Biophysical insights into the molecular pharmacology of angiotensin II receptor heteromers

Elizabeth K. M. Johnstone
B.Sc. (Hons) Pharmacology

This thesis is presented for the degree of Doctor of Philosophy of The University of Western Australia

School of Medicine and Pharmacology

2016
Biophysical insights into the molecular pharmacology of angiotensin II receptor heteromers
DECLARATION FOR THESES CONTAINING PUBLISHED WORK AND/OR WORK PREPARED FOR PUBLICATION

The examination of the thesis is an examination of the work of the student. The work must have been substantially conducted by the student during enrolment in the degree.

Where the thesis includes work to which others have contributed, the thesis must include a statement that makes the student’s contribution clear to the examiners. This may be in the form of a description of the precise contribution of the student to the work presented for examination and/or a statement of the percentage of the work that was done by the student.

In addition, in the case of co-authored publications included in the thesis, each author must give their signed permission for the work to be included. If signatures from all the authors cannot be obtained, the statement detailing the student’s contribution to the work must be signed by the coordinating supervisor.

Please sign one of the statements below.

<table>
<thead>
<tr>
<th>1. This thesis does not contain work that I have published, nor work under review for publication.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Student signature ..........................................................................................................................</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>2. This thesis contains only sole-authored work, some of which has been published and/or prepared for publication under sole authorship. The bibliographical details of the work and where it appears in the thesis are outlined below.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Student signature ..........................................................................................................................</td>
</tr>
</tbody>
</table>
3. This thesis contains published work and/or work prepared for publication, some of which has been co-authored. The bibliographical details of the work and where it appears in the thesis are outlined below. The student must attach to this declaration a statement for each publication that clarifies the contribution of the student to the work. This may be in the form of a description of the precise contributions of the student to the published work and/or a statement of percent contribution by the student. This statement must be signed by all authors. If signatures from all the authors cannot be obtained, the statement detailing the student’s contribution to the published work must be signed by the coordinating supervisor.

A) Application of BRET to monitor ligand binding to GPCRs.

Data from this publication is presented in Chapter 4.

Elizabeth Johnstone was directly involved in the development of the BRET ligand binding assay. She contributed Figure 2i and Supplementary Figure 9 to this paper. She was also involved in the design of the study and the analysis of the data.

B) Functional interaction between angiotensin II receptor type 1 and chemokine (C-C motif) receptor 2 with implications for chronic kidney disease.

Data from this publication is presented in Chapter 7.

Elizabeth Johnstone contributed Figure 2 to this paper. She was also involved in the design of the study, the analysis of the data and the writing of the text.
C) Bioluminescence Resonance Energy Transfer Approaches to Discover Bias in GPCR Signaling.
Johnstone E.K.M., Pfleger K.D.G.

Literature and methods from this publication are presented in Chapter 1 and Chapter 2, respectively.

Elizabeth Johnstone wrote the first draft of this paper. Revisions and edits were jointly worked on with Kevin Pfleger.

D) Receptor-Heteromer Investigation Technology and its application using BRET.
Johnstone E.K.M., Pfleger K.D.G.

Literature from this publication is presented in Chapter 1.

Elizabeth Johnstone wrote the first draft of this paper. Revisions and edits were jointly worked on with Kevin Pfleger.
Statement of Candidate Contribution

This thesis has been completed during my enrolment at The University of Western Australia. The research described within has been composed in its entirety by the author, with the exception of the generation of cDNA constructs used in Chapter 3 by Rekhati Abhayawardana and Chapter 5 by Ruth Seeber.

I have not previously submitted any work documented in this thesis for any other degree or qualification. For work that has been co-published with other authors, I have the permission of all co-authors to include such work in this thesis. All publications arising from this thesis prior to submission are recorded in Appendix II. This thesis complies with the guidelines set out by the University of Western Australia and the Faculty of Medicine, Dentistry and Health Sciences.

Elizabeth Johnstone
(PhD Candidate)

7th April 2016

Kevin Pfleger
(Coordinating Supervisor)
Abstract

The renin-angiotensin system (RAS) is a vital regulatory system involved in the maintenance of blood pressure. Its principle hormone is angiotensin II (AngII), which mediates its effects through two G protein-coupled receptors (GPCRs), the AngII type 1 (AT1) and type 2 (AT2) receptors. The AT1 receptor mediates most of the classical actions of the RAS, while the molecular and physiological effects of the AT2 receptor remain incompletely characterised, though it is often believed to counteract many AT1-mediated functions.

The ability of GPCRs to form both homomers and heteromers has revealed new complexity within GPCR signalling systems. GPCR homo- and heteromerisation has been described for numerous receptors, and can cause alterations to every facet of GPCR pharmacology. Within the RAS, several GPCR heteromers containing the AT1 or the AT2 have been described, and this thesis has investigated the pharmacology of some of these: the AT1-AT2 heteromer, the AT1-bradykinin (BK) type 2 (B2) heteromer and the AT2-B2 heteromer. Work from this thesis also contributed to the characterisation of the previously unreported AT1-chemokine (C-C) receptor 2 (CCR2) heteromer. Additionally, this thesis has investigated the potential existence of an AT1-AT2-B2 heteromer and provided initial research into the existence of a potential AT1-B2-CCR2 heteromer.

The predominant approach used throughout this thesis is Receptor-Heteromer Investigation Technology, which has most commonly been applied to GPCRs (GPCR-HIT). The GPCR-HIT assay enables monitoring of the proximity of two receptors, as well as interactions with GPCR signalling and regulatory proteins. In this thesis, the GPCR-HIT assay has been applied using bioluminescence resonance energy transfer (BRET), as well as a dual BRET/biomolecular fluorescence complementation (BiFC) approach. This has enabled investigation of β-arrestin recruitment and trafficking of the receptors and heteromers. Additionally, as part of this thesis, a novel adaptation of the GPCR-HIT assay has been developed. Through the fusion of a new, bright luciferase (NanoLuc) to the N terminus of a GPCR, binding of a fluorescent ligand has enabled receptor-ligand binding to be monitored using BRET for the first time. Adaptation of
this assay using the GPCR-HIT configuration has enabled the proximity between the AT₁ and the AT₂ receptor to be revealed using the BRET ligand binding approach.

This thesis has provided further evidence for the existence of the AT₁-AT₂ heteromer, as well as the AT₂-B₂ heteromer, showing that they both recruit β-arrestin in an AngII- or BK-dependent manner, respectively. While the AT₂-B₂ heteromer subsequently underwent internalisation, the AT₁-AT₂ heteromer did not. Additionally, both heteromers appear to traffic to the cell surface upon treatment with their respective ligands, and there was also initial evidence of increased formation of the heteromers upon prolonged agonist exposure.

The existence of the AT₁-B₂ heteromer is contentious. The results of this study support the existence of this heteromer, revealing that it recruits β-arrestin in an AngII- and BK-dependent manner, whereby the receptors subsequently co-internalise. AngII-induced internalisation of the heteromer occurred at a more rapid rate than the BK-induced internalisation, and dual agonist treatment resulted in a reduced level of internalisation of the heteromer. Unlike the AT₁-AT₂ heteromer and the AT₂-B₂ heteromer, there was no evidence of an increase in the expression of the heteromers upon prolonged agonist exposure.

In a recent publication I co-authored using research from this thesis, the AT₁ receptor was shown to form a heteromer with CCR2. Activation of the AT₁ receptor inhibited CCR2-Gαi₁-coupling and induced recruitment of β-arrestin proximal to CCR2. My contribution to this study revealed that there was no modulation of Gαq signalling upon heteromerisation, indicating a biased effect of heteromerisation compared to Gαi₁. I have also investigated the trafficking of the heteromer, showing that like the AT₁-B₂ heteromer, there was a reduced level of internalisation upon dual agonist treatment.

Using BRET/BiFC approaches, this study has also revealed the first evidence for the existence of a trimeric complex containing the AT₁, the AT₂ and the B₂ receptor. Additionally, evidence for the existence of a B₂-CCR2 heteromer was revealed, establishing a basis for further research to test the hypothesis that the AT₁, the B₂ and CCR2 form a trimeric complex. Understanding all these intricate receptor interactions is crucial to further elucidation of the complex physiology associated with the RAS.
# Table of Contents

**Statement of Candidate Contribution** vi

**Abstract** vii

**Acknowledgements** xiii

**List of Tables** xiv

**List of Figures** xv

**List of Abbreviations** xviii

## Chapter 1. General Literature Review 1

1.1. Introduction to GPCRs 1
   1.1.1. GPCR activation and G protein-dependent signalling 2
   1.1.2. GPCR heterologous and homologous desensitisation 4
   1.1.3. GPCR regulation by β-arrestin adaptor proteins 6
   1.1.4. GPCR trafficking and internalisation 8
   1.1.5. Non-canonical aspects of GPCR pharmacology 10

1.2. GPCR Heteromerisation 13
   1.2.1. Functional significance of GPCR receptor heteromerisation 16
   1.2.2. In vivo evidence and physiological functions of GPCR heteromerisation 18
   1.2.3. Mechanisms of GPCR heteromerisation 21

1.3. BRET 23
   1.3.1. BRET methodologies 25
   1.3.2. BRET assays 27
   1.3.3. Advantages and constraints of BRET 29

1.4. Angiotensin II and its Receptors 31
   1.4.1. The renin-angiotensin system 31
   1.4.2. Structure of angiotensin II receptors 36
   1.4.3. Expression and function of angiotensin II receptors 37
   1.4.4. Activation and regulation of angiotensin II receptors 39
   1.4.5. Angiotensin II receptor signalling 40
   1.4.6. Angiotensin II receptor heteromers 45

1.5. Summary 57
   1.5.1. Hypotheses 58
   1.5.2. Organisation of thesis 60
Chapter 2. General Materials and Methods

2.1. Introduction 61

2.2. Recombinant DNA Techniques 61
   2.2.1. Plasmid construction 61
   2.2.2. Plasmid cDNA preparation 68
   2.2.3. Plasmid cDNA preparation 68

2.3. Tissue Culture Procedures 69
   2.3.1. Cell lines and reagents 69
   2.3.2. Cell storage and recovery 70
   2.3.3. Transient transfection 70
   2.3.4. Generation of MAS1 receptor stable cell line 71

2.4. BRET Assays 75
   2.4.1. NanoBRET assays 75
   2.4.2. eBRET assays 75

2.5. Inositol Phosphate Cell Signalling Assays 76

2.6. Enzyme-Linked Immunosorbent Assays 78

2.7. Preparation of Compounds 78

2.8. Data Presentation and Statistical Analyses 78

Chapter 3. BRET Ligand Binding – Development and Optimisation

3.1. Introduction 80

3.2. Materials and Methods 83
   3.2.1. Materials 83
   3.2.2. cDNA constructs 84
   3.2.3. Mammalian cell transfections 84
   3.2.4. BRET assays 84
   3.2.5. IP1 assays 85
   3.2.6. Spectral scans 85
   3.2.7. Data presentation and statistical analyses 86

3.3. Development and Optimisation 86
   3.3.1. Assessment of pharmacology and spectra of fluorescent ligands and luciferases 86
   3.3.2. Fluorescent ligand binding assay tests 90
   3.3.3. Ligand binding assay optimisation 95
   3.3.4. AR ligand binding tests 98

3.4. Discussion 101
   3.4.1. Titration assays 103
   3.4.2. Competition binding assays with various AT1 receptor ligands 105
   3.4.3. Other results 107
   3.4.4. Summary 108
## Chapter 4. Angiotensin II Receptor and Heteromer Ligand Binding

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1. Introduction</td>
<td>109</td>
</tr>
<tr>
<td>4.2. Materials and Methods</td>
<td>112</td>
</tr>
<tr>
<td>4.2.1. Materials</td>
<td>112</td>
</tr>
<tr>
<td>4.2.2. cDNA constructs</td>
<td>113</td>
</tr>
<tr>
<td>4.2.3. Mammalian cell transfections</td>
<td>113</td>
</tr>
<tr>
<td>4.2.4. BRET assays</td>
<td>113</td>
</tr>
<tr>
<td>4.2.5. IP&lt;sub&gt;1&lt;/sub&gt; assays</td>
<td>114</td>
</tr>
<tr>
<td>4.2.6. Data presentation and statistical analyses</td>
<td>114</td>
</tr>
<tr>
<td>4.3. Results</td>
<td>114</td>
</tr>
<tr>
<td>4.3.1. AT&lt;sub&gt;1&lt;/sub&gt; and AT&lt;sub&gt;2&lt;/sub&gt; receptor ligand binding</td>
<td>114</td>
</tr>
<tr>
<td>4.3.2. AT&lt;sub&gt;1&lt;/sub&gt;-AT&lt;sub&gt;2&lt;/sub&gt; receptor heteromer ligand binding</td>
<td>118</td>
</tr>
<tr>
<td>4.4. Discussion</td>
<td>128</td>
</tr>
</tbody>
</table>

## Chapter 5. β-arrestin Recruitment to AT<sub>1</sub>, AT<sub>2</sub> and B<sub>2</sub> Receptors and Heteromers

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1. Introduction</td>
<td>135</td>
</tr>
<tr>
<td>5.2. Materials and Methods</td>
<td>138</td>
</tr>
<tr>
<td>5.2.1. Materials</td>
<td>138</td>
</tr>
<tr>
<td>5.2.2. cDNA constructs</td>
<td>138</td>
</tr>
<tr>
<td>5.2.3. Mammalian cell transfections</td>
<td>139</td>
</tr>
<tr>
<td>5.2.4. BRET assays</td>
<td>139</td>
</tr>
<tr>
<td>5.2.5. BiFC fluorescence assessment</td>
<td>140</td>
</tr>
<tr>
<td>5.2.6. Data presentation and statistical analyses</td>
<td>140</td>
</tr>
<tr>
<td>5.3. Results</td>
<td>140</td>
</tr>
<tr>
<td>5.3.1. β-arrestin&lt;sub&gt;2&lt;/sub&gt; recruitment to AT&lt;sub&gt;1&lt;/sub&gt;, AT&lt;sub&gt;2&lt;/sub&gt; and B&lt;sub&gt;2&lt;/sub&gt; receptors and heteromers with BRET</td>
<td>140</td>
</tr>
<tr>
<td>5.3.2. β-arrestin&lt;sub&gt;2&lt;/sub&gt; recruitment to AT&lt;sub&gt;1&lt;/sub&gt;, AT&lt;sub&gt;2&lt;/sub&gt; and B&lt;sub&gt;2&lt;/sub&gt; receptors with BRET and BiFC</td>
<td>143</td>
</tr>
<tr>
<td>5.4. Discussion</td>
<td>151</td>
</tr>
<tr>
<td>5.4.1. AT&lt;sub&gt;1&lt;/sub&gt;-B&lt;sub&gt;2&lt;/sub&gt; receptor heteromer</td>
<td>151</td>
</tr>
<tr>
<td>5.4.2. AT&lt;sub&gt;1&lt;/sub&gt;-B&lt;sub&gt;2&lt;/sub&gt; receptor heteromer</td>
<td>152</td>
</tr>
<tr>
<td>5.4.3. Interactions between the AT&lt;sub&gt;1&lt;/sub&gt;, the AT&lt;sub&gt;2&lt;/sub&gt; and the B&lt;sub&gt;2&lt;/sub&gt; receptor</td>
<td>153</td>
</tr>
</tbody>
</table>

## Chapter 6. Internalisation and Trafficking of AT<sub>1</sub>, AT<sub>2</sub> and B<sub>2</sub> Receptors and Heteromers

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.1. Introduction</td>
<td>156</td>
</tr>
<tr>
<td>6.2. Materials and Methods</td>
<td>158</td>
</tr>
<tr>
<td>6.2.1. Materials</td>
<td>158</td>
</tr>
<tr>
<td>6.2.2. cDNA constructs</td>
<td>158</td>
</tr>
<tr>
<td>6.2.3. Mammalian cell transfections</td>
<td>159</td>
</tr>
<tr>
<td>6.2.4. BRET assays</td>
<td>159</td>
</tr>
</tbody>
</table>
Acknowledgements

I would like to thank all my past and present colleagues who have helped me over the years. First and foremost, my supervisors, A/Prof Kevin Pfleger and A/Prof Livia Hool. Kevin, your continued sound advice, encouragement and enthusiasm created an environment in which I was able to flourish scientifically. To Livia, your guidance has always given new perspective to my work and provided valuable insight. I am also grateful to Ruth Seeber, Ethan See, and Werner Jaeger, whose expertise and assistance has been invaluable. There are of course many other colleagues who also deserve acknowledgement, yet unfortunately far too little space to do so.

I extend my deepest gratitude to my family and friends for their love and encouragement. To my sister Vicky, for your never-ending guidance with every aspect of my thesis, my career and my life, I am forever grateful. To Ceri and Bart, your support in everything I do means the world to me.

To my husband Rhys, thank you for your patience and understanding over the past three and a half years. Without your love and support, this would not have been possible.

I dedicate this thesis to the memory of my mother and father, Valerie and Raymond Johnstone. Their curiosity and boundless passion for knowledge ignited my own desire for learning, and they will forever remain an inspiration.
List of Tables

Table 1.1. Spectral and biochemical characteristics of different BRET generations. ..............26
Table 1.2. Evidence for putative angiotensin II receptor heteromers (GPCRs only). ..........45
Table 2.1. cDNAs and constructs used throughout this thesis. ..................................................62
Table 3.1. cDNA constructs used in this chapter........................................................................84
Table 3.2. CLARIOstar settings for fluorescence spectral scans.................................................85
Table 3.3. CLARIOstar settings for luminescence spectral scans..............................................86
Table 3.4. Luminescence peak and maximum intensity for Nluc-AT1 and Rluc8-AT1. ..........87
Table 3.5. Fluorescent AngII ligands pEC50 values for AT1-mediated IP1 production.............88
Table 3.6. Excitation and emission maxima of fluorescent AngII ligands.................................89
Table 3.7. pIC50 values for displacement of FITC-AngII binding by two ARBs. .........................91
Table 3.8. pIC50 values for TAMRA-AngII displacement by various AT1 ligands....................93
Table 3.9. pIC50 values from Figure 3.11 for displacement of TAMRA-AngII binding by a range of AT1 ligands..........................................................................................................................96
Table 3.10. pIC50 values for displacement of CA200700 and CA200689 binding by propranolol.................................................................................................................................101
Table 4.1. cDNA constructs used in this chapter........................................................................113
Table 4.2. pIC50 values from Nluc-AT1 competition binding experiments.................................116
Table 4.3. pIC50 values from AT1-AT2 receptor heteromer ligand binding experiment.........122
Table 5.1. cDNA constructs used in this chapter.........................................................................139
Table 6.1. cDNA constructs used in this chapter.........................................................................158
Table 6.2. pEC50 values for dose-response curves in Figure 6.8B and Figure 6.9B, C...............170
Table 7.1. cDNA constructs used in this chapter.........................................................................180
Table 7.2. pEC50 data for IP1 production shown in Figure 7.1..................................................184
List of Figures

Figure 1.1. GPCR activation and G protein mediated signalling pathways. ........................................... 4
Figure 1.2. Desensitisation and internalisation following GPCR activation. ........................................ 7
Figure 1.3. Heteromeric receptors and receptor heteromers. ................................................................. 15
Figure 1.4. Comparison of the principles of FRET and BRET. ............................................................ 24
Figure 1.5. Illustration of the GPCR-HIT assay. ....................................................................................... 29
Figure 1.6. Overview of the Classical RAS. .............................................................................................. 33
Figure 1.7. Diagram illustrating signalling in the RAS. ............................................................................. 35
Figure 2.1. Diagram of pcDNA3 and pcDNA3.1+ vectors showing the restriction enzyme sites in the multiple cloning site. ........................................................................................................... 62
Figure 2.2. Cell surface expression of human and rat AT1 and AT2 constructs. ................................. 65
Figure 2.3. Validation of Nluc-AT1 plasmid. .......................................................................................... 66
Figure 2.4. Testing MAS1 receptor-induced IP1 production. ............................................................... 72
Figure 2.5. Testing for constitutive IP1 production in cells transiently or stably transfected with MAS1 receptor. .......................................................................................................................... 74
Figure 2.6. Diagram of IP production and its detection using the CisBio IP-One HTRF® assay. ........................................................................................................................................................................... 77
Figure 3.1. FRET-based approaches for monitoring ligand binding. .................................................. 81
Figure 3.2. Illustration of the BRET ligand binding assay. ................................................................. 83
Figure 3.3. Comparison of Nluc-AT1 and Rluc8-AT1 luminescence spectra. ........................................ 87
Figure 3.4. IP dose response with fluorescent AngII ligands. ............................................................. 88
Figure 3.5. Excitation and emission spectra of fluorescent AngII ligands superimposed on the Nluc-AT1 emission spectrum. ........................................................................................................ 89
Figure 3.6. FITC-AngII fluorescence and BRET saturation binding assay. ......................................... 90
Figure 3.7. FITC-AngII fluorescence and BRET competition binding assay. ...................................... 91
Figure 3.8. TAMRA-AngII fluorescence and BRET saturation binding assay. ................................... 92
Figure 3.9. TAMRA-AngII fluorescence and BRET competition binding assay. ............................. 93
Figure 3.10. TAMRA-AngII fluorescence and BRET competition binding assay with AT1 and AT2 antagonists. ........................................................................................................................................ 94
Figure 3.11. Displacement of TAMRA-AngII with various AT1 receptor ligands using BRET competition binding assay. ........................................................................................................................................................................ 95
Figure 3.12. Nluc-AT1 cDNA titration with furimazine. ......................................................................... 97
Figure 3.13. pIC50 values from Nluc-AT1 cDNA titration with furimazine. ........................................ 98
Figure 3.14. Fluorescence and BRET β2AR saturation binding assay. ............................................. 99
Figure 3.15. Fluorescence and BRET β2AR competition binding assay. ............................................ 100
Figure 3.16. Affinity estimates for various ARBs at mammalian AT1 receptors, as determined in competition radioligand binding studies. ................................. 106
Figure 4.1. Amino acids involved in AngII activation of the AT₁ and the AT₂ receptor. 111
Figure 4.2. Illustration of the GPCR-HIT ligand binding assay using BRET. 112
Figure 4.3. AT₁ receptor competition binding experiments. 115
Figure 4.4. pIC₅₀ values from Nluc-AT₁ competition binding experiments. 116
Figure 4.5. AT₂ receptor competition binding. 117
Figure 4.6. Maximal TAMRA-AngII binding to AT₁ and AT₂ receptors. 118
Figure 4.7. AT₁ receptor AngII-induced IP₃ production. 119
Figure 4.8. AT₁-AT₂ receptor heteromer ligand binding: homogenous and non-homogenous assay. 121
Figure 4.9. AT₁-AT₂ receptor heteromer ligand binding: homogenous and non-homogenous assay (1 μM TAMRA-AngII removed). 123
Figure 4.10. AT₁-AT₂ receptor heteromer ligand binding – non-homogenous assay (raw BRET ratios). 125
Figure 4.11. AT₁-AT₂ receptor heteromer ligand binding – non-homogenous assay (normalised data). 126
Figure 4.12. Heteromer ligand binding with V₂ receptor as negative control. 127
Figure 5.1. GPCR-HIT β-arrestin assays used for detection of receptor heteromers. 138
Figure 5.2. β-arrestin2 GPCR-HIT between the AT₁ receptor and the B₂ receptor. 141
Figure 5.3. β-arrestin2 GPCR-HIT between the AT₂ receptor and the AT₁a receptor or the B₂ receptor. 142
Figure 5.4. Fluorescence from BiFC-tagged receptor constructs. 144
Figure 5.5. GPCR-HIT with BRET and BiFC between β-arrestin2-Rluc8 and AT₁-AT₂ heteromer. 145
Figure 5.6. GPCR-HIT with BRET and BiFC between β-arrestin2-Rluc8 and AT₁-B₂ heteromer. 146
Figure 5.7. GPCR-HIT with BRET and BiFC between β-arrestin2-Rluc8 and AT₂-B₂ receptor heteromer. 147
Figure 5.8. GPCR-HIT with BRET and BiFC between AT₁-Rluc8, AT₂, B₂ and β-arrestin2. 149
Figure 5.9. GPCR-HIT with BRET and BiFC between B₂-Rluc8, AT₁a, AT₂ and β-arrestin2. 150
Figure 6.1. Application of GPCR-HIT to monitor internalisation and trafficking of receptor heteromers. 157
Figure 6.2. Internalisation of AT₁, AT₂, and B₂ monomers/homomers. 161
Figure 6.3. Internalisation of AT₁-AT₂ heteromers. 162
Figure 6.4. Internalisation of AT₁-B₂ heteromers. 164
Figure 6.5. Endocytic rate constants of AT₁ and B₂ receptor trafficking. 165
Figure 6.6. BK-induced endosomal sequestration of the AT₁-Rluc8. 166
Figure 6.7. Internalisation of AT₂-B₂ heteromers. 167
Figure 6.8. Ligand-induced proximity between AT₂ and B₂ receptors. 168
Figure 6.9. Ligand-induced proximity between AT$_{1a}$ and AT$_2$ receptors. ........................................ 169
Figure 6.10. Lack of ligand-induced interaction between the AT$_1$ receptor and the B$_2$ receptor. ........................................................................................................................................ 171
Figure 7.1. IP1 production in cells co-expressing AT1a receptor and CCR2. ........................................... 183
Figure 7.2. Internalisation of AT$_{1a}$ and CCR2 monomers/homomers....................................................... 185
Figure 7.3. Internalisation of AT$_{1a}$-CCR2 heteromers............................................................................. 186
Figure 7.4. Ligand-induced change in proximity with Venus-Kras................................................................. 187
Figure 7.5. β-arrestin2 GPCR-HIT between the B$_2$ receptor and CCR2.................................................... 188
Figure 7.6. BK-induced β-arrestin2-Venus recruitment to CCR2-Rluc8. .................................................... 189
Figure 7.7. Internalisation of B$_2$ and CCR2 monomers/homomers.............................................................. 190
Figure 7.8. Internalisation of B$_2$-CCR2 heteromers.................................................................................. 191
Figure 7.9. Endosomal sequestration of CCR2-Rluc8.................................................................................. 192
Figure 7.10. BK-induced modulation of CCR2-Rluc8 plasma membrane expression......................... 192
Figure 8.1. Model depicting proposed trafficking and arrestin recruitment timeline of the AT$_1$-AT$_2$ heteromer. ................................................................................................................. 199
Figure 8.2. Model depicting proposed trafficking and arrestin recruitment timeline of the AT$_2$-B$_2$ heteromer. ......................................................................................................................... 200
Figure 8.3. Model depicting proposed trafficking and arrestin recruitment timeline of the AT$_1$-B$_2$ heteromer. ......................................................................................................................... 205
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>6'-GNTI</td>
<td>6'-guanidinonaltrindole</td>
</tr>
<tr>
<td>AA</td>
<td>arachidonic acid</td>
</tr>
<tr>
<td>ACE</td>
<td>angiotensin converting enzyme</td>
</tr>
<tr>
<td>ACE2</td>
<td>angiotensin converting enzyme 2</td>
</tr>
<tr>
<td>Ang1-7</td>
<td>angiotensin 1-7</td>
</tr>
<tr>
<td>AngI</td>
<td>angiotensin I</td>
</tr>
<tr>
<td>AngII</td>
<td>angiotensin II</td>
</tr>
<tr>
<td>AngIII</td>
<td>angiotensin III</td>
</tr>
<tr>
<td>AP2</td>
<td>adaptor protein 2</td>
</tr>
<tr>
<td>ARAP1</td>
<td>AT1 receptor associated protein</td>
</tr>
<tr>
<td>ARB</td>
<td>AT1 receptor blocker</td>
</tr>
<tr>
<td>AT1 receptor</td>
<td>angiotensin II type 1 receptor</td>
</tr>
<tr>
<td>AT2 receptor</td>
<td>angiotensin II type 2 receptor</td>
</tr>
<tr>
<td>AT4 receptor</td>
<td>angiotensin II type 4 receptor</td>
</tr>
<tr>
<td>ATBP</td>
<td>AT1 receptor-binding protein (also known as ATIP1)</td>
</tr>
<tr>
<td>ATIP</td>
<td>AT1 receptor-interacting protein</td>
</tr>
<tr>
<td>ATRAP</td>
<td>AT1 receptor-associated protein</td>
</tr>
<tr>
<td>β2AR</td>
<td>β2 adrenergic receptor</td>
</tr>
<tr>
<td>B2 receptor</td>
<td>bradykinin type 2 receptor</td>
</tr>
<tr>
<td>BiFC</td>
<td>bimolecular fluorescence complementation</td>
</tr>
<tr>
<td>BiLC</td>
<td>bimolecular luminescence complementation</td>
</tr>
<tr>
<td>BK</td>
<td>bradykinin</td>
</tr>
<tr>
<td>BRET</td>
<td>bioluminescence resonance energy transfer</td>
</tr>
<tr>
<td>c-Src</td>
<td>cellular-sarcoma tyrosine kinase</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine 5'-monophosphate</td>
</tr>
<tr>
<td>CCR2</td>
<td>chemokine (C-C) receptor 2</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CKD</td>
<td>chronic kidney disease</td>
</tr>
<tr>
<td>CNK</td>
<td>connector enhancer of Ksr</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>eBRET</td>
<td>extended BRET</td>
</tr>
<tr>
<td>EET</td>
<td>epoxyeicosatrienoic acids</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ESCRT</td>
<td>endosomal sorting complexes required for transport</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum</td>
</tr>
<tr>
<td>FRET</td>
<td>fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>G protein</td>
<td>guanine nucleotide-binding protein</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GASP</td>
<td>GPCR-associated sorting protein</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GLP</td>
<td>guanine nucleotide exchange factor-like protein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>GPCR-HIT</td>
<td>GPCR-Heteromer Investigation Technology</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>GPER</td>
<td>G protein-coupled oestrogen receptor</td>
</tr>
<tr>
<td>GRK</td>
<td>GPCR kinase</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks' Balanced Salt Solution</td>
</tr>
<tr>
<td>HEK293</td>
<td>human embryonic kidney 293</td>
</tr>
<tr>
<td>HEK293FT</td>
<td>human embryonic kidney 293 FT</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HTRF</td>
<td>homogenous time-resolved FRET</td>
</tr>
<tr>
<td>IL6</td>
<td>interleukin 6</td>
</tr>
<tr>
<td>IP</td>
<td>inositol phosphate</td>
</tr>
<tr>
<td>IP1</td>
<td>inositol-1-phosphate</td>
</tr>
<tr>
<td>IP3</td>
<td>inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun-N-terminal kinase</td>
</tr>
<tr>
<td>KKS</td>
<td>kallikrein-kinin system</td>
</tr>
<tr>
<td>LB broth</td>
<td>Luria-Bertani broth</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor-κ B</td>
</tr>
<tr>
<td>Nluc</td>
<td>NanoLuc</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PDGF R</td>
<td>platelet-derived growth factor receptor</td>
</tr>
<tr>
<td>PGE2</td>
<td>prostaglandin E2</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3 kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLA2</td>
<td>phospholipase A2</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PLD</td>
<td>phospholipase D</td>
</tr>
<tr>
<td>PLZF</td>
<td>promyeolytic leukaemia zinc finger</td>
</tr>
<tr>
<td>PTH</td>
<td>parathyroid hormone-related peptide</td>
</tr>
<tr>
<td>RAS</td>
<td>renin-angiotensin system</td>
</tr>
<tr>
<td>Receptor-HIT</td>
<td>Receptor-Heteromer Investigation Technology</td>
</tr>
<tr>
<td>RET</td>
<td>resonance energy transfer</td>
</tr>
<tr>
<td>RGS</td>
<td>regulator of G protein signalling</td>
</tr>
<tr>
<td>RH</td>
<td>RGS homology</td>
</tr>
<tr>
<td>Rluc</td>
<td>Renilla luciferase</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen specie</td>
</tr>
<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>SHP-1</td>
<td>Src homology 2 domain-containing tyrosine phosphatase 1</td>
</tr>
<tr>
<td>SII</td>
<td>[Sar1,Ile4,Ile8]-angiotensin II</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>SLP</td>
<td>self-labelling protein</td>
</tr>
<tr>
<td>SP</td>
<td>signal peptide</td>
</tr>
<tr>
<td>STNx</td>
<td>subtotally nephrectomised</td>
</tr>
<tr>
<td>TIMP-3</td>
<td>tissue inhibitor of metalloproteinases-3</td>
</tr>
<tr>
<td>TR-FRET</td>
<td>time-resolved FRET</td>
</tr>
<tr>
<td>TSH</td>
<td>thyroid-stimulating hormone</td>
</tr>
<tr>
<td>YFP</td>
<td>yellow fluorescent protein</td>
</tr>
</tbody>
</table>
Biophysical insights into the molecular pharmacology of angiotensin II receptor heteromers
Chapter 1. General Literature Review

1.1. Introduction to GPCRs

Signal transduction is one of life’s most fundamental biological processes. It enables precise coordination of cellular physiology in all organisms. To allow communication between the external and internal cellular environments, cells possess receptor proteins that transduce extracellular signals to effector molecules within the interior of the cell. Subsequent changes in cellular physiology allow the cell to respond appropriately to the initial external stimuli.

The largest group of cell surface receptors are the G protein-coupled receptors (GPCRs). Over 800 human GPCR genes have been identified (Fredriksson et al., 2003), accounting for approximately 2% of the genes encoded within the genome (Dorsam et al., 2007). GPCRs are involved in the regulation of a vast array of physiological processes including neurological (Gainetdinov et al., 2004), endocrinological (Vassart et al., 2011) and immunological (Sun et al., 2012) functions, olfaction (Spehr et al., 2009) and light (Smith, 2010) perception and embryogenesis (Schulte et al., 2007). It then follows that dysregulation of GPCR expression or function is involved in a variety of pathologies, including cancer (Dorsam et al., 2007) and cardiovascular disease (Smith et al., 2006). Their importance is further highlighted by the fact that they represent the largest group of drug targets, with an estimated 30% of current drugs modulating GPCR function (Wise et al., 2004).

GPCRs all share a common membrane topology consisting of an extracellular N terminus, a cytoplasmic C terminus and seven transmembrane helices separated by six alternating extracellular and intracellular loops. Despite this consistency in basic topology, members of the GPCR superfamily are highly diverse in primary structure. This diversity results in GPCRs being the most versatile of all the cell surface receptors (Hill, 2006), able to detect a myriad of sensory and chemical stimuli such as photons, odorants, taste ligands, peptides, lipids and nucleotides. Their diversity in primary
structure has also resulted in their phylogenetic classification into five main families: rhodopsin (family A), secretin (family B), glutamate (family C), adhesion and Frizzled/Taste2 (Schiöth et al., 2005).

The rhodopsin family is the largest family and contains ~700 members including the photo-sensing receptor rhodopsin and ~400 olfactory receptors (Bjarnadóttir et al., 2006). Other members of this family include the amine receptors (e.g. muscarinic and adrenergic receptors), peptide binding receptors (e.g. angiotensin, bradykinin and vasopressin receptors) and the chemokine receptors. Despite being heterogeneous in primary structure, members of the rhodopsin family exhibit a high level of conservation of a few sequence motifs. These include the conserved NPxxY motif in transmembrane 7 and the DRY motif at the border between transmembrane 3 and intracellular loop 2 (Fredriksson et al., 2003; Mirzadegan et al., 2003). These characteristics are thought to be involved in stabilisation of different receptor activation states (Urizar et al., 2005; Kobilka et al., 2007). The other four classes of receptors (which are not the major focus of this thesis) exhibit substantial diversity from one another, particularly in their N terminal domain and their extracellular loop regions (Katritch et al., 2013).

1.1.1. GPCR activation and G protein-dependent signalling

GPCRs exert their effects on cellular physiology by regulating the activity of downstream effector molecules within the plasma membrane, the cytosol and the nucleus. GPCR ligands typically bind to the extracellular face of the receptor or within the transmembrane region (Kristiansen, 2004). Ligand binding affects the structure of the receptor by altering intramolecular bonding between key residues, particularly those in the transmembrane domains (Nygaard et al., 2009). In the traditional two-state GPCR signalling model, binding of an agonist ligand stabilises the receptor in an ‘active’ conformation, while binding of an inverse agonist stabilises the ‘inactive’ receptor conformation (Leff, 1995; Strange, 2002). Although this model is now understood to be a substantial oversimplification, it remains a useful concept for understanding GPCR function. The updated model will be described in more detail in Section 1.1.5.

GPCRs were named as such because their classical signalling partners are the heterotrimeric guanine nucleotide-binding proteins (G proteins), which are composed of
α, β and γ subunits. The α subunit (Ga) contains the GDP/GTP binding site and the β and γ subunits (Gβγ) form an effectively undissociable complex that is associated with GDP-bound Ga. In this conformation, the G protein is inactive (Wettschureck et al., 2005). When a GPCR is in an ‘active’ conformation the structural arrangement of the helices results in an increased affinity for the G protein. This allows rapid dissociation of GDP and replacement with GTP on the Ga subunit. The exchange of the guanine nucleotides leads to reduced affinity of the Ga subunit for the Gβγ complex and the subsequent functional dissociation of the heterotrimer frees both complexes to interact with effector molecules. This activated state remains until GTP is hydrolysed to GDP through the intrinsic GTPase activity of the Ga subunit. Additionally, GTP hydrolysis can be accelerated by GTPase-activating proteins such as regulators of G protein signalling (RGS) proteins (Hendriks-Balk et al., 2008). GDP-bound Ga is again in an inactive conformation, resulting in its dissociation from the effector and functional reassociation with Gβγ.

There are four major families of G proteins that are classified based on the primary sequence similarity of their α subunits: Gs, Gi, Gq and G12/13. As shown in Figure 1.1, these Ga proteins have a variety of downstream effectors such as adenylyl cyclase, phospholipase C (PLC) and protein kinase A (PKA) and C (PKC). Additionally, the Gβγ complex is able to interact with many of the same effectors (Khan et al., 2013). The G protein effectors mediate a diverse number of intracellular signalling cascades such as mitogen-activated protein kinase (MAPK) activation and release of intracellular Ca2+ (Kristiansen, 2004; Wettschureck et al., 2005), leading to a range of physiological outcomes.
1.1.2. GPCR heterologous and homologous desensitisation

Binding of an agonist to a GPCR not only aids in its activation, it also triggers cellular events that lead to its desensitisation. Desensitisation is the attenuation of a receptor’s responsiveness to an agonist, protecting the cell against both acute and chronic receptor overstimulation (Ferguson, 2001). Desensitisation occurs as a result of the uncoupling of a GPCR from G protein signalling, and largely involves three families of regulatory proteins: second messenger-regulated kinases (e.g. PKA and PKC), GPCR kinases (GRKs) and arrestins.

The process of desensitisation begins with phosphorylation of the intracellular region of the GPCR by second messenger-regulated kinases or by GRKs. These kinases phosphorylate distinct serine and threonine residues in the intracellular loops and/or the
carboxyl tail of the receptor (Hendriks-Balk et al., 2008) at regions thought to directly interfere with G protein-coupling (Kristiansen, 2004). As second messenger-regulated kinases such as PKA and PKC are activated in response to GPCR-G protein coupling activity, this process of desensitisation is a classical form of negative feedback. However, second messenger-regulated kinases are also able to phosphorylate other, non-activated GPCRs, resulting in this process being termed ‘heterologous desensitisation’. The molecular and physiological outcomes of heterologous desensitisation have been described for various receptors; for example, activation of members of the opioid and chemokine receptor families results in desensitisation of the chemotactic activities of receptors from the other family (Rogers et al., 2000). It has been suggested that heterologous desensitisation may be more important at low agonist concentrations (Tobin, 1997) and interestingly, a switch in G protein coupling from Gs to Gi has been observed following PKA phosphorylation of the β2 adrenergic receptor (β2AR; Daaka et al., 1997).

In contrast to phosphorylation by second messenger-regulated kinases, GRKs are thought to only phosphorylate agonist-activated receptors, and this process is thus termed ‘homologous desensitisation’. The mammalian GRK family consists of seven members, two of which are localised to the visual system (GRK1, 7). With the exception of GRK4 that is localised to the testis (Premont et al., 1996), the remaining members (GRK2, 3, 5, 6) are expressed ubiquitously throughout the body (Magalhaes et al., 2012). While GPCRs and GRKs do show some specificity for one another, in general the GRKs regulate large, overlapping portfolios of receptors (Pierce et al., 2002).

GRKs all exhibit three common functional domains. The C terminal domain ensures localisation at the plasma membrane. This localisation can occur constitutively through palmitoylation of cysteine residues (GRK4, 6; Stoffel et al., 1994; Premont et al., 1996) or through the interaction of basic residues with membrane phospholipids (GRK5; Kunapuli et al., 1994; Premont et al., 1994). Plasma membrane localisation can also occur through agonist-dependent mechanisms including association of the activated βγ subunit of the G protein (GRK2, 3; Pitcher et al., 1992; Boekhoff et al., 1994). The central catalytic domain of the GRK is necessary for phosphorylation of the receptor, while the N terminal is the GPCR-binding domain and contains an RGS homology (RH) domain (Ferguson, 2001). As well as interacting with the GPCR at their N terminal tail,
several GRKs have been shown to interact with G protein α and βγ subunits via their RH domain (Carman et al., 1999; Sallese et al., 2000; Day et al., 2003; Tesmer et al., 2005). Subsequent actions as a GTPase-activating protein accelerate GTP hydrolysis on the Ga subunit and aid in desensitisation of the receptor. Indeed, GRKs are able to initiate desensitisation of several GPCRs in a phosphorylation-independent manner (Freedman et al., 1997; Carman et al., 1999; Sallese et al., 2000; Usui et al., 2000; Dhami et al., 2002). Furthermore, unlike second messenger-regulated kinase phosphorylation that is sufficient for receptor desensitisation (Kendall et al., 2009), GRK-mediated phosphorylation alone is insufficient to mediate desensitisation of many GPCRs (Magalhaes et al., 2012). Instead, most receptors require the subsequent recruitment of the adaptor protein arrestin.

1.1.3. GPCR regulation by β-arrestin adaptor proteins

Arrestin proteins were named as such because they were identified as the final component required for complete ‘arrest’ of rhodopsin (Wilden et al., 1986), and later β2AR (Benovic et al., 1987) signalling following GRK-mediated phosphorylation. Since then, they have been established as fundamental components of GPCR regulation and signalling.

The arrestin family has four members, two of which are found exclusively in the retina: visual rod arrestin (arrestin 1) and cone arrestin (arrestin 4). The non-visual arrestins, β-arrestin1 (arrestin 2) and β-arrestin2 (arrestin 3), are expressed ubiquitously in all non-retinal cells (Pierce et al., 2002), with variable expression ratios in different tissues (Sterne-Marr et al., 1993). All four arrestins share sequence and structural homology, with human β-arrestin1 and β-arrestin2 having 78% amino acid sequence identity (DeWire et al., 2007). Furthermore, arrestin primary sequence is highly conserved throughout different species (Palczewski, 1994).

Both visual and non-visual arrestins act to desensitise receptors in a similar manner, though they show specificity for either visual or non-visual receptors, respectively (Gurevich et al., 2006). Following agonist-induced activation and receptor phosphorylation, β-arrestins are rapidly recruited from the cytosol where they bind to the intracellular region of the receptor (see Figure 1.2). This enables complete
uncoupling of G protein signalling by sterically hindering access to the receptor’s binding domains (Shenoy et al., 2011). Although β-arrestins typically bind agonist-activated GRK-phosphorylated receptors, there is some evidence for binding following heterologous phosphorylation by PKC (Namkung et al., 2004).

As well as sharing sequence homology, all arrestins are structurally very similar, consisting of an N terminal domain and a C terminal domain that are connected by a polar core region. The solving of inactive and active arrestin crystal structures, in conjunction with data from spectroscopic and mutational studies, has enabled elucidation of arrestin’s mechanism of action. Simplistically, arrestin is restrained in an inactive conformation through clamping of the C terminal tail by the polar core and other regions. Rotation of the N terminal and C terminal domains relative to one another leads to release of the C terminal tail, whereby it is replaced with the phosphorylated cytoplasmic tail of the GPCR (Ostermaier et al., 2014).

Figure 1.2. Desensitisation and internalisation following GPCR activation. (Pierce et al., 2002)
As well as uncoupling GPCRs from G protein signalling, β-arrestins also act as scaffold proteins, recruiting numerous proteins with a variety of functions. For example, they have been shown to scaffold enzymes that degrade G protein-mediated second messengers, resulting in a dampening of GPCR signalling (Perry et al., 2002; Nelson et al., 2007). β-arrestins also recruit proteins integral for GPCR endocytosis (see Section 1.1.4) and non-canonical forms of GPCR signalling (see Section 1.1.5).

1.1.4. GPCR trafficking and internalisation

GPCRs are synthesised and folded in the endoplasmic reticulum, where they may undergo post-translational modifications and associate with various accessory proteins. From there, they traffic through the Golgi apparatus where protein maturation and vesicular targeting to the cell surface occurs (Jean-Alphonse et al., 2011). Once at the surface they are able to mediate classical G protein-coupled signalling. Upon activation following agonist binding, most GPCRs undergo internalisation. Receptor internalisation is an important regulatory mechanism that allows for fine-tuning of magnitude and duration of signalling.

Several mechanisms of GPCR internalisation have been described, which can be broadly classified as clathrin-dependent and clathrin-independent. Clathrin-independent internalisation can occur through localisation of receptors in lipid rafts or caveolae (Chini et al., 2004). The best characterised mechanism for GPCR internalisation is clathrin-mediated endocytosis (see Figure 1.2). Clathrin is a triskelion structural protein that forms a lattice coat around budding membrane vesicles (McMahon et al., 2011). The C terminal tail of β-arrestin contains a clathrin binding site (Goodman et al., 1996) and release of the C terminal tail upon interaction with the GPCR allows binding of clathrin proteins to β-arrestin (Nobles et al., 2007). β-arrestin’s C terminal tail also contains a binding site for the β2 subunit of adaptor protein 2 (AP2; Laporte et al., 1999). AP2 is a critical component of the clathrin-mediated endocytic machinery and without it mature vesicles cannot form and endocytosis does not occur (Motley et al., 2003; Boucrot et al., 2010). Following recruitment of clathrin and AP2 to β-arrestin, clathrin polymerises forming the lattice coat around the developing vesicle. The GTPase dynamin is then recruited to the neck of the forming vesicle, where it polymerises and...
upon GTP hydrolysis induces membrane scission (McMahon et al., 2011). The vesicle, termed an early endosome, is now separated from the plasma membrane and the receptor begins its endocytic path.

The fate of the receptor at this stage is determined by the stability of the GPCR-β-arrestin complex as well as the level of receptor ubiquitination. There are two main features that determine stability of the GPCR-β-arrestin complex: the presence of a serine/threonine motif in the receptor C terminal tail (Oakley et al., 1999; Oakley et al., 2001) and the pattern of agonist-induced β-arrestin ubiquitination (Shenoy et al., 2003). GPCRs are broadly classified into two groups based on the stability of their interaction with β-arrestin (Oakley et al., 2000) and their patterns of internalisation (Oakley et al., 1999). Class B receptors such as the angiotensin II type 1 (AT₁) and vasopressin type 2 (V₂) receptor contain the C terminal serine/threonine motif (Oakley et al., 2001), forming tight complexes with β-arrestins and displaying sustained β-arrestin ubiquitination (Shenoy et al., 2003). Such receptors are typically trafficked from early to late endosomes, where they are either targeted to lysosomes for degradation or recycled back to the plasma membrane via slow recycling endosomes. Receptor ubiquitination is usually, but not always, required for lysosomal targeting (Alonso et al., 2013). In contrast, Class A receptors such as the β₂AR, contain different phosphorylation sites other than the serine/threonine motif. They form less stable complexes with β-arrestin and display transient β-arrestin ubiquitination. These receptors are typically targeted to fast recycling endosomes where they are trafficked back to the cell surface (Seachrist et al., 2003). Alternatively, after prolonged agonist exposure, Class A receptors may be targeted to lysosomes (Hanyaloglu et al., 2008), though the half-life of this degradative process is substantially increased from that of Class B receptor lysosomal targeting (Marchese et al., 2008).

Spatiotemporal trafficking of receptors is further regulated by various groups of proteins that interact with the GPCR-β-arrestin complex. GPCR-associated sorting proteins (GASPs) are thought to regulate post-endocytic sorting of receptors (Magalhaes et al., 2012) while endosomal sorting complexes required for transport (ESCRT) proteins support transport of receptors to degradative pathways (Marchese et al., 2008). PDZ proteins are involved in regulating the subcellular localisation of receptors, as well as having roles in GPCR signalling and receptor stability (Magalhaes et al., 2012). Rab GTPases are a large family of small GTPases that are involved in almost all membrane
trafficking processes in eukaryotic cells. They act to aid in cargo sorting and vesicle budding, motility, fusion and uncoating (Stenmark, 2009). Many Rab GTPases are able to interact directly with the C terminal tail of GPCRs (Seachrist et al., 2002; Hamelin et al., 2005; Parent et al., 2009; Reid et al., 2010; Esseltine et al., 2011) and can compete with one another for binding at the same C terminal site in the receptor (Esseltine et al., 2011).

Receptor internalisation is necessary to allow resensitisation of receptors through receptor recycling, and conversely to allow downregulation of receptor expression through receptor degradation. Downregulation of receptor expression also occurs through decreased levels of receptor mRNA, as a result of suppressed gene expression or destabilisation of mRNA (Hadcoc et al., 1988; Hadcock et al., 1989). As well, chronic treatment with antagonists or inverse agonists can increase GPCR cell surface expression, likely due to increased protein synthesis, mRNA stabilisation or cell surface trafficking (MacEwan et al., 1996; Yoburn et al., 2004).

1.1.5. Non-canonical aspects of GPCR pharmacology

Traditionally it was believed that a ligand stimulates or inhibits all receptor functions equally and consistently, leading to the model of GPCR signalling comprising an ‘active’ and an ‘inactive’ state. It is now understood that this theory is inadequate to explain the complex pharmacology observed with numerous ligands. In reality, GPCRs likely exist in numerous conformational states, with individual conformations having unique affinities for the various signalling and regulatory interacting proteins (Urban et al., 2006). It then follows that ligands will stabilise different receptor conformations, leading to ligand-specific or ligand-biased pharmacological outputs. This ligand-directed signalling is known as ‘functional selectivity’ or ‘biased signalling’. There are now numerous examples of biased ligands which have selectivity for many aspects of GPCR pharmacology including G protein coupling (Urban et al., 2007; Dewire et al., 2013), β-arrestin recruitment (Rajagopal et al., 2006; Wisler et al., 2007) and internalisation (van der Westhuizen et al., 2014; Conroy et al., 2015).

Further complexity of GPCR pharmacology can occur through allosteric modulation. Allosteric modulators bind to sites that are distinct from the receptor’s...
orthosteric site (where the endogenous ligand binds). This induces conformational changes in the receptor that modify the response of the receptor for its orthosteric ligand (Gao et al., 2006). The modulation is reciprocal, in that orthosteric ligand binding also modulates allosteric sites (Müller et al., 2012). Allosteric modulators can be positive, negative or neutral and can display biased signalling in the same manner as orthosteric ligands (Müller et al., 2012). Allosteric modulators are commonly thought of as ligands, however GPCR interacting proteins (including other receptors) can also be classified as allosteric modulators. Furthermore, ‘off-target’ allosteric modulation is a mechanism by which the allosteric modulator binds to orthosteric or allosteric sites on a GPCR interacting protein (Ballesteros et al., 2006), the allostery therefore being transferred through this intermediate.

As well as the classical GPCR signalling that occurs as a result of G protein coupling at the plasma membrane, it is now well established that GPCRs are able to regulate numerous non-canonical signalling paths. The most well established of these is the signalling that occurs as part of GPCR-β-arrestin complexes. For over a decade β-arrestins have been known to act as scaffolds for a wide variety of signalling complexes. In particular, signalling to the MAPK cascades has been well described. This was first shown when it was discovered that β-arrestin1 could recruit cellular-sarcoma tyrosine kinase (c-Src) to the β2AR resulting in activation of extracellular signal-regulated kinases 1/2 (ERK1/2; Luttrell et al., 1999). It was then discovered that β-arrestins could scaffold other components of the ERK pathway, including MEK1 and Raf (DeFea et al., 2000; Luttrell et al., 2001b), as well as components of the other MAPK pathways, c-Jun-N-terminal kinase 3 (JNK3; McDonald et al., 2000) and p38 (Sun et al., 2002). Importantly, β-arrestin-scaffolding of the MAPK cascades leads to retention of the active kinases in the cytosol thereby inhibiting the traditional signalling of these kinases in the nucleus (DeFea et al., 2000; McDonald et al., 2000; Luttrell et al., 2001b). In addition to MAPKs, β-arrestins can signal to other kinases such as phosphatidylinositol 3 kinase (PI3K) and Akt (Goel et al., 2002; Beaulieu et al., 2005). Furthermore, they are able to recruit other signalling and regulatory molecules such as small GTPases (Godin et al., 2010), guanine nucleotide exchange factors (Ma et al., 2012) and ubiquitin ligases (Shenoy et al., 2001). As well, β-arrestins are able to regulate transcription, either indirectly, such as through activation of nuclear receptors (Piu et al., 2006) or directly,
through nuclear translocation and interaction with proteins involved in transcriptional regulation (Kang et al., 2005).

From all these studies it has become evident that GPCR-β-arrestin-mediated signalling is separated both spatially and temporally from classical GPCR-G protein coupling. A well studied example of this is the spatiotemporal differences in ERK1/2 activation. G protein-dependent activation of ERK1/2 is initiated at the plasma membrane via the traditional G proteins and effectors. This leads to a rapid and transient activation of ERK1/2 resulting in nuclear translocation of the kinase (Ahn et al., 2004). In contrast, β-arrestin-mediated activation of ERK1/2 occurs in endosomes following the scaffolding of MAPK complexes. This results in a more sustained level of ERK1/2 activation and its retention in the cytosol (Ahn et al., 2004).

While it is now well established that GPCR signalling via β-arrestins can occur following receptor internalisation, it is becoming apparent that internalised GPCRs are also able to signal to G proteins, challenging the longstanding paradigms of receptor signalling and desensitisation. The α and βγ subunits of G proteins have been observed in intracellular compartments for many years (Jamora et al., 1997; Weiss et al., 1997; Park et al., 1999), however their roles in mediating GPCR signalling from these locations is only just beginning to be revealed. Studies of the thyroid-stimulating hormone (TSH) receptor (Calebiro et al., 2009) and the parathyroid hormone-related peptide (PTH) receptor (Ferrandon et al., 2009) have demonstrated sustained, endosomal Gαs signalling in addition to the conventional transient, plasma membrane Gαs signalling. Acute Gαs signalling has also been observed in endosomes following stimulation of the D1 dopamine receptor (Kotowski et al., 2011) and the β2AR receptor (Irannejad et al., 2013). How this G protein signalling occurs when the receptor is β-arrestin bound is still unclear, but a possible mechanism was revealed in a follow-up study on the PTH receptor (Wehbi et al., 2013). The study showed the formation of an ‘alternate’ signalling complex composed of β-arrestin-bound receptors together with the entire G protein heterotrimer in which the Gαs had been stabilised in an activated state.

