

# Behavioral, neuroendocrine and neurochemical studies on agomelatine in social isolation reared rats

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## 1. Introduction

There is currently a large body of evidence suggesting that stressful early life experiences, such as maternal separation and social isolation, may play a role in the development of major depressive disorder (MDD) (Fone & Porkess, 2008). Such experience adversely affects brain development as well as multiple neurobiological systems (aan het Rot et al., 2009) that lead to maladaptive behaviour in adulthood (Weiss et al., 2003). The understanding of MDD is currently based on the classic monoamine-deficiency hypothesis which proposes that MDD follows as a result of sustained deficits in the monoamine transmitters, serotonin (5-HT), noradrenalin (NA) and dopamine (DA) (Cai et al., 2015). However, the relationship between stress and affective disorders can be more fully understood from an integrative perspective (Jurueña, 2014). In fact, over and above the above-mentioned monoamine deficits, the impact of psychosocial stress on brain-derived neurotrophic factor, inflammatory, redox and metabolic processes, as well as structural brain changes (Palazidou 2012) are now considered important contributory factors in MDD. During psychological stress, adaptive physiological responses occur through activation of the hypothalamic-pituitary-adrenal (HPA) axis (Cai et al., 2015) culminating in an increase in cortisol (Jurueña 2011, Martins et al., 2011). Cortisol in turn is known to disturb a number of the above-mentioned processes, also mediating structural brain changes such as hippocampal shrinkage (Campbell et al., 2014). Disturbances in the circadian release of cortisol, as well as other neuroendocrine messengers, is now strongly implicated in depressive symptomatology and pathology, so that targeting the central biorhythm centre of the brain has important significance for novel antidepressant drug action.

The social isolation rearing (SIR) model in rats produces various symptoms and deficits in line with MDD, such as anhedonic-like behavior (Hall et al., 1997), depressive-like behaviors (Hall et al., 1998), cognitive deficits (Bianchi et al., 2006) and monoaminergic alterations (as reviewed by (Fone & Porkess, 2008). Agomelatine is a new generation antidepressant with melatonin (MT1 and MT2) receptor agonist and 5-HT<sub>2c</sub> receptor antagonist properties, exerting its antidepressant effects through the re-entrainment of altered circadian rhythms and a very specific action on frontal cortical monoamines (Cai et al., 2015). To the best of our knowledge, the antidepressant capabilities of agomelatine have not been studied in a neurodevelopmental animal model of depression, viz. SIR.

## 2. Methods

Male Sprague-Dawley (SD) rats (12 rats/group) were used. The study was divided into two arms consisting of (1) a behavioral cohort, and (2) a neurochemical and neuroendocrine cohort. Eight

groups of rats, divided into 4 behavioral and 4 neurochemical groups, were randomly separated at weaning (postnatal day (PND 21), and exposed to either 8 weeks of SIR or 8 weeks social rearing.

Agomelatine (40mg/kg/day) or 1% hydroxyethylcellulose (HEC) vehicle was administered at 16h00 by intraperitoneal injection for the last 14 days of rearing (PND 63-77). Behavioral analysis of anhedonia, memory, locomotor activity and behavioural despair were analysed on the final 4 days of rearing, using the sucrose preference test (SPT), novel object recognition test (NORT), open field test (OFT) and forced swim test (FST), respectively. The remaining groups were sacrificed at 8 weeks and plasma and brain tissue harvested for analysis of regional brain monoamine concentrations and lipid peroxidation (LPX) by high-performance liquid chromatography (HPLC) and enzyme-linked immunosorbent assay (ELISA), respectively, and plasma corticosterone and superoxide dismutase (SOD) activity both done by ELISA respectively. A one-way ANOVA with a suitable post-hoc test were performed.

### 3. Results

SIR significantly increased immobility in the FST without affecting locomotor activity, and showed a trend in reducing swimming and climbing behaviors, although no significant effects were seen in the NORT. SIR also showed a significant decrease in sucrose preference compared to the social control. Cortico-hippocampal monoamines remained unaffected, except for a trend towards reduced striatal 5-HT and increased hippocampal 5-HIAA. No changes in LPX, SOD or CORT levels were found in SIR rats. For the most, agomelatine treatment had minimal to no effect in socially reared animals. Agomelatine had no effect on locomotor activity, but significantly reduced immobility in SIR rats, and selectively increased struggling behaviour without affecting swimming behaviour. Agomelatine significantly decreased sucrose preference vs. socially reared rats receiving vehicle, but did not affect memory. Agomelatine significantly increased both LPX activity and basal CORT levels in SIR rats vs. SIR rats receiving vehicle, but had no effect on SOD activity. Agomelatine significantly increased striatal dihydroxyphenylacetic acid (DOPAC) levels in SIR rats vs. SIR rats receiving vehicle.

### 4. Conclusion and Discussion

SIR produced depressive-like bio-behavioral changes in rats, specifically behavioural despair with reduced behavioural coping strategies, anhedonia-like manifestations and regional brain monoamine alterations. However, it failed to engender cognitive deficits, or alter lipid peroxidation, superoxide dismutase activities or basal corticosterone levels. These findings establish some albeit weak face validity for MDD. Agomelatine demonstrated antidepressant-like effects in this model, indicative of a reversal of SIR-related immobility and attenuated coping strategies, especially reduced struggling

(NAergic-related) behaviors. Interestingly, the latter corresponds with its purported noradrenergic mode of action. On this point, agomelatine showed no obvious 5-HTergic activity with respect to behaviour in the FST (swimming) as well as 5-HT related changes in the frontal cortex, striatum and hippocampus of SIR rats. Rather agomelatine decreased hippocampal 5-HT levels in the social reared rats. Although plasma corticosterone was unaltered by SIR, agomelatine increased basal corticosterone levels in SIR rats, an unexpected observation. However, this action could be linked to both cognitive deficits in the NORT and an increase in lipid peroxidation observed in SIR rats receiving agomelatine. Agomelatine significantly decreased sucrose preference in both social and SIR rats, which is contradictory to its clinical profile in treating anhedonia, as well as its supposedly dopaminergic profile. Finally, agomelatine did not affect SOD activity in either social or SIR animals. Thus, agomelatine does show antidepressant-like effects in the SIR model, especially in the FST, with evidence of cortico-hippocampal monoamine changes related to the monoamine hypothesis of MDD. However, failure of the SIR model to reproduce the desired MDD-related pathology in other behavioural tests, as well as with respect to corticosterone and redox analysis, precludes a more comprehensive interpretation of the findings, and further study is warranted.

**Keywords:** depression, early life stress, HPA-axis, circadian rhythm, agomelatine, frontal cortex, striatum, hippocampus

## 1. Inleiding

Daar is tans menige bewyse wat voorstel dat stresvolle gebeure soos moederlike skeiding en sosiale isolasie gedurende vroeë ouderdomme, 'n rol mag speel in die ontwikkeling van erge depressie (ED) (Fone & Porkess, 2008). Sulke stresvolle gebeure affekteer brein ontwikkeling sowel as veelvuldige neurobiologiese sisteme (aan het Rot et al., 2009) wat kan lei tot gedragsafwykings in volwassenes (Weiss et al., 2003). Die teorie van ED word tans gebaseer op die monoamien wanbalans hipotese wat voorstel dat ED veroorsaak word deur volgehoue tekorte aan die volgende monoamien oordragstowwe: serotonien (5-HT), noradrenalin (NA) en dopamin (DA) (Cai et al., 2015). Maar die verhouding tussen stres en gemoedsversteurings kan beter verstaan word vanaf 'n geïntegreerde perspektief (Jurueña, 2014). In werklikheid, bo en behalwe die bogenoemde monoamien tekorte, word die impak van psigologiese stres op brein-afkomstige neurotrofiese faktor (BANF), inflammasie, redoks en metaboliese prosesse sowel as strukturele brein veranderinge (Palazidou, 2012) ook nou oorweeg as bydraende faktore tot die ontwikkeling van ED. Gedurende psigologiese stres, vind daar ondersteunende prosesse plaas deur aktivering van die hipotalamus-pituitêre-adrenale (HPA) aksis (Cai et al., 2015) wat opbou tot die verhoogde vrystelling in kortisol (Jurueña, 2011; Martins et al., 2011). Kortisol op sy beurt kan lei tot krimpings van die hippokampus (Campbell et al., 2014). Versteurings in sirkadiese ritmes vanaf kortisol vrystelling, asook ander neuroendokriene boodskappers, word duidelik benadruk by depressiewe simptomologie en patologie, dus het die sentrale bio-ritme sentrum van die brain 'n belangrike teiken geword het vir nuwer antidepressante werking.

Die stres-geïnduseerde isolasie (SSI) model in rotte lei tot verskeie simptome en versteurings wat geassosieer word met ED, soos anhedoniese gedrag (Hall et al., 1997), depressiewe gedrag (Hall et al., 1998), kognitiewe versteurings (Bianchi et al., 2006) en monoamien versteurings (Fone en Porkess 2008). Agomelatien is 'n nuwe generasie antidepressant met melatonien (MT1 en MT2) reseptor agonistiese en 5-HT<sub>2c</sub> antagonistiese eienskappe, wat sy antidepressiewe werking uitbeeld deur die versteurde sirkadiese ritmes reg te stel asook 'n baie spesifieke werking op frontale-kortikale monoamiene (Cia et al., 2015). Sover on kennis dra, is daar nog geen studies wat die antidepressiewe vermoë van agomelatien bewys het in 'n neuro-ontwikkelende dieremodel vir depressie nie.

## 2. Metodes

Manlike Sprague-Dawley (SD) rotte (12 rotte/groep) is gebruik. Die studie is verdeel in 2 arms wat bestaan het uit (1) 'n gedragsarm, en (2) 'n neurochemiese en neuroendokriene arm. 8 Groepe rotte

was verdeel in 4 gedragsgroepe, en 4 neurochemiese groepe, wat lukraak verdeel was tydens spening (Post-natale dag (PND) 21) en blootgestel aan of 8 weke SSI, of 8 weke normale sosiale omstandighede.

Agomelatien (40mg/kg/dag) of 'n 1% hidrosie-etielsellulose mobiele fase is toegedien 16H00 deur die intraperitoneale roete vir die laaste 14 dae van isolasie. Gedragsanalises van anhedonie, geheue, lokomotor aktiwiteit en gedragswanhoop was geanaliseer op die laaste 4 dae van behandeling deur gebruik te maak van die sukrose voorkeur toets (SVT), voorwerp herkenningstoets (VHT), oopveldtoets (OFT) en geforseerde swemtoets (GST), onderskeidelik. Die oorblywende groepe was onthoof aan die einde van die 8 weke waarna plasma en brein weefsel verkry was vir die analisering van brein monoamien en lipiedperoksiedase konsentrasies met behulp van hoë werkverrigting vloeistof kromatografie (HVC), en ensiem-gekoppelde immunosorberende toets (EGIT), onderskeidelik. Plasma kortikosteron en superoksied dismutase (SOD) aktiwiteit was ook bepaal met EGIT. 'n Een-weg ANOVA met die geskikte post-hoc toetse was uitgevoer.

### 3. Resultate

SSI het 'n beduidende verhoging in immobiliteit veroorsaak in die GST sonder om die lokomotor aktiwiteit te beïnvloed, en het ook 'n neiging gewys tot die verlaging in beide die swem en klim gedrag, alhoewel geen verskil gesien was in die VHT nie. SSI het ook 'n beduidende vermindering in sukrose voorkeur gewys in vergelyking met die sosiale kontrole groep. Kortiko-hippokampus monoamiene her ongeaffekteerd gebly, behalwe vir die neiging tot 'n vermindering in striatale 5-HT en 'n verhoogde die 5-HT metaboliet (5-HIAA) in die hippokampus. Geen veranderinge in lipied peroksiedase, SOD of kortikosteron in SSI rotte was gevind nie. Agomelatien behandeling het minimaal tot geen effekte in normale sosiale rotte gehad nie. Ook het agomelatien geen effek gehad op lokomotor aktiwiteit nie, maar het die immobiliteit in SSI rotte beduidend verlaag en het selektief die klim gedrag verhoog sonder om die swem gedrag te affekteer. Agomelatien het beduidend die sukrose voorkeur verlaag in SSI rotte in verglyking met normale sosiale rotte, maar het geen effek gehad op geheue nie. Agomelatien het ook beduidend die lipied peroksidase aktiwiteit en basale kortikosteron vlakke verhoog in SSI rotte i.v.m. SSI rotte wat die mobiele fase ontvang het. Striatale dihidrosie-fenielasynsuur (DOPAC) vlakke was beduidend vrhoog in SSI rotte i.v.m SSI rotte wat die mobiele fase ontvang het.

### 4. Gevolgtrekking en Bespreking

SSI het depressiewe, bio-gedragsverandering in rotte veroorsaak, spesiek gedragswanhoop met verminderde hanterings-strategie, anhedoniese manifestasies en streeks brein monoamien

veranderinge. Maar, SSI het nie geslaag om kognitiewe versteurings te induuser of lipied peroksidase, SOD of basale kortkosteron vlakke te verander nie. Die bevindinge wys matige, maar swak gesigsvaliditeit vir ED. Agomelatien het wel antidepressiewe effekte gedemonstreer in die model, wat aandui op 'n omkering van SSI-verwante immobiliteit en opbering van hanterings-strategieë, veral die sukkel (noradrenergies-verwante) gedrag. Wat interessant voorkom, is dat beide die bevindinge ooreenstem met die voorgestelde noradrenerge meganisme van werking, alhoewel geen skeinbare 5-HT verwante aktiwiteit m.b.t swem-gedrag in die GST sowel as 5-HT-verwant veranderinge in die frontale korteks, striatum of hippokampus gesien was in SSI rotte nie. Agomelatien het wel 5-HT vlakke in die hippokampus van sosiale rotte verminder. Alhoewel die plasma kortkosteron vlakke onveranderd was in SSI, het agomelatien die basale kortkosteron vlakke verhoog in SSI rotte wat onverwags is. Hierdie aksie kan wel gekoppel word aan beide die kognitiewe versteurings in die VHT asook die verhoogde lipied peroksidase wat gesien was in SSI rotte. Agomelatien het beduidend die suiker voorkeur in die SVT verminder in beide die SSI en sosiale rotte, wat teenstrydig is met agomelatien se kliniese profiel wat die behandeling van anhedonie aanbetref asook sy voorgestelde dopamienergiese profiel. Ter afsluiting het agomelatien nie SOD aktiwiteit in beide SSI of sosiale rotte verander nie. Dus wys agomelatien antidepressiewe effekte in die SSI-model, veral in die GST, met duidelike veranderinge in die kortiko-hippokampus verwante monoamien hipotese van ED. Maar, die faal van die SSI-model op die gewenste MD-verwante patologie te herproduseer in die ander gedragstoetse, asook die kortkosteron en redoksanalises, dui dit op 'n meer omvattende interpretasie van die bevindinge, en verdere navorsing is dus nodig in die geval.

**Sleutelwoorde: depressie, vroeë ontwikkelings-stress, sirkadiese ritmes, agomelatien, frontale korteks, striatum, hippokampus**

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Effect of chronic morning vs. evening agomelatine administration on depressive-like behaviors in a neurodevelopmental animal model of depression.

(Paper presented as podium presentation at the South African Society for Basic and Clinical Pharmacology (SASBCP) at Wits University, Johannesburg, South Africa, 31<sup>st</sup> August 2015 – 2<sup>nd</sup> September 2015)

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**Chapter 1**

Figure 1:

Study design consisting of two arms: 1. Behavioral studies including a sucrose preference test (SPT), novel object recognition test (NOR), open field test (OFT) and a forced swim test (FST); 2. Neurochemical and neuroendocrine studies.

**Chapter 2**

Figure 1:

Structural and functional abnormalities in patients with MDD. Structural cortical and subcortical abnormalities have been observed in patients with MDD (aan het Rot et al., 2009)

Figure 2:

(A) Depiction of ventromedial prefrontal cortex (vmPFC) (in red) in midline views of each hemisphere. (B) Depiction of dorsolateral prefrontal cortex (dlPFC) (in blue) in later views of each hemisphere (Koenigs & Grafman, 2009).

Figure 3:

Schematic representation of the pathways by which alterations and/or quantity of maternal care and sensory stimuli, influence HPA-axis activity. Although ELS is often studied as an independent factor, it can be modulated by the same environmental conditions. ELS can exert lasting effects on neuronal plasticity in the hippocampus, which in turn results in permanent alterations in hippocampus-dependent cognitive function (Image adapted from Lucassen et al, 2013).

Figure 4:

Consequences of early life experience and adult stress on the HPA system and depression. The left hand side shows normal brain development and HPA axis functioning. The right hand side shows: (a) high levels of maternal care lead to a suppressed and reduced depression and anxiety-like behavior in adulthood; trauma, neglect and stress, during development (b) or during adulthood (c) may lead to a hyperactive HPA system with increased depression and anxiety-like behavior in adulthood (Ansorge et al., 2007).

Figure 5:

Regulation of the HPA-Axis. CRF is released and acts on the corticotrophins to release ACTH which reaches the adrenal cortex via the bloodstream to stimulate the release of glucocorticoids. Glucocorticoids, with its many functions (including synthetic forms such as dexamethasone) suppress CRH and ACTH synthesis and release thus inhibiting their own synthesis. At higher levels, glucocorticoids also impair, and may even damage the hippocampus (Nestler et al., 2002).

## Figure 6:

Regulation of the HPA axis activity. The hypothalamic PVN receives circadian inputs from the SCN and homeostatic/stress inputs from the brain stem and limbic areas. The PVN projects to the median eminence where it releases CRF into the portal circulation and passes to corticotrophs in the anterior pituitary which then releases ACTH into the venous circulation which then reaches the adrenal cortex where it activates the synthesis and secretion of cortisol in man and corticosterone in rodents. This in turn feeds back to the release of ACTH and later to CRF from the hypo (Walker, Terry et al., 2010).

## Figure 7:

Mechanisms of oxidative/nitrosative damage which may ultimately lead to depression. 5-HT (serotonin), HPA (hypothalamus-pituitary-adrenal), BDNF (brain-derived neurotrophic factor), ROS (reactive oxygen species), RNS (reactive nitrogen species), IDO (indolamine 2,3-dioxygenase), TPH (tryptophan hydroxylase), NK- $\kappa$ B (nuclear factor kappa-light-chain-enhancer of activated B cells), TNF (tumor necrosis factor), IL (interleukin) and IFN (interferon). Red arrows indicate increased production of cytokines, ROS and RNS, kynurenine, quinolinic acid, decreased 5-HT and glutamate excitotoxicity or an increased activity of IDO and TPH. Blue bars indicate an inhibitory effect of internal and external stressors on BDNF as well as inflammatory cytokines on activity of the hippocampus. Black crosses are indicative of no effect, whereas the hippocampus has no negative feedback in the HPA-axis. (Image adapted Lee et al., 2013)

## Figure 8:

The red arrows indicate the metabolism pathway of TRP along the kynurenine pathway. IDO is induced by cytokines and other immune mediators, whereas tryptophan 2, 3-dioxygenase is induced by TRP itself. Black arrows show alternative pathways of TRP converted into 5-HT and then to melatonin. Kynurenine aminotransferase II (KAT II) converts kynurenine into kynurenic acid, an NMDA receptor antagonist and kynurenine monooxygenase (KMO) converts kynurenine to quinolinic acid, an NMDA receptor agonist. (Image adapted from Molteni et al., 2013; Stone et al., 2002)

## Figure 9:

5-HT<sub>2C</sub> antagonists increase DA and NA release. When serotonergic 5-HT<sub>2C</sub> receptors on GABAergic interneurons in the brainstem are blocked by a 5-HT<sub>2C</sub> antagonist such as agomelatine, this prevents inhibition of downstream DA and NA release in the PFC (Stahl, 2014).

**Chapter 3**

## Figure 1:

Depressive-like behaviors (A-C) in socially and SIR rats receiving vehicle and SIR rats receiving agomelatine: (A), immobility (B), struggling and (C), swimming.

Figure 2:

5-HT and 5-HIAA concentrations (ng/mg brain) in (A, D) frontal cortex, (B, E) striatum and (C, F) hippocampus of the socially reared rats and SIR receiving vehicle and SIR receiving agomelatine.

Figure 3:

DA and DOPAC concentrations (ng/mg brain) in (A, D) frontal cortex, (B, E) striatum and (C, F) hippocampus of the socially reared rats and SIR receiving vehicle and SIR receiving agomelatine.

Figure 4:

NA concentrations (ng/mg brain) in (A) frontal cortex, (B) striatum and (C) hippocampus of the socially reared rats and SIR receiving vehicle and SIR receiving agomelatine.

## Chapter 5

Figure 1:

Behavioral, neuroendocrine and neurochemical treatment cohorts of SIR and socially reared rats receiving either vehicle, or agomelatine (Ago) treatments.

Figure 2:

Typical calibration curve and data for determination of the concentration of CORT in unknown samples.

Figure 3:

Principle of the SOD Assay kit. (Sigma-Aldrich®, 2004).

Figure 4:

Typical calibration curve obtained for the determination of % SOD activity.

Figure 5:

Typical calibration curve for determination of the concentration of MDA in unknown samples.

Figure 6:

% Sucrose preference for social and SIR rats receiving vehicle or agomelatine treatment

Figure 7:

Percentage time spent at the novel object in the NORT in social and SIR rats receiving either vehicle or agomelatine treatment

Figure 8:

Plasma corticosterone concentrations in social and SIR rats receiving either vehicle or agomelatine treatment.

Figure 9:

Percentage superoxide dismutase activity in social and SIR rats receiving either vehicle or agomelatine treatment.

Figure 10:

Concentration of malondialdehyde (MDA) nmol/ $\mu$ L in (A) frontal cortex, (B) striatum, (C) hippocampus in social and SIR rats receiving either vehicle or agomelatine treatment

5-HIAA – 5-hydroxy indole acetic acid  
5HT – Serotonin  
ACTH – Adrenocorticotrophic Hormone  
AD – Antidepressant  
AGO – Agomelatine  
ANOVA – Analysis of Variance  
ATP – Adenosine Triphosphate  
BDNF – Brain Derived Neurotrophic Factor  
CNS – Central Nervous System  
CMS – Chronic Mild Stress  
CORT – Corticosterone  
CRH – Corticotrophic Releasing Hormone  
CSF – Cerebrospinal Fluid  
DA – Dopamine  
DBS - Deep Brain Stimulation  
dlPFC – Dorsolateral Prefrontal Cortex  
DOPAC – Dihydroxyphenylacetic acid  
DSM – Diagnostic and Statistical Manual  
DST – Dexamethasone Suppression Test  
ECT – Electroconvulsive Therapy  
ED – Erge Depressie  
ELISA – Enzyme-linked Immunosorbent Assay  
ELS – Early Life Stress  
FC – Frontal Cortex  
FSL – Flinder Sensitive Line  
FST – Forced Swim Test  
GABA –  $\gamma$ -Amino-Butyric Acid  
GAD – General Anxiety Disorder  
GSH – Glutathione  
GST – Geforseerde Swem toets  
HPA – Hypothalamic-Pituitary-Adrenal

HEC – Hydroxyethylcellulose  
HVC – Hoë-werkverrigting vloeistof chromatografie  
IDO – Indolamine-2, 3 Dioxygenase  
IL – Interleukin  
IF – Interferon  
KMO – Kynurenine-3-monooxygenase  
KYNA – Kynurenine  
LOCF – Last Observed Carried Forward  
LPS – Lipopolysaccharides  
LPX – Lipid Peroxidation  
MDA – Malondialdehyde  
MDD – Major Depressive Disorder  
METH – Methamphetamine  
MOI – Monoamine Oxygenase (A/B) Inhibiter  
MRI – Magnetic Resonance Imaging  
MT – Melatonin  
NAcc – Nucleus Accumbens  
NA – Noradrenalin  
NAT – Noradrenalin Transporter  
NF – Nucleus Factor  
NMDA – N-Methyl-D-Aspartate  
NO-cGMP – Nitric Oxide-cyclic Guanosine Monophosphate  
NORT – Novel Object Recognition Test  
NSB – No sample blank  
OFT – Open Field Test  
OVT - Oopveldtoets  
PND – Post Natal Day  
PTSD – Post Traumatic Stress Disorder  
PVN – Paraventricular Nucleus  
PPI – Prepulse Inhibition  
PT – Pars Tuberalis

PFC – Prefrontal Cortex  
QA – Quinolinic Acid  
REM – Rapid Eye Movement  
ROS – Reactive Oxygen Species  
RNS – Reactive Nitrogen Species  
SSRI – Selective Serotonin Reuptake Inhibitor  
SNRI – Serotonin Noradrenaline Reuptake Inhibitor  
SWS – Slow Wave Sleep  
SHRP – Stress Hyporesponsive Period  
SCN – Suprachiasmatic Nucleus  
SOD – Superoxide Dismutase  
SPT – Sucrose Preference Test  
SSI – Stress-geïnduceerde social isolatie  
SN – Substantia Nigra  
SD – Sprague-Dawley  
SIR – Social Isolation Rearing  
SVT – Suiker voorkeur toets  
TCA – Tricyclic Antidepressant  
TNF – Tumor Necrosis Factor  
TRP – Tryptophan  
vmPFC – Ventromedial Prefrontal Cortex  
VTA – Ventral Trigeminal Area  
WHO – World Health Organisation  
WST – Water soluble tetrazolium salt

## 1. Problem statement.

Major depression (MD) is a commonly diagnosed mental disorder among adults (Richards, 2011) and still remains one of the most devastating illnesses (Wells et al., 1989). Previously seen as being an acute and self-limiting illness, many now consider depression as a chronic, lifelong illness (Richards, 2011). Chronic psychosocial and environmental stressors play a major role in the development of depression with genetic variability determining the susceptibility of the individual (Fone & Porkess, 2008; Harvey, 2008). Studies comparing concordance rates for MD suggests a heritability rate of about 37% (Manji et al., 2001), which is much lower when compared to bipolar disorder and schizophrenia (Belmaker et al., 2008).

Effective treatments, including drugs and psychotherapy, have drawn attention to the rather high frequency of negative outcomes (Paykel, 1994), which in turn has stimulated research in determining whether specific patient characteristics may possibly predict favorable vs. unfavorable outcomes (Black et al., 1988; Hirschfield et al., 1998). The diagnosis of depression requires a distinct change of mood, characterized by sadness or irritability and accompanied by at least several psychophysiological changes, such as sleep disturbances, loss of appetite, decrease in sexual desire, constipation, anhedonia, crying, suicidal thoughts as well as slowing of speech and actions (Belmaker et al., 2008). These changes must last for a minimum of 2 weeks and significantly interfere with work and family relations (Belmaker et al., 2008).

There are many promising hypotheses on the development of depression and antidepressant (AD) action which include the monoamine hypothesis, dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis which in turn affects corticotropin-releasing factor (CRF) and glucocorticoids as well as deficits in neurotrophic factors such as brain-derived neurotrophic factor (BDNF) (Nestler et al., 2002). Other hypotheses include the inflammatory cytokine hypothesis as well as abnormal glutamate receptors and circadian rhythm alterations (Cai et al., 2015). The noradrenergic (NA) and serotonergic (5-HT) systems originate in the brain stem and from there spread to almost the entire brain, which suggests a system capable of modulating many areas of feeling, thinking and behaving (Belmaker et al., 2008). The neurotrophin hypothesis of depression states that a deficiency in neurotrophic support may contribute to hippocampal pathology, such as hippocampal shrinkage (Savitz & Drevets, 2009; Nestler et al., 2002). This, together with disruptions in inhibitory-excitatory  $\gamma$ -amino butyric acid (GABA)-glutamate signaling, culminates in structural changes in critical brain regions regulating the stress response, eventually leading to deficits in memory and other cognitive processes (see Harvey et al., 2003; Krishnan and Nestler, 2008; Savitz & Drevets 2009; Renoir et al., 2012; Harvey et al., 2013).

However, the above brief overview of our current knowledge of the disorder clearly indicates that the neurobiology of depression is much more complex. Importantly, an abnormal cortisol response, such as a flatter diurnal cortisol pattern, implies an abnormal stress reactivity that correlates with a greater severity of depression (Sou tre et al., 1989; Hsiao et al., 2010; Doane et al., 2013), suggesting that altered circadian rhythms occupy a critical role in how the brain copes with stressful experiences and ultimately in regulating mood. In depressive subjects, alterations have also been detected in circadian rhythms linked to alterations in body temperature and several endocrine-metabolic parameters such as the secretion of cortisol, thyroid stimulating hormone, melatonin and monoamines, in comparison with healthy individuals (Soria & Urretavizcaya, 2009). It is therefore clear that altered circadian rhythm is a core biological manifestation of depression (McClung, 2013) that may mediate a variety of neuroendocrine abnormalities that in turn may muster the involvement of numerous biological processes, particularly cardiovascular, immune and metabolic function that are also disordered in depression. Activation of the HPA-axis is a mechanism by which the brain reacts to acute and chronic stress (Nestler et al., 2002) which stimulates the release of adrenocorticotrophic hormone (ACTH) that in turn stimulates cortisol release in humans or corticosterone in rodents (Nestler et al., 2002). What is relevant is that elevated levels of glucocorticoids under prolonged and severe stress not only impacts cardiovascular, immune and metabolic function noted above, but may damage hippocampal neurons (Nestler et al., 2002). In fact, hypercortisolemia has been advocated as an explanation for hippocampal shrinkage evident in imaging studies in depressed patients (Savitz & Drevets, 2009).

Exposing humans or animals to early-life adverse events, such as maternal separation or isolation, profoundly affects brain development and behavior in adulthood and may contribute to the occurrence of psychiatric disorders such as depression and schizophrenia (Fone & Porkess, 2008). Early-life social isolation rearing (SIR) of rat pups is known to produce late-life behavioral and biological changes consistent with the neurodevelopmental hypothesis of depression as well as schizophrenia (Fone & Porkess, 2008; Pryce and Klaus, 2013). At the behavioral level, SIR induces neophobia, aggression and cognitive rigidity, and impairs sensorimotor gating and social interaction (Fone & Porkess, 2008). Neuro-biologically these animals demonstrate reduced prefrontal cortical volume and decreased cortical and hippocampal synaptic plasticity (Fone & Porkess, 2008), while more recent work from our laboratory has demonstrated that SIR induces altered glutamate receptor binding (Toua et al., 2010) as well as oxidative stress and mitochondrial and immune-inflammatory dysfunction (M ller et al., 2011, 2013a), hallmark characteristics of depression.

