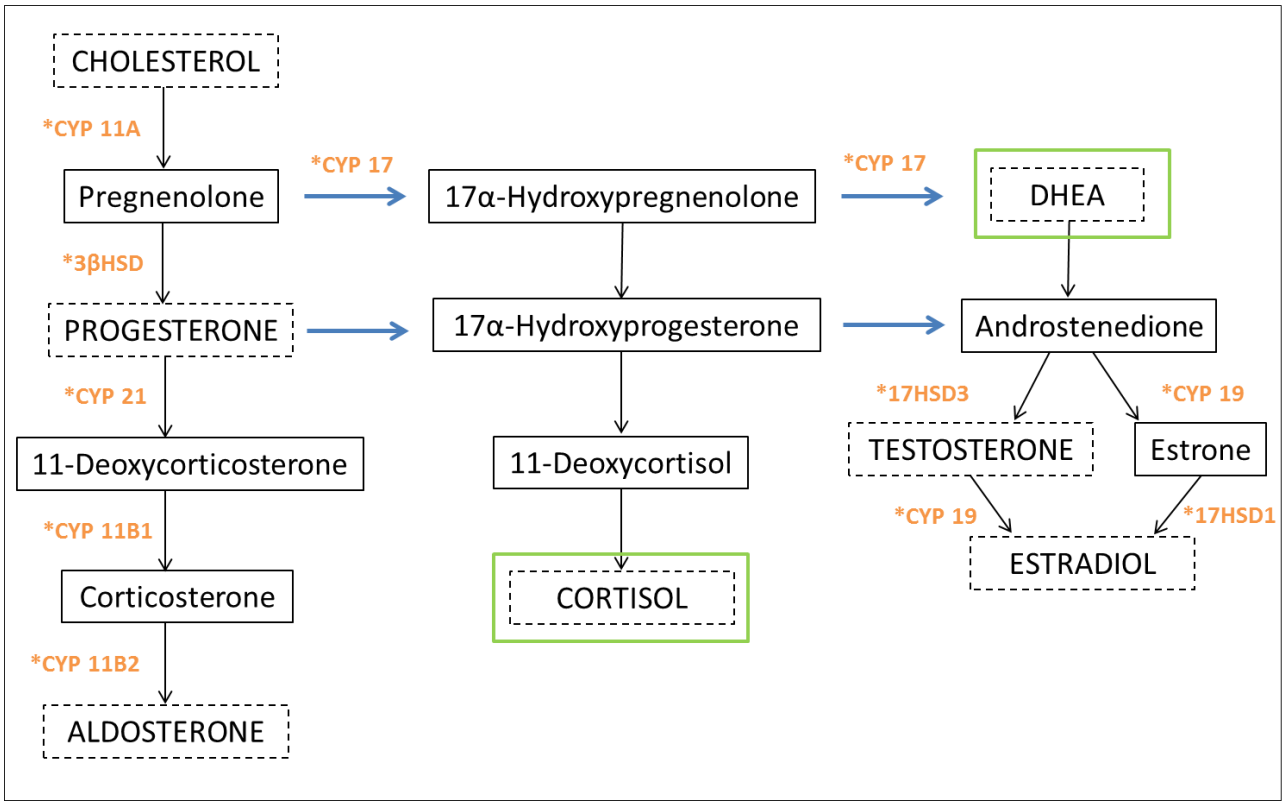

Figure 2-8: Biosynthesis of steroid hormones in adrenal glands and gonads, highlighting the possible ways DHEA can compete with the synthesis of cortisol. Illustration adapted from (Payne & Hales, 2004).

2.1.4.5.3 Oxytocin and Vasopressin

An important aspect in the neuroendocrine component of anxiety is the involvement of several neuropeptides such as oxytocin and vasopressin (Garner *et al.*, 2009). Altered circadian fluctuation of these peptides has also been described in chronic anxiety states, especially vasopressin (Kalsbeek *et al.*, 2012; McClung, 2013). The stimulation of vasopressin and oxytocin receptors exerts opposite effects on fear and anxiety-related behaviours (Huber *et al.*, 2005; Legros, 2001). Vasopressin enhances aggressiveness, anxiety and stress in rats (Huber *et al.*, 2005). Another study showed an increase in peripheral plasma vasopressin, ACTH and cortisol after a 2 hour mild stress test in 10 out of 25 non-depressed patients with a high state of anxiety (Legros, 2001). The latter study confirmed the role of central vasopressin in regulating ACTH during psychological stress in non-pathological conditions (Legros, 2001). Moreover, previous results indicated that the vasopressin V_{1B} receptor antagonist, SR149415, is effective in reversing anxiety-like behaviour in animal models of depression (Griebel *et al.*, 2002). Oxytocin on the other hand, attenuates glucocorticoid secretion (Windle *et al.*, 1997), and has anxiolytic effects in animals (Heinrichs *et al.*, 2003). In humans oxytocin shows similar anxiolytic effects (Heinrichs *et al.*, 2003; Legros, 2001; Uvnäs-Moberg, 1998), especially during breast feeding (Altemus, 1995). Oxytocin could therefore be considered as an endogenous anxiolytic neuropeptide in humans

(Legros, 2001). In addition to the "fight-or-flight" response to stress mediated by cortisol, humans also demonstrate a "tend-and-befriend" response to stress (Taylor, 2006) where oxytocin seems to play a key role in this behavioural response to stress (Taylor, 2006; Taylor *et al.*, 2000). Tending involves nurturing activities intended to protect and promote safety in order to reduce distress; befriending is forming and maintaining social networks that may assist in stressful situations (Taylor *et al.*, 2000). Thus, oxytocin is implicated in the seeking of social contact in response to stress in humans and animals (Cardoso *et al.*, 2013; Taylor, 2006). Oxytocin in combination with social relationships may attenuate psychological and biological stress responses, whereas oxytocin with no supportive contacts may exacerbate psychological and biological stress responses (Taylor, 2006). Studies in PTSD have also revealed the important role of oxytocin to increase social interaction and social support in order to reduce anxiety-associated symptoms in patients with PTSD (Olff, 2012; Olff *et al.,* 2010).

2.1.5 Current treatment for anxiety disorders

A treatment plan may be based on the patient's preference, severity of illness, comorbidity, concomitant medical illnesses, complications like substance abuse or suicide risk, the history of previous treatments, cost issues and the availability of types of treatment in a given area (Bandelow *et al.*, 2002). Treatment options include pharmacological treatment and psychological therapy (Bandelow *et al.*, 2002). Patients with anxiety disorders require supportive therapy, therefore psychological and pharmacological treatments are often concomitant therapies, rather than alternative therapies (Bandelow *et al.*, 2002). Naturally, drugs used in anxiety disorders have side-effects such as sexual dysfunction and weight gain, for e.g. SSRI associated side-effects can jeopardize compliance, and ultimately drug treatment may fail (Bandelow *et al.*, 2002). Compliance with drug treatment may be improved when the advantages and disadvantages of the drugs are explained carefully to patients (Bandelow *et al.*, 2002). Treatment should continue for at least 6 – 24 months after remission has occurred in order to reduce the risk of relapse, and may be stopped only if all, or almost all, symptoms disappear (Bandelow *et al.*, 2002).

2.1.5.1 Antidepressants

The current pharmacological guidelines recommend antidepressants as first-line treatment for the chronic treatment of anxiety disorders, in particular SSRIs and 5-HT and NA reuptake inhibitors (SNRIs) (Nuss, 2015). Interesting is that acute administration of SSRIs in humans and animals cause anxiety, which disappears after chronic treatment, emphasizing the delayed effectiveness of SSRIs in the treatment of anxiety states (Bagdy *et al.*, 2001). Benzodiazepines are generally used for 4-6 weeks at the start of treatment together with SSRIs to reduce the initial anxiogenic effects of these antidepressants (Nuss, 2015). SSRIs provoke anxiety in rodents via the indirect activation of 5-HT_{2C} receptors, while chronic administration down-regulates 5-HT_{2C} receptors and

reduces anxiety (Millan *et al.*, 2005). The key involvement of 5-HT in anxiety is demonstrated by evidence that administration of a 5-HT_{1A} and a 5-HT_{1B} autoreceptor antagonist together with a SSRI (that increases synaptic 5-HT) hastens the onset of anxiolytic activity (Garner *et al.*, 2009). Moreover, co-administration of a 5-HT_{2C} receptor antagonist with a SSRI may relieve the anxiety symptoms induced by the latter and improve sleep disturbances (Bagdy *et al.*, 2001; Garner *et al.*, 2009). The partial 5-HT_{1A} agonist, buspirone, has also been shown to be effective in GAD (Baldwin *et al.*, 2005) as well as for anxiety-like behaviour in rats (Kumar *et al.*, 2014).

Tricyclic antidepressants (TADs) have also been proven effective for treating GAD, although their use is limited due to their anticholinergic side-effects, weight gain and sedation (Zohar & Westenberg, 2000).

2.1.5.2 Benzodiazepines

GABA neurotransmission, as targeted by benzodiazepines and related drugs (Lydiard, 2003), are anxiolytic by decreasing the activation of the amygdala (Del-Ben *et al.*, 2012; Paulus *et al.*, 2005). Although benzodiazepines are potent anxiolytics, their use is restricted to the acute treatment of GAD due to their association with physical and psychological dependence (Davidson, 2001). Non-benzodiazepine approaches may therefore be useful in treating anxiety, such as indirectly enhancing the effects of GABA by (1) increasing its synthesis e.g. with valproate, (2) inhibiting its breakdown e.g. with vigabatrin, (3) inhibiting its reuptake e.g. with tiagabine, (4) using GABA analogues such as pregablin or gabapentin (Bech, 2007; Garner *et al.*, 2009) as well as (5) targeting GABA receptor subunits with drugs such as zaleplon, zolpidem and zopiclone which exerts anxiolytic effects (Drover, 2004).

2.1.5.3 Diverse drugs

The antihistamine, hydroxyzine, is effective in GAD, but due to its sedating effects should only be used when other medications have not been successful or well tolerated (Baldwin *et al.*, 2005; Bandelow *et al.*, 2002). However, when a sedating effect is desirable, this antihistamine is a better option than benzodiazepines (Bandelow *et al.*, 2002). Atypical antipsychotics such as quetiapine have also been shown to be an effective anxiolytic in anxiety disorders, and can be used as monotherapy in GAD or as add-on treatment for non-responsive cases of anxiety disorders (Baldwin *et al.*, 2005; Bandelow *et al.*, 2002). The anxiolytic properties of quetiapine seem to be due to the antagonism of the H_1 receptor as well as the α_{2C} receptor, and treating anxiety requires lower doses than necessary for schizophrenia (Schwartz & Stahl, 2011). Although most of the second generation antipsychotic drugs have similar mechanisms of action, they are still unique in their pharmacodynamic profile; hence they can't all be used for anxiety (e.g. ziprasidone) (Schwartz & Stahl, 2011).

Moreover, pregabalin, a calcium channel modulator, has been found to be effective in GAD (Bandelow *et al.*, 2002). The anxiolytic effects of pregablin are attained due to its binding at the α_2-δ subunit protein of voltage-gated calcium channels in CNS tissues (Bandelow *et al.*, 2002). Voltage-gated calcium channels (α_2-δ subunit protein) are situated, amongst others, in the hippocampus (Davies *et al.*, 2007). Binding on these calcium channels reduces calcium influx on nerve terminals and subsequently modulates the release of neurotransmitters (e.g. glutamate) (Bandelow *et al.*, 2008). The onset of efficacy occurs in the first days of treatment, which is an advantage over treatment with antidepressants (Bandelow *et al.*, 2002).

Optimal anxiolytic treatment requires several characteristics, which includes: effectiveness across a wide range of anxiety symptoms, the ability to achieve remission, few side-effects, few interactions and no discontinuation symptoms (Dell'Osso *et al.*, 2010). Given the possibility of serious side-effects of these agents, which causes problems with adherence in the treatment of anxiety disorders (Dell'Osso *et al.*, 2010), novel approaches for the treatment of GAD as well as anxiety in depression or other psychiatric disorders are needed and should be investigated. One such option is to target circadian rhythms, discussed below, especially the mechanism regulating these processes seated in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus.

2.2 Circadian rhythms

The SCN is considered the body's master biological clock (Buijs *et al.*, 2003; Liu *et al.*, 2007) and regulates all circadian rhythms in mammals, including physiologic processes, metabolic processes (e.g. hormone production), behaviour as well as the sleep-wake cycle (Sipilä *et al.*, 2010). The relation between anxiety and the circadian rhythms have also been confirmed in various studies (McClung, 2013; Sipilä *et al.*, 2010; Tapia-Osorio *et al.*, 2013; Verma *et al.*, 2010). A recent preclinical study found that rats exposed to constant light for 8 weeks had significantly disrupted circadian rhythms, including decreased plasma melatonin levels and increased grooming as well as increased fecal boli in the OFT suggestive of a high anxiety-like state (Tapia-Osorio *et al.*, 2013). Human genetic studies have also concluded that circadian rhythm disruptions are causally related to mood disorders, rather than being an effect of a mood disorder, although the one probably aggravates the other (McClung, 2013). For example, conditioned or psychophysiological insomnia, a common form of insomnia, follows when anxiety concentrated on sleeplessness causes alertness at bed time (Berk, 2009). This anxiety concerning sleeplessness maintains the cycle that prolongs the insomnia (Berk, 2009). Circadian rhythm disruptions can lead to mood disorders in various ways, such as altered monoamine signalling, immune dysfunction, altered neurogenesis and HPA axis dysfunction (McClung, 2013).

The circadian system also regulates monoamine transmission involved in mood and anxiety via circadian genes that directly regulate genes involved in monoamine synthesis and release (McClung, 2013). Monoamine transmission such as DA (ventral tegmental area), 5-HT (dorsal raphe) and NA (locus coeruleus) are also mediated through indirect connections originating from the SCN (McClung, 2013). The SCN also regulates hormone systems involved in mood and anxiety such as the HPA axis. One in particular being vasopressin which acts in the paraventricular nucleus to release CRH, the latter in turn stimulating the secretion of ACTH in the anterior pituitary and the subsequent release of cortisol (McClung, 2013). Thus, altered cortisol secretion can occur as a result of disrupted circadian rhythms (Son *et al.*, 2011), or as part of a stress response.

Pre-clinically it has been shown that female rats secrete more corticosterone in resting conditions than males, while the circadian rhythm secretion of corticosterone is more frequent and with higher pulses in females (Ter Horst *et al.*, 2012). Circadian genes contribute to the sleep–wake cycle and mood (McClung, 2013) and patients with anxiety disorder often suffer from sleep disturbances (American Psychiatric Association, 2013). Furthermore, a clinical study found that altered circadian genes play a role in the genetic predisposition to anxiety disorders (Sipilä *et al.*, 2010). It is therefore apparent that targeting circadian rhythms may be an important approach to treating anxiety disorders. One pharmacological treatment option that may do this is the antidepressant agomelatine.

2.3 Agomelatine

2.3.1 Mechanism of action and pharmacological profile

The efficacy of agomelatine in anxiety disorders has become a main focus of investigation since studies have observed its anxiolytic effect in patients with depression (De Berardis *et al.*, 2015) and GAD (Stein *et al.*, 2008a). Agomelatine is a high affinity melatonin MT_1/MT_2 agonist and a moderate 5-HT_{2C} antagonist (Figure 2-9) (Guardiola-Lemaitre *et al.*, 2014), and has no affinity for adenosine, adrenergic, DA, GABA, muscarinic, nicotine, histamine, excitatory amino acid, benzodiazepine and sigma receptors as well as sodium, potassium or calcium channels (Guardiola-Lemaitre *et al.*, 2014). Additionally, agomelatine acts as a weak antagonist on the 5-HT_{2B} receptor, although the functional significance of this activity is uncertain (Guardiola-Lemaitre *et al.*, 2014). Important, agomelatine has negligible affinity on 5-HT_{2A} receptors (Guardiola-Lemaitre *et al.*, 2014), indicating that only the 5-HT_{2C} antagonistic property of agomelatine is of functional significance (Guardiola-Lemaitre *et al.*, 2014).

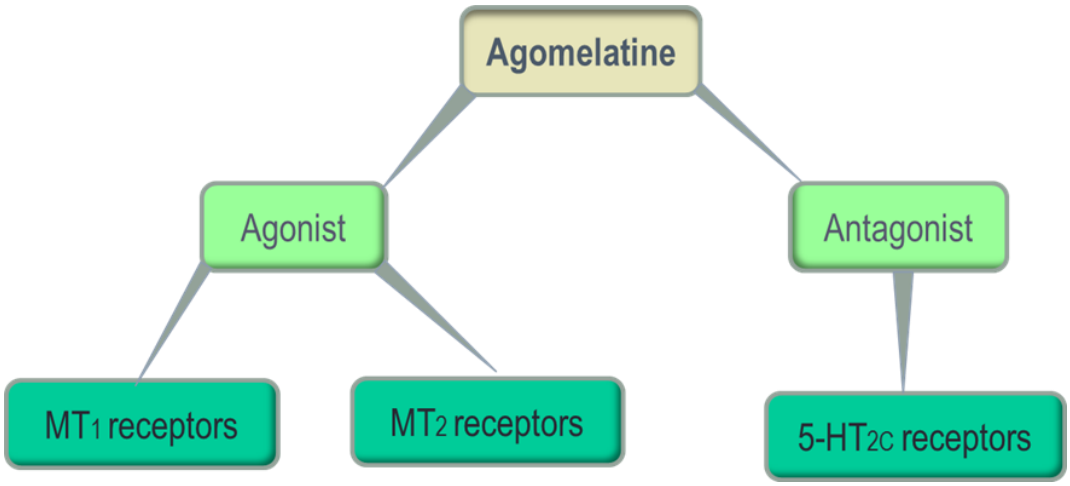

Figure 2-9: The mechanism of action of agomelatine, indicating the agonism at the MT_1/MT_2 receptors and the antagonism at the 5-HT_{2C} receptors. Illustration adapted from Guzman (2009).

Clinical studies have demonstrated that pharmacological agents that target more than one neurotransmitter system are more effective than agents that target a single system, presumably due to synergistic mechanisms (Ballenger, 2001). These drugs that modulate more than one neurochemical system have a broader spectrum of action and may facilitate the attainment of remission among patients with moderate to severe GAD, who are likely to have comorbid psychiatric illnesses such as depression (Ballenger, 2001). Moreover, agomelatine is anxiolytic in animals (Millan *et al.*, 2005) and has shown anxiolytic properties in numerous anxiety-like behavioural tests in rodents (EPM, the Vogel conflict test and the social defeat test) (San & Arranz, 2008). Moreover, 5-HT_{2C} receptors are concentrated in the prefrontal cortex, hippocampus and amygdala (Millan *et al.*, 2003) and the involvement of these receptors in anxiety is widely evident (Bagdy *et al.*, 2001; De Berardis *et al.*, 2015; Millan *et al.*, 2005). For example, mice genetically lacking 5-HT_{2C} receptors show reduced anxiety (De Berardis *et al.*, 2015); while 5-HT_{2C} receptor agonists show anxiogenic properties (De Berardis *et al.*, 2015) and antagonists have anxiolytic properties (Bagdy *et al.*, 2001). Interestingly, the acute anxiogenic effects of SSRIs are reversed by pre-treatment with low doses of a selective 5-HT_{2C} antagonist, suggesting that the anxiogenic effects of SSRIs are mediated by the 5-HT_{2C} receptor (Bagdy *et al.*, 2001). Thus, the anxiolytic effects of agomelatine may be mediated via antagonising the 5-HT_{2C} receptor, especially in the amygdala and in the hippocampus (De Berardis *et al.*, 2015).

However, this antagonistic mechanism on the 5-HT_{2C} receptor also increases the levels of DA and NA in the prefrontal cortex (Millan *et al.*, 2003; Zupancic & Guilleminault, 2006) as well as decreases stress-induced glutamate release in the prefrontal cortex (Racagni *et al.*, 2011) that may contribute to its anxiolytic properties. Studies suggest that elevated NA levels in the prefrontal cortex are anxiolytic (Arnsten, 2009; Nakane *et al.*, 1994). This effect of agomelatine on NA

release seems to manifest as an acute response after 2 days of administration and disappears after chronic (14 day) administration (Chenu *et al.*, 2013). Furthermore, acute administration of agomelatine increase NA in the locus coeruleus (Millan *et al.*, 2003), but is dampened back to normal after chronic administration because of the enhanced negative feedback exerted by up-regulated 5-HT neurotransmission (Chenu *et al.*, 2013). This mechanism could possibly explain why agomelatine doesn't induce anxiety at first, like the SSRIs. Although agomelatine indirectly increases 5-HT after chronic administration (Chenu *et al.*, 2013) it does not provoke the release of 5-HT after acute administration (Chenu *et al.*, 2013). In fact it is devoid of serotonergic side-effects, including the evoking of 5-HT mediated anxiety and sleep disturbances at the start of treatment (Harvey & Slabbert, 2014; Racagni *et al.*, 2011).

The tree shrew chronic social stress model is used to study the behavioural, endocrine, and neurobiological changes that may underlie stress related disorders (Schmelting *et al.*, 2014). This study found that NA concentration was elevated in the urine, indicating sympathetic hyperactivity, and was normalized with chronic agomelatine treatment (Schmelting *et al.*, 2014). Corticosterone concentration was also elevated in the urine and returned to basal levels after agomelatine treatment (Schmelting *et al.*, 2014). Other preclinical studies have also concluded that agomelatine normalizes stress-induced increases in urinary corticosterone (Popoli, 2009).

A central component of the action of agomelatine is that it can resynchronise disrupted circadian rhythms in animal models and in humans (Lemoine *et al.*, 2007). Given that circadian rhythms are implicated in anxiety regulation (Sipilä *et al.*, 2010; Tapia-Osorio *et al.*, 2013; Verma *et al.*, 2010), this action of agomelatine could contribute to its anxiolytic properties. Polysomnographic studies, which are used to diagnose and investigate sleep disorders, is a graphical recording of sleep patterns as well as brain wave patterns, amongst others (Mosby's Dictionary of Medicine, 2009). Polysomnographic studies have found that 25 mg/day of agomelatine normalizes the distribution of slow-wave sleep, and improves the continuity and quality of sleep throughout the night (Salva *et al.*, 2007). Preclinical studies show that administration of agomelatine also increases monoaminergic activity. That this action may be blocked with concomitant administration of a melatonin antagonist confirms the important role of melatonin in monoaminergic activity and links circadian rhythms and monoamine systems to the action of agomelatine (McClung, 2013). Moreover, a previous study observed that melatonin enhances open arms exploration in the EPM in rats (Papp *et al.*, 2006), suggesting that the anxiolytic effects of agomelatine may also be mediated via the activation of melatonin (MT_1 and MT_2) receptors (De Berardis *et al.*, 2015). Interestingly, melatonin has been shown to reduce pre-operative anxiety in humans (Caumo *et al.*, 2009). However, the anxiolytic effects of melatonin is inferior to that of agomelatine (Tuma *et al.*, 2005), indicating a synergistic response with 5-HT_{2C} receptor

antagonism in the case of agomelatine. However, agomelatine's anxiolytic effects, when administered in the morning, was not inhibited by a melatonin antagonist, indicating that stimulating the MT_1/MT_2 receptors is involved but not essential to sustain the efficacy of agomelatine (Papp *et al.*, 2006). Important is that the anxiolytic action of agomelatine is absent in SCN-lesioned animals, suggesting a direct action of agomelatine within the SCN (Tuma *et al.*, 2005). Thus, agomelatine's anxiolytic efficacy depends on the synergy between melatonin agonism and $5\text{-}HT_{2C}$ antagonism (Papp *et al.*, 2006; Racagni *et al.*, 2011), especially in the SCN (illustrated in Figure 2-10) (Racagni *et al.*, 2011).

As noted earlier, oxidative stress and inflammation play a role in the development of anxiety disorders (Saavedra *et al.*, 2011). Importantly, melatonin (Galano *et al.*, 2011; Kumar *et al.*, 2014) and agomelatine (Aguiar *et al.*, 2013) have antioxidant properties that may be beneficial in treating anxiety disorders (Kumar *et al.*, 2014). Chronic agomelatine treatment significantly reduced pro-inflammatory cytokines (IL-1β and IL-6) in rats peripherally and in the CNS tissue (Guardiola-Lemaitre *et al.*, 2014). Thus, agomelatine affects the molecular systems involved in the inflammatory response (Guardiola-Lemaitre *et al.*, 2014), which could contribute in treating anxiety disorders.

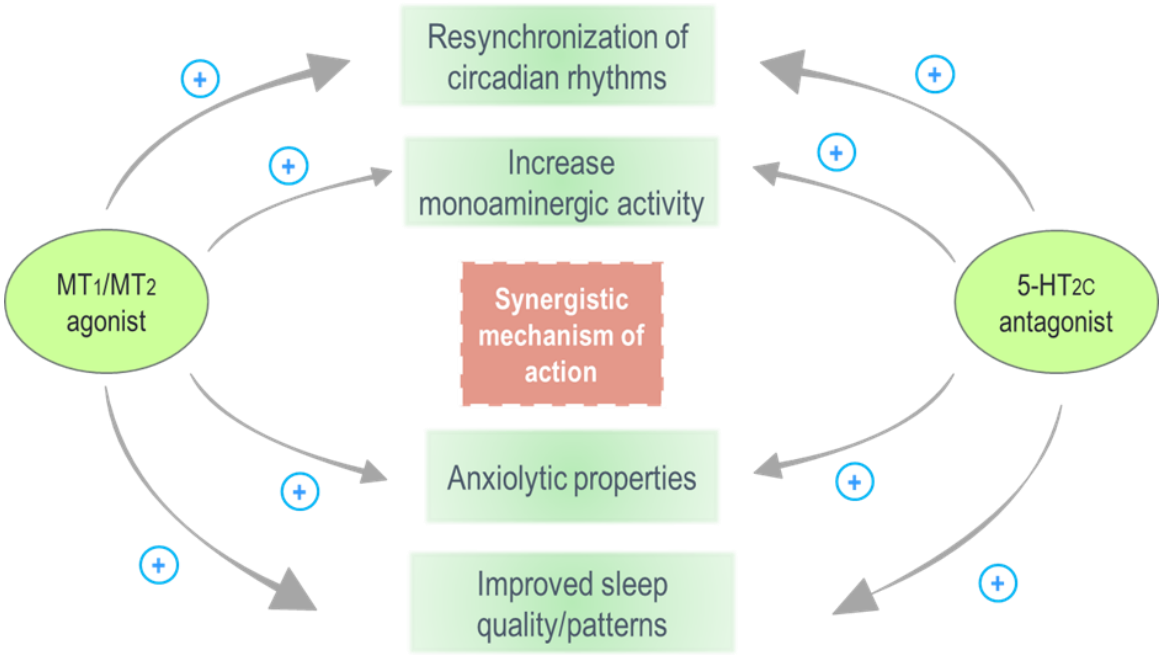

Figure 2-10: The synergistic mechanism of the agonism on the MT_1/MT_2 receptors and the antagonism of the $5\text{-}HT_{2C}$ receptors are responsible for certain effects of agomelatine. (Illustration adapted from (De Bodinat *et al.*, 2010).