There is now also evidence that some GPCRs may even be able to signal during the biosynthetic pathway. Vasopressin V2 receptor mutants that are unable to traffic to the plasma membrane can be activated by membrane permeable agonists but not by the endogenous membrane impermeable agonists (Robben et al., 2009). As well, there is evidence that upon activation the G protein-coupled oestrogen receptor (GPER,
previously GPR30) can signal from the endoplasmic reticulum (Revankar et al., 2005). Furthermore, the discovery that the β2AR traffics through the endoplasmic reticulum/Golgi apparatus pre-associated with its heterotrimeric G protein and effector enzyme, adenylyl cyclase (Dupré et al., 2007) supports the concept that GPCR signalling complexes are ready to be activated early in the biosynthetic pathway. Perhaps even more surprising is the discovery that GPCRs, G proteins and a variety of their regulatory and signalling proteins have not only been found in the nuclear membrane, but are able to be activated and produce classic second messengers such as cAMP (Tadevosyan et al., 2012). While these signalling outputs have been observed in several studies it is not known whether GPCRs and their interacting proteins are trafficked to the nuclear membrane from the biosynthetic pathway or as a result of receptor internalisation (Tadevosyan et al., 2012). At present it is not clear how widespread the phenomenon of non-plasma membrane G protein-coupled signalling is, and whether it results in distinct physiological outcomes from classical plasma membrane signalling.

With so many examples of non-traditional G protein-mediated signalling, it is clear that GPCR signalling systems are far more complex than originally imagined. Adding to this complexity is the concept of GPCR oligomerisation, resulting in the formation of homomeric or heteromeric complexes.

1.2. GPCR Heteromerisation

While it is well established that many receptors exist as oligomeric complexes composed of two or more receptors (Heldin, 1995; Ferré et al., 2009; Lemmon et al., 2010), historically GPCRs were thought to only act as single monomeric units. Early evidence for GPCR oligomerisation arose from studies with β adrenergic receptors (Limbird et al., 1975) and neuropeptide and monoamine receptors (Fuxe et al., 1983) that showed cooperativity between different receptors. As well, ligand-mediated aggregation of receptor units was observed for the gonadotropin-releasing hormone receptor (Conn et al., 1982). Further evidence came from Maggio et al. (1993) who were able to restore the function of chimeric adrenergic/muscarinic receptors upon their
coexpression. In the same year, the existence of heterodimers composed of different GPCR units was proposed (Zoli et al., 1993).

In the late 1990s, the first obligate GPCR heterodimers were discovered (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998). Heteromerisation of gamma-aminobutyric acid (GABA) seven-transmembrane proteins GABA_B1 and GABA_B2 was found to be necessary for cell surface trafficking and G protein activation. GABA_B1 contains an endoplasmic reticulum retention motif that prevents expression at the plasma membrane (Margeta-Mitrovic et al., 2000). Interaction with GABA_B2 masks this motif and allows trafficking of the complex to the cell surface. Additionally, GABA_B1 contains the ligand-binding domain (Galvez et al., 1999; Kniazeff et al., 2002) while GABA_B2 is necessary for activation of the G protein (Galvez et al., 2001; Margeta-Mitrovic et al., 2001; Robbins et al., 2001; Duthey et al., 2002). Since the discovery of the GABA_B1-GABA_B2 heteromeric receptor, similar obligate heteromers have been discovered for sweet (Nelson et al., 2001) and umami (Nelson et al., 2002) taste receptors.

In 2003, the first direct visualisation of GPCR oligomers was provided when infrared-laser atomic-force microscopy images of retinal disc membranes revealed rows of densely packed rhodopsin dimers (Fotiadis et al., 2003). This provided compelling evidence in support of the existence of GPCR oligomers. In the following decade many more examples of both homomeric and heteromeric GPCR oligomers were to be described, including heteromeric complexes of receptors from different families. This has resulted in the development of a classification system for receptor oligomers (see Figure 1.3) (Ferré et al., 2009). ‘Homomeric receptors’ and ‘heteromeric receptors’ are composed of identical or different receptor units, respectively, which require oligomerisation for the formation of a functional receptor unit. Receptor tyrosine kinases (RTKs) are the classic example of homomeric and heteromeric receptors, as are the GABA_B receptor and taste receptors mentioned previously. ‘Receptor homomers’ and ‘receptor heteromers’ are composed of identical or different (respectively) receptor units that are functional without oligomerisation. Importantly, oligomerisation of receptor homomers and receptor heteromers produces complexes with pharmacology that is distinct from the constituent monomers (Ferré et al., 2009). Because of this attribute, the existence of receptor homomers and heteromers adds a further level of complexity to cell signalling systems. As well, the unique pharmacology associated
with receptor homomers and receptor heteromers indicates they can be viewed as a new class of drug targets, with the aim of developing pharmaceuticals with increased specificity and reduced side effects.

![Figure 1.3. Heteromeric receptors and receptor heteromers.](image)

**Figure 1.3. Heteromeric receptors and receptor heteromers.** (A) Heteromeric receptors are obligate heteromers composed of receptor protomers that are only functional upon heteromerisation, such as the GABA\(_{B1}\)-GABA\(_{B2}\) heteromeric receptor. (B) Receptor heteromers are composed of functional receptor protomers that acquire novel pharmacology as a result of heteromerisation, such as the adenosine A\(_{2A}\)-dopamine D\(_2\) receptor heteromer. Adapted from Ferré *et al.* (2009).

While there has been much research into the pharmacology of both receptor homomers and receptor heteromers, the latter has received the most attention. However, the existence of GPCR receptor heteromers has proved to be a controversial issue, with many of the assays and systems used having the potential to produce artefactual results (Prinster *et al.*, 2005). Despite the controversy, over the past decade growing evidence has resulted in the existence of receptor heteromers becoming widely accepted. Elucidation of the function and mechanism of formation of receptor heteromers has provided support for their existence (see Sections 1.2.1 and 1.2.3, respectively). However, due to difficulties investigating receptor heteromers in native tissues, the applicability of information from heterologous expression systems to native tissues needs to be demonstrated (see Section 1.2.2). Additionally, because most current approaches for studying receptor heteromers cannot distinguish between dimers and
higher order oligomers, the stoichiometry of most of these complexes is still unknown (Agnati et al., 2010).

1.2.1. Functional significance of GPCR receptor heteromerisation

While the function of oligomerisation is clear for obligate GPCR complexes (e.g. GABA$_{B1}$-GABA$_{B2}$ heteromeric receptor) the relevance is less clear for non-obligate GPCR oligomers. This is particularly evident since the discovery that single GPCR monomers are able to functionally interact with G proteins, GRKs and arrestins (Whorton et al., 2007; Bayburt et al., 2011). However, by definition (Ferré et al., 2009) receptor homomers and receptor heteromers have pharmacology that is distinct from that of their constituent protomers. Thus, non-obligate GPCR oligomerisation provides a means for enhancing specificity and selectivity of cellular signalling.

Evidence has been provided for receptor heteromerisation modulating GPCR pharmacology at all levels of signalling and regulation. Several studies have described the importance of heteromerisation in receptor cell surface targeting. A study using an endoplasmic reticulum-retained $\alpha_2B$ adrenergic receptor mutant caused retention of wild type $\alpha_2A$ and $\alpha_2B$ adrenergic receptors (Zhou et al., 2006), suggesting that heteromerisation occurs early in the biosynthetic pathway. Furthermore, the association of the $\alpha_{1D}$ adrenergic receptor with other adrenergic receptor subtypes enhances its usually poor cell surface expression and function (Uberti et al., 2005).

Receptor heteromerisation has also been shown to alter the pharmacology of ligand binding. This is a means of ‘off target’ allosteric modulation of one protomer on another’s orthosteric binding site. Negative allosterism was observed between the chemokine CCR5 and CCR2b receptors in a reciprocal manner: treatment with the CCR5 ligand inhibited binding of the CCR2b radiolabelled ligand, and treatment with the CCR2b ligand inhibited binding of the CCR5 radiolabelled ligand (El-Asmar et al., 2005). Examples of positive allosterism have been observed for the dopamine receptors: the D$_1$ receptor displayed increased ligand binding potency upon coexpression of the D$_3$ receptor (Fiorentini et al., 2008), and the D$_2$ receptor displayed increased ligand binding potency upon coexpression of the D$_1$ receptor (Rashid et al., 2007). There have also been examples of receptor protomers exerting opposite effects on one another’s ligand
binding, as is the case with the heteromer between the somatostatin SST5 receptor and the dopamine D2 receptor (Rocheville et al., 2000). Treatment with a high concentration of the dopamine antagonist sulpiride resulted in decreased binding potency of the somatostatin agonist SST-14 while treatment with a high concentration of SST-14 increased the binding potency of sulpiride.

Receptor heteromerisation can modulate G protein coupling, either altering the extent of coupling to the same G protein or even altering G protein-coupling preference. In a study on α2A adrenergic-µ opioid receptor heteromers, a decrease in G protein signalling was observed upon dual activation with clonidine and morphine, while activation of only one receptor resulted in an increase in G protein signalling (Jordan et al., 2003). Using [35S]GTPγS binding assays on the δ-µ opioid receptor heteromer an increase in agonist-mediated µ opioid receptor G protein signalling following δ opioid receptor antagonism was observed (Gomes et al., 2004). Furthermore, this heteromer was later shown to switch G protein-coupling preference from Ga_i to Ga_z (Hasbi et al., 2007). Another heteromer that is able to switch G protein-coupling preference is between the δ opioid receptor and the sensory neuron-specific receptor-4. Upon activation of both protomers the δ opioid receptor switches signalling from Ga_i to Ga_q (Breit et al., 2006).

Receptor heteromerisation can also modulate interactions between receptors and other GPCR interacting partners such as RGS proteins, GRKs and β-arrestins. Heteromerisation of the melatonin MT2 receptor with the melatonin MT1 receptor resulted in strong binding of RGS20, despite the MT2 protomer only weakly associating with RGS20 (Maurice et al., 2010). GRK-mediated cross-phosphorylation within heteromers has been described for heteromers between the chemokine CCR5 receptor and the C5a receptor (Hüttenrauch et al., 2005), the µ opioid receptor and the somatostatin SST2A receptor (Pfeiffer et al., 2002), and the neurokinin 1 receptor and the µ opioid receptor (Pfeiffer et al., 2003). Conversely the α2A-α2C adrenergic receptor heteromer displays reduced GRK-mediated phosphorylation and β-arrestin recruitment with subsequent reductions in downstream Akt phosphorylation (Small et al., 2006). There are also examples of heteromers which switch from a predominantly G protein-mediated to a β-arrestin-mediated pathway. In the case of the δ-µ opioid receptor heteromer this switch in bias to a β-arrestin-mediated signalling results in cytoplasmic
retention of MAPKs and subsequent differential regulation of transcription factors (Rozenfeld et al., 2007).

Receptor heteromerisation can also result in altered internalisation profiles. In many instances activation of only one protomer is required for co-internalisation of both receptors. This is the case for the α1A-α1B adrenergic receptor heteromer (Stanasila et al., 2003) and the δ-μ opioid receptor heteromer (Hasbi et al., 2007). There are also examples of heteromers in which internalisation is inhibited, such as the β2-β3 adrenergic receptor heteromer. The β3 adrenergic receptor is resistant to internalisation and it is likely that heteromerisation inhibits the traditional internalisation of the β2AR (Breit et al., 2004).

In summary, it is clear that receptor heteromerisation can have broad-ranging consequences for GPCR pharmacology, in both a qualitative and quantitative manner. This vastly increases the complexity of GPCR signalling systems and enables fine-tuning and specification of signals. With this large body of evidence for the existence of GPCR heteromers in in vitro systems, it is crucial to correlate these molecular findings with outcomes from in vivo research. This will enable elucidation of the associated physiology and/or pathology, and may aid in the development of pharmaceuticals with improved specificity and reduced side effects.

1.2.2. In vivo evidence and physiological functions of GPCR heteromerisation

Examples of GPCR heteromerisation have been found throughout the body and efforts to pharmacologically target them as new species is already underway. The opioid receptors have been extensively studied in terms of receptor heteromerisation with the final aim of developing heteromer-specific opioid ligands that retain potent analgesic properties while minimising the unwanted side effects such as respiratory depression and dependence. The most successful ligand discovered so far is 6’-guanidinonaltrindole (6’-GNTI), which has been shown to selectively activate the δ-κ opioid receptor heteromer (Waldhoer et al., 2005). In vivo, 6’-GNTI mediated analgesia in the mouse, while in vitro it simulated G protein coupling that was selective for δ-κ opioid receptor heteromers. Interestingly, 6’-GNTI activity induced analgesia when it was administered
intrathecally but not intracerebroventricularly, suggesting the existence of the δ-κ opioid receptor heteromer in the spinal chord but not the brain (Waldhoer et al., 2005). This heteromer-specific tissue distribution provides further promise for the selectivity of receptor heteromers as new drug targets. More recently, δ-κ opioid receptor heteromers have been found in cultured rat peripheral sensory neurons and 6'-GNTI treatment was able to inhibit prostaglandin E2 (PGE2) stimulated cAMP levels following pre-treatment with an inflammatory mediator (Berg et al., 2012). 6'-GNTI also completely inhibited PGE2-induced thermal allodynia in rat hind paws following administration of an inflammatory mediator.

Another example of an opioid receptor heteromer in which the physiological relevance has been revealed is between the cannabinoid CB1 receptor and the μ opioid receptor. It has been known for many years that the major psychoactive constituent of marijuana, 9-tetrahydrocannabinol, enhances the potency of opioids (Ghosh et al., 1979; Welch et al., 1992). In vitro and in native tissues a reciprocal antagonistic relationship has been demonstrated for the two receptors on G protein coupling and ERK1/2 activation (Rios et al., 2006), and their direct interaction has been observed using fluorescence resonance energy transfer (FRET) and co-immunoprecipitation (Hojo et al., 2008). Furthermore the two receptors have also been found colocalised in catecholaminergic neurons in the locus coeruleus, the centre of opiate addiction and withdrawal (Scavone et al., 2010).

Dopamine receptor heteromers have been implicated in a variety of pathologies such as Parkinson’s disease and dyskinesia. Using co-immunoprecipitation and confocal microscopy the dopamine D1 and D2 receptors have been found colocalised in rat and human brain as well as in heterologous cells (Lee et al., 2004). The same study revealed that the heteromer switched G protein coupling from Gs and Gi (D1 and D2 receptors, respectively) to Gq. A later study revealed that the Gq11 co-activation resulted in increased levels of calcium/calmodulin-dependent protein kinase IIα in the nucleus accumbens suggesting an involvement in synaptic plasticity (Rashid et al., 2007). The D1 and D2 receptors have also been shown to form heteromers with the histamine H3 receptor (Ferrada et al., 2008). Selective activation of the H3 receptor inhibited D1- and D2-mediated locomotion in reserpinised (dopamine-depleted) mice, while also decreasing the binding affinity of D2 receptors ex vivo. An interaction between the D1
and H\textsubscript{1} receptors \textit{in vitro} was demonstrated using bioluminescence resonance energy transfer (BRET).

Heteromerisation of the adenosine A\textsubscript{2A} receptor and the dopamine D\textsubscript{2} receptor has also been described. Their interaction has been demonstrated using co-immunoprecipitation and BRET (Canals \textit{et al.}, 2003; Kamiya \textit{et al.}, 2003) and treatment with agonists for one or both receptors results in co-desensitisation and co-internalisation (Hillion \textit{et al.}, 2002). In reserpinised mice A\textsubscript{2A} receptor antagonists cause increased locomotion (Tanganelli \textit{et al.}, 2004) and it has been suggested that the tolerance observed to the Parkinson’s drug L-DOPA may be partly attributable to the A\textsubscript{2A}-D\textsubscript{2} receptor heteromer (Kroeger \textit{et al.}, 2003).

Heteromerisation between opioid receptors and the β\textsubscript{2}AR has also been observed. The existence of opioid receptors in the heart has been known for many years (Ventura \textit{et al.}, 1989; Tai \textit{et al.}, 1991), and selective opioid ligands have been shown to inhibit noradrenaline-mediated actions in the heart (Pepe \textit{et al.}, 1997). Jordan \textit{et al.} (2001) showed an interaction of the β\textsubscript{2}AR with both the δ and κ opioid receptors using co-immunoprecipitation in heterologous cells. They further demonstrated that coexpression of the β\textsubscript{2}AR with the δ opioid receptor resulted in internalisation of each receptor upon treatment with the others agonist. Conversely, the β\textsubscript{2}AR did not undergo opioid- or isoprenaline-induced internalisation upon coexpression with the κ opioid receptor. Moreover, coexpression of κ but not δ opioid receptor resulted in inhibition of MAPK phosphorylation suggesting a mechanism for the observed inhibition of noradrenaline-mediated physiology in the heart.

Investigating GPCR heteromerisation \textit{in vivo} is more difficult than assessing their pharmacology in native tissues and heterologous recombinant systems. However, the development of new approaches, such as single-molecule imaging (Kasai \textit{et al.}, 2014) and time-resolved FRET with fluorescent ligands (Albizu \textit{et al.}, 2010) provide new tools to study them. Additionally, the heteromer-specific pharmacology (“biochemical fingerprint”) (Ferré \textit{et al.}, 2009) discovered from \textit{in vitro} assays can be used in the identification of heteromers \textit{in vivo}. The further development of heteromer-selective or -biased ligands (Mustafa \textit{et al.}, 2010) will aid in the \textit{in vivo} detection of heteromers, and may also provide the basis for designing pharmaceuticals that target heteromer-related pathologies.
1.2.3. Mechanisms of GPCR heteromerisation

The molecular mechanisms underpinning the formation of GPCR heteromers are not well understood. Many GPCR heteromers appear to form constitutively early in the biosynthetic pathway such as the α adrenergic receptor heteromers described previously (Uberti et al., 2005; Zhou et al., 2006). There is, however, some evidence that GPCR oligomerisation can be modulated in a ligand-dependent manner as seen with the somatostatin SSTR5-dopamine D2 receptor heteromer (Rocheville et al., 2000) and the ghrelin-dopamine D2 receptor heteromer (Jiang et al., 2006).

Several sites of interaction have been proposed for the formation and/or stabilisation of GPCR oligomers: extracellular, intracellular and the transmembrane domains. The formation of extracellular covalent bonds is thought to be particularly relevant for family C oligomers, which are stabilised by disulphide bridges in their extracellular N terminal Venus flytrap domains (Romano et al., 1996; Ray et al., 1999; Ray et al., 2000). Interactions between intracellular domains are thought to occur through coiled-coil loop interactions (Kammerer et al., 1999) or epitope-epitope electrostatic interactions (Woods et al., 2005) at the C terminal tails. Interactions between the transmembrane domains are proposed to occur either through domain swapping or through direct contact, whilst maintaining the ternary structure of the protomer (Kroeger et al., 2003). While some motifs and receptor regions have been found to be important for GPCR oligomerisation (Hebert et al., 1996; Trettel et al., 2003), no conserved motifs across different GPCRs have been found (Lambert et al., 2014). This suggests that different complexes recognise different motifs and use different mechanisms for oligomerisation (Ferre et al., 2007).

It has also been suggested that the inability to identify consensus oligomer interfaces may be due to many oligomeric interactions being transient in nature (Lambert et al., 2014). Indeed, using single fluorescent-molecule video imaging, several studies have now revealed homomeric interactions lasting in the range of 0.1 – 10 seconds. This has been shown for the muscarinic M1 receptor (Hern et al., 2010), the N-formyl peptide receptor (Kasai et al., 2011) as well as β1 and β2 adrenergic receptors (Calebiro et al., 2013). If this transient nature is widely applicable to most GPCR oligomeric complexes, then it is likely that the oligomeric interface will be more ‘non-
specific’ than initially anticipated, such as through weak attractive forces (e.g. hydrogen bonding; Lambert, 2010). This non-specificity and transiency is consistent with the seemingly promiscuous nature of GPCR oligomerisation.

The stoichiometry of most GPCR oligomeric complexes is yet to be resolved. Different studies on the same oligomers have shown the formation of monomers, dimers and even tetramers (Herrick-Davis et al., 2013). It has been suggested that receptor expression levels may influence the oligomeric state, with low expression levels favouring monomers and increasing expression levels favouring higher order oligomers (Hern et al., 2010; Lambert, 2010; Calebiro et al., 2013; Patowary et al., 2013). Additionally, transient β2AR oligomers appear to exist in a dynamic equilibrium of monomers, dimers and higher order oligomers (Calebiro et al., 2013). It has also been suggested that G proteins have two GPCR docking sites with a separation distance larger than the width of a single GPCR, supporting a preferred stoichiometry of 2:1 GPCR to G protein ratio (Liang et al., 2003; Filipek et al., 2004). Furthermore, there is emerging evidence that GPCR oligomerisation may provide a larger platform for the scaffolding of the numerous proteins required for GPCR signalling and regulation (Maurice et al., 2011). For example, it has been suggested that a single GPCR-β-arrestin complex can only accommodate a maximum of six β-arrestin-interacting proteins (Gurevich et al., 2006). Receptor dimerisation or higher order oligomerisation could therefore substantially increase the scaffolding ability and potential number of interacting partners, enhancing the signalling capabilities of the complex (Gurevich et al., 2008). Following from this, there is now evidence that as well as oligomerising with other receptors, GPCRs may be pre-coupled to signalling partners and other regulatory proteins (Nobles et al., 2005; Galés et al., 2006; Dupré et al., 2007; Halls, 2012). This has led to the development of the concept of ‘receptor mosaics’ (Fuxe et al., 2010) or ‘signalosomes’ (Ferré, 2015). Thought to enable efficient targeting and integration of complex GPCR signalling, signalosomes are believed to be microdomains consisting of oligomeric receptors and their various signalling and regulatory partners.
1.3. BRET

Although there are numerous techniques used in the identification and profiling of GPCR oligomers, resonance energy transfer (RET) techniques have become one of the predominant tools in the field, enabling characterisation of a variety of GPCR-protein interactions. RET involves the transfer of energy from a donor molecule to an acceptor molecule in a non-radiative manner as a result of dipole-dipole coupling (Pfleger et al., 2005). It is used to study protein-protein interactions by labelling each of the proteins of interest, one with the donor molecule and the other with an acceptor fluorophore. If the two proteins are in close proximity, energy from the donor will be transferred to the acceptor fluorophore and then emitted at a characteristic wavelength. Monitoring the light emission enables determination of the energy transfer, which is indicative of protein-protein interaction, although there are caveats. The efficiency of the energy transfer is inversely proportional to the distance between the donor and acceptor molecules by the sixth power, indicating a distance of less than 10 nm (Pfleger et al., 2006a; Dacres et al., 2012). The energy transfer process also depends on the orientation of the donor and acceptor molecules relative to one another, as well as the degree of overlap between the donor emission spectrum and the acceptor absorption spectrum.

There are two main forms of RET which differ in the type of donor molecule used. FRET uses a fluorophore whose excitation by light at a characteristic wavelength results in the transfer of energy to the acceptor molecule. While a powerful technique, there are some associated problems that occur as a result of the need for excitation of the donor molecule such as autofluorescence, photobleaching, cell damage and signal loss (Kroeger et al., 2003). Because of these issues, the sister technique, BRET, is sometimes the preferred method. BRET overcomes the issues associated with external excitation by utilising a luciferase enzyme as the energy donor (see Figure 1.4).
Chapter 1. General Literature Review

Figure 1.4. Comparison of the principles of FRET and BRET. (A) FRET requires external illumination for excitation of the donor fluorophore and RET to the acceptor fluorophore. (B) BRET requires oxidation of the luciferase substrate for RET to the acceptor fluorophore. Adapted from Pfleger et al. (2005).

BRET is a naturally occurring phenomenon present in marine organisms such as the jellyfish *Aequorea victoria* and the sea pansy *Renilla reniformis* (Pfleger et al., 2006a). In its adaptation as a pharmacological research tool, the two proteins of interest are labelled with either the donor luciferase or the acceptor fluorophore. Oxidation of the luciferase’s substrate results in the emission of light. If the donor is proximal to the acceptor, less donor light is emitted, and instead, energy is transferred to the fluorophore. This results in excitation of the fluorophore and emission of light at a characteristic wavelength. The resulting BRET signal (quantified as the ratio of light emission from the acceptor over the light emission from the donor) indicates that the two fusion proteins are in close proximity, indicative of the two proteins of interest being in the same complex (Pfleger et al., 2006a).
1.3.1. BRET methodologies

There are now several generations of BRET that are differentiated by the donors, acceptors, and substrates used (see Table 1.1). The original donor enzyme, termed Rluc, was based on the luciferase from the sea pansy *Renilla reniformis* (Xu et al., 1999). Subsequently, variants such as Rluc2 and Rluc8 were developed which had improved stability and increased light output (Loening et al., 2006; De et al., 2007). Rluc8 in particular tends to be the luciferase of choice for the Rluc-based BRET methodologies, of which BRET\(^1\), BRET\(^2\) and extended BRET (eBRET) are the most common (De et al., 2007; Kocan et al., 2008). While these forms of BRET all use an Rluc-based luciferase, they are classified by the particular variant of green fluorescent protein (GFP) and luciferase substrate used. Each generation of BRET has particular features that make it advantageous in specific circumstances. Out of the three common Rluc-based BRET methodologies BRET\(^1\) produces the brightest luminescence while BRET\(^2\) has the greatest spectral resolution and thus lower background noise (Hamdan et al., 2005; Kocan et al., 2008). However, due to issues of substrate instability with BRET\(^1\) and BRET\(^2\), measurements can only be recorded for ~1 h. In contrast, eBRET uses a protected luciferase substrate that enables detection of BRET over many hours, despite producing substantially lower luminescence (Pfleger et al., 2006b). With the development of improved yellow shifted fluorescent proteins such as Venus (Nagai et al., 2002), BRET\(^1\) and eBRET have become the preferred methodologies for most researchers, with the determining factor for assay choice being measurement run-time. Despite this, there may still be applications for which BRET\(^2\) is particularly suitable, such as measuring ligand-induced intramolecular rearrangements within GPCRs (Dacres et al., 2011). Additionally, there are newer generations of Rluc-based BRET with red shifted fluorophores that have enabled monitoring of protein-protein interactions in *in vivo* (De et al., 2009; Dragulescu-Andrasi et al., 2011).
Table 1.1. Spectral and biochemical characteristics of different BRET generations.

<table>
<thead>
<tr>
<th>Property</th>
<th>BRET¹</th>
<th>BRET²</th>
<th>eBRET</th>
<th>NanoBRET</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SUBSTRATE</strong></td>
<td>Coelenterazine</td>
<td>Coelenterazine</td>
<td>EnduRen</td>
<td>Furimazine</td>
</tr>
<tr>
<td><strong>SUITABLE MEASUREMENT TIME FRAME</strong></td>
<td>Up to 2 h</td>
<td>Up to 1 h</td>
<td>Many hours</td>
<td>Up to 1 h</td>
</tr>
<tr>
<td><strong>DONOR ENZYME</strong></td>
<td>Rluc2/Rluc8</td>
<td>Rluc2/Rluc8</td>
<td>Rluc2/Rluc8</td>
<td>Nluc</td>
</tr>
<tr>
<td><strong>DONOR EMISSION PEAK</strong></td>
<td>~480</td>
<td>~400</td>
<td>~480</td>
<td>~450</td>
</tr>
<tr>
<td><strong>SUITABLE DONOR MEASUREMENT FILTER RANGE (nm)</strong></td>
<td>400-475</td>
<td>370-450</td>
<td>400-475</td>
<td>Dependent on acceptor e.g. 400–470 (yellow) 410–490 (red)</td>
</tr>
<tr>
<td><strong>ACCEPTOR FLUOROPHORE</strong></td>
<td>YFP/Venus</td>
<td>GFP²/GFP10</td>
<td>YFP/Venus</td>
<td>Variable e.g. YFP/Venus or BODIPY630</td>
</tr>
<tr>
<td><strong>ACCEPTOR EMISSION PEAK (nm)</strong></td>
<td>~530</td>
<td>~510</td>
<td>~530</td>
<td>Venus: ~530 BODIPY630: ~650</td>
</tr>
<tr>
<td><strong>DONOR/ACCEPTOR EMISSION PEAK SEPARATION (nm)</strong></td>
<td>~50</td>
<td>~110</td>
<td>~50</td>
<td>Venus: ~80 BODIPY630: ~200</td>
</tr>
</tbody>
</table>

Recently, a new generation of BRET has been established through the development of an engineered luciferase called NanoLuc (Nluc; Hall et al., 2012; Machleidt et al., 2015). Nluc is based on the secreted luciferase from the deep sea shrimp Oplophorus and is substantially smaller than Rluc and its derivatives (19 kDa vs. 36 kDa, respectively). Importantly, the emission peak of Nluc is ~20 nm left shifted from that of Rluc in BRET¹ and eBRET, which indicates there would be improved spectral separation with GFPs and yellow fluorescent proteins (YFPs). Furthermore, Nluc produces significantly brighter luminescence than all previous BRET luciferases.
(with a specific activity 30-fold greater than BRET\(^1\) with Rluc8), suggesting that a substantial amount of energy transfer is possible even with red shifted fluorophores.

### 1.3.2. BRET assays

BRET is used in GPCR pharmacology to monitor protein-protein proximity by labelling each of the proteins of interest with the donor enzyme or the acceptor fluorophore. BRET assays are now common practice for investigating GPCR interactions with G proteins (Ayoub et al., 2009; Ayoub et al., 2010b) and β-arrestins (Kocan et al., 2009; Mustafa et al., 2012), and have also been used to investigate GRK recruitment (Hollins et al., 2009; Breton et al., 2010) and ubiquitination processes (Dalrymple et al., 2011; Armstrong et al., 2013; Jaeger et al., 2014b). Most recently, BRET trafficking and internalisation assays have been developed using specific membrane-associated cellular location markers (Lan et al., 2011; Lan et al., 2012).

BRET has also been used extensively to study GPCR oligomerisation. Traditionally this has been done by labelling one receptor with the luciferase and the second receptor with the acceptor fluorophore. A limitation to this approach is that specific receptor interactions need to be differentiated from 'bystander BRET’ caused from intracellular overcrowding of receptors (Mercier et al., 2002). Attempts have been made to address this by employing BRET saturation or competition assays (reviewed in Ayoub et al., 2010a). In a BRET saturation assay a constant amount of the donor fusion protein is coexpressed with increasing amounts of the acceptor fusion protein. In theory, if the receptor interaction is specific, a hyperbolic curve should be obtained with an asymptote tending toward the maximal BRET value (BRET\(_{\text{max}}\)). In contrast, non-specific interactions should theoretically show a near-linear increase in the BRET signal with increasing quantity of acceptor fusion protein. Similarly, BRET competition assays involve the coexpression of constant amounts of donor and acceptor fusion proteins with increasing amounts of untagged receptor. Specific interactions should result in a decrease in the BRET signal, while non-specific interactions should not alter the BRET signal.

BRET saturation and competition assays are somewhat laborious and require numerous controls and careful interpretation to avoid false positives. Furthermore,
recent reports have seriously called in to question the interpretation and validity of many BRET saturation studies (Szalai et al., 2014; Lan et al., 2015). Assessing ligand-dependence of BRET signals is a further method for confirming the specificity of interaction between receptors, however it has been shown that many GPCR oligomers form constitutively. A recently developed technique called Receptor-Heteromer Investigation Technology (Receptor-HIT) allows assessment of ligand-dependent functional outcomes specific for the receptor heteromer (See et al., 2011; Johnstone et al., 2012). Receptor-HIT has been most thoroughly applied to the study of GPCR heteromers (GPCR-HIT), however heteromers from all receptor classes can be assessed using this approach, such as RTKs (RTK-HIT; Ayoub et al., 2013). Receptor-HIT utilises proximity-based reporter systems, which enables its use on a variety of platforms including BRET, FRET, protein fragment complementation systems (and varieties thereof) and proteolysis-based reporter systems (Johnstone et al., 2012).

The Receptor-HIT scheme comprises three elements, which are illustrated for GPCR-HIT in Figure 1.5: i) GPCR A, fused to a proximity-based first reporter component, ii) unlabelled GPCR B and iii) a GPCR-interacting group labelled with the complementary second reporter component. The GPCR-interacting group can be any biomolecule whose interaction with the receptor is modulated upon binding a ligand that is selective for the untagged receptor or the heteromer. Commonly, the GPCR-interacting group is a protein, such as β-arrestin (Figure 1.5A), however, a fluorescent GPCR ligand could also be used (Figure 1.5B), though this has not been previously attempted with BRET. Upon coexpression of these three elements, the proximity of the two receptors can be monitored via ligand-dependent modulation of the interactions with the GPCR-interacting group. Generation of a BRET signal upon treatment with a GPCR B-selective or heteromer-selective ligand indicates interaction of GPCR B with the GPCR-interacting group and thus proximity with GPCR A. If the two receptors are not proximal, no signal will be generated. Additionally, only heteromeric populations of receptors can produce BRET signals, enabling elucidation of heteromer-specific pharmacology.
Chapter 1. General Literature Review

Figure 1.5. Illustration of the GPCR-HIT assay. GPCR A is fused to the first reporter component, GPCR B is unlabelled with respect to the reporter system and a GPCR-interacting group is linked to the complementary second reporter component. (A) GPCR A is C-terminally fused to the first reporter component, enabling monitoring of interactions with intracellular GPCR-interacting proteins such as β-arrrestin. (B) Theoretically, fusion of the first reporter component to the N terminus of GPCR A should enable monitoring of binding of ligands fused to the complementary second reporter component. (Jaeger et al., 2014a)

1.3.3. Advantages and constraints of BRET

One of the major advantages that RET-based methodologies have over conventional biochemical approaches for investigating GPCR oligomerisation is that protein-protein interactions can be monitored in live cells at 37°C and in real time. This enables
monitoring of the dynamics of the interactions, which can be quantified in kinetic assays through calculation of apparent association/dissociation rates (Hamdan et al., 2005). Other standard pharmacological parameters can also be readily generated from BRET assays, such as affinity, potency and efficacy, as well as quantification of parameters associated with ligand bias and allosterism. While some of these pharmacological parameters can be measured through conventional biochemical approaches, significantly more post-assay processing is required, making it far more laborious and potentially resulting in processing-related artefacts. Moreover, BRET is highly amenable to miniaturisation and screening, generating Z’ values which indicate high assay performance (Kocan et al., 2008; See et al., 2011).

One of the current major limitations of BRET is that it can only be performed in recombinant systems with exogenously expressed fusion proteins of interest. Time-resolved FRET has recently been used to assess GPCR homomers in native tissue using fluorescently labelled oxytocin ligands on lactating rat mammary gland tissue (Albizu et al., 2010). While a fluorescent ligand could be used as a BRET acceptor (Jaeger et al., 2014a), currently the only method for labelling with the luciferase is through genetic fusion to the protein of interest. Genetic fusion of luciferases and fluorophores has the potential to alter the molecular function of the proteins of interest, however this issue can be resolved by pharmacological validation, and if necessary, altering various aspects of the fusion protein constructs (such as position of the label and length of linker region between label and protein; Johnstone et al., 2015). Additionally, BRET assays are typically done in cell lines that may not necessarily contain all the signalling and regulatory molecules required for exact replication of receptor pharmacology. Furthermore, the cell lines may contain endogenous levels of the proteins under investigation that could potentially compete with the BRET labelled proteins. Finally, there is the potential to overexpress proteins of interest in cell lines beyond physiologically relevant levels, however the development of extremely bright luciferases (Hall et al., 2012) will enable the detection of BRET signals even at very low expression levels.

While there are some limitations to using recombinant systems for studying GPCR oligomerisation, these can be minimised by various means as discussed above. Additionally, the aim of assessing GPCR oligomerisation in cell lines is to characterise the ‘biochemical fingerprint’ of the heteromer in a relatively straightforward manner.
This biochemical fingerprint can then be used to demonstrate the presence of the heteromer in native tissues. Thus, BRET remains a robust and sensitive approach for the identification and characterisation of GPCR oligomers.

1.4. **Angiotensin II and its Receptors**

1.4.1. **The renin-angiotensin system**

One mechanism by which multicellular organisms regulate their internal environment is through modulation of extracellular volume or solute concentration. A key regulator of this in vertebrates is the renin-angiotensin system (RAS). The RAS maintains blood pressure and fluid homeostasis through integration of the actions of numerous enzymes, hormones and receptors. It is thought to have evolved ~400 million years ago with the development of bony fish, with some of the first components appearing in animals as early as ~700 million years ago (Fournier *et al.*, 2012).

Insights into the regulation of blood pressure began with the discovery of a pressor mediator that was termed “renin” by Tigerstedt and Bergman in 1897 (Tigerstedt *et al.*, 1898; de Gasparo *et al.*, 2000). Several decades later two groups independently isolated the vasopressor peptide (Braun-Menendez *et al.*, 1940; Page *et al.*, 1940) which they later jointly termed angiotensin (now known as angiotensin II; AngII; Braun-Menendez *et al.*, 1958). Over the coming decades, other key regulators of blood pressure were identified, such as angiotensin converting enzyme (ACE) and various angiotensin precursors and metabolites. In the late 1980’s, with the development of receptor selective ligands, it was established that there were at least two receptor types that bound the conventional angiotensin peptide analogues (Chiu *et al.*, 1989; Whitebread *et al.*, 1989; Speth *et al.*, 1990), and in 1995 these were classified as the AngII type 1 (AT1) and type 2 (AT2) receptor (de Gasparo *et al.*, 1995). Since then, other new elements of the renin-angiotensin system have been discovered, including angiotensin converting enzyme 2 (ACE2) and the MAS1 GPCR.
In addition to its effects on blood pressure, the RAS is now known to be involved in numerous other physiological processes including proliferation, hypertrophy, hyperplasia, fibrosis and remodelling, angiogenesis, inflammation, cellular differentiation, stem cell programming and haematopoiesis (George et al., 2010). It then follows that dysregulation of the RAS is linked to numerous pathologies, including many cardiovascular and renal diseases, cancer and malignancy, as well as being involved with the inflammatory aspects of diseases such as diabetes and atherosclerosis (George et al., 2010). The importance of the system is further highlighted by the widespread use of pharmaceuticals that target it; notably the majority of prescriptions for cardiovascular diseases target either the RAS or the adrenergic system (Salazar et al., 2007).

In general, the RAS acts to maintain cardiovascular homeostasis by increasing blood volume and pressure. The classic RAS system begins with the release of renin into the circulation from the juxtaglomerular apparatus in the kidney (see Figure 1.6). Renin activation and release are tightly controlled by various mechanisms including baroreflex activity (Carey et al., 1997) and solute delivery (Lorenz et al., 1991; Bell et al., 2003). Upon its secretion, renin cleaves circulating angiotensinogen into the inactive decapeptide angiotensin I (AngI; Fournier et al., 2012). Angiotensinogen is produced primarily in the liver and is the precursor to all the downstream angiotensin peptides. Angiotensin I is then cleaved to the octapeptide AngII by ACE that is present at particularly high levels in pulmonary epithelial cells as well as being found in other tissues (Fournier et al., 2012). AngII acts predominantly on AT1 receptors to produce all the classic AngII-mediated functions: vasoconstriction; release of aldosterone (which mediates sodium reabsorption); stimulation of hypothalamic thirst sensors and release of arginine vasopressin (which leads to water conservation); increased sympathetic activity and myocardial contractility; stimulation of renal tubular sodium reabsorption; and regulation of tubular glomerular feedback (Crowley et al., 2012; Fournier et al., 2012; Macia-Heras et al., 2012). Taken together, all these functions act to increase blood pressure and volume.
Over the past two decades the RAS has been revealed to be increasingly complex, with multiple pathways that provide balance for the classical AngII-mediated functions (see Figure 1.7). The major countervailing arm of the RAS was first revealed with the discovery of ACE2 (Donoghue et al., 2000; Tipnis et al., 2000), which cleaves the C terminal amino acid from AngII to produce angiotensin 1-7 (Ang1-7; Vickers et al., 2002). Ang1-7 was discovered to be the endogenous agonist for the MAS1 GPCR (Santos et al., 2003), and has been shown to antagonise many AngII-mediated functions, producing vasodilatory, antihypertrophic, antiproliferative, antifibrotic, and antithrombotic effects (Santos et al., 2008). A further countervailing component of the RAS is the AT2 receptor that mediates antagonistic effects of the classical RAS in an AngII-dependent manner (Maciá-Heras et al., 2012). In addition to Ang1-7, there is increasing evidence that other angiotensin metabolites may also have biological activity such as angiotensin III (AngIII; the 2-8 peptide), which is generated by cleavage of AngII by aminopeptidase A and appears to have similar though less potent functions as AngII (Vauquelin et al., 2002). AngIII binds with moderately greater affinity to AT2 receptors than AT1 receptors (Bosnyak et al., 2011), and there is evidence that AngIII may be the endogenous ligand for the AT2 receptor, at least in the mediation of...
natriuresis (Padia et al., 2006; Padia et al., 2008). Further metabolism of angiotensin III to angiotensin IV (the 3-8 peptide) occurs via aminopeptidase N. Angiotensin IV binds the novel angiotensin type 4 (AT4) receptor (also known as insulin-regulated aminopeptidase) and is involved in cognition and memory, as well as having cardiovascular effects (Vanderheyden, 2009). In contrast to the AT1, the AT2 and the MAS1 receptor, the AT4 receptor is not a GPCR, and is instead a membrane-anchored zinc-dependent metalloproteinase (Albiston et al., 2001). Other metabolites with recently discovered biological activity include angiotensin 1-12, angiotensin 1-9, angiotensin 3-7, angiotensin 5-8, angiotensin 5-7 and angiotensin A, as well as angioprotectin and alamandine (Balakumar et al., 2014b). Additionally, renin and its precursor prorenin have been shown to bind a renin receptor (Nguyen et al., 2002) stimulating profibrotic and proinflammatory pathways (Nguyen et al., 2010), as well as having functions independent of the RAS (Crowley et al., 2012). ACE and ACE2 have also been found to display receptor-like behaviour, mediating AngII-independent signalling pathways (Lambert et al., 2010).
Our understanding of the RAS as a purely endocrine system has also evolved as a result of the discovery of ‘tissue’ or ‘local’ RAS. The full complement of RAS components have been found in many tissues, demonstrating the potential for local AngII synthesis and RAS activation (Paul et al., 2006). Locally produced AngII acts in an autocrine or paracrine manner and local RASs may act either synergistically or independently of the circulating RAS (George et al., 2010). To add further complexity, the existence of an intracellular RAS has also been proposed. The presence of all the components required to produce AngII have been found intracellularly, including AT₁ and AT₂ receptors bound at the membrane of various organelles such as the nucleus and the mitochondria (Kumar et al., 2012). So far this intracellular RAS has been proposed
to be involved in cardiac hypertrophy and remodelling, cardiac electrical function and cardiac gene expression (Tadevosyan et al., 2012).

1.4.2. Structure of angiotensin II receptors

While there is now a complex array of signalling pathways and physiological outcomes associated with the RAS, the best established and the most pharmacologically targeted is that mediated via the AT₁ receptor. The first AT₁ receptors to be cloned were bovine (Sasaki et al., 1991) and rat (Murphy et al., 1991). They were later shown to have 95% amino acid sequence homology with human AT₁ receptors (Curnow et al., 1992) and have since been classified as part of the γ class of receptors in the rhodopsin family (Fredriksson et al., 2003). The human AT₁ receptor has been localised on the q22 band of chromosome 3 and is 359 amino acids in length (Curnow et al., 1992; Davies et al., 1994). The extracellular regions contain three N-glycosylation sites (Jayadev et al., 1999) as well as four cysteine residues that form two disulphide bonds, one of which is essential for binding of non-peptide antagonists (Ohyama et al., 1995; Dasgupta et al., 2011). Additionally, like most GPCRs there are numerous serine and threonine residues in the cytoplasmic tail. Very recently, crystal structures of the human AT₁ receptor bound to the antagonists ZD7155 (Zhang et al., 2015b) and olmesartan (Zhang et al., 2015a) were published, revealing new insights into the structural features of ligand binding and receptor activation. As predicted for small molecule non-peptide AT₁ antagonist ligands, they bound in the transmembrane region of the receptor, forming interactions with several residues in helices I, II, III and IV, as well as unexpectedly forming a critical interaction with Arg167 in extracellular loop 2. In conjunction with mutagenesis studies, the crystal structures also revealed key residues that are involved in binding of peptide ligands as well as receptor activation.

The first AT₂ receptors to be cloned were from mouse (Nakajima et al., 1993) and rat (Ichiki et al., 1994), and shared only about 35% amino acid sequence homology with the species equivalent AT₁ receptor. Cloning of the human AT₂ receptor revealed 93% sequence homology with the rat AT₂ receptor (Tsuzuki et al., 1994) and 30% with the human AT₁ receptor. The human AT₂ receptor is located on chromosome X at position q22 and is 363 amino acids in length (Tissir et al., 1995). Like the AT₁
receptor, it is also grouped in the $\gamma$ class of receptors in the rhodopsin family (Fredriksson et al., 2003). The majority of the homology between AT\(_1\) and AT\(_2\) receptors is localised to the transmembrane domains that contain most of the residues important for ligand binding. This explains the similar affinities of AngII for both AT\(_1\) and AT\(_2\) receptors (de Gasparo et al., 2000). The other regions of the receptors display high sequence divergence, particularly in intracellular loop 3 and the C terminal tail. The AT\(_2\) receptor has five potential N-glycosylation sites and unlike the AT\(_1\) receptor does not require the formation of extracellular disulphide bonds for ligand binding (de Gasparo et al., 2000). Furthermore, the AT\(_2\) receptor does not contain the Ser-Thr-Leu motif that is highly conserved in mammalian AT\(_1\) receptors and is necessary for AT\(_1\) receptor internalisation (de Gasparo et al., 2000).

1.4.3. Expression and function of angiotensin II receptors

The AT\(_1\) receptor is widely expressed throughout the body, and is found in all organs including the liver, adrenals, brain, lung, kidney, heart and the vasculature (Lyngsø et al., 2009; Dasgupta et al., 2011). In the cardiovascular system it is expressed in all tissues: endothelial cells, smooth muscle cells, fibroblasts, cardiomyocytes, monocytes and macrophages (Dasgupta et al., 2011). In the kidney and adrenal gland it is expressed in numerous cell types (Dinh et al., 2001) and is also ubiquitously expressed throughout the nervous system (Dasgupta et al., 2011). In contrast, it is generally believed that although the AT\(_2\) receptor is highly expressed in foetal tissues, its expression throughout the body rapidly declines after birth (Dasgupta et al., 2011). However, recent studies suggest that in many tissues and organs, expression of the AT\(_2\) receptor may actually increase throughout life (Yu et al., 2010; Gao et al., 2012; Yu et al., 2014). In either case, the AT\(_2\) receptor is expressed at detectable levels in various adult tissues including uterus, ovary, adrenals, heart, blood vessels and brain (Bottari et al., 1993). AT\(_2\) receptor expression levels are known to be dependent on a variety of factors. AT\(_1\):AT\(_2\) receptor ratios are lower in females than males (Silva-Antonelli et al., 2004; Sampson et al., 2008), and while the AT\(_1\) receptor is generally believed to be expressed at higher levels than the AT\(_2\) receptor, different species have also been shown to have different expression ratios (Chang et al., 1991). Additionally, the AT\(_2\) receptor...
is downregulated in pregnancy (de Gasparo et al., 1994) and upregulated under various pathological conditions such as heart failure (Nio et al., 1995; Ohkubo et al., 1997), vascular injury (Nakajima et al., 1995) and wound healing (Viswanathan et al., 1994; Viswanathan et al., 1997).

As described above, the AT₁ receptor mediates most of the classical AngII actions, such as vasoconstriction, fluid retention, hypertrophy and inflammation. Correspondingly, most of the pathologies associated with AngII also arise through AT₁ receptor-dependent activation, such as endothelial cell dysfunction and cardiovascular remodelling (Dasgupta et al., 2011). In contrast, the AT₂ receptor is generally believed to counteract most of the AT₁-mediated actions. In the vasculature the AT₂ receptor typically mediates vasodilation, although some studies have reported vasoconstrictive responses in old normotensive (Pinaud et al., 2007) and spontaneously hypertensive rats (Touyz et al., 1999b; You et al., 2005; Moltzer et al., 2010). The AT₂ receptor also has beneficial effects on vascular remodelling (Nakajima et al., 1995) (Akishita et al., 2000a; Wu et al., 2001; Suzuki et al., 2002) and development of cardiovascular hypertrophy and fibrosis in the heart (van Kesteren et al., 1997; Akishita et al., 2000a; Kurisu et al., 2003). Again, however, different studies report conflicting results: the AT₂ receptor has been shown to either have no effect (van Esch et al., 2010) or to potentiate (Senbonmatsu et al., 2000; Ichihara et al., 2001) hypotrophy and fibrosis in the heart. In the kidney the AT₂ receptor antagonises the AT₁ receptor by mediating natriuresis (Gross et al., 2000) in an AngIII-dependent manner (Padia et al., 2006; Padia et al., 2008). In the brain, the AT₂ receptor appears to have a role in brain development and cognitive function (Vervoort et al., 2002), while also working in concert with the AT₁ receptor to increase thirst (Li et al., 2003). Additionally, upregulated AT₂ receptors after brain injury are involved in the promotion of cell differentiation, neuronal regeneration and reduction of oxidative stress (Lucius et al., 1998; Iwai et al., 2004; Lu et al., 2004; Li et al., 2005). There is also growing evidence that AT₂ receptors are involved in nociception, although some studies report anti-analgesic properties (Anand et al., 2013; Smith et al., 2013; Muralidharan et al., 2014; Rice et al., 2014) while others report analgesic properties (Marion et al., 2014). Finally, the AT₂ receptor appears to also have anti-inflammatory actions (Akishita et al., 2000b; Wu et al., 2001; Rompe et al., 2010).

Most of the drugs targeting the RAS do so through antagonism of AT₁ receptor signalling. ACE inhibitors and AT₁ receptor blockers (ARBs) are used in the treatment
Chapter 1. General Literature Review

of hypertension, heart failure, stroke prevention, atherosclerosis and diabetes (Dasgupta et al., 2011). ACE inhibitors (and renin inhibitors) reduce the availability of AngII, thereby indirectly blocking AT1 receptor signalling. ARBs directly antagonise AT1 receptor signalling, and their effectiveness is partly attributed to an increase in AT2 receptor signalling resulting from increased concentration of circulating AngII upon AT1 blockade (Widdop et al., 2003). As many non-human studies show a beneficial effect of AT2 receptor stimulation it is speculated that AT2 receptor agonists could be beneficial in treating a variety of diseases, such as hypertension, stroke, aneurysm formation, inflammation, myocardial fibrosis (Verdonk et al., 2012). However, as the physiological role of the AT2 receptor is not always clear, pharmacological targeting of the AT2 receptor in humans in most diseases requires further preclinical research. Despite this, the first clinical trial of an AT2 agonist is currently underway for the treatment of hypertension (Mitsubishi Tanabe Pharma, 2015). Conversely, a recently completed phase 2 clinical trial has shown superior pain relief upon treatment with an AT2 antagonist in comparison with placebo (Rice et al., 2014).

1.4.4. Activation and regulation of angiotensin II receptors

Binding of an agonist to the AT1 receptor releases it from a constrained, inactive confirmation (Balakumar et al., 2014a). Like most GPCRs, AT1 receptor activation results in classical G protein-coupling and subsequent desensitisation. The AT1 receptor has been shown to be phosphorylated by GRK2, 3 and 5 in overexpression transfected systems (Oppermann et al., 1996b; Oppermann et al., 1996a), while in HEK293 cells siRNA studies suggested the GRK2 was the primary mediator of AT1 phosphorylation (Kim et al., 2005). Phosphorylation of the C terminal tail results in the recruitment of β-arrestin. The AT1 receptor is classified as a class B receptor in terms of its desensitisation profile: it shows equal affinity for both β-arrestin1 and β-arrestin2 (Oakley et al., 2000) and forms stable β-arrestin complexes (Oakley et al., 2001). AT1 receptors are rapidly endocytosed where they are retained in endosomes and remain phosphorylated (Zhang et al., 1999; Hunyady et al., 2002). As well, AT1 receptors are able to recycle via fast or slow recycling endosomal paths (Hein et al., 1997; Hunyady et al., 2002; Dale et al., 2004; Esseltine et al., 2011). AT1 receptor internalisation occurs
predominantly via clathrin-coated pits in a β-arrestin- and dynamin-dependent manner (Anborgh et al., 2000; Gáborik et al., 2001). However under particular conditions it appears to be able to internalise in a β-arrestin- and dynamin-independent manner (Zhang et al., 1996a) as well as ligand-independent manner (Anborgh et al., 2000). Finally, interactions with various Rab GTPases regulate AT₁ receptor intracellular trafficking and internalisation (Seachrist et al., 2002; Dale et al., 2004; Zhang et al., 2009; Esseltine et al., 2011).

In contrast to the AT₁ receptor and most other GPCRs, the AT₂ receptor is generally believed to not undergo classical G protein coupling. In addition, several studies have found that it is resistant to AngII-induced desensitisation and internalisation (Pucell et al., 1991; Mukoyama et al., 1995; Hein et al., 1997; Widdop et al., 2002; Turu et al., 2006). Despite this, the AT₂ receptor can interact with a variety of other signalling and regulatory proteins via its C terminal tail (Rodrigues-Ferreira et al., 2010). For example, the transcription factor promyeolytic leukaemia zinc finger (PLZF) protein induces AT₂ receptor internalisation in an AngII-dependent manner (Senbonmatsu et al., 2003). Additionally, AT₂ receptor-interacting protein 1 (ATIP1; also known as AT₂ receptor-binding protein; ATBP) regulates the trafficking of the AT₂ receptor from the Golgi apparatus to the plasma membrane (Wruck et al., 2004). Furthermore, there is evidence that the AT₂ receptor may be constitutively active (Miura et al., 2000; Jin et al., 2002; Su et al., 2002; D'Amore et al., 2005). While the AT₁ receptor is constrained in an inactive state until binding of an agonist, the AT₂ receptor is believed to be in a ‘relaxed’ conformation even in the absence of agonist (Miura et al., 1999).

1.4.5. Angiotensin II receptor signalling

1.4.5.1. AT₁ receptor signalling

The classic vasoconstrictor actions of AngII described in Section 1.4.1 are predominantly mediated by G protein-dependent pathways (Dasgupta et al., 2011). Activated AT₁ receptors are primarily coupled to Ga_{q/11} proteins that stimulate the PLC pathway via the two second messengers diacylglycerol (DAG) and inositol 1,4,5-
trisphosphate (IP$_3$; Lassègue et al., 1993; Ushio-Fukai et al., 1998). The IP$_3$-dependent release of Ca$^{2+}$ through this pathway is essential for the smooth muscle contractions that mediate vasoconstriction (Mehta et al., 2007). DAG is a potent activator of PKC, which is an effector in the ERK1/2 pathway. This MAPK activation contributes to vasoconstriction as well as growth promotion (Balakumar et al., 2014b). The AT$_1$ receptor also couples to $G_{q/16}$ and $G_{12/13}$ in some tissues (de Gasparo et al., 2000) resulting in inhibition of the adenylyl cyclase activity and activation of small monomeric G proteins such as Rho, respectively. AT$_1$ receptor activation also stimulates phospholipase D (PLD) and this is primarily mediated by activation of $G_{q/16}$ subunits (Ushio-Fukai et al., 1999).

AT$_1$ receptors can also transactivate RTKs such as the epidermal growth factor receptor (EGFR; Olivares-Reyes et al., 2005) and the platelet-derived growth factor receptor (PDGFR; Linseman et al., 1995; Heeneman et al., 2000). This may occur through several mechanisms but the most well established is the ‘triple-membrane-passing-signalling’ model, which involves the GPCR-dependent activation of membrane metalloproteinases (Wetzker et al., 2003). In the case of the EGFR, AT$_1$ receptor-induced $G_q$-mediated stimulation of metalloproteinases results in the cleavage and release of heparin binding-epidermal growth factor (EGF) and subsequent activation of the EGFR (Uchiyama-Tanaka et al., 2001). Recently, a functional siRNA screen of the human kinome has revealed novel kinases that appear to be involved in mediating AT$_1$-EGFR transactivation (George et al., 2013). AT$_1$ receptor-mediated RTK transactivation results in the stimulation of MAPK cascades such as ERK1/2 and p38 and is involved in many of the pro-growth effects of AngII (Balakumar et al., 2014b).