SIR is associated with a number of monoaminergic deficits akin to depression and schizophrenia (M ller et al., 2013b). The latter includes hyper-function of mesolimbic DA systems, enhanced

presynaptic DA and 5-HT function in the nucleus accumbens (NAcc), attenuated mesocortical DA activity as well as reduced PFC and striatal 5-HT function (Fone & Porkess, 2008; Möller et al., 2013a, 2013b). Importantly, SIR-induced behavioral changes can be reversed with an AD (fluoxetine) (Brenes & Fornaguera, 2009) or an antipsychotic (Möller et al., 2011, 2013a), confirming its predictive validity for depression and schizophrenia, respectively. Interestingly, we have found that the anti-oxidant N-acetyl cysteine (NAC) may bolster the actions of clozapine in reversing the bio-behavioral effects following SIR in rats (Möller et al., 2013a, 2013b), thereby emphasizing a possible augmenting action when combining an antioxidant with traditional pharmacotherapy. One compound worth considering is therefore an antidepressant that not only targets circadian rhythms but also has antioxidant capabilities.

Agomelatine is a new generation AD presenting with melatonin (MT) M1/2 receptor agonist and 5HT<sub>2C</sub> receptor antagonist properties. Its primary mode of action is the re-entrainment of circadian rhythms by targeting SCN function, and the selective release of dopamine (DA) and NA in the frontal cortex (FC) without affecting that of 5-HT (Dremencov et al., 2015). Moreover, recent studies have shown that, like melatonin (Pandi-Perumal et al., 2006), agomelatine too has antioxidant properties (Aguar et al., 2013). Melanergic receptors are widely distributed in the brain with the highest density of melatonin (MT)<sub>1</sub> and MT<sub>2</sub> receptors found in the suprachiasmatic nucleus (SCN) and pars tuberalis (PT), but also in the FC, prefrontal cortex (PFC), cerebellar cortex, basal ganglia, substantia nigra (SN), hippocampus (HPC), ventral tegmental area (VTA), nucleus accumbens (NAcc), thalamus and the retina (Hardeland et al., 2011; Tardito et al., 2012). 5-HT<sub>2C</sub> receptors enjoy a similar distribution, but are located predominantly as somatodendritic heteroreceptors on GABAergic, glutamatergic, NAergic and DAergic neurons, so that tonic release of 5-HT and activation of 5-HT<sub>2C</sub> receptors will excite GABAergic neurons in the brainstem leading to inhibition of NA and DA release in the PFC (Millan et al., 2003; De Berardis et al., 2011). The expression of 5-HT<sub>2C</sub> and MT<sub>1</sub> receptors also shows a diurnal rhythmicity in the SCN and in the HPC (reviewed in Racagni et al., 2011; Tardito et al., 2012). The SCN targets hypothalamic nuclei that in turn modulates brain stem monoamine nuclei, implying that monoamines are indirectly affected by changes in SCN activity (McClung, 2013; Harvey & Slabbert, 2014). The circadian system therefore regulates multiple monoaminergic brain regions that control mood, anxiety, and motivated behaviors through local expression of clock genes, as well as indirect connections originating from the SCN (McClung, 2013). Moreover, the circadian system regulates many hormones and peptides in the brain and periphery that impact mood and reward, especially the HPA axis (McClung, 2013).

Appropriately targeting reward in depression is becoming increasingly important, with anhedonia one of the last symptoms to improve (Boyer et al., 2000). Moreover, serotonergic active antidepressants are less effective in managing hedonic symptoms (Nutt et al., 2007), while also worsening cognitive and emotional functioning (McCabe et al., 2010; Sansone & Sansone, 2010). These symptoms are related to 5-HT-directed suppression of DA-directed reward processing (Alén et al., 2013). Novel agents such as agomelatine that improve fronto-cortical DA without increasing 5-HT may have significant benefit in addressing anhedonia and thus provide an improved option to treating depression.

To the best of our knowledge, the ability of agomelatine to reverse SIR-induced bio-behavioral changes has not been undertaken. In our hands this model has demonstrated excellent validity for modeling the neurobiology and behavioral profile of schizophrenia (Toua et al., 2010; Trabace et al., 2012; Möller et al., 2011, 2012, 2013a, 2013b), while other laboratories have established its relevance for depression (Fone & Porkess, 2008; Pryce & Klaus, 2013). It is relevant then that depression forms an important co-morbid illness with schizophrenia (Buckley et al., 2009). This project will investigate the association between biological rhythms and specific pathology known to play a causal role in depression, namely immune-inflammatory, redox and cellular resilience pathways, and whether this is amenable to treatment with a novel AD agent designed to target circadian rhythms. This study is unique in its approach in that these questions will be addressed using a neurodevelopmental animal model never before used for this purpose, one that has robust validity for depression, but also schizophrenia, viz. the SIR model. The study will incorporate behavioral, endocrine and neurochemical analyses that will provide new knowledge on the role of circadian rhythms in depression, while also having some relevance for schizophrenia. Harnessing this knowledge may then be exploited to change how we see these illnesses and inform on approaches to improve current treatment strategies. This study will also extend the validity of agomelatine as a novel AD compound, while at the same time allowing a better understanding of its mode of action at behavioral and biologic levels.

## 2. Research problem, Aims and Objectives.

### 2.1 Hypothesis:

The efficacy of current ADs is disappointing at best, being about 55-60% effective in most cases (Nestler et al., 2002). These treatments are restricted to actions on monoaminergic systems, such as the 5-HT, DA or NA reuptake inhibitors, monoamine oxidase inhibitors (Nestler et al., 2002), and various atypical agents such as mirtazepine and trazodone. The circadian system plays a major role to ensure optimal functioning of the organism and its adaptation to environmental changes (Mairesse et

al., 2012). Disruption of circadian rhythms is a core symptom and therapeutic target in mood disorders (Mairesse et al., 2012). Although optimal AD treatment should therefore act as a resynchronizer of disorganized circadian rhythms, most ADs only partially meet this requirement (Mairesse et al., 2012), while serotonergic antidepressants can actually worsen sleep rhythms. By virtue of its rationale and targeted action on melatonergic and serotonergic receptors, agomelatine resynchronizes circadian rhythms with either a direct and/or an indirect action on regional brain monoamines. Furthermore, actions on stress-associated glutamate release (Morley-Fletcher et al., 2011) and oxidative stress (Aguilar et al., 2013) may contribute to its therapeutic properties. Despite the unique effects of agomelatine on frontal cortical monoamine release, the precise contribution of the drug's direct regulation of monoamine vs. melatonergic activity on the one hand as opposed to its indirect action on monoaminergic activity via re-entrainment of biological rhythms on the other hand still requires clarification. Similarly, the possible role of a putative antioxidant action for the drug in both humans and translational animal models requires further study.

Based on this, we will make the following hypothesis:

SIR will induce cognitive, anhedonic and depressive-like behavioral manifestations vs. socially housed animals that will be reversed or attenuated by agomelatine treatment. Moreover, SIR will be accompanied by altered basal corticosterone as well as hippocampal and cortico-striatal monoamines and redox status that too will be reversed by agomelatine.

## 2.2 Aims and Objectives:

- The first aim of this study was to establish that SIR induces cognitive, anhedonic and depressive-like behavioral manifestations in SIR vs. socially housed animals.
- To demonstrate that chronic afternoon agomelatine treatment reverses SIR-induced cognitive, anhedonic and depressive-like behavioral manifestations.
- To demonstrate altered basal corticosterone release, as well as altered hippocampal and cortico-striatal redox status consisting of lipid peroxidation and superoxide dismutase in SIR vs. socially housed animals, and whether any such changes can be reversed by agomelatine.
- To demonstrate altered brain monoamine levels in SIR vs. socially housed animals, and whether these changes can be reversed by agomelatine.

### 3. Project layout.

This study will comprise two arms, a behavioral study and a neurochemical and neuroendocrine study. Male Sprague-Dawley rats (12/group) will be used throughout the study.

- The behavioral study will evaluate changes in anhedonia, locomotor activity, visual learning and memory as well as depressive-like behaviors (Expt 1), as illustrated in Fig 1.
- The neurochemical study will evaluate regional brain monoamine levels and lipid peroxidation, while the neuroendocrine-peripheral study will evaluate plasma corticosterone and superoxide dismutase (Expt 2), as illustrated in Fig 1.

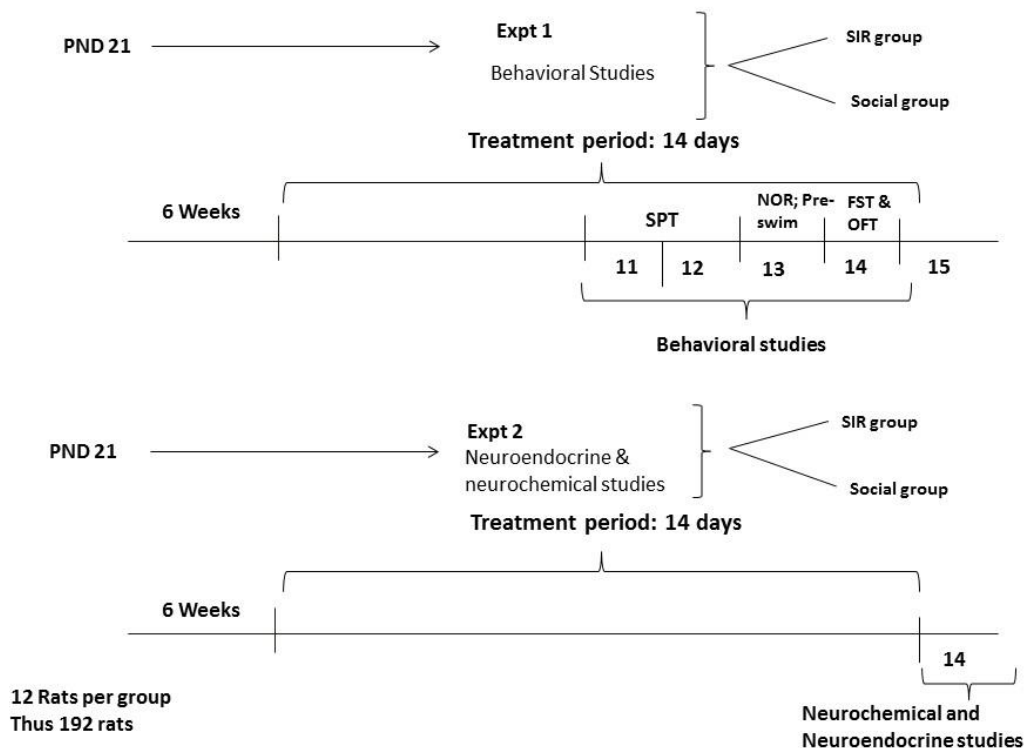


Fig. 1. Study design consisting of two arms: 1. Behavioral studies including a sucrose preference test (SPT), novel object recognition test (NOR), open field test (OFT) and a forced swim test (FST); 2. Neurochemical and neuroendocrine studies.

## 4. General points

This dissertation has been written and submitted in the article format for thesis/dissertation submission, as approved by the North-West University. The format includes an introductory chapter (chapter 1), a chapter covering the relevant literature overview (chapter 2), a chapter containing one full length article for submission to a peer-review neuroscience journal (chapter 3), a chapter describing the conclusion of the study as well as providing recommendations for future studies (chapter 4), and a list of addendums. The article chapter has been prepared to present the novelest and impactful data from the study. To this end, the article will be prepared according to the house style and author instructions of that particular journal. The house style and the instructions to authors are provided through a web-link to the journal home page. All the work performed during the course of the study will be presented either in the journal article or in the addenda. Consequently, data from the behavioral studies (the OFT and FST assessments) as well as the monoamine analyses, will be the focus of a full length research paper intended for submission to *Acta Neuropsychiatrica* (Cambridge University Press). Additional work performed during the course of the study, or data deemed unsuitable for the article, will be presented in the Addenda.

### Study responsibilities:

D Coutts performed all behavioral procedures, including treatment of the animals, performed all neurochemical analyses, undertook the statistical analysis, and prepared the first draft version of the manuscript. BH Harvey devised the concept of the study as well as the layout of the study and of the concept article. However, during the course of the study, Mr Coutts was assisted by Mr Walter Dreyer (lipid peroxidation, superoxide dismutase and corticosterone analysis), Mr Francois Viljoen (monoamine analysis), Mr Hylton Bunting (animal breeding and welfare) and Mrs Antoinette Fick (decapitation and brain dissection).

### Study site:

All aspects of the work were performed in the vivarium (behavioral analysis), the Laboratory for Analytical and Molecular Biology (LAMB) (lipid peroxidation, superoxide dismutase and corticosterone analysis) and the Analytical Technology Laboratory (ATL) (monoamine analysis) of the NWU.

## 1. Introduction

Depression represents a range of environmental, genetic and neurochemical determinants that each occupies a distinct role in the etiology, progression and treatment response of disorders which range from depression on one end, to psychosis on the other (Harvey, 2008). Depression can have profound effects on the ability to function and has immediate and long-term detrimental effects both when regarded categorically as a disorder and dimensionally as a continuum of symptoms (Rice et al., 2002; Bujoreanu et al., 2011). Major depressive disorder (MDD) can span the life time of a patient, but more importantly, depression in childhood and adolescence is strongly predictive of adult MDD (Rice et al., 2002). Depression compromises memory, diminishes hope and alters perception of oneself and others, and intrudes upon fundamental biological processes that regulate sleep, appetite, sexual activity, autonomic function and neuroendocrine regulation (Meyer et al., 2001).

Taking into account the natural history, mental suffering, and medical morbidity associated with MDD, the World Health Organization (WHO) estimated that during any 12-month period, about 34 million depressed individuals worldwide are untreated (Richards & Richardson, 2012), and ranked this disorder as the third leading contributor to the global disease burden (Huang, Lu et al., 2014), thus if left untreated in early life there is a significant risk that the disorder may persist into adulthood as MDD, or be associated with substance abuse, recurrence of depression and suicidal behaviors (Bujoreanu et al., 2011).

Current treatments for depression are inadequate for many individuals, while progress in new drug development is slow, much of which can be ascribed to our inherent poor understanding of the neurobiology of the illness (Nestler et al., 2002). Selective serotonin reuptake inhibitors (SSRI's), which are the most prescribed antidepressants (ADs) (Nestler et al., 2002), have a better side-effect profile and are easier to prescribe in comparison with other AD's, especially the tricyclic antidepressants (TCA) (Nestler et al., 2002). Newer medications essentially have the same mechanism of action as the older TCA's, resulting in no better efficacy in treating depressed patients (Nestler et al., 2002). Nevertheless, the improved tolerability of the SSRI's has improved compliance and may in this way lead to an overall better outcome. Only 50% of depressed patients treated with an antidepressant, regardless of class, achieve complete remission (Dagytė et al., 2010), highlighting that the search for a suitable biological target of MDD remains elusive. Moreover, poor tolerability and late onset of therapeutic efficacy further increase the risks of unsuccessful treatment (Dagytė et al., 2010).

Circadian rhythm disturbances are a common feature of MDD, including sleep homeostasis such as insomnia/hypersomnia, non-restful sleep, diurnal variation in mood, decreased slow wave sleep (SWS), and reduced sleep latency (Huang et al., 2014). Resynchronizing the circadian system is a proposed approach with which to treat MDD, and includes light therapy, restructuring of sleep/wake timing and melatonin treatment (Huang et al., 2014). Since monoamine-based AD's do not directly affect depression-associated circadian disorders, treatments targeted at the resynchronization of the circadian system may be effective in treating the disorder (Huang et al., 2014).

## 2. Diagnosis of depression

Since the 1960's, depression has been diagnosed as "major depression" based on the symptomatic criteria set forth in the Diagnostic and Statistical Manual (DSM-IV, 2000) (Nestler et al., 2002). However, according to the new DSM-V (American Psychiatric Association 2013) it is clear that neither the core criterion symptoms applied to the diagnosis for MDD nor the requisite duration of at least 2 weeks has changed from the DSM-IV. Criteria A for MDD in the DSM-V is identical to that of the DSM-IV for MDD as is the requirement for clinically significant distress or impairment in social, occupational, or other areas of life although this is now listed as Criterion B rather than Criterion C. (American Psychiatric Association, 2013). Criterion B for MDD are symptoms that cause clinically significant distress or impairment in social, occupational or other important areas of functioning as mentioned above, while Criterion C, are symptoms which are not due to direct physiological effects of a substance (e.g. a drug of abuse, a medication) or a general medical condition (e.g. hypothyroidism) (American Psychiatric Association, 2013). Consequently, depression should not be seen as a single disease, but as a syndrome comprised of numerous diseases of distinct causes and pathophysiologies (Nestler et al., 2002).

The common feature of "Depressive Disorders" and "Bipolar and Related Disorders", as described in DSM-V, is that all these disorders present with sad, empty or irritable mood accompanied by somatic and cognitive changes that significantly affects the individual's capacity to function. Persistent depressive disorder (dysthymia), a more chronic form of depression, can be diagnosed when the mood disturbances continue for at least 2 years in adults or 1 year in children (American Psychiatric Association 2013). This diagnosis, which is new in the DSM-V, includes both the DSM-IV diagnostic categories of chronic major depression and dysthymia (American Psychiatric Association 2013).

## 3. Genetic and environmental causes of depression

MDD is a common and prevalent disorder (Saveanu & Nemeroff 2012). Epidemiologic studies show that roughly 40-50% of the risk for MDD is genetic (Nestler et al., 2002), which makes it a highly

heritable disorder, at least as heritable as type II diabetes, hypertension, asthma and certain cancers (Nestler et al., 2002). The search for specific genes that confer this risk has been frustrating due to depression being a complex phenomenon with many genes possibly involved. In addition, vulnerability to MDD is only partly genetic, with non-genetic factors, such as chronic psychosocial and environmental stressors determining the susceptibility of the individual (Fone & Porkess, 2008; Harvey et al, 2008; Nestler et al., 2002). A number of studies have shown that the onset of mood disorders such as MDD is undoubtedly impacted by stressful life events that occur in childhood (Saveanu & Nemeroff, 2012). Individuals with a history of childhood abuse are not only at higher risk for developing MDD, but also posttraumatic stress disorder (PTSD), panic disorder, generalized anxiety disorder (GAD), bipolar disorder and schizophrenia (Saveanu & Nemeroff, 2012).

Early life trauma has also been shown to impact the clinical course of depression. Patients with depression who have a history of childhood trauma have (a) lower rates of remission and recovery, (b) longer episodes of depression, (c) a more chronic disease course, and (d) earlier onset of depressive symptoms (Saveanu & Nemeroff, 2012).

## 4. Pathophysiology

### 4.1 Neuroanatomy

Although there is little doubt that various neurotransmitter systems are pathologically involved in MDD, no single neurotransmitter system seems to be solely responsible (Saveanu & Nemeroff, 2012). A more recent conceptual approach to the biology of MDD is to consider it a disorder involving several critical brain regions and associated pathways (Saveanu & Nemeroff 2012). Thus, if we consider behavioral despair as well as anhedonia as characteristics of MDD (Martinowich & Lu, 2008), these sets of behaviors are likely controlled by two interacting brain systems: the stress system (hippocampus-hypothalamus pituitary adrenal (HPA)-pathway) and the reward system (ventral tegmentum area – nucleus accumbens (VTA-Nac), and VTA-prefrontal cortex (PFC) (Martinowich & Lu, 2008). The hippocampal circuitry includes functional components for learning and memory as well as negative regulation of the HPA-mediated stress pathway, which are both altered in MDD (see section: 4.3.3) (Martinowich & Lu, 2008). Structural brain neuroimaging using magnetic resonance imaging (MRI), which has allowed the identification of distinct brain regions and associated circuits, has revealed altered volumes of several brain regions in patients with MDD, most notably a reduction in the hippocampal and caudate nucleus size and an increase in pituitary volume (Saveanu & Nemeroff, 2012).

The development of neuroimaging techniques has opened up the potential to investigate structural and functional abnormalities in living depressed patients (Hasler, 2010). Because of the diversity of techniques used, the relatively small and heterogeneous study samples, as well as the limited overlap of results across imaging paradigms, it is nonetheless difficult to reliably identify neuronal regions or networks with consistently abnormal structure or function in MDD (Hasler, 2010). A technique known as function-location meta-analysis aims to determine the nature of consistent activity across experiments within a certain class of imaging studies, and involves searching for locations of functional agreement among statistically significant effects (Fox et al., 1998). Functional imaging studies such as symptom provocation and resting state studies provide the most limited overlap of findings, due to many different symptoms that may contribute to the diagnosis of MDD (Linden, 2006).

A recent meta-analytic study found the best evidence for abnormal brain activity in MDD to be in the lateral frontal and temporal cortices, insula and cerebellum (Hasler, 2010). In these brain regions, activity was found to be decreased at rest, showed a relative lack of activation during induction of negative emotions, and an increase in activity following treatment with SSRI's (Hasler, 2010). Opposite changes may exist in ventromedial frontal areas, striatum and possibly other subcortical brain regions (Fitzgerald et al., 2008). Altered volumes in several brain regions in patients with MDD, most notably a reduction in hippocampal volume and caudate nucleus size as well as an increase in pituitary volume, may be more likely caused by early life stress during a critical period in brain development than to depression per se (Saveanu & Nemeroff, 2012). The subgenual cingulate has become a particular area of interest in depression research and implicate it as a focus of dysfunction (Greicius et al., 2007). Increased metabolism in the subgenual cingulate declines towards the normal range in patients with MDD who responds to treatment (Kennedy et al., 2001; Mayberg et al., 2000), while it was the most active region in a group of healthy controls when they evaluated emotional valence of pleasant and unpleasant words (Maddock et al., 2003).

Moreover, humans with lesions in the subgenual prefrontal cortex showed abnormal autonomic responses to social stimuli, while studies in rats have shown that left-sided lesions to this region have increased sympathetic arousal and corticosterone responses to restraint stress (Hasler, 2010). Despite the considerable heterogeneity of findings from neuroimaging studies, there is convergent evidence for the presence of abnormalities in the subgenual prefrontal cortex in MDD, as illustrated in Fig. 1 (Hasler, 2010).

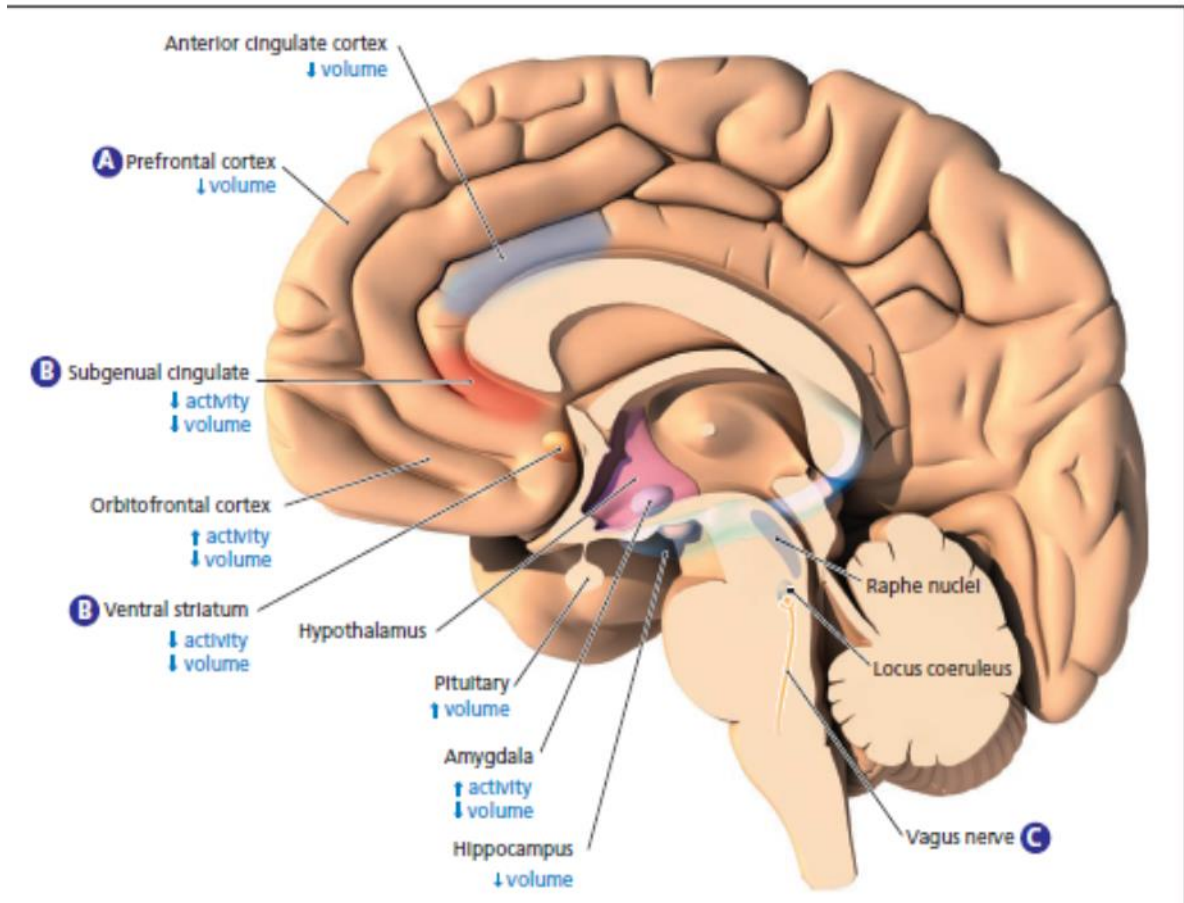


Fig. 1: Structural and functional abnormalities in patients with MDD. Structural cortical and subcortical abnormalities have been observed in patients with MDD (Figure from aan het Rot et al., 2009).

Furthermore, the prefrontal cortex consists out of the ventromedial prefrontal cortex (vmPFC) and the dorsolateral prefrontal cortex (dlPFC) (Fig.2A and 2B respectively). The vmPFC includes the hypo and periaqueductal grey which mediate the visceral autonomic activity associated with emotion, and the ventral striatum which signals reward and motivational value (Koenigs & Grafman 2009). The vmPFC (Fig. 2A) also has dense reciprocal connections with the amygdala, which is involved in threat detection and fear conditioning (Koenigs & Grafman 2009). In contrast, the dlPFC (Fig.2B) includes portions of the middle and superior frontal gyri on the lateral surface of the frontal lobes, which receives input from specific sensory cortices, and has dense interconnections with premotor areas, the frontal eye fields and lateral parietal cortex (Koenigs & Grafman 2009).

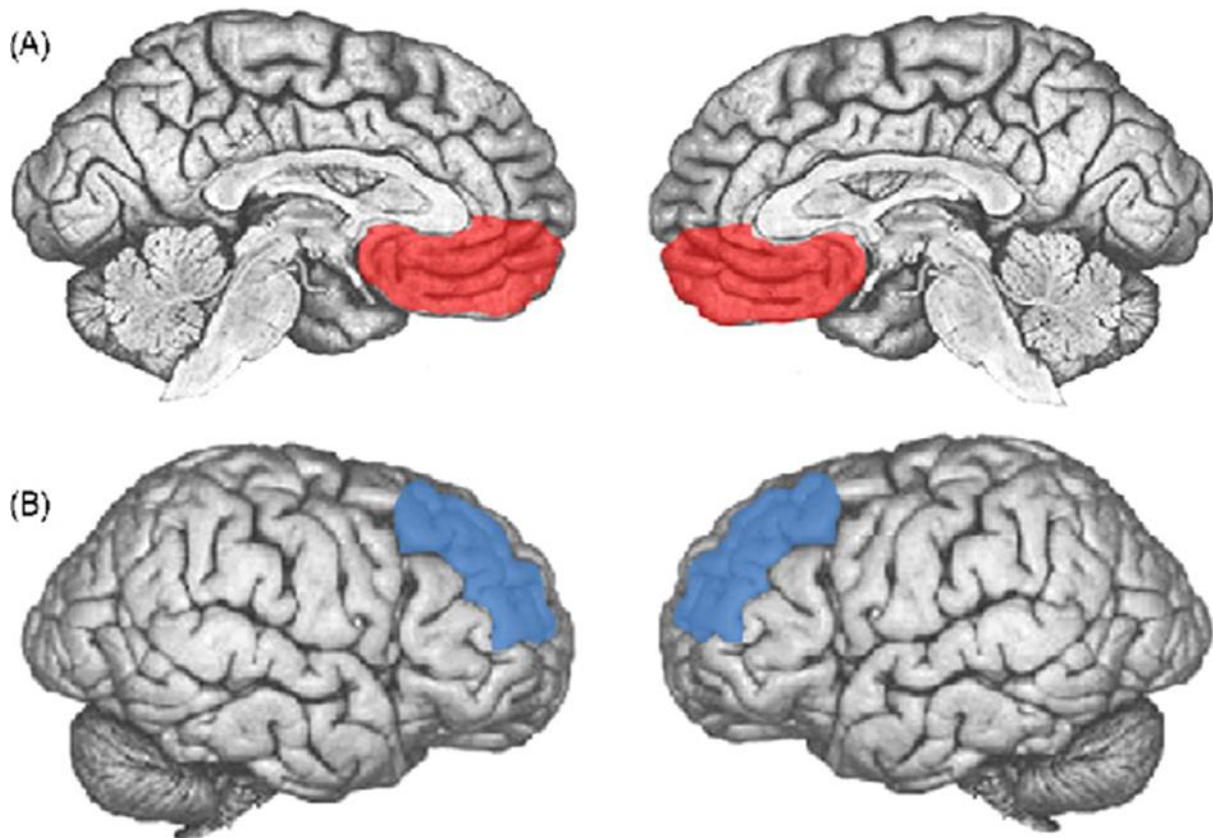


Fig. 2: (A) Depiction of ventromedial prefrontal cortex (vmPFC) (in red) in midline views of each hemisphere. (B) Depiction of dorsolateral prefrontal cortex (dlPFC) (in blue) in lateral views of each hemisphere (Figure from Koenigs & Grafman, 2009).

A study performed by (Koenigs et al., 2008) using the “lesion method” (an approach whereby a focal area of brain damage is associated with the development of a change in some aspect of cognition or behavior), directly addressed the question of whether the vmPFC and/or dlPFC play a critical role in the development of depression. If the vmPFC hyperactivity and dlPFC hypoactivity are indeed involved in the pathogenesis of depression as revealed by functional imaging studies, then damage to either area would presumably affect the development of depression (Koenigs & Grafman 2009). Lesions to the vmPFC would deliberate resistance to depression, while lesion to the dlPFC would deliberate vulnerability to depression (Koenigs & Grafman, 2009). And indeed, (Koenigs et al., 2008), did find opposite effects of vmPFC and dlPFC damage on depression. Veterans with bilateral vmPFC damage reported significantly lower depression severity as to veterans with damage involving other areas of the brain or those with no brain damage, predominantly for the cognitive/affective symptoms of depression (Koenigs et al., 2008).