Considering the prominent role for disordered neuroplasticity in depression and its impact on neurotrophin release and function noted earlier, agomelatine has the ability to stimulate progenitor

cell proliferation in the dorsal (AlAhmed & Herbert, 2010) and ventral (Banasr *et al.*, 2006) dentate gyrus in adult rats, thus enhancing hippocampal neurogenesis (AlAhmed & Herbert, 2010; Banasr *et al.*, 2006). This ability to increase neurogenesis seems to be through its action on 5-HT_{2C} and not melatonin receptors (AlAhmed & Herbert, 2010). 5-HT_{2C} receptors may regulate neurogenesis directly (AlAhmed & Herbert, 2010) or via DA release. DA plays a role in neurogenesis in the subventricular zone (O'Keeffe *et al.*, 2009), but whether DA has a role in neurogenesis after agomelatine administration, particularly in the dentate gyrus, needs further investigation (AlAhmed & Herbert, 2010). A recent study indicates that repeated restraint stress decreased total dendritic length, reduced total branch points and decreased dendritic surface, all of which could be inhibited by daily administration of agomelatine (Grillo *et al.*, 2015). Moreover, agomelatine may prevent and reverse stress-induced morphological and neurochemical changes caused by glutamate (Grillo *et al.*, 2015); therefore confirming that, like other known antidepressants, its distal mode of action involves neuroplasticity and morphological changes in neurons and synapses that culminate in behavioural and neurochemical homeostasis. Furthermore, studies show that agomelatine treatment reduces the release of glutamate (by 30%) in the rat hippocampus, indicating a dampening of excitatory neurotransmission after agomelatine treatment (Popoli, 2009). Studies have shown that acute agomelatine administration increases BDNF in the prefrontal cortex and chronic administration of agomelatine increases BDNF in the hippocampus in rats (Guardiola-Lemaitre *et al.*, 2014). The role of neuroplasticity has been described in section 2.1.4.2, indicating that agomelatine's mechanism of action on neuroplasticity is beneficial in anxiety disorders.

2.3.2 Side-effects

Since agomelatine's mechanism of action isn't directly associated with an increase in 5-HT levels, the side-effects of agomelatine does not include weight gain, sexual dysfunction, psychomotor agitation, anxiety and serotonin syndrome as is frequently observed with SSRIs and SNRIs (De Berardis *et al.*, 2015). Moreover, the absence of pronounced serotonergic effects may explain why it does not induce a discontinuation syndrome (Harvey & Slabbert, 2014). Furthermore, binding studies indicate that agomelatine has no affinity for adrenergic, histamine, dopaminergic, cholinergic or benzodiazepine receptors (Jhanjee *et al.*, 2010). The above mentioned contributes to a better adverse drug reaction profile and better compliance. However, hepatotoxic effects and elevations of the transaminase enzymes have been reported with agomelatine (De Berardis *et al.*, 2015; Levitan *et al.*, 2015). Therefore, patients on agomelatine should test and monitor their liver function regularly and agomelatine is contra-indicated in patients with impaired liver function as well as patients over 75 years (De Berardis *et al.*, 2015; Levitan *et al.*, 2015). Apart from the hepatotoxicity, the safety profile of agomelatine remains satisfactory (De Berardis *et al.*, 2015). The most common side-effects observed are headaches, dizziness, somnolence, diarrhoea,

nausea, fatigue and insomnia and all of these were reported in the mild-to-moderate range (De Berardis *et al.*, 2015).

2.3.3 Pharmacokinetics

Agomelatine is absorbed rapidly (0.5 – 4 h) and well (80%) after oral administration. However, its bioavailability is relatively low (< 5%) at therapeutic oral doses due to the high first-pass metabolism (De Berardis *et al.*, 2015). Furthermore, agomelatine has a moderate volume of distribution (35 L), a plasma protein binding of 90% - 94% (albumin and alpha 1-acid glycoprotein) and a short plasma half-life time (1 – 2h) (De Berardis *et al.*, 2015). Agomelatine is metabolized by cytochrome P450 (CYP) 1A2, CYP 2C9 and CYP 2C19 enzymes and eliminated via urinary and fecal excretion (De Berardis *et al.*, 2015). Drug interactions may occur with medications that interact with isoenzymes CYP 1A2, CYP 2C9 and CYP 2C19 and may increase/decrease the plasma concentrations of agomelatine (Levitan *et al.*, 2015). Examples include fluvoxamine, a potent CYP 1A2 and moderate CYP 2C9 inhibitor, while CYP 1A2 inhibitors such as ciprofloxacin, amiodarone, mexiletine and zileuton should also be avoided, as well as moderate CYP 1A2 inhibitors such as oestrogens (Levitan *et al.*, 2015). Interestingly, despite its short half-life, it is only dosed once a day and is not prone to causing a withdrawal syndrome after discontinuation, an effect that has been explained by virtue of its short occupancy time needed on $5HT_{2C}$ and MT_1/MT_2 receptors to activate circadic-dependent events in the SCN (Harvey & Slabbert, 2014).

2.4 Animal models in anxiety disorders

Animal models make it possible to investigate the neurobiology and treatment of a neuropsychiatric illness such as depression and anxiety. They offer the possibility of simulating conditions under controlled circumstances that will enable us to study symptoms seen in humans as they develop and to test prospective treatments in a model that closely resembles human physiology, pathophysiology and behaviour (Van der Staay, 2006). Furthermore, the use of novel pharmacological and therapeutic drug entities with unknown biological effects can be tested in these animal models to ascertain if there is value in pursuing their potential therapeutic benefits in humans. For example, and relevant to this study, agomelatine (a registered antidepressant), has shown promise as an anxiolytic agent in animals as well as in patients with depression and GAD (Stein *et al.*, 2008a). However, its exact mode of action in this indication requires further study, particularly how these actions relate to neuroendocrine/neurochemical changes.

2.4.1 Clinical applications of animal models

The focus of preclinical animal models is to establish face validity, construct validity and predictive validity in order to closely resemble the psychiatric disorder and to allow translation to humans (Uys *et al.*, 2003). This is known as the validity criteria of McKinney and Bunny (1969) for an

animal model (Willner, 1984). Face validity includes reproducing the clinical symptoms relevant to humans and construct validity involves the theoretical rational regarding the illness (e.g. alterations to specific bio-markers observed in the illness and the animal model) (Willner, 1984). Predictive validity refers to the ability to predict human response from an animal model and whether treatment may improve the disorder observed in humans (Willner, 1984). Preclinical models have aided in the discovery of the anxiolytic properties of several known and unknown drugs as well as neuropeptide agonists and antagonists (Garner *et al.*, 2009).

Face validity is measured during behavioural testing, the latter being designed to evaluate anxiety-like behaviours based on approach or avoidance tasks (Cryan & Holmes, 2005). For example, animals are subjected to an aversive environment such as the open and elevated arms of the EPM (Cryan & Holmes, 2005). The animal is required to choose whether to explore (approach) the open, exposed arms of the maze, which are potentially dangerous but also potentially rewarding or to stay in the safe, enclosed sections (avoidance) (Bannerman *et al.*, 2014). In the OFT the aversive environment is the lighted centre area of the arena where the inherent fear of predation is exacerbated in the animal. Here avoidance behaviour is expressed as whether the rat spends more time in the centre of the open field or more time against the walls of the OFT which are less stressful (Garner *et al.*, 2009; Gould *et al.*, 2009). In each test increased avoidance indicates anxiety-like behaviour (Garner *et al.*, 2009). This behaviour correlates with anxiety disorders since human anxiety is reflected in behavioural disturbances such as avoidance, escape, non-verbal vocalization and hypervigilance (Palanza, 2001).

2.4.2 Types of animal models

2.4.2.1 Knockout mice

“Knockout” describes an animal, usually a mouse, where a target gene has been deleted so that the product of the mutant gene is not synthesized in the offspring (Crawley, 1999). Knockout mice target specific genes that are purported to be dysfunctional in the human disease, and provide excellent animal models of the behavioural traits characterizing a human genetic disorder (Crawley, 2007). Behavioural anomalies observed in these mice provide quantifiable surrogate markers for the symptoms observed in the human disorder (Crawley, 2007). Thus, such an animal model is deemed as showing good face validity. Prevention or reversal of these behavioural anomalies in these animals can be used as a preclinical endpoint to assess the efficacy of new treatments for a specific genetic-related disease (Crawley, 2007).

Dysfunctional serotonergic neurotransmission is implicated in anxiety disorders (as described in section 2.1.4.4.3), and to investigate the involvement of 5-HT in anxiety disorders, mice

genetically lacking 5-HT_{1A} and 5-HT_{1B} receptors would represent an animal model of anxiety disorders (Zhuang *et al.*, 1999). These receptors are localized on serotonergic neurons where they act as autoreceptors and on non-serotonergic neurons (Zhuang *et al.*, 1999). As a result, the 5-HT_{1A} and 5-HT_{1B} receptors control the tone of the serotonergic system and mediate some of the postsynaptic effects of 5-HT (Zhuang *et al.*, 1999). Homologous recombination is used to generate mice lacking specific 5-HT receptors (Ramboz *et al.*, 1998). Studies have shown that mice lacking the 5-HT_{1A} receptors display decreased exploratory activity, increased fear of aversive environments (open or elevated spaces) and are less aggressive (Ramboz *et al.*, 1998; Zhuang *et al.*, 1999). These results demonstrate that 5-HT_{1A} receptors are involved in the modulation of exploratory and fear-related behaviours and suggest that reductions in 5-HT_{1A} receptor density due to genetic defects or environmental stressors might result in heightened anxiety (Ramboz *et al.*, 1998).

Another knockout model for anxiety is the 5-HT_{2C} knockout mouse (Bagdy *et al.*, 2001). The role of the 5-HT_{2C} receptor in anxiety is evident through numerous studies (Bagdy *et al.*, 2001; Heisler *et al.*, 2007) and is discussed in section 2.3.1. Knockout studies have indicated that mice lacking the 5-HT_{2C} receptor show a decrease in anxiety as measured in the OFT (Heisler *et al.*, 2007) and SIT (Bagdy *et al.*, 2001). A study also found that 5-HT_{2C} knockout mice present a dampening of amygdala CRH release in response to an anxious stimulus (Heisler *et al.*, 2007). Thus, 5-HT_{2C} receptors are critically involved in regulating anxiety-like behaviours (Heisler *et al.*, 2007).

2.4.2.2 Genetic animal models

The Flinders sensitive line (FSL) rats were initially bred from Sprague Dawley rats in order to display hyper cholinergic activity (Overstreet *et al.*, 2005). Researchers observed that these animals displayed enhanced sensitivity to environmental stressors. The Flinders resistant line rats (FRL) do not display this enhanced sensitivity to stress and is regarded as the healthy control of the FSL rat. Stress sensitivity of the FSL rat includes the display of behaviours that resemble human depression (Overstreet *et al.*, 2005), including decreased general activity, changes in REM-sleep and appetite, anhedonia, increased stress responsiveness and enhanced response to antidepressants (Neumann *et al.*, 2011). After these observations, the FSL rats were validated as an internationally approved animal model for depression (Neumann *et al.*, 2011; Overstreet *et al.*, 2005).

However, depression and anxiety are invariably comorbid disorders, while the animal's increased sensitivity to stress makes it suitable as an animal model of anxiety (Liebenberg *et al.*, 2010; Liebenberg *et al.*, 2012). In the latter studies, we observed that FSL rats had lower baseline social interaction compared to FRL rats, which was reversed with chronic treatment of fluoxetine

(Liebenberg *et al.*, 2010; Liebenberg *et al.*, 2012). This observation is consistent with a study from Overstreet and colleagues where increased anxiety was noted in FSL rats when performing certain tasks such as the SIT (Overstreet, 2002). Although the rats exhibited anxiogenic behaviour in the SIT, there were no differences between the FSL and FRL rats in the EPM, which is a classical test of anxiety-like behaviour (Neumann *et al.*, 2011; Overstreet *et al.*, 2005). Furthermore, treatment with benzodiazepines did not differ between the two strains (Neumann *et al.*, 2011). Thus, anxiety is not a prominent feature of the FSL strain (Neumann & Landgraf, 2012; Overstreet *et al.*, 2005). The anxiogenic behaviour in the SIT may suggest that they exhibit enhanced social anxiety (Neumann *et al.*, 2011). These aspects make it a less robust model for evaluating possible novel anxiolytic treatments.

2.4.2.3 Social isolation reared (SIR) animal model

Exposure to adverse and stressful experiences in early-life increases the vulnerability to develop a psychiatric disorder, including anxiety, depression and schizophrenia (Lukkes *et al.*, 2009). Social isolation rearing (SIR), whereby postnatal animals are separated from their littermates immediately after weaning (one animal/cage) for several weeks, is well-recognised as a neurodevelopmental animal model of early-life stress that results in several behavioural alterations related to those observed in humans with early-life stressful experiences (Yorgason *et al.*, 2013). A review by Lukkes *et al.*, 2009 concluded that SIR results in long-lasting increases in anxiety-like behaviour that cannot be reversed by re-socialization, provided that SIR occurred during postnatal day (PND) 21 to 56 (pre- to mid-adolescence). Moreover, socially reared rats which are isolated as adults do not develop anxiety, emphasising a critical time period for adverse early life stress where neurodevelopmental changes can propagate an anxiety disorder (Yorgason *et al.*, 2013). The Social Zeitgeber Theory postulates that stressful or adverse life events lead to changes in the sleep-wake cycle and other circadian rhythms, which then leads to mood disorders (Grandin *et al.*, 2006; McClung, 2013) and possibly anxiety disorders (Brown *et al.*, 1996; Shear *et al.*, 1994), thus making the SIR model suitable for explorative studies relating mood/anxiety to disordered circadian rhythms.

In this study we will use the SIR animal model to emulate a neurodevelopmental abnormality that culminates in late-life bio-behavioural changes, resembling the pathophysiology of anxiety. In our laboratory this model has revealed itself to be a reliable and well validated model presenting with numerous bio-behavioural alterations related to schizophrenia (Möller *et al.*, 2011; Möller *et al.*, 2012; Möller *et al.*, 2013) and depression (Coutts, 2015). However, other laboratories have established its neurochemical and behavioural translation with respect to anxiety (Bledsoe *et al.*, 2011; Lukkes *et al.*, 2012b; Lukkes *et al.*, 2009; Yorgason *et al.*, 2013). Moreover, neurochemical systems involved in the regulation of stress responses (mood and anxiety), such as 5-HT, DA and

CRH are not fully developed until early-to-late adolescence in humans and rodents (Lukkes *et al.*, 2009). The latter emphasises again that this is a critical period in which to evoke earlier life adversity in order to engender psychopathology-related behaviours later in life (Lukkes *et al.*, 2009).

The role of 5-HT in anxiety has been described (see section 2.1.4.4.3) as well as the effects of SIR on 5-HT levels in various regions of the limbic system in rodents (Lukkes *et al.*, 2009). SIR increases 5-HTergic activity in the medial prefrontal cortex such as increased 5-HT_{1A} and 5-HT_{2A} receptor binding (Günther *et al.*, 2008), and increases 5-HT turnover (Brenes *et al.*, 2008) and tissue concentration (Lukkes *et al.*, 2009). Decreased 5-HT levels are also noted in the hippocampus after SIR (Lukkes *et al.*, 2009), which may be related to the neuropathology of anxiety (Lapiz *et al.*, 2003). In the amygdala, specifically the centromedial amygdala complex, increased 5-HTergic activity has been described in various preclinical SIR studies (reviewed by Lukkes *et al.*, 2009), as well as increased 5-HT activity in the BLA (Lehmann *et al.*, 2003). Alterations to DA activity after SIR is seen in the same anxiety-related limbic structures as 5-HT, and there is a clear connection between 5-HT and DA activity in these regions (Lukkes *et al.*, 2009). In the medial prefrontal cortex DA levels are generally decreased but increased in the nucleus accumbens and striatum after SIR (Lukkes *et al.*, 2009; Möller *et al.*, 2013). Behavioural deficits seen in adult rats after SIR may be a consequence of altered interactions between 5-HT and DA in the medial prefrontal cortex and nucleus accumbens (Lukkes *et al.*, 2009). For example, SIR enhances 5-HT in the nucleus accumbens, which facilitates increased DA in this region via presynaptic mechanisms (Lukkes *et al.*, 2009). In the medial prefrontal cortex interactions are also noted, with an increase in 5-HT_{1A} receptor binding and 5-HT turnover observed in the prefrontal cortex after SIR, resulting in inhibited DA release in this region (Diaz-Mataix *et al.*, 2005).

CRH involvement in SIR-induced anxiety is also well-described. Rats exposed to SIR exhibit an increase in CRH type 2 (CRH_2) receptor expressions in the dorsal raphe nucleus (Bledsoe *et al.*, 2011). However, SIR causes no changes to the expression of CRH type 1 (CRH_1) receptors, suggesting that the increase in CRH_2 receptor expression in the dorsal raphe nucleus is responsible for the heightened anxiety states evident in SIR rats (Bledsoe *et al.*, 2011). In fact, CRH_2 receptor antagonists decrease anxiety (e.g. increased open arm entries in the EPM) in SIR rats without an effect in social reared rats (Bledsoe *et al.*, 2011).

Earlier (section 2.1.4.4.1) the key role of GABA-glutamate signalling in anxiety was discussed. Rats subjected to SIR demonstrate an up regulation of $GABA_A$ receptors in the frontal cortex as well as alterations to GABA signalling (Harte *et al.*, 2007; Hickey *et al.*, 2012). Moreover, SIR reduces the total GABAergic interneurons within the hippocampus (Harte *et al.*, 2007) which may

lead to an increase in $GABA_A$ receptor expression, since the expression of these receptors increases in response to chronic $GABA_A$ receptor hypofunction (Hickey *et al.*, 2012). Thus, the up regulation of $GABA_A$ receptors may be a compensatory mechanism in response to a decrease in GABAergic neurotransmission (Hickey *et al.*, 2012). Furthermore, it seems that SIR rats have a reduced function of the $GABA_A$ receptor (Serra *et al.*, 2000). The fact that GABA can reduce anxiety-like behaviours after SIR in the SIT and EPM strengthens the connection between GABA, anxiety and SIR (Wongwitdecha & Marsden, 1996). A previous study in female rats suggests that post-weaning SIR restricts the normal, adaptive activation of the inhibitory GABAergic interneurons in response to a stress-related stimulus within the basolateral amygdala, which may lead to an increased vulnerability to anxiety-related responses in adulthood (Lukkes *et al.*, 2012a). Chronic anxiety-like states in female rats exposed to adolescent SIR may be due to dysregulation of resilience mechanisms involving serotonergic activation of $5\text{-}HT_{2A}$ receptor expressing GABAergic interneurons in the basolateral amygdala (Hale *et al.*, 2010; Lukkes *et al.*, 2012a). In line with the latter studies, although not performed in SIR rats, an earlier study indicated that blocking the $GABA_A$ receptors in the basolateral amygdala results in an increase in anxiety-like behaviours in rats as measured in the SIT (Sajdyk & Shekhar, 1997).

We have earlier shown that SIR induces altered expression of NMDA receptors in the frontal cortex that are differentially modulated by the typical and atypical antipsychotics, haloperidol and clozapine (Toua *et al.*, 2010). Interestingly, SIR decreases glutamate NMDA receptors expression in the striatum and prefrontal cortex, but increased its level in the hippocampus (Zhao *et al.*, 2009). Consistent with those studies, a study showed that SIR remarkably decreases NMDA receptors in the frontal cortex in female rats (Hermes *et al.*, 2011). Glutamate is an important mediator of synaptic plasticity and previous studies have shown that SIR may decrease synaptic plasticity in rats (Hermes *et al.*, 2011).

Although women are more likely to develop anxiety disorders (as previously discussed), preclinical studies are mainly based on male animals (Ter Horst *et al.*, 2012) and there are therefore limited studies available that have used female animals. The strongest argument for not using female rats is that fluctuating sex hormones affect the behaviour of these rats and therefore may influence the results (Ter Horst *et al.*, 2012). However, such studies may provide important insights. For example, SIR in female rats is not dependant on a critical period of development, implying that SIR in female rats may induce anxiety-like behaviour at any age (Lukkes *et al.*, 2009). Pre-clinical studies in other animal models have also confirmed that female rats are more prone to anxiety than male rats (Ter Horst *et al.*, 2012). Differences in the neuroendocrine system have also been observed between female and male rats following SIR, with for example males presenting with high basal levels of ACTH and enhanced release of ACTH and corticosterone following stress, whereas HPA axis dysfunction was not observed in SIR female rats (Weiss *et*

al., 2004). However, another SIR study found that female rats had higher plasma concentrations of corticosterone than males, but no differences were observed in plasma corticosterone between SIR and socially reared rats (Fone & Porkess, 2008). SIR increased social interaction and aggressive behaviour in both female and male rats (Wall *et al.*, 2012), which is in contrast with another study where only male rats presented with increased social interaction after SIR (Ferdman *et al.*, 2007). Another SIR study showed that SIR induces anxiogenic behaviour in the EPM (a decrease in open arm entries) in male but not female rats (Weiss *et al.*, 2004). The inconsistencies in the literature with respect to female behaviour after SIR may be due to their fluctuating oestrous cycle. In fact, females in proestrus phase (high levels of oestrogen and progesterone) have higher levels of corticosterone during resting and stressful situations than females in diestrus phase (low levels of sex hormones) (Ter Horst *et al.*, 2012). The fact that proestrus female rats show less anxiety-like behaviour and longer social interaction time than females in diestrus phase (Ter Horst *et al.*, 2012) highlights the importance of controlling for the menstrual cycle when performing SIR studies in females. Apart from these discrepancies, SIR remains a valid model in evaluating anxiety in female rats since post-weaning SIR has been shown to sensitize the dorsal raphe nucleus-basolateral amygdala system in female rats (Lukkes *et al.*, 2012b). This increases the sensitivity to stress-related stimuli, which may increase susceptibility to develop an anxiety-related disorder later in adulthood (Lukkes *et al.*, 2012b).

In summary, employing SIR animals to model anxiety symptoms is a valid model for neuroscience and pharmacological research as it models not only key behavioural symptoms akin to anxiety, but also embraces many of its known neurobiological constructs.

2.5 Synopsis

Anxiety disorders are characterized by a chronic progression and associated with high rates of comorbidity (Dell'Osso *et al.*, 2010) with a significant increased risk for suicide (Garner *et al.*, 2009). Furthermore, severe anxiety disorders are relentlessly debilitating and some clinicians have compared the impairment of quality of life of these patients to those of patients with schizophrenia (Dell'Osso *et al.*, 2010). The current treatment regimens of anxiety disorders focus on the role of enhanced serotonergic and noradrenergic neurotransmission and the altered function of the GABA-benzodiazepine chloride complex (Garner *et al.*, 2009). Moreover, anxiety disorders require long-term treatment while the above mentioned treatment options present with side-effects that may jeopardise treatment (Dell'Osso *et al.*, 2010; Garner *et al.*, 2009). The frequent partial response to standard treatments reflects unmet clinical need in terms of overall response, remission rates and treatment tolerability (Dell'Osso *et al.*, 2010). Altered circadian rhythms have been shown to be involved in the etiology of a number of anxiety disorders, although few treatments have been developed that target the processes that regulate this system.

Agomelatine, a 5-HT_{2C} antagonist and MT_1/MT_2 agonist, is recognized as a circadian rhythm modulator that may be an important drug for the treatment of anxiety disorders (De Berardis *et al.*, 2015). Studies have shown that agomelatine has anxiolytic properties in rats as well as in humans (Millan *et al.*, 2005; Stein *et al.*, 2008a). Indeed, the above-described synergistic mechanism of action reduces anxiety, corrects altered circadian rhythms (McClung, 2013), improves quality of sleep (Lemoine *et al.*, 2007) as well as enhances neurogenesis (AlAhmed & Herbert, 2010).

Research on anxiety disorders was among the first to show how genetic and environmental variation can change neuronal circuitry (Stein & Nesse, 2011). At the same time, many challenges remain due to the complexity of these conditions (Stein & Nesse, 2011). Thus, much still needs to be uncovered regarding the neurodevelopmental aspects of anxiety disorders and more effective treatment. SIR is a neurodevelopmental animal model in rats that will allow the evaluation of environmental influences on developing an anxiety disorder later in life and how this may be modulated by selected drug therapy (Lukkes *et al.*, 2009). This study will therefore utilize the SIR model in evaluating anxiety-like behaviour as measured in the OFT, SIT and EPM, and how these behavioural changes are related to central neuroendocrine systems linked to anxiety, e.g. corticosterone as well as the effects on GABA and glutamate systems. Thereafter the response to chronic agomelatine treatment on the SIR-induced bio-behavioural changes will be investigated. Finally, we will consider the role of gender in SIR-associated anxiety and how it may influence the response to agomelatine.

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CHAPTER 3

MANUSCRIPT

This chapter presents a concept article for submission to Behavioural Brain Research, an appropriate peer-reviewed scientific journal. The current chapter was prepared according to the instructions to the author for this journal (Addendum D).

The guidelines for the preparation of the article manuscript are outlined on the journal website: http:/www.journals.elsevier.com/behavioural-brain-research/, under “Guidelines for Authors”.

The manuscript title, contributing authors and affiliations will appear on the following page. The abstract, highlights and keywords will be presented on a single page, followed by the main body of the manuscript according to the following structure: Introduction, Materials and Methods, Results and Discussion, Conclusions, Acknowledgements, References, Legends to Tables, Legends to Figures. To benefit the reader all figures have been inserted in the text and not at the end of the manuscript as required by the journal. The heading numbers and page numbers for this chapter will align with the dissertation.

W. Regenass assisted with the design of the study with the help of B.H. Harvey and M. Möller-Wolmarans, conducted the behavioural experiments, undertook the statistical analyses and prepared the first draft of the manuscript. B.H. Harvey and M. Möller-Wolmarans supervised the study and assisted in the interpretation of the study data, as well as finalized the manuscript for publication.

All co-authors granted permission for the article to be submitted for the purpose of the MSc.

Title

The effect of sub-chronic agomelatine treatment on anxiety-like behaviours and plasma corticosterone levels in male and female social isolation reared rats

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Highlights

- Social isolation rearing increased locomotor activity in male and female rats
- Social isolation rearing increased anxiety-like behaviours equally in both genders
- Agomelatine reduced anxiety-like behaviours in both genders
- Anxiety-like behaviours were more resistant to treatment in female rats
- Social isolation rearing decreased corticosterone in both genders

Abstract

Anxiety disorders are severely disabling disorders, often difficult to treat. Delayed onset of action, low remission rates and side-effect concerns of current available treatment complicates treatment and compliance. This emphasizes the need for identifying new biological targets and developing more effective anxiolytics. The anxiolytic efficacy of agomelatine, a melatonin (MT_1/MT_2) agonist and serotonin (5-HT_{2C}) antagonist, has been explored in preclinical and clinical studies. The present study examined its anxiolytic activity in social isolation reared (SIR) rats, a neurodevelopmental animal model of anxiety, as assessed in the open field test (OFT), social interaction test (SIT) and elevated plus maze (EPM). Since glucorticoids follow a distinct circadic rhythm while being causally implicated in anxiety disorders, the effect of SIR and agomelatine treatment on cortiocosterone was determined. Since gender has been suggested to affect illness severity and treatment response, gender differences were also considered. SIR increased locomotor activity in the OFT in both male and female rats, which was reversed by agomelatine in both genders. SIR reduced the time spent in active social interactions in the SIT in both genders. Agomelatine increased social interaction, thus decreasing avoidance behaviour. In the EPM, SIR significantly increased anxiety-like behaviour (less time and entries onto the open arms) in both genders, with agomelatine reversing SIR-related anxiety-like behaviours. Female and male rats submitted to SIR develop anxiety-like behaviours to a similar degree, with response to agomelatine found to be superior in male vs. female rats. SIR decreased plasma corticosterone in both genders, although were higher in females compared to males. Agomelatine had no effect on corticosterone in either gender. In conclusion, SIR is a useful neurodevelopmental animal model of anxiety for assessing drug treatment response. Agomelatine is anxiolytic in this animal model, although gender-related differences in treatment response are evident.

Keywords

Agomelatine, social isolation rearing, female, male, anxiety-like behaviours, corticosterone

3.1 Introduction

According to Darwin's evolutionary theory, anxiety may represent adaptive states that provoke behaviour intended to assist the individual to manage threatening situations (Spielberger, 2010). Anxiety can be defined as an emotional state which includes fear, tension, nervousness and worry, accompanied by physical arousal (Spielberger, 2010). However, prolonged anxiety reflects an unresolved stress-response that is no longer beneficial. Such anxiety should be considered pathological when the manifestations are excessive, uncontrollable, caused by no specific threat and results in changes in behaviour and cognition (American Psychiatric Association, 2013). The latter would include self-centred worry and task-irrelevant thoughts that interfere with attention and performance (Spielberger, 2010). Furthermore, anxiety disorders are characterised by excessive fear and avoidance in response to objects or situations that don't possess true danger for the individual (Shin & Liberzon, 2010).

Anxiety disorders are particularly common in the general population (Shin & Liberzon, 2010), while anxiety is comorbid in many psychiatric disorders (Spielberger, 2010) such as depression (McEvoy *et al.*, 2011) and schizophrenia (Braga *et al.*, 2013), amongst others. Importantly, anxiety disorders adversely affect the prognosis of other medical disorders (Martinowich *et al.*, 2007) as well as the response to treatment (Rommelse *et al.*, 2009; Simon *et al.*, 2004). Indeed depression comorbid in patients with posttraumatic stress disorder (PTSD), for example, is very often treatment resistant (Green *et al.*, 2006). Considering their high comorbidity with other disorders, as well as invariably running a chronic course, anxiety disorders are described as the most disabling of medical disorders (Martinowich *et al.*, 2007). Epidemiological studies also indicate that women are diagnosed twice as often with anxiety disorders (McLean *et al.*, 2011; Ter Horst *et al.*, 2012), while also responding differently to treatment (Ter Horst *et al.*, 2012). Although sex differentiation and its effect on response is important clinically, few preclinical studies have looked at the effect of fluctuating circulating sex hormones and their influence on behaviour and treatment response (Ter Horst *et al.*, 2012). Even so, available animal studies indicate differences in behavioural responses between males and females (Palanza, 2001; Weiss *et al.*, 2004).

γ–Amino butyric acid (GABA) neurotransmission, bolstered by benzodiazepines (Lydiard, 2003), exerts an anxiolytic effect by decreasing the activation of the amygdala, a brain region central to fear and anxiety response (Del-Ben *et al.*, 2012; Paulus *et al.*, 2005). Although benzodiazepines are potent anxiolytics, their use is restricted to the acute treatment of anxiety due to their association with physical and psychological dependence (Davidson, 2001). The chronic treatment of anxiety disorders, on the other hand, includes a wide range of antidepressants such as selective serotonin (5-HT) reuptake inhibitors (SSRIs), 5-HT and noradrenalin (NA) reuptake inhibitors (SNRIs) (Nuss, 2015) and tricyclic antidepressants (TAD) (Zohar & Westenberg, 2000).

The partial 5-HT_{1A} agonist, buspirone, has also been shown to be effective in anxiety disorders (Baldwin *et al.*, 2005). However, non-response to current treatment regimens can be as high as 30% (Heisler *et al.*, 2007), often requiring additional anxiolytic treatment. Furthermore, side-effects like sedation, weight gain and sexual dysfunction often jeopardize compliance and contribute to failed response (Bandelow *et al.*, 2002). Non-response, side effects and poor long-term outcome emphasizes the need for identifying new biological targets for anxiolytic action and the development of novel treatments with improved efficacy.

The GABA/benzodiazepine receptor complex has significant prominence in the development and treatment of anxiety states (Bergink *et al.*, 2004), while neuroendocrine messengers such as oxytocin (Heinrichs *et al.*, 2003), vasopressin (Griebel *et al.*, 2002; Legros, 2001), corticotrophin (McEwen *et al.*, 2012) and glucocorticoids (Heim & Nemeroff, 2001) are realizing an ever increasing role in causation and treatment. Glucocorticoids are important for adaption to stress, while decreased levels seem to be associated with anxiety-like behaviour (Arborelius, 1999). However, the effect of prolonged glucorticoid secretion may be harmful to the central nervous system and other organs (Bermudo-Soriano *et al.*, 2012; Heim & Nemeroff, 2001). Monoamines, especially 5-HT and NA, are important, with anxiety disorders being associated with increased levels of these two neurotransmitters (Leonard, 2004). Also recognized as an important yet poorly defined contributor to anxiety disorders are altered circadian rhythms (McClung, 2013; Sipilä *et al.*, 2010; Tapia-Osorio *et al.*, 2013; Verma *et al.*, 2010). Interestingly, illness susceptibility has also been linked to gender-based differences in circadian rhythms (Ter Horst *et al.*, 2012).

Agomelatine is a recently introduced antidepressant that shows promise as an anxiolytic drug in clinical (De Berardis *et al.*, 2015; Stein *et al.*, 2008; Stein *et al.,* 2012; Stein *et al.,* 2014) and preclinical studies (Guardiola-Lemaitre *et al.*, 2014). Agomelatine is a 5-HT_{2C} receptor antagonist and a melatonin (MT_1/MT_2) receptor agonist, while its primary mode of action has been linked to the re-entraining of disordered circadian rhythms (Guardiola-Lemaitre *et al.*, 2014). Preclinical studies have indicated that 5-HT_{2C} receptor agonism heightens anxiety-like behaviour, whereas antagonizing this receptor is anxiolytic (De Berardis *et al.*, 2015). Furthermore, melatonin has also been associated with anxiolytic effects (De Berardis *et al.*, 2015; Papp *et al.*, 2006), implicating a possible dual role for these receptors in agomelatine's anxiolytic effects (Guardiola-Lemaitre *et al.*, 2014). Although agomelatine has shown anxiolytic effects in preclinical screening tests such as the elevated plus maze (EPM), social interaction test (SIT) (Millan *et al.*, 2005) and open field test (OFT) (Rainer *et al.*, 2012), it has yet to be studied in a translational (pathological) neurodevelopmental animal model of anxiety.

Post-weaning social isolation rearing (SIR) is a well-described neurodevelopmental rat model with face, construct and predictive validity for schizophrenia and depression (Brenes *et al.*, 2008; Fone & Porkess, 2008). Anxiety is highly comorbid in either of the above illnesses (Braga *et al.*, 2013; Judd *et al.*, 1998; McEvoy *et al.*, 2011). Since glucorticoids follow a distinct circadic rhythm (Buckley & Schatzberg, 2005; Kalsbeek *et al.,* 2012) while being causally implicated in anxiety disorders (Arborelius, 1999; Boyer, 2000; Schuder; 2005), we set out to determine whether sub-chronic treatment with agomelatine is able to reverse SIR-mediated corticosterone levels, locomotor activity and anxiety behaviours, as determined in the EPM and SIT. Finally, given the increased interest in ascertaining a possible interaction between treatment response and gender, we also considered whether any sex-related differences are evident in the response to agomelatine.

3.2 Methods and Materials

3.2.1 Animals

A total of 72 Sprague-Dawley (SD) rats (36 males and 36 females) were provided by the Vivarium of the North West University (NWU). This strain shows no spontaneous hyperactivity, which is an important confounding factor when evaluating anxiety-like behaviours linked to locomotor activity, such as the EPM (Weiss *et al.*, 2004). Animals were allocated randomly to groups consisting of 12 rats per group. At weaning, post-natal day (PND) 21, the animals were randomized to either SIR (1 animal per cage) or social rearing (3 animals per cage) for 8 weeks (PND 77) (Möller *et al.*, 2011; Möller *et al.*, 2012; Möller *et al.*, 2013). All the animals were reared in solid bottom cages with corncob bedding. Socially reared animals were housed in polysulphone individually ventilated cages (395 mm (w) x 346 mm (d) x 213 mm (h)) and SIR rats in 230 mm (h) x 380 mm (w) x 380 mm (l) cages. The rats were reared under identical conditions: temperature (21 ± 2 ºC), humidity (50 ± 10%), white light (350 - 400 lux), 12 h light/dark cycle and free access to food and water (Möller *et al.*, 2011).

Male and female rats were housed in separate cages, but in the same room. The isolated animals had visual, auditory and olfactory social contact and no environmental enrichment (Fone & Porkess, 2008). The socially housed animals had environmental enrichment in their cages, in the form of PVC pipes. Animals were only disturbed for cleaning purposes, which consisted of changing the cage once a week and replenishing bedding material. Apart from routine husbandry, animals were not handled during the period before experimental studies commenced. With regards to the specific oestrous cycle of the female rats, since regular handling during the isolation period reduces the effects of SIR and alters the response to stress, vaginal smears were not performed (Fone & Porkess, 2008; Wall *et al.*, 2012). Therefore, the specific oestrous cycle was

not determined in female animals. 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 NWU. All experiments were approved by the AnimCare animal research ethics committee (NHREC reg. number AREC-130913-015) of the NWU. 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-00347-15-S5).

3.2.2 Drug treatment

Agomelatine was provided through a kind sponsorship from Servier, Suresnes, France. The drug was freshly prepared in a suspension with the vehicle, 1% hydroxyethylcellulose (HEC) (pH = 6.4). Agomelatine (40 mg/kg) or HEC was administered intraperitoneally (i.p.) (Banasr *et al.*, 2006) for 16 days from PND 61 (15 days + 1 day to complete behavioural analysis) (Möller *et al.*, 2011; Möller *et al.*, 2012; Möller *et al.*, 2013) at 16:00, as previously described (Coutts, 2015). We (Coutts, 2015) and others (Banasr *et al.*, 2006; Norman *et al.*, 2011; Papp *et al.*, 2006) have found this time of administration to be the most appropriate for therapeutic response. In all instances, an injection volume of 1ml/kg was deployed.

3.2.3 Body weight

The body weights of all animals were measured on PND 21 and subsequently on each day of drug treatment, this to determine the daily dose of agomelatine and to confirm equal development of the animals across all the treatment groups over the study period.

3.2.4 Behavioural testing

Behavioural testing was performed within the first two hours of the dark cycle. Prior to testing all the animals were moved in their home cages to the experimental room and allowed to acclimatize for 15 min before the start of the experiments. All the animals were subjected to the OFT (on day 13 of treatment; PND 74), SIT (on day 14 of treatment; PND 75) and the EPM (on day 15 of treatment; PND 76). The behavioural tests were performed from the least stressful to most stressful in order to reduce the likelihood that the behavioural outcomes of a test would be confounded by the carry-over effects of a preceding test (Weiss *et al.*, 2004). The rats were euthanized (via decapitation) 36 hours after the last behavioural test. The behavioural tests were recorded with a digital camera and scored using EthoVision© XT software (Noldus Information Technology, Wageningen, Netherlands). After each trial and specific behavioural test, the arenas were cleaned with 10% ethanol to remove all odours.

3.2.4.1 Open field test (OFT)

The OFT measures general locomotor behaviour of the animal and is used to ensure that alterations regarding mobility of the animal are not due to the procedures enforced on the animals (i.e. SIR) and/or drug treatment. The OFT was performed as previously described (Liebenberg *et al.,* 2010). Briefly, the rat was placed in the centre of an open field arena ($1m^2$) and allowed to explore for 5 min. The total distance moved (cm) was digitally recorded and scored as an indicator of locomotor behaviour using EthoVision© XT software (Noldus Information Technology, Wageningen, Netherlands). This test was performed under dim red light (40 lux).

3.2.4.2 Social interaction

Two rats of the same gender, treatment group and similar body weight (body weight did not differ by more than 10 g) were placed in an open field arena ($1m^2$) for 10 min (Möller *et al.*, 2011). Socially housed rats were tested with a rat from a different cage, thus all the rats were tested with an unknown conspecific. The arena was illuminated with dim red light (40 lux) with the two rats placed in the centre of the arena. The following social interactive behaviours were recorded: time spent anogenital sniffing, times approaching each other and time spent together. Self-directed behaviour (self-grooming) as well as exploratory behaviour (rearing) was also recorded. The total distance moved (cm) was also measured and served as an indication of locomotor activity during the SIT. The test was performed under dim red light conditions (40 lux) as this promotes social interaction, i.e. decreases anxiety-like behaviour (see review by File and Seth 2003).

3.2.4.3 Elevated plus maze

The EPM apparatus is a plus shaped maze that consists of two open arms, 50 x 10 cm (length x width) and two closed arms 50 x 50 x 10 cm (length x width x height), elevated 50 cm from the ground. The EPM was performed as described previously (Walf & Frye, 2007) under dim white light (10 lux; Walf & Frye, 2007). Rats were placed in the centre zone of the apparatus facing the open arm opposite the investigator and allowed to explore the maze for 5 min under surveillance of a digital camera. Thereafter rats were immediately returned to their home cage and the maze cleaned thoroughly with 10% ethanol to eliminate odour trails. The following behaviours were scored by Noldus EthoVision© XT: time and entries into the open arms; time and entries into the closed arms. These behaviours were expressed as a percentage of entries into open arms (% EOA) and a percentage time spent on the open arms (% TOA). Arm entries were scored only when all four paws entered the arm. A 1 cm transparent Plexiglas rim attached to the open arms prevented the rats from falling off (Carlini *et al.*, 2002).

3.2.5 Corticosterone analysis

The day after the last day of drug treatment (PND 78) the rats were euthanized (via decapitation) with no prior anaesthesia and trunk blood collected in pre-chilled, 4 ml vacutainer tubes (SGVac) containing K_2EDTA solution as anti-coagulant. The blood was centrifuged at 20 000 xg at 4 °C for 10 min and the plasma stored at -80 °C until the day of analysis. On the day of analysis plasma samples were thawed on ice, centrifuged again as described above, and the plasma used for the analysis of corticosterone.

The method of Hariharan *et al.* (1992) and Viljoen *et al.* (2012) was used with minor adjustments. Solid-phase extraction (SPE) was used to quantify corticosterone in the plasma. Briefly, Bond Elut C18 (1CC, 100mg) SPE cartridges was preconditioned with 3 ml methanol and then washed with 3 ml distilled water. Thereafter, 1.5 ml plasma was added to the cartridges and allowed to elute slowly. The cartridges were subsequently washed with 2 ml distilled water, followed by 4 ml of a 20% acetone solution. The SPE cartridges were then air-dried for 10 minutes. Methanol (2 ml) was added to the cartridges to elute the corticosterone and the internal standard (dexamethasone) into glass tubes. The methanol was evaporated under dry air at ± 45°C. The residue in the glass tubes were reconstituted with 125 µl of the mobile phase, and vortexed for 1 min. The glass tubes were then centrifuged for 2 minutes at 20 000 xg. The sample was then pipetted into conical glass inserts inside amber high-performance liquid chromatography (HPLC) vials. The vials were placed into the HPLC auto sampler and 100 µl of the sample was injected.

3.3 Statistical analysis

Graphpad Prism version 6 for windows (Graphpad Software, San Diego, USA) was used for all statistical analysis and graphical presentations. Normality of the data was determined using the Shapiro-Wilk test. Data was analysed by two-way analysis of variance (ANOVA) with respect to gender and rearing conditions to establish whether SIR does in fact engender corticosterone alterations and anxiety-like behaviours in male and female rats. Thereafter a two-way ANOVA was performed with respect to gender and treatment to determine the anxiolytic effects of agomelatine. Where there was an interaction and/or simple main effects found in the ANOVA analysis, it was followed by a Bonferroni post-hoc analysis. In all cases, data was expressed as the mean ± standard error of the mean (SEM), with a p value of < 0.05 deemed statistically significant. Finally, in cases where no interaction and/or simple main effects was observed following the ANOVA or no statistical significance was evident, statistical analysis was subsequently followed by Cohen's d calculations to establish the effect-size and practical significance. Cohen's d indicates the standardized difference between two means, describing medium ($0.5 \geq d < 0.8$), large ($0.8 \geq d < 1.3$) and very large ($d \geq 1.3$) effect sizes.

3.4 Results

3.4.1 Body weight

All groups showed a significant and equal amount of growth over the period of the study, with no significant group differences observed with respect to treatment and rearing condition (data not shown).

3.4.2 The effects of SIR on locomotor activity

3.4.2.1 The open field test (OFT)

The Shapiro-Wilk test indicated that the assumption of normality was true for all the OFT data, so that the ordinary two-way ANOVA could be applied as appropriate.

A two-way ANOVA indicated no significant gender*rearing condition interaction ($F [1, 44] = 1.358$, $p = 0.2501$) with regards to total distance moved. However, a significant main effect with respect to rearing condition was observed ($F [1, 44] = 19.29$, $p < 0.0001$), but not for gender ($F [1, 44] = 0.9627$, $p = 0.3319$).

A large effect-size ($d = 0.85$) and a very large effect-size ($d = 1.67$) was seen between the social vehicle- and the SIR vehicle-treated animals in female (Figure 3-1; left panel) and male (Figure 3-1, right panel) rats, respectively, indicating that SIR tends to increase locomotor activity in either gender.

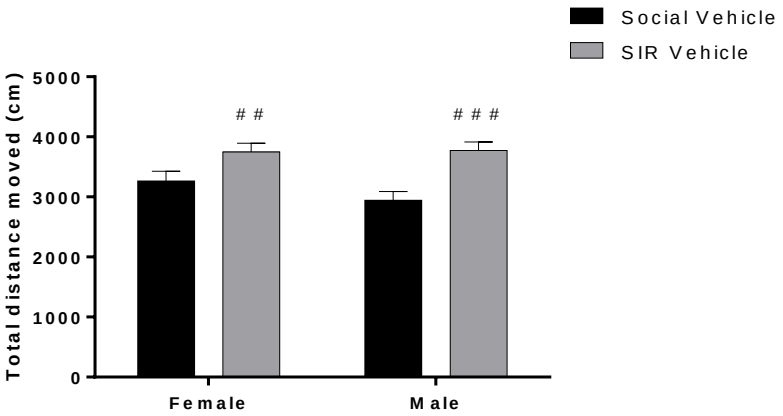

Figure 3-1: The effect of SIR on locomotor activity (total distance moved) measured in the OFT in female (left panel) and male (right panel) rats, ##d= 0.8 ≥ d < 1.3 vs. Social Vehicle, ###d ≥ 1.3 vs. Social Vehicle (Cohen's d value).

3.4.3 The effects of SIR on anxiety-like behaviours

3.4.3.1 Social interaction test

The Shapiro-Wilk test indicated that the assumption of normality was true for the SIT data, except for time spent together. However, an ordinary two-way ANOVA was still applied.

Time spent anogenital sniffing (Figure 3-2A)

A two-way ANOVA of the data indicated no significant gender*rearing condition interaction ($F [1, 44] = 2.449$, $p = 0.1248$) regarding time spent anogenital sniffing. There was, however, a significant main effect regarding gender ($F [1, 44] = 5.140$), $p = 0.0283$) and rearing condition ($F [1, 44] = 52.95$, $p < 0.0001$). This indicates that, regardless of rearing conditions, female rats spent more time engaged in anogenital sniffing compared to male rats.

A Bonferroni post-hoc analysis indicated a significant decrease in anogenital sniffing in both female (Figure 3-2A; left panel; $p < 0.00001$) and male (Figure 3-2A; right panel; $p < 0.0001$) SIR vehicle vs. social vehicle-treated rats.

Times approaching each other (Figure 3-2B)

A two-way ANOVA revealed no significant interaction between gender and rearing condition ($F [1, 44] = 3.638e-007$, $p = 0.9995$) regarding times approaching each other. However, a significant main effect with respect to rearing condition was observed ($F [1, 44] = 14.82$, $p = 0.0004$), but not a gender effect ($F [1, 44] = 0.7872$, $p = 0.3798$).

A Bonferroni post-hoc analysis revealed a significant decrease in times approaching each other in female SIR vehicle- vs. social vehicle-treated rats (Figure 3-2B; left panel; $p = 0.02$) as well as in male SIR vehicle vs. social vehicle-treated rats (Figure 3-2B; right panel; $p = 0.02$).

Time spent together (Figure 3-2C)

A two-way ANOVA of the data revealed no significant gender*rearing condition interaction ($F [1, 20] = 0.4313$, $p = 0.5188$) with respect to time spent together. However, a significant main effect regarding rearing condition ($F [1, 20] = 40.27$, $p < 0.0001$), but not a gender effect ($F [1, 20] = 0.03949$, $p = 0.8445$), was observed.

A Bonferroni post-hoc test indicated a significant decrease in time spent together in SIR vehicle-treated female (Figure 3-2C; left panel; $p < 0.001$) and male rats (Figure 3-2C; right panel; $p < 0.0001$) compared to social vehicle-treated rats.

Rearing Behaviour (Figure 3-2D)

A two-way ANOVA of the data revealed no significant gender*rearing condition interaction regarding rearing behaviour ($F [1, 44] = 0.1122$, $p = 0.7393$). Nevertheless, a significant main effect was observed regarding the effect of rearing condition on rearing behaviour ($F [1, 44] = 34.94$, $p < 0.0001$). There was no significant gender effect observed ($F [1, 44] = 3.379$, $p = 0.0728$).

A Bonferroni post-hoc analysis observed a significant decrease with respect to rearing (exploratory behaviour), with SIR significantly reducing the time spent rearing compared to social vehicle-treated animals in both female (Figure 3-2D; left panel; $p < 0.0001$) and male (Figure 3-2D; right panel; $p < 0.0001$) rats.

Self-grooming (Figure 3-2E)

A two-way ANOVA revealed no significant gender*rearing condition interaction with respect to self-grooming ($F [1, 44] = 0.06325$, $p = 0.8026$), as well as no rearing condition ($F [1, 44] = 3.075$, $p = 0.0865$) or gender effect ($F [1, 44] = 0.8323$, $p = 0.3666$).

No statistical or practical significance was observed regarding self-grooming in either of the gender groups.

Locomotor activity (Figure 3-2F)

A two-way ANOVA of the data revealed no significant gender*rearing condition interaction ($F [1, 44] = 0.1767$, $p = 0.6763$) with regards to locomotor activity (total distance moved). However, a significant main effect was seen regarding treatment ($F [1, 44] = 142.9$, $p < 0.000$), but no significant gender effects were observed ($F [1, 44] = 0.03947$, $p = 0.8434$).

A Bonferroni post-hoc test indicated that SIR significantly increased locomotor activity in female SIR vehicle vs. social vehicle-treated rats (Figure 3-2F; left panel; $p < 0.00001$) as well as in the male SIR vs. social vehicle-treated rats (Figure 3-2F; right panel; $p < 0.00001$).

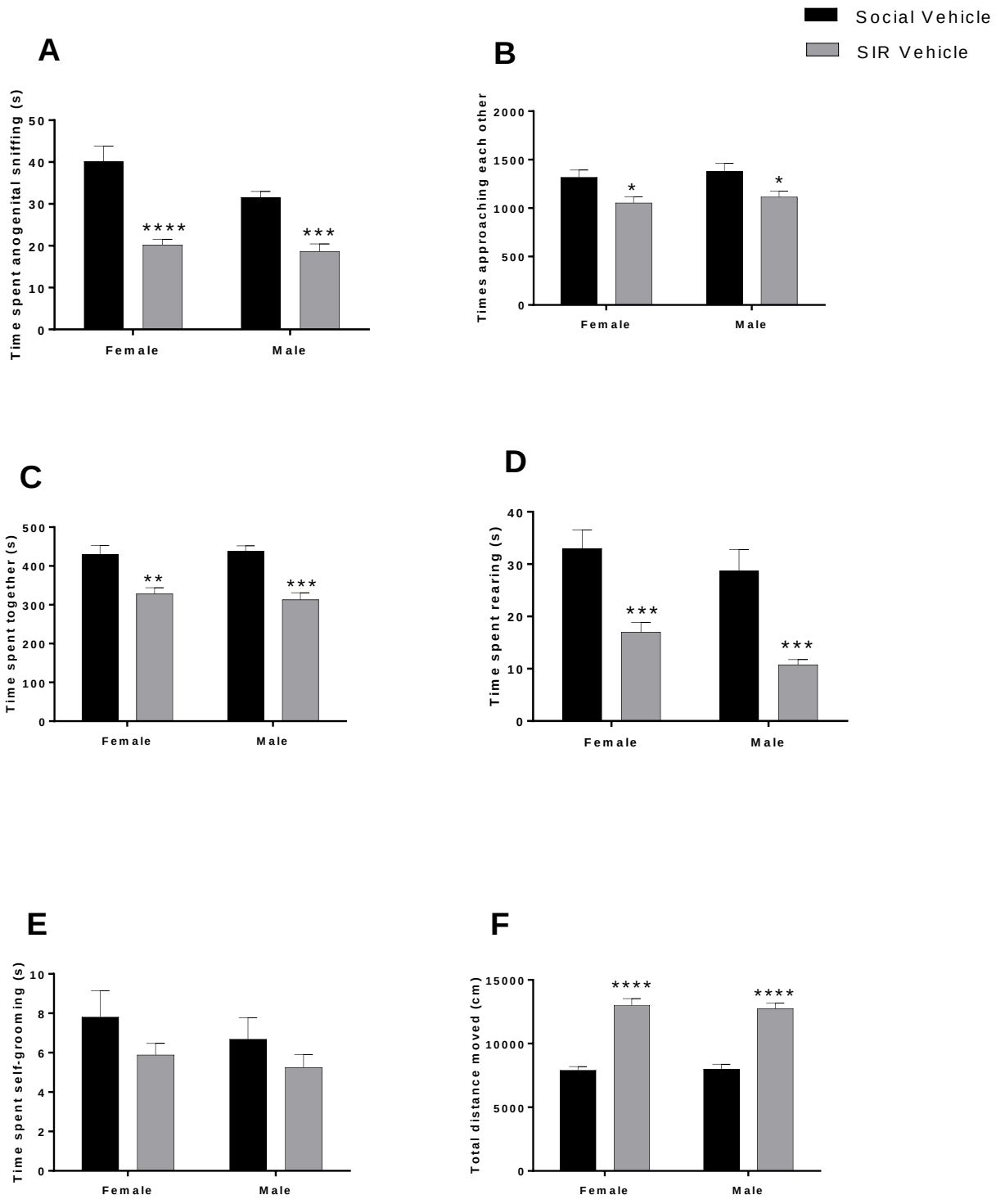

Figure 3-2: The effect of SIR in female (left panel) and male (right panel) rats in the SIT, with respect to A: Time spent anogenital sniffing, B: Times approaching each other, C: Time spent together, D: Time spent rearing, E: Time spent self-grooming, F: Total distance moved. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$ and, ****$p < 0.00001$ vs. Social Vehicle (Two-way ANOVA, Bonferroni post-hoc test)

3.4.3.2 Elevated plus maze

One male rat from the socially housed rats had to be excluded from the statistical analysis because it fell off from the maze during the experiment, reducing the group to 11 rats.

The Shapiro-Wilk test indicated that the assumption of normality was true for all the EPM data, allowing the ordinary two-way ANOVA to be applied as appropriate.

Entries into open arms (% EOA)

A two-way ANOVA indicated no significant gender*rearing condition interaction ($F [1, 43] = 0.3278$, $p = 0.5699$) with regards to the % EOA. Nevertheless, a significant main effect regarding rearing condition was seen ($F [1, 43] = 17.30$, $p = 0.0001$), but not for gender ($F [1, 43] = 0.5509$, $p = 0.4620$).

A Bonferroni post-hoc test revealed that SIR significantly decreased the % EOA compared to socially reared rats in female (Figure 3-3A; left panel; $p = 0.03$) and male rats (Figure 3-3A; right panel; $p < 0.001$).

Time spent on the open arms (% TOA)

A two-way ANOVA indicated no significant interaction between gender and rearing condition regarding the % TOA ($F [1, 43] = 0.1439$, $p = 0.7063$). Although, a significant main effect with respect to rearing condition was seen ($F [1, 43] = 16.43$, $p = 0.0002$), no significant gender effect was observed ($F [1, 43] = 0.1922$, $p = 0.6633$).

A Bonferroni post-hoc analysis revealed a significant decrease in % TOA in SIR vehicle vs. social vehicle-treated female (Figure 3-3B; left panel; $p = 0.02$) and male (Figure 3-3B; right panel; $p < 0.001$) rats.

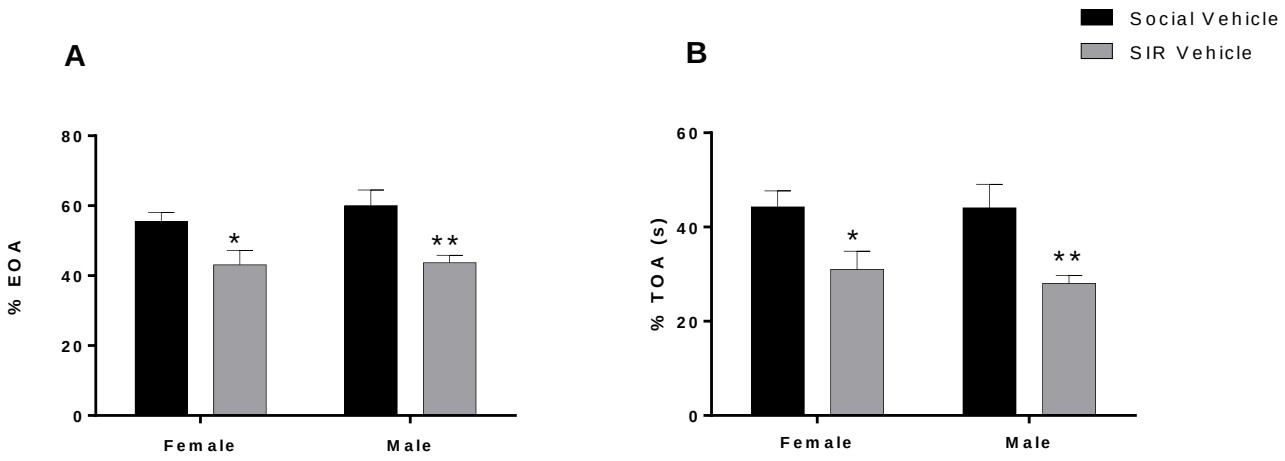

Figure 3-3: The effects of SIR in female (left panel) and male (right panel) rats regarding activity in the EPM, A: % EOA, B: % TOA. *p < 0.05 and **p < 0.001 vs. Social Vehicle (Two-way ANOVA, Bonferroni post-hoc test)

3.4.4 The effect of SIR on plasma corticosterone concentrations

The Shapiro-Wilk test indicated that the assumption of normality was true for all the corticosterone data, so that the ordinary two-way ANOVA could be applied as appropriate.

A two-way ANOVA of the data showed no significant rearing condition*treatment interaction ($F [1, 44] = 0.1037$, $p = 0.7490$) with regards to plasma corticosterone concentrations. However, a significant main effect regarding the effect of rearing condition was observed ($F [1, 44] = 22.11$, $p < 0.0001$), but no significant gender effect ($F [1, 44] = 3.434$, $p = 0.0706$).

A Bonferroni post-hoc test showed a significant decrease in plasma corticosterone in female SIR vs. social vehicle-treated rats (Figure 3-4; left panel; $p < 0.001$), as well as in male SIR vs. social vehicle-treated rats (Figure 3-4; right panel; $p < 0.001$). The difference between female SIR vehicle vs. male SIR vehicle did not reach statistical significance ($p = 0.0794$), although a medium effect-size was observed (Figure 3-4; $d = 0.74$).

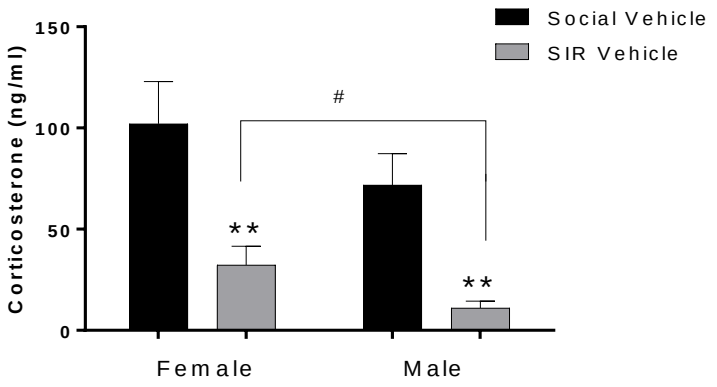

Figure 3-4: Plasma corticosterone concentrations in female (left panel) and male (right panel) rats subjected to SIR or social housing conditions, **p < 0.001 vs. social vehicle (two-way ANOVA, Bonferroni post-hoc test), #d = 0.5 ≥ d < 0.8 (Cohen's d value).

3.4.5 The effects of agomelatine treatment on locomotor activity

3.4.5.1 Open field test (OFT)

The Shapiro-Wilk test supported the assumption of normality for all the OFT data, thus allowing the ordinary two-way ANOVA to be applied as appropriate.

A two-way ANOVA revealed no significant gender*treatment interaction (F [1, 44] = 0.03360), p = 0.8554) with regards to total distance moved. Although a main effect regarding treatment was observed (F [1, 44] = 13.99, p = 0.0005), no significant gender effect was observed (F [1, 44] = 0.1294, p = 0.7208).

Agomelatine significantly decreased the total distance moved in the female SIR rats (Figure 3-5; left panel; p = 0.02) vs. SIR vehicle-treated rats as well as in the male SIR groups (Figure 3-5; p = 0.03) vs. SIR vehicle-treated rats.

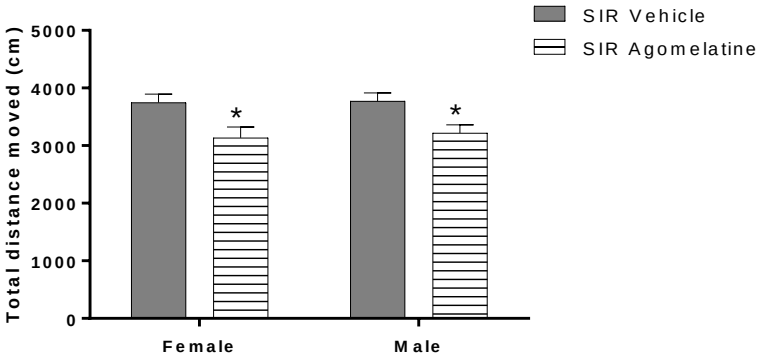

Figure 3-5: The effects of agomelatine treatment on locomotor activity in SIR female (left panel) and male (right panel) rats. *p < 0.05 vs. SIR Vehicle (Two-way ANOVA, Bonferroni post-hoc test)

3.4.6 The effects of agomelatine treatment on anxiety-like behaviours

3.4.6.1 Social interaction test (SIT)

The Shapiro-Wilk test confirmed that the assumption of normality was true for the SIT data, except for time spent together. Nevertheless, an ordinary two-way ANOVA was applied throughout, as described earlier.

Time spent anogenital sniffing (Figure 3-6A)

A two-way ANOVA of the time spent anogenital sniffing indicated a significant gender*treatment interaction ($F [1, 44] = 5.512$, $p = 0.0234$) as well as a significant main effect regarding treatment ($F [1, 44] = 9.100$, $p = 0.0042$), but not a gender effect ($F [1, 44] = 2.276$, $p = 0.1386$).

A Bonferroni post-hoc test showed that agomelatine significantly increased anogenital sniffing in male SIR rats compared to SIR vehicle-treated rats (Figure 3-6A; right panel; $p < 0.0001$), but was without effect in female animals. A large effect-size was seen between male and female SIR agomelatine-treated rats with respect to time spent anogenital sniffing (Figure 3-6A; $d = 0.88$).

Times approaching each other (Figure 3-6B)

A two-way ANOVA of the data indicated no significant gender*treatment interaction ($F [1, 44] = 1.046$, $p = 0.3121$) as well as no gender ($F [1, 44] = 3.883$, $p = 0.0551$) or treatment ($F [1, 44] = 3.624$, $p = 0.0635$) effect regarding times approaching each other.

A medium effect size ($d = 0.7$) was observed in the male rats concerning the ability of agomelatine to increase the times of the two rats approaching each other (Figure 3-6B; right panel).

Time spent together (Figure 3-6C)

A two-way ANOVA indicated no significant gender*treatment interaction ($F [1, 20] = 1.545$, $p = 0.2282$) regarding time spent together. Although a significant main effect concerning treatment ($F [1, 20] = 20.54$, $p = 0.0002$) was observed, no significant gender effect was evident ($F [1, 20] = 0.1571$, $p = 0.6960$).

A very large effect size ($d = 1.38$) was observed regarding agomelatine's ability to increase time spent together in female SIR rats compared to SIR vehicle-treated rats (Figure 3-6C; left panel) as well as in the male SIR rats vs. SIR vehicle-treated rats (Figure 3-6C; right panel; $d = 2.06$). A medium effect-size with regards to time spent together (Figure 3-6C; $d = 0.58$) was observed between male and female SIR agomelatine treated rats.

Rearing Behaviour (Figure 3-6D)

A two-way ANOVA of the data revealed no significant gender*treatment interaction regarding rearing behaviour ($F [1, 44] = 0.3640$, $p = 0.5494$). That said, a significant main effect concerning treatment ($F [1, 44] = 31.18$, $p < 0.0001$) and gender ($F [1, 44] = 11.11$, $p = 0.0017$) was observed.

A Bonferroni post-hoc test indicated that agomelatine significantly increased the time spent rearing in female SIR rats (Figure 3-6D; left panel; $p < 0.001$) as well as in male SIR rats (Figure 3-6D; right panel; $p < 0.0001$) compared to SIR vehicle-treated animals.

Self-grooming (Figure 3-6E)

A two-way ANOVA of the data indicated no significant gender*treatment interaction regarding self-grooming ($F [1, 44] = 1.088$, $p = 0.3026$). No significant main effects regarding treatment ($F [1, 44] = 1.857$, $p = 0.1799$) or gender ($F [1, 44] = 2.900$, $p = 0.0956$) was seen.

No statistical or practical significance was observed regarding agomelatine treatment on self-grooming in either of the gender groups (Figure 3-6E).

Locomotor activity (Figure 3-6F)

A two-way ANOVA of the data revealed no significant gender*treatment interaction ($F [1, 44] = 0.6655$, $p= 0.4190$). A significant main effect was observed regarding treatment ($F [1, 44] = 43.43$, $p < 0.0001$) but not with regard to gender ($F [1, 44] = 1.86$, $p = 0.1786$).

A Bonferroni post-hoc analysis indicated a significant decrease in locomotor activity in the SIR agomelatine-treated rats vs. SIR vehicle-treated rats in both female (Figure 3-6F; left panel; $p < 0.0001$) and male (Figure 3-6F; right panel; $p < 0.00001$) animals.

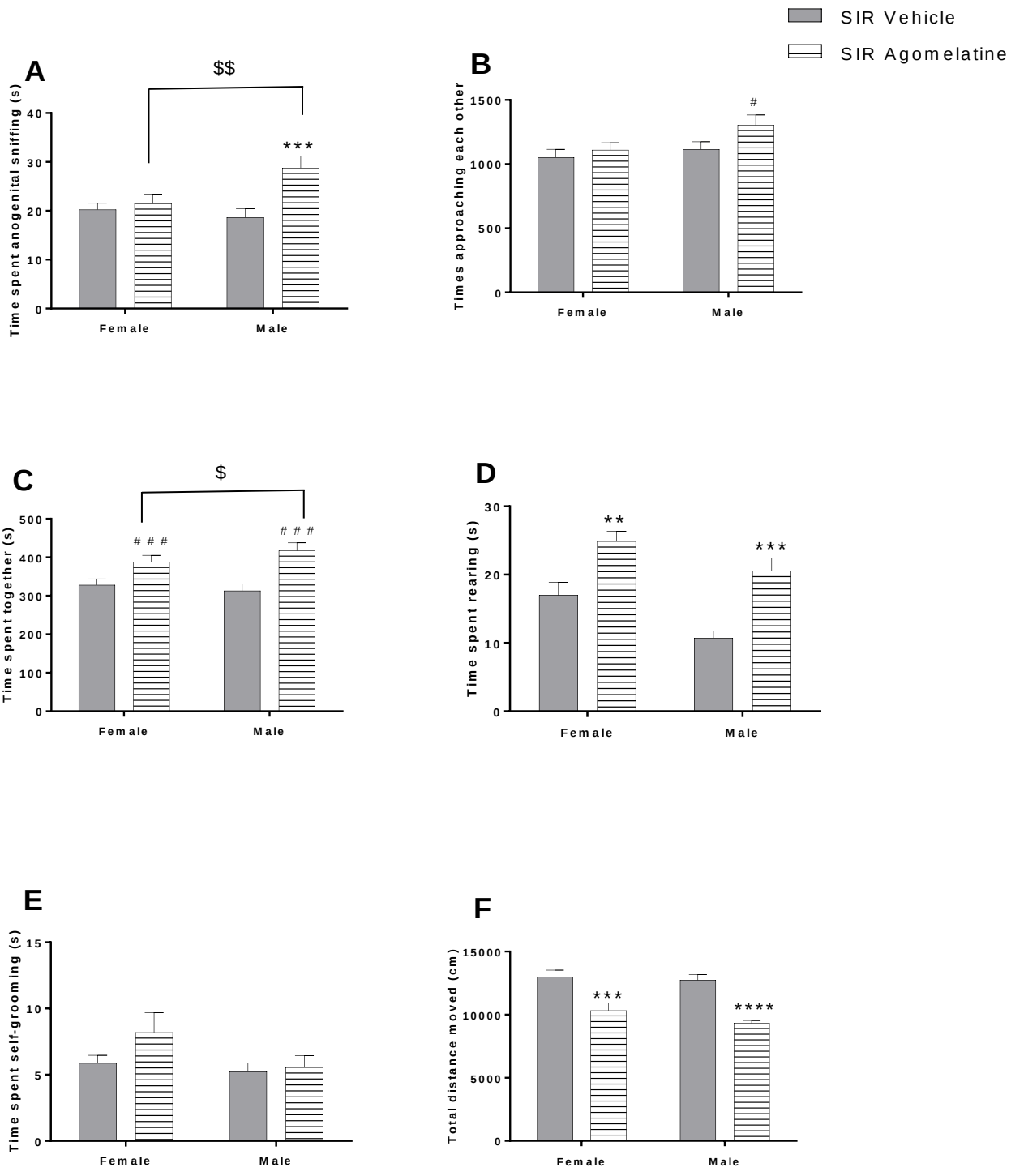

Figure 3-6: The effect of agomelatine treatment in SIR female (left panel) and male (right panel) rats in the SIT, with respect to A: Time spent anogenital sniffing, B: Times approaching each other, C: Time spent together, D: Time spent rearing, E: Time spent self-grooming, F: Total distance moved. *p < 0.05 vs. SIR Vehicle, **p < 0.001, ***p < 0.0001 and ****p < 0.00001 vs. SIR Vehicle (Two-way ANOVA, Bonferroni post-hoc test), $^{\#}$d = 0.5 ≥ d < 0.8 and $^{\#\#\#}$d ≥ 1.3 vs. SIR Vehicle (Cohen's d value), $^{\$}$d =0.5 ≥ d < 0.8 and $^{\$\$}$d = 0.8 ≥ d < 1.3 vs. SIR Agomelatine male (Cohen's d value).

3.4.6.2 Elevated plus maze (EPM)

The Shapiro-Wilk test confirmed the assumption of normality for all the EPM data, thereby allowing the ordinary two-way ANOVA to be applied as appropriate.

Entries into open arms (% EOA)

A two-way ANOVA showed no significant gender*treatment interaction (F [1, 44] = 1.170, p = 0.2853) with respect to the % EOA. However, a significant main effect with respect to treatment was seen (F [1, 44] = 5.486, p = 0.0238), but not with regard to gender (F [1, 44] = 1.598, p = 0.2129).

A very large effect-size (d = 1.03) was observed in the male SIR agomelatine-treated rats compared to their SIR vehicle-treated controls (Figure 3-7A; right panel). A medium effect-size was seen between male and female SIR agomelatine-treated rats (Figure 3-7A; d = 0.69).

Time spent on the open arms (% TOA)

A two-way ANOVA revealed no significant gender*treatment interaction with respect to % TOA (F [1, 44] = 0.02184, p = 0.8832). However, a significant main effect was observed with respect to treatment (F [1, 44] = 4.143, p = 0.0479) but not gender (F [1, 44] = 1.114, p = 0.2971).

A medium effect-size was seen between the female SIR agomelatine-treated rats (Figure 3-7B; left panel; d = 0.53) and their vehicle-treated controls. A large effect-size was observed between the male SIR agomelatine- and SIR vehicle-treated rats (Figure 3-7B; right panel; d = 0.88).

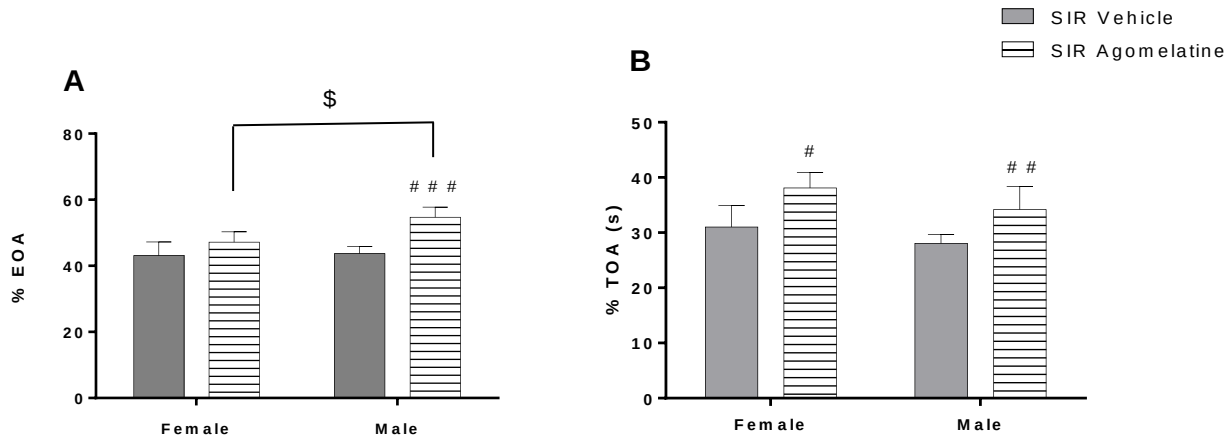

Figure 3-7: The effects of agomelatine treatment on activity in the EPM in female (left panel) and male (right panel) SIR rats. A: % EOA, B: % TOA. $^{\#}d = 0.5 \geq d < 0.8$, $^{\#\#}d = 0.8 \geq d < 1.3$ and $^{\#\#\#}d \geq 1.3$ vs. SIR Vehicle (Cohen's d value), $^{\$}d = 0.5 \geq d < 0.8$ vs. SIR Agomelatine male (Cohen's d value).

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3.4.7 The effects of agomelatine treatment on SIR-induced corticosterone changes

The Shapiro-Wilk test indicated that the assumption of normality was true for all the corticosterone data, so that the ordinary two-way ANOVA could be applied as appropriate.

A two-way ANOVA revealed no significant gender*rearing condition interaction (F [1, 44] = 0.2306, p= 0.6334). However, a significant main effect regarding gender was observed (F [1, 44] = 17.07, p = 0.0002), but no significant treatment effect (F [1, 44] = 0.04779, p = 0.8280).

A Bonferroni test failed to reveal any significant difference in plasma corticosterone following agomelatine treatment in either gender in SIR-exposed rats. However, corticosterone levels was significantly higher in the female SIR agomelatine-treated rats compared to the male SIR agomelatine-treated rats (Figure 3-8; p = 0.0129).

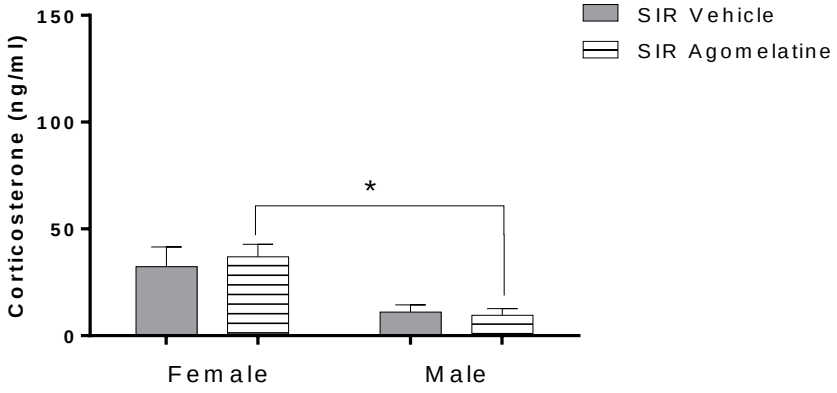

Figure 3-8: Plasma corticosterone concentrations in female (left panel) and male (right panel) SIR-exposed rats, *p < 0.05 vs. female SIR Agomelatine (two-way ANOVA, Bonferroni post-hoc test).

3.5 Discussion

The key findings of this study are that SIR significantly increased locomotor activity in both genders, as measured in the SIT (Figure 3-2F), and that such behaviour was fully reversed by agomelatine treatment (Figure 3-5F). Although locomotor activity was not significantly increased by SIR in the OFT (Figure 3-1), it was significantly reduced in both genders by agomelatine treatment (Figure 3-5). Considering social interaction, SIR significantly reduced anogenital sniffing (Figure 3-2A), approaching (Figure 3-2B) time spent together (Figure 3-2C) and rearing behaviour (Figure 3-2D) in females and males, as well as increased distance moved in both sexes (Figure 3-2F), without markedly affecting self-grooming (Figure 3-2E). Agomelatine significantly reversed SIR-associated changes in anogenital sniffing (Figure 3-6A), time together (Figure 3-6C), rearing (Figure 3-6D) and distance moved (Figure 3-6F) in male cohorts, with such efficacy displayed in females only with respect to rearing (Figure 3-6D) and distance moved (Figure 3-6F).

In the EPM, SIR significantly reduced the % EOA (Figure 3-3A) and % TOA (Figure 3-3B) in both genders, with agomelatine showing a noteworthy effect size in reversing said changes, especially in males (Figure 3-7A and Figure 3-7B). SIR also significantly decreased corticosterone consentrations in females and males compared to socially reared rats (Figure 3-4), although this was not reversed by agomelatine (Figure 3-8).

Locomotor activity: An increase in locomotor activity in the OFT and SIT are two of the most robust observations reported in SIR rats compared to socially housed rats (Brenes *et al.*, 2008; Elliott & Grunberg, 2005; Fone & Porkess, 2008; Varty *et al.*, 2000). This response has been described as a lack in normal habituation in a novel environment, known as neophobia, as well as less time spent resting (Fone & Porkess, 2008). Neophobia has been implicated in anxiety disorders (Griebel *et al.*, 1993), with patients presenting with generalized social anxiety disorders exhibiting high novelty seeking traits (Kashdan & Hofmann, 2008). A neophobia-induced increase in locomotor activity may in turn increase activity in an open field arena, and thus be representative of anxious behaviour. However, neophobia can also indicate avoidance behaviour in a social setting indicating fear and anxiety in rodents and humans (Cavigelli & McClintock, 2003). A clinical study also revealed a greater activation of the amygdala after patients were shown a picture of an unknown person compared to a picture of a familiar person (Schwartz *et al.*, 2003). These results indicate these patients have an increased risk for the development of generalized social phobia (Schwartz *et al.*, 2003). The latter is a psychiatric disorder characterized by persistent and pervasive fear of interacting with strangers and avoiding situations where such interactions are expected (Schwartz *et al.*, 2003). The increase in locomotor activity in the SIT (Figure 3-2F) and the decrease in social interaction behaviour (Figure 3-2A, 3-2B and 3-2C) may suggest that these rats avoided social interaction, further indicating anxiety-like behaviour.

Socially housed animals, on the other hand, habituate faster to a novel environment and subsequently groom themselves instead of exploring their environment (Brenes *et al.*, 2008; Elliott & Grunberg, 2005). However, we did not observe any significant differences with respect to self-grooming between social vehicle-treated rats and SIR vehicle-treated rats in either gender cohort (Figure 3-2E). Another study observed no significant differences regarding self-grooming between SIR rats and socially reared female rats as measured in the SIT (Ferdman *et al.*, 2007). Important to consider is that self-grooming may have two opposite functions in rodent, one being described as ‘comfort’ grooming while the other is ‘stress-evoked’ grooming, since mild stress such as exposure to novel environment has also been known to induce grooming in rats (Kalueff & Tuohimaa, 2005). The exact purpose of self-grooming should be investigated at a deeper level since studies have shown that an increase in self-grooming in a novel environment may reflect either faster habituation to the environment (Brenes *et al.*, 2008; Elliott & Grunberg, 2005), or be indicative of an anxiety-like state (Kalueff & Tuohimaa, 2005). In fact, a previous study from our

group indicated that SIR increased self-grooming in the SIT (Möller *et al.*, 2011), while the current study indicated no significant differences (Figure 3-2E), thus re-emphasizing the importance of further study in this regard.

In both these behavioural measures, agomelatine significantly reversed hyperactivity in both male and female rats (Figure 3-5 and Figure 3-6F). In fact, agomelatine has earlier been shown to reduce locomotor hyperactivity in the OFT in rodents (Loiseau *et al.*, 2006) without affecting total arm entries on the EPM (Loiseau *et al.*, 2006). Agomelatine was also able to reverse locomotor hyperactivity in mice exposed to ultra-mild chronic stress (Boulle *et al.*, 2014). Therefore, locomotor data presented in the SIR model using the OFT and SIT are congruent in ascribing an anxiolytic-like profile for agomelatine.

Social interaction: Earlier we noted the correlation between increased locomotor activity and decreased social interaction in the SIT, suggestive of social avoidance and anxiety. Niesink and Van Ree (1982) proposed that isolating a rat for a short period (4 - 7 days) increases social interaction, compared to socially housed rats, ascribed as a reaction to social deprivation (File & Seth, 2003). However, isolating rodents for a longer period and during a critical window of development (pre- to mid-adolescence) causes lasting changes in behavioural responses (Arakawa, 2003; Lukkes *et al.*, 2009b). SIR causes deficits in social interaction behaviour in both male and female rats (Lukkes *et al.*, 2009a; Lukkes *et al.*, 2009b), and are corroborated in our results with respect to time spent anogenital sniffing, times approaching each other and time spent together (Figure 3-2A; 3-2B; 3-2C). On the contrary, SIR during pre- to mid-adolescence has also been found to *increase* social interaction in male and female SD rats (Wall *et al.*, 2012), while another study observed that SIR during that same period decreased social interaction (Lukkes *et al.*, 2009a). However, Wall *et al.* (2012) evaluated the later effects of SIR on social interaction on PND 49 (late adolescent) with Lukkes *et al.* (2009a) evaluating such behaviour on PND 56 (early adulthood), confirming that behavioural deficits due to SIR are indeed observed during adulthood and *not* adolescence, although the time of behavioural testing is critical to the outcome. This observation is supported in our results (Figure 3-2A; 3-2B; 3-2C). Previous studies in our laboratory also indicated that SIR reduced social interaction behaviours in rats (Möller *et al.*, 2011). Former studies often report that SIR increases aggressive behaviour (File & Seth, 2003; Wongwitdecha & Marsden, 1996), although we did not observe any aggressive behaviour in this study (viz. biting, boxing or threatening partners). A study from Ferdman *et al.* (2007) observed the same trend.

This study further indicated that the SIR rats spent significantly less time rearing compared to socially reared rats in both genders (Figure 3-2D), as well as less time self-grooming in both genders, although not significantly so (Figure 3-2E). Since rearing behaviour could indicate

exploration behaviour (Palanza, 2001), this decrease in a natural rodent behaviour emphasizes the presence of a pathological state. Therefore, the decrease in social interaction observed in SIR rats is not the consequence of spending more time involved in other activities, such as exploring the arena, but rather the presence of inherent anxiety. These findings are in line with another study (File & Seth, 2003). Our social interaction data indicates a pronounced increase in anxiety-like behaviour in SIR rats, a characteristic behaviour evident in this model (Schrijver *et al.*, 2002; Weiss *et al.*, 2004).

From an intervention point of view, agomelatine reversed hyperactivity in female SIR rats (distance moved; Figure 3-6F); with a trend to increase time spent together (Figure 3-6C) and significantly increased the time spent rearing (Figure 3-6D). In the male SIR rats, however, agomelatine significantly increased the time spent anogenital sniffing (Figure 3-6A), time spent together (Figure 3-6C), rearing (Figure 3-6D) while reducing hyperactivity (distance moved; Figure 3-6F). Moreover, it showed a trend to increase the times approaching each other (Figure 3-6B). These social interactive data confirm agomelatine to have noteworthy anxiolytic effects in male animals and are in line with previous results (Millan *et al.,* 2005; Tuma *et al.,* 2005).

Elevated plus maze: The results of this study show that SIR significantly decreased the % EOA (Figure 3-3A) and % TOA (Figure 3-3B) in both genders, compared to their socially reared counterparts. A greater preference for the closed arms and/or an associated lack of exploration in the open arms reflects anxiety-like behaviour (Walf & Frye, 2007), thus supportive of the findings described in the SIT. These results suggest avoidance behaviour in these rats, an important aspect of anxiety disorders (Shin & Liberzon, 2010). As observed in previous studies (Millan *et al.*, 2005; Morley-Fletcher *et al.*, 2011), we found agomelatine to have modest efficacy in reversing SIR-associated anxiety-like behaviours in the EPM. Agomelatine showed a notable trend in increasing the % EOA (Figure 3-7A) and % TOA (Figure 3-7B) in male rats, and a trend to increasing % EOA in female rats (Figure 3-7A).

Corticosterone: A significant decrease in plasma corticosterone in both male and female rats subjected to SIR were observed (Figure 3-4). Behavioural deficits observed in SIR rats have been linked with abnormalities in the endocrine response (Serra *et al.*, 2007). However, the exact action of corticosterone during chronic stress is inconsistent. SIR is well described as a chronic psychosocial stressor with corticosterone in SIR studies shown to be either elevated (Serra *et al.*, 2000), unaltered (Scaccianoce *et al.*, 2006) or decreased (Weiss *et al.*, 2004). Studies suggest that isolating rats in standard cages does not evoke an increase in corticosterone levels (Schrijver *et al.*, 2002), while isolation in more aversive cages, such as cages with wire floors, can increase corticosterone (Heidbreder *et al.*, 2000).

However, other animal models have shown that acute stress increases corticosterone while chronic stress decreases its secretion (Cyr & Romero, 2007; Harvey *et al.*, 2006; Oosthuizen, 2003). The decrease in corticosterone may be due to a dysfunctional hypothalamus–pituitary–adrenal (HPA) axis negative feedback response and a constant hyper secretion of corticotrophin-releasing hormone (CRH) (Baker *et al.*, 1999; Dedovic & Ngiam, 2015). The constant hyper secretion of CRH results in a diminished adrenocorticotropic hormone (ACTH) response and a decrease in the release of corticosterone (Dedovic & Ngiam, 2015; Liberzon *et al.*, 1997). This mechanism suggests that chronic stress, such as SIR, increases the sensitivity of the pituitary to CRH, resulting in decreased corticosterone levels over time (Serra *et al.*, 2005). It therefore appears that SIR, being a chronic stressor, induces a long-term adaptive response and alterations with regards to the responsiveness of the HPA-axis, as evidenced here.

Evidence from clinical studies have shown that anxiety and stress-related disorders such as GAD and PTSD is associated with increased CRH and hypocortisolemia (Arborelius *et al.,* 1999; Boyer, 2000; Schuder, 2005; Steudte *et al.,* 2011). This indicates that anxiety and stress-related disorders may be charactarized by hypocortisolemia. As suggested in our results the hypocortisolemia may be responsible or partly responsible for behavioural deficits observed in patients with anxiety disorders and anxiety-like behaviour in rodents. Of interest is that agomelatine did not reverse SIR-induced hypocortisolemia (Figure 3-8), indicating that agomelatine's anxiolytic effects are unrelated to normalization of the HPA-axis, at least under our conditions of study. Previous studies in tree shrews have indicated that agomelatine treatment reduces corticosterone after chronic stress, although here corticosterone was measured in urine (Schmelting *et al.*, 2014).

The anxiolytic mechanism of agomelatine: This study has for the first time evaluated the anxiolytic activity of agomelatine in a neurodevelopmental animal model presenting with profound anxiety manifestations. The latter anxiety-like behaviour is likely a result of neurodevelopmental changes brought about by adverse events experienced early in life (Fone & Porkess, 2008). Indeed, adverse early life environmental conditions profoundly affect neuronal growth and differentiation and as such impacts on a number of neurotransmitter systems of relevance here, such as serotonin and the HPA axis (Heim & Nemeroff, 2001; McEwen *et al.*, 2012). Early life adversity is also deemed an important risk factor for the later development of psychopathology, in particular anxiety, mood disorders (Lukkes *et al.*, 2009b), and schizophrenia (Fone & Porkess, 2008).

Agomelatine acts to resynchronise disrupted circadian rhythms in animal models and in humans (Lemoine *et al.*, 2007; McClung, 2013; Salva *et al.*, 2007), and which has been ascribed to a dual action at melatonergic and serotonergic receptors (De Berardis *et al.*, 2015; Guardiola-Lemaitre

et al., 2014). Of interest is that SIR did in fact demonstrate an HPA-axis ryhthm abnormality, viz. hypocortisolemia, but that wasn't corrected by agomelatine. Interestingly, 5-HT_{2C} receptor antagonism may directly underlie a reduction in neophobia (Fone *et al.*, 1996), while serotonergic agents such as SSRIs increase neophobia in mice as well as induce anxiety-like behaviours (Griebel *et al.*, 1994), which has immediate relevance to the anxiety-related data presented here. Moreover, acute treatment with a serotonergic agent, such as an SSRI, *reduces* social interaction behaviour in rodents (Bagdy *et al.*, 2001), while this effect can be reversed by pre-treatment with a selective 5-HT_{2C} antagonist. Importantly, the lack of serotonergic effects of agomelatine coupled with prominent 5-HT_{2C} receptor antagonism (Harvey & Slabbert, 2014) may explain agomelatine's reversal of SIR-related deficits in social interaction. An important component of the neuronal effects of agomelatine is a resultant increase in frontal cortical dopamine (Millan *et al.*, 2003; Harvey and Slabbert, 2014) known to play an important role in cognition and social behaviour (Guardiola-Lemaitre *et al.*, 2014). Furthermore, the $5HT_{2C}$ receptor has an important role in the SIT, where 5-HT_{2C} agonists have been found to decrease social interaction and antagonists the opposite effect (File & Seth, 2003). When considering melatonin and the contribution of MT_1/MT_2 receptors, melatonin tends to increase the time spent in active social interaction, although not significantly so (Millan *et al.*, 2005), while increasing open arm exploration in the EPM (Papp *et al.*, 2006). What is important to note is that although agomelatine significantly reduces anxiety-like behaviours in the EPM, this effect is not shared by either melatonin or a selective 5-HT_{2C} antagonist (Millan *et al.*, 2005). This demonstrates the co-dependence of its anxiolytic effects on the synergistic interplay between MT_1/MT_2 and 5-HT_{2C} receptors. Moreover, the anxiolytic effects of agomelatine are not inhibited by a melatonin antagonist (Papp *et al.*, 2006), indicating that stimulating the MT_1/MT_2 receptors is involved but not sufficient to sustain the anxiolytic efficacy of agomelatine.

This study shows much more robust anxiolytic effects for agomelatine in the SIT compared to the EPM, an effect that could relate to the differential role of the 5-HT_{2C} receptor in these behavioural tests. Preclinical studies have shown moderate anxiolytic effects for 5-HT_{2C} antagonists (Millan, 2003; Millan *et al.*, 2005; Rodgers *et al.*, 1997) as well as moderate anxiogenic effects of 5-HT_{2C} agonists (Alves *et al.*, 2004; Martin *et al.*, 1998) in the EPM, while the SIT shows a strong correlation between the 5-HT_{2C} receptor and social interaction behaviour (Bagdy *et al.*, 2001; Dekeyne *et al.*, 2000; Millan, 2003; Millan *et al.*, 2005). The correlation between increased locomotor activity and decreased social interaction noted in the SIT suggests deficits in social cohesion, and thus possibly a bias in agomelatine's response toward a social anxiety disorder. In fact, agomelatine *is* effective in social phobia (Crippa *et al.*, 2010). Nevertheless preclinical (De Berardis *et al.*, 2015; Guardiola-Lemaitre *et al.*, 2014; Millan *et al.*, 2005) and clinical studies have indicated that agomelatine is anxiolytic in patients with generalized anxiety disorders (GAD) (Stein

et al., 2008; Stein *et al.*, 2013; Stein *et al.,* 2014), panic disorders (Fornaro, 2011), and PTSD (De Berardis *et al.*, 2012). There are therefore some inconsistencies and further investigation is needed. Taken together, these results may reveal that agomelatine has differentiating effects on various anxiety disorders as well as in certain anxiety related behavioural tests. Also confounding is its inability to reverse hypocortisolemia induced by SIR, which contradicts its well-described ability to correct biorhythm disturbances (McClung, 2013; Racagni *et al.*, 2011), in particular that associated with the stress-HPA-axis. This finding is corroborated in a previous study in mice, where agomelatine did re-entrain the circadian cycle of body temperature in the mice, but had no effect on corticosterone, ACTH or CRH (Barden *et al.,* 2005). Indicating that the anxiolytic effects of agomelatine is probably not associated with corrections to the HPA axis (Barden *et al.,* 2005).

Gender effects: When overviewing the differences in anxiety-like traits in male and female rats subjected to SIR, no clear differentiation is evident. With regard to hyperactivity, data in the OFT and SIT demonstrated an increase in locomotor activity in both genders (Figure 3-1 and Figure 3-2F). With regard to social interaction, we found a significant main effect between gender and rearing condition with regards to time spent anogenital sniffing (Figure 3-2A). No significant rearing condition*gender interactions or simple main effects concerning gender were observed with respect to time approaching (Figure 3-2B) and time spent together (Figure 3-2C). These findings suggests that female rats, regardless of rearing condition, spent significantly more time anogenital sniffing compared to male rats. Thus, females are more social compared to males, not unusual considering their tendency to “tend and befriend” (Taylor *et al.*, 2000). Thus, male and female rats don’t share the same social interaction behaviours, and which has a biological basis (Taylor *et al.*, 2000), there may be distinct implications for treatment response.

Preclinical studies have observed gender-related behavioural differences in the SIT, indicating a different function in male and female rats in as far as social interaction is concerned (File & Seth, 2003; Wall *et al.*, 2012). However, adult female rats display similar social interaction to that of castrated male rats (Primus & Kellogg, 1990). Gonadal function during puberty is thus required for the development of neural systems underlying social interaction in rats (Primus and Kellogg (1990)). However, our data show that SIR may change these behavioural responses.

Contrary to the social interaction data described above, the EPM data (Figure 3-3A and Figure 3-3B) demonstrated no significant gender-based differences in response to SIR. Here female rats did not experience more profound anxiety than males, as is assumed from the literature (Altemus *et al.*, 2014; McLean *et al.*, 2011). However, gender differences in isolated Wistar rats have been described in the EPM (Da Silva *et al.*, 1996; Weiss *et al.*, 2004), while socially housed female but not male mice exhibit more rearing behaviour (Palanza, 2001), indicating an effect of strain and species (Lukkes *et al.* 2009b). Interestingly, individually housed male mice display more rearing

compared to individually housed females (Palanza, 2001), while our results (Figure 3-2D) describe significant differences in rearing behaviour between genders, irrespectively of rearing conditions.

Although previous studies have shown that SIR male rats have a greater elevation in corticosterone compared to female SIR rats (Weiss *et al.*, 2004), the present study suggests that SIR female rats tend towards a greater elevation in corticosterone compared to male SIR rats (Figure 3-4, d = 0.74). The latter is consistent with the literature, where female rats have been noted to secrete more corticosterone, even in resting conditions (Ter Horst *et al.*, 2012).

Although no significant gender-related differences regarding SIR-associated anxiety-like behaviours were evident, these symptoms appear to be more resistant to agomelatine treatment in female compared to male rats. A medium effect-size was seen in male vs. female SIR agomelatine-treated rats with regards to % EOA (Figure 3-7A; d = 0.69). SIR-induced deficits in social behaviour, such as time spent anogenital sniffing, times approaching each other and time spent together, are more resistant to agomelatine treatment in female than male rats (Figure 3-6A; B; C). A large effect-size was seen between male and female SIR agomelatine-treated rats with respect to time spent anogenital sniffing (Figure 3-6A; d = 0.88) and a medium effect-size with regards to time spent together (Figure 3-6C; d = 0.58). Furthermore, a significant gender-treatment interaction was observed regarding time spent anogenital sniffing (Figure 3-6A), and time spent rearing (Figure 3-6D), implicating gender-based differences in response to agomelatine treatment.

Gender differences should thus be considered when predicting an anxiolytic response to agomelatine treatment. Preclinical and clinical studies implicate oestrogen and progesterone in behavioural responses (Altemus *et al.*, 2014; Ter Horst *et al.*, 2012), while illness susceptibility has also been linked to gender-based differences in circadian rhythms (Ter Horst *et al.*, 2012). On this point, an increase in oestrogen and progesterone has been associated with a decrease in anxiety-like and depressive-like symptoms in female rodents (Hrubá *et al.*, 2012; Palanza, 2001; Ter Horst *et al.*, 2012), while preclinical and clinical studies have implicated significant gender-treatment interactions in the response to psychoactive drugs (Palanza, 2001).

3.6 Conclusion

Post-weaning SIR in both male and female rats presents with significant anxiety-like behaviour, which for the most part were significantly reversed by agomelatine in male and female rats. Yet, contrary to current dogma, female rats did not present with a greater degree of anxiety-like behaviours than males following SIR, while the anxiolytic effect of agomelatine is more

pronounced in male rats. However, since all the female rats were arguably not at the same point in their oestrous cycle, this might have introduced an uncontrolled influence on the effect of agomelatine in the female group. This study also revealed evidence that SIR may in fact decrease corticosterone, which was not reversed with agomealtine treatment, indicating that anxiety and stress disorders might co-present with hypocortisolemia but may not be equally responsive to treatment. This study extends the evidence base for the anxiolytic activity of agomelatine, this time in a neurodevelopmental model. Nevertheless, several remaining questions should be addressed in future studies, in particular unravelling the roles of the respective sex-hormones on anxiety-like behaviours and their influence on treatment response.

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CHAPTER 4

SUMMARY, CONCLUSION AND RECOMMENDATIONS

This chapter will summarize and discuss the results (as presented in Chapter 3 and Addendum A), in order to provide a comprehensive overview of the study. I will take into account the hypothesis and objectives of the study in order to present a final conclusion and to suggest future study.

4.1 Summary of results

It has been established that adversities in early-life increase the vulnerability to develop psychiatric disorders such as anxiety disorders (Heim & Nemeroff, 2001). This study evaluated whether social isolation rearing (SIR), an animal model that resembles early-life adversities experienced in humans (Lukkes *et al.*, 2009), induces anxiety-like behavioural deficits in male and female rats. The study established that SIR does in fact increase anxiety-like behaviours as measured in the open field test (OFT), social interaction test (SIT) and the elevated plus-maze (EPM) (Table 4-1). Subsequently we evaluated the anxiolytic effects of the novel antidepressant and circadian rhythm regulator, agomelatine, noting that agomelatine reduced anxiety-like behaviours induced by SIR (Table 4-1). Although agomelatine has been shown to treat anxiety in patients with depression (Stein *et al.*, 2013) as well as reduce anxiety-like behaviours in preclinical studies (Millan *et al.*, 2005; Millan *et al.*, 2003), this is the first assessment of these properties using a translational animal model of anxiety.

Given the increasing interest in the gender-related contribution to psychiatric illness, I also studied the contribution of gender to the presentation of anxiety-like behaviour in this model, and thereafter how gender may affect treatment response to agomelatine. Contrary to the clinical picture where females are more prone to developing anxiety symptoms (Altemus *et al.*, 2014; McLean *et al.*, 2011), the results from this study indicate that female rats did not develop anxiety-like behaviours to a greater degree than males, although moderating these anxiety-like behaviours with pharmacological treatment seems to be more recalcitrant in female rats (discussed in Chapter 3).

I also evaluated the effects of SIR on certain neuroendocrine bio-markers purported to be involved in anxiety disorders and anxiety-like behaviours, viz. corticosterone, γ-amino butyric acid (GABA) and glutamate. However, SIR had no significant effect on GABA levels or glutamate N-methyl-D-aspartate (NMDA) receptor density in the frontal cortex of male rats (Table 4-2, Addendum A), although significantly reduced plasma corticosterone levels in females (Table 4-2, Chapter 3).

Despite this, agomelatine treatment presented with a trend to increase frontal cortical GABA and NMDA receptor density in male SIR rats (Table 4-2, Addendum A), but failed to alter plasma corticosterone levels (Table 4-2, Chapter 3).

Table 4-1: Summary of behavioural analysis in male and female Sprague-Dawley rats with regards to treatment and housing conditions, ↑ = increase; ↓ = decrease; ↔ = no change observed. Anxiety-like behaviours were measured in the open field test (OFT), elevated plus maze (EPM) and social interaction test (SIT). Abbreviations: social isolation rearing (SIR); open arm entries (EOA); time in open arms (TOA). (*) indicates a significant change ($p < 0.05$, Two-way ANOVA, Bonferroni post-hoc test), where no (*) is indicated it demonstrates a trend as distinguished by Cohen-d calculations.

Treatment Groups		**Anxiety-like behaviour**									
Gender	Comparison	OFT		SIT						EPM	
		Locomotor activity	Centre entries	Time spent anogenital sniffing	Times approaching	Time spent together	Time spent rearing	Time spent self-grooming	Locomotor activity	% EOA	% TOA
Female	*SIR vehicle vs. Social vehicle*	↑	↔	*↓	*↓	*↓	*↓	↔	*↑	*↓	*↓
Female	*SIR agomelatine vs. SIR vehicle*	*↓	↔	↔	↔	↑	*↑	↔	*↓	↔	↑
Male	*SIR vehicle vs. Social vehicle*	↑	*↑	*↓	*↓	*↓	*↓	↔	*↑	*↓	*↓
Male	*SIR agomelatine vs. SIR vehicle*	*↓	*↓	*↑	↑	↑	*↑	↔	*↓	↑	↑

Table 4-2: Summary of neurochemical and neuroendocrine analysis in male and female Sprague-Dawley rats with regards to treatment and housing conditions, ↑ = increase; ↓ = decrease; ↔ = no change observed. Corticosterone was determined in the plasma of male and female rats. The concentration of γ-amino butyric acid (GABA) and N-methyl-D-aspartate (NMDA) receptor density was determined in the frontal cortex, only in the male rats. (*) indicates a significant change ($p < 0.05$), where no (*) is indicated it demonstrates a trend as distinguished by Cohen-d calculations**.**

Treatment Groups		**Neurochemical analysis**		
Gender	Comparison	Corticosterone	GABA	NMDA receptor density
Female	*SIR vehicle vs. Social vehicle*	*↓		
Female	*SIR agomelatine vs. SIR vehicle*	↔		
Male	*SIR vehicle vs. Social vehicle*	*↓	↔	↔
Male	*SIR agomelatine vs. SIR vehicle*	↔	↑	↑

4.2 Primary objectives with their relevant outcomes

◙ ***To establish the severity of 8 weeks SIR in rats on various anxiety-like behavioural manifestations, as determined in the EPM, SIT and OFT, compared to socially reared control animals***

SIR significantly increased locomotor activity in both genders, possibly relating to neophobia, which may represent anxiety in a novel environment. Social interaction behaviours were significantly decreased in SIR rats compared to socially reared animals in both genders. SIR rats also presented with significantly more anxiety-like behaviour in the EPM, with a decrease in activity on the open arms. A brief overview of these results are presented in Table 4-1 and thoroughly discussed in Chapter 3.

◙ ***To investigate any gender-specific differences in SIR-induced anxiety-like behaviour***

The behavioural tests revealed no gender-specific differences with regards to anxiety-like behaviour, as measured in the SIT and EPM (Table 4-1 and discussed in Chapter 3). No differences between genders was observed regarding locomotor activity, as illustrated in Table 4-1 and discussed in Chapter 3.

◙ ***To investigate whether SIR induced altered release of corticosterone (as determined in plasma), in comparison to socially reared controls and whether these alterations were gender-specific***

SIR altered release of corticosterone in plasma compared to socially housed animals. SIR rats presented with significantly less corticosterone compared to socially housed rats (Table 4-2). This may possibly relate to altered negative feedback caused by chronic stress as described in Chapter 3. Gender-specific differences were observed with regards to SIR-associated corticosterone release, with female rats secreting more corticosterone compared to male rats irrespective of treatment and rearing conditions.

◙ ***To determine whether the observed behavioural and corticosterone alterations induced by SIR (if any) could be reversed by sub-chronic treatment with agomelatine***

Agomelatine reversed the behavioural alterations induced by SIR as seen in the normalization of locomotor activity, an increase in social interaction behaviour and an increase in activity on the open arms of the EPM (Table 4-1 and Chapter 3). However, endocrine (corticosterone) anomalies

induced by SIR were not corrected by agomelatine treatment, as seen in Table 4-2 and discussed in Chapter 3.

◙ ***To investigate any gender specific improvements in SIR-induced bio-behavioural alterations after agomelatine treatment***

Gender differences regarding improvement in SIR-induced anxiety-like behavioural alterations after treatment intervention were observed. A trend was observed that anxiety-like behaviours in SIR female rats were more resistant to agomelatine treatment in comparison to their male counterparts, as discussed in Chapter 3. There were no gender-related differences regarding the corticosterone improvements after agomelatine treatment (Chapter 3).

4.3 Secondary objective and the relevant outcome

◙ ***To investigate evidence for SIR evoked changes in the male rats with regards to NMDA receptor binding and GABA levels in the frontal cortex***

SIR did not alter GABA release or NMDA receptor density compared to socially reared animals (Table 4-2, Addendum A). Agomelatine did show a trend to increase the concentration of GABA as well as the density of NMDA receptors in the frontal cortex in SIR male rats (Table 4-2, Addendum A).

4.4 Recommendations

While the current study supports previous findings regarding the anxiogenic effects of SIR, as well as the anxiolytic activity of agomelatine, albeit this time in a translational animal model, there are several remaining questions which should be addressed in prospective studies. These are discussed below:

- Although agomelatine did show anxiolytic effects in the SIR model, it would be valuable to assess its efficacy relative to a known anxiolytic e.g. a benzodiazepine, especially since previous studies have shown that benzodiazepines may decrease anxiety-like behaviours after SIR (Haller & Halász, 1999; Wongwitdecha & Marsden, 1996).

- Although this study did indicate a difference between male and female treatment response with regards to agomelatine, the exact mechanism was not investigated. While we have only been able to speculate that sex-hormones may be the driver of these differences, future studies should investigate the precise role of these hormones in agomelatine treatment response.

- Future studies should investigate the effects of the sex hormones (oestrogen and progesterone) on female behaviour and how this may influence susceptibility in developing an anxiety-like behaviour, and its influence on symptom severity.

- While monoamines and other neurotransmitters may have a prominent role in anxious behaviour, it is imperative that the contribution by neuropeptides such as oxytocin, vasopressin and endogenous opioids are carried out. Indeed, hormones such as oxytocin play a dominant role in the biobehavioural mechanism of social interaction (Taylor *et al.*, 2000), which in turn determines how the animal will respond to aversive conditions.

- Since a genetic component underlies the development of an anxiety disorder (Garner *et al.*, 2009), studies investigating the anxiolytic effects of agomelatine in a genetic animal model of depression with anxiety, such as the Flinders sensitive line (FSL) rat (Overstreet, 2002; Overstreet *et al.*, 2005) may be of value. Since depression is often co-morbid with anxiety, such a model would provide confirmation of its anxiolytic capabilities in a typical clinical scenario.

- Anxiety disorders have an early onset and persist into adulthood (Kessler *et al.*, 2005; McEvoy *et al.*, 2011), indicating that treatment should focus on pharmacotherapy in the youth. Thus, agomelatine treatment should also be investigated in rats from post-natal day (PND) 21 to PND 28 which corresponds to pre-adolescence, as well from PND 28 to PND 34, corresponding to early adolescence (Lukkes *et al.*, 2009). This work could offer important insights on agomelatine response in children and adolescents, and its possible effect in the young, developing brain.

- Agomelatine is a 5-HT_{2C} antagonist and a melatonin (MT) receptor agonist and activity at both these receptors has been suggested to be involved in its antidepressant and anxiolytic properties (Racagni *et al.*, 2011). Other studies have suggested its anxiolytic effects can be linked to an antioxidant effect (Aguiar *et al.*, 2013; Guardiola-Lemaitre *et al.*, 2014), an increase in brain-derived neurotrophic factor (BDNF) (Guardiola-Lemaitre *et al.*, 2014) and neural proliferation (AlAhmed & Herbert, 2010; Banasr *et al.*, 2006), and most importantly its ability to resynchronize disrupted circadian rhythms (Lemoine *et al.*, 2007; McClung, 2013; Salva *et al.*, 2007). Thus, further research is warranted to investigate how these mechanisms may be involved in the anxiolytic effects of agomelatine.

- Unfortunately, in this study we could not evaluate the effect of SIR and agomelatine treatment on GABA transmission and NMDA receptor density in the frontal cortex of female rats due to financial aspects. Since the data suggest differences regarding response to treatment between males and females, and agomelatine did have an effect on GABA and NMDA receptors, this could possibly be valuable and attribute to our understanding of how males and females respond differently to treatment and should be investigated in the future. Although this study was unsuccessful in delineating a role for GABA and/or glutamate pathways in SIR and the response to agomelatine, further dedicated studies with these specific objectives in mind should be pursued in the future.

4.5 Novel findings and Conclusion

This study demonstrated that SIR is a robust and reliable neurodevelopmental animal model for anxiety and useful in drug development studies to assess anxiolytic properties. However, we were unable to demonstrate the involvement of altered GABA and glutamate signalling in the genesis of these behaviours. Yet, since altered NMDA receptor binding has been noted in SIR rats (Toua *et al.*, 2010), we believe further work in this regard is needed. On that note, a single animal model cannot accurately resemble every aspect of an anxiety disorder.

This study also revealed that corticosterone may not necessarily be the most accurate bio-marker to evaluate the effect of chronic stress, for example SIR, on the hypothalamic-pituitary-adrenal (HPA) axis. As described in Chapter 3, chronic stress may alter the negative feedback mechanism on the HPA axis, resulting in decreased corticosterone concentrations which in turn causes defective responses to stress.

Considering treatment intervention, this study established that agomelatine has pronounced anxiolytic effects in a neurodevelopmental animal model of anxiety. This study did not explore all the possible mechanisms how agomelatine may exert these anxiolytic effects, although it was apparent that agomelatine did not have an effect on SIR-induced changes in plasma corticosterone. Although agomelatine did show a trend to increase GABA transmission and decrease NMDA receptor density, SIR associated changes in these bio-markers could not be demonstrated/ This complicates and at best nullifies any attempt at linking these pathways to how agomelatine induces its anxiolytic effects. In fact, earlier studies have already described a role for glutamate in the response to agomelatine (Popoli, 2009; Tardito *et al.*, 2010). Finally, this study revealed that gender differences may predict the anxiolytic treatment response to agomelatine.

4.6 References

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ADDENDUM A

ADDITIONAL RESULTS

This addendum contains and discusses additional results obtained during the study but those are not presented in Chapter 3, and are provided as additional supporting material for the study. Initially the study was destined to include various behavioural tests as well as neurochemical data, but for reasons that will be disclosed some of these data sets were excluded from the concept article.

The open field test (OFT) can be used to assess locomotor activity (viz. distance moved) and anxiety behaviour (viz. time in the centre arena). Although locomotor data obtained from this test was effectively deployed in Chapter 3, the test failed to demonstrate the same anxiety-like behavioural observed in the elevated plus maze (EPM) and in the social interaction test (SIT), and used in the preparation of the article (Chapter 3). Since the goal of the concept article is to present data that may be publishable, this data was deemed unsatisfactory and henceforth exclude from the article (Chapter 3). For completion sake, these data will be presented and briefly discussed in this addendum.

Similarly, the neurochemical analysis (frontal cortical GABA concentration and NMDA receptor density) did not complement the focus of the article (Chapter 3) and is thus also presented in this addendum. It should be noted that quantification of GABA and NMDA receptor density was only undertaken in male rats, as was explained in Chapter 4.

Addendum A will thus consist of:

- Additional results as measured in the OFT
- The effects of SIR and agomelatine treatment on frontal cortical GABA concentrations, in the male rat
- The effects of SIR and agomelatine treatment on NMDA receptor density in the frontal cortex, in male rats

5.1 Introduction

Patients with anxiety disorders have the tendency to overestimate “dangerous” situations linked to a possible dysfunctional bias in emotional processing (Garner *et al.*, 2009). Consequently, anxiety disorders are characterised by excessive fear and avoidance in response to objects or situations that do not harbour true danger for the individual (Shin & Liberzon, 2010). This trait is used for developing behavioural tests for anxiety in animals, where the increase in avoidance behaviour reflects greater anxiety-like behaviours (Cryan & Holmes, 2005; Garner *et al.*, 2009). The OFT relies on the rodent’s natural aversion for well-lit open spaces where there is a risk of predation. Thus, avoidance behaviour towards the centre arena of the OFT is regarded as anxiety-like behaviour.

At a neurobiological level, these anxiety-like behaviours involve GABAergic and glutamatergic transmission. Moreover, an increase in GABAergic activity results in a decrease in anxiety-like behaviour as shown in various animal models of anxiety (Leonard, 2004; Nuss, 2015), while the blockade of the GABA receptor induces anxiety (Leonard, 2004). The inhibitory actions of GABA are opposed by the excitatory effects of glutamate, resulting in an increase in anxiety-like behaviours (Bergink *et al.,* 2004).

5.2 Animals and Drug treatment

The same animals were used for the data presented in Chapter 3 and the addendum. Briefly, male and female Sprague-Dawley (SD) rats (36 males and 36 females) were provided by the Vivarium of the North-West University. On post-natal day (PND) 21, the animals were randomized to either SIR (1 animal per cage) or social rearing (3 animals per cage) for 8 weeks (PND 77), as described in Chapter 3 (Möller *et al.*, 2011; Möller *et al.*, 2012; Möller *et al.*, 2013). 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 NWU. All experiments were approved by the AnimCare animal research ethics committee (NHREC reg. number AREC-130913-015) of the NWU. 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-00347-15-S5).

Drug treatment with agomelatine continued for 16 days (from PND 61), with dosing at 16:00 based on earlier work in our laboratory (Coutts, 2015) as well as others (Banasr *et al.*, 2006; Norman *et al.*, 2011; Papp *et al.*, 2006). Agomelatine (40 mg/kg) or the vehicle, 1% hydroxyethylcellulose (HEC), was freshly prepared daily and administered intraperitoneally (i.p.) (Banasr *et al.*, 2006).

5.3 Behavioural testing

Following sub-chronic vehicle or agomelatine treatment, the animals were subjected to three behavioural tests on consecutive nights; the OFT described below, as well as the social interaction test (SIT) and the elevated plus maze (EPM) described in Chapter 3. Behavioural tests were performed within the first two hours of the dark cycle, thus between 18:00 and 20:00. The animals were moved in their home cages to the experimental group prior to testing. The behavioural tests were performed from the least stressful to the most stressful to reduce the likelihood that the behavioural outcomes of the one test would be confounded by the carry-over effects of a preceding test (Weiss *et al.*, 2004). The behavioural tests were recorded digitally and scored using Noldus EthoVision©. After each trial and behavioural test the arenas were wiped with 10% ethanol to remove all odour trails.

5.3.1 Open field test (OFT)

The set-up of the OFT is described in Chapter 3. In addition to measuring locomotor activity in the OFT (as described in Chapter 3), anxiety-like behaviour can also be assessed by scoring the time spent in the centre zone of the arena (Liebenberg *et al.*, 2010).

5.4 Neurochemical analysis

5.4.1 Sample preparation for neurochemical analysis

After euthanasia (via decapitation), the whole brain was removed and placed in ice-cold saline. The brain was dissected into the right and left cerebral hemispheres. Thereafter the olfactory bulb was removed, the frontal cortex identified using the Paxinos brain atlas and dissected out with the corpus callosum anterior tip being the external limit (Paxinos *et al.,* 1980; Toua *et al.,* 2010). The frontal cortices were macro-dissected out on an ice-cooled dissection slab, snap frozen in liquid nitrogen and stored at -80°C until the day of analysis, as described previously (Harvey *et al.*, 2006).

5.4.2 GABA analysis

GABA levels in the frontal cortex were estimated according to the method described by Harvey *et al.* (2002). This method and HPLC conditions are thoroughly described in Addendum B. On the day of analysis approximately 10 mg of the frontal cortex of each rat was placed into separate Pony vials. 1 ml Ice-cold 0.05 perchloric acid ($HClO_4$) was added to the vials and thawed. The tissue was ruptured by sonication (2 x 12 seconds, at amplitude of 14 µ) and the tubes were allowed to stand on ice for 20 minutes to complete perchlorate precipitation of protein and extraction of neurotransmitters. Subsequently, the samples were centrifuged at 4 °C for 20 min

at 20 000 xg. It should be emphasized that during the preparation of the supernatant, all vials were kept on ice. The pH of the samples was adjusted to approximately 9.0 by adding two drops of 10 M potassium acetate. The supernatant (200 µl) was placed in amber Eppendorf vials whereafter 20 µl of 5 µg/ml DL-Homoserine (internal standard) was added to the sample and vortexed. Subsequently, 50 µl was then pipetted into an insert and placed into an HPLC vial, and the latter placed into the HPLC auto sampler. The software's injector program was programmed for pre-column orto-phtaldialdehyde derivatization and a final sample of 10µl was injected onto the HPLC column. The mobile phase consisted of 0.1 M Na_2HPO_4, 0.13 mM Na_2EDTA and 28% methanol (pH 6) (Harvey *et al.*, 2002).

5.4.3 NMDA receptor density analysis

The determination of NMDA receptor binding was done by using radioligand binding techniques. Radioligand binding studies are based on the following principles: a chemical with high affinity for a specific receptor (NMDA) is marked radioactively with tritium [^{3}H]. The brain homogenate is incubated with the radioligand at a certain temperature until equilibrium is reached. The total amount of specific radioligand that binds per milligram of the brain sample is an indication of receptor density (Enna, 1984).

NMDA receptor binding was determined using a method previously described by Harvey *et al.* (2004). The brain tissue (2 pooled frontal cortices) was homogenized in excess HTS buffer (5 mM HEPES/4.5 mM Tris, pH = 7.8) with a Polytron homogenizer (setting 6 for 20 seconds). The suspension was centrifuged at 20 000 xg for 20 min at 4 °C. The supernatant was decanted; excess buffer was added to the pellet and centrifuged again. The resulting pellet was re-suspended again in excess buffer and centrifuged for 20 min at 20 000 xg. The pellet was now re-suspended in 60 volumes buffer. The homogenate was kept on ice throughout the procedure and 50 µl of this final suspension was used to determine protein, according to the Bradford method.

Total binding was determined by adding 300 µl of the brain homogenate, glycine (50 µl 300 µM) and l-glutamate (50 µl 100 µM) to activate the receptor channel. Finally, 50 µl of the respective [^{3}H]-MK801 concentration was added as well as 50 µl buffer to a final incubation volume of 500 µl. Non-specific binding was determined by adding 300 µl brain homogenate, 50 µl of 300 µM glycine, 50 µl of 100 µM l-glutamate, 50 µl of 20 µM MK801 and 50 µl of the respective [^{3}H]-MK801 concentration. The radioligand ([^{3}H]-MK801) was added in ten concentrations ranging from 0.1 nM to 20 nM. These mixtures were incubated in a shaking water bath for 90 min at 25 °C. The reaction was terminated by rapid filtration through Whatman GF/B filters pre-soaked in buffer. The filters were rinsed twice with 4 ml ice-cold HTS buffer and placed in polypropylene

tubes, with 4 ml scintillation fluid. The binding data were analysed using Graphpad Prism version 6 for windows (Graphpad Software, San Diego, USA) to obtain values for the maximal number of binding sites (B_{max}; expressed as fmol/mg protein) and the binding affinity (K_d; expressed in µM) (Harvey *et al.*, 2004).

5.5 Statistical analysis

Graphpad Prism version 6 for windows (Graphpad Software, San Diego, USA) was used for all statistical analysis and graphical presentations. Normality of the data was determined using the Shapiro-Wilk test. Data was analysed by two-way analysis of variance (ANOVA) with respective to gender and rearing conditions. Thereafter a two-way ANOVA was done between gender and treatment. Where there was an interaction and/or simple main effects found in the ANOVA analysis, it was followed by a Bonferroni post-hoc analyses. In all cases, data was expressed as the mean ± standard error of the mean (SEM), with a p value of < 0.05 deemed statistically significant. When comparing only two data points and where normality was not violated, a Student t-test was used, under Welch's correction. When the assumption of normality was not confirmed, a non-parametric Mann-Whitney t-test was performed. Statistical analysis was followed by Cohen's d calculations in order to establish the practical significance of effect magnitude where only a trend towards significance was apparent. Cohen's d is an effect size used to indicate the standardized difference between two means, where effect sizes were considered medium when $0.5 \geq d < 0.8$, large when $0.8 \geq d < 1.3$ and very large when $d \geq 1.3$.

5.6 Results

5.6.1 Open field test (OFT)

The Shapiro-Wilk test indicated that the assumption of normality was true for all OFT data, so that the ordinary two-way ANOVA could be applied as appropriate.

5.6.1.1 The effect of SIR on anxiety-like behaviour in OFT

A two-way ANOVA of the data showed no significant rearing condition*treatment interaction ($F [1, 44] = 1.329$, $p = 0.2553$) with regards to the time spent in the centre arena of the OFT. However, a significant main effect regarding the effect of rearing condition was observed ($F [1, 44] = 7.992$, $p = 0.0070$) although no significant gender effect was seen ($F [1, 44] = 2.029$, $p = 0.1614$).

A Bonferroni post-hoc test indicated a significant increase in time spent in the centre arena in male SIR vs. social vehicle-treated rats (Figure 5-1; right panel; $p = 0.04$).

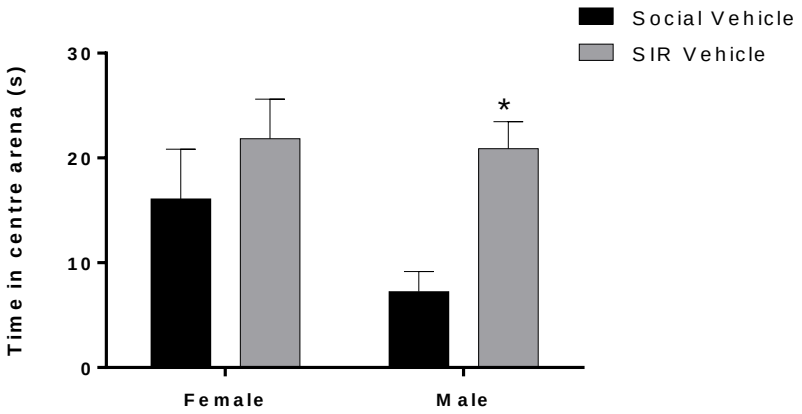

Figure 5-1: The effect of SIR on time spent in the centre arena as measured in the OFT in female (left panel) and male (right panel) rats *p < 0.05 vs. social vehicle-treated animals (two-way ANOVA, Bonferroni post-hoc test).

5.6.1.2 The effect of agomelatine treatment on SIR-induced anxiety-like behaviour in OFT

A two-way ANOVA revealed no significant gender*rearing condition interaction (F [1, 44] = 2.210, p = 0.1443) with respect to time spent in the centre arena of the OFT. Nevertheless, a significant main effect regarding rearing condition was seen (F [1, 44] = 9.658, p = 0.0033), but no significant gender effect (F [1, 44] = 3.242, p = 0.0786).

A Bonferroni post-hoc analysis showed that agomelatine significantly decreased the time spent in the centre arena in the male SIR vs. SIR vehicle-treated rats (Figure 5-2; right panel; p < 0.001).

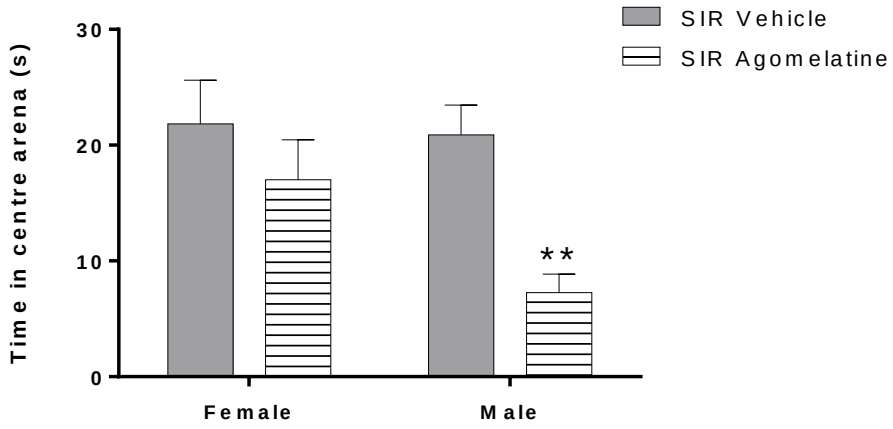

Figure 5-2: The effect of agomelatine treatment in SIR female (left panel) and SIR male (right panel) rats with regards to time spent in the centre arena in the OFT, *p < 0.001 vs. SIR vehicle (two-way ANOVA, Bonferroni post-hoc test).

5.6.2 GABA

5.6.2.1 The effect of SIR on GABA concentrations

The Shapiro-Wilk test indicated that the assumption of normality was true for all the GABA data. A Student's t-test revealed no statistical significant differences between social vehicle vs. SIR vehicle-treated animals (Figure 5-3; p = 0.8915).

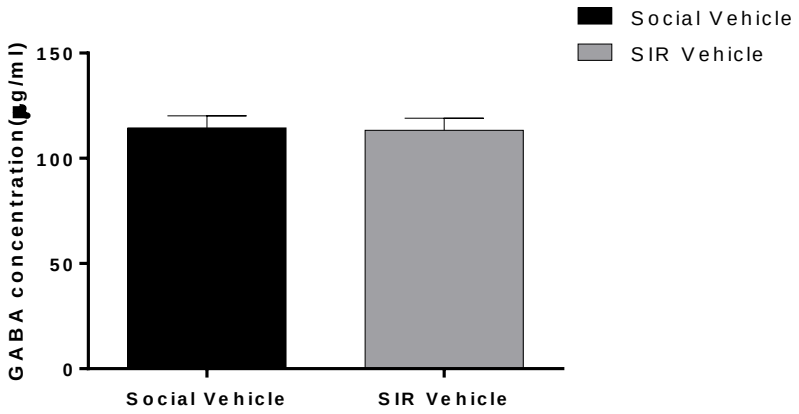

Figure 5-3: The effect of SIR on GABA concentrations in the frontal cortex of male SIR vs. socially housed rats, no statistical significance (Student's t-test).

5.6.2.2 The effects of agomelatine treatment on GABA concentrations in SIR rats

The Shapiro-Wilk test indicated that the assumption of normality was true for all the GABA data. A Student's t-test revealed no statistical significance regarding GABA concentrations between SIR vehicle and SIR agomelatine-treated animals (p = 0.1194). However, a medium effect size (d = 0.721) (increase in GABA) was seen between the SIR vehicle vs. SIR agomelatine-treated rats (Figure 5-4).

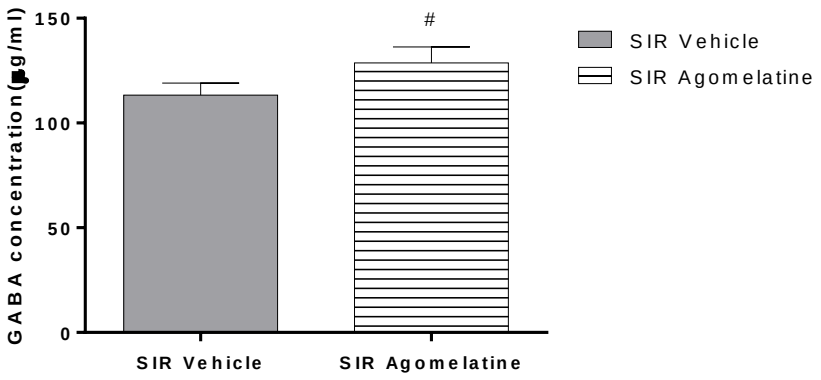

Figure 5-4: The effect of agomelatine treatment on GABA concentrations in the frontal cortex of male rats after SIR. $^{\#}d = 0.5 \geq d < 0.8$ (Cohen's d-value).

5.6.3 NMDA receptor density

5.6.3.1 The effects of SIR on NMDA receptor density

The Shapiro-Wilk test indicated that the assumption of normality was violated regarding the data for the NMDA receptor density. Therefore, a non-parametric Mann-Whitney t-test was performed.

The student's t-test indicated no statistical differences regarding NMDA receptor density between male social vs. SIR vehicle-treated animals (Figure 5-5; p = 0.6571).

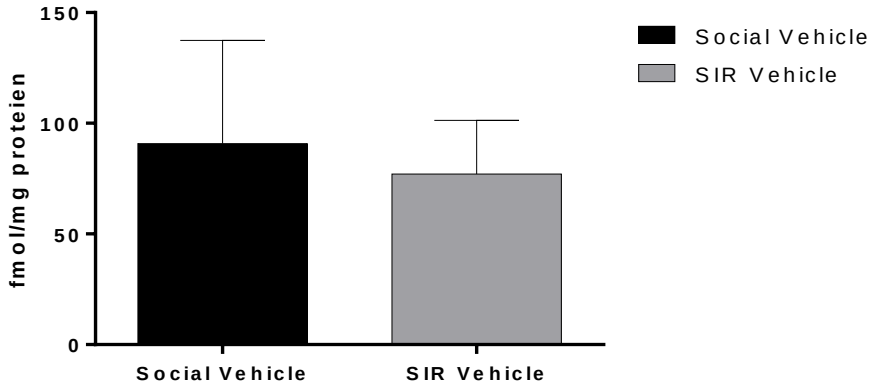

Figure 5-5: The density of NMDA receptors in the frontal cortex of male SIR and socially reared rats. No statistical or practical differences.

5.6.3.2 The effects of agomelatine treatment on NMDA receptor density in SIR rats

The Shapiro-Wilk test indicated that the assumption of normality was violated regarding the data for the NMDA receptor density. Therefore, a non-parametric Mann-Whitney t-test was done.

The student's t-test indicated no statistical differences regarding NMDA receptor density between SIR vehicle and SIR agomelatine-treated animals (p = 0.4857). However, a large effect size (d = 0.824) was seen between the SIR vehicle vs. SIR agomelatine-treated rats (Figure 5-6).

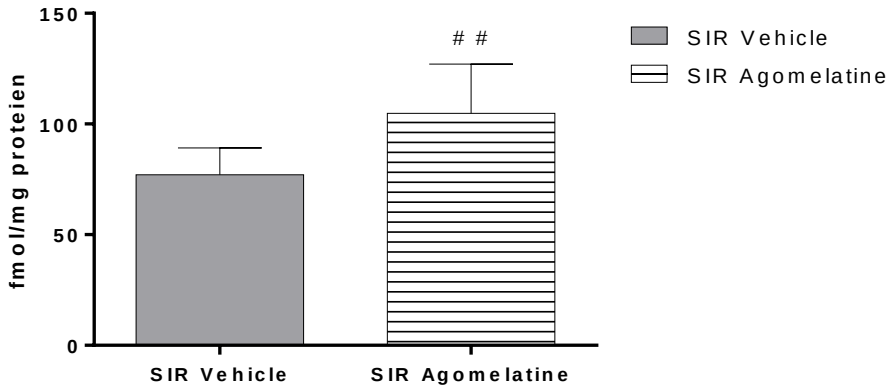

Figure 5-6: The effect of agomelatine treatment on NMDA receptor density in the frontal cortex of male rats after SIR, ##d = 0.8 ≥ d < 1.3 vs. SIR vehicle (Cohen's d-value).

5.6.3.3 The effects of SIR and agomelatine treatment on NMDA receptor affinity

The Shapiro-Wilk test indicated that the assumption of normality was violated regarding the data for the NMDA receptor affinity. Therefore, a non-parametric Mann-Whitney t-test was done.

The student's t-test indicated no statistical differences regarding NMDA receptor affinity between social vs. SIR vehicle-treated rats (Table 5-1, p = 0.48). No statistical significance were found between SIR vehicle and SIR agomelatine-treated animals (Table 5-1; p = 0.83).

Table 5-1: Frontal cortical NMDA receptor affinity (K_d) in male SIR and socially reared rats following agomelatine treatment.

Group	K_d: mean ± SEM (µM)	*N* value
Social vehicle	2.179 ± 0.4328	4
SIR vehicle	4.022 ± 1.637	4
SIR agomelatine	3.445 ± 0.9411	4

5.7 Discussion

The results from the OFT suggests that SIR rats do not demonstrate an anxiety-like profile, in fact presenting an anxiolytic action (male SIR vehicle rats spent significantly *more* time in the centre arena) (Figure 5-1). However, this is not what is demonstrated in the EPM, where SIR rats exhibited a decrease in activity on the open arms, hence an anxiogenic response (Chapter 3).

Previous studies also evaluating the anxiety-like effects of SIR have indicated an increase locomotor activity in the OFT (Elliott & Grunberg, 2005; Lapiz *et al.*, 2003; Varty *et al.*, 2000; Weiss *et al.*, 2004), or an anxiogenic response similar to that described in Chapter 3. However, these latter authors did not present any data on the time spent in the center arena, thus making it impossible to draw a comparison between this study and the aforementioned. This apparent paradox with respect to anxiety in the OFT (centre zone activity) and the EPM (open arm activity) requires further study. Nevertheless, we speculate that hyper-locomotor activity in the OFT *may* underlie these unexpected results, as explained below.

Socially reared rats habituate faster to novel environments (see Chapter 3), presenting as reduced locomotor activity in the OFT (less anxiety-like), while SIR vehicle-treated rats display neophobia suggestive of enhanced responsiveness to a novel environment. Thus, since SIR vehicle-treated rats presented with hyper-locomotor activity, they moved around more during the test and consequently spent more time in the center arena. An earlier study indicated that SIR decreased the number of centre entries in rats, although a light shining on the centre arena made it more aversive for the rats (Lukkes *et al.*, 2009). The latter authors proposed this to be a behavioural test indicating an aversion for light and not open spaces as advocated in our study. Another study also concluded that SIR rats show latency to enter the centre of a brightly lit open field (Schrijver *et al.*, 2002). Despite the paradoxical response in as far as anxiety is concerned, agomelatine significantly *reduced* the time spent in the centre arena in male rats (Figure 5-2). However, this may possibly relate to agomelatine reducing locomotor hyperactivity (Chapter 3). No significant differences were observed in the female rats. Concluding, the EPM is one of the most widely used screening tests for anxiety in animals (Carobrez & Bertoglio, 2005; Rodgers *et al.*, 1997) and may offer more superior validity than the OFT for the evaluation of anxiety-like behaviours. For this reason we opted to use the OFT only for assessing locomotor activity, as applied in Chapter 3.

The results indicate that SIR does not affect GABA transmission in the frontal cortex (Figure 5-3). Previous studies have found that SIR up regulates the $GABA_A$ receptors in the frontal cortex, possibly due to a decrease in GABAergic transmission (Hickey *et al.*, 2012; Serra *et al.*, 2008). Although similar designed studies have successfully quantified regional brain GABA levels using HPLC methods (Harvey *et al.*, 2002; Harvey *et al.*, 2004), such methods may not be sensitive enough for this purpose (Van der Zeyden *et al.*, 2008), even microdialysis have shown not to be sensitive enough for quantification of GABA (Timmerman & Westerink, 1997; Van der Zeyden *et al.*, 2008). On face value, the results obtained in this study suggest that the anxiety-like behavioural deficits observed in SIR rats might not be associated with defective GABA transmission. Although a role for altered GABA could not be demonstrated in SIR rats *per se*, it

is interesting that agomelatine tended to increase GABA levels in the frontal cortex in SIR rats (Figure 5-4). The effect of agomelatine on GABA transmission has not been extensively studied and the findings are somewhat inconsistent, with one study showing no significant increase in GABA after stress and agomelatine treatment (Tardito *et al.*, 2010), and another suggesting agomelatine has beneficial effects on GABA transmission (Aguiar *et al.*, 2013; Reagan *et al.*, 2012). Melatonin, however, may increase GABA transmission (Cheng *et al.*, 2012). Clearly, more studies delineating the actions of agomelatine on GABAergic transmission are advocated.

Very few studies have examined the effect of SIR on glutamatergic neurotransmission in the brain, while these findings are inconsistent. The results from this section revealed that SIR had no significant effect on NMDA receptor density (B_{max}) (Figure 5-5) or receptor affinity (K_d) (Table 5-1) in the frontal cortex of male rats. These results indicate that SIR-associated anxiety-like behaviours described in Chapter 3 might not be related to glutamatergic dysfunction. However, previous studies have shown a decrease in NMDA gene expression after SIR (Hall *et al.*, 2002; Zhao *et al.*, 2009); while previous studies in our laboratory showed that SIR increased frontal cortical NMDA B_{max} and decreased K_d (Toua *et al.*, 2010). The exact role of glutamate as a pathological mediator following SIR therefore requires further study. Although no longer suitable for drawing any conclusion regarding a role for glutamate following treatment, agomelatine tended to increase NMDA receptor density in the frontal cortex (Figure 5-6; d = 0.824). Previous studies showed that agomelatine may decrease glutamate transmission (Popoli, 2009; Tardito *et al.*, 2010), which in turn may up-regulate the receptor density.

Since the presence of disordered GABA-glutamate dysfunction could not be demonstrated in the SIR model, it was no longer feasible to use these data to attempt to draw any conclusion on whether the anxiolytic activity of agomelatine may involve correction of GABAergic and/or glutamatergic deficits. For this reason, the GABA and NMDA receptor binding data were excluded from the article (Chapter 3).

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ADDENDUM B

HPLC SYSTEM SUITABILITY

The following addendum is included to supply valuable and additional information concerning the methods and experimental procedures regarding the measurements of corticosterone in the plasma and γ-amino butyric acid (GABA) in the frontal cortex.

The methods described in this addendum are maintained and validated by the Analytical Technology Laboratory (ATL) of the Centre of Excellence for Pharmaceutical Sciences (PharmaCen) of the North-West University, Potchefstroom Campus. The high performance liquid chromatography (HPLC) methods were previously validated, therefore only system suitability was done for this study. The objective of system suitability is to demonstrate that the method and analytical instrument will still produce the same accurate and valid results as what it did with the original method validation. The analytical parameters necessary for system suitability of biomolecules are: linearity, range, and repeatability, lower limit of quantification (LLOQ) and lower limit of detection (LLOD).

The linearity of an analytical procedure is the ability to obtain test results in a specific range which are directly proportional to the concentration in the sample. The range can be defined as the interval between the upper and the lower concentration in the sample (Chan *et al.*, 2004). The acceptable criteria for regression (r^2), the coefficient of determination for biomolecules must be at least 0.95 or greater (Shabir, 2005). Repeatability is a measure of the precision and accuracy under the same operating conditions over a short interval of time and the coefficient of variation (CV) is a parameter of repeatability (Chan *et al.*, 2004). Repeatability is determined at 3 concentrations (low, medium and high) over the calibration curve with 3 repetitions each (Chan *et al.*, 2004). The repeatability is determined at each concentration level and should not exceed 15% of CV except for the LLOQ, where it should not exceed 20% of the CV (FDA, 2001). Sensitivity is described as the lowest concentration that can be measured accurately; LLOQ is the measurement of sensitivity (Chan *et al.*, 2004; FDA, 2001).

This addendum will thus consist of:

B.1: The HPLC method for the determination of corticosterone in plasma

B.2: The HPLC method for the determination of GABA in the frontal cortex

Addendum B.1

HPLC method for corticosterone determination in plasma

Corticosterone concentrations in the plasma were determined by means of HPLC with ultraviolet (UV) detections. The method is a combination of two methods; the method from Hariharan *et al.* (1992) and Viljoen *et al.* (2012).

6.1 Results for the system suitability of corticosterone

6.1.1 Linearity and range

The linearity used in this validation process comprised of the following standard concentrations: 10, 25, 50, 100, 250 and 500 ng/ ml. The linear regression value determined for corticosterone was $r^2 = 0.997$ (indicated in Figure 6-1).

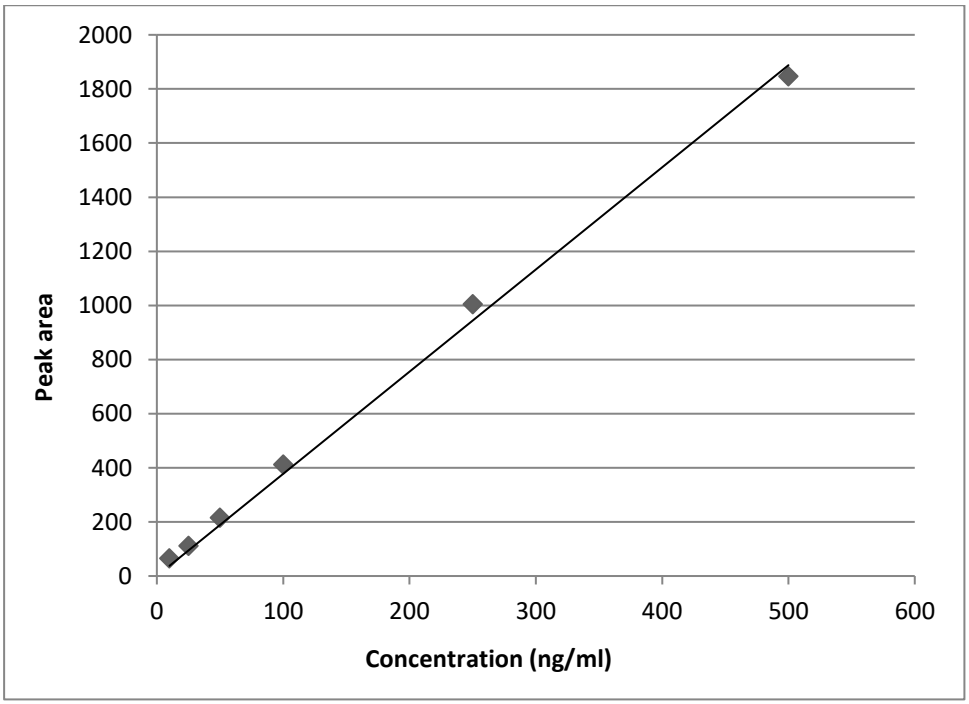

Figure 6-1: Line regression graph of corticosterone at 10, 25, 50, 100, 250 and 500 ng/ml concentrations.

6.1.2 Repeatability

The 3 concentrations used for the repeatability were 10, 50 and 500 ng/ml. The % CV for each of these concentrations was 9.15%, 3.04% and 5.51% respectively.

6.1.3 Sensitivity

The LLOQ was 10 ng/ml, which was also the lowest concentration on the calibration curve. The LLOD was determined as 2.5 ng/ml.

6.2 Mobile phase

The mobile phase for this chromatographic procedure consisted out of the following: distilled water and acetonitrile (60:40 v/v) and the pH were adjusted to ± 3.50 with glacial acetic acid.

6.3 Chemicals

Distilled water was obtained from a Milli-Q® water system, the methanol, dichloromethane and acetonitrile were of HPLC grade and purchased from Merck, as well as the glacial acetic acid. The raw materials for corticosterone as the standard and the dexamethasone (DX) as the internal standard were purchased from Sigma-Aldrich.

6.4 Chromatographic conditions (indicated in Table 6-1)

Table 6-1: The chromatographic conditions for the determination of corticosterone

Analytical Instrument	Agilent 1100 series HPLC, equipped with a quaternary pump, auto sampler, diode array detector (DAD) and Chemstation Rev. A.06.02 data acquisition and analysis software.
Column	Synergi 4µ Max-RP 80Å, 250 x 4.6 mm from Phenomenex purchased from Separations
Flow rate	1.0ml/ min
Injection volume	100 µl
DAD settings	Wavelength set at 245 nm
Run Time	± 25 minutes
Retention times	Internal standard ± 6 minutes, corticosterone ± 7 minutes

6.5 Preparation of standard solutions

6.5.1 Standards

Corticosterone (1 mg) was weighed and dissolved in 10ml of 20% methanol in an amber volumetric flask. This served as the stock solution (SS) with a concentration of 1000 ng/ml and was stored at 2 – 8 °C. From the SS a concentration range of 500 - 10 ng/ ml were prepared in distilled water as described in Table 6-2 below and stored between 2 – 8 °C.

Table 6-2: Standard solutions were prepared from a 1000 ng/ ml stock solution (SS).

Standard number	Concentration (ng/ml)	Dilution Volume	+	Distilled Water	=	Total Volume
SS	1000	100 µl	+	9900 µl	=	2 ml
1	500	1000 µl (SS)	+	1000 µl	=	2 ml
2	250	500 µl (SS)	+	1500 µl	=	2 ml
3	100	200 µl (SS)	+	1800 µl	=	2 ml
4	75	150 µl (SS)	+	1850 µl	=	2 ml
5	50	100 µl (SS)	+	1900 µl	=	2 ml
6	25	50 µl (SS)	+	1950 µl	=	2 ml
7	10	20 µl (SS)	+	1980 µl	=	2 ml

6.5.2 Internal standard

For the internal standard 1 mg DX was weighed and dissolved in 10 ml of 20% methanol in an amber volumetric flask. This was used as the SS with a concentration of 100 µg/ml and stored at 2 – 8 °C. The stock solution (50 µl) was made up to 2 ml in 2 ml amber Eppendorf tube with distilled water. This was the working internal standard with a concentration of 2.5 µg/ml and was also stored at 2 – 8 °C.

6.6 Sample preparation

Sample preparation was done as discussed in Addendum A.

6.7 Corticosterone chromatograms (indicated in Figures 6-2 to 6-4)

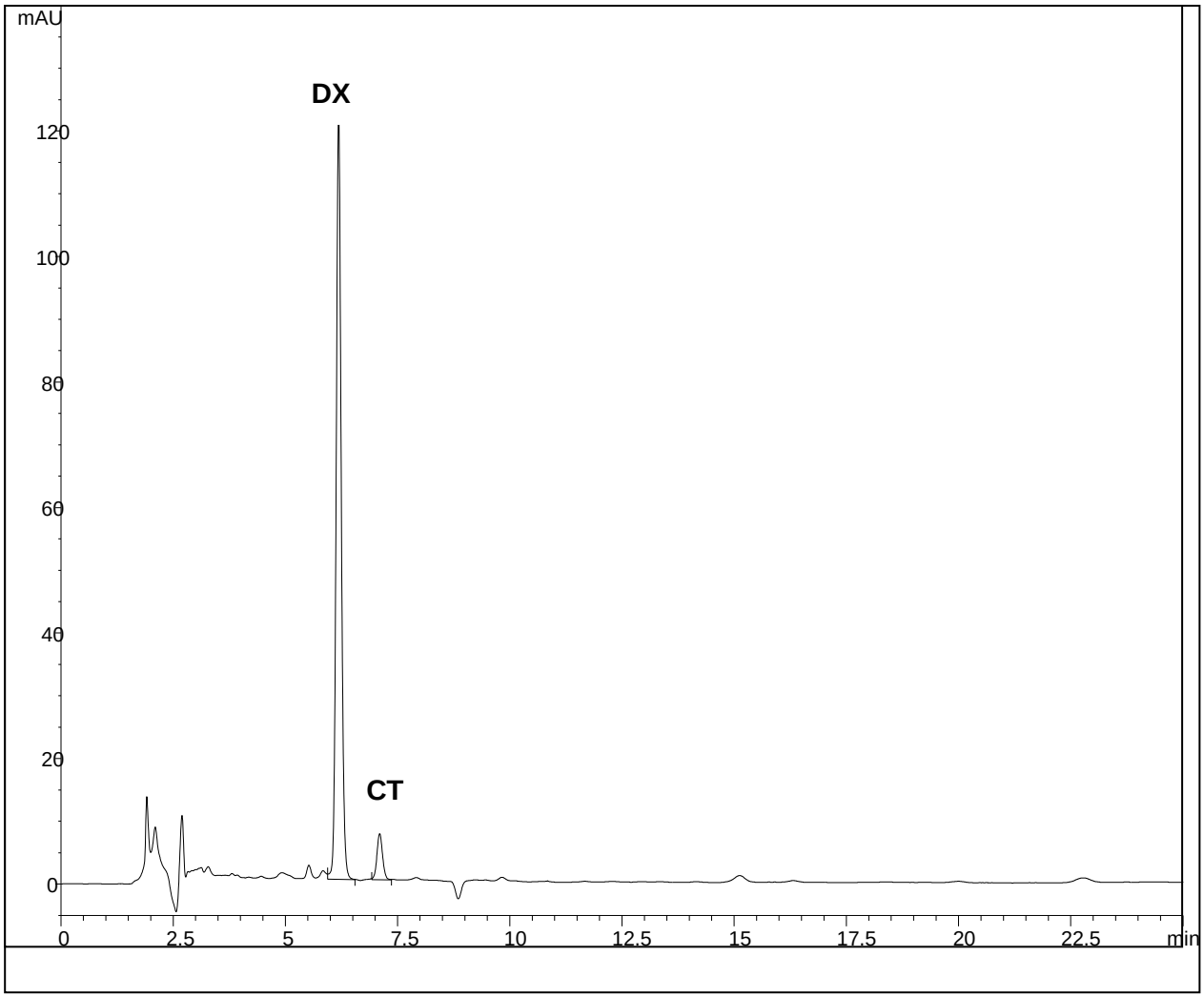

Figure 6-2: Corticosterone standard 10 ng/ ml measured in milli absorption units (mAU). Retention time: dexamethasone (DX) ± 6 minutes and corticosterone (CT) ± 7 minutes.

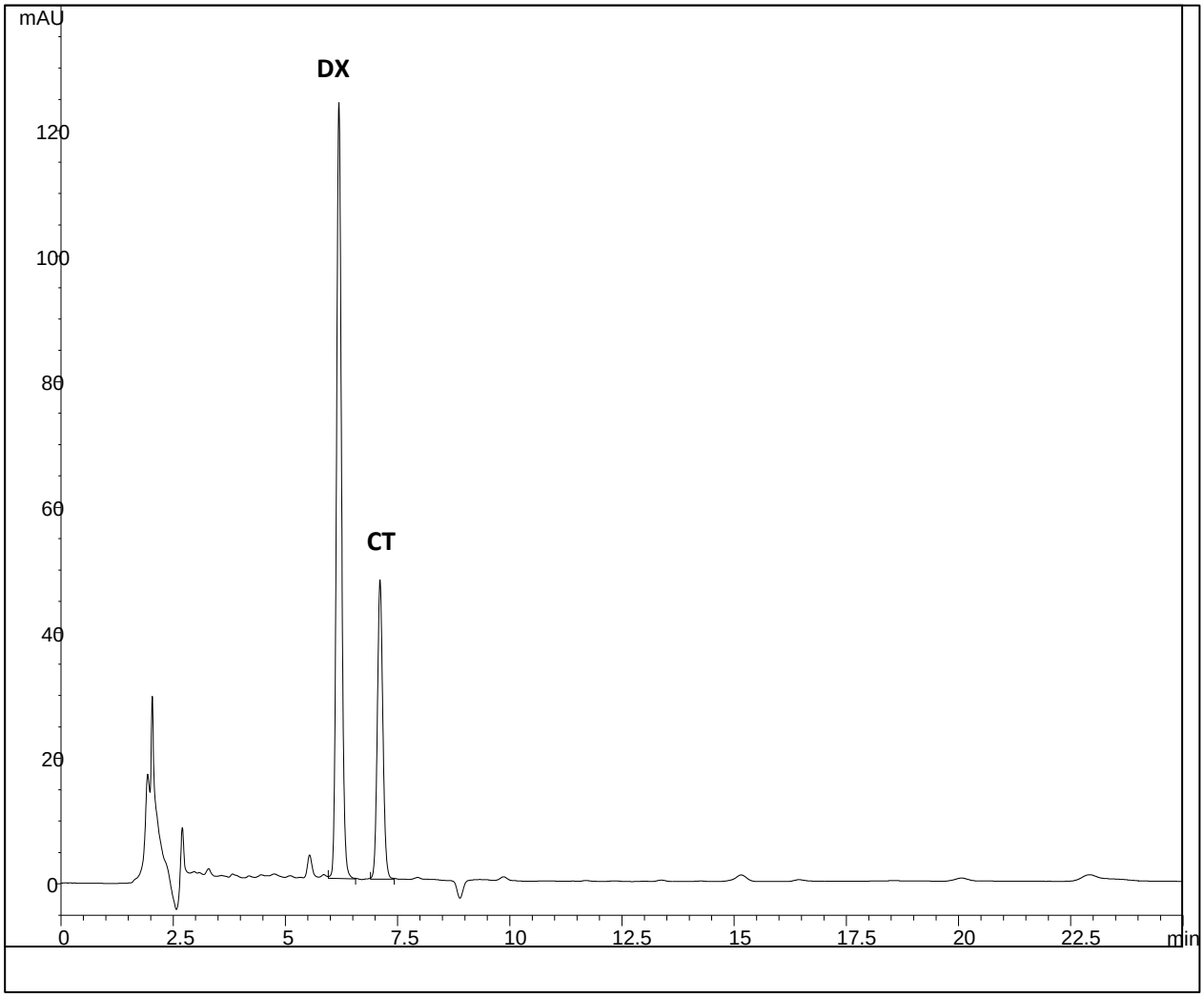

Figure 6-3: Corticosterone standard 100 ng/ ml measured in milli absorption units (mAU). Retention time: dexamethasone (DX) ± 6 minutes and corticosterone (CT) ± 7 minutes.

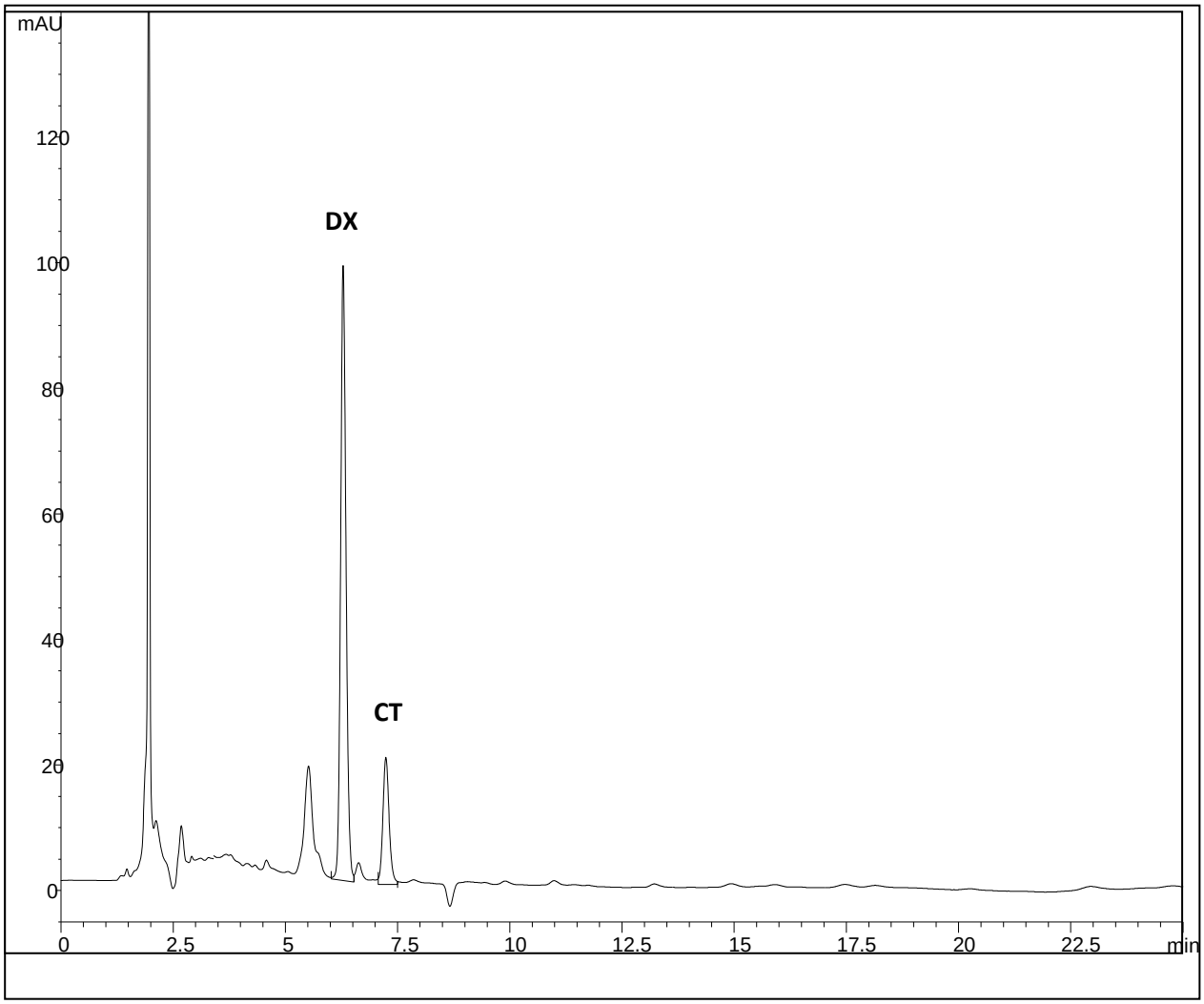

Figure 6-4: Corticosterone in a plasma sample measured in milli absorption units (mAU). Retention time: dexamethasone (DX) ± 6 minutes and corticosterone (CT) ± 7 minutes.

Addendum B.2

The HPLC method for the determination of GABA in the frontal cortex

GABA concentrations in the frontal cortex were determined by means of HPLC with electrochemical (EC) detection. This method was proven to be selective and sensitive enough under our laboratory conditions to detect Nano molar quantities of GABA (Harvey *et al.*, 2002).

6.8 Results for the system suitability of GABA

6.8.1 Linearity and range

The linearity used in this validation process comprised of the following standard concentrations: 0.25, 0.5, 1.0, 2.5, 5.0, 7.5 and 10.0 µg/ml. The linear regression value determined for GABA was $r^2 = 0.996$ (indicated in Figure 6-5).

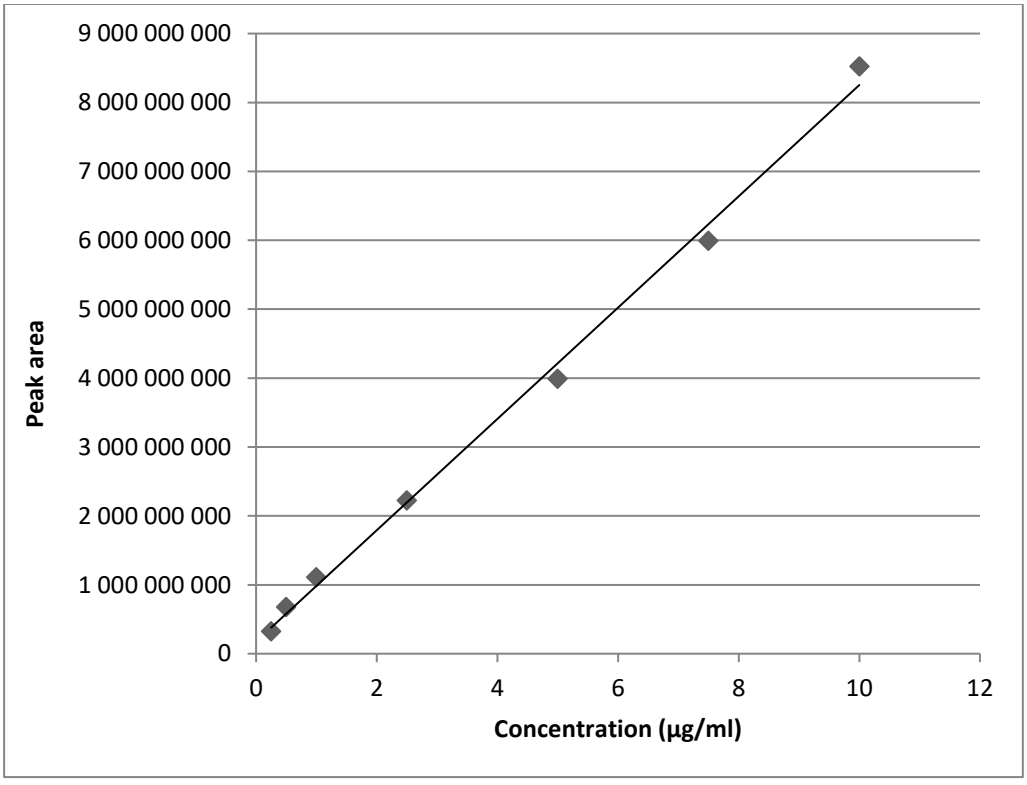

Figure 6-5: Line regression graph of GABA at 0.25, 0.5, 1.0, 2.5, 5.0, 7.5 and 10 µg/ml.

6.8.2 Repeatability

The 3 concentrations used for the repeatability were 0.25, 1.0 and 7.5 µg/ ml. The CV for each of these concentrations was 3.95%, 2.74% and 2.90% respectively.

6.8.3 Sensitivity

The LLOQ was 0.25 µg/ml, which was also the lowest concentration on the calibration curve. The LLOD was 0.1 µg/ml.

6.9 Mobile phase

For the mobile phase 14.20 g sodium phosphate dibasic ($Na_2HPO_4.2H_2O$) and 0.05 g ethylenediaminetetraacetic acid (Na_2EDTA) were weighed and dissolved in 700 ml distilled H_2O. Subsequently, 300 ml of 30% methanol was added and mixed thoroughly. Thereafter ortophosphoric acid (85%) was used to adjust the pH of the mobile phase to ± 6.9.

6.10 Chemicals

Distilled water was obtained from a Milli-Q® water system, the methanol (HPLC grade), sodium phosphate dibasic; ethylenediaminetetraacetic acid and ortophosphoric acid were purchased from Merck. The raw materials for GABA as the standard and the DL-Homoserine as the internal standard were purchased from Sigma-Aldrich.

6.11 Chromatographic conditions

The specific chromatographic conditions for the GABA method are indicated in Table 6-3.

Table 6-3: The chromatographic conditions for the determination of GABA

Analytical Instrument	Agilent 1200 series HPLC, equipped with an isocratic pump, auto sampler, coupled to an ESA Coulochem III Electrochemical detector (with coulometric detection; ESA Analytical Cell 5011A and Guard Cell 5020) and Chromeleon® Chromatography Management System version 6.8.
Column	Luna C18-2 column, 75 x 4.6 mm, 5µm, 100 Å pores, 17.8% carbon load, end capped, Phenomenex, Torrance, CA (Column L1, USP 24, 2000, p 1925).
Flow rate	1.0 ml/min
Injection volume	10µl
ECD Detector settings	ESA Analytical Cell 5011A, coulometric detection, Cell potential settings: Test Electrode 1 (E1): -150mV; gain range: 1mA

	Test Electrode 2 (E2): 600mV; gain range: 500nA Guard Cell 5020 set at 350mV
Run time	± 25 minutes
Retention time	Internal standard ± 5 minutes, GABA ± 12.5 minutes.

6.12 Preparation of standard solutions

6.12.1 Standard solutions

For the standard solutions 1mg GABA were weighed and dissolved in 10ml of solution A. Solution A was an ice-cold 0.1 M Perchloric acid solution, which is made from 2.72 ml of a 60% Perchloric acid and made up to a volume of 250 ml with distilled water. This solution served as a SS used for the standard solutions (indicated in Table 6-4) with a concentration of 100 µg/ml and was stored at 2 – 8 °C.

Table 6-4: Standard solutions were prepared from a 100 µg/ ml stock solution (SS).

Standard number	Concentration (µg/ml)	Dilution Volume	+	Solution A	=	Total Volume
1	10	200 µl	+	1800 µl		2 ml
2	7.5	150 µl	+	1850 µl		2 ml
3	5	100 µl	+	1900 µl		2 ml
4	2.5	50 µl	+	1950 µl		2 ml
5	1.0	20 µl	+	1980 µl		2 ml
6	0.5	10 µl	+	1990 µl		2 ml
7	0.25	5 µl	+	1995 µl		2 ml

6.12.2 Internal standard

DL-Homoserine (1 mg) was weighed and dissolved in solution A. The concentration of this solution was 100 µg/ml, 100µl from the latter solution was made up to a volume of 2 ml with solution A. The final concentration of the internal standard was 5 µg/ml.

6.13 Preparation of brain samples

The sample preparations were done as described in Addendum A. Consequently, 50 µl was then pipetted into an insert and placed into an HPLC vial. The vial was placed into the HPLC auto

sampler. A final sample of 10µl was injected onto the HPLC column after the pre-column orto-phtaldialdehyde derivatization as indicated in Table 6-5.

Table 6-5: The software's injector program was programmed as follows for pre-column orto-phtaldialdehyde derivatization.

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

6.14 Chromatograms (indicated in Figures 6-6 to 6-8)

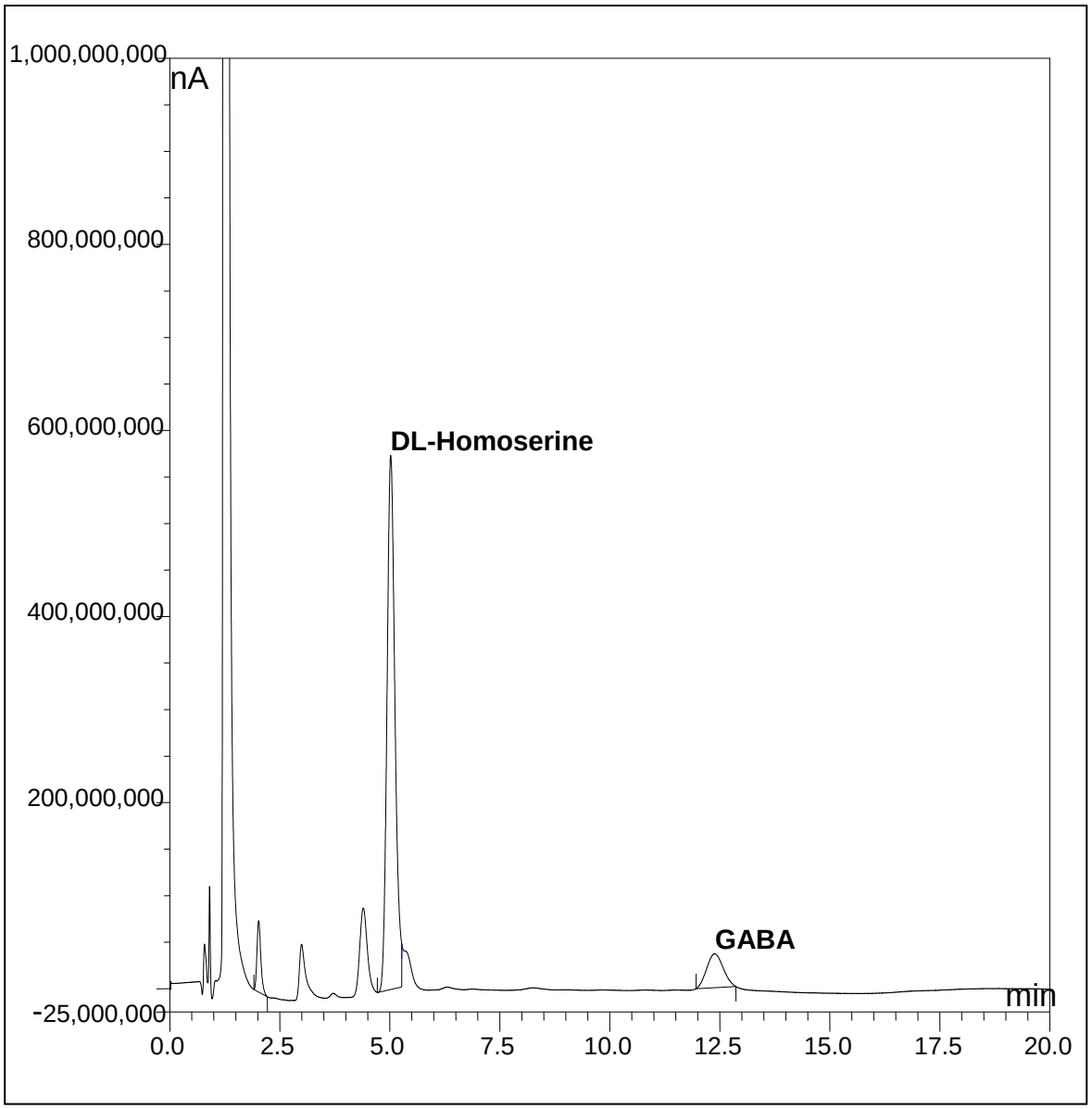

Figure 6-6: GABA standard 1.0 µg/ml measured in nano ampere (nA). Retention time: DL-Homoserine ± 5 minutes and GABA ± 12.5 minutes.

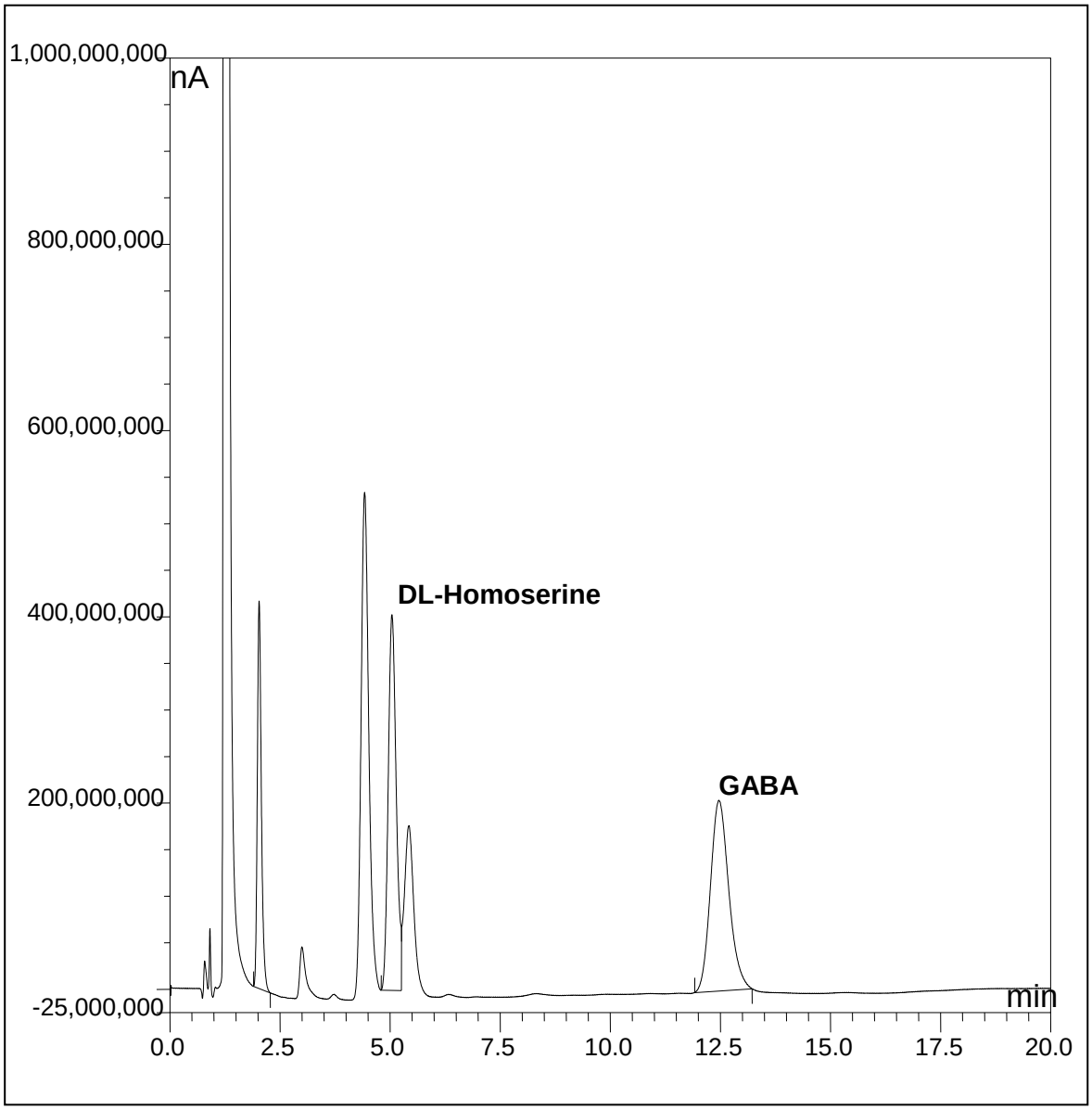

Figure 6-7: GABA standard 7.5 µg/ml measured in nano ampere (nA). Retention time: DL-Homoserine ± 5 minutes and GABA ± 12.5 minutes

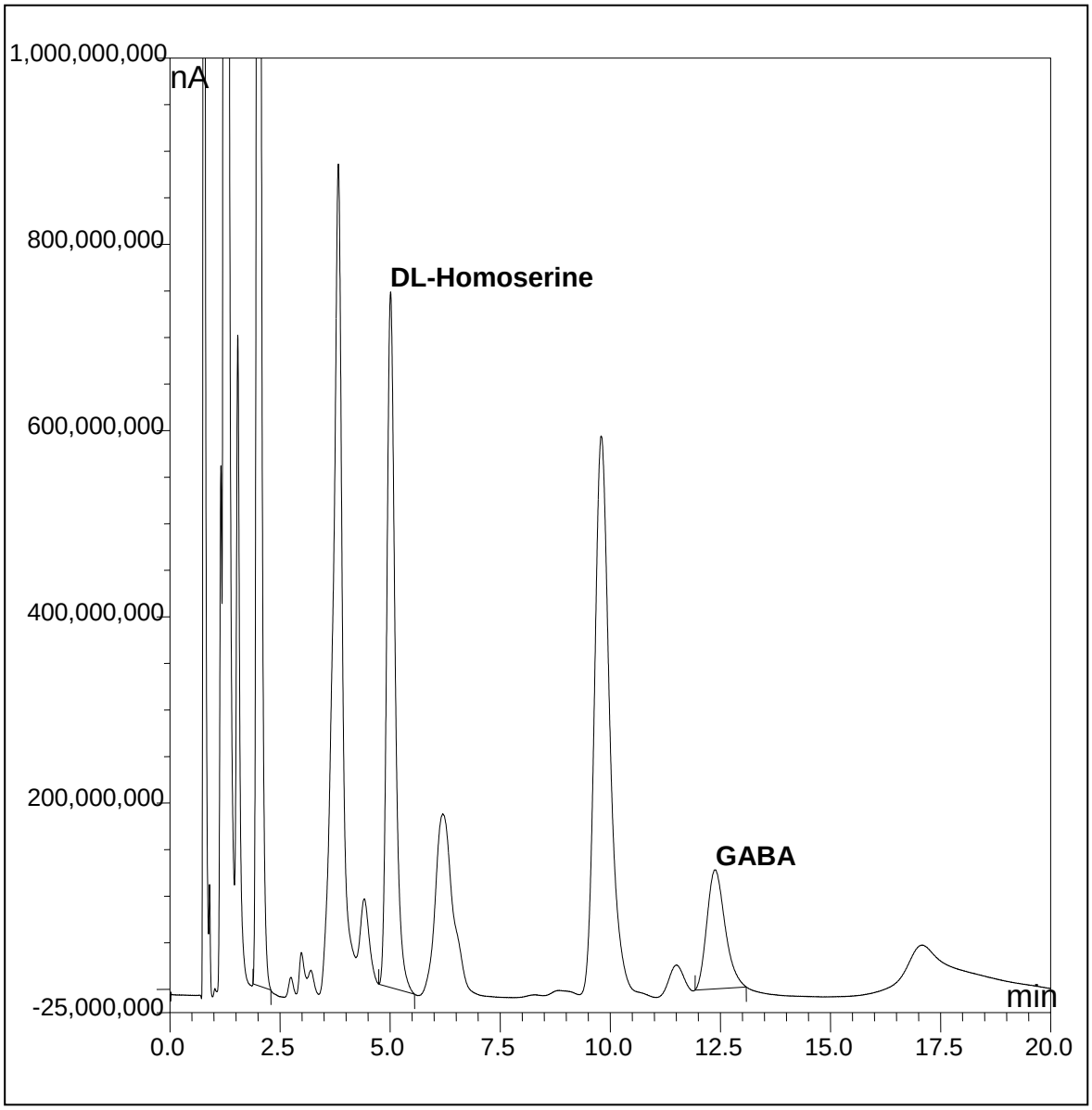

Figure 6-8: GABA in a tissue sample measured in nano ampere (nA). Retention time: DL-Homoserine ± 5 minutes and GABA ± 12.5 minutes.

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ADDENDUM C

CONGRESS ABSTRACT

Evaluation of agomelatine treatment on anxiety-like behaviours in social isolation reared rats, and its relation with gender

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Purpose: Anxiety disorders are the most prevalent of psychiatric disorder, and present with an estimated 30% non-response rate to current treatment regimens. Anxiety is comorbid in many psychiatric disorders, and is associated with disturbances in circadian rhythms. Considering the latter, agomelatine is a new antidepressant acting as melatonin (MT_1/MT_2) agonist and $5HT_{2C}$ antagonist, with known circadian rhythm regulating effects that has been purported to be involved in its antidepressant and anxiolytic actions. Accordingly, possible circadian dysrhythmia, gender and sex-hormone related biorhythms may indirectly affect treatment response. Social isolation rearing (SIR) has been suggested to present with anxiety-like manifestations in rodents. We investigated this property and whether agomelatine may have anxiolytic properties in SIR rats. Finally, we also determined whether any treatment response to agomelatine may be related to gender differences.

Methods: Ethics approval was obtained (NWU-00347-15-S5). A total of 72 Sprague-Dawley rats (36 males and 36 females) were used and randomly allocated to groups consisting of 12 rats per group. At weaning, post-natal day (PND) 21, the animals were randomized to either SIR (1 animal per cage) or social rearing (3 animals per cage) for 8 weeks. Agomelatine (40 mg/kg) or 1% hydroxyethylcellulose was administered intraperitoneally (i.p.) for 16 days. All the animals were subjected to the open field test (OFT) (on day 13 of treatment), social-interaction test (SIT) (on day 14 of treatment) and the elevated plus maze (EPM) (on day 15 of treatment). Two-way ANOVA with Bonferroni post hoc test was performed with a p-value of <0.05 deemed statistically significant.

Results: The OFT data revealed that SIR significantly increased locomotor activity in both male and female rats, which was reversed by agomelatine in both genders. In the SIT socially housed rats spent more time together and more time involved in active social interaction (e.g. anogenital sniffing) compared to SIR. Female socially housed rats exhibited increased social interaction compared to male socially housed rats. Furthermore, agomelatine reversed SIR-related social interaction deficits (anogenital sniffing and time spent together) in male but not female animals. The EPM revealed that in both genders SIR significantly increased anxiety-like behaviour (more entries into the closed arms of the EPM) whereas female and male socially housed rats spent significantly more time on the open arms. Agomelatine treatment significantly reversed SIR-related anxiety-like behaviour in the EPM in male rats.

Conclusions: SIR induced significant anxiety-like behaviour, as measured in the OFT, SIT and EPM. Agomelatine shows promise as an anxiolytic drug, although treatment response was significantly better in males than females with regards to most of the anxiety-like behaviours measured.

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