



## **Terms and Conditions of Use of Digitised Theses from Trinity College Library Dublin**

### **Copyright statement**

All material supplied by Trinity College Library is protected by copyright (under the Copyright and Related Rights Act, 2000 as amended) and other relevant Intellectual Property Rights. By accessing and using a Digitised Thesis from Trinity College Library you acknowledge that all Intellectual Property Rights in any Works supplied are the sole and exclusive property of the copyright and/or other IPR holder. Specific copyright holders may not be explicitly identified. Use of materials from other sources within a thesis should not be construed as a claim over them.

A non-exclusive, non-transferable licence is hereby granted to those using or reproducing, in whole or in part, the material for valid purposes, providing the copyright owners are acknowledged using the normal conventions. Where specific permission to use material is required, this is identified and such permission must be sought from the copyright holder or agency cited.

### **Liability statement**

By using a Digitised Thesis, I accept that Trinity College Dublin bears no legal responsibility for the accuracy, legality or comprehensiveness of materials contained within the thesis, and that Trinity College Dublin accepts no liability for indirect, consequential, or incidental, damages or losses arising from use of the thesis for whatever reason. Information located in a thesis may be subject to specific use constraints, details of which may not be explicitly described. It is the responsibility of potential and actual users to be aware of such constraints and to abide by them. By making use of material from a digitised thesis, you accept these copyright and disclaimer provisions. Where it is brought to the attention of Trinity College Library that there may be a breach of copyright or other restraint, it is the policy to withdraw or take down access to a thesis while the issue is being resolved.

### **Access Agreement**

By using a Digitised Thesis from Trinity College Library you are bound by the following Terms & Conditions. Please read them carefully.

I have read and I understand the following statement: All material supplied via a Digitised Thesis from Trinity College Library is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of a thesis is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form providing the copyright owners are acknowledged using the normal conventions. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone. This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.

**Neuronal Nitric oxide synthase:  
a novel target for antidepressant activity**



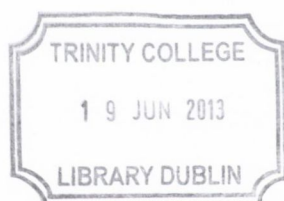
**Thesis submitted for the degree of  
Doctor of Philosophy  
at the University of Dublin, Trinity College  
by**

**Valentina Gigliucci**

**Supervised by Dr. Andrew Harkin**

Thesis submitted December 2012

School of Pharmacy and Pharmaceutical Sciences and Trinity College Institute of  
Neuroscience,  
Trinity College Dublin,  
Dublin 2



Thesis 10131

## I. Declaration

I declare that this thesis is submitted for the degree of Doctor of Philosophy at the University of Dublin, Trinity College and has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work.

I agree to deposit this thesis in the University's open access institutional repository or allow the library to do so on my behalf, subject to Irish Copyright Legislation and Trinity College Library conditions of use and acknowledgement.

Valentina Gigliucci

Valentina Gigliucci

## II. Acknowledgements

First of all I would like to thank my supervisor Dr. Andrew Harkin for all the help and the guidance during all the time I spent in Ireland. Thank you for being kind to me even before I moved here and for trying to make me look at the bright side of my project when I was seeing only disasters.

I would also like to thank Trinity College Dublin and the Health Research Board of Ireland for their generosity and for giving me the chance to live this experience in Dublin. I really couldn't have made it without some money.

Many grateful thanks to Jennifer Rouine and Shane Gormley for their help with the MRI scans and analysis, and for their dedication to my project.

I would like to thank all the students I supervised, in particular Eoin Sherwin, without whom my PhD would have needed an extra year to finish.

I can't help but thank all the amazing wonderful people, past and present, from the TC/AH lab. Starting from Natacha, Lorna and Karen who were the "experts to look at" when I started, and Alessia for making me feel at home every time. Then Áine, Jen R., Katie and Jen D., my "equals": thank you for sharing all these years and for sharing your doubts and your ideas with me, I really never felt alone. Then Raasay, Éimear and Martina, thank you for the lovely time and for never giving up at inviting me out for drinks. A special thanks to Martina for the "late late chats" after work, I'll miss that! A big thanks to the guys, Barry and Shane, for doubling the fun and making me lough even in the worst days and a very special thanks to Sinéad and Éadaoin, the two lighthouses of my PhD. Thank you for saving my life more than a couple of times with one of your genial ideas or even better using your time to help me out. I also would like to thank Tom, for always making clear that you were there for me if I ever needed it. It really made my day sometimes!

I have to thank all the friends I met here in Dublin: Christine, Alessia, Valerie, Valeria, Nella. Thank you for being my family outside work.

I would like to thank all the people at home waiting for me, in particular my parents, my granny and all the uncles, aunties and cousins. I was always happy to come home to join you. And all the friends, no matter how many times we heard each other while I was away.

At last I would like to thank Gio, for I can say you are the reason why I made it until the end. Thank you for keeping me on track when I was losing my way, for your support and for giving your best with me every time.

### III. Summary

Major depressive disorder (MDD) is one of the most common psychiatric disorders and its origin has been attributed to dysfunction of the monoamine systems, particularly noradrenergic and serotonergic, making them the main targets of the majority of antidepressants currently available. However a number of unmet clinical needs remain, such as a delay of several weeks from the first treatment to manifest antidepressant effects, low remission rates and high rates of relapse and recurrence. Fast acting antidepressant activity and efficacy in treatment-resistant cases has been reported with drugs that inhibit the glutamate N-methyl-D-aspartic acid (NMDA) receptor. However, this pharmacological approach is problematic, as it leads to motor and psychotic side effects. Inhibitors of nitric oxide synthase (NOS), a downstream target of NMDA receptors, produce antidepressant effects in the Porsolt test of antidepressant activity in laboratory rodents, without the adverse effects associated with NMDA receptor antagonism. Given the role of neuronal NOS (nNOS) in mediating glutamatergic transmission in the brain, this enzyme represents a novel putative target for the development of a faster acting and more efficacious antidepressant treatment. Here the efficacy of nNOS inhibitors in preclinical models of depression is further assessed. Specifically new insights are provided into the role of 5-HT in the antidepressant-related actions of both ketamine and the NOS inhibitor N<sup>o</sup>-Nitro-L-arginine (L-NA). L-NA shows antidepressant-related effects in the olfactory bulbectomised (OB) rat model of depression and attenuates the characteristic hyperactivity with a more rapid onset of action when compared to the tricyclic antidepressant imipramine. The selective nNOS inhibitor 1-(2-trifluoro-methyl-phenyl) imidazole (TRIM) also showed a faster onset of action in the model. Finally the effect of L-NA on stress-induced neuronal activation

determined by immediate early gene expression was examined to identify those brain regions involved in the antidepressant-like properties of the NOS inhibitor. L-NA provoked a 5-HT-independent region specific bi-directional regulation of neuronal activation associated with antidepressant-like behavioural effects in the FST providing insight into the underlying neurocircuitry where functional changes in the prelimbic cortex and lateral septum may result in regulation of transmission throughout an extended subcortical brain circuit that includes the hippocampus, amygdala, hypothalamus, striatum and brain stem. These studies support the potential of nNOS as a novel target for the treatment of depression and possibly other stress-related disorders. The experiments have advanced the knowledge and understanding of antidepressant action in animal models of stress and depression. Given the prevalence of depression and the significant problems associated with resistance to conventional antidepressants coupled with slow onset of therapeutic action, examination of the viability of novel non-monoaminergic targets for antidepressant activity is an imperative.

## IV. Table of contents

<b>I. Declaration</b> .....	<b>3</b>
<b>II. Acknowledgements</b> .....	<b>4</b>
<b>III. Summary</b> .....	<b>5</b>
<b>IV. Table of contents</b> .....	<b>7</b>
<b>V. List of figures</b> .....	<b>15</b>
<b>VI. List of tables</b> .....	<b>19</b>
<b>VII. List of abbreviations</b> .....	<b>21</b>

### **Introduction**

<b>1. Major Depressive Disorder (MDD)</b> .....	<b>27</b>
1.1 Biological mechanisms underlying the pathophysiology of MDD .....	28
<b>2. Animal models of depression</b> .....	<b>30</b>
2.1 Stress and animal models of depression .....	31
2.2 Despair-based models of depression .....	32
2.2.1 Forced swimming test.....	33
2.3 The olfactory bulbectomised rodent model of depression .....	36
<b>3. Beyond the monoamine hypothesis: other biological mechanisms involved in depression</b> .....	<b>38</b>
3.1 Depression and the hypothalamic-pituitary-adrenal (HPA) axis .....	38
3.2 Depression and the immune system .....	39
3.3 Depression and neurotrophic factors .....	41
3.4 A role for glutamate in the pathophysiology of MDD .....	44
3.5 Targeting the glutamatergic system for antidepressant activity.....	51



<b>4.</b>	<b><i>The NMDA receptor – nNOS – NO pathway.....</i></b>	<b>54</b>
4.1	<i>The NMDA receptor .....</i>	55
4.2	<i>The post synaptic density protein of 95 kDa (PSD-95).....</i>	56
4.3	<i>Neuronal nitric oxide synthase (nNOS) .....</i>	57
4.4	<i>Intracellular targets of nNOS activation and effects of NO production .....</i>	58
<b>5.</b>	<b><i>Is there a role for NMDA-R coupled NO in the pathophysiology of depression?59</i></b>	
5.1	<i>A role for NO in depression .....</i>	59
5.2	<i>NO and neuroprotection.....</i>	61
5.3	<i>Antidepressant-like properties of nitric oxide synthase inhibitors.....</i>	63
5.4	<i>Stress and NOS.....</i>	68
<b>6.</b>	<b><i>Aim and objectives .....</i></b>	<b>73</b>

## **Materials and Methods**

<b>1.</b>	<b><i>Materials.....</i></b>	<b>77</b>
1.1	<i>Animals.....</i>	77
1.2	<i>Equipment.....</i>	77
1.3	<i>Special equipment .....</i>	78
1.4	<i>Anaesthesia and operative care .....</i>	79
1.5	<i>Consumables .....</i>	79
1.6	<i>Chemicals and reagents .....</i>	80
1.7	<i>Kits .....</i>	82
1.8	<i>Drugs.....</i>	82
1.9	<i>Hardware and software.....</i>	82
<b>2.</b>	<b><i>Methods .....</i></b>	<b>85</b>
2.1	<i>Subjects.....</i>	85
2.2	<i>In vivo procedures .....</i>	85
2.2.1	<i>Surgical procedures .....</i>	85
2.2.1.1	<i>Bilateral olfactory bulbectomy (OB).....</i>	86
2.2.1.2	<i>Implantation of biotelemetry transponders .....</i>	87
2.2.2	<i>Central 5-HT depletion.....</i>	88
2.2.3	<i>Immobilisation stress .....</i>	88

2.2.4	<i>Combination of 5-HT depletion and immobilisation stress</i> .....	89
2.2.5	<i>Behavioural assessments</i> .....	89
2.2.5.1	<i>Randomisation of subjects</i> .....	89
2.2.5.2	<i>Forced Swimming Test (FST)</i> .....	90
2.2.5.3	<i>Modified Forced Swimming Test</i> .....	90
2.2.5.4	<i>Open field test</i> .....	92
2.2.5.5	<i>Home cage activity</i> .....	93
2.2.6	<i>Magnetic Resonance Imaging (MRI)</i> .....	93
2.3	<i>Ex vivo procedures</i> .....	97
2.3.1	<i>High Performance Liquid Chromatography (HPLC)</i> .....	97
2.3.2	<i>Quantification of cortical and hippocampal gene expression</i> .....	98
2.3.2.1	<i>Total RNA extraction</i> .....	98
2.3.2.2	<i>cDNA synthesis</i> .....	99
2.3.2.3	<i>Real-Time PCR</i> .....	99
2.3.3	<i>c-FOS immunoreactivity quantification</i> .....	100
2.3.3.1	<i>Intracardiac perfusion</i> .....	100
2.3.3.2	<i>c-FOS immunohistochemistry</i> .....	101

## **Results**

<b>1.</b>	<b><i>Results chapter 1</i></b> .....	<b>107</b>
1.1	<i>Introduction</i> .....	109
1.2	<i>Materials and Methods</i> .....	113
1.2.1	<i>Subjects and drug treatment</i> .....	113
1.2.2	<i>Study design</i> .....	114
1.2.2.1	<i>The effect of ketamine on immobility in the FST</i> .....	114
1.2.2.2	<i>The effect of ketamine on locomotor activity and anxiety related parameters in the open field test</i> .....	114
1.2.2.3	<i>The effect of ketamine on immobilisation stress-induced immobility in the FST</i> .....	115
1.2.2.4	<i>The effect of central 5-HT depletion on antidepressant-like effects of ketamine in the FST</i> .....	116

1.2.3	<i>Determination of cortical 5-HT concentrations</i>	117
1.2.4	<i>Statistical analysis</i>	117
1.3	<i>Results</i>	119
1.3.1	<i>Dose and time dependent effects of ketamine in the rat FST</i>	119
1.3.2	<i>The effects of ketamine on locomotor activity</i>	119
1.3.3	<i>The effects of ketamine on anxiety related behaviour in the open field</i>	121
1.3.4	<i>Effects of immobilisation stress in the FST</i>	123
1.3.5	<i>Ketamine attenuates stress-induced immobility in the FST</i>	124
1.3.6	<i>5-HT depletion attenuates the antidepressant-like effects of ketamine in the FST</i>	125
1.3.7	<i>Combination of 5-HT depletion and repeated restraint stress induces a persistent depressive-like state in the FST</i>	127
1.3.8	<i>Ketamine does not counteract the pro-depressant effects of the combination of 5-HT depletion and repeated restraint stress in the FST</i>	130
1.4	<i>Discussion</i>	133
1.4.1	<i>The acute and sustained antidepressant response to ketamine in the FST</i>	133
1.4.2	<i>Ketamine attenuates stress-induced immobility in the FST</i>	135
1.4.3	<i>5-HT depletion attenuates the antidepressant response to ketamine</i>	136
1.4.4	<i>Combination of 5-HT depletion and restraint stress provokes a long-term increase in immobility in the FST</i>	137
1.4.5	<i>Can ketamine overcome immobility produced by a combination of 5-HT depletion and immobilisation stress in the FST?</i>	137
1.4.6	<i>Ketamine promotes molecular and cellular changes which enhance the synaptic efficacy of central 5-HT</i>	139
1.4.7	<i>Conclusions</i>	140
<b>2.</b>	<b><i>Results chapter 2</i></b>	<b>143</b>
2.1	<i>Introduction</i>	145
2.2	<i>Materials and Methods</i>	147

2.2.1	<i>Subjects and drug treatment</i> .....	147
2.2.2	<i>Study design</i> .....	147
2.2.2.1	<i>Influence of NSD-1015 on the antidepressant-like activity of L-NA in the FST</i> .....	147
2.2.2.2	<i>The effect of combining sub-active doses of L-NA and fluoxetine in the FST</i> .....	148
2.2.2.3	<i>Dose related effects of metergoline on the antidepressant-like activity of L-NA in the FST</i> .....	149
2.2.2.4	<i>The effect of ketanserin, RO-430440, WAY-100635 or GR-127935 on the antidepressant-like activity of L-NA in the FST</i> .....	149
2.2.3	<i>Locomotor activity</i> .....	150
2.2.4	<i>Determination of regional biogenic amine concentrations</i> .....	150
2.2.5	<i>Statistical analysis</i> .....	151
2.3	<i>Results</i> .....	153
2.3.1	<i>Influence of NSD-1015 on the antidepressant-like activity of L-NA in the FST</i> .....	153
2.3.2	<i>L-NA increases regional brain 5-HTP accumulation and 5-HT metabolism following FST exposure</i> .....	155
2.3.3	<i>L-NA increases 5-HT metabolism in the striatum following NSD-1015 administration</i> .....	157
2.3.4	<i>L-NA augments the activity of fluoxetine in the FST</i> .....	159
2.3.5	<i>Effects of metergoline on the antidepressant-like activity of L-NA in the FST</i> .....	161
2.3.6	<i>Treatment with ketanserin and RO-430440 provoke a similar response to metergoline in the FST attenuated by co-treatment with L-NA</i> .....	163
2.3.7	<i>Co-treatment with WAY-100635 or GR-127935 do not influence the antidepressant-like activity of L-NA in the FST</i> .....	167
2.3.8	<i>The effects of metergoline, ketanserin, RO-430440 and L-NA alone or in combination on locomotor activity</i> .....	169

2.4	<i>Discussion</i> .....	171
2.4.1	<i>L-NA increases 5-HT synthesis and metabolism</i> .....	171
2.4.2	<i>L-NA shows characteristics typical of SSRIs</i> .....	173
2.4.3	<i>5-HT receptors involvement in the antidepressant-like action of L-NA in the FST</i> .....	174
2.4.4	<i>Conclusions</i> .....	178
<b>3.</b>	<b><i>Results chapter 3</i></b> .....	<b>179</b>
3.1	<i>Introduction</i> .....	181
3.2	<i>Materials and Methods</i> .....	185
3.2.1	<i>Subjects and drug treatment</i> .....	185
3.2.2	<i>Experimental design</i> .....	186
3.2.2.1	<i>The effects of fluoxetine and L-NA on the depressive state induced by central 5-HT depletion and restraint stress in the FST</i> .....	186
3.2.2.2	<i>The effects of FST, combined 5-HT depletion and restraint stress and L-NA on brain regional neuronal activation</i> .....	186
3.2.3	<i>Determination of cortical 5-HT concentrations</i> .....	187
3.2.4	<i>Intracardiac perfusion and c-FOS immunohistochemistry</i> .....	187
3.2.5	<i>c-FOS immunolabelling analysis</i> .....	188
3.2.6	<i>Statistical analysis</i> .....	189
3.3	<i>Results</i> .....	191
3.3.1	<i>Antidepressant-like action of L-NA in the FST</i> .....	191
3.3.2	<i>Cortical 5-HT content</i> .....	191
3.3.3	<i>Regional c-FOS expression</i> .....	193
3.4	<i>Discussion</i> .....	197
3.4.1	<i>Antidepressant related behaviour of L-NA in the FST</i> .....	198
3.4.2	<i>Combination of 5-HT depletion and stress does not alter regional c-FOS FLI following exposure to the FST</i> .....	199

3.4.3	<i>Region specific c-FOS expression associated with the antidepressant-like properties of N<sup>ω</sup>-nitro-L-arginine</i>	201
3.4.4	<i>Conclusions</i>	206
<b>4.</b>	<b><i>Results chapter 4</i></b>	<b>209</b>
4.1	<i>Introduction</i>	209
4.2	<i>Materials and Methods</i>	215
4.2.1	<i>Subjects and drug treatment</i>	215
4.2.2	<i>Bilateral olfactory bulbectomy (OB)</i>	216
4.2.3	<i>Experimental design</i>	216
4.2.4	<i>Open field test</i>	217
4.2.5	<i>Magnetic Resonance Imaging (MRI)</i>	218
4.2.6	<i>Determination of cortical NOS expression</i>	219
4.2.7	<i>Effects of TRIM in the OB rat model</i>	220
4.2.8	<i>Statistical analysis</i>	220
4.3	<i>Results</i>	223
4.3.1	<i>OB related hyperactivity in the open field test</i>	223
4.3.2	<i>The increased speed of bout in the OB rats is not determined by the alteration of a single speed-related parameter</i>	224
4.3.3	<i>L-NA attenuates the habituation of distance moved to repeated open field testing in OB rats and the OB related increase in activity bout speed and also shows a faster onset of action when compared to the tricyclic antidepressant imipramine</i>	225
4.3.4	<i>MR neuroimaging markers in the OB rat model</i>	227
4.3.4.1	<i>OB is associated with changes in ventricular volume</i>	227
4.3.4.2	<i>L-NA treatment is associated with regional changes in resting state blood perfusion</i>	228
4.3.4.3	<i>L-NA treatment is associated with changes in T1 and T2 relaxation times</i>	232
4.3.5	<i>OB is not associated with changes in cortical NOS expression</i>	236

4.3.6	<i>TRIM attenuates OB related hyperactivity in the open field but fails to influence open field induced hyperthermia or tachycardia.....</i>	237
4.4	<i>Discussion .....</i>	241
4.4.1	<i>The speed per bout parameter withstands habituation to repeated open field testing.....</i>	241
4.4.2	<i>NOS inhibitors show a faster antidepressant action compared to imipramine in the OB model of depression .....</i>	243
4.4.3	<i>TRIM fails to influence heart rate and body temperature responses to stress in the OB model of depression.....</i>	245
4.4.4	<i>OB surgery does not induce the expression of NOS genes .....</i>	246
4.4.5	<i>MR imaging markers in the OB model of depression.....</i>	247
4.4.6	<i>Conclusions.....</i>	250

## ***General Discussion and Future Directions***

1.	<b><i>The NMDA-NO pathway: a locus for antidepressant activity .....</i></b>	255
1.1	<i>Ketamine.....</i>	255
1.2	<i>L-NA.....</i>	257
2.	<b><i>NO: a non-synaptic extension of glutamate .....</i></b>	260
2.1	<i>NO and 5-HT modulation.....</i>	261
3.	<b><i>Ongoing development of animal models of depression.....</i></b>	263
4.	<b><i>Translational perspective on the regionally-dependent effects of L-NA .....</i></b>	266
5.	<b><i>Future directions.....</i></b>	267

	<b><i>References.....</i></b>	271
--	-------------------------------	-----

	<b><i>VIII. Appendix.....</i></b>	313
--	-----------------------------------	-----

	<b><i>IX. Publications.....</i></b>	315
--	-------------------------------------	-----

## V. List of figures

### **Introduction**

<i>Fig. 1 Glutamatergic pathways in the brain</i> .....	45
<i>Fig. 2 The glutamatergic synapse</i> .....	46
<i>Fig. 3 Influence of glial dysfunction on the glutamatergic system and neurotrophic factors</i> .....	49
<i>Fig. 4 The neurotrophic hypothesis of depression</i> .....	50
<i>Fig.5 The NMDA-R-NO-cGMP signalling pathway</i> .....	54

### **Materials and Methods**

#### **Methods**

<i>Fig. 6 Open field arena</i> .....	92
<i>Fig. 7 The rodent Bruker Biospec system</i> .....	94

### **Results**

#### **Results Chapter 1**

<i>Fig. 1.1 Effects of acute ketamine administration in the FST and open field test</i> .....	120
<i>Fig. 1.2 Effects of acute ketamine administration on anxiety related parameters in the open field test</i> .....	122
<i>Fig. 1.3 Exposure to acute and repeated restraint stress transiently increase immobility time in the FST</i> .....	123
<i>Fig. 1.4 Effect of ketamine on immobilisation stress-induced immobility in the FST</i> .....	124



<i>Fig. 1.5 5-HT depletion attenuates the antidepressant-like activity of ketamine in the FST</i> .....	126
<i>Fig. 1.6 Prolonged effects of the combination of 5-HT depletion and restraint stress in the FST</i> .....	128
<i>Fig. 1.7 Combination of 5-HT depletion and immobilisation stress does not alter locomotor activity but induces anxiety-like behaviour in the open field test</i> .....	129
<i>Fig. 1.8 Effect of ketamine on FST immobility induced by a combination of 5-HT depletion and stress</i> .....	131

## **Results Chapter 2**

<i>Fig. 2.1 Effects of NSD-1015 pre-treatment on acute L-NA effect in the FST</i> .....	154
<i>Fig. 2.2 Effects of the combination of sub-active doses of L-NA and fluoxetine in the FST</i> .....	160
<i>Fig. 2.3 Effects of co-treatment with metergoline on the effects of L-NA in the FST</i> .....	162
<i>Fig. 2.4 Effects of co-treatment with ketanserin on the effects of L-NA in the FST</i> .....	164
<i>Fig. 2.5 Effects of co-treatment with RO-430440 on the effects of L-NA in the FST</i> .....	166
<i>Fig. 2.6 Effects of co-treatment with WAY-100635 on the effects of L-NA in the FST</i> .....	168

## **Results Chapter 3**

<i>Fig. 3.1 Effect of L-NA and fluoxetine on FST immobility induced by a combination of 5-HT depletion and stress</i> .....	192
---	-----

<i>Fig. 3.2 Diagrams adapted from “The rat brain” representing the brain regions analysed in the study .....</i>	<i>194</i>
<i>Fig. 3.3 Images representative of c-FOS immunostaining .....</i>	<i>195</i>
<i>Fig. 3.4 Effects of L-NA administration on neuronal activation in cortical and subcortical regions .....</i>	<i>196</i>

#### **Results Chapter 4**

<i>Fig. 4.1 Effects of repeated open field testing on locomotor activity parameters in the bulbectomised rat model of depression .....</i>	<i>223</i>
<i>Fig. 4.2 Characterisation of speed related parameters in the bulbectomised rat model of depression .....</i>	<i>224</i>
<i>Fig. 4.3 Effects of imipramine and L-NA administration in the bulbectomised rat model of depression .....</i>	<i>226</i>
<i>Fig. 4.4 Representative high resolution MRI scans depicting enlarged ventricles following bulbectomy .....</i>	<i>227</i>
<i>Fig. 4.5 Representative blood perfusion map depicting decreased cerebral blood perfusion following L-NA treatment in OB rats .....</i>	<i>229</i>
<i>Fig. 4.6 Rapid behavioural effects of TRIM in the bulbectomised rat model of depression .....</i>	<i>237</i>
<i>Fig. 4.7 Physiological responses to stress in the bulbectomised rat model of depression .....</i>	<i>240</i>



## VI. List of tables

### **Introduction**

<i>Table 1 Selected examples of clinical studies implicating a role for glutamate in depression .....</i>	<i>47</i>
<i>Table 2 Selected trials of glutamatergic drugs for the treatment of depression .....</i>	<i>52</i>
<i>Table 3 Antidepressant-like properties of NOS inhibitors in preclinical studies .....</i>	<i>65</i>

### **Results**

#### **Results Chapter 2**

<i>Table 2.1 Effects of L-NA on regional changes to 5-HTP, 5-HIAA, 5-HT and the 5-HIAA:5-HT ratio following FST exposure in animals pretreated with NSD-1015 .....</i>	<i>156</i>
<i>Table 2.2 Effects of L-NA on regional changes to 5-HTP, 5-HIAA, 5-HT and the 5-HIAA:5-HT ratio following FST exposure in non-swimmied animals pretreated with NSD-1015 .....</i>	<i>158</i>

#### **Results Chapter 4**

<i>Table 4.1 Regional brain volume and regional resting state blood perfusion in the OB model of depression .....</i>	<i>230</i>
<i>Table 4.2 Regional T1 and T2 relaxation times in the bulbectomised rat model of depression .....</i>	<i>234</i>
<i>Table 4.3 Effects of 16 days of imipramine treatment on expression of NOS isoforms and glial related markers in the bulbectomised rat model of depression .....</i>	<i>236</i>



## VII. List of abbreviations

### Most used abbreviations

5-HIAA = 5-hydroxyindoleacetic acid

5-HT = 5-hydroxytryptamine (serotonin)

5-HTP = 5-hydroxytryptophan

7-NI = 7-nitroindazole

AMPA =  $\alpha$ -amino-3-hydroxy-5-methylisoxazole-4-propionic acid

AMPA-R =  $\alpha$ -amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor

ANOVA = Analysis of Variance

a.u. = arbitrary units

BDNF = brain derived neurotrophic factor

BLA = basolateral amygdala

btASL = bolus-tracking arterial spin labeling

CBF = cerebral blood flow

CBV = cerebral blood volume

cGMP = cyclic guanosine monophosphate

CMS = chronic mild stress

CNS = central nervous system

CRH = corticotropin-releasing hormone

CTT = capillary transit time

DA = dopamine

dDG = dorsal dentate gyrus

DRN = dorsal raphe nucleus

eNOS = endothelial nitric oxide synthase

FLI = c-FOS-like immunoreactivity

FST = forced swimming test

GABA = gamma aminobutyric acid

GC = guanylate cyclase

GFAP = glial fibrillary acidic protein

GR = glucocorticoid receptor

HPA = hypothalamic-pituitary-adrenal axis

HPLC = high performance liquid chromatography

iNOS = inducible nitric oxide synthase  
KP = kynurenine pathway  
L-AAA = L-alpha-aminoadipic acid  
L-NAME = N<sup>o</sup>-nitro-L-arginine methyl ester  
L-NMMA = N<sup>o</sup>-nitro-L-monomethylarginine  
LS = lateral septum  
MAOI = monoamine-oxidase inhibitors  
MDD = major depressive disorder  
MeA = medial amygdala  
mGlu-R = metabotropic glutamate receptor  
MRI = magnetic resonance imaging  
mTOR = mammalian target of rapamycin  
MTT = mean transit time  
NA = noradrenaline  
NAc = nucleus accumbens  
NMDA = N-Methyl-D-aspartic acid  
NMDA-R = N-Methyl-D-aspartic acid receptor  
nNOS = neuronal nitric oxide synthase  
NO = nitric oxide  
NOS = nitric oxide synthase  
NOSi = nitric oxide synthase inhibitors  
OB = olfactory bulbectomy/bulbectomised  
PCP = 1-(1-phenylcyclohexyl)piperidine or phencyclidine  
*p*CPA = *para*-chlorophenylalanine  
PDE = phosphodiesterase  
PKA = protein kinase A  
PKC = protein kinase C  
PLCx = prelimbic cortex  
PSD-95 = post-synaptic density protein of 95 kDa  
PVN = paraventricular nucleus of the hypothalamus  
ROI = region of interest  
RS = restraint stress  
SEM = standard error of the mean

SNRI = serotonin/noradrenalin re-uptake inhibitor

SSRI = selective serotonin re-uptake inhibitor

TCA = tricyclic antidepressants

TFI = total FOS immunoreactivity

TPH = tryptophan hydroxylase

TRIM = 1-2-trifluoromethylphenyl imidazole

Trp = tryptophan

vCA1 = ventral cornu ammonis 1





# ***Introduction***



## **1. Major Depressive Disorder (MDD)**

Major depressive disorder (MDD) is a mood disorder and, together with anxiety, is one of the most common psychiatry disorders, affecting up to 25% of the general population worldwide. It is highly prevalent and often associated with a negative impact on medical health and quality of life (Baune et al., 2007; Kessler et al., 2006). The symptoms of depression include both psychological and physiological aspects, e.g. alterations in mood and perception, loss of motivation, social withdrawal which are accompanied by loss of appetite, changes in body weight and disruption in sleep patterns (Berton and Nestler, 2006). To the present day, diagnosis of depression is made based on an evaluation of the symptoms and their recurrence in accordance with the guidelines outlined in the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV, published in 1994 and revised in 2000). In the past 10 years it has been recognised that depression is a complex disorder, which can arise in co-morbidity with anxiety (Lamers et al., 2011; Moffitt et al., 2007), metabolic disorders, e.g. type II diabetes (Ali et al., 2006; Knol et al., 2006), cardiovascular disease (Machado-Vieira and Mallinger, 2012; Zahn et al., 2013), neurodegenerative pathologies (Barnes et al., 2012) and many others.

A genetic predisposition for depression has an important role (Northoff, 2013) as it is known that familial inheritance is a risk factor for development of MDD (Sullivan et al., 2000). In addition the environment plays a critical part in predisposition and vulnerability to depression. Personal experiences such as early life traumas or an impairing injury or illness interact to a lesser or greater extent with genetic, psychological and physiological backgrounds of the individual resulting in the

insurgence of the clinically relevant depressive symptoms (Roh et al., 2012; Han et al., 2011).

Within the brain, depression and mood disorders in general are correlated to the neuronal circuitry of emotional processing, referred to as the “limbic system”, composed of prefrontal cortex, anterior and medial thalamus, cingulate gyrus, hippocampus, amygdala and specific areas in the hypothalamus related to visceral control (Price and Drevets, 2010). There are two overlapping and interconnected circuits in the limbic system: the medial prefrontal cortico–striato–pallido–thalamic and the amygdalo–striato–pallido–thalamic loops. The prefrontal cortex and amygdala in particular are pivotal regions in the cortico-limbic system involved in the processing of emotion which are dysregulated in depression (Diener et al., 2012; Matthews et al., 2008).

### **1.1 Biological mechanisms underlying the pathophysiology of MDD**

From the action of pharmacological antidepressants currently available, it is proposed that depressed patients have alterations in the content and/or functionality of different neurotransmitter systems in the brain, even though the depletion of monoamines such as serotonin (5-HT), noradrenaline (NA) and dopamine (DA) does not seem to affect mood in healthy controls (Ruhè et al., 2007). For many years the prevalent hypothesis for the pathophysiology of depression was the so called “monoamine hypothesis”, attributing the occurrence of the disorder to dysfunctions of the monoamine systems (mainly noradrenergic and serotonergic). In support of the monoamine hypothesis antidepressants have been reported to alter transcription or activity of monoaminergic receptors (Artigas, 2013) and genetic studies have correlated specific alleles of the 5-HT

transporter gene with treatment-resistant depression (Bonvicini et al., 2010) or with suicidal behaviour and an increased risk of MDD following exposure to stressful events in life (Daniele et al., 2011).

Commonly prescribed antidepressants fall into the monoaminergic category and are classified as tricyclic antidepressants (TCAs), monoamine-oxidase inhibitors (MAOIs), selective 5-HT re-uptake inhibitors (SSRIs), NA re-uptake inhibitors (NRIs) and 5-HT and NA re-uptake inhibitors (SNRIs). Re-uptake inhibitors block the plasma membrane transporters for 5-HT and/or NA, leading to an increase in the synaptic availability of the neurotransmitters and their persistence in the synapse augmenting neurotransmission of the two systems (Berton and Nestler, 2006). Older antidepressants such as TCAs (e.g. imipramine) and MAOIs (e.g. moclobemide) also act by increasing monoamine availability in the synaptic cleft by inhibiting the re-uptake or inhibiting the degradation of the neurotransmitters respectively (Frazer, 1997).

Despite the clinical benefit obtained with antidepressant medications, a number of unmet clinical needs remain. The most important is that several weeks of continuous treatment are needed for antidepressant actions to manifest, even if they raise monoamine availability in the brain within a few hours following administration. Remission in half of the patients often requires six months of treatment and two antidepressant trials (Trivedi et al., 2006) which is a major problem as it has been shown that the risk of suicide in depressed patients is increased during the first month of antidepressant treatment, particularly during the first week or nine days, regardless of the class of antidepressant used (Jick et al., 2004; Simon et al., 2006). The existence of this delayed onset of action of standard antidepressants has led to the hypothesis that a

cascade of downstream events is ultimately responsible for their therapeutic effects which follow on from a sustained increase in intra-synaptic levels of 5-HT and/or NA. In addition, these medicines produce low remission rates and a high rate of relapse and recurrence (Thase, 2011). They also produce physiological side effects which can lead the patients to drop out from the treatment, such as sedation, weight gain, drowsiness, sexual dysfunction, constipation or blurred vision. These largely arise due to the antagonistic action of the antidepressants on muscarinic, histaminergic and adrenergic receptors (Stahl, 1998).

## **2. Animal models of depression**

In the process that goes from the discovery of a new compound to the standardisation of a therapeutic treatment for clinicians, the understanding of the pathophysiology of a disease and the identification of possible new targets for drugs are fundamental. Along with this, another very important step is the assessment of the potential efficacy of the new drug and its possible side effects. Preliminary screenings in this direction are usually performed in animal models. The ideal animal model for a human disorder should provide good approximations of the dysfunctions seen in the disease in humans. Nonetheless, in the research of new medications for psychological disorders such as depression, the wide spectrum of disruptions involved highlights the difficulty in mimicking the disorder in laboratory animals, especially because symptoms such as suicidal thoughts are technically impossible to assess. To overcome this difficulty, researchers' approach has been to create animal models in which only one or two specific traits of the disorder are reproduced. More specifically, Willner et al. (1997) proposed that the perfect animal model of a mood disorder should present three main

characteristics: 1. *face validity*, meaning that it should reproduce some of the symptoms of depression observed in humans, 2. *construct validity*, as that the symptoms seen in the animals should be mediated by equivalent neurobiological mechanisms as in humans and 3. *predictive validity*, meaning that currently used treatments for depression should modulate the behavioural alterations presented by the animal model with a clear cut behavioural readout which can be reliable and reproducible between different laboratories. An additional ideal characteristic is the *usability* of the model i.e. that it should be easy to produce and manipulate.

Multiple animal models are employed in order to make significant contributions to understanding the aetiology and treatment of clinical depression.

## **2.1 Stress and animal models of depression**

Clinical observations support the hypothesis that stress is one of the major precipitating factors in the onset of depression (e.g. Gilbert et al., 2004), and many animal models of depression utilise stressors to induce distinctive behavioural and neurobiological deficits. For example the chronic mild stress (CMS) model is a chronic model of depression based on exposure of mice and rats to a series of mild unpredictable stressors such as cage tilting, soiled bedding, forced swimming and inversion of the light/dark cycle. Behavioural anhedonia develops in such animals who exhibit a decreased capacity to respond to rewarding stimuli including preference for palatable sweet solutions or places associated with the availability of food rewards. Anhedonic behaviour is prominent in human depression particularly in melancholic depression. Other symptoms suggestive of depression are also evident in the model such as despair in the FST, weight loss and reduction in self care/grooming. Chronic but not acute



antidepressant treatments reverse such behaviours in animals exposed to CMS mimicking the delay in onset of therapeutic action observed in the clinic (for review of CMS see Willner, 1997).

Specific changes in brain structure and function have been identified in clinical depression where reductions in hippocampal volume are evident relative to healthy controls. In this regard, exposure to chronic restraint stress (CRS) or psychosocial stress in rats induces neuronal atrophy in the hippocampus mediated by elevated circulating levels of glucocorticoids and central glutamate release. Such atrophy is accompanied also by behavioural changes and both may be prevented with antidepressant drug treatment. It has been suggested that a similar psychopathology may account for changes observed in hippocampal volume associated with clinical depression. NMDA receptor antagonists also prevent hippocampal atrophy following exposure of rats to CRS (Wood et al., 2004; see also Lee et al., 2009; McKittrick et al., 2000; Magariños et al., 1997).

## **2.2 Despair-based models of depression**

Along with the induction of depression related neurobiological changes, stressful stimuli are used to produce a depressive state in animals in order to create behavioural animal models of depression. In most of cases these models involve the presentation of the animal with an uncontrollable stressor such as inescapable shock, immobilisation stress or other treatments unpleasant to the animals, and the assumption that rats previously exposed to an uncontrollable stress would subsequently show some behavioural deficits, referred to as “learned helplessness” (Cryan et al., 2002). The restraint stress model is one of the most commonly employed models to mimic potent

physical and psychological stress in laboratory animals (Glavin et al., 1994), due to the simplicity of its procedure as it is performed by placing rodents into a well ventilated plastic transparent tube for several hours. A single episode of restraint stress has been shown to be enough for rats to exhibit a decrease in 24 hr cumulative food intake and growth rate (Haleem and Parveen, 1994) and an increased HPA axis activity, reflected in increased circulating levels of corticosterone, noradrenaline and glucose (Samad et al., 2006). 5-HT metabolism in the brain is also increased (Samad et al., 2006; Haleem et al., 2007). Unfortunately, models based on learned helplessness have been shown to lose the depressive-like symptomatology within two or three days following cessation of the stress, thus allowing to test only for preventive action of antidepressants on the induced depression-like features, but this drawback seems to be overcomeable by repeated administration of a mild stressor (Gambarana et al., 2001). Repeated exposure to a stressor can trigger a sort of adaptive change in the brain to cope with the stress, thus accounting for the dissipation of behavioural deficits produced for instance by a regime of repeated immobilisation stress (Haleem and Parveen, 1994). A change in 5-HT receptor responsiveness has been suggested to be involved in this type of adaptation (Haleem et al., 2007).

### ***2.2.1 Forced swimming test***

The forced swimming test (FST), originally described by Porsolt et al. (1978), is one of the most widely used tests to screen for acute pharmacological antidepressant activity. The test is based upon the observation that rodents, when placed in a cylinder full of water, eventually adopt an immobile posture and stop active escape related behaviours,

such as climbing or swimming, considered a state of learned helplessness referred to as “behavioural despair”. Traditionally in rats it is carried out in naïve animals where an exposure to an inescapable swim session is used to make the animals acquire the “behavioural despair” which is then measured in a subsequent test session 24 hr later. Treatment with antidepressants reduces the amount of time the animals spend immobile and prolongs the active escape related behaviours. The FST is able to detect behavioural effects common to very different antidepressant treatments with very diverse pharmacological and physiological actions, and these include clinically effective treatments such as tricyclic antidepressants, monoamine oxidase inhibitors and atypical antidepressants (Borsini and Meli, 1988).

Despite its common use as a test to screen for antidepressant activity, the FST does present with some weaknesses. One of them is the fact that results generated from the test depend on a performance based on physical exercise, so they can be affected by psychomotor stimulant drugs which can induce false positive reductions in immobility time. For this reason the swim test is usually accompanied by common locomotor activity tests such as open field exploration or locomotor activity chambers. Another major drawback of the standard FST is its unreliability in detecting the antidepressant-like effects of SSRIs, and possibly all similar serotonergic drugs (reviewed in Cryan et al., 2005). However, Detke and colleagues (1995) were able to develop a modified version of the test, characterised by an increased water depth (from depths of 15–18 to 30 cm) and scoring of multiple behaviours within the session. In particular, along with the immobility time, swimming and climbing episodes are recorded and the actual scoring system is based on the prevalence of the different behaviours over 5 seconds intervals during the test session. This version of the FST allows for the discrimination

between antidepressant drugs with different acute neuropharmacological effects, on the basis of the behaviour predominantly induced by the drug. In fact swimming behaviour is increased by SSRIs, whereas climbing behaviour is increased by antidepressant drugs with selective effects on catecholamine transmission (Cryan et al., 2005).

Usually the potential antidepressant effect of a new drug is deduced by its ability to reduce immobility time in the test when administered in naïve rats, and by comparing its effects in the FST to those of clinically prescribed antidepressants. In this paradigm antidepressant drugs are altering the stress response of healthy animals to the inescapable swim, and if it was to be transposed into humans it would be the situation of administering antidepressant drugs to healthy subjects. For this reason, more recently the FST has been looked at as a possible way to detect a depressive phenotype induced in animals following various manipulations including exposure to stressors (Kim et al., 2006; 2012; Veena et al., 2009; Armario et al., 1991) or with pre-existing genetic vulnerability to stress and depression (Solomon et al., 2012; Jacobson and Cryan, 2007; Cryan and Mombereau, 2004). The manipulations performed prior to the test are believed to bring about a depressive endophenotype in the animals which results in an increase in immobility time during the FST, thus resembling the human condition where depression develops before providing any pharmacological treatment. After almost 35 years, the versatility, easiness and speed of the FST in detecting both anti- or pro-depressant effects of treatments, make it remain one of the most popular behavioural test to evaluate depression-like behaviour and antidepressant efficacy in rodents.

### **2.3 The olfactory bulbectomised rodent model of depression**

The olfactory bulbectomised (OB) rat or mouse, is a lesion-based animal model of depression which is obtained by surgical removal of the olfactory bulbs from the animals, thus resulting in an anterograde and retrograde degeneration of the neurons that project to and from the main and accessory olfactory bulbs (Kelly et al., 1997). This leads to remodelling of brain areas linked to the olfactory bulbs, such as cortex, hippocampus, amygdala and locus coeruleus and brings about a series of behavioural and physiological changes referred to as the “OB syndrome” (reviewed by Harkin et al., 2003 and Song and Leonard, 2005). Such changes are thought not to be associated with anosmia and include: increased locomotor activity in a novel environment; high corticosterone plasma concentrations under basal and stressful conditions; altered circadian rhythm and changes in basal locomotor activity, body temperature, heart rate and heart rate variability (Vinkers et al., 2009); altered physiological responses to stress compared to sham-operated controls (Vinkers et al., 2009; Roche et al., 2007); hyperemotionality, indicated by stronger responses to some minor stimuli such as a rod, a stream of air, handling or pinching of the tail (Pandey et al., 2008; 2010) and increased aggressiveness, e.g. muricide behaviour. The dysinhibition of the amygdala due to neurodegeneration and remodelling, and the lack of detection of pheromones due to anosmia may be pivotal in the development of the OB syndrome (Song and Leonard, 2005). Structural changes in the brain of OB animals are evident including enlarged ventricular volumes analogous to those reported in depressed patients. Changes in the central concentrations of NA and in particular 5-HT have been reported, such that the model is related to a hyposerotonergic state (Kelly et al., 1997). It is noteworthy that pharmacological agents acting on specific 5-HT receptor subtypes have

antidepressant related activity in the OB rat model (Mnie-Filali et al., 2011; Pandey et al., 2010). A reduction in central glutamate concentrations and in the density of the NMDA receptors has been also reported in these animals which may link to the cognitive deficits associated with the model (Ho et al., 2001; Robichaud et al., 2001). Neurotrophins such as BDNF and NGF, and also the immune system are affected by bulbectomy (Song et al., 2009; Hellweg et al., 2007; Connor et al., 2000).

The OB rat exhibits changes in behaviour and neurotransmitter function which are attenuated following chronic antidepressant treatment only, mimicking the time course required for therapeutic efficacy in depressed patients. This feature sets it apart from alternative models and is a useful characteristic for determination of the onset of antidepressant activity over repeated treatments. The issue of a slow onset of action of antidepressant activity of commonly prescribed medications is still a major problem in the treatment of depression and testing for potentially faster-acting antidepressant drugs is a promising strategy. The OB model, alongside with other chronic models of depression, poses as a useful model in which the onset of antidepressant activity of test compounds may be evaluated and compared to activity associated with more conventional antidepressants.

As yet, the neuro-regenerative capacity of antidepressants via the induction of neurotrophic factors in the model has been rarely put to the test (Freitas et al., 2013). Nonetheless such an explanation for the mechanism of action of antidepressants in the model would complement the current understanding that bulbectomy can damage specific vulnerable populations of neurons which underlie the OB syndrome. Several studies have reported alterations in glutamatergic transmission through the N-methyl-D-

aspartate (NMDA) receptor following bulbectomy, which may contribute to the associated behavioural changes. Moreover NMDA receptor antagonists display antidepressant activity in the model (reviewed by Harkin et al., 2003).

### **3. Beyond the monoamine hypothesis: other biological mechanisms involved in depression**

Limitations presented by the monoamine hypothesis of depression and unmet needs associated with monoaminergic-based antidepressants have prompted further research to explore alternative mechanisms involved in the aetiology and pathophysiology of the illness with increased attention towards glutamatergic, GABAergic and neuropeptidergic systems which give rise to changes in neuronal plasticity or alterations in the hypothalamic-pituitary-adrenal (HPA) axis associated with depression. The immune system has also been implicated in the pathophysiology of the illness. Such efforts have led to the development of several new hypotheses being proposed for the pathophysiology of depression (Masi and Brovedani, 2011; Rakofsky et al., 2009; Dantzer et al., 2008).

#### **3.1 Depression and the hypothalamic-pituitary-adrenal (HPA) axis**

Depressed patients commonly exhibit hyperactivity of the HPA axis and raised circulating cortisol concentrations may relate to hypersecretion of corticotropin-releasing hormone (CRH) and/or impaired sensitivity to endogenous glucocorticoids, which are potent negative regulators of the HPA axis activity itself (reviewed by Pariante and Miller, 2001). Drugs targeting the CRH<sub>1</sub> and CRH<sub>2</sub> receptors for

antidepressant action, particularly CRH<sub>1</sub> as it is the predominant subtype, are showing encouraging results (Ising and Holsboer, 2007; Künzel et al., 2003; Zobel et al., 2000). Cortisol produces its effects through the glucocorticoid receptor (GR) and considerable efforts have focused on elucidation of a role for this receptor in depression (Pariante and Miller, 2001). Genetic polymorphisms of both GR and its chaperone protein FKBP5 have been linked to susceptibility to depression (Szczepankiewicz et al., 2011; Zimmermann et al., 2011; Binder et al., 2004), thus supporting a role for GR receptor regulation and HPA axis alterations in the pathophysiology of the illness.

### **3.2 Depression and the immune system**

Depression, stress and immunological activation and the role of the immune system in depressive illness have been the subject of a number of reviews to date (Krishnadas and Cavanagh, 2012; Littrell, 2012; Raison and Miller, 2011). Smith (1991) proposed a macrophage theory of depression suggesting that hypersecretion of macrophage cytokines IL-1, TNF- $\alpha$  and IFN- $\alpha$  may play a causal role in depressive illness. Cytokines are a heterogeneous group of polypeptide hormones produced and secreted by immune cells and play a role in activation of the immune system and inflammatory responses. Cytokines and their receptors are also located in the peripheral and central nervous systems (CNS) and can affect neurotransmission within the CNS. MDD is often associated with raised inflammatory markers including high sensitivity c-reactive protein (CRP), IL-6, IL-1, TNF- $\alpha$  and soluble IL-2 receptor (Dowlati et al., 2010; Howren et al., 2009) and changes in inflammatory markers are more profound in treatment-resistant depression. When taken together with evidence that administration of immunotherapeutic cytokines such as IFN- $\alpha$  or IL-2 to psychiatrically normal



individuals can provoke depression and that there is an increased prevalence of depression in autoimmune and inflammatory disorders, there is growing support for a role of inflammation in the pathogenesis of the illness (reviewed by Krishnadas and Cavanagh, 2012). Recent observations report an inflammatory signature associated with depression and support a role for the activation of brain microglia, resident monocytic-like cells in the brain capable of phagocytosis which are responsible for the production of inflammatory cytokines and are capable of responding to them (Beumer et al., 2012; Dantzer et al., 2008; Raison and Miller, 2011). Patients with autoimmune or infectious diseases show a much greater extent of induction of pro-inflammatory molecules than patients with depressive disorders, in which case increases are modest, typically just two to three times higher compared to healthy controls (Dowlati et al., 2010). In this regard inflammation may act as a predisposing factor for depression overlapping with other mechanisms implicated in the disorder including activation of the HPA axis and reduced neurotrophin expression/neurogenesis leading to impaired synaptic plasticity and possibly contributing to neurodegeneration where neuroinflammation may promote neuronal cell loss (Krishnadas and Cavanagh, 2012).

Increased circulating concentrations of inflammatory cytokines and acute phase proteins returning to normal following antidepressant treatment suggest that such markers may be state rather than trait markers of the illness (Hannestad et al., 2011; Hernández et al., 2008). Moreover there is clinical evidence for antidepressant efficacy against cytokine (IFN- $\alpha$ )-induced depression (Musselman et al., 2001; Schäfer et al., 2000). Interestingly, there seems to be a differentiation between short and long term effects of antidepressants on inflammation where short term use reduces inflammation and ameliorates depression and prolonged use is associated with a loss of efficacy and the

promotion of a pro-inflammatory state (Littrell, 2012). Cytokine antagonists too have shown potential for the treatment of depression where the microglial inhibitor minocycline or the IL-1 receptor antagonist (IL-1ra) exhibit antidepressant-like properties in animal models (Pae et al., 2008). Conversely it has also been reported that IL-1ra administration to human subjects may promote depression related symptoms and that depressed patients exhibit higher circulating concentrations of IL-1ra (Jonville-Bera et al., 2011). A recent randomised controlled trial of the TNF- $\alpha$  antagonist infliximab was reported to be effective in a group of treatment-resistant depressed patients where it was also shown that only those patients with an inflammatory phenotype responded to treatment (Raison et al., 2013). Other anti-inflammatory agents including cyclooxygenase inhibitors have also been reported to possess antidepressant properties (Abbasi et al., 2012; Müller et al., 2006). Thus targeting the immune system in some cases has proved to be an effective approach in treating depression, in particular where depression is present with evidence of an activated immune system and inflammation.

### **3.3 Depression and neurotrophic factors**

It has been proposed that antidepressant action may relate to an up-regulation of neurotrophic factors and in particular brain derived neurotrophic factor (BDNF) which has been reported consistently in the hippocampus of laboratory animals following chronic antidepressant treatment. Such a mechanism is further supported by evidence that exposure to chronic uncontrollable stress provokes reduction in hippocampal BDNF (Xu et al., 2006; Luo et al., 2005) and several antidepressants counteract these effects (Della et al., 2012). Reduced BDNF levels have been also associated with vulnerability to depression (Blugeot et al., 2011) and with reduced resilience to chronic

stress (Taliaz et al., 2011). A role for BDNF is also supported by evidence that antidepressants increase activation of the BDNF receptor TrkB (Rantamäki et al., 2007; Saarelainen et al., 2003) and in animal models where TrkB agonists display antidepressant-like activity (Blugeot et al., 2011).

Reduced neurotrophic support ties in well with neuroimaging studies in humans where reduced hippocampal and amygdaloid volumes are reported in depressed patients (McKinnon et al., 2009; Kronenberg et al., 2009; Colla et al., 2007; Frodl et al., 2003) indicating that structural remodelling may contribute to the pathophysiology of depression and reductions in BDNF may contribute to hippocampal atrophy and reductions in hippocampal volume (Videbech and Ravnkilde, 2004). Antidepressant treatments reverse or prevent stress-induced reductions in hippocampal BDNF expression and it is notable that other treatments including NMDA receptor antagonists that are known to have antidepressant efficacy also increase the expression of BDNF in the hippocampus. These neurotrophic actions of antidepressants could reverse neuronal atrophy and thereby contribute to the therapeutic effects of these treatments (reviewed by Duman and Monteggia, 2006).

On foot of neurotrophic deficits observed in the hippocampus considerable attention has been paid to the effects of stress and antidepressants on hippocampal neurogenesis (Ehninger and Kempermann, 2008). Stress related impairments in hippocampal neurogenesis which are opposed by antidepressant treatment may contribute to hippocampal atrophy and contribute to mechanisms underlying neuronal atrophy and the reduction of neuronal processes (Duman, 2004; 2002). The role of astrocytic glial cells in adult neurogenesis by secreting appropriate neurotrophic factors and maintaining a “neurogenic permitting” environment is also significant and will be

returned to later within the context of a role for glutamatergic transmission in the pathophysiology of depression (Morrens et al., 2012).

Reduced BDNF expression in the hippocampus is proposed to be mediated by stress related increase in glucocorticoid concentrations leading to reduced dendritic arborisation and neuronal atrophy in the hippocampus. Along with BDNF other growth factors, such as the vascular endothelial growth factor (VEGF) and the insulin-like growth factor 1 (IGF-1), have been linked to depression (Masi and Brovedani, 2011) in a similar fashion. Inflammatory cytokines promote activation of the HPA axis (reviewed by Dunn, 2000) and systemic administration to rats is associated with reduced cortical and hippocampal BDNF and TrkB expression (Guan and Fang, 2006; Tanaka et al., 2006). A role for IL-1 is also reported in mediating stress related reduction in hippocampal BDNF where reduced expression of the neurotrophin following social isolation stress was blocked by intra-hippocampal administration of IL-1ra (Barrientos et al., 2003). Such reports compliment evidence of a role for inflammatory mediators as casual factors in depression and promote the concept of an integrated pathophysiological basis for the disorder comprising central transmitting, endocrine and immunological factors.

Over the past decade there have been several significant advances in the understanding of the role of CNS transmitters and in particular the involvement of neurotrophic factors, endocrine and immune systems which underlie pathophysiological and behavioural changes related to stress and depression. The bigger picture is likely to involve the integration of the relative contribution of each system as they co-exist and work in concert although it may be possible to identify different subtypes of the

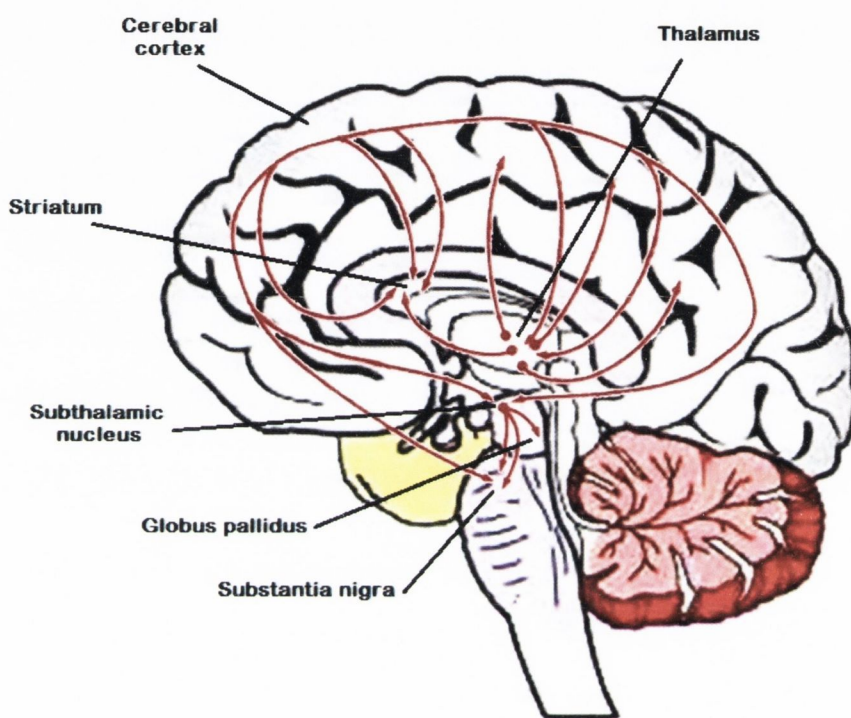
disorder depending on the extent to which dysregulation within any individual system prevails. It is important to consider how any immunological/endocrine theory of depression might fit with more established hypotheses including the monoamine hypothesis where depression is proposed to be related to a deficit in synaptic availability of monoamine transmitters. In this regard it might be pivotal to be aware of and investigate different metabolic pathways which can alter the availability of monoamine precursors, thus reducing monoamine production. For instance the major pathway responsible for the metabolism of tryptophan (Trp) in the CNS, the kynurenine pathway (KP), may represent such a link where neurotransmission is influenced by endocrine and/or immune factors: in fact the rate limiting enzymes in this pathway are indoleamine 2,3 dioxygenase (IDO), which is induced by inflammatory cytokines, and tryptophan 2,3 dioxygenase (TDO), which is induced by stress hormones such as glucocorticoids.

One unifying theme across multisystem dysregulation in depression is the role the excitatory amino acid transmitter glutamate plays in neuronal plasticity, neuroendocrine regulation and neuroimmune interactions. In addition, on account of new leads which suggest that targeting this system promotes rapid onset action and efficacy in treatment-resistant cases of depression, the glutamatergic system has been singled out for special consideration.

### **3.4 A role for glutamate in the pathophysiology of MDD**

The prominent glutamatergic pathways in the brain are cortico-cortical, cortico-striatal, thalamo-cortical and projections from the cortex to subcortical regions such as the locus

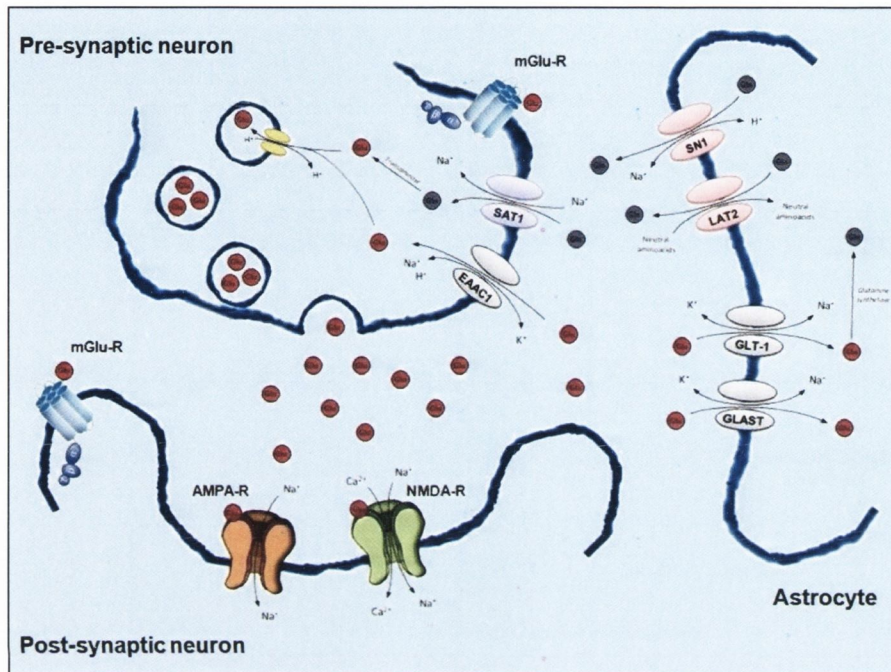
coeruleus, raphe and substantia nigra where monoaminergic pathways are modulated [Fig.1]. Glutamate is charged with homeostatic regulation and integration of cognitive and emotional processes.



**Fig. 1:** Glutamatergic pathways in the brain.

Glutamate is synthesised from glutamine and is sequestered into vesicles at the nerve terminus. Following synaptic release glutamate is taken up by astrocytic glial cells via the glutamate transporters GLAST and GLT-1 and is converted back to glutamine by glutamine synthetase and recycled for the synthesis of new glutamate [Fig.2]. Once glutamate is released from the synapse it activates glutamate receptors characterised into 4 main types: N-methyl-D-aspartic acid (NMDA),  $\alpha$ -amino-3-hydroxy-5-

methylisoxazole-4-propionic acid (AMPA), kainate and metabotropic (mGluR). mGluRs are also located on the presynaptic terminal where they act as inhibitors autoceptors in part by a reduction in  $Ca^{2+}$  flux [Fig.2].



**Fig. 2:** The glutamatergic synapse. Glutamate is released from the pre-synaptic neuron into the synaptic cleft and can activate post-synaptic glutamate receptors (AMPA-R, NMDA-R and mGlu-R). glutamate can also activate pre-synaptic mGlu-R which regulate glutamate release. Excessive glutamate is taken up by pre-synaptic and astrocytic transporters to be recycled. Synaptic transmission plays key functional roles in the CNS including neuronal development and plasticity and is frequently implicated in the aetiology of neurodegenerative disorders as glutamate in excess, or excitotoxicity, represents a primary pathway of neuronal cell death. Glu = glutamate; Gln = glutamine; EAAC1 = excitatory amino acids transporter 1; GLT-1 = glutamate transporter 1; GLAST = glutamate aspartate transporter; LAT2, SAT1 and SN1 = glutamine transporters.

There is growing evidence that glutamate is involved in the pathophysiology and treatment of depression [Table 1]. To date a number of studies carried out on blood and

*post mortem* brain have reported evidence implicating a role for glutamate in major depressive illness (reviewed by Sanacora et al., 2008). Of particular note is the greater intraplatelet  $\text{Ca}^{2+}$  response to glutamate stimulation reported in depressed patients when compared to healthy controls, suggesting that glutamate receptors on platelets are supersensitive and may be a possible peripheral marker of glutamate function in depression.

**Table 1:** Selected examples of clinical studies implicating a role for glutamate in depression

<b>Biomarker</b>	<b>Patient group and outcome – peripheral and <i>post-mortem</i> markers</b>	<b>Reference</b>
<b>Serum or plasma glutamate</b>	Elevated in medicated depressed patients	Altamura, 1993
	Reduced levels after antidepressant treatment	Maes et al., 1998
	Platelet glutamate receptor sensitivity ( $\text{Ca}^{2+}$ ) in depressed patients	Berk et al., 2001
	Increased CSF glutamine levels in depressed patients	Levine et al., 2000
	No significant abnormality in frontal cortex of neurosurgical samples from depressed patients	Francis et al., 1989
<b>Receptor binding</b>	Reduced NMDA binding in suicide victims and subjects with bipolar/unipolar mood disorders	Nowak et al., 1995 Nudmanmud-Thanoi and Reynolds, 2004
	Increased AMPA receptor binding in the striatum of suicide victims	Freed et al., 1993 Noga et al., 1997
<b>Receptor composition</b>	NR <sub>2C</sub> subunit of NMDA receptor elevated in the locus coeruleus in depressed patients	Karolewicz et al., 2005
	Decreased expression of striatal GLU <sub>A1</sub> mRNA (bipolar disorder)	Meador-Woodruff et al., 2001

Several studies have applied proton magnetic resonance spectroscopy to the *in vivo* measurement of amino acids in the brain which have been reviewed elsewhere (Yildiz-Yesiloglu and Ankerst, 2006). In one report there was a 15% increase in occipital cortex

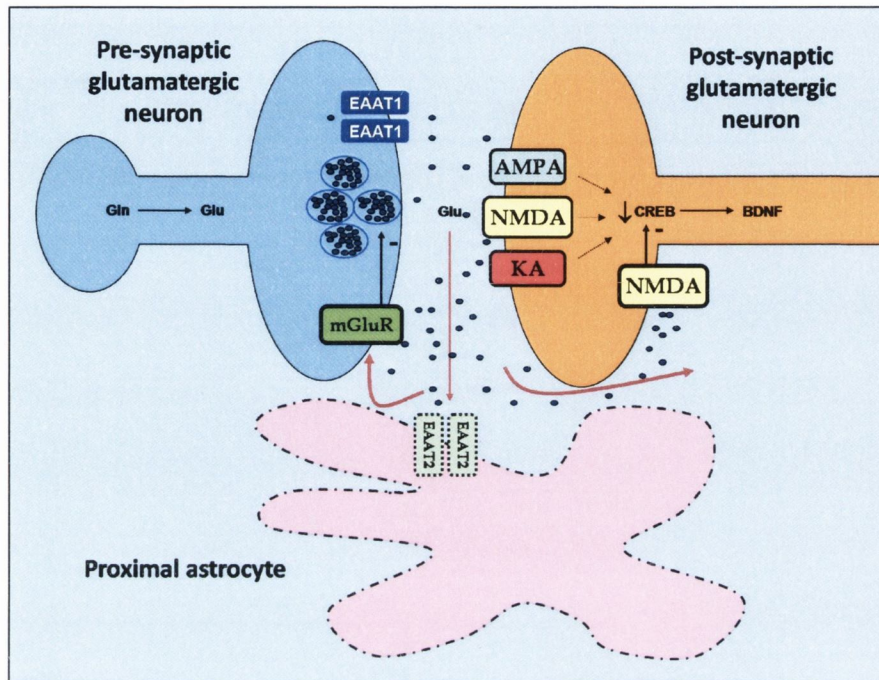


glutamate concentrations in medication-free patients with unipolar depression when compared to age and gender matched healthy controls (Sanacora et al., 2004). Changes were accompanied by reductions in cortical GABA in the same patients. Such evidence supports an hypothesis that regulation of amino acid systems are altered in subjects with depression, indicated by raised Glu:GABA ratios in cortical areas.

The mechanisms that underlie and contribute to the dysregulation of amino acid systems in the cortex of depressed patients are currently unknown. Given the role of glia in maintaining proper glutamate function it is important to take note of several recent studies reporting glial cell abnormalities in depressed patients. Reduced immunostaining of the astrocytic glial marker glial fibrillary acidic protein (GFAP) in the dorsolateral prefrontal cortex has been reported in depressed patients (reviewed by Rajkowska, 2003). The disruption of glia may have a substantial influence on glutamate and GABA function in the brains of individuals with depression.

Since astrocytes play a role in removal of glutamate from the synapse it has been proposed that an astrocytic deficit may account for alterations in glutamate neurotransmission in depression (Kugaya and Sanacora, 2005). Studies employing *in vivo* magnetic resonance spectroscopy have provided functional evidence in support of this hypothesis, where reduced glutamine/glutamate ratios are suggestive of reduced glutamate conversion to glutamine by glial cells (Yüksel and Öngür, 2010). The following elements are proposed in the model: 1. Reduced glial number/function, reduced glutamate uptake, reduced metabolism of glutamate and availability of glutamine for GABA biosynthesis and 2. Increased extracellular glutamate and activation of extrasynaptic NMDA receptors couple to cell death pathways, reduced

neurotrophic support and reduced pre-synaptic release and activation of synaptic receptors via activation of presynaptic mGlu-R [Fig. 3].

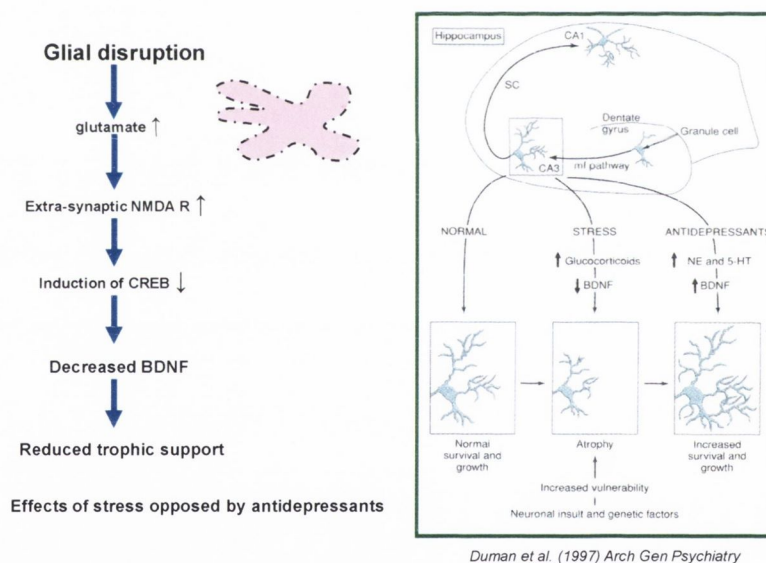


**Fig. 3:** Influence of glial dysfunction on the glutamatergic system and neurotrophic factors. When astrocytes are impaired, excessive glutamate into the synaptic cleft can hyperactivate post-synaptic AMPA-R, NMDA-R, kainate receptors and extra-synaptic NMDA-R, leading to reduced BDNF production.

The mechanisms which contribute to the proposed glial cell pathology associated with depression are currently not understood. Such mechanisms may include genetic predisposition or represent a developmental phenomenon. Moreover stress, glucocorticoids and neurotrophins may modify glial cell number and/or function. A report from the Duman group (Banasr and Duman, 2008) showed a reduction in the density of GFAP-positive cells in the prefrontal cortex of animals subjected to chronic unpredictable stress compared to home cage controls. These changes were accompanied

by anhedonia, anxiety and helplessness characteristic of this rodent model of depression. Administration of the astroglial specific toxin L-alpha-aminoadipic acid (L-AAA) into the prefrontal cortices induced anhedonia, anxiety and helplessness similar to those observed with chronic unpredictable stress. Thus reduced glial number in the prefrontal cortex is sufficient to induce depression-like behaviour and supports the hypothesis that loss of glia contributes to the core symptoms of depression.

A role for abnormal glutamate neuronal transmission in mood disorders may be proposed in line with Duman's neurotrophin hypothesis (originally proposed by Duman et al., 1997) [Fig.4].



**Fig. 4:** Reduced neurotrophic support in response to dysregulated glutamate in accordance with the neurotrophin hypothesis of depression and antidepressant action. Stress related glucocorticoids suppress BDNF in the hippocampus to promote neuronal atrophy. Such atrophy is consistent with brain imaging studies where reduced hippocampal and cortical volumes have been reported in stress-related neuropsychiatric disorders and in particular major depression (reviewed by Lorenzetti et al., 2009 and Duman, 2004). The effects of antidepressants on glial dysfunction are currently unknown although the effects of stress are opposed by antidepressants to promote increased survival and growth through the up-regulation of BDNF.

The delay in onset of antidepressant action may be on account of neurobiological adaptation and changes to synaptic plasticity which take place following repeated antidepressant administration. Following 50 years of antidepressant drug use, there is up to a 30% non response rate and together with a poor understanding of mechanism of action, new drug development will likely depend on an assessment of alternative neurotransmitter systems to provide novel targets for antidepressants working beyond the monoamine hypothesis. In this regard there is a large body of evidence that antidepressants in use effect changes to glutamate neuronal transmission, to suggest that antidepressant may be working in this way (reviewed by Kugaya and Sanacora, 2005). Chronic antidepressant treatments can (1) effect changes to the binding properties of NMDA receptors; (2) alter the expression of NMDA receptor subunits and (3) up-regulate AMPA receptors. Here NMDA receptors play an important role in trafficking of AMPA receptor subunits to the membrane. Antidepressants (4) alter the expression of group 1 and 2 mGluRs and (5) increase the expression of the vesicular glutamate transporter VGLUT1 in the cortex and hippocampus. Antidepressants also (6) reduce depolarisation-evoked glutamate release and (7) can overcome the effects of stress on long term potentiation.

### **3.5 Targeting the glutamatergic system for antidepressant activity**

As antidepressants effect changes to glutamate, the possibility of targeting glutamate receptors directly for therapeutics effect presents. Consistent with a role for glutamate in depression, anti-glutamatergic agents have demonstrated antidepressant efficacy both clinically and in several animal models of antidepressant action (Skolnick et al., 2009). In this regard a number of drugs have shown some activity. Reports to date however are

largely limited to case reports and open label trials and large placebo controlled double blind studies are warranted (see Table 2).

**Table 2:** Selected trials of glutamatergic drugs for the treatment of depression

<b>Drug</b>	<b>Mechanism of action</b>	<b>Patient group and outcome</b>	<b>Reference</b>
<b>D-cycloserine</b>	NMDA-glycine partial agonist	Unsuspected antidepressant action in TB patients. Improvement in mood, insomnia and appetite	Crane, 1959
<b>Amantadine</b>	Low affinity non-competitive NMDA antagonist	Antidepressant activity in PD patients	Parkes et al., 1980
<b>Memantine</b>	Low affinity NMDA receptor antagonist	Positive effects on mood and motor activity in mild to moderate dementia syndrome  No significant effect in double blind placebo controlled study in MDD	Gortelmey and Erbler, 1992  Zarate et al., 2006
<b>KETAMINE</b>	Non-competitive NMDA antagonist	Rapid antidepressant effect for >72 hr  Patients with treatment-resistant MDD showed robust and rapid response to i.v. ketamine for 1 week	Berman et al., 2000  Zarate et al., 2006
<b>Lamotrigine</b>	Inhibits glutamate release (Na <sup>+</sup> , Ca <sup>2+</sup> and K <sup>+</sup> channels)	Monotherapy/augmentation in bipolar, unipolar and treatment-resistant depression (preliminary)	Maltese, 1999 Barbeck and Janhour, 2002
<b>Riluzole</b>	Increases glutamate astrocytic uptake; inhibits glutamate release	Improvement of symptoms in treatment-resistant depression	Zarate et al., 2004

Most notable are the clinical studies that have reported a rapid improvement in depressive symptoms after intravenous administration of ketamine, a non-competitive NMDA receptor antagonist at a dose of 0.5 mg/kg (Berman et al., 2000; Zarate et al., 2006). Two additional reports have more recently confirmed these effects. Using a similar treatment regime to earlier investigations, Phelps and co-workers (2009) reported in an open label study that ketamine reduced depressive symptoms in treatment-resistant patients with a significantly higher response in individuals with a confirmed family history of alcohol abuse when compared to patients with no family history. Moreover Price et al. (2009) reported a rapid and robust antidepressant action of

ketamine using the Montgomery-Asberg Depression Rating Scale (MADRS-SI) in a cohort of treatment-resistant depressed patients and determined that suicidal ideation was significantly reduced, suggesting that ketamine may be useful in acutely suicidal depressed patients. Although beneficial effects have been observed for up to 7 days (Zarate et al., 2006), the use of ketamine in the clinic is hampered due to its adverse effects. A significant drawback associated with the use of ketamine and related drugs is that they produce a series of side effects in particular dissociative effects and perceptual disturbances. Cognitive deficits and euphoria are however transient following ketamine infusion whereas improvement in mood lasts for several days. Nevertheless the use of such drugs as therapeutics is limited by the psychotomimetic effects and potential for abuse (Berman et al., 2000). Although there are other drugs in clinical use which also target the NMDA-R such as memantine and metapramine, they are less effective as antidepressants compared to ketamine probably on account of a lack of potency (Zarate et al., 2006b). Moreover intravenous administration of ketamine seems to be required to achieve a robust antidepressant effect (Zarate et al., 2006a) which is less desirable by comparison to oral medication. Ultimately any medication which modulates NMDA-R functioning, particularly where long term administration is required, is likely to be problematic due to the extensive presence of these receptors throughout the CNS. It may however be possible to target the NMDA-R and related signalling for antidepressant effect in a manner which does not result in blockade of the ion channel yet maintains the antidepressant properties of NMDA-R antagonists like ketamine. To explore this possibility further the NMDA-R-NO intracellular signalling pathway will be discussed as a strategy which shows early promise to harness the potential of the NMDA-R without the significant risk of related adverse effects.

#### 4. The NMDA receptor – nNOS – NO pathway

The NMDA-R is an ionotropic glutamate receptor subtype that is widely distributed in mammalian brain. Stimulation of the receptor results in entry of  $\text{Ca}^{2+}$  into the receptive neuron and it binds to a calmodulin complex which in turn stimulates neuronal nitric oxide synthase (nNOS) leading to production of nitric oxide (NO) [Fig. 5]. NO can exert different actions within the cells, from permanent chemical modification of molecules (S-nitrosylation or SNO), to activation of intracellular signalling (via NO-sensitive guanylate cyclase or NO-GC), to paracrine signalling by diffusion to neighbouring cells.

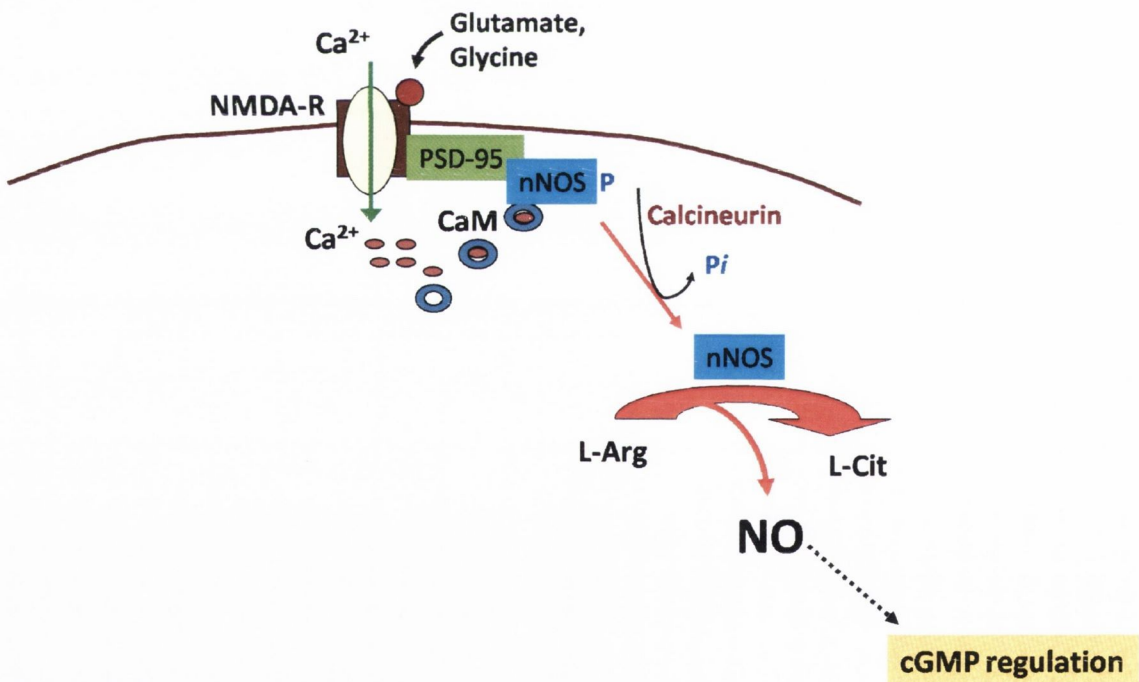


Fig. 5: The NMDA-R-NO-cGMP signalling pathway

## 4.1 The NMDA receptor

The NMDA-R is a complex comprising different subunits, mainly NR1 and NR2, and more rarely NR3. These subunits build a transmembrane ion channel that allows  $\text{Ca}^{2+}$  and  $\text{Na}^+$  influx in the postsynaptic cell and  $\text{K}^+$  to efflux from it. The majority of NMDA-Rs in the CNS are made up of two NR1 subunits and two NR2 subunits, forming a tetramer. There are seven subtypes of NR1 (NR1A-G), four of NR2 (NR2A-D) and two of NR3 (NR3A-B) and a functional NMDA-R requires expression of at least one NR1 subunit and one NR2 subunit (Paoletti and Neyton, 2007). It is suggested that the NR3A subunit may serve as a regulatory factor in NMDA-Rs activation by controlling the amplitude of response and  $\text{Ca}^{2+}$  influx through the channel (Dingledine et al., 1999).

The NMDA-R subunits consist of a modular structure composed by four distinct domains: the extracellular amino-terminal domain (ATD), the extracellular ligand binding domain, the transmembrane domain and the intracellular carboxyl-terminal domain (Hansen et al., 2010). The ATD is involved in trans-synaptic protein-protein interactions, receptor assembly and control of the receptor activation. ATDs have been recently looked at with interest as candidate targets for new NMDA-R modulating drugs, as in some specific subunits they can carry binding sites for allosteric modulators (Mony et al., 2009).

Activation of the receptor is dependent on simultaneous binding of the two co-agonists glutamate and glycine to the specific binding sites allocated on the NR2 and the NR1 subunits respectively, while its intracellular signalling is mediated by the carboxyl terminus which provides multiple sites of interaction for intracellular proteins (Mayer,



2006). At resting conditions endogenous  $Mg^{2+}$  is bound within the channel and prevents the passage of ions. There are also additional binding sites within the channel such as the  $Zn^{2+}$  binding site and the 1-(1-phenylcyclohexyl)piperidine (PCP or phencyclidine) binding site, at which use-dependent antagonists such as ketamine and MK-801 can also bind.

#### **4.2 The post synaptic density protein of 95 kDa (PSD-95)**

The post synaptic density protein of 95 kDa (PSD-95) is a scaffolding protein that links NMDA-R to a number of molecules via its various domains. Particular motifs in PSD-95 structure are known as the PSD-95/discs large/ZO-1 homologous (PDZ) domains, which are made by ~90 residues and act as elements for protein-protein interactions through both homo- or heterodimerisation. There are three PDZ domains in PSD-95 and specifically, the first and the second one (PDZ1PSD95 and PDZ2PSD95) have been both shown to be able to interact with the carboxyl terminal of NMDA-R, but PDZ2PSD95 is also able to interact with the nNOS PDZ domain (PDZnNOS) to form a heterodimer. Thus through PSD95, nNOS is kept in close proximity to the NMDA receptor to achieve maximal efficiency in coupling NMDA-induced  $Ca^{2+}$  influx to the activation of nNOS. Under resting conditions the majority of NOS molecules are found in the cytoplasm (Rothe et al., 1998) and adaptor proteins (syntrophin, PSD-95/SAP-90, PSD-93 and CAPON) are required to carry it to the cell membrane when needed (reviewed by Doucet et al., 2012 and Zhou and Zhu, 2009).

### 4.3 Neuronal nitric oxide synthase (nNOS)

Nitric oxide synthases have an almost ubiquitous distribution in the body. They are a family of enzymes and four subtypes of NOS exist including endothelial NOS (eNOS or NOS3), neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2) and mitochondrial NOS (mtNOS). These enzymes catalyse the oxidation of L-arginine to citrulline and NO in the presence of NADPH and O<sub>2</sub> upon dimerisation in the presence of cofactors such as tetrahydrobiopterin (BH<sub>4</sub>), Zn<sup>2+</sup>, iron protoporphyrin IX (heme), flavin adenine dinucleotide (FAD) and flavin adenine mononucleotide (FMN). nNOS and eNOS are constitutively expressed in mammalian cells and are both Ca<sup>2+</sup>-calmodulin dependent, while the inducible isoform is not (reviewed by Guix et al., 2005, Zhou and Zhu, 2009). nNOS derives its name from the fact that it was first identified in neuronal tissue, but it is also present in skeletal, cardiac and smooth muscle. nNOS constitutes the predominant source of NO in neurons, usually localising to synaptic spines, and it is expressed in both immature and mature neurons and also in astrocytes. NOS containing neurons have a diffuse distribution throughout the brain and are found in the cerebral cortex, the olfactory system, caudate-putamen, hippocampus, amygdala, thalamus, hypothalamus, raphe nucleus, ventral tegmental area, substantia nigra, cerebellum, pons and medulla oblongata (Rodrigo et al., 1994; Vincent and Kimura, 1992).

Dimerisation is fundamental for activation as it creates high-affinity binding sites for L-arginine and BH<sub>4</sub> and protects the enzyme from proteolysis; it is known that the endogenous protein inhibitor of NOS (PIN) acts through destabilisation of the NOS dimer. Phosphorylation also plays an important role in nNOS activation and in response

to extra- and intracellular stimuli several kinases and phosphatases, e.g. protein kinase A (PKA), protein kinase C (PKC) and phosphatase 1, can modulate NOS activity in order to create an integrated system with NMDA-R activation (Zhou and Zhu, 2009).

#### **4.4 Intracellular targets of nNOS activation and effects of NO production**

nNOS in the cytoplasm is bound to different adaptor proteins. One of them is the cytoplasmic protein CAPON, which binds nNOS with its carboxyl-terminal PDZ-binding domain and which also possesses an amino-terminal phosphotyrosine binding (PTB) domain that interacts with the small monomeric G protein Dexras1 (Fang et al., 2000). Dexras1 can alternate between an 'inactive' GDP-bound state and an 'active' GTP-bound state thus influencing the activity of MAP kinase signalling pathways in the brain.

In the brain NO exerts numerous physiological effects mainly through two separate mechanisms: a biochemical reaction called S-nitrosylation and the guanylate cyclase/cGMP-dependent pathway. S-nitrosylation is a post-translational protein modification involving the covalent attachment of a nitrogen monoxide group to the thiol side chain of cysteine. This chemical modification is involved in the regulation of different receptors, including NMDA-R, and their downstream pathways (Wang et al., 2012). The second mechanism involves the activation of NO-sensitive guanylate cyclase (NO-GC) and the generation of cGMP, which in turn activates Protein Kinase G-I and G-II (PKGI and PKGII respectively). NO, via NO-GC, is thought to mediate interneuronal communication and long-term potentiation (LTP) by its action as retrograde messenger, in fact NO is produced after NMDA-R activation in the post-

synaptic neuron and then it can diffuse to influence the pre-synaptic neuron causing long lasting increases in neurotransmitter release (Friebe and Koesling, 2009). cGMP/PKG-induced phosphorylation is implicated in neuronal differentiation, in gene expression and in the regulation of intracellular  $\text{Ca}^{2+}$  homeostasis and of the release and uptake of various neurotransmitters (Eguchi et al., 2012; Yuasa et al., 2012; Wong et al., 2012; Gallo and Iadecola, 2011; Kawai and Miyachi, 2001). cGMP in turn is degraded by phosphodiesterase-5 (PDE-5) (Mullershausen et al., 2003).

## **5. Is there a role for NMDA-R coupled NO in the pathophysiology of depression?**

NO is a highly diffusible gas, sufficiently small and hydrophobic to readily cross cytoplasm, membrane and extracellular fluid, and thus it can act intracellularly and intercellularly on surrounding neurons, glia and vasculature (Kiss and Vizi, 2001). It is able to influence the serotonergic, dopaminergic, cholinergic and other neurotransmitting systems (Prast and Philippu, 2001), and it can also modulate the neuroendocrine system (Orlando et al., 2008).

### **5.1 A role for NO in depression**

A potential role for NO in affective disorders has been proposed, as patients with major depression show altered plasma nitrate and/or nitrite levels (Talarowska et al., 2012; Chrapko et al., 2004; Suzuki et al., 2001) that seem to be associated with suicide attempt (Kim et al., 2006). Moreover, post-mortem studies have provided evidence for a reduction (Gao et al., 2012; Karolewicz et al., 2004; Xing et al., 2002) or an increase

(Oliveira et al., 2008) in NOS protein and activity in specific brain regions in depressed patients. There is also a strong association between major depression and cardiovascular disease (CVD) and considering the fact that NO is a key mediator of blood vessels smooth muscle relaxation and a physiological inhibitor of platelet adhesion and aggregation, it might represent a mechanism to account for this association (Chrapko et al., 2004). Le Melleo et al. (2004) have proposed that factors such as the stress hormone cortisol or the inflammatory marker c-reactive protein may mediate the dysregulation of NO production in depression.

Some commonly prescribed antidepressant drugs have been shown to alter NOS activity. It is common knowledge that sexual dysfunction is one of the side effects when treating depressed patients with SSRIs and this is believed to be due to the induced increase of 5-HT on one hand and to the inhibition of NOS activity on the other. Antidepressants of this family, such as paroxetine and citalopram, have indeed been linked to modulation of NOS expression and activity (Kadioglu et al., 2010; Angulo et al., 2001). With regard to neuronal tissue, citalopram, paroxetine and imipramine, all commercially available antidepressants, have been shown to dose dependently decrease hippocampal NOS activity *in vitro* albeit at concentrations which are considered too high to be of therapeutic relevance (Wegener et al., 2003; Finkel et al., 1996). Wegener and colleagues (2003) reported that local, but not systemic, *in vivo* citalopram, paroxetine and imipramine administration significantly decreased NOS activity in the rat ventral hippocampus. More recently Crespi (2010) reported similar effects with fluoxetine in the rat striatum. It might be that the action of antidepressants on NOS may be exerted only under pathological conditions, as chronic systemic administration of

citalopram, alone or in combination with lithium, did not affect NOS activity in hippocampus, cerebellum and frontal cortex *ex vivo* (Wegener et al., 2004).

Nitric oxide metabolites (NO<sub>x</sub>) levels have been found to be significantly increased in plasma of healthy humans after paroxetine administration which normalised after paroxetine discontinuation (Lara et al., 2003). By contrast paroxetine was reported to significantly decrease NO<sub>x</sub> plasma levels in ischaemic heart disease patients, an effect absent with the tricyclic antidepressant nortriptyline (Finkel et al., 1996). From such reports it is evident that some antidepressants can modulate NOS activity.

Although the evidence for a role for NO in the pathophysiology of depression is limited, its role is further implicated secondary to evidence in support of a role for the NMDA-R given its close association with this glutamatergic signalling pathway. Thus NMDA-R-NO has provided researchers with a novel platform on which to further explore the potential of glutamatergic signalling for antidepressant action.

## **5.2 NO and neuroprotection**

As discussed earlier MRI studies in depressed patients have reported reductions in the volume of a number of brain areas relative to healthy controls, especially in limbic structures such as the prefrontal cortex, cingulate gyrus, caudate nucleus and hippocampus (Videbech and Ravnkilde, 2004). Antidepressants and NMDA-R antagonists prevent hippocampal atrophy following exposure of rats to restraint stress (Wood et al., 2004). Since NO is proposed to play an important role in mediating the physiological and pathophysiological actions of glutamate, it is tempting to speculate

that nNOS inhibitors would block stress-induced hippocampal atrophy. In this regard Reagan and co-workers (1999) have reported that the glutamate release inhibitor and anticonvulsant phenytoin inhibits glucocorticoid-induced atrophy of CA3 pyramidal neurons concomitant with a reduction in the expression of nNOS in hippocampal interneurons.

NO has both neuroprotective and neurodestructive potential which may be of relevance in the pathophysiology of stress related disorders like depression. Such properties have been reviewed in detail elsewhere (Lipton et al., 1998; Nelson et al., 2003; Pacher et al., 2007) and involve S-nitrosylation of the NR2A subunit of the NMDA-R, thus reducing intracellular  $\text{Ca}^{2+}$  influx (see Nelson et al., 2003 for review) and superoxide radicals ( $\text{O}_2^-$ ) generation, leading to reduced production of destructive peroxynitrite ( $\text{ONOO}^-$ ) and promoting neuronal survival (see Pacher et al., 2007). NO can also confer cytoprotection by S-nitrosylating cysteines of the catalytic site of caspase enzymes reducing their enzymatic activity (Liu and Stamler, 1999). Inhibition of NO production using the broad spectrum NOS inhibitor L-NAME results in progressive apoptotic death of cerebellar granule cells which can be rescued by the addition of NO donors/cGMP analogues (Ciani et al. 2002). Inhibition of NOS in cells was associated with down regulation of the Akt/Glycogen synthase kinase-3 (GSK-3) system and the transcription factor CREB, which were proposed as converging cellular pathways that may mediate the survival role of NO (Ciani et al., 2002). Finally NO induces heme oxygenase 1 in microglia and astrocytes (Mancuso, 2004). Heme oxygenase 1 has a neuroprotective function in the brain resulting in the production of bilirubin, a molecule with antioxidant and anti-nitrosative properties (Mancuso, 2004; Mancuso et al., 2003).

### 5.3 Antidepressant-like properties of nitric oxide synthase inhibitors

NOS inhibitors (NOSi) are drugs able to block NOS activity and reduce NO production. A major problem with NO modulation is that the three isoforms of NOS have significantly different physiological functions, tissue distributions and mechanisms of regulation, and consequently the disease states associated with overproduction of NO are often isoform specific (Paige and Jaffrey, 2007). Development of isoform-specific NOS inhibitors is extremely important nowadays, especially because the endothelial NOS regulates arterial blood pressure and blood vessel relaxation, and its inhibition could theoretically lead to adverse cardio- or cerebrovascular effects. For this reason, despite their potential usefulness in treating different types of pathologies, NOS inhibitors are rarely tested in humans. To my knowledge only in a very few studies have NOS inhibitors been administered to humans, either as a topical application (Kellogg et al., 2009; Goldsmith et al., 1996) or an intravenous infusion (Cherney et al., 2012; Delles et al., 2002; White et al., 1998), and psychotropic effects were not the focus of any of these investigations. Most of the studies administered NOS inhibitors topically on skin, and this approach is probably not so relevant in light of a possible treatment of depression. White and colleagues (1998) however administered N<sup>o</sup>-nitro-L-arginine methyl ester (L-NMMA) intravenously, proving its safety under systemic administration, although they reported that it was able to reduce basal cerebral blood flow. Cherney and colleagues (2012) instead administered L-NMMA intravenously in both diabetic type 1 patients and healthy controls to study renal hyperfiltration and did not report any safety-related concern. This might lead the way to clinical experimentation of NOS inhibitors in humans, opening the possibility for these drugs to be studied in depressed patients. There are few clinical studies which provide direct evidence in support of targeting NO



or NOS for antidepressant effects. Naylor et al. (1987) reported an improvement in patients with severe depressive illness treated with methylene blue at a dose of 15 mg/day in a 3 week placebo controlled trial suggesting that methylene blue had potent antidepressant properties worthy of further clinical evaluation. Methylene blue is a non toxic dye which is known to interact with iron containing enzymes including soluble guanylate cyclase, reducing its sensitivity to activation by NO. Significant antidepressant efficacy was also reported with methylene blue in patients with bipolar affective disorder when used in addition to lithium in the long-term treatment of manic depressive psychosis (Naylor et al., 1986).

Experiments in animals have been carried out to assess the psychoactive properties of a number of NOS inhibitors and evidence in support of antidepressant-like properties of NOS inhibitors is abundant (see Table 3). L-arginine-derived inhibitors of NOS dose-dependently and stereo-selectively produce antidepressant activity in mice which are reversed by pretreatment with the NO precursor L-arginine (Harkin et al., 1999a). Some L-arginine-derived inhibitors display a biphasic response in the FST suggesting a dual effect of NO on behaviour in the test (Harkin et al., 1999a; Yildiz et al., 2000a). A possible explanation for this trend may be that NO bi-directionally regulates NMDA-R activity, playing both a positive (via activation of guanylate cyclase) and negative (via feedback resulting in decreased NMDA-R and NO synthase activity) role in NMDA-R-mediated events (Lei et al., 1992; Manzoni and Bockaert, 1993). L-arginine too exhibits a U-shaped effect on behaviour in the FST inducing both pro- and antidepressant-like effects and in the presence of the NOS inhibitor N<sup>o</sup>-nitro-L-arginine (L-NA) these effects of L-arginine disappear, suggesting that both effects are due to the production of

NO and providing further support for a bi-directional regulation by NO on behaviour in the test (Da Silva et al., 2000; Inan et al., 2004; Ergun and Ergun, 2007).

**Table 3:** Antidepressant-like properties of NOS inhibitors in preclinical studies.

NOS selectivity	Drug	Class of compound	Outcome	Reference
Non selective	<b>N-nitro-L-arginine methyl ester (L-NAME)</b>	L-arginine-derived	Antidepressant-like effects in rat FST, reversed by L-arginine  Enhancement of the antidepressant-like activity of sub-active doses of lithium in mice FST, reversed by L-arginine	Jefferys and Funder (1996)  Ghasemi et al., 2008
	<b>N<sup>ω</sup>-nitro-L-arginine (L-NA)</b>	L-arginine-derived	Antidepressant-like effects in mice FST, reversed by L-arginine  Enhancement of the behavioural effects of sub-active doses of imipramine and fluoxetine in mice FST	Harkin et al., 1999a Yildiz et al., 2000a  Harkin et al., 2004
nNOS selective	<b>N<sup>ω</sup>-propyl-L-arginine (NPLA)</b>	L-arginine-derived	Enhancement of the antidepressant-like activity of sub-active doses of lithium in mice FST, reversed by L-arginine	Ghasemi et al., 2008
	<b>1-(2-trifluoromethyl-phenyl)imidazole (TRIM)</b>	Imidazole	Antidepressant-like effects in mice FST, reversed by L-arginine  Enhancement of the behavioural effects of sub-active doses of imipramine, fluoxetine and citalopram in rat FST	Volke et al., 2003  Ulak et al., 2008
	<b>7-nitroindazole (7-NI)</b>	Indazole	Antidepressant-like effects in mice FST, not reversed by L-arginine  Prevention of stress-induced increase in immobility in FST (intrahippocampal injection)	Volke et al., 2003 Harkin et al., 2003a  Joca and Guimarães (2006)

As the early studies on NOS inhibitors did not address the issue of specificity, attention was focused on the determination of the effects of inhibitors of the nNOS isoform specifically (see table 3). Targeting nNOS is important as inhibition of eNOS, which regulates arterial blood pressure, could theoretically lead to adverse cardio or cerebrovascular effects. In this regard, three selective inhibitors of nNOS 7-nitroindazole (7-NI) , 1-(2-trifluoro-methyl-phenyl) imidazole (TRIM) and N<sup>0</sup>-propyl-L-arginine (NPLA) display antidepressant properties in the FST (Harkin et al., 2003a; Volke et al., 2003; Ghasemi et al., 2008). Pretreatment with L-arginine abolished the effect of TRIM but not 7-NI in the test (Volke et al., 2003) suggesting that the activity of L-arginine may be accounted for by a differential mechanism of interaction with the NOS enzyme. *In vivo*, 7-NI selectively inhibits nNOS and is without effect on arterial blood pressure in a range of species including the rat. TRIM is a selective inhibitor of nNOS with weak inhibition against eNOS. *In vivo* studies have reported antinociceptive and neuroprotective properties of both 7-NI and TRIM, without cardiovascular effects in animal models of pain and stroke respectively (Coert et al., 2003; Haga et al., 2003; Handy and Moore, 1998). Joca and Guimarães (2006) reported that local inhibition of NO synthesis following direct injection of 7-NI into the hippocampus prevented the development of stress-induced immobility in the FST, suggesting that hippocampal NO may contribute to the development of stress related behavioural changes. Together these findings suggest a role for NOS in the pathophysiology of depression as well as antidepressant-sensitive behaviour and neurochemical responses (Karolewicz et al., 2004).

The behavioural profile obtained with NOS inhibitors in the rat FST parallels that previously shown with SSRIs, the most widely prescribed class of antidepressant drug

for the treatment of depression (Harkin et al. 2003a). Depletion of endogenous 5-HT blocked L-NA- and 7-NI-induced reductions in immobility and increases in swimming behaviour during the FST, suggesting that NO synthase inhibitors elicit their antidepressant-like activity in the FST through a 5-HT dependent mechanism.

Moreover, ineffective doses of L-NA and 7-NI were able to increase the response to ineffective doses of conventional antidepressants with a preferential effect on 5-HT re-uptake inhibition, when administered in combination (Harkin et al., 2004). Similar synergistic effects were reported when combining antidepressant drugs with the nNOS-selective inhibitor TRIM (Ulak et al., 2008).

Modulation of the activity of antidepressants that enhance catecholaminergic function following inhibition of NOS has also been reported. Dhir and Kulkarni (2007) showed that the antidepressant action of bupropion, a catecholamine re-uptake inhibitor, could be enhanced by sub-active doses of 7-NI or methylene blue and attenuated by pretreatment with L-arginine or the PDE-5 inhibitor sildenafil. Such effects of sildenafil are suggestive of the involvement of NO-cGMP signalling in the antidepressant activity associated with bupropion and are supported by observations where L-arginine was shown to attenuate the antidepressant activity of imipramine (Harkin et al., 1999a; 2004) and venlafaxine (Krass et al., 2011) in the FST. Venlafaxine is regarded as an inhibitor of both 5-HT and noradrenaline transporters. As previously described for bupropion, imipramine and fluoxetine, low doses of 7-NI or methylene blue enhance, while sildenafil attenuates, the activity of venlafaxine in the test. Taken together these reports provide evidence for an involvement of the L-arginine-NO-cGMP signalling pathway in the behavioural effects of some selected antidepressants in the FST. NO has also been proposed to play a role in the antidepressant-like effects of lithium in the

mouse FST where NOS inhibitors L-NAME or L-NPA enhance the antidepressant activity of sub-active doses of lithium which in turn may be prevented by pretreatment with L-arginine (Ghasemi et al., 2008).

#### **5.4 Stress and NOS**

Expression of NOS increases in limbic brain regions following acute restraint stress in rats (Madrigal et al., 2003; 2001). Echeverry and co-workers (2004) reported similar findings where acute restraint stress provoked an increase in the number of neurons expressing nNOS and NADPH-diaphorase in the amygdaloid complex initially with an additional increase reported five days after single or repeated exposure to stress in the CA1 and CA3 fields of the hippocampus and entorhinal cortex. Others have reported that NOS activation is sustained for 3 weeks post-stress in rat hippocampus (Harvey et al., 2004). Moreover, attenuating steroid synthesis using ketoconazole, a steroid synthesis inhibitor, blocks the stress related increase in NOS activity, suggesting that adrenal steroids mediate these effects. By contrast increased levels of corticosterone have been previously shown to lead to decreased NOS expression in rat hippocampus (Lopez-Figueroa et al., 1998; Reagan et al., 1999). In this regard it has previously been reported that glucocorticoids induce a down regulation of eNOS in rat and bovine endothelial cells in addition to provoking a decrease in plasma nitrite levels (Rogers et al., 2002; Wallerath et al., 1999). One potential factor that may account for the differential response to stress is the release of other factors such as glutamate, which work in concert with stress hormones to influence NOS expression and activity in the hippocampus.

Most research to date has examined the effects of NOS inhibitors in the FST which is best described as an acute stress model predictive of antidepressant activity. In order to develop the hypothesis that nNOS inhibitors have antidepressant activity, such compounds should be tested in more sophisticated animal models of chronic stress and depression. To date a number of studies have been undertaken to evaluate the effects of NO synthase inhibition in the chronic mild stress (CMS) model of depression. nNOS-knockout mice appear more resilient to the behavioural effects of exposure to CMS. Zhou and co-workers (2007) reported that nNOS deletion or treatment with 7-NI prevents CMS-induced behavioural changes including increased behavioural despair in the FST and related tail suspension test. Mutlu and co-workers (2009) confirmed the antidepressant activity associated with inhibition of NO production in the CMS model. Treatment of mice with TRIM over 5 weeks subjected to CMS reversed stress-induced behaviours including disturbed coat state, grooming behaviours and reduced aggression when compared to the stressed control group.

Palumbo et al. (2007) reported a reduction in the expression and activity of nNOS in the hippocampus of male Balb/c mice exposed to the CMS procedure. It was of interest to note that there was a concomitant increase in eNOS expression and activity suggestive of a compensatory mechanism to balance NO production in the hippocampus. In tandem, CMS was associated with reduced neuronal numbers in the CA1 and CA3 subfields and deficits in learning determined in open field and passive avoidance tasks. The authors proposed a link between the stress related cognitive deficit and reduced activity of hippocampal nNOS. Moreover differential patterns of protein kinase C (PKC) activity were identified in the hippocampus of control and CMS exposed animals and proposed to relate to reduced nNOS activity associated with CMS. By contrast stress did not affect learning performance or NO production in C57BL/6 mice although

changes in the expression of PKC isoforms were evident in the hippocampus (Palumbo et al., 2009). Such differences between strains are indicative of a differential stress related regulation of NOS in learning and memory, considering that Balb/c represent a stress-sensitive strain of mice, and point to an important role for NO in stress related cognitive deficit.

A genetic animal model of depression, the Flinders sensitive line (FSL) rats, has been used to further confirm this role of NO (Wegener et al., 2009). These animals present the characteristic features of depression as well as increased stress responsiveness, and respond to chronic antidepressant treatment when tested in the FST. In this study they show how exposure of FSL rats to escapable/inescapable stress leads to an increase in nNOS mRNA, protein and activity in the hippocampus.

El-Faramawy and colleagues (2009) have recently reported that in rats exposed to CMS the reduction in hippocampal glutamate metabolism and increased tau phosphorylation, a hallmark of Alzheimer's type dementia in aged rats, can be attenuated with chronic administration of 7-NI. The authors suggested that there may be a causal relationship between stress-induced activation of NOS and the hyperphosphorylation of tau and that regulation of nNOS may be a target for pharmacological manipulation of tau phosphorylation, especially when the latter is associated with reductions in glutamate metabolism. It is also interesting to note that intra-hippocampal administration of the iNOS inhibitor aminoguanidine during CMS exposure in rats suppressed stress-induced behavioural changes including anhedonia, weight change, increased immobility in the FST and reduced exploration and grooming in the open field test (Wang et al., 2008). Results from this study suggest a role for iNOS in the hippocampus in mediating stress-induced depression-like behaviours.

An increase in the expression of iNOS has been noted to occur in cortical and limbic brain regions following repeated restraint stress in rats (Olivenza et al., 2000; Madrigal et al. 2001; 2002; 2003). In addition Olivenza and co-workers (2000) reported that immobilisation stress for 6 hr over 3 weeks can increase immunoreactivity for nitrotyrosine, a nitration product of NO, and lead to an accumulation of NO metabolites in the cortex in parallel with increased lactate dehydrogenase (LDH) release and an impairment in glutamate uptake in synaptosomes. Treatment with the iNOS inhibitor aminoguanidine could prevent these changes suggesting that the production of NO via the induction of iNOS following exposure to stress may be responsible for some of the stress related neurodegenerative changes. Madrigal et al. (2001) reported that treatment with the NMDA receptor antagonist MK-801 or the nuclear factor kappa B (NF- $\kappa$ B) inhibitor pyrrolidine dithiocarbamate (PDTC) at the onset of stress inhibits the stress-induced increase in iNOS expression. As glutamate has been shown to induce iNOS in the brain via an NF- $\kappa$ B-dependent mechanism, Madrigal and co-workers proposed that stress-induced glutamate release and NF- $\kappa$ B activation are likely to account for the stress related increase in iNOS in the cerebral cortex. Further work by this group has shown that acute exposure to restraint stress (immobilisation for 6 hr) also induced cortical iNOS expression. An increase in TNF- $\alpha$ -convertase (TACE) and the cytokine TNF- $\alpha$  were found to precede induction of iNOS. Moreover treatment with the NMDA receptor antagonist MK-801 or the TACE inhibitor BB1101 inhibited iNOS expression induced by stress. BB1101 also inhibited the stress-induced translocation of NF- $\kappa$ B to the nucleus. Taken together Madrigal and co-workers (2002) proposed that glutamate receptor activation provoked an up-regulation of TACE and a subsequent increase in TNF- $\alpha$  and that these events account for stress-induced iNOS expression via NF- $\kappa$ B activation. Such a mechanism may also generalise to other regions of the brain which



contain NOS-positive neurons. Shirakawa and colleagues (2004) showed that immobilisation stress in rats induced acute NO production in the paraventricular nucleus (PVN) of the hypothalamus that could be reduced by inhibition of NOS and ionotropic glutamate receptors, suggestive of a role for glutamate receptors in basal and stress-induced NO production in this region of the brain.

In 2004, Harvey and co-workers reported that stress–restress in a model of time dependent sensitization (TDS), an animal model of human post traumatic stress disorder (PTSD), evokes an immediate and sustained increase in hippocampal NOS activity driven primarily by iNOS. As the inhibitor of steroid synthesis ketoconazole attenuated the stress-related increase in NOS activity, they proposed that stress-induced glucocorticoid release may contribute to the activation of NOS. The participation of iNOS in depression related behaviour has also more recently been confirmed, since repeated intra-hippocampal microinjections of the iNOS inhibitor aminoguanidine significantly suppressed depression-like behavioural changes induced by CMS (Wang et al., 2008).

Astrocytic-derived NO has been associated with modifications in stress related behaviours in animals. Buskila and co-workers (2007) investigated a role for astroglial iNOS in normal brain function in a iNOS mutant mouse (B6;129P2-NOS2tm1Lau/J, Jackson Laboratory) that show increased basal NOS activity in brain astrocytes. The astroglial mutation was associated with distinct behavioural abnormalities characterised by an increase in stress related behaviours including a suppression in grooming in novel environments, increased freezing and reduced exploratory behaviours in the open field and behaviour suggestive of increased anxiety on the elevated plus maze. Abu-Ghanem

and colleagues (2008) extended these findings and reported that these mutants exhibited higher anxiety-like behaviour in the elevated plus maze, increased acoustic startle responses and higher plasma corticosterone levels following exposure to predator scent when compared to controls. Systemic administration of the NOS inhibitor L-NAME reversed these stress related effects. These reports are in agreement with previous studies showing an association between increased NO levels and enhanced anxiety-like behaviours. It is interesting to note that the mutant mice performed better in the Morris water maze prior to stress exposure but not in the object recognition task, suggestive of enhanced stress reactivity rather than enhanced cognitive ability given that water maze may involve a stress exposure influence on motivation to perform in the task.

## **6. Aim and objectives**

Through the following series of experiments further characterisation and validation of the novel antidepressant properties of nNOS inhibitors will be sought in animal models of stress and depression. These experiments will help determine if NOS inhibition represents an advantageous approach in relation to antidepressant onset or efficacy, identify those brain regions involved in the antidepressant-like properties of nNOS inhibitors, and elucidate if 5-HT may play a role in mediating their antidepressant-like activity. Specifically the objectives of the project were to:

**I.** Elucidate a role for 5-HT in the antidepressant related actions of ketamine, the effects of ketamine administration were investigated in the rat FST in naïve and in 5-HT depleted animals.

**II.** Determine regional 5-HT synthesis and metabolism changes associated with the NOS inhibitor N<sup>o</sup>-nitro-L-arginine (L-NA) and the influence of 5-HT receptor activation in the antidepressant-like actions of L-NA in the rat FST.

**III.** Evaluate the regional pattern of immediate early gene (c-fos) activation following exposure to the FST and determine those regions in which neuronal activation is influenced by prior administration of L-NA in order to identify brain circuits/systems involved in mediating the antidepressant-like activity.

**IV.** Further characterise the OB rat model of depression as a test of antidepressant onset incorporating behavioural, molecular, physiological and neuroimaging markers and to assess the effects of the NOS inhibitors L-NA and TRIM in the model.

# *Materials and Methods*



## 1. Materials

### 1.1 Animals

Male Sprague-Dawley rats

Harlan Laboratories,  
UK

### 1.2 Equipment

Beakers

Pipettes

Thermometer

Stopwatch

Fine-tipped paintbrush

Stirrer

Orbital oscillator

Sonicator

Polytron homogeniser

Weighing scales

Vacuum suction pump

Centrifuge

NanoDrop®

Thermo Scientific,  
IRL

Thermocycler PTC-200

MJ Research

Reverse phase HPLC column Kinetex™ Core Shell  
Technology (specific area 100 mm x 4.6 mm, particle size 2.6  
µm)

Phenomenex, IT

Electrochemical detector

Antec Decade

ABI Prism 7300 PCR instrument

Applied Biosystems,  
IRL

Cryostat

Fume hood

-20 °C freezer

-80 °C freezer

### 1.3 Special equipment

Polypropylene cages (41x24 cm)	Bioresources TCD,
Polypropylene cages (55x30 cm)	Bioresources TCD,
Stereotactic frame	
Surgical drill	
Drill bits (2 mm diameter)	
Shaver	
Surgical staples	Bioresources TCD,
Steel sutures (Steelex®)	Bbraun Aesculap, IRL
Polyester thread (PremiCron®)	Bbraun Aesculap, IRL
Radiotelemetric transponders PDT-4000 E-Mitter (Emitter series)	Mini Mitter Oregon, USA
Radiotelemetric receivers (Series 4000)	Mini Mitter Oregon, USA
Plexiglass adjustable restrainers (35 cm length x 7 cm internal diameter)	Harvard Apparatus, UK
Clear glass tank (40 cm high and 20 cm in diameter)	
Open field arena	
Floor lamps (200-250 lux)	
Activity monitor cages (32 cm length x 20 cm width x 18 cm height)	Benwick Electronics, UK
Rodent Bruker Biospec system	Bruker Biospin, D
Perfusion grid	
Perfusion pump	
Perfusion cannula	
Dissection kit	

#### 1.4 Anaesthesia and operative care

Isoflurane (IsoFlo)	Abbott Animal Health, UK
Urethane	Sigma Aldrich, IRL
Surgical scrub (Betadine)	Medlock Medical, UK
Haemostatic sponge (Haemofibrine)	Specialities Septodont, FR
Antibiotic powder (Cicatrín)	GlaxoSmithKline, IRL
Antiseptic cream (Savlon)	Novartis, IRL
Analgesia (Disprol)	Reckitt Benckiser Healthcare Ltd., UK

#### 1.5 Consumables

Pipette tips	
Needles 26G	Bioresources TCD,
Syringes (1 mL)	Bioresources TCD,
Nylon syringe microfilters 0.45 µm	Nalgene, UK
Petri dishes	Sarstedt, D
RNase-free tubes	
RNaseZap® wipes	Invitrogen, IRL
Glass slides	Lennox, IRL
Glass coverslips	Lennox, IRL
Netwells	Corning Life Sciences, FR
Netwell tray	Corning Life Sciences, FR
96-well optical reaction plate	Applied Biosystems, IRL
HPLC vials	Fisher Scientific, UK



## 1.6 Chemicals and reagents

De-ionised H <sub>2</sub> O	
NaCl	Sigma Aldrich, IRL
NaOH pellets	Sigma Aldrich, IRL
Na <sub>2</sub> HPO <sub>4</sub>	Sigma Aldrich, IRL
NaH <sub>2</sub> PO <sub>4</sub>	Sigma Aldrich, IRL
Citric acid	Sigma Aldrich, IRL
EDTA	Sigma Aldrich, IRL
Octane-1-sulphonic acid	Sigma Aldrich, IRL
HPLC grade water (double-distilled, re-filtered, NANOpure H <sub>2</sub> O)	Fisher Chemical, UK
Methanol	Fisher Chemical, UK
N-methyl serotonin	Sigma Aldrich, IRL
Maple syrup	Newforge, IRL
Tween-20	Sigma Aldrich, IRL
Triton-X (TX)	Sigma Aldrich, IRL
Liquid nitrogen	
RNase-free water	Gibco, IRL
β-mercaptoethanol	
TaqMan <sup>®</sup> Gene Expression Assay eNOS (Rn02132634)	Applied Biosystems, IRL
TaqMan <sup>®</sup> Gene Expression Assay nNOS (Rn00583793)	Applied Biosystems, IRL
TaqMan <sup>®</sup> Gene Expression Assay iNOS (Rn00561646)	Applied Biosystems, IRL
TaqMan <sup>®</sup> Gene Expression Assay Glial fibrillary acidic protein (GFAP) (Rn00566603)	Applied Biosystems, IRL
TaqMan <sup>®</sup> Gene Expression Assay CD11b (Rn00709342)	Applied Biosystems, IRL
TaqMan <sup>®</sup> Gene Expression Assay CD40 (Rn01423583)	Applied Biosystems, IRL

TaqMan <sup>®</sup> Gene Expression Assay IL-1 $\beta$ (Rn00580432)	Applied Biosystems, IRL
TaqMan <sup>®</sup> Gene Expression Assay IL-6 (Rn99999011)	Applied Biosystems, IRL
TaqMan <sup>®</sup> Gene Expression Assay TNF- $\alpha$ (Rn93699071)	Applied Biosystems, IRL
TaqMan <sup>®</sup> Gene Expression Assay IFN- $\gamma$ (Rn00594078)	Applied Biosystems, IRL
TaqMan <sup>®</sup> Gene Expression Assay $\beta$ -actin (4352340E)	Applied Biosystems, IRL
2X TaqMan <sup>®</sup> Universal PCR Master Mix (No AmpErase <sup>®</sup> UNG)	Applied Biosystems, IRL
Paraformaldehyde (PFA)	Sigma Aldrich, IRL
Sucrose	Sigma Aldrich, IRL
Ethylene glycol	Fisher Scientific, UK
Hydrogen peroxide (H <sub>2</sub> O <sub>2</sub> )	Sigma Aldrich, IRL
Normal goat serum (NGS)	Sigma Aldrich, IRL
Rabbit anti-c-Fos primary antibody	Santa Cruz Biotechnology Inc., D
Biotinylated goat anti-rabbit antisera (Elite Vectastain ABC kit)	Vector Laboratories, UK
Avidin-biotinylated peroxidase complex (Elite Vectastain ABC kit)	Vector Laboratories, UK
3,3'-diaminobenzidine(DAB)	Dako Diagnostics, IRL
Absolute Ethanol 100%	Sigma Aldrich, IRL
Xylene	Sigma Aldrich, IRL
DPX mountant for microscopy	Fisher Scientific, UK
Gelatine	Sigma Aldrich, IRL
Chromium(III) potassium sulfate	Fisher Scientific, UK

## 1.7 Kits

Nucleospin RNA II kit	Macherey-Nagel, D
ABI High Capacity cDNA kit	Applied Biosystems, IRL

## 1.8 Drugs

Ketamine hydrochloride (100 mg/ml)	Vétoquinol Ltd., UK
Fluoxetine hydrochloride	Clonmel Healthcare Ltd., IRL
Imipramine hydrochloride	Sigma Aldrich, IRL
N <sup>o</sup> -Nitro-L-arginine (L-NA)	Sigma Aldrich, IRL
1-(2-trifluoro-methyl-phenyl) imidazole (TRIM)	Alfa Aesar, UK
DL-4-Chlorophenylalanine ethyl ester hydrochloride (pCPA)	Alfa Aesar, UK
m-hydroxybenzylhydrazine dihydrochloride (NSD-1015)	Sigma Aldrich, IRL
Metergoline	Sigma Aldrich, IRL
N-[4-methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)-1,1'-biphenyl-4-carboxamide hydrochloride] (GR-127935)	Glaxo Wellcome, UK
4-fluoro-N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-2-pyridinyl benzamide (WAY-100635) maleate	Wyeth, UK
Ketanserin tartrate	Sigma Aldrich, IRL
Benzofuran-2-carboxamidine (RO-430440)	Hoffman-La Roche, CH

## 1.9 Hardware and software

CCD camera (high resolution monochrome VCB 3372)	Sanyo, IRL
AM1051 data logger	Benwick Electronics, UK
VitalView <sup>TM</sup> software	Mini Mitter Oregon, USA
Ethovision analysis system	Noldus IT, NL

Menu2020 software

ParaVision 4.0 software

MIPAV 5.4 software

Class-VP software package

7300 System Software

GB-Stat v.10

GraphPad Prism v.4.0

HVS Image Ltd., UK

Bruker Biospin, D

JHU Medic, USA

Shimadzu, Japan

Applied Biosystems,

IRL

Dynamic

Microsystems Inc.,

USA

GraphPad Software,

USA



## **2. Methods**

### **2.1 Subjects**

All studies were performed on male Sprague-Dawley rats purchased from Harlan Laboratories (UK) and were housed in groups of 4 under standard laboratory conditions (12 hr:12 hr light:dark cycle with lights on from 08.00 hr to 20.00 hr, room temperature 20–22 °C) for one week in order to allow them to acclimatise to their new environment. Food and water were available *ad libitum*. For studies requiring single housing, 3 days before the beginning of the study rats were singly housed in standard medium sized polypropylene cages (41x24 cm).

On the first day of the study subjects were randomly assigned to a control or a treatment group based on body weight, so that all the body weights in the range were equally distributed throughout the different groups.

All procedures were approved by the Animal Ethics Committee Trinity College Dublin and were in accordance with the European Council Directive 1986 (86/806/EEC).

### **2.2 *In vivo* procedures**

#### **2.2.1 *Surgical procedures***

Surgical procedures were performed in a dedicated surgery room, paying particular attention to hygiene and sterility. Post-operative care was provided in any circumstance

and euthanasia was performed whenever the animals in recovery showed signs of excessive pain or distress which could not be ameliorated otherwise.

### ***2.2.1.1 Bilateral olfactory bulbectomy (OB)***

The surgical removal of both the right and left olfactory bulbs was performed as previously described (Kelly et al. 1997). Briefly, rats were deeply anaesthetised with 3.5% isoflurane (IsoFlo, Abbott Animal Health, UK) in 100% oxygen, mounted on a stereotactic frame and maintained under anaesthesia with 1.5-2% isoflurane. The head was shaven, prepared with surgical scrub (Betadine, Medlock Medical) and a midline sagittal incision was made with a scalpel to the skin overlying the skull. Two burr holes of 2 mm diameter were then drilled 7.5-8 mm rostral to bregma, and 2 mm lateral to the midline on each side. The olfactory bulbs were aspirated using a vacuum suction pump. After the operation, bleeding was controlled with haemostatic sponge (Haemofibrine, Specialities Septodont, France). For sham animals the procedure was identical and the dura mater was carefully pierced without aspirating the bulbs. Once the bleeding was contained the skin was closed with surgical staples, dusted with antibiotic powder (Cicatrín, GlaxoSmithKline) and antiseptic cream was applied (Savlon, Novartis). Animals were returned to their home cage after recovery from anaesthesia. Analgesia (disprol containing aspirin and saccharin in the drinking water) was provided and wet food was placed inside the cage for the first day post-surgery. The general appearance of the animals and their body weights were monitored daily. Rats were given 14 days recovery prior to any further treatment or behavioural tests and were handled daily

throughout the recovery period to eliminate any aggressiveness that would otherwise arise.

### ***2.2.1.2 Implantation of bioradiotelemetric transponders***

In order to evaluate physiological parameters some of the animals were implanted with radiotelemetric transponders (Emitter series, Mini Mitter, Oregon, USA) one week following OB surgery. Signals from the transponders were detected by receivers (Series 4000) placed underneath the home cages and data acquisition was controlled and monitored by the VitalView<sup>TM</sup> software (Mini Mitter, Oregon, USA). The transponders (PDT-4000 E-Mitter) are fully implantable, inserted in a bio-inert and sealed capsule made of laboratory grade glass with a biocompatible silastic coating, and allow for the continuous monitoring of activity, core body temperature and heart rate in freely behaving animals. A pair of helically wound stainless steel wires, insulated with silicone tubing, extended from the core housing and allowed for heart rate data acquisition. The devices were implanted in the abdomen of the animals and their presence is known not to affect locomotor activity and behaviour (Harkin et al., 2002). Implantation was performed as previously described (Harkin et al., 2002; Roche et al., 2007). Briefly, the animals were anaesthetised again, the abdomen and the thorax shaved and swabbed with surgical scrub (Betadine, Medlock Medical, UK). A 2 cm midline abdominal skin incision was made approximately 1 cm below the diaphragm and the core body of the transponder was slipped into the abdominal cavity. The heart leads were threaded through the abdominal wall and passed under the skin using a trochar and sleeve to reach two small incisions made on the thorax skin: the first one on



the right side of the animals, near the clavicle, and the second one on the left side lower towards the abdomen, with an angle of approximately 45° respect to the previous one. Leads were secured on the thorax muscle with metal sutures (Steelex<sup>®</sup>, Bbraun Aesculap, IRL) and hearth rate signal reception was checked. After a regular and high ECG-like waveform was seen, the abdominal opening and the two lead access points were sutured with polyester thread (PremiCron<sup>®</sup>, Bbraun Aesculap, IRL). Animals were then placed on a heating blanket to help the recovery from anaesthesia and returned to their home cages once awake. The animals were allowed to recover for an additional week following radiotransponder implantation prior to any further treatment.

### ***2.2.2 Central 5-HT depletion***

For experiments involving central 5-HT depletion, DL-4-Chlorophenylalanine ethyl ester hydrochloride (*p*CPA) (Alfa Aesar, UK) was dissolved in saline solution to a concentration of 75 mg/ml. *p*CPA was injected i.p. at a volume of 2 ml/kg body weight to yield a dose of 150 mg/kg. *p*CPA or vehicle were administered once daily for three consecutive days as described by Harkin and co-workers (2003).

### ***2.2.3 Immobilisation stress***

Each immobilisation stress session was performed by confining the animals in clear plexiglass adjustable restrainers (35 cm length x 7 cm internal diameter; Harvard Apparatus, UK) for 2 hr. These restrainers are provided with aeration holes to prevent suffocation and to help to disperse the heat. Rats were moved to a separate room and

subjected to restraint stress in their home cages. Control animals were handled at equivalent times but not exposed to immobilisation stress.

#### ***2.2.4 Combination of 5-HT depletion and immobilisation stress***

When the combination of 5-HT depletion and restraint stress was performed, rats were initially subjected to 5-HT depletion as previously described. 48 hr following the final *p*CPA treatment, rats were exposed to 2 hr restraint stress once daily for three consecutive days. 24 hr following the final period of stress animals were exposed to the 15 min pre-FST session and FST 24 hr later.

#### ***2.2.5 Behavioural assessments***

All behavioural readouts were performed in dedicated rooms, paying particular attention to avoid noises or other types of interferences which could alter or bias the data collected during the performances of the animals in the tests.

##### ***2.2.5.1 Randomisation of subjects***

In all the studies the sequence of behavioural testing was randomised throughout the experiment so as to minimise any confounding effects of order of testing and the individual scoring the behaviour was blind to the treatments administered to the animals.

### ***2.2.5.2 Forced Swimming Test (FST)***

The FST was performed in accordance with the protocol described by Porsolt (Porsolt et al., 1978). On the first day, rats were removed from their home cage, placed individually into a clear glass tank (40 cm high and 20 cm in diameter) filled with 30 cm of water (22-23°C) and allowed to swim for 15 min (pre-FST session). At the end, the animals were removed from the water, dried with paper towel and returned to their home cage. 1 hr after pre-FST exposure, rats received their first vehicle/drug treatment. A second and a third injection were administered 5 hr and 1 hr prior to the 5 min FST, which was performed 24 hr after the first swim exposure. The drug administration protocol followed is standard for testing pharmacological agents in the rat FST (see Cryan and Lucki, 2000; Detke et al., 1995; Porsolt et al., 1978). In the test session, rats were allowed to swim for 5 minutes, and the amount of time (seconds) spent immobile was recorded with a stopwatch. The animals were considered immobile when floating in the water without struggling and making only those movements necessary to keep their heads above the water.

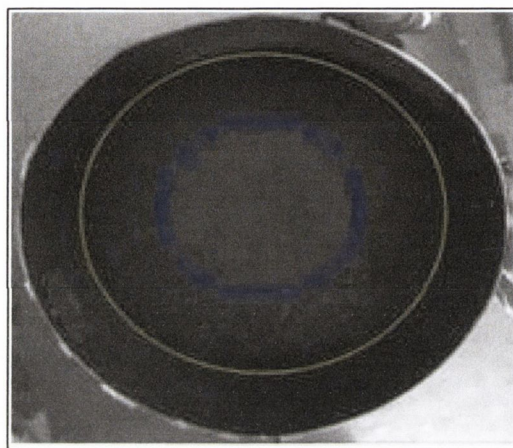
### ***2.2.5.3 Modified Forced Swimming Test***

This test was performed using a modification of the method described by Detke et al. (1995). As previously described, on the first day of the experiment the rats were placed individually into a clear glass tank (40 cm high and 20 cm in diameter) filled with 30 cm of water (22-23°C) and allowed to swim for 15 min (pre-FST session). At the end, the animals were removed from the water, dried with paper towel and returned to their

home cage. 1 hr after pre-FST exposure, rats received their first vehicle/drug treatment. A second and a third injection were administered 5 hr and 1 hr prior to the 5 min FST, which was performed 24 hr after the first swim exposure. In the second exposure to the FST, rats' behaviour was videotaped from above using a CCD (Sanyo VCB 3372 high resolution monochrome) camera. The sequence of testing was randomised throughout the experiment so as to minimise any confounding effects of order of testing and behavioural scoring was performed using the manual event recording capability of the Ethovision, videotracking and behavioural analysis system (Noldus IT, The Netherlands). Keys (computer keyboard) were assigned to individual behaviours and used to record the duration of these behaviours in the test from video footage. We use event duration when scoring as opposed to a time sampling technique used by others (Cryan and Lucki, 2000; Detke et al., 1995). The main difference between these techniques is that time sampling provides an approximation of the behaviours scored. Behaviours scored included immobility, swimming and climbing. Immobility was defined as the animal floating in the water without struggling and making only those movements necessary to keep its head above the water. Swimming was scored as the animal making active swimming motions more than necessary to keep its head above water and involved moving horizontally or crossing the swim tank. Climbing behaviour consisted of the animal making active upward movements with forepaws in and out of the water directed against the walls of the swim chamber. These behaviours were scored in a mutually exclusive fashion throughout the trial.

#### **2.2.5.4 Open field test**

The open field apparatus consisted of a black-bottomed circular arena, 1 meter in diameter with the wall surrounding the base constituted by a mirrored reflective surface [Fig. 6].



**Fig. 6:** Open field arena.

Normal houselighting was provided when assessing for basal locomotor activity, while stronger illumination was provided by floor lamps positioned in the room (200-250 lux) when testing for locomotor activity in OB animals. Such conditions have previously been described as optimal for the determination of OB-related hyperactivity (Mar et al., 2002; Kelly et al., 1997). Floor lights were positioned to provide an equal dispersion of light and to avoid casting shadows in the open field. Each animal was placed in the centre of the open field and left free to explore the arena. The distance moved was recorded by a video camera placed above the arena and analysed by the Menu2020

software (HVS Image Ltd, UK) videotracking system. All tests were conducted in the morning between 10.00 and 13.00 hr. Each animal was exposed to the open field for a duration of 15 minutes. The arena was cleaned with 70% ethanol after each trial to remove olfactory cues between individual tests.

#### ***2.2.5.5 Home cage activity***

In one of the studies basal locomotor activity was evaluated by monitoring activity in a new cage. Animals were removed from their home cage and placed individually into activity monitor cages (32 cm length x 20 cm width x 18 cm height) which were connected to an AM1051 data logger (Benwick Electronics). Each activity monitor was equipped with 2 sets of horizontal infrared beams, positioned 3 cm and 15 cm above the base of the cage. Both sets of beams consist of a 12 beam x 7 beam matrix, forming a grid of 66 x 2.54 cm<sup>2</sup> cells within the cage. Activity is recorded as the number of times a beam changes from unbroken to broken. Activity of the rats was monitored over a 20 minute period.

#### ***2.2.6 Magnetic Resonance Imaging (MRI)***

A multimodal approach including an assessment of regional volumetric changes and blood perfusion was adopted to identify possible neuroimaging markers related to the OB rat model and the response to imipramine and L-NA. MRI scans were carried out in a rodent Bruker Biospec system (Bruker Biospin, Germany) with a 7 Tesla magnet and

a 30 cm diameter core, equipped with a 20 cm actively-shielded gradient system [Fig. 7].



**Fig. 7:** The rodent Bruker Biospec system with a 7T magnet and a 30 cm diameter core (Bruker Biospin, Germany) used for functional and volumetric MR imaging in rats.

Data construction and analysis was performed with the ParaVision 4.0 software (Bruker Biospin). Animals were anaesthetised with isoflurane as previously described and a high resolution anatomical scan (T2-weighted Rapid Acquisition with Relaxation Enhancement (RARE) of a coronal brain section corresponding to bregma -4.3 mm (Paxinos and Watson rat brain atlas) was generated using the following parameters: slice thickness = 1.5 mm, repetition time (TR) = 3134.511 ms, echo time (TE) = 12 ms, RARE factor = 8, RF flip angle = 90°/180°, field of view (FOV) = 3.0 x 3.0 cm, image matrix = 128 x 128, total scan time = 50 s.

Bolus-tracking arterial spin labelling (btASL) is a contrast agent-free approach to the measurement of regional cerebral blood perfusion with MRI. A continuous arterial spin labelling (cASL) sequence was applied to the section imaged at high resolution as previously described by Kelly and colleagues (2009). Briefly, the sequence consists of a 5 second preparation interval which contained the inversion pulse followed by snapshot

fast low angle shot (FLASH) acquisition. The sequence was used to provide signal-time curves of the passage of a 3 second bolus through a region of interest. The following acquisition parameters were used: slice thickness = 2 mm, TR = 6.938 ms, TE = 2.63 ms, RF flip angle = 30°, FOV = 3.0 x 3.0 cm, image matrix = 128 x 64. btASL-MRI assesses cerebral perfusion through the calculation of two transit times: mean transit time (MTT) which represents time taken for labelled arterial blood to traverse the vasculature and reach the imaging plane and capillary transit time (CTT) which represents time taken for the blood to disperse at the imaging plane. MTT is inversely proportional to cerebral blood flow (CBF), while CTT is inversely proportional to CBF squared. A third quantifiable output is the btASL signal amplitude, which is derived from the area under the signal-time ASL curve and has been interpreted as being proportional to cerebral blood volume (CBV) (see Kelly et al., 2009; 2010).

High resolution anatomical images were also acquired using T1-weighted MR axial images and were collected using a RARE sequence. The following acquisition parameters were used: slice thickness = 0.5 mm, number of slices = 54, TR = 6.26 s, TE = 36.00 ms, FOV = 4.00 x 3.00 cm, image matrix = 266 x 200. T1 and T2 relaxation time changes were evaluated as they have been proposed to be potential markers for changes in tissue water content and possible astrocytic activation (Cowley et al., 2012; Sibson et al., 2008). T1 relaxation times were calculated from a rapid acquisition with relaxation enhancement (RARE) with variable repetition time (RARE-VTR) image. The following acquisition parameters were used: slice thickness = 1.5 mm, TE = 25.3 ms, RF flip angle = 180°, FOV = 3.0 x 3.0 cm, image matrix = 128 x 128 with varying TR, using values of 300.0, 589.12, 942.3, 1396.1, 2032.0, 3103.1 and 8000.0 ms. T2 relaxation times were calculated from a multi-slice multi-echo (MSME) image. The



following acquisition parameters were used: slice thickness = 1.5 mm, TR = 2000 ms, RF flip angle = 180°, FOV = 3.0 x 3.0 cm, image matrix = 128 x 128 with varying TE, using values of 8.06, 16.12, 24.18, 32.24, 40.29, 48.35, 56.41, 64.47, 72.53, 80.59 88.65 and 96.71 ms. An echo train of 12 values was used to ensure signal was at noise level by the last echo times to ensure accuracy of T2 estimation. Only the central 3 sections of the MSME scan were used for analysis of T2 relaxation times. All analysis was performed using the image sequence analysis (ISA) tool in the Bruker Paravision 4.0 Software package. Regions of interest selected using the ROI tool included the auditory cortex, hippocampus, parietal association cortex, retrosplenial cortex, thalamus and visual cortex bilaterally as well as the third ventricle.

Ventricular and hippocampal volumes were assessed using a method adapted from Kalisch et al. (2006). Briefly tissue borders were defined using a standardised rat brain atlas (Paxinos and Watson, 1998) for reference and regions of interest were manually traced out along the coronal plane in the T1-weighted structural images in the MIPAV 5.4 (Medical Image Processing, Analysis and Visualisation software, JHU Medic, USA) software package. The third ventricle was traced to form a three dimensional mask which was then quantified by using MIPAV tools. The volume obtained was normalised for brain size by calculating the relative percentage volume of the region in relation to the total intracranial volume (see Kalisch et al., 2006).

## 2.3 *Ex vivo* procedures

### 2.3.1 *High Performance Liquid Chromatography (HPLC)*

In experiments involving the analysis of biogenic amines content in the brain, the animals were euthanised immediately after behavioural testing by decapitation and regions of interest were dissected on an ice-cold plate for neurotransmitter measurements. 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA), L-dihydroxyphenylalanine (L-DOPA) dopamine and noradrenaline concentrations were measured by high performance liquid chromatography (HPLC) coupled to electrochemical detection as previously described (Durkin et al., 2008). Tissue samples were weighed (50-80 mg of tissue) and homogenised by sonication in 500  $\mu$ L of homogenisation buffer composed of mobile phase [0.1 M citric acid, 0.1 M  $\text{NaH}_2\text{PO}_4$ , 0.1 mM EDTA, 1.4 mM octane-1-sulphonic acid and 10% (v/v) methanol in HPLC grade water (double-distilled, re-filtered NANOpure  $\text{H}_2\text{O}$ , Fisher Chemical, UK), pH 2.8] spiked with 5 ng/20  $\mu$ L of N-methyl serotonin (Sigma Aldrich, Ireland) as internal standard. Samples were centrifuged at 15,000g for 15 min at 4°C, the supernatants were filtered with syringe microfilters (Nalgene, UK, 0.45  $\mu$ m, Nylon) and 10  $\mu$ L of filtrate was injected onto a reverse phase column (Kinetex™ Core Shell Technology column, specific area 100mm x 4.6mm, particle size 2.6  $\mu$ m, Phenomenex) at a flow rate of 0.8 ml/min for separation of the neurotransmitters. 5-HT and 5-HIAA concentrations were quantified by electrochemical detection (Antec Decade) and chromatograms were generated using the Class-VP software package (Shimadzu, Japan). Final results were expressed as ng neurotransmitter/g wet weight of tissue.

### ***2.3.2 Quantification of cortical and hippocampal gene expression***

#### ***2.3.2.1 Total RNA extraction***

Rats were euthanised by decapitation, the brains were rapidly removed and the frontal cortex and hippocampus were dissected on an ice cold plate and placed in RNase-free tubes, snap frozen in liquid nitrogen and transferred to a  $-80^{\circ}\text{C}$  freezer until further processing could be undertaken. Total RNA extraction was performed using Nucleospin RNA II kits (Macherey-Nagel) as per the manufacturer's instructions. Briefly, tissue samples were weighted out so that all the specimens didn't overcome 30 mg of tissue, and placed into new RNase-free tubes containing homogenisation buffer constituted by 100 parts of RA1 buffer (provided in the kit) and 1 part of  $\beta$ -mercaptoethanol. After mechanical disruption of the tissue with a polytron homogeniser, the lysate was filtered through Nucleospin filter units and diluted 1:2 with 70% ethanol before being loaded onto a Nucleospin II column, which selectively binds nucleic acids. The membrane was desalted with the provided buffer and DNA was digested directly on the column with a DNase solution. The column was then washed with the provided buffers and RNA was finally eluted from the column with RNase-free water. Total RNA was then quantified with a NanoDrop® (Thermo Scientific) micro-volume spectrophotometer and all the RNA samples were equalised with RNase-free water to the lowest detected concentration.

### **2.3.2.2 cDNA synthesis**

cDNA was synthesised using the ABI High Capacity cDNA kit (Applied Biosystems) as per the protocol provided. A 2X master-mix solution containing reverse transcription buffer, dNTPs, random primers and MultiScribe™ reverse transcriptase was made up in RNase-free water and stored on ice. For each volume of the RNA samples, an equal volume of the master mix was added. The reverse transcription reaction was performed in a thermocycler (PTC-200, MJ Research) with a 3-step program: 10 minutes at 25 °C followed by 120 minutes at 37 °C and a final 5 minutes step at 85 °C. The machine was set to cool down automatically to 4 °C after the end of the final step. cDNA samples were then used immediately for real time PCR or frozen and stored at –20 °C until needed.

### **2.3.2.3 Real-Time PCR**

Real-Time PCR was performed in a multiplex fashion using TaqMan® Gene Expression Assays in accordance with the manufacturer's instructions (Applied Biosystems). For each gene the assays provide a set of forward and reverse primers and a fluorescence-labeled MGB TaqMan® probe. The target genes eNOS (Rn02132634), nNOS (Rn00583793), iNOS (Rn00561646), the astrocytic activation marker glial fibrillary acidic protein (GFAP) (Rn00566603) and the microglial activation marker CD11b (Rn00709342) were identified by FAM-labelled probes, while the housekeeping gene of reference,  $\beta$ -actin (4352340E), was identified by a VIC-labelled probe. Multiplex was chosen because it allows for normalisation of gene expression data to the

endogenous control within the same reaction mixture. The mixture was prepared by adding a 1:5 dilution of the cDNA sample obtained from the previous reaction into a 96-well optical reaction plate (Applied Biosystems), followed by 2X TaqMan® Universal PCR Master Mix (No AmpErase® UNG, Applied Biosystems) and 20X primers for both the target gene and  $\beta$ -actin (to reach a final 1X concentration). The plate was then loaded onto an ABI Prism 7300 instrument (Applied Biosystems) and the amplification reaction was achieved with a 3-stage protocol: 2 minutes at 50 °C followed by 10 minutes at 95 °C, followed again by 40 repetitions of a 2-step cycle composed by 15 seconds at 95 °C (denaturation) and 1 minute at 60 °C (annealing and extension). At the end of the reaction, data analysis was performed with the 7300 System Software (Applied Biosystems) and RQ values ( $2^{-\Delta\Delta CT}$ , where CT is the threshold cycle) of the target genes relative to their own endogenous control were obtained. The RQ values were then converted into fold change values relative to control group.

### ***2.3.3 c-FOS immunoreactivity quantification***

#### ***2.3.3.1 Intracardiac perfusion***

90 minutes after the FST animals were deeply anaesthetised with an intraperitoneal injection of urethane (Sigma Aldrich, Ireland, 2 g/kg). The forelimbs were fixed to a perfusion grid and the skin of the abdomen removed to show the muscular layer of the abdomen. The abdominal cavity was then opened to expose the sternum of the animal, the diaphragm pierced to gain entry to the thoracic cavity and the ribs were pulled back to expose the heart. Finally, a cannula connected to a perfusion pump was inserted into

the left ventricle and the right atrium was pierced. Blood was rinsed out of the body by perfusing 10 mM phosphate buffered saline (PBS) solution (1.9 mM NaH<sub>2</sub>PO<sub>4</sub>, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub> and 0.9% w/v NaCl, all powders from Sigma Aldrich, Ireland, pH 7.4, in de-ionised H<sub>2</sub>O) in the systemic circulation of the rat. The solution was passed through the body for at least 5 minutes before switching to a paraformaldehyde (PFA, Sigma Aldrich, Ireland) fixative solution (4% w/v PFA in 10 mM PBS, pH 7.4) which was run for at least 10 minutes before ending the procedure. At the end the brain of the animal was removed and immersed 4% PFA solution at 4°C for 48 hr. After this post-fixation period, the brain was cryo-protected in sucrose solution (30% w/v sucrose, Sigma Aldrich, Ireland, in 10 mM PBS, pH 7.4) at 4°C for 3 or 4 days. Once the brains sunk, they were removed from sucrose and snap frozen in dry ice-cold isopentane. Brains were then stored at -80°C until sectioning.

### ***2.3.3.2 c-FOS immunohistochemistry***

Frozen brains were sectioned at -21°C in a cryostat. Serial 40 µm thick slices of the whole brain from the frontal cortex to the cerebellum were collected in a dedicated freezing solution (30% v/v ethylene glycol, 30% w/v sucrose in 10 mM PBS) and stored at -80°C prior to further use. In preparation for staining brain sections were defrosted to room temperature and free-floating immunohistochemistry staining was conducted. Using a fine-tipped paintbrush, tissue slices were transferred into netwells (Corning Life Sciences, France) immersed in 10 mM PBS in a netwell tray. For negative controls, 3 or 4 tissue slices were isolated into an additional netwell. All the incubation steps were performed under constant oscillation to allow reagents to penetrate better into the tissue.

Briefly, the sections were washed in 10 mM PBS and endogenous peroxidase activity was quenched by incubation in a 0.75% v/v hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>, Sigma Aldrich, Ireland) solution for approximately 20 minutes. Unreacted H<sub>2</sub>O<sub>2</sub> was washed off with 10 mM PBS. Non-specific binding sites were blocked by incubation in 10% v/v normal goat serum (NGS, Sigma Aldrich, Ireland) for 20 minutes and as c-FOS is localised to the nucleus (Kovacs, 2008), permeabilisation of the cell membrane was achieved by adding 0.05% v/v Triton-X (TX, Sigma Aldrich, Ireland) to the solution. Blocking with NGS was followed by incubation with a solution containing rabbit anti-c-Fos primary antibody (Santa Cruz Biotechnology Inc, Germany, 1:6500 dilution in 3% v/v NGS, 0.05% v/v TX and 10 mM PBS) for at least 16 hr or overnight. For the negative controls the same solution lacking the c-Fos antibody was prepared. The next day the sections were incubated with a biotinylated goat anti-rabbit antisera (Elite Vectastain ABC kit, Vector Laboratories, UK, 1:200 dilution in 3% v/v NGS and 10 mM PBS) for approximately 90 min followed by a further 90 min incubation with an avidin-biotinylated peroxidase complex (ABC, Elite Vectastain ABC kit, Vector Laboratories, UK) solution formulated in 10 mM PBS as described by the manufacturer. The sections were then moved from the netwells to clean petri dishes and incubated for 5 minutes with a 10% w/v 3,3'-diaminobenzidine solution (DAB, 0.4% v/v H<sub>2</sub>O<sub>2</sub> in 10 mM PBS). The reaction was terminated by addition of de-ionised H<sub>2</sub>O into the petri dishes. The sections were rinsed in 10 mM PBS and transferred onto gelatine-coated glass slides where they were allowed to dry over night at room temperature. Finally, the sections were dehydrated in an increasing gradient of alcohols (ethanol 70% v/v, 90% v/v and 100%) after desalting them into de-ionised H<sub>2</sub>O. The last dehydration step was done with xylene (Sigma-Aldrich, Ireland) and then the slides were mounted and sealed with

a mixture of distyrene, a plasticiser and xylene (DPX) mountant for microscopy (Fisher Scientific, UK) to preserve and enhance immunostaining.

Immunolabelled sections were visualised using an Olympus BX51 microscope, and photomicrographs were collected with an Olympus DP72 digital camera and the Olympus Cell^D computer software. Neuroanatomical areas were identified according to the Paxinos and Watson (1998) rat brain atlas and all the c-FOS positive cells within the region of interest were counted bilaterally. Where no differences between the two hemispheres were found an average total c-FOS-like immunoreactivity (TFI) per region was calculated.





# *Results*



# *Results Chapter 1*

*Ketamine elicits sustained antidepressant-like activity via a 5-HT-dependent mechanism*



## 1.1 Introduction

Ketamine, a glutamatergic N-methyl-D-aspartate receptor (NMDA-R) antagonist, produces rapid and sustained antidepressant effects in patients with severe and treatment resistant depression (Berman et al., 2000; Cornwell et al., 2012; Koike et al., 2011; Maeng et al., 2008; Tizabi et al., 2012; Zarate et al., 2006). Therapeutic effects are immediate and last for several days following a single sub-anaesthetic intravenous dose. The antidepressant effects of ketamine tie in with a proposed role for the glutamatergic neuronal system in the pathophysiology of depression (Bernard et al., 2011; Bonanno et al., 2005; Zarate et al., 2003) although the mechanisms remain to be fully characterised. It remains unclear as to whether monoaminergic systems are involved and if rapid and/or sustained antidepressant effects may be achieved through these transmitter mechanisms.

Reports of the rapid antidepressant activity of ketamine have spurred efforts to model such effects in laboratory animals so that the underlying neurobiological mechanisms might be examined. To date a number of studies have demonstrated that ketamine produces antidepressant-like behavioural effects in a variety of animal paradigms including the learned helplessness paradigm (Koike et al., 2011; Maeng et al., 2008), the forced swimming and tail suspension tests (Autry et al., 2011; Bechtholt-Gompf et al., 2011a; Engin et al., 2009; Garcia et al., 2008a; Li et al., 2011; Maeng et al., 2008) and reversal of behavioural and physiological alterations induced by chronic mild stress in rats (Garcia et al., 2009; Li et al., 2011). In some cases such effects have been reported to be sustained for up to two weeks following a single dose of ketamine (Maeng et al., 2008). In tandem with antidepressant related behavioural effects,

molecular and cellular plasticity changes within hippocampal and cortical neurocircuitry have also been described (for review see Murrough, 2012). Identification of the types of circuits and neurotransmitters involved remains to be elucidated. The AMPA subtype of glutamate receptor has emerged as a potential mechanism in the antidepressant-like effect of ketamine (Tizabi et al., 2012; Koike et al., 2011) where it has been proposed that enhanced AMPA relative to NMDA throughput leads to activation of the mammalian target of rapamycin (mTOR) that is involved in trafficking AMPA receptor subunits to the membrane (Wang et al., 2006) leading to increased synaptogenesis (Li et al., 2010).

As described in the introductory section, the forced swimming test (FST) is one of the most widely employed screening tests for antidepressants in rodents (Borsini, 1995; Borsini and Meli, 1988; Porsolt et al. 1978). A variety of antidepressant drugs increase escape-oriented behaviour and reduce “behavioural despair” in the test. Traditionally the FST in rats is carried out in naïve animals, however more recently the FST has been adopted to assess an endophenotype associated with animal models of depression where immobility in the FST is increased by prior exposure to stressors (Armario et al., 1991; Kim et al., 2006; 2012; Veena et al., 2009) and by genetic factors which may increase vulnerability to stress and depression (Cryan and Mombereau, 2004; Jacobson and Cryan, 2007; Solomon et al., 2012).

It is presently unclear whether 5-HT transmission is involved in the antidepressant-like effects of ketamine in the FST. There are a number of early reports which indicate that ketamine, albeit at higher doses than those used in the present investigation, influence brain 5-HT concentration (Kari et al., 1978), uptake (Martin et al., 1982), synthesis and

metabolism (Martin and Smith, 1982) following systemic administration to rats. Tso and co-workers (2004) reported that ketamine stimulated 5-HT efflux and uptake in isolated superfused slices of the rat dorsal raphe nucleus. Ketamine promotes the efflux of 5-HT in the medial prefrontal cortex (mPFC) following systemic administration of sub-anaesthetic dose to rats indicating that increased 5-HT transmission in the mPFC is a functional consequence of NMDA-R blockade (Amargos-Bosch, et al., 2006; Lindefors et al., 1997). Evidence that inhibition of neuronal nitric oxide synthase (NOS) elicits antidepressant-like activity through a 5-HT dependent mechanism (Harkin et al., 2003, 2004; Ulak et al., 2008) suggest that NMDA-R blockade may act through a similar 5-HT dependent mechanism.

To elucidate a role for 5-HT in the antidepressant related actions of ketamine, the effects of ketamine administration were investigated in the rat FST in naïve and in 5-HT depleted animals. Ketamine provoked both rapid (1 hr) and sustained (24 hr) antidepressant-like effects in naïve animals. Sustained but not immediate antidepressant effects were attenuated by prior central 5-HT depletion. The ability of ketamine to block stress-induced immobility in the FST was also diminished in 5-HT depleted animals indicative of a role for 5-HT in mediating sustained antidepressant activity in the rat FST.





## 1.2 Materials and Methods

### 1.2.1 Subjects and drug treatment

Male Sprague-Dawley rats weighing 280-320 g at the beginning of the studies were singly housed in standard medium sized polypropylene cages (41x24 cm) after acclimatisation to the new environment. Food and water were available *ad libitum*.

Ketamine hydrochloride was prepared with saline solution to final concentrations of 10 and 25 mg/ml. Ketamine 10 mg/ml has been reported to provoke antidepressant-like effects in the rat FST (Li et al., 2010). The higher dose of 25 mg/kg was selected as it is a quite high subanaesthetic dose but at the same time the hyperlocomotion and stereotyped behaviours induced by it have been shown to dissipate 60 min after systemic injection (Razoux et al., 2007). Ketamine was injected intraperitoneally (i.p.) at a volume of 1 ml/kg body weight. For experiments involving central 5-HT depletion, DL-4-Chlorophenylalanine ethyl ester hydrochloride (*p*CPA) was dissolved in saline solution to a concentration of 75 mg/ml. *p*CPA was injected i.p. at a volume of 2 ml/kg body weight to yield a dose of 150 mg/kg.

All procedures were approved by the Animal Ethics Committee Trinity College Dublin and were in accordance with the European Council Directive 1986 (86/806/EEC).

## ***1.2.2 Study design***

### ***1.2.2.1 The effect of ketamine on immobility in the FST***

The FST was performed as described in the Methods section. To characterise the effects of ketamine in the FST, rats were assigned to one of five treatment groups: group 1: Vehicle, group 2: Ketamine 10 mg/kg 1 hr prior to FST, group 3: Ketamine 25 mg/kg 1 hr prior to FST, group 4: Ketamine 25 mg/kg 24 hr prior to FST or group 5: Ketamine 25 mg/kg 24-5-1 hr prior to FST. All the rats received a total of three injections 24, 5 and 1 hr prior to FST where rats receiving only a single ketamine treatment were administered 2 saline injections at the other time points.

### ***1.2.2.2 The effect of ketamine on locomotor activity and anxiety related parameters in the open field test***

Locomotor activity was measured in animals in the open field. The subanaesthetic doses of ketamine 10 and 25 mg/kg have been reported to increase locomotor activity in rats after injection, however locomotor behaviour already normalised 110 min after drug administration (Littlewood et al., 1997). Together with the report by de Oliveira and colleagues (2011) that alterations in locomotor activity after chronic ketamine (25 mg/kg once daily for 7 consecutive days) vanish 6 hr following the last drug injection, and considering that a single anaesthetic dose of ketamine (100 mg/kg) decreases locomotor activity 24 hr following administration (Prudian et al., 1997), it was

considered safe to assume that a single dose of ketamine 10 or 25 mg/kg would not increase locomotor activity in rats 24 hr after drug administration. Locomotor activity was therefore assessed only in animals receiving the injection (i.p.) 1 hr prior to test and rats were assigned to one of three treatment groups: group 1: Vehicle, group 2: Ketamine 10 mg/kg or group 3: Ketamine 25 mg/kg.

### ***1.2.2.3 The effect of ketamine on immobilisation stress-induced immobility in the FST***

To characterise the effects of restraint stress on immobility time in the FST rats were subjected to 2 hr restraint stress, as described in the Methods section, once or for 10 consecutive days and were subsequently exposed to the 15 min pre-FST session 2 hr following the last restraint stress session, received a saline injection 1 hr later and then were tested in the FST further 24 hr later. Animals were assigned to one of four groups: group 1: Control, group 2: Stress (once), group 3: Control (10 days), group 4: Stress (10 days). To test if the pro-depressant effects of stress in the FST were protracted over time, a similar experiment to the previous one was conducted, allowing a longer interval of time (24 hr instead of 2 hr) between the final stress session and the adaptation session of the FST. Rats received a saline injection 1 hr after the pre-FST session and then were tested in the FST 24 hr later. Again, animals were assigned to one of four groups: group 1: Control, group 2: Stress (once), group 3: Control (10 days), group 4: Stress (10 days). The effect of ketamine was subsequently assessed. Rats were subjected to 2 hr restraint stress for 2 hr daily over 10 days. 2 hr following the final restraint stress session animals were exposed to the 15 min pre-FST session. Animals subsequently received ketamine 1

hr following the pre-FST session and were tested in the FST 24 hr later. Animals were assigned to one of four groups: group 1: Control + Vehicle, group 2: Stress + Vehicle, group 3: Control + Ketamine (25 mg/kg), group 4: Stress + Ketamine (25 mg/kg).

#### ***1.2.2.4 The effect of central 5-HT depletion on antidepressant-like effects of ketamine in the FST***

*p*CPA (150mg/kg, i.p.) or vehicle (saline) were administered once daily for three consecutive days as previously described. 72 hr after the last *p*CPA administration, rats were exposed to the 15 min pre-FST session. Rats were assigned to one of six groups: group 1: Vehicle + Vehicle, group 2: Vehicle + Ketamine (25 mg/kg, 1 hr prior to FST), group 3: Vehicle + Ketamine (25 mg/kg, 24 hr prior to FST), group 4: *p*CPA + Vehicle, group 5: *p*CPA + Ketamine (25 mg/kg, 1 hr prior to FST), group 6: *p*CPA + Ketamine (25 mg/kg, 24 hr prior to FST).

The effects of 5-HT depletion were assessed on stress-induced immobility in the FST. Rats were subjected to 5-HT depletion followed by 2 hr restraint stress once daily for three consecutive days as previously described. 24 hr following the final period of stress animals were exposed to the 15 min pre-FST session and FST 24 hr later. Rats were assigned to one of four experimental groups: group 1: Vehicle + Control, group 2: Vehicle + Stress, group 3: *p*CPA + Control, group 4: *p*CPA + Stress. In a following experiment to evaluate the persistence of the depressive-like state induced in the FST by central 5-HT depletion followed by restraint stress, the same protocol was repeated and the pre-FST session performed 72 hr after cessation of stress. The FST was then

conducted 24 hr later. Rats were assigned to two experimental groups: group 1: Control, group 2: *p*CPA + Stress.

The effects of the combination of 5-HT depletion and repeated restraint stress were also assessed in the open field, which was performed 24 hr following the final stress session. Rats were again assigned to two experimental groups: group 1: Control, group 2: *p*CPA + Stress.

In a final experiment the effects of ketamine administration were established. Rats were subjected to 5-HT depletion and immobilisation stress and subsequently sub-divided into drug treatment groups as follows: group 1: Control + Vehicle, group 2: Control + Ketamine, group 3: *p*CPA/Stress + Vehicle, group 4: *p*CPA/Stress + Ketamine. Ketamine (25 mg/kg, i.p.) or saline was administered 1 hr after the 15 minute pre-FST session 24 hr prior to FST.

### ***1.2.3 Determination of cortical 5-HT concentrations***

Immediately following these tests the animals were euthanised in order to verify the depletion of 5-HT induced by *p*CPA in the frontal cortex by HPLC. Final results were expressed as ng neurotransmitter/g wet weight of tissue.

### ***1.2.4 Statistical analysis***

Data are expressed as group mean with standard error of the mean (SEM) and were analysed by one factor or two factor or two factor repeated measure analysis of variance

(ANOVA) where appropriate. If any statistically significant change was found, *post hoc* comparisons were performed using a Dunnett's (following 1 factor ANOVA) or a Student Newman-Keuls (following 2 factor ANOVA) test. Data were deemed significant when  $p < 0.05$ . All analysis was carried out with GB-Stat v10 statistical package.

## 1.3 Results

### 1.3.1 Dose and time dependent effects of ketamine in the rat FST

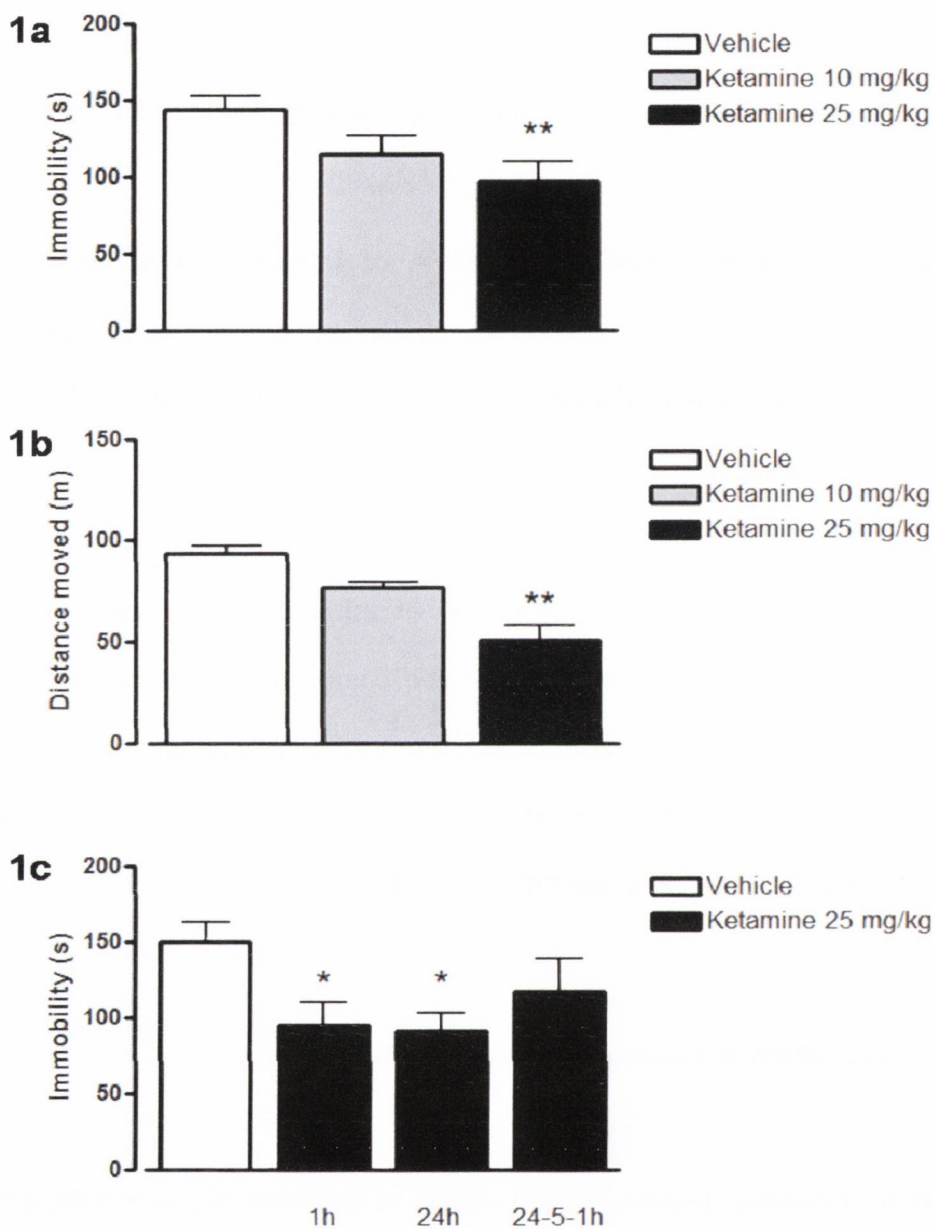
In the dose response experiment ANOVA of immobility time showed effects of ketamine [ $F_{(2,50)} = 4.98$ ,  $p=0.01$ ]. *Post hoc* comparison revealed that ketamine 25 mg/kg, but not 10 mg/kg, when administered 1 hr prior to FST reduced immobility time compared to vehicle treated controls ( $p<0.01$ ) [Fig. 1.1a].

In the time course experiment ANOVA of immobility time showed an effect of ketamine [ $F_{(3,46)} = 2.81$ ,  $p<0.05$ ]. *Post hoc* comparison revealed that ketamine (25 mg/kg) reduced immobility time compared to vehicle treated controls when given 1 hr or 24 hr prior to FST ( $p<0.05$ ), but this effect was lost when ketamine was injected three times (24, 5 and 1 hr) prior to the FST [Fig. 1.1c].

### 1.3.2 The effects of ketamine on locomotor activity

ANOVA of distance moved showed effects of ketamine [ $F_{(2,95)} = 8.96$ ,  $p<0.01$ ]. *Post hoc* comparisons revealed that ketamine 25 mg/kg, but not 10 mg/kg, reduced locomotor activity in the open field when compared to vehicle treated controls when given 1 hr prior to FST ( $p<0.01$ ) [Fig. 1.1b].

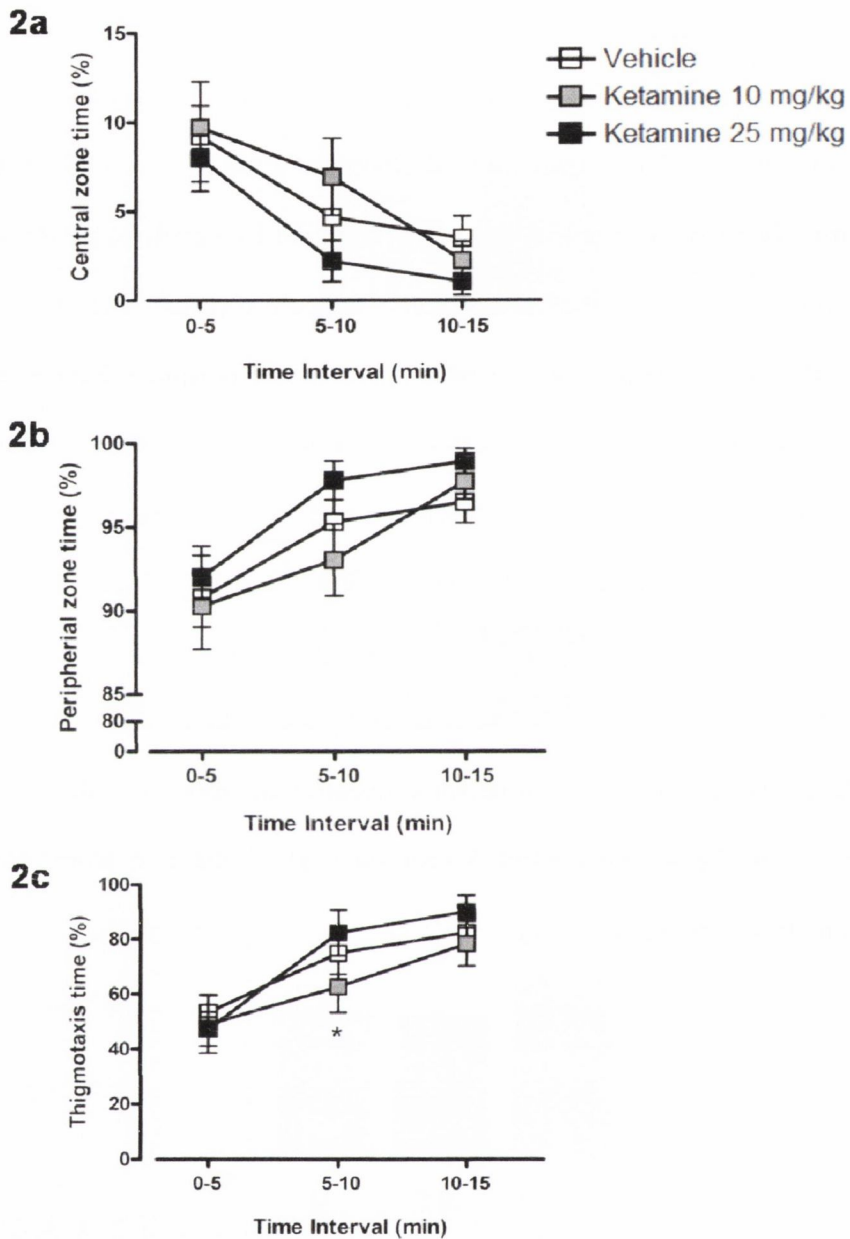




**Fig. 1.1** Effects of acute ketamine administration in the FST and open field test. Rats were exposed to a 15 min adaptation session of the FST and administered ketamine (10 or 25 mg/kg, i.p.) 24 hr later 1hr prior to test. Data is expressed as mean and SEM of 16-20 animals (Fig. 1.1a). Separate groups of rats were exposed to the open field for 15 min 1hr following ketamine (10 or 25 mg/kg i.p.) administration. Data is expressed as mean and SEM of 6 animals (Fig. 1.1b). Rats were exposed to a 15 min adaptation session of the FST and treated with ketamine (25 mg/kg, i.p.) 1 hr, 24 hr and 1, 5 and 24 hr prior to test. Data are expressed as mean and SEM of 10-16 animals (Fig. 1.1c). \* $p < 0.05$ ; \*\* $p < 0.01$  compared to vehicle controls.

### ***1.3.3 The effects of ketamine on anxiety related behaviour in the open field***

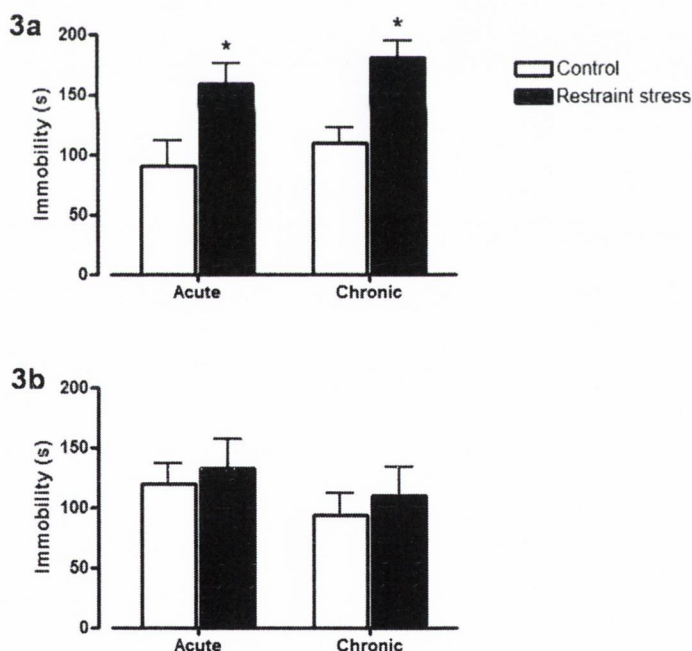
Repeated measure ANOVA of the percentage of time spent in the central zone of the open field arena over a 15 min trial showed effects of time [ $F_{(2,30)} = 28.15, p < 0.0001$ ]. *Post hoc* comparisons did not reveal any effect induced by ketamine at any of the time intervals analysed when compared to respective vehicle controls [Fig. 1.2a]. Repeated measure ANOVA of the percentage of time spent in the peripheral zone of the open field arena over a 15 min trial showed effects of time [ $F_{(2,30)} = 28.15, p < 0.0001$ ]. *Post hoc* comparisons did not reveal any effect induced by ketamine at any of the time intervals analysed when compared to respective vehicle controls [Fig. 1.2b]. Analysis of the percentage of time spent at the edges of the open field arena over a 15 min trial showed effects of time [ $F_{(2,30)} = 75.78, p < 0.0001$ ] and a ketamine x time interaction [ $F_{(2,30)} = 2.82, p < 0.05$ ]. *Post hoc* comparisons revealed an anxiolytic-like effect induced by ketamine 10 mg/kg in the central 5 min interval of the test when compared to respective vehicle controls [Fig. 1.2c].



**Fig. 1.2** Effects of acute ketamine administration on anxiety related parameters in the open field test. Rats were given ketamine (10 or 25 mg/kg, i.p.) 1 hr prior exposure to 15 min open field test. Ketamine does not influence time spent in both the central (Fig.1.2a) and the peripheral (Fig.1.2b) zones of the arena. At a dose of 10 mg/kg it is able to reduce time spent on the edges of the arena only in the second 5 min interval of the test (Fig.1.2c). Data are expressed as mean and SEM of 6 animals. \* $p < 0.05$  compared to Vehicle control group.

### 1.3.4 Effects of immobilisation stress in the FST

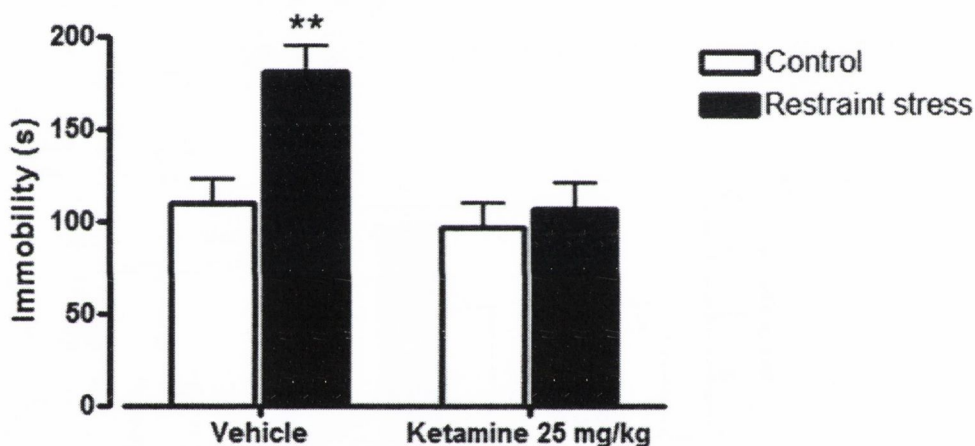
Analysis of immobility time of animals exposed to the adaptation session of the FST 2 hr following cessation of single or repeated immobilisation stress showed effects of stress exposure [ $F_{(1,28)} = 17.19, p < 0.01$ ]. *Post hoc* comparisons revealed that immobility time was increased by both acute (single, 2 hr/day once) and chronic (repeated, 2 hr/day for 10 days) restraint stress when compared to non stressed controls [Fig. 1.3a]. Analysis of immobility time of animals exposed to the adaptation session of the FST 24 hr following cessation of single or repeated immobilisation stress did not show effects neither of stress exposure nor of duration of stress [Fig. 1.3b].



**Fig. 1.3** Exposure to acute and repeated restraint stress transiently increases immobility time in the FST. Rats were exposed to acute (2 hr/day once) or chronic (2 hr/day for 10 days) restraint stress and then exposed to the 15 min adaptation session of the FST either 2 hr (Fig. 1.3a) or 24 hr (Fig. 1.3b) after the last stress session. Data are expressed as mean and SEM of 7-8 animals. \* $p < 0.05$  compared to corresponding control group.

### 1.3.5 Ketamine attenuates stress-induced immobility in the FST

Analysis of immobility time of animals exposed to the FST following cessation of repeated immobilisation stress showed effects of stress exposure [ $t$ -test;  $t_{(14)} = 3.7$ ,  $p < 0.01$ ]. In a subsequent experiment to assess the effect of ketamine, no effect of an identical stress regime was observed in ketamine treated animals [Fig. 1.3].

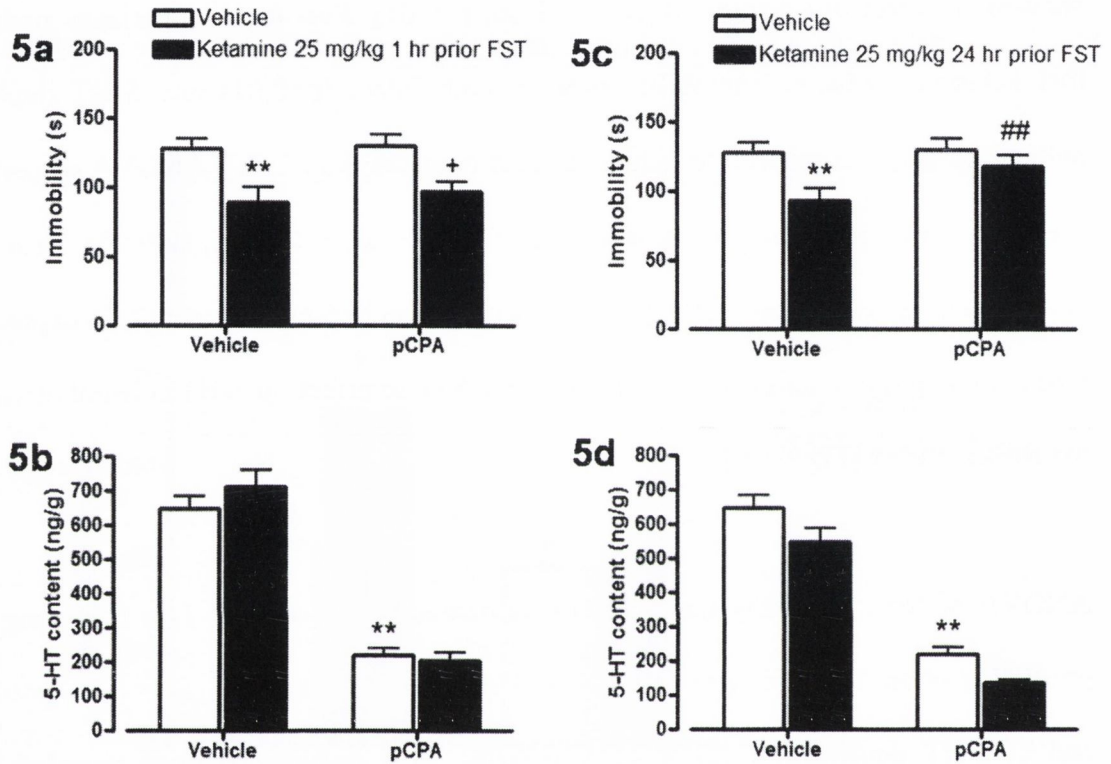


**Fig. 1.4** Effect of ketamine on immobilisation stress-induced immobility in the FST. In the first experiment rats were exposed to repeated (2 hr/day for 10 days) immobilisation stress and subsequently subjected to a 15 min FST adaptation session 2 hr following the cessation of stress and the FST 24 hr later. In a second experiment the stress regime was repeated and rats were treated with ketamine (25 mg/kg, i.p.) 1 hr following the pre-FST session. Data are expressed as mean and SEM of 8-9 animals. \* $p < 0.05$ , \*\* $p < 0.01$  compared to vehicle control group.

### ***1.3.6 5-HT depletion attenuates the antidepressant-like effects of ketamine in the FST***

ANOVA of the immobility time of animals treated with ketamine 1 hr prior to FST showed an effect of ketamine [ $F_{(1,61)} = 18.36$ ,  $p < 0.01$ ]. *Post hoc* comparisons revealed that ketamine reduced immobility time in both intact ( $p < 0.01$ ) and 5-HT depleted animals ( $p < 0.05$ ) compared to vehicle treated controls [Fig. 1.5a]. ANOVA of cortical 5-HT concentrations showed an effect of *p*CPA [ $F_{(1,61)} = 206.03$ ,  $p < 0.01$ ]. *Post hoc* comparisons revealed that *p*CPA produced a reduction in 5-HT content (70% depletion) when compared to controls ( $p < 0.01$ ). Ketamine had no effect on 5-HT concentrations in the frontal cortex [Fig. 1.5b].

ANOVA of the immobility time of animals treated with ketamine 1 hr following the pre-FST session, 24 hr prior to FST, showed effects of ketamine [ $F_{(1,67)} = 8.75$ ,  $p < 0.01$ ] and of 5-HT depletion [ $F_{(1,67)} = 4.24$ ,  $p < 0.05$ ]. *Post hoc* comparisons revealed that ketamine reduced immobility time compared to vehicle treated controls in non-depleted animals ( $p < 0.01$ ). 5-HT depletion did not affect immobility time when compared to non-depleted vehicle treated controls, but attenuated the antidepressant-like effect of ketamine ( $p < 0.01$ ) [Fig. 1.5c]. ANOVA of cortical 5-HT concentrations showed effects of *p*CPA [ $F_{(1,67)} = 198.50$ ,  $p < 0.01$ ]. *Post hoc* comparisons revealed that *p*CPA produced a reduction in 5-HT content when compared to controls ( $p < 0.01$ ) [Fig. 1.5d].

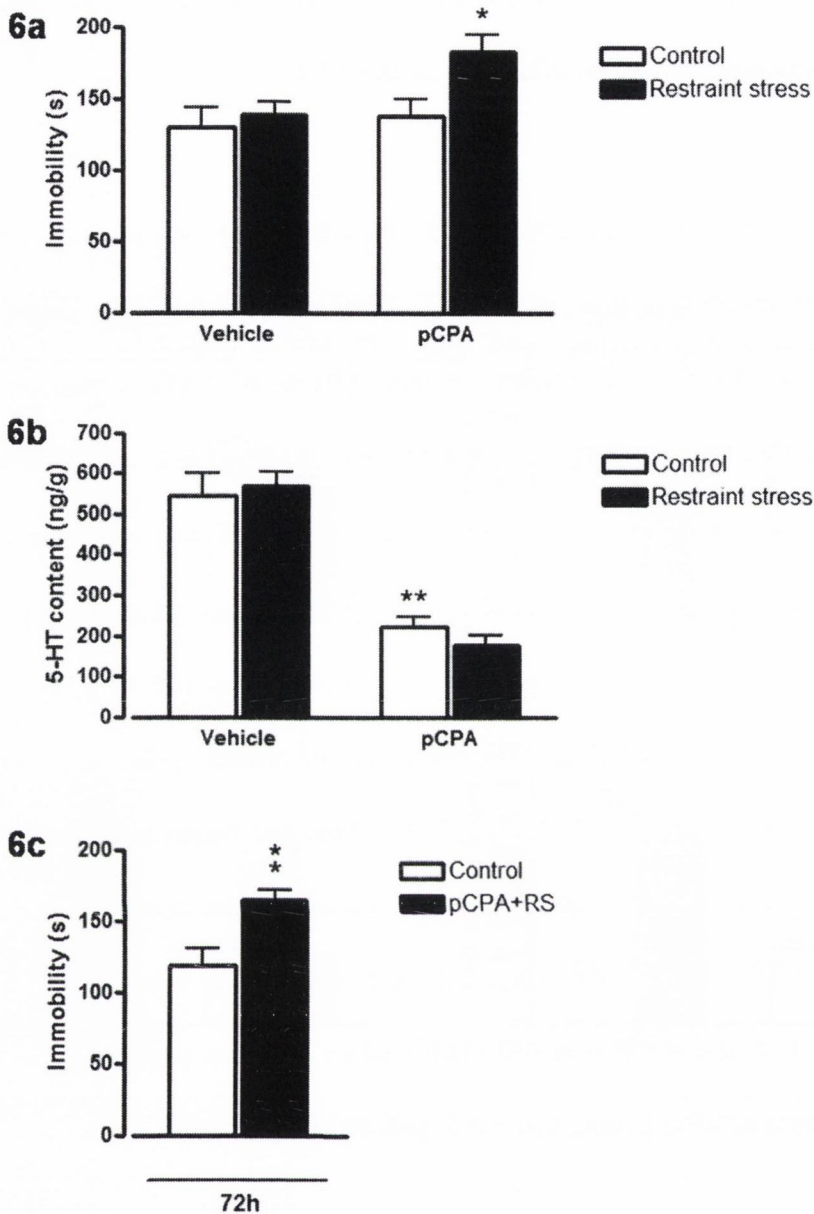


**Fig. 1.5** 5-HT depletion attenuates the antidepressant-like activity of ketamine in the FST. Rats were treated with pCPA (150 mg/kg i.p., once daily for 3 days). 3 days later the animals were exposed to a 15 min adaptation session of the FST and administered ketamine (25 mg/kg, i.p.) 1hr (Fig. 1.5a) or 24 hr (Fig. 1.5c) prior to test. Cortical 5-HT concentrations were determined immediately following the test (Fig. 1.5b and 1.5d respectively). Data are expressed as mean and SEM of 13-18 (Fig. 1.5a and 1.5b) and 17-18 (Fig. 1.5c and 1.5d) animals. \* $p < 0.05$ , \*\* $p < 0.01$  compared to vehicle control group, + $p < 0.05$  compared to pCPA + vehicle group, ## $p < 0.01$  compared to vehicle + ketamine administered 24 hr prior to FST group.

### ***1.3.7 Combination of 5-HT depletion and repeated restraint stress induces a persistent depressive-like state in the FST***

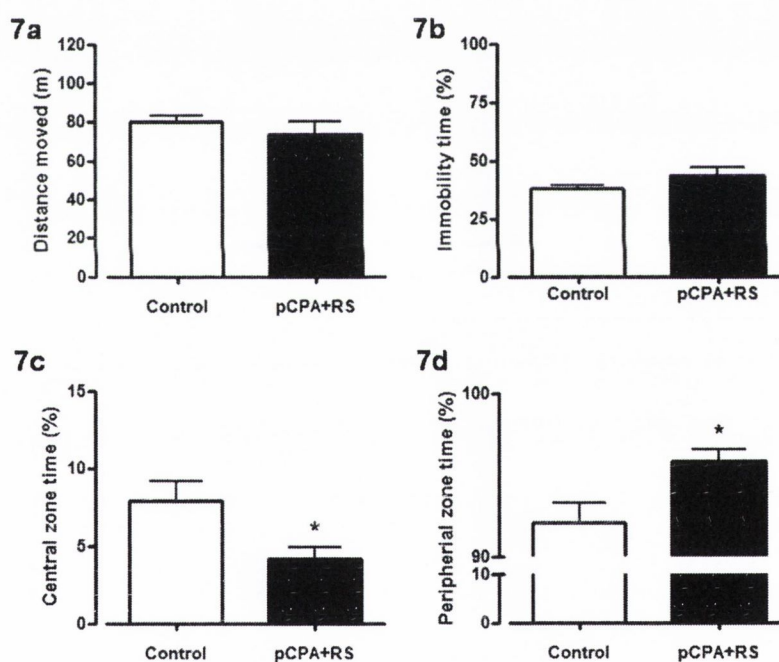
Combination of 5-HT depletion and immobilisation stress provoked a persistent increase in immobility time in the FST. ANOVA of immobility times recorded 48 hr following the final stress session showed effects of 5-HT depletion [ $F_{(1,39)} = 4.27$ ,  $p < 0.05$ ] and restraint stress [ $F_{(1,39)} = 4.58$ ,  $p < 0.05$ ]. *Post hoc* comparisons revealed that neither 5-HT depletion nor restraint stress alone influenced immobility time when compared to vehicle treated controls. 5-HT depletion followed by restraint stress provoked an increase in immobility time when compared to either stress or 5-HT depletion alone ( $p < 0.05$ ) [Fig. 1.6a]. ANOVA of cortical 5-HT concentrations showed an effect of *p*CPA [ $F_{(1,39)} = 103.83$ ,  $p < 0.01$ ]. *Post hoc* comparisons revealed a reduction in 5-HT concentrations in *p*CPA treated groups when compared to vehicle treated controls ( $p < 0.01$ ) [Fig. 1.6b]. The increase in immobility time in the FST provoked by 5-HT depletion and stress was still visible when FST was performed 96 hr after the last restraint stress session [*t*-test;  $t_{(15)} = 3.5$ ,  $p < 0.01$ ] [Fig. 1.6c].





**Fig. 1.6** Prolonged effects of the combination of 5-HT depletion and restraint stress in the FST. Rats were treated with pCPA (150 mg/kg i.p., once daily for 3 days). 2 days later the animals were exposed to immobilisation stress (2 hr/day for 3 days). 24 hr following the last stress session, rats were subjected to a 15 min adaptation session of the FST and tested 24 hr later (Fig. 1.6a). Cortical 5-HT concentrations were determined following the test (Fig. 1.6b). The treatment regime of pCPA and stress was repeated. Animals were exposed to a 15 min adaptation session of the FST 72 hr after the last stress session, followed by the 5 min FST 24 hr later (Fig. 1.6c). Data are expressed as mean and SEM of 7-13 animals. \* $p < 0.05$ , \*\* $p < 0.01$  compared to respective vehicle controls.

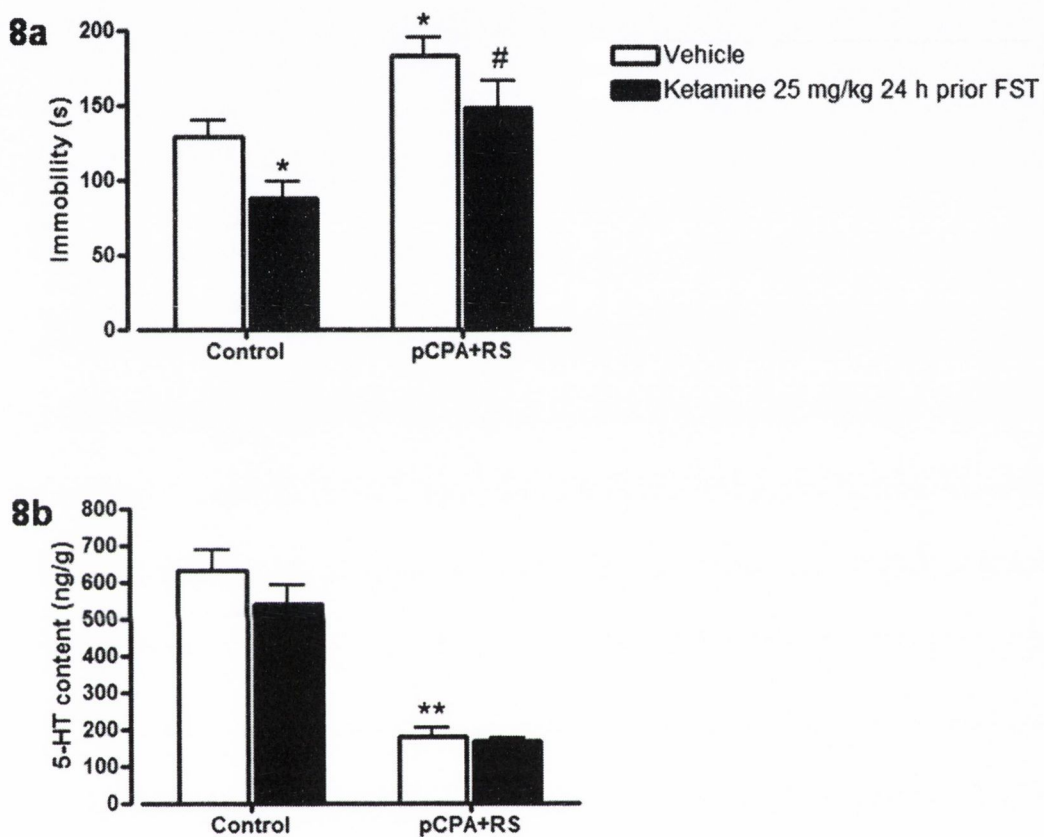
In a separate experiment the effects of the combination of 5-HT depletion and restraint stress on locomotor activity and anxiety parameters in the open field were assessed. Analysis of distance moved and immobility time during the test failed to show any difference between control and 5-HT depleted and stressed rats [Fig. 1.7a and 1.7b]. Analysis of percentage of time spent in the central zone and in the peripheral zone of the open field arena over a 15 min trial highlighted anxiogenic effects of 5-HT depletion and restraint stress [ $t$ -test;  $t_{(10)} = 2.6, p < 0.05$  and  $t_{(10)} = 2.6, p < 0.05$  respectively] [Fig. 1.7c and 1.7d].



**Fig.1.7** Combination of 5-HT depletion and immobilisation stress does not alter locomotor activity but induces anxiety-like behaviour in the open field test. Rats were treated with pCPA (3 x 150 mg/kg, i.p.), left to recover for 48 hr and then exposed to restraint stress (2 hr/day for 3 days). 24 hr after the last stress session rats were exposed to the 15 min open field test. Locomotor activity parameters path length (Fig. 1.7a) and immobility time (Fig. 1.7b) are unaffected by combination of 5-HT depletion and immobilisation stress. Anxiety-related parameters central zone time (Fig. 1.7c) and peripheral zone time (Fig. 1.7d) reveal an anxiogenic-like effect of the combination of 5-HT depletion and stress in the test. Data are expressed as mean and SEM of 6 animals. \* $p < 0.05$  compared to control group.

### ***1.3.8 Ketamine does not counteract the pro-depressant effects of the combination of 5-HT depletion and repeated restraint stress in the FST***

Subsequent experiments were carried out to determine the effects of ketamine on immobility induced by the combination of 5-HT depletion and stress. ANOVA of immobility time of animals treated with ketamine 24 hr prior to FST showed a combined effect of 5-HT depletion and restraint stress [ $F_{(1,39)} = 17.52$ ,  $p < 0.01$ ] and an effect of ketamine [ $F_{(1,39)} = 7.65$ ,  $p < 0.01$ ]. *Post hoc* comparisons revealed that ketamine reduced immobility time when compared to non-depleted and non-stressed vehicle treated controls ( $p < 0.05$ ). The combination of 5-HT depletion and stress provoked an increase in immobility when compared to non-depleted non-stressed controls ( $p < 0.05$ ) [Fig. 1.8a]. Ketamine failed to significantly attenuate this increase. Moreover an increase in immobility time was apparent in 5-HT depleted and stress exposed animals treated with ketamine when compared to the non-depleted non-stressed ketamine treated group ( $p < 0.05$ ) [Fig. 1.8a]. ANOVA of cortical 5-HT concentrations showed effects of the 5-HT depletion and restraint stress combination [ $F_{(1,39)} = 104.83$ ,  $p < 0.01$ ]. *Post hoc* comparisons confirmed that *p*CPA provoked a reduction in 5-HT content when compared to vehicle treated controls ( $p < 0.01$ ) [Fig. 1.8b].



**Fig. 1.8** Effect of ketamine on FST immobility induced by a combination of 5-HT depletion and stress. Rats were treated with pCPA (150 mg/kg i.p., once daily for 3 days). 2 days later the animals were exposed to immobilisation stress (2 hr/day for 3 days). 24 hr following the last stress session, rats were subjected to a 15 min adaptation session of the FST were subsequently treated with ketamine (25 mg/kg, i.p.) 24 hr prior to test (Fig. 1.8a). Cortical 5-HT concentrations were determined following the test (Fig. 1.8b). Data are expressed as mean and SEM of 7-13 animals. \* $p < 0.05$ , \*\* $p < 0.01$  compared to vehicle control group; # $p < 0.05$  compared to control + ketamine treated group.



## **1.4 Discussion**

In the present study ketamine produced a dose dependent reduction in immobility in the rat FST. Such an action is indicative of antidepressant activity in the test and was obtained in the absence of drug-induced psychomotor stimulation. The response observed following acute administration was sustained for 24 hr yet dissipated following sub-acute administration over the same period suggestive of pharmacological tolerance. The sustained antidepressant response was further characterised by the ability of ketamine to attenuate a stress-related increase in immobility, considered a depression related state, in the FST. Central 5-HT depletion attenuated the sustained, but not acute, reduction in immobility obtained with ketamine in the FST. The ability of ketamine to attenuate stress-induced immobility in the FST was also diminished following central 5-HT depletion indicating a role for 5-HT in the antidepressant response.

### ***1.4.1 The acute and sustained antidepressant response to ketamine in the FST***

The results are in line with previous reports of the antidepressant activity of ketamine in the FST. Previous investigations have shown antidepressant-like activity at doses of 10 and 15 mg/kg following acute administration of ketamine in the rat FST (Garcia et al., 2008a; Li et al., 2010). In the current investigation a single acute dose of 10 mg/kg reduced immobility time albeit non-significantly. Differences between reports may be accounted for by factors such as strain, age or weight of rats tested in addition to variations in test conditions between laboratories.

Active doses of ketamine in the FST cannot be attributed to a psychomotor stimulant action of the drug. In fact, ketamine reduced locomotor activity at a dose and time post administration that displays anti-immobility effects in the FST. Interestingly many tricyclic antidepressants also suppress locomotor activity at doses that are behaviourally active in the FST (Borsini and Meli, 1988). Other investigators have suggested that the motor effects of ketamine can be dissociated from its antidepressant-like effects (Engin et al., 2009). Several antidepressants display anxiolytic-like properties in rodents (Javelot et al., 2011; Pinheiro et al., 2008), a characteristic which was not exhibited by ketamine in the present study. Indeed ketamine has been shown to possess anxiogenic-like effects in rodents (da Silva et al., 2010; Silvestre et al., 1997), although reports on the topic are rare. Anxiety-modulating properties of ketamine were not further investigated in the presented work, as depression was the primary and main focus of the study.

Typically antidepressants are administered on 3 occasions over a 24 hr period prior to determining immobility time in the rat FST (Porsolt, 1978). When such a treatment regime was used however, the antidepressant effects of ketamine dissipated indicative of tolerance following sub-acute administration. Tolerance to ketamine has been shown to develop after repeated administration of the drug in rats (Popik et al., 2008), even if contrasting reports have been published (Garcia et al., 2008b). Surprisingly tolerance to sub-anaesthetic doses of ketamine is largely unreported in pharmacological studies. Rapid tolerance has been reported in ocular motor paradigms in macaque monkeys (Pouget et al., 2010) and in the suppression of cortical spreading depression in rats (Rashidy-Pour et al., 1995).