## 4.2 Neurodevelopmental aspects of depression

There is clear evidence that MDD is passed on in families (Meyer et al., 2001). However, growing consensus indicates that genes cannot entirely account for the transmission of MDD across generations (Meyer et al., 2001). Indeed, there is widespread agreement that complex psychiatric illnesses, such as MDD, are heterogeneous in their etiology and course (Meyer et al., 2001). Early life stress is an established predictor of adverse outcomes across the lifespan encompassing neurocognitive, behavioral, health and psychiatric domains (Nugent et al., 2011). Investigations of early life stress (ELS) in humans have examined a wide range of adverse life experiences such as natural disaster, childhood maltreatment (sexual/physical abuse, severe neglect) or adverse family environment (maternal depression, parental loss, divorce) (Nugent et al., 2011), and have concluded that ELS is an important risk factor for several psychiatric disorders. However, ELS does not invariably lead to dysfunction, nor is it a specific risk factor for any particular disorder (Nugent et al., 2011). Such divergent outcomes can be explained in part by gene-environment interactions, in which genetic differences influence the likelihood that exposure to ELS will result in psychopathology (Nugent et al., 2011).

During prenatal and early postnatal development, the stress system undergoes dramatic changes, with various components developing at different rates (Meyer et al., 2001). Adrenocorticotrophic hormone (ACTH) has been detected in fetal life as early as 7 weeks, and by 26 weeks, levels are comparable to those of a newborn (Meyer et al., 2001). In response to stress the fetal hypothalamus increases the production of corticotropin-releasing hormone (CRH), triggering heightened secretion of ACTH, which in turn stimulates the production of adrenal cortisol, which inhibits the activity of the hypothalamus and pituitary (Meyer et al., 2001). It has been suggested that exposure to environmental stress in prenatal and early postnatal life is likely to result in permanent biological changes (Fig. 3) (Meyer et al., 2001) and that prenatal stress exposure may permanently alter neural circuitry, with regard to its impact on serotonin (5-HT).

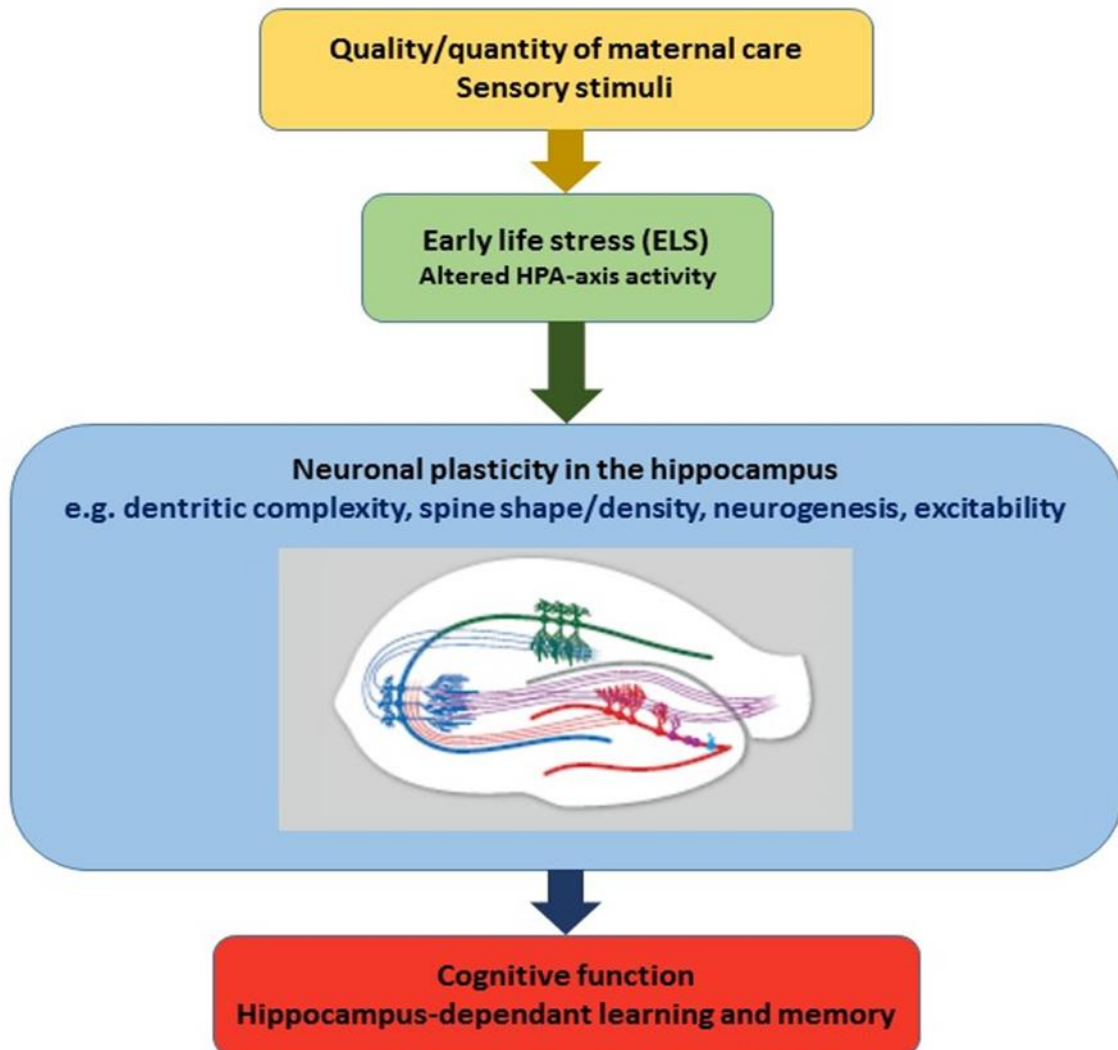


Fig 3: Schematic representation of the pathways by which alterations and/or quantity of maternal care and sensory stimuli, influence HPA-axis activity. Although ELS is often studied as an independent factor, it can be modulated by the same environmental conditions. ELS can exert lasting effects on neuronal plasticity in the hippocampus, which in turn results in permanent alterations in hippocampus-dependent cognitive function (Figure adapted from Lucassen et al., 2013).

Moreover, a preclinical study suggest that increased glucocorticoid levels may be related to a stress-induced decrease in activity of placental  $11\beta$ -hydroxysteroid dehydrogenase, a critical enzyme in prenatal development which converts glucocorticoids to inactive products and thereby reducing harmful effects to the fetus (Meyer et al., 2001).

Other preclinical studies observed that the stress hypo-responsive period (SHRP) (a period between approximately post-natal day (PND) 4 through 14 that presents with markedly reduced adrenocortical

response to mild stress (Sapolsky et al., 1985) of an animal model could be used for exploring the regulatory function of early maternal care (Meyer et al., 2001). The SHRP is characterized by relative inactivity of the stress response (Meyer et al., 2001). This down-regulation of the stress system appears to be dependent upon maternal proximity (Meyer et al., 2001). Even as infants become increasingly autonomous, parenting continues to serve an important regulatory function (Meyer et al., 2001).

In a 20-year follow-up study of children whose mothers were diagnosed with unipolar depression, bipolar 1 or 2, or had no history of psychiatric illness, Meyer and colleagues showed that adolescents whose mothers displayed a highly angry or irritable parenting style during childhood were more likely to exhibit exaggerated ACTH activation (Meyer et al., 2001). On the other hand, mothers who used considerate control strategies imparted a significant buffer effect on their children's stress responses, as evidenced by a less profound increase in ACTH levels following CRH challenge (Meyer et al., 2001).

Present research builds on existing life-stress models by adopting a transactional developmental perspective that considers the mechanisms through which children contribute to their environment (Rudolph et al., 2000). Specifically, traditional stress-exposure models conceptualize depression merely as a reaction to stress and, therefore, highlight the impact of context on children's development (Rudolph et al., 2000). Understanding the association between the individuals' contributions to the stressful circumstances in which they live and their experience of psychopathology is particularly important in youth, given that early life experiences set the stage for future adaptive or maladaptive functioning (Rudolph et al., 2000). Adopting a transactional approach may help to elucidate the mechanisms underlying the continuity of depression across the life span (Rudolph et al., 2000). Early programming of neurobiological systems that are implicated in regulating emotion and stress responses appears to mediate increased stress vulnerability and depression risk later in life (Heim & Binder 2012).

#### 4.3 Neurochemistry

One of the earliest theories attempting to explain the pathogenesis of depression was the monoamine hypothesis stating that depression may be a result of decreased availability of monoamine neurotransmitters such as 5-HT and NA in the central nervous system (CNS) (Haase & Brown, 2015). There are many other promising hypotheses of depression and AD action, also including dysregulation of the HPA axis with subsequent effects on CRF and glucocorticoids, and deficits in neurotrophic factors such as brain-derived neurotrophic factor (BDNF) (Nestler et al., 2002). The neurotrophin hypothesis of depression states that a deficiency in neurotrophic support may contribute to hippocampal pathology, such as hippocampal shrinkage (Savitz & Drevets, 2009; Nestler et al., 2002).

This, together with disruptions in inhibitory-excitatory  $\gamma$ -amino butyric acid (GABA)-glutamate signalling, culminates in structural changes in critical brain regions regulating the stress response, eventually leading to deficits in memory and other cognitive processes (see Harvey et al., 2003; Krishnan & Nestler, 2008; Savitz & Drevets, 2009; Renoir et al., 2012; Harvey et al., 2013).

In particular, many cases of depression stem at least in part from disturbances in brain circuits that convey signals through certain neurotransmitters of the monoamine class (Nemeroff, 1998). These biochemical pathways, all derived from amino acids, include 5-HT, NA and DA (Nemeroff, 1998). The monoamine hypothesis explains that depletion of the aforementioned monoamines in the hippocampus, limbic system and frontal cortex are responsible for depressive symptoms (Girish et al., 2010). Serotonergic, noradrenergic and dopaminergic cell bodies are located in the midbrain and brainstem and project to distal areas of the brain (Hasler, 2010), especially the basal ganglia, thalamus and brain stem (Nemeroff, 1998).

#### 4.3.1 The monoamine hypothesis

##### 4.3.1.1 Serotonin

5-HT is a simple monoamine synthesized from tryptophan and is distributed throughout the body with almost 90% produced by enterochromaffin cells in the gastrointestinal tract (Berger et al., 2009), while most of the remaining 5-HT is produced in the 5-HTergic neurons of the raphe nuclei and are restricted to a relatively small area of the brain (Hornung, 2003). Evidence from an impressive array of studies supports a role for 5-HT systems in depression. Post-mortem, cerebrospinal fluid (CSF) and neuroendocrine studies have demonstrated reduced activity of serotonergic neurons in depressed patients (Saveanu & Nemeroff, 2012). The most direct evidence for an abnormally reduced function of central 5-HTergic system comes from studies using tryptophan depletion, which leads to a reduction in central 5-HT synthesis (Hasler, 2010). Such a reduction leads to the development of depressive symptoms in subjects with an increased risk for depression (i.e. subjects with MDD in full remission, healthy subjects with a family history of MDD), possibly mediated by increased 5-HT brain metabolism in the ventromedial prefrontal cortex (Neumeister et al., 2002; Neumeister et al., 2004).

Defects in 5-HTergic circuits have also been found to dampen NA signaling (Nemeroff, 1998), indicating the important co-operation between these two monoamines. 5-HT-producing neurons interact with those that secrete or control the release of NA (Nemeroff, 1998). Thus, 5-HT depletion might contribute to depression by affecting other neurocircuits as well; 5-HT-producing cells extend into the brain regions thought to participate in depressive symptoms – including the amygdala (area involved in emotions), the hypothalamus (involved in appetite, libido and sleep), and cortical areas that participate in cognition and other higher processes (Nemeroff, 1999).

There is also evidence for abnormalities of 5-HT receptors in depression, with the most solid evidence pointing to the 5-HT<sub>1A</sub> receptor, which regulates 5-HT function (Hasler, 2012). Decreased availability of this receptor has been found in multiple brain areas of patients with MDD, although this abnormality is not highly specific for MDD and has been found in patients with panic disorder and temporal lobe epilepsy (Hasler, 2012). Furthermore, 5-HT<sub>1B</sub> are present in many parts of the brain; basal ganglia, striatum and frontal cortex and is thought to act as an autoreceptor, inhibiting the release of 5-HT, but has a secondary role as a controlling terminal heteroreceptor of secretion of other neurotransmitters such as acetylcholine, glutamate, DA, NA and GABA (Pytliak et al., 2011). Ex vivo studies have indicated a possible interaction between the 5-HT transporter (SERT) and 5-HT<sub>2B</sub> receptors (Bevilacqua et al., 2011) and the control of SERT in the raphe neurons (Launay et al., 2006), while in vivo studies confirmed a contribution to behavioral and physiological effects of 5-HT releasers such as ecstasy and dexfenfluramine (Doly, et al., 2008, 2009; Banas et al., 2011).

The blockade of the 5-HT<sub>2C</sub> receptors are under investigation for the treatment of schizophrenia, depression and anxiety disorders as well as Parkinson's disease (Hamon & Blier, 2013). Systemic exposure to a 5-HT<sub>2C</sub> receptor agonist, suppresses 5-HT and DA firing in the dorsal raphe nucleus and ventral tegmental area (Boothman et al., 2006). A study by Millan et al., (2003) demonstrated that agomelatine, a melatonin (MT<sub>1/2</sub>) receptor agonist and 5-HT<sub>2C</sub> receptor antagonist increases the firing of NA neurons and NA release in the PFC, and showed that this effect of agomelatine was not reversed by melatonin antagonists which indicates that the NA effect of agomelatine is mediated via 5-HT rather than its melatonin agonistic properties. It can thus be stated that the 5-HT<sub>2C</sub> receptors negatively regulate monoamine transmission in the brain through suppression of firing of monoamine neurons and/or through local inhibition of transmitter release from the terminals of monoamine neurons (Dremencov et al., 2011).

A major reason for the continuing interest in 5-HT and depression is the fact that SSRI's are useful in treating depression in some patients (Cowen & Browning, 2015). Basic studies have shown intriguing results of repeated SSRI administration in animals such as an increase in hippocampal cell proliferation and enhanced expression of proteins such as BDNF relating to neuroplasticity (Sharp & Cowen, 2011). 5-HT neurons exert an inhibitory influence on DA neurons in the VTA (Ugedo et al., 1989) of which the mean firing rate is suppressed by escitalopram through enhanced activation of the 5-HT<sub>2C</sub> receptors likely in GABA neurons (Dremencov et al., 2009). The lack of therapeutic benefits of SSRI's in some depressed patients could be a result of the attenuation of DA transmission in the presence of 5-HT levels (Blier & El Mansari, 2013a). The inhibitory effects of SSRI's on catecholamine neurons may account for the residual symptoms often observed after remission with SSRI's (Blier & El Mansari, 2013a).

Apart from AD treatment, it is very interesting that light may influence mood through its ability to rapidly induce increases in 5-HT turnover (Stephenson et al., 2012). 5-HT production in the brain is slowest in the winter and directly related to the prevailing duration of bright sunlight with 5-HT transporter binding values being higher in individuals in the fall and winter compared to those investigated in the spring and summer (Stephenson et al., 2012). Pharmacogenetic studies on the influence of a 5-HT transporter polymorphism on antidepressant response to 5-HTergic treatment also support the hypothesis that light therapy shares the neurobiological mechanisms of action of drugs targeting the 5-HT system (Serretti et al., 2005).

#### 4.3.1.2 Noradrenaline

The major NAergic nucleus on the brain is the locus coeruleus (LC) in the brain stem and projects to almost all areas of the brain involved in the pathophysiology of depressive states such as the PFC or the hippocampus (Haenisch & Bönisch, 2011). Dysfunction of the central NAergic system has been hypothesized to play a role in the pathophysiology of MDD (Saveanu & Nemeroff, 2012), based upon evidence of increased activity of tyrosine hydroxylase (Ordway et al., 1994), and decreased density of the NA transporter (NAT) in the locus coeruleus of depressed patients (Klimek et al., 1997). The initial catecholamine hypothesis of affective disorders proposed that abnormally low availability of NA may lead to depressive symptoms, whereas elevated NA availability may lead to euphoric or manic symptoms (Ressler & Nemeroff, 2000). Measurements of NA receptor kinetics have also been consistently altered in depression and anxiety states (Ressler & Nemeroff, 2000). Early recognition of altered  $\alpha_2$  receptor binding in depression led to the “ $\alpha_2$  receptor supersensitivity hypothesis” purported to result in an overall decreased activity of the LC (Charney et al., 1981). However, these changes could also be seen as evidence for supersensitive post-synaptic NA activity.

One of the notable and puzzling aspects of treating depression is the fact that enhancement of either 5-HT or NA transmission can uplift mood (Wiles et al., 2012). However, part of the reason why NA acting AD's have not received as much attention as SSRI's is over safety concerns such as increased blood pressure and heart rate regulated by NA (Nutt & McAllister-Williams, 2013).

#### 4.3.1.3 Dopamine

DA innervation and receptor expression are present in early development, maturing during adolescence and forms stable patterns in young childhood (Money & Stanwood 2013). Although DA-containing neuronal pathways have been largely implicated in the pathophysiology of schizophrenia, much evidence now supports an important role for CNS DA circuits in depression (Saveanu & Nemeroff, 2012). In the CSF and jugular vein plasma, levels of DA metabolites have been found to be consistently reduced in depression, suggesting decreased DA turnover (Lambert et al., 2000). Striatal

DA transporter binding and DA uptake are also reduced in MDD, consistent with a reduction in DA neurotransmission (Meyer et al., 2001b).

In line with these findings is the fact that many investigators suggest that suboptimal therapeutic responses to SSRIs and SNRIs may be due in part to their relative lack of effects on brain DA circuits (Saveanu & Nemeroff, 2012). In fact, SSRI's inhibit DA release in the frontal cortex and as such may be counterproductive to an antidepressant effect.

Significant portions of patients receiving SSRI or SNRI treatments fail to respond to or achieve remission with symptoms such as avolition (lack of interest), psychomotor retardation, poor concentration and anhedonia, likely to persist even after symptoms such as a depressed mood and anxiety has been resolved (Hori & Kunugi 2013). Moreover, clinical studies observed that DA reuptake inhibitors (e.g., nomifensine) and DA receptor agonists (e.g., pramipexole) have antidepressant effects in MDD compared to a placebo-control (Hasler 2010). Chiba et al., (2010) conducted a preclinical study to investigate the possible anti-depressant like effect of cabergoline (a DA receptor agonist) as monotherapy. This study demonstrated that cabergoline reduced immobility in the forced swim test, screening for depressive-like behaviors in both acute and chronic treatments (Chiba et al., 2010). Cabergoline also demonstrated a slight anxiolytic effect at low doses (Chiba et al., 2010).

Pleasure, whether associated with eating, social, or sexual behavior, is primarily mediated by activation of the DA neurons as part of the DA reward pathways (Saveanu & Nemeroff, 2012; Nestler & Carlezon Jr, 2006). Thus, the emergence of a DA hypothesis of depression is not surprising in view of the fact that the inability to experience pleasure or anhedonia, is considered by many to be the most important pathognomonic symptom of depression (Saveanu & Nemeroff, 2012). A variety of findings implicate ventral striatal regions, especially the nucleus accumbens (NAcc), in coding the incentive properties of stimuli and reward prediction errors (Pizzagalli, 2014). Studies done in non-human primates have shown that striatal DA neurons code reward-related prediction errors using phasic bursts of DA when the animal receives an unpredicted award, increasing firing and DA release, while expected rewards lead to transient neural and DA suppression (Pizzagalli, 2014).

Another avenue that could explain the involvement of DA in MDD is the abuse of amphetamines. Amphetamine is a psychostimulant drug which increases arousal and alertness, and induces hyperactivity, elevates mood and causes racing thoughts, high energy and restlessness (Drevet et al., 2001) with a potential of abuse and a great risk for addiction (Che et al., 2013). Dysphoric symptoms such as fatigue, agitation, anhedonia, sleep alterations, social inhibition, cognitive dysfunction and depressive-like symptoms are most common in prolonged use of amphetamine abusers (Koob & Le Moal 1997; Shoptaw et al., 2009). Chronic methamphetamine (METH) leads to neurotoxicity which

alters cognition, mood and causes motor impairments (Gouzoulis-Mayfrank & Daumann, 2009; Carvalho et al., 2012). METH users revealed reduced levels of DAergic nerve terminal markers in the striatum (Kitamura et al., 2007), while abstinent METH users showed a reduction in 5-HT transporter density and regional cerebral metabolic abnormalities which are associated with depressive symptoms (Semple et al., 2005, Panenka et al., 2013).

Few tests distinguish the effects of AD's that facilitate NA or DA from 5-HT transmission unless employing pharmacological depletion (Carr & Lucki, 2011). A decrease of 5-HT and NA monoamines in the hippocampus, limbic system and the frontal cortex are responsible for the depressive symptoms (Charles et al., 2009) and despite the above evidence, interindividual variation in these symptoms is great and the existing antidepressants which act by increasing DA, 5-HT and NA levels are notoriously ineffective, being effective in less than 50% of patients (Popoli, 2009). Thus the monoamine hypothesis fails to fully explain the pathophysiology of depression resulting in the search for newer drug therapies with novel mechanisms of action (Girish et al., 2010).

#### 4.3.2 Glutamate and gamma-aminobutyric acid (GABA) hypothesis

Glutamate mediates the vast majority of fast excitatory transmission in the brain, with GABA responsible for inhibitory neurotransmission (Sanacora et al., 2012). In humans, the neocortex represents about 85% of the total brain mass (Sanacora et al., 2012), with up to 80% of neurons in this region forming 85% of all synapses, while approximately 20% of these neurons are inhibitory, contributing towards 15% of synapses (Sanacora et al., 2012). This data indicates that glutamate neurons and synapses by far outnumber all other neurotransmitter systems in the brain, with the exception of the GABAergic system (Sanacora et al., 2012). Therefore, from a chemical neurotransmission point of view, the brain is largely in a state of excitation via glutamate but counter-regulated by a smaller GABAergic inhibitory component as well as modulation by a much smaller number of neurons releasing a variety of other neurotransmitters including monoamines (Sanacora et al., 2012).

Multiple studies have reported findings of elevated glutamate content, and a trend for decreased plasma glutamine/glutamate ratios in the plasma of depressed patients compared to healthy comparison subjects (Sanacora et al., 2012). A series of MRS studies consistently showed reductions in total GABA concentrations in the prefrontal and occipital cortex in acute depression (Hasler, 2010; Müller & Schwarz 2007). This may reflect acute stress effects, since psychological stress seems to induce presynaptic down-regulation of prefrontal GABAergic neurotransmission (Hasler, 2010). In addition, chronic stress may reduce GABA<sub>A</sub> receptor function, possibly through changes in

neuroactive steroid synthesis (Hasler, 2010), especially 3 $\alpha$ -reduced pregnane steroids, which are potent positive allosteric modulators (Eser et al., 2006).

A remarkable feature of glutamate synapses is their ability to undergo structural, as well as functional changes in response to environmental stimuli (Sanacora et al., 2012). Environmental stress has a heavy impact on brain tissue morphology and is considered a risk factor for mood/anxiety disorders (Sanacora et al., 2012). It is well known that higher than normal extracellular concentrations of glutamate cause excitotoxicity, neuronal degeneration and death, a phenomenon that is central to the pathophysiology of neurodegenerative diseases (Sanacora et al., 2012), and that may have relevance to the shrinkage of certain brain regions observed in patients with depression (Sanacora, Treccani et al., 2012).

#### 4.3.3 Dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis (Hormonal abnormalities)

Neurons in the paraventricular nucleus of the hypothalamus secrete CRH, which stimulates the release of ACTH (illustrated in Fig. 4) that in turn stimulates cortisol release in humans or corticosterone in rodents (Nestler et al., 2002). CRH is released from the hypothalamus (Fig. 6) in response to the perception of psychological stress by cortical brain regions (Hasler, 2010). Although MDD is considered as a stress disorder, most subjects treated for MDD have no evidence of dysfunction of the HPA axis (Belmaker & Agam, 2008). However, some subjects with MDD do show abnormalities of the HPA-axis (Pariante & Lightman, 2008).

The importance of HPA axis dysfunction as a function of antidepressant action is therefore a matter of debate. Nevertheless, there is convergent evidence to suggest that CRH plays a major role in the pathogenesis of certain types of depression, one being the elevated levels of CRH in the CSF of some depressed subjects (Hasler, 2010). It is the existence of cortisol hyper-secretion and alterations in the circadian rhythm, with an advance in the lowest point of the cortisol and ACTH rhythms (Soria & Urretavizcaya, 2009), that have been consistently described in depression. These alterations in cortisol secretion have been related to HPA-axis hyper-activity (Soria & Urretavizcaya, 2009). Importantly, an abnormal cortisol response, such as a flatter diurnal cortisol pattern, implies an abnormal stress reactivity that correlates with a greater severity of depression (Sou  tre et al., 1989; Hsiao et al., 2010; Doane et al., 2013), suggesting that altered circadian rhythms occupy a critical role in how the brain copes with stressful experiences and ultimately in regulating mood.

Early life stressful events, such as childhood trauma and neglect, are associated with depression and anxiety disorders and sustained changes in the HPA system (Fig. 4), as mentioned earlier in section 4.2

(Ansorge et al., 2007). These associations demonstrate that developmental environmental factors can produce enduring changes in HPA system physiology and emotional behavior (Ansorge et al., 2007).

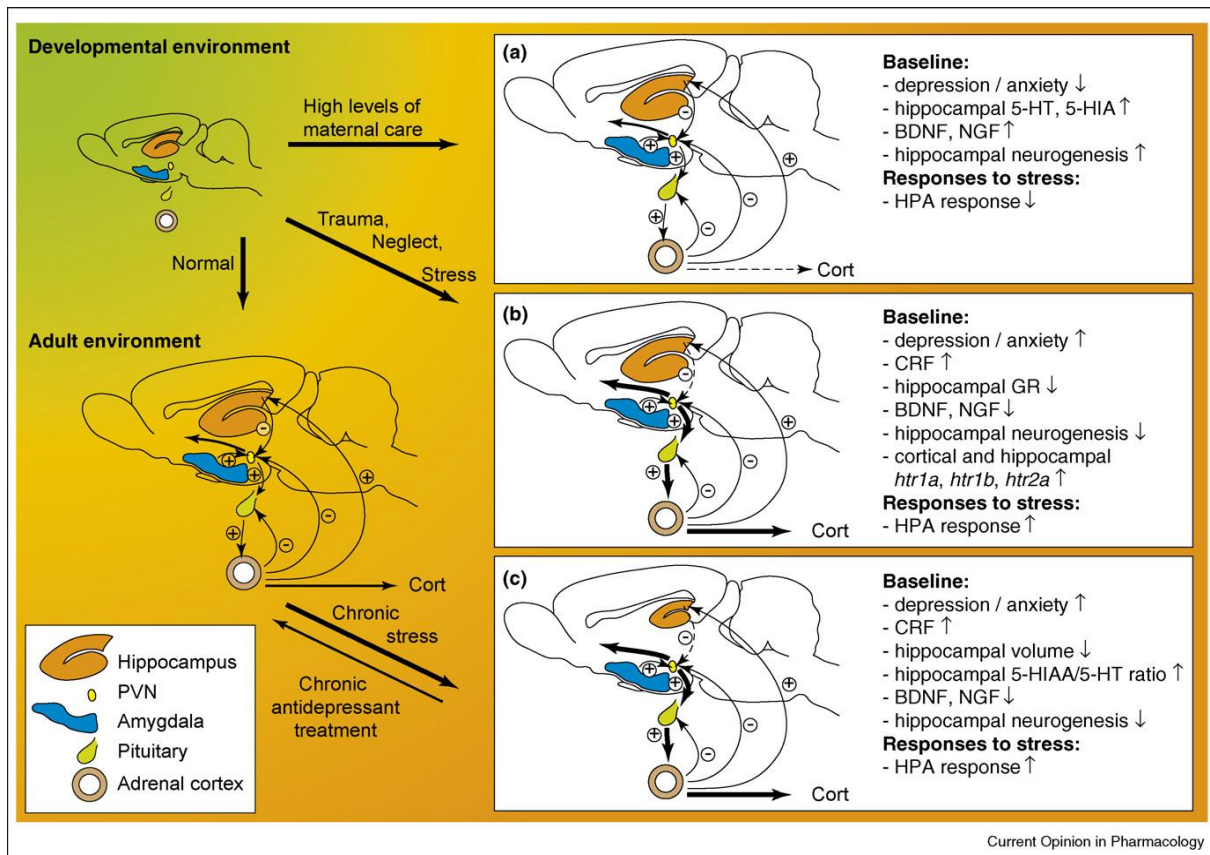


Fig 4: Consequences of early life experience and adult stress on the HPA system and depression. The left hand side shows normal brain development and HPA axis functioning. The right hand side shows: (a) high levels of maternal care lead to a suppressed and reduced depression and anxiety-like behavior in adulthood; trauma, neglect and stress, during development (b) or during adulthood (c) may lead to a hyperactive HPA system with increased depression and anxiety-like behavior in adulthood (Figure from Ansorge et al., 2007).

Activation of the HPA-axis is a mechanism by which the brain reacts to acute and chronic stress (Fig. 6) (Nestle et al., 2002). Activity of the HPA-axis is controlled by the hippocampus (inhibitory influences) and the amygdala (direct excitatory influences) (Nestler et al., 2002). In turn, glucocorticoids (Fig. 5; Fig 6) potentially regulate the hippocampus and paraventricular nucleus (PVN) and exert powerful feedback effects on the HPA-axis. What is especially relevant is that elevated levels of glucocorticoids under prolonged and severe stress may damage hippocampal neurons (Nestler et al., 2002), and has been advocated as an explanation for hippocampal shrinkage evident in imaging studies in depressed patients (Savitz & Drevets, 2009).