The AT$_1$ receptor is also involved in the production of reactive oxygen species (ROS). AT$_1$-mediated ROS activation occurs in a G protein-dependent manner through the stimulation of various second messengers including PLC/PKC (Seshiah et al., 2002) and PLD (Touyz et al., 1999a). These second messengers activate nicotinamide adenine dinucleotide phosphate (NADPH) oxidases that result in the production of ROS. Under physiological conditions the ROS can act as signalling molecules (Martínez-Ruiz et al., 2011), which contribute to the maintenance of cardiovascular homeostasis, however chronic stimulation can cause inflammation contributing to the initiation and progression of cardiovascular disease (Balakumar et al., 2014b).
Chapter 1. General Literature Review

The G protein-independent paths of AT₁ receptor signalling have also been investigated. β-arrestin-AT₁ receptor complexes are sequestered in endosomes with ERK and components of the ERK cascade including Raf-1 and MEK (Luttrell et al., 2001a). This results in reduced nuclear ERK expression and diminished transcriptional responses (Tohgo et al., 2002). β-arrestin-dependent signalling has also been reported to account for 30% of AngII-mediated cardiomyocyte functional responses (Rajagopal et al., 2006), however another study reported almost no involvement of AT₁ receptor-β-arrestin signalling in contraction in Langendorff-perfused hearts (Aplin et al., 2007). Furthermore, in contrast to the often unfavourable vasoconstrictive and hypertrophic responses mediated by G protein-dependent paths, there is evidence that β-arrestin-dependent signalling may regulate some beneficial processes such as cardioprotection (Zhai et al., 2005; Aplin et al., 2007; Ahn et al., 2009; Hostrup et al., 2012).

The AT₁ receptor also interacts via its C terminal tail with signalling and regulatory proteins other than G proteins and β-arrestin, including Janus kinase 2 (JAK2; Ali et al., 1997) and PLCγ1 (Venema et al., 1998). Additionally, interaction of the AT₁ receptor with the AT₁ receptor-associated protein (ATRAP; Daviet et al., 1999; Tsurumi et al., 2006) results in a downregulation of AT₁ receptor signalling and surface expression (Cui et al., 2000; Lopez-Ilasaca et al., 2003). This association also leads to a decrease in AT₁ receptor-mediated cardiomyocyte hypertrophy (Tanaka et al., 2005) and vascular remodelling (Oshita et al., 2006). Conversely, increased recycling and subsequent hypertension and hypertrophy occur as a result of the interaction of the AT₁ receptor with the AT₁ receptor associated protein (ARAP1; Guo et al., 2003; Guo et al., 2006), while hypertrophy was also observed as a result of the interactions with the guanine nucleotide exchange factor-like protein (GLP; Guo et al., 2004).

1.4.5.2. AT₂ receptor signalling

Although the AT₂ receptor displays many classic GPCR motifs and residues, there is little evidence that G protein coupling is an important signalling mechanism. Limited evidence suggests that it can couple to Ga₁₁₀ (Huang et al., 1995; Hayashida et al., 1996; Zhang et al., 1996b; Hansen et al., 2000; Senbonmatsu et al., 2003; Lara et al., 2006; Marion et al., 2014), as well as Ga₃ (Feng et al., 2002). Its interaction with Ga₃ has been
implicated in inhibition of the proximal tubule \(\text{Na}^+\)-ATPase (Lara et al., 2006) and increases in nitric oxide synthase (NOS) expression (Li et al., 2007a). Additionally, \(\text{G}_i\) is thought to be involved in the internalisation of the \(\text{AT}_2\) receptor-PLZF complex resulting in the subsequent translocation of PLZF into the nucleus (Senbonmatsu et al., 2003). The interaction between the \(\text{AT}_2\) receptor and PLZF is reportedly involved in the development of cardiac hypertrophy (Senbonmatsu et al., 2003) and neuroprotection (Seidel et al., 2011).

One of the most well established signalling pathways of the \(\text{AT}_2\) receptor is the stimulation of nitric oxide (NO) production by NOS (Siragy et al., 1997). NO acts as a signalling molecule by activating soluble guanylyl cyclase to produce cGMP which then regulates the activity of various downstream effectors. In the cardiovascular system cGMP mediates vasorelaxation through regulation of contractile proteins as well as induction of gene transcription (Martinez-Ruiz et al., 2011). Furthermore, NO itself can directly mediate vasodilation through activation of calcium-dependent potassium channels in a cGMP-independent manner (Bolotina et al., 1994). The \(\text{AT}_2\) receptor upregulates NOS expression via the activation of calcineurin/nuclear factor of activated T-cell pathway (Ritter et al., 2003). The \(\text{AT}_2\) receptor can also produce NO through activation of NOS either directly (Cervenka et al., 2001; Abadir et al., 2003) or indirectly through a pathway involving the bradykinin (BK) type 2 (B2) receptor. This can occur as a result of the \(\text{AT}_2\)-dependent release of endogenously formed BK (Tsutsumi et al., 1999; Katada et al., 2002). Additionally, there is evidence that the \(\text{AT}_2\) receptor and the B2 receptor form a heteromer that may be involved in \(\text{AT}_2\)-mediated NO signalling (see Section 1.4.6.) In either case, NO signalling via the \(\text{AT}_2\) receptor and B2 receptor is known to mediate vasodilation, natriuresis and inhibit renin production (Madrid et al., 1997; Siragy et al., 2007).

The \(\text{AT}_2\) receptor has also long been known to activate phosphatases including MAPK phosphatase 1, Src homology 2 domain-containing tyrosine phosphatase 1 (SHP-1) and serine/threonine phosphatase 2A (Bottari et al., 1992; Huang et al., 1995; Bedecs et al., 1997; Matsubara et al., 2001). These actions are thought to be involved in the inhibitory effects of the \(\text{AT}_2\) receptor on \(\text{AT}_1\) receptor-induced growth responses (Nakajima et al., 1995; Fischer et al., 1998; Akishita et al., 1999; Horiuchi et al., 1999; Elbaz et al., 2000). However, there are some contradictory reports of \(\text{AT}_2\) receptor phosphatase activity, showing no growth effects and inhibition of phosphatase activity.
(Warnecke et al., 2001) as well as MAPK activation and pro growth responses (D’Amore et al., 2005). Kinase dephosphorylation is also implicated in the pro-apoptotic effects of the AT2 receptor (Horiuchi et al., 1997), although AT2-mediated MAPK activation has been implicated in both pro-apoptotic responses (Miura et al., 2000) and anti-apoptotic responses (Miyamoto et al., 2008). The anti-inflammatory actions of the AT2 receptor are also thought to be mediated by phosphatases with one mechanism occurring through decreased activation of nuclear factor-κ B (NF-κB; Wu et al., 2004; Rompe et al., 2010).

The AT2 receptor is also able to activate phospholipase A2 (PLA2), which results in the release of arachidonic acid (AA; Lokuta et al., 1994; Jacobs et al., 1996; Dulin et al., 1998). One outcome of this is the activation of the Na+/HCO₃⁻ symporter and subsequent increased intercellular alkalinity and polarisation in cardiac cells (Kohout et al., 1995). Additionally, AA is metabolised into epoxyeicosatrienoic acids (EETs) which induce inflammation through activation of MAPKs (Dulin et al., 1998), providing paradoxical signalling responses to those produced by phosphatase activation. Furthermore, stimulation of AA and EETs have been implicated in NOS-independent vasodilation by the AT2 receptor (Arima et al., 1997).

Finally, ATIPs are a class of GPCR-interacting proteins that all contain a consensus AT2 receptor interacting domain and are involved in the regulation of receptor trafficking and signalling (Nouet et al., 2004; Wruck et al., 2004). As previously mentioned, ATIP1 is involved in the plasma membrane localisation of the AT2 receptor (Wruck et al., 2004). In addition, it also has a role in AT2 receptor induced signalling. ATIP1 is highly expressed in the brain (Di Benedetto et al., 2006b) where its association with the AT2 receptor and SHP-1 is involved in neural differentiation (Li et al., 2007b). Furthermore, in vitro ATIP1 interactions with the AT2 receptor results in anti-growth effects such as inhibition of growth factor-induced ERK2 activation and cell proliferation (Nouet et al., 2004). In vivo, similar anti-growth effects were seen in the context of vascular remodelling (Fujita et al., 2009). The other members of the family have not yet been shown to interact with the AT2 receptor, however regulatory actions on microtubule networks (Rodrigues-Ferreira et al., 2009) and involvement in breast cancer have so far been revealed for ATIP3 (Di Benedetto et al., 2006a; Frank et al., 2007; Rodrigues-Ferreira et al., 2009).
1.4.6. Angiotensin II receptor heteromers

The AT\(_1\) and AT\(_2\) receptor are known to form both homomers and heteromers with other GPCRs. AT\(_1\) receptor homomerisation occurs constitutively and appears to result in increased G protein signalling, desensitisation and internalisation (AbdAlla et al., 2001b; AbdAlla et al., 2004; Hansen et al., 2004; Karip et al., 2007). AT\(_2\) homomers also form constitutively and may be important for regulation of constitutive activity of the receptor (AbdAlla et al., 2001b; Miura et al., 2005). AT\(_1\) and AT\(_2\) receptors both form heteromers with a variety of other GPCRs including several adrenergic and dopamine receptors (see Table 1.2).

### Table 1.2. Evidence for putative angiotensin II receptor heteromers (GPCRs only).

<table>
<thead>
<tr>
<th>Heteromer</th>
<th>Physical interaction</th>
<th>Functional interaction</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT(_1)-APJ (Apelin receptor)</td>
<td>Apelin-induced heteromer formation (co-IP and saturation BRET).</td>
<td>Inhibition of AT(_1) ligand binding. Inhibition of AT(_1)-induced IP accumulation and (\beta)-arrestin recruitment.</td>
<td>(Siddiquee et al., 2013)</td>
</tr>
<tr>
<td>AT(_1)-(\alpha_2)AR ((\alpha_2)C adrenergic receptor)</td>
<td>Constitutive heteromer formation (saturation BRET). Plasma membrane colocalisation (BIFC with HA-tagged receptors).</td>
<td>Induction of distinct heteromer conformations with different ligands. Dual agonist treatment potentiated (\beta)-arrestin recruitment and a switch in G protein-coupling to (G_\alpha). Prolonged co-internalisation with dual agonist treatment. NA potentiation of AngII-induced NA release in neuronal culture and mice.</td>
<td>(Bellot et al., 2015)</td>
</tr>
<tr>
<td>Heteromer</td>
<td>Physical interaction</td>
<td>Functional interaction</td>
<td>References</td>
</tr>
<tr>
<td>-----------</td>
<td>---------------------</td>
<td>------------------------</td>
<td>------------</td>
</tr>
<tr>
<td>AT(_1)-(\alpha)(_D)AR ((\alpha)(_D) adrenergic receptor)</td>
<td>Increased expression of heteromer in PIH rats (co-IP). Colocalisation of receptors in smooth muscle layer of aorta (confocal microscopy).</td>
<td>In cardiomyocytes, propranolol and selective (\beta)(_1)AR blockade inhibited AngII-induced contractility. AT(_1) blockade inhibited isoprenaline-induced contractility.</td>
<td>(González-Hernández et al., 2010)</td>
</tr>
<tr>
<td>AT(_1)-(\beta)(_1)AR, AT(_1)-(\beta)(_2)AR ((\beta)(_1) and (\beta)(_2) adrenergic receptor)</td>
<td>Constitutive heteromer formation (co-IP).</td>
<td>In cardiomyocytes, propranolol and selective (\beta)(_1)AR blockade inhibited AngII-induced contractility. AT(_1) blockade inhibited isoprenaline-induced contractility. In cardiac membranes, (\beta)(<em>1)AR blockade inhibited AngII-induced (G</em>\beta)(_7)-coupling and AT(<em>1) blockade inhibited isoprenaline-induced (G</em>\beta)(_7)-coupling. In COS7 cells, propranolol and selective (\beta)(_2)AR blockade inhibited AngII-induced ERK activation and AT(_1) blockade inhibited isoprenaline-induced ERK activation. Induction or inhibition of AT(_1) internalisation upon treatment with isoprenaline or propranolol, respectively. Inhibition of (\beta)(_2)AR internalisation with AT(_1) blockade. AT(_1) blockade inhibited isoprenaline-stimulated increase in heart rate.</td>
<td>(Barki-Harrington et al., 2003)</td>
</tr>
<tr>
<td>AT(_1)-(\beta)(_2)AR ((\beta)(_2) adrenergic receptor)</td>
<td>Constitutive heteromer formation (co-IP).</td>
<td>AT(_1) glycosylation/maturation dictates interactions of heteromer with chaperone proteins. ER-retained receptors (due to mutation or inhibition of glycosylation) dictate interactions of heteromer with chaperone proteins. Involvement of the chaperone ERp57 in the formation of the heteromer.</td>
<td>(Hammad et al., 2010)</td>
</tr>
<tr>
<td>AT(_1)-AT(_2)</td>
<td>Constitutive heteromer formation (co-IP and BiFC).</td>
<td>Inhibition of AT(<em>1)-induced G(</em>{q11})/G(_{i_0})-coupling and IP accumulation. Inhibition of AT(_1)-induced IP accumulation occurs via AT(_2) receptor’s 3rd intracellular loop but not the C terminal tail. Intracellular retention of AT(_2) upon coexpression of ER-retained AT(_1) mutant.</td>
<td>(AbdAlla et al., 2001b) (Kumar et al., 2002) (Zhang et al., 2009)</td>
</tr>
<tr>
<td>AT(_1)-AT(_2)</td>
<td>Constitutive heteromer formation (co-IP and BiFC).</td>
<td>Inhibition of AT(<em>1)-induced G(</em>{q11})/G(_{i_0})-coupling and IP accumulation.</td>
<td>(AbdAlla et al., 2001b)</td>
</tr>
<tr>
<td>AT(_1)-AT(_2)</td>
<td>Intracellular retention of AT(_2) upon coexpression of ER-retained AT(_1) mutant.</td>
<td>High and low [AngII] causes dissociation or maintenance of heteromers, respectively (IP/WB).</td>
<td>(Zhang et al., 2009)</td>
</tr>
</tbody>
</table>

(Axelband et al., 2009)
<table>
<thead>
<tr>
<th>Heteromer</th>
<th>Physical interaction</th>
<th>Functional interaction</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constitutive heteromer</td>
<td>Constitutive heteromer formation and receptor colocalisation (BifC).</td>
<td>AngII-induced β-arrestin recruitment but inhibition of internalisation.</td>
<td>(Porrello et al., 2011)</td>
</tr>
<tr>
<td>AT₂-B₂ (bradykinin type 2</td>
<td>Colocalisation of receptors that was increased with AT₂ agonism (co-IP, confocal</td>
<td>AT₂ agonism decreased AT₁ expression via a NO/cGMP pathway and dephosphorylation of a</td>
<td>(Yang et al., 2012)</td>
</tr>
<tr>
<td>receptor)</td>
<td>microscopy).</td>
<td>regulator of AT₁ transcription. AT₂ agonism decreased AngII-induced stimulation of Na⁺-K⁺-ATPase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AngII induced increase in levels of heteromer (IP/WB).</td>
<td></td>
<td>(Ferrão et al., 2012)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Constitutive heteromer</td>
<td>Constitutive heteromer formation, sensitive to reduction (co-IP).</td>
<td>Altered endocytic pathway from dynamin-independent to dynamin-dependent. Dual agonist</td>
<td>(AbdAlla et al., 2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>treatment potentiated G_{q/11}/G_{iso}-coupling and IP accumulation but was impaired</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>by coexpression of a G protein uncoupled B₂.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(AbdAlla et al., 2001a)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Increased B₂ expression correlates with increased heteromer formation on platelets</td>
<td>Increased AngII-induced [Ca^{2+}] and IP signalling in cotransfected cells and pre-eclampt</td>
<td></td>
</tr>
<tr>
<td></td>
<td>and omental vessels from pre-eclamptic patients (Co-IP).</td>
<td>ic platelets. H₂O₂ inhibition of AT₁ inhibited by heteromerisation.</td>
<td>(AbdAlla et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>Increased heteromers on mesangial cells from hypertensive rats compared to normotensive</td>
<td>Increased endothelin-1 secretion and G_{q/11} coupling in mesangial cells from hypertensive</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rats (Co-IP).</td>
<td>rats compared to normotensive rats.</td>
<td>(AbdAlla et al., 2009)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calreticulin chaperone</td>
<td>Calreticulin chaperone promotes B₂ maturation and heteromerisation with AT₁. (Co-IP)</td>
<td>Downregulation of calreticulin inhibited AngII-induced hyperresponsiveness from heteromers.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(AbdAlla et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>Calreticulin chaperone promotes B₂ maturation and heteromerisation with AT₁.</td>
<td>Co-internalisation of receptors.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heteromer</td>
<td>Physical interaction</td>
<td>Functional interaction</td>
<td>References</td>
</tr>
<tr>
<td>-----------</td>
<td>----------------------</td>
<td>------------------------</td>
<td>------------</td>
</tr>
<tr>
<td>Constitutive heteromer formation (co-IP and fluorescence microscopy and FRET).</td>
<td>GRK-induced co-internalisation of receptors.</td>
<td>(Quitterer et al., 2011)</td>
<td></td>
</tr>
<tr>
<td>Constitutive heteromer formation (saturation BRET).</td>
<td>SII-induced inhibition of B₂ [Ca²⁺] signalling and Gα₂-coupling upon coexpression of AT₁. No effect of SII on B₂ ligand affinity or β-arrestin recruitment. SII-induced AT₁-depentant internalisation of B₂, with kinetics favouring BK/B₂ rather than AngII/AT₁ internalisation.</td>
<td>(Wilson et al., 2013)</td>
<td></td>
</tr>
<tr>
<td>Colocalisation of receptors in aortic smooth muscle (immunohistology and confocal microscopy and FRET).</td>
<td>Reduced AT₁-induced vasopressor response in mice B₂ KO mice.</td>
<td>(Quitterer et al., 2014)</td>
<td></td>
</tr>
<tr>
<td>AT₁-CB₁ (cannabinoid type 1 receptor)</td>
<td>Colocalisation of receptors (co-IP, confocal microscopy, BRET, heteromer specific antibodies) CB₁ potentiates AngII-induced G protein-coupling, β-arrestin-mediated signalling, [Ca²⁺] mobilisation and ERK, p38 and JAK activation. Switch in AT₁ G protein-coupling from Gq to Gαi/o. Potentiation of AT₁ activity involved in profibrogenic actions.</td>
<td>(Rozenfeld et al., 2011)</td>
<td></td>
</tr>
<tr>
<td>AT₁-D₁ (dopamine D₁ receptor)</td>
<td>Colocalisation of receptors (co-IP confocal microscopy). AngII/AT₁-induced increase in D₁ expression in normotensive but not hypertensive rats and correlated with heteromer expression. AngII/AT₁-induced internalisation of D₁ in normotensive but not hypertensive rats. Identification of interacting motif and a reduction in interaction upon treatment with agonists (co-IP). D₁ activation induced AT₁ internalisation and AT₁ activation induced D₁ internalisation. D₁ activation inhibited AT₁ [Ca²⁺] signalling and AT₁ activation inhibited D₁ cAMP signalling. AT₁ and D₁ interact in a complex with Na⁺-K⁺-ATPase. Colocalisation of receptors (co-IP) Losartan increased plasma membrane expression of D₁ and interaction with AT₁. Losartan increased D₁ cAMP signalling upon coexpression with AT₁. D₁ antagonism inhibited the antihypertensive effect of losartan in vivo.</td>
<td>(Zeng et al., 2005c) (Khan et al., 2008) (Li et al., 2012)</td>
<td></td>
</tr>
</tbody>
</table>
### Table 1.1: Heteromer Interactions and Functional Implications

<table>
<thead>
<tr>
<th>Heteromer</th>
<th>Physical interaction</th>
<th>Functional interaction</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT(_1)-D(_2)</td>
<td>Constitutive heteromer formation and colocalisation of receptors (confocal microscopy, saturation BRET, in situ PLA)</td>
<td>AT(_1) antagonism inhibited D(_1) G(_i) signalling, β-arrestin recruitment and ERK1/2 phosphorylation. AT(_1) antagonism of D(_1) signalling was also observed in neuronal primary cultures and rat striatal slices. D(_2) coexpression delayed AT(_1) Ca(^{2+}) signalling and D(_2) antagonism blunted AT(_1) Ca(^{2+}) signalling.</td>
<td>(Martínez-Pinilla et al., 2015)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AT(_1) antagonism inhibited D(_3) expression, which was potentiated SHR and correlated with heteromer expression.</td>
<td>(Zeng et al., 2003)</td>
</tr>
<tr>
<td>AT(_1)-D(_3)</td>
<td>Colocalisation of receptors (co-IP).</td>
<td>D(_3) agonism decreased AT(_1) expression in normotensive rats but increased it in SHR.</td>
<td>(Zeng et al., 2006)</td>
</tr>
<tr>
<td>AT(_1)-D(_4)</td>
<td>Colocalisation of receptors (co-IP and confocal microscopy).</td>
<td>D(_4) agonism decreased AT(_1) expression in normotensive rats via a Ca(^{2+}) pathway but increased it in SHR. D(_4) agonism decreased AngII-induced stimulation of Na(^+)-K(^-)-ATPase activity in normotensive rats but increased it in SHR.</td>
<td>(Chen et al., 2015)</td>
</tr>
<tr>
<td>AT(_1)-D(_5)</td>
<td>Colocalisation of receptors (co-IP).</td>
<td>AngII/AT(_1)-induced decrease in D(_5) expression in both normotensive and SHR.</td>
<td>(Zeng et al., 2005d)</td>
</tr>
<tr>
<td>AT(_1)-ETB</td>
<td>Colocalisation of receptors (co-IP and confocal microscopy).</td>
<td>ETB agonism decreased AT(_1) expression in normotensive rats but had no effect in SHR. ETB agonism decreased heteromer formation in SHR but had no effect in normotensive rats. ETB agonism increased AT(_1) Ca tail phosphorylation in normotensive rats but decreased it in SHR.</td>
<td>(Zeng et al., 2005b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AngII/AT(_1) increased ETB expression in normotensive rats but had no effect in SHR. AngII/AT(_1) increased heteromer formation in normotensive rats but had no effect in SHR. AngII/AT(_1) decreased ETB Ca tail phosphorylation in both normotensive and SHR.</td>
<td>(Zeng et al., 2005a)</td>
</tr>
<tr>
<td>AT(_1)-MAS1</td>
<td>Constitutive heteromer formation (saturation BRET and co-IP).</td>
<td>Inhibition of AngII-induced IP, [Ca(^{2+})]. Increased surface expression of AT(_1). Inhibition of AngII-induced contraction in mesenteric microvessels.</td>
<td>(Kostenis et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>Ligand binding restored to mutant AT(_1) upon MAS1 coexpression.</td>
<td>Inhibition of AngII-induced extracellular acidification and IP (wild type receptors). Functional rescue to wild type levels of mutant AT(_1) upon coexpression of MAS1.</td>
<td>(Santos et al., 2007)</td>
</tr>
<tr>
<td>Heteromer</td>
<td>Physical interaction</td>
<td>Functional interaction</td>
<td>References</td>
</tr>
<tr>
<td>-----------</td>
<td>----------------------</td>
<td>------------------------</td>
<td>------------</td>
</tr>
<tr>
<td>AT1-PGF2A (prostaglandin F2α prostaglandin receptor)</td>
<td>Colocalisation of receptors (co-IP, saturation BRET, BiFC, confocal microscopy)</td>
<td>Potentiation of agonist-induced aortic contraction when pretreated with the other receptors agonist. Agonist-induced IP production was inhibited by antagonism of the other receptor. Agnili-induced DNA and protein synthesis was inhibited by PGF2α antagonism. PGF2α-induced ERK activation was potentiated by AT1 antagonism via transactivation of EGFR. Agnili-induced β-arrestin recruitment and co-internalisation. AT1 expression increased affinity and dissociation rate of PGF2α for the PGF2α receptor. Agonist or antagonist occupancy of PGF2α increased affinity of AngII for AT1.</td>
<td>(Goupil et al., 2015)</td>
</tr>
<tr>
<td>AT1-SCTR (secretin receptor)</td>
<td>Colocalisation of receptors (saturation BRET, confocal FRET). Synthetic peptides for TM regions inhibited heteromerisation.</td>
<td>AT1 inhibited SCT-mediated cAMP production, however dual receptor agonism reversed this inhibition. Agonist induced co-internalisation of receptors. Synthetic peptides for TM regions inhibited SCT-mediated cAMP production. The heteromer is involved in the mediation of thirst under hyperosmotic stress.</td>
<td>(Lee et al., 2014)</td>
</tr>
<tr>
<td>AT2-B2 (bradykinin type 2 receptor)</td>
<td>Colocalisation of receptors and constitutive heteromer formation (confocal FRET, co-IP).</td>
<td>Heteromer formation was linked to phosphorylation of JNK, PTP, IκBα, and ATF2 and dephosphorylation of p38 and p42/44 MAPK and STAT3, as well as potentiation of NO-cGMP.</td>
<td>(Abadir et al., 2006)</td>
</tr>
<tr>
<td>AT2-D1 (dopamine D1 receptor)</td>
<td>Colocalisation of receptors (co-IP and fluorescence microscopy, FRET)</td>
<td>D1 activation increased plasma membrane expression of AT2 via a cAMP- and PP2A-dependent mechanism, and inhibited AngII-mediated ERK activation in an AT2-dependent manner. The heteromer was associated with increased cAMP and cGMP production, PP2A activation, Na+K+-ATPase internalisation, and sodium transport inhibition.</td>
<td>(Gildea et al., 2012)</td>
</tr>
<tr>
<td>AT2-MAS1</td>
<td>Colocalisation of receptors (FRET).</td>
<td></td>
<td>(Villela et al., 2012)</td>
</tr>
<tr>
<td>AT2-RXFP1 (relaxin family peptide receptor 1)</td>
<td>Colocalisation of receptors and constitutive heteromer formation (saturation BRET).</td>
<td>AT3 antagonism blocks H2 relaxin-induced antifibrotic actions in cultured renal myofibroblasts (numerous signalling outputs investigated). AT2 antagonism or knockout blocks H2 relaxin-induced antifibrotic actions in mice.</td>
<td>(Chow et al., 2014)</td>
</tr>
</tbody>
</table>
1.4.6.1. AT1 receptor and the chemokine (C-C) receptor 2

The chemokine (C-C) receptor 2 (CCR2) binds a class of four chemokine proteins called monocyte chemoattractant proteins: CCL2, CCL7, CCL8, and CCL13 (MCP-1, MCP-2, MCP-4 and MCP-3, respectively). In the immune system CCR2 is expressed on monocytes, immature dendritic cells and memory T cells (Charo et al., 2006). It is also expressed in numerous other tissues, including the vasculature (endothelial and smooth muscle cells), brain, lung and kidney (Hayes et al., 1998; Jiang et al., 1998; Salcedo et al., 2001; Banisadr et al., 2005). The major role of CCR2 signalling is in the mediation of the egress of monocytes from the bone marrow to sites of inflammation (Tsou et al., 2007; Wisniewski et al., 2010). CCR2s have been implicated in a broad range of diseases such as rheumatoid arthritis, multiple sclerosis, atherosclerosis, restenosis, pulmonary fibrosis, diabetes, diabetic kidney disease, and cancer (Carter, 2013).

Previously, an interaction between AngII and CCL2 signalling has been found in the inflammation and vascular remodelling associated with hypertension (Bush et al., 2000; Dai et al., 2007), hypertensive nephropathy (Liao et al., 2008) and atherosclerosis and abdominal aortic aneurysms (Ishibashi et al., 2004; Daugherty et al., 2010). Furthermore, functional interactions between the receptors themselves have been revealed with studies showing that dual receptor antagonism significantly improves outcomes of ischemic brain damage (Tsukuda et al., 2011) and renal injury in antglomerular basement membrane nephritis (Urushihara et al., 2011). Recently, our laboratory investigated the involvement of the AT1 receptor and CCR2 in chronic kidney disease (CKD; Ayoub et al., 2015). The AT1 receptor and CCR2 are both coexpressed in several cell types in the kidney, including podocytes (Durvasula et al., 2004; Burt et al., 2007; Nam et al., 2012), in which upregulation of expression of both receptors has been linked with pathology (Hoffmann et al., 2004; Burt et al., 2007). Our study demonstrated that dual receptor blockade significantly reduced proteinuria, macrophage infiltration and podocyte loss, all of which are implicated in the pathogenesis of CKD (Ayoub et al., 2015). In addition, some of the molecular mechanisms potentially underscoring this synergistic beneficial effect were revealed for the first time. The GPCR-HIT assay demonstrated functional AT1-CCR2 heteromers in which activation of the AT1 receptor inhibited the conformational change associated
with CCR2-Gαᵢ₁-coupling. Furthermore, dual agonist treatment resulted in increased levels of β-arrestin2 recruitment that was reversed with dual antagonist treatment. Further discussion of results related to this heteromer will be discussed in Chapter 7.

1.4.6.2. AT₁, AT₂ and the MAS receptor

Within the RAS, both the AT₁ and the AT₂ receptor have been shown to functionally interact with the MAS1 receptor. The human MAS1 receptor is expressed in the brain, testis, heart, kidney, lung, liver, spleen, tongue and skeletal muscle (Bader et al., 2014) and shares only about 25% sequence identity with the human AT₁ and human AT₂ receptors. Similar to the AT₂ receptor, the MAS1 receptor exerts mainly countervailing actions to the classic AT₁ receptor AngII-mediated actions, producing anti-inflammatory, anti-fibrotic, neuroregenerative, vasodilatory and beneficial metabolic effects (Villela et al., 2015). However, like the AT₂ receptor, it sometimes appears to act in concert with the AT₁ receptor rather than opposing it, such as having pro-inflammatory effects in the kidney (Esteban et al., 2009). The MAS1 receptor also shares some similar signalling pathways with the AT₂ receptor such as inhibition of MAPK activation (Su et al., 2006), SHP-1 activation (Gava et al., 2009) and NO signalling (Pinheiro et al., 2004). Again, however, it also signals through some similar paths as the AT₁ receptor, such as Gαq-coupling (Canals et al., 2006) and activation of MAPKs (Zimpelmann et al., 2009). Ang1-7 is believed to be the endogenous agonist for the MAS1 receptor (Santos et al., 2003) and has been shown to antagonise AT₁ receptor signalling (Sampaio et al., 2007) and actions on nociception (Nemoto et al., 2014) and cell proliferation (Xue et al., 2012). Additionally, both the AT₁ receptor and the MAS1 receptor are known to colocalise in several tissues including lumbar superficial dorsal horn (Nemoto et al., 2014) and rat renal mesangial cells (Xue et al., 2012). Several studies have provided evidence that antagonism of the AT₁ receptor occurs via a MAS1 receptor-dependent mechanism (Wolf et al., 1992; Von Bohlen und Halbach et al., 2000; Castro et al., 2005; Su et al., 2006), with some evidence that this occurs as a result of receptor heteromerisation (Kostenis et al., 2005; Santos et al., 2007). As well as its antagonistic actions on AT₁ receptor signalling, the MAS1 receptor has been shown to cause upregulation of AT₁ receptor expression (Kostenis et al.,
2005). However there is evidence that this, and also some AT_1 receptor antagonism, may be a result of heterologous desensitisation by the constitutively active MAS1 receptor (Canals et al., 2006).

As previously mentioned, the MAS1 receptor has been shown to have similar actions to the AT_2 receptor. Additionally, numerous studies have reported that the actions of Ang1-7 can be blocked (Jaiswal et al., 1992; Gorelik et al., 1998; De Souza et al., 2004; Roks et al., 2004; Walters et al., 2005; Tesanovic et al., 2010; Bosnyak et al., 2012) or enhanced (Castro et al., 2005) by AT_2 receptor antagonists, while there is evidence that actions of the AT_2 agonist Compound 21 can be blocked by the MAS1 receptor antagonist A779 (Villela et al., 2015). This effect could potentially be due to a lack of specificity of AT_2 receptor and MAS1 receptor ligands, and indeed Ang1-7 does bind to the AT_2 receptor, albeit with low affinity in comparison to AngII (Ang1-7 IC_{50} = 2x10^{-7} M, AngII IC_{50} = 5x10^{-10} M) (Bosnyak et al., 2011). In this study, the affinities of A779 and the MAS1 agonist AVE0991 for the AT_2 receptor were so low that IC_{50} values could not be determined. The affinity of AT_2 receptor ligands for the MAS1 receptor is currently unknown (Villela et al., 2015). As a consequence of the uncertainty of the specificity of the AT_2 receptor and MAS1 receptor ligands, as well as the fact that in some studies AT_2 antagonists were only able to cause partial antagonism of Ang1-7 effects (De Souza et al., 2004; Walters et al., 2005; Tesanovic et al., 2010; Bosnyak et al., 2012), it has been suggested that the AT_2 receptor and the MAS1 receptor may form a heteromeric complex (Villela et al., 2015). Some preliminary evidence of a direct interaction between these two receptors has been described using FRET (Villela et al., 2012; conference abstract), however no peer reviewed paper has yet been published.

In addition to the MAS1 receptor, the AT_1 receptor and AT_2 receptor are known to form a heteromeric complex with one another. Through in vivo studies it had long been known that the AT_2 receptor was able to antagonise the functions of the AT_1 receptor (Hein et al., 1995; Ichiki et al., 1995; Nakajima et al., 1995; Masaki et al., 1998; Horiuchi et al., 1999; Matsubara et al., 2001). In 2001 AbdAlla et al. (2001b) reported that coexpression of the AT_2 receptor inhibited AT_1-induced inositol phosphate (IP) production and G_\alpha_q/11 and G_\alpha_i/o activation, independent of the activation state of the AT_2 receptor. They also demonstrated co-immunoprecipitation of the two receptors from recombinant cells, foetal fibroblasts and myometrium tissue, and showed that heteromer formation was AngII-independent. Shortly thereafter, the third intracellular
loop of the AT2 receptor was shown to mediate the inhibition of AT1-induced IP signalling (Kumar et al., 2002). The constitutive nature of the interaction between the AT1 receptor and the AT2 receptor was later supported by a study which showed that the AT2 receptor was unable to traffic to the cell surface upon coexpression with a mutant AT1 receptor, which was arrested in the endoplasmic reticulum (Zhang et al., 2009). In contrast, a more recent study reported an increase in the level of AT1-AT2 heteromers upon treatment with AngII (Ferrão et al., 2012). Furthermore, high concentrations of AngII were shown to induce dissociation of AT1-AT2 heteromers while low concentrations maintained them (Axelband et al., 2009). AT2 agonism with CGP 42112 resulted in increased levels of AT1-AT2 heteromers while also decreasing AT1 expression via a NO/cGMP-dependent pathway (Yang et al., 2012). This study also showed a CGP42114-induced inhibition of AngII-mediated Na⁺-K⁺-ATPase activity. A possible mechanism for the antagonism of the AT1 receptor by the AT2 receptor was revealed using a combination of bimolecular fluorescence complementation (BiFC) and GPCR-HIT (Porrello et al., 2011). This study showed that although the heteromer was able to recruit β-arrestin it was unable to subsequently internalise. While the antagonistic actions of the AT2 receptor on the AT1 receptor are not in doubt, the mechanisms for this are less certain. One study showed that the functional antagonism likely occurs through downstream crosstalk rather than through the formation of heteromers, and the formation of AT1 and AT2 homomers was much more prevalent than heteromers (Miura et al., 2010).

1.4.6.3. AT1, AT2 and the B2 receptor

The kallikrein-kinin system (KKS) is a regulatory system whose cardiovascular actions are generally in opposition to the hypertensive arm of the RAS. The major effector of the KKS is BK and it decreases blood pressure through vasodilation and water and salt loss (Blaes et al., 2013). These actions are primarily mediated through the B2 receptor, which shares 28% sequence identity with the human AT1 receptor and 29% sequence identity with the human AT2 receptor. The B2 receptor is expressed in many similar tissues and cell types as the AT1 and AT2 receptors, including the vasculature (endothelial and smooth muscle), heart, kidney, adrenal gland, liver, brain and the
trachea (Leeb-Lundberg et al., 2005; Costa-Neto et al., 2014). The B₂ receptor is primarily coupled to G\(\alpha_q/11\) and G\(\alpha_i\) proteins (Yano et al., 1985; Liao et al., 1993; Quitterer et al., 1999), although it has also been linked with G\(\alpha_s\) (Liebmann et al., 1996) and G\(\alpha_{12/13}\) (Gohla et al., 1999). In addition, like the AT₂ receptor, it can directly activate NOS leading to NO signalling and vasodilation (Berguer et al., 1993). Like most GPCRs, the B₂ receptor undergoes agonist-induced β-arrestin recruitment and subsequent desensitisation and internalisation (Austin et al., 1997; Lamb et al., 2001; Simaan et al., 2005).

The first evidence for a direct interaction between the AT₁ receptor and the B₂ receptor was demonstrated by Fior et al. (1993) who showed that BK receptors were involved in altering the affinity of AT₁ receptors for AngII after treatment with BK in the rat brain. This research was continued by a group who reportedly found evidence for the existence of the heteromer between the AT₁ receptor and the B₂ receptor in smooth muscle cells (AbdAlla et al., 2000). They suggested that heteromer formation in HEK293 cells was agonist-independent, and that the heteromer had an altered endocytic pathway. Most interestingly, they reported that angiotensin II had increased potency and efficacy in cells coexpressing both receptors. AbdAlla et al. followed this study with an important paper reporting that the AT₁-B₂ receptor heteromer contributed to the hypertension involved in pre-eclampsia (AbdAlla et al., 2001a). They provided evidence for platelets and omental vessels from pre-eclamptic women having heightened expression of B₂ receptors, which appeared to correlate with an increased level of AT₁-B₂ receptor heteromer formation. The authors also reported enhanced AngII-mediated signalling in these tissues, resulting in increased G\(\alpha_q\) coupling. AbdAlla et al. had thus provided considerable evidence that the AT₁-B₂ receptor heteromer contributed to the AngII hypersensitivity found in pre-eclampsia. In the subsequent years, the same group has continued their research, indicating that the heteromer contributes to the AngII-hypersensitivity seen in experimental hypertension (AbdAlla et al., 2005). They also reported that the chaperone calreticulin enhanced B₂ receptor maturation and heteromerisation with the AT₁ receptor (AbdAlla et al., 2009; AbdAlla et al., 2010). Using FRET they have confirmed the close proximity of the two receptors in HEK293 cells and have shown that they co-internalise in a GRK-dependent manner upon treatment with either AngII or BK (Quitterer et al., 2011). Finally, again
using FRET they have shown the colocalisation of the receptors *ex vivo* in mouse aorta (Quitterer *et al.*, 2014). Recently, a different group has also demonstrated that the β-arrestin-selective agonist [Sar1,Ile4,Ile8]-AngII (SII) inhibits B₂ receptor signalling in an AT₁-dependent manner (Wilson *et al.*, 2013). SII was also able to promote co-internalisation of the AT₂ receptor and the B₂ receptor.

Despite these numerous studies supporting the existence of the AT₁-B₂ receptor heteromer, a collaboration of several groups was unable to find any evidence for the existence of the heteromer (Hansen *et al.*, 2009). In three different cell lines they investigated possible functional modulation of IP accumulation, ERK activation and β-arrestin2 recruitment. To investigate any direct physical interaction between the receptors they employed competition BRET assays. However, they were not only unable to detect the AT₁-B₂ receptor heteromer, they were also unable to find any evidence of the altered pharmacology reported by AbdAlla *et al*.

This heteromer has also been investigated in our laboratory using the GPCR-HIT assay. See *et al.* (2011) looked for β-arrestin2-Venus recruitment in HEK293 cells coexpressing AT₁ receptor and B₂-Rluc8. The data gave support to the findings of Hansen *et al.*, as there was no detection of β-arrestin2-Venus recruitment to B₂-Rluc8 after treatment with AngII. However it is still possible that the heteromer was present in the system but the B₂-Rluc8-AT₁-β-arrestin2-Venus complex was undetectable due to unfavourable donor-acceptor distance or orientation. Additionally it may be that the heteromer has gained novel pharmacology and does not interact with β-arrestin2 at a detectable level. Thus the existence of the AT₁-B₂ receptor heteromer remains unresolved and requires further study.

Prior to evidence of an interaction between the AT₂ receptor and the B₂ receptor was revealed, it was unexpectedly found that ARBs resulted in a greater level of cGMP production than ACE inhibitors (Gohlke *et al.*, 1993; Gohlke *et al.*, 1996). ACE not only generates AngII from AngI but also breaks down BK. Thus ACE inhibitors, but not ARBs, were expected to cause increased BK levels and B₂ receptor activation and signalling. The ARB-induced stimulation of cGMP could be blocked by treatment with AT₂ receptor or B₂ receptor antagonists (Gohlke *et al.*, 1997). Later it was shown that overexpression of the AT₂ receptor in mice promoted BK generation through activation of kininogenases and subsequent paracrine B₂ receptor activation and NO/cGMP signalling (Tsutsumi *et al.*, 1999), and these results were confirmed in later models.
(Siragy et al., 1999a; Kurisu et al., 2003; Zhu et al., 2012). As well, AT$_2$ receptor knock out mice have been shown to have low basal BK levels (Siragy et al., 1999b). In mice overexpressing AT$_2$ receptor the vasoconstrictive effects of AngII are diminished but can be restored upon treatment with a B$_2$ receptor antagonist or NO scavenger (Tsutsumi et al., 1999). Thus the NO signalling mediated by the AT$_2$ receptor requires, in part, activation of the B$_2$ receptor.

While one pathway for this interaction has been described, involving the sequential activation of SHP-1, prolylcarboxypeptidase/kallikrein/BK release/B$_2$ receptor activation (Zhu et al., 2012), there is also evidence that the AT$_2$ receptor and the B$_2$ receptor may form a heteromer. Confocal FRET showed that the distance between the two receptors in PC12W cell membranes was 50±5 Å (Abadir et al., 2006). This heteromerisation was linked to phosphorylation of JNK, phosphotyrosine phosphatase, activating transcription factor 2, inhibitory protein κBα and dephosphorylation of p38 and p42/44 MAPK and STAT3.

1.5. Summary

The AT$_1$ receptor is a fairly well characterised receptor whose signalling and regulatory mechanisms are characteristic of standard GPCRs. It regulates most of the classic AngII-mediated physiological effects and is associated with many of the deleterious pathological RAS outcomes. In contrast, the physiological functions of the AT$_2$ receptor are often believed to be in opposition to the AT$_1$ receptor, and are generally associated with the protective arm of the RAS. Additionally, the signalling and regulatory mechanisms of the AT$_2$ receptor are still not well established.

The complexity of the RAS is further enhanced through the formation of receptor heteromers. Both the AT$_1$ and AT$_2$ receptor are believed to form heteromers with several GPCRs, and the molecular mechanisms and physiology of these interactions is beginning to be elucidated. This thesis aims to investigate several aspects of angiotensin II receptor heteromer pharmacology.
1.5.1. Hypotheses

This study will firstly investigate the use of BRET as a technique to measure receptor-ligand binding (detailing results published in Stoddart et al., 2015). This assay will then be adapted using the GPCR-HIT configuration to determine if the proximity between two receptors can be monitored through ligand binding.

**Hypothesis 1:** The development of a GPCR-HIT BRET ligand binding assay will reveal a novel method for detecting the proximity of receptor heteromers in living cells.

The AT\textsubscript{1}-AT\textsubscript{2} heteromer has been shown to result in an inhibition of AT\textsubscript{1} receptor signalling. The heteromer is able to recruit β-arrestin but does not subsequently internalise. This study will investigate the proximity of the AT\textsubscript{1}-AT\textsubscript{2} heteromer in living cells using the newly developed GPCR-HIT BRET ligand binding assay. Additionally, the interactions between the two receptors will be further investigated using BRET/BiFC approaches, and GPCR-HIT trafficking assays.

**Hypothesis 2:** The close proximity of AT\textsubscript{1} and AT\textsubscript{2} receptors in living cells will be confirmed with the GPCR-HIT BRET ligand binding assay. As a consequence of its inability to internalise, there will be no endosomal sequestration of the AT\textsubscript{1}-AT\textsubscript{2} heteromer.

Despite considerable evidence showing functional crosstalk between the AT\textsubscript{2} and the B\textsubscript{2} receptor, the only evidence for heteromerisation between the two receptors is from their close proximity in cell membranes using confocal FRET. This study will investigate the existence of the AT\textsubscript{2}-B\textsubscript{2} heteromer using various GPCR-HIT and BRET/BiFC assays.

**Hypothesis 3:** The AT\textsubscript{2} and the B\textsubscript{2} receptor form a functional heteromer with unique β-arrestin recruitment and trafficking profiles.

Although previous studies suggested functional crosstalk between the AT\textsubscript{1} receptor and CCR2, the first evidence for heteromerisation of the two receptors was not revealed until the recent publication by my group and our collaborators (Ayoub et al., 2015). This study details my contributions to that paper as well as investigating the trafficking and internalisation properties of the heteromer.
Hypothesis 4: The AT\textsubscript{1} receptor and CCR2 form a functional heteromer with a unique pharmacological profile.

Heteromerisation with the MAS1 receptor has been shown to occur constitutively and result in an inhibition of AT\textsubscript{1} receptor signalling. The only evidence for heteromerisation between the AT\textsubscript{2} and the MAS1 receptor has come from a conference abstract describing their proximity using FRET.

Hypothesis 5: The MAS1 receptor forms functional heteromers with the AT\textsubscript{1} and the AT\textsubscript{2} receptors, resulting in unique pharmacological profiles. Note: due to time constraints, this hypothesis was not investigated beyond the development stages (see Section 2.3.4).

Although several studies have reported the existence of the AT\textsubscript{1}-B\textsubscript{2} heteromer, its existence remains highly contentious. This study will investigate the existence of the AT\textsubscript{1}-B\textsubscript{2} heteromer using various GPCR-HIT and BRET/BiFC assays.

Hypothesis 6: The AT\textsubscript{1} and the B\textsubscript{2} receptor form a functional heteromer with unique β-arrestin recruitment and trafficking profiles.

Hypothesis 7: The controversy surrounding the existence of the AT\textsubscript{1}-B\textsubscript{2} heteromer is due to complex interactions and competition with other receptors (such as AT\textsubscript{2} and CCR2) and heteromers.
1.5.2. Organisation of thesis

The pharmacology to be investigated will be separated into the different chapters, with the first chapter outlining the general materials and methods used throughout:

- Chapter 2: General Materials and Methods
- Chapter 3: BRET Ligand Binding – Development and Optimisation
- Chapter 4: Angiotensin II Receptor and Heteromer Ligand Binding
- Chapter 5: β-arrestin Recruitment to AT₁, AT₂ and B₂ Receptors and Heteromers
- Chapter 6: Internalisation and Trafficking of AT₁, AT₂ and B₂ Receptors and Heteromers
- Chapter 7: AT₁, CCR2 and B₂ Receptors and Heteromers
- Chapter 8: General Discussion
Chapter 2. General Materials and Methods

2.1. Introduction

This chapter outlines the general materials and techniques used throughout this study. Methods that are specific to individual chapters will be described in the relevant sections.

2.2. Recombinant DNA Techniques

2.2.1. Plasmid construction

Apart from exceptions in Chapter 3 and Chapter 4, all cDNA constructs used in this thesis were assembled in pcDNA3 or pcDNA3.1+ vectors. The structure and function of each vector is similar, however they differ in their multiple cloning sites (see Figure 2.1). These restriction enzyme sites were used to sub-clone cDNA in frame into either vector. Table 2.1 lists all of the cDNAs and constructs used throughout this thesis, and they are described in more detail below.
Figure 2.1. Diagram of pcDNA₃ and pcDNA₃.₁+ vectors showing the restriction enzyme sites in the multiple cloning site. Adapted from technical notes (Invitrogen, 2015).

Table 2.1. cDNAs and constructs used throughout this thesis. Constructs are listed in the order in which they appear within the thesis.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Species</th>
<th>Genbank Accession No.</th>
<th>Constructs</th>
<th>Chapter</th>
<th>Source</th>
<th>Validation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiotensin II receptor type 1</td>
<td>Human</td>
<td>AY221090.1</td>
<td>Nluc-AT₁, Rluc8-AT₁</td>
<td>3, 4</td>
<td>Promega</td>
<td>Figure 2.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Made by R. Abhayawardana</td>
<td></td>
</tr>
<tr>
<td>Angiotensin II receptor type 2</td>
<td>Human</td>
<td>U10273.1</td>
<td>Nluc-AT₂, SP-Flag-AT₂</td>
<td>4</td>
<td>Made from AT₂ from Missouri S&amp;T</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Made from AT₂ from Missouri S&amp;T</td>
<td>-</td>
</tr>
<tr>
<td>Vasopressin receptor type 2</td>
<td>Human</td>
<td>NM_000054.4</td>
<td>Nluc-V₂, Flag-V₂</td>
<td>4</td>
<td>Promega</td>
<td>External</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>B. Feldman</td>
<td></td>
</tr>
<tr>
<td>Angiotensin II receptor type 1</td>
<td>Rat</td>
<td>NM_030985</td>
<td>AT₁₂-Rluc8, HA-AT₁α, AT₁₂-V1, AT₁₂-V2, AT₁₂-Venus</td>
<td>5, 6, 7</td>
<td>Previously made from AT₁₂-Rluc from W. Thomas</td>
<td>Prior to thesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>W. Thomas</td>
<td>External</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>W. Thomas</td>
<td>External</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>W. Thomas</td>
<td>External</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>W. Thomas</td>
<td>External</td>
</tr>
<tr>
<td>Receptor Family</td>
<td>Species</td>
<td>Accession Number</td>
<td>Fusion Protein</td>
<td>Source/Notes</td>
<td>Additional Notes</td>
<td></td>
</tr>
<tr>
<td>---------------------------------</td>
<td>-----------</td>
<td>------------------</td>
<td>---------------</td>
<td>--------------------------------------------------</td>
<td>-----------------</td>
<td></td>
</tr>
<tr>
<td>Angiotensin II receptor type 2</td>
<td>Rat</td>
<td>NM_012494</td>
<td>AT₂-Rluc8</td>
<td>5, 6</td>
<td>Previously made from AT₁a-Rluc from W Thomas</td>
<td>Prior to thesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HA-AT₂</td>
<td>5, 6</td>
<td>W. Thomas</td>
<td>External</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AT₂-V1</td>
<td>5</td>
<td>W. Thomas</td>
<td>External</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AT₂-V2</td>
<td>5</td>
<td>W. Thomas</td>
<td>External</td>
</tr>
<tr>
<td>Bradykinin receptor type 2</td>
<td>Human</td>
<td>AY275465</td>
<td>B₂-Rluc8</td>
<td>5, 6, 7</td>
<td>Previously made from B₂ from Missouri S&amp;T</td>
<td>Prior to thesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HA-B₂</td>
<td>5, 6, 7</td>
<td>Missouri S&amp;T</td>
<td>Prior to thesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B₂-V1</td>
<td>5</td>
<td>Made from B₂- Rluc8 with R. Seeber</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B₂-V2</td>
<td>5</td>
<td>Made from B₂- Rluc8 with R. Seeber</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B₂-Venus</td>
<td>6</td>
<td>Previously made from B₂- Rluc8</td>
<td>-</td>
</tr>
<tr>
<td>β-arrestin2</td>
<td>Human</td>
<td>NM_004313</td>
<td>β-arrestin2-Venus</td>
<td>5, 7</td>
<td>Previously made from pCS2-Venus from A. Miyawaki</td>
<td>Prior to thesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>β-arrestin2-Rluc8</td>
<td>5</td>
<td>Previously made using Rluc8 from S. Gambhir</td>
<td>Prior to thesis</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>NM_012911</td>
<td>β-arrestin2-V2</td>
<td>5</td>
<td>W. Thomas</td>
<td>External</td>
</tr>
<tr>
<td>Kras GTPase</td>
<td>Human</td>
<td>NM_004985.4</td>
<td>Venus-Kras (25 amino acid C terminal fragment of Kras)</td>
<td>6, 7</td>
<td>N. Lambert</td>
<td>External</td>
</tr>
<tr>
<td>Rab5a GTPase</td>
<td>Human</td>
<td>NM_004162.4</td>
<td>Venus-Rab5</td>
<td>6, 7</td>
<td>N. Lambert</td>
<td>External</td>
</tr>
<tr>
<td>Rab7 GTPase</td>
<td>Human</td>
<td>AF498942.1</td>
<td>Venus-Rab7</td>
<td>6, 7</td>
<td>N. Lambert</td>
<td>External</td>
</tr>
<tr>
<td>Rab11 GTPase</td>
<td>Human</td>
<td>X56740.1</td>
<td>Venus-Rab11</td>
<td>6, 7</td>
<td>N. Lambert</td>
<td>External</td>
</tr>
<tr>
<td>Chemokine (C-C motif) receptor 2</td>
<td>Human</td>
<td>NM_001123396.1</td>
<td>wt CCR2</td>
<td>7</td>
<td>Made from CCR2-Rluc from A. Chakera</td>
<td>Prior to thesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CCR2-Rluc8</td>
<td>7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.2.1.1. Receptor constructs used throughout this thesis

Throughout the majority of this thesis (Chapter 5, Chapter 6 and Chapter 7), rat AngII receptor constructs were used (rat AT\textsubscript{1a} (rAT\textsubscript{1a}) and rat AT\textsubscript{2} (rAT\textsubscript{2})). Rat AngII receptors are often predominantly used in research, as they express better than the human constructs (Figure 2.2). The rat AT\textsubscript{1} receptor has two isoforms (AT\textsubscript{1a} and AT\textsubscript{1b}) that have 95% amino acid sequence identity and display similar ligand binding and activation properties, but different tissue distribution, chromosomal localisation, genomic structure and transcriptional regulation (de Gasparo et al., 2000).

Most of the rat AT\textsubscript{1a} and AT\textsubscript{2} receptor constructs used in this thesis were kindly provided by Walter Thomas (University of Queensland): HA-rAT\textsubscript{1a}, HA-rAT\textsubscript{2}, rAT\textsubscript{1a}-Venus and the split Venus constructs rAT\textsubscript{1a}-V1, rAT\textsubscript{1a}-V2, rAT\textsubscript{2}-V1 and rAT\textsubscript{2}-V2. Although not used in this thesis, rAT\textsubscript{1a}-Rluc and rAT\textsubscript{2}-Rluc were also provided by Walter Thomas (University of Queensland), and were used to generate rAT\textsubscript{1a}-Rluc\textsubscript{8} and rAT\textsubscript{2}-Rluc\textsubscript{8} by replacing the Rluc coding region with Rluc\textsubscript{8} from pcDNA\textsubscript{3.1}.Rluc\textsubscript{8} kindly provided by Andreas Loening and Sanjiv Gambhir (Stanford University, USA; Loening et al., 2006). All these constructs were previously generated and validated before commencing work on this thesis. In all instances where wild type receptors were to be used, HA-tagged receptor constructs were used instead, however when labelling graphs the HA will not be included in figure legends for simplicity. Additionally, the HA-rAT\textsubscript{1a} and the HA-rAT\textsubscript{2} constructs have an Asn4*Asp mutation which does not alter function (Yamano et al., 1992) but inhibits glycosylation of this residue, which could potentially interfere with access to the HA tag.
Chapter 2. General Materials and Methods

Figure 2.2. Cell surface expression of human and rat AT1 and AT2 constructs. HEK293FT cells were transfected with HA-tagged AT1 or AT2 receptors (100 ng) and cell surface expression was determined using Enzyme-linked immunosorbent assay (method as described in Section 2.6). Cells were fixed, blocked and incubated with the primary and then secondary antibodies, and chemiluminescence was measured. Data from one experiment are presented as mean ± SEM of quadruplicates.