As sustained effects were found with a 25 mg/kg dose of ketamine, this dose was used in subsequent experiments. Higher doses of ketamine in excess of 40 mg/kg are questionable as they have been associated with behavioural activating effects (Bechtholt-Gompf et al., 2011b; Yang et al., 2011) and neurotoxicity (Jevtovic-Todorovic et al., 2001) although there are reports of antidepressant effects associated with such doses in rats (Koike et al., 2011; Popik et al., 2008). The sustained activity of ketamine detected in the present investigation is also in accordance with previous reports in mice (Koike et al., 2011; Maeng et al., 2008).

#### ***1.4.2 Ketamine attenuates stress-induced immobility in the FST***

To date the antidepressant related effects of ketamine have been more widely reported in naïve mice and rats and to a lesser extent in animal models showing behaviours characteristic of depression. In this regard, as previously stated, the FST is being increasingly used in animal models of depression where increased immobility times are reported as characteristic of depression related behaviour in response to provocative stimuli or genetically determined vulnerability. In the current investigation, single or repeated daily sessions of immobilisation stress provoked an increase in immobility time in the FST in rats. A single administration of ketamine attenuated the stress-induced increase in immobility in the FST. Such effects are in keeping with sustained antidepressant effects described in naïve animals providing additional evidence that ketamine produces antidepressant effects in animals where an increase in immobility is provoked. The results are consistent with those of other investigators who report that acute administration with ketamine rapidly ameliorate anhedonic and anxiogenic



behaviours in rats exposed to chronic unpredictable stress (Garcia et al., 2009; Li et al., 2011) and depression related behaviour in a nerve injury model of neuropathic pain (Wang et al., 2011).

#### ***1.4.3 5-HT depletion attenuates the antidepressant response to ketamine***

In order to test if the antidepressant effects obtained with ketamine are mediated by 5-HT, the consequence of 5-HT depletion with the tryptophan hydroxylase inhibitor *p*CPA was determined. It has previously been reported that the behavioural effects of the SSRI fluoxetine in the FST (Page et al., 1999) and the novel antidepressant-like properties of NO synthase inhibitors are blocked by 5-HT depletion (Harkin et al., 2003). In the current investigation *p*CPA produced in excess of a 70% depletion of 5-HT in the frontal cortex and attenuated the ketamine-induced reduction in immobility when administered 24 hr, but not 1 hr, prior to the FST. The sustained behavioural response to ketamine 24 hr prior to test may thus be differentiated from the immediate response obtained 1 hr following drug administration on the basis of a dependency on central 5-HT. 5-HT depletion did not influence baseline immobility in the current investigation or that previously reported by Page and colleagues (1999) or Harkin and co-workers (2003). The lack of impact of depletion on baseline behaviour may indicate that different neurobiological mechanisms mediate immobility under baseline conditions compared to following antidepressant treatment. Similarly, noradrenergic lesions do not alter baseline immobility in the FST, but block the effects of noradrenergic antidepressants (Cryan et al., 2002).

#### ***1.4.4 Combination of 5-HT depletion and restraint stress provokes a long-term increase in immobility in the FST***

In a series of parallel tests in the laboratory it was established that repeated stress-induced increase in immobility time in the FST is not observed in animals provided with an interval of 24 hr between the stress exposure and the pre-FST session when compared to non-stressed controls. Thus the stress related increase in immobility is a transient effect from which the animals recover rapidly. When stress is preceded by 5-HT depletion however, the stress-induced increase in immobility in the FST is maintained for up to 72 hr following cessation of the stress. Such a persistent change in behaviour is more akin to a depression related state as it endures over time. The interaction observed between 5-HT depletion and stress is of interest and indicates that the 5-HT depleted state may increase vulnerability to stress. This hypothesis is also supported by evidence that in rats the combination of 5-HT depletion and restraint stress selectively induces anxiogenic-like traits in the open field test. The model has construct validity as deficits in 5-HT transmission are associated with susceptibility to depression in humans (Carver et al., 2008; Daniele et al., 2011) and reduced antidepressant responsivity (Delgado, 2004; Nutt, 2002).

#### ***1.4.5 Can ketamine overcome immobility produced by a combination of 5-HT depletion and immobilisation stress in the FST?***

In light of recent clinical reports of the efficacy of ketamine in depressed patients who failed to respond to conventional antidepressant treatments and given the role of 5-HT

in mediating behavioural response to antidepressants, it was of interest to determine if ketamine would produce antidepressant activity in the combined depletion and stress exposure model developed in the laboratory. If ketamine produced significant antidepressant activity in this model, such an outcome would indicate a likely 5-HT independent and putatively novel mechanism underlying the antidepressant response. By contrast, if ketamine was inactive in the model, previous observations in naïve animals in line with a 5-HT dependent mechanism would be confirmed. On the one hand, ketamine failed to significantly attenuate the increase in immobility associated with the combination of 5-HT depletion and stress. Furthermore an increase in immobility was observed following 5-HT depletion and stress in ketamine treated animals when compared to non-depleted and non-stressed ketamine treated controls. Such effects are indicative of a lack of antidepressant efficacy of ketamine in 5-HT depleted animals and supportive of a 5-HT dependent mechanism underlying the antidepressant response. On the other hand, immobility scores were reduced in ketamine treated animals exposed to 5-HT depletion and stress such that there was no significant difference in immobility time when compared to vehicle treated controls. This could be argued as a partial antidepressant response yet given the incomplete nature of 5-HT depletion, it is not unreasonable to suggest that residual 5-HT may be sufficient to produce such a partial, albeit non significant, antidepressant response in the model. When taken together the results support an interpretation that the antidepressant actions of ketamine in the FST are 5-HT dependent.

In parallel experiments immobility time of animals treated with ketamine 1 hr prior to each session of immobilisation stress session subsequent to the *p*CPA treatment regime were determined. Here too ketamine failed to attenuate increased immobility time

produced by the combination of 5-HT depletion and stress (data not shown). Thus ketamine administered concurrently with stress does not affect the emergence of this depression related behavioural phenotype.

#### ***1.4.6 Ketamine promotes molecular and cellular changes which enhance the synaptic efficacy of central 5-HT***

To date the mechanisms mediating the rapid and sustained antidepressant actions of ketamine have been partially resolved as preclinical studies have reported that AMPA receptor (AMPA-R) activation is required for the antidepressant behavioural response (Koike et al., 2011; Maeng et al., 2008; Tizabi et al., 2012). Ketamine enhances AMPA-R activity relative to NMDA-R blockade by increased expression and trafficking of AMPA-R subunits and via the activation of the mammalian target of rapamycin (mTOR), expression of synaptic proteins and increased density of dendritic spines in the prefrontal cortex (PFC) (Li et al., 2010). Ketamine promotes synaptogenesis in tandem with antidepressant responses in behavioural tests (Li et al., 2010) and has been reported to reverse both chronic unpredictable stress (CUS)-induced behavioural deficits in rats and reduced spine density and synaptic function of PFC neurons. It has been proposed accordingly that ketamine reverses the atrophy of spines in the PFC associated with stress and leads to a reconnection of neurons that underlie rapid behavioural responses (for review see Duman and Voleti, 2012). Notably stress provoked a reduction in amplitude as well as frequency of 5-HT induced excitatory post synaptic current (EPSC) in tissue slices prepared from the PFC. A single dose of ketamine completely reversed the 5-HT-induced EPSC deficit provoked by exposure to stress (Li et al.,

2011). Such mechanisms indicate a causal relationship between morphological, physiological and behavioural responses and suggest that a functional change in the synaptic efficacy of 5-HT plays a role in the antidepressant effects of ketamine.

#### ***1.4.7 Conclusions***

The high proportion of depressed patients not responding to conventional antidepressant treatments leads to a necessity for new antidepressant drugs to be discovered and developed. Animal models are critical to this process. The current investigation characterises the antidepressant effects of ketamine in the rat FST and further indicates that the behavioural effects are dependent on endogenous 5-HT. Furthermore, they confirm the utility of the FST as a tool to observe stress-induced depression related behaviour in rats and assess the role of 5-HT in the emergence of depression or antidepressant related responses.

The use of ketamine as an antidepressant looks really promising especially for treatment-resistant patients, however it is fundamental to highlight major contraindications to its use in the clinics. Ketamine induces psychotomimetic and dissociative effects, and the extensive presence of the NMDA-R in the central and peripheral nervous systems leads to a high potential for side effects related to NMDA-R blockade. Moreover ketamine raises concerns about its potential for abuse, as it is illegally used as a recreational drug. To improve the safety profile of an NMDA-R-based antidepressant treatment, it would be ideal to identify specific downstream components of the NMDA-R signalling pathway mainly involved in the antidepressant

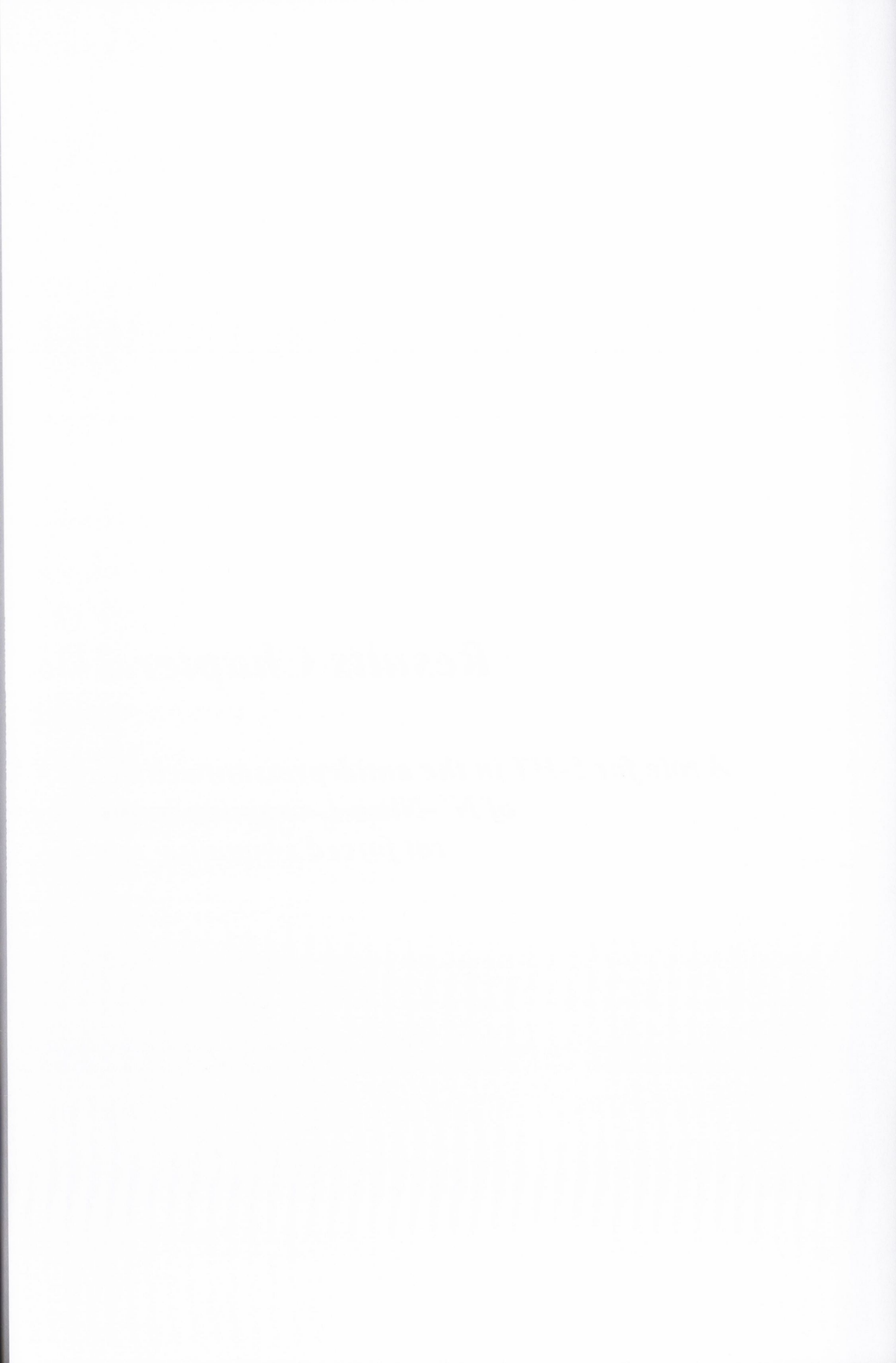
effects induced by the blockade of the NMDA-R. These molecules could be used as targets for antidepressant activity without interfering with the other signalling components modulated by NMDA-R activation. One such molecule is the neuronal isoform of the nitric oxide synthase (nNOS) enzyme which is bound to the intracellular side of the NMDA-R by the post-synaptic density protein of 95 kDa (PSD-95) and its inhibition has been shown to possess antidepressant-like properties in pre-clinical studies (Mutlu et al., 2009; Ulak et al., 2008; Harkin et al., 1999).



## ***Results Chapter 2***

***A role for 5-HT in the antidepressant activity  
of N<sup>ω</sup>-Nitro-L-arginine in the  
rat forced swimming test***





## 2.1 Introduction

In the previous chapter it was described how NMDA-R blockade can induce antidepressant-like effects in rats and how problematic this strategy can be if applied to depressed patients. Targeting a downstream effector of the NMDA-R signalling pathway, such as nitric oxide synthase (NOS), seems to be a more promising way to pursue in order to reduce the side effects and improve the safety profile of antidepressant treatments interfering with the NMDA-R.

To date a number of studies have demonstrated that inhibition of NOS produces anxiolytic and antidepressant-like behavioural effects in a variety of animal paradigms (da Silva et al. 2000; Harkin et al. 1999; 2003; 2004; Jefferys and Funder, 1996; Mutlu et al., 2009; Spiacci et al., 2008; Spolidório et al., 2007; Ulak et al., 2008; Yildiz et al. 2000a,b). However, it is presently unclear whether monoaminergic systems are involved in the antidepressant-like effects of NOS inhibitors.

The behavioural tool employed to assess the role of monoamines in antidepressant action is the modified FST. Lucki and colleagues (Detke et al. 1995; Lucki, 1997) adapted the original FST introduced by Porsolt and colleagues in 1978 to score distinct active behaviours elicited by different classes of antidepressant drugs. Specifically, selective 5-HT re-uptake inhibitors (SSRIs) and 5-HT receptor agonists provoke swimming behaviour (Cryan and Lucki, 2000; Detke et al. 1995; Page et al. 1999), whereas tricyclic antidepressants (TCAs) and selective noradrenaline and dopamine re-uptake inhibitors elicit climbing behaviour in the FST (Cryan et al. 2002b; Detke et al. 1995; Page et al. 1999; Reneric and Lucki, 1998).

A more detailed analysis of the behavioural effects of NOS inhibitors in the FST offers clues to their potential mechanism of action which may involve monoamine neurotransmitters. The behavioural profile of NOS inhibitors in the adapted FST for rats parallels that obtained with the SSRI, fluoxetine. Moreover, depletion of endogenous 5-HT blocks the antidepressant-like activity associated with NOS inhibitors in the test (Harkin et al., 2003). From these observations it was proposed that NOS inhibitors may elicit their antidepressant-like activity in the FST through a 5-HT dependent mechanism. To further explore this mechanism I examined the ability of L-NA to influence the 5-HT synthetic pathway and 5-HT metabolism in the FST. Moreover, I examined if antagonists to 5-HT receptor subtypes could block the antidepressant-like activity of L-NA in the paradigm.

## **2.2 Materials and Methods**

### **2.2.1 Subjects and drug treatment**

Male Sprague-Dawley rats weighing 250-300 g at the beginning of the studies were housed in groups of four. Food and water were available *ad libitum*.

L-NA, NSD-1015, metergoline, fluoxetine hydrochloride, GR 127935 and WAY 100635 maleate were dissolved in saline and administered intraperitoneally (i.p.) in an injection volume of 2 ml/kg. Ketanserin tartrate and RO 430440 were dissolved in 0.5% tween saline and administered i.p. in an injection volume of 1 ml/kg.

All experiments were in compliance with the European Community's Council directive, 1986 (86/609/EEC). All experiments were performed using independent groups of animals.

### **2.2.2 Study design**

#### **2.2.2.1 Influence of NSD-1015 on the antidepressant-like activity of L-NA in the FST**

In these studies the modified version of the FST was performed, as described in the Methods section. Rats were assigned to one of four groups: group 1: Vehicle, group 2: L-NA (5 mg/kg), group 3: L-NA (10 mg/kg), group 4: L-NA (20 mg/kg). Immediately prior to the last treatment of L-NA, the groups were subdivided into those receiving co-treatment with NSD-1015 or vehicle control. Animals receiving NSD-1015 were

euthanised immediately following the FST on test day and regional brain biogenic amine concentrations were determined. A parallel experiment was carried out with identical NSD-1015 treatment groups for comparison purposes, where regional brain biogenic amine concentrations were determined in animals not subjected to the FST.

Both 5-HTP and L-DOPA usually occur as transient intermediates and are not normally detected in brain tissue, but administration of NSD-1015 prevents the decarboxylation step common to both compounds and thus allows them to accumulate *in vivo* (Carlsson et al., 1972; Conley et al., 2007; Evans et al., 2009). The selection of this dose and time were determined from reports indicating that NSD-1015 (100 mg/kg, i.p.) produces optimal increases in 5-HTP and L-DOPA concentrations in rat brain 1 hour after administration without altering behaviour and well-being of the animal (Nolan et al., 2000).

#### ***2.2.2.2 The effect of combining sub-active doses of L-NA and fluoxetine in the FST***

Rats were assigned to one of four groups: group 1: Vehicle + Control, group 2: L-NA (1 mg/kg) + Control; group 3: Fluoxetine (2.5 mg/kg) + Control; group 4: L-NA (1 mg/kg) + Fluoxetine (2.5 mg/kg). The selection of doses was determined from previously reported dose response studies (Harkin et al., 2003a).

### ***2.2.2.3 Dose related effects of metergoline on the antidepressant-like activity of L-NA in the FST***

Metergoline is a non selective 5-HT receptor antagonist and was used at doses effective in blocking the *in vivo* effects induced by 5-HT receptor agonists in rats (Golozoubova et al., 2006; Mokler et al., 1983; Stachowicz et al., 2007). Rats were assigned to one of eight groups: group 1: Vehicle + Control, groups 2-4: Metergoline (1, 2 and 4 mg/kg) + Control, group 5: L-NA (20 mg/kg) + Control, groups 6-8: L-NA (20 mg/kg) + Metergoline (1, 2 and 4 mg/kg).

### ***2.2.2.4 The effect of ketanserin, RO-430440, WAY-100635 or GR-127935 on the antidepressant-like activity of L-NA in the FST***

In a separate series of experiments, the involvement of the 5-HT receptor subtypes in the antidepressant-like activity of L-NA in the FST was examined. Rats were co-treated with L-NA and ketanserin, a preferential 5-HT<sub>2A</sub> receptor antagonist (Van Oekelen et al., 2003), RO-430440, a selective 5-HT<sub>2C</sub> receptor antagonist [RO-430440 has been shown to have a selectivity for 5-HT<sub>2C</sub> (pKi=7.1) over 5-HT<sub>2A</sub> (pKi=5.3) receptors (International application published under the Patent Cooperation Treaty, Benzofuryl derivatives and their use WO 97/42183; See also Cryan et al., 2000)], WAY-100635, a 5-HT<sub>1A</sub> receptor antagonist (Fletcher et al., 1996) or GR-127935 (a 5-HT<sub>1B/D</sub> receptor antagonist) (Skingle et al., 1996). In each experiment rats were assigned to one of 4 groups: group 1: Vehicle + Control, group 2: Ketanserin (5 mg/kg) or RO-430440 (5 mg/kg) or WAY-100635 (0.3 mg/kg) or GR-127935 (4 mg/kg) + Control, group 3: L-

NA (20 mg/kg) + Control, group 4: L-NA + each antagonist outlined in group 2. A single dose of each antagonist, obtained from the literature, was used. These doses have previously been shown to block 5-HT receptor mediated behaviours in a range of test paradigms in rats (Cryan et al., 2000; Hawkins et al., 2008; Martinez-Mota et al., 2002).

### ***2.2.3 Locomotor activity***

Animals were removed from their home cage and placed individually into activity monitor cages as described in the Methods section. On the day of testing animals were placed in one of five test chambers and their activity was monitored over a 20 minute period. The effects of metergoline, ketanserin, RO-430440 alone and in combination with L-NA were examined in groups of animals independent of those exposed to the FST. The doses of drugs and the treatment regime used were as described for the FST.

### ***2.2.4 Determination of regional biogenic amine concentrations***

Immediately following the FST, animals treated with NSD 1015 were euthanised by decapitation and the frontal cortex, striatum, hippocampus and amygdala were dissected on an ice cold plate for biogenic amine measurements by HPLC as previously described. 5-HT, 5-HIAA, L-DOPA, DA and NA concentrations were measured by HPLC coupled with electrochemical detection as previously described. Final results were expressed as ng neurotransmitter/g wet weight of tissue.

### **2.2.5 Statistical analysis**

Data are expressed as group mean with standard errors and were analysed using a one factor or two factor analysis of variance (ANOVA). If any statistically significant change was found, *post hoc* comparisons were performed using a Dunnett's or Student Newman Keuls test respectively. Data were deemed significant when  $p < 0.05$ .





## 2.3 Results

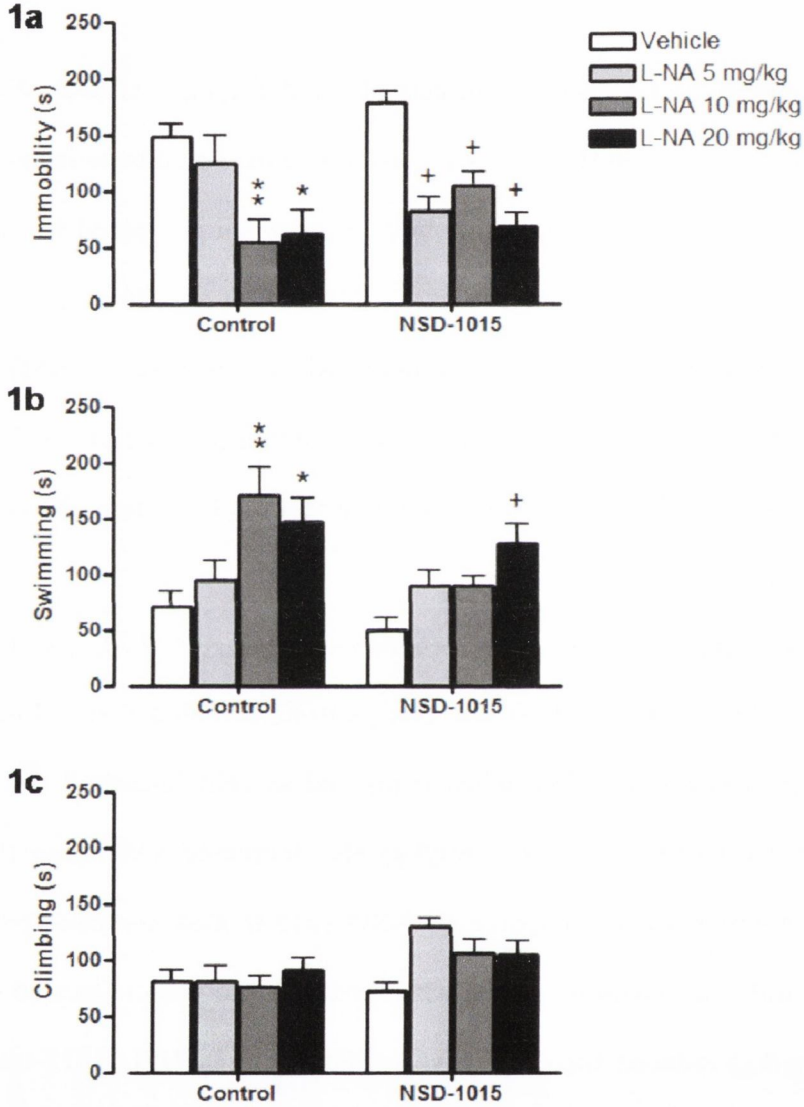
### 2.3.1 Influence of NSD-1015 on the antidepressant-like activity of L-NA in the FST

ANOVA of immobility time showed effects of L-NA [ $F_{(3,63)} = 14.84$ ,  $p < 0.01$ ] and a L-NA x NSD-1015 interaction [ $F_{(3,63)} = 3.17$ ,  $p < 0.05$ ]. *Post hoc* comparisons revealed that L-NA (10 and 20 mg/kg) reduced immobility time when compared to vehicle treated controls ( $p < 0.01$  and  $p < 0.05$  respectively). L-NA (5, 10 and 20 mg/kg) also reduced immobility time in NSD-1015-treated animals when compared to NSD-1015-treated controls ( $p < 0.05$ ). NSD-1015 did not influence immobility time or the L-NA-induced reduction in immobility time when compared to their non NSD-1015-treated counterparts [Fig. 2.1a].

ANOVA of swimming time showed effects of L-NA [ $F_{(3,63)} = 9.73$ ,  $p < 0.01$ ] and NSD-1015 [ $F_{(1,63)} = 7.91$ ,  $p < 0.01$ ]. *Post hoc* comparisons revealed that L-NA (10 and 20 mg/kg) increased swimming time when compared to vehicle-treated controls ( $p < 0.01$  and  $p < 0.05$  respectively). L-NA (20 mg/kg) also increased swimming time in NSD-1015-treated animals when compared to NSD-1015-treated controls ( $p < 0.01$ ). NSD-1015 did not influence swimming time when compared to vehicle-treated controls. The L-NA (10 mg/kg)-induced increase in swimming time in NSD-1015-treated animals was reduced when compared to their non NSD-1015-treated counterparts ( $p < 0.01$ ), although co-treatment with NSD-1015 did not influence the response to L-NA (5 and 20 mg/kg) [Fig. 2.1b].

ANOVA of climbing time showed effects of NSD-1015 [ $F_{(1,63)} = 7.49$ ,  $p < 0.01$ ] only. There were no effects of L-NA on the time spent climbing. *Post hoc* comparisons

revealed that NSD-1015 did not influence climbing time when compared to vehicle-treated controls [Fig. 2.1c].



**Fig. 2.1** Effects of NSD-1015 pre-treatment on acute L-NA effect in the FST. Rats were exposed to a 15 min adaptation session of the FST and administered L-NA (5, 10 and 20 mg/kg, i.p.) 24 and 5 hr, and were co-treated with NSD-1015 (100 mg/kg, i.p.) 1 hr, prior to test. Immobility (Fig. 2.1a), swimming (Fig. 2.1b) and climbing (Fig. 2.1c) times are expressed as mean and SEM of 7-10 animals. \* $p < 0.05$ , \*\* $p < 0.01$  compared to vehicle controls; + $p < 0.05$  compared to vehicle + NSD-1015 group.

### ***2.3.2 L-NA increases regional brain 5-HTP accumulation and 5-HT metabolism following FST exposure***

ANOVA of 5-HTP, 5-HT, 5-HIAA concentrations and the 5-HIAA:5-HT metabolism ratio failed to show effects of L-NA in the frontal cortex or striatum. A trend towards an increase in cortical 5-HTP concentrations following L-NA (20 mg/kg) administration was observed when compared to vehicle-treated controls. However this failed to achieve significance [ $F_{(3,33)} = 2.55$ ,  $p = 0.07$ ].

ANOVA of 5-HTP and 5-HT concentrations in the hippocampus failed to show effects of L-NA. Significant effects were observed for 5-HIAA [ $F_{(3,36)} = 4.5$ ,  $p < 0.01$ ] and 5-HIAA:5-HT [ $F_{(3,36)} = 9.03$ ,  $p < 0.01$ ]. *Post hoc* comparisons revealed that 5-HIAA concentrations and 5-HIAA:5-HT ratios were increased in the L-NA (5, 10 and 20 mg/kg)-treated groups when compared to vehicle-treated controls (Table 1).

ANOVA of 5-HTP concentrations in the amygdaloid cortex showed effects of L-NA [ $F_{(3,34)} = 8.63$ ,  $p < 0.01$ ]. *Post hoc* comparisons revealed a dose-related increase in 5-HTP concentrations following L-NA (10 and 20 mg/kg) when compared to vehicle-treated controls. Significant effects for 5-HIAA [ $F_{(3,34)} = 3.59$ ,  $p < 0.05$ ] and 5-HIAA:5-HT [ $F_{(3,34)} = 6.39$ ,  $p < 0.01$ ] were also observed in the amygdaloid cortex. *Post hoc* comparisons revealed that 5-HIAA concentrations and 5-HIAA:5-HT ratios were increased in the L-NA (5, 10 and 20 mg/kg)-treated groups when compared to vehicle-treated controls (Table 1). There were no effects of L-NA observed for L-DOPA, dopamine, its metabolites HVA and DOPAC or dopamine metabolism ratios in any of the brain regions tested with one exception.

ANOVA of L-DOPA concentrations in the amygdaloid cortex showed effects of L-NA [ $F_{(3,34)} = 5.6$ ,  $p < 0.05$ ]. *Post hoc* comparisons revealed a dose-related increase ( $p < 0.05$ ,

Dunnett's test) in L-DOPA concentrations following L-NA (10 and 20 mg/kg;  $373 \pm 18$  ng/g and  $408 \pm 22$  ng/g) when compared to vehicle-treated controls ( $301 \pm 10$  ng/g).

**Table 2.1** Effects of L-NA on regional changes to 5-HTP, 5-HIAA, 5-HT and the 5-HIAA:5-HT ratio following FST exposure in animals pre-treated with NSD-1015

		Vehicle	L-NA 5 mg/kg	L-NA 10 mg/kg	L-NA 20 mg/kg
Frontal cortex	5-HTP (ng/g)	$179 \pm 10$	$221 \pm 21$	$216 \pm 11$	$223 \pm 6$
	5-HIAA (ng/g)	$108 \pm 6$	$130 \pm 6$	$118 \pm 8$	$118 \pm 5$
	5-HT (ng/g)	$532 \pm 28$	$512 \pm 18$	$530 \pm 20$	$506 \pm 22$
	5-HIAA:5-HT (a.u.)	$0.207 \pm 0.017$	$0.259 \pm 0.020$	$0.223 \pm 0.011$	$0.235 \pm 0.009$
Striatum	5-HTP (ng/g)	$364 \pm 37$	$431 \pm 40$	$398 \pm 28$	$404 \pm 49$
	5-HIAA (ng/g)	$439 \pm 33$	$496 \pm 49$	$458 \pm 36$	$442 \pm 45$
	5-HT (ng/g)	$753 \pm 42$	$751 \pm 76$	$707 \pm 53$	$685 \pm 71$
	5-HIAA:5-HT (a.u.)	$0.584 \pm 0.034$	$0.671 \pm 0.039$	$0.653 \pm 0.035$	$0.656 \pm 0.037$
Hippocampus	5-HTP (ng/g)	$246 \pm 12$	$286 \pm 18$	$275 \pm 10$	$280 \pm 15$
	5-HIAA (ng/g)	$183 \pm 16$	$254 \pm 16^{**}$	$227 \pm 9^*$	$233 \pm 14^*$
	5-HT (ng/g)	$553 \pm 39$	$572 \pm 17$	$522 \pm 19$	$526 \pm 29$
	5-HIAA:5-HT (a.u.)	$0.330 \pm 0.014$	$0.443 \pm 0.022^*$	$0.437 \pm 0.013^*$	$0.446 \pm 0.023^*$
Amygdala	5-HTP (ng/g)	$344 \pm 13$	$403 \pm 27$	$411 \pm 10^*$	$465 \pm 16^*$
	5-HIAA (ng/g)	$172 \pm 11$	$223 \pm 14^*$	$200 \pm 8^*$	$212 \pm 12^*$
	5-HT (ng/g)	$748 \pm 38$	$773 \pm 25$	$738 \pm 23$	$752 \pm 31$
	5-HIAA:5-HT (a.u.)	$0.229 \pm 0.007$	$0.288 \pm 0.015^*$	$0.271 \pm 0.008^*$	$0.282 \pm 0.011^*$

**Table 2.1** Dose related effects of L-NA on regional changes to 5-HTP, 5-HT and 5-HIAA concentrations in NSD-1015 treated animals. Rats were exposed to a 15 min adaptation session of the FST and administered L-NA (5, 10 and 20 mg/kg, i.p.) 24 and 5 hr, and were co-treated with NSD-1015 (100 mg/kg, i.p.) 1 hr prior to test. Brain regions were dissected immediately after the FST. Data are expressed as mean and SEM of 8-10 animals. \* $p < 0.05$ , \*\* $p < 0.01$  compared to vehicle controls. 5-HTP = 5-hydroxytryptophan, 5-HIAA = 5-hydroxyindoleacetic acid, 5-HT = 5-hydroxytryptamine, a.u. = arbitrary units.

### ***2.3.3 L-NA increases 5-HT metabolism in the striatum following NSD-1015 administration***

ANOVA of 5-HTP, 5-HT, 5-HIAA concentrations and the 5-HIAA:5-HT metabolism ratio failed to show effects of L-NA in the frontal or amygdaloid cortex. ANOVA of 5-HTP, 5-HT and 5-HIAA concentrations in the striatum and hippocampus also did not show drug effects. Significant effects were observed for the 5-HIAA:5-HT ratio in the striatum [ $F_{(3,25)} = 3.01, p < 0.05$ ] and there was a non significant trend in the hippocampus [ $F_{(3,26)} = 2.68, p = 0.07$ ]. *Post hoc* comparisons revealed that 5-HT metabolism was increased in the striatum following L-NA (20 mg/kg) administration when compared to vehicle-treated controls (Table 2). There were no effects of L-NA observed for L-DOPA, dopamine, its metabolites HVA and DOPAC or dopamine metabolism ratios in any of the brain regions tested.

**Table 2.2** Effects of L-NA on regional changes to 5-HTP, 5-HIAA, 5-HT and the 5-HIAA:5-HT ratio in non-swimmied animals pre-treated with NSD-1015

		Vehicle	L-NA 5 mg/kg	L-NA 10 mg/kg	L-NA 20 mg/kg
Frontal cortex	5-HTP (ng/g)	158 ± 32	233 ± 51	160 ± 21	184 ± 22
	5-HIAA (ng/g)	109 ± 16	160 ± 24	138 ± 18	157 ± 17
	5-HT (ng/g)	463 ± 74	553 ± 90	446 ± 48	502 ± 41
	5-HIAA:5-HT (a.u.)	0.245 ± 0.021	0.296 ± 0.015	0.322 ± 0.045	0.318 ± 0.029
Striatum	5-HTP (ng/g)	284 ± 59	341 ± 30	235 ± 37	241 ± 27
	5-HIAA (ng/g)	434 ± 84	438 ± 28	340 ± 36	462 ± 27
	5-HT (ng/g)	837 ± 145	799 ± 33	620 ± 70	674 ± 37
	5-HIAA:5-HT (a.u.)	0.495 ± 0.033	0.556 ± 0.046	0.562 ± 0.052	0.698 ± 0.055*
Hippocampus	5-HTP (ng/g)	246 ± 38	282 ± 45	255 ± 35	276 ± 36
	5-HIAA (ng/g)	202 ± 35	258 ± 38	236 ± 33	298 ± 32
	5-HT (ng/g)	452 ± 65	529 ± 66	463 ± 63	519 ± 50
	5-HIAA:5-HT (a.u.)	0.431 ± 0.024	0.482 ± 0.022	0.528 ± 0.047	0.572 ± 0.042
Amygdala	5-HTP (ng/g)	402 ± 54	415 ± 48	297 ± 17	398 ± 32
	5-HIAA (ng/g)	214 ± 23	281 ± 44	237 ± 39	308 ± 24
	5-HT (ng/g)	793 ± 90	862 ± 80	657 ± 65	875 ± 63
	5-HIAA:5-HT (a.u.)	0.276 ± 0.017	0.326 ± 0.042	0.361 ± 0.038	0.359 ± 0.032

**Table 2.2** Dose related effects of L-NA on regional changes to 5-HTP, 5-HT and 5-HIAA concentrations in NSD-1015 treated animals. Rats were administered L-NA (5, 10 and 20 mg/kg, i.p.) 24 and 5 hr, and were co-treated with NSD-1015 (100 mg/kg, i.p.) 1 hr prior to animal sacrifice. Brain regions were dissected immediately. Data are expressed as mean and SEM of 7-8 animals.  $p < 0.05$  compared to vehicle controls. 5-HTP = 5-hydroxytryptophan, 5-HIAA = 5-hydroxyindoleacetic acid, 5-HT = 5-hydroxytryptamine, a.u. = arbitrary units.

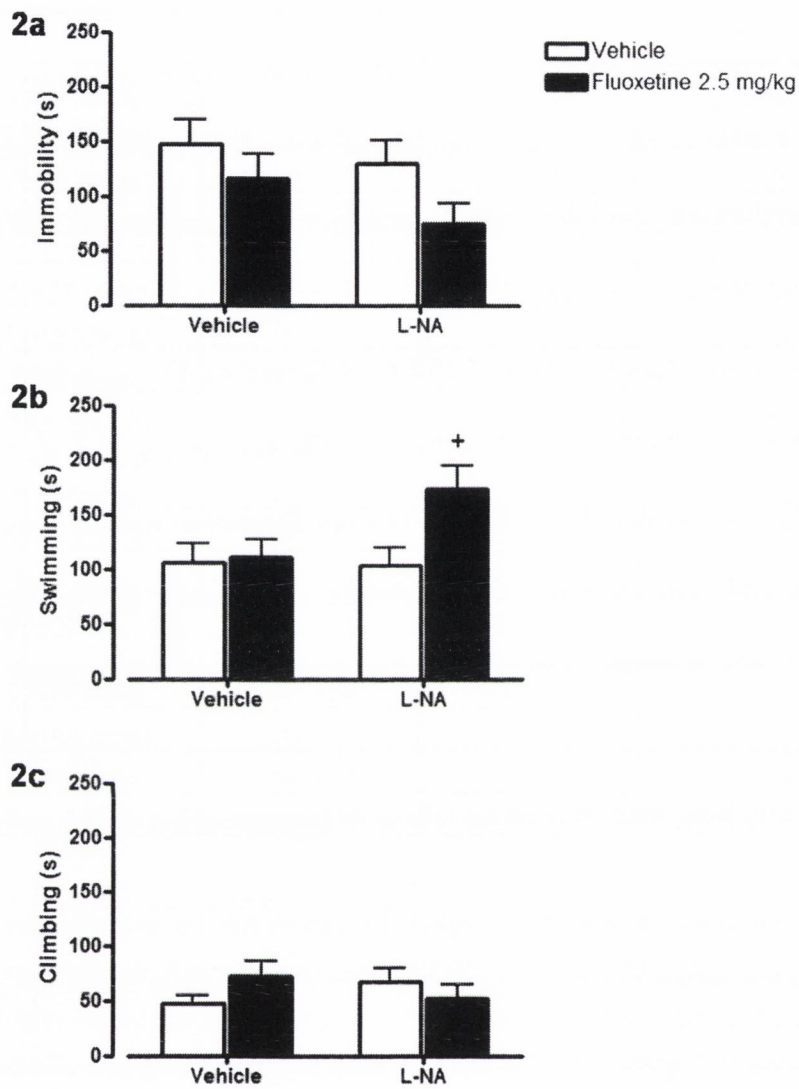
#### **2.3.4 L-NA augments the activity of fluoxetine in the FST**

ANOVA of immobility time showed effects of fluoxetine only [ $F_{(1,36)} = 4.30, p < 0.05$ ]. *Post hoc* comparisons revealed no significant differences between the groups. Fluoxetine induced a small decrease in immobility time and a trend was observed for L-NA to potentiate this effect [Fig. 2.2a].

ANOVA of swimming time showed effects of fluoxetine [ $F_{(1,36)} = 4.50, p < 0.05$ ] and a L-NA x fluoxetine interaction approaching significance [ $F_{(1,36)} = 3.36, p = 0.075$ ]. *Post hoc* comparisons revealed that neither L-NA nor fluoxetine had an effect on swimming time. L-NA and fluoxetine together provoked an increase in swimming time when compared to vehicle-treated controls and L-NA or fluoxetine treatments alone ( $p < 0.05$ ) [Fig. 2.2b].

ANOVA of climbing time showed no effects of treatment [Fig. 2.2c].





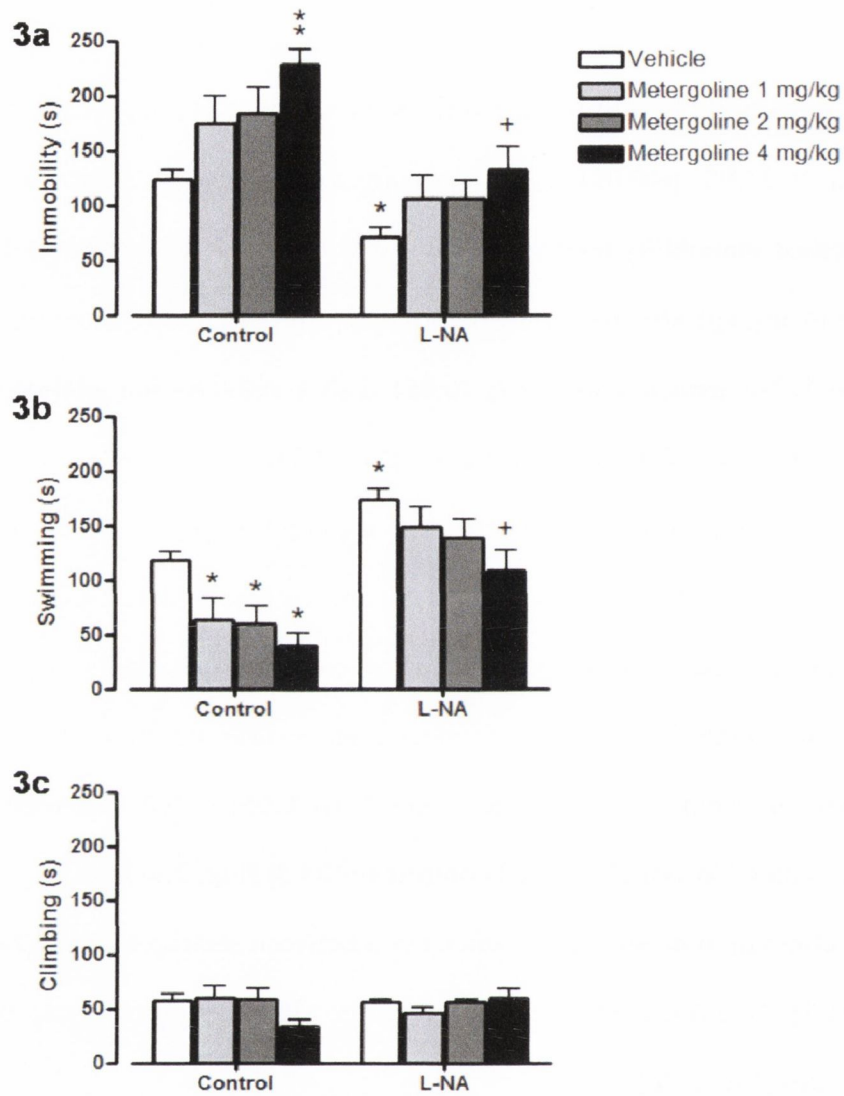
**Fig. 2.2** Effects of the combination of sub-active doses of L-NA and fluoxetine in the FST. Rats were exposed to a 15 min adaptation session of the FST and received L-NA (1 mg/kg, i.p.) and fluoxetine (2.5mg/kg, i.p.) alone and in combination 24, 5 and 1 hr prior to test. Immobility (Fig. 2.2a), swimming (Fig. 2.2b) and climbing (Fig. 2.2c) times are expressed as mean and SEM of 10 animals. +p<0.05 compared to vehicle controls and L-NA and fluoxetine alone-treated groups.

### 2.3.5 Effects of metergoline on the antidepressant-like activity of L-NA in the FST

ANOVA of immobility time showed effects of metergoline [ $F_{(3,70)} = 6.63$ ,  $p < 0.01$ ] and L-NA [ $F_{(1,70)} = 31.95$ ,  $p < 0.01$ ]. *Post hoc* comparisons revealed that metergoline (4 mg/kg) increased immobility time when compared to vehicle-treated controls ( $p < 0.01$ ). Metergoline (4 mg/kg) also increased immobility time in L-NA-treated animals when compared to L-NA-treated controls ( $p < 0.05$ ). L-NA reduced immobility time when compared to vehicle-treated controls ( $p < 0.05$ ) [Fig. 2.3a].

ANOVA of swimming time showed effects of metergoline [ $F_{(3,70)} = 7.33$ ,  $p < 0.01$ ] and L-NA [ $F_{(1,70)} = 43.27$ ,  $p < 0.01$ ]. *Post hoc* comparisons revealed that metergoline (1, 2 and 4 mg/kg) decreased swimming time when compared to vehicle-treated controls ( $p < 0.05$ ). Metergoline (4 mg/kg) also decreased swimming time in L-NA-treated animals when compared to L-NA-treated controls ( $p < 0.05$ ). L-NA increased swimming time when compared to vehicle-treated controls ( $p < 0.05$ ) [Fig. 2.3b].

ANOVA of climbing time showed an interaction between metergoline and L-NA [ $F_{(3,70)} = 2.79$ ,  $p < 0.05$ ]. *Post hoc* comparisons revealed no significant differences between the treatment groups [Fig. 2.3c].



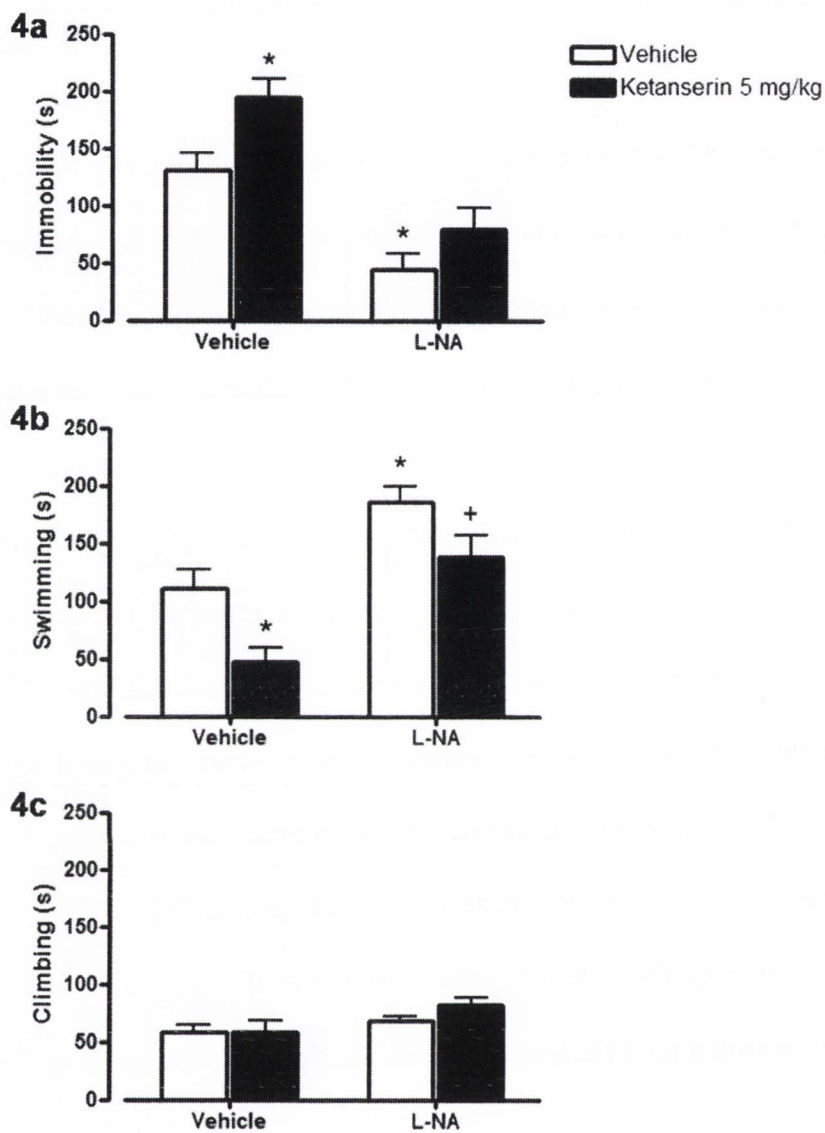
**Fig. 2.3** Effects of co-treatment with metergoline on the effects of L-NA in the FST. Rats were exposed to a 15 min adaptation session of the FST and received L-NA (20 mg/kg, i.p.) and metergoline (1, 2 and 4 mg/kg, i.p.) alone and in combination 24, 5 and 1 hr prior to test. Immobility (Fig. 2.3a), swimming (Fig. 2.3b) and climbing (Fig. 2.3c) times are expressed as mean and SEM of 9-10 animals. \* $p < 0.05$ , \*\* $p < 0.01$  compared to vehicle controls; + $p < 0.05$  compared to vehicle-L-NA group.

### ***2.3.6 Treatment with ketanserin and RO-430440 provoke a similar response to metergoline in the FST attenuated by co-treatment with L-NA***

ANOVA of immobility time showed effects of ketanserin [ $F_{(1,36)} = 9.77$ ,  $p < 0.01$ ] and L-NA [ $F_{(1,36)} = 40.21$ ,  $p < 0.01$ ]. *Post hoc* comparisons revealed that ketanserin increased immobility time when compared to vehicle-treated controls ( $p < 0.01$ ). There was a reduction in immobility time following L-NA treatment when compared to vehicle-treated controls ( $p < 0.01$ ) [Fig. 2.4a].

ANOVA of swimming time showed effects of ketanserin [ $F_{(1,36)} = 13.46$ ,  $p < 0.01$ ] and L-NA [ $F_{(1,36)} = 30.31$ ,  $p < 0.01$ ]. *Post hoc* comparisons revealed that ketanserin decreased swimming time when compared to vehicle-treated controls ( $p < 0.01$ ). Ketanserin reduced swimming time in L-NA-treated animals when compared to L-NA-treated controls ( $p < 0.05$ ). There was an increase in swimming time following L-NA treatment when compared to vehicle-treated controls ( $p < 0.01$ ) [Fig. 2.4b].

ANOVA of climbing time showed effects of L-NA [ $F_{(1,36)} = 6.23$ ,  $p < 0.01$ ]. *Post hoc* comparisons revealed no differences between the treatment groups [Fig. 2.4c].

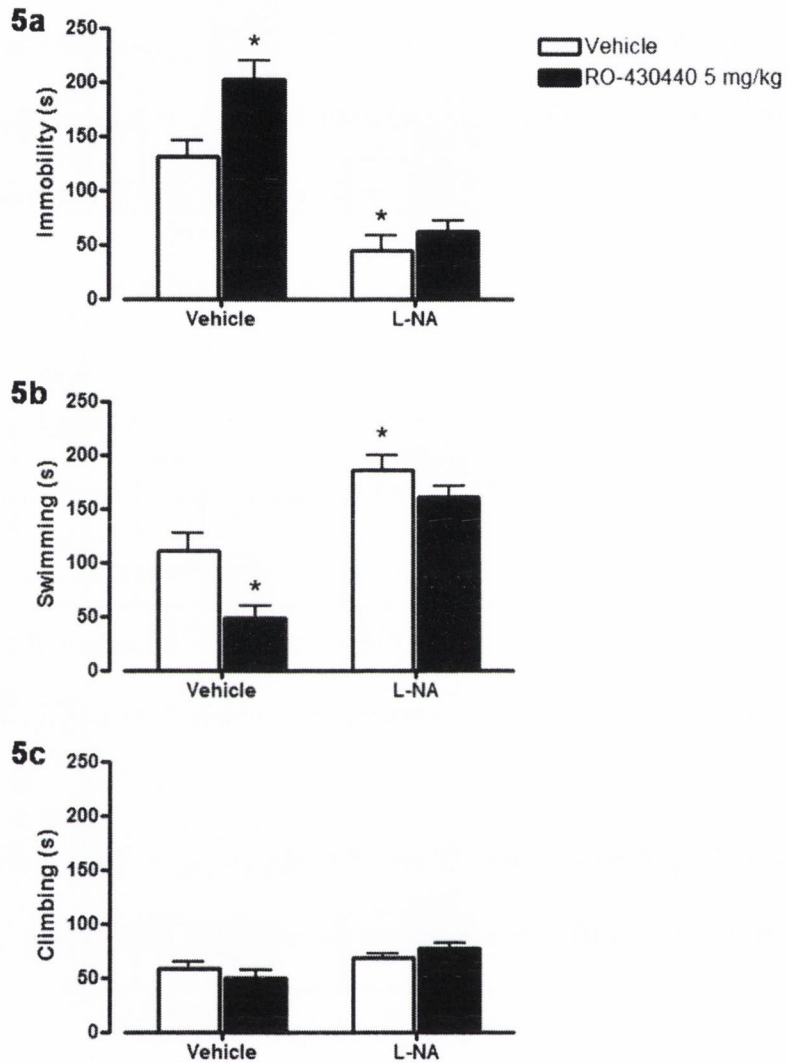


**Fig. 2.4** Effects of co-treatment with ketanserin on the effects of L-NA in the FST. Rats were exposed to a 15 min adaptation session of the FST and received L-NA (20 mg/kg, i.p.) and ketanserin (5 mg/kg, i.p.) alone and in combination 24, 5 and 1 hr prior to test. Immobility (Fig. 2.4a), swimming (Fig. 2.4b) and climbing (Fig. 2.4c) times are expressed as mean and SEM of 10 animals. \* $p < 0.05$  compared to vehicle controls; + $p < 0.05$  compared to vehicle-L-NA group.

ANOVA of immobility time showed effects of RO-430440 [ $F_{(1,36)} = 9.49$ ,  $p < 0.01$ ] and L-NA [ $F_{(1,36)} = 61.95$ ,  $p < 0.01$ ]. *Post hoc* comparisons revealed that RO-430440 increased immobility time when compared to vehicle-treated controls ( $p < 0.01$ ). There was a reduction in immobility time following L-NA treatment when compared to vehicle-treated controls ( $p < 0.01$ ). RO-430440 failed to influence the L-NA-induced reduction in immobility time when compared to vehicle-L-NA-treated controls [Fig. 2.5a].

ANOVA of swimming time showed effects of RO-430440 [ $F_{(1,36)} = 11.62$ ,  $p < 0.01$ ] and L-NA [ $F_{(1,36)} = 53.14$ ,  $p < 0.01$ ]. *Post hoc* comparisons revealed that RO-430440 decreased swimming time when compared to vehicle-treated controls ( $p < 0.01$ ). There was an increase in swimming time following L-NA treatment when compared to vehicle-treated controls ( $p < 0.01$ ). RO-430440 failed to influence the L-NA-induced increase in swimming time when compared to vehicle-L-NA-treated controls [Fig. 2.5b].

ANOVA of climbing time showed effects of L-NA [ $F_{(1,36)} = 10.70$ ,  $p < 0.01$ ]. *Post hoc* comparisons revealed no differences between the treatment groups [Fig. 2.5c].



**Fig. 2.5** Effects of co-treatment with RO-430440 on the effects of L-NA in the FST. Rats were exposed to a 15 min adaptation session of the FST and received L-NA (20 mg/kg, i.p.) and RO-430440 (5 mg/kg, i.p.) alone and in combination 24, 5 and 1 hr prior to test. Immobility (Fig. 2.5a), swimming (Fig. 2.5b) and climbing (Fig. 2.5c) times are expressed as mean and SEM of 10 animals. \* $p < 0.05$  compared to vehicle controls; + $p < 0.05$  compared to vehicle-RO-430440 group.

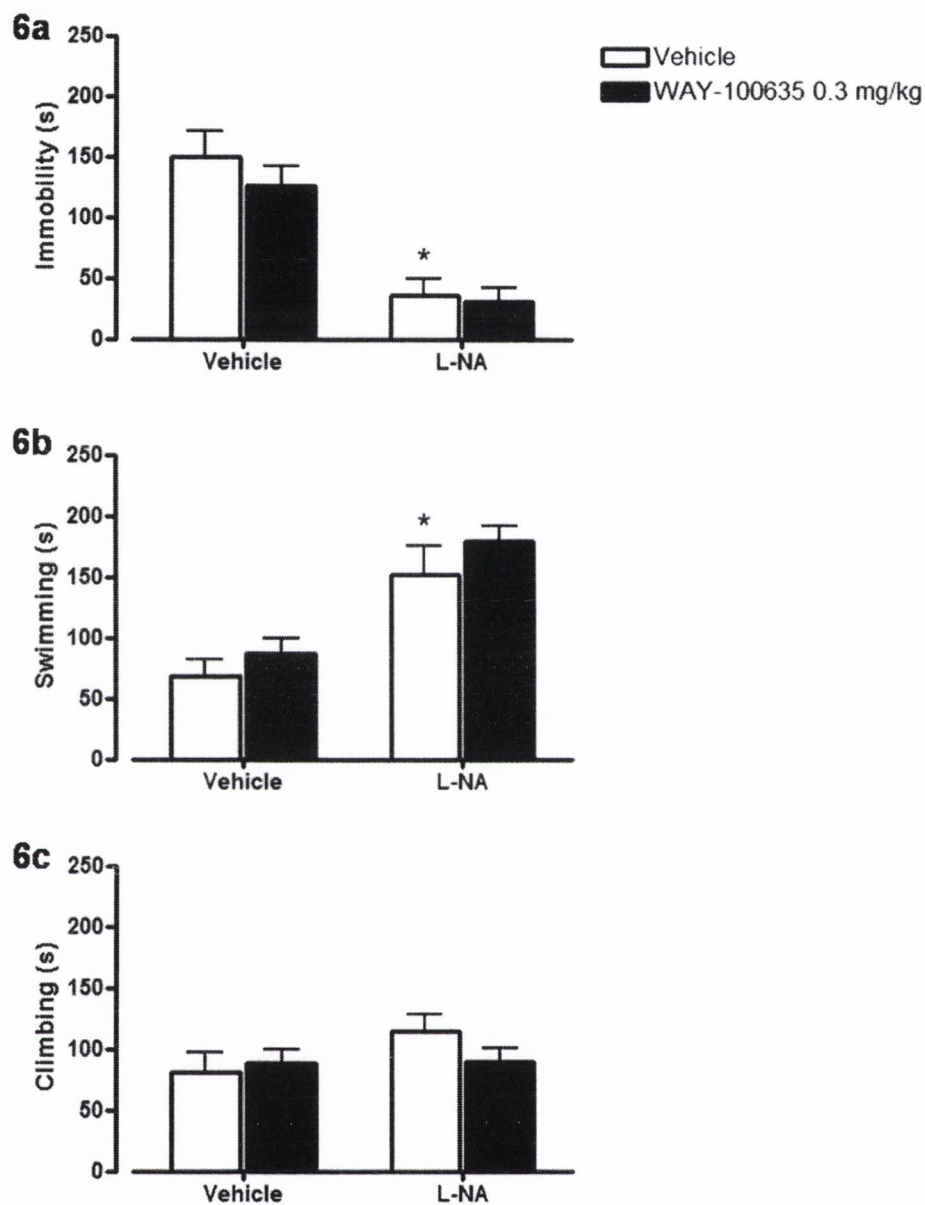
### ***2.3.7 Co-treatment with WAY-100635 or GR-127935 do not influence the antidepressant-like activity of L-NA in the FST***

ANOVA of immobility time showed effects of L-NA [ $F_{(1,36)} = 43.45$ ,  $p < 0.01$ ]. *Post hoc* comparisons revealed that L-NA reduced immobility time when compared to vehicle-treated controls ( $p < 0.01$ ). WAY-100635 failed to influence immobility time or the L-NA-induced reduction in immobility time when compared to L-NA-treated counterparts [Fig. 2.6a].

ANOVA of swimming time showed effects of L-NA [ $F_{(1,36)} = 30.99$ ,  $p < 0.01$ ]. *Post hoc* comparisons revealed that L-NA increased swimming time when compared to vehicle-treated controls ( $p < 0.01$ ). WAY-100635 failed to influence swimming time or the L-NA-induced increase in swimming time [Fig. 2.6b].

ANOVA of climbing time showed no effects of treatments [Fig. 2.6c].





**Fig. 2.6** Effects of co-treatment with WAY-100635 on the effects of L-NA in the FST. Rats were exposed to a 15 min adaptation session of the FST and received L-NA (20 mg/kg, i.p.) and WAY-100635 (0.3 mg/kg, i.p.) alone and in combination 24, 5 and 1 hr prior to test. Immobility (Fig. 2.6a), swimming (Fig. 2.6b) and climbing (Fig. 2.6c) times are expressed as mean and SEM of 10 animals. \* $p < 0.05$  compared to vehicle controls.

For GR-127935, ANOVA of immobility time showed effects of L-NA [ $F_{(1,34)} = 41.14$ ,  $p < 0.01$ ]. *Post hoc* comparisons revealed that L-NA reduced immobility time when compared to vehicle-treated controls ( $p < 0.01$ ). GR-127935 failed to influence immobility time or the L-NA-induced reduction in immobility time when compared to L-NA-treated counterparts. Mean immobility times for each treatment group were as follows: Vehicle + Control:  $167 \pm 18$ ; GR-127935 + Control:  $169 \pm 17$ ; Vehicle + L-NA:  $47 \pm 17^*$ ; GR-127935 + L-NA:  $60 \pm 19^*$  (\* $p < 0.05$  compared to vehicle control group).

ANOVA of swimming time showed effects of L-NA [ $F_{(1,34)} = 58.56$ ,  $p < 0.01$ ]. *Post hoc* comparisons revealed that L-NA increased swimming time when compared to vehicle-treated controls ( $p < 0.01$ ). GR-12793 failed to influence swimming time or the L-NA-induced increase in swimming time. Mean swimming times for each treatment group were as follows: Vehicle + Control:  $65 \pm 13$ ; GR-127935 + Control:  $51 \pm 8$ ; Vehicle + L-NA:  $181 \pm 14^*$ ; GR 127935 + L-NA:  $152 \pm 20$  (\* $p < 0.05$  compared to vehicle control group).

ANOVA of climbing time showed no effects of treatments.

### ***2.3.8 The effects of metergoline, ketanserin, RO-430440 and L-NA alone or in combination on locomotor activity***

Placement of the animals in the activity chambers resulted initially in exploratory activity followed by a gradual acclimatisation to the novel surroundings over the 20 min test period. ANOVA of activity counts following ketanserin or L-NA administration alone or in combination showed effects of ketanserin [ $F_{(1,16)} = 4.47$ ,  $p = 0.05$ ], L-NA

[ $F_{(1,16)} = 14.11, p < 0.01$ ] and a ketanserin x L-NA interaction [ $F_{(1,16)} = 5.43, p < 0.05$ ]. *Post hoc* comparisons revealed that ketanserin ( $2285 \pm 311$  cnts) and L-NA ( $1790 \pm 252$  cnts) reduced activity when compared to vehicle-treated controls ( $3622 \pm 381$  cnts) ( $p < 0.05$ ). Ketanserin failed to influence the L-NA related reduction in activity ( $1855 \pm 240$  cnts) when compared to L-NA treatment alone. There were 5 animals per treatment group. The numbers in parentheses represent mean activity counts over the 20 minute observation period with standard error of the mean. Metergoline or RO-430440 did not affect activity over the trial period when compared to vehicle-treated controls. Neither metergoline nor RO-430440 influenced the response to L-NA (data not shown).

## 2.4 Discussion

L-NA produced a reduction in immobility together with an increase in active swimming behaviour without any significant change in climbing consistent with previous reports of its antidepressant-like activity in the FST (Harkin et al., 2003). This behavioural profile is similar to that elicited by SSRIs or serotonergic agonists (Cryan and Lucki, 2000; Detke et al., 1995, Page et al., 1999). In the present investigation, co-treatment with NSD-1015 did not influence the antidepressant-like activity of L-NA in the FST. With or without NSD-1015, L-NA produced a dose-dependent reduction in immobility that occurs as a result of a selective increase in swimming behaviour. NSD-1015 inhibits the 5-HT biosynthetic pathway, although the acute treatment regime of NSD-1015 employed was not sufficient to provoke a depletion of endogenous 5-HT. In this regard, it has been previously shown that depletion of 5-HT, via treatment with the tryptophan hydroxylase inhibitor *p*-chlorophenylalanine (*p*CPA), attenuates the antidepressant-like properties of NOS inhibitors in the FST (Harkin et al., 2003).

### 2.4.1 *L-NA increases 5-HT synthesis and metabolism*

By determination of the accumulation of 5-HTP following NSD-1015 administration, the results of the present study indicate that tryptophan hydroxylase is activated in response to L-NA administration. L-NA provoked an increase in 5-HTP concentrations in the amygdaloid cortex suggesting that NOS inhibitors may act in a region specific manner. Moreover, the effects obtained were dose dependent and evident in FST exposed animals only. Inactivation of brain tryptophan hydroxylase by NO via

nitrosylation has been previously described (Kuhn and Arthur, 1996; 1997) and such a mechanism may account for the increase in tryptophan hydroxylase activity observed in the current study. Raised glutamate and nitrergic neuronal transmission in response to exposure to a stressful stimulus such as the FST (Joca et al., 2007; Kirby et al., 2007; Shirakawa et al., 2004) may increase NO-dependent nitrosylation of TPH leading to its inactivation. L-NA, in turn, may reverse this process by limiting the availability of NO, reducing the inactivation of tryptophan hydroxylase. Further experiments however are required to verify this mechanism linking stress-induced NO to the regulation of tryptophan hydroxylase activity.

L-NA also provoked a regional increase in 5-HIAA concentrations and 5-HT metabolism indicative of a possible enhancement of 5-HT release, re-uptake and/or metabolism to 5-HIAA. These effects were also largely dependent on exposure to the FST. Consistent with this are reports that NOS inhibitors increase extracellular levels of 5-HT in the rat brain after local or systemic administration (Kiss, 2000; Smith and Whitton, 2000; Segieth et al., 2001; Wegener et al., 2000). Inhibition by NO of 5-HT uptake into rat brain synaptosomes (Asano et al., 1997) and the human 5-HT transporter (Bryan-Lluka et al., 2004) has been previously described. More recently accounts of a physical interaction between the 5-HT transporter and neuronal NOS that underlies a reciprocal modulation of their activity provides further evidence for the regulation of 5-HT by NO (Chanrion et al., 2007; Garthwaite, 2007). NO may also inhibit monoamine oxidase activity (Muriel and Pérez-Rojaset, 2003) although the mechanism by which NO inhibits monoamine oxidase remains unknown at present. There are therefore a number of mechanisms by which NOS inhibitors may influence 5-HT metabolism. Overall, as 5-HT function is implicated in stress related disorders such as depression, it

is tempting to speculate that chemically reactive species like NO influence conditions where 5-HT deficits have been identified. The current study provides evidence in support of enhanced 5-HT synthesis and increased 5-HT metabolism indicative of overall 5-HT activation which may account for the antidepressant-like properties of NOS inhibitors.

#### ***2.4.2 L-NA shows characteristics typical of SSRIs***

NOS inhibitors have been previously reported to augment the behavioural effects of SSRIs and the tricyclic antidepressant imipramine in the mouse FST, but not antidepressants affecting noradrenergic transmission (Harkin et al., 2004). In the current study this has been extended in the rat FST where a combination of sub-active doses of fluoxetine and L-NA produced an antidepressant response in the test. The ability of L-NA to increase the activity of fluoxetine is consistent with the qualitative similarities between the antidepressant-like activity of NOS inhibitors and SSRIs and the dependency of these effects on endogenous 5-HT (Harkin et al., 2003; Page et al., 1999). Coupled to the aforementioned evidence of enhanced 5-HT synthesis and metabolism, it is not unreasonable to suggest that NOS inhibitors may produce their antidepressant augmenting properties by modulating the availability and release of 5-HT. In this regard, it is also of interest to note that some antidepressants can inhibit the activity of NOS. For example, the SSRI paroxetine inhibits NOS activity at concentrations comparable to those achieved in clinical therapy (Finkel et al., 1996; Wegener et al., 2003). Such studies have raised the possibility that NOS inhibition may be a clinically relevant feature of at least some antidepressants.

### ***2.4.3 5-HT receptors involvement in the antidepressant-like action of L-NA in the FST***

In a separate series of experiments, the involvement of the 5-HT receptor subtypes in the antidepressant-like activity of L-NA in the FST was studied. To this end, rats were co-treated in turn with the non selective 5-HT receptor antagonist metergoline, the preferential 5-HT<sub>2A</sub> receptor antagonist ketanserin, the selective 5-HT<sub>2C</sub> receptor antagonist RO-430440, the selective 5-HT<sub>1A</sub> receptor antagonist WAY-100635, the 5-HT<sub>1B/D</sub> receptor antagonist GR-127935 and L-NA. Here, involvement of 5-HT receptor subtypes in the antidepressant-like effect of L-NA is supported by the demonstration that metergoline and ketanserin attenuate the antidepressant-like effect of L-NA and L-NA attenuates the increase in immobility and reduction in escape oriented behaviours induced by these 5-HT receptor antagonists in the FST.

Co-treatment with metergoline dose dependently attenuated L-NA-induced antidepressant-like activity in the FST. Metergoline alone provoked an increase in immobility and reduction in swimming behaviours consistent with a pro-depressant-like action of the drug in the test. Co-treatment with L-NA reduced these behavioural effects. In rats, metergoline can influence components of behaviour including a suppression of rearing, sniffing and locomotion at doses of 2 mg/kg and higher (Halford and Blundell, 1996; Mokler et al., 1983; Wilson et al., 1998). Thus the question arises, if the ability of higher doses of metergoline to reduce locomotor and other general behaviours might confound any conclusions regarding a reduction in the antidepressant-like activity of L-NA. In the current investigation any effect of metergoline (4 mg/kg) on locomotor activity was observed. It is known that active doses of L-NA in the FST

cannot be attributed to any psychomotor stimulant action (Harkin et al., 2003), yet L-NA attenuates the ability of metergoline to increase immobility in the FST. Taken together with the ability of metergoline to reverse the antidepressant-like actions of L-NA, these findings are consistent with the participation of 5-HT receptors in the antidepressant-like activity of L-NA in the FST.

Co-treatment with ketanserin produced a qualitatively similar response to that obtained with metergoline. Ketanserin alone provoked an increase in immobility and reduction in swimming behaviours consistent with a pro-depressant-like action and these behavioural effects were reduced by co-treatment with L-NA. Moreover, ketanserin attenuated L-NA-induced swimming activity in the FST. In the current investigation, by contrast to metergoline, ketanserin reduced locomotor activity. Co-administration with L-NA failed to influence this response suggesting that the ability of L-NA to attenuate the actions of ketanserin in the FST are related to an inhibition of the depressive phenotype independent of locomotor activity. Other investigators have not reported effects of ketanserin alone in the FST but this may be related to the lower doses used and differences in the manner in which the FST was carried out and scored (Martínez-Mota et al., 2002; Savegnago et al., 2007). The dose of ketanserin in the present study was selected based on the observed lack of interaction of lower doses of ketanserin with L-NA in pilot experiments undertaken in the laboratory. Similar doses have been employed by others where ketanserin (1-5 mg/kg) showed no effect on behavioural measures in the open field but was sufficient to block 5-HT agonist-induced behaviours (Hawkins et al., 2008).



Activation of 5-HT<sub>2C</sub> receptors has previously been reported to provoke antidepressant-like activity in the FST. Moreover the effects of the SSRI fluoxetine may be blocked by pretreatment with the 5-HT<sub>2C</sub> receptor antagonist SB-206533 (Cryan and Lucki, 2000). It was therefore of interest in the current study to determine if blockade of 5-HT<sub>2C</sub> receptors might influence the antidepressant-like response to L-NA. Co-treatment with RO-430440 produced a similar response to that obtained with ketanserin although RO-430440 did not significantly attenuate L-NA-induced antidepressant-like activity in the FST. Like ketanserin, RO-430440 alone provoked an increase in immobility and reduction in swimming behaviours consistent with a pro-depressant like action and these behavioural effects were attenuated by co-treatment with L-NA. By contrast to ketanserin, RO-430440 did not affect locomotor activity in the current study. The dose of RO-430440 was selected based on observations that pretreatment with RO-430440 at doses not less than 2.5 mg/kg can block fenfluramine-induced hypophagia and hypothermia which have previously been reported to be mediated via the stimulation of 5-HT<sub>2C</sub> receptors (Cryan et al., 2000; Gibson et al., 1993).

The apparent differences between responses obtained with metergoline, ketanserin and RO-430440 suggest that the 5-HT<sub>2A</sub> receptor is the predominant receptor subtype involved in the attenuation of the antidepressant-like activity of L-NA and that 5-HT<sub>2A/2C</sub> receptors play a role in promoting pro-depressant-like behaviours in the FST which may be attenuated by co-treatment with L-NA. To clarify the role of 5-HT<sub>2</sub> receptors further, treatment with the selective 5-HT<sub>1A</sub> and 5-HT<sub>1B/1D</sub> receptor antagonists, WAY-100635 and GR-127935 respectively, failed to influence behaviours in the FST either alone or in combination with L-NA. WAY-100635 is a full antagonist at both pre- and post-synaptic 5-HT<sub>1A</sub> receptors and doses of 0.1 mg/kg or lower are required to block both the pre- and post-synaptic effects of 8-OH DPAT (Critchley et

al., 1994; Forster et al., 1995; Moser and Sanger, 1999). Both agents have previously been reported to lack activity in the FST (De Vry et al., 2004; Tatarczyńska et al., 2004) although co-treatment with WAY-100635 (0.3 mg/kg) can attenuate the effects of the selective 5-HT<sub>1A</sub> receptor agonist 8-OH-DPAT in the FST (DeVry et al., 2004; Moser and Sanger 1999). Moreover WAY-100635 (0.3 mg/kg) has previously been reported to partially attenuate the antidepressant activity of the SSRI fluoxetine in the rat FST. Whilst higher doses have been reported to demonstrate a potential role of 5-HT<sub>1A</sub> receptors in the FST, these experiments have used alternative strains (Martínez-Mota, 2002), female rats (Estrada-Camarena et al., 2006a) and/or rats which have been ovariectomised (Estrada-Camarena, 2006b) and direct comparisons are not possible with the present study.

Similarly, pretreatment with the 5-HT<sub>1B</sub> antagonist GR-127935 (at doses up to 4 mg/kg) attenuates the behavioural effects of 5-HT<sub>1B</sub> receptor agonists in various rat behavioural paradigms (de Boer and Koolhaas, 2005; Chaouloff et al., 1999; Tomkins and O'Neill, 2000) but could not influence the antidepressant-like effect of L-NA in the present experiment. Given the evidence to implicate 5-HT in the antidepressant-like activity of L-NA, it is perhaps surprising that inhibition of pre-synaptic autoreceptors by WAY-100635 or GR-127935 did not facilitate the effects of L-NA in the FST, even if post-synaptic 5-HT<sub>1A</sub> receptors are not involved. This outcome suggests that L-NA may modulate synaptic 5-HT availability by mechanisms independent of pre-synaptic autoreceptors. Support for a role of 5-HT<sub>1A</sub> and/or 5-HT<sub>1B</sub> receptors in the antidepressant-like effects of L-NA in the FST is currently lacking, although additional studies with selective compounds might be worthwhile.

#### **2.4.4 Conclusions**

In conclusion the current data further demonstrate the antidepressant-like potential of the NOS inhibitor L-NA, and also suggest that the behavioural effects have a 5-HT related mechanism of action due to the additive effects of low doses of L-NA and fluoxetine and the influence of L-NA in swimming but not climbing behaviour. Interpretation of the results obtained with the non selective and 5-HT<sub>2A</sub> receptor antagonists must be approached with caution since the antagonists caused effects by themselves that were still apparent following co-administration with L-NA. The antidepressant-like effects of L-NA however were not modified by RO-430440 or WAY-100635 suggesting that 5-HT<sub>2C</sub> and 5-HT<sub>1A</sub> receptors are not involved. The fact that WAY-100635 can block both pre and post-synaptic receptors may contribute to the lack of effects observed with this compound.

The FST in the presented investigation was carried out in naïve rats in order to highlight the ability of L-NA to work as an antidepressant, however in humans antidepressant drugs are prescribed only after depression has developed. L-NA proved its ability to work as a 5-HT related antidepressant in the FST, therefore it would be of interest to test its pharmacological properties in a model of depression characterised by an induced depressive-like state accompanied by reduced 5-HT availability, such as the combination of 5-HT depletion and restraint stress which results in an increased immobility time in the rat FST, as described in results chapter 1.

## ***Results Chapter 3***

***Region specific modulation of neuronal activation associated with the antidepressant-like properties of N<sup>ω</sup>-Nitro-L-arginine in the rat FST***



### 3.1 Introduction

The forced swimming test (FST) is a primary tool for screening for potential antidepressant activity of compounds. In the previous chapter it was demonstrated that inhibition of NOS by N<sup>ω</sup>-nitro-L-arginine (L-NA) produces antidepressant-like effects in the rat FST and that this is achieved through influence on 5-HT synthesis and metabolism. The 5-HT<sub>2</sub> receptor subtype also seems to be involved in the antidepressant-like action of L-NA in the FST. It is yet to be established if this compound would show similar antidepressant-like properties in rats exhibiting a depressive-like state detectable in the FST, induced by the combination of central 5-HT depletion and repeated restraint stress as previously described (see results chapter 1).

Immediate early gene (IEG) products are induced in response to stressful stimuli and have been used extensively as a tool to map neuronal activation in cortical and limbic brain centres in response to stress and modulation of the stress response by psychotherapeutic agents (Kovács, 1998, Miyata et al., 2005). c-FOS, the protein product of the *c-fos* gene, is considered to be a marker of neuronal activation, as its expression is induced in response to changes in afferent synaptic inputs and is often used to delineate regional patterns of neuronal activation (Kovács, 2008). Previous studies have shown that many areas of the brain, especially those involved in the general stress response, increase their activity and *c-fos* expression levels when animals are exposed to a variety of physiological and psychological stressors (see Senba and Ueyama, 1997 for review). Psychoactive drugs in naïve animals can modulate *c-fos* expression in a drug class-selective fashion (Slattery et al., 2005; Sumner et al., 2004).

In some animal models of depression altered cellular activation in response to stress has been reported in brain regions related to the neurobiology of the illness, and antidepressant drugs can counteract these changes (Silva et al., 2012; Bechtholt et al., 2008; Roche et al., 2007). Specifically in relation to the antidepressant-like properties of NOS inhibitors, Amir and co-workers (1997) reported that treatment of rats with 7-nitroindazole (7-NI) or N<sup>ω</sup>-nitro-L-arginine methyl ester (L-NAME) blocked c-FOS production in the paraventricular nucleus (PVN) of the hypothalamus 60 minutes following exposure to immobilisation stress indicating that NO is involved in the regulation of c-FOS in stress-activated neurons. In rats, the selective nNOS inhibitor 7-NI has been reported to attenuate FST-induced c-FOS in a number of cortical and limbic brain regions in a similar fashion to the antidepressants fluoxetine and venlafaxine indicating that NOS inhibitors may share common neurobiological substrates with typical antidepressive agents (Silva et al., 2012). To further characterise neuronal substrates involved in NO/stress interactions Salchner et al. (2004) used c-FOS expression as a marker and examined the pattern of neuronal activation in response to FST in nNOS knockout mice. Deletion of nNOS leads to an antidepressant-like behavioural phenotype in the FST which is accompanied by increased c-FOS expression in the medial amygdala, periventricular hypothalamic nucleus, dentate gyrus, CA1 field of the hippocampus and infralimbic cortex (Salchner et al., 2004).

Central 5-HT depletion when combined with sub-acute immobilisation stress provokes an increase in immobility in the FST suggestive of a depressive-like state which can be attenuated by a single administration of ketamine as previously described (Gigliucci et al., 2013). Neither 5-HT depletion nor restraint stress alone were able to increase immobility time in the test. Given the aforementioned role of 5-HT in mediating the

antidepressant-like properties of NOS inhibitors, it was of interest to characterise neuronal activation by c-FOS mapping in order to determine if any changes obtained in c-FOS expression may depend on endogenous 5-HT. Moreover the combination of 5-HT depletion with stress provided a model in which to identify brain regions which may be associated with depression related immobility in addition to the antidepressant-like properties of NOS inhibitors. In the present study the antidepressant-like effects of the NOS inhibitor N<sup>0</sup>-Nitro-L-arginine (L-NA) were determined in naïve and in the 5-HT depleted rats which were exposed to immobilisation stress. Moreover the recruitment of specific brain regions in the antidepressant-like actions of L-NA was assessed through evaluation of regional neuronal activation in response to the FST by means of c-FOS immunohistochemistry.





## 3.2 Materials and Methods

### 3.2.1 Subjects and drug treatment

Male Sprague-Dawley rats weighing 280-320 g at the beginning of the studies were singly housed in standard medium sized polypropylene cages (41x24 cm) after acclimatisation to the new environment. Food and water were available *ad libitum*.

Fluoxetine hydrochloride was dissolved in de-ionised water to achieve a concentration of 10 mg/ml and was administered intraperitoneally (i.p.) to deliver a dose of 20 mg/kg. This dose of fluoxetine has been previously shown to decrease immobility counts and increase swimming counts, both indications of antidepressant-like effect in the rat FST (Cryan and Lucki, 2000b).

L-NA was prepared in a similar manner with the help of sonication to produce an injectable suspension at a final concentration of 5 mg/ml. L-NA was administered i.p. in an injection volume of 2 ml/kg to deliver a dose of 10 mg/kg. It was previously reported that this dose of L-NA proved to be effective in the rat FST (Gigliucci et al., 2010).

DL-4-Chlorophenylalanine ethyl ester hydrochloride (*p*CPA) was prepared by dissolving in saline solution to a concentration of 75 mg/ml. *p*CPA was administered i.p. in an injection volume of 2 ml/kg to deliver a dose of 150 mg/kg.

All procedures were approved by the Animal Ethics Committee Trinity College Dublin and were in accordance with the European Council Directive 1986 (86/806/EEC).

### **3.2.2 Experimental design**

#### **3.2.2.1 The effects of fluoxetine and L-NA on the depressive state induced by central 5-HT depletion and restraint stress in the FST**

Restraint stress and FST were performed as described in the Methods section. To characterise the effects of fluoxetine and L-NA on the 5-HT depletion and restraint stress-induced increase in immobility time in the FST, rats were subdivided into controls and 5-HT depleted and stressed animals. Depletion of central 5-HT was achieved by repeated *p*CPA administration as previously described, followed by 48 hr recovery, finally followed by exposure to three sessions of 2 hr immobilisation stress (once a day for 3 consecutive days). 24 hr after the last stress session, rats were exposed to the 15 min adaptation session of the FST and assigned to one of six treatment groups according to the pre-treatment received: group 1: Vehicle + Vehicle, group 2: Vehicle + Fluoxetine (20 mg/kg), group 3: Vehicle + L-NA (10 mg/kg), group 4: *p*CPA/stress + Vehicle, group 5: *p*CPA/stress + Fluoxetine (20 mg/kg), group 6: *p*CPA/stress + L-NA (10 mg/kg). Drugs or vehicle were administered 24, 5 and 1 hr prior to test, with the first administration being 1 hr after the adaptation session of swim.

#### **3.2.2.2 The effects of FST, combined 5-HT depletion and restraint stress and L-NA on brain regional neuronal activation**

In a follow up experiment in a separate group of rats, mapping of c-FOS immunolabelling was undertaken with the same protocol, where only saline or L-NA

was administered 24, 5 and 1 hr prior to FST. To assess for basal expression of c-FOS a smaller number of rats underwent the *p*CPA/stress protocol and were subject to the drug administration regime but did not undergo FST. Rats were assigned to one of eight treatment groups: group 1: Control + Vehicle No FST, group 2: Control + Vehicle FST, group 3: Control + L-NA No FST, group 4: Control + L-NA FST, group 5: *p*CPA/Stress + Vehicle No FST, group 6: *p*CPA/Stress + Vehicle FST, group 7: *p*CPA/Stress + L-NA No FST, group 8: *p*CPA/Stress + L-NA FST.

### ***3.2.3 Determination of cortical 5-HT concentrations***

Immediately following these tests the animals were euthanised in order to verify the depletion of 5-HT induced by *p*CPA in the frontal cortex by HPLC. Final results were expressed as ng neurotransmitter/g wet weight of tissue.

### ***3.2.4 Intracardiac perfusion and c-FOS immunohistochemistry***

90 minutes after the FST animals were deeply anaesthetised with an intraperitoneal injection of urethane and intracardially perfused as described in the Methods section. Brains were cryo-protected in sucrose, snap frozen in dry ice-cold isopentane and stored at -80°C until sectioning. c-FOS immunohistochemistry was performed on the sections as described in the Methods section.

### 3.2.5 *c-FOS immunolabelling analysis*

c-FOS positive cells were visualised using an Olympus BX51 microscope, and photomicrographs were collected with an Olympus DP72 digital camera and the Olympus Cell^D computer software. Neuroanatomical areas according to the Paxinos and Watson (1998) rat brain atlas were identified on the sections with the help of neuroanatomical markers such as white matter bundles and ventricle edges. The anterior-posterior (AP) levels from bregma were as follows: prelimbic cortex (PLCx, AP: 2.70 mm), lateral septum (LS, AP: 0.70 mm), nucleus accumbens (NAc, AP: 0.70 mm), paraventricular nucleus of the hypothalamus (PVN, AP: -1.80 mm), medial amygdaloid nucleus (MeA, AP: -2.80 mm), basolateral amygdaloid nucleus (BLA, AP: -2.80 mm), dorsal dentate gyrus (dDG, AP: -3.30), ventral cornu ammonis 1 of the hippocampus (vCA1, AP: -4.80 mm), dorsal raphe nucleus (DRN, AP: -7.64) [Fig. 4.2]. 200X magnification was used for the identification and manual quantification of c-FOS immunoreactivity. All c-FOS positive nuclei, defined as circular spots with a dark brown intensity, within the region of interest being analysed were counted. The total c-FOS-like immunoreactivity (TFI) per region analysed was recorded. Each brain region of interest was analysed in the appropriate section bilaterally, and where there was no clear difference between the two hemispheres the average number of c-FOS positive nuclei between the two hemispheres was taken as the definitive value.

### 3.2.6 *Statistical analysis*

Data are expressed as group mean with standard error of the mean (SEM) and were analysed by two factor, two factor repeated measure or three factor repeated measure analysis of variance (ANOVA) where appropriate. If any statistically significant change was found, *post hoc* comparisons were performed using a Student Newman-Keuls or a Fisher's LSD test. When only two groups were to be compared, Student's *t*-test was performed. In all cases data were deemed significant when  $p < 0.05$ .



### 3.3 Results

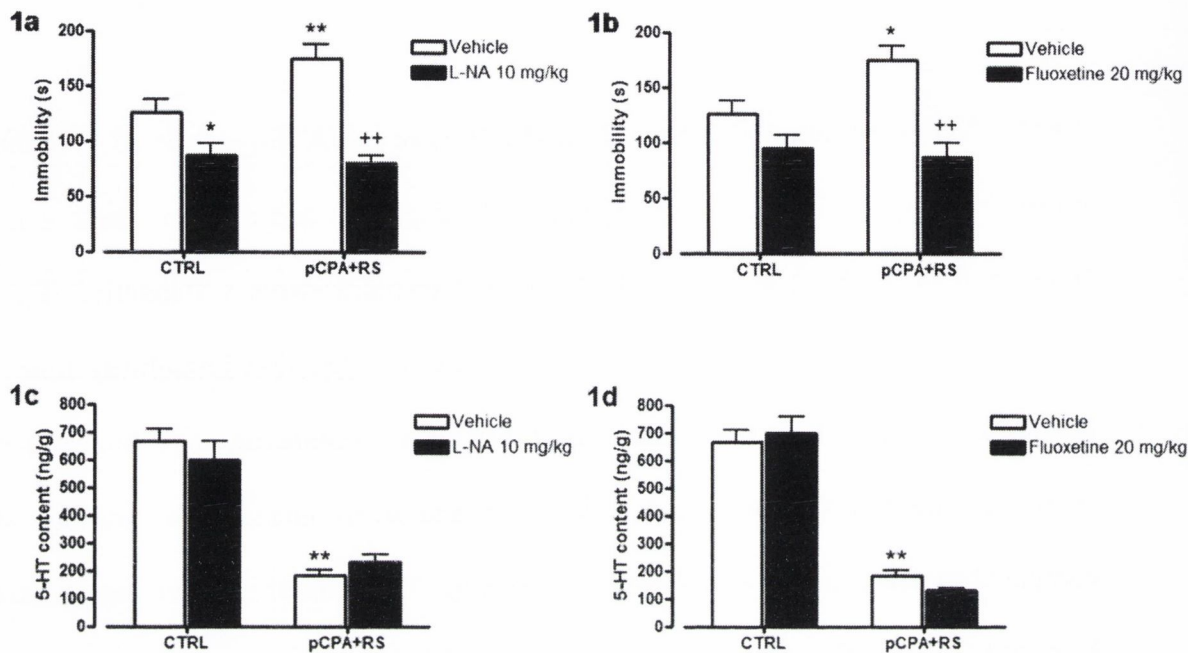
#### 3.3.1 Antidepressant-like action of L-NA in the FST

2 way ANOVA of immobility time showed effects of L-NA [ $F_{(1,28)} = 36.87, p < 0.0001$ ], fluoxetine [ $F_{(1,32)} = 22.40, p < 0.0001$ ] and 5-HT depletion and restraint stress x L-NA [ $F_{(1,28)} = 6.34, p < 0.05$ ] and 5-HT depletion and restraint stress x fluoxetine [ $F_{(1,32)} = 5.07, p < 0.05$ ] interactions. *Post hoc* comparisons revealed that immobility time was decreased in control animals receiving L-NA when compared to vehicle controls ( $p < 0.05$ ). The combination of 5-HT depletion and stress increased immobility when compared to non-depleted non-stressed controls ( $p < 0.01$ ), and this effect was attenuated by both L-NA and fluoxetine administration ( $p < 0.01$ ) [Fig. 3.1a and 3.1b].

#### 3.3.2 Cortical 5-HT content

2 way ANOVA of cortical 5-HT concentrations showed an effect of 5-HT depletion and restraint stress [ $F_{(1,28)} = 83.46, p < 0.001$ ]. *Post hoc* comparisons revealed that 5-HT content was significantly reduced in 5-HT depleted rats compared to vehicle-treated controls ( $p < 0.01$ ), an effect not altered by L-NA [Fig. 3.1c] or fluoxetine [Fig. 3.1d].



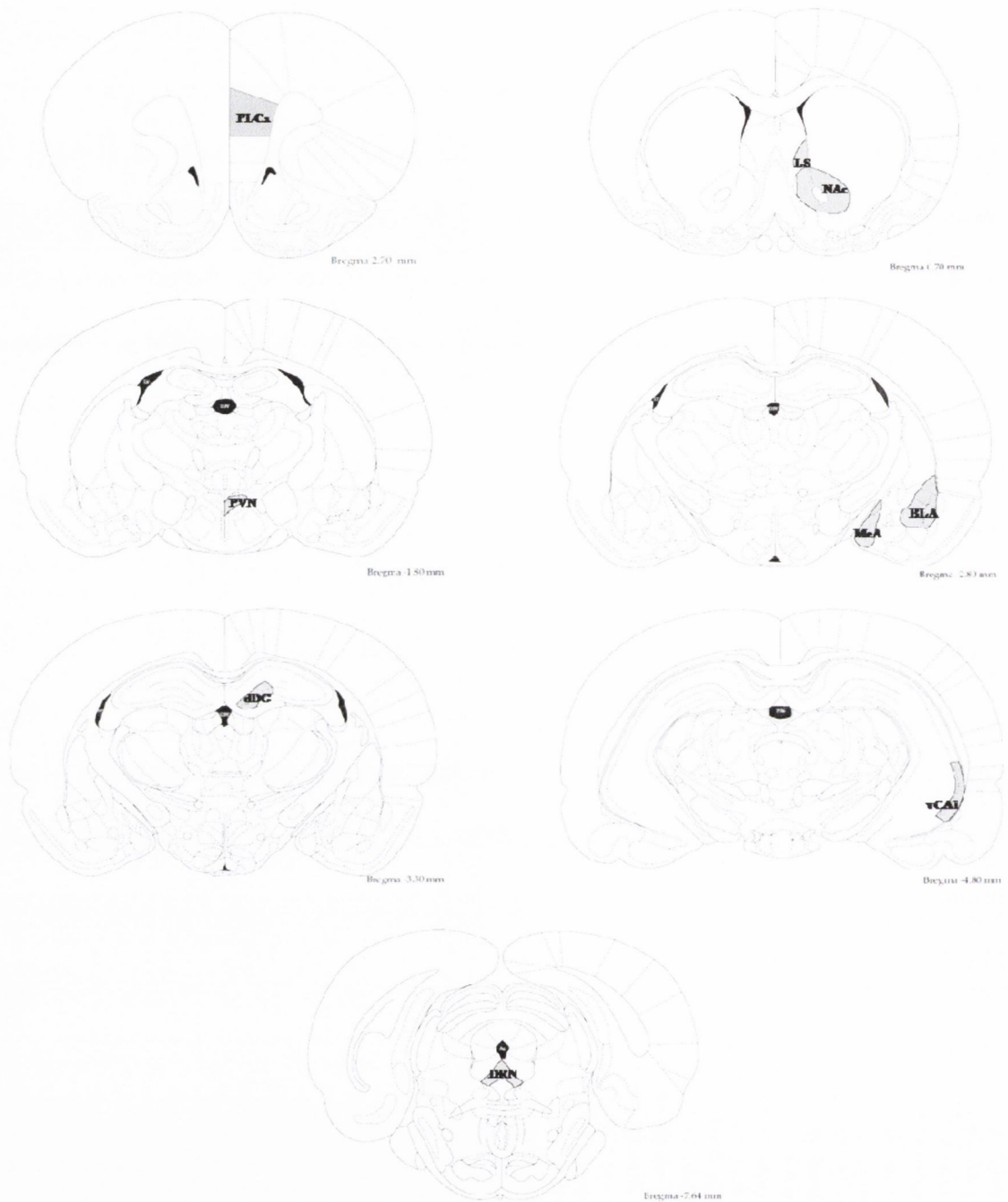


**Fig. 3.1** Effect of L-NA and fluoxetine on FST immobility induced by a combination of 5-HT depletion and stress. Rats were exposed to a 15 min adaptation session of the FST and administered L-NA (10 mg/kg, i.p.) or fluoxetine (20 mg/kg, i.p.) 24, 5 and 1 hr prior to test 24 hr after the adaptation session (Fig. 3.1a and 3.1b respectively). Cortical 5-HT concentrations were determined immediately following the test (Fig. 3.1c and 3.1d). Data are expressed as mean and SEM of 7-10 animals. \* $p < 0.05$ , \*\* $p < 0.01$  compared to vehicle control group; ++ $p < 0.01$  compared to pCPA + stress + vehicle treated group.

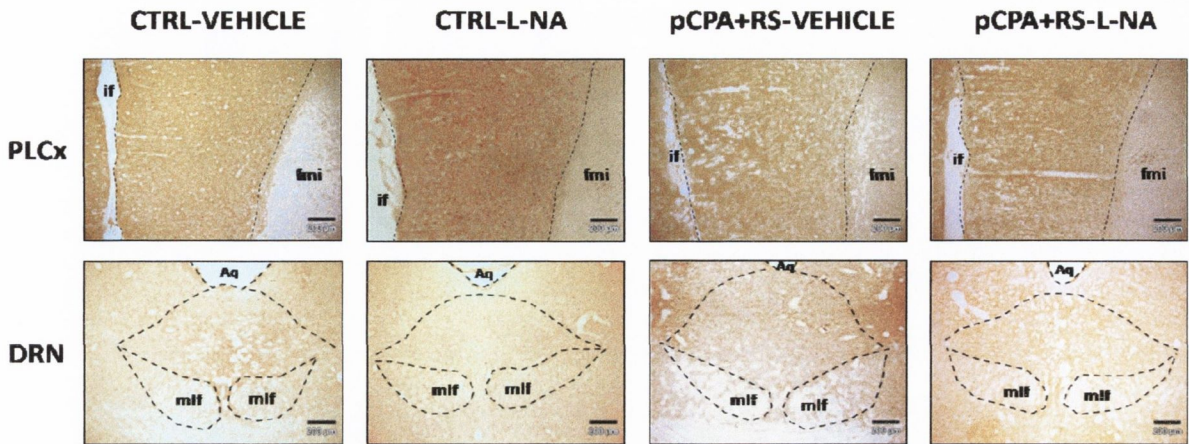
### 3.3.3 Regional c-FOS expression

2 way ANOVA of immobility time in the FST of the group of rats dedicated to c-FOS immunostaining analysis showed effects of the combination of 5-HT depletion and restraint stress [ $F_{(1,20)} = 12.51$ ,  $p < 0.01$ ] and L-NA [ $F_{(1,20)} = 4.17$ ,  $p = 0.05$ ]. *Post hoc* comparisons revealed that the immobility time in the FST was significantly increased in 5-HT depleted stressed rats (165.7 s  $\pm$  11.20) compared to non-depleted non-stressed controls (98.00 s  $\pm$  13.51) ( $p < 0.01$ ), and that this effect was attenuated by L-NA administration (117.7 s  $\pm$  8.60) when compared to vehicle-treated controls ( $p < 0.05$ ) (data not shown). In this study we could not replicate the effects of L-NA in reducing immobility times in sham animals when compared to vehicle controls, as the difference between the two groups did not achieve statistical significance.

Fig. 3.2 shows diagrams modified from Paxinos and Watson (Paxinos 1997) representing the brain regions where c-FOS like immunoreactivity was analysed. Immunoreactivity was detected in the regions analysed following FST exposure (see Fig. 3.3 as an example).

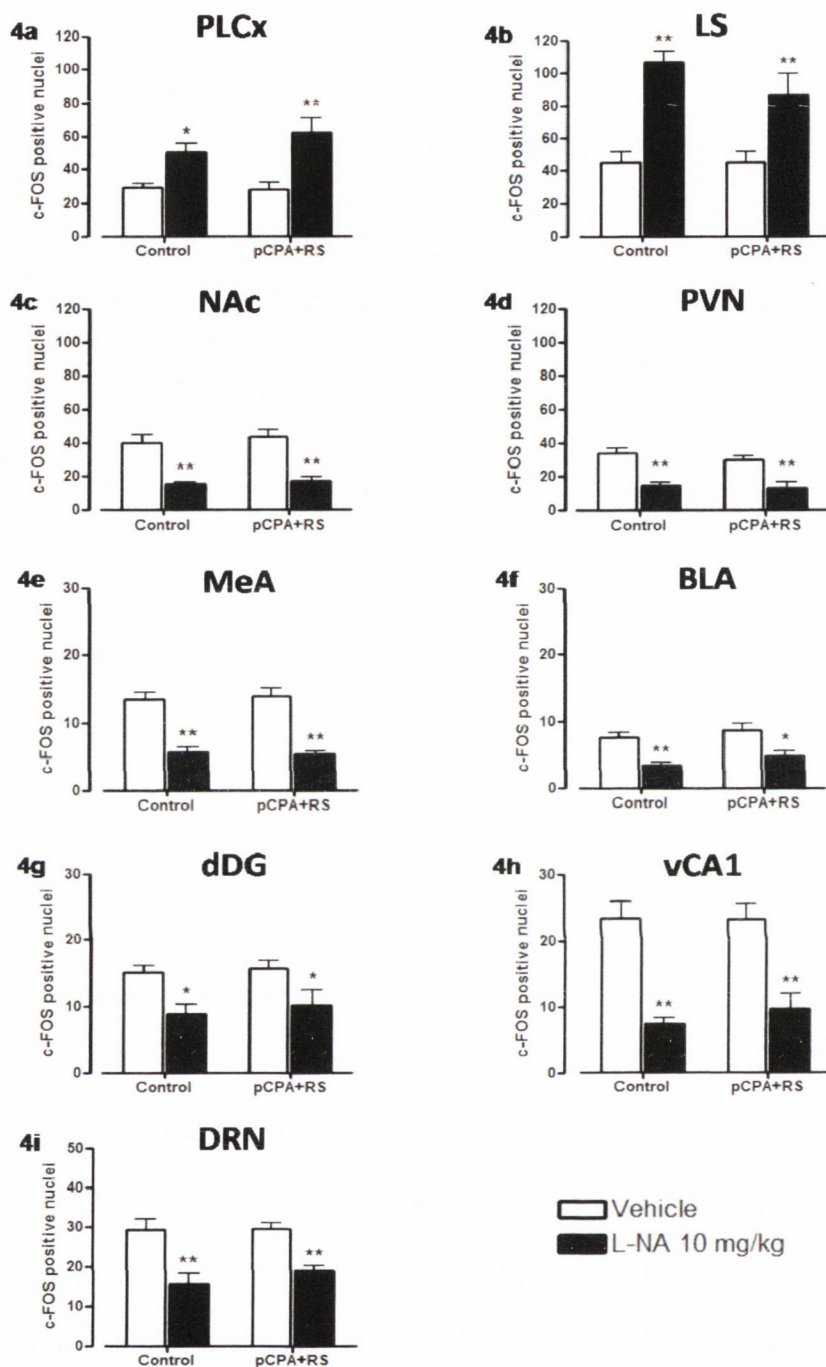


**Fig. 3.2** Diagrams adapted from “The rat brain” Fourth Edition (Paxinos and Watson, 1998) representing the brain regions analysed in the study (grey areas). PLCx = prelimbic cortex; LS = lateral septum; NAc = nucleus accumbens; PVN = paraventricular nucleus of the hypothalamus; MeA = medial amygdala; BLA = basolateral amygdala; dDG = dorsal dentate gyrus of the hippocampus; vCA1 = ventral cornu ammonis 1; DRN = dorsal raphe nucleus.



**Fig. 3.3** Images representative of c-FOS immunostaining. L-NA treatment induced increased c-FOS expression in the PLCx (top row) and reduced c-FOS expression in the DRN (bottom row) of rats exposed to FST. if = interhemispheric fissure; fmi = forceps minor of the corpus callosum; Aq = aqueduct (Sylvius); mlf = medial longitudinal fasciculus.

In the prelimbic cortex [ $F_{(1,18)} = 27.48$ ,  $p < 0.01$ ; Fig. 3.4a] and lateral septum [ $F_{(1,19)} = 34.92$ ,  $p < 0.01$ ; Fig. 3.4b], L-NA enhanced c-FOS reactivity in both control and 5-HT depleted/stressed groups when compared to vehicle treated counterparts. In the nucleus accumbens [ $F_{(1,19)} = 46.14$ ,  $p < 0.01$ ; Fig. 3.4c], paraventricular nucleus of the hypothalamus [ $F_{(1,18)} = 38.25$ ,  $p < 0.01$ ; Fig. 3.4d], medial amygdaloid nucleus [ $F_{(1,20)} = 83.03$ ,  $p < 0.01$ , Fig. 3.4e], basolateral amygdaloid nucleus [ $F_{(1,20)} = 27.76$ ,  $p < 0.01$ ; Fig. 3.4f], dentate gyrus of the hippocampus [ $F_{(1,18)} = 16.06$ ,  $p < 0.01$ ; Fig. 3.4g], hippocampal CA1 [ $F_{(1,19)} = 51.50$ ,  $p < 0.01$ ; Fig. 3.4h] and dorsal raphe nucleus [ $F_{(1,18)} = 29.54$ ,  $p < 0.01$ ; Fig. 3.4i] L-NA attenuated c-FOS immunoreactivity in both control and 5-HT depleted/stressed groups when compared to their vehicle treated counterparts ( $p < 0.05$ ). In all regions the combination of 5-HT depletion and stress did not influence c-FOS when compared to non-depleted non-stressed controls subjected to FST [Fig. 3.4].



**Fig. 3.4** Effects of L-NA administration on neuronal activation in cortical and subcortical regions in control and in 5-HT depleted and stressed rats. Rats were exposed to a 15 min adaptation session of the FST and administered L-NA (10 mg/kg, i.p.) or vehicle 24, 5 and 1 hr prior to test 24 hr after the adaptation session. Brains were collected 90 minutes after the test and immunolabeled for c-FOS protein. PLCx (Fig. 3.4a), LS (Fig. 3.4b), NAc (Fig. 3.4c), PVN (Fig. 3.4d), MeA (Fig. 3.4e), BLA (Fig. 3.4f), dDG (Fig. 3.4g), vCA1 (Fig. 3.4h) and DRN (Fig. 3.4i) were analysed under a light microscope and c-FOS-positive nuclei counted. Data are expressed as mean and SEM of 5-6 animals. \* $p < 0.05$ , \*\* $p < 0.01$  compared to vehicle control group.

### 3.4 Discussion

L-NA produced a characteristic antidepressant related reduction in immobility time in the FST in line with previous reports (Harkin et al., 2003; Gigliucci et al., 2010). The increase in immobility provoked by the combination of 5-HT depletion and stress which has also been reported previously (Gigliucci et al., 2013) was attenuated by fluoxetine and also by L-NA. In the present study only two rats per non-FST group were processed for c-FOS immunoreactivity due to time limitations, thus it was not possible to conclude if 5-HT depletion with stress and/or L-NA treatment induced changes in c-FOS in any of the brain regions examined in non-FST exposed animals. Analysis of neuronal activation in these animals is currently being carried out. After FST exposure c-FOS-like immunoreactivity (FLI) was found in all the brain regions analysed: the prelimbic cortex, the lateral septum, the nucleus accumbens, the paraventricular nucleus of the hypothalamus, the medial and basolateral amygdala, the hippocampus (CA1 and dentate gyrus) and the dorsal raphe nucleus. Prior 5-HT depletion and stress failed to influence this pattern of c-FOS induction following FST exposure in any of the brain regions examined. L-NA treatment attenuated FLI in all of the brain regions examined except the prelimbic cortex and lateral septum where an increase in immunoreactivity was observed. L-NA related changes in c-FOS were similar in rats exposed to the combination of 5-HT depletion and stress when compared to non-depleted non-stressed controls.

### ***3.4.1 Antidepressant related behaviour of L-NA in the FST***

Combined 5-HT depletion and stress was used as a manipulation to promote a despair related increase in immobility in the FST which can be attenuated by acute administration of the glutamate NMDA receptor antagonist ketamine (Gigliucci et al., 2013). Here these findings may be extended to include L-NA. L-NA also decreased immobility in non stressed animals, confirming its antidepressant-like action in naïve rats in the FST as previously reported (Gigliucci et al., 2010). As NOS is a downstream target of the NMDA receptor, the ability of L-NA to promote a ketamine-like response in the test may indicate a common mechanism related to modulation of glutamate NMDA-NO related signalling. Surprisingly, treatment with the typical antidepressant fluoxetine too reduced the increase in immobility obtained following the combination of 5-HT depletion and stress characteristic of an antidepressant response in the test. In control animals however fluoxetine tended, but not significantly achieved, to decrease immobility time in the FST. Fluoxetine has been extensively reported to possess antidepressant-like properties in the FST in naïve animals, although a modified version of the test is needed to better highlight these effects (Harkin et al., 2004; Cryan and Lucki, 2000a; Detke et al., 1995). In the study presented there was no focus on escape-oriented behaviours, as this was beyond the aim of the study to confirm that depletion of 5-HT followed by repeated restraint stress can induce a depressive-like state in the animals in the FST and that this model might represent a putative model of SSRI-resistant depression.

It is of interest that 5-HT depletion failed to attenuate the antidepressant related activity of L-NA or fluoxetine as previous reports have indicated that the actions of both agents are 5-HT dependent in the FST (Harkin et al., 2003 and Page et al., 1999 respectively).

It is important to point out however that the extent of cortical 5-HT depletion achieved and reported following *p*CPA administration varies across the different studies reported to date, from 85-90% depletion in the investigations of Harkin and colleagues (2003) and Page and colleagues (1999) to a 70% depletion in cortical 5-HT in the present investigation, at the time at which the animals were subjected to the FST. *p*CPA is an irreversible inhibitor of tryptophan hydroxylase (TPH), the rate limiting enzyme in the synthesis of 5-HT. Recovery in the activity of TPH after interruption of *p*CPA administration leads towards normalisation of 5-HT levels in the brain over time, thus it is possible that a lower availability of 5-HT compared to controls is sufficient to promote a depression related increase in immobility on the one hand, and yet mediate a reduction in immobility due to antidepressant treatment on the other, despite the overall state of 5-HT depletion. Moreover it is possible that the reduction in 5-HT availability may alter the balance between activation and/or binding properties of the different subtypes of 5-HT receptors (Winstanley et al., 2004) which may ultimately favour the antidepressant-like action of fluoxetine and L-NA in the FST. Further studies would be needed to elucidate which mechanisms could be mediating the actions of fluoxetine and L-NA on the 5-HT depletion and stress-induced increase in immobility time in the test.

#### ***3.4.2 Combination of 5-HT depletion and stress does not alter regional c-FOS FLI following exposure to the FST***

c-FOS immunostaining in brains from vehicle control rats subjected to FST revealed neuronal activation in all the regions analysed: prelimbic cortex (PLCx), lateral septum (LS), nucleus accumbens (NAc), medial amygdala (MeA), basolateral amygdala (BLA),



paraventricular nucleus of the hypothalamus (PVN), dorsal dentate gyrus (dDG), ventral cornu ammonis 1 (vCA1) and dorsal raphe nucleus (DRN). c-FOS immunoreactivity induced by exposure to the FST within cortical and subcortical regions of the limbic system was not altered in animals subjected to the combined regime of 5-HT depletion and stress when compared to non-depleted non-stressed counterparts. The results indicate that although antidepressant related reductions in immobility are associated with changes in regional c-FOS immunoreactivity, the same is not the case in relation to stress related increases in immobility. The outcome however is in line with a previous report where 5-HT depletion achieved by intracerebroventricular injection of 5,7-dihydroxytryptamine (5,7-DHT), did not alter c-FOS expression in response to social defeat stress in most of the brain areas analysed including the prefrontal cortex (PFC), LS, bed nucleus of the stria terminalis (BNST) and dorsal paraventricular nucleus (dPVN) (Chung et al., 1999). In addition to this, the lack of impact of prior exposure to immobilisation stress on the c-FOS response to FST is consistent with other reports where a single swim exposure was able to induce the same extent of c-FOS expression in several brain regions in both naïve and restraint-habituated animals (Melia et al., 1994). c-FOS induction in response to the FST is robust and likely to be close to a ceiling response in drug-free animals, so it remains possible that the response to a milder stressor, such as a brief exposure to the “open field”, may be influenced by the combination of 5-HT depletion and repeated restraint stress and further experiments are necessary to confirm this. Moreover the combination of 5-HT depletion and stress might be influencing other IEGs, such as *fos B*, *c-jun* or *zif268*, when compared to control rats, thus a more extensive analysis is required before excluding any influence of 5-HT depletion and stress on general neuronal response to the FST.

### ***3.4.3 Region specific c-FOS expression associated with the antidepressant-like properties of N<sup>o</sup>-nitro-L-arginine***

The effects of L-NA on c-FOS FLI were apparent in animals exposed to the FST. The response to L-NA was bi-directional and region specific whereby c-FOS immunoreactivity was enhanced in the PLCx and LS and reduced in all the other subcortical brain regions analysed. Enhanced activation within the cortex and septum indicate that these regions may be regulated by NO under stressful conditions. The PFC and the LS have both been implicated previously in the modulation of stress responses (Sheehan et al., 2004; Weinberg et al., 2010). These regions are connected as the medial PFC sends projections to the LS (Sesack et al., 1989), although it seems that the LS can modulate some stress responses independently from the PFC (Figueiredo et al., 2003). Rats that exhibit behavioural despair in the FST show reduced activation in the LS in response to stress when compared to control animals (Contreras et al., 2004), although some contrasting reports have been published (Silva et al., 2012), and rats and mice that acquire learned helplessness present reduced activation of the LS after tail-shock administration (Steciuk et al., 1999; Huang et al., 2004). Here, the analysis of FLI in the LS of rats displaying a depressive behavioural profile in the FST, induced by the combination of 5-HT depletion and restraint stress, did not highlight alterations in the activation pattern of the LS in vehicle-treated rats. Several factors may be contributing to these discrepancies, such as differences in the induction of the learned helplessness (e.g. training with electrical shock vs. swim) which might activate different subtypes of neurons in response to stress. Moreover, as previously suggested, it might be that the interaction between serotonin depletion and stress influences other IEGs that were not under investigation in the current study.

However, it is of interest to note within the context of the present investigation that fluoxetine can increase LS firing to correspond with antidepressant activity in the FST (Contreras et al., 2001). Enhanced activation within the LS is also evident following L-NA administration in the present investigation. Thus increased activation of the LS seems to play an important role in appropriate stress-coping responses. Projections from the LS extend to various regions of the limbic system including the nucleus accumbens (NAc), the amygdala and the hypothalamus (Sheenan et al., 2004). The lateral septum is known to relay inhibitory GABAergic fibres to the amygdaloid complex and the PVN of the hypothalamus, therefore it can be hypothesised that if the ability of L-NA to enhance neuronal activity in the septum, which is in keeping with the properties of other antidepressants in this region in response to the FST, is localised to those GABAergic neurons this could account for the reduction in c-FOS immunoreactivity observed in the amygdala and PVN. Other more selective and specific analyses, such as a dual immunostaining approach, would be decisive to support or discard this hypothesis of stress response modulation by the LS.

A further possibility is that L-NA-induced effects would be mediated by the modulation of 5-HT as it has been reported that the behavioural effects induced by L-NA in the FST are dependent on endogenous 5-HT or the blockade of 5-HT receptors (Gigliucci et al., 2010; Harkin et al., 2003). However despite the role elucidated for 5-HT in mediating the behavioural effects of L-NA in the FST, 5-HT depletion combined with restraint stress had no effect on the amplitude of c-FOS response when compared to intact animals. Even though c-FOS immunostaining alone does not provide information on which neuronal subtypes are activated in response to the FST in the different treatment groups, the presented data may suggest that mechanisms independent of 5-HT are

involved in the regulation of c-FOS-mediated neuronal activation following L-NA administration.

An alternative mechanism by which L-NA promotes a reduction in neuronal activation within these regions may relate to reduced glutamate transmission on foot of inhibiting postsynaptic NMDA related NO production. NMDA receptors are localised on hypothalamic neurones that express NOS (Bhat et al., 1995). Stress-induced activation of c-FOS in the PVN has been shown to involve glutamatergic stimulation of NMDA receptors (Wan et al., 1994). Such observations together with the finding that L-NA reduces c-FOS in the PVN following FST suggest that NO may be part of the NMDA receptor-coupled cellular mechanism responsible for stress activation of c-FOS expression in the PVN. Such mechanisms have been proposed previously (Amir et al., 1997). In the hippocampus glutamate is a prominent neurotransmitter and the ability of L-NA to attenuate FST related neuronal activation within the hippocampus (CA1 field and dentate gyrus) was not unexpected. A previous report has indicated that blockade of NMDA receptors or inhibition of downstream target ERK1/2 in the granule layer of the DG results in an attenuation of swim stress-induced c-FOS expression coupled with a reduction in behavioural immobility in the FST (Chandramohan et al., 2008). This study suggests that expression of c-FOS within the DG following FST is glutamate dependent and that NMDA-mediated transmission facilitates the learned response underlying immobility. The dorsal raphe nucleus (DRN) is known to receive glutamatergic projections from the medial PFC (mPFC) which predominantly synapse on GABAergic neurons (Jankowski and Sesack, 2004), thus it is not unreasonable to suggest that interferences within the glutamate pathway following L-NA administration

may contribute to the alterations in DRN neuronal activation found following FST stress.

The basolateral and medial amygdala (BLA and MeA respectively) showed reduced c-FOS induction following FST exposure after L-NA administration. These data replicate what has been previously reported that c-FOS production in the amygdala after swim stress can be inhibited by the traditional antidepressants fluoxetine and venlafaxine and the selective nNOS inhibitor 7-NI (Silva et al., 2012). On the one hand this parallelism confirms the involvement of the amygdaloid complex in the stress response induced by the FST and its role as a target region for drugs inducing antidepressant-like effects in the test, and on the other hand it further supports the potential for NOS inhibitors to be used as potential antidepressant treatments in the clinic.

Antidepressant drugs influence neuronal activation of common brain regions (Slattery et al., 2005). A single administration of the tricyclic antidepressant imipramine, as well as the SSRI fluoxetine, the noradrenaline and 5-HT-selective mirtazapine, and the mood stabilizer lithium chloride, increase c-fos mRNA in the central amygdala, in the anterior insular cortex and reduce expression within the septum when compared to saline-treated controls. There were also specific regions influenced only by one particular drug (e.g. strong c-fos mRNA induction was noted throughout the dorsal and ventral striatum, including the nucleus accumbens core and shell regions only after fluoxetine treatment). Clinically such agents require chronic administration in order to elicit their antidepressant effects and it may be that patterns of neuronal activation would differ following repeated administration. Silva and co-workers (2012) propose that NOS inhibitors and antidepressants share common neurobiological substrates where FST induced c-FOS expression in a number of brain regions in rats including the medial

prefrontal cortex, nucleus accumbens, locus coeruleus, raphe nuclei, striatum, hypothalamic nucleus, periaqueductal grey, amygdala, habenula, paraventricular nucleus of the hypothalamus and bed nucleus of the stria terminalis. Such effects on c-FOS were attenuated by pretreatment with the NOS inhibitor 7-NI, and the antidepressants venlafaxine and fluoxetine showing that 7-NI produces similar neuronal activation effects to those of typical antidepressants.

The effects of NOS inhibitors on neuronal activation in response to stress have been examined in other paradigms. For instance, Aguiar and Guimarães (2009) confirmed previous findings that exposure of rats to a live cat induce an increase in c-FOS expression in the dorsolateral periaqueductal gray (dIPAG), the dorsal premmammillary nucleus of hypothalamus (PMd) and the paraventricular hypothalamic nucleus (PVNp). In addition they reported that microinjection of the NOS inhibitor N-propyl-L-arginine (NPLA) into the dIPAG reduced defensive behavior induced by predator exposure and at the same time reduced FOS IR (neuronal activation) in this region (for a complete description about the role of nitric oxide in brain regions related to defensive reactions, please refer to Guimarães et al., 2005). It is interesting that a very similar experiment carried out by Moreira and Guimarães (2008) showed a lack of influence of the 5-HT re-uptake inhibitor and antidepressant clomipramine on predator stress related c-FOS expression, corroborating also at a molecular level the possibility for NOS inhibitors to be used as pharmacological intervention in cases of treatment-resistant depression.

In conclusion, L-NA produced antidepressant related activity in the FST accompanied by region specific changes in c-FOS activation. These changes occurred independently of 5-HT and prior exposure to immobilisation stress. Increased neuronal activation is

seen in cortical regions such as the PLCx and the LS following L-NA treatment which paralleled with reduced neuronal activation in sub-cortico-limbic structures directly or indirectly innervated by the frontal cortex. Further work is required to address some open questions such as: the influence of FST itself on the regional c-FOS response in the brain (indeed brains from rats not subjected to the FST are under current analysis and results will subsequently be compared to the results presented in this chapter); the identification of specific neuronal subtypes expressing c-FOS after exposure to the FST in both controls and 5-HT depleted stressed rats, and L-NA-induced changes to this neuronal activation pattern; the influence of 5-HT depletion and restraint stress and L-NA administration on other IEGs' activation. Nonetheless, the results presented here implement the knowledge of the role of NOS in the regulation of stress responses in the brain and the potential as a novel therapeutic target for the treatment of stress related disorders such as depression.

#### ***3.4.4 Conclusions***

Inhibition of NOS by L-NA administration was able to attenuate the depressive-like state induced in the FST by the combination of 5-HT depletion and repeated restraint stress. This effect was comparable to the one provoked by the traditional antidepressant fluoxetine in the same model of depression, thus supporting L-NA as valuable new potential antidepressant drug. L-NA's antidepressant-like action seemed to be correlated with a 5-HT independent region specific regulation of neuronal activation in the brain in response to the FST, as evaluated by c-FOS immunostaining. In particular

hyperactivation of cortical and septal limbic regions and a concurrent hypoactivation of sub-cortical limbic regions were detected.

The FST can be used to detect a depressive-like state and allows to observe if this state is influenced by a test compound. However there are some limitations to its use, the main one being that it is primarily a test for the detection of acute antidepressant-like activity of compounds, although some reports have used it to assess the effects of chronic antidepressant treatment (Dulawa et al., 2004; Vázquez-Palacios et al., 2004). Other established animal models of depression such as the olfactory bulbectomised rat present behavioural alterations which selectively respond to chronic antidepressant treatment, a condition which best parallels the human pharmacological treatment of depression. Therefore it would be advisable to test L-NA in such models to provide a better evaluation of L-NA's antidepressant-like action *in vivo*.





## *Results Chapter 4*

*Antidepressant related action of  
N<sup>ω</sup>-Nitro-L-arginine in the olfactory  
bulbectomised rat model of depression*



## 4.1 Introduction

In Results chapters 3 and 4 it was established that NOS inhibition can produce antidepressant-like effects in the forced swimming test (FST), in line with numerous other reports of the antidepressant-like activity of NOS inhibitors in preclinical tests (Doucet et al., 2013; Ghasemi et al., 2008; Harkin et al., 2003; 2004; Volke et al., 2003). Despite their fundamental contribution to the process of assessment of potential antidepressant-like activity of new compounds, such tests are subject to some limitations. In particular, antidepressant activity of well known antidepressants including tricyclic antidepressant and selective monoamine re-uptake inhibitors are observed in the tests following acute or sub-acute treatment, in contrast to the clinical therapeutic effects of antidepressants which are apparent following chronic treatment only. Given the difficulties with acute tests, the present investigation examines the effects of NOS inhibitors in the olfactory bulbectomised (OB) rat model of depression which responds to chronic but not acute antidepressant treatment (Harkin et al., 2003b).

The OB rat has been developed as an animal model of depression. It exhibits many behavioural alterations that are similar to depressive-like symptoms such as irritability and agitation, reduced sexual behaviour, anhedonia and alterations in food motivated behaviours, cognitive deficits and many *ex vivo* measures of endocrine and immune function. Many of the deficits associated with the OB syndrome, in particular hyperactivity in a novel environment such as the “open field”, are reversed by chronic, but not acute, antidepressant treatments (reviewed by Harkin et al., 2003; Song and Leonard, 2005). Such face similarity with the onset of antidepressant activity in the clinics adds validity to the OB rat model. Moreover comparisons of onset of the

antidepressant response can be drawn between test compounds and conventional antidepressants. Traditionally this is achieved by exposing the animals to the open field at different time points during drug treatment (Harkin et al., 1999; Cryan et al., 1998), or repeatedly exposing the same animals to the test over time (Breuer et al., 2008; Cryan et al., 1999). An advantage of the latter approach is that it facilitates a reduction in the number of animals required to test a compound in the model, but is limited on account of habituation to the open field environment which may mask the OB related hyperactivity.

In order to develop the translational relevance of the OB rat model magnetic resonance imaging (MRI) may be employed to investigate both structural and functional changes in the brain associated with the OB syndrome and the antidepressant response obtained in the model. MRI studies in depressed patients have reported alterations in the volume of a number of brain areas such as the hippocampus (McKinnon et al., 2009; Colla et al., 2007) and the amygdala (Kronenberg et al., 2009; Frodl et al., 2003) when compared to non-psychiatric healthy controls. To date there is a single MRI report of structural and tissue related changes in OB rats where ventricular enlargement and alterations in the water content of the extracellular tissue space in the cortex, hippocampus, caudate and amygdaloid complex were described suggestive of altered tissue volume or tissue compression and/or damage (Wrynn et al., 2000). In light of this, MR imaging was undertaken in the current investigation to seek neuroimaging markers of interest alongside the behavioural responses obtained with antidepressants and test compounds in the model.

Although L-arginine-derived inhibitors cannot be considered isoform selective, of these inhibitors N<sup>ω</sup>-nitro-L-arginine (L-NA) has been reported to be the most selective in inhibiting brain NO synthesis (Lambert et al., 1991; Moncada et al., 1991). It has been demonstrated *in vivo* that L-NA can compete for the active sites on the NO synthase and inhibit the generation of NO from L-arginine (Ishii et al, 1990; Moore et al., 1990). 1-(2-trifluoro-methyl-phenyl) imidazole (TRIM) is a selective inhibitor of nNOS with weak inhibition against the endothelial NOS (eNOS). *In vivo* studies have reported anti-nociceptive and neuroprotective properties of TRIM, without cardiovascular effects in animal models of pain and stroke respectively (Coert et al., 2003; Haga et al., 2003; Handy and Moore, 1998). Moreover, it has been reported that TRIM displays antidepressant-like properties in preclinical tests predictive of antidepressant activity (Doucet et al., 2012; Harkin et al., 2003a; Volke et al., 2003).

The aim of the present study was to assess the antidepressant potential of L-NA in comparison to imipramine in the OB rat model of depression. Behavioural and neuroimaging parameters were measured to further explore a role for L-NA in producing an antidepressant related response in the model. In order to fulfil the aim, there were a number of objectives including (1) the development of a behavioural parameter related to OB hyperactivity which is not subject to habituation following repeated exposure to the “open field”, enabling onset of antidepressant activity to be determined in the same animals by repeated exposure to the test environment; (2) to determine onset of action of L-NA in the “open field” test by comparison to the reference antidepressant and positive control imipramine; (3) to investigate the expressional levels of the three isoforms of the NOS enzyme to determine if antidepressant-like effects of NOS inhibition might be related to an enhanced expression of a specific NOS isoform induced by the OB procedure; (4) to assess the

selective nNOS inhibitor TRIM for antidepressant activity in the model; (5) to develop neuroimaging markers in the model using a multimodal MRI approach and (6) to determine physiological responses to stress in the model using radiotelemetric devices.

## 4.2 Materials and Methods

### 4.2.1 Subjects and drug treatment

Male Sprague-Dawley rats weighing 250-320 g at the beginning of the studies were housed in groups of four. Food and water were available *ad libitum*.

Imipramine hydrochloride was prepared as a solution in maple syrup to a final concentration of 20 mg/ml and administered orally in a volume of 1 ml/kg to deliver a dose of 20 mg/kg. L-NA was also prepared in maple syrup to a final concentration of 5 mg/ml and administered orally in a volume of 2 ml/kg to deliver a dose of 10 mg/kg.

As drugs had to be administered for almost 20 days, oral administration was preferred over daily i.p. injections.

TRIM was dissolved by sonication in saline solution to reach a final concentration of 50 mg/ml which was administered subcutaneously at 1 ml/kg in a dose of 50 mg/kg. Imipramine and L-NA were administered once daily for 17 days. On the day of open field tests the drugs were administered at least 20 hr prior to test on the previous day. TRIM was administered once daily for 7 days and the open field test was carried out 24 hr following the last treatment. Doses of the drugs were chosen on the basis that they had been previously reported to possess antidepressant-like activity in rats in the FST and TST (Doucet et al., 2012; Koike et al., 2011; Gigliucci et al., 2010; Réus et al., 2011; Volke et al., 2003).

All procedures were approved by the Animal Ethics Committee Trinity College Dublin and were in accordance with the European Council Directive 1986 (86/806/EEC).



#### **4.2.2 Bilateral olfactory bulbectomy (OB)**

The surgical removal of both the right and left olfactory bulbs was performed as described in the methods section. Rats were given 14 days recovery prior to any further treatment or behavioural tests and were handled daily throughout the recovery period to eliminate any aggressiveness that would otherwise arise. Rats receiving implantation of biotelemetry devices were given 7 days recovery after OB surgery before undergoing telemetry surgery.

#### **4.2.3 Experimental design**

All the behavioural studies started two weeks after bulbectomy surgery. To investigate the persistence of hyperactivity of OB rats in a regime of repeated open field testing, a preliminary study with straight comparison between drug-free shams and OBs was carried out. The open field was performed every 7 days from day 14 up to day 28 post-surgery [Fig. 3.1].

In studies involving drug treatments, following the initial open field test rats in each study were subdivided into treatment groups such that there were no differences in activity within the sham or OB subgroups.

For the Imipramine/L-NA experiment, subgroups were as follows: group 1: Sham + Vehicle, group 2: Sham + Imipramine (20 mg/kg), group 3: Sham + L-NA (10 mg/kg), group 4: OB + Vehicle, group 5: OB + Imipramine (20 mg/kg), group 6: OB + L-NA (10 mg/kg). Animals received imipramine, L-NA or vehicle orally for 17 days and once treatment commenced the open field test was repeated every four days. On the days of

testing, drug administration was delayed until the open field test was carried out such that all open field testing was conducted at least 20 hr following prior drug administration.

For the TRIM experiment, following the initial open field test animals were subdivided into treatment groups as follows: group 1: Sham + Vehicle, group 2: Sham + TRIM (50 mg/kg), group 3: OB + Vehicle and group 4: OB + TRIM (50 mg/kg). Animals received TRIM (s.c.) or vehicle once daily for 7 days and the open field was performed at least 20 hr following the last drug injection.

#### **4.2.4 *Open field test***

Open field test was carried out as described in the Methods section. Illumination was provided by floor lamps positioned in the room to provide an equal dispersion of light (200-250 lux) and to avoid casting shadows in the open field as these conditions are optimal for the determination of OB related hyperactivity (Mar et al., 2002; Kelly et al., 1997).

Behaviour in the open field was recorded and distance moved was measured in the first 3 minutes using the Menu2020 software (HVS Image Ltd, UK). With repeated exposure of the same animal to the open field, OB-related hyperactivity falls and habituates to the test environment such that it is not distinguishable from the activity levels of sham-operated controls [Fig. 3.1a]. Repeated testing with the parameter distance moved is of limited value therefore especially where the objective of repeated testing is to determine the onset of antidepressant activity in the same animals over time. However from

observation, despite habituation with repeated exposure, it remains clear that bulbectomised animals continue to behave in an agitated manner best described as engaging in short bursts of rapid locomotion. By taking the distance of individual bursts or bouts of activity over the observation period and calculating the speed of these individual bouts it was possible to devise a new parameter which relates to the hyperactive nature of the behaviour exhibited by the OB rat which does not habituate upon repeated exposure [Fig. 3.1b].

Speed of bout was recorded by calculating the distance moved during individual bouts of horizontal activity and dividing by the duration in seconds of the bout over the course of the 3 minute test. A bout of movement was defined as the rat moving continuously to a point at least 30 cm away from the starting point of origin. Activity shorter than 1 second was not scored as the software Menu2020 accepts 1 second as the minimal accessible time unit for analysis. Speed of bout for each rat was calculated by averaging speeds of all bouts of activity calculated during the trial. In all cases behavioural analysis was carried out by a single investigator who was blind to the group treatments.

#### ***4.2.5 Magnetic Resonance Imaging (MRI)***

MRI scans were carried out on the day following the last open field test, 20-24 hr after the last drug treatment. The protocol used was the one described in the Methods section.

Bolus tracking arterial spin labelling (btASL) was adopted to determine if cerebral perfusion was changed in the OB rat model and if drug treatments might affect this measure. Mean transit time (MTT) and capillary transit time (CTT) were analysed as

MTT is inversely proportional to CBF, while CTT is inversely proportional to CBF squared. The analysis also included btASL signal amplitude, as it represents an indication of CBV (see Kelly et al., 2009; 2010).

T1 and T2 relaxation time changes were evaluated as they have been proposed to be potential markers for changes in tissue water content and possible astrocytic activation (Cowley et al., 2012; Sibson et al., 2008). All analysis was performed using the image sequence analysis (ISA) tool in the Bruker Paravision 4.0 Software package. Regions of interest selected using the ROI tool included the auditory cortex, hippocampus, parietal association cortex, retrosplenial cortex, thalamus and visual cortex bilaterally as well as the third ventricle.

Ventricular and hippocampal volumes were assessed as well as per the protocol previously described in the methods section.

#### ***4.2.6 Determination of cortical NOS expression***

Rats were euthanised by decapitation 24 hr following the MRI scan. The brains were rapidly removed and checked for signs of cortical damage or incomplete removal of the olfactory bulbs. The frontal cortex was dissected on an ice cold plate and placed in RNase-free tubes, snap frozen in liquid nitrogen and transferred to a  $-80^{\circ}\text{C}$  freezer until further processing for total RNA extraction, cDNA synthesis and Real-Time PCR performance as described in the Methods section. Target genes were eNOS, nNOS, iNOS, the astrocytic activation marker glial fibrillary acidic protein (GFAP) and the microglial marker CD11b, and they were identified by FAM-labeled probes, while the housekeeping gene of reference,  $\beta$ -actin, was identified by a VIC-labeled probe. At the

end the RQ values obtained from the 7300 System Software were converted into fold change values relative to control group.

#### ***4.2.7 Effects of TRIM in the OB rat model***

An independent experiment was carried out to determine the effects of TRIM. In order to evaluate physiological parameters all animals were implanted with radiotelemetric transponders one week following OB surgery as described in the Methods section. The animals were allowed to recover for an additional week following radiotransponder implantation prior to testing in the open field. The open field test was carried out as described above 24 hr following the 7 day treatment schedule. Body temperature and heart rate were continuously assessed except during the open-field test, on account of a technical limitation of the telemetric receiving devices, and an average computed at 5-min intervals for 15 min before and 60 min following open-field exposure. For determination of the change in temperature and heart rate, a baseline was calculated by averaging samples collected before open-field exposure, which was subtracted from all values to determine changes from baseline average. Data post-stressor exposure was compared to the baseline average.

#### ***4.2.8 Statistical analysis***

Data are expressed as group mean with SEM and were analysed by two factor, two factor repeated measures or three factor repeated measure analysis of variance (ANOVA) where appropriate. When datasets did not meet the normality criteria,

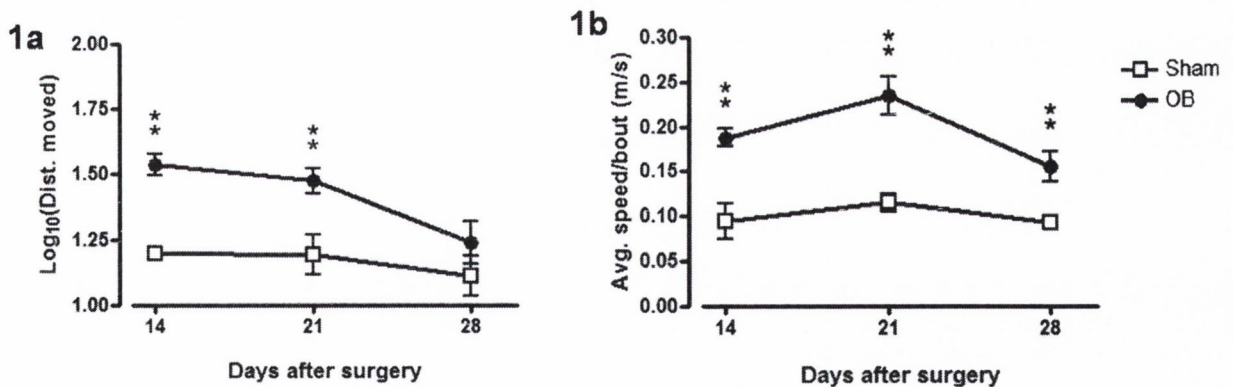
logarithmic transformation of the data was performed before applying the appropriate statistical test. If any statistically significant change was found, *post hoc* comparisons were performed using a Fisher's LSD test. In all cases data were deemed significant when  $p < 0.05$ . All analysis was carried out with GB-Stat v10 statistical package.



### 4.3 Results

#### 4.3.1 OB related hyperactivity in the open field test

ANOVA of the logarithmic transformation of distance moved showed effects of OB [ $F_{(1,16)} = 25.50, p < 0.01$ ] and time [ $F_{(2,12)} = 1.63, p < 0.05$ ]. *Post hoc* comparisons revealed that OB rats moved over a greater distance when compared to sham-operated controls on days 14 and 21 ( $p < 0.01$ ) but not 28 days following surgery [Fig. 4.1a]. By contrast ANOVA of bout speed showed effects of OB [ $F_{(1,16)} = 47.89, p < 0.01$ ] and time [ $F_{(2,12)} = 6.91, p < 0.01$ ] where *post hoc* comparisons revealed that the average speed of activity bout was greater in OB rats when compared to sham-operated controls at all of the time points analysed ( $p < 0.01$ ) [Fig. 4.1b]. Speed of bout was not subject to habituation in a similar fashion to distance moved in the test arena.

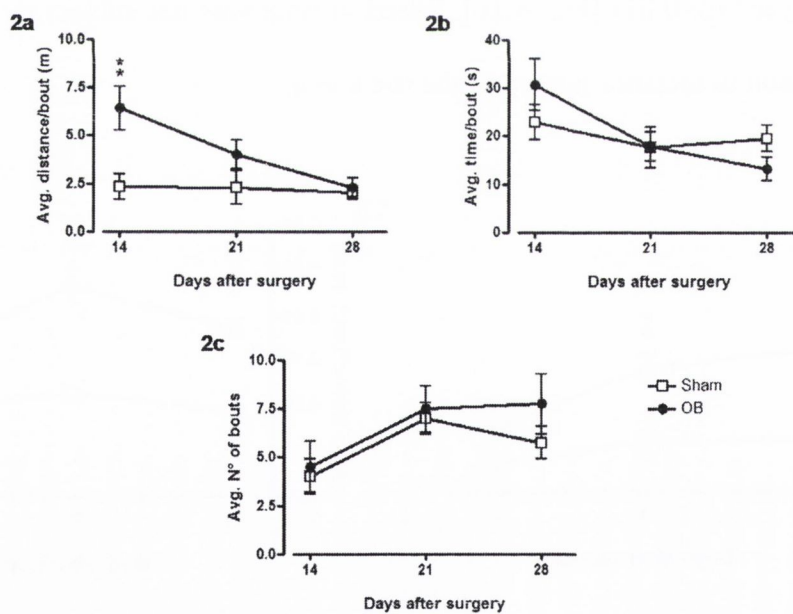


**Fig. 4.1** Effects of repeated open field testing on locomotor activity parameters in the bulbectomised rat model of depression. The first 3 min the rats spent in the open field were analysed for both distance moved (Fig. 4.1a) and average speed/bout (Fig. 4.1b). Logarithmic transformation of distance moved data was performed to meet normality. Data are expressed as mean and SEM of 4 animals. \*\* $p < 0.01$  compared to Sham-operated controls.



### 4.3.2 The increased speed of bout in the OB rats is not determined by the alteration of a single speed-related parameter

ANOVA of average distance per bout showed effects of OB [ $F_{(1,16)} = 14.36, p < 0.01$ ] and time [ $F_{(2,12)} = 3.99, p < 0.05$ ]. *Post hoc* comparisons revealed that OB rats covered a greater distance for each bout of movement 14 days after surgery when compared to sham-operated controls ( $p < 0.01$ ), but this parameter normalised with repeated open field testing [Fig. 4.2a]. ANOVA of average time per bout showed effects of time only [ $F_{(2,12)} = 4.19, p < 0.05$ ]. *Post hoc* comparisons did not reveal any differences in OB rats when compared to sham-operated controls at any of the time points analysed [Fig. 4.2b]. ANOVA of average number of bouts in 3 minutes showed no effects of neither OB nor time [Fig. 4.2c].

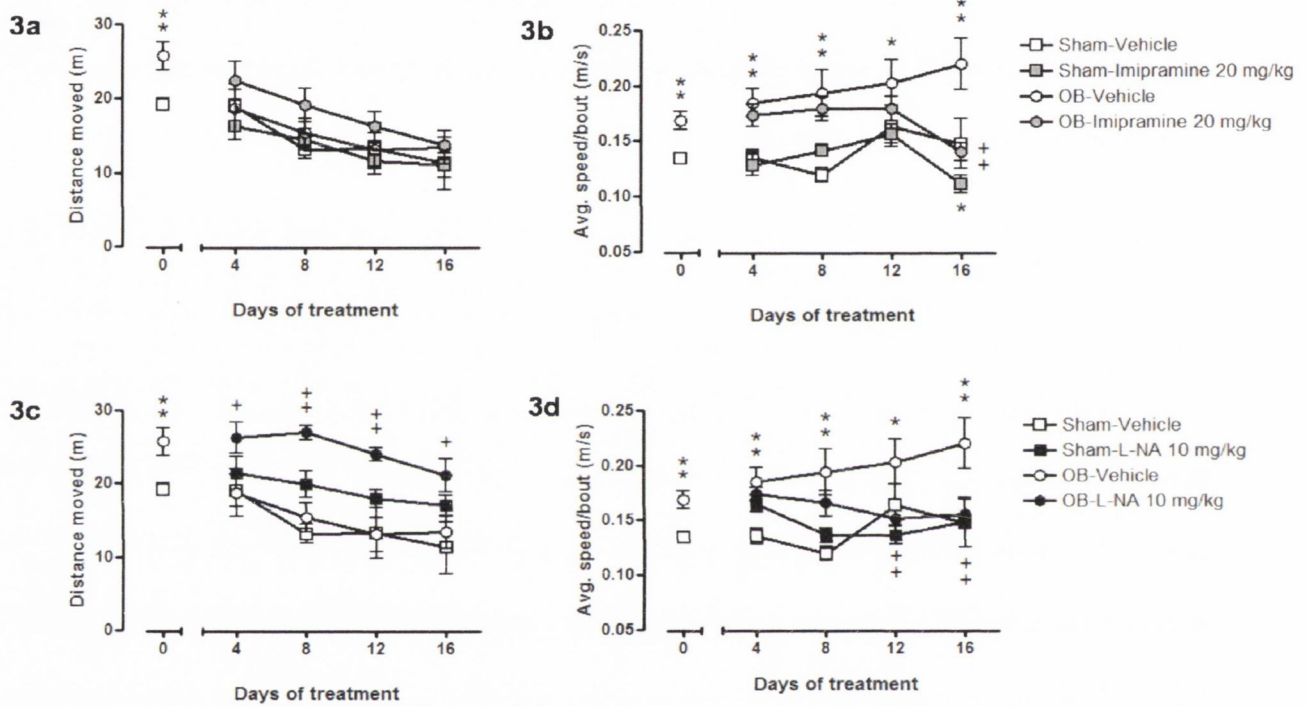


**Fig. 4.2** Characterisation of speed related parameters in the bulbectomised rat model of depression. The first 3 min the rats spent in the open field were analysed for average distance moved (Fig. 4.2a) and average duration (Fig. 4.2b) of each bout of movement. The average number of bouts performed over the 3 min interval was also analysed (Fig. 4.2c). Data are expressed as mean and SEM of 4 animals. \*\* $p < 0.01$  compared to Sham-operated controls.

**4.3.3 L-NA attenuates the habituation of distance moved to repeated open field testing in OB rats and the OB related increase in activity bout speed and also shows a faster onset of action when compared to the tricyclic antidepressant imipramine**

Prior to drug treatment there was higher average distance moved (Student's *t*-test; [ $t_{(43)} = 3.11, p < 0.01$ ]) and bout speed (Student's *t*-test; [ $t_{(43)} = 3.83, p < 0.01$ ]) in OB rats when compared to sham-operated controls. ANOVA of distance moved showed effects of OB [ $F_{(1,39)} = 8.55, p < 0.01$ ], drug [ $F_{(2,39)} = 12.58, p < 0.0001$ ] and time [ $F_{(3,117)} = 23.38, p < 0.0001$ ]. *Post hoc* comparisons revealed no differences between any of the groups analysed with the exception of OB rats treated with L-NA, which showed a persistent significantly higher distance moved when compared to OB-vehicle controls ( $p < 0.05$ ) [Fig. 4.3a and c].

ANOVA of bout speeds showed effects of OB [ $F_{(1,40)} = 40.51, p < 0.0001$ ], drug [ $F_{(2,40)} = 4.52, p < 0.05$ ], an OB x drug [ $F_{(2,40)} = 4.69, p < 0.05$ ] and a drug x time interaction [ $F_{(6,120)} = 6.28, p < 0.0001$ ]. *Post hoc* comparisons revealed that speeds were increased in OB rats compared to sham-operated controls at all the time points analysed ( $p < 0.05$ ) and that imipramine attenuated the OB related effect following 16 days of treatment ( $p < 0.01$ ) [Fig. 4.3b]. 16 days of imipramine treatment also decreased speed of bout in sham-operated rats when compared to sham-operated controls ( $p < 0.05$ ) [Fig. 4.3b]. L-NA also attenuated the OB related effect on speed of bout, following 12 and 16 days of treatment ( $p < 0.01$ ), thus L-NA provoked an earlier onset of action when compared to imipramine [Fig. 4.3d].



**Fig. 4.3** Effects of imipramine and L-NA administration in the bulbectomised rat model of depression. After the first open field test (left part of the graphs) rats were subdivided into the 6 groups and received daily oral imipramine (Fig. 4.3a and b) or L-NA (Fig. 4.3c and d) for 16 days. The first 3 min the rats spent in the open field were analysed for distance moved (Fig. 4.3a and c) and average speed/bout (Fig. 4.3b and d). Data are expressed as mean and SEM of 6-9. \* $p < 0.05$ ; \*\* $p < 0.01$  compared to Sham-vehicle group; + $p < 0.05$ , ++ $p < 0.01$  compared to OB-vehicle group.

#### 4.3.4 MR neuroimaging markers in the OB rat model

Statistical analysis of MRI data was performed on the imipramine and L-NA datasets separately, as in some cases the effects of the two drug treatments were masking each other.

##### 4.3.4.1 OB is associated with changes in ventricular volume

ANOVA of ventricular volume showed effects of OB [ $F_{(1,23)} = 21.62, p < 0.01$ ] and an OB x L-NA interaction [ $F_{(1,23)} = 5.34, p < 0.05$ ]. *Post hoc* comparisons revealed that ventricular volume was increased in OB-L-NA rats compared to OB-vehicle controls only ( $p < 0.05$ ) [Table 4.1]. An example of enlarged ventricles provoked by OB surgery is shown in Fig. 4.4.

Analysis of hippocampal volume did not show any difference between OB rats and sham-operated controls and no effects of drug treatment between the groups [Table 4.1].

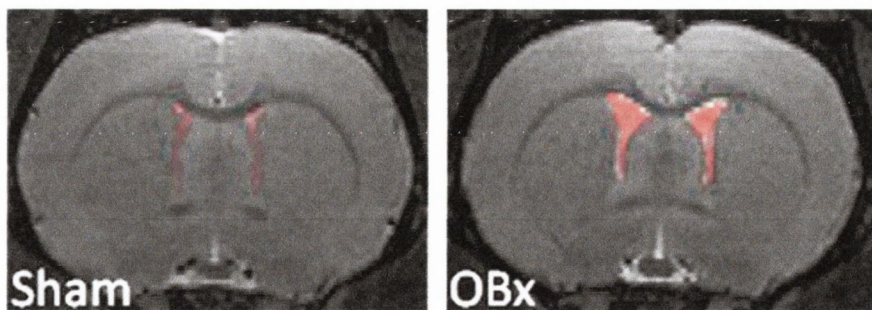


Fig. 4.4 Representative high resolution MRI scans depicting enlarged ventricles following bullectomy.

#### ***4.3.4.2 L-NA treatment is associated with regional changes in resting state blood perfusion***

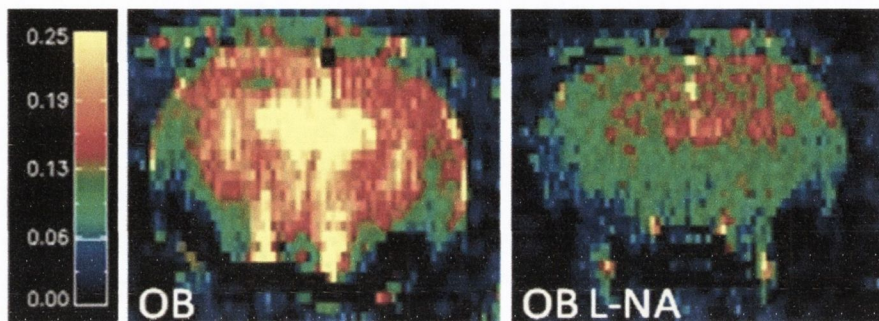
ANOVA of MTT times showed effects of L-NA in right auditory cortex [ $F_{(1,24)} = 6.13$ ,  $p < 0.05$ ] and right [ $F_{(1,24)} = 8.19$ ,  $p < 0.01$ ] and left [ $F_{(1,24)} = 4.75$ ,  $p < 0.05$ ] hippocampus. An OB x L-NA interaction was found in the left auditory cortex [ $F_{(1,24)} = 5.34$ ,  $p < 0.05$ ], while OB x imipramine [ $F_{(1,27)} = 4.49$ ,  $p < 0.05$ ] and OB x L-NA [ $F_{(1,23)} = 6.21$ ,  $p < 0.05$ ] interactions were obtained in the ventricle. *Post hoc* comparisons revealed that MTT time was significantly decreased by L-NA in the left auditory cortex in sham-L-NA rats when compared to sham-vehicle controls ( $p < 0.01$ ) [Table 4.1]. In the ventricle, MTT was increased by imipramine in OB rats when compared to OB-vehicle controls ( $p < 0.05$ ). A similar effect in OB rats was provoked by L-NA in right and left hippocampus when compared to OB-vehicle controls ( $p < 0.01$ ) [Table 4.1].

ANOVA of CTT times showed significant effects of OB in the left thalamus [ $F_{(1,27)} = 4.50$ ,  $p < 0.05$ ], of L-NA in the right hippocampus [ $F_{(1,24)} = 7.23$ ,  $p < 0.05$ ], and OB x imipramine [ $F_{(1,27)} = 4.55$ ,  $p < 0.05$ ] and OB x L-NA [ $F_{(1,23)} = 5.17$ ,  $p < 0.05$ ] interactions in the ventricle. *Post hoc* comparisons revealed that CTT time was increased by L-NA in the right hippocampus and in the ventricle when compared to OB-vehicle controls ( $p < 0.05$  and  $p < 0.01$  respectively) [Table 4.1].

ANOVA of amplitude showed significant effects of L-NA in the left auditory cortex [ $F_{(1,23)} = 9.14$ ,  $p < 0.01$ ], right [ $F_{(1,24)} = 4.93$ ,  $p < 0.05$ ] and left [ $F_{(1,24)} = 4.96$ ,  $p < 0.05$ ] thalamus and in the ventricle [ $F_{(1,24)} = 6.01$ ,  $p < 0.05$ ]. *Post hoc* comparisons revealed that L-NA significantly increased amplitude in the left auditory cortex in sham L-NA-treated rats when compared to sham-vehicle controls ( $p < 0.05$ ), while it reduced it in the right

and left thalamus of OB L-NA-treated rats when compared to OB-vehicle controls ( $p < 0.05$ ) [Table 4.1].

A representative cerebral blood perfusion map depicting effects of L-NA treatment on cerebral blood perfusion in OB rats can be seen in Fig. 4.5.



**Fig. 4.5** Representative cerebral blood perfusion map depicting decreased cerebral blood perfusion following L-NA treatment in OB rats.

**Table 4.1** Regional brain volume and regional resting state blood perfusion in the OB model of depression (Continues on next page)

		Sham			OB		
		Vehicle	Imipramine	L-NA	Vehicle	Imipramine	L-NA
Hippocampal volume (% of control %volume)		100.00 ± 6.186	95.07 ± 2.606	105.20 ± 3.790	99.99 ± 4.492	99.25 ± 2.284	102.90 ± 4.148
Ventricular volume (% of control % volume)		100.00 ± 8.249	86.24 ± 4.899	79.46 ± 8.483	146.70 ± 15.70	200.60 ± 31.37	<b>218.00 ± 37.31 +</b>
Right Auditory Cortex	MTT (s)	2.033 ± 0.132	1.911 ± 0.102	1.735 ± 0.117	1.938 ± 0.100	1.909 ± 0.098	1.678 ± 0.092
	CTT (s)	1.624 ± 0.130	1.579 ± 0.102	1.490 ± 0.097	1.547 ± 0.104	1.481 ± 0.109	1.430 ± 0.066
	Amplitude (a.u.)	0.080 ± 0.008	0.091 ± 0.010	0.099 ± 0.002	0.086 ± 0.009	0.087 ± 0.009	0.093 ± 0.004
Left Auditory Cortex	MTT (s)	2.190 ± 0.095	1.951 ± 0.119	<b>1.598 ± 0.101 **</b>	1.933 ± 0.135	1.962 ± 0.080	2.013 ± 0.237
	CTT (s)	1.727 ± 0.163	1.525 ± 0.094	1.391 ± 0.203	1.518 ± 0.146	1.567 ± 0.283	1.669 ± 0.340
	Amplitude (a.u.)	0.074 ± 0.005	0.090 ± 0.010	<b>0.095 ± 0.003 *</b>	0.078 ± 0.008	0.081 ± 0.008	0.092 ± 0.006
Right Hippocampus	MTT (s)	1.573 ± 0.068	1.523 ± 0.082	1.725 ± 0.096	1.558 ± 0.107	1.610 ± 0.097	<b>2.000 ± 0.138 ++</b>
	CTT (s)	1.394 ± 0.065	1.398 ± 0.087	1.504 ± 0.089	1.416 ± 0.105	1.452 ± 0.098	<b>1.841 ± 0.132 ++</b>
	Amplitude (a.u.)	0.095 ± 0.007	0.113 ± 0.011	0.097 ± 0.003	0.112 ± 0.013	0.099 ± 0.001	0.090 ± 0.054
Left Hippocampus	MTT (s)	1.574 ± 0.074	1.567 ± 0.104	1.716 ± 0.095	1.594 ± 0.136	1.606 ± 0.083	<b>1.891 ± 0.035 +</b>
	CTT (s)	1.436 ± 0.089	1.565 ± 0.132	1.486 ± 0.087	1.491 ± 0.160	1.402 ± 0.076	1.693 ± 0.024
	Amplitude (a.u.)	0.097 ± 0.008	0.113 ± 0.013	0.099 ± 0.003	0.093 ± 0.009	0.086 ± 0.009	0.092 ± 0.004
Right Thalamus	MTT (s)	1.459 ± 0.087	1.408 ± 0.053	1.757 ± 0.190	1.482 ± 0.099	1.497 ± 0.082	1.491 ± 0.047
	CTT (s)	1.379 ± 0.117	1.214 ± 0.087	1.428 ± 0.098	1.354 ± 0.096	1.312 ± 0.082	1.329 ± 0.054
	Amplitude (a.u.)	0.115 ± 0.010	0.129 ± 0.013	0.104 ± 0.005	0.133 ± 0.017	0.122 ± 0.013	<b>0.094 ± 0.004 +</b>
Left Thalamus	MTT (s)	1.600 ± 0.120	1.434 ± 0.074	1.500 ± 0.062	1.519 ± 0.089	1.712 ± 0.133	1.497 ± 0.063
	CTT (s)	1.330 ± 0.077	1.271 ± 0.064	1.355 ± 0.079	1.442 ± 0.095	1.664 ± 0.166	1.346 ± 0.053
	Amplitude (a.u.)	0.113 ± 0.010	0.125 ± 0.013	0.099 ± 0.003	0.125 ± 0.015	0.115 ± 0.013	<b>0.092 ± 0.003 +</b>
Ventricle	MTT (s)	2.070 ± 0.172	1.870 ± 0.117	1.653 ± 0.158	1.689 ± 0.120	<b>2.069 ± 0.138 +</b>	2.150 ± 0.258
	CTT (s)	1.597 ± 0.253	1.305 ± 0.195	1.356 ± 0.065	1.084 ± 0.186	1.584 ± 0.121	<b>1.656 ± 0.170 +</b>
	Amplitude (a.u.)	0.143 ± 0.020	0.157 ± 0.022	0.103 ± 0.004	0.159 ± 0.027	0.135 ± 0.022	0.109 ± 0.002

**Table 4.1 (Continued)** Regional resting state blood perfusion in the OB model of depression

		Sham			OB		
		Vehicle	Imipramine	L-NA	Vehicle	Imipramine	L-NA
Right Visual Cortex	MTT (s)	1.924 ± 0.050	1.748 ± 0.110	1.853 ± 0.072	1.785 ± 0.128	1.866 ± 0.072	1.892 ± 0.065
	CTT (s)	1.604 ± 0.052	1.453 ± 0.094	1.489 ± 0.166	1.661 ± 0.115	1.625 ± 0.090	1.609 ± 0.102
	Amplitude (a.u.)	0.083 ± 0.006	0.099 ± 0.010	0.096 ± 0.003	0.092 ± 0.009	0.087 ± 0.006	0.090 ± 0.005
Left Visual Cortex	MTT (s)	2.034 ± 0.101	1.848 ± 0.100	2.032 ± 0.114	2.009 ± 0.153	1.997 ± 0.053	1.898 ± 0.162
	CTT (s)	1.710 ± 0.156	1.595 ± 0.124	1.735 ± 0.191	1.661 ± 0.147	1.723 ± 0.079	1.634 ± 0.194
	Amplitude (a.u.)	0.082 ± 0.006	0.096 ± 0.009	0.097 ± 0.003	0.082 ± 0.008	0.082 ± 0.007	0.097 ± 0.009
Right Parietal Association Cortex	MTT (s)	1.632 ± 0.066	1.625 ± 0.090	1.635 ± 0.069	1.742 ± 0.052	1.748 ± 0.075	1.654 ± 0.052
	CTT (s)	1.462 ± 0.051	1.394 ± 0.081	1.329 ± 0.141	1.433 ± 0.053	1.437 ± 0.053	1.491 ± 0.050
	Amplitude (a.u.)	0.087 ± 0.007	0.100 ± 0.010	0.103 ± 0.003	0.091 ± 0.008	0.088 ± 0.008	0.095 ± 0.004
Left Parietal Association Cortex	MTT (s)	1.727 ± 0.064	1.751 ± 0.105	1.599 ± 0.113	1.896 ± 0.090	1.833 ± 0.093	<b>1.611 ± 0.083 +</b>
	CTT (s)	1.443 ± 0.061	1.479 ± 0.112	1.302 ± 0.171	1.680 ± 0.113	1.502 ± 0.075	1.422 ± 0.064
	Amplitude (a.u.)	0.090 ± 0.007	0.094 ± 0.008	0.099 ± 0.003	0.090 ± 0.008	0.084 ± 0.008	0.096 ± 0.006
Right Retrosplenial Cortex	MTT (s)	1.889 ± 0.136	1.835 ± 0.130	2.098 ± 0.151	1.807 ± 0.118	1.917 ± 0.126	2.095 ± 0.153
	CTT (s)	1.569 ± 0.138	1.442 ± 0.094	1.796 ± 0.112	1.462 ± 0.126	1.596 ± 0.122	1.816 ± 0.127
	Amplitude (a.u.)	0.091 ± 0.007	0.101 ± 0.012	0.099 ± 0.004	0.094 ± 0.010	0.091 ± 0.010	0.094 ± 0.004
Left Retrosplenial Cortex	MTT (s)	1.849 ± 0.109	1.742 ± 0.119	1.853 ± 0.099	1.749 ± 0.116	1.820 ± 0.094	1.780 ± 0.074
	CTT (s)	1.540 ± 0.110	1.549 ± 0.134	1.499 ± 0.088	1.563 ± 0.164	1.574 ± 0.112	1.597 ± 0.101
	Amplitude (a.u.)	0.088 ± 0.007	0.106 ± 0.012	0.095 ± 0.004	0.093 ± 0.009	0.086 ± 0.009	0.092 ± 0.004



**Table 4.1** Summary of MR imaging markers for regional brain volume and regional resting state blood perfusion in the OB model of depression following chronic oral treatment with imipramine and L-NA. MTT = mean transitory time, CTT = capillary transitory time, a.u. = arbitrary units. Data are expressed as mean and SEM of 6-8 animals. \* $p < 0.05$ , \*\* $p < 0.01$  compared to Sham-vehicle group, + $p < 0.05$ , ++ $p < 0.01$  compared to OB-vehicle group.

#### **4.3.4.3 L-NA treatment is associated with changes in T1 and T2 relaxation times**

ANOVA of T1 relaxation times showed a significant effect of L-NA in right visual cortex [ $F_{(1,24)} = 9.63$ ,  $p < 0.01$ ], right parietal association cortex [ $F_{(1,24)} = 4.25$ ,  $p = 0.05$ ], right [ $F_{(1,24)} = 6.10$ ,  $p < 0.05$ ] and left [ $F_{(1,24)} = 6.42$ ,  $p < 0.05$ ] retrosplenial cortex, right [ $F_{(1,23)} = 4.81$ ,  $p < 0.05$ ] and left [ $F_{(1,24)} = 5.26$ ,  $p < 0.05$ ] hippocampus and right [ $F_{(1,23)} = 23.40$ ,  $p < 0.01$ ] and left [ $F_{(1,24)} = 9.69$ ,  $p < 0.01$ ] thalamus. Moreover, an OB x L-NA interaction was found in left visual cortex [ $F_{(1,24)} = 4.15$ ,  $p = 0.05$ ] and left hippocampus [ $F_{(1,24)} = 5.26$ ,  $p < 0.05$ ]. *Post hoc* comparisons revealed that T1 time was increased in sham-L-NA rats compared to sham-vehicle controls in the right parietal association cortex and the right thalamus ( $p < 0.05$ ). T1 times were significantly higher in OB-L-NA rats when compared to OB-vehicle controls in right and left visual cortex, right and left retrosplenial cortex, right and left hippocampus and right and left thalamus ( $p < 0.05$  and  $p < 0.01$ ) [Table 4.2].

ANOVA of T2 relaxation times showed a significant effect of OB surgery in right [ $F_{(1,28)} = 8.57$ ,  $p < 0.01$ ] and left [ $F_{(1,28)} = 8.84$ ,  $p < 0.01$ ] visual cortex, left retrosplenial cortex [ $F_{(1,28)} = 9.60$ ,  $p < 0.01$ ], left hippocampus [ $F_{(1,28)} = 4.87$ ,  $p < 0.05$ ] and left thalamus [ $F_{(1,28)} = 5.50$ ,  $p < 0.05$ ]. Imipramine effect was only seen in the right hippocampus [ $F_{(1,28)} = 5.27$ ,  $p < 0.05$ ], while L-NA effect was seen in the right visual cortex [ $F_{(1,24)} = 9.95$ ,  $p < 0.01$ ], right retrosplenial cortex [ $F_{(1,24)} = 7.10$ ,  $p < 0.05$ ], right

hippocampus [ $F_{(1,23)} = 15.74$ ,  $p < 0.01$ ] and right [ $F_{(1,23)} = 4.82$ ,  $p < 0.05$ ] and left [ $F_{(1,24)} = 12.09$ ,  $p < 0.01$ ] thalamus. An OB x imipramine interaction was found only in the right hippocampus [ $F_{(1,28)} = 7.49$ ,  $p < 0.05$ ], while OB x L-NA interactions were seen in left retrosplenial cortex [ $F_{(1,24)} = 4.66$ ,  $p < 0.05$ ], right hippocampus [ $F_{(1,23)} = 10.74$ ,  $p < 0.01$ ] and left thalamus [ $F_{(1,24)} = 5.95$ ,  $p < 0.05$ ]. *Post hoc* comparisons revealed that T2 time was decreased in OB rats when compared to sham-vehicle controls in the right and left visual cortex, left retrosplenial cortex and right hippocampus ( $p < 0.05$  and  $p < 0.01$ ). T2 times were normalised in OB rats by imipramine in the right hippocampus and by L-NA in the right visual cortex, the right and left retrosplenial cortex and the right hippocampus when compared to OB-vehicle controls ( $p < 0.05$  and  $p < 0.01$ ). Moreover L-NA increased T2 time in OB-LNA treated animals when compared to OB vehicle-treated controls in the right and left thalamus ( $p < 0.01$ ) [Table 4.2].

**Table 4.2** Regional T1 and T2 relaxation times in the OB model of depression (Continues on next page)

		Sham			OB		
		Vehicle	Imipramine	L-NA	Vehicle	Imipramine	L-NA
Right Visual Cortex	T1 (ms)	1942.00 ± 23.60	1915.00 ± 28.01	2008.00 ± 37.25	1914.00 ± 19.27	1918.00 ± 16.58	<b>2069.00 ± 60.66 ++</b>
	T2 (ms)	52.17 ± 0.42	52.27 ± 0.36	52.69 ± 0.25	<b>51.08 ± 0.28 *</b>	51.41 ± 0.27	<b>52.58 ± 0.30 ++</b>
Left Visual Cortex	T1 (ms)	1967.00 ± 35.68	1971.00 ± 33.37	1937.00 ± 25.48	1930.00 ± 24.80	2001.00 ± 23.73	<b>2027.00 ± 39.45 +</b>
	T2 (ms)	52.53 ± 0.25	52.69 ± 0.32	52.51 ± 0.38	<b>51.62 ± 0.31 *</b>	51.81 ± 0.30	52.64 ± 0.92
Right Retrosplenial Cortex	T1 (ms)	1812.00 ± 21.94	1814.00 ± 20.30	1859.00 ± 35.77	1793.00 ± 11.66	1811.00 ± 20.07	<b>1882.00 ± 38.71 +</b>
	T2 (ms)	48.86 ± 0.46	48.65 ± 0.43	49.41 ± 0.33	47.75 ± 0.35	48.38 ± 0.27	<b>49.33 ± 0.46 +</b>
Left Retrosplenial Cortex	T1 (ms)	1820.00 ± 33.77	1824.00 ± 17.36	1839.00 ± 21.94	1792.00 ± 11.30	1843.00 ± 27.31	<b>1899.00 ± 29.84 ++</b>
	T2 (ms)	49.64 ± 0.71	48.96 ± 0.32	49.43 ± 0.51	<b>47.65 ± 0.27 **</b>	48.16 ± 0.43	<b>49.82 ± 0.69 +</b>
Right Hippocampus	T1 (ms)	1884.00 ± 16.46	1901.00 ± 27.64	1933.00 ± 22.59	1889.00 ± 20.28	1904.00 ± 14.95	<b>1988.00 ± 63.98 +</b>
	T2 (ms)	52.28 ± 0.35	52.16 ± 0.25	52.65 ± 0.35	<b>51.24 ± 0.25 *</b>	52.64 ± 0.27 ++	<b>55.13 ± 1.03 ++</b>
Left Hippocampus	T1 (ms)	1930.00 ± 25.76	1909.00 ± 16.63	1918.00 ± 14.26	1893.00 ± 29.62	1943.00 ± 23.84	<b>2029.00 ± 34.54 +</b>
	T2 (ms)	53.51 ± 0.41	53.31 ± 0.40	53.79 ± 0.46	52.73 ± 0.43	52.51 ± 0.18	53.97 ± 0.83
Right Thalamus	T1 (ms)	1623.00 ± 21.14	1623.00 ± 23.75	<b>1694.00 ± 16.24 *</b>	1601.00 ± 21.12	1650.00 ± 21.06	<b>1709.00 ± 12.49 ++</b>
	T2 (ms)	46.11 ± 0.38	46.52 ± 0.46	47.65 ± 0.10	46.74 ± 0.57	46.37 ± 0.26	<b>48.09 ± 0.35 ++</b>
Left Thalamus	T1 (ms)	1639.00 ± 29.77	1643.00 ± 14.95	1687.00 ± 22.95	1610.00 ± 23.76	1663.00 ± 15.69	<b>1710.00 ± 10.15 ++</b>
	T2 (ms)	46.65 ± 0.29	46.83 ± 0.24	47.10 ± 0.21	46.12 ± 0.25	46.18 ± 0.23	<b>48.03 ± 0.46 ++</b>
Ventricle	T1 (ms)	1995.00 ± 68.60	1908.00 ± 61.51	2041.00 ± 56.97	2004.00 ± 32.85	2025.00 ± 88.03	2066.00 ± 95.54
	T2 (ms)	66.17 ± 2.41	65.26 ± 3.23	60.79 ± 2.98	63.53 ± 2.33	66.04 ± 3.01	64.18 ± 2.39

**Table 4.2 (Continued)** Regional T1 and T2 relaxation times in the OB model of depression

		Sham			OB		
		Vehicle	Imipramine	L-NA	Vehicle	Imipramine	L-NA
Right Auditory Cortex	T1 (ms)	1802.00 ± 29.72	1842.00 ± 29.16	1856.00 ± 18.88	1868.00 ± 12.61	1820.00 ± 23.18	1851.00 ± 20.73
	T2 (ms)	49.62 ± 0.39	50.07 ± 0.28	50.32 ± 0.43	49.59 ± 0.26	49.37 ± 0.23	50.44 ± 0.30
Left Auditory Cortex	T1 (ms)	1824.00 ± 37.34	1842.00 ± 40.66	1839.00 ± 23.54	1859.00 ± 29.70	1844.00 ± 23.12	1892.00 ± 33.13
	T2 (ms)	50.79 ± 0.52	50.42 ± 0.37	50.93 ± 0.47	50.62 ± 0.55	49.55 ± 0.24	50.46 ± 0.22
Right Parietal Association Cortex	T1 (ms)	1839.00 ± 15.56	1874.00 ± 30.21	<b>1939.00 ± 33.12 *</b>	1907.00 ± 20.65	1865.00 ± 18.08	1915.00 ± 34.15
	T2 (ms)	50.44 ± 0.30	50.49 ± 0.31	50.57 ± 0.37	50.43 ± 0.30	50.61 ± 0.25	50.13 ± 0.45
Left Parietal Association Cortex	T1 (ms)	1921.00 ± 37.29	1915.00 ± 31.55	1904.00 ± 20.78	1906.00 ± 12.00	1911.00 ± 25.15	1895.00 ± 29.15
	T2 (ms)	51.06 ± 0.31	51.43 ± 0.30	50.95 ± 0.29	51.06 ± 0.34	50.68 ± 0.31	51.06 ± 0.72

**Table 4.2** Summary of MR imaging markers for regional T1 and T2 relaxation times in the OB model of depression following chronic oral treatment with imipramine and L-NA. Data are expressed as mean and SEM of 6-8 animals. \*p<0.05, \*\*p<0.01 compared to Sham-vehicle group, +p<0.05, ++p<0.01 compared to OB-vehicle group.

### 4.3.5 OB is not associated with changes in cortical NOS expression

$\beta$ -actin was used as the reference housekeeping gene in this study and its validity was assessed by comparison of the cycle threshold (CT) between all the groups in the frontal cortex. No alterations in the expression of  $\beta$ -actin were found (Sham-Vehicle CT =  $19.74 \pm 0.07$ ; Sham-Imipramine CT =  $19.89 \pm 0.16$ ; OB-Vehicle CT =  $19.79 \pm 0.10$ ; OB-Imipramine CT =  $19.63 \pm 0.08$ ). No changes in the expression of n, e and iNOS were found in the frontal cortex of OB rats when compared to sham-operated controls [Table 4.3]. OB was associated with an up-regulation of the astrocytic marker GFAP in the frontal cortex [ $F_{(1,25)} = 7.92$ ,  $p < 0.01$ ] when compared to sham-operated controls. Treatment with imipramine reduced the OB related increase in expression of this marker whereas imipramine treatment alone failed to significantly influence its expression in sham-operated controls [Table 4.3]. The expression of the microglial marker CD11b was not affected neither by surgery nor imipramine [Table 4.3].

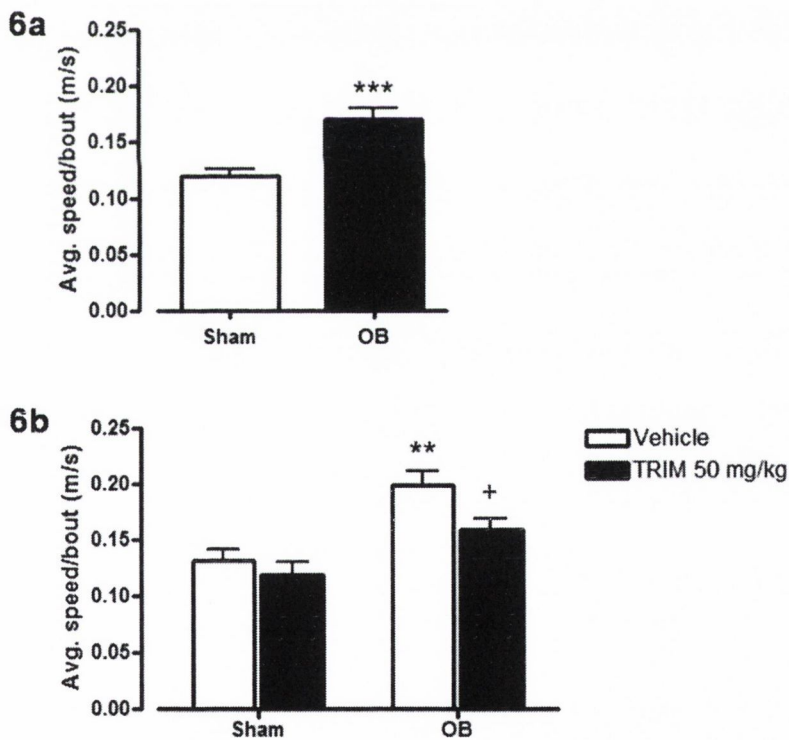
**Table 4.3** Effects of 16 days of imipramine treatment on expression of NOS isoforms and glial related markers in the bulbectomised rat model of depression.

Target	Sham-Vehicle	Fold Change vs Sham-Vehicle controls		
		Sham –Imipramine 20 mg/kg	OB-Vehicle	OB –Imipramine 20 mg/kg
<i>eNOS</i>	1.000 $\pm$ 0.038	1.096 $\pm$ 0.082	1.004 $\pm$ 0.037	0.898 $\pm$ 0.051
<i>nNOS</i>	1.000 $\pm$ 0.125	1.411 $\pm$ 0.161	1.033 $\pm$ 0.088	1.056 $\pm$ 0.125
<i>iNOS</i>	1.000 $\pm$ 0.155	2.144 $\pm$ 0.554	2.030 $\pm$ 0.350	1.911 $\pm$ 0.340
<i>GFAP</i>	1.000 $\pm$ 0.112	1.087 $\pm$ 0.074	<b>2.175 <math>\pm</math> 0.471 *</b>	1.399 $\pm$ 0.211
<i>CD11b</i>	1.000 $\pm$ 0.103	1.049 $\pm$ 0.029	1.197 $\pm$ 0.103	0.987 $\pm$ 0.106

**Table 4.3** mRNA expression profile for NOS isoforms and glial related markers in the bulbectomized rat model of depression after 16 days of imipramine treatment. mRNA expression levels were measured by quantitative PCR in cortical samples. Data are expressed as mean and SEM of 6-9 animals. \* $p < 0.05$  compared to Sham-Vehicle group, + $p < 0.05$  compared to OBx-Vehicle group.

#### 4.3.6 TRIM attenuates OB related hyperactivity in the open field but fails to influence open field induced hyperthermia or tachycardia

Prior to drug treatment there was a higher average bout speed in OB rats when compared to sham operated controls (Student's t-test;  $t_{(22)} = 4.47$ ,  $p < 0.01$ ) [Fig. 4.6a]. ANOVA of bout speed following 7 days of treatment showed effects of OB [ $F_{(1,20)} = 23.17$ ,  $p < 0.0001$ ] and TRIM [ $F_{(1,20)} = 5.79$ ,  $p < 0.05$ ]. *Post hoc* comparisons revealed that bout speed was increased in OB rats compared to sham-operated controls ( $p < 0.01$ ) which was attenuated in the OB-TRIM treated group ( $p < 0.05$ ). TRIM alone failed to influence bout speed in sham-operated controls [Fig. 4.6b].



**Fig. 4.6** Rapid behavioural effects of TRIM in the bulbectomised rat model of depression. After the first open field test (Fig. 4.3a), rats were subdivided into the 4 groups and received daily s.c. TRIM/vehicle administration for 7 days. 24 hr after the 7<sup>th</sup> injection, rats were tested again in the open field (Fig. 4.3b). The first 3 min the rats spent in the open field were analysed for average speed/bout. Data are expressed as mean and SEM of 5-6 animals. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared to Sham-vehicle group; + $p < 0.05$  compared to OBx-vehicle group.

To exclude any possible effect of TRIM on resting body temperature and heart rate, the 24 hr prior to the second open field exposure were observed at 1 hr intervals and no effects of TRIM were detected on both parameters (data not shown).

ANOVA of change in body temperature following an initial drug-free open field exposure showed effects of OB [ $F_{(1,22)} = 11.23, p < 0.01$ ], time [ $F_{(14,308)} = 11.19, p < 0.0001$ ] and an OB x time interaction [ $F_{(14,308)} = 5.76, p < 0.0001$ ]. *Post hoc* comparisons revealed an increase in body temperature in sham animals following open field exposure which persisted for 40 minutes when compared to baseline ( $p < 0.05$ ). A similar increase was not apparent in OB rats and was significantly reduced at all the time points analysed when compared to their sham-operated counterparts ( $p < 0.05$ ) [Fig. 4.7a].

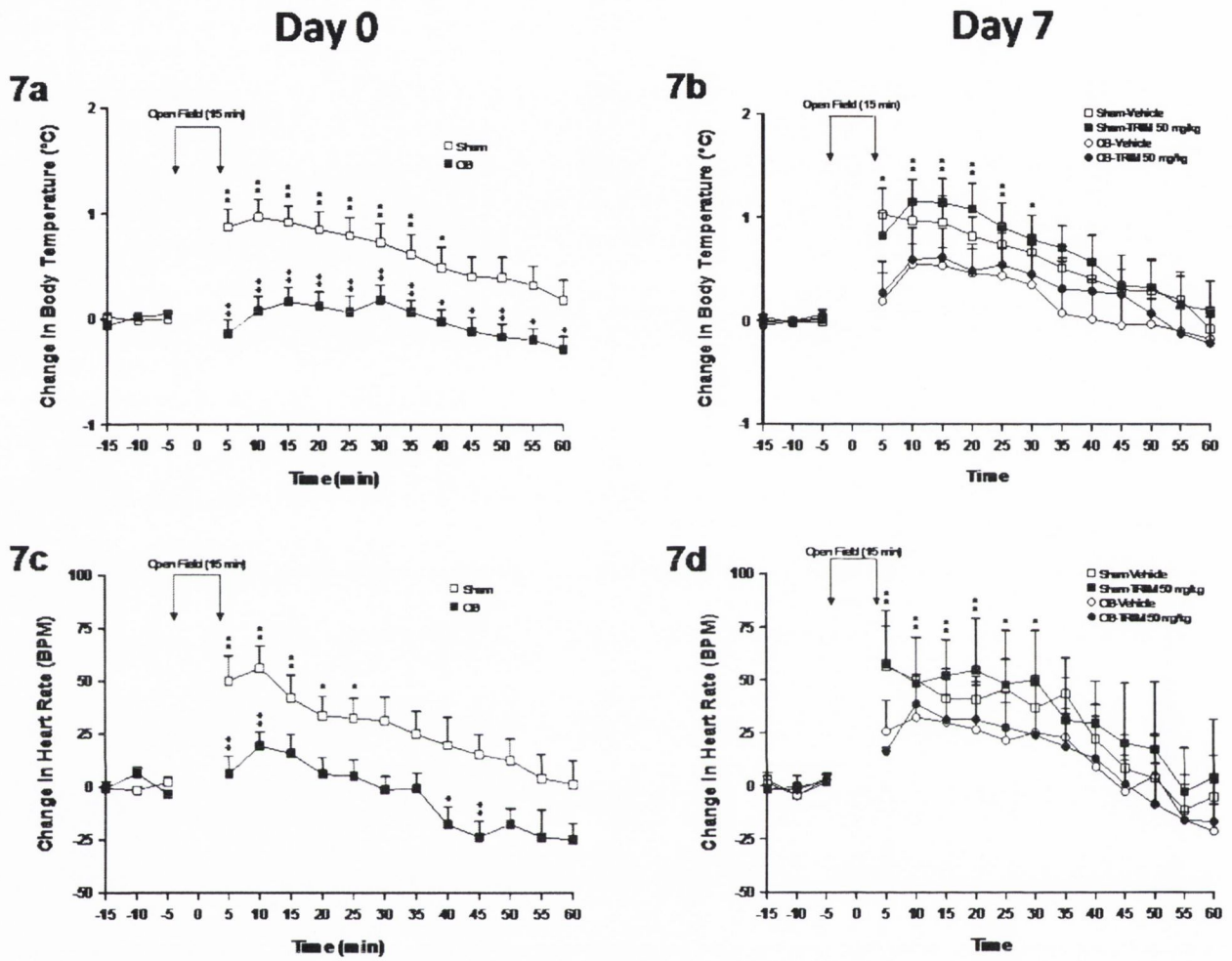
ANOVA of change in body temperature in response to open field re-exposure following 7 days of TRIM administration showed effects of time [ $F_{(14,266)} = 20.88, p < 0.001$ ] and an OB x time interaction [ $F_{(14,266)} = 2.00, p < 0.05$ ]. *Post hoc* comparisons revealed an increase in temperature in sham-operated controls following open field exposure for 30 minutes when compared to baseline ( $p < 0.05$ ). A reduced temperature response was evident in OB rats throughout when compared to sham-operated controls ( $p < 0.05$ ) and this response was not affected by TRIM. TRIM also failed to influence the increase in temperature observed in sham-operated controls [Fig. 4.7b].

ANOVA of change in heart rate following the initial open field exposure showed effects of OB [ $F_{(1,22)} = 9.63, p < 0.01$ ], time [ $F_{(14,308)} = 11.17, p < 0.0001$ ] and an OB x time interaction [ $F_{(14,308)} = 2.63, p < 0.01$ ]. *Post hoc* comparisons revealed an increase in heart rate in sham-operated controls for 25 minutes when compared to baseline ( $p < 0.05$ ). A similar increase was not apparent in OB rats and heart rate was significantly lower when

compared to sham-operated controls 10, 40 and 45 minutes following exposure to the open field ( $p < 0.05$ ) [Fig. 4.7c].

ANOVA of change in heart rate in response to open field re-exposure following 7 days of TRIM administration showed an effect of time only [ $F_{(14,266)} = 17.64$ ,  $p < 0.0001$ ]. *Post hoc* comparisons revealed an increase in heart rate in sham-operated controls for 30 minutes in response to exposure to the open field when compared to baseline ( $p < 0.05$ ). A reduced increase in heart rate response was evident in OB rats throughout when compared to sham-operated controls ( $p < 0.05$ ) and this response was not influenced by TRIM. TRIM also failed to influence the increase in heart rate observed in sham-operated controls [Fig. 4.7d].





**Fig. 4.7** Physiological responses to stress in the bulbectomised rat model of depression. After the first open field test (Fig. 4.7a and 4.7c), rats were subdivided into the 4 groups and received daily s.c. TRIM/vehicle administration for 7 days. 24 hr after the 7<sup>th</sup> injection, rats were tested again in the open field (Fig. 4.7b and 4.7d). Body temperature (Fig. 4.7a and 4.7b) and heart rate (Fig. 4.7c and 4.7d) were recorded every 5 minutes for 1 hr following the end of the test. Data are expressed as mean and SEM of 5-6 animals. \* $p < 0.05$ , \*\* $p < 0.01$  compared to respective baseline values; + $p < 0.05$ , ++ $p < 0.01$  compared to Sham-vehicle group.

## 4.4 Discussion

L-NA reduced OB related hyperactivity in the open field in a similar fashion to imipramine treatment following 16 days of treatment, although the response was also evident with L-NA following 12 days of treatment indicating a faster onset of action. The selective nNOS inhibitor TRIM also proved to be effective in the model, reducing OB-induced hyperactivity after only 7 days of administration. Attenuation of the behavioural hyperactivity of OB rats is a property shared by a variety of typical and atypical antidepressants (Kelly et al., 1997). To my knowledge, this is the first time that NOS inhibitors have been tested in the OB rat model of depression. The results add support to accumulating evidence in favour of the antidepressant related properties of NOS inhibitors as reported in a range of acute pre-clinical paradigms to date (Ghasemi et al., 2008; Harkin et al., 2004; 2003; Mutlu et al., 2009; Volke et al., 2003).

### ***4.4.1 The speed per bout parameter withstands habituation to repeated open field testing***

Hyperactivity of OB rats in a novel environment compared to their sham-operated counterparts is one of the most tested features of the OB syndrome (reviewed by Harkin et al., 2003). Chronic antidepressant treatment consisting of a minimum of 14 consecutive days of administration is normally required for traditional antidepressants to normalise the behavioural syndrome (Harkin et al., 1999; Cryan et al., 1998; Norman et al., 2012; Pandey et al., 2010). To assess onset of action however it becomes necessary to test animals at intervals leading up to 14 days which may be achieved

either by treating different groups of animals which are tested at difference times (Harkin et al., 1999; Cryan et al., 1998) or by treating a single group of animals and exposing them to the test at multiple times. As shown in this chapter when evaluating distance moved as indicator of hyperactivity, the latter strategy has proved to be less successful on account of habituation to the test environment, although there are reports where repeated testing in the open field have produced few signs of adaptation in either OB or sham-operated animals (Breuer et al., 2008; Cryan et al., 1999). To illustrate the problem a clear adaptation of distance moved in the test arena is evident when the animals are tested once per week over a three week period (see Fig. 4.1a). Despite this adaptation however OB rats remain behaviourally agitated in the open field with elements of hyperactivity present to the observer. This may be best described as the animals showing more sporadic movements, with sudden and fast bouts of random movement rather than the more natural homogeneous and inquisitive patterns of locomotion typically exhibited by rats in the open field. To capture this behaviour a new hyperactivity parameter was devised based on the speed of bouts of activity rather than distance moved representative of the average speed of the rats' horizontal movement in the arena independent of the number of bouts of movement performed and of the amount of time the rats spend mobile during the test. For example, although OB rats may spend a considerable amount of time either mobile or immobile during the test, they move more rapidly in the test arena, a characteristic reflected by a higher bout speed when compared to sham-operated controls. What is essential for antidepressant screening is that bout speed does not adapt and is not subject to the same degree of habituation when compared to the more commonly adopted distance moved parameter (see Fig. 4.1b). As distance moved per bout habituated over time and the duration of bouts did not show any major differences between shams and OBs and considering also

that the number of bouts was similar between the two groups throughout all the time points analysed, we can conclude that the increase in speed per bout exhibited by OB rats is not determined by the alteration of one specific parameter, but rather from an altered combination of distance moved and duration per bout (see Fig. 4.2).

#### ***4.4.2 NOS inhibitors show a faster antidepressant action compared to imipramine in the OB model of depression***

As previously described, L-NA reduced OB related hyperactivity represented as speed of bout in the open field test. To my knowledge, this is the first time that NOS inhibitors have been tested in the OB rat model of depression. The results add support to accumulating evidence in favour of the antidepressant related properties of NOS inhibitors as reported in a range of acute pre-clinical paradigms to date (Ghasemi et al., 2008; Harkin et al., 2004; 2003; Mutlu et al., 2009; Volke et al., 2003). When looking at distance moved, however, L-NA prevented the habituation of the OB rats to the regime of repeated open field testing. The nitric oxide synthase inhibitor L-NAME has been repeatedly reported to impair spatial learning and memory in healthy rats (Tanaka et al., 2009; Bannerman et al., 1994; Chapman et al., 1992), however in this study sham animals did not show impairments in habituating to the open field. The doses of L-NAME used in the aforementioned reports are consistently higher (100 mg/kg and 75 mg/kg) compared to the dose of 10 mg/kg of L-NA used in the current study, and injections were administered 1 hr prior to training/behavioural testing, while in the current protocol L-NA was provided at least 20 hr prior to testing. These differences in dose and time of administration might explain why sham rats behaved normally over

time in the open field. Bulbectomy in rodents is known to induce impaired learning (Mucignat-Caretta et al., 2006; Grecksch et al., 1997; Yamamoto et al., 1997) and this predisposition combined with a possible learning impairment induced by L-NA administration, even though at a very low dosage, might explain why OB-L-NA rats did not display a rate of habituation to the open field similar to that displayed by OB control rats. Further and more focused experiments are needed to support this hypothesis, however this data confirm the unreliability of distance moved as a reference parameter to evaluate the onset of antidepressant-like action of drugs in the OB model of depression.

In support of the antidepressant related response to L-NA, sub-acute treatment with the nNOS inhibitor TRIM also reduced OB related hyperactivity in the open field. TRIM has been previously reported to produce a fast onset of action when compared to fluoxetine in the chronic unpredictable mild stress paradigm in mice (Mutlu et al., 2009), where an improvement of the coat state occurs 2 weeks earlier than that observed in fluoxetine-treated animals.

From these results it seems that nNOS might be the target of selection for an antidepressant-like activity over general inhibition of NOS isoforms. As no alteration in the levels of expression of the NOS enzymes was found (see Table 4.3), it appears that in some way eNOS and/or iNOS-derived NO might favour an antidepressant-like action in the OB model of depression. This might be due to differences in regional and cellular localisation of each NOS isoform, particularly the constitutive ones, in the brain thus leading to different downstream effects of NO (Buchholzer and Kline, 2002; Kano et al., 1998).

#### ***4.4.3 TRIM fails to influence heart rate and body temperature responses to stress in the OB model of depression***

The present investigation also assessed OB-induced alterations in heart rate and core body temperature and the impact of treatment with TRIM on these parameters. Heart rate and body temperature were increased in sham-operated rats following open field exposure, a predictable response to stress which was significantly reduced in OB rats. Such a reduced stress response has been previously reported in the OB rat model (Roche et al., 2007). Treatment with TRIM failed to influence the heart rate and body temperature response in either OB or sham-operated rats. This is in contrast to the effects reported by Roche et al. (2007) where chronic treatment with the 5-HT re-uptake inhibitor fluoxetine attenuated all of the OB associated changes. The effects of nNOS inhibitors on the regulation of core body temperature in response to stress have been previously reported in rats (Soszynski, 2001; 2006) although such effects are associated with acute inhibition and the effects of chronic NOS inhibition on this parameter are less well characterised. The lack of effect of TRIM on heart rate despite the antidepressant effect on behaviour in the open field may also relate to the duration of the drug treatment schedule. Perhaps a longer treatment is required to restore physiological responses to stress. Regardless of the absence of an attenuation of the abnormal stress response in OB rats, the lack of effects on heart rate and body temperature suggests that the test drug does not provoke adverse effects on these physiological parameters and renders the drug safer to use. The combination of a positive effect on a behavioural parameter (hyperactivity) and absence of effect on physiological parameters (body temperature and heart rate) suggests that different

mechanisms are associated with the regulation of these stress sensitive parameters as proposed by Vinkers et al. (2009).

#### ***4.4.4 OB surgery does not induce the expression of NOS genes***

Removal of the olfactory bulbs was not associated with a change in the expression of any of the NOS isoforms indicating that the overproduction of NO is unlikely to be associated with the OB syndrome, as there is no excess of NOS expression in the brain of OB rats. However to rule out completely an involvement of NO in the OB syndrome, an assessment of the activity rates of the NOS enzymes would be required. This is also supported by previous reports that neither bulbectomy nor treatment with the tricyclic antidepressant desipramine (5 mg/kg, i.p. for 21 days) influences cortical NOS activity when compared to sham-operated or vehicle-treated controls respectively (Harkin et al., 1999). By contrast, systemic L-NA administration (1-10 mg/kg, i.p. for 21 days) produced a dose related reduction in cortical NOS activity (>90%) measured by the conversion of [<sup>3</sup>H]arginine to [<sup>3</sup>H]citrulline in cortical cytosol in a pilot study carried out in naïve rats (unpublished observations).

A small increase in the expression of GFAP (2-fold higher compared to  $\beta$ -actin levels used as reference) was observed in the cortex of OB rats when compared to their sham-operated counterparts, which was attenuated following imipramine treatment. It is worth noting that these changes, whilst modest, have been reproduced in a number of separate experiments in the laboratory to date. The results are consistent with other reports of an anti-inflammatory action of imipramine (Yang et al., 2010). In particular, it appears that

imipramine is able to exert this anti-inflammatory action through inhibition of NF- $\kappa$ B (Lee et al., 2012; Yang et al., 2010) which is known to be implicated in GFAP transcription (Hwang et al., 2010). GFAP expression may be considered a marker of astrogliosis (Eng and Ghirnikar, 1994) and may be associated with aberrant dysregulation within the glutamatergic system given the role of astrocytes in the regulation of synaptic glutamate. Traditionally the neurochemical abnormalities underlying the behavioural deficits in OB rats were assumed to be mediated through the 5-HT system although there are a number of reports where central glutamate and glycine concentrations and the binding properties of NMDA receptors are altered in the model (Collos, 1984; Scholfield et al., 1983; Harvey et al., 1975; Dennis et al., 1994). Moreover Redmond et al. (1996; 1997) reported that glycine, a modulator at NMDA-R, and the use-dependent NMDA-R antagonist MK-801 exhibit antidepressant-like activity in the OB rat. Such effects were apparent at doses that do not produce any change in other behavioural activities or body weight. As the NMDA-R gates  $\text{Ca}^{2+}$  which interacts with calmodulin to subsequently activate NOS (see Fig. 5), such a mechanism may underlie the antidepressant related activity of NOS inhibitors in the model although further work will be required to support this.

#### ***4.4.5 MR imaging markers in the OB model of depression***

The MRI data collected in the present investigation include an analysis of regional blood perfusion changes at rest to assess effects of the OB lesion, volumetric analysis of the third ventricle and hippocampus and T1 and T2 relaxation times which are reported



as potential indicators of astrocytic activation (Cowley et al., 2010). To my knowledge, this is the first time this type of MRI data was collected in the OB rat model.

In the first instance bulbectomy failed to influence cerebral blood perfusion as assessed by bt-ASL MRI. The lesion has been previously reported not to alter resting regional glucose consumption, an indirect way to evaluate brain activity (Skelin et al., 2010). It seems that a challenge with a stressor is fundamental to elicit differences in regional neuronal activation in comparison with sham-operated animals, as bulbectomy provokes altered neuronal activation patterns in response to the open field (Roche et al., 2007). Despite the lack of change to resting state perfusion in OB rats, L-NA administration produced a reduction in resting state blood perfusion to the hippocampal regions in OB rat but not in sham-operated controls. As both nNOS and eNOS have a role in regulating cerebrovascular tone (Koehler et al., 2009) and L-NA is a non selective inhibitor of NOS, it is not possible to know by which mechanism L-NA exerts its action on resting state cerebral blood perfusion. It is tempting to speculate that the reason why L-NA provokes changes in OB rats only may relate to astrocytic activation and dysregulated perfusion which becomes apparent when these animals are treated with L-NA. Future imaging work may involve the use of a specific nNOS inhibitor to try to discern if these effects on cerebrovascular tone are primarily due to the inhibition of nNOS or eNOS.

Enlarged ventricles have previously been reported in OB rats (Wrynn et al. 2000), and this parallels evidence of enlarged ventricles in depressed patients (Elkis et al, 1995). In the current investigation there was a statistical trend towards increased ventricular volume following bulbectomy ( $p < 0.05$  versus sham-operated controls *t*-test). Both L-

NA and imipramine treatment resulted in a non significant increase in total ventricular volume in OB animals when compared to their sham-operated counterparts. As this increase is not associated with changes in hippocampal volume, it can be suggested that alterations in perfusion or an excessive drainage within the region might account for an excessive accumulation of cerebrospinal fluid into the ventricular cavity. Anyway a reversal of the increase in ventricular volume is not evident following conventional or experimental antidepressant treatment indicating that changes to ventricular volume are of limited value in the model as a marker for detection of antidepressant activity.

Relaxometry times T1 and T2 in MRI represent the times the magnetised water protons take to revert back to equilibrium, and they are tissue-specific. Changes in T1 relaxation times have previously been shown to be associated with acute astrocytic activation (Cowley et al. 2012; Sibson et al. 2008), while changes in T2 relaxation times have been associated with microglial activation (Justicia et al., 2008). Although it provokes an increase in GFAP expression in the frontal cortex as evaluated by Real-Time PCR (see Table 4.3), OB surgery seems not to affect T1 relaxation times in any of the regions analysed. Here it was shown that treatment with L-NA increases T1 relaxation times in cortical, thalamic and limbic regions analysed compared to vehicle-treated animals and may indicate that L-NA treatment is influencing astrocytic activity within these regions. Astrocytes become reactive in response to neural injury or disease and are believed to be protective for neurons and oligodendrocytes by limiting glutamate excitotoxicity after injury, and restricting inflammatory processes to the damaged area in the brain (for a review see Sofroniew, 2005). The anti-depressant like effects of L-NA in the OB model of depression may be related to increased astrocytic activation relative to microglial activation.

OB surgery resulted in a significant decrease in T2 relaxation times in central cortical and limbic structures analysed when compared to the sham-operated control rats. L-NA proved to be effective in attenuating the observed decrease in T2 relaxation times in both cortical (visual and retrosplenial cortex) and periventricular regions including the hippocampus. Reductions in T2 relaxation time have been associated with microglial activation (Justicia et al., 2008) and also with an increased density of brain parenchyma indicating an increased packing of brain cells in brain regions that show normal morphology (Ding et al., 2008). Changes in cell morphology and structural organisation might be the factors accounting for the tendency in decreased T2 relaxation time in OB-vehicle rats, which L-NA is able to reverse. Indeed olfactory bulbectomy has been shown to reduce dendritic spines in the hippocampus (Norrholm and Ouimet, 2001) and to enhance neurogenesis in the basolateral amygdala where the new cells were selectively neuronal precursors and not glial cells (Keilhoff et al., 2006), events which might contribute to changes in the density and structure of the parenchyma.

#### **4.4.6 Conclusions**

In conclusion L-NA produces antidepressant related activity in the OB rat model of depression. Such effects are shared with the nNOS inhibitor TRIM and tricyclic antidepressant imipramine. Evaluation of the onset of action of L-NA was made possible by the introduction of a new behavioural index of hyperactivity in the open field, namely speed of activity bout, which was used to indicate a faster onset of activity associated with the NOS inhibitor. As neither OB nor imipramine treatment affect NOS expression it is difficult to speculate on a role for NOS in the neurobiological

mechanisms that underlie the behavioural abnormalities in the model despite the antidepressant-like properties of L-NA and TRIM. It may be that the antidepressant activity emerges from a mechanism that is somewhat different from conventional pharmacological treatments and in this regard a role for glutamate and NMDA-R related signalling may be implicated.

This work also reports for the first time on a series of OB and treatment related MR neuroimaging markers. Removal of the olfactory bulbs induces an increase in ventricular volume which is not ameliorated by drug treatment, thus limiting its value as a marker for detection of antidepressant activity of drugs. OB is related to a decrease in T2 relaxation times in cortical and hippocampal regions analysed, which is attenuated by L-NA treatment. L-NA treatment is associated with an increase in T1 relaxation times in limbic and cortical regions analysed. L-NA treatment is also shown to decrease resting state hippocampal blood perfusion in OB animals which may be a factor in the antidepressant action of L-NA although such a link remains to be proven. Future work will have to be carried out to discern if this change in resting state cerebral blood perfusion is caused primarily via inhibition of the nNOS or the eNOS isoform.



*General Discussion  
and Future Directions*



## **1. The NMDA-NO pathway: a locus for antidepressant activity**

The present thesis combines a number of preclinical tests, models and behavioural, biochemical and molecular approaches to detect and characterise the antidepressant-like actions of ketamine and the NOS inhibitor N<sup>ω</sup>-nitro-L-arginine (L-NA).

### **1.1 Ketamine**

The NMDA-R antagonist ketamine produced a characteristic antidepressant-like reduction in immobility time in the rat FST. Depletion of 5-HT blocked this reduction in immobility when ketamine was administered 24 hr prior FST, indicative of 5-HT dependency. Further confirmation of a link between the antidepressant related actions of ketamine and 5-HT was evident where ketamine failed to attenuate a depressive-like phenotype in the FST induced by prior combination of 5-HT depletion and restraint stress. These observations are consistent with a role for 5-HT in mediating a sustained antidepressant activity of ketamine in the FST (see Results chapter 1).

There are extensive interactions between glutamatergic and monoaminergic systems, which have significant behavioural and neurochemical effects (Drago et al., 2011). Molecular and cellular changes induced by ketamine may produce a rapid adaptation of 5-HT transmission which underlies the antidepressant response. Ketamine has been reported to modulate 5-HT release and uptake in isolated superfused slices of rat dorsal raphe nucleus (Tso et al., 2004), and 5-HT transmission is facilitated by acute, and even more by prolonged, NMDA-R blockade (Amargos-Bosch, et al., 2006; Lindfors et al., 1997). A functional synergism may exist between monoamine related antidepressants



and NMDA-R antagonists (Hashimoto, 2011), which raises an interesting question as to whether the glutamatergic system may be targeted to enhance the therapeutic effects of antidepressant drugs. On the contrary, given that the acute neurochemical effects of many antidepressants involve aminergic neurotransmission, changes in such transmission may be important precursors of antidepressant-induced alterations to the NMDA-R complex and overall glutamatergic neurotransmission mediating the antidepressant action. Evidence that the NMDA-R complex may underlie the mechanisms of action of antidepressants originally derived from observations that chronic administration of imipramine and ECT alter the ligand binding properties of the NMDA-R complex in both mice and rats (Paul et al., 1993). Such effects generalise across antidepressant drug classes and are specific to drugs with antidepressant properties (Paul et al., 1994). Moreover antidepressant-induced adaptable changes in the ligand binding properties of the NMDA-R complex are dose-dependent requiring 10-21 days of treatment and persist for 5-10 days following cessation of treatment (Paul et al., 1994). Further characterisation would be needed to clarify the exact mechanisms by which modulation of the glutamatergic system can produce antidepressant actions, however the results presented in this thesis highlight that ketamine possesses a dual antidepressant-like action in the rat FST. This is characterised by an immediate effect which results to be 5-HT-independent, accompanied by a prolonged effect which is 5-HT-dependent instead.

Since NMDA-R antagonists possess antidepressant properties in humans accompanied by several side effects, I hypothesised that an effector down-stream from the receptor such as nNOS may represent a target for antidepressant drug action that lacks the problems associated with direct inhibition of the receptor.

## 1.2 L-NA

The non selective NOS inhibitor L-NA also produced an antidepressant-like reduction in immobility time in the rat FST, in line with previous reports (Harkin et al., 2003). In light of the evidence that the antidepressant-like effects of NMDA-R blockade in the FST are dependent on endogenous 5-HT, I aimed to characterise a possible interaction between the nitroergic and serotonergic systems. L-NA promoted an increase in regional 5-HT metabolism and it was able to attenuate the pro-depressant-like effects elicited in the FST by 5-HT<sub>2A/C</sub> receptor blockade (see Results chapter 2). When taken together with a previous report that the antidepressant-like actions of L-NA are dependent on endogenous 5-HT (Harkin et al., 2003) the results provide further support for a 5-HT-dependent mechanism underlying the antidepressant-like properties of NOS inhibitors.

As activation of the NMDA-R leads to the activation of NOS a potential role for NO in depression and antidepressant action was originally proposed by Harvey (1996). Harvey implicated NO as an ideal regulator of both short and long term adaptive changes as well placed to play a role in the phenomenon of neuronal adaptation to antidepressant drugs. Targets of NO include guanylate cyclase, G proteins, phosphodiesterase, ion channel conductance, monoamine/neuropeptide release and 5-HT synthesis and re-uptake (Bartus et al., 2013; Nelson et al., 2003; Hanafy et al., 2001; Kiss, 2000; Kuhn and Arthur, 1996; 1997). NO-mediated cGMP synthesis also mediates modulation of immediate early gene expression including c-fos, c-jun, and map2, which are implicated in long term synaptic changes (Tegenge et al., 2009; Chan et al., 2004; Park et al., 2000). NO producing neurons in rodent brain are activated during stress and given the role of stress as a predisposing factor in psychiatric illness, it is not unreasonable to

suggest that stress related NO production may play a role in the induction of depressive symptoms.

In the presented work, the regional effects of NOS inhibition by L-NA in the FST were mapped using c-FOS as a marker of neuronal activation. Acute L-NA treatment attenuated c-FOS expression in the majority of the brain regions examined except the prelimbic cortex and lateral septum, where an increased expression was observed. Similar changes were observed in both naïve and 5-HT-depleted and stressed rats showing that L-NA provokes a 5-HT-independent region specific regulation of neuronal activation associated with antidepressant-like behavioural effects in the FST (see Results chapter 3). The pattern of activation following L-NA administration provides insights into the areas of the brain involved in the response to swim stress in the presence or absence of the NOS inhibitor, and with more specific analysis, such as the identification of the neuronal subtypes activated within each region, the possible neurocircuitry of L-NA's antidepressant-like action in the FST might be clarified.

To move away from assessing the antidepressant-like effects of acute L-NA administration, the drug was put to test in a model of depression which selectively requires chronic antidepressant treatment in order to achieve normalisation of the behaviour (see Results chapter 4). Repeated administration of L-NA attenuated the OB related behavioural hyperactivity in the open field test typical of antidepressant response in the model. Examination of the onset of the antidepressant effect demonstrated that L-NA reduced the behavioural hyperactivity more rapidly (following 12 days) than the tricyclic antidepressant imipramine (following 16 days). The faster acting antidepressant related properties of the NOS inhibitor in the model were further supported by the additional findings of an independent study where treatment with the nNOS inhibitor 1-

2-trifluoromethylphenyl imidazole (TRIM) also attenuated the OB related hyperactivity in the open field following 7 days of treatment. This is the first account of the antidepressant related properties of NOS inhibitors in the olfactory bulbectomised rat model of depression.

Together these studies further the understanding of the role of NOS and in particular nNOS in depression related behaviour and further its potential as a novel therapeutic target for the treatment of stress related disorders such as depression. Very recently two compounds that disrupt the PSD-95/nNOS interaction have been identified. IC87201 (2-((1H-benzo [d] [1,2,3] triazol-5-ylamino) methyl)-4,6-dichlorophenol) was an effective anti-nociceptive after intraperitoneal injection, with an  $EC_{50}$  of 0.1 mg/kg. In rats, IC87201 abolished mechanical allodynia when administered intrathecally (at 50 and 100 nmol doses) or intraperitoneally (2 mg/kg) (Florio et al., 2009). ZL006 (5-(3,5-dichloro-2-hydroxy-benzylamino)-2-hydroxybenzoic acid) is a molecule structurally related to IC87201, which contains a hydrophobic ring, a hydrophilic ring with a carboxyl group and a linker between the rings that enhance the flexibility of the compound. ZL006 inhibits NMDA-R-dependent NO synthesis in cortical neurons with an  $IC_{50}$  of 82 nM and crosses the blood brain barrier after systemic administration (Zhou et al., 2010). Both IC87201 and ZL006, have attractive properties to investigate the PSD-95/nNOS interface as a drug target for the treatment of depression.

## **2. NO: a non-synaptic extension of glutamate**

NO is a highly diffusible gas and is sufficiently small and hydrophobic to readily cross cytoplasm, membrane and extracellular fluid, allowing it to act intracellularly or intercellularly on other neurons, glia and vasculature (Kiss and Vizi, 2001). The half-life of NO is a few seconds and within this period it can diffuse a few hundred micrometers and exert an influence on the function of a large number of neurons in the sphere surrounding the synapse and modulate presynaptic neurochemical transmission. For instance, NO has been proposed as a non-synaptic chemical messenger in mediating long-range interactions between glutamate and monoaminergic systems (see Kiss and Vizi, 2001).

Low NO tissue levels appear to reduce glutamate release whereas higher endogenous NO levels enhance its release, as NO donors enhance glutamate release and NOS inhibitors reduce glutamate release. Both the increase and decrease in glutamate release has been attributed to exocytotic processes mediated by cGMP (Segieth et al., 1995; Prast and Philippu, 2001). GABA, like glutamate, is also bi-phasically regulated by endogenous NO. Low NO concentrations reduce GABA release whereas high NO concentration increases GABA release (Getting et al., 1996). NO-induced GABA release is mediated by Ca<sup>2+</sup>-dependent and reverse process Na<sup>+</sup>-dependent transmitter carrier-mediated mechanisms. Furthermore, the peroxynitrate ONOO<sup>-</sup>, produced from the reaction of one molecule of NO and one molecule of superoxide, also stimulates Ca<sup>2+</sup>-dependent GABA release (Ohkuma et al., 1996). NO-induced release of other transmitters including acetylcholine (Prast and Philippu, 1992, Prast and Philippu, 2001), dopamine (West and Galloway, 1998; Prast and Philippu, 2001), noradrenaline

(Lonart et al., 1992), 5-HT (Sinner et al., 2001 ) and histamine (Prast et al., 1996) depend on NO modulation of both glutamate and GABA release (reviewed by Prast and Philippu, 2001).

## **2.1 NO and 5-HT modulation**

NO produced from NMDA-R activation has been proposed to facilitate 5-HTergic transmission by inhibition of the 5-HT transporter (SERT) in some brain areas (see Kiss and Vizi, 2001 for review). Inhibition by NO of 5-HT uptake into rat brain synaptosomes (Asano et al., 1997) and of the human 5-HT transporter (Bryan-Lluka et al., 2004) has been described. NO may also inhibit monoamine oxidase activity (Muriel and Perez-Rojas, 2003, Girgin Sagin et al., 2004) although the mechanism by which NO inhibits this enzymes remains unknown at present. These reports seem to be in contrast with reports of inactivation of brain tryptophan hydroxylase (TPH) by NO via nitrosylation (Kuhn and Arthur, 1996; 1997). Pharmacological inhibition of nNOS produces an increase in the immunoreactivities of TPH and 5-HT in the median raphe nucleus and dentate gyrus of the hippocampus in rats (Park et al., 2004). As the median raphe nucleus supplies the dentate gyrus with dense 5-HT innervation, these changes are likely to provoke an enhancement of 5-HT function in the hippocampus. Consistent with this are reports that NOS inhibitors influence 5-HT metabolism in the mouse forebrain (Karolewicz et al., 2001) and increase the extracellular levels of 5-HT in rat hippocampus after local or systemic administration (Segieth et al., 2001; Wegener et al., 2000). In Results chapter 2 of this thesis it was demonstrated that inhibition of NOS by L-NA induced TPH activity, as evidenced by the greater accumulation of the 5-HT synthesis intermediate metabolite 5-HTP in presence of L-NA compared to vehicle

controls (see Table 2.1). It is possible that L-NA relieves TPH from the inhibition exerted by NO, thus boosting the 5-HT synthetic pathway in response to swim stress.

Novel protein interactions with the C-terminal PSD-95/discs large/ZO-1 homologous (PDZ)-like sequence of the SERT have been identified and include a physical association with nNOS (Chanrion et al., 2007). This association leads to a reduction in the delivery of SERT to the plasma membrane and influences maximal 5-HT uptake (Steiner et al., 2009). SERT can also bind to Sec23A and Sec24C, which function to recognise and bind to newly made proteins in the endoplasmic reticulum destined for export to the plasma membrane (Dev et al., 2004). Therefore, nNOS may compete with Sec23A-Sec24C for binding to the C-terminus of SERT, resulting in a reduction in the export of SERT to the cell membrane. Reciprocally, 5-HT uptake induces NO production from nNOS physically linked to membrane bound SERT, through a calmodulin (CaM)-dependent mechanism. It has been proposed that NO formed in association with 5-HT transport might, through cGMP and cGMP-dependent protein kinase (PKG), phosphorylate SERT, increasing its activity (Garthwaite, 2007; Ramamoorthy et al., 2007). A role for the inhibitory influence of nNOS on the activity of SERT in serotonergic terminals in the pathogenesis of psychiatric disorders including depressive states has been proposed (Karolewicz et al., 2004). NOS inhibitors may interfere with this regulatory system leading to an overall decrease in SERT activity. In fact nNOS may reduce SERT uptake activity, while the reduction in NO production prevents cGMP/PKG-mediated SERT stimulation.

There are therefore a number of mechanisms by which NOS inhibitors may influence 5-HT metabolism. It is tempting to speculate that certain mechanisms might prevale above

others in specific brain areas and/or neurocircuitries. Overall, as 5-HT function is implicated in stress related disorders such as depression, modulation of chemically reactive species like NO might be used as a strategy to influence conditions where 5-HT alterations have been identified.

### **3. Ongoing development of animal models of depression**

In order to test new compounds for antidepressant activity and their potential for clinical use, animal models of depression are required. Despite some limitations, animal models of psychological disorders can provide useful insights of the dysfunctions seen in the disease in human and models of depression are being continuously subjected to refinement in order to enhance their validity, applicability and usefulness. In the present body of work the focus was placed on further innovating the chronic restraint stress and the olfactory bulbectomised rat models, as described as follows:

- I. Either single or repeated exposure to restraint stress induced a depressive-like state in the animals subjected to the FST, however recovery was very rapid, back to control non-stressed levels, when an interval of 24 hr was introduced between the last stress exposure and the pre-FST session. The transient nature of the depression related behaviour is somewhat problematic, as in humans the depressed condition persists over time. In the experiments described in this thesis, a state of 5-HT depletion resulted in a depressive-like phenotype in the FST which was persistent and enduring beyond cessation of the stress regime. If such an interaction translates to human experience, a person in a low 5-HT state leading a stressful lifestyle may increase his/her risk of developing a depressive episode. This ties in with clinical



evidences that in humans acute depletion of the 5-HT precursor tryptophan reduces 5-HT availability in the brain (Nishizawa et al., 1997) and can induce a relapse of depressive symptoms in remitting depressed patients (Moreno et al., 2000) and low mood in persons with a familial risk for depression (Benkelfat et al., 1994).

- II. The introduction of 5-HT depletion was also an attempt to establish a condition of treatment resistance to conventional antidepressants in the animals. Surprisingly an enhanced efficacy of fluoxetine was observed in 5-HT depleted stressed animals. This evidence arguably serves to increase the predictive validity of the model, allowing for direct comparison between commonly prescribed antidepressants and novel test compounds. Anyway further studies involving the depletion of other monoamines such as noradrenaline combined with repeated restraint stress will be of interest in order to characterise the contribution of the different systems to the depression related phenotype induced in the FST.
  
- III. Animal models of depression that enable the antidepressant response to be monitored during the course of treatment are necessary. To assess the issue of onset of action an adaptation in the determination of hyperactivity characteristic of the olfactory bulbectomised rat was introduced. In the presented study a clear adaptation of the distance travelled after exposing the animals to the open field for three consecutive times was evident. To overcome this problem a new activity related parameter, average speed per bout of movement, was developed. As shown in Results chapter 4 this new parameter withstood habituation to repeated open field exposure and proved to be more reliable than distance moved in characterising the antidepressant-like effects of L-NA in the OB rat model of depression. If the

predictive validity of speed per bout will be confirmed by other conventional antidepressants known to reduce hyperactivity in the model, the introduction of this parameter as routine would help researchers to comply with legislation concerning the welfare of laboratory animals, which require efforts to be made to reduce the number of animals used in drug development investigations, as several time points can be observed in one single set of animals.

- IV. Attempts were made to develop a MR neuroimaging marker related to the OB rat syndrome and antidepressant response in the model. Structural volumes, resting-state blood perfusion and relaxation times were assessed. Although only some of these parameters revealed to be affected by OB surgery and/or by antidepressant treatment, and although further neuroimaging work is required to clarify such effects, the presented work highlights that MRI has a potential as a translational technique which can be applied to both humans and animals in order to make comparisons between the two species. In fact, the observed reduction in hippocampal blood perfusion associated with L-NA treatment in OB rats is in line with reduced hippocampal resting-state activation determined by BOLD fMRI reported in healthy human subjects treated with SSRIs (McCabe and Mishor, 2011; McCabe et al., 2011) and also hippocampal T2 relaxation times have been found to be altered in MDD in humans (Cho et al., 2010).

#### **4. Translational perspective on the regionally-dependent effects of L-NA**

*Post mortem* and MRI studies to date highlight structural abnormalities occurring in the brain of depressed patients when compared to healthy controls (reviewed in Sheline et al., 2002). Changes in structure does not necessarily indicate altered functionality, in fact compensatory mechanisms might develop to restore a functional level of activity in a structurally compromised field. With the development of non invasive functional neuroimaging researchers have focused on studying regional brain activity in resting conditions or in response to specific stimuli in order to detect alterations in neuronal functionality or connectivity of relevance to depression. For instance, a recent fMRI study by Zhu and colleagues (2012) showed altered resting-state functional activation and connectivity within the default mode network (DMN, a neuronal network involved in self-referential activity and emotional regulation that includes a specific set of brain regions such as prefrontal and cingulate cortices, and medial, lateral, and inferior parietal regions) in first episode treatment-naïve depressed patients. It is therefore important to discover and understand the origin of altered connectivity associated with depression and to know if antidepressant treatment can restore normal connectivity pivotal to its antidepressant activity.

A first step in this direction has been undertaken in the present investigation by c-FOS immunostaining to detect regional neuronal activation in response to the FST. Although the combination of 5-HT depletion and restraint stress (inducing a depressive-like state in the FST) did not influence the pattern of c-FOS activation in the brain, L-NA reduced neuronal activation in subcortical regions in response to FST exposure indicating a

different cortical-subcortical involvement in the antidepressant related effects of L-NA in the test. Taken together these results suggest that the antidepressant action of L-NA might be exerted through diverse yet complementary actions in different brain regions, so that a balance in neuronal connectivity is achieved in the brain to promote the antidepressant related response. Analysis of the expression profile of other IEGs activated by stress exposure, such as c-jun, or the transcription factor cAMP response element binding protein (CREB) would serve to broaden the scope of the results generated thus far. Moreover it would be of considerable interest to identify the cell phenotype by double-immunostaining with antibodies directed against individual neurotransmitters including glutamate and GABA.

## **5. Future directions**

Following from the work presented in this thesis, several studies could be performed to further explore the potential of NOS inhibitors as antidepressants.

- I. Antidepressant related changes to the NMDA-R complex may underlie a common mechanism for antidepressant drugs. In order for antidepressant-induced changes in the ligand binding properties of the NMDA-R to be considered a neural adaptation, it should be shown that these changes are accompanied by alterations in the functionality of the receptor. Future studies on the effects of antidepressant treatment on NMDA-R-stimulated subcellular cGMP and NO production would be of interest.

II. With the development and availability of more selective inhibitors of various isoforms of NOS, future studies on the effects of non-arginine derived inhibitors in animal models should be examined. In addition, the effect of NO scavengers and inhibitors of the production of guanylate cyclase could be explored. The post synaptic density protein 95 (PSD-95) is a scaffolding protein that links a number of molecules via its various domains including nNOS to NMDA-R. Disruption of the PSD-95/nNOS interaction has been achieved with both peptide fragments and with small-molecule inhibitors (for review see Doucet et al., 2012). As other functions of the NMDA-R remain intact, adoption of this selective approach as a strategy to further test the hypothesis for the development of a novel glutamatergic-based treatment for depression can be proposed. As previously described, compounds such as IC87201 and ZL006 look very promising, however before uncoupling of nNOS from NMDA-R can move to further stages of development as an antidepressant treatment the effects of these compounds on behaviour need to be determined.

III. Preclinical studies have reported that AMPA-R activation is required for the antidepressant-like actions of ketamine. Specifically the AMPA-R antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX) blocks the effects of ketamine in tests predictive of antidepressant potential including the FST, TST and learned helplessness (LH) model in rodents (Koike et al., 2011; Maeng et al., 2008). Moreover AMPA-R is known to play a central role in cellular studies of synaptic protein synthesis and plasticity (see Li et al., 2010) consistent with the hypothesis that ketamine induction of synaptogenesis occurs via stimulation of glutamate transmission via AMPA-R activation (see Li et al., 2010). More recently

ketamine was reported to rapidly reverse both CUS-induced behavioural deficits in rats and reduced spine density and synaptic function of PFC neurons (Li et al., 2011; reviewed by Duman and Voleti, 2012). Together these studies indicate that ketamine reverses the atrophy of spines in the PFC and leads to a reconnection of neurons that underlie rapid behavioural responses. Density of dendritic spines thus provides a morphological endpoint that is relevant to the atrophy of PFC in depression (Drevets et al., 2008). A role for NO and cGMP-dependent protein kinase, under the regulation of the NMDA-R, in AMPA-R trafficking and synaptic plasticity has been reported (Serulle et al., 2008) linking the various signalling components most likely implicated in the antidepressant activity associated with NMDA-R antagonists. It would be therefore of interest to study and characterise the interaction between NO and the AMPA-R and the effects that NOS inhibition might have on AMPA-R activity and on synaptic plasticity.

- IV. Ongoing development of animal models is essential in order to enhance their validity and improve their predictability if useful antidepressive agents are to be developed for clinical use to address current unmet needs. Further testing of NOS inhibitors in additional animal models such as early life deprivation, genetic inbred rodent models of depression and chronic social defeat stress is warranted.



# *References*





- Abbasi SH, Hosseini F, Modabbernia A, Ashrafi M, Akhondzadeh S (2012) Effect of celecoxib add-on treatment on symptoms and serum IL-6 concentrations in patients with major depressive disorder: randomized double-blind placebo-controlled study. *J Affect Disord* 141: 308-14.
- Abu-Ghanem Y, Cohen H, Buskila Y, Grauer E, Amitai Y (2008) Enhanced stress reactivity in nitric oxide synthase type 2 mutant mice: findings in support of astrocytic nitrosative modulation of behavior. *Neuroscience* 156: 257-65.
- Aguiar D, Guimarães F (2009) Blockade of NMDA receptors and nitric oxide synthesis in the dorsolateral periaqueductal gray attenuates behavioral and cellular responses of rats exposed to a live predator. *J Neurosci Res* 87: 2418-29.
- Ali S, Stone MA, Peters JL, Davies MJ, Khunti K (2006) The prevalence of co-morbid depression in adults with Type 2 diabetes: a systematic review and meta-analysis. *Diabet Med* 23: 1165-73.
- Altamura CA, Mauri MC, Ferrara A, Moro AR, D'Andrea G, Zamberlan F (1993) Plasma and platelet excitatory amino acids in psychiatric disorders. *Am J Psychiatry* 150: 1731-33.
- Amargós-Bosch M, López-Gil X, Artigas F, Adell A (2006) Clozapine and olanzapine, but not haloperidol, suppress serotonin efflux in the medial prefrontal cortex elicited by phencyclidine and ketamine. *Int J Neuropsychopharmacol* 9: 565-73.
- American Psychiatric Association (2000) *Diagnostic and Statistical Manual of Mental Disorders* (4th ed., text rev.). Washington, DC: Author.
- Amir S, Rackover M, Funk D (1997) Blockers of nitric oxide synthase inhibit stress activation of c-fos expression in neurons of the hypothalamic paraventricular nucleus in the rat. *Neuroscience* 77: 623-27.
- Angulo J, Peiró C, Sanchez-Ferrer C, Gabancho S, Cuevas P, Gupta S, Sáenz de Tejada I (2001) Differential effects of serotonin reuptake inhibitors on erectile responses, NO-production, and neuronal NO synthase expression in rat corpus cavernosum tissue. *Br J Pharmacol* 134: 1190-94.
- Armario A, Gil M, Marti J, Pol O, Balasch J (1991) Influence of various acute stressors on the activity of adult male rats in a holeboard and in the forced swim test. *Pharmacol Biochem Behav* 39: 373-77.
- Artigas F (2013) Serotonin receptors involved in antidepressant effects. *Pharmacol Ther* 137: 119-31.

- Asano S, Matsuda T, Nakasu Y, Maeda S, Nogi H, Baba A (1997) Inhibition by nitric oxide of the uptake of [3H]serotonin into rat brain synaptosomes. *Jpn J Pharmacol* 75: 123-28.
- Autry AE, Adachi M, Nosyreva E, Na ES, Los MF, Cheng PF, Kavalali ET, Monteggia LM (2011) NMDA receptor blockade at rest triggers rapid behavioural antidepressant responses. *Nature* 475: 91-95.
- Banasr M, Duman RS (2008) Glial loss in the prefrontal cortex is sufficient to induce depressive-like behaviors. *Biol Psychiatry* 64: 863-70.
- Bannerman DM, Chapman PF, Kelly PA, Butcher SP, Morris RG (1994) Inhibition of nitric oxide synthase does not impair spatial learning. *J Neurosci* 14: 7404-14.
- Barbee JG, Jamhour NJ (2002) Lamotrigine as an augmentation agent in treatment-resistant depression. *J Clin Psychiatry* 63: 737-41.
- Barnes DE, Yaffe K, Byers AL, McCormick M, Schaefer C, Whitmer RA (2012) Midlife vs late-life depressive symptoms and risk of dementia: differential effects for Alzheimer disease and vascular dementia. *Arch Gen Psychiatry* 69: 493-98.
- Barrientos RM, Sprunger DB, Campeau S, Higgins EA, Watkins LR, Rudy JW, Maier SF (2003) Brain-derived neurotrophic factor mRNA downregulation produced by social isolation is blocked by intrahippocampal interleukin-1 receptor antagonist. *Neuroscience* 121: 847-53.
- Bartus K, Pigott B, Garthwaite J (2013) Cellular targets of nitric oxide in the hippocampus. *PLoS One* 8: e57292.
- Baune B, Adrian I, Jacobi F (2007) Medical disorders affect health outcome and general functioning depending on comorbid major depression in the general population. *J Psychosom Res* 62: 109-18.
- Bechtholt-Gompf AJ, Smith KL, John CS, Kang HH, Carlezon WA, Cohen BM, Ongür D (2011a) CD-1 and Balb/cJ mice do not show enduring antidepressant-like effects of ketamine in tests of acute antidepressant efficacy. *Psychopharmacology (Berl)* 215: 689-95.
- Bechtholt-Gompf AJ, Smith KL, John CS, Kang HH, Carlezon WA Jr, Cohen BM, Ongür D (2011b) Re: concerns about the antidepressant-like effects of high dose ketamine in mice. *Psychopharmacology (Berl)* 215(4): 815-16.
- Benkelfat C, Ellenbogen MA, Dean P, Palmour RM, Young SN (1994) Mood-lowering effect of tryptophan depletion. Enhanced susceptibility in young men at genetic risk for major affective disorders. *Arch Gen Psychiatry* 51: 687-97.

- Berk M, Plein H, Ferreira D (2001) Platelet glutamate receptor supersensitivity in major depressive disorder. *Clin Neuropharmacol* 24: 129-32.
- Berman R, Cappiello A, Anand A, Oren D, Heninger G, Charney D, Krystal J (2000) Antidepressant effects of ketamine in depressed patients. *Biol Psychiatry* 47: 351-54.
- Bernard R, Kerman IA, Thompson RC, Jones EG, Bunney WE, Barchas JD, Schatzberg AF, Myers RM, Akil H, Watson SJ (2011) Altered expression of glutamate signaling, growth factor, and glia genes in the locus coeruleus of patients with major depression. *Mol Psychiatry* 16: 634-46.
- Berton O, Nestler E (2006) New approaches to antidepressant drug discovery: beyond monoamines. *Nat Rev Neurosci* 7: 137-51.
- Beumer W, Gibney SM, Drexhage RC, Pont-Lezica L, Doorduyn J, Klein HC, Steiner J, Connor TJ, Harkin A, Versnel MA, Drexhage HA (2012) The immune theory of psychiatric diseases: a key role for activated microglia and circulating monocytes. *J Leukoc Biol* 92: 959-75.
- Bhat GK, Mahesh VB, Lamar CA, Ping L, Aguan K, Brann DW (1995) Histochemical localization of nitric oxide neurons in the hypothalamus: association with gonadotropin-releasing hormone neurons and co-localization with N-methyl-D-aspartate receptors. *Neuroendocrinology* 62: 187-97.
- Binder EB, Salyakina D, Lichtner P, Wochnik GM, Ising M, Pütz B, Papiol S, Seaman S, Lucae S, Kohli MA, Nickel T, Künzel HE, Fuchs B, Majer M, Pfennig A, Kern N, Brunner J, Modell S, Baghai T, Deiml T, Zill P, Bondy B, Rupprecht R, Messer T, Köhnllein O, Dabitz H, Brückl T, Müller N, Pfister H, Lieb R, Mueller JC, Löhmussaar E, Strom TM, Bettecken T, Meitinger T, Uhr M, Rein T, Holsboer F, Muller-Myhsok B (2004) Polymorphisms in FKBP5 are associated with increased recurrence of depressive episodes and rapid response to antidepressant treatment. *Nat Genet* 36: 1319-25.
- Blugeot A, Rivat C, Bouvier E, Molet J, Mouchard A, Zeau B, Bernard C, Benoliel JJ, Becker C (2011) Vulnerability to depression: from brain neuroplasticity to identification of biomarkers. *J Neurosci* 31: 12889-99.
- Bonanno G, Giambelli R, Raiteri L, Tiraboschi E, Zappettini S, Musazzi L, Raiteri M, Racagni G, Popoli M (2005) Chronic antidepressants reduce depolarization-evoked glutamate release and protein interactions favoring formation of SNARE complex in hippocampus. *J Neurosci* 25: 3270-79.
- Bonvicini C, Minelli A, Scassellati C, Bortolomasi M, Segala M, Sartori R, Giacomuzzi M, Gennarelli M (2010) Serotonin transporter gene polymorphisms and treatment-resistant depression. *Prog Neuropsychopharmacol Biol Psychiatry* 34: 934-39.

- Borsini F (1995) Role of the serotonergic system in the forced swimming test. *Neurosci Biobehav Rev* 19: 377-95.
- Borsini F, Meli A (1988) Is the forced swimming test a suitable model for revealing antidepressant activity? *Psychopharmacology (Berl)* 94: 147-60.
- Breuer ME, Chan JS, Oosting RS, Groenink L, Korte SM, Campbell U, Schreiber R, Hanania T, Snoeren EM, Waldinger M, Olivier B (2008) The triple monoaminergic reuptake inhibitor DOV 216,303 has antidepressant effects in the rat olfactory bulbectomy model and lacks sexual side effects. *Eur Neuropsychopharmacol* 18: 908-16.
- Bryan-Lluka L, Papacostas M, Paczkowski F, Wanstall J (2004) Nitric oxide donors inhibit 5-hydroxytryptamine (5-HT) uptake by the human 5-HT transporter (SERT). *Br J Pharmacol* 143: 63-70.
- Buchholzer ML, Klein J (2002) NMDA-induced acetylcholine release in mouse striatum: role of NO synthase isoforms. *J Neurochem* 82: 1558-60.
- Buskila Y, Abu-Ghanem Y, Levi Y, Moran A, Grauer E, Amitai Y (2007) Enhanced astrocytic nitric oxide production and neuronal modifications in the neocortex of a NOS2 mutant mouse. *PLoS One* 2: e843.
- Carlsson A, Davis JN, Kehr W, Lindqvist M, Atack CV (1972) Simultaneous measurement of tyrosine and tryptophan hydroxylase activities in brain in vivo using an inhibitor of the aromatic amino acid decarboxylase. *Naunyn Schmiedebergs Arch Pharmacol* 275: 153-68.
- Carver CS, Johnson SL, Joormann J (2008) Serotonergic function, two-mode models of self-regulation, and vulnerability to depression: what depression has in common with impulsive aggression. *Psychol Bull* 134: 912-43.
- Chan SH, Chang KF, Ou CC, Chan JY (2004) Nitric oxide regulates c-fos expression in nucleus tractus solitarius induced by baroreceptor activation via cGMP-dependent protein kinase and cAMP response element-binding protein phosphorylation. *Mol Pharmacol* 65: 319-25.
- Chandramohan Y, Droste SK, Arthur JS, Reul JM (2008) The forced swimming-induced behavioural immobility response involves histone H3 phosphorylation and c-Fos induction in dentate gyrus granule neurons via activation of the N-methyl-D-aspartate/extracellular signal-regulated kinase/mitogen- and stress-activated kinase signalling pathway. *Eur J Neurosci* 27: 2701-13.
- Chanrion B, Mannoury la Cour C, Bertaso F, Lerner-Natoli M, Freissmuth M, Millan M, Bockaert J, Marin P (2007) Physical interaction between the serotonin

- transporter and neuronal nitric oxide synthase underlies reciprocal modulation of their activity. *Proc Natl Acad Sci U S A* 104: 8119-24.
- Chaouloff F, Courvoisier H, Moisan MP, Mormède P (1999) GR 127935 reduces basal locomotor activity and prevents RU 24969-, but not D-amphetamine-induced hyperlocomotion, in the Wistar-Kyoto hyperactive (WKHA) rat. *Psychopharmacology (Berl)* 141: 326-31.
- Chapman PF, Atkins CM, Allen MT, Haley JE, Steinmetz JE (1992) Inhibition of nitric oxide synthesis impairs two different forms of learning. *Neuroreport* 3: 567-70.
- Cherney DZ, Reich HN, Jiang S, Har R, Nasrallah R, Hébert RL, Lai V, Scholey JW, Sochett EB (2012) Hyperfiltration and effect of nitric oxide inhibition on renal and endothelial function in humans with uncomplicated type 1 diabetes mellitus. *Am J Physiol Regul Integr Comp Physiol* 303: R710-18.
- Cho ZH, Kim YB, Han JY, Kim NB, Hwang SI, Kim SJ, Cho SJ (2010) Altered T2\* relaxation time of the hippocampus in major depressive disorder: implications of ultra-high field magnetic resonance imaging. *J Psychiatr Res* 44: 881-86.
- Chrapko W, Jurasz P, Radomski M, Lara N, Archer S, Le Mellédo J (2004) Decreased platelet nitric oxide synthase activity and plasma nitric oxide metabolites in major depressive disorder. *Biol Psychiatry* 56: 129-34.
- Chung KK, Martinez M, Herbert J (1999) Central serotonin depletion modulates the behavioural, endocrine and physiological responses to repeated social stress and subsequent c-fos expression in the brains of male rats. *Neuroscience* 92: 613-25.
- Ciani E, Virgili M, Contestabile A (2002) Akt pathway mediates a cGMP-dependent survival role of nitric oxide in cerebellar granule neurones. *J Neurochem* 81: 218-28.
- Coert B, Anderson R, Meyer F (2003) Is neuroprotective efficacy of nNOS inhibitor 7-NI dependent on ischemic intracellular pH? *Am J Physiol Heart Circ Physiol* 284: H151-59.
- Cogswell JP, Godlevski MM, Wisely GB, Clay WC, Leesnitzer LM, Ways JP, Gray JG (1994) NF-kappa B regulates IL-1 beta transcription through a consensus NF-kappa B binding site and a nonconsensus CRE-like site. *J Immunol* 153: 712-23.
- Colla M, Kronenberg G, Deuschle M, Meichel K, Hagen T, Bohrer M, Heuser I (2007) Hippocampal volume reduction and HPA-system activity in major depression. *J Psychiatr Res* 41: 553-60.

- Conley RK, Li J, Ivarsson M, Hutson PH (2007) Effect of the beta3 adrenoceptor agonist CL 316243 on hypothalamic 5-HT synthesis and suppression of REM sleep in the rat. *J Psychopharmacol* 21: 597-602.
- Connor TJ, Harkin A, Kelly JP, Leonard BE (2000) Olfactory bulbectomy provokes a suppression of interleukin-1beta and tumour necrosis factor-alpha production in response to an in vivo challenge with lipopolysaccharide: effect of chronic desipramine treatment. *Neuroimmunomodulation* 7: 27-35.
- Contreras CM, Chacón L, Rodríguez-Landa JF, Bernal-Morales B, Gutiérrez-García AG, Saavedra M (2004) Spontaneous firing rate of lateral septal neurons decreases after forced swimming test in Wistar rat. *Prog Neuropsychopharmacol Biol Psychiatry* 28: 343-48.
- Contreras CM, Rodríguez-Landa JF, Gutiérrez-García AG, Bernal-Morales B (2001) The lowest effective dose of fluoxetine in the forced swim test significantly affects the firing rate of lateral septal nucleus neurones in the rat. *J Psychopharmacol* 15: 231-36.
- Cornwell BR, Salvadore G, Furey M, Marquardt CA, Brutsche NE, Grillon C, Zarate CA (2012) Synaptic Potentiation Is Critical for Rapid Antidepressant Response to Ketamine in Treatment-Resistant Major Depression. *Biol Psychiatry* 72: 555-61.
- Cowley TR, O'Sullivan J, Blau C, Deighan BF, Jones R, Kerskens C, Richardson JC, Virley D, Upton N, Lynch MA (2012) Rosiglitazone attenuates the age-related changes in astrocytosis and the deficit in LTP. *Neurobiol Aging* 33: 162-75.
- Crane GE (1959) Cycloserine as an antidepressant agent. *Am J Psychiatry* 115: 1025-26.
- Crespi F (2010) The selective serotonin reuptake inhibitor fluoxetine reduces striatal in vivo levels of voltammetric nitric oxide (NO): a feature of its antidepressant activity? *Neurosci Lett* 470: 95-99.
- Critchley DJ, Childs KJ, Middlefell VC, Dourish CT (1994) Inhibition of 8-OH-DPAT-induced elevation of plasma corticotrophin by the 5-HT1A receptor antagonist WAY100635. *Eur J Pharmacol* 264: 95-97.
- Cryan J, Lucki I (2000a) Antidepressant-like behavioral effects mediated by 5-Hydroxytryptamine(2C) receptors. *J Pharmacol Exp Ther* 295: 1120-26.
- Cryan JF, Lucki I (2000b) 5-HT4 receptors do not mediate the antidepressant-like behavioral effects of fluoxetine in a modified forced swim test. *Eur J Pharmacol* 409: 295-99.

- Cryan J, Valentino R, Lucki I (2005) Assessing substrates underlying the behavioral effects of antidepressants using the modified rat forced swimming test. *Neurosci Biobehav Rev* 29: 547-69.
- Cryan JF, Harkin A, Naughton M, Kelly JP, Leonard BE (2000) Characterization of D-fenfluramine-induced hypothermia: evidence for multiple sites of action. *Eur J Pharmacol* 390: 275-85.
- Cryan JF, Markou A, Lucki I (2002a) Assessing antidepressant activity in rodents: recent developments and future needs. *Trends Pharmacol Sci* 23: 238-45.
- Cryan JF, McGrath C, Leonard BE, Norman TR (1998) Combining pindolol and paroxetine in an animal model of chronic antidepressant action--can early onset of action be detected? *Eur J Pharmacol* 352: 23-28.
- Cryan JF, McGrath C, Leonard BE, Norman TR (1999) Onset of the effects of the 5-HT<sub>1A</sub> antagonist, WAY-100635, alone, and in combination with paroxetine, on olfactory bulbectomy and 8-OH-DPAT-induced changes in the rat. *Pharmacol Biochem Behav* 63: 333-38.
- Cryan JF, Mombereau C (2004) In search of a depressed mouse: utility of models for studying depression-related behavior in genetically modified mice. *Mol Psychiatry* 9: 326-57.
- Cryan JF, Page ME, Lucki I (2002b) Noradrenergic lesions differentially alter the antidepressant-like effects of reboxetine in a modified forced swim test. *Eur J Pharmacol* 436: 197-205.
- da Silva G, Matteussi A, dos Santos A, Calixto J, Rodrigues A (2000) Evidence for dual effects of nitric oxide in the forced swimming test and in the tail suspension test in mice. *Neuroreport* 11: 3699-702.
- da Silva FC, do Carmo de Oliveira Cito M, da Silva MI, Moura BA, de Aquino Neto MR, Feitosa ML, de Castro Chaves R, Macedo DS, de Vasconcelos SM, de França Fonteles MM, de Sousa FC (2010) Behavioral alterations and pro-oxidant effect of a single ketamine administration to mice. *Brain Res Bull* 83: 9-15.
- Daniele A, Divella R, Paradiso A, Mattioli V, Romito F, Giotta F, Casamassima P, Quaranta M (2011) Serotonin transporter polymorphism in major depressive disorder (MDD), psychiatric disorders, and in MDD in response to stressful life events: causes and treatment with antidepressant. *In Vivo* 25: 895-901.
- Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW (2008) From inflammation to sickness and depression: when the immune system subjugates the brain. *Nat Rev Neurosci* 9: 46-56.



- de Boer S, Koolhaas J (2005) 5-HT1A and 5-HT1B receptor agonists and aggression: a pharmacological challenge of the serotonin deficiency hypothesis. *Eur J Pharmacol* 526: 125-39.
- de Oliveira L, Fraga DB, De Luca RD, Canever L, Ghedim FV, Matos MP, Streck EL, Quevedo J, Zugno AI (2011) Behavioral changes and mitochondrial dysfunction in a rat model of schizophrenia induced by ketamine. *Metab Brain Dis* 26: 69-77.
- De Vry J, Schreiber R, Melon C, Dalmus M, Jentsch KR (2004) 5-HT1A receptors are differentially involved in the anxiolytic- and antidepressant-like effects of 8-OH-DPAT and fluoxetine in the rat. *Eur Neuropsychopharmacol* 14: 487-95.
- Delgado PL (2004) How antidepressants help depression: mechanisms of action and clinical response. *J Clin Psychiatry* 65 Suppl 4: 25-30.
- Della FP, Abelaira HM, Réus GZ, Ribeiro KF, Antunes AR, Scaini G, Jeremias IC, dos Santos LM, Jeremias GC, Streck EL, Quevedo J (2012) Tianeptine treatment induces antidepressant-like effects and alters BDNF and energy metabolism in the brain of rats. *Behav Brain Res* 233: 526-35.
- Delles C, Jacobi J, Schlaich MP, John S, Schmieder RE (2002) Assessment of endothelial function of the renal vasculature in human subjects. *Am J Hypertens* 15: 3-9.
- Detke M, Rickels M, Lucki I (1995) Active behaviors in the rat forced swimming test differentially produced by serotonergic and noradrenergic antidepressants. *Psychopharmacology (Berl)* 121: 66-72.
- Dev K (2004) Making protein interactions druggable: targeting PDZ domains. *Nat Rev Drug Discov* 3: 1047-56.
- Dhir A, Kulkarni S (2007) Involvement of nitric oxide (NO) signaling pathway in the antidepressant action of bupropion, a dopamine reuptake inhibitor. *Eur J Pharmacol* 568: 177-85.
- Dhir A, Kulkarni SK (2011) Nitric oxide and major depression. *Nitric Oxide* 24: 125-31.
- Diener C, Kuehner C, Brusniak W, Ubl B, Wessa M, Flor H (2012) A meta-analysis of neurofunctional imaging studies of emotion and cognition in major depression. *Neuroimage* 61: 677-85.
- Ding XQ, Fiehler J, Kohlschütter B, Wittkugel O, Grzyska U, Zeumer H, Ullrich K (2008) MRI abnormalities in normal-appearing brain tissue of treated adult PKU patients. *J Magn Reson Imaging* 27: 998-1004.

- Dingledine R, Borges K, Bowie D, Traynelis SF (1999) The glutamate receptor ion channels. *Pharmacol Rev* 51: 7-61.
- Doucet MV, Harkin A, Dev KK (2012) The PSD-95/nNOS complex: new drugs for depression? *Pharmacol Ther* 133: 218-29.
- Dowlati Y, Herrmann N, Swardfager W, Liu H, Sham L, Reim EK, Lanctôt KL (2010) A meta-analysis of cytokines in major depression. *Biol Psychiatry* 67: 446-57.
- Drago A, Crisafulli C, Sidoti A, Serretti A (2011) The molecular interaction between the glutamatergic, noradrenergic, dopaminergic and serotonergic systems informs a detailed genetic perspective on depressive phenotypes. *Prog Neurobiol* 94: 418-60.
- Drevets WC, Price JL, Furey ML (2008) Brain structural and functional abnormalities in mood disorders: implications for neurocircuitry models of depression. *Brain Struct Funct* 213: 93-118.
- Dulawa SC, Holick KA, Gundersen B, Hen R (2004) Effects of chronic fluoxetine in animal models of anxiety and depression. *Neuropsychopharmacology* 29: 1321-30.
- Duman RS (2002) Pathophysiology of depression: the concept of synaptic plasticity. *Eur Psychiatry* 17 Suppl 3: 306-10.
- Duman RS (2004) Depression: a case of neuronal life and death? *Biol Psychiatry* 56: 140-45.
- Duman RS, Heninger GR, Nestler EJ (1997) A molecular and cellular theory of depression. *Arch Gen Psychiatry* 54: 597-606.
- Duman RS, Monteggia LM (2006) A neurotrophic model for stress-related mood disorders. *Biol Psychiatry* 59: 1116-27.
- Duman RS, Voleti B (2012) Signaling pathways underlying the pathophysiology and treatment of depression: novel mechanisms for rapid-acting agents. *Trends Neurosci* 35: 47-56.
- Dunn AJ (2000) Cytokine activation of the HPA axis. *Ann N Y Acad Sci* 917: 608-17.
- Durkin S, Prendergast A, Harkin A (2008) Reduced efficacy of fluoxetine following MDMA ("Ecstasy")-induced serotonin loss in rats. *Prog Neuropsychopharmacol Biol Psychiatry* 32: 1894-901.

- Echeverry MB, Guimarães FS, Del Bel EA (2004) Acute and delayed restraint stress-induced changes in nitric oxide producing neurons in limbic regions. *Neuroscience* 125: 981-93.
- Eguchi K, Nakanishi S, Takagi H, Taoufiq Z, Takahashi T (2012) Maturation of a PKG-dependent retrograde mechanism for exoendocytic coupling of synaptic vesicles. *Neuron* 74: 517-29.
- Ehninger D, Kempermann G (2008) Neurogenesis in the adult hippocampus. *Cell Tissue Res* 331: 243-50.
- El-faramawy Y, El-banouby M, Sergeev P, Mortagy A, Amer M, Abdel-tawab A (2009) Changes in glutamate decarboxylase enzyme activity and tau-protein phosphorylation in the hippocampus of old rats exposed to chronic mild stress: reversal with the neuronal nitric oxide synthase inhibitor 7-nitroindazole. *Pharmacol Biochem Behav* 91: 339-44.
- Elkis H, Friedman L, Wise A, Meltzer HY (1995) Meta-analyses of studies of ventricular enlargement and cortical sulcal prominence in mood disorders. Comparisons with controls or patients with schizophrenia. *Arch Gen Psychiatry* 52: 735-46.
- Eng LF, Ghirnikar RS (1994) GFAP and astrogliosis. *Brain Pathol* 4: 229-37.
- Engin E, Treit D, Dickson CT (2009) Anxiolytic- and antidepressant-like properties of ketamine in behavioral and neurophysiological animal models. *Neuroscience* 161: 359-69.
- Ergün Y, Ergün U (2007) Prevention of pro-depressant effect of L-arginine in the forced swim test by NG-nitro-L-arginine and [1H-[1,2,4]Oxadiazole[4,3-a]quinoxalin-1-one]. *Eur J Pharmacol* 554: 150-54.
- Estrada-Camarena E, Fernández-Guasti A, López-Rubalcava C (2006a) Participation of the 5-HT1A receptor in the antidepressant-like effect of estrogens in the forced swimming test. *Neuropsychopharmacology* 31: 247-55.
- Estrada-Camarena E, López-Rubalcava C, Fernández-Guasti A (2006b) Facilitating antidepressant-like actions of estrogens are mediated by 5-HT1A and estrogen receptors in the rat forced swimming test. *Psychoneuroendocrinology* 31: 905-14.
- Evans AK, Heerkens JL, Lowry CA (2009) Acoustic stimulation in vivo and corticotropin-releasing factor in vitro increase tryptophan hydroxylase activity in the rat caudal dorsal raphe nucleus. *Neurosci Lett* 455: 36-41.

- Fang M, Jaffrey S, Sawa A, Ye K, Luo X, Snyder S (2000) Dexas1: a G protein specifically coupled to neuronal nitric oxide synthase via CAPON. *Neuron* 28: 183-93.
- Figueiredo HF, Bruestle A, Bodie B, Dolgas CM, Herman JP (2003) The medial prefrontal cortex differentially regulates stress-induced c-fos expression in the forebrain depending on type of stressor. *Eur J Neurosci* 18: 2357-64.
- Finkel M, Laghrissi-Thode F, Pollock B, Rong J (1996) Paroxetine is a novel nitric oxide synthase inhibitor. *Psychopharmacol Bull* 32: 653-58.
- Fletcher A, Forster EA, Bill DJ, Brown G, Cliffe IA, Hartley JE, Jones DE, McLenachan A, Stanhope KJ, Critchley DJ, Childs KJ, Middlefell VC, Lanfumey L, Corradetti R, Laporte AM, Gozlan H, Hamon M, Dourish CT (1996) Electrophysiological, biochemical, neurohormonal and behavioural studies with WAY-100635, a potent, selective and silent 5-HT<sub>1A</sub> receptor antagonist. *Behav Brain Res* 73: 337-53.
- Florio SK, Loh C, Huang SM, Iwamaye AE, Kitto KF, Fowler KW, Treiberg JA, Hayflick JS, Walker JM, Fairbanks CA, Lai Y (2009) Disruption of nNOS-PSD95 protein-protein interaction inhibits acute thermal hyperalgesia and chronic mechanical allodynia in rodents. *Br J Pharmacol* 158: 494-506.
- Forster EA, Cliffe IA, Bill DJ, Dover GM, Jones D, Reilly Y, Fletcher A (1995) A pharmacological profile of the selective silent 5-HT<sub>1A</sub> receptor antagonist, WAY-100635. *Eur J Pharmacol* 281: 81-88.
- Francis PT, Poynton A, Lowe SL, Najlerahim A, Bridges PK, Bartlett JR, Procter AW, Bruton CJ, Bowen DM (1989) Brain amino acid concentrations and Ca<sup>2+</sup>-dependent release in intractable depression assessed antemortem. *Brain Res* 494: 315-24.
- Frazer A (1997) Pharmacology of antidepressants. *J Clin Psychopharmacol* 17 Suppl 1: 2S-18S.
- Freed WJ, Dillon-Carter O, Kleinman JE (1993) Properties of [<sup>3</sup>H]AMPA binding in postmortem human brain from psychotic subjects and controls: increases in caudate nucleus associated with suicide. *Exp Neurol* 121: 48-56.
- Freitas AE, Machado DG, Budni J, Neis VB, Balen GO, Lopes MW, de Souza LF, Dafre AL, Leal RB, Rodrigues AL (2013) Fluoxetine modulates hippocampal cell signaling pathways implicated in neuroplasticity in olfactory bulbectomized mice. *Behav Brain Res* 237: 176-84.
- Friebe A, Koesling D (2009) The function of NO-sensitive guanylyl cyclase: what we can learn from genetic mouse models. *Nitric Oxide* 21: 149-56.

- Frodl T, Meisenzahl EM, Zetzsche T, Born C, Jäger M, Groll C, Bottlender R, Leinsinger G, Möller HJ (2003) Larger amygdala volumes in first depressive episode as compared to recurrent major depression and healthy control subjects. *Biol Psychiatry* 53: 338-44.
- Gallo EF, Iadecola C (2011) Neuronal nitric oxide contributes to neuroplasticity-associated protein expression through cGMP, protein kinase G, and extracellular signal-regulated kinase. *J Neurosci* 31: 6947-55.
- Gambarana C, Scheggi S, Tagliamonte A, Tolu P, De Montis MG (2001) Animal models for the study of antidepressant activity. *Brain Res Brain Res Protoc* 7: 11-20.
- Gao SF, Qi XR, Zhao J, Balesar R, Bao AM, Swaab DF (2012) Decreased NOS1 Expression in the Anterior Cingulate Cortex in Depression. *Cereb Cortex*. [In press]
- Garcia LS, Comim CM, Valvassori SS, Réus GZ, Andreazza AC, Stertz L, Fries GR, Gavioli EC, Kapczinski F, Quevedo J (2008a) Chronic administration of ketamine elicits antidepressant-like effects in rats without affecting hippocampal brain-derived neurotrophic factor protein levels. *Basic Clin Pharmacol Toxicol* 103: 502-06.
- Garcia LS, Comim CM, Valvassori SS, Réus GZ, Barbosa LM, Andreazza AC, Stertz L, Fries GR, Gavioli EC, Kapczinski F, Quevedo J (2008b) Acute administration of ketamine induces antidepressant-like effects in the forced swimming test and increases BDNF levels in the rat hippocampus. *Prog Neuropsychopharmacol Biol Psychiatry* 32: 140-44.
- Garcia LS, Comim CM, Valvassori SS, Réus GZ, Stertz L, Kapczinski F, Gavioli EC, Quevedo J (2009) Ketamine treatment reverses behavioral and physiological alterations induced by chronic mild stress in rats. *Prog Neuropsychopharmacol Biol Psychiatry* 33: 450-55.
- Garthwaite J (2007) Neuronal nitric oxide synthase and the serotonin transporter get harmonious. *Proc Natl Acad Sci U S A* 104: 7739-40.
- Getting SJ, Segieth J, Ahmad S, Biggs CS, Whitton PS (1996a) Biphasic modulation of GABA release by nitric oxide in the hippocampus of freely moving rats in vivo. *Brain Res* 717: 196-99.
- Ghasemi M, Sadeghipour H, Mosleh A, Sadeghipour H, Mani A, Dehpour A (2008) Nitric oxide involvement in the antidepressant-like effects of acute lithium administration in the mouse forced swimming test. *Eur Neuropsychopharmacol* 18: 323-32.

- Gibson EL, Kennedy AJ, Curzon G (1993) d-Fenfluramine- and d-norfenfluramine-induced hypophagia: differential mechanisms and involvement of postsynaptic 5-HT receptors. *Eur J Pharmacol* 242: 83-90.
- Gigliucci V, Buckley K, Nunan J, O'Shea K, Harkin A (2010) A role for serotonin in the antidepressant activity of N(G)-Nitro-L-arginine, in the rat forced swimming test. *Pharmacol Biochem Behav* 94: 524-533.
- Gigliucci V, O'Dowd G, Casey S, Egan D, Gibney S, Harkin A (2013) Ketamine elicits sustained antidepressant-like activity via a serotonin-dependent mechanism. *Psychopharmacology (Berl)*. [In press]
- Gilbert P, Gilbert J, Irons C (2004) Life events, entrapments and arrested anger in depression. *J Affect Disord* 79: 149-60.
- Girgin Sagin F, Sozmen EY, Ersoz B, Menten G (2004) Link between monoamine oxidase and nitric oxide. *Neurotoxicology* 25: 91-99.
- Glavin GB, Paré WP, Sandbak T, Bakke HK, Murison R (1994) Restraint stress in biomedical research: an update. *Neurosci Biobehav Rev* 18: 223-49.
- Goldsmith PC, Leslie TA, Hayes NA, Levell NJ, Dowd PM, Foreman JC (1996) Inhibitors of nitric oxide synthase in human skin. *J Invest Dermatol* 106: 113-18.
- Golozoubova V, Strauss F, Malmjöf K (2006) Locomotion is the major determinant of sibutramine-induced increase in energy expenditure. *Pharmacol Biochem Behav* 83: 517-27.
- Grecksch G, Zhou D, Franke C, Schröder U, Sabel B, Becker A, Huether G (1997) Influence of olfactory bulbectomy and subsequent imipramine treatment on 5-hydroxytryptaminergic presynapses in the rat frontal cortex: behavioural correlates. *Br J Pharmacol* 122: 1725-31.
- Guan Z, Fang J (2006) Peripheral immune activation by lipopolysaccharide decreases neurotrophins in the cortex and hippocampus in rats. *Brain Behav Immun* 20: 64-71.
- Guillemin GJ, Kerr SJ, Smythe GA, Smith DG, Kapoor V, Armati PJ, Croitoru J, Brew BJ (2001) Kynurenine pathway metabolism in human astrocytes: a paradox for neuronal protection. *J Neurochem* 78: 842-53.
- Guimarães F, Beijamini V, Moreira F, Aguiar D, de Lucca A (2005) Role of nitric oxide in brain regions related to defensive reactions. *Neurosci Biobehav Rev* 29: 1313-22.

- Guix F, Uribealago I, Coma M, Muñoz F (2005) The physiology and pathophysiology of nitric oxide in the brain. *Prog Neurobiol* 76: 126-52.
- Görtelmeyer R, Erbler H (1992) Memantine in the treatment of mild to moderate dementia syndrome. A double-blind placebo-controlled study. *Arzneimittelforschung* 42: 904-13.
- Haga K, Gregory L, Hicks C, Ward M, Beech J, Bath P, Williams S, O'Neill M (2003) The neuronal nitric oxide synthase inhibitor, TRIM, as a neuroprotective agent: effects in models of cerebral ischaemia using histological and magnetic resonance imaging techniques. *Brain Res* 993: 42-53.
- Haleem D, Parveen T (1994) Brain regional serotonin synthesis following adaptation to repeated restraint. *Neuroreport* 5: 1785-88.
- Haleem D, Samad N, Perveen T, Haider S, Haleem M (2007) Role of serotonin-1A receptors in restraint-induced behavioral deficits and adaptation to repeated restraint stress in rats. *Int J Neurosci* 117: 243-57.
- Halford JC, Blundell JE (1996) Metergoline antagonizes fluoxetine-induced suppression of food intake but not changes in the behavioural satiety sequence. *Pharmacol Biochem Behav* 54: 745-51.
- Han PP, Holbrook TL, Sise MJ, Sack DI, Sise CB, Hoyt DB, Coimbra R, Potenza B, Anderson JP (2011) Postinjury depression is a serious complication in adolescents after major trauma: injury severity and injury-event factors predict depression and long-term quality of life deficits. *J Trauma* 70: 923-30.
- Hanafy KA, Krumenacker JS, Murad F (2001) NO, nitrotyrosine, and cyclic GMP in signal transduction. *Med Sci Monit* 7: 801-19.
- Handy R, Moore P (1998) Effects of selective inhibitors of neuronal nitric oxide synthase on carrageenan-induced mechanical and thermal hyperalgesia. *Neuropharmacology* 37: 37-43.
- Hannestad J, DellaGioia N, Bloch M (2011) The effect of antidepressant medication treatment on serum levels of inflammatory cytokines: a meta-analysis. *Neuropsychopharmacology* 36: 2452-59.
- Hansen KB, Furukawa H, Traynelis SF (2010) Control of assembly and function of glutamate receptors by the amino-terminal domain. *Mol Pharmacol* 78: 535-49.
- Harkin A, Bruce K, Craft B, Paul I (1999a) Nitric oxide synthase inhibitors have antidepressant-like properties in mice. 1. Acute treatments are active in the forced swim test. *Eur J Pharmacol* 372: 207-13.

- Harkin A, Kelly JP, McNamara M, Connor TJ, Dredge K, Redmond A, Leonard BE (1999b) Activity and onset of action of reboxetine and effect of combination with sertraline in an animal model of depression. *Eur J Pharmacol* 364: 123-32.
- Harkin A, O'Donnell J, Kelly J (2002) A study of VitalView for behavioural and physiological monitoring in laboratory rats. *Physiol Behav* 77: 65-77.
- Harkin A, Connor TJ, Walsh M, St John N, Kelly JP (2003a) Serotonergic mediation of the antidepressant-like effects of nitric oxide synthase inhibitors. *Neuropharmacology* 44: 616-23.
- Harkin A, Kelly J, Leonard B (2003b) A review of the relevance and validity of olfactory bulbectomy as a model of depression. *Clin Neurosci Res* 3: 253–262.
- Harkin A, Connor T, Burns M, Kelly J (2004) Nitric oxide synthase inhibitors augment the effects of serotonin re-uptake inhibitors in the forced swimming test. *Eur Neuropsychopharmacol* 14: 274-81.
- Harvey BH (1996) Affective Disorders and Nitric Oxide: A Role in Pathways to Relapse and Refractoriness? *Hum Psychopharm* 11: 309-19.
- Harvey B, Oosthuizen F, Brand L, Wegener G, Stein D (2004) Stress-restress evokes sustained iNOS activity and altered GABA levels and NMDA receptors in rat hippocampus. *Psychopharmacology (Berl)* 175: 494-502.
- Hashimoto K (2011) The role of glutamate on the action of antidepressants. *Prog Neuropsychopharmacol Biol Psychiatry* 35: 1558-68.
- Hawkins MF, Uzelac SM, Hearn JK, Baumeister AA (2008) Effects of selective serotonin<sub>2</sub> ligands on behaviors evoked by stress in the rat. *Pharmacol Biochem Behav* 90: 632-39.
- Hellweg R, Zueger M, Fink K, Hörtnagl H, Gass P (2007) Olfactory bulbectomy in mice leads to increased BDNF levels and decreased serotonin turnover in depression-related brain areas. *Neurobiol Dis* 25: 1-7.
- Hernández ME, Mendieta D, Martínez-Fong D, Loria F, Moreno J, Estrada I, Bojalil R, Pavón L (2008) Variations in circulating cytokine levels during 52 week course of treatment with SSRI for major depressive disorder. *Eur Neuropsychopharmacol* 18: 917-24.
- Ho Y, Liu T, Tai M, Wen Z, Chow RS, Tsai Y, Wong C (2001) Effects of olfactory bulbectomy on NMDA receptor density in the rat brain: . *Brain Res* 900: 214-18.
- Howren MB, Lamkin DM, Suls J (2009) Associations of depression with C-reactive protein, IL-1, and IL-6: a meta-analysis. *Psychosom Med* 71: 171-86.



- Huang YH, Cheng CY, Hong CJ, Tsai SJ (2004) Expression of c-Fos-like immunoreactivity in the brain of mice with learned helplessness. *Neurosci Lett* 363: 280-83.
- Hwang J, Lee HJ, Lee WH, Suk K (2010) NF- $\kappa$ B as a common signaling pathway in ganglioside-induced autophagic cell death and activation of astrocytes. *J Neuroimmunol* 226: 66-72.
- Inan S, Yalcin I, Aksu F (2004) Dual effects of nitric oxide in the mouse forced swimming test: possible contribution of nitric oxide-mediated serotonin release and potassium channel modulation. *Pharmacol Biochem Behav* 77: 457-64.
- Ishii K, Chang B, Kerwin JF, Huang ZJ, Murad F (1990) N omega-nitro-L-arginine: a potent inhibitor of endothelium-derived relaxing factor formation. *Eur J Pharmacol* 176: 219-23.
- Ising M, Holsboer F (2007) CRH-sub-1 receptor antagonists for the treatment of depression and anxiety. *Exp Clin Psychopharmacol* 15: 519-28.
- Jacobson LH, Cryan JF (2007) Feeling strained? Influence of genetic background on depression-related behavior in mice: a review. *Behav Genet* 37: 171-213.
- Jankowski MP, Sesack SR (2004) Prefrontal cortical projections to the rat dorsal raphe nucleus: ultrastructural features and associations with serotonin and gamma-aminobutyric acid neurons. *J Comp Neurol* 468: 518-29.
- Javelot H, Weiner L, Terramorsi R, Rougeot C, Lalonde R, Messaoudi M (2011) Efficacy of chronic antidepressant treatments in a new model of extreme anxiety in rats. *Depress Res Treat* 2011: article ID 531435, 10 pages.
- Jefferys D, Funder J (1996) Nitric oxide modulates retention of immobility in the forced swimming test in rats. *Eur J Pharmacol* 295: 131-35.
- Jevtovic-Todorovic V, Wozniak DF, Benshoff ND, Olney JW (2001) A comparative evaluation of the neurotoxic properties of ketamine and nitrous oxide. *Brain Res* 895: 264-67.
- Jick H, Kaye J, Jick S (2004) Antidepressants and the risk of suicidal behaviors. *JAMA* 292: 338-43.
- Joca S, Ferreira F, Guimarães F (2007) Modulation of stress consequences by hippocampal monoaminergic, glutamatergic and nitrenergic neurotransmitter systems. *Stress* 10: 227-49.

- Joca S, Guimarães F (2006) Inhibition of neuronal nitric oxide synthase in the rat hippocampus induces antidepressant-like effects. *Psychopharmacology (Berl)* 185: 298-305.
- Jonville-Bera AP, Guilmot JL, Aspe G, Autret-Leca E, Magnant J (2011) Is exogenous administration of IL-1ra (anakinra) likely to induce severe depression? *Eur J Clin Pharmacol* 67: 213-14.
- Justicia C, Ramos-Cabrer P, Hoehn M (2008) MRI detection of secondary damage after stroke: chronic iron accumulation in the thalamus of the rat brain. *Stroke* 39: 1541-47.
- Kadioglu M, Muci E, Ozyavuz R, Yaris E, Kesim M, Kalyoncu NI (2010) Paroxetine inhibited the relaxations induced by EFS in mice corpus cavernosum: is it a NOS inhibition? *Fundam Clin Pharmacol* 24: 55-61.
- Kalisch R, Schubert M, Jacob W, Kessler MS, Hemauer R, Wigger A, Landgraf R, Auer DP (2006) Anxiety and hippocampus volume in the rat. *Neuropsychopharmacology* 31: 925-32.
- Kano T, Shimizu-Sasamata M, Huang PL, Moskowitz MA, Lo EH (1998) Effects of nitric oxide synthase gene knockout on neurotransmitter release in vivo. *Neuroscience* 86: 695-99.
- Kari HP, Davidson PP, Kohl HH, Kochhar MM (1978) Effects of ketamine on brain monoamine levels in rats. *Res Commun Chem Pathol Pharmacol* 20: 475-88.
- Karolewicz B, Paul I, Antkiewicz-Michaluk L Effect of NOS inhibitor on forced swim test and neurotransmitters turnover in the mouse brain. *Pol J Pharmacol* 53: 587-96.
- Karolewicz B, Stockmeier CA, Ordway GA (2005) Elevated levels of the NR2C subunit of the NMDA receptor in the locus coeruleus in depression. *Neuropsychopharmacology* 30: 1557-67.
- Karolewicz B, Szebeni K, Stockmeier C, Konick L, Overholser J, Jurjus G, Roth B, Ordway G (2004) Low nNOS protein in the locus coeruleus in major depression. *J Neurochem* 91: 1057-66.
- Kawai F, Miyachi E (2001) Modulation by cGMP of the voltage-gated currents in newt olfactory receptor cells. *Neurosci Res* 39: 327-37.
- Keilhoff G, Becker A, Grecksch G, Bernstein HG, Wolf G (2006) Cell proliferation is influenced by bulbectomy and normalized by imipramine treatment in a region-specific manner. *Neuropsychopharmacology* 31: 1165-76.

- Kellogg DL, Zhao JL, Wu Y (2009) Roles of nitric oxide synthase isoforms in cutaneous vasodilation induced by local warming of the skin and whole body heat stress in humans. *J Appl Physiol* 107: 1438-44.
- Kelly J, Wrynn A, Leonard B (1997) The olfactory bulbectomized rat as a model of depression: an update. *Pharmacol Ther* 74: 299-316.
- Kelly ME, Blau CW, Griffin KM, Gobbo OL, Jones JF, Kerskens CM (2010) Quantitative functional magnetic resonance imaging of brain activity using bolus-tracking arterial spin labeling. *J Cereb Blood Flow Metab* 30: 913-22.
- Kelly ME, Blau CW, Kerskens CM (2009) Bolus-tracking arterial spin labelling: theoretical and experimental results. *Phys Med Biol* 54: 1235-51.
- Kessler R, Akiskal H, Ames M, Birnbaum H, Greenberg P, Hirschfeld R, Jin R, Merikangas K, Simon G, Wang P (2006) Prevalence and effects of mood disorders on work performance in a nationally representative sample of U.S. workers. *Am J Psychiatry* 163: 1561-68.
- Kim KS, Han PL (2006) Optimization of chronic stress paradigms using anxiety- and depression-like behavioral parameters. *J Neurosci Res* 83: 497-507.
- Kim KS, Kwon HJ, Baek IS, Han PL (2012) Repeated Short-term (2h×14d) Emotional Stress Induces Lasting Depression-like Behavior in Mice. *Exp Neurobiol* 21: 16-22.
- Kim Y, Paik J, Lee S, Yoon D, Han C, Lee B (2006) Increased plasma nitric oxide level associated with suicide attempt in depressive patients. *Prog Neuropsychopharmacol Biol Psychiatry* 30: 1091-96.
- Kirby LG, Pan YZ, Freeman-Daniels E, Rani S, Nunan JD, Akanwa A, Beck SG (2007) Cellular effects of swim stress in the dorsal raphe nucleus. *Psychoneuroendocrinology* 32: 712-23.
- Kiss J, Vizi E (2001) Nitric oxide: a novel link between synaptic and nonsynaptic transmission. *Trends Neurosci* 24: 211-15.
- Kiss JP (2000) Role of nitric oxide in the regulation of monoaminergic neurotransmission. *Brain Res Bull* 52: 459-66.
- Knol MJ, Twisk JW, Beekman AT, Heine RJ, Snoek FJ, Pouwer F (2006) Depression as a risk factor for the onset of type 2 diabetes mellitus. A meta-analysis. *Diabetologia* 49: 837-45.
- Koehler RC, Roman RJ, Harder DR (2009) Astrocytes and the regulation of cerebral blood flow. *Trends Neurosci* 32: 160-69.

- Koike H, Iijima M, Chaki S (2011) Involvement of AMPA receptor in both the rapid and sustained antidepressant-like effects of ketamine in animal models of depression. *Behav Brain Res* 224: 107-11.
- Kovács KJ (1998) c-Fos as a transcription factor: a stressful (re)view from a functional map. *Neurochem Int* 33: 287-97.
- Kovács KJ (2008) Measurement of immediate-early gene activation- c-fos and beyond. *J Neuroendocrinol* 20: 665-72.
- Krass M, Wegener G, Vasar E, Volke V (2011) The antidepressant action of imipramine and venlafaxine involves suppression of nitric oxide synthesis. *Behav Brain Res* 218: 57-63.
- Krishnadas R, Cavanagh J (2012) Depression: an inflammatory illness? *J Neurol Neurosurg Psychiatry* 83: 495-502.
- Kronenberg G, Tebartz van Elst L, Regen F, Deuschle M, Heuser I, Colla M (2009) Reduced amygdala volume in newly admitted psychiatric in-patients with unipolar major depression. *J Psychiatr Res* 43: 1112-17.
- Kugaya A, Sanacora G (2005) Beyond monoamines: glutamatergic function in mood disorders. *CNS Spectr* 10: 808-19.
- Kuhn D, Arthur RJ (1996) Inactivation of brain tryptophan hydroxylase by nitric oxide. *J Neurochem* 67: 1072-77.
- Kuhn D, Arthur RJ (1997) Molecular mechanism of the inactivation of tryptophan hydroxylase by nitric oxide: attack on critical sulfhydryls that spare the enzyme iron center. *J Neurosci* 17: 7245-51.
- Künzel HE, Zobel AW, Nickel T, Ackl N, Uhr M, Sonntag A, Ising M, Holsboer F (2003) Treatment of depression with the CRH-1-receptor antagonist R121919: endocrine changes and side effects. *J Psychiatr Res* 37: 525-33.
- Lambert LE, Whitten JP, Baron BM, Cheng HC, Doherty NS, McDonald IA (1991) Nitric oxide synthesis in the CNS endothelium and macrophages differs in its sensitivity to inhibition by arginine analogues. *Life Sci* 48: 69-75.
- Lamers F, van Oppen P, Comijs HC, Smit JH, Spinhoven P, van Balkom AJ, Nolen WA, Zitman FG, Beekman AT, Penninx BW (2011) Comorbidity patterns of anxiety and depressive disorders in a large cohort study: the Netherlands Study of Depression and Anxiety (NESDA). *J Clin Psychiatry* 72: 341-48.
- Lara N, Archer S, Baker G, Le Mellédo J (2003) Paroxetine-induced increase in metabolic end products of nitric oxide. *J Clin Psychopharmacol* 23: 641-45.

- Le Mellédo J, Mahil N, Baker G (2004) Nitric oxide: a key player in the relation between cardiovascular disease and major depressive disorder? *J Psychiatry Neurosci* 29: 414-16.
- Lee T, Jarome T, Li S, Kim J, Helmstetter F (2009) Chronic stress selectively reduces hippocampal volume in rats: a longitudinal magnetic resonance imaging study. *Neuroreport* 20: 1554-58.
- Lee YH, Kim SH, Kim Y, Lim Y, Ha K, Shin SY (2012) Inhibitory effect of the antidepressant imipramine on NF- $\kappa$ B-dependent CXCL1 expression in TNF $\alpha$ -exposed astrocytes. *Int Immunopharmacol* 12: 547-55.
- Lei S, Pan Z, Aggarwal S, Chen H, Hartman J, Sucher N, Lipton S (1992) Effect of nitric oxide production on the redox modulatory site of the NMDA receptor-channel complex. *Neuron* 8: 1087-99.
- Levine J, Panchalingam K, Rapoport A, Gershon S, McClure RJ, Pettegrew JW (2000) Increased cerebrospinal fluid glutamine levels in depressed patients. *Biol Psychiatry* 47: 586-93.
- Li N, Lee B, Liu RJ, Banasr M, Dwyer JM, Iwata M, Li XY, Aghajanian G, Duman RS (2010) mTOR-dependent synapse formation underlies the rapid antidepressant effects of NMDA antagonists. *Science* 329: 959-64.
- Li N, Liu RJ, Dwyer JM, Banasr M, Lee B, Son H, Li XY, Aghajanian G, Duman RS (2011) Glutamate N-methyl-D-aspartate receptor antagonists rapidly reverse behavioral and synaptic deficits caused by chronic stress exposure. *Biol Psychiatry* 69: 754-61.
- Lindfors N, Barati S, O'Connor WT (1997) Differential effects of single and repeated ketamine administration on dopamine, serotonin and GABA transmission in rat medial prefrontal cortex. *Brain Res* 759: 205-12.
- Lipton S, Choi Y, Sucher N, Chen H (1998) Neuroprotective versus neurodestructive effects of NO-related species. *Biofactors* 8: 33-40.
- Littlewood CL, Jones N, O'Neill MJ, Mitchell SN, Tricklebank M, Williams SC (2006) Mapping the central effects of ketamine in the rat using pharmacological MRI. *Psychopharmacology (Berl)* 186: 64-81.
- Littrell JL (2012) Taking the Perspective that a Depressive State Reflects Inflammation: Implications for the Use of Antidepressants. *Front Psychol* 3: article ID 297.
- Liu L, Stamler J (1999) NO: an inhibitor of cell death. *Cell Death Differ* 6: 937-42.

- Lonart G, Wang J, Johnson K (1992) Nitric oxide induces neurotransmitter release from hippocampal slices. *Eur J Pharmacol* 220: 271-72.
- Lorenzetti V, Allen N, Fornito A, Yücel M (2009) Structural brain abnormalities in major depressive disorder: a selective review of recent MRI studies. *J Affect Disord* 117: 1-17.
- Lucki I (1997) The forced swimming test as a model for core and component behavioral effects of antidepressant drugs. *Behav Pharmacol* 8: 523-32.
- Luo C, Xu H, Li XM (2005) Quetiapine reverses the suppression of hippocampal neurogenesis caused by repeated restraint stress. *Brain Res* 1063: 32-39.
- López-Figueroa MO, Itoi K, Watson SJ (1998) Regulation of nitric oxide synthase messenger RNA expression in the rat hippocampus by glucocorticoids. *Neuroscience* 87: 439-46.
- Machado-Vieira R, Mallinger AG (2012) Abnormal function of monoamine oxidase-A in comorbid major depressive disorder and cardiovascular disease: pathophysiological and therapeutic implications (review). *Mol Med Report* 6: 915-22.
- Machado-Vieira R, Salvatore G, Diazgranados N, Zarate CJ (2009) Ketamine and the next generation of antidepressants with a rapid onset of action. *Pharmacol Ther* 123: 143-50.
- Madrigal J, García-Bueno B, Moro M, Lizasoain I, Lorenzo P, Leza J (2003) Relationship between cyclooxygenase-2 and nitric oxide synthase-2 in rat cortex after stress. *Eur J Neurosci* 18: 1701-05.
- Madrigal J, Hurtado O, Moro M, Lizasoain I, Lorenzo P, Castrillo A, Boscá L, Leza J (2002) The increase in TNF- $\alpha$  levels is implicated in NF- $\kappa$ B activation and inducible nitric oxide synthase expression in brain cortex after immobilization stress. *Neuropsychopharmacology* 26: 155-63.
- Madrigal J, Moro M, Lizasoain I, Lorenzo P, Castrillo A, Boscá L, Leza J (2001) Inducible nitric oxide synthase expression in brain cortex after acute restraint stress is regulated by nuclear factor  $\kappa$ B-mediated mechanisms. *J Neurochem* 76: 532-38.
- Maeng S, Zarate CA, Du J, Schloesser RJ, McCammon J, Chen G, Manji HK (2008) Cellular mechanisms underlying the antidepressant effects of ketamine: role of  $\alpha$ -amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors. *Biol Psychiatry* 63: 349-52.

- Maes M, Verkerk R, Vandoolaeghe E, Lin A, Scharpé S (1998) Serum levels of excitatory amino acids, serine, glycine, histidine, threonine, taurine, alanine and arginine in treatment-resistant depression: modulation by treatment with antidepressants and prediction of clinical responsivity. *Acta Psychiatr Scand* 97: 302-08.
- Magariños A, Verdugo J, McEwen B (1997) Chronic stress alters synaptic terminal structure in hippocampus. *Proc Natl Acad Sci U S A* 94: 14002-8.
- Maltese TM (1999) Adjunctive lamotrigine treatment for major depression. *Am J Psychiatry* 156: 1833.
- Mancuso C (2004) Heme oxygenase and its products in the nervous system. *Antioxid Redox Signal* 6: 878-87.
- Mancuso C, Bonsignore A, Di Stasio E, Mordente A, Motterlini R (2003) Bilirubin and S-nitrosothiols interaction: evidence for a possible role of bilirubin as a scavenger of nitric oxide. *Biochem Pharmacol* 66: 2355-63.
- Manzoni O, Bockaert J (1993) Nitric oxide synthase activity endogenously modulates NMDA receptors. *J Neurochem* 61: 368-70.
- Mar A, Spreekmeester E, Rochford J (2002) Fluoxetine-induced increases in open-field habituation in the olfactory bulbectomized rat depend on test aversiveness but not on anxiety. *Pharmacol Biochem Behav* 73: 703-12.
- Martin LL, Bouchal RL, Smith DJ (1982) Ketamine inhibits serotonin uptake in vivo. *Neuropharmacology* 21: 113-18.
- Martin LL, Smith DJ (1982) Ketamine inhibits serotonin synthesis and metabolism in vivo. *Neuropharmacology* 21: 119-25.
- Martínez-Mota L, Estrada-Camarena E, López-Rubalcava C (2002) Indorenate produces antidepressant-like actions in the rat forced swimming test via 5-HT1A receptors. *Psychopharmacology (Berl)* 165: 60-66.
- Masi G, Brovedani P (2011) The hippocampus, neurotrophic factors and depression: possible implications for the pharmacotherapy of depression. *CNS Drugs* 25: 913-31.
- Matthews SC, Strigo IA, Simmons AN, Yang TT, Paulus MP (2008) Decreased functional coupling of the amygdala and supragenual cingulate is related to increased depression in unmedicated individuals with current major depressive disorder. *J Affect Disord* 111: 13-20.
- Mayer ML (2006) Glutamate receptors at atomic resolution. *Nature* 440: 456-62.

- McCabe C, Mishor Z (2011) Antidepressant medications reduce subcortical-cortical resting-state functional connectivity in healthy volunteers. *Neuroimage* 57: 1317-23.
- McCabe C, Mishor Z, Filippini N, Cowen PJ, Taylor MJ, Harmer CJ (2011) SSRI administration reduces resting state functional connectivity in dorso-medial prefrontal cortex. *Mol Psychiatry* 16: 592-94.
- McKinnon MC, Yucel K, Nazarov A, MacQueen GM (2009) A meta-analysis examining clinical predictors of hippocampal volume in patients with major depressive disorder. *J Psychiatry Neurosci* 34: 41-54.
- McKittrick C, Magariños A, Blanchard D, Blanchard R, McEwen B, Sakai R (2000) Chronic social stress reduces dendritic arbors in CA3 of hippocampus and decreases binding to serotonin transporter sites. *Synapse* 36: 85-94.
- Meador-Woodruff JH, Hogg AJ, Smith RE (2001) Striatal ionotropic glutamate receptor expression in schizophrenia, bipolar disorder, and major depressive disorder. *Brain Res Bull* 55: 631-40.
- Melia KR, Ryabinin AE, Schroeder R, Bloom FE, Wilson MC (1994) Induction and habituation of immediate early gene expression in rat brain by acute and repeated restraint stress. *J Neurosci* 14: 5929-38.
- Miyata S, Hamamura T, Lee Y, Miki M, Habara T, Oka T, Endo S, Taoka H, Kuroda S (2005) Contrasting Fos expression induced by acute reboxetine and fluoxetine in the rat forebrain: neuroanatomical substrates for the antidepressant effect. *Psychopharmacology (Berl)* 177: 289-95.
- Mnie-Filali O, Faure C, Lambás-Señas L, El Mansari M, Belblidia H, Gondard E, Etiévant A, Scarna H, Didier A, Berod A, Blier P, Haddjeri N (2011) Pharmacological blockade of 5-HT<sub>7</sub> receptors as a putative fast acting antidepressant strategy. *Neuropsychopharmacology* 36: 1275-88.
- Moffitt TE, Harrington H, Caspi A, Kim-Cohen J, Goldberg D, Gregory AM, Poulton R (2007) Depression and generalized anxiety disorder: cumulative and sequential comorbidity in a birth cohort followed prospectively to age 32 years. *Arch Gen Psychiatry* 64: 651-60.
- Mokler DJ, Commissaris RL, Warner MR, Rech RH (1983) Blockade of the behavioral effects of lysergic acid diethylamide, 2,5-dimethoxy-4-methylamphetamine, quipazine and lisuride by 5-hydroxytryptamine antagonists. *J Pharmacol Exp Ther* 227: 557-62.
- Moncada S, Palmer RM, Higgs EA (1991) Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev* 43: 109-42.



- Mony L, Kew JN, Gunthorpe MJ, Paoletti P (2009) Allosteric modulators of NR2B-containing NMDA receptors: molecular mechanisms and therapeutic potential. *Br J Pharmacol* 157: 1301-17.
- Moore PK, al-Swayeh OA, Chong NW, Evans RA, Gibson A (1990) L-NG-nitro arginine (L-NOARG), a novel, L-arginine-reversible inhibitor of endothelium-dependent vasodilatation in vitro. *Br J Pharmacol* 99: 408-12.
- Moreira F, Guimarães F (2008) Lack of effects of clomipramine on Fos and NADPH-diaphorase double-staining in the periaqueductal gray after exposure to an innate fear stimulus. *Physiol Behav* 94: 316-21.
- Moreno FA, Heninger GR, McGahuey CA, Delgado PL (2000) Tryptophan depletion and risk of depression relapse: a prospective study of tryptophan depletion as a potential predictor of depressive episodes. *Biol Psychiatry* 48: 327-29.
- Morrens J, Van Den Broeck W, Kempermann G (2012) Glial cells in adult neurogenesis. *Glia* 60: 159-74.
- Moser PC, Sanger DJ (1999) 5-HT<sub>1A</sub> receptor antagonists neither potentiate nor inhibit the effects of fluoxetine and biefloxatone in the forced swim test in rats. *Eur J Pharmacol* 372: 127-34.
- Mucignat-Caretta C, Bondí M, Caretta A (2006) Time course of alterations after olfactory bulbectomy in mice. *Physiol Behav* 89: 637-43.
- Mullershausen F, Friebe A, Feil R, Thompson WJ, Hofmann F, Koesling D (2003) Direct activation of PDE5 by cGMP: long-term effects within NO/cGMP signaling. *J Cell Biol* 160: 719-27.
- Muriel P, Pérez-Rojas J (2003) Nitric oxide inhibits mitochondrial monoamine oxidase activity and decreases outer mitochondria membrane fluidity. *Comp Biochem Physiol C Toxicol Pharmacol* 136: 191-97.
- Murrough JW, Iacoviello B, Neumeister A, Charney DS, Iosifescu DV (2011) Cognitive dysfunction in depression: neurocircuitry and new therapeutic strategies. *Neurobiol Learn Mem* 96: 553-63.
- Murrough JW (2012) Ketamine as a novel antidepressant: from synapse to behavior. *Clin Pharmacol Ther* 91: 303-09.
- Musselman DL, Lawson DH, Gumnick JF, Manatunga AK, Penna S, Goodkin RS, Greiner K, Nemeroff CB, Miller AH (2001) Paroxetine for the prevention of depression induced by high-dose interferon alfa. *N Engl J Med* 344: 961-66.

- Mutlu O, Ulak G, Laugeray A, Belzung C (2009) Effects of neuronal and inducible NOS inhibitor 1-[2-(trifluoromethyl) phenyl] imidazole (TRIM) in unpredictable chronic mild stress procedure in mice. *Pharmacol Biochem Behav* 92: 82-87.
- Müller N, Schwarz MJ, Dehning S, Douhe A, Cerovecki A, Goldstein-Müller B, Spellmann I, Hetzel G, Maino K, Kleindienst N, Möller HJ, Arolt V, Riedel M (2006) The cyclooxygenase-2 inhibitor celecoxib has therapeutic effects in major depression: results of a double-blind, randomized, placebo controlled, add-on pilot study to reboxetine. *Mol Psychiatry* 11: 680-84.
- Naylor G, Martin B, Hopwood S, Watson Y (1986) A two-year double-blind crossover trial of the prophylactic effect of methylene blue in manic-depressive psychosis. *Biol Psychiatry* 21: 915-20.
- Naylor G, Smith A, Connelly P (1987) A controlled trial of methylene blue in severe depressive illness. *Biol Psychiatry* 22: 657-59.
- Nelson E, Connolly J, McArthur P (2003) Nitric oxide and S-nitrosylation: excitotoxic and cell signaling mechanism. *Biol Cell* 95: 3-8.
- Nishizawa S, Benkelfat C, Young SN, Leyton M, Mzengeza S, de Montigny C, Blier P, Diksic M (1997) Differences between males and females in rates of serotonin synthesis in human brain. *Proc Natl Acad Sci U S A* 94: 5308-13.
- Noga JT, Hyde TM, Herman MM, Spurney CF, Bigelow LB, Weinberger DR, Kleinman JE (1997) Glutamate receptors in the postmortem striatum of schizophrenic, suicide, and control brains. *Synapse* 27: 168-76.
- Nolan Y, Connor TJ, Kelly JP, Leonard BE (2000) Lipopolysaccharide administration produces time-dependent and region-specific alterations in tryptophan and tyrosine hydroxylase activities in rat brain. *J Neural Transm* 107: 1393-401.
- Norman TR, Cranston I, Irons JA, Gabriel C, Dekeyne A, Millan MJ, Mocaër E (2012) Agomelatine suppresses locomotor hyperactivity in olfactory bulbectomised rats: a comparison to melatonin and to the 5-HT(2c) antagonist, S32006. *Eur J Pharmacol* 674: 27-32.
- Norrholm SD, Ouimet CC (2001) Altered dendritic spine density in animal models of depression and in response to antidepressant treatment. *Synapse* 42: 151-63.
- Northoff G (2013) Gene, brains, and environment-genetic neuroimaging of depression. *Curr Opin Neurobiol* 23: 133-42.
- Nowak G, Ordway GA, Paul IA (1995) Alterations in the N-methyl-D-aspartate (NMDA) receptor complex in the frontal cortex of suicide victims. *Brain Res* 675: 157-64.

- Nudmamud-Thanoi S, Reynolds GP (2004) The NR1 subunit of the glutamate/NMDA receptor in the superior temporal cortex in schizophrenia and affective disorders. *Neurosci Lett* 372: 173-77.
- Nutt DJ (2002) The neuropharmacology of serotonin and noradrenaline in depression. *Int Clin Psychopharmacol* 17 Suppl 1: S1-12.
- Ohkuma S, Katsura M, Guo J, Narihara H, Hasegawa T, Kuriyama K (1996) Role of peroxynitrite in [3H] gamma-aminobutyric acid release evoked by nitric oxide and its mechanism. *Eur J Pharmacol* 301: 179-88.
- Oliveira R, Guimarães F, Deakin J (2008) Expression of neuronal nitric oxide synthase in the hippocampal formation in affective disorders. *Braz J Med Biol Res* 41: 333-41.
- Olivenza R, Moro M, Lizasoain I, Lorenzo P, Fernández A, Rodrigo J, Boscá L, Leza J (2000) Chronic stress induces the expression of inducible nitric oxide synthase in rat brain cortex. *J Neurochem* 74: 785-91.
- Orlando G, Wolf G, Engelmann M (2008) Role of neuronal nitric oxide synthase in the regulation of the neuroendocrine stress response in rodents: insights from mutant mice. *Amino Acids* 35: 17-27.
- Pacher P, Beckman J, Liaudet L (2007) Nitric oxide and peroxynitrite in health and disease. *Physiol Rev* 87: 315-424.
- Pae CU, Marks DM, Han C, Patkar AA (2008) Does minocycline have antidepressant effect? *Biomed Pharmacother* 62: 308-11.
- Page M, Detke M, Dalvi A, Kirby L, Lucki I (1999) Serotonergic mediation of the effects of fluoxetine, but not desipramine, in the rat forced swimming test. *Psychopharmacology (Berl)* 147: 162-67.
- Paige J, Jaffrey S (2007) Pharmacologic manipulation of nitric oxide signaling: targeting NOS dimerization and protein-protein interactions. *Curr Top Med Chem* 7: 97-114.
- Palumbo M, Fosser N, Rios H, Zorrilla Zubilete M, Guelman L, Cremaschi G, Genaro A (2007) Loss of hippocampal neuronal nitric oxide synthase contributes to the stress-related deficit in learning and memory. *J Neurochem* 102: 261-74.
- Palumbo M, Zorrilla Zubilete M, Cremaschi G, Genaro A (2009) Different effect of chronic stress on learning and memory in BALB/c and C57BL/6 inbred mice: Involvement of hippocampal NO production and PKC activity. *Stress* 12: 350-61.

- Pandey D, Mahesh R, Kumar A, Rao V, Arjun M, Rajkumar R (2010) A novel 5-HT(2A) receptor antagonist exhibits antidepressant-like effects in a battery of rodent behavioural assays: approaching early-onset antidepressants. *Pharmacol Biochem Behav* 94: 363-73.
- Pandey D, Rajkumar R, Mahesh R, Radha R (2008) Depressant-like effects of parthenolide in a rodent behavioural antidepressant test battery. *J Pharm Pharmacol* 60: 1643-50.
- Paoletti P, Neyton J (2007) NMDA receptor subunits: function and pharmacology. *Curr Opin Pharmacol* 7: 39-47.
- Pariante CM, Miller AH (2001) Glucocorticoid receptors in major depression: relevance to pathophysiology and treatment. *Biol Psychiatry* 49: 391-404.
- Park C, Cho K, Ryu J, Shin K, Kim J, Ahn H, Huh Y (2004) 7-Nitroindazole upregulates phosphorylated cAMP response element binding protein, polysialylated-neural cell adhesion molecule and tryptophan hydroxylase expression in the adult rat hippocampus. *Brain Res* 1008: 120-25.
- Park HS, Huh SH, Kim MS, Lee SH, Choi EJ (2000) Nitric oxide negatively regulates c-Jun N-terminal kinase/stress-activated protein kinase by means of S-nitrosylation. *Proc Natl Acad Sci U S A* 97: 14382-87.
- Paul IA, Layer RT, Skolnick P, Nowak G (1993) Adaptation of the NMDA receptor in rat cortex following chronic electroconvulsive shock or imipramine. *Eur J Pharmacol* 247: 305-11.
- Paul IA, Nowak G, Layer RT, Popik P, Skolnick P (1994) Adaptation of the N-methyl-D-aspartate receptor complex following chronic antidepressant treatments. *J Pharmacol Exp Ther* 269: 95-102.
- Paxinos G, Watson C (1998) *The rat brain in stereotaxic coordinates*, 4th edn. Academic Press Inc., San Diego, CA, USA.
- Phelps LE, Brutsche N, Moral JR, Luckenbaugh DA, Manji HK, Zarate CA (2009) Family history of alcohol dependence and initial antidepressant response to an N-methyl-D-aspartate antagonist. *Biol Psychiatry* 65: 181-84.
- Pinheiro SN, Del-Ben CM, Zangrossi H, Graeff FG (2008) Anxiolytic and panicolytic effects of escitalopram in the elevated T-maze. *J Psychopharmacol* 22: 132-37.
- Popik P, Kos T, Sowa-Kućma M, Nowak G (2008) Lack of persistent effects of ketamine in rodent models of depression. *Psychopharmacology (Berl)* 198: 421-30.

- Porsolt R, Anton G, Blavet N, Jalfre M (1978) Behavioural despair in rats: a new model sensitive to antidepressant treatments. *Eur J Pharmacol* 47: 379-91.
- Pouget P, Wattiez N, Rivaud-Péchoux S, Gaymard B (2010) Rapid development of tolerance to sub-anaesthetic dose of ketamine: an oculomotor study in macaque monkeys. *Psychopharmacology (Berl)* 209: 313-18.
- Prast H, Lamberti C, Fischer H, Tran M, Philippu A (1996) Nitric oxide influences the release of histamine and glutamate in the rat hypothalamus. *Naunyn Schmiedebergs Arch Pharmacol* 354: 731-35.
- Prast H, Philippu A (1992) Nitric oxide releases acetylcholine in the basal forebrain. *Eur J Pharmacol* 216: 139-40.
- Prast H, Philippu A (2001) Nitric oxide as modulator of neuronal function. *Prog Neurobiol* 64: 51-68.
- Price JL, Drevets WC (2010) Neurocircuitry of mood disorders. *Neuropsychopharmacology* 35: 192-216.
- Price RB, Nock MK, Charney DS, Mathew SJ (2009) Effects of intravenous ketamine on explicit and implicit measures of suicidality in treatment-resistant depression. *Biol Psychiatry* 66: 522-26.
- Prudian F, Gantenbein M, Pelissier AL, Attolini L, Bruguerolle B (1997) Daily rhythms of heart rate, temperature and locomotor activity are modified by anaesthetics in rats: a telemetric study. *Naunyn Schmiedebergs Arch Pharmacol* 355: 774-78.
- Raison CL, Miller AH (2011) Is depression an inflammatory disorder? *Curr Psychiatry Rep* 13: 467-75.
- Raison CL, Rutherford RE, Woolwine BJ, Shuo C, Schettler P, Drake DF, Haroon E, Miller AH (2013) A randomized controlled trial of the tumor necrosis factor antagonist infliximab for treatment-resistant depression: the role of baseline inflammatory biomarkers. *JAMA Psychiatry* 70: 31-41.
- Rajkowska G (2003) Depression: what we can learn from postmortem studies. *Neuroscientist* 9: 273-84.
- Rakofsky JJ, Holtzheimer PE, Nemeroff CB (2009) Emerging targets for antidepressant therapies. *Curr Opin Chem Biol* 13: 291-302.
- Ramamoorthy S, Samuvel DJ, Buck ER, Rudnick G, Jayanthi LD (2007) Phosphorylation of threonine residue 276 is required for acute regulation of serotonin transporter by cyclic GMP. *J Biol Chem* 282: 11639-47.

- Rantamäki T, Hendolin P, Kankaanpää A, Mijatovic J, Piepponen P, Domenici E, Chao MV, Männistö PT, Castrén E (2007) Pharmacologically diverse antidepressants rapidly activate brain-derived neurotrophic factor receptor TrkB and induce phospholipase-Cgamma signaling pathways in mouse brain. *Neuropsychopharmacology* 32: 2152-62.
- Rashidy-Pour A, Motaghd-Larijani Z, Bures J (1995) Tolerance to ketamine-induced blockade of cortical spreading depression transfers to MK-801 but not to AP5 in rats. *Brain Res* 693: 64-69.
- Razoux F, Garcia R, Léna I (2007) Ketamine, at a dose that disrupts motor behavior and latent inhibition, enhances prefrontal cortex synaptic efficacy and glutamate release in the nucleus accumbens. *Neuropsychopharmacology* 32: 719-27.
- Reagan LP, McKittrick CR, McEwen BS (1999) Corticosterone and phenytoin reduce neuronal nitric oxide synthase messenger RNA expression in rat hippocampus. *Neuroscience* 91: 211-19.
- Redmond AM, Kelly JP, Leonard BE (1996) Behavioural effects of chronic glycine in the olfactory bulbectomized rat in the "open field" test and on phencyclidine (PCP)-induced hyperactivity. *Med Sci Research* 24: 55-56
- Redmond AM, Kelly JP, Leonard BE. (1997) Behavioural and neurochemical effects of dizocilpine in the olfactory bulbectomized rat model of depression. *Pharmacol Biochem Behav* 58: 355-59.
- Rénéric JP, Lucki I (1998) Antidepressant behavioral effects by dual inhibition of monoamine reuptake in the rat forced swimming test. *Psychopharmacology (Berl)* 136: 190-97.
- Réus GZ, Stringari RB, Ribeiro KF, Ferraro AK, Vitto MF, Cesconetto P, Souza CT, Quevedo J (2011) Ketamine plus imipramine treatment induces antidepressant-like behavior and increases CREB and BDNF protein levels and PKA and PKC phosphorylation in rat brain. *Behav Brain Res* 221: 166-71.
- Robichaud M, Beauchemin V, Lavoie N, Dennis T, Debonnel G (2001) Effects of bilateral olfactory bulbectomy on N-methyl-D-aspartate receptor function: autoradiographic and behavioral studies in the rat. *Synapse* 42: 95-103.
- Roche M, Commons KG, Peoples A, Valentino RJ (2003) Circuitry underlying regulation of the serotonergic system by swim stress. *J Neurosci* 23: 970-77.
- Roche M, Harkin A, Kelly J (2007) Chronic fluoxetine treatment attenuates stressor-induced changes in temperature, heart rate, and neuronal activation in the olfactory bulbectomized rat. *Neuropsychopharmacology* 32: 1312-20.

- Rodrigo J, Springall D, Uttenthal O, Bentura M, Abadia-Molina F, Riveros-Moreno V, Martínez-Murillo R, Polak J, Moncada S (1994) Localization of nitric oxide synthase in the adult rat brain. *Philos Trans R Soc Lond B Biol Sci* 345: 175-221.
- Rogers KM, Bonar CA, Estrella JL, Yang S (2002) Inhibitory effect of glucocorticoid on coronary artery endothelial function. *Am J Physiol Heart Circ Physiol* 283: H1922-28.
- Roh YS, Chung HS, Kwon B, Kim G (2012) Association between depression, patient scar assessment and burn-specific health in hospitalized burn patients. *Burns* 38: 506-12.
- Rothe F, Canzler U, Wolf G (1998) Subcellular localization of the neuronal isoform of nitric oxide synthase in the rat brain: a critical evaluation. *Neuroscience* 83: 259-69.
- Ruhé HG, Mason NS, Schene AH (2007) Mood is indirectly related to serotonin, norepinephrine and dopamine levels in humans: a meta-analysis of monoamine depletion studies. *Mol Psychiatry* 12: 331-59.
- Saarelainen T, Hendolin P, Lucas G, Koponen E, Sairanen M, MacDonald E, Agerman K, Haapasalo A, Nawa H, Aloyz R, Ernfors P, Castrén E (2003) Activation of the TrkB neurotrophin receptor is induced by antidepressant drugs and is required for antidepressant-induced behavioral effects. *J Neurosci* 23: 349-57.
- Salchner P, Lubec G, Engelmann M, Orlando GF, Wolf G, Sartori SB, Hoeger H, Singewald N (2004) Genetic functional inactivation of neuronal nitric oxide synthase affects stress-related Fos expression in specific brain regions. *Cell Mol Life Sci* 61: 1498-506.
- Samad N, Perveen T, Haider S, Haleem M, Haleem D (2006) Inhibition of restraint-induced neuroendocrine and serotonergic responses by buspirone in rats. *Pharmacol Rep* 58: 636-42.
- Sanacora G, Gueorguieva R, Epperson CN, Wu YT, Appel M, Rothman DL, Krystal JH, Mason GF (2004) Subtype-specific alterations of gamma-aminobutyric acid and glutamate in patients with major depression. *Arch Gen Psychiatry* 61: 705-13.
- Sanacora G, Zarate CA, Krystal JH, Manji HK (2008) Targeting the glutamatergic system to develop novel, improved therapeutics for mood disorders. *Nat Rev Drug Discov* 7: 426-37.

- Savegnago L, Jesse CR, Pinto LG, Rocha JB, Nogueira CW, Zeni G (2007) Monoaminergic agents modulate antidepressant-like effect caused by diphenyl diselenide in rats. *Prog Neuropsychopharmacol Biol Psychiatry* 31: 1261-69.
- Scholfield CN, Moroni F, Corradetti R, Pepeu G (1983) Levels and synthesis of glutamate and aspartate in the olfactory cortex following bulbectomy. *J Neurochem* 41: 135-38.
- Schwarcz R, Pellicciari R (2002) Manipulation of brain kynurenines: glial targets, neuronal effects, and clinical opportunities. *J Pharmacol Exp Ther* 303: 1-10.
- Schäfer M, Schmidt F, Amann B, Schlösser S, Loeschke K, Grunze H (2000) Adding low-dose antidepressants to interferon alpha treatment for chronic hepatitis C improved psychiatric tolerability in a patient with schizoaffective psychosis. *Neuropsychobiology* 42 Suppl 1: 43-45.
- Segieth J, Getting S, Biggs C, Whitton P (1995) Nitric oxide regulates excitatory amino acid release in a biphasic manner in freely moving rats. *Neurosci Lett* 200: 101-04.
- Segieth J, Pearce B, Fowler L, Whitton P (2001) Regulatory role of nitric oxide over hippocampal 5-HT release in vivo. *Naunyn Schmiedebergs Arch Pharmacol* 363: 302-06.
- Senba E, Ueyama T (1997) Stress-induced expression of immediate early genes in the brain and peripheral organs of the rat. *Neurosci Res* 29: 183-207.
- Serulle Y, Arancio O, Ziff EB (2008) A role for cGMP-dependent protein kinase II in AMPA receptor trafficking and synaptic plasticity. *Channels (Austin)* 2: 230-32.
- Sesack SR, Deutch AY, Roth RH, Bunney BS (1989) Topographical organization of the efferent projections of the medial prefrontal cortex in the rat: an anterograde tract-tracing study with Phaseolus vulgaris leucoagglutinin. *J Comp Neurol* 290: 213-42.
- Sheehan TP, Chambers RA, Russell DS (2004) Regulation of affect by the lateral septum: implications for neuropsychiatry. *Brain Res Brain Res Rev* 46: 71-117.
- Sheline YI, Mittler BL, Mintun MA (2002) The hippocampus and depression. *Eur Psychiatry* 17 Suppl 3: 300-05.
- Shirakawa T, Mitome M, Kikuri T, Nakamura W, Oshima S, Hasegawa T, Shindoh M, Oguchi H (2004) Immobilization induces acute nitric oxide production in the rat hypothalamus: a role of ionotropic glutamate receptors in the paraventricular nucleus. *Endocrinology* 145: 3603-07.



- Sibson NR, Lowe JP, Blamire AM, Martin MJ, Obrenovitch TP, Anthony DC (2008) Acute astrocyte activation in brain detected by MRI: new insights into T(1) hypointensity. *J Cereb Blood Flow Metab* 28: 621-32.
- Silva M, Aguiar DC, Diniz CR, Guimarães FS, Joca SR (2012) Neuronal NOS inhibitor and conventional antidepressant drugs attenuate stress-induced fos expression in overlapping brain regions. *Cell Mol Neurobiol* 32: 443-53.
- Silvestre JS, Nadal R, Pallarés M, Ferré N (1997) Acute effects of ketamine in the holeboard, the elevated-plus maze, and the social interaction test in Wistar rats. *Depress Anxiety* 5: 29-33.
- Simon G, Savarino J, Operskalski B, Wang P (2006) Suicide risk during antidepressant treatment. *Am J Psychiatry* 163: 41-47.
- Sinner C, Kaehler S, Philippu A, Singewald N (2001) Role of nitric oxide in the stress-induced release of serotonin in the locus coeruleus. *Naunyn Schmiedeberg Arch Pharmacol* 364: 105-09.
- Skelin I, Sato H, Diksic M (2010) Acute challenge with d-fenfluramine decreases regional cerebral glucose utilization in Sham, but not in OBX, rats: an autoradiographic study. *Brain Res* 1310: 162-71.
- Skingle M, Beattie DT, Scopes DI, Starkey SJ, Connor HE, Feniuk W, Tyers MB (1996) GR127935: a potent and selective 5-HT<sub>1D</sub> receptor antagonist. *Behav Brain Res* 73: 157-61.
- Skolnick P, Popik P, Trullas R (2009) Glutamate-based antidepressants: 20 years on. *Trends Pharmacol Sci* 30: 563-69.
- Slattery D, Morrow J, Hudson A, Hill D, Nutt D, Henry B (2005) Comparison of alterations in c-fos and Egr-1 (zif268) expression throughout the rat brain following acute administration of different classes of antidepressant compounds. *Neuropsychopharmacology* 30: 1278-87.
- Smith J, Whitton P (2000) Nitric oxide modulates N-methyl-D-aspartate-evoked serotonin release in the raphe nuclei and frontal cortex of the freely moving rat. *Neurosci Lett* 291: 5-8.
- Smith RS (1991) The macrophage theory of depression. *Med Hypotheses* 35: 298-306.
- Sofroniew MV (2005) Reactive astrocytes in neural repair and protection. *Neuroscientist* 11: 400-07.
- Solomon MB, Furay AR, Jones K, Packard AE, Packard BA, Wulsin AC, Herman JP (2012) Deletion of forebrain glucocorticoid receptors impairs neuroendocrine

stress responses and induces depression-like behavior in males but not females. *Neuroscience* 203: 135-43.

- Song C, Leonard B (2005) The olfactory bulbectomised rat as a model of depression. *Neurosci Biobehav Rev* 29: 627-47.
- Song C, Zhang XY, Manku M (2009) Increased phospholipase A2 activity and inflammatory response but decreased nerve growth factor expression in the olfactory bulbectomized rat model of depression: effects of chronic ethyl-eicosapentaenoate treatment. *J Neurosci* 29: 14-22.
- Soszynski D (2001) The inhibition of nitric oxide synthase suppresses LPS- and psychological-stress-induced fever in rats. *Physiol Behav* 72: 65-72.
- Soszynski D (2006) Molecular mechanism of emotional fever--the role of nitric oxide. *J Physiol Pharmacol* 57 Suppl 8: 51-59.
- Spiacci AJ, Kanamaru F, Guimarães F, Oliveira R (2008) Nitric oxide-mediated anxiolytic-like and antidepressant-like effects in animal models of anxiety and depression. *Pharmacol Biochem Behav* 88: 247-55.
- Spolidório P, Echeverry M, Iyomasa M, Guimarães F, Del Bel E (2007) Anxiolytic effects induced by inhibition of the nitric oxide-cGMP pathway in the rat dorsal hippocampus. *Psychopharmacology (Berl)* 195: 183-92.
- Stachowicz K, Chojnacka-Wójcik E, Kłak K, Pilc A (2007) Anxiolytic-like effect of group III mGlu receptor antagonist is serotonin-dependent. *Neuropharmacology* 52: 306-12.
- Stahl SM (1998) Basic psychopharmacology of antidepressants, part 1: Antidepressants have seven distinct mechanisms of action. *J Clin Psychiatry* 59 Suppl 4: 5-14.
- Steciuk M, Kram M, Kramer GL, Petty F (1999) Decrease in stress-induced c-Fos-like immunoreactivity in the lateral septal nucleus of learned helpless rats. *Brain Res* 822: 256-59.
- Steiner JA, Carneiro AM, Wright J, Matthies HJ, Prasad HC, Nicki CK, Dostmann WR, Buchanan CC, Corbin JD, Francis SH, Blakely RD (2009) cGMP-dependent protein kinase Ialpha associates with the antidepressant-sensitive serotonin transporter and dictates rapid modulation of serotonin uptake. *Mol Brain* 2: 26.
- Sullivan PF, Neale MC, Kendler KS (2000) Genetic epidemiology of major depression: review and meta-analysis. *Am J Psychiatry* 157: 1552-62.

- Sumner BE, Cruise LA, Slattery DA, Hill DR, Shahid M, Henry B (2004) Testing the validity of c-fos expression profiling to aid the therapeutic classification of psychoactive drugs. *Psychopharmacology (Berl)* 171: 306-21.
- Suzuki E, Yagi G, Nakaki T, Kanba S, Asai M (2001) Elevated plasma nitrate levels in depressive states. *J Affect Disord* 63: 221-24.
- Szczepankiewicz A, Leszczyńska-Rodziewicz A, Pawlak J, Rajewska-Rager A, Dmitrzak-Węglarz M, Wilkosc M, Skibinska M, Hauser J (2011) Glucocorticoid receptor polymorphism is associated with major depression and predominance of depression in the course of bipolar disorder. *J Affect Disord* 134: 138-44.
- Talarowska M, Gałeczki P, Maes M, Orzechowska A, Chamielec M, Bartosz G, Kowalczyk E (2012) Nitric oxide plasma concentration associated with cognitive impairment in patients with recurrent depressive disorder. *Neurosci Lett* 510: 127-31.
- Taliaz D, Loya A, Gersner R, Haramati S, Chen A, Zangen A (2011) Resilience to chronic stress is mediated by hippocampal brain-derived neurotrophic factor. *J Neurosci* 31: 4475-83.
- Tanaka K, Yagi T, Shimakoshi R, Azuma K, Nanba T, Ogo H, Tamura A, Asanuma M (2009) Effects of galantamine on L-NAME-induced behavioral impairment in Y-maze task in mice. *Neurosci Lett* 462: 235-38.
- Tanaka S, Ide M, Shibutani T, Ohtaki H, Numazawa S, Shioda S, Yoshida T (2006) Lipopolysaccharide-induced microglial activation induces learning and memory deficits without neuronal cell death in rats. *J Neurosci Res* 83: 557-66.
- Tatarczyńska E, Kłodzińska A, Stachowicz K, Chojnacka-Wójcik E (2004) Effect of combined administration of 5-HT<sub>1A</sub> or 5-HT<sub>1B/1D</sub> receptor antagonists and antidepressants in the forced swimming test. *Eur J Pharmacol* 487: 133-42.
- Tegenge MA, Stern M, Bicker G (2009) Nitric oxide and cyclic nucleotide signal transduction modulates synaptic vesicle turnover in human model neurons. *J Neurochem* 111: 1434-46.
- Thase ME (2011) Treatment-resistant depression: prevalence, risk factors, and treatment strategies. *J Clin Psychiatry* 72: e18.
- Tizabi Y, Bhatti BH, Manaye KF, Das JR, Akinfiresoye L (2012) Antidepressant-Like Effects of Low Ketamine Dose is Associated with Increased Hippocampal Ampa/Nmda Receptor Density Ratio in Female Wistar-Kyoto Rats. *Neuroscience* 213: 72-80.

- Tomkins DM, O'Neill MF (2000) Effect of 5-HT(1B) receptor ligands on self-administration of ethanol in an operant procedure in rats. *Pharmacol Biochem Behav* 66: 129-36.
- Trivedi M, Rush A, Wisniewski S, Nierenberg A, Warden D, Ritz L, Norquist G, Howland R, Lebowitz B, McGrath P, Shores-Wilson K, Biggs M, Balasubramani G, Fava M, Team SDS (2006) Evaluation of outcomes with citalopram for depression using measurement-based care in STAR\*D: implications for clinical practice. *Am J Psychiatry* 163: 28-40.
- Tso MM, Blatchford KL, Callado LF, McLaughlin DP, Stamford JA (2004) Stereoselective effects of ketamine on dopamine, serotonin and noradrenaline release and uptake in rat brain slices. *Neurochem Int* 44: 1-7.
- Ulak G, Mutlu O, Akar F, Komsuoğlu F, Tanyeri P, Erden B (2008) Neuronal NOS inhibitor 1-(2-trifluoromethylphenyl)-imidazole augment the effects of antidepressants acting via serotonergic system in the forced swimming test in rats. *Pharmacol Biochem Behav* 90: 563-68.
- Ulak G, Mutlu O, Tanyeri P, Komsuoğlu FI, Akar FY, Erden BF (2010) Involvement of serotonin receptor subtypes in the antidepressant-like effect of TRIM in the rat forced swimming test. *Pharmacol Biochem Behav* 95: 308-14.
- Van Oekelen D, Luyten WH, Leysen JE (2003) 5-HT<sub>2A</sub> and 5-HT<sub>2C</sub> receptors and their atypical regulation properties. *Life Sci* 72: 2429-49.
- Vázquez-Palacios G, Bonilla-Jaime H, Velázquez-Moctezuma J (2004) Antidepressant-like effects of the acute and chronic administration of nicotine in the rat forced swimming test and its interaction with fluoxetine [correction of flouxetine]. *Pharmacol Biochem Behav* 78: 165-69.
- Veena J, Srikumar BN, Raju TR, Shankaranarayana Rao BS (2009) Exposure to enriched environment restores the survival and differentiation of new born cells in the hippocampus and ameliorates depressive symptoms in chronically stressed rats. *Neurosci Lett* 455: 178-82.
- Videbech P, Ravnkilde B (2004) Hippocampal volume and depression: a meta-analysis of MRI studies. *Am J Psychiatry* 161: 1957-66.
- Vincent S, Kimura H (1992) Histochemical mapping of nitric oxide synthase in the rat brain. *Neuroscience* 46: 755-84.
- Vinkers C, Breuer M, Westphal K, Korte S, Oosting R, Olivier B, Groenink L (2009) Olfactory bulbectomy induces rapid and stable changes in basal and stress-induced locomotor activity, heart rate and body temperature responses in the home cage. *Neuroscience* 159: 39-46.

- Volke V, Wegener G, Bourin M, Vasar E (2003) Antidepressant- and anxiolytic-like effects of selective neuronal NOS inhibitor 1-(2-trifluoromethylphenyl)-imidazole in mice. *Behav Brain Res* 140: 141-47.
- Wallerath T, Witte K, Schäfer S, Schwarz P, Prellwitz W, Wohlfart P, Kleinert H, Lehr H, Lemmer B, Förstermann U (1999) Down-regulation of the expression of endothelial NO synthase is likely to contribute to glucocorticoid-mediated hypertension. *Proc Natl Acad Sci U S A* 96: 13357-62.
- Wan W, Wetmore L, Sorensen CM, Greenberg AH, Nance DM (1994) Neural and biochemical mediators of endotoxin and stress-induced c-fos expression in the rat brain. *Brain Res Bull* 34: 7-14.
- Wang D, An SC, Zhang X (2008) Prevention of chronic stress-induced depression-like behavior by inducible nitric oxide inhibitor. *Neurosci Lett* 433: 59-64.
- Wang J, Goffer Y, Xu D, Tukey DS, Shamir DB, Eberle SE, Zou AH, Blanck TJ, Ziff EB (2011) A single subanesthetic dose of ketamine relieves depression-like behaviors induced by neuropathic pain in rats. *Anesthesiology* 115: 812-21.
- Wang JQ, Chu XP, Guo ML, Jin DZ, Xue B, Berry TJ, Fibuch EE, Mao LM (2012) Modulation of ionotropic glutamate receptors and Acid-sensing ion channels by nitric oxide. *Front Physiol* 3: 164.
- Wang Y, Barbaro MF, Baraban SC (2006) A role for the mTOR pathway in surface expression of AMPA receptors. *Neurosci Lett* 401: 35-39.
- Wegener G, Bandpey Z, Heiberg I, Volke V, Trabace L, Rosenberg R, Harvey B (2004) Combined chronic treatment with citalopram and lithium does not modify the regional neurochemistry of nitric oxide in rat brain. *J Physiol Pharmacol* 55: 575-86.
- Wegener G, Harvey B, Bonefeld B, Müller H, Volke V, Overstreet D, Elfving B (2009) Increased stress-evoked nitric oxide signalling in the Flinders sensitive line (FSL) rat: a genetic animal model of depression. *Int J Neuropsychopharmacol*: 1-13.
- Wegener G, Volke V, Harvey B, Rosenberg R (2003) Local, but not systemic, administration of serotonergic antidepressants decreases hippocampal nitric oxide synthase activity. *Brain Res* 959: 128-34.
- Wegener G, Volke V, Rosenberg R (2000) Endogenous nitric oxide decreases hippocampal levels of serotonin and dopamine in vivo. *Br J Pharmacol* 130: 575-80.

- Weinberg MS, Grissom N, Paul E, Bhatnagar S, Maier SF, Spencer RL (2010) Inescapable but not escapable stress leads to increased struggling behavior and basolateral amygdala c-fos gene expression in response to subsequent novel stress challenge. *Neuroscience* 170: 138-48.
- West A, Galloway M (1998) Nitric oxide and potassium chloride-facilitated striatal dopamine efflux in vivo: role of calcium-dependent release mechanisms. *Neurochem Int* 33: 493-501.
- White RP, Deane C, Vallance P, Markus HS (1998) Nitric oxide synthase inhibition in humans reduces cerebral blood flow but not the hyperemic response to hypercapnia. *Stroke* 29: 467-72.
- Willner P (1997) Validity, reliability and utility of the chronic mild stress model of depression: a 10-year review and evaluation. *Psychopharmacology (Berl)* 134: 319-29.
- Wilson AW, Neill JC, Costall B (1998) An investigation into the effects of 5-HT agonists and receptor antagonists on ethanol self-administration in the rat. *Alcohol* 16: 249-70.
- Winstanley CA, Theobald DE, Dalley JW, Glennon JC, Robbins TW (2004) 5-HT<sub>2A</sub> and 5-HT<sub>2C</sub> receptor antagonists have opposing effects on a measure of impulsivity: interactions with global 5-HT depletion. *Psychopharmacology (Berl)* 176: 376-85.
- Wong A, Zhang YW, Jeschke GR, Turk BE, Rudnick G (2012) Cyclic GMP-dependent stimulation of serotonin transport does not involve direct transporter phosphorylation by cGMP-dependent protein kinase. *J Biol Chem* 287: 36051-58.
- Wood G, Young L, Reagan L, Chen B, McEwen B (2004) Stress-induced structural remodeling in hippocampus: prevention by lithium treatment. *Proc Natl Acad Sci U S A* 101: 3973-78.
- Wrynn AS, Mac Sweeney CP, Franconi F, Lemaire L, Pouliquen D, Herlidou S, Leonard BE, Gandon J, de Certaines JD (2000) An in-vivo magnetic resonance imaging study of the olfactory bulbectomized rat model of depression. *Brain Res* 879: 193-99.
- Xing G, Chavko M, Zhang L, Yang S, Post R (2002) Decreased calcium-dependent constitutive nitric oxide synthase (cNOS) activity in prefrontal cortex in schizophrenia and depression. *Schizophr Res* 58: 21-30.
- Xu H, Chen Z, He J, Haimanot S, Li X, Dyck L, Li XM (2006) Synergetic effects of quetiapine and venlafaxine in preventing the chronic restraint stress-induced

decrease in cell proliferation and BDNF expression in rat hippocampus. *Hippocampus* 16: 551-59.

- Yamamoto T, Jin J, Watanabe S (1997) Characteristics of memory dysfunction in olfactory bulbectomized rats and the effects of cholinergic drugs. *Behav Brain Res* 83: 57-62.
- Yang J, Qu JM, Summah H, Zhang J, Zhu YG, Jiang HN (2010) Protective effects of imipramine in murine endotoxin-induced acute lung injury. *Eur J Pharmacol* 638: 128-33.
- Yang J, Zhou Z, Yang C (2011) Concerns about the antidepressant-like effects of high-dose ketamine in mice. *Psychopharmacology (Berl)* 215: 813; author reply 815-16.
- Yildiz F, Erden B, Ulak G, Utkan T, Gacar N (2000a) Antidepressant-like effect of 7-nitroindazole in the forced swimming test in rats. *Psychopharmacology (Berl)* 149: 41-44.
- Yildiz F, Ulak G, Erden BF, Gacar N (2000b) Anxiolytic-like effects of 7-nitroindazole in the rat plus-maze test. *Pharmacol Biochem Behav* 65: 199-202.
- Yildiz-Yesiloglu A, Ankerst DP (2006) Neurochemical alterations of the brain in bipolar disorder and their implications for pathophysiology: a systematic review of the in vivo proton magnetic resonance spectroscopy findings. *Prog Neuropsychopharmacol Biol Psychiatry* 30: 969-95.
- Yuasa K, Nagame T, Dohi M, Yanagita Y, Yamagami S, Nagahama M, Tsuji A (2012) cGMP-dependent protein kinase I is involved in neurite outgrowth via a Rho effector, rhotekin, in Neuro2A neuroblastoma cells. *Biochem Biophys Res Commun* 421: 239-44.
- Yüksel C, Öngür D (2010) Magnetic resonance spectroscopy studies of glutamate-related abnormalities in mood disorders. *Biol Psychiatry* 68: 785-94.
- Zahn D, Petrak F, Uhl I, Juckel G, Neubauer H, Hägele AK, Wiltfang J, Herpertz S (2013) New pathways of increased cardiovascular risk in depression: A pilot study on the association of high-sensitivity C-reactive protein with pro-atherosclerotic markers in patients with depression. *J Affect Disord* 146: 420-25.
- Zarate CA, Payne JL, Quiroz J, Sporn J, Denicoff KK, Luckenbaugh D, Charney DS, Manji HK (2004) An open-label trial of riluzole in patients with treatment-resistant major depression. *Am J Psychiatry* 161: 171-74.
- Zarate CJ, Du J, Quiroz J, Gray N, Denicoff K, Singh J, Charney D, Manji H (2003) Regulation of cellular plasticity cascades in the pathophysiology and treatment

of mood disorders: role of the glutamatergic system. *Ann N Y Acad Sci* 1003: 273-91.

Zarate CJ, Singh J, Carlson P, Brutsche N, Ameli R, Luckenbaugh D, Charney D, Manji H (2006a) A randomized trial of an N-methyl-D-aspartate antagonist in treatment-resistant major depression. *Arch Gen Psychiatry* 63: 856-64.

Zarate CJ, Singh J, Quiroz J, De Jesus G, Denicoff K, Luckenbaugh D, Manji H, Charney D (2006b) A double-blind, placebo-controlled study of memantine in the treatment of major depression. *Am J Psychiatry* 163: 153-55.

Zhou L, Li F, Xu HB, Luo CX, Wu HY, Zhu MM, Lu W, Ji X, Zhou QG, Zhu DY (2010) Treatment of cerebral ischemia by disrupting ischemia-induced interaction of nNOS with PSD-95. *Nat Med* 16: 1439-43.

Zhou L, Zhu D (2009) Neuronal nitric oxide synthase: structure, subcellular localization, regulation, and clinical implications. *Nitric Oxide* 20: 223-30.

Zhou Q, Hu Y, Hua Y, Hu M, Luo C, Han X, Zhu X, Wang B, Xu J, Zhu D (2007) Neuronal nitric oxide synthase contributes to chronic stress-induced depression by suppressing hippocampal neurogenesis. *J Neurochem* 103: 1843-54.

Zhu X, Wang X, Xiao J, Liao J, Zhong M, Wang W, Yao S (2012) Evidence of a dissociation pattern in resting-state default mode network connectivity in first-episode, treatment-naive major depression patients. *Biol Psychiatry* 71: 611-17.

Zimmermann P, Brückl T, Nocon A, Pfister H, Binder EB, Uhr M, Lieb R, Moffitt TE, Caspi A, Holsboer F, Ising M (2011) Interaction of FKBP5 gene variants and adverse life events in predicting depression onset: results from a 10-year prospective community study. *Am J Psychiatry* 168: 1107-16.

Zobel AW, Nickel T, Künzel HE, Ackl N, Sonntag A, Ising M, Holsboer F (2000) Effects of the high-affinity corticotropin-releasing hormone receptor 1 antagonist R121919 in major depression: the first 20 patients treated. *J Psychiatr Res* 34: 171-81.





## VIII. Appendix

### – *Solutions*

#### Saline solution

0.89% NaCl in de-ionised H<sub>2</sub>O

#### Phosphate buffered saline solution (PBS) 10 mM

NaH<sub>2</sub>PO<sub>4</sub>                      1.9 mM

Na<sub>2</sub>HPO<sub>4</sub>                      8.1 mM

NaCl                              0.89%

pH 7.4

#### Paraformaldehyde 4% solution

PFA                              4%

NaOH pellets                  as needed

in PBS 10 mM

pH 7.4

#### Sucrose 30% solution

Sucrose                          30%

in PBS 10 mM

#### Cryoprotectant freezing solution

Sucrose                          30%

Ethylene glycole              30%

in PBS 10 mM

Gelatine coating solution

Gelatine 0.5%

CrK(SO<sub>4</sub>)<sub>2</sub> 0.05%

in de-ionised H<sub>2</sub>O

HPLC mobile phase

Citric acid 0.1 M

NaH<sub>2</sub>PO<sub>4</sub> 0.1 M

EDTA 0.1 mM

Octane-1-sulphonic acid 1.4 mM

Methanol 10%

in HPLC grade water

pH 2.8

## IX. Publications

**Gigliucci V.**, Buckley K., Nunan J., O'Shea K., Harkin A. (2010) *A role for serotonin in the antidepressant activity of N(G)-Nitro-L-arginine, in the rat forced swimming test.* Pharmacol Biochem Behav 94: 524-533.

**Gigliucci V.**, O'Dowd G., Casey S., Egan D., Gibney S., Harkin A. (2013) *Ketamine elicits sustained antidepressant-like activity via a serotonin dependent mechanism.* Psychopharmacology (Berl). [In press]

Rouine J., Gobbo O., Campbell M., **Gigliucci V.**, Ogden I., McHugh-Smith K., Duffy P., Behan B., Byrne D., Kelly M., Blau C., Kerskens C., Harkin A. (2013) *MDMA "Ecstasy" increases cerebral cortical perfusion determined by bolus-tracking arterial spin labelling (btASL) MRI.* British Journal of Pharmacology. [In press]

### Published abstracts

**Gigliucci V.**, Buckley K.N., Nunan J., O'Shea K., Harkin A. *A role for serotonin in the antidepressant activity of N<sup>ω</sup>-Nitro-L-arginine, in the rat forced swimming test.* J of Psychopharmacol 24 suppl.3:A39.

**Gigliucci V.**, Gibney S., Casey S., Harkin A. *Combination of stress and central 5-HT depletion promotes a depression related phenotype in a putative model of treatment resistant depression.* J of Psychopharmacol 25(8) suppl.:A23.

### International conferences

**Anxiety and depression 2011**, Washington DC (USA) – poster presentation

**Gigliucci V.**, Gibney S., Casey S., Egan D., Harkin A. *An assessment of the antidepressant-like activity of ketamine in a rodent model of depression-related behaviour unresponsive to conventional antidepressants.*