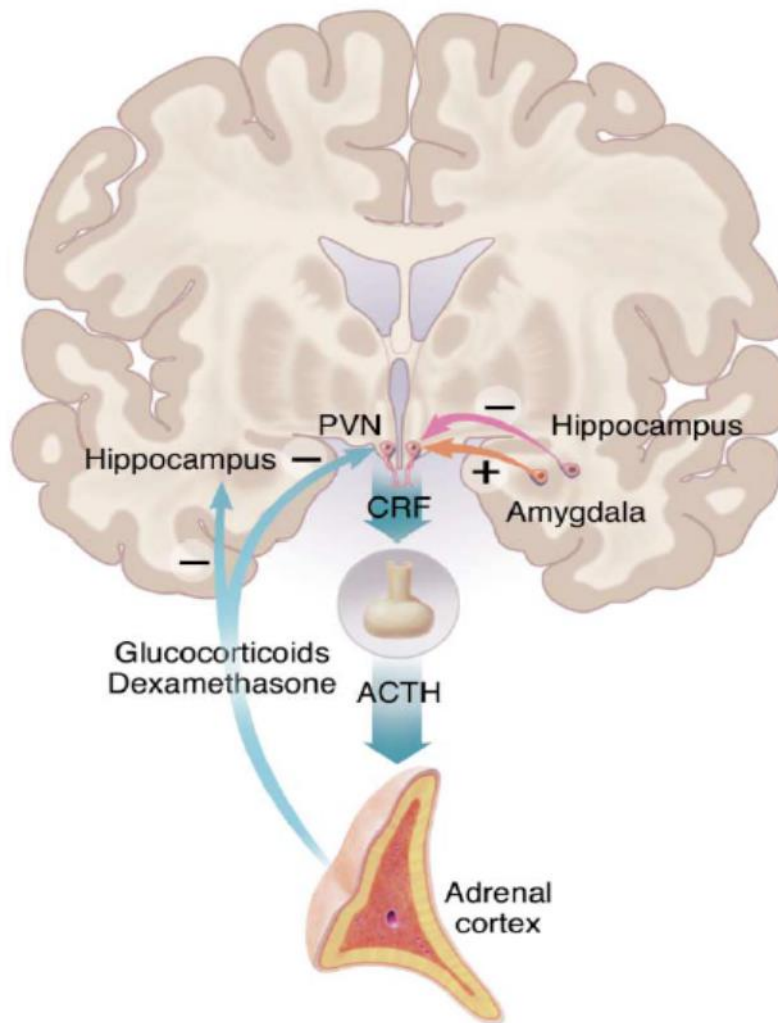


Fig. 5: Regulation of the HPA-Axis. CRF is released and acts on the corticotrophins to release ACTH which reaches the adrenal cortex via the bloodstream to stimulate the release of glucocorticoids. Glucocorticoids, with its many functions (including synthetic forms such as dexamethasone) suppress CRH and ACTH synthesis and release thus inhibiting their own synthesis. At higher levels, glucocorticoids also impair, and may even damage the hippocampus (Figure from Nestler et al., 2002).

Many drug-free patients with depression fail to suppress cortisol secretion after administration of dexamethasone (the so-called dexamethasone suppression test (DST) (Saveanu & Nemeroff, 2012). Failure to suppress plasma cortisol concentrations after dexamethasone administration suggests impaired feedback regulation and hyperactivity of the HPA axis (Saveanu & Nemeroff, 2012). As seen in Figure 5, dexamethasone acts primarily on the anterior pituitary corticotrophins to reduce the secretion of adrenocorticotrophic hormone (ACTH), resulting in a decrease in the synthesis and release of cortisol from the adrenal cortex (Saveanu & Nemeroff, 2012). However, dexamethasone non-suppression as a biological diagnostic test for depression is neither reliable nor specific as patients

with other diagnoses also exhibit DST non-suppression, including those with eating disorders, Alzheimer disease and bipolar disorder (Saveanu & Nemeroff, 2012). DST non-suppression has generally been found to be associated with depression severity, and when persistent, with a significant risk of relapse (Saveanu & Nemeroff, 2012).

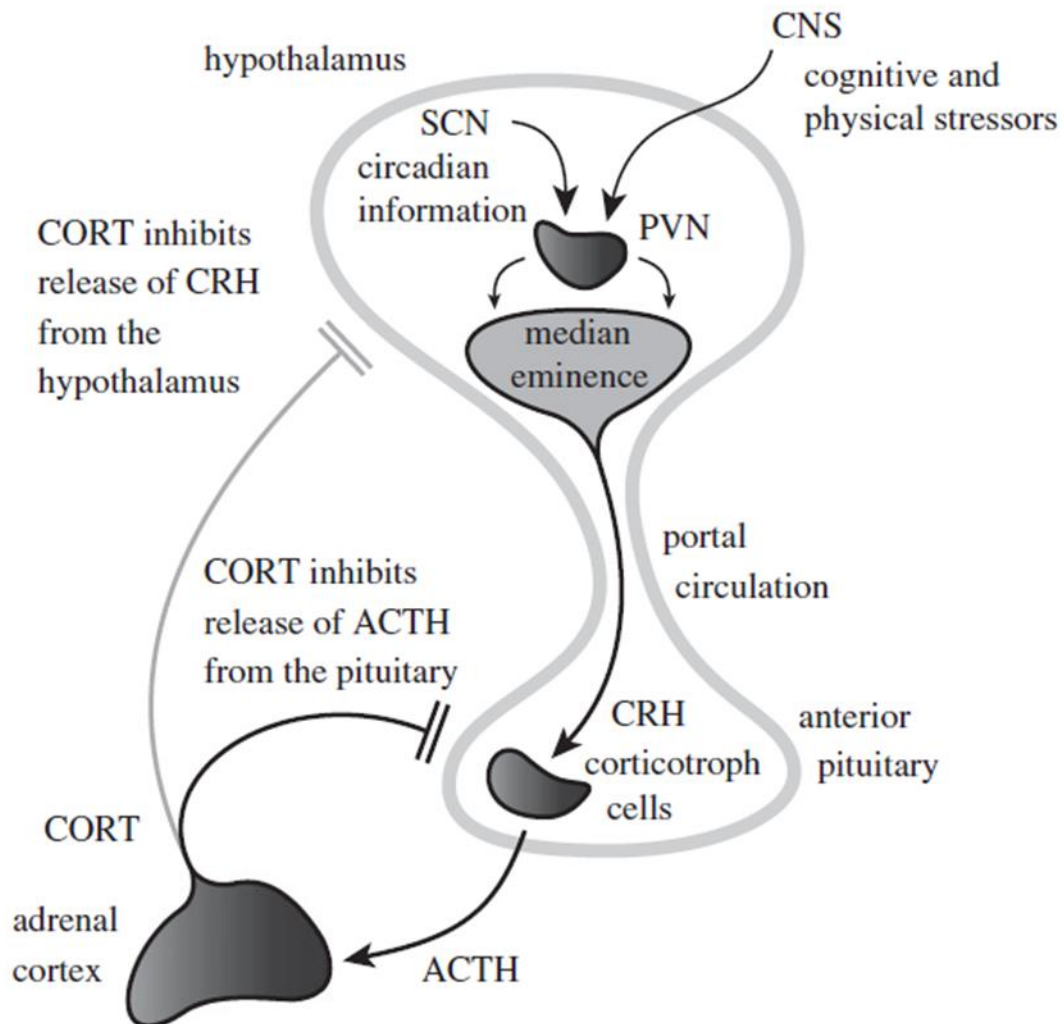


Fig 6: Regulation of the HPA axis activity. The hypothalamic PVN receives circadian inputs from the SCN and homeostatic/stress inputs from the brain stem and limbic areas. The PVN projects to the median eminence where it releases CRF into the portal circulation and passes to corticotrophs in the anterior pituitary which then releases ACTH into the venous circulation which then reaches the adrenal cortex where it activates the synthesis and secretion of cortisol in man and corticosterone in rodents. This in turn feeds back to the release of ACTH and later to CRF from the hypo (Figure from Walker et al., 2010).

#### 4.3.3 The neurotrophic hypothesis – BDNF

Neurotrophins eg. brain derived neurotrophic factor (BDNF) is the most profuse and widely distributed neurotrophin in the CNS and plays an important role in regulating a wide range of functions (Martinowich & Lu., 2008). Several lines of evidence suggest that reduced neurotrophic factor

signaling in the adult brain may be implicated in the pathophysiology of depression (Ansorge et al., 2007). Findings indicate that BDNF production is decreased during depressive episodes and tends to be normalized following effective treatments including AD's, mood stabilizers and electroconvulsive therapy (ECT) (Noto et al., 2011). The DAergic VTA-Nacc pathway plays a crucial role in reward and motivation, while it appears that BDNF elicits opposite effects on these two systems (Martinowich & Lu, 2008). Infusions of BDNF in the hippocampus produce antidepressant effects (Shirayama et al., 2002) while it plays a depressogenic role in the VTA-Nacc reward pathway (Eisch et al., 2003).

Substantial evidence suggests that BDNF promotes the development and function of 5-HTergic neurons (Martinowich & Lu, 2008). In rodents, acute and chronic stress decreases the levels of BDNF expression in the dentate gyrus and pyramidal cell layer of the hippocampus (Nestler et al., 2002, Martinowich & Lu, 2008). This reduction seems to be partly due to stress induced release of glucocorticoids and partly other mechanisms such as alterations in serotonergic and glutamatergic transmission (Nestler et al., 2002, Martinowich & Lu, 2008). On the other hand, BDNF infusions into the adult rat brain have been shown to produce sprouting of 5-HT nerve terminals and to accelerate the regrowth of damaged serotonergic fibers (Naert et al., 2011).

BDNF and 5-HT are known to regulate synaptic plasticity, neurogenesis and neuronal survival in the adult brain (Mattson et al., 2004). These two signals co-regulate one another; 5-HT stimulates the expression of BDNF and BDNF enhances the growth and survival of 5-HT neurons (Mattson et al., 2004). Similarly, rapid increases in BDNF expression precede the activation of CRH expressing neurons in response to stress, where centrally administered BDNF substantially modifies HPA axis activity (Naert et al., 2011). However, the above brief overview of our current knowledge of the disorder clearly indicates that the neurobiology of depression is much more complex. In fact, there is evidence to suggest that BDNF may in certain conditions be counter-productive, possibly even worsening the neuropathology and symptoms of depression (Harvey et al., 2013). Indeed, some animal studies have found BDNF to be depressogenic (Harvey et al., 2013).

#### 4.3.4 Circadian rhythm dysfunction

The sleep-wake cycle is the most obviously evident circadian rhythm in human beings. In mammals, these rhythms are controlled by a hierarchy of cellular oscillators, at the top of which are pacemaker cells in the suprachiasmatic nucleus (SCN) in the hypothalamus (Li et al., 2013). Local oscillators throughout the body coordinate daily cycles by integrating signals from the SCN with other internal and external time cues (Li et al., 2013). Many lines of evidence in humans as well as in animal models clearly demonstrate the relationship between depression and circadian rhythms (Kronfeld-Schor & Einat, 2012).

Mood disorders, especially, are associated with changes in various circadian rhythms (Germain & Kupfer, 2008). It is estimated that approximately 80% of depressive patients suffer from insomnia, whether expressed as trouble falling asleep, trouble remaining asleep through the night, and/or waking up too early (Soria & Urretavizcaya, 2009; Kronfeld-Schor & Einat, 2012). Many lines of evidence in humans as well as in animal models clearly demonstrate the relationship between depression and circadian rhythms (Kronfeld-Schor & Einat, 2012). These lines of evidence stem from disease patterns, mechanisms and models as well as treatment effects in patients and in animal models (Kronfeld-Schor & Einat, 2012). Regarding disease patterns, some important and established connections include: daily patterns of depression, changes in physiological and behavioral daily rhythms in depressed patients and in animal models of depression, seasonal affective disorder, recently established connections between circadian system genes and depression, and the relationship between sleep disorders and depression (Kronfeld-Schor & Einat, 2012).

Regarding the effects of antidepressant treatment, the evidence includes: the antidepressant effect of sleep deprivation, the antidepressant effect of bright light exposure, the effects of antidepressant drugs in sleep and daily rhythms and the circadian system in animal models (Kronfeld-Schor & Einat, 2012). Melatonin, the hormone from the pineal gland regulates various circadian rhythms, such as body temperature, cortisol secretion, sleep-wake cycles, rapid eye movement (REM) and slow wave sleep (Girish et al., 2010). In depressive subjects, alterations have also been detected in the circadian rhythms of body temperature and several endocrine-metabolic parameters, such as the secretion of cortisol, thyroid stimulating hormone, melatonin and monoamines, in comparison with healthy individuals (Soria & Urretavizcaya, 2009). It is clear that altered circadian rhythm is a core biological manifestation of depression (McClung, 2013; Girish et al., 2010). Melatonin secretion is elevated at night (Kronfeld-Schor & Einat, 2012), while this rhythm is absent in depressed patients. Some studies suggest that depressed patients are internally desynchronized (Kronfeld-Schor & Einat, 2012).

#### 4.3.6 Immune-inflammatory hypothesis

Evidence is growing that current AD's may exert their therapeutic effect in other ways than monoamine modulation, and with growing evidence for the role of oxidative stress in MDD, treatment strategies aimed at decreasing oxidative stress in chronic stress animal models has gained interest (Lee et al., 2013). Such approaches may involve suppressing the production of pro-inflammatory cytokines and reactive oxygen species (ROS)/ reactive nitrogen species (RNS) or enhancing antioxidant defenses such as superoxide dismutase (SOD) levels (Lee et al., 2013).

An immunological model of MDD is "sickness behavior", the non-specific reaction of the organism to infection and inflammation (Müller & Schwarz, 2007). Pro-inflammatory cytokines are known to

activate as well as inhibit the HPA axis (Fig. 7) (Lee et al., 2013). The stimulating effects of cytokines and impairment of hippocampal inhibition on the HPA axis together contribute to the altered HPA axis in depression (Lee et al., 2013). Immune-inflammatory dysfunction and oxidative stress are important pathological components of depression (Ng et al., 2008), while factors that induce cellular oxidative stress are known to evoke monoaminergic changes that in turn may mediate psychiatric manifestations (Garcia-Cazorla et al., 2008). The sickness-related psychopathology and symptomatology during infection and inflammation is mediated by pro-inflammatory cytokines such as interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)- $\alpha$  and interferon (IFN)- $\gamma$  (Fig. 7) (Müller & Schwarz, 2007). Moreover, evidence that cytokine-mediated inflammatory processes play an important role in the development of depression is also strong (Zunszain et al., 2011).

Lipopolysaccharide (LPS), a bacterial endotoxin and potent activator of pro-inflammatory cytokines, has been studied in human volunteers and was found to induce mild fever, anorexia, anxiety, depressed mood and cognitive impairment (Müller & Schwarz, 2007). Moreover, the levels of anxiety, depression and cognitive impairment could be related to the levels of circulating cytokines (Müller & Schwarz, 2007). Once cytokines reach the brain, they have the capacity to influence the synthesis, release and reuptake of mood-relevant neurotransmitters including the monoamines (Miller et al., 2009). Numerous animal studies demonstrate that the administration of cytokines or cytokine inducers can profoundly affect the metabolism of 5-HT, NA and DA (Miller et al., 2009).

Regarding the mechanism involved, much attention has focused on the enzyme indolamine 2, 3 dioxygenase (IDO) (Miller, et al., 2009). Moreover, oxidative stress, altered glutamate N-Methyl-D-aspartate (NMDA) receptor signaling and associated changes in down-stream nitric oxide-cyclic guanosine monophosphate (NO-cGMP) activity (Wegener et al., 2010; Dhir and Kulkarni, 2011), as well as altered kynurenine metabolism (Maes et al., 2011), are known pro-oxidative mechanisms involved in the pathology of depression. Through stimulation of multiple inflammatory signaling pathways, cytokines can activate IDO that in turn breaks down tryptophan to form kynurenine (Fig. 8) (Miller et al., 2009).

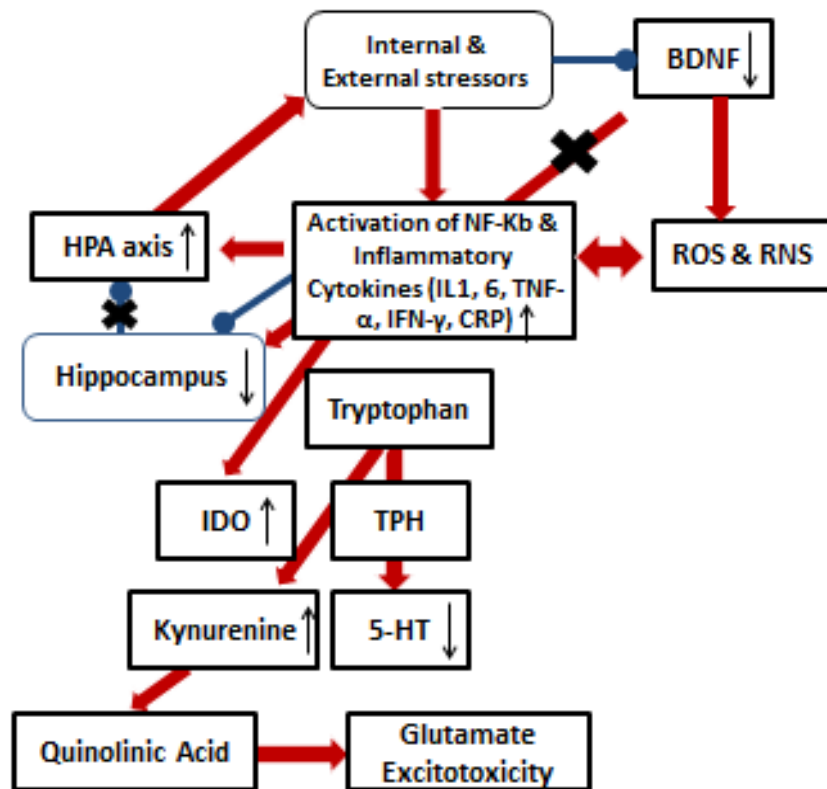


Fig 7: Mechanisms of oxidative/nitrosative damage which may ultimately lead to depression. 5-HT (serotonin), HPA (hypothalamus-pituitary-adrenal), BDNF (brain-derived neurotrophic factor), ROS (reactive oxygen species), RNS (reactive nitrogen species), IDO (indolamine 2,3-dioxygenase), TPH (tryptophan hydroxylase), NK-kB (nuclear factor kappa-light-chain-enhancer of activated B cells), TNF (tumor necrosis factor), IL (interleukin) and IFN (interferon). Red arrows indicate increased production of cytokines, ROS and RNS, kynurenine, quinolinic acid, decreased 5-HT and glutamate excitotoxicity or an increased activity of IDO and TPH. Blue bars indicate an inhibitory effect of internal and external stressors on BDNF as well as inflammatory cytokines on activity of the hippocampus. Black crosses are indicative of no effect, whereas the hippocampus has no negative feedback in the HPA-axis (Figure adapted from Lee et al., 2013).

The essential amino acid tryptophan is not only the precursor of 5-HT and melatonin, but also of the so-called kynurenine (KYN) pathway (Müller & Schwarz, 2007). This prominent metabolic pathway of tryptophan (Fig. 8) includes several neuroactive intermediates, of which kynurenic acid (KYNA) is a NMDA receptor antagonist, and quinolinic acid (QA), a NMDA receptor agonist (Müller & Schwarz, 2007). In particular, altered kynurenine metabolism with an associated elevation in neurotoxic QA and a reduction in neuroprotective KYNA is known to promote oxidative stress and inflammation and is increasingly being viewed as an important contributing factor to the development of depression (Maes et al., 2011). KYNA has been shown to inhibit the release of glutamate, which, by extension, may inhibit the release of DA (Miller et al., 2009). In contrast, QA promotes glutamate release through

activation of the NMDA receptor thereby inducing oxidative stress, which in combination with glutamate release may contribute to CNS excitotoxicity (Miller et al., 2009).

Treatment of up to 24 weeks with IFN- $\alpha$  in patients with chronic hepatitis C, increased the kynurenine over tryptophan (TRP) ratio while at the same time increasing symptoms of depression (Wichers et al., 2005). The kynurenine over KYNA ratio increased mainly due to an increase in kynurenine levels and the lack of KYNA, which was interpreted as a decrease in neuroprotection, while not being restricted to the periphery in these patients (Dantzer et al., 2011). Increases of kynurenine and QA in the CSF in these patients were correlated with an increase in depressive symptoms (Raison et al., 2010). Gabbay et al (2010) proposed that the clinical form of depression itself could play a role since decreased TRP and elevated kynurenine ratios were found in adolescent patients with melancholic depression with but not-melancholic features independent of treatment.

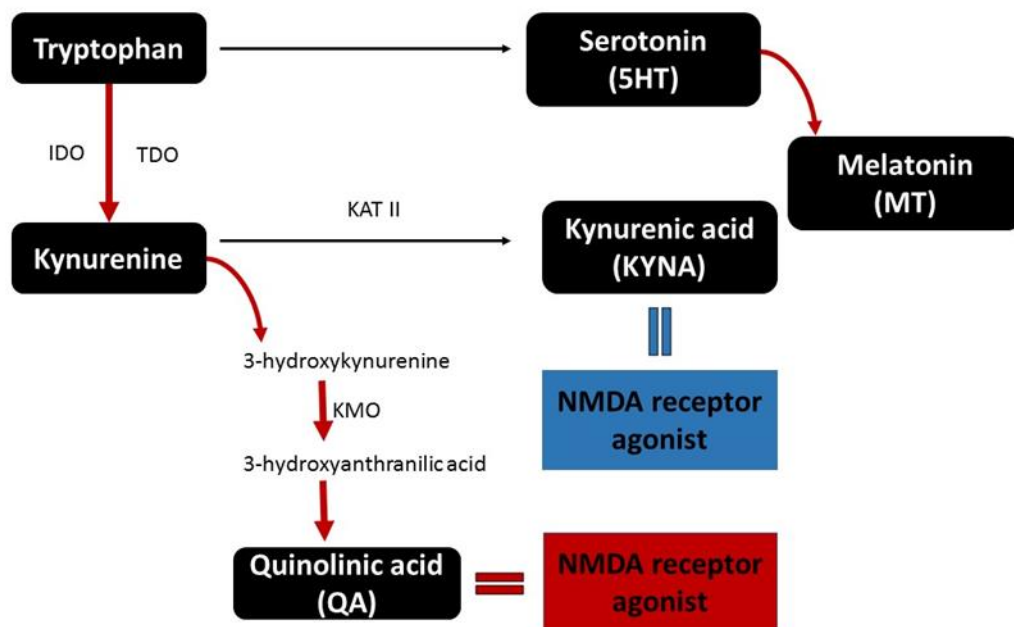


Fig. 8. The red arrows indicate the metabolism pathway of TRP along the kynurenine pathway. IDO is induced by cytokines and other immune mediators, whereas tryptophan 2, 3-dioxygenase is induced by TRP itself. Black arrows show alternative pathways of TRP converted into 5-HT and then to melatonin. Kynurenine aminotransferase II (KAT II) converts kynurenine into kynurenic acid, an NMDA receptor antagonist and kynurenine monoxygenase (KMO) converts kynurenine to quinolinic acid, an NMDA receptor agonist. Adapted from Molteni et al., 2013; Stone et al., 2002

With regards to AD treatment and oxidative stress, it has been observed that SSRI's such as paroxetine inhibit nitric-oxide synthase (NOS) enzyme activity (Lee et al., 2013), as have ADs of varying classes (Wegener et al., 2003), while AD withdrawal increases NOS activity (Harvey et al., 2006). Khanzode et al., reported that patients with MDD displayed a significant increase in serum SOD, malondialdehyde (MDA) and a decrease in plasma ascorbic acid levels compared to those of a match control (Lee et al., 2013). Moreover, treatment with SSRI's have been found to significantly decrease nitric oxide (NO), xanthine oxide (XO) and lipid peroxidation levels (Herken et al., 2007). Furthermore, both affective symptoms and cognitive deficits noted in depression can be ameliorated by SSRI's through an antioxidative mechanism (Lee et al., 2013).

Glutamate released by astrocytes has preferential access to extrasynaptic NMDA receptors, which mediates excitotoxicity and decreases production of trophic factors including BDNF (Miller et al., 2009). Cytokines, including TNF- $\alpha$  and IL-1, can also induce both astrocytes and microglia to release ROS that in combination with QA can amplify oxidative stress and further endanger relevant cell types, including neurons and oligodendrocytes, which are especially vulnerable to oxidative damage (Miller et al., 2009). It is noteworthy that the major mechanism whereby immune stimuli activate the HPA axis is the same as that involved in the responses to psychological and physical stressors, namely activation of the CRF-containing neurons in the paraventricular nucleus of the hypothalamus, and subsequent secretion of anterior pituitary ACTH (Dunn et al., 2005).

Of relevance is that melatonin has noteworthy antioxidant and anti-inflammatory properties (Anderson and Maes, 2012), with recent evidence suggesting the same for agomelatine (Molteni et al., 2013). In fact, agomelatine is able to alter the expression of enzymes related to the kynurenine pathway, viz. kynurenine-3-monooxygenase (KMO) and kynurenine-aminotransferase II (KAT II) (illustrated in Fig. 8) (Molteni et al., 2013). A study done by Molteni et al., (2008) indicated that agomelatine prevented an LPS-dependent increase in KMO which converts kynurenine to QA (Fig. 8).

## 5. Treatment

### 5.1 Psychopharmacology

Despite our limited understanding of depression, there are many AD treatments available (Nestler et al., 2002). The first AD's were discovered over 40 years ago by serendipity and only much later was it determined that these agents acted by either inhibiting the enzyme monoamine oxidase (MOA) or by blocking the reuptake of NA and 5-HT (Stahl, 1998). The mechanism of action of MOA inhibitors (MOAI) is to increase the availability of monoamine neurotransmitters NA, DA and 5-HT by blocking their metabolism (Stahl, 1998). Tricyclic AD's can be regarded as multi-target agents, being: (1) a SRI; (2) a NRI; (3) an anticholinergic-antimuscarinic drug (M1); (4) an  $\alpha$ 1-adrenergic antagonist; and (5) an

antihistamine (H1) (Stahl, 1998). The discovery of these mechanisms of action led to the development of numerous second generation medications, such as the SSRI'S and SNRI'S (Nestler et al., 2002). Pre-clinical behavioral tests in animals represent important tools with which to study brain function under normal conditions and to identify a range of proteins in the brain that might serve as targets for novel AD treatments, although progress has been limited (Nestler et al., 2002). Even after combination therapy with a variety of AD's, patients show no improvement and although AD's rapidly increase monoamine levels in the CNS, it may often take two weeks or longer for AD efficacy (Cai et al., 2015). This evidence indicates that deficiencies in monoamines only partly explain the pathogenesis of depression (Cai et al., 2015).

Newer medications, such as the SSRI's, have essentially the same mechanism of action as the older tricyclic ADs, although from a compliance point of view they may be better due to fewer side effects (Nestler et al., 2002). As a result, the efficacy of the newer agents and the range of depressed patients they treat are no better than the older medications (Nestler et al., 2002). The mechanism of action of AD's is far more complex than their direct mode of action might suggest (Nestler et al., 2002). Thus selectivity for example at a monoamine transporter may not translate into selectivity in the synapse or in the subcellular domain, this due to the ubiquitous presence of neurotransmitter and subcellular cross talk mechanisms (Harvey, 1997). All available AD's exert their mood-elevating effects after prolonged administration, which means that enhancing 5-HT and NA neurotransmission is not per se responsible for the clinical actions of these drugs, but rather developing adaptations to these enhancements over a long period is now seen as the critical requirement for psychotropic activity (Nestler et al., 2002).

There are several other strategies to treat MDD that may not directly target monoamine systems in order to trigger an antidepressant response (Blier & El Mansari, 2013b). Sleep deprivation, for instance, may produce a rapid antidepressant response, although very transient (Blier & El Mansari, 2013b). This strategy can indirectly affect the function of monoamine systems, and as a novel antidepressant strategy produce strikingly rapid therapeutic benefits in MDD, providing credence to the theory that neuronal hyperactivity in limbic structures may account in large part for the pathology of MDD (Blier & El Mansari, 2013b). Another strategy is the injection of a sub-anesthetic dose of the glutamate NMDA antagonist ketamine, which may produce a robust antidepressant response within hours, presumably in part by blocking excitatory NMDA receptors (Blier & El Mansari, 2013b). Furthermore, ketamine may produce a dampening of the excessive neuronal activity in limbic structures within hours that traditional ADs normally take weeks to attain by enhancing inhibitory monoaminergic transmission (Blier & El Mansari, 2013b).

Deep brain stimulation (DBS) is another novel, yet still experimental approach to treat refractory MDD (Blier & El Mansari, 2013b). Consistent with the attenuated hyperactivity of limbic structures following effective AD treatments; DBS also produces the same phenomenon (Blier & El Mansari, 2013b). DBS is an invasive neurosurgical intervention being investigated for the treatment of resistant depression (Holtzheimer & Mayberg, 2011). It is achieved by implanting one or more electrode leads into a specific region of the brain through holes in the skull using neuroimaging-guided stereotactic neurosurgical techniques (Holtzheimer & Mayberg, 2011). However, electroconvulsive therapy (ECT), remains the most effective treatment option for treatment-resistant depression (Cassidy et al., 2010), with its mechanism of action being unknown, although many studies have shown that the release of hormones associated with the HPA-axis, such as adrenocorticotropin, is altered by ECT (Bolwig, 2011).

### 5.1.1 Agomelatine

Agomelatine is the subject of this study, and while it is an AD, it contrasts with other conventional AD's through a novel non-monoaminergic mechanism of action (De Bodinat et al., 2010), acting as a MT1 and MT2 receptor agonist and a 5-HT<sub>2C</sub> receptor antagonist (Fig. 9a) (Fornaro et al., 2010; Demyttenaere, 2011).

#### 5.1.1.1 Chemistry

Agomelatine (N-[2-(7-methoxybaphtalen-1yl) ethyl] acetamide is a synthetic analog of the hormone melatonin (Girish et al., 2010).

#### 5.1.1.2 Pharmacokinetics

The absorption of agomelatine is not markedly altered by food and is rapidly absorbed, with a bioavailability of more than 78% following oral administration and peaking in plasma after 1 to 2 hours (Girish et al., 2010). It is more than 95% plasma protein bound with 35% bound to albumin and 36% bound to  $\alpha$ -1 acid glycoprotein with a volume of distribution of 35L (Girish et al., 2010). According to in vitro studies, it is unlikely to cause displacement of highly protein bound drugs, although in vivo studies are lacking (Girish et al., 2010).

Agomelatine is metabolized by cytochrome P450 1A2 to the 7-O – demethylated and hydroxylated inactive metabolites, with 61 to 81% of the dose excreted as metabolites in the urine over the first 24h with the mean terminal half-life of 2 to 3h (Girish et al., 2010). It is also metabolized to 3, 4-dihydrodiol which is excreted in the faeces (Girish et al., 2010). Moderate hepatic impairment drastically (100 times) increases the plasma levels of agomelatine, while renal impairment only causes a small increase in the plasma levels of agomelatine (>25%) (Girish et al., 2010).