In Chapter 3 and Chapter 4, human AT1 and human AT2 receptors were used. The human receptors could be used in these chapters as they were tagged with N terminal signal peptides that enhanced their cell surface expression. The human AT1 receptor constructs used were Nluc-AT1 and (Signal Peptide-Flag-)Rluc8-hAT1 (Rluc8-AT1. Nluc-AT1 was kindly provided by Promega and cloned by inserting human AT1 receptor cDNA into pF-sNnK vector (Promega), which encodes a fusion of the secretory signal peptide sequence of interleukin 6 (IL6) on the N terminus of Nluc, as described by Stoddart et al. (2015). The G$_q$ coupling function of the Nluc-AT1 construct was validated using an inositol-1-phosphate (IP$_1$) accumulation assay (Figure 2.3; method as described in Section 2.5). The Rluc8-AT1 construct was generated by Rekhati Abhayawardana (Molecular Endocrinology and Pharmacology, Harry Perkins Institute of Medical Research). Two human AT2 receptor constructs were used: signal peptide (SP)-Flag-hAT2 and Nluc-AT2. SP-Flag-hAT2 was generated by firstly inserting hAT2 (ending with a stop codon) in frame into previously generated pcDNA3.SP-Flag-hAT2-Rluc8 (hAT2 was from Missouri S&T cDNA Resource Center). This resulted in insertion of a stop codon after the receptor. The resulting construct was pcDNA3.SP-
Flag-hAT2-STOP-Rluc8, and the SP-Flag-hAT2-STOP portion was then inserted into empty pcDNA3 vector to remove the Rluc8 coding region. Nluc-AT2 was generated from the above pcDNA3.SP-Flag-hAT2-STOP construct by inserting the hAT2-STOP portion into the pF-sNnK vector encoding a fusion of secretory signal peptide sequence of IL6 on the N terminus of Nluc.

![Image of graph](image.png)

**Figure 2.3. Validation of Nluc-AT1 plasmid.** HEK293FT cells were transfected with rAT1a or Nluc-hAT1 (100 ng). IP1 production was measured on the EnVision after 30 min incubation at 37°C and 5% CO2 with increasing doses of AngII. Data are shown as a percentage of AngII-induced IP1 production in cells transfected with rAT1a. Data from one experiment are presented as mean ± SEM of duplicates.

The other receptor used throughout much of this thesis was the B2 receptor. Human B2 receptor cDNA and HA-B2 were obtained from the Missouri S&T cDNA Resource Center. B2-Rluc was previously produced by PCR amplification of B2 receptor cDNA to remove the stop codon and ligation into pcDNA3 containing Rluc. The Rluc coding region was replaced with Rluc8 from pcDNA3.1.Rluc8 kindly provided by Andreas Loening and Sanjiv Gambhir (Stanford University, USA; Loening et al., 2006), as described previously for other constructs (Kocan et al., 2008). These two constructs were previously generated and validated in the laboratory before commencing work on this thesis. In all instances, HA-B2 was used rather than wild type
B₂, however when labelling graphs the HA will not be included in figure legends for simplicity. B₂-Venus was previously generated by subcloning the B₂ receptor from B₂-Rluc8 into pcDNA₃.Venus. The split Venus constructs B₂-V1 and B₂-V2 were generated with assistance from Ruth Seeber (Molecular Endocrinology and Pharmacology, Harry Perkins Institute of Medical Research) by replacing the Rluc8 coding region from B₂-Rluc8 with the V1 or the V2 coding region.

In Chapter 4, the vasopressin receptor constructs Nluc-V₂ and Flag-V₂ were used. Nluc-V₂ was kindly provided by Promega. As with Nluc-AT₁, the V₂ cDNA was cloned into the pF-sNnK vector encoding the IL6 secretory signal peptide on the N terminus of Nluc. Flag-V₂ was kindly provided by Brian Feldman (Stanford University, USA).

In Chapter 7, CCR2 was used, both wild type and Rluc8 tagged. Wild type CCR2 and CCR2-Rluc were kindly provided by Aron Chakera (Harry Perkins Institute of Medical Research, Australia). CCR2-Rluc8 was generated by replacing the Rluc coding region of CCR2-Rluc with Rluc8 from pcDNA₃.₁.Rluc8 kindly provided by Andreas Loening and Sanjiv Gambhir (Stanford University, USA; Loening et al., 2006), as described previously for other constructs (Kocan et al., 2008).

2.2.1.1. GPCR-HIT interacting protein constructs

Several different interacting proteins were used for GPCR-HIT studies within this thesis. The human β-arrestin2-Venus cDNA construct was prepared previously from pCS2-Venus kindly provided by Atsushi Miyawaki (RIKEN Brain Science Institute, Japan; Kocan et al., 2008). The β-arrestin2-Rluc8 cDNA construct was previously generated by subcloning Rluc8 cDNA (kindly provided by S. Gambhir, Stanford University, USA) into pcDNA₃ containing β-arrestin2 with the stop codon removed (Porrello et al., 2011). The split Venus construct, rat β-arrestin2-V₂ was kindly provided by Walter Thomas (University of Queensland). The four other interacting proteins were: Venus-Kras, Venus-Rab5, Venus-Rab7 and Venus-Rab11, and were kindly provided by Nevin Lambert (Georgia Health Sciences University, USA).
2.2.2. Plasmid cDNA preparation

Concentrated preparations of purified cDNA were obtained by bacterial replication of plasmids using either electrocompetent or heat shock cells. TOP10 Electrocomp™ *E. coli* cells (Invitrogen) were transformed with plasmid cDNA by electroporation at a potential difference of 1.8 V. Subcloning Efficiency™ DH5α™ *E. coli* cells (Invitrogen) were transformed by heating cells to 42°C for 20 sec. In both cases, cells were then incubated in SOC medium (Sigma Aldrich) at 37°C with shaking for 1 hour, and then spread on to Luria-Bertani (LB) -agar plates containing the appropriate antibiotic (ampicillin for pcDNA3, pcDNA3.1+ and pZIP-NeoSV (Section 2.3.4); kanamycin for pF-sNnK). Following overnight incubation at 37°C, single colonies were added to 5 mL LB broth (Difco™ LB Broth, BD Biosciences) containing the appropriate antibiotic and incubated overnight at 37°C with shaking.

During initial sub-cloning and verification stages of cDNA synthesis, this culture was used to generate small preparations of plasmid cDNA using a QIAprep Spin Miniprep Kit (Qiagen). Following cDNA sequence verification or when transforming from previously verified preparations, this culture was added to 100 mL of LB broth containing appropriate antibiotic (0.1 mg/ml) and grown overnight at 37°C with shaking. From this culture, large preparations of cDNA were purified using a Qiagen Plasmid Plus Maxiprep Kit and eluted DNA was stored at -20°C. The resulting plasmid cDNA obtained was quantified using a NanoDrop 1000™ Spectrophotometer (Thermo Fisher Scientific) and the 260/280 ratios consistently remained between 1.8 and 2.0.

2.2.3. Plasmid cDNA preparation

Following plasmid purification and throughout the experimental period cDNA sequences were verified to ensure the nucleotide sequences were, and remained, in-frame and correct in comparison to published sequences. Purified DNA samples containing appropriate primers were sent to the Australian Genome Research Facility (Perth) according to the following volumes and concentrations:
1) 600 ng template cDNA
2) 1 µL primer (9.6 pmol, 9.6 pmol/µL)
3) ddH$_2$O to a total volume of 12 µL

DNA sequences were labelled and sequenced using Big Dye Terminator reagents and an AB 3730xl DNA analyser (Applied Biosystems). Sequence and trace files were analysed using Serial Cloner 2.6 (Serial Basics, Franck Perez) and 4Peaks (Nucleobytes Inc.).

### 2.3. Tissue Culture Procedures

#### 2.3.1. Cell lines and reagents

With one exception (Figure 2.5), all experiments using transient transfections were conducted in human embryonic kidney 293 cells (HEK293) that stably express the SV40 large T antigen (HEK293FT; Invitrogen, Australia) resulting in increased transfection efficiency. HEK293 cells were used for transient transfections in Figure 2.5 and for generation of MAS1 receptor stable cells. HEK293 cells stably expressing Nluc tagged β$_2$AR (Nluc-β$_2$AR) were from Promega. All cells were maintained at 37°C, 5% CO$_2$ in complete Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen, Australia) containing 10% foetal calf serum (FCS; Gibco, Australia), 0.3 mg/mL L-glutamine, 100 IU/mL penicillin and 100 µg/mL of streptomycin (Gibco). For continual selection of the SV40 large T antigen in HEK293FT cells, or the MAS1 receptor or Nluc-β$_2$AR in HEK293 cells, Geneticin (0.4 mg/mL; Gibco) was added to the media.
2.3.2. Cell storage and recovery

All HEK293 and HEK293FT cells were briefly stored at -80°C on initiation of the freezing process before storing in the gas phase of liquid nitrogen at -180°C. To maintain low passage cell populations, cells were frozen down upon recovery from liquid nitrogen. To freeze, cells were centrifuged at 190 x g for 5 min at room temperature. After aspirating the supernatant the cell pellet was resuspended to $1 \times 10^5$ cells/mL in a freezing mixture consisting of 10% dimethyl sulfoxide (Sigma Aldrich) in 10% FCS complete DMEM. 1 mL of this mixture was aliquoted into a CryoTube™ vial (Nunc) and placed into a freezing container (Cryo 1°C Freezing Container, Nalgene) in a -80°C freezer to reduce the temperature by approximately 1°C/min. Cells were recovered by immediately defrosting samples in a 37°C water bath, centrifuging at 190 x g for 5 min and then resuspending the cell pellet in 5 mL of 10% FCS complete DMEM. Cells were then incubated at 37°C, and Geneticin was added after approximately 1 day when the cells had settled and adhered to the flask. Geneticin was not added to HEK293 cells.

2.3.3. Transient transfection

Cells were transfected directly into poly-L-lysine-coated 96-well plates with plasmids containing cDNA for the various proteins using GeneJuice (Novagen) or FuGENE (Promega). Briefly, plasmid cDNA was incubated for 5-15 min at room temperature with a mix of 0.5 μL of transfection reagent and 50 μL of serum-free DMEM (pre-incubated at room temperature for 5 minutes). Cells ($10^5$ in 150 μL/well) in 10% FCS complete DMEM were then incubated with the final DNA-transfection reagent mix (50 μL/well). Experiments were carried out 48 hours post transfection.
2.3.4. Generation of MAS1 receptor stable cell line

2.3.4.1. Subcloning and validation of MAS1 receptor construct

Human MAS1 receptor cDNA in pZIP-NeoSV vector was kindly provided by Walter Thomas (University of Queensland). After generation of a MAS1 receptor cDNA mini prep using DH5α™ E. coli cells, the MAS1 receptor was excised from the pZIP-NeoSV vector and inserted into pcDNA3 using the BamHI restriction enzyme site. To prevent self-ligation of pcDNA3, the 5’ and 3’ ends were dephosphorylated using calf intestinal alkaline phosphatase (New England Biolabs) according to manufacturer’s instructions.

To validate the functionality of the pcDNA3.MAS1 construct, IP₁ assays were performed (method as described in Section 2.5). The MAS1 receptor constitutively couples to G₉ proteins (Canals et al., 2006; Zhang et al., 2012) and this was observed in cells transiently transfected with the MAS1 receptor (Figure 2.4). Furthermore, IP₁ production was not altered by Ang1-7 or AngII treatment, and was still evident upon coexpression of either the AT₁ or AT₂ receptor.
Figure 2.4. Testing MAS1 receptor-induced IP$_1$ production. HEK293FT cells were transiently transfected with 100 ng of each receptor or pcDNA3 (to make a total of 200 ng of plasmid). IP$_1$ production was measured (method as described in Section 2.5) after 30 min incubation at 37°C and 5% CO$_2$ with AngII, Ang1-7 or both. Data are shown as a percentage of AngII-induced IP$_1$ production in cells transfected with AT$_{1a}$ alone. * indicates significant difference (p < 0.05). ** indicates significant difference (p < 0.01). ns indicates no significant difference (p > 0.05). Data analysed using two-way ANOVA followed by Tukey’s test for multiple comparisons. Not all significant differences are indicated on graph. AT$_{1a}$ data were excluded from analysis. Data are presented as mean ± SEM of three independent experiments performed in triplicate.

2.3.4.2. Generation of MAS1 receptor stable cell line

The MAS1 receptor stable cell line was generated using HEK293 cells. Firstly, the pcDNA3.MAS1 cDNA was linearised using a single-cutting restriction enzyme (PvuI). The linearised cDNA was added to a mix of 4 µL of FuGENE and 100 µL of serum-free DMEM (pre-incubated at room temperature for 5 minutes) and incubated for 15 min. This mix was added to HEK293 cells that had been seeded (5 X 10$^5$ cells seeded) 1 day previously in a 6-well plate. Cells were then incubated for 48 hours at 37°C, 5% CO$_2$. Media was subsequently removed and the cells were washed with phosphate buffered saline (PBS) and trypsinsised (200 µL) for 1 min before adding 1 mL of 10% FCS
complete DMEM. Cells were centrifuged at 190 x g for 5 min and the pellet was resuspended in 10% FCS complete DMEM. Cells were split into two 10 cm dishes (10 mL/dish). 24 hours later, the media was aspirated and replaced by 10% FCS complete DMEM containing Geneticin. Cells were maintained at 37°C, 5% CO2, replacing the media every 48 hours.

After two weeks, cells were washed with PBS, trypsinsised, centrifuged and resuspended in 10% FCS complete DMEM. They were then plated onto 10 cm dishes at a range of cell concentrations to generate sparse cell densities. Cells were again maintained at 37°C, 5% CO2 in 10% FCS complete DMEM containing Geneticin, replacing media every 48 hours, over a 2-3 week period. Growth of potential colonies was observed closely. Colonies were chosen based on their origin from a single cell, and were subsequently picked using 3 mm cloning disks (Sigma Aldrich), and placed in 24-well plates containing 10% FCS complete DMEM and Geneticin. Selected colonies were grown up and subsequently stored and recovered as previously described (Section 2.3.2.)

2.3.4.3. Testing clonal cells for MAS1 receptor expression.

To test for MAS1 receptor expression in clonal cells, IP1 assays were performed (methods as described in Section 2.5). Figure 2.5 shows that of the six clonal cell lines generated, only one constitutively produced IP1 to a similar extent as cells transiently expressing the MAS1 receptor. These cells were sent to collaborators for further testing and experiments, however due to time restraints, they were not used for further research in this thesis.
Figure 2.5. Testing for constitutive IP$_1$ production in cells transiently or stably transfected with MAS1 receptor. HEK293 cells were transiently transfected with pcDNA3 or MAS1 receptor (100 ng), or MAS1 stably transfected clonal cells were transiently transfected with pcDNA3 (100 ng). Cells were assayed for the level of ligand-independent IP$_1$ production. (A, B, C) are individual experiments performed on separate days. Data are shown as a percentage of IP$_1$ production in cells transiently transfected with MAS1 receptor, mean ± SEM of duplicates.
2.4. BRET Assays

2.4.1. NanoBRET assays

NanoBRET assays were used in Chapter 3 and Chapter 4. They were conducted 48 hours post transfection (see Section 2.3.3) by firstly carefully removing the media from cells. Following this, the cells were incubated with ligands at 37°C, 5% CO₂. Immediately prior to measuring BRET, furimazine (Promega) was added to the cells. Ligands and furimazine were diluted in Hanks' Balanced Salt Solution (HBSS) and the ligands were added to a correct final concentration prior to addition of furimazine. Furimazine was added to a final concentration of 10 µM, and NanoBRET was measured at 37°C using either the CLARIOstar or PHERAstar FS (PHERAstar) plate reader (BMG Labtech). Two filters were used to measure short and long wavelength luminescence corresponding to donor and acceptor emission wavelengths, respectively. Filter wavelengths are described in the relevant sections. Filtered light emissions were simultaneously measured for 1 s per filter before moving to the next well. Work with furimazine and fluorescent ligands was done in the dark or in red or green light as appropriate.

NanoBRET data are presented as the “raw BRET ratio” (the ratio of the long wavelength emission over the short wavelength emission) or the “relative BRET ratio” (normalised as described in figure legends).

2.4.2. eBRET assays

eBRET assays were used in Chapter 5, Chapter 6 and Chapter 7. They were conducted 48 hours post transfection (see Section 2.3.3) by firstly carefully removing the media from cells. Cells were then incubated at 37°C, 5% CO₂ for 2 hours with 30 µM EnduRen (Promega) to ensure substrate equilibrium was reached. eBRET measurements were taken at 37°C using the VICTOR Light plate reader with Wallac 1420 software (PerkinElmer). Two filters were used to measure short (400–475 nm) and long (520–540 nm) wavelength luminescence corresponding to donor and acceptor
emission wavelengths, respectively. Filtered light emissions were sequentially measured for between 0.5-2 s per filter before moving to the next well. Subsequent re-measurements were taken in the same order either immediately or after a pre-set time interval. Luminescence values were measured for 5-20 min before adding ligand to establish a baseline BRET signal. Ligand was added to the correct final concentration required after establishment of a baseline BRET level. EnduRen and ligands were diluted in either HBSS or serum-free DMEM containing 25 mM HEPES buffer, using the same diluent for both EnduRen and ligand in each experiment. Work with EnduRen and Venus-tagged proteins was done in the dark or in green light.

All acquired eBRET data were analysed comparing a ligand-treated and vehicle-treated cell population that were identically transfected. Using this method, the vehicle-treated cell sample represents the background, eliminating the requirement for measuring a donor-only control sample (Pfleger et al., 2006a; Kocan et al., 2008). The equation used to calculate the BRET signal labelled as “ligand-induced BRET” in all relevant assays is as follows:

\[
\text{Ligand-induced BRET} = \frac{\text{long wavelength (ligand-treated)}}{\text{short wavelength (ligand-treated)}} - \frac{\text{long wavelength (vehicle-treated)}}{\text{short wavelength (vehicle-treated)}}
\]

2.5. Inositol Phosphate Cell Signalling Assays

Inositol phosphates (IPs) are downstream species formed by activation of the Gi protein (see Figure 2.6). IP was measured using the IP-One HTRF® assay (CisBio Bioassays). IP assays were carried out 48 hours post transfection. After removing media, 50 µL of agonist diluted in 1x stimulation buffer (supplied in kit) was added to cells and incubated at 37°C, 5% CO₂ for 30 min. Assays which included antagonist stimulation were pretreated for 30 min with antagonist (diluted in serum-free DMEM containing 25 mM HEPES buffer), which was removed prior to treatment with agonist. Following agonist stimulation, 12.5 µL of IP1-d2 (supplied) was added to each well, followed by addition of 12.5 µL of anti-IP1 labelled with terbium cryptate (supplied). Both reagents were diluted according to manufacturer’s instructions in lysis buffer (supplied). Cells were incubated at room temperature in the dark for 1 hour and then
fluorescence was measured using the EnVision Multilabel Plate Reader (PerkinElmer) (excitation 340 nm, emission 620 nm and 665 nm). Data were analysed using the following calculation:

\[
\frac{\text{recorded signal (665 nm)} \times 10000}{\text{recorded signal (620 nm)}}
\]

As this assay measures a competitive interaction, the maximal and minimal values were subsequently normalised by inverting the curve (i.e. maximal value = 0%, minimal value = 100%).

Figure 2.6. Diagram of IP production and its detection using the CisBio IP-One HTRF® assay. The IP-One HTRF® assay utilises an anti-IP₁ monoclonal antibody labelled with a long-life lanthanide fluorophore Lumi4-Tb™ terbium cryptate (620 nm emission). Endogenous IP₁ competes with d2 fluorophore labelled IP₁ (665 nm emission) for binding to the anti-IP antibody. Upon binding of the anti-IP₁ monoclonal antibody to the d2 fluorophore labelled IP₁, UV stimulation (340 nm) excites the cryptate fluorophore and time-resolved FRET is used to resolve a signal indicating the detection of exogenous IP₁. However, upon binding to cell-generated IP₁, FRET is not permissible, resulting in a lack of signal attributable to endogenous IP₁. LiCl inhibits the degradation of IP₁ to myo-inositol, resulting in a measurable accumulation of IP₁ over time.
2.6. **Enzyme-Linked Immunosorbent Assays**

Enzyme-linked immunosorbent assays (ELISAs) were used to determine cell surface expression of AT₁ and AT₂ receptors. 48 hours post transfection media was removed and cells were fixed with 4% paraformaldehyde/PBS for 10 min. Cells were then washed once with PBS, and then blocked with 1% FCS/PBS for 30 min. Without removing blocker, anti-HA antibody (Sigma Aldrich) was added (2 x 1/2500 dilution in 1% FCS/PBS) and incubated for 30 min. Cells were then washed twice with 1% FCS/PBS. The final wash was left in wells and incubated for 15 min at room temperature. After removing the wash, anti rabbit IgG horseradish peroxidase-linked whole antibody (GE Healthcare) was added (1/1000 dilution in 1% FCS/PBS) and incubated for 30 min. Cells were then washed three times with 1% FCS/PBS, and the substrate added (1:1 ratio of SuperSignal™ ELISA Femto Luminol/Enhancer and Stable Peroxide; Thermo Scientific). The plate was gently mixed for one minute in the dark and then total luminescence was measured on the VICTOR Light plate reader. All steps were conducted at room temperature, and all volumes added were 100 µL. PBS was supplemented with Ca²⁺ and Mg²⁺ to minimise cell detachment and subsequent loss.

2.7. **Preparation of Compounds**

The compounds used throughout this thesis will be described in specific chapters. All compounds were prepared from initial stocks to suitably concentrated aliquots.

2.8. **Data Presentation and Statistical Analyses**

All data were presented and analysed using GraphPad Prism 6.0. Competition binding data and concentration-response data were fitted using logarithmic nonlinear regression (three parameter). Saturation binding data were fitted with one-site nonlinear regression. Rate constants were generated by fitting one-phase association nonlinear regression curves.
When the means of three or more groups were compared, one-way or two-way ANOVA tests were used as applicable. ANOVA tests with repeated measures were used with kinetic data from the same samples at multiple time points, allowing comparisons of the means at each time point. Where mean pEC$_{50}$ values, pIC$_{50}$ values or rate constants were calculated from several experiments, the pEC$_{50}$/pIC$_{50}$/rate constant from each experiment was used to determine mean and SEM for analysis. When post-hoc analysis was undertaken, the test details are outlined in the relevant Figure or Table legend.
Chapter 3. BRET Ligand Binding – Development and Optimisation

3.1. Introduction

The ability to measure the binding of a ligand to its cognate protein is one of the most fundamental tools in pharmacology. The first ligand binding assay was developed by Yalow and Berson, who used radiolabelled insulin to measure binding to insulin-binding antibodies, enabling quantification of endogenous insulin levels (Berson et al., 1959; Yalow et al., 1960). After a decade of extensive use of ligand binding immunoassays, the first receptor-ligand binding assays using radiolabelled ligands were developed (Lefkowitz et al., 1970; Rodbell et al., 1971). The use of these assays resulted in the broadening of our understanding of hormone and neurotransmitter systems by providing novel insights into the molecular pharmacology of receptors. Additionally, they enabled the discovery and characterisation of the multitudes of receptors and their subtypes.

The traditional approach to investigate receptor-ligand binding using radiolabelled ligands has been successfully used for decades to probe receptor targets in native tissues with high sensitivity. However, due to the nature of the radioligands, these assays are costly and time consuming, requiring numerous measures to monitor and minimise exposure to ligands and waste. Because of these issues new approaches using fluorescently labelled ligands have been developed. Direct measurement of bound fluorescent ligands is not ideal due to low signal to noise ratios resulting from cellular autofluorescence (Cottet et al., 2011). Additionally, these assays generally require media removal and wash steps, and therefore cannot be performed homogenously. Fluorescence polarisation, which is based on the premise that bound fluorophores emit highly polarised light in comparison to unbound fluorophores, enables homogenous measurement of ligand binding (Allen et al., 2000). However due to its narrow
measurement window and potential to get false negatives, it has not been used extensively to monitor ligand binding (Cottet et al., 2011).

Increasingly, FRET-based approaches have been successfully applied to ligand binding studies (Figure 3.1). In these assays, ligand binding is indicated by measurement of the FRET between a fluorophore linked at the N terminus of the receptor, and bound fluorescent ligand. Theoretically, the N terminus of the receptor could be fluorophore-labelled through the use of receptor-specific antibodies, which would enable this approach to be used in native tissues. However due to the difficulty in obtaining receptor antibodies with high affinity and selectivity, the receptor is typically genetically altered to enable fluorophore labelling. This is either through direct fusion with the fluorophore (Figure 3.1A) (Ilien et al., 2003), epitope labelling of the receptor to enable binding of a fluorescent antibody (Figure 3.1B) (Emami-Nemini et al., 2013) or through conjugation with a self-labelling tag (such as a SNAP-tag; Figure 3.1C) (Zwier et al., 2010). Fusion of the fluorophore to the N terminus of the receptor reduces the non-specific background, as FRET signals are only generated when the two fluorophores are proximal. Additionally, the use of lanthanides as acceptor fluorophores has further improved the specificity of the signal. Unlike classical fluorophores that have fluorescence lifetimes in the range of nanoseconds, lanthanides have lifetimes up to several milliseconds (Cottet et al., 2011). This enables temporal separation of non-specific background fluorescence and the specific fluorescence of the bound fluorophore, and is therefore known as time-resolved FRET (TR-FRET).

Figure 3.1. FRET-based approaches for monitoring ligand binding. (A) Fusion of a fluorophore to the N terminus of a receptor enables FRET with bound fluorescent ligand.
Internalised receptors remain fluorescently tagged and may impact assay sensitivity. (B) Fusion of an epitope to the N terminus of a receptor enables fluorescent antibody binding, and subsequent FRET between the fluorescent antibody and the bound fluorescent ligand. The large size of antibodies can cause steric hindrance. Additionally, assay equilibrium is dependent on binding of the antibody as well as binding of the fluorescent ligand. (C) Receptors are N terminally fused to a self-labelling protein (SLP; such as SNAP-tag) which transfers the fluorescence moiety of its substrate to itself. FRET is then measured between the SLP and bound fluorescent ligand. Adapted from Cottet et al. (2013).

While FRET-based approaches have now been used fairly extensively to monitor ligand binding, the use of BRET has not previously been explored. Previous BRET energy donors (Rluc and its derivatives) are relatively large and fusion to the N terminus of a receptor would likely inhibit correct trafficking and export to the cell surface. Following the development of the substantially smaller Nluc luciferase (Hall et al., 2012), my studies, in collaboration with the University of Nottingham and Promega, have enabled investigation into the use of BRET to monitor ligand binding for the first time (Stoddart et al., 2015). My collaborators confirmed that while the N terminally Rluc8-tagged β2AR was unable to traffic to the cell surface, the N terminally Nluc-tagged β2AR was clearly localised at the cell membrane. In addition to the small size of Nluc relative to Rluc, this capacity to traffic to the cell surface is also likely due to the secretory nature of the luciferase from which Nluc is derived (Stoddart et al., 2015). This chapter will describe the development and optimisation of the BRET ligand binding assay, as illustrated in Figure 3.2. Consequently, most of the data in this chapter are n = 1, but with multiple conditions being tested. Chapter 4 will then apply this assay to the investigation of AT₁ and AT₂ receptor ligand binding, as well as binding to AT₁-AT₂ heteromers.
Figure 3.2. Illustration of the BRET ligand binding assay. (A) Fusion of Nluc to the N-terminus of a receptor enables BRET with bound fluorescent ligand. (B) Addition of a non-fluorescent competitor ligand blocks binding of the fluorescent ligand and thus inhibits the BRET signal. (C) Saturation assays use a constant amount of competitor ligand that is displaced with increasing concentrations of fluorescent ligand. (D) Competition assays use a constant amount of fluorescent ligand that is displaced with increasing concentrations of competitor ligand.

3.2. Materials and Methods

3.2.1. Materials

Ligands used were AngII, ICI 118551, propranolol, PD 123319 (Sigma Aldrich), fluorescein isothiocyanate-AngII (FITC-AngII) and Alexa488-AngII (Molecular
Probes®, TAMRA-AngII (AnaSpec), olmesartan, eprosartan, candesartan, losartan (Zhou Fang Pharm Chemical), CA200700 and CA200689 (CellAura).

3.2.2. cDNA constructs

The cDNA constructs used in this chapter are listed in Table 3.1, and detailed in Section 2.2.1.

Table 3.1. cDNA constructs used in this chapter.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Species</th>
<th>Genbank Accession No.</th>
<th>Constructs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiotensin II receptor type 1</td>
<td>Human</td>
<td>AY221090.1</td>
<td>Nluc-AT₁,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Rluc8-AT₁</td>
</tr>
</tbody>
</table>

3.2.3. Mammalian cell transfections

Transient transfections were carried out in HEK293FT cells as previously described in the General Materials and Methods Chapter (Section 2.3.3). cDNA quantities are described in figure legends. Nluc-β₂AR ligand binding assays used stably transfected cells from Promega.

3.2.4. BRET assays

All assays in this chapter were carried out using NanoBRET, as described previously in the General Materials and Methods Chapter (Section 2.4.1). Both the CLARIOstar and the PHERAstar were used, as well as various filter settings for the donor and acceptor channels (detailed in figure legends).
3.2.5. **IP₁ assays**

All IP₁ assays were carried out as described previously in the General Materials and Methods Chapter (Section 2.5).

3.2.6. **Spectral scans**

The excitation and emission spectra of the fluorescent ligands (concentrations detailed in the figure legends) was determined on the CLARIOstar using the fluorescence scanning option (see Table 3.2 for protocol details). For determination of the emission spectra of Nluc-AT₁ and Rluc8-AT₁, 48 hours post transfection media was removed and replaced with furimazine (10 μM) or coelenterazine h (5 μM), respectively. The emission spectra were measured immediately on the CLARIOstar using the luminescence scanning option (see Table 3.3 for protocol details).

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Scan</th>
<th>Ex Wavelength (nm)</th>
<th>Ex Bandwidth (nm)</th>
<th>Em Wavelength (nm)</th>
<th>Em Bandwidth (nm)</th>
<th>Resolution (nm)</th>
<th>Gain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa488-AngII</td>
<td>Ex</td>
<td>420-520</td>
<td>10</td>
<td>548</td>
<td>16</td>
<td>1</td>
<td>1450</td>
</tr>
<tr>
<td></td>
<td>Em</td>
<td>449</td>
<td>24</td>
<td>484-600</td>
<td>15</td>
<td>1</td>
<td>1719</td>
</tr>
<tr>
<td>FITC-AngII</td>
<td>Ex</td>
<td>420-520</td>
<td>10</td>
<td>548</td>
<td>16</td>
<td>1</td>
<td>1752</td>
</tr>
<tr>
<td></td>
<td>Em</td>
<td>449</td>
<td>24</td>
<td>484-600</td>
<td>15</td>
<td>1</td>
<td>2596</td>
</tr>
<tr>
<td>TAMRA-AngII</td>
<td>Ex</td>
<td>472-590</td>
<td>10</td>
<td>646</td>
<td>72</td>
<td>1</td>
<td>1856</td>
</tr>
<tr>
<td></td>
<td>Em</td>
<td>496</td>
<td>22</td>
<td>527-707</td>
<td>10</td>
<td>1</td>
<td>1942</td>
</tr>
</tbody>
</table>
Table 3.3. CLARIOstar settings for luminescence spectral scans. Em, Emission.

<table>
<thead>
<tr>
<th>Luciferase</th>
<th>Scan</th>
<th>Em Wavelength (nm)</th>
<th>Em Bandwidth (nm)</th>
<th>Resolution (nm)</th>
<th>Gain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nluc-AT₁</td>
<td>Em</td>
<td>370-700</td>
<td>20</td>
<td>2</td>
<td>3000</td>
</tr>
<tr>
<td>Rluc8-AT₁</td>
<td>Em</td>
<td>370-700</td>
<td>20</td>
<td>2</td>
<td>3000</td>
</tr>
</tbody>
</table>

3.2.7. Data presentation and statistical analyses

All curve fitting and statistical analysis were carried out as previously described in Section 2.8).

3.3. Development and Optimisation

3.3.1. Assessment of pharmacology and spectra of fluorescent ligands and luciferases

Before beginning testing of the BRET ligand binding assays, the spectral properties of Nluc were compared with Rluc8. Cells expressing either Nluc-AT₁ or Rluc8-AT₁ were treated with furimazine or coelenterazine, respectively and the luminescence spectra were measured on the CLARIOstar. The luminescence peak of Nluc-AT₁ was left shifted 32 nm from the luminescence peak of Rluc8-AT₁ (Figure 3.3A and Table 3.4). As well, Nluc-AT₁ produced significantly greater luminescence than Rluc8-AT₁ (Figure 3.3B and Table 3.4), with the maximum intensity being approximately 40-fold greater (Table 3.3).
Figure 3.3. Comparison of Nluc-AT$_1$ and Rluc8-AT$_1$ luminescence spectra. HEK293FT cells were transfected with Nluc-AT$_1$ or Rluc8-AT$_1$ (100 ng) and spectra were measured on the CLARIOstar after addition of furimazine or coelenterazine $h$, respectively. Data are presented as (A) normalised luminescence, or (B) raw luminescence values in relative light units (RLU). Data are presented as mean ± SEM of three experiments. * indicates significant difference (p < 0.05, two-way ANOVA with repeated measures followed by Sidak’s test for multiple comparisons).

Table 3.4. Luminescence peak and maximum intensity for Nluc-AT$_1$ and Rluc8-AT$_1$. Data taken from Figure 3.3.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Luminescence Peak (nm)</th>
<th>Maximum Luminescence (RLU ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nluc-AT$_1$</td>
<td>454</td>
<td>5 994 334 ± 613 017</td>
</tr>
<tr>
<td>Rluc8-AT$_1$</td>
<td>486</td>
<td>151 420 ± 37 539</td>
</tr>
</tbody>
</table>

Following testing of the two luciferases, several fluorescent AngII ligands were assessed for their potency in mediating AT$_1$-induced IP production. All of the fluorescent ligands had lower potencies than AngII, with the rank order of potency being Alexa488-AngII > TAMRA-AngII = FITC-AngII (Figure 3.4 and Table 3.5).
Figure 3.4. IP dose response with fluorescent AngII ligands. HEK293FT cells were transfected with AT1α (50 ng). IP1 production was measured on the EnVision after 30 min incubation at 37°C and 5% CO2 with increasing doses of ligand. Data are normalised to $10^{-13}$ M as 0% and $10^{-6}$ M as 100%. Data are presented as mean ± SEM of one experiment performed in duplicate.

Table 3.5. Fluorescent AngII ligands pEC$_{50}$ values for AT1-mediated IP1 production. pEC$_{50}$ values from Figure 3.4.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>pEC$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AngII</td>
<td>9.1</td>
</tr>
<tr>
<td>Alexa488-AngII</td>
<td>8.2</td>
</tr>
<tr>
<td>FITC-AngII</td>
<td>7.1</td>
</tr>
<tr>
<td>TAMRA-AngII</td>
<td>7.2</td>
</tr>
</tbody>
</table>

Next, the absorption and emission spectra of Alexa488-AngII, FITC-AngII and TAMRA-AngII were determined using the CLARIOstar, and the data were superimposed on the emission spectra of Nluc-AT1 (Figure 3.5 and Table 3.6). All fluorescent ligands produced spectra as expected for those fluorophores. The superimposition illustrated that while the excitation spectrum of the green ligands had substantially greater overlap with Nluc-AT1 emission than TAMRA-AngII, their emission spectra also overlapped to a far greater extent.
Figure 3.5. Excitation and emission spectra of fluorescent AngII ligands superimposed on the Nluc-AT1 emission spectrum. (A) Alexa488-AngII (4 µM), (B) FITC-AngII (10 µM) and (C) TAMRA-AngII (4 µM). Data are presented as normalised fluorescence from one experiment. Nluc-AT1 emission data are taken from Figure 3.3A.

Table 3.6. Excitation and emission maxima of fluorescent AngII ligands. Data taken from Figure 3.5.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Excitation Maximum</th>
<th>Emission Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa488-AngII</td>
<td>488</td>
<td>517</td>
</tr>
<tr>
<td>FITC-AngII</td>
<td>488</td>
<td>522</td>
</tr>
<tr>
<td>TAMRA-AngII</td>
<td>545</td>
<td>579</td>
</tr>
</tbody>
</table>
3.3.2. Fluorescent ligand binding assay tests

The first BRET ligand binding experiments utilised the green AngII ligands, Alexa488-AngII and FITC-AngII. FITC-AngII produced better results than Alexa488-AngII, so only the FITC-AngII results will be displayed in this thesis. Figure 3.6 shows an example saturation binding assay using FITC-AngII and the ARB olmesartan as the competitive ligand. While there is some evidence of displacement of FITC-AngII binding in the direct fluorescence assay (Figure 3.6A), this is less evident in the BRET assay (Figure 3.6B). A saturation binding curve was not able to be fitted to the BRET data.

![Figure 3.6. FITC-AngII fluorescence and BRET saturation binding assay.](image)

An example competition binding assay using FITC-AngII and two ARBs is illustrated in Figure 3.7. While there is evidence of displacement of FITC-AngII binding in both the fluorescence assay (Figure 3.7A) and the BRET assay (Figure 3.7B), data from both assays display large error.
Chapter 3. BRET Ligand Binding – Development and Optimisation

Figure 3.7. FITC-AngII fluorescence and BRET competition binding assay. HEK293FT cells transfected with Nluc-AT₁ (70 ng) were incubated with vehicle or antagonist for 30 min and then FITC-AngII for a further 30 min. After removing ligand, fluorescence was measured at 535-30 nm following excitation at 488-14 nm (A). Immediately after addition of furimazine, luminescence was measured at 475-30 and 535-30 nm to assess BRET (B). Recordings were measured on the CLARIOstar. Data are presented as mean ± SEM of one experiment performed in duplicate.

Table 3.7. pIC<sub>50</sub> values for displacement of FITC-AngII binding by two ARBs. pIC<sub>50</sub> values from Figure 3.7.

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Fluorescence pIC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>BRET pIC&lt;sub&gt;50&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eprosartan</td>
<td>6.6</td>
<td>7.0</td>
</tr>
<tr>
<td>Olmesartan</td>
<td>7.2</td>
<td>6.5</td>
</tr>
</tbody>
</table>

After testing the green AngII ligands, TAMRA-AngII was assessed for its ligand binding capacity at AT₁ receptors. Figure 3.8 shows an example saturation binding assay using TAMRA-AngII and two ARBs. The direct fluorescence assay showed a moderate level of displacement of TAMRA-AngII by the competitors (Figure 3.8A). This was far more evident in the BRET assay, where there was almost complete inhibition of TAMRA-AngII binding (Figure 3.8B).
Figure 3.8. TAMRA-AngII fluorescence and BRET saturation binding assay. HEK293FT cells transfected with Nluc-AT\(_1\) (70 ng) were incubated with vehicle or AT\(_1\) antagonists for 30 min and then TAMRA-AngII for a further 30 min. After removing ligand, fluorescence was measured at 585-30 nm following excitation at 535-20 nm (A). Immediately after addition of furimazine, luminescence was measured at 475-30 and 585-30 nm to assess BRET (B). Recordings were measured on the CLARIOstar. Data are presented as mean ± SEM of one experiment performed in duplicate.

TAMRA-AngII was then used in a competition binding assay with various AT\(_1\) receptor competitor ligands. In both the fluorescence and the BRET assays the AT\(_1\) receptor ligands were able to displace binding of TAMRA-AngII (Figure 3.9). The pIC\(_{50}\) values from the fluorescence assay were slightly left shifted from those in the BRET assays, however they also had larger error (Table 3.8). pIC\(_{50}\) values from the BRET assay were similar between the CLARIOstar and the PHERAstar (statistical analysis could not be performed as this was a single experiment).
Figure 3.9. TAMRA-AngII fluorescence and BRET competition binding assay. HEK293FT cells transfected with Nluc-AT1 (70 ng) were incubated with AT1 ligands for 30 min and then TAMRA-AngII (1 µM) for a further 30 min. After removing ligand, fluorescence was measured at 585-30 nm following excitation at 535-20 nm on the CLARIOstar (A). Immediately after addition of furimazine, BRET was measured using filters for 475-30 and 585-30 nm on the CLARIOstar (B) and then 460-80 nm and 610-longpass on the PHERAstar. Data are presented as mean ± SEM of one experiment performed in duplicate.

Table 3.8. pIC₅₀ values for TAMRA-AngII displacement by various AT1 ligands. pIC₅₀ values from Figure 3.9.

<table>
<thead>
<tr>
<th>Competitor</th>
<th>Fluorescence pIC₅₀</th>
<th>BRET CLARIOstar pIC₅₀</th>
<th>BRET PHERAstar pIC₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>AngII</td>
<td>8.4</td>
<td>7.2</td>
<td>7.3</td>
</tr>
<tr>
<td>Eprosartan</td>
<td>8.8</td>
<td>8.1</td>
<td>8.3</td>
</tr>
<tr>
<td>Olmesartan</td>
<td>9.0</td>
<td>8.2</td>
<td>8.2</td>
</tr>
</tbody>
</table>
The reverse experiment was then tested. Here, various doses of TAMRA-AngII were added to constant concentrations of competitor ligands olmesartan and PD123319 (AT2 receptor selective antagonist). While there was no displacement of TAMRA-AngII binding evident in the fluorescence assay (Figure 3.10A), olmesartan but not PD123319 displaced TAMRA-AngII binding in the BRET assay (Figure 3.10B). Furthermore, this assay demonstrated the very low affinity of TAMRA-AngII, as no specific binding could be observed until 1 \( \mu \)M TAMRA-AngII was used. However, despite its low affinity, TAMRA-AngII produced superior results to the green fluorescent ligands, and was thus used for the rest of the ligand binding experiments.

Figure 3.10. TAMRA-AngII fluorescence and BRET competition binding assay with AT1 and AT2 antagonists. HEK293FT cells transfected with Nluc-AT1 (50 ng) were incubated with olmesartan or PD 123319 (AT2 antagonist) for 30 min and then increasing doses of TAMRA-AngII were added for a further 30 min. Before removing ligand, fluorescence was measured at 585-30 nm following excitation at 535-20 nm on the CLARIOstar (A). Media was then removed and BRET immediately measured (using 475-30 and 585-30 nm filters on the CLARIOstar) after addition of furimazine (B). Data are presented as mean ± SEM of one experiment performed in duplicate.
3.3.3. Ligand binding assay optimisation

TAMRA-AngII was then used to assess a range of AT$_1$ receptor selective ligands (Figure 3.11). The various ligand potencies ranged from -8.0 ± 0.1 for AngII to -9.3 ± 0.2 for olmesartan (Table 3.9). As it had the highest affinity, olmesartan was selected as the competitor of choice for the following ligand binding assays.

Additionally, while previous experiments had been performed non-homogenously, in this assay BRET was measured without removal of the ligand-containing media. The results demonstrate that ligand binding can be successfully measured homogenously, and thus most of the following experiments were performed using this approach.

Figure 3.11. Displacement of TAMRA-AngII with various AT$_1$ receptor ligands using BRET competition binding assay. HEK293FT cells transfected with Nluc-AT$_1$ (5 ng) were incubated with AT$_1$ ligands for 30 min and then TAMRA-AngII (1 µM) for a further 30 min. Without removing ligand, furimazine was added and BRET measured on the PHERAstar using 460-80 nm and 610-longpass filters. Data are presented as raw BRET ratio, mean ± SEM of one experiment performed in duplicate.
Table 3.9. pIC$_{50}$ values from Figure 3.11 for displacement of TAMRA-AngII binding by a range of AT$_1$ ligands.

<table>
<thead>
<tr>
<th>Competitor</th>
<th>pIC$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AngII</td>
<td>8.0</td>
</tr>
<tr>
<td>Candesartan</td>
<td>8.0</td>
</tr>
<tr>
<td>Eprosartan</td>
<td>8.2</td>
</tr>
<tr>
<td>Losartan</td>
<td>8.5</td>
</tr>
<tr>
<td>Olmesartan</td>
<td>9.3</td>
</tr>
</tbody>
</table>

The following experiment investigated ligand binding with a range of transfected Nluc-AT$_1$ cDNA quantities. For consistency, competition binding was first assessed at a constant gain level of 1600 (Figure 3.12A, C, E, G, I). Using this approach, displacement curves were observed down to 0.5 ng cDNA with 682 nM and 205 nM TAMRA-AngII. The same cells were then assessed after automatic adjustment of the gain to optimum levels (Figure 3.12B, D, F, H, J). This method produced displacement curves down to 0.005 ng cDNA with 682 nM and 205 nM TAMRA-AngII. Interestingly, when the data for 682 nM and 205 nM TAMRA-AngII (adjusted gain only) was replotted on separate graphs, a shift in pIC$_{50}$ was observed with decreasing cDNA quantity, but not with decreasing TAMRA-AngII concentration (Figure 3.13).
Figure 3.12. Nluc-AT₁ cDNA titration with furimazine. HEK293FT cells were transfected with decreasing amounts of Nluc-AT₁ cDNA (as described on graphs) and pcDNA3 (to make a total...
of 50 ng of plasmid). After a 30 min incubation with olmesartan, TAMRA-AngII (various concentrations as indicated) was added for a further 30 min. Without removing ligand, furimazine was added and BRET measured on the PHERAstar using 460-80/610-longpass filters. Gain was either set at a constant value of 1600 (A, C, E, G, I) or adjusted to optimal value (B, D, F, H, J). Data are presented as raw BRET ratio, mean ± SEM of one experiment performed in duplicate.

**Figure 3.13.** pIC\textsubscript{50} values from Nluc-AT\textsubscript{1} cDNA titration with furimazine. Data with optimal gain from Figure 3.12 replotted to allow comparison of olmesartan competition binding curves (A, B) and pIC\textsubscript{50} values (C). TAMRA-AngII 682 nM (A) and TAMRA-AngII 205 nM (B) were used for comparison.

### 3.3.4. β\textsubscript{2}AR ligand binding tests

Lastly, as proof of principle that the BRET ligand binding assay would be amenable to use with stable transfection, it was tested on the β\textsubscript{2}AR using stably transfected Nluc-β\textsubscript{2}AR cells. The β\textsubscript{2}AR antagonists ICI 118551 and propranolol were used to test the displacement of red fluorescent β\textsubscript{2}AR ligands CA200700 (salbutamol derivative) and
CA200689 (propranolol derivative). The direct fluorescent binding assay did not show any evidence of displacement of CA200700 (Figure 3.14A) or CA200689 binding (Figure 3.14B). In contrast, the BRET binding assays showed complete inhibition of CA200700 (Figure 3.14C, E) and CA200689 binding (Figure 3.14D, F) by ICI 118551 and propranolol.

Figure 3.14. Fluorescence and BRET $\beta_2$AR saturation binding assay. HEK293 cells stably transfected with Nluc-$\beta_2$AR were incubated with vehicle or $\beta_2$AR antagonists ICI 118551 or propranolol for 30 min. Cells were then incubated for a further 30 min with fluorescent $\beta_2$AR.
ligands CA200700 (agonist - salbutamol; A, C, E) or CA200689 (antagonist - propranolol; B, D, F). After removing ligand, fluorescence was measured at 659-11 nm following excitation at 621-13 nm on the CLARIOstar (A, B). Immediately after addition of furimazine, BRET was measured using 460-80/610-LP filters on the PHERAstar (C, D) or 475-30/650-15 filters on the CLARIOstar (E, F). Data are presented as mean ± SEM of one experiment performed in duplicate.

A propranolol competition binding experiment was then conducted. Again, no displacement of the fluorescent ligands was evident when the direct fluorescence was assessed (Figure 3.15A). The BRET assay showed displacement of CA200700 and CA200689 binding (Figure 3.15B, C) with extremely low error (Table 3.10).

Figure 3.15. Fluorescence and BRET β₂AR competition binding assay. HEK293 cells stably transfected with Nluc-β₂AR were incubated with propranolol for 30 min followed by a further 30 min incubation with fluorescent β2AR ligands CA200700 (500 nM) or CA200689 (500 nM). After removing ligand, fluorescence was measured at 680-30 nm following excitation at 625-30 nm.
(A). Immediately after addition of furimazine, BRET was measured using 475-30/650-40 filters on the CLARIOstar (B) or 460-80/610-LP filters on the PHERAstar (C). Data are presented as mean ± SEM of one experiment performed in duplicate.

Table 3.10. pIC\textsubscript{50} values for displacement of CA200700 and CA200689 binding by propranolol. pIC\textsubscript{50} values from Figure 3.15.

<table>
<thead>
<tr>
<th>Fluorescent Ligand</th>
<th>CLARIOstar pIC\textsubscript{50}</th>
<th>PHERAstar pIC\textsubscript{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA200700</td>
<td>8.73</td>
<td>8.75</td>
</tr>
<tr>
<td>CA200689</td>
<td>7.31</td>
<td>7.55</td>
</tr>
</tbody>
</table>

3.4. Discussion

In discussion of the results of this chapter, it is important to keep in mind that all data presented, with the exception of Figure 3.3 (Comparison of Nluc-AT\textsubscript{1} and Rluc8-AT\textsubscript{1} luminescence spectra.), were performed as single experiments. This is because these experiments were designed to guide assay development and optimisation. All data in the following chapters were generated from multiple independent experiments. The following discussion of results therefore assumes the n of 1 data is an accurate representative sample.

The main finding of this chapter is that, of those tested, the most successful fluorescent AngII ligand to monitor binding to AT\textsubscript{1} receptors was TAMRA-AngII. In both saturation and competition assays displacement of bound TAMRA-AngII by various AT\textsubscript{1} receptor ligands was evident. The superiority of the yellow TAMRA-AngII in comparison to the green Alexa488-AngII and FITC-AngII is likely due to the increased spectral separation of donor (Nluc-AT\textsubscript{1}) and acceptor (TAMRA-AngII) emission. Although this increased spectral separation coincides with decreased overlap of donor emission with acceptor excitation, the intense brightness of Nluc overcomes this loss. This is further evidenced by the extremely precise data generated in the β\textsubscript{2}AR
ligand binding assays. A major contributing factor to this precision is the improved signal to background ratio resulting from the greater spectral separation with red fluorescent ligands. This effect was clearly demonstrated in my collaborators’ work with Nluc-β2AR ligand binding assays, comparing a green (propranolol-BYFL) with a red (propranolol-BY630) ligand (Stoddart *et al.*, 2015). While the β2AR competitors produced clear, dose-dependent displacement of both fluorescent ligands, a lower signal to background ratio was observed for green propranolol-BYFL than for red propranolol-BY630. These results all illustrate that the BRET ligand binding assay can be used successfully with a broad range of fluorescent acceptors, and confirms that fluorophores with longer wavelength emissions produce more optimal results.

In this study, the superiority of TAMRA-AngII was evident in both the BRET assays as well as the direct fluorescence assays, although distinctly better results were achieved when measuring BRET. The direct fluorescence assays had larger error and pIC50 values were left shifted from those of the BRET assays. This confirms that as with FRET assays, BRET is superior to measuring direct fluorescence when monitoring ligand binding. This was further confirmed in the β2AR ligand binding assays with the stable cell line, where exceptionally precise data were obtained in the BRET assays but no specific binding could be measured in the fluorescence assays. This distinctly poor fluorescence result is likely due to the lipophilic nature of these particular fluorescent ligands. This allows their diffusion through the cell membrane and intracellular accumulation, resulting in high levels of non-specific background. The use of BRET eliminates this problem as signals are only generated when the fluorophore is proximal to the Nluc-tagged receptor.

The superior luminescence of Nluc to Rluc8 was confirmed in this study, whereby Nluc-AT1 produced 40-fold greater luminescence than Rluc8-AT1. Additionally, the emission spectrum was approximately 30 nm left shifted from the Rluc8-AT1 spectrum, enabling further increases in spectral separation between Nluc and the acceptor emission. Again, the intense brightness of Nluc overcomes the loss of overlap between the donor emission and the acceptor excitation spectrum.
3.4.1. Titration assays

The titration assays carried out in this study revealed that there was no shift in olmesartan pIC$_{50}$ with increasing TAMRA-AngII concentration. As increasing concentration of labelled agonist should decrease the pIC$_{50}$ of competitor binding (Cheng et al., 1973), a lack of shift implies that even at the highest concentration of TAMRA-AngII used, there is still only minimal TAMRA-AngII binding. This is supported by the TAMRA-AngII saturation assay, which showed no specific binding of TAMRA-AngII until 1 µM was used (Figure 3.10). As a consequence of this, the observed olmesartan pIC$_{50}$ values should be approximately equal to pKi.

The estimation of olmesartan’s affinity needs to be applied with caution, however, as the titration assays also indicated that decreased Nluc-AT$_1$ expression resulted in an increased pIC$_{50}$ of olmesartan binding to the AT$_1$ receptor. After a thorough search of the literature, no reports of this effect in other studies appears to have been published, perhaps because studies using potentially sub-physiological levels of receptors are uncommon. Despite this, there are several potential causes which can be speculated upon. As has been suggested in other studies (Hern et al., 2010; Lambert, 2010; Calebiro et al., 2013; Patowary et al., 2013), it is possible that the decrease in receptor expression causes an increase in the proportion of monomers relative to homomers. If this is the case, any effects of negative cooperativity within AT$_1$ homomers would be diminished in the system, resulting in an increase in the apparent affinity of olmesartan binding. Negative cooperativity of ligand binding has been observed for several GPCR oligomers including the dopamine D$_2$ homomer (Armstrong et al., 2001), oxytocin receptor homomers (Albizu et al., 2010) and the $\alpha_{2A}$ adrenergic-µ opioid receptor heteromer (Vilardaga et al., 2008). While no studies have conclusively confirmed negative cooperativity of ligand binding within the AT$_1$ homomer, early evidence showed that binding of the antagonist [Sar$^1$Ile$^8$]-AngII negatively modulated AngII and AngIII binding, with the suggested mechanism being homomerisation (Moore et al., 1989). However, these results need to be interpreted with caution as [Sar$^1$Ile$^8$]-AngII shows little selectivity for AngII receptor subtypes (McMullen et al., 2002), and the presence of AT$_2$ receptors would therefore confound results. To determine if the increased affinity of olmesartan binding in this study is a result of increased levels of AT$_1$ monomers, the BRET ligand binding assay could be
combined with bimolecular luminescence complementation (BiLC). BRET with split Rluc8 constructs has previously been used to investigate oligomer-specific interactions with G proteins (Urizar et al., 2011; Armando et al., 2014), and recently, split Nluc constructs have been used to determine protein-protein interactions (Dixon et al., 2015).

If the complementary Nluc portions can be N terminally fused to AT1 receptor constructs while maintaining correct receptor surface expression and luciferase function, this would ensure only homomer-specific signals were generated. If this method produced affinities that were comparable to those from high receptor expression levels in this study, this would support the hypothesis that the increased apparent affinity is due to an increased ratio of monomers to homomers. Additionally, if the homomer-specific affinities did not increase with reduced receptor expression, this would provide further support for this hypothesis.

