Molecular and cellular effects of glucocorticoids, inflammation and antidepressant compounds in a cellular model of depression

Horowitz, Mark Abie

Awarding institution:
King's College London

The copyright of this thesis rests with the author and no quotation from it or information derived from it may be published without proper acknowledgement.

END USER LICENCE AGREEMENT

Unless another licence is stated on the immediately following page this work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International licence. https://creativecommons.org/licenses/by-nc-nd/4.0/

You are free to copy, distribute and transmit the work

Under the following conditions:

- Attribution: You must attribute the work in the manner specified by the author (but not in any way that suggests that they endorse you or your use of the work).
- Non Commercial: You may not use this work for commercial purposes.
- No Derivative Works - You may not alter, transform, or build upon this work.

Any of these conditions can be waived if you receive permission from the author. Your fair dealings and other rights are in no way affected by the above.

Take down policy

If you believe that this document breaches copyright please contact librarypure@kcl.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.
Molecular and cellular effects of glucocorticoids, inflammation and antidepressant compounds in a cellular model of depression

By

Mark Horowitz

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy at King's College London

Department of Psychological Medicine

Institute of Psychology, Psychiatry and Neuroscience

King's College London
Acknowledgements

I would like to thank my first supervisor, Carmine Pariante, for giving me the opportunity to work in his excellent lab, for his help and savvy guidance throughout my PhD, for his enduring patience through all the peaks and troughs of the process, and for his efforts in injecting liveliness into the lab.

I would also like to thank my second supervisor, Patricia Zunszain, who has been nothing short of remarkable. She has reliably provided both insightful feedback regarding everything from misplaced commas to fundamental errors of logic, with a near-inhuman attention to detail, and also compassionate mentorship, in equal measure. I would not have produced the work I did without her support.

Angela Bithell was patient and insightful in her supervision while we toiled through the valleys of chromatin immunoprecipitation. Anna Cattaneo has been delightful and brilliant in helping me with microarray analysis. Sandrine Thuret provided expert guidance and advice on all things cellular, and neurogenic. I am very thankful to Brenda Williams, Graham Cocks, Greg Anderson and Alex Maruszak for all their help in several tight spots. Jack Price was a reliably critical eye in presentations. Thanks to Abdul Hye for all his help using the Luminex machine.

I would like to thank all the members of the CBU (and latterly, the CCBB) for the reagents they lent me, and the ones I took, their advice, and for their trench solidarity on the football field, in the Sun and crowded round the sink. Christos, Helen, Yewande, Andrew, Thomas, Ksenia and others provided tennis, parties, paraformaldehyde, plays, running trips and good times.

I would like to thank all my litter-mates in the SPI lab: the sensible older (age-independent) siblings, Alice and Nilay, for making sure my shoes matched; my desk-mates, Dom, Jasmin, Andrea, Zuzanna and Martin, for providing many diverting moments and discussions; my tissue culture- and lab-mates, Julia, Natalia, Luis, Naghneh and Alessandra, with whom I weathered many a divine plague wrought on our incubators and hoods; and everyone else who made conferences, meetings and seminars cheerful events.

I would also like to thank Chris for originally teaching me cell and molecular biology, and Sue Pawlby for her warmth.

I would also like to thank Tytus and Natalia for their comraderie in all things, and Zoe for her forbearance and support, without which I could not have persevered.

Lastly, I would like to thank my long-suffering parents and my sisters for their support during what - I promise - is my very final degree.

I would like to dedicate this thesis to my grandmothers, Gitia and Tessa, who did not live to see its end, but who always encouraged me in all my endeavours.
Abstract

Chronic psychological stress has been thought to induce depression through increased activity of both the innate immune system and the hypothalamic-pituitary-adrenal (HPA) axis. The co-existence of high levels of pro-inflammatory cytokines and cortisol, produced by these systems, respectively, has presented a conundrum, given the potent anti-inflammatory properties of cortisol. Recently noted pro-inflammatory effects of glucocorticoids, or the presence of glucocorticoid resistance have been proposed to explain this relationship. In turn, antidepressant compounds (monoaminergic and polyunsaturated fatty acids (PUFAs)) may work by counteracting inflammation and its effects on the brain – including altering levels of inflammation, and reversing the effects of inflammation on hippocampal neurogenesis and the kynurenine metabolic pathway. I have used human hippocampal progenitor cells (HPC03A/07) to investigate these processes in a cell model with relevance to depression.

While glucocorticoids were anti-inflammatory upon co-incubation with inflammatory stimulation in these cells, they were pro-inflammatory when their treatment preceded inflammatory stimulation. Pre-treatment with dexamethasone potentiated subsequent inflammatory response by 72.2% and pre-treatment with cortisol potentiated responses by 49.3%, as measured by IL-6 secreted into the supernatant following IL-1β stimulation. These effects were dose-dependent, such that intermediate doses produced the greatest effects, while higher and lower doses produced smaller effects; they were also time-dependent, with an interval between glucocorticoid treatment and inflammatory stimulation of 24 hours producing a significant potentiation effect, with 1 hour, 12 hours and 48 hours producing no effect. Inflammatory potentiation was also dependent on the glucocorticoid receptor (GR) as it could be abrogated by
treatment with the GR antagonist, RU486. These potentiation effects were observed for a variety of cytokines and chemokines secreted in response to IL-1β treatment - IL-8, IP-10, IL-1RA, RANTES, and MCP-1. There was some evidence that the underlying mechanism involved the induction of glucocorticoid resistance as there was a reduction in the ability of glucocorticoids to inhibit inflammation upon re-exposure, and GR-target genes were down-regulated following dexamethasone pre-treatment. There was also evidence that glucocorticoid treatment up-regulated immune pathways, including the important stress sensing protein from the NOD-like receptor family, pyrin domain containing 6 gene (NLRP6).

A systematic literature review of clinical studies that measured glucocorticoid levels, pro-inflammatory cytokines and glucocorticoid resistance found a strong association between the presence of glucocorticoid resistance and increased levels of pro-inflammatory cytokines, quantified by meta-analysis (Fisher’s z = 0.38), though not between cortisol levels and pro-inflammatory cytokines.

The effects of the monoaminergic antidepressants (venlafaxine and sertraline) and the PUFAs (EPA and DHA) on stimulated levels of inflammation, IL-1β-induced reductions in neurogenesis, and associated increases in the neurotoxic kynurenine metabolite, quinolinic acid (QUIN) were investigated. The antidepressant compounds diverged in their effect on immune processes, with venlafaxine and EPA reducing the amount of cytokines and chemokines secreted in response to IL-1β stimulation, while DHA and sertraline potentiated this response. Interestingly, all compounds reduced the activity of NF-κB. Sertaline, venlafaxine and DHA all reversed IL-1β-induced reductions in hippocampal neurogenesis, and increases in QUIN; conversely, EPA did not affect levels of neurogenesis or levels of QUIN.
Overall, these results indicate that glucocorticoids can exert pro-inflammatory effects in human hippocampal cells, by a mechanism involving upregulated immune pathways and glucocorticoid resistance, potentially explaining the coexistence of HPA axis and innate immune dysfunction in depression. Antidepressant compounds act through different mechanisms in human hippocampal cells, with EPA largely affecting immune processes directly, while DHA, venlafaxine and sertraline affected inflammation-induced changes to neurogenesis, potentially through their effect on the kynurenine metabolic pathway.
Statement of Originality

Patricia Zunszain performed the gel electrophoresis presented in Section 3.2.1. Anna Cattaneo performed microarray processing, quality control and pathway analysis of the gene arrays presented in Section 3.3.5.

Luis Tojo helped perform the quantification of neurogenesis upon treatment with IL-1β and antidepressants in Section 3.5.1. Luis Tojo, Danhui Zhu and Katherine O’Farrell helped with PCRs presented in Section 3.5.2. Silvia Alboni performed the analysis of quinolinic acid levels presented in Section 3.5.2. Angela Bithell helped perform immunoprecipitation steps of the chromatin immunoprecipitation, and qPCRs for detection of enrichment presented in Section 3.6.3.

All other experiments were conducted by Mark Horowitz, under the supervision of Carmine Pariante and Patricia Zunszain. The human hippocampal progenitor cells were kindly provided by Professor Jack Price, on behalf of ReNeuron. The studentship which funded this work was provided by the King’s College London Graduate School.
Publications related to this PhD thesis


Oral communications of this PhD thesis

Psychoneuroimmunology Research Society (PNIRS), annual meeting, San Diego, USA (2012).

British Association for Psychopharmacology (BAP), Harrogate, UK (2014), Hannah Steinberg Award lecture.

Poster communications of this PhD thesis

British Association for Psychopharmacology (BAP), Harrogate, UK (2014).


British Association for Psychopharmacology (BAP). Harrogate, UK (2013).

Psychoneuroimmunology Research Society (PNIRS), annual meeting, San Diego, USA (2012).

British Association for Psychopharmacology (BAP). Harrogate, UK (2012).

**Other publications during the course of this PhD thesis**


Barbosa, Izabela Guimarães, Natália Pessoa Rocha, Moisés Evandro Bauer, Aline Silva de Miranda, Rodrigo Barreto Huguet, Helton José Reis, Patricia A. Zunszain, Mark A. Horowitz, Carmine M. Pariante, and Antônio Lúcio Teixeira.
“Chemokines in bipolar disorder: trait or state?.” European Archives of Psychiatry and Clinical Neuroscience 263, no. 2 (2013): 159-165.


# Table of Contents

Acknowledgements .............................................................................................................. 2
Abstract ................................................................................................................................ 3
Statement of Originality ........................................................................................................... 6
Publications related to this PhD thesis .................................................................................. 7
Oral communications of this PhD thesis .............................................................................. 7
Poster communications of this PhD thesis .......................................................................... 7
Other publications during the course of this PhD thesis ....................................................... 8
Table of Contents .................................................................................................................. 10
Table of Figures ..................................................................................................................... 16
Table of Tables ...................................................................................................................... 18
List of Abbreviations ............................................................................................................ 20

1 Introduction ....................................................................................................................... 25
  1.1 Major Depressive Disorder ............................................................................................ 26
    1.1.1 Definition of Major Depressive Disorder ............................................................... 26
    1.1.2 Prevalence of Major Depression ........................................................................... 26
    1.1.3 Environmental factors in depression .................................................................... 27
  1.2 The Biological Basis of Depression ............................................................................. 28
    1.2.1 The hippocampus and depression ....................................................................... 29
    1.2.2 Hypothalamic-Pituitary-Adrenal (HPA) axis ......................................................... 32
    1.2.3 Glucocorticoid receptor dysfunction .................................................................... 35
    1.2.4 The innate immune system .................................................................................. 37
    1.2.5 Adult hippocampal neurogenesis ....................................................................... 41
    1.2.6 Inflammation decreases neurogenesis ................................................................. 43
    1.2.7 Kynurenine Metabolites ...................................................................................... 45
    1.2.8 Other biological domains affected in depression ................................................ 49
  1.3 Synthesis of glucocorticoid and inflammatory theories of depression ......................... 49
    1.3.1 Tension between glucocorticoid and cytokine theories of depression ............... 49
    1.3.2 The potential role of glucocorticoid resistance ................................................... 50
1.3.3 The potential role of pro-inflammatory glucocorticoids ........................................54
1.4 The biological basis of antidepressant action: effects on immune processes,
neurogenesis and the kynurenine pathway ....................................................................61
  1.4.1 Monoamines and antidepressant action .................................................................62
  1.4.2 Immunomodulatory properties of antidepressant compounds .............................64
  1.4.3 Antidepressants and AHN ......................................................................................69
1.5 The role of the GR in neurogenesis in stress and antidepressant treatment ..............75
  1.5.1 The glucocorticoid receptor ....................................................................................75
  1.5.2 The GR and neurogenesis .....................................................................................76
  1.5.3 Glucocorticoids, neurogenesis and the GR ............................................................76
  1.5.4 Antidepressants, the GR and neurogenesis .............................................................77
  1.5.5 Outstanding issues ..................................................................................................78
1.6 Human hippocampal progenitor cells as a research tool to study depression ..........80
1.7 Aims and Hypotheses of Study ..................................................................................82
  1.7.1 Aim 1: To determine whether glucocorticoid resistance or pro-inflammatory
properties of glucocorticoids are associated with depression in clinical literature
examining the glucocorticoid and cytokine systems via systematic review and meta-
analysis 82
  1.7.2 Aim 2: To determine whether glucocorticoids can be pro-inflammatory in
human neural cells, and the mechanisms underlying these effects .............................83
  1.7.3 Aim 3: To determine whether antidepressant compounds modulate
inflammation in human neural cells, and the underlying mechanism of action ..........84
  1.7.4 Aim 4: To determine whether antidepressant compounds affect neurogenesis
via effects on kynurenine metabolites ...........................................................................85
  1.7.5 Aim 5: To optimise chromatin immunoprecipitation in the human progenitor
cells in order to develop a tool that can be used to determine whether activation of the
glucocorticoid receptor by distinct compounds has specific genomic targets underlying
divergent effects on neurogenesis ..................................................................................87
2 Methods .......................................................................................................................89
  2.1 Systematic Literature Review ...................................................................................89
  2.1.1 Search procedure ....................................................................................................89
2.1.2 Inclusion & exclusion Criteria ................................................................. 90
2.2 Meta-analysis .............................................................................................92
2.3 Cellular Study Design...............................................................................92
2.4 Cell Culture................................................................................................95
2.5 Experimental cellular paradigms ...............................................................101
  2.5.1 Assays involving glucocorticoid and inflammatory treatments ..........101
  2.5.2 Assays with Antidepressant Compounds ........................................108
  2.5.3 Differentiation assay ...........................................................................110
2.6 Secreted protein quantification .................................................................112
  2.6.1 Collection of supernatant .................................................................112
  2.6.2 Cell quantification ............................................................................112
  2.6.3 ELISA .................................................................................................113
  2.6.4 Multiplex protein quantification using Luminex platform ...............113
2.7 Gene expression analysis ..........................................................................117
  2.7.1 RNA isolation......................................................................................117
  2.7.2 Primer Design....................................................................................118
  2.7.3 Reverse Transcription Polymerase Chain Reaction (RT-PCR) ..........118
  2.7.4 Quantitative Real-Time PCR (qPCR) ................................................119
2.8 NF-κB transactivation analysis ................................................................121
  2.8.1 Cytoplasmic and nuclear protein extraction .......................................121
  2.8.2 Protein quantification .......................................................................121
  2.8.3 NF-κB transactivation assay .............................................................122
2.9 Gene array .................................................................................................124
  2.9.1 RNA Isolation, Microarray Processing, Quality Control ..................124
  2.9.2 Pathway Analysis ..............................................................................124
2.10 Immunocytochemistry ............................................................................125
  2.10.1 Neuronal differentiation ..................................................................125
  2.10.2 Cell counting ...................................................................................126
2.11 Kynurenine pathway quantification .......................................................126
  2.11.1 Sample preparation ..........................................................................126
  2.11.2 Liquid chromatography ...................................................................126
2.12 Chromatin immunoprecipitation ............................................................127
2.12.1 Chromatin immunoprecipitation by sonication ..................................................127
2.12.2 Chromatin immunoprecipitation by enzymatic digestion.................................136
2.13 Drugs and Reagents ............................................................................................139
2.14 Statistical Analysis .............................................................................................139
3 Results ....................................................................................................................141
3.1 Systematic Literature Review and Meta-analysis of Clinical Literature assessing HPA axis and inflammation in patients .................................................................141
  3.1.1 Systematic literature review ............................................................................141
  3.1.2 Meta-analysis ..................................................................................................153
3.2 Inflammatory properties of human hippocampal progenitor cells .......................156
  3.2.1 Human hippocampal progenitor cells possess cytokine receptors ...............156
  3.2.2 Production of cytokines in response to inflammatory stimulation in a time- and dose-dependent manner ........................................................................158
  3.2.3 Wide expression of pro- and anti-inflammatory cytokines and chemokines in response to stimulation ................................................................................161
3.3 Interaction between glucocorticoid and inflammatory systems in human hippocampal stem cells .........................................................................................163
  3.3.1 Effect of co-treatment of glucocorticoids and inflammatory stimulus .............163
  3.3.2 Potentiation of Inflammation by pre-treatment with glucocorticoids ..............165
  3.3.3 Role of the glucocorticoid receptor ..................................................................175
  3.3.4 Mechanism underlying glucocorticoid potentiation of inflammation ..............183
  3.3.5 Genome-wide gene expression and pathway analysis underlying effects of glucocorticoids on inflammatory responses .........................................................187
3.4 Modulation of inflammation by antidepressant compounds ................................203
  3.4.1 Antidepressant compounds and PUFAs ..........................................................203
3.5 Modulation of neurogenesis by antidepressant compounds ................................215
  3.5.1 Effect of antidepressant compounds on neurogenesis ...................................215
  3.5.2 The effect of antidepressant compounds on the kynurenine pathway ..........219
3.6 Chromatin Immunoprecipitation of glucocorticoid receptor targets ..................224
  3.6.1 Optimisation of chromatin in human hippocampal stem cells by shearing ...226
3.6.2 Optimisation of chromatin in human hippocampal stem cells by MNase digestion 229
3.6.3 Optimisation of immunoprecipitation .......................................................... 231

4 Discussion ........................................................................................................... 238
4.1 Summary of findings ......................................................................................... 238
4.1.1 Aim 1 ............................................................................................................ 238
4.1.2 Aim 2 ............................................................................................................ 239
4.1.3 Aim 3 ............................................................................................................ 241
4.1.4 Aim 4 ............................................................................................................ 242
4.1.5 Aim 5 ............................................................................................................ 242
4.2 Systematic review of studies in depressed patients reveals robust association between glucocorticoid resistance and increased inflammation ........................................ 243
4.2.1 Overview of findings .................................................................................... 243
4.2.2 Shift in balance/glucocorticoid resistance theory supported ......................... 244
4.2.3 Theory of pro-inflammatory glucocorticoids largely unsupported ............... 248
4.2.4 Mechanisms by which glucocorticoid resistance gives rise to increased inflammation .............................................................................................................. 249
4.2.5 Bi-directional interaction between glucocorticoid sensitivity and inflammation 252
4.2.6 Limitations of systematic review .................................................................. 253
4.3 Interaction of innate immune system and glucocorticoid system in vitro ........ 255
4.3.1 Overview of findings .................................................................................... 255
4.3.2 Glucocorticoids potentiate inflammation ..................................................... 256
4.3.3 GR dependency of the pro-inflammatory action of glucocorticoids ............ 272
4.3.4 Insight into mechanisms underlying glucocorticoid potentiation of inflammatory processes ........................................................................................................ 273
4.3.5 Potential translational relevance to depression .......................................... 278
4.4 Antidepressant compounds as immunomodulators .................................... 285
4.4.1 Overview of findings .................................................................................... 285
4.4.2 Anti-inflammatory effects .......................................................................... 285
4.4.3 Pro-inflammatory effects ............................................................................ 286
4.4.4 No immunomodulatory effect ..................................................................... 287
Table of Figures

Figure 1 The components of the HPA axis and its feedback loops ........................................ 33
Figure 2 The role of the innate immune system in stress and depression .......................... 40
Figure 3 The kynurenine pathway ......................................................................................... 48
Figure 4 Shift in balance hypothesis .................................................................................... 52
Figure 5 A model for pro-inflammatory effects of glucocorticoids .................................... 55
Figure 6 Search procedure for meta-analysis ....................................................................... 91
Figure 7 Experimental overview ......................................................................................... 94
Figure 8 ReNeuron’s HPC03A/07 cells with c-mycER technology ....................................... 98
Figure 9 Co-incubation paradigms for dexamethasone and IL-1β ..................................... 102
Figure 10 Pre-treatment of cells with dexamethasone before inflammatory stimulation ...... 105
Figure 11 Blockade of glucocorticoid effects by RU486 in dexamethasone pre-treatment paradigm ............................................................................................................ 107
Figure 12 Co-incubation of antidepressant compounds and inflammatory stimulation ...... 109
Figure 13 Differentiation Assay ............................................................................................ 111
Figure 14 Cytokine Human Magnetic 25-Plex Panel protocol .......................................... 115
Figure 15 TransAM NF-κB procedure .................................................................................. 123
Figure 16 Chromatin immunoprecipitation protocol ......................................................... 138
Figure 17 Correlation between GR resistance and inflammation in clinical studies .......... 155
Figure 18 Human hippocampal progenitor cells constitutively express cytokine receptors .... 157
Figure 19 Time- and dose-dependent inflammatory response of human hippocampal progenitor cells ................................................................................................................................. 160
Figure 20 Dexamethasone inhibits release of IL-6 dose-dependently upon co-incubation...... 164
Figure 21 Effect of time interval between DEX pre-treatment and IL-1β stimulation........... 168
Figure 22 Time-dependent effects of glucocorticoid treatment .......................................... 169
Figure 23 Time-dependent effects of glucocorticoid treatment on multiple chemokines and cytokines ......................................................................................................................... 170
Figure 24 Dose-dependent effects of dexamethasone priming .......................................... 172
Figure 25 Dose-dependent effects of cortisol priming ....................................................... 174
Figure 26 GR-dependent effects at all doses of pre-treatment with dexamethasone ......... 176
Figure 27 Dose-dependent inhibition of dexamethasone effects by GR antagonist RU486 .... 178
Figure 28 The pro-inflammatory effects of dexamethasone are GR-dependent .................. 181
Figure 29 The pro-inflammatory effects of dexamethasone are induced during pre-treatment 182
Figure 30 No change in gene expression of GR or NF-κB in glucocorticoid potentiation ........ 184
Figure 31 Glucocorticoid resistance with and without glucocorticoid pre-treatment............. 186
Figure 32 Experimental paradigms used for genome-wide gene expression analysis............ 188
Figure 33 Overlap between genes differentially regulated by dexamethasone pre-treatment and dexamethasone co-treatment with immune treatment.............................................. 197
Figure 34 Effects of antidepressant compounds on protein expression in hippocampal progenitor cells................................................................. 205
Figure 35 Antidepressant compounds have differential effects upon IL-6 mRNA expression. 208
Figure 36 Antidepressant compounds have differential effects upon IL-1 mRNA expression. 209
Figure 37 All four antidepressant compounds reduced NF-κB activity.............................. 211
Figure 38 IL-1β decreases neurogenesis in human hippocampal cells........................... 216
Figure 39 Monoaminergic antidepressants and PUFAs modulate neurogenesis in human hippocampal cells................................................................. 218
Figure 40 Antidepressant compounds modulate IL-1β-induced changes to the kynurenine pathway......................................................................................... 221
Figure 41 Antidepressant compounds reduce the rise in quinolinic acid caused by IL-1β treatment......................................................................................... 223
Figure 42 Shearing of samples by sonication to optimal size........................................... 228
Figure 43 Optimisation of fragmentation of DNA using micrococcal nuclease................. 230
Figure 44 Confirmation of chromatin formation using validated antibody, assessed as compared to input............................................................................. 232
Figure 45 Confirmation of chromatin formation using validated antibody, assessed as compared to IgG control.......................................................................... 233
Figure 46 Enrichment of GR-target genes........................................................................ 236
Figure 47 Enrichment of GR target genes – second repetition........................................ 237
Figure 48 Potentiation of inflammatory responses in macrophages shows a similar time-dependent course to the present study......................................................... 260
Figure 49 Potentiation of inflammatory responses in humans is dose-dependent consistent with the present study.............................................................. 263
Figure 50 Dose-dependent glucocorticoid pre-treatment potentiation of inflammatory gene expression in the frontal cortex of rats ................................................................. 265
Figure 51 A dose-dependent model of glucocorticoid actions ................................................................. 268
Figure 52 A proposed model of glucocorticoid effects, synthesizing time and dose dependency .............................................................................................................. 271
Figure 53 Increased inflammatory response to stress by depressed patients with increased early life stress ........................................................................................................ 284

Table of Tables

Table 1 Components added to DMEM for proliferation media................................................................. 99
Table 2 Components added to DMEM for differentiation media ................................................................. 100
Table 3 Cytokines and chemokines measured by the Human Cytokine Magnetic 25-Plex Panel ..................................................................................................................... 116
Table 4 Primer pairs used in PCR experiments ......................................................................................... 120
Table 5 Buffers used in chromatin immunoprecipitation ......................................................................... 133
Table 6 Glucocorticoid receptor antibodies used to immunoprecipitate GR-bound DNA ........... 134
Table 7 Primers used in chromatin immunoprecipitation detection of enrichment ......................... 135
Table 8 Depressed patients with GR resistance, increased innate immunity and hypercortisolaemia .................................................................................................................. 144
Table 9 Depressed patients with GR resistance, increased innate immunity, and normal cortisol levels ........................................................................................................................................ 148
Table 10 Depressed patients with GR resistance, no changes in immune function and normal cortisol ..................................................................................................................... 150
Table 11 Evidence of pro-inflammatory states in depressed patients without any endocrine changes 152
Table 12 Pattern of chemokine and cytokine secretion in unstimulated and IL-1β-stimulated hippocampal progenitor cells ........................................................................................................... 162
Table 13 Genes most strongly up-regulated by IL-1β treatment ............................................................... 191
Table 14 Pathways significantly regulated by IL-1β treatment ................................................................. 193
Table 15 Genes most strongly inhibited by dexamethasone co-incubation ........................................ 201
Table 16 Gene pathways significantly regulated by dexamethasone co-incubation ................... 202
Table 17 Modulation of cytokines and chemokines by antidepressant compounds.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>AHN</td>
<td>Adult hippocampal neurogenesis</td>
</tr>
<tr>
<td>Akt</td>
<td>RAC-alpha serine/threonine-protein kinase</td>
</tr>
<tr>
<td>ALA</td>
<td>Alpha-linolenic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AP1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin Immunoprecipitation</td>
</tr>
<tr>
<td>ChIP-seq</td>
<td>ChIP-sequencing</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CORT</td>
<td>Cortisol/ corticosterone</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotropin-releasing hormone</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>DCX</td>
<td>Doublecortin</td>
</tr>
<tr>
<td>DEX</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DSCAM</td>
<td>Down Syndrome Cell Adhesion Molecule</td>
</tr>
<tr>
<td>DSM-V</td>
<td>Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5)</td>
</tr>
<tr>
<td>DST</td>
<td>Dexamethasone suppression test</td>
</tr>
<tr>
<td>ECT</td>
<td>Electroconvulsive therapy</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>ER</td>
<td>Oestrogen receptor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>FC</td>
<td>Fold change</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>GBD</td>
<td>Global burden of disease</td>
</tr>
<tr>
<td>GFAP</td>
<td>Gial fibrillary acidic protein</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GC</td>
<td>Glucocorticoid</td>
</tr>
<tr>
<td>GILZ</td>
<td>Glucocorticoid-Induced Leucine Zipper Protein</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>GRE</td>
<td>Glucocorticoid response element</td>
</tr>
<tr>
<td>HAM-D</td>
<td>Hamilton Rating Scale for Depression</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic–pituitary–adrenal axis</td>
</tr>
<tr>
<td>HPC</td>
<td>Hippocampal progenitor cells</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IDO</td>
<td>Indoleamine 2,3-dioxygenase</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon-alpha</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-1RA</td>
<td>Interleukin 1 receptor antagonist</td>
</tr>
<tr>
<td>IP-10</td>
<td>Interferon gamma-induced protein 10</td>
</tr>
<tr>
<td>JAK-STAT</td>
<td>Janus kinase-Signal Transducer and Activator of Transcription signalling pathway</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinases</td>
</tr>
<tr>
<td>KAT</td>
<td>Kynurenine aminotransferase</td>
</tr>
<tr>
<td>KMO</td>
<td>Kynurenine 3-Monoxygenase</td>
</tr>
<tr>
<td>KYN</td>
<td>Kynurenine</td>
</tr>
<tr>
<td>KYNA</td>
<td>Kynurenine Acid</td>
</tr>
<tr>
<td>KYNU</td>
<td>Kynureninase</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography-tandem mass spectrometry</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAO-I</td>
<td>Monoamine oxidase inhibitor</td>
</tr>
<tr>
<td>MAP2</td>
<td>Microtubule-associated protein 2</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemotactic protein 1</td>
</tr>
<tr>
<td>mD</td>
<td>Minor depressed</td>
</tr>
<tr>
<td>MD</td>
<td>Major depressed</td>
</tr>
<tr>
<td>MDD</td>
<td>Major depressive disorder</td>
</tr>
<tr>
<td>MIG</td>
<td>Monokine induced by interferon-γ</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage inflammatory protein</td>
</tr>
<tr>
<td>MR</td>
<td>Mineralocorticoid receptor</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MT2A</td>
<td>Metallothionein 2A</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NFIL3</td>
<td>Nuclear factor interleukin-3-regulated protein</td>
</tr>
<tr>
<td>NGS</td>
<td>Normal goat serum</td>
</tr>
<tr>
<td>NLRP3</td>
<td>NOD-like receptor family, pyrin domain containing 3</td>
</tr>
<tr>
<td>NLRP6</td>
<td>NOD-like receptor family, pyrin domain containing 6</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate receptor</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide-binding oligomerization domain</td>
</tr>
<tr>
<td>p27Kip1</td>
<td>Cyclin-dependent kinase inhibitor 1B</td>
</tr>
<tr>
<td>p57Kip2</td>
<td>Cyclin-dependent kinase inhibitor 1C</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PBMCs</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PGE2</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohaemagglutinin</td>
</tr>
<tr>
<td>PIC</td>
<td>Pro-inflammatory cytokine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3-Kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acid</td>
</tr>
<tr>
<td>PVN</td>
<td>Paraventricular nucleus of hypothalamus</td>
</tr>
<tr>
<td>QUIN</td>
<td>Quinolinic acid</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated on Activation, Normal T Cell Expressed and Secreted</td>
</tr>
<tr>
<td>RIP A</td>
<td>Radioimmunoprecipitation assay buffer</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RMM</td>
<td>Reduced modified media</td>
</tr>
<tr>
<td>RU486</td>
<td>Mifepristone</td>
</tr>
<tr>
<td>SAP/JNK</td>
<td>Stress-activated protein kinases-Jun amino-terminal kinases signalling pathway</td>
</tr>
<tr>
<td>SGK1</td>
<td>Serum and glucocorticoid-regulated kinase 1</td>
</tr>
<tr>
<td>SLC19A2</td>
<td>Solute carrier family 19 member 2</td>
</tr>
<tr>
<td>SMD</td>
<td>Standardised mean difference</td>
</tr>
<tr>
<td>SNRI</td>
<td>Serotonin–norepinephrine reuptake inhibitor</td>
</tr>
<tr>
<td>SOX2</td>
<td>SRY (sex determining region Y)-box 2</td>
</tr>
<tr>
<td>SSRI</td>
<td>Selective serotonin reuptake inhibitor</td>
</tr>
<tr>
<td>STAR-D</td>
<td>Sequenced Treatment Alternatives to Relieve Depression</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricyclic antidepressant</td>
</tr>
<tr>
<td>TDO</td>
<td>Tryptophan 2,3-dioxygenase</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA buffer solution</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF-associated factor</td>
</tr>
<tr>
<td>TRD</td>
<td>Treatment resistant depression</td>
</tr>
<tr>
<td>TRP</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>TSV</td>
<td>Topical steroid-induced vasoconstriction</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>3-HK</td>
<td>3-hydroxykynurenine</td>
</tr>
<tr>
<td>4-OHT</td>
<td>4-Hydroxytamoxifen</td>
</tr>
<tr>
<td>-------</td>
<td>---------------------</td>
</tr>
<tr>
<td>5-HT</td>
<td>Serotonin</td>
</tr>
<tr>
<td>5-HTT</td>
<td>Serotonin transporter</td>
</tr>
</tbody>
</table>
1 Introduction

This thesis explores elements of the pathogenesis of depression and the action of antidepressant compounds in a cellular model with relevance to depression - human hippocampal progenitor cells. A particular focus is on the seeming contradiction between over-activity of both the HPA axis and inflammation in chronic stress and depression, in the context of cortisol’s well known anti-inflammatory properties. This issue will be explored through a systematic literature review and meta-analysis before looking at possible interactions of these systems in human neural cells. This thesis will also explore the mechanisms by which monoaminergic antidepressants and polyunsaturated fatty acids may exert their antidepressant effects by investigating their influence on inflammation, neurogenesis and the kynurenine pathway in human hippocampal cells, trying to discern critical areas of overlap in the action of these varied compounds to clarify targets for treatment. Finally, this thesis will outline the process of optimising chromatin immunoprecipitation, a technique for determining the genomic targets of transcriptional activation, in this cell system, to allow future investigation of the mechanisms underlying antidepressant and glucocorticoid effects on hippocampal neurogenesis.

The introduction will briefly describe the major hypotheses of depression, with relevance to this thesis, with a particular focus on the cytokine and glucocorticoid hypotheses, in the context of hippocampal abnormalities in depression. It will then describe the biological domains thought to be affected by effective antidepressant treatment, with a particular focus on hippocampal neurogenesis, inflammation and the kynurenine pathway, before outlining the aims of this thesis.
1.1 Major Depressive Disorder

1.1.1 Definition of Major Depressive Disorder

Depression is the illness of our age. It is defined in the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) as a mental disorder consisting of:

- depressed mood,
- markedly diminished interest or pleasure,
- significant unintentional increase or decrease in appetite and weight,
- insomnia or hypersomnia,
- psychomotor agitation or retardation,
- fatigue,
- feelings of worthlessness or excessive or inappropriate guilt,
- diminished ability to think or concentrate or indecisiveness, and
- recurrent thoughts of death and suicidal ideation or plan for suicide, present for at least two weeks (DSM-5, 2013).

These symptoms must cause clinically significant distress or impairment in social, occupational, or other important areas of functioning, and are not due to drug abuse, general medical condition or bereavement (DSM-5, 2013).

1.1.2 Prevalence of Major Depression

With the decline of illness due to infectious diseases, and cardiovascular diseases, the proportion of disease burden attributed to mental illnesses, and especially depression, has steadily increased. When using the metric years of life lived with disability (YLDs) in the 1990 global burden of disease (GBD) study
depression was ranked as the fourth leading cause of disease burden after lower respiratory tract infections, diarrheal diseases and conditions arising from the perinatal period (Ferrari et al., 2013). In the 2000 GBD study, depressive disorders were the third leading cause of disease burden behind lower respiratory infections and diarrheal diseases. In the latest iteration of the study, conducted in 2010, depressive disorders now rank second in terms of global disability burden, with 106 million people around the world suffering from depression, accounting for 8.2% of all global YLDs in 2010 (Ferrari et al., 2013). It was also the eleventh leading cause of global burden (measured by disability-adjusted life years (DALYs)) in 2010, accounting for 2.4% of global DALYs.

The burden of depressive disorders is higher in women than men and the largest proportion of YLDs from depressive disorders occurs among adults of working age. The global burden of depressive disorders increased by 37.5% between 1990 and 2010, largely because of population growth and aging (Ferrari et al., 2013). Depression would account for an additional 16 million DALYs and 4 million DALYs when it is considered as a risk factor for suicide and ischemic heart disease, respectively. Depression increases the risk of suicide by 20-fold and the risk of ischaemic heart disease by 60%. This "attributable" burden increased the overall burden of depressive disorders to 3.8% of global DALYs (Ferrari et al., 2013).

1.1.3 Environmental factors in depression

Depression, like many complex illnesses, is thought to arise from the interaction of genetic and environmental factors. The heritability of depression, determined to be approximately 37% from meta-analysis of genetic epidemiological studies (Sullivan et al., 2000), indicates that there is a large environmental component to depression (significantly larger than other psychiatric illnesses such as bipolar,
autism or schizophrenia). Most recognised environmental risk factors for depression are related to chronic psychological stress, particularly at sensitive periods. Stressful life events (such as job loss, marital difficulties, loss of close personal relationships and severe health problems) (Kendler et al, 1999, 2004; Kessler, 1997), as well as low social status and low social support (situations of chronic stress) all present significant risks for developing depression (Fava and Kendler, 2000). Stressful events in childhood (for example, adverse childhood experiences, including poor relationship of the child to their parents, parents’ divorce, and physical or sexual abuse) have particular relevance to influencing risk of depression in later adulthood (Anda et al, 2006).

Several studies have demonstrated that gene-environment interactions are likely to be important in the risk of depression. Personality traits, like neuroticism (Fava and Kendler, 2000), which are largely genetically determined, and particular genetic polymorphisms (such as polymorphisms of the serotonin transporter promoter and key proteins in the regulation of glucocorticoids levels) (Caspi et al, 2003; van Rossum et al, 2006) increase sensitivity to stress and increase the risk of depression. The experience of stress in susceptible individuals therefore seems central to the pathogenesis of depression.

1.2 The Biological Basis of Depression

The biological abnormalities found in depressed patients are consistent with the notion that stress is central to the pathogenesis of depression. The biological domains implicated in depression are those that have a role in responding to stress or are particularly sensitive to the effects of stress-activated systems. In the following section I briefly outline the biological domains – physiological processes and anatomical locations – implicated in the pathogenesis of depression relevant to this thesis.
1.2.1 The hippocampus and depression

The diverse symptoms of depression in affective, cognitive and behavioural domains are thought to reflect abnormalities of several brain regions, and have led researchers to examine which portions of the brain demonstrate gross abnormalities. Human imaging and post mortem studies have revealed anatomical abnormalities in the prefrontal cortex, the cingulate cortex, the striatum, the amygdala, the thalamus and the hippocampus (Drevets et al, 1997; Liotti and Mayberg, 2001; Nestler et al, 2002; Samuels and Hen, 2011). Together, these brain regions interact in function, and disturbance of their individual and common functionality may explain the complexity of depressive symptoms (Samuels and Hen, 2011).

The hippocampus, in particular, a central structure in the limbic network, has emerged as a key region for understanding the pathogenesis of depression and the effects of antidepressant treatment. It has been implicated in mood, regulation of the stress response, is found to be shrunken in depressed patients, and this reduction in size be reversed by antidepressant treatment (Campbell and MacQueen, 2004; Sheline et al, 2003). Several biological domains thought to be dysfunctional in depression, detailed below in Sections 1.2.2 – 1.2.8, interact with the hippocampus, both affecting it, as well as being regulated by hippocampal function. The hippocampus therefore represents an important point of convergence between several biological systems important in depression and antidepressant effects. This thesis will focus on understanding how these different biological systems affect cells of the hippocampus, and the ability of antidepressant compounds to modulate these effects.
1.2.1.1 The function of the hippocampus

The hippocampus is a major component of the limbic system, which also includes the amygdala, the olfactory cortex and the cingulate cortex, brain regions critically involved in emotion processing (Phelps, 2004). Although the hippocampus has been primarily associated with forming and recalling memories and spatial navigation, it has been increasingly associated with stress regulation and mood, and been implicated in neurodegenerative diseases and mental illnesses (Egeland et al, 2015).

There is now a broad base of evidence suggesting that hippocampal function is divided across two major mental domains – cognition, and ‘cold’ memory (declarative memory) on the one hand, and emotion and stress regulation on the other – corresponding to dorsal and ventral anatomical portions of the hippocampus, respectively (Fanselow and Dong, 2010). This is supported by data showing that lesions of the ventral hippocampus, but not the dorsal hippocampus, alter emotional behaviour and response to stress (Henke, 1990).

1.2.1.2 Hippocampal changes in depression

One of the first indications of the involvement of the hippocampus in depression was the detection of a reduction in its size in depressed patients. It has been consistently reported that total hippocampal volume is decreased in depressed patients (8% on the left side and 10% on the right side in a meta-analysis) (Videbech and Ravnkilde, 2004) and in animal models of chronic stress and depression, suggesting a potential functional impairment of the hippocampus (Czéh et al, 2001).

It has been hypothesised that this hippocampal volume loss may explain the long lasting mood and memory disturbances which occur in depression (Gould
et al, 2007; Sahay and Hen, 2007). Consistent with this notion, the domains of memory function that are found to be impaired in depressed patients are those that are most dependent on hippocampal function (MacQueen and Frodl, 2011). The hippocampus has also been implicated in depression by the finding that larger hippocampal volume predicts a better response to antidepressant treatment (MacQueen et al, 2008), and successful antidepressant treatment can reverse the reduction in hippocampal volume in depression (Sheline et al, 2003). It has also been noted that subjects at risk for major depressive disorder (MDD) have reduced hippocampal volumes before the clinical onset of illness (MacQueen and Frodl, 2011). There is intense debate regarding the nature of the underlying cellular changes that constitute this volume loss detected in depression (MacQueen and Frodl, 2011). It has been thought that this volume loss may be due to atrophy and retraction of apical dendrites of hippocampal pyramidal cells, a reduction in the amount of neuropil, hippocampal neuronal death, decreases in neurogenesis or combinations of these changes (MacQueen and Frodl, 2011; Zunszain et al, 2011).

In addition to the hippocampus being regularly found to be reduced in size, implicated in the function of mood and emotional regulation, it is also the part of the brain most sensitive to environmental changes, particularly to stressful stimuli (Thomas et al, 2007), mirroring the strong relationship between depression and environmental stressors (Kessler, 1997). Stress, including psychological or psychosocial stress, is associated with structural changes to the hippocampus (MacQueen and Frodl, 2011). It has also been observed in humans that in conditions of chronic hypercortisolaemia, as in Cushing’s disease, atrophy of the hippocampus is observed and that these changes are reversed on normalisation of cortisol levels, indicating that the human
hippocampus is sensitive to increased levels of cortisol as observed in animal models of chronic stress and depression (MacQueen and Frodl, 2011). Additionally, the hippocampus is also linked to the pathogenesis of depression because it controls the regulation of the hypothalamic-pituitary-adrenal (HPA) axis, which, as discussed below, plays a key role in the stress response, and has been implicated in depression (Jacobson and Sapolsky, 1991). Lastly, a recently discovered property of the hippocampus – the formation of new neurons in this region throughout adult life – has been thought to contribute to the pathogenesis of depression, and will be discussed in Section 1.2.5.

1.2.2 Hypothalamic-Pituitary-Adrenal (HPA) axis

One of the systems most implicated in the pathogenesis of depression is the HPA axis, the system canonically identified with the biological response to stress (Figure 1). Cortisol, the systemically active human glucocorticoid, is released by this axis in response to stress and allows for a number of adaptive responses in the context of threat – both physiological and psychological (Kudielka et al, 2004). An inability of the HPA axis to respond to stressful stimuli conveys a reduced survival advantage to the organism.
The paraventricular nucleus (PVN) of the hypothalamus contains neuroendocrine neurons that synthesize and secrete corticotropin-releasing hormone (CRH). In turn, this stimulates the release of adrenocorticotropic hormone (ACTH) from the anterior lobe of the pituitary gland. ACTH acts on the cortex of the adrenal gland. Cortisol produced in the adrenal cortex will negatively feedback to inhibit both the hypothalamus and the pituitary gland. This reduces the secretion of CRH, and also reduces the cleavage of proopiomelanocortin (POMC) into ACTH and β-endorphins. Cortisol, and other glucocorticoids, also have the additional effect of reducing neuroplasticity and neurogenesis in the hippocampus. Reproduced with permission (Anacker et al, 2011a).
Consistent with the psychological literature regarding the relationship between stress and depression, abnormalities of both steady state and dynamic responses of the HPA axis are common biological findings in depression (Pariante and Lightman, 2008). Evidence of HPA axis hyperactivity, including increased pituitary and adrenal gland volume (Pariante, 2009), increased levels of cortisol (in saliva, CSF, blood and urine) are found in many studies of depressed subjects, with greater incidence in those with more severe depression (Juruena et al, 2006; Pariante, 2009). There is a degree of uncertainty as to how common such findings are with estimates in the literature ranging between 20-80% (Stetler and Miller, 2011).

A recent meta-analysis of aspects of the HPA axis in depression found evidence of modest increases in cortisol in depressed patients (an effect size of 0.6 (Cohen’s d)), though this was reduced by half (Cohen’s d=0.33) when restricted to higher quality studies (Stetler and Miller, 2011). ACTH levels were also found to be elevated in this meta-analysis, though not CRH levels. Interestingly, atypical depression was associated with decreased levels of cortisol compared to non-atypical depression, and there was evidence that atypically depressed patients had even lower cortisol levels than controls, though the numbers of studies was small (Stetler and Miller, 2011). There was also evidence of a gradient in cortisol levels associated with severity of depression. Blunted cortisol levels in response to stress and impaired stress recovery are also found in depressed patients, especially in more severe cases, further suggesting functional impairments to the HPA axis (Burke et al, 2005).

Chronically elevated glucocorticoid levels can induce the changes thought to be present in depressed individuals, such as decreased neurogenesis, monoaminergic system dysfunction, volume reduction in limbic structures, and
impaired synaptic plasticity, also observed in animal and in vitro work (Anacker et al, 2011b; Herbert et al, 2006; de Kloet et al, 2005). Correspondingly, administration of glucocorticoids to animals induces depression-like behaviour. These findings have led to the formulation of the glucocorticoid theory of depression, whereby chronic stress causes abnormally high activity of the HPA axis in susceptible individuals, involving the production of excess cortisol, contributing to the neuropathology and psychological disturbances manifest in depression (Holsboer, 2000; Pariante and Lightman, 2008). This hypothesis has widely informed research into depression - including an exploration of medications that can affect GR function and cortisol levels, the testing of glucocorticoid agonists and antagonist drugs as novel antidepressants and an intense search for biomarkers of depression based on HPA axis dysfunction (Pariante and Lightman, 2008).

1.2.3 Glucocorticoid receptor dysfunction

There has been a particular focus in depression on dysfunction of a central component of the HPA axis – the glucocorticoid receptor (GR). The GR is a transcription factor that when activated by glucocorticoid ligation causes the transcription of multiple genes, transducing the effect of glucocorticoids throughout the body (Anacker et al, 2011a). The GR, extensively distributed in the hypothalamus, the pituitary and the hippocampus, is also responsible for negative feedback mechanisms that regulate the HPA axis by inhibiting release of CRH and ACTH in response to increased levels of glucocorticoids (McEwen, 1982). (Further details of GR activation will be discussed in Section 1.5).

Glucocorticoid function is normally measured by the ability of exogenous glucocorticoids to suppress the HPA axis through negative feedback, or through the ability of glucocorticoids to inhibit inflammation in vivo or ex vivo. Decreased
responsiveness to glucocorticoids, known as glucocorticoid resistance, attributed largely to functional impairment of the glucocorticoid receptor (GR), is a common finding in depression. It is reported in between 25 to 80% of depressed patients, depending on age, sex, severity of depression and the type of test employed (Pace and Miller, 2009; Pariante and Miller, 2001), with more severely depressed patients demonstrating higher incidences of glucocorticoid resistance (Holsboer, 2000; Miller et al, 2002; Pariante and Miller, 2001).

The most commonly employed tests for glucocorticoid sensitivity are the dexamethasone suppression test (DST) or the DEX/CRH combined suppression test in which the sensitivity of the HPA axis to feedback inhibition is tested. Other tests involve inhibiting stimulated peripheral immune cells with glucocorticoids, and topical application of glucocorticoids to the skin. Recently, a novel molecular measure of GR resistance, observing reduced expression of glucocorticoid receptor-target genes in whole blood following in vivo dexamethasone administration, suggests that GR resistance may be even more prevalent in depression than previously thought, based on the DST (Menke et al, 2012).

This body of evidence has led to the glucocorticoid receptor hypothesis of depression. This theory is distinguished from the glucocorticoid theory of depression in that it postulates that although circulating concentrations of glucocorticoids may be increased (or normal), intracellular signalling through the GR is reduced (Raison and Miller, 2003). In this interpretation, insufficient glucocorticoid signalling rather than an excess is critical to the pathophysiology of depression. Such a theory has been closely related to the finding of increased activity of the innate immune system in depression, outlined below. The direction of causality between increased cortisol and GR resistance is
intensely debated – with suggestions that hypercortisolaemia might induce resistance of the GR, or that GR resistance, induced by other means, including increased inflammation, may give rise to hypercortisolaemia as a compensatory mechanism (Pace and Miller, 2009; Pariante and Lightman, 2008; Raison and Miller, 2003), and will be discussed further below in Section 1.3.