### 5.1.1.3 Dosing and drug safety

The effective dose for agomelatine is 25 mg per day given once at bedtime for two weeks, increased thereafter to 50mg per day in patients with inadequate responses (Girish et al., 2010). Night time dosing is recommended because agomelatine improves the quality of sleep without day time sedation (Girish et al., 2010). Due to phasic binding to the MT1/MT2 and 5-HT2C receptors, agomelatine correlates with its required dosing at night (Harvey & Slabbert, 2014). Specific data on safety during pregnancy and lactation is not available, although animal studies have not shown any risk (Girish et al., 2010). It is not recommended for use in people under the age of 18 years and should be used with caution in the elderly (>66 yrs of age) due to the lack of clinical data (Girish et al., 2010). Patients receiving 50mg agomelatine had a history of cholecystitis, gallbladder disorder or hepatic stenosis and agomelatine is therefore contraindicated in patients with hepatic impairment (Demyttenaere, 2011). At initiation of treatment, liver function tests should be performed in all patients and then periodically after 6 weeks, 12 weeks and 24 weeks and then afterwards when clinically indicated (Demyttenaere, 2011).

### 5.1.1.4 Drug interactions

Agomelatine does not induce or inhibit the cytochrome P450 enzymes, although inducers such as omeprazole and nicotine can decrease the serum levels of agomelatine (Howland, 2006; 2009). Using the latter drugs together with agomelatine therefore necessitates an increase in dose to 50 mg per day (Girish et al., 2010). Fluvoxamine and oestrogens have been found to increase the levels of agomelatine due to CYP enzyme inhibition, while other enzyme inhibitors like paroxetine, fluconazole, lithium, lorazepam, alcohol and valproic acid have insignificant effects on its kinetics (Girish et al., 2010).

### 5.1.1.5 Mechanism of action

Over and above its antidepressant effects, it also presents with anxiolytic properties in animals (Morley-Fletcher et al., 2011; Banasr et al., 2006) and humans (Kennedy & Rizvi, 2010). Studies have also shown that agomelatine modulates the expression of BDNF to enhance adult neurogenesis in the hippocampal dentate gyrus, and to reduce stress-induced glutamate release in the prefrontal/frontal cortex (Morley-Fletcher et al., 2011).

Through a synergistic interaction between its MT1/MT2 and 5-HT2C receptor components, agomelatine modulates glutamate signalling, engaging time-dependent modifications of receptors and transporters in circumscribed regions involved in the regulation of mood, circadian rhythms and cognition (Guardiola-Lemaitre et al., 2014). This was observed in a preclinical study where an acute administration of agomelatine (40mg/kg i.p.) abolished restraint stress-induced increase in

extracellular glutamate efflux in limbic structures such as the basolateral and central nuclei of the amygdala and the hippocampus (Guardiola-Lemaitre et al., 2014). Its primary mode of action has been attributed to re-entrainment of disordered circadian rhythms in the SCN of the hypothalamus and the facilitation of frontal cortical DAergic and NAergic activity via complimentary actions on the above two receptors (Fig. 9B) (Racagni et al., 2011). Furthermore, unlike other currently marketed ADs, agomelatine does not provoke the release of 5-HT and is devoid of serotonergic side effects (Racagni et al., 2011). Numerous findings suggest that it is its antagonistic properties on brain stem 5-HT<sub>2C</sub> receptors that are responsible for its concomitant effects on DA and NA release in the FC (Huang et al., 2014; Chenu et al., 2013; Norman, 2012). These actions have a fundamental effect on frontal cortical function and as a result on mood, cognition and emotion, all factors contributing to depression symptomology.

Because DAergic and NAergic mechanisms in the FC modulate cognitive and attention performances, mood and motor behaviors, all of which are deeply perturbed in depressive states, it can be reasonably assumed that the stimulant effects of agomelatine on these monoaminergic systems contribute not only to its AD action, but also to improve neurocognitive functions, especially memory, attention and problem-solving (Guardiola-Lemaitre et al., 2014).

In addition to its direct modulatory effects on the circadian clock in the SCN, as noted above, agomelatine is also able to indirectly modulate brain stem activity of 5-HTergic (raphae) DAergic (ventral tegmentum) and NAergic (locus coeruleus) nuclei via SCN-brain stem projections (Fig 9) (Harvey & Slabbert, 2014), thereby emphasizing both a direct and an indirect action on central monoaminergic transmission.

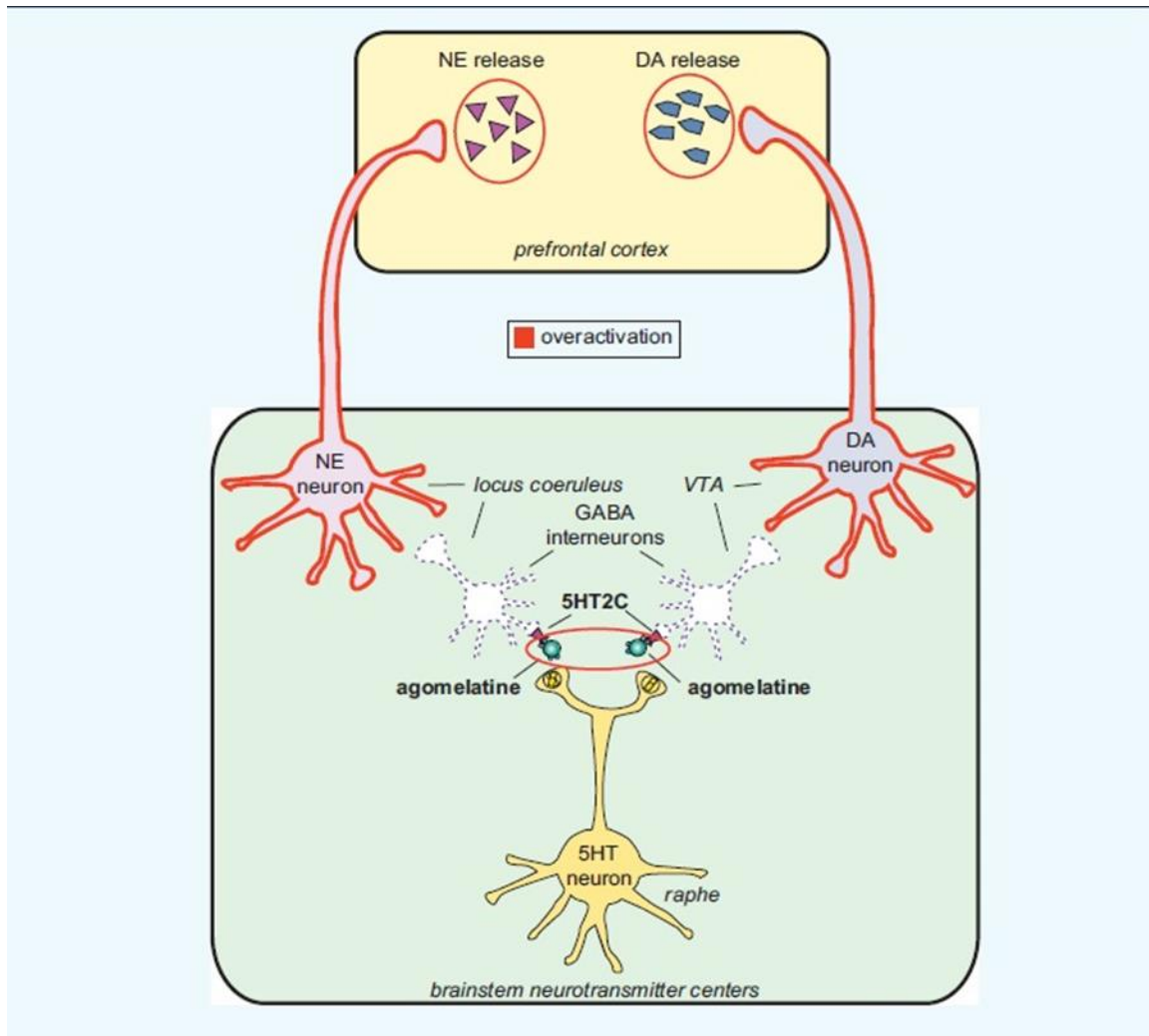


Fig 9: 5-HT<sub>2C</sub> antagonists increase DA and NA release. When serotonergic 5-HT<sub>2C</sub> receptors on GABAergic interneurons in the brainstem are blocked by a 5-HT<sub>2C</sub> antagonist such as agomelatine, this prevents inhibition of downstream DA and NA release in the PFC (Figure from Stahl, 2014).

Specific components of the immune/inflammatory system (as discussed in section 4.3.6) also play a crucial role in AD response and thereby in depression aetiopathology (Guardiola-Lemaitre et al., 2014). Chronic treatment with agomelatine significantly reduced LPS-induced up-regulation of the pro-inflammatory cytokines IL-1 $\beta$  and IL-6 at both a peripheral level as well as in different brain regions of rats (Molteni et al., 2013). As previously mentioned (see section 4.3.6) agomelatine also altered the expression of enzymes within the kynurenine pathway, also linked to important mediators associated with inflammation-related depression (Guardiola-Lemaitre et al., 2014).

Recent clinical evidence suggests that serotonergic ADs, such as SRI and SNRI, are less effective in managing the hedonic symptoms of depression (Nutt et al., 2007). In fact, SSRI's induce cognitive and emotional blunting (McCabe et al., 2010; Sansone & Sansone, 2010), with improvements in anhedonia

often the last group of symptoms to remit (Boyer et al., 2000). This response may be due to 5-HTergic drugs adversely affecting the neural processing of reward and aversive stimuli (Alén et al., 2013). However, agomelatine does not induce emotional blunting and displays a better profile regarding these symptoms in depressed patients (Harmer et al., 2011; Corruble et al., 2013), while clinical evidence confirms that it improves anhedonia (Quera-Salva et al., 2011; Di Giannantonio et al., 2011; Martinotti et al., 2012). Agomelatine's beneficial effects on anhedonia and lack of emotional blunting can be accredited to its DAergic properties in the FC and lack of serotonergic actions (Harvey & Slabbert, 2014).

In order to fully understand agomelatine's AD mechanism of action and how this involves the circadian system, it is important to first evaluate the localization, function and properties of the different melatonergic and serotonergic receptors involved. Melatonergic receptors are widely distributed in the brain with the highest density of MT1 and MT2 receptors found in the SCN and pars tuberalis (PT), but also in the FC, prefrontal cortex (PFC), cerebellar cortex, basal ganglia, substantia nigra (SN), hippocampus (HPC), ventral tegmental area (VTA), NAcc, thalamus and the retina (Hardeland et al., 2011; Tardito et al., 2012).

The expression of 5-HT<sub>2C</sub> and MT1 receptors also shows a diurnal rhythmicity in the SCN and in the hypothalamus (reviewed in Racagni et al., 2011; Tardito et al., 2012). That SCN targets within the hypothalamus in turn modulate brain stem monoamine nuclei, implies that monoamines are indirectly affected by changes in SCN activity (McClung, 2013; Harvey & Slabbert, 2014). The circadian system therefore regulates multiple monoaminergic brain regions that control mood, anxiety, and motivated behaviors through local expression of clock genes, as well as indirect connections originating from the SCN (McClung, 2013). Moreover, the circadian system regulates many hormones and peptides in the brain and periphery that impact mood and reward, especially the HPA axis (McClung, 2013).

#### 5.1.1.6 Preclinical studies

Agomelatine mimics the actions of melatonin in the synchronization of circadian rhythm patterns in rodents (San & Arranz, 2008). It resynchronizes circadian rhythms in animal models of delayed sleep-phase syndrome, restores the phase shifts, and resynchronizes circadian rhythms in free-running rats kept in constant darkness (San & Arranz, 2008). Agomelatine also seems to influence daily patterns of locomotor activity, running-wheel activity and fluctuations in body temperature (San & Arranz, 2008). These behavioral effects of agomelatine do not seem to be associated with changes in HPA axis activity (San & Arranz, 2008). Resynchronization of circadian rhythms in animals appears to occur following brief exposure to agomelatine, an effect that is consistent with its short half-life (1-2h), while prolonged exposure is much less effective (San & Arranz, 2008).

Agomelatine has shown anxiolytic properties in animal models of anxiety, including the elevated plus maze paradigm, the Vogel conflict test and the social defeat test, and may therefore be expected to have putative benefits in the treatment of anxiety disorders (San & Arranz, 2008; Guardiola-Lemaitre et al., 2014). In a study done by Millan et al., (2005), agomelatine displayed anxiolytic-like activity, which was comparable to that of the benzodiazepine, clorazepate and a selective 5-HT<sub>2C</sub> receptor antagonist. In contrast to melatonin which showed modest activity only in the evening, agomelatine showed anxiolytic-like action independent of the time of administration which may confirm that the existence of a 5-HT<sub>2C</sub> component may deprive agomelatine of chronobiotic dependence (Papp et al., 2006). Given that pre-treatment with a selective melatonergic antagonist blocked the anxiolytic effect of agomelatine in the evening but not in the morning, it has been postulated that this anxiolytic effect is related to both the melatonergic agonism and the 5-HT<sub>2C</sub> antagonistic property of the drug (San & Arranz, 2008).

Agomelatine has demonstrated AD properties in animal models of depression, including learned helplessness, chronic mild stress (CMS), forced swimming and psychosocial stress model in tree shrews, with the AD-like activity of agomelatine being superior to that of melatonin (San & Arranz, 2008; Schmelting et al., 2014). The CMS model appears to be appropriate for studying compounds with chronobiotic properties for the reason that animals exposed to CMS show an advanced phase shift of diurnal rhythms, diurnal variation with symptoms worst at the start of the dark phase, and a variety of sleep disorders which are characteristic of depression (Papp et al., 2006). Agomelatine also seems to increase cell proliferation and neurogenesis in the ventral dentate gyrus of the hippocampal formation (Banar et al., 2006), a region implicated in the response to anxiety and emotion (San & Arranz, 2008). Studies in translational animal models of depression are relatively sparse and further work in models such as the Flinders Sensitive Line (FSL) and social isolation rearing (SIR) models are warranted.

#### 5.1.1.7 Clinical studies

In a double-blind, placebo-controlled, cross-over study in elderly healthy men, agomelatine 50mg/day significantly phase-advanced the 24-hour profiles of body temperature, cortisol and thyroid-stimulating hormone levels at the end of a 15-day treatment period, and stimulated growth hormone secretion and prolactin levels during the wake period (San & Arranz, 2008). One possible explanation for this effect may lie in agomelatine antagonizing the 5-HT<sub>2C</sub> receptor, which is involved in circadian rhythm (Varcoe et al., 2003) resynchronization and in mood regulation (Yamada & Sugimoto, 2001; Gurevich et al., 2002). Kennedy and Emsley (2006), showed that agomelatine in a flexible-dose of 25-50mg was significantly more effective in treating both moderate and severe MDD than placebo. In a double-blind, randomized study (Olivier & Allgulander, 2012), patients with GAD maintained on

agomelatine treatment of 25-50mg/d for 6 months had a reduced risk of relapse compared to patients switched to placebo. This supports that in clinical practice, agomelatine will have an efficacy at least equivalent to other available treatments.

Loss of interest, also termed as anhedonia, is considered a core symptom of MDD, and is associated with dysfunctions within the brain reward system (Di Giannantonio & Martinotti, 2012; Keedwell et al., 2005). Anhedonia is commonly measured by the Snaith-Hamilton Pleasure Scale (SHAPS) (Snaith et al., 1995). In a study done by Di Giannantonio and Martinotti in 2012, depressed patients were started on an agomelatine dosage regime which involved the administration of 25mg/d at 08:00 pm. In the event of no clinical response the dosage could be increased to 50mg/d administered as a single dose.

Agomelatine has few adverse effects and is associated with early resolution of depressive symptoms (Cardinali et al., 2012). Findings from a study done by Harmer et al., (2011), suggests that both anxiety and depression are sensitive to 25mg agomelatine and that the drug does not have the same generalized blunting effect on emotional perception as SSRI's. The superior efficacy of agomelatine over other ADs, including paroxetine, venlafaxine, fluoxetine and sertraline is supported by several clinical investigations (Girish et al., 2010). Agomelatine is unique because it has a chronobiological basis for its action and acts differentially at different circadian phases of the sleep/wake cycle (Cardinali et al., 2012).

#### 5.1.1.8 Efficacy and tolerability of agomelatine

Agomelatine has demonstrated AD properties in an extensive international dose-ranging study involving 711 depressed patients, meeting the DSM-IV criteria for MDD or bipolar II disorder (Olie & Kasper, 2007), where its efficacy was shown to be similar to that of SSRI's and with a relapse rate less than other AD'S (Howland, 2009). Agomelatine has been shown to have a good acceptability and safety profile (Olie & Kasper, 2007; San & Arranz, 2008; Girish et al., 2010). In these short-term studies, the percentage of patients treated with agomelatine (25-50mg/day) reporting at least one emergent adverse event was low, as were the number of adverse events during the treatment period, which was comparable to placebo (Kennedy & Emsley, 2006; Loo et al., 2002). Treatment emergent adverse events that were reported as mild to moderate (mainly occurring within the first 2 weeks) were usually transient and did not require any intervention (San & Arranz, 2008). The most common emergent adverse events were headache, nausea and fatigue (San & Arranz, 2008; Girish et al., 2010). Clinically relevant changes in body weight or serotonergic syndrome were not observed, as well as any effect on sexual function, while its cardiovascular safety was comparable to placebo (San & Arranz, 2008). Additional clinical advantages of agomelatine include weight neutrality and, unlike many current AD

agents, is agomelatine not associated with discontinuation symptoms such as dizziness, fatigue, insomnia, gastrointestinal disorders and influenza symptoms (Olie & Kasper, 2007; Girish et al., 2010).

Assessment of discontinuation symptoms after discontinuing AD treatment can be complicated by the reappearance of depressive symptoms (Montgomery et al., 2004). Discontinuation symptoms were assessed in one double-blind, placebo-controlled report involving 192 patients receiving either agomelatine 25mg/day or paroxetine 20mg/day (Montgomery et al., 2004). Discontinuation symptoms were measured 1 and 2 weeks after abruptly stopping 12 weeks of treatment and clearly showed no signs of discontinuation symptoms (Montgomery et al., 2004). Another study also observed no statistically significant difference in the number of emergent discontinuation symptoms seen 1 or 2 weeks after treatment interruption between patients discontinuing agomelatine and those continuing agomelatine (Olie & Kasper, 2007).

## 6. Animal models of depression

Depressive illness is uniquely found in humans, although many of its symptoms can be modelled in rats (Schmidt et al., 2011). A first important decision with regards to modelling depression is the choice of the model system and secondly is whether a complex disease such as depression can be modelled in rats (Schmidt et al., 2011). A number of core symptoms of depression do have an equivalent in animals such as anhedonia, low locomotor activity and anxiety related behaviors, altered sleep/activity patterns, cognitive deficits and hyperactivity of the stress system which makes it possible to reach a certain level of face validity in animal models of depression (Schmidt et al., 2011). Construct and predictive validity are two other often considered criteria for a possible animal model, where construct validity implies that the hypothetical rationale and psychological mechanisms are similar in human and model (Schmidt et al., 2011). Predictive validity addresses the ability of successful treatment options in humans to improve the symptomatology in the animal model (Schmidt et al., 2011).

Most animal models of depression involve exposure to an acute or chronic stressor to elicit the symptoms of the disorder (McGonigle, 2014). The forced swim test (FST) is one of the most popular used models of depression and is based on the observation that a rat placed in an inescapable cylinder will eventually adopt an immobile posture (Cryan et al., 2002; Porsolt et al., 1977). The chronic mild stress (CMS) model involves repeated unpredictable mild stressors such as temporary food and water deprivation, temperature changes and housing changes over a 4-week period (Willner, 1997; Cryan & Mombereau, 2004; Katz, 1982).

Exposing humans or animals to early-life adverse events, such as maternal separation or isolation, profoundly affects brain development and behaviour in adulthood and may contribute to the occurrence of psychiatric disorders such as depression and schizophrenia (Fone & Porkess, 2008) (See section 4.2). Rearing rats under social isolation (SIR) or environmental enrichment produces long-term effects on brain development and adult behavior (Brenes Sáenz et al., 2006). One of the most robust observations reported in SIR rats is a lack of normal habituation following placement in a novel arena, characterized by motor hyperactivity compared with group housed controls (Fone & Porkess, 2008). This hyperactive response is also reduced in bright light and absent when the environment becomes familiar (Fone & Porkess, 2008).

Early-life SIR of rat pups is known to produce late-life behavioral and biological changes consistent with the neurodevelopmental hypothesis of depression as well as schizophrenia (Fone & Porkess, 2008; Pryce & Klaus, 2013). At the behavioral level, SIR induces neophobia, aggression and cognitive rigidity, and impairs sensorimotor gating and social interaction (Fone & Porkess, 2008). Prepulse inhibition (PPI), which refers to the inhibitory influence of a weak stimulus is typically measured by an acoustic eliciting stimulus (Fone & Porkess, 2008). This is a useful operational index of pre-attentive sensorimotor gating mechanisms essential for the integration of cognitive and sensory information (Geyer et al., 2001) which shows a similar neurobiology and neuropharmacology in rat and man (Fone & Porkess, 2008). Impairments in PPI are often reported in patients with schizophrenia as well as other psychiatric disorders such as depression, and this may reflect stimulus overload-induced cognitive fragmentation (Fone & Porkess, 2008).

Neuro-biologically, these animals demonstrate reduced prefrontal cortical volume and decreased cortical and hippocampal synaptic plasticity (Fone & Porkess, 2008), while more recent work from our laboratory has demonstrated that SIR induces altered glutamate receptor binding (Toua et al., 2010) as well as oxidative stress, and mitochondrial and immune-inflammatory dysfunction (Möller et al., 2011, 2013a). SIR is associated with a number of monoaminergic deficits as well as glutamate-redox-inflammation related changes present in depression and schizophrenia (Möller et al., 2013b). Finally, SIR-induced behavioral changes are reversed with an AD (fluoxetine) (Brenes & Fornaguera, 2009) and an antipsychotic (Möller et al., 2011, 2013a) and interestingly, the anti-oxidant N-acetyl cysteine (NAC) may strengthen the actions of clozapine in reversing the bio-behavioral effects following SIR in rats (Möller et al., 2013a, 2013b), which emphasizes a possible augmenting action when combining an antioxidant with traditional pharmacotherapy.

Several laboratory animal studies have demonstrated that SSRI ADs can reverse certain neurobiological effects brought on by trauma (Saveanu & Nemeroff, 2012). Moreover, elevated CNS

CRF mRNA expression as well as serum ACTH and corticosterone concentration following maternal deprivation is reduced by the SSRI paroxetine (Saveanu & Nemeroff, 2012). These neuroendocrine changes then revert to pre-treatment levels on discontinuation of the SSRI (Saveanu & Nemeroff, 2012).

## 7. Conclusion / Summary

It is relevant that depression forms an important co-morbid illness with schizophrenia (Fone & Porkess, 2008; Möller et al., 2013b). This study is unique in its approach in that these questions will be addressed using a neurodevelopmental animal model never before used for this purpose, one that has robust validity for depression, viz. the SIR model. The study will incorporate behavioral, endocrine and neurochemical analyses that will provide new knowledge on the role of circadian rhythms in depression. Harnessing this knowledge may then be exploited to change how we see this illness and inform on approaches to improve current treatment strategies. This study will also extend the validity of agomelatine as a novel AD compound, while at the same time allowing a better understanding of its mode of action at behavioral and biologic levels.

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The manuscript will begin with the title, contributing authors and affiliations on a separate page, followed by an Abstract on a single page. Thereafter will follow the main body of the manuscript, including Introduction, Experimental procedures, Results, Discussion, Acknowledgements, References, and Legends to Figures and Tables. As per the journal submission format, all figures are separate and placed at the end of the manuscript.

Effect of chronic agomelatine administration on depressive-like behaviours and limbic monoamines in a neurodevelopmental animal model of depression

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**Conflicts of interest:** This work was partly sponsored by Servier (SA) who is the manufacturer of agomelatine.

## Abstract:

Early-life adversity profoundly affects brain development and behavior in adulthood, consistent with the neurodevelopmental hypothesis of major depressive disorder (MDD). In this study we demonstrate using a behavioral sampling technique, that social isolation rearing (SIR), a neurodevelopmental animal model, can produce distinct depressive-like behaviors in the forced swim test (FST), as well as associated limbic monoamine alterations. The antidepressant action of agomelatine has been studied in the chronic mild stress translational model as well as the prenatal stress model of MDD but remains to be studied in an early life trauma model. SIR significantly increased immobility in the FST without affecting locomotor activity, as well as non-significantly reduced swimming and climbing behaviors. Cortico-hippocampal monoamines remained unaffected, except for non-significant reductions in striatal 5-HT and increased hippocampal 5-HIAA. Agomelatine (40mg/kg/day at 16:00 x 14 days) significantly reversed immobility in SIR rats and selectively increased noradrenergic-directed (struggling) behaviors but without markedly affecting limbic noradrenaline levels. Agomelatine did not alter serotonergic directed swimming behaviors and significantly increased serotonin (5-HT) turnover in SIR rats (decreasing 5-HT in the hippocampus; increasing 5-hydroxy indole acetic acid (5-HIAA) in the cortex and hippocampus, the main areas involved in mood). Agomelatine also significantly increased striatal dihydroxyphenylacetic acid (DOPAC) levels in SIR animals, suggesting elevated dopaminergic activity. For the most, agomelatine treatment had minimal to no effect in socially reared animals. In conclusion, SIR is associated with depressive-like symptoms and few neurochemical changes and some of these effects are attenuated by chronic agomelatine treatment, in this a neurodevelopmental animal model of MDD.

**Keywords:** circadian rhythm, agomelatine, fronto-cortical-striatal, animal model, early-life adversity

## 1. Introduction

By 2030, major depressive disorder (MDD) will be the leading contributor to the worldwide burden of disease, making it the most common psychiatric disease in terms of years lost to disability (1). The first episode of MDD generally manifests against a background of vulnerability that is determined by genetic and environmental factors. The initiator can usually be traced to some form of chronic stress that overburdens inherent psychological coping mechanisms leading to a state described as allostatic load (2). Where the body would normally strive for allostasis (balance), this compromised state leads to maladaptive behavior with progression over time to MDD (2).

The biogenic amine hypothesis, which describes MDD as a state of insufficient monoamine signalling in limbic brain circuits, still remains the foremost theory for describing the pathological basis for MDD, and its reversal by antidepressants (3). Nevertheless, on-going research has now established that a rise in monoamine levels is only one part of a complex series of events that underlies how antidepressants work, involving glutamate, neurotrophins, and metabolic and inflammatory cascades (3). However, one well recognized biological change in MDD is altered circadian rhythms (4) leading to dysfunction of a number of important physiological processes, in particular the hypothalamic pituitary adrenal (HPA) axis and its subsequent role in regulating the stress response, mood and cognitive functioning (5). Moreover, since the central regulation of circadian rhythm provides access to many fundamental processes that are either directly or indirectly involved in mood regulation, including neuroendocrine, metabolic and immune function, it provides a well-positioned neurobiological target for multi-target antidepressant action (5).

A core diagnostic feature of MDD are sleep disruptions consistent with symptoms of insomnia such as prolonged sleep latency, frequent nocturnal awakenings and early morning awakenings (6,7). The phase-shift hypothesis of MDD proposes that mood disturbances result from a phase advance or delay of the suprachiasmatic nucleus (SCN) leading to pathological changes in subservient circadian rhythms that regulate temperature, melatonin, cortisol and other neuroendocrine responses, and including subcellular processes involved in neuroplasticity (3,8). Importantly, a flatter diurnal cortisol pattern implies abnormal stress reactivity that correlates with a greater severity of depression (3). Thus, altered circadian rhythms will predetermine how the brain copes with stressful experiences and ultimately in regulating mood (3). Consequently, agents that target circadian rhythms represent novel and exciting prospects in the treatment of MDD. Since, activation of 5HTergic pathways induce circadian phase shifts by conveying non-photic timing stimuli to the SCN (8), and that selective serotonin reuptake inhibitors (SSRI) cause significant disruption of sleep architecture, this suggests that appropriate targeting of serotonin may positively influence circadian rhythms, thereby effecting an antidepressant response.

Pre-, peri- and post-natal early life stress (ELS) is known to lay the foundation for later psychiatric illness through neurodevelopmental changes in brain structure, connectivity and function (9). A number of meta-analyses have confirmed the link between traumatic or stressful life events during childhood and adult psychopathology (10-12). Social isolation in early life exerts long term effects on brain development and adult behavior both in animals and humans (13). In humans it can be linked to enduring changes in emotional behaviors that manifest in adulthood as MDD (9,14,15). This correlation between species provides a means to modeling the illness in animals, thereby providing a platform for research into the neurobiology and treatment of MDD. Post-natal social isolation rearing (SIR) is a neurodevelopmental animal model that induces a number of bio-behavioral changes related to MDD such as altered response to reward-related stimuli, cognitive deficits, anxiety- and depressive-like behaviors as well as monoaminergic alterations reviewed in (16).

Agomelatine is a new generation antidepressant presenting with melatonin (MT) MT<sub>1/2</sub> receptor agonist and 5HT<sub>2c</sub> receptor antagonist properties. Its primary mode of action is the re-entrainment of circadian rhythms by targeting SCN function, and the selective release of dopamine (DA) and NA in the frontal cortex (FC) without affecting that of 5-HT (17,18). Although the antidepressant action of agomelatine has been studied in several animal models of depression, including the chronic mild stress model of MDD (18-20), it is imperative that it be studied in other animal models, especially one that emphasizes early life trauma. In this line the effects of agomelatine were studied in the prenatal stress model of depression (21). The aim of this study is therefore to further evaluate the antidepressant-like effects of agomelatine in the SIR, neurodevelopmental animal model of MDD. In addition, we will relate any behavioral change to effects on cortico-hippocampal (limbic) and striatal monoamine levels pre- and post-treatment.

## 2. Experimental procedures

### 2.1 Animals

Male Sprague-Dawley (SD) rats, initially weighing  $\pm$  170g were obtained from the Vivarium (Northwest-University Potchefstroom Campus). All groups consisted of 12 rats. At weaning (postnatal day 21), the animals were randomly subjected to either 8 weeks SIR (one animal / cage) or social rearing (3-4 animals / cage) according to our earlier protocol (22). The rats were reared in identical cages containing sawdust (23) and housed under controlled conditions, as follows: temperature,  $21 \pm 5^\circ\text{C}$ ; humidity,  $50 \pm 10\%$ ; full spectrum cold white light (350 – 400 lux) over a 12-hour light/dark cycle; positive air with air filtration of 99.7% effective for a particle size of 2 micron and 99.9% for a particle size of 5 micron. Free access to food and water was allowed. During the period of isolation, animals were maintained with minimal handling, and no environmental enrichment. Cages were changed once a week with fresh sawdust. Behavioral testing was carried out over the last 2 days of the applied rearing condition during the dark phase of the cycle (starting at 19h00). Ethics approval was obtained from the Animal Ethics Committee of the North-West University (Ethics approval

number NWU-0054-14-S5 (2014-10-07)), and all animals were handled according to the code of ethics in research, training and testing of drugs in South Africa.

## 2.2 Drugs and drug treatment protocol

Agomelatine (Servier, France) was suspended in 1% hydroxyethylcellulose (HEC) solution and administered intraperitoneally once a day at a dose of 40 mg/kg ip, according to a previous protocol (24) while control groups received a 1% HEC solution. Agomelatine or HEC were administered at 16h00 each day during the final 2 weeks of the respective housing condition. An injection volume of 0.3 ml was used and drugs were freshly prepared each day before experimental testing and stored in a sealable glass bottle.

## 2.3 Experimental design

The study comprised a behavioral study consisting of 4 groups of animals, each randomly separated at weaning (PND 21). Two of these groups were destined for 8 weeks SIR or social rearing and received HEC treatment, with the remaining SIR group receiving agomelatine. On day 13 of treatment, the four groups of animals were subjected to behavioral testing in the open field test (OFT) and a pre-swim in the forced swim test (FST) (4 groups; see below). On the final day of treatment (day 14) the 4 groups were subjected to final testing in the FST, as described below and took place at the NWU Vivarium.

## 2.4 Behavioral analysis

### 2.4.1 Open Field Test (OFT)

The OFT measures locomotor activity, which is used as a parameter to test the general ability of the animal to move and negotiate its surroundings. The apparatus used for the test consists of a 1 m<sup>2</sup> test arena, marked with sixteen 25 x 25 cm square blocks, surrounded by opaque, vertical walls (25). Light intensity is maintained at 80 lux during the test.

On the day of testing, i.e. day 14, the rat was placed in the arena and allowed to explore the environment for 5 minutes under low light conditions (25). During this time the rat's behavior was video-taped and scored by a researcher blind to the treatment groups.

### 2.4.2 Forced Swim Test (FST)

Depressive-like symptoms were assessed using the FST on days 13 and 14, as described previously (25). All the animals received their injections at the same time each day for 14 days. On the penultimate day of treatment (day 13), i.e. 24 h prior to the final swim test, the rats were placed in the test room (under the same controlled conditions as the holding facility) to habituate for 60 min. Thereafter they were subjected to 15 min of pre-swimming in transparent Perspex cylinders (30 cm (d) x 40 cm (h)) containing 30 cm of clean water (25 ° ± 2C). On the final day of treatment (day 14), and after a 20 min habituation period in the test room, the rats were assessed in the OFT to determine locomotor activity (as described above). An hour later, the rats were reintroduced to the swimming cylinders for a final 7 min swim test in the FST apparatus. The first and last

minute of the recording was omitted from the analysis. Following this, the rats were immediately dried and returned to their home cages. The following parameters were measured:

- a. Immobility: No additional activity is observed other than that required to keep the animal's head above water.
- b. Climbing (or struggling): Upward directed movements of the forepaws along the side of the cylinder.
- c. Swimming: Either horizontal or downward movements throughout the cylinder.

## 2.5 Neurochemical analysis

### 2.5.1 Preparation of brain tissue

24 hours after the final day of drug treatment, the rats were decapitated and the frontal cortex, striatum and hippocampus immediately dissected out on an ice-cooled glass slab. After removing the olfactory bulb from the cortex and then cutting around the anterior tip of the corpus callosum, the frontal cortical tissue was dissected (26). When dissecting the striatum, the dorsal side of the brain was placed side up. The two cerebral hemispheres were split and the striatum dissected with the corpus callosum as external limits and the external walls of the lateral ventricles as internal limits (26). Finally, the hippocampus was dissected. The brain regions were then fixed in liquid nitrogen and stored at -80°C until the day of monoamine analysis. On the day of assay, the brain regions were removed from the freezer, weighed and allowed to thaw on ice.

### 2.5.2 Regional brain monoamine analysis

Frozen brain tissue collected after sacrifice was used for quantification of DA, 5-HT and NA levels in the selected brain regions using a high performance liquid chromatography (HPLC) system with electrochemical detection (HPLC-EC), as previously described (27). Monoamine concentrations in the samples were determined by comparing the area under the peak to that of the monoamine standard (isoprenaline; range 5-50 ng/ml; Chemstation Rev. A 06.02 data acquisition and analysis software). Linear standard curves (regression coefficient greater than 0.99) were found in this range. Monoamine concentrations were expressed as ng/mg wet weight of tissue.

## 3. Statistical analysis

Graphpad Prism version 6 for windows (Graphpad software, San Diego, USA) was used for statistical analysis and graphical presentations. For the behavioral and neurochemical analysis, a one-way analysis of variance (ANOVA) was performed, followed by a Bonferroni post-hoc test.

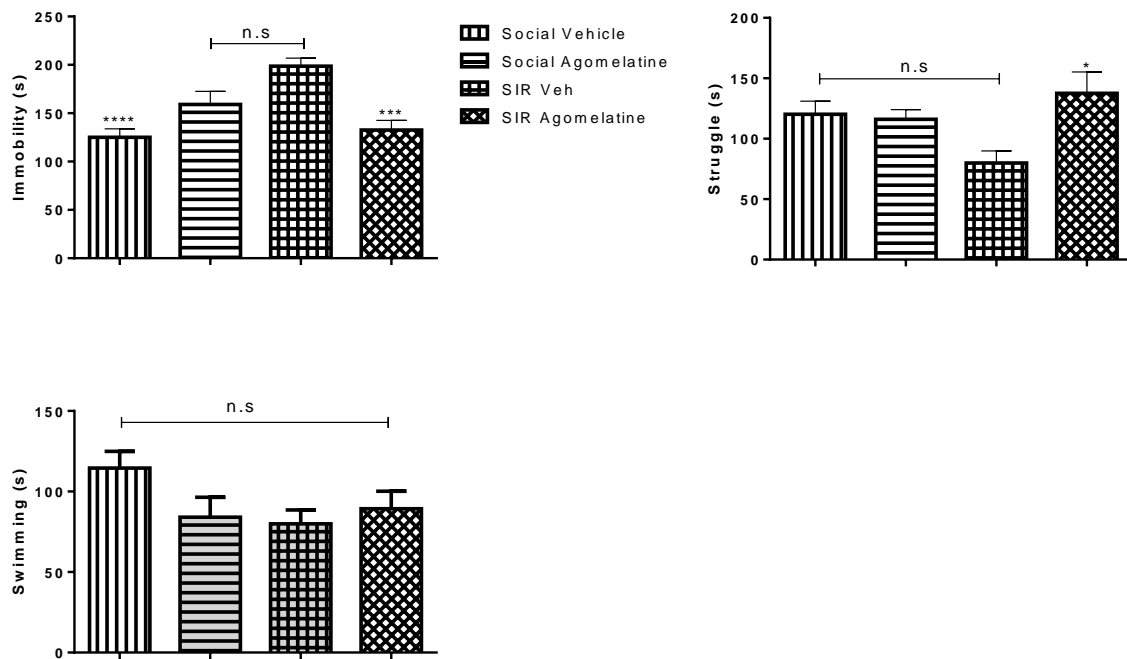


Fig. 1. Behaviors as determined in the FST in social and SIR rats receiving vehicle or agomelatine, with respect to the following behaviors: A, Immobility; B, Struggling; C, Swimming. (n=12): (A) immobility (\*\*\*)  $p < 0.001$  vs. SIR vehicle; \*\*\*\*  $p < 0.0001$  vs. SIR vehicle); (B) Struggling (\*  $p < 0.01$  vs. SIR vehicle; n.s, no significant interaction); (C) Swimming (n.s, no significant interaction). (One-way ANOVA and Bonferroni post-hoc test).

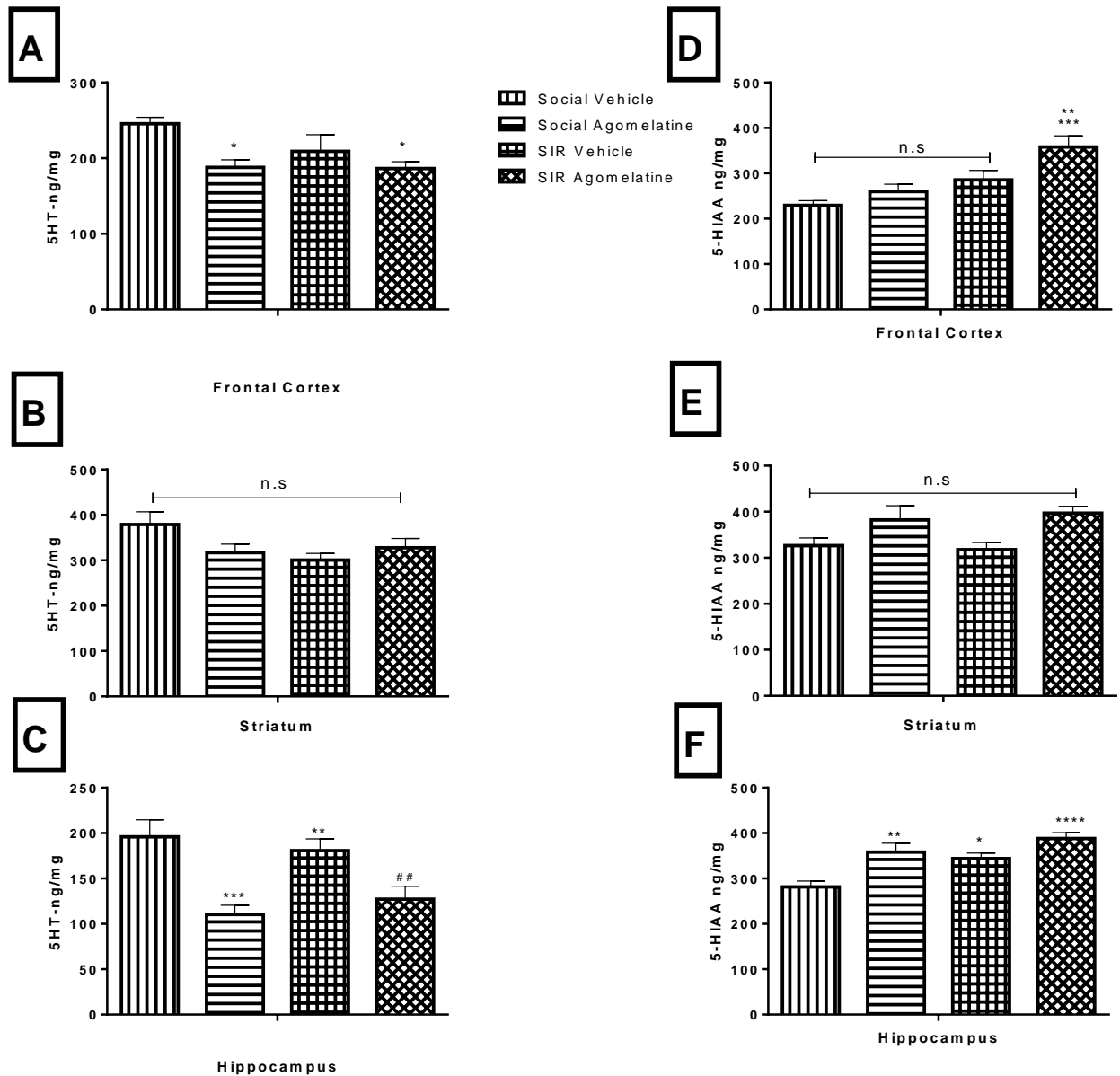


Fig. 2. 5-HT and 5-HIAA concentrations (ng/mg brain) in (A, D) frontal cortex, (B, E) striatum and (C, F) hippocampus in social and SIR rats receiving vehicle or agomelatine ( $n=12$  / group). (A) ( $* p < 0.01$  vs. social vehicle), (B) (n.s no significance), (C) ( $*** p < 0.001$  vs. social vehicle;  $** p < 0.001$  vs. social agomelatine  $## p < 0.001$  vs. social vehicle), (D) ( $*** p < 0.001$  vs. social vehicle;  $** p < 0.001$  vs. social agomelatine), (E) (n.s no significance), (F) ( $**** p < 0.0001$  vs. social vehicle;  $** p < 0.001$  vs. social vehicle;  $* p < 0.01$  vs. social vehicle). (One-way ANOVA and Bonferroni post-hoc test).

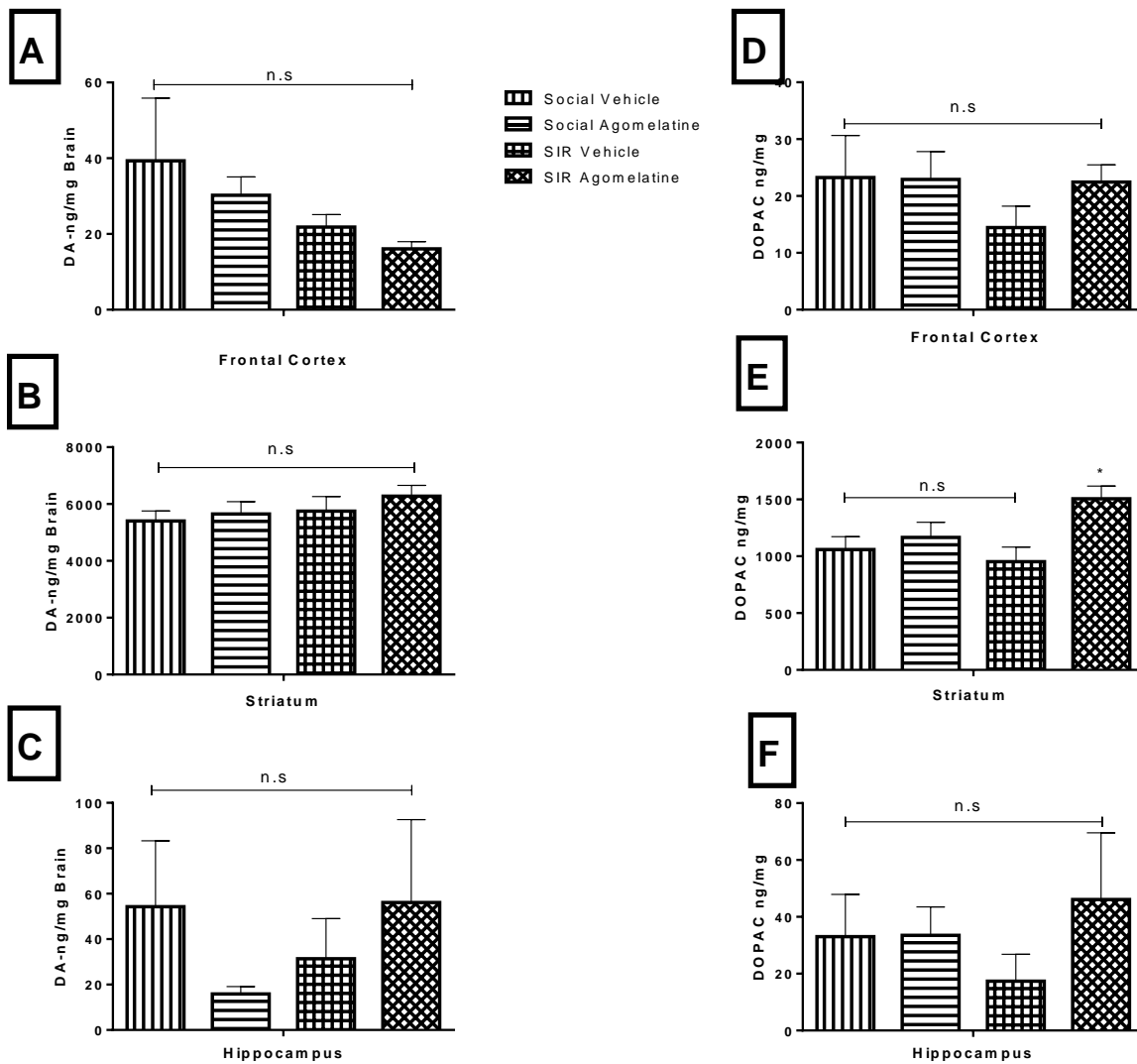


Fig. 3. DA and DOPAC concentrations (ng/mg brain) in (A, D) frontal cortex, (B, E) striatum and (C, F) hippocampus in social and SIR rats receiving either vehicle or agomelatine ( $n=12$  / group). (A-D, F) (n.s interactions); (E) (\*  $p < 0.01$  vs. SIR vehicle). (One-way ANOVA and Bonferroni post-hoc test).

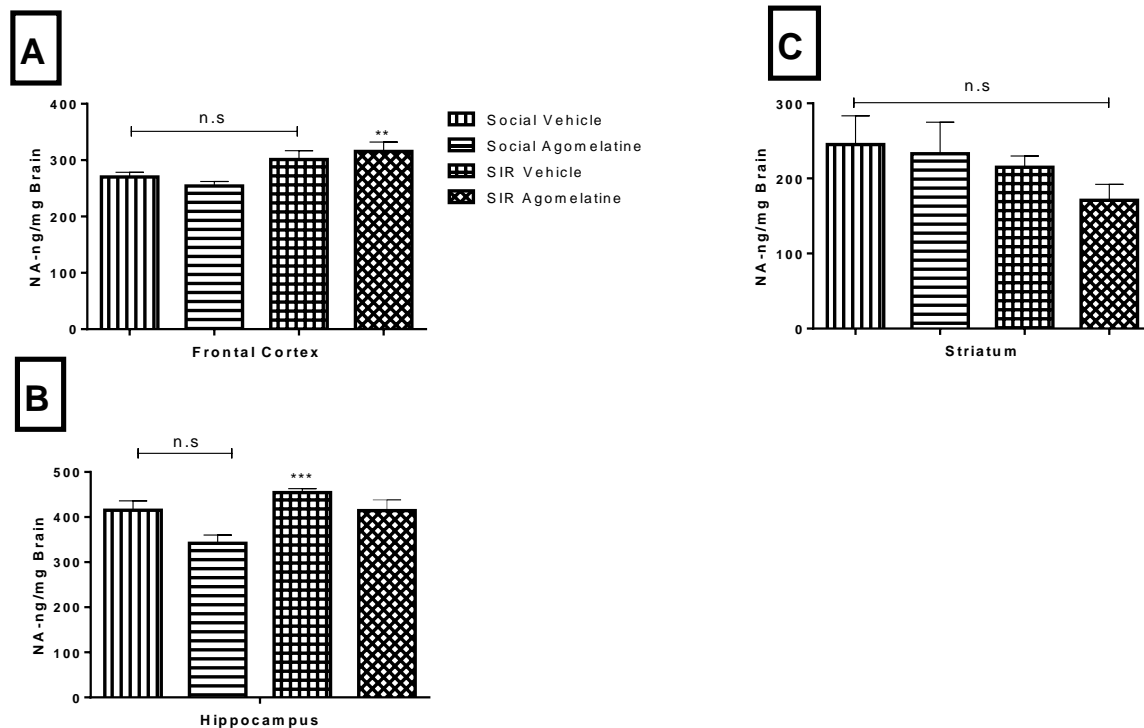


Fig. 4. NA concentrations (ng/mg brain) in (A) frontal cortex, (B) striatum and (C) hippocampus in social and SIR rats receiving vehicle or agomelatine treatment ( $n=12$  / group). (A) (\*\*  $p < 0.001$  vs social agomelatine), (B) (n.s significance), (C) (\*\*\*)  $p < 0.001$  vs .social agomelatine). (One-way ANOVA and Bonferroni post-hoc test).

## 4. Results

### 4.1. Behavioral studies

#### 4.1.1 Open field test (OFT) – locomotor activity

One-way ANOVA revealed no significant treatment\*locomotor activity interaction ( $F [3, 44] = 0.20, p = 0.90$ ) in SIR and social rats receiving drug or vehicle (data not shown).

#### 3.1.2 Forced swim test (FST)

**Immobility:** One-way ANOVA revealed a significant treatment\*immobility time interaction ( $F [3, 44] = 10.24, p < 0.0001$ ). Bonferroni post-hoc test revealed a significant increase in immobility in SIR-vehicle vs. social vehicle groups ( $p < 0.0001$ ). While agomelatine did not significantly affect immobility in socially reared animals, a significant decrease in immobility time in SIR rats receiving agomelatine vs the SIR-vehicle group was observed ( $p < 0.001$ ), with agomelatine bringing immobility time down to that of the social vehicle control (Fig. 1A).

**Struggling:** One-way ANOVA revealed a significant treatment\*struggle time interaction ( $F [3, 44] = 3.91, p = 0.02$ ). Bonferroni post-hoc test showed a trend towards reduced struggling behaviours in SIR-vehicle vs. social vehicle treated animals (n.s; Fig 1B). However, while agomelatine did not significantly affect struggling in

socially reared animals, agomelatine significantly improved struggling behaviours in SIR animals vs. SIR vehicle treated animals ( $p < 0.01$ ) and similar to that observed in social vehicle and social agomelatine treated animals (Fig. 1B).

*Swimming*: One-way ANOVA revealed no significant treatment\*swimming time interaction ( $F [3, 44] = 2.14, p = 0.11$ ) (Fig 1C).

## 4.2 Monoamine studies

### 4.2.1 Serotonin, 5-HIAA

One-way ANOVA revealed a significant treatment\*serotonin interaction in the frontal cortex ( $F [3, 44] = 4.10, p = 0.01$ ), striatum ( $F [3, 44] = 2.61, p = 0.06$ ) and hippocampus ( $F [3, 44] = 8.19, p = 0.0002$ ), as well as a significant treatment\*5-HIAA interaction in the frontal cortex ( $F [3, 44] = 8.40, p = 0.0002$ ), striatum ( $F [3, 44] = 3.67, p = 0.02$ ) and hippocampus ( $F [3, 44] = 9.15, p < 0.0001$ ).

In the frontal cortex, Bonferroni post-hoc test revealed no effect of SIR on 5-HT levels in SIR-vehicle vs. social vehicle treated animals. However, a significant ( $p < 0.01$ ) decrease in 5-HT levels in the frontal cortex was observed between social agomelatine vs. social vehicle treated animals, and SIR agomelatine vs. social vehicle treated animals ( $p < 0.01$ ) (Fig 2A).

5-HT levels were not significantly altered by SIR in the striatum of SIR vehicle vs. social vehicle animals, as well as in agomelatine treated animals (Fig. 2B;  $p < 0.01$ ) (Fig 2B; 2E).

In the hippocampus, Bonferroni post-hoc test showed significant differences in hippocampal 5-HT levels between SIR vehicle and social vehicle treated rats (Fig 2C). Furthermore, agomelatine significantly decreased hippocampal 5-HT levels in social reared agomelatine treated rats compared to social vehicle treated rats ( $p < 0.001$ ), as well as significantly decreased ( $p < 0.001$ ) 5-HT levels in SIR rats compared to social vehicle treated rats (Fig. 2C).

Concerning 5-HIAA, Bonferroni post-hoc analysis showed that SIR significantly increased 5-HIAA in the frontal cortex (Fig. 2D  $p < 0.001$ ) and hippocampus (Fig. 2F  $p < 0.01$ ) vs. social vehicle treated animals, with a similar trend noted in the striatum albeit not significant (Fig. 2E). With regard to treatment response, agomelatine induced a significant increase in 5-HIAA in the frontal cortex compared to socially reared vehicle-treated rats (Fig. 2D;  $p < 0.001$ ), with no significant differences in the striatum (Fig 2E) of SIR or socially reared rats. In the hippocampus, agomelatine induced a non-significant increase in 5-HIAA compared to SIR vehicle (Fig. 2F) but a significant increase vs. social vehicle-treated rats (Fig. 2F;  $p < 0.0001$ ). Social reared rats receiving agomelatine treatment also showed a significant increase ( $p < 0.001$ ) in 5-HIAA levels in the hippocampus compared to social reared vehicle treated rats (Fig. 2F.)

#### 4.2.2. Dopamine, DOPAC

One-way ANOVA revealed no significant treatment\*dopamine interactions in the frontal cortex ( $F [3, 44] = 1.32, p = 0.28$ ), striatum ( $F [3, 44] = 0.76, p = 0.52$ ) and hippocampus ( $F [3, 44] = 0.60, p = 0.62$ ), as well as no treatment\*DOPAC interactions in the frontal cortex ( $F [3, 44] = 0.70, p = 0.56$ ), striatum ( $F [3, 44] = 3.85, p = 0.02$ ) and hippocampus ( $F [3, 44] = 0.58, p = 0.63$ ). Subsequently, no significant differences were evident in any of the groups with respect to dopamine in the frontal cortex, striatum and hippocampus (Fig. 3A, B, C), and with respect to DOPAC in the frontal cortex and hippocampus (Fig. 3D, F). However, Bonferroni post-hoc analysis revealed a significant increase in striatal DOPAC levels in SIR agomelatine treated rats when compared to SIR vehicle treated rats (Fig. 3E;  $p = 0.05$ ).

#### 4.2.3. Noradrenaline

One-way ANOVA revealed the following treatment\*noradrenaline interactions in the frontal cortex ( $F [3, 44] = 4.74, p = 0.006$ ), striatum ( $F [3, 44] = 1.09, p = 0.36$ ) and hippocampus ( $F [3, 44] = 6.22, p = 0.001$ ). Bonferroni post-hoc analysis however showed a significant increase ( $p < 0.001$ ) in NA levels in the frontal cortex of SIR agomelatine treated groups compared to social reared agomelatine treated groups (Fig. 4A), as well as a significant increase in hippocampal NA levels in SIR vehicle treated rats compared to social reared agomelatine treated rats (Fig. 4B). No significance effects on NA in the striatum was found (Fig. 4C). MHPG levels were unfortunately below the detection limit.

## 5. Discussion and conclusion

The most important findings of this study are that SIR significantly increased immobility in the FST, without any concomitant change in locomotor activity, anhedonia-like manifestations thus indicative of a depressed-like state. In addition, swimming and climbing behaviors were reduced, although not significantly. These behavioral effects in SIR rats were not associated with monoamine changes in the frontal cortex and hippocampus, although significantly increased 5-HIAA in the hippocampus was observed. Chronic agomelatine significantly reversed the aforementioned immobility in SIR rats, but did not significantly alter immobility time in social reared rats. However, these antidepressant-like effects could not be immediately related to a bolstering of serotonergic behaviors (swimming), but rather agomelatine significantly increased noradrenergic behavior of SIR rats. In concert with these behavioral effects, agomelatine significantly reduced hippocampal 5-HT in SIR and social reared animals vs. social vehicle treated animals as well as increased 5-HIAA in the frontal cortex and hippocampus, and increased striatal DOPAC levels vs. SIR vehicle treated animals. None of the treatments altered locomotor activity, thus disassociating any antidepressant effects of the drug with increased locomotor activity.

Early life adversity is a major contributor to the development of MDD (14,15). In fact, a number of studies in children have demonstrated the association between chronic stress during adolescence with later

psychopathology (28,29). Likewise, young rodents reared from weaning in social isolated conditions show a range of maladaptive behaviors and neuropathology later in life, including depressive-like symptoms, anxiety, social and cognitive deficits etc. (30,31). With the biogenic amine hypothesis being a central construct in our understanding of MDD, it is important to know whether monoamine changes occurring in the two species as a consequence of early life stress are congruent. In this regard, clinical studies have shown that stressors in early life may influence and interact with catecholamine neurotransmitter systems via genetic polymorphisms to influence response to antidepressant treatment (32).

The literature pertaining to monoamine changes in SIR rats is varied, with evidence often contradictory (16). Post-mortem studies in rats have also found little consistency with regard to alterations in tissue levels of DA and DOPAC (33,34), and 5-HT or 5-HIAA in the striatum although basal prefrontal cortex DA levels may be elevated (33). In a recent study performed in our laboratory (22), we noted that SIR increases striatal DA with a reduction in frontal cortical DA, which links to the increased striatal DOPAC observed in this study. In the current study, SIR rats showed a significantly greater immobility time when compared to socially reared rats receiving vehicle, as well as reduced swimming and climbing behaviors (although not significantly). Social isolation therefore diminishes the attempt of the animal to escape and also impairs active coping strategies (eg. swimming and climbing). Since the latter behaviors have been linked to serotonergic and noradrenergic mechanisms, respectively (35), these findings support the idea that adaptive coping mechanisms are overcome in SIR animals leading to reduced monoaminergic drive culminating in learned helplessness and behavioral despair, i.e. depressive-like manifestations (36). At the same time, however, we failed to identify any simultaneous changes in monoamines in the frontal cortex, striatum as well as the hippocampus. That behavioral pathology, as described here, is not immediately correlated with alterations in all monoamines is not unusual. In fact, an earlier study (37) using Wistar Kyoto (WKY) rats, an animal model of depression, showed that the majority of monoaminergic alterations in these animals is restricted to 5-HTergic and NAergic systems with few differences in dopaminergic systems. Nevertheless, the observed changes in 5-HT in SIR animals herein described is noteworthy and has value in explaining the observed behavioral changes in these animals and following response to agomelatine.

Immobility, the most important variable in the FST has been interpreted as an index of depression because of the ability of antidepressants to reduce time spent in this position (38). Since mechanistically, agomelatine is well-known to selectively enhance the release of noradrenaline and dopamine but not serotonin in limbic areas by *in vivo* microdialysis (39), it was therefore important to undertake the FST using a behavioral sampling method, i.e. measuring swimming and climbing sub-scores, as well as measuring total tissue levels of limbic monoamines. This would allow correlation between behavior and monoamine levels with agomelatine's purported ability to increase noradrenergic related behavior, yet limit serotonergic activity (40).

As noted above, no noteworthy differences with respect to noradrenaline were observed in any of the brain regions studied. Moreover, no differences were observed with respect to agomelatine-treated social or SIR animals that could otherwise be linked to the significantly increased climbing behavior observed with agomelatine in SIR rats (Fig. 1B). Few studies have specifically measured behavioural changes in the FST following chronic antidepressant treatment with subsequent linkage to total tissue levels of monoamines, this possibly due to *in vivo* intra-cerebral microdialysis being a more preferred method of monoamine analysis (41). Interestingly, a decrease in swimming behaviors in SIR rats receiving agomelatine correlates with its apparent inability to increase 5HT, as can be seen in Fig. 2 (A-C). Even agomelatine treatment of social animals resulted in non significant attenuated swimming behaviors (Fig. 1C). In fact, agomelatine significantly *increased* cortical 5-HIAA levels in SIR rats (but not the striatum) as well as increased 5-HIAA levels in social and SIR rats receiving agomelatine vs. socially reared receiving vehicle treatment. These findings confirm earlier work that despite it not acutely increasing 5-HT release, agomelatine still bolsters 5-HT after chronic treatment (42,43). On the other hand, lower 5-HT in the hippocampus but elevated 5-HIAA as described above seems to suggest an increased turnover of 5-HT following agomelatine treatment. Finally, although agomelatine did not increase DA levels in the frontal cortex in either SIR or social rats, it did induce a significant increase in DOPAC levels in the striatum of SIR agomelatine treated rats vs. SIR vehicle treated rats, which could be linked to its dopaminergic profile described both in pre-clinical and clinical studies (43,44).

Agomelatine selectively increased noradrenergic-directed (struggling) behaviors yet did not markedly affect limbic NA levels, while at the same time it increased 5-HT turnover (decreasing 5-HT; increasing 5-HIAA) in selected brain regions of SIR animals without altering serotonergic directed swimming behaviors. The literature indicates that one of the primary sub-cellular actions of agomelatine rests on modulating 5-HT-NA cross-talk in the brain stem, especially by attenuating 5-HT activity (through inhibition of 5-HT<sub>2c</sub> receptors) leading to disinhibition of NA'ergic activity, most notably in the frontal cortex (43). As recently described by Chenu and colleagues (43), this complex array of monoamine interactions may be evident in our results, with increased 5-HT turnover implying increased 5-HT'ergic activity after chronic agomelatine treatment that culminates in a *normalization* of NA'ergic activity, hence the absence of obviously elevated limbic NA levels but an improvement in NA-driven coping strategies (i.e. struggling behavior).

Please include small discussion showing that the effects of agomelatine on behavior un the SIR paper are in line with AD like effect observed in prenatal stressed rats, another neurodevelopmental models of depression (21).

In conclusion, this study has confirmed that SIR presents with depressive-like symptoms in rats, together with reduced behavioral coping techniques that underscores some deficits in monoaminergic signaling, especially serotonin and noradrenaline. Secondly, agomelatine demonstrates antidepressant-like properties in SIR rats

that allow it to attenuate the above-mentioned depressive-like symptoms and to restore some of the altered monoaminergic activity. For the most, agomelatine treatment has minimal to no effect in socially reared animals, which confirms the predictive validity of these findings.

### Acknowledgments

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### Conflicts of interest

The authors declare that over the past three years, Brian Harvey has participated in advisory boards and received honoraria from Servier<sup>®</sup>, and has received research funding from Servier and Lundbeck. The authors declare that, except for income from the primary employer and research funding to BHH from the NRF, and the above-mentioned exceptions, no financial support or compensation has been received from any individual or corporate entity over the past three years for research or professional services, and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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## List of Figures

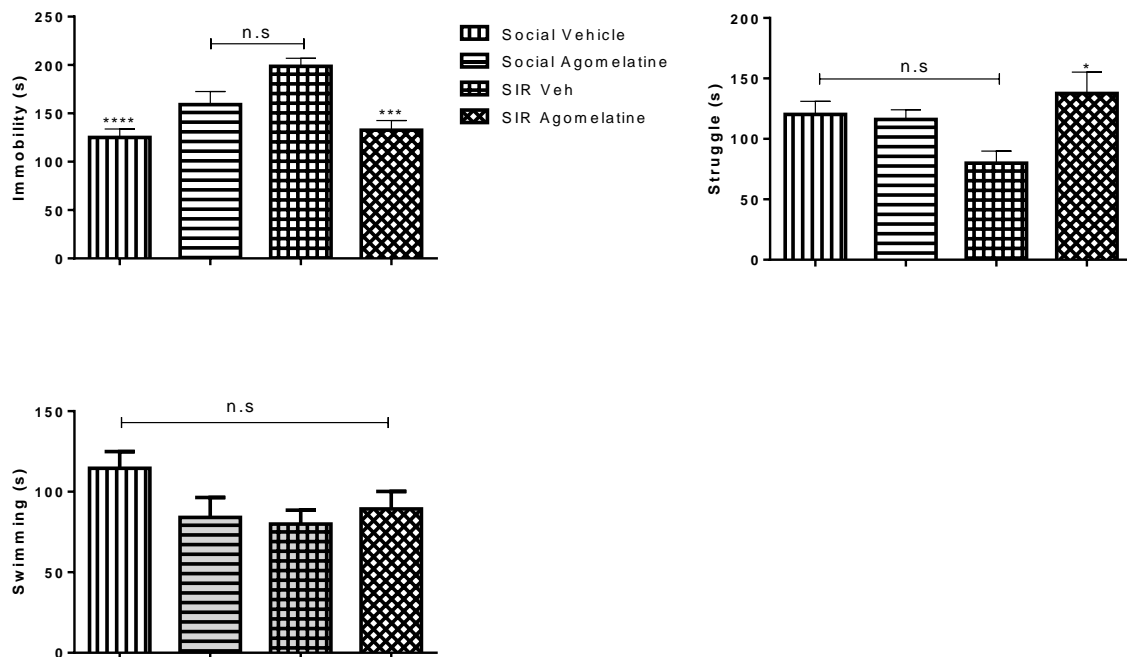


Fig. 1. Behaviors as determined in the FST in social and SIR rats receiving vehicle or agomelatine, with respect to the following behaviors: A, Immobility; B, Struggling; C, Swimming. (n=12): (A) immobility (\*\* $p < 0.001$  vs. SIR vehicle; \*\*\*\*  $p < 0.0001$  vs. SIR vehicle); (B) Struggling (\*  $p < 0.01$  vs. SIR vehicle; n.s, no significant interaction); (C) Swimming (n.s, no significant interaction). (One-way ANOVA and Bonferroni post-hoc test).

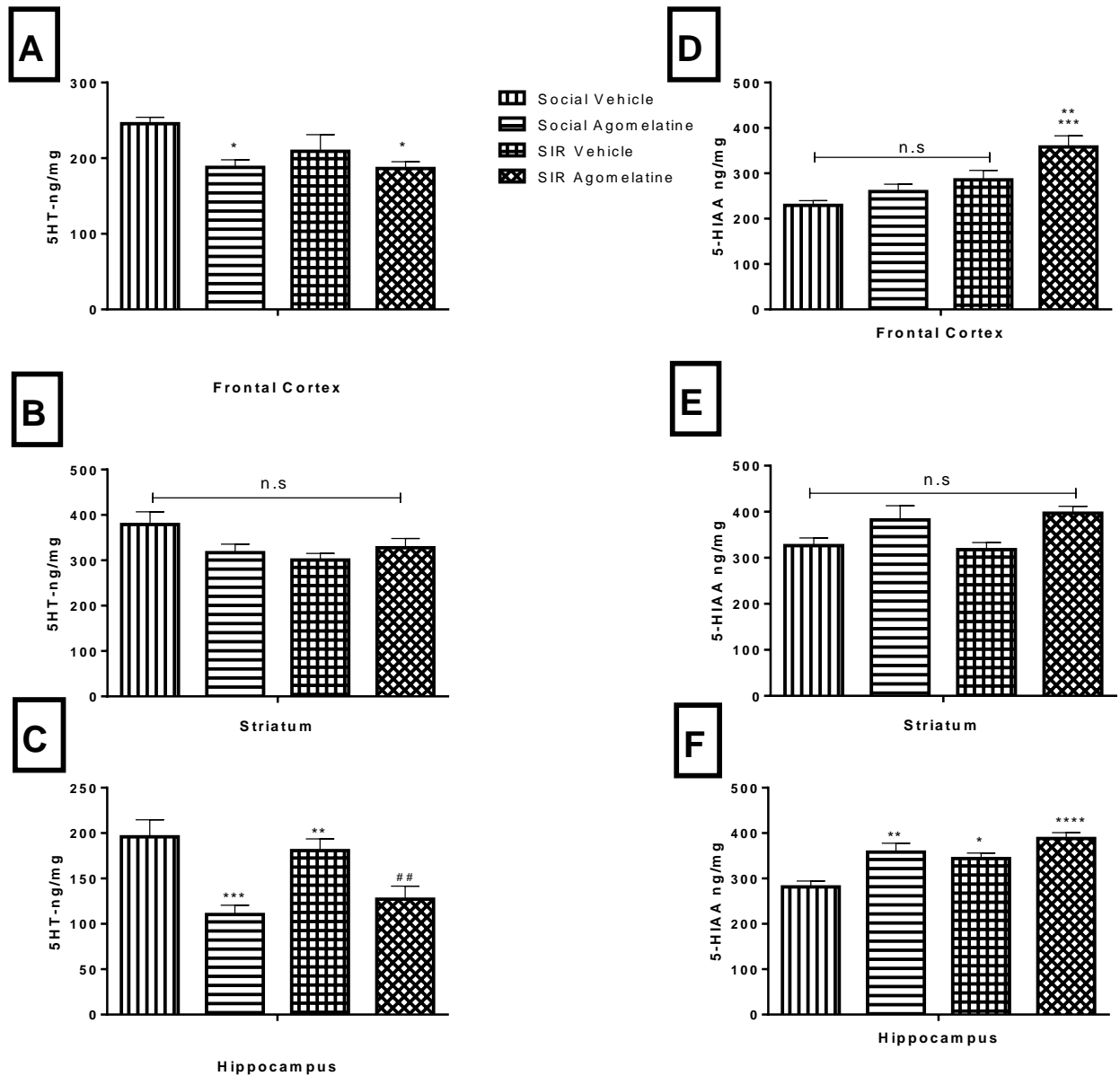


Fig. 2. 5-HT and 5-HIAA concentrations (ng/mg brain) in (A, D) frontal cortex, (B, E) striatum and (C, F) hippocampus in social and SIR rats receiving vehicle or agomelatine (n=12 / group). (A) (\*  $p < 0.01$  vs. social vehicle), (B) (n.s no significance), (C) (\*\* $p < 0.001$  vs. social vehicle; \*\*  $p < 0.001$  vs. social agomelatine ##  $p < 0.001$  vs. social vehicle), (D) (\*\* $p < 0.001$  vs. social vehicle; \*\*  $p < 0.001$  vs. social agomelatine), (E) (n.s no significance), (F) (\*\*\*\*  $p < 0.0001$  vs. social vehicle; \*\* $p < 0.001$  vs. social vehicle; \*  $p < 0.01$  vs. social vehicle). (One-way ANOVA and Bonferroni post-hoc test).

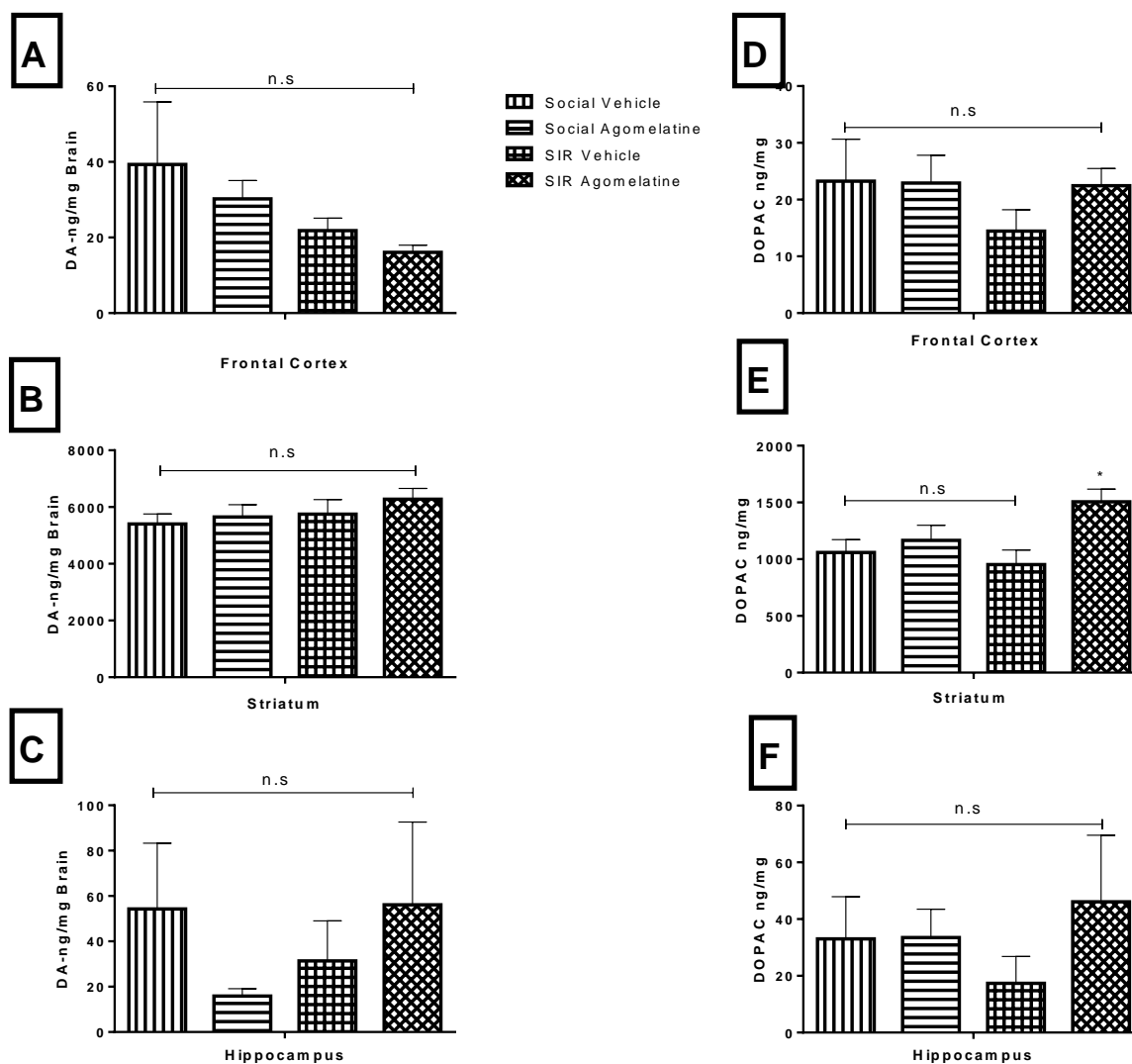


Fig. 3. DA and DOPAC concentrations (ng/mg brain) in (A, D) frontal cortex, (B, E) striatum and (C, F) hippocampus in social and SIR rats receiving either vehicle or agomelatine ( $n=12$  / group). (A-D, F) (n.s interactions); (E) (\*  $p < 0.01$  vs. SIR vehicle). (One-way ANOVA and Bonferroni post-hoc test).

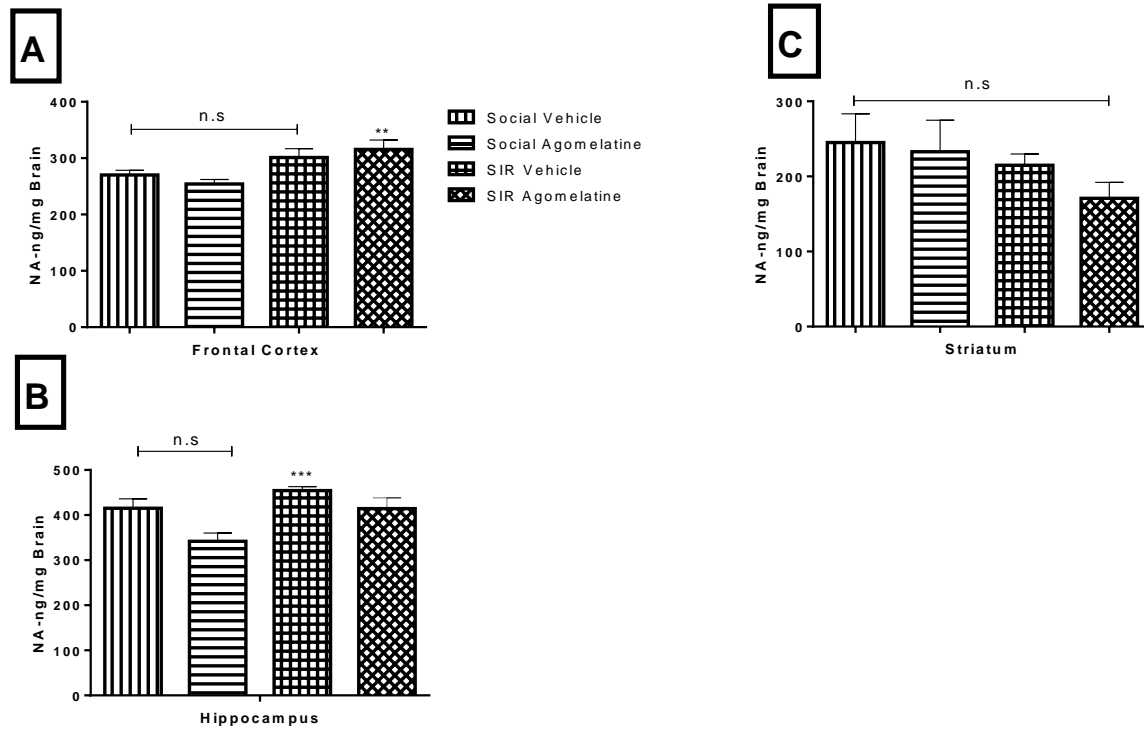


Fig. 4. NA concentrations (ng/mg brain) in (A) frontal cortex, (B) striatum and (C) hippocampus in social and SIR rats receiving vehicle or agomelatine treatment (n=12 / group). (A) (\*\*  $p < 0.001$  vs social agomelatine), (B) (n.s significance), (C) (\*\*\*)  $p < 0.001$  vs .social agomelatine). (One-way ANOVA and Bonferroni post-hoc test).

**Figure 1**

Behaviors as determined in the FST in social and SIR rats receiving vehicle or agomelatine, with respect to the following behaviors: A, Immobility; B, Struggling; C, Swimming.

**Figure 2**

5-HT and 5-HIAA concentrations (ng/mg brain) in (A, D) frontal cortex, (B, E) striatum and (C, F) hippocampus in social and SIR rats receiving vehicle or agomelatine

**Figure 3**

DA and DOPAC concentrations (ng/mg brain) in (A, D) frontal cortex, (B, E) striatum and (C, F) hippocampus in social and SIR rats receiving either vehicle or agomelatine

**Figure 4**

NA concentrations (ng/mg brain) in (A) frontal cortex, (B) striatum and (C) hippocampus in social and SIR rats receiving vehicle or agomelatine treatment

Major depressive disorder (MDD) or depression accounts for 4.4 % of years lived with disability worldwide with the second highest cause of morbidity (Kessler et al., 2005). Among factors associated with depression in adulthood is exposure to stressors such as death of a parent, maternal deprivation, and parental abandonment in early life stages (Jurueña, 2014). Childhood maltreatment is a major social problem and a complex global phenomenon which can affect a child's mental health well into adulthood (Martins et al., 2011). There are large numbers of clinical and basic studies providing new hypotheses for the pathogenesis of MDD (Cai et al., 2015). Stress and adversity experienced over a protracted period has led us to focus on the effects of both recent and remote stressors on the hypothalamic-pituitary-adrenal (HPA) axis, brain function and structure, as well as the clinical manifestation of MDD (Palazidou, 2012). The primary protagonists are suggested to be low brain-derived neurotrophic factor (BDNF) concentrations, raised cytokines, cortical and subcortical functional and structural brain changes, as well as interactions between these factors (Palazidou, 2012).

Social isolation rearing has been suggested as a useful neurodevelopmental animal model for depression with aetiological, face, construct and predictive validity (Pryce et al., 2005). The novel antidepressant agomelatine, which presents with both melatonergic and serotonergic mechanistic properties, has previously been used in various animal models including chronic mild stress (Papp et al., 2003), as well as transgenic mouse models (Cardinali et al., 2012), although has to date not been evaluated in a neurodevelopmental animal model of depression. Therefore, this study has investigated whether SIR can be established as an animal model of MDD in our laboratory, and whether it may be used to demonstrate the antidepressant properties of a novel antidepressant such as agomelatine. Furthermore, the study will assess the efficacy of agomelatine to reverse behavioral, neurochemical and neuroendocrine alterations induced by the SIR model.

## 1. Outcomes

2. SIR induced anhedonic- despair and learned helplessness -like manifestations vs. socially housed animals but did not affect cognitive function, as well as reduced active coping mechanisms, viz. struggle and swimming behaviors.
3. Chronic agomelatine treatment reversed the above depressive-like manifestations, without affecting cognition but worsened anhedonia in both social and SIR rats.
4. Chronic agomelatine treatment selectively increased noradrenergic-directed coping behaviors (i.e. struggle) without affecting serotonergic behaviors (swimming).

5. Basal corticosterone levels were unaltered in SIR animals, whereas agomelatine induced a significant increase in corticosterone levels in these animals.
6. Chronic agomelatine treatment increased plasma corticosterone levels in SIR rats, but not in socially reared animals.
7. SIR did not affect NA in any of the brain regions, although 5-HTergic changes have been observed, with increases in cortical 5-HIAA levels. SIR also showed no DA or DOPAC level alterations in any of the brain regions.
8. Agomelatine did not affect 5-HT levels in the frontal cortex, striatum or hippocampus in SIR animals, although decreased hippocampal 5-HT levels.
9. SIR did not alter brain lipid peroxidation (LPX) or superoxide dismutase (SOD) activity.
10. Agomelatine did not affect SOD activity, but increased striatal frontal-cortical lipid peroxidation in SIR rats.

## 2. Recommendations for future studies

- In order to fully understand the neurobiology of depression, it is imperative that the SIR model be further validated for MDD in order to enable more comprehensive study, and to learn why its face, construct and predictive validity were somewhat compromised in this study.
- The high levels of LPX in the frontal cortex following agomelatine treatment needs to be repeated and studied further. Further markers of oxidative stress need to be studied to give more insight on agomelatine's effects on redox status.
- The elevated plasma CORT levels following agomelatine treatment needs to be repeated and studied further. Further markers of circadian rhythm, over and above CORT, need to be studied in SIR rats to provide more insight on how agomelatine affects biological rhythms after chronic treatment.
- Utilize a serotonin (5-HT<sub>2c</sub>) receptor antagonist (eg. SB 242084), a melatonin antagonist (eg. luzindole) and a melatonin agonist (eg. ramelteon) separately and in comparison to agomelatine in order to learn more about the contributing pharmacological characteristics that contribute to the bio-behavioral effects of agomelatine in this model.
- In order to improve on the SPT results in SIR rats delivered in this study, attempt should be made to evaluate anhedonic-like behaviors over an extended time interval, ranging from post-natal day 66, 94 and 109 as described in (Brenes, Fornaguera 2008).

- In order to improve on the NORT results in SIR rats delivered in this study, longer exploration time would be of value, with bigger arenas, and possibly more prominent differences in objects.
- Instead of measuring total tissue monoamine levels, as done here, in-vivo microdialysis studies should be considered in future studies as this technique can be used more effectively to assess brain monoamine alterations in live animals, with and without drug treatment.
- In order to assess the importance of when agomelatine is administered and how this may affect its response, time-dependent dosing of agomelatine in morning vs. evening and their subsequent effects on behavioral, neurochemical and neurochemical parameters in SIR rats should be considered.

### 3. Novel findings and conclusion

No single animal model can fully replicate the overall pathology of depression which itself is a highly co-morbid disorder. The model used in this study however, demonstrated that early life stress (social isolation) can produce late-life changes in behavior and neurochemistry that are strongly reminiscent of MDD. These behaviors include significant deficits in the forced swim test, indicating behavioral despair, as well as reduced sucrose preference in the sucrose preference test, indicating anhedonic-like behaviors. In line with agomelatine's efficacy, neurochemical and neuroendocrine evidence in support of its mode of action were varied, although the absence of swimming behaviors could be related to its lack of effect on 5-HT, although on the other hand evidence in support of increased 5-HT turnover may support an increased serotonergic activity following chronic exposure. Lack of brain changes in NA, but evidence for increased noradrenergic behaviors in the FST suggest possible normalization or stabilization of NA after long-term treatment. That agomelatine increased basal plasma corticosterone in SIR animals (but not in social animals) is important as it suggests that SIR and agomelatine may together illicit an activation of the HPA axis, resulting in lipid membrane damage as observed in the LPX assay, which appears contradictory to previous studies on agomelatine.

To conclude, SIR produces selected behaviors related to MDD, thus providing us with a possible valuable approach to further investigate the neurodevelopmental etiology of MDD, this in order to identify new possible biomarkers as well as novel treatments for MDD. Moreover, we have for the first time demonstrated that the novel antidepressant, agomelatine, exerts distinct antidepressant-like effects in this model, based on behavioral and neurochemical analysis.

## 1. Introduction

This addendum contains supporting data and material to the main study where agomelatine did not demonstrate the anticipated effects and was thus removed from the concept article (Chapter 3). Consequently, the sucrose preference test (SPT), novel object recognition test (NORT), corticosterone, lipid peroxidation (LPX) and superoxide dismutase (SOD) data will be presented here.

This addendum will thus consist of:

A: The effects of agomelatine in the assessment of anhedonia, cognitive deficits and neurochemical analysis of corticosterone, LPX and SOD activities.

## 2. Materials and Methods

### 2.1 Animals

Male Sprague-Dawley (SD) rats, initially weighing  $\pm$  170g were obtained from the Vivarium (Northwest-University Potchefstroom Campus). All groups consisted of 12 rats. At weaning (postnatal day 21), the animals were randomly subjected to either SIR (one animal / cage) or social rearing (3-4 animals / cage) for 8 weeks. The rats were reared in identical cages containing sawdust (Weiss and Feldon, 2001) and housed under controlled conditions as described by (Moller et al., 2013). Ethics approval was obtained from the Animal Ethics Committee of the North-West University (Ethics approval number NWU-0054-14-S5 (2014-10-07)), and all animals were handled according to the code of ethics in research, training and testing of drugs in South Africa.

### 2.2 Drugs and drug treatment protocol

Agomelatine (Servier, France) was suspended in 1% hydroxyethylcellulose (HEC) solution and administered intraperitoneally once a day at a dose of 40 mg/kg i.p, according to a previous protocol (Papp et al., 2006), while control groups received a 1% HEC solution. Agomelatine or HEC were administered at 16h00 each day during the final 2 weeks of the respective housing condition. An injection volume of 0.3 ml was used and drugs were freshly prepared each day before experimental testing and stored in a sealable glass bottle.

### 2.3 Experimental design

The study comprised a behavioral, neurochemical and neuroendocrine study consisting of 8 groups of animals, each randomly separated at weaning (PND 21). These groups were destined for 8 weeks SIR or social rearing and received HEC vehicle or agomelatine treatment. On day 11 and 12 of treatment, the same 4 groups of animals as in chapter 3, were subjected to behavioral testing in the sucrose preference test (SPT), and on day 13 the novel object recognition test (NORT) (see description below). The remaining 4 groups were sacrificed on 24H after the last treatment on day 14 where trunk blood was collected and brain regions dissected where after analysis took place in our own laboratories under the supervision of the laboratory technicians (Fig. 1).

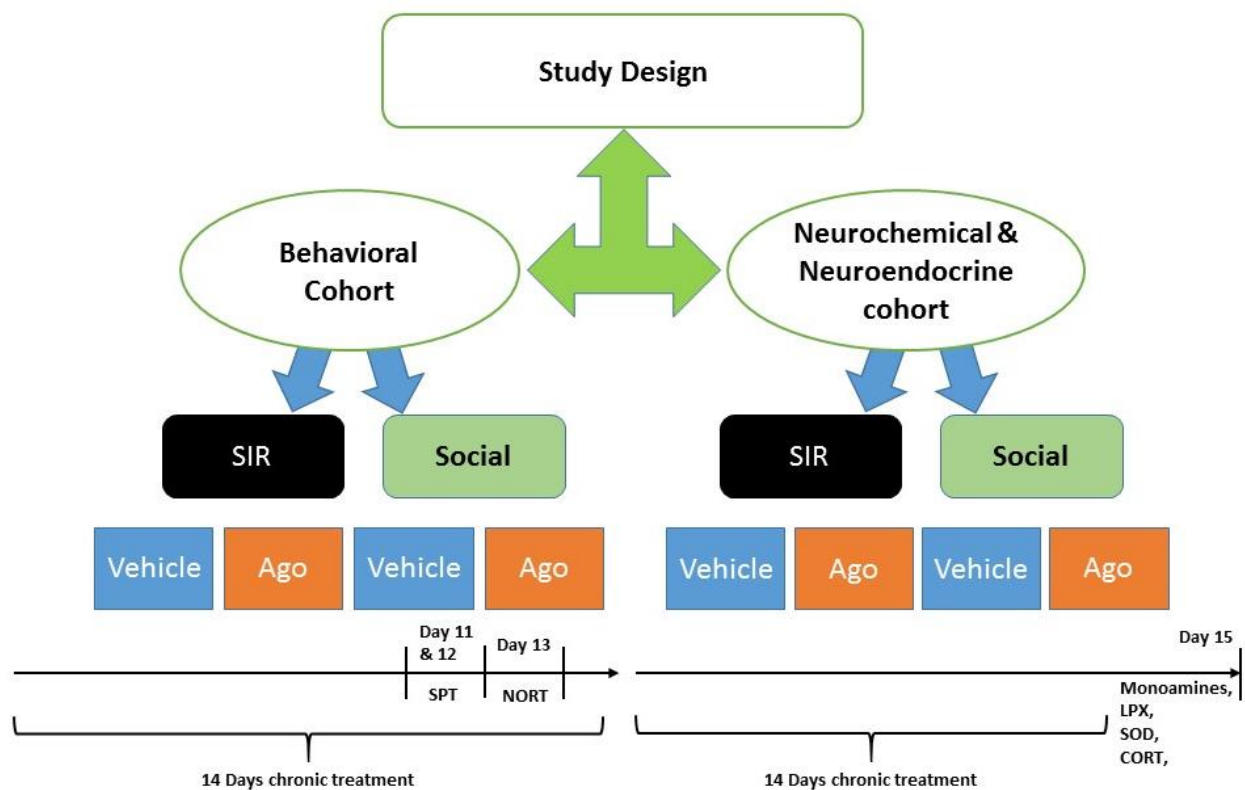


Fig. 1. Behavioral, neuroendocrine and neurochemical treatment cohorts of SIR and socially reared rats receiving either vehicle, or agomelatine (Ago) treatments.

## 3. Behavioral paradigm

### 3.1 Assessment of anhedonia

Anhedonia is one of many core symptoms of depression in humans (Moreau, 1997; Loas, 1996), while stressful conditions in rodents can lead to various behavioral changes, including anhedonia (decreased preference for sucrose) which may indicate a desensitization of the brain reward circuitry (Rygula et

al., 2005). In this study anhedonia was assessed using the sucrose preference test (SPT) as described by Brenes and Fornaguera (2009). At the beginning of the test all the groups were singly housed for 48h in individual cages. Two bottles were available in each cage, one with 200ml of 32% sucrose (w/v) and the other with 200ml of tap water. The preference was determined by measuring the following: 1. Sucrose consumption (ml), from a maximum volume of 200ml. 2. Water consumption (ml), from a maximum volume of 200ml and 3. Total sucrose and water consumption. % Sucrose consumption was calculated over the total liquid consumption x 100. At the end of 48 hours, the bottles were removed, the consumption noted and the animals returned to their previous housing conditions (Brenes et al., 2006).

### 3.2 Cognitive assessment

Stress-related psychiatric disorders are invariably associated with memory disturbances (Bremner, Narayan 1998, Bremner et al., 2003). Stress stimulates the hypothalamic-pituitary-adrenal axis which stimulates the secretion of glucocorticoids from the adrenal cortex (Aisa et al., 2007). It is well documented that cortisol (in humans) or corticosterone (in rodents) modulate learning and memory (Aisa et al., 2007), with high levels of corticosterone in rats having an inhibitory effect on learning and memory (Bodnoff et al., 1995, Roozendaal, McReynolds & McGaugh 2004, Roozendaal et al., 2003, Roozendaal et al., 2004). Novel object recognition was evaluated on day 13 as an expression of declarative recognition memory, as described previously by Moller (Möller et al., 2013a). In short the rats were exposed to a 5-min habituation session in a NOR box, made of Plexiglas (70cm x 70cm x 40cm), followed by a series of experimental trials where each rat was exposed to 2 identical objects (A1 and A2) for a period of 5 min. The rats were then returned to their home cage for a 1.5h inter-trial interval. The entire box was then cleaned with ethanol (35% vol.), both objects removed and replaced with an identical familiar copy and one with a novel unfamiliar object. Following the 1.5h inter-trial interval, the rats were returned to explore the familiar and the novel object (B) in the test box for a 5 min retention trial. The experiments were filmed and video recorded for subsequent behavioral analysis (Möller et al., 2013). Behavioral scoring was measured as follows: Time spent at familiar object + time spent at novel object / time spent at novel object x 100.

## 4. Neurochemical paradigm

### 4.1 Plasma corticosterone (CORT) analyses

#### 4.1.1 Preparation of working solution for corticosterone analysis

Plasma corticosterone levels were measured using a corticosterone enzyme-linked immunosorbent assay (ELISA) kit (Enzo Life Sciences, Telluride, Colorado) following the manufacturer's instructions. In

short, all solutions were allowed to adjust to room temp before use. Thereafter 10µl of each sample were aliquoted into microfuge tubes. A 1ml 1:100 steroid displacement reagent (SDR) was prepared immediately before use with deionized water supplied in a 2ml container. The SDR is a special formulated displacer to inhibit the steroid from binding to proteins. 10µl 1:100 SDR was added to each sample tube. The samples were then vortexed and left to stand for > 5min before diluting with ELISA buffer which was formulated using 10ml of the supplied concentrate and diluting it with 90ml deionized water. 380µl of ELISA assay buffer was added to each plasma tube and vortexed. The final dilution was 1:40. The samples were then analyzed spectrophotometrically using a Spectronic 20 spectrophotometer plate reader, as described below. For buffer ingredients, please see the manufacturer's instructions.

#### 4.1.2 Calibration of CORT curve

The percentage corticosterone bound to the antibody vs concentration of CORT was plotted using the corticosterone standards provided. A straight line was approximated through the points and the concentration of corticosterone in the unknown samples determined by interpolation, as seen in Fig. 2.

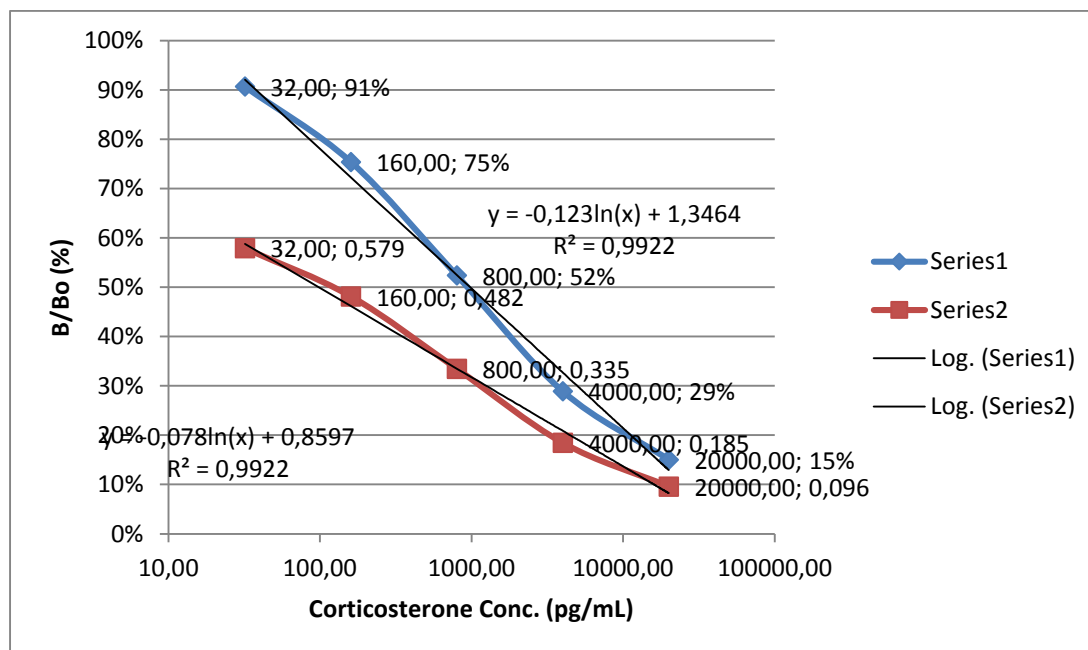


Fig. 2. Typical calibration curve and data for determination of the concentration of CORT in unknown samples.

#### 4.2 Plasma superoxide dismutase (SOD) activity

Plasma SOD activity was determined using a sensitive SOD assay kit (Sigma-Aldrich®), that uses a water soluble tetrazolium salt (WST) to produce a water-soluble formazan dye upon reduction with the superoxide anion in the sample. The rate of reduction with the superoxide anion is linearly related to

the xanthine oxidase (XO) activity present in the plasma which is inhibited by sample SOD, and can be determined by a colorimetric method. The basic biochemistry of this reaction is depicted in Fig. 3.

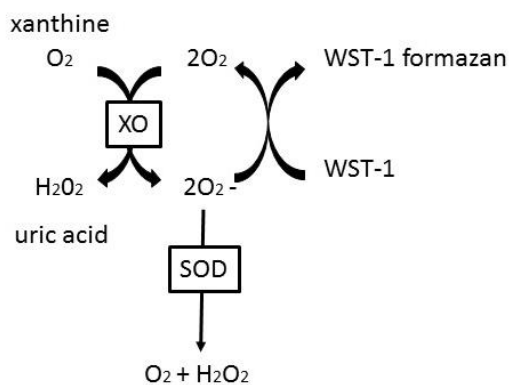


Fig. 3. Principle of the SOD Assay kit. (Sigma-Aldrich®, 2004).

#### 4.2.1 Preparation of blood samples

Blood was collected using citrate or EDTA and centrifuged at 1,000 x g for 10 min at 4°C. The plasma layer was transferred to a new tube without disturbing the buffy layer and stored at -80°C until later analysis. The buffy layer was removed from the red cell pellet and the erythrocytes re-suspended in 5x volume of ice cold distilled water and centrifuged at 10,000 x g for 10 min to pellet the erythrocyte membranes. The supernatant was then stored at -80°C until ready for analysis.

#### 4.2.2 Preparation of working solution for the SOD activity assay

**Water-soluble tetrazolium salt (WST) Working Solution:** 1 ml of WST solution was diluted with 19 ml of Assay Buffer Solution. The diluted solution is stable for up to 2 months at 4°C.

**Enzyme Working Solution:** The Enzyme working Solution provided was vortexed for 5 seconds. A pipette was used to mix the solution. 15 µl of the solution was diluted with 2.5 ml of Dilution Buffer. The diluted enzyme solution is stable for up to 3 weeks at 4°C. For buffer ingredients, please see the manufacturer's instructions.

#### 4.2.3 Determination of SOD activity in blood samples

Blanks are standards which do not contain any SOD samples and are subtracted from the SOD sample. SOD activity is determined using the following formula:

$$\text{SOD Activity (inhibition rate \%)} = \frac{(A_{\text{blank 1}} - A_{\text{blank 3}}) - (A_{\text{sample}} - A_{\text{blank 2}})}{(A_{\text{blank 1}} - A_{\text{blank 3}})} \times 100$$

#### 4.2.4 Calibration curve of SOD standards

The percentage inhibition (as mentioned above) vs. SOD Activity (U/ml) was plotted using the standards provided. A straight line was approximated through the points to determine the unknown values by interpolation Fig. 4).

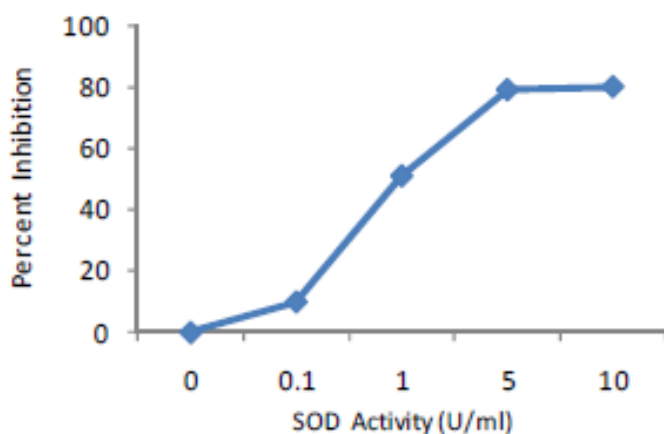


Fig. 4. Typical calibration curve obtained for the determination of % SOD activity.

### 4.3 Brain lipid peroxidation assay

#### 4.3.1 Determination of MDA present in brain samples

Lipid peroxidation is a degradation product of lipids that occurs as a result of oxidative damage and is a useful marker for oxidative stress. A well-defined chain reaction with the production of malondialdehyde (MDA) occurs in polyunsaturated lipids susceptible to oxidative attack. In this lipid peroxidation kit, lipid peroxidation is determined by the reaction of MDA with thiobarbituric acid (TBA) to form a colorimetric (532nm) product, proportional to the amount of MDA present. The values obtained from the appropriate MDA standards are obtained by subtracting the blank (0 data) from the appropriate MDA standards to plot a standard curve from which the amount of MDA present in the samples is determined by interpolation. A typical calibration curve is depicted in Fig. 5.

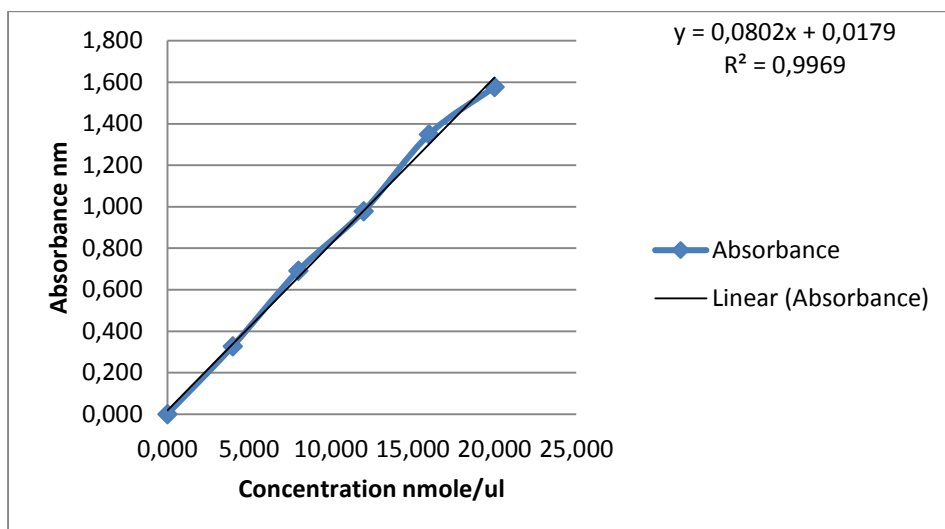


Fig. 5. Typical calibration curve for determination of the concentration of MDA in unknown samples.

#### 4.3.2 Brain preparation

Brain dissection of the frontal cortex, striatum and hippocampus are fully described in “Chapter 3”. Brain samples were homogenized on ice in 300 $\mu$ L of the MDA Lysis Buffer (see below). The samples were then centrifuged to remove insoluble materials. 200 $\mu$ L of the supernatant from each sample was then placed into a microcentrifuge tube for further processing. For buffer ingredients, please see the manufacturer’s instructions.

#### 4.3.3 Preparation of working solution for MDA standards for colorimetric detection

10 mL of the 4.17 M MDA Standard Solution was diluted with 407 mL of water to prepare a 0.1 M MDA Standard Solution. Further 20 mL of the 0.1 M MDA Standard Solution was diluted with 980 mL of water to prepare a 2 mM MDA Standard. 0, 2, 4, 6, 8, and 10 mL of the 2 mM MDA Standard Solution was added to separate microcentrifuge tubes, generating 0 (blank), 4, 8, 12, 16, and 20 nmole standards. Water was then added to each tube to bring the volume to 200 mL.

## 5. Statistical analysis

Graphpad Prism version 6 for windows (Graphpad software, San Diego, USA) was used for statistical analysis and graphical presentations. One-way ANOVA with Bonferroni post-hoc analysis was used throughout the study.

## 6. Results

### 6.1 Behavioral analysis

#### 6.1.1 Assessment of anhedonia

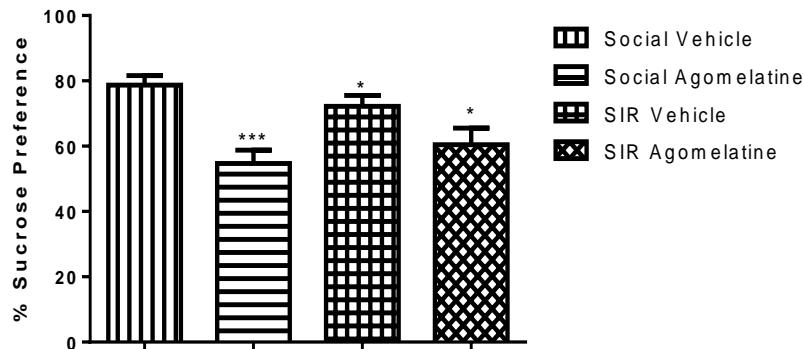


Fig. 6. % Sucrose preference for social and SIR rats receiving vehicle or agomelatine treatment. (\*\*\*)  $p < 0.001$  vs. socially reared vehicle; \*  $p < 0.01$  vs. socially reared vehicle;  $p < 0.01$  vs. socially reared agomelatine treatment). (One-way ANOVA and Bonferroni post-hoc test).

#### 6.1.2 Cognitive assessment

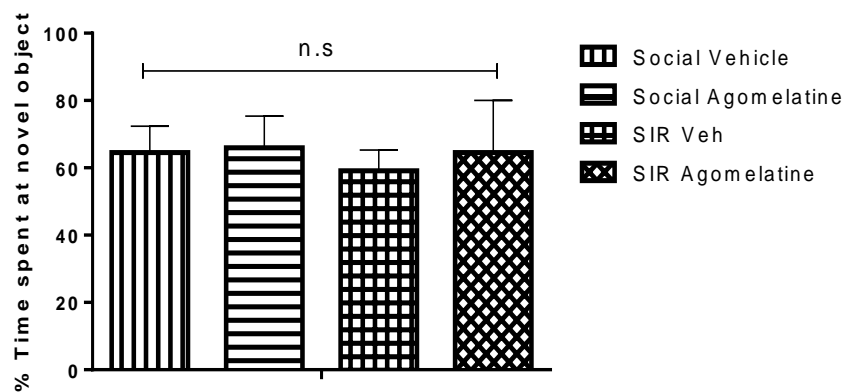


Fig. 7. Percentage time spent at the novel object in the NORT in social and SIR rats receiving either vehicle or agomelatine treatment. (n.s.=no-significance. (One-way ANOVA and Bonferroni post-hoc test).

## 6.2 Neuroendocrine and neurochemical analysis

### 6.2.1 Plasma corticosterone analysis

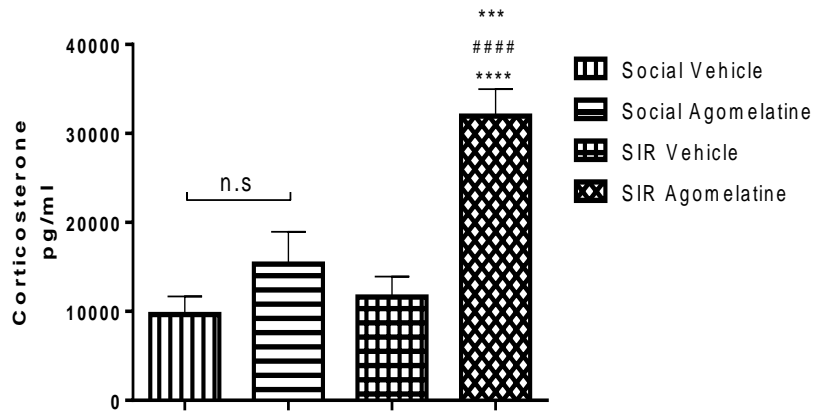


Fig. 8. Plasma corticosterone concentrations in social and SIR rats receiving either vehicle or agomelatine treatment. (\*\*\*\*  $p < 0.0001$  vs. social vehicle; #####  $p < 0.0001$  vs. SIR vehicle; \*\*\*  $p < 0.001$  vs. social agomelatine). (One-way ANOVA and Bonferroni post-hoc test).

### 6.2.2 Plasma SOD activity

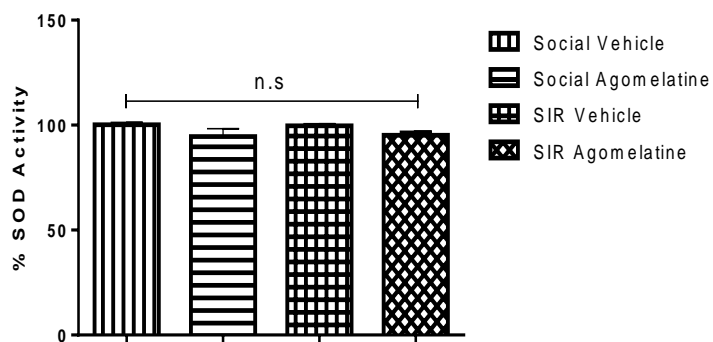


Fig. 9. Percentage superoxide dismutase activity in social and SIR rats receiving either vehicle or agomelatine treatment. (n.s.=no-significance). (One-way ANOVA and Bonferroni post-hoc test).

## 6.2.3 Regional brain lipid peroxidation

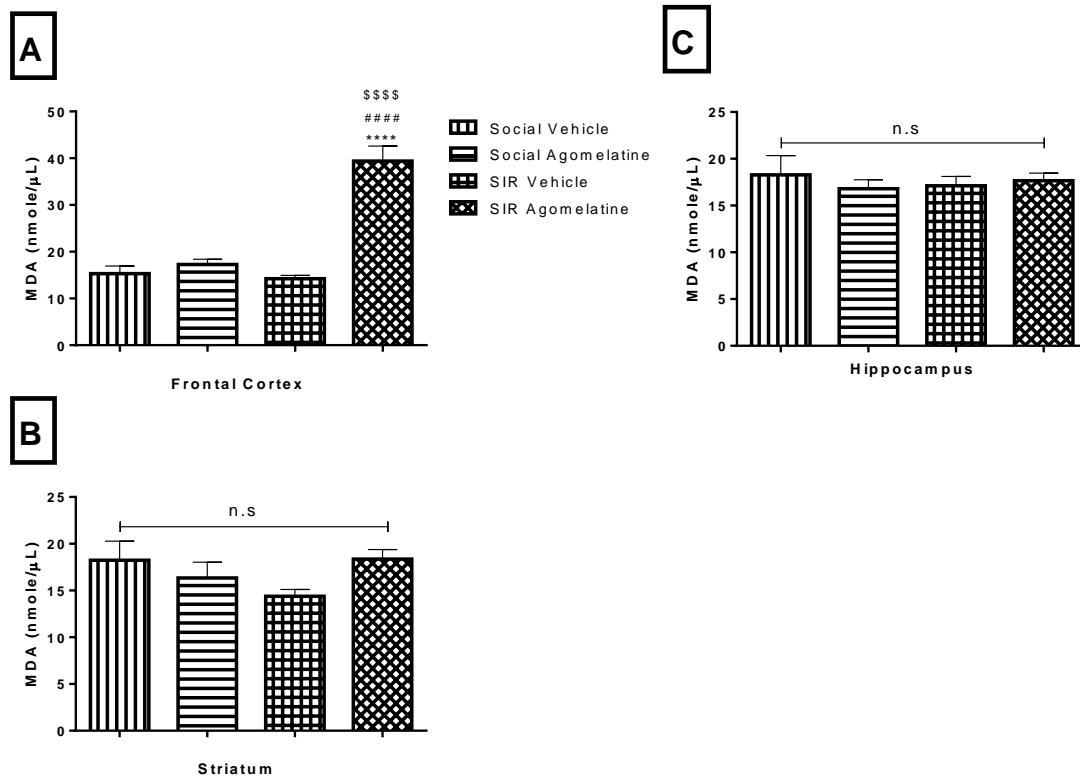


Fig. 10. Concentration of malondialdehyde (MDA) nmol/μL in (A) frontal cortex, (B) striatum, (C) hippocampus in social and SIR rats receiving either vehicle or agomelatine treatment. (A) (\*\*\*\*  $p < 0.0001$  vs. social vehicle; #####  $p < 0.0001$  vs. social agomelatine; §§§§  $p < 0.0001$  vs. SIR vehicle); (B) (n.s = no-significance); (C) (n.s = no significance). (One-way ANOVA and Bonferroni post-hoc test).

**Sucrose preference:** One-way ANOVA revealed a significant treatment \* % sucrose consumption interaction ( $F(2, 33) = 5.72, p = 0.007$ ). Bonferroni post-hoc analysis revealed that SIR vehicle-treated animals had a significantly lower sucrose consumption vs. social vehicle treated animals (Fig. 6). However, a significant reduction in SC ( $p < 0.01$ ) was noted in both social agomelatine and SIR agomelatine treated rats when compared to the social vehicle control group.

**Novel object recognition test:** One-way ANOVA revealed no significant treatment \* time exploring the novel object interaction ( $F[2, 33] = 0.57, p = 0.57$ ) (Fig. 7).

**Corticosterone analysis:** One-way ANOVA revealed a significant treatment \* corticosterone interaction ( $F[2, 33] = 24.51, p < 0.0001$ ). Bonferroni post-hoc analysis revealed there to be no difference between SIR vehicle and social vehicle treated animals (Fig. 8). However, a significant increase in CORT ( $p <$

0.0001) was observed in SIR agomelatine treated rats when compared to socially reared rats receiving vehicle, and well as compared to SIR vehicle treated rats ( $p < 0.00001$ ) (Fig. 8).

*Superoxide dismutase activity:* One-way ANOVA revealed no significant treatment\* SOD activity interaction ( $F [3, 44] = 2.03, p = 0.12$ ) (Fig. 9).

*Lipid peroxidation:* One-way ANOVA revealed a significant treatment\*MDA concentration interaction in the frontal cortex ( $F [3, 44] = 38.78, p < 0.0001$ ), although no significant interactions were observed in the striatum ( $F [3, 44] = 1.63, p = 0.20$ ) and hippocampus ( $F [3, 44] = 0.25, p = 0.86$ ). Bonferroni post-hoc analysis revealed there to be no difference between SIR vehicle vs social vehicle treated animals. However, a significant increase in MDA ( $p < 0.0001$ ) was observed in the frontal cortex of SIR agomelatine treated rats compared to socially reared vehicle treated rats, as well as compared to SIR agomelatine treated rats ( $p < 0.0001$ ) and compared to social agomelatine treated rats ( $p < 0.0001$ ) (Fig. 10A). No significance was shown for any of the treatment groups in the striatum and hippocampus (Fig. 10 B-C).

## 7. Discussion and Conclusion

The most important findings of this study are that no differences were observed between SIR vehicle and social vehicle treated animals in any of the measured parameters, except in the sucrose preference test where SIR induced a significant reduction on sucrose consumption. These data suggest that for the most SIR did not present with the predicted pathological changes akin to depression or oxidative stress, thus in essence complicating the interpretation of any changes brought about by agomelatine. These findings with agomelatine are in stark contrast to that noted in the FST (see chapter 3).

SIR vehicle treated rats showed a significant decrease in sucrose preference when compared to the socially reared vehicle controls rats, which is an indication of anhedonic-like behaviors and in line with the clinical presentation of anhedonia in patients with MDD (Bogdan, Pizzagalli 2006). Moreover, earlier studies have found that SIR decreases sucrose consumption (Hall et al., 1998a, Hall et al., 1998b), although data were not always consistent with variances being ascribed to strain differences and sucrose paradigms (Hall et al., 1997). Paradoxically however, agomelatine significantly *reduced* sucrose preference in both socially reared and SIR rats compared to the socially reared vehicle treatment group, an effect stronger than that noted in the SIR vehicle treated group. That agomelatine significantly *lowered* sucrose consumption contradicts clinical evidence where agomelatine has been found to improve hedonic behavior in patients with MDD (Di Giannantonio, Martinotti 2012) an increased sucrose consumption in the chronic mild stress animal models of

depression (Papp et al 2003). As can be seen in Chapter 3, agomelatine in the same experiment did not significantly increase DA concentration in the frontal cortex compared to the social vehicle control group, which can possibly explain the failure to increase sucrose preference noted here. However, the reason for this discrepancy with the clinical literature requires further investigation.

SIR also failed to evoke any changes in object recognition memory (Fig. 7). However, earlier work has shown that SIR does indeed induce deficits in the NORT (Moller et al., 2013).

Glucocorticoid hormones protect the brain against adverse events and are essential for cognitive performance (Sandi 1998). However, in most literature, their central action has mostly been depicted as damaging and disruptive to memory formation (de Kloet, Oitzl & Joëls 1999). In our study, no differences in plasma corticosterone were observed between SIR vehicle and social vehicle treated animals, indicating an absence of dyscortisolemia, especially hypercortisolemia that is typical of depression (Zhang et al., 2012). With the biorhythm of glucocorticoids known to be disorganized in MDD (Keller et al., 2006), our findings suggest that this particular cohort of SIR animals did not present with altered circadian rhythms. Nevertheless, earlier work in SIR rats has found that plasma CORT can be either normal (Benton, Brain 1981; Haller & Halász, 1999, Holson et al., 1991; Misslin et al., 1982) or reduced (Sánchez et al., 1998; Jessop & Bayer, 1989; Miachon et al., 1993). Oddly enough, agomelatine treated rats showed a significant increase in corticosterone, which is a paradoxical finding for an antidepressant (Holsboer, 2000). However, since plasma CORT did not differ in SIR vehicle treated animals vs. social vehicle treated animals, the agomelatine findings have limited translational relevance. Interestingly, Barden and colleagues (Barden et al., 2005) also failed to find any effect for agomelatine on corticosterone concentrations in a transgenic mouse model of depression. With such an increase in CORT following agomelatine administration, one would have expected other behavioral and/or neurochemical anomalies to be evident, yet agomelatine treated rats showed no changes in cognitive function (Fig. 7), which often shows deficits under states of continued hypercortisolemia eg, Cushing's syndrome (Sirois, 2003).

SIR failed to evoke any effects on SOD activity (plasma) or lipid peroxidation (brain), thereby indicating a relative absence of oxidative stress in SIR animals. This is opposite to that noted in MDD, where elevated lipid peroxidation levels (increased MDA concentration) have been described in patients diagnosed with recurrent depressive disorder (Rybka et al., 2013), with MDA concentrations found to be positively correlated to a prior history of recurrent episodes (Stefanescu & Ciobica 2012). As noted earlier, the evidence of hypercortisolemia in SIR animals receiving agomelatine is intriguing, especially since antidepressants are known to reduce hypercortisolemia (Pariante et al., 2012). However, that agomelatine also evoked an *increase* in cortical lipid peroxidation may be a direct consequence of

agomelatine-associated hypercortisolemia, since sustained high levels of glucocorticoids is damaging to neurons (Lee et al., 2002). In fact, hypercortisolemia has been linked to increased lipid peroxidation (oxidative stress) in MDD (Wolkowitz et al., 2010). However, this too is unusual since agomelatine has been shown to promote neurogenesis in the rat hippocampus (Banasr et al., 2006; Dagytė et al., 2010, Soumier et al., 2009) as well as the prefrontal cortex (De Berardis et al., 2013). Indeed, its antidepressant properties are related to beneficial effects on neurogenesis, cell survival, brain-derived neurotrophic factor (BDNF), activity-regulated cytoskeleton associated protein (Arc) and stress-induced glutamate release (De Berardis et al., 2013).

This work has documented the absence of certain aspects of face validity for SIR. While chapter 3 demonstrated behavioral despair in the FST following SIR, here we were only able to demonstrate the presence of anhedonia-like behavior yet failed to demonstrate cognitive deficits. Regarding construct validity, chapter 3 described the effects of SIR on regional brain monoamines which align with the monoamine hypothesis of MDD. These data suggest that SIR presents with more robust validation with respect to the FST and SPT (sucrose preference test), and with regards to the monoamine hypothesis of MDD.

Concluding, we have demonstrated contradicting results with the antidepressant-like properties of agomelatine in an early life stress model (social isolation rearing) of depression with regards to the restoration of reward processing in the SPT, thus in disagreement with the FST data presented in chapter 3. However, unexplained possibly paradoxical findings were that agomelatine *worsened* anhedonia-like symptoms as well as induced an increase in corticosterone release and an increase in lipid peroxidation, responses not typically associated with chronic antidepressant administration. It is possible that other underlying mechanisms are responsible for this and further work is required. Nevertheless, these controversial findings with agomelatine, as well as the above-mentioned deficits in face validity, complicate the interpretation of the agomelatine data. Consequently, only the FST and monoamine data were included in the chapter 3.

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