Another potential explanation for the increase in olmesartan pIC$_{50}$ with decreased receptor expression is that the interaction between olmesartan, TAMRA-AngII and the AT1 receptor may not be completely competitive, or the system may not be at equilibrium. Indeed, although olmesartan rapidly binds to AT1 receptors, it has a slow rate of dissociation (T$_{1/2}$ of 65 ± 11 min) and also displays a level of insurmountability (Le et al., 2007). This fact, coupled with the low affinity of TAMRA-AngII suggests that an equilibrium between TAMRA-AngII and olmesartan binding may not be completely achieved in the time frame used in this assay (60 min incubation with olmesartan and 30 min incubation with TAMRA-AngII). To test this hypothesis, a variety of incubation times could be investigated. Additionally, adding TAMRA-AngII prior to olmesartan could provide further insight into the association and dissociation kinetics, however the potential for TAMRA-AngII-induced AT1 receptor internalisation needs to be taken into account using this approach. Detailed information on the rates of association and dissociation of the different ligands would also be achieved through kinetic ligand binding assays.

It should also be considered that the increased pIC$_{50}$ of olmesartan binding with decreased receptor expression may be an artefact of using BRET as an approach to monitor ligand binding. Investigations with other fluorescent and competitor ligands, as well as other Nluc-tagged receptors would enable determination of the ligand- and receptor specificity of this effect. Additionally, it would be interesting to investigate whether a decrease in cell number/density increases the pIC$_{50}$ in a similar manner to a
decrease in receptor expression. Whatever the cause of this shift in displacement potency, it is important to keep in mind when interpreting the BRET ligand binding data from this study.

### 3.4.2. Competition binding assays with various AT₁ receptor ligands

Various ARBs were tested for their ability to displace binding of TAMRA-AngII. The rank order of affinity in this study was: olmesartan > losartan > eprosartan > candesartan. Olmesartan was picked as the ARB of choice for further studies as it had the highest affinity. Recently, a meta-analysis of all reported affinity estimates for various ARBs in competition radioligand binding studies was published (Figure 3.16) (Michel et al., 2013). The studies were from a range of cell types, tissues and species, as well as from cells heterologously expressing human AT₁ receptor. Figure 3.16 shows that there is a broad range of affinity estimates for most of the ARBs in the meta-analysis, likely due to the various sample origins and assay conditions used. As well, both pKi and pIC₅₀ values were included in the analysis. As pIC₅₀ is only an estimate of pKi, and changes with varying conditions (such as concentration of labelled ligand), inclusion of pIC₅₀ values will increase the range of results in this analysis. Comparison of the pIC₅₀ values generated in my study with those from the meta-analysis shows that most are similar to previously reported values (Figure 3.16). The slightly higher pIC₅₀ of olmesartan is likely due to the much smaller sample size in the meta-analysis than the three other antagonists used in my study.
Figure 3.16. Affinity estimates for various ARBs at mammalian AT₁ receptors, as determined in competition radioligand binding studies. Figure is taken from a meta-analysis comparing pKᵢ or pIC₅₀ values from studies using a wide range of cell types, tissues and species (canine excluded as ARB affinity values tend to be lower than in other mammals), as well as heterologously expressed human AT₁ receptor. Red text and circles indicate pIC₅₀ values generated in my study. Adapted from Michel et al. (2013).

The pIC₅₀ of AngII displacement in this study (8.0 ± 0.1) was approximately ten-fold lower than the potency of AngII-induced IP₁ production at Niuc-AT₁ (pIC₅₀ ± SEM = 9.3 ± 0.2; Figure 2.3). This is not necessarily surprising, as receptor reserve and signal amplification may result in binding affinity being less than the potency of functional responses (Ruffolo, 1982). The pIC₅₀ of AngII displacement in this study is similar to other previously reported estimated affinities (Kostenis et al., 2005; Sanni et al., 2010; Bosnyak et al., 2011) and approximately ten-fold lower than others (Miura et al., 1999; Griessner et al., 2009; Siddiquee et al., 2013). Differences in estimated affinities are likely to be due to different experimental conditions, such as sample origin and assay temperature. For example, radioligand binding assays are often conducted at 4°C to prevent receptor internalisation. As AngII induces internalisation of the AT₁ receptor, binding studies done at 4°C may have considerably different results to those performed at room temperature or at 37°C.
The competition binding assay with various ligands was also the first in this chapter to be performed homogenously. This revealed that BRET ligand binding could be successfully performed without the need for separation of bound and unbound ligand. This is not surprising as resonance energy transfer can only occur to acceptors within 10 nm, with the diameter of a GPCR being approximately 4 nm (Gurevich et al., 2008). Thus, there should be minimal non-specific background from unbound fluorescent ligands. Indeed, some early FRET ligand binding assays have also been successfully performed homogenously (Ilien et al., 2003; Maurel et al., 2004), however most require removal of the donor substrate prior to measurement (Maurel et al., 2008; Zwier et al., 2010; Emami-Nemini et al., 2013; Hounsou et al., 2014; Oueslati et al., 2015).

3.4.3. Other results

Each of the fluorescent AngII ligands were assessed for their ability to mediate AT₁ receptor-induced IP₁ production. All three ligands had lower potency than AngII, and this is not uncommon upon conjugation of a relatively large fluorophore to a ligand (Vernall et al., 2014). Although TAMRA-AngII had moderate potency for IP production (pEC₅₀ = 7.2 ± 0.1) it had very low binding affinity (too low to allow comprehensive saturation ligand binding assay analysis and calculation of K_D, see Figure 3.10). As previously mentioned in regards to olmesartan, this discrepancy is likely due to receptor reserve and signal amplification, resulting in binding affinity being less than the potency of functional responses.

The β₂AR experiments with the stable cell line showed extremely precise data. As mentioned previously, this is likely to be due to the use of red shifted fluorophores and the use of stably transfected cells rather than transiently transfected cells, which produce less variability in receptor expression levels. The β₂AR experiments also demonstrated that both the CLARIOstar and the PHERAstar performed equally well in these ligand binding studies.
3.4.4. Summary

This chapter has illustrated some of the results from the development and optimisation of the BRET ligand binding assay. After testing various parameters, optimal conditions were determined. TAMRA-AngII and olmesartan were selected as the ligands of choice for further studies on AT$_1$ receptor binding. The use of red shifted fluorescent ligands and stably transfected cells was shown to improve the precision of results. In the development of these assays it was also revealed that they can successfully be performed homogenously, eliminating the need to remove unbound ligands. Finally, both the PHERAstar and the CLARIOstar performed equally well at monitoring ligand binding.
Chapter 4. Angiotensin II Receptor and Heteromer Ligand Binding

4.1. Introduction

The previous chapter discussed the development and optimisation of the BRET ligand binding assay. This chapter will more thoroughly investigate ligand binding to both AT₁ and AT₂ receptors, and then adapt the assay to investigate ligand binding to the AT₁-AT₂ receptor heteromer.

The precursor decapeptide AngI and its metabolite AngII binds the AT₂ receptor with moderately greater affinity than the AT₁ receptor, and this AT₂ selectivity is enhanced with the various downstream metabolites (Timmermans et al., 1991; Miura et al., 1999; Bosnyak et al., 2011). The first selective AngII receptor ligands were developed more than two decades ago, and included the non-peptide antagonists losartan (AT₁ selective; Chiu et al., 1989) and PD 123319 (AT₂ selective; Dudley et al., 1990), as well as peptide ligands such as CGP 42114 (AT₂ selective agonist; Whitebread et al., 1989). Numerous derivatives of losartan have since been developed and are now widely used clinically.

Both peptide and non-peptide ligands occupy the same ligand binding pocket in the AT₁ and the AT₂ receptor (Balakumar et al., 2014a). AngII is believed to bind both receptors via a similar mechanism (Figure 4.1) (Prokop et al., 2013), in which the final binding site results in AngII being in a vertical position with its C terminus within the transmembrane domain and its N terminus across the extracellular loops (Fillion et al., 2013). As a consequence of AngII residing in the binding pocket in this position, it is unlikely that fusion of fluorescent labels to anywhere other than the N terminus of the peptide would allow maintenance of even moderate binding affinity. Consequently, the three fluorescently tagged AngII peptides used in this study are all tagged at the N terminus.
Chapter 4. Angiotensin II Receptor and Heteromer Ligand Binding

**AngII**

\[
\text{Angiotensin II} \quad \text{(H}_{2}\text{N-Asp-Arg-Val-Tyr-His-Pro-Phe-COOH)}
\]

**AT\(_1\) receptor**

**AT\(_2\) receptor**
The molecular pharmacology of the AT$_1$-AT$_2$ receptor heteromer has been reasonably well characterised, showing that it has an inhibitory effect on AT$_1$ signalling (AbdAlla et al., 2001b; Kumar et al., 2002; Yang et al., 2012) and that it does not internalise subsequent to recruitment of β-arrestin (Porrello et al., 2011). Despite this, the ligand binding properties of the heteromer have not been previously investigated. Following a study of the binding properties of the individual receptors, this chapter will then adapt the BRET ligand binding assay using GPCR-HIT to investigate binding to the AT$_1$-AT$_2$ heteromer (Figure 4.2).
Figure 4.2. Illustration of the GPCR-HIT ligand binding assay using BRET. Blockade of Nluc-AT\(_1\) with olmesartan and coexpression with untagged AT\(_2\) receptor ensures BRET signals are only obtained from AT\(_2\) receptors bound with TAMRA-AngII. (A) If the receptors are not within a complex, energy transfer between Nluc on AT\(_1\) and TAMRA-AngII bound to AT\(_2\) will not occur. (B) If AT\(_1\) and AT\(_2\) are proximal to one another, BRET between Nluc-AT\(_1\) and AT\(_2\)-bound TAMRA-AngII should be measurable. The specificity of this interaction can be confirmed using an AT\(_2\) specific ligand (PD 123319).

4.2. Materials and Methods

4.2.1. Materials

Ligands used were AngII, PD 123319 (Sigma Aldrich), TAMRA-AngII (AnaSpec), olmesartan, candesartan, (Zhou Fang Pharm Chemical).
4.2.2. cDNA constructs

The cDNA constructs used in this chapter are listed in Table 4.1, and detailed in Section 2.2.1.

Table 4.1. cDNA constructs used in this chapter.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Species</th>
<th>Genbank Accession No.</th>
<th>Constructs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiotensin II receptor type 1</td>
<td>Human</td>
<td>AY221090.1</td>
<td>Nluc-AT₁</td>
</tr>
<tr>
<td>Angiotensin II receptor type 2</td>
<td>Human</td>
<td>U10273.1</td>
<td>Nluc-AT₂</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SP-Flag-AT₂</td>
</tr>
<tr>
<td>Vasopressin receptor type 2</td>
<td>Human</td>
<td>NM_000054.4</td>
<td>Nluc-V₂</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Flag-V₂</td>
</tr>
</tbody>
</table>

4.2.3. Mammalian cell transfections

Transient transfections were carried out in HEK293FT cells as previously described in the General Materials and Methods Chapter (Section 2.3.3). cDNA quantities are described in figure legends.

4.2.4. BRET assays

All assays in this chapter were carried out using NanoBRET, as described previously in the General Materials and Methods Chapter (Section 2.4.1). Both the CLARIOstar and the PHERAstar were used, with filter settings of 475-30/585-40 and 460-80/610-LP, respectively.
4.2.5. IP1 assays

IP1 assays were carried out as described previously in the General Materials and Methods Chapter (Section 2.5).

4.2.6. Data presentation and statistical analyses

All curve fitting and statistical analysis were carried out as previously described in Section 2.8).

4.3. Results

4.3.1. AT1 and AT2 receptor ligand binding

Before investigating ligand binding to the AT1-AT2 receptor heteromer, binding to the individual receptors was assessed. Following the preliminarily ligand binding tests at AT1 receptors in the previous chapter, more thorough analysis was undertaken. Competition binding assays were used exclusively throughout this chapter, as the affinity of TAMRA-AngII is too low to allow comprehensive saturation binding assay analysis and KD determination.

Firstly, AngII, candesartan and olmesartan were used to displace increasing concentrations of TAMRA-AngII at AT1 receptors (Figure 4.3). Figure 4.3A, C, E shows the raw BRET ratios, illustrating the dose-dependent displacement of TAMRA-AngII binding to AT1 receptors. Specific binding was observed to 100 nM TAMRA-AngII. Normalisation reduced the error associated with these data (Figure 4.3B, D, F). Plotting the pIC50 values of these data illustrated a rank order of affinity: olmesartan > candesartan > AngII (Figure 4.4 and Table 4.2). Despite the appearance of a potential trend in left shift of the pIC50 values for AngII, no significant difference was observed for the four concentrations of TAMRA-AngII analysed. In contrast, the pIC50 values for each competitor at 1 µM were significantly different.
Figure 4.3. AT₁ receptor competition binding experiments. HEK293FT cells transfected with Nluc-AT₁ (1 ng) and pcDNA₃ (49 ng) were incubated with AT₁ ligands: AngII (A, B), candesartan (C, D) or olmesartan (E, F) for 30 min. Various concentrations of TAMRA-AngII were then added and incubated for a further 30 min. Without removing ligands, furimazine was added and BRET measured immediately on the PHERAstar. Data are presented as raw BRET ratio (A, C, E) and relative BRET ratio (B, D, F; normalised as 100% being TAMRA-AngII 1 µM at minimum competitor concentration and 0% being TAMRA-AngII 0 nM at maximum competitor concentration). Data are presented as mean ± SEM of three experiments performed in duplicate.


**Figure 4.4. pIC\textsubscript{50} values from Nluc-AT\textsubscript{1} competition binding experiments.** pIC\textsubscript{50} values from each experiment in Figure 4.3. * and ns indicate significant (p < 0.05) or no significant difference (p > 0.05) between means, respectively (using two-way ANOVA followed by Tukey’s test for multiple comparisons; solid lines compare different TAMRA-AngII concentrations within one competitor treatment while dashed lines compare the same TAMRA-AngII concentrations between two different competitor treatments).

**Table 4.2. pIC\textsubscript{50} values from Nluc-AT\textsubscript{1} competition binding experiments.** pIC\textsubscript{50} values from Figure 4.3. Data are presented as mean ± SEM.

<table>
<thead>
<tr>
<th>Competitor</th>
<th>[TAMRA-AngII] nM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1000</td>
</tr>
<tr>
<td>Angiotensin II</td>
<td>7.7 ± 0.1</td>
</tr>
<tr>
<td>Candesartan</td>
<td>8.4 ± 0.2</td>
</tr>
<tr>
<td>Olmesartan</td>
<td>9.3 ± 0.2</td>
</tr>
</tbody>
</table>

A similar experiment was then carried out for TAMRA-AngII binding to AT\textsubscript{2} receptors. Again, there was clear, dose-dependent displacement of TAMRA-AngII by
the AT₂ receptor antagonist PD 123319 (Figure 4.5A), however a much lower affinity was observed than for displacement of TAMRA-AngII by AT₁ receptor antagonists. Affinity was only calculated for 100 nM TAMRA-AngII (pIC₅₀ ± SEM = 6.3 ± 0.1), as it was not clear that the other concentrations had reached maximal displacement. Similar to the AT₁ receptor competitor binding assay, specific binding was observed to 100 nM TAMRA-AngII. Normalisation again reduced the error associated with the data (Figure 4.5B). One difference between the AT₁ receptor binding assay and the AT₂ receptor binding assay is that the maximal BRET signal (at 1 µM TAMRA-AngII and minimum competitor concentration) was lower in the AT₂ binding assay (Figure 4.6). While there was no significant difference between the maximal BRET signal in all three AT₁ competitor assays, there was significantly less BRET in the PD 123319 displacement assay when compared to AngII and candesartan assays.

**Figure 4.5. AT₂ receptor competition binding.** HEK293FT cells transfected with Nluc-AT₂ (50 ng) and pcDNA₃ (100 ng) were incubated with AT₂ antagonist PD 123319 for 30 min. Various concentrations of TAMRA-AngII were then added and incubated for a further 30 min. Without removing ligands, furimazine was added and BRET measured immediately on the PHERAstar. Data are presented as raw BRET ratio (A) and relative BRET ratio (B; normalised as 100% being TAMRA-AngII 1 µM at 10⁻¹¹ M PD 123319 and 0% being TAMRA-AngII 0 nM at 10⁻⁴ M PD 123319). Data are presented as mean ± SEM of three experiments performed in duplicate.
Chapter 4. Angiotensin II Receptor and Heteromer Ligand Binding

Figure 4.6. Maximal TAMRA-AngII binding to AT$_1$ and AT$_2$ receptors. Maximum TAMRA-AngII binding values were taken from Figure 4.3A, C, E and Figure 4.5A. The maximum was determined as the point at minimal competitor concentration, 1 $\mu$M TAMRA-AngII. * and ns indicate significant ($p < 0.05$) or no significant difference ($p > 0.05$) between means, respectively (using one-way ANOVA followed by Tukey’s test for multiple comparisons).

4.3.2. AT$_1$-AT$_2$ receptor heteromer ligand binding

The GPCR-HIT assay for assessing ligand binding at the AT$_1$-AT$_2$ receptor heteromer involved measuring binding to the untagged AT$_2$ receptor through its proximity with the antagonist-blocked Nluc-AT$_1$. Before undertaking these experiments it was necessary to confirm that the AT$_2$ receptor antagonist PD 123319 does not bind with reasonable affinity to the AT$_1$ receptor. Preliminary evidence of this was provided in Figure 3.10B, whereby 2 $\mu$M olmesartan, but not 2 $\mu$M PD 123319 was able to block binding of TAMRA-AngII to AT$_1$ receptors. To further support the lack of PD 123319 binding to AT$_1$ receptors, an IP$_1$ AngII dose-response assay was conducted. Figure 4.7 shows a significant decrease in potency of AngII-induced IP$_1$ production in the presence of olmesartan, but not PD 123319. Additionally, this experiment suggested that the AT$_1$ receptor may have a basal level of constitutive coupling to G$_{q}$, as olmesartan significantly decreased IP$_1$ accumulation, even at negligible concentrations of AngII. This constitutive coupling to G$_{q}$ has previously been reported for the rat, mouse and
human AT₁ receptor, both in vitro (Zou et al., 2004; Miura et al., 2006) and ex vivo (Yasuda et al., 2012).

As mentioned, binding to the AT₁-AT₂ heteromer was investigated by measuring TAMRA-AngII binding to the AT₂ receptor through its interaction with the blocked Nluc-tagged AT₁ receptor. This experiment was conducted by measuring ligand binding both before and after removal of the ligand-containing media. Figure 4.8A shows olmesartan displacement of TAMRA-AngII binding to Nluc-AT₁, as seen in previous studies. The affinity of olmesartan (Table 4.3) was right shifted from the monomer study (Figure 4.3 and Table 4.2), likely due to the increased quantity of Nluc-AT₁ used in the heteromer experiment (as discussed in Section 3.4.1). Treatment with
up to 100 µM PD 123319 did not cause displacement of TAMRA-AngII binding (Figure 4.8C). When the untagged AT₂ receptor was coexpressed with Nluc-AT₁, treatment with 1 µM olmesartan and increasing doses of PD 123319 did not immediately reveal displacement of TAMRA-AngII binding (Figure 4.8E), however upon normalisation of these data, there was initial evidence of PD 123319-induced displacement of TAMRA-AngII binding (Figure 4.8G).

To determine if a clearer signal could be obtained through reduction in the background noise, the media was then removed from the samples and BRET was measured with new furimazine. As seen prior to ligand removal, displacement of TAMRA-AngII binding to Nluc-AT₁ was evident with olmesartan (Figure 4.8B) but not PD 123319 (Figure 4.8D), and the non-specific background was now removed from the olmesartan binding assay. Importantly, in cells expressing olmesartan-blocked Nluc-AT₁ and AT₂, there was now robust evidence of PD 123319-induced displacement of TAMRA-AngII binding with (Figure 4.8H) or without (Figure 4.8F) normalisation of the data. At 100 nM TAMRA-AngII, the pIC₅₀ of this displacement was not significantly different to that observed by PD 123319 at AT₂ monomers in Figure 4.5 (Nluc-AT₂ = 6.3 ± 0.1; Nluc-AT₁ + AT₂ = 6.5 ± 0.1). Additionally, the maximum BRET level was significantly lower in the heteromer assay (Nluc-AT₁ + AT₂, 0.00145 ± 0.00000404) than in the monomer/homomer assay (PD 123319 displacement curve; 0.00219 ± 0.000193, p < 0.05).
Figure 4.8. AT₁-AT₂ receptor heteromer ligand binding: homogenous and non-homogenous assay. HEK293FT cells were transfected with Nluc-AT₁ (50 ng) and either pcDNA₃ (A-D) or AT₂ (E – H) (100 ng). Cells were incubated for 30 min with increasing concentrations of AT₁ antagonist olmesartan (A, B), increasing concentrations of AT₂ antagonist PD 123319 (C, D), or 1 µM olmesartan + increasing concentrations of PD 123319 (E – H). Various concentrations of TAMRA-AngII were then added and incubated for a further 30 min. Without removing ligands, furimazine was added and BRET measured immediately on the PHERAstar (A, C, E, G). Following removal of ligand/media new furimazine was added and BRET immediately measured (B, D, F, H). (G) and (H) are normalised (100% being TAMRA-AngII 1 µM at 10⁻¹¹ M competitor and 0% being TAMRA-AngII 1 nM at maximum competitor
concentration) data from (E) and (F), respectively. Data are presented as mean ± SEM of three experiments performed in duplicate.

Table 4.3. pIC$_{50}$ values from AT$_1$-AT$_2$ receptor heteromer ligand binding experiment. pIC$_{50}$ values from Figure 4.8. * indicates significant difference (p < 0.05; one-way ANOVA followed by Tukey’s test for multiple comparisons) from 100 nM TAMRA-AngII for that transfection and treatment. No significant differences were observed for pIC50 values from Nluc-AT$_1$ experiment. pIC$_{50}$ values are presented as mean ± SEM.

<table>
<thead>
<tr>
<th>Transfection and Treatment</th>
<th>[TAMRA-AngII] nM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1000</td>
</tr>
<tr>
<td>Nluc-AT$_1$ olmesartan pre ligand removal</td>
<td>7.7 ± 0.3</td>
</tr>
<tr>
<td>Nluc-AT$_1$ olmesartan post ligand removal</td>
<td>7.0 ± 0.3</td>
</tr>
<tr>
<td>Nluc-AT$_1$ + AT$_2$ PD 123319 + olmesartan pre ligand removal</td>
<td>ND</td>
</tr>
<tr>
<td>Nluc-AT$_1$ + AT$_2$ PD 123319 + olmesartan post ligand removal</td>
<td>ND</td>
</tr>
</tbody>
</table>

To more clearly visualise the PD 123319-induced displacement of TAMRA-AngII bound to AT$_2$ receptors, the same data were replotted with the 1 μM TAMRA-AngII values removed. As before, displacement of TAMRA-AngII binding to Nluc-AT$_1$ was evident with olmesartan (Figure 4.9A, B) but not PD 123319 (Figure 4.9C, D). PD 123319-induced displacement of TAMRA-AngII bound to AT$_2$ receptors was now more evident prior to ligand removal (Figure 4.9E), particularly upon normalisation of the data (Figure 4.9G). As before, upon ligand removal, there was clear evidence of PD 123319-induced TAMRA-AngII displacement (Figure 4.9F, H).
Figure 4.9. AT₁-AT₂ receptor heteromer ligand binding: homogenous and non-homogenous assay (1 µM TAMRA-AngII removed). Data from Figure 4.8 replotted without 1 µM TAMRA-AngII data.
This experiment was then repeated without the initial BRET measurement before removing the media. Figure 4.10 shows the raw BRET ratios measured on both the PHERAstar and the CLARIOstar. As with the previous experiment, there is clear, dose-dependent displacement of TAMRA-AngII binding by olmesartan (Figure 4.10A, B) and none by PD 123319 (Figure 4.10C, D). PD 123319 again shows displacement of TAMRA-AngII binding upon coexpression of AT\textsubscript{2} receptors (Figure 4.10E, F). In this experiment, the PHERAstar produced more precise data than the CLARIOstar. When these same data were normalised, the PHERAstar and the CLARIOstar both displayed similarly precise results (Figure 4.11). Intriguingly however, there was now evidence of a dose-dependent increase in TAMRA-AngII binding to Nluc-AT\textsubscript{1} upon treatment with PD 123319 (Figure 4.11C, D).
Figure 4.10. AT₁-AT₂ receptor heteromer ligand binding – non-homogenous assay (raw BRET ratios). HEK293FT cells were transfected with Nluc-AT₁ (50 ng) and either pcDNA₃ (A-D) or AT₂ (E, F) (100 ng). Cells were incubated for 30 min with increasing concentrations of AT₁ antagonist olmesartan (A, B), increasing concentrations of AT₂ antagonist PD 123319 (C, D), or 1 µM olmesartan + increasing concentrations of PD 123319 (E, F). Various concentrations of TAMRA-AngII were then added and incubated for a further 30 min. After removing ligands, furimazine was added and BRET measured immediately on the PHERAstar then the CLARIOstar. Data are presented as mean ± SEM of three experiments performed in duplicate.
Figure 4.11. AT$_1$-AT$_2$ receptor heteromer ligand binding – non-homogenous assay (normalized data). HEK293FT cells were transfected with Nluc-AT$_1$ (50 ng) and either pcDNA$_3$ (A-D) or AT$_2$ (E, F) (100 ng). Cells were incubated for 30 min with increasing concentrations of AT$_1$ antagonist olmesartan (A, B), increasing concentrations of AT$_2$ antagonist PD 123319 (C, D), or 1µM olmesartan + increasing concentrations of PD 123319 (E, F). Various concentrations of TAMRA-AngII were then added and incubated for a further 30 min. After removing ligands, furimazine was added and BRET measured immediately on the PHERAstar then the CLARIOstar. Data are presented as relative BRET ratio (normalized as 100% being TAMRA-AngII 1000 nM at 10$^{-11}$ M competitor concentration and 0% being TAMRA-AngII 1 nM at maximum competitor concentration). Data are presented as mean ± SEM of three experiments performed in duplicate.

In the previous chapter it was shown that decreasing the expression of Nluc-AT$_1$ caused an increased displacement pIC$_{50}$ by the competitor ligand (Figure 3.12). Thus, it
was important to confirm that the PD 123319-induced displacement of TAMRA-AngII when Nluc-AT1 was coexpressed with AT2 was not due to reduced expression of the AT1 receptor and subsequent increased potency of PD 123319. To investigate this possibility, a similar experiment was conducted whereby Nluc-AT1 was coexpressed with untagged vasopressin V2 receptor. Treatment of these cells with 1 \( \mu \)M olmesartan and TAMRA-AngII did not result in a specific BRET signal (Figure 4.12A, B), indicating there was no binding of TAMRA-AngII to, or proximal to Nluc-AT1. Correspondingly, increasing doses of PD 123319 had no effect on the level of BRET produced. Finally, cells expressing Nluc-V2 also underwent the same treatment, confirming that TAMRA-AngII was not able to bind to the V2 receptor.

**Figure 4.12. Heteromer ligand binding with V2 receptor as negative control.** HEK293FT cells were transfected with Nluc-AT1 (A, B) or Nluc-V2 (C, D) (50 ng) and V2 (A, B) or pcDNA3 (C, D) (100 ng). Cells were incubated for 30 min with 1 \( \mu \)M olmesartan + increasing concentrations of PD 123319. Various concentrations of TAMRA-AngII were then added and incubated for a further 30 min. Without removing ligands, furimazine was added and BRET measured immediately (A, C). Following removal of ligand/media, new furimazine was added
and BRET measured (B, D). Data were measured on the PHERAstar and are presented as mean ± SEM of three experiments performed in duplicate.

4.4. Discussion

The results of the AT$_1$-AT$_2$ receptor heteromer ligand binding assay demonstrate the close proximity of the AT$_1$ and the AT$_2$ receptor, as PD 123319 produced clear, dose-dependent displacement of TAMRA-AngII binding upon coexpression of Nluc-AT$_1$ and AT$_2$. This displacement was specifically from TAMRA-AngII bound to AT$_2$ protomers as the AT$_1$ receptor was blocked with 1 µM olmesartan. Several other lines of evidence confirm that the displacement was from TAMRA-AngII bound specifically to AT$_2$ receptors and not AT$_1$ receptors: i) PD 123319 was unable to displace binding of TAMRA-AngII when Nluc-AT$_1$ was expressed without the AT$_2$ receptor, ii) PD 123319 did not inhibit AngII-induced IP$_1$ production mediated by the AT$_1$ receptor, and iii) the pIC$_{50}$ of PD 123319-induced TAMRA-AngII displacement in the AT$_1$-AT$_2$ heteromer assay was not significantly different to that observed in the Nluc-AT$_2$ binding assay.

The previous chapter revealed that reduced expression of Nluc-AT$_1$ increased the pIC$_{50}$ of olmesartan-induced displacement of TAMRA-AngII, with suggested potential causes being: i) reduced proportion of homomers displaying negative cooperativity, relative to monomers, ii) interactions between olmesartan, TAMRA-AngII and Nluc-AT$_1$ not being completely competitive, or the system not being at equilibrium, and iii) an artefact of the BRET assay. As it was not clear which of these was the cause of the observed potency shift, it was necessary to confirm that the PD 123319-induced displacement of TAMRA-AngII in the heteromer assay was not an artefact of altered ligand affinity, resulting from reduced Nluc-AT$_1$ expression upon coexpression of the AT$_2$ receptor. To test this, the heteromer assay was repeated with replacement of the AT$_2$ receptor with the vasopressin V$_2$ receptor. If the observed PD 123319-induced displacement of TAMRA-AngII was merely due to altered ligand affinity upon reduced Nluc-AT$_1$ expression, a similar reduction in Nluc-AT$_1$ expression
through coexpression with a control receptor that does not bind TAMRA-AngII would confirm this. The results showed that coexpression of the V2 receptor with Nluc-AT1 did not cause binding of TAMRA-AngII proximal to Nluc, providing further confirmation that TAMRA-AngII was binding specifically to the AT2 receptor within the AT1-AT2 heteromer.

Evidence for GPCR heteromerisation from ligand binding studies has traditionally come from radioligand binding assays that reveal ligand cooperativity upon coexpression of the two receptors of interest (Birdsall, 2010). Indeed, there are numerous examples of GPCR oligomers that display either positive (Rashid et al., 2007; Fiorentini et al., 2008; Marcellino et al., 2008) or negative (Ferre et al., 1991; Armstrong et al., 2001; El-Asmar et al., 2005; Vilardaga et al., 2008) ligand cooperativity. While this approach enables elucidation of functional consequences of oligomerisation, careful interpretation of the data is required to ensure the observed results are actually a consequence of heteromer-mediated cooperativity. For example, as GPCRs can have different affinities for ligands when they are bound to G proteins, dual receptor activation could limit the supply of G proteins, leading to altered ligand affinities and thus false ligand cooperativities (Birdsall, 2010). Furthermore, assessment of ligand cooperativity does not provide direct evidence for proximity between receptors, and nor will it enable identification of ligand interactions with neutral cooperativity. In this study, the pIC50 of the PD123319-induced displacement of TAMRA-AngII at AT1-AT2 heteromers was not significantly different to AT2 monomers/homomers, indicating no positive or negative effects of ligand cooperativity between the two protomers. Thus, investigating this heteromer using similar ligands in a radioligand binding assay would not reveal any evidence for heteromerisation between the two receptors. This highlights a major advantage of the GPCR-HIT ligand binding assay for detecting heteromers, as I have demonstrated using BRET.

Recently, a similar approach using TR-FRET has been utilised to investigate ligand binding to the dopamine D1-D3 heteromer. Hounsou et al. (2014) coexpressed SNAP-tag-labelled D1 receptors (SNAP-D1) with D3 receptors, and treated with a fluorescent D3 receptor selective ligand. Using this approach they were able to measure TR-FRET between SNAP-D1 and the fluorescent ligand bound to the D3 receptor. Similar to my study with the AT1-AT2 heteromer, they were able to confirm that the fluorescent ligand was binding specifically to the D3 receptor through displacement with
D₃ selective ligands. Furthermore, they showed that the affinity of these competitor ligands was similar (although statistical significance was not assessed) to their affinities at D₃ monomers/homomers. This indicates neutral cooperativity of ligand binding within the heteromer, as was seen in my study with the AT₁-AT₂ heteromer.

In the experiments by Hounsou et al. (2014), clear displacement of fluorescent ligand binding occurred without removal of the fluorescent and competitor ligands. In my study, although displacement was observable without removal of the ligands (particularly upon normalisation of the data), precision was not achieved until the media was removed. Rather than being a consequence of the sensitivity of the BRET approach in comparison to the TR-FRET approach, this is almost certainly due to the low affinity of TAMRA-AngII. While the TR-FRET study used 1 nM fluorescent ligand for their competition binding experiments, the low affinity of TAMRA-AngII required the use of concentrations up to 1 µM. Although this produced optimal binding and displacement of TAMRA-AngII, the presence of excess, unbound ligand will have resulted in a substantially greater background signal. Use of a fluorescent AngII ligand with greater affinity would overcome this issue, and should eliminate the need for media removal. This would then render the BRET heteromer ligand binding assay a truly homogenous assay in comparison to the TR-FRET method, as although both approaches require the addition of substrates for the energy donor (furimazine for Nluc and SNAP-Lumi4-Tb for TR-FRET; Oueslati et al., 2015), the TR-FRET method requires removal of the SNAP-tag substrate and several washes (Hounsou et al., 2014).

The heteromer ligand binding assay also revealed that there was a significantly greater level of maximum BRET in the monomer/homomer assay when compared to the heteromer assay. The major cause of this increase is likely to be due to the decreased distance between Nluc and TAMRA-AngII when bound to AT₁ receptors in comparison to TAMRA-AngII bound to AT₂ receptors heteromerised with AT₁ receptors. BRET efficiency is inversely proportional to the distance between donor and acceptor molecules to the sixth power, resulting in energy transfer than occurs over distances less than 10 nm (Dacres et al., 2012). As GPCRs are estimated to be ~4 nm in diameter (Gurevich et al., 2008), this highlights the sensitivity of the BRET assay at detecting small changes in proximity between donor and acceptor molecules.

Another potential contributing factor to the increased level of BRET in the monomer/homomer assay is that the proportion of heteromers in the system may be
lower than monomers/homomers. Although most studies suggest that GPCRs display a similar propensity to form homomers or heteromers (Ayoub et al., 2010a), there are examples of receptors that have greater (Ramsay et al., 2004; Goin et al., 2006) or lesser (Ayoub et al., 2004; Small et al., 2006) proclivity to form homomers relative to heteromers. Furthermore, the transient nature of many GPCR homomers and heteromers (Kasai et al., 2014) implies that the proportion of monomers within a system at any given time will be greater than oligomers. Thus, the existence of a greater proportion of AT1 monomers/homomers relative to AT1-AT2 heteromers may also contribute to the lower level of BRET in the heteromer assay.

The AT1-AT2 receptor has been shown to result in an inhibition of AT1 receptor signalling (AbdAlla et al., 2001b; Kumar et al., 2002; Yang et al., 2012). The lack of ligand cooperativity in this study does not conflict with these functional consequences of heteromerisation for two reasons. Firstly, cooperativity can be probe dependent and/or display ligand bias (Müller et al., 2012), so that different ligands can produce qualitatively or quantitatively different cooperativities, as seen with the chemokine CCR2b-CCR5 heteromer (El-Asmar et al., 2005). Thus the heteromerisation-mediated inhibition of AngII-induced AT1 receptor signalling may not occur with TAMRA-AngII. Secondly, the inhibition of AT1 receptor signalling does not necessarily occur at the level of ligand binding. It has been shown that although the AT1-AT2 heteromer is able to recruit β-arrestin, it is unable to subsequently internalise (Porrello et al., 2011). Retention of desensitised β-arrestin-bound AT1 receptors at the cell surface would inhibit their recycling and resensitisation, and thus provides a basis for the observed AT1 inhibition that does not occur at the level of ligand cooperativity.

The heteromer ligand binding assay also revealed that there was a small PD 123319 dose-dependent increase in the BRET signal when the AT1 receptor was expressed alone. While the lack of TAMRA-AngII displacement confirms PD 123319 is not entering the ligand binding pocket, it still appears to be modulating the level of BRET between Nluc and the bound TAMRA-AngII. A potential explanation for this result is that PD 123319 may be blocking non-specific binding sites that TAMRA-AngII would otherwise bind to. Thus, as the concentration of PD 123319 increases, there will be reduced non-specific binding of TAMRA-AngII, and consequently a greater effective concentration of TAMRA-AngII available to bind to Nluc-AT1. This
would therefore cause the dose-dependent increase in BRET signal observed with increasing PD 123319 concentration.

The results of the monomer/homomer ligand binding assays reveal that there is a significantly greater level of BRET between Nluc-AT\(_1\) and TAMRA-AngII than between Nluc-AT\(_2\) and TAMRA-AngII. This could be due to different positioning of Nluc on the two receptors, resulting in an increased distance for energy transfer between TAMRA-AngII and Nluc-AT\(_2\) in comparison to Nluc-AT\(_1\). Indeed, the N terminus of the AT\(_2\) receptor is longer than the AT\(_1\) receptor (de Gasparo et al., 2000), supporting this hypothesis. Another contributing factor could be that the AT\(_2\) receptor is expressed at a lower level than the AT\(_1\) receptor. Indeed, my work with these two receptors has often shown this, as seen in the ELISA in Chapter 2, which measured the cell surface expression of HA tagged rat and human receptors (Figure 2.2). Thus, it may be that despite using 50-fold more Nluc-AT\(_2\) cDNA (50 ng) than Nluc-AT\(_1\) cDNA (1 ng) the AT\(_1\) receptor still had a higher level of cell surface expression. As a consequence of its potentially lower expression, double the amount of AT\(_2\) receptor cDNA (100 ng) was used in the heteromer ligand binding assays.

As discussed, the results of the ligand binding assay titrations in the previous chapter revealed that there was an increase in olmesartan displacement pIC\(_{50}\) of TAMRA-AngII binding upon decreased expression of the AT\(_1\) receptor. In this chapter, 1 ng of Nluc-AT\(_1\) cDNA was used in the monomer/homomer study while 50 ng was used in the heteromer study. This increase in Nluc-AT\(_1\) quantity was because a ratio of 1:2 (50 ng:100 ng) Nluc-AT\(_1\):AT\(_2\) was deemed to be more appropriate than 1:100. The pIC\(_{50}\) of olmesartan displacement of 1 \(\mu\)M TAMRA-AngII from 50 ng Nluc-AT\(_1\) in the heteromer study (7.7 \pm 0.3) was similar to that of 50 ng Nluc-AT\(_1\) in the titration study from the previous chapter (8.2 \pm 0.2, with 682 nM TAMRA-AngII), however statistical significance could not be determined as the titration assay experiment was only performed once. In contrast, the pIC\(_{50}\) of olmesartan displacement of 1 \(\mu\)M TAMRA-AngII from 50 ng Nluc-AT\(_1\) in the heteromer study was significantly different to that observed for 1 ng Nluc-AT\(_1\) in the monomer/homomer study (9.3 \pm 0.2; p < 0.05), confirming the increase in potency with reduced Nluc-AT\(_1\) expression observed in the previous chapter.
In contrast to the shift in olmesartan displacement pIC\textsubscript{50} between AT\textsubscript{1} monomers/homomers and the AT\textsubscript{1}-AT\textsubscript{2} heteromers, there was no significant difference between the pIC\textsubscript{50} of PD 123319 displacement of 100 nM TAMRA-AngII binding to AT\textsubscript{2} monomers (50 ng Nluc-AT\textsubscript{2}, 6.3 ± 0.1) and AT\textsubscript{1}-AT\textsubscript{2} heteromers (100 ng AT\textsubscript{2}, 6.5 ± 0.1). This provides support to the hypothesis that the shift in olmesartan displacement pIC\textsubscript{50} is ligand- and/or receptor- specific, potentially caused by negative cooperativity within AT\textsubscript{1} homomers.

The AT\textsubscript{1}-AT\textsubscript{2} heteromer study also revealed that there was a significant increase in the pIC\textsubscript{50} of PD 123319-induced displacement with decreasing TAMRA-AngII concentration (Table 4.3). While this is an expected trend upon treatment with decreasing concentrations of labelled ligand, this was not observed for competitor ligands at AT\textsubscript{1} monomers/homomers (Figure 4.4; also seen in the titration assays in the previous chapter). This lack of left increase in pIC\textsubscript{50} suggests that TAMRA-AngII binds to AT\textsubscript{1} receptors with low (albeit specific) affinity and thus the observed olmesartan displacement pIC\textsubscript{50} is close to its pKi. In contrast, the increase in PD 123319 displacement pIC\textsubscript{50} with decreasing TAMRA-AngII concentration upon coexpression with the AT\textsubscript{2} receptor (Table 4.3) suggests that TAMRA-AngII may have greater affinity for AT\textsubscript{1}-AT\textsubscript{2} heteromers than to AT\textsubscript{1} monomers/homomers. Unfortunately, this effect could not be determined at AT\textsubscript{2} monomers/homomers, as maximal displacement was only reached with 100 nM TAMRA-AngII, and thus pIC\textsubscript{50} values could not be calculated. To investigate any shift in pIC\textsubscript{50} with AT\textsubscript{2} monomers/homomers, further serial dilutions below 100 nM TAMRA-AngII should be performed.

The pIC\textsubscript{50} of PD 123319 displacement of 100 nM TAMRA-AngII to AT\textsubscript{2} monomers/homomers (6.3 ± 0.1) was somewhat lower than pIC\textsubscript{50} or pKi values reported in previous studies. In cells expressing rat AT\textsubscript{2} receptors, pIC\textsubscript{50} or pKi values of 8.2 ± 0.4 (Miura et al., 1999), ~7.5 (Mukoyama et al., 1995) and 8.2 (Bosnyak et al., 2011) have been reported, while in human myometrium membranes a pKi of 7.35 ± 0.04 (Bouley et al., 1998) was observed. As discussed above, the pIC\textsubscript{50} will vary depending on the concentration of labelled ligand, which likely accounts for much of the variation in the above values. Furthermore, if the affinity of TAMRA-AngII for AT\textsubscript{2} receptors is similar regardless of heteromerisation with AT\textsubscript{1} receptors, further decreases in the
concentration of TAMRA-AngII will likely reduce the pIC$_{50}$ of PD 123319 displacement to a level closer to its pK$_i$.

The results of both the monomer/homomer assays and the heteromer assays highlight the robustness of the BRET ligand binding assay. In particular, the monomer/homomer assays produced precise data with clear displacement, evident even before normalisation of the data. The assays were highly sensitive, able to measure specific binding of TAMRA-AngII down to 100 nM for the AT$_1$ and the AT$_2$ receptor and 1 nM for the AT$_1$-AT$_2$ heteromer, despite the low affinity of TAMRA-AngII. Additionally, although the PHERAstar and CLARIOstar performed equally well in the monomer/homomer assays, the PHERAstar produced more precise results than the CLARIOstar in the heteromer assays. However, this could be overcome through normalisation of the data.
Chapter 5.  β-arrestin Recruitment to AT₁, AT₂ and B₂ Receptors and Heteromers

5.1. Introduction

Recruitment of β-arrestin to activated and phosphorylated GPCRs is critical to allow receptor desensitisation (in terms of G protein signalling) and subsequent internalisation, protecting the cell against agonist overstimulation. In addition, its actions as a scaffold enable the initiation of β-arrestin-dependent signalling. The AT₁ receptor is a typical GPCR in that it rapidly recruits β-arrestin upon stimulation with agonist. It is classified as a class B GPCR in terms of its internalisation profile: it has similar affinity for both β-arrestin1 and β-arrestin2 (Oakley et al., 2000), and forms stable β-arrestin complexes which co-internalise into endocytic vesicles (Oakley et al., 2001). In contrast, the AT₂ receptor does not recruit β-arrestin upon agonist stimulation (Turu et al., 2006). The B₂ receptor resembles a class B GPCR in that has a similar affinity for both β-arrestin1 and β-arrestin2 (Willets et al., 2015) and co-internalises with β-arrestin into endosomes (Simaan et al., 2005). It has been suggested that it dissociates from β-arrestins and recycles more rapidly than typical class B GPCRs (Simaan et al., 2005), however several studies report a recycling kinetic profile similar to the AT₁ receptor (Kalenga et al., 1996; Hein et al., 1997; Hunyady et al., 2002; Guo et al., 2003; Enquist et al., 2007).

Although the monomeric/homomeric AT₂ receptor does not recruit β-arrestin, upon heteromerisation with the AT₁ receptor, β-arrestin is recruited proximal to the AT₂ receptor in an AngII-induced manner (Porrello et al., 2011). Three studies have previously investigated β-arrestin recruitment to the controversial AT₁-B₂ receptor heteromer. Hansen et al. (2009) observed no modulation of AngII-induced β-arrestin recruitment to the AT₁ receptor upon coexpression of the B₂ receptor. Using the GPCR-HIT assay, our lab demonstrated that β-arrestin2 was not recruited proximal to B₂-Rluc8
upon coexpression and activation of the AT₁ receptor (See et al., 2011). Wilson et al. (2013) showed that the β-arrestin pathway-selective AT₁ receptor ligand SII did not modulate BK-induced β-arrestin recruitment in cells expressing B₂-YFP and β-arrestin2-Rluc, however it did cause reduced B₂ receptor signalling and promoted B₂ receptor internalisation in a β-arrestin-dependent manner. Thus, the β-arrestin recruitment profile of the putative AT₁-B₂ heteromer has not been completely explored, particularly in terms of BK-induced recruitment to the AT₁ protomer. β-arrestin recruitment to the putative AT₂-B₂ receptor heteromer has not previously been investigated.

To investigate β-arrestin recruitment to the AT₁-B₂ heteromer and the AT₂-B₂ heteromer, three different GPCR-HIT assays were used. Figure 5.1A shows the configuration of the standard β-arrestin GPCR-HIT assay, whereby a ligand-induced BRET signal indicates recruitment of β-arrestin2-Venus to the untagged receptor and proximity to the Rluc8-tagged receptor. This is indicative of receptor heteromerisation. Figure 5.1B and C show modified configurations of the standard BRET GPCR-HIT assay, which include BiFC using split Venus. In both configurations, a ligand-induced BRET signal indicates recruitment of β-arrestin to the receptor heteromer. It is important to note that complementation of the two parts of Venus is effectively irreversible, so the two associated fusion proteins are also irreversibly joined. This needs to be taken into account when interpreting data from such experiments. The modified GPCR-HIT assays may enable monitoring of weak or transient interactions, as well as potentially providing information on interactions between all three receptors.
Chapter 5. \(\beta\)-arrestin Recruitment to AT\(_1\), AT\(_2\) and B\(_2\) Receptors and Heteromers

A. GPCR-HIT assay with BRET

- Receptor A
- Receptor B
- Rluc8
- Arrestin
- Venus

Ligand-selective for Receptor B or Heteromer

B. GPCR-HIT assay with BRET/BiFC - \(\beta\)-arrestin2-Rluc8

- Receptor A
- Receptor B
- Receptor C
- V1
- V2
- Arrestin
- Rluc8

Ligand-selective for Receptor A or B (modified GPCR-HIT assay)

Ligand-induced BRET

C. GPCR-HIT assay with BRET/BiFC - Receptor-Rluc8

- Receptor A
- Receptor B
- Receptor C
- Rluc8
- Arrestin
- Venus

Ligand-selective for Receptor A or B (modified GPCR-HIT assay)

Ligand-induced BRET
Figure 5.1. GPCR-HIT β-arrestin assays used for detection of receptor heteromers. (A) The standard GPCR-HIT assay monitors receptor-receptor interactions through recruitment of β-arrestin to the heteromer. Receptor A is tagged with Rluc8 while Receptor B remains untagged with respect to the system. β-arrestin2 is tagged with the Venus fluorophore. A BRET signal upon addition of a ligand selective for untagged Receptor B is indicative of receptor heteromerisation. (B) The BRET/BiFC assay includes Venus fluorophore complementation. In this configuration Receptor A and Receptor B are tagged with the complementary parts of Venus (V1 and V2), while β-arrestin2 is tagged with Rluc8. A ligand-induced BRET signal indicates recruitment of β-arrestin2-Rluc8 proximal to the Receptor A- Receptor-B heteromer in which V1 and V2 have formed a functional Venus fluorophore. Addition of untagged Receptor C enables monitoring of a heteromer involving three receptors, a modified form of GPCR-HIT. (C) The second BRET/BiFC assay again uses Venus complementation. In this configuration one part of Venus (V1) is fused to Receptor B while β-arrestin2 is tagged with the complementary part (V2). Receptor A is tagged with Rluc8 while Receptor C remains untagged. If the two parts of Venus come together through recruitment of β-arrestin2 they are able to form a functional Venus fluorophore. When Receptor A and Receptor B are in close proximity the resulting BRET signal is indicative of receptor interactions. Addition of Receptor C again enables monitoring of a heteromer involving three receptors, which is again a modified form of GPCR-HIT.

5.2. Materials and Methods

5.2.1. Materials

Ligands used were AngII and BK (Sigma Aldrich).

5.2.2. cDNA constructs

The cDNA constructs used in this chapter are listed in Table 5.1, and detailed in Section 2.2.1.
Table 5.1. cDNA constructs used in this chapter.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Species</th>
<th>Genbank Accession No.</th>
<th>Constructs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiotensin II receptor type 1</td>
<td>Rat</td>
<td>NM_030985</td>
<td>AT₁-Rluc8, HA-AT₁, AT₁-V1, AT₁-V2</td>
</tr>
<tr>
<td>Angiotensin II receptor type 2</td>
<td>Rat</td>
<td>NM_012494</td>
<td>AT₂-Rluc8, HA-AT₂, AT₂-V1, AT₂-V2</td>
</tr>
<tr>
<td>Bradykinin receptor type 2</td>
<td>Human</td>
<td>AY275465</td>
<td>B₂-Rluc8, HA-B₂, B₂-V1, B₂-V2</td>
</tr>
<tr>
<td>β-arrestin2</td>
<td>Human</td>
<td>NM_004313</td>
<td>β-arrestin2-Venus, β-arrestin2-Rluc8</td>
</tr>
<tr>
<td>β-arrestin2</td>
<td>Rat</td>
<td>NM_012911</td>
<td>β-arrestin2-V2</td>
</tr>
</tbody>
</table>

5.2.3. Mammalian cell transfections

Transient transfections were carried out in HEK293FT cells as previously described in the General Materials and Methods Chapter (Section 2.3.3). cDNA quantities are described in figure legends.

5.2.4. BRET assays

All assays in this chapter were carried out using eBRET, as described previously in the General Materials and Methods Chapter (Section 2.4.2).
5.2.5. BiFC fluorescence assessment

In the BRET/BiFC assays with β-arrestin2-Rluc8 and two receptors tagged with the complementary portions of Venus, fluorescence measurements were recorded to determine the constitutive level of fluorescence complementation. Extra samples from the same transfections as the BRET assays were transfected into black 96 well plates. 48 hours following transfection, the media was replaced with PBS and fluorescence was measured on the EnVision Multilabel Plate Reader (excitation 485, emission 535).

5.2.6. Data presentation and statistical analyses

All curve fitting and statistical analysis were carried out as previously described in Section 2.8.

5.3. Results

5.3.1. β-arrestin2 recruitment to AT₁, AT₂, and B₂ receptors and heteromers with BRET

Initial investigation of β-arrestin recruitment to the AT₁ and the B₂ receptor showed, as expected, β-arrestin2-Venus was recruited strongly to the receptor monomers and/or homomers when treated with their endogenous ligand (Figure 5.2A, C). Coexpression with the AT₁a receptor resulted in β-arrestin2-Venus recruitment proximal to B₂R-Rluc8 when treated with BK but not AngII (Figure 5.2B), as seen previously (See et al., 2011). However, in the reverse configuration β-arrestin2-Venus was recruited proximal to AT₁-Rluc8 when co-expressed with B₂ and treated with either AngII or BK (Figure 5.2D). While the AngII-induced signal may well be largely the result of β-arrestin2 recruitment to monomers/homomers, the BK-induced signal suggests that β-arrestin2 is being recruited to AT₁-B₂ heteromers. Additionally, dual agonist treatment caused an increased level of β-arrestin2 recruitment proximal to the AT₁ receptor.
Chapter 5. \(\beta\)-arrestin Recruitment to AT\(_1\), AT\(_2\) and B\(_2\) Receptors and Heteromers

Figure 5.2. \(\beta\)-arrestin2 GPCR-HIT between the AT\(_1\) receptor and the B\(_2\) receptor. HEK293FT cells were transfected with receptor-Rluc8 (75 ng), \(\beta\)-arrestin2-Venus (50 ng) and pcDNA3 or untagged receptor (75 ng) as described on graphs. Basal BRET measurements were recorded for 10 min before adding agonists and measuring for a further 60 min. Data are presented as mean ± SEM of three independent experiments performed in triplicate.

Figure 5.3A confirmed the lack of AngII-induced \(\beta\)-arrestin recruitment to the AT\(_2\) receptor. As well, the previously reported AngII-induced \(\beta\)-arrestin2 recruitment to the AT\(_2\) receptor upon coexpression of the AT\(_1\) receptor was confirmed (Figure 5.3B). Unlike the AT\(_1\)-AT\(_2\) heteromer, recruitment of \(\beta\)-arrestin to the AT\(_2\)-B\(_2\) receptor has not been investigated previously. Coexpression of the B\(_2\) receptor caused BK-dependent recruitment of \(\beta\)-arrestin2-Venus proximal to AT\(_2\)-Rluc8 (Figure 5.3C).
Figure 5.3. β-arrestin2 GPCR-HIT between the AT₂ receptor and the AT₁a receptor or the B₂ receptor. HEK293FT cells were transfected with AT₂-Rluc8 (75 ng), β-arrestin2-Venus (50 ng) and pcDNA3 or untagged receptor (75 ng) as described on graphs. Basal BRET measurements were recorded for 25 min before adding agonists and measuring for a further 115 min. Data are presented as mean ± SEM of four independent experiments performed in triplicate.
5.3.2. β-arrestin2 recruitment to AT₁, AT₂, and B₂ receptors with BRET and BiFC

5.3.2.1. β-arrestin2-Rluc8 assays

In the following experiments, interactions between all three receptors were investigated. In the first set of BRET experiments utilising BiFC, β-arrestin2 was tagged with Rluc8 while two of the receptors were tagged with the complementary parts of Venus. The third receptor remained untagged with respect to the system. Through unification of the complementary parts of Venus, this assay enabled monitoring of β-arrestin2 recruitment specifically to the heteromers. Introduction of the third, untagged receptor allowed investigation of functional interactions between all three receptors.

Before assessing the results of the BRET experiments, the fluorescence from each of the samples was measured. Figure 5.4 shows that only small increases in fluorescence from background was measured from most of the transfections, indicating low levels of fluorescence complementation. Some BiFC receptor combinations were more successful than others, such as AT₂-V1 + AT₁a-V2.
Figure 5.4. Fluorescence from BiFC-tagged receptor constructs. β-arrestin2-Rluc8 (60 ng; not shown on graph), receptor-V1 (60 ng) and receptor-V2 (60 ng) plasmids were transfected into HEK293FT cells with either pcDNA3 or untagged receptor (60 ng) as described. Mock samples were not transfected. Data are presented as mean ± SEM of three independent experiments performed in triplicate.