1.2.4 The innate immune system

More recently, there has been increasing recognition of the role of the innate immune system in depression (Dantzer et al, 2008). The innate immune system responds in a stereotyped, non-specific manner to a number of different immunological and physical insults, providing immediate protection against infection, and found in all classes of animal and plant life (Medzhitov and Janeway, 2002). It is now well understood that the immune system also responds acutely to the experience of psychological stress (Steptoe et al, 2007). This has been observed in a number of components of the immune system, but most robustly in increased levels of inflammatory molecules. These findings have extended the understanding of what constitutes the ‘stress response’ to include activation of the innate immune system. Following psychological stress (commonly studied by paradigms like the Trier Social Stress Test) there is now robust evidence, confirmed on meta-analysis, that circulating levels of interleukin-6 (IL-6) and IL-1β rise in the period following acute psychological stress (20-120 minutes), with marginal effects for C-reactive protein (CRP) (Steptoe et al, 2007). Of note, this acute rise in innate immune activity following psychological stress is similar to the observed rise in cortisol that established the HPA axis as the canonical stress response system (Dickerson and Kemeny, 2004).
The involvement of the innate immune system in the response to stress is consistent with the finding that the innate immune system is abnormal in the context of chronic stress and demonstrates over-activity in depressed individuals, confirmed in meta-analyses looking at peripheral levels of immune proteins (Dowlati et al, 2010; Howren et al, 2009). This finding has been recently strengthened by the detection of up-regulation of IL-6 pathways in genome-wide gene expression studies of depressed patients (Jansen et al, 2015). Dysfunction of other elements of the innate immune system is also evident in depressed patients, with observations of increased levels of acute phase proteins, chemokines, adhesion molecules, and inflammatory mediators such as prostaglandins (Miller et al, 2009a) and increased numbers of innate immune cells like monocytes and neutrophils (Maes, 1999).

As with cortisol, pro-inflammatory cytokines can produce many of the neurological changes thought to be characteristic of depression in animal and cellular models, including decreased neurogenesis, regional brain abnormalities, monoamine changes, decreased neural plasticity and neurodegeneration (Miller et al, 2009a; Zunszain et al, 2013) (Figure 2). Along with converging lines of evidence - the overlap between cytokine-induced sickness behaviour and depression, the increased incidence of depression in the medically ill, the induction of depression in humans and animals by stimulating cytokine production and preliminary evidence that reducing levels of cytokines has antidepressant effects - the cytokine theory of depression has been formulated (Dantzer et al, 2008; Miller et al, 2009a), suggesting that pro-inflammatory cytokines might be key molecules in transducing chronic psychological stress into the neurological changes thought to underlie
depression. There is also the possibility that depression may give rise to increased levels of inflammation, although a recent meta-analysis demonstrating the presence of increased levels of inflammation preceding the development of depression support a causal role for inflammation in depression (Valkanova et al, 2013).
Figure 2 The role of the innate immune system in stress and depression

Activation of the immune system by stress affects the brain through various pathways and affects many biological systems thought to be perturbed in depression.
Further evidence for the role of inflammation in depression has also been derived from observations linking levels of inflammation to outcomes in depression. Depressed patients who fail to respond to antidepressants are more likely to exhibit increased inflammatory markers prior to treatment, and reduced inflammation during antidepressant treatment has been associated with responsiveness to therapy in some studies (Lanquillon et al, 2000; Raison et al, 2006). Furthermore, selective serotonin reuptake inhibitor (SSRI) pre-treatment and symptomatic treatment has been effective in cytokine-induced depression, as shown in hepatitis-C patients exposed to IFN-α (Sockalingam and Abbey, 2009), suggesting that major depressive disorder and inflammation-induced depression have overlapping neurobiological abnormalities.

The ability of anti-inflammatory medications to exert antidepressant effects has also supported the role of inflammation in the pathogenesis of depression. A recent meta-analysis of anti-inflammatory medication in depression found that anti-inflammatory treatment reduced depressive symptoms (standardised mean difference (SMD) = -0.34) compared with placebo (Köhler et al, 2014). The authors note that there was a high risk of bias and high heterogeneity in the ten publications reviewed, however, they do highlight evidence that an anti-inflammatory strategy, particularly celecoxib, may be useful in treating depression.

1.2.5 Adult hippocampal neurogenesis
A recently discovered neuroplastic property of the hippocampus has received particular attention for its role in stress, depression and the action of antidepressants: the formation of new neurons in this region of the brain
throughout adult life, called adult hippocampal neurogenesis (AHN), as mentioned above. This process appears to be key in the role that the hippocampus plays in the regulating and responding to stress, and in the response to antidepressants.

The hippocampus is now recognised as the only region of the adult brain in which new neurons are constantly being born throughout adulthood. The recent confirmation of adult hippocampal neurogenesis (AHN) in humans has overturned dogma that no new neurons are formed in the adult human brain (Spalding et al, 2013). AHN represents a novel and powerful form of neuroplasticity whereby new neurons are generated and incorporated into neural networks in response to changes in environmental stimuli (Clelland et al, 2009; Deng et al, 2010).

Stress reduces AHN and in turn reduced AHN leads to a dysregulated stress response, increasing the chance of developing depression-like behaviour in animals (Egeland et al, 2015). Experimental paradigms with animals demonstrate that exposure to acute or chronic stress reduces AHN and leads to the development of depressive-like symptoms (Schoenfeld and Gould, 2012). While there are currently no non-invasive methods available to study neurogenesis in human subjects, reduced AHN has also been detected in the brains of unmedicated depressed patients on post mortem, consistent with findings in chronically stressed animals (Boldrini et al, 2009), though the samples size examined was small and an earlier, smaller study found no effect (Reif et al, 2006). Furthermore, the biological systems that are thought to be dysfunctional in depression have also been shown to regulate AHN – that is, glucocorticoids (Schoenfeld and Gould, 2012), inflammation (Goshen et al,
(discussed in further depth in Section 1.2.7) and monoamine signalling (Mahar et al., 2014).

Whether decreasing neurogenesis is enough in itself to induce depression is a subject of intense debate, mirroring the debate regarding the importance of neurogenesis in the action of antidepressants (to be discussed further in Section 1.4.4). It seems that experimental reduction of neurogenesis, in the absence of stress does not of itself induce depression, but rather that reduced neurogenesis contributes to the depression-like symptoms in animals by impairing stress modulation (Petrik et al., 2012). This notion is supported by two studies that have demonstrated that rodents with reduced neurogenesis – induced by genetic means or exposure to radiation - demonstrate increased levels of stress hormones in response to stress (Snyder et al., 2011; Surget et al., 2011). Interestingly, these studies also indicated an important role for AHN in regulation of the HPA axis, as neurogenically depleted animals demonstrate glucocorticoid resistance, further implicating the role of AHN in the stress systems perturbed in depression. Reduced AHN may be part of a vicious cycle whereby the experience of psychological stressors leads to the development of depression (Egeland et al., 2015).

1.2.6 Inflammation decreases neurogenesis

As indicated above, one of the focuses of this thesis is examining points of convergence between different biological domains implicated in the pathogenesis of depression. One such interaction is between inflammation, particularly the pro-inflammatory cytokine IL-1β, and reduced AHN. For example, mice subjected to chronic mild stress for five weeks demonstrated depressive-like symptoms, increased IL-1β expression in the hippocampus and decreased levels of hippocampal neurogenesis (Goshen et al., 2008). Excluding
the effect of IL-1β by knocking out the IL-1 receptor gene in a transgenic line prevented the increase in depression-like behaviour and abrogated the decrease in hippocampal neurogenesis, suggesting IL-1β is necessary for the decrease in hippocampal neurogenesis and the induction of depression. Furthermore, administration of IL-1β directly to mice produced the same increase in depressive-like behaviour and decrease in neurogenesis as chronic mild stress (Goshen et al, 2008).

These findings were confirmed in another study demonstrating that the use of an inhibitor for the IL-1 receptor or IL-1R1 null mice blocked the effects of chronic stress on reduction of hippocampal neurogenesis and increased anhedonia, suggesting IL-1β is a critical mediator of decreased hippocampal neurogenesis in the context of chronic stress (Koo and Duman, 2008). Other studies also support this findings - treatment with IL-1 inducers, like lipopolysaccharide (LPS) and radiation, resulted in marked suppression of hippocampal neurogenesis in rats (Ekdahl et al, 2003; Monje et al, 2003). Furthermore, intrahippocampal transplantation of neural precursor cells that overexpress the IL-1 receptor antagonist, therefore blocking IL-1 signalling by chronically elevating levels of the antagonist, completely abolishes the detrimental effect of stress on neurogenesis (Ben Menachem-Zidon et al, 2007).

Following these studies in animal models, the effects of IL-1β on decreasing neurogenesis have been supported by direct in vitro incubation of both animal and human hippocampal progenitor cells with IL-1β. In vitro incubation of neural stem cells from mice brains with IL-1β showed epigenetic modifications of the neural progenitor cell marker NeuroD, consistent with decreased hippocampal neurogenesis (Kuzumaki et al, 2010). Furthermore, work in our lab has shown that incubation of human hippocampal progenitor cells with IL-1β
decreases DCX-positive neuroblasts and MAP2-positive neurons, extending the ability of IL-1β to reduce hippocampal neurogenesis to human hippocampal cells (Zunszain et al, 2012). Both increased pro-inflammatory cytokines (Dowlati et al, 2010; Howren et al, 2009) and reduced hippocampal neurogenesis (Boldrini et al, 2009) are also found in depressed patients, and the above mechanistic work in animals suggests the connection may be causal.

1.2.7 Kynurenine Metabolites

Further work has examined the potential mechanisms that connect increased levels of pro-inflammatory cytokines and reduced hippocampal neurogenesis, and alterations to the kynurenine metabolic pathway have been implicated. The kynurenine pathway (Figure 3) involves conversion of tryptophan (TRP) into kynurenine, a precursor of both quinolinic acid (QUIN), a potentially neurotoxic metabolite, and kynurenic acid (KYNA), a potentially neuroprotective metabolite. The first step is catalysed by the enzyme tryptophan-2,3-dioxygenase (TDO) and, especially in response to inflammation, also by indolamine-2,3-dioxygenase (IDO) (Dantzer et al, 2011). There is evidence of perturbations to the kynurenine pathway in patients with major depression. In one study, depressed patients showed a shift towards kynurenine metabolism from tryptophan, away from serotonin, and a reduction in the neuroprotective arm of the pathway (Myint et al, 2007). A recent, exhaustive review of studies in depressed patients that have measured kynurenine metabolites found abnormalities were commonly found – including findings of increased QUIN, increased KYNA, and increased kynurenine, in depressed patients compared with controls, with some studies showing correlations between severity of symptoms and levels of these metabolites (Réus et al, n.d.).
Inflammation-induced depression in animals and humans have further emphasised the importance of perturbation of the kynurenine pathway in the link between inflammation and depression. In a model of depression induced by inflammatory stimulus in animals, IDO is activated, and the depressive symptoms can be prevented by administration of an IDO antagonist (O’Connor et al, 2009). Indeed, simply administering kynurenine to animals induces depression-like behaviour (O’Connor et al, 2009). Furthermore, in the equivalent model in humans, that of INF-α-induced depression, levels of kynurenine and QUIN are increased in both blood and cerebrospinal fluid, and correlate with depressive symptoms (Capuron et al, 2002; Raison et al, 2010). Consistent with these findings, another study found that in patients treated with INF-α the KYN/TRP ratio was increased, as was the KYN/KYNA ratio, indicating reduced neuroprotective metabolites, which also correlated with depressive symptoms (Wichers et al, 2005).

Two possible effects of inflammatory perturbation of the kynurenine pathway have been thought to have relevance for the pathogenesis of depression (and neurodegeneration) - the reduction in available serotonin, putatively linked to increased metabolism of tryptophan into kynurenine by activated IDO, as well as increased neurotoxicity, due to a change in the relative balance of QUIN and KYNA (Maes et al, 2009; Myint and Kim, 2003; Myint et al, 2007; Wichers et al, 2005). These pathways may also affect hippocampal neurogenesis, and, indeed, tryptophan-2,3-dioxygenase (TDO) knockout mice show increased neurogenesis (Kanai et al, 2009).

Recently, out lab has also shown that inflammation (in the form of the pro-inflammatory cytokine IL-1β) reduces neurogenesis in human hippocampal progenitor cells by a mechanism involving a shift in the balance of the
kynurenine pathway towards neurotoxic metabolites (Zunszain et al, 2012). This involved up-regulation of IDO, as well as two enzymes in the neurotoxic arm of the pathway - kynurenine 3-monooxygenase (KMO), and kynureninase (KYNU). The reduction in hippocampal neurogenesis could be blocked in part by inhibition of KMO, suggesting a causal role for this enzyme in the effect of inflammation reducing hippocampal neurogenesis. The possibility that antidepressant compounds might exert their effects in part by reversing the effects of inflammation on the kynurenine pathway and hippocampal neurogenesis will be addressed in Aim 4 of this thesis, and will be discussed further in Section 1.4.3.4.
Figure 3 The kynurenine pathway

A key element of this pathway appears to be the balance between neurotoxic metabolites (quinolinic acid) and neuroprotective metabolites (kynurenic acid).
1.2.8 Other biological domains affected in depression

Other biological domains have also been implicated in the pathogenesis of depression – including changes to neurotrophic factors, synaptic plasticity, oxidative and nitrosative stress pathways, and monoamine and glutamatergic signalling - but fall beyond the scope of this thesis. It is interesting that many of these domains interact (often bi-directionally) with glucocorticoid signalling, immune processes and neurogenesis, suggesting that biological perturbations in more than one of these systems may arise together, likely in the context of chronic stress in vulnerable individuals.

1.3 Synthesis of glucocorticoid and inflammatory theories of depression

1.3.1 Tension between glucocorticoid and cytokine theories of depression

As outlined above, several processes have been implicated in transducing the experience of chronic stress into the biological changes that manifest as depression. Two of the most important systems implicated in the connection between stress and depression are the HPA axis and the innate immune systems, as outlined above in Sections 1.2.2, 1.2.3 and 1.2.4. While the dysfunction of each of these systems in stress and depression has been extensively studied, an ever more sophisticated understanding of the myriad and multi-level interactions between these two systems (Bellavance and Rivest, 2014; Zunszain et al, 2011) has made investigation of either in isolation increasingly untenable. I have evaluated this interaction in a recent paper (Horowitz and Zunszain, 2015), which I briefly outline this issue below.

The innate immune system affects the HPA axis at many levels: pro-inflammatory cytokines induce the release of CRH and ACTH, and can induce
GR resistance by direct interaction with the GR via various mechanisms, including disruption of GR translocation, GR-DNA binding through protein-protein interactions and alterations in GR phosphorylation status (Pace and Miller, 2009). In turn, the HPA axis has profound effects upon the innate immune system, largely understood to be inhibitory, by preventing the transcriptional activity of one of the key inflammatory transcription factor nuclear factor light chain kappa beta (NF-κB) and suppressing production and secretion of pro-inflammatory cytokines (Silverman and Sternberg, 2012a).

Given the robust inhibitory actions of cortisol on the innate immune system, two theories have been proposed to account for the apparently paradoxical over-activity of both the innate immune system (as manifest in pro-inflammatory cytokine levels) and the HPA axis (as manifest in hypercortisolaemia) in depressed patients. The first highlights the role played by glucocorticoid resistance, and the second proposes hitherto under-appreciated pro-inflammatory properties of glucocorticoids. I will outline the evidence for both theories.

1.3.2 The potential role of glucocorticoid resistance

One theory suggests that the co-existence of hypercortisolaemia and increased levels of pro-inflammatory cytokines can be explained by the mediating presence of glucocorticoid resistance, lessening the potency of each molecule of cortisol, and therefore freeing the innate immune system from a degree of inhibitory control, normally exerted by the glucocorticoid system.

This theory has been in part derived from evidence in populations that are chronically stressed (with mechanistic relevance to depression) that homeostatic balance is tipped in favour of immune signallling, away from
glucocorticoid signalling (Miller, 2008) (Figure 4). For example, peripheral blood cells from the caregivers of brain cancer sufferers express reduced numbers of transcripts bearing response elements to the glucocorticoid receptor, compared with controls; they also show an increased number of transcripts containing NF-κB response elements, along with an increase in inflammatory molecules, such as CRP and IL-1RA (a molecule released by monocytes to neutralize the pro-inflammatory activities of IL-1) (Miller et al, 2008). This finding has been replicated in people with low early life social class and lonely people (Cole, 2008; Miller et al, 2009b); these groups also demonstrate a dose-dependent relationship between degree of loneliness and glucocorticoid resistance (Cole, 2008). Interestingly, these studies identified evidence of glucocorticoid resistance in the absence of increased levels of cortisol (Miller et al, 2008).

Further support for the relationship between chronic stress and glucocorticoid resistance has been derived from studies of humans and animals (Avitsur et al, 2001; Sheridan et al, 2000; Wirtz et al, 2003) in which chronic stressors have been shown to diminish the capacity of glucocorticoids to suppress endotoxin-stimulated cytokine production. For example, mice subjected to a social defeat stress paradigm demonstrate both glucocorticoid resistance and increased inflammation (Avitsur et al, 2009; Sheridan et al, 2000). Immune cells from the spleens of these mice display glucocorticoid resistance, through a lack of sensitivity to corticosterone inhibition following LPS simulation (Avitsur et al, 2009). Additionally, monocytes from these animals secrete higher levels of tumour necrosis factor-α (TNF-α) and IL-6 in response to LPS, linked with an increased likelihood of developing endotoxic shock (Avitsur et al, 2009). These studies suggest that an overactive immune system results from chronic stress inducing glucocorticoid resistance.
Figure 4 Shift in balance hypothesis

This hypothesis suggests that a balance between glucocorticoid signalling and inflammatory signalling is pushed towards inflammation, away from glucocorticoid activity by the experience of chronic stress. Glucocorticoid resistance is thought to be a key component of this ‘shift in balance’. Adapted with permission (Miller, 2008)
This link between stress and enhanced innate immunity, mediated by glucocorticoid resistance, has been extended to dynamic responses of the innate immune system in humans (Cohen et al, 2012). In comparison to non-stressed controls, stressed individuals demonstrate increased glucocorticoid resistance, an increased likelihood to develop a cold after rhinovirus seeding, and increased levels of pro-inflammatory cytokines (IL-1β, TNF-α, and IL-6) in nasal secretions in response to the virus. A strong correlation between glucocorticoid resistance and levels of pro-inflammatory cytokines was detected. This work provides evidence that increased inflammatory responses in glucocorticoid resistant individuals, secondary to stress exposure, may underlie the increased susceptibility to illness of stressed individuals, and may also be pertinent to depressed patients (Cohen et al, 2012).

1.3.2.1 Evidence for a shift in balance between glucocorticoid and immune signalling in depressed patients

The glucocorticoid resistance hypothesis predicts that in depressed individuals, increased levels of pro-inflammatory cytokines and high levels of cortisol will be present only in the context of glucocorticoid resistance. On investigating the literature I found that a few studies had examined this possibility but no systematic examination of this literature had been conducted, and consequently, as outlined below, Aim 1 of this thesis was to undertake a systematic literature review and meta-analysis of clinical studies that had examined these three biological characteristics (cortisol levels, innate immune function and glucocorticoid resistance) in depressed patients.
1.3.3 The potential role of pro-inflammatory glucocorticoids

A competing theory to the glucocorticoid resistance theory, that also may explain the co-existence of increased levels of pro-inflammatory cytokines and cortisol in depressed patients, focuses on emerging evidence that glucocorticoids are not uniformly anti-inflammatory, as has been previously supposed, but can be both pro- and anti-inflammatory depending on circumstance (Frank et al, 2013; Sorrells et al, 2009). In particular, this hypothesis holds that glucocorticoids can be pro-inflammatory, under specific conditions (Frank et al, 2013). The temporal relationship between glucocorticoid actions and inflammatory insult is thought to be critical: glucocorticoid actions which coincide with inflammatory insult or follow it are thought to be anti-inflammatory, while those which precede it are likely to be pro-inflammatory (Figure 5).
Figure 5 A model for pro-inflammatory effects of glucocorticoids

At baseline glucocorticoid levels are low; upon exposure to a threat they rise sharply inducing anti-inflammatory effects; afterwards as glucocorticoid levels drop immune cells, like microglia, are in a primed state where they exhibit potentiated responses to inflammatory stimuli. Adapted with permission (Frank et al, 2013)
This is consistent with an evolutionary narrative that emphasises the utility of immunosuppressant effects during the period of an acute threat, accompanied by increased levels of glucocorticoids, given the energetic demands of a fight or flight response. This is followed by a post-threat period in which recovery from damage sustained is likely to be prioritised, accompanied by a normalisation of glucocorticoid levels and an enhancement of immune function (Frank et al, 2013). In this view, an incident of stress and the attendant rise in glucocorticoid hormones is seen as a ‘neuroendocrine warning signal’ priming central and peripheral inflammatory cells for potential danger (Frank et al, 2013).

Microglial cells have been thought to be key cells in the CNS in the temporally bimodal response to this ‘neuroendocrine warning signal’ (Frank et al, 2013). Microglia respond strongly to inflammatory stimuli by producing pro-inflammatory cytokines and other inflammatory mediators, responses that are inhibited by glucocorticoid administration or stress applied following inflammatory stimulation (Drew and Chavis, 2000; Tanaka et al, 1997). Chronic stress has been shown to increase the density of microglia in the hippocampus, while priming these cells into their active, more pro-inflammatory state (Nair and Bonneau, 2006; Tynan et al, 2010). Glucocorticoids have a similar effect (Nair and Bonneau, 2006; Tynan et al, 2010). Interestingly, minocycline, a microglial inhibitor, has been shown to block the stress-induced potentiation of hypothalamic IL-1β (Blandino Jr. et al, 2006), suggesting microglial actions are necessary for these pro-inflammatory effects.
1.3.3.1 Evidence from *in vivo* animal and human studies

Evidence for a temporally bimodal action of glucocorticoids has been found in animals, as well as preliminary evidence in human studies. Exposure to stress or glucocorticoids after an inflammatory insult in animals suppresses the inflammatory response, consistent with conventional notions of the anti-inflammatory effects of glucocorticoids (Goujon *et al*., 1995). However, when rodents are exposed to acute, sub-acute or chronic stress before inflammatory stimulus, a potentiated inflammatory response is observed, as measured by levels of pro-inflammatory cytokines (Argu *et al*., 2006; Espinosa-Oliva *et al*., 2011; Frank *et al*., 2010; Johnson *et al*., 2002; Munhoz *et al*., 2006, 2010). For example, exposure to inescapable tail-shock (Johnson *et al*., 2002) or to daily sessions of unpredictable chronic stress (Munhoz *et al*., 2006) potentiates the increase in hippocampus and frontal cortex expression of pro-inflammatory mediators, like IL-1β, induced by a peripheral injection of lipopolysaccharide (LPS) given 24 hours after the stressor regimen. This phenomenon is glucocorticoid- and glucocorticoid receptor-dependent as it can be abolished by adrenalectomy or administration of RU486 (Argu *et al*., 2006; Espinosa-Oliva *et al*., 2011; Munhoz *et al*., 2006; Nair and Bonneau, 2006). Interestingly, glucocorticoid treatment administered too short a period before an inflammatory insult produces anti-inflammatory effects, emphasising the importance of timing, consistent with the proposed existence of a window of pro-inflammatory effects (Frank *et al*., 2010; Smyth *et al*., 2004).

There is also preliminary evidence that this phenomenon occurs in humans. An infusion of hydrocortisone (synthetic cortisol) given just before or concomitant with LPS administration in healthy subjects completely abrogated a rise in TNF-
α, whereas hydrocortisone given 12 or 144 hours before LPS caused large increases in levels of TNF-α and IL-6, compared with LPS alone (Barber et al, 1993). A more recent study found that intermediate doses of hydrocortisone (corresponding to stress levels) infused 24 hours before LPS administration potentiated the rise of IL-6 levels compared with LPS alone, while high doses failed to (Yeager et al, 2011). These studies provide evidence that the pro-inflammatory effects of glucocorticoid pre-treatment are not restricted to animals but are also present in humans and influenced by similar temporal factors.

1.3.3.2 Evidence from in vitro and ex vivo studies

Ex vivo and in vitro studies have also provided evidence that glucocorticoids can be pro-inflammatory. For example, glucocorticoid pre-treatment in rodent macrophages enhances the subsequent response to inflammatory stimulation as measured by phagocytic activity, cytokine release and NF-κB activation (Smyth et al, 2004; Zhang and Daynes, 2007). Ex vivo experiments probing inflammatory responses have shown that hippocampal microglia isolated from rodents subjected to stress paradigms or glucocorticoid administration demonstrate signs of increased activation and potentiated pro-inflammatory cytokine responses to inflammatory stimuli. Inescapable tail-shock resulted in a potentiated pro-inflammatory cytokine response in isolated hippocampal microglia, as measured by increased expression of IL-1β, IL-6 and NF-κB1α (Frank et al, 2011). Prior corticosterone administration in vivo potentiated the response in microglial IL-1β and TNF-α to LPS exposure ex vivo (Frank et al, 2010).
1.3.3.3 Proposed mechanisms for the pro-inflammatory effects of glucocorticoids

Several components of immunological pathways are up-regulated following acute or chronic stress, or glucocorticoid treatment, from cell surface receptors to intracellular signalling molecules, which may underlie the pro-inflammatory effects of glucocorticoids. Glucocorticoids have been shown to induce or enhance the expression of several cytokine receptors in peripheral immune cells including those for TNF-α, IL-1, IL-2, IL-6, IFN-γ and GM-CSF (Wiegers and Reul, 1998) in a GR-dependent process that is abolished by the GR antagonist RU486 (Re et al., 1994). A wide variety of cell types are responsive to glucocorticoid-induced stimulation of cytokine-receptor expression including: epithelial cells, hepatic cell lines, lymphocytes, monocytes, and neutrophils (Yeager et al, 2004a). In the brains of rodents subjected to acute or chronic stress critical immune signalling molecules have been shown to be up-regulated, including NF-κB, MAPK, ERK1/2, SAP/JNK and AKT (Frank et al, 2010; Munhoz et al, 2006; Zhang and Daynes, 2007), enzymes responsible for activating inflammatory responses – for example, TNF-α convertase, COX-1, COX-2 (Horowitz et al, 2013) and the NLRP3 inflammasome (Busillo et al, 2011). In addition to pro-inflammatory pathways being enhanced by glucocorticoid treatment, anti-inflammatory pathways, like PI3K/Akt, are down-regulated (Zhang and Daynes, 2007).

As mentioned above, in response to glucocorticoids, microglia develop a 'primed' immunophenotype (Walkera et al, 2013). Several molecules have been implicated in this priming, including toll-like receptors (TLRs). TLRs are pattern recognition receptors, which respond to pathogen associated molecular
patterns (PAMPs), like LPS, and transduce signals leading to activation of NK-
 kB and inflammatory pathways (Kawai and Akira, 2010). Pertinently, TLR2 and
TLR4, expressed on microglia (Aravalli et al, 2007) are upregulated by
glucocorticoids (Frank et al, 2013), and, in particular, TLR2 is upregulated in the
CNS following such exposure (Frank et al, 2010). TLR activation by PAMPs
leads to the formation of the inflammasome, a multi-protein assemblage that is
responsible for activation of inflammatory processes, including promoting the
maturation of pro-inflammatory cytokines. In particular, the inflammasome
nucleotide-binding domain, leucine-rich repeat, pyrin containing protein 3
(NLRP3) is known to require a priming stimulus modulated by glucocorticoids
(Hornung and Latz, 2010). Indeed, glucocorticoids have been shown to
increase NLRP3 at the level of mRNA and protein, in association with an
increased inflammatory response (Busillo et al, 2011) suggesting this may be a
key pro-inflammatory mechanism for glucocorticoids.

Following this debate with respect to the relationship of the innate immune
system and the glucocorticoid system Aim 1 of this thesis is to undertake a
systematic literature review and meta-analysis of clinical studies that have
looked at both these systems in the same depressed patients to determine
which of the above hypotheses is best supported by the literature. Aim 2 is to
model some of the interactions between these two systems in an vitro model of
human hippocampal progenitor cells to generate insights into how these
systems might interact in depression and to further test which of the above two
hypotheses is supported.
1.4 The biological basis of antidepressant action: effects on immune processes, neurogenesis and the kynurenine pathway

So far I have discussed the biological aspects of the pathogenesis of depression; I now turn to the biology underlying antidepressant action. As with research into the pathogenesis of depression, the study of antidepressant action has focused on the biological changes detected in successful antidepressant treatment in animal models and clinical populations, and attempted to explore mechanisms underlying these changes by investigating molecular and cellular effects in vitro. Clarifying the identity of these pathways would allow for rational drug design that may yield more targeted agents, with greater efficacy and less off target side effects.

To this end, researchers have investigated the effect of compounds with proven antidepressant properties on the biological domains thought to be disrupted in depression. An organising principle has been the idea that antidepressants, despite being from differing chemical classes, would converge in correcting perturbations in biological pathways that were necessary to antidepressant effects. For example, finding that compounds with antidepressant properties as widely differing in chemical structure and known receptor specificity as polyunsaturated fatty acids, melatonergic agonists, SSRIs, serotonin–norepinephrine reuptake inhibitors (SNRIs) and monoamine oxidase inhibitor (MAO-I)s all converged on normalising perturbed immune processes or neurogenesis or the kynurenine pathway would lend credence to the notion that such pathways were important common pathways in antidepressant action.

I have taken this approach in this thesis: examining the effects of a number of antidepressant compounds on selected processes known to be disturbed in
depression and alleviated by antidepressants in clinical or animal studies in order to discern which, if any, are affected similarly by all medication classes. Alternatively, such an approach might reveal that different antidepressant compounds have quite distinct effects upon each of these processes, suggesting that these medications act in distinct ways, or that there are final common pathways as yet unknown. One particular limitation to previous studies investigating the effects of antidepressant medication, addressed by this thesis, has been the use of animal models, or peripheral samples and imaging approaches, in clinical populations, to study changes in neural tissues, without direct examination of the effects of antidepressants on human brain cells, the intended target of these medications. The human hippocampal progenitor cells used in the present study allow the effects of antidepressant compounds on human brain cells to be examined directly.

I have focused on three different biological domains thought to be affected by antidepressant compounds: effects on the innate immune system, effects on neurogenesis and effects on the kynurenine pathway. Before focusing on these three processes, given the dominance of a monoamine-based theory of antidepressant action for many decades, I will briefly outline the utility of this theory, and its limitations. In the following section I briefly explain what is known about the effect of antidepressant compounds on these processes, as well as inter-relationships between these domains.

1.4.1 Monoamines and antidepressant action

The discovery that medications with antidepressant effects increased monoamine levels in the neuronal synapse led to the formulation of the monoamine hypothesis of antidepressant action (and depression pathogenesis) (Schildkraut, 1965). This hypothesis proposes that the underlying biological
basis for depression is a deficiency of central noradrenergic and/or serotonergic systems and that targeting this neuronal lesion with an antidepressant would tend to restore normal function in depressed patients (Hirschfeld, 2000).

However, several aspects of antidepressant effects and depression have indicated that the monoamine hypothesis does not offer an exhaustive account and that aspects beyond monoamine signalling are involved in depression and antidepressant effect. Firstly, monoamine depletion induces lowered mood only in those patients with previous experience or family history of depression, but not control subjects, suggesting that there are factors beyond levels of monoamines that affect mood (Ruhe et al, 2007). Secondly, mirtazapine and tianeptine are both effective antidepressants, which antagonise monoamine receptors, and increase serotonin uptake (reducing its activity), respectively (Hindmarch, 2002). Polyunsaturated fatty acids and anti-inflammatory drugs also demonstrate antidepressant effects, without direct action on monoamine receptors (Lin and Su, 2007). Ketamine, which acts through antagonism of the NMDA receptor, appears to be a highly effective antidepressant with quicker onset of action than monoaminergic antidepressants and even to be effective in patients unresponsive to SSRIs, implicating pathways other than monoamine signalling in antidepressant action (Skolnick et al, 2009).

Additionally, although recent studies have suggested that antidepressant show positive effects on mood and cognition earlier than previously thought, the lag time of days to weeks before full antidepressant effects are realised from monoaminergic medications, while monoamine levels are modified in hours, suggests that there are effects downstream of monoamine signalling that contribute to the antidepressant effect. Furthermore, monoaminergic agents have limited efficacy in treating depression, as evident from the STAR-D study.
(Warden et al, 2007). As a consequence of the limitations of the monoamine hypothesis, attention has turned to other processes that are affected by monoaminergic antidepressants, as well as the modes of action of non-monoaminergic antidepressants, in order to understand antidepressant effects.

1.4.2 Immunomodulatory properties of antidepressant compounds

One area that has gained significant attention is the possibility that immunomodulation presents an effective antidepressant strategy. As outlined above (Section 1.2.4) there is much evidence implicating increased activity of the innate immune system in depression. Consistent with this, there is accumulating evidence that antidepressant compounds may act through immunomodulatory mechanisms. In particular, evidence suggests that two groups of compounds with demonstrated antidepressant properties, monoaminergic antidepressants and omega-3 polyunsaturated fatty acids (ω-3 PUFAs), may share modulation of the inflammatory response as a common pathway of action.

1.4.2.1 PUFAs are effective in depression

PUFAs have been recently identified to possess antidepressant properties. Long-chain PUFAs make up one fifth of the brain’s dry weight, and are critical for healthy brain development and function because of their role in membrane structure and cytokine regulation. Although there are numerous PUFAs, the omega-3 fatty acids EPA (eicosapentaenoic acid, 20:5ω3) and DHA (docosahexaenoic acid, 22:6ω3) have been found to be particularly importance in brain function, along with levels of ALA (α-linolenic acid, 18:3ω3), the precursor molecule to these two compounds (Beltz et al, 2007). These compounds must be obtained largely from dietary sources because humans cannot synthesise them de novo.
There is evidence that abnormalities in fatty acid metabolism may play a part in a range of psychiatric disorders (Beltz et al., 2007). For example, several studies support a connection between dietary intake of omega-3 fatty acids and the prevalence of depressive illnesses (Beltz et al., 2007). Furthermore, recent evidence suggests that PUFAs, and, in particular EPA and DHA, can be effective antidepressant agents. Although there has been some debate about the efficacy of PUFAs as antidepressant agents, a recent, authoritative, meta-analysis demonstrated that PUFA preparations with EPA accounting for greater than 60% of their composition were effective against depression, while those preparations with less than 60% EPA were ineffective (Sublette and Ellis, 2011). In particular, EPA may be more effective in patients with high levels of baseline inflammation. A recent trial found that patients with higher levels of baseline inflammation (high IL-1RA, high CRP or low adiponectin) showed a stronger antidepressant response to PUFA treatment high in EPA compared to placebo (Rapaport et al., 2015). Indeed, patients given PUFA treatment high in DHA content actually did worse than those patients on placebo.

1.4.2.2 Antidepressant compounds are immunomodulatory in peripheral clinical samples

There is now evidence that both PUFAs and monoaminergic antidepressants have immunomodulatory properties. A number of studies have investigated the ability of these compounds to affect levels of inflammatory cytokines in human blood samples. These studies have generated findings broadly indicating anti-inflammatory properties for both monoaminergic antidepressants (Bielecka et al., 2010; Hannestad et al., 2011; Kenis and Maes, 2002; Obuchowicz et al., 2006; Tynan and Weidenhofer, 2012; Xia et al., 1996), and ω-3 PUFAs (Lu et al., 2010; Novak et al., 2003; Rangel-Huerta et al., 2012), though pro-inflammatory effects,
or an absence of effect, have been reported as well (Diamond et al, 2006; Jazayeri et al, 2010; Kubera et al, 2005; Maes et al, 2007; Tynan and Weidenhofer, 2012).

A recent meta-analysis has shown that antidepressant treatment reduces serum levels of IL-1β, while an effect on IL-6 levels was also found using a less stringent model (Hannestad et al, 2011). This finding was supported by a meta-analysis showed a significant decrease in IL-6 and CRP after antidepressant treatment (Hiles et al, 2012). However, in addition to wide methodological variations amongst studies, complicating the drawing of overall conclusions, a major limitation in this field has been a lack of studies in human brain cells; an issue to be addressed in this thesis.

1.4.2.3 Antidepressants can be immunomodulatory in animals

These studies in humans are also supported by findings in the animal literature - administration of the tricyclic noradrenaline reuptake inhibitor antidepressant desipramine decreased levels of TNF-α in rats (Reynolds et al, 2005). Bupropion, a noradrenaline dopamine reuptake inhibitor, reduced TNF-α, IFN-γ and IL-1β, upon LPS induced inflammation in a mouse model (Brustolim et al, 2006).

Further studies have looked at the ability of antidepressants to inhibit the expression of inflammatory mediators in rodent glial cells. Several types of antidepressants have been studied using in vitro cultures of astrocytes and microglial cells derived from rodents. Moclobemide, a reversible selective inhibitor of monoamine oxidase-A, diminished LPS-stimulated IL-1β and TNF-α mRNA and protein expression in rat glial cells and inhibited the LPS-induced translocation of NF-κB to the nucleus (Bielecka et al, 2010). Pre-treatment with
the selective serotonin reuptake inhibitor fluvoxamine, the relatively selective noradrenaline reuptake inhibitor reboxetine, or the non-selective monoaminergic reuptake inhibitor imipramine, significantly inhibited IL-6 induced by treatment of murine microglia with IFN-γ (Hashioka et al, 2007). The exposure of mixed glial culture to amitriptyline and nortriptyline led to a decrease in both IL-1β and TNF-α release (Obuchowicz et al, 2006). Interestingly, although amitriptyline reduced the secretion of both cytokines, the drug did not affect their mRNA levels.

In another microglial cell model, fluoxetine significantly inhibited LPS-induced production of TNF-α and IL-6, along with a reduction in their mRNA levels (Liu et al, 2011). Several SSRIs have been shown to decrease LPS-induced production of NO and TNF-α in a murine microglial line (Tynan and Weidenhofer, 2012). Desipramine and fluoxetine inhibited the release of TNF-α from LPS-stimulated human monocytes (Roumestan et al, 2007). However, the effect of antidepressants on cytokine production in human brain cells has not yet been studied.

1.4.2.4 Anti-inflammatory drugs demonstrate antidepressant effects

Preliminary findings that treatment of depressed patients with anti-inflammatory drugs can reduce depressive symptoms also supports the notion that immunomodulation may be a successful antidepressant strategy. Anti-inflammatory drugs such as aspirin, a COX-1, COX-2 and prostaglandin inhibitor (Mendlewicz et al, 2006), celecoxib, a COX-2 inhibitor (Müller et al, 2006) and etanercept, a TNF-α blocker (Tyring et al, 2006), have been shown to have antidepressant effects or improve antidepressant response when combined with monoaminergic medications. As mentioned above, a recent systematic review and meta-analysis has found evidence that anti-inflammatory medication can exert antidepressant effects (Köhler et al, 2014). An SMD of -
0.54 was found on meta-analysis of efficacy in patients with depression, with sub-analyses indicating that the COX-2 inhibitor, celecoxib, increasing the chance of remission by an odds ratio (OR) of 7.89. The risk of bias and high heterogeneity limit the conclusions that can be drawn from this study, but it confirms that a body of evidence exists supporting immune modulation as a potential antidepressant strategy.

1.4.2.5 Mechanism of immunomodulation

There is some understanding of how monoaminergic antidepressants and PUFAs may exert their immunomodulatory effects, with regulation of NF-κB emerging as a common mechanism of action. NF-κB is one of the primary transcription factors involved in the synthesis and release of pro-inflammatory cytokines (Tak and Firestein, 2001). It has been identified as a key mediator of the effects of stress on depressive behaviour (Koo et al, 2010) and found to be upregulated in depressed patients (Pace et al, 2006). Monoaminergic antidepressants have been shown to reduce NF-κB activity in rat glial cultures (Bielecka et al, 2010) and brains (Zhu et al, 2008). Moclobemide inhibited the LPS-induced translocation of NF-κB to the nucleus in rat glial cells, while also reducing pro-inflammatory cytokines (Bielecka et al, 2010). Fluoxetine also inhibited, phosphorylation and nuclear translocation of the p65 subunit of NF-κB, as well as IκB-α (an inhibitor of NF-κB, that keeps it sequestered in the cytoplasm, preventing nuclear localisation), and degradation and phosphorylation of p38 MAPK in the LPS-stimulated microglia (Liu et al, 2011). Mirtazapine reduced NF-κB activation in rat brains induced by physical injury, while also reducing levels of pro-inflammatory cytokines (Zhu et al, 2008).

Similarly, ω-3 PUFAs have been thought to decrease production of inflammatory cytokines by down regulating the expression of inflammatory
cytokine genes via an influence on NF-κB, and other receptors such as peroxisome proliferator-activated receptor-γ (Calder, 2013). ω-3 PUFAs reduce NF-κB activity in murine macrophages (Novak et al, 2003) and microglia (Moon et al, 2007), as well as in human aortic endothelial cells (Wang et al, 2011). In addition to effects on inflammatory cytokines, there have also been described a number of different means by which ω-3 PUFAs might affect inflammation, including reducing leucocyte chemotaxis, decreasing the production of eicosanoids, altering endocannabinoids, increasing production of resolvins and protectins and decreasing T cell reactivity (Calder, 2013). These effects can be produced by PUFAs either by increased membrane content of EPA and DHA, down-regulation of relevant receptors and adhesions molecules, or disruption of membrane rafts (Calder, 2013).

Aim 3 of this thesis will be to determine whether monoaminergic antidepressants and PUFAs can decrease inflammation in human hippocampal progenitor cells and whether this process involves an effect on NF-κB.

1.4.3 Antidepressants and AHN
Recent work has suggested that antidepressants increase adult hippocampal neurogenesis and this may be one means by which they exert their antidepressant effects; some studies have suggested that enhancing neurogenesis may be a necessary step for successful antidepressant action in animal models.

1.4.3.1 Antidepressants increase AHN in animals
The role of neurogenesis in antidepressant action was first suggested by experiments in mice where SSRIs, SNRIs, MAO-Is and even electroconvulsive seizures, the most effective antidepressant treatment, all increase progenitor
proliferation and neurogenesis in the hippocampus of non-stressed animals (Malberg et al, 2000). These results have been extended to non-human primates as well (Perera et al, 2007).

Furthermore, the decrease in cell proliferation and neuronal differentiation, which is induced by chronic stress or by elevated glucocorticoid levels (as discussed in Section 1.2.5.5), can be reversed by chronic antidepressant treatment (David et al, 2009; Liu et al, 2008) suggesting that antidepressants counteract the detrimental effects of stress and glucocorticoids on hippocampal neurogenesis, and thereby contribute to changes in behaviour. However, negative findings have also been reported, which suggest that the effects of antidepressants on several behavioural paradigms are already present after 14 days of fluoxetine treatment without concomitant changes in cell proliferation or neuronal differentiation (Huang et al, 2008).

The limited number of post mortem studies in depressed patients have provided evidence that humans show changes consistent with findings in animal models of depression. One study found decreased levels of neurogenesis in untreated depressed patients compared with control subjects, with levels restored to normal in patients treated with SSRIs and greatly exceeding control levels in patients treated with MAO-Is (Boldrini et al, 2009). It is possible that antidepressant effects on AHN are present in humans, and may explain antidepressant effects. However, another small study only observed a trend towards decreased hippocampal cell proliferation in depressed patients and no effect of antidepressant treatment (Reif et al, 2006). Further studies involving larger number of samples, though difficult, would be instructive.
1.4.3.2 AHN may be a necessary component of antidepressant action

Recent studies have suggested that AHN may be a necessary component of antidepressant action. A pivotal study demonstrated that the antidepressant fluoxetine reversed the depression-like behaviour of animals induced by chronic mild stress, but failed to do so in the same paradigm if hippocampal neurogenesis was blocked (by X ray irradiation of the hippocampus) (Santarelli et al, 2003). This finding was also confirmed in a mouse model (Surget et al, 2008) and a non-human primate model (Perera et al, 2011) suggesting that AHN is necessary for the action of antidepressants. However, other studies have suggested that there may be neurogenesis-dependent and -independent mechanisms of antidepressant action, as some behavioural effects of antidepressants were still produced even when AHN was inhibited (David et al, 2009). The debate as to whether AHN is a necessary, or sufficient, condition for antidepressant action is ongoing (Hill et al, 2015) but significant evidence suggests that it is important in antidepressant action.

1.4.3.3 PUFAs can increase AHN

There is also evidence that ω-3 PUFAs, along with monoaminergic antidepressants can increase AHN. For example, ω-3 PUFA supplementation increases neurogenesis in lobsters (Beltz et al, 2007). DHA also reverses the anti-neurogenic effect of LPS-activated microglia in mouse neural progenitor cells (Antonietta Ajmone-Cat et al, 2012). DHA supplementation has also been shown to partially alleviate age-related reductions in levels of neurogenesis in rats (Dyall et al, 2010), and DHA-deficient embryonic rat brains show decreased neurogenesis (Coti Bertrand et al, 2006), while transgenic mice with high levels of endogenous DHA show increased neurogenesis (He et al, 2009).
Although DHA is known to be linked to neurogenesis, the mechanism behind its role has yet to be fully established (Crupi et al, 2013). It has been suggested that ω-3 PUFAs might exert a modulatory effect on AHN through multiple mechanisms. For example, ω-3 PUFAs can regulated neural stem cell proliferation and differentiation through increased expression of basic helix-loop-helix (bHLH) transcription factors promoting cell cycle exit (Wada et al, 2006). ω-3 PUFAs have also been shown to have a neurotrophic effect in rat neuroblastoma cells (Shrivastava et al, 2005), and DHA increases neurite outgrowth of immature neurons in embryonic hippocampal cells (Cao et al, 2009). Consistent with these observations, a ω-3 PUFA enriched diet promotes hippocampal plasticity and an increase in BDNF expression in mice (Venna et al, 2009).

Additionally, DHA can increase the levels of certain phospholipids in cell membranes such as phosphatidylserine and phosphatidylethanolamine, and DHA is itself incorporated into the cell phospholipids (Cao et al, 2009). As the production of phospholipids and other membrane components is a pre-requisite for neurite growth this may represent a pathway by which PUFAs are neurotrophic. PUFAs can also modulate the binding of serotonin to neuronal membranes by affecting the structure of cell membranes, which may in turn increase neurogenesis (Crupi et al, 2013). PUFAs may also affect neurogenesis through the intermediate step of affecting secretion of pro-inflammatory cytokines (as described in Section 1.4.2 and 1.2.6). Finally, PUFA supplementation can increase cholesterol in cell membranes, rendering it more resilient to membrane oxidation (Crupi et al, 2013).
1.4.3.4 Kynurenine metabolites in the action of antidepressant agents

As discussed previously there are several points of interaction between the different biological domains perturbed in depression, which may, in turn, be relevant targets for antidepressant action. In particular, increased levels of inflammation have multiple effects on the brain, including decreasing hippocampal neurogenesis. As also discussed above, there is evidence that one mechanism underlying this process is perturbation of the kynurenine pathway. Increased levels of inflammation induce metabolic enzymes in this pathway, leading to an increase in neurotoxic metabolites, as compared to neuroprotective metabolites. As mentioned above, recent work from our lab has shown that a reduction in neurogenesis in hippocampal progenitor cells is associated with an increase in key enzymes that increase neurotoxic products of this pathway (Zunszain et al, 2012). The ability to partially reverse the effects of inflammation on neurogenesis with inhibitors of these enzymes suggests that this may be a potential antidepressant strategy (Zunszain et al, 2012).

Accordingly, it is possible that antidepressant compounds may reverse the effects of inflammation in reducing hippocampal neurogenesis in part by reversing changes to the kynurenine pathway. As outlined above, there is considerable evidence that monoaminergic antidepressants can increase neurogenesis, and can reverse the reduction in neurogenesis caused by stress or treatment with glucocorticoids. There is also some evidence that ω-3 PUFAs, especially DHA, can exert the same protective effects on neurogenesis. Given that IL-1β is a critical mediator of the anti-neurogenic effects of stress (Koo and Duman, 2008), and that IL-1β reduces neurogenesis through a mechanism in part reliant on perturbations to the kynurenine pathway, antidepressants may reverse the effect of IL-1β on neurogenesis by affecting kynurenine metabolites.
There has been limited investigation of whether antidepressant compounds can affect kynurenine metabolites. One study found that a reduced neuroprotective ratio (kynurenic acid/kynurenine) observed in depressed patients, increased significantly following successful treatment with monoaminergic antidepressants (Myint et al, 2007). Interestingly, this happened in those subjects with a first episode, but not those patients with multiple episodes. Another study detected no effect of monoaminergic antidepressants on levels of kynurenine metabolites secreted by blood cultures, when stimulated by LPS in vitro (Krause et al, 2012). A recent review of studies examining the effects of antidepressants on kynurenine metabolites presents a mixed picture, thought with overall support for the ability of antidepressants to modulate the kynurenine pathway away from neurotoxic metabolites towards neuroprotective ones (Réus et al, n.d.). A study investigating the effect of escitalopram on kynurenine metabolites in depressed subjects found that treatment reduces the amount of neurotoxic metabolites, as well as ratios of neurotoxic to neuroprotective metabolites, compared to their baseline values (which were elevated compared with healthy controls) (Halaris et al, 2015).

Overall, there is some evidence derived from investigation of peripheral samples in depressed subjects that antidepressant can affect kynurenine metabolites, suggesting the possibility that this may be one mechanism by which they exert their effects. However, no study has examined whether monoaminergic antidepressants and PUFAs can affect the influence of inflammation on neurogenesis through an effect on the kynurenine pathway in human neural cells, which will form Aim 4 of this thesis.
1.5 The role of the GR in neurogenesis in stress and antidepressant treatment

Adult hippocampal neurogenesis, thought to be a key process in the pathogenesis of depression and recovery from depression, is decreased by glucocorticoids and increased by antidepressants. Work from our lab and others suggest that the effects of both glucocorticoids and antidepressants on hippocampal neurogenesis are transduced by activation of the glucocorticoid receptor. Blocking the GR with antagonists can abolish the effects of both glucocorticoids and antidepressants on hippocampal neurogenesis and, conversely, enhancement of its downstream activity can potentiate their effects.

1.5.1 The glucocorticoid receptor

Activation of the glucocorticoid receptor (GR) is the key process by which glucocorticoids affect almost every cell in the body. The GR, as described briefly in Section 1.2.3, is a transcription factor, normally resident in the cytoplasm, which, upon activation, by binding of glucocorticoids, translates to the nucleus. In the nucleus, it binds to DNA and induces transactivation and transrepression of complex patterns of genes. The GR binds to highly conserved glucocorticoid response elements (GREs) on DNA to activate gene transcription (known as transactivation) and also interrupts the binding of other transcription factors (for example, NF-κB or AP1) to DNA, generally repressing gene transcription (known as transrepression). The GR can also repress gene transcription by binding to negative GREs (for example, in glucocorticoid-mediated negative feedback inhibition).

Rather than exhibit a single set pattern of transactivation and transrepression upon activation the GR can, under different conditions, bind to the promaters of
some GR target genes but not to others, exhibit different transactivation potential, different subcellular localisation of the GR, and differences in protein stability (Blind and Garabedian, 2008; Chen et al, 2008; Davies et al, 2008). These differential patterns of transactivation and transrepression have been attributed to several factors, but a key element appears to be differential phosphorylation of serine residues in the GR (Ismaili and Garabedian, 2004; Rogatsky et al, 1998). Consistent with this, distinct patterns of GR phosphorylation have been associated with distinct patterns of gene transcription (Galliher-Beckley and Cidlowski, 2009).

1.5.2 The GR and neurogenesis

Our lab has shown that activation of the GR and the consequent patterns of gene transcription affect hippocampal neurogenesis. Of great interest, GR activation and its transcriptional sequelae appear to be involved in both the decrease in neurogenesis induced by glucocorticoid treatment and the increase in neurogenesis induced by antidepressant treatment (Anacker et al, 2011b). This finding is of particular importance because it indicates a convergence in two biological domains highly implicated in the pathogenesis and recovery from depression – the HPA axis and hippocampal neurogenesis.

1.5.3 Glucocorticoids, neurogenesis and the GR

The relationship between stress and glucocorticoids, and reduced neurogenesis was outlined in Section 1.2.5.5. Activation of the GR by glucocorticoids has been thought important in this effect of reduced neurogenesis. It has been shown that the GR agonist, dexamethasone, decreases adult hippocampal neurogenesis in rats – both in vivo and in vitro (Hwang et al, 2008; Wagner et al, 2009; Xi et al, 2011). The importance of the role of glucocorticoids in modulating neurogenesis is further supported by the observation that
adrenalectomy leads to an increase in hippocampal progenitor cell proliferation due to lower levels of circulating glucocorticoids and lower GR activation (Fischer et al, 2002).

Studies have also emphasised the role of the GR in the effect of glucocorticoids on neurogenesis. For example, brief treatment with the GR antagonist, RU486, abrogates the reduction in neurogenesis upon exposure to stress or glucocorticoids in the adult hippocampus of rats (Mayer et al, 2006; Oomen et al, 2011). Our lab has further extended this finding by demonstrating that the effect of dexamethasone treatment in reducing neurogenesis in human hippocampal progenitor cells can be completely abolished by RU486 treatment (Anacker et al, 2011a). RU486 co-treatment along with dexamethasone abolished the effect of dexamethasone treatment on cell proliferation as well as its effect on the development of immature neurons and mature neurons, implicating the GR in each of these processes.

1.5.4 Antidepressants, the GR and neurogenesis

As discussed above antidepressants from different classes increase the neurogenesis in animal models of chronic stress and depression, a finding consistent with human post-mortem studies of depressed individuals (Section 1.4.4). The means by which antidepressants might increase hippocampal neurogenesis is an area of intense study. Activation of the glucocorticoid receptor is one proposed mechanism.

There are now several studies demonstrating an interaction between antidepressants and the glucocorticoid receptor. Antidepressants can affect levels of GR expression and GR binding affinity (Okugawa et al, 1999; Peiffer et al, 1991; Pepin et al, 1989), and also induce nuclear translocation of the GR, in
the absence of glucocorticoids (Funato et al., 2006; Herr et al., 2003; Pariante et al., 1997, 2003). Other studies have shown that they can also inhibit GR-mediated gene transcription (Augustyn et al., 2005; Budziszewska et al., 2000). Our lab has shown that antidepressants both increase and decrease GR-mediated gene transcription dependent on the cell type, the function of PGP, and the incubation time (Carvalho et al., 2008; Pariante and Miller, 2001; Pariante et al., 1997, 2003).

Recently, our lab demonstrated that glucocorticoid receptor activation appears to be a key step in transducing the effects of antidepressants on hippocampal neurogenesis in human cells. Treatment with the SSRI, sertraline, alone increased hippocampal neurogenesis, and abrogated the reduction in neurogenesis caused by treatment with dexamethasone, consistent with findings in animals. The effect of sertraline on neurogenesis was dependent on activation of the GR, as the effect could be blocked by co-treatment with RU486 (Anacker et al., 2011b).

Furthermore, treatment with sertraline alone, dexamethasone alone or co-treatment with dexamethasone and sertraline had distinct effects on the pattern of serine phosphorylation in the GR and GR-dependent gene transcription. Distinct temporal patterns of GR phosphorylation at serine residues 203 and 211 (implicated as important in GR nuclear translocation and GR-dependent gene transcription) are induced by each of these three treatments (Anacker et al., 2011b).

1.5.5 Outstanding issues

Adult hippocampal neurogenesis appears to be critically affected in depression and upon successful treatment with antidepressants, with glucocorticoids and
antidepressants having opposite effects on neurogenesis, as demonstrated in animal models and now in human hippocampal progenitor cells. These effects on neurogenesis have been shown to be dependent on activation of the GR in human hippocampal progenitor cells. Interestingly, the glucocorticoid dexamethasone and the antidepressant sertraline have differential effects on the pattern of activation of the GR, as manifest in distinct patterns of serine phosphorylation and GR-dependent gene expression. Given that patterns of serine phosphorylation of the GR are known to associate with different patterns of GR-target gene activation, it is likely that differential activation of the GR by dexamethasone or sertraline leads to differential patterns of GR-target gene expression regulation, which in turn regulates the process of hippocampal neurogenesis.

The next step in extending this understanding of how antidepressants increase neurogenesis would be identifying the gene networks underlying this process, as regulated by the GR. Understanding the genetic network activated by antidepressants that leads to an increase in neurogenesis would provide insight into the mechanisms by which antidepressants increase hippocampal neurogenesis, and perhaps identify novel targets more directly affecting this process. As outlined in Aim 5 below, this thesis will seek to identify these gene networks by immunoprecipitation of the GR, fixed to its genomic targets, when activated by these different conditions – dexamethasone alone, sertraline alone and dexamethasone and sertraline co-treatment. This process will allow identification of the differential gene targets of the GR when transducing increased versus decreased neurogenesis.
1.6 *Human hippocampal progenitor cells as a research tool to study depression*

Human hippocampal progenitor cells offer the opportunity to study the interactions between biological domains implicated in the pathogenesis of depression, including the molecular mechanisms underlying this interaction, in a cellular model with particular relevance to the clinical condition. These cells also provide a controlled model in which to explore the effects of antidepressant compounds on these processes.

The hippocampus has been critically implicated in the pathogenesis and recovery from depression (MacQueen and Frodl, 2011). It is the most plastic part of the brain, responding most profoundly to perturbations caused by exposure to stressful stimuli, such as glucocorticoid changes and inflammation (Koo and Duman, 2008; MacQueen and Frodl, 2011). Furthermore it is the only part of the brain that undergoes the process of adult neurogenesis, a process critically implicated in depression and antidepressant effect. In addition, these cells are themselves immunologically active, producing the innate immune signalling molecules, like cytokines and chemokines (Pugazhenthi et al, 2013; Zunszain et al, 2012), implicated in depression.

Previous work aimed at understanding the role of the glucocorticoid and inflammatory systems in the pathogenesis of depression, and the ability of monoaminergic antidepressants and PUFAs to modify these domains has been limited to an examination of peripheral samples from clinical populations or an investigation of the brain in animal models. However, both approaches, while useful, do not allow a species-specific, cell-specific understanding that would
allow a translation to clinical practice, offered by investigation of the present model.

Additionally, the study of neurogenesis in depression has been restricted to investigation of post-mortem samples (Boldrini et al, 2009; Eriksson et al, 1998; Reif et al, 2006) which do not allow investigation of the molecular pathways underlying any observed differences. The promise of in vivo human neuroimaging of neurogenesis failed to manifest (Manganas et al, 2007).

The embryonic hippocampal progenitor cells employed in the current study exhibit the same characteristics as adult hippocampal progenitor cells: they express specific progenitor markers, such as GFAP, nestin and SOX2, they proliferate indefinitely, and they have the potential to develop into the main cell types of the human central nervous system: neurons, astrocytes and oligodendrocytes.

The model employed throughout this thesis is based on exposing human neural progenitor cells to a variety of depressogenic insults, leading to changes in cell phenotypes that resemble those described in the brains of depressed patients as well as in the brains of animals exposed to chronic stress (Anacker et al, 2011b, 2013a, 2013b; Zunszain et al, 2012). IL-1β was used as an inflammatory stimulus because it is increased in depressed patients (Howren et al, 2009) and is critical to the pathogenesis of depression (Koo and Duman, 2008). Moreover, we have previously shown that these neural progenitor cells are responsive to IL-1β (Zunszain et al, 2012).
1.7 **Aims and Hypotheses of Study**

1.7.1 **Aim 1:** To determine whether glucocorticoid resistance or pro-inflammatory properties of glucocorticoids are associated with depression in clinical literature examining the glucocorticoid and cytokine systems via systematic review and meta-analysis.

As outlined in Section 1.3, over-activity of the HPA axis and the innate immune system have both been implicated as central to the pathogenesis of depression. However, the co-existence of over-activity of both these systems appears paradoxical given the anti-inflammatory properties of cortisol. Two theories have been proposed to resolve this apparent contradiction: one focusing on the importance of glucocorticoid resistance and the other focused on recently discovered pro-inflammatory properties of glucocorticoids. Evidence consistent with both theories has been derived from studies in animals and stressed humans. However, examination of the literature revealed that few clinical studies had examined both the HPA axis and the innate immune system in the same group of patients allowing for assessment of their relationship in depression and no systematic review of this literature has been undertaken.

Therefore, before exploring the interaction of these systems in vitro, the first aim of this thesis was to systematically review the clinical literature that measured cortisol, pro-inflammatory cytokines and glucocorticoid resistance in depressed patients to determine which theory (glucocorticoid resistance or pro-inflammatory glucocorticoids) was best supported by the clinical literature. A meta-analysis of the relationship between glucocorticoid resistance and levels of pro-inflammatory cytokines was also performed.
1.7.2 Aim 2: To determine whether glucocorticoids can be pro-inflammatory in human neural cells, and the mechanisms underlying these effects

Following the conflicting theories raised in Aim 1 about the interaction of the glucocorticoid and innate immune systems in depression I wished to investigate the nature of this interaction in an in vitro model with relevance to depression and to determine which of these theories best accounts for their interaction.

It has been reported in animal studies and a small number of human studies looking at peripheral samples that glucocorticoids can be pro-inflammatory, particularly when glucocorticoid treatment precedes inflammatory stimulation. A different approach examining chronically stressed humans has demonstrated that there is evidence of glucocorticoid resistance in combination with increased inflammatory signalling, suggesting a shift in balance from glucocorticoid to immune signalling in chronic stress, potentially arising from glucocorticoid resistance. However, there has been no investigation of pro-inflammatory effects of glucocorticoids in human neural cells, and no study that has examined whether glucocorticoid resistance is responsible for the pro-inflammatory effects detected in pre-treatment paradigms.

Therefore, I wanted to establish whether glucocorticoids could exhibit pro-inflammatory effects in human neural cells, particularly in hippocampal cells, with relevance to depression, and what were the mechanisms underlying these effects. To do so I adopted the paradigm employed in animal studies and pre-treated cells with glucocorticoids before inflammatory stimulation and measured the effects of this pre-treatment by looking at levels of secreted cytokines in the supernatant. I also examined the effects of dose and timing of glucocorticoid treatment on pro-inflammatory effects.
In order to investigate the mechanisms underlying this process I also used the GR antagonist, RU486, to determine whether the process was GR-dependent and which periods were critical for producing the pro-inflammatory effects. I also looked at changes in gene expression levels using a genome-wide microarray to determine whether pro-inflammatory pathways were upregulated (consistent with the proposed pro-inflammatory properties of glucocorticoids) or whether glucocorticoid-target genes were down regulated (consistent with glucocorticoid resistance). Furthermore, I conducted a functional in vitro test of glucocorticoid resistance by examining the ability of glucocorticoids to inhibit inflammation after glucocorticoid pre-treatment.

1.7.3 Aim 3: To determine whether antidepressant compounds modulate inflammation in human neural cells, and the underlying mechanism of action

As outlined in Section 1.4.2, significant evidence suggests that antidepressant compounds – both monoaminergic antidepressants and ω-3 PUFAs – have immunomodulatory properties, which may present a common final pathway of antidepressant action. However, studies of the immunomodulatory properties of antidepressant compounds have been so far conducted in animals or in the peripheral blood samples of depressed individuals, and no studies have yet examined whether these compounds can be immunomodulatory in human hippocampal cells, a likely target of these compounds in clinical practice.

To explore the possibility that immunomodulation may be a common pathway of antidepressant action I chose to investigate a wide variety of antidepressant compounds, an SSRI (sertraline), an SNRI (venlafaxine), an MAO-I
(moclobemide) and a novel melatonergic antidepressant (agomelatine), as well as the two principal ω-3 PUFAs, EPA and DHA, all of which have demonstrated anti-inflammatory properties in previous studies (Bielecka et al, 2010; Lu et al, 2010; Molteni et al, 2013; Novak et al, 2003; Tynan and Weidenhofer, 2012; Vollmar et al, 2008). In order to model the environment that is thought to be present in depression we stimulated the cells with the pro-inflammatory cytokine, IL-1β.

In order to determine the effect of these antidepressant compounds and to elucidate their mechanism of action, I measured levels of secreted cytokines and chemokines in the supernatant, gene expression of IL-6 and IL-1β, as well as NF-κB activation, thought to be a likely target for immunodulatory activity by antidepressants.

1.7.4 Aim 4: To determine whether antidepressant compounds affect neurogenesis via effects on kynurenine metabolites

As outlined in Section 1.2.6, the mechanism by which IL-1β decreases neurogenesis is still unclear, but evidence has implicated perturbations of the kynurenine pathway, caused by inflammation. Increases in the neurotoxic metabolites in this pathway, activated by inflammatory molecules, are found in inflammation-induced depression and blocking the effects of inflammation on this pathway can abrogate its depressogenic effects. Blocking the kynurenine pathway in mice also increases neurogenesis.

Evidence exists that antidepressant compounds – both monoaminergic antidepressants and ω-3 PUFAs – may exert their effects by increasing hippocampal neurogenesis. Most in vitro studies have examined the ability of
antidepressants to abrogate the reduction in neurogenesis caused by glucocorticoids, but given the critical importance attributed to IL-1β in the pathogenesis of depression, including in affecting hippocampal neurogenesis I wanted to see whether antidepressant compounds could counteract the effects of IL-1β on hippocampal neurogenesis.

Therefore, I co-incubated the progenitor cells during three days of proliferation, and seven days of differentiation with IL-1β and antidepressant compounds from different classes, as above, – sertraline, venlafaxine, EPA and DHA – to determine their ability to counteract the effect of IL-1β on hippocampal neurogenesis.

Furthermore, I wanted to see whether antidepressant compounds could reverse IL-1β-induced changes to the kynurenine pathway, a potential mechanism by which they might reverse the effect of IL-1β on neurogenesis.

Therefore, I examined the effects of co-incubation of each of these antidepressant compounds along with IL-1β, for three days of proliferation and seven days of proliferation, on gene expression of the critical kynurenine pathway enzymes and on the protein level expression of the key neurotoxic metabolite, quinolinic acid (QUIN) using quantitative Real-Time PCR, and high performance liquid chromatography, respectively.
1.7.5 Aim 5: To optimise chromatin immunoprecipitation in the human progenitor cells in order to develop a tool that can be used to determine whether activation of the glucocorticoid receptor by distinct compounds has specific genomic targets underlying divergent effects on neurogenesis.

As outlined in Sections 1.2.5.5 and 1.4.4 stress and glucocorticoids induce reduced hippocampal neurogenesis and depression-like behaviour in animals, while effective antidepressant treatment increases hippocampal neurogenesis, which may be necessary for antidepressant action. Our lab has recently extended these findings to shown that the glucocorticoid dexamethasone decreases neurogenesis in human hippocampal progenitor cells, and the antidepressant sertraline increased neurogenesis.

Furthermore, both these effects are dependent on activation of the glucocorticoid receptor (GR), and dexamethasone and sertraline activate the GR in distinct ways, manifest in differential patterns of serine phosphorylation, to transduce these differential effects. The GR exerts its effects on cells through transcription of genomic targets, the nature of which is determined by its pattern of phosphorylation status. The differential pattern of gene regulation transduced by the GR upon dexamethasone treatment alone, sertraline treatment alone or co-treatment of both is likely to underlie the differential effects on neurogenesis.

As the GR is known to bind directly to glucocorticoid response elements in the promoter regions of its target genes, its gene targets under different conditions can be identified by fixation of the GR to its genomic targets, breakage of the DNA into fragments and immunoprecipitation of the GR attached to its genomic targets. This process is called chromatin immunoprecipitation.
Therefore, Aim 5 of this thesis was to optimise this complex technique in human hippocampal progenitor cells. This was done by the identification of known GR-target genes upon immunoprecipitation of GR when activated by dexamethasone, compared with vehicle treatment. Two different methods were used to break DNA: sonication through the physical impact of sounds waves; and enzymatic digestion. Validation that DNA fragments are of appropriate size was achieved through DNA purification of fragments, reverse crosslinking and conducting gel electrophoresis. Following this, optimisation of conditions and antibodies for immunoprecipitation was conducted, verified by enrichment of quantitative real time PCR.

Optimisation of this technique would allow for future comparisons of the genomic targets of the GR when activated by glucocorticoids and antidepressants, shedding light on the genomic networks underlying the effects of these compounds on hippocampal neurogenesis.
2 Methods

2.1 Systematic Literature Review

The purpose of this review was to assess the interaction between HPA-axis function and innate immune system activity in depressed patients. Specifically, I wanted to identify studies that had examined cortisol levels, measures of innate immunity and measures of glucocorticoid receptor function in depressed patients. This was in order to determine whether glucocorticoid resistance is a necessary feature of patients that demonstrate high levels of glucocorticoids and pro-inflammatory cytokines or whether the clinical literature was consistent with posited pro-inflammatory properties of glucocorticoids.

2.1.1 Search procedure

All search terms were fully exploded to catch all the relevant results, and the truncation symbol ‘$’ was used. Multiple overlapping synonyms were used to produce the broadest possible search. The search terms used were as follows:

“Depression OR Major Depressive Disorder OR Depressive Episode OR Depressive Symptoms” AND “Cortisol OR Corticosteroid$ OR HPA axis OR Glucocorticoid$ OR Hypothalamic-Pituitary-Adrenal Axis” OR “Glucocorticoid Resistance OR GR resistance OR Dexamethasone Suppression Test OR DST OR DEX/CRH Suppression Test” AND “Inflammation OR Inflammator$ OR Cytokines OR Interleukin$ OR IL-6$ OR IL-1$ OR Macrophage OR Interferon$ OR TNF$ OR IFN$”

Each set was searched individually, and the results combined using the ‘AND’ function. This left 97 studies from a search of PsycINFO, 499 from an Embase search, 214 from Medline and 1098 from Web of Knowledge. Combining the
results from all databases produced 1898 studies. Six studies were removed as they were book chapters and 316 were found to be duplicates and removed, leaving 1576. Screening abstracts for inclusion criteria reduced this number to 23. Full text examination reduced this number to 8 studies. In particular, studies were restricted to those which measured HPA axis in two ways: (1) a measure of cortisol and (2) a measure of GR function, either centrally or peripherally; and measured levels of pro-inflammatory cytokines, either baseline or stimulated (Figure 6).

2.1.2 Inclusion & exclusion Criteria

Studies were restricted to human studies, published in English, investigating patients without significant co-morbidities. Because of the limited number of appropriate studies and lack of previous reviews no restrictions were placed on the year of publication.
Figure 6 Search procedure for meta-analysis

Studies identified were filtered for inclusion and exclusion criteria.
2.2 Meta-analysis

In addition to a systematic review, given the qualitative association of GR resistance with inflammatory markers, a meta-analysis was used to quantify the association. Effect size data for meta-analysis was extracted from eligible papers, and authors were contacted when data was not available. Four studies had appropriate data available for meta-analysis. Due to the small number of studies, measures of basal and stimulated inflammation were combined in the analysis. All effect sizes were converted to Fisher’s z before being entered into the analysis. Specifically, Fisher's z was chosen as recommended by Borenstein et al. (2009), to reflect the continuous nature of both levels of inflammatory markers as well as GR function, whilst decreasing the risk of bias associated with Pearson’s r. Since we hypothesised that the true effect sizes would differ depending on sample and biomarker characteristics, random-effects models were chosen for the meta-analysis. All statistical procedures were carried out using Stata, using the metan package for meta-analyses, and the metafunnel and metabias packages for assessment of publication bias. P-values below 0.05 were accepted as being statistically significant, and values below 0.1 reported as trends.

2.3 Cellular Study Design

This thesis examines the effects of glucocorticoid hormones, inflammation and antidepressant compounds (monoaminergic and polyunsaturated fatty acids (PUFAs)) on several processes thought to be integral to the development and recovery from depression: inflammatory and glucocorticoid signalling pathways, the kynurenine pathway and neurogenesis in a cell model with particular
relevance to depression - human hippocampal neural progenitor cells (HPC03A/07, ReNeuron, UK). The effects of glucocorticoids, inflammation and antidepressant compounds on the cell fate of the human hippocampal progenitor cells and the molecular effects on RNA expression, secreted immunoproteins, transcription factor activation, and transactivation of genomic targets, were studied with a range of cellular and molecular techniques.

HPC03A/07 cells were expanded in culture and subsequently probed for inflammatory responses by measuring levels of secreted immunoproteins using ELISA and multiplex immunoassays after inflammatory stimulation, glucocorticoid co-treatment and pre-treatment, and co-treatment with antidepressant compounds (Figure 7). To measure changes in gene expression, whole cell RNA was isolated and quantitative real-time PCR was performed, as well as gene microarrays. To measure NF-κB transactivation, nuclear protein lysates were obtained and NF-κB binding to the NF-κB sequence was analysed using a commercially available transactivation kit (Active Motif). To measure kynurenine metabolites, supernatant samples were extracted and analysed with high performance liquid chromatography and mass spectrometry. To determine the genomic targets of the glucocorticoid receptor chromatin immunoprecipitation was attempted. To detect changes in differentiation, immunocytochemistry was performed using specific antibodies immature and mature neurons.
Figure 7 Experimental overview

Overview of experimental techniques employed in this PhD thesis. All techniques will be described in detail below.
2.4 **Cell Culture**

The human embryonic hippocampal progenitor cell line HPC03A/07 (provided by ReNeuron Ltd., Surrey, UK) was used for all experiments. This cell line is a conditionally immortalised, multipotent progenitor cell line. Multipotent stem cells are unspecialised cells that have the ability to self-renew for long periods of time and to differentiate into specialised cells with specific functions. In contrast to totipotent or pluripotent stem cells, multipotent stem cells are limited in their ability to differentiate, such that the multipotent hippocampal progenitor cells can give rise to neural cells and glia, but not to other cell types. HPC03A/07 cells therefore have the potential to proliferate indefinitely before they differentiate into a target cell (neuron, astrocyte or oligodendrocyte), depending on the cell culture conditions applied (Figure 8).

HPC03A/07 cells were generated by ReNeuron Ltd. as described previously (Pollock *et al.*, 2006). Briefly, primary cells were isolated from the hippocampal region of a 12-week-old male foetus and transfected with the c-myc-ERTM gene. The c-myc-ERTM technology was developed by Littlewood and colleagues and is based on a fusion protein comprising the growth promoting gene c-myc and the genetically modified murine estrogen receptor (ER) G525R (Danielian *et al.*, 1993; Littlewood *et al.*, 1995). The mutant oestrogen receptor is exclusively responsive to the synthetic steroid 4-hydroxytamoxifen (4-OHT) and will activate the c-myc gene only in the presence of 4-OHT (Danielian *et al.*, 1993). HPC03A/07 cells containing the c-myc-ERTM thus proliferate indefinitely in the presence of 4-OHT whereas proliferation is ceased whenever 4-OHT is removed from the cell culture media (Pollock *et al.*, 2006; Littlewood *et al.*, 1995).
Cells were cultured as described by Johansson et al. (2008) with minor modifications. Working stock cultures of $2 \times 10^6$ cells/vial, passaged 13 times after they were generated (p13), were stored in 10% DMSO solution in liquid nitrogen at -120°C. Upon removal from the liquid nitrogen tank, cells were quickly thawed in a waterbath at 37°C under constant agitation, transferred to a microbiological safety cabinet and resuspended in 5 ml of fresh Reduced Modified Media (RMM) consisting of Dulbecco’s Modified Eagle’s Media/F12 (DMEM:F12, Invitrogen, Paisley, UK) supplemented with 0.03% human albumin (Baxter Healthcare, Compton, UK), 100 μg/ml human apo-transferrin, 16.2 μg/ml human putrescine DiHCl, 5 μg/ml human rec. insulin, 60 ng/ml progesterone, 2 mM L-glutamine and 40 ng/ml sodium selenite. To maintain proliferation, 10 ng/ml human bFGF, 20 ng/ml human EGF and 100 nM 4-OHT were added (Table 1). To induce differentiation, cells were cultured in RMM without bFGF, EGF or 4-OHT (RMM (-)) (Table 2). This cell culture media is free of any glucocorticoids unless dexamethasone or cortisol is used as a treatment. Cells were centrifuged at 900 rpm for 5 min, media supernatant was aspirated, and cells were resuspended in fresh media to remove any residual DMSO. Cells were counted using a standard Neubauer hemocytometer and $1.2 \times 10^6$ cells were plated on laminin pre-coated 75 cm$^2$ tissue culture flasks (Nunclon) in 12 ml RMM.

During normal expansion in a humidified cell culture incubator at 37°C and 5% CO$_2$, HPC03A/07 cells proliferate with a doubling time of 72 hours (Johansson et al., 2008). Cells were thus passaged every 72 hours at approximately 80% confluence. For passaging, cell culture media was aspirated and cells were incubated in 3 ml pre-warmed Accutase (Sigma) for 5 min at 37°C to detach cells from the flask. The bottom of the flask was then washed with 3 ml pre-
warmed RMM (ratio Accutase to RMM is 1:1) and the cell suspension was collected in 15 ml centrifuge tubes (Corning). Cells were centrifuged at 900rpm for 5min, supernatant was aspirated, cells were resuspended in RMM and re-seeded at a cell density of 1.2 x 10^6 cells per 75cm^2 tissue culture flask.
Figure 8 ReNeuron’s HPC03A/07 cells with c-mycER technology

Human embryonic hippocampal progenitor cells stably expressing the c-mycER transgene proliferate in the presence of the growth factor EGF and bFGF, and the synthetic compound 4-hydroxytamoxifen (4-OHT) which activates the c-myc ER transgene. Upon removal of EGF, bFGF and 4-OHT, HPC03A/07 cells cease to proliferate and start to differentiate into neurons, astrocytes and oligodendrocytes. Picture source: www.reneuron.com
<table>
<thead>
<tr>
<th><strong>Compound</strong></th>
<th><strong>Concentration</strong></th>
<th><strong>Supplier</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM:F12</td>
<td>1:1</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>human albumin solution</td>
<td>0.03%</td>
<td>Baxter Healthcare, Ltd</td>
</tr>
<tr>
<td>human apo-transferrin</td>
<td>100 μg/ml</td>
<td>Scipac</td>
</tr>
<tr>
<td>human putrescine DiHCl</td>
<td>16.2 μg/ml</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>human rec. insulin</td>
<td>5 μg/ml</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>progesterone</td>
<td>60 ng/ml</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>2 mM</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>sodium selenite</td>
<td>40 ng/ml</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>human bFGF</td>
<td>10 ng/ml</td>
<td>Peprotech EC</td>
</tr>
<tr>
<td>human EGF</td>
<td>20 ng/ml</td>
<td>Peprotech EC</td>
</tr>
<tr>
<td>4-OHT</td>
<td>100nM</td>
<td>Sigma Aldrich</td>
</tr>
</tbody>
</table>

**Table 1 Components added to DMEM for proliferation media**

(DiHCl, dihydrochloride; rec, recombinant; FGF, fibroblast growth factor; EGF, epidermal growth factor; 4-OHT, 4-hydroxytamoxifen)
<table>
<thead>
<tr>
<th><strong>Compound</strong></th>
<th><strong>Concentration</strong></th>
<th><strong>Supplier</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM:F12</td>
<td>1:1</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>human albumin solution</td>
<td>0.03%</td>
<td>Baxter Healthcare, Ltd</td>
</tr>
<tr>
<td>human apo-transferrin</td>
<td>100 µg/ml</td>
<td>Scipac</td>
</tr>
<tr>
<td>human putrescine DiHCl</td>
<td>16.2 µg/ml</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>human rec. insulin</td>
<td>5 µg/ml</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>progesterone</td>
<td>60 ng/ml</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>2 mM</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>sodium selenite</td>
<td>40 ng/ml</td>
<td>Sigma Aldrich</td>
</tr>
</tbody>
</table>

**Table 2 Components added to DMEM for differentiation media**

(DiHCl, dihydrochloride; rec, recombinant; FGF, fibroblast growth factor; EGF, epidermal growth factor; 4-OHT, 4-hydroxytamoxifen)
2.5 **Experimental cellular paradigms**

2.5.1 **Assays involving glucocorticoid and inflammatory treatments**

One of the aims of this thesis was to understand the interaction of the neuroendocrine and neuroimmune pathways in depression by modelling aspects of this interaction in human hippocampal progenitor cells. Two main paradigms were used to understand the interaction of glucocorticoids and inflammation in these cells – a co-incubation paradigm and a pre-treatment paradigm.

2.5.1.1 Co-incubation of glucocorticoids and inflammatory stimulation

The optimal dose and duration of treatment of IL-1β required to provoke a robust inflammatory was first determined by examining multiple doses and treatment periods (see **Section 3.2**). Then experiments were conducted involving the co-incubation of dexamethasone and IL-1β (see **Section 3.3.1**). Cells were plated in 6 well plates (Nunclon) at a density of 300,000 cells per well in 2mL of RMM and allowed to firmly attach for 24 hours. Subsequently cells were co-incubated with IL-1β (10 ng/mL) and dexamethasone at a dose of 1nM, 10nM, 100nM, 1μM or 10μM for 24 hours before supernatant from the cells was collected to analyse IL-6 secretion (**Figure 9a**). Experiments were also conducted involving co-incubation of RU486, the GR antagonist, with IL-1β and dexamethasone to determine the ability of RU486 to abrogate dexamethasone-induced changes (see **Section 3.3.2.3; Figure 9b**).
Figure 9 Co-incubation paradigms for dexamethasone and IL-1β

(a) Doses of dexamethasone from 1nM to 10μM were co-incubated with IL-1β (10 ng/mL) for 24 hours before supernatant was collected for measurement of IL-6 by ELISA. (b) Cells were co-incubated with IL-1β (10 ng/mL), dexamethasone (10μM) and doses of RU486 from 50nM to 1μM to determine the optimal dose of RU486 for blockade of glucocorticoid effects.
2.5.1.2 Pre-treatment with glucocorticoids before inflammatory stimulation

The second paradigm employed was a pre-treatment paradigm where dexamethasone treatment of 24 hours duration preceded inflammatory stimulation of the cells to investigate for the presence of reported pro-inflammatory effects of glucocorticoids (see Section 3.3.2). A period of 24 hour treatment with dexamethasone was selected as this period of treatment has significant anti-inflammatory effects upon co-incubation (see Section 3.3.1), to induce significant changes to the GR (Anacker et al, 2011b) and to induce pro-inflammatory effects in animal studies (Walker et al, 2013).

Based on experiments in animals the time duration between glucocorticoid pre-treatment and inflammatory stimulation has been reported to be critical for determining the effect of glucocorticoids on subsequent inflammatory responses (Frank et al, 2013). Studies have suggested a broad range of time periods that may be effective for inducing pro-inflammatory effects, with a delay of 24 hours following glucocorticoid treatment before inflammatory stimulation demonstrating potentiating effects in several studies (Espinosa-Oliva et al, 2011; Johnson et al, 2002; Munhoz et al, 2006). However, the time periods we could explore was limited by the time taken for proliferating cells to reach confluence (approximately 72-96 hours from commencing the experiment). Therefore, we explored as broad a range of time periods as possible for the delay after glucocorticoid treatment – from 1 hour to 48 hours (1, 12, 24 and 48 hours) – to determine the effect on subsequent inflammatory response (Figure 10). The final stage of the paradigm was treatment of the cells with IL-1β (10 ng/mL) for a period of 24 hours, aimed to induce a robust inflammatory response. Supernatant from the cells was collected following IL-1β treatment for analysis of IL-6 levels by ELISA or chemokines and cytokines by multiplex
immunoassay. RNA was extracted from cells at this time period to allow for measurement of mRNA changes.
Two characteristics were varied in the glucocorticoid pre-treatment paradigms: the dose of dexamethasone used in pre-treatment and the time interval between glucocorticoid pre-treatment and the introduction of an inflammatory stimulus. (a) This paradigm was used to investigate the effect of differing doses of dexamethasone incubated in cells before inflammatory stimulation. 24 hours of treatment with differing doses of dexamethasone (1nM, 10nM, 100nM, 1μM, 10μM), followed by thorough washing (to remove all glucocorticoids from the cell medium) and 24 hours with no treatment. Cells were then incubated with IL-1β (10 ng/mL) for 24 hours, before supernatant was collected and cells fixed, or RNA extracted. Vehicle pre-treatment served as the control condition. (b, c) demonstrate examples of the experiments in which the time interval between glucocorticoid and inflammatory treatment were varied (while the period of dexamethasone treatment and IL-1β stimulation were held constant at 24 hours each). Time intervals were varied - 1 hour, 12 hours, 24 hours and 48 hour - each using a dose of 1μM of dexamethasone. Supernatant was collected from the cells at the end of this period and cells were fixed for cell counting. Vehicle pre-treatment served as the control condition in each case.

**Figure 10 Pre-treatment of cells with dexamethasone before inflammatory stimulation**
A second variation of this pre-treatment protocol was also employed to assess the role of dose of glucocorticoid in determining the effects of glucocorticoid pre-treatment (Figure 10a). The large range of doses of dexamethasone - from 1nM to 10μM - was selected to cover a range equivalent to doses of cortisol that have been thought to exhibit differential effects on inflammation in human and animal experiments (http://www.ncbi.nlm.nih.gov/pubmed/22013396). Dexamethasone was explored, in addition to the endogenous human glucocorticoid, cortisol, because of its purer targeting of the GR compared with the mixed targeting of cortisol for the GR and MR.

Finally, the role of the glucocorticoid receptor was investigated in this pre-treatment paradigm, involving co-treatment of the cells with RU486 either during the pre-treatment period with dexamethasone, the period in between dexamethasone pre-treatment and inflammatory stimulation or both periods (Figure 11). Both 12 well plates (Nunclon) seeded with 240,000 cells and 6 well plates (Nunclon) seeded with 300,000 cells were used in this paradigm, with supernatant collected at the end of the paradigm, and cells fixed for cell counting.
These experiments were designed to assess the role of the glucocorticoid receptor in the pro-inflammatory effects of glucocorticoids. (a) RU486, the GR antagonist, was co-incubated with dexamethasone in the pre-treatment and period, and introduced into the media following washing for the rest interval. (b) RU486 was only co-incubated during the pre-treatment period or (c) only during the rest interval.
2.5.2 Assays with Antidepressant Compounds

To determine the effects of monoaminergic antidepressants from different classes and ω-3 polyunsaturated fatty acids (PUFAs) on the inflammatory cascade in human neural progenitor cells (Section 3.4) cells were plated in 6 well plates (Nunclon) at a density of 300,000 cells per well in 2mL of RMM and allowed to firmly attach for 24 hours. Cells were then co-incubated with IL-1β (10 ng/mL) either alone or in combination with each of the monoaminergic antidepressants, agomelatine, venlafaxine, moclobemide or sertraline, or one of the ω-3 PUFAs, EPA or DHA, for a further period of 24 hours (Figure 12). This paradigm was used for all experiments in Section 3.4, unless otherwise stated. Treatment doses were informed by previous in vitro literature for antidepressants (Maes et al., 1999; Vollmar et al., 2008) and ω-3 PUFAs (Lu et al., 2010) as well as therapeutic levels where known (Schulz et al., 2012) before performing viability assays in our cells. All treatments had the same vehicle (including 0.01% DMSO and 0.1% ethanol) to exclude the possibility of any differences observed being the consequence of differing concentrations of solvents. Supernatants were collected or RNA extracted (not from the same experiments to allow for cell number measurements and correction for secreted protein levels) and stored at -80°C for subsequent measurement.
Six antidepressant compounds were co-incubated with IL-1β (10 ng/mL) for 24 hours. Cell supernatant was collected to analyse levels of secreted chemokines and cytokines by ELISA and Luminex multiplex immunoassay in the cell supernatant. RNA was extracted to examine gene expression of inflammatory molecules and nuclear protein was extracted to assess NF-κB activation.

**Figure 12 Co-incubation of antidepressant compounds and inflammatory stimulation**

Venlafaxine (100nM or 1μM), Sertraline (100nM or 1μM), Agomelatine (100nM or 1μM), Moclobemide (100nM or 1μM), EPA (1μM or 10μM), or DHA (1μM or 10μM)
2.5.3 Differentiation assay

To assess whether changes in neuronal differentiation caused by inflammation could be abrogated by antidepressant compounds, HPC03A/07 cells were plated on black-sided 96 well plates (Nunclon) at a density of 12,000 cells per well in 100μl RMM media. After 24 hours, cells were cultured in RMM media (i.e. in the presence of EGF, bFGF and 4-OHT) for 72 hours. After this initial proliferation phase, cells were washed twice for 15 min in RMM (i.e. media without EGF, bFGF or 4-OHT), and then cultured in RMM(-) media for subsequent 7 days. Cells were treated with IL-1β (10 ng/mL) either alone or in combination with each of the antidepressant compounds venlafaxine, sertraline, EPA or DHA for this entire period (Figure 13). At the end of the total incubation time (10 days), supernatant was collected and stored at -80°C for subsequent measurement of cytokines and kynurenine metabolites, before cells were fixed with 4% PFA for 20 min at room-temperature. Cells were washed three times in PBS and stored at 4°C in PBS containing 0.2% sodium azide before further processing by immunocytochemistry. This experimental paradigm was also conducted in 6 well plates so that RNA could be extracted to investigate the gene expression changes underlying effects on neurogenesis.
HPC03A/07 cells were plated on 96 well plates and allowed to adhere to the plastic for 24h. Cells were cultured in RMM media (i.e. in the presence of EGF, bFGF and 4-OHT) for 72 hours. After this initial proliferation phase, cells were washed twice for 15 min in RMM (-) media without EGF, bFGF or 4-OHT, and then cultured in RMM(-) media for subsequent 7 days. Cells were treated during the proliferation phase and during the differentiation phase (total treatment of 10 days) with vehicle, IL-1β alone or IL-1β in combination with the four antidepressant compounds. At the end of the 10 days incubation period, immunocytochemistry was performed.
2.6 Secreted protein quantification

2.6.1 Collection of supernatant

Experiments were conducted as outlined in Section 2.5 in either 6 well plates (with 2 mL of treatment solution per well) or 12 well plates (with 1 mL per well) or 96 well plates (four replicates of 200 uL per condition). At the end of the incubation period the cell supernatant was collected and stored in two aliquots (1 mL each for 6 well plates, and 0.5 mL each for 12 well and 96 well plates) to avoid future multiple freeze/thaw cycles. Supernatants were immediately transferred to storage at -80°C for later measurement.

2.6.2 Cell quantification

In order to correct values of secreted proteins for variation based on cell number in different experimental conditions cells were fixed and quantified using crystal violet stain. Briefly, cells grown and treated in 6 or 12 well plates were fixed with paraformaldehyde (PFA) 4% for 20 minutes. After washing, the plates were allowed to air dry and were stained by addition of 750 uL (for 6 well plates) or 375 uL (for 12 well plates) of 10% crystal violet (Pro-lab, Cheshire, UK) solution to each well. After 20 minutes of shaking, cells were vigorously washed and allowed to air dry before being treated with 750 uL (for 6 well plates) or 375 uL (for 12 well plates) of 10% acetic acid. Absorbance, proportional to cell number, was measured at 595 nm on a DTX 880 Multimode Detector (Beckman-Coulter, Brea, CA, USA) and used to normalise relative levels of soluble proteins measured via ELISA and multiplex arrays.
2.6.3 **ELISA**

IL-6 secreted into the supernatant was quantified using the human IL-6 Quantikine ELISA kit (R&D Systems, Abingdon, UK). The procedure was performed according to the manufacturer’s instructions. Briefly, cell supernatant and standard dilutions were incubated in wells pre-coated with IL-6 antibody for two hours. IL-6 conjugate, a polyclonal antibody specific for human IL-6 conjugated to horseradish peroxides, was then added and incubated for two hours. Finally, a substrate solution of TMB was incubated for thirty minutes causing a colour change, before being terminated with a stop solution of sulphuric acid. Absorbance was read at 450 nm using a DTX 880 Multimode Detector (Beckman-Coulter). Data were analysed using a four parameter logistic algorithm to derive concentrations of samples from known standards using SoftMax Pro (Molecular Devices, Sunnyvale, CA, USA). All concentrations were then corrected by number of cells as determined by crystal violet (above). At least three independent experiments were conducted on three independent cultures, and each sample was tested in duplicate. Data is presented as absolute concentrations when comparing vehicle and IL-1β treatment and as percentage change when comparisons are made between IL-1β alone and co-incubation with IL-1β and antidepressant compounds.

2.6.4 **Multiplex protein quantification using Luminex platform**

Cell supernatants were also run on the Human Cytokine Magnetic 25-Plex Panel (Invitrogen) according to the manufacturer’s instructions. Briefly, samples were incubated with 25μL of a suspension of antibody beads containing 25 antibodies to different chemokines and cytokines (Table 3) for two hours, along with standard curves, formulated by serial dilution (containing all 25 immunoproteins assayed). Antibodies form primary complexes in proportion to
the amount of soluble immunoprotein specific to that antibody. Each antibody is covalently bound to magnetic microspheres which are dyed with red and infrared fluorospheres (known as a bead region), which allows for differentiation of one bead (and therefore the bound antibody) from another using infrared lasers. Samples were then incubated with a biotinylated detector antibody (100uL per well) for one hour. The final incubation step consists of 100uL of Streptavidin-RPE solution in each well. The samples were then read on a Luminex 200 platform which using the principle of flow cytometry to read individual beads on two characteristics: their bead region (determined by infrared lasers) and their fluorescent intensity (proportional to the amount of original immunoprotein (Figure 14).

Data was analysed with a four parameter logistic algorithm to derive concentrations of the samples from known standards using SoftMax Pro (Molecular Devices) and is presented semi-quantitatively for comparisons between vehicle treatment and IL-1β (Table 12), and as percentage change for comparisons between IL-1β alone and co-incubation with IL-1β and antidepressant compounds (Table 17). Levels of seven chemokines and cytokines are reported in Table 17 as sixteen of them were below and one was above the detection limits of the assay, while values for IL-1β were considered confounded due to IL-1β being added to the supernatant. Values were normalised to cell number determined by crystal violet.
Samples and standards were incubated in turn with antibody-bound beads to 25 immunoproteins, followed by incubation with a biotinylated detector antibody and finally with a streptavidin-RPE solution that produces a fluorescent signal in proportion to the amount of original immunoprotein. This signal is then read, along with an infrared marker distinguishing immunoprotein antibodies from each other, by the Luminex xMAP system working on the principle of flow cytometry.
### Cytokines

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Chemokines</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF</td>
<td>Eotaxin</td>
</tr>
<tr>
<td>IFN-α</td>
<td>IP-10</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>MCP-1</td>
</tr>
<tr>
<td>IL-1β</td>
<td>MIG</td>
</tr>
<tr>
<td>IL-1RA</td>
<td>MIP-1α</td>
</tr>
<tr>
<td>IL-2</td>
<td>MIP-1β</td>
</tr>
<tr>
<td>IL-2R</td>
<td>RANTES</td>
</tr>
<tr>
<td>IL-4</td>
<td></td>
</tr>
<tr>
<td>IL-5</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td></td>
</tr>
<tr>
<td>IL-7</td>
<td></td>
</tr>
<tr>
<td>IL-8</td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td></td>
</tr>
<tr>
<td>IL-12 (p40/p70)</td>
<td></td>
</tr>
<tr>
<td>IL-13</td>
<td></td>
</tr>
<tr>
<td>IL-15</td>
<td></td>
</tr>
<tr>
<td>IL-17</td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td></td>
</tr>
</tbody>
</table>

Table 3 Cytokines and chemokines measured by the Human Cytokine Magnetic 25-Plex Panel
2.7 Gene expression analysis

2.7.1 RNA isolation

For analysis of gene expression changes, RNA was isolated using RNeasy mini kit (Qiagen, Crawley, UK) according to the manufacturer’s instructions. HPC03A/07 cells were grown in a monolayer in 75 cm² tissue culture flasks (Nunclon) until ~80% confluence and the cells were lysed in 350μl lysis buffer RLT (Qiagen) and physically removed from the plates. One volume (350μl) of 70% molecular biology grade ethanol (Sigma) was added to the lysate and the solution was mixed well by pipetting. Each sample (700μl) was transferred to an RNeasy spin column. The spin column was placed into a 2ml collection tube and centrifuged for 1min at 10,000rpm. The flow through was discarded and 700μl of wash buffer RW1 were added to the RNeasy spin column. The spin column was centrifuged for 1 min at 10,000rpm and 500μl buffer RPE were added and centrifuged once for 1 min at 10,000rpm. Another 500μl of buffer RPE were added and spin columns were centrifuged for 2min at 10,000rpm. The spin columns were placed into a clean 2 ml collection tube and centrifuged for 1min at 13,000rpm to eliminate any possible carryover of buffer RPE, or any residual flowthrough remains on the outside of the spin column which may impair RNA quality. To elute the RNA from the spin column, spin columns were then placed into 1.5ml collection tubes and 30μl of RNase-free water were added. Columns were centrifuged for 1min at 10,000rpm and the RNA-containing eluate was stored at -80°C until further processing.
2.7.2 Primer Design

For each gene of interest, the human cDNA sequence was obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/nuccore). Primers were designed using primer3 software (http://frodo.wi.mit.edu/primer3/). Primers were designed to span at least one exon boundary within the coding sequence of the mRNA sequence of the gene of interest. For quantitative real-time PCR, the length of the amplicon was designed to be within the range of 100 to 150 base pairs (bp). Primer length was set to 18 to 21 bp. Primer melting temperature was set to 60±2°C. To confirm specificity of the designed primer pairs for the desired gene of interest, in silico PCRs were performed on the UCSC website (http://genome.ucsc.edu/cgi-bin/hgPcr?org=Human&db=hg18&hgsid=129542060) and with Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

For each target primer set, a validation experiment was performed to demonstrate that PCR efficiencies were within the range of 90-100% and approximately equal to the efficiencies of the reference genes.

