Bio-behavioral characterisation of a selective α_{2C}-receptor antagonist in animal models of schizophrenia and depression

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I can do all things through Christ
who strengthens me

I dedicate this work to my husband and friend, Nelis Uys, and to my serendipitous neighbours and ever-supporting, ever-loving friends, Anke and Theunis Cloete. You have always been there to encourage and strengthen me.

"Twee is beter as een. Saam bereik hulle meer in hulle werk. As een mens val, kan sy vriend hom ophelp. Maar as een val wat alleen is, is daar niemand om hom op te help nie. 'n Driedubbele tou breek nie maklik nie"

– Pred 9:10, 12 –

Abstract

Purpose

Schizophrenia and depression are neuropsychiatric disorders characterised by affective and cognitive dysfunction and are associated with altered monoaminergic and neurotrophic function. Social isolation rearing (SIR) is a neurodevelopmental rodent model of schizophrenia that reflects many of the behavioural and neurochemical features of schizophrenia, while the Flinders Sensitive Line (FSL) genetic rodent model of depression reflects various behavioural and neurochemical features of the human disorder. The α_2-adrenoceptor (α_2-AR) is a well-established neurobiological target for antipsychotic and antidepressant drug design, with a number of clinically used drugs presenting with α_2-AR antagonism in their pharmacological profile. Selective α_{2C}-adrenoceptor (α_{2C}-AR) antagonism has been suggested to present with superior neuropsychiatric effects vs. non-selective α_2-AR antagonism. The purpose of this study was to assess cognitive and antipsychotic-like effects of α_{2C}-AR antagonism in the SIR model of schizophrenia, as well as cognition and antidepressant-like effects in the FSL model of depression. These pharmacological effects were compared to various reference agents, such as clozapine (CLOZ) and imipramine (IMI), as well as the non-selective α_2-AR antagonist, idazoxan (IDAZ). Additionally, the behavioural and neurotrophic effects of augmenting D_2-antagonist therapy with α_{2C}-AR antagonism in the SIR model were investigated. Finally this study assessed the effects of α_{2C}-AR antagonism on striatal (SIR) and hippocampal (FSL) monoamine levels and brain-derived neurotrophic factor (BDNF) in the respective animal models.

Methods

Three separate studies were conducted employing chronic treatment with the novel, highly selective α_{2C}-AR antagonist ORM-10921. In the first study, male Sprague Dawley rats were either reared socially (SOC) or reared in social isolation (SIR) for 8 weeks following weaning. SIR rats received either vehicle (1 ml/kg), CLOZ 5 mg/kg , IDAZ 3 mg/kg or one of various doses of ORM-10921 (0.3 – 1mg/kg) subcutaneously (SC) once daily for 14 days, where after behaviour in the prepulse inhibition (PPI) test and novel object recognition test (NORT) were assessed. Post-mortem striatal monoamine levels were determined by high performance liquid chromatography (HPLC), while total striatal BDNF levels were determined by enzyme-linked immunosorbent assay (ELISA). In study 2, SIR animals received either vehicle, CLOZ, haloperidol (HAL) 2 mg/kg, ORM-10921 (0.01 or 0.03 mg/kg) or HAL + ORM-10921 (0.01 or 0.03 mg/kg) for 14 days SC. Again behaviour in the PPI test and NORT were assessed, where after post-mortem striatal BDNF levels were determined as above. The third study employed 11-week old

male FSL rats that were treated with either vehicle, ORM-10921 (0.01 – 1mg/kg), IMI 15 mg/kg or IDAZ 3mg/kg administered SC or intraperitoneally for 14 days. Behaviour in the forced swim test (FST) and the NORT were subsequently assessed. In a separate group of drug treated animals, post-mortem hippocampal monoamine and BDNF levels were determined as described above.

Results

α_{2C}-AR-antagonism with ORM-10921 reversed SIR-induced deficits in PPI and object recognition memory comparable to CLOZ treatment but superior to the non-selective α_2-antagonist, IDAZ. ORM-10921 increased striatal noradrenaline (NA) and decreased striatal dopamine (DA) in SIR rats, while CLOZ, but not ORM-10921 or IDAZ increased striatal BDNF. Augmentation of HAL with ORM-10921 bolstered the effects of HAL on PPI and object recognition memory, while also elevating striatal BDNF, an effect not obtained with monotherapy of either drug. ORM-10921 improved object recognition memory and decreased immobility in the FST in FSL rats. These behaviours were comparable to IMI, but superior to that of IDAZ. ORM-10921 increased serotonergic-driven swimming more than noradrenergic-driven climbing behaviour in the FST. ORM-10921 did not alter hippocampal BDNF levels after 14 days of treatment, but did increase hippocampal levels of DA, NA and serotonin.

Conclusions

α_{2C}-AR-antagonism with ORM-10921 presents with pro-cognitive, antipsychotic-like and antidepressant-like effects in the SIR and FSL translational models of schizophrenia and depression. These behavioural effects were associated with beneficial effects on dysfunctional monoamine levels in the striatum and hippocampus of SIR and FSL rats, respectively, albeit not with immediate effects on striatal and hippocampal BDNF levels. Furthermore, beneficial effects of α_{2C}-AR-antagonism on the outcomes of D_2-antagonist therapy on sensorimotor gating and cognition was evident, while these effects were correlated to increased striatal BDNF levels. The results thus suggest that α_{2C}-AR-antagonism is a potentially valuable therapeutic strategy in the treatment of neuropsychiatric disorders characterised by cognitive and affective dysfunction associated with monoaminergic alterations, as evidenced in two translational models of neuropsychiatric illnesses. α_{2C}-AR-antagonism also shows potential as augmentation strategy to typical antipsychotic treatment.

Keywords: α_{2C}-adrenoceptor, α_{2C}-antagonism, schizophrenia, depression, social isolation rearing, Flinders Sensitive Line rat, prepulse inhibition, object recognition memory, forced swim test, hippocampus, striatum, BDNF, monoamines

Opsomming

Doelstelling

Skisofrenie en depressie is neuropsigiatriese steurnis wat geassosieer word met afwykings in monoaminergiese en neurotrofiese funksies en gekenmerk word deur affektiewe en kognitiewe versteurings. Sosiale isolasie-geïnduseerde stres, oftewel, sosiale isolasie-stres (SIS) is 'n neuro-ontwikkelingsmodel van skisofrenie in rotte, en dit reflekteer baie van die neurochemiese en gedragversteurings wat waargeneem word in skisofrenie. Aan die ander kant is die Flinders se Sensitiewe Lyn- (FSL-) rot 'n genetiese knaagdiermodel van depressie, wat verskeie neurochemiese en gedragseienskappe van menslike depressie weerspieël. Die adrenergiese α_2-reseptor (α_2-AR) is 'n goedgevestigde neurobiologiese teiken vir die ontwerp van farmakologiese middels wat as antipsigotikums en antidepressante kan optree, en 'n aantal middels wat tans klinies aangewend word beskik oor α_2-AR-antagonisme as deel van hul farmakologiese profiel. Daar word aangevoer dat selektiewe antagonisme van die adrenergiese α_{2C}-reseptor (α_{2C}-AR) verbeterde neuropsigiatriese effekte kan toon teenoor nieselektiewe α_2-reseptorantagonisme. Die doel van hierdie studie was om die kognitiewe en antipsigotiese effekte van α_{2C}-AR-antagonisme in die SIS-model van skisofrenie en die kognitiewe en antidepressantagtige effekte van α_{2C}-AR-antagonisme in die FSL-model van depressie te ondersoek. Hierdie farmakologiese effekte is vergelyk met die kliniese antipsigotikum, klosapien (KLOS), die kliniese antidepressant, imipramien (IMI) en die nieselektiewe α_2-AR-antagonis, idasoksaan (IDAS). Daarbenewens is die effekte van kombinasieterapie met D_2-antagonism en α_{2C}-AR-antagonisme op gedrag en neurotrofiese effekte in die SIS-model ondersoek. Laastens het hierdie studie ook die effekte van α_{2C}-AR-antagonisme op breinmonoamienvlakke en brainafkomstige neurotrofiese faktor (BDNF) in die striatum (SIS) en hippokampus (FSL) in die onderskeie dieremodelle ondersoek.

Metodes

Drie afsonderlike studies het chroniese behandeling met die nuwe, hoogs selektiewe α_{2C}-AR antagonist, ORM-10921, geïmplementeer. In die eerste studie is Sprague Dawley-mannetjiesrotte toegelaat om óf sosiaal (SOS), óf sosiaal geïsoleerd (SI) te ontwikkel na spening. SI-rotte is eenmaaldaaglikse onderhuidse soutoplossing (1ml/kg), KLOS 5 mg/kg, IDAS 3 mg/kg of een van 'n aantal dosisse van ORM-10921 (0.3-1mg/kg) toegedien vir 14 dae, waarna gedrag in die prepulsinhibisie-toets (PPI) en voorwerpherkenningstoets (VHT) geëvalueer is. Nadoodse striatale monoamienvlakke is bepaal d.m.v. die hoogsdoeltreffende vloeistofchromatografie (HDVC) metode en totale striatale BDNF-vlakke is

m.b.v. die ensiemgekoppelde immuunsorberende essai (ELISA)-metode bepaal. In die tweede studie het SI-diere vir 14 dae onderhuidse soutoplossing, KLOS, haloperidol (HAL) 2mg/kg, ORM-10921 (0.01 of 0.03 mg/kg) of HAL+ORM-10921 (0.01 of 0.03 mg/kg) ontvang. Gedrag is weereens in die PPI en VHT geëvalueer, waarna nadoodse striatale BDNF-vlakke bepaal is soos hierbo beskryf. Die derde studie het van 11-weekoue mannetjies FSL-rotte gebruik gemaak. FSL-rotte is vir 14 dae behandel met onderhuidse of intraperitoneale soutoplossing, ORM-10921 (0.01 – 1 mg/kg), IMI 15 mg/kg of IDAS 3mg/kg. Gedrag in die geforseerde swemtoets (FST) en die VHT is daarna waargeneem. In 'n afsonderlike groep diere is nadoodse monoamien- en BDNF-vlakke in die hippokampus bepaal soos hierbo beskryf.

Resultate

α_{2C}-AR-antagonisme met ORM-10921 het die SIS-geïnduseerde afwykings in PPI en voorwerpherkenningsgeheue omgekeer op 'n wyse wat vergelykbaar was met die effekte van KLOS, maar wat meer effektief was as die nieselektiewe α_2-antagonis, IDAS. ORM-10921 het striatale dopamienvlakke in SIS rotte verlaag en striatale noradrenalienvlakke in SIS rotte verhoog. KLOS het striatale BDNF-vlakke verhoog, maar nie ORM-10921 óf IDAS het striatale BDNF-vlakke beïnvloed nie. Kombinering van HAL met ORM-10921 het die effekte van HAL op PPI en voorwerpherkenningsgeheue verbeter en ook striatale BDNF-vlakke verhoog. Laasgenoemde is nie 'n effek wat deur monoterapie met HAL of ORM-10921 verkry kon word nie. ORM-10921 het voorwerpherkenningsgeheue verbeter in FSL-rotte en ook FST-immobiliteit in hierdie diere verlaag. Hierdie gedragseffekte was vergelykbaar met IMI, maar meer doeltreffend as die effekte van IDAS. ORM-10921 het meer betekenisvolle effekte op serotonergies-gedrewe swemgedrag in die FST gehad as wat dit op noradrenergies-gedrewe klimgedrag gehad het. ORM-10921 het nie BDNF-vlakke in die hippokampus van FSL-rotte beïnvloed na 14 dae se behandeling nie, maar het wel vlakke van dopamien, noradrenalien en serotonien in die hippokampus van FSL-rotte verhoog.

Gevolgtrekkings

α_{2C}-AR-antagonisme met ORM-10921 blyk dus voordelige effekte te hê op kognisie en beide antipsigotiese en antidepressantagtige effekte uit te oefen in die SIS- en FSL-modelle van skisofrenie en depressie onderskeidelik. Hierdie effekte op gedrag kan gekoppel word aan voordelige effekte op monoamienvlakke in die striatum en hippokampus van SIS- en FSL-diere onderskeidelik, alhoewel dit nie met onmiddellike effekte op BDNF-vlakke in hierdie breindele geassosieer kon word nie. Verder blyk α_{2C}-AR-antagonis-kombinasieterapie voordelige effekte te hê op die uitkomstes van D_2-antagonisterapie op PPI en kognisie, terwyl hierdie effekte 'n positiewe korrelasie getoon het met striatale BDNF-vlakke. Die resultate uit twee dieremodelle van neuropsigiatriese siektes dui dus daarop

dat α_{2C}-AR-antagonisme ‘n belowende terapeutiese strategie kan wees in die behandeling van neuropsigiatriese versteurings wat deur kognitiewe en affektiewe disfunksie gekenmerk word. α_{2C}-AR-antagonisme toon ook potensiaal as ‘n ondersteuningstrategie in kombinasie met tipiese antipsigotika.

Sleutelwoorde: adrenergiese α_{2C}-reseptor, α_{2C}-antagonisme, skisofrenie, depressie, sosiale isolasie-stres, Flinders se Sensitiewe Lyn-rotmodel, prepuls-inhibisie, voorwerpherkenningsgeheue, geforseerde swemtoets, hippokampus, striatum, BDNF, monoamiene

Congress Proceedings

Findings from this study were presented at two international congresses (poster presentations) and one national congress (podium presentation)

1) Madeleine Uys, Brian H. Harvey, Mohammed Shahid, Jukka Sallinen (2016). An investigation into the antipsychotic and pro-cognitive properties of α_{2C}-adrenoceptor antagonism in social isolation reared rats (*podium presentation*). The 2nd African College of Neuropsychopharmacology Congress, Stellenbosch, South Africa (30 – 31 July 2016).

2) Madeleine Erasmus*, Mohammed Shahid, Jukka Sallinen, Brian H. Harvey (2015). α_{2C}-selective AR-antagonism with ORM-10921 decreases behavioural despair and improves cognition in the Flinders Sensitive Line rat model of depression (*poster presentation*). The 28th Congress of the European College of Neuropsychopharmacology, Amsterdam, Netherlands (29 August – 1 September 2015).

3) Madeleine Erasmus*, Mohammed Shahid, Jukka Sallinen, Brian H. Harvey (2014). The selective α_{2C}-AR-antagonist ORM-10921 improves recognition memory and shows modest antipsychotic-like effects in an animal model of schizophrenia (*poster presentation*) The 17th World Congress of Basic and Clinical Pharmacology, Cape Town, South Africa (13 – 18 July 2014).

*The maiden name of Madeleine Uys was *Erasmus*

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"No man is an island, entire of itself"

-John Donne

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" My maer hande steek vir jou 'n kers aan,
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omdat jy my maat bly,
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en my vashou teen jou bors.
….
Kyk hoe bewe my kers nou.
Hy sê dankie, en hy bid vir jou."
-Antjie Krog-

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Chapter 1: Introduction

1. Thesis Layout

This PhD thesis has been compiled in the article format as approved by the North-West University. The main experimental findings from this study are therefore presented in three research articles, two are already published in international peer-reviewed journals, and a third is in preparation. A fourth article is an extensive literature review of the therapeutic potential of the α_{2C} receptor and of selective α_{2C}-AR ligands in depression and schizophrenia, and is currently in submission. Additional findings pertaining to certain unpublished dose-response data as well as other relevant data are presented as addenda. Fig 1-1 and Figure 1-2 at the end of this chapter provide a graphical representation of the thesis layout.

Chapter 1: Introduction

In Chapter 1, the reader is introduced to how the thesis has been structured and presented, including the problem statement, the study hypothesis and aims, and the study layout.

Chapter 2: Literature review

In Chapter 2, a literature review relevant to the background necessary to formulate the hypotheses and aims of this study is provided. This literature review more comprehensively covers the literature background of the studies presented in Manuscripts A, B and C. Due to the paucity of available literature on the α_{2C} receptor, which is the focus of this study, appropriate detail from this chapter has by necessity been incorporated in Manuscript D, a literature review and update of the therapeutic potential of targeting the α_{2C} receptor in neuropsychiatric illness.

Chapters 3-6: Manuscripts prepared for peer-reviewed publication

In Chapters 3 to 6, full-length articles are presented that have either been published in, or are in submission or in preparation for submission to a peer-reviewed international journal. The manuscripts are presented as they appear in the respective journals, or according to their submission criteria if unpublished at the time the thesis is submitted for examination.

- **Chapter 3** presents the main findings regarding the antipsychotic-like, pro-cognitive and neurotrophic effects of a selective α_{2C} AR-antagonist in an animal model of schizophrenia. This full-length article (**Manuscript A**) has been published in *Progress in Neuro-Psychopharmacology and Biological Psychiatry* (Elsevier).

- **Chapter 4** presents the main findings regarding the antidepressant-like and pro-cognitive effects of a selective α_{2C}-AR-antagonist in an animal of depression (**Manuscript B**). This article has been published in *Behavioural Pharmacology* (Wolters Kluwer).
- **Chapter 5** presents the main findings regarding the effects of the selective α_{2C} AR-antagonist on hippocampal monoamine levels in an animal model of depression (**Manuscript C**). This manuscript has been prepared as a concept article for submission to an international peer-reviewed journal.
- **Chapter 6** provides an in-depth overview of current knowledge regarding the α_{2C} receptor as a therapeutic target in schizophrenia and depression, and is the first comprehensive review on the subject (**Manuscript D**). This manuscript has been submitted to *Frontiers in Psychiatry, Molecular Psychiatry* section (Frontiers Media).

Chapter 7: Conclusion and Recommendations

Chapter 7 overviews and discusses the findings presented and/or published in international peer-reviewed scientific journals (Chapters 3-6), as well as unite these findings with those presented as addenda. Chapter 7 will also provide a final conclusion on the study as well as report on shortcomings and limitations, while at the same time providing recommendations and directions for future research.

Additional Data (Addenda)

The addenda contain important data that, although not included in any of the published manuscripts or manuscripts prepared for submission to an international peer-reviewed journal (Chapters 3-6), provide the necessary basis upon which various decisions were made during the study.

- **Addendum A** reports the dose-response effects of ORM-10921 on prepulse inhibition and recognition memory in SIR rats; these data were an essential component that led to the publication of **Manuscript A** (Chapter 3).
- **Addendum B** reports the dose-response effects of ORM-10921 on striatal BDNF and monoamine levels in SIR rats; these data were an essential component that led to the data published in **Manuscript A** (Chapter 3).
- **Addendum C** reports additional dose-response effects of ORM-10921 alone and as augmentation to haloperidol on prepulse inhibition and recognition memory in SIR rats; these data were essential to the outcome of **Manuscript A** (Chapter 3).
- **Addendum D** reports the dose-response data of ORM-10921 in the FSL animal model of depression and the effects in the forced swim test and object recognition memory; these data were essential to the generation of the data published in **Manuscript B** (Chapter 4).

- **Addendum E** reports additional methodology and data reporting the effects of ORM-10921 on hippocampal BDNF levels in the FSL model, relating to **Manuscript B**.

References for Chapters 3-6 are presented in each of the respective chapters, while the bibliography for Chapters 1, 2 and 7 and Addendum A to E are provided at the end of the thesis.

2. Problem Statement

Depression and schizophrenia are neuropsychiatric disorders presenting with various symptom similarities. Genetic, neurodevelopmental and environmental factors affect the development and/or progress of both disorders (Fava and Kendler, 2000; Kendler et al., 2001; Sigurdsson, 2015), while monoaminergic dysfunction remain the main target of conventional antidepressant and antipsychotic therapy (Brand et al., 2015; El-Hage et al., 2013; Millan et al., 2015). The role of the presynaptic α_2-adrenoceptors (α_2-ARs) as auto- and heteroreceptors in regulating the release and synaptic availability of not only norardenaline (NA), dopamine (DA) and serotonin (5-HT), but also of excitatory and inhibitory amino acids and acetylcholine, suggests that this receptor could be a beneficial target in the pharmacotherapy of both disorders (Gilsbach and Hein, 2012). In support of this, augmenting antipsychotic (Litman et al., 1993; Litman et al., 1996; Marcus et al., 2005; Marcus et al., 2010b) or antidepressant therapy (Blier et al., 2009; Blier et al., 2010; Sanacora et al., 2004) with an α_2-AR-antagonist have produced improved responses in both preclinical and clinical studies in schizophrenia and depression. Furthermore, while mirtazapine and mianserin are antidepressants that present with α_2-AR-antagonist properties (Anttila and Leinonen, 2001; Marshall, 1983), atypical antipsychotics are almost all antagonists at the α_2-AR (Svensson, 2003).

Early studies in transgenic mice have produced evidence demonstrating distinct and often opposing effects of α_{2A}-ARs and α_{2C}-ARs on various cognitive parameters, depressive-like and psychotic-like symptoms, as well as on neurotransmitter release and regulation (Gilsbach and Hein, 2012; Philipp et al., 2002; Scheinin et al., 2001; Schramm et al., 2001). These studies, and more recent studies with highly subtype selective ligands, have highlighted that subtype selective α_{2C}-AR-antagonism might present with significantly enhanced antidepressant- and antipsychotic like activity as opposed to subtype non-selective antagonism (Sallinen et al., 1999; Sallinen et al., 2007; Sallinen et al., 2013a). This finding is interesting, considering that although the addition of α_2-AR-antagonism to antidepressant and antipsychotic treatment has produced improved symptom reduction in a few studies, these findings have not translated into currently applied clinical protocols. Selective targeting of the α_{2A}-ARs and α_{2C}-AR subtypes may therefore be necessary to obtain sustained preclinical and clinical improvements of

depressive and schizophrenic symptoms. Indeed targeting specific α_2-subreceptors has been suggested as an attractive therapeutic option for investigation in central nervous system (CNS) disorders, especially in mood disorders and disorders of cognition (Scheinin et al., 2001). The following section will briefly outline background information on the α_{2C}-AR, schizophrenia and depression, and the respective psychiatric animal models that have been applied in this study.

The distinct role of the α_{2C}-AR

The presynaptic α_2-AR consists of 3 subtypes that are conserved across mammalian species, identified as the α_{2A}, α_{2B} and α_{2C}-AR-subtypes (MacDonald et al., 1997; Starke, 2001). In the CNS, the α_{2B} receptor is mainly expressed in the thalamus and does not seem to contribute to CNS auto- and heteroreceptor function (Hein et al., 1999; Trendelenburg et al., 2001). On the other hand, the α_{2A}-ARs and α_{2C}-AR are the main α_2-ARs modulating neurotransmission in the CNS (Scheibner et al., 2001a, 2001b; Trendelenburg et al., 2001). While the α_{2A}-AR is widely expressed throughout the CNS, α_{2C}-ARs are mainly expressed in the striatum, hippocampus and olfactory tube, but also in the frontal cortex where their expression is less dense (Holmberg et al., 2003; Holmberg et al., 1999; Scheinin et al., 1994; Winzer-Serhan et al., 1997). Of note is that these areas are specifically involved in cognitive functions and in mood disorders such as depression and schizophrenia (Sapolsky, 2001; Simpson et al., 2010). The location of the α_{2C}-AR therefore seems to point to an important role in the neurobiology of these neuropsychiatric disorders.

For many years, a paucity in the availability of sufficiently subtype selective α_2-AR ligands have limited our understanding of the roles of α_2-AR subtypes in cell signalling to information gleaned from transgenic mouse models. In these models α_2-AR subtype deletion or knockout (KO) is assumed to reflect the effect of chronic administration of a subtype-selective antagonist, whereas α_2-receptor subtype overexpression (OE) is said to mimic the effects of chronic subtype selective agonist treatment (Scheinin et al., 2001). These models have indicated that the α_{2C}-AR modulates noradrenaline release at low endogenous NA concentrations, as opposed to the α_{2A}-AR which regulates NA release at high endogenous NA levels. Additionally, the potency and affinity of NA and DA is higher at the α_{2C}-AR than at the α_{2A}-AR (Bunemann et al., 2001; Hein et al., 1999; Link et al., 1992). Behaviourally α_{2C}-OE mice present with a depressive-like phenotype, compared to the opposite noticed in α_{2C}-KO mice (Sallinen et al., 1999) and specific differences in sensorimotor-gating have been observed in KO vs. OE mice (Sallinen et al., 1998). Furthermore, α_{2C}-AR overexpression seems to detrimentally affect memory processes, while the opposite has been demonstrated in α_{2C}-AR deletion (Björklund et al 1998, 1999a, 1999b, 2001). Most of these findings in transgenic mice have recently been corroborated using novel highly selective α_{2C}-AR subtype antagonists (Sallinen et al., 2007; Sallinen et al., 2013a; Sallinen et al., 2013b).

However, the latter studies have revealed that transgenic mice might not in all cases predict effects of pharmacological α_{2C}-AR antagonism (Sallinen et al., 1998; Sallinen et al., 2007; Sallinen et al., 2013a). It is therefore imperative to explore and verify the data from transgenic models with new studies employing selective α_{2C} receptor ligands and using more naturalistic animal models with good validity for the chosen human disorder.

ORM-10921 is one such novel highly selective α_{2C}-AR antagonist, developed by OrionPharma. It has an $\alpha_{2C/2A}$ selectivity ratio of ~100 in rodents, is well-tolerated and produces antidepressant-like, antipsychotic-like and pro-cognitive effects in rodents (Sallinen et al., 2013a). These findings have been produced after acute treatment in Sprague Dawley and Han-Wistar rats in either NMDA-antagonist pharmacological models of schizophrenia or in normal animals. The aforementioned models have limited translational validity and therefore these findings need to be corroborated in translational animal models with good face, construct and predictive validity for the chosen human disorder. In this study, we have studied this compound in two separate models of relevance for schizophrenia and depression. Thus ORM-10921 was deployed in a *chronic* treatment paradigm in a neurodevelopmental animal model of schizophrenia, the social isolation rearing model (SIR) and in a genetic animal model of depression, the Flinders Sensitive Line (FSL) rat, comparing the behavioural and pro-cognitive effects of ORM-10921 respectively to a reference antipsychotic, clozapine (CLOZ), or to the reference antidepressant, imipramine (IMI). Moreover, we have used the non-selective α_2-AR antagonist, idazoxan (IDAZ), in order to investigate the role of selectively targeting the α_{2C}-AR. Using the SIR model we have investigated the benefits of supplementing a first generation antipsychotic, haloperidol (HAL) with ORM-10921 to assess its augmenting actions vs. a highly regarded reference agent (CLOZ). We also investigated the effect of subchronic treatment with ORM-10921 and its comparators on regional brain monoamines and brain-derived neurotrophic factor (BDNF), and how these may relate to effects on behaviour.

Investigating the role of the α_{2C}-AR in an animal model of schizophrenia

The α_{2C}-AR is very densely expressed in the striatum, a brain area that is prominent in the pathophysiology of schizophrenia (Reynolds, 2008). Schizophrenia is a neurodevelopmental neuropsychiatric disorder presenting with (1) positive symptoms (including fractured thought processes, delusions and psychosis), (2) negative symptoms (including affective flattening, alogia, avolition and social withdrawal), and (3) various cognitive impairments (Elvevag and Goldberg, 2000; Kahn and Keefe, 2013; Tsapakis et al., 2015). While an arsenal of first generation antipsychotics, presenting mainly with antidopaminergic activity, have shown efficacy in treating positive psychotic-like symptoms (Wadenberg et al., 2001), the atypical antipsychotics, which present with lower dopamine D_2-blocking activity and multiple receptor antagonist properties, have shown broader efficacy in

treating both positive and various negative symptoms of schizophrenia (Tsapakis et al., 2015). The cognitive deficits observed in schizophrenia are poorly responsive to currently available antipsychotics (Kahn and Keefe, 2013; Vreeker et al., 2015), although atypical antipsychotics are proposed to more beneficially affect cognitive parameters (Keefe et al., 2004; Swartz et al., 2008). Improvement of cognitive impairment however is largely refractory to treatment and investigating treatment options that would improve this parameter is imperative to improve treatment outcome (Bowie and Harvey, 2006; Keefe and Harvey, 2012).

Atypicality of antipychotics have been proposed to strongly revolve around α_2-AR modulation (Svensson, 2003), which has been suggested to contribute to stabilisation of dysregulated dopaminergic activity. The pathophysiology of schizophrenia includes alterations in monoaminergic, GABAergic, glutamatergic and neurotrophic function (Favalli et al., 2012; Reynolds, 2008; Tsapakis et al., 2015), while the dopamine paradox of hyperdopaminergic mesolimbic activity, revolving around the striatum, and hypodopaminergic mesocortical transmission has been postulated as a central paradigm (Moller et al., 2015; Reynolds, 2008), with noradrenergic (Yamamoto and Hornykiewicz, 2004) and serotonergic dysfunction interconnected with dopaminergic deficits (Reynolds, 2008). Furthermore, loss of tonic inhibitory GABAergic input on glutamatergic neurons compromise tonic control over subcortical dopaminergic neurons, culminating in excessive mesolimbic (striatal) dopamine release (Schwartz et al., 2012). The deactivation of the α_{2C}-AR seems to disinhibit striatal GABA release (Zhang and Ordway, 2003), while α_{2C}-AR antagonism reverses behavioural and dopaminergic deficits induced by models of glutamatergic dysfunction in rodents (Sallinen et al., 2007; Sallinen et al., 2013a). α_{2C}-AR antagonism could therefore present with beneficial effects on various dysfunctional processes in schizophrenia. Striatal monoaminergic dysfunction is a core feature of schizophrenia, while impairment in brain-derived neurotophic factor (BDNF) signalling (Buckley et al., 2007a; Buckley et al., 2007b) has been linked to neurodegenerative phenomena in this brain region (Autry and Monteggia, 2012b; Steen et al., 2006). In addition to behavioural parameters discussed below, this study therefore set out to determine the effect of sub-chronic administration of the selective α_{2C}-AR antagonist, ORM-10921 on striatal monoamine levels and BDNF levels in the SIR model of schizophrenia.

Post-weaning social isolation rearing (SIR) of rodents is a well-described neurodevelopmental animal model of schizophrenia with good face, construct and predictive validity (Fone and Porkess, 2008; Jones et al., 2011). SIR produces long-lasting behavioural alterations in rodents resembling various features of the human disorder, including deficient sensorimotor gating, recognition and working memory and impaired cognitive function, locomotor hyperactivity, decreased social interaction and aggressive behaviour (Fone and Porkess, 2008; Jones et al., 2011). SIR also induces various neurochemical changes that strongly correlates with that of schizophrenia, including dopaminergic and glutamatergic

dysfunction (Heidbreder et al., 2000; King et al., 2009; Toua et al., 2010), and disordered immune-inflammatory function (Möller et al., 2013a). The model also presents with good predictive response to antipsychotic agents (Möller et al., 2011, 2012; 2013a, 2013b; Toua et al., 2010).

On a behavioural level, augmentation of D_2-receptor antagonist antipsychotic treatment with non-selective α_2-AR antagonists bolsters the efficacy of the antipsychotic to resemble the effects of the atypical antipsychotic, CLOZ (Hertel et al., 1999a; Marcus et al., 2005; Marcus et al., 2010b). CLOZ is the most effective antipsychotic in refractory schizophrenia and is the most potent antagonist at the α_{2C}-AR among all antipsychotics (Kalkman and Loetscher, 2003; Shahid et al., 2009; Swartz et al., 2008). Recent findings with selective α_{2C}-AR antagonists have shown antipsychotic-like and pro-cognitive effects in pharmacological animal models of schizophrenia (Sallinen et al., 2007; Sallinen et al., 2013a; Sallinen et al., 2013b). These findings suggest that α_{2C}-AR antagonism could be involved in the mechanism underlying clozapine's superior clinical antipsychotic and pro-cognitive actions. In addition, all antipsychotics bind to the α_{2C}-AR, and a higher α_{2C}/D_2 receptor selectivity ratio has been proposed to underlie antipsychotic efficacy (Kalkman and Loetscher, 2003; Shahid et al., 2009). The α_{2C}-AR is thus emerging as an important therapeutic target in schizophrenia. Although highly selective α_{2C}-AR antagonists have shown antipsychotic-like effects in pharmacological animal models of schizophrenia, no studies have to date been conducted in a non-pharmacological, neurodevelopmental model of schizophrenia.

This study therefore set out to determine the effect of sub-chronic administration of the selective α_{2C}-AR-antagonist, ORM-10921, on sensorimotor gating, which has been described as being deficient in schizophrenia (Braff and Geyer, 1990; Braff et al., 1992; Geyer and Braff, 1987; Möller et al., 2011). Moreover, sensorimotor gating can be used to assess the efficacy of antipsychotic drugs (Powell and Geyer, 2002). Schizophrenia is also associated with impaired visual object recognition memory (Guillaume et al., 2015), while this impairment is also seen in SIR rats (McLean et al., 2010; Möller et al., 2013a). We therefore also examined the effect of subchronic ORM-10921 on object recognition memory in SIR rats. The ability of ORM-10921 to augment the effects of the D_2-antagonist antipsychotic, HAL, was also assessed to confirm the hypothesis that a higher degree of α_{2C} vs D_2 receptor binding might predict greater antipsychotic efficacy resembling the atypical character of CLOZ.

The SIR neurodevelopmental animal model of schizophrenia represents a more naturalistic model in which to corroborate the effects of α_{2C}-AR antagonists reported in pharmacological models of schizophrenia (Jones et al., 2011; Nestler and Hyman, 2010). Assessing the antipsychotic-like, pro-cognitive and neurochemical effects of sub-chronic administration of a novel α_{2C}-AR antagonist, ORM-10921, in this model compared to that of the atypical antipsychotic, CLOZ, and to that of a non-selective α_2-AR antagonist, idazoxan (IDAZ), as well as augmentation of the typical antipsychotic, HAL , will

provide valuable translational data which could add valuable information to our understanding of the role of the α_{2C}-AR in the treatment of schizophrenia.

Investigating the role of the α_{2C}-AR in an animal model of depression

The α_{2C}-AR is also densely expressed in the hippocampus, an area that is prominent in the pathophysiology of depression (Sapolsky, 2001). Major depressive disorder, commonly known as depression, is one of the most common neuropsychiatric disorders, with a lifetime prevalence of 8-12% (Andrade et al. 2003), and presents with symptoms of depressed mood and motivation, fatigue, sleep abnormalities, anhedonia and impaired working and declarative memory (Krishnan and Nestler, 2008). Depression generally involves deficits in monoamine neurotransmission and HPA-axis over-activity with reduced negative feedback and hypercortisolaemia (Kharade et al., 2010). Aside from an important role on mood and motivation, the hippocampus plays an important role in learning and memory, functions that strongly depend on BDNF (Brand et al., 2015). Reduced hippocampal BDNF levels along with hippocampal atrophy have been described in depression (Brunoni et al., 2008; Neto et al., 2011) and might account for cognitive dysfunction and mood symptoms (Kharade et al., 2010; Sapolsky, 2001). These hippocampal and BDNF phenomena can however be reversed by antidepressants to accompany symptom improvement (Dunham et al., 2009; Neto et al., 2011).

The Flinders Sensitive Line (FSL) rat is an in-bred line of Sprague Dawley rat that displays enhanced sensitivity to environmental stressors. The Flinders resistant line rat (FRL) does not display this enhanced sensitivity to stress and is regarded as the healthy control of the FSL rat, although Sprague Dawley rats are also used as controls for FSLs (Overstreet et al., 2005; Overstreet and Wegener, 2013). FSL rats present with good face validity, in that they model various bio-behavioural characteristics of depression (Overstreet and Wegener, 2013), including altered monoaminergic function (Overstreet et al., 2005), naturally decreased immobility in the forced swim test (FST), a screening test for antidepressant activity (Overstreet et al., 2005; Petit-Demouliere et al., 2005), impaired declarative memory (Abildgaard et al., 2011) and reduced BDNF levels compared to FRL controls (Elfving et al., 2010a). Moreover, these deficits are reversed by chronic, but not acute treatment with antidepressants, making the model a very good predictive model for assessing antidepressant activity (Overstreet et al., 2005). Considering the focus of this study, FSL rats also present with distinct changes in α_2-AR expression (Lillethorup et al., 2015). This animal model is therefore a good translational model to establish whether novel compounds present with pro-cognitive and antidepressant-like activity.

While most antidepressants generally increase the levels of noradrenaline, serotonin and dopamine to varying extents (Krishnan and Nestler, 2008), about 40% of patients do not respond to mainline

conventional antidepressants (Rush et al., 2006; Thase et al., 2001). Considering that α_{2C}-ARs have a fairly dense expression in the hippocampus, this adrenoceptor subtype might be a potential tool to address hippocampal-related disturbances in depression. Upregulation and increased density of α_2-AR expression in depressive disorders is widely described in the literature (Cottingham and Wang, 2012). Additionally, mirtazapine and mianserin, at least in part, exert their antidepressant effects via antagonism of the noradrenergic α_2 auto- and heteroreceptor (Anttila and Leinonen, 2001; Marshall, 1983) leading to increased noradrenaline and serotonin release (Blier, 2003). However, α_2-AR-antagonists do not consistently exert antidepressant effects in the FST (Rénéric et al., 2001; Zhang et al., 2009). Additionally, transgenic studies suggest that α_{2A}-antagonism would *increase* immobility in the FST, suggesting increased depressive-like behaviour (Schramm et al., 2001) while α_{2C}-antagonism would *decrease* depressive-like behaviour in this test (Sallinen et al*.,* 1999). Thus α_{2C}-antagonism may be more suited to exert antidepressant-like effects than non-selective α_2-modulating drugs.

Depression also presents with cognitive deficits, which are often the most difficult symptoms to treat (Conradi et al., 2011). Opposing effects on cognition have been reported for α_{2A}-ARs and α_{2C}-ARs, with the pro-cognitive effects of α_2-AR-agonists (Cai et al., 1993; Carlson et al., 1992) reportedly associated with α_{2A}-AR agonism (Arnsten and Leslie, 1991; Björklund et al., 2001; Franowicz et al., 2002), while α_{2C}-AR agonism however might present with detrimental effects on cognition (Björklund et al*.,* 1999a; Björklund et al*.,* 1999b), suggesting that α_{2C}-AR antagonism might possibly elicit beneficial effects in cognitive tasks. Supportive of this hypothesis, highly selective α_{2C}-AR-antagonists have shown improvements of age-related memory impairment (Rinne et al., 2013; Rouru et al., 2013; Sallinen et al., 2013b), while also showing significant antidepressant-like activity (Sallinen et al., 2007; Sallinen et al., 2013a). The animals that have been used in the above studies are however not considered translation animal models for depression, and while pharmacologic antidepressant treatment is invariably chronic (Blier, 2003, 2016), the majority of these preclinical studies employed acute treatment paradigms. This study therefore set out to determine the effect of chronic treatment with ORM-10921 on depressive-like behaviour in the FST and declarative memory in the novel object recognition test (NORT) in FSL animals, and to compare these effects with that of the reference antidepressant, IMI and the non-selective α_2-AR-antagonist, IDAZ. Furthermore, the effect of these treatments on hippocampal monoamine levels and metabolism and BDNF levels were assessed to determine whether behavioural effects could be correlated to certain altered neurochemical parameters at the core of the pathophysiology of depression.

In summary, the principle objectives of this study are as follows:

1a) via a dose range study, to determine whether the α_{2C}-selective antagonist ORM-10921 induces antipsychotic-like and pro-cognitive-like effects in a neurodevelopmental animal model of schizophrenia, the SIR rat, and

1b) whether these behavioural effects can be associated with changes in striatal BDNF and mononamine levels.

(2a) via a dose range study, to determine whether the α_{2C}-selective antagonist ORM-10921 can induce antidepressant-like and pro-cognitive-like effects in a genetic animal model of depression, the FSL rat, and

(2b) whether these behavioural effects can be associated with changes in hippocampal BDNF and mononamine levels.

These objectives have, to the best of my knowledge, never been studied in the models described above, and are outlined in more detail in section 3 below.

3. Study Hypothesis and aims

3.1 Hypothesis

The main hypothesis of this study is that sub-chronic selective α_{2C}-AR-antagonism will induce antidepressant-like, antipsychotic-like and pro-cognitive effects in animal models of A) schizophrenia and B) depression:

A. Antipsychotic-like effects of ORM-10921

1. I propose that the selective α_{2C}-antagonist, ORM-10921, will exert beneficial effects on behavioural and neurochemical deficits observed in the SIR neurodevelopmental animal model of schizophrenia, including:

- Reduced SIR-induced sensorimotor-gating deficits.
- Improved SIR-induced visual recognition memory deficits.
- Reverse SIR-associated disturbances in striatal dopamine, serotonin and noradrenalin levels.
- Reverse altered striatal BDNF levels evident in SIR rats.

2. I propose that any reversal of behavioural and/or neurochemical effects following SIR described above by ORM-10921 will mirror the effects of sub-chronic treatment with a reference antipsychotic, viz. CLOZ.

3. I propose that any of the above behavioural and neurochemical effects following SIR described above will not be observed following sub-chronic treatment with the non-selective α_2-antagonist, IDAZ.

4. I propose that addition of ORM-10921 to the antidopaminergic antipsychotic HAL will enhance the efficacy of HAL on the above behavioural and neurochemical parameters similar to that of the atypical antipsychotic, CLOZ.

B. Anti-depressant-like effects of ORM-10921

1. I propose that sub-chronic treatment with the selective α_{2C}-antagonist, ORM-10921, will exert beneficial effects on behavioural and neurochemical deficits observed in the FSL genetic animal model of depression, including:

- Reduced FSL-related depressive-like behaviour in the rat forced swim test, and increased escape-related behaviour.
- Improved FSL-related deficits in visual recognition memory.
- Reverse FSL-associated disturbances in hippocampal serotonin, noradrenalin and dopamine levels.
- Reverse altered hippocampal BDNF levels evident in FSL rats.

2. I propose that any reversal of behavioural and/or neurochemical effects in FSL rats described above by ORM-10921 will mirror the effects of sub-chronic treatment with a reference antidepressant, IMI.

3. I propose that any of the above behavioural and neurochemical effects described above in FSL rats will not be observed with sub-chronic treatment of the non-selective α_2-AR-antagonist, IDAZ.

3.2 Study aims

A. IN AN ANIMAL MODEL OF SCHIZOPHRENIA

1. Establish face, predictive and construct validity of the SIR neurodevelopmental model of schizophrenia.

1.1 Establish face validity of the SIR neurodevelopmental model of schizophrenia.

Establish whether SIR rats present with sensorimotor-gating and cognitive deficits vs. socially reared controls (SOC).

1.2. Establish predictive validity of the SIR neurodevelopmental model of schizophrenia.

Establish whether behavioural deficits in (A.1.1) above can be reversed by a known reference antipsychotic, CLOZ, while a clinically *ineffective* drug and non-selective α_2-antagonist, IDAZ, will have little to no efficacy in reversing the above behavioural deficits.

1.3. Establish construct validity of the SIR neurodevelopmental model of schizophrenia.

Investigate whether altered behaviour in SIR vs. SOC rats are associated with altered striatal DA, 5-HT and NA levels, as well as altered striatal BDNF levels.

2. Establish whether ORM-10921 can reverse sensorimotor gating and cognitive deficits in SIR animals and to delineate the dosage of ORM-10921 required for efficacy in this paradigm.

3. Establish if ORM-10921 exerts its effects on the above psychotic-like symptoms by beneficially affecting striatal monoamine levels as well as striatal BDNF levels.

4. Compare the behavioural and neurochemical effects of ORM-10921 to the reference antipsychotic CLOZ to establish parity in antipsychotic-like activity.

5. Compare the behavioural and neurochemical effects of ORM-10921 to the non-selective α_2-AR antagonist, IDAZ, to establish whether the effects of selective α_{2C}-AR antagonism with ORM-10921 are superior to non-selective α_2-AR antagonism with IDAZ.

Developed from data accrued above in A.2 to A.5, the following additional objectives will be considered:

6. Establish whether adding a low dose of ORM-10921 to a standard dose of an antidopaminergic antipsychotic that is devoid of alpha-lytic activity, viz. HAL, in SIR rats would be more effective in exerting antipsychotic-like behavioural effects compared to either drug alone.

7. Establish whether the neurotrophic effects of the typical antipsychotic, HAL, can be enhanced by augmentation with a selective α_{2C}-AR antagonist.

B. IN AN ANIMAL MODEL OF DEPRESSION

1. Establish face, predictive and construct validity of the FSL genetic animal model of depression.

1.1 Establish face validity of the FSL genetic animal model of depression

Establish whether FSL rats present with depressive-like behaviours as well as evidence of cognitive deficits vs. FRL controls.

1.2. Establish predictive validity of the FSL genetic animal model of depression.

Establish whether behavioural deficits in (B.1.1) above can be reversed by a known reference antidepressant, IMI, while a clinically ineffective drug and non-selective α_2-AR antagonist, IDAZ, demonstrates inefficacy in this regard.

1.3. Establish construct validity of the FSL genetic animal model of depression.

Investigate whether altered behaviour in FSL rats vs. FRL rats are associated with altered hippocampal DA, 5-HT and NA levels, as well as altered hippocampal BDNF levels.

2. Establish whether ORM-10921 can reverse depressive-like and cognitive deficits in FSL animals and to delineate the dosage or ORM-10921 required for efficacy in this paradigm.

3. Establish whether ORM-10921 reverses the above depressive-like symptoms by beneficially affecting hippocampal monoamine and BDNF levels.

4. Compare the behavioural and neurochemical effects of ORM-10921 to the reference antidepressant IMI to establish parity in antidepressant-like activity.

5. Compare the behavioural and neurochemical effects of ORM-10921 to the non-selective α_2 AR antagonist, IDAZ, to establish whether the effects of selective α_{2C}-AR antagonism with ORM-10921 are superior to non-selective α_2-AR-antagonism with IDAZ.

4. Study Layout

This thesis comprises five studies that are directly related to the above aims. These studies are presented either as published or submitted manuscripts or as addenda as follows:

- A dose-ranging study of the antipsychotic-like and pro-cognitive effects of ORM-10921 in the SIR animal model of schizophrenia (**Addendum A**).
- Response of the most effective dose of ORM-10921 (above) on striatal monoamine and BDNF levels in SIR rats (**Addendum B**).
- Antipsychotic like effects of low-dose ORM-10921 augmentation of HAL on sensorimotor gating, recognition memory and striatal BDNF in SIR rats (**Manuscript A** and **Addendum C**).
- A dose-ranging study of the antidepressant-like and pro-cognitive effects of ORM-10921 in the FSL animal model of depression (**Manuscript B** and **Addendum D**).
- Response of the most effective doses of ORM-10921 on hippocampal monoamine and BDNF levels in FSL rats (**Manuscript C** and **Addendum E**).

The findings from this study are presented as follows in the thesis and are depicted graphically in Figure 1-1 and Figure 1-2 below on page 17 and 18 of section 5 of this chapter (also refer to section 1: Thesis Layout):

4.2 Article 1: Manuscript A

The α_{2C}-adrenoceptor antagonist, ORM-10921, has antipsychotic-like effects in social isolation reared rats and bolsters the response to HAL

This article reports the efficacy of low-dose ORM-10921 in reversing SIR-induced deficits in sensorimotor-gating, recognition memory and striatal BDNF, while demonstrating comparable efficacy to CLOZ and superiority to HAL. Furthermore, this article reports the bolstering effect of low-dose ORM-10921 on the response to HAL to match the effects of CLOZ, thereby establishing the promise of selective α_{2C} antagonism as an important design concept in novel drug discovery in schizophrenia.

4.3 Article 2: Manuscript B

The α_{2C}-adrenoceptor antagonist, ORM-10921, exerts antidepressant-like effects in the Flinders Sensitive Line rat

This article reports the antidepressant-like and pro-cognitive effects of ORM-10921 in the FSL animal model of depression by decreasing FST immobility time and increasing novel object recognition memory, in comparison to IMI and IDAZ. This article establishes the superior efficacy of α_{2C}-AR-antagonism over non-selective α_2-AR-antagonism as antidepressant, and is thus supportive of further pre-clinical and clinical development of this and similar compounds in this therapeutic area.

4.4 Article 3: Manuscript C

The effects of selective α_{2C}-AR-antagonism on hippocampal monoamine levels in the Flinders Sensitive Line rodent model of depression

This article reports on the effects of subchronic treatment with ORM-10921, IMI and IDAZ on hippocampal levels of NA, 5-HT and DA in FSL rats, and in comparison with reference agent, IMI. The findings assist in delineating the mechanisms underlying the efficacy of ORM-10921 on behavioural parameters described in **Manuscript B**.

4.4 Article 4: Manuscript D

Therapeutic potential of targeting the α_{2C}-adrenoceptor in cognition, depression and schizophrenia – new developments and future perspective

Amidst a paucity of information on the subject, this article provides an extensive literature study on the therapeutic potential of α_{2C}-adrenoceptor antagonism in neuropsychiatric illness. The paper constitutes the first comprehensive review on the subject and will enable future researchers to more easily access the available literature, to identify gaps in knowledge and to design appropriate studies that will uplift the state of the art.

4.5 Addendum A

Face validity of the SIR model of schizophrenia is reported here through the assessment of PPI and novel object recognition memory in SOC vs. SIR rats. Predictive validity for the model is established by assessing whether the behavioural deficits in SIR rats can be reversed by the atypical antipsychotic CLOZ, but not by the clinically ineffective α_2-AR antagonist, IDAZ. After establishing face and predictive validity of the model, the results from the dose-ranging study of ORM-10921 on PPI and object recognition memory in SIR rats is reported here, compared to IDAZ and CLOZ. These results were fundamental in producing the results published in **Manuscript A**.

4.6 Addendum B

Construct validity of the SIR model is assessed vs. SOC animals through assessing striatal monoamine and BDNF levels. Effects of CLOZ, IDAZ and selected doses of ORM-10921 (based on behavioural efficacy) on striatal monoamine and BDNF levels are also reported. These results were fundamental in producing the results that led to the publication **of Manuscript A**.

4.7 Addendum C

The lowest effective dose of ORM-10921 in Addendum A, viz. 0.03mg/kg, and a lower dose, viz. 0.01mg/kg, are used alone or in combination with HAL to establish whether antipsychotic efficacy in the PPI test can be evoked by lower doses of ORM 10921 and how these doses differ with respect to augmenting the action of HAL. The results for ORM 0.01 mg/kg are reported in **Manuscript A**, while Addendum C reports the results in the PPI, NORT and on striatal BDNF obtained with the 0.03 mg/kg dose alone, or in combination with HAL, compared to CLOZ.

4.8 Addendum D

Face validity of the FSL model of depression is reported by assessing FST immobility and novel object recognition memory in FSL vs. FRL rats. Predictive validity is established by assessing whether the behavioural deficits in FSL rats can be reversed by the IMI, but not by the clinically ineffective α_2-AR antagonist, IDAZ. Following the above validation, the results from the dose-ranging study of ORM-10921 that led to the findings published in **Manuscript B,** are reported here.

4.9 Addendum E

Effects of 14-day treatment with selected doses of ORM-10921 on hippocampal BDNF levels are reported here, while construct validity of the FSL vs. FRL animals is also reported and discussed. This data was eventually not included in **Manuscript B** in order to retain the emphasis on the behavioural data.

5. Graphical presentation integrating the Study Layout and Thesis Layout

On page 17 and 18 I have used flow diagrams (Figure 1-1 and Figure 1-2) to bring together the Study Layout and how the study outputs are presented in the Layout of this Thesis. This will enable the reader to place the various Manuscripts and Addenda into perspective. The study layouts depicted in each diagram are described in the figure legends. Additionally a time-line is provided to clarify the chronology of experimental procedures in Figure 1-3.

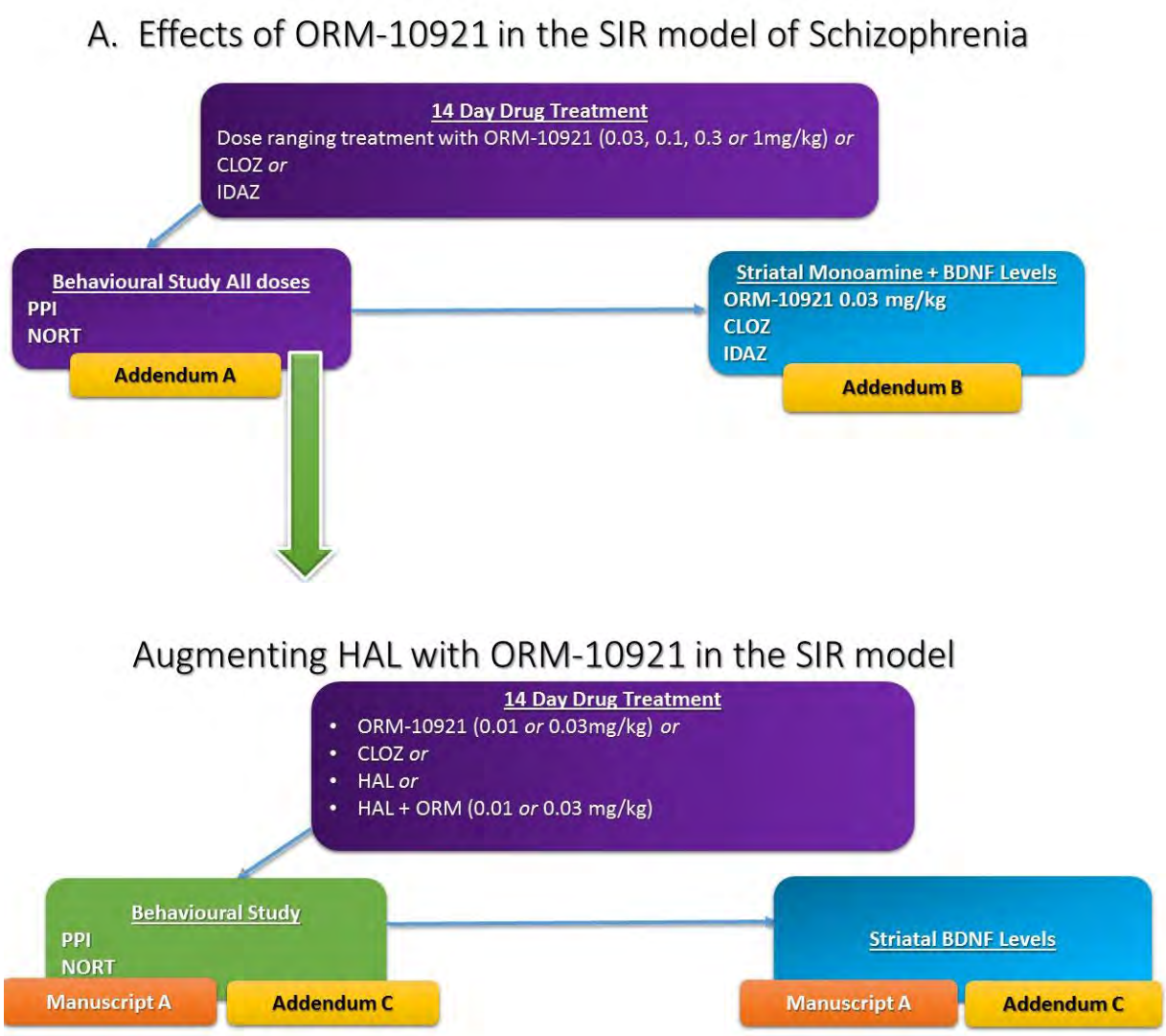


Fig 1-1. Graphical representation of the layout of Study A, which examined the effects of ORM-10921 in the SIR model of schizophrenia. The aims for this study are set out in section 3.2 A of this chapter. Study A consisted of the initial dose-response analysis, and a subsequent sub-study investigating antipsychotic augmentation with ORM-10921. In the initial dose-response analysis, SIR animals received either vehicle, ORM-10921 (0.03, 0.1, 0.3 or 1 mg/kg), CLOZ 5 mg/kg or IDAZ 3 mg/kg for 14 days SC. Behaviour was assessed in the NORT and PPI. These results are reported in **Addendum A**. Based on behavioural dose-response outcomes, the 0.03 mg/kg dose of ORM-10921 was selected for further post-mortem striatal monoamine and BDNF analyses, the findings of which are reported in **Addendum B.** Following on observations from the behavioural data, ORM-10921 was employed as augmentation strategy to HAL 2mg/kg using an additional lower dose (0.01 mg/kg) and the effective dose from the dose ranging study (0.03mg/kg). Behaviour in the PPI and NORT were assessed, and post-mortem striatal BDNF levels analysed. These data are reported in **Addendum C and Manuscript A**.

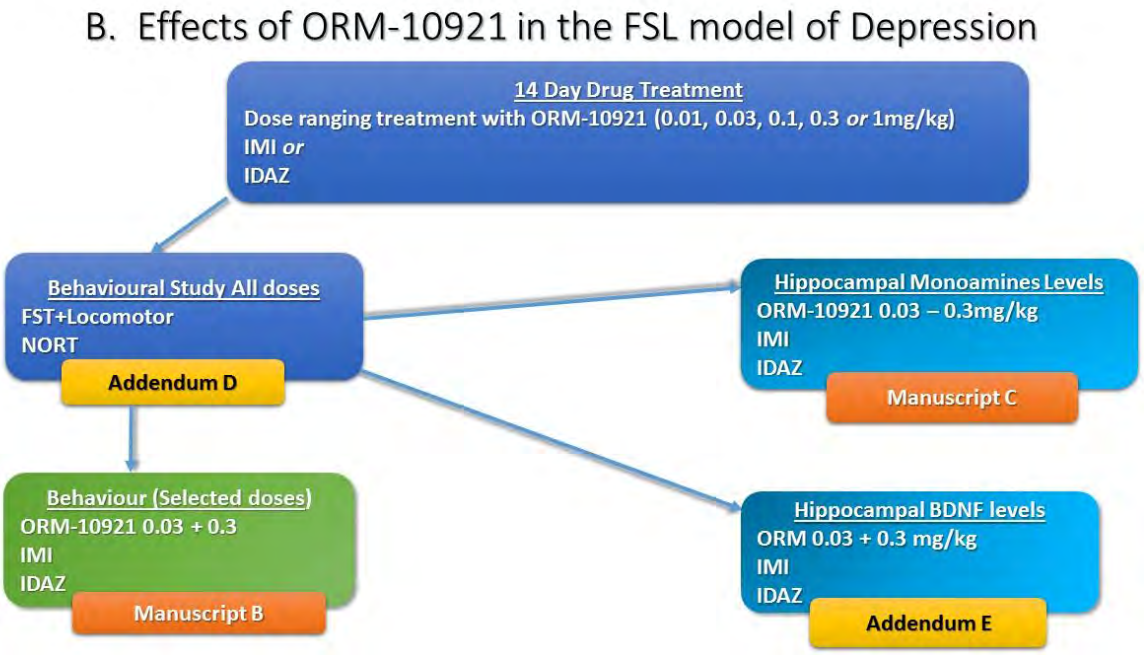


Fig 1-2. Graphical presentation of the layout of Study B, which examined the effects of ORM-10921 in the FSL model of depression. The aims for this study are set out in section 3.2 B of this chapter. Study B consisted of a dose-response analysis employing treatment with either vehicle, ORM-10921, IMI 15 mg/kg or IDAZ 3 mg/kg for 14 days, with subsequent behavioural assessment in the NORT and FST. These results are reported in **Addendum D**. The doses demonstrating the most pronounced effects on behaviour were selected for comparison with IDAZ and IMI and reported in **Manuscript B**. Post-mortem hippocampal BDNF levels was assessed in the same drug treatment groups and the findings are reported in Addendum E. Based on behavioural outcomes, the 0.03 - 0.3 mg/kg doses of ORM-10921 was selected for determination of hippocampal monoamine levels and the results are reported in **Manuscript C.**

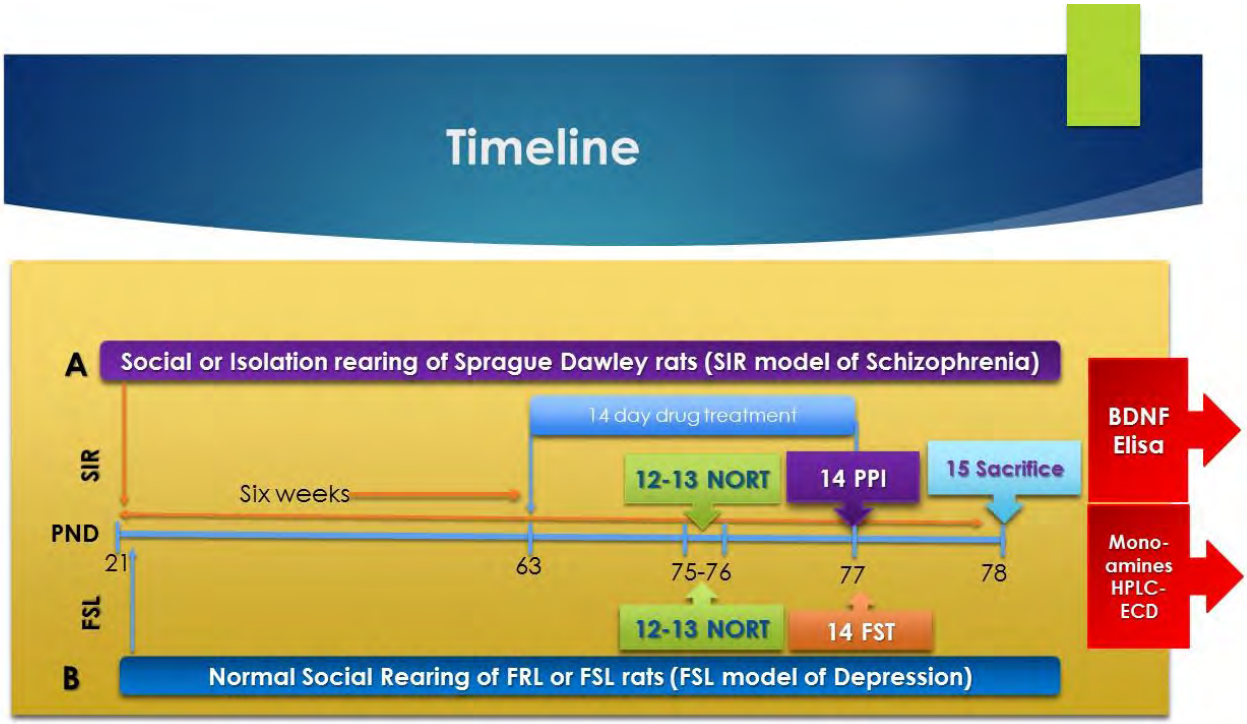


Fig 1-3. Timeline for drug administration and behavioural tests in both studies. For study A, Sprague Dawley animals were weaned at post-natal day (PND) 21 and reared socially or in isolation for six weeks. Drug treatment commenced for 14 days and the NORT was conducted on day 12 and 13 of drug treatment, while the PPI was conducted on day 14. Behavioural tests were conducted at least 12 hours after drug administration. Animals were sacrificed on day 15 after drug treatment commenced and 24 hours after the final drug administration, with brain tissue collected for analysis of striatal BDNF and monoamine levels. Drug treatment of FSL rats commenced at 8-9 weeks of age, for 14 days. Behaviour was assessed in the NORT on day 12-13 and in the FST on day 14, 12 hours after drug treatment. Analysis of hippocampal BDNF and monoamine levels were conducted in separate animals, with animals sacrificed on day 15, 24 hours after the last drug treatment.

6. Ethical Considerations

This project employed two translational rodent models of neuropsychiatric illness as well as three behavioural assessments that should be evaluated with regards to animal welfare. Rodents are an excellent model in studies of cognition, memory and neuropsychiatric processes, since the physiology involved in these processes in rats strongly correspond to the human conditions (Iannaccone and Jacob, 2009). The rat is also an intelligent species and is capable of learning various tasks that correlate to the tasks used to assess human behaviour (Iannaccone and Jacob, 2009). The established and well-validated rodent models of depression and schizophrenia employed in this project have been described in section

2 of this chapter, and the necessity of using a model that can be translated to clinical relevance for the human condition was also highlighted in section 2. Since rats are considered to excellently model various neuropsychiatric processes (Ellenbroek and Youn, 2016) and considering that the distribution of α_2-ARs in human and rodent brains are analogous and have been well-studied (Fagerholm et al., 2008; Finnema et al., 2015; Holmberg et al., 2003; Lehto et al., 2015), the replacement with a lower-order animal was not viable in this translational study. Furthermore the application of translational animal models refines the investigation so that the applicability to the human condition is largely increased. Responsibility for the welfare of the animals was espoused in efforts to understand and monitor stress and discomfort or suffering for the animals throughout the study. Daily monitoring of the animals for signs of pain, discomfort or stress were done by the investigator and by the staff of the Vivarium, including signs of ruffled fur, weight loss, altered food or water intake, biting of feet or tails. If signs of such distress were noted in these animals, the animal was euthanized. The procedures used in this study may cause various levels of negligible/mild, mild or moderate stress to the animals. These are outlined below.

Negligible to Mild Stressors

The NORT and Locomotor Activity tests are considered to cause negligible discomfort in the animals, although mild anxiety in a novel environment may be experienced, but is transient and is not considered consequential.

Mild Stressors

- Daily SC injections: The animals received daily SC injections. This does not cause significant pain to the animals if done correctly, and the researcher is well-trained in injecting animals via the SC route. If done correctly, minimal momentary discomfort during injection could be experienced in the same way as humans might experience a routine injection. Injection sites were alternated to prevent repetitive tissue insult at the same injection site.
- The Prepulse Inhibition test (PPI) – The PPI can evoke discomfort in the rodent due to the auditory startle stimulus. The PPI is not considered a stressful procedure to the animals, other than the period of mild restraint for half an hour in the assessment chamber. The exposure to a variety of sound pulses at between 72 and 115 dB exposure time not considered to harm the animal's hearing (Pace and Zhang, 2013). This procedure has been applied in this laboratory for many years.

Moderate Stressors

- Forced Swim Test (FST) – The FST involves placing the animal into an inescapable cylinder filled with water, requiring the animal to swim for 7 minutes. Rats are good natural swimmers, and respond intuitively to this procedure. This procedure subjects the animal to a brief period of

moderate stress, since the swimming time and thus resolution of the stressor is unknown to the animal (Castagné et al., 2009). To minimise discomfort, the animals are dried and warmed after removal from the water. This test is well-validated as a screening study for possible antidepressant activity and has been used in this laboratory for many years.

- Social isolation rearing (SIR) – rearing animals in social isolation for 8 weeks constitutes a mental and emotional stressor, as seen in animal behaviour tested after SIR. However, this procedure does not physically harm the animal and is an essential element in modelling the neurodevelopmental processes involved in schizophrenia in humans. This procedure has been conducted in many studies in the Vivarium, is a well-documented, well-validated and very effective procedure to induce schizophrenia–like behaviours in animals.

In consultation with the NWU Statistical Consultation Service, the amount of animal subjects necessary for producing scientifically sound data was determined and hereby efforts were made to reduce the amount of animal subjects that were necessary for inclusion in this project.

Animals were bred, supplied and housed at the Vivarium (SAVC reg no. FR15/13458; SANAS GLP compliance no. G0019) of the Pre-clinical Drug Development Platform of the North-West University (NWU). In accordance with the above ethical considerations, all experiments were approved by the AnimCare animal research ethics committee (NHREC reg. number AREC-130913-015) at the NWU. All animals were maintained and procedures performed in accordance with the code of ethics in research, training and testing of drugs in South Africa, and complied with national legislation (ethics approval number: NWU-00050-13-A5).

Chapter 2: Literature Review

1. The potential of the α_{2C}-adrenoceptor as a treatment strategy in neuropsychiatric illness

1.1 Introduction

The central presynaptic α_2-adrenoceptor (α_2-AR) acts as an auto- and heteroreceptor on noradrenergic, serotonergic and dopaminergic nerve terminals. As an autoreceptor it is sensitive to the synaptic concentrations of noradrenaline (NA) thereby mediating a negative feedback mechanism to regulate the synthesis and release of NA (Svensson et al., 1975). As heteroreceptors, α_2-ARs are also located on serotonergic nerve terminals whereby NA modulates the release and synthesis of serotonin (5-HT) (Esteban et al., 1996; Scheibner et al., 2001b; Trendelenburg et al., 1994). Furthermore, α_2-heteroreceptors play an important role in mediating the release of dopamine (DA) and NA from noradrenergic terminals (Devoto et al., 2004), and are indirectly involved in DA release from dopaminergic terminals (Bücheler et al., 2002). The interconnectivity of monoaminergic neurons suggests that alterations in one system would lead to compensatory responses of other monoamine systems, as well as changes or compensations in numerous neurotransmitter systems that are directly or indirectly regulated by the altered monoamine system, such as gamma-aminobutyric acid (GABA), cholinergic and glutamatergic systems (Ordway et al., 2002). Considering this important and integrated role of α_2-ARs on these major neurotransmitter systems, it is not surprising that pharmacological modulation of the α_2-AR is involved in the clinical therapeutic approach to various neuropsychiatric illnesses characterised by dysfunctional monoaminergic neurotransmission (Langer, 2015). While α_2-AR agonists are useful in the treatment of attention deficit hyperactivity disorder (ADHD) (Sallee et al., 2013), the treatment of major depressive disorder by α_2-AR antagonists mianserin and mirtazapine have seen widespread use. An earlier onset of antidepressant action has been suggested for mirtazapine by targeting synaptic and somatodendritic NA auto- and heteroreceptors (Blier, 2003). Additionally, almost all atypical antipsychotics display moderate to potent levels of α_2-AR antagonism (see Figure 11, section *3.1*) (Kalkman and Loetscher, 2003; Shahid et al., 2009), while this mechanism has also been suggested to underlie the atypical profile of antipsychotics such as clozapine, quetiapine, risperidone, asenapine etc. (Svensson, 2003). Furthermore, augmentation of conventional antipsychotics (Litman et al., 1993; Litman et al., 1996; Marcus et al., 2005; Marcus et al., 2010b) and antidepressants (Blier et al., 2009; Blier et al., 2010; Dhir and Kulkarni, 2007; Grossman et al., 1999; Rénéric et al., 2001; Sanacora et al.,

2004a) with an α_2-AR antagonist has been shown to bolster antidepressant, antipsychotic and cognitive outcomes.

However, findings from transgenic mouse studies have indicated distinct and sometimes opposing roles of the two major α_2-AR subtypes involved in the regulation of central nervous system (CNS) neurotransmission, namely the α_{2A}-AR and α_{2C}-AR (Bücheler et al., 2002; Hein et al., 1999; Philipp et al., 2002; Scheinin et al., 2001). Before the availability of sufficiently subtype selective ligands, transgenic mouse studies have suggested a potential therapeutic role for selectively targeting the α_{2C}-AR in depression, schizophrenia and related symptoms of cognitive decline (Björklund et al., 1998; Björklund et al., 1999a; Björklund et al., 2001; Sallinen et al., 1999; Sallinen et al., 1998a; Scheinin et al., 2001). More recently, the use of highly selective α_{2C}-AR antagonists have confirmed the antipsychotic-like, antidepressant-like and pro-cognitive effects of this treatment strategy in animal models (Sallinen et al., 2007; Sallinen et al., 2013a; Sallinen et al., 2013b; Uys et al., 2016). This study therefore set out to determine the therapeutic efficacy of selective α_{2C}-AR antagonism in translational animal models of schizophrenia and depression.

This literature review will provide an overview of the differential roles of the α_{2A}-AR and α_{2C}-AR in CNS function (section *1.2*), followed by a review of the epidemiology (section *2.1*), etiology (section *2.1*), symptoms (section *2.2*) and pathophysiology (section *2.3*) of schizophrenia and depression with focus on systems relevant to the current study. In section *3*, current treatment options for these neuropsychiatric illnesses will be reviewed in order to contextualise the possible role of α_{2C}-AR antagonism as a treatment strategy and the current knowledge on the therapeutic potential of such treatment will be reviewed and summarised. In section *4*, the reader will be introduced to the animal models and behavioural tests applied in this study, while these models and tests will be placed into context by a short review of other models and tests applied in schizophrenia and depression research. Finally, section *5* reviews findings that suggest a role for the α_{2C}-AR in behavioural paradigms predictive of antipsychotic-like, antidepressant-like and pro-cognitive effects.

1.2 Central effects of α_2-AR subtypes in neuropsychiatric illness: a case for selectively targeting the α_{2C}-AR

A review on the current state of the art regarding the pharmacological and physiological role of the α_{2C}-AR and the therapeutic potential of targeting this receptor subtype in neuropsychiatric illness is sorely needed. Part of the outcomes of this work was therefore to produce such a review, which is included in this thesis as Manuscript D in Chapter 6 and is currently under review at *Frontiers in Psychiatry,*

Molecular Psychiatry. The reader is therefore referred to that chapter for any additional details, since the overview provided in this chapter only provides a focussed summary of the topic.

Central α_2-ARs consist of 3 subtypes that are conserved across mammalian species, namely the α_{2A}, α_{2B} and α_{2C}-AR-subtypes (MacDonald et al., 1997; Starke, 2001). The distribution of α_2-ARs in human, monkey and rodent brains are analogous (Fagerholm et al., 2008; Finnema et al., 2015; Holmberg et al., 2003; Lehto et al., 2015), implying that neuropharmacological data from animal models may be relevant for humans also. The α_2-AR subtypes have different tissue distribution patterns, related to separate functional systems (Bücheler et al., 2002; Gilsbach and Hein, 2012; MacDonald et al., 1997). While the α_{2A}-ARs and α_{2C}-ARs are the main α_2-ARs modulating neurotransmission in the CNS, the α_{2B} receptor only makes up a very small fraction of central α_2-ARs and is mainly expressed in the thalamic nuclei and it does not seem to contribute to CNS auto- and heteroreceptor function (Scheibner et al., 2001a, b; Trendelenburg et al., 2001).

The α_{2A}-AR constitutes 90% of central α_2-ARs and is widely distributed throughout the CNS (Bücheler et al., 2002; Scheinin et al., 1994). On the other hand, the α_{2C}-AR only constitutes approximately 10 % of α_2-ARs in the CNS (Bücheler et al., 2002). Furthermore, the expression of the α_{2C}-AR is much more distinct and is focussed in specific areas involved in cognitive functions and in the regulation of mood and affect (Holmberg et al., 2003; Holmberg et al., 1999; Scheinin et al., 1994; Winzer-Serhan et al., 1997). The densest expression of α_{2C}-ARs is found in the ventral and dorsal striatum, where it supercedes the expression of the α_{2A}-AR, while its expression in the hippocampus and olfactory tubercle is also prominent (Fagerholm et al., 2008; Finnema et al., 2015; MacDonald et al., 1997; Scheinin et al., 1994). The α_{2C}-AR is also expressed in the frontal cortex and in the cell bodies of noradrenergic neurons located in the locus coeruleus (LC), although its expression here is more subtle than in the limbic and midbrain regions (Rosin et al., 1996; Scheinin et al., 1994). This distinct distribution pattern suggests that the α_{2C}-AR is involved in limbic, mesolimbic and mesocortical functions, systems which are dysregulated in schizophrenia and depression (section *2.3.1.1* and *2.3.2.1*). The distinct distribution patterns of the α_{2C}-AR and α_{2A}-AR are represented in Figure 1.

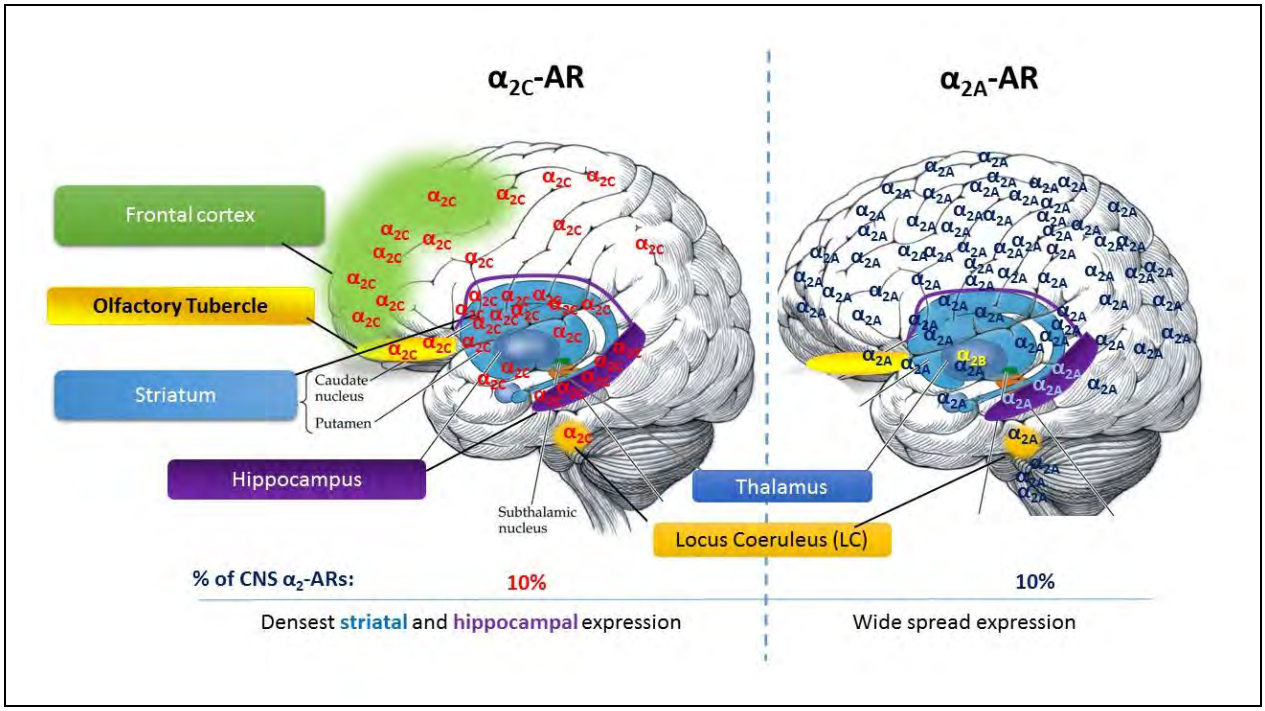


Figure 1. Distribution patterns of α_2-AR subtypes. α_{2C}-ARs represent ~10% of α_2-ARs in the CNS are densely expressed in the hippocampus, olfactory tubercle and striatum, and less densely expressed in the frontal and cerebral cortex and locus coeruleus. The α_{2A}-ARs (~90% of CNS α_2-ARs) are widely expressed throughout the central nervous system. α_{2B}-ARs expression is limited to thalamic nuclei More details are provided in the text. Figure of brain structures adapted from *Biological Psychology* (Breedlove and Watson, 2013).

Due to the unavailability of sufficiently subtype-selective ligands, the current pool of knowledge has mainly been built on the back of transgenic mouse models that employ targeted genetic deletion or overexpression of the α_{2A}-AR and/or α_{2C}-AR. In order to investigate the pharmacological role of α_2-AR subtypes, the assumption has been made that *α_2-AR subtype deletion or* ***knockout (α_{2x}–KO)*** reflects the effect of *chronic* administration *of a subtype-selective* ***antagonist***, whereas *α_2-receptor subtype* ***overexpression (α_{2x}-OE)*** mimics the effects of *chronic* subtype selective ***agonist*** treatment (Scheinin, 2001).

The α_{2C}-AR is associated with various effects on monoamine turnover. While treatment with a non-selective α_2-AR agonist is usually associated with *decreased* monoamine levels due to negative feedback inhibition at auto- and heteroreceptors, α_2-AR agonist treatment is associated with *increased* central DA, NA and 5-HT levels in α_{2C}-KO mice, and *absent responses* in α_{2C}-OE mice (Sallinen et al., 1997). α_{2C}-AR antagonism might thus facilitate central monoamine transmission, which could be of benefit in disorders where monoamine dysfunction is apparent.

Various studies report a higher potency and affinity of NA at the α_{2C}-AR than at the α_{2A}-AR (Bunemann et al., 2001; Hein et al., 1999a; Link et al., 1992) and that the α_{2C}-AR inhibits NA release at low endogenous concentrations of NA, compared to the α_{2A}-AR which inhibits NA release at high

endogenous concentrations (Bücheler et al., 2002; Hein et al., 1999a). Additionally, α_{2C}-AR-mediated presynaptic inhibition occurs much more slowly than that mediated by α_{2A}-ARs (Bücheler et al., 2002), while the α_{2C}-AR displays slower deactivation upon removal of NA compared to the faster deactivation of the α_{2A}-AR (Bunemann et al., 2001). The density of α_{2C}-ARs appears to be strongly regulated by synaptic availability of NA, while α_{2A}-AR density appears to not be as sensitive to the synaptic availability of NA. Figure 2 (which appears in **Manuscript D** in Chapter 6 of this thesis and is repeated here for the sake of clarity) depicts the differential regulation on NA feedback and receptor pharmacodynamics mediated by α_{2A}-ARs and α_{2C}-ARs. Furthermore, α_{2C}-AR have been suggested to more potently modulate the synthesis of the DA and NA precursor 3,4-dihydroxyphenylalanine (DOPA) than the α_{2A}-AR (Esteban et al., 1996). The effect of synaptic availability on α_{2C}-AR expression might imply an important role in a disorder like depression characterised by hyponoradrenergic activity and increased α_2-AR expression (Cottingham and Wang, 2012), while dysregulated noradrenergic function in schizophrenia (Yamamoto and Hornykiewicz, 2004) might also be amenable to strategic targeting of the α_{2C}-AR.

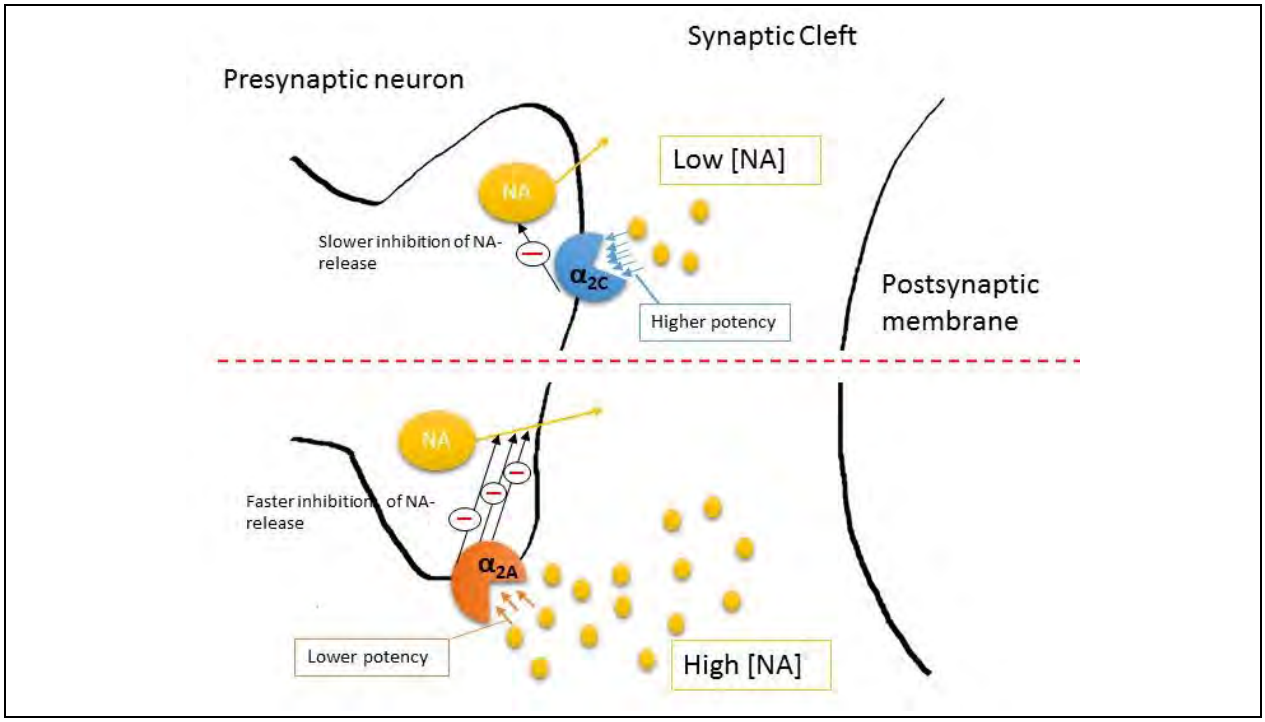


Figure 2. Differential presynaptic inhibition of NA release by the α_{2C}-AR and the α_{2A}-AR. At low endogenous noradrenaline concentrations (top panel), the α_{2C}-AR is responsible for inhibition of NA release, while the α_{2A}-AR inhibits NA release at high endogenous NA concentrations (bottom panel). α_{2C}-AR-mediated inhibition of NA release (top panel) is a slower process than α_{2A}-AR mediated inhibition of NA release (bottom panel). Furthermore, the potency and affinity of NA is higher at the α_{2C}-AR (top panel) than at the α_{2A}-AR. See text for more detail. NA = noradrenaline, Θ = speed of inhibitory processes (Figure courtesy of Uys et al., 2016, in submission; Chapter 6)

The densest expression of α_{2C}-ARs is found in the striatum, suggesting a functional role for this receptor in subcortical DA transmission (Bücheler et al., 2002; Ihalainen et al., 2001). Indeed, DA appears to present with an increased selectivity for the α_{2C}-AR (Sallinen et al., 2013a) and is capable of activating

striatal α_{2C}-ARs (Zhang et al., 1999) with high potency (Sallinen et al., 2013a). Furthermore, selective antagonism of the α_{2C}-AR appears to increase prefrontal cortical (PFC) but not striatal synaptic DA concentrations in rodents (Sallinen et al., 2013a). Decreased striatal DA turnover has been shown in α_{2C}-KO mice, while increased cortical DA turnover is seen in α_{2C}-OE mice (Sallinen et al., 1997). Additionally DA-mediated behaviours and cognitive effects are increased in α_{2C}-KO mice (Ihalainen et al., 2001; Sallinen et al., 1998b), suggesting that α_{2C}-AR antagonism would mediate DA-mediated actions. Figure 3 depicts the effects of DA at the α_{2C}-AR and the effects of α_{2C}-antagonism on mesocortical DA transmission. Dopamine thus has potent actions at the α_{2C}-AR, and antagonism of this receptor (which is mainly found in subcortical areas) appears to mediate alterations in mesolimbic-mesocortical DA signalling, DA levels and DA-mediated behaviours. Such actions could have therapeutic potential in conditions characterised by deficient cortical dopaminergic transmission, such as schizophrenia, depression (Mizoguchi et al., 2002) and cognitive deficits associated with these illnesses (Sheynikhovich et al., 2013).

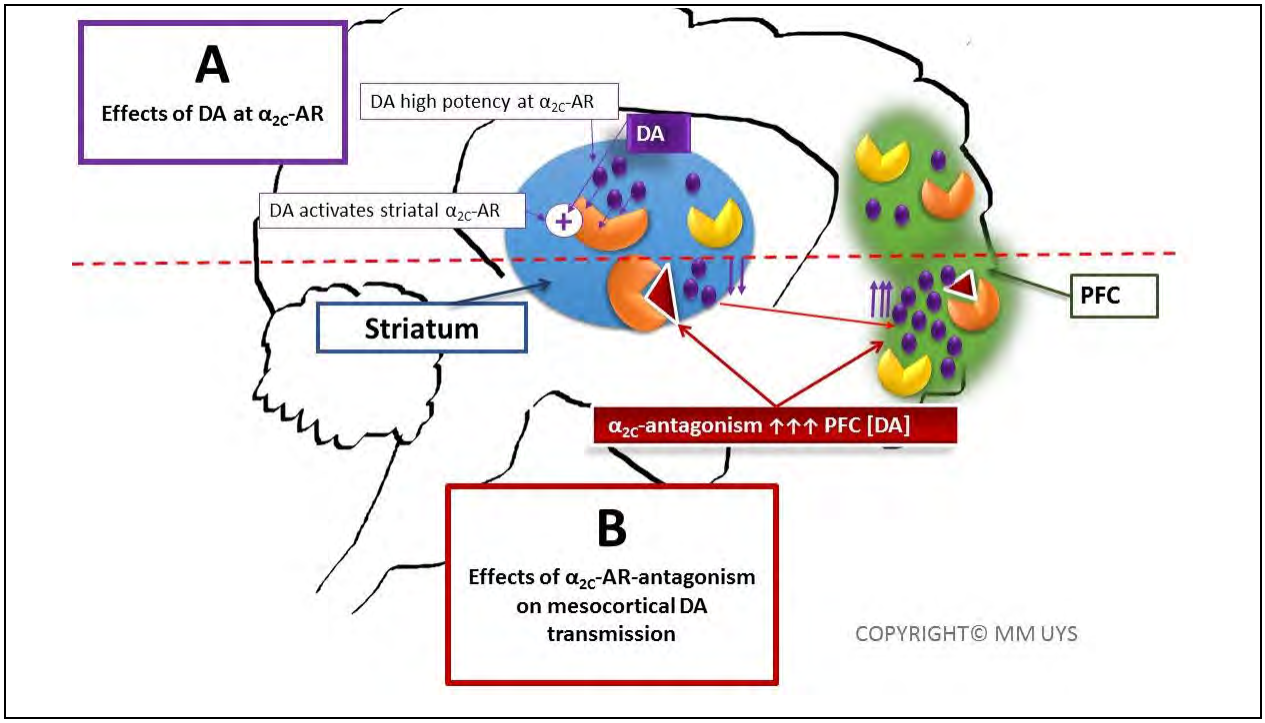


Figure 3. Effects of DA at α_{2C}-ARs (A, top panel), and of α_{2C}-AR-antagonism on mesocortical DA (B, bottom pannel). DA is a high potency agonist at the α_{2C}-ARs, where it may have significant implications for DA release in the striatum and PFC. α_{2C}-AR antagonism increases PFC DA levels, but not striatal DA levels. See text for details.

The role of the α_{2C}-AR in serotonergic transmission is less clear, and while both both α_{2A}-ARs and α_{2C}-ARs inhibit 5-HT release, the α_{2C}-AR appears to exert a more subtle effect on 5-HT release (Scheibner et al., 2001b) and 5-HT synthesis (Esteban et al., 1996). α_{2C}-KO mice present with lower disinhibition of agonist-induced 5-HT release in hippocampal and cortical slices compared to α_{2A}-KO mice (Scheibner et al., 2001b). The α_{2A}-AR is therefore the main α_2-AR regulating 5-HT release and possibly 5-HT synthesis,

although selective antagonism of the α_{2C}-AR could result in subtle, yet meaningful increases in region-specific 5-HT synthesis and release, which could be of importance in disorders characterised by altered serotonergic neurotransmission such as schizophrenia and depression (section *2.3.1.2.3* and *2.3.2.2.1*).

Monoaminergic systems are reciprocally interconnected to GABAergic, glutamatergic and cholinergic systems (Carlsson et al., 2001; Ordway et al., 2002; Scarr et al., 2013), and an imbalance between glutamatergic excitation and GABergic inhibition in the CNS has been suggested to underlie both schizophrenia and depression (Chiapponi et al., 2016; Cohen et al., 2015; Möhler, 2012). While α_{2C}-ARs and α_{2A}-ARs are located on different striatal neurons, almost all GABAergic projection neurons from the striatum contains α_{2C}-ARs (Holmberg et al., 1999), and the α_{2C}-AR appears to be an important mediator of striatal, but not hippocampal GABA release (Zhang and Ordway, 2003). These authors suggest that α_{2C}-AR antagonism could disinhibit GABA release in brain regions with dense dopaminergic innervation and low noradrenergic innervation. Since GABAergic interneurons tonically regulate glutamate release, the α_{2C}-AR may thus also have an indirect effect on glutamatergic neurotransmission in the striatum (Schwartz et al., 2012). Supporting this theory, altered behaviour and impaired cognition induced by glutamate receptor N-methyl-d-aspartate (NMDA)-antagonists in animals has been reversed by novel highly selective α_{2C}-AR antagonists (Sallinen et al., 2007; Sallinen et al., 2013a; Sallinen et al., 2013b). By potentially altering excitatory vs. inhibitory neurotransmission, α_{2C}-AR antagonism may thus support therapeutic efficacy in depression and schizophrenia (Cohen et al., 2015; Möhler, 2012).

Dysfunctional central cholinergic transmission has also been implicated in the underlying pathophysiology of mood disorders, cognitive dysfunction and schizophrenia (Scarr et al., 2013), while various drugs target the cholinergic system in an attempt to improve symptoms of depression, schizophrenia and impaired cognition (Friedman, 2004; Furey and Drevets, 2006; Scarr et al., 2007). α_2-adrenergic heteroreceptors, as well as D_2 receptors are involved in the inhibition of acetylcholine (ACh) release (Langer, 2015). Considering that ACh modulates GABA release (Raiteri et al., 1990) and that the α_{2C}-AR has been implicated in mediating striatal GABA release, the α_{2C}-AR has been suggested to be located on striatal cholinergic neurons, potentially modulating GABA release via ACh release (Zhang and Ordway, 2003; Zhang et al., 1999). These actions are purportedly mediated by dopaminergic activation of the α_{2C}-AR (Zhang et al., 1999). Thus, a complex interplay between GABAergic, cholinergic and dopaminergic systems in neuropsychiatric illness may be modulated by α_{2C}-ARs. In a disorder like schizophrenia characterised by excessive striatal DA and thus potentially excessive α_{2C}-AR activation, α_{2C}-AR antagonism may facilitate disinhibited ACh and GABA release, which in turn may be involved in improved illness outcomes (Cohen et al., 2015; Scarr et al., 2013).

Central neurotrophic dysfunction, especially altered brain-derived neurotrophic (BDNF) activity and altered synaptic plasticity is associated with various neuropsychiatric disorders associated with cognitive impairment (Autry and Monteggia, 2012b). C-fos and JunB are markers of neuronal activity, and play an important role in synaptic function (Tuvikene et al., 2016; West et al., 2002). The expression of c-fos in the CNS is induced by various stimuli, including learning and memory processes, various neurotransmitters and neurotrophic growth factors (Alberini, 2009), while Jun-B is involved in the regulation of emotional memory (Radwanska et al., 2015). BDNF plays a role in neuroplasticity and has been shown to restore expression of both c-fos and JunB after neuronal insult (Hsieh et al., 1998). Interestingly, cortical and hippocampal levels of c-fos and JunB mRNA are increased in α_{2C}-KO mice, but not α_{2C}-OE mice, compared to wildtype-controls (Sallinen et al., 1999). These findings imply increased neuronal activity in α_{2C}-KO mice that is correlated with an antidepressant-like phenotype and improved cognitive parameters (Björklund et al., 1999b; Björklund et al., 2001; Sallinen et al., 1999). The association of α_{2C}-AR modulation with the expression of BDNF has however not yet been investigated, although certain non-selective α_2-AR antagonists have been associated with neurogenesis and increased BDNF levels in the hippocampus (Rizk et al., 2005; Yanpallewar et al., 2011). Noradrenergic (Francis et al., 2012; Mannari et al., 2008), dopaminergic (Kuppers and Beyer, 2001), serotonergic (Martinowich and Lu, 2007) and GABA-glutamate (Marmigere et al., 2003) interactions are involved in the expression of BDNF. Considering that the α_{2C}-AR acts as a heteroreceptor affecting the release of many of these neurotransmitters, this receptor may play an indirect role in altering the expression of BDNF.

Thus, the α_{2C}-ARs is uniquely located in limbic areas that present with pronounced functional impairment in both schizophrenia and depression. It appears to play an important role in homeostatic regulation of monoaminergic, GABAergic and possibly glutamatergic and cholinergic neurotransmission, while antagonism of this receptor subtype might be associated with increased neuronal activity. Furthermore, its distinct effects on systems important in neuropsychiatric illness and cognition sets it apart from the α_{2A}-AR and emphasizes selective α_{2C}-AR antagonism as an important potential neurobiological target in neuropsychiatric disorders.

With this in mind, this review will now focus on **schizophrenia** and **depression** (section *2*) and their pathophysiological underpinnings to demonstrate the potential role of selective α_{2C}-AR antagonism in these disorders. The behavioural evidence for a role of the α_{2C}-AR in these disorders is presented in section *5* after the reader has been introduced to animal models used to study the underpinnings of these neuropsychiatric disorders in section *4*.

2. Schizophrenia and Depression

2.1 Epidemiology and etiology

2.1.1 Schizophrenia

Schizophrenia is estimated to present with a world-wide lifetime prevalence of 1-4% (Ayano, 2016; van Os et al., 2010) that can be correlated to risk-factors in around 10-20% of the general healthy population (van Os et al., 2010). In more than 90% of cases the disorder manifests before 40 years of age, although late onset of schizophrenia and psychosis has also been reported (Girard and Simard, 2008). The disorder does not present with ethnic preference (Ayano, 2016), and while both men and women are equally affected, juvenile onset is more prevalent in men (Abel et al., 2010). Immigrants to developed countries have an increased incidence of schizophrenia in both the first and second generation, while non-migrant minority groups are also more predisposed to the illness (van Os et al., 2010).

Schizophrenia presents with high heritability estimates, indicating a strong genetic influence, and the risk of developing schizophrenia rises substantially if a first or second-order family member is affected (Tandon et al., 2010). In monozygotic twins, the affliction of one twin with schizophrenia increases the risk of the other twin by 50-70% (Goldberg et al., 1995).

Schizophrenia is considered a neurodevelopmental disorder, and early life stress and various environmental factors are associated with its development (van Os et al., 2010). Prenatal factors including prenatal maternal stress, nutritional deficiency (such as Vitamin D) and specific maternal viral and bacterial infections as well as various complications of pregnancy and birth constitute risk factors for schizophrenia (Cannon et al., 2002), while childhood trauma, including abuse and neglect, constitute a very high vulnerability for the disorder (Read et al., 2001). Environmental risk factors for schizophrenia include growing up in an urban environment (urbanicity), substance abuse (with a very high risk associated with cannabis use) (van Os et al., 2010), as well as exposure to infections or toxic substances during early development (Lewis and Levitt, 2002). The etiology of schizophrenia therefore involves various genetic vulnerabilities and environmental and developmental insults which adversely affect the normal neurodevelopment of brain circuitry.

2.1.2 Depression

Depression presents with a significant disease burdern, with estimates for European Union (EU) member states reporting a lifetime prevalence of approximately 6.9% for major depression (Wittchen et al., 2011), while world-wide prevalence of the disorder is reported to be between 8-12% (Andrade et al., 2003). The mood disorder is more prevalent in women (ratio of 2.3) than in men and affects about 30 million people in the EU alone (Wittchen et al., 2011). The average age of onset is usually in the early to mid twenties, with risk for depression already beginning to appear in early adolescence (Andrade et al., 2003). Urbanicity is highly correlated with disease burden (Andrade et al., 2003), while certain ethnic

groups, including those of European descent display a higher incidence of depression (Andrade et al., 2003; Riolo et al., 2005).

Akin to schizophrenia, depression is associated with a strong genetic vulnerability. In monozygotic twins, the estimated heritability liability for depression is 39% (Kendler and Prescott, 1999), while the offspring of depressed parents have a 3-fold higher risk of developing depression than the offspring of non-depressed parents, persisting into the next generation (Hirschfeld and Weissman, 2002).

Trauma, early life and chronic stress are strongly implicated in the etiology of depression. Acute and chronic stressors that increase the risk of developing depression include bereavement, assault, intense work-related stress and marital discord/divorce (Hirschfeld and Weissman, 2002). Early life trauma, including childhood physical and sexual abuse and neglect adversely affect important limbic processes and constitute major risk factors for depression (Daskalakis et al., 2015; Hirschfeld and Weissman, 2002), while various adverse neuroendocrinological processes have similarly been linked to chronic, uncontrollable and unpredictable stress (Hampton, 2012; Tafet and Bernardini, 2003). Additionally, as with schizophrenia, prenatal factors, such as gestational stress and maternal immune activation has also been proposed to contribute to the risk of developing depression in adulthood (Braithwaite et al., 2014; Khan et al., 2014). Thus, various genetic vulnerabilities, environmental insults and early life and chronic stress have been associated with the development of depression.

2.2 Symptoms and diagnosis

2.2.1 Schizophrenia

The clinical features of schizophrenia are diverse and include distortions of thinking and perception, motor abnormalities, avolition, affective flattening and cognitive impairments. The main symptom clusters that describe the symptomatology of schizophrenia are divided into positive, negative and cognitive symptoms (Tandon et al., 2009) and are described below.

Positive symptoms revolve around distorted thought processes and perceptions of reality, and include psychotic-like symptoms such as delusions and sensory hallucinations (visual, tactile, auditory, olfactory, visceral) (Geyer and Vollenweider, 2008; Tandon et al., 2009). Delusions are erroneous beliefs due to exaggerated reasoning or misinterpretation of perceived experiences (Geyer and Vollenweider, 2008). The most frequent delusions include persecutory delusions and delusions of grandeur, while auditory hallucinations (accusatory or threatening voices speaking to the patient or conversing among themselves) are more common than other sensory hallucinations. While some patients only experience one short episode of psychosis, others will progress onto chronic schizophrenia, displaying recurrent psychotic manifestations including mania, psychotic depression and mixed affective psychosis (Tandon et al., 2009). Mesolimbic dopaminergic hyperactivity is proposed to underlie positive symptoms, and these symptoms are the most responsive to antipsychotic therapy (Keshavan et al., 2008).

Negative symptoms include various affective disturbances that resemble that of depression, including impaired affective perception and expression, anhedonia, loss of motivation and avolition, apathy, poverty of speech and social withdrawal (Tandon et al., 2009). The pathophysiology underlying negative symptoms is complex and poorly understood (Keshavan et al., 2008). Mesocortical hypodopaminergic activity along with hypoglutamatergic activity has been implicated as underlying negative symptoms, and while certain atypical antipsychotics are more effective in treating these symptoms than typical agents (Miyamoto et al., 2012), treatment outcomes for negative symptoms are less successful than for positive symptoms (Keshavan et al., 2016).

Cognitive symptoms of schizophrenia are so pervasive that schizophrenia has been described as a cognitive illness (Kahn and Keefe, 2013). The latter authors posit that cognition is the core component of the disorder, and that diagnostic criteria should emphasize the altered cognitive function that occurs earlier in the development of the disorder, thereby facilitating early treatment before psychosis emerges. However, antipsychotic treatment poorly addresses these cognitive outcomes (Keshavan et al., 2016). Diagnostic criteria for schizophrenia focus mainly on positive and negative symptoms (American Psychiatric Association, 2013b), although cognitive impairment is already visibly in the early stages of the illness (Kahn and Keefe, 2013) and may be very severe (Keefe and Harvey, 2012). Cognitive domains that are affected include vigilance and attention (e.g. inability to follow social conversations or instructions), verbal and visual learning and memory (related to social outcomes), processing speed, working memory (which is strongly related to functional outcomes such as employment status) and social cognition (e.g inability to infer another's intentions). Cognitive symptoms severely affect occupational and social functioning in schizophrenia and are an area of unmet pharmacological and clinical need.

A summary of the diagnostic criteria for schizophrenia, as set out in the DSM-5 (American Psychiatric Association, 2013a), is provided in Table 1 on the next page.

Table 1. Diagnostic criteria for schizophrenia, as set out in the DSM-5 (American Psychiatric Association, 2013a).

A. At least two of the following symptoms present for at least 1 month, of which one of the two symptoms must be either delusions, hallucinations of disorganized speech: 1. delusions 2. hallucinations 3. disorganized speech (derailment of incoherent speech) 4. grossly abnormal psychomotor behaviour, including catatonia 5. negative symptoms (e.g. avolition, diminished affect)	**B.** The level of functioning must be significantly lower than before onset of symptoms or, if presenting in childhood or adolescence it must be lower than the expected level of functioning. Withdrawal from social activity is included. Furthermore, criterion C-F state that psychosis due to drugs or medical conditions must be excluded, while schizophrenia and autism spectrum disorder should be clearly distinguished.

2.2.2 Depression

Depression is a heterogeneous disorder presenting with several symptoms manifesting differently in various individuals. The core symptoms of depression however include depressed mood, feelings of despair, worthlessness and guilt, anhedonia (reduced ability to experience pleasure) decreased motivation, recurrent thoughts of death and suicidal ideation (Nestler et al., 2002; Ward and Irazoqui, 2010). Other symptoms are heterogeneously expressed, including sleep disturbances (hypersomnia or insomnia), disturbances in appetite (weight gain or weight loss) and disturbed motor function (psychomotor retardation or agitation) (American Psychiatric Association, 2013b; Ward and Irazoqui, 2010). These symptoms are amenable to treatment with most classes of antidepressants that bolster biogenic amine levels in central synapses, although antidepressant treatment failure rates are high (Al-Harbi, 2012; Conradi et al., 2011; Ward and Irazoqui, 2010). Cognitive deficits are also evident in depression, but is notorious for being inadequately addressed by currently available antidepressants (Conradi et al., 2011), often persisting during periods of symptom remission (Lam et al., 2014). Meta-analytic studies report declarative memory deficits in depression, with the largest effect on recollection memory and episodic memory (encoding and retrieving daily experiences and events), while semantic fluency is also affected (Zakzanis et al., 1998). Impaired attention and inferior processing speed may partially underlie various cognitive deficits in depression, although memory processes have been shown to be impaired independently of attention (Zakzanis et al., 1998). Depression is thus a disorder presenting with various symptoms displaying dysfunctional limbic and cognitive processes. Diagnosing depression includes subjective self-reported presence of at least 5 of the above-mentioned symptoms,

of which at least one symptom should be either depressed mood or loss of interest in pleasure (American Psychiatric Association, 2013b). Depression is associated with other psychiatric disorders such as anxiety disorders and schizophrenia, which complicates the diagnosis. Diagnostic criteria for depressive disorders are set out in the DSM-V (American Psychiatric Association, 2013b) and are summarised in Table 2.

Table 2. Diagnostic criteria for depression as set out in the DSM-V (American Psychiatric Association, 2013b).

A. At least 5 of the following symptoms have to be present for at least 14 consecutive days, nearly every day for the greater part of the day, of which at least one symptoms is either 1) depressed mood or 2) loss of interest in pleasure. 1. Depressed mood (feelings of sadness, hopelessness, emptiness, tearfulness, or irritability) 2. Diminished interest or pleasures in daily activities (anhedonia) 3. Significant alterations in weight or appetite 4. Insomnia or hypersomnia 5. Psychomotor alterations 6. Fatigue 7. Feelings of worthlessness or inappropriate guilt 8. Diminished cognitive clarity, concentration, indeciseveness 9. Recurrent thoughts of death or suicide ideation	**B.** The symptoms reported under criterion A cause significant distress or impairment in social, occupational or other daily functioning. **C.** The episode is not attributable to another medical condition or to substance use. **D.** The depressive episode is not better explained by any schizophrenic or schizoaffective or any other psychotic disorder. **E.** There has never been a manic or hypomanic episode.

2.3 Pathophysiology

2.3.1 Pathophysiology of Schizophrenia

The pathophysiology of schizophrenia is multifactorial and complex, involving various hypotheses relating to a number of different, but interconnected systems. The following section outlines the main theories of the neurobiological underpinnings of schizophrenia. It will focus on those theories that more specifically relate to this study, and is not an exhaustive review.

2.3.1.1 Neuroanatomical disturbances

Adverse early life and prenatal insults may contribute to deficits in development of certain brain areas and neurocircuitry that have been shown to underlie dysfunctional connectivity and neurotransmission in schizophrenia (Fallon et al., 2003). The neuroanatomical alterations underlying dysfunctional neurocircuitry in schizophrenia are complex, but can be summarised according to a few main

observations. Fronto-striatal dysfunction lies at the core of schizophrenia pathology (Fallon et al., 2003), and is depicted in Figure 4. A deficit in the subcortical neurons that project from the ventral tegmental area (VTA) (the origin of various dopaminergic cell bodies) to the frontal cortex results in reduced neuronal connectivity to the prefrontal cortex (PFC) (pathway 2 in Figure 4)(Kasai et al., 2002). The PFC has reciprocal connections to other cortical areas, as well as to limbic and basal ganglia structures (pathway 1 in Figure 4), of which hippocampal and striatal dysfunction is prominent in schizophrenia (Kasai et al., 2002). Hypofrontality describes hypofunction of dopaminergic and glutamatergic pathways connecting to the frontal cortex, discussed in section *2.3.1.2.1* and *2.3.1.2.2* below and depicted in Figure 5. The PFC exerts top-down control over sub-cortical regions, and hypofrontality is thus associated with dysfunctional regulation of the limbic system and striatal structures. Excessive striatal dopaminergic stimulation is thus received from the VTA and permeates limbic structures, resulting in various alterations in connectivity and neurotransmission (Fallon et al., 2003; Kasai et al., 2002). Structural alterations have been described in neuroimaging studies, reporting enlargement of the ventricles (fluid-filled compartments) which impinge on various surrounding structures and is accompanied by overall reductions in cortical grey matter volume (Goldman et al., 2008) and striatal and hippocampal volume reductions in schizophrenic patients (Deshmukh et al., 2005; Koch et al., 2014; MacDonald and Schulz, 2009).

2.3.1.2 Neurochemical and neuromolecular disturbances

2.3.1.2.1 Dopamine hypothesis

The hypothesis of dysfunctional dopaminergic transmission has traditionally defined the pathology of schizophrenia, and has prompted the development of D_2 antagonists as effective antipsychotics. However, a multifactorial view has since been adopted (Carlsson et al., 2001). The initial hypothesis was based on the ability of dopamine-releasing drugs, like amphetamine, to induce psychosis, and was further supported by the fact that almost all antipsychotics are antagonists of the dopamine D_2 receptor (Reynolds, 2008). Dopaminergic pathways are divided into the nigrostriatal, mesolimbic, mesocortical and tuberoinfundibular pathways (Figure 4). Dopaminergic impairments in schizophrenia result from dysfunctions in the substantia nigra, VTA, striatum, PFC and hippocampus (Lodge and Grace, 2011; Yoon et al., 2013). The mesolimbic tract projects from the VTA in the midbrain to the nucleus accumbens of the ventral striatum, the hippocampus and amygdala (pathway 1 in Figure 4). Increased DA activity in this pathway is associated with positive symptoms of schizophrenia. The mesocortical pathway projects from the VTA to the frontal cortex (pathway 2 in Figure 4). This pathway is involved in the regulation of cognitive, emotional and motivational aspects of behaviour. Dopaminergic hypoactivity in this pathway is associated with negative and cognitive symptoms of schizophrenia. The nigrostriatal pathway projects from the substantia nigra in the mid-brain to the striatum and is part of the extrapyramidal nervous system that coordinates motor movements (pathway 3 in Figure 4). Antipsychotic D_2 antagonism

adversely affects this pathway, which results in the typical motor side-effects associated with antipsychotic drugs. The tuberoinfundibular pathway (pathway 4 in Figure 4) involves hypothalamic dopaminergic projection neurons which, in the face of imbalanced D_2 and 5-HT_{2A} receptor activity, may result in altered release of prolactin, which may cause endrocrinological side-effects with antipsychotic use (Stahl, 2013).

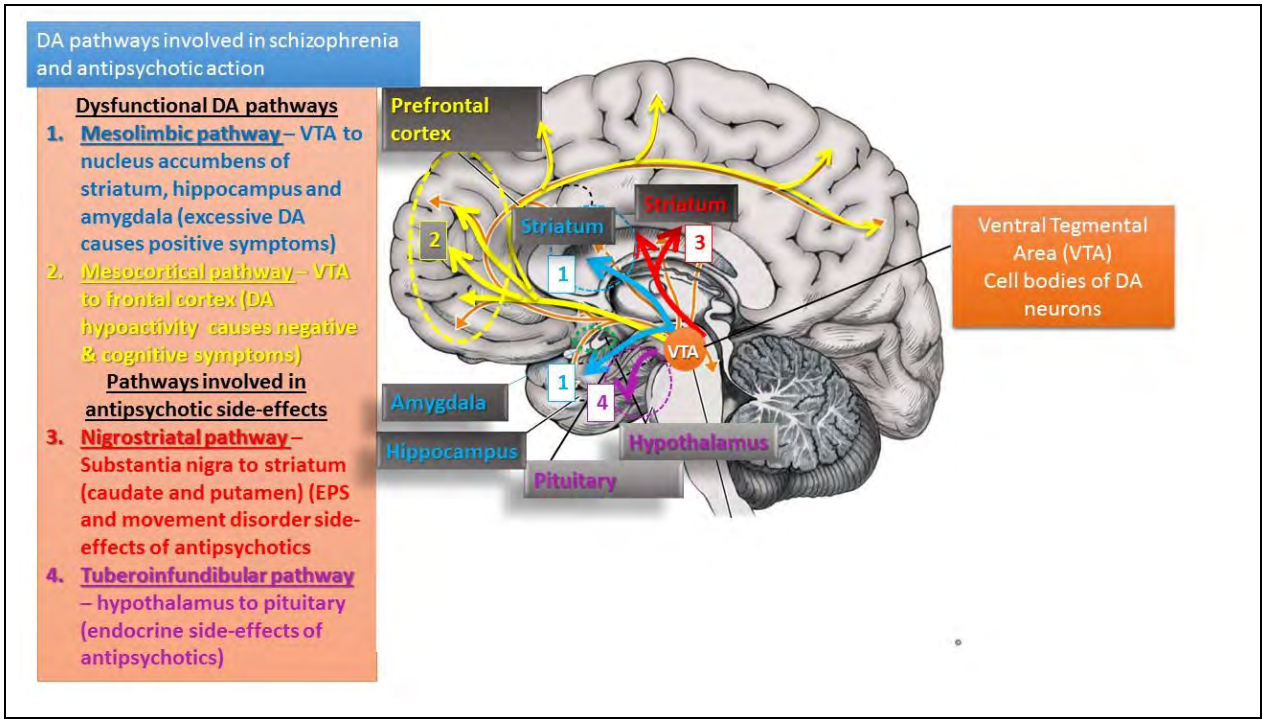


Figure 4. Dopaminergic pathways thought to be involved in the pathophysiology of schizophrenia and the side-effects of antidopaminergic drugs. More detail in the text and in section *2.3.1.1.* Figure adapted from *Biological Psychology* (Breedlove and Watson, 2013)

Increased striatal and limbic DA synthesis and release have been associated with schizophrenia and have been correlated to the severity of positive symptoms (Abi-Dargham et al., 2000; Grace, 2012; Heinz and Schlagenhauf, 2010). Elevated striatal D_2 receptor sensitivity is also proposed to underlie the hyperdopaminergic-driven positive symptoms of schizophrenia as well as breakthrough psychosis during on-going antipsychotic treatment (Samaha et al., 2007; Seeman, 2011; Seeman et al., 2006). Additionally, dysfunctional frontal cortical D_1 receptors and a subsequent imbalance between cortical and subcortical DA levels are associated with negative symptoms and cognitive dysfunction (Brisch et al., 2014). The latter symptoms have been correlated with *reduced* PFC DA transmission (Knable and Weinberger, 1997), while a general *decrease* of frontal cortical D_1 receptors have been described in medication naïve schizophrenics (Okubo et al., 1997). The D_1 receptor is involved in regulating the affinity of the D_2 receptor for DA, and therefore reduction in the density of D_1 receptors could result in mesolimbic D_2 *supersensitivity* and subsequent dopaminergic hyperactivity (Seeman, 2011; Seeman et

al., 1989; Seeman et al., 2006; Suhara and Miyoshi, 2007). Further to this point, augmenting D_1 receptor stimulation has been associated with improvement of cognitive and negative effects of antipsychotics (Goldman-Rakic et al., 2004). On the other hand, compensatory upregulation of D_1-receptors in schizophrenia secondary to mesocortical DA dysfunction has also been described, although this upregulation appears to be ineffective in altering the manifestation of D_1-D_2 imbalance (Abi-Dargham et al., 2002) (Figure 5). A key behavioural parameter employed in this study to assess impaired sensorimotor gating function in schizophrenia, the prepulse inhibition test, is a measure of imbalanced mesolimbic-mesocortical dopaminergic function (Brisch et al., 2014).

The imbalance in mesolimbic-mesocortical dopaminergic function is a cornerstone of antipsychotic therapy (Miyamoto et al., 2005). However, dopaminergic deficits are highly interconnected to serotonergic, GABAergic, glutamatergic, adrenergic and cholinergic deficits and cannot be viewed in isolation (Brisch et al., 2014). For this reason, typical D_2-antagonists alone do not effectively address the negative and cognitive symptoms of schizophrenia. The efficacy of atypical antipsychotics and newer drugs that also target muscarinic, alpha-adrenergic, serotonergic and glutamatergic systems supports the role of multiple interconnected neurotransmitter systems in the pathophysiology of schizophrenia (Miyamoto et al., 2012)

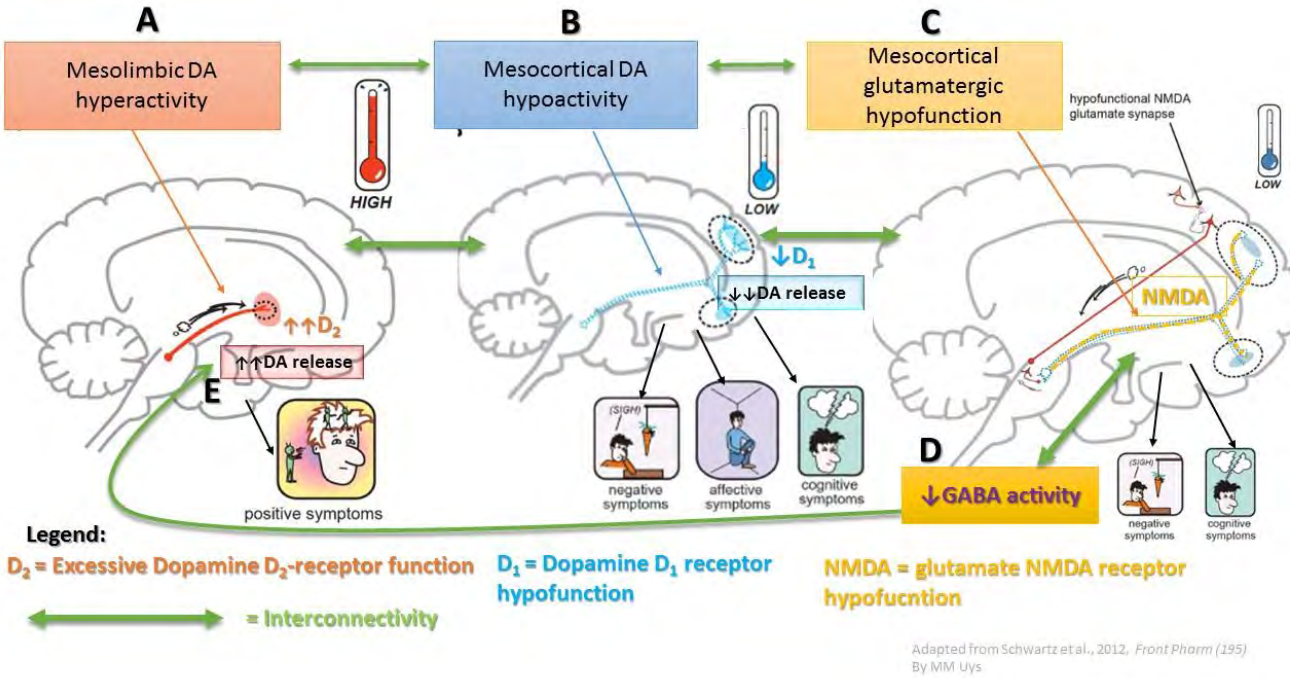


Figure 5. A. Excessive mesolimbic dopaminergic activity is associated with excessive D_2 sensitivity and activation and with positive symptoms of schizophrenia. **B**. Diminished mesocortical dopaminergic activity is associated with frontal D_1 receptor dysfunction. This dysfunctional pathway is associated with negative, affective and cognitive symptoms of schizophrenia. **C**. Mesocortical hypoglutamatergic signalling is associated with NMDA-receptor dysfunction and is associated with negative and cognitive symptoms of schizophrenia. **D**. Reduced NMDA-receptor function is purported to lead to disruption of GABAergic neurotransmission and dysfunctional cortical glutamate-GABA-glutamate neurotransmission. **E.** The latter purportedly disinhibits subcortical DA release, perpetuating the cycle of dysfunction (Heinz and Schlagenhauf, 2010). More details are provided in the text. This figure has been created by adaptions to several figures in Schwartz et al., 2012.

2.3.1.2.2 Glutamate and GABA dysfunction hypothesis

Dopaminergic activity is regulated by both the inhibitory amino acid neurotransmitter, GABA and the excitatory amino acid neurotransmitter, glutamate (Schwartz et al., 2012). Corticostriatal glutamatergic pathways cross-talk with dopaminergic nerve terminals and specific striatal glutamate receptors are sensitive to stimulation by DA (Javitt, 2012). DA reduces the release of PFC glutamate, while PFC glutamate dysfunction in turn increases the release of striatal DA (Seeman, 2009). These systems interact to result in an overall hypoglutamatergic state in schizophrenia, which is purported to underlie cognitive and negative symptoms of the illness (Schwartz et al., 2012). This theory is supported by the fact that glutamate NMDA receptor antagonists induce various behavioural alterations in rodents akin to the positive, negative and cognitive deficits in schizophrenia (Seeman, 2009). Glutamatergic actions at NMDA receptors play an important role in stimulating GABA release, so it is not surprising that, in addition to reduced NMDA receptor function, impaired GABAergic activity has also been described in schizophrenia (Chiapponi et al., 2016; Cohen et al., 2015). Loss of GABAergic output onto secondary glutamatergic cortical neurons, required for tonic control over sub-cortical dopaminergic neurons,

result in increased mesolimbic dopaminergic firing (↑striatal DA release) and consequently the presentation of psychotic symptoms (Schwartz et al., 2012). Thus, glutamatergic, GABAergic and dopaminergic dysfunction form part of an interconnected cycle of neurotransmitter dysfunction, with dysfunction of one system originating from and/or leading to dysfunction of the other systems. These dysfunctional systems are depicted in Figure 5. As discussed in section 1.2, α_{2C}-AR deactivation could play a role in disinhibiting GABA release (Zhang and Ordway, 2003), while a role of α_{2C}-AR antagonism in improving NMDA-antagonist induced behavioural and cognitive deficits as well as frontal cortical DA release has been demonstrated (Sallinen et al., 2007; Sallinen et al., 2013a), supporting a possible therapeutic role for this receptor in treatment.

2.3.1.2.3 Serotonin hypothesis

The nature of serotonergic imbalance in schizophrenia is not very well understood (Miyamoto et al., 2012; Reynolds, 2008). The hallucinogenic action of 5-HT_{2A} agonists, such as lysergic acid diethylamide (LSD), initially suggested a hyperserotonergic hypothesis for schizophrenia (Quednow et al., 2009). This idea was later supported by the ability of clozapine, which has actions at multiple serotonergic receptors, to much more effectively address cognitive and negative symptoms of schizophrenia compared to traditional antidopaminergic antipscyhotics (Meltzer, 1999; Reynolds, 2004). Schizophrenia has been associated with cortical 5-HT hypofunction (Breier, 1995), as reflected by decreased frontal cortical 5-HT reuptake sites and reduced cortical serotonergic innervation (Svensson, 2003), while augmentation of cortical serotonergic neurotransmission has been associated with increased cortical synaptic DA (reviewed in Svensson, 2003). On the other hand, *increased* central serotonergic neurotransmission has been demonstrated in subcortical brain regions, including the putamen, nucleus accumbens and globus pallidus (Crow et al., 1979; Farley et al., 1980). Thus, while a general increase in central serotonergic activity has initially been proposed, various contradictory findings have been reported and it is now accepted that neurobiological abnormalities involving various 5-HT receptors might affect multiple neurotransmitter systems, including dopaminergic, noradrenergic, GABAergic and glutamatergic systems (Quednow et al., 2009). A dysregulation of 5-HT_2 and D_2 receptor-mediated neurotransmission has been related to the pathophysiology of schizophrenia (Meltzer, 1989), while many atypical antipsychotics demonstrate pronounced activity at the D_2 and 5-HT_{2A} receptors. However, these drugs are active at various 5-HT receptors (Miyamoto et al., 2012; Reynolds, 2004). Indeed, 5-HT_{2C} agonism, 5-HT_3, 5-HT_6 and 5-HT_7 antagonism has been associated with antipsychotic-like and pro-cognitive effects in animal models of schizophrenia (Miyamoto et al., 2012). 5-HT_{2A} antagonism may contribute to the 'normalisation' of DA levels (Miyamoto et al., 2012; O'Neill et al., 1999), while 5-HT_{1A} agonists or partial agonists may potentiate the antipsychotic activity of DA antagonists via post-synaptic actions and thereby enhance certain cognitive domains (Sumiyoshi et al., 2014; Sumiyoshi et al., 2007). Various serotonergic imbalances may thus underlie the mesolimbic-mesocortical

dopaminergic imbalance (O'Neill et al., 1999). However, the initial hypothesis that a serotonergic mechanism underlies the therapeutic superiority of atypical agents has not sufficiently been supported by evidence, and other theories abound. In this regard, α_2-AR mechanisms have been proposed to underlie the atypicality of antipsychotics such as clozapine, quetiapine, asenapine and risperidone (Shahid et al., 2009; Svensson, 2003).

2.3.1.2.4 Noradrenergic hypothesis

The interconnectivity of the noradrenergic and dopaminergic systems, as well as that many atypical antipsychotics possess α_2-AR antagonist properties, has prompted greater realization of the important role of noradrenergic dysregulation in schizophrenia (van Kammen and Kelley, 1991; Yamamoto and Hornykiewicz, 2004). The atypical antipsychotic, clozapine has been described as the most effective antipsychotic in refractory schizophrenia, and addresses various aspects of positive, negative and cognitive symptoms (Lewis et al., 2006a; Lewis et al., 2006b; Meltzer et al., 2003; Swartz et al., 2008) while its efficacy is purported to be related to its prominent action at the α_2-AR (Kalkman and Loetscher, 2003; Shahid et al., 2009; Svensson, 2003). α_2-AR antagonists have been shown to selectively increase the variability of the firing pattern of dopaminergic neurons in the VTA (Svensson, 2003). Additionally, presynaptic α_2-AR antagonism has been associated with increased DA output in the PFC, but not in the striatum (Hertel, P. et al., 1999a; Hertel, P. et al., 1999c; Marcus et al., 2005), while augmenting antidopaminergic antipsychotics with α_2-AR antagonists increases PFC glutamatergic transmission (Marcus et al., 2005). This effect is associated with increased synaptic NA, as illustrated by similar effects achieved with NA reuptake inhibition as augmentation strategy.(Marcus et al., 2010a) Adjunctive use of the NA reuptake inhibitor, reboxetine with both the D_2-antagonist raclopride and the atypical antipschotic, olanzapine, has shown similar prominent improvement of affective and negative symptom outcomes in schizophrenic patients and in animal models (Linner et al., 2002; Marcus et al., 2010a; Raedler et al., 2004). These studies thus suggest that increasing central synaptic availability of NA could improve antipsychotic efficacy. These same effects have also been shown for clozapine (Marcus et al., 2005; Moghaddam and Bunney, 1990), which has a very high affinity for α_2-ARs and specifically the α_{2C}-AR (Kalkman and Loetscher, 2003; Shahid et al., 2009). α_2-AR antagonism may also facilitate cortical 5-HT release (Hertel et al., 1997; Hertel, P. et al., 1999b), which is associated with increased cortical DA transmission as described above. Noradrenergic modulation therefore appears to address the mesolimbic-mesocortical dopaminergic and glutamatergic imbalances associated with schizophrenia as well as related cognitive and negative symptoms, (Hertel, Peter et al., 1999; Marcus et al., 2010a; Marcus et al., 2005; Marcus et al., 2010b), which is supportive of a proposal for deficient noradrenergic function in schizophrenia (Yamamoto and Hornykiewicz, 2004). However, the nature of noradrenergic dysfunction in the various brain regions involved in schizophrenia is complex and the NA hypothesis is still poorly understood and therefore relatively poorly defined (Yamamoto and Hornykiewicz, 2004).

However, while augmentation with non-selective α_2-AR antagonists present with enhanced outcomes in schizophrenia as described above, α_2-AR antagonism *per se* does not present with antipsychotic-like efficacy. On the other hand, α_{2C}-AR selective antagonism stand-alone therapy has recently shown antipsychotic and pro-cognitive effects in NMDA-antagonist models of schizophrenia (Sallinen et al., 2007; Sallinen et al., 2013a), indicating that subtype selective α_2-AR modulation could be involved in the pathophysiology of schizophrenia, and therefore a valid new approach for pharmacological intervention. A moderate association of a genetic polymorphism of the α_{2C}-AR adrenoceptor with schizophrenia further lends credence to this proposal (Rivero et al., 2016).

2.3.1.2.5 Cholinergic hypothesis

Alterations in the cholinergic system have also been implicated in the pathophysiology of schizophrenia, built on a proposed imbalance between cholinergic and dopaminergic systems (Scarr et al., 2013). This hypothesis has been supported by evidence of decreased muscarinic receptors in post-mortem brain tissue of schizophrenics, by neuroimaging studies (Raedler et al., 2003; Scarr et al., 2007) and by evidence that muscarinic receptor antagonists, such as scopolamine, induce psychotic-like symptoms (Barak and Weiner, 2006; Barak and Weiner, 2009). Dysfunctional cholinergic neurotransmission will affect various neurotransmitters implicated in schizophrenia, including the monoamines and excitatory and inhibitory amino acids (Scarr et al., 2013). Muscarinic and nicotinic agonists may lead to enhanced GABAergic interneuron function (Miyamoto et al., 2012), while acetylcholine esterase inhibitors may enhance cognitive parameters of schizophrenia (Miyamoto et al., 2012). Thus, cholinergic involvement in schizophrenia is also an active field of interest and viable therapeutic avenue to explore (Scarr et al., 2013).

2.3.1.2.6 HPA-axis involvement

Considering the importance of the HPA-axis in the stress response (Walker and Diforio, 1997), and that schizophrenia is related to early life adversity, HPA-axis dysregulation has also been suggested to underlie aspects of the disorder (Read et al., 2001). Glucocorticoid receptors are densely expressed in the hippocampus and this brain structure is vital in the feedback system modulating HPA-axis activation. Persistent exposure to stress may permanently alter HPA-axis function, dampening the negative feedback system that should normally shut down HPA-axis activation. Schizophrenics have higher baseline cortisol levels and pre-existing HPA-axis hyperactivation, while cortisol release is associated with severity of schizophrenia symptoms (Walker and Diforio, 1997). HPA-axis activation increases DA receptor sensitivity, DA synthesis and dopaminergic neurotransmission, while DA also synergistically enhances HPA-axis activation (Walker and Diforio, 1997). Early life environmental stressors (e.g. childhood neglect and abuse) have been associated with excessive HPA-axis activation and thus with the altered DA activity seen in schizophrenia, especially with excessive altered hippocampal and striatal DA activity leading to DA hypoactivity in the frontal cortex (Read et al., 2001).

2.3.1.2.7 Neurotrophic dysfunction

Neurotrophins promote the growth and differentiation of developing neurons as well as the survival of neuronal cells in response to stress (Buckley et al., 2007). Key neurotrophins include nerve growth factor (NGF), vascular endothelial growth factor (VEGF) and brain-derived neurotrophic factor (BDNF) (Buckley et al., 2007; Rosenstein and Krum, 2004; Sofroniew et al., 2001). BDNF is the most prevalent neurotrophic growth factor in the CNS, where it is especially important in regulating synaptic plasticity and various aspects underlying cognitive performance, memory and mood (Autry and Monteggia, 2012a; Lu et al., 2014). Binding of BDNF to its TrkB receptor (tropomyosin receptor kinase B) activates phosphatidylinositol 3-kinase, mitogen-activated protein kinase (MAPK) and phospholipase C-γ pathways, ultimately facilitating the expression of cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) and leading to cell survival pathways (Autry and Monteggia, 2012b; Buckley et al., 2007). Thus, the normal biological responses of neurotrophins include proliferation, survival, axonal and dendritic growth, synapse formation and synaptic function plasticity (Buckley et al., 2007).

Schizophrenia is associated with neurodegenerative processes and progressive tissue loss over the course of the illness, including reduced hippocampal and temporal lobe volumes (Velakoulis et al., 2006) and reduced volume of striatal structures, including the putamen and nucleus accumbens (Deshmukh et al., 2005). Of note is that reduced hippocampal volumes have been associated with decreased serum BDNF levels in schizophrenic patients (Rizos et al., 2011), suggesting that reduced neurotrophin levels might be associated with central attrition in schizophrenia. Additionally, loss of striatal grey matter (Koch et al., 2014), temporal lobe grey matter (Pantelis et al., 2003) and frontal cortical gray matter (Vidal et al., 2006) have been observed in schizophrenic patients. Thus, these findings highlight the deficient neurotrophic promotion of cell proliferation and cell survival in schizophrenia.

As described in section *2.1.1*, pre-natal insults and early-life stress may underlie vulnerability for the development of schizophrenia (van Os et al., 2010). Rodent studies have shown that prenatal maternal restraint stress and postnatal maternal deprivation, which constitute developmental animal models of schizophrenia, produce persistent reductions in frontal cortical and striatal expression of BDNF in adulthood (Fumagalli et al., 2004; Roceri et al., 2004), while various other models of schizophrenia produce reduced cortical and hippocampal BDNF (Angelucci et al., 2007; Pillai, 2008). Furthermore, transgenic mice presenting with genetically modified alterations of central BDNF activity present with deficits in sensorimotor gating and cognitive impairment that strongly correlate to positive and cognitive symptoms in schizophrenia (Autry and Monteggia, 2012b). Sensorimotor gating deficits in animal models of schizophrenia have also been correlated with decreased hippocampal and striatal

BDNF levels (Lippmann et al., 2007). Animal models therefore describe an important role of BDNF in the symptoms and neuropathology associated with schizophrenia.

Similarly, there is evidence of disrupted neurotrophin levels in clinical studies of schizophrenia (Shoval and Weizman, 2005). Amidst various studies often reporting conflicting findings, a meta-analysis of 17 different studies supports the notion that schizophrenic patients have reduced BDNF blood levels vs. healthy controls (Green et al., 2011; Nieto et al., 2013). Although postmortem studies of regional BDNF expression in brain tissue of schizophrenic patients have reported conflicting results, the overall picture suggests decreased cortical, subcortical and limbic BDNF levels, which is in agreement with the reduction in plasma BDNF levels described above (Durany et al., 2001; Durany and Thome, 2004; Ray et al., 2014; Ray et al., 2011; Takahashi et al., 2000; Weickert et al., 2003; Weickert et al., 2005). Post-mortem CNS findings are however complicated by multiple antipsychotic use in the case subjects (Autry and Monteggia, 2012), so that findings from post-mortem studies are difficult to interpret. However, low serum BDNF levels have been correlated with reduced hippocampal volume at the onset of schizophrenia (Martinotti et al., 2012; Rizos et al., 2011), suggesting a correlation between neuronal atrophy and decreased serum BDNF levels in schizophrenic patients. Thus, clinical and preclinical studies point to correlations of blood and brain levels of BDNF in schizophrenia, which are variably altered by chronic antipsychotic treatment (Buckley et al., 2007; Fumagalli et al., 2003; Pedrini et al., 2011a; Pillai et al., 2007; Pillai et al., 2006).

Interestingly, various studies report that the typical antidopaminergic antipsychotic, haloperidol (HAL), is associated with *decreased* striatal and hippocampal BDNF and NGF levels (Parikh et al., 2004; Pillai et al., 2006; Weickert et al., 2003). These effects have been attributed to excess striatal DA release induced by long term block of striatal D_2 receptors and are associated with motor side-effects that might be mediated via striatal oxidative stress and neurodegeneration (Moghaddam and Bunney, 1990; Pillai et al., 2006; Tan et al., 2005; Westerink et al., 2001). These adverse effects of chronic haloperidol on NGF and BDNF can however be reversed by long-term treatment with the atypical antipsychotic olanzapine (Fumagalli et al., 2003; Parikh et al., 2004; Pillai et al., 2006). Some atypical antipsychotics appear to elevate central BDNF levels, although long term treatment may reverse this elevation (Buckley et al., 2007). Higher plasma levels of NGF have been observed in medicated schizophrenics compared to a drug-naïve first episode schizophrenia sample, while plasma NGF levels were significantly higher in patients on atypical antipsychotics than those on typical antipsychotics (Buckley et al., 2007). On the other hand less robust effects for typical antipsychotics have been reported on serum BDNF levels. Moderate increases in serum BDNF levels have been reported in patients after 6 weeks of antipsychotic treatment with clozapine, but not haloperidol (Pedrini et al., 2011b; Pirildar et al., 2004). Evidence from various studies also suggest reduced BDNF and NGF levels in acutely psychotic unmedicated patients

(Toll and Mané, 2015). Reduced serum BDNF levels have been positively associated with the negative symptoms and cognitive deficits in schizophrenia (de Azua et al., 2013; Niitsu et al., 2011; Zhang et al., 2012), while *improvement* of cognition in schizophrenic patients is associated with *increased* plasma BDNF levels (Vinogradov et al., 2009).

Although antipsychotics affect plasma levels of neurotrophins in schizophrenic patients as described above, the effect of antipsychotics on brain levels of neurotrophins in schizophrenics is unclear, since evidence from post mortem studies is confounded by multiple antipsychotic use, as described earlier (Autry and Monteggia, 2012a). However, considering that a correlation of brain and blood levels of neurotrophins in schizophrenic patients has been described (Martinotti et al., 2012; Pillai et al., 2010; Rizos et al., 2011), and that such a correlation of brain and blood levels of BDNF have also been reported in rodent models of schizophrenia (Autry and Monteggia, 2012a; Buckley et al., 2007; Nieto et al., 2013), the effects of antipsychotics on the brain region-specific expression of neurotrophins in rodent models are thought to provide useful translational information (Buckley et al., 2007).

Thus, given that schizophrenia presents with cognitive and neurodegenerative impairments coupled with reduced neurotrophic support, typical antipsychotics appear to be less capable in re-establishing such support to the extent afforded by the atypical compounds. When investigating novel antipsychotic compounds, it will be prudent to investigate the effect of drug treatment on neurotrophic parameters, since these proteins not only are associated with antipsychotic-like and pro-cognitive effects, but in addition promote neuronal growth and survival in an illness characterised by neurodegeneration.

2.3.1.2.8 Hypothesis of neuroinflammation

Immune-inflammatory responses are implicated in the etiology and pathophysiology of schizophrenia (Watanabe et al., 2010). Abnormal expression of specific cytokines, including interleukins, neuregulin-1 and epidermal growth factor has been described in brain and peripheral blood of schizophrenics (Watanabe et al., 2010). These cytokines have been proposed to perturb structural aspects of brain development by transmission of immune/inflammatory signals to immature brain tissue. The neurodegenerative changes observed in schizophrenia have thus been associated with an immune-inflammatory state and increased production of pro-inflammatory cytokines (Monji et al., 2013). The environmental impact of prenatal maternal infection and perinatal infection as vulnerability factors for schizophrenia further support a role of altered immune-inflammatory state in the disorder (Brown and Derkits, 2010).

Schizophrenia is thus a complex disorder characterised by multiple interconnecting pathophysiological presentations, including disordered monoaminergic, GABAergic, glutamatergic, cholinergic, neurotrophic, endocrine and immune-inflammatory processes that have a variable yet distinct role in its progression and clinical presentation (Davis et al., 2014).

2.3.2 Pathophysiology of Depression

The pathophysiology of depression remains poorly understood amidst a vast pool of research and various hypotheses as to its neurobiological basis. The following section will outline the main theories as they relate to this study, and is not intended to be an exhaustive review.

2.3.2.1 Neuroanatomical disturbances

Various brain structures are implicated in mood and cognition. Central to the underpinnings of depression are functional abnormalities of the PFC and the limbic structures, especially the hippocampus, amygdala and ventral striatum (nucleus accumbens and olfactory tubercle)(Nestler et al., 2002). Dysfunction of the hypothalamus is also suspected, as well as dysregulation of the hypothalamic-pituitary-adrenal axis (HPA), brain regions and systems critical to the stress response (Nestler et al., 2002). Monoaminergic dysfunction is a core underpinning of depression (see section *2.3.2.2.1*). Noradrenergic projection neurons originating from the locus coeruleus (LC), serotonergic projection neurons from the raphe nuclei and dopaminergic projection neurons from the VTA, all originating from the midbrain, project to all the aforementioned limbic and cortical brain areas, resulting in dense monoaminergic innervation of brain structures implicated in the pathophysiology of depression (Stahl, 2013). Furthermore, deficient monoaminergic and GABAergic signalling are intricately related to excessive and dysregulated glutamatergic and glucocorticoid signalling thought to result in altered functionality, connectivity and neurodegeneration of the above brain structures (Nestler et al., 2002). Figure 6 presents a simplistic summary of the most affected brain regions and neurotransmitter pathways.

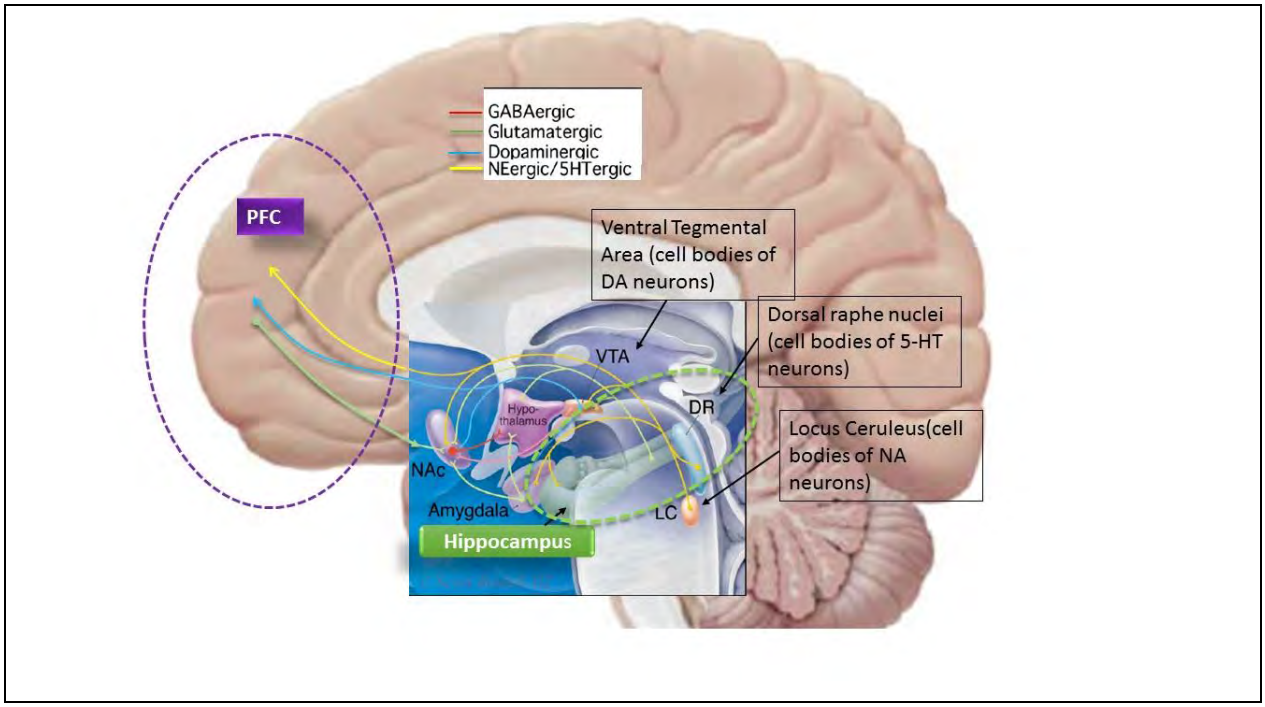


Figure 6. Brain structures and neurotransmitter pathways associated with altered neurocircuitry in depression. Prominent structures include the prefrontal cortex (PFC), hippocampus, nucleus accumbens (NAc) of the striatum and the amygdala. The cell bodies of noradrenergic (LC), dopaminergic (VTA) and serotonergic (DR, dorsal raphe nuclei) projection neurons are also indicated. Adapted from Nestler et al., 2002.

Depression is associated with neurodegenerative processes specifically involving hippocampal atrophy (Nestler et al., 2002; Sapolsky, 2000a), including reduced hippocampal volume, reduced hippocampal grey matter and reduced dendritic branching (Sheline, 2003), changes that are associated with glucocorticoid-induced toxicity (see section *2.3.2.2.4*), altered BDNF signalling (see section *2.3.2.2.5*) and deficient monoaminergic neurotransmission (see section *2.3.2.2.1*) (Nestler et al., 2002). Various studies suggest that antidepressants may beneficially alter these aversive processes and increase hippocampal neurogenesis and synaptic plasticity (Anacker et al., 2011). Neurotransmitter-coupled structural anomalies and associated effects of antidepressants in hippocampal neurons are depicted in Figure 7. Although hippocampal dysfunction is prominent, frontal cortical and amygdalar grey matter loss and volume reduction as well as altered striatal volumes have also been described in depression, amid various contradictory reports (Sheline, 2003).

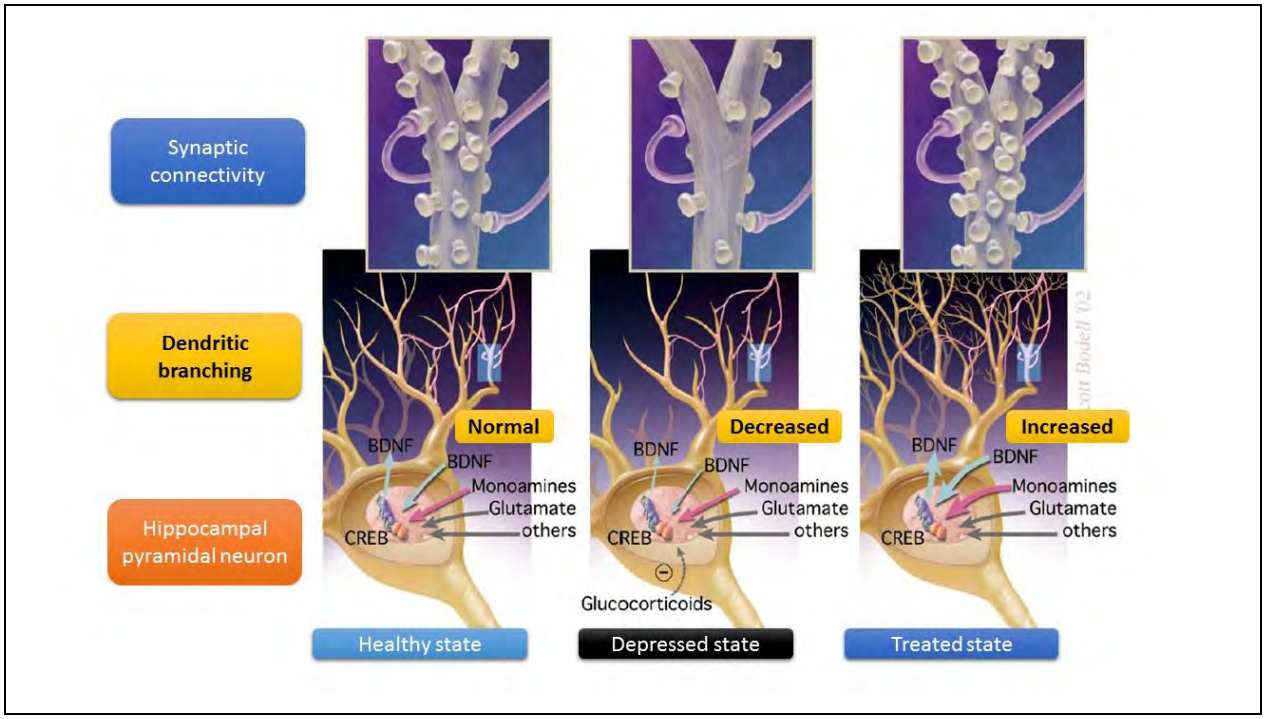


Figure 7. Proposed structural and trophic abnormalities associated with hippocampal atrophy (adapted from Nestler et al, 2002). Decreased dendritic branching and diminished synaptic connectivity and synaptic plasticity is associated with the depressed state, while antidepressants reverse these structural and functional abnormalities. These processes are described in sections *2.3.2.2.1*, *2.3.2.2.4* and *2.3.2.2.5*.

2.3.2.2 Neurochemical and neuromolecular disturbances

2.3.2.2.1 Biogenic amine hypothesis

The hypothesis that depression is associated with a monoamine-deficiency, including decreased levels and dysfunctional neurotransmission of NA, DA and 5-HT, has been the earliest hypothesis of depression and has led to the development of the vast majority of currently available antidepressants (Krishnan and Nestler, 2008; Nestler et al., 2002). This hypothesis was serendipitously born from the discovery of the antidepressant qualities of drugs that increase the brain levels of NA and 5-HT, namely the tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (Schildkraut, 1965). Furthermore, the monoamines regulate various functions that are dysregulated in depression, including motivation, affect, appetite, sleep and reward (Stahl, 2013). Multidirectional relationships among 5-HT, NA and DA systems are evident and thus a dysfunction in one system translates into altered functionality of the other systems (Guiard et al., 2008). 5-HT has been the most widely studied monoamine in depression, but evidence of the importance and interrelated actions of dopaminergic and noradrenergic dysfunction promotes a hypothesis of monoaminergic deficiency in depression (Ruhe et al., 2007).

The raphe nuclei in the brainstem contain the cell bodies of serotonergic neurons that project to the brain regions comprising the limbic system (Ordway et al., 2002). Serotonergic deficits have been widely reported in depression, although findings are often heterogenous. Several studies report reduced CSF

levels of the 5-HT metabolite, 5-hydroxyindole acetic acid (5-HIAA) in depressed patients, correlating with the degree of symptom severity (Van Praag, 1977). Significantly increased 5-HT_{2A} receptor binding, decreased 5-HT_{1A} receptor binding and decreased 5-HIAA have been reported in post-mortem brain tissue of depressives (Drevets et al., 2007; Ferrier et al., 1986; McKeith et al., 1987). Additionally, depletion of the 5-HT precursor, tryptophan, causes affective disturbances in healthy volunteers, while causing relapse in remitted depressives (Delgado, 2000). An early finding reported that, regardless of the primary pharmacological mechanism of action of various antidepressant treatments, including electroconvulsive shock, all effective antidepressant treatments increase serotonergic neurotransmission (Blier et al., 1990). Today, the selective serotonin reuptake inhibitors (SSRIs) are the most commonly prescribed antidepressants (Kharade et al., 2010) and act to increase serotonergic neurotransmission by inhibiting the synaptic removal of 5-HT.

Dysfunctional noradrenergic neurotransmission and central noradrenergic deficiency has been described in depression (Moret and Briley, 2011). This hypothesis is supported by a number of observations. Firstly, limbic brain regions that play an important role in mood and cognition, including the hippocampus and amygdala, are densely innervated by noradrenergic neurons from the locus coeruleus (Ordway et al., 2002). Secondly, depletion of NA results in a relapse of depression after successful remission with antidepressant therapy (Charney, 1998). Thirdly, many antidepressants promote the release of, or inhibit the reuptake of NA (Whiskey and Taylor, 2013). Fourthly, various alterations in the noradrenergic system have been described in depressed patients. While the α_2-AR modulates the negative feedback response of NA, altered α_2-AR expression is widely described in depressive disorders (Cottingham and Wang, 2012 for review). Increased α_2-AR density is found in platelets and in post-mortem brain tissue of depressed suicide completers in the locus coeruleus, temporal and frontal cortex, hippocampus and hypothalamus (De Paermentier et al., 1997; González et al., 1994; Ordway et al., 2003; Ordway et al., 1994). Moreover, α_2-AR receptor up-regulation and increased sensitivity has been specifically associated with the α_{2A}-AR subtype in depressed states (Callado et al., 1998; Javier Meana et al., 1992; Meana and García-Sevilla, 1987). Altered β-receptor binding has also been described in the frontal cortex of suicide completers (Mann et al., 1986), along with alterations in the expression and binding of the NA transporter (Klimek et al., 1997). Finally, genetic deletion of the NA transporter in mice, which results in increased synaptic levels of NA, result in a resilience to stress-induced depressive-like behaviours (Haenisch et al., 2009). Certain symptoms of depression respond poorly to treatment with serotonergic antidepressants, including fatigue, anhedonia, and cognitive impairment (Chamberlain and Robbins, 2013; Ruhe et al., 2007), which subsequently prompted the development of noradrenergic or serotonergic-noradrenergic active antidepressants such as reboxetine, duloxetine, mirtazapine and venlafaxine. There is evidence suggesting that antidepressants that additionally increase noradrenergic neurotransmission, such as the

tricyclic antidepressants, mirtazapine and noradrenaline reuptake inhibitors (Moret and Briley, 2011) are more likely to improve these depressive symptoms, especially regarding cognitive paramaters (Chamberlain and Robbins, 2013). In fact, that venlafaxine and mirtazapine is today widely regarded as important in combination therapies, especially in treatment resistance (Al-Harbi, 2012; Kennedy et al., 2011), is unequivocal evidence of the added importance of NA in the biology and treatment of depression.

DA plays an essential role in regulating motivation, pleasure and attention, behaviours which are often impaired in depression (Takamura et al., 2014). Several studies have pointed to a relative hypodopaminergic state in depression. These include reports of lower levels of the DA metabolite, homovanillic acid (HVA) in the CSF of depressive patients and altered central binding of dopamine D_2/D_3 receptors and of the DA transporter (Meyer et al., 2001; Shah et al., 1997). Stress-induced DA reductions have been correlated to depressive states and cognitive impairment (Mizoguchi et al., 2002). On the other hand, increasing DA neurotransmission has been reported to produce antidepressant-like effects in rodent models of depression (Kitamura et al., 2008; Kitamura et al., 2010), while some clinically effective antidepressants, such as bupropion exert beneficial effects on DA release and reuptake (Al-Harbi, 2012). DA has also been suggested to be involved in various steps of adult hippocampal neurogenesis. Section *2.3.2.2.5* describes the neurotrophic hypothesis of depression, which includes diminished hippocampal neurogenerative processes. Depletion of DA in rodents results in decreased hippocampal neuronal proliferation and survival (Hoglinger et al., 2004), while other studies support a role of DA in adult hippocampal neurogenesis (Takamura et al., 2014). Thus decreased hippocampal DA might play a role in the hippocampal atrophy observed in depression, while antidepressant drugs that increase DA neurotransmission may exert their antidepressant effects by augmenting hippocampal neurotrophic processes (Takamura et al., 2014). Reduced PFC dopaminergic function has also been associated with neural processes involved in the pathophysiology of mood and cognitive symptoms of depression (Mizoguchi et al., 2002). That chronic stress appears to reduce PFC DA neurotransmission correlates well with the premised etiological theories of depression and is associated with depressive-like cognitive impairment (Mizoguchi et al., 2000). Decreased serotonergic and glucocorticoid responses have been proposed to be involved in this PFC dopaminergic dysregulation (Mizoguchi et al., 2002), emphasizing the interconnectivity of various central mechanisms underlying the pathology of depression.

Deficient DA, 5-HT and NA neurotransmission is thus proposed to underlie the pathophysiology of depression. The purported role of the α_{2C}-AR on these systems is described in section *1.2*, although exactly how these actions correlate to the pathophysiology of depression is still poorly understood and warrants further investigation.

2.3.2.2.2 Cholinergic disturbances

The cholinergic system plays an important role in the regulation of CNS function, including that of the monoaminergic system (Dagyte et al., 2011). Cholinergic neurons widely innervate the brain, including the frontal cortex and hippocampus, where it regulates various aspects of memory and mood (Dagyte et al., 2011). Depression has been associated with cholinergic dominance, or excessive cholinergic activity, as initially evidenced by the finding that cholinesterase inhibitors induce depressive-like symptoms in healthy subjects (Janowsky et al., 1974). Neuroimaging studies have reported increased brain levels of the acetylcholine precursor, choline in depressive patients, which is reversed after recovery from depression (Dagyte et al., 2011). Cholinergic hypersensitivity is thus purported to underlie depressive pathogenesis and symptomatology, while co-administered centrally acting anticholinergics have demonstrated therapeutic value in managing depression (Drevets et al., 2013; Furey and Drevets, 2006). This phenomenon was the basis for the development of the FSL animal model of depression, which demonstrates cholinergic hypersensitivity and enhanced sensitivity to environmental stressors (Overstreet et al., 2005). A wealth of evidence also points to a role of dysfunctional cholinergic neurotransmission in the neurodegenerative processes underlying hippocampal atrophy in depression (Dagyte et al., 2011).

2.3.2.2.3 GABA and Glutamate dysfunction

The involvement of dysregulated inhibitory and excitatory neurotransmitter systems has also been reported in depression. While stress increases glutamatergic signalling, up-regulated glutamatergic transmission is purported to underlie depressive states (Zarate et al., 2003). Evidence of the efficacy of the glutamate NMDA receptor antagonist, ketamine, in treatment resistant depression suggest that aberrant glutamatergic neurotransmission at the NMDA receptor is involved in the pathophysiology of depression (Al-Harbi, 2012). Moreover, its efficacy and rapid onset of action in treatment resistant depression in contrast to all other pharmacological antidepressant treatment modalities has attracted a great deal of clinical and scientific interest (Zarate and Niciu, 2015). Part of ketamine's action has been attributed to disinhibition of GABAergic control over synaptic glutamatergic release, resulting in a 'glutamate surge' (Moghaddam et al., 1997). Due to the blockade of postsynaptic NMDA-receptors, the elevated glutamate preferentially binds to alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, initiating various second messenger pathways that ultimately increase BDNF translation and release, stimulating synaptic plasticity (Zarate and Niciu, 2015) (see section *2.3.2.2.5* for details on the involvement of BDNF in depression).

Following on excitatory amino acid dysfunction, inhibitory amino acid (GABAergic) pathophysiology is also said to underlie depression (Möhler, 2012). Reduced plasma, CSF and cortical levels of GABA have been described in depression (Sanacora et al., 2000). Interestingly, lower cortical GABA levels are correlated to increased glutamatergic markers (Sanacora et al., 2004b), suggesting that an altered

excitatory/inhibitory ratio is present in depression. Indeed, it is well known that the GABA-glutamate shunt (which is a closed-loop metabolic pathway of GABA) involves the formation of GABA from glutamate via the enzyme glutamic acid decarboxylase (GAD) (Hertz, 2013; Olsen and DeLorey, 1999), cementing their close association both biochemically and physiologically (Figure 8). GABAergic deficits have been associated with a causal predisposition for depression, as evidenced in various transgenic animal models of GABAergic deficiency (Möhler, 2012). Furthermore, the majority of hippocampal 5-HT_{1A} receptors are located on GABAergic interneurons such that a GABAergic mechanism has been suggested to underlie the antidepressant action of serotonergic antidepressants (Möhler, 2012). Chronic antidepressant treatment has also been shown to enhance GABAergic neurotransmission (Okamoto et al., 2010), while increased GABAergic transmission has been associated with hippocampal neuronal survival (Methippara et al., 2010) and enhanced BDNF signalling (Saarelainen et al., 2003). Finally, the $GABA_A$ allosteric modulator, alprazolam, reportedly has effective antidepressant properties (van Marwijk et al., 2012).

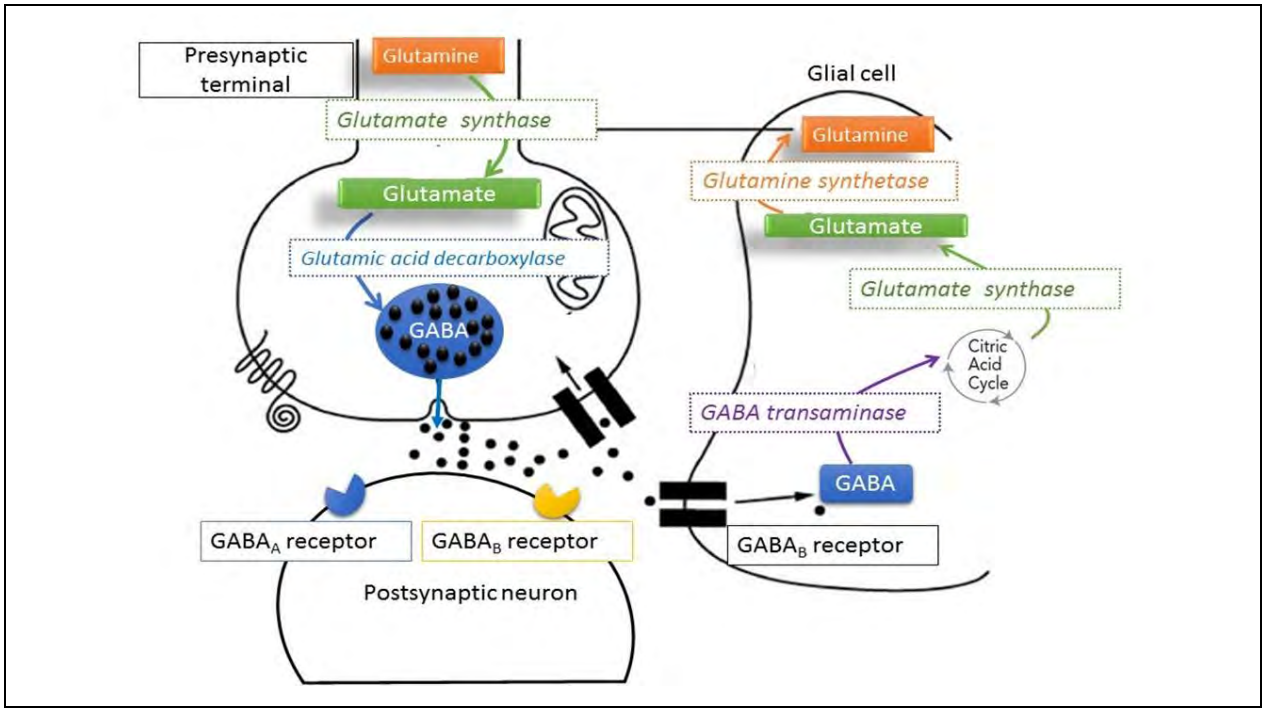


Figure 8. Closed-loop GABA-glutamate shunt. Glutamine is converted into glutamate via glutamate synthase. Glutamate is converted into GABA via glutamic acid decarboxylase. GABA transaminase catalyzes a series of metabolic cascades that eventually culminate in the formation of glutamate via glutamine synthetase, where after the cycle is perpetuated.

Dysregulated excitatory and inhibitory amino acid signalling is therefore associated with the pathophysiology of depression, while pharmacological targeting of these systems may result in improved symptom outcome and neurotrophic benefits.

2.3.2.2.4 HPA-axis dysregulation and hippocampal atrophy

Major depressive disorder is thought to be characterised by diminished inhibitory neural control of the hippocampus and PFC over the HPA-axis, resulting in HPA-axis over-activity with reduced negative feedback and hypercortisolaemia (Kharade et al., 2010). Stress activates the HPA-axis, triggering the release of glucocorticoids (cortisol in humans and corticosterone in rodents) and adrenaline from the adrenal cortices and also triggers the release of limbic DA and NA (Read et al., 2001). The release of the latter suppresses cortical top-down inhibitory control over sub-cortical structures, while heightening the ability of the hippocampus to store emotional-driven contextual memory (Read et al., 2001). The hippocampus has a high density of glucocorticoid receptors and is vital in the negative feedback system that "turns off" the stress response by modulating HPA-axis activation (Sapolsky, 2000a). Persistent exposure to stressors and heightened glucocorticoid release may permanently dampen HPA-axis negative feedback, resulting in a persistent state of HPA-axis activation.

Patients with depression consistently display elevated CSF, plasma and urinary levels of cortisol, corticotropin-releasing hormone (CRH) and adrenocorticotrophic hormone (ACTH) (Sapolsky, 2000a), while elevated post-mortem CRH levels have been found in limbic structures in suicide completers (Merali et al., 2004). Chronic stress is related to reduced glucocorticoid receptor sensitivity resulting in insufficient negative feedback to turn off the stress response (Raison and Miller, 2003). Additionally, hypercortisolaemia and altered glucocorticoid receptor sensitivity has been associated with detrimental effects on hippocampal neuronal plasticity (Manji et al., 2003). The HPA-axis theory therefore posits that disrupted glucocorticoid receptor signalling, hypercortisoleamia and elevated hippocampal glucocorticoid levels may contribute to hippocampal atrophy and compromised hippocampal functioning (Nestler et al., 2002). Indeed, hippocampal atrophy is positively correlated to rates of hypercortisoleamia (Sapolsky, 2000a; Sapolsky, 2001). Interestingly, corticosterone treatment reduces hippocampal BDNF expression, indicating that hippocampal BDNF expression is regulated via glucocorticoids. Chronically elevated cortisol levels may therefore induce hippocampal atrophy via down-regulating BDNF signalling (Lee and Kim, 2010). Figure 9 at the end of section *2.3.2.2.6* depicts the interactions of the HPA-axis, BDNF, monoamines and pro-inflammatory markers on hippocampal function. Interestingly, while antidepressants ameliorate HPA-axis hyperactivity, they have been shown to increase hippocampal neurogenesis by normalizing desensitized glucocorticoid receptors (Anacker et al., 2011). The α_{2C}-AR is densely expressed in the hippocampus, and may possibly modulate corticosterone responses to stress (Sallinen et al., 1999). Additionally, α_{2C}-KO mice display consistently

lower plasma corticosterone levels after repeated stress compared to wild-type controls, whereas α_{2C}-OE mice reportedly display elevated corticosterone levels after repeated stress (Sallinen et al., 1999), suggesting that α_{2C}-AR antagonism might beneficially alter aberrant glucocorticoid responses in depression, highlighting its potential therapeutic benefit.

2.3.2.2.5 Neuroplasticity and neurodegeneration

Considering the evidence of hippocampal atrophy and volume loss described above, the role of neurotrophic factors in depression has been intensely investigated (Autry and Monteggia, 2012b). The effects and purpose of the neurotrophic growth factor, BDNF, on cell proliferation and neuronal survival have been described in section *2.3.1.2.7.* To reiterate, BDNF is essential in the maintenance of synaptic plasticity and in processes underlying learning and memory (Autry and Monteggia, 2012b). Presynaptic BDNF signalling also promotes neurotransmitter release, while postsynaptic BDNF signalling enhances ion channel function, including the function of the NMDA-receptor as well as transient receptor potential cation channels modulating resting membrane potential, neuronal excitability and synaptic transmission (Rose et al., 2004). Its role in cognition and mood-related behaviour is well documented (Autry and Monteggia, 2012b). As described in section *2.1.2*, vulnerability for developing depression is greatly predicted by early life trauma and acute and chronic stress (Charney and Manji, 2004). BDNF on the other hand can also be described as a molecular substrate of stress, and since its expression is reduced by stress, this phenomenon is an important risk factor for the development of depression (Martinowich et al., 2007). Thus, it is not surprising that altered BDNF signalling is postulated to underlie the pathophysiology of depression.

Hippocampal atrophy in depressed patients has been associated with excessive HPA-axis and glucocorticoid toxicity (Sapolsky, 2000a, b; Sapolsky, 2001) as well as altered BDNF levels (Autry and Monteggia, 2012b). Post-mortem studies of suicide completers or depressed patients reveal reductions in BDNF and its TrkB receptor in various regions of the hippocampus (Ray et al., 2011) and cortex (Ray et al., 2014), as well as being reduced in serum of depressed patients (Molendijk et al., 2011; Molendijk et al., 2014; Shimizu et al., 2003). A positive correlation between decreased serum BDNF levels and depressive symptom severity has been demonstrated (Shimizu et al., 2003). Intriguingly, it seems that BDNF expression is increased in the nucleus accumbens of the striatum and in the amygdala, with imbalanced BDNF signalling correlated to stress hyper-responsivity (Autry and Monteggia, 2012b). On the other hand meta-analyses report that antidepressant therapy increases reduced serum BDNF levels in depressed patients (Sen et al., 2008; Shimizu et al., 2003) and in post-mortem hippocampal tissue (Chen et al., 2001; Duman and Monteggia, 2006).

Findings in rodents have mirrored clinical reports of altered BDNF in depression, showing that chronic treatment with various antidepressants increase corticolimbic BDNF expression, particularly in the

hippocampus, but also in the frontal cortex (Altar et al., 2003; Balu et al., 2008; Duman and Monteggia, 2006; Nibuya et al., 1995). Additionally, infusion of BDNF into the midbrain and hippocampus produces antidepressant-like effects in rodent behavioural models such as the learned helplessness and forced swim test (see section *4.2*) (Hoshaw et al., 2005; Shirayama et al., 2002). Conversely, animal models of deficient BDNF expression have shown an inability to respond to antidepressants (Adachi et al., 2008; Monteggia et al., 2007; Saarelainen et al., 2003). Hippocampal neurogenesis, including increased neuronal proliferation, has been shown to coincide with the onset of therapeutic effects of antidepressants (Malberg and Blendy, 2005; Santarelli et al., 2003a).

Interplay between monoaminergic systems and BDNF signalling has been proposed, due to the beneficial effect of antidepressant treatments on monoaminergic signalling and the subsequent correction of BDNF signalling (Monteggia et al., 2007). Most investigations have focussed on the functional connection between the serotonergic systems and BDNF signalling in the wake of widespread efficacy of drugs that increase serotonergic signalling. BDNF undergoes retrograde transport from the hippocampus to the raphe nuclei, suggesting a complex interplay of BDNF and serotonergic function (Anderson et al., 1995). In this regard, mice with gene-targeted BDNF alterations show altered serotonergic innervation of cortical and hippocampal regions in conjunction with depressive-like behaviours (Lyons et al., 1999). Interplay between BDNF signalling and the 5-HT transporter and 5-HT receptors have also been demonstrated. BDNF dose-dependently decreases 5-HT reuptake from the synaptic cleft, reminiscent of the effects of various antidepressants and suggesting a role of BDNF in modulating 5-HT neurotransmission (Mossner et al., 2000). Additionally, activation of the 5-HT_{1A} receptor stimulates hippocampal neurogenesis (Martinowich and Lu, 2007; Santarelli et al., 2003b). BDNF may therefore be involved in the functional modulation of the serotonergic system, especially in the hippocampus, which might explain how its expression might be related to the development of depression and the response to antidepressant therapy. Additional studies investigating the interplay between BDNF and other neurotransmitter systems are warranted.

That depression presents with deficient hippocampal neurotrophic signalling, and that antidepressant therapy is associated with increased hippocampal neurogenesis and BDNF signalling is thus well-reported in the literature. Figure 9 depicts the inter-related effects of dysregulated HPA-axis, monoaminergic and immune-inflammatory function on the hippocampus. Investigating the effects of a novel antidepressant compound on the expression of BDNF and markers of neurogenesis is thus a helpful tool in understanding drug effects on behavioural parameters.

2.3.2.2.6 Neuroinflammation

Severe depressive episodes have been correlated to immune activation and increased levels of pro-inflammatory cytokines, while depression has been described as a neuroinflammatory disorder

(Anisman et al., 2002; Leonard, 2010). A high comorbidity of depression with inflammatory illnesses such as irritable bowel syndrome, arthritis, type-2 diabetes and autoimmune disorders also exemplifies this fact (Leonard, 2010). Additionally, psychosocial stress is a known risk factor for depression and is also associated with peripheral and central inflammatory processes (Leonard, 2010). Serotonergic deficits have been associated with HPA-axis hyperactivity and correlated to increased release of adrenaline and NA, which in turn promotes the release of pro-inflammatory cytokines (Anisman and Merali, 2003; Capuron et al., 2008). The increased expression of pro-inflammatory cytokines has been associated with various symptoms seen in depression, including cognitive disturbances, fatigue, somnolence and anxiety (Anisman and Merali, 2003) and to engender alterations in neuroendocrine function and central neurotransmission similar to that observed in depression, including altered brain DA, 5-HT and NA levels (Anisman et al., 2007). Furthermore, cytokines activate the HPA-axis, which, as described in section *2.3.2.2.4* is already hyperactive in depression (Anisman et al., 2002). Both glucocorticoids and pro-inflammatory cytokines promote the conversion of the 5-HT precursor, tryptophan, to various neurotoxins which contribute to apoptotic processes and neurodegeneration (Leonard, 2010), in particular products of the kynurenine pathway. Thus, depression is associated with immune activation and neuroinflammation associated with serotonergic and noradrenergic alterations to the stress response, and which ultimately contributes to neurodegenerative processes.

Figure 9 depicts how pro-inflammatory cytokines may act on monoaminergic, neurotrophic and glucocorticoid systems to alter limbic function.

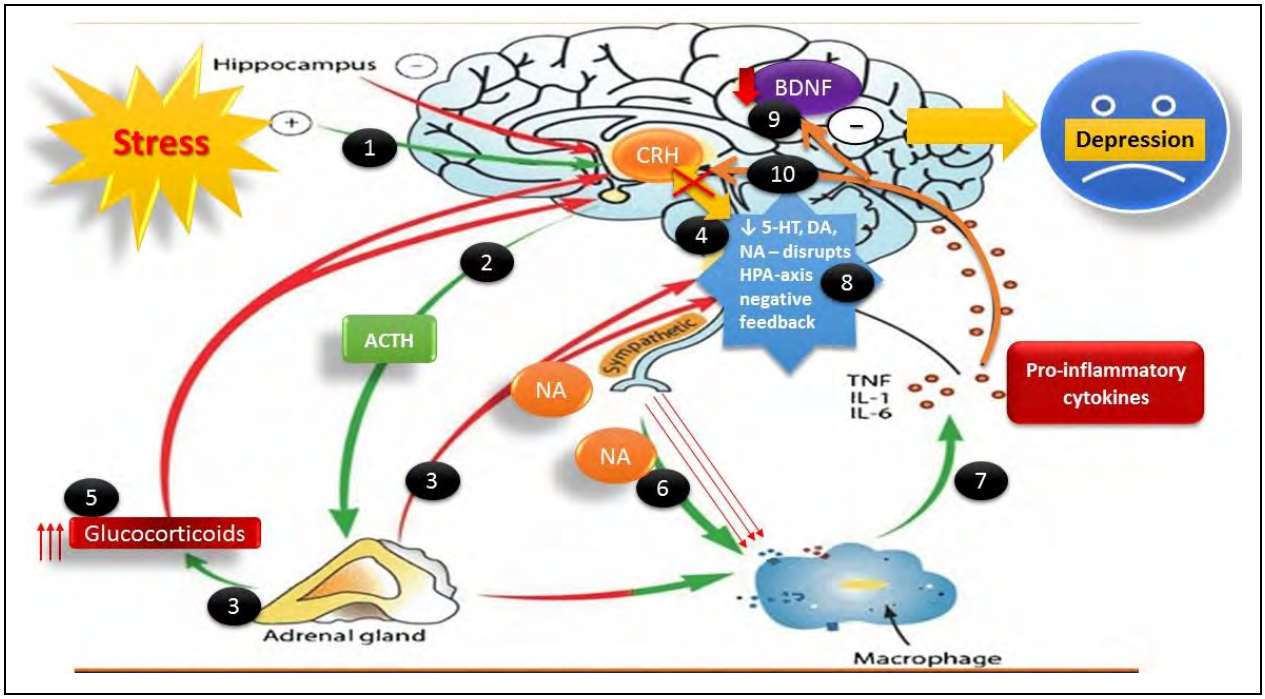


Figure 9. Effect of chronic and acute stress, the HPA-axis and inflammation on limbic function and neurodegenerative processes. **(1)** The hypothalamus releases CRH which **(2)** stimulates the pituitary to release ACTH. **(3)** ACTH stimulates the release of NA and glucocorticoids from the adrenal glands. **(4)** Failure of the HPA-axis to shut down the stress response via negative feedback, supposedly due to disruption of 5-HT, NA and DA signalling (as postulated in depression) may result in excessive glucocorticoid activity **(5)**. Excessive noradrenergic sympathetic stimulation **(6)** contributes to immune activation and the release of pro-inflammatory cytokines **(7)**. Cytokines exacerbate monoaminergic disruption **(8)** and down-regulates neurotrophic BDNF signalling **(9)** and may diminish corticosteroid receptor sensitivity, leading to further HPA-axis over-activity **(10)**. Adapted from Maletic et al., 2007; Raison et al., 2006.

2.3.2.2.7 Circadian rhythm hypothesis

The observation that depression is associated with disturbed sleeping patterns and altered HPA-axis activity has led to the hypothesis that depression is associated with a disturbed circadian rhythm or 'biological clock' (Quera Salva et al., 2011). This hypothesis has been supported by the introduction of agomelatine, an antidepressant with specific 'chronobiotic' effects. By acting as a 5-HT_{2C} antagonist and melatonin M_1/M_2 agonist, agomelatine re-entrains disordered circadian rhythms to evoke pronounced clinical antidepressant and anxiolytic effects (De Berardis et al., 2011; Stein et al., 2014) recently also demonstrated in our laboratory using a rodent model (Coutts, 2015; Regenass, 2016). Interestingly, agomelatine shows an earlier onset of action compared to other antidepressants and is said to be very effective against severe manifestations of depression with residual symptoms (De Berardis et al., 2011). Thus, antidepressants with intrinsic chronobiotic properties represent a novel therapeutic approach to depression.

3. Treatment options

3.1 Antipsychotics

Schizophrenia is a lifelong illness and requires continued treatment throughout the patient's lifetime. While positive symptoms are often responsive to treatment, atypical antipsychotics are more effective in addressing negative symptoms, while cognitive symptoms are often refractory (Bowie and Harvey, 2006; Miyamoto et al., 2012).

Since the introduction of the first generation of antidopaminergic antipsychotics, such as chlorpromazine and haloperidol, up to the development of the atypical antipsychotics, the mainstay of antipsychotic therapy still remains dopamine D_2 antagonism, although this view has been challenged over the years (Millan et al., 2015; Miyamoto et al., 2012). Antipsychotics have been proposed to work via DA receptor modulation, 5-HT receptor modulation, NMDA-receptor modulation, α-AR modulation and various actions on histaminergic and muscarinic neurotransmitter systems (Miyamoto et al., 2012; Svensson, 2003). Figure 10 provides a summary of the sites of action of various antipsychotic treatment modalities. Here only the main effects and actions of antipsychotics will be summarised for the benefit of the reader within the context of the antipsychotics and candidate antipsychotic compound employed in this study.

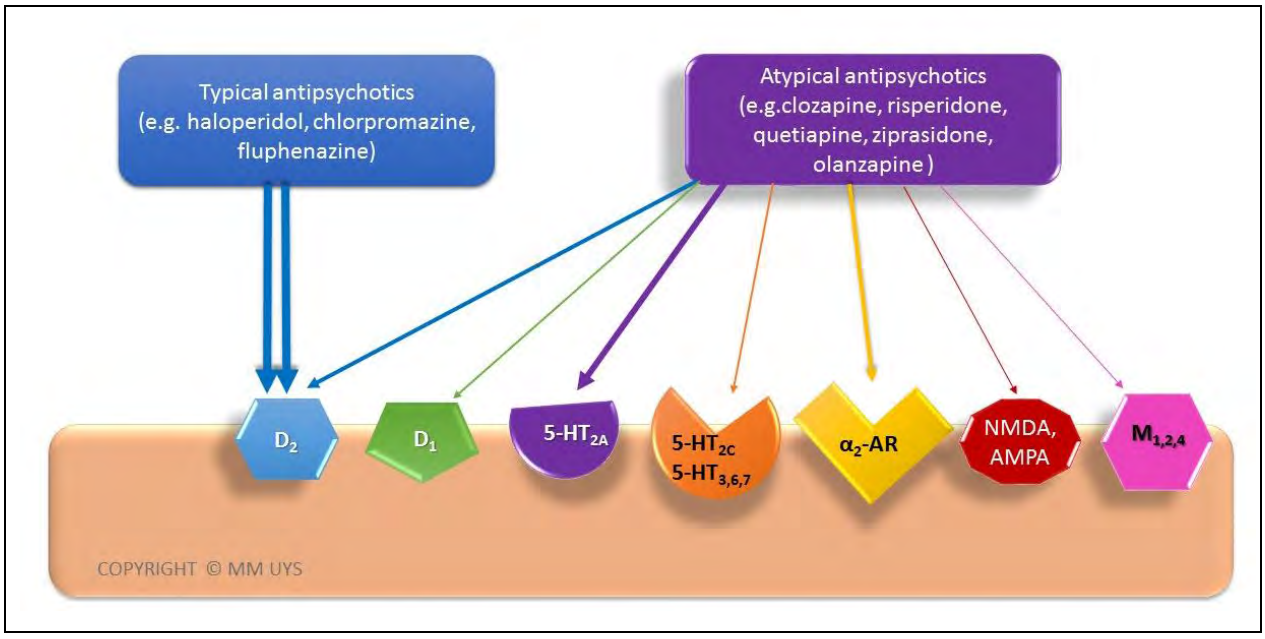


Figure 10. Sites of antipsychotic drug action. Thicker arrows portray more potent action at the receptor subtype.

Antipsychotics were initially divided into typical (first generation) antipsychotics and atypical (second generation) antipsychotics based on their ratio of affinities for either the D_2 receptor (typicals, including haloperidol, chlorpromazine) or the 5-HT_{2A} receptor (e.g. clozapine, olanzapine, quetiapine, ziprasidone) (Meltzer et al., 1989). More recently modulation of the α_2-AR has been proposed as a mechanism of antipsychotic atypicality (Svensson, 2003).

D_2-receptor antagonism is aimed at modulating mesolimbic hyperactivity and thus mainly addresses psychotic symptoms. All antipsychotics block the D_2 receptor, the typical compounds more so than the atypicals. This mechanism however does not address prefrontal hypodopaminergic function and therefore has poor outcomes on cognitive and negative symptoms (Abi-Dargham et al., 2002; Keshavan et al., 2016). Most typical antipsychotics require striatal D_2-occupancy of 65-70% in order to evoke their desired antipsychotic effects (Farde and Nordström, 1992; Farde et al., 1989). However, D_2-antagonism at this high occupancy is associated with D_2 binding throughout the brain, but especially in the nigrostriatal and tuberoinfundibular tracts (refer to Figure 4 in section *2.3.1.2.1*) resulting in severe extrapyramidal side-effects (EPS) and endocrine abnormalities (Millan et al., 2015).D_2-antagonist induced striatal toxicity that is linked to these side-effects (Dalgalarrondo and Gattaz, 1994; Mion et al., 1991; Tan et al., 2005) also appears to be linked to compromised neurotrophic activity after long-term treatment (Pillai et al., 2006; Tost et al., 2010). This could negatively affect both the progression of the disorder as well as negative and cognitive treatment outcomes.

Atypicality of an antipsychotic drug reflects a decreased EPS side-effect profile as well as various degrees of improved outcomes for negative and cognitive symptoms of schizophrenia, in addition to their efficacy against positive symptoms (Grunder et al., 2009). Atypical antipsychotics such as clozapine and quetiapine exhibit <60% striatal D_2 occupancy at clinically effective doses, accounting for lower EPS and indicating that antipsychotic efficacy can be attained at lower D_2 occupancies with drugs that exhibit additional serotonergic and adrenergic activity (Miyamoto et al., 2012). The atypicals (including clozapine, olanzapine, paliperidone, quetiapine, risperidone, iloperidone, ziprasidone) generally have a higher affinity for 5-HT_2 receptors and specifically for 5-HT_{2A} receptors compared to typical antipsychotics. 5-HT_{2A}-antagonism contributes to the 'normalisation' of DA release (O'Neill et al., 1999), including (1) increased dopaminergic transmission in the nigrostriatal pathways, thus reducing the risk of EPS, (2) increased dopaminergic transmission in the tuberoinfundibular pathway, reducing the risk of neuroendocrine side-effects and (3) by increasing DA release in the PFC, thus more effectively addressing negative and cognitive symptoms (Miyamoto et al., 2012). The addition of the 5-HT_{2A}-antagonist, ritanserin to typical antipsychotics demonstrates increased efficacy against negative symptoms, although monotherapy with selective 5-HT_{2A} antagonists present with insufficient antipsychotic efficacy (Miyamoto et al., 2012). The atypical antipsychotics also have variably affinites at

the 5-HT_{2C} (e.g. ziprasidone, clozapine, olanzapine) the 5-HT_6 (e.g. clozapine, olanzapine) and 5-HT_7 (e.g. paliperidone, risperidone, ziprasidone, aripiprazole) receptors, while directly or indirectly activating the presynaptic 5-HT_{1A} autoreceptor (e.g. ziprasidone, quetiapine, aripiprazole). Agonism of 5-HT_{1A} receptors inhibits the firing rate of 5-HT neurons and is said to enhance certain cognitive domains of antipsychotics while potentiating the activity of D_2-antagonists (Miyamoto et al., 2012). The 5-HT_{2C} receptor also plays a critical role in tonically regulating DA release and mediating interaction between serotonergic and dopaminergic systems (Sumiyoshi et al., 2007).

Additionally, indirect actions of atypical antipsychotics to correct glutamate NMDA-receptor hypofunction is evident from animal studies employing NDMA-antagonists (Miyamoto et al., 2005). *Acute* clozapine and olanzapine reverse phenylcyclidine (PCP)-induced deficits on sensorimotor gating, social behaviour and neurotoxicity, while *chronic* haloperidol is necessary to reverse PCP-induced sensorimotor gating deficits (Millan, 2005). The therapeutic action of antipsychotics may therefore involve the amelioration of glutamatergic hypofunction, although the mechanism whereby this occurs is poorly understood (Miyamoto et al., 2012).

Apart from action at 5-HT receptors as set out above, atypicality has also been proposed to strongly revolve around α-AR modulation, with α_1 and α_2-AR antagonism suggested to contribute to stabilisation of dysregulated mesolimbic-mesocortical dopaminergic activity (Svensson, 2003). To this end, many atypical antipsychotics present with much higher α_2/D_2 binding ratios compared to typical antipsychotics (Kalkman and Loetscher, 2003; Shahid et al., 2009), while a pharmacological profile specifically presenting with a higher α_{2C}/D_2 receptor selectivity ratio has been suggested to mediate the improved efficacy of drugs like clozapine that exhibit lower D_2-receptor occupancy (Kalkman and Loetscher, 2003; Shahid et al., 2009). Figure 11 depicts the α_{2A}/D_2 and α_{2C}/D_2 ratios of various atypical antipsychotics and of haloperidol, as reported by Shahid et al., 2009. Thus reduced D_2-receptor occupancy might be possible in antipsychotic therapy partly because of the beneficial effects of α_2-AR antagonism on dopaminergic function, allowing for improved efficacy with less motor side-effect profiles. Indeed, studies employing non-selective α_2-AR antagonists (with idazoxan) as augmentation to atypical antipsychotic treatment in rodent models of schizophrenia has shown evidence of enhanced antipsychotic-like effects (Hertel, P. et al., 1999a; Marcus et al., 2005; Marcus et al., 2010b), while also enhancing cortical glutamatergic and dopaminergic neurotransmission, with subsequent improvement in cognitive parameters (Marcus et al., 2005). These effects were comparable to that of the atypical antipsychotic clozapine, which presents with a very high α_2/D_2 binding affinity ratio (Kalkman and Loetscher, 2003).

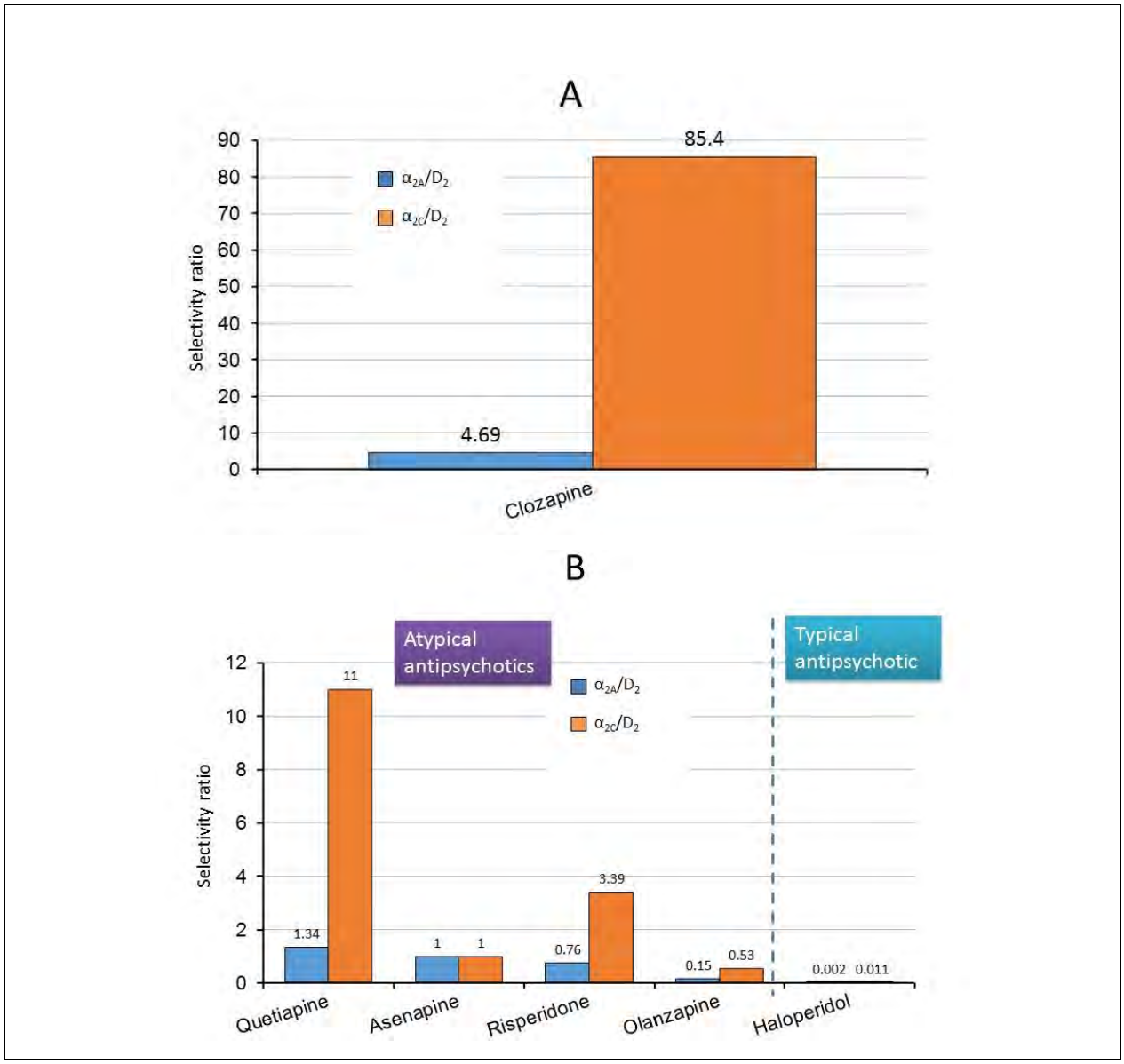


Figure 11. Human α_2-AR subtype/ D_2 selectivity ratios of various antipsychotics. Selectivity ratios were determined by dividing the D_2 K_i value by the applicable α_2 receptor K_i value. Clozapine displays a much higher α_{2C}/D_2 ratio compared to other atypicals, while the typical antispychotic haloperidol clearly has negligible α_2-activity. These graphs have been adapted from Shahid et al., 2009 and Figure 5A in **Manuscript D**.

Clozapine is reported to be the most effective antipsychotic in refractory cases of schizophrenia (Swartz et al., 2008) through more effectively managing positive and negative symptoms and certain cognitive deficits. Its superiority has been demonstrated versus typical (Kane et al 1988, Rozenheck et al 1997) and atypical antipsychotics (Lewis et al., 2006a; Lewis et al., 2006b; Meltzer et al., 2003). Clozapine has potent activity at multiple receptors, while its high 5-HT_2/D_2 ratio has been proposed to allow frontal cortical dopamine D_1 receptor disinhibition and increased frontal cortical NMDA and DA neurotransmission (Englisch and Zink, 2012; Galletly et al., 2005; Lahti et al., 2003; Miyamoto et al., 2012). Its efficacy has been related to lower D_2 antagonism, neuroprotective effects (van Haren et al.,

2012) and beneficial effects on central NA transmission (Bürki et al. 1974, Elman et al. 1999, Lee et al. 1999, McMillen, Shore 1978, Kalkman en Loetsher), proposedly by antagonism of the α_2-adrenoceptor (Svensson, 2003). Specifically, clozapine has the most prominent activity of all antipsychotics at the α_{2C}-AR and by far the highest α_{2C}/D_2 ratio (Kalkman and Loetscher, 2003; Shahid et al., 2009) (refer to Figure 11). Unfortunately, the of risk of leukopenia and agranulocytosis and the accompanying necessity for regular blood cell monitoring reduces its use as first line agent in clinical practice (Cirulli, 2005; Hasegawa et al., 1994; Warnez and Alessi-Severini, 2014).

α_{2C}-AR antagonism may thus be a novel treatment strategy in schizophrenia (Scheinin et al., 2001). Indeed, α_{2C}-KO mice indicate a beneficial effect of α_{2C}-AR deactivation on monoamine levels and on cognitive and negative domains associated with schizophrenia (Sallinen et al., 1999; Sallinen et al., 1998a, b; Sallinen et al., 1997; Scheibner et al., 2001b). Furthermore, treatment with novel highly selective α_{2C}-AR antagonists, JP-1302, ORM-10921 and ORM-12741 has shown antipsychotic-like and pro-cognitive effects in NMDA-antagonist rodent models of schizophrenia (Sallinen et al., 2007; Sallinen et al., 2013a; Sallinen et al., 2013b), which has been related to increased PFC DA release (Sallinen et al., 2013a).

3.2 Antidepressants

While various novel classes of antidepressants have been introduced over the last 60 years, 50-60% of depressed patients remain only partially responsive to first line antidepressant therapy (Fava, 2003), necessitating second-line or adjunctive treatment. About 30% of patients do not respond to therapy, of which 10-30% develop treatment-resistant symptoms (Al-Harbi, 2012) that often present with suicidal episodes (Ward and Irazoqui, 2010).

The vast majority of antidepressants act by increasing synaptic levels of NA, 5-HT or DA (Kennedy et al., 2011). These include the TCAs (e.g. amitriptyline, IMI) that reduce 5-HT and NA reuptake, the SSRIs (e.g. fluvoxamine, fluoxetine), the NA reuptake inhibitor, reboxetine, the SNRIs (e.g. venlafaxine, duloxetine, milnacipran), the DA reuptake inhibitor and DA and NA releaser, bupropion, the monoamine oxidase inhibitors (MAO-I) (e.g. tranylcypromine, moclobemide) and the α_2-AR antagonists mianserin and mirtazapine (Kennedy et al., 2011). The circadian modulator, agomelatine, is the first non-monoaminergic antidepressant introduced onto the market that offers a completely new approach in the treatment of depression by mainly acting as a melatonin receptor agonist (as discussed in section *2.3.2.2.7*) (Hickie and Rogers, 2011). Based on evidence of circadian rhythm disruption in depression, agomelatine, acts as a melatonin-1 and melatonin-2 agonist and 5-HT_{2C} antagonist and is the first melatonergic antidepressant to be clinically applied. Its chronobiotic actions lead to increased 5-HT, DA and NA signalling (Chenu et al., 2013; Millan et al., 2003). It displays similar efficacy to standard antidepressant treatments (Hickie and Rogers, 2011; Taylor et al., 2014).

Treatment outcomes for depression are related to a fine balance of efficacy and tolerability. Evidence from meta-analyses report similar outcomes for SSRIs, TCAs and MAOIs, although inpatient studies have reported superior outcomes with TCAs vs. SSRIs, while the poor tolerability of TCAs is however associated with higher discontinuation rates (Anderson, 2000; Geddes et al., 2000). Thus the TCAs and MAOIs are generally reserved for second-line therapy and for treatment-resistance (Kennedy et al., 2011). Some studies demonstrate higher remission rates with the SNRIs compared to SSRIs and TCAs, although others demonstrate superiority of SSRIs over SNRIs. A comprehensive network meta-analysis of 12 new-generation antidepressants have concluded that mirtazapine, venlafaxine, and the SSRIs escitalopram and sertraline generally have higher efficacy profiles, while bupropion demonstrates a favourable balance between efficacy and tolerability (Al-Harbi, 2012; Kennedy et al., 2011). Venlafaxine has been found to be slightly more efficacious in severely ill patients vs. SSRIs (Bauer et al., 2013; Nemeroff et al., 2008), and is used as one of the reference options for treatment resistance in the STAR*D study (Rush et al., 2006; Warden et al., 2007).

Bupropion is one of the few antidepressants that mainly act by increasing synaptic DA (Guiard et al., 2009). However, the contribution of DA to antidepressant therapy is being investigated further in the form of triple reuptake inhibitors that inhibit the uptake of NA, 5-HT and DA, (SNDRIs) (El Mansari et al., 2010; Guiard et al., 2009). The benefits of this novel group of antidepressants vs. conventional treatment has been suggested to lie in additional neurotrophic effects and a hastened antidepressant response (Guiard et al., 2009). The first drug in this class to be investigated in clinical trials (GSK372475) unfortunately did not demonstrate enhanced efficacy over paroxetine or venlafaxine and was not well tolerated (Learned et al., 2012). Another SNDRI, ansofaxine hydrochloride (Zhang et al., 2014) has shown enhanced synaptic monoamine levels vs. desvenlafaxine and has been approved for Phase II and III clinical trials (Zhang et al., 2014).

Vilazodone is a novel antidepressant that, apart from inhibiting 5-HT reuptake, is a partial agonist at the 5-HT_{1A} receptor (Pierz and Thase, 2014). This action is thought to promote rapid autoreceptor desensitisation leading to a faster onset of antidepressant action (Pierz and Thase, 2014). Vortioxetine is another novel antidepressant that increases 5-HT neurotransmission by binding with high affinity to the 5-HT transporter (D'Agostino et al., 2015). Additionally vortioxetine increases 5-HT neurotransmission by acting as an agonist at the 5-HT_{1A} autoreceptor and as a partial agonist at the 5-HT_{1B} autoreceptor, while antagonism at the 5-HT_7 receptor is said to potentiate its inhibitory effects on the 5-HT transporter (D'Agostino et al., 2015). Vortioxetine has been shown to display similar efficacy to other antidepressants with a marginally improved tolerability profile as well as having purported benefits on cognitive parameters (D'Agostino et al., 2015).

The clinical benefits of augmenting standard antidepressant therapy with antiglutamatergic drugs such as riluzole and ketamine have also been described in the literature, but currently their application is restricted to treatment resistant depression (Aan Het Rot et al., 2012; Murrough et al., 2013; Sanacora et al., 2007) (Zarate et al., 2003). As described in section *2.3.2.2.3*, the glutamate NMDA receptor antagonist, ketamine, demonstrates efficacy in treatment resistant depression and has a rapid onset of action (Zarate and Niciu, 2015). However, ketamine's penchant to cause hallucinatory events (Hasselmann, 2014) and that it needs to be administered under the care of an anesthesiologist, greatly limits its application in the clinic (Al-Harbi, 2012). Nevertheless, recent clinical trials have reported the safety and efficacy of intranasal ketamine (Lapidus et al., 2014). The role of oxidative stress in depression has led to the testing of the antioxidant and glutathione precursor, n-acetyl cysteine, in both preclinical (Ferreira et al., 2008) and clinical (Berk et al., 2014) studies, while the tetracycline antibiotic, minocycline, has similarly attracted interest based on reported anti-inflammatory and pro-oxidant properties, as well as effects on glutamatergic and neurotrophic mechanisms (Dean et al., 2014).

On the other hand, the combination of antidepressant therapies possessing different mechanisms of action have been reported to markedly increase response and remission rates vs. the respective monotherapy approaches, while also preventing relapse (Kennedy et al., 2011). The most common combinations are TCA+SSRI's and venlafaxine+SSRI/TCA, while venlafaxine+mirtazapine is the combination most frequently used in clinical practice (Al-Harbi, 2012). Of note, studies investigating augmentation of SSRI's, venlafaxine and buproprion with the α_2-AR antagonist mirtazapine, report enhanced clinical efficacy, earlier antidepressant response and markedly higher remission rates compared to monotherapy (Blier et al., 2009; Blier et al., 2010). However, draw-backs to such combinations include a higher incidence of side-effects (Malhi et al., 2008).

Although most classes of antidepressants have been associated with a modest degree of cognitive improvement (Lam et al., 2014), cognitive dysfunction still remains a refractory and often persistent residual symptom of depression (Conradi et al., 2011). The SNRIs, duloxetine and venlafaxine, have been suggested to exert more pronounced effects on cognitive dysfunction with the strongest evidence for improved verbal and visual memory provided for vortioxetine. Evidence has also been provided for possible beneficial effects of bupropion on visual memory and processing speed (Lam et al., 2014). However, a paucity of randomised placebo-controlled studies that objectively measure cognitive parameters as a primary endpoint limits our knowledge of the cognitive effects of certain antidepressant therapies (Lam et al., 2014). Studies with candidate antidepressant compounds are thus being designed to include cognitive function as a key outcome (Conradi et al., 2011; Lam et al., 2014), considering that this is an important unmet need in antidepressant efficacy.

Figure 12 provides a succinct overview of the mechanism of action of most antidepressants on noradrenergic, dopaminergic and serotonergic mechanisms.

Furthermore, an adequate trial of antidepressant therapy should span a minimum of 6 weeks, while 10-12 weeks of treatment may be required to elicit a full therapeutic response (Al-Harbi, 2012). The development of antidepressant therapies that have more rapid onset of action are thus an important research goal. Interestingly, while ketamine has a very rapid onset of action, α_2-AR augmentation of SSRIs or SNRIs reportedly hastens antidepressant response (Blier et al., 2009; Blier et al., 2010; Sanacora et al., 2004a). A role of α_{2C}-AR selective antagonism has been suggested by the observation of the depressive phenotype of α_{2C}-OE mice, in contrast to α_{2C}-KO mice who display antidepressive-like and pro-cognitive behaviour, as well as attenuated plasma corticosterone responses to stress (Björklund et al., 1999b; Sallinen et al., 1999). Recently, treatment with the novel highly selective α_{2C}-AR antagonists JP-1302, ORM-10921 and ORM-12741 have demonstrated antidepressant-like and pro-cognitive effects in animals (Sallinen et al., 2007; Sallinen et al., 2013a; Sallinen et al., 2013b). Thus, this study investigated the antidepressant-like and pro-cognitive effects of ORM-10921 in a genetic animal model of depression, while also assessing the effects of chornic treatment on BDNF and hippocampal monoamine levels.

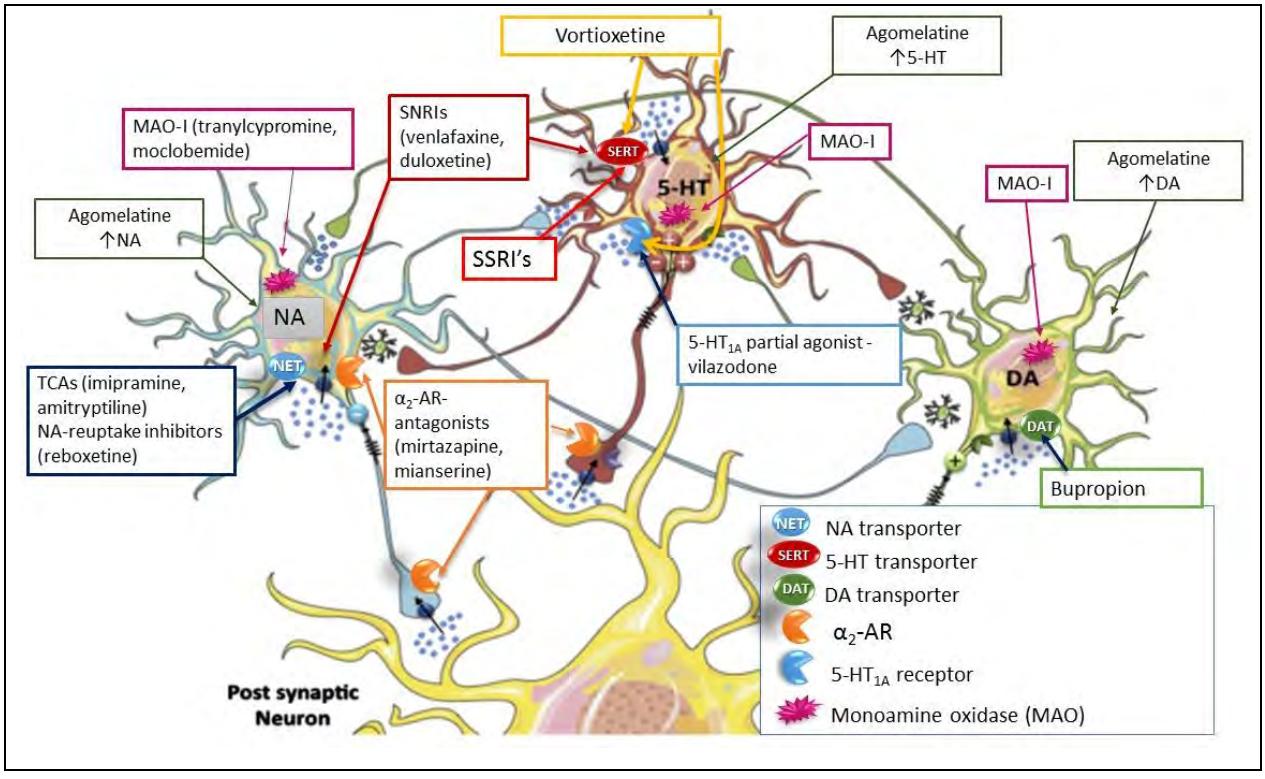


Figure 12. Sites of action of various antidepressants that target or affect monoaminergic neurotransmission. Adapted from Hamon and Blier, 2013.

4. Animal models of schizophrenia and depression

Animal models are an important tool with which the underlying substrates and neurobiological mechanisms of neuropsychiatric illness can be studied (van der Staay et al., 2009). Joseph van der Staay defines a valid animal model as being an organism that has "biological and/or clinical relevance in the behavioural neurosciences" and can be used to study the relationship between the brain and behaviour under controlled conditions, the goal being to 1) accumulate evidence that provides insight into these brain-behaviour dynamics in order to 2) enable predictions about the etiology and thus the 3) prevention and/or early detection and 4) treatment of the disorder in humans (van der Staay, 2006). Firstly, it is important to distinguish between an animal model of a neuropsychiatric illness, and a behavioural test that would be used to assess whether the animal model produces altered behaviour and whether appropriate treatment can reverse this altered behaviour (Cryan and Slattery, 2007). An animal model of a neuropsychiatric illness would thus be expected to present with various behavioural alterations ("symptoms") and neurochemical alterations ("signs") akin to the specific disorder, while a behavioural test would be able to model these "symptoms" and demonstrate the effects of pharmacological treatment thereon (Cryan and Slattery, 2007; O'Leary and Cryan, 2013). Examples of animal models of neuropsychiatric illness are the Flinders Sensitive Line (FSL) genetic animal model of depression and the Social Isolation Rearing (SIR) model of schizophrenia used in this study, while tests that predict psychotic-like and depressive-like behaviour would include the prepulse inhibition test (PPI) or the forced swim test (FST) respectively, also applied in this study. Both animal models and tests that model aspects of the human disorder have to be validated in order to be able to translate observations from these models and tests to the clinic.

4.1 Validation of Animal Models: Relevance for schizophrenia and depression

Although no animal model can possibly fully replicate all aspects of the human disorder, various animal models simulate different elements of the human disorder, making it possible to investigate and identify new drug targets and prospective treatments as well as delineate their mechanisms of action (van der Staay et al., 2009). Ultimately, a robust animal model or behavioural test should include qualities that enable the translation of new insights from "bench to bedside", i.e. from preclinical to clinical research (Belzung and Lemoine, 2011). Thus, for animal models or behavioural tests to possess translational value, they are validated according to the following criteria:

- *Face validity* describes the accuracy with which the animal model reproduces and resembles the *symptoms* of the human condition (van der Staay et al., 2009), which would include aspects

like behaviour (such as motivation-driven, social and stereotypical behaviours etc.) and cognition (Belzung and Lemoine, 2011; Willner and Mitchell, 2006).

- *Construct validity* assesses whether the model thoroughly describes similarity to the *theoretical underpinnings* and *pathophysiological features* of the human disorder (Belzung and Lemoine, 2011). Theoretic construct validity should align pathophysiological underpinnings of the model with the disordered behaviour that it exhibits (Willner and Mitchell, 2003). While construct validity can be assessed to a large and meaningful extent in both models of depression and schizophrenia, this criterion is often confounded by inconsistent literature and incomplete understanding of the biochemical and neurochemical abnormalities underpinning the human disorder (Marcotte et al., 2001; Willner and Mitchell, 2002).
- *Etiological validity* assesses causality of the disordered behavioural and neurochemical features of the model. Thus etiological validity would pertain to *how the symptoms and features of the model originated and progress*, and how this can be related to the origins and progression of the human disorder (Belzung and Lemoine, 2011). For example, both depression and schizophrenia have been causally related to stressful early life events (Fava and Kendler, 2000; Kendler et al., 2001; Sigurdsson, 2015) and thus animal models that include early-life stressors or behavioural tests that include stressors may possess good etiological validity. Depression is also associated with monoaminergic depletion (Kharade et al., 2010), and thus a model that induces monoaminergic depletion would be considered to present with good etiological validity (McArthur and Borsini, 2006).
- *Predictive validity* implies that pharmacological agents that are known to alter or alleviate the symptoms and/or pathophysiological state in humans, should have similar *treatment response* in the animal model, while a lack of pharmacological response is expected from agents that are *not* clinically effective (Willner and Mitchell, 2002). Although various influential researchers differ on the hierarchical order of the concepts of validity, predictive validity is considered by some as first in the hierarchy (van der Staay, 2006), provided that construct validity and face validity has been established to a desirable extent. Thus, if several known antipsychotics or antidepressants are able to attenuate or reverse the behaviour and/or pathophysiological features exhibited by the animal model, this would confer good predictive validity to an animal model of schizophrenia or depression, respectively (Marcotte et al., 2001; Willner and Mitchell, 2002). If an animal model shows good predictive validity, novel compounds can be investigated in these models to investigate potential antidepressant-like or antipsychotic-like properties.

Etiological validity is often considered an aspect of construct validity (Willner and Mitchell, 2002). Therefore face, construct and predictive validity are considered by many to be the main criteria determining whether an animal model presents with translational value (Belzung and Lemoine, 2011; Marcotte et al., 2001).

The following sections (*4.2-4.4*) will give background on various tests that assess cognitive, psychotic-like and depressive-like behaviours as well as various animal models of neuropsychiatric illness. A more detailed overview will be provided under the relevant sections where the animal models that have been applied in this study are discussed, namely the SIR model of schizophrenia and the FSL model of depression and the behavioural tests applied in these models. The face, construct and predictive validity of these models will be dissected and a brief overview of alternative models of schizophrenia and depression is given in order for the reader to contextualise the SIR and FSL models within the scientific arena. Some of these models and behavioural tests are referenced in this thesis, and the reader is therefore shortly introduced to them. Figure 13 provides a summative overview of animal models of schizophrenia and depression and some relevant behavioural tests employed to assess psychotic-like and depressive like-behaviour in these models.

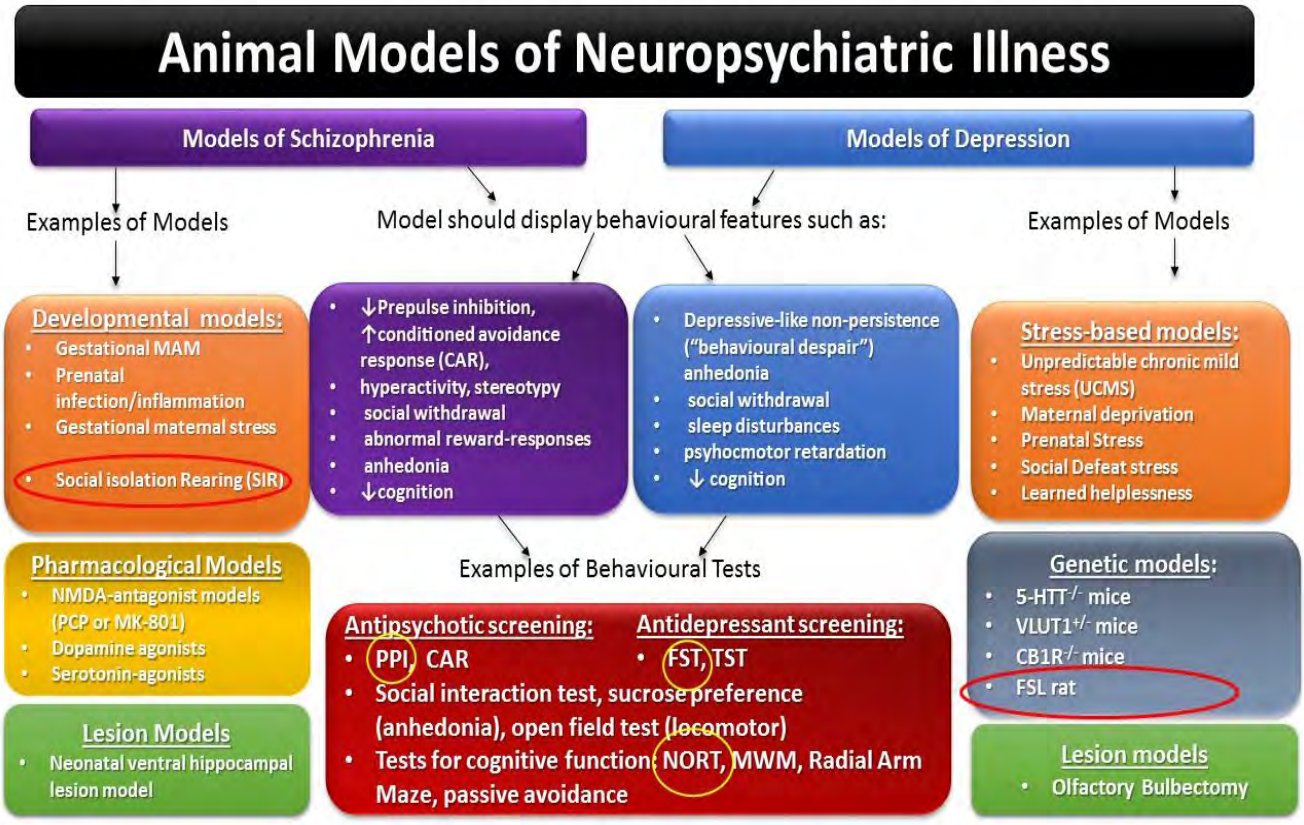


Figure 13. Schematic summary of various animal models of schizophrenia and depression and the behavioural tests used to study psychotic-like and depressive-like behaviour in these animal models, as described in sections 4.2-4.4. The animal models used in this study have been encircled in red, while the behavioural tests applied in this study have been encircled in yellow.