Figure 5.5 shows AngII-induced β-arrestin2 recruitment to the AT₁-AT₂ heteromer, with a weaker signal than observed in the standard GPCR-HIT assay (Figure 5.3B). As well, the signal between AT₂-V1 and AT₁-V2 (Figure 5.5C) was slightly stronger than between AT₁-V1 and AT₂-V2 (Figure 5.5A). Coexpression of the B₂ receptor did not alter the level of AngII-induced recruitment but also caused a similar level of BK-induced recruitment (Figure 5.5B, D).
Chapter 5. $\beta$-arrestin Recruitment to AT$_1$, AT$_2$ and B$_2$ Receptors and Heteromers

Figure 5.5. GPCR-HIT with BRET and BiFC between $\beta$-arrestin2-Rluc8 and AT$_1$-AT$_2$ heteromer. $\beta$-arrestin2-Rluc8 (60 ng), receptor-V1 (60 ng) and receptor-V2 (60 ng) plasmids were transfected into HEK293FT cells with either pcDNA3 or untagged receptor (60 ng) as described on graphs. Basal BRET measurements were recorded for 20 min before adding agonists and measuring for a further 90 min. Data are from the same experimental samples as in Figure 5.4. Data are presented as mean ± SEM of three independent experiments performed in triplicate.

$\beta$-arrestin2 recruitment to the AT$_1$-B$_2$ heteromer is illustrated in Figure 5.6. As seen in the standard GPCR-HIT assay (Figure 5.2D), there was similar, albeit weaker BK-induced $\beta$-arrestin2 recruitment to the heteromer (Figure 5.6A, C). There was a similar level of AngII-induced $\beta$-arrestin2 recruitment, as well as an increased level of recruitment with dual agonist treatment. Here, both configurations produce similar levels of $\beta$-arrestin2 recruitment (AT$_1$-V1 + B$_2$-V2, Figure 5.6A and B$_2$-V1 + AT$_1$-V2,
Figure 5.6C). Introduction of the AT$_2$ receptor does not alter the response to either single or dual agonist treatment (Figure 5.6B, D).

![Graphs showing ligand-induced BRET over time for different receptor configurations](image)

**Figure 5.6.** GPCR-HIT with BRET and BiFC between β-arrestin2-Rluc8 and AT$_1$ + AT$_2$ heteromer. β-arrestin2-Rluc8 (60 ng), receptor-V1 (60 ng) and receptor-V2 (60 ng) plasmids were transfected into HEK293FT cells with either pcDNA3 or untagged receptor (60 ng) as described on graphs. Basal BRET measurements were recorded for 20 min before adding agonists and measuring for a further 90 min. Data are from the same experimental samples as in Figure 5.4. Data are presented as mean ± SEM of three independent experiments performed in triplicate.

As seen in the standard GPCR-HIT assay, again with a weaker signal, (Figure 5.3C), the AT$_2$-B$_2$ heteromer recruited β-arrestin2 in a BK- but not AngII-dependent...
manner (Figure 5.7A, C). A more robust and sustained signal was observed between AT2-V1 and B2-V2 (Figure 5.7A) than between B2-V1 and AT2-V2 (Figure 5.7C). Coexpression of the AT1 receptor caused a moderate reduction in the level of BK-induced β-arrestin2 recruitment, while also causing a small level of AngII-induced recruitment (Figure 5.7B, D).

Figure 5.7. GPCR-HIT with BRET and BiFC between β-arrestin2-Rluc8 and AT2-B2 receptor heteromer. β-arrestin2-Rluc8 (60 ng), receptor-V1 (60 ng) and receptor-V2 (60 ng) plasmids were transfected into HEK293FT cells with either pcDNA3 or untagged receptor (60 ng) as described on graphs. Basal BRET measurements were recorded for 20 min before adding agonists and measuring for a further 90 min. Data are from the same experimental samples as in Figure 5.4. Data are presented as mean ± SEM of three independent experiments performed in triplicate.
5.3.2.1. Receptor-Rluc8 assays

In the next set of BRET experiments utilising BiFC, β-arrestin2 was tagged with the V2 part of Venus while one receptor was tagged with the complementary part. The two remaining receptors were tagged with either Rluc8 or were untagged with respect to the system. This configuration provided information on the different strengths of interactions through monitoring of the ligand-induced BRET signals with a single Rluc8-tagged receptor. Again, introduction of the third, untagged receptor allowed investigation of interactions between all three receptors.

Similar to previous experiments, treatment of cells expressing AT1-Rluc8, AT2-V1 and β-arrestin2-V2, caused AngII-induced recruitment of β-arrestin2 to the AT1-AT2 heteromer (Figure 5.8A). Addition of the B2 receptor into this system caused a reduction in the AngII-induced BRET signal, as well as also causing BK-induced β-arrestin2-V2 recruitment (Figure 5.8B).

The interaction between the AT1 and the B2 receptor resulted in a weaker AngII-induced BRET signal than observed for the AT1-AT2 heteromer (Figure 5.8A), regardless of whether they were coexpressed with (Figure 5.8D) or without (Figure 5.8C) the AT2 receptor. As in the standard GPCR-HIT assay, BK treatment caused β-arrestin2 recruitment to the AT1-B2 heteromer.
Investigation of the AT₂-B₂ heteromer using this assay again resulted in a BK-induced recruitment of β-arrestin2 to the heteromer (Figure 5.9A). This signal was moderately reduced by the presence of the AT₁ receptor, particularly when activated (Figure 5.9B).

A weaker signal was observed between the AT₁ and the B₂ receptor (Figure 5.9C, D) than seen in the AT₂-B₂ heteromer (Figure 5.9A). There was no AngII-induced
β-arrestin2 recruitment observed in this configuration, and coexpression of the AT\textsubscript{2} receptor did not alter the response (Figure 5.9D).

Figure 5.9. **GPCR-HIT with BRET and BiFC between B\textsubscript{2}-Rluc8, AT\textsubscript{1a}, AT\textsubscript{2} and β-arrestin2.** B\textsubscript{2}-Rluc8 (60 ng) and β-arrestin2-V\textsubscript{2} (60 ng) plasmids were transfected into HEK293FT cells with receptor-V\textsubscript{2} (60 ng) and either pcDNA\textsubscript{3} or untagged receptor (60 ng) as described on graphs. Basal BRET measurements were recorded for 20 min before adding agonists and measuring for a further 90 min. Data are presented as mean ± SEM of three independent experiments performed in triplicate.
5.4. Discussion

5.4.1. AT-B2 receptor heteromer

This study provides evidence for the existence of the AT2-B2 receptor heteromer in transfected cells. This is shown by the GPCR-HIT signal obtained through the translocation of β-arrestin2 proximal to the AT2 receptor upon activation of the B2 receptor. This finding reveals that the heteromer has novel pharmacology from that of the monomeric/homomeric AT2 receptor, which does not recruit β-arrestin upon treatment with agonist.

Recruitment of β-arrestin to a GPCR has a variety of effects on the functioning of the receptor. β-arrestin-bound GPCRs are unable to couple to G proteins and are desensitised from G protein signalling. The AT2 receptor is an unusual GPCR in that it does not readily signal through G proteins, although there is some evidence it can couple to Gaαi/o (Huang et al., 1995; Hayashida et al., 1996; Zhang et al., 1996b; Hansen et al., 2000; Senbonmatsu et al., 2003; Lara et al., 2006) and Gaαs (Zhang et al., 1996b; Feng et al., 2002). It is, however, known to interact with other signalling and regulatory proteins at its intracellular face. Interactions with the ErbB3 epidermal growth factor receptor (Knowle et al., 2000), the scaffold protein connector enhancer of Ksr (CNK; Fritz et al., 2005) and tissue inhibitor of metalloproteinases-3 (TIMP-3; Kang et al., 2008) are implicated in AT2 receptor-mediated antigrowth effects, while interactions with the transcription factor promyelocytic zinc finger protein (PLZF) are involved in the mediation of cardiac hypertrophy (Senbonmatsu et al., 2003). Interactions with the Na+/H+ exchanger NHE6 are important for AT2 receptor regulation of sodium levels (Pulakat et al., 2005). Finally, interactions with ATIP1 result in antigrowth effects (Nouet et al., 2004; Fujita et al., 2009) and neural differentiation (Li et al., 2007b). It is possible that when the AT2 receptor is heteromerised with the B2 receptor (or the AT1 receptor) β-arrestin recruitment could cause inhibition of the above AT2 receptor interactions leading to alterations in signalling. In addition, many studies reveal that the AT2 receptor is constitutively active (Miura et al., 1999; Miura et al., 2000; Jin et al., 2002; Su et al., 2002; D’Amore et al., 2005). If β-arrestin recruitment to the AT2-B2 heteromer were able to block the interaction between the AT2 receptor and its various...
signalling partners, this would be a mechanism of reducing the constitutive activity observed for this receptor.

In addition to their role in receptor desensitisation, it is now well established that endocytosed GPCR-bound β-arrestins are able to aid in the initiation of signalling cascades through their function as scaffold proteins. Numerous signalling molecules are regulated through this property of β-arrestins, in particular the MAPKs ERK, JNK, and p38. The AT₂ receptor generally exerts inhibitory effects on MAPK cascades through activation of phosphatases (Bottari et al., 1992; Huang et al., 1995; Bedecs et al., 1997; Matsubara et al., 2001). The potential for additional signalling through β-arrestin scaffolds adds another, unanticipated level of complexity to AT₂ receptor signalling. Furthermore, this heteromerisation-mediated recruitment of β-arrestin could explain some of the contradictory studies that report AT₂-mediated activation of MAPKs (Miura et al., 2000; Miyamoto et al., 2008).

5.4.2. AT₁-B₂ receptor heteromer

Using the GPCR-HIT assay, this study confirmed previous research showing that coexpression of the AT₁ and the B₂ receptor does not alter BK- or AngII-induced β-arrestin recruitment proximal to the B₂ receptor (See et al., 2011; Wilson et al., 2013). Similarly, there was no modulation to the AngII-induced β-arrestin recruitment to the AT₁ receptor upon coexpression of the B₂ receptor, as reported previously by Hansen et al. (2009) as part of their evidence against the existence of the AT₁-B₂ heteromer. However, BK-induced β-arrestin recruitment proximal to the AT₁ receptor had not been previously investigated. The results of my study showed that upon coexpression of the B₂ receptor and treatment with BK, β-arrestin2-Venus was recruited proximal to AT₁-RLuc8. This GPCR-HIT signal not only supports the existence of the controversial AT₁-B₂ receptor heteromer but also reveals novel pharmacology in terms of BK-induced β-arrestin recruitment to an AT₁ receptor complex. Furthermore, dual agonist treatment with both AngII and BK resulted in an increased level of β-arrestin recruitment, likely due to the additive effect of AngII-induced recruitment to AT₁ monomers/homomers and BK-induced recruitment to AT₁-B₂ heteromers. A similar additive response was
observed with respect to IP$_1$ production upon dual agonist treatment of AT$_1$ and B$_2$ receptor-expressing cells by AbdAlla et al. (2000).

Both the AT$_1$ and B$_2$ receptors resemble class B GPCRs in terms of their affinities for β-arrestin1 and β-arrestin2 (Oakley et al., 2000; Willets et al., 2015) and their colocalisation in endocytic vesicles with β-arrestins (Oakley et al., 2001; Simaan et al., 2005). Additionally, it has been suggested that the B$_2$ receptor dissociates from β-arrestins and recycles more rapidly than typical class B receptors (Simaan et al., 2005). Conversely, other studies report a recycling kinetic profile similar to that of the AT$_1$ receptor (Kalenga et al., 1996; Hein et al., 1997; Hunyady et al., 2002; Guo et al., 2003; Enquist et al., 2007). Endosomal β-arrestin-dependent signalling of the AT$_1$ receptor is often reported to mediate beneficial cardioprotective processes (Zhai et al., 2005; Aplin et al., 2007; Ahn et al., 2009; Hostrup et al., 2012), while G protein-dependent signalling mediates the majority of the vasoconstrictive response (Rajagopal et al., 2006; Aplin et al., 2007). If BK-induced β-arrestin recruitment shifts the endosomal sequestration of the AT$_1$ receptor-β-arrestin complex so that it resembles the rapid recycling profile of the B$_2$ receptor, this could shift the balance of the AT$_1$ receptor-mediated signalling output. Quitterer and colleagues have repeatedly reported increased G protein signalling upon heteromerisation of the AT$_1$ and B$_2$ receptor (AbdAlla et al., 2000; AbdAlla et al., 2005; AbdAlla et al., 2009), suggesting that it is involved in AngII-induced hypertension (AbdAlla et al., 2001a; Quitterer et al., 2014). Increased rates of recycling by the AT$_1$ receptor when complexed with the B$_2$ receptor would reduce the level of β-arrestin-mediated endosomal signalling by the AT$_1$ receptor and increase the level of G protein-mediated signalling, fitting the observations of Quitterer and colleagues.

5.4.3. Interactions between the AT$_1$, the AT$_2$ and the B$_2$ receptor

The first general observation of the BRET/BiFC β-arrestin assays is that in all cases the BRET signals were smaller than those observed in the standard GPCR-HIT assay. This is attributable to two factors: the expression of four, rather than three constructs, causing a reduction in the overall expression of all the transfected plasmids; and the requirement for Venus complementation, which results in there being less functional fluorophores
available for BRET. Additionally, it is likely that complemented Venus may not function as effectively as normal Venus fluorophores. Despite this, similar trends in β-arrestin recruitment to the three heteromers were observed. The similar trends observed in both the BRET and the BRET/BiFC GPCR-HIT assays suggests that Venus complementation is not forcing formation of the heteromers in the endoplasmic reticulum. Additionally, as both assays are run over similar time courses, it is unlikely that disassociation of the heteromers is being inhibited by the irreversibility of Venus complementation, at least within the time frame assessed within the experiments.

The second set of experiments, using Receptor-Rluc8 and β-arrestin2-V2, enabled the monitoring of the different strength of interactions between the receptors within the three heteromers. The AT2 receptor appeared to form robust interactions with both the AT1 and the B2 receptor, as indicated by the AngII- and BK-induced β-arrestin recruitment to the AT1-AT2 heteromer and the AT2-B2 heteromer, respectively. In these assays, addition of the third receptor into the system produced substantial reductions in the level of β-arrestin recruitment, likely due to competition from the new heteromers that are formed in the presence of the third receptor. In contrast to the strong interactions observed between the AT2 receptor and both the AT1 and the B2 receptor, smaller BRET signals were generated between β-arrestin and the AT1 and the B2 receptor. This suggests that the interaction between these two receptors may be of a weaker or more transient nature. Addition of the AT2 receptor into these experiments had no effect on the β-arrestin recruitment profiles, likely due to low expression levels of the AT2 receptor (as seen in Figure 2.2, Figure 4.6). Importantly, these AT1-B2 heteromer assays show AngII- as well as BK-induced β-arrestin recruitment to the heteromer. The heteromer-specificity of the BRET/BiFC assay suggests that both ligands are able to cause recruitment of β-arrestin to the heteromer. The lack of AngII-induced β-arrestin recruitment to B2-Rluc8 in the standard GPCR-HIT assay has two potential explanations: i) B2 receptors form monomers/homomers preferentially to AT1-B2 heteromers, making the GPCR-HIT signal negligible, and ii) an inability to detect the B2-Rluc8/β-arrestin2-Venus/AT1 complex due to unfavourable donor-acceptor distance and/or orientation.

Perhaps the most interesting result of the BRET/BiFC assays is the AngII- or BK-induced β-arrestin recruitment to AT2-B2 or AT1-AT2 heteromers upon coexpression of untagged AT1 or B2 receptor, respectively. For the AT1-AT2 heteromer,
this was observed in the BK-induced β-arrestin2-V2 recruitment to the AT1-Rluc8/AT2-V2 complex when it was coexpressed with the untagged B2 (Figure 5.8B) and the BK-induced β-arrestin2-Rluc8 recruitment to the AT1-AT2 split Venus complexes when coexpressed with the untagged B2 (Figure 5.5B, D). For the AT2-B2 heteromer this was observed in the AngII-induced β-arrestin2-Rluc8 recruitment to the AT2-B2 split Venus complexes when coexpressed with the untagged AT1 receptor (Figure 5.7B, D). These results suggest the possibility that these three receptors may all be in a complex with one another. Similar approaches using combinations of BRET, BiFC and/or BiLC have previously been used to investigate the stoichiometry of oligomeric GPCR complexes. These studies have revealed higher order oligomers of calcitonin receptor-like receptor complexes (Héroux et al., 2007), CXCR4 homo-oligomers (Hamatake et al., 2009) and CXCR4-CCR2 heteromers (Armando et al., 2014). Furthermore, this approach has also been used to investigate complexes containing three different receptor protomers: the adenosine A2A-dopamine D2-metabotropic glutamate type 5 receptor heteromer (Cabello et al., 2009), and the CCR5-CD4-CXCR4 heteromer (Martínez-Muñoz et al., 2014). So far, these two heteromers, plus the adenosine A2A-cannabinoid CB1-dopamine D2 receptor heteromer (Navarro et al., 2008) and the GPR39-serotonin 5-HT1A-galanin GalR1 receptor heteromer (Tena-Campos et al., 2015) are the only examples of trimeric receptor heteromers to be described. While the evidence from this study does suggest the existence of oligomers containing the AT1, the AT2 and the B2 receptor, further evidence is required to support this hypothesis. In addition, due to the permanent nature of the unification of the split Venus constructs, there is the potential to get false positives with this assay. Thus, the four studies above describing trimeric receptor heteromers all used other supporting techniques to confirm the existence of the heteromers, such as sequential BRET/FRET (Carriba et al., 2008), and similar approaches should be taken to confirm the existence of AT1-AT2-B2 receptor heteromers. In any case, the results of these BRET/BiFC assays provide insight into the potential complicated nature of interactions and competition between all three receptors in this system. As these receptors are often expressed in the same cells, it is likely that this complex interplay may underpin some of the controversy and inconsistency surrounding the AT1-B2 heteromer.
Chapter 6. Internalisation and Trafficking of AT$_1$, AT$_2$ and B$_2$ Receptors and Heteromers

6.1. Introduction

Recruitment of β-arrestin to a receptor not only completes the process of G protein signalling desensitisation, it also initiates internalisation of the receptor. As mentioned, the AT$_1$ receptor (Oakley et al., 2001) and the B$_2$ receptor (Simaan et al., 2005) both co-internalise in endosomes with β-arrestins. The AT$_1$ receptor remains β-arrestin bound and sequestered in endosomes for sustained periods (Oakley et al., 2001). It has been suggested that the B$_2$ receptor dissociates from β-arrestins and recycles more rapidly than typical class B receptors (Simaan et al., 2005), however other studies report a recycling kinetic profile similar to that of the AT$_1$ receptor (Kalenga et al., 1996; Hein et al., 1997; Hunyady et al., 2002; Guo et al., 2003; Enquist et al., 2007). In contrast to the AT$_1$ and the B$_2$ receptor, not only does the AT$_2$ receptor not recruit β-arrestin, it is also unable to subsequently internalise (Hein et al., 1997; Turu et al., 2006).

In Chapter 5 it was demonstrated that β-arrestin is recruited proximal to the AT$_2$ receptor in an AngII- or BK-dependent manner upon coexpression of the AT$_1$ or the B$_2$ receptor, respectively. It has previously been reported that this AngII-induced β-arrestin recruitment to the AT$_1$-AT$_2$ heteromer does not result in subsequent internalisation (Porrello et al., 2011). The internalisation profile of the AT$_2$-B$_2$ heteromer has not previously been investigated. The controversial AT$_1$-B$_2$ heteromer has been shown to internalise upon treatment with AngII and BK (AbdAlla et al., 2010; Quitterer et al., 2011), as well as the β-arrestin pathway-selective AT$_1$ receptor agonist SII (Wilson et al., 2013).

To investigate the internalisation and trafficking profiles of the three heteromers a relatively new variant of the GPCR-HIT assay was utilised (Figure 6.1). Here, the Venus-labelled interacting proteins are specific membrane-associated protein markers.
This enables monitoring of the trafficking of the receptor/heteromer through proximity with the Venus-tagged markers (Lan et al., 2011; Balla et al., 2012; Lan et al., 2012). The cell surface marker is a 25 amino acid C terminal fragment of the oncogenic small GTPase Kras (Friday et al., 2005). This C terminal fragment contains a polybasic motif that targets it to the plasma membrane (Hancock et al., 1990; Chiu et al., 2002). The three other membrane-associated markers are from the Rab family of small GTPases. Rab5 is localised to early endosomes while Rab7 and Rab11 are localised to late and slow recycling endosomes, respectively (Stenmark, 2009). Use of these endosomal markers enables generation of a detailed internalisation and trafficking profile, allowing monitoring of receptor localisation in distinct cellular compartments.

Figure 6.1. Application of GPCR-HIT to monitor internalisation and trafficking of receptor heteromers. The GPCR-HIT assay allows for monitoring of the heteromer’s cellular trafficking profile through proximity with specific membrane-associated protein markers. Here, Receptor A is tagged with the Rluc8 donor enzyme while Receptor B remains untagged. The membrane-associated proteins are tagged with Venus. The cell surface membrane-associated protein marker is a C terminal fragment of Kras and a reduction in BRET signal upon addition of a ligand selective for Receptor B or Heteromer.
ligand selective for the untagged Receptor B is indicative of receptor heteromerisation and co-
internalisation. Similarly, when the membrane associated protein is endosomal, such as Rab5 
(early endosome), Rab7 (late endosome) and Rab11 (slow recycling endosome), an increase in 
BRET signal upon addition of a ligand selective for the untagged Receptor B is indicative of 
receptor heteromerisation and co-sequestration into endosomes.

6.2. Materials and Methods

6.2.1. Materials

Ligands used were AngII, BK, CGP 42114, PD 123319 (Sigma Aldrich), HOE140 
(Tocris Bioscience).

6.2.2. cDNA constructs

The cDNA constructs used in this chapter are listed in Table 6.1, and detailed in Section 
2.2.1.

Table 6.1. cDNA constructs used in this chapter.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Species</th>
<th>Genbank Accession No.</th>
<th>Constructs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiotensin II receptor type 1</td>
<td>Rat</td>
<td>NM_030985</td>
<td>AT₁α-Rluc8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HA-AT₁α</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AT₁α-Venus</td>
</tr>
<tr>
<td>Angiotensin II receptor type 2</td>
<td>Rat</td>
<td>NM_012494</td>
<td>AT₂-Rluc8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HA-AT₂</td>
</tr>
</tbody>
</table>
6.2.3. Mammalian cell transfections

Transient transfections were carried out in HEK293FT cells as previously described in the General Materials and Methods Chapter (Section 2.3.3). cDNA quantities are described in figure legends.

6.2.4. BRET assays

All assays in this chapter were carried out using eBRET, as described previously in the General Materials and Methods Chapter (Section 2.4.2).

6.2.5. Data presentation and statistical analyses

All curve fitting and statistical analysis were carried out as previously described in Section 2.8.
6.3. Results

Receptor internalisation and trafficking was investigated using GPCR-HIT with membrane-bound proteins associated with specific cellular compartments. Venus tagged Kras is a plasma membrane-associated protein while Venus tagged Rab5a, Rab7 and Rab11 are associated with early, late and slow recycling endosomes, respectively (Figure 6.1) (Stenmark, 2009; Lan et al., 2011; Lan et al., 2012).

Figure 6.2 illustrates the robustness of this assay at investigating receptor localisation, as all three receptors display their expected agonist-induced internalisation profiles. Upon stimulation with AngII, cells expressing AT1-Rluc8 and Venus-Kras show a large reduction in the ligand-induced BRET signal (Figure 6.2A), which indicates internalisation of the AT1 receptor away from the plasma membrane bound Kras. Figure 6.2B, C, D all show AngII-induced increases in BRET, indicating the endosomal trafficking of the AT1 receptor as it becomes proximal with the Rab5, Rab7 and Rab11 markers. The B2 receptor followed a very similar internalisation (Figure 6.2I) and endosomal trafficking path (Figure 6.2J, K, L) upon treatment with BK, albeit with a marginally slower kinetic profile. It is important to note that these BRET internalisation assays are done in the continued presence of agonist, and thus the receptors are expected to be continually internalising and recycling. The resulting data then shows the receptors plateauing at a dynamic steady state.

In contrast to the AT1 and the B2 receptor, cells expressing AT2-Rluc8 and Venus-Kras (Figure 6.2E), Venus-Rab5 (Figure 6.2F), Venus-Rab7 (Figure 6.2G) or Venus-Rab11 (Figure 6.2H) had no change in ligand-induced BRET, indicating there was no internalisation or sequestration of the receptor into endosomes, as expected for this receptor (Pucell et al., 1991; Mukoyama et al., 1995; Hein et al., 1997; Turu et al., 2006).
Chapter 6. Internalisation and Trafficking of AT_1, AT_2 and B_2 Receptors and Heteromers

Figure 6.2. Internalisation of AT_1, AT_2, and B_2 monomers/homomers. Venus-tagged membrane protein plasmids (50 ng) were transfected into HEK293FT cells with either AT_1-Rluc8 (75 ng; A – D) or AT_2-Rluc8 (100 ng; E – H) or B_2-Rluc8 (75 ng; I – L) and pcDNA3 (to make a total of 200 ng of plasmid). Basal BRET measurements were recorded for 20 min before adding agonists and measuring for a further 110 min. Data are presented as mean ± SEM of ≥ three independent experiments performed in triplicate. PM, plasma membrane; EARLY, early endosome; LATE, late endosome; RECYCLING, slow recycling endosome.

Using this approach to investigate the cellular trafficking profiles of the heteromers required the addition of the second, untagged receptor into the system, as seen in the previous GPCR-HIT assays. Although the AT_1-AT_2 heteromer recruits β-arrestin2 upon treatment with AngII, it has previously been reported to not subsequently
internalise (Porrello et al., 2011). Whilst in these GPCR-HIT assays, coexpression of the AT2 receptor did not inhibit internalisation of the AT1 receptor (Figure 6.3A) into endosomes (Figure 6.3B, C and D), this is expected to be due to a relative excess of AT1 monomers or homomers compared to heteromers. This is likely the case, as although there was no internalisation observed for the AT2 monomer/homomer (Figure 6.2E, F, G and H), co-expression with the AT1 receptor changed the trafficking profile of the AT2 receptor. Unexpectedly, in this configuration AngII treatment caused a gradual increase in the surface expression of the AT2 receptor (Figure 6.3E). The lack of sequestration into endosomes (Figure 6.3F, G and H) even after the surface expression has increased, further confirms the hypothesis that this heteromer does not internalise upon AngII-induced β-arrestin recruitment.

Figure 6.3. Internalisation of AT1-AT2 heteromers. Venus-tagged membrane protein plasmids (50 ng) were transfected into HEK293FT cells with either AT1-Rluc8 (75 ng) and AT2 (75 ng) (A – D) or AT2-Rluc8 (100 ng) and AT1a (50 ng) (E – H). Basal BRET measurements were recorded for 20 min before adding agonists and measuring for a further 110 min. Data are presented as mean ± SEM of ≥ three independent experiments performed in triplicate. PM, plasma membrane; EARLY, early endosome; LATE, late endosome; RECYCLING, slow recycling endosome.
The AT\textsubscript{1}-B\textsubscript{2} heteromer has been observed internalising in both an AngII-induced and a BK-induced manner (Quitterer \textit{et al.}, 2011). More recently, it has been shown to recruit \(\beta\)-arrestin2 and internalise upon treatment with SII (Wilson \textit{et al.}, 2013). In this study, coexpression of the AT\textsubscript{1} receptor did not alter the BK-induced internalisation profile of the B\textsubscript{2} receptor (Figure 6.4E-H) from what was seen in the monomer/homomer assays (Figure 6.2I-L). In contrast, the rate of AngII-induced AT\textsubscript{1} receptor sequestration into early and recycling endosomes was significantly increased upon coexpression of the B\textsubscript{2} receptor (Figure 6.4B, D and Figure 6.5B, D). No significant change was observed for the AngII-induced internalisation and late endosomal sequestration of the AT\textsubscript{1} receptor upon coexpression of the B\textsubscript{2} receptor (Figure 6.4A, C and Figure 6.5A, C). In both receptor configurations (AT\textsubscript{1}-R\textsubscript{Luc}8 + B\textsubscript{2} and B\textsubscript{2}-R\textsubscript{Luc}8 + AT\textsubscript{1}) and in all assays, dual agonist treatment resulted in a reduced level of internalisation, with only the early endosomal sequestration reaching the same level as the endogenous agonist (Figure 6.4B, F). Additionally, the internalisation of the AT\textsubscript{1} receptor upon dual agonist treatment had an altered kinetic profile: the rate of internalisation and sequestration into early, late and recycling endosomes was significantly reduced from AT\textsubscript{1} receptor expression alone (Figure 6.5). Interestingly, in all configurations this rate was not significantly different from that seen with BK-induced internalisation of the B\textsubscript{2} receptor (Figure 6.5). Lastly, while treatment with AngII had negligible effect on internalisation of the B\textsubscript{2} receptor upon coexpression of the AT\textsubscript{1} receptor (Figure 6.4E-H), there was small, but significant BK-induced-internalisation of the AT\textsubscript{1} receptor upon coexpression of the B\textsubscript{2} receptor (Figure 6.4A-D and Figure 6.6).
Figure 6.4. Internalisation of AT₁-B₂ heteromers. Venus-tagged membrane protein plasmids (50 ng) were transfected into HEK293FT cells with either AT₁-Rluc8 (75 ng) and B₂ (75 ng) (A – D) or B₂-Rluc8 (75 ng) and AT₁a (75 ng) (E – H). Basal BRET measurements were recorded for 20 min before adding agonists and measuring for a further 110 min. Data are presented as mean ± SEM of ≥ three independent experiments performed in triplicate. PM, plasma membrane; EARLY, early endosome; LATE, late endosome; RECYCLING, slow recycling endosome.
Chapter 6. Internalisation and Trafficking of AT$_1$, AT$_2$ and B$_2$ Receptors and Heteromers

Figure 6.5. Endocytic rate constants of AT$_1$ and B$_2$ receptor trafficking. Data from Figure 6.2 and Figure 6.4 were used to calculate the rate of internalisation and endosomal sequestration. * indicates significant difference (p < 0.05). ** indicates significant difference (p < 0.01). ns indicates no significant difference (p > 0.05). # indicates significant difference (p < 0.05) from all other conditions. † indicates significant difference (p < 0.05) from all other conditions except B$_2$-Rluc8 (both treatments). All analysis conducted using one-way ANOVA followed by Tukey's test for multiple comparisons. Data are presented as mean ± SEM of ≥ three independent experiments performed in triplicate.
Chapter 6. Internalisation and Trafficking of AT₁, AT₂ and B₂ Receptors and Heteromers

Figure 6.6. BK-induced endosomal sequestration of the AT₁-Rluc8. Data from Figure 6.2 and Figure 6.4 at final time point (110 min). Black bars show BK-induced sequestration of AT₁-Rluc8 monomers/homomers into Rab5-, Rab7- and Rab11-positive endosomes. Blue bars show BK-induced endosomal sequestration of AT₁-Rluc8 when coexpressed with the B₂ receptor. * indicates significant difference (p < 0.05). ** indicates significant difference (p < 0.01). Analysis conducted using two-way ANOVA followed by Sidak’s test for multiple comparisons. Data are presented as mean ± SEM of three independent experiments performed in triplicate.

The internalisation profile of the AT₂-B₂ receptor heteromer has not been previously investigated. Treatment with BK caused a gradual increase in the surface expression of the AT₂ receptor (Figure 6.7A), similar to what was seen with AngII treatment of the AT₁-AT₂ heteromer (Figure 6.3). Interestingly, and in contrast to the AT₁-AT₂ heteromer, after approximately 30 min of BK treatment, the AT₂ receptor began to be observed in early (Figure 6.7B) and late (Figure 6.7C) endosomes. There was no appearance of the AT₂ receptor in recycling endosomes (Figure 6.7D). Again, similar to the AT₁ in the AT₁-AT₂ heteromer, there was no change in the internalisation (Figure 6.7E) or endosomal sequestration (Figure 6.7F, G and H) profile of the B₂ receptor in these GPCR-HIT assays, likely due to a relative excess of B₂ monomers or homomers compared to heteromers.
Chapter 6. Internalisation and Trafficking of AT$_1$, AT$_2$ and B$_2$ Receptors and Heteromers

Figure 6.7. Internalisation of AT$_2$-B$_2$ heteromers. Venus-tagged membrane protein plasmids (50 ng) were transfected into HEK293FT cells with either AT$_2$-Rluc8 (100 ng) and B$_2$ (50 ng) (A–D) or B$_2$-Rluc8 (75 ng) and AT$_2$ (75 ng) (E–H). Basal BRET measurements were recorded for 20 min before adding agonists and measuring for a further 110 min. Data are presented as mean ± SEM of ≥ three independent experiments performed in triplicate. PM, plasma membrane; EARLY, early endosome; LATE, late endosome; RECYCLING, slow recycling endosome.

To further investigate the increase in surface expression of the AT$_2$ receptor upon coexpression and activation of either the AT$_1$ or the B$_2$ receptor, the ligand-induced interaction between these two heteromers was investigated using a more conventional BRET approach with both receptors tagged with the BRET donor and acceptor. Although the validity of this approach has been questioned recently, particularly in regards to BRET saturation assays (Szalai et al., 2014; Lan et al., 2015), investigating ligand-dependent signals using this method supports the specificity of the observed interactions.

Firstly, AT$_2$-Rluc8 was coexpressed with B$_2$-Venus and treated with AngII or BK for 5 hours. BK but not AngII treatment caused an increase in the proximity between the AT$_2$ and the B$_2$ receptor, and by 150 min this was significantly different
from what was observed with AngII treatment (Figure 6.8A). Further confirmation of
the specificity of this interaction was revealed through dose response analysis, which
showed a BK dose-dependent increase in the interactions between the two receptors
(Figure 6.8B). No dose-dependent interactions were observed upon treatment with
AngII, CGP 42114 (AT₂ receptor selective agonist) or HOE140 (B₂ IP-biased ligand).

Next, AT₂-Rluc8 was coexpressed with AT₁-Venus. Here, both BK and AngII
treatment caused an increase in the proximity between the AT₁ and the AT₂ receptor,
however, the AngII-induced increase was significantly greater than the BK-induced
increase (Figure 6.9A). Furthermore, while AngII treatment caused a potent, dose-
dependent increase in proximity, the dose dependence of the BK-induced BRET signal
could not be determined (Figure 6.9B and Table 6.2). Additionally, CGP 42114 caused
a dose-dependent increase in proximity between the AT₁ and the AT₂ receptor, albeit
with a low potency.
One possibility for the observed BK-induced dose-dependent interaction could be heteromerisation of the AT$_2$-Rluc8 and AT$_1$-Venus with endogenous B$_2$ receptors. If all three receptors are in a complex with one another, BK could conceivably cause the increased proximity observed. The small signal would correlate with the relatively low level of endogenous B$_2$ receptors. Endogenous B$_2$ receptors have previously been reported in HEK293 cells (Kramarenko et al., 2009), and their presence in HEK293FT cells was confirmed in this study using an IP$_1$ accumulation assay. BK caused potent dose-dependent IP$_1$ production in cells transfected with the B$_2$ receptor (Figure 6.9C and Table 6.2). Untransfected cells also produced BK dose-dependent IP$_1$ production, albeit with a lower potency and efficacy.

Figure 6.9. Ligand-induced proximity between AT$_{1a}$ and AT$_2$ receptors. (A, B) HEK293FT cells were transfected with AT$_2$-Rluc8 (100 ng) and AT$_{1a}$-Venus (50 ng). (A) Basal BRET measurements were recorded for 15 min before adding agonists and measuring for a further 300 min. * indicates significance was observed (p < 0.05) between means of BK and either

---

169
AngII or BK + AngII treatment between 156 and 300 min (using two-way ANOVA followed by Sidak’s test for multiple comparisons). (B) Dose-response curves for ligand-induced BRET at 200 min. CGP, CGP 42114. Data are presented as mean ± SEM of ≥ three independent experiments performed in triplicate (A, B). (C) HEK293FT cells were transfected with B₂ (50 ng) or were untransfected. IP₁ production was measured after 30 min incubation at 37°C and 5% CO₂ with increasing doses of BK. Data are shown as a percentage of BK-induced IP₁ production in cells transfected with B₂. Data are presented as mean ± SEM of four independent experiments performed in duplicate. pEC₅₀ values are shown in Table 6.2.

Table 6.2. pEC₅₀ values for dose-response curves in Figure 6.8B and Figure 6.9B, C.

<table>
<thead>
<tr>
<th>Transfection</th>
<th>Ligand</th>
<th>pEC₅₀ ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT₂-Rluc8 + B₂-Venus</td>
<td>BK</td>
<td>7.9 ± 0.2</td>
</tr>
<tr>
<td>AT₂-Rluc8 + AT₁-Venus</td>
<td>AngII</td>
<td>9.9 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>BK</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>CGP</td>
<td>5.9 ± 0.1</td>
</tr>
<tr>
<td>B₂</td>
<td>BK</td>
<td>8.3 ± 0.1</td>
</tr>
<tr>
<td>Untransfected</td>
<td>BK</td>
<td>7.0 ± 0.5</td>
</tr>
</tbody>
</table>

Although a ligand-dependent increase in surface expression was not observed for the AT₁ and the B₂ receptor upon their coexpression (Figure 6.4A, E), the same approach was used to investigate any ligand-dependent changes in their proximity to one another. Coexpression of AT₁-Rluc8 with B₂-Venus did not result in any significant ligand-dependent changes in receptor proximity (Figure 6.10). This lack of effect was unlikely to be due to suboptimal cDNA ratios or BRET label positioning as numerous cDNA ratios were tested in both donor/acceptor configurations (data not shown).
6.4. Discussion

6.4.1. Internalisation of AT₁-AT₂ and AT₁-B₂ heteromers

Recruitment of β-arrestin to an activated GPCR initiates the process of receptor internalisation into endosomes. The internalised receptor can then be either targeted to lysosomes for degradation or recycled back to the plasma membrane, allowing resensitisation of the receptor. This study confirmed previous observations that the AT₁-AT₂ heteromer is unable to internalise upon treatment with AngII (Porrello et al., 2011). In contrast, the AT₂-B₂ heteromer internalised in a BK-dependent manner.

Coexpression with either the AT₁ or the B₂ receptor also resulted in a rapid AngII- or BK-induced (respectively) increase in surface expression of the AT₂ receptor. These results are similar to what is seen with the AT₂-D₁ heteromer, in which fenoldopam-induced activation of the dopamine D₁ receptor results in increased surface expression of the AT₂ receptor. Gildea et al. (2012) showed that this occurred with a
strikingly similar kinetic profile to what is seen in this study with the AT₁-AT₂ and
AT₂-B₂ heteromer: the AT₂ receptor reached maximal plasma membrane expression at
10 min (T₁/₂ of 5 min) which began to decline at 60 min. D₁-mediated plasma
membrane recruitment of the AT₂ receptor was dependent on microtubule
polymerisation (Padia et al., 2012) and interestingly, the three hour infusion of
fenoldopam used also caused a parallel increase in surface expression in the D₁ receptor
(Salomone et al., 2007), despite the D₁ receptor typically undergoing conventional
agonist induced internalisation (Ng et al., 1995; Dumartin et al., 1998).

The concurrent increase in surface expression of D₁ receptors with AT₂
receptors suggests that internalised D₁ receptors could be replaced at the cell surface
with AT₂-D₁ heteromers. Similarly, it is also possible that replacement of internalised
AT₁ or B₂ monomers/homomers with AT₁-AT₂ or AT₂-B₂ heteromers (respectively)
could result in the increased surface expression of AT₂ receptors observed in this study.
This theory is supported by the results of the GPCR-HIT β-arrestin assay (Chapter 5)
with both the AT₁-AT₂ and the AT₂-B₂ heteromer, in which it takes approximately 20
min for β-arrestin recruitment to reach maximal levels. In contrast, we see that maximal
β-arrestin recruitment to AT₁ and B₂ monomers/homomers occurs in less than five
minutes. Furthermore, there was a delayed endosomal sequestration of the AT₂-B₂
heteromer, with the AT₂ receptor observed in endosomes after approximately 1 hour
treatment with BK. This delayed endosomal sequestration likely follows sequentially
from the initial increase in surface expression of the AT₂ receptor after 10 min, followed
by β-arrestin recruitment to the AT₂-B₂ heteromer after 20 min. Once the heteromer has
recruited β-arrestin it is able to begin the process of internalisation.

An increased surface expression of the AT₂ receptor upon treatment with similar
concentrations of AngII has been reported previously in smooth muscle cells of the rat
internal anal sphincter (de Godoy et al., 2006). As these cells express both the AT₁ and
the AT₂ receptor, this effect may well be the result of heteromerisation of the receptors.
Although the study by de Godoy et al. (2006) also reported a concurrent decrease in
surface expression of the AT₁ receptor, it is possible that the antibodies used did not
have specificity for the AT₁ receptor when heteromerised with the AT₂ receptor, and
may have therefore been unable to detect their localisation.
The AT\textsubscript{1}-AT\textsubscript{2} heteromer is known to result in an inhibition of AT\textsubscript{1} signalling (AbdAlla \textit{et al.}, 2001b; Kumar \textit{et al.}, 2002; Yang \textit{et al.}, 2012). While a lack of internalisation of the heteromer upon β-arrestin recruitment likely accounts for much of this inhibition, it is possible that this increased AT\textsubscript{2} surface expression also contributes. If the balance between monomeric/homomeric AT\textsubscript{1} receptors and AT\textsubscript{1}-AT\textsubscript{2} heteromers shifts in favour of the heteromers, this would result in a further decrease in the level of AT\textsubscript{1} signalling. In the study on the rat internal anal sphincter, it was found that the high concentrations of AngII used not only increased the surface expression of the AT\textsubscript{2} receptor and reduced the surface expression of the AT\textsubscript{1} receptor, but that this shifted the AngII-induced effect from a contractile response (at low concentrations) to relaxation. This could therefore be a mechanism for protecting against excess contraction in response to surplus AngII.

Similar to the AT\textsubscript{1}-AT\textsubscript{2} heteromer, an increase in the surface expression of AT\textsubscript{2}-B\textsubscript{2} heteromers could shift the balance from predominantly monomeric/homomeric receptors. As the AT\textsubscript{2}-B\textsubscript{2} heteromer is thought to cause enhanced NO/cGMP signalling (Abadir \textit{et al.}, 2006), this shift could further potentiate the vasodilatory response of these receptors. Furthermore, the endosomal sequestration of the AT\textsubscript{2}-B\textsubscript{2} heteromer suggests that it may be able to recycle and subsequently resensitise upon recruitment of β-arrestin. This would enable continual signalling of the heteromer, unlike the AT\textsubscript{1}-AT\textsubscript{2} heteromer, which is unable to internalise. While the AT\textsubscript{2}-B\textsubscript{2} heteromer did not appear in slow recycling endosomes, localisation in fast recycling endosomes was not investigated in this study. The use of a fast recycling endosomal marker, such as Rab4 GTPase would enable elucidation of the recycling profile of this heteromer.

Although the results suggest an increase in surface expression of the AT\textsubscript{2} receptor upon coexpression and activation of the AT\textsubscript{1} receptor or the B\textsubscript{2} receptor, further experiments would be required to confirm this. This could be done with BRET through comparison of the BRET ratio from a donor only control with the BRET ratio calculated in the Venus-Kras assays. Additionally, other approaches such as the BRET ligand binding assay, confocal microscopy and ELISA should enable determination of the level of ligand-induced increases in surface expression of the AT\textsubscript{2} receptor.
6.4.1.1. Ligand-induced interactions between AT2-Rluc8 and AT1-Venus or B2-Venus

The ligand-induced increase in surface expression of the AT2 receptor was further investigated by coexpression of AT2-Rluc8 with either AT1-Venus or B2-Venus. In both experiments, this resulted in a prolonged ligand-induced increase in the BRET signal, indicating an increase in the proximity between AT2-Rluc8 and either AT1-Venus or B2-Venus. This increase in proximity, indicative of interaction, was delayed (occurring after approximately 1 hour) in comparison with the relatively rapid increase in cell surface expression of AT2 heteromers observed in the trafficking assay. As the cell surface tracking occurs within only ten minutes, these heteromers must be preformed in the biosynthetic pathway, and thus no change in the level of BRET between AT2-Rluc8 and either AT1-Venus or B2-Venus would be expected during this time period. The increase in the BRET signal between AT2-Rluc8 and either AT1-Venus or B2-Venus after one hour is likely due to the formation of new heteromers either within the biosynthetic pathway or at the plasma membrane. It is unlikely that the increase in BRET signal is merely due to an accumulation of internalised receptors, as no ligand-induced increase in proximity was seen between the AT1 receptor and the B2 receptor, even upon dual agonist treatment.

The increase in expression of the AT2-B2 heteromer occurred in a BK- but not AngII-, CGP 42114- or HOE140-dependent manner. In their study on the AT2-B2 heteromer, Abadir et al. (2006) found that although the heteromer formed constitutively, its expression could be modulated through prolonged (1 day) treatment with different ligands: CGP 42114, HOE140, CGP 42114 + HOE140 and CGP 42114 + BK all caused increases in the expression of the heteromer, while BK, PD 123319, BK + PD123319 and PD 123319 + HOE140 caused decreases in the expression. In my study, only BK caused an increase in heteromer expression, and this is likely due to the substantially different temporal profile that was investigated.

While all of the studies on the AT1-AT2 heteromer report that it forms constitutively, most of those studies report various ligand-induced modulations in the expression level of the heteromer. Yang et al. (2012) reported that 30 min treatment with 100 nM CGP 42114 caused an increase in the expression of heteromers while Ferrão et al. (2012) reported that both 1 µM and 0.1 nM AngII caused an increase in the
expression. Axelband et al. (2009) reported that 30 min treatment with 0.1 nM AngII caused dissociation of the heteromer, while 100 nM AngII maintained the expression level of heteromers. Two studies have also reported no effect of AngII-treatment on the expression level of heteromers: Porrello et al. (2011) treated with 0.1 µM AngII for 45 min and AbdAlla et al. (2001b) did not specify concentration or time of treatment. My study reported an AngII dose-dependent ($\text{pEC}_{50} \pm \text{SEM} = 9.9 \pm 0.4$) increase in the expression of heteromers over a sustained period, with an observable increase beginning after approximately 1 hour. My study also observed a CGP 42114-dependent increase in the level of heteromers, as described previously (Yang et al., 2012), as well as a small, BK-induced increase in the level of heteromers. The BK-induced effect was hypothesised to be due to the presence of endogenous B2 receptors which could form a complex with the AT1-AT2 heteromers. As was observed in Chapter 5, there is evidence that a heteromer of all three receptors may be able to form, and thus, this trimeric heteromer may also be regulated in a ligand-dependent manner. The lack of an AngII-induced BRET signal between AT2-Rluc8 and B2-Venus is likely due to the absence of endogenous AT1 receptors in HEK293FT cells (Figure 2.4) (Atwood et al., 2011).

### 6.4.1.2. Summary

Unlike the AT1 and the B2 receptor that are highly expressed in most adult tissue, expression of the AT2 receptor is generally believed to be quite low, although it is found at detectable levels in several tissues (Bottari et al., 1993) and upregulated in certain pathologies (Viswanathan et al., 1994; Nakajima et al., 1995; Nio et al., 1995; Ohkubo et al., 1997; Viswanathan et al., 1997). Due to the constitutively active nature of the AT2 receptor it has been proposed that a major determinant of AT2 receptor signalling may be its surface expression (Porrello et al., 2009). If the agonist-induced increase in surface expression of the AT2 receptor when coexpressed with either the AT1 or the B2 receptor is confirmed, this aspect of AT2 receptor trafficking may play a major role in the regulation of AT2 receptor signalling.
6.4.2. Internalisation of AT₁-B₂ heteromer

The controversial AT₁-B₂ heteromer has previously been reported to internalise in an AngII- and BK-dependent manner (AbdAlla et al., 2010; Quitterer et al., 2011), as well as an SII-dependent manner (Wilson et al., 2013). Hansen et al. (2009), whose collaboration reportedly found no evidence for the existence of the AT₁-B₂ heteromer, did not investigate receptor internalisation in their study. My study also used the GPCR-HIT assay to investigate the internalisation properties of the putative AT₁-B₂ heteromer. In concurrence with the GPCR-HIT β-arrestin assay, there was a significant level of BK-induced internalisation of AT₁-Rluc8 when coexpressed with the B₂ receptor, however no AngII-induced internalisation of B₂-Rluc8 when it was coexpressed with the AT₁ receptor. This provides further support that this heteromer is activated in a BK-dependent manner. Although there was no observable AngII-induced internalisation of the B₂ receptor when coexpressed with the AT₁ receptor, the heteromer may still undergo AngII-induced internalisation. As seen with the internalisation profiles of the AT₁ and the B₂ receptor when coexpressed with the AT₂ receptor, a relative excess of monomers/homomers can mask the relatively small signal from heteromers.

In contrast to the dual agonist-induced increase in β-arrestin recruitment to AT₁-Rluc8 when coexpressed with the B₂ receptor, there was a reduced level of BRET between AT₁-Rluc8 and the Venus-tagged compartment markers upon dual agonist treatment. Although an increased level of β-arrestin recruitment suggests there may be increased internalisation, this is not seen for the AT₁-AT₂ heteromer, which does not internalise subsequent to β-arrestin recruitment. It is possible that dual receptor activation may result in allosteric cross-inhibition in terms of internalisation but not β-arrestin recruitment. However, it is also possible that the reduction in BRET signal could be an artefact of the assay rather than a reflection of the level of heteromer internalisation. For example, the BRET signal could be reduced due to molecular crowding within endocytic compartments and causing separation between AT₁-Rluc8 and Venus-tagged proteins. Additionally, there could be a reduction in the level of internalisation due to competition for the endocytic machinery by activated AT₁ and B₂ monomers/homomers. While these hypotheses are supported by the observation that dual agonist treatment also causes a reduced level of BRET between B₂-Rluc8 the
Venus-tagged compartment markers when coexpressed with the AT$_1$ receptor, this trend is not always seen upon coexpression and dual receptor activation (as will be seen in Chapter 7).

In addition to the reduced level of internalisation seen with dual agonist treatment, the rate of internalisation of AT$_1$-RLuc8 upon coexpression of the B$_2$ receptor and dual agonist treatment was significantly reduced from AT$_1$-RLuc8 alone. Interestingly, in all assays (Kras, Rab5, Rab7 and Rab11) this reduced rate was not significantly different to the rate observed for BK-induced internalisation of B$_2$-RLuc8. This shift in kinetics to a more B$_2$ receptor-like profile is reminiscent of what is seen in the study on the AT$_1$-B$_2$ heteromer using SII. In that study, Wilson et al. (2013) observed that SII-induced AT$_1$ receptor internalisation occurred at a faster rate than BK-induced B$_2$ receptor internalisation, similar to what was seen in this study with AngII-induced internalisation of the AT$_1$ receptor. However, coexpression of the AT$_1$ receptor caused SII-induced internalisation of the B$_2$ receptor, and the kinetics of this resembled BK-induced B$_2$ receptor internalisation rather than SII-induced AT$_1$ receptor internalisation. The authors suggest that the presence of the B$_2$ receptor in the complex dictates the kinetics of the heteromer’s trafficking, supporting the results of my study.

The same study also reported that the SII-induced endosomal sequestration of AT$_1$ receptors was more short-lived than the BK-induced endosomal sequestration of B$_2$ receptors. This is in contrast to some (Simaan et al., 2005) but not all (Enquist et al., 2007) studies, which suggest that the B$_2$ receptor dissociates from β-arrestins and recycles more rapidly than typical class B receptors. In either case, the SII-induced endosomal sequestration of B$_2$ receptors upon coexpression of AT$_1$ receptors was also reported to be prolonged. In my study, coexpression of the B$_2$ receptor resulted in a dual agonist-induced internalisation and endosomal sequestration profile of AT$_1$ receptors that resembled BK-induced internalisation of the B$_2$ receptor. However, the duration of the endosomal sequestration was not investigated as my study only assessed the steady state of receptor localisation.

In addition to the modulation in BK- and AngII + BK-induced internalisation observed for the AT$_1$ receptor when coexpressed with the B$_2$ receptor, there was also an increase in the rate of AngII-induced sequestration into early and recycling endosomes, but not late endosomes. These results suggest that heteromerisation with the B$_2$ receptor may increase the rate and level of resensitisation of the AT$_1$ receptor in an AngII-
induced manner, through increased recycling and reduced degradation. As mentioned previously, an increase in the rate of recycling of the AT1 receptor would shift the balance of AT1-mediated signalling in favour of G protein-dependent over β-arrestin-dependent. As the AT1-mediated G protein-dependent signalling is associated with the majority of the vasoconstrictive response (Rajagopal et al., 2006; Aplin et al., 2007), this could at least partially explain the AngII-induced hypertension associated with the AT1-B2 heteromer (AbdAlla et al., 2001a; Quitterer et al., 2014).

AbdAlla et al. (2000) previously investigated the mechanism of endosomal sequestration of the AT1-B2 heteromer. They found that although the individual receptors endocytosed via dynamin- and clathrin-independent paths, upon heteromerisation with one another the receptors internalised via dynamin- and clathrin-dependent paths. It is interesting to speculate that the modulated rates and levels of internalisation observed in this study could be at least partially attributable to an altered endocytic pathway of the heteromer.

Finally, the AT1-B2 heteromer was also investigated for any ligand-induced modulations in the interactions between the two receptors. Unlike the AT1-AT2 and AT2-B2 heteromers, there was no evidence of any ligand-induced modulation in the interaction between AT1-Rluc8 and B2-Venus. This supports the lack of ligand-induced increase in surface expression of either the AT1 or the B2 receptor upon heteromerisation with one another, as was seen with the AT2 receptor. This suggests that although preformed AT1-B2 heteromers are able to recycle and maintain a steady state level of receptor/heteromer at the cell surface, the number of new heteromers formed in a ligand-dependent manner does not appear to significantly exceed the number of heteromers degraded. There appears to be an equilibrium, and perhaps the seemingly sinusoidal nature of the signal hints at this, even if the change is not significantly different from baseline.
7.1. Introduction

Crosstalk between AT₁ receptor and CCR2 signalling has been implicated in several pathologies (Bush et al., 2000; Ishibashi et al., 2004; Dai et al., 2007; Liao et al., 2008; Daugherty et al., 2010), with some studies reporting significant benefits to dual receptor antagonism (Tsukuda et al., 2011; Urushihara et al., 2011). Recently, my colleagues and I have published an investigation into the mechanisms of this crosstalk, and its involvement in CKD (Ayoub et al., 2015). Using the GPCR-HIT assay to investigate Gαᵢ-coupling and β-arrestin recruitment, evidence was provided for heteromerisation of the two receptors. AT₁ receptor activation antagonised CCR2-Gαᵢ₁-coupling and mediated AngII-induced CCR2-β-arrestin2 proximity. My contribution to this publication involved investigation of the Gα₉-coupling of the heteromer, which will be detailed in this chapter. Additionally, I have also investigated the internalisation profile of this heteromer using the GPCR-HIT assay.