2.7.3 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

To reverse transcribe 1μg of total RNA into complementary DNA (cDNA), an appropriate volume containing 1μg of extracted RNA was added to 0.5μg/μl random primers (Invitrogen), 5μl of 5x First Strand Buffer (Invitrogen), 20mM Dithiothreitol (DTT), 0.2 μl RNase OUT (Invitrogen), 10mM dNTP mix in a total volume of 25μl. Samples were heated for 5min at 42°C before 1μl of Superscript III enzyme (Invitrogen) was added per 25μl sample to reverse transcribe 1μg of RNA. The PCR cycles consisted of 50min at 42°C and 15min at 70°C on a G-Storm PCR thermocycler (G-Storm, Byfleet, UK).
2.7.4 Quantitative Real-Time PCR (qPCR)

Quantitative Real-Time PCR (qPCR) was performed using HOT FIREPol® EvaGreen® qPCR Mix (Solis BioDyne, Tartu, Estonia) according to the SYBR Green method. qPCR was run on an Opticon Monitor PCR cycler, and PCR cycles consisted of an initial heating step at 95 °C for 1 5 min to activate the polymerase, 45 PCR cycles were performed. Each cycle consisted of a denaturation step at 95 °C for 30 s, a primer annealing step at 60 °C for 30 s and an elongation step at 72 °C for 30 s. Each sample was assayed in duplicate and each target gene (IL-6, IL-1β, GR, NF-κB, KMO, KYNU, IDO) was normalized to the geometric mean of the two reference genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and beta-actin (ACTB). The Pfaffl Method (Pfaffl, 2001) was used to determine relative target gene expression. Data are expressed as fold change from the vehicle treated control condition. Primer sequences are listed in below in Table 4.
<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6, forward</td>
<td>GAAAGCAGCAAAAGAGGCAC</td>
</tr>
<tr>
<td>IL-6, reverse</td>
<td>TTTCCACCAGGCAAGTCTCCT</td>
</tr>
<tr>
<td>IL-1β, forward</td>
<td>ACGATGCACCTGTACGATCA</td>
</tr>
<tr>
<td>IL-1β, reverse</td>
<td>GAACACCACCTTGTGGCTCCT</td>
</tr>
<tr>
<td>NF-κB, forward</td>
<td>GTGAGGATGGGATCTGCACT</td>
</tr>
<tr>
<td>NF-κB, reverse</td>
<td>CTCTGTCATTCTGCTTCCA</td>
</tr>
<tr>
<td>KMO, forward</td>
<td>AGAGGACGACAAACCTTGAA</td>
</tr>
<tr>
<td>KMO, reverse</td>
<td>TCCCATAGGGAATTGCAGAC</td>
</tr>
<tr>
<td>KYNU, forward</td>
<td>ATGCGGATGATAAAGCCAAG</td>
</tr>
<tr>
<td>KYNU, reverse</td>
<td>GCTTTTGATGGGAGGAAT</td>
</tr>
<tr>
<td>IDO, forward</td>
<td>GTGTTTCAACAAATCCACGA</td>
</tr>
<tr>
<td>IDO, reverse</td>
<td>CTGATAGCTGGGGGTGTC</td>
</tr>
<tr>
<td>Beta-actin (ACTB), forward</td>
<td>CTCTTCCAGCCTCTCTCCT</td>
</tr>
<tr>
<td>Beta-actin (ACTB), reverse</td>
<td>AGCACTGTGTGGGCGTACAG</td>
</tr>
<tr>
<td>Glyceraldehyde 3-phosphate</td>
<td>CAATGACCCCTTCATTGACC</td>
</tr>
<tr>
<td>dehydrogenase (GAPDH) forward</td>
<td>CAATGACCCCTTCATTGACC</td>
</tr>
<tr>
<td>Glyceraldehyde 3-phosphate</td>
<td>TGGAAGATGGTGATGGGATT</td>
</tr>
<tr>
<td>dehydrogenase (GAPDH) reverse</td>
<td>AACTCTGCCTGGTGTGCTCT</td>
</tr>
<tr>
<td>Glucocorticoid receptor (GR) forward</td>
<td>GCTGTCCTTCCACTGCTCTT</td>
</tr>
<tr>
<td>Glucocorticoid receptor (GR) reverse</td>
<td>GCTGTCCTTCCACTGCTCTT</td>
</tr>
</tbody>
</table>

**Table 4 Primer pairs used in PCR experiments**
2.8 **NF-κB transactivation analysis**

2.8.1 **Cytoplasmic and nuclear protein extraction**

Nuclear extracts were obtained using a Nuclear Extraction kit (Active Motif, Rixensart, Belgium) according to the manufacturer’s instructions with slight modifications. Cells were transferred to the cold room and washed once in 5mL ice cold PBS. Cells were then scraped in 5ml ice-cold PBS, transferred to 15ml centrifuge tubes and centrifuged at 3000 rpm for 10 min in a pre-cooled centrifuge at 4°C. The supernatant was carefully discarded and each cell pellet was gently re-suspended in 400μl Hypotonic Buffer. Samples were transferred to 1.5ml microcentrifuge tubes and incubated for 15 min on ice. 20μl detergent was added and samples were vortexed for 10 sec at the highest setting. The suspensions were then centrifuged at 14,000 x g for 2 min at 4°C and the supernatant (cytoplasmic fraction) was transferred to a fresh microcentrifuge tube and stored at -80°C until further analysis. Cell pellets (nuclear pellets) were resuspended in 50 μl Complete Lysis Buffer containing protease inhibitors (Active Motif) and vortexed for 30 sec at the highest setting. The samples were incubated for 30 min at 4°C on a rocking platform at 400 rpm. Afterwards, the lysates were vortexed for 1 min at the highest setting and centrifuged for 10 min at 14,000 x g in a pre-cooled microcentrifuge at 4°C. The supernatants (nuclear fraction) were transferred to a fresh microcentrifuge tube and stored at -80°C until further analysis.

2.8.2 **Protein quantification**

Protein concentrations were quantified using a bicinchoninic acid (BCA) colorimetric assay system (Novagen). Protein samples were diluted 1:10 in
protein lysis buffer and incubated with the kit reaction mixture in a ratio of 1:8 (sample: reaction mixture) for 30 min at 37°C. Absorbance was measured with a microplate reader (DTX 880 Multimode Detector, Beckman Coulter, Brea, USA) at 562nm and the protein concentration of each sample in duplicate was determined by comparison with a bovine serum albumin (BSA) standard curve (0μg/ml, 1.25μg/ml, 5μg/ml, 10μg/ml, 25μg/ml, 75μg/ml, 125 μg/ml, 250μg/ml, 500μg/ml, 750μg/ml).

2.8.3 NF-κB transactivation assay

Nuclear extracts were obtained after treatment with IL-1β alone and with antidepressant compounds, as explained above. NF-κB binding to the NF-κB consensus sequence (5’-GGGACTTTCC-3’) was analysed using the TransAM NF-κB p65 assay (Active Motif). Protein concentrations were measured using the BCA assay (Section 2.8.2) and 5μg nuclear protein extracts were added to a 96 well plate which contains the immobilized NF-κB oligonucleotide (Active Motif). Nuclear protein extracts were incubated with the NF-κB oligonucleotide in binding buffer for 1 hour and sequentially incubated with G rant antibody (1:1000, Active Motif) for 1 hour at room temperature and with HRP-conjugated antibody (1:1000, Active Motif) for 1 hour at room temperature. Plates were washed three times with Washing Buffer and developed for 5 min before absorbance was read with a spectrophotometer (DTX 880 Multimode Detector, Beckman Coulter, Brea, USA) at 450 nm (Figure 15). Specificity of the assay was confirmed using wild-type and mutated oligonucleotide sequences.
**Figure 15 TransAM NF-κB procedure**

Flow chart of NF-κB transactivation assay using the NF-κB TransAM kit from Active Motif. Nuclear protein extracts were incubated with the NF-κB response element oligonucleotide on the assay plate and sequentially incubated with NF-κB-antibody and with HRP-conjugated antibody for 1 hour at room temperature. Absorbance was measured to quantify the amount of NF-κB bound to the NF-κB sequence on the plate. Picture source: www.activemotif.com
2.9 **Gene array**

2.9.1 **RNA Isolation, Microarray Processing, Quality Control**

RNA of human progenitor cells was isolated using RNeasy mini kit (Qiagen, Crawley, UK) and subsequently DNase treated (Ambion, Warrington, UK). Gene expression microarray assays were performed using Human Gene 2.1st Array Strips or on GeneAtlas platform (Affymetrix) following the WT Expression Kit protocol described in the Affymetrix GeneChip Expression Analysis Technical Manual (http://media.affymetrix.com/support/downloads/manuals/geneatlas_WTExpression_expkit_manual.pdf). Briefly 250ng RNA were used to synthesize second strand cDNA with the Ambion Express Kit (Ambion, Life technologies). Subsequently 5.5µg of purified cDNA were then fragmented, labelled and hybridized onto HuGene2.1 Array strips. The reactions of hybridation, fluidics and imaging were performed on the Affymetrix Gene Atlas instrument according to the manufacturer’s protocol.

2.9.2 **Pathway Analysis**

Affymetrix CEL files were imported into Partek Genomics Suite version 6.6 for data visualization and statistical testing. Quality control assessment and Principal-component analysis (PCA) was performed to identify outliers. All samples passed the criteria for hybridization controls, labelling controls and 3'/5' Metrics. Background correction was conducted using Robust Multi-strip Average (RMA) (Irizarry et al, 2003) to remove noise from auto fluorescence. After background correction, normalisation was conducted using Quantiles normalization (Bolstad et al, 2003) to normalise the distribution of probe intensities among different microarray chips. Subsequently, a summarisation
step was conducted using a linear median polish algorithm (Tukey, 1977) to integrate probe intensities in order to compute the expression levels for each gene transcript.

Upon data upload, pre-processing of CEL data for the complete data set (total of thirty samples; six biological replicates per sample for veh/veh/veh (1), DEX/veh/veh (2), DEX/veh/IL-1β (3), veh/veh/IL-1β (4), veh/veh/IL-1β+DEX (5)) was performed using ANOVA to assess treatment effects between conditions (4) and (1), (5) and (4), (4) and (3), and (2) and (1), to assess for differences in treatment. Differential gene expression across treatment was assessed by applying a p-value filter (for treatment) of p<0.05 to the ANOVA results.

2.10 Immunocytochemistry

2.10.1 Neuronal differentiation

Neuronal differentiation was assessed by doublecortin (DCX), and microtubulin associated protein-2 (MAP2). Our lab has previously confirmed the specificity of the MAP2 antibody for mature neurons in our cell culture using co-labelling experiments and Western Blotting, demonstrating that the MAP2 antibody used for our experiments specifically detects the neuronal isoforms MAP2a and MAP2b, but not the isoforms MAP2c, which is also expressed by oligodendrocytes and undifferentiated progenitor cells.

For immunocytochemistry of differentiated cells, PFA-fixed cells were incubated in blocking solution (10% NGS in PBS containing 0.3% Triton-X) for 2 hours at room temperature, and with primary antibodies (rabbit anti-DCX, 1:1000; mouse anti-MAP-2 [HM], 1:500, Abcam, Cambridge, UK) at 4°C overnight. Cells were incubated sequentially in blocking solution for 30min, secondary antibodies (Alexa 594 goat anti-rabbit; 1:1000; Alexa 488 goat anti-mouse, 1:500,
Invitrogen) for 1 hour, and 0.02 mg/ml Hoechst33342 dye for 5 min at room temperature.

2.10.2 Cell counting
As the Hoechst33342 dye labels all cells on the culture plate, the fraction of DCX and MAP2-positive cells over Hoechst33342 positive cells was used to determine the percentage of newly born neurons and, mature neurons, respectively. This count was performed by a Cell Insight Personal Cell Imager with upper and lower intensity thresholds set for detection of positive cell events to allow unbiased quantification of cell type.

2.11 Kynurenine pathway quantification

2.11.1 Sample preparation
Perchloric acid 0.5M (HClO₄), was used to extract analytes from cell culture medium. 1mL of cell supernatant from each experimental condition was centrifuged (2000 x rpm, 5 min) to pellet and remove dead cells. 100 µL of each supernatant sample was collected and combined with an equal volume of ice cold solvent (1M HClO₄) fortified with IS (final concentration 1µM); then samples were centrifuged (15000 x g, 10 min) and the supernatants were collected and stored at -80 °C until HPLC analysis. The supernatants were directly injected into the liquid chromatography-tandem mass spectrometry (LC-MS/MS) device.

2.11.2 Liquid chromatography
The analysis were performed using an Agilent HP 1200 liquid chromatograph (Agilent, Milan) consisting of a binary pump, an autosampler and a thermostated column compartment. Chromatographic separations were carried out using a Discovery HS-F5 column (150 x 4.6 mm, 5 µm) using 0.1% formic
acid in water (solvent A) and acetonitrile (solvent B) as mobile phase. The HPLC analyses were carried out using a linear elution profile of 15 minutes from 5% to 90% of acetonitrile (ACN). The column was washed with 90% of acetonitrile for 3.5 min followed by the equilibration of the column for 5 min with 5% ACN. The flow rate was 0.5 mL/min. The injection volume was 40 µL. An Agilent 6410 triple quadrupole-mass spectrometer with an electrospray ion source operated in positive mode was used for detection. Flow injection analysis was used to optimise the fragmentor and source parameters. The optimized source parameters for MS analysis were: drying gas temperature 350 °C and gas flow 12 L/min, nebulizer gas flow pressure 35 psi and capillary voltage 4500 V.

2.12 Chromatin immunoprecipitation

In order to investigate the genomic targets of the differentially activated GR a chromatin immunoprecipitation protocol was adapted to the human hippocampal neural progenitor cells. Two separate protocols were used – one using sonication for fragmentation of the DNA and one using enzyme digestion (illustrated in Figure 16).

2.12.1 Chromatin immunoprecipitation by sonication

2.12.1.1 Cell culture and fixation

HPC03A/07 cells were grown to approximately 90% confluence in three T175-cm² Nunc tissue culture flasks (approximately 60 million cells) for each condition and treated 1 hour prior to fixation with dexamethasone (1µM) or vehicle (0.01% ethanol). After washing with warm PBS, cells were subjected to a fixation step with 1% formaldehyde for five minutes. Formaldehyde cross-links DNA and proteins (including the glucocorticoid receptor) in the cells. This process was
then quenched by the addition of glycine to a final concentration of 125mM for 5 minutes.

2.12.1.2 Lysis of cells

The cells were then rinsed twice with ice-cold PBS, scraped off the flasks in a solution of ice-cold PBS and protease inhibitor (Roche) solution (to prevent digestion of the protein). The cells were then centrifuged at 1300 rpm for 5 minutes at 4°C and re-suspended in lysis buffer (see Table 5) and protease inhibitors and incubated on ice for thirty minutes. The lysis buffer has the effect of lysing the cell membrane whilst leaving the nuclear membrane intact. Nuclei were obtained because the structure of interest – the GR-DNA interaction – occurs after translocation of the GR to the nucleus where it can bind to the promoter regions of its target genes. The resulting nuclei were pelleted by centrifugation and resuspended in shearing buffer (Table 5) and protease inhibitors.

2.12.1.3 Shearing by sonication

Chromatin was then sheared into small fragments through the process of sonication using a Biorupter® Standard UCD-200 sonication device (Diagenode, Liege, Belgium). Sonication involves the application of ultrasound waves through a sonication probe to the sample which is situated in a waterbath that reflects these sound waves for a thirty second cycle. The energy from these sound waves breaks the chromatin into smaller fragments. An average DNA length of 200-500 base pairs is required as the final product to enable pinpointing the location of the DNA sequences of interest (Das et al, 2004). The length of sonicated samples was quantified by a 1% agarose gel electrophoresis, using a 2-log DNA ladder (New England BioLabs, Ipswich, Massachusetts, USA) as a reference scale. The process for optimising this step
of the protocol in human hippocampal progenitor cells is outlined in Section 3.6.1.

2.12.1.4 Binding to antibody

The process of optimising the antibody used in this step is outlined in Section 3.6.3. Briefly, sonicated samples (20mg) were incubated in a solution of modified radioimmunoprecipitation (RIPA) buffer containing glucocorticoid receptor antibodies (Table 6). Protein G magnetic beads (Invitrogen) (which were washed in modified RIPA buffer previous to use), and protease inhibitor overnight at 4°C on a rotator. This step involves binding of the magnetic beads to the GR antibody which selectively binds to the GR, which is in turn bound to its DNA target regions. A control condition was run with non-specific IgG (2ug) in the place of the GR antibody.

2.12.1.5 Immunoprecipitation and reverse crosslinking

Immunoprecipitation was then performed - the antibody-bound chromatin fragments and protein G beads were pelleted on a magnetic separation rack (New England BioLabs) and the supernatant removed. The beads were washed twice with wash buffer 1, once with wash buffer 2 and twice with Tris-ethylenediaminetetraacetic acid (TE) buffer (Table 5).

The washed beads were resuspended in elution buffer and cross-links were reverted by incubation of the sample at 65°C for four hours with 0.2M sodium chloride solution, RNAase (10 mg, Sigma) and TE buffer, followed by ninety minutes of incubation with proteinase K (1mg, Sigma) at 42°C (to remove the protein from the solution, leaving DNA). Magnetic beads were again pelleted on a magnetic stand and supernatant removed. DNA was purified from the supernatant using QIAquick PCR purification system (QIAGEN, Valencia,
California, USA) according to the manufacturer's instructions. Input samples were treated by the same procedure except that no immunoprecipitation step was performed.

2.12.1.6 Detection of enrichment

To assess enrichment of DNA from the ChIP procedure DNA was quantified using quantitative real-time PCR. The quantity of DNA was evaluated by a NanoDrop ND-1000 spectrometer (NanoDrop Technologies, Wimington, USA). Quantitative real-time PCR was performed using Hot FIREPol® EvaGreen® qPCR Mix (no ROX) (Solis BioDyne, Tartu, Estonia), a standard curve of DNA dilutions and specific primers for solute carrier family 19 (thiamine transporter) member 2 (SLC19A2), serum/glucocorticoid regulated kinase 1 (SGK1), the glucocorticoid-induced leucine zipper gene (GILZ) and FK506 binding protein 5 (FKBP5) according to the SYBR Green method, using a MyIQ Single Color Real-Time PCR Detection System (Bio-Rad, Hercules, California, USA), analysed using Bio-Rad IQ5 Optical Software System Version 2.0 (Bio-Rad). 45 PCR cycles were performed. Each cycle consisted of a denaturation step at 95°C for 30 seconds, an annealing step at 60°C and an elongation step at 72°C for 30s. Genes known to possess functional glucocorticoid response elements (GREs) in human cell lines were selected for enrichment – SLC19A2 (primers from So et al. 2007), SGK1 (primers from Luca et al. 2009), MT2A and GILZ (primers from So et al. 2007) (Table 7). The gene Down syndrome cell adhesion molecule (DSCAM) which has no known GRE and has been used successfully as a control in a previous ChIP study (Paakinaho et al. 2010) was used as our control gene.

Target gene expression was calculated using the Pfaffl formula, and expression of each target gene was calculated as a percentage of expression in the input
sample (in which no immunoprecipitation was conducted). Furthermore, the relative fold change of target genes for the GR antibody condition compared with the non-specific IgG condition was calculated. Successful enrichment is indicated by increased enrichment of GR-target genes compared with the control gene (DSCAM) for GR antibody immunoprecipitates.
<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysis buffer</td>
<td>5mM PIPES pH 8.0&lt;br&gt;85mM KCl&lt;br&gt;0.5% NP-40</td>
</tr>
<tr>
<td>Shearing buffer</td>
<td>50mM Tris (Trizma base) pH 8.1&lt;br&gt;10 mM EDTA, disodium salt&lt;br&gt;0.1% SDS&lt;br&gt;0.5% Sodium deoxycholate</td>
</tr>
<tr>
<td>Wash Buffer 1</td>
<td>20mM T pH 8.1&lt;br&gt;50mM NaCl&lt;br&gt;2mM EDTA&lt;br&gt;1% TX-100&lt;br&gt;0.1% SDS</td>
</tr>
<tr>
<td>Wash Buffer 2</td>
<td>10mM T pH 8.1&lt;br&gt;150mM NaCl&lt;br&gt;1 mM EDTA&lt;br&gt;1% NP40&lt;br&gt;1% Na deoxycholate&lt;br&gt;250 mM LiCl</td>
</tr>
<tr>
<td>Elution Buffer</td>
<td>0.1M NaHCO3&lt;br&gt;1% SDS</td>
</tr>
<tr>
<td>Modified RIPA Buffer</td>
<td>140mM NaCl&lt;br&gt;10mM Tris pH7.5&lt;br&gt;1mM EDTA&lt;br&gt;0.5mM EGTA</td>
</tr>
<tr>
<td>Table 5 Buffers used in chromatin immunoprecipitation</td>
<td></td>
</tr>
<tr>
<td>-----------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td><strong>ChIP-IT Protein G Magnetic Beads</strong> (Active Motif 53014)</td>
<td></td>
</tr>
<tr>
<td>Proteinase K</td>
<td>0.5 μg/μl</td>
</tr>
<tr>
<td>RNase A</td>
<td>10 μg/μl</td>
</tr>
<tr>
<td>Antibody name</td>
<td>Manufacturer</td>
</tr>
<tr>
<td>---------------</td>
<td>------------------</td>
</tr>
<tr>
<td>GR E-20</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>GR 59</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>GR D8H2 XP</td>
<td>Cell signalling</td>
</tr>
<tr>
<td>GR PA1-511A</td>
<td>Thermo Scientific</td>
</tr>
</tbody>
</table>

Table 6 Glucocorticoid receptor antibodies used to immunoprecipitate GR-bound DNA
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLC19A2</td>
<td>CCGGAATGTCCATTCACTTT</td>
<td>TCCTGGGCTTCTGATGTCTT</td>
</tr>
<tr>
<td>SGK1</td>
<td>CCCCTCCCCTTCGCTTGGTT</td>
<td>GGAAGAAGTACAATCTGCATTTCACT</td>
</tr>
<tr>
<td>GILZ</td>
<td>GTGCCTGGAGACCAACTCAT</td>
<td>ACCCTTGATGCTGAGCAAGT</td>
</tr>
<tr>
<td>DSCAM</td>
<td>ACGTTGAACAAACCCATGCT</td>
<td>GGTCACCAAGGAACACTCAG</td>
</tr>
<tr>
<td>MT2A</td>
<td>GACGATTCGGCTGAGCTAGA</td>
<td>AGGGCCTTAGATCGTCAACC</td>
</tr>
</tbody>
</table>

**Table 7** Primers used in chromatin immunoprecipitation detection of enrichment
2.12.2 Chromatin immunoprecipitation by enzymatic digestion

This protocol was conducted according to the manufacturer’s protocol (SimpleChIP® Chromatin Immunoprecipitation Protocol (Magnetic Beads), Cell Signaling) with minor variations. The process for optimising enzymatic digestion is outlined in Section 3.6.2.

2.12.2.1 Cell culture and fixation

As above, HPC03A/07 cells were grown to approximately 90% confluence in three T175-cm² Nunc tissue culture flasks (approximately 60 million cells) for each condition and treated 1 hour prior to fixation with dexamethasone (1μM) or vehicle (0.01% ethanol). After washing with warm PBS, cells were subjected to a fixation step with 1% formaldehyde for five minutes and this process was quenched by the addition of glycine to a final concentration of 125mM for 5 minutes.

Cells were lysed by re-suspension in ice-cold buffer, and nuclei were isolated by centrifugation. Micrococcal nuclease was added to suspended cells at 37°C with regular inversion (determination of optimal conditions for this step outlined in Section 3.6.2, involving reverse crosslinking and running DNA fragments on an agarose gel). Digestion of the sample was stopped by the addition of 0.5 M EDTA. Nuclei were pelleted and suspended in ChIP buffer and the nuclear membrane was broken by several pulses of sonication by the Biorupter® Standard UCD-200 sonication device (Diagenode, Liege, Belgium). Lysates were clarified and stored for later immunoprecipitation at -80°C.
2.12.2.2 Chromatin Immunoprecipitation

10μg of cross-linked chromatin was re-suspended in ChIP buffer and 2% of this sample removed as the ‘input’ (that is, not immunoprecipitated). Samples were then incubated with either the GR antibody or the non-specific IgG and ChIP Grade Protein G Magnetic Beads and incubated for 2 hours at 4°C with rotation. Magnetic Beads were pelleted by placing the tubes on a magnetic separation rack. Beads were then washed three times with a low salt wash and then once with a high salt wash. The chromatin was then eluted from the antibody and magnetic beads by incubation with an elution buffer for 30 minutes at 65°C with gentle vortexing. Chromatin, including the input sample, was reverse crosslinked using sodium chloride, Proteinase K. DNA was purified using spin columns and quantification of DNA by PCR was performed as in Section 2.12.1.6, using the same primer pairs.
Figure 16 Chromatin immunoprecipitation protocol

(1) Live cells were treated with formaldehyde which crosslinks proteins (coloured shapes) to DNA (including the transcription factor protein the glucocorticoid receptor (orange circle). (2) Chromatin is fragmented by either physical means (sonication) or enzymatic digestions (with micrococcal nuclease). ‘Input’ DNA – consisting of DNA not immunoprecipitated is removed at this point to allow comparison to immunoprecipitated DNA. (3) Fragments of DNA cross-linked to the GR are precipitated by means of a GR antibody, linked to a magnetic bead. Control immunoprecipitation is performed by using non-specific IgG in place of the specific GR. (4) The precipitated DNA is reverse crosslinked (by means of enzymatic digestion of protein and RNA, leaving just DNA fragments). DNA precipitated with a GR antibody should be enriched with GR-target genes. From http://compbio.pbworks.com/w/page/16252885/ChIPonchip%20on%20Tiling%20Microarrays
2.13 Drugs and Reagents

All drugs and reagents were purchased from Sigma–Aldrich (St Louis, MO, USA), unless otherwise stated. Growth factors EGF and bFGF were purchased from Peprotech (London, UK). Sertraline hydrochloride, venlafaxine hydrochloride, moclobemide, agomelatine, EPA (>98% pure, extracted and purified from fish), DHA (>98% pure, extracted and purified from algal vegetable oil), dexamethasone and RU486 were dissolved in 100% ethanol (EtOH), and IL-1β was dissolved in RMM.

2.14 Statistical Analysis

For measurement of cytokine levels in the supernatant by ELISA or Luminex multiplex immunoassay for antidepressant co-incubation and dexamethasone co- and pre-treatment paradigms, at least four independent experiments (biological replicates, indicated as ‘n’) were conducted for each set of experiments. Data in each individual experiment was normalized to the average value of the vehicle condition for experimental conditions involving IL-1β, and to the average value of the IL-1β condition for experimental conditions involving co-incubation with antidepressants. The % change from the vehicle condition or the IL-1β condition is presented as the mean value ± SEM. For differentiation assays, at four independent experiments (biological replicates, indicated as ‘n’) with 4 technical replicates (four different wells on the same plate in the same experiment) were conducted for each set of experiments. Data in each individual experiment was normalized to the average value of the vehicle condition. The % change from the vehicle condition is presented as the mean
value ± SEM. One RT-PCR was conducted per sample per experiment, and quantitative Real-Time PCR was conducted in duplicates for each sample. For the NF-κB transactivation assay, at least six independent experiments were conducted. All statistical analyses were performed with GraphPad Prism 4.03 (Graph Pad Inc., La Jolla, CA, USA) on independent biological replicates (indicated as ‘n’). One-Way ANOVA with Dunnett’s post hoc test was used for multiple comparisons between treatment groups and controls. Two-Way ANOVA was used to compare treatment effects of RU486 on dexamethasone pre-treatment on inflammatory response for varying doses of dexamethasone. Student’s t-test was used to compare means of two independent treatment groups. P-values <0.05 were considered significant.
3 Results

3.1 Systematic Literature Review and Meta-analysis of Clinical Literature assessing HPA axis and inflammation in patients

In this thesis I wanted to explore some of the pathomechanisms of depression in a relevant cell model to generate insights that might be clinically relevant. Two of the most widely explored theories of the pathogenesis of depression are the neuroimmune or cytokine theory and the neuroendocrine or glucocorticoid theory. As outlined above, there is a seeming contradiction in these theories in that cortisol the end product of the HPA axis, is inhibitory to inflammation, limiting the production and secretion of pro-inflammatory cytokines. There are two theories that aim to make sense of this contradiction – one focusing on the importance of glucocorticoid resistance as a pre-requisite for the co-existence of high levels of glucocorticoids and the other focusing on recently discovered pro-inflammatory properties of glucocorticoids. I wished to explore the interaction of these two important systems in our cellular model of depression, but before doing so I realised there had been no systematic review of clinical literature that had explored both these systems in the same set of patients to understand their interaction in depression.

3.1.1 Systematic literature review

Although there are a large number of studies that have examined a particular biological system in a group of depressed patients (compared with a control group) there are few studies that have examined levels of glucocorticoids, pro-inflammatory cytokines and glucocorticoid resistance in the same group of patients. It is these studies that might shed light on the nature of the interaction
between the immune system and the HPA axis in depression. I therefore carried out a systematic literature review and meta-analysis of all studies that had explored all three of these biological characteristics in the same group of depressed patients.

A key discriminator between the theories proposed to resolve the apparent paradox of hypercortisolaemia and high levels of pro-inflammatory cytokines – a ‘shift in balance’ theory posited on the presence of glucocorticoid resistance, and a theory focused on the pro-inflammatory properties of glucocorticoids – is the presence or absence of glucocorticoid resistance in the context of high levels of cortisol and inflammation. In the shift in balance theory glucocorticoid resistance is predicted and in the pro-inflammatory glucocorticoid theory it is not.

Only eight studies examining all three variables were identified and their findings with respect to these competing hypotheses are outlined.

3.1.1.1 Depressed patients with GR resistance, increased innate immunity and hypercortisolaemia

Increased levels of cortisol and increased markers of innate immune activity were found in association with glucocorticoid resistance in two studies (Table 8). One study found that depressed patients demonstrated higher spontaneous secretion of IL-6 from peripheral blood mononuclear cells (PBMCs) and were more likely to have higher levels of 24hr free urinary cortisol compared with controls (Humphrey 2006). Central GR resistance was also more likely to be present in depressed patients than controls, as indicated by level of suppression of plasma cortisol in a dexamethasone suppression test (using
either 4mg/dL or 1.8 mg/dL as a threshold value). However, there was no difference in levels of LPS-stimulated IL-1β and TNF-α, and lower levels of IL-6 were stimulated in PBMCs from depressed patients. There is recognised difficulty in comparing findings from ex vivo stimulation studies to the commonly reported finding of increased serum levels of cytokines (Humphrey 2006) and high levels of spontaneous IL-6 secretion may provide a more relevant comparison.

Another study found that depressed patients had higher plasma cortisol and circulating IL-6 levels, as well as higher unstimulated IL-6 levels in vitro, than controls, although there was no difference in LPS-stimulated levels of IL-6 (Carvalho et al. 2008). Although there was no absolute difference in the ability of glucocorticoids to inhibit LPS-induced IL-6 release between the two groups, as this study was conducted in whole blood the samples of depressed patients were tested in the presence of higher levels of endogenous cortisol. This is consistent with glucocorticoid receptor resistance in the depressed sample. Furthermore, in a subset of patients and controls IC50s were calculated revealing significantly higher levels for patients over controls (Carvalho et al., 2009). These depressed patients therefore demonstrated increased levels of cortisol, the pro-inflammatory cytokine IL-6, along with evidence of glucocorticoid resistance.
<table>
<thead>
<tr>
<th>Author</th>
<th>Subjects</th>
<th>Method</th>
<th>HPA-axis activity</th>
<th>Immune system activity</th>
<th>GR resistance</th>
<th>Conclusions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Humphreys et al (2006)</td>
<td>9 MD patients, 11 controls</td>
<td>24hr free urinary cortisol (FUC) measured, and plasma cortisol basal/stimulated PBMC cytokine production, Dexamethasone suppression test (DST)</td>
<td>More pts than control showed FUC above 70ug/24hrs No difference in plasma cortisol</td>
<td>Pts had higher circulating IL-6 but reduced LPS-induced IL-6 production</td>
<td>More depressed pts showed non-suppression to dex (2/9) than controls (1/11)</td>
<td>MD Pts show central GR resistance with higher IL-6 and greater FUC cortisol over 24 hours</td>
</tr>
<tr>
<td>Carvalho et al (2008)</td>
<td>15 pts – TRD, inpatients, 28 controls</td>
<td>Morning plasma cortisol, Morning plasma IL-6, Basal and LPS-stimulated whole blood IL-6, GC inhibition of LPS-induced IL-6 secretion in vitro</td>
<td>Pts showed higher morning cortisol</td>
<td>Pts show higher plasma IL-6</td>
<td>In the presence of higher levels of endogenous cort for pts, pts and controls showed similar dex inhibition of IL-6, indicating relative GR resistance in pts</td>
<td>Pts show increased cortisol, increased IL-6 (in vitro and in vivo) and in vitro GR resistance.</td>
</tr>
</tbody>
</table>

Table 8 Depressed patients with GR resistance, increased innate immunity and hypercortisolaemia
3.1.1.2 Depressed patients with GR resistance, increased innate immunity, and normal cortisol levels

A further four studies found increases in inflammatory cytokines in depressed patients associated with GR resistance, without evidence of raised cortisol (Table 9). In one study peripheral GR receptor function, as measured by the topical steroid vasoconstriction (TSV) assay, was found to be decreased in depressed patients, along with higher circulating TNF-α and IL-6 levels (Fitzgerald 2006). There was no difference between groups in plasma cortisol levels. The TSV assay measures GR function by measurement of the degree of vasoconstriction induced in response to three different potencies of topical glucocorticoids. The reduction in vasoconstriction in depressed patients was very pronounced – for example, with a moderately potent topical steroid 100% of controls showed a full vasoconstriction response, while no patients showed a full response. The degree of GR resistance in the patient group, correlated with HAM-D scores and, interestingly, with levels of plasma TNF-α. Of note, morning plasma cortisol levels were not different between controls and patients, and serum cortisol levels did not correlate with IL-6, TNF-α or GR resistance.

Another study found that 28 depressed patients showed higher levels of IL-1β in the supernatant of mitogen-stimulated PBMCs and post-DST plasma cortisol, though not significantly different baseline cortisol levels compared with 10 healthy control subjects (Maes et al, 1993a). Furthermore, there was a significant positive correlation between post-dexamethasone cortisol and levels of stimulated IL-1β, suggesting a relationship between GR resistance and levels of pro-inflammatory cytokines. This correlation held for depressed patients, normal controls and both groups considered together. Indeed, this correlation
remained significant even after allowing for the effects of basal cortisol level, dexamethasone level (following the DST) and age in multiple regression analysis. Cortisol non-suppressors showed greatly increased IL-1β production than suppressors (mean 2906 pg/mL vs mean 1331 pg/mL). Baseline cortisol measures were slightly lower for depressed patients, but there was no significant difference from control subjects.

In a similar study by the same group melancholic major depressed patients showed significantly higher levels of IL-6 produced by PHA-stimulated PBMCs, a trend towards higher levels of glucocorticoid resistance in a DST, more pronounced with more severe depression, and no significant difference in levels of cortisol (Maes et al, 1993b). Consistent with an account that link GR resistance with levels of inflammation, the degree of GR resistance correlated strongly with levels of IL-6 produced by PBMCs. Indeed, even after controlling for baseline levels of cortisol, levels of dexamethasone following dexamethasone administration and age the correlation between IL-6 levels and post-dexamethasone levels remained significant. Subjects with increased levels of IL-6 production showed almost twice the level of GR resistance (mean post-dex cortisol - 7.42 ug/dL) than those with low IL-6 production (mean post-dex cortisol – 3.84ug/dL). Notably, cortisol levels did not differ between patients and controls.

A recent study found that monocytes from drug-free depressed patients showed a trend towards increased gene expression levels of IL-1β and IL-6 following stimulation with LPS, although no difference was found in plasma levels of CRP
(Lisi et al, 2013). While there was no difference in the profiles of daily salivary cortisol levels there was a difference in incidence of GR resistance, as measured by non-suppression of salivary cortisol following a low dose DST. All controls demonstrated normal suppression in the DST, but only 7/10 patients did so. The increase in gene expression of IL-1β and TNF-α was approximately two-fold greater in depressed patients compared with controls, but considerable variability prevented significance being reached. This study is ongoing and findings with a larger number of participants will be interesting.
<table>
<thead>
<tr>
<th>Author</th>
<th>Subjects</th>
<th>Method</th>
<th>HPA-axis activity</th>
<th>Immune system activity</th>
<th>GR resistance</th>
<th>Conclusions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fitzgerald et al (2006)</td>
<td>19 pts – TRD MD 19 controls</td>
<td>Morning plasma cortisol</td>
<td>No difference in baseline cortisol</td>
<td>Pts showed higher circulating TNF-α and IL-6 levels.</td>
<td>Pts showed markedly reduced vasoconstriction, representing dermal GR resistance</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Morning plasma IL-6 and TNF-α</td>
<td></td>
<td></td>
<td>This measure of GR resistance correlated with HAMD scores and TNF-alpha levels</td>
<td>No difference between groups in morning cortisol</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Topical steroid vasoconstriction (TSV) assay</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maes et al (1993) (1)</td>
<td>28 pts – 19 MD, 9 dysthmic/md 10 control</td>
<td>Morning plasma cortisol</td>
<td>No difference in baseline cortisol</td>
<td>MD pts showed higher circulating IL-1β which correlated in all groups with post-DST cortisol.</td>
<td>MD pts show higher post-DST cortisol than controls (md pts were intermediate)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Stimulated PMBC IL-1β levels</td>
<td></td>
<td></td>
<td></td>
<td>Pts showed increased central GR resistance and stimulated IL-1β but no difference in morning cortisol.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DST</td>
<td></td>
<td></td>
<td></td>
<td>There was a significant positive correlation between GR resistance and stimulated IL-1β levels.</td>
</tr>
<tr>
<td>Maes et al (1993) (2)</td>
<td>24 pts - 7 MD, 7 MD, 8 MDM 8 controls</td>
<td>Morning plasma cortisol</td>
<td>No difference in baseline cortisol</td>
<td>Stimulated IL-6 was higher in MDM pts and correlated with post-DEX cortisol.</td>
<td>There is a trend towards higher post-DEX cortisol in MDM pts.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>PHA-stimulated PBMC IL-6 production</td>
<td></td>
<td></td>
<td></td>
<td>Pts had higher baseline and stimulated cytokine levels, with evidence of increased GR resistance in melancholically depressed pts. IL-6 levels correlated strongly with level of GR resistance.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DST</td>
<td></td>
<td></td>
<td></td>
<td>No difference in levels of cortisol</td>
</tr>
<tr>
<td>Lisi et al 2013</td>
<td>10 out-patient MDD 10 controls</td>
<td>Diurnal salivary cortisol rhythm (8am, 4pm and 11pm)</td>
<td>No difference in daily cortisol rhythm</td>
<td>IL-1β and IL-6 mRNA were increased in MDD after stimulation (trend)</td>
<td>DST – 10/10 controls HPA suppressed 7/10 pts supressed</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>DST</td>
<td></td>
<td></td>
<td></td>
<td>Pts showed increased incidence of GR resistance, higher stimulated cytokine response, but no difference in cortisol levels</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LPS-induced PIC mRNA expression in PBMCs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 9 Depressed patients with GR resistance, increased innate immunity, and normal cortisol levels
3.1.1.3 Depressed patients with GR resistance, no changes in immune function and normal cortisol

Another study provided more equivocal results, finding GR resistance without other immune or endocrine abnormalities (Bauer 2003) (Table 10). 36 patients with treatment resistant depression were significantly more likely to be DST non-suppressors, compared with 31 controls (10/36 vs 1/31). This GR resistance was reflected in vitro by a reduced sensitivity to glucocorticoid suppression of leukocyte proliferation and cytokine production following stimulation with either PHA or LPS. However, no difference was found between patients and controls in morning salivary cortisol, or stimulated secretion of IL-2 or TNF-α from PBMCs. Notably, when patients were divided into suppressors and non-suppressors based on their response to the DST, PBMCs from suppressors showed higher levels of TNF-α than non-suppressors, indicating a possible relationship between GR resistance and inflammatory responses.
<table>
<thead>
<tr>
<th>Author</th>
<th>Subjects</th>
<th>Method</th>
<th>HPA-axis activity</th>
<th>Immune system activity</th>
<th>GR resistance</th>
<th>Conclusions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bauer et al (2003)</td>
<td>36 treated in-patients MDD, 31 controls</td>
<td>Hourly salivary cortisol samples Basal/stimulated PBMC cytokine production DST</td>
<td>No difference in baseline cortisol</td>
<td>No difference in stimulated IL-2 or TNF-α production</td>
<td>Pts more likely to be DST non-suppressors (10/36 vs 1/31) Pts show reduced lymphocyte GC sensitivity</td>
<td>Pts show central and peripheral GR resistance. No difference in cortisol No difference in immune system activity</td>
</tr>
</tbody>
</table>

**Table 10** Depressed patients with GR resistance, no changes in immune function and normal cortisol
3.1.1.4 Evidence of pro-inflammatory states in depressed patients without any endocrine changes

The final study captured found immune changes in the absence of endocrine changes in a depressed group of patients (Landmann et al, 1997) (Table 11). Plasma levels of cytokines and cortisol, as well as monocyte responses in vitro were examined. Increased monocyte counts were detected in depressed patients, as well as increased levels of plasma IFN-γ levels (30+/-8 vs 17+/-4 pg/mL) though this was not found to be significant. In particular, depressed patients were overly represented in the highest values of IFN-γ with five patients and only two controls having values greater than 50 pg/mL (both groups consisted of 22 individuals). Both unstimulated and stimulated levels of TNF-α detected in the supernatant of monocytes were similar between patients and controls. Using conservative criteria the authors report no difference in levels of glucocorticoid resistance between patients and controls (with 21/22 individuals from both groups demonstrating a decrease in cortisol levels 9 hours after dexamethasone administration). Interestingly, the mean plasma cortisol levels 16 hours after dexamethasone administration was higher in patients (63+/-22 nmol/L) than in controls (46+/-5 nmol/L) suggesting some evidence of glucocorticoid receptor resistance may have been present in the patient group. No difference in baseline morning plasma cortisol was found between controls and patients.
<table>
<thead>
<tr>
<th>Author</th>
<th>Subjects</th>
<th>Method</th>
<th>HPA-axis activity</th>
<th>Immune system activity</th>
<th>GR resistance</th>
<th>Conclusions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Landmann et al (1997)</td>
<td>22 pts – chronic MD 22 controls.</td>
<td>Morning plasma cortisol Morning plasma interferon-γ; TNF-α levels in the supernatant of mononuclear leucocytes, at baseline and after IFN-γ and LPS-stimulation DST</td>
<td>No difference in baseline cortisol</td>
<td>Greater proportion of pts with &gt;100pg/ml IFN-γ levels (5/22 vs 2/22), though stimulated monocyte levels were not different between the groups. No difference in TNF-α levels</td>
<td>No difference in DST response was found between the groups.</td>
<td>No evidence of GR resistance Pts had higher proportion of extreme IFN-γ levels</td>
</tr>
</tbody>
</table>

Table 11  Evidence of pro-inflammatory states in depressed patients without any endocrine changes
3.1.1.5 Evidence for pro-inflammatory glucocorticoids

It is worth noting that none of the studies captured reported increased cortisol levels, increased pro-inflammatory cytokines, without GR resistance in depressed patients. Although the numbers of captured studies was small, such a group of patients might be expected if the key driver of increased levels of pro-inflammatory cytokines detected in depression was due to the pro-inflammatory effects of cortisol. In this hypothesis GR resistance is not a necessary pre-condition.

3.1.2 Meta-analysis

Overall, the systematic review had suggested a pronounced association between glucocorticoid resistance and increased pro-inflammatory cytokines. Six out of eight studies found increased pro-inflammatory cytokines in association with increased glucocorticoid resistance, with three of these explicitly reporting a significant correlation between glucocorticoid resistance and levels of inflammation. In contrast, only two studies reported increased cortisol in association with pro-inflammatory cytokines in depressed patients, and both these studies also reported increased glucocorticoid resistance in the depressed patients. No study reported a significant correlation between cortisol levels and pro-inflammatory cytokines.

Following this suggestion raised by the qualitative examination of the literature, I conducted a formal meta-analysis on the association between glucocorticoid resistance and inflammatory markers in all those papers with relevant data available (Figure 17). The association between GR resistance and inflammatory markers was significantly positive (Fisher’s z = 0.38, p = 0.002,
95%-CI = 0.144 - 0.619). A trend for heterogeneity was observed (Q = 52.05, df = 4, p = 0.06, I^2 = 55.9%, T2 = 0.0403). The number of studies precluded analysis of the association of GR resistance with individual cytokines, so that cytokines were grouped together. Visual inspection of funnel plots suggested no overall evidence of publication bias, and Egger's test for publication bias was not significant (p = 0.102).
Figure 17 Correlation between GR resistance and inflammation in clinical studies

Meta-analysis of all studies with appropriate data from those captured showed a significant association between measures of GR resistance and levels of pro-inflammatory cytokines. Due to the small number of studies captured, basal and stimulated measurements of cytokine levels were considered together and individual cytokines were grouped together.
3.2 Inflammatory properties of human hippocampal progenitor cells

In order to investigate the interaction of the glucocorticoid and inflammatory systems in depression in human hippocampal progenitor cells I first established that these cells were immunocompetent – that they could respond to, and produce, inflammatory molecules. I focused on two molecules that are routinely found increased in the blood of depressed patients, and have been implicated in the pathogenesis of depression - the pro-inflammatory cytokines IL-1β and IL-6.

3.2.1 Human hippocampal progenitor cells possess cytokine receptors

I first demonstrated by PCR that the human hippocampal stem cells constitutively express the key molecules for recognition of these two cytokines: the IL-1 receptor; and the two components of the IL-6 receptor complex - the IL-6 binding subunit (IL-6R, gp80) and the transmembrane protein gp130, required for signal transduction (Figure 18).
Figure 18 Human hippocampal progenitor cells constitutively express cytokine receptors

Human hippocampal stem cells constitutively express IL-1 receptor (A), the two components of the IL-6 receptor complex: IL-6 binding subunit (IL-6R, gp80) (D) and the transmembrane protein gp130 (C), required for signal transduction. B is the genomic DNA of the IL-1 receptor.
3.2.2 Production of cytokines in response to inflammatory stimulation in a time- and dose-dependent manner

In order to demonstrate that the cells could functionally respond to inflammatory stimuli I also sought to demonstrate that the cells secreted cytokines in response to immune stimulation. Lipopolysaccharide (LPS) is widely used as an inflammatory stimulant in vitro with peripheral immune cells, however, the hippocampal progenitor cells failed to respond to LPS stimulation, suggesting they do not possess a functional TLR-4 receptor, necessary for detecting LPS. Consequently, IL-1β was used as the inflammatory stimulant because it has been implicated strongly in the pathogenesis of depression (Koo and Duman, PNAS), and has been found to be elevated in the serum of depressed patients in meta-analysis.

Immunocompetent cells respond to inflammatory stimulation by activation of intracellular signalling mechanisms, leading to the transcription of a number of cytokines and their secretion from the cell. In particular, stimulation with IL-1β, leads to the secretion of a number of pro-inflammatory cytokines, including IL-6.

I demonstrated that the cells secreted IL-6 into the supernatant in response to treatment with IL-1β. At baseline IL-6 protein levels detected in the supernatant were near to the lower detection limit of the ELISA assay used for measurement (0.7pg/mL). Treatment with 0.1 ng/mL, 1 ng/mL and 10 ng/mL of IL-1β produced a dose-dependent rise in IL-6 levels detected, with a maximal increase of 674-fold (p<0.01) compared to vehicle treatment in response to 10ng/mL of IL-1β (Figure 19a). The increase in secreted IL-6 was also time-dependent, showing a cumulative increase after 30 minutes, 1 hour, 3 hour, 6 hours, 12 hours and 24
hours, with a maximal response at 24 hours of 166 fold (p<0.01) compared to vehicle treatment (Figure 19b).

Given the robust response to IL-1β at a dose of 10ng/mL for 24 hours of treatment this dose and duration of treatment was used for all subsequent experiments.
Figure 19 Time- and dose-dependent inflammatory response of human hippocampal progenitor cells

Human hippocampal progenitor cells HPC03A/07 responded to immune stimulation. (a) Incubation of the cells for 24 hours led to a dose-dependent increase in IL-6 detected in the supernatant. (b) The cells also responded in a time-dependent manner to stimulation with IL-1β (10ng/mL). Data are shown as mean ± SEM, *p<0.05, **p<0.01, compared with vehicle treatment. At least three independent experiments were conducted on at least three independent cultures (n=3-4).
3.2.3 Wide expression of pro- and anti-inflammatory cytokines and chemokines in response to stimulation

In order to confirm that immune stimulation with IL-1β produced wide expression of inflammatory molecules I measured levels of pro- and anti-inflammatory cytokines and chemokines in the supernatant of unstimulated cells and cells stimulated with IL-1β (10ng/mL) for 24 hours using a multiplex immunoassay.

As seen in Table 12 below, at baseline IL-8, IL-15 and IL-1RA were detected at low levels (<40pg/mL), with MCP-1 detected at high levels (>4,000pg/mL). Stimulation with IL-1β (10ng/mL) led to increases in the levels of the cytokines IFN-α, IL-6, IL-8, IL-15, the cytokine antagonist IL-1RA and the chemokines MCP-1, IP-10 and RANTES.

These findings are consistent with the notion that human hippocampal stem cells are immunocompetent and produce a broad range of inflammatory molecules in response to inflammatory stimulation.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Unstimulated</th>
<th>Stimulated</th>
<th>Protein</th>
<th>Unstimulated</th>
<th>Stimulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCP-1</td>
<td>+++</td>
<td>++++</td>
<td>MIP-1α</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IL-8</td>
<td>-</td>
<td>+++</td>
<td>GM-CSF</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IL-15</td>
<td>+</td>
<td>++</td>
<td>MIP-1β</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IL-1RA</td>
<td>+</td>
<td>++</td>
<td>IL-5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IP-10</td>
<td>-</td>
<td>+++</td>
<td>IFN-γ</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IL-1β</td>
<td>-</td>
<td>++</td>
<td>IL-10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IL-6</td>
<td>-</td>
<td>+</td>
<td>IL-2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RANTES</td>
<td>-</td>
<td>+</td>
<td>IL-7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IFN-α</td>
<td>-</td>
<td>+</td>
<td>IL-2R</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TNF-α</td>
<td>-</td>
<td>-</td>
<td>MIG</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IL-13</td>
<td>-</td>
<td>-</td>
<td>IL-4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>-</td>
<td>-</td>
<td>IL-12</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IL-17</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 12 Pattern of chemokine and cytokine secretion in unstimulated and IL-1β-stimulated hippocampal progenitor cells**

The pattern of chemokine and cytokine expression indicated as: -(0-40 pg/mL); +(40-400 pg/mL); ++(400-4 000 pg/mL), +++(4 000-20 000 pg/mL), ++++(>40 000 pg/mL).
3.3 Interaction between glucocorticoid and inflammatory systems in human hippocampal stem cells

I wished to generate insights into the relationship between the neuroimmune and neuroendocrine systems in depression by exploring their interaction of these systems in the cell model of hippocampal progenitor cells. There are two major interactions I wished to explore – the first is the well-known inhibitory effects of glucocorticoids on inflammatory processes (including cytokine release) and the second the more novel pro-inflammatory effects. I examined each in turn and tried to establish which characteristics of the treatment paradigm determined whether glucocorticoids were pro- or anti-inflammatory and the molecular mechanisms underlying these disparate effects.

3.3.1 Effect of co-treatment of glucocorticoids and inflammatory stimulus

Glucocorticoids are widely employed clinically for their anti-inflammatory properties, including an inhibitory effect on the synthesis and release of pro-inflammatory cytokines (De Bosscher and Haegman 2003). I wanted to determine whether these properties applied in human hippocampal progenitor cells so I treated the progenitor cells with IL-1β (10ng/mL) alone and then in combination with 1nM, 10nM, 100nM, 1μM and 10μM of dexamethasone for 24 hours. Co-incubation demonstrated a dose-dependent inhibition of IL-1β-induced IL-6 secretion into the supernatant, maximal for a dose of 10μM of dexamethasone, yielding a 43% ( +/- 0.1%, p<0.01) inhibition of IL-6 released (Figure 20).
Figure 20 Dexamethasone inhibits release of IL-6 dose-dependently upon co-incubation

(a) Cells were co-incubated with dexamethasone and IL-1β (10ng/mL) for a period of 24 hours.
(b) Dexamethasone reduced the large increase in IL-6 detected in the supernatant upon IL-1β treatment alone. The inhibitory effect of dexamethasone was dose-dependent. Maximal effect was achieved with DEX 10μM, reducing IL-6 by 43% (p<0.01). Results are presented as a fraction of IL-6 detected in the supernatant upon IL-1β treatment alone ± SEM **p<0.01 compared to IL-1β treated condition. n=3-11.
3.3.2 Potentiation of Inflammation by pre-treatment with glucocorticoids

I also wanted to determine whether glucocorticoids could be pro-inflammatory in certain circumstances. Pre-treatment with glucocorticoids before immune stimulation is the most widely used paradigm to discern pro-inflammatory effects of glucocorticoids in animal brains and human peripheral cells. In these studies both the timing of glucocorticoid treatment with respect to inflammatory stimulation and the dose of glucocorticoid treatment have been reported to be important in determining the pro-inflammatory effects of glucocorticoids. I examined the influence of timing and dose in paradigms involving pre-treatment of cells with glucocorticoids before inflammatory stimulation.

3.3.2.1 Time dependent effects

The time interval between glucocorticoid treatment and inflammatory stimulation has been demonstrated to be an important variable in determining the pro-inflammatory effects of glucocorticoids. This has been thought to be consistent with an evolutionary narrative whereby acute stressors necessitate cessation of non-vital bioenergetic processes like immune responses during evasive action, followed by a post-stressor period where the importance of immune responses are likely to be enhanced due to injuries sustained.

Therefore, I treated the cells for 24 hours with dexamethasone at a dose of 1μM, before thorough washing and a variable period of no treatment (1 hour, 12 hours, 24 hours and 48 hours), before stimulating the cells with 24 hours of IL-1β (10ng/mL). Pre-treatment with dexamethasone did potentiate subsequent inflammatory response compared to vehicle pre-treatment, and, interestingly,
only did so for a time interval of 24 hours between glucocorticoid treatment and inflammatory stimulation (One-way ANOVA, p<0.05, F1,4=2.953, n=7-12; for 24 hour interval +37.6%, p<0.05, n=12) (Figure 21). This is consistent with the notion that the pro-inflammatory effects of glucocorticoids are influenced by their temporal relation to inflammatory stimulation.

I next wanted to establish whether the differential effect of co-treatment with dexamethasone versus pre-treatment on IL-6, was generalised to other cytokines and chemokines. To do this I measured the same supernatant with a luminex multiplex immunoassay kit. The results generated from the luminex kit were very similar to those derived from the ELISA kit (Figure 22), demonstrating 36% reduction in IL-6 on co-incubation with dexamethasone (p<0.05), with 40% increase in IL-6 with pre-treatment 24 hours before immune stimulation (p<0.05), compared with IL-1β stimulation alone, with no significant effect of dexamethasone pre-treatment one hour before immune stimulation (Figure 22c). Differing timing of dexamethasone treatment had similar effects on the other measurable cytokines and chemokines (IL-8, IP-10, IL-1RA, RANTES and MCP-1), with co-treatment reducing all cytokines and chemokines RANTES, reaching significance for RANTES (-45%, p<0.001, compared with IL-1β stimulation alone). Pre-treatment 24 hours before immune stimulation potentiated the increase in all cytokines and chemokines IL-8 (+23%), IP-10 (+28%), IL-1RA (+82%), RANTES (+29%), MCP-1 (+10%), compared with IL-1β stimulation alone, reaching significance for IL-1RA (p<0.01), and RANTES (p<0.05), after multiple correction. The effect of pre-treatment with dexamethasone only one hour before immune stimulation was intermediate between the effect of co-treatment or the effect of 24 hours before immune
stimulation, consistent with the notion of time-dependent effects of glucocorticoid treatment on immune responses.
Figure 21 Effect of time interval between DEX pre-treatment and IL-1β stimulation

(a) Hippocampal progenitor cells were treated with vehicle or DEX (1μM) for 24 hours, before an interval of no treatment. This interval was varied from 1 hour to 48 hours before subsequent stimulation with IL-1β (10ng/mL). (b) Only a time interval of 24 hours significantly potentiated levels of IL-6. A time interval of 1 hour, 12 hours and 48 hours did not significantly potentiate subsequent inflammatory responses. Data is presented as percentage change of IL-6 detected in the supernatant from vehicle pre-treatment before IL-1β stimulation +/-SEM. *p<0.05 compared to vehicle pre-treatment before inflammatory stimulation. n=7-12
Figure 22 Time-dependent effects of glucocorticoid treatment

a) Four different conditions are compared: IL-1β (10μg/mL) treatment alone; co-treatment with IL-1β and DEX (1μM); pre-treatment with DEX (1μM), with a one hour no-treatment interval before IL-1β stimulation; and pre-treatment with DEX (1μM), with a 24 hour no-treatment interval before IL-1β stimulation. b) Results from Figure 20 and 21 displayed in sequence, showing inhibition on co-treatment, potentiation on pre-treatment, with this effect of greater magnitude when interval is 24 hours. c) The same conditions measured by luminex multiplex immunoassay, demonstrating a similar pattern of effect. Data is presented as percentage change from levels of immunoprotein detected in the IL-1β alone treatment condition. * p<0.05, ** p<0.01, *** p<0.001, compared to IL-1β treatment alone, or the indicated condition. n= 6-8.
Figure 23 Time-dependent effects of glucocorticoid treatment on multiple chemokines and cytokines

a) Four different conditions as in Figure 22. b-f) The effect of timing on five different immunoproteins. Data is presented as percentage change from levels of immunoprotein detected in the IL-1β alone treatment condition. * p<0.05, ** p<0.01, *** p<0.001, compared to IL-1β treatment alone, or the indicated condition. n= 6-8.
3.3.2.2 Dose dependent effects of glucocorticoid priming

Previous studies have demonstrated that the pro-inflammatory effects of glucocorticoids also depend on dosage. Some studies find that intermediate doses of glucocorticoids (equivalent to stress levels), as opposed to low (baseline) or high (clinically used) doses will be pro-inflammatory. Therefore, I also conducted pre-treatment experiments, varying the dose of glucocorticoid used, while holding the time interval between glucocorticoid treatment and inflammatory stimulation constant (at 24 hours). That is, the treatment paradigm, consisted of 24 hours of dexamethasone treatment, using doses of 1nM, 10nM, 100nM, 1μM, and 10μM (followed by thorough washing), 24 hours of no treatment, and 24 hours of treatment with IL-1β (10ng/mL).

All doses of dexamethasone potentiated subsequent inflammatory response, with doses of 100nM and 1μM achieving significance (One-way ANOVA, p<0.05, F1,5=3.894, n=5; DEX 100nM by +72.2%, p<0.01, n=5; DEX 1μM by 48.9%, p<0.05, n=5) (Figure 24). This is nominally consistent with a thesis that intermediate doses of glucocorticoids are pro-inflammatory.
Hippocampal progenitor cells were treated with doses of dexamethasone varying from 1nM to 10μM for 24 hours, before thorough washing and 24 hours of no treatment. Cells were then treated with IL-1β (10ng/mL) for 24 hours and the amount of IL-6 produced in the supernatant measured. All doses of dexamethasone used for pre-treatment increased the IL-6 secreted into the supernatant, with significance reached for doses of 100nM and 1μM. Data is presented as percentage change from levels of IL-6 detected in the vehicle pre-treatment condition +/- SEM. *p<0.05, **p<0.01 compared with vehicle pre-treatment condition.
This experimental paradigm was also employed using cortisol (at doses of 1nM, 10nM, 100nM, 1μM, and 10μM), the endogenous glucocorticoid in humans, to establish whether it could produce similarly dose-dependent pro-inflammatory effects. Pre-treatment with cortisol for 24 hours produced significant potentiation of subsequent inflammatory responses, reaching significance at doses of 10nM, 100nM and 1μM (One-way ANOVA, p<0.0001, F1,5=9.089, n=5; cortisol 10nM by +25.9%, p<0.01, n=5; cortisol 100nM by +49.3%, p<0.01, n=5; cortisol 1μM by 30.2%, p<0.01, n=5) (Figure 25). This further demonstrates consistency with a theory that intermediate doses of glucocorticoids have pro-inflammatory effects.
Figure 25 Dose-dependent effects of cortisol priming

(a) Hippocampal progenitor cells were treated with doses of cortisol varying from 1nM to 10μM for 24 hours, before thorough washing and 24 hours of no treatment. Cells were then treated with IL-1β (10ng/mL) for 24 hours and the amount of IL-6 produced in the supernatant measured. (b) All doses of cortisol used for pre-treatment increased the IL-6 secreted into the supernatant, with significance reached for doses of 10nM, 100nM, and 1μM. Data is presented as percentage change from levels of IL-6 detected in the vehicle pre-treatment condition. * p<0.05, ** p<0.01 compared with vehicle pre-treatment condition.
3.3.3 Role of the glucocorticoid receptor

In animal models the ability of glucocorticoids to be pro-inflammatory has been shown to be dependent on activation of the GR. In order to determine whether this was the case with human neural cells I repeated the dexamethasone pre-treatment paradigm outlined above (24 hours DEX, 24 hours no treatment, 24 hours IL-1β) with varying doses of DEX (from 1nM to 10μM), co-incubated with 50nM of RU486 during the dexamethasone treatment period, a dose of RU486 that blocks the effect of glucocorticoids on the neural progenitor cells in previous experiments in our lab (Anacker et al. 2011).

This co-treatment with RU486 reduced the ability of glucocorticoids to be pro-inflammatory (2 way ANOVA, main effect RU486, F(1,5)=4.855, p<0.05) (Figure 26).
Figure 26 GR-dependent effects at all doses of pre-treatment with dexamethasone

(a) Human hippocampal cells were treated with 24 hours of dexamethasone (doses from 1nM to 10μM) with or without RU486 (50nM), before thorough washing, and a period of 24 hours with no treatment. Cells were then treated with IL-1β (10ng/mL) for 24 hours and the amount of IL-6 produced in the supernatant measured. (b) Co-treatment with RU486 significantly reduced the potentiation of IL-6 secretion by dexamethasone pre-treatment (2-way ANOVA, main effect between +RU486 (a) and -RU486 (b), F(1,5)=4.855, p<0.05). Data is presented as percentage change from levels of IL-6 detected in the vehicle pre-treatment condition +/-SEM. n=6 in each condition.
However, given the lack of full abrogation of the effect by co-incubation with RU486 I wished to establish whether fuller blockade of the GR could be achieved with higher doses of RU486. In order to establish the optimal dose for blockade of the effects of glucocorticoids I co-treated cells with IL-1β (10ng/mL), dexamethasone 10μM and RU486 at doses of 50nM, 100nM, 500nM and 1μM. RU486 reduced the effect of dexamethasone on IL-1β-induced IL-6 secretion (One-way ANOVA, p<0.05, F$_{1,4}$=4.058, n=4; 50nM RU486 by 19%, n.s.; 100nM RU486 by 29%, n.s.; 500nM RU486 by 28%, n.s.; 1μM RU486 by 40%, p<0.01) (Figure 27).
Figure 27 Dose-dependent inhibition of dexamethasone effects by GR antagonist RU486

(a) Human neural progenitor cells were co-incubated with IL-1β (10ng/mL), DEX 10μM and doses of RU486 from 50nM to 1μM for 24 hours before measurement of IL-6 in the supernatant. (b) DEX 10μM (black column) inhibits secretion of IL-6 into the supernatant upon co-incubation with IL-1β, compared with IL-1β treatment alone (white column). RU486, a glucocorticoid receptor antagonist, dose-dependently inhibits this effect of DEX on IL-1β-induced IL-6 secretion (grey columns). The effect of DEX was completely abrogated by a dose of 1μM of RU486 (right-most grey column).
Consequently, I used higher doses of RU486 to more completely block GR-dependent effects of glucocorticoids to establish whether the pro-inflammatory effects of glucocorticoids were GR-dependent. In the first experiment I repeated the paradigm above with dexamethasone pre-treatment at a dose of 1μM, before IL-1β, with RU486 at a dose of 1μM during both dexamethasone treatment and the interval between dexamethasone treatment and inflammatory stimulation, as well as appropriate controls. The significant potentiation of the inflammatory response following dexamethasone pre-treatment (73+/− 27%, p<0.05, n=12, Figure 28, column three), was almost completely abolished by co-treatment with RU486 1μM (no significant difference compared with vehicle pre-treatment; 55+/− 27% decrease compared with DEX pre-treatment, p=0.0586, n=6, Figure 28, column four). Treatment with RU486 alone showed no significant difference to vehicle treatment. These results are consistent with the pro-inflammatory effects of glucocorticoids being GR-dependent, similarly to the anti-inflammatory effects of glucocorticoids.

I performed a further experiment to help clarify the time period during which the pro-inflammatory effects of glucocorticoids were transduced. It is possible that molecular changes that occur during dexamethasone treatment, or in the time interval between dexamethasone treatment and inflammatory stimulation, or both, are responsible for the potentiated response to inflammation evident. In order to understand this I conducted experiments where RU486 was added to the cells during pre-treatment only (and not present in the interval between dexamethasone and inflammatory stimulation) or not during pre-treatment and only during the interval between dexamethasone and inflammatory stimulation. Co-incubation with RU486 during dexamethasone pre-treatment abolished the
potentiating effects of dexamethasone pre-treatment (-25+/-8% compared with DEX pre-treatment, p=0.012, n=6; no significant difference compared with vehicle pre-treatment) (Figure 29). Co-incubation with RU486 during the interval between dexamethasone treatment and inflammatory stimulation decreased the potentiation of inflammation but did not reach significance (-13+/-12%, p=0.29). This finding is consistent with the notion that the pro-inflammatory effects produced by glucocorticoid pre-treatment are largely induced during the period of pre-treatment.
Figure 28 The pro-inflammatory effects of dexamethasone are GR-dependent

(a) Cells were co-treated with RU486 (1μM) during DEX (1μM) pre-treatment and the period of no treatment before inflammatory stimulation. (b) RU486 co-treatment (fourth column) abolishes the pro-inflammatory effect of DEX pre-treatment (third column). Treatment with RU486 alone during the first 48 hours of the paradigm has no significant effect (second column). *p<0.05 compared with vehicle pre-treatment or DEX pre-treatment, where indicated. V- vehicle treatment; R- RU486 (500nM), D- DEX 1μM; I- IL-1β (10ng/mL).
Figure 29 The pro-inflammatory effects of dexamethasone are induced during pre-treatment

(a) Cells were either co-incubated with RU486 during DEX pre-treatment or (b) during the interval between DEX pre-treatment and inflammatory stimulation. (c) Co-incubation of RU486 (500nM) during DEX (1μM) pre-treatment (second column from right) significantly reduced the pro-inflammatory effect of DEX pre-treatment (black column), while treatment with RU486 during the interval between DEX pre-treatment and inflammatory stimulation (right-most column) showed a non-significant reduction. RU486 alone in either period (left-most grey columns) showed no significant effect upon inflammatory response. * p<0.05, ** p<0.01 compared with vehicle pre-treatment. V- vehicle treatment, R – RU486 (500nM), D - DEX 1μM; I- IL-1β (10ng/mL).
3.3.4 Mechanism underlying glucocorticoid potentiation of inflammation

There have been two major mechanisms proposed to explain the ability of glucocorticoids to be pro-inflammatory in certain circumstances. The first proposes that glucocorticoid treatment activates anti-inflammatory pathways acutely, but also upregulates pro-inflammatory pathways whose influence predominates following the acute period. The second mechanism proposed is that glucocorticoid treatment induces glucocorticoid resistance, leading to less inhibitory control, potentiating subsequent inflammatory stimulation.

In order to examine the former proposed mechanism I measured expression of the critical inflammatory transcription factor NF-κB after the paradigm outlined above (DEX 24 hours, no treatment 24 hours, IL-1β 24 hours), using varied doses of DEX from 1nM to 10μM. There was no significant difference in expression of NF-κB in any of the conditions, compared with vehicle pre-treatment (Figure 30c).
Figure 30 No change in gene expression of GR or NF-κB in glucocorticoid potentiation

(a) mRNA was extracted from experiments conducted as previously (24 hours DEX, 24 hours no treatment, 24 hours IL-1β) with the dose of DEX varied from 1nM to 10μM. (b) No significant difference in GR mRNA expression was found between DEX pre-treatment at any dose and vehicle pre-treatment. (c) No significant difference in NF-κB mRNA was found between DEX pre-treatment at any dose and vehicle pre-treatment. Data are shown as fractional change from vehicle pre-treatment ± SEM. n=4
In order to examine the alternative hypothesis I also explored the possibility that the glucocorticoid system might demonstrate resistance that underpinned the potentiation of subsequent inflammatory stimulation. Firstly, I measured the expression of GR mRNA and found no significant difference between any dose of dexamethasone in pre-treatment and vehicle treatment (Figure 30b). Because resistance may exist on a functional level but not be reflected in changes in expression of GR, I examined a functional test of glucocorticoid function – its ability to inhibit IL-1β-induced IL-6 secretion – before and after glucocorticoid treatment. In order to allow comparison, cells were either left for 48 hours before co-treatment with IL-1β (10ng/mL) and DEX 1μM, or pre-treated (for 24 hours) with DEX 1μM before 24 hours of no treatment, followed by co-treatment with IL-1β (10ng/mL) and DEX 1μM. Dexamethasone exhibited a reduced ability to suppress IL-1β-induced IL-6 secretion following previous treatment with dexamethasone, but the change was not significant (for an n=6) (Figure 31), with pronounced variation in the dexamethasone pre-treatment group. This experiment did not yield clear evidence for glucocorticoid resistance but was underpowered.
Figure 31 Glucocorticoid resistance with and without glucocorticoid pre-treatment

a) Glucocorticoid sensitivity was measured by determining the percentage inhibition of IL-1β (10μg/mL)-induced IL-6 secretion by DEX 1μM. The VEH condition entailed no pre-treatment, and the DEX condition involved DEX 1μM pre-treatment, 24 hours before co-treatment, analogous to the paradigm employed above. b) DEX inhibited IL-6 secretion by 50.5% (compared with IL-1β stimulation alone) in the VEH pre-treatment condition, and 45.4% in the DEX pre-treatment condition, though there was no difference in significance. Data is presented as percentage change from IL-1β stimulation alone ± SEM. n=6.
3.3.5 Genome-wide gene expression and pathway analysis underlying effects of glucocorticoids on inflammatory responses

In order to further investigate the mechanisms underlying the ability of dexamethasone to potentiate subsequent inflammatory responses to inflammation, I examined the genes and gene pathways differentially regulated in association with this effect. In order to contrast the inhibitory effects of glucocorticoids upon co-incubation with these potentiating effects, co-treatment with dexamethasone and IL-1β was also analysed. Finally, the effect of dexamethasone treatment without subsequent inflammatory stimulus was also analysed to determine whether pathways underlying potentiation could be discerned from this condition. Five conditions were therefore analysed with Affymetrix gene arrays: vehicle, vehicle, vehicle (VVV); vehicle, vehicle, IL-1β (VVI); dexamethasone, vehicle, IL-1β (DVI); dexamethasone, vehicle, vehicle (DVV); and vehicle, vehicle, IL-1β + dexamethasone (VVI+D) (Figure 32).
Figure 32 Experimental paradigms used for genome-wide gene expression analysis
3.3.5.1 The effect of inflammatory stimulus on genome-wide gene expression (VVI vs VVV)

To understand the effect of inflammatory stimulation on genome-wide gene expression in the hippocampal progenitor cells, VVI treatment was compared with VVV treatment. There were found to be 5117 differentially regulated genes (cut off: fold change (FC)>1.2, p<0.05). Of interest, there was a group of differentially regulated genes (90 genes) that were highly significant (p<0.00015) with large fold changes in expression (FC >5); the 30 genes with the greatest fold change are shown below in Table 13. Many of these highly up-regulated genes are known inflammatory genes. For example, IP-10 was up-regulated 470 fold (p=7.21E-06), reflecting large changes at the level of protein expression as seen in Table 12. Most of the genes for which increases in protein expression were detected on luminex (Table 12) were also found to be up-regulated at the level of gene expression, including IL-6 (p=0.00020538, FC=2.08154), IL-8 (p= 8.50E-08, FC=10.4024), IL-1RA (p=0.00353, FC=1.43562), RANTES (p=2.35E-07, FC= 5.4196), and MCP-1 (p= 3.65E-06, FC=2.80727). Additionally, chemokines, several interferon-induced proteins and genes from the tumour necrosis factor family were strongly up-regulated, as was IL-1β (p= 0.001452, FC=1.56673) itself, consistent with its known auto-induction. Kynurenine enzymes known to be up-regulated by inflammation were also detected by the gene array – IDO (p=9.05E-06, FC=2.01365), and KYNU (p=1.46E-05, FC=1.63188) (See Section 3.5.2).

Interestingly, several genes were significantly down-regulated by IL-1β treatment, reflecting known effects of inflammation on neurogenesis (MAP2, p=6.57E-06, FC=-2.30329; DCX, p=0.00028372, FC=-2.81558) (Zunszain et al, 2012) and the cannabinoid system (CNR1, cannabinoid receptor 1 (brain),
p=0.00031888, FC=-3.84618); however, glutamate systems (GRIK3, glutamate receptor, ionotropic, kainate 3, p=2.04E-06, FC=-3.73854); GRIA2, glutamate receptor, ionotropic, AMPA 2, p=0.000839394, FC=-3.09746) were also down-regulated, contrary to findings in other cell types (Carlton and Coggeshall, 1999). Interestingly, SGK1, a protein implicated in the effect of glucocorticoids on neurogenesis was also up-regulated (p=0.00102665, FC=1.39487). Of note, gene expression of the glucocorticoid receptor was not significantly affected by treatment with IL-1β.

Pathway analysis revealed several pathways affected, as shown below (Table 14), including several well known to be activated by inflammatory stimulation, including MAPK and chemokine signalling pathways. Of note, the apoptotic pathway was the pathway most strongly regulated by IL-1β stimulation, consistent with the neurotoxic potential of inflammation.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene name</th>
<th>p-value</th>
<th>Fold-Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL10</td>
<td>chemokine (C-X-C motif) ligand 10</td>
<td>7.21E-06</td>
<td>470.262</td>
</tr>
<tr>
<td>CXCL11</td>
<td>chemokine (C-X-C motif) ligand 11</td>
<td>1.6E-06</td>
<td>117.544</td>
</tr>
<tr>
<td>OAS2</td>
<td>2'-5'-oligoadenylate synthetase 2</td>
<td>2.75E-06</td>
<td>91.8663</td>
</tr>
<tr>
<td>HERC6</td>
<td>HECT and RLD domain containing E3 ubiquitin protein ligase family</td>
<td>2.31E-07</td>
<td>90.4366</td>
</tr>
<tr>
<td>IFIT1</td>
<td>interferon-induced protein with tetratricopeptide repeats 1</td>
<td>1.85E-06</td>
<td>80.2093</td>
</tr>
<tr>
<td>IFIT3</td>
<td>interferon-induced protein with tetratricopeptide repeats 3</td>
<td>9.83E-07</td>
<td>66.7388</td>
</tr>
<tr>
<td>MX1</td>
<td>myxovirus (influenza virus) resistance 1, interferon-inducible p</td>
<td>3.34E-06</td>
<td>62.7647</td>
</tr>
<tr>
<td>OAS3</td>
<td>2'-5'-oligoadenylate synthetase 3</td>
<td>8.93E-07</td>
<td>53.5393</td>
</tr>
<tr>
<td>IFI44</td>
<td>interferon-induced protein 44</td>
<td>2.36E-07</td>
<td>50.5046</td>
</tr>
<tr>
<td>IFIH1</td>
<td>interferon induced with helicase C domain 1</td>
<td>2.76E-07</td>
<td>48.618</td>
</tr>
<tr>
<td>BST2</td>
<td>bone marrow stromal cell antigen 2</td>
<td>4.94E-07</td>
<td>40.0724</td>
</tr>
<tr>
<td>TNFAIP2</td>
<td>tumor necrosis factor, alpha-induced protein 2</td>
<td>2.93E-07</td>
<td>36.846</td>
</tr>
<tr>
<td>ICAM1</td>
<td>intercellular adhesion molecule 1</td>
<td>2.63E-06</td>
<td>35.5504</td>
</tr>
<tr>
<td>OAS1</td>
<td>2'-5'-oligoadenylate synthetase 1</td>
<td>3.23E-07</td>
<td>35.1349</td>
</tr>
<tr>
<td>IFITM1</td>
<td>interferon induced transmembrane protein 1</td>
<td>3.85E-06</td>
<td>34.1707</td>
</tr>
<tr>
<td>SAMD9</td>
<td>sterile alpha motif domain containing 9</td>
<td>4.71E-07</td>
<td>33.0732</td>
</tr>
<tr>
<td>TNFAIP6</td>
<td>tumor necrosis factor, alpha-induced protein 6</td>
<td>3.91E-07</td>
<td>31.7215</td>
</tr>
<tr>
<td>XAF1</td>
<td>XIAP associated factor 1</td>
<td>3.36E-06</td>
<td>28.9437</td>
</tr>
<tr>
<td>SAMD9L</td>
<td>sterile alpha motif domain containing 9-like</td>
<td>1.89E-06</td>
<td>26.8874</td>
</tr>
<tr>
<td>MMP7</td>
<td>matrix metallopeptidase 7</td>
<td>3.94E-07</td>
<td>26.8041</td>
</tr>
<tr>
<td>C3</td>
<td>complement component 3</td>
<td>8.96E-07</td>
<td>26.5512</td>
</tr>
<tr>
<td>TNFSF10</td>
<td>tumor necrosis factor (ligand) superfamily, member 10</td>
<td>1.58E-06</td>
<td>26.4914</td>
</tr>
<tr>
<td>TNFAIP3</td>
<td>tumor necrosis factor, alpha-induced protein 3</td>
<td>5.52E-08</td>
<td>26.3095</td>
</tr>
<tr>
<td>HERC5</td>
<td>HECT and RLD domain containing E3 ubiquitin protein ligase 5</td>
<td>3.35E-07</td>
<td>21.928</td>
</tr>
<tr>
<td>IFI44L</td>
<td>interferon-induced protein 44-like</td>
<td>1.01E-06</td>
<td>21.3751</td>
</tr>
<tr>
<td>VCAM1</td>
<td>vascular cell adhesion molecule 1</td>
<td>4.85E-08</td>
<td>20.5139</td>
</tr>
<tr>
<td>DDX60L</td>
<td>DEAD (Asp-Glu-Ala-Asp) box polypeptide 60-like</td>
<td>1.94E-07</td>
<td>20.442</td>
</tr>
<tr>
<td>DDX58</td>
<td>DEAD (Asp-Glu-Ala-Asp) box polypeptide 58</td>
<td>4.77E-07</td>
<td>19.9505</td>
</tr>
<tr>
<td>IFI27</td>
<td>interferon, alpha-inducible protein</td>
<td>3.81E-07</td>
<td>17.7912</td>
</tr>
<tr>
<td>IFI6</td>
<td>interferon, alpha-inducible protein 6</td>
<td>3.08E-06</td>
<td>17.2564</td>
</tr>
</tbody>
</table>

Table 13 Genes most strongly up-regulated by IL-1β treatment
<table>
<thead>
<tr>
<th>Pathway</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptosis</td>
<td>8.70E-06</td>
</tr>
<tr>
<td>RNA polymerase</td>
<td>3.50E-04</td>
</tr>
<tr>
<td>Purine metabolism</td>
<td>3.80E-04</td>
</tr>
<tr>
<td>Amyotrophic lateral sclerosis (ALS)</td>
<td>4.90E-04</td>
</tr>
<tr>
<td>Pathways in cancer</td>
<td>6.80E-04</td>
</tr>
<tr>
<td>ErbB signaling pathway</td>
<td>7.60E-04</td>
</tr>
<tr>
<td>Cytosolic DNA-sensing pathway</td>
<td>9.60E-04</td>
</tr>
<tr>
<td>MAPK signaling pathway</td>
<td>1.20E-03</td>
</tr>
<tr>
<td>Wnt signaling pathway</td>
<td>1.60E-03</td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>1.70E-03</td>
</tr>
<tr>
<td>Acute myeloid leukemia</td>
<td>2.40E-03</td>
</tr>
<tr>
<td>p53 signaling pathway</td>
<td>3.10E-03</td>
</tr>
<tr>
<td>Axon guidance</td>
<td>3.30E-03</td>
</tr>
<tr>
<td>Small cell lung cancer</td>
<td>3.40E-03</td>
</tr>
<tr>
<td>Endometrial cancer</td>
<td>5.70E-03</td>
</tr>
<tr>
<td>Circadian rhythm</td>
<td>6.30E-03</td>
</tr>
<tr>
<td>Phosphatidylinositol signaling system</td>
<td>1.20E-02</td>
</tr>
<tr>
<td>Alanine, aspartate and glutamate metabolism</td>
<td>1.30E-02</td>
</tr>
<tr>
<td>Insulin signaling pathway</td>
<td>1.50E-02</td>
</tr>
<tr>
<td>Fc gamma R-mediated phagocytosis</td>
<td>1.50E-02</td>
</tr>
<tr>
<td>Basal cell carcinoma</td>
<td>2.50E-02</td>
</tr>
<tr>
<td>Chronic myeloid leukemia</td>
<td>2.70E-02</td>
</tr>
<tr>
<td>NOD-like receptor signaling pathway</td>
<td>2.80E-02</td>
</tr>
<tr>
<td>Pancreatic cancer</td>
<td>2.80E-02</td>
</tr>
<tr>
<td>Melanogenesis</td>
<td>2.90E-02</td>
</tr>
<tr>
<td>Arginine and proline metabolism</td>
<td>3.20E-02</td>
</tr>
<tr>
<td>Chemokine signaling pathway</td>
<td>3.30E-02</td>
</tr>
<tr>
<td>Valine, leucine and isoleucine degradation</td>
<td>3.70E-02</td>
</tr>
<tr>
<td>Pathway</td>
<td>Score</td>
</tr>
<tr>
<td>--------------------------------------------------</td>
<td>---------</td>
</tr>
<tr>
<td>Inositol phosphate metabolism</td>
<td>3.90E-02</td>
</tr>
<tr>
<td>Methane metabolism</td>
<td>4.00E-02</td>
</tr>
<tr>
<td>GnRH signaling pathway</td>
<td>4.10E-02</td>
</tr>
<tr>
<td>Propanoate metabolism</td>
<td>4.30E-02</td>
</tr>
<tr>
<td>Pyrimidine metabolism</td>
<td>4.40E-02</td>
</tr>
<tr>
<td>Glycerophospholipid metabolism</td>
<td>4.50E-02</td>
</tr>
<tr>
<td>Glycerolipid metabolism</td>
<td>4.50E-02</td>
</tr>
<tr>
<td>Glutathione metabolism</td>
<td>6.30E-02</td>
</tr>
<tr>
<td>Adipocytokine signaling pathway</td>
<td>6.50E-02</td>
</tr>
<tr>
<td>Ubiquitin mediated proteolysis</td>
<td>6.70E-02</td>
</tr>
<tr>
<td>Endocytosis</td>
<td>6.90E-02</td>
</tr>
<tr>
<td>Lysine degradation</td>
<td>7.10E-02</td>
</tr>
<tr>
<td>VEGF signaling pathway</td>
<td>7.70E-02</td>
</tr>
<tr>
<td>B cell receptor signaling pathway</td>
<td>7.70E-02</td>
</tr>
<tr>
<td>Taurine and hypotaurine metabolism</td>
<td>8.30E-02</td>
</tr>
<tr>
<td>Focal adhesion</td>
<td>8.50E-02</td>
</tr>
<tr>
<td>Bladder cancer</td>
<td>9.10E-02</td>
</tr>
<tr>
<td>Glioma</td>
<td>9.90E-02</td>
</tr>
<tr>
<td>ECM-receptor interaction</td>
<td>1.00E-01</td>
</tr>
</tbody>
</table>

**Table 14 Pathways significantly regulated by IL-1β treatment**
3.3.5.2 The effect of dexamethasone co-incubation with IL-1β on gene expression (VVI+D vs VVI)

To understand the effect of dexamethasone co-incubation on inflammatory processes the condition VVI+D was compared with the condition VVI. 478 gene were differentially regulated between these two conditions (cut off: FC> 1.2, p<0.05), with 285 down-regulated, and 193 up-regulated. Many of the genes most strongly down-regulated (Table 15) were cytokine or chemokine genes, including TNF-α, IL-1α, IL-11, chemokine ligand 1 (CCL1), chemokine receptor 4 (CXCR4), chemokine (C-X-C motif) ligands 5, 9, 12, and 16, and other immune related genes - T cell receptor alpha joining 17, T cell receptor delta joining 1, 2, 4 and 17, T cell receptor delta constant and colony stimulating factor 2 (granulocyte-macrophage) (CSF2 or GM-CSF), reflecting the known inhibitory influence of dexamethasone co-incubation on inflammatory processes. Of note, IL-6 was not found to be differentially regulated by dexamethasone, despite the pronounced inhibition observed at the level of secreted protein, perhaps because of different time line of changes at the mRNA and protein levels (Section 3.3.1). Interestingly, several immune related genes were also up-regulated by dexamethasone co-incubation including chemokine (C-X-C motif) ligand 5 (CXCL5), NOD-like receptor family, pyrin domain containing 6 (NLRP6), and chemokine ligand 20 (CCL20). The up-regulation of NLRP6 is particularly notable as the NOD-like receptors have been strongly implicated as a mechanism by which glucocorticoids can potentiate inflammation (Busillo et al, 2011; Frank et al, 2013).

13 gene pathways were also differentially regulated by dexamethasone treatment (Table 16), including inflammatory pathways such as cytokine-cytokine receptor interaction, TGF-beta signalling, chemokine signalling, toll-like
receptor signalling and JAK-STAT signalling. Interestingly, dexamethasone down-regulated the apoptotic pathway, which was strongly up-regulated by IL-1β treatment alone, consistent with the protective effects ascribed to glucocorticoids in the context of inflammation.

3.3.5.3 Pre-treatment with dexamethasone gene expression (DVI vs VVI)

To determine if dexamethasone had differential effects when it preceded immune stimulation, compared with co-incubation, I compared gene expression changes of DVI compared with VVI, and compared the genes regulated in this comparison compared to the above comparison (VVI+D vs VVI). There were 102 genes differentially regulated by DVI compared with VVI (cut off: FC> 1.2, p<0.05), with 59 genes up-regulated and 43 genes down-regulated. Interestingly, immune genes were down-regulated in the condition involving dexamethasone pre-treatment, before IL-1β stimulation, compared with no pre-treatment – including the interleukin 18 receptor 1 (p=0.0101997, FC=-1.262), TNF receptor-associated factor 1 (p=0.0393691, FC=-1.24428), nuclear factor, interleukin 3 regulated (p=0.0426072, FC=-1.20182) and colony stimulating factor 2 (CSF2 or GM-CSF) (p=0.0465768, FC=-1.22921).

There were also immune genes up-regulated by dexamethasone pre-treatment, including NLRP6 (p=0.0019415, FC=1.39546), a NOD-like receptor, which has been strongly implicated in the potentiation by glucocorticoid pre-treatment (Frank et al, 2013). In addition, T cell receptor genes, TRAV1, TRBV5-3, TRAV9-1 were also significantly upregulated, as were other immune system-related genes such as HLA-DQA2 and the immunoglobulin gene IGKV3D-11.

There were two gene pathways differentially regulated by dexamethasone pre-treatment - haematopoietic cell lineage (p=5.70E-02) and vascular smooth
muscle contraction \((p=9.00E-02)\). Interestingly, the haematopoietic cell lineage pathway was also regulated by dexamethasone co-incubation, and the direction of regulation in both dexamethasone treatment paradigms was downwards.

In comparing the genes regulated by dexamethasone pre-treatment and those regulated by dexamethasone co-incubation, there were some interesting observations. Of the 102 genes regulated by dexamethasone pre-treatment and the 478 genes regulated by dexamethasone co-incubation there were 31 differentially regulated genes in common (Figure 33). Of these 31 genes, all were regulated in the same direction in both circumstances. That is, dexamethasone regulated 31 genes in the same direction whether dexamethasone treatment preceded or coincided with immune stimulation. This indicates that no genes that were down-regulated by dexamethasone co-incubation were up-regulated by dexamethasone pre-treatment; nor any genes up-regulated by dexamethasone co-treatment that were down-regulated by dexamethasone pre-treatment. This seems to argue against a ‘rebound’ effect of dexamethasone pre-treatment, which would hold that genes down-regulated during dexamethasone treatment ‘rebound’ into up-regulation in the period following dexamethasone treatment, underpinning the potentiation of immune processes. For example, NLRP6 was significantly up-regulated by both co- and pre-treatment, and nuclear factor, interleukin 3 regulated (NFIL3) and TNF-receptor associated factor 1 (TRAF1) were decreased in both conditions.

There were also 71 genes that dexamethasone pre-treatment regulated uniquely compared with dexamethasone co-treatment, including the up-regulated immune-related genes outlined above - TRAV1, TRBV5-3, TRAV9-1, HLA-DQA2, IGKV3D-1 - that may explain the differential effects of dexamethasone co- and pre-treatment.
Figure 33 Overlap between genes differentially regulated by dexamethasone pre-treatment and dexamethasone co-treatment with immune treatment

This figure indicates that of the 478 genes differentially regulated by dexamethasone co-treatment (VVI+D vs VVI), 31 genes are also differentially regulated by dexamethasone pre-treatment (DVI vs VVI). Of interest, all 31 genes were regulated in the same direction by both treatments. Dexamethasone pre-treatment differentially regulated 71 unique genes (genes not regulated by dexamethasone co-treatment).
3.3.5.4 Gene expression changes due to dexamethasone treatment alone (DVV vs VVV)

In order to understand the effect of dexamethasone pre-treatment on inflammatory processes, I also examined a simpler comparison, between DVV and VVV, which would allow identification of changes due to dexamethasone treatment alone, after a period of delay of 48 hours, with no further treatment. This comparison is simpler than DVI compared with VVI because it doesn't include the added complication of interaction effects with inflammatory stimulation. Dexamethasone treatment alone (DVV) differentially regulated 94 genes compared with the control condition (FC<1.2, p<0.05), with 34 genes up-regulated by dexamethasone pre-treatment and 60 genes down-regulated. The only gene pathway differentially regulated was the cell adhesion molecule pathway (p=9.4E-02), a pathway not differentially regulated in other comparisons.

To evaluate the glucocorticoid resistance hypothesis at the genomic level, I wished to determine whether GR-target genes were under-expressed 48 hours following dexamethasone treatment. In order to examine this hypotheses, I used the Transcription Element Listening System (TELiS) bioinformatics analysis (Cole *et al*, 2005) to quantify the prevalence of transcription factor-binding motifs (TFBMs) in the promoters of differentially expressed genes. This analysis can detect if the target genes of a particular transcription factor are over-expressed in a group of differentially regulated genes. I found that amongst the genes that were upregulated by previous dexamethasone treatment there was no transcription factor with response elements in those genes that was under- or over-represented. However, I found that there was a trend for response elements to the glucocorticoid receptor to be over-
represented in the down-regulated genes. Indeed there was a 19.2 fold greater incidence of genes bearing a response element to the GR in the group of genes down-regulated by dexamethasone pre-treatment (p=0.0508). This indicates a down-regulation of GR-target genes following previous dexamethasone treatment, a finding considered evidence of glucocorticoid resistance (Miller et al., 2009b). However, given the small number of genes analysed (20 genes recognised in the TELiS database), this finding should be cautiously interpreted. Additionally, in the genes down-regulated differentially in DVI vs VVI, GR binding sites were not over-represented, though with 19 genes recognised in the TELiS database this finding is also under-powered.

Overall, from these preliminary analyses some insights could be derived regarding the underlying mechanism of the potentiation of inflammatory processes by dexamethasone pre-treatment. Some suggestive findings include that although dexamethasone, upon co-incubation with immune stimulation (VVI+D vs VVI), largely down-regulated immune-related genes, it also up-regulated a small number of immune-related genes including chemokines and NLRP6. Immune-related genes are also upregulated by dexamethasone pre-treatment before immune stimulation (DVI vs VVI), including, notably, NLRP6, amongst other adaptive immune system-related genes. In comparing genes regulated by dexamethasone pre-treatment with those regulated by co-treatment there were 31 genes in common, and 71 genes uniquely regulated by pre-treatment. The 31 genes regulated by both treatments were regulated in the same direction. Finally, there was some evidence that glucocorticoid pre-treatment (DVV vs VVV) down-regulated GR-target genes, potentially consistent with glucocorticoid resistance, though this was not found when dexamethasone pre-treatment was following by immune stimulation. Further
analysis is required to establish whether glucocorticoid resistance or the up-regulation of inflammatory pathways by glucocorticoid pre-treatment is responsible for pro-inflammatory effects of glucocorticoids.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene name</th>
<th>p-value</th>
<th>fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRAF1</td>
<td>TNF receptor-associated factor 1</td>
<td>0.000215</td>
<td>-1.59822</td>
</tr>
<tr>
<td>HAS2</td>
<td>hyaluronan synthase 2</td>
<td>0.040962</td>
<td>-1.60264</td>
</tr>
<tr>
<td>IL1A</td>
<td>interleukin 1, alpha</td>
<td>0.034604</td>
<td>-1.61198</td>
</tr>
<tr>
<td>ITGA4</td>
<td>integrin, alpha 4</td>
<td>0.014226</td>
<td>-1.61304</td>
</tr>
<tr>
<td>SNORD114-11</td>
<td>small nucleolar RNA, C/D box 114-11</td>
<td>0.043014</td>
<td>-1.62993</td>
</tr>
<tr>
<td>CD38</td>
<td>CD38 molecule</td>
<td>0.008452</td>
<td>-1.64297</td>
</tr>
<tr>
<td>COL1A1</td>
<td>collagen, type I, alpha 1</td>
<td>3.92E-06</td>
<td>-1.64452</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
<td>0.007267</td>
<td>-1.64639</td>
</tr>
<tr>
<td>TRAJ17</td>
<td>T cell receptor alpha joining 17</td>
<td>0.009417</td>
<td>-1.65444</td>
</tr>
<tr>
<td>CXCR4</td>
<td>chemokine (C-X-C motif) receptor 4</td>
<td>3.31E-05</td>
<td>-1.70631</td>
</tr>
<tr>
<td>RRAD</td>
<td>Ras-related associated with diabetes</td>
<td>0.00096</td>
<td>-1.71265</td>
</tr>
<tr>
<td>LOX</td>
<td>lysyl oxidase</td>
<td>0.00174</td>
<td>-1.7556</td>
</tr>
<tr>
<td>CCL1</td>
<td>chemokine (C-C motif) ligand 1</td>
<td>0.010425</td>
<td>-1.7801</td>
</tr>
<tr>
<td>CSF2</td>
<td>colony stimulating factor 2 (granulocyte-macrophage)</td>
<td>1.77E-05</td>
<td>-1.79984</td>
</tr>
<tr>
<td>IGFBP5</td>
<td>insulin-like growth factor binding protein 5</td>
<td>4.98E-07</td>
<td>-1.80951</td>
</tr>
<tr>
<td>TRDJ2</td>
<td>T cell receptor delta joining 2</td>
<td>0.005128</td>
<td>-1.85491</td>
</tr>
<tr>
<td>MAMDC2</td>
<td>MAM domain containing 2</td>
<td>3.66E-08</td>
<td>-1.99977</td>
</tr>
<tr>
<td>GBP4</td>
<td>guanylate binding protein 4</td>
<td>0.028628</td>
<td>-2.12321</td>
</tr>
<tr>
<td>TRDC</td>
<td>T cell receptor delta constant</td>
<td>0.000349</td>
<td>-2.17042</td>
</tr>
<tr>
<td>IL11</td>
<td>interleukin 11</td>
<td>0.004042</td>
<td>-2.1708</td>
</tr>
<tr>
<td>TRDJ4</td>
<td>T cell receptor delta joining 4</td>
<td>0.000395</td>
<td>-2.26032</td>
</tr>
<tr>
<td>CHRNA1</td>
<td>cholinergic receptor, nicotinic, alpha 1 (muscle)</td>
<td>0.000682</td>
<td>-2.26351</td>
</tr>
<tr>
<td>TRDJ1</td>
<td>T cell receptor delta joining 1</td>
<td>0.000405</td>
<td>-2.27053</td>
</tr>
<tr>
<td>KRT75</td>
<td>keratin 75</td>
<td>0.000942</td>
<td>-2.49433</td>
</tr>
<tr>
<td>MMP13</td>
<td>Matrix metalloproteinase 13 (collagenase 3)</td>
<td>0.000961</td>
<td>-3.65363</td>
</tr>
</tbody>
</table>

*Table 15 Genes most strongly inhibited by dexamethasone co-incubation*
<table>
<thead>
<tr>
<th>Gene pathway</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokine-cytokine receptor interaction</td>
<td>9.60E-08</td>
</tr>
<tr>
<td>TGF-beta signaling pathway</td>
<td>3.00E-04</td>
</tr>
<tr>
<td>Hematopoietic cell lineage</td>
<td>1.30E-03</td>
</tr>
<tr>
<td>Focal adhesion</td>
<td>1.50E-03</td>
</tr>
<tr>
<td>Chemokine signaling pathway</td>
<td>7.10E-03</td>
</tr>
<tr>
<td>Intestinal immune network for IgA production</td>
<td>7.30E-03</td>
</tr>
<tr>
<td>Dilated cardiomyopathy</td>
<td>8.10E-03</td>
</tr>
<tr>
<td>Amyotrophic lateral sclerosis (ALS)</td>
<td>1.00E-02</td>
</tr>
<tr>
<td>ECM-receptor interaction</td>
<td>1.90E-02</td>
</tr>
<tr>
<td>Hypertrophic cardiomyopathy (HCM)</td>
<td>2.00E-02</td>
</tr>
<tr>
<td>Toll-like receptor signaling pathway</td>
<td>4.10E-02</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>6.70E-02</td>
</tr>
<tr>
<td>Jak-STAT signaling pathway</td>
<td>9.50E-02</td>
</tr>
</tbody>
</table>

**Table 16 Gene pathways significantly regulated by dexamethasone co-incubation**
3.4 **Modulation of inflammation by antidepressant compounds**

Given the strong association of increased measures of inflammation in depressed patients and the ability of inflammation to predict response to treatment, inflammation has become a target of antidepressant treatment. Studies of the effect of antidepressant compounds on levels of inflammation in animal models of depression and in peripheral samples from clinical populations have yielded mixed findings. A major limitation to these previous studies is that anti-inflammatory properties of antidepressant compounds have not been investigated in human brain tissues or cells – the intended target of these medications. Consequently, an aim of this thesis was to explore the ability of antidepressant compounds to modify immune processes in the human hippocampal progenitor cells, in order to understand whether an immunomodulatory effect may present a common final pathway for antidepressant treatment, thereby clarifying molecular targets for treatment.

3.4.1 **Antidepressant compounds and PUFAs**

Antidepressant compounds targeting the monoamine system are the mainstay of treatment, with drugs from different chemical classes having similar efficacy in depression. More recently, the therapeutic effect of PUFAs on depression has been identified. One possible shared mechanism of action is the ability of these compounds to exert immunomodulatory effects on neural cells. In order to investigate this possibility I co-treated the progenitor cells for 24 hours with IL-1β (10ng/mL) and two doses of four antidepressant compounds with distinct monoamine targets – moclobemide (a monoamine oxidase inhibitor), sertraline
(a selective serotonin re-uptake inhibitor), venlafaxine (a serotonin noradrenaline reuptake inhibitor) and agomelatine (a melatonergic antidepressant) and the two key ω-3 PUFAs – EPA and DHA.

3.4.1.1 IL-6 protein secretion

In order to determine whether these antidepressants had immunomodulatory effects I measured IL-6 in the cell supernatant at two doses for each antidepressant after 24 hours of co-treatment with IL-1β (Figure 34). The presence of venlafaxine (1µM) decreased IL-6 protein levels secreted in the supernatant by 14% compared to IL-1β alone (p<0.001), whereas sertraline (1µM) showed the opposite effect, with a 27% increase in IL-6 levels (p<0.001). Agomelatine and moclobemide had no significant effect on the IL-6 levels at either of the doses tested.

I also sought to investigate the effects of the ω-3 PUFAs, EPA and DHA, on immune processes. Upon co-incubation with IL-1β, EPA (10µM) decreased IL-6 secretion by 8% (p<0.01), whereas DHA (10µM) resulted in an increase of 12% (p<0.01). Smaller doses of EPA or DHA had no significant effect on the levels of IL-6 secreted.
Figure 34 Effects of antidepressant compounds on protein expression in hippocampal progenitor cells.

Modulation of IL-1β-induced IL-6 secretion in the human hippocampal progenitor cells by monoaminergic antidepressants or ω-3 PUFAs. Cells were incubated with IL-1β (10ng/mL) in the absence and presence of each drug for 24 hours. The levels of IL-6 secreted in the supernatant are presented as percentage change from IL-1β treatment alone. (a)-(d) Venlafaxine (Ven, 1µM) exerted anti-inflammatory effects, decreasing IL-6 secretion compared with IL-1β alone; sertraline (Sert, 1µM) was pro-inflammatory, enhancing the inflammatory response to IL-1β; agomelatine (Ago) and moclobemide (Moc) had no significant immunomodulatory effects at the doses tested. Eight independent experiments were conducted (n=8). (e), (f) EPA was anti-inflammatory, significantly reducing the inflammatory response to IL-1β, while DHA had the opposite effect. At least six independent experiments were conducted (n=6-8). Data are shown as mean ± SEM, *p<0.05, **p<0.01, ***p<0.001 compared with IL-1β treatment alone.
3.4.1.2 Cytokine gene expression

In order to investigate whether the mechanisms underlying the changes detected in IL-6 secretion were due to differences in gene expression, I examined the effect of co-incubation with IL-1β and the two antidepressants shown to affect IL-6 protein levels (venlafaxine, sertraline, DHA and EPA) and the two ω-3 PUFAs on levels of IL-6 mRNA expression. On its own, IL-1β (10ng/mL) stimulation time-dependently induced IL-6 mRNA levels, with the greatest effect occurring at 24 hours of incubation (36-fold increase, p<0.01, Figure 35a). Co-incubation with sertraline (1µM) for 24 hours increased IL-6 mRNA expression by 17% (p<0.05, Figure 35b) compared with IL-1β alone, consistent with the direction and magnitude of change of levels of IL-6 protein at the same time point (Figure 34d, right hand column). There was no significant effect of co-incubation of venlafaxine (1µM) on IL-6 mRNA levels (Figure 35b). Co-incubation of EPA (10µM) with IL-1β decreased IL-6 mRNA levels by 20% (p<0.01, Figure 35b), in line with changes in IL-6 protein (Figure 34e, right hand column). However, co-incubation of DHA (10µM) along with IL-1β had no significant effect on IL-6 mRNA levels (Figure 35b).

Although secretion of IL-1β protein into the supernatant could not be used as a measure of inflammatory response (as it would be confounded by the addition of IL-1β directly to the supernatant), gene expression of IL-1β is not confounded in the same manner and so was also measured in this paradigm to investigate the effects of the antidepressant compounds on inflammatory gene expression. IL-1β treatment time-dependently induced its own expression, with maximal expression evident after 24 hours of treatment (372 fold increase, p<0.05, n=6). Only DHA (10µM) decreased the expression of IL-1β mRNA at this time point,
with the other antidepressant compounds having no significant effect (Figure 36).
Antidepressant compounds have differential effects upon IL-6 mRNA expression.

(a) The induction of IL-6 gene expression by IL-1β (10ng/mL) was time-dependent. Results are expressed as fold change vs time zero, and IL-6 levels were normalised using beta-actin and GAPDH. At least four independent experiments were conducted (n=4-6). (b) The four antidepressant compounds with modulatory effects on IL-6 protein levels were co-incubated with IL-1β (10ng/mL) for 24 hours and levels of IL-6 mRNA measured. Results are presented as percentage change from IL-1β incubation alone. (b) Venlafaxine (1µM) had no significant effect, while sertraline (1µM) increased IL-6 mRNA by 17% (p<0.05). Co-incubation with EPA (10µM) decreased IL-6 mRNA levels by 20% (p<0.01), while DHA (10µM) had no significant effect. At least seven independent experiments were conducted (n=7-12). Data are shown as mean ± SEM, *p<0.05, **p<0.01 compared with IL-1β treatment alone.
Antidepressant compounds have differential effects upon IL-1 mRNA expression.

(a) Treatment with IL-1β (10ng/mL) time-dependently increases IL-1 mRNA expression. (b) 10μM DHA upon co-incubation with IL-1β significantly reduces IL-1 mRNA expression, while EPA, sertraline and venlafaxine have no significant effect. Data are presented as percentage change from IL-1β (10ng/mL) treatment alone. * p<0.05 compared with IL-1β treatment alone. n=3-6
3.4.1.3 Transcriptional regulation

In order to determine whether the antidepressant compounds modulated immune processes via mechanisms involving NF-κB, a key transcriptional regulator of cytokine gene expression, we studied NF-κB transactivation by quantifying activated NF-κB. IL-1β (10ng/mL) induced NF-κB activation in a time-dependent manner, producing a 7-fold increase at 24 hours (p<0.001, Figure 37a). Co-incubation with venlafaxine (1µM) reduced NF-κB transactivation by 22% (p<0.05, Figure 37b), consistent with changes to IL-6 protein secretion (Figure 34a, right hand column). Co-incubation with EPA (10µM) also decreased NF-κB transactivation by 15% (p<0.001, Figure 37b), again, consistent with reduced IL-6 protein and mRNA levels (Figure 34e, right hand column). However, in contrast with the increase of protein and/or mRNA levels of IL-6, co-incubation of IL-1β with either sertraline (1µM) or DHA (10µM) decreased NF-κB DNA binding (by 12% and 38%, respectively; p<0.001, Figure 37b).
Figure 37 All four antidepressant compounds reduced NF-κB activity.

(a) Stimulation of the neural stem cells with IL-1β (10ng/mL) alone produced a time-dependent increase in NF-κB transactivation and DNA binding. Results are presented as fold change vs baseline levels of activation. At least twelve independent experiments were conducted (n=12-14). (b) All four immunomodulatory antidepressant compounds reduced NF-κB activity when co-incubated with IL-1β (10ng/mL). Results are presented as percentage change of NF-κB activity from IL-1β alone. At least six independent experiments were conducted (n=6-15). Data are shown as mean ± SEM, *p<0.05, **p<0.01, ***p<0.001 compared with IL-1β treatment alone.
3.4.1.4 Effect of antidepressant compounds on cytokines and chemokines

As the effects of these antidepressant compounds was somewhat inconsistent across IL-6 protein secretion, inflammatory gene expression and NF-κB expression I wanted to see whether broader anti- or pro-inflammatory effects of these antidepressant compounds could be more clearly determined by looking at wider modulation of chemokine and cytokine secretion. Therefore, I compared the wider inflammatory profiles (cytokines and chemokines) stimulated by IL-1β with and without each of the four immunomodulatory antidepressant compounds (Table 17). With regards to IL-6, the direction of changes detected in the multiplex array were identical to those detected using ELISA (in the experiments described above), and the magnitude very similar; discrepancies in statistical significance were likely due to the superior accuracy of ELISA.

Co-incubation with sertraline (1µM) increased levels of all cytokines and chemokines that were expressed in detectable levels (range +4% to 24%), showing the same direction of effect demonstrated on IL-6 levels via ELISA, reaching significance for IFN-α (+19%, p<0.05), with the exception of the chemokine RANTES (-15%, n.s.). Co-incubation with EPA (10µM) decreased the levels of all chemokines and cytokines measured, again consistent with the findings of secreted IL-6 protein via ELISA, reaching significance for IL-15 (-17%, p<0.01), IL-1RA (-23%, p<0.01), IP-10 (-16%, p<0.05), and IL-6 (-14%, p<0.01). Co-incubation with DHA (10µM) increased the levels of all cytokines and chemokines, again consistent with changes in secreted IL-6 levels via ELISA, reaching significance for IL-1RA (+29%, p<0.01), IFN-α (+16%, p<0.01), IL-15 (+12%, p<0.05) and IL-6 (+9.2%, p<0.01), with the exception of RANTES.
(-14%, p<0.001, Table 17). Co-incubation with venlafaxine (1µM) had mixed effects, with changes in both directions, though none reached significance.
Table 17 Modulation of cytokines and chemokines by antidepressant compounds.

Modulation of cytokines and chemokines by antidepressant compounds is largely consistent with IL-6 changes. The effects of antidepressant compounds are shown as percentage change in levels of the seven cytokines and chemokines in detectable levels in the supernatant compared with IL-1β (10ng/mL) treatment alone. At least six independent experiments were conducted (n=6-8). Data are presented as mean values, *p<0.05, **p<0.01, ***p<0.001 compared with IL-1β treatment alone.

<table>
<thead>
<tr>
<th>Protein</th>
<th>IL-1β + 1µM Ven</th>
<th>IL-1β + 1µM Sert</th>
<th>IL-1β+ 10µM DHA</th>
<th>IL-1β + 10µM EPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8</td>
<td>-10.2 %</td>
<td>3.7 %</td>
<td>9.2 %</td>
<td>-13.4 %</td>
</tr>
<tr>
<td>IL-15</td>
<td>7.7 %</td>
<td>11.6 %</td>
<td>12.2 % *</td>
<td>-16.7 % **</td>
</tr>
<tr>
<td>IL-1RA</td>
<td>0.5 %</td>
<td>23.9 %</td>
<td>29.4 % **</td>
<td>-23.3 % **</td>
</tr>
<tr>
<td>IFN-α</td>
<td>1.4 %</td>
<td>18.6 % *</td>
<td>15.8 % **</td>
<td>-4.5 %</td>
</tr>
<tr>
<td>IP-10</td>
<td>-15.5 %</td>
<td>5.8 %</td>
<td>3.5 %</td>
<td>-16.1 % *</td>
</tr>
<tr>
<td>IL-6</td>
<td>-6.6 %</td>
<td>17.1 %</td>
<td>9.2 % **</td>
<td>-13.9 % **</td>
</tr>
<tr>
<td>RANTES</td>
<td>-2.5 %</td>
<td>-15.1 %</td>
<td>-13.9 % ***</td>
<td>-9.6 %</td>
</tr>
</tbody>
</table>
3.5 Modulation of neurogenesis by antidepressant compounds

The ability of antidepressant compounds to abrogate the decrease in neurogenesis caused by depressogenic insults in animals has led to the possibility that this mechanism also underlies their efficacy in humans. Our lab has previously demonstrated that antidepressants increase neurogenesis and can abolish the decrease in neurogenesis induced by glucocorticoids. Furthermore, our lab has also demonstrated that IL-1β reduces neurogenesis in the human hippocampal progenitor cells. Here I wished to extend these findings by investigating whether monoaminergic antidepressants and PUFAs could abrogate the decreased neurogenesis caused by inflammation in human hippocampal progenitor cells, and to investigate the mechanism by which they may exert these effects.

3.5.1 Effect of antidepressant compounds on neurogenesis

In order to confirm the effect of inflammation on neurogenesis in the human hippocampal progenitors I used a protocol of treatment with vehicle or IL-1β (10ng/mL) for three days of proliferation, followed by seven days of differentiation. The percentage of cells that stained positive for DCX (a marker of immature neurons) and MAP2 (a marker of mature neurons) was used to establish the degree of neurogenesis in the cell population. IL-1β treatment reduced neurogenesis significantly (MAP2: -54.2+/−8.2%, p<0.0001, n=4; DCX: -65.6+/−11.4%, p<0.0001, n=4, Figure 38), consistent with previous work implicating IL-1β as negative regulator of neurogenesis.
Figure 38 IL-1β decreases neurogenesis in human hippocampal cells

Data is presented here as percentage change compared with vehicle treated cells. (a) Treatment with IL-1β decreased the percentage of MAP2-positive cells (mature neuronal phenotype) in differentiated cultures; and (b) IL-1β induced decrease in percentage of DCX-positive cells (immature neuronal phenotype). ***p<0.001 compared to IL-1β treated condition. n=4
I next wanted to establish whether the monoaminergic antidepressants examined previously (venlafaxine and sertraline, at doses of 1μM) and the PUFAs (EPA and DHA, at doses of 10μM) would be able to reverse the effects of IL-1β-induced reduction of neurogenesis. The hippocampal progenitor cells were incubated with IL-1β, with or without the antidepressant compounds throughout three days of proliferation and seven days of differentiation, at which point neurogenesis was assessed by quantification of MAP2-positive and DCX-positive cells. Co-incubation of venlafaxine (+56.3+/−19.9%, p<0.01, n=4), sertraline (+38.7+/−12.2%, p<0.01, n=4) and DHA (+71.7+/−16.4%, p<0.001, n=4) along with IL-1β all increased the expression of MAP2-positive neurons compared with IL-1β treatment alone, while co-incubation with EPA had no significant effect (Figure 39a). These results were in part reflected in changes in DCX-positive neurons, where co-incubation with venlafaxine (+26.5+/−10.9%, p<0.05, n=4) and DHA (71.7+/− 16.4%, p<0.001, n=4) showed increases compared with IL-1β alone (Figure 39b). These findings are consistent with the notion that antidepressant compounds may exert their effects by reversing the effects of inflammation on neurogenesis.
Figure 39 Monoaminergic antidepressants and PUFAs modulate neurogenesis in human hippocampal cells

Data is presented here as percentage change compared with IL-1β treated cells. (a) Treatment with venlafaxine, sertraline and DHA partially blocked IL-1β-induced decreases in percentage of MAP2-positive cells (mature neuronal phenotype); (b) venlafaxine and DHA also partially blocked IL-1β-induced decreases in percentage of DCX-positive cells (immature neuronal phenotype). *p<0.05, **p<0.01, ***p<0.001 compared to IL-1β-treated condition.
3.5.2 The effect of antidepressant compounds on the kynurenine pathway

It has previously demonstrated that alterations to the kynurenine pathway are likely to be important in the effect of IL-1β on reducing neurogenesis. Treatment with IL-1β upregulates several components of the neurotoxic arm of the kynurenine pathway and blockade of this pathway can partially ameliorate the effect of IL-1β on neurogenesis. I therefore wished to investigate whether the ability of these antidepressant compounds to abrogate the effects of IL-1β on neurogenesis involved effects on the kynurenine pathway. I did this in two ways – looking at modulation of mRNA key enzymes in the kynurenine metabolic pathway – IDO, KMO and KYNU – and looking at the key neurotoxic metabolite of the kynurenine pathway – quinolinic acid (QUIN).

I found that IL-1β (10ng/mL) upregulated the expression of three enzymes in the kynurenine pathway in human hippocampal progenitor cells: IDO (216.8+/-64.9 fold change, p<0.05, n=4), KMO (11.0+/-3.1 fold change, p<0.01, n=4) and KYNU (26.4+/-9.7 fold change, p<0.05, n=6) (Figure 40a), consistent with previous findings in our lab (Zunszain et al 2012). Upregulation of these genes contributes to a shift in balance towards the kynurenine pathway away from serotonin, and in turn towards the neurotoxic end-products of the kynurenine pathway.

In order to understand whether the antidepressant compounds of interest (venlafaxine, sertraline, EPA and DHA) might modulate the effects of inflammation on neurogenesis by reversing the effects of IL-1β on the kynurenine pathway I co-incubated these compounds with IL-1β for three days of proliferation and seven days of differentiation. DHA had the most significant modulating effects on IL-1β-induced changes to the kynurenine pathway.
enzymes, decreasing IL-1β-induced IDO upregulation by 34+/-7% (p<0.01, n=4) and decreasing KMO upregulation by 27+/-7% (P<0.01, n=4) while upregulating KYNU by 78+/-24% (p<0.05, n=4) (Figures 40b,c,d). Sertraline also reduced the upregulation of KMO by 30+/-9% (p<0.01, n=6), had no significant effect on IDO regulation and significantly upregulated KYNU by 21+/-8% (p<0.05. n=6) (Figures 40b,c,d). Venlafaxine had no significant modulatory effect on any of the three kynurenine metabolic genes, while EPA showed no significant effect on IDO or KYNU but significantly up-regulated KMO by 22+/-6% (Figures 40b,c,d).
Figure 40 Antidepressant compounds modulate IL-1β-induced changes to the kynurenine pathway

(a) Treatment of the progenitor cells with IL-1β (10ng/mL) for three days of proliferation and seven days of differentiation significantly upregulated gene expression of all three enzymes in the kynurenine pathway. (b) Co-treatment of the cells for this period with 10μM of DHA or 1μM of sertraline significantly increased expression of KYNU, while co-treatment with 1μM of venlafaxine or 10μM of EPA had no significant effect. (c) Co-treatment with 10μM DHA significantly decreased expression of IDO, while none of the other compounds had a significant effect. (d) Co-treatment with 10μM of DHA or 1μM of sertraline significantly reduced the upregulation of KMO produced by IL-1β stimulation alone, while co-incubation of 10μM EPA significantly increased the upregulation of KMO, and 1μM of venlafaxine had no significant effect.
In order to further explore the mechanism underlying the ability of antidepressant compounds to abrogate the effects of inflammation on neurogenesis I looked at the potentially neurotoxic end product of the kynurenine metabolic pathway – quinolinic acid – which has been related to changes in neurogenesis previously (Figure 41). Treatment with IL-1β (10ng/mL) for three days during proliferation and seven days during differentiation increased quinolinic acid (QUIN) levels detected in the supernatant by 123.5 +/-32% (P<0.01, n=4) compared with vehicle treatment. Co-incubation with sertraline (1μM) completely abrogated this rise in QUIN (-123.8+/-35% compared with IL-1β treatment alone, p<0.05, n=4) and co-incubation with DHA significantly reduced this rise in QUIN (-98+/-24% compared with IL-1β treatment alone, p<0.01, n=4). Venlafaxine showed a trend towards decreasing QUIN (-79+/-38%, p=0.079, n=4), while EPA showed a non-significant reduction in QUIN levels.
Antidepressant compounds reduce the rise in quinolinic acid caused by IL-1β treatment

After treatment with IL-1β treatment during three days of proliferation and seven days of differentiation there was a significant increase in QUIN detected in the supernatant. Co-incubation with all four antidepressant compounds – venlafaxine (1μM), sertraline (1μM), EPA (10μM) and DHA (10μM) – decreased the amount of QUIN produced, reaching significance for sertraline and DHA. * p<0.05, ** p<0.01 compared with vehicle treatment or IL-1β treatment alone, as indicated.
3.6 **Chromatin Immunoprecipitation of glucocorticoid receptor targets**

We have previously demonstrated in our lab that the SSRI, sertraline, and dexamethasone both affect neurogenesis in hippocampal progenitor cells through a mechanism involving the glucocorticoid receptor (Anacker 2011). Furthermore, treatment with sertraline or dexamethasone, and co-treatment with sertraline and dexamethasone induce distinct patterns of phosphorylation of serine residues in the glucocorticoid receptor (known to regulate GR nuclear translocation and GR-dependent gene transcription) and, consistent with this, have differential effects on the GR-target genes, p27Kip1 and p57Kip2, which promote cell cycle exit and increase neuronal differentiation in the developing rat brain.

To more fully understand the genetic programs initiated by activation of the GR by dexamethasone, sertraline and their co-treatment I wanted to identify the differential genomic targets of the GR during treatment by chromatin immunoprecipitation (ChIP). This technique involves fixation of the GR to its genomic targets, breaking DNA molecules into smaller fragments and selectively enriching those portions of the DNA which have bound GR by means of a GR antibody using a magnetic process. This technique would allow identification of the differential targets of the GR when activated by antidepressants, glucocorticoids or co-treatment with both. Delineation of the genetic pathways for increasing or decreasing neurogenesis might present novel antidepressant target genes.
Chromatin immunoprecipitation is widely regarded as challenging to optimise in new cellular specimens and so I attempted to optimise a protocol for chromatin immunoprecipitation in the human neural progenitor cell line. Validation of an effective GR ChIP protocol involves demonstration of enrichment of known GR-target genes in the immunoprecipitated sample (meaning that fragments of the DNA that contain response elements to the GR have been successfully enriched by means of selective immunoprecipitation). For example, SGK1 is known to be upregulated by dexamethasone treatment and to contain glucocorticoid response elements within its sequence, while the gene DSCAM is not upregulated by dexamethasone treatment, nor does it contain GRE response elements. The demonstration that SGK1 is more highly expressed than DSCAM in a dexamethasone-treated DNA sample immunoprecipitated by GR antibody, in comparison to control conditions (vehicle-treated cells, and immunoprecipitation with non-specific IgG antibody) would indicate effective immunoprecipitation. Further characterisation of the immunoprecipitated gene fragments (by sequencing) would then allow identification of unknown targets of the GR, when activated by glucocorticoids, sertraline or by co-treatment with both.

There are several key steps in this procedure requiring optimisation for a particular cell type, treatment and antibody. Following fixation of the cells (causing cross-linking of the GR to its genomic targets), DNA must be divided into smaller fragments. If the fragments formed are too large then the resolution of the ChIP assay will be decreased. For example, if a portion of a gene that contains a GR binding site is separated by 1200 base pairs from a portion of a gene that does not contain a GR binding site, DNA fragments of larger than this size will prevent detection of the enrichment of the GR-target gene. On the
other hand, if the fragments are too small then it is difficult to amplify the target fragment (the amplimer) using primer sequences. Consequently, it is considered optimal to fragment DNA into sections between 200 and 500 base pairs in length, especially for the purpose of sequencing of fragments (Das et al, 2004).

There are two principle means employed to break chromatin into optimally sized fragments – using either sonication or enzymatic digestion. Given the difficulty in achieving effective fragmentation in ChIP I pursued both means for fragmentation of DNA.

3.6.1 Optimisation of chromatin in human hippocampal stem cells by shearing

After culturing of hippocampal progenitor cells (30 million cells in each condition) and treatment with dexamethasone (1μM) or vehicle for one hour (determined by maximal effects on GR serine residue phosphorylation (Anacker et al, 2011)), cells were fixed for 5 minutes with 1% formaldehyde. Following fixation of the GR to its genomic targets the chromatin must be fragmented.

One commonly used method for fragmenting DNA is through sonication, whereby pulsed sounds waves cause physical breakage of the DNA molecules. Sonication cycles consist of 30 seconds of pulsed sound waves followed by 30 seconds of rest. As chromatin configuration may vary based on drug treatment optimal shearing conditions had to be established not just for each cell type, but also for each treatment condition individually.
3.6.1.1 Shearing of chromatin

The factors affecting sonication include the cell type, cell culture conditions used, quantity of cells, volume of the sample sonicated, the depth of the sonication probe, sonication strength, degree of crosslinking and the duration of sonication (Das et al., 2004; Lee et al., 2006).

Undersonification can be identified by the size of the de-crosslinked, purified DNA fragments being too large on gel electrophoresis. Oversonification is somewhat harder to identify as there is evidence that there is a physical limit to sonication and that additional sonication past this point will not result in further visible shearing on a gel but may affect results on analysis (Lee et al., 2006). However, the best way to initially establish adequate sonication is through gel electrophoresis of de-crosslinked purified sonicated DNA.

In order to optimise the amount of shearing I performed titration experiments with increasing numbers of sonication cycles, while keeping the other variables as constant as possible (30 million cells per condition, cultured and treated under uniform conditions, five minutes of cross-linking with 1% formaldehyde, 0.5 mL of sonication volume with probe at constant depth, sonicator on highest setting). After each block of cycles a sample of chromatin was taken (25 uL in each case), de-cross-linked, purified and run on a 1% agarose gel to determine the range of chromatin sizes. A representative example is shown below with increasing number of sonication cycles associated with shortening of the DNA fragments – both the range of sizes (the height of the fluorescent band) and the most common size of fragments (represented by the portion of the band with greatest intensity) (Figure 42).
Figure 42 Shearing of samples by sonication to optimal size

(a) $3 \times 10^7$ human progenitor cells were treated with DEX 1μM or vehicle for one hour (DEX samples shown here), crosslinked for five minutes with 1% formaldehyde, and then sonicated under the following conditions: Bioruptor UCD-200; power maximum; 40 cycles (30 seconds sonication, 30 seconds rest). Samples were removed after every 5 cycles of sonication, de-crosslinked and DNA-purified, and run on a 1% agarose gel with ethidium bromide. Lanes with molecular weight ladders (each step represents a 100 base pair (bp) interval, with the first and second more intense steps of the ladder representing 500 bp and 1000 bps (from bottom to top)) sit on both sides of the gel and number of sonications after which samples were removed is labelled under each lane (from left to right). For example, Lane 2 contains a sample of chromatin subjected to 10 sonication cycles. Some increased intensity of signal can be seen from 100 to 600 bps in length, but the signal is distributed throughout the entire spectrum of the ladder. This indicates a wide distribution of the different fragments from 100 bps or less, up to hundreds of bps in length, consistent with inadequate fragmentation. Each lane towards the right contains a sample subjected to a further five cycles of sonication. As the number of sonication cycles increases, a greater intensity of the band between 100 and 400 bps is evident, representing a greater proportion of the DNA fragments of the desired length. Lane 9 contains a sample with the maximum number of sonication cycles performed (40) and contains fragments of length 100 to 300 bps, smaller than optimal. Based on this graded shearing, 15 sonication cycles yielding high representation of 100 to 500 bp length fragments was considered optimal for this treatment. (b) This gel shows two samples, one treated with DEX 1μM for one hour, and the other with vehicle for one hour. Otherwise, DNA was subject to the same treatment. The greatly increased shearing of the vehicle sample compared to the DEX samples is due to the greater amount of DNA harvested from the DEX sample. This image serves to emphasise the importance of optimising sonication for each sample of chromatin.
3.6.2 Optimisation of chromatin in human hippocampal stem cells by MNase digestion

An alternative method used for the fragmentation of DNA involves using the enzyme micrococcal nuclease. This enzyme makes double-stranded cuts between nucleosomes. As a nucleosome consist of approximately 147 base pairs of DNA (wrapped around a histone octamer), this digestion yields fragments of integer number multiples of 147 base pairs (that is, mononucleosomes, dinucleosomes, trinucleosomes, etc) (Yamasaki et al., 2007).

A preponderance of mono- and dinucleosomes is considered optimal for ChIP-seq (Zhao, private communication). Figure 43 illustrates the process of optimising conditions to yield the desired fragment length.
Figure 43 Optimisation of fragmentation of DNA using micrococcal nuclease

All samples presented were treated with DEX 1μM for one hour (with the exception of the final sample in (d) treated with vehicle for one hour), cross-linked for 5 minutes using 1% formaldehyde, prepared according to the manufacturer's protocol before digestion with micrococcal nuclease (MNase). After digestion, and de-crosslinking, the DNA samples were purified and run on a 1% agarose gel. (a) Samples were treated with 0.8 uL (left lane) and 1.2 uL (right lane) of MNase per million cells for twenty minutes. Fragments of DNA from bottom to top represent mononucleosomes (approximately 147 base pairs of DNA), dinucleosomes, trinucleosomes, etc. The increased proportional intensity of the lower weight nucleosome fragments in the right lane represents increased digestion of the DNA fragments by an increased dose of MNase. However, optimal conditions would produce fragments with a greater proportion of mono- and di-nucleosomes. (b) Samples prepared under the same conditions were digested with (from left to right) 1.6, 3.2 or 4.8 uL of MNase per million cells for twenty minutes. An increased proportion of shorter DNA fragments is evident in this gel. However, there is too great a proportion of DNA fragments accounted for by mono-nucleosomes and so these samples were considered over-digested. (c) Samples prepared under the same conditions as above were digested with 1.6uL of MNase per million cells for 10 minutes (left lane) and 15 minutes (right lane). (d) Samples treated with DEX 1μM (left lane) and vehicle (right lane) both digested by the same dose of enzyme for the same period of time show differences in extent of digestion, largely due to amount of DNA in each sample, demonstrating the need to optimise digestion for each sample separately.
3.6.3 Optimisation of immunoprecipitation

Following fixation and fragmentation of the DNA, DNA fragments are immunoprecipitated with a specific antibody. There are two major aspects of immunoprecipitation requiring optimisation: determining an effective antibody for immunoprecipitation, and determining the optimal conditions for efficient antibody immunoprecipitation (involving variation of salt concentrations, and washing conditions). Efficient immunoprecipitation can be validated by identification of enrichment of target genes, compared with control genes, in the immunoprecipitated samples through qPCR.

In order to confirm first that good quality chromatin had been formed in the previous steps, a ChIP quality antibody to RE-1 silencing transcription factor (REST) was employed. REST has a known target region in the synaptosomal-associated protein 25 (SNAP25) gene and no known target region in the M4 cholinergic muscarinic receptor gene (M4), used as a control gene. Immunoprecipitation with the anti-REST antibody demonstrated pronounced enrichment for SNAP25 compared with M4 for both DEX-treated and VEH-treated chromatin, an enrichment not present in IgG-treated samples (Figures 44 and 45). This experiment confirms the formation of high quality chromatin of adequate shearing.
After fixation and sonication a sample of DNA is removed (referred to as the 'input'). Other DNA was immunoprecipitated with the anti-REST antibody or a non-specific IgG antibody. In the REST immunoprecipitated samples (DEX- and vehicle-treated) there is a greater amount of the REST-target gene SNAP25 detected than the control gene M4. This enrichment is not present in samples immunoprecipitated with non-specific IgG antibody, demonstrating its specificity to REST-immunoprecipitated samples. Gene expression is represented as percentage of expression in input samples.
Figure 45 Confirmation of chromatin formation using validated antibody, assessed as compared to IgG control

Samples were the same as presented in Figure 44 but are here presented as fold change compared with the matching IgG antibody-immunoprecipitated samples. Samples treated with the REST antibody demonstrate clear enrichment of target genes over control genes.
The final step of validation of the ChIP involves demonstration of enrichment of GR-target genes compared with control genes in anti-GR antibody treated samples, compared with non-specific IgG-treated samples. Several genes were identified in the literature as GR-target genes containing GRE binding regions: MT2A, SGK1, SLC19A2, and GILZ. Primers for the regions in these genes adjacent to the GRE binding regions were either taken from papers where they had successfully been used for GR ChIP experiments or designed using Primer3. DSCAM was used as a control gene. Several protocols, involving variations in a number of variables were attempted. The variation that produced the best results involved pre-clearing the chromatin (that is, adding magnetic protein beads to the chromatin, incubating and removing before using the beads to immunoprecipitate in order to reduce non-specific binding to the beads), pre-blocking the beads (multiple rounds of washing with buffers containing BSA to block non-specific sites on the beads before use in the immunoprecipitation), incubating chromatin and antibody overnight without beads) and increasing the length of the wash steps.

The two most recent qPCR experiments are presented below (Figure 46 and Figure 47), using two different antibodies, one from Thermo Scientific and one from Diagenode, using sonicated samples. Significant enrichment (normally defined as a two-fold increase) was identified in the first instance using these genes compared with DSCAM, between GR-immunoprecipitated samples and IgG-immunoprecipitated samples. In the first experiment, enrichment of GR-target genes was clear, with SGK1 enriched in the dexamethasone-treated sample by 8.6 fold with the Thermo Scientific antibody, and 7.4 fold with the Diagenode antibody, compared with non-specific IgG, evidence of specific
enrichment of genomic fragments attached to the GR (Figure 46). There was also clear enrichment of SGK1 in the dexamethasone-treated sample compared with the ethanol-treated sample (8.6 fold vs 1.15 fold with the Thermo Scientific antibody, and 7.44 fold vs 1.77 fold with the Diagenode antibody), evidence of a treatment-specific enrichment as expected. There was similar evidence of 2- to 4-fold enrichment in dexamethasone-treated samples for SLC19A2, GILZ and MT2A for both antibodies, compared with IgG and with ethanol-treatment samples (Figure 46). There was, however, some evidence of enrichment of DSCAM, suggesting that it is not an internal control gene in our samples. The second repeat of this qPCR experiment does not provide evidence of enrichment, with similar levels of expression in the dexamethasone- and ethanol-treated samples for all four GR-target genes and DSCAM, suggesting that there was higher background in the second repeat (Figure 47). This degree of variability necessitates further repeats in order to confirm enrichment, but the results of the first repeat allows the tentative conclusion that the ChIP is enriching GR target genes.
Figure 46 Enrichment of GR-target genes

Data are presented as fold enrichment of expression compared with IgG immunoprecipitation of the same condition. Level of expression for IgG immunoprecipitation is set to 1 in each instance. This repeat shows clear enrichment of SGK1 in dexamethasone-treated samples compared with ethanol-treated samples. This enrichment is also clear compared with DSCAM enrichment in dexamethasone-treated samples. However, DSCAM does appear, unexpectedly, to be enriched in dexamethasone-treated samples. Consequently, SLC19A2, GILZ and MT2A show marked enrichment in the dexamethasone-treated condition compared with the ethanol-treated condition but not compared to the internal control DSCAM. These effects are evident for both antibodies, but more strongly for the Thermo Scientific GR antibody. Overall, this is evidence of enrichment but suggests DSCAM is not a control.
Figure 47 Enrichment of GR target genes – second repetition

Data are presented as above. Enrichment of GR-target genes in the dexamethasone-treated condition are not clearly present compared with either the internal control or compared with ethanol-treated samples. It is likely that this variation compared to the previous experiment is due to higher background and necessitates further repeats of the experiment.
4 Discussion

4.1 Summary of findings

In this thesis I have investigated the relationship between the glucocorticoid system and inflammation in human hippocampal progenitor cells, with potential clinical relevance to depression, and have also conducted a systematic review of the literature and meta-analysis to examine the relationship between these two systems in depressed subjects. In addition, I have investigated the action of monoaminergic antidepressants and polyunsaturated fatty acids in these human neural cells on immune processes, neurogenesis and the kynurenine pathway. Furthermore, I have established an important technique – chromatin immunoprecipitation – in the hippocampal progenitor cells which will be vital for exploring the activity of the glucocorticoid receptor after activation by glucocorticoids or antidepressants, a process found to be involved in their effect on hippocampal neurogenesis.

The main findings of this thesis are summarised below:

4.1.1 Aim 1

Aim 1 of this thesis was to review the clinical literature to determine whether clinical studies in depressed subjects were consistent with a theory holding that glucocorticoid resistance, or, alternatively, that the pro-inflammatory properties of glucocorticoids, accounted for the seeming contradiction of high levels of cortisol and pro-inflammatory cytokines detected in depressed patients. The large majority of studies (six out of eight that met criteria for inclusion) found evidence of increased activity of the innate immune system, either at baseline or upon stimulation, in association with evidence of glucocorticoid resistance. Only two of these six studies detected increased levels of cortisol in addition to
glucocorticoid resistance and increased inflammation. Of the two remaining studies, one found glucocorticoid resistance without evidence of increased cortisol or increased inflammation, and the other reported increased inflammation, without evidence of any abnormalities in cortisol levels of glucocorticoid resistance. Notably, no study detected increased cortisol and increased inflammation, without detecting glucocorticoid resistance: the circumstance predicted by a theory focused on pro-inflammatory glucocorticoid actions.

Overall, the co-occurrence of glucocorticoid resistance and increased immunity was the most striking finding in the review, with three studies reporting significant positive associations between these characteristics. Meta-analysis of this relationship in four studies, with relevant data available, demonstrated a strong positive association, though a trend towards heterogeneity was observed, indicating variation in the size of the association between studies. Within the constraints of interpretation imposed by the small number of studies captured, these findings suggest that glucocorticoid resistance associates with increased levels of inflammation in depressed patients, supporting the notion that glucocorticoid resistance is a key driver of increased inflammation in these patients. Conversely, there was little evidence found to support pro-inflammatory properties of glucocorticoids as the driver of inflammation in depressed subjects.

4.1.2 Aim 2

Aim 2 of this thesis was to gain mechanistic insights into the pathogenesis of depression by investigating the interaction of neuroimmune and neuroendocrine signalling in a cell model relevant to depression. I wanted to establish whether glucocorticoids could be pro-inflammatory in human neural cells, understand the
determinants of whether they exert pro- or anti-inflammatory effect, and determine whether the mechanism underlying pro-inflammatory effects is consistent with glucocorticoid resistance or up-regulation of pro-inflammatory pathways.

When glucocorticoids were co-incubated with inflammatory stimulation they exhibited canonical anti-inflammatory properties in a dose-dependent manner. However, I found that glucocorticoids could exhibit pro-inflammatory properties when their administration preceded the inflammatory stimulus, whereby the inflammatory response to IL-1β was potentiated, as measured by increased levels of secreted cytokines – IL-6, IL-8, IP-10, IL-1RA, RANTES and MCP-1 – in the supernatant. The degree of potentiation of the inflammatory response (as measured by IL-6) was dose-dependent, with intermediate doses of both dexamethasone and cortisol inducing the greatest potentiation of the inflammatory response, while smaller and larger doses had smaller effects. The pro-inflammatory properties of glucocorticoids was critically time-dependent with potentiation effects only exhibited when the interval between glucocorticoid treatment and inflammatory stimulation was 24 hours (for a dexamethasone dose of 1μM), and not present for time intervals of lesser or greater periods.

The potentiation effects of glucocorticoid pre-treatment was dependent on activation of the GR during glucocorticoid pre-treatment, as co-treatment with RU486 during this period abrogated the potentiation effect, with a non-significant trend towards reduction for treatment with RU486 during the rest interval alone. No change in mRNA expression of NF-κB or the GR was seen at any dose of dexamethasone pre-treatment. There was a non-significant trend towards increased glucocorticoid resistance, as measured by the ability of dexamethasone to inhibit inflammatory response, following incubation with
dexamethasone, indicating some evidence that this induced resistance may be a mechanism by which pro-inflammatory effects are produced. Genome-wide gene expression analysis demonstrated some evidence that glucocorticoid treatment upregulated immune-related genes, both after 24 hours of dexamethasone treatment in combination with immune stimulation, and when immune stimulation followed 48 hours after dexamethasone treatment. There was also some evidence that glucocorticoid resistance was induced by 24 hours of dexamethasone treatment 48 hours before gene expression was analysed. This suggests that both up-regulation of immune pathways and the induction of glucocorticoid resistance may underpin the potentiation effects of glucocorticoid pre-treatment, though further research is needed.

4.1.3 Aim 3

Aim 3 of this thesis was to investigate the hypothesis that antidepressant compounds from different chemical classes might converge in similar immunomodulatory properties as a final common pathway of action. I found that the six different compounds investigated exhibited differing effects on the immune responses of the human hippocampal progenitor cells. The SNRI, venlafaxine, and the ω-3 PUFA, EPA, exerted anti-inflammatory effects on the neural cells; while the SSRI, sertraline, and the ω-3 PUFA, DHA, exerted pro-inflammatory effects. The melatonergic agonist, agomelatine, and the MAO-I, moclobemide, demonstrated no immunomodulatory properties (as measured by IL-1β-induced IL-6). Interestingly, all four compounds that exerted immunomodulatory effects decreased NF-κB activation, a critical step in the transcription of pro-inflammatory cytokines, suggesting the possibility that other transcription factors are involved in the divergent effects of these compounds.
4.1.4 Aim 4

Aim 4 of this thesis was to investigate the ability of monoaminergic antidepressants and ω-3 PUFAs to affect inflammation-induced reductions in neurogenesis and induction of the neurotoxic limb of the kynurenine pathway. IL-1β was found to induce a reduction in neurogenesis, in association with induction of the neurotoxic end-product of the kynurenine pathway, quinolinic acid (QUIN), and up-regulation of the enzymes involved in its production – IDO, KYNU and KMO. I also found that three antidepressant compounds with very different chemical structures – venlafaxine, sertraline, and DHA - could partially reverse the anti-neurogenic effects of IL-1β, in association with reductions in the IL-1β-induced increase in QUIN, though with more mixed effects on expression of the kynurenine pathway enzymes. Conversely, EPA had no effect on IL-1β-induced reductions in neurogenesis or increase in QUIN.

4.1.5 Aim 5

Aim 5 of this thesis was to optimise the technique of chromatin immunoprecipitation in human hippocampal progenitor cells. Different conditions were evaluated and successfully optimised for division of DNA into fragments – physical shearing through sonication, and enzymatic digestion, using micrococcal nuclease. Following this, conditions for immunoprecipitation with a GR antibody, linked to magnetic beads, were optimised – including selecting an antibody, stringency of washes and minimisation of background enrichment. There was preliminary evidence of enrichment of several fold of GR-target genes SGK1, SLC19A2, GILZ, and MT2A by qPCR compared with both vehicle-treated samples (demonstrating specificity for dexamethasone treatment), and dexamethasone-treated samples precipitated with a non-specific control IgG (demonstrating specificity for the antibody). However,
enrichment of the supposed control gene DSCAM and variability in the
detection of enrichment necessitates further validation of the protocol, though
preliminary evidence exists that this complex technique has been successfully
adapted for use in the human hippocampal progenitor cells. This technique will
be instrumental in identifying the differential genomic targets of the
glucocorticoid receptor when activated by antidepressants and glucocorticoids,
yielding insight into how these treatments differentially regulate hippocampal
neurogenesis.

4.2 Systematic review of studies in depressed patients reveals
robust association between glucocorticoid resistance and
increased inflammation

4.2.1 Overview of findings
I attempted to determine which of two potential explanations for the apparently
paradoxical co-existence of increased levels of cortisol and pro-inflammatory
cytokines in depression was most consistent with findings in the clinical
literature. A key discriminator between the two proposed theories is the
importance placed on glucocorticoid resistance. In the shift in
balance/glucocorticoid resistance hypothesis increased inflammatory signalling
is attributed to the presence of glucocorticoid resistance, with an attendant
decrease in intracellular glucocorticoid signalling, and disinhibition of
inflammatory signalling as a consequence. In contrast, the competing theory
emphasises the ability of glucocorticoids to be pro-inflammatory in certain
circumstances; of note, a functional glucocorticoid receptor is necessary for this
process. Consequently, I focused the review on those studies that had
measured levels of cortisol, levels of pro-inflammatory cytokines and glucocorticoid sensitivity in the same patients.

The first observation to be made is that there are only a small number of studies that have evaluated these different variables in the same group of patients, despite the large numbers of studies that have focused on either the HPA axis or the innate immune system. The increasingly recognised interconnectedness of the neuroendocrine and neuroimmunological systems, amongst other systems implicated in depression, should lead to study designs that incorporate multiple variables measured in the same group of patients.

4.2.2 Shift in balance/glucocorticoid resistance theory supported

The findings in the eight studies examined in this systematic review reflect observations in the broader clinical literature. Seven out of eight studies reported evidence of greater innate immune system activity in depressed patients compared to controls, consistent with meta-analyses identifying an increase in pro-inflammatory cytokines in depressed patients (Dowlati et al., 2010; Howren et al., 2009) and recent genome-wide expression studies (Jansen et al., 2015). Seven out of eight studies found that glucocorticoid resistance was more likely to be present in depressed patients than in controls, and the eighth study showed some evidence of increased glucocorticoid resistance in depressed patients. This is in line with studies that identify glucocorticoid resistance in 25-80% of depressed patients, with greater incidence in severely depressed patients (Holsboer, 2000; Miller et al., 2002; Pariante and Miller, 2001), with molecular measures of glucocorticoid resistance suggesting it may even be more common (Menke et al., 2012). Only two out of eight studies found overt differences in plasma cortisol levels between patients and controls, and one found a trend to lower levels of cortisol in depressed patients (Maes et al.,
This observation is consistent with a recent systematic review and meta-analysis which found that the effect size of depression on glucocorticoid resistance was much larger than on increased levels of cortisol in older depressed participants (Belvederi Murri et al., 2014). A possible explanation for this finding will be discussed further below.

Only two studies out of the eight captured detected increased levels of cortisol and pro-inflammatory cytokines in the same group of patients. Indeed, this finding draws attention to a more fundamental weakness in the literature – though studies have found that depressed patients demonstrate increased cortisol levels and increased inflammation, there exists no systematic review to determine whether the same depressed patients demonstrate both these characteristics. On the other hand, there was a pronounced co-occurrence of glucocorticoid resistance and increased pro-inflammatory cytokines in depressed patients, reported in six out of eight studies. Furthermore, two studies found that the correlation between glucocorticoid resistance and inflammation remained significant even after controlling for potentially confounding factors such as age, baseline levels of cortisol, and dexamethasone levels (after the DST) (Maes et al., 1993a, 1993b). This finding is consistent with a notion that glucocorticoid resistance is a critical factor in increased levels of inflammation detected in depressed patients.

Indeed, no study found increased levels of pro-inflammatory cytokines as well as increased level of cortisol without also finding glucocorticoid resistance, further supporting the importance of glucocorticoid resistance in this relationship. Meta-analysis detected a significant positive association between glucocorticoid resistance and increased levels of pro-inflammatory cytokines, though this was based on data from only four studies. Overall, this is consistent
with the shift in balance/glucocorticoid resistance hypothesis which suggests that reduced intracellular glucocorticoid signalling increases innate immune signalling, by a relative reduction in its usual inhibitory influence (Miller, 2008).

The small number of studies captured precluded separating studies into those which measured baseline levels of cytokines and those which measured stimulated levels, as well as looking at individual cytokines. The most positive correlations were detected between GR resistance and stimulated levels of IL-1β (Maes et al, 1993a) and IL-6 (Maes et al, 1993b) from PBMCs. Although in another study there was a correlation found between LPS-stimulated IL-6 from whole blood and glucocorticoid resistance it failed to reach significance (effect size = 0.288, 95% CI (-0.022, 0.60)) (Carvalho et al, 2008), perhaps explained by the difference in response between PBMCs and whole blood samples. This study also showed no significant correlation between basal levels of IL-6 from PBMCs and glucocorticoid resistance (Carvalho et al, 2008). However, another study found plasma levels of TNF-α were found to correlate with GR resistance (Fitzgerald et al, 2006a). Although the small number of studies makes it difficult to discern which factors are likely to explain these discrepant results, it is likely that differences in cell type measured, basal vs stimulated levels, and the particular cytokine evaluated all play a role, as discussed further below. It will be an interesting direction for future studies to allow for evaluation of individual cytokines.

There was one study in those captured in which patients demonstrated an increase in glucocorticoid resistance without evidence of increased inflammation (Bauer et al, 2003). However, in this study, it was notable that PBMCs from those patients who were non-suppressors to a DST (10 out of 36 subjects) produced a greater TNF-α response to stimulation than patients who
suppressed normally to a DST, suggesting a relationship between glucocorticoid resistance and inflammatory responses. The remaining study captured reported the converse finding: depressed patients demonstrated evidence of increased inflammation but no difference in glucocorticoid resistance (determined by cortisol suppression 9 hours after dexamethasone administration) (Landmann et al., 1997). However, in this study there was evidence of increased resistance to the effect of glucocorticoids in the depressed group when cortisol levels were compared 16 hours after dexamethasone, suggesting some degree of glucocorticoid resistance.

It should be noted that not all depressed subjects demonstrated glucocorticoid resistance in the studies that reported increased resistance in the depressed group, but simply that there was a greater preponderance than in control subjects – for example, 2 out of 9 patients showed dexamethasone non-suppression in one cohort (Humphreys et al., 2006). However, it is possible that the dexamethasone suppression test is not the most sensitive means of detecting glucocorticoid resistance. For example, the topical vasoconstriction test employed in one study with a moderately potent steroid found that while all controls showed a full vasoconstriction response, no depressed patients showed a full response (Fitzgerald et al., 2006b). Furthermore, it was the more severely depressed patients that demonstrated glucocorticoid resistance and increased inflammation: for example, levels of glucocorticoid resistance correlated significantly with both HAM-D scores (and TNF-α levels) and those antidepressant resistant patients had the highest levels of glucocorticoid resistance (and inflammation) (Fitzgerald et al., 2006b). This suggests that this relationship might be most pronounced in more severe depression, and less so
in milder forms of depression, perhaps accounting for some of the discrepancies between different study findings.

Other clinical studies (not captured in this review because of a lack of a measure of cortisol) also support a relationship between GR resistance and levels of pro-inflammatory cytokines in depressed populations. For example, a recent study found that several inflammatory molecules were raised in depressed patients, compared with controls, including the cytokines and chemokines IL-8, MCP-1, IL-1β, IL-6, as well as ICAM and VEGF, in association with down-regulation of GRα (the functional GR isoform) expression, and a trend towards up-regulation of GRβ (a non-functional and perhaps inhibitory GR isoform) (Carvalho et al., 2014). This finding may reflect glucocorticoid resistance in association with increased inflammatory markers in depressed patients, a notion reinforced by the finding that GRα levels negatively correlated with IL-8 levels, and GRα/GRβ positively correlated with MCP-1 levels (though other cytokines did not demonstrate such associations) (Carvalho et al, 2014).

4.2.3 Theory of pro-inflammatory glucocorticoids largely unsupported

It is worth noting that predictions derived from a theory based on the pro-inflammatory effects of glucocorticoids were largely unsupported by the studies captured. For example, cortisol might be expected to correlate with levels of inflammation, given that increased levels of glucocorticoids, exerting pro-inflammatory effects, should co-occur with increased levels of inflammation. However, the majority of studies did not detect increased levels of cortisol in the depressed group, along with increased levels of inflammation, failing to support this prediction. One study reported that there was no correlation between cortisol levels and IL-6 or TNF-α levels (Fitzgerald et al, 2006b). As noted above, the two studies that detected increased levels of cortisol and increased
levels of cytokines also found increased glucocorticoid resistance (Carvalho et al, 2008; Humphreys et al, 2006). The finding that glucocorticoid resistance was detected in most of the studies along with increased inflammation, together with the well understood mechanisms by which these two states might be connected (outlined below), suggests that glucocorticoid resistance, and not increased cortisol, is the driver of increased inflammation. Indeed, it is plausible that glucocorticoid resistance may also drive the increased cortisol, found in chronically stressed and depressed subjects.

However, given the importance placed on pre-treatment in the pro-inflammatory effects of glucocorticoids it is also plausible to suggest that this theory might predict previous periods of hypercortisolaemia in depressed patients leading to increased levels of inflammation, even after resolution of hypercortisolaemia. Nevertheless, such an explanation does not account for the strong association between glucocorticoid resistance and inflammation evident in these studies, given that pro-inflammatory effects of glucocorticoids are not attributed to glucocorticoid resistance but rather to up-regulation of inflammatory pathways. Another possibility, previously raised by other authors (Frank et al, 2013), is that a shift in balance hypothesis and the ability of glucocorticoids to be pro-inflammatory are not mutually exclusive. While this possibility is theoretically plausible, clarification of the role of these two processes is required, and would require further molecular studies (addressed in Section 4.3).

4.2.4 Mechanisms by which glucocorticoid resistance gives rise to increased inflammation

The studies captured in this systematic review and those above are consistent with the idea that impaired glucocorticoid signalling may allow disinhibited inflammation, contributing to illness with significant inflammatory components,
including depression (Silverman and Sternberg, 2012b). The role of glucocorticoids in suppressing peripheral and central inflammatory responses is well known (Besedovsky and Del Rey, 1996; Nadeau and Rivest, 2003; Silverman and Sternberg, 2012b), consistent with the idea that reduced glucocorticoid signalling might release inflammation from inhibition, contributing to long term increased inflammation. There are various mechanisms by which reduce glucocorticoid signalling might give rise to increased inflammation. For example, upon dimerisation, the GR is known to bind to negative GREs in the promoter regions of cytokine genes (Ray et al, 1990; Zhang et al, 1997), to up-regulate the transcription of genes that inhibit cytokine mRNA stability (Amano et al, 1993; Smoak and Cidlowski, 2006) and to increase the expression of anti-inflammatory molecules, such as IL-10, IκBα and the IL-1R2 receptor (Silverman et al, 2013). Therefore, reduced glucocorticoid signalling would give rise to increased expression of cytokine genes, increased cytokine mRNA stability and less anti-inflammatory molecules, leading to greater inflammation.

This prediction is supported by a recent study involving mice, genetically modified to model glucocorticoid resistance by impairment of GR-dependent transcriptional activity, known as GR(dim) mice (Silverman et al, 2013). These glucocorticoid resistant mice demonstrate elevated and prolonged plasma levels of IL-6 (and IL-10) following LPS stimulation, increased early expression of TNFα, IL-6 and IL-1β mRNA levels, as well as exaggerated sickness behaviour. Of interest, these mice also demonstrated increased and prolonged levels of plasma corticosterone in response to LPS, such that increased levels of both pro-inflammatory cytokines and corticosterone co-occurred, in the context of glucocorticoid resistance; these mice also demonstrated higher levels of baseline corticosterone. This study supports a causative role for
glucocorticoid resistance in enhancing inflammatory responses, behaviours associated with inflammatory responses (in this case sickness behaviour) and increased levels of corticosterone, abnormalities often found in chronically stressed animals, with equivalent changes in human populations (Avitsur et al, 2009; Cole et al, 2007; Miller et al, 2008; Quan et al, 2003). This finding suggests that the increased cortisol levels detected in a subset of depressed patients may arise as a result of glucocorticoid resistance.

A recent study in humans confirms the finding that the presence of glucocorticoid resistance gives rise to a larger inflammatory response and increased likelihood of illness (and importantly, that glucocorticoid resistance precedes enhanced inflammatory responses), and connects the presence of glucocorticoid resistance to the experience of chronic stress (Cohen et al, 2012). This study, mentioned previously, found that in a group of healthy volunteers that those exposed to long-term stressful experiences demonstrated increased glucocorticoid resistance (at baseline) and increased likelihood to develop a cold following viral challenge. Subjects with greater glucocorticoid resistance produced greater amounts of nasal pro-inflammatory cytokines following viral challenge, emphasising the connection between glucocorticoid resistance and increased inflammation (Cohen et al, 2012).

Taken together, these studies indicate that chronic stress can give rise to glucocorticoid resistance, which, in turn, increases the magnitude of subsequent inflammatory responses and, in some cases, baseline levels of inflammation (Miller et al, 2008), as well as the risk for illness, including depressive symptoms. This may account for the increased levels of inflammation detected in depressed patients. It may also account for the increased co-morbidity of a variety of illnesses with depression as reduced glucocorticoid signalling has
been implicated as a causative mechanism in illnesses characterised by prolonged inflammation (such as autoimmune diseases, chronic fatigue, PTSD, cardiovascular diseases, type 2 diabetes, the metabolic syndrome and osteoporosis), in addition to depression (Silverman and Sternberg, 2012b). Indeed, depression has been shown to predict exacerbations in diseases with inflammatory components, like rheumatoid arthritis and multiple sclerosis (Raison and Miller, 2003).

4.2.5 Bi-directional interaction between glucocorticoid sensitivity and inflammation

A complicating factor to the suggested model - whereby glucocorticoid resistance allows increased inflammation, with increased risks for illnesses with inflammatory components, including depression - is that inflammation can induce glucocorticoid resistance (Silverman and Sternberg, 2012b). The means by which this is possible include reduced expression or affinity of the GR, alteration to translational properties, increased interaction with inflammatory transcription factors, like NF-κB, or increased interaction with GRβ (Pace et al, 2007). As impaired GR function can contribute to enhanced inflammatory signalling, this suggests the possibility of a vicious cycle arising which might perpetuate increased inflammatory signalling and impaired glucocorticoid feedback. Of note, this model also suggests that chronic treatment with a glucocorticoid antagonist in depression may increase inflammation and impair GR function as a consequence, a potential drawback to such a strategy of antidepressant treatment. As acute and chronic stress are associated with both increased inflammation and increased glucocorticoid resistance, both are present in depressed subjects, and each can induce the other it remains to be elucidated if they arise sequentially, or in concert. Accordingly, longitudinal data
would be invaluable in detecting whether increased inflammation or glucocorticoid resistance arises first in stressed and then depressed subjects.

4.2.6 Limitations of systematic review

There are several limitations of this review. The small number of studies limits the conclusions that can be drawn; although this was somewhat offset by the striking association, qualitatively and quantitatively, of glucocorticoid resistance with increased inflammation. However, further studies examining multiple endocrine and immune variables in the same cohort of depressed patients are needed to confirm the relationship between these systems. This will also allow for a separate examination of baseline and stimulated inflammatory activity, and differences between individual cytokines.

There were differing methodologies used in the different studies for each variable of interest: glucocorticoid resistance was measured in some studies by central means (the DST) and in other studies by peripheral means (the topical vasoconstriction assay, and in vitro glucocorticoid inhibition of the cytokine secretion of stimulated PBMCs); inflammatory responses were measured either in vivo or ex vivo, with or without stimulation, and examining different cytokine levels (IL-6, IL-1β, TNF-α, IL-2 and IFN-γ), generally at the protein level but also at the level of mRNA; and lastly, cortisol levels were measured in some studies by a one-off morning plasma level, in other studies with three or more samples during the day and in one study by urinary cortisol excretion, in addition to plasma levels.

There was a trend (p=0.06) towards heterogeneity detected in the meta-analysis, reflecting variation in the size of the association between studies. An I² of 55.9% has been tentatively assigned to the moderate range (Higgins et al,
2003). This heterogeneity likely reflects the methodological variability outlined above. However, due to the small number of studies, it was not possible to divide studies into different categories to determine the source of the heterogeneity. It is of note that such widely differing methodological approaches yielded similar findings with respect to the relationship of glucocorticoid resistance and inflammation. However, future studies adopting more standardised measures in depressed populations may yield even more coherent findings – such an approach may involve a central and peripheral measure of glucocorticoid resistance, including a molecular measure (Menke et al, 2012), along with in vivo, and ex vivo measurements of baseline and stimulated levels of the cytokines most often associated with depression, as well as multiple measures of cortisol.

Lastly, there were some discrepancies in the observations made between baseline and stimulated cytokine production. The most commonly reported finding was of increased baseline cytokine levels, consistent with the wider literature. Some studies reported lower levels of stimulated inflammatory responses in depressed patients while others reported the opposite. For example, one study found that although there was higher baseline levels of IL-6 from PBMCs isolated from depressed patients, there was no difference in levels of LPS-stimulated IL-1β or TNF-α and lower levels of IL-6 from these PBMCs (Humphreys et al, 2006). Other studies found that stimulated PBMCs from depressed subjects released higher levels of IL-1β and IL-6 than controls (Maes et al, 1993a, 1993b). Decreased ex vivo production of cytokines by PBMCs from depressed patients has been reported previously (Krause et al, 2012) and has been attributed to these cells being refractory to stimulation because of a pre-existing pro-inflammatory state in depression. The mechanism that might
underlie this refractory state, and the differences between patients who exhibit exaggerated responses to inflammatory stimulation versus reduced response have yet to be elucidated.

4.3 **Interaction of innate immune system and glucocorticoid system**

*in vitro*

4.3.1 **Overview of findings**

Following on from my systematic review, examination of the interaction of glucocorticoids and inflammation in human neural cells demonstrated a more complex relationship than previously thought. In the human hippocampal progenitor cells glucocorticoids suppressed inflammation, in a dose-dependent manner, upon co-incubation. However, when glucocorticoid treatment preceded inflammatory stimulation, inflammatory responses were potentiated, indicating that glucocorticoids can demonstrate pro-inflammatory effects in human neural cells. The degree of potentiation depended on the dose of glucocorticoid – for either dexamethasone or cortisol – with an increase in effect seen from small to medium doses, and a decline in effect from medium to high doses. The effect was also clearly dependent on the time period between glucocorticoid treatment and inflammatory stimulation, with a maximal effect at 24 hours and non-significant effects at periods smaller or greater than this interval. The time-dependency of the potentiating effect was clear in a wide variety of cytokines and chemokines produced by the progenitor cells, including IL-8, IP-10, IL-1RA, RANTES, and MCP-1. The effect was also GR-dependent, because blockade of the GR abrogated the effect. Blockade of the GR also indicated that the critical period for induction of the potentiation effects was during glucocorticoid treatment, with a non-significant reduction of the potentiation effect caused by
GR blockade during the rest interval. There was no change in expression levels of the GR or NF-κB, but there was a non-significant increase in GR resistance induced by the pre-treatment interval, suggesting that GR resistance may be involved in the potentiation effects. Genome-wide gene expression analysis demonstrated some evidence that glucocorticoid treatment upregulated immune-related genes, and could induce glucocorticoid resistance, suggesting that both processes might contribute to the ability of pre-treatment with glucocorticoids to potentiate subsequent inflammation.

4.3.2 Glucocorticoids potentiate inflammation

My results showing that glucocorticoids can be pro-inflammatory in human neural cells fit into an emerging literature demonstrating similar effects in animals and in some human studies looking at peripheral samples. These studies indicate that exposure to stress or glucocorticoids before inflammatory stimulation can potentiate the subsequent inflammatory response. For example, exposure to inescapable tail-shock (Johnson et al, 2002) or to daily sessions of unpredictable chronic stress (Munhoz et al, 2006) potentiates the increase in the hippocampus and frontal cortex expression of pro-inflammatory mediators, like IL-1β, induced by a peripheral injection of LPS given 24 hours after the stressor regimen.

A key aspect of the studies demonstrating pro-inflammatory effects due to glucocorticoids is that glucocorticoid treatment (or the stress paradigm) precedes inflammatory simulation. This is distinct from the well-known suppressive effects of glucocorticoid treatment that coincides with inflammatory stimulus. For example, animals that are adrenalectomised show enhanced behavioural and thermoregulatory response to IL-1β, which was reversed by supplementation of corticosterone that coincided with IL-1β infusion, indicating
the suppressive effects of glucocorticoids (Goujon et al., 1995). The distinction between pre- and co-treatment is demonstrated in a more recent study where corticosterone administered to rats 2 hours or 24 hours before an LPS challenge potentiates IL-1β, TNF-α and IL-6 mRNA expression in the hippocampus, but suppresses the expression of these same cytokines when corticosterone is administered one hour after LPS stimulation (Frank et al., 2010). This reflects the distinct effects observed in the present study on co-incubation of IL-1β and dexamethasone, in which glucocorticoids were anti-inflammatory, compared with pre-treatment of dexamethasone (and cortisol) before IL-1β, in which glucocorticoids were pro-inflammatory.

Pro-inflammatory effects of glucocorticoids have also been demonstrated in humans, similarly dependent on whether glucocorticoids preceded or coincided with inflammatory stimulus. An infusion of hydrocortisone given just before or concomitant with LPS administration in healthy subjects completely abrogated a rise in TNF-α, whereas hydrocortisone given 12 or 144 hours before LPS caused large increases in levels of TNF-α and IL-6, compared with LPS alone (Barber et al., 1993). A more recent study in humans found that intermediate doses of hydrocortisone (corresponding to stress levels) infused 24 hours before LPS administration potentiated the rise of IL-6 levels compared with LPS alone, while high doses failed to do so (Yeager et al., 2011). I have extended these findings to include human neural cells, demonstrating that their production of a wide variety of chemokines and cytokines (IL-8, IP-10, IL-1RA, RANTES, and MCP-1) are potentiated by pre-treatment with glucocorticoids.

4.3.2.1 Potentiating effects are time-dependent

A key question that arises regarding these differential effects of glucocorticoids on inflammatory processes is what are the determinants of whether
glucocorticoids are pro- or anti-inflammatory? As above, timing is important – as co-incubation yields anti-inflammatory properties and pre-treatment yields pro-inflammatory properties. However, there seems to be a more fine-grained effect of time as previous literature indicates that the duration of the interval between glucocorticoid treatment and inflammatory stimulation is important in determining the size of the potentiation effect. There are reports in the literature of time periods between glucocorticoid and inflammatory treatment that were too large to generate a pro-inflammatory effect, as well as reports of intervals that were too small. For example, one study found that mice subjected to a chronic stress paradigm exhibited reduced inflammatory responses to LPS challenge administered two weeks after completion of a stress paradigm, as indicated by reduced levels of TNF-α and IL-1 mRNA in the midbrain and hippocampus (Barnum et al., 2012).

In the human study mentioned above, subjects who were infused with hydrocortisone showed a large potentiation of peripheral TNF-α and IL-6 levels when injected with LPS 12 hours or 144 hours after hydrocortisone infusion was ceased, but suppression of TNF-α and IL-6 levels when LPS injection was given along with the hydrocortisone infusion or just six hours after the infusion was ceased (Barber et al., 1993). There was a similar effect for CRP – which showed potentiation after an interval of 12 hours; however at 144 hours of interval there was a slight suppression of CRP, and no effect either way after an interval of 6 hours. Indeed, different time lines of effect on the different immunoproteins (IL-6 was potentiated more strongly at 144 hours than 12 hours, opposite to the effects on TNF-α) suggests differential effects of glucocorticoid treatment, similarly to the findings in my study (where some immunoproteins demonstrated
increases with a treatment interval of one hour, while others did not demonstrate increases with this interval).

Other studies generally found a potentiating effect of glucocorticoid pre-treatment on inflammation when inflammatory stimulation was administered within 24 hours of the stress paradigm or glucocorticoid administration (Espinosa-Oliva et al, 2011; Johnson et al, 2002; Munhoz et al, 2006; de Pablos et al, 2006). One study found that inflammatory responses were potentiated one day and four days after a stress paradigm, but not after 10 or 21 days (Johnson et al, 2002). Taken together, these studies indicate that there is a window of potentiation following glucocorticoid treatment, starting hours after treatment and lasting for days.

The time-dependent nature of glucocorticoid potentiation of inflammatory responses has also been detected in vitro. Murine macrophage-like cells, after incubation with corticosterone, demonstrate a potentiation of TNF-α, IL-6 and nitrite levels in the supernatant in a time-dependent manner, maximal between 12 and 24 hours (Figure 48) (Smyth et al, 2004). These findings are consistent with the present observations in the human neural cells in which potentiation effects were detected at an interval of 24 hours, but not at 1 hour, 12 hours or 48 hours. However, with respect to the 48 hour time interval, cells achieved confluence by this time point and this may have altered the nature of their inflammatory response (perhaps leading to reductions in response due to reduced exposure of each cell to IL-1β stimulation). In any case, findings in the cells extend the time-dependency of the glucocorticoid potentiation effect to human neural cells.
Figure 48 Potentiation of inflammatory responses in macrophages shows a similar time-dependent course to the present study

Similarly to the present study, there is a window of potentiation for TNF-α, nitrite and IL-6 secretion in macrophages following pre-treatment with corticosterone. TNF-α and IL-6 are potentiated when corticosterone treatment preceded immune stimulation by at least 12 hours. Nitrite is potentiated when corticosterone treatment preceded by 12 hours, but not shorter or greater periods. Adapted with permission (Smyth et al, 2004)
The pro-inflammatory effects of glucocorticoid pre-treatment also extended to a variety of cytokines and chemokines – IL-8, IP-10, IL-1RA, RANTES and MCP-1. The time course for each of these immunoproteins was consistent with that observed for IL-6: co-treatment was inhibitory, pre-treatment with an interval of 24 hours was stimulatory, with intermediate effects with an interval of one hour. Previous studies in animals, peripheral human samples and in vitro models have not examined as wide a spectrum of immunoproteins as in the present study but have found pro-inflammatory effects in a number of different immunoproteins - TNF-α, IL-1β, IL-6, nitrite (Espinosa-Oliva et al, 2011; Frank et al, 2012) - consistent with the findings in the present study. (IL-1β secretion into the supernatant was not examined in my study because it was used as the immune stimulus, and thus introduced into the supernatant).

The importance of timing in the effect of glucocorticoids on immune responses has been attributed to an evolutionary narrative holding that inflammatory responses are unnecessary and energetically expensive during acute stress (perhaps involving pursuit from a predator, etc) but become important in the period following acute threat, when injuries or infections sustained require an immune response (Frank et al, 2013). This model is derived from an older behavioural model, called the perceptual-defensive-recuperative (PDR) model, that contrasted behavioural responses during threats, with those that occur after threats (Frank et al, 2013). This model has been extended to incorporate a description of the innate immune system, with the innate immune response an example of recuperative behaviour at the cellular level. The steep rise in glucocorticoids that occurs during a stressor is seen as a key signal of threat to the innate immune system, causing subsequent potentiation (Frank et al, 2013). This model predicts that when the threat is removed, and glucocorticoid levels
have fallen, innate immune responses will be enhanced to any persisting injury or infection.

4.3.2.2 Potentiating effects are dose-dependent

Other studies have also reported dose-related aspects to the potentiation phenomenon and have proposed that dose is an important determinant of whether glucocorticoids are pro- or anti-inflammatory. These studies have often distinguished between baseline levels of glucocorticoids, as would be found in a non-stressed subject, stress levels and pharmacological levels of glucocorticoids. For example, in human subjects, depletion of cortisol action by infusion of the adrenal cortisol synthesis inhibitor, etomidate, and administration of RU486, did not alter the response of monocytes stimulated \textit{ex vivo} by LPS, as measured by TNF-\(\alpha\), IL-6 and IL-10 levels, suggesting that basal levels of cortisol may not be suppressive (Yeager \textit{et al}, 2011). Another paradigm in the same study involved administration of either saline, stress levels of hydrocortisone (to achieve plasma levels of ~30-50\(\mu\)g/dL) or pharmacological doses of hydrocortisone to healthy human subjects, who were then infused with LPS the next day and their inflammatory response measured by serial testing of plasma IL-6 for 8 hours. The saline groups and the pharmacological groups demonstrated small inflammatory responses, while those subjects with ‘stress’ levels of cortisol demonstrated potentiated inflammatory responses (\textit{Figure 49}) (Yeager \textit{et al}, 2011).
Consistent with the present study, human subjects with intermediate levels of cortisol measured after a six hour infusion of hydrocortisone had the greatest potentiation of IL-6 in response to LPS injection given 24 hours after the end of the hydrocortisone infusion. Re-printed with permission (Yeager et al, 2011).
This pattern of findings has also been demonstrated in animal models. For example, adrenalectomised rats implanted with corticosterone pellets for three days to induce basal, ‘mild stress’, or ‘moderate/major stress’ before LPS stimulation one day later demonstrated large potentiation of inflammatory responses in the hippocampus, as measured by TNF-α and IL-1β mRNA levels, for the intermediate levels of corticosterone, while these effects were not present for the higher and lower levels (Figure 50) (Munhoz et al, 2010).
Figure 50 Dose-dependent glucocorticoid pre-treatment potentiation of inflammatory gene expression in the frontal cortex of rats

Intermediate levels of glucocorticoids given to rats potentiated the expression of inflammatory genes in the hippocampus (and frontal cortex), more so than higher or lower levels, similar to the results found in cells in the present study. Re-printed with permission (Munhoz et al, 2010).
This data shows a remarkable consistency with the response I found in human hippocampal cells, where low and high doses of both cortisol and dexamethasone demonstrated non-significant effects on inflammatory responses, while intermediate doses demonstrated pronounced potentiation of inflammatory responses. One of the advantages of the in vitro model employed is that it excludes complex systemic explanations for the observed effects, suggesting that the effects observed must be due to intracellular signalling pathways rather than more complex interactions between the immune system, HPA axis and other interacting systems.

The data supporting dose-dependent glucocorticoid potentiation of inflammation has been formulated into a hypothesis whereby glucocorticoids effects on inflammation follow an inverted U-shaped pattern of activity based on dose of glucocorticoids: at low levels, including those experienced in normal diurnal variation, they are permissive to inflammatory processes; at stress levels of glucocorticoids they are stimulatory to inflammatory processes; and at pharmacological doses they are suppressive to inflammatory processes (Figure 51) (Sorrells et al, 2009; Yeager et al, 2004b). According to this model, normal diurnal concentrations of glucocorticoids permit function of the innate immune system, while levels experienced during stress acutely suppress inflammation. Additionally, a time-dependent effect is proposed whereby stress-levels of glucocorticoids also exert a delayed pro-inflammatory effect (Yeager et al, 2011). Supraphysiological doses of glucocorticoid are strongly suppressive as per their clinical usage. Such a model supports time- and dose-dependent influences on whether glucocorticoids are pro- or anti-inflammatory but does not propose a mechanism for dose-dependent effects, or explain the relationship
between time and dose effects. Consequently, an iteration of this model is proposed below.
This model proposes that at low concentrations glucocorticoids exert primarily permissive effects. As the glucocorticoid concentration increases up to a peak that is close to plasma cortisol levels observed during normal diurnal variation, glucocorticoids are stimulatory. At high stress levels, glucocorticoids inhibit or suppress defense responses in order to prevent injury caused by over-activity of the stress-induced response. Note that time is not a feature of this model (Yeager et al, 2004b)
4.3.2.3 A potential synthesis of dose- and time-dependency

My findings and the above literature would suggest that the effects of glucocorticoids are both dose- and time-dependent. I would like to speculate a more parsimonious model that incorporates both time- and dose-dependency (Figure 52). The most plausible explanation for the pro- and anti-inflammatory effects is centred primarily on different timings, rather than doses. All the studies so far mentioned have induced inflammatory responses after treatment with glucocorticoids. No studies have reported pro-inflammatory effects in paradigms involving co-treatment of glucocorticoids and inflammatory stimuli, at any dose of glucocorticoids. Timing, therefore, seems of paramount importance.

I speculate that a biphasic action of glucocorticoids includes a period of suppression followed by a period of potentiation, and the duration of the suppressive period is determined by the dose of glucocorticoid. It is already well known, with regards the pharmacokinetics of dexamethasone in humans, that higher doses cause a longer duration of inflammatory suppression, in addition to a greater degree of suppression (Loew et al., 1986). This is highly consistent with the notion that there would be greater time delays for higher doses of glucocorticoid before inflammatory potentiation effects were seen. This may explain the dose-dependent effects of glucocorticoid potentiation of inflammation observed in animal and human studies, and in the present cellular study. This model makes predictions that can be tested. Specifically, it suggests that the apparent decrease in potentiation induced by higher doses of glucocorticoids compared with intermediate doses – in animals, humans and in human hippocampal cells – would be reversed if a longer time period was observed before inflammatory stimulation (Figure 52). It might be speculated that such a model makes evolutionary sense, whereby a greater stressor might
necessitate a greater period of monopoly of energetic demands, before a recovery period is initiated.
Figure 52 A proposed model of glucocorticoid effects, synthesizing time and dose dependency

This model proposes that greater doses of glucocorticoids inhibit inflammation to a greater degree and for a longer period of time than smaller doses, consistent with well-established characteristics of glucocorticoids. After a period of time (greater for larger doses) glucocorticoids exert pro-inflammatory effects, again greater for larger doses. This model accounts for the dose-dependent findings in some papers: for example, measuring inflammatory response 24 hours after glucocorticoid treatment would show greatest effects for a dose of 1μM, compared with 100nM and 10μM. However, at a later time (perhaps 30 hours), the larger dose of 10 μM would show greater effects on potentiation than smaller doses.
4.3.3 GR dependency of the pro-inflammatory action of glucocorticoids

A number of other studies have found that, in addition to the well-characterised GR-dependency of the anti-inflammatory effects of glucocorticoids, the pro-inflammatory effects are also GR-dependent. For example, a number of studies have blocked the stress-induced priming of pro-inflammatory responses in the CNS, by treatment with the GR antagonist, RU486, during the stress paradigm (Espinosa-Oliva et al, 2011; Munhoz et al, 2006; Nair and Bonneau, 2006; de Pablos et al, 2006). Stress induced priming of inflammatory responses demonstrated by ex vivo inflammatory stimulation of microglia isolated from stressed animals can also be blocked by treatment with RU486 during the stress paradigm (Frank et al, 2012). These studies are consistent with my findings in human hippocampal cells in which co-incubation of dexamethasone with RU486 blocked the potentiating effects of pre-treatment. In these animal studies, RU486 was administered during or just before the stress paradigm, and, to my knowledge, none of these studies treated animals with RU486 during the period between stress and inflammatory stimulation, so it is unclear whether my finding that priming effects were largely unaffected by RU486 during this period is also the case in animal brains.

This point is of interest as it would help to define when changes occur that produce pro-inflammatory effects. The finding that the duration of the time interval between glucocorticoid treatment and inflammatory stimulation is of critical importance suggests that molecular processes occurring during this period are responsible for the potentiation of inflammatory responses subsequently. If the changes that occurred during the glucocorticoid pre-treatment period were sufficient to potentiate inflammation then exaggerated inflammatory responses would be present directly after this period – a finding
not supported in the current study or those discussed above. In this context, the finding that the potentiation effect appears not to be blocked by RU486 treatment in the no treatment interval, suggests that the changes that give rise to the potentiated inflammatory response must be downstream of the GR during this period. This is consistent with the function of the GR, as a transcription factor, activating and repressing expression of genes with downstream effects. Overall, one might preliminarily conclude that molecular processes initiated during glucocorticoid treatment continue, downstream of the GR, during the rest interval, giving rise to potentiated immune responses hours (in the present study, 24 hours) after glucocorticoids have been removed.

4.3.4 Insight into mechanisms underlying glucocorticoid potentiation of inflammatory processes

4.3.4.1 GR and NF-κB mRNA expression

An attempt was made to discern the mechanisms underlying the ability of dexamethasone pre-treatment to potentiate subsequent immune responses. As mentioned above, the two leading hypotheses attribute these effects either to the up-regulation of inflammatory pathways (Frank et al, 2013) or to the induction of glucocorticoid resistance (Miller, 2008). Given that a balance between glucocorticoid signalling and immune signalling is at the most simple level influenced by the key transcription factors GR and NF-κB, the level of expression of mRNA for both these proteins was measured for different doses of dexamethasone pre-treatment compared with no pre-treatment. No significant difference was found in expression level of either for any dose of dexamethasone pre-treatment, indicating that a change in their level of expression did not explain the potentiation effect of dexamethasone pre-treatment. This is consistent with other studies that have not found gross
changes in the level of gene expression of these key proteins. For example, in one study comparing subjects of high and low socioeconomic status (SES), there was significant evidence that subjects from low SES had potentiated inflammatory processes – greater stimulated production of IL-6, greater expression of inflammatory genes, as well as evidence of reduced expression of glucocorticoid receptor-target genes – analogous to the current study (Miller et al, 2009b). However, there was no difference in levels of mRNA of GR or NF-κB across these two groups, findings strongly consistent with the present study, leading the authors to speculate that it was likely functional changes, such as altered phosphorylation by MAP kinases of the GR, reducing its sensitivity to cortisol and so facilitating NF-κB signalling, or post-translational modification of NF-κB, that explained the enhanced inflammatory activity in the low SES group (Miller et al, 2009b).

4.3.4.2 Functional glucocorticoid resistance

Although there was no change in expression levels of mRNA for the GR, there are other mechanisms by which glucocorticoid resistance might be induced. In order to test for the existence of glucocorticoid resistance I employed a functional test – whereby the ability of glucocorticoids to suppress inflammation (that is, the fraction of IL-1β-induced IL-6 inhibited by dexamethasone co-treatment) was examined with or without glucocorticoid pre-treatment. There was a trend observed towards glucocorticoid resistance following dexamethasone pre-treatment but this did not reach significance. This finding would fit with literature detecting glucocorticoid resistance in association with increased inflammation (as discussed in Aim 1). Indeed, it has been suggested that pro-inflammatory effects in animals following glucocorticoid pre-treatment might be attributed in part to the induction of glucocorticoid resistance (Frank et
al, 2013), perhaps, in addition to the up-regulation of inflammatory pathways. As these were the final experiments conducted in the thesis, time precluded further repeats, but a higher-powered investigation for the presence of glucocorticoid resistance would be instructive.

4.3.4.3 Genome-wide gene expression

Following these equivocal findings, a genome-wide gene expression approach was taken to allow the widest possible exploration of mechanisms. As expected, dexamethasone, upon co-incubation with an inflammatory stimulus, down-regulated a number of immune-related genes, including a number of cytokines and chemokines. However, interestingly, dexamethasone in this circumstance also up-regulated some immune genes, including two chemokine ligands, 5 and 20, and NOD-like receptor family, pyrin domain containing 6 (NLRP6). The importance of NLRP6 was emphasised by finding that this gene was still up-regulated 48 hours after dexamethasone treatment in the pre-treatment condition (dexamethasone/vehicle/IL-1β vs vehicle/vehicle/IL-1β).

NLRP6 is a member of the NOD-like receptor family of pattern recognition receptors. They are not as well understood as the Toll-like receptor family (TLRs) but are known to sense microbial or danger stimuli in the cytoplasm, in the form of pathogen-associated molecular patterns (PAMPs). Structurally, NLRP6 resembled the more well characterised NLRP3 member of the NOD-like receptor family (Anand and Kanneganti, 2013). NLRP3 forms a multimeric structure known as the ‘inflammasome’ leading to inflammatory events, including activation of pro-caspase-1, which in turn leads to cleaving of IL-1β and IL-18 into their biologically active forms (Anand and Kanneganti, 2013).
NLRP6, similarly to NLRP3, is known to activate caspase-1, and, in turn, increase production of mature of IL-1β and IL-18, although its activator is currently unknown (Vanaja et al., 2015).

There has been significant attention directed at the involvement of NLRP3 in glucocorticoid priming of inflammatory responses. The NLRP3 inflammasome is known to require a priming stimulus that is modulated by glucocorticoids and so has presented a plausible mechanism for glucocorticoid-potentiated inflammatory processes. Glucocorticoid treatment in macrophages has been shown to increase mRNA levels and protein of NLRP3 (Busillo et al., 2011). This up-regulation has functional consequences as this glucocorticoid-dependent induction of NLRP3 sensitised the macrophages to ‘danger signals’ like extracellular ATP, leading to an enhanced release of IL-1β, TNF-α and IL-6 in response to increased ATP. This potentiation of inflammatory processes was also shown to be glucocorticoid- and glucocorticoid receptor-dependent (Busillo et al., 2011) showing pronounced similarities to the present study in hippocampal progenitor cells. Furthermore, in the macrophages, glucocorticoid treatment increased NLRP3 mRNA and protein for periods sustained beyond treatment with dexamethasone (dexamethasone (100nM) treatment for four hours sustained increases in NLRP3 for up to eight hours), similarly to the present experiments where up-regulation of NLRP6 appeared to be sustained for at least 48 hours following 24 hours treatment with dexamethasone.

It is therefore plausible that NLRP6 may also be involved in the enhancement of inflammatory activity following previous exposure to glucocorticoids. Although NLRP6 is involved in activation of the inflammatory pathways, involving caspase-1, IL-1β, IL-18, similarly to NLRP3, it has also been implicated as a negative regulator of inflammation (Anand and Kanneganti, 2013). Recent work
has demonstrated that NLRP6 protects against colitis, and that it inhibited TLR2 and TLR4-dependent activation of the NF-κB and MAP-kinase pathways (such that NLRP6 deficient mice produced increased levels of TNF-α and IL-6 following induction of colitis) (Anand and Kanneganti, 2013). However, the role of NLRP6 has so far been characterised largely in the intestine and its role in the brain has not been investigated. It is possible that, similarly to NLRP3, it may be involved in the potentiation of inflammation following exposure to increased levels of glucocorticoids.

The competing hypothesis holds that the induction of glucocorticoid resistance by dexamethasone treatment accounts for subsequent potentiation of inflammatory processes (Miller, 2008). There was some, albeit limited, evidence of glucocorticoid resistance following dexamethasone treatment. Dexamethasone treatment down-regulated a number of genes, in which response elements to the glucocorticoid receptor was over-represented, indicating that GR-target genes were less expressed followed dexamethasone treatment. This is consistent with the presence of glucocorticoid resistance and similar findings have been reported in a number of populations that have been exposed to stress in various forms – low SES (Miller et al, 2009b), loneliness (Cole et al, 2007) and caregiving stress (Miller et al, 2008). One limitation to this interpretation is, however, that glucocorticoid resistance is normally understood in terms of a response to glucocorticoid stimulation and the importance of finding less GR-target gene expression at baseline conditions (that is, without glucocorticoid administration or stress) is somewhat less clear. An interesting approach to further clarify this issue would be, in cells, to re-introduce dexamethasone after pre-treatment with dexamethasone, or, in chronically stressed populations, to administer a small amount of dexamethasone, before
genome-wide gene expression analysis, similar to the approach employed in a previous study (Menke et al, 2012). Two other limitations to the evidence of glucocorticoid resistance was the small number of genes analysed for GR response elements, limiting conclusions that can be drawn, and the fact that the dexamethasone/vehicle/IL-1β condition did not demonstrate a similarly reduced expression of GR-target genes. Higher powered analysis of these conditions would yield further insights into the mechanisms underlying potentiation, but there seems to be evidence of a role for both upregulated inflammatory pathways and glucocorticoid resistance.

4.3.5 Potential translational relevance to depression
The finding that glucocorticoids can be pro-inflammatory under certain conditions would be of only academic interest if it were not for the potential for this finding to help explain the seemingly contradictory aspects of the pattern of biological perturbations in humans under chronic stress. Large numbers of studies indicate evidence for over-activity of the HPA axis, as well as over-activity of the innate immune system. Effector molecules of both systems – cortisol, and cytokines, respectively – can produce many of the changes thought to be present in depression and so provide plausible candidates for inducing pathogenesis in depression. Indeed, these abnormalities have become targets for therapeutic strategies for depression – cortisol blocking agents like RU486, and cortisol synthesis inhibitors – such as metyrapone – have and are continuing to be investigated as antidepressant treatments (Gallagher et al, 2008; McAllister-Williams et al, 2013; Sigalas et al, 2012). Treatments targeting inflammation, such as etanercept and minocycline, as well as other anti-inflammatory strategies are also being trialled (Raison et al, 2013), and a recent systematic review and meta-analysis of anti-inflammatory medications in
depression found positive effects for these treatments, particularly emphasising the COX-2 inhibitor celecoxib (but with a high risk of bias and high heterogeneity) (Köhler et al, 2014).

However, taking into account the interaction between these two targeted systems raises issues: for example, blocking cortisol in the context of heightened inflammation may exacerbate problems caused by the inflammatory state; and blocking inflammation, which may impose regulation of the HPA axis, by the induction of glucocorticoid resistance, may exacerbate problems attributed to hypercortisolaemia. The findings of the present study may shed light on this dilemma. The ability of glucocorticoids to be pro-inflammatory in the human brain may help to make sense of the seeming paradox of increased cortisol and pro-inflammatory cytokines in depression, and inform rational targeting in novel antidepressant strategies.

While the present in vitro study exposed cells to glucocorticoids for relatively short periods of time, it is possible that they could provide insight into the effects of chronic stress on the glucocorticoid and innate immune systems of chronically stressed humans. My study, and similar studies in animals (outlined above), support the hypothesis that chronic stress, which induces hypercortisolaemia, may, in turn, induce a state of increased inflammatory responsiveness of the innate immune system as a secondary effect. Interestingly, in my study, and similar studies in animals, this potentiated inflammatory responsiveness exists even when glucocorticoid levels return to baseline. In animal models, for example, corticosterone levels drop to baseline within two hours of exposure to a chronic stress (Barnum et al, 2012) and in my experiments glucocorticoids were absent from the media (after stringent washing of cells) when heightened immune responses were demonstrated. One
might speculate that periods of transient hypercortisolaemia (perhaps repeated or for long duration) may leave their mark by alterations to the inflammatory system and glucocorticoid sensitivity that persist for periods, perhaps quite prolonged, after normalisation of cortisol levels. This may explain the observation that hypercortisolaemia is not as common a finding in depressed patients as increased levels of pro-inflammatory cytokines, or glucocorticoid insensitivity.

Such an explanation would be consistent with the observation, now confirmed on meta-analysis, that adults who have suffered childhood trauma demonstrated significantly elevated baseline levels of TNF-α, IL-6 and CRP (Baumeister et al., 2015). This is also true of depressed patients with increased early life stress who demonstrate increased baseline and stimulated immune responses in adult life (Pace et al., 2006). Indeed, elevated immune system activity in chronically stressed and depressed subjects might be even more evident in exaggerated immune response to stimulation rather than in baseline levels, despite the latter finding attracting more recent attention. For example, adult subjects with a history of childhood trauma demonstrate exaggerated immune responses to inflammatory provocation (Miller and Chen, 2010). This study followed participants for 1.5 years, testing in vitro responses of white bloods cell to LPS and sensitivity to cortisol inhibition. They found that over time the inflammatory response to stimulation increased, along with decreasing sensitivity to cortisol inhibition for those participants that had been exposed to the most harsh family environments (Miller and Chen, 2010). Interestingly, family environment strongly predicted glucocorticoid resistance and inflammatory response to provocation, but not baseline levels of inflammation.
This matches findings in newborn infants, where those exposed to greater stress in utero had larger responses to microbial challenge ex vivo than those without exposure to maternal stress (Wright, 2010). Adults who were raised in lower socioeconomic environments also display larger ex vivo cytokine response to microbial challenge (Miller et al, 2009b). Interestingly, maternal warmth could reduce the immune priming effect of low SES childhoods on ex vivo stimulation of IL-6 levels in whole blood (Chen et al, 2011). Another study, mentioned previously, demonstrated an increased propensity to illness and increased cytokine responses to viral challenge in those patients who were most stressed (and possessed the greatest degree of glucocorticoid resistance) (Cohen et al, 2012). Subjects who reported more depressive symptoms showed larger increases in serum IL-6 levels 2 weeks after vaccination against influenza-virus infection, than those with few or no depressive symptoms (Glaser et al, 2003). Women with a history of major depression showed increased IL-6 and sIL-1RA responses to the stress of childbirth than control women (Maes et al, 2001). This potentiation of immune responses in stressed subjects is also consistent with the increased propensity of disease in subjects previously exposed to chronic stress (reviewed in (Glaser and Kiecolt-Glaser, 2005)).

This is also true of innate immune responses to psychological stress provocation, in addition to bacterial stimulation, extending the innate immune sensitivity of stressed subjects to include more ethologically relevant stimuli for depression. Subjects with a history of greater childhood psychological stress demonstrate exaggerated IL-6 responses to the Trier Social Stress Test, despite having similar levels of baseline inflammation (Carpenter et al, 2010). Similarly, people with a history of childhood abuse responded with greater
increases in IL-6 levels (though not TNF-α or CRP) to daily stressors (Gouin et al, 2012). This suggests that enhanced responsiveness to inflammatory or psychological stress may be characteristic of chronic stress conditions (along with, or perhaps more markedly than, increased baseline inflammation), analogous to findings in animal models of chronic stress and in the present human hippocampal progenitor cell study. Extensive studies of the immune responsiveness in depressed subjects has not been conducted, but one study conducted is very interesting. Subjects with major depression demonstrated significantly larger inflammatory responses to stress in the TSST, as evidenced by increased IL-6 plasma levels and increased NF-κB binding in PBMCs, than control subjects (Figure 53) (Pace et al, 2006). Depression scores correlated significantly with both the increase in IL-6 and the increase in NF-κB binding.

Studies in humans suggest that the mechanism that connects chronic stress to enhanced inflammatory response may be glucocorticoid resistance. For example, one study has shown that glucocorticoid resistance increases in female participants one hour after exposure to the Trier Social Stress Test (although men demonstrated an increase in glucocorticoids sensitivity), demonstrating that changes to glucocorticoid resistance can occur even after short stressors (Rohleder et al, 2001). Other studies have shown that greater exposure to chronic stressors predicts the presence of increased glucocorticoid resistance. Glucocorticoid resistance increased longitudinally in subjects who had a history of exposure to harsh family environments, along with increased inflammatory response to immune stimulation (Miller and Chen, 2010). Glucocorticoid resistance in another group of otherwise healthy subjects was correlated to stress exposure and was in turn correlated to immune response and illness following viral challenge (Cohen et al, 2012).
Taken together, these studies indicate evidence for increased immune responsiveness – to both physical and psychological stressors – that may arise due to chronic stress, potentially in a sequential manner, over numerous life stressors, which cause transient hypercortisolaemia, and which may account for the relationship between chronic stress and depression. Such a description accounts for the increased immune activity in chronically stressed and depressed subjects, in the absence of robust detection of hypercortisolaemia, and may also account for the presence of glucocorticoid resistance, as one of the mechanisms of a potentiated immune system. Following on from this model, cortisol lowering treatments in depressed patients might be attending to an empty stable; perhaps cortisol blocking treatments, such as RU486, might present effective preventative treatment during periods of intense stress, to prevent potential sequelae. This model does suggest that anti-inflammatory treatments may be more relevant to treating depression, but the underlying cause of increased immune responsiveness, like glucocorticoid resistance, might present the most rational target for treatment, as a simplistic reduction of inflammatory activity might be inadequate to modify the underlying abnormalities.
Increased inflammatory response to stress by depressed patients with increased early life stress

Depressed patients with increased early life stress demonstrate both increased baseline levels of IL-6, and also greater potentiation of the immune response to the Trier Social Stress Test, as illustrated by higher levels of IL-6 after exposure. The potentiation was also found in increased activation of NF-κB (right hand panel). These findings mirror those found in the human neural cells, where previous exposure to glucocorticoids potentiated subsequent inflammatory responses, suggesting that the similar mechanisms may be involved in immune processes in depressed subjects. Adapted with permission (Pace et al., 2007).

Figure 53 Increased inflammatory response to stress by depressed patients with increased early life stress

\(^{a}\) Significant difference from baseline \((p<0.05)\).

\(^{b}\) Significant difference between groups \((p<0.05)\).
4.4 **Antidepressant compounds as immunomodulators**

4.4.1 **Overview of findings**

I report that antidepressant compounds, commonly found to be anti-inflammatory in animal brain or human blood samples, diverge in their influence on immune processes in human hippocampal progenitor cells, an observation which may be pertinent to their clinical efficacy, as I have reported in a recently published paper (Horowitz *et al*, 2014). In particular, I found that venlafaxine and EPA exerted an anti-inflammatory influence, with a corresponding decrease in NF-κB activity. In contrast, sertraline and DHA demonstrated pro-inflammatory effects, despite a reduction in NF-κB activity, suggesting alternative mechanisms underlying their immunomodulatory effects. Agomelatine and moclobemide had no immunomodulatory effects, as measured by IL-6 secretion into the supernatant.

4.4.2 **Anti-inflammatory effects**

I found that venlafaxine and EPA both demonstrated anti-inflammatory effects as measured by a decrease in secreted IL-6 levels in the context of IL-1β stimulation. This is consistent with previous studies that report venlafaxine to be anti-inflammatory in depressed patients (Lee and Kim, 2006), in an animal astroglia-microglia co-culture model (Vollmar *et al*, 2008) as well as in murine T cell clones and peritoneal macrophages in vitro (Vollmar *et al*, 2009). Indeed, this matches reports finding that other noradrenaline reuptake inhibitors are also anti-inflammatory in rat primary cortical glial cells (O’Sullivan *et al*, 2009). I also found that EPA reduced secretion of IL-15, IL-1RA and IP-10 and down-regulated IL-6 mRNA. This finding is in line with previous research showing
EPA to exert anti-inflammatory effects in stimulated murine macrophages (Novak et al, 2003), human macrophages (Hao et al, 2010) and rat microglia (Lu et al, 2010). Our study confirms and extends these previous findings to a clinically relevant model of human neural cells.

4.4.3 Pro-inflammatory effects

Contrary to our expectations, we found that both sertraline and DHA exerted pro-inflammatory effects in human hippocampal cells. Although sertraline and DHA have been reported to be anti-inflammatory in previous studies (Antonietta Ajmone-Cat et al, 2012; Lu et al, 2010; Maes et al, 1999; Sutcigil et al, 2008; Tynan and Weidenhofer, 2012) it is clear that their immunomodulatory effects are cell type-specific: for example, sertraline is anti-inflammatory in human PBMCs (Maes et al, 1999) while pro-inflammatory in rat neutrophils (Paschoal et al, 2013). Indeed, DHA has shown anti-inflammatory effects in murine microglia (Lu et al, 2010), while demonstrating pro-inflammatory effects, as measured by an increased IFN-γ/IL-10 ratio, in diluted whole blood (Maes et al, 2007).

Another important determinant of immunomodulatory effects is dose of treatment. For example, sertraline at a dose of 2.5µM, similar to the range employed in the present study, produced pro-inflammatory effects in murine microglia (Tynan and Weidenhofer, 2012), with anti-inflammatory effects only evident at concentrations above 10µM, unlikely to correspond to clinically therapeutic levels (Schulz et al, 2012). Consistent with this notion, sertraline has been observed to increase the serum levels of cytokines in depressed patients (Marques-Deak et al, 2007). The same dose-related effects are recognised for DHA: in rat neutrophils, DHA increased TNF-α production at a dose of 25µM,
whereas it caused a reduction when used at 50µM (Paschoal et al, 2013). We selected dosages of antidepressant compounds informed by both previous studies and likely therapeutic concentrations (Lu et al, 2010; Maes et al, 1999; Schulz et al, 2012; Vollmar et al, 2008) but we cannot exclude anti-inflammatory effects at concentrations larger than those employed here.

4.4.4 No immunomodulatory effect

The finding that agomelatine and moclobemide did not demonstrate immunomodulatory effects in human neural stem cells as measured by IL-6 protein was somewhat distinct from findings in peripheral blood cells and animal models. Although there are no previous studies investigating the immunomodulatory effects of agomelatine in vitro, there have been studies in the periphery and brains of rats demonstrating an anti-inflammatory effect (Molteni et al, 2013). A more pertinent issue, only discovered after experiments were completed, was a paper demonstrating that the antidepressant properties of agomelatine are likely to be clinically insignificant when taking into account unpublished trials on its efficacy; its reported positive effects were found to be a result of a disturbing degree of publication bias (Koesters et al, 2013). Moclobemide has also been found to be anti-inflammatory in primary rat mixed glial cell culture (Bielecka et al, 2010) and in whole blood samples of healthy volunteers, as measured by IL-8 and TNF-α (but, interestingly, it had no effect on IL-6 levels) (Scharpe et al, 2000). This suggests the possibility that either these are cell type-specific effects, or that moclobemide may have immunomodulatory effects on cytokines other than IL-6 that may not have been identified in the present study.
4.4.5 Potential clinical relevance

The contrast in the effects of sertraline (pro-inflammatory) and venlafaxine (anti-inflammatory), as well as in the effects of DHA (pro-inflammatory) and EPA (anti-inflammatory) are intriguing. These differences seem particularly pertinent in light of recent meta-analyses emphasising the importance of EPA over DHA in the antidepressant efficacy of fish oil preparations (Lin et al., 2010; Martins et al., 2012). While, to our knowledge, there are no direct comparisons of monoaminergic antidepressants in inflammation-induced depression, a meta-analysis found that venlafaxine is more effective than other SSRIs in treating depression (Smith, 2002), perhaps reflecting the anti-inflammatory properties identified in the present study. Moreover, a recent clinical trial has shown that EPA, but not DHA, reduces the incidence of depression in patients treated with IFN-α, a context in which pro-inflammatory cytokines are most clearly implicated in the development of depression (Su et al., 2014).

In addition, a recent study has reported that depressed patients with high levels of baseline inflammation (as indicated by high IL-1RA, high CRP or low adiponectin) showed significantly improved effects with ω-3 PUFA treatment, with high EPA content, compared to placebo (with a difference of effect size of 0.39) (Rapaport et al., 2015). Interestingly, this ‘high inflammation’ group showed a significantly worse response to DHA than to placebo (effect size difference of 0.21), and a markedly worse response of DHA as compared to EPA (effect size difference of 0.60). Although inflammatory biomarkers were only measured at baseline in these patients and not after treatment, it is possible that the greater efficacy of EPA in these inflamed patients reflects its ability to be anti-inflammatory, in contrast to the pro-inflammatory effects of DHA, as reflected in the present study in human neural cells.
4.4.6 Mechanism of action

Perhaps surprisingly, considering that venlafaxine, sertraline, EPA and DHA demonstrated different effects on the expression of cytokines and chemokines, all four compounds reduced the activity of NF-κB. NF-κB has been implicated as a key target underlying the anti-inflammatory effects of many monoaminergic antidepressants (Bielecka et al., 2010; Zhu et al., 2008) but, to our knowledge, no previous study has examined the ability of venlafaxine to inhibit NF-κB activity, as demonstrated here. EPA has been previously observed to reduce endotoxin-induced activation of NF-κB in human macrophages (Novak et al., 2003) and in BV2 microglia (Moon et al., 2007), in line with our findings. Interestingly, both monoaminergic antidepressants and ω-3 PUFAs may affect NF-κB activity through interactions with the neuronal cell membrane (Allen et al., 2007; Czysz and Rasenick, 2013). Specifically, these compounds have been thought to liberate G-protein alpha subunits from membrane-associated lipid rafts leading to a cascade of intracellular events, involving increases in adenylyl cyclase, cAMP and PKA signalling, known to inhibit NF-κB activity (Czysz and Rasenick, 2013; Donati and Rasenick, 2005; Parry and Mackman, 1997).

The finding of decreased NF-κB activity in the context of pro-inflammatory effects with DHA is especially interesting. DHA has been observed to inhibit NF-κB in macrophages (Mullen et al., 2010), and to do so even more strongly than EPA (Weldon et al., 2007), as seen in the present study. However, DHA and EPA are well recognised to have distinct effects in a variety of cell types, in the pattern of cytokines affected, the time course of NF-κB inhibition (Mullen et al., 2010) and in the effects on cell membranes and intracellular signalling pathways (Gorjão et al., 2009). Therefore, a different time-course of the effects of these compounds on NF-κB may explain the divergent effects on IL-6 levels.
A further consideration is raised by recent work demonstrating anti-inflammatory effects of paroxetine in murine microglia without a significant effect on NF-κB; instead the authors identified JNK1/2 and ERK1/2 pathways as more relevant (Liu et al, 2014). Furthermore, there are alternative pathways that have been implicated in the ability of ω-3 PUFAs to be anti-inflammatory, including the inhibition of p38 MAPK phosphorylation and activation of the nuclear receptor peroxisome proliferator activated receptor (PPAR)-γ (Antonietta Ajmone-Cat et al, 2012).

In summary, I have observed divergent effects of antidepressant compounds in a human, clinically-relevant, cellular model of depression, suggesting a possible explanation for the differences in efficacy seen in the different compositions of fish oil preparations, and perhaps in the putative variations of outcomes with monoaminergic antidepressants. An increased understanding of the molecular mechanisms underlying these effects may allow for more effective personalisation of antidepressant choice based on the inflammatory status of depressed patients.

4.5 Antidepressant compounds effect on neurogenesis and the kynurenine pathway

4.5.1 Summary of findings

I found that IL-1β induces a reduction in neurogenesis, in part, by its modulation of the kynurenine pathway towards neurotoxic metabolites, away from neuroprotective end products of the pathway, as evidenced by change in expression of key regulatory enzymes, consistent with a previous study in these cells (Zunszain et al, 2012). The connection between the kynurenine pathway
and decreased neurogenesis in IL-1β treatment was extended in the present study by demonstration of a significant increase in the putative neurotoxic end product of the kynurenine pathway, quinolinic acid (QUIN), consistent with the induction of the enzymes IDO, KMO and KYNU, enzymes that catalyse the formation of QUIN. This extends previous work in our laboratory that demonstrated that blocking the kynurenine enzymes induced by IL-1β, partially reversed IL-1β-induced reduction in neurogenesis (Zunszain et al, 2012).

I also found that three antidepressants with very different chemical structures – venlafaxine, sertraline, and DHA - could partially reverse the anti-neurogenic effects of IL-1β, while the ω-3 PUFA, EPA, had no effect on neurogenesis. This effect was most strongly seen in the effect on mature neurons (MAP2 positive) but in the case of venlafaxine and DHA, also on immature neurons (DCX positive).

The effect of the antidepressant compounds on kynurenine enzyme gene expression was mixed, while effects on protein expression of quinolinic acid were consistent with each compound’s effect on neurogenesis. DHA partially reversed the effect of IL-1β on IDO and KMO, but increased the effect of IL-1β on KYNU. However, importantly, at the protein level, DHA almost completely abolished the induction of QUIN by IL-1β, consistent with its effect on IL-1β-induced reduction in neurogenesis. Sertraline completely abrogated the IL-1β-induced increase in QUIN, consistent with its reversal of the effects of IL-1β on neurogenesis, while having mixed effects on kynurenine enzymes – decreasing KMO, while increasing KYNU, and having no effect on IDO. Venlafaxine showed a trend to attenuate IL-1β-induced increases in QUIN, along with its reversal of the effects of IL-1β on neurogenesis, with no significant effect on kynurenine enzymes. Interestingly, EPA demonstrated both no effect on
neurogenesis, and no effect on QUIN levels, with mixed effects on kynurenine enzymes, including an increase in KMO.

Overall, three out of the four antidepressant compounds tested partially reversed the effect of IL-1β on reducing neurogenesis and increasing QUIN, and the single compound that did not affect neurogenesis also did not affect QUIN levels. Given findings from our lab that a shift towards neurotoxic metabolites of the kynurenine pathway is in part responsible for the anti-neurogenic effects of IL-1β treatment (Zunszain et al, 2012), the ability of these antidepressant compounds to block changes to quinolinic acid production by IL-1β may be the mechanism by which they block the IL-1β-induced reduction in neurogenesis.

4.5.2 IL-1β increases the neurotoxic kynurenine metabolite, QUIN, further strengthening perturbation of the kynurenine pathways as an anti-neurogenic mechanism

Consistent with the findings of the present study, it is well established in animals that administration of inducers of pro-inflammatory cytokines, or pro-inflammatory cytokines themselves, can result in suppression of hippocampal neurogenesis (along with depressive-symptoms) (Ekdahl et al, 2003; Goshen et al, 2008; Koo and Duman, 2008; Monje et al, 2003). It has also been shown that other pro-inflammatory cytokines, like IFN-α decrease neurogenesis through modulation of IL-1 (Kaneko et al, 2006). In this study IFN-α-induced reduction in neurogenesis could be completely abolished by co-administration of an IL-1 receptor antagonist, indicating that IL-1 signalling could represent a common pathway by which inflammation reduces neurogenesis. Our lab has also shown that the induction of kynurenine enzymes that direct the kynurenine pathways
towards neurotoxic end products by inflammation accounts for, in part, its anti-neurogenic effects (Zunszain et al, 2012).

I have extended these findings in the present study by demonstrating that IL-1β greatly increases production of quinolinic acid, the principal neurotoxic product of the kynurenine pathway. This has not previously been measured in human hippocampal progenitor cells, although the connection between inflammation and increased quinolinic acid has been observed in other cell types (Schwarcz et al, 2012). Quinolinic acid, an NMDA receptor agonist, can increase neurotoxicity by various pathways, most notably by over-activation of NMDA receptors and increasing cytosolic calcium ions, leading to mitochondrial dysfunction, cytochrome c release, ATP exhaustion, free radical formation and oxidative damage (Cruz et al, 2012; Guillemin, 2012). This study has demonstrated that the increase in kynurenine enzymes (IDO, KMO, KYNU) induced by inflammation leads to an increase in neurotoxic end products, and strengthens the notion that perturbation of the kynurenine pathway is one of the mechanisms by which inflammation reduces neurogenesis.

4.5.3 Antidepressants can reverse the anti-neurogenic effects of IL-1β

This study has demonstrated that monoaminergic antidepressants (venlafaxine and sertraline) and the ω-3 PUFA, DHA, can reverse the anti-neurogenic effects of inflammation in human hippocampal progenitor cells. Monoaminergic antidepressants have previously been shown to be pro-neurogenic in animal models, both in control and stressed animals, in human cellular models and through post-mortem studies of depressed subjects (Section 1.4.3). ω-3 PUFAs also have considerable evidence that they can be pro-neurogenic in
animals, as well as in vitro, including in studies where inflammation was used as the depressogenic stimulus, with an emphasis on DHA as the causative agent, consistent with findings in the present study (Section 1.4.3.3). Indeed, it has previously been shown that DHA can reverse the anti-neurogenic effects of inflammation-activated microglia on mouse neural progenitor cells (Antonietta Ajmone-Cat et al, 2012). DHA could inhibit cytokine production in microglial cells when co-incubated with LPS or IFN-γ. In addition, when media from the cultured microglia was placed on rat hippocampal progenitor cells DHA+LPS-treated microglia reduced hippocampal neurogenesis significantly less than LPS-treated microglia. The ability for DHA to be pro-neurogenic is also supported by studies in older rats (Dyall et al, 2010) and mice engineered to express high levels of DHA (He et al, 2009), as well as by a study showing that DHA-deficient embryonic rats show reduced neurogenesis (Coti Bertrand et al, 2006).

Interestingly, a recent study demonstrated that both EPA and DHA could increase neuronal differentiation in rat neural progenitor cells, but seemed to do so by alternative mechanisms (Katakura et al, 2013). In this study in rat neural progenitor cells, both EPA and DHA affected different cell cycle regulating genes to induce cell cycle arrest and induce neuronal differentiation. It is possible that in human neural progenitor cells the effect of these compounds is even more distinct leading to the divergent effects on neurogenesis observed in the present study. Further investigation of the effect of these compounds on cell cycle genes would be instructive.

In general, glucocorticoids have been used to simulate stressors in animal and cellular experiments. For example, in the human hippocampal progenitor cells, sertraline was able to reverse dexamethasone-induced reduction of
neurogenesis (Anacker et al., 2011b). In numerous animal models glucocorticoid administration has been used to simulate stress paradigms, and have been shown to induce depressive-like behaviour, and reduced neurogenesis, both of which can be reversed by monoaminergic antidepressant treatment (David et al., 2009; Qiu et al., 2007). Fewer studies have explored the ability of monoaminergic antidepressants to reverse neurogenic and behavioural changes induced by inflammatory stimulation, despite work demonstrating that IL-1β is integral to the anhedonic and anti-neurogenic effects of stress (Koo and Duman, 2008). This in vitro study provides evidence that antidepressant compounds, both monoamine-based and ω-3 PUFAS, can reverse the anti-neurogenic effects of inflammation in cells relevant to depression and this may have relevance to clinical treatment of depression, given the importance attributed to increased inflammation in the pathogenesis of depression. Indeed, given the debate surrounding inflammation versus glucocorticoids as the pathogenic molecules in depression, it may be that a compound’s ability to reverse the anti-neurogenic effects of inflammation is more clinically relevant than the ability to reverse the anti-neurogenic effects of glucocorticoids. I will therefore briefly discuss the mechanism by which antidepressants might reverse the effects of inflammation on neurogenesis.

4.5.4 Mechanism by which these antidepressant compounds block the anti-neurogenic effects of IL-1β

Inflammation has been thought to affect neurogenesis via three potential pathways – by activating the HPA axis to release cortisol, in turn, reducing neurogenesis; affecting glial cell function, with a particular emphasis on neurotrophic factors produced by astrocytes; and lastly, by direct effects on neural progenitor cells (Song and Wang, 2011). Most studies that have looked
at the ability of compounds to reverse the effect of inflammation on neurogenesis have focused on the ability of anti-inflammatory drugs to reduce the inflammatory activation or response of glial cells, especially microglia. For example, the anti-inflammatory drug indomethacin can block the effects of inflammation on reducing neurogenesis (Monje et al, 2003), by decreasing microglial activation induced by LPS. The authors also point out that indomethacin may also reduce HPA axis activation, caused by LPS administration, or reduce newborn cell death (Monje et al, 2003). Another recent study demonstrated that iptakalim, an ATP-sensitive potassium channel opener, can reverse the neuroinflammation and reduced neurogenesis, induced by chronic mild stress in mice (Lu et al, 2014). This compound appeared to achieve this effect by suppressing the activation of the inflammatory pathway – the NLRP3-inflammasome/caspase-1/IL-1β axis - in hippocampal microglia.

However, the present in vitro study excludes systemic effects, such as changes to the HPA axis, and indirect effects on microglia, or other glial cells, so allows for examination of direct effects upon progenitor cells, and therefore suggests that the monoaminergic antidepressant compounds venlafaxine and sertraline, and the ω-3 PUFA, DHA, exert direct effect upon progenitor cell development. Furthermore, the present study also suggests that this ability of antidepressants involves mechanisms other than direct anti-inflammatory effects. Such a conclusion can be drawn from the observation that the antidepressant compounds that blocked the effects of IL-1β on neurogenesis had either pro-inflammatory effects (sertraline, DHA) or small anti-inflammatory effects (venlafaxine) (Section 3.4), while exerting large pro-neurogenic effects. This suggests that the mechanism by which these compounds act to be pro-neurogenic is not through directly reducing inflammation. Indeed, the most
strongly anti-inflammatory compound, EPA, had no effect on neurogenesis. One potential explanation is that these compounds act downstream of IL-1β signalling, to modulate the kynurenine pathway that contributes to the effect of IL-1β on reduced neurogenesis, discussed below. The present study’s ability to isolate the direct effects of these compounds on neurogenesis is both a strength, as we can focus on direct effects on progenitor cells, and a limitation, as there may be other indirect effects, as outlined in studies above, that may also contribute to the overall effect of antidepressant compounds on neurogenic processes influenced by inflammation.

4.5.5 Antidepressant effects on the kynurenine pathway

This study demonstrated that the three antidepressant compounds that attenuated the effect of inflammation on neurogenesis, also reduced the neurotoxic products of the kynurenine pathway, suggesting this may be the mechanism of this attenuation. This is consistent with studies demonstrating that compounds which can reverse the effects of inflammation on the kynurenine pathway exert antidepressant effects. For example, the IDO antagonist, 1-methyltryptophan, can reduce the depression-like behaviour induced by inflammation (via administration of the BCG vaccination) in animals (O’Connor et al., 2009). Pertinently, this effect on the kynurenine pathway and its attendant antidepressant effect occurs in the absence of any change to levels of inflammation suggesting that the antidepressant effects were directly related to IDO antagonism (O’Connor et al., 2009). Considering the role that activation of the kynurenine pathway has been thought to play in reducing neurogenesis (Kanai et al., 2009; Zunszain et al., 2012), this suggests that the kynurenine pathway might be a pathway, downstream of inflammation, in part
responsible for both the anti-neurogenic and behavioural effects of inflammation that might be directly targeted by antidepressant compounds.

Additionally, previous studies have shown that antidepressants can affect the kynurenine pathway, consistent with the findings in the present study. For example, sertraline reduces the KYN/MEL (melatonin) ratio (melatonin being a downstream metabolite of serotonin) and 3-hydroxykynurenine (3-OHKY)/MEL ratio in depressed patients who respond to this treatment (compared with pre-treatment measures) but not in those who do not respond (Zhu et al., 2013), demonstrating changes consistent with the present study. Furthermore, polymorphisms in the genes for IDO1 and IDO2 have been shown to associate with outcomes from treatment with citalopram (Cutler et al., 2012), suggesting a role for the kynurenine pathway in antidepressant effects. A recent study demonstrated that escitalopram can reduce levels of quinolinic acid and 3-OHKY in the plasma of depressed patients, in association with minimal changes in inflammatory cytokines (a trend to decrease in TNF-α, no change in other pro-inflammatory cytokines) and clinical antidepressant response (Halaris et al., 2015). This study provides further evidence that antidepressants can affect the kynurenine pathway, without significant effect on inflammation, as in the present study.

The ability of antidepressants to shift the kynurenine pathway away from neurotoxic metabolites towards neuroprotective metabolites is also suggested by in vitro studies. For example, SSRIs and TCAs increase production of KYNA and diminish 3-HK synthesis (at 24 and 48 hours) in rat glial cultures, representing a shift from the neurotoxic pathway towards the neuroprotective (Kocki et al., 2012). Interestingly, the largest effects on kynurenine metabolites and gene expression of kynurenine enzymes for the antidepressants tested
were at 48 hours (Kocki et al, 2012). This suggests the possibility that the equivocal changes to mRNA levels of the kynurenine metabolites (at 24 hours) found in the present study may have been more consistent with changes seen to levels of quinolinic acid at the protein level (at 10 days) had they measured at a later time point.

No studies have looked directly at the effects of venlafaxine on the kynurenine pathway but studies in animals with other monoaminergic antidepressants provide some evidence that these compounds can affect the kynurenine pathway. Paroxetine reverses the increase in KYN and the reduction in KYNA induced by tryptophan dietary depletion in rats, but did not alter the depressive behaviour induced (Franklin et al, 2012). Citalopram exerted antidepressant effects and inhibited IDO activity, increasing turnover of serotonin, in the hippocampus and other brain regions of stressed rats (Ara and Bano, 2012). In BV-2 microglia, fluoxetine attenuated the increase in expression of IDO (and the depletion in serotonin) caused by LPS stimulation, along with reducing levels of IL-1β (Yang et al, 2014). These effects on the kynurenine pathways have also been extended to other antidepressant compounds – imipramine reversed the elevated KYN/TRP ratio (and depressive behaviour) in rats exposed to LPS and chronic mild stress (Elgarf et al, 2014), and agomelatine has also been shown to prevent the LPS-dependent increase in KMO in the brain and periphery of rodents (Molteni et al, 2013).

There has been less attention paid to the ability of PUFAs to affect the kynurenine pathway. A single study shows that rats subjected to spinal cord surgery previously fed a diet high in ω-3 PUFAs show an increase in kynurenine compared to rats fed a control diet (Figueroa and De Leon, 2014), though levels of neurotoxic or neuroprotective end products were not measured. However, it
would appear from the present study that the ability of ω-3 PUFAs to affect the kynurenine pathway and to affect inflammatory processes dissociate. EPA exerted broadly inflammatory effects in the human hippocampal cells, as measured by levels of IL-8, IL-15, IL-1RA, IFN-α, IP-10, IL-6 and RANTES (Section 3.4.1), while DHA exerted pro-inflammatory effects on most of these chemokines and cytokines. Conversely, EPA showed no significant effect on IL-1β-induced increased in QUIN, while DHA showed a highly significant reversal of this effect. This is also consistent with the effect that these differing compounds exerted on neurogenesis, with EPA showing no effect on IL-1β-induced reductions in neurogenesis, while DHA showed a highly significant, large reversal of this effect.

A greater number of studies have found evidence for DHA influencing neurogenesis (Antonietta Ajmone-Cat et al, 2012; Coti Bertrand et al, 2006; Dyall et al, 2010; He et al, 2009), with less studies focused on the ability of EPA to affect such changes. However, one recent study found that both EPA and DHA could increase neuronal differentiation in rat neural progenitor cells, although there seemed to be alternative mechanisms involved (Katakura et al, 2013). The difference between this study and the present study suggests that DHA may have direct effects on progenitor differentiation, while EPA may affect progenitor cells indirectly, perhaps through their anti-inflammatory effects on glial cells.

4.5.6 Antidepressant effects on the kynurenine pathway as a mechanism for increasing neurogenesis, and alternative mechanisms
There have been limited studies on the mechanisms by which monoaminergic antidepressants might affect the kynurenine pathway. It has been suggested that antidepressants might be able to act at more than one point in the kynurenine pathway – including reversing the release of inflammatory mediators from various cell types, inhibition of IDO and reduction of kynurenine levels and its metabolites (Réus et al, n.d.) There was partial support found for inhibition of IDO in the present study, as DHA which strongly reduced QUIN, also showed a significant down-regulation of IDO mRNA, but sertraline and venlafaxine, which also reduced QUIN levels, showed no significant effect upon IDO mRNA levels. As discussed previously, this may be due to missing effects after 24 hours on mRNA levels of the kynurenine metabolic enzymes, and studies at further time points would be instructive. This is consistent with findings that antagonism of IDO, by either minocycline (which reduced IDO mRNA) or the IDO antagonist 1-methyltryptophan, has antidepressant effects in rodents (Henry et al, 2008; O’Connor et al, 2009). Consistent with this, citalopram and fluoxetine both can attenuate the increase in IDO activity in rats, induced by stress or LPS stimulation (Ara and Bano, 2012; Yang et al, 2014), suggesting IDO antagonism might be an important mechanism of action for antidepressant compounds.

There are also means other than affecting the kynurenine pathway that antidepressants might reverse the anti-neurogenic effects of inflammation that were not explored in the present study. These include the ability of antidepressants to reverse the effects of inflammation on neurotrophic factors like BDNF, known to be reduced by IL-1β and to negatively affect neurogenesis (Littrell, 2012). Other than direct effects on reducing inflammatory molecules released by immunologically active cells in the brain, antidepressants may reverse the effect of inflammation on neurogenesis by affecting cell cycle
genes, the HPA axis, the glucocorticoid receptor or levels of monoamines, amongst other proposed mechanisms (Anacker et al, 2011b; Dranovsky and Hen, 2006).

As mentioned above, there is only one previous study that has examined the mechanism by which ω-3 PUFAs can affect the kynurenine pathway. Rats fed ω-3 PUFAs showed higher levels of kynurenine when compared to animals fed control diets, however, levels of kynurenic acid and quinolinic acid were not measured so comparison to the present study is difficult (Figueroa and De Leon, 2014). It is possible that ω-3 PUFAs may affect the kynurenine pathway via similar means to monoaminergic antidepressants as outlined above, but further work is needed to explore potential mechanisms. Alternatively, PUFAs may also affect neurogenesis by mechanisms other than modulation of the kynurenine pathway. Some existing hypotheses have focused on the ability of ω-3 PUFAs to increased basic helix-loop-helix transcription factors which promote cell cycle exit, or the ability of ω-3 PUFAs to increase expression of synaptophysin and BDNF (Crupi et al, 2013).

4.6 Chromatin immunoprecipitation

The aim of this section of the thesis was to establish the technique of chromatin immunoprecipitation (ChIP) for discerning the genomic targets of the activated glucocorticoid receptor. This technique, though difficult to optimise, will be critical for determining the different patterns of genomic activation transduced by either glucocorticoid or antidepressant treatment of the hippocampal progenitor cells, which is likely to underpin their opposite effects of hippocampal neurogenesis.
I validated this technique in three steps. Firstly, I demonstrated that the chromatin fragments were sheared to optimal lengths (approximately 200-500 base pairs) by both sonication and enzymatic degradation. Secondly, I demonstrated that the chromatin fragments formed were of good quality by demonstration of enrichment of known targets of the REST antibody, an antibody already validated for use in ChIP. Lastly, I demonstrated preliminary evidence of enrichment of known targets of the glucocorticoid-activated glucocorticoid receptor in the human hippocampal progenitor cells upon treatment of the cells with dexamethasone. Specifically, I demonstrated several-fold enrichment of the GR-target genes SGK1, SLC19A2, GILZ and MT2A in nuclei treated with dexamethasone 1μM for one hour, compared with samples treated with vehicle for one hour, as well as by comparison of samples immunoprecipitated with the GR antibody versus the control non-specific IgG. However, evidence of enrichment of DSCAM, which was selected as a control gene, suggests that this gene may not be an effective control gene. The variability of the qPCR results also requires repetition to confirm validation of the protocol.

This technique will be instrumental in discerning the pathways by which glucocorticoids and antidepressants influence hippocampal neurogenesis in opposite directions. It is well known in animal models that glucocorticoids reduce hippocampal neurogenesis, while antidepressants can increase it, and also reverse the effects of glucocorticoids on this process, though the mechanisms underlying this process are poorly understood. Our lab recently extended this finding to human hippocampal progenitor cells, with the novel finding that the glucocorticoid receptor was a key point of interaction in this process as blockade of this receptor by the glucocorticoid antagonist, RU486
was able to block the effects of antidepressant and glucocorticoids on neurogenesis (Anacker et al, 2011b).

Furthermore, treatment with dexamethasone alone, sertraline alone or co-treatment with dexamethasone and sertraline induced specific conformational changes of the GR, evident in differential phosphorylation of serine residues, which have been linked to differential actions of the GR, including distinct patterns of genomic transactivation and transrepression (Chen et al, 2008). There is also evidence that these structural changes to the GR have functional consequences to the activity of the GR, as the cell cycle regulating genes, p27 and p57, were differentially induced (Anacker et al, 2011b). Therefore, it is likely that transcription or repression of the different genomic targets of the GR, when activated by dexamethasone alone, sertraline alone or in combination, underpin its differential effects on hippocampal neurogenesis. The technique optimised in this thesis for use in the human hippocampal cells will allow these genomic targets to be identified, potentially yielding insights into the mechanisms by which hippocampal neurogenesis is regulated by different compounds, which has been thought to be a key process in the pathogenesis and treatment of depression.

4.7 **Methodological considerations**

There are a few methodological limitations that should be considered when interpreting the findings obtained in this thesis. The limitations to the systematic review and meta-analysis were discussed in Section 4.2.6, so the limitations below pertain to the cellular work conducted.
4.7.1 The use of an in vitro system

I have used a conditionally immortalised human embryonic hippocampal progenitor cell line (HPC03A/07) to examine the effects of glucocorticoid hormones, inflammation and antidepressants on immune processes, the kynurenine pathway and neurogenesis on hippocampal progenitor cells. As with all in vitro models, a limitation of this system lies in the challenge to extrapolate findings from cells to the whole organism. As isolated cells in culture are not embedded in their natural environment, such a system necessarily provides a simplistic representation of the whole organism. Data obtained from in vitro studies must therefore be interpreted with caution in order not to draw erroneous conclusions about the more complex, entire organism. However, the cells used in the present study represent one of the only means to investigate the processes relevant to the pathogenesis of depression and the effect of antidepressants in human neural cells, and, in particular, hippocampal progenitor cells with pertinent relevance to depression. Previously, studies have looked at the interaction of the HPA axis and inflammation, the effect of antidepressants on inflammation, neurogenesis and the kynurenine pathway either in animal models or in peripheral samples from humans. These approaches have been instructive, but there are difficulties translating effects found in rodents to humans, due to the possibility of species-specific effects, and findings from peripheral human samples are also difficult to extrapolate to the CNS, given that ultimately human neural cells are of primary interest. The present model allows examination of effects directly on human neural cells, and the associated molecular mechanisms, which, in turn enables comparison to findings in animal brains and peripheral human samples, providing a complementary set of findings.
Previous findings from this cell model have been replicated in human samples, and effects thought to occur in depressed subjects have been re-capitulated in this cell model, suggesting that the model possesses translational relevance and may be a useful system for generating insights into the brain of depressed subjects. For example, we have identified that antidepressants up-regulate the pro-neurogenic gene, p11, (Anacker et al, 2011b) and then replicated this finding in peripheral blood mRNA of depressed patients before and after eight weeks of antidepressants (Cattaneo et al, 2013); we have demonstrated that IL-1β (Zunszain et al, 2012) and dexamethasone (Anacker et al, 2011b) (both considered depressogenic insults) decrease neurogenesis in the present cell model. Furthermore, dexamethasone treatment of these cells demonstrate epigenetic and gene expression changes in the stress-regulated gene, FKBP5, that are identical to those identified in peripheral blood mRNA of patients who have experienced childhood maltreatment (Klengel et al, 2013). Lastly, transcriptomic analysis of these cells treated with cortisol identify gene expression pathways regulating human neurogenesis (Anacker et al, 2013a), which were replicated in brain transcriptomics of rats exposed to stress as well as candidate gene expression analyses of peripheral blood mRNA in patients with depression (Anacker et al, 2013a, 2013b).

Furthermore, the simplicity of in vitro systems also has advantages, as it allows examination of the contribution of specific factors, such as glucocorticoid hormones or antidepressants, without confounding influences which cannot be eliminated in vivo, but simply added or omitted in a cell culture system. This has been particularly interesting in examining phenomena, like the effect of antidepressants on inflammation-induced reductions in neurogenesis, which have been thought to act either through indirect systemic effects or direct
effects on progenitor cell fate, by isolating only direct effects. This is similarly true for the examination of the interaction between inflammation and glucocorticoids which could affect by various indirect and direct mechanisms, and has allowed conclusions to be drawn about intracellular interactions of these systems from the present experiments.

Notably, findings in animal brains with regards to the effect of glucocorticoid treatment preceding inflammatory stimulus show remarkable consistency with the findings in human progenitor cells, and may help explain the mechanisms underlying peripheral findings in stressed and depressed humans. In the context of previous studies in these cells demonstrating effects of glucocorticoids, inflammation and antidepressants on neurogenesis, also consistent with findings in animal brains and supported by post-mortem findings in humans, these cells offer a means to investigate processes relevant to depression without the need for animals and with species-specific relevance.

4.7.2 Cell type

A more particular methodological consideration is the cell type used in the present study. With respect to immune responses, glia, and, in particular, microglia have been regarded as the most relevant cell type to investigate. Microglia are known to show pronounced phenotypic changes in response to inflammatory stimuli and to produce inflammatory mediators (Nayak et al, 2014). Microglia have also been thought to be a critical cell type in the apparently pro-inflammatory effect of glucocorticoids, with a growing body of evidence demonstrating that they can be ‘primed’ to produce potentiated inflammatory responses by preceding exposure to stress or glucocorticoids (Walker et al, 2013). Some authors have regarded them as the key transducer of the pro- or anti-inflammatory effects of glucocorticoids (Frank et al, 2013;
Walker et al, 2013). I have extended these findings in a perhaps unexpected manner – including hippocampal progenitor cells as cell types that can be differentially affected by glucocorticoids with respect to their inflammatory responses.

Hippocampal progenitor cells are known to respond to and produce inflammatory signalling (Johansson et al, 2008; Kokaia et al, 2012; Zunszain et al, 2012). However, they have been generally considered to be affected by inflammation rather than effectors of it. Indeed, it has been shown that stem cells can exert immunomodulatory effects on microglia, for example an expanding body of evidence examining the ability of bone marrow derived progenitor cells to modulate the immune responses of microglial cells (McGuckin et al, 2013; Ohtaki et al, 2008), drawing attention to the ability of progenitor cells to be effectors of co-ordinated immune responses. In addition, work over the last few years has demonstrated that activation of endogenous neural stem and progenitor cells (or delivery of these cells to the brain) offer potential as treatment for CNS diseases because of their ability to influence immune cells (Kokaia et al, 2012). In this study we have shown that hippocampal progenitor cells can directly be primed by glucocorticoid pre-treatment, in a manner similar to microglia, suggesting this phenomenon is more generalised than previously considered, and identifies hippocampal progenitor cells as potential influencers of their milieu, in addition to being influenced by it. Additionally, it has been assumed that antidepressants are immunomodulatory largely because of their effects on microglia (Lu et al, 2010; Tynan and Weidenhofer, 2012) but I have demonstrated here that antidepressants, both monoaminergic and ω-3 PUFAs are also
immunomodulatory directly to hippocampal progenitor cells, extending the scope of cells in which antidepressants can modulate immune processes.

Human microglial cell lines are difficult to obtain, but it would be interesting to build on the model used in this thesis to introduce more complex interactions. For example, it would be interesting to explore the effect of co- or pre-treatment with glucocorticoid on the effect of inflammation on microglial phenotype and cytokines secretion; and then instructive to see microglia from these different conditions co-incubated with hippocampal progenitor cells to determine their effect on neurogenesis, cell survival and modulation of the kynurenine pathway. Additionally it would be interesting to establish whether treatment of either the hippocampal progenitor cells or the microglia with antidepressants would protect the hippocampal progenitor cells from the effects of variously activated microglia. Based on data from animal studies it is likely that DHA exerts larger anti-inflammatory effect on stimulated microglia than it does in human hippocampal progenitor cells (Antonietta Ajmone-Cat et al, 2012).

4.7.3 Proliferating cells compared with differentiated cells

Most experiments in this thesis have used progenitor cells in their proliferation phase, rather than after differentiation into neurons, astrocytes and oligodendrocytes (except for experiments investigating the effects of neurogenesis). There are two reasons why this choice was made. Firstly, progenitor cells are thought to have an impact on the brain milieu, far greater than might be predicted by their relatively small number, thought to be due to their unique characteristics, for example, their hyper-excitability (Piatti et al, 2013). Secondly, they are also more sensitive to environmental cues than other cell types – this has been demonstrated in animals by showing the myriad ways that neurogenesis can be affected by diet, exercise, stress, inflammation and
other changes, suggesting that they are the most plastic elements of the brain (Balu and Lucki, 2009; Kropff et al, 2015); indeed neurogenesis has been thought of as a pronounced form of neuroplasticity. In experiments conducted with differentiated cells in our model, inflammatory responses were found to be diminished compared with those of proliferating cells. This is consistent with previous findings in these cells, where treatment with glucocorticoids or antidepressants during the proliferation phase but not the differentiation phase affected cell fate, indicating greater sensitivity to environmental cues during the proliferative phase (Anacker et al, 2011b). There may also be interesting aspects of brain responses to glucocorticoids, inflammation and antidepressants to be derived from studies in differentiated cells, but we chose to examine progenitor cell responses because of their centrality in the response to stress and antidepressants. While it is difficult to be certain as to how readily translatable the findings in hippocampal progenitor cells are to processes in the human brain, studies continue to emphasise their importance in memory processes and other functions (Kropff et al, 2015).

4.7.4 Statistical power for gene array analysis

One limitation to the interpretation of the gene array results presented in this thesis is that they were not corrected for multiple comparisons, leading to the possibility of false positives. The power of this analysis is limited because only six biological repeats were analysed due to the cost of the array technology. The risk of a false positive is reduced for those genes (highlighted in the results section) with very small p-values, many of which would survive genome wide correction. Additionally, pathway analysis limits the risk of false positives by considering genes to be acting in networks - this entails fewer comparisons (there are less total pathways than total number of genes) and each regulated
pathway is derived from several data points. However, the lack of power in this analysis should constrain the conclusions derived from these analyses.

### 4.8 Future directions

In relation to the interaction of glucocorticoids and the innate immune system, future work should seek to understand the underlying mechanisms and translate the work into human subjects. A definitive analysis of the presence of glucocorticoid resistance should be conducted, both by a greater powered examination of the ability of glucocorticoids to inhibit inflammation after previous treatment, as well as a time series of gene arrays following glucocorticoid treatment. This would entail treatment with dexamethasone and extraction of RNA 1, 12, 24 and 48 hours after treatment for gene array analysis to observe the evolution of changes. This may reveal the induction of glucocorticoid resistance over time (evinced by reduced expression of GR-target genes). It may also demonstrate upregulation of immune pathway genes in a time-dependent fashion, reflecting the time-dependent changes seen in secreted immunoproteins. The model proposed above in Section 4.3.2.3 could also be further tested by looking at different dosages of glucocorticoids with different time intervals before immune stimulation.

Furthermore, this phenomena could be explored in microglia in vitro. Potentiation of inflammatory responses has been observed in rodent microglia removed after stress paradigms but, to my knowledge, has not been examined in vitro. Both rodent and human microglia could be investigated. Another gap in the research which should be closed, as suggested by work in this thesis, is a test of glucocorticoid resistance in animal models that demonstrate the potentiation phenomenon, as there has been far more focus on the up-
regulation of inflammatory pathways. It is possible, as found in this thesis, that both mechanisms are involved.

Finally, this phenomenon should be further studied in depressed subjects. These findings predict that depressed patients are likely to have potentiated responses to both inflammatory stimuli (like LPS or vaccination) as well as psychological stress paradigms. These responses may also show a correlation with glucocorticoid resistance, or upregulation of certain immune genes, such as NOD-like receptors. There are a small number of studies that have explored this possibility, with positive results, but this research should be expanded. Furthermore, as suggested by the systematic literature review further studies in depressed subjects should evaluate a variety of immune and HPA axis characteristics to further understand the interaction of these systems in depression (and a systematic review and meta-analysis of the co-existence of hypercortisolaemia and increased pro-inflammatory cytokines is warranted).

Longitudinal studies in people exposed to chronic stress would be instructive, perhaps demonstrating the development of glucocorticoid resistance and increased inflammatory responses over time, and, potentially, allowing identification of the sequence of the development of these abnormalities.

In relation to the action of antidepressants, further exploration of the time course of action of the compounds explored in this thesis would be interesting, given their uniform effect on reducing NF-κB activity, presenting the possibility that they may exert immunomodulatory effects over differing time periods. A determination of their effect on other pathways implicated in the immunomodulatory action of antidepressants, such as JNK1/2 and ERK1/2 pathways, would also be instructive. Following on from the demonstrated effects of these compounds on quinolinic acid, a fuller characterisation of their effects
on kynurenine metabolites would be interesting, and an exploration of their effects on gene expression of kynurenine enzymes at longer times points might reveal more consistent findings.

As mentioned previously, after final confirmation of enrichment of GR-target genes by chromatin immunoprecipitation, the next step would be to examine differences in GR activation by dexamethasone alone, sertraline alone, and both in combination given their divergent effects on neurogenesis. This would reveal the genomic networks transducing these effects, allowing understanding of the mechanism by which antidepressants can reverse the effect of glucocorticoids on neurogenesis, a potentially critical process.

4.9 Conclusion

I have shown in this thesis for the first time that glucocorticoids can be pro-inflammatory in human neural cells, extending findings in animals and peripheral human samples. I have also shown that this phenomenon is dose- and time-dependent: in particular, intermediate doses of glucocorticoids potentiate subsequent inflammatory responses to a greater degree than low or high doses; and there appears to be a window of potentiation effects following glucocorticoid pre-treatment. I have shown that this effect, like the anti-inflammatory effects of glucocorticoids, is dependent on the glucocorticoid receptor. I have also generated evidence that the mechanism underlying this effect may involve both an induction of glucocorticoid resistance and the up-regulation of immune pathways, in particular, NLRP6, a NOD-like receptor, a family of receptors implicated in the pro-inflammatory effect of glucocorticoids in animals.
These findings may shed light on the pathogenesis of depression and suggest that exposure to stress and associated hypercortisolaemia may induce a potentiated innate immune system, primed to respond strongly to activating stimuli (including immunogens and psychological stress), accounting for the increased illness, including depression, in stressed subjects. This notion was supported by a systematic literature review of clinical studies which found that glucocorticoid resistance is associated with increased inflammation, but not increased levels of cortisol, suggesting that increased glucocorticoid resistance and increased inflammation may be residues of previous exposure to stress (and its attendant hypercortisolaemia).

I also found that antidepressants from differing classes had common as well as differentiated effects on inflammation and its consequences. Sertraline, venlafaxine and DHA all reversed inflammation-induced reductions in hippocampal neurogenesis and increases in the neurotoxic metabolite of the kynurenine pathway, while having pro-inflammatory (sertraline and DHA) or limited anti-inflammatory (venlafaxine) effects on immune responses to IL-1β. Conversely, EPA demonstrated significant anti-inflammatory effects on immune responses to IL-1β, without significant effects on inflammation-induced neurogenesis or induction of neurotoxic products of the kynurenine pathway.

Finally, a significant step was taken to understanding the genomic programs underlying divergent effects on hippocampal neurogenesis by antidepressants and glucocorticoids via the glucocorticoids receptor, by validating chromatin immunoprecipitation in human hippocampal progenitor cells.
References


Maes M, Ombelet W, Jongh, R De, Kenis G, Bosmans E (2001). The inflammatory response following delivery is amplified in women who previously suffered from major depression, suggesting that major depression is accompanied by a sensitization of the inflammatory response system. *J Affect Disord* 63: 85–92.