4.2 Behavioural tests to model symptoms of depression, schizophrenia and cognitive function

4.2.1 Behavioural models predicting antipsychotic-like activity

Various behavioural tests that model illness-specific aspects of schizophrenia have been developed. The prepulse inhibition (PPI) of startle and conditioned avoidance response (CAR) tests are most commonly used as screening tests for the antipsychotic-potential of novel compounds, although tests of stereotypy, hyperactivity and latent inhibition can also provide valuable additional information. In this study, the PPI has been employed as a putative and reliable screening test for antipsychotic activity in SIR rats (Geyer et al., 1993), and which has been used on numerous occasions in work from this laboratory (Möller et al., 2011; Möller et al., 2013a; Strauss et al., 2014). I will therefore describe this test in more detail, while briefly providing context on the other tests that measure psychotic-like behaviour.

4.2.1.1 Prepulse inhibition test (PPI)

Schizophrenic patients display deficient processing of thoughts and sensory information reflecting an inability to unconsciously filter or "gate" sensory stimuli and irrelevant thoughts (Geyer and Moghaddam, 2002). This feature is also known as sensorimotor gating and has been suggested to underlie the fragmentation of reality evident in schizophrenia (Braff and Geyer, 1990). A behavioural measure that models sensorimotor gating in both humans and animals revolves around the concept of the prepulse inhibition (PPI) of the startle response (Braff and Geyer, 1990; Swerdlow et al., 2000). While a startling stimulus usually elicits an involuntary startle response from both humans and animals, the pre-presentation of a lower amplitude of the stimulus results in an attenuated startle response to the subsequent presentation of the startling stimulus. The PPI of the acoustic startle response thus refers to the ability of the animal to attenuate its startle response to an auditory pulse when presented with a prepulse of smaller amplitude. A smaller startle response to the prepulse will translate into a higher % PPI (Braff and Geyer, 1990; Geyer and Braff, 1987). Figure 14 gives a graphical representation of acoustic PPI. PPI in rodents is usually measured in a startle chamber equipped with speakers to transmit the auditory stimuli, while the rodent's movement is limited by an animal enclosure. The startle response is measured by a piezo-electric (or similar) stabilimeter which is very sensitive to movement (Braff and Geyer, 1990; Geyer et al., 2001; Swerdlow et al., 2000). In humans, a typical example of the PPI test employs the somatosensory eye blink reflex in response to acoustic, tactile (e.g. air puffs) or light stimuli (Braff and Geyer, 1990; Flaten, 2002; Geyer et al., 1990). Figure 15 on page 67 depicts examples of apparatus used to assess PPI in rodents and in humans.

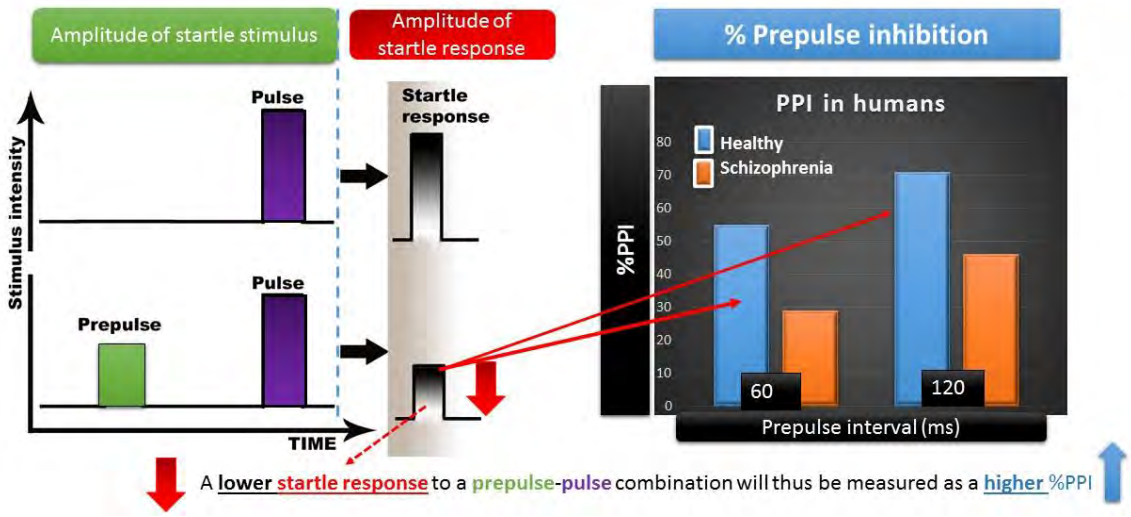


Figure 14. Schematic representation of the prepulse inhibition of the startle response. The presentation of an acoustic startle (PULSE) will elicit an individual startle response. If subsequently a PREPULSE of lower amplitude than the PULSE is presented shortly before the PULSE, the startle response will display a decrease in amplitude. This decreased startle amplitude (to the PREPULSE) is divided by the amplitude of the startle response to the PULSE in order to give an index of the prepulse inhibition (PPI) as a percentage (%). A higher PPI is therefore correlated to a lower startle amplitude to the presentation of a prepulse. Schizophrenic patients have lower % PPI than healthy patients.

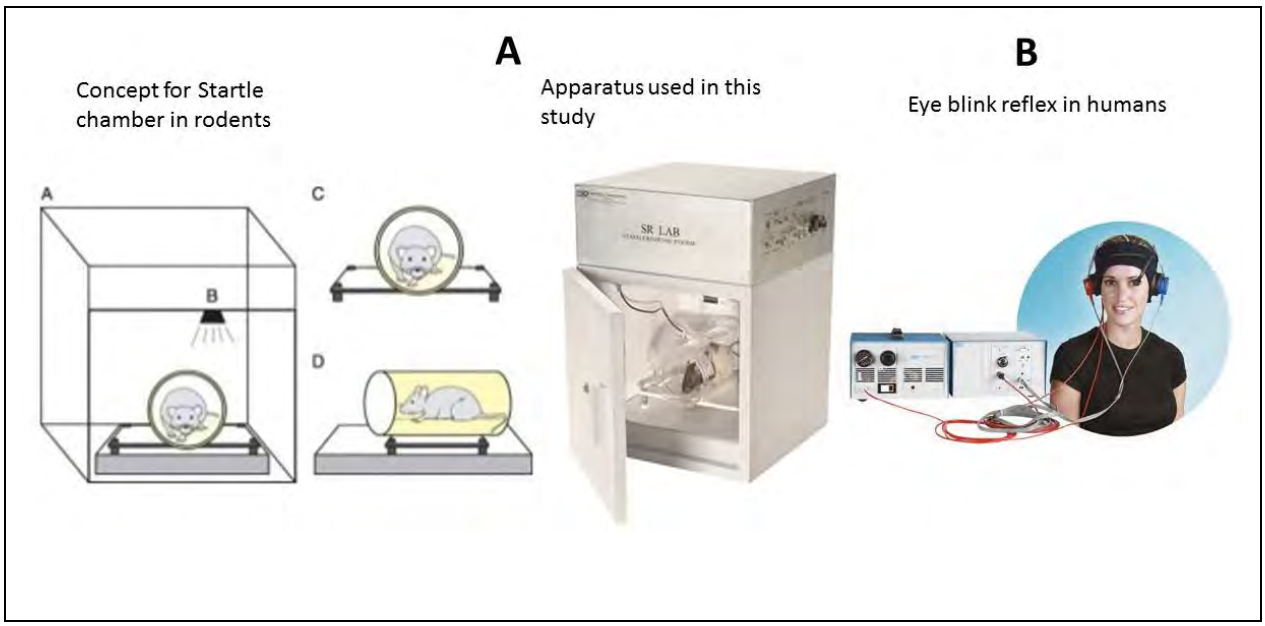


Figure 15. Apparatus used to assess PPI in **A**) rodents and **B**) humans. In humans the eye-blink reflex of the oculomotor muscles is measured by using electromyographic recording. Images were obtained from the website of San Diego Instruments, which manufactures startle response apparatus (www.sandiegoinstruments.com) and from www.slideshare.net

A PPI deficit can be induced in humans and animals by challenges that simulate a psychotic event, such as exposure to various psychotomimetic drugs, including dopaminergic and antiglutamatergic drugs. Animal models of schizophrenia, such as SIR (Bakshi et al., 1994; Geyer et al., 1993; Varty et al., 1995) and various transgenic models including mice with altered dopamine, serotonin and glutamate receptor

expression (Geyer et al., 2002), present with deficits in PPI. Importantly, antipsychotic drugs normalize disrupted PPI in animals and humans (Csomor et al., 2014; Depoortere et al., 1997; During et al., 2014; Swerdlow et al., 2006; Vollenweider et al., 2006), while atypical antipsychotics, including clozapine, quetiapine, asenapine, olanzapine and risperidone seem to be more effective, the typical D_2 antagonists, such as haloperidol, are also effective, but not in all models, e.g. the MK-801 NMDA-antagonist model (Feifel and Priebe, 1999; Jones et al., 2011; Möller et al., 2011; Möller et al., 2013a; Möller et al., 2013b; Swerdlow and Geyer, 1993). The neural substrates underlying PPI are complex, but includes excessive dopaminergic activity, and altered serotoninergic and glutamatergic signalling in the mesolimbic-mesocortical pathway and the amygdala (Swerdlow et al., 2000), pathways that are widely accepted to underlie psychotic behaviour (Brand et al., 2015). These qualities provide good construct validity for applying this behavioural test in schizophrenia research.

SIR robustly induces PPI deficits in rodents, which can be reversed by a range of typical and atypical antipsychotics (Weiss and Feldon, 2001), although atypical antipsychotics seem to present with greater effects on PPI in SIR rats, a finding mirroring superior atypical antipsychotic efficacy in certain clinical studies (Wynn et al., 2007). The PPI as applied in SIR is therefore a very robust screening agent for novel antipsychotic compounds, presenting with good face, construct and predictive validity (Weiss and Feldon, 2001).

4.2.1.2 Latent inhibition

Latent inhibition (LI) refers to the ability of the brain to ignore irrelevant stimuli (Gray and Snowden, 2005) and is also related to the gating theories of schizophrenia (Geyer and Moghaddam, 2002). Thus, repeated exposure to a non-noxious stimulus reduces the likeliness of a response to this stimulus. Although deficient LI has been reported in schizophrenics, it seems to be associated with acute episodes, and this test therefore has not attracted much interest (Geyer and Moghaddam, 2002).

4.2.1.3 Conditioned Avoidance Response (CAR)

The conditioned avoidance response (CAR) test is one of the earliest tests predictive of antipsychotic activity (Gobira et al., 2013). Animals are placed in a two-compartment shuttle box and trained to avoid an aversive stimulus (usually an electric foot shock) by associating it with a neutral conditioned stimulus, in the form of an auditory tone or a light. The behavioural conditioned avoidance response would usually include moving to the other side of the test arena upon presentation of the neutral stimulus, and the latency to display this behaviour is taken as an indication of a CAR. Animal models of schizophrenia usually have a decreased latency in the CAR test, while antipsychotics usually decrease this escape response to the neutral stimulus, although they still respond to the foot shock by escaping to the other side of the shuttle box (Smith et al., 2004). This model has very good and reliable predictive validity for a variety of antipsychotic drugs with different modes of action (Wadenberg, 2010).

4.2.1.4 Stereotypies

Stereotypy is a distinct feature of schizophrenia, which manifests in humans as repetitive functionless motor behaviour (Morrens et al., 2006) and in animals as persistent paw licking, smelling and biting of the cage bars (Gobira et al., 2013). Stereotypies are thought to be a product of excessive striatal D_2-stimulation, and typically worsens as the condition progresses (Morrens et al., 2006). Thus this test is also useful in assessing antipsychotic-like activity, although the applicability is mostly limited to drugs that potently alter striatal D_2-receptor stimulation (Gobira et al., 2013), hence atypical antipsychotics like clozapine that possess less D_2 antagonist activity perform poorly in this test (Tschanz and Rebec, 1988). With the increased search for agents with greater atypical vs. typical properties (see section *3.1* for discussion), such a test would be counter-intuitive.

4.2.1.5 Hyperlocomotion/ Hyperactivity

Hyperlocomotion is said to mimic positive symptoms of schizophrenia and results from excessive mesolimbic and striatal dopaminergic activity (Iversen, 1987). Hyperactivity in animals are said to be a behavioural measurement of the disorganized behaviour and agitation akin to psychosis (Forrest et al., 2014). This behaviour can be attenuated by various antipsychotics that influence elevated striatal dopamine levels by indirect or direct measures, so that assessing hyperlocomotion is a useful supplementary test in antipsychotic screening (Forrest et al., 2014; Gobira et al., 2013) Again, one needs to be mindful of the role of the striatum in this test which, as noted above, may disadvantage agents that don't target striatal function, such as the atypical antipsychotics.

4.2.2 Models predicting antidepressant-like activity

Various behavioural tests that model illness-specific aspects of depression that can be reversed by antidepressants have been developed. The FST (and its counterpart in mice, the tail suspension test (TST) and learned helplessness (LH) are commonly used as screening tests for the antidepressant-potential of novel compounds. The aforementioned tests assess stress-induced failure to persist in escape orientated behaviour, although tests of anhedonia (eg. sucrose preference test), and psychomotor retardation (bar-pressing for food rewards), to name but a few, can also be used as supplementary tests to assess antidepressant-like effects of compounds in animal models of depression. In this study, the FST was employed as a putative and reliable screening test for antidepressant activity in FSL rats (Overstreet et al., 2005). I will thus discuss the FST in more detail as well as give an overview of other tests that measure aspects of depressive-like states.

4.2.2.1 Forced Swim Test

Considering that stressful events often predispose to depression, and that depression is associated with an altered stress response, acute inescapable stressors with unpredictable duration can be used to assess depressive-like behaviours in rodents (O'Leary and Cryan, 2013). A presumed state of depression is induced by these uncontrollable stressors as evidenced by reduced efforts to escape the stressor

following a brief re-exposure (McArthur and Borsini, 2006). Porsolt's forced swim test is a classic example of such a test (Porsolt et al., 1978), although the terminology used to describe the translational aspect modelled in this test has led to quite a strong division among scientists, with some describing it as modelling "resignation" (Belzung and Lemoine, 2011), others as decreased motivation to persist (Willner and Mitchell, 2006), and still others as "behavioural despair" (Lucki, 2010; Porsolt et al., 1978). This test is however purported to model aspects of depression that are clinically described as the psychological feeling of "entrapment" and replacement of active coping strategies with passivity or an unwillingness to actively attempt to escape a perceived inescapable situation (Cryan and Mombereau, 2004; Holmes, 2003; Petit-Demouliere et al., 2005). A lack in motivational drive has been related to cognitive and social dysfunction in depression (Huang et al., 2015; Radke et al., 2014)

Rodents are placed in a cylinder of water from which they cannot escape. The animals will then typically try to escape or "struggle" by vigorous escape movements, such as climbing up against the walls of the cylinder, swimming around and across the water filled cylinder and diving beneath the surface. After a period of time though, as well as with a subsequent exposure, these escape-directed behaviours will decrease and eventually the rodents will adopt an "immobile" posture, meaning that only sufficient movements are made that enables the animal to keep its head above the water (Castagné et al., 2011). The test usually consists of a pre-swim or 15-20 minutes, followed the next day by the test swim in which immobility and escape-directed strategies are more pronounced. The adoption of an immobile posture during re-exposure is thought to reflect failure in persistent escape-directed behaviour, with an increase in immobility time considered to reflect the aforementioned depressive-like manifestations (Petit-Demouliere et al., 2005) and is the most robust measure of face validity in this test. However, certain rodent strains, like the FSL animal, naturally adopt this immobile posture earlier on and for longer periods of time compared controls, making a pre-swim unnecessary (Overstreet et al., 2005; Overstreet and Wegener, 2013) Figure 16 shows a graphical representation of the FST and its counterpart, the TST. However, a limitation of this test is that antidepressants can reverse immobility after acute *and* chronic treatment, while response to acute antidepressant treatment has limited to no translational value as antidepressants do not produce rapid symptom improvement in the clinic (O'Leary and Cryan, 2013). For maximal translational value this screening test has maximal relevance following application in an animal model of depression that causes increased FST immobility that is only reversed by chronic antidepressant treatment. The FSL model fits this criterion (Overstreet et al., 2005). Thus application of the FST in the FSL rat presents with very good predictive validity for antidepressant-like compounds (Overstreet et al., 2005; Overstreet and Wegener, 2013; Petit-Demouliere et al., 2005), and virtually all classes of antidepressants decrease immobility in the FST under this challenge. Additionally, the climbing/struggling (repeated attempts to escape from the cylinder by trying to climb up the walls) or swimming (actively swimming around in the cylinder) based escape strategies in the FST also have

translational relevance. Increased climbing behaviour is proposed to be mediated by antidepressants that have a predominant noradrenergic mechanism of action, while increased swimming has been suggested to be mediated by antidepressants with a predominant serotonergic action (Detke et al., 1997).

The FST is thus a suitable screening test for assessing antidepressant-like activity of existing and novel investigational compounds, especially if built on the platform of the FSL model of depression.

4.2.2.2 Tail suspension test

The tail suspension test (TST) is based on the same principles of the FST, but in this test the rodent's tail is fixed to an overhead bar and is suspended in the air by its tail. This test is used almost exclusively in mice as rats are too heavy to hang from their tails. The test is graphically depicted in Figure 16. Struggling and immobility is recorded as in the FST, with the same level of predictive validity as the FST. However, this test is seen as a lessor stressor compared to the FST, with the animal resuming normal spontaneous activity immediately after the test, while animals take longer to resume normal activity following the FST (Castagné et al., 2011).

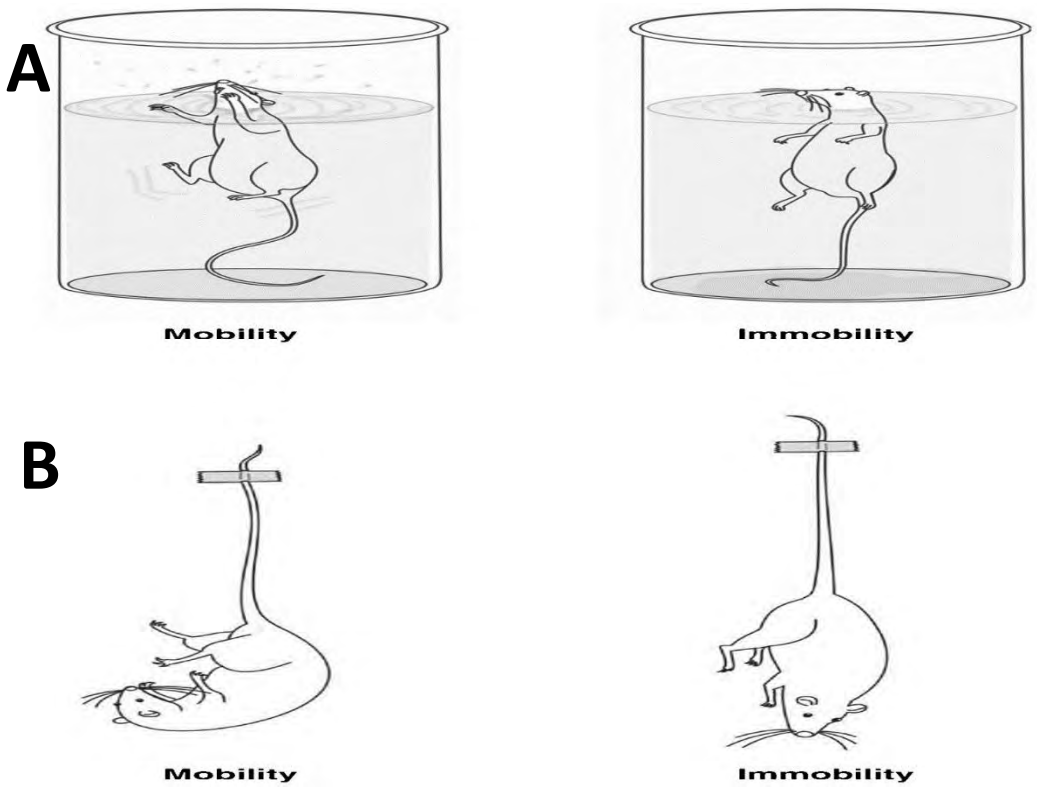


Figure 16. Schematic representation of A) the Forced Swim Test and B) the Tail Suspension Test. Adapted from Abelaira et al., 2013

4.2.2.3 Sucrose preference

The sucrose preference test is purported to reflect symptoms of anhedonia in depression (O'Leary and Cryan, 2013). Most rodents, when given a choice of sucrose solution or water to drink, will choose the sucrose solution, possibly due to its rewarding actions via the dopamine system (Powell et al., 2011). However, animal models of depression, such as the FSL model or the chronic mild stress model present with decreased sucrose preference (Powell et al., 2011), and has been suggested to reflect the anhedonic symptoms of depression (O'Leary and Cryan, 2013). This phenomenon can be reversed by antidepressant treatment, and thus presents with good face and predictive validity. However, this test is strain-dependent, as it relies on the expression of the Tas1r3 taste receptor gene which allows rodents the ability to taste sucrose (Powell et al., 2011). The FSL rat is an example of a strain not displaying altered sucrose preference (Overstreet et al., 2005).

4.2.2.4 Novelty-suppressed feeding

Another test built on the anhedonic symptoms of depression is novelty-suppressed feeding, which assesses the latency of a rodent to approach a food reward in a novel environment. This test therefore assesses anhedonia in the context of increased anxiety-like behaviour. This test is also sensitive to chronic, but not to acute antidepressant treatment (Dulawa and Hen, 2005). Although this test seems to present with good face and predictive validity, it is not as widely used or regarded as a screening method for antidepressant treatment, although it is useful in investigating the neurobiology underlying antidepressant response (Dulawa and Hen, 2005).

4.2.3 Behavioural models of cognitive function

Cognitive dysfunction in both depression and schizophrenia includes deficits in attention, executive functioning, working, episodic, reference and associative memory (Darcet et al., 2016). There are many tests that measure these cognitive domains with relevance to depression and schizophrenia, and I will briefly elaborate on the tests that are cited in this thesis. Working and reference memory is very often assessed in the Morris Water Maze (MWM) and the radial arm maze. The MWM is a navigational task reliant on intact hippocampal function by requiring the rodent to learn and remember the location of an escape platform in a water arena in order to locate a hidden (submerged) platform in subsequent trials by using various spacial cues. The escape latency is a measure of spatial working memory (Vorhees and Williams, 2006). The radial arm maze requires of the rodent to remember the location of food rewards hidden in various radial arm target sites, requiring intact functioning of the prefrontal cortical, hippocampal and striatal interconnections (Floresco et al., 1997). Associative memory can be measured by classical conditioning tasks, including contextual or cued fear conditioning and passive avoidance. These tests rely heavily on intact hippocampal function (Darcet et al., 2016) and require the animals to learn to associate a conditioned stimulus (tone, context or light) with an aversive stimulus, such as a foot shock. Recognition memory is a form of declarative or episodic memory, and this type of memory

is impaired in both schizophrenia and depression (Darcet et al., 2016; Kahn and Keefe, 2013). This test is discussed in more detail below.

4.2.3.1 Novel Object Recognition Test (NORT)

The novel object recognition test (NORT) is a two-trial behavioural measure that relies on the rodent's innate preference to explore novel objects more than familiar objects (Ennaceur and Delacour, 1988). This enables measurement of recognition memory by introducing a novel object and measuring exploration time vs. a familiar object (Antunes and Biala, 2012; Ennaceur and Delacour, 1988). After habituation to the test arena, two identical objects are placed in the test arena and the animal is allowed to explore them. In the recognition trial, one familiar object is replaced with a new object that is visually distinct from the novel objects. The time that the rodent spends exploring the novel object vs. the familiar object is regarded as an index of recognition memory (Antunes and Biala, 2012). This procedure is graphically depicted in Figure 17. The declarative memory processes underlying the NORT relies on the perirhinal cortex and the hippocampal complex (Broadbent *et al.*, 2010; Cohen and Stackman, 2015; Reger *et al.*, 2009), hippocampal function being especially compromised in depression (Sapolsky, 2001) and schizophrenia (Harrison, 2004). Both the SIR model and the FSL model present with deficits in the NORT (Abildgaard et al., 2011; Gomez-Galan et al., 2013; McLean et al., 2010; Möller et al., 2013a). Typical, and more so atypical antipsychotic treatment reverses deficits in the NORT (Möller et al., 2011; Rajagopal et al., 2014), while antidepressants that specifically inhibit noradrenaline reuptake improves object recognition memory in this test (Feltmann et al., 2015).

The NORT is therefore a valid construct reflecting an aspect of cognitive impairment evident in both schizophrenia and depression and is amenable to both antipsychotic and antidepressant treatments. This test was employed in this study to determine whether α_{2C}-AR-antagonism could improve recognition memory in animal models of depression and schizophrenia.

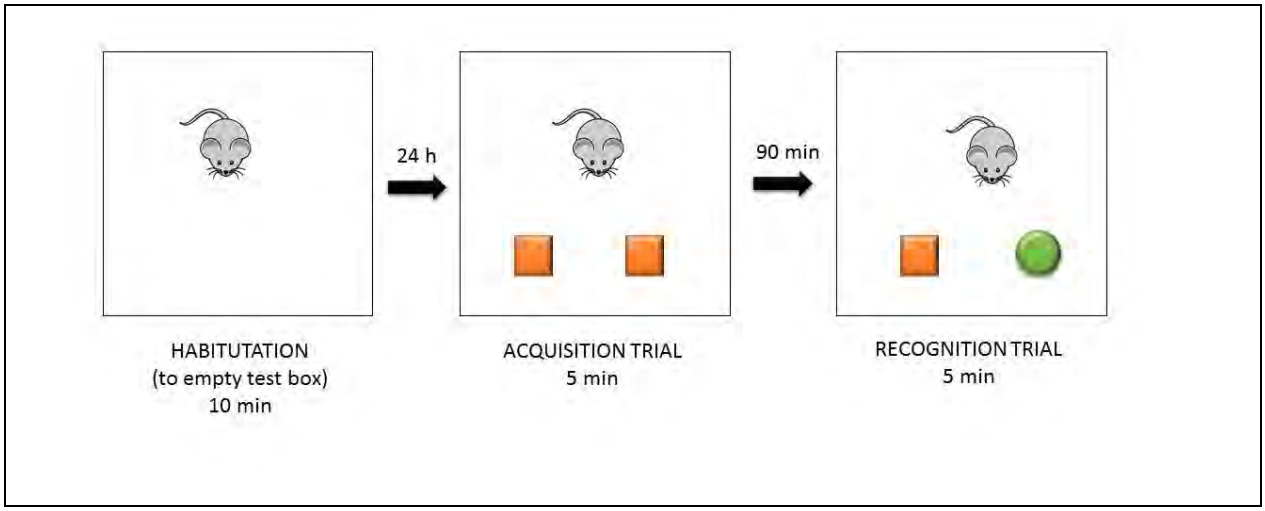


Figure 17. Schematic representation of a typical NORT procedure, as described in Chapters 3 and 4 of this thesis.

4.3 Modelling Schizophrenia in Animals

According to the criteria provided in section *4.1*, an animal model of schizophrenia should mimic the core symptoms of schizophrenia, thus presenting with behavioural abnormalities akin to positive symptoms (e.g. decreased PPI, hyperactivity, hyperlocomotion, stereotypy), negative symptoms (e.g. decreased social interaction, abnormal response to reward) and cognitive impairments. These behavioural abnormalities should supposedly have a post-pubertal onset and be aligned with pathophysiological traits including corticolimbic dopaminergic dysfunction, cortical glutamatergic dysfunction and neurotrophic alterations. Finally, the majority of clinically effective antipsychotics should be able to reverse most aspects of these behavioural and neurochemical alterations (Jones et al., 2011). More than 20 different animal models of schizophrenia have been published, fitting into four different categories: developmental, pharmacological (drug-induced), brain lesioning or genetic manipulation (Carpenter and Koenig, 2008). In this study, a developmental model of schizophrenia, namely the SIR model, was used. The advantage of a developmental model is that epidemiological studies have provided compelling evidence that the risk of developing schizophrenia is greatly increased by exposure of the neonate to environmental adversity, including maternal stress or immune activation or an early-life adverse event (Lewis and Levitt, 2002). A neurodevelopmental model of schizophrenia thus presents with very good etiological construct validity. Furthermore, neurodevelopmental models present with very robust presentation of sensorimotor gating deficits in the PPI (Jones et al., 2011), considered to be a very important translational instrument to assess psychotic-like and antipsychotic-like interventions (Gobira et al., 2013; Swerdlow et al., 2000). These models generally present with a broader replication of the symptom triad compared to non-developmental models, although there are individual exceptions (Jones et al., 2011).

Examples of developmental models include the gestational methylazoxymethanol acetate (MAM) model, which employs administration of the anti-mitotic agent, MAM, to pregnant dams. This agent targets central neuroblast proliferation thereby selectively conferring dysfunctional development in the CNS in the off-spring (Flagstad et al., 2004). Exposure of pregnant dams to infections that lead to elevated circulatory inflammatory mediators also affects neurodevelopment of the offspring, and is based on the associated of such conditions with increased risk of schizophrenia in humans (Brown and Derkits, 2010; Jones et al., 2011). Other examples of neurodevelopmental models include exposure of pregnant dams to unpredictable stress and ventral hippocampal lesioning (Jones et al., 2011).

Lesion models are a subset of neurodevelopmental models as they employ neonatal lesioning of the ventral hippocampus, leading to marked deficits in the development of the nervous system leading to post-pubertal behavioural abnormalities, abnormal architectural integrity of regions of the frontal cortex and striatum and deficits in social interaction, cognitive function and sensorimotor gating, to

name but a few (Beninger et al., 2009; Jones et al., 2011; Lipska et al., 1995). However, this method is very sensitive to methodological errors and leads to significant mortality rate (15%) in animals shortly after the procedure (Jones et al., 2011; Richtand et al., 2006).

Pharmacological models employ a pharmacological agent that would induce the central neurochemical alterations observed in schizophrenics. Based on the dopaminergic dysfunction noted in schizophrenia, and that amphetamine-induced psychosis has been described in humans and is related to exaggerated dopamine release (Geyer and Moghaddam, 2002), chronic administration of amphetamine and dopamine agonists such as apomorphine are popular as inducing agents. The induced behaviour typically represents positive symptoms of schizophrenia (hyperactivity, hyperlocomotion), while also impairing PPI, although hippocampal dependent memory processes seem to be unaffected (Jones et al., 2011). Serotonin-agonist models are also employed on the basis of the psychotomimetic effects of the 5-HT_{2A} agonist drug, lysergic acid d-ethylamide (LSD) and is supported by clinical evidence that atypical antipsychotics are antagonists of this receptor (Geyer and Moghaddam, 2002). The predictive validity of this model is however limited as displayed by PPI enhancing effects of the 5-HT releasing recreational drug, ecstacy, in humans, as opposed to disrupting PPI in rodents (Geyer and Moghaddam, 2002). Based on cortical glutamatergic dysfunctions noted in schizophrenia, glutamate NMDA-receptor antagonists are also employed as pharmacological models, using either PCP or MK-801 (Jones et al., 2011). Chronic administration of these drugs induce several neurochemical changes that correlate to the human disorder, including mesolimbic dopaminergic hyper-responsivity, cortical glutamatergic hypoactivity and reduced cortical and hippocampal neuronal connectivity (Beninger et al., 2010). Although acute administration of an NMDA-antagonist leads to impaired PPI, this deficit is not always sustained with chronic administration, which limits the assessment of novel antipsychotic compounds in this model within a chronic treatment paradigm (Egerton et al., 2008). Furthermore, chronic PCP or MK-801 treatment results in impaired working and recognition memory and impaired attention and informational processing (Egerton et al., 2005). These cognitive deficits seem to be reversed by atypical, but not typical antipsychotics, although this reversal is not always reproducible across different laboratories and is thus not a very robust finding. In addition, the effects of these NMDA-antagonists seem to be dependent on the rodent strain used. For example Sprague Dawley's don't display consistent PCP-induced deficits in spatial learning and attentional set-shifting, while these deficits are produced in Listar hooded and Long-Evans rats (Jones et al., 2011). This model has also been criticized for producing a high rate of "false positive" results on parameters for antipsychotic efficacy, while it also does not reliably model negative symptoms of schizophrenia (Egerton et al., 2008; Jones et al., 2011). However, considering that all animal models have their relevant shortcomings, the NMDA-antagonist models are very good models for investigating novel antipsychotic-like compounds, while their limitations can be

overcome by supplementing such findings with studies in neurodevelopmental or lesion models of schizophrenia.

4.3.1 Social Isolation rearing model of schizophrenia

In this study, the extensively studied Social Isolation Rearing (SIR) model of schizophrenia has been employed to assess antipsychotic-like effects of ORM-10921. SIR is a neurodevelopmental model based on the deprivation of social structure and social interaction during the critical phases of the developing brain (Fone and Porkess, 2008; Jones et al., 2011). This is done by removing pups from their littermates at the age of weaning (usually on post-natal day 21), and placing them alone in a cage to develop in isolation. This social deprivation causes significant alterations in their neural development, resulting in various behavioural and biochemical alterations at adulthood (Fone and Porkess, 2008).

Behavioural abnormalities induced by SIR include various aspects representing the symptom triad of schizophrenia, including positive symptoms (van den Buuse, 2010), spontaneous locomotor hyperactivity as well as sensorimotor gating deficits (Cilia et al., 2005), while negative symptoms are also well-modelled in this model. Social withdrawal and maladaptive social cognition is one of the first manifestations of the illness in humans (Strous et al., 2004). SIR induces decreased social interaction (Möller et al., 2011), increased aggressive behaviour and social dominance (Arakawa, 2007). The period of isolation has also been positively correlated to muricidal behaviour (killing of a newly introduced mouse by breaking its neck) (Valzelli and Garattini, 1972). Additionally, increased anxiogenic behaviour has been reported in SIR rats (Jones et al., 2011; Weiss et al., 2004). A point of dispute regarding face validity for some negative symptoms in SIR rats lies in the abnormal response to reward as reflected by hyperphagia, increased ethanol preference, increased sucrose consumption and increased reward sensitivity, behaviours which seem to be at odds with the avolition often observed in schizophrenics (Fone and Porkess, 2008), although it could reflect hyperdopaminergic processes in the striatum which falls within the construct of schizophrenia (Báez-Mendoza and Schultz, 2013; Fone and Porkess, 2008).

Cognitive symptoms of schizophrenia have reliably been demonstrated in the SIR model. Deficient conditioned learning (Weiss et al., 2004), rule-learning and behavioural inflexibility as assessed by attentional set-shifting (Fone and Porkess, 2008; Jones et al., 1991; Krech et al., 1962) has been demonstrated in SIR rats. Furthermore, SIR rats display context-independent episodic and visual recognition memory deficits in the NORT, cognitive parameters that are impaired in schizophrenia (McClure et al., 2007; Nestor et al., 2007). Since SIR induces reliable and pronounced deficits in the NORT (Fone and Porkess, 2008; Möller et al., 2013a), which are reversed by atypical antipsychotic treatment (Jones et al., 2011; Möller et al., 2011; Möller et al., 2013a), this test was applied in this study. While schizophrenia also presents with visuo-spatial working memory deficits (Bozikas et al., 2006) SIR

mirrors this dysfunctional cognitive domain as demonstrated in the MWM test (Fone and Porkess, 2008), and which can be reversed by antipsychotic treatment (Jones et al., 2011).

The SIR model has also demonstrated various neurochemical alterations that mirror the theoretical construct of pathophysiological alterations found in schizophrenic patients (Fone and Porkess, 2008), although it must be noted that an in-depth literature study has revealed various inconsistencies and discrepancies (Fone and Porkess, 2008; Jones et al., 2011). Although contradictory findings have been reported in the literature, various studies have reported dopaminergic alteration in SIR rats, including increases in basal DA turnover in the amygdala (Heidbreder et al., 2000) and increased basal synaptic (Hall et al., 1998) and striatal tissue levels of DA (Möller et al., 2013b), increased sensitivity to the effects of dopaminergic agents (Jones et al., 1992) and decreased frontal cortical dopamine D_1 receptor binding (Toua et al., 2010). Moreover, depletion of striatal dopamine reversed SIR-induced PPI deficits (Powell et al., 2003), strengthening construct and predictive validity of this model. While deficient serotonergic neurotransmission is proposed to underlie the pathophysiology of schizophrenia, the mechanisms remain unclear (Reynolds, 2008). SIR does however present with serotonergic deficits, including increased 5-HT_{2A} receptor binding and responsiveness (Preece et al., 2004; Wright et al., 1991), while stress reduces 5-HT release in limbic regions of SIR rats (Muchimapura et al., 2003). Recently, findings from our laboratory has demonstrated altered redox (Möller et al., 2011) and tryptophan metabolism (Möller et al., 2012) in SIR rats, and well as evidence for an overall imbalance in mitochondrial-immune-inflammatory function (Möller et al., 2013a), all of which being reversed by sub-chronic clozapine treatment (Möller et al., 2011, 2012, 2013a). Less evidence is available on noradrenergic dysfunction in SIR rats, although a prominent finding is that SIR rats display elevated presynaptic α_2 autoreceptor sensitivity (Fulford and Marsden, 1997). Considering that α_2–AR antagonism has been suggested to underlie antipsychotic atypicality (Svensson, 2003), this finding provides important construct validity for assessing the effects of an α_{2C}-AR antagonist in this study.

Hypoglutamatergic frontal cortical function and impaired GABAergic inhibitory control is an important construct in schizophrenia (Reynolds, 2008), and these characteristics are reflected in the SIR model as decreased frontal cortical NMDA-receptor binding and receptor expression (Hall et al., 2002; Toua et al., 2010), although strain-dependent differences are evident (Hall et al., 2002). Additionally, sub-chronic administration of an NMDA-antagonist seems to enhance SIR-induced behavioural deficits (Lapiz et al., 2003). Furthermore, alterations in HPA-axis functionality and corticosterone response have also been reported in SIR rats (Fone and Porkess, 2008). There is very little information on the effects of post-weaning SIR on neurotrophic function. Decreased limbic BDNF levels have however been reported in rats isolated during adulthood (Djouma et al., 2006; Scaccianoce et al., 2006), but neurotrophic deficits in rats reared in isolation from weaning still need to be investigated.

The SIR model of schizophrenia therefore presents with very good face and construct validity, while various behavioural and neurochemical alterations, including deficient PPI, impaired cognition and disordered monoaminergic and glutamatergic functioning can be reversed by various antipsychotics (Jones et al., 2011; Möller et al., 2012; Möller et al., 2013a; Möller et al., 2013b). Core findings supporting face, construct and predictive validity for the SIR model are summarised in Figure 18. Thus, the SIR is a suitable model for evaluating the antipsychotic-like and pro-cognitive effects of a novel compound. Considering that the SIR presents with deficient PPI and impaired object recognition memory, the PPI and NORT was applied in this study.

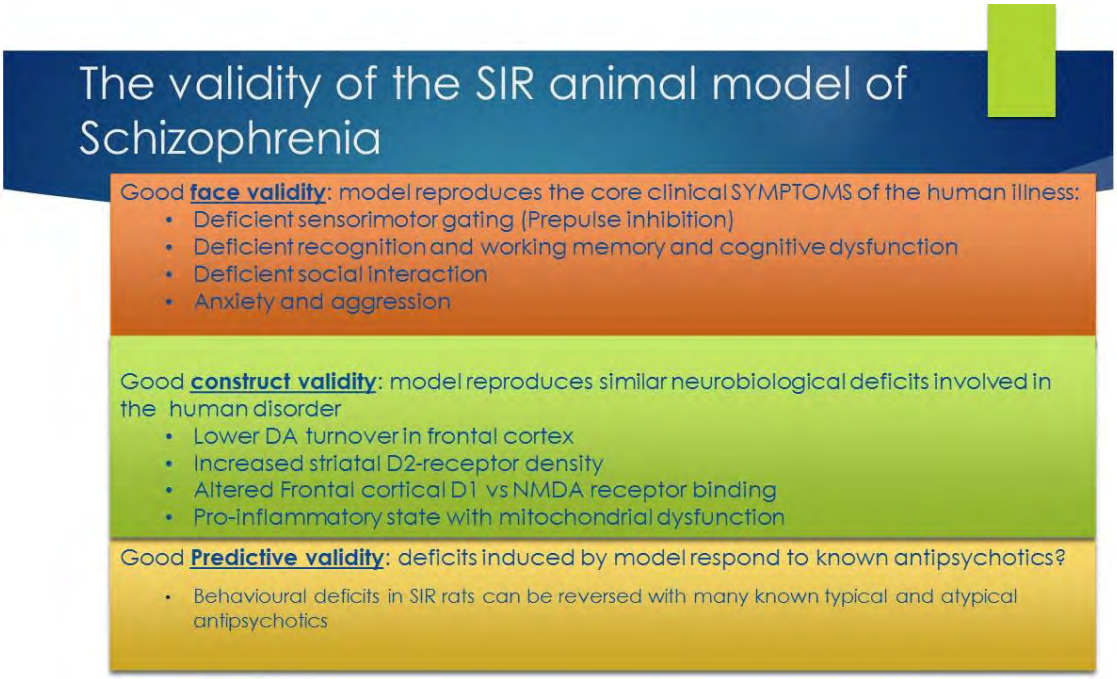


Figure 18. Summary of the core findings in SIR rats supporting face, construct and predictive validity as an animal model of schizophrenia.

4.4 Modelling Depression in Animals

According to the criteria of face, construct and predictive validity, an animal model of depression should present with behavioural abnormalities that present with anhedonia, depressed mood, feelings of worthlessness and guilt, decreased motivation, fatigue, sleep disturbances and cognitive impairments (Willner and Mitchell, 2002). However, modelling symptoms that are usually subjectively reported by depressed patients, such as depressed mood and feelings of worthlessness, for example, is an obvious limitation as the animal cannot communicate these “feelings”. Motivation-driven responses to stress can however be measured by the FST and the TST (Slattery and Cryan, 2012). Sleep disturbances, anhedonia and cognitive impairments can be modelled, while fatigue may be modelled by tests that require persistent efforts (Willner and Mitchell, 2002). In order for construct validity to be established, the model should mirror various molecular and biochemical changes observed in depression, such as

HPA-axis hyperactivity, disrupted circadian rhythms, cholinergic supersensitivity, monoaminergic and GABAergic alterations, as well as hippocampal dysfunction and reduced levels of key neurotrophins (Overstreet, 2012; Overstreet et al., 2005). Finally, the majority of clinically effective antidepressant treatments should be able to reverse most aspects of these behavioural and physiological alterations. Importantly however, since clinical improvement of depressive symptoms can take several weeks or even months to present after the initiation of treatment, predictive validity in an animal model therefore is strengthened when chronic, but not acute antidepressant treatment attenuates behavioural alterations (Overstreet et al., 2005). Various animal models of depression have been investigated over the years, and these can roughly be divided into three main categories, viz. stress-based models, models of genetic predisposition and limbic circuitry lesion models (O'Leary and Cryan, 2013).

Stress-based models are based on the etiological construct that stress is a predisposing factor for depression (O'Leary and Cryan, 2013). The main models in this paradigm are the unpredictable chronic mild stress model (UCMS), maternal deprivation, prenatal stress, social defeat stress and learned helplessness. UCMS exposes the animal to a series of mild stressors that are presented in a random fashion over a time course of several weeks (Willner, 1997). This model induces features of anhedonia as measured by the sucrose preference test, a behaviour which can only be reversed by chronic but not acute antidepressant treatment (Willner, 1997). Although the model has good construct and predictive validity, it is very labour intensive and difficult to reproduce across laboratories (Willner, 1997). Learned helplessness employs a series of inescapable foot shocks that eventually induces a state of "resignation" in the animal, as observed in eventual failure to attempt escape, an effect reduced by chronic antidepressant treatment (Pryce et al., 2011). Maternal separation involves the early post-natal separation of pups from their mother, which can cause depressive-like phenotypes in adulthood (Pryce and Feldon, 2003) and also shows response to antidepressant treatments (Nestler and Hyman, 2010).

Limbic circuitry lesion models mainly comprise olfactory bulbectomy (O'Leary and Cryan, 2013). This model involves removing the olfactory bulb in rodents, producing long-lasting neurochemical, physiological, endocrine and behavioural changes in rats that resemble the alterations of depression in humans (Skelin et al., 2011). Most antidepressants are able to reverse depressive-like behaviours and neurochemical alterations in this model, and therefore the model has very good construct, face and predictive validity for depression (Kelly et al., 1997).

Genetic models of depression often employ transgenic mouse models that induce altered expression of genes associated with the pathophysiology of depression (Abelaira et al., 2013). For example, a targeted deficiency of the 5-HT transporter (5-HTT$^{-/-}$ mice) models major adaptive changes in 5-HT neurotransmission that mimic elements of serotonergic dysfunction in depression (Lesch and Mossner,

2006). Aberrant expression of the vesicular glutamate transporter (VGLUT) mRNA has also been described in depressive disorder (Uezato et al., 2009) 1 gene (VLUT1$^{+/-}$), thus modelling excitatory inhibitory imbalances observed in depressed patients (Tordera et al., 2007). Another model includes the targeted disruption of the gene that encodes for the cannabinoid 1 receptor (CB1R$^{-/-}$), based on the contribution of the endocannabinoid system to neuromodulatory control of emotional behaviour (Hill and Gorzalka, 2005). This disruption causes depressive-like symptoms and stress-sensitivity as well as disturbances in 5-HT transmission (Aso et al., 2009). These genetically modified animals are helpful to model certain aspects of the human disorder, especially since genetic manipulation can build on a particular construct of its pathology. However, depression includes multiple genetic vulnerabilities, while these models by design are only able to model certain aspects of these alterations (Abelaira et al., 2013). Another cause of concern is that such genetic "knock-in" or "knock-out" models may introduce adaptive neuronal changes later in life to accommodate for the new or deficient protein introduced into the off-spring and that may invalidate its translational relevance for the human illness. Another approach to depression is by employing natural genetic models, based on the finding that depressive disorders have a very strong genetic basis (McArthur and Borsini, 2006). The most prominent and widely researched natural genetic model of depression is the Flinders Sensitive Line rat (FSL) (Overstreet and Wegener, 2013). This model presents with excellent face, good construct and very good predictive validity for depression (Overstreet et al., 2005). Moreover, the prominent natural immobility in the FST demonstrated by FSL rats makes this model especially suitable for assessing the antidepressant-like effects of a novel compound. This model is discussed in detail below.

4.4.1 The Flinders sensitive line model of depression

Depression is characterized by cholinergic supersensitivity and enhanced sensitivity to stressors (Manji et al., 2001). The FSL rat has been in-bred from the Sprague Dawley rat to display cholinergic supersensitivity as well as enhanced sensitivity to environmental stressors (Overstreet et al., 2005). The Flinders resistant line rat (FRL) does not display this enhanced sensitivity to stress and is regarded as the healthy control of the FSL rat, although Sprague Dawley rats are also often used as controls for FSL rats (Knapp et al., 2014; Overstreet et al., 2005; Overstreet and Wegener, 2013). This animal model presents with many bio-behavioural, neurochemical and neuroendocrine abnormalities associated with depression, while many of these abnormalities can be reversed by chronic, but not acute antidepressant treatment (Overstreet and Wegener, 2013).

The key behavioural abnormality modelled in the FSL rat is its natural immobility in the FST, which can be reversed by a variety of chronic antidepressant treatments (Overstreet et al., 2005; Overstreet and Wegener, 2013). This model is therefore well-suited to test antidepressant-like activity of novel compounds. Psychomotor retardation has been demonstrated in the FSL rat, in that it executes food-

reward related bar-pressing at a much lower rate than its FRL controls (Overstreet and Russell, 1982), is less active in novel open fields and takes much longer to learn cognitive behaviours in operant tasks (Bushnell et al., 1995). Altered appetite is a common feature of depression, with reduced appetite being more common than increased appetite (American Psychiatric Association, 2013a). FSL rats appear to also present with decreased appetite, as evidenced by smaller sized food pellets necessary to train these rats for food rewards (Bushnell et al., 1995). While disrupted circadian rhythm and disrupted rapid eye movement (REM) sleep is a prominent feature of depression (American Psychiatric Association, 2013a), the FSL rat similarly demonstrates alterations in circadian rhythm as well as altered sleep patterns, including elevated REM sleep and reduced REM sleep latency (Overstreet et al., 2005). Anxiety is evident in certain aspects of behaviour in depression, while the FSL rat demonstrates social anxiety in the social interaction task (Overstreet et al., 2005). On the other hand, this model does not demonstrate any marked disturbances in parameters suggestive of anhedonia (Overstreet et al., 2005).

The FSL rat does not model the cognitive deficits of depression very well either (Overstreet et al., 2005), although it does take longer to learn cognitive spatial working and reference memory tasks than its FRL counterpart, where after they perform equally well (Bushnell et al., 1995). However, the FSL rat does present with recognition memory deficits in the NORT (Abildgaard et al., 2011; Gomez-Galan et al., 2013), although the effects of antidepressant treatment on recognition memory deficits have not been studied. Indeed, this study will provide the first report of predictive validity for this test in these animals.

Depression has been associated with alterations in monoaminergic, glutamatergic and GABAergic functioning (Kharade et al., 2010). The FSL model reflects alteration in a number of monoaminergic parameters. Although various serotonergic alterations do not mimic the human disorder, reduced limbic serotonin synthesis (Hasegawa et al., 2006) and serotonergic subsensitivity (Zangen et al., 2001) relate to the human disorder, while chronic antidepressant treatment generally reverse these changes (Overstreet et al., 2005; Zangen et al., 2001; Zangen et al., 1997). Dopaminergic deficits in FSLs are evidenced by decreased basal DA levels (Zangen et al., 2001), decreased limbic DA neurotransmission (Friedman et al., 2007; Friedman et al., 2005) and low extracellular DA due to decreased DA release (Roth-Deri et al., 2009). The latter has been correlated to increased FST immobility and is reversed by antidepressants (Dremencov et al., 2004; Roth-Deri et al., 2009). Current evidence suggests that noradrenergic alterations in FSL rats revolve around α_2-AR receptor alterations. Increased α_2-AR density has been reported in FSL animals (Landau et al., 2015; Lillethorup et al., 2015b), consistent with the human disorder (De Paermentier et al., 1997), while electroconvulsive shock reduces α_2-AR binding (Lillethorup et al., 2015a).

Other alterations in the FSL rat that mimic the human disorder include decreased levels of neuropeptide Y (Jimenez-Vasquez et al., 2000), an important peptide involved in the modulation and release of NA

and 5-HT (Finta et al., 1992; Overstreet et al., 2005), as well as disordered glutamate-nitric oxide signalling (Wegener et al., 2010), a pathway well-studied in the neurobiology of mood and antidepressant action (Wegener and Volke, 2010). Finally, hippocampal atrophy and reduced hippocampal neurogenesis and neurotrophic support is evident in depression (Brand et al., 2015; Campbell and MacQueen, 2004). Reduced hippocampal levels of BDNF and vascular endothelial growth factor has been described in FSL rats (Elfving et al., 2010a; Elfving et al., 2010b), while chronic administration of a neurotrophin called nerve growth factor (NGF) reduces FST immobility in FSL rats (Overstreet et al., 2010).

The FSL model therefore provides a very well-established translational platform for studies into the efficacy of novel treatments of depression, and in particular the objectives of this study, viz. to investigate the effect of chronic α_{2C}-AR-antagonism on depressive-like behaviour, cognition, hippocampal monoamines and hippocampal BDNF levels within a construct of depression.

Figure 19 below provides a summary of various animal models used to model depression and schizophrenia as well as various behavioural tests employed to assess behaviour in these models. The figure also highlights the behavioural tests and animal models employed in this study.

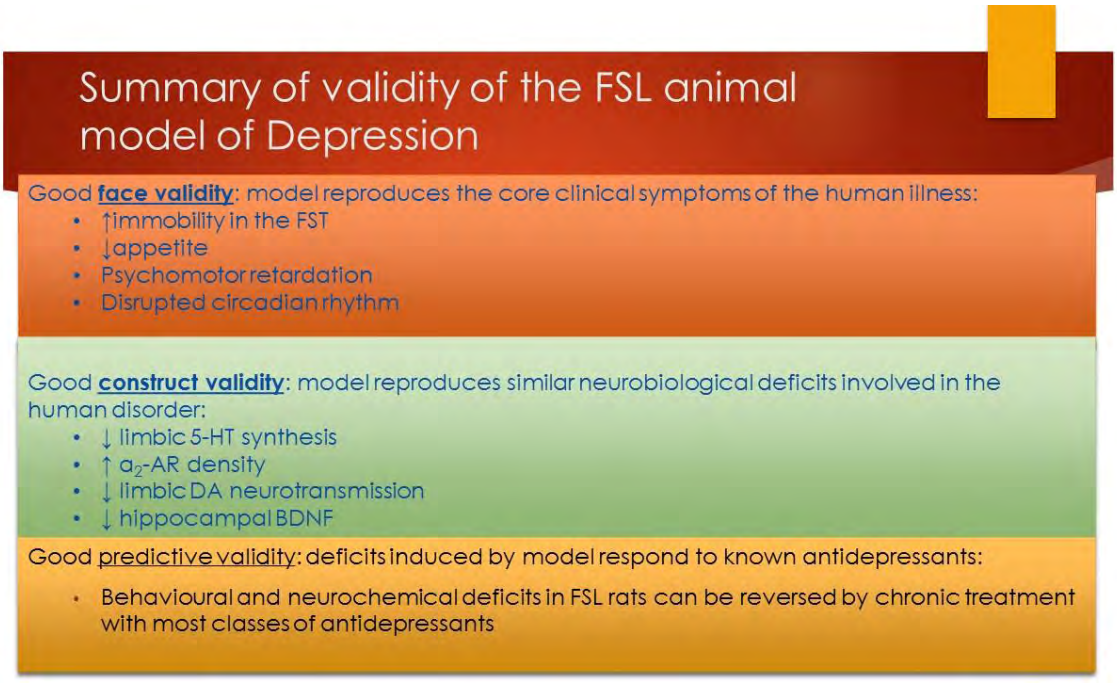


Figure 19. Summary of the core findings in FSL rats supporting face, construct and predictive validity as an animal model of depression.

5. Role of α_{2C}-AR in antipsychotic-like, antidepressant-like and pro-cognitive behaviour in animal models

The previous section discussed various behavioural tests predicting antipsychotic-like and antidepressant-like effects, while section *1.2* reviewed the effects of the α_{2C}-AR on neurochemical correlates of neuropsychiatric illness. Here, behavioural findings that imply a distinct role for the α_{2C}-AR in schizophrenia and depression are described.

Regarding sensorimotor gating, the contribution of non-selective α_2-blockade to the modulation of PPI has been proposed, although the literature is somewhat inconclusive in this regard (Larrauri and Levin, 2012; Ozcetin et al., 2016). In fact some papers have suggested that antagonism of the α_{2A}-AR does not contribute to enhancement of PPI (Lähdesmäki et al., 2004; Larrauri and Levin, 2012; Ozcetin et al., 2016; Powell et al., 2005). On the other hand, studies in transgenic α_{2C}-KO and α_{2C}-OE mice have indicated that the α_{2C}-AR *is* involved in regulation of the PPI, although these studies have suggested that α_{2C}-AR-agonism would improve deficits in PPI (Sallinen et al., 1998a). This extrapolation from transgenic studies has since been disproven by the use of the highly selective α_{2C}-AR antagonists JP-1302, ORM-10921 and ORM-12741, which improve PPI in NMDA-antagonist rodent models of schizophrenia (Sallinen et al., 2007; Sallinen et al., 2013a; Sallinen et al., 2013b).

In the FST, the α_2-AR has been implicated in mediating the antidepressant (or anti-immobility) effects of TCAs, while activation of the α_{2A}-AR subtype seems especially involved (Cottingham and Wang, 2012; Schramm et al., 2001). Interestingly, the α_{2C}-AR seems to play an opposite role in regulating antidepressant effects in the FST. Early studies in α_{2C}-OE models in mice have suggested that α_{2C}-AR activation has a detrimental effect on FST immobility, with α_{2C}-OE mice displaying increased immobility compared to wild type-controls (Sallinen et al., 1999), an effect not attributed to altered locomotor activity (Sallinen et al., 1997). On the other hand, α_{2C}-KO mice (i.e. inactivation of the α_{2C}-AR) demonstrate an antidepressive phenotype (Sallinen et al., 1999). These findings might explain why relatively non-selective α_2-AR agonists (Cervo and Samanin, 1991; Cottingham et al., 2012; Stone et al., 2011) and certain non-selective α_2-AR *antagonists* have both shown antidepressant-like effects in the FST. Mirtazapine is an α_2-AR antagonist that has shown a putative early-onset antidepressant effect (Blier, 2003) and which increases serotonergic neurotransmission via antagonism of presynaptic α_2-ARs (Davis and Wilde, 1996). Dhir and Kulkarni (2007) demonstrated that augmentation with the non-selective α_2-AR antagonist yohimbine potentiated the anti-immobility effects of both fluoxetine and venlafaxine in the mouse FST (Dhir and Kulkarni, 2007). This effect is mirrored in the clinic, where addition of yohimbine to SSRI treatment hastens antidepressant response and increases the number of responders compared to SSRI treatment alone (Sanacora et al., 2004a). Recently an important role for the α_{2C}-AR in these findings has been demonstrated in rodents using subtype selective α_{2C}-AR

antagonists. Acute administration of JP-1302 (Sallinen et al., 2007), ORM-10921 (Sallinen et al., 2013a) and ORM-12741 (Sallinen et al., 2013b) in Sprague Dawley and Han-Wistar rats was found to decrease immobility in the FST.

Behavioural studies have also supported the selective targeting of the α_{2C}-AR to improve *cognitive parameters*. Non-selective α_2-AR antagonists have previously shown improved recognition memory in the NORT, although not in an animal model of depression (Chen et al., 2014; Chopin et al., 2002), and the role of the α_{2C}-AR in this paradigm is unknown. α_{2C}-OE mice display impaired escape strategies in the MWM, which can be reversed, to a greater extent than in wild-type mice, by the administration of an α_2-AR antagonist and (Björklund et al., 1998; Björklund et al., 1999a; Björklund et al., 1999b). MWM navigation relies on intact striatal and hippocampal function (Annett et al., 1989; Marston et al., 1993) and the dense expression of the α_{2C}-AR in these brain regions thus suggest a role for the α_{2C}-AR in modulating cognition in this test. In the radial arm maze, the non-selective α_2-AR agonist dexmedetomidine improves working memory, which is enhanced in α_{2C}-KO mice, suggesting that the absence of α_{2C}-AR agonism (by inference α_{2C}-AR antagonism) might result in enhanced effects on working memory in this test (Björklund et al., 2001). Recently, the above findings have been corroborated using the novel highly selective α_{2C}-AR antagonists ORM-10921 and ORM-12741, supporting the notion that selective α_{2C}-AR antagonism has pro-cognitive effects in animals (Sallinen et al., 2013a; Sallinen et al., 2013b). Additionally the efficacy of ORM-12741 has also been reported on cognitive parameters in clinical trials of Alzheimer's disease (Rinne et al., 2013).

6. Conclusions

This literature review provided a focussed overview on the key neuroanatomical and neurochemical alterations associated with schizophrenia and depression. Furthermore, an overview of various animal models of schizophrenia and depression and behavioural tests employed to assess antipsychotic-like, antidepressant-like and pro-cognitive behaviour was provided, in order to place the models and tests employed in this study into context. In addition, what we know about the role that the α_{2C}-AR plays in the regulation of monoaminergic, GABAergic, glutamatergic and glucocorticoid related mechanisms as well as neuronal activity was also discussed, providing a basis on how α_{2C}-AR selective antagonism might mediate antipsychotic and antidepressant-like effects. Although recent studies have employed novel selective α_{2C}-AR antagonists to demonstrate antipsychotic-like, antidepressant-like and pro-cognitive effects, very little is known about the mechanisms underlying the efficacy of these novel compounds. Furthermore, the purported neuropsychiatric effects of these compounds need to be corroborated in translational animal models of schizophrenia and depression. Therefore this study investigated the effect of chronic (14-day treatment) of the novel α_{2C}-AR antagonist, ORM-10921 in the SIR model of schizophrenia and the FSL model of depression, assessing effects on sensorimotor gating (PPI),

behavioural “despair” (or depressive-like non-persistence) (FST) and recognition memory (NORT). Furthermore, the effects of 14-day treatment with ORM-10921 on striatal or hippocampal monoamine and BDNF levels were investigated to ascertain possible mechanism by which ORM-10921 exerts its neuropsychiatric effects. ORM-10921 displays ~100-fold selectivity for the α_{2C}-AR vs. the α_{2A}-AR and α_{2B}-AR, shows high central permeability and is safe and well-tolerated in rodents (Sallinen et al., 2013a). While dose-differences have been noted for the application of drugs in different strains of animals (Campbell et al., 1988; Lopez-Rubalcava and Lucki, 2000; Swerdlow et al., 2004), and since anti-psychotic-like and antidepressant-like effects may be exerted at different doses, this study incorporated a dose-response design. Furthermore, efficacy was compared with the known antipsychotics clozapine and haloperidol and the known antidepressant imipramine, while the non-selective α_2-AR antagonist, idazoxan, was employed to conservatively determine selective vs. non-selective effects at the α_2-AR. The subsequent chapters of this thesis will therefore report and discuss the results from these investigations as well as the final observations.

Chapter 3: Manuscript A

The following article has been published in ***Progress in Neuro-Psychopharmacology & Biological Psychiatry***, and is entitled:

"The α_{2C}-adrenoceptor antagonist, ORM-10921, has antipsychotic-like effects in social isolation reared rats and bolsters the response to haloperidol"

Preamble

This chapter presents the full-length manuscript published in *Progress in Neuro-Psychopharmacology & Biological Psychiatry*, published by Elsevier. The manuscript is presented in the final form as published by Elsevier, and can be accessed on-line via the journal's website:

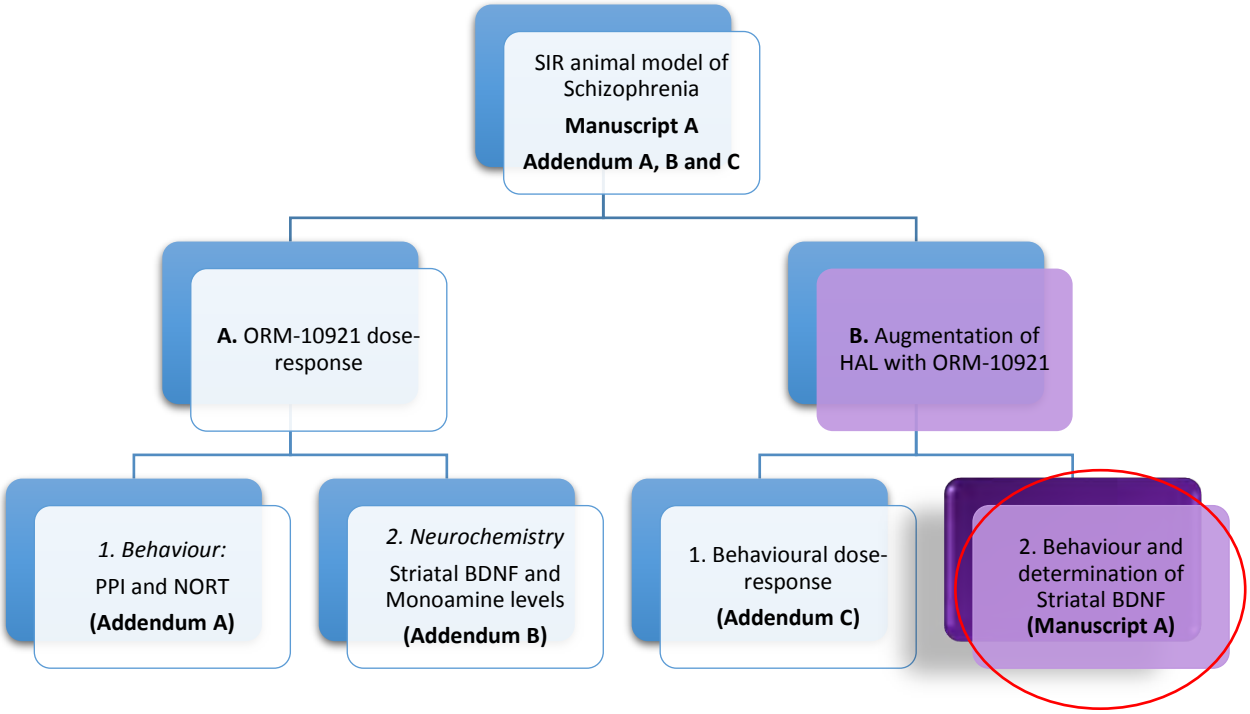
http://www.sciencedirect.com/science/article/pii/S0278584616301051

This article reports the efficacy of low-dose ORM-10921 in reversing SIR-induced deficits in sensorimotor-gating, object recognition memory and striatal BDNF, while demonstrating comparable efficacy to clozapine and superiority to haloperidol. Furthermore, this article reports the bolstering effect of low-dose ORM-10921 on the response to haloperidol to match the effects of clozapine, thereby establishing the promise of selective α_{2C} antagonism as an important design concept in novel drug discovery in schizophrenia. The diagram on the next page depicts where this manuscript fits into the overall SIR study design and into the thesis layout, as indicated by the red circle.

Authors' contributions

1. *M Uys* contributed towards the study design and undertook all behavioural, neurochemical and statistical analyses
2. *BH Harvey* designed the study and the original protocol and contributed towards the preparation of the manuscript and finalised it for publication
3. *M Shahid* and *J Sallinen* contributed towards the study design and preparation of the manuscript
4. *W Dreyer* assisted in performing the BDNF assay
5. *M Cockeran* assisted and advised on statistical data analyses

All co-authors provided permission to use this manuscript as part of M. Uys' Ph.D thesis, the letters of confirmation are annexed at the end of the thesis.


SIR animal model of Schizophrenia
Manuscript A
Addendum A, B and C
A. ORM-10921 dose-response
B. Augmentation of HAL with ORM-10921
1. Behaviour:
PPI and NORT
(Addendum A)
2. Neurochemistry
Striatal BDNF and Monoamine levels
(Addendum B)
1. Behavioural dose-response
(Addendum C)
2. Behaviour and determination of Striatal BDNF
(Manuscript A)

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The α_{2C}-adrenoceptor antagonist, ORM-10921, has antipsychotic-like effects in social isolation reared rats and bolsters the response to haloperidol

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ABSTRACT

Early studies suggest that selective α_{2C}-adrenoceptor (AR)-antagonism has anti-psychotic-like and pro-cognitive properties. However, this has not been demonstrated in an animal model of schizophrenia with a neurodevelopmental construct. The beneficial effects of clozapine in refractory schizophrenia and associated cognitive deficits have, among others, been associated with its α_{2C}–AR modulating activity. Altered brain-derived neurotrophic factor (BDNF) has been linked to schizophrenia and cognitive deficits. We investigated whether the α_{2C}-AR antagonist, ORM-10921, could modulate sensorimotor gating and cognitive deficits, as well as alter striatal BDNF levels in the social isolation reared (SIR) model of schizophrenia, comparing its effects to clozapine and the typical antipsychotic, haloperidol, the latter being devoid of α_{2C}–AR-activity. Moreover, the ability of ORM-10921 to augment the effects of haloperidol on the above parameters was also investigated. Animals received subcutaneous injection of either ORM-10921 (0.01 mg/kg), clozapine (5 mg/kg), haloperidol (0.2 mg/kg), haloperidol (0.2 mg/kg) + ORM-10921 (0.01 mg/kg) or vehicle once daily for 14 days, followed by assessment of novel object recognition (NOR), prepulse inhibition (PPI) of startle response and striatal BDNF levels. SIR significantly attenuated NOR memory as well as PPI, and reduced striatal BDNF levels vs. social controls. Clozapine, ORM-10921 and haloperidol + ORM-10921, but *not* haloperidol alone, significantly improved SIR-associated deficits in PPI and NOR, with ORM-10921 also significantly improving PPI deficits vs. haloperidol-treated SIR animals. Haloperidol + ORM-10921 significantly reversed reduced striatal BDNF levels in SIR rats. α_{2C}-AR-antagonism improves deficits in cognition and sensorimotor gating in a neurodevelopmental animal model of schizophrenia and bolsters the effects of a typical antipsychotic, supporting a therapeutic role for α_{2C}-AR-antagonism in schizophrenia.

1. Introduction

Schizophrenia is a severe neuropsychiatric disorder involving environmental, neurodevelopmental and genetic factors (Sigurdsson, 2016), presenting with positive symptoms (disordered thoughts, delusions, hallucinations, psychosis), negative symptoms (affective flattening, social withdrawal) and cognitive impairments (deficits in executive functioning, working, declarative and recognition memory and attention) (Kahn and Keefe, 2013; Tsapakis et al., 2015). Monoaminergic, glutamatergic and GABAergic mechanisms are implicated in its pathology (Reynolds, 2008; Tsapakis et al., 2015), as is impaired neurotrophin function e.g. brain-derived neurotrophic factor (BDNF) (Favalli et al., 2012; Nieto et al., 2013). Dopamine D_2-receptor antagonism remains a prominent target for antipsychotic efficacy (Millan et al., 2015), with most typical antipsychotics requiring at least 70% D_2-occupancy for clinical efficacy (Farde and Nordström, 1992; Farde et al., 1989). However, D_2-antagonism is associated with severe movement and endocrine disorders (Millan et al., 2015), while D_2-induced striatal toxicity (Dalgalarrondo and Gattaz, 1994; Mion et al., 1991; Tan et al., 2005) appears linked to compromised neurotrophic activity (Pillai et al., 2006; Tost et al., 2010). Indeed, much of the adverse effects of typical

Abbreviations: DI, discrimination index; BDNF, brain-derived neurotrophic factor; α_2-AR, α_2-adrenoceptor; SIR, social isolation rearing/social isolation reared; PPI, prepulse inhibition; NOR, novel object recognition; CLOZ, clozapine; HAL, haloperidol; ORM, ORM-10921; SOC, socially reared controls; ANOVA, analysis of variance; FAM, familiar.

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antipsychotics, as well as being less effective in treating the cognitive and negative symptoms of schizophrenia (Mishara and Goldberg, 2004), may be associated with neurodegenerative effects (Andreassen et al., 1998; Chakos et al., 2005).

In contrast, atypical antipsychotics such as clozapine (CLOZ) present with notably lower rates of D_2 occupancy (~45%) and are associated with better therapeutic outcomes for negative and cognitive symptoms (Keefe et al., 2004; Swartz et al., 2003). Lower D_2-occupancy is also associated with less extrapyramidal side-effects (Harvey et al., 1999), less neurodegenerative effects (Chakos et al., 2005), and may even reverse such effects induced by typical agents (Nel and Harvey, 2003; Pillai et al., 2006). Additional receptor binding profiles, including serotonergic receptor antagonism and α_2-adrenoceptor (α_2-AR) antagonism, could lower the threshold of D_2-occupancy necessary for clinical efficacy (Kalkman and Loetscher, 2003; Kapur et al., 2000; Shahid et al., 2009). CLOZ's more effective management of positive, negative and cognitive symptoms (Swartz et al., 2008) has been linked to effects on central noradrenaline turnover (McMillen and Shore, 1978; Pickar et al., 1992) and antagonism of the α_2-AR (Baldessarini et al., 1992; Kalkman and Loetscher, 2003; Svensson, 2003).

Additional α_2-AR-lytic activity improves antipsychotic-like effects in humans and animals, while the non-selective α_2-AR-antagonist, idazoxan, augments sub-therapeutic doses of antidopaminergic antipsychotics in humans (Litman et al., 1993; Litman et al., 1996) and animals (Marcus et al., 2005, 2010). Improved cognitive performance with a combined antidopaminergic-α_2-AR-lytic approach has been correlated to higher α_{2C}-AR vs. α_{2A}-AR and D_2 receptor occupancy (Kalkman and Loetscher, 2003), and demonstrated as comparable to CLOZ (Marcus et al., 2005). Asenapine, with its greater α_2-AR vs. D_2 affinity (Shahid et al., 2009), elicits similar beneficial effects (Elsworth et al., 2012). However, all α_2-AR-subtypes might not contribute equally to purported antipsychotic and pro-cognitive effects.

α_{2A} and α_{2C}-AR subtypes are widely distributed in the central nervous system (CNS) and are involved in presynaptic inhibition of neurotransmitter release (Hein et al., 1999; Starke, 2001), often having different and even opposing effects on CNS function. Regarding cognition, while non-selective α_2-AR agonists improve cognition in animals (Cai et al., 1993; Carlson et al., 1992), transgenic mouse studies suggest these beneficial effects are due to α_{2A}-AR agonism (Arnsten and Leslie, 1991; Björklund et al., 2001; Franowicz et al., 2002). Conversely, α_{2C}-AR agonism could have detrimental effects on cognition (Björklund et al., 1998; Björklund et al., 1999a; Björklund et al., 1999b). Evidence in humans (Rouru et al., 2013) and rodents (Sallinen et al., 2013a, 2013b; Erasmus et al., 2015) concur that α_{2C}-AR antagonism would improve cognition. Furthermore, the selective α_{2C}-antagonists, JP-1302, ORM-10921 and ORM-12741, also display antipsychotic-like effects (Sallinen et al., 2007; Sallinen et al., 2013a, 2013b). In fact CLOZ exhibits the highest α_{2C}/D_2 ratio among atypical antipsychotics, and markedly higher than haloperidol (HAL) (α_{2C}/D_2 ratio 12 vs. 0.0005) (Kalkman and Loetscher, 2003; Shahid et al., 2009). α_{2C}-AR antagonism might underscore the improved antipsychotic and pro-cognitive effects of CLOZ and makes a provocative case for the role of α_{2C}-AR antagonism in improving treatment outcomes in schizophrenia.

Post-weaning social isolation rearing (SIR) of rodents is a well-described neurodevelopmental animal model of schizophrenia with noteworthy face, construct and predictive validity (Fone and Porkess, 2008; Jones et al., 2011). We set out to determine whether sub-chronic treatment with the selective α_{2C}-AR antagonist ORM-10921 (ORM) is able to reverse SIR-mediated deficits in sensorimotor gating and declarative memory vs. CLOZ and HAL, and whether additional α_{2C}-AR-lytic activity might improve the response to HAL. ORM displays selective α_{2C}-AR binding at appropriate dosages, viz. 10 (binding assay) to 30 (functional assay) fold higher selectivity for rodent α_{2C}- vs. α_{2A}-ARs (Sallinen et al., 2013a). Considering the prominent role of striatal dysfunction in schizophrenia, as well as the neurodegenerative effects of typical antipsychotics (Andreassen et al., 1998; Chakos et al., 2005), and that α_{2C}-ARs are extensively expressed in the striatum (Rosin et al., 1996; Scheinin et al., 1994), striatal BDNF levels were analysed to determine treatment effects on neurotrophin activity.

2. Methods

2.1. Animals

Male Sprague Dawley rats (160–190 g) were bred and cared for at the Vivarium of North-West University, Potchefstroom, South Africa. At weaning (post-natal day 21) the animals were removed from their home cages and blindly randomized to SIR (1 animal/cage) or social rearing (SOC; 3–4 rats/cage) for 8 weeks (until post-natal day 77), whereafter SIR animals were randomized to treatment groups. The rats were reared under identical conditions: cages with sawdust (Möller et al., 2013) and dimensions 230 (h) × 380 (w) × 380 (l) (mm), temperature (21 ± 2 °C), humidity (55 ± 10%), white light (350–400 lx), 12 h light/dark cycle and food and water ad libitum. Animals were bred, supplied, and housed at the Vivarium (SAVC reg. no. FR15/13458; SANAS GLP compliance no. G0019) of the Pre-Clinical Drug Development Platform of the North-West University. All experiments were approved by the AnimCare animal research ethics committee (NHREC reg. no. AREC-130913-015) of the North-West University, and all animals were maintained and all procedures performed in studies involving animals were in accordance to the code of ethics in research, training and testing of drugs in South Africa and complied with national legislation (ethics number: NWU-00050-13-A5). Animals received equal amounts of daily handling, with sawdust changed twice weekly. A qualified animal technologist routinely monitored the animals for evidence of distress or ill health, with no ill effects observed.

2.2. Drug treatment

Drugs were injected subcutaneously (s.c.) (1 ml/kg, pH 4.5–5.5) once daily for 14 days. Early antipsychotic response in schizophrenia is an accurate predictor of later response (Kapur et al., 2005), and thus a 14 day administration period was deemed adequate. CLOZ (Pharmaplan, Johannesburg, South Africa) was dissolved in 1 M glacial acetic acid and administered at a dose of 5 mg/kg (Bakshi et al., 1994; Möller et al., 2011, 2012, 2013; Toua et al., 2010; Zhang et al., 1999b). This dose presents with D_2-receptor occupancy of approximately 25–30% (Natesan et al., 2007, 2008). HAL (Sigma Aldrich) was dissolved in water containing 0.3% tartaric acid (Binder et al., 2001; Ishiwari et al., 2007; Singh et al., 2013) and administered at a non-cataleptic dose of 0.2 mg/kg (unpublished data). This dose is capable of 80% D_2 receptor occupancy (Natesan et al., 2007; Wadenberg et al., 2001), presents with robust antipsychotic activity in animals (Depoortere et al., 1997; Hadamitzky et al., 2007; Kusljic et al., 2006; Varty and Higgins, 1995), and approximates clinically effective doses (Castner et al., 2000). ORM-10921 ((-)-1-methoxymethyl-1(beta)-methyl-1,3,4,5,6,11b(alpha)-hexahydro-2H-11-oxa-4a-aza-benzo[a]fluorine), a gift from Orion Pharma (Orion Corporation, Turku, Finland), was dissolved in saline and administered at a dose of 0.01 mg/kg (Sallinen et al., 2013a) and corroborated in a suitable dose ranging study (0.01–1 mg/kg; unpublished data). Controls received physiological saline (pH 5.5).

2.3. Behavioural tests

Behavioural tests were performed in the same animals beginning with the least stressful test, viz. NOR test on days 12 and 13 with prepulse inhibition (PPI) on day 14, the last day of drug treatment. However as PPI more specifically relates to schizophrenia, this behavioural measure is presented first followed by the NOR test.

2.3.1. Prepulse inhibition (PPI) test

Sensorimotor gating describes the ability to filter and process incoming sensory information, deficits of which may reflect the cognitive fragmentation evident in schizophrenia (Braff and Geyer, 1990). PPI, a behavioural expression of sensorimotor gating, refers to the ability of the animal to attenuate a startle response to an auditory pulse when preceded by a pulse of smaller amplitude (prepulse). Under normal conditions, the presentation of a prepulse inhibits the startle response to the next higher amplitude pulse. In schizophrenia, however, sensorimotor gating mechanisms that regulate incoming sensory stimuli to the brain may be compromised (Geyer et al., 1993; Möller et al., 2011), resulting in attenuation of PPI (Dawson et al., 2000; Li et al., 2009; Young et al., 2009). PPI was assessed in two illuminated and ventilated, sound-attenuated startle chambers containing a Plexiglas cylinder on a platform mounted on a piezo-electric sensor (SR-LAB, San Diego Instruments, San Diego, USA). The sound bursts were delivered and startle responses detected and captured by the SR-Lab software running on a personal computer. Data was processed by an investigator blind to the study. Startle amplitudes are defined as the average of 100 × 1 ms stabilimeter readings collected at stimulus onset. The stabilimeter was calibrated before each session. The protocol is adapted from previously described methods (Geyer and Swerdlow, 2001; Möller et al., 2011; Wang et al., 2003).

PPI was assessed 12 h after the last drug dose on day 14 of drug treatment. The startle session began with a 5-min acclimatization period, during which a 68-dB white background noise was delivered from a speaker on the ceiling of the chamber. This background noise level was maintained throughout the session. The first 10 trials consisted of a single 40 ms 115 dB white-noise startle stimulus (Block 1). The test session then continued with 70 trials of randomly delivered pulses: 20 trials of 115 dB PULSE-ALONE trials (Block 2 and Block 3), 40 PREPULSE trials and 10 trials during which no pulse was delivered. A final 10 trials of single 40 ms 115 dB PULSE-ALONE startle stimuli was delivered (Block 4). Startle responses to Block 1 to 4 of the PULSE ALONE stimuli was used to obtain a measure of response habituation to the repeated delivery of startling pulses. The prepulse trials consisted of a single 115 dB pulse which was preceded (with a time-interval of 80 ms) by a 20 ms non-startling prepulse-stimulus with intensities of 72, 76, 80 or 84 dB (4, 8, 12 and 16 dB over baseline). Thus, a total of 90 trials of startle stimuli were delivered, with an average, but not consistent interval of 25 s.

2.3.2. Novel object recognition (NOR) test

Declarative memory is compromised in schizophrenia (Bowie and Harvey, 2006). The NOR test is a measure of declarative recognition memory that relies on the innate preference of rats to explore novel rather than familiar objects (Ennaceur and Delacour, 1988). The NOR test was performed during the dark cycle 12 h after the last drug administration on the 12th and 13th day of drug treatment, using a black NOR test box (40 × 40 × 70 cm) as previously described (Möller et al., 2013). On day 12, animals were placed into the empty box and allowed to habituate for 10 min. Twenty four hours later, on day 13, the animals were re-introduced to the box for 5 min during the acquisition trial, this time with two identical objects placed opposite each other in the box. The rats were then returned to their home cages and the NOR test box and objects cleaned with soapy water and 70% alcohol to remove any olfactory cues. After 90 min, the recognition trial was conducted in which one of the familiar objects was removed and a new object introduced in its place. Once again the rat was given 5 min to explore the box and objects. Digital video cameras placed above the boxes recorded the animals' activity, with object exploration scored by hand by investigators who were blind to treatment and rearing conditions. Object exploration was considered as active sniffing, licking or physical exploration of the object. Time spent exploring the novel vs. the familiar object was recorded.

More exploration time at the novel object reflects recognition of the familiar object, prompting greater exploration of the novel object. Although time spent at the novel vs. the familiar object can be used to determine recollection within a specific cohort, in order to make more accurate across group comparisons, it is necessary to correct for the total time spent exploring both objects (Broadbent et al., 2010). This correction is done by calculating the so-called "discrimination index" (DI), expressed here as a percentage and calculated according to the formula [(time spent at novel object − time spent at familiar object) / (total time spent exploring both objects) × 100] (Antunes and Biala, 2012).

2.4. Assessment of striatal brain-derived neurotrophic factor (BDNF) levels

BDNF plays an important role in synaptic plasticity, implicated in the processes underlying learning, memory and mood. Striatal BDNF levels were analysed using a Biosensis® BDNF *Rapid*™ ELISA Kit (DivBio, Netherlands). This test has been found to produce highly reliable and accurate measurement of total BDNF (Polacchini et al., 2015). Twenty four hours after the last drug administration, animals were sacrificed by decapitation and the whole brain quickly removed and briefly placed in ice cold double distilled water, with the striata dissected out immediately thereafter on an ice cooled slab. The striata were snap frozen in liquid nitrogen and transferred to a −70 °C freezer. On the day of the analysis, the tissue was thawed on ice, weighed and homogenized in acid extraction buffer (100 μl/10 mg brain tissue) according to the manufacturer's instructions. Sample protein content was determined by the Bradford Protein Assay. Sample supernatants were diluted 40 times and assayed in duplicate. The plate was read in a BioTek SYNERGY/HT microplate reader at 450 nM. Unknown sample BDNF concentrations were calculated using a software program capable of generating a four parameter logistic (4PL) curve fit (regression coefficient > 0.99) derived from the known reference BDNF concentrations supplied by the manufacturer and multiplied by the dilution factor. Final BDNF concentrations were expressed as pg/mg soluble protein.

2.5. Statistical analyses

GraphPad Prism® version 6.01 (GraphPad Software, San Diego California USA, www.graphpad.com) was used for parametric and nonparametric statistical analyses and graphical presentations. Normality of data sets was determined using the Shapiro-Wilk test. Habituation of the startle response to the PULSE-ALONE stimulus was determined using the average startle amplitude values from Block 1 to Block 4 and analysed with a repeated measures two-way analysis of variance (rmANOVA) with Tukey's multiple comparison test for the SIR treatment cohorts and multiple *t*-tests for the SIR vs. SOC cohorts. The percentage PPI (% PPI) for the four prepulse intensities were calculated for each individual rat according to the following formula: % PPI = [(startle response for PULSE ALONE trial) − (startle response for PREPULSE + PULSE trial) / (startle response for PULSE ALONE trial) × 100] (Van den Buuse and Eikelis, 2001). The % PPI across all four prepulse intensities was analysed with unpaired Student's *t*-test for the average % PPI in SIR vs. SOC cohorts and rmANOVA with Tukey's multiple comparison test for the average % PPI for SIR treatment groups (Gacsályi et al., 2013). The DI in the NOR test for the SOC vs. SIR groups were analysed with unpaired Student's *t*-test, and for the SIR treatment groups using Kruskal-Wallis one-way ANOVA for non-parametric data with Dunn's post hoc multiple comparisons. Exploration time for novel vs. familiar objects in the NOR test was compared using unpaired Student's *t*-tests, with Welch's correction applied where the standard deviations were unequal (parametric data) and Mann Whitney *U* tests (for non-parametric data). BDNF data was analysed using Mann-Whitney *U* test for SOC vs. SIR controls and Kruskal-Wallis ANOVA with Dunn's multiple comparisons for SIR treatment groups.

3. Results

3.1. SOC vs. SIR

The mean startle amplitudes of SOC vs. SIR controls during the four blocks of 10 PULSE ALONE (115 dB) stimuli are shown in Fig. 1a. Repeated measures ANOVA showed no significant interaction on mean startle response between the SIR and SOC housed groups (Fig. 1a; $F(1,25) = 0.0008$, $p = 0.98$). Fig. 1b depicts the average % PPI over all 4 prepulse intensities for SOC vs. SIR animals. Unpaired Student's *t*-test showed a significant average decrease in % PPI in SIR vs. SOC animals (Fig. 1b; $t = 5.486$, $df = 25$, $p < 0.0001$).

Fig. 2a depicts the average time spent exploring the novel vs. the familiar object for SOC and SIR animals. SOC animals spent significantly more time exploring the novel vs. the familiar object (Fig. 2a; $U = 30$, $p = 0.004$), while SIR animals showed no difference in preference for the novel vs. familiar object (Fig. 2a; $U = 79$, $p = 0.79$). The DI (%) for SOC vs. SIR animals is depicted in Fig. 2b. Unpaired Student's *t*-test revealed a significant decrease in DI (Fig. 2b; $t = 3.41$, $df = 24$, $p = 0.002$) in SIR vs. SOC controls.

Fig. 3 depicts striatal BDNF levels in SOC vs. SIR animals. Mann-Whitney *U* test indicates that striatal BDNF levels are significantly decreased in SIR vs. SOC animals (Fig. 3; $U = 0.0$, $p = 0.002$).

3.2. Effects of various drug treatments in SIR animals

3.2.1. Prepulse inhibition of the startle response

Fig. 4a depicts the mean startle amplitudes of SIR animals following the various drug treatments during the four PULSE ALONE blocks. Repeated measures ANOVA showed an overall significant effect of drug treatment on startle amplitude ($F(4, 48) = 2.686$, $p = 0.042$) with

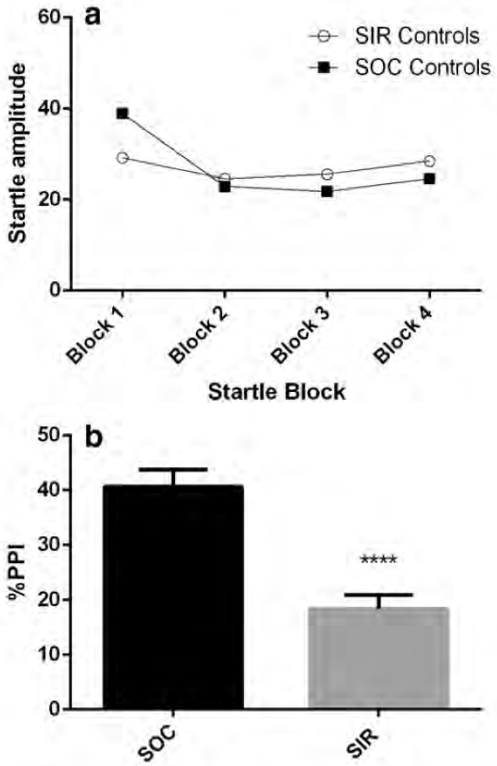

Fig. 1. (a) Startle response habituation to the four startle blocks throughout the startle session in vehicle treated SOC vs. SIR animals (n = 13–14). Repeated measures ANOVA. SOC = socially reared controls, SIR = social isolation reared. (b) Average percentage prepulse inhibition of the startle response in vehicle treated SOC or SIR animals (n = 13–14). Unpaired *t*-test ****p < 0.0001 vs. SOC. SOC = socially reared controls, SIR = social isolation reared controls.

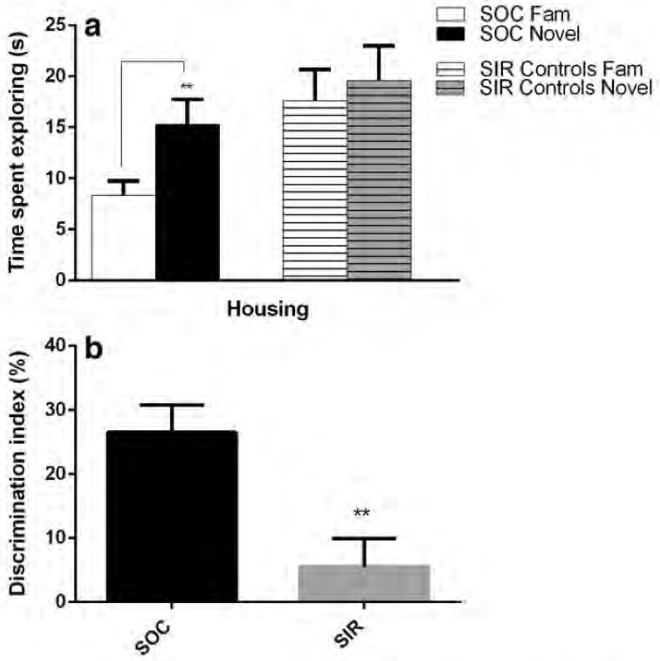

Fig. 2. (a) Average time spent exploring novel and familiar objects in the NOR test in vehicle treated SOC and SIR animals (n = 13–14). Unpaired Student's *t*-tests for novel vs. familiar objects **p < 0.01. Fam = familiar, SOC = socially reared controls, SIR = social isolation reared. (b). DI in vehicle treated SOC or SIR animals (n = 13–14). Unpaired Student's *t*-test. **p < 0.01 drug treatments vs. SOC. SOC = socially reared controls, SIR = social isolation reared.

Tukey's multiple comparison indicating that CLOZ-treated animals had higher startle responses in block 1 vs. other treatment groups (p-values < 0.0001), while there were no differences between groups in blocks 2 to 4, indicating equal habituation to the PULSE-ALONE trial over time with all drug treatments.

The average % PPI for SIR animals over all four prepulse intensities following the various drug treatments is depicted in Fig. 4b. Repeated measures ANOVA showed a significant effect of drug treatment on average % PPI ($F(4, 48) = 7.639$, $p < 0.0001$). Tukey's multiple comparison test showed that CLOZ ($p = 0.04$), ORM ($p = 0.0003$) and ORM + HAL ($p = 0.01$) but NOT HAL significantly increased the % PPI in SIR animals compared to SIR controls (Fig. 4b). Furthermore, both ORM treatment ($p = 0.002$) and HAL + ORM treatment ($p = 0.04$), but not CLOZ ($p = 0.12$) increased % PPI to a significantly higher extent compared to HAL treatment alone (Fig. 4b).

3.2.2. Novel object recognition test

Fig. 5a depicts the average time spent exploring the novel vs. the familiar object for SIR animals receiving drug treatment. Animals treated with CLOZ (Fig. 5a; $t = 2.54$, $df = 9.57$, $p = 0.03$) and ORM (Fig. 5a;

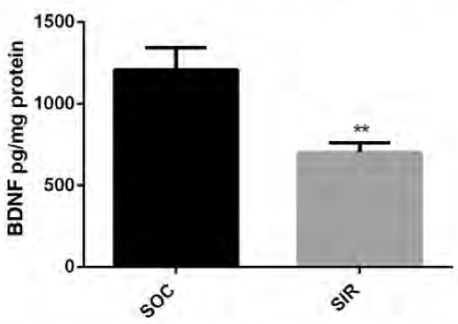

Fig. 3. Striatal BDNF in vehicle treated SOC and SIR animals. (n = 6) Mann-Whitney *U* test. **p < 0.01, vs. SOC. SOC = socially reared controls SIR = social isolation reared.

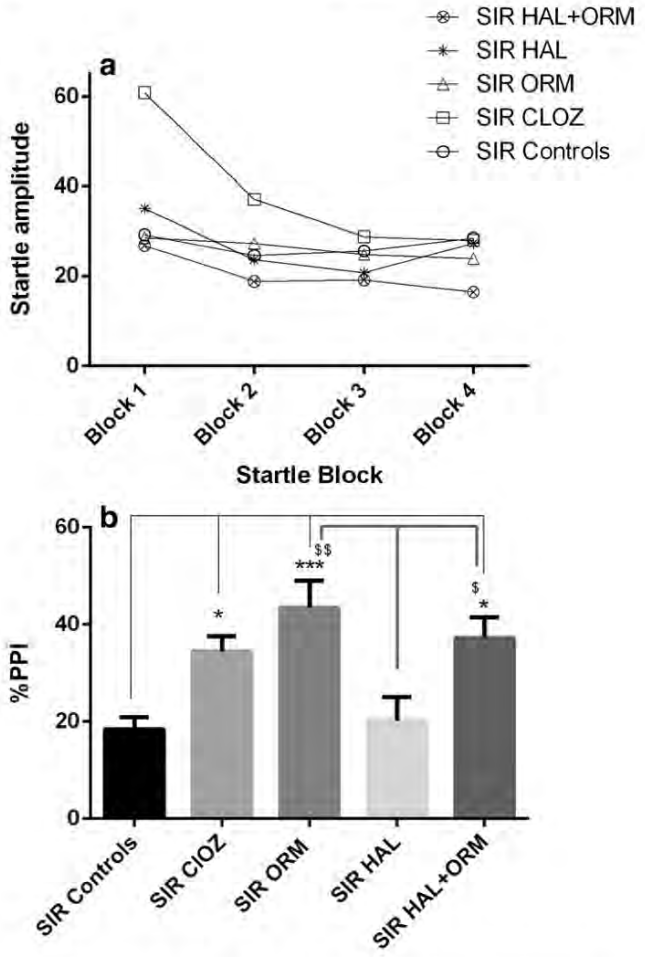

Fig. 4. (a) Startle response habituation to the four startle blocks throughout the startle session for SIR animals following the various drug treatments, as indicated (n = 10–13). SIR = social isolation reared, CLOZ = clozapine 5 mg/kg, ORM = ORM-10921 0.01 mg/kg, HAL = haloperidol 0.2 mg/kg. (b) Average percentage prepulse inhibition of the startle response in SIR animals following the various drug treatments, as indicated (n = 10–13). rmANOVA and Tukey's Multiple comparisons. $^{*}p < 0.05$, $^{***}p < 0.001$ vs. SIR controls, $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ vs. HAL treatment. SIR = social isolation reared, CLOZ = clozapine 5 mg/kg, ORM = ORM-10921 0.01 mg/kg, HAL = haloperidol 0.2 mg/kg.

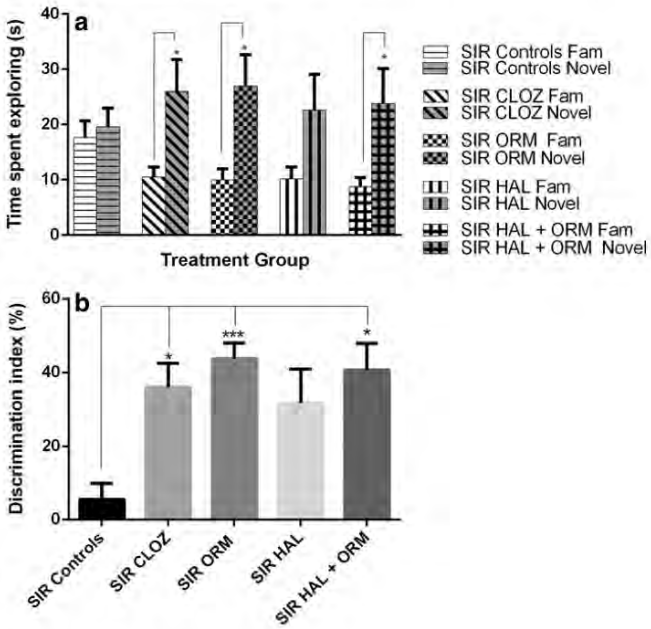

Fig. 5. (a) Average time spent exploring novel and familiar objects in the NOR test in SIR animals following the various drug treatments, as indicated (n = 7–14). Unpaired Student's *t*-tests for novel vs. familiar objects for each drug treatment. $^{*}p < 0.05$; $^{**}p < 0.01$. Fam = familiar, SIR = social isolation reared, CLOZ = clozapine 5 mg/kg, ORM = ORM-10921 0.01 mg/kg, HAL = haloperidol 0.2 mg/kg. (b) NOR test DI in SIR animals following the various drug treatments, as indicated (n = 7–14). Kruskal-Wallis ANOVA and Dunn's Multiple Comparison test. $^{*}p < 0.05$, $^{***}p < 0.001$ drug treatments vs. SIR Controls. SIR = social isolation reared, CLOZ = clozapine 5 mg/kg, ORM = ORM-10921 0.01 mg/kg, HAL = haloperidol 0.2 mg/kg.

$t = 2.76$, df = 9.91, $p = 0.02$) spent significantly more time exploring the novel object (*t*-test with Welch's correction), whilst HAL + ORM-10921 (Fig. 5a; $U = 5$, $p = 0.01$) induced significantly more exploration time of the novel object but not HAL alone (Fig. 5a: $U = 13$, $p = 0.16$) (Mann-Whitney *U* test). However, a trend to improve the exploration time was observed with HAL alone (Fig. 5a).

The DI (%) is depicted in Fig. 5b. Kruskal-Wallis ANOVA indicated a significant effect of treatment with respect to DI (Fig. 5b; Kruskal-Wallis statistic = 20.93, $p = 0.0003$). Dunn's multiple comparisons test showed that CLOZ (Fig. 5b; $p = 0.02$), ORM (Fig. 5b; $p = 0.0004$) and HAL + ORM (Fig. 5b; $p = 0.02$), but not HAL alone (Fig. 5b; $p = 0.14$), significantly increased the DI vs. SIR controls.

3.2.3. Brain-derived neurotrophic factor

Striatal BDNF levels in SIR animals treated with the respective drug treatments is depicted in Fig. 6. Kruskal-Wallis ANOVA indicated a significant effect of treatment on striatal BDNF levels (Fig. 6; Kruskal-Wallis statistic 17.23, $p = 0.002$). Dunn's multiple comparison indicate that striatal BDNF levels are significantly increased in SIR animals treated with HAL + ORM vs. SIR controls (Fig. 6; $p = 0.03$) vs. SIR animals treated only with ORM (Fig. 6; $p = 0.03$), and numerically greater than SIR-CLOZ and SIR-HAL animals. Although CLOZ tended to increase striatal BDNF levels in SIR animals, this did not reach significance (Fig. 6; $p = 0.055$).

4. Discussion

SIR is a neurodevelopmental animal model of schizophrenia that produces long-lasting behavioural alterations in rodents resembling several features of the human disorder, including deficient sensorimotor gating, object recognition and working memory, locomotor hyperactivity, increased anxiety and aggression, decreased social interaction, and impairments in cognitive functioning (Fone and Porkess, 2008;

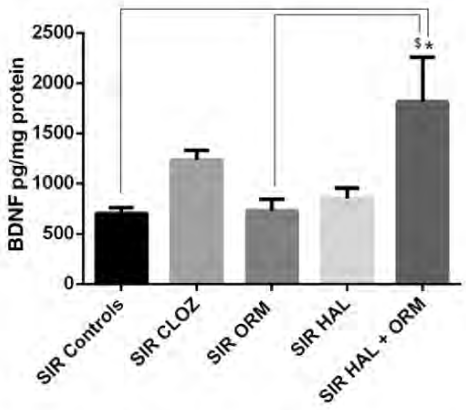

Fig. 6. Striatal BDNF levels in SIR animals following the various treatments, as indicated (n = 6–8). Kruskal-Wallis ANOVA and Dunn's Multiple Comparison test. $^{*}p < 0.05$, drug treatments vs. SIR Controls. $^{\$}p < 0.05$ ORM vs. HAL + ORM. SIR = social isolation reared, CLOZ = clozapine 5 mg/kg, ORM = ORM-10921 0.01 mg/kg, HAL = haloperidol 0.2 mg/kg.

Jones et al., 2011). SIR also induces various neurochemical changes that strongly correlates with that of schizophrenia, including lower frontal cortical DA turnover (Heidbreder et al., 2000), altered frontal cortical D_1- vs. glutamate NMDA receptor binding (Toua et al., 2010), increased striatal D_2-receptor density (King et al., 2009) as well as disordered immune-inflammatory function (Möller et al., 2013). The model also presents with good predictive response to antipsychotic agents (Möller et al., 2011, 2012, 2013; Toua et al., 2010).

4.1. Validation of the social isolation rearing model

Deficits in sensorimotor gating have been suggested to express a pathological process underlying schizophrenia (Braff and Geyer, 1990). This can be measured by assessing PPI, deficits of which have been described in schizophrenia and associated animal models (Braff and Geyer, 1990; Braff et al., 1992; Geyer and Braff, 1987; Möller et al., 2011). Moreover, PPI can be used to assess the efficacy of antipsychotic drugs (Powell and Geyer, 2002). Schizophrenia is also associated with impaired visual object recognition memory (Guillaume et al., 2015). Such impairment in object recognition is also seen in SIR rats (McLean et al., 2010; Möller et al., 2013). Schizophrenia is also known to present with impairment in neurotrophic (BDNF) signalling (Buckley et al., 2007a; Buckley et al., 2007b) that has been linked to neurodegenerative phenomena (Autry and Monteggia, 2012; Steen et al., 2006). In this study, SIR animals presented with significant deficits in sensorimotor gating and declarative memory, as well as reduced striatal BDNF levels compared to SOC controls, confirming its validity for application in the current study.

4.2. Effect of drug treatment on behavioural parameters

The main findings of this study were that CLOZ, ORM and ORM + HAL but not HAL alone were able to improve deficits in both PPI and declarative memory in SIR animals. CLOZ reversed SIR-induced PPI deficits and bolstered object recognition memory, which is in line with earlier pre-clinical (Möller et al., 2011; Möller et al., 2013) and clinical (Sharma et al., 2003; Vollenweider et al., 2006) findings. That ORM-10921 alone improved both PPI and recognition memory is in line with earlier studies (Sallinen et al., 2007; Sallinen et al., 2013a, 2013b). This observation challenges the notion that D_2-receptor antagonism alone represents the primary target in treating psychosis (Millan et al., 2015), especially when considering the relatively low affinity of ORM-10921 for the D_2 receptor (Sallinen et al., 2013a). That CLOZ presents with superior clinical efficacy as well as a much higher α_{2C}/D_2 affinity ratio lends further credence to this proposal.

Although some acute treatment studies have reported improved PPI with HAL (Binder et al., 2001; Varty and Higgins, 1995), here HAL failed to improve deficits in PPI in SIR animals after *sub-chronic* dosing. However, findings described in the literature at higher and lower doses show mixed results for improving PPI in several models of schizophrenia (Hadamitzky et al., 2007; Kusljic et al., 2006; Le Pen and Moreau, 2002; Lipska et al., 1995). Furthermore, although HAL tended to improve recognition memory in this work, it did not do so significantly, which is similar to cognitive effects observed in other schizophrenia models (Horiguchi et al., 2012; Redrobe et al., 2010; Snigdha et al., 2010) and in the clinic (Babin et al., 2011; Davidson et al., 2009). That HAL alone did not improve PPI or NOR deficits could be related to dose. However, HAL is known to compromise frontal cortical function via down-regulation of D_1 receptors, which might also explain these findings (Castner et al., 2000; Harvey et al., 1999). Nevertheless, the aim of this study was to determine whether α_{2C}-AR antagonism could improve the antipsychotic-like effects of HAL. Importantly, the same dose of HAL that proved ineffective on SIR-induced deficits in sensorimotor gating and memory resulted in significantly improved PPI and recognition memory when combined with ORM-10921. This finding suggests that, whatever the exact basis for its lack of efficacy with respect to these parameters, adding α_{2C}-AR-antagonism profoundly bolsters this response. Importantly, the combined response to ORM + HAL was comparable to that of CLOZ, congruent with earlier studies describing combined D_2 plus α_2-AR antagonism (Hertel et al., 1999a; Litman et al., 1996; Marcus et al., 2010).

That both ORM alone and ORM + HAL significantly improved PPI compared to HAL alone further supports a beneficial role for improved antipsychotic and cognitive outcomes with additional α_{2C}-AR antagonism. Such an improvement has been suggested to be mediated by enhanced medial prefrontal cortical (mPFC) dopamine output (Hertel et al., 1999a; Hertel et al., 1999b; Wadenberg et al., 2007) and its prominent role in cognitive function (Arnsten et al., 1994; Sawaguchi and Goldman-Rakic, 1994). CLOZ, as opposed to conventional antipsychotics, also increases dopaminergic output in the rodent mPFC (Moghaddam and Bunney, 1990), suggesting that CLOZ's high affinity for the α_{2C}-AR could partly contribute to its ability to increase cortical dopaminergic output (Svensson, 2003). Furthermore, the addition of α_2-lytic activity to D_2-antagonism facilitates prefrontal cortical glutamatergic transmission similar to that seen with CLOZ, an effect mediated via D_1 receptors, while also effectively reversing NMDA-antagonist induced impairment in working memory similar to CLOZ (Marcus et al., 2005). The addition of ORM-10921 to HAL could therefore benefit cortical dopaminergic and glutamatergic transmission with an improvement in antipsychotic-like and pro-cognitive effects. Thus α_{2C}-AR engagement might reduce the need for high D_2-receptor occupancy in antipsychotic therapy, which would also have important implications with regards to side effects and treatment tolerability. It is now recognized that excessive dopamine release and metabolism mediated by HAL induces oxidative stress that would drive degenerative changes, as noted below (Raudenska et al., 2013).

4.3. Effect of drug treatment on striatal BDNF

Impaired function of neurotrophins such as BDNF has been proposed to contribute to the pathogenesis of schizophrenia (Favalli et al., 2012; Nieto et al., 2013) while long term block of striatal D_2 receptors is associated with motor side-effects that might be mediated via striatal oxidative stress and neurodegeneration (Pillai et al., 2006). That CLOZ, ORM and HAL failed to significantly alter striatal BDNF levels in SIR animals should thus be carefully interpreted, since a more protracted treatment period as well as an extended time line for evaluation might be needed to establish the long term effects of these drugs on neurotrophic changes (Andreassen et al., 1998; Pillai et al., 2006). Clinically CLOZ, but not HAL treatment, is associated with modest increases in serum BDNF levels (Pedrini et al., 2011). How this finding correlates to brain BDNF expression remains unclear, considering that conflicting post-mortem CNS findings are complicated by multiple antipsychotic use in case subjects (Autry and Monteggia, 2012). Nevertheless, we found that CLOZ showed a strong tendency to increase striatal BDNF levels in SIR rats ($p = 0.055$). However, augmentation of HAL with ORM significantly increased striatal BDNF vs. SIR controls in this study. This finding correlates with that of Pillai et al. (2006), who demonstrated that reduced striatal BDNF levels following long term (90–180 days) HAL treatment could be partially reversed by co-administered risperidone or olanzapine, that respectively show α_{2C}/D_2 ratios of 80 and 8 times higher than that of HAL (Kalkman and Loetscher, 2003). Chronic HAL use is associated with excess dopamine release in the striatum (Moghaddam and Bunney, 1990; Westerink et al., 2001), with associated degenerative effects, while striatal α_{2C}-ARs have been proposed to be subtly stimulated by dopamine (Zhang et al., 1999a). Adjunctive α_{2C}-AR antagonism with ORM-10921 might therefore inhibit the detrimental actions of excess dopamine in this region, which in turn could affect BDNF levels as noted here. However, further long term studies on biomarkers of regional brain neuroplasticity and cytoarchitecture are needed, including the striatum, frontal cortex and hippocampus.

5. Conclusion

This study presents the first evidence for the beneficial effects of α_{2C}-AR antagonism (alone and combined with D_2-antagonism) to engender an antipsychotic-like and pro-cognitive response in a neurodevelopmental animal model of schizophrenia. This work supports the argument that the superior antipsychotic effects of CLOZ might be related to combined α_{2C}-AR/D_2 antagonism. Furthermore, preliminary evidence suggests a positive effect of additional α_{2C}-AR antagonism on striatal BDNF levels, especially that following HAL treatment, which may have distinct clinical benefits. Finally, ORM-10921 represents a valuable tool with which to study the effects of α_{2C}-AR antagonism in schizophrenia, while at the same time announcing α_{2C}-AR antagonism as a valuable therapeutic approach in schizophrenia as well as in antipsychotic drug-design.

Author contribution

MU contributed towards the study design, conducted all experiments and processed the data, WD assisted in performing the BDNF assay. Marike Cockeran assisted and advised on all data analyses. BHH designed the study and the original protocol, MS and JS contributed towards the study design and preparation of the manuscript.

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Conflicts of interest

The authors declare that over the past three years, BHH has participated in advisory boards and received honoraria from Servier®, and has received research funding from Servier® and Lundbeck®. ORM-10921 for this study was sponsored by Orion Pharma. Mohammed Shahid and Jukka Sallinen are employees of Orion Pharma. BHH declares that, except for income from the primary employer and research funding from the above-mentioned organisations and agencies, 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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Chapter 4: Manuscript B

The following article has been published online and is currently *in press* in ***Behavioural Pharmacology***, and is entitled:

"The α_{2C}-adrenoceptor antagonist, ORM-10921, exerts antidepressant-like effects in the Flinders Sensitive Line rat"

Preamble

This chapter presents the full-length manuscript as published online ahead of print in *Behavioural Pharmacology*, published by *Wolters Kluwer*. The manuscript is presented in the final form supplied by *Wolters Kluwer*, and can be accessed on-line via the journal's website:

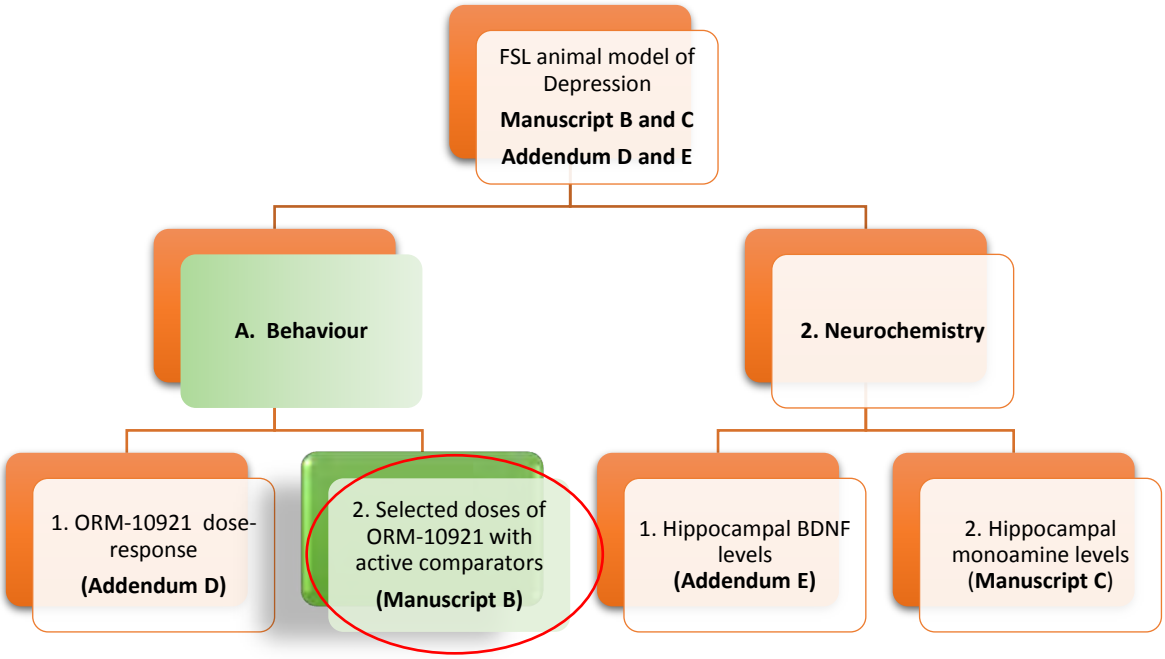
http://journals.lww.com/behaviouralpharm/Abstract/publishahead/The__alpha_2C_adrenoceptor_antagonist,_ORM_10921,.99436.aspx

This article reports the antidepressant-like and pro-cognitive effects of ORM-10921 in the Flinders Sensitive Line (FSL) rat, an animal model of depression, in comparison to imipramine and idazoxan. The superior efficacy of α_{2C}-AR-antagonism over non-selective α_2-AR-antagonism as antidepressant is reported, as well as showing equal efficacy versus a reference antidepressant, imipramine. These data thus support the pre-clinical and clinical development of ORM-10921 and similar α_{2C}-AR-antagonists in the treatment of depression. The diagram on the next page depicts where this manuscript fits into the FSL study design and into the thesis layout, as indicated by the red circle.

Authors' contributions

1. *M Uys* contributed towards the study design and undertook all behavioural and statistical analyses
2. *BH Harvey* designed the study and the original protocol and contributed towards the preparation of the manuscript and finalised it for publication
3. *M Shahid* and *J Sallinen* contributed towards the study design and preparation of the manuscript

All co-authors provided permission to use this manuscript as part of M. Uys' Ph.D thesis, the letters of confirmation are annexed at the end of the thesis.


FSL animal model of Depression
Manuscript B and C
Addendum D and E
A. Behaviour
2. Neurochemistry
1. ORM-10921 dose-response
(Addendum D)
2. Selected doses of ORM-10921 with active comparators
(Manuscript B)
1. Hippocampal BDNF levels
(Addendum E)
2. Hippocampal monoamine levels
(Manuscript C)

The α_{2C}-adrenoceptor antagonist, ORM-10921, exerts antidepressant-like effects in the Flinders Sensitive Line rat

Madeleine M. Uys[a], Mohammed Shahid[c], Jukka Sallinen[d] and Brian H. Harvey[b]

Depression involves deficits in monoaminergic neurotransmission. Differential roles for $\alpha_{2A, B \text{ and } C}$ subtypes of the α_2-adrenoceptor (AR) are evident, with selective α_{2C}-AR antagonists purported to have antidepressant and procognitive properties. However, this has not been demonstrated in a genetic animal model of depression. The role of the α_{2C}-AR in modulating two key depression-related behaviours in the Flinders Sensitive Line (FSL) rat was studied using a dose–response analysis following subcutaneous administration with the selective α_{2C}-AR antagonist ORM-10921 (0.03; 0.3 mg/kg), the nonselective α_2-AR antagonist idazoxan (3 mg/kg), or vehicle once daily for 14 days. Behaviour in the novel object recognition test, forced swim test (FST) and locomotor activity test was assessed. To ratify the validity of the FSL model, the reference tricyclic antidepressant imipramine (15 mg/kg, intraperitoneally) was used as a comparator drug in the FST. FSL rats demonstrated significantly increased immobility and recognition memory deficits versus Flinders Resistant Line controls, with imipramine significantly reversing said immobility. Similarly, ORM-10921 at both doses but not idazoxan significantly reversed immobility in the FST as well as attenuated cognitive deficits in FSL animals. We conclude that selective α_{2C}-AR antagonism has potential as a novel therapeutic strategy in the treatment of depression and cognitive dysfunction.

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Keywords: α_2-adrenoceptor antagonism, α_{2C} selectivity, antidepressant, forced swim test, genetic animal model, learning and memory, procognitive, rat

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Introduction

Major depressive disorder affects more than 300 million people worldwide (Marcus *et al.*, 2012), presenting with symptoms of depressed mood, despair, fatigue, sleep abnormalities, irritability, changes in appetite, poor concentration and anhedonia (Krishnan and Nestler, 2008). Despite the moderate efficacy of current antidepressants, impairments of memory, concentration and cognition remain a persistent problem (McIntyre *et al.*, 2013) and are often the most difficult to treat (Conradi *et al.*, 2011). Amidst new theories regarding the neurobiology of depression, most antidepressants directly or indirectly increase synaptic release and/or accumulation of monoamine neurotransmitters, thereby consolidating the construct and predictive validity of the biogenic amine hypothesis (Brand *et al.*, 2015).

α_{2C}-Adrenoceptors (α_{2C}-ARs) are extensively expressed in the striatum and hippocampus, with similar distribution in humans and rodents (Scheinin *et al.*, 1994; Rosin *et al.*, 1996; Fagerholm *et al.*, 2004; Fagerholm *et al.*, 2008). The role of the α_2-adrenoceptor (α_2-AR) in antidepressant action is well recognized (Cottingham and Wang, 2012). Mirtazapine and mianserin, at least in part, exert their antidepressant effects through antagonism of the noradrenergic α_2-autoreceptor and -heteroreceptor (Anttila and Leinonen, 2001), leading to increased noradrenaline and serotonin release (Blier, 2003). Similarly, postsynaptic α_2-ARs are implicated in mediating behavioural effects of the tricyclic antidepressants (TCAs) in a chronic stress paradigm (Yalcin *et al.*, 2005) and in the forced swim test (FST) (Cervo *et al.*, 1990; Rénéric *et al.*, 2001; Zhang *et al.*, 2009). Of note, the effects of TCAs have been shown to be specifically mediated by the intact α_{2A}-AR subtype (Schramm *et al.*, 2001; Cottingham and Wang, 2012). On the other hand, some studies show further decreases in immobility in the FST when fluoxetine and venlafaxine are augmented with an α_2-AR antagonist (Dhir and Kulkarni, 2007). However, whereas some acute FST studies demonstrate the antidepressant-like effect of α_2-AR antagonism *per se* (Castagné *et al.*, 2009), these effects have not consistently been duplicated in acute studies (Rénéric *et al.*, 2001; Wesolowska, 2007; Zhang *et al.*, 2009), and neither have they been corroborated in chronic studies.

Studies in transgenic mice, where the α_{2A}-AR and α_{2C}-AR are overexpressed or deleted, indicate that α_{2A}-AR knockout (KO) increases immobility in the FST (Schramm *et al.*, 2001), whereas α_{2C}-AR KO decreases immobility (Sallinen *et al.*, 1999; Scheinin *et al.*, 2001). These findings suggest that nonselective α_2-AR blockade may have a weakened antidepressant effect compared with selective α_2-AR

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modulation. Although both the α_{2A}-AR and the α_{2C}-AR modulate presynaptic neurotransmitter release in the central nervous system, the α_{2A}-AR constitutes the majority of α_2-AR in the central nervous system (~90%) (Bücheler *et al.*, 2002) and emerges as the primary autoreceptor and heteroreceptor (Hein *et al.*, 1999; Starke, 2001). However, the potency and affinity of noradrenaline at the α_{2C}-AR exceeds that of the α_{2A}-AR (Hein *et al.*, 1999), while cerebral α_{2C}-AR density but not α_{2A}-AR density is increased by very low synaptic availability of noradrenaline (Ordway, 1995). These findings suggest that in conditions characterized by deficits in noradrenergic transmission, the α_{2C}-AR subtype could be a viable therapeutic target. Preliminary studies in rodents have associated α_{2C}-AR antagonism, but not α_{2A}-AR antagonism, with increased synthesis of the noradrenaline and dopamine precursor 3,4-dihydroxyphenylalanine (Esteban *et al.*, 1996). Furthermore, activation of α_2-ARs on serotoninergic terminals inhibits serotonin release (Starke and Montel, 1973), and, whereas α_{2A}-ARs are more dominant than α_{2C}-ARs in this response, α_{2C}-AR antagonism also beneficially influences serotonin release (Scheibner *et al.*, 2001). This response is especially relevant in the treatment of depression. Thus, α_{2C}-AR antagonism (but not α_{2A}-AR antagonism) may better harness antidepressant-like effects than nonselective α_2-AR-modulating drugs. In agreement, the selective α_{2C}-AR antagonists JP-1302 and ORM-10921 have significant antidepressant-like activity in Sprague Dawley rats (Sallinen *et al.*, 2007, 2013a). Concerning cognition, although α_2-AR agonists clonidine and medetomidine improve cognition (Carlson *et al.*, 1992; Cai *et al.*, 1993), these effects seem to be mediated more by α_{2A}-AR than by α_{2C}-AR agonism (Arnsten and Leslie, 1991; Björklund *et al.*, 2001; Franowicz *et al.*, 2002). Moreover, studies in transgenic mice suggest detrimenral effects for α_{2C}-AR agonism (Björklund *et al.*, 1999a, 1999b), raising the possibility that α_{2C}-AR antagonism might elicit beneficial effects in cognitive tasks. Indeed, the highly selective α_{2C}-AR antagonist, ORM-12741, improves memory in both aged rats (Sallinen *et al.*, 2013b) and in humans with Alzheimer's disease (Rouru *et al.*, 2013).

Flinders Sensitive Line (FSL) rats model certain biobehavioural characteristics of depression (Overstreet and Wegener, 2013), including impaired declarative memory (Abildgaard *et al.*, 2011). Moreover, increased α_2-AR density has been described in FSL rats versus Sprague Dawley controls (Lillethorup *et al.*, 2015), the latter findings being congruent with postmortem studies in depressed humans (González *et al.*, 1994; de Paermentier *et al.*, 1997). FSL rats therefore offer a valuable translational platform with which to study the therapeutic prospects of α_{2A}-AR versus α_{2C}-AR modulation.

Considering the paradoxical findings for α_{2A}-AR versus α_{2C}-AR in mood and cognition, the pharmacological effects of selective α_{2C}-AR antagonism on these parameters warrants reappraisal. ORM-10921 displays a 100-fold higher selectivity for rodent α_{2C}-AR over α_{2A}-ARs (Sallinen *et al.*, 2013a), showing selective α_{2C}-AR binding at appropriate dosages. We have investigated the effect of subchronic ORM-10921 on immobility and escape strategies in the FST, and on novel object recognition (declarative) memory in FSL rats, comparing this response to the nonselective α_2-AR antagonist, idazoxan. Flinders Resistant Line (FRL) rats were used as control. To futther validate the FST findings, the reference TCA, imipramine, was used as an additional comparator to reinforce predictive validity.

Methods

Subjects

The original colonies of FSL and FRL rats were obtained from Dr David H Overstreet, University of North Carolina, Chapel Hill, North Carolina, USA. Eight-week-old male FSL and FRL rats were used in the study. The rats were reared under identical conditions: cages [230 × 380 × 380 mm (height × width × length)], temperature (21 ± 5°C), humidity (50 ± 10%), white light (350–400 lux), 12 h light/dark cycle and free access to food and water. Animals were bred, supplied and housed at the Vivarium (SAVC reg no. FR15/13458; SANAS GLP compliance no. G0019) of the Preclinical Drug Development Platform of the North-West University (NWU). All experiments were approved by the AnimCare animal research ethics committee (NHREC reg. number AREC-130913-015) at NWU. All animals were maintained and procedures performed in accordance with the code of ethics in research, training and testing of drugs in South Africa, and complied with national legislation (ethics approval number: NWU-00050-13-A5).

FRL rats are widely used as the control for FSL rats, particularly with respect to their response in the FST (Knapp *et al.*, 2014; Overstreet *et al.*, 1994). FRLs did not receive any treatment other than vehicle, and were included to confirm the depressive phenotype of the FSL rat. As FSL rats generally require a chronic antidepressant treatment paradigm to evoke a noteworthy reduction in immobility in the FST (Overstreet and Wegener, 2013), a 14-day treatment paradigm was chosen, which also simulates the equivalent human therapeutic response (Overstreet, 2012).

Locomotor activity

Locomotor activity was determined immediately before the FST on day 14 of drug treatment to ensure that changes observed in immobility were not attributable to alterations in locomotor activity. General locomotor activity was assessed for 5 min using a Digiscan Animal Activity Monitor (Omnitech Electronics, Columbus, Ohio, USA). Each monitor consisted of a 42 × 42 × 30.5 cm clear Plexiglas box with a grid of 16 infrared beams 2.5 cm apatt from front to back and from side to side. Monitors were connected to a Digiscan Analyser that collated the transmitted activity from beam breaks in digital form with subsequent analysis using a personal computer. Horizontal

activity was measured as the distance travelled in cm as determined by horizontal beam breaks.

Forced swim test

The FST is a well-described predictive model for antidepressant drug screening (Porsolt *et al.*, 1978; Petit-Demouliere *et al.*, 2005). The FST was conducted 12 h after the final drug administration on day 14 of drug treatment. On the test day, animals were habituated to the test room for 30 min, after which they were placed in individual inescapable cylinders containing 30 cm of water (25°C). As is commonly done in other studies (Gomez-Galan *et al.*, 2013; du Jardin *et al.*, 2016; Eskelund *et al.*, 2016), no preswim was applied in this study as FSL rats innately show higher immobility than FRL controls (Schiller *et al.*, 1992; Pucilowski and Overstreet, 1993; Overstreet *et al.*, 2005). Swim activity was recorded digitally for 7 min, with the first and last minute of the recording discarded, and 5 min of swim activity recorded by a researcher blind to the treatment groups. Afterwards the animals were dried and warmed and returned to their home cages. Observations were divided into 5 s intervals during which the times spent indulging in immobility and swimming or climbing/escape/struggling behaviour were recorded according to the guidelines of Lucki (1997). Swimming is deemed an expression of serotonergic-driven behaviour with climbing an indication of noradrenergic-driven behaviour (Detke *et al.*, 1995).

Novel object recognition test

The novel object recognition test (NORT) is a measure of declarative recognition memory, which relies on the innate preference of rats to explore novel objects more than familiar objects (Ennaceur and Delacour, 1988). The NORT was performed during the dark cycle on days 12 and 13 of drug treatment (ending on the day before the FST), 12 h after the last drug administration, as previously described (Möller *et al.*, 2013). The NORT box was black with dimensions 40 × 40 × 70 cm. On day 12, animals were allowed to explore the empty box for 10 min during the habituation session. Twenty-four hours later, they were reintroduced to the box during the acquisition trial, this time with two identical objects placed opposite each other, which they were allowed to explore for 5 min. Thereafter the rats were returned to their home cages. Ninety minutes later, one familiar object was placed opposite a novel object in the NORT box and the rat was once again allowed to explore the box for 5 min. The entire box and both the familiar and novel objects were removed and cleaned with soapy water and 70% alcohol before and after each test trial to remove any olfactory cues that might adversely affect unbiased exploration in the subsequent trial (Antunes and Biala, 2012).

The time spent exploring the novel versus the familiar object was scored using video recording and hand scoring. Object exploration was considered as active sniffing, licking or physical exploration of the object. Animals that failed to accumulate a total exploration time of 6 s or more or that failed to explore both objects were excluded from the study (du Jardin *et al.*, 2016). Data are expressed as the discrimination index (DI), which was calculated according to the formula [(time spent at novel object – time spent at familiar object)/(total time spent exploring both objects)] × 100 (Antunes and Biala, 2012). Essentially, more exploration time at the novel object reflects accurate recollection that the familiar object has been presented before, thus prompting greater exploration of the novel object. Although the latter can be used to determine recollection within a specific drug cohort, the DI corrects for the time spent exploring both objects, allowing for comparisons between groups. A larger DI indicates superior declarative memory.

Drugs

Drugs were dissolved in physiological saline, and drug or vehicle (saline) was injected subcutaneously or intraperitoneally once a day for 14 days at an injection volume of 1 ml/kg. ORM-10921, a gift from Orion Pharma (Orion Corporation, Turku, Finland), was administered subcutaneously at doses of 0.03 and 0.3 mg/kg, on the basis of an earlier study (Sallinen *et al.*, 2013a). Idazoxan hydrochloride (IDAZ) (Sigma Aldrich, Johannesburg, South Africa) was administered subcutaneously at a dose of 3 mg/kg, on the basis of previously observed anti-immobility effects in acute studies at 1, 4 and 8 mg/kg (Rénéric *et al.*, 2001; Castagné *et al.*, 2009). Imipramine hydrochloride (IMI) (Sigma Aldrich) was administered intraperitoneally at a dose of 15 mg/kg, based on its well-described antidepressant-like effects in the FST (Chen *et al.*, 2010; Castagné *et al.*, 2011; de Morais *et al.*, 2014).

Statistical analyses

Normality of data was determined using the Shapiro–Wilk test. To establish the face and predictive validity of the FSL model, FSL controls, FRL controls and FSL + IMI groups were compared with one way analysis of variance (ANOVA) with regards to the FST and locomotor activity. Since FSL + IMI rendered the NORT invalid (see Results for details), FSL controls and FRL controls were compared in the NORT using the unpaired Student's *t*-test to validate the impaired cognition observed in FSL rats relative to the FRL controls. Welch's correction was applied to *t*-tests with unequal standard deviations. Exploration time for novel versus familiar objects in the NORT was compared using paired Student's *t*-tests (parametric data). Where the Shapiro–Wilk normality test indicated a non-Gaussian distribution, the Wilcoxon matched-pairs signed rank test was conducted. Statistical differences between FSL controls and FSL drug treatment groups in the FST and NORT were analysed using ANOVA with Tukey's post-hoc multiple

comparisons. All data are expressed as mean ± SEM unless otherwise stated, with *P*-value less than or equal to 0.05 denoting statistical significance and NS denoting no statistically significant difference between the relevant groups. GraphPad Prism 6 (GraphPad Software Inc., La Jolla, California, USA) was used for data representation and all statistical analyses.

Results

Validation of the Flinders Sensitive Line model of depression

Table 1 presents evidence in support of the face and predictive validity of the FSL rat in modelling depression under our conditions of study, compared with its FRL control. Here three behavioural tests of relevance to depression are presented – namely, the FST, locomotor activity and the NORT – to demonstrate face validity. Predictive validity is presented in the form of treatment response of FSL rats to subchronic exposure to IMI, as assessed in the animal activity cages and the FST. Unfortunately IMI-treated FSL animals were excluded from the NORT analyses because they did not meet the minimum requirements for exploratory activity (see later for detail).

One-way ANOVA showed a significant difference in locomotor activity between the different treatment groups [$F(2, 28) = 7.97$, $P < 0.002$; Table 1]. Tukey's post-hoc test indicated no significant difference in locomotor activity between FSL and FRL controls. However, the FSL + IMI group showed significantly attenuated locomotor activity compared with FSL ($P < 0.002$; Tukey's test) and FRL ($P < 0.02$; Tukey's test).

In the FST, ANOVA indicated a significant treatment effect on climbing [$F(2, 28) = 8.10$, $P < 0.002$] and immobility [$F(2, 28) = 6.26$, $P < 0.01$], but not on swimming [$F(2, 28) = 0.18$, NS]. Tukey's post-hoc analysis indicated that FSL controls displayed significantly increased immobility ($P < 0.01$) and decreased climbing ($P < 0.002$) versus FRL (Table 1). On the other hand, FSL animals treated for 14 days with IMI showed significantly decreased immobility ($P < 0.02$) and increased climbing ($P < 0.01$) versus FSL controls, although no significant difference in climbing ($P = 0.87$) or immobility ($P = 0.7$) versus FRL controls (Table 1).

In the NORT, FSL controls displayed a significantly lower DI compared with FRL controls ($P < 0.001$; Table 1, Student's *t*-test with Welch's correction), although both FSL and FRL controls spent significantly more time exploring the novel object than the familiar object (Fig 4; $P < 0.002$ for both groups, Wilcoxon matched-pairs signed rank test).

Effects of drug treatment on behaviour of Flinders Sensitive Line animals

Figures 1–4 present the comprehensive drug treatment challenges in FSL rats. Here the dose response to ORM-10921 is compared with vehicle treatment (control), the nonselective α_2-AR antagonist IDAZ, and the reference antidepressant IMI. Results for the IMI group are only reported for the FST and for locomotor activity as only four out of 11 IMI-treated rats met the minimum criteria for total exploration time in the NORT, i.e. more than 6 s (Table 1), five of which showed no exploration whatsoever. The remaining IMI-treated subjects showed a higher DI versus FSL controls ($P < 0.002$, data not shown), although the small number of subjects ($n = 4$) was deemed unsuitable for inclusion in a multiple comparison analysis alongside the other FSL drug treatment groups.

Locomotor activity

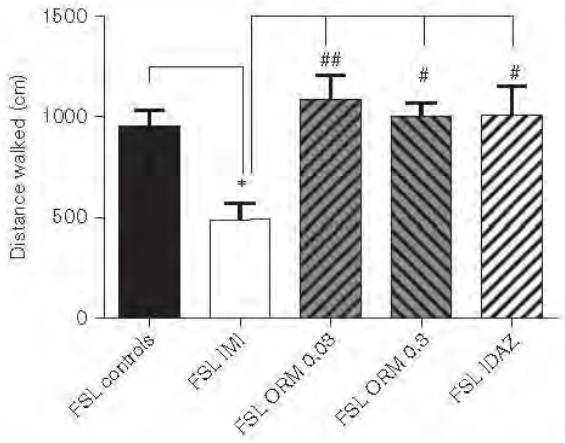
Locomotor activity results for FSL treatment groups are presented in Fig. 1. ANOVA showed significant differences in locomotor activity between all FSL treatment groups [Fig. 1; $F(4,45) = 4.72$; $P < 0.005$]. Tukey's multiple comparison showed significant differences between IMI-treated

Table 1 Validation of the Flinders Sensitive Line animal model of depression

	Validity (mean ± SEM)				
	Face		Predictive		
Behavioural tests	FRL	FSL	FSL + IMI	Statistical analysis	*P*-value
Locomotor activity (cm)	838.20 ± 96.95	953.50 ± 80.47	490.0 ± 77.68	ANOVA and Tukey's post-hoc	0.020* FRL vs. FSL + IMI 0.002** FSL vs. FSL + IMI
FST: immobility (s)	156.40 ± 10.16	211.00 ± 12.06	160.5 ± 14.05	ANOVA and Tukey's post-hoc	0.009** FSL vs. FRL 0.019* FSL vs. FSL + IMI
Climbing (s)	113.60 ± 8.18	61.00 ± 7.00	106.5 ± 13.79	ANOVA and Tukey's post-hoc	0.002** FSL vs. FRL 0.010* FSL vs. FSL + IMI
Swimming (s)	30.00 ± 5.64	28.00 ± 6.33	33.00 ± 5.333	ANOVA	NS
NORT (discrimination index)	35.99 ± 4.54	13.09 ± 2.25	Test invalidated	Unpaired *t*-test with Welch's correction (FSL vs. FRL)	0.0006***

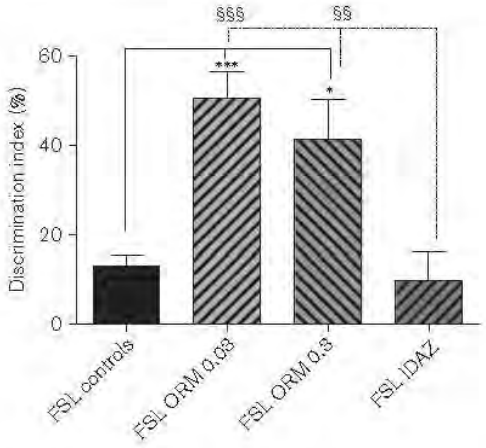
Face (depressive phenotype) validity of the FSL rat is described, as assessed in the FST and NORT, whereas predictive (treatment response) validity is shown following the response to IMI, as assessed in the FST, as well as effect of strain/treatment on locomotor activity.
ANOVA, analysis of variance; FRL, Flinders Resistant Line; FSL, Flinders Sensitive Line; FST, forced swim test; IMI, imipramine hydrochloride; NORT, novel object recognition test; NS, non-significant.
*$P < 0.05$.
**$P < 0.01$.
***$P < 0.001$ ($n = 10$–11).

Fig. 1



Locomotor activity of FSL animals treated with saline vehicle or with the various drug treatments as indicated ($n = 10$ per group). Analysis of variance and Tukey's post-hoc comparison. $^{*}P < 0.05$ versus FSL controls, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus IMI. FSL, Flinders Sensitive Line; IDAZ, idazoxan 3 mg/kg; IMI, imipramine 15 mg/kg; ORM, ORM-10921.

Fig. 3



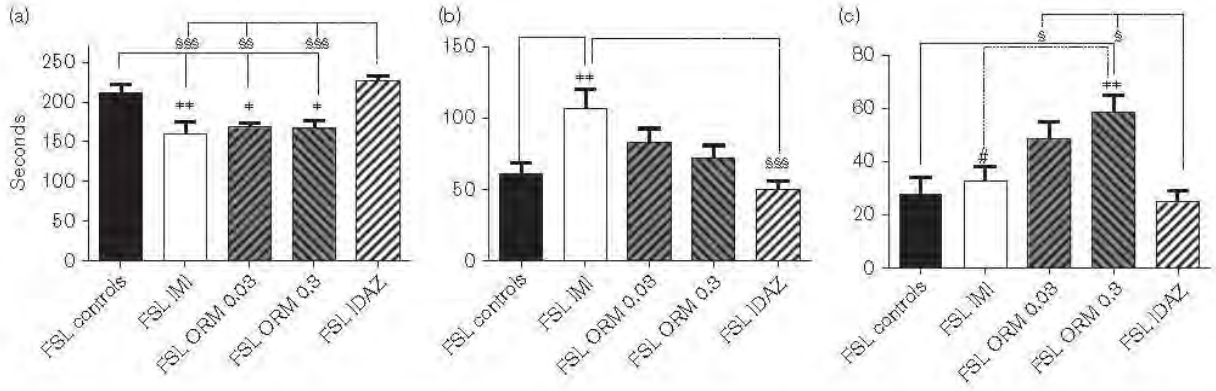
Discrimination index in the NORT in FSL animals following the various drug treatments as indicated ($n = 7$–10). Analysis of variance and Tukey's multiple comparison. $^{*}P < 0.05$, $^{***}P < 0.001$ versus FSL controls. $^{\S\S}P < 0.01$, $^{\S\S\S}P < 0.001$ versus IDA. Controls, Flinders Sensitive Line controls; FSL, Flinders Sensitive Line; IDAZ, idazoxan 3 mg/kg; NORT, novel object recognition test; ORM, ORM-10921.

animals versus FSL controls (Fig. 1; $P < 0.05$) as well as versus ORM 0.03 ($P < 0.005$), ORM 0.3 ($P < 0.02$) and IDAZ ($P < 0.02$). While ORM 0.03, ORM 0.3 and IDAZ treatment groups did not show altered locomotor activity versus FSL controls or versus any other treatment group (Fig. 1), IMI significantly reduced said activity ($P < 0.01$).

Forced swim test

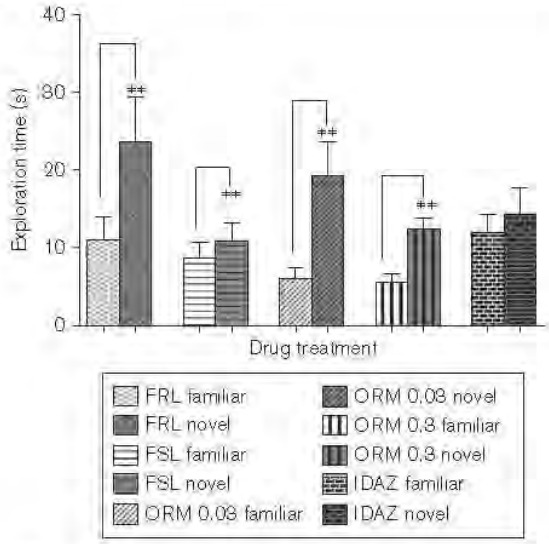
FST results for FSL treatment groups are depicted in Fig. 2. ANOVA showed significant differences between all FSL treatment groups with respect to immobility [Fig. 2a; $F(4,45) = 8.81$; $P < 0.001$], climbing [Fig. 2b; $F(4,45) = 5.48$; $P < 0.001$] and swimming behaviour [Fig. 2c; $F(4,45) = 6.22$; $P < 0.001$]. IMI significantly decreased immobility versus FSL controls (Fig. 2a; $P < 0.01$) and versus IDAZ ($P < 0.0001$), while also significantly increasing climbing versus FSL controls (Table 1 and Fig. 2b; $P < 0.01$) and IDAZ ($P = 0.0008$). ORM-10921 at 0.03 and 0.3 mg/kg significantly decreased immobility in FSL animals compared with FSL controls (Fig. 2a; $P < 0.05$ for both doses, Tukey's test) and compared with IDAZ ($P < 0.002$ for both doses, Tukey's test). Although climbing behaviour was not significantly increased in animals

Fig. 2



Immobility (a), climbing (b) and swimming (c) in the FST in FSL animals treated subcutaneously for 14 days with the various drug treatments, as indicated ($n = 10$ per group). Analysis of variance and Tukey's multiple comparison. $^{*}P < 0.05$, $^{**}P < 0.01$ versus FSL controls, $^{\S}P < 0.05$, $^{\S\S}P < 0.01$, $^{\S\S\S}P < 0.001$ versus IDAZ 3 mg/kg drug treatment, $^{\#}P < 0.05$ versus IMI 15 mg/kg treatment. FSL, Flinders Sensitive Line control; FST, forced swim test; IDAZ, idazoxan 3 mg/kg; IMI, imipramine 15 mg/kg; ORM, ORM-10921.

Fig. 4



Time spent exploring novel and familiar objects in the NORT following the various drug treatments as indicated ($n = 7$–10). FRL controls received saline vehicle. All other drug treatments were conducted in FSL animals. Paired *t*-tests or Wilcoxon matched-pairs test for novel versus familiar objects for each drug treatment. $^{**}P < 0.01$. Familiar, familiar object; FRL, Flinders Resistant Line controls; FSL, Flinders Sensitive Line controls; IDAZ, idazoxan 3 mg/kg; NORT, novel object recognition test; novel, novel object; ORM, ORM-10921.

treated with either dose of ORM-10921 compared with FSL controls, ORM-10921 0.3 mg/kg significantly increased swimming behaviour compared with FSL controls (Fig. 2c; $P = 0.005$, Tukey's test) and versus IMI-treated FSL rats (Fig. 2c; $P < 0.03$, Tukey's test), whereas ORM-10921 0.03 mg/kg tended to increase swimming behaviour compared with FSL controls. Furthermore, ORM-10921 0.03 mg/kg and ORM 0.3 mg/kg both significantly increased swimming behaviour compared with IDAZ 3 mg/kg (Fig. 2b; $P < 0.05$ and $P < 0.001$, respectively, Tukey's test). IMI did not affect swimming in FSL animals vs. control, while IDAZ failed to alter behaviour in any of the three parameters analysed compared with FSL controls.

Novel object recognition test

In the NORT, the DI corrects for the total exploration time to give an index of preference for the novel object. Only four of the 11 subjects treated with IMI met the minimum inclusion criteria of total exploration time of more than 6 s, five of which showed zero exploration activity. These data effectively invalidated the NORT as a means of behavioural assessment for this treatment group, and thus the IMI data are not included in the data analyses for the NORT. One-way ANOVA of the total exploration time of all other FSL treatment groups did not show any significant differences [$F(3, 31) = 2.13$, NS] (data not shown).

The DI in all FSL treatment groups is presented in Fig. 3, with ANOVA showing significant group differences ([$F(3,31) = 12.33$; $P < 0.0001$]. Post-hoc analysis indicated that both doses of ORM-10921 significantly increased the DI compared with FSL controls [Fig. 3; $P < 0.001$ (0.03 mg/kg) and $P < 0.02$ (0.3 mg/kg), Tukey's test], as well as significantly increased the DI versus IDAZ treatment [Fig. 3; $P < 0.001$ (0.03 mg/kg) and $P < 0.005$ (0.3 mg/kg), Tukey's test]. IDAZ did not significantly affect object recognition memory compared with FSL controls (Fig. 3).

Figure 4 shows the time spent exploring the novel versus familiar object for all treatment groups. Here, Wilcoxon matched-pairs signed rank test showed no significant difference between exploration time for novel versus familiar objects for IDAZ 3 mg/kg cohorts (Fig. 4; $P = 0.38$, NS). However, paired *t*-tests showed a statistically significant difference in novel versus familiar object exploration time in both ORM-10921 cohorts (Fig. 4; 0.03 mg/kg: $P < 0.005$ and 0.3 mg/kg: $P < 0.005$).

Discussion

As a genetic animal model of depression, FSL rats have distinct disturbances in serotonergic and noradrenergic systems that parallel depressive symptoms in humans (Overstreet and Wegener, 2013), such as reduced limbic serotonin synthesis (Hasegawa *et al.*, 2006; Ruhé *et al.*, 2007), altered noradrenergic activity and increased α_2-AR binding (González *et al.*, 1994; González-Maeso *et al.*, 2002; Escribá *et al.*, 2004; Lillethorup *et al.*, 2015). FSL rats also show reductions in the vesicular monoamine transporter in limbic regions (Schwartz *et al.*, 2003) and altered noradrenergic levels that are normalized after antidepressant treatment (Zangen *et al.*, 1999). In this study, FSL rats displayed significant swim test immobility and deficits in recognition memory relative to FRL controls (Table 1), in line with the literature (Overstreet *et al.*, 2005; Abildgaard *et al.*, 2011; Overstreet and Wegener, 2013), whereas increased immobility in the FST was significantly abrogated by subchronic IMI treatment (Table 1). Adopting an immobile posture in the FST reflects failure in persistent escape-directed behaviour, for example, swimming and climbing (Lucki, 1997), variations of which are extensively used to predict antidepressant drug response in rodents (Petit-Demouliere *et al.*, 2005). IMI is widely regarded as a reference antidepressant with well-described antidepressant-like effects in the FST (Kitamura *et al.*, 2002, 2008; Morley-Fletcher *et al.*, 2004; Chen *et al.*, 2010; Ferreira *et al.*, 2012) in FSL but not in FRL animals after chronic (Schiller *et al.*, 1992; Chen *et al.*, 2010) but not acute (Schiller *et al.*, 1992; Overstreet *et al.*, 2005) treatment. Here we have reaffirmed this attribute following a 14-day treatment regimen, strengthening the predictive validity not only of the FSL model but also of the FST procedure as a suitable research platform for use in this study.

Subchronic treatment with the α_{2C}-AR antagonist ORM-10921 but not the nonselective α_2-AR antagonist IDAZ reversed swim test immobility as well as deficits in object

recognition memory in these animals, without any associated effects on locomotor activity. Interestingly, the antidepressant-like effects of ORM-10921 in the FST were related to increased swimming, a serotonergic-mediated behaviour (Detke *et al.*, 1995; Lucki, 1997). When using IMI as a reference comparator, we found its antidepressant effects to be unimpaired by its apparent hypolocomotor properties, although in many animals IMI abolished the exploratory activity necessary for validation in the NORT. Although findings in the literature are inconsistent (Strekalova *et al.*, 2013), chronic treatment with IMI does generally decrease locomotor activity in rodents (Kos *et al.*, 2006; Diniz *et al.*, 2011) and in felines (Zagrodzka *et al.*, 1987), a factor that would negatively affect exploratory activity (Mogensen *et al.*, 1994) as observed here in the NORT. Cerebral concentrations of IMI are reduced to below detection levels 10 h after acute drug administration (Daniel *et al.*, 1981), thus making it improbable that hypolocomotion noted here could be an acute effect. Instead, chronic IMI dosing is eliminated more slowly (Daniel *et al.*, 1981), thereby possibly allowing its sedative effects to impair locomotor activity required for exploration in the NORT, albeit unaffecting brain functions required in the FST. Importantly, both doses of ORM-10921 engendered similar anti-immobility (antidepressant-like) effects in the FST as did IMI, although IMI presented with more pronounced climbing (noradrenergic) and ORM-10921 with more pronounced swimming (attributed to a serotonergic mechanism) activities.

Although early clinical evidence with IDAZ support possible efficacy in depression (Osman *et al.*, 1989; Grossman *et al.*, 1999), and later studies have demonstrated the benefit of α_2-AR antagonism as an augmentation strategy (Sanacora *et al.*, 2004), the effects of IDAZ in preclinical models of depression are mixed, with reduced immobility in the FST described in some acute preclinical studies (Castagné *et al.*, 2009) but not in others (Cervo *et al.*, 1990; Rénéric *et al.*, 2001; Wesolowska 2007; Zhang *et al.*, 2009). As augmentation to combined desipramine and fluoxetine therapy, IDAZ further reduces swim test immobility in a response said to be attributed to additional α_2-AR antagonism (Zhang *et al.*, 2009). However, in the current study IDAZ was employed as a nonselective α_2-AR antagonist comparator to the effects of selective α_{2C}-AR antagonism with ORM-10921, rather than as an augmentation strategy. Although IDAZ is also an imidazoline I_2 receptor antagonist, this action is unlikely to contribute to the present results as selective imidazoline I_2 antagonists do not alter activity in the FST (O'Neill *et al.*, 2001). To the best of our knowledge, the current study provides the first evaluation of IDAZ under subchronic treatment conditions, and employing a genetic animal model of depression. In this instance we were unable to demonstrate antidepressant-related activity for IDAZ, confirming the notion that α_2-AR antagonists do not *per se* exert antidepressant effects in the FST (Cervo *et al.*, 1990; Rénéric *et al.*, 2001; Wesolowska, 2007; Zhang *et al.*, 2009). Thus, under our more stringent testing condition, viz. subchronic exposure, application in a genetic animal model of depression, and using a dose in a range found to be effective under acute treatment conditions, we found IDAZ to be ineffective as an antidepressant in the FSL model. Our findings, however, do not exclude its potential value as an augmentation strategy, as has been described elsewhere (Rénéric *et al.*, 2001). Nevertheless, studies in α_{2A}-AR-deficient animals suggest that α_{2A}-AR antagonism would increase immobility in the FST (Schramm *et al.*, 2001), whereas α_{2C}-AR-deficient animals display decreased immobility (Sallinen *et al.*, 1999). The nonselective antagonism of both α_{2A}-ARs and α_{2C}-ARs by IDAZ might therefore compromise effects on immobility in the FST, which could explain our findings.

On the other hand, selective α_{2C}-AR antagonism (with ORM-10921) did produce anti-immobility effects in this paradigm, suggesting superior antidepressant-like potential compared with nonselective α_2-AR antagonism. These data are in accordance with earlier work in Sprague Dawley rats describing the anti-immobility effects of ORM-10921 (Sallinen *et al.*, 2013a) as well as other α_{2C}-AR antagonists (JP-1302, ORM-12741) (Sallinen *et al.*, 2007, 2013b). That we have extended these findings to a genetic animal model of depression is noteworthy as FSL rats display many attributes of the human disorder (Overstreet *et al.*, 2005; Overstreet and Wegener, 2013) that are not seen in the strains used in earlier work (Sallinen *et al.*, 1999, 2007, 2013a). In FST studies, swimming and climbing behaviour have been correlated to increased serotonergic or noradrenergic signalling, respectively (Detke *et al.*, 1995). ORM-10921 increased swimming but not climbing behaviour relative to controls, which could indicate that its anti-immobility effects might be mediated through a more pronounced serotonergic mechanism. Indeed α_{2C}-AR deletion is associated with disinhibition of agonist-induced serotonin release (Scheibner *et al.*, 2001), and consequently ORM-10921 may elicit its anti-immobility response in the FST by increasing serotonin through selective targeting of α_{2C}-heteroreceptors on serotonin neurons (Stahl, 2013). However, further studies are needed to support this claim. As α_2-AR antagonism is geared to bolster noradrenaline release, it is noteworthy that the lower dose of ORM-10921 did tend to elevate climbing behaviour, indicating a possible noradrenergic action as well. Although we did not perform an acute-dose challenge to demonstrate the absence of an acute response so as to validate chronic effects, the fact that the effect of ORM-10921 was equivalent to that of IMI in the FST, and that such antidepressant effects in FSL animals are for the most part evident following chronic or subchronic treatments (Overstreet and Wegener, 2013), indirectly supports a chronic treatment response to this compound. Additionally, the pharmacokinetic elimination profiles of IMI (Daniel *et al.*, 1981), IDAZ (Valles *et al.*, 1995) and ORM-10921 (Sallinen *et al.*, 2013a) make the possibility of a

12-h delayed acute effect on behaviour improbable. Moreover, our earlier work has demonstrated a favourable effect of 14 days' exposure to ORM-10921 add-on therapy on striatal brain-derived neurotrophic factor levels (Uys *et al.*, 2016), a well-described drug effect dependent on long-term drug exposure (Nibuya *et al.*, 1995; Pillai *et al.*, 2006).

Depression presents with cognitive deficits such as impaired memory and concentration, including impaired declarative memory (Campbell and MacQueen, 2004; Hammar and Årdal, 2009; Gotlib and Joormann, 2010). Deficits in declarative memory have also been demonstrated in FSL versus FRL rats (Abildgaard *et al.*, 2011). The NORT measures declarative memory and relies strongly on medial temporal lobe structures such as the perirhinal cortex and the hippocampal complex (Reger *et al.*, 2009; Broadbent *et al.*, 2010; Cohen and Stackman, 2015), the hippocampus being especially important in the proposed pathophysiology of depression (Sapolsky, 2001). We have recently shown that ORM-10921 reverses deficits in object recognition memory in rats reared in social isolation and to upregulate neurotrophin levels during adjunctive therapy with haloperidol (Uys *et al.*, 2016). Indeed, our results in FSL rats show that both doses of ORM-10921, but not IDAZ, improved declarative memory in the NORT. These procognitive effects seem to be more prominent at lower doses (0.03 mg/kg), although this difference was not statistically significant. It is possible that the higher dose (0.3 mg/kg) of ORM-10921 engages with non-α_{2C}-ARs, such as α_{2A}-ARs (Sallinen *et al.*, 2013a, 2013b). Indeed, the receptor binding profile of ORM-10921 shows K_i values for α_{2C} (1.4 nmol/l) versus α_{2A}-ARs (9.2 nmol/l) (Sallinen *et al.*, 2013a) that may reflect dual engagement at higher doses and as such may attenuate positive effects on cognition. Similarly it is possible that the lack of effect of IDAZ in the NORT may indicate pronounced α_{2A}-AR antagonism, especially when one considers the abrogated mnemonic performance demonstrated in α_{2A}-AR KO mice (Franowicz *et al.*, 2002), whereas various studies have described the procognitive effects of α_2-AR agonists (and not antagonists) in rats (Carlson *et al.*, 1992; Cai *et al.*, 1993; Björklund *et al.*, 2001; Franowicz *et al.*, 2002). Additional studies employing various doses of ORM-10921 and IDAZ in rodents and in transgenic mice (α_{2A}-AR KO and overexpressed), measuring aspects of working and declarative memory, and coupled with receptor occupancy studies, might shed more light on these findings and on the role of α_{2A}-AR antagonism, especially to explain the effects of IDAZ on swim test immobility and object recognition.

Conclusion

This report provides the first evidence for antidepressant-like effects of a selective α_{2C}-AR antagonist as compared with a reference antidepressant (IMI) following subchronic treatment in a genetic animal model of depression, utilizing two behavioural measures of relevance for depression, the FST and NORT. That said, although the FST is a reliable screening tool for antidepressant activity and presents with good predictive validity (Borsini and Meli, 1988; Holmes, 2003) the test has moderate face but minimal construct validity (Petit-Demouliere *et al.*, 2005). Therefore further studies on ORM-10921 need to be undertaken using additional screening tools, such as the sucrose preference test (reflecting anhedonic-like symptoms of depression) and chronic mild stress (Papp *et al.*, 1996). These data have important implications for the therapeutic potential of α_{2C}-AR antagonists in the treatment of depression. Further preclinical studies on the utilization of α_{2C}-AR antagonists alone and as augmentation therapy in treating depression and/or cognitive dysfunction are therefore warranted to confirm the current findings and to elucidate the mechanisms underlying the behavioural data.

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MU contributed towards the study design, conducted all experiments and processed the data. BHH designed the study and the original protocol, MS and JS contributed towards the study design and preparation of the manuscript.

Conflicts of interest

M.S. and J.S. are employees of Orion Pharma. The authors declare that over the past three years, BHH has participated in advisory boards and received honoraria from Servier, and has received research funding from Servier and Lundbeck. BHH declares that, except for income from the primary employer and research funding from the above-mentioned organisations and agencies, 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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Chapter 5: Manuscript C

The following article will be prepared for submission to *Acta Neuropsychiatrica*, and is entitled:

"**The effects of selective α_{2C}-AR-antagonism on hippocampal monoamine levels in the Flinders Sensitive Line rat**"

Preamble

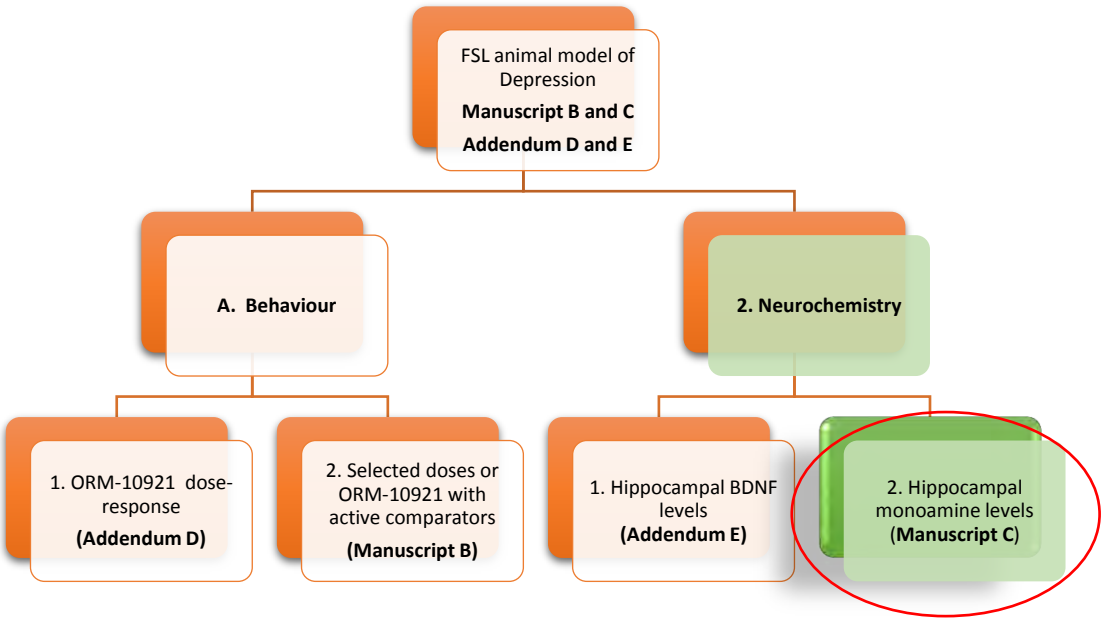
This chapter presents the full-length manuscript that will be submitted for publication in *Acta Neuropsychiatrica* via the journal's website:

http://onlinelibrary.wiley.com/journal/10.1111/(ISSN)1601-5215

This manuscript reports the effects of chronic treatment with ORM-10921 on hippocampal tissue levels of noradrenaline, serotonin and dopamine, as well as on the metabolism of these monoamines in the Flinders Sensitive Line (FSL) animal model of depression. The effects of ORM-10921 are reported in relation to that of idazoxan and imipramine. Although this data would have added value to the behavioural data reported in **Manuscript B**, it was not included in that manuscript due to the disparity of dose-response effects on behaviour as opposed to that of the hippocampal monoamines. When applying the same statistical analyses used to analyze the behavioural data in FSL rats described in Manuscript B to the dose-response effects on monoamines reported here, the dose-response data on hippocampal monoamines versus that on behaviour did not align with one another. This lack of congruency seriously compromised the ability to interpret the behavioural data, in particular the swimming and climbing data (Manuscript B). The behavioural data was considered to be robust and meaningful and thus the decision was made to present the hippocampal monoamine data separately. One reason for the disparity between dose-response effects on behaviour versus hippocampal monoamine levels could have been that neurochemistry and behaviour was conducted in different animals. Since the forced swim test is a noteworthy acute stressor for rats (Castagné et al., 2009), we were concerned that this test would introduce unnecessary confounders into the effects of treatment on hippocampal monoamine responses, especially since monoamine responses are very sensitive to stressful events. Although specific doses of ORM-10921 did not necessarily show correlations between behaviour and neurochemistry, the overall effect of various doses of ORM-10921 on hippocampal monoamines satisfactorily explain the behavioural data within the paradigm of antidepressant mechanisms, which will make a valuable contribution to the scientific literature. We report this data

here in **Manuscript C**, making use of a statistical analysis more suited to dose-response data, as detailed in the manuscript.

The diagram below depicts where this manuscript fits into the FSL study design and into the thesis layout, as indicated by the red circle.



Authors' contributions

1. *M Uys* contributed towards the study design and undertook all experimental animal and laboratory work, as well as the statistical analyses
2. *BH Harvey* designed and supervised the study and the original protocol and contributed towards the preparation of the manuscript and finalised it for publication
3. *F Viljoen* assisted in all aspects of the analytical laboratory analyses

All co-authors provided permission to use this manuscript as part of M Uys' Ph.D thesis, the letters of confirmation are annexed at the end of the thesis.

The effects of selective α_{2C}-AR-antagonism on hippocampal monoamine levels in the Flinders Sensitive Line rat.

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Short title (Running head): Selective vs. non-selective α_{2C} antagonism on hippocampal monoamines

Abstract

Major depressive disorder is characterized by mood, motivational and cognitive impairment, with dysfunction of various neurotransmitters, including the monoamines dopamine (DA), noradrenaline (NA) and serotonin (5-HT) underlying the complex pathophysiology. Dysfunctional hippocampal activity is a core element in limbic dysfunction, with hippocampal volume depletion and reduced neurotrophic function often ameliorated by successful antidepressant treatment. The role of the α_2-adrenoceptor (α_2-AR) in the pathophysiology of depression is established, including increased α_2-AR density and its reversal with antidepressants. Certain antidepressants, such as mirtazapine, exert their antidepressant effects via α_2-AR antagonism. However, few selective α_2-AR antagonists have shown enduring clinical efficacy as antidepressants. Evidence for different and often opposing roles of the α_{2A}-AR and α_{2C}-AR subtypes on noradrenergic neurotransmission and behavioural parameters of depression have been described, suggesting a positive role for α_{2C}-AR antagonism but not α_{2A}-AR antagonism in depression and related cognitive deficits. We have previously shown antidepressant-like and pro-cognitive effects for the selective α_{2C}-AR antagonist, ORM-10921, using the Flinders Sensitive Line (FSL) genetic rodent model of depression. These actions were superior to idazoxan, a non-selective α_2-AR antagonist, but equivalent to the tricyclic antidepressant, imipramine. In order to establish a possible mechanism of action for α_{2C}-AR antagonism in this model, we investigated the effect of chronic (14 day SC administration) treatment with ORM-10921, idazoxan and imipramine on hippocampal levels of noradrenaline (NA), dopamine (DA) and serotonin (5-HT) in the FSL model of depression. FSL rats had significant reductions in hippocampal 5-HT and DA, but not NA vs. FRL controls. ORM-10921 and idazoxan increased hippocampal NA and DA levels, while ORM-10921 also increased hippocampal 5-HT levels. These effects were correlated with decreased monoamine turnover rates. Imipramine increased hippocampal 5-HT levels, but not NA or DA. α_{2C}-AR antagonism with ORM-10921 exerts its pharmacological effects via noradrenergic and dopaminergic mechanisms, which may explain its antidepressant and pro-cognitive properties.

Keywords: Flinders Sensitive Line, depression, hippocampus, monoamines, α_{2C}-AR antagonism,

1. Introduction

Major depressive disorder is characterized by the dysfunction of various neurotransmitter systems, including the monoamines noradrenaline (NA), dopamine (DA) and serotonin (5-HT) (Manji et al 2001). The monoamine deficiency hypothesis posits that deficient limbic and cortical levels and/or neurotransmission of 5-HT, DA and NA give rise to the symptoms of depressed mood, decreased motivation, cognitive impairment, anhedonia and sleep disorders noted in depression (Delgado, 2000). While disrupted synthesis, storage or release of these neurotransmitters may be involved, their concentrations may also appear normal in which case postsynaptic monoamine receptors may be impaired, resulting in insufficient or ineffective neurotransmission (Kharade et al., 2010). Increasing the availability of brain monoamines has thus been a pillar of antidepressant therapy, including inhibition of reuptake from the synaptic cleft, inhibition of monoamine metabolism or antagonism of auto- and heteroreceptors effecting negative feedback loops on the release of these neurotransmitters (Kharade et al., 2010; Kiss, 2008).

The hippocampus is a prominent brain structure intimately involved in the neurocircuitry of depression (Campbell and MacQueen, 2004). This brain region forms part of the limbic system and plays a major role in the regulation of mood and the execution of various cognitive functions, while hippocampal dysfunction has been related to emotional symptoms of depression, including depressed mood, feelings of despair, guilt and worthlessness (American Psychiatric Association, 2013). Cognitive deficits observed in depression, such as impaired memory processes are also associated with hippocampal dysfunction (Krishnan and Nestler, 2008). Hippocampal atrophy and volume depletion has been suggested to be related to the underlying pathophysiology of depression (Sapolsky, 2001; Videbech and Ravnkilde, 2004), while antidepressants have been shown to reverse these structural deficits. The hippocampus is extensively innervated by monoaminergic neurons, and hippocampal atrophy subsequently results in reduced monoamine levels, which suggest that events driving the development of depression could drive downstream monoamine dysfunction (Pittaluga et al., 2007). However, increases in monoamine levels again lead to increased hippocampal neurogenesis (Santarelli et al., 2003) establishing the intricate relationship between monoaminergic function and hippocampal volume.

While various antidepressants, such as the selective 5-HT reuptake inhibitors (SSRIs), 5-HT/NA reuptake inhibitors (SNRI's) and tricyclic antidepressants (TCAs) exert their antidepressant effects by inhibiting the reuptake of NA and/or 5-HT (Kharade et al., 2010), thereby increasing synaptic availability of these monoamines, drugs like mirtazapine and mianserin (at least in part) increase NA and 5-HT levels via antagonism of the noradrenergic α_2-adrenergic hetero- and autoreceptors situated on noradrenergic and serotonergic nerve terminals (Anttila and Leinonen, 2001; Hamon and Blier, 2013; Mongeau et al., 1993). The α_2-adrenoceptor (α_2-AR) has a well-recognized role in depression and in antidepressant

action (Cottingham and Wang, 2012). While α_2-ARs seem to be up-regulated in platelets of depressed patients and in key brain areas of depressed suicide completers, such as the frontal cortex and hippocampus, chronic treatment with antidepressants are associated with down-regulation of these receptors (Cottingham and Wang, 2012). Up-regulated α_2-ARs are associated with excessive inhibitory effects on the release of NA, which indirectly decreases the release of 5-HT, resulting in deficient levels of both monoamines (Blier, 2003; Cottingham and Wang, 2012). α_2-AR antagonism by an antidepressant such as mirtazapine therefore results in disinhibition of the negative feedback loop, thus increasing NA release with a resultant indirect effect on 5-HT release, thereby resulting in an early-onset of antidepressant effects (Blier, 2003). Moreover, augmenting antidepressant therapy with mirtazapine or the α_2-AR antagonist yohimbine, appears to bolster response to a wide variety of antidepressants, in particular SSRI's, SNRI's and the dopamine reuptake inhibitor, bupropion (Besson et al., 2000; Blier et al., 2009; Blier et al., 2010; Sanacora et al., 2004).

However, not all α_2-AR antagonists are, like mirtazapine and mianserin, clinically effective antidepressants, despite early reports that the α_2-AR-antagonist, idazoxan has antidepressant effects (Grossman et al., 1999; Osman et al., 1989). While mirtazapine and mianserin may also exert their antidepressant effects via additional actions involving 5-HT receptors, such as 5-$HT_{2A/2C}$ and 5-HT_3 receptors (Anttila and Leinonen, 2001; Blier, 2003; Duka, 2010; Marshall, 1983), targeting of α_2-AR subtypes have been suggested to possibly engender more potent antidepressant effects (Scheinin et al., 2001). α_2-ARs are subdivided into α_{2A}, α_{2B} and α_{2C} subtypes, of which the α_{2A}-AR and α_{2C}-ARs are the main receptors involved in presynaptic feedback inhibition in the central nervous system (CNS) (Bücheler et al., 2002; Hein et al., 1999). The α_{2B}-AR is mainly expressed in the thalamus, while the α_{2A}-AR is widely expressed throughout the CNS (Rosin et al., 1996; Scheinin et al., 1994). The α_{2C}-AR, however, has a distinct distribution in areas of the brain involved in stress-related mood and psychiatric disorders, especially the hippocampus and striatum, but also the frontal cortex (Fagerholm et al., 2008; Rosin et al., 1996; Scheinin et al., 1994). Furthermore, transgenic mouse studies employing targeted deletion or overexpression of the α_{2A}-AR or α_{2C}-AR have suggested that α_{2C}-AR antagonism may have antidepressant-like effects, while α_{2A}-AR agonism is most probably more related to antidepressant-like effects (Sallinen et al., 1999; Schramm et al., 2001), and that non-selective antagonism could present with a weakened antidepressant-like effect. Similarly, opposing effects of the α_{2A}-AR and α_{2C}-ARs have been suggested on cognition, with α_{2A}-AR agonism suggested to mediate pro-cognitive effects (Arnsten and Leslie, 1991; Björklund et al., 2001; Franowicz et al., 2002) in contrast to α_{2C}-AR agonism which has been related to detrimental effects on memory (Björklund et al., 1998; Björklund et al., 1999a; Björklund et al., 1999b). A paucity of sufficiently subtype selective ligands have hampered further research in this area. Recently, however, a series of highly selective α_{2C}-AR antagonists have become available that have

shown antidepressant-like, antipsychotic-like and pro-cognitve effects in rodents (Rinne et al., 2013; Rouru et al., 2013; Sallinen et al., 2007; Sallinen et al., 2013a; Sallinen et al., 2013b).

We recently reported that the highly selective α_{2C}-AR antagonist, ORM-10921, exerts antidepressant-like and pro-cognitive effects in the Flinders Sensitive Line (FSL) rodent model of depression (Uys et al., 2016a). The FSL rat is outbred from Sprague Dawley rats to exhibit cholinergic supersensitivity and is a genetic animal model of depression presenting with good face, predictive and construct validity for the disorder (Overstreet et al., 2005; Overstreet and Wegener, 2013). The Flinders Resistant Line rat (FRL) was also selectively bred from Sprague Dawley rats and are often used inter-changeably with Sprague Dawley rats as control for FSL rats (Overstreet et al., 2005; Overstreet and Wegener, 2013; Skelin et al., 2011). FSL rats present with behavioural deficits akin to depression, such as impaired escape-directed behaviour, impaired motivation, anhedonia, sleep disturbances and impaired declarative memory (Abildgaard et al., 2011; Overstreet et al., 2005; Overstreet and Wegener, 2013). Furthermore these animals demonstrate reduced limbic serotonin synthesis (Hasegawa et al., 2006), reduced limbic neurotrophic support (Elfving et al., 2010a; Elfving et al., 2010b), increased α_2-AR density (Lillethorup et al., 2015) as well as decreased basal dopamine levels (Zangen et al., 2001).

This study builds on our previous study reporting on the antidepressant-like and pro-cognitive effects of sub-chronic treatment with the selective α_{2C}-AR antagonist, ORM-10921 in FSL rats (Uys et al., 2016a). ORM-10921 has a 100-fold selectivity for the α_{2C}-AR over the α_{2A}-AR (Sallinen et al., 2013a), with very low activity at other receptor types, and is thus an ideal drug to explore the neuropharmacological effects of α_{2C}-AR selective antagonism. Here we have investigated the dose-related effects of sub-chronic ORM-10921 administration on hippocampal levels of NA, DA, 5-HT and their turnover rates, comparing these data to the non-selective α_2-AR antagonist, idazoxan, and the reference tricyclic antidepressant, imipramine.

2. Methods

2.1 Animals and drug treatment

Eight week old male FSL and FRL rats were bred and cared for at the Vivarium of the North-West University. The original colonies were obtained from Dr. David H. Overstreet, University of North Carolina, USA. The rats were reared under identical conditions: cages (230(h) x 380(w) x 380(1) mm), temperature (21 ± 5 ˚C), humidity (50 ± 10%), white light (350-400 lux), 12 h light/dark cycle and food and water *ad libitum*. Animals were bred, supplied and housed at the Vivarium (SAVC reg no. FR15/13458; SANAS GLP compliance no. G0019) of the Pre-clinical Drug Development Platform of the North-West University (NWU). All experiments were approved by the AnimCare animal research ethics committee (NHREC reg. number AREC-130913-015) at the NWU. All animals were maintained and

procedures performed in accordance with the code of ethics in research, training and testing of drugs in South Africa, and complied with national legislation (ethics approval number: NWU-00050-13-A5).

FRL rats are widely used as the control for FSL rats (Overstreet et al., 2005; Overstreet and Wegener, 2013). FRLs were included to confirm the depressive phenotype of the FSL rats, and received vehicle treatment but no drug treatment. Drug or vehicle (saline) was injected subcutaneously (s.c.) once daily for 14 days. ORM-10921 (ORM), a gift from Orion Pharma (Orion Corporation, Turku, Finland), was administered at doses of 0.03, 0.1 and 0.3mg/kg, based on earlier studies (Sallinen et al., 2013a; Uys et al., 2016a) and dissolved in physiological saline to an injection volume of 1 ml/kg. Idazoxan hydrochloride (IDAZ) (Sigma Aldrich, South Africa) was administered at a dose of 3 mg/kg, based on earlier studies (Castagné et al., 2009; Rénéric et al., 2001) as well as our earlier behavioural study (Uys, et al., 2016a). Imipramine hydrochloride (IMI) (Sigma Aldrich, South Africa) was administered at a dose of 15 mg/kg, based on its well-described antidepressant-like effects at this dose (Castagné et al., 2011; Chen et al., 2010; de Morais et al., 2014).

2.2 Brain homogenate preparation and monoamine analyses

Animals were sacrificed by decapitation 24 hours after the final drug treatment. The brain was dissected into right and left hemispheres, whereafter the olfactory bulb was removed and total hippocampus dissected out on an ice-cooled dissection slab. The hippocampi were snap frozen in liquid nitrogen and stored in Eppendorf™ tubes at -70^{o}C. On the day of the analysis, brain samples were thawed and weighed, whereafter 1 ml of 0.1 M perchloric acid solution was added to each tube, sonicated and left on ice for 20 minutes to complete perchlorate precipitation. Samples were then centrifuged at 4^{o}C and 24 000 x g for 20 min. 200uL of the supernatant was withdrawn and 20 uL of the internal standard (isoprenaline HCl) added and mixed. pH was adjusted to pH 5 with 10 M potassium acetate. Quantification of hippocampal NA, 5-HT, DA, 3-methoxy-4-hydroxyphenylglycol (MHPG), 5-hydroxyindoleacetic acid (5-HIAA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were performed by high-performance liquid chromatography (HPCL) with electrochemical detection (ECD, HPLC-ECD), according to a previously described method (Harvey et al., 2006; Möller et al., 2013a; Möller et al., 2013b). An Agilent 1200 series HPLC, equipped with an isocratic pump and autosampler and coupled to an ESA Coulochem Electrochemical detecter with Chromeleon® Chromatography Management System (version 6.8), was used. Sample monoamine concentrations were determined by the response ratio (area under the peak for each monoamine/ the area under the peak of the internal standard for each sample) and calculated according to the regression curves for the response ratio of the monoamine standards (range 1.25 ng/ml – 50 ng/ml) and that of the internal standard. Linear standard regression curves (regression coefficient >0.98) were generated. Monoamine concentrations were expressed as ng/g wet hippocampal tissue (mean ± SEM). Monoamine turnover for NA and 5-HT

are expressed as the ratio of MHPG (ng/g) ÷ NA (ng/g) and 5-HIAA (ng/g) ÷ 5-HT (ng/g), respectively. Due to hippocampal DA levels often being below the lower limit of detection, DA turnover is not presented. Rather DA (where possible), DOPAC and HVA are presented and interpreted separately.

2.3 Statistical analyses

Normality of data was determined using the Shapiro Wilk test, which has very good statistical power to detect a non-Gaussian population and is well-suited to the n-range of data reported in this study (Ghasemi and Zahediasl, 2012; Razali and Wah, 2011). Differences in monoamine levels in FSL vs. FRL rats were analyzed with student's unpaired t-tests or Mann-Whitney U-tests in the case of non-parametric data sets. In the case unequal standard deviations, Welch's corrected p-value was applied to t-tests. Comparison of the effects of drug treatments on hippocampal monoamines in FSL rats were performed using one-way analysis of variance (ANOVA). Fisher's Least Significant Difference (LSD) post hoc test was applied to indicate where treatment groups differed significantly. This test is often used in the literature when mutliple doses of a compound are analyzed (Bachtell et al., 2005; Debrah et al., 2005; Kufahl et al., 2011; Labonte et al., 2012; Newman and Beardsley, 2006; Ruxton and Beauchamp, 2008). Where the criteria of equality of variances for ANOVA was not met as indicated by the Brown-Forsythe test, Kruskal-Wallis ANOVA and Dunn's post hoc multiple comparison test was employed. Significance was set at a 5% level ($p<0.05$). Cohen's *d*-value was calculated as a measure of effect size to indicate the practical significance (if applicable) of results demonstrating statistical significance on a 10% significance level ($p\leq0.1$). An effect size of ~0.2 to ~0.49 is considered a small effect, ~0.5 to ~0.79 a medium effect showing a trend for practical significance and effect sizes of ~.08 and greater are considered as large and practically significant (Cohen, 1988).

3. Results

3.1 Hippocampal 5-HT levels and 5-HT turnover

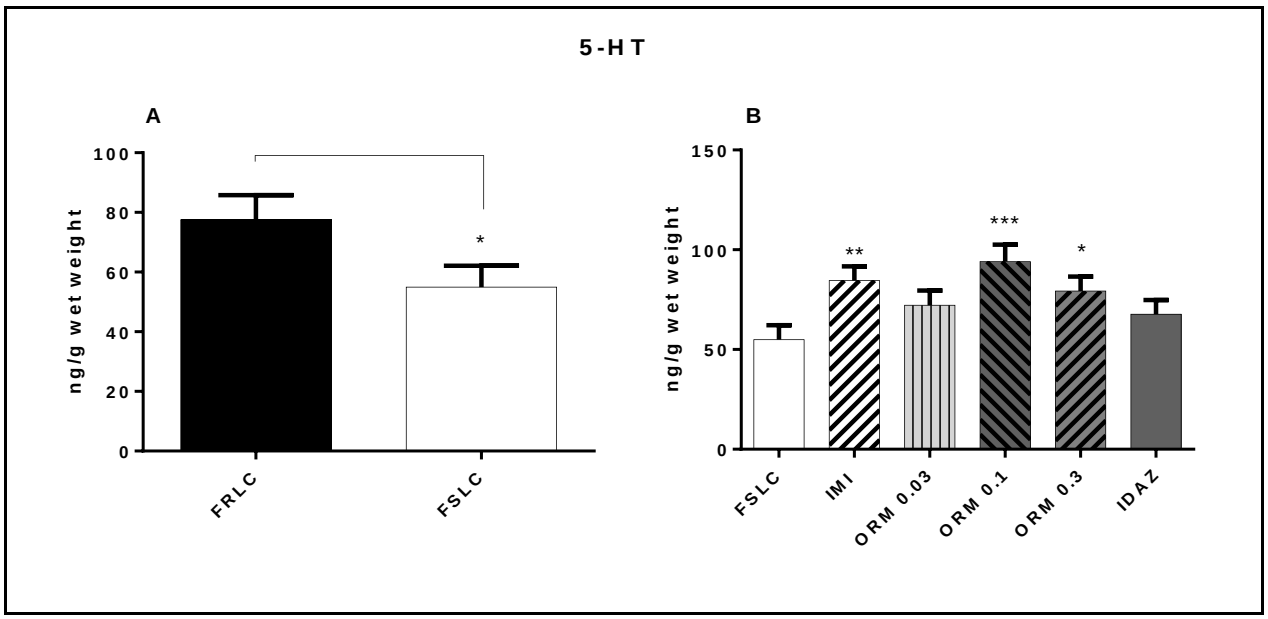

Fig 1. Hippocampal 5-HT levels in FSL vs. FRL controls (A) or in FSL animals treated with the drug treatments as indicated (B). *p<0.05, **p<0.01, ***p<0.001 vs. FSL controls. n=7-10. FRLC = FRL controls, FSLC = FSL controls, IMI = imipramine 15 mg/kg, ORM= ORM-10921 0.03, 0.1 or 0.3 mg/kg, IDAZ = idazoxan 3mg/kg

Unpaired t-test indicated that FSL animals presented with significantly lower 5-HT levels vs. their FRL controls ($p=0.03$) (Fig 1A). ANOVA indicated that drug treatment in FSL animals induced significant differences in hippocampal 5-HT levels ($F(5,52) = 3.228$, $p = 0.01$). Fisher's LSD test indicated that ORM 0.1 ($p=0.0006$), ORM 0.3 ($p=0.03$) and IMI ($p=0.009$) significantly increased hippocampal 5-HT levels vs. FSL controls. ORM 0.03 ($p=0.11$) and IDAZ ($p=0.24$) did not affect hippocampal 5-HT levels in these animals (Fig 1B).

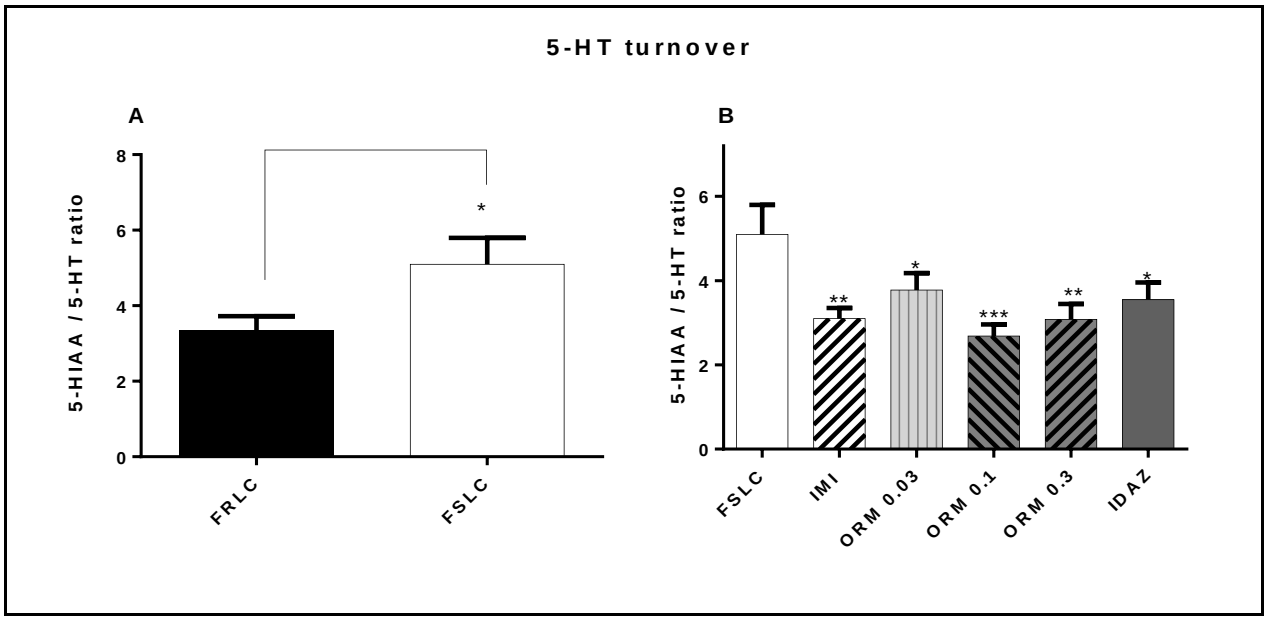

Fig 2. Hippocampal 5-HT turnover rates in FSL vs. FRL controls (**A**) or in FSL animals treated with the drug treatments as indicated (**B**), and expressed as 5-HIAA (ng/g)/5-HT (ng/g). *p<0.05, **p<0.01, ***p<0.001 vs. FSL controls. n=7-10. FRLC = FRL controls, FSLC = FSL controls, IMI = imipramine 15 mg/kg, ORM= ORM-10921 0.03, 0.1 or 0.3 mg/kg, IDAZ = idazoxan 3mg/kg

Unpaired t-test indicated that FRL controls had lower 5-HT turnover ratios than FSL controls (p=0.04) (Fig 2A), while ANOVA indicated significant differences of drug treatment on 5-HT turnover levels in FSL animals ($F(5,52)= 4.037$, p=0.003). Post-hoc analysis indicated that all drug treatments decreased 5-HT turnover vs. FSL controls (ORM 0.03, p=0.03; ORM 0.1, p=0.0002; ORM 0.3, p=0.001; IMI, p=0.001; IDAZ, p=0.01) (Fig 2B).

3.2 Hippocampal NA levels and NA turnover

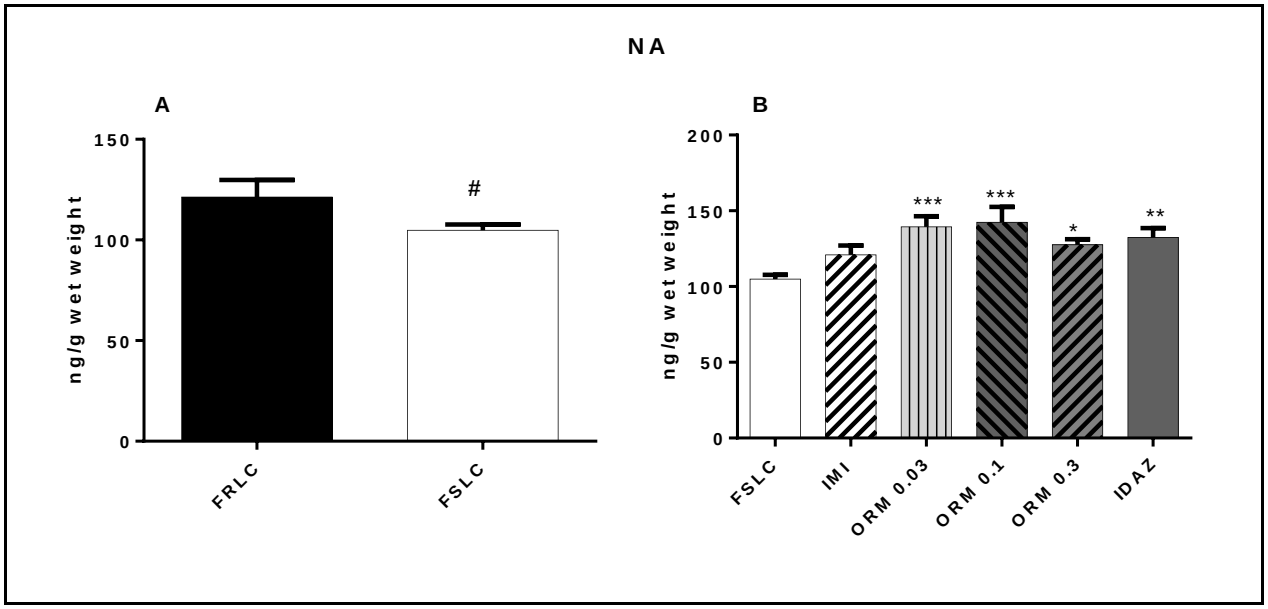

Fig 3. Hippocampal NA levels in FSL vs. FRL controls (**A**) or in FSL animals treated with the drug treatments as indicated (**B**). *p<0.05, **p<0.01, ***p<0.001 and # practical significance vs. FSL controls. n=7-10. FRLC = FRL controls, FSLC = FSL controls, IMI = imipramine 15 mg/kg, ORM= ORM-10921 0.03, 0.1 or 0.3 mg/kg, IDAZ = idazoxan 3 mg/kg.

Unpaired t-test showed a tendency for FSL animals to display lower hippocampal NA levels vs. FRL animals, and although this difference was not statistically significant at the 5% significance level, it was statistically significant at a 10% significance level (p= 0.07, Fig 3A). The effect size for this analysis (*d*= 1.17) was found to exceed Cohen's convention for a large effect (>0.8), indicating a practical significant difference for NA levels in FRL vs. FSL rats. One-way ANOVA indicated a significant difference between FSL rats treated with either vehicle or the respective drug treatments F (5,52)= 4.51, p=0.002, (Fig 3B). Fisher's LSD test indicated that ORM 0.03 (p=0.0003), ORM 0.1 (p=0.0001), ORM 0.3 (p=0.02) and IDAZ (p=0.005) treatment resulted in significantly higher hippocampal NA levels vs. FSL controls (Fig 3B). Although the response to IMI treatment did not show a statistically significant increase in NA levels, this increase did show statistical significance at the 10% level (p=0.09). In fact treatment with IMI showed a medium effect size (*d*=0.5) indicating a trend towards practical significance.

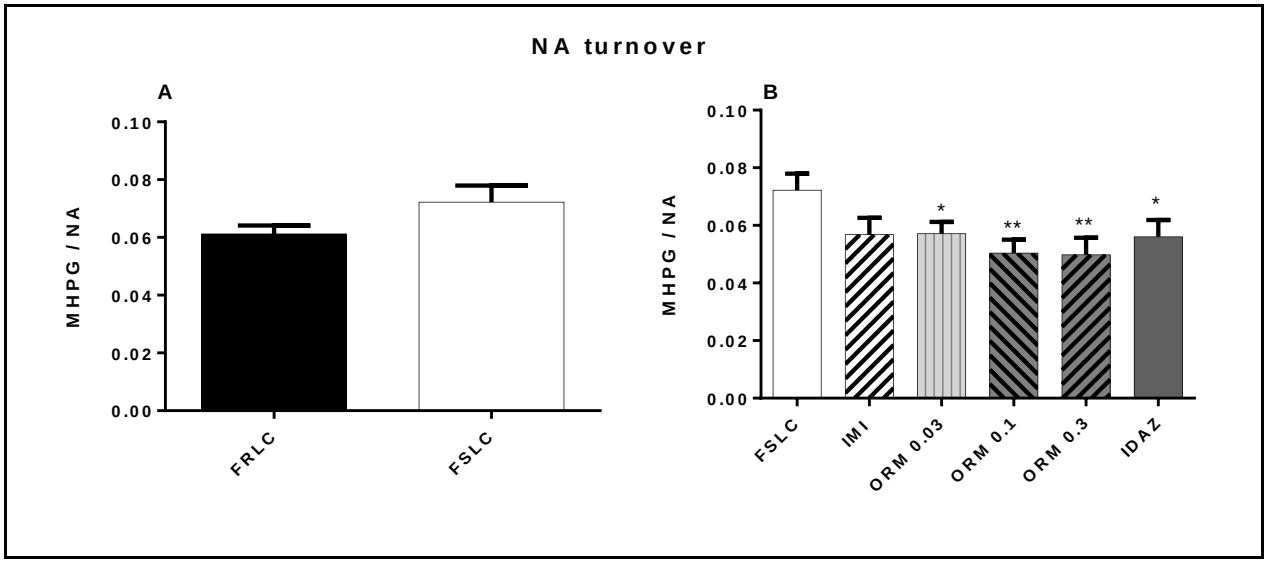

Fig 4. Hippocampal NA turnover in FSL vs. FRL controls (**A**) or in FSL animals treated with the drug treatments as indicated (**B**). NA turnover is expressed as MHPG (ng/g)/NA (ng/g). *p<0.05, **p<0.01, # practical significance vs. FSL controls. n=7-10. FRLC = FRL controls, FSLC = FSL controls, IMI = imipramine 15 mg/kg, ORM= ORM-10921 0.03, 0.1 or 0.3 mg/kg, IDAZ = idazoxan 3 mg/kg

Unpaired t-test with Welch's correction indicated no statistically significant differences in NA turnover between FRL and FSL controls on a 5% significance level (p=0.1), although the effect size met Cohen's convention for a large effect size (*d*=0.8) (Fig 4A). ANOVA indicated that drug treatment in FSL animals induced significant differences in hippocampal NA turnover (F (5,52) = 2.446, p = 0.04). Fisher's LSD indicated that ORM 0.03 (p=0.04), ORM 0.1 (p-0.004), ORM 0.3 (p=0.004) and IDAZ (p=0.04) significantly decreased hippocampal NA turnover in FSL rats (Fig 4B). IMI strongly tended to decrease NA turnover in the hippocampi of FSL rats, closely missing statistical significance at the 5% significance level (p= 0.05). However the effect size (*d*=1.1) exceeds Cohen's convention for a large effect, demonstrating practical significance (Fig 4B).

3.3 Hippocampal DA levels and DA metabolites

The hippocampus is not highly innervated by dopaminergic neurons, and in this study DA levels in the hippocampus was very low, being below the lower limit of detection in FSL control rats. Values for FRL controls and for the drug treatment groups, however, were within the detection limit (n=5-7 per group). In order to present values for the FSL controls for comparison with FRL and FSL treatment groups, the lowest measured value for DA in the HPLC-ECD procedure was applied to FSL samples, calculated according to the regression formula and divided by the respective wet weights. Non-parametric data was produced and are reported here.

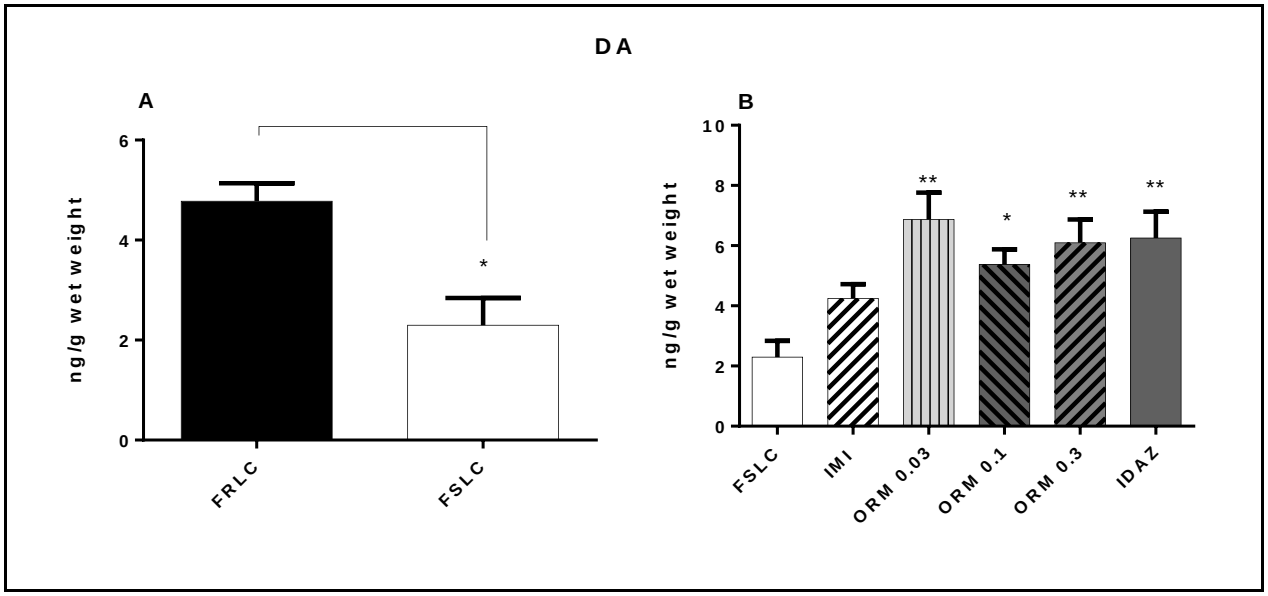

Fig 5. Hippocampal DA levels in FSL vs. FRL controls (**A**) or in FSL animals treated with the drug treatments as indicated (**B**). *p<0.05, **p<0.01, vs. FSL controls. n=5-7. FRLC = FRL controls, FSLC = FSL controls, IMI = imipramine 15 mg/kg, ORM= ORM-10921 0.03, 0.1 or 0.3 mg/kg, IDAZ = idazoxan 3mg/kg.

Mann-Whitney U test showed a significant difference between hippocampal DA levels of FRL vs. FSL animals (p=0.02; Fig 5A). Kruskal-Wallis ANOVA indicated a significant difference between FSL rats treated with the respective drug treatments (Kruskal-Wallis statistic 17.27, p=0.0015). Post hoc Dunn's comparison indicated that ORM 0.03 (p=0.003), ORM 0.1 (p=0.01), ORM 0.3 (p=0.006) and IDAZ (p=0.004), but not IMI treatment (p>0.9), resulted in significantly higher hippocampal DA levels vs. FSL controls (Fig 5B).

Due to the low detection of DA in FSL controls, determining DA turnover by dividing the metabolites DOPAC and HVA by the values obtained for DA resulted in a skewed data set that presented evidence of elevated DA turnover for FSL controls. The result being that all cohorts display disproportionately decreased DA turnover compared to FSL controls. Due to this, DA turnover was not represented as the conversion indices of the metabolites to DA, but are represented independently to clarify the findings and to interpret it accordingly.

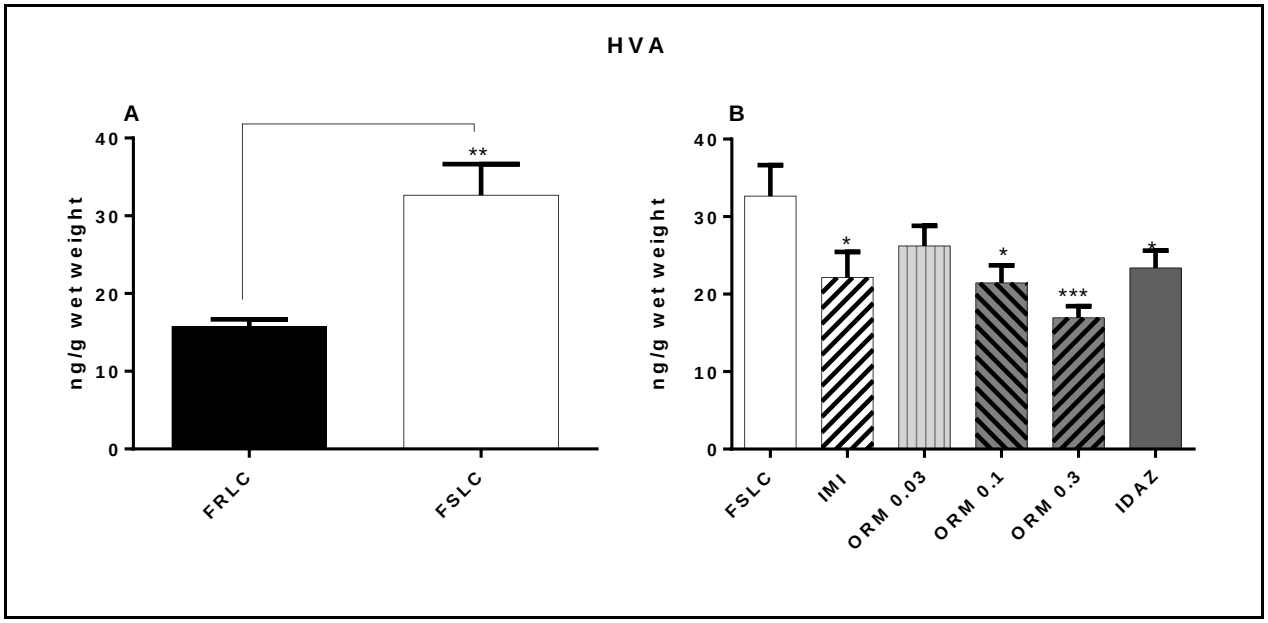

Fig 6. Hippocampal HVA levels in FSL vs. FRL controls (**A**) or in FSL animals treated with the drug treatments as indicated (**B**). *p<0.05, ***p<0.001 vs. FSL controls. n=7-10. FRLC = FRL controls, FSLC = FSL controls, IMI = imipramine 15 mg/kg, ORM= ORM-10921 0.03, 0.1 or 0.3 mg/kg, IDAZ = idazoxan 3mg/kg

Mann-Whitney U-test indicated significantly higher HVA levels in FSL controls compared to FRL controls (p=0.002; Fig 6A), while ANOVA indicated significant differences between FSL treatment groups ($F(5,53) = 3.66$, p=0.006), with Fisher's LSD post hoc test indicating that all drug treatments, except ORM 0.03 (p=0.1) decreased HVA levels compared to FSL controls: ORM 0.1 (p=0.01), ORM 0.3 (p=0.0002), IMI (p=0.01) and IDAZ (p=0.02) (Fig 6B).

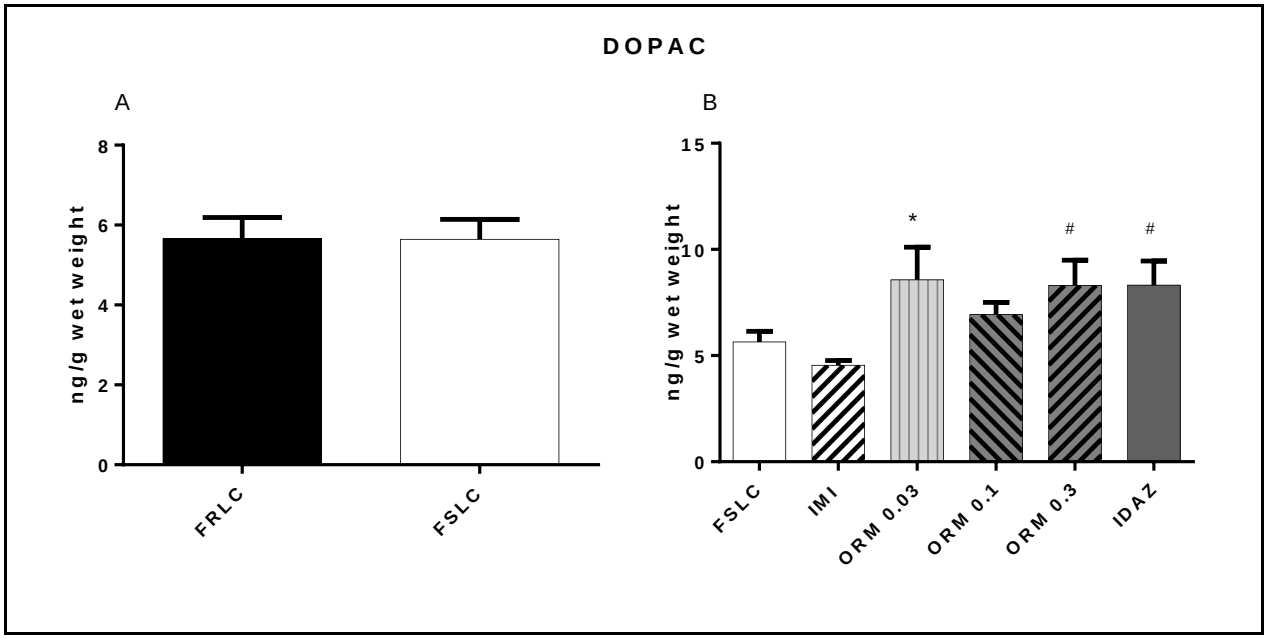

Fig 7. Hippocampal DOPAC levels in FSL vs. FRL controls (**A**) or in FSL animals treated with the drug treatments as indicated (**B**). *p<0.05 and # practical significance vs. FSL controls. n=7-10. FRLC = FRL controls, FSLC = FSL controls, IMI = imipramine 15 mg/kg, ORM= ORM-10921 0.03, 0.1 or 0.3 mg/kg, IDAZ = idazoxan 3mg/kg

DOPAC levels didn't differ significantly between FSL and FRL controls (p=0.9; Fig 7A), while ANOVA indicated significant differences between FSL treatment groups (F (5,51) = 2.96, p=0.02; Fig 7B), with Fisher's LSD indicating that ORM 0.03 (p=0.05; *d*=0.9), ORM 0.3 (p=0.06; *d*=0.9) and IDAZ (p=0.06; *d*=0.9) tended to increase DOPAC levels compared to FSL controls on a 10% significance level, while the effect sizes for all these comparisons exceeded Cohen's convention for a large effect size (>0.8) (Cohen, 1988).

4. Discussion

Serotonin and serotonin turnover

FSL rats reportedly present with reduced 5-HT sensitivity and reduced 5-HT synthesis, correlating with the human disorder (Hasegawa et al., 2006; Overstreet et al., 2005). In this study, FSL rats displayed reduced hippocampal 5-HT levels vs. FRL rats, with increased 5-HT turnover, in agreement with the theoretical model of serotonergic deficiency in humans (Kharade et al., 2010). However, these findings are contrary to previous studies reporting that FSL rats display increased 5-HT levels and reduced 5-HT-turnover (5-HIAA/5-HT ratio) in various limbic areas vs. SD controls, including the hippocampus (Zangen et al., 1997). However, findings in FSL rats vs. SD controls cannot be directly extrapolated to FSL vs. FRL rats. FRL rats present with decreased 5-HT synthesis in various brain regions, including the ventral hippocampus, vs. SD controls, while FSL rats display reduced 5-HT synthesis in the hippocampus vs. both FRL rats and SD rats (Hasegawa et al., 2006). Thus, our findings that the FSL rat presents with lower total

5-HT tissue levels vs. FRL controls might reflect this reduced 5-HT synthesis noted in FSL vs. FRL rats. The relationship between 5-HT turnover, serotonergic neurotransmission and the synthesis of 5-HT is unclear, although 5-HT synthesis is one of the most important steps in serotonergic neurotransmission (Commissiong, 1985; Nelson, 1993). Furthermore, relating the tissue levels of 5-HT and its metabolite (5-HIAA) in FSL animals to depressed patients is difficult, since mixed findings of increased, decreased or unaffected 5-HIAA levels have been reported in the CSF of depressed patients (Asberg et al., 1984; Reddy et al., 1992; Roy et al., 1985). However, in post-mortem brain samples of depressed suicide patients, levels of 5-HT was lower than that in brain samples of non-depressed patients (Mann et al., 1989), while FSL rats also present with reduced 5-HT-turnover (Zangen et al., 1997). Additionally, the FSL rat also presents with serotonergic subsensitivity (Zangen et al., 2001) That the FSL rat does therefore present with serotonergic abnormalities is evident from literature, although these alterations do not always mimic that found in the clinic (Overstreet et al., 2005). However, chronic antidepressant treatment does normalize altered serotonergic activity in FSL animals (Overstreet et al., 2005; Zangen et al., 2001; Zangen et al., 1997), and here we provide more evidence that this is indeed the case.

The tricyclic antidepressant, IMI, increased hippocampal 5-HT levels vs. FSL controls in this study, while also decreasing 5-HT turnover, which is in line with previous studies (Alpers and Himwich, 1972; Sugrue, 1983). IMI acts by increasing the synaptic availability of 5-HT by inhibition of its reuptake from the synaptic cleft (Felton et al., 2003; Vetulani and Nalepa, 2000). Although the method applied in this study does not distinguish between extracellular and intracellular 5-HT levels, these findings are in line with the expectation that IMI would increase serotonergic activity.

Selective α_{2C}-AR antagonism with ORM-10921 also increased hippocampal 5-HT levels and decreased 5-HT turnover, while non-selective α_2-AR antagonism with IDAZ reduced 5-HT turnover without increasing 5-HT levels. These findings support our recent findings that ORM-10921 produces serotonergic driven antidepressant-like effects in the FST while this is not evident with IDAZ (Uys et al., 2016a), thus making a strong case for the therapeutic benefits of α_{2C}-AR selective vs. non-selective α_2-AR antagonism, particularly with respect to antidepressant-like behavioural effects. Llado and colleauges reported that IDAZ decreases 5-HT synthesis in the rodent hippocampus (Llado et al., 1996), which seemed to be mediated by the 5-HT_{1A} autoreceptor. Although no data is available on the effect of ORM on 5-HT synthesis, that selective α_{2C}-AR antagonism (at doses that display antidepressant-like activity) but not non-selective α_2-AR antagonism (at a dose that doesn't display antidepressant or pro-cogntive activity; (Uys et al., 2016a) increase 5-HT levels suggests that ORM does not produce the same measure of inhibition on 5-HT synthesis, although supportive evidence using autoradiographic analysis of 5-HT synthesis is needed to validate this proposal.

Noradrenaline and noradrenaline turnover

Our study failed to show that FRL and FSL animals differ statistically on a 5% significance level with respect to total hippocampal NA levels, although the large effect size (*d*=1.17) confirms its practical significance that FSL animals do in fact present with lower NA levels. Zangen et al (1999) reported higher NA levels in FSL rats, although compared to SD controls. Considering the above differences noted with respect to 5-HT levels and 5-HT synthesis in SD and FRL animals, these NA data should be interpreted within the same context. Recent studies reported decreased *in vivo* α_2-AR binding in FSL animals vs. FRL animals, which might be due to increased endogenous synaptic NA or due to receptor down-regulation (Landau et al 2015). Either way, the authors report a possible noradrenergic over-activity in FSL animals compared to FRLs, which suggests that while behaviour in these animals are amenable to antidepressant effects vs. the FRL controls, not all physiological underpinnings might mimic the human disorder. However, although NA levels are reduced in some but not all patients presenting with depression, altered function of NA in depression could be related to disruptions in storage, release or synthesis of the monoamines, as well as dysfunction of postsynaptic receptors or second messenger activity (Kharade et al., 2010). Since FSL and FRL rats did not demonstrate a robust statistical change in NA, thus supporting the construct validity of the model, there is questionable value in attempting to explain the effects of drug treatment thereon.

Nonetheless, when considering the effects of drug treatment in FSL animals, 14-day treatment with IMI also did not affect NA levels in FSL animals to a robust level of statistical significance in this study, its effect size (*d*=0.5) suggests medium practical significance. This is not in line with early studies reporting statistically significant alteration of NA after chronic treatment in SD rats (Chung et al., 1993). IMI is widely regarded as an inhibitor of both 5-HT and NA reuptake and thus to present with serotonergic and nordrenergic related antidepressant properties (Hamon and Blier, 2013). This is at odds with the monoamine data presented here. Interestingly, this dose of IMI *increases* climbing behaviour in the Porsolt forced swim test (FST) in FSL animals (Uys et al., 2016a), widely considered to be a noradrenergic-motivated behaviour (Detke et al., 1997). That IMI did not statistically increase NA levels in FSL animals or robustly alter NA turnover suggests that IMI may exert its noradrenergic effects in a manner that is not sensitive to detection by measuring total tissue NA levels by HPLC-ECD analysis. While previous studies reported no significant effects of chronic IMI on extracellular MHPG levels, pre-synaptic α_2-AR sensitivity was however decreased by IMI treatment (Sugrue, 1983), indicating that decreased presynaptic negative feedback could be responsible for increased extracellular NA release. Accordingly, in vivo microdialysis studies have demonstrated increased extracellular NA levels following acute and chronic application of the IMI metabolite, desimipramine, in the dorsal hippocampus, effects which were also related to down-regulated α_2-AR sensitivity (Sacchetti et al., 2001).

Both ORM-10921 and IDAZ increased total NA tissue levels vs. FSL controls, an effect which seems to be associated with a decrease in NA turnover. Previous studies in our laboratory have demonstrated that sub-chronic IDAZ does *not* present with antidepressant-like or pro-cognitive effects in FSL animals, in contrast to ORM-10921 (Uys et al., 2016a). These data would suggest that increased hippocampal levels of NA in FSL animals are not related to antidepressant and pro-cognitive efficacy, and that effects on 5-HT and/or DA are possibly involved. The increase in NA levels by ORM and IDAZ however does show that both α_{2C}-AR selective and α_2-AR non-selective antagonism presents with increased hippocampal NA levels in FSL animals, which is to be expected from antagonists at the α_2-AR autoreceptor (Leonard, 2003). Microdialysis studies measuring extracellular levels of the catecholamines might be more helpful to delineate the extracellular noradrenergic mechanisms underlying the antidepressant-like effects of ORM-10921.

Dopamine and dopamine turnover

DA is involved in both hedonic and motivational behavior, deficits of which are core symptoms in depression (Grace, 2016). Depression presents with deficits in dopaminergic signalling, while drugs that increase DA are associated with antidepressant effects (El Mansari et al., 2010; Grace, 2016; Guiard et al., 2009). Although dopaminergic innervation of the hippocampus is minimal, noradrenergic fibres may be the primary source of DA release in the hippocampus (Smith and Greene, 2012), since the dopaminergic input to the hippocampus from the ventral tegmental area is limited. Considering the effects that α_{2C}-AR antagonism might have on DA activity (Sallinen et al., 2013a; Sallinen et al., 1999; Sallinen et al., 1998; Sallinen et al., 1997), as well as the involvement of the hippocampal dopaminergic circuitry in depression (Grace, 2016), it was incumbent to also investigate the effects of ORM-10921 on hippocampal DA levels.

FSL rats present with a diminished ability of 5-HT to release DA (Zangen et al., 2001), and with decreased limbic DA neurotransmission (Friedman et al., 2007; Friedman et al., 2005), a phenomenon that is associated with depressive-like behaviour in the FST and reversed by treatment with certain antidepressants (Dremencov et al., 2004; Roth-Deri et al., 2009). FSL rats also exhibit low extracellular DA levels as a consequence of low release (Roth-Deri et al., 2009). Although the lower limit of detection compromised this analysis, we report lower total DA levels and higher levels of the DA metabolite, HVA, in FSL rats vs. FRL controls, which is in line with the altered dopaminergic levels described in the literature for these animals. In fact, reduced plasma HVA levels have been found in the plasma of depressed patients (Altshuler et al., 2001), while such reduced levels have been associated with reduced dopaminergic activity in the brains of depressed suicide completers (Pitchot et al., 2001).

Treatment with IMI did not significantly increase hippocampal DA levels, although it did decrease HVA levels indicative of a decrease in DA metabolism. IMI mainly acts by decreasing reuptake of 5-HT and

NA, although effects on dopaminergic systems have been reported, albeit via an indirect mechanism, including increasing functional activity at DA synapses (Muscat et al., 1990). Chronic imipramine treatment has been associated with supersensitive post-synaptic DA receptors (Dziedzicka-Wasylewska and Rogoz, 1998) and with increased extracellular mesolimbic DA output (Rossetti et al., 1993). In this study, however, no effect on total hippocampal tissue levels could be demonstrated.

On the other hand, all doses of ORM-10921 increased the levels of DA in the hippocampus, which seemed to be correlated with decreased HVA levels. Effects on DOPAC were minimal but practically significant (*d*=0.9), with the 0.03 and 0.3mg/kg doses seemingly increasing DOPAC vs. controls. Increased DA transmission has been shown to play a role in decreasing immobility in the FST, a predictive animal model of antidepressant-like activity (Perona et al., 2008; Renoir et al., 2012), while exposure to extended periods of swim-stress results in long-lasting depletion of mesolimbic DA output, which could partially be reversed by antidepressant pre-treatment (Rossetti et al., 1993). Although we previously reported that ORM-10921 displays antidepressant-like effects in the FST showing behavioural evidence for the involvement of both noradrenergic and serotonergic signalling (Uys et al., 2016a), it is possible that elevation of hippocampal DA levels may also play a yet undisclosed role in its antidepressant-like effects. Previously, Sallinen and co-workers reported that ORM-10921 increases extracellular DA levels in rodent prefrontal cortex (Sallinen et al., 2013a). The effects of α_{2C}-AR antagonism on DA activity in various brain regions may therefore contribute to the antidepressant-like and pro-cognitive effects reported previously for α_{2C}-AR antagonists (Sallinen et al., 2007; Sallinen et al., 2013a; Uys et al., 2016a; Uys, et al., 2016b). IDAZ also increased hippocampal DA levels and decreased HVA levels, while moderately increasing DOPAC levels, in line with previously reported effects in the hippocampus and frontal cortex (Borgkvist et al., 2012; Matsumoto et al., 1998). However, these elevations of hippocampal DA did not translate into antidepressant-like or pro-cognitive effects for IDAZ in our earlier study (Uys, et al., 2016a), once again emphasizing the importance of determining synaptic availability of the monoamines via in vivo microdialysis to ascertain how tissue levels and extracellular availability can be related to effects on behaviour.

Conclusions

While selective α_{2C}-AR antagonism with ORM-10921 was associated with increased levels of hippocampal DA, NA and 5-HT, non-selective α_2-AR antagonism appeared to follow a similar pattern, albeit not significantly increasing hippocampal 5-HT. The pro-cognitive and antidepressant-like effects reported for ORM-10921 might therefore be related to its elevation of hippocampal DA, NA and 5-HT levels. The interpretations of this study are limited by the inability to determine extracellular vs. intracellular monoamine levels, since increasing monoaminergic synaptic availability is associated with antidepressant-like effects. Since extracellular vs. intracellular levels cannot be determined by assessing

whole tissue levels with HPLC, no inferences can be made regarding synaptic availability of NA, DA and 5-HT induced by the respective drug treatments. Microdialysis studies assessing monoamine release after acute and chronic treatment in freely moving rats could provide more information on extracellular levels of monoamines. Autoradiographical studies could also be useful in revealing more on how monoamines and monoamine synthesis are affected by ORM-10921. Elevations in whole tissue levels of the monoamines, as reported here, suggest increased synthesis vs. control rats, and therefore these findings do highlight potential effects of ORM-10921 on altered monoamine levels. There is a confounder though. All drugs tested here, viz. IMI, ORM-10921 and IDAZ, have a well-described theoretical basis for modifying NA while they also demonstrated such effects in this study. However the moderate difference in hippocampal NA levels in FSL vs. FRL rats complicates the interpretation of these data. While this work has revealed some direction as to how ORM-10921 may exert its pro-cognitive and antidepressant-like effects, as described in Chapter 4 (**Manuscript B**), more elaborate neurochemical analyses are necessary to better understand these underlying mechanisms.

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Chapter 6: Manuscript D

This manuscript has been submitted for publication to *Frontiers in Psychiatry,* section *Molecular Psychiatry,* and is entitled:

"***Therapeutic potential of targeting the α_{2C}-adrenoceptor in cognition, depression and schizophrenia – new developments and future perspective***"

Preamble

This chapter presents the full-length manuscript submitted for publication in *Frontiers in Psychiatry,* section *Molecular Psychiatry*, published by Frontiers Media. This article brings together an extensive literature review conducted during the course of this research project on the therapeutic potential of α_{2C}-adrenoceptor antagonism in neuropsychiatric illness, with specific focus on schizophrenia and depression. The paper constitutes the first comprehensive review on the subject matter of selective α_{2C}-antagonism. The paper will enable future researchers to more easily access the relevant literature, to identify gaps in knowledge and to design appropriate studies that will uplift the state of the art.

The manuscript is presented in the prescribed submission format as outlined in the *Author Guidelines* on the journal website, with exceptions as noted below:

http://journal.frontiersin.org/journal/psychiatry/section/molecular-psychiatry#author-guidelines

The manuscript begins with the the Title Page showing that the article is currently in review at *Frontiers in Psychiatry, Molecular psychiatry,* followed by a second page produced during the submission process containing statements for Conflict of Interests, Contributions of Authors and the Funding Statement. This page also contains the Abstract and Keywords. On the following pages, the main body of the text is provided in keeping with the format of the rest of the thesis. Providing a slightly amended format of the submission document as stipulated by the Frontiers enables a simplified layout making it easier for the reader to relate the text to the Figures in the Manuscript. *Frontiers* requires the Figure legends and Tables to be included after the references and at the end of the manuscript, followed by the Figures themselves. To enable easier reading, I have inserted the figures and tables into the text at the appropriate places.

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Therapeutic potential of targeting the a2C-adrenoceptor in cognition, depression and schizophrenia - new developments and future perspective

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Conflict of interest statement

The authors declare a potential conflict of interest and state it below

Mohammed Shahid is an employee of Orion Pharma. No funding was received by Orion Pharma for this work. The authors declare that over the past three years, BHH has participated in advisory boards and received honoraria from Servier®, and has received research funding from Servier® and Lundbeck®. ORM-10921, which was used in recent studies cited in this work was sponsored by Orion Pharma. BHH declares that, except for income from the primary employer and research funding from the above-mentioned organisations and agencies, 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. The authors declare no other conflicts of interest.

Author contribution statement

MM Uys prepared the first draft of the manuscript, prepared all the figures and tables, as well as managed all subsequent changes and formatting. M Shahid reviewed the manuscript and provided input on the manuscript design and content, as well as on the figures and tables. BH Harvey was the study leader and student supervisor to MMU, developed the article concept and design, and finalized the manuscript for submission.

Keywords

Alzheimer's disease, α2C-antagonism, Schizophrenia, Depression, Cognition

Abstract

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α2-Adrenoceptor (AR) modulators are used in the therapy of various neuropsychiatric disorders, including attention deficit hyperactivity disorder (ADHD), depression and schizophrenia. While augmentation of conventional antidepressant and antipsychotic therapy with non-selective α2-AR antagonism presents with some indication of improved therapeutic efficacy, early studies have shown distinct and often opposing roles for α2A- and α2C-ARs, the two main α2-AR subtypes involved in central nervous system (CNS) function. Lack of α2-AR selectivity may thus be a potential limiting factor. More recent work in animal models suggests that α2C-AR selective antagonism presents with antidepressant-like, antipsychotic-like and pro-cognitive effects. On the other hand, α2A-AR selective antagonism is not associated with the above effects and may indeed compromise α2C-AR antagonism-mediated beneficial effects. The α2A-AR is widely distributed throughout the CNS, while α2C-AR expression is localised most notably in the striatum and hippocampus, and to a lesser extent the prefrontal cortex. These areas are closely involved in the pathophysiology of a number of mood, psychotic and cognitive disorders, including schizophrenia, schizo-affective and depressive disorders, and Alzheimer's disease. As an autoreceptor, the α2C-AR is responsible for regulating noradrenaline negative feedback particularly during states of low endogenous noradrenaline activity. Heteroreceptor mediated modulation of dopaminergic, serotonergic, cholinergic and GABAergic neurons as well as evidence showing attenuation of NMDA-receptor-mediated effects provide further support for the therapeutic potential of selective α2C-AR antagonist in these illnesses. This review will summarize our current understanding and future prospects of the α2C-AR as a neuropsychiatric drug target, focussing on the potential of α2C-AR antagonism in treating cognitive dysfunction, depression and schizophrenia, as well as presenting challenges, new prospects and future directions of investigation.

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Chapter 6: Manuscript D

1. Introduction

Drugs that target the central presynaptic α_2-adrenoceptor (α_2-AR), which plays an important role in modulating the release of noradrenaline as well as various other neurotransmitters, have clinical use in several major neuropsychiatric disorders [1]. While α_2-AR agonists clonidine and guanfacine are used in the treatment of attention deficit hyperactivity disorder (ADHD) [2], α_2-AR antagonists mianserin and mirtazapine have seen widespread use in the therapy of major depressive disorder. Additionally, the unique clinical profile of atypical antipsychotics like clozapine and asenapine has, at least in part, been related to potent antagonism of α_2-ARs [3; 4; 5]. Importantly, both conventional antipsychotics [6; 7; 8] and antidepressants [9; 10; 11] show improved efficacy following augmentation with an α_2-AR antagonist. Furthermore, the α_2-AR antagonist idazoxan has been shown to improve cognitive performance in patients with frontal dementia [12]. However, the role of selectively targeting α_2-AR subtypes and specifically the α_{2C}-AR subtype have introduced the possibility of additional therapeutic benefits in the treatment of the above illnesses [13; 14; 15; 16]. On the back of early transgenic mouse models that have indicated an important and distinctive role for α_{2C}-AR in several models of neuropsychiatric illness and function [17; 18; 19], this receptor has subsequently been associated with the mechanism of action of antipsychotics [4]. More recently genetic polymorphism of the α_{2C}-AR has been associated with emotional dysfunction in major depressive disorder [20] and ADHD [21]. With the first highly selective α_{2C}-AR subtype antagonist, ORM-12741, in further clinical development following successful completion of initial Phase IIa evaluation for improvement of cognitive parameters in Alzheimer's disease [22], and coupled with a recently published series of animal studies using a related chemical entity (ORM-10921) [16;48], the potential therapeutic benefit of selectively blocking α_{2C}-ARs for the treatment cognitive dysfunction, and its application in mood and psychotic disorders has attracted renewed interest. This review will in particular focus on depression and schizophrenia, and will also highlight the progress in development of α_{2C}-AR related tools and technology to facilitate future basic and clinical research.

2. Distinct roles for α_2-AR subtypes

Firstly, it is worthwhile to outline the physiological characteristics that indicate a distinct role for the α_{2C}-AR in the treatment of neuropsychiatric disorders.

The α_2-AR couples to heterotrimeric $G_{i/o}$ proteins when activated by their endogenous agonist, leading to inhibition of adenylyl cyclase and voltage-gated calcium channels, and activation of mitogen-activated protein kinase (MAPK) signalling cascades. The presynaptic α_2-AR autoreceptor inhibits noradrenaline (NA) synthesis and release and as such plays an important role in negative feedback,

while presynaptic α_2-AR heteroreceptors located on dopaminergic, serotoninergic, glutamatergic and other terminals regulate the release of these latter transmitters [23; 24].

The presynaptic α_2-AR consists of three subtypes which are conserved across mammalian species, identified as the $\alpha_{2A/D}$, α_{2B} and α_{2C}-AR-subtypes; the $\alpha_{2A/D}$ designation refers to a small difference in amino acid sequence in rodents (α_{2D}) as opposed to that in humans and rabbits (α_{2A}) [25; 26]. The rodent α_{2D}-AR however is presumed to reflect the same physiological processes and pharmacological outcomes as the α_{2A}-AR, and studies on this receptor in rodents is therefore reported as findings for the α_{2A}-AR. The α_2-ARs have different tissue distribution patterns, with different physiological and pharmacological profiles [26; 27]. While all three receptors are present in the central nervous system (CNS), the α_{2B} receptor is mainly expressed in the thalamus and does not seem to contribute to CNS auto- and heteroreceptor function [28]. The α_{2A}-ARs and α_{2C}-ARs, on the other hand, are the main α_2-ARs modulating neurotransmission in the CNS [28; 29; 30], with the α_{2C}-AR recognized to play a very distinct and specific role in memory, cognition and mood disorders in a manner different to that of the α_{2A}-AR. These separate effects will become evident in this review.

The α_{2C}-AR only constitutes approximately 10 % of α_2-ARs in the CNS compared to the remaining 90% contributed by the α_{2A}-AR [31]. Nevertheless, the α_{2C}-AR seems to play a very important role in neurotransmission and potentially in the dysregulation observed in neuropsychiatric illness. The α_{2A}-AR is widely distributed in the CNS, while the expression of the α_{2C}-AR is more distinct and focussed in specific areas involved in cognitive functions and in the regulation of mood [32; 33; 34; 35]. Thus α_{2C}-ARs densely populate the ventral and dorsal striatum and the hippocampus in humans [26; 34; 36], monkeys and rodents [37]. Dense population of the olfactory tubercle is also evident, while cortical expression is evident albeit more subtle [34; 38]. The cerebellum is devoid of these receptors. This specific distribution pattern suggests that the α_{2C}-AR could be involved in illnesses involving hippocampal and striatal dysfunction, such as schizophrenia and depression, and in conditions characterized by cognitive deficits and cognitive decline involving these cortico-limbic structures (e.g. Alzheimer's disease).

The distribution of α_{2C}-ARs in human, monkey and rodent brains are analogous [32; 36; 37; 39], implying that neuropharmacological data from transgenic mouse models and from rodent animal models may be relevant for humans also. Due to the paucity of sufficiently subtype-selective ligands, of which only a few have become available for preclinical investigation during the last decade [13; 14; 40], transgenic mouse models have predominantly been used to shed light on the physiology and pharmacology of the different α_2-AR subtypes, with transgenic work in zebrafish also contributing to

this pool of knowledge [41; 42]. Transgenic mouse models employ targeted genetic deletion or overexpression of the α_{2A}-AR and/or α_{2C}-AR. In order to investigate the pharmacological role of these α_2-AR subtypes, the assumption has been made that α_2-AR subtype deletion or knockout (α_{2x}–KO) would reflect the effect of chronic administration of a subtype-selective antagonist, whereas α_2-receptor subtype overexpression (α_{2X}-OE) would mimic the effects of chronic subtype selective agonist treatment [43]. Findings from these transgenic mouse models have suggested distinct and often seemingly opposing CNS roles for the α_{2A}-AR and α_{2C}-AR, with the potential implication that non-selective α_2-AR modulation might negate potential beneficial effects which could be attained by subtype selective targeting.

Using rodent models predicting antipsychotic-, antidepressant- and procognitive-like effects in genetically modified mice, an important role for the α_{2C}-AR has been brought to light, illustrated by a modulation of behaviour and neurotransmission associated with neuropsychiatric conditions such as depression, schizophrenia and cognitive decline [18; 19; 43; 44; 45; 46; 47]. However, transgenic mouse studies may suffer from the unknown contribution by physiological compensatory changes that take place in the lifelong absence or overexpression of α_2-ARs [13]. For example Sallinen et al [19] demonstrated deficient sensorimotor gating in α_{2C}-KO mice, suggesting that α_{2C}-AR antagonism may induce effects akin to psychotomimetic agents such as phencyclidine (PCP). This contradicts recent findings described in the social isolation rearing (SIR) model of schizophrenia where ORM-10921 *improved* sensorimotor gating deficits in this model [16; 48]. Similarly, improved sensorimotor gating has been demonstrated in NMDA-antagonist models that induce sensorimotor gating deficits applied in Sprague Dawley and Wistar rats treated with the α_{2C}-AR selective antagonists JP-1302, ORM-10921 and ORM-12741 [13; 14; 15]. This type of anomaly underscores the necessity to verify results obtained using transgenic mouse models with studies employing selective α_{2C}-AR ligands in more naturalistic animal models with good validity for the chosen human disorder.

3. Role of the α_{2C}-AR in regulating monoamines, excitatory and inhibitory amino acids and neurotrophic activity

Despite a number of new theories that have been put forward to explain the underlying biology and development of mood and psychotic disorders, targeting monoaminergic transmission as a construct towards understanding and treating these disorders remains a relevant subject of investigation (reviewed in [49]). The latter review emphasizes that while oxidative stress, neuroinflammation and neuroplastic/degenerative events are implicated, selectively and appropriately targeting

monoaminergic processes remains a core construct in novel drug development. The α_{2C}-AR is associated with various effects on monoamine turnover. When treated with the subtype non-selective α_2-AR agonist, dexmedetomidine, agonist-induced decreases in monoamine levels were absent in α_{2C}-OE mice, while concentrations of dopamine (DA), noradrenaline (NA) and serotonin (5-HT) were shown to be increased in the brains of α_{2C}-KO mice [47]. Deactivation of α_{2C}-ARs might thus facilitate increased CNS monoamine levels, which could be of benefit in disorders where monoamine dysfunction is apparent.

3.1 Noradrenaline

The potency and affinity of NA has been shown to be higher at the α_{2C}-AR than at the α_{2A}-AR [50; 51; 52], and evidence from peripheral and CNS tissue demonstrates that the α_{2C}-AR would inhibit NA release at low endogenous concentrations of NA as opposed to high concentrations for the α_{2A}-AR [31; 51]. Deactivation kinetics also differs for the α_{2A}-AR and α_{2C}-AR, with the α_{2C}-AR displaying much slower deactivation upon removal of NA than the α_{2A}-AR [52]. Despite their modest presentation in the CNS, α_{2C}-ARs have distinct effects on various neurotransmitters. Along with the α_{2A}-ARs, the α_{2C}-ARs are involved in the presynaptic negative feedback loop on NA release in the cortex, although α_{2C}-AR-mediated presynaptic inhibition occurs much more slowly than that mediated by α_{2A}-ARs [31]. Figure 1 depicts this proposed differential regulation on NA feedback and receptor pharmacodynamics mediated by α_{2A}-ARs and α_{2C}-ARs. Ordway and co-workers (1995) demonstrated that the density of α_{2C}-AR binding sites increases 3 weeks after the destruction of NA terminals in the rodent cerebral cortex, which suggests that α_{2C}-AR density is regulated by the synaptic availability of NA. In contrast, altered α_{2A}-AR density was not observed under the same conditions [53]. This effect of synaptic availability on α_{2C}-AR expression might imply a unique role for the α_{2C}-AR in disorders characterized by hypo- or hyperadrenergic states, such as depression and schizophrenia.

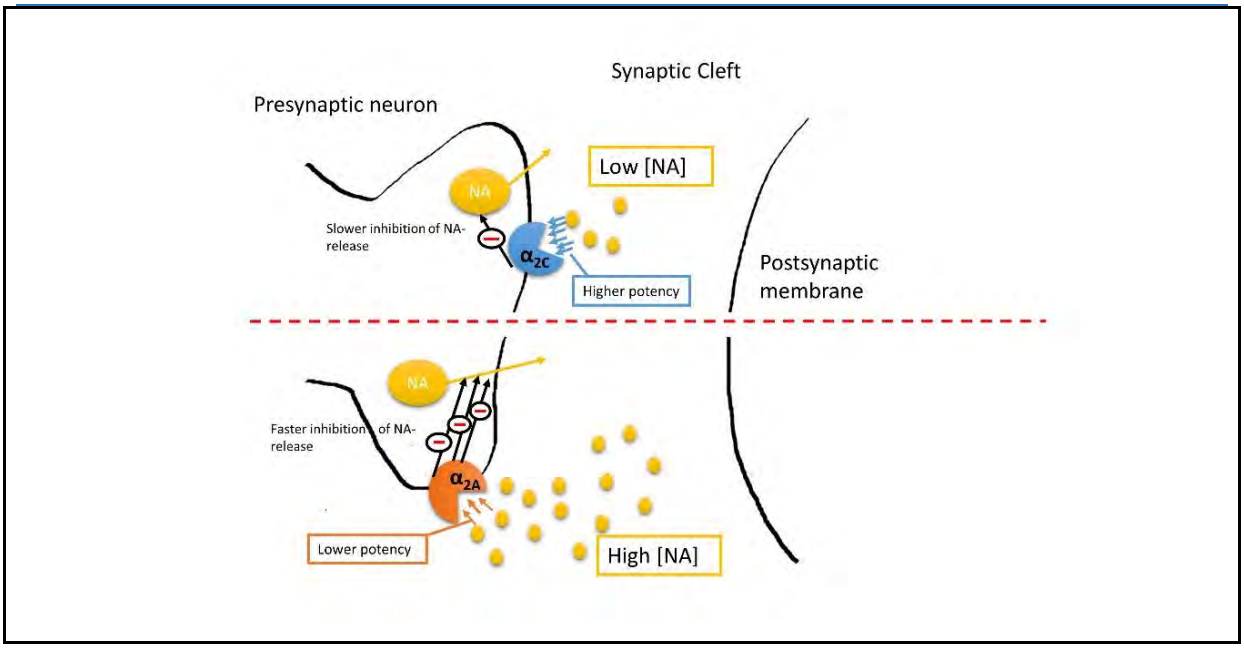

Fig 1. Differential presynaptic inhibition of noradrenaline release by the α_{2C}-AR (top panel) and the α_{2A}-AR (bottom panel). At low endogenous noradrenaline concentrations, the α_{2C}-AR is responsible for inhibition of noradrenaline release, while the α_{2A}-AR inhibits noradrenaline release at high endogenous NA concentrations. α_{2C}-AR-mediated inhibition of NA release is a slower process than that of α_{2A}-AR mediated inhibition, although the potency and affinity of NA is higher at the α_{2C}-AR than at the α_{2A}-AR. See text for more detail. NA = noradrenaline, Θ = inhibition

The α_{2C}-AR has also been implicated in α_2-autoreceptor-mediated modulation of hippocampal and cortical DA and NA synthesis via feed-back inhibition on tyrosine hydroxylase, which converts tyrosine to the DA precursor 3,4-dihydroxyphenylalanine (DOPA) [54]. These authors used early subtype-specific antagonists and agonists to measure levels of DOPA and NA in rodent hippocampus and cerebral cortex, with $\alpha_{2B/C}$-AR antagonists increasing synthesis of DOPA and $\alpha_{2B/C}$-AR agonists decreasing its synthesis. Although the ligands used in this study were $\alpha_{2B/C}$-AR specific ligands, the expression of α_{2B}-ARs is limited to the hypothalamus and doesn't seem to contribute to auto- and heteroreceptor function in the CNS [28]. The modulation of DOPA levels and consequently NA and DA levels in this early study using marginally selective α_2-AR subtype-specific ligands, with α_{2A}-AR specific antagonism and agonism devoid of such effects, are depicted in Figure 2. The limitation of this study is however that the subtype-specific ligands used here also have some antagonist activity at $5HT_{1A}$ receptors [55]. α_{2C}-AR selective antagonism could therefore play a role in increasing DA and NA levels and thus be of benefit in the treatment of neuropsychiatric illness. Nevertheless, these findings need to be confirmed using novel, highly subtype selective α_2-AR ligands.

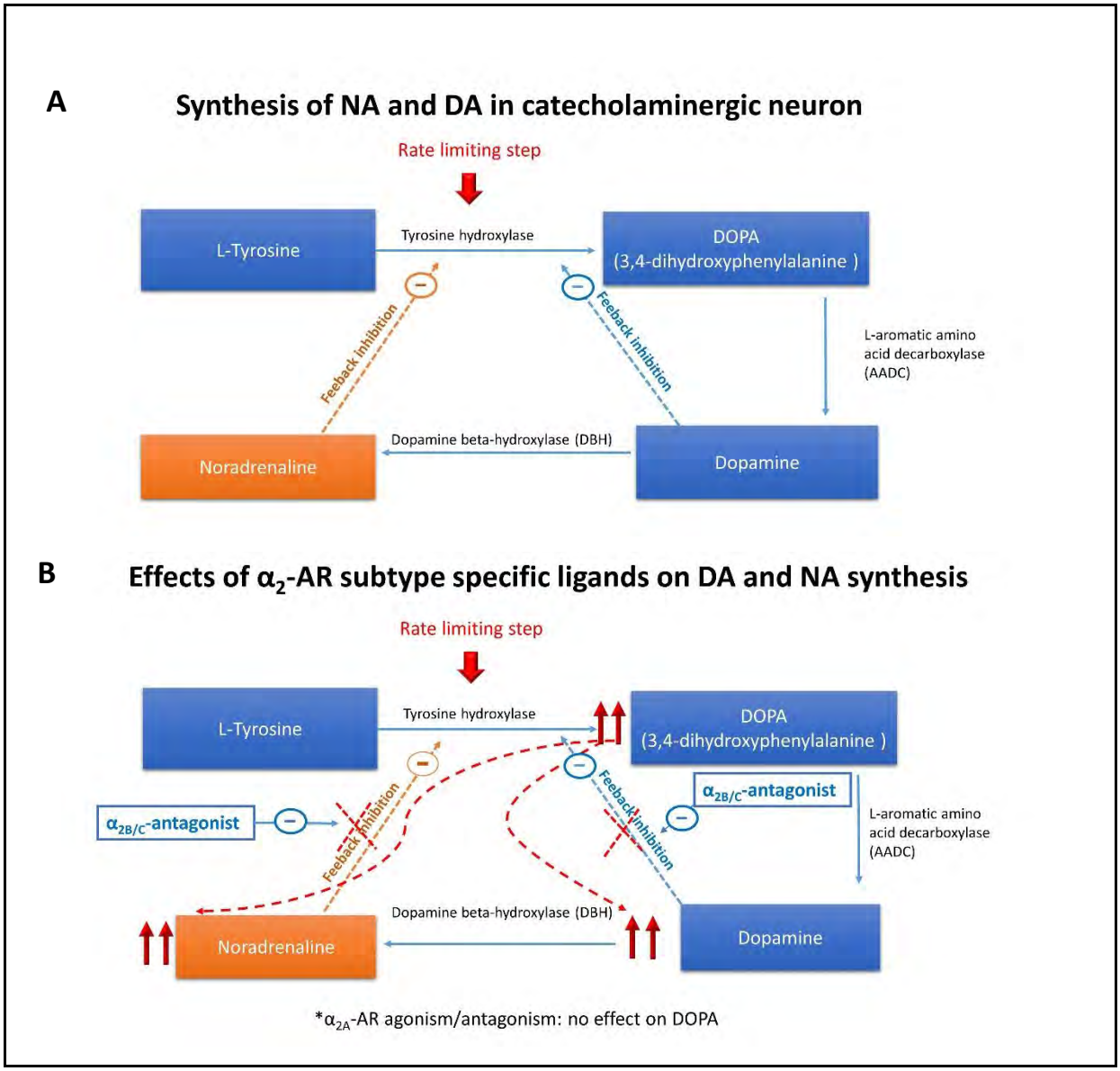

Fig 2. Negative feedback of DA and NA on catecholamine synthesis (A), showing the results from early studies on the effect of $\alpha_{2B/C}$-AR selective ligands (although not highly selective) on the synthesis of DOPA and consequently NA and DA levels [54] in rodent hippocampus and cortex (B). While a specific $\alpha_{2B/C}$-AR antagonist increases the synthesis of DOPA via inhibiting the negative feedback on the rate-limiting tyrosine hydroxylase enzyme, this increased levels of DOPA could result in increased synthesis of DA and NA. α_{2A}-AR specific agonism and antagonism did not influence the levels of DOPA in rodent hippocampus and cortex. These pharmacological characteristics provide a rationale for the use of α_{2C}-AR selective ligands in the treatment of psychiatric disorders involving disturbances in NA and DA. NA = noradrenaline, DA= dopamine Θ = inhibition

3.2 Dopamine

The high expression of α_{2C}-ARs in the striatum allows it to modulate presynaptic DA release and DA-mediated behaviours [31]. Zhang and co-workers (1999) [44] provided early evidence for the remarkable ability of DA to function as an activating ligand on striatal α_{2C}-ARs, while Sallinen and co-

workers (2013) [14] used a novel α_{2C}-AR selective antagonist (ORM-10921) to show increased *in vitro* α_{2C}-AR potency and selectivity ratios in the presence of DA as agonist. These authors also reported that ORM-10921 increases extracellular DA levels in the rodent prefrontal cortex. Changes in brain DA metabolism have been observed in α_{2C}-KO and α_{2C}-OE mice [47]. α_{2C}-OE mice show higher levels of the DA metabolite homovanillic acid (HVA) in the frontal cortex but not in the striatum compared to wild type controls, whereas α_{2C}-KO animals showed lower HVA concentrations in the striatum [47], although not in the frontal cortex. These findings suggest decreased striatal but not frontal cortical DA turnover in response to α_{2C}-AR deactivation and increased cortical DA turnover in response to α_{2C}-AR stimulation (schematically represented in Figure 3). Therefore an important relationship between DA and α_{2C}-ARs exists. The therapeutic potential of this can be realized in the targeting of α_{2C}-ARs in disorders characterized by mesolimbic-cortical DA imbalance, such as schizo-affective conditions.

Another example of DA modulation by α_{2C}-AR's is the latter's effects on D-amphetamine induced hyperlocomotion. D-amphetamine administration is associated with increased DA and NA release in the caudate nucleus and nucleus accumbens of the dorsal and ventral striatum, respectively, as well as in the prefrontal cortex, with the animals presenting with hyperactive behaviour [56; 57; 58]. Hyperlocomotion was further increased in α_{2C}-KO mice following D-amphetamine administration, while D-amphetamine-induced hyperlocomotion was attenuated in α_{2C}-OE mice [46]. Furthermore, subsequent studies with methylphenidate, a drug which also increases DA release and blocks DA and NA reuptake, showed increased response rates in a cognitive task sensitive to alterations in striatal DA levels in α_{2C}-KO mice [59]. The effects of drugs that increase synaptic DA could therefore be enhanced by antagonism of the α_{2C}-AR, further emphasizing the role of α_{2C}-ARs in regulating DA release and metabolism.

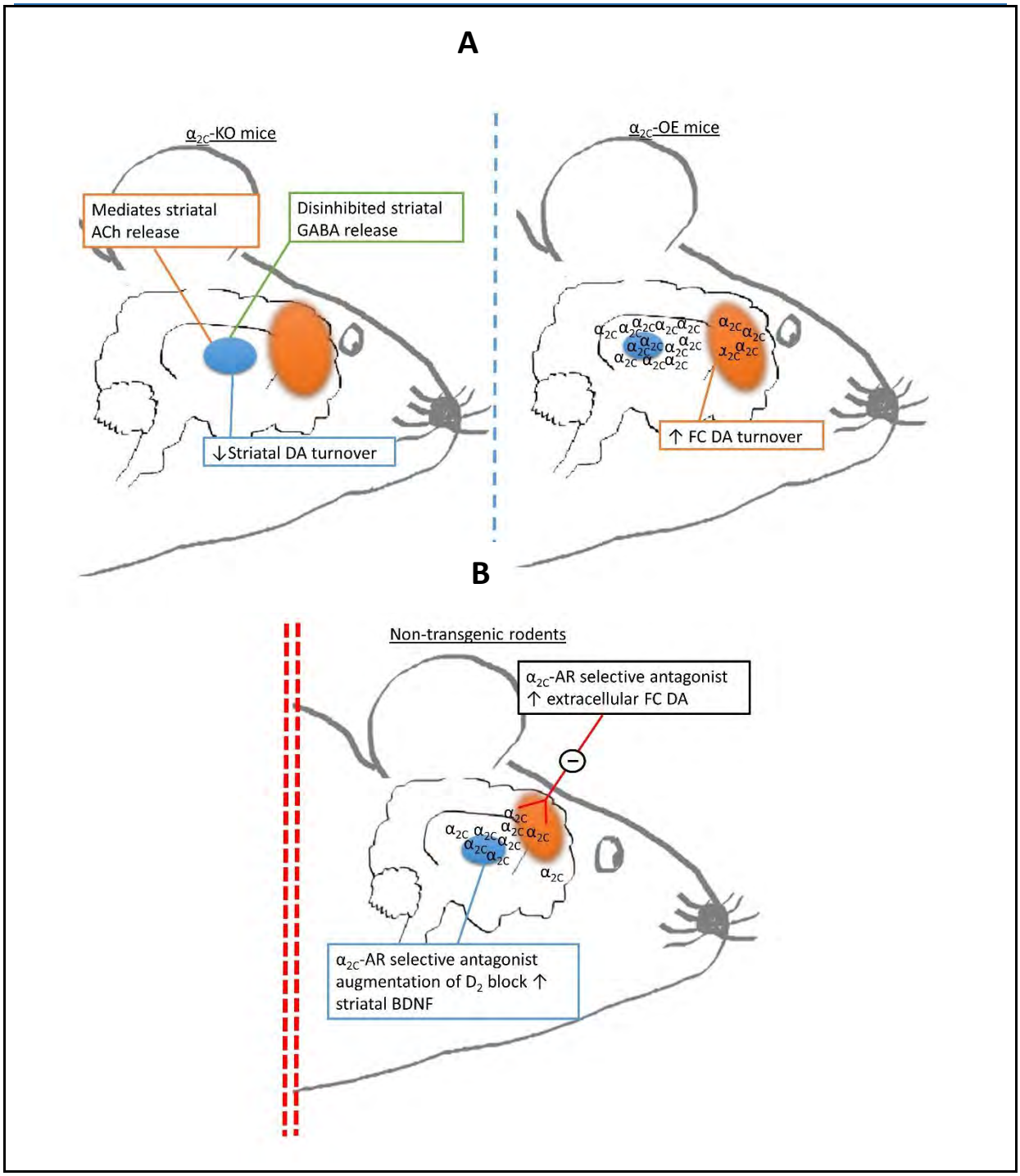

Fig 3. Schematic outline of findings relating to dopaminergic, GABA-ergic and cholinergic transmission in the striata and frontal cortices of (A) α_{2C}-OE and α_{2C}-KO mice and in (B) non-transgenic rodents treated with a selective α_{2C}-AR antagonist. **A.** HVA levels are increased in the frontal cortex of α_{2C}-OE mice, while HVA concentrations are decreased in the striata of α_{2C}-KO mice. Furthermore, α_2-AR agonist induced inhibition of striatal GABA release is disinhibited in α_{2C}-KO mice, while studies in α_{2C}-KO mice indicate a role of the α_{2C}-AR in mediating acetylcholine release. **B.** Microdialysis assays show that treatment with the α_{2C}-AR selective antagonist, ORM-10921, increases extracellular DA levels in the frontal cortex of Han-Wistar rats, while augmentation of haloperidol with ORM-10921 increased BDNF in striatal brain tissue of SIR rats. Ach = Acetylcholine, FC = frontal cortical, SIR= social isolation reared. Θ = inhibition

3.3 Serotonin

Fewer evidence is available to delineate the role of the α_{2C}-AR on serotonergic function. The hippocampal and cortical synthesis of the serotonin (5-HT) precursor, 5-hydroxytryptophan (5-HTP), via the rate-limiting enzyme tryptophan hydroxylase, seems to be dependent on both α_{2A}-ARs and α_{2C}-ARs in the rodent, with α_{2A}-ARs emerging as the main α_2-AR modulating 5-HT synthesis [54]. Non-selective α_2-AR agonism decreases 5-HTP levels in rodent hippocampus and cerebral cortex, while an increase in cortical 5-HTP levels seems to be largely induced by α_{2A}-specific antagonism, with a $\alpha_{2B/C}$-AR antagonist producing an *increase* in 5-HTP levels (a quarter of the increase induced by a α_{2A}-AR antagonist). These effects were not mirrored in the hippocampus, although $\alpha_{2B/C}$-AR specific antagonism decreased hippocampal 5-HTP levels in this brain region [54] (see Figure 4 for a schematic representation). Similarly, α_2-AR-agonist induced inhibition of 5-HT release is dependent on both α_{2A}-ARs and α_{2C}-ARs, although the α_{2C}-AR exerts a more subtle effect on 5-HT release [30]. These authors demonstrated that α_{2C}-KO mice present with lower disinhibition of agonist-induced 5-HT release in hippocampal and occipito-parietal cortex slices compared to α_{2A}-KO mice. The α_{2A}-AR is therefore the main α_2-AR regulating 5-HT release and possibly 5-HT synthesis. Nevertheless, selective antagonism of the α_{2C}-AR could result in meaningful increases in 5-HT release and region-specific 5-HT synthesis, which could be of importance in various neuropsychiatric illnesses characterised by altered serotonergic neurotransmission, such as obsessive compulsive disorder, depression and schizophrenia. Confirmation of these findings using highly selective α_{2C}-AR subtype ligands is warranted to enhance our understanding of this receptor's effect on central serotoninergic transmission.

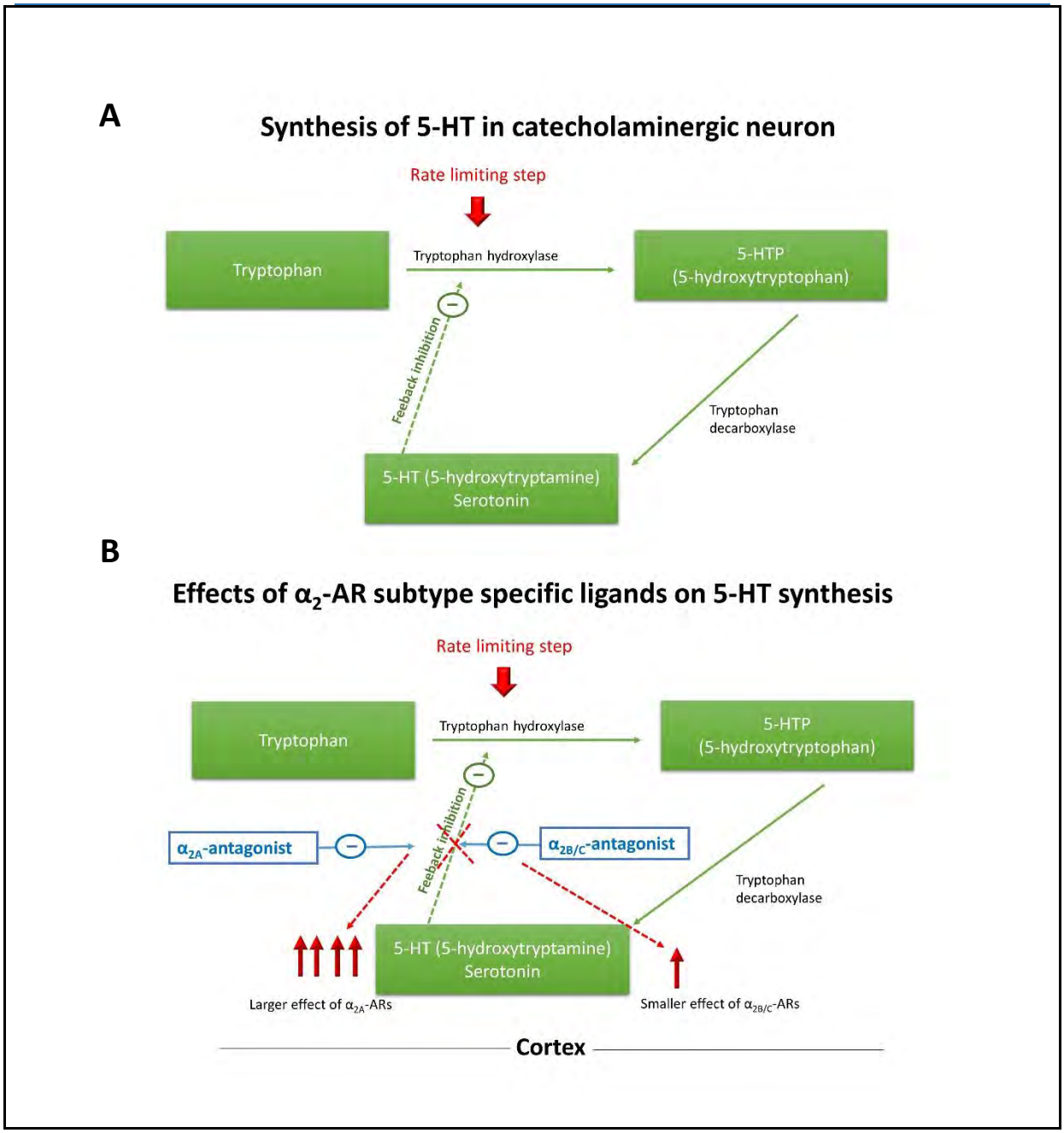

Fig 4. Negative feedback of 5-HT on catecholamine synthesis (A), showing the results from early studies employing α_{2A}-AR and $\alpha_{2B/C}$-AR specific ligands (although not highly selective) on the synthesis of 5-HTP and consequently by implication on 5-HT levels [54] in rodent cortex (B). Specific α_{2A}-AR antagonism has the largest effect on the synthesis of 5-HTP via inhibiting the negative feedback on the rate-limiting tryptophan hydroxylase enzyme, while an $\alpha_{2B/C}$-AR specific antagonist mirrors this effect but to a lesser extent. These pharmacological characteristics provide a rationale for the use of α_{2C}-AR selective ligands in the treatment of psychiatric disorders involving disturbances in 5-HT. Θ = inhibition

3.4 GABA

Apart from effects on the synthesis and release of monoamines, the α_{2C}-AR is an important mediator of striatal, but not hippocampal GABA release [45]. While α_{2C}-ARs and α_{2A}-ARs are located on different

striatal neurons, almost all GABAergic projection neurons in the striatum contains α_{2C}-ARs [33], which project to the globus pallidus and substantia nigra [60]. Inhibition of striatal GABA release by an α_2-AR antagonist (RX821002) is completely blocked in α_{2C}-KO mice, while enhancement of striatal GABA release by an α_2-AR agonist is maintained in these mice, suggesting that inhibition of striatal GABA release is strongly mediated by the α_{2C}-AR [45]. This response was not found with respect to hippocampal GABA release [45]. These findings could suggest that blockade of the α_{2C}-ARs disinhibits GABA release in brain regions with dense dopaminergic innervation and low noradrenergic innervation (Zhang and Ordway 2003). Considering the presence of α_{2C}-ARs in the striatum (particularly the reward centres), and the role of GABAergic transmission in mania and the action of mood-stabilizers [61], selective α_{2C}-ARs antagonism could be of value in disorders like schizophrenia in which deficient GABAergic transmission plays a pathophysiological role [62].

3.5 Glutamate

Limited information is available on the specific role of the α_{2C}-AR on central glutamatergic neurotransmission. Non-selective α_2-AR antagonism per se does not seem to be beneficial in reversing glutamate receptor NMDA-antagonist induced cognitive impairment [63], while non-selective α_2-AR agonism may ameliorate these impairments [64; 65; 66]. However, combined α_2-AR antagonism and dopamine $D_{2/3}$ receptor antagonism does reverse NMDA-antagonist induced cognitive dysfunction, while enhancing cortical glutamate transmission mediated through prefrontal cortical DA release and activation of frontal D_1 receptors [8; 67]. Contrasting the aforementioned findings, α_{2C}-AR selective antagonists JP-1302, ORM-10921 and ORM-12741 reverse cognitive and social dysfunction in NMDA-antagonist induced animal models of neuropsychiatric illness [13; 14; 15], indicating a beneficial role of selective α_{2C}-AR antagonism (and *not* agonism) in attenuating symptoms induced by hypoglutamatergic states, although the mechanism is uncertain.

Non-selective activation of α_2 heteroreceptors on glutamatergic neurons by NA reduces glutamate release in various brain areas implicated in depression and schizophrenia, including the frontal cortex, ventral tegmental area, hippocampus and nucleus accumbens [68; 69]. Moreover, the treatment arsenal of both depression and schizophrenia include drugs that are α_2-AR antagonists that would thus facilitate disinhibition of glutamate release. In support of this notion, the addition of a non-selective α_2-AR-antagonist to a D_2-blocker *increases* frontal cortical glutamatergic neurotransmission in rodents to a similar extent as the atypical antipsychotic clozapine, while at the same time improving cognitive and negative symptoms [4; 8; 67]. Notably, clozapine has a 3-4 fold α_{2C}/α_{2A} ratio and one of the highest α_{2C}/D_2 ratios of any antipsychotic. The novel antipsychotic, asenapine, which also presents increased

affinity for the α_{2C}-AR [3] as well as good efficacy in treating both positive and negative symptoms of schizophrenia, has also been shown to enhance frontal cortical glutamate transmission via dopaminergic activation of D_1 receptors [70], as seen with clozapine and following the combination of a α_2-AR lytic with a dopamine antagonist [8; 67]. In PCP-treated Sprague Dawley rats, Sallinen and co-workers (2013) [14] measured striatal NMDA currents in medium spiny neurons after administration of ORM-10921, reporting that ORM-10921 did not reverse PCP-induced NMDA channel block in these neurons. Considering the above described effects of α_2-lytic activity on prefrontal cortical glutamatergic transmission [67], measuring frontal cortical NMDA currents in NMDA-antagonist model of schizophrenia might elucidate the effects whereby α_{2C}-AR selective antagonists improve NMDA-induced behavioural deficits.

Thus, the above findings suggest that α_{2C}-AR antagonism allows the regulation of cortical glutamatergic transmission, which may underscore a therapeutic option in schizophrenia and cognitive dysfunction in particular. The involvement of α_{2C}-ARs in the inhibition of striatal GABA release as mentioned above [45], could also indicate an indirect role of the α_{2C}-AR in glutamate release, since glutamate release is also tonically regulated by GABAergic interneurons [71].

3.6 Acetylcholine

Dysfunctional central cholinergic transmission has been implicated in the underlying pathophysiology of mood disorders, cognitive dysfunction and schizophrenia (reviewed in [72]), while various drugs target the cholinergic system in an attempt at improving the above symptoms [73; 74; 75]. α_2-adrenergic heteroreceptors, as well as D_2 receptors, inhibit the release of acetylcholine [1]. Considering its regulation of dopaminergic, serotoninergic, GABAergic and possibly glutamatergic transmission as described above, the α_{2C}-AR might similarly be involved in the presynaptic regulation of cholinergic transmission. Since acetylcholine inhibits GABA release [76], Zhang and Ordway (2003) [45] have posited that α_{2C}-AR effects on striatal GABA release (described above) might be attributed to the location of α_{2C}-AR on striatal cholinergic neurons. These authors have also reported that the α_{2C}-AR mediates inhibition of striatal adenylyl cyclase and acetylcholine release, while these effects might be related to tonic activation of the α_{2C}-AR by DA [44; 45]. A selective α_{2C}-AR antagonist might thus disinhibit striatal acetylcholine release that in turn may decrease extracellular striatal DA [72]. The findings of Zhang and Ordway (2003) [45]might thus be applicable to a neuropsychiatric disorder characterized by striatal dopaminergic over-activity, such as schizophrenia.

A complex interplay of cortico-striatal cholinergic, GABAergic and glutamatergic transmission has been described in the pathophysiology of schizophrenia [72], along with cholinergic regulation of dopaminergic and serotoninergic transmission and vice versa. However, more evidence in this regard

using α_{2C}-AR selective ligands is required to make more definitive conclusions regarding the interplay of the α_{2C}-AR, the cholinergic system and the effect of this interplay in neuropsychiatric disorders.

The α_{2C}-AR thus seems to play a distinct role in monoaminergic, GABAergic, glutamatergic and possibly cholinergic neurotransmission, making it a promising target in several neuropsychiatric illnesses characterized by deficits in monoaminergic and/or aminergic neurotransmission, such as depression, schizophrenia and conditions associated with cognitive decline. The potential therapeutic role of the α_{2C}-AR in these conditions is discussed below, with a specific focus on therapeutic potential in depression and schizophrenia.

4. The α_{2C}-AR and cognition

Many neuropsychiatric illnesses, including depression and schizophrenia present with cognitive deficits and memory impairments [77; 78; 79; 80]. With the α_{2C}-AR has been shown to be involved in cognitive deficits evident in non-pathological animal models [17; 81; 82; 83], these findings imply a significant role in the treatment of cognitive deficits in a number of neuropsychiatric disorders. Although α_2-AR agonists are associated with improved cognitive processing in humans and animals [84; 85; 86; 87; 88] and in the treatment of cognitive decline associated with ageing [89], these effects have been shown to be mediated via activation of the α_{2A}-AR [83; 87; 90], which is also responsible for sedative and hypotensive effects [26; 91]. In contrast, a beneficial role for genetic deletion of the α_{2C}-AR subtype or, by extrapolation, for selective α_{2C}-AR antagonism has been demonstrated in improving memory and cognition.

The Morris Water Maze (MWM) is a spatial water navigation task requiring the rodent to learn and remember the location of an escape platform in a water arena in order to locate a hidden (submerged) platform in subsequent trials by using various spatial cues. The escape latency is a measure of spatial working memory. The test is a reliable tool correlating with hippocampal synaptic plasticity as well as intact glutamate NMDA-receptor function [92]. In early transgenic mouse studies, α_{2C}-OE mice showed impaired spatial and non-spatial escape strategies and search patterns in the MWM. Since these impairments could be reversed to a greater extent in α_{2C}-OE than in wild type mice by the administration of an α_2-AR antagonist, this suggests that the α_{2C}-AR might play a more important role than the α_{2A}-AR in brain areas involved in spatial navigation [17; 81; 82]. Considering the dense expression of the α_{2C}-AR in the hippocampus and striatum and that hippocampal [93] and striatal lesions [94] impair aspects of MWM navigation, α_{2C}-AR selective antagonism may be important in information processing and behavioural adaptation to environmental change. α_{2C}-OE mice display normal passive avoidance learning, suggesting that impaired water maze navigation in α_{2C}-OE mice does not reflect defective stimulus-response learning but is involved in complex organization of escape

behaviour [17]. This effect of α_{2C}-AR antagonism might partially explain previous findings for pro-cognitive effects of the non-selective α_2-AR idazoxan on planning, attention, episodic memory and verbal fluency in patients with frontal lobe dysfunction [95].

The radial arm maze is a test used to measure reference and working memory in rodents and relies on intact functioning of the prefrontal cortical, hippocampal and striatal interconnections to locate food rewards hidden in various radial arm target sites [96]. Björklund and co-workers (2001) [83] demonstrated that the non-selective α_2-AR agonist dexmedetomidine improved working memory in the radial arm maze, and that this improvement was enhanced in α_{2C}-KO mice, suggesting that the absence of α_{2C}-AR agonism or α_{2C}-AR antagonism might result in enhanced effects on working memory in this test. Moreover the benefits of selective α_{2C}-AR antagonism on cognitive parameters have been corroborated in animal models of schizophrenia, depression and age-related cognitive impairment [14; 15] [16; 48] as well as in clinical trials investigating novel therapy for Alzheimer's disease [22]. These findings will be discussed in sections 5, 6 and 7 under the relevant neuropsychiatric illness.

Brain derived neurotrophic factor (BDNF) is the most prevalent neurotrophic growth factor in the CNS where it is especially important in regulating synaptic plasticity and various aspects underlying cognitive performance, memory and mood [97; 98]. Acute and chronic stress purportedly has detrimental effects on rodent BDNF expression in the hippocampus, while altered BDNF levels are evident in depressive disorders [49; 99] and in schizophrenia [100; 101]. While both antipsychotics and antidepressants alter BDNF levels to varying extents [102; 103; 104; 105; 106], non-selective α_2-AR antagonism has also been associated with neurogenesis and increased BDNF levels in the hippocampus [107; 108]. Noradrenergic [105; 109], dopaminergic [110], serotonergic [111] and GABA-glutamate [112] interactions are involved in the expression of BDNF. With the α_{2C}-AR acting as a heteroreceptor to modulate the release of many of the aforementioned neurotransmitters, this receptor might play an indirect role in altering the expression of BDNF. In social isolation reared rats, an animal model of schizophrenia presenting with *reduced* striatal BDNF [16], combining haloperidol with the selective α_{2C}-AR antagonist ORM-10921 increased striatal BDNF levels vs. haloperidol alone, although α_{2C}-AR antagonism per se did not reverse lowered striatal BDNF levels on its own [16]. The ability of ORM-10921 to augment the haloperidol response was also correlated with significantly improved object recognition memory in rats following 14 day treatment vs. haloperidol alone [16].

C-fos and JunB are markers of neuronal activity, and play an important role in synaptic function [113; 114]. Up-regulation of c-fos mRNA indicates recent neuronal activity while its expression in the CNS is induced by various stimuli, including noxious stimuli, neurotransmitters, neurotrophins and other growth factors as well as learning and memory processes [115], Jun-B is also involved in the regulation

of emotional memory [116]. BDNF restores the expression of these transcription factors after neuronal insult [117], indicating a role for BDNF in neuroplasticity at gene-transcription level. Interestingly, cortical and hippocampal levels of c-fos and JunB mRNA are increased in α_{2C}-KO mice compared to wild type-controls [18], while this is not the case in α_{2C}-OE mice. Whether this increase is associated with altered BDNF levels in α_{2C}-KO mice has not been investigated. Nevertheless, the increase in neuronal activity in α_{2C}-KO mice is of interest considering the pro-cognitive behavioural characteristics of this transgenic strain.

Considering these findings and the beneficial effects of α_{2C}-AR antagonism on cognitive parameters (discussed above and in sections 5 to 7), incorporating selective α_{2C}-AR antagonism as an augmentation strategy might present with additional neurotrophic effects, as evident when combined with haloperidol [13].

5. The α_{2C}-AR and depression

The α_{2C}-AR is densely expressed in the hippocampus, an area that is prominent in the pathophysiology of depression [118]. Major depressive disorder is thought to be characterized, at least in some patients, by deficits in monoamine activity and diminished inhibitory neural control of the hippocampus and prefrontal cortex (PFC) over the hypothalamic-adrenal-pituitary-axis (HPA-axis), resulting in HPA-axis over-activity with reduced negative feedback and hypercortisolaemia [119]. Additionally sleep alterations, deficient neurotrophic support and the effects of chronic stress on neurotrophic factors and hippocampal atrophy has been hypothesised to underlie the complex pathophysiology of depression [120; 121]. Aside from limbic function, the hippocampus plays an important role in learning and memory, and hippocampal atrophy could account for the cognitive deficits that accompany major depressive disorder [118].

Antidepressants generally increase the levels of noradrenaline, serotonin and dopamine to varying extent depending on the class of antidepressant (e.g. TCA, SSRI, SNRI) [122], although about 40% of patients do not respond to the most commonly used conventional antidepressants [123; 124]. Considering that α_{2C}-ARs have a fairly dense expression in the hippocampus, this adrenoceptor subtype might be a potential tool to address hippocampal-related disturbances in depression. α_2-AR dysregulation in depressive disorders is widely described in the literature ([23] for review) with increased α_2-AR density found in platelets and in post-mortem brain tissue of depressed suicide completers in the locus coeruleus, temporal and frontal cortex, hippocampus and hypothalamus [125; 126; 127; 128]. Moreover, receptor up-regulation has been specifically associated with the α_{2A}-AR subtype in depressed states [129; 130; 131]. The role of the α_2-AR in the action of antidepressants is

also fairly well described in the literature, with α_2-AR down-regulation induced by antidepressants such as the TCAs and mirtazapine in rodents and depressed humans (brain and platelets), although regional differences in α_2-AR down-regulation have been noted in the CNS (findings reviewed in Cottingham and Wang (2012) [23].

The rodent forced swim test (FST) is a well-described predictive model for antidepressant drug screening [11; 132; 133; 134; 135; 136; 137]. In this test, rodents are exposed to inescapable swim stress where the adoption of an immobile posture during re-exposure is thought to reflect failure in persistent escape-directed behaviour, purported to model certain behavioural aspects of depression such as modelling the psychological feeling of "entrapment" and the replacement of active coping strategies with passivity or an unwillingness to actively attempt to escape a perceived inescapable situation [132; 138; 139]. Specifically, an increase in immobility time is considered to reflect the aforementioned depressive-like manifestations. Various antidepressants reduce immobility time in the FST [132].

The α_2-AR has been implicated in mediating the antidepressant (or anti-immobility) effects of TCAs in the FST, while activation of the α_{2A}-AR subtype seems especially involved [23; 140]. Interestingly, the α_{2C}-AR seems to play an opposite role in regulating antidepressant effects in the FST. Early studies in α_{2C}-OE models in mice have suggested that α_{2C}-AR activation has a detrimental effect on FST immobility, with α_{2C}-OE mice displaying increased immobility compared to wild type-controls [18], an effect not attributed to altered locomotor activity [47]. On the other hand, α_{2C}-KO mice (i.e. inactivation of the α_{2C}-AR) demonstrate an antidepressive phenotype [18]. These findings might explain why relatively non-selective α_2-AR agonists [141; 142; 143] and certain non-selective α_2-AR *antagonists* have both shown antidepressant-like effects in the FST. Mirtazapine is an α_2-AR antagonist that has shown a putative early-onset antidepressant effect [144] and which increases serotonergic neurotransmission via antagonism of presynaptic α_2-ARs [145]. Similarly, the antidepressant mianserin is also an antagonist at α_2-ARs. Dhir and Kulkarni (2007) demonstrated that augmentation with the non-selective α_2-AR antagonist yohimbine potentiated the anti-immobility effects of both fluoxetine and venlafaxine in the mouse FST [10]. This effect is mirrored in the clinic, where addition of yohimbine to SSRI treatment hastens antidepressant response and increases the number of responders compared to SSRI treatment alone [146]. Enhanced clinical response to SSRI's, venlafaxine and bupropion is also evident following augmentation with the α_2-AR antagonist antidepressant mirtazapine, resulting in an almost doubling of the remission rates in patients on mirtazapine add-on therapy [147; 148].

The novel object recognition test (NORT) is a two-trial behavioural measure that relies on the rodent's innate preference to explore novel objects over familiar objects, thereby enabling measurement of recognition memory [149; 150]. This cognitive function is deficient in patients with depression [151; 152]. Recently an important role for the α_{2C}-AR in this test has been demonstrated in rodents using subtype selective α_{2C}-AR antagonists [16; 48]. The declarative memory processes underlying the NORT relies on the perirhinal cortex and the hippocampal complex [153; 154; 155], with hippocampal function being compromised in depression [118]. Recently we reported that sub-chronic ORM-10921 reduced immobility time in the FST *as well as* increased novel object recognition memory in the Flinders Sensitive Line (FSL) rat, a genetic rodent model of depression [48]. Moreover, these effects were not seen with the non-selective α_2-AR antagonist idazoxan[48]. Although non-selective α_2-AR antagonists have shown improved recognition memory in the NORT, these data have never been corroborated in a translational animal model of depression [156; 157]. The finding of improved novel object memory and reduced immobility in the FST in FSL animals constitutes the first findings for an antidepressant-like effect of an α_{2C}-AR antagonist under relevant translational conditions, viz. evaluation under pathological conditions akin to depression and following chronic treatment [43]. Considering the above-mentioned beneficial effect of α_{2A}-AR agonism on immobility in the FST and the increased immobility of α_{2C}-OE mice observed in this test, emphasizes that both the absence/minimization of α_{2A}-AR antagonism and the presence of α_{2C}-AR antagonism might be required for antidepressant-like effects.

Altered circadian rhythm is a well-recognised biomarker of major depressive disorder [49], with HPA-axis dysregulation and hypercortisolaemia underlying the pathophysiology of the disorder [119]. Since stress and depression are causally linked, stress-induced increases in glucocorticoids have been suggested to mediate hippocampal atrophy and neurodegeneration evident in depressed individuals [77; 118]. This incapacitation of the hippocampus leads to impaired cognitive function as well as a perpetuation of the stress response, the latter due to an inability of the hippocampus to exert top-down control over the HPA-axis [77]. Long-term exposure to elevated cortisol levels induces regional up-regulation of α_2-ARs [158], which in turn could result in further decreased noradrenaline levels in depressive disorders. In this regard, the α_2 antagonist antidepressant mirtazapine has been associated with amelioration of HPA-axis hyperactivity in depressed patients [159; 160], although this amelioration was not necessarily related to clinical improvement. Interestingly this amelioration of HPA-axis hyperactivity is not mirrored in rodents [161]. In healthy volunteers, the acute administration of the α_2 antagonist idazoxan has been associated with an attenuated normal diurnal fall in plasma cortisol, although this effect seemed to disappear with chronic treatment [162]. Earlier studies on the other hand have shown that depressed patients exhibited much greater cortisol responses to

yohimbine than controls [163]. Therefore hypercortisolism in depression may underscore a central dysfunctional adrenocortical feedback mechanism, and while non-selective α_2-AR antagonism aids in down-toning HPA-hyperactivity, the α_{2C}-AR subtype too seems important in regulating glucocorticoid responses.

The α_{2C}-KO mouse demonstrates attenuated plasma corticosterone elevations vs. wild type controls following different stressors, while α_{2C}-OE mice show more intense corticosterone responses compared to α_{2C}-KO [18]. Interestingly, non-selective α_2-AR antagonism seems to elevate plasma corticosterone levels and to potentiate corticosterone responses to restraint stress in rodents [164]. More selective α_{2C}-AR antagonism might therefore elicit beneficial effects on HPA-axis functioning in depressive states. Previous studies have shown that both inhibition of corticosterone synthesis and injection of glucocorticoid receptor antisense oligonucleotides into the dentate gyrus of the hippocampus decreases immobility in the FST [165; 166]. That the α_{2C}-AR is the only α_2-AR subtype expressed in this region in mice [47], together with the effects of α_{2C}-AR modulation on corticosterone levels and FST immobility, consolidates a valuable role for α_{2C}-AR antagonism in the treatment of depression. Furthermore, a genetic polymorphism of the α_{2C}-AR has been associated with emotional dysfunction in major depressive disorder [20], further prompting investigations into the therapeutic potential of targeting this receptor in depression.

This is an important area for further investigation, given the growing recognition of stress and inflammation pathways in neuropsychiatric and neurological disorders. Additional studies with highly selective α_{2C}-AR antagonists in various animal models of depression, especially etiological models such as the FSL rat and the chronic stress paradigm, will be valuable in increasing our understanding of the therapeutic potential of these subtype selective antagonists in depression.

6. The α_{2C}-AR and schizophrenia

α_{2C}-ARs are widely distributed in the striatum, including humans [167], where they are thought to play an inhibitory role [168]. In fact, 40% of striatal α_2-ARs are of the α_{2C}-AR subtype, which is 4 times higher than the total representation of the α_{2C}-AR in the CNS. This role has distinct importance when striatal dysfunction in schizophrenia is considered, especially its intricate connection to frontal cortical cognitive deficits [169]. The α_{2C}-AR therefore represents a potentially beneficial pharmacological approach to modulate striatal deficits in schizophrenia. The prefrontal cortex (PFC), striatum and hippocampus are implicated in schizophrenia, where noradrenergic and dopaminergic terminals presenting with α_{2C} auto and heteroreceptors are well-represented in these brain regions [31; 32; 33;

169]. Despite the prominence of the dopamine hypothesis of schizophrenia, a hypothesis for noradrenergic dysfunction in schizophrenia is strongly supported in the literature [170].

The dopamine paradox is well-described in schizophrenia [171], with mesolimbic hyperdopaminergic and mesocortical hypodopaminergic states being postulated. Excessive striatal dopamine is linked to positive symptoms, while cortical dopaminergic deficits are linked to cognitive dysfunction [172]. In section 3 we discussed findings that suggest decreased striatal but not frontal cortical dopamine turnover in response to α_{2C}-AR deactivation, while increased cortical dopamine turnover is noted in response to α_{2C}-AR stimulation [47]. These early findings suggest a positive role for α_{2C}-AR antagonism in regulating mesolimbic-cortical dopaminergic imbalances, which may have therapeutic value in schizophrenia. GABAergic and glutamatergic deficits are also implicated in disease pathology, where loss of GABAergic output onto secondary glutamatergic cortical neurons, required for tonic control over sub-cortical dopaminergic neurons, results in increased mesolimbic dopaminergic firing (increased striatal dopamine release) and consequently the presentation of psychotic symptoms [71]. As discussed earlier, the α_{2C}-AR strongly mediates striatal GABA release, while α_{2C}-AR deactivation seems to disinhibit α_2-AR antagonist-induced inhibition of GABA release [45]. Here α_{2C}-AR subtype selective antagonism might present with more beneficial effects on striatal GABA release when applied in the treatment of schizophrenia than non-selective α_2-AR antagonism.

The atypicality of antipsychotic drugs primarily reflects their reduced risk of extra-pyramidal side effects and to some extent improved efficacy against negative and cognitive symptoms of schizophrenia [173], over and above their efficacy against positive symptoms. Atypicality has, apart from actions at serotonergic receptors, been proposed to strongly revolve around α-AR modulation, with α_1 and α_2-AR antagonism suggested to contribute to stabilisation of dysregulated dopaminergic activity [5]. Indeed, in a thorough comparative study employing human receptor binding data, Shahid et al., (2009) [3] have shown that a number of atypical antipsychotics (clozapine, quetiapine, asenapine, risperidone, ziprasidone) possess significant α_2-AR antagonist properties. Furthermore, quetiapine and in particular clozapine showed prominent α_{2C}/D_2 as well as α_{2C}/α_{2A} receptor selectivity. A pharmacological profile constituting a higher α_2/D_2 receptor binding ratio [5] and specifically a higher α_{2C}/D_2 receptor selectivity ratio [3; 4] has been suggested to mediate the improved efficacy of drugs like clozapine that exhibit lower D_2-receptor occupancy. The α_2/D_2 receptor subtype selectivity ratios for various antipsychotics as well as the α_{2C}-AR selective antagonist, ORM-10921 (which as described below has shown antipsychotic-like activity in animal models) are depicted in Figure 5. Thus reduced D_2-receptor occupancy might be possible in therapy because of the beneficial effects of α_2-AR antagonism on dysregulated dopaminergic activity, allowing for improved efficacy with less motor side-effect profiles. Support for this hypothesis has been demonstrated in studies employing non-

selective α_2-AR antagonists (eg. idazoxan) as augmentation to D_2-receptor antagonist antipsychotic treatment [8; 67; 174]. While this combination of α_2-AR and D_2 receptor antagonism presented with improved antipsychotic-like effects in mouse models of schizophrenia, it also resulted in enhanced cortical glutamatergic transmission and increased dopaminergic output in the PFC, with subsequent improvement in cognitive parameters in rats [67]. The effects of this augmentation strategy were comparable to that of clozapine. While clozapine requires approximately 45% D_2 receptor occupancy compared to >70% required by other D_2 receptor antagonists for antipsychotic efficacy [175; 176], the combination of idazoxan with a D_2 receptor antagonist exhibited potent antipsychotic effects similar to that of clozapine at similar low D_2 receptor occupancy rates [67].

Sensorimotor gating refers to the ability to integrate and process sensorimotor information, deficits of which are suggested to underlie the fragmentation of reality evident in schizophrenia [177]. The prepulse inhibition (PPI) of startle test refers to the attenuation of a startle response produced by the presentation of a smaller prepulse, and is used to study the gating of sensorimotor information by the brain [177; 178]. A typical example of the PPI test in humans employs the somatosensory eye blink reflex in response to acoustic, tactile (e.g. air puffs) or light stimuli [177; 179; 180]. A PPI deficit can be induced in humans and animals by various psychotomimetic drugs, including dopaminergic and antiglutamatergic drugs. Animal models of schizophrenia, such as social isolation rearing (SIR) [181; 182; 183] and various transgenic models including mice with altered dopamine, serotonin and glutamate receptor expression [184], present with deficits in PPI. Importantly, antipsychotic drugs normalize disrupted PPI in animals and humans [185; 186; 187; 188; 189]. While the contribution of non-selective α_2-blockade to modulation of PPI has been proposed, the literature is somewhat inconclusive in this regard. In fact some papers have suggested that antagonism of the α_{2A}-AR does not contribute to enhancement of the PPI [190; 191; 192; 193].

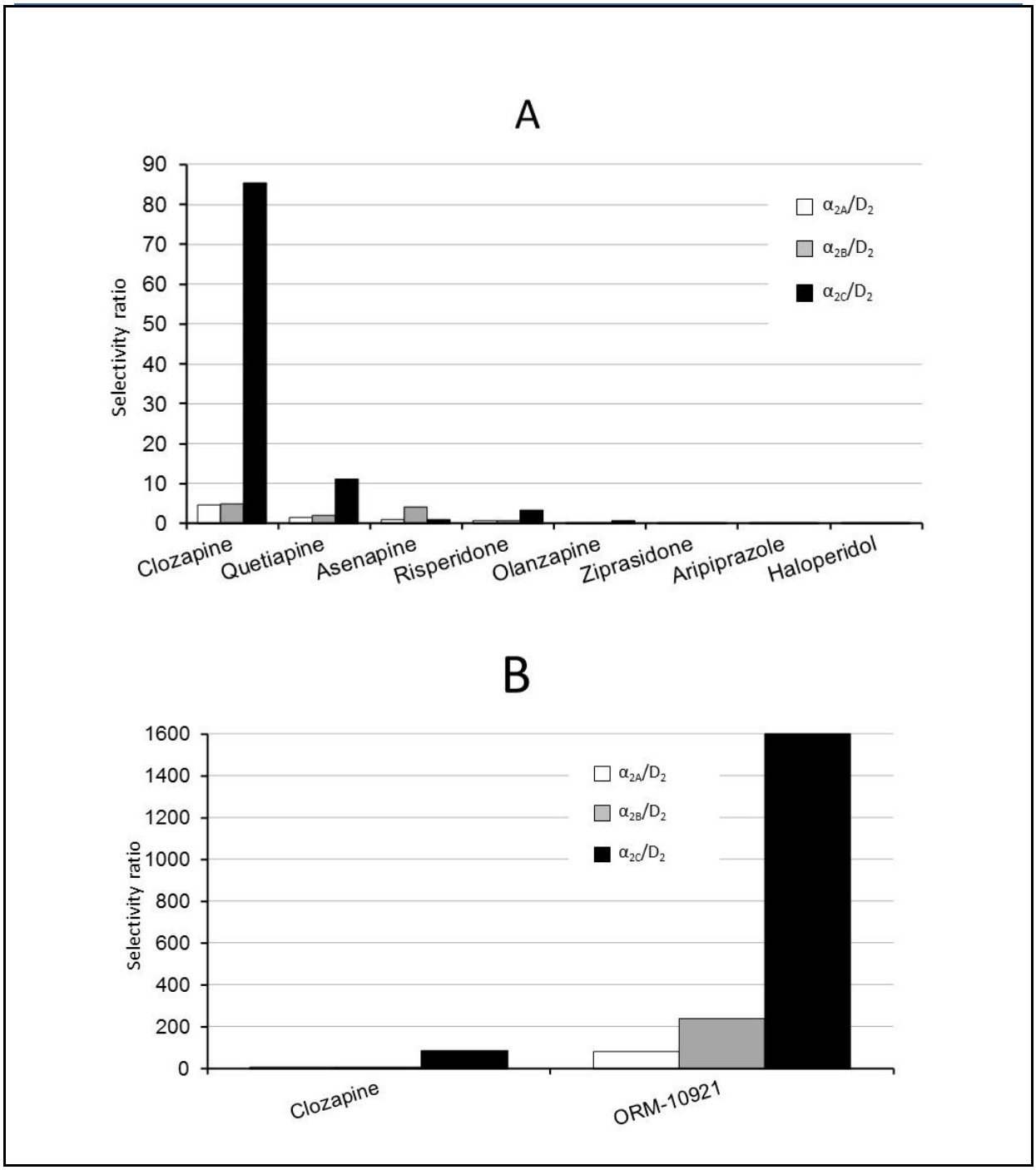

Figure 5. Human α_2-AR subtype/ D_2 selectivity ratios of various antipsychotics, adapted from Shahid et al., 2009 [3]. **A**. Comparative overview of the subtype selectivities of various antipsychotics. The α_{2C}/D_2 receptor selectivity ratios are as follows: clozapine, 85; quetiapine, 11; risperidone, 3.4; asenapine, 1; olanzapine, 0.53; ziprasidone, 0.02; haloperidol, 0.011. **B**. Comparison between the subtype selective ratios of clozapine and the α_{2C}-AR antagonist ORM-10921, which has shown antipsychotic-like effects in preclinical studies [14;16]. The α_{2C}/D_2 receptor selectivity ratio for ORM-10921 is 1600. Selectivity ratios were determined by dividing the D_2 K_i value by the applicable α_2 receptor K_i value.

Considering the important role for α_2-AR antagonism in managing schizophrenia [5], it is interesting to review earlier studies in transgenic mouse models where antipsychotic-like effects were shown to be rather sub-type dependent. In this regard, α_{2C}-KO mice demonstrated clear PPI deficits compared to wild type controls, while α_{2C}-OE mice had markedly higher PPI scores than their wild type controls [19], suggesting that α_{2C}-receptor agonism may induce antipsychotic-like effects. However, this extrapolation from transgenic mouse studies has since been disproven following experiments with α_{2C}-AR antagonists. JP-1302, ORM-10921 and ORM-12741 consistently show improved PPI in Sprague Dawley and Wistar rats in NMDA-antagonist induced models of schizophrenia [13; 14; 15]. More recent findings in SIR rats, a putative neurodevelopmental model of schizophrenia [194; 195], corroborate these earlier findings, with ORM-10921 found to significantly improve SIR-associated PPI deficits in a manner comparable to clozapine [16]. Moreover, ORM-10921 enhanced the effects of haloperidol on the above-mentioned deficits in PPI [16]. This discrepancy between findings from transgenic models and pharmacological treatment highlights the need for studying the physiological and pharmacological role of the α_{2C}-AR using subtype selective ligands, as transgenic mice might suffer from unknown physiological compensatory changes that may be misleading as to how the various α_2-AR subtypes are involved.

Cognitive impairment is an important cause of functional disability in patients with schizophrenia and is for the most part refractory to treatment. These impairments include deficits in working, recognition and spatial memory, cognitive flexibility, learning and attention [78; 79; 196]. Such deficits can be measured in rodent models of schizophrenia using the MWM, NORT, 8-arm Radial Maze, 5-choice serial reaction time task and attentional set-shifting task [194]. Using these tests, cognitive performance is severely impaired in various rodent models of schizophrenia [194; 197; 198; 199; 200; 201; 202; 203; 204]. However, antipsychotic treatments are not always reproducibly effective in reversing these cognitive deficits [204; 205; 206; 207; 208], which in facts reflects the relative lack of efficacy displayed by antipsychotics in treating cognitive impairment in the clinic [78; 196], or variations in protocol used from one group to another. In this regard, the NEWMEDS initiative is making progress at harmonising protocols and animal models in order to develop standard procedures with good translation to the clinic [209].

Recently, the highly selective α_{2C}-AR antagonist ORM-12741 showed improved effects on NMDA-antagonist induced disruptions in working memory and spatial learning, navigation and memory in rodents [15]. NMDA-antagonist models include the administration of the glutamate NMDA-receptor antagonists dizolcipine (MK-801) or phenylcyclidine (PCP) which are known to induce behavioural, cognitive and neurochemical disruptions in behaviour akin to those seen in schizophrenia [210]. ORM-12741 attenuates the disruption of learning in the MWM induced by MK-801, while also improving

PCP-induced memory deficits in the 8-ARM radial maze [15]. Similar findings were reported for the selective α_{2C}-AR antagonist ORM-10921 which attenuates MK-801-induced spatial navigation in the MWM [14], a finding consistent with effects described for atypical [206; 211] but not typical antipsychotics such as haloperidol [212]. Additionally, ORM-10921 significantly improved novel object recognition memory in SIR rats, comparable to the atypical antipsychotic clozapine, while also significantly improving the efficacy of haloperidol in this regard [16]. Cognitive deficits in schizophrenia are for the most highly refractory to treatment [79] and make up some of the core elements of the disorder [213]. Evidence of improved cognition in NMDA-antagonist and neurodevelopmental models of schizophrenia with novel highly selective α_{2C}-AR antagonists therefore provides important information that demonstrates the therapeutic potential of targeting the α_{2C}-AR in treating cognitive deficits associated with schizophrenia.

Another interesting observation concerns the neurotrophic hypothesis of schizophrenia, where alterations in BDNF signalling are widely evident in the illness [100; 101], as well as being associated with the above-mentioned cognitive deficits [49]. As noted earlier, although ORM-10921 alone did not significantly reverse lowered BDNF levels in SIR rats on its own after 14 days of treatment, haloperidol plus ORM-10921 showed a significant increase in BDNF levels that exceeded that of either drug alone [16]. These preliminary results further support a therapeutic role for α_{2C}-AR antagonism in improving cognitive symptoms in schizophrenia.

Social isolation, decreased social cognition and impaired social skills form part of the negative symptoms of schizophrenia and are refractory to most antipsychotic treatments [214]. The social interaction test measures deficits in social motivation and self-directed behaviour in rats and is used to measure predictive validity of antipsychotics in rodent models of schizophrenia [215]. Although there are mixed results, generally atypical antipsychotics are more effective than typical antipsychotics at attenuating social deficits in rodent models of schizophrenia [215; 216]. In this regard, the α_{2C}-AR antagonists ORM-10921 and ORM-12741 significantly attenuate PCP-induced deficits in social interaction in short-term single-housed (Sallinen et al 2013) and pair-housed rats (Sallinen et al 2013a).

Considering the above-mentioned role of the α_{2C}-AR in addressing symptoms of schizophrenia, it is of note that most antipsychotic drugs have activity at the α_{2C}-AR. Using functional assays performed using cloned receptors in Chinese hamster ovary (CHO) cell lines, Kalkman and Loetscher (2003) noted α_{2C}/α_{2A} receptor selectivity ratios for clozapine, chlorpromazine, risperidone, quetiapine and iloperidone to be between 3 and 12 [4]. Interestingly, the novel antipsychotics asenapine and lurasidone both present with potent α_{2C}-AR binding affinity [3; 217]. As mentioned earlier, the α_{2C}/D_2

selectivity ratio has been suggested to be an important factor in antipsychotic efficacy [4], and here clozapine, arguably the most efficacious antipsychotic in treatment refractory schizophrenia [218], presents with an α_{2C}/D_2 selectivity ratio of 85 compared to ratios of 0.01 – 11 for other tested antipsychotics [3] (see Figure 5). Haloperidol, on the other hand, has the lowest potency at the α_{2C}-AR as well as the lowest α_{2C}/D_2 ratio of tested compounds [3; 4]. In fact, the above-mentioned results demonstrating bolstered antipsychotic-like and pro-cognitive effects of haloperidol when combined with a selective α_{2C}-AR-antagonist supports the notion that an increased α_{2C}-AR/D_2 ratio *will* translate to superior antipsychotic effects.

Taken together, α_{2C}-AR antagonism therefore seems to be involved in the mechanism of improved sensorimotor gating, recognition and spatial working memory as well as social functioning in pharmacological and neurodevelopmental models of schizophrenia. Therefore current pharmacological, behavioural and neurochemical evidence are indicative of a potential therapeutic role for α_{2C}-AR antagonism in the treatment of schizophrenia, and further study with more subtype selective ligands is encouraged.

To summarize and enable quick comparison between findings from transgenic mouse models and those gained from treatment with α_{2C}-subtype selective ligands, Table 1 summarizes neurochemical and behavioural findings reported in transgenic mice and in various rodent models predicting pro-cognitive-like, antipsychotic-like and antidepressant-like effects.

Table 1. Summary of neurochemical and behavioural findings in transgenic α_{2C}-OE or α_{2C}-KO mice and in available data in rodent and human studies employing highly selective novel α_{2C}-AR antagonists.

Parameter investigated	Findings in transgenic α_{2C}-OE mice	Findings in transgenic α_{2C}-KO mice	Findings in rodents and humans using highly selective α_{2C}-AR antagonists
Monoamine levels	α_2-agonist-induced decreases in whole brain DA, NA and 5-HT levels is absent in α_{2C}-OE mice and OE-wt controls[1] Stress-induced elevations in whole brain HVA and 5-HIAA responses are attenuated in α_{2C}-OE mice vs. OE-wt controls[2]	*In*creased levels of DA, NA and 5-HT in whole brains of α_{2C}-KO mice and KO-wt mice after treatment with α_2-agonist [1] Stress-induced elevations in whole brain HVA and 5-HIAA in α_{2C}-KO mice was similarly to KO-wt controls[2]	-
Dopamine turnover	Increased cortical DA turnover in α_{2C}-OE mice (higher HVA levels) vs. OE-wt mice[1] Increased whole brain HVA levels in α_{2C}-OE mice vs. OE-wt controls with a trend towards increased DOPAC[2]	Decreased striatal DA turnover in α_{2C}-KO mice (lower HVA levels) vs. KO-wt mice[1] Decreased whole brain DOPAC and HVA concentrations in α_{2C}-KO mice vs. KO-wt controls[2]	ORM-10921 increases extracellular DA in rodent prefrontal cortex[3]
Markers of neuronal activity	α_{2C}-OE mice do not present with altered cortical and hippocampal levels of JunB and c-fos mRNA vs. OE-wt-controls[2]	α_{2C}-KO mice have increased cortical and hippocampal levels of JunB and c-fos mRNA vs. KO-wt controls. This difference disappears after stress [2]	-

Parameter investigated	Findings in transgenic α_{2C}-OE mice	Findings in transgenic α_{2C}-KO mice	Findings in rodents and humans using highly selective α_{2C}-AR antagonists
Dopaminergic drug induced hyperlocomotion	D-amphetamine induced hyperlocomotion is attenuated in α_{2C}-OE mice vs. OE-wt controls[4]	D-amphetamine induced hyperlocomotion is further increased in α_{2C}-KO mice vs. KO-wt controls[4]	-
Dopaminergic drug induced cognitive reward responses	-	Increased response rates to methylphenidate in cognitive task sensitive to altered striatal DA in α_{2C}-KO mice vs. KO-wt controls [5]	-
Striatal GABA release	-	α_2-AR antagonist-induced inhibition of striatal GABA release is disinhibited in α_{2C}-KO mice vs. KO-wt mice[6]	-
Working memory in MWM	α_{2C}-OE mice show impaired MWM navigation strategies vs. OE-wt-controls. Impaired MWM navigation can be reversed by an α_2-AR antagonist [7,8,9]	-	ORM-12741 and ORM-10921 attenuates MK-801-disrupted learning in the MWM[3,10]
Working memory in radial arm maze	-	α_2-AR agonist-induced working memory improvements in radial arm maze are more pronounced in α_{2C}-KO mice vs. KO-wt controls[11]	ORM-12741 attenuates PCP-disrupted working memory in the radial arm maze[10] ORM-12741 attenuates age-related memory and learning deficits in the radial arm maze[10] ORM-12741 improves episodic memory in Alzheimer's patients with a tendency to improve working memory[12]

Parameter investigated	Findings in transgenic α_{2C}-OE mice	Findings in transgenic α_{2C}-KO mice	Findings in rodents and humans using highly selective α_{2C}-AR antagonists
Response learning in T-maze	-	α_2-AR agonist does not induce improvements in response learning in the T-maze in α_{2C}-KO or KO-wt control mice, and no differences were noted in drug naive α_{2C}-KO vs. wt-control mice in the T-maze [11]	-
Passive Avoidance learning	α_{2C}-OE mice show normal passive avoidance behaviour vs. OE-wt controls[7]	-	-
FST immobility	Increased FST immobility in α_{2C}-OE mice vs. OE-wt mice[2]	Decreased FST immobility time in α_{2C}-KO mice vs. KO-wt controls[2]	JP-1302 decreases FST immobility time in Sprague Dawley rats[13] ORM-12741 decreases FST immobility time in Sprague Dawley rats [10] ORM-10921 decreases FST immobility time in Sprague Dawley rats[3] ORM-10921 decreases FST immobility time in FSL rats[14]
Plasma corticosterone levels	Stress-induced plasma corticosterone elevations are more intense in α_{2C}-OE mice vs. OE-wt controls after repeated, but not acute stress[2]	Stress-induced plasma corticosterone elevations are attenuated in α_{2C}-KO mice vs. KO-wt controls[2]	-
Recognition memory in NORT	-	-	ORM-10921 improves object recognition memory (declarative memory) in the NORT in FSL rats[14]

Parameter investigated	Findings in transgenic α_{2C}-OE mice	Findings in transgenic α_{2C}-KO mice	Findings in rodents and humans using highly selective α_{2C}-AR antagonists
PPI	α_{2C}-OE mice present with higher PPI vs. OE-wt controls[15]	α_{2C}-KO mice present with deficient PPI vs. KO-wt controls[15]	JP-1302 reverses PCP-induced PPI deficits in Wistar and Sprague Dawley rats[13] ORM-12741 reverses PCP-induced PPI deficits in Sprague Dawley rats[10] ORM-10921 reverses SIR-induced PPI deficits in Sprague Dawley rats and augments the effect of haloperidol on PPI to a similar extent as clozapine [16]
Social interaction	-	-	ORM-10921 and ORM-12741 attenuates PCP-induced social interaction deficits in Sprague Dawley rats[3,10]
Recognition memory in NORT	**-**	-	ORM-10921 improves object recognition memory (declarative memory) in the NORT in SIR rats and augments the effect of haloperidol in the NORT to a similar extent as clozapine[16]

SIR= social isolation reared, MWM = Morris water maze, FST = forced swim test, NORT = novel object recognition test, FSL = Flinders Sensitive Line, PCP = phenylcyclidine, MK-801 = dizolcipine, KO = receptor knockout, OE = receptor overexpression, wt= wild type. References: 1. Sallinen et al., 1997 [47]; 2. Sallinen et al 1999 [18]; 3. Sallinen et al., 2013a [14] ; 4. Sallinen et al., 1998b [46]; 5. Ihalainen et al., 2001 [59]; 6. Zhang and Ordway, 2003 [45]; 7. Björklund et al., 1998 [17]; 8. Björklund et al., 1999a [81]; 9. Björklund et al., 1999b [82]; 10. Sallinen et al., 2013b [15]; 11. Björklund et al., 2001 [83]; 12. Rinne et al., 2013 [22]; 13. Sallinen et al., 2007 [13]; 14. Uys et al., 2016b [48]. 15. Sallinen et al., 1998a [19]; 16. Uys et al., 2016a [48]

7. Evidence for targeting the α_{2C}-AR in other neuropsychiatric disorders

This review has focussed on the therapeutic potential of targeting the α_{2C}-AR subtype in depression and schizophrenia. However, recent preclinical and preliminary clinical evidence has revealed the promising therapeutic role for the α_{2C}-AR in age-related cognitive decline, particularly Alzheimer's disease. These new developments nevertheless have relevance for depression and schizophrenia as cognitive dysfunction is common in patients with Alzheimer's disease, depression and schizophrenia, while symptoms of the latter two illnesses in turn permeate through to patients suffering from Alzheimer's disease.

ORM-12741 is a novel highly selective α_{2C}-AR antagonist with a 4000-fold selectivity for α_{2C}-AR over that of the $\alpha_{2A/B}$-AR [15]. Age-related memory and learning, as assessed in the rodent 8-ARM radial maze (measuring spatial working memory and reference memory), was attenuated by sub-chronic administration of ORM-12741 [15]. The beneficial effect of this highly selective α_{2C}-AR antagonist was confirmed in a phase IIa randomized, double-blind, placebo-controlled clinical study in patients with moderate Alzheimer's Disease and associated behavioural symptoms [22]. Here ORM-12741 was used as an add-on drug in patients already receiving donepezil, galantamine, rivastigmine or memantine. Significant improvements in episodic memory were observed with ORM-12741 add-on therapy, while a tendency to improve working memory was also observed. Additional to the benefits on cognition, ORM-12741 produced statistically significant improved scores on the Neuropsychiatric Inventory (NPI) questionnaire distress score, which assesses the caregiver's perceived levels of distress with respect to symptoms of delusions, agitation and aggression, depression, anxiety, disinhibition and other behavioural symptoms in the patient. Moreover, there was a positive trend to improve the total NPI score which would also reflect symptom severity and frequency [219]. These findings indicate that improvements in cognitive performance in Alzheimer's disease patients are supported by amelioration of co-presenting behavioural impairments, of which various aspects are reminiscent of the symptomatology of depression and schizophrenia.

Although beyond the scope of this review, some early evidence has also suggested a potential therapeutic role for selective targeting of the α_{2C}-AR subtype in attention deficit hyperactivity disorder and possibly in bipolar disorder. A study in coloboma mice, a mouse model of ADHD [220], reported that the α_{2C}-subtype preferring α_2-AR antagonist MK912 (~10 fold selectivity over α_{2A}-AR and α_{2B}-AR) ameliorated noradrenaline-dependent hyperactivity [221], while α_{2A}-AR and α_{2B}-AR subtype-preferring drugs were ineffective. Considering the pronounced expression of the α_{2C}-ARs in the basal ganglia, the authors suggest that α_{2C}-AR antagonism might be a useful treatment for locomotor-related and hyperactivity functions in coloboma mice and by implication a potential therapeutic target for ADHD.

These effects need to be corroborated using subtype selective ligands with higher selectivity ratios, and subsequent testing on cognition in models of ADHD.

Considering the potential therapeutic role of targeting the α_{2C}-AR in both depression and schizophrenia, a final comment on the role of this receptor subtype in bipolar disorder is warranted. Bipolar disorder is a mood disorder characterized by mixed symptoms of depression and mania, with both antidepressants [222] and antipsychotics in combination with mood stabilizers as standard first-line treatment [223]. Quetiapine is a second generation (atypical) antipsychotic with a favourable α_{2C}/α_{2A} and a fairly high α_{2C}/D_2 ratio [3; 4] (see Figure 5) that has shown marked clinical efficacy in treating mania and depression in bipolar disorder [223; 224]. In the light of evidence provided in the afore going sections on the possible therapeutic value of targeting the α_{2C}-AR in symptoms akin to those seen in depression and schizophrenia, future studies investigating the therapeutic potential of targeting the α_{2C}-AR in bipolar disorder using α_{2C}-AR selective ligands could provide valuable insights into the mechanism by which quetiapine exerts its beneficial effects in this disorder and whether a new avenue for drug targets in this disorder could be explored.

8. Future perspective: what do we have and what do we need?

Recent developments and the current state of knowledge continue to support the therapeutic potential of selective targeting of the α_{2C}-AR in the treatment of cognitive dysfunction, depression and schizophrenia. Treatment benefits are likely to include broader/enhancement of efficacy as well as reduced side-effects. There is, however, limited clinical data in this respect and further patient trials are needed for stronger validatory evidence. In addition, despite recent progress there are still significant gaps in knowledge base relating to the function, physiology and pharmacology of α_{2C}-ARs. Some areas requiring further research include:

- α_{2C}-AR signal transduction pathways and trafficking: for instance what is the relative role of the cAMP and non-MAPK pathways in brain tissue from normal and disease model animals?
- α_{2C}-AR receptor regulation: do the levels change in human disease tissue or material from animal models? Furthermore do existing treatments, e.g. for schizophrenia and depression, alter α_{2C}-AR density?
- Distribution and cellular localisation of α_{2C}-ARs at noradrenergic and non-adrenergic synapses. Do these receptors play an extra-synaptic role?
- Heteroreceptor function and mode of modulation of non-adrenergic neurotransmitter release, particularly in the hippocampus and frontal cortex.

- Insight towards putative receptors (e.g. $5HT_{1A}$, D_1, AMPA receptors) that may be involved in mediating the *in vivo* central effects of selective α_{2C}-AR receptor antagonism.
- Contribution towards modulation of stress and inflammation-linked pathways.
- Evaluation in animal models with strong disease construct (e.g. genetic, age, stress) and applying more translationally relevant approaches (e.g. chronic treatment, combination with existing drugs).
- Further experimental medicine with new tools (e.g. PET ligand ORM-13070) to learn about the role of the α_{2C}-AR in human brain in healthy subjects and patients.
- Considering the high comorbidity of anxiety in these illnesses, and that it can significantly affect prognosis and treatment response [225; 226], further study into the anxiolytic capabilities of α_{2C}-ARs in appropriate models is required.

An array of tools is now available to facilitate further research. Highly selective α_{2C}-AR subtype ligands, and specifically α_{2C}-AR selective antagonists, have been generated over the past decade. Before that, drugs with marginal selectivity were used to delineate pharmacological effects of the α_2-AR subtypes. For example, although BMY7378 is mainly an α_{1D}-AR antagonist, it also presents with a 10-fold selectivity for α_{2C}-ARs vs. α_{2A}-ARs [227]. Another example of an antagonist drug with marginal α_{2C}-AR selectivity is MK912, which also exhibits an approximate 10-fold greater selectivity for α_{2C}-ARs vs α_{2A}-AR and α_{2B}-ARs [228; 229] and has been used to delineate the role of the α_{2C}-AR on hyperactive behaviour in a mouse model of ADHD [221].

In 2008 N-{2-[4-(2,3-dihydro-benzo[1,4]dioxin-2-ylmethyl)-[1,4]diazepan-1-yl]-ethyl}-2-phenoxy-nicotinamide was synthesized, and found to display >100 fold selectivity for α_{2C}-AR vs. α_{2A}-AR, with excellent binding affinity and functional activity at the α_{2C}-AR in rats. Although low selectivity vs. α_{2B}-ARs was shown, the α_{2B}-ARs have negligible distribution in the CNS. This compound displayed excellent binding affinity and functional activity for α_{2C}-ARs in rats, with adequate CNS penetration [230]. Further animal studies with this promising compound are eagerly awaited.

In 2007 Orion Pharma reported that their novel selective α_{2C}-AR antagonist, JP-1302, presented with a minimum 50-fold selectivity for the α_{2C}-AR with an $\alpha_{2C/2A}$ ratio of 93 [13]. However, this compound does not optimally enter the CNS. In 2013 another Orion Pharma compound, ORM-10921, was characterized with an $\alpha_{2C/2A}$ ratio of about 100 in rodents, although this ratio was found to be lower in human cells (~29) [14]. Both JP-1302 and ORM-10921 have since been used safely in preclinical studies in rodent models of neuropsychiatric illness. On the other hand, the novel α_{2C}-AR antagonist, ORM-12741, has been tested for safety and efficacy in both rodents and humans [15; 22] and presents with a 4000-fold selectivity for the α_{2C}-AR vs. α_{2A}-AR and α_{2B}-AR. This highly selective α_{2C}-AR antagonist is currently in clinical trials for the treatment of symptoms associated with Alzheimer's disease.

A very important recent development has been the development of ORM-13070, a selective α2C-AR which is amenable to labelling with 11C and has been successfully used as α2C-AR PET tracer which readily enters the CNS [231]. This compound has a binding affinity selectivity of over 200-fold vs. the α_{2A}-AR, with weak or no activity at more than 100 other potential target sites and receptors, and will be highly valuable for facilitating forward and reverse translational between animal and human system research. An obvious application is determination of target engagement, through conducting receptor occupancy studies, for novel drug candidate molecules for preclinical and clinical studies [39; 231; 232]. However, it could also be used to gain more precise insight on the relative α2C-AR occupancy for antipsychotic (e.g. clozapine) and antidepressant (e.g. mirtazapine) agents at clinical doses thus enabling a better understanding on the mode of action of these drugs. The tracer could also be potentially used to investigate disease-related changes in receptor density and effects on neurotransmitter activity. The latter aspect has been investigated and in line with evidence that the α2C-AR is sensitive to low synaptic concentrations of noradrenaline, $^{[11C]}$ORM-13070 shows increased CNS binding in response to decreased synaptic noradrenaline [167].

On the other side of the spectrum, novel α_{2C}-AR agonists have also been characterized recently. [N-[3,4-dihydro-4-(1H-imidazol-4-ylmethyl)-2H-1,4-benzoxazin-6-yl]-N-ethyl-N'-methylurea] or "Compound A" and a chemically similar "Compound B" were found to be highly selective for the α_{2C}-AR, albeit with poor brain penetration. These compounds are being investigated for effects on peripheral vasoconstriction [233; 234].

The recent availability of highly subtype selective α_{2C}-AR-ligands that effectively penetrate the CNS should stimulate further investigation into the potential of targeting this receptor in neuropsychiatric disorders. Genetic and molecular biology driven approaches will also be critical in this regard. Mice over-expressing or lacking the α_{2C}-AR have been generated but have been phenotyped to a limited extent. Further behavioural but in particular biological characterisation, for example using –omics type molecular profiling, would deliver more insight. Moreover, more regionally restricted genetic manipulation using genetic deletion technology in rats would yield valuable data. The zebrafish is another platform of discovery that may provide a powerful model in which to study developmental and genetic factors that underlie human disease [235]. Work in zebrafish has shown that the zebrafish α_2-AR subtypes are markedly conserved compared to mammalian α_2-AR subtypes with similar pharmacological profiles and functional effects compared to human α_2-AR subtypes [41; 42]. This model might also be beneficial in future studies when characterizing novel subtype selective α_2-AR ligands.

9. Conclusion

This review has provided an overview of recent developments and future direction in research investigating the role of the α_{2C}-AR in neuropsychiatric illness and therapy, with specific focus on the effects of α_{2C}-AR antagonism in cognition, depression and schizophrenia. Targeting this receptor could present beneficial therapeutic effects when used alone or as augmentation strategy in the treatment of depressive disorders, schizo-affective disorders, and disorders presenting with cognitive decline, such as Alzheimer's disease. The recent advent of clinical grade subtype selective α_{2C}-AR antagonists will assist in further delineating the role of this receptor in neuropsychiatric therapy and studies employing these novel highly selective α_{2C}-AR ligands in putative translationally relevant animal models of psychiatric illness to inform further experimental medicine evaluation in humans will be vital in strengthening our understanding of the α_{2C}-AR as a therapeutic target.

10. Abbreviations

ADHD:	attention-deficit hyperactivity disorder
AR:	adrenoceptor
BDNF:	brain-derived neurotrophic factor
cAMP:	cyclic adenosine monophosphate
CNS:	central nervous system
DOPA:	3,4-dihydroxyphenylalanine
DA:	dopamine
FST:	forced swim test
FSL:	Flinders Sensitive Line
FRL:	Flinders Resistant Line
GABA:	gamma-aminobutyric acid
HPA-axis:	hypothalamic-pituitary-adrenal axis
HVA:	homovanilic acid
KO:	knockout
MAPK:	mitogen-activated protein kinase
MK-801:	dizolcipine
MWM:	Morris Water Maze
NA:	noradrenaline
NMDA:	N-methyl-D-aspartate
NORT:	novel object recognition test
OE:	overexpressing
PCP:	phenylcyclidine
PPI:	prepulse inhibition
SIR:	social isolation reared / social isolation rearing
SSRI:	selective serotonin reuptake inhibitor
SNRI:	serotonin and noradrenaline reuptake inhibitor
TCA:	tricyclic antidepressant
5-HIAA:	5-hydroxyindoleacetic acid

5-HT: serotonin
5-HTP: 5-hydroxytryptophan

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12. References

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Chapter 7: Conclusions and Recommendations

This chapter serves to summarise the results from the research project as a whole. The key findings will be discussed, as well as how these findings have advanced our knowledge in this field. Furthermore, I will provide recommendations on directions that future studies can take in order to build on the findings from this project.

1. Introduction

Depression and schizophrenia are neuropsychiatric disorders sharing various symptom similarities that have significant detrimental effects on daily functioning, including affective flattening, social withdrawal and deficits in memory and cognition (Kahn and Keefe, 2013; Krishnan and Nestler, 2008; Tsapakis et al., 2015). While genetic, neurodevelopmental and environmental factors affect the development or progress of both disorders (Kendler et al., 2001; Sigurdsson, 2015), prominent monoaminergic dysfunction is currently the main target of conventional antidepressant and antipsychotic treatment (El-Hage et al., 2013; Millan et al., 2015). The treatment arsenals of both disorders include drugs that act as α_2-AR-antagonists (Kharade et al., 2010; Svensson, 2003), while augmentation of various antidepressants and antipsychotics with an α_2-AR-antagonist has demonstrated enhanced treatment response (Blier et al., 2010; Marcus et al., 2005; Marcus et al., 2010b; Sanacora et al., 2004). While various treatment regimens are available, treatment outcome in both disorders remains inadequate for many patients (Millan et al., 2015; Rush et al., 2006; Thase et al., 2001), with cognitive deficits remaining especially refractory to treatment (Bowie and Harvey, 2006; McIntyre et al., 2013). This disparity drives the need for improved pharmacological understanding of both disorders in order to develop superior treatment options.

Early studies in transgenic mice described distinct and in some instances opposing roles for the α_{2A}-AR and α_{2C}-AR, these being the main α_2-AR subtypes regulating neurotransmission in the CNS (Bücheler et al., 2002; Hein et al., 1999). While α_{2C}-AR antagonism has been associated with antidepressant-like, antipsychotic-like and pro-cognitive effects, α_{2A}-AR-antagonism is associated with the opposite effects (Björklund et al., 2001; Sallinen et al., 1999; Sallinen et al., 2007; Sallinen et al., 2013a; Sallinen et al., 2013b; Scheinin et al., 2001), suggesting that non-selective α_2-AR modulation might negate potential beneficial effects that could be attained by subtype selective targeting (Scheinin et al., 2001). Recently, and for the first time highly selective α_{2C}-AR subtype antagonists have become available as tools to investigate the role of targeting this receptor in neuropsychiatric illness. Thus far, the effects of α_{2C}-AR

antagonists have been reported in only one animal model of schizophrenia with translational value for the human disease, namely the glutamate NMDA receptor antagonist model (Sallinen et al., 2007; Sallinen et al., 2013a), while no such studies have been performed in a translational animal model of depression. Other findings have to date been gleaned from animals with very little translational value for the human disorder, while acute treatment paradigms were employed in the majority of studies, in contrast to the necessity of chronic antidepressant or antipsychotic therapy in the clinic (Kharade et al., 2010; Millan et al., 2015). Therefore this study set out to determine the bio-behavioural effects of chronic treatment with a selective α_{2C}-AR antagonist, ORM-10921 in translational models of depression and schizophrenia. ORM-10921 presents with ~100 fold selectivity for α_{2C}-ARs vs. α_{2A}-ARs, has good CNS penetration and tolerability in rodents, and presents with a back-drop of earlier studies supporting its possible antipsychotic and/or antidepressant-like effect in rodents (Sallinen et al., 2013a), thereby making it suitable for assessment in the translational animal models applied here.

The social isolation rearing (SIR) model of schizophrenia and the Flinders Sensitive Line (FSL) model of depression are putative models of neuropsychiatric disease that present with good face, predictive and construct validity for their respective disorders. Post-weaning SIR of rodents for 8-weeks is a putative neurodevelopmental animal model of schizophrenia with exceptional face, construct and predictive validity (Jones et al., 2011; King et al., 2008). This model therefore presents with monoaminergic and neurochemical alterations that strongly correlate with the disturbances seen in schizophrenia, while deficient sensorimotor gating, impaired cognition (including impaired visual object recognition memory) and altered neurotrophic function evident in schizophrenia has repeatedly been demonstrated in SIR rats (Fone and Porkess, 2008; Jones et al., 2011; Möller et al., 2013a). Furthermore, these deficits can reliably be reversed by antipsychotic agents, making the model suitable for assessing the antipsychotic potential of a novel compounds, particularly when using sensorimotor gating and cognitive deficits as behavioural endpoints (Jones et al., 2011; Möller et al., 2013a). This study therefore investigated the dose-response effects of 14 day treatment with ORM-10921 on sensorimotor gating in the prepulse inhibition (PPI) test (Martinez et al., 2002; Weiss and Feldon, 2001)) and visual object recognition memory in the novel object recognition test (NORT), relating these effects to the reference antipsychotic, clozapine (CLOZ), and to the non-selective α_2-AR antagonist, idazoxan (IDAZ) (**Addendum A**). This choice of comparative agents was to establish parity of antipsychotic efficacy and to investigate therapeutic difference between α_{2C}-AR selective vs. non-selective α_2-AR antagonism. This dose ranging study enabled us to select an effective dose of ORM-10921 at a behavioural level to assess its effects on striatal BDNF and monoamine levels (**Addendum B**). It also prompted a sub-study that investigated an additional lower dose of ORM-10921 in comparison to the D_2-antagonist antipsychotic, haloperidol (HAL), the atypical antipsychotic, CLOZ as well as a combination of ORM-10921 + HAL to determine if this pharmacological combination would demonstrate greater therapeutic efficacy due to the α_{2C}/D_2

ratio, as postulated in literature (Kalkman and Loetscher, 2003; Svensson, 2003) (**Manuscript A**). These findings also enabled us to investigate the hypothesis that the beneficial α_{2C}/D_2 ratio of CLOZ might in part underlie its superior antipsychotic efficacy (Swartz et al., 2008) (**Manuscript A** and **Addendum A**).

The FSL rat is a genetic animal model of depression that displays depressive-like impairment in escape strategies and motivation, as mirrored in decreased immobility in the forced swim test (FST), a screening test for antidepressant activity (Overstreet et al., 2005; Petit-Demouliere et al., 2005). Furthermore, cognitive deficits and reduced hippocampal BDNF levels in these animals, as well as altered monoaminergic function akin to that in depression, has also been demonstrated (Overstreet et al., 2005; Overstreet and Wegener, 2013). These deficits are reversed by chronic, but not acute antidepressant treatment, making the model a good predictive model for assessing antidepressant activity of novel and known compounds (Overstreet et al., 2005). This study therefore investigated the dose-response effects of 14-day treatment with ORM-10921 on depressive-like behaviour of the FSL rat in the FST and on declarative memory in the NORT, relating these effects to the reference antidepressant, imipramine (IMI) and to IDAZ, in order to establish parity of antidepressant efficacy and to investigate therapeutic difference between α_{2C}-AR selective vs. non-selective α_2-AR antagonism (**Manuscript B** and **Addendum D**). Furthermore, this dose ranging study enabled us to select doses effectively altering depressive-like behaviour to assess effects of ORM-10921 on hippocampal monoamines and BDNF levels (**Manuscript C** and **Addendum E**).

Therefore, this study investigated antidepressant-like, antipsychotic-like and pro-cognitive effects of α_{2C}-AR antagonism with ORM-10921 in appropriate translational animal models, while at the same time assessing whether behavioural and cognitive improvements could be related to either hippocampal or striatal alterations in monoamines and neurotrophic support. Below I provide the summary of these results.

2. Summary of results

The results of this research project has been presented in separate sections either as manuscripts that have been published, are in submission, or are in preparation for submission to an international peer-reviewed journal (Chapter 3, 4, 5 and 6), or presented in the form of various addenda to this thesis (Addenda A to E). Therefore, a cohesive but succinct summary of all the results produced will be provided here, especially their relation with each other and the original study objectives. The results will be discussed according to the aims as outlined in Chapter 1.

Chapter 7: Conclusions and Recommendations

A. FINDINGS IN THE SIR NEURODEVELOPMENTAL ANIMAL MODEL OF SCHIZOPHRENIA

1. Establish validity of SIR as a translational model for schizophrenia

Face validity of SIR: Rodents reared in social isolation (SIR) for eight weeks post-weaning displayed prominent deficits in sensorimotor gating (as assessed by the %PPI of the startle response) as well as deficits in visual recognition memory (as assessed in the NORT) compared to their socially reared controls (**Manuscript A** and **Addendum A**).

Construct validity of SIR: SIR induced significant reduction of striatal BDNF levels vs. socially reared rats (**Manuscript A**). However, I was not successful in demonstrating altered striatal monoamine levels in SIR rats vs. socially reared controls, this being a well-established response in these animals in other laboratories (Fone and Porkess, 2008; Jones et al., 2011) as well as our own (Möller et al., 2013a; Möller et al., 2013b; Strauss et al., 2014) (**Addendum B**). However, drug treatment in the SIR group was able to alter striatal monoamines according to the theoretical paradigms (see below).

Predictive validity of SIR: CLOZ effectively attenuated SIR-induced deficits in PPI and in the NORT, demonstrating improved sensorimotor gating (PPI) and enhanced visual object recognition memory (NORT) (**Manuscript A** and **Addendum A**). CLOZ increased striatal BDNF and mediated a decrease in striatal 5-HT, although it did not alter striatal DA or NA in SIR rats (**Addendum B**). The expectation was that CLOZ would decrease striatal DA and 5-HT, however we were unsuccessful in demonstrating decreased DA levels with CLOZ, regardless of the prominent behavioural effects produced by CLOZ in the behavioural studies.

2. Establish whether ORM-10921 could reverse behavioural deficits in SIR rats in comparison to CLOZ and IDAZ

ORM-10921 (ORM) reversed deficits in %PPI and in the NORT in SIR animals (Manuscript A and Addendum A), while the lowest dose of 0.03 mg/kg was identified as the most appropriate dose to pursue neurochemical assessment (Addendum B). The efficacy of ORM in the PPI showed dependence on lower doses (≤ 0.03mg/kg) while a dose range of 0.03mg/kg - 0.3mg/kg was effective in attenuating NORT deficits in SIR, but not the highest dose (1 mg/kg) (**Addendum A**). Non-selective α_2-AR antagonism with IDAZ did not attenuate SIR induced behavioural deficits (**Addendum A**), while CLOZ reversed SIR induced deficits in the PPI superior to ORM 0.03 mg/kg, with cognitive enhancement by ORM 0.03 mg/kg in the NORT appearing to be comparable to that of CLOZ (**Addendum A**).

3. Establish whether ORM-10921 induces behavioural effects via effects on striatal BDNF in comparison to CLOZ and IDAZ

ORM 0.03mg/kg did not induce any alterations of striatal BDNF levels vs. SIR controls, and neither did IDAZ. CLOZ however increased striatal BDNF levels vs. controls (**Addendum B**).

4. Establish how ORM-10921 affects striatal monoamine level in comparison to CLOZ and IDAZ

ORM at 0.03mg/kg significantly increased striatal NA levels and decreased striatal DA levels but did not affect striatal 5-HT levels in SIR rats. ORM-10921 significantly decreased striatal DA levels vs. IDAZ treatment. ORM-10921 did not affect the turnover rates of NA, 5-HT or DA (**Addendum B**). CLOZ did not alter striatal NA levels, although it resulted in lower NA levels vs. ORM, and lower NA turnover vs. IDAZ. CLOZ curiously also did not decrease striatal DA levels vs. SIR controls, although increased DA turnover was noted vs. IDAZ. However, CLOZ significantly decreased striatal 5-HT levels vs. all other treatments, while increasing 5-HT turnover vs. IDAZ (**Addendum B**). IDAZ increased NA turnover, but not NA levels and decreased DA turnover vs. both controls and CLOZ. IDAZ didn't alter 5-HT levels or 5-HT turnover vs. controls (**Addendum B**).

Following findings that behavioural parameters were more responsive to lower doses of ORM-10921, we studied the effects of a lower dose of ORM-10921 on behavioural effects and striatal BDNF levels (**Manuscript A** and **Addendum C**). Additionally, we investigated how augmentation of HAL with ORM-10921 would affect the behavioural and neurotrophic response to HAL (**Manuscript A** and **Addendum C**), as follows:

5. Establish whether an additional low dose of ORM-10921 would be more effective in exerting antipsychotic-like effects in SIR animals

The lower dose of ORM (0.01mg/kg) presented with visibly higher improvement of %PPI than the 0.03 mg/kg dose and even numerically higher than CLOZ, although the effects of ORM 0.01mg/kg in the NORT remained similar to the 0.03 mg/kg dose of ORM (**Addendum C**).

6. Establish whether the behavioural and neurotrophic effects of a typical antidopaminergic antipsychotic devoid of alpha-lytic activity could be enhanced by augmenting treatment with selective α_{2C}-AR antagonism

While CLOZ effectively attenuated SIR-associated PPI and NORT deficits, HAL proved to be ineffective in this regard (**Manuscript A** and **Addendum C**). However, the addition of ORM 0.01 mg/kg or ORM 0.03 mg/kg therapy to the same dose of HAL significantly bolstered the effects of HAL on PPI and NORT compared to HAL monotherapy, although these improvements did not exceed the effects of ORM

monotherapy (**Manuscript A** and **Addendum C**). Furthermore, striatal BDNF was significantly increased by ORM + HAL combined treatment at the 0.01 mg/kg doses of ORM (**Manuscript A**)

B. FINDINGS IN THE FSL GENETIC ANIMAL MODEL OF DEPRESSION

1. Establish validity of the FSL model as a translational model for depression

Face validity of SIR: FSL rats displayed significantly increased immobility in the FST, while also demonstrating deficits in recognition memory in the NORT compared to FRL controls (**Manuscript B**).

Construct validity of SIR: FSL rats displayed decreased hippocampal BDNF levels vs. FRL controls (**Manuscript E**). Additionally, FSL rats displayed lower DA and 5-HT levels compared to FRL controls (**Manuscript C**). The lower DA levels could be related to increased turnover of DA in FSL rats, as homovanllic acid (HVA) levels were significantly increased compared to FRL controls. FSL rats did not display differences in levels of NA vs. controls, nor did FSL rats differ from FRL rats regarding 5-HT or NA turnover.

Predictive validity of SIR: IMI effectively reversed the behavioural deficits in FSL animals, decreasing immobility time and increasing noradrenergic-driven climbing behaviour in the FST, while also improving object recognition memory in the NORT vs. FRL controls (**Manuscript B**). IDAZ did not affect deficits of FSL rats in the FST or NORT (**Manuscript B**). IMI increased hippocampal 5-HT levels and decreased 5-HT turnover in FSL rats, but did not affect either NA or DA levels or metabolism (**Manuscript C**). IDAZ increased hippocampal levels of DA and NA, but not 5-HT levels (**Manuscript C**). IDAZ also decreased NA and 5-HT turnover, but increased DA turnover. Finally, we were unable to demonstrate increased hippocampal BDNF levels following IMI treatment in FSL animals vs. FRL animals, despite the above-mentioned behavioural efficacy of IMI. IDAZ did not increase hippocampal BDNF in FSL animals (**Addendum E**).

2. Establish whether ORM-10921 could reverse behavioural deficits in FSL rats in comparison to IMI and IDAZ

ORM only at 0.03 and 0.3 mg/kg significantly decreased immobility time in the FST in FSL rats, while at the same time increasing serotonergic-driven swimming behaviour (**Manuscript B** and **Addendum D**). ORM 0.03mg/kg also increased noradrenergic-driven climbing behaviour in the FST. Most doses of ORM improved memory in the NORT, viz. 0.03, 0.1, 0.3 and 1 mg/kg, with only the lowest dose of 0.01 mg/kg not effective (**Addendum D**). These effects were not related to altered locomotor activity. Moreover, effects on immobility were comparable to the effects of IMI. IDAZ did not alter FST immobility and presented with significantly higher immobility vs. IMI and ORM 0.03 and 0.3mg/kg (**Manuscript B** and **Addendum D**). IMI detrimentally affected locomotor activity, which decreased exploration in the NORT.

However, when correcting for this factor, IMI increased object recognition memory (**Addendum D**). IDAZ did not affect memory in FSL rats in the NORT (**Manuscript B** and **Addendum D**).

3. Establish whether ORM-10921 induces behavioural effects via effects on hippocampal BDNF in comparison to IMI and IDAZ

Neither IMI, IDAZ or treatment with ORM 0.03 mg/kg altered hippocampal BDNF levels vs. FSL controls. (**Addendum E**).

4. Establish how ORM-10921 affects hippocampal monoamine levels in comparison to IMI and IDAZ

ORM increased hippocampal NA levels and decreased NA turnover, as did IDAZ, although IMI failed in this regard. ORM 0.1 and 0.3 mg/kg increased hippocampal 5-HT levels, as did IMI, but not IDAZ, while all drugs decreased 5-HT turnover. ORM 0.03 – 0.3 mg/kg increased hippocampal DA, as did IDAZ, but not IMI. ORM 0.1 and 0.3 mg/kg, as well as IDAZ and IMI decreased the DA metabolite, HVA in the hippocampi of FSL rodents, while the 0.03 mg/kg dose was the only drug to increase DOPAC (**Manuscript C**).

3. Novel findings and conclusion

Although previous studies have reported the effect of the α_{2C}-AR antagonists, JP-1302, ORM-10921 and ORM-12741 in the FST and PPI test (Sallinen et al., 2007; Sallinen et al., 2013a; Sallinen et al., 2013b), none of these studies employed a chronic treatment paradigm. Furthermore, while NMDA-antagonist models were employed to assess antipsychotic-like effects on PPI and certain cognitive parameters, Sprague-Dawley and Wistar rats were employed to determine antidepressant-like effects of these α_{2C}-AR antagonists. These rodents are not considered to be translational animal models of depression, while the NMDA-antagonist model of schizophrenia has various limitations as a drug-induced model (Bubenikova-Valesova et al., 2008; Jones et al., 2011; Overstreet, 2012). Thus, this is the first study reporting the effects of *chronic* administration of α_{2C}-AR antagonism on behavioural and neurochemical markers in a robust translational animal model of depression, and also the first study to report such findings in a *neurodevelopmental* animal model of schizophrenia. Thus, the majority of findings from this research project has broadened the current pool of knowledge regarding the therapeutic potential of targeting the α_{2C}-AR in neuropsychiatric illness, and have set the stage for further studies on α_{2C}-AR antagonists in these and other animal models. Firstly, as opposed to the above studies, this study:

a) provided the first evidence of pro-cognitive, antidepressant-like and antipsychotic-like effects of *chronic* treatment with an α_{2C}-AR antagonist
b) provided the first report of the effect of α_{2C}-AR antagonism on declarative memory in the novel object recognition test

c) provided the first comparison of effects exerted with an α_{2C}-AR antagonist vs. that of a non-selective α_2-AR antagonist
d) provided the first report of the effect of chronic α_{2C}-AR antagonism on striatal and hippocampal BDNF levels as applied in translation animal models
e) provided the first report of the effect of chronic α_{2C}-AR antagonism on total striatal and hippocampal monoamine levels as applied in translational animal models
f) provided the first evidence that chronic augmentation of ORM-10921 bolsters the antipsychotic-like, pro-cognitive and neurotrophic effects of HAL in a translational animal model of schizophrenia.

The impact of this research therefore lies in establishing the dose ranging effects of ORM-10921 in two translational platforms to further our understanding of the underlying mechanisms involving α_{2C}-AR antagonism in antidepressant and antipsychotic action.

The advent of atypical antipsychotics have introduced new horizons of antipsychotic efficacy across the symptom triad (Svensson, 2003; Swartz et al., 2008), although remission is only partially obtained in approximately 40% of patients, while the number of patients not reaching full functional recovery, is even larger (Englisch and Zink, 2012). Furthermore, cognitive improvements are the most difficult to attain (Kahn and Keefe, 2013; Millan et al., 2015). In this study we not only provided evidence that α_{2C}-AR antagonism induces antipsychotic-like and pro-cognitive effects, but also that α_{2C}-AR antagonism is an effective cognitive-enhancer for D_2-antagonist therapy. While this has implications for enhancing antipsychotic treatment strategies, it provides the first evidence in a translational animal model of schizophrenia that antipsychotic efficacy in domains of both positive and cognitive symptoms is dependent on the ratio of α_{2C}/D_2 blockade (Kalkman and Loetscher, 2003; Shahid et al., 2009). Considering that clozapine has the highest α_{2C}/D_2 ratio among antipsychotics (Kalkman and Loetscher, 2003) and has been associated with efficacy in refractory schizophrenia (Swartz et al., 2008) and in cognitive deficits (Englisch and Zink, 2012), our data reinforces evidence that the α_{2C}-AR affinity of clozapine might underscore its clinical efficacy in all symptom domains. While α_{2C}-AR antagonism as monotherapy did not alter striatal BDNF levels in this study, augmentation of D_2 antagonist therapy increased striatal BDNF levels vs. monotherapy with either drug. This could be of importance in addressing the striatal neurodegenerative effects that have been observed with the use of the typical antipsychotics (Buckley et al., 2007a; Pillai et al., 2006). The pro-cognitive effects of α_{2C}-AR antagonism was evident at a much broader dose range compared to its effects on sensorimotor gating, which was more pronounced at lower doses. This observation might underlie the sensitivity of selectively targeting α_{2C}-AR in achieving antipsychotic efficacy, since higher doses might present with increasing α_{2A}-AR engagement. This however needs to be verified with receptor occupancy studies. On the other hand, ORM-10921 increased striatal tissue levels of NA, and decreased striatal tissue levels of DA, while no

immediate effect on striatal 5-HT levels was observed. Since schizophrenia is associated with a relative hyponoradrenergic state and excessive striatal DA acitivity (Reynolds, 2008; Yamamoto and Hornykiewicz, 2004), these findings might point to a possible mechanism underlying the antipsychotic and pro-cognitive effects of ORM-10921 in the SIR model. However, a limitation of these findings is that extracellular (synaptic) availability of these monoamines cannot reliably be inferred from altered tissue levels (Finlay and Smith, 2000), although it does provide rudimentary information regarding the effects of ORM-10921 on regional monoamine levels. An important foundation has however been laid for future studies. Such studies should include methods such as microdialysis or *in vivo* patch clamping, which has the ability to measure dynamic release of neurotransmitters in the freely moving animal (Connelly, 1999; Lee et al., 2014). Optogenetics is also a very useful tool to assess the effects of drug treatment on neuronal activity and transmitter release (Carter et al., 2010; Xiao et al., 2014).

Amidst a veritable arsenal of drugs available for the treatment of major depressive disorder, some 40% of patients do not respond adequately, while 10-30% of patients become resistant to treatment (Al-Harbi, 2012). Additionally, cognitive impairment in depression is very refractory to treatment (Conradi et al., 2011; McIntyre et al., 2013). The finding that α_{2C}-AR antagonism with ORM-10921 has antidepressant-like effects with prominent pro-cognitive effects in FSL rats, while also increasing hippocampal monoamine levels, is therefore a very important step forward in the process of developing new therapeutic strategies that also present with beneficial cognitive outcomes. The pro-cognitive effects of ORM-10921 seem to be evident over a broader dose range than its antidepressant-like effects. Additionally, while augmentation with non-selective α_2-AR-antagonists has been reported to bolster antidepressant treatment outcomes (Blier et al., 2010; Sanacora et al., 2004), our findings suggest that α_{2C}-AR antagonism might translate into a more effective tool for augmentation in antidepressant therapy. That these effects could not be related to beneficial effects on hippocampal BDNF is an important point for further study, since BDNF is intricately involved in the pathophysiology of depression (Brunoni et al., 2008) as well as in the processes underlying learning, memory and mood (Neto et al., 2011), while antidepressant treatment is associated with increases in hippocampal neurogenesis and BDNF levels (Dunham et al., 2009; Neto et al., 2011). However, further studies incorporating extended treatment timelines, measurement of BDNF gene expression as well as the expression of other neurotrophic factors (such as neural growth factor (NGF) and vascular endothelial growth factor (VEGF)) and transcription factors related to neurotrophic processes suc as cyclic AMP response element binding protein (CREB) (Vinet et al., 2004) should be targeted in future studies. On the other hand, this study reported increased hippocampal tissue levels of NA, 5-HT and DA, monoamines that typically are depleted in depression (Kharade et al., 2010). As described above, a limitation of these findings includes the inability of this method to demonstrate synaptic availability or synthesis rates of the monoamines. A critical foundation has however been laid down for future studies

that employ methods that are better designed to assess neurotransmitter release and dynamics, such as microdialysis, patch clamping and optogenetics.

While lower doses were necessary for antipsychotic-like effects in the PPI, higher doses were necessary to demonstrate antidepressant-like effects of ORM-10921 in the FST. However, similar doses of ORM-10921 exerted cognitive enhancing effects in both models, thus reaffirming that the cognitive effects of ORM-10921 are more robust, regardless of pathological condition. In support of this notion, another highly selective α_{2C}-AR antagonist, ORM-12741, is currently undergoing clinical trials for treating cognitive impairment in Alzheimer's Disease as an augmentation strategy, with beneficial effects reported on cognitive parameters after 12 weeks of treatment (Rinne et al., 2013; Rouru et al., 2013). Therefore, apart from the promising antidepressant-like and antipsychotic-like effects of α_{2C}-AR antagonism, this study has highlighted the important therapeutic value of α_{2C}-AR antagonism in treating the cognitive effects in depression and schizophrenia.

While no single animal model can possibly replicate the symptoms, neurochemistry or neurobiology of a neuropsychiatric illness, there is a metaphorical "strength in numbers": when investigating the pharmacological effect of a novel compound, it is necessary to provide evidence of its pharmacological effects on behaviour and physiology in several translational animal models of the illness under scrutiny, in order to verify and strengthen the findings in one model with evidence from a model with a different etiology. In this study, pro-cognitive, antidepressant-like and antipsychotic-like effectivity of chronic α_{2C}-AR antagonist treatment in the FSL model of depression and the SIR model of schizophrenia have been added to the current pool of knowledge. These findings cannot be viewed in isolation or interpreted as *proving* that α_{2C}-AR antagonism has therapeutic benefits in neuropsychiatric disorders. However, these findings make a valuable contribution to the contingent scientific evaluation of the therapeutic role of the α_{2C}-AR in translational animal models of depression and schizophrenia, and hopefully will stimulate further work land-mark studies in pre-clinical and clinical research.

4. Recommendations for future studies

The literature on the effects of selective α_{2C}-AR in neuropsychiatric models is extremely limited, and the bulk of information is provided by results from transgenic studies. There are thus numerous studies spanning a wide range of applications that can still be performed to delineate the role of α_{2C}-AR antagonism in neuropsychiatric disorders. In order to retain focus, I will therefore limit my recommendations to studies that will build on the findings described in this thesis.

1. Building on evidence of improved therapeutic outcomes following non-selective α_2-AR-antagonism of antidepressant therapy (Blier et al., 2009; Blier et al., 2010; Sanacora et al., 2004) and taking into account the benefits of selectively targeting the α_{2C}-AR as discussed in this chapter, employing ORM-

10921 as augmentation strategy to antidepressant therapy should be investigated, especially as add-on therapy to SSRI's, venlafaxine and bupropion, considering the evidence in the literature for the benefits of augmenting these agents with a non-selective α_2-AR antagonist.

2. The FST as a tool to assess antidepressant activity is very reliable across laboratories, providing very good predictive validity in FSL rodents (Borsini and Meli, 1988; Holmes, 2003; Overstreet and Wegener, 2013) and moderate to good face validity (Petit-Demouliere et al., 2005), although its construct validity is perhaps questionable. Therefore, applying ORM-10921 in additional tests that are associated with other symptoms of depression would significantly enhance the findings described here. Such tests should include the sucrose preference test, which is a putative test of anhedonia in rodents (Overstreet, 2012) and the novelty-suppressed feeding test, which assess anhedonia in the context of increased anxiety like behaviour (Dulawa and Hen, 2005).
3. Although the PPI test presents with very good face, construct and predictive validity for antipsychotic-like activity (Geyer et al., 2001; Geyer and Swerdlow, 2001), additional behavioural tests predicting antipsychotic efficacy within different behavioural constructs of the disorder should be employed to enhance the antipsychotic-like profile of α_{2C}-AR antagonism with ORM-10921. Such tests may include the conditioned avoidance response test (CAR), tests of stereotypy, hyperlocomotion as well as the social interaction test (Gobira et al., 2013).
4. Considering the over-activity of the HPA-axis, hypercortisolaemia and hippocampal atrophy associated with the pathophysiology of depression (Campbell and MacQueen, 2004; Sapolsky, 2000) as well as evidence of attenuated plasma corticosterone responses in α_{2C}-KO mice (Sallinen et al., 1999), measuring basal plasma corticosterone levels as well as plasma corticosterone responses to various stressors following treatment with ORM-10921 would elucidate whether ORM-10921 elicits its antidepressant-like effects via the HPA-axis. While the FSL rat is not a suitable model for assessing altered HPA-axis function (Overstreet et al., 2005), the olfactory bulbectomized rat is a suitable candidate model in which to assess such a research question (Willner and Mitchell, 2002)
5. While the benefits of augmenting D_2-antagonist therapy with ORM-10921 have been described in this study, these effects have not been compared head-to-head with non-selective α_2-AR-antagonism. Since antipsychotic augmentation with α_2-AR-antagonists have produced enhanced antipsychotic-like effects vs. monotherapy with either drug (Marcus et al., 2005; Marcus et al., 2010b), it should be investigated whether such effects could be further enhanced with α_{2C}-selective agents, or whether the extent of improvement with selective vs. non-selective α_2-AR antagonism is similar.
6. Studies employing techniques such as microdialysis, patch-clamping and optogenetics could be useful to investigate the effects of α_{2C}-AR antagonism on extracellular synaptic availability of NA, 5-HT and DA in translational animal models and regional brain slices. This would aid in delineating the

mechanism of antidepressant and antipsychotic action observed with chronic treatment with ORM-10921 in this study, as well as reveal regional brain differences in the release and dynamics of monoamine transmitters. Additionally, such investigations should also be conducted on the effects of α_{2C}-AR antagonist augmentation of antidepressant and antipsychotic therapy. These investigations would add tremendously to our understanding of how add-on therapy with ORM-10921 would augment antidepressant and antipsychotic response.

7. More protracted treatment timelines with ORM-10921 should be employed in translational animal models to determine long-term effects on neuronal growth factors such as VEGF, NGF, BDNF as well as transcription factors that are involved in the eventual production of BDNF, such as CREB, mitogen-activated protein kinase (MAPK) and protein kinase A (PKA) among others (Xue et al., 2016). These growth factors should be assessed in plasma, as growth factors including BDNF are invariably assessed in blood plasma of depressed and schizophrenic patients (Autry and Monteggia, 2012a; Buckley et al., 2007b; Grillo et al., 2007). Animal studies looking specifically atbrain areas that present with the most pronounced dysfunction in depression and schizophrenia, such as the hippocampus, striatum and frontal cortex, and including sub-regions of the hippocampus and striatum, are also needed in order to gauge how cognitive benefits of α_{2C}-AR antagonism are exerted on a regional molecular level.
8. Receptor occupancy studies will be essential in determining how behavioural changes at the various doses translate into receptor occupancy of the α_{2C}-AR and α_{2A}-AR and to determine wether receptor occupancy is optimal in treating the various disorders. It would also be valuable in explaining why higher doses of ORM-10921 seem to lose antipsychotic and antidepressant efficacy in the animal models applied in this study. One explanation could be related to higher doses of ORM-10921 engaging the α_{2A}-ARs, which is said to have opposing effects to that of the α_{2C}-AR, thus leading to different and even abrogated bio-behavioral responses. ORM-13070 has recently been developed as an α_{2C}-AR selective antagonist amenable to labelling with ^{11}C, and which has successfully been used as an α_{2C}-AR positron emission tomography (PET) tracer in humans and animals (Finnema et al., 2015; Lehto et al., 2015a; Lehto et al., 2016; Lehto et al., 2015b). This compound will be helpful in determining α_{2C}-AR occupancy, while ^{11}C MPTQ has been described as a potential PET tracer for α_{2A}-ARs (Prabhakaran et al., 2010).
9. The pro-cognitive effects of ORM-10921 are stable across a wide dose range in both models applied in this study. The pro-cognitive potential of ORM-10921 might therefore be a reliable and robust effect of this drug, and while the NORT is helpful in assessing declarative and visual recognition memory dependent on hippocampal and entorhinal cortical function (Antunes and Biala, 2012; Broadbent et al., 2010; Cohen and Stackman, 2015), it is only capable of reflecting effects on declarative memory or visual recognition memory associated with recall. Additional tests of

cognitive performance reliant on other learning processes and various additional brain regions should therefore be investigated, such as assessing spatial working memory (e.g. the Morris water maze), reference memory (e.g. the radial arm maze, Y-maze, T-maze) and stimulus-response learning (e.g. the passive avoidance test) among others.

New developments have revealed exciting avenues of investigation into studying the role of the α_{2C}-AR in neuropsychiatric illness. The recent availability of centrally-active highly selective α_{2C}-AR antagonists have opened up the arena to assess a variety of functions that might be amenable to the effects of α_{2C}-AR antagonism in treating neuropsychiatric illness.

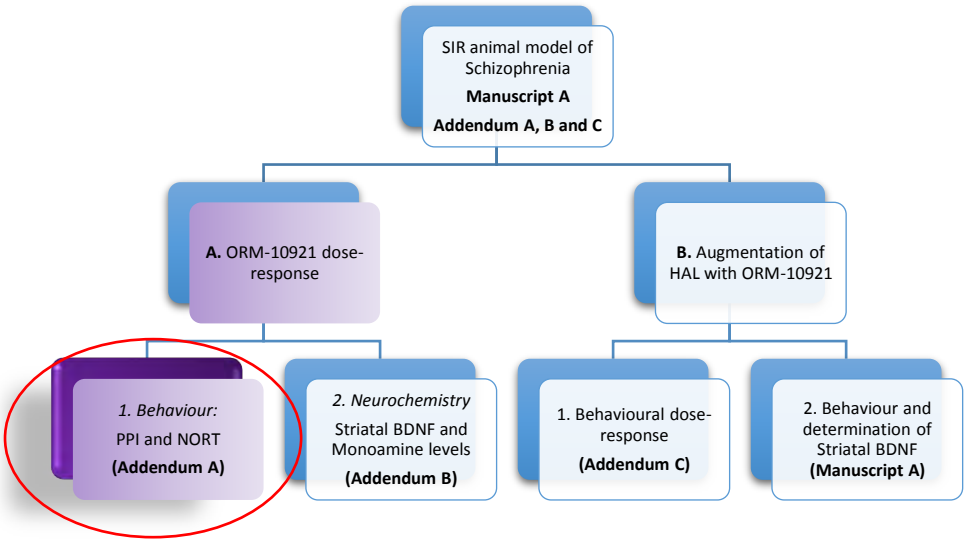
Addendum A

Antipsychotic-like and pro-cognitive effects of various doses of ORM-10921 in the social isolation rearing (SIR) animal model of schizophrenia

Preamble

This addendum will report and discuss the dose-response data with respect to the behavioural effects of ORM-10921 used to design and prepare **Manuscript A** and **Addendum C**. Four doses of ORM-10921 were assessed in the social isolation rearing (SIR) model of schizophrenia, and compared to clozapine (CLOZ) and idazoxan (IDAZ). This section contains a validation of the SIR model with regards to face and predictive validity, assessing whether deficits in the SIR animals could be reversed by the atypical antipsychotic, CLOZ, but not by the non-selective α_2-AR antagonist, IDAZ. This dose-response data led us to conclude that an additional lower dose of ORM-10921 should be employed along with an augmentation study using a typical antidopaminergic antipsychotic in order to more definitively demonstrate the therapeutic benefits of additional α_{2C}-AR-antagonism, in the latter case to bolster the antipsychotic-like effects of haloperidol (reported in **Addendum C**). This information was later used to develop Manuscript A.

The diagram below depicts where each addendum or manuscript relating to the SIR study is reported in this thesis, and how this fits into the overall study design. The circled box indicates the section of the SIR study that is reported here, as described above.



1. Aims

The main aim of this investigation was to employ an animal model of schizophrenia to investigate whether chronic treatment with ORM-10921 has antipsychotic-like and pro-cognitive effects, as assessed in the SIR neurodevelopmental animal model of schizophrenia. The study aims were as follows:

1.1. Validation of SIR as an animal model of schizophrenia

We needed to validate:

1. Whether SIR animals present with psychotic-like sensorimotor gating deficits in the prepulse inhibition (PPI) test and cognitive impairment in the novel object recognition test (NORT), compared to socially reared animals (SOC) (face validity),

2. Whether deficits in the PPI and NORT in SIR animals can be reversed by a known reference antipsychotic, CLOZ (predictive validity), and

3. Whether the non-selective α_2-AR-antagonist, IDAZ, which does not present with clinical antipsychotic activity, would exert any therapeutic behavioural effects in the PPI and NORT in SIR rats (predictive validity).

1.2 To determine whether α_{2C}-AR selective antagonism with ORM-10921 has antipsychotic-like and pro-cognitive effects in SIR animals

1. A four-tier dose-response analysis of ORM-10921 was conducted to determine which (if any) of the doses would display the desired therapeutic effects with respect to PPI and NORT in SIR rats.

2. To select the most effective doses of ORM-10921, and thereafter to compare the effects of sub-chronic ORM-10921 treatment to that of the atypical antipsychotic, CLOZ, and the non-selective α_2-AR-antagonist, IDAZ, using PPI and the NORT as behavioural outcome markers.

2 Methods

2.1 Animals and drug treatment

Animals were bred and cared for at the Vivarium of the North-West University, Potchefstroom. Sprague Dawley rats were weaned on post natal day 21 and either placed in a cage with 3 other rodents (social rearing, 4 animals per cage) or placed alone (isolation rearing, one animal per cage) for 8 weeks (until post-natal day 77). The animals were handled according to the code of ethics in research, training and testing of drugs in South Africa, and ethical approval for this study was obtained from the AnimCare Animal Research Committee of the North-West University (ethics approval number: NWU-00050-13-A5). Early antipsychotic response is deemed an accurate predictor of later response, and therefore a 14 day sub-chronic administration period was deemed adequate (Kapur et al., 2005; Möller et al., 2011, 2012; Möller et al., 2013a; Toua et al., 2010). Once daily sub-cutaneous (SC) drug administration (1

ml/kg, pH 4.5-5.5) commenced on week 7 (post natal day 63) of isolation rearing and continued for 14 days. CLOZ was dissolved in 1 M glacial acetic acid and administered at a dose of 5 mg/kg. The latter dose has shown robust antipsychotic-like and pro-cognitive effects in animal models of schizophrenia (Bakshi et al., 1994; Möller et al., 2011, 2012, 2013; Toua et al., 2010; Zhang et al., 1999b), and also presents with α_{2A} and α_{2C} AR occupancies of 65 and 95%, respectively (Marcus et al., 2005). IDAZ was dissolved in normal saline and administered at a dose of 3mg/kg. This dose presents with α_{2A} and α_{2C} AR occupancies of ~85 and ~90% (Marcus et al., 2005) and presents with an α_{2C}-AR occupation equal to that of CLOZ 5 mg/kg. Moreover, this dose allows similar α_{2A}-AR vs. α_{2C}-AR occupancy (Marcus et al., 2005) and has demonstrated similar effects to that of CLOZ on prefrontal cortical dopamine levels (Matsumoto et al., 1998; Moghaddam and Bunney, 1990). Additionally, this dose is not associated with confounding anxiogenic effects (Redfern and Williams, 1995) or with decreased locomotor activity as observed at higher doses (Rosu et al., 2015). ORM-10921 (0.03; 0.1; 0.3 and 1 mg/kg) was dissolved in saline (Sallinen et al., 2013a). The doses of ORM-10921 falls within a dose range applied previously to assess antipsychotic-like activity (Sallinen et al., 2013a), albeit in a different rodent model. Receptor occupancy data for ORM-10921 has not yet been described in the literature at any given dose.

2.2 Prepulse inhibition of startle (PPI)

PPI was assessed 12 hours after the last drug treatment on day 14 in two illuminated, ventilated, sound-attenuated startle chambers containing a Plexiglas cylinder on a platform mounted on a piezo-electric sensor (SR-LAB, San Diego Instruments, San Diego, USA). The startle session was conducted as described in Manuscript B against a 68dB background white noise level with prepulse trials consisting of a single 115 dB pulse (with a time-interval of 80 ms) preceded by a 20 ms non-startling prepulse-stimulus with intensities of 72, 76, 80 or 84 dB. Startle responses to 40 115dB PULSE ALONE trials were used to determine habituation to repeated delivery of startling pulses. Briefly, startle responses to a 40ms 115dB PULSE ALONE trial was measured as 10 consecutive trials at the beginning (BLOCK 1) and end (BLOCK 4) of the test session. 70 trials consisting of 40 PREPULSE+PULSE trials, 20 PULSE ALONE trials (BLOCK 2 and 3) and 10 no-stimulation trials were randomly delivered between BLOCK 1 and BLOCK 4. The percentage PPI was calculated for each individual subject according to the formula: %PPI = [(startle reponse for PULSE ALONE trial) – (startle reponse for PREPULSE +PULSE trial)/(startle repsone for PULSE ALONE trial) x 100] (Van den Buuse and Eikelis, 2001). The average %PPI across all four prepulse intensities was then determined (Gacsalyi et al., 2013). A higher % PPI correlates to improved sensorimotor gating. The protocol is adapted from previously described methods (Geyer and Swerdlow, 2001; Möller et al., 2011; Wang et al., 2003).

2.3 Novel object recognition test (NORT)

The NORT is a measure of declarative recognition memory, which relies on the innate preference of rats to explore novel objects more than familiar objects (Ennaceur and Delacour, 1988). The NORT was performed during the dark cycle on days 12 and 13 of drug treatment (ending on the day before the PPI), 12 hours after the last drug administration, as previously described (Möller *et al.,* 2013). The NORT procedure is described in detail in Manuscript A and B, and the reader is referred to these chapters for further details. Time spent exploring the novel vs. the familiar object was scored using video recording and hand scoring. Object exploration was considered as active sniffing, licking or physical exploration of the object. Animals that failed to accumulate a total exploration time of ≥6 seconds or who failed to explore both objects were excluded from the study (du Jardin *et al*., 2016). Data are expressed as the percentage of the discrimination index (DI), which was calculated according to the formula [(time spent at novel object – time spent at familiar object)/(total time spent exploring both objects)] x100 (Antunes and Biala, 2012). Essentially, more exploration time at the novel object reflects accurate recollection that the familiar object has been presented before, thus prompting greater exploration of the novel object. Although the latter can be used to determine recollection within a specific drug cohort, the DI corrects for the time spent exploring both objects, allowing for comparisons between groups. A larger DI indicates superior declarative memory

2.4 Statistical analysis

Data are expressed as mean ± SEM except for Startle Habituation data expressed as mean. GraphPad Prism® version 6.01 (GraphPad Software, San Diego California USA, www.graphpad.com) was used for parametric and nonparametric statistical analyses and graphical presentations. Normality of data sets was determined using the Shapiro-Wilk test, since this test is highly recommended for the n-range of data included in this section and has very good statistical power to detect a non-guassian population (Ghasemi and Zahediasl, 2012; Razali and Wah, 2011). Habituation of the startle response to the PULSE-ALONE stimulus was determined using the average startle amplitude values from Block 1 to Block 4 and analysed with a repeated measures two-way analysis of variance (rmANOVA) and the appropriate post-hoc test as described below. Exploration time for novel vs. familiar objects in the NORT within a cohort was compared using paired student's t-tests, or Wilcoxon's matched pairs rank test for non-parametric data. Total exploration times were compared using ANOVA and Tukey's post hoc test. Statistical significance was set at $p<0.05$.

2.4.1 Validation of the SIR model

Unpaired student's t-tests were used to determine whether SIR and SOC controls displayed significant differences in the PPI test and NORT. To analyze whether SIR animals treated for 14 days with either

vehicle, IDAZ or CLOZ displayed differences in the PPI and NORT, SIR treatment groups were analyzed with ANOVA and Tukey's multiple comparison test. Tukey's multiple comparison test is powerful and the preferred post-hoc parametric test when doing pairwise comparisons (Kim, 2015). It is also effective for differing group sizes and when the investigation places emphasis on diminishing the chance for a Type I statistical error (false positive discovery rate)(McHugh, 2011).

2.4.2 Dose-response

For the dose-response of ORM-10921 compared to SIR controls, ANOVA was used with Fisher's Least Significant Difference test (LSD), which does not correct for multiple comparisons like the Tukey test. The rationale of not correcting for multiple comparisons with a Tukey test for a simple dose-response analysis is that statistical power to detect real differences is compromised, thus realising a Type II statistical error (false negative) (Banerjee et al., 2009). Since the aim is to establish which of the various applied doses of the drug is effective vs. control, and not to determine if different doses of the drug are more effective than other doses, Fisher's LSD test was used. This test is similar to a set of individual t-tests, but computes the pooled standard deviations from all the groups as opposed to only two groups (as in a t-test), thereby gaining statistical power. This test is often used when multiple doses of the same compound are tested for efficacy (Bachtell et al., 2005; Debrah et al., 2005; Kufahl et al., 2011; Labonte et al., 2012; Newman and Beardsley, 2006; Ruxton and Beauchamp, 2008). We decided that favouring a type I statistical error (false positive) in our preliminary dose-response analyses by not correcting for multiple comparisons would enable us to decide whether this drug is indeed effective. The rationale was to determine effective drug doses, where after these chosen drug doses would be subjected to more rigourous statistical analysis compared to the positive and negative controls (CLOZ and IDAZ) in a post hoc test that corrects for multiple comparisons, the Tukey test, as described below (Nickerson, 2000).

2.4.3 Comparison of ORM-10921 to IDAZ and CLOZ

After selecting the most effective drug doses in order to employ a more rigorous statistical analysis, the two most effective doses of ORM-10921 were compared statistically to the effects of CLOZ and IDAZ. ANOVA and Tukey's post hoc comparison was employed to determine differences between the effects of drug treatment on behaviour in the PPI and NORT. Startle habituation analysis and total exploration time were performed as described above, while the time spent exploring the novel vs. the familiar object was not repeated, as the within-group analysis is performed for all applicable drugs in section 3.1 and 3.2 of the results.

3. Results

3.1 Validation of the SIR model

3.1.1 Prepulse inhibition test (PPI)

Fig A-1 depicts the startle response habituation of SOC vs SIR animals across the four startle blocks. No significant differences in startle habituation was observed between SOC and SIR animals ($F(1,11) = 0.75$, $p=0.4$).

Fig A-2 depicts the startle response habituation of SIR animals treated with vehicle, CLOZ or IDAZ. No significant difference was observed between the startle habituations of any of the treatment groups ($F(2, 28) = 0.965$, $p=0.39$)

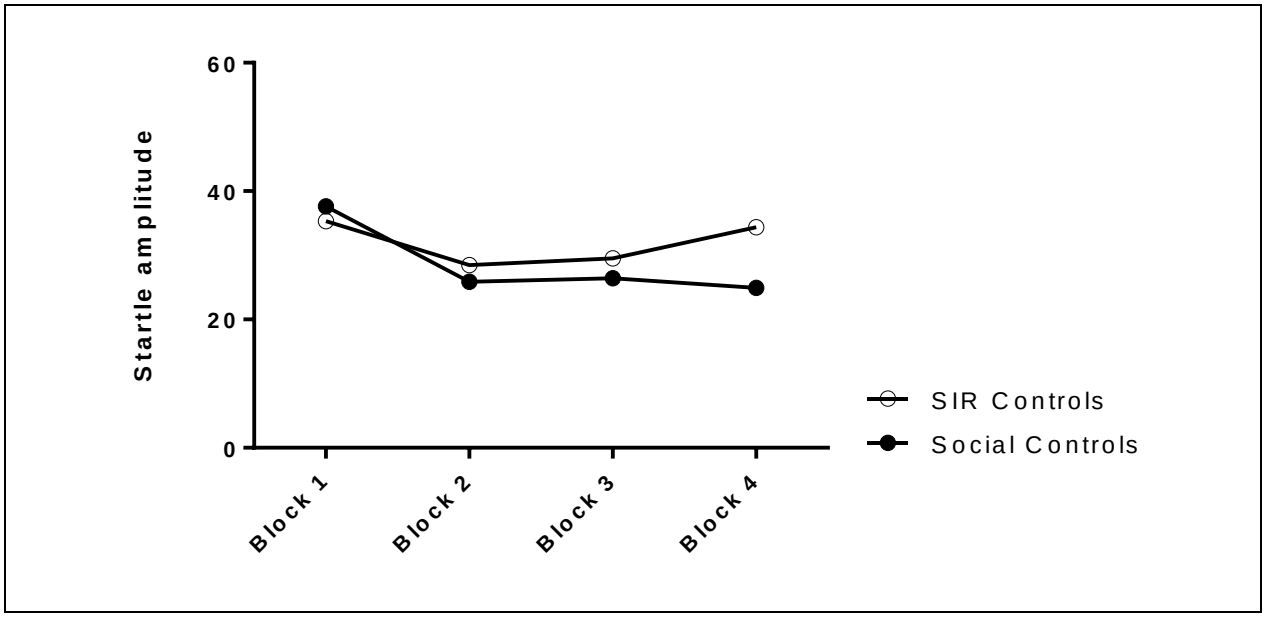


Fig A-1 Startle response habituation to the four startle blocks throughout the startle session in vehicle treated SOC vs. SIR animals (n=10-11). Repeated Measures Anova. SOC = Socially Reared Controls, SIR = Social Isolation Reared.

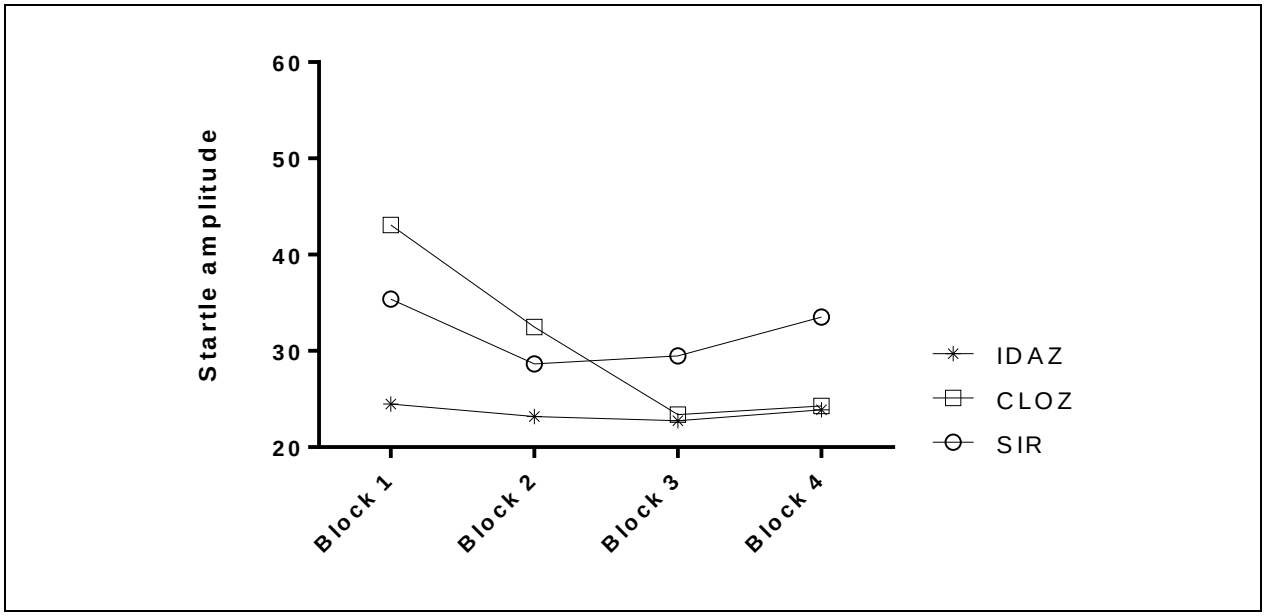


Fig A-2 Startle response habituation to the four startle blocks throughout the startle session in vehicle treated SIR animals treated with vehicle, IDAZ or CLOZ (n=9-11). Repeated Measures Anova. SIR = Social Isolation Reared controls, IDAZ = SIR+idazoxan 3 mg/kg, CLOZ = SIR+clozapine 5 mg/kg.

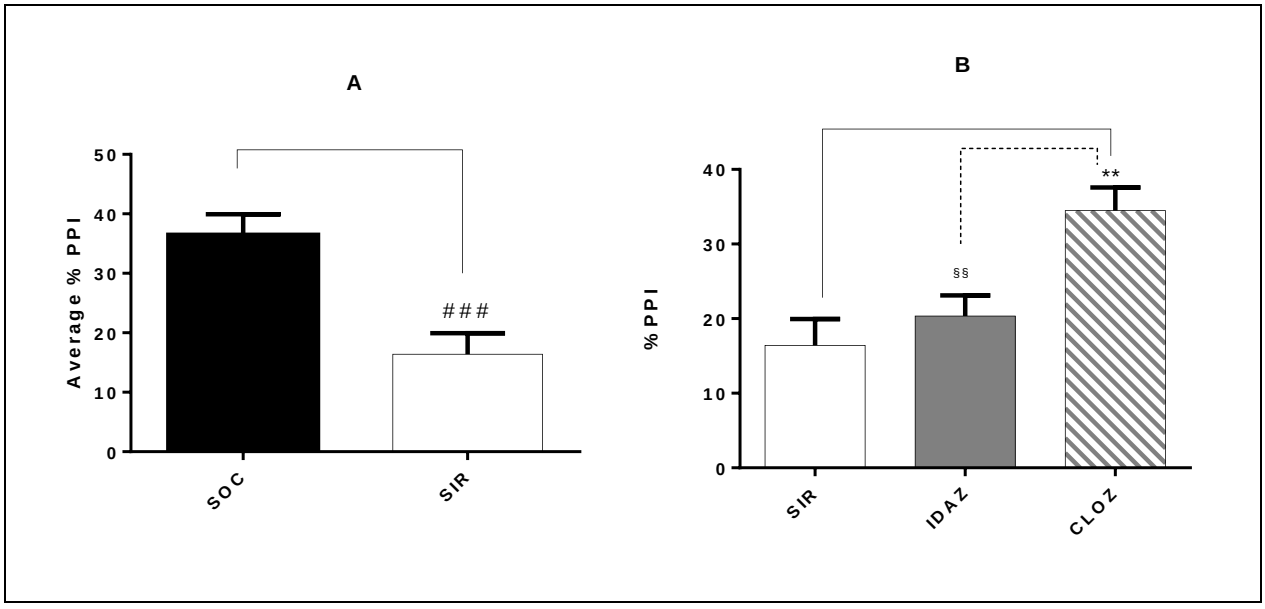


Fig A-3. Average percentage PPI of the startle response in vehicle treated SOC or SIR animals (A), and in SIR animals treated for 14 days with either IDAZ or CLOZ (n=9-11) (B). Unpaired t-test $^{###}p<0.001$ vs. Social Controls, ANOVA and Tukey post hoc **p<0.01 vs. SIR controls, $^{§§}p<0.01$ vs. CLOZ. SOC = Socially Reared Controls, SIR = Social Isolation Reared controls, IDAZ = SIR+idazoxan 3 mg/kg, CLOZ = SIR+clozapine 5 mg/kg.

Fig A-3-A depicts the average percentage PPI in SOC vs. SIR animals. Unpaired t-test indicated a significantly lower PPI in SIR vs. SOC animals (p=0.0008). Fig A-3-B depicts the average percentage PPI

in SIR animals following treatment with IDAZ or CLOZ. ANOVA indicated a significant difference between groups ($F(2,26)=9.334$, $p=0.0009$). Tukey's post hoc test indicated that CLOZ but not IDAZ significantly increased PPI vs. SIR controls ($p=0.001$). CLOZ also significantly increased PPI vs. IDAZ in SIR rats ($p=0.008$).

3.1.2 Novel Object Recognition Test (NORT)

In the NORT, a number of parameters can be assessed. The time spent exploring the familiar object vs. the time spent exploring the novel object can be determined using paired-tests or Wilcoxon ranked matched-pairs tests (non-parametric data) to assess whether a certain cohort spent more time exploring the novel object. This measure is however not helpful when making between group comparisons, since it does not correct for the total time spent exploring both objects. However, the discrimination index, or DI, is a measure of the difference in exploration time for the two objects, corrected for the total time spent exploring both objects by dividing this difference by the total exploration time and allows for between-group comparisons (Antunes and Biala, 2012; Broadbent et al., 2010). The DI is a much more robust index of memory retention for the familiar object and thus preference to explore the novel object. A minimum total exploration time of 6 seconds was an inclusion criterion for the test, as well as exploration of both objects (du Jardin et al., 2016).

Total exploration times of the novel and familiar objects for SOC+vehicle, SIR+vehicle, SIR+CLOZ and SIR+IDAZ are depicted in Fig A-4. Unpaired t-test indicated no significant difference in exploration times for SOC vs. SIR animals ($p=0.56$). ANOVA indicated no significant differences between the exploration times for SIR animals treated with either vehicle, CLOZ or IDAZ ($F(2,22) = 1.6$, $p=0.22$).

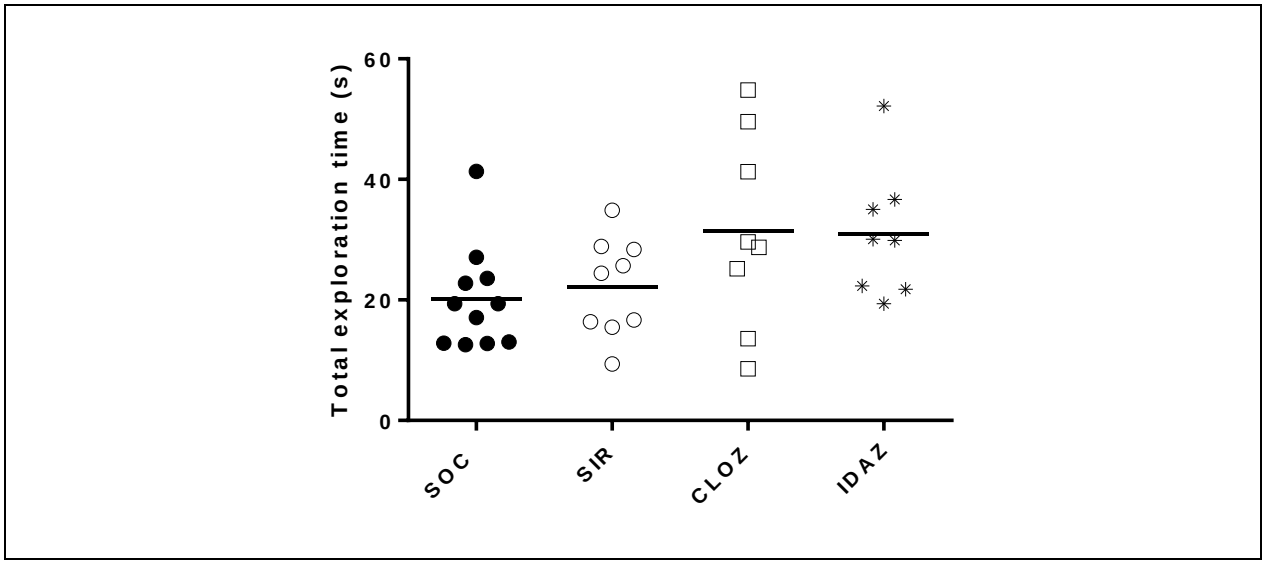


Fig. A-4 Total exploration times for subjects that met the inclusion criteria in the recognition trial of the NORT (n=8-10). SOC = Social+vehicle controls, SIR = SIR+vehicle controls, CLOZ = SIR+clozapine 5 mg/kg, IDAZ = SIR+idazoxan 3 mg/kg.

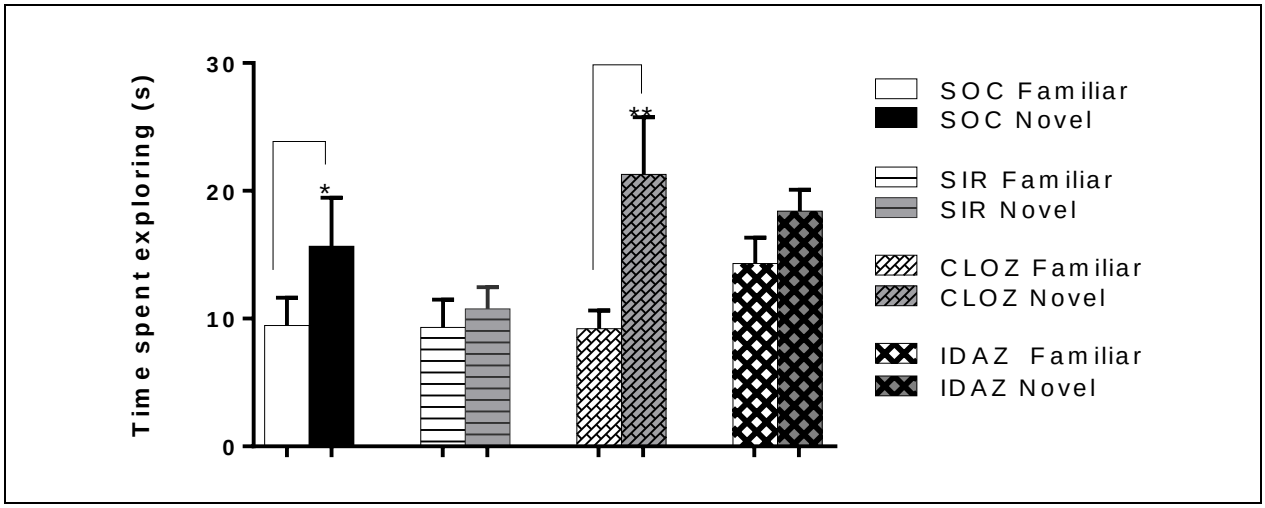


Fig. A-5. Time spent exploring novel and familiar objects in the NORT following the various drug treatments as indicated (n=8-10). SOC Controls received saline vehicle. All other drug treatments were conducted in SIR animals. Paired t-tests were conducted for novel vs. familiar objects for each drug treatment. *p<0.05, **p<0.01 novel vs. familiar object epxloration time. SOC = Social Controls, SIR = SIR Controls, CLOZ = SIR+clozapine 5 mg/kg, IDAZ = SIR+idazoxan 3 mg/kg. Novel = Novel object, Familiar = Familiar object.

Figure A-5 shows the time spent exploring the novel and the familiar object. Paired t-tests were conducted to determine whether more time was spent exploring the novel object for each individual subject (within group comparison). SOC Controls (p= 0.02) but not SIR controls (p=0.42) spent significantly more time exploring the novel object than the familiar object. SIR animals treated with

CLOZ for 14 days (p=0.006) but not IDAZ (p=0.07) spent significantly more time exploring the novel object than the familiar object.

Figure A-6 shows the effects of rearing condition (SOC vs. SIR, Fig A-6-A) and treatment with IDAZ or CLOZ (Fig A-6-B) in SIR animals on recognition memory in the NORT. Unpaired student's t-test indicates a significantly lower DI for SIR vs. SOC controls (Fig A-6A, p=0.02). ANOVA of the SIR treatment groups showed a significant effect of drug treatment on the DI in SIR animals ($F(2,23)=6.164$, p=0.075). Tukey's post hoc multiple comparison indicated that SIR animals treated with CLOZ but not IDAZ showed significantly increased recognition memory vs. SIR controls (p=0.01). CLOZ also showed significantly increased recognition memory vs. SIR animals treated with IDAZ (p=0.01) (Fig a-6-B).

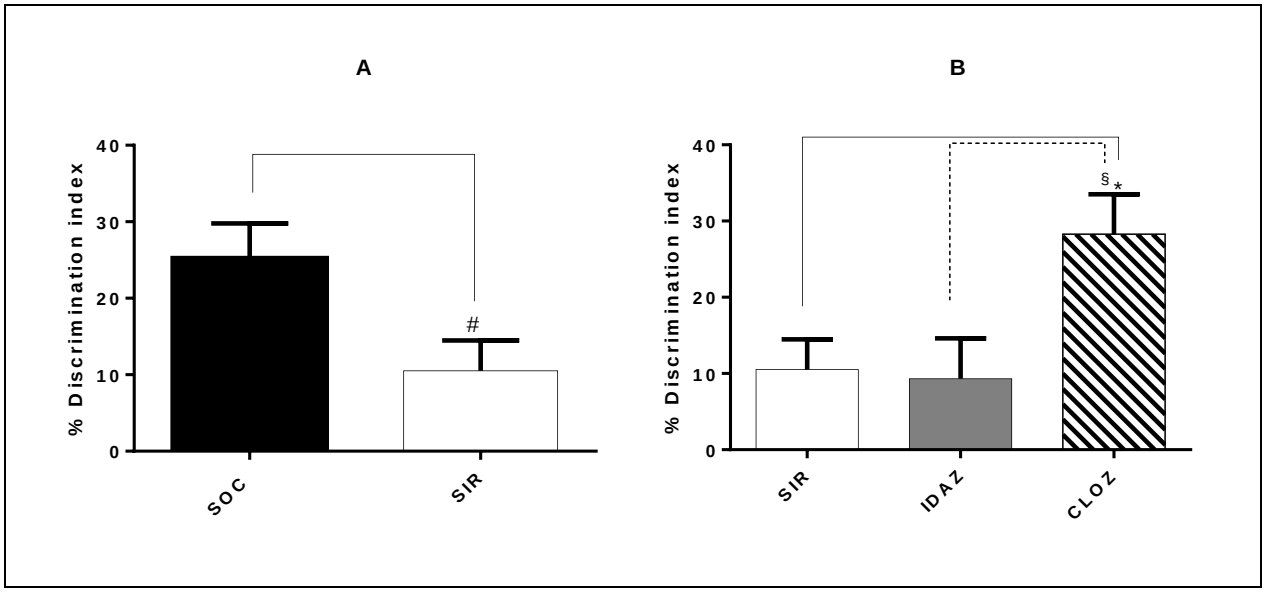


Fig A-6. DI of SIR and SOC controls (A) and SIR animals treated for 14 days with either vehicle, CLOZ or IDAZ (B) (n=7-11 per group). Unpaired student's t-test $^{\#}p<0.05$ vs. SOC Controls (A). ANOVA and Tukey's Multiple Comparison test, #p<0.05 vs. SOC controls, *p<0.05 vs. SIR Controls, $^{\S}p<0.01$ vs. CLOZ treatment in SIR animals. SOC = Social vehicle controls, SIR = SIR vehicle controls, CLOZ = SIR+clozapine 5 mg/kg, IDAZ = SIR+idazoxan 3 mg/kg.

3.2. Dose-response analysis of ORM-10921

3.2.1 PPI

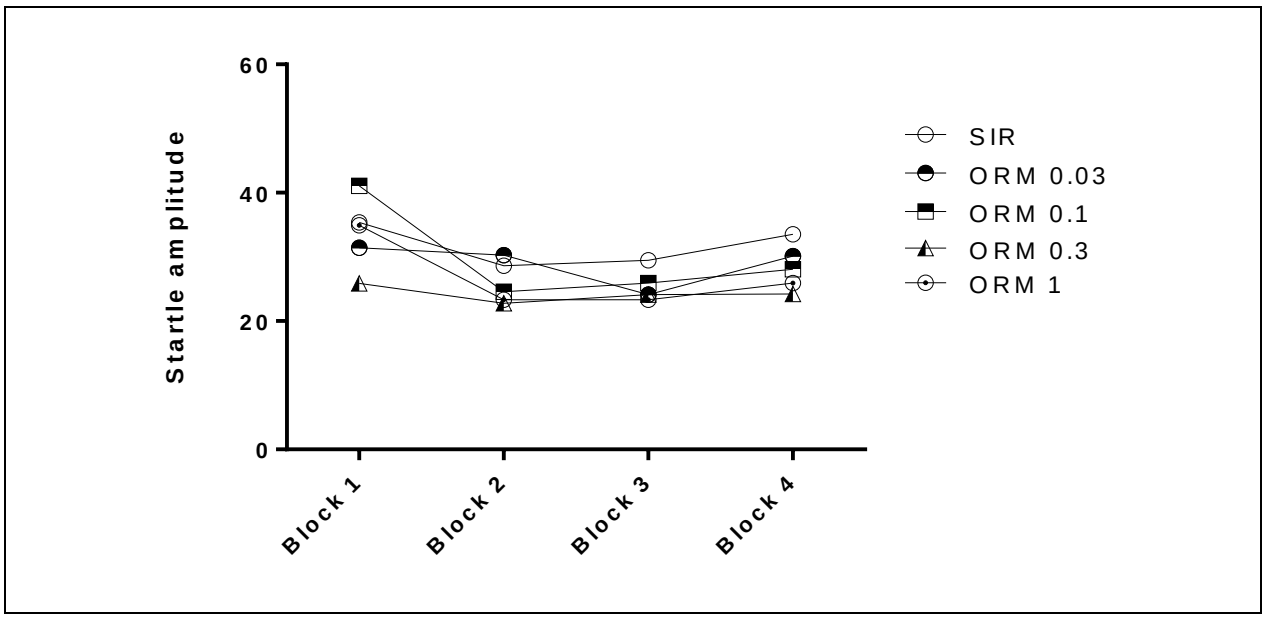


Fig A-7 Startle response habituation to the four startle blocks throughout the startle session in vehicle treated SIR animals treated with vehicle or various doses of ORM-10921 (n=9-11). Repeated Measures Anova. SIR = Social Isolation Reared, ORM = ORM-10921 at 0.03; 0.1; 0.1; 0.3 and 1 mg/kg.

The startle habituation of SIR animals treated with vehicle or various doses of ORM-10921 is indicated in Fig A-7. ANOVA indicated no significant differences in startle habituation between treatment groups ($F(4,40)= 0.9$, $p=0.4$, ns).

The average % PPI in SIR animals treated with the different doses of ORM-10921 is indicated in Fig A-8. ANOVA indicated significant effect of treatment ($F(4,40)=2.659$, $p=0.04$). Fisher's LSD indicated a significant increase in %PPI in SIR rats treated with ORM 0.03 ($p= 0.008$) and ORM 0.1 ($p=0.04$) vs. SIR controls.

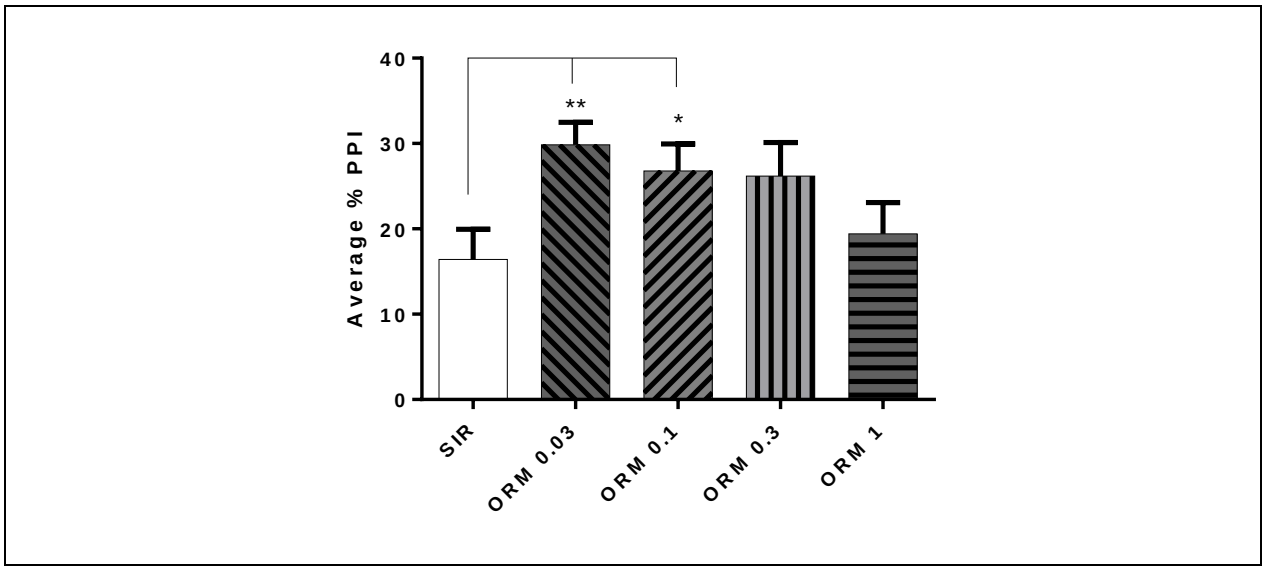


Fig A-8. Average % PPI in SIR animals treated with various doses of ORM-10921. ANOVA and Fisher's LSD test. *p<0.05, **p<0.01 vs. SIR controls. SIR = SIR controls, ORM= SIR+ORM-10921 0.03, 0.1, 0.3 or 1mg/kg.

3.2.2 NORT

The total exploration times of SIR animals treated with vehicle or various doses of ORM-10921 is depicted in figure A-9. One-way ANOVA showed there to be a significant difference in exploration time between the different treatment groups ($F(4,37)=3,793$; $p=0.01$). Fisher's LSD indicated that SIR rats treated with ORM 1 mg/kg spent significantly more time exploring both objects compared to SIR controls ($p=0.01$) and compared to SIR rats treated with ORM 0.03mg/kg ($p=0.03$). Interestingly, while SIR rats treated with ORM 1 mg/kg spent the most time exploring, this did not result in improved object recognition (Fig A-10).

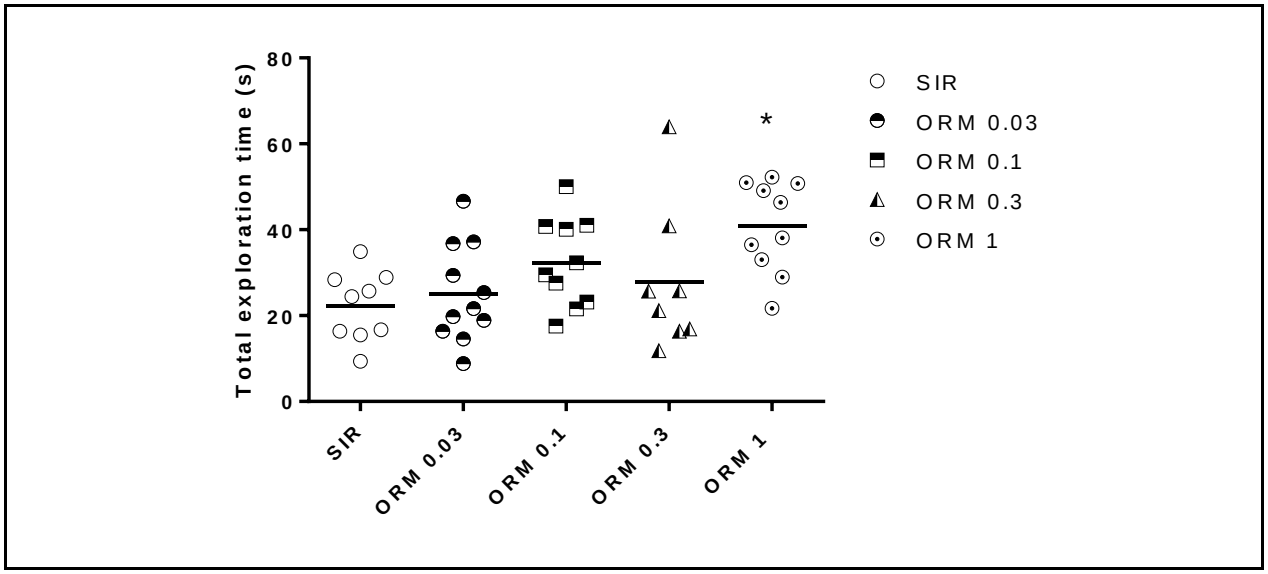


Fig. A-9. Total exploration times for subjects that met the inclusion criteria in the recognition trial of the NORT (n=7-10) SOC = Social vehicle controls, SIR = SIR vehicle controls, ORM = SIR+ORM-10921 at 0.03; 0.1; 0.1; 0.3 and 1 mg/kg.

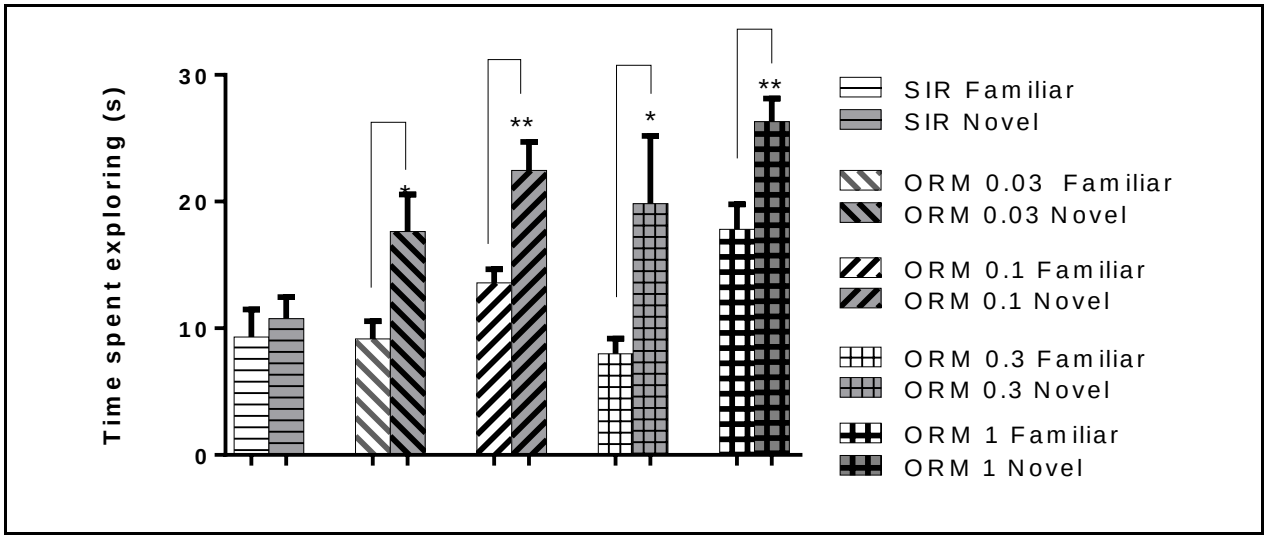


Fig. A-10. Time spent exploring novel and familiar objects in the NORT following the various drug treatments as indicated (n=7-10). All drug treatments were conducted in SIR animals. Paired t-tests or Wilcoxon matched pairs test for novel vs. familiar objects for each drug treatment. *p<0.05, **p<0.01. SIR= SIR vehicle controls, ORM=SIR+ORM-10921 0.03, 0.1, 0.3 or 1 mg/kg. Novel = Novel object, Familiar = Familiar object.

Fig A-10 depicts the time spent exploring the novel vs. familiar object for SIR animals treated with either vehicle or one of various doses of ORM-10921, as indicated. Here, paired t-test showed a statistically significant increase in novel vs. familiar object exploration time for ORM-10921 0.03; 0.3 and 1 mg/kg cohorts (Fig A-10; 0.03mg/kg: p=0.005 and 0.3mg/kg: p=0.004 and p=0.03), while Wilcoxon matched-pairs signed rank test showed that ORM 0.1mg/kg also spent more time exploring the novel object (p=0.0039).

The DI in SIR animals treated with saline vehicle or several doses of ORM-10921 is depicted in Fig A-11, with ANOVA showing significant group differences ($F(4, 37)=4.876$; $p=0.002$). Fisher's LSD test indicated that all doses of ORM-10921 except the highest dose of 1 mg/kg ($p=0.11$) significantly increased the DI for the novel object compared to the SIR controls. ORM 0.03mg/kg ($p=0.001$), 0.1 mg/kg ($p=0.01$) and 0.3 mg/kg ($p=0.005$) all improved the DI significantly compared to SIR controls.

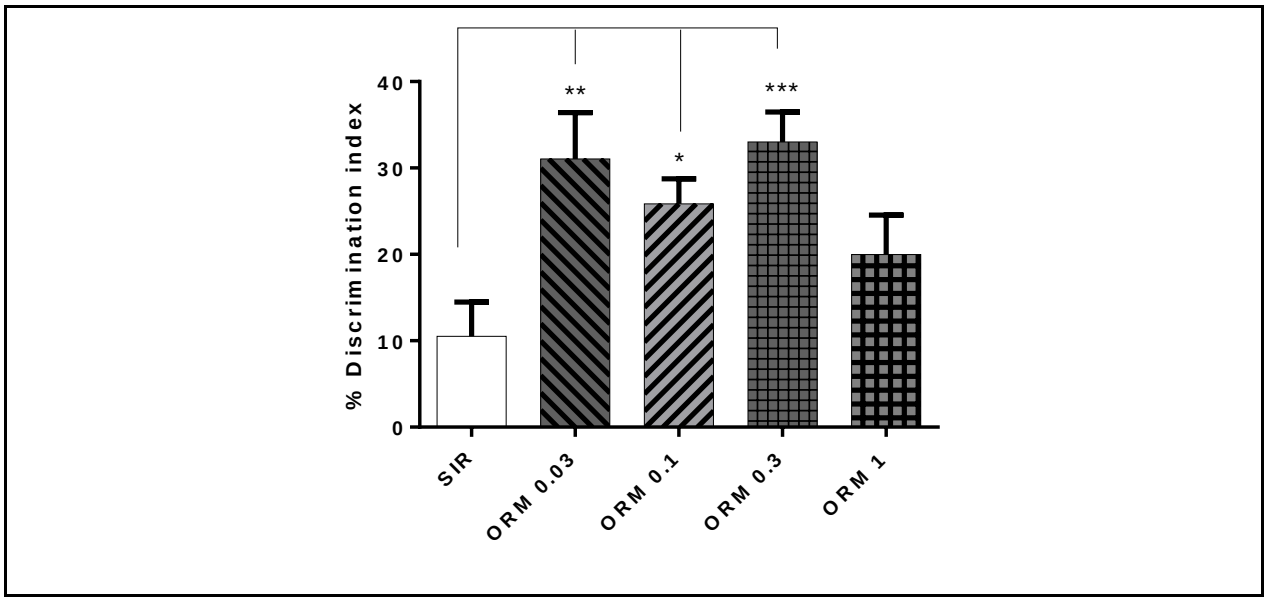


Fig. A-11 DI in the NORT in SIR animals treated with the various doses of ORM-10921 (n=7-10). ANOVA and Fisher LSD. * $p<0.05$, ** $p<0.01$, ***$p<0.001$ vs. SIR. SIR = SIR vehicle controls, ORM = SIR+ORM-10921 0.03, 0.1, 0.3 or 1 mg/kg.

3.3 Comparison of ORM-10921 vs. IDAZ and CLOZ

3.3.1 PPI

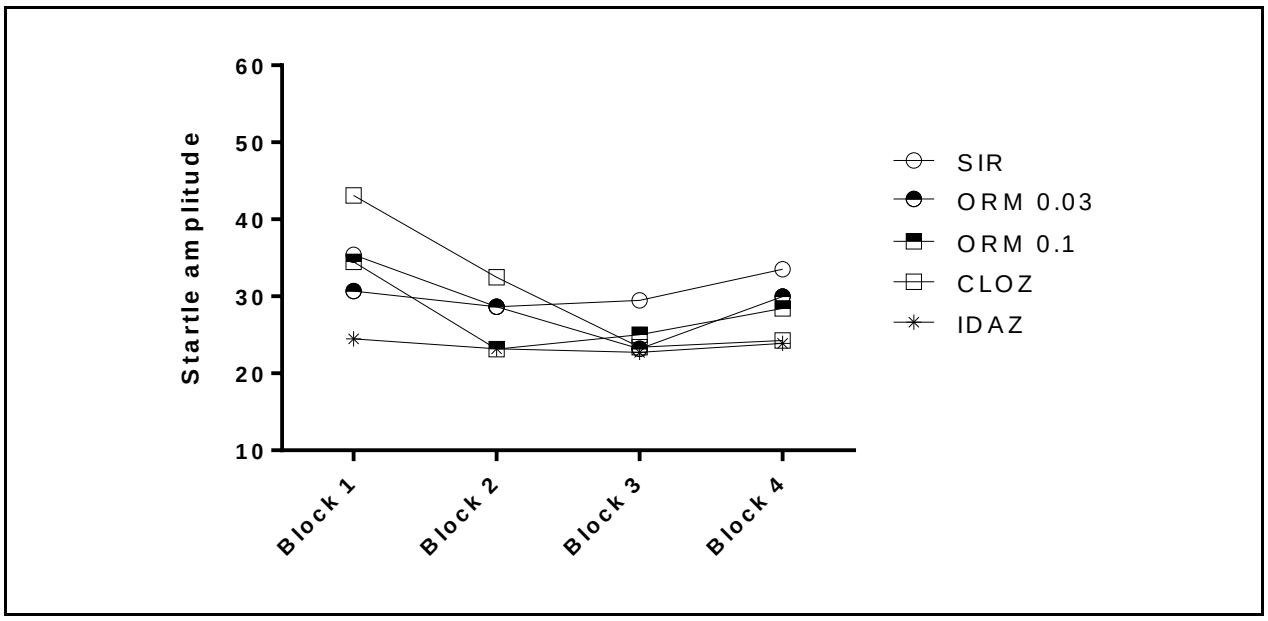


Fig A-12 Startle response habituation to the four startle blocks throughout the startle session in SIR animals treated with vehicle, CLOZ, IDAZ or either ORM-10921 0.03mg/kg or 0.1mg/kg (n=9-11). Repeated Measures Anova. SOC = Socially Reared Controls, SIR = Social Isolation Reared.

Fig A-12 depicts the habituation of the startle response to continuous presentation of the PULSE in the various cohorts as indicated. rmANOVA indicated no differences in startle response between drug treatment groups in any of the four startle blocks ($F(4,45) = 0.627$, $p=0.64$).

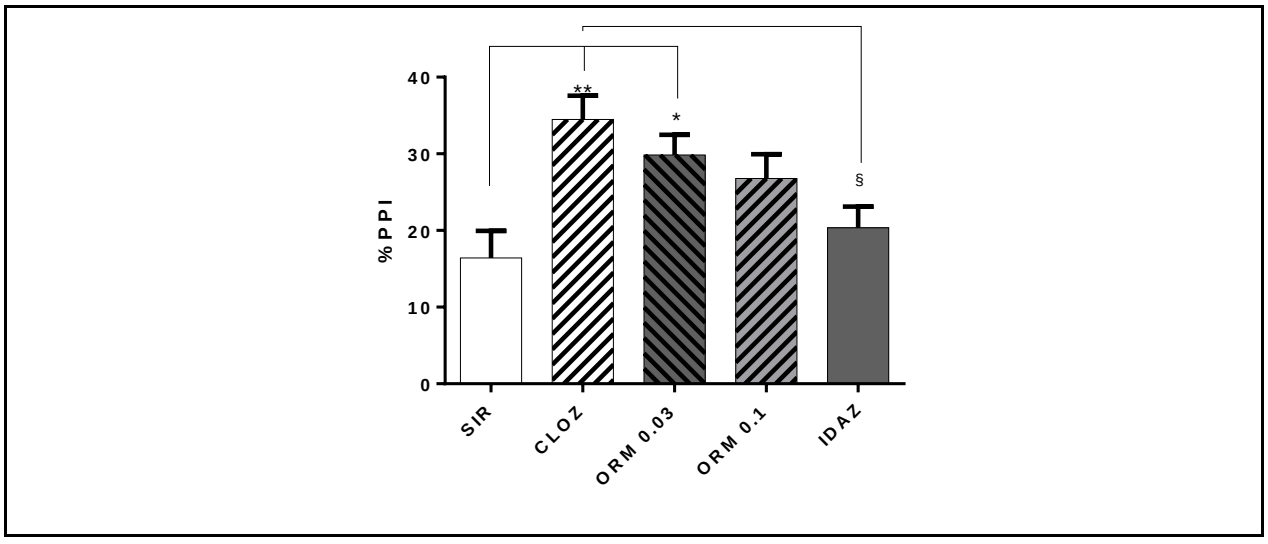


Fig A-13 Average percentage PPI of the startle response in SIR animals following the various drug treatments (n=10-13). ANOVA and Tukey's Multiple comparisons. $^{*}p<0.05$, $^{***}p<0.001$ vs SIR Controls, $^{\$}p<0.05$, vs. IDAZ. SIR = Social Isolation Reared, CLOZ = SIR+clozapine 5 mg/kg, ORM = SIR+ORM 10921 0.03 or 0.1 mg/kg, IDAZ = SIR+idazoxan 3mg/kg.

Fig A-13 depicts the average PPI in SIR animals treated with either vehicle, CLOZ, IDAZ or ORM-10921 0.03 or 0.01mg/kg. ANOVA indicated a significant effect of drug treatment on average PPI ($F(4,45)=5.728$, $p=0.0009$). Tukey's post hoc test indicated that CLOZ ($p=0.0012$) and ORM 0.03 mg/kg ($p=0.03$), but neither ORM 0.1mg/kg ($p=0.15$) nor IDAZ ($p=0.89$), significantly increased PPI compared to vehicle-treated SIR controls. Furthermore, CLOZ significantly increased PPI vs. IDAZ ($p=0.012$).

3.3.2 NORT

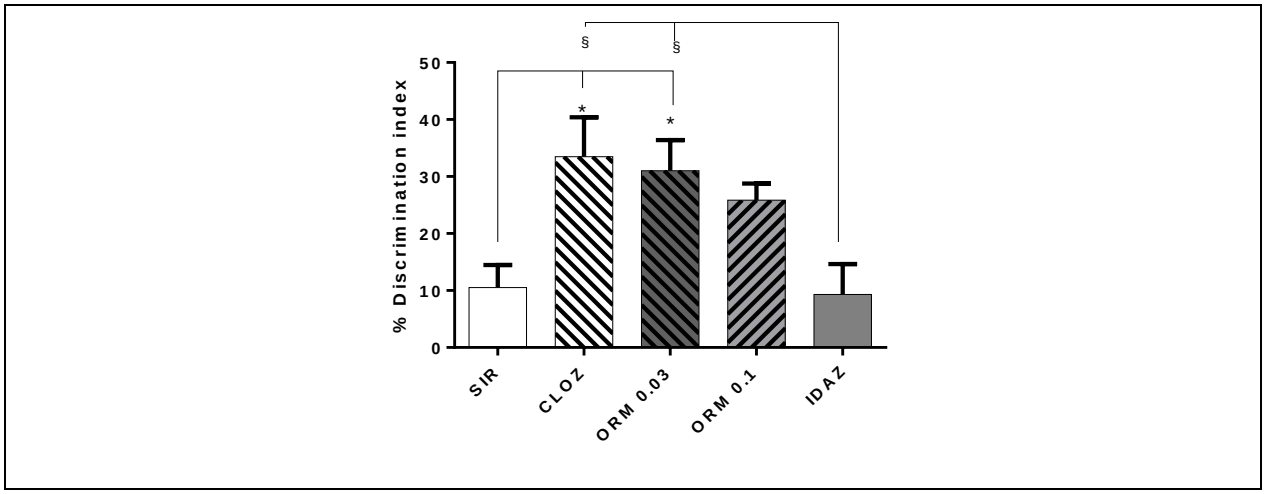


Fig. A-14 DI in the NORT in SIR animals treated with CLOZ, IDAZ and ORM-10921 0.03 and 0.1 mg/kg (n=8-10). ANOVA and Tukey. * $p<0.05$, vs. SIR. § $p<0.05$ vs. IDAZ. SIR = SIR vehicle controls, CLOZ=SIR+clozapine 5 mg/kg, ORM = SIR+ORM-10921 0.03, 0.1 mg/kg, IDAZ = SIR+idazoxan 3 mg/kg.

Fig A-14 depicts the DI in the NORT of SIR animals treated with either vehicle, CLOZ, IDAZ or ORM-10921 0.03 or 0.1mg/kg. ANOVA indicated a significant effect of drug treatment on DI in the NORT ($F(4,36)=5.177$, $p=0.002$). Tukey's post hoc test indicated that treatment with CLOZ ($p=0.02$) and ORM 0.03 ($p=0.04$), but not ORM 0.1 ($p=0.16$, ns) or IDAZ ($p=0.99$, ns) significantly increased the DI (object recognition memory) vs. SIR controls. Furthermore, both CLOZ ($p=0.02$) and ORM ($p=0.03$) induced a significantly higher DI vs. IDAZ treated SIR animals.

4. Discussion

4.1 Validation of the SIR model

Patients with schizophrenia show impairments in sensorimotor gating, as demonstrated by impaired PPI (Swerdlow et al., 2006a; Swerdlow et al., 2007; Swerdlow et al., 2006b) as well as in visual and episodic memory (Guillaume et al., 2015; McClure et al., 2007; Nestor et al., 2007). SIR is a neurodevelopmental animal model of schizophrenia that, apart from neurochemical alterations akin to schizophrenia, produces long-lasting impairments in PPI and object recognition memory, as well as

deficits in working memory and executive functioning and decreased social interaction (Fone and Porkess, 2008; Jones et al., 2011). Moreover, these behavioural deficits in SIR animals can be reversed by antipsychotic agents (Jones et al., 2011; King et al., 2004; Marsden et al., 2011; Möller et al., 2011; Möller et al., 2013a; Swerdlow et al., 2006b; Watson et al., 2012), indicating good predictive validity for this model.

Our findings that SIR rats presents with impaired PPI and impaired object recognition memory vs. socially reared controls is thus in agreement with the literature (Cilia et al., 2001; Weiss and Feldon, 2001), and provides good face-validity for using this model to assess antipsychotic-like drug effects. Moreover, PPI can be used to assess the efficacy of antipsychotic drugs, deficits of which are reversed by various antipsychotics in animals (Geyer et al., 2001) and humans (Hamm et al., 2001; Kumari et al., 2002). Deficits in object recognition memory in rodent models of schizophrenia can also be reversed by various antipsychotic compounds (Marsden et al., 2011; Watson et al., 2012), making it a valuable behavioural marker in drug treatment studies. Here we have shown that the atypical antipsychotic, CLOZ, but NOT the non-selective α_2-antagonist IDAZ, reversed both PPI and NORT deficits in SIR rats. CLOZ is a highly effective atypical antipsychotic widely used in the treatment of schizophrenia (Stroup et al., 2016; Swartz et al., 2008), and was similarly found effective in reversing PPI and NORT deficits in the SIR model. IDAZ, on the other hand is not only a non-selective α_2-AR antagonist, but is also ineffective when applied as monotherapy in the treatment of schizophrenia (Litman et al., 1993; Litman et al., 1996), while it was also unable to reverse SIR induced PPI and NORT deficits in this study. These findings provided robust predictive validity for using this model to assess the antipsychotic and pro-cognitive like effects of ORM-10921. With both face and predictive validity established for the SIR model, we could thus commence assessing whether the selective α_{2C}-antagonist, ORM-10921, could improve PPI and object recognition deficits in this model, thus assessing its antipsychotic-like effects.

4.2 Dose-response analysis of ORM-10921

Here we have provided evidence that 14 day sub-chronic treatment with ORM-10921 was able to reverse deficits in sensorimotor gating deficits and object recognition memory in a neurodevelopmental model of schizophrenia, the SIR rat. That a selective α_{2C}-AR antagonist (ORM-10921) but <u>not</u> a non-selective α_2-AR antagonist (IDAZ, see above), was able to engender these effects is significant, and will be specifically addressed in Section 4.3. These findings are in accordance with studies showing that ORM-10921, JP-1302 and ORM-12741, all highly selective α_{2C}-AR antagonists, reverse sensorimotor gating and cognitive deficits in NMDA-antagonist models of schizophrenia (Sallinen et al., 2007; Sallinen et al., 2013a; Sallinen et al., 2013b). We now extend these findings beyond pharmacologically induced models of schizophrenia to that of a neurodevelopmental model, which has superior etiological validity.

The observed effects of ORM-10921 would appear to be dose-dependent, with the cognitive deficits in this model demonstrating greater sensitivity to ORM-10921 treatment compared to the sensorimotor gating deficits. Whilst the two lower doses of 0.03 and 0.1 mg/kg improved PPI vs. controls, with this effect waning as the dosage increases, object recognition could be enhanced by 0.03, 0.1 and 0.3 mg/kg of ORM-10921, thus spanning a ten-fold dose increase. This is a very valuable finding, considering that cognitive deficits in schizophrenia are often more refractory to treatment, but are considered to be the best predictors of functional outcomes in patients (Bowie and Harvey, 2006; Kahn and Keefe, 2013; Keefe and Harvey, 2012). While various antipsychotics might improve sensorimotor-gating in animals, cognitive improvement with these antipsychotics is lacking (Swerdlow et al., 2008; Young et al., 2009). α_{2C} antagonism may therefore be a valuable therapeutic tool in addressing this aspect of schizophrenia symptomology.

An observation throughout is that increasing doses of ORM-10921 results in decreased antipsychotic-like and pro-cognitive effects in SIR animals. A probable explanation for this dose-response curve could be that higher doses of ORM-10921 start to engage α_{2A}-ARs, thus reducing the effect of selective targeting of the α_{2C}-AR. Indeed, some studies have suggested that antagonism of the α_{2A}-AR does not contribute to enhancement of the PPI response (Lähdesmäki et al., 2004; Larrauri and Levin, 2012; Ozcetin et al., 2016; Powell et al., 2005), while antagonism of the α_{2A}-AR has also been associated with *detrimental* effects on cognition (Franowicz et al., 2002). That IDAZ, which engages both α_{2C}-ARs and α_{2A}-ARs, is also unable to improve PPI and NORT deficits in this model, further supports the argument that loss of efficacy of ORM-10921 at higher doses could be related to dual engagement of α_{2C}-ARs and α_{2A}-ARs. ORM-10921 has a 100-fold selectivity for α_{2C} vs. α_{2A}-ARs, and while the lowest dose of 0.03mg/kg ORM-10921 was effective in both PPI and NORT, the 30-fold dose increase to the highest dose of 1 mg/kg may constitute a loss of α_{2C}-AR selectivity and an associated loss in efficacy. Such a loss in efficacy in the PPI test with increasing doses is similar to that reported elsewhere for ORM-10921 (Sallinen et al., 2013a), although a correlation to a specific effective doses was not made. This is not unexpected, since pharmacological models of schizophrenia along with differing rodent strains could very much affect the dosages necessary to effect symptom remission (Swerdlow et al., 1998).

An important conclusion from this dose ranging study was that lower doses of ORM-10921 are more likely to produce robust effects on aberrant PPI in a rodent developmental model of schizophrenia.

4.3 Comparison of ORM-10921 vs. IDAZ and CLOZ

In order to establish whether the effects of ORM-10921 on PPI and object recognition memory are due to α_{2C}-AR selective antagonism, the most effective doses of ORM-10921, namely 0.03 and 0.1 mg/kg,

were compared to that of the non-selective α_2-AR antagonist, IDAZ. ORM-10921 significantly improved PPI vs. SIR controls and object recognition memory vs. both SIR controls and vs. IDAZ treated rats, with IDAZ having no effect on these parameters. While α_2-AR antagonism has been suggested as playing an important role in the mechanism of atypical antipsychotics in managing positive and negative symptoms of schizophrenia (Svensson, 2003), α_2-AR antagonists do not *per se* reverse psychotic-like symptoms in animal models of schizophrenia (Marcus et al., 2005; Marcus et al., 2010b), nor do they present with any clinical value as an antipsychotic *per se* (Litman et al., 1993; Litman et al., 1996). However, augmenting antidopaminergic antipsychotic treatments with an α_2-AR antagonist has been reported to enhance antipsychotic-like and pro-cognitive effects in animal models of schizophrenia (Hertel et al., 1999a; Marcus et al., 2005; Marcus et al., 2010b; Wadenberg et al., 2007), presumably by enhancing prefrontal dopamine release (Hertel et al., 1999a; Hertel et al., 1999b; Matsumoto et al., 1998), while early clinical studies have also reported the benefit of α_2-AR antagonism when added to typical antipsychotic treatment (Litman et al., 1993; Litman et al., 1996). However, these studies report that α_2-AR antagonism monotherapy is not sufficient to effect antipsychotic-like and pro-cognitive effects, and that additional D_2 antagonism is required in human schizophrenics and in animal models of schizophrenia to exert therapeutic effects. Furthermore chronic IDAZ in healthy subjects does not reliably improve various cognitive domains (Smith et al., 1992). When considering the literature, it therefore isn't surprising that IDAZ did not exert antipsychotic-like or pro-cognitive effects in this animal model of schizophrenia, while there is evidence that monotherapy with selective α_{2C}-AR antagonism does produces antipsychotic-like and pro-cognitive effects in NMDA-antagonist animal models of schizophrenia (Sallinen et al., 2007; Sallinen et al., 2013a). Here we provide further evidence that selective α_{2C}-AR antagonism with ORM-10921 produces antipsychotic-like and pro-cognitive effects in this model as a stand-alone treatment, which suggests that non-selective engagement of both α_{2C}-ARs and α_{2A}-ARs may explain IDAZ's inefficacy in this regard. At least in this paradigm, the data certainly suggests that α_{2C}-AR selective antagonism has superior antipsychotic-like and pro-cognitive effects compared to non-selective antagonism.

Furthermore, the beneficial effects of ORM-10921 in the PPI test and NORT were comparable to the effects of CLOZ, which has a 3-fold α_{2C} /α_{2A} selectivity ratio and the highest α_{2C}/D_2 ratio of all available antipsychotics (Kalkman and Loetscher, 2003; Shahid et al., 2009) (refer to Figure 5 in **Chapter 6, Manuscript D**). This data therefore suggests that the clinical efficacy of CLOZ in part might be related to significant antagonism at the α_{2C}-AR. Considering that almost all antipsychotics have antagonist properties at this receptor (Kalkman and Loetscher, 2003) and that the α_{2C}/D_2 ratio has been proposed to mediate the improved efficacy of drugs like CLOZ and quetiapine, our findings support the theory that α_{2C}-AR antagonism might be a beneficial therapeutic target in schizophrenia.

A final important observation was that the most marked beneficial effects of ORM-10921 in a multiple comparison analysis seemed to be exerted at the lowest dose of 0.03mg/kg. This observation raises the possibility that lower doses of ORM-10921 might present with more marked psychotropic effects. When considering that non-selective α_2-AR antagonism as an add-on treatment may thus improve the effects of an antipsychotic, and that the α_{2C}/D_2 ratio of antipsychotics may inform on clinical efficacy (Kalkman and Loetscher, 2003; Shahid et al., 2009; Stroup et al., 2016; Swartz et al., 2008), the question arises whether ORM-10921 might enhance the response to a D_2-antagonist antipsychotic devoid of α_2-AR activity, such as haloperidol, in an animal model such as SIR.

This study has thus delivered four important outcomes: (i) it demonstrated that ORM-10921 does indeed exert antipsychotic and pro-cognitive effects in an animal model of schizophrenia, (ii) these effects are superior to that of the non-selective α_2 antagonist, IDAZ, (iii) the antipsychotic-like effects seem to be exerted at very low doses of ORM-10921, while the pro-cognitive effects seemed to be present over a broader dose range and (iv) these effects are comparable to that of the atypical antipsychotic, CLOZ. Furthermore it enabled us to identify an effective dose with which to study the neurochemical effects of drug treatment in SIR rats (reported in **Addendum B**). Following the outcome of this study, we were prompted to investigate the effect of a lower dose of ORM-10921, namely 0.01 mg/kg, on PPI and NORT as well as the ability of low-dose ORM-10921 to augment the response to haloperidol (HAL), a D_2-antagonist. The results reported here for CLOZ and ORM-10921 0.03mg/kg were also used in the preparation of **Manuscript A** and the HAL augmentation study (**Addendum C**). Since the HAL augmentation study reported in **Manuscript A** and **Addendum C** was an extension of this study, the time period over which the study was performed was also extended, and the reason for incorporating additional SOC and SIR controls to control for variability in animal response over time (**Manuscript A**). Given that ORM-10921 *per se* did not affect BDNF levels (**Addendum B**) and that the interpretation of the effect of ORM-10921 on striatal monoamine levels was limited by an inability to demonstrate altered monoamine levels in SOC vs. SIR animals (**Addendum B**), the HAL augmentation study was favoured for publication (**Manuscript A**). Although the findings reported in this Addendum are novel and important, we felt that the follow-up study with HAL reported in **Manuscript A** presented with greater impact, and thus the findings reported in this Addendum have not yet been submitted for publication. However, blood samples collected during this investigation are available for further analyses and for future publication purposes. This study also led to the inclusion of the additional lower dose of 0.01 mg/kg ORM-10921 in the FSL study (**Addendum D**).

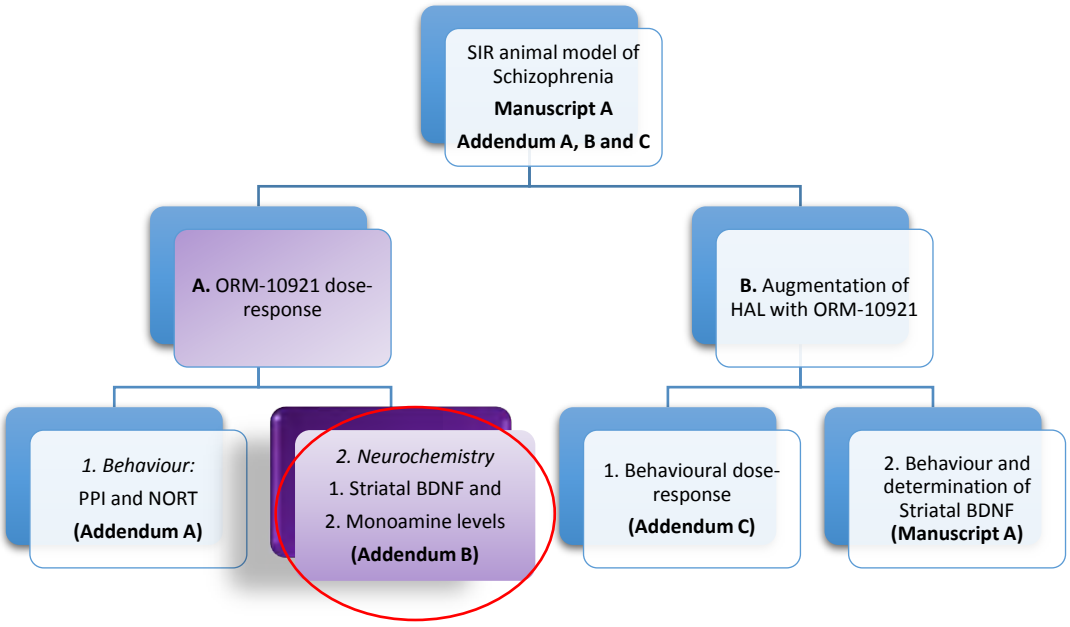
Addendum B

ORM-10921 beneficially alters striatal monoamine levels, but not striatal BDNF expression, in the SIR animal model of schizophrenia

Preamble

The following section will report and discuss the neurochemical effects of ORM-10921 in an animal model of schizophrenia. The effect of subchronic ORM-10921 on striatal monoamines and striatal brain derived neurotropic factor (BDNF) levels are reported here. The most effective dose of ORM-10921 as reported in **Addendum A** (0.03mg/kg) is applied here and compared to treatment with clozapine (CLOZ) and idazoxan (IDAZ). However socially reared (SOC) and social isolation reared (SIR) animals did not show differences in certain key monoamines, despite evidence that alterations in SOC vs. SIR animals is well-documented (Fone and Porkess, 2008) and despite pronounced behavioural differences observed between these two groups described in **Addendum A**. The data describing treatment effects on striatal monoamines may have been compromised by factors affecting the housing of the animals at the time, rendering the data unusable. Monoamines are extremely sensitive to environmental factors such as stress. This particular arm of the study commenced shortly after the Animal Research Center, where the animals were bred and housed, had been subjected to major upgrades to later become the Vivarium of the Preclinical Drug Development Platform (PCDDP). The new building suffered a number of set-backs after the animals were reintroduced into the center. These problems could have had a detrimental effect on the animals, and subsequently on the interpretation of the striatal monoamine data. Since we were unable to validate the SIR monoamine data in terms of SOC controls, the monoamine data could not be considered for inclusion in the publication. Nevertheless, these data are presented here for the sake of completion.

The diagram on the next page graphically depicts where this addendum fits into the study design in the SIR model and in this thesis, as indicated by the red circle.



1. The effects of ORM-10921 on striatal BDNF

The striatum plays an important role in the pathophysiology of schizophrenia and in the underlying cognitive deficits of the disorder (Simpson et al., 2010), while the prominent role of BDNF in processes underlying cognition and memory suggests that various cognitive manifestations in schizophrenia may be correlated to altered BDNF (Favalli et al., 2012). Further to this point, impaired neurotrophic signalling forms part of the proposed underlying pathogenesis of schizophrenia (Favalli et al., 2012; Nieto et al., 2013).

Previous studies have reported decreased BDNF levels in limbic structures of SIR rats (Djouma et al., 2006; Scaccianoce et al., 2006), but not in the striatum. Striatal volume reductions have been observed in drug-naive schizophrenia patients (Keshavan et al., 1998; Shihabuddin et al., 1998) and in certain animal models of schizophrenia (Simpson et al., 2010). Typical antipsychotics seem to decrease striatal BDNF levels in rodents (Pillai et al., 2006), possibly due to neurodegenerative effects following sustained D_2-antagonism and a reactive increase in dopamine release (Moghaddam and Bunney, 1990). On the other hand, clozapine (CLOZ) is more associated with *increased* serum BDNF levels in patients compared to those receiving typical antipsychotics (Grillo et al., 2007), while it seems to aid reversal of typical antipsychotic-induced decreases in striatal and hippocampal BDNF in rodents (Pillai et al., 2006). The α_{2C}-AR is most densely expressed in the striatum, amidst very little noradrenergic innervation of this region (Rosin et al., 1996; Scheinin et al., 1994), while CLOZ has the highest affinity for the α_{2C}-AR of all antipsychotics (Kalkman and Loetscher, 2003). In **Manuscript A**, we show that ORM-10921 at a dose of

0.01 mg/kg failed to alter striatal BDNF, yet significantly bolstered the response to haloperidol. However, in **Addendum A** we show that a higher dose of ORM-10921 (0.03mg/kg) exerts behavioural effects in SIR animals. The aim of this study was therefore to determine whether such a higher dose of ORM-10921 would offer any benefits as a monotherapy on striatal BDNF levels, compared to that of a reference antipsychotic, CLOZ, and to that of the reference non-selective α_2-AR antagonist, IDAZ.

Construct and predictive validity regarding striatal BDNF levels in the SIR model is reported in **Manuscript A** and will not be reported here again.

1.1. Aims

1.1.1 Validation of construct validity of the SIR animal model of schizophrenia

The aim in this study was to determine:

1. Whether a higher dose of ORM-10921 than that used in Manuscript A, and which effectively improved behavioural parameters (**Addendum A**), alters striatal BDNF levels in any way.

2. Whether the effects of ORM-10921 on striatal BDNF compares to that of CLOZ and IDAZ.

1.2 Methods

1.2.1 Animals and drug treatment

SIR animals were reared according to the description in **Addendum A**, and treated with ORM-10921 0.03 mg/kg, CLOZ 5mg/kg or IDAZ 3mg/kg for 14 days. Drugs were prepared and administered as described in **Addendum A**.

1.2.2 Brain homogenate preparation

24 hours after the last drug administration, animals were sacrificed by decapitation and the whole brain quickly removed and briefly placed in ice cold double distilled water, with the brain tissue dissected out immediately on an ice cooled slab. The frontal cortex was removed, whereafter the brain was placed with the dorsal side facing upward. The two cerebral hemispheres were split and the striatum dissected out by applying the external walls of the lateral ventricles as internal boundaries and the corpus callosum as external boundary, as described previously (Möller et al., 2013a; Möller et al., 2013b). The striata were snap-frozen in liquid nitrogen and transferred to a -70˚C freezer. On the day of the analysis, brain tissue was thawed on ice, weighed and then homogenized in acid extraction buffer (100µl/10mg brain tissue) according to the kit manufacturer's instructions (see ***Appendix 1*** at the end of this Addendum for additional details). One *cOmplete™ Protease Inhibitor Coctail* tablet (Roche, South Africa) was added to 50ml extraction buffer immediately prior to use. The brain tissue and extraction buffer was sonicated in 7-second bursts on ice with a probe sonicator and left to incubate on ice for 30 minutes, where after sonification and ice incubation was repeated. Homogenates were centrifuged for 30

minutes at 11 000 *g* and 4˚C and the supernatants transferred to clean Eppendorf™ tubes. Sample protein content was determined by the Bradford Protein Assay.

1.2.3 Bradford protein assay

The Bradford protein determination assay was carried out (Bradford, 1976) to determine the protein content of each brain homogenate sample in order to express BDNF content in terms of sample protein content according to the BDNF kit manufacturer's instructions.

Principles of the assay

This is a colorimetric assay that makes use of spectrophotometry to analyse the concentration of protein in a sample and is based on a shift in the absorbance of the Coomassie Brilliant Blue-G dye under acidic conditions. When the cationic (unbound) red form of the dye is added to a sample containing soluble protein, the non-polar region of the unbound dye binds non-covalently to hydrophobic components in the protein's tertiary structure. This stabilizes the anionic, blue form of the dye, the amount of which can be determined by measuring the absorbance of the sample. This amount is proportional to the protein concentration of the sample (Bradford, 1976; Ernst and Zor, 2010).

Procedure

Two 96-well plates were needed to accommodate the amount of samples analysed. Bradford Reagent (Sigma Aldrich) was tilted back and forth to ensure contents were mixed. Bovine serum albumin was dissolved in double distilled water and prepared at a concentration of 5mg/ml, whereafter a series of 100µl dilutions were performed to produce a protein standard concentration range between 0.5 and 2 mg/ml.

Brian tissue homogenates were prepared as described above (section *1.2.2*). 100 µl of the samples and standards were added to the 96-well plate in duplicate. Cohorts were divided into subgroups of 3-4 samples, which were distributed across two 96 well plates so that each plate and each vertical and horizontal row on both plates were equally represented by each study cohort. 250µl of Bradford reagent was then added to each well and immediately placed on the plate reader's shaker for 30 seconds. The plate was incubated at room temperature for 30 seconds, where after the plate reader determined the absorbance using a 560nm filter. The average absorbance of the blank was subtracted from the average absorbance of each sample. A standard linear concentration curve was generated from the absorbances of the protein standard. Sample protein content for each plate (A and B) was determined using the linear regression formula generated by the respective standard curves, as depicted in figure B-2.

1.2.4 BDNF ELISA

Striatal BDNF levels were analyzed using a Biosensis® BDNF *Rapid*™ enzyme linked immunoassay (ELISA) Kit obtained from DivBio, Netherlands, capable of measuring mature BDNF and recently demonstrated to be a highly reliable and accurate commercial kit for measuring BDNF in biological samples (Polacchini et al., 2015). This assay kit measures total BDNF in a concentration range of 7.8-500 pg/ml, with the lowest limit of detection at 2 pg/ml.

Principles of the procedure

This kit employs a sandwich ELISA. The term "sandwich" refers to the quantification of the antigen between two layers of antibodies, namely the capture and detection antibody. The procedure is graphically presented in Fig B-1. The 96-well kit plates are pre-coated with a highly specific and sensitive mouse monoclonal anti-BDNF capture antibody. When the sample is added, any antigen (BDNF) present in the sample binds to the capture antibody. The biotinylated anti-BDNF detection antibody is added, which also binds to the antigen, "sandwiching" the BDNF antigen between these two antibodies. Streptavidin binds with very high affinity to biotin. Thus, the addition of horseradish peroxidase (HRP)-conjugated streptavidin (HRP-streptavidin), which acts as the enzyme-linked secondary antibody, results in the streptavidin domain binding with very high affinity to the biotinylated detection antibody. 3,3',5,5'-tetramethylbenzidine (TMB) is a chromogenic substrate which acts as a hydrogen donor for the reduction of hydrogen peroxide to H_2O by peroxidase enzymes. This results in the formation of a blue-coloured diimine. When the TMB substrate is thus added to the well, the horseradish peroxidase mediates a blue reaction product which is directly proportional to the amount of BDNF in the sample. This reaction is halted by the addition of sulfuric acid (the stop agent), causing the TMB to turn yellow. This colour is read in the spectrophotometer at a wavelength of 450 nm as per the Manufacturer's instructions.

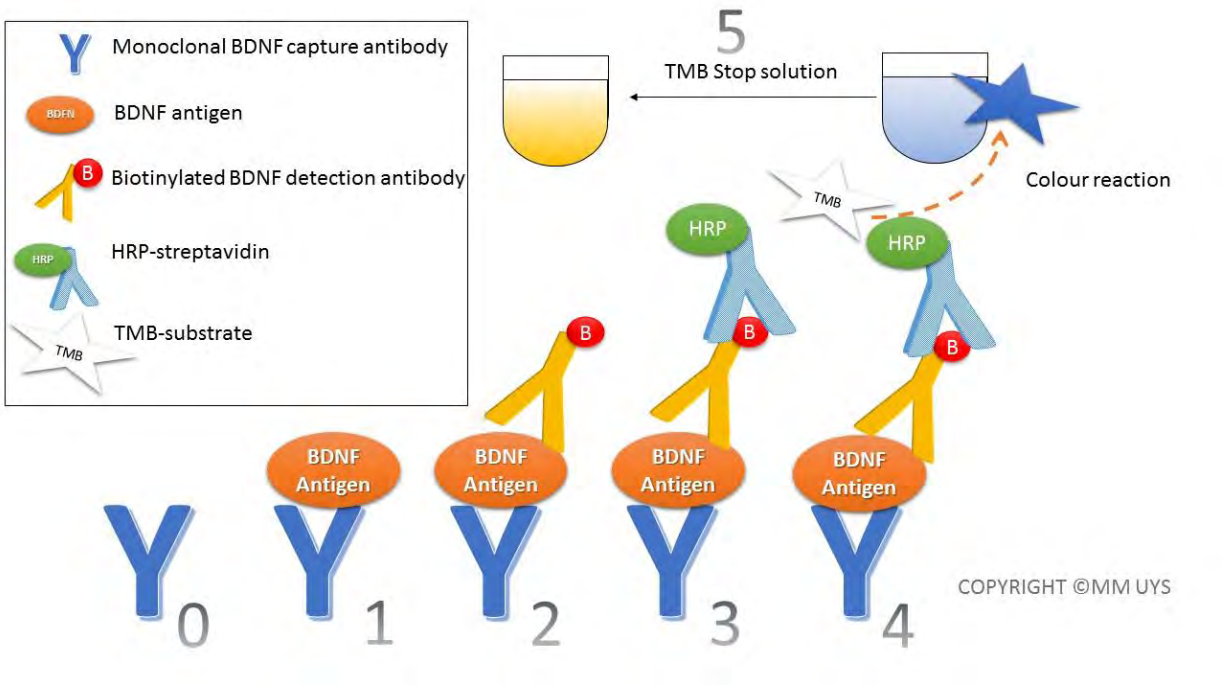


Fig B-1 The priniples of the BDNF sandwich ELISA. Step 0: 96-well plates are purchased pre-coated with monoclonal BDNF capture antibody. Step 1: Sample containing the antigen (BDNF) is added to the wells. Step 2: Biotinylated BDNF detection antibody is added to the well. Step 3: Horseradish peroxidase-streptavidin complex (HRP-streptavidin) is added to the well. Step 4: TMB-substrate is added to the well, which mediates a colour reaction, causing the contents of the well to develop a blue colour. Step 5: After 5 minutes, the colour reaction is stopped with the addition of the TMB stop solution (sulfuric acid). This causes the contents of the well to turn yellow. The colour proportionately represents the BDNF content of the sample. The absorbance is read at 450nm.

Method

After a preliminary run to approximate the concentration range of the sample pool, supernatants (prepared as described in section *1.2.2*) were diluted to dilution factor of 40 with the sample diluent (see ***Appendix 1*** at the end of this Addendum for a complete list of diluent contents and assay reagents). Cohorts were divided into subgroups of 3-4 samples, which were distributed across two 96 well plates so that each plate, and each vertical and horizontal row on both plates were equally represented by each study cohort. All samples and standards were assayed in duplicate. A 100 µl sample was loaded into microplate wells pre-coated with BDNF-antibodies and incubated at room temperature on a plate shaker for 45 minutes at 140 rpm. After 5 washes a biotinylated BDNF detection antibody was added to the wells and incubated for 30 minutes as above. Streptavidin-horse radish peroxidase was added as secondary antibody and the plate incubated again as above for 30 minutes. TMB-substrate was added to visualize antibody reactivity and incubated for 8 minutes in the dark until sufficient colour development. The plate was read in a BioTek SYNERGY/HT microplate reader at 450nM. Unknown sample BDNF concentrations were calculated using a software program capable of generating a four paramater logistic (4PL) curve fit (regression coefficient >0.99) derived from the known reference BDNF

concentrations supplied by the manufacturer and multiplied by the dilution factor. Final BDNF concentrations were expressed as pg/mg protein, according to the kit Manufacturer's instructions.

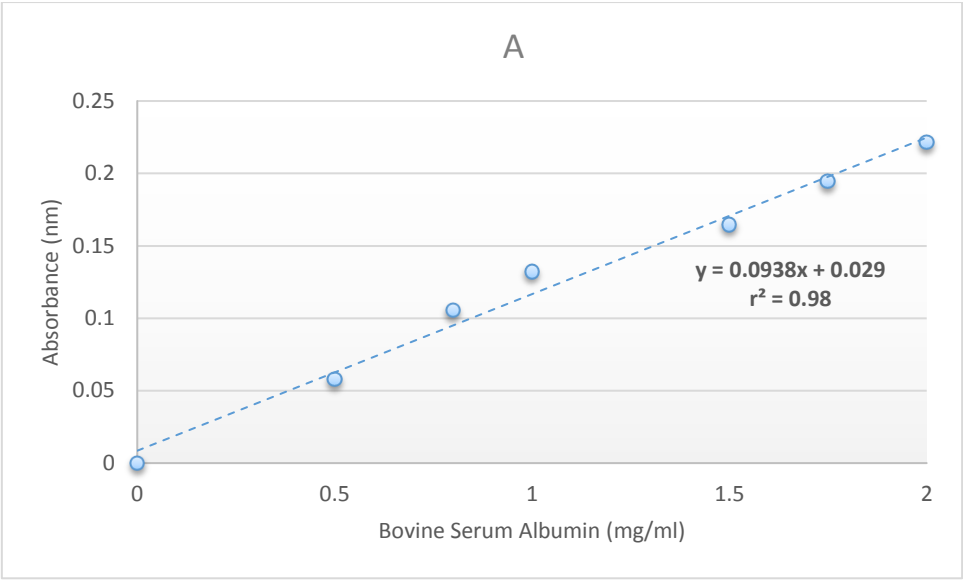
1.2.5 Statistical analysis

Normality of data was determined using the Shapiro Wilk test as motivated in **Addendum A**. To analyze whether SIR animals treated for 14 days with either vehicle, IDAZ, ORM-10921 or CLOZ displayed altered BDNF levels, SIR treatment groups were analyzed with the non-parametric counterparts of ANOVA and Tukey's multiple comparison test, the Kruskal-Wallis ANOVA and Dunn's multiple comparison test, as motivated in **Addendum A**. Effect sizes for non-parametric analyses were calculated to indicate the practical significance (if applicable) of results demonstrating statistical significance on a 10% significance level ($p \leq 0.1$). An effect size of ~0.2 to ~0.49 is considered to be a small effect, ~0.5 to ~0.79 a medium effect showing a trend for practical significance and effect sizes of ~.08 and greater are considered as a large and practically significant effect (Cohen, 1988).

1.3 Results

1.3.1 Generation of standard protein curves

The standard protein concentration curves generated are depicted in Figure B-1. A coefficient of determination (r^2) of >0.95 is required for biological samples (Health Canada and Directorate, 1994; Shabir, 2006).The standard protein concentration curves indicated a linear regression of 0.98 and 0.99 for plates A and B respectively with the formulas as indicated in Fig B-1-A and B-1-B, respectively, used to calculate the protein content of each brain tissue homogenate sample.



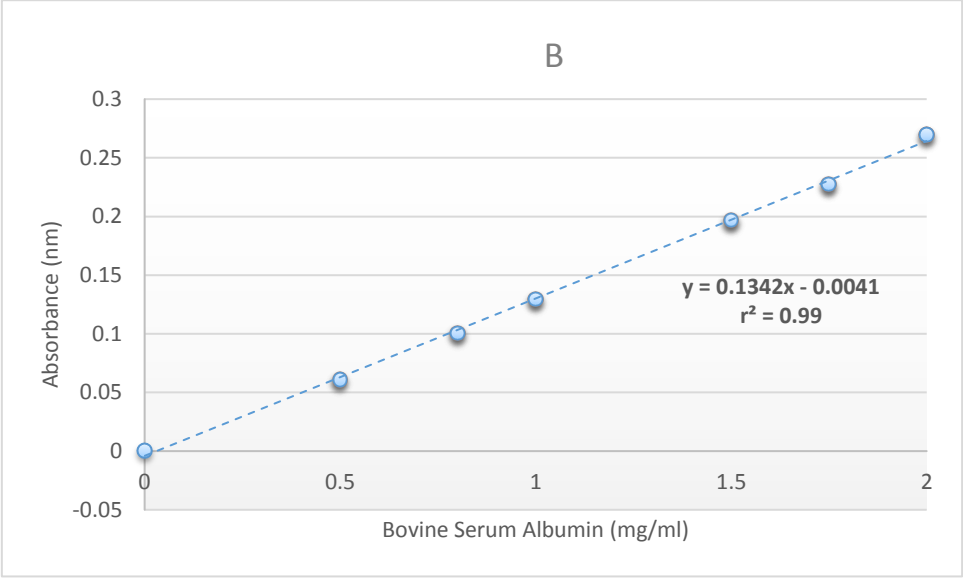
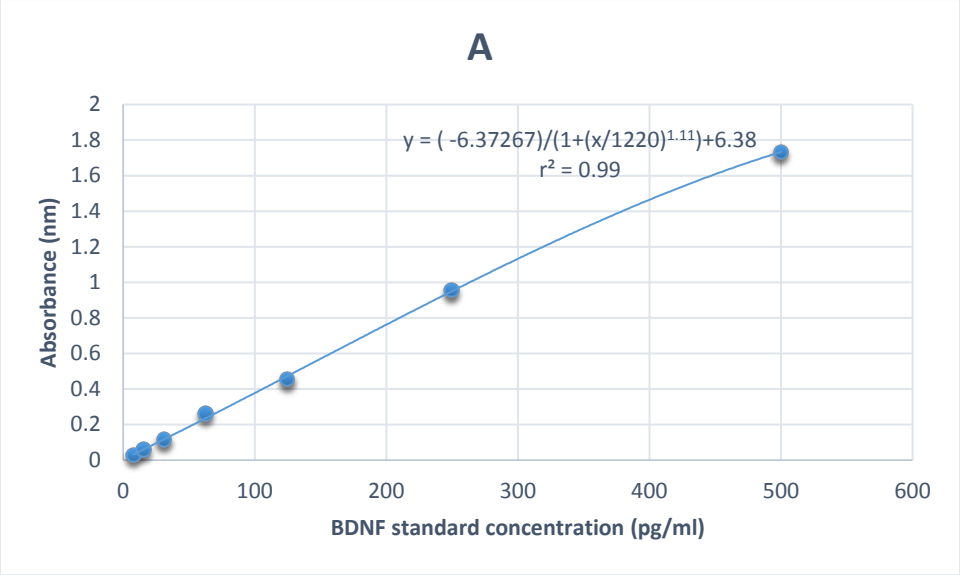


Fig B-2. Standard calibration curves generated for bovine serum albumin to determine striatal sample protein content in SOC and SIR animals in the Bradford Protein assay, depicting A) plate 1 and B) plate two.

1.3.2 Generation of standard BDNF concentration curves

The standard BDNF concentration curves are depicted in Figure B-3. The standard BDNF concentration curves indicated a linear regression of 1 for plates A and B respectively with the formulas as indicated in Fig B-3-A and B-3-B respectively used to calculate the BDNF content of each brain tissue homogenate sample (this was done automatically byt the BioTek software.



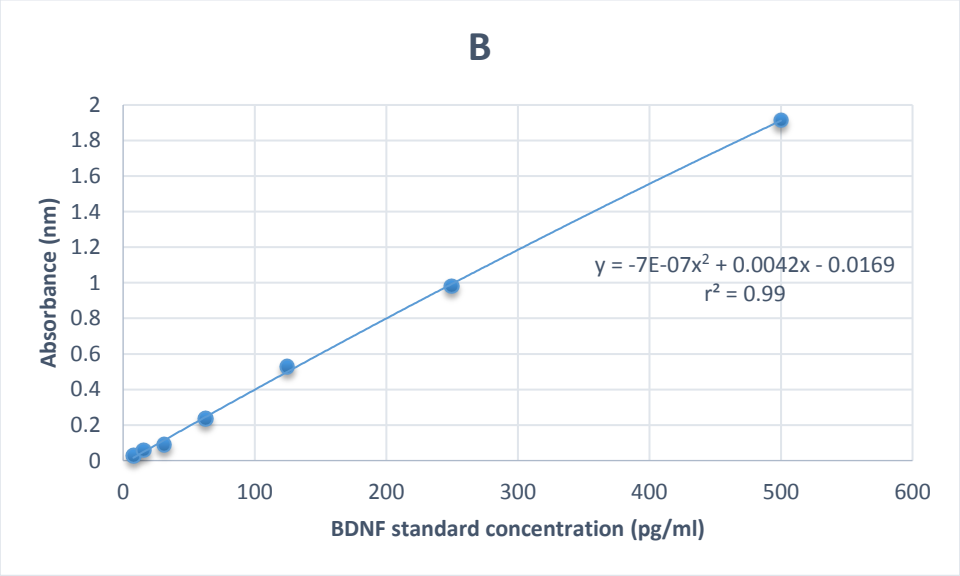


Fig B-3. Standard calibration curves generated for BDNF standards in order to determine sample BDNF concentrations for the ELISA assay, depicting A) plate 1 and B) plate 2.

1.3.3 Striatal BDNF levels

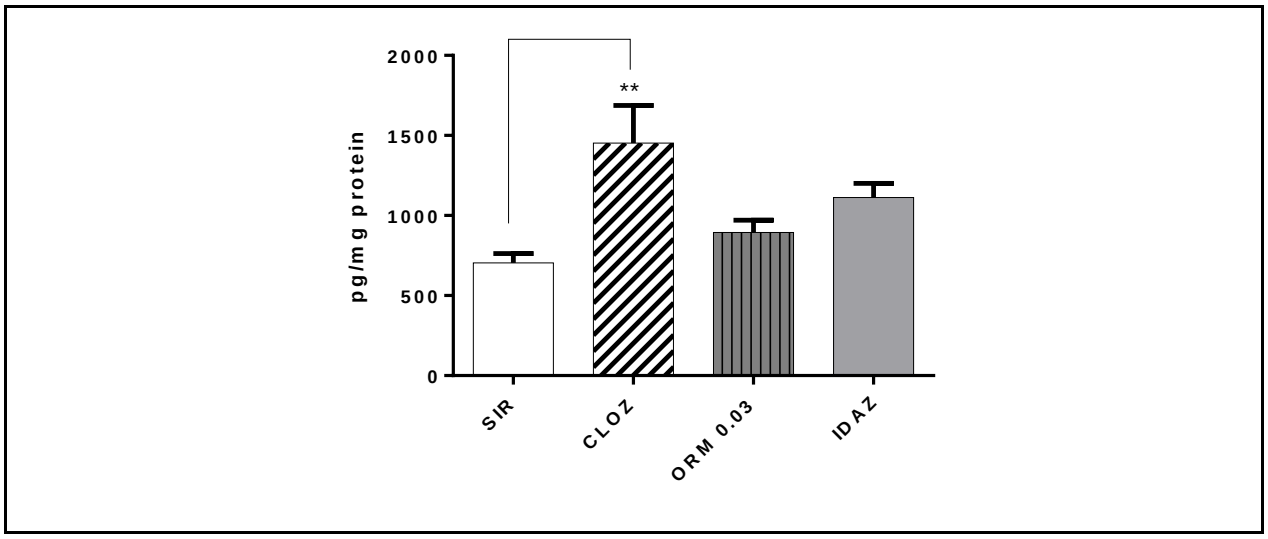


Fig B-4. Striatal total BDNF values as determined by ELISA in SIR animals treated with either vehicle or CLOZ, IDAZ or ORM-10921 0.03 mg/kg (n=7-8), as shown, and reported as pg BDNF per mg protein. SIR= Social isolation reared controls, ORM = SIR+ORM-10921, IDAZ = SIR+idazoxan 3mg/kg, CLOZ = SIR+clozapine 5mg/kg

The effect of 14 day treatment of SIR animals with either vehicle, CLOZ, IDAZ or ORM-10921 0.03mg/kg on striatal BDNF levels is depicted in Fig B-4. Kruskal-Wallis ANOVA indicated significant differences between treatment cohorts (Kruskal-Wallis statistic: 15.47; p=0.0015). Dunn's multiple comparison test indicated that CLOZ treated animals displayed significantly elevated striatal BDNF levels vs. SIR controls (p=0.009), while no alterations in BDNF levels were evident for ORM-10921 (p>0.9) treatment. IDAZ treatment did not increase BDNF levels significantly on a 5% significance level (p=0.06), while the effect size (0.7) indicates a medium effect which is not correlated strongly to practical significance (Cohen, 1988).

1.4 Discussion

1.4.1 Validation of construct validity

Schizophrenia is a neurodevelopmental illness characterized by altered neurotransmission and its development can be strongly influenced by environmental factors (Reynolds, 2008; van Os et al., 2010), such as developmental trauma (e.g. childhood neglect and abuse), prenatal maternal stress and infections, maternal rhesus incompatibility, pregnancy and birth complications, growing up in an urban environment (urbanicity) and being part of a migrant minority (van Os et al., 2010). BDNF is involved in the development of the nervous system and in neurotransmission, and its expression can be altered by

noxious or beneficial environmental stimuli. The expression of BDNF is therefore thought to be an important factor in the underlying pathology of schizophrenia (Autry and Monteggia, 2012a).

Many animal models of schizophrenia point to alterations in BDNF expression (Autry and Monteggia, 2012a), while animals subjected to extended social isolation later in life present with altered neurogenesis, altered neurotrophic activity and decreased limbic BDNF levels (Fone and Porkess, 2008; Meng et al., 2011). BDNF is essential in processes underlying synaptic plasticity, cognitive performance, learning, memory and mood (Autry and Monteggia, 2012a; Lu et al., 2014). Apart from the role of the striatum in classic psychotic-like positive symptoms of schizophrenia, deficits in striatal function have also been proposed to play a role in the pathogenesis of cognitive symptoms of the disorder (Simpson et al., 2010). Reduced striatal volume has been observed in treatment naive schizophrenics, while studies in D_2-overexpressing animals also demonstrate decreased striatal volume (Simpson et al., 2010). Also important to note is that typical (e.g. haloperidol) and atypical (e.g. CLOZ) antipsychotics have different fingerprints with regard to how they affect the integrity of cortico-limbic structures (Buckley et al., 2007a). Thus, chronic haloperidol demonstrates neurodegenerative effects which are associated with long-term block of striatal D_2 receptors (Angelucci et al., 2005; Angelucci et al., 2000; Pillai et al., 2006) while atypical antipsychotics like olanzapine and CLOZ has shown the opposite (Maeda et al., 2007; Parikh et al., 2004). The latter drugs also reverse haloperidol-induced reduction in limbic BDNF (Pillai et al., 2006), although there are various inconsistencies in the literature (Buckley et al., 2007a). That CLOZ, but not haloperidol, has pronounced antagonistic actions at the α_{2C} AR (Kalkman and Loetscher, 2003) might be involved in these different effects on neurogenesis.

Our finding that SIR animals presented with significantly decreased striatal BDNF levels vs. SOC animals (**Manuscript A**) is in line with previous studies reporting decreased BDNF levels in limbic structures of SIR rats (Djouma et al., 2006; Scaccianoce et al., 2006). However, while we demonstrate decreased striatal BDNF levels in **Manuscript A**, the latter authors reported unaltered striatal BDNF levels in SIR rats (Scaccianoce et al., 2006). Differences in study protocol may explain such discrepancies. While our socially housed rats were housed in groups of 4, Scaccianoce and colleagues housed their social groups in pairs of 2. Although we are not aware of literature comparing isolation rearing to different group sizes of socially housed controls, the difference in group dynamics in rodents reared in larger social groups might be a factor influencing development. Although findings in the literature regarding striatal BDNF levels in schizophrenia is mixed(Buckley et al., 2007a), our findings are in line with striatal volume reduction observed in humans and animal models (Simpson et al., 2010) and concur with the hypothesis of insufficient neurotrophic support in schizophrenia (Nieto et al., 2013).

The atypical antipsychotic, CLOZ, reversed the decreased striatal BDNF levels evident in SIR rodents to the level of SOC housed rodents, while IDAZ did not exert this effect. These observations strengthen the

predictive validity of our findings, since CLOZ has been reported to increase serum BDNF levels in humans (Grillo et al., 2007) and to reverse typical antipsychotic-induced decreases in striatal BDNF (Pillai et al., 2006). Importantly, the decrease in striatal BDNF in SIR animals correlates with the sensorimotor gating deficits and recognition memory deficits in SIR animals (**Manuscript A** and **Addendum A**), while both these parameters were also improved by CLOZ treatment but not by IDAZ. That CLOZ, but not IDAZ, restored striatal BDNF levels in SIR animals to that of socially reared animals provides the necessary predictive validity for this model in order to assess the bio-behavioural effects of ORM-10921.

IDAZ tended to increase BDNF levels on a medium practical level, albeit not statistically significant. Although the increase in NA levels expected to follow IDAZ treatment would theoretically be expected to increase BDNF levels, postsynaptic α_2 antagonism may negate such effects (Chen et al., 2007). Another α_2 antagonist, dexefaroxan, has been shown to increase limbic BDNF and neurogenesis after 28 days of treatment (Rizk et al., 2005), suggesting that duration of treatment may also have impacted these findings.

1.4.2 Effects of ORM-10921 on BDNF compared to CLOZ and IDAZ

Contrary to what was expected from the beneficial effects of ORM-10921 0.03mg/kg on sensorimotor gating and cognition (**Addendum A**), this dose of ORM-10921 did not alter reduced striatal BDNF levels in SIR animals. In **Manuscript A**, we report the effects of a lower dose of ORM-10921 on striatal BDNF levels, which was also left unaltered by the drug. However, augmentation of haloperidol with low dose ORM-10921 (see **Manuscript A**) significantly increased the striatal BDNF levels vs. SIR controls.

Both IDAZ and CLOZ appear to be more effective at increasing BDNF levels in the striatum than ORM-10921, although this was not significant. In addition to its action at 5-HT-receptors, CLOZ also blocks D_2 receptors at a receptor occupancy of 25-30% at the dose tested (Natesan et al., 2007, 2008), and was able to reverse reduced striatal BDNF levels in SIR rats. Considering that **Manuscript A** reports that ORM-10921 is able to enhance striatal BDNF levels only when haloperidol is added to therapy, the argument can therefore be made that combined α_{2C}/D_2 blocking activity (as also seen in the mechanism of action of CLOZ) impacts striatal neurotrophin expression levels, but not α_{2C} antagonism or D_2-antagonism alone (**Manuscript A**).

However, this study has its limitations, and the results should be interpreted with the necessary scientific caution. Although CLOZ increased striatal BDNF levels in this specific study, drug induced neurotrophic alterations may follow a much more protracted time-line (Andreassen et al., 1998; Pillai et al., 2006), as noted above with regard to α_2 antagonist effects on BDNF (Rizk et al., 2005). Pillai and co-workers noticed a progressively growing effect of antipsychotics on striatal and hippocampal BDNF levels after 90 and 180 days of treatment. It might therefore be that while combined α_{2C} /D_2 antagonism

exerts early effects on BDNF expression in the striatum, both IDAZ and ORM-10921 may (or may not) only affect neurotrophic factors over a more extended timeline. Furthermore, drugs that affect BDNF levels might initially first alter cAMP response element binding protein (CREB), a transcription factor involved in the expression of BDNF (Vinet et al., 2004), before alterations in BDNF become evident. It would thus be prudent to assess such transcription factors, as well as the BDNF TrkB receptor in future studies, in order to gather a better perspective on how ORM-10921 may affect the many factors determining neurotrophic activity. Another important difference with respect to the findings described in **Manuscript A**, is that in Manuscript A CLOZ narrowly missed significance in increasing BDNF following SIR (p=0.06), yet here we report a marked effect of CLOZ on BDNF levels (p=0.009). This could be attributed to the analysis of a smaller number of cohorts in this Addendum (with different means and standard deviations) compared to those reported in **Manuscript A**, since reducing the amount of groups in a multiple comparison increases the power of the Tukey test to detect statistical differences (Ruxton and Beauchamp, 2008). Furthermore, the effect size for CLOZ vs. SIR controls is 0.8, indicating a practical significant effect of CLOZ on striatal BDNF levels applicable here and in **Manuscript A**.

2. The effects of ORM-10921 on striatal monoamines

The striatum is a focal point engendering dysfunctional monoamine transmission in schizophrenia, effects which are causally and functionally interrelated to hippocampal and frontal cortical alterations in neurotransmission (Reynolds, 2008) (see Figure 4 and 5 in Chapter 2, section *2.3.1.2.1*). Although the striatum is mainly innervated by dopaminergic neurons, both serotonergic and noradrenergic effects play vital roles in this region (Carlsson et al., 2001; Nicola and Malenka, 1998). The dopamine (DA) hypothesis describes hyperdopaminergic and hypodopaminergic function in the striatum and cortex, respectively, mediating the positive, negative and cognitive deficits of the disorder (Meyer-Lindenberg et al., 2002; Millan et al., 2015). Excessive striatal DA has been linked to psychotic symptoms and to ineffective sensorimotor gating leading to positive symptoms (Howes and Kapur, 2009) (Swerdlow et al., 2001; Zhang et al., 2000), but also indirectly to cognitive dysfunction (Simpson et al., 2010). Transgenic studies suggest a mediatory role for α_{2C}-AR deactivation in cortico-striatal DA metabolism (Sallinen et al., 1997). The serotonin (5-HT) hypothesis of schizophrenia developed from the observation that hallucinogens like lysergic acid diethylamide (LSD) and psilobycin are agonists of the 5-HT_{2A} receptor (Vollenweider and Geyer, 2001) and has gained circumstantial support due to the efficacy of atypical antipsychotics that have prominent antagonist activity at this receptor, to ameliorate the negative symptoms of schizophrenia (Reynolds, 2004). Functional deficits of DA release during development may ultimately lead to serotonergic afferent hyperinnervation of the striatum accompanied by

supersensitivity of 5-HT_{2A} and 5-HT_{2C} receptors and enhanced release of striatal 5-HT later in adulthood (Jackson and Abercrombie, 1992). The interrelated effects of DA and 5-HT in this brain region are thus evident. Lastly, a hypothesis for noradrenaline (NA) dysfunction in schizophrenia has also been proposed (Yamamoto and Hornykiewicz, 2004), while preclinical studies have supported the role of drugs that increase synaptic NA levels (such as NA reuptake inhibitors and α_2-AR-antagonists) in enhancing the effects of antipsychotic drugs (Marcus et al., 2010a; Marcus et al., 2005; Marcus et al., 2010b). Furthermore, the role of α_2-AR-antagonism has been highlighted as basis for the improved efficacy of atypical antipsychotics (Svensson, 2003), with a specific role of α_{2C}-AR antagonism (Kalkman and Loetscher, 2003). The α_{2C}-AR is most densely expressed in the striatum, and thus we investigated the effects of antagonism of this receptor on tissue levels and metabolism of NA, DA and 5-HT in SIR rats. These data would aid in delineating the mechanisms by which α_{2C}-AR-antagonism with ORM-10921 exerts its antipsychotic-like and pro-cognitive effects, as described in **Addendum A** and **Manuscript A** and in the literature (Sallinen et al., 2013a)**.** The dose of 0.03 mg/kg ORM-10921 was selected for this study as this was the most effective dose in the initial dose-response study reported in **Addendum A**.

2.1 Aims

1. To determine construct validity of the SIR animal model by assessing striatal monoamine levels vs. SOC controls

2. To determine the effects of drug treatment with ORM-10921, CLOZ and IDAZ on striatal levels of DA, NA and 5-HT and their metabolites, DOPAC, HVA, MHPG and 5-HIAA.

2.2 Methods

2.2.1 Animals and drug treatment

SIR animals were reared as described in Addendum A, and treated with ORM-10921 0.03 mg/kg, CLOZ 5mg/kg or IDAZ 3mg/kg for 14 days. Drugs were prepared and administered as described in **Addendum A**.

2.2.2 Brain homogenate preparation

24 hours after the last drug administration, animals were sacrificed by decapitation and the whole brain quickly removed and briefly placed in ice cold double distilled water, with the striata dissected out immediately on an ice cooled slab. The frontal cortex was dissected out, whereafter the brain was placed with the dorsal side facing upward. The two cerebral hemispheres were split and the striata dissected out by applying the external walls of the lateral ventricles as internal boundaries and the corpus callosum as external boundary, as described previously (Möller et al., 2013a; Möller et al., 2013b). The striata were snap-frozen in liquid nitrogen and transferred to a -70^{o}C freezer. On the day of analysis, brain tissue was thawed on ice, weighed and sonicated in 1 ml of a pre-prepared homogenization

solution (refer to ***Appendix 1*** at the end of this addendum for details), whereafter it was left on ice for 20 minutes to complete perchlorate precipitation of protein and extraction of monoamines. Samples were then centrifuged for 20 min at 4^{o}C at 24 000*g*. The supernatant was withdrawn and pipetted in to an Eppendorf™ tube. The pH of the supernatant was adjusted to pH 5 with the addition of 1 drop/ml of 10 M potassium acetate. 200µl of this tissue extract was withdrawn into a new tube, and 20µl of the internal standard, isoprenaline, added to the sample. The sample was vortexed and centrifuged for another 5 minutes at 21 000*g*. 200µl of the resulting supernatant was pipetted into a 300µl glass insert for the HPLC system. The HPLC-ECD system injects 20µl onto the HPLC column. This original method was developed and validated in our laboratory (Basson et al., 1988) and has since undergone refinement and improvement as described in subsequent studies (Harvey et al., 2006; Möller et al., 2013a; Möller et al., 2013b).

2.2.3 Striatal Monoamine analyses

Striatal levels of DA and its metabolites homovanillic acid (HVA), 3,4-dihydroxyphenylacetic acid, NA and its metabolite, MHPG and 5-HT and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA), were determined by high performance liquid chromatography (HPLC) with electrochemical detection (HPLC-ECD) (see validation and set up described in Addendum F). The analytical instrument was an Agilent 1200 series HPLC, equipped with an isocratic pump and autosampler coupled to an ESA Coulochem II electrochemical detector with a coulometric flow cell and Chromeleon® Chromatography Management System (version 6.8). The constituents of the mobile phase as well as the full validated method are described in ***Appendix 1*** at the end of this addendum. The pH of the mobile phase was adjusted to approximately 3.85 with orthophosphoric acid (85%) and the flow rate set at 0.5 ml/min. The monoamine concentration of each analyte was determined by dividing the area under the peak for each monoamine by the area under the peak of the internal standard and then calculating the respective monoamine concentrations via linear regression according to the standard concentration curves generated for each monoamine standard (regression coefficient >0.98). Monoamine turnover rates are expressed as the ratio of MHPG (ng/g) ÷ NA (ng/g), 5-HIAA (ng/g) ÷ 5-HT (ng/g), and the ratio of (HVA+DOPAC ng/g) ÷ DA (ng/g).

2.2.4 Statistical Analyses

Normality of data was determined using the Shapiro Wilk test, as motivated elsewhere in this addendum (section *1.2.5*). Striatal monoamine levels and turnover rates between SOC and SIR rats were analysed with unpaired student's t-test, or where Shapiro Wilk indicated non-parametric data, the Mann-Whitney U test was employed. Effects of 14 day treatment with either vehicle, ORM-10921, IDAZ or CLOZ in SIR animals was determined using one-way ANOVA or Tukey's multiple comparison test, as motivated in section *1.2.5* of this addendum. Where the assumption for ANOVA of equal variances was

not met (as indicated by the Brown-Forsythe test for unequal variances) or where Shapiro-Wilk's normality test indicated non-guassian distribution of data, ANOVA and Tukey's non-parametric counterparts - the Kruskal-Wallis ANOVA and Dunn's multiple comparison test were employed. Significance was set at a 5% level ($p<0.05$). Effect sizes were calculated to indicate the practical significance (if applicable) of results demonstrating statistical significance on a 10% significance level ($p\leq0.1$). An effect size of ~0.2 to ~0.49 is considered to be a small effect, ~0.5 to ~0.79 a medium effect showing a trend for practical significance and effect sizes of ~.08 and greater are considered as a large and practically significant effect (Cohen, 1988).

2.3. Results

2.3.1 Validation of SIR vs. SOC

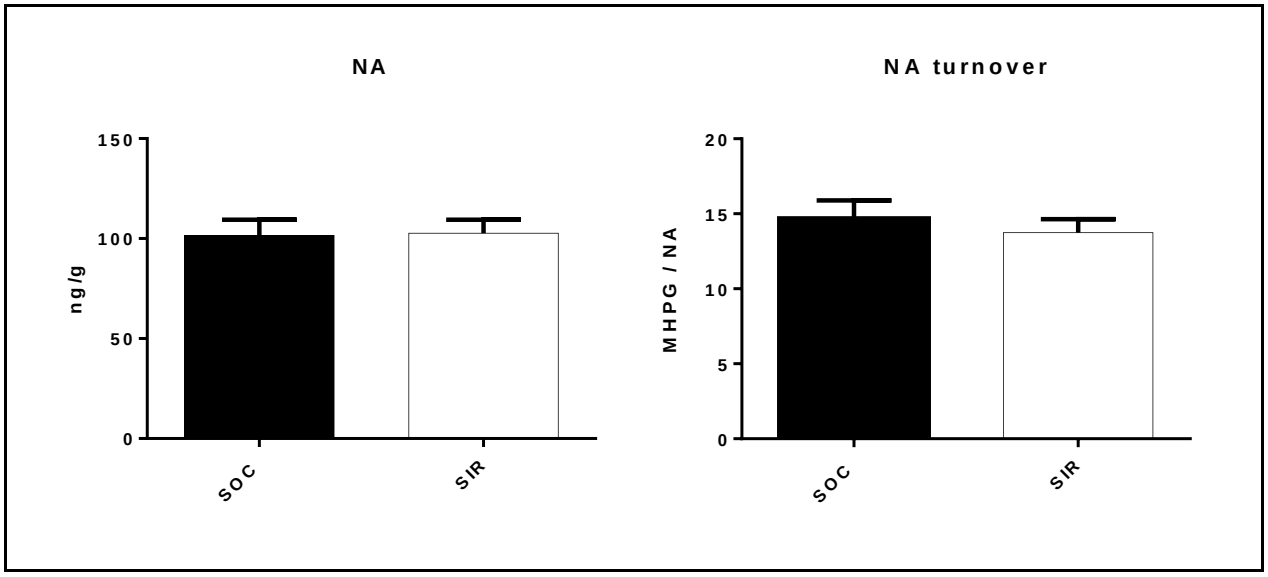


Fig B-5. Striatal NA levels and NA turnover in SOC vs. SIR rats (n=8-10). SOC = socially reared controls, SIR = Social isolation reared controls.

Fig B-5 to Fig B-7 depicts the striatal NA, DA and 5-HT concentrations and turnover rates in SOC vs. SIR rats. Unpaired t-tests indicated no differences between SOC vs. SIR animals for any of the monoamines (NA, p=0.9; DA, p=0.8; 5-HT, p=0.5) or their respective turnover rates (NA turnover, p=0.5; DA turnover, p=0.4, 5-HT turnover, p=0.5).

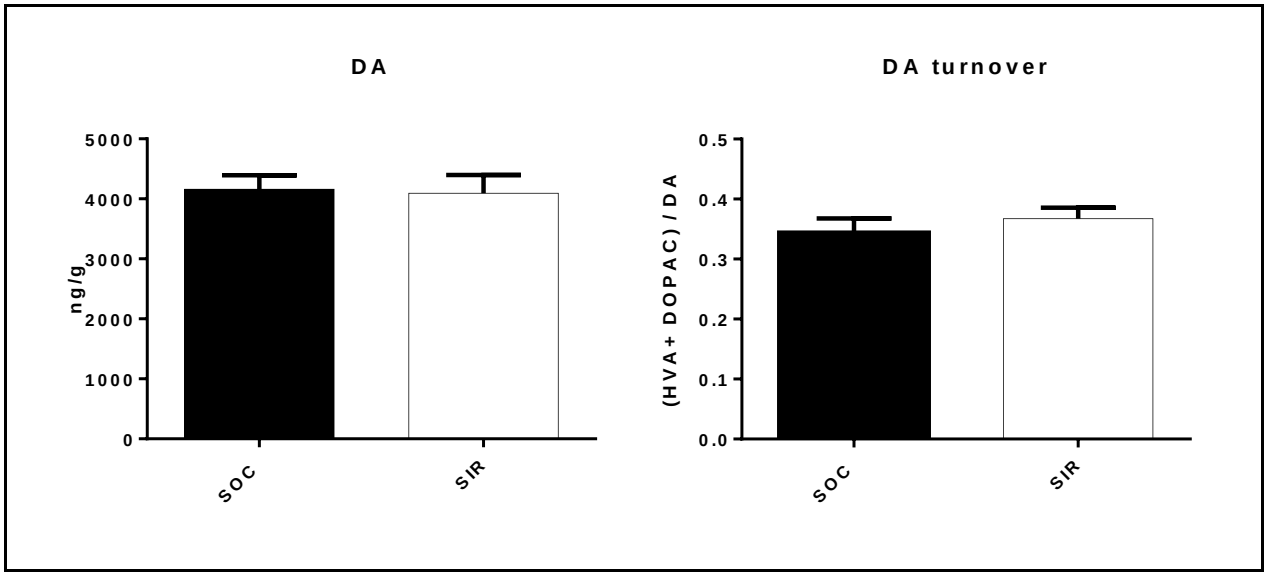


Fig B-6. Striatal DA levels and DA turnover in SOC vs. SIR rats (n=10). SOC = socially reared controls, SIR = Social isolation reared controls.

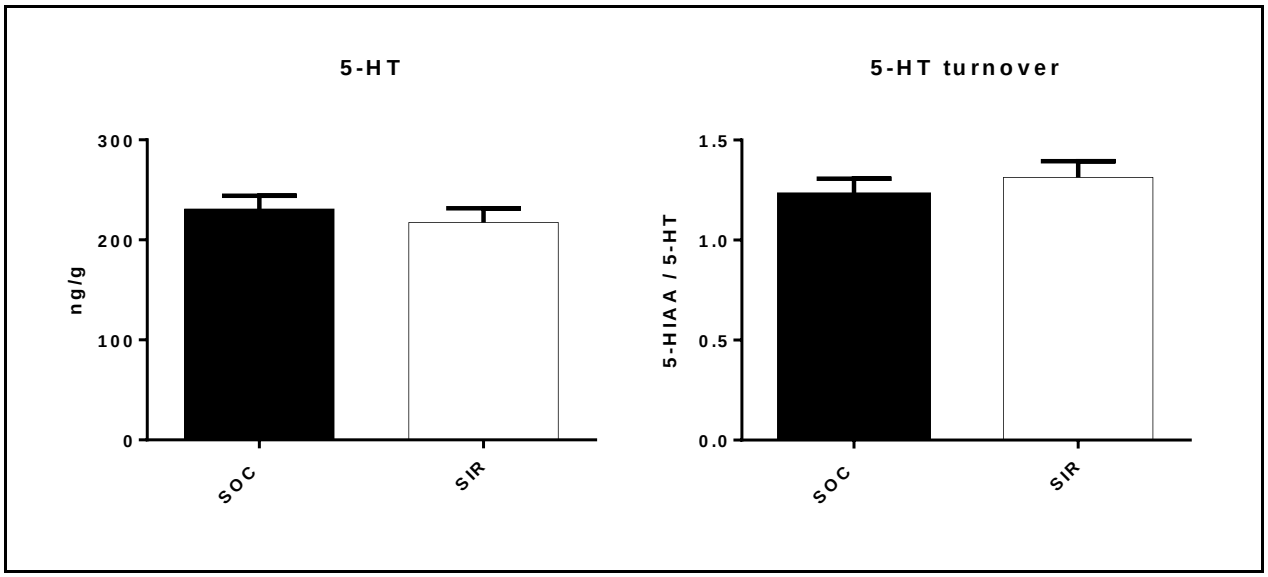


Fig B-7. Striatal 5-HT levels and 5-HT turnover in SOC vs. SIR rats (n=10). SOC = socially reared controls, SIR = Social isolation reared controls.

2.3.2 Effects of drug treatment on striatal monoamine levels and turnover.

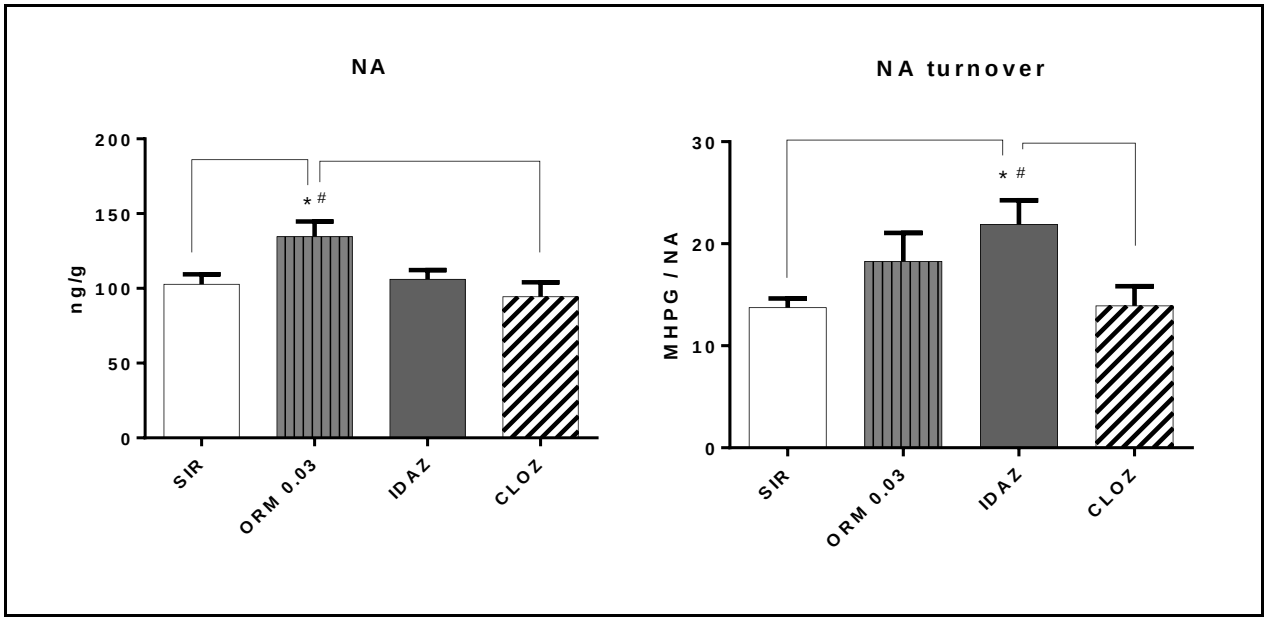


Fig B-8. Striatal NA levels and NA turnover rates in SIR animals treated with vehicle, ORM-10921 0.03mg/kg, IDAZ or CLOZ. *p<0.05 vs. SIR controls, #p<0.05 vs. CLOZ (n=9-11). SIR = SIR controls, ORM = SIR+ORM-10921 0.03mg/kg, IDAZ = SIR+idazoxan 3mg/kg, CLOZ = SIR+clozapine 5mg/kg.

Effects of drug treatment on striatal NA levels and NA turnover rates in SIR animals are depicted in Fig B-8. ANOVA indicated significant effects of drug treatment on striatal NA levels $F(3,38) = 3.86$, $p=0.01$) and NA turnover rates ($F(3,38) = 4.372$, $p=0.001$). Tukey's post hoc test indicated that treatment with ORM-10921 significantly increased striatal NA levels vs. SIR controls ($p=0.04$) and vs. CLOZ treated SIR animals ($p=0.005$). While neither ORM-10921 ($p=0.15$) nor CLOZ ($p=0.1$) significantly affected NA turnover in SIR animals vs. SIR controls, IDAZ significantly increased NA turnover vs. SIR controls ($p=0.03$) and vs. CLOZ ($p=0.04$).

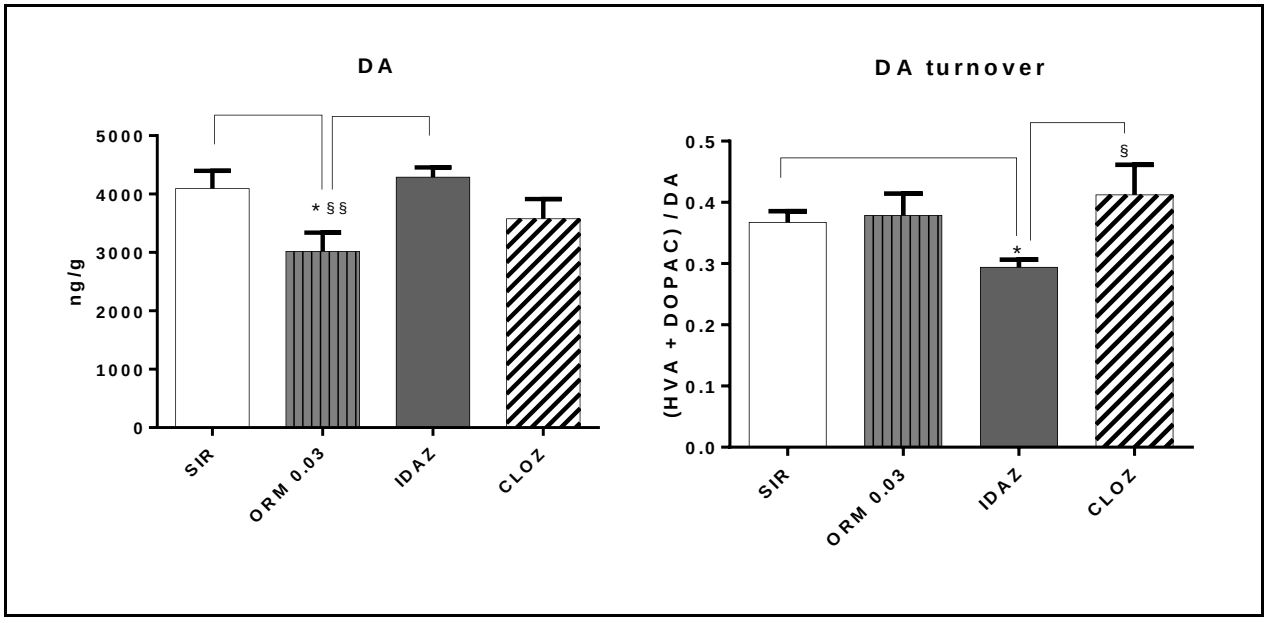


Fig B-9. Striatal DA levels and DA turnover rates in SIR animals treated with vehicle, ORM-10921 0.03mg/kg, IDAZ or CLOZ. *p<0.05 vs. SIR controls, §p< 0.05 and §§p<0.01 vs. IDAZ (n=9-11). SIR = SIR controls, ORM = SIR+ORM-10921 0.03mg/kg, IDAZ = SIR+idazoxan 3mg/kg, CLOZ = SIR+clozapine 5mg/kg.

Effects of drug treatment on striatal DA levels and DA turnover rates in SIR animals are depicted in Fig B-9. ANOVA indicated significant effects of drug treatment on striatal DA levels $F(3,36)=5.86$, $p=0.0024$) while Kruskal-Wallis ANOVA also indicated significant effects of drug treatment on DA turnover rates (Kruskal-Wallis statistic=10.13, $p=0.0175$). Tukey's post hoc test indicated that treatment with ORM-10921 significantly decreased striatal DA levels vs. SIR controls ($p=0.04$) and vs. IDAZ treated animals ($p=0.013$), while CLOZ ($p=0.6$) and IDAZ (0.9) did not. Dunn's multiple comparison test indicated that IDAZ significantly decreased DA turnover vs. SIR controls ($p=0.04$) and vs. CLOZ treatment ($p=0.04$). Neither ORM-10921 ($p=0.15$) nor CLOZ ($p=0.1$) significantly affected DA turnover in SIR animals vs. SIR controls, although IDAZ significantly increased NA turnover vs. SIR controls ($p=0.03$) and vs. CLOZ ($p=0.04$).

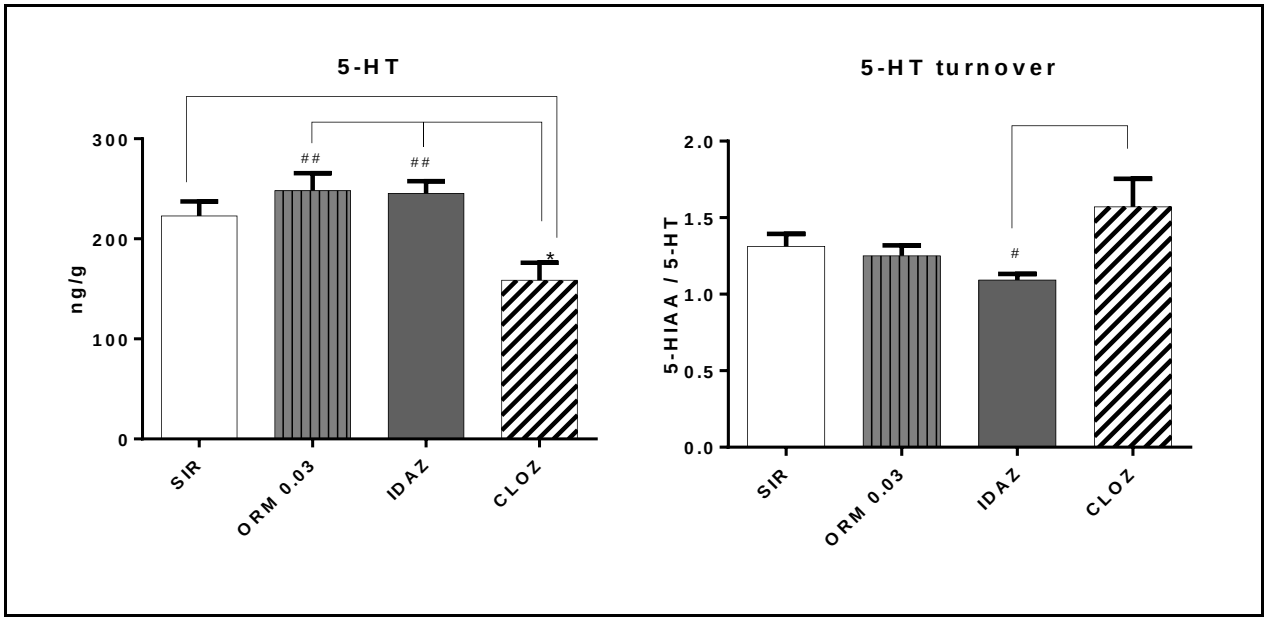


Fig B-10. Striatal 5-HT levels and 5-HIAA turnover rates in SIR animals treated with vehicle, ORM-10921 0.03mg/kg, IDAZ or CLOZ. *p<0.05 vs. SIR controls, #p<0.05 and ##p<0.01 vs. CLOZ (n=9-11). SIR = SIR controls, ORM = SIR+ORM-10921 0.03mg/kg, IDAZ = SIR+idazoxan 3mg/kg, CLOZ = SIR+clozapine 5mg/kg.

Effects of drug treatment on striatal 5-HT levels and 5-HT turnover rates in SIR animals are depicted in Fig B-10. ANOVA indicated significant effects of drug treatment on striatal 5-HT levels ($F(3,38) = 7.008$, $p=0.0006$) while Kruskal-Wallis ANOVA also indicated significant effects of drug treatment on 5-HT turnover rates (Kruskal-Wallis statistic= 8.839, $p=0.03$). Tukey's post hoc test indicated that treatment with CLOZ significantly decreased striatal 5-HT levels vs. SIR controls ($p=0.03$) and vs. IDAZ (0.001) and ORM-10921 ($p=0.001$) treated animals. Dunn's multiple comparison test indicated that treatment with CLOZ significantly increased striatal 5-HT turnover vs. IDAZ treated animals ($p=0.02$). No drug treatment altered 5-HT turnover significantly vs. SIR controls.

2.4. Discussion

2.4.1. Validation of SOC vs. SIR

The monoamine results obtained for SOC vs. SIR animals demonstrated no differences between socially reared and isolation reared animals. This is not in line with the expected outcomes, since differences in striatal monoamines have previously been reported in our laboratory (Möller et al., 2013a; Möller et al., 2013b) as well as in the literature (reviewed in Fone and Porkess, 2008). Furthermore, in this study SIR rats displayed marked behavioural deficits compared to SOC controls (**Addendum A**), thus expectant of at least some changes in striatal monoamine values. Although findings regarding monoaminergic alterations in SIR rats are mixed and sometimes contradictory (Fone and Porkess, 2008), the overall picture points towards enhanced striatal dopaminergic activity (Fone and Porkess, 2008; Fulford and Marsden, 1998), decreased 5-HT turnover (Heidbreder et al., 2000) and increased 5-HT release (Fone

and Porkess, 2008), findings that are similar to that described in schizophrenia (Reynolds, 2008). Additionally, SIR-induced enhanced presynaptic α_2 AR function (Fulford et al., 1994) could lead to decreased striatal NA, although increased striatal NA has also been reported (Möller et al., 2013b). Previously in this laboratory, we have previously reported increased striatal DA and DA metabolism, increased striatal 5-HT and 5-HIAA and increased striatal NA and MHPG levels in SIR vs. SOC controls (Möller et al., 2013b). Thus, although findings in the literature are mixed, overall SIR is widely expected to induce alterations in monoamine levels vs. SOC animals, especially in the striatum, which was not observed in this study.

We believe that this disparity might be related to renovations to our animal research centre. Although these renovations were completed 6 months prior to the study, a number of unforeseen problems developed after the completion date. During this period, animals were exposed to an unexpected level of movement, noise and disturbances in their daily routine. Although the animals used in this study were born 6 months after the major renovations were completed, the parents of these animals bore the brunt of these environmental stressors that could have had major effects in utero, even though attempts were made to attenuate its impact on the animals. The pups born to these stress-exposed parents could thus have been victim of a generational effect of stress (Champagne and Meaney, 2006, 2007). Both the enduring effects of maternal stress (Champagne and Meaney, 2006, 2007) and the long-lasting effects of prenatal stress on the unborn pups (Boersma and Tamashiro, 2015; Weinstock, 2008) could have significantly affected brain neurochemistry in the animals examined in this study and compromised its later response to SIR. Moreover, these events could specifically have compromised the "stress free" environment that should be maintained for group housed rats. Pups born to dams who show altered postpartum maternal care present with marked anxiety-like behaviour (Champagne and Meaney, 2006), which ultimately will also affect levels of 5-HT, DA and NA in a stress-related manner (Marrocco et al., 2012; Pittaluga et al., 2001; Stahl, 2007). These effects might explain why socially reared animals did not show altered monoamine levels from that of SIR animals. Although building renovations seemed to not have affected behavioural differences between SOC and SIR animals in this study (**Addendum A** and **Manuscript A**), when these data are compared to that of previous studies (Möller et al., 2011; Möller et al., 2013a) it is apparent that the behavioural differences between SOC and SIR animals were indeed less pronounced. Nevertheless, while behavioural alterations between SOC and SIR animals was statistically very significant (**Addendum A**), that we were not successful in correlating these behavioural differences to the expected alterations in brain monoamines prevented verification of construct validity for the model and interpretation of the findings within the SIR context. So while drug treatment on SIR animals presented with marked behavioural effects (**Addendum A** and **Manuscript A**) and effects on striatal monoamines vs. SIR control animals, these findings are not supported by proof of construct validity in this particular study. That being said, these effects will be

discussed below in terms of expected findings for the various drug treatments and for SIR animals in the literature (based on proof of altered behaviour as reported in Addendum A and Manuscript A), as described above.

2.4.2 Effects of ORM-10921, CLOZ and IDAZ on striatal monoamine levels

SIR rats demonstrate enhanced presynaptic α_2-AR function (Fulford et al., 1994), which would result in increased noradrenergic negative feedback and thus reduced synaptic NA levels. This could explain why α_2 antagonism has become an important mechanism of action underlying the efficacy of atypical antipsychotics (Svensson, 2003). Furthermore, SIR demonstrates increased striatal DA levels akin to findings in human schizophrenics (Fone and Porkess, 2008) as well as increased striatal 5-HT levels (Fone and Porkess, 2008; Heidbreder et al., 2000; Möller et al., 2013b). These findings demonstrate marked correlations with the human disorder (Reynolds, 2008).

So while SOC vs. SIR comparisons were uninformative, comparisons between vehicle and drug-treated SIR animals did provide some interesting observations. Using a dose of ORM-10921 that was effective in reversing sensorimotor gating deficits and object recognition memory deficits in the same SIR rats (**Addendum A**), the main findings of this study were that ORM-10921 presented with significantly increased striatal NA levels, while also decreasing striatal DA levels significantly compared to SIR controls. No effect on 5-HT or its metabolism was observed for ORM-10921.

Effects on striatal noradrenaline and noradrenaline turnover

ORM-10921 increased striatal NA levels vs. SIR controls and vs. CLOZ-treated SIR animals, while neither CLOZ nor IDAZ altered striatal NA levels. Schizophrenic patients seem to present with decreased levels of central NA (Risch, 1996), while drugs that increase central NA appear to improve the negative symptoms of schizophrenia (Yamamoto and Hornykiewicz, 2004). The α_{2C} AR inhibits NA release at low endogenous levels of NA, as opposed to the α_{2A}-AR which inhibits NA release at high endogenous levels of NA (Bücheler et al., 2002; Hein et al., 1999). The potency and affinity of NA is also higher at the α_{2C}-AR. Low endogenous NA levels in SIR animals (animals that also present with heightened α_2 autoreceptor functioning) would thus theoretically favour α_{2C}-mediated inhibition of NA release, further decreasing endogenous NA. This could explain why α_{2C} antagonism with ORM-10921 demonstrated a marked increase in striatal NA. Both IDAZ and CLOZ have much lower α_{2C}-AR antagonist activity compared to ORM-10921 (Kalkman and Loetscher, 2003; Sallinen et al., 2013a; Shahid et al., 2009), and thus their effects on low striatal NA levels via the α_{2C}-AR was probably insufficient to effect alterations in NA levels. Although noradrenergic innervation of the striatum is limited, striatal NA is highly responsive to environmental stressors (Ihalainen et al., 1999). Thus, increased striatal NA levels induced by ORM-10921 might have beneficially affected behaviour in this animal model of schizophrenia, which involves a stressful rearing paradigm. Furthermore, noradrenaline has been shown to modulate increased

striatal dopamine metabolism (Weinstock et al., 1980), which might have played a role in the decrease in striatal DA effected by ORM-10921 (see below). These altered levels of NA and DA may account for the beneficial effects of ORM-10921 on sensorimotor gating and recognition memory as described in **Addendum A**.

The finding that IDAZ does not increase NA levels is confounding when one takes into account that α_2-AR antagonism should essentially increase central NA levels (Leonard, 2003), although it is consistent with some reports in the literature for brain regions with higher noradrenergic innervation than the striatum, including the hippocampus (Sacchetti et al., 1999). Once again, low NA levels favour α_{2C} mediated inhibition of NA release, and insufficient engagement with α_{2C}-ARs might mediate the inability of IDAZ to alter NA levels in the striatum. However IDAZ was the only drug that increased NA turnover, in line with its effects reported in literature (Walter et al., 1984). This implies that the ratio of NA to its metabolite, MHPG, was lower. Deficient noradrenergic function has been proposed to indirectly underlie the behavioural deficits in schizophrenia (Svensson, 2003; Yamamoto and Hornykiewicz, 2004). Increased metabolism of NA might thus have influenced IDAZ's lack of efficacy on behaviour as reported in **Addendum A**.

That CLOZ did not affect NA metabolism is in line with the literature (McMillen and Shore, 1978). Although the potency of CLOZ at the α_2-AR and specifically at the α_{2C}-ARs has been proposed to underlie its superior efficacy (Kalkman and Loetscher, 2003; Svensson, 2003), we did not show an immediate effect of CLOZ on striatal NA levels or turnover. Such effects might be visible in areas such as the hippocampus and frontal cortex, areas with higher noradrenergic innervation. CLOZ's effects might thus be mainly mediated via dopaminergic and serotonergic mechanisms in the corpus striatum, as discussed below.

Effects on striatal dopamine and dopamine turnover

The DA hypothesis describes hyperdopaminergic striatal function contrasted with cortical hypodopaminergic function, mediating positive, negative and cognitive functions of the disorder (Meyer-Lindenberg et al., 2002; Millan et al., 2015). Excessive striatal DA has been linked to psychotic symptoms and to deficient sensorimotor gating (Howes and Kapur, 2009) (Swerdlow et al., 2001; Zhang et al., 2000), but also indirectly to cognitive dysfunction (Simpson et al., 2010). Transgenic studies suggest a positive effect of α_{2C}-AR deactivation on cortico-striatal DA metabolism (Sallinen et al., 1997).

Schizophrenia is characterised by excessive striatal dopaminergic activity (Reynolds, 2004; Reynolds, 2008) and increased striatal D_2 receptor density (Sedvall, 1990). Accordingly, SIR is also associated with increased striatal DA levels and DA release (Fone and Porkess, 2008; Han et al., 2011; Möller et al., 2013a; Yorgason et al., 2016) as well as increased and hypersensitive striatal D_2 receptors, which

accentuate the hyperdopaminergic state in the striatum (King et al., 2009; Seeman et al., 2006). Thus, the finding that ORM-10921, which has negligible D_2 antagonist activity (Sallinen et al., 2013a), decreases striatal DA in SIR rats suggests that the improvements in PPI and object recognition memory (**Addendum A**) elicited by selective α_{2C}-AR antagonism may in part be related to indirect actions on excessive striatal dopaminergic activity. There are reports that very high doses of α_2-AR antagonism decreases baseline synaptic DA in models of striatal atrophy (Buck et al., 2010), however these effects are not shown at conventionally used doses (Buck et al., 2010; Invernizzi et al., 2003). That non-selective α_2-AR antagonism with IDAZ did not influence striatal DA at the dose used in this study is in line with studies reporting its inability to alter striatal DA release (Invernizzi et al., 2003) but to selectively increase prefrontal cortical DA levels (Hertel et al., 1999b; Matsumoto et al., 1998). Furthermore, in this study, IDAZ decreased striatal DA turnover vs. SIR controls and CLOZ. In a model characterised by excessive striatal dopaminergic activity, decreased striatal DA metabolism and unaltered DA levels after IDAZ treatment could explain its lack of efficacy in attenuating behavioural deficits in SIR rats vs. the beneficial effects of ORM-10921.

CLOZ did not decrease striatal DA as expected from previous studies (Bürki et al., 1975; Möller et al., 2013a), although in vivo microdialysis studies have also not been able to report altered striatal DA in CLOZ treated rats (Hernandez and Hoebel, 1995). Nevertheless, increased DA turnover is reported following CLOZ treatment (Batool et al., 2010; Westerink and Korf, 1975), which was also observed in this study. However, CLOZ's effects on behaviour can also be explained by its additional D_2 antagonist effects, which would address striatal hyperdopaminergic activity, while its positive effect on cognition is said to be associated with its ability to enhance cortical and hippocampal DA release through stimulation of the 5-HT_{1A} receptor (Chung et al., 2004) (Ichikawa et al., 2001).

Thus, while non-selective α_2-AR antagonism with IDAZ does not decrease striatal DA in SIR animals, selective α_{2C}-AR antagonism with ORM-10921 does decrease striatal DA levels in SIR animals, although not via decreased DA turnover. This may be one of the key mechanisms whereby ORM-10921 exerts its antipsychotic-like and procognitive effects, i.e. by rectifying dysregulated mesolimbic dopaminergic activity (Brisch et al., 2014; Simpson et al., 2010). Although CLOZ only tended to decrease striatal DA levels, it has a much broader receptor binding profile compared to ORM-10921, and its beneficial effects on behaviour could be related to a combination of various mechanisms. IDAZ did however decrease striatal DA turnover vs. both SIR controls and vs. CLOZ. IDAZ's inefficacy in improving behavioural deficits could thus also be related to decreased metabolism of DA.

Effects on striatal serotonin and serotonin turnover

The serotonin hypothesis of schizophrenia suggests that increased 5-HT transmission via 5-HT_{2A} receptors mediate positive and negative symptoms of schizophrenia (Vollenweider and Geyer, 2001)

(Reynolds, 2004). Serotonergic hyper-innervation of the ventral tegmental area projecting to the striatum has been related to deficient sensorimotor gating and may be related to striatal dopaminergic hyperactivity as seen in schizophrenia (Jackson and Abercrombie, 1992; Klejbor et al., 2009). SIR is associated with increased 5-HT levels in the nucleus accumbens of the striatum (Han et al., 2011) which was attenuated here by CLOZ, in accordance with previous studies (Batool et al., 2010; Burnet et al., 1996), possibly via actions on glutamate-GABA pathways which exert inhibitory control over striatal monoamine release (Stahl, 2007). However, neither ORM-10921 nor IDAZ affected 5-HT levels or 5-HT turnover in the striatum vs. SIR controls.

In conclusion this study demonstrated that selective α_{2C}-AR antagonism with ORM-10921 decreases striatal DA levels and increases striatal NA levels, without effects on striatal 5-HT, in contrast to non-selective α_2-AR antagonism. Further studies should investigate the effects of ORM-10921 on hippocampal and frontal cortical monoamines.

However, there is an important limitation to interpreting these results within the SIR construct. SIR rats did display significant deficits in PPI and object recognition memory vs. SOC animmals, as reported in **Addendum A** and **Manuscript A**, yet these behavioural deficits could not be correlated to altered monoamine levels vs. SOC rats, which questions the validity of the model-specific inferences made with regards to drug treatments. Moreover, the same animals used for behavioural analyses were used for neurochemical analyses, which introduces a significant confounding variable to these results. The lack of altered monoamine activity vs. SOC rats may be attributable to factors relating to renovation of the Animal Research Center, as discussed earlier. The interpretation of the effects of drug treatment in the SIR model is therefore compromised by this disparity. Nevertheless since drug treatment significantly improved SIR-induced behavioural deficits akin to antipsychotic therapy (**Addendum A** and **Manuscript A**), we felt it would be useful to report the effects of drug treatment (and ORM-10921 specifically) on monoamine levels in SIR animals as it does relate to the behavioural paradigm of schizophrenia, but noting the limitations mentioned. Also, the effect of ORM-10921 has not previously been studied with respect to striatal monoamine levels in a neurodevelopmental animal model of schizophrenia, so these data do provide some food for thought. These monoamine data therefore still present a possible mechanism of action of ORM-10921 on behaviour as noted, although cannot be considered suitable for publication in their current form.

Appendix 1

The materials and methods used to perform the 1) analyses of regional brain tissue *monoamine content* using *high-performance liquid chromatography (HPCL) with electrochemical detection (*ECD, HPLC-ECD) (**Manuscript C** and **Addendum B**) and 2) *BDNF enzyme-linked immunosorbent assay (ELISA)* analyses (**Manuscript A** and **Addendum B** and **E**) are described in more detail in this appendix.

1. Determination of tissue monoamine levels using a high-performance liquid chromatography (HPLC) system with electrochemical detection (HPLC-ECD)

This section describes the full validated method used to determine striatal and hippocampal tissue levels of noradrenaline (NA) and its main metabolite, 3-methoxy-4-hydroxyphenylglycol (MHPG), serotonin (5-HT) and its main metabolite, 5-hydroxyindole-3-acetic acid (5-HIAA) and dopamine (DA) and its main metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) using a HPLC-ECD system. The method forms part of the standard methods of the Analytical Technology Laboratory (ATL) of the Centre of Excellence for Pharmaceutical Sciences (Pharmacen) of the North-West University, Potchefstroom. The method is maintained and validated by the ATL and has been described in the literature (Harvey et al., 2006; Möller et al., 2011; Möller et al., 2013)

1.1. Chromatographic conditions

Analytical Instrument:	Agilent 1200 series HPLC, equipped with an isocratic pump, autosampler, coupled to an ESA Coulochem III Electrochemical detector (with Coulometric flow cell) and Chromeleon® Chromatography Management System version 6.8.
Column/Stationary phase:	Kinetix C18, 4.6 x 150mm, 2.6µm, 100Å (Phenomenex, Torrance, CA, USA).
Guard column:	4.0 x 3.0 mm C18 SecurityGuard™, HPLC Guard Cartridge System (Phenomenex, Torrance, CA, USA).
Mobile Phase:	0.1 M (6.801g/l) Sodium formate buffer, 5 mM (1.01125g/l) sodium heptane sulphonic acid, 0.17 mM (20 mg/l) ethylenediaminetetraacetic acid (EDTA disodium salt Na_2EDTA), 60 ml

	methanol (6%) and 40ml (4%) acetonitrile. The pH of the mobile phase was set at ± pH 3.5-4.1 with orthophosphoric acid (85%).
Flow rate:	0.50 ml/min.
Injection volume:	20 µl
EC Detector settings:	ESA 5011A Analytical Cell Potential settings Coulometric Electrochemical Detection Volts: E1: -150mV, E2: +750mV Gain range: 20nA Polarity: Positive Reaction: Oxidation Guardcell Potential setting: +350mV

1.2. Chemicals and reagents

The reagents for the mobile phase and the homogenization solution were purchased from Merck (Pty) Ltd., Modderfontein, Gauteng, South Africa.

The monoamine salts and the internal standard, isoprenaline, were purchased from Sigma-Aldrich Pty. Ltd., Johannesburg, Gauteng, South Africa.

1.2.1 Preparation of the Homogenization solution

All standards and samples are prepared with this solution.

0.5 mM sodium metabisulphite

0.3 mM Na_2EDTA

0.1 M perchloric acid (60% strong solution).

Preparation: 0.09505 g sodium metabisulphite and 0.111672 g Na_2EDTA is weighed off and dissolved in 800 ml distilled water. 10.87 ml perchloric acid is then added to the above solution and made up to 1000 ml.

1.2.2. Preparation of Monoamine Standards

The monoamine standards were each prepared in 10ml of the homogenization solution and made up to a stock solution with a concentration of 100µg/ml for each analyte. **Table 1** reports the substances and amounts used to prepare the stock solution for each analyte. The stock solution was subsequently used to prepare a series of dilutions to produce a standard concentration range between 1.25 – 50 ng/ml.

Table 1. Preparation of the various monoamine standard stock solutions

Analyte	**Molar Mass (g/mol)**	**Substance**	**Molar Mass (g/mol)**	**Amount dissolved in 10ml homogenization solution (mg)**	**Final concentration of analyte**
MHPG	184.09	3-methoxy-4-hydroxyphenylglycol hemipiperazinium salt	454.5	2.47	100µg/ml
NA	169.18	*l*-noradrenaline hydrochloride	205.6407	1.22	
DOPAC	168.15	3,4-dihydroxyphenylacetic acid	168.15	1	
DA	153.18	3-hydroxytyramine hydrochloride	189.64	1.24	
HVA	182.18	homovanillic acid	182.18	1	
5-HT	176.2	5-hydroxytryptamine creatinine sulphate	405.43	2.3	
5-HIAA	191.19	5-Hydroxyindole-3-acetic acid	191.19	1	

1.2.3. Preparation of Internal Standard

The internal standard is a known concentration of a substance possessing physico-chemical characteristics as close as possible to that of the analyte of interest (United Nations Office on Drugs and Crime, 2009). Internal standards are used to correct for variability in the sample preparation process and are usually employed as a measure to improve the precision of a quantitative analysis. The internal standard should ideally provide a response that is similar to that of the analyte, but provide a signal that is distinct from the signal of the analyte. The rationale is that factors that could affect the signal of the analyte will similarly affect the signal of the internal standard. The ratio of the two signals should therefore exhibit a lower degree of variability than the signal of the analyte alone (Center for Drug Evaluation and Research, 1994; Magee and Herd, 1999; United Nations Office on Drugs and Crime, 2009).

1mg of the internal standard, isoprenaline (n-isopropyl-dl-noradrenaline hydrochloride), was dissolved in 10 ml homogenization solution to make up a stock solution with a concentration of 100µg/ml. To prepare a working internal standard solution of 1500 ng/ml, 30µl of this stock solution was added to 1970 µl of the homogenization solution.

1.3. Sample preparation of brain tissue and determination of monoamine content

1. Following dissection, brain tissue (hippocampus or striatum, dissected as described in **Addendum B** and **E**) of each animal is placed individually into polypropylene tubes, marked and snap frozen with liquid nitrogen. The samples are stored at -70°C until the day of analyses.
2. On the day of analysis, samples are weighed, thawed and 1 ml of homogenization solution is added to each tube. The tissue in each tube is then ruptured by sonication (2 x 12 seconds, at amplitude of 14 µ) (Keller et.al. 1976).
3. The tubes are left to stand on ice for a period of 20 minutes to complete perchlorate precipitation of protein and extraction of monoamines.
4. Following this period, samples were centrifuged at 4°C in an ultra-centrifuge for 20 minutes at 16 000 revolutions per minute (rpm) (24 000 *g*).
5. The supernatant of the tissue extract (± 1ml) is pipetted into a 2 ml amber polypropylene tube.
6. The pH of the sample is adjusted to pH 5 with the addition of 1 drop/ml of 10 M potassium acetate.
7. 200 µl of the tissue extract sample or standard is pipetted into another 1.5 ml polypropylene tube.
8. 20 µl of the internal standard working solution is added to the sample.
9. This final sample is vortexed and then centrifuged again for 5 minutes.
10. The whole sample is pipetted into a 300 µl glass insert, which fits into an amber HPLC glass vial. The vial is then placed into the sample tray of the Agilent 1200 series autosampler.
11. The instrument's software is programmed to inject 20 µl of the sample onto the HPLC column.
12. The peak area data of each sample is collected, divided by the peak area of the internal standard, and converted to a concentration value in ng/ml by making use of the relevant analyte's standard linear calibration curves (regression >0.98) (see **Table 3 on page 255**).
13. The concentration value of the monoamine is expressed in terms of ng/g wet tissue weight.

1.4. HPLC Chromatographic Information

The retention times for the respective analytes are reported in **Table 2**, with **Figure 1** representing a typical chromatogram generated by the solution containing all the monoamine standards at a monoamine standard concentration of 10ng/ml.

Table 2: Retention times of the various analytes

Analyte	Retention time (minutes)
MHPG	± 5.97
NA	± 6.15
DOPAC	± 10.61
DA	± 13.95
5-HIAA	± 20.18
Internal Standard	± 22.90
HVA	± 24.93
5-HT	± 39.45

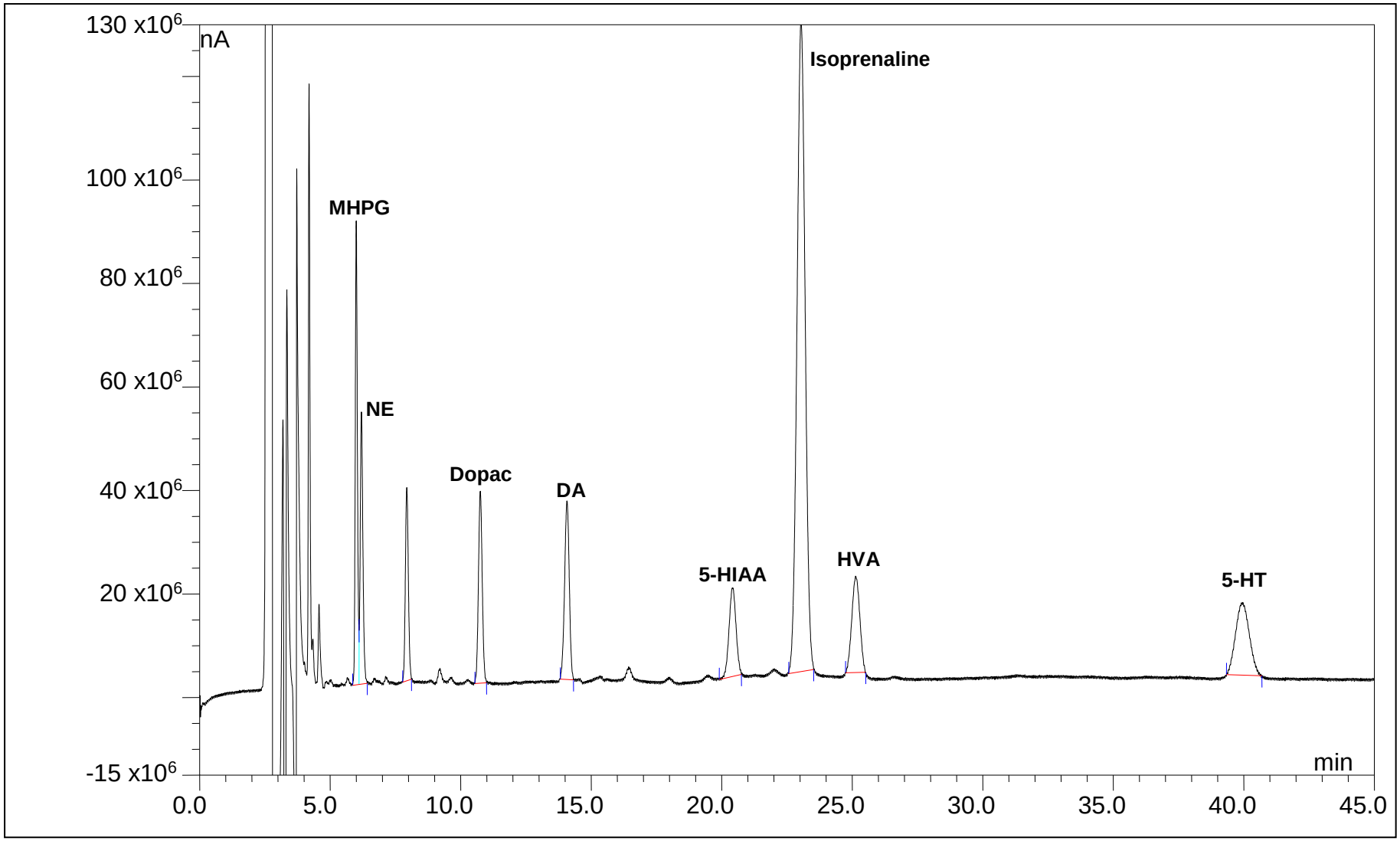


Figure 1: Representative chromatogram of monoamine standard, isoprenaline (10ng/ml), as well as all other analyte standards (10 ng/ml) in a typical brain sample.

1.5. Mini method validation

A mini method validation, otherwise referred to as analytical method verification, is performed before starting routine analyses of samples with the objective of demonstrating that the method and analytical instrument used will still provide the same accurate and valid results as generated with the original validation of the analytical method (Geetha *et.al.* 2012). This method has been validated in the Analytical Technology Laboratory (ATL) of the Centre of Excellence for Pharmaceutical Sciences (Pharmacen) of the North-West University, Potchefstroom.(Basson et al., 1988).

1.5.1 Mini method validation parameters

The analytical parameters used in this mini method validation are as follows: a) **linearity (**calibration curve), b) **repeatability**, c) **lower limit of quantification** (LLOQ) and d) **lower limit of detection** (LLOD).

1.5.2 Results for system suitability

a) Linearity / Calibration curve

According to a guidance document released by Health Canada on methods validation (Health Canada and Directorate, 1994)*,,* the acceptable criteria for regression (r^2), the coefficient of determination for biomolecules (biologics), must be at least 0.95 or greater (Shabir, 2006). The linearity/calibration curve used in this validation process comprised of the following 6 standard concentrations for each analyte: 1.25, 2.5, 5.0, 10, 20 and 50.0 ng/ml.

The internal standard method was used to set up the calibration curve as well as to calculate the test sample results according to the following response ratio:

$$Response\ factor\ ratio\ (x) = \frac{Peak\ Area\ of\ Standard}{Peak\ Area\ of\ Internal\ Standard}$$

The calibration curve was set up with the 6 standard concentrations of the specific analyte on the x-axis of the graph with the corresponding response factor ratio on the y-axis. These linear regression results for the 7 analytes are reported in **Table 3** on the next page.

b) Repeatability

The repeatability measures the precision of the method as reported by the % coefficient of variation (CV) and is determined at 3 concentrations (lowest, middle and highest concentrations) and using 3 repetitions each (Huber, 2010). The % CV is determined by dividing the standard deviation of the 3 repetitions by their mean and multiplying this number by 100. The repeatability determined at each of

the 3 concentration levels should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV.

The 3 concentrations used to measure repeatability were 1.25, 5.0 and 50 ng/ml. See the repeatability results in the table below.

Table 3: Linear regression and repeatability (%CV) for each analyte

Analyte	*Calibration curve* *Linear Regression (r^2)*	*Concentration* *(ng/ml)*	*Average CV (%)*
NA	0.996	1.25	4.48
		10.0	4.01
		50.0	2.63
MHPG	0.994	1.25	4.82
		10.0	2.09
		50.0	0.28
DA	0.992	1.25	2.50
		10.0	4.85
		50.0	2.44
DOPAC	0.994	1.25	7.94
		10.0	3.96
		50.0	4.14
HVA	0.984	1.25	3.58
		10.0	7.68
		50.0	4.51
5-HT	0.985	1.25	5.22
		10.0	4.91
		50.0	4.19
5-HIAA	0.987	1.25	6.40
		10.0	1.99
		50.0	4.34

c) Lower limit of quantification (LLOQ)

The lower limit of quantification was found to be 1.25 ng/ml, which corresponds with the lowest concentration on the calibration curve.

d) Lower limit of detection (LLOD)

The lower limit of detection was found to be 1.0 ng/ml.

1.6. Limitations

Since this analytical procedure analyses biological samples, a few unknown peaks are produced on the chromatogram. Furthermore, the monoamine molecules are structurally (physico-chemically) very similar to each other, resulting specifically in the retention times of MHPG and of NA to lie very close to each other in this HPLC method.

The validation of analytical methods for endogenous molecules/biomarkers (such as the monoamines) has been complicated by the absence of official guidelines (van de Merbel, 2008). Most researchers apply "The method-validation principles for the analysis of drugs" issued by the US Food and Drug Administration (Center for Drug Evaluation and Research, 2001) to their methods when measuring endogenous molecules/biomarkers, to ensure that their results have an acceptable and comparable level of quality (van de Merbel, 2008). The FDA principles were however meant for drug analysis and not for endogenous molecules/biomarkers and therefore direct application of these principles are not possible, requiring analyte-specific modifications (van de Merbel 2008).

2. Determination of hippocampal and striatal BDNF levels using ELISA

This section describes the materials and procedures used to determine regional brain levels of BDNF using the Biosensis® BDNF *Rapid*™ ELISA kit (Catalogue number BEK-2211-2P). This kit has been developed and validated by the manufacturer for quantitative determination of mature BDNF in rat and mouse brain extracts and blood. The kit provides a method for generating a BDNF standard concentration curve of 7.8-500 pg/ml, with a minimum detection limit of 2 pg/ml.

2.1 Materials

Kit components:
BDNF antibody-coated 96-well microplates
Assay Diluent
Recombinant BDNF standard
BDNF Detection Antibody
Streptavidin-horseradish peroxidase (HRP) conjugate
Wash Buffer
3,3',5,5'-tetramethylbenzidine (TMB) substrate
TMB Stop Solution
Plate sealer

The kit provides instructions for preparing an acid-extraction buffer and sample diluent developed to release bound BDNF in brain tissue samples, as the Assay Diluent provided is not suited for use in brain tissue analyses. These buffers are based on the protocol of Kolbeck and co-workers (Kolbeck et al., 1999) and the method has been validated by the manufacturer (Biosensis®).

Acid-extraction Buffer

This buffer was used to prepare brain homogenates.

50 mmol/l sodium acetate (4.102 g in 1000 ml double-distilled water)
1mol/l NaCl (58.44 g in 1000 ml double-distilled water)
0.1% Triton X100 (1ml in 999 ml double-distilled water)
Acetic acid is added until pH 4.0 is reached.
Immediately before use, add 1 *cOmplete™ protease inhibitors* cocktail tablet (Roche®) per 50ml of extraction buffer.

Neutralization Buffer

0.1 mol/l phophate buffer (0.1 mol/l KH_2PO_4 + 01. mol/l Na_2HPO_4) made up to ph 7.6

Thus 13,69 g KH_2PO_4 was mixed in 1000 ml distilled water and 14,195 g Na_2HPO_4 was mixed in 1000 ml distilled water. The KH_2PO_4 solution was added to 500ml of Na_2HPO_4 solution until pH 7.6 was attained.

Acid-extraction Sample Diluent

This diluent was used to prepare serial dilutions of the standards and samples as per manufacturer's instructions.

Acid-extraction buffer and neutralization buffer are mixed at a ratio of 1 mL extraction buffer and 3 ml neutralization buffer.

2.2 Assay procedure

All steps are performed at room temperature (20-25^{o}C) as per the manufacturer's instructions. A graphical summary of the main steps and principles of the procedure is given in Figure B-2 (**Addendum B**)

1. Brain homogenates were prepared as described in **Addendum B** *section 1.2.2* and **Addendum E** *section 2.2* using the acid-extraction buffer and sample diluent described above.
2. BDNF standards are prepared with the acid-extraction sample diluent by making an initial 1000 pg/ml stock solution, which is vortexed and then left to stand for 15 minutes. 1:2 serial dilutions are then prepared to generate BDNF standards with concentrations of 500, 250, 125, 62.5, 31.3, 15.6 and 7.8 pg/ml.
3. 100µl of diluted BDNF standards, samples and blanks (consisting of the acid-extraction sample diluent only) are added to the pre-coated microplate wells in duplicate, with samples distributed across the plate so that each cohort is represented equally across the vertical and horizontal dimensions of the plate.
4. The plate is sealed with the plate sealer and incubated on a plate shaker at 140 rpm for 45 minutes.
5. The contents of the wells are discarded and 5 washes are performed with 200µl wash buffer per well, using a BioTek Elx50™ Automated Strip Washer (BioTek Instruments Inc., USA)
6. 100 µl of the detection antibody is added to the wells.
7. The plate is sealed and incubated on the plate shaker for 30 minutes at 140 rpm.
8. The contents of the wells are discarded and 5 washes are performed with 200µl wash buffer per well as described in step 5.
9. 100 µl streptavidin-HRP conjugate is added to each well.
10. The plate is sealed with the plate sealer and incubated on a plate shaker at 140 rpm for 30 minutes.
11. The contents of the wells are discarded and 5 washes are performed with 200µl wash buffer per well as described in step 5.
12. 100 µl TMB is added to each well and the plate is incubated at room temperature in the dark without shaking.
13. After 5 minutes the reaction is stopped by adding 100 µl TMB stop solution to each well. The blue color changes to yellow and the absorbance is read in a BioTek SYNERGY/HT microplate reader at 450nM (BioTek Instruments Inc., USA).
14. The average readings for each BDNF standard, blank and sample was taken and the average values of the blank subtracted. The BioTek SYNERGY/HT software then generates a four

parameter logistic (4-PL) curve-fit and performs a regression analysis to calculate the concentration of BDNF in the samples and multiplies the result by the dilution factor as reported in **Addendum B** and **E**.

15. The repeatability for the four plates used in this study was measured as the % coefficient of variation (CV) and performed according to the principles set out by Polacchini and co-workers (Polacchini et al., 2015). The desired %CV was set at <10% (Polacchini et al., 2015), and we report an intra-assay %CV of 6.08%.

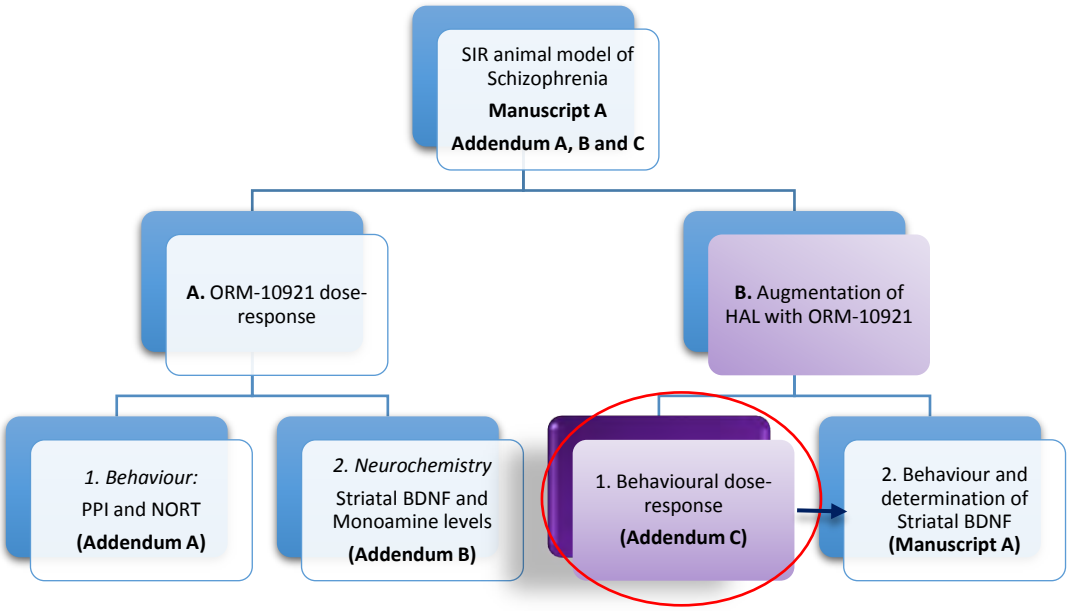
Addendum C

Antipsychotic-like and pro-cognitive effects of augmenting haloperidol with ORM-10921 in the SIR model of schizophrenia

Preamble

The following section will report and discuss the behavioural data employing ORM-10921 in an augmentation study with haloperidol (HAL). This sub-study eventually led to the publication of **Manuscript A**. The most effective dose from the dose-response analysis in **Addendum A** (0.03mg/kg ORM-10921), which assessed the antipsychotic-like and pro-cognitive effects of ORM-10921 in the SIR model of schizophrenia, was used along with an additional lower dose of ORM-10921 (0.01mg/kg). Additionally, these doses were used as augmentation to HAL therapy to determine whether this combination would result in enhanced antipsychotic-like effects vs. ORM-10921 and HAL alone. **Manuscript A** reports data using the most effective dose in this regard, ORM-10921 0.01mg/kg. Here we will report the data generated with ORM-10921 0.03mg/kg that was not included in **Manuscript A**. We will report the data in comparison to ORM 0.03mg/kg in order to demonstrate why the ORM 0.01 mg/kg dose was selected for the study culminating in **Manuscript A**. To this end, the HAL monotherapy data reported in **Manuscript A** is reported here in order to provide a comparator for the augmentation studies. Because this study was an extension of the study reported in **Addendum A**, the ORM 0.03 mg/kg data is reported here again. Furthermore, this extension of the originally planned dose-response study also resulted in an extension of the time period over which the study was performed. Thus, additional vehicle treated SOC and SIR controls were incorporated into this HAL augmentation study, to ensure that behavioural data generated in the final drug treatment cohorts displayed the same baseline control levels in order to ensure comparability to and ORM-10921 0.03 mg/kg.

The diagram on the next page depicts how this addendum relates to the SIR study as reported in this thesis. The circled box indicates the section of the SIR study that is reported here, as described above.



1. Aims

The main aims of this investigation was to employ an animal model of schizophrenia to investigate whether chronic treatment with ORM-10921 in combination with HAL would result in enhanced antipsychotic-like and pro-cognitive effects as compared to either drug alone. This study has four main aims:

1. Determine whether a lower dose of ORM-10921 (0.01mg/kg) would demonstrate more pronounced antipsychotic-like and pro-cognitive effects in the SIR model of schizophrenia.
2. Determine the effects of HAL on PPI and NORT in the SIR model of schizophrenia.
3. Determine whether augmentation of HAL with two low doses of ORM-10921 (0.01 mg/kg and 0.03 mg/kg) in SIR rats presents with improved effects in the NORT and PPI test vs. HAL or ORM-10921 alone.
4. Compare the effects of ORM-10921 augmentation of HAL in SIR rats to the effects of clozapine. This objective is reported in **Manuscript A** and will not be reported here again.

The validation of SIR vs. SOC animals is described in **Manuscript A** and will not be repeated here.

2. Methods

2.1 Animals and drug treatment

Male Sprague Dawley rats were weaned on post natal day 21 and reared alone (one animal per cage) for 8 weeks, as described in **Manuscript A**. The animals were handled according to the code of ethics in research, training and testing of drugs in South Africa, and ethical approval for this study was obtained

from the AnimCare Animal Research Committee of the North-West University (ethics approval number: NWU-00050-13-A5). Once daily sub-cutaneous (SC) drug administration commenced on week 7 of isolation rearing and continued for 14 days, according to the rationale described in **Addendum A** and **Manuscript A**. Rats received either vehicle, HAL 0.2mg/kg and/or ORM-10921 (0.01 or 0.03mg /kg), prepared as described in **Manuscript A**. HAL (Sigma Aldrich) was dissolved in water containing 0.3% tartaric acid (Binder et al., 2001; Ishiwari et al., 2007; Singh et al., 2013) and administered at a non-cataleptic dose of 0.2 mg/kg (unpublished data) capable of 80% D_2 receptor occupancy (Natesan et al., 2007; Wadenberg et al., 2001) and robust antipsychotic activity in animals (Depoortere et al., 1997; Hadamitzky et al., 2007; Kusljic et al., 2006; Varty et al., 1995) and approximating clinically effective doses (Castner et al., 2000). ORM-10921 was a kind donation from Orion Pharma, Turku, Finland.

2.2 PPI

The PPI was conducted on day 14, 12 hours after drug treatment, as described in **Addendum A** and **Manuscript B**. Briefly, startle responses to a 40ms 115dB PULSE ALONE trial was measured as 10 consecutive trials at the beginning (BLOCK 1) and end (BLOCK 4) of the test session. 70 trials consisting of 40 PREPULSE+PULSE trials 20 PULSE ALONE trials (BLOCK 2 and 3) and 10 no-stimulation trials were randomly delivered between BLOCK 1 and BLOCK 4. The percentage PPI was calculated for each individual subject according to the formula: %PPI = [(startle reponse for PULSE ALONE trial) – (startle response for PREPULSE +PULSE trial)/(startle response for PULSE ALONE trial) x 100]. The average %PPI across all four prepulse intensities was then determined (Gacsalyi et al., 2013). A higher % PPI correlates with improved sensorimotor gating.

2.3 NORT

The NORT was conducted on day 12 and 13, 12 hours after drug treatment as described in **Addendum A** and **Manuscript A**. Briefly, 24 hours after a 10 minute habituation exposure to the NORT box, a 5 minute acquisition trial allowed the subject to explore two identical objects. The animal was returned to its home cage and 90 minutes later the recognition trial allowed the subject to explore one familiar object and one novel object. Time spent exploring the novel and familiar objects was recorded. Object exploration was considered as active sniffing, licking or physical exploration of the object. Animals that failed to accumulate a total exploration time of ≥6 seconds or who failed to explore both objects were excluded from the study (du Jardin *et al*., 2016). Essentially, more exploration time at the novel object reflects accurate recollection that the familiar object has been presented before, thus prompting greater exploration of the novel object. Object recognition was expressed as the discrimination index (DI) calculated according to the formula: % DI = [(time spent exploring novel object – time spent

exploring familiar object)/(total time spent exploring both objects) x100]. A higher DI indicates improved declarative recognition memory.

2.4 Statistical analysis

Data are expressed as mean ± SEM except for Startle Habituation data, expressed as the mean. GraphPad Prism® version 6.01 (GraphPad Software, San Diego California USA, www.graphpad.com) was used for statistical analyses and graphical presentations. Normality of data sets was determined using the Shapiro-Wilk test, as described in **Addendum A**. All statistical analyses were conducted as described in **Addendum A.** Briefly, analysis of variance (ANOVA) and Fisher's LSD test was used to determine dose-response effects of HAL and ORM-10921 (ORM) monotherapy as well as HAL+ORM augmentation at the various doses. Effect sizes were calculated to indicate the practical significance (if applicable) of results demonstrating statistical significance on a 10% significance level ($p \leq 0.1$), as described in **Addendum B**. The rationale was to determine effective drug doses or augmentation regimens. The most effective drug and augmentation regimen doses would be subjected to a more rigourous statistical analysis with the Tukey post hoc test, which robustly controls for the type I statistical error (false positive discovery rate), in a comparison including the atypical antipsychotic CLOZ. This comparison is reported in **Manuscript A**.

3. Results

3.1. Dose-response analysis of two doses of ORM-10921 alone and as augmentation to HAL

3.1.1 PPI

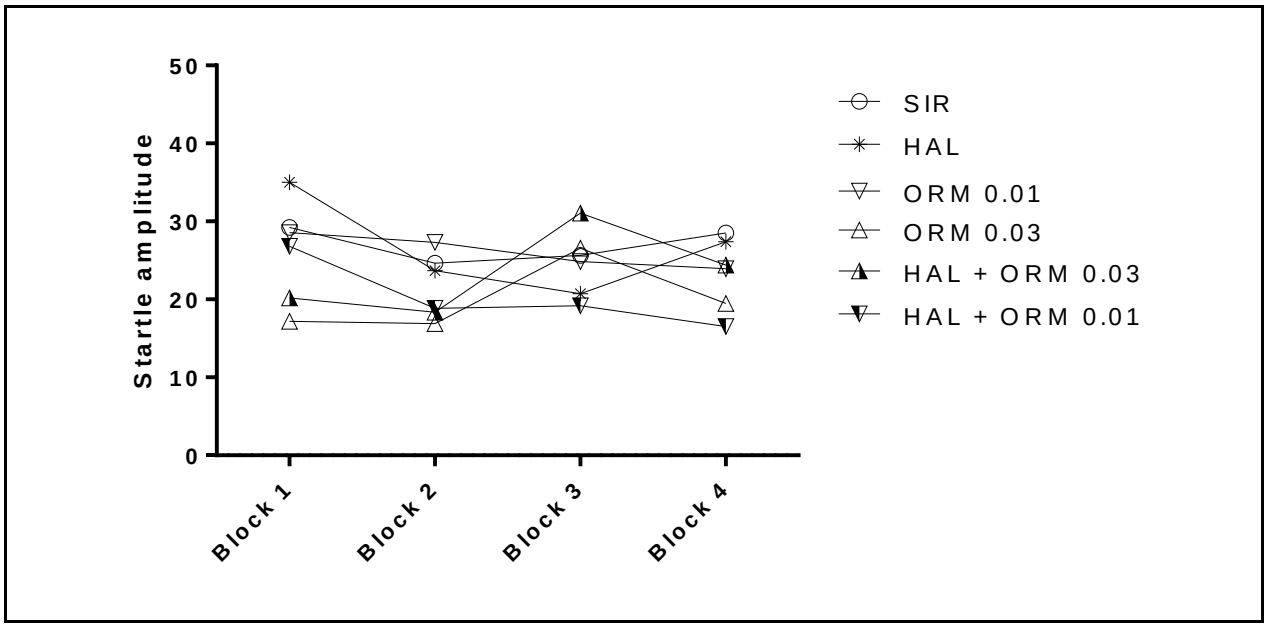


Fig C-1 Startle response habituation to the four startle blocks throughout the startle session in SIR animals treated with vehicle, and the various drug treatments, as indicated (n=9-13). SIR = Social Isolation Reared, ORM = ORM-10921 at 0.01 or 0.03 mg/kg, HAL= haloperidol 0.2mg/kg.

The startle habituation of SIR animals treated with vehicle or the various drug treatments is indicated in Fig C-1. ANOVA indicated no significant differences in startle habituation between treatment groups ($F(5,52)= 1.141$ $p=0.35$).

The average % PPI in SIR animals treated with the various drug treatments is indicated in Fig C-2. ANOVA indicated a significant effect of drug treatment ($F(5,52)=5.561$, $p=0.0004$) on the PPI. Fisher's LSD test indicated that treatment with ORM 0.01 ($p=0.0002$), ORM 0.03 ($p=0.02$) as well as HAL+ORM 0.01 ($p=0.004$) and HAL+ORM 0.03 ($p=0.02$) significantly improved PPI compared to SIR controls (Fig C-2). HAL however did not significantly alter PPI vs. SIR controls ($p= 0.94$). All treatment groups also exhibited significantly higher % PPI vs. HAL (Fig C-2: ORM 0.01, $p=0.0001$; ORM 0.03, $p=0.02$; HAL+ORM 0.01, $p=0.004$; HAL+ORM 0.03, $p=0.02$).

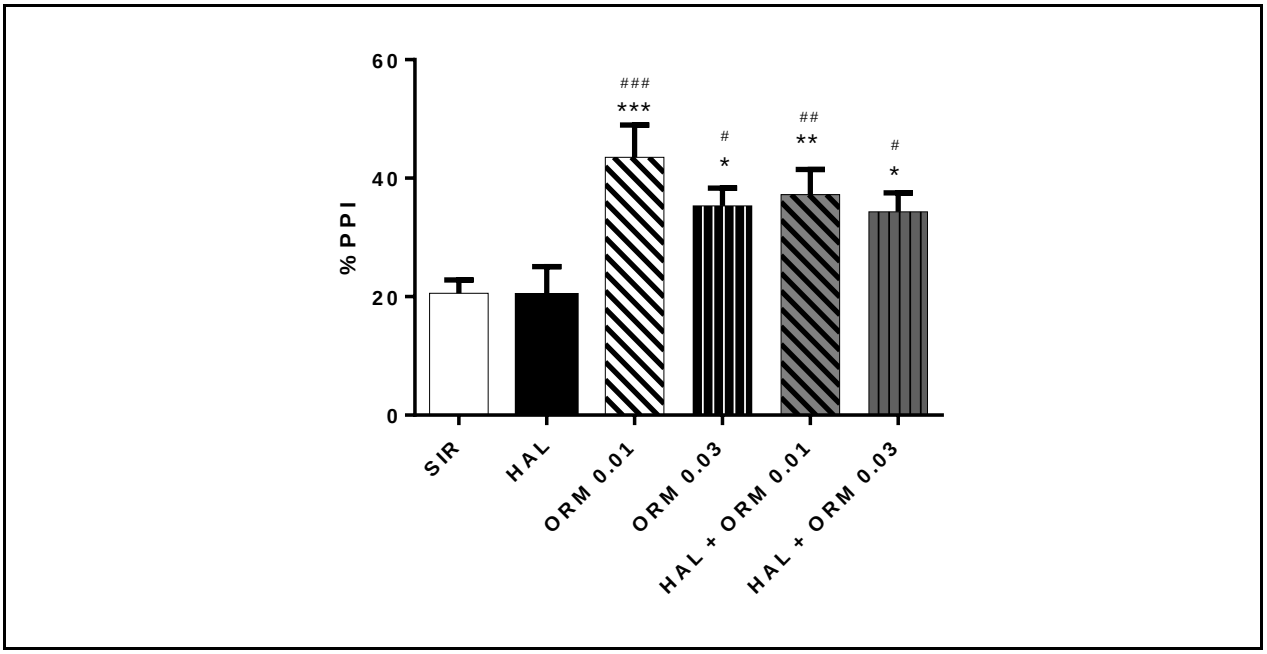


Fig C-2. Average % PPI of the startle response in SIR animals following the various drug treatments, as indicated (n=9-13). $^{*}p<0.05$, ** $p<0.01$, $^{***}p<0.001$ vs. SIR Controls, $^{\#}p<0.05$, $^{\#\#}p<0.01$ and $^{\#\#\#}<0.001$ vs. HAL treatment. SIR = Social Isolation Reared, ORM = ORM-10921 0.01 or 0.03 mg/kg, HAL = haloperidol 0.2 mg/kg.

3.1.2 NORT

The total exploration times of all SIR cohorts treated with vehicle or drug treatment is depicted in figure C-3. One-way ANOVA showed that there was no significant difference between the exploration times of any of the treatment cohorts ($F(5,50)= 0.54$; $p= 0.74$), indicating that total object exploration time was not significantly influenced by any of the drug treatments.

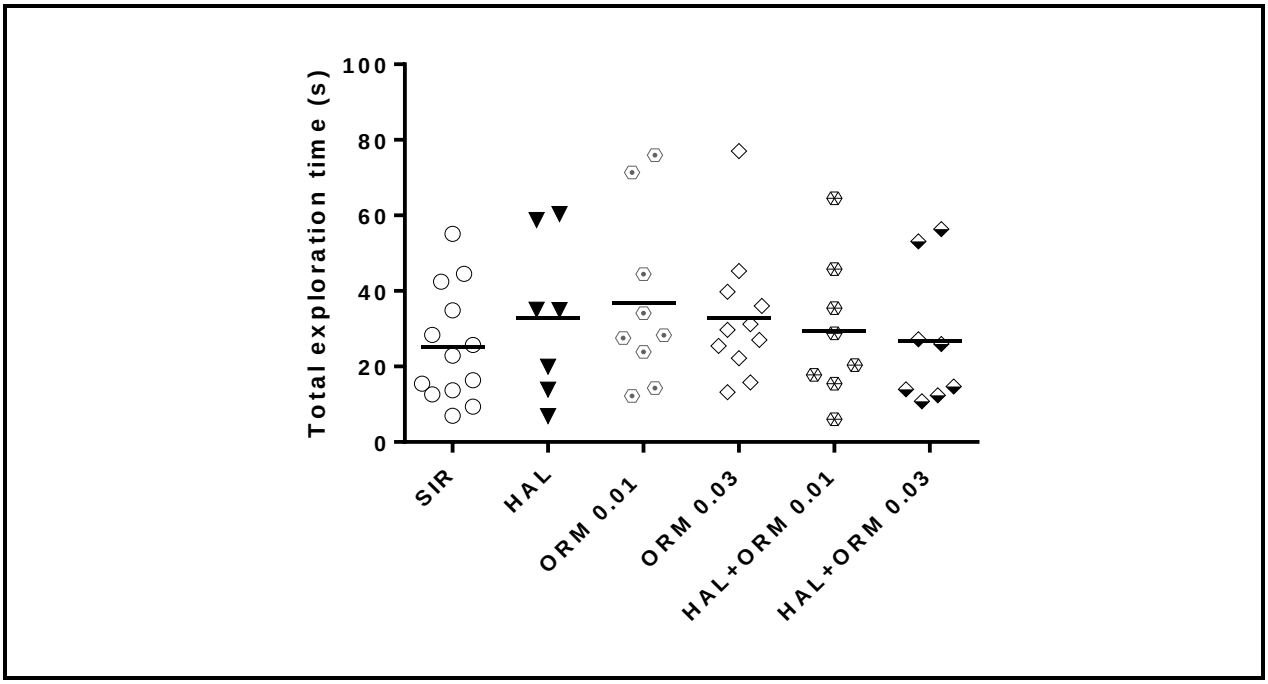


Fig. C-3. Total exploration times for subjects that met the inclusion criteria in the recognition trial of the NORT (n=7-13). SIR = Social Isolation Reared, ORM = ORM-10921 0.01mg/kg, HAL = haloperidol 0.2mg/kg.

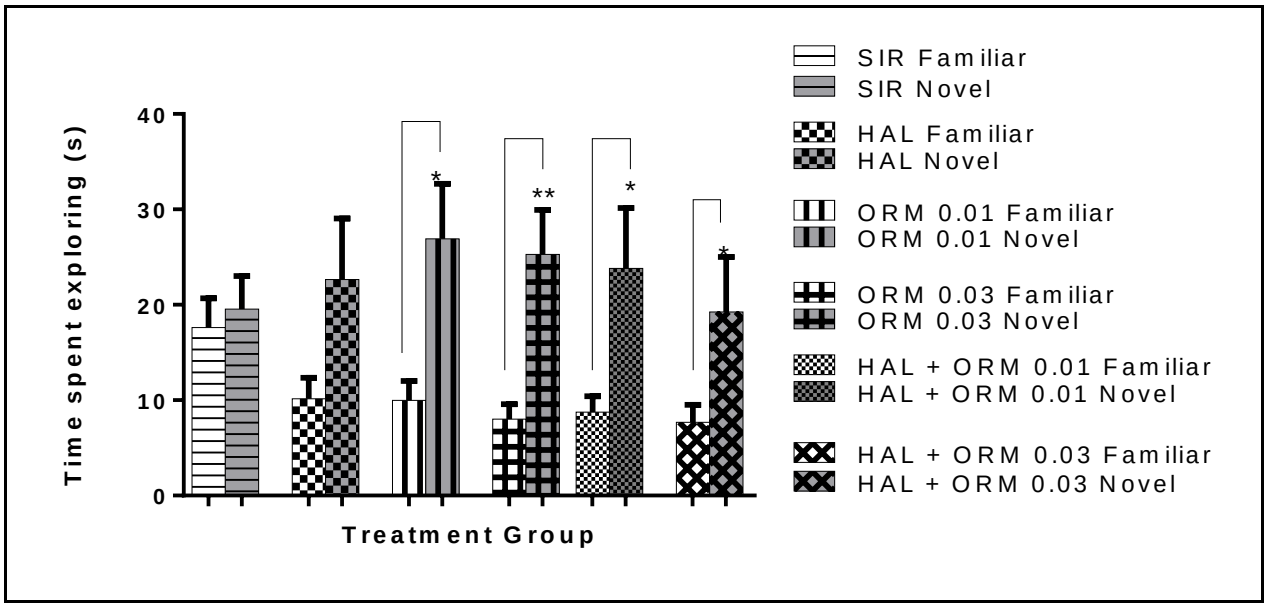


Fig. C-4. Time spent exploring novel and familiar objects in the NORT following the various drug treatments as indicated (n=7-13). All drug treatments were conducted in SIR animals. Paired t-tests or Wilcoxon matched pairs test for novel vs. familiar objects for each drug treatment. *p<0.05, **p<0.01. SIR = Social Isolation Reared, ORM = ORM-10921 0.01 or 0.03 mg/kg, HAL = haloperidol 0.2mg/kg.

Fig C-4 depicts the time spent exploring the novel vs. familiar object for SIR animals treated with either vehicle, one of the various doses of ORM-10921 or ORM-10921+HAL, as indicated. SIR controls (p=0.2) and SIR HAL-treated animals failed to spend more time at the novel object (Wilcoxon), although the

HAL-treated group barely missed statistical significance on a 5% significance level (p=0.06), displaying significance on a 10% significance level. However, the effect size (*d*=0.9) exceeds Cohen's convention for a large effect size, and thus this difference is deemed to be practically significant. Paired t-test showed a statistically significant difference in novel vs. familiar object exploration time for ORM-10921 0.01 and 0.03 mg/kg (Fig C-4; ORM 0.01mg/kg: p= 0.03; ORM 0.03mg/kg: p=0.002), while Wilcoxon matched-pairs signed rank test showed that HAL + ORM 0.03 (p=0.015) as well HAL + ORM 0.01 (p=0.015) also spent significantly more time at the novel object.

The discrimination index (DI) in SIR animals treated with saline vehicle or drug treatment is depicted in Fig C-5, with Kruskal Wallis Anova showing significant group differences (Kruskal-Wallis statistic = 23.01; p=0.0005). Dunn's multiple comparison test indicated that ORM 0.01 (p=0.0005), ORM 0.03 (p=0.0004), HAL+ORM 0.01 (p=0.02) and HAL+ORM 0.03 (p=0.03) significantly improved the DI vs. SIR control animals, whilst HAL was ineffective (p=0.19).

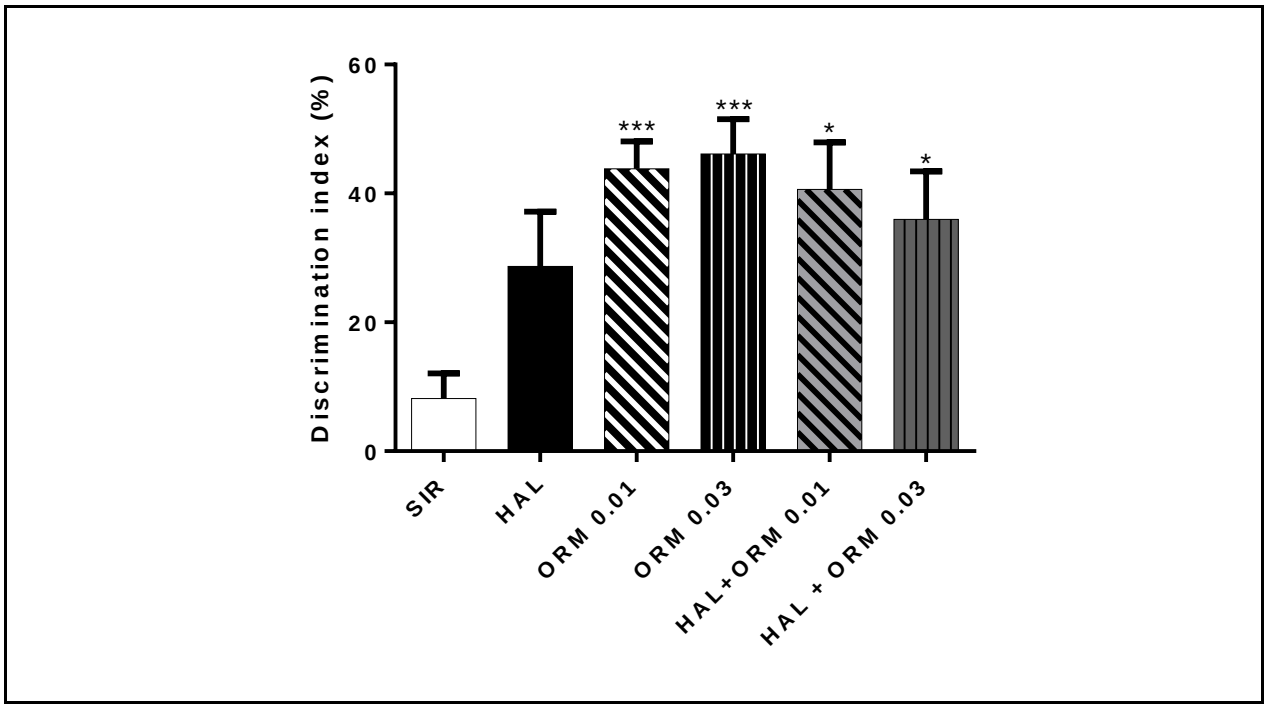


Fig. C-5 Discrimination index (DI) in the NORT in SIR animals following the various drug treatments, as indicated (n=7-13). Kruskal-Wallis ANOVA and Dunn's Multiple Comparison test. *p<0.05, ***p<0.001, drug treatments vs. SIR Controls. SIR = Social Isolation Reared, ORM = ORM-10921 0.01 or 0.03 mg/kg, HAL = haloperidol 0.2mg/kg.

4. Discussion

Manuscript A already reports the antipsychotic-like and pro-cognitive efficacy of a lower dose of ORM-10921 (0.01 mg/kg). This dose-response analysis of ORM-10921 0.01 mg/kg and 0.03 mg/kg including

augmentation of HAL with these doses confirmed the conclusion made in **Addendum A** that lower doses of ORM-10921 are more conducive to improving PPI in rodents reared in isolation. Here we employed an additional dose a third lower than that tested previously, i.e. 0.01 vs. 0.03 mg/kg. Although activity in the NORT was not improved using the lower dose of ORM-10921 compared to the 0.03 mg/kg dose, the lower dose engendered a more robust improvement in the PPI test in SIR animals than the 0.03 mg/kg dose.

The possible explanations for efficacy of a lower dose is discussed in detail in **Addendum A***,* section *4.2* and can be summarised here as pertaining to a more selective α_{2C} vs α_{2A} engagement profile as the dosage decreases. All animals displayed equal baseline startle responses and habituation to startle response and displayed equal duration of total exploration activity in the NORT, indicating that the drug effects on the DI weren't confounded by all over lower or higher exploratory activity.

The first conclusion from this study is therefore that lower doses of ORM-10921 produce more robust effects on PPI, while cognitive parameters involved in recognition memory are less sensitive to alterations in dose.

The literature describes mixed results for improving PPI at a several higher and lower doses of HAL in various other models of schizophrenia or models of sensorimotor gating impairement (Hadamitzky et al., 2007; Kusljic et al., 2006; Le Pen and Moreau, 2002; Lipska et al., 1995). HAL tended to improve the recognition memory index, but not significantly, a finding similar to effects described elsewhere (Horiguchi et al., 2012; Redrobe et al., 2010; Snigdha et al., 2010). Nevertheless, Watson and co-workers suggested that D_2-antagonism alone is sufficient to reverse object recognition impairment in SIR rats (Watson et al., 2012). However, despite the relative lack of efficacy for HAL in this model, augmenting HAL with ORM-10921 provided a robust improvement in sensorimotor gating and recognition memory deficits in SIR rodents compared to controls (**Manuscript A**), while sensorimotor gating deficits were also significantly improved compared to HAL treatment alone (also reported in **Manuscript A**). However, this improvement was not greater than that of ORM-10921 alone and at any dose. In fact PPI and DI was numerically lower when ORM-10921 was augmented with HAL than in animals treated with ORM-10921 monotherapy. This finding has important implications. *Firstly*, it challenges the notion that D_2-antagonism (Millan et al., 2015) remains a central therapeutic target in treating schizophrenia, since ORM-10921 presents with very low activity at the D_2 receptor (Sallinen et al., 2013a) and was able to exert antipsychotic-like and pro-cognitive effects when administered as monotherapy. *Secondly*, these findings support the notion that an increased α_{2C}/D_2 receptor selectivity ratio could present with improved antipsychotic-like and pro-cognitive effects in schizophrenia (Kalkman and Loetscher, 2003). *Thirdly*, this data suggests that the α_{2C}-AR plays an important role in earlier findings reporting beneficial effects of adding non-selective α_2-AR antagonist activity to antipsychotic treatment (Marcus et al., 2005;

Marcus et al., 2010b). Further studies employing augmentation of HAL with non-selective α_2 antagonists, selective α_{2A} antagonists and selective α_{2C} antagonists will be valuable in validating this notion. The impact and relevance of these findings pertaining to the potent effects of ORM-10921 0.01mg/kg is discussed in more detail in **Manuscript A**.

This study also demonstrated that the effects of combined D_2 and α_{2C}-AR antagonist activity is dose-related, with a lower dose of ORM-10921 presenting with more pronounced improvements in HAL effects on the PPI and NORT than the 0.03mg/kg dose. Once again, this phenomenon might be attributable to a lower dose possibly engaging α_{2A}-ARs to a lesser extent than higher doses (see **Addendum A** for detailed discussion).

This study thus delivered three important outcomes: (i) it demonstrated that a lower dose of ORM-10921 (0.01mg/kg, used in **Manuscript A**) does indeed present with more pronounced antipsychotic-like activity compared to a 3-fold higher dose (0.03 mg/kg), (ii) it demonstrated that the pharmacological effects of a D_2-antagonist can be significantly enhanced by additional α_{2C}-antagonism, supporting the idea that an increased α_{2C} /D_2 ratio might be related to antipsychotic efficacy (this was subsequently reported in **Manuscript A**), and (iii) it enabled us to select a dose for assessing striatal BDNF levels for publication purposes, namely the 0.01 mg/kg dose. This data is reported in **Manuscript A**.

This addendum was thus vital in producing the data as reported in **Manuscript A**.

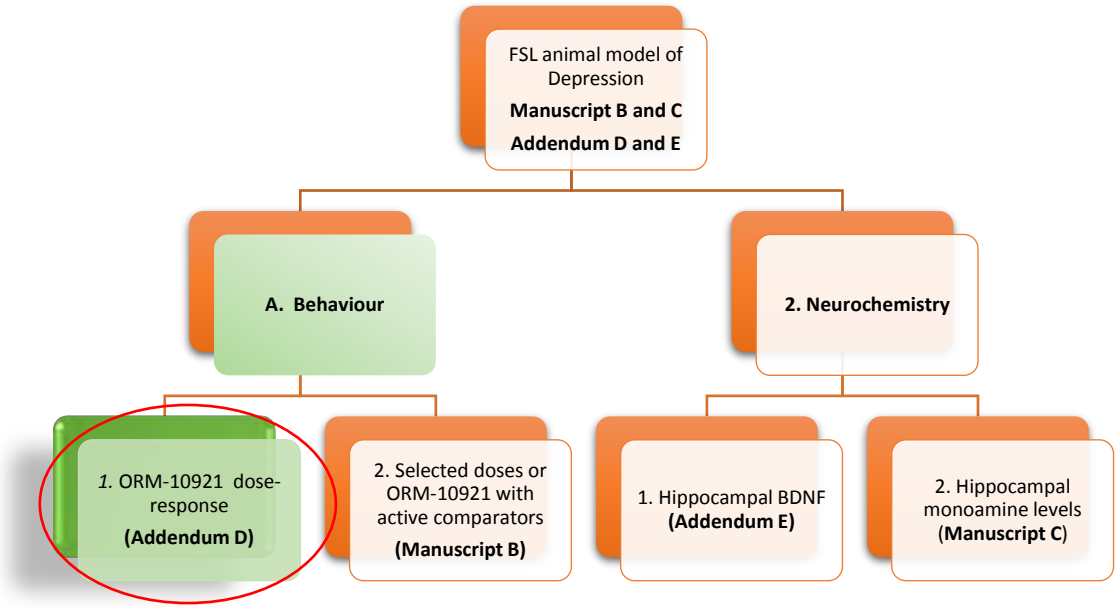
Addendum D

Antidepressant-like and pro-cognitive effects of ORM-10921 in the FSL animal model of depression

Preamble

The following section will report and discuss the behavioural dose-response data leading to that reported in **Manuscript B**. Five doses of ORM-10921 were assessed, of which the two most effective doses (0.03 and 0.3 mg/kg) were reported in comparison with imipramine (IMI) and idazoxan (IDAZ) treatment in **Manuscript B**. The FSL controls necessary for this dose-response study was also applied in the preparation of **Manuscript B**. However, in order to report on the dose-response effects of ORM-10921, FSL controls, ORM 0.03, and ORM 0.3 mg/kg are reported here again. The FSL vs. FRL validation in the forced swim test (FST) and novel object recognition test (NORT) was reported in **Manuscript B**, and will therefore not be reported here again. Furthermore, predictive validity for the FSL model was reported in **Manuscript B**, with IMI decreasing immobility, while IDAZ did not affect immobility in FSL animals. This section contains an additional validation of the FSL animal model with regards to predictive validity in the NORT, assessing whether FSL animals would respond to a known antidepressant, IMI, but not to a drug that is ineffective clinically for the treatment of depression, viz. IDAZ. This additional validation in the NORT was not included in **Manuscript B** due to the adverse locomotor effects of IMI on object exploration in the NORT, as described in **Manuscript B**. This resulted in a low number of IMI-treated animals meeting the inclusion criteria. This number was deemed to not be adequate for inclusion in a multiple analysis for international publication.

The diagram on the next page depicts where this addendum fits into the FSL study design and into the thesis layout, as indicated by the red circle.



1. Aims

The main aims of this investigation was to employ an animal model of depression to investigate whether chronic treatment with ORM-10921 has antidepressant-like and pro-cognitive effects in a translational preclinical paradigm. The Flinders Sensitive Line (FSL) genetic animal model of depression was employed. This study had two main aims:

1.1. Predictive validity of the FSL model of depression

1. To determine whether depressive-like behaviour and cognitive impairment in the NORT in drug-naïve FSL animals, as reported in **Manuscript B**, are reversed by a known reference antidepressant, imipramine (IMI) in FSL animals, but not by the non-selective α_2-AR-antagonist, idazoxan (IDAZ). IDAZ is not known for its clinical antidepressant activity and is thus not expected to exert any therapeutic effects in the NORT. The validation in the FST was reported in **Manuscript B** and will not be reported here again. These findings will validate the later application of the model to explore a dose-response analysis for ORM-10921.

1.2 To determine whether α_{2C}-AR selective antagonism with ORM-10921 has antidepressant-like and pro-cognitive effects in FSL animals

1. To conduct a dose-response analysis of 5 doses of ORM-10921 in order to determine which (if any) of the doses would display antidepressant-like effects in FSL animals in the FST and pro-cognitive effects in the NORT.

2. To select the most effective doses of ORM-10921 for comparison with the reference antidepressant, IMI and the non-selective α_2-AR-antagonist, IDAZ, following chronic treatment on antidepressant-like and pro-cognitive behaviour in the FST and NORT, respectively. These results were reported and published in **Manuscript B** and are thus not reported in this addendum**.**

2. Methods

2.1 Locomotor activity

Altered locomotor activity might be a complicating factor in interpreting strain or drug effects in the FST, since decreased or increased locomotor activity could increase or decrease (respectively) immobile behaviour in this test (Slattery and Cryan, 2012).

Procedures for determining locomotor activity have been reported in **Manuscript B**. Briefly, locomotor activity was determined immediately prior to the FST on day 14. General locomotor activity was assessed for 5 minutes using a Digiscan Animal Activity Monitor (*Omnitech* Electronics, Columbus, OH, USA) as described in **Manuscript B**. Horizontal activity was measured as the distance travelled in cm as determined by horizontal beam breaks.

2.2 FST

The FST is a well-described predictive model for antidepressant drug screening (Petit-Demouliere *et al.,* 2005; Porsolt *et al.,* 1978). The procedure applied in conducting the FST is described in **Manuscript B.** Briefly, the FST was conducted 12 hours after the final drug administration on day 14 of drug treatment. As is commonly done in other FSL studies (du Jardin *et al*., 2016; Eskelund *et al*., 2016; Gomez-Galan *et al*., 2013), no pre-swim was applied in this study since FSL rats innately show higher immobility than FRL controls (Overstreet *et al.,* 2005; Pucilowski and Overstreet 1993; Schiller *et al.,* 1992). Swim activity was recorded digitally for 7 min, with the 1st and last minute of the recording discarded, and 5 minutes of swim activity recorded by a researcher blind to the treatment groups. Observations were divided into 5 s intervals during which the time spent displaying immobility and swimming or climbing/escape/struggling behaviour, were recorded according to the guidelines of Lucki (1997). Swimming is deemed an expression of serotonergic-driven behaviour with climbing being deemed noradrenergic-driven behaviour (Detke *et al.,* 1995).

2.3 NORT

The NORT is a measure of declarative recognition memory, which relies on the innate preference of rats to explore novel objects more than familiar objects (Ennaceur and Delacour, 1988). The NORT was conducted on day 12 and 13, 12 hours after drug treatment as described in **Addendum A** and

Manuscript A and **B**. Briefly, 24 hours after a 10 minute habituation exposure to the NORT box, a 5 minute acquisition trial allowed the subject to explore two identical objects. The animal was returned to its home cage and 90 minutes later the recognition trial allowed the subject to explore one familiar object and one novel object. Time spent exploring the novel and familiar objects were recorded. Object exploration was considered as active sniffing, licking or physical exploration of the object. Animals that failed to accumulate a total exploration time of ≥6 seconds or who failed to explore both objects were excluded from the study (du Jardin *et al*., 2016). Essentially, more exploration time at the novel object reflects accurate recollection that the familiar object has been presented before, thus prompting greater exploration of the novel object. Object recognition was expressed as the discrimination index (DI) calculated according to the formula: % DI = [(time spent exploring novel object – time spent exploring familiar object)/(total time spent exploring both objects) x100]. A higher DI indicates improved declarative recognition memory.

2.4 Statistical analysis

2.4.1 Predictive validity of the FSL model

Normality of data was determined using the Shapiro Wilk test, since this test is highly recommended for the n-range of data included in this section and has very good statistical power to detect a non-guassian population (Ghasemi and Zahediasl, 2012; Razali and Wah, 2011), as motivated in Addenda A-C. To analyze whether FSL animals treated for 14 days with either vehicle, ORM-10921, IDAZ or IMI displayed differences in recognition memory, ANOVA and Tukey's non-parametric counterparts - the Kruskal-Wallis ANOVA and Dunn's multiple comparison test, were used as motivated by the prinicples described in **Addendum A**, section *2.4.1*.

2.4.2 Dose-response

For the dose-response, ANOVA was used with Fisher's Least Significant Difference post-hoc multiple comparison test (LSD), which does not correct for multiple comparisons as the Tukey test does. The rationale of not correcting for multiple comparisons with a Tukey test in a simple dose-response analysis, is given in Addendum A, section *2.4.*2. Briefly, Tukey increases the chance of making a Type II statistical error (false negative) (Banerjee et al., 2009), while the aim of this dose reponse study was to establish which doses of the drug is effective vs. control, and not to determine if different doses of the drug were more effective compared to other doses. Fisher's LSD test was deemed most appropriate to this end, since it decreases the chance of making a Type II error, and is often used in the literature when multiple doses of the same compound are tested for efficacy (Bachtell et al., 2005; Debrah et al., 2005;

Kufahl et al., 2011; Labonte et al., 2012; Newman and Beardsley, 2006; Ruxton and Beauchamp, 2008). Favouring a Type I statistical error (false positive) in the dose-response analyses would enable us to decide identify (an) effective dose(s) to submit to more rigourous multiple comparisons including the positive and negative controls. This would allow preparation of the data for publication (Nickerson, 2000), and is reported in **Manuscript B.**

3. Results and Discussion

3.1. Predictive Validity for the Novel Object Recognition Test (NORT) in the FSL model

In the NORT, a number of parameters can be assessed, as described in **Addendum A**, section *3.1.2*. The time spent exploring the familiar object vs. the time spent exploring the novel object can be determined using paired t-tests or Wilcoxon ranked matched-pairs tests (non-parametric data) to assess whether a certain cohort spent more time exploring the novel object. This measure is however not helpful for between-group comparisons, since it does not correct for the total time spent exploring both objects. However, the discrimination index (DI) is a measure of the difference in exploration time for the two objects, corrected for the total time spent exploring both objects by dividing this difference by the total exploration time and allows for between-group comparisons (Antunes and Biala, 2012; Broadbent et al., 2010). This index of discrimination is a much more robust index of memory retention for the familiar object and thus preference to explore the novel object. A minimum total exploration time of 6 seconds was used as an inclusion criterion for the test, as well as exploration of both objects (du Jardin et al., 2016).

Total exploration times of FSL and FRL controls are depicted in Fig D-1-A. Total exploration times for FSL vs. FRL animals are not reported in Manuscript B, and are therefore reported here. Unpaired t-test indicated no significant differences between total exploration times of FRL vs. FSL animals (p=0.2). The total exploration times of FSL animals treated with vehicle, IMI or IDAZ are reported in Fig D-1-B. Kruskal-Wallis ANOVA indicated a difference in exploration times of FSL animals treated with vehicle, IMI or IDAZ, with Dunn's post hoc comparison test indicating that IMI-treated animals spent significantly less time exploring than IDAZ-treated animals (p=0.02)

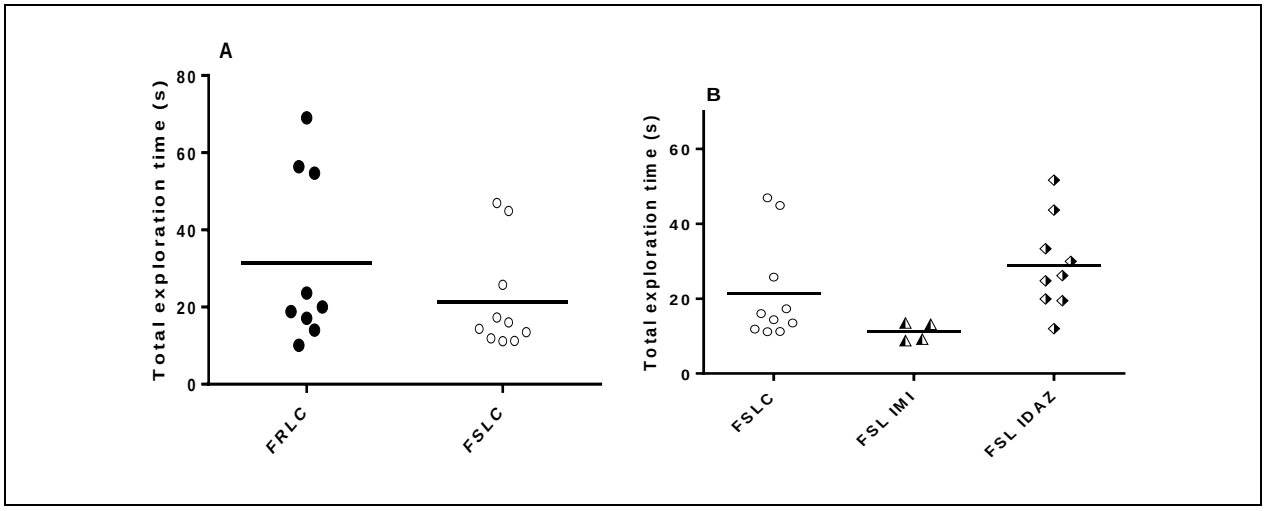


Fig. D-1 Total exploration times for **A**) FRL and FSL controls and **B**) FSL treatment groups for subjects that met the inclusion criteria in the recognition trial of the NORT (n=4-10) FRLC = FRL Controls, FSLC = FSL Controls, IMI = FSL+imipramine 15 mg/kg, ORM = FSL+ORM-10921, IDAZ = FSL+idazoxan 3 mg/kg.

Only 4 of the 11 subjects treated with IMI met the minimum inclusion criteria of total exploration time of more than 6 seconds, while 5 of the IMI subjects showed zero exploration activity. This low amount of data points compromised our ability to interpret the validity of the results for the IMI group when compared to other cohorts and thus invalidated the NORT as a means of behavioural assessment for this treatment group. Although the effects of IMI in the NORT was therefore excluded from **Manuscript B** due to this reason, in the interest of reporting all relevant findings a multiple comparison analysis including IMI is reported in this addendum (Fig D-3), However, the small amount of animals are explicitly stated here as a limitation in interpreting the findings from this treatment group as depicted in Fig D-3.

Figure D-2 shows the time spent exploring both the novel and the familiar objects. Paired t-tests or Wilcoxon matched pairs test (non-parametric data) was conducted to determine whether more time was spent exploring the novel object for each individual subject. FSL Controls ($p=0.002$, Wilcoxon) and FSL animals treated with IMI for 14 days ($p=0.02$, Wilcoxon) spent significantly more time exploring the novel object than the familiar object. This was not true for the IDAZ FSL treatment group ($p=0.33$, paired t-test).

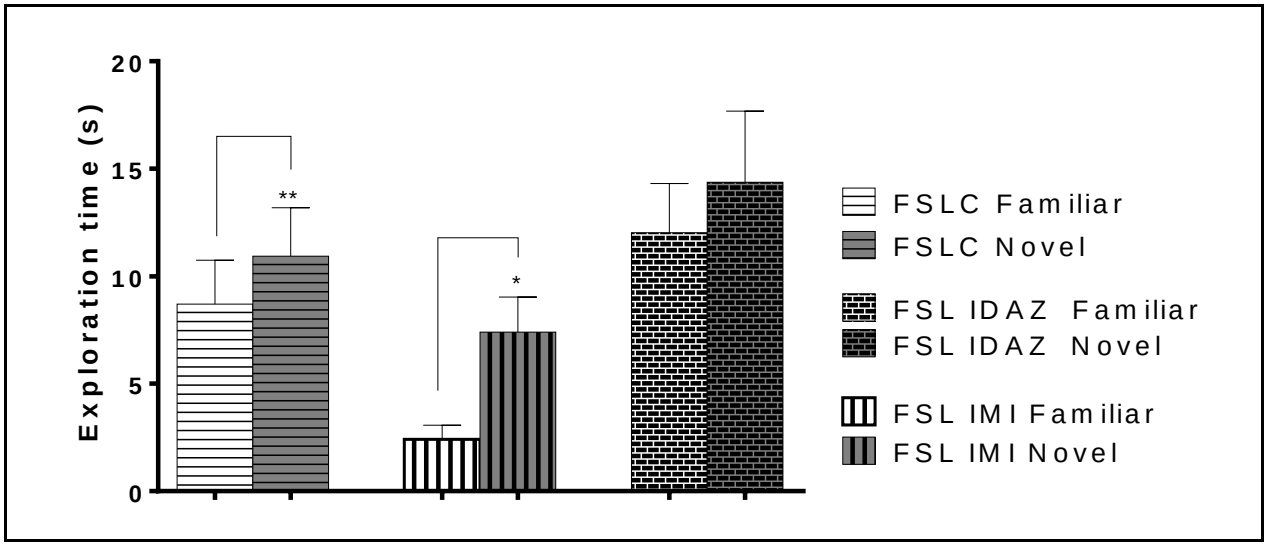


Fig. D-2 Time spent exploring novel and familiar objects in the NORT following the various drug treatments as indicated (n=4-10). FRL Controls received saline vehicle. All other drug treatments were conducted in FSL animals. Paired t-tests or Wilcoxon matched pairs test for novel vs. familiar objects for each drug treatment. *p<0.05, **p<0.01., FSLC= FSL Controls, IDAZ = FSL+idazoxan 3mg/kg, IMI= FSL+imipramine 15 mg/kg, Novel = Novel object, Familiar = Familiar object.

Figure D-3 shows the effects of treatment with IDAZ and IMI in FSL rats on recognition memory in the NORT in terms of the discrimination index (DI). Kruskal-Wallis ANOVA showed a significant effect of drug treatment on the DI in FSL animals (Kruskal-Wallis statistic 8.91; p=0.006). Dunn's post hoc multiple comparison indicated that FSL animals treated with IMI showed significantly improved recognition memory vs. FSL controls (p=0.013) and vs. FSL animals treated with IDAZ (p=0.02).

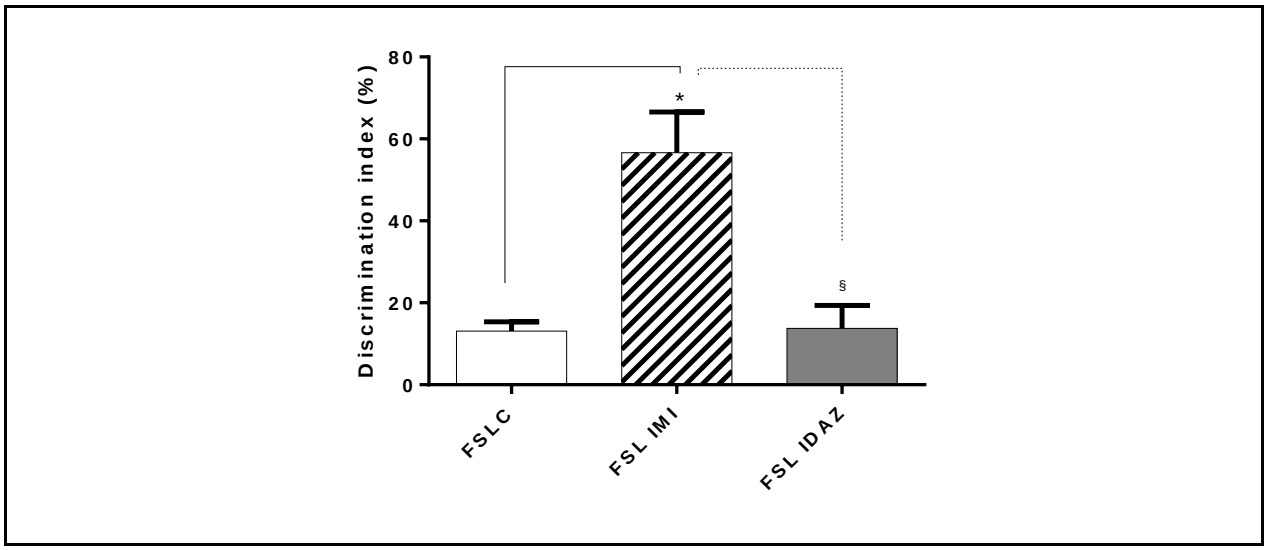


Fig D-3. Discrimination indices of animals treated for 14 days with either vehicle, IMI or IDAZ (n=4-10 per group). Kruskal Wallis and Dunn's Multiple Comparison test *p<0.05 vs. FSL Controls, §p<0.05 vs. IDAZ drug treatment in FSL. FSLC = FSL Controls, IMI = FSL+imipramine 15 mg/kg, IDAZ = FSL+idazoxan 3 mg/kg.

3.2. Dose-response analysis of ORM-10921

3.2.1.1 Locomotor activity

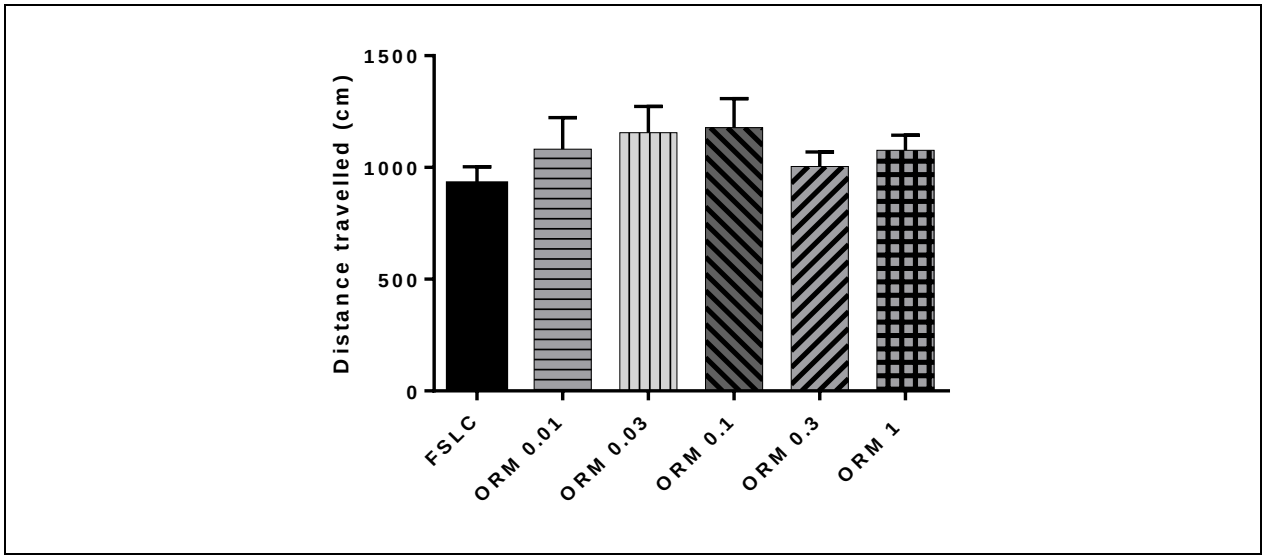


Fig D-4 Locomotor activity of FSL animals treated with saline vehicle or the various doses of ORM-10921 as indicated (n=9-11 per group). ANOVA and Tukey's post hoc comparison. ORM = FSL+ORM-10921 0.01; 0.03; 0.1; 0.3 and 1 mg/kg.

Fig D-4 indicates the locomotor activities of FSL animals treated with either saline vehicle or with various doses of ORM-10921. One-way ANOVA showed no significant differences in locomotor activity between the various groups ($F(5,54)=0.809$, $p=0.548$).

3.2.1.2 Forced Swim Test

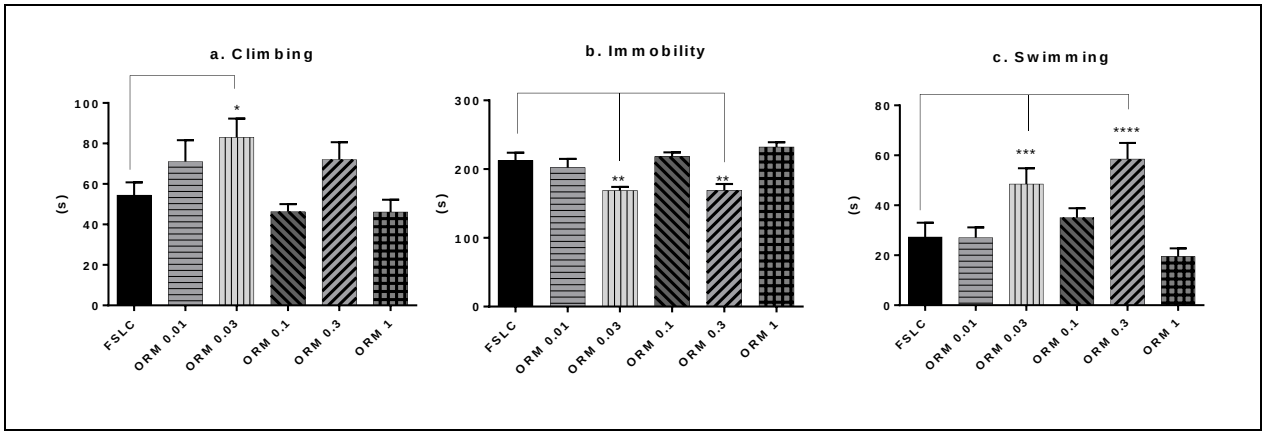


Fig D-5. Climbing (a), immobility (b) and swimming (c) in the FST in FSL animals treated subcutaneously with vehicle or the various drug treatments, as indicated (n=9-11 per group). ANOVA and Tukey's Multiple Comparison * p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs. FSLC. FSLC = FSL controls, ORM = FSL+ORM-10921 0.01; 0.03; 0.1; 0.3 and 1 mg/kg.

Figure D-5 depicts time spent displaying climbing (a), immobility (b) and swimming (c) behaviour in the FST. One-way ANOVA showed a significant effect of drug treatment on climbing ($F(5,54)=3.527$; $p=0.008$), with Fisher's LSD test indicating that ORM-10921 0.03 mg/kg significantly increased climbing behaviour vs. FSL controls ($p=0.01$). Regarding immobility, ANOVA showed a significant effect of drug treatment ($F(5,54)=7.78$; $p<0.0001$), with the 0.03 and 0.3 mg/kg doses of ORM-10921 decreasing immobility significantly vs. FSL controls ($p=0.001$ for both doses). One-way ANOVA showed a significant effect of drug treatment on swimming ($F(5,54)=8.148$; $p<0.0001$) with Fisher's LSD test indicating that the 0.03 mg/kg dose ($p=0.005$) and the 0.3 mg/kg dose ($p<0.0001$) significantly increased swimming vs. FSL controls. ORM-10921 0.01; 0.1 and 1 mg/kg did not significantly affect climbing, immobility or swimming in the FST.

3.2.1.3 NORT

The total exploration times are shown in figure D-6. One-way ANOVA showed that there was a significant difference between the exploration time of the different treatment groups ($F(5,46)=2.880$; $p=0.024$), with Fisher's LSD indicating that ORM 0.01 mg/kg spent significantly less time exploring both objects compared to ORM 0.03mg/kg ($p=0.04$). Since this difference did not affect the outcome of the DI in the NORT and was not correlated with altered locomotor activity (Fig D-4), it was assumed that this would not affect the outcome of the statistical analysis. No groups showed any statistically significant difference compared to FSL Controls.

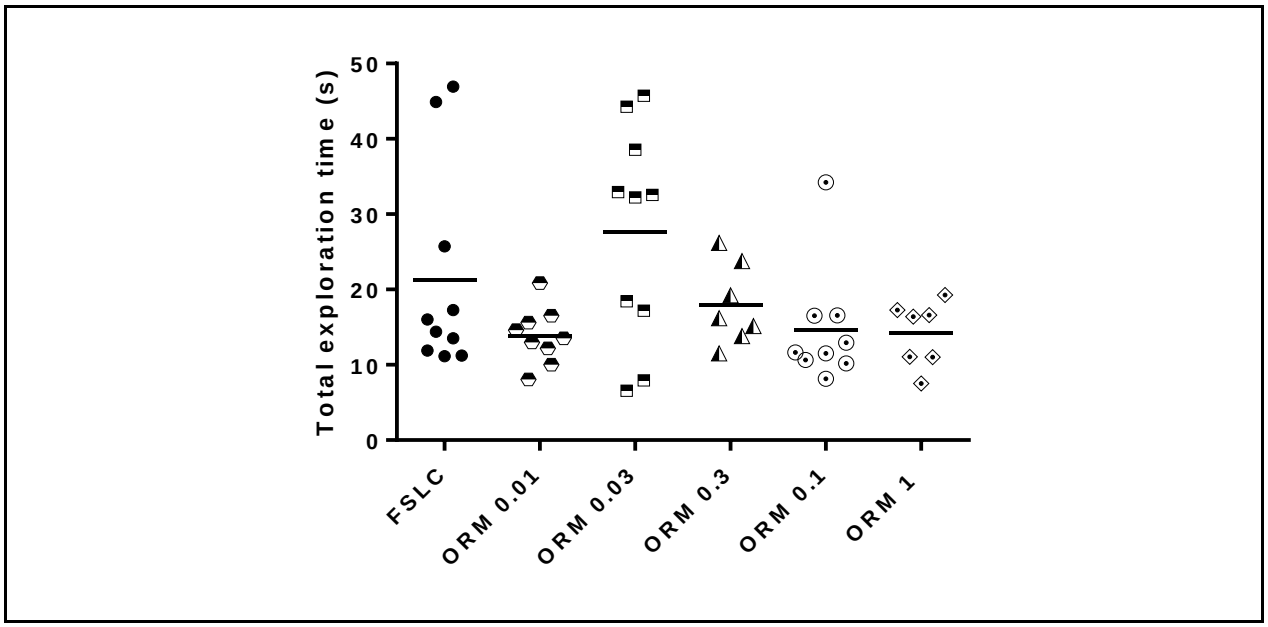


Fig. D-6 Total exploration times for subjects that met the inclusion criteria in the recognition trial of the NORT (n=7-10). FSLC = FSL Controls, ORM = FSL+ORM-10921 at 0.01; 0.03; 0.1; 0.3 and 1 mg/kg.

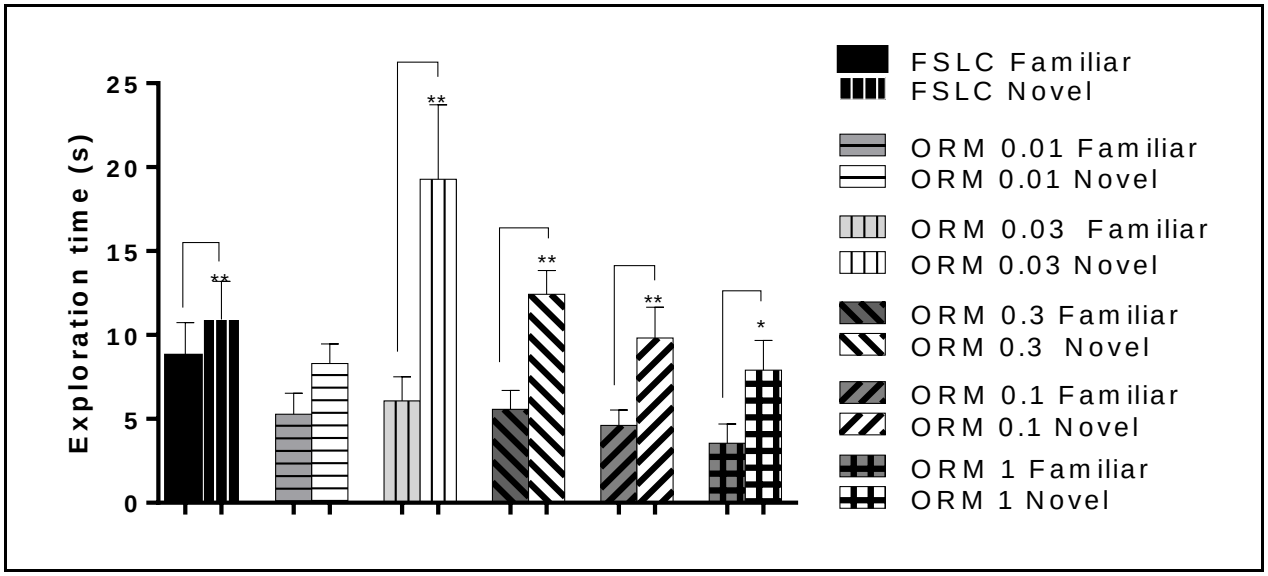


Fig. D-7 Time spent exploring novel and familiar objects in the NORT following the various drug treatments as indicated (n=7-10). All drug treatments were conducted in FSL animals. Paired t-tests or Wilcoxon matched pairs test for novel vs. familiar objects for each drug treatment. *p<0.05, **p<0.01. FSLC= FSL Controls, ORM= FSL+ORM-10921 0.01; 0.03; 0.1; 0.3 and 1mg/kg, Novel = Novel object, Familiar = Familiar object.

Fig D-7 depicts the time spent exploring the novel vs. familiar object for all treatment groups. Here, paired t-test showed no significant difference between exploration time for novel vs. familiar objects in the ORM 0.01 mg/kg dose cohort (Fig 4; p=0.17, ns). However, paired t-test showed a statistically significant difference in novel vs. familiar object exploration time for ORM-10921 0.03; 0.3 and 1 mg/kg cohorts (0.03mg/kg: p=0.005 and 0.3 mg/kg: p=0.004 and ORM 1 mg/kg p=0.03), while Wilcoxon matched-pairs signed rank test showed that ORM 0.1 mg/kg also spent more time exploring the novel object. FSLC object exploration is reported in section 3.1 of this addendum.

The DI in FSL animals treated with saline vehicle or several doses of ORM-10921 is depicted in Fig D-8, with ANOVA showing significant effects of drug treatment on object recognition DI ($F(5,46)=4.451$; $p=0.002$). Fisher's LSD test indicated that ORM 0.01 mg/kg (p=0.02), ORM 0.03mg/kg (p=0.001), 0.1mg/kg (p=0.004), 0.3 mg/kg (p=0.004) and 1 mg/kg (p=0.004) all improved the DI significantly compared to FSL controls.

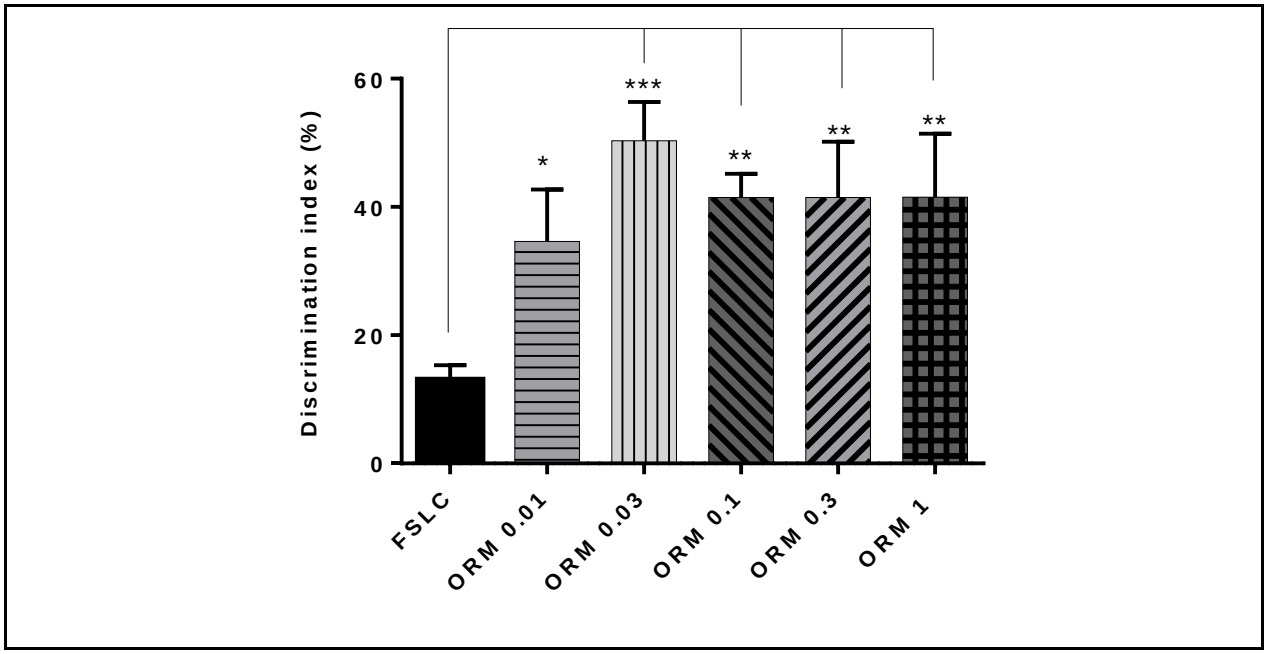


Fig. D-8 Discrimination index (DI) in the NORT in FSL animals treated with the various doses of ORM-10921 (n=7-10). ANOVA and Fisher LSD.*p<0.05, ** p<0.01, ***p<0.001 vs. FSLC. FSLC = FSL Controls, ORM = FSL+ORM-10921 at 0.01; 0.03; 0.1; 0.3 and 1 mg/kg.

4. Discussion

4.1 Validation of the FSL model

Although the main findings for face and predictive validity of the FSL model have been discussed in **Manuscript B**, what was not addressed there was predictive validity for IMI in FSL rats in the NORT, due to the low number of FSL IMI-treated subjects meeting the minimum criteria for exploratory activity. Thus, in **Manuscript B** face validity was established by demonstrating that FSL rats presented with increased immobility and decreased climbing in the FST vs. FRL rats, as well as impaired recognition memory in the NORT, while the effects on FST immobility could be reversed by chronic treatment with IMI in FSL animals. Here we will discuss the predictive validity for FSL rats as demonstrated in the NORT. Apart from increased swim test immobility, the FSL rat presents with deficits in object recognition memory vs. its non-depressive-like phenotype, the FRL control rat (Abildgaard et al., 2011; Overstreet et al., 2005; Overstreet and Wegener, 2013). This deficit is reported in **Manuscript B**, and found to be independent of any confounding effects on locomotor activity, constituting face validity for the use of this model.

While major depressive disorder also presents with declarative memory deficits (Campbell and MacQueen, 2004), we wanted to extend our predictive validation to the NORT, considering that this

study aimed to establish whether a novel compound elicits pro-cognitive effects in a model of depression.

Manuscript B elaborates on the effects of IMI on locomotor activity and the effect of altered locomotor activity on NORT exploration. In **Manuscript B**, however, the effects of IMI on object memory in the NORT was not included since the low number of animals (4) that met the inclusion criteria were deemed to not be adequate for inclusion in a multiple comparison test required for international publication. To briefly summarise the discussion in **Manuscript B** (Chapter 4), although findings in the literature are not always consistent (Strekalova et al., 2013), chronic treatment with IMI *does* decrease locomotor activity in rodents (Diniz et al., 2011; Kos et al., 2006) and in felines (Zagrodzka et al., 1987) and for that matter also causes sedation in humans, factors that would negatively affect exploration activity (Mogensen et al., 1994) as observed in the NORT. Although there was a low exploration rate in IMI-treated animals, the animals that did explore in the NORT showed a significant increase in object recognition memory, in agreement with the pro-cognitive effects of IMI described in the literature (Papp et al., 2016; Rozza et al., 2012) and with its clinical effects in depressives (Peselow et al., 1991). That being said, these pro-cognitive findings have not been consistently corroborated in other tests for cognition (Naudon et al., 2007; Zarrindast et al., 2003). Nevertheless, the small amount of animals that produced these findings for IMI compromises the interpretation of these data significantly. However, IDAZ failed to reverse the NORT memory deficit in FSL animals, as discussed in **Manuscript B.** While the beneficial effects of non-selective α_2 agonists, like guanfacine, clonidine and medetomidine in aspects of memory and cognition are well-documented (Arnsten et al., 1988; Arnsten and Cai, 1993; Björklund et al., 2001; Cai et al., 1993; Carlson et al., 1992; Franowicz et al., 2002), the beneficial effects of α_2 antagonists, including atipamezole and IDAZ have recently been reported in rodent models of Alzheimer's disease (Chen et al., 2014; Gannon et al., 2015). Less information is available on the cognitive effects of a α_2-AR antagonism in an animal model of depression. The current finding that IDAZ does not improve memory in the NORT is in agreement with the literature reporting a beneficial effect of α_2 agonism on cognitive parameters as cited above. Lack of improvement in NORT memory in FSL animals receiving IDAZ also strengthens predictive validity of the FSL model as presented in this study. **Manuscript B** elaborates on possible α_2-AR subtype mechanisms that might explain why IDAZ did not improve NORT memory.

Face and predictive validity of the FSL model has thus been established, enabling us to determine whether selective α_{2C}-AR antagonism with ORM-10921 presents with antidepressant-like and pro-cognitive effects in this animal model of depression, and that now can be exploited for further study into the pharmacological response to ORM-10921.

4.2 Antidepressant-like and pro-cognitive effects of ORM-10921 in FSL animals

14 day treatment with ORM-10921 showed anti-depressant-like and pro-cognitive effects in the FST and NORT respectively, not attributable to altered locomotor activity. This confirms findings in transgenic mice that α_{2C}-AR deletion (Sallinen et al., 1999), but not α_{2A}-AR deletion (Schramm et al., 2001), produces antidepressant like effects in the FST as well as beneficial effects on cognitive parameters (Björklund et al., 2001; Franowicz et al., 2002). It is also in line with antidepressant-like and pro-cognitive effects reported for highly selective α_{2C}-AR antagonists JP-1302, ORM-10921 and ORM-12741 in Han-Wistar and Sprague Dawley rats. These findings have now been extended for the first time to a translational animal model of depression, the FSL genetic model, providing evidence of antidepressant-like effects of α_{2C}-AR antagonism in a model with good face, predictive and construct validity and therefore improved translational value.

While all doses of ORM-10921 improved recognition memory in the NORT in FSL animals, only the 0.03 mg/kg and 0.3 mg/kg doses were effective in decreasing immobility in the FST. Moreover, these effects seemed to be associated with increased climbing and increased swimming behaviour, indicating a possible noradrenergic and serotonergic mechanism, respectively (Detke et al., 1997). The results obtained with ORM-10921 0.03 and 0.3 mg/kg are discussed in more detail in **Manuscript B**, where these effective doses are also compared to non-selective α_2-AR antagonism with IDAZ, as well as to the effects of the reference antidepressant, IMI. While the lack of efficacy of the lower dose of 0.01mg/kg in the FST might be ascribed to a too low dose, lack of efficacy at the higher dose of 1 mg/kg in the FST might be ascribed to increased engagement of α_{2A}-AR resulting in dual engagement at both α_{2A}- and α_{2C}-AR's thereby decreasing the efficacy of α_{2C}-AR selective antagonism on FST immobility (Schramm et al., 2001). ORM-10921 demonstrates a 100-fold selectivity for α_{2C} vs. α_{2A}-ARs, and it is possible that across the 100-fold dose increase (0.01 mg/kg – 1mg/kg) this selectivity may at a point become compromised (Sallinen et al., 2013a). However, it is more difficult to explain why the middle dose of 0.1mg/kg did not decrease immobility in the FST, especially since it proved equally effective in improving recognition memory in the NORT compared to the 0.03 and 0.3 mg/kg doses. It may have basis in the differential expression of α_{2C}-ARs in brain regions controlling mood and cognition, or specifically those regions implicated in behavioural responses prompted by exposure of the animal to the FST and NORT. Further study into this suggestion is warranted.

From these data it is clear that the dose-response relationship of ORM-10921 appears not to follow a linear relationship. When the results in the NORT are considered, ORM-10921 demonstrates an inverted U-shaped curve with the lowest dose being marginally effective in improving memory, while the highest dose is ineffective. Possibly there is a threshold concentration that needs to be reached in order for the α_{2C}-AR to be occupied at a rate sufficient to induce beneficial effects on memory and depressive-like

behaviour. This is speculative however, and needs to be corroborated with pharmacokinetic studies of ORM-10921 in FSL animals, since the rate of metabolism in these animals could influence response to various doses, with minimum plasma concentrations required for therapeutic efficacy perhaps not reached at the lower dose of ORM-10921. The higher the dosage, however, the more α_{2C}-ARs are theoretically likely to be engaged. An increased dose would also eventually lead to an increased amount of α_{2A}-AR-engagement, antagonism of which could be detrimental both in the FST (Schramm et al., 2001) and in parameters assessing cognition (Franowicz et al., 2002), as the higher dose of ORM-10921 (1 mg/kg) would indicate in this study. Further to this point, studies in rodents and primates have suggested that cognitive function follows an inverted U-shaped pattern with regards to prefrontal cortical noradrenergic activity (Montoya et al., 2016), with low noradrenergic levels activating α_2-ARs and strengthening neural inputs that facilitate cognitive processes, while conditions resulting in reduced NA release into the PFC will reduce these neural inputs and result in impairment of cognitive function (Hains and Arnsten, 2008). During conditions of stress, on the other hand, excessive NA release will activate α_1-ARs and β-ARs which may impair cognitive function (Arnsten, 2009). Higher doses of ORM-10921 and dual engagement of both α_{2C}-ARs and α_{2A}-ARs could therefore lead to excessive increases in synaptic NA release that may compromise any beneficial effects on cognition afforded by the more subtle noradrenergic effects of selective α_{2C}-AR antagonism (Bücheler et al., 2002; Hein et al., 1999). In vivo microdialysis studies that assess extracellular neurotransmitter release will be vital in determining the underlying mechanisms resulting in this inverted U-shaped dose-response curve. Additionally, receptor occupation studies will be equally necessary to establish the role of receptor subtype engagement in the observed dose-response effect of ORM-10921.

Although the above-mentioned inverted U-shaped dose-response curve was not replicated in the FST due to the lack of efficacy of the 0.1 mg/kg dose of ORM-10921, the dose-response curve seen in the FST cannot be easily explained without additional information on receptor occupation and monoamine release at this dose. Moreover, this phenomenon may also represent an unexplained anomaly that is sometimes observed when studying behaviour in animals, especially when considering sample sizes employed in animal research. This highlights the importance of replicating these findings in further studies, perhaps using a larger sample size.

This study thus delivered two key outcomes: it demonstrated that ORM-10921 does indeed demonstrate antidepressant-like and pro-cognitive effects in an animal model of depression, effects which are closely related to dose. These effective doses were subsequently applied in **Mansucript B.** Furthermore, it enabled us to identify dose ranges in which to study neurochemical effects on brain tissue. The doses effective in the FST and NORT, i.e. 0.03 mg/kg, and 0.3 mg/kg, were chosen to assess effects on hippocampal BDNF levels, with the controversial 0.1 mg/kg dose included as it occupies the

middle of the dose range between the aforementioned two effective doses. These results are reported in **Manuscript C** (Chapter 5).

Addendum E

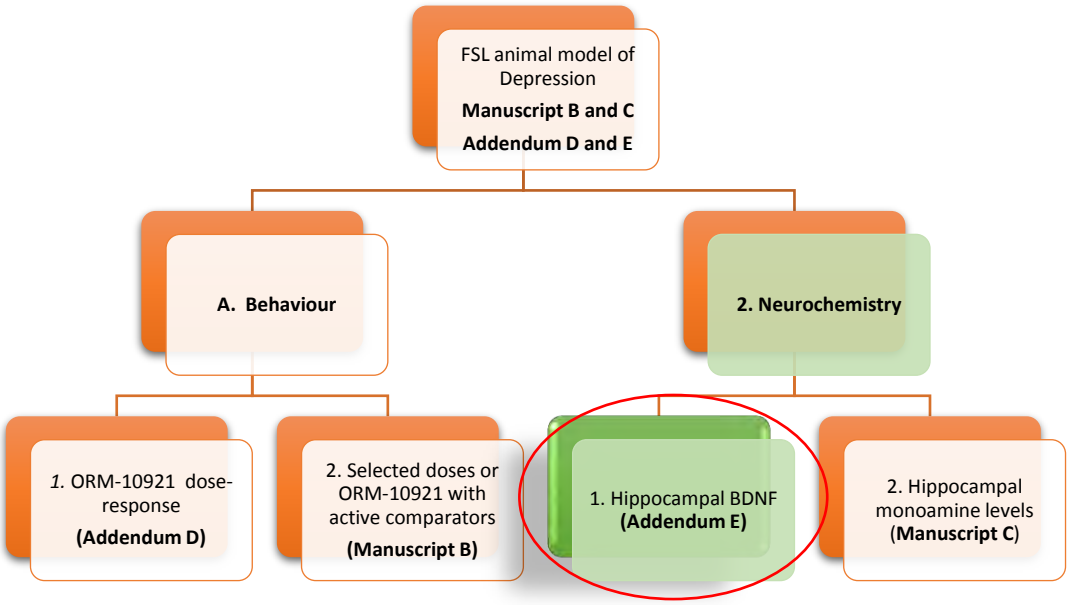
Analysis of effects of ORM-10921 on hippocampal BDNF levels in FSL rats

Preamble

During the preparation of the thesis it became apparent that a disparity between the behavioural effects demonstrated by ORM-10921 and its effects on hippocampal BDNF levels prevented its inclusion in **Manuscript B**. It was therefore decided to exclude the BDNF data from **Manuscript B** in order to retain maximum impact on the behavioural data.

Therefore, the BDNF data from the FSL study is represented in its entirety in this addendum. Of the 5 doses of ORM-10921 employed in the behavioural studies (see **Addendum D**), the 3 most effective doses were employed in the assessment of the hippocampal BDNF response, while the dose with the most prominent effect on BDNF and behaviour was selected for a comparative analysis with imipramine (IMI) and idazoxan (IDAZ).

The diagram below depicts where this addendum fits into the FSL study design and into the thesis layout.



1. Aims

1.1 Validation of construct validity of the FSL animal model of depression

BDNF plays an important role in synaptic plasticity, being implicated in the processes underlying aspects of cognition, memory and the pathology of depression (Brand et al., 2015; Neto et al., 2011). To date, only one study has reported that FSL rats display reduced hippocampal BDNF levels compared to FRL rats (Elving et al. 2010a), so that more studies in this regard are needed to add to the construct validity of the model as a translational model of depression. The aim in this study was to determine:

1. Whether FSL animals present with reduced hippocampal BDNF levels compared to FRL rats.
2. Whether alterations in hippocampal BDNF levels in FSL animals can be reversed by the reference tricyclic antidepressant, IMI.
3. Whether, the non-selective α_2-AR-antagonist, IDAZ, which is not clinically used as an antidepressant and not known to increase BDNF levels in animal models of depression, would alter hippocampal BDNF levels in FSL animals. The latter data would reinforce the use of IDAZ as a negative control in a comparative study versus ORM-10921 described in **Manuscript B** and **C**.

1.2 To determine whether α_{2C}-AR selective antagonism with ORM-10921 affects the expression of hippocampal BDNF in FSL animals

1. To determine whether various doses of ORM-10921 alter hippocampal BDNF levels in any way.
2. To determine how the effects of a selected dose of ORM-10921 compares to that of IMI and IDAZ.

In order to achieve the aims of this investigation we also needed:

- To generate standard protein concentration curves
- To generate BDNF standard concentration curves

2. Methods

2.1 Animals and drug treatment

FRL and FSL animals were reared according to the description in **Manuscript B** and **C**. FRL and FSL control animals were treated with saline vehicle as described in **Manuscript B** and **C**. FSL animals were treated with IMI 15 mg/kg, IDAZ 3 mg/kg and ORM-10921 0.03, 0.1 and 0.3 mg/kg as described in **Manuscript B** and **C.**

2.2 Brain homogenate preparation

24 hours after the last drug administration, animals were sacrificed by decapitation and the whole brain quickly removed and briefly placed in ice cold double distilled water, with the brain tissue dissected out

immediately on an ice cooled slab. After the frontal cortex was removed the brain was placed on its ventral side. The hindbrain was removed and a saggital incision made to separate the cerebral hemispheres. These were gently lifted with the cortex side now facing down. Non-cortical brain tissue was removed and the hippocampi were rapidly dissected out (Spijker, 2011). The hippocampi were snap-frozen in liquid nitrogen and transferred to a -70^{o}C freezer. On the day of the analysis, brain tissue was thawed on ice, weighed and then homogenized in acid extraction buffer (100µl/10mg brain tissue) according to the kit Manufacturer's instructions. The constituents of this buffer, as well as finer details on this method is reported in ***Appendix 1*** at the end of **Addendum B**. One *cOmplete™ Protease Inhibitor Coctail* tablet (Roche, South Africa) was added to 50 ml extraction buffer immediately prior to use. The brain tissue and extraction buffer was sonicated in 7-second bursts on ice with a probe sonicator and left to incubate on ice for 30 minutes, whereafter sonification and ice incubation was repeated once. Homogenates were centrifuged for 30 minutes at 11 000 *g* and 4^{o}C and the supernatants transferred to clean Eppendorf™ tubes. Sample protein content was determined by the Bradford Protein Assay.

2.3 Bradford protein assay

The Bradford protein assay (Bradford, 1976) was performed to determine protein content of samples. Two 96-well plates were needed to accommodate the amount of samples analysed. Cohorts were divided into groups of 3-4 samples, which were distributed across the 96 well plates so that each plate, each row and each column on both plates were equally represented by each study cohort. The Bradford assay was performed according to the methods and principles set out in **Addendum B**. Sample protein content for each plate (A and B) was determined using the linear formula generated by the respective standard curves as depicted in Figure E-1.

2.4 BDNF ELISA

Hippocampal BDNF levels were analyzed using a Biosensis® BDNF *Rapid*™ELISA Kit obtained from DivBio, Netherlands, capable of measuring tissue levels of mature BDNF and recently demonstrated to be a very reliable and accurate commercial kit for measuring BDNF in biological samples (Polacchini et al., 2015).

After a preliminary run to approximate the concentration range of BDNF in the sample pool, supernatants (prepared as described in 2.1 of this addendum) were diluted 100 times with the brain tissue sample diluent (refer to ***Appendix 1*** at the end of **Addendum B** for full method). Cohorts were divided into subgroups of 3-4 samples, which were distributed across two 96 well plates so that each plate, each row and each column on both plates were equally represented by each study cohort. All samples and standards were assayed in duplicate. The BDNF ELISA procedure was conducted as set out in **Addendum B** and ***Appendix 1*** of **Addendum B** and BDNF levels were expressed in terms of pg BDNF per mg sample protein, according to the kit Manufacturer's instructions.

2.5 Statistical analysis

2.5.1 Validation of the FSL model

Normality of data was determined using the Shapiro Wilk test as motivated in Addendum A - D. Student's unpaired t-tests were used to determine whether FSL and FRL controls displayed significant differences in hippocampal BDNF levels. Welsch's correction was applied to the t-test in the case of unequal standard deviations. To analyze whether FSL animals treated for 14 days with either vehicle, IDAZ or IMI altered BDNF levels, FSL treatment groups were analyzed with ANOVA and Tukey's multiple comparison test, as motivated in **Addendum D**. In the case of non-guassian data or where the assumption of equality of variances for ANOVA was not met (as indicated by the Brown-Forsythe test) data sets were analyzed with ANOVA and Tukey's non-parametric counterparts - the Kruskal-Wallis ANOVA and Dunn's multiple comparison test.

2.5.2 Dose-response effects of ORM-10921 and comparison to IDAZ and IMI

For the dose-response, ANOVA was used with Fischer's Least Significant Difference test (LSD), as motivated in Addendum A and D. The 0.03 mg/kg dose of ORM-10921 was then employed versus its comparators, IMI and IDAZ in FSL controls using ANOVA and Tukey's multiple comparison test.

3. Results

3.1 Generation of standard protein curves

The standard protein concentration curves are depicted in Figure E-1. The standard protein concentration curves indicated a linear regression of 0.983 and 0.97 for plates A and B respectively with the formulas as indicated in Fig E-1-A and E-1-B respectively used to calculate the protein content of each brain tissue homogenate sample. A coefficient of determination (r^2) of >0.95 is required for biological samples (Health Canada and Directorate, 1994; Shabir, 2006).

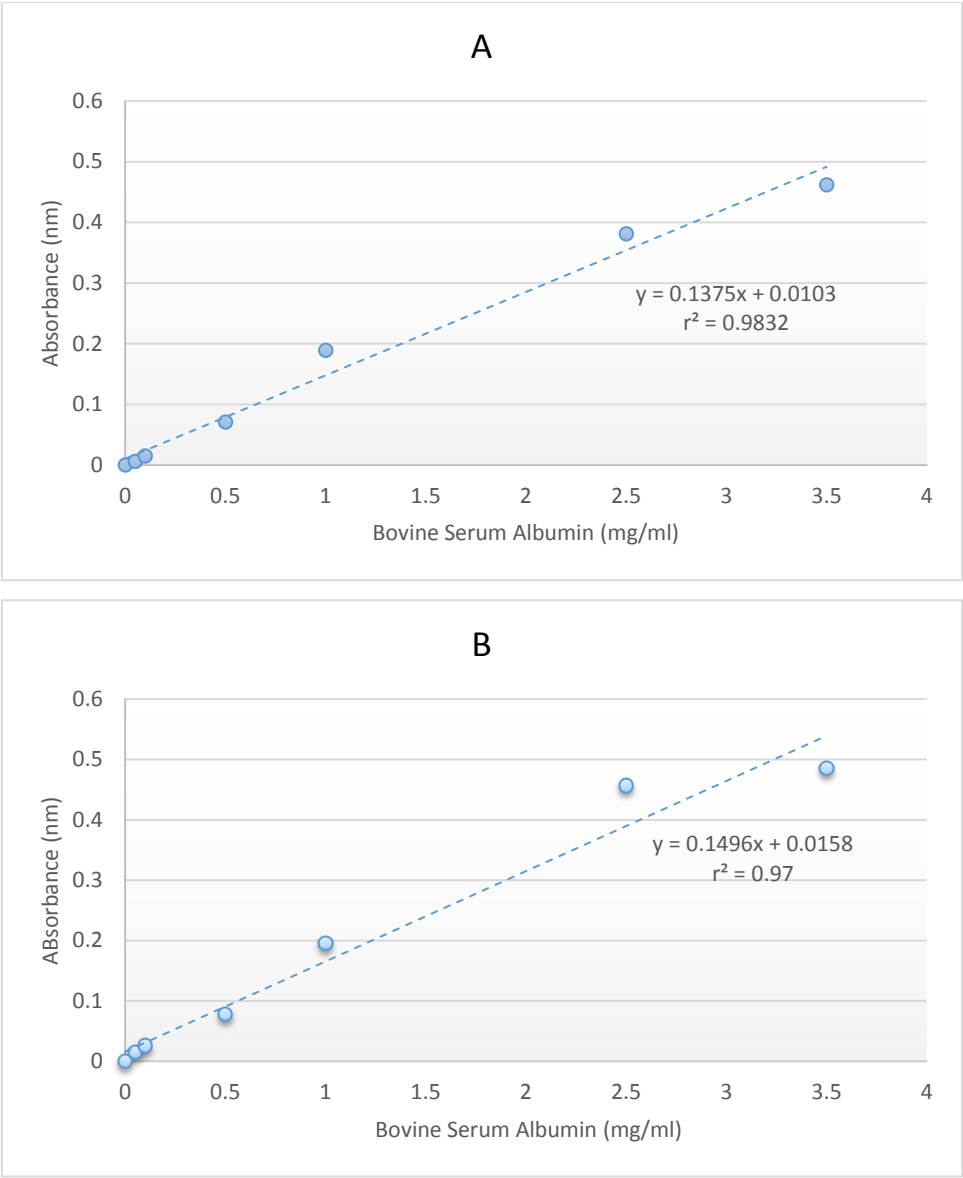
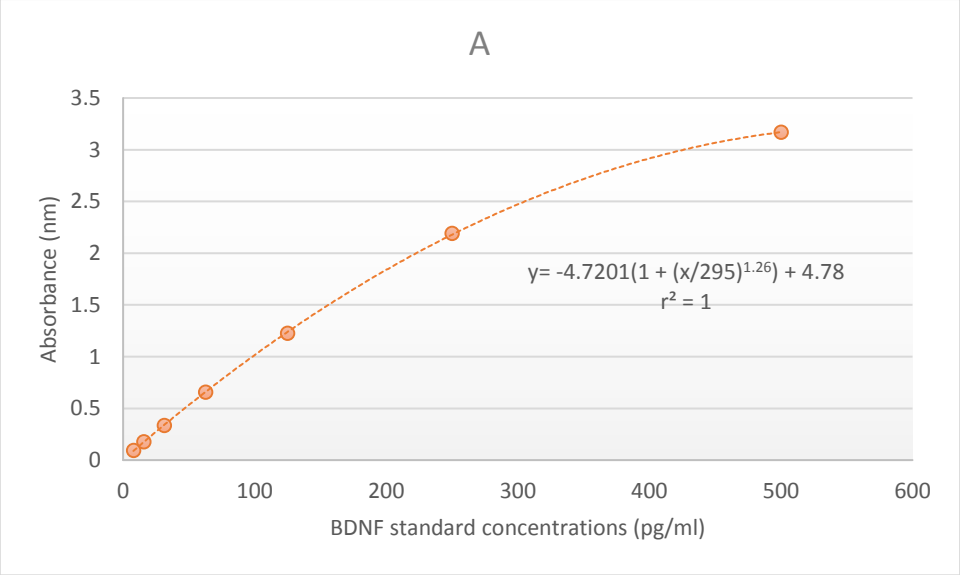


Fig E-1. Standard calibration curves for bovine serum albumin in order to determine hippocampal sample protein content in FSL and FRL animals in the Bradford Protein assay, depicting A) plate 1 and B) plate 2.

3.2 Generating standard BDNF concentration curves

The standard BDNF concentration curves are depicted in Figure E-2. The standard BDNF concentration curves indicated a linear regression of 1 for plates A and B respectively with the formulas as indicated in Fig E-1-A and E-1-B respectively used to calculate the protein content of each brain tissue homogenate sample.



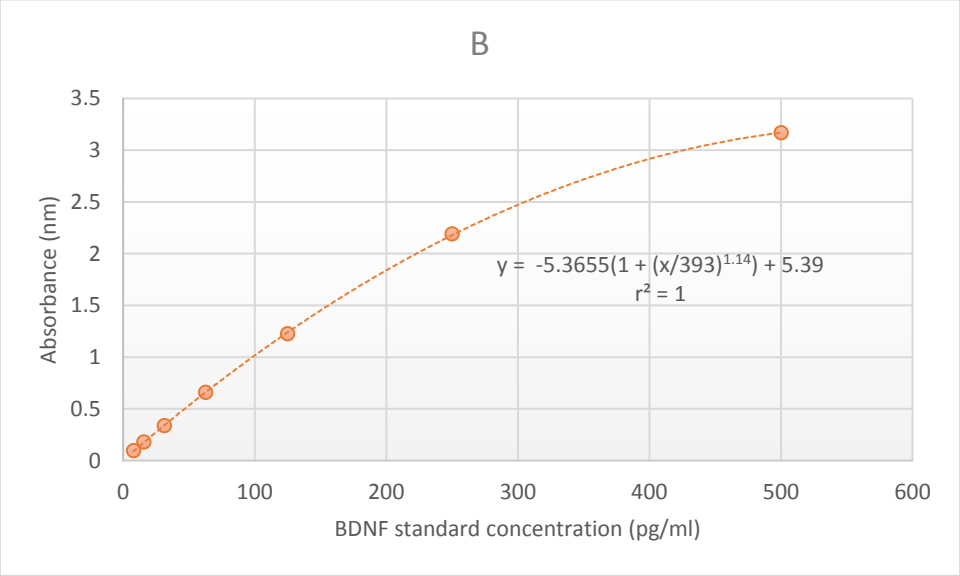


Fig E-2. Standard calibration curves for BDNF standards in order to determine sample BDNF concentrations for the ELISA assay, depicting A) plate 1 and B) plate two.

3.3 Hippocampal BDNF levels

3.3.1 Validation of the FSL model

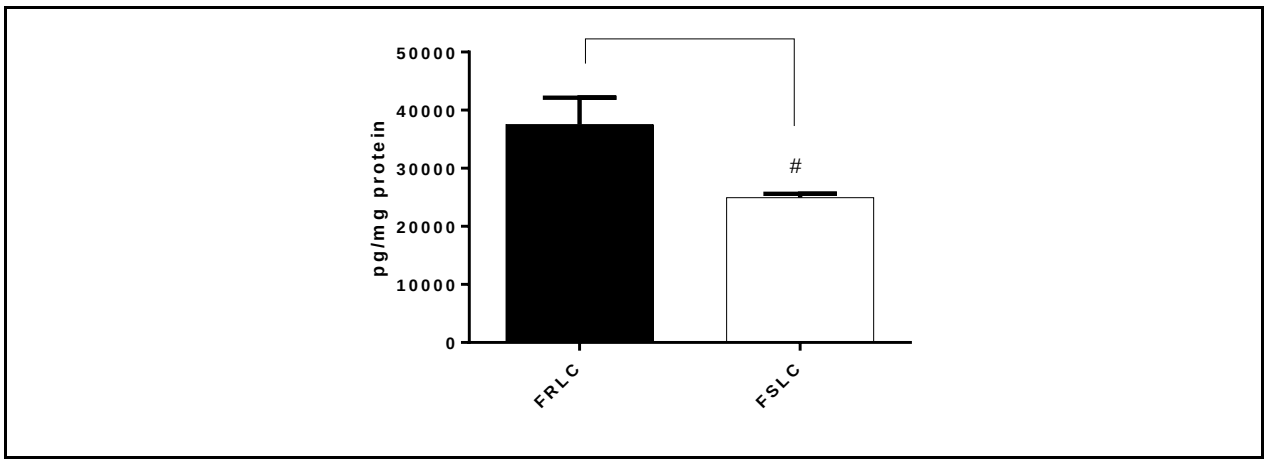


Fig E-3 Hippocampal total BDNF levels as determined by ELISA in FSL and FRL control animals (n=7) reported as pg BDNF per mg protein. FRLC = FRL Controls, FSLC = FSL Controls

The effect of strain on hippocampal BDNF levels is depicted in Fig E-3. Unpaired t-test with Welch's correction indicated that total hippocampal BDNF levels in FSL controls was significantly lower compared to FRL controls (Fig E-3; p=0.035).

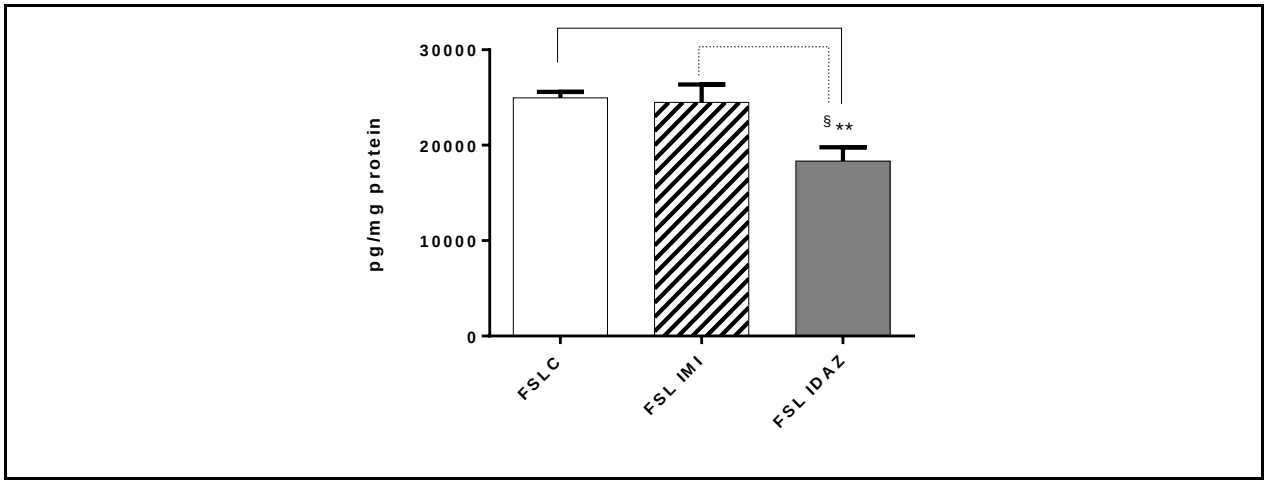


Fig E-4 Hippocampal total BDNF levels as determined by ELISA in FSL animals treated with vehicle, IMI or IDAZ (n=7) reported as pg BDNF per mg protein. FSLC = FSL Controls, IMI = FSL+imipramine 15mg/kg, IDAZ = FSL+idazoxan 3mg/kg.

The effect of 14 day treatment of FSL animals with either vehicle, IMI or IDAZ on hippocampal BDNF levels is depicted in Fig E-4. One-way ANOVA indicated a significant difference in hippocampal BDNF in FSL animals treated with either vehicle, IMI or IDAZ ($F(2,18)=6.82$, $p=0.006$), with Tukey's multiple comparison test indicating that IMI-treated animals demonstrated significantly higher hippocampal

BDNF levels vs. IDAZ (p=0.02), but not vs. FSL controls (p=0.97) (Fig E-4). IDAZ treatment resulted in significantly lower hippocampal BDNF levels vs. FSL controls (p=0.01) (Fig E-4).

3.3.2 Dose-response effects of ORM-10921 and comparison to IDAZ and IMI

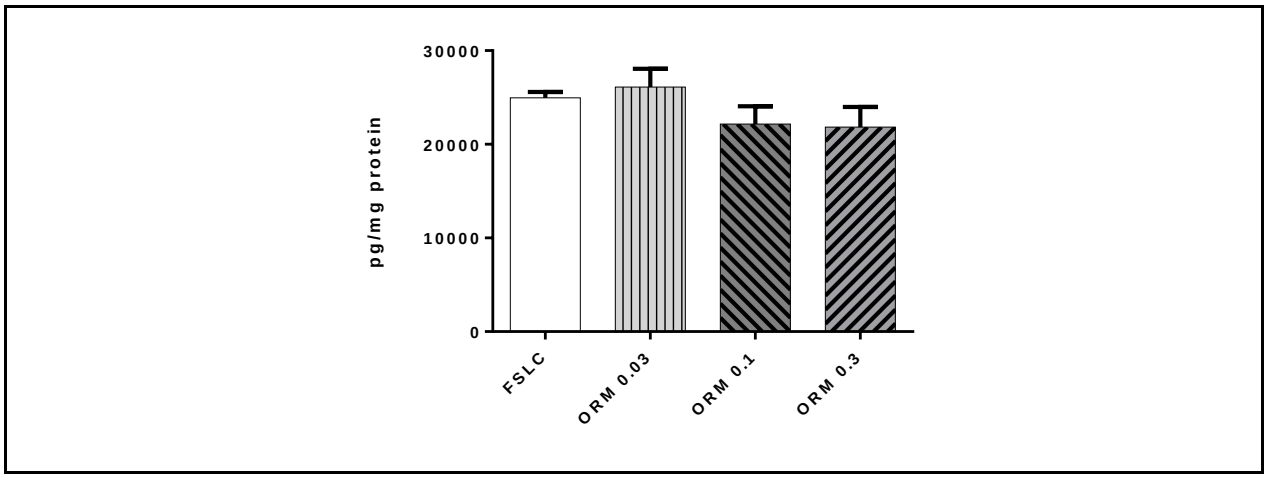


Fig E-5 Hippocampal total BDNF values as determined by ELISA in FSL animals treated with either vehicle or ORM-10921 0.03, 0.1 or 0.3 mg/kg (n=7-8) reported as pg BDNF per mg protein. FSLC = FSL Controls, ORM = FSL+ORM-10921

The effect of 14 day treatment of FSL animals with either vehicle or ORM-10921 0.03, 0.1 or 0.3 mg/kg on hippocampal BDNF levels is depicted in Fig E-5. One-way ANOVA indicated no significant differences between cohorts treated with various doses of ORM-10921 and FSL controls ($F(3,28)=1.363$, $p=0.27$).

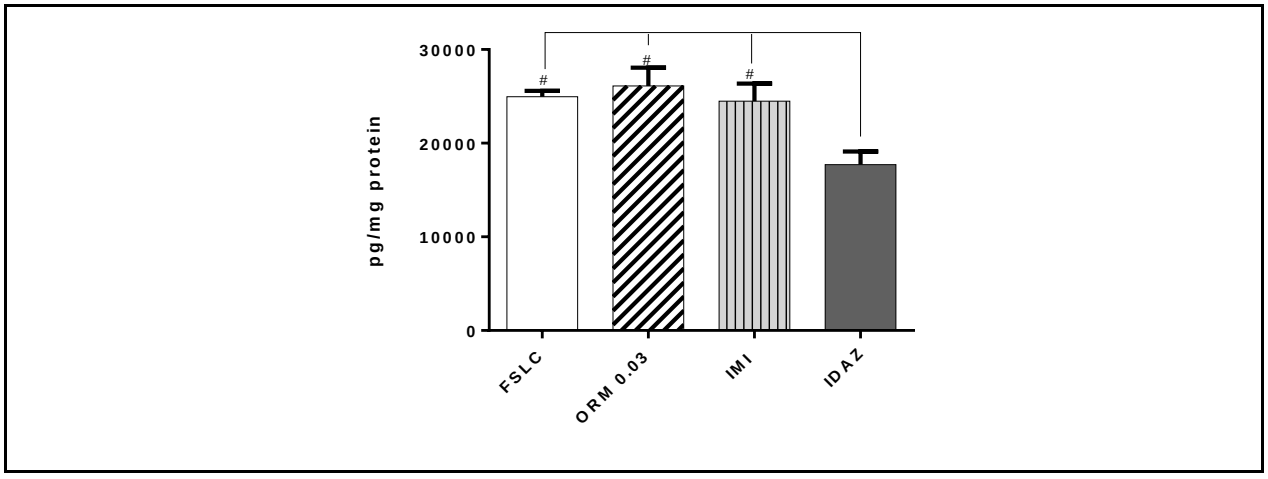


Fig E-6 Hippocampal total BDNF values as determined by ELISA in FSL animals treated with vehicle, or ORM-10921 0.03, IMI and IDAZ (n=7-8) reported as pg BDNF per mg protein. FSLC = FSL Controls, ORM = ORM-10921, IMI= imipramine 15 mg/kg, IDAZ= idazoxan 3 mg/kg

Since the above dose-response study with ORM-10921 did not alter hippocampal BDNF levels as expected, we selected a dose of 0.03 mg/kg for the comparator study, based on its more pronounced behavioural effects in both the NORT and FST. The effect of 14 day treatment of FSL animals with either vehicle, ORM-10921 0.03 mg/kg, IMI or IDAZ on hippocampal BDNF levels is depicted in Fig E-6. ANOVA indicated that FSL animals treated with IMI, IDAZ or ORM-10921 0.03 mg/kg demonstrated significant differences in hippocampal BDNF protein ($F(3,27)=5.74$, $p=0.004$). Tukey's multiple comparison test indicated that IDAZ treated animals demonstrated significantly lower BDNF levels compared to FSL controls ($p=0.02$), ORM 0.03 ($p=0.004$) and IMI ($p=0.03$). ORM-10921 and IMI were without effect compared to FSL controls.

4. Discussion

Cognitive decline in depression is associated with neuroplastic deficits, among others a decrease in BDNF levels (Brand et al. 2015; Neto et al. 2011). Hippocampal atrophy is implicated in the pathophysiology of depressive disorders (see Campbell and MacQueen (2004) and Brand et al. (2015) for review) while antidepressant therapy has been associated with increased BDNF levels in post mortem hippocampi of depressed patients (Chen et al. 2001; Dunham et al. 2009,) and in animal models of depression (Brand et al., 2015; Campbell and MacQeen 2004, Neto et al. 2011). Similarly, the expression of neurotrophic factors such as BDNF and vascular endothelial growth factor (VEGF) are decreased in FSL rats compared to FRL controls (Elfving et al., 2010b, c), suggesting that these animals could be a suitable translational model to determine medication-induced changes in BDNF and their possible therapeutic benefits in depressive disorders.

4.1 Validation of FSL animal model

FSL animals in this study demonstrated significantly lower hippocampal BDNF protein levels compared to FRL animals, providing good construct validity for the use of this genetic animal model, and is in agreement with the literature (Elfving et al., 2010a; Overstreet and Wegener, 2013). Since increased hippocampal BDNF levels have also been correlated with pro-cognitive effects as well as reduced immobility time in the FST (Shirayama et al. 2002), the observed reduction in hippocampal BDNF levels noted here in FSL rats is in-line with the depressive-phenotype of these animals reported in **Manuscript B** and **Addendum D**.

Observing a trend of depressive-like behaviour in this study and its correlation with reduced hippocampal BDNF levels in FSL control rats, the expectation was that chronic treatment with IMI would reverse lowered hippocampal BDNF levels in FSL animals, while IDAZ would not, in accordance with the behavioural effects of these drugs reported in **Manuscript B** and **Addendum D**. However, while IDAZ

further reduced BDNF levels, IMI was without effect. Although this finding with IMI seems not to be in agreement with current literature, it does, however reflect the inconsistent findings regarding the effect of antidepressants on BDNF levels in FSL animals. In fact, several studies in FSL rats have reported beneficial effects of chronic antidepressant treatment on behavioural deficits in FSL rats but without being correlated with altered hippocampal BDNF levels (Angelucci et al., 2003; Hansson et al., 2011), while other studies in Sprague Dawley rats have however reported increased hippocampal BDNF levels (De Foubert et al., 2004). Chronic nortriptyline or escitalopram has been noted to actively improve behavioural parameters in FSL rats, but without any associated changes in hippocampal BDNF mRNA (Hansson et al., 2011). Another study also demonstrated that nortryptiline did not affect hippocampal cell proliferation as measured by the number of BrdU positive cells (Petersen et al., 2009). Furthermore, using electroconvulsive shock as antidepressant treatment (which is deemed to be highly effective in improving depressive-like symptoms), no correlation between improvement in behaviour and hippocampal BDNF could be found in FSL animals (Angelucci et al., 2003). Important to note is that this disparity between antidepressant-like behavioural effects and hippocampal BDNF levels has also been observed in other animal models. In mice with a genetically-induced glucocorticoid receptor dysfunction, chronic fluoxetine and desimipramine were found to induce an increase in hippocampal BDNF mRNA levels, but not in wild-type mice (Vinet et al., 2004), while in Wistar rats chronic IMI failed to enhance hippocampal BDNF protein levels in spite of decreasing FST immobility (Garcia et al., 2008). In normal mice, Kos and colleagues found that IMI increased cortical BDNF mRNA after 14 days of treatment, but not hippocampal BDNF mRNA (Kos et al., 2006). Vinet et al (2004) offered a possible explanation for the absence of antidepressant-induced increases in hippocampal BDNF. In their study chronic fluoxetine or desimipramine did not increase hippocampal BDNF mRNA levels in wildtype mice, although it did up-regulate CREB, the transcription factor related to the expression of BDNF (Vinet et al., 2004). Vinet and colleagues (2004) offer the suggestion that the induction of transcription factors for other genes could eventually affect BDNF levels, which would arguably then only be visible at a later stage.

Methodological differences may also affect the detection of increased BDNF levels following antidepressant therapy. The above studies made use of Northern Blot, *in situ* hybridization and immunohistochemistry to report altered BDNF gene expression, and ELISA to report alterations in BDNF protein levels. Where ELISA was applied, BDNF was expressed in terms of the wet weight or in terms of the ml homogenate obtained per sample (Garcia et al 2008), or in terms of a % of the control group, without a unit for expression being disclosed (De Foubert et al., 2004). In other studies investigating brain BDNF protein levels in rats, BDNF is expressed in pg/mg protein (Katoh-Semba et al., 1997; Klein et al., 2011). It is therefore possible that methodological differences could influence findings regarding the effects of antidepressant treatment on BDNF. An additional factor could be the duration of

treatment. De Foubert and co-workers demonstrated that fluoxetine treatment only induced increased BDNF protein levels in the hippocampus after 21 days of treatment (BDNF expressed in terms of a percentage of control) (De Foubert et al., 2004). Various factors, including, strain, method, terms of expression and duration of treatment could therefore affect the outcome of antidepressant therapy on hippocampal BDNF levels in rodents, and may explain our unexpected results.

IDAZ failed to show positive effects on BDNF compared to FSL controls, which is in line with its failure to alter depressive-like behaviour and impaired cognition in FSL rats as reported in **Addendum D** and **Manuscript B**. Furthermore, IDAZ treatment was associated with significantly lower BDNF levels compared to FSL controls. No information is available in the literature regarding the effects of IDAZ on hippocampal BDNF, although 28-day treatment with another non-selective α_2 antagonist, dexefaroxan, in healthy rodents has been associated with increased hippocampal BDNF and neurogenesis (Rizk et al., 2005). However, evidence from studies by Chen and co workers (2007) in embryonic hippocampal cell cultures show that while increased noradrenaline (NA) levels bolster BDNF levels, the non-selective α_2 antagonist, yohimbine (which increases NA levels) reduces BDNF levels in these cells. The authors hypothesize that although presynaptic α_2-AR inhibition increases NA levels, which in turn should theoretically result in increased hippocampal BDNF protein *in vitro*, simultaneous postsynaptic blockade of α_2-ARs might negate any positive effects of increased NA on BDNF protein (Chen et al., 2007). IDAZ is not clinically effective as an antidepressant or as a drug that should aid cognitive functioning, therefore its inferior effect on BDNF vs. IMI is not unexpected. Our findings regarding the effects of IDAZ on BDNF levels is therefore not out of line with our behavioural findings reported in **Manuscript B** or with currently available literature.

4.2 Effect of ORM-10921 on hippocampal BDNF levels in FSL animals

None of the doses of ORM-10921 produced any noteworthy effects on hippocampal BDNF levels. ORM-10921 at the lower dose of 0.03 mg/kg was therefore selected for active comparison with IMI and IDAZ based on the behavioural data described in **Manuscript B** and **Addendum D**. Importantly, this dose of ORM-10921 did present with significantly higher BDNF levels compared to the non-selective α_2-AR antagonist, IDAZ (Fig E-6). Once again, in view of the beneficial behavioural effects effected with 14-day administration of ORM-10921 as reported in **Manuscript B** and **Addendum D**, the lack of beneficial effect of ORM-10921 on hippocampal BDNF was unexpected. Although the same arguments could be made here as for IMI noted earlier, namely that a more protracted treatment timeline, the induction of transcription factors for genes that eventually affect BDNF levels and the measurement of BDNF gene expression vs. protein expression might be able to provide more answers as to how ORM-10921 affects the expression of BDNF. Alternatively, the beneficial effects of ORM-10921 with respect to its antidepressant-like effects in the FST and deficits in recognition memory could however be mediated

via a mechanism that is independent of hippocampal BDNF. Measuring transcription factors that directly affect the eventual expression of BDNF, such as CREB, as well as other neurotrophic factors such as NGF and VEGF could produce valuable information in clarifying the mechanism whereby ORM-10921 exerts its beneficial effects on behaviour. Alternatively, improvements in BDNF levels might follow a more protracted time-line (De Foubert et al. 2004), emphasizing a need for extended treatment in order to observe changes at a neuromolecular level. Indeed preliminary evidence suggests that 28 day treatment with ORM-10921 can enhance hippocampal neurogenesis reliant on augmentation of neurotrophic factor activity (unpublished data, Orion Pharma). Another possible limitation is that BDNF levels in this study were analysed in homogenate of whole hippocampus. Future BDNF analyses on specific sub-regions of the hippocampus might shed more light on these findings, since it is possible that subtle changes in BDNF in specific regions of the hippocampus might not have been amenable to detection using whole hippocampal extract (Vinet et al., 2004).

In conclusion, the findings from this investigation strengthened the construct validity of the FSL animal model of depression, while also reporting the differential effects of 14 day drug treatment with IMI, IDAZ and ORM-10921 in these animals on hippocampal BDNF levels. While the FSL animal does present with decreased hippocampal BDNF levels, IMI treatment did not induce a corresponding increase in hippocampal BDNF levels correlating to the improvements in depressive-like behaviour (**Manuscript B** and **Addendum D**). Likewise ORM-10921, although very effective in reversing depressive-like behavioural and cognitive deficits as reported in **Addendum D** and **Manuscript B**, did not induce an associated increase in hippocampal BDNF levels. Treatment time-line, methodology, assessment of gene expression vs. protein expression and the simultaneous measurement of related neurotrophins and transcription factors that affect neurogenesis are all factors that could influence the interpretation of these BDNF data. Future studies investigating BDNF levels in FSL animals should employ an extended treatment timeline and look at transcription factors associated with BDNF as well as TrkB, the BDNF receptor.

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List of Abbreviations

	Numerical	
5-HIAA	-	5-hydroxyindoleacetic acid
5-HT	-	Serotonin
5-HTP	-	5-hydroxytryptophan
	A	
α_2-AR	-	Alpha-2-adrenceptor
α_{2A}-AR	-	Alpha-2A -adrenoceptor
α_{2B}-AR	-	Alpha-2B-adrenoceptor
α_{2C}-AR	-	Alpha-2C-adrenoceptor
α_{2C}-KO	-	α_{2C}-receptor knockout (deletion)
α_{2C}-OE	-	α_{2C}-receptor overexpression
ACTH	-	Adrenocorticotrophic hormone
ADHD	-	Attention deficit hyperactivity disorder
AMP	-	Adenosine monophosphate
AMPA receptor	-	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA	-	Analysis of variance
	B	
BDNF	-	Brain-derived neurotrophic factore
BrdU	-	Bromodeoxyuridine
	C	
cAMP	-	Cyclic adenosine monophosphate
CLOZ	-	Clozapine
CNS	-	Central Nervous System
CREB	-	cAMP response element binding protein
CRH	-	Corticotropin-releasing
CSF	-	Cerebrospinal fluid
CV	-	Coefficient of variation
	D	
DA	-	Dopamine
DOPA	-	3,4-dihydroxyphenylalanine

DOPAC	-	3,4-dihydroxyphenylacetic acid
		E
ELISA	-	Enzyme-linked immunosorbent assay
		F
Fisher's LSD	-	Fisher's Least Significant Difference
FRL	-	Flinders Resistant Line
FSL	-	Flinders Sensitive Line
FST	-	Forced swim test
		G
GABA	-	Gamma-aminobutyric acid
GAD	-	Glutamic acid decarboxylase
		H
HAL	-	Haloperidol
HPA-axis	-	Hypothalamic-pituitary-adrenal axis
HPLC	-	High-performance liquid chromatography
HPLC-ECD	-	High-performance liquid chromatography with electrochemical detection
HRP	-	Horseradish peroxidase
HVA	-	Homovanillic acid
		I
IDAZ	-	Idazoxan
IMI	-	Imipramine
		K
KO	-	Knockout (receptor deletion)
		L
LC	-	Locus coeruleus
LLOD	-	Lower limit of detection
LLOQ	-	Lower limit of quantification
LSD	-	Lysergic acid diethylamide
		M
MAPK	-	Mitogen-activated protein kinase
MAOI	-	Monoamine oxidase inhibitor
MHPG	-	3-methoxy-4-hydroxyphenylglycol
MK-801	-	Dizolcipine

MWM	-	Morris water maze
		N
NA	-	Noradrenaline
NGF	-	Neural growth factor
NMDA	-	N-methyl-d-aspartate glutamate receptor
NOR	-	Novel object recognition
NORT	-	Novel object recognition test
		O
ORM	-	ORM-10921
		P
PCP	-	Phenylcyclidine
PFC	-	Prefrontal cortex / prefrontal cortical
PPI	-	Prepulse inhibition
		R
rpm	-	Revolutions per minute
		S
SIR	-	Social isolation rearing/social isolation reared
SNDRI	-	Serotonin/noradrenaline/dopamine reuptake inhibitor
SNRI	-	Serotonin/noradrenaline reuptake inhibitor
SOC	-	Socially reared /group housed
SSRI	-	Selective serotonin reuptake inhibitor
		T
TCA	-	Tricyclic antidepressant
TMB	-	3,3',5,5'-tetramethylbenzidine
TrkB	-	Tropomyosin receptor B
		U
US	-	United States
USA	-	United States of America
		V
VEGF	-	Vascular endothelial growth factor
VTA	-	Ventral tegmental area

Co-authors Letters of Consent

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Dear examiner

16 November 2016

PHD THESIS – MM UYS
PERMISSION TO INCLUDE MANUSCRIPTS FOR EXAMINATION PURPOSES

As study leader and senior corresponding author on four manuscripts first authored by Ms Madeleine Monique Uys, I hereby approve that the manuscripts listed below be included as part of the requirements for fulfillment of the PhD degree, and that these manuscripts be submitted for examination of Ms Uys's thesis.

The four articles are as follows:

Manuscript A
The α_{2C}-adrenoceptor antagonist, ORM-10921, has antipsychotic-like effects in social isolation reared rats and bolsters the response to haloperidol
Published in *Progress in Neuro-Psychopharmacology & Biological Psychiatry* (Elsevier)

Manuscript B
The α_{2C}-adrenoceptor antagonist, ORM-10921, exerts antidepressant-like effects in the Flinders Sensitive Line rat
Published online and currently *in press* in *Behavioural Pharmacology* (Wolters Kluwer)

Manuscript C
The effects of selective α_{2C}-AR-antagonism on hippocampal monoamine levels in the Flinders Sensitive Line rat
In preparation for submission to *Acta Neuropsychiatrica* (John Wiley & Sons)

Manuscript D
Therapeutic potential of targeting the α_{2C}-adrenoceptor in cognition, depression and schizophrenia – new developments and future perspective
In submission to *Frontiers in Psychiatry*, section *Molecular Psychiatry* (Frontiers Media)

A letter of confirmation of submission or acceptance for publication from the respective journal editorial offices for manuscripts A, B and D is included in the thesis, and appear at the start of each of the article chapters.

I trust that you will find this in order.

Sincerely,

Brian H Harvey, PhD
Study leader, Co-Author

16 November 2016

RE: PhD THESIS – MM UYS
LETTER OF PERMISSION TO INCLUDE MANUSCRIPTS FOR EXAMINATION PURPOSES

Dear Sir/Madam

As co-author on the three manuscripts first authored by Ms Madeleine Monique Uys, I hereby give permission for the following three manuscripts to be included in the PhD thesis that Ms Uys will submit as part of the requirements for fulfillment of the PhD degree, and that these manuscripts may be submitted for examination of Ms Uys's thesis.

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Yours sincerely

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Yours sincerely

Jukka Sallinen

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Co-author

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Yours sincerely

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Yours sincerely

Francois P. Viljoen

Co-author