This thesis has previously revealed evidence that the AT₁ receptor may form a trimeric complex with both the AT₂ and the B₂ receptors. It was suggested that the complex interactions between the three receptors may underpin some of the controversy surrounding the existence of the AT₁-B₂ heteromer. This chapter will also begin investigations into the possibility that as well as the AT₁ receptor, CCR2 also interacts with the B₂ receptor, potentially further adding to the complexity of the AT₁-B₂ heteromer pharmacology and controversy. The AT₁, the B₂ and CCR2 are all expressed in several of the same tissues and cell types, including the vasculature, the kidney and the lung (Hayes et al., 1998; Jiang et al., 1998; Salcedo et al., 2001; Leeb-Lundberg et al., 2005; Gao et al., 2012; Costa-Neto et al., 2014). Additionally, there is overlap and crosstalk in some of the physiological processes they mediate, most notably, they are all major mediators of inflammation. Before investigating the possibility that the three
receptors form a trimeric complex, the B2 receptor and CCR2 were investigated for functional interactions. Numerous studies have reported functional interactions between BK/B2 receptor and CCL2/CCR2 signalling systems. Specifically, it has repeatedly been demonstrated that B2 receptor activation modulates the expression of CCL2 (Koyama et al., 1998; Koyama et al., 1999; Chen et al., 2003; Dos Santos et al., 2007; Yao et al., 2007; Marney et al., 2009; Shaw et al., 2011; Takemura et al., 2011; Xu et al., 2014), however the possibility of heteromerisation between these two receptors has not been previously explored. This chapter will use the GPCR-HIT β-arrestin and trafficking assays to investigate interactions between the B2 receptor and CCR2, however due to time constraints, BRET/BiFC assays looking for interactions between all three receptors were not conducted.

7.2. Materials and Methods

7.2.1. Materials

Ligands used were AngII, BK, (Sigma Aldrich), CCL2 (PeproTech).

7.2.2. cDNA constructs

The cDNA constructs used in this chapter are listed in Table 7.1, and detailed in Section 2.2.1.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Species</th>
<th>Genbank Accession No.</th>
<th>Constructs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiotensin II receptor type 1</td>
<td>Rat</td>
<td>NM_030985</td>
<td>AT1a-Rluc8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HA-AT1a</td>
</tr>
</tbody>
</table>
Chapter 7. AT₁, CCR2 and B₂ Receptors and Heteromers

<table>
<thead>
<tr>
<th>Bradykinin receptor type 2</th>
<th>Human</th>
<th>AY275465</th>
<th>B₂-Rluc8 HA-B₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemokine (C-C motif) receptor 2</td>
<td>Human</td>
<td>NM_001123396.1</td>
<td>wt CCR2 CCR2-Rluc8</td>
</tr>
<tr>
<td>β-arrestin2</td>
<td>Human</td>
<td>NM_004313</td>
<td>β-arrestin2-Venus</td>
</tr>
<tr>
<td>Kras GTPase</td>
<td>Human</td>
<td>NM_004985.4</td>
<td>Venus-Kras (25 amino acid C terminal fragment of Kras)</td>
</tr>
<tr>
<td>Rab5a GTPase</td>
<td>Human</td>
<td>NM_004162.4</td>
<td>Venus-Rab5</td>
</tr>
<tr>
<td>Rab7 GTPase</td>
<td>Human</td>
<td>AF498942.1</td>
<td>Venus-Rab7</td>
</tr>
<tr>
<td>Rab11 GTPase</td>
<td>Human</td>
<td>X56740.1</td>
<td>Venus-Rab11</td>
</tr>
</tbody>
</table>

7.2.3. Mammalian cell transfections

Transient transfections were carried out in HEK293FT cells as previously described in the General Materials and Methods Chapter (Section 2.3.3). cDNA quantities are described in figure legends.

7.2.4. BRET assays

All assays in this chapter were carried out using eBRET, as described previously in the General Materials and Methods Chapter (Section 2.4.2).

7.2.5. IP₁ assays

IP₁ assays were carried out as described previously in the General Materials and Methods Chapter (Section 2.5).
7.2.6. Data presentation and statistical analyses

All curve fitting and statistical analysis were carried out as previously described in Section 2.8.

7.3. Results

7.3.1. AT1 and CCR2 receptors and heteromers

In our study, my colleagues showed that heteromerisation with the activated AT1 receptor inhibited the conformational change associated with CCR2-Go11-coupling. I also investigated the heteromer’s ability to regulate Goq signalling. The AT1 receptor predominantly couples to Goq while CCR2 has been shown to regulate Ca2+ signalling in a pertussis toxin sensitive and insensitive manner (Mellado et al., 1998; Armando et al., 2014). Treatment of cells expressing the AT1 receptor with AngII resulted in potent, dose-dependent IP1 production, which was not altered upon co-treatment with CCL2 (Figure 7.1A, B and Table 7.2). Cells expressing CCR2 produced CCL2-induced IP1, with a lower potency than observed for AngII/AT1-mediated IP1 production (Figure 7.1C, D and Table 7.2). Coexpression of the AT1 receptor and CCR2 did not modulate the IP1 response of either receptor (Figure 7.1E, F and Table 7.2).
Figure 7.1. IP$_1$ production in cells co-expressing AT$_{1a}$ receptor and CCR2. HEK293FT were transfected with pcDNA3 (to make a total of 200 ng of plasmid) and AT$_{1a}$ receptor (50 ng; A, B) or CCR2 (150 ng; C, D) or both AT$_{1a}$ receptor and CCR2 (50 ng and 150 ng respectively; E, F). Agonist-induced IP$_1$ production was measured after 30 min incubation at 37°C and 5% CO$_2$ with increasing doses of CCL2 in the presence of 0, 10 or 1000 nM AngII (A, C, E) or with increasing doses of AngII in the presence of 0, 10 or 1000 nM CCL2 (B, D, F). Data are shown as a percentage of AngII-induced IP$_1$ production in cells expressing AT$_{1a}$ receptor alone. Data are presented as mean ± SEM of five independent experiments performed in duplicate. pEC$_{50}$ values are shown in Table 7.2.
Table 7.2. pEC\textsubscript{50} data for IP\textsubscript{1} production shown in Figure 7.1. Data are mean ± SEM. Co-treatment with 10 or 1000 nM AngII did not significantly alter the CCL2 pEC\textsubscript{50} with cells expressing CCR2 and likewise, co-treatment with 10 or 1000 nM CCL2 did not significantly alter the AngII pEC\textsubscript{50} with cells expressing either AT\textsubscript{1a} receptor or both AT\textsubscript{1a} receptor and CCR2 (p > 0.05). In cells expressing AT\textsubscript{1a} receptor and CCR2, a significant difference was observed between the CCL2 pEC\textsubscript{50} and AngII pEC\textsubscript{50} in the absence of the other ligand (p < 0.05). ND, not determined. All analysis conducted using one-way ANOVA followed by Bonferroni’s test for multiple comparisons).

<table>
<thead>
<tr>
<th>Receptor</th>
<th>[AngII] (nM)</th>
<th>CCL2 pEC\textsubscript{50}</th>
<th>[CCL2] (nM)</th>
<th>AngII pEC\textsubscript{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT\textsubscript{1a}</td>
<td>0</td>
<td>ND</td>
<td>0</td>
<td>8.61 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>ND</td>
<td>10</td>
<td>8.65 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>ND</td>
<td>1000</td>
<td>8.85 ± 0.06</td>
</tr>
<tr>
<td>CCR2</td>
<td>0</td>
<td>7.13 ± 0.06</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>7.37 ± 0.09</td>
<td>10</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>7.41 ± 0.03</td>
<td>1000</td>
<td>ND</td>
</tr>
<tr>
<td>AT\textsubscript{1a} + CCR2</td>
<td>0</td>
<td>7.31 ± 0.07</td>
<td>0</td>
<td>8.81 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>ND</td>
<td>10</td>
<td>8.86 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>ND</td>
<td>1000</td>
<td>9.02 ± 0.06</td>
</tr>
</tbody>
</table>

Internalisation of the AT\textsubscript{1}-CCR2 heteromer has not previously been investigated. The BRET assay was first used to investigate the internalisation profiles of the monomers/homomers. As seen in Figure 6.2A-D, the AT\textsubscript{1} receptor underwent AngII-induced internalisation away from the plasma membrane and then into early, late and recycling endosomes (Figure 7.2A-D). Similarly, the CCR2 underwent CCL2-induced internalisation and sequestration into early and late endosomes (Figure 7.2E-G). In contrast however, the CCR2 did not localise in slow recycling endosomes (Figure 7.2H), supporting previous research showing that the CCR2 is predominantly targeted to lysosomes for degradation (García Lopez \textit{et al.}, 2009).
Coexpression of CCR2 with the AT₁ receptor did not alter its internalisation profile (Figure 7.3A-D). The small CCL2-induced increase in surface expression is not statistically significant within the time period assessed (Figure 7.4A). In contrast, coexpression and activation of the AT₁ receptor caused a reduced level of CCR2 internalisation away from the plasma membrane and into early and late endosomes (Figure 7.3E-H). As with CCR2 monomers/homomers, coexpression of the AT₁ receptor did not result in trafficking into recycling endosomes (Figure 7.3H). Additionally, similar to that observed with AT₁-Rluc8 upon coexpression of CCR2, the small increase in surface expression of CCR2-Rluc8 upon coexpression and activation of the AT₁ receptor was not statistically significant within this time period (Figure 7.4B).
Figure 7.3. Internalisation of AT1-CR2 heteromers. Venus-tagged membrane protein plasmids (50 ng) were transfected into HEK293FT cells with either AT1a-Rluc8 (75 ng) and CCR2 (75 ng) (A – D) or CCR2-Rluc8 (75 ng) and AT1a (75 ng) (E – H). Basal BRET measurements were recorded for 15 min before adding agonists and measuring for a further 160 min. Data are presented as mean ± SEM of ≥ three independent experiments performed in triplicate. PM, plasma membrane; EARLY, early endosome; LATE, late endosome; RECYCLING, slow recycling endosome.
Figure 7.4. Ligand-induced change in proximity with Venus-Kras. Data from Figure 7.2 and Figure 7.3 showing (A) CCL2-induced proximity between AT1α-Rluc8 and Venus-Kras, with or without CCR2 coexpression, and (B) AngII-induced proximity between CCR2-Rluc8 and Venus-Kras, with or without AT1α coexpression. ns indicates no significant difference (p > 0.05; using two-way ANOVA followed by Sidak’s test for multiple comparisons). Data are presented as mean ± SEM of ≥ three independent experiments performed in triplicate.

7.3.2. B2 and CCR2 receptors and heteromers

The B2 receptor and CCR2 were first assessed for their β-arrestin recruitment profiles. As seen previously (Figure 5.2A), BK treatment caused rapid recruitment of β-arrestin2 to the B2 receptor (Figure 7.5A). Similarly, CCR2 recruited β-arrestin2 upon treatment with CCL2, however a more delayed kinetic profile was observed (Figure 7.5C). Coexpression with CCR2 did not alter the level of β-arrestin2 recruitment to the B2 receptor (Figure 7.5B), similar to previous observations upon coexpression of the AT1 receptor (Figure 5.2B). Interestingly, coexpression of the B2 receptor reduced CCL2-induced β-arrestin2 recruitment, which was restored upon dual agonist treatment (Figure 7.5D). As well, there was a small, but significant level of BK-induced β-arrestin2 recruitment to CCR2 upon coexpression of the B2 receptor (Figure 7.6).
Figure 7.5. β-arrestin2 GPCR-HIT between the B₂ receptor and CCR2. HEK293FT cells were transfected with receptor-Rluc8 (75 ng), β-arrestin2-Venus (50 ng) and pcDNA3 or untagged receptor (75 ng) as described on graphs. Basal BRET measurements were recorded for 20 min before adding agonists and measuring for a further 115 min. Data are presented as mean ± SEM of three independent experiments performed in triplicate.
Chapter 7. AT₁, CCR2 and B₂ Receptors and Heteromers

Figure 7.6. BK-induced β-arrestin2-Venus recruitment to CCR2-Rluc8. Data from Figure 7.5 showing BK-induced recruitment of β-arrestin2-Venus to CCR2-Rluc8 monomers/homomers (black) and CCR2-Rluc8 upon coexpression of the B₂ receptor (blue). Statistically significant differences (p < 0.05) were observed at 37, 80, 88, 94 and 102 min (using two-way ANOVA followed by Sidak’s test for multiple comparisons). Data are presented as mean ± SEM of three independent experiments performed in triplicate.

Next, the internalisation profiles of B₂ and CCR2 monomers/homomers was investigated. As seen previously (Figure 6.2I-L), the B₂ receptor underwent internalisation away from the plasma membrane and was sequestered into early, late and recycling endosomes upon treatment with BK (Figure 7.7A-D). CCL2 treatment caused CCR2 internalisation and sequestration into early and late endosomes, but negligible sequestration into recycling endosomes (Figure 7.7E-H), as seen earlier in the chapter (Figure 7.2E-H).
Chapter 7. AT₁, CCR2 and B₂ Receptors and Heteromers

B₂-Rluc8

A

PM: Venus-Kras

B

EARLY: Venus-Rab5

C

LATE: Venus-Rab7

D

RECYCLING: Venus-Rab11

Figure 7.7. Internalisation of B₂ and CCR2 monomers/homomers. Venus-tagged membrane protein plasmids (50 ng) were transfected into HEK293FT cells with either B₂-Rluc8 (75 ng; A – D) or CCR2-Rluc8 (75 ng; E – H) and pcDNA₃ (75 ng). Basal BRET measurements were recorded for 15 min before adding agonists and measuring for a further 115 min. Data are presented as mean ± SEM of three independent experiments performed in triplicate. PM, plasma membrane; EARLY, early endosome; LATE, late endosome; RECYCLING, slow recycling endosome.

Using the GPCR-HIT assay to investigate the trafficking profiles of the putative B₂-CCR2 heteromer, showed that coexpression of CCR2 did not alter the internalisation or endosomal sequestration of B₂-Rluc8 (Figure 7.8A-D). In contrast, coexpression of the B₂ receptor resulted in a significant reduction of CCL2-induced CCR2-Rluc8 internalisation and endosomal sequestration, with or without co-treatment of BK (Figure 7.8E-G and Figure 7.9). Additionally, by the end of the assay measurement period, coexpression and activation of the B₂ receptor resulted in a significant increase in CCR2-Rluc8 cell surface expression (Figure 7.10).
**Figure 7.8. Internalisation of B2-CCR2 heteromers.** Venus-tagged membrane protein plasmids (50 ng) were transfected into HEK293FT cells with either B2-Rluc8 (75 ng) and CCR2 (75 ng) (A – D) or CCR2-Rluc8 (75 ng) and B2 (75 ng) (E – H). Basal BRET measurements were recorded for 15 min before adding agonists and measuring for a further 115 min. Data are presented as mean ± SEM of three independent experiments performed in triplicate. PM, plasma membrane; EARLY, early endosome; LATE, late endosome; RECYCLING, slow recycling endosome.
Figure 7.9. Endosomal sequestration of CCR2-Rluc8. Data from Figure 7.7 and Figure 7.8 at final time point (115 min). Black and grey bars show sequestration of CCR2-Rluc8 monomers/homomers. Blue bars show sequestration of CCR2-Rluc8 upon coexpression of the B₂ receptor. * indicates significant difference (p < 0.05). ns indicates no significant difference (p > 0.05). Data analysed using two-way ANOVA followed by Sidak’s test for multiple comparisons. Data are presented as mean ± SEM of three independent experiments performed in triplicate.

Figure 7.10. BK-induced modulation of CCR2-Rluc8 plasma membrane expression. Data from Figure 7.7 and Figure 7.8 showing cell surface expression of CCR2-Rluc8 monomers/homomers (black) and CCR2-Rluc8 upon coexpression of the B₂ receptor (blue). Statistically significant difference (p < 0.05) was observed at final time point (115 min; using two-way ANOVA followed by Sidak’s test for multiple comparisons). Data are presented as mean ± SEM of three independent experiments performed in triplicate.
7.4. **Discussion**

7.4.1. **AT\(_1\)-CCR2 heteromer**

Recently, I have co-authored a publication showing that the AT\(_1\) receptor forms a heteromer with CCR2 (Ayoub *et al.*, 2015). Activation of the AT\(_1\) receptor was shown to antagonise CCR2-G\(_{\alpha_i}\) coupling and induce recruitment of \(\beta\)-arrestin proximal to CCR2. Dual agonist treatment resulted in a \(\beta\)-arrestin recruitment BRET signal that was more than additive, but did not significantly alter potency. AT\(_1\) antagonism or CCR2 antagonism resulted in partial blockade of the dual agonist-induced \(\beta\)-arrestin recruitment, which was essentially completely blocked with dual antagonist treatment. In subtotally nephrectomised (STNx; a model of progressive CKD) rats, dual antagonist treatment was beneficial in comparison to monotherapy, resulting in decreased proteinuria, reduced podocyte loss and reduced macrophage infiltration (Ayoub *et al.*, 2015).

As part of this study I investigated G\(_{\alpha_q}\) coupling of the heteromer (Figure 7.1 and Table 7.2) (Ayoub *et al.*, 2015). While the AT\(_1\) receptor is predominantly coupled to G\(_{\alpha_q/11}\) (Lassègue *et al.*, 1993; Ushio-Fukai *et al.*, 1998) CCR2 is primarily coupled to G\(_{\alpha_i/o}\) (Jiménez-Sainz *et al.*, 2003). This was confirmed in my experiments whereby potent IP\(_1\) production was produced by AngII activation of the AT\(_1\) receptor, while less potent IP\(_1\) production occurred as a result of CCL2 activation at the CCR2 receptor. Coexpression of the AT\(_1\) receptor and CCR2 did not result in any modulation of IP\(_1\) production upon treatment with either AngII, CCR2 or dual agonist treatment. Thus, the heteromerisation of the AT\(_1\) receptor with CCR2 results in a biased G protein signalling profile, whereby CCR2-mediated G\(_{\alpha_i}\) coupling is antagonised with no effect on G\(_{\alpha_q}\) coupling. Similar biased profiles have been described for other heteromers, including the dopamine D\(_1\)-D\(_2\) heteromer. In comparison to the D\(_2\) homomer, the D\(_2\) selective antagonist NPA displayed increased potency for inhibition of G\(_{\alpha_i}\) coupling with no shift in potency being observed for inhibition of G\(_{\alpha_q}\)-coupling (Urizar *et al.*, 2011).
I also investigated the internalisation profile of the AT₁-CCR2. There was no modulation of AT₁ receptor internalisation upon coexpression of CCR2 and treatment with AngII, CCL2 or AngII + CCL2. This is in contrast to the altered AT₁ receptor internalisation profile observed upon coexpression of the B₂ receptor when treated with either AngII, BK or AngII + BK. However, the lack of observable modulation could also be the result of a relative excess of AT₁ monomers/homomers masking the heteromer signal, and a similar lack of effect occurred for AT₁ and B₂ receptor internalisation upon coexpression of the AT₂ receptor. Unlike what was observed for the AT₁ receptor, dual agonist treatment resulted in a reduced level of internalisation of CCR2 upon heteromerisation with the AT₁ receptor, similar to what was seen for the AT₁-B₂ heteromer. Furthermore, both the AT₁-CCR2 and the AT₁-B₂ heteromer undergo potentiated β-arrestin recruitment upon dual receptor activation. This similarity in β-arrestin recruitment and internalisation profiles suggest common mechanisms may underpin these processes in both heteromers. Interestingly, as previously mentioned, heteromerisation reportedly alters the endocytic pathway of the AT₁ receptor and the B₂ receptor (AbdAlla et al., 2000). It is possible that a similar shift in endocytic pathway occurs for the internalisation of the AT₁-CCR2 heteromer.

The results of the CCR2 internalisation profile support the specificity and sensitivity of the BRET internalisation assay. Rather than being recycled back up to the plasma membrane, the CCR2 monomer/homomer is predominantly targeted to lysosomes for degradation (García Lopez et al., 2009). This was confirmed in this study by the lack of sequestration into recycling endosomes. Additionally, in contrast to AT₁ and B₂ receptors that remain sequestered to a constant level in early endosomes, CCR2 sequestration into early endosomes declines after reaching peak sequestration at approximately 10 min. This provides further evidence that endocytosed CCR2s are not being recycled.

7.4.2. B₂-CCR2 heteromer

The B₂ receptor and CCR2 are both major mediators of inflammation. While numerous studies have reported functional interactions between B₂ and CCR2 signalling systems,
to my knowledge this study is the first to investigate potential functional interactions at the level of the receptors. The GPCR-HIT assays revealed that upon coexpression of the B$_2$ receptor, there were substantial changes to the $\beta$-arrestin recruitment and trafficking profiles of CCR2, providing the first evidence of possible heteromerisation between these two receptors. Support for the existence of a B$_2$-CCR2 heteromer came from the small GPCR-HIT signal of BK-induced $\beta$-arrestin recruitment proximal to CCR2 upon coexpression of the B$_2$ receptor. Additionally, a small BK-induced GPCR-HIT signal was observed for modulation of the cell surface expression of CCR2. These two results indicate the close proximity of the B$_2$ receptor and CCR2, and suggest that CCR2 pharmacology is modulated upon activation of the B$_2$ receptor.

In addition to these GPCR-HIT signals, the $\beta$-arrestin2 assays also showed a reduced level of CCL2-induced $\beta$-arrestin2 recruitment proximal to CCR2, which was restored upon dual receptor activation. This $\beta$-arrestin2 recruitment profile is similar to what was previously observed for the AT$_1$-B$_2$ heteromer (Figure 5.2) as well as the AT$_1$-CCR2 heteromer (Ayoub et al., 2015), albeit with lower BRET signals from the B$_2$-CCR2 assays. The internalisation profile of CCR2 when coexpressed with the B$_2$ receptor also shared similarities with the AT$_1$-B$_2$ and the AT$_1$-CCR2 heteromer, showing a reduced level of internalisation of CCR2. However, unlike the AT$_1$-B$_2$ and the AT$_1$-CCR2 heteromer, in which reduced internalisation occurred only upon dual agonist treatment, CCL2-induced internalisation was also markedly reduced upon coexpression of the B$_2$ receptor. This result provides further evidence against the possibility that the reduced level of internalisation of these heteromers is due to competition for the endocytic machinery between the two receptors, or reduced BRET due to molecular crowding.

In contrast to the altered CCR2 pharmacology observed upon coexpression of the B$_2$ receptor, there appeared to be no modulation of B$_2$ receptor pharmacology upon coexpression of CCR2. Similar results have previously been seen in this thesis, whereby altered pharmacology is only observed in one configuration of the GPCR-HIT assay. For example, while BK-induced $\beta$-arrestin2-Venus recruitment to the AT$_1$ receptor was observed on coexpression of the B$_2$ receptor, there appeared to be no AngII-induced $\beta$-arrestin2-Venus recruitment to the B$_2$-Rluc8 upon coexpression of the AT$_1$ receptor. However, in the heteromer-specific BRET/BiFC assays, both AngII- and BK-induced
β-arrestin recruitment to the AT1-B2 complex. As was suggested for the AT1-B2 heteromer in the standard GPCR-HIT assay, it is possible that the lack of modulation to B2 receptor pharmacology upon coexpression of CCR2 is due to a relative excess of monomers/homomers, masking the relatively small signal from heteromers. Use of the heteromer-specific BRET/BiFC assays would enable more definite characterisation of modulations to B2 receptor pharmacology upon coexpression of CCR2.

Reports of functional interactions between B2 receptor and CCR2 signalling systems all involve BK/B2 receptor modulation of CCL2 expression levels. Most studies show an increase in CCL2 expression upon B2 receptor activation (Koyama et al., 1998; Koyama et al., 1999; Dos Santos et al., 2007; Yao et al., 2007; Marney et al., 2009; Shaw et al., 2011; Takemura et al., 2011; Xu et al., 2014), however reduced CCL2 expression has also been reported (Chen et al., 2003). At this initial stage of investigation it is not possible to determine whether the altered CCR2 pharmacology observed in my study is involved in the B2 receptor modulation of CCL2 expression levels. The mechanism of B2 receptor modulation of CCL2 expression has not yet been characterised, although B2 receptor-induction of other inflammatory mediators has been shown to occur through activation of NF-κB and cAMP response element-induced cyclooxygenase-2 (Blaes et al., 2013). Thus, it is possible that B2 receptor modulation of CCL2 expression occurs through a similar path, rather than through the altered CCR2 pharmacology observed in my study. Additional investigation is required to confirm this, as well as to further probe the existence and consequences of heteromerisation of the B2 receptor and CCR2.

Although interactions between the AT1, the B2 and CCR2 were not investigated in my study, the formation of dimeric heteromers with unique pharmacological profiles between each of these receptors provides an initial basis for further investigation of a potential trimeric complex. It is also notable that the AT1-B2, the AT1-CCR2 and the B2-CCR2 heteromers all show similarities in their β-arrestin recruitment and trafficking profiles. Upon further characterisation of the B2-CCR2 heteromer, research into interactions between all three receptors can be undertaken, applying similar BRET/BiFC approaches used in Chapter 5 of this thesis.
Chapter 8. General Discussion

8.1. Dimeric receptor heteromers

8.1.1. AT₁-AT₂ and AT₂-B₂ heteromer

While the AT₁ receptor is a fairly well characterised and prototypical GPCR, the pharmacology and physiology of the AT₂ receptor remains incompletely understood. Evidence for an interaction between the AT₁ and the AT₂ receptor has previously come from co-immunoprecipitation, BiFC, BRET and dominant negative receptor studies. Additionally, functional assays have enabled elucidation of some of the signalling and regulatory consequences of heteromerisation. For the first time, this study has investigated the ligand binding properties of the AT₁-AT₂ heteromer. The BRET ligand binding heteromer assay has demonstrated the close proximity of the AT₁ and the AT₂ receptor in living cells. Displacement of TAMRA-AngII by the AT₂ selective antagonist PD 123319 occurred with the same estimated affinity at AT₁-AT₂ heteromers as at AT₂ monomers/homomers, indicating neutral ligand cooperativity within the heteromer. The lack of ligand cooperativity within the AT₁-AT₂ heteromer does not conflict with the known functional antagonism of the AT₁ receptor when heteromerised with the AT₂ receptor, as cooperativity can be probe dependent and/or display bias, or the antagonism can occur further downstream from ligand binding.

Indeed, it has previously been shown that the AT₁-AT₂ heteromer does not internalise following recruitment of β-arrestin, providing a basis for the inhibition of AT₁ receptor signalling upon heteromerisation with the AT₂ receptor. This study has further confirmed that the AT₁-AT₂ heteromer recruits β-arrestin in an AngII-dependent manner. Additionally, the GPCR-HIT internalisation assay has provided further information on the trafficking profile of the heteromer, confirming that it does not internalise or undergo endosomal sequestration. Interestingly however, AngII treatment
appeared to cause an increase in the surface expression of the AT$_2$ receptor upon coexpression of the AT$_1$ receptor. A very similar trafficking profile has previously been observed for the AT$_2$-D$_1$ heteromer, in which activation of the D$_1$ receptor resulted in an increase in surface expression of both the AT$_2$ receptor and the D$_1$ receptor. As a consequence of both these results, it is proposed that the apparent increase in AT$_2$ receptor surface expression occurs as a result of the replacement of internalised AT$_1$ (or D$_1$) receptors with AT$_1$-AT$_2$ heteromers (or AT$_2$-D$_1$ heteromers).

The results of this study on the AT$_1$-AT$_2$ heteromer suggest a time course of events that occur upon treatment with AngII (Figure 8.1). Firstly, AT$_1$ monomers/homomers are rapidly internalised (~5 min). In the following 5 min, there appears to be an increase in surface expression of AT$_1$-AT$_2$ heteromers that are preformed in the biosynthetic pathway. Over the next 10 min, β-arrestin is recruited to AT$_1$-AT$_2$ heteromers where the complex is retained at the cell surface, unable to internalise. Following this, it is speculated that there is an increase in the overall expression of AT$_1$-AT$_2$ heteromers (as seen in the AT$_1$-Venus + AT$_2$-Rluc8 studies), potentially due to increased transcription or translation.
Chapter 8. General Discussion

This study has also used similar approaches to investigate the putative AT2-B2 heteromer. In all of the GPCR-HIT assays used, coexpression of the B2 receptor resulted in BK-induced modulation of AT2 receptor pharmacology, providing considerable evidence for the existence of the AT2-B2 heteromer. Interestingly, the study has revealed that some aspects of the AT2-B2 heteromer’s pharmacology is strikingly similar to the AT1-AT2 heteromer, however there are also distinct differences. A model for the time course of events upon activation of the AT2-B2 heteromer is depicted in Figure 8.2. Similar to the AT1-AT2 heteromer, it is proposed that upon rapid internalisation of the B2 receptor, there is cell surface translocation of AT2-B2 heteromers which are preformed in the biosynthetic pathway. In the 20 min following initial BK treatment, β-arrestin is recruited to the heteromer. Next, and in contrast to the AT1-AT2 heteromer, the AT2-B2 heteromer is internalised over the following 30 min.
Finally, similar to the AT$_1$-AT$_2$ heteromer, there appears to be an increase in the overall expression of the AT$_2$-B$_2$ heteromer, potentially due to increased transcription or translation.

While this study has provided considerable evidence to support these two models, further investigations are required to fully elucidate the consequences of the interactions between the receptors. For example, recruitment of β-arrestin proximal to AT$_2$ receptors heteromerised with AT$_1$ or B$_2$ receptors may inhibit interactions with its signalling and regulatory proteins such as PLZF (Senbonmatsu et al., 2003), ATIP1 (Nouet et al., 2004; Li et al., 2007b; Fujita et al., 2009) and TIMP-3 (Kang et al., 2008). Conversely, recruitment of β-arrestin proximal to AT$_2$ receptors provides the potential for new signalling opportunities, such as activation of MAPK cascades. Investigating
how coexpression with AT₁ or B₂ receptors affects these signalling pathways, potentially with the use of β-arrestin knockdown siRNA (Ahn et al., 2003) will provide valuable information on the outcomes of heteromerisation-induced β-arrestin recruitment to the AT₂ receptor.

The two models suggest that the apparently increased surface expression of the AT₂ receptor is due to translocation of AT₁-AT₂ or AT₂-B₂ heteromers to the cell surface. Additionally, the subsequent internalisation of the AT₂ receptor (in the presence of the B₂ receptor) is believed to be due to internalisation of AT₂-B₂ heteromers. To provide further confirmation that these receptors are co-trafficking with one another, a combined BRET/BiFC or BRET/BiLC approach could be used. Fusion of the complementary parts of Venus or luciferase to each receptor would ensure generation of heteromer-specific signals.

As mentioned, support for the hypothesis that the increased AT₂ surface expression occurs as a result of translocation of AT₁-AT₂ or AT₂-B₂ heteromers to the cell surface comes from studies of the AT₂-D₁ heteromer (Salomone et al., 2007; Gildea et al., 2012; Padia et al., 2012). These studies show an increase in both AT₂ and D₁ receptor surface expression upon activation of the D₁ receptor, with a kinetic profile similar to what was observed in my studies with the AT₁-AT₂ and the AT₂-B₂ heteromer. A similar mechanism may explain the AngII-induced increase in surface expression of the AT₂ receptor in smooth muscle cells coexpressing both AT₁ and AT₂ receptors (de Godoy et al., 2006). In that study, AT₂ receptor translocation could be blocked with losartan, but not PD 123319, indicating the requirement of AT₁ receptor activation. It may be that the AT₂ receptor is predominantly located intracellularly, as suggested by several studies (de Godoy et al., 2006; Jiang et al., 2012), and that activation of a heteromeric partner may assist in cell surface trafficking of the receptor. Indeed, in the study by Jiang et al. (2012), monomeric AT₂ receptors were predominantly localised intracellularly while higher order AT₂ homomers were found predominantly at the cell surface.

Due to the constitutively active nature of the AT₂ receptor, as well as its upregulated expression under certain pathologies, it has been suggested that a major determinant of AT₂ receptor signalling may be its expression level (Porrello et al., 2009). Increased expression of the AT₂ receptor when heteromerised with the AT₁ or the B₂ receptor may therefore contribute greatly to regulation of AT₂ receptor
signalling. The AT$_2$ receptor is known to antagonise AT$_1$ receptor signalling and potentiate B$_2$ receptor NO-cGMP signalling. An increase in the proportion of heteromers relative to AT$_1$ or B$_2$ monomers/homomers will likely reinforce these inhibitory or enhancing actions.

While the proposed models suggest that the increase in interaction between AT$_2$-Rluc8 and AT$_1$-Venus or B$_2$-Venus are due to increased transcription or translation of the receptors, this is currently speculation that needs further verification. This could be achieved by monitoring these interactions in the presence of inhibitors of transcription (Bensaude, 2011) or translation (Schneider-Poetsch et al., 2010). Additionally, a more complete model of the trafficking of these heteromers could be investigated through use of other cellular compartment markers (such as markers of fast recycling endosomes, exocytic vesicles or the Golgi apparatus) as well as inhibitors of the different stages of trafficking (various exocytic, endocytic and recycling inhibitors are available).

The AT$_2$ receptor is a poorly characterised GPCR, and despite decades of research, still one of the least understood aspects of the RAS (Porrello et al., 2009). Numerous studies have described conflicting results and the physiological functions and signalling mechanisms of the AT$_2$ receptor remain incompletely defined. However, the importance of the AT$_2$ receptor is highlighted through two recent clinical trials for an AT$_2$ agonist (Mitsubishi Tanabe Pharma, 2015) and an antagonist (Rice et al., 2014). Additionally, the biotech company that developed this AT$_2$ antagonist has recently been acquired by Novartis for US$200 million (Williamson, 2015), further highlighting the importance of the AT$_2$ receptor as a therapeutic target. Heteromerisation with the AT$_1$ receptor and the B$_2$ receptor establishes an additional important role for the AT$_2$ receptor in both the RAS and the KKS. Further research will likely reveal that AT$_2$ receptor heteromerisation is an integral part of its molecular functioning, possibly explaining the previous difficulties encountered when characterising its pharmacology.

8.1.2. AT$_1$-B$_2$ heteromer

Despite being first characterised over a decade ago, the existence of the AT$_1$-B$_2$ heteromer remains contentious. In 2000 AbdAlla et al. (2000) reported that it was a constitutive heteromer which caused enhanced AT$_1$ receptor signalling. The same group
went on to link the AT1-B2 heteromer with pre-eclamptic hypertension (AbdAlla et al., 2001a) and experimental hypertension (AbdAlla et al., 2005). The controversy arose when these results were challenged by a collaboration of four independent research groups which were unable to find any evidence for the existence of the AT1-B2 heteromer (Hansen et al., 2009). Despite this, Quitterer’s group continued to further characterise the pharmacology of the heteromer both in vitro and in vivo (Quitterer et al., 2011; Quitterer et al., 2014), while also providing explanations for why Hansen et al. (2009) were unable to detect the heteromer. For example, they showed that the chaperone calreticulin is required for B2 receptor maturation, and in its absence the AT1 and the B2 receptor do not heteromerise (AbdAlla et al., 2009; AbdAlla et al., 2010).

Two other studies have investigated the existence of the AT1-B2 heteromer. Wilson et al. (2013) investigated the effect of the arrestin-selective agonist SII on the heteromer, providing supporting evidence for its existence. See et al. (2011) used GPCR-HIT to investigate AngII-induced β-arrestin recruitment to B2-Rluc8 when coexpressed with the AT1 receptor. Though no GPCR-HIT signal was observed, this was not necessarily indicative of a lack of heteromerisation, as donor-acceptor orientation and/or distance may have been unfavourable. Alternatively, it was suggested that the heteromer may not recruit β-arrestin in an AngII dependent manner.

This study has confirmed the previous GPCR-HIT result, showing no detectable AngII-dependent β-arrestin recruitment to a putative B2-Rluc8/AT1 complex. However, the reverse configuration showed BK-dependent β-arrestin recruitment to an AT1-Rluc8/B2 complex. Additionally, the BRET/BiFC assay, which detects heteromer-specific signals, showed not only BK-dependent recruitment but also AngII-dependent recruitment. These results support the existence of the AT1-B2 heteromer in transfected cells, and suggest that it recruits β-arrestin upon treatment with both AngII and BK. The lack of AngII-dependent recruitment in the standard GPCR-HIT assay may then be due to unfavourable donor-acceptor orientation and/or distance. Additionally, it could be that B2 receptors form monomers/homomers preferentially to AT1-B2 heteromers, making any GPCR-HIT signal negligible in one orientation.

This study also investigated the internalisation properties of the AT1-B2 heteromer. From all these results, the β-arrestin recruitment and trafficking profile of the heteromer has been modelled (Figure 8.3). As mentioned, the heteromer appears to
be able to recruit β-arrestin in both an AngII- and BK-dependent manner. Subsequently, it also appears to be able to internalise into endosomes upon treatment with both AngII and BK (as previously observed; AbdAlla et al., 2010; Quitterer et al., 2011), however the profiles of internalisation upon different treatments vary. The AngII-induced internalisation of the heteromer occurs more rapidly than the AngII-induced internalisation of AT1 monomers/homomers. As an increased rate of sequestration into early and recycling, but not late endosomes suggests an increase in recycling and resensitisation, this would shift the signalling balance to less β-arrestin-mediated and greater G protein-mediated signalling. As AT1-induced vasoconstriction is mostly mediated by G protein signalling (Rajagopal et al., 2006; Aplin et al., 2007), this supports the AngII-induced hypertension associated with the AT1-B2 heteromer. In contrast, BK and dual agonist treatment causes a reduced level of internalisation, and dual agonist treatment results in internalisation kinetics which resemble the B2 receptor rather than the AT1 receptor. This is reminiscent of the results of Wilson et al. (2013), whereby SII-induced internalisation of the AT1-B2 heteromer resembled B2 receptor kinetics. Similar to the AngII-induced internalisation of the heteromer, adoption of B2 receptor internalisation and trafficking kinetics could increase the level of G protein-mediated signalling, as the B2 receptor appears to dissociate from β-arrestin and recycle more rapidly than typical Class B receptors (Simaan et al., 2005).
The AT₁-B₂ heteromer model generated from this study suggests several differences from the AT₁-AT₂ heteromer and the AT₂-B₂ heteromer models. Firstly, there appears to be no ligand-induced increase in the cell surface trafficking of the AT₁-B₂ heteromer, and no ligand-induced increase in the formation of new heteromers (however, it should be noted that in both assays, the receptors are likely to be in a state of dynamic equilibrium, resulting from the continual internalisation and recycling of the heteromer, as well as continual degradation and synthesis). Additionally, the results of the BRET/BiFC assays suggest that the interactions between the AT₁ and the B₂ receptor may be weaker or more transient than the interactions between the AT₁ and the AT₂ receptor or the AT₂ and the B₂ receptor. Taken together, these results suggest that the AT₁-AT₂ heteromer and the AT₂-B₂ heteromer form relatively strong and prolonged interactions that may begin early in the biosynthetic pathway. In contrast, I speculate...
that the AT$_1$ and the B$_2$ receptor interact in a more transient fashion, perhaps only occurring upon colocalisation at the cell surface.

### 8.1.3. AT$_1$-CCR2 heteromer

CKD is a major cause of morbidity in the developed world, affecting 10% of the population in Europe and the United States (Hallan et al., 2006). It is characterised by systemic hypertension, proteinuria, interstitial fibrosis, peritubular capillary loss, a decline in glomerular filtration rate and tubular atrophy leading to destruction of functioning nephrons (Eddy, 2005; Metcalfe, 2007). The RAS is well known to be involved in the initiation and acceleration of CKD (Metcalfe, 2007; Kelly et al., 2015) through the hypertrophic, inflammatory and fibrotic actions of AngII (Remuzzi et al., 2005). Additionally, the CCL2/CCR2 system has also been implicated in various renal pathologies (Yokoyama et al., 1998; Tesch, 2008; Nam et al., 2012). Recent research from my group and our collaborators has revealed an involvement of both the AT$_1$ receptor and the CCR2 in CKD (Ayoub et al., 2015). Treatment of STNx rats with dual antagonist therapy resulted in improved outcomes in comparison to monotherapy. Further investigation using the GPCR-HIT assay revealed that the AT$_1$ receptor forms a functional heteromer with the CCR2. Activation of the AT$_1$ receptor was shown to inhibit CCR2-G$_{\alpha_i}$ coupling and induce recruitment of $\beta$-arrestin to CCR2. Dual agonism potentiated $\beta$-arrestin recruitment which was completely blocked with dual but not single, antagonist treatment.

My contribution to this study revealed that in contrast to the modulation in G$_{\alpha_i}$ coupling, there was no modulation of G$_{\alpha_q}$ coupling, indicating that AT$_1$-CCR2 heteromerisation results in a biased G protein signalling profile. I also investigated the internalisation profile of this heteromer, showing that similar to the AT$_1$-B$_2$ heteromer and the B$_2$-CCR2 heteromer, but not the AT$_1$-AT$_2$ or the AT$_2$-B$_2$ heteromer, there was a reduced level of internalisation upon dual agonist treatment. Together, these studies have revealed for the first time the distinct pharmacological profile of the AT$_1$-CCR2 heteromer. Further research will enable elucidation of the underlying pharmacology causing the in vivo beneficial synergism from dual receptor blockade.
8.1.4. \( \beta \)-CCR2 heteromer

This study has provided the first evidence of a functional interaction between the \( \beta_2 \) receptor and CCR2. It was shown that coexpression of the \( \beta_2 \) receptor resulted in BK-induced \( \beta \)-arrestin recruitment and internalisation of CCR2. Additionally, the heteromer displayed similar \( \beta \)-arrestin recruitment and trafficking profiles upon dual agonist treatment as the \( AT_1 \)-\( \beta_2 \) heteromer and the \( AT_1 \)-CCR2 heteromer. Although functional interactions between \( \beta_2 \) and CCR2 signalling systems have previously been reported, further investigation is required to determine if the heteromerisation-induced modulated pharmacology observed in this study contributes to those functional interactions. Additionally, further research is required to more thoroughly elucidate the physiological consequences of \( \beta_2 \)-CCR2 heteromerisation.

8.2. Putative higher order heteromers

From their initial characterisation as functional monomeric receptors, our understanding of GPCR quaternary structure has evolved to the now well accepted paradigm that most GPCRs form dimers, as well as higher order oligomers. Furthermore, the discovery that many GPCRs are ‘pre-coupled’ to signalling partners and effectors, has lead to the development of the concept of ‘receptor mosaics’ (Fuxe et al., 2010) or ‘signalosomes’ (Ferré, 2015). These are believed to be microdomains that consist of oligomeric receptors and their various signalling and regulatory partners, enabling efficient targeting and integration of complex GPCR signalling.

The existence of GPCR signalosomes suggests that higher order heteromeric GPCR complexes may be relatively common, however to date, only four examples of GPCR heteromers containing more than two different receptors have been described. These are the adenosine \( A_{2A} \)-cannabinoid CB\(_1\)-dopamine D\(_2\) receptor heteromer (Navarro et al., 2008), the adenosine \( A_{2A} \)-dopamine D\(_2\)-metabotropic glutamate type 5 receptor heteromer (Cabello et al., 2009), the CCR5-CD4-CXCR4 heteromer (Martínez-Muñoz et al., 2014) and the GPR39-serotonin 5-HT\(_{1A}\)-galanin GalR\(_1\) receptor
heteromer (Tena-Campos et al., 2015). The lack of reports of other higher order GPCR heteromers is likely due to the more complex nature of probing their existence and pharmacology, however approaches such as those used in this study provide novel means of investigating them.

Using combined BRET/BiFC approaches, this study has provided the first evidence that the AT1, the AT2 and the B2 receptor may form a functional heteromer in transfected cells. The modified GPCR-HIT assays with BiFC showed AngII-induced β-arrestin recruitment to AT2-B2 heteromers upon coexpression of the AT1 receptor, and BK-induced recruitment of β-arrestin to AT1-AT2 heteromers upon coexpression of the B2 receptor. To further explore the hypothesis of the existence of an AT1-AT2-B2 trimer, additional studies will be required. Application of the BRET/BiFC assays to other aspects of receptor pharmacology, such as internalisation and trafficking or GRK recruitment, will provide further insights. It would also be interesting to investigate the existence of the trimer using a modified version of the BRET ligand binding assay. Generation of a BRET signal upon fusion of complementary parts of Nluc to the N terminus of the AT1 and the AT2 receptor and treatment with a fluorescent B2 ligand would provide strong evidence of the proximity of all three receptors. In addition to combined BRET/BiLC or BRET/BiFC approaches, other techniques should also be applied, such as the sequential BRET/FRET approach used for some of the previously reported trimeric heteromers.

Although not investigated in this study, the existence of an AT1-B2-CCR2 heterotrimer is also hypothesised. This study has revealed evidence for the existence of AT1-B2, AT1-CCR2 and B2-CCR2 heteromers. This provides an initial basis to begin investigations into the existence of a trimeric complex containing all three receptors. Additionally, it is possible that complexes containing all four receptors (AT1, AT2, B2, CCR2) may exist, perhaps in the context of signalosomes with other GPCR interacting proteins. Application of the approaches described above will be required to test these hypotheses.

The AT1, the AT2 and the B2 receptor, as well as CCR2, are all expressed in several of the same cell types, such as endothelial and smooth muscle cells of the vasculature. While this supports the possibility of the existence of higher order complexes containing these receptors in vivo, further confirmation of their coexpression and colocalisation in native tissue will be required. Whether or not the in vivo existence...
of higher order heteromers is eventually confirmed, their coexpression within the same cell types likely confounds many studies of the individual receptors, as well as the dimeric heteromers. Cells expressing these receptors will contain a heterogeneous population of monomers, homomers, heteromers and potentially even higher order oligomers. As the results of the BRET/BiFC assays demonstrate, under these conditions there will be complicated interactions and competition between each of these oligomeric populations. It is likely that this complex interplay underpins much of the controversy and inconsistency surrounding the AT$_1$-B$_2$ heteromer.

8.3. BRET assays

8.3.1. BRET ligand binding assay

This study involved the development and application of the BRET ligand binding assay, demonstrating a new method for investigating one of the most fundamental aspects of molecular pharmacology. As with FRET ligand binding assays, the BRET technique has several advantages when compared to traditional radioligand binding assays. Firstly, the BRET/FRET approach enables monitoring of these biomolecular interactions with greater ease and efficiency, while reducing expense and hazard exposure. As well, the BRET/FRET assays are conducted under more physiologically relevant conditions, being performed in live cells, in real time, at 37°C. Additionally, the BRET/FRET assays are able detect to GPCR oligomers with neutral ligand cooperativity, which cannot be achieved with radioligand binding assays alone. In addition, the BRET approach also has a distinct advantage over the FRET approach, in that it can be performed homogenously, without the need for removal of the energy donor’s substrate. While this homogeneity was less evident for the BRET heteromer ligand binding assay, this was almost certainly due to the low affinity of the TAMRA-AngII ligand, and the resulting high concentrations and high background signal obtained. Indeed, normalisation of the homogenously performed heteromer assay clearly revealed PD 123319-induced displacement of TAMRA-AngII bound to the AT$_2$ protomer in the AT$_1$-AT$_2$ heteromer.
The robustness and sensitivity of the BRET ligand binding assay is revealed through the generation of precise results when using a low affinity ligand like TAMRA-AngII. Despite using up to 1 µM of the fluorescent AngII ligand, specific signals could still be easily separated from the non-specific background. The results of this study have also revealed that the BRET ligand binding assay can be used successfully with a wide range of different coloured fluorescent ligands. While fluorophores with longer wavelength emissions produced superior results due to greater spectral separation between donor and acceptor emissions, specific ligand binding was still evident with the shorter wavelength green ligands.

8.3.2. Assessment of receptor expression levels

A limitation of all the BRET approaches used in this study is that the relative expression levels of the receptors, particularly upon their coexpression, was not assessed. For example, the lack of modulation to the BRET signal upon coexpression of the AT2 receptor in many assays is believed to be due to the low level of expression of the AT2 receptor. Understanding the relative expression levels of all the receptors within a transfection is important when teasing apart the modulations of one receptor on another receptor’s pharmacology, and thus assessment of expression would enable more comprehensive interpretation of the results.

In all of the BRET assays conducted in this study, one receptor or protein was tagged with the luciferase while an interacting biomolecule was tagged with the fluorophore (or two proteins tagged with complementary portions of Venus in the BRET/BiFC assays). Determination of the relative levels of each of these fusion molecules would be fairly straightforward, through assessment of the emitted luminescence and fluorescence from cells from the same samples as the BRET assays. In the heteromer assays using the GPCR-HIT configuration, a third receptor was coexpressed, which in most cases was tagged with an HA epitope. Assessment of the expression level of the these receptors could have been determined through an immunoassay approach such as ELISA.

Additionally, the BRET ligand binding assay could be used to assess changes to Nluc-tagged receptor levels upon coexpression of a second, untagged receptor. While
this information would provide less direct information about expression levels in each of the specific transfections in the BRET assays, it would be a simple method to provide general information about changes to expression. For example, titrations could be carried out with varying cDNA ratios, giving general indications of the effect of coexpression of one receptor on another.

8.3.3. Application of other techniques to assess heteromerisation

With the exception of BiFC and time-resolved FRET (IP$_1$ assay), BRET has been used almost exclusively throughout this thesis to investigate modulations to receptor pharmacology upon heteromerisation. Although a powerful technique for characterising heteromer pharmacology, particularly when used in the GPCR-HIT configuration, the use of other approaches would provide support to the conclusions drawn from the results of this study. For example, more comprehensive use of the BRET/BiFC system, and/or a BRET/BiLC system, would enable detection of heteromer-specific signals which may have been masked in the presence of excess monomers/homomers. This was believed to be the case for β-arrestin recruitment to the B$_2$ receptor upon coexpression of the AT$_1$ receptor, as well as internalisation of the AT$_1$ receptor upon coexpression of the AT$_2$ receptor.

Microscopy approaches would provide direct evidence of colocalisation of receptors and could potentially provide support to the BRET β-arrestin recruitment and trafficking assays. An interesting approach to investigate heteromerisation would involve adaptation of the GPCR-HIT configuration using a microscopy assay. For example, coexpression of a fluorophore-tagged receptor and an untagged receptor, and subsequent treatment with a fluorescent ligand for the untagged receptor, would enable monitoring of the localisation of the two fluorophores in parallel. If the receptors colocalise or cointernalise, this would be visualised by colocalisation and/or cointernalisation of the two fluorophores.

To thoroughly assess receptor heteromer pharmacology, a suite of G protein and downstream signalling assays, such as cAMP, calcium accumulation and ERK assays would be required. Additionally, interactions with other adapter proteins such as GRKs could be investigated.
Finally, all these various assay systems need to be tested in different cellular backgrounds, with an eventual application to \textit{in vivo} systems. This would enable confirmation of the heteromer’s existence \textit{in vivo}, as well as determination of its physiological relevance. Identification of heteromer-selective compounds or antibodies would help \textit{in vivo} evaluation enormously (Gomes \textit{et al.}, 2016).

### 8.4. Summary

The RAS is a vital regulatory system which is involved in the maintenance of blood pressure, as well as numerous other physiological and pathological processes. The system functions through the interactions of a variety of enzymes, hormones and receptors, many of which have been well characterised over the past several decades. The potential for GPCR heteromerisation has revealed further complexity within the RAS, particularly for the two receptors which mediate the effects of the system’s primary hormone, AngII. This study has provided evidence for the existence in transfected cell lines of the AT$_1$-AT$_2$ heteromer, the AT$_1$-B$_2$ heteromer, the AT$_2$-B$_2$ heteromer, the AT$_1$-CCR2 heteromer and the B$_2$-CCR2 heteromer. The various biophysical approaches used have revealed some of the distinct pharmacology associated with each heteromer. Additionally, evidence for interactions between AT$_1$, AT$_2$ and B$_2$ receptors has been provided, and further research will be undertaken to determine if similar interactions occur between AT$_1$, B$_2$ and CCR2. It is hypothesised that complex interactions such as these, whether the result of heteromerisation or not, underpin some of the controversy surrounding the existence of the AT$_1$-B$_2$ heteromer. Understanding the consequences of receptor heteromerisation is fundamental to further elucidation of the complex physiology and pathology associated with the RAS.
References


AbdAlla S, Lother H, Abdel-tawab AM, Quitterer U (2001b). The angiotensin II AT2 receptor is an AT1 receptor antagonist. The Journal of Biological Chemistry 276: 39721-39726.


References


Cheng Y, Prusoff WH (1973). Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem. Pharmacol.* 22: 3099-3108.


References


References


References


Knowle D, Ahmed S, Pulakat L (2000). Identification of an interaction between the angiotensin II receptor sub-type AT2 and the ErbB3 receptor, a member of the epidermal growth factor receptor family. *Regul. Peptides* 87: 73-82.


Lambert NA, Javitch JA (2014). CrossTalk opposing view: Weighing the evidence for class A GPCR dimers, the jury is still out. *J. Physiol. (Lond.*) 592: 2443-2445.


References


References


Touyz RM, Schiffrin EL (1999a). Ang II-stimulated superoxide production is mediated via phospholipase D in human vascular smooth muscle cells. Hypertension 34: 976-982.

References


Appendix I – Materials

BACTERIAL MEDIA

LB (Luria-Bertani) broth
10 g LB powder (containing 10 g Bacto-tryptone, 5 g yeast extract and 5 g NaCl per 1 L of LB broth; BD Bioscience) was added per 400 mL of ddH₂O and then autoclaved. Antibiotics were added to aliquots just prior to use.

LB (Luria-Bertani) agar
10 g LB-agar powder (containing 10 g Bacto-tryptone, 5 g yeast extract, 5 g NaCl and 15 g Bacto-agar per 1 L of LB broth; BD Bioscience) was added per 400 mL of ddH₂O and then autoclaved. The solutions were cooled to ~60°C before adding antibiotics and plating.

ANTIBIOTICS

Ampicillin
From Sigma Aldrich. 50 mg/mL in ddH₂O. Aliquots of stock solutions were stored at -20°C.

Kanamycin
From Sigma Aldrich. 50 mg/mL in ddH₂O. Aliquots of stock solutions were stored at -20°C.
GENERAL REAGENTS

Agarose gels
1 g of agarose (Promega) was added per 100 mL of ddH₂O (1%) and dissolved by slow heating. Upon complete dissolution 2 µL of ethidium bromide was added per 40 mL before pouring.

TAE electrophoresis buffer
10x TAE (Ambion) was diluted in ddH₂O. Final concentrations in 1x TAE were 40 mM Tris-Acetate and 1 mM Na₂EDTA.

Phosphate buffered saline (PBS)
Powdered PBS (Gibco) was added to ddH₂O and autoclaved. Final concentrations were 0.2 g/L KCl, 0.2 g/L KH₂PO₄, 8 g/L NaCl, 1.15 g/L Na₂PO₄.

PBS supplemented with Ca²⁺ and Mg²⁺
Supplied from Sigma Aldrich. Contains 0.133 g/L CaCl₂, 0.1 g/L MgCl₂, 0.2 g/L KCl, 0.2 g/L KH₂PO₄, 8 g/L NaCl, 1.15 g/L Na₂PO₄.

Hanks’ balanced salt solution (HBSS)
Supplied from Gibco. Contains 0.4 g/L KCl, 0.06 g/L KH₂PO₄, 0.35 g/L NaHCO₃, 8 g/L NaCl, 0.048 g/L Na₂PO₄ (anhydrous), 1 g/L D-glucose (dextrose).

HBSS supplemented with Ca²⁺ and Mg²⁺
Supplied from Gibco. Contains 0.14 g/L CaCl₂ (anhydrous), 0.1 g/L MgCl₂-6H₂O, 0.1 g/L MgSO₄-7H₂O, 0.4 g/L KCl, 0.06 g/L KH₂PO₄, 0.35 g/L NaHCO₃, 8 g/L NaCl, 0.048 g/L Na₂PO₄ (anhydrous), 1 g/L D-glucose (dextrose).
TISSUE CULTURE SOLUTIONS

Complete DMEM medium
5 mL of L-glutamine (0.3 mg/mL final concentration; Gibco), 5 mL penicillin/streptomycin (100 IU/mL final concentration of penicillin, 100 µg/mL of streptomycin; Gibco) was added to 500 mL of Dulbecco’s modified Eagle’s medium (DMEM; Gibco). 50 mL of foetal calf serum (FCS; Gibco) was added if required (10% final concentration).

Trypsin-EDTA solution
Trypsin-EDTA (Gibco) was supplied as a 10x liquid solution containing 5 g/L of trypsin (0.5% w/v), 2 g/L EDTA and 8.5 g/L of NaCl in 100 mL. 5 mL aliquots (stored at -20°C) were diluted in 45 mL of PBS and stored at 4°C.

Poly-L-lysine
Sterile poly-L-lysine hydrobromide (70-150 000 MW; Sigma Aldrich) was diluted in ddH₂O to a final concentration of 0.1 mg/mL and stored at 4°C.

ELISA SOLUTIONS

Fixing solution
16% paraformaldehyde (ProSciTech) was diluted to 4% in PBS + Ca²⁺ and Mg²⁺.

Blocking solution
1 mL FCS was added per 100 mL of PBS + Ca²⁺ and Mg²⁺ (1% final concentration).
Appendix II – Publications and Abstracts

Refereed Journal Articles


Review Articles


Abstracts for Oral Presentations


**Abstracts for Poster Presentations**


Oral Presentations

1. ASCEPT Molecular Medicine Symposium: “The molecular pharmacology of angiotensin and bradykinin receptor heteromers”. March 2016, Harry Perkins Institute of Medical Research, Nedlands, Australia.

2. Final PhD Seminar: “Biophysical insights into the molecular pharmacology of angiotensin II receptor heteromers”. September 2015, Harry Perkins Institute of Medical Research, Nedlands, Australia.

3. UWA Pharmacology Department Seminar: “Pharmacological profiling of angiotensin II receptor heteromers”. June 2014, University of Western Australia, Nedlands, Australia.
4. **WA Australian Society for Medical Research Scientific Symposium 2014:** 
   “Monitoring the internalisation profiles of angiotensin II receptor heteromers”. June 2014, Edith Cowan University, Mt Lawley, Australia.

5. **Drug Discovery and Cell Signalling Mini-Symposium – New Perspectives on Receptors:** “Characterisation of angiotensin II receptor heteromers”. April 2014, University of Nottingham, Nottingham, United Kingdom.


7. **WA Australian Society for Medical Research Scientific Symposium 2013:** “Investigating angiotensin II and bradykinin receptor heteromers using a novel approach combining bioluminescence resonance energy transfer and bimolecular fluorescence complementation”. June 2013, Edith Cowan University, Mt Lawley, Australia.

8. **Preliminary PhD seminar:** “Identification and pharmacological profiling of novel angiotensin receptor complexes”. December 2012. WA Institute for Medical Research, Perth, Australia.

9. **Renin-Angiotensin System Pre-International Society of Hypertension Satellite Meeting:** “Heteromerisation with the bradykinin type 2 receptor enables β-arrestin recruitment to the angiotensin II type 2 receptor”. September 2012, Hunter Valley, Australia.

10. **WA Australian Society for Medical Research Scientific Symposium 2012:** “β-arrestin recruitment to the angiotensin II type 2 receptor is enabled by heteromerisation with the bradykinin type 2 receptor”. June 2012, Edith Cowan University, Mt Lawley, Australia.
11. **ASCEPT Molecular Pharmacology Perth Symposium**: “Heteromerisation with the bradykinin type 2 receptor alters β-arrestin recruitment and internalisation of the angiotensin II type 2 receptor”. February 2012. WA Institute of Medical Research, Nedlands, Australia.

**Awards**

1. Finalist in the ASCEPT Neville Percy Prize for best poster communication at the 2014 ASCEPT Annual Scientific Meeting.

2. Winner of the Lions Eye Institute and the Ear Science Institute of Australia’s Oral Communication Award at the 2013 WA Australian Society for Medical Research Scientific Symposium, Perth, Australia.


**Travel Grants**

1. ASCEPT travel award to present at the 2014 Joint ASCEPT-MPGPCR Annual Scientific Meeting in Melbourne, Australia.

2. Harry Perkins Institute of Medical Research travel award to present at the 2014 EMBL Australia PhD Symposium in Sydney, Australia.

3. British Pharmacological Society Bain Memorial Bursary Fund travel award to present at the 2014 BPS 5th Focused Meeting on Cell Signalling, Leicester, United Kingdom.
4. University of Western Australia travel award to present at the 2014 BPS 5th Focused Meeting on Cell Signalling, Leicester, United Kingdom.

5. ASCEPT travel award to present at the 2013 ASCEPT Annual Scientific Conference, Melbourne, Australia.

6. Monash University travel award to present at the 2012 Molecular Pharmacology of G Protein-Coupled Receptors 7th International Meeting, Melbourne, Australia.

7. ASCEPT travel award to present at the 2012 Joint ASCEPT-Australian Pharmaceutical Science Association Annual Scientific Conference, Sydney, Australia.


9. WA Institute for Medical Research 2012 Travel Award to present at the Renin-Angiotensin System Pre-International Society of Hypertension Satellite Meeting, Hunter Valley, Australia.
Application of BRET to monitor ligand binding to GPCRs

Leigh A Stoddart1, Elizabeth K M Johnstone2,3, Amanda J Wheal3, Joëlle Goulding3, Matthew B Robers4, Thomas Machledt5, Keith V Wood6, Stephen J Hill5,6,7 & Kevin D G Pfleger2,3,5

Bioluminescence resonance energy transfer (BRET) is a well-established method for investigating protein-protein interactions. Here we present a BRET approach to monitor ligand binding to G protein-coupled receptors (GPCRs) on the surface of living cells made possible by the use of fluorescent ligands in combination with a bioluminescent protein (NanoLuc) that can be readily expressed on the N terminus of GPCRs.

The ability to monitor protein-protein or drug-protein interactions with ease and sensitivity is the cornerstone of cell biology and pharmacology. BRET is dependent upon energy transfer between a bioluminescent donor (luciferase emitting its substrate) and fluorescent acceptor. It has become the proximity assay of choice for many researchers owing to its ease of use and capacity for real-time monitoring in live cells.1–3. Renilla luciferase variant Rluc8 and GFP variant Venus are a notable example of a BRET combination; however, various donor-acceptor pairs have been used successfully for different applications.4 To date, drug-protein interactions have not been directly studied using BRET, although it has recently been shown that BRET can be used to detect drug concentration using bioluminescent sensor proteins.5 The development of many different fluorescent agonists and antagonists for GPCRs enables their use as energy acceptors to measure BRET between a fluorescently labeled ligand and luciferase-tagged receptor.

We have developed an assay that can measure ligand binding to GPCRs using BRET in living cells. We initially investigated whether β2-adrenergic receptors (β2ARs) tagged on their extra-cellular N terminus with a luminescent protein can be expressed in living cells. We assessed Rluc8 and the recently described NanoLuc (Nluc)6 luciferase engineered from the luciferase found in deep sea shrimp, Opheliasaurus gracilis. In HEK293 cells, increasing levels of transiently transfected cDNA in the presence of luciferase substrates coexpressed Rluc8 and furmazine for Nluc caused concurrent increases in luminescence with both Rluc8- and Nluc-tagged β2AR. Nluc-β2AR produced substantially greater luminescence signals than Rluc8-β2AR, and with a spectrum left shifted by ~20 nm (Supplementary Fig. 1). We initially selected a TAMRA-labeled β2AR antagonist (alprenolol-TAMRA) as a fluorescent acceptor ligand because it has spectral characteristics theoretically amenable both for accepting energy from either of the bioluminescent protein donors (peak excitation at 565 nm) and for emitting light at wavelengths that can be clearly distinguished from luciferase light emission (peak emission at 580 nm). We added increasing concentrations of TAMRA-labeled alprenolol to HEK293 cells transiently transfected with luciferase-tagged β2AR before direct addition of luciferase substrate in the continued presence of fluorescent ligand. We could readily detect receptor-specific binding using Nluc as the BRET donor but not when Rluc8 was used (Fig. 1a,b). The main reason for this is likely to be the capacity of these N-terminally Nluc-tagged receptors to traffic appropriately to the plasma membrane (Supplementary Fig. 2). Thus, we observed a clear concentration-dependent ligand-binding BRET signal in cells expressing Nluc-β2AR, which was completely prevented by competition with a high concentration (10 μM) of unlabeled alprenolol (Fig. 1b).

To test compatibility of this NanoBRET assay with various fluorophores, we used fluorescent propranolol derivatives conjugated to either BODIPY-630/650 (excitation 630 nm, emission 650 nm; propranolol-BY630) or BODIPY-FL (excitation 503 nm, emission 512 nm; propranolol-BYFL) combined with Nluc-β2AR (Fig. 1c,d). We observed specific binding with both fluorescent ligands, which was inhibited by the agonist isoprenaline and the antagonists propranolol, ICI 118551 and CGP12177. Therefore, Nluc has a substantial dynamic range compatible with excitation wavelengths of both BODIPY-630/650 and BODIPY-FL fluorophores. We observed a lower signal-to-background ratio with propranolol-BYFL (Supplementary Fig. 3), which is due to the large degree of donor background present in the BRET acceptor channel. Consequently, BODIPY-630/650 is generally a preferred choice of acceptor. However, despite suboptimal performance with the BODIPY-FL, our results support use of a variety of fluorescent dyes as potentially robust tracers for ligand binding applications for GPCRs. We calculated equilibrium dissociation constant (Kd) values indicating the affinity of propranolol-BY630 and propranolol-BYFL (mean ± s.e.m.) from saturation binding assays as 18.9 ± 4.1 nM (n = 6) and 42.8 ± 10.8 nM (n = 8) respectively. Subsequently, we calculated the respective pKi values indicating the affinity of propranolol, ICI 118551 and CGP12177 from the corresponding half-maximal inhibitory concentration (IC50) values using the Cheng-Prusoff equation: 8.13 ± 0.05, 8.04 ± 0.04 and 8.32 ± 0.03 (competing with propranolol-BY630) and 8.89 ± 0.09, 8.69 ± 0.14 and 8.92 ± 0.03 (competing with propranolol-BYFL).

1Cell Signalling Research Group, School of Life Sciences, The University of Nottingham Medical School, Nottingham, UK. 2Molecular Endocrinology and Pharmacology, Harry Perkins Institute of Medical Research, Nedlands, Western Australia, Australia. 3Centre for Medical Research, The University of Western Australia, Crawley, Western Australia, Australia. 4Promega Corporation, Madison, Wisconsin, USA. 5These authors jointly directed this work. Correspondence should be addressed to K.D.G.P. (kevin.pfleger@perkins.uwa.edu.au) or S.J.H. (stephen.hill@nottingham.ac.uk).

RECEIVED 30 DECEMBER 2014; ACCEPTED 4 APRIL 2015; PUBLISHED ONLINE 1 JUNE 2015; DOI:10.1038/NMETH.3398

© 2015 Nature America, Inc. All rights reserved.
propranolol-BYFL) (Online Methods). These values (particularly those obtained with propranolol-BYFL) are comparable to those obtained by Baker.

As with distinct radioligands acting on the same receptor, different fluorophores may result in a ligand exhibiting slightly different affinities and binding modes. This could potentially shift apparent affinities of competing ligands owing to the probe dependency of cooperative interactions between protomers within a receptor complex. This factor may also influence fluorophore choice, opening up interesting avenues of potential research into cooperativity mechanisms through the use of multiple fluorescent ligands (see below).

To further exemplify BRET from ligand binding with multiple fluorescent ligands, we used HEK293 cells stably transfected with N-terminally Nluc-labeled adenosine A1 or A3 receptors and treated them with increasing concentrations of the BODIPY labeled antagonist CA200645 (ref. 11). We determined nonspecific binding using a high concentration of unlabeled antagonist (DPCPX for Nluc-A1 or MRS 1220 for Nluc-A3) and measured BRET after direct furimazine addition. We observed a saturable signal for both receptors with low nonspecific binding (Fig. 2a,b) across the full concentration range of fluorescent ligand. $K_d$ values for specific binding were $7.5 \pm 2.4 \text{nM}$ for Nluc-A1 and $7.6 \pm 3.7 \text{nM}$ for Nluc-A3 (mean $\pm$ s.e.m. of $n = 4$), which is consistent with values for unmodified receptors. In addition, we examined kinetics of CA200645 binding to Nluc-A1 (Supplementary Fig. 4), which yielded a similar $K_d$ to that obtained with saturation binding ($20.4 \pm 6.9 \text{nM}, n = 3, P > 0.05$ for unpaired t-test vs. saturation $K_d$). As affinity values for CA200645 at both adenosine receptors were very similar, it was important to confirm that the specific ligand-binding BRET signals generated had the appropriate pharmacology for the specific receptor under study and were not simply a consequence of nonspecific membrane interactions due to the lipophilicity of BODIPY. We used an A1-selective fluorescent ligand, AV039 (compound 19 in ref. 13), containing the same BODIPY fluorophore and tested its ability to bind to Nluc-A1- and Nluc-A3-expressing cells (Fig. 2c,d). We did not detect a saturable specific BRET signal at Nluc-A1 at

![Figure 1](image1.png)

**Figure 1** Suitability of NanoLuc for BRET binding studies. (a,b) BRET ligand-binding assay for transiently transfected Rluc8-AR (a) and Nluc-βAR (b) treated with increasing concentrations of alprenolol-TAMRA in the absence or presence of 10 nM unlabeled alprenolol. Data are mean $\pm$ s.e.m. of three experiments performed in quadruplicate. (c,d) inhibition of the BRET signal for HEK293 cells stably expressing Nluc-βAR treated with 10 nM propranolol-BYFL (c) or propranolol-BYFL (d) and increasing concentrations of unlabeled ligands as shown. Each data point represents mean $\pm$ s.e.m. of three experiments (in duplicate, o) or four (others in d) separate experiments. In each experiment we made triplicate determinations for each data point.

![Figure 2](image2.png)

**Figure 2** Extending the use of NanoBRET. (a,b) BRET signal for Nluc-A1 (a) and Nluc-A3 (b) treated with increasing CA200645 concentrations, with nonspecific binding established with 1 µM DPCPX for Nluc-A1 (a) and 1 µM MRS 1220 for Nluc-A3 (b). (c,d) BRET signal for Nluc-A1 (c) and Nluc-A3 (d) with increasing AV039 concentrations in the absence and presence of 1 µM DPCPX (c) or 1 µM MRS 1220 (d). In c, the only statistically significant difference was at 250 nM AV039 (two-way ANOVA; $P < 0.01$). (e,f) BRET signal for Nluc-A1 (e) and Nluc-A3 (f) cells treated with 25 nM CA200645 and increasing concentrations of unlabeled ligand. (g) Saturation BRET binding curves for binding fluorescent agonist ABEA-X-BY630 to Nluc-A1 in the absence or presence of 1 µM MRS 1200. (h) Ability of increasing concentrations of DPCPX, SCH 58261, MRS 1220 and CGS 15943 to decrease BRET between Nluc-A1 and ABEA-X-BY930. (i) BRET signal for Nluc-AT1 treated with 1 µM TAMRA-AngII and increasing concentrations of angiotensin II, candesartan and olmesartan. We measured BRET after furimazine addition. Data in a,d,g represent four experiments (in triplicate; error bars are s.e.m. of triplicate points). Data in e,f,h,i represent mean $\pm$ s.e.m. of three experiments (in duplicate, i) or four experiments (in triplicate, e,f,h). Exceptions are DPCPX in e, which is mean $\pm$ s.e.m. of five experiments (in triplicate) and MRS 1220 in f, which is mean $\pm$ s.e.m. of three experiments (in triplicate).
concentrations of AV039 up to 500 nM (Fig. 2c). In contrast, we detected clear specific binding at Nluc-A1 (Fig. 2d), which yielded a $K_D$ for AV039 of 24.6 ± 8.3 nM.

We then investigated the ability of a panel of ligands to inhibit specific binding of CA200645 to Nluc-A1 and Nluc-A3 (Fig. 2ef and Supplementary Fig. 5) and calculated affinity (pK$_A$) values (Supplementary Table 1). Notably, the A$_1$-selective antagonist DPCPX showed high affinity at Nluc-A1 and low affinity at Nluc-A3, conversely, the A$_2$-selective antagonist MR81220 showed high affinity at Nluc-A3 and lower affinity at Nluc-A1. Furthermore, affinities were comparable to those obtained using radioligand binding assays (Supplementary Table 1). We also obtained comparable results with the A$_3$-selective ligand AV039 in Nluc-A3–expressing cells (Supplementary Fig. 6 and Supplementary Table 2).

To determine whether the BRET ligand-binding assay was applicable to fluorescent agonists, we also undertook experiments with a fluorescent adenosine receptor agonist, ABEA-X-BY630 (ref. 15). In saturation binding experiments, we observed a clear saturable BRET signal at both Nluc-A1 (Fig. 2g) and Nluc-A3 (Supplementary Fig. 7a). ABEA-X-BY630 had a higher affinity for Nluc-A3 ($K_D = 38.4 ± 13.7$ nM) than for Nluc-A1 ($K_D = 167.0 ± 74.4$ nM). Competition binding assays with ABEA-X-BY630 and the panel of eight ligands used above yielded comparable affinities to those obtained with the antagonist fluorescent ligands (Fig. 2h, Supplementary Fig. 7 and Supplementary Table 2). Again, affinities were comparable to those obtained using radioligand binding assays (Supplementary Table 2). However, we obtained subtle differences in pK$_A$ values for nonfluorescent competing ligands with different fluorescent ligands, particularly in the case of Nluc-A1 (Supplementary Fig. 8). This would be in keeping with known allosterism across the A$_3$ homodimer interface 10.

Interestingly, we observed a similar phenomenon with the β2AR (Fig. 1) that is also known to form homodimers 16.

We used a similar fluorescent agonist strategy to investigate ligand binding to a peptide receptor (angiotensin II receptor type 1, AT$_1$) using a TAMRA-labeled angiotensin II (TAMRA-AngII). We performed competition BRET binding assays to investigate the ability of three AT$_1$ ligands (angiotensin II, candesartan and olmesartan) to lower the BRET signal. We observed a clear, concentration-dependent decrease in BRET signal in the presence of competing ligands (Fig. 2i), with olmesartan exhibiting highest affinity. To demonstrate assay sensitivity, we carried out competition binding assays at Nluc-AT$_1$ using varying concentrations of TAMRA-AngII and found concentrations down to 100 nM TAMRA-AngI, a specific signal was still observed that could be reduced by all three competing compounds (Supplementary Fig. 9).

Traditionally, binding assays have used radiotracer ligands to probe targets; however, this has become increasingly costly and undesirable for practical reasons 17,18. Furthermore, for technical reasons, many of the ligand-receptor affinities published in the literature have been derived using cell membranes assayed at 4 °C, with assumptions made about applicability to receptors in live cells at 37 °C. More recently, fluorescence resonance energy transfer (FRET) ligand-binding assays have been developed and successfully used 17,18, setting a precedent for applicability of resonance energy transfer approaches. However, there are a number of reasons why BRET is distinct from FRET and, indeed, is often used in preference 1. This study has demonstrated that BRET ligand-binding assays provide an exciting alternative to radioligand binding assays, with the notable advantage of being able to monitor ligand-receptor interactions in live cells, at 37 °C and in real time. Separation of free and bound fluorescent ligand is not required owing to the exquisite distance-dependence of BRET 19, nor is an additional step of conjugating a fluorophore to the N-terminal domain of the receptor of interest, as is required with SNAP or CLIP technology commonly used with time-resolved FRET 20. Indeed, as illustrated by our data, no wash steps or lysis are needed, thereby making this approach truly homogenous. Consequently, BRET ligand-binding assays have considerable potential for future drug discovery and profiling applications.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS

The authors thank C. Corona and P. Meinsenheimer (Promega) for providing the adenosine-TAMRA fluorescent ligand and J. Denman (The University of Western Australia) for assistance with generating the NanoLuc-adenosine receptor expression constructs. This project was funded by the Australian Research Council (ARC) Linkage Grant LP110100037. The University of Nottingham, Promega Corporation and BNG Labtech Pty Ltd provided funding as partner organizations of this grant. E.K.M.J. was funded by the Richard Walter Golden Medical Research Scholarship from The University of Western Australia. Work at S.J.H.’s laboratory was funded by the UK Medical Research Council (G0800006). K.D.G.P. was funded by an ARC Future Fellowship (FT100100271) and subsequently a National Health and Medical Research Council of Australia IID Bright Future (1008442). S.J.H. thanks the Balfour Foundation for a Visiting Research Fellowship at The University of Western Australia.

AUTHOR CONTRIBUTIONS


COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.
ONLINE METHODS

cDNA constructs. We cloned β2AR and AT1 receptor cDNAs into a pF-sNnK vector (Promega), encoding a fusion of the secretory signal peptide sequence of IL6 on the N terminus of NanoLuc (Nluc). The resulting open reading frame (ORF) therefore encoded a fusion of secreted Nluc at the N terminus of β2AR or AT1 receptor, with Gly-Ser-Ser-Gly linkers between the Nluc ORF and the GPCR ORF. N-terminally Rluc8-labeled β2AR was generated by substituting the Nluc ORF for the Rluc ORF. We generated Nluc-labeled adenosine receptor constructs by amplifying the full-length sequence of Nluc luciferase (as provided by Promega) and fusing it in-frame with the membrane signal sequence of the SH3 domain receptor within pcDNA3.1 to yield sig-Nluc. We then fused the full-length human sequence of the adenosine receptor of choice (with the methionine start signal removed) to the 3’ end of the sig-Nluc in pcDNA3.1. This gave the constructs designated as Nluc-AT1 receptor and Nluc-AT2 receptor, both of which include the signal sequence.

Ligands. CA200645, propranolol-BY630 (propranolol-β-alanine-β-alanine-X-BODIPY-630/650) and propranolol-BYFL (propranolol-β-alanine-X-BODIPY-FL) were synthesized by the University of Nottingham as described by Vernall et al. and Middleton et al. Alprenolol-TAMRA was synthesized by Promega. TAMRA-Ing was from AnaSpec. Alprenolol and angiotensin II were from Sigma. Candesartan and olmesartan were from Zhou Fang Pharm Chemical. DPCPX, SCH 58261, MBS 1220, CGS 19454, ZM 241385, XAC, PSB 603, isoprenaline, propranolol, IC3 118551 and CGP 12177 were from Tocris.

Stable cell-line generation. We maintained HEK293 cells (for Nluc-AT1 receptor, from ATCC) or HEK293G cells (GloSensor cAMP HEK293 for Nluc-AT2 receptor) in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS) and 2 mM l-glutamine at 37 °C. We then added the required substrate (furimazine (Promega). For each well we incubated 1 ng of Nluc-AT1 receptor construct using Lipofectamine (Life Technologies) according to the manufacturer’s instructions and then subjecting the cells to selective pressure (1 mg/ml G418) for 2–3 weeks. We then diluted-cloned the Nluc-AT1 and AT2 receptor cell lines to obtain cell lines originating from a single cell. The Nluc-β2AR stable cell line was from Promega. We confirm that all cell lines used were mycoplasma free.

BRET β2AR-ligand binding assays. We carried out transient transfections of HEK293 cells (ATCC) using FuGENE (Promega) at a 3:1 lipid:DNA ratio. We then seeded cells and lipid-DNA complexes into 96-well plates at a density of 20,000 cells per well (in DMEM supplemented with 10% FCS (Gibco)), with 50 ng of DNA per well. 24 h post-transfection, we removed the medium from each well and replaced it with OptiMEM without phenol red (Gibco). For experiments using alprenolol-TAMRA, we then incubated for 180 min in OptiMEM (without phenol red). We then added serially diluted alprenolol-TAMRA in the absence or presence of 10 μM alprenolol and incubated for 120 min at room temperature. We then added the required substrate (furimazine for Nluc-β2AR and coelenterazine h for Rluc8-β2AR) to a final concentration of 10 μM. We then measured BRET using the CLARIOstar plate reader (BMG Labtech) at room temperature.

We sequentially measured filtered light emissions at 450 nm (80-nm bandpass) and >610 nm (longpass) and calculated the raw BRET ratio by dividing the >610-nm emission by the 450-nm emission. For competition experiments using fluorescent-propranolol derivatives, we incubated Nluc-β2AR-stably transfected HEK293 cells with 10 nM propranolol-BY630 or propranolol-BYFL and the required concentration of competing ligand diluted in HBSS buffered saline solution (HBSS, 25 mM HEPES, 10 mM glucose, 146 mM NaCl, 5 mM KCl, 1 mM MgSO4, 2 mM sodium pyruvate, 1.3 mM CaCl2) for 1 h at 37 °C. We then measured the luminescence and fluorescence using the PHERAstar FS plate reader (BMG Labtech) at room temperature. We measured filtered light emissions at 460 nm (80-nm bandpass) and 535 nm (60-nm bandpass) for propranolol-BYFL and at 460 nm (80-nm bandpass) and >610 nm (longpass) for propranolol-BY630. We calculated the raw BRET ratio by dividing the >610-nm emission or 535-nm emission by the 460-nm emission. We have adopted the term "raw BRET ratio" as no background ratio has been subtracted.

BRET AT1 receptor-ligand binding assays. We performed saturation and competition binding assays on stably transfected cells that we seeded 24 h before experimentation in white Thermo Scientific Matrix 96-well microplates. We removed the medium from each well and replaced it with HBSS with the required concentration of fluorescent ligand and competing ligand. For preincubation experiments with ABEA-X-BY630, we incubated competing unlabeled ligand for 30 min before the addition of 250 nM ABEA-X-BY630 for Nluc-AT1-expressing cells and 50 nM ABEA-X-BY630 for Nluc-AT2-expressing cells. For saturation and competition experiments, upon the addition of fluorescent-labeled ligand, we incubated cells for 1 h at 37 °C (no CO2) and then added the Nluc substrate furimazine (Promega) to a final concentration of 10 μM. For association kinetics experiments on Nluc-AT1-expressing cells, we removed medium from each well, replaced it with HBSS containing 10 μM furimazine and incubated for 15 min at room temperature in the PHERAstar FS plate reader (BMG Labtech) to allow the signal to reach equilibrium. We then added the required concentration of CA200645, immediately reinerted the plate and read every well once per minute for 60 min. For all experiments, we measured the luminescence and resulting BRET using the PHERAstar FS plate reader (BMG Labtech) at room temperature. We again sequentially measured filtered light emissions at 460 nm (80-nm bandpass) and >610 nm (longpass) and calculated the raw BRET ratio by dividing the >610-nm emission by the 460-nm emission.
cDNA and 49 ng of pcDNA3 (Life Technologies) for 10 min at room temperature with a mix of 0.5 μl of FuGENE and 49.5 μl of serum-free DMEM (preincubated at room temperature for 5 min). We then incubated cells (10^5 in 150 μl per well) in DMEM supplemented with 10% FCS with the final DNA-FuGENE mix (50 μl/well). We carried out assays 48 h post-transfection after removing medium. We treated cells with competitor ligand for 30 min and then incubated them with TAMRA-AngII for a further 30 min. We carried out ligand incubations at 37 °C, 5% CO₂. We then added the Nluc substrate furimazine (Promega) to a final concentration of 10 μM and measured luminescence immediately. We measured BRET at 37 °C using the PHERATstar FS plate reader (BMG Labtech). We sequentially measured filtered light emissions at 460 nm (80-nm bandpass) and >610 nm (longpass) and calculated the raw BRET ratio by dividing the >610-nm emission by the 460-nm emission.

Measurement of Nluc and Rluc8 emission spectra. To determine the emission spectra of Nluc and Rluc8, we transiently transfected HEK293 cells (ATCC) with the expression constructs for Nluc-β2AR and Rluc8-β2AR as described above for the β2AR-ligand binding assays. 24 h post-transfection, we removed the medium from each well and replaced it with OptiMEM without phenol red (Gibco), which was followed by incubation for 180 min at 37 °C. Immediately before measurement we added the luciferase substrates furimazine (Nluc) or coelenterazine h (Rluc8) at a final concentration of 10 μM. We then determined the emission spectra with a CLARIOstar plate reader (BMG Labtech) using the luminescence scanning option (20-nm bandwidth, 1-nm resolution, integration time, 500 ms, gain, 3,000).

Bioluminescence imaging of Nluc-β2AR and Rluc8-β2AR. We performed bioluminescence imaging experiments to determine the localization of the Nluc-β2AR and Rluc8-β2AR fusion proteins. We performed all imaging experiments using the Olympus LV2000 bioluminescence microscope equipped with a Hamamatsu ImagEM electron-multiplying charge-coupled device (EMCCD) camera and a 100×/1.4 U PlansApo. We transiently transfected HEK293 cells with expression constructs for Nluc-β2AR and Rluc8-β2AR, plated in 35-mm optically clear dishes (Ibidi) at a density of 200,000 cells per dish in 2 ml growth medium (DMEM supplemented with 10% FCS), and incubated for 24 h in a tissue culture incubator. We then replaced the growth medium with 1 ml OptiMEM and incubated the cells for 180 min at 37 °C. Immediately before image acquisition, we replaced the media with OptiMEM including the luciferase substrates furimazine (Nluc) or coelenterazine h (Rluc8) at a final concentration of 10 μM. We identified suitable fields of view based on images using an EM gain of 200 and exposure times of 1 s (Nluc-β2AR) and 20 s (Rluc8-β2AR). We acquired all images using Olympus cellSens software and performed image processing with ImageJ software.

Data presentation and statistical analysis. We presented and analyzed data using Prism software (GraphPad).

We simultaneously fitted the total and nonspecific saturation binding curves using the following equation

\[
BRET = \frac{B_{\text{max}} \times [B]}{[B] + K_D + ([M] \times [B]) + C}
\]

where \(B_{\text{max}}\) is the maximal response, \([B]\) is the concentration of fluorescent ligand in nM, \(K_D\) is the equilibrium dissociation constant in nM, \([M]\) is the slope of the nonspecific binding component, and \(C\) is the intercept with the y axis.

We fitted the competition binding curves to calculate the \(K_i\) of the unlabeled ligands using the Cheng-Prusoff equation

\[
K_i = \frac{K_D}{1 + \frac{[L]}{K_D}}
\]

where \([L]\) is the concentration of fluorescent ligand in nM and \(K_D\) is the \(K_D\) of fluorescent ligand in nM. The calculated \(K_i\) values used were as calculated from the saturation binding experiments. The \(K_{cat}\) is calculated from the following equation

\[
\% \text{ inhibition of specific binding} = \frac{100 \times [A]}{[A] + K_{cat}}
\]

where \([A]\) is the concentration of competing drug and the \(K_{cat}\) is the molar concentration of ligand required to inhibit 50% of the specific binding of concentration \([L]\) of the fluorescent ligand. We also used this equation to fit concentration-inhibition data where the affinity of the labeled ligand is unknown.

From association kinetic data, we obtained \(k_{on}\), \(k_{off}\) and \(K_D\) values from the following equation

\[
K_D = \frac{k_{off}}{k_{on}}
\]

where \(K_D\) is the equilibrium dissociation constant and \(k_{off}\) is the dissociation rate constant of the ligand in min⁻¹, \(k_{on}\) is the association rate constant in M⁻¹ min⁻¹ and is calculated as follows

\[
k_{on} = \frac{k_{on} + k_{off}}{[L]}
\]

where \([L]\) is the ligand concentration in M and \(k_{on}\) is calculated from global fitting of the data to the following monoeponential association function

\[
Y = Y_{\text{max}} (1 - e^{-kt})
\]

Here \(Y_{\text{max}}\) equals levels of binding at infinite time (t), and \(k_{on}\) is the rate constant for the observed rate of association.

We carried out statistical analysis using unpaired t-test or ANOVA as appropriate (P < 0.05). The n values in the text refer to the number of separate repeat experiments. In our experience, a minimum of three repeat experiments, a power of 98% and a P value of 0.05 will give a standardized difference of interest (for example, a difference in p-values) of approximately 0.5 using the NanoBRET ligand binding assay.
Supplementary Figure 1

Comparison of Rluc8-β2AR and Nluc-β2AR luminescence spectra

We generated luminescence spectra with HEK293 cells transiently transfected with Rluc8-β2AR or Nluc-β2AR following addition of coelenterazine h or furimazine substrate respectively. (a) Presentation as normalized luminescence illustrates that the Nluc emission peak is left-shifted by about 20 nm compared to Rluc8, thus enabling better spectral separation from the acceptor emission. (b) Presentation of the same spectra in terms of measured luminescence in relative light units (RLU) without normalization to the peak emission. This illustrates the substantially greater luminescence, and therefore energy transfer potential, of Nluc compared to Rluc8 despite the spectrum being left-shifted (Nluc peak (462 nm): 113,909 RLU; Rluc8 peak (480 nm): 1642 RLU). This becomes more relevant as more red-shifted energy acceptors are utilized, and therefore the spectral overlap of the donor emission spectrum with the acceptor excitation spectrum diminishes. The high luminescence output of Nluc means that this reduced overlap is much less of an issue compared with Rluc8. The data shown here are representative of three independent experiments.
Supplementary Figure 2

Microscopy images comparing the cellular localization of Rluc8-\(\beta_2\)AR and Nluc-\(\beta_2\)AR

(a) Images of HEK293 cells transiently transfected with Rluc8-\(\beta_2\)AR indicate that this fusion protein is not appropriately trafficked to the plasma membrane. (b) This is in contrast to Nluc-\(\beta_2\)AR that is clearly localized at the plasma membrane. We acquired images of eight different fields of view per sample and two representative fields are shown. Furthermore, the data are representative of three independent experiments. Nluc is derived from the luciferase expressed in deep sea shrimp Oplophorus gracilirostris. The native luciferase is secreted by the shrimp in bright luminescent bursts to ward off predators. It has therefore evolved to be secreted and therefore pass through cellular membranes. This is not the case for Renilla luciferase. Indeed multiple attempts have been made to generate a secreted Renilla luciferase. However, addition of the signal peptide of human interleukin-2 resulted in a secreted form of Rluc with 15 times less activity than cytosolic Rluc in mammalian cells. The reason for this was unclear, but one suggested possibility was that addition of the signal peptide resulted in misfolding in the endoplasmic reticulum.

Nature Methods: doi:10.1038/nmeth.3398
Supplementary Figure 3

BRET binding assessed with propranolol-BY630 and propranolol-BYFL

(a,b) We treated HEK293 cells stably expressing Nluc-β2AR with 10 nM propranolol-BY630 (a) or 10 nM propranolol-BYFL (b) and increasing concentrations of unlabeled ligands as shown. In the presence of 10 µM propranolol we observed a decrease of 69.2 ± 1.3% in propranolol-BY630 BRET signal, whereas with propranolol-BYFL we only achieved a 25.6 ± 1.2% decrease in signal. Each data point represents mean ± s.e.m. of five (all curves in (a) and propranolol in (b)) or four (b) separate experiments. In each experiment we made triplicate determinations for each data point.

Nature Methods: doi:10.1038/nmeth.3398
Inhibition of BRET between NanoLuc and CA200645 at the adenosine A1 and A3 receptors by four additional compounds

(a,b) We treated HEK293 cells stably expressing Nluc-A1 receptor (a) or Nluc-A3 receptor (b) with 25 nM CA200645 and increasing concentrations of unlabeled ligands as shown. We monitored the resulting concentration dependent decrease in BRET and each data point represents mean ± s.e.m. of five (a: propranolol, ZM241385), four (a: XAC, PSB 603; b: PSB 603) or three (b: propranolol, ZM241385, XAC) experiments performed in triplicate.
We treated HEK293 cells stably expressing Nluc-A₁ receptor with various concentrations of CA200645. We measured BRET between Nluc and the fluorescent ligand every min for 60 min at room temperature. The data shown are representative of three independent experiments performed in triplicate. From global fitting of the data, the kinetic parameters for CA200645 at Nluc-A₁ receptor are $k_{on} = 9.64 \pm 0.32 \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$ and $k_{off} = 0.019 \pm 0.005 \text{ min}^{-1}$ with a resulting $K_D$ of $20.4 \pm 6.9 \text{ nM}$ (mean ± s.e.m., n=3).

**Supplementary Figure 4**

Kinetic measurements of binding of CA200645 to Nluc-A₁ receptor

We treated HEK293 cells stably expressing Nluc-A₁ receptor with various concentrations of CA200645. We measured BRET between Nluc and the fluorescent ligand every min for 60 min at room temperature. The data shown are representative of three independent experiments performed in triplicate. From global fitting of the data, the kinetic parameters for CA200645 at Nluc-A₁ receptor are $k_{on} = 9.64 \pm 0.32 \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$ and $k_{off} = 0.019 \pm 0.005 \text{ min}^{-1}$ with a resulting $K_D$ of $20.4 \pm 6.9 \text{ nM}$ (mean ± s.e.m., n=3).
Inhibition of BRET between NanoLuc and CA200645 at the adenosine A<sub>1</sub> and A<sub>3</sub> receptors by four additional compounds

(a,b) We treated HEK293 cells stably expressing Nluc-A<sub>1</sub> receptor (a) or Nluc-A<sub>3</sub> receptor (b) with 25 nM CA200645 and increasing concentrations of unlabeled ligands as shown. We monitored the resulting concentration dependent decrease in BRET and each data point represents mean ± s.e.m. of five (a: propranolol, ZM241385), four (a: XAC, PSB 603; b: PSB 603) or three (b: propranolol, ZM241385, XAC) experiments performed in triplicate.
Supplementary Figure 6

Inhibition of BRET between NanoLuc and AV039 at the adenosine A3 receptor by a panel of eight GPCR antagonists

(a,b) We treated HEK293 cells stably expressing Nluc-A3 receptor with 10 nM AV039 and increasing concentrations of unlabeled ligands as shown. The resulting concentration dependent decrease in BRET was monitored and each data point represents mean ± s.e.m. of four experiments performed in triplicate.
Supplementary Figure 7

Saturation and competition ligand binding with ABEA-X-BY630 at Nluc-A₁ and Nluc-A₃ receptors

(a) We treated HEK293 cells stably expressing Nluc-A₁ receptor with increasing concentrations of ABEA-X-BY630. We established non-specific binding in the presence of 1 µM DPCPX and measured the resulting BRET ratios after 1 h incubation at 37 °C. (b–d) We also performed competition BRET binding assays on Nluc-A₁ (b, c) and Nluc-A₃ (d) receptor-expressing HEK293 cells treated with 250 nM ABEA-X-BY630 (b, c) or 50 nM ABEA-X-BY630 (d), along with increasing concentrations of unlabeled ligands as shown. Panel a is a representative graph of three experiments performed in triplicate. Data points in b, c and d represent mean ± s.e.m. of four experiments performed in triplicate.

Nature Methods: doi:10.1038/nmeth.3398
Supplementary Figure 8

A comparison of pK values obtained at the adenosine A₁ and A₃ Niuc-tagged receptors using three different fluorescent ligands.

(a,b) We obtained pK values for (a) the Niuc-A₁ receptor and (b) the Niuc-A₃ receptor with the non-selective fluorescent antagonist CA200645, non-selective fluorescent agonist ABEA-X-BY630 and A₃-selective fluorescent antagonist AV039. We have taken pK values from Supplementary Tables 1 and 2 (see tables for n numbers). * indicates values which are significantly different (p<0.05) using an unpaired t-test (a) or one-way ANOVA (b). In (b) the ANOVA analysis shows that for most competing ligands (with the exception of PSB 603) the data for each competing ligand cannot be described by a single pK value.
Supplementary Figure 9

Competition ligand binding at Niuc-AT1 receptor with varying concentrations of TAMRA-AngII

(a–c) We treated cells transiently expressing Niuc-AT1 receptor with 1, 0.6, 0.3, 0.1 or 0 µM TAMRA-AngII and increasing concentrations of (a) angiotensin II, (b) candesartan and (c) olmesartan. We measured BRET between Niuc and TAMRA-AngII. Data points represent mean ± s.e.m. of three experiments performed in duplicate.
Appendix II – Publications and Abstracts

Supplementary Table 1

<table>
<thead>
<tr>
<th>Phospho-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nluc-A1 receptor</td>
</tr>
<tr>
<td>$pK_i$</td>
</tr>
<tr>
<td>DPCPX</td>
</tr>
<tr>
<td>SCH 38261</td>
</tr>
<tr>
<td>MRS 1220</td>
</tr>
<tr>
<td>CGS 15943</td>
</tr>
<tr>
<td>Propranolol</td>
</tr>
<tr>
<td>ZM 241385</td>
</tr>
<tr>
<td>XAC</td>
</tr>
<tr>
<td>PSB 603</td>
</tr>
</tbody>
</table>

a We obtained $pK_i$ values (mean ± s.e.m.) in the NanoBRET binding assay using whole, live HEK293 cells expressing Nluc-A1 or Nluc-A3 receptor and 25 nM CA200645.
b Previously published $pK_i$ values and associated references as listed in IUPHAR/BPS Guide to Pharmacology (www.guidetopharmacology.org).

Note: Using $^3$H-DPCPX in live CHO cells expressing the wild-type A1 receptor, we have previously published $pK_i$ values for DPCPX, XAC and CGS15943 of 8.37 ± 0.03, 7.25 ± 0.02 and 8.35 ± 0.05 respectively. These are very similar to the respective values quoted for Nluc-A1 receptor with CA200645 above.

Supplementary Table 2

**pK$_i$ values at Nluc-A$_1$ and Nluc-A$_3$ receptors using different fluorescent ligands**

<table>
<thead>
<tr>
<th></th>
<th>Nluc-A$_1$ receptor</th>
<th>Nluc-A$_3$ receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABEA-X-BY630</td>
<td>pK$_i$</td>
<td>pK$_i$</td>
</tr>
<tr>
<td>Co-addition</td>
<td>8.17 ± 0.25</td>
<td>8.20 ± 0.12</td>
</tr>
<tr>
<td>Preincubation</td>
<td>7.54 ± 0.19</td>
<td>7.87 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>7.13 ± 0.14</td>
<td></td>
</tr>
<tr>
<td>MRS 1220</td>
<td>6.97 ± 0.23</td>
<td>7.31 ± 0.12</td>
</tr>
<tr>
<td>SCH 58261</td>
<td>6.54 ± 0.21</td>
<td>6.12 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>5.09 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>DPCPX</td>
<td>8.49 ± 0.13</td>
<td>8.56 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>6.02 ± 0.11</td>
<td>6.54 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>5.47 ± 0.17</td>
<td></td>
</tr>
<tr>
<td>PSB 603</td>
<td>5.70 ± 0.13</td>
<td>5.93 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>5.91 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>XAC</td>
<td>7.40 ± 0.12</td>
<td>7.50 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>6.96 ± 0.10</td>
<td>7.44 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>6.64 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>ZM 241385</td>
<td>6.33 ± 0.12</td>
<td>6.03 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>5.49 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>propranolol</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

We obtained pK$_i$ values (mean ± s.e.m.; n = 4) in the NanoBRET binding assay using whole, live HEK293 cells expressing Nluc-A$_1$ or Nluc-A$_3$ receptor. We used 250 nM ABEA-X-BY630 in Nluc-A$_1$ receptor-expressing cells, 50 nM ABEA-X-BY630 in Nluc-A$_3$ receptor-expressing cells and 10 nM AV039 in Nluc-A$_3$ receptor-expressing cells. We added unlabeled ligand simultaneously with fluorescent ligand (Co-addition). Alternatively, in order to check for differences in pK$_i$ values determined with the fluorescent agonist ABEA-X-BY630 due to simultaneous addition, we also investigated the values obtained for four antagonists following a 30 min preincubation with the antagonist prior to addition of ABEA-X-BY630 (Preincubation).

Note: Using $^3$H-DPCPX in live CHO cells expressing the wild-type A$_1$ receptor, we have previously published pK$_i$ values for DPCPX, XAC and CGS15943 of 8.37 ± 0.03, 7.25 ± 0.02 and 8.35 ± 0.05 respectively. These are very similar to the respective values quoted for Nluc-A$_1$ receptor with ABEA-X-BY630 above.

RESEARCH ARTICLE

Functional Interaction between Angiotensin II Receptor Type 1 and Chemokine (C-C Motif) Receptor 2 with Implications for Chronic Kidney Disease

Mohammed Akli Ayoub1,2,*, Yuan Zhang3,*, Robyn S. Kelly2, Heng B. See1,3, Elizabeth K. M. Johnstone1,3, Elizabeth A. McCall4, James H. Williams5, Darren J. Kelly3,*, Kevin D. G. Pfleger1,2,*, 1 Molecular Endocrinology and Pharmacology, Harry Perkins Institute of Medical Research, QEII Medical Centre, Nedlands, Western Australia, Australia, 2 Centre for Medical Research, The University of Western Australia, Crawley, Western Australia, Australia, 3 Department of Medicine, St. Vincent’s Hospital, The University of Melbourne, Melbourne, Victoria, Australia, 4 Dimerix Bioscience Limited, Nedlands, Western Australia, Australia

* These authors contributed equally to this work.
‡ These authors also contributed equally to this work.

Abstract

Understanding functional interactions between G protein-coupled receptors is of great physiological and pathophysiological importance. Heteromerization provides one important potential mechanism for such interaction between different signalling pathways via macro-molecular complex formation. Previous studies suggested a functional interplay between angiotensin II receptor type 1 (AT1) and Chemokine (C-C motif) Receptor 2 (CCR2). However the molecular mechanisms are not understood. We investigated AT1-CCR2 functional interaction in vitro using bioluminescence resonance energy transfer in HEK293 cells and in vivo using subtotal-nephrectomized rats as a well-established model for chronic kidney disease. Our data revealed functional heteromers of these receptors resulting in CCR2-Gαi1 coupling being sensitive to AT1 activation, as well as apparent enhanced β-arrestin2 recruitment with agonist co-stimulation that is synergistically reversed by combined antagonist treatment. Moreover, we present in vivo findings where combined treatment with AT1- and CCR2-selective inhibitors was synergistically beneficial in terms of decreasing proteinuria, reducing podocyte loss and preventing renal injury independent of blood pressure in the subtotal-nephrectomized rat model. Our findings further support a role for G protein-coupled receptor functional heteromerization in pathophysiology and provide insights into previous observations indicating the importance of AT1-CCR2 functional interaction in inflammation, renal and hypertensive disorders.
Appendix II – Publications and Abstracts

AT1-CCR2 Functional Interaction and Chronic Kidney Disease

Introduction
The interplay between different hormones, neurotransmitters and chemokines targeting G protein-coupled receptors (GPCRs) has been reported in many cases. To finely integrate signals transduced via different pathways, cells have established various mechanisms of interactions between receptor systems such as functional crosstalk and receptor heteromerization [1]. Heteromerization has been reported for many classes and subtypes of GPCRs, both in vitro and in vivo, where either a direct or indirect interaction between two different receptors in a macromolecular complex results in one protomer changing the function of another protomer with respect to receptor maturation/trafficking, ligand binding, G protein coupling and/or desensitization/internalization [2]. A receptor heteromer is defined as a "macromolecular complex composed of at least two (functional) receptor units with biochemical properties that are demonstrably different from those of its individual components" [3]. Note that two GPCRs in such a heteromer complex, which is likely to include multiple other proteins, can influence each other’s function without physically touching [4].

The heteromerization concept has evolved rapidly over recent years, bringing more evidence for its importance in physiology and pathology [5]. One important aspect of studying GPCR heteromerization is to investigate whether any of the functional interaction between two hormones/neurotransmitters/chemokines observed in vivo could potentially be mediated, at least in part, at the level of their specific receptors. In this context, the functional interaction in the kidney between the receptors for CC chemokine ligand 2 (CCL2, also known as monocyte chemoattractant protein 1 or MCP-1) and angiotensin II (AngII), the main effector peptide of the renin-angiotensin system (RAS), constitutes an important model. Indeed, several lines of evidence suggest a relationship between the angiotensin system and the immune system [5–7]. In addition, the link between AngII and CCL2 signalling has been suggested in multiple situations [8–11]. More interestingly, evidence for a potential functional interaction between CCL2 and AngII cognate receptors (CCR2 and AT1 receptor, respectively) has only recently emerged, with studies using specific antagonists showing that the combined blockade of the two receptors markedly attenuates renal injury (crescentic glomerulonephritis) [12] and ischemic brain damage [13]. Moreover, a number of studies provide evidence for expression of AT1 receptor [14,15] and CCR2 [16,17] in kidney cells, including both podocytes and mesangial cells [15,17]. Indeed, overexpression of both of these receptors in podocytes is associated with pathology [16,18]. These findings support our hypothesis that AT1 receptor and CCR2 influence each other’s function, with consequent implications for mediating kidney disease progression.

Chronic kidney disease (CKD) is a major cause of morbidity, recurrent hospitalisation and accelerated death, affecting 10–11% of the population in both Europe and the United States [19]. Histopathologically, interstitial inflammatory cell infiltration, cell apoptosis, capillary rarefaction, and fibrosis are the characteristic features of progressive CKD [20]. These structural changes, in turn, result in a loss of glomerular filtration rate (GFR) that is frequently accompanied by progressive proteinuria [20]. The pathological role of AngII has been well documented in the initiation and progression of CKD [21]. Despite current treatments including control of hypertension and blockade of RAS, a considerable proportion of CKD patients continues to progress in association with interstitial macropage accumulation, suggesting the need for additional immunotherapy [22]. On the other hand, CCL2 has been implicated in the development of a variety of renal diseases including chronic rejection of renal transplantation, lupus nephritis, IgA nephropathy, crescentic glomerulonephritis and diabetic nephropathy by promoting circulating monocellular cells, as well as tissue macrophage recruitment and activation in the kidney interstitium [23–27]. More importantly, in addition to its role as a mediator of monocyte recruitment, recent studies on both experimental and human diabetic nephropathy...
have shown that the CCL2/CCR2 system plays a pathological role in the depletion of podocytes and the development of proteinuria [17,28]. Conversely, the blockade of CCL2/CCR2 interaction by either neutralization of CCL2 or CCR2 antagonists has been shown to attenuate progressive kidney damage [29,30].

In this study, we investigated the functional interactions between AT1 receptor and CCR2 both in vitro, using HEK293FT cells, and in vivo, using the sub-total nephrectomized (STNx) rat model characterized by extensive renal mass ablation associated with glomerular RAS upregulation, glomerular hypertension, development of podocyte loss, progressive proteinuria and declining GFR associated with interstitial macrophage infiltration, glomerulosclerosis, and tubulointerstitial fibrosis [31–33]. We carried out in vivo experiments to investigate the effect of AT1 receptor and CCR2 coexpression on their complex formation, heterotrimeric G protein coupling and β-arrestin2 recruitment. In particular, we utilized the GPCR Heteromer Identification Technology (GPCR-HIT) configuration [4,34–39], the most established version of the Receptor-HIT assay [40] and built upon our recent work assessing receptor-G protein proximity using bioluminescence resonance energy transfer (BRET) [41,42]. Individual and combined treatments with agonists as well as antagonists were performed to investigate the pharmacological profile of AT1 receptor-CCR2 complexes. We then investigated in vivo whether inhibition of both receptor signalling pathways with a combination of Irbesartan (Irb; AT1 receptor antagonist) and Propagermanium (PPG; CCR2 pathway inhibitor) could potentially have a synergistic benefit for CKD treatment, which would be consistent with functional interaction of these receptor signalling pathways.

Materials and Methods

Materials

AngII and PPG were from Sigma-Aldrich (Castle Hill, Australia). Irb was from Zhou Fang Pharm Chemical (Shanghai, China). RS504393 was from Tocris. CCL2 was from PeproTech (Rocky Hill, NJ, USA).

Plasmid Construction

AT1 receptor-Rluc8 and CCR2-Rluc8 cDNA constructs were generated from plasmids containing AT1 receptor-Rluc and CCR2-Rluc kindly provided by Walter Thomas (University of Queensland) and Aron Chakera (University of Western Australia) respectively. The Rluc coding region was replaced with Rluc8 cDNA from pcDNA3.1-Rluc8 kindly provided by Andreas Loening and Sanjiv Gambhir (Stanford University, CA), as described previously for other GPCR constructs [43]. CCR2-Topaz yellow fluorescent protein (YFP) was also previously generated in the laboratory from CCR2-Rluc. Non-BRET tagged AT1 receptor and CCR2 were also provided by Walter Thomas and Aron Chakera respectively. The β-arrestin 2-Venus cDNA construct was prepared previously from pCs2-Venus kindly provided by Atsushi Miyawaki (RIKEN Brain Science Institute, Wako-city, Japan) [43]. Gαi1-Rluc8 was generated from the original Gαi1-Rluc construct kindly provided by Jean-Philippe Pin and previously reported [41,44]. All constructs were confirmed by DNA sequencing at the Australian Genome Research Facility (Adelaide, Australia).

Cell culture and transfection

HEK293FT cells were maintained at 37°C, 5% CO2 in complete medium (Dulbecco’s modified Eagle’s medium (DMEM) containing 0.3 mg ml⁻¹ glutamine, 100 IU ml⁻¹ penicillin, and 100 μg ml⁻¹ streptomycin) (Gibco BRL, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS;
Appendix II – Publications and Abstracts

AT1-CCR2 Functional Interaction and Chronic Kidney Disease

Gibco). Transient transfections were carried out using GeneJuice (Merck, Kilsyth, Australia) or FuGENE (Promega, Alexandria, Australia) according to the manufacturer’s instructions and the experiments were performed 48 hours post-transfection.

GPCR-HIT BRET assays

HEK293FT cells were transfected as specified in the figure legends using GeneJuice (Merck, Kilsyth, Australia) or FuGENE (Promega, Alexandria, Australia) as per the manufacturer’s instructions. 48 h post-transfection, BRET measurements were carried out in white 96-well plates using coelenterazine h (5 μM final), or following incubation at 37°C, 5% CO₂ for 2 h with 30 μM EnduRen (Promega, Alexandria, Australia) for assessing arrestin recruitment kinetics, as described previously [4]. BRET detection was carried out in live cells at 37°C by measuring sequential light emissions at 400–475 nm and 520–540 nm using the VICTOR Light plate reader with Wallac 1420 software (PerkinElmer, Melbourne, Australia). The ligand-induced BRET signal was calculated by subtracting the ratio of 520–540 nm emission over 400–475 nm emission for a vehicle-treated cell sample from the same ratio for a second aliquot of the same cells treated with ligand (ligand-induced BRET), as described previously [43]. With kinetic data, the final pre-treatment measurement is presented at the zero timepoint (time of ligand or vehicle addition).

Measurement of inositol-1-phosphate (IP₁) production

The determination of IP₁ accumulation was performed using the IP-One HTRF assay (CisBio Bioassays, Bagnol sur Ceze, France), as described previously [45]. Briefly, cells were transfected and seeded into white 96-well plates. 48 h post-transfection cell media was replaced with 50 μl stimulation buffer containing agonists as indicated. After a 30 min incubation at 37°C, 5% CO₂, cells were lysed with 12.5 μl of the supplied conjugate-lysis buffer containing d2-labeled IP₁. This was immediately followed by addition of 12.5 μl of conjugate-lysis buffer containing terbium cryptate-labeled anti-IP₁ antibody. Following a 1 h incubation at room temperature, fluorescence was measured at 620 and 665 nm 50 μs after excitation at 337 nm using an EnVision 2102 plate reader (PerkinElmer).

Animal Ethics Statement

All animal experiments were conducted with approval from the St Vincent’s Hospital Animal Ethics Committee (AEC) in accord with the National Health and Medical Research Council Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. The approved AEC code was 005/12.

Animal experimental design and surgery

Six week old, male Sprague-Dawley (SD) rats weighing 200–250g were sourced from the Animal Resources Centre (Western Australia). All rats received normal rat chow (Certified Rodent Diet #5002, LabDiet, USA) and drinking water ad libitum. All animals were housed in a stable environment maintained at 22 ± 1°C with a 12-hour light/dark cycle commencing at 6am. STNx surgery was performed in the operating theatre at St Vincent’s Experimental Surgical Unit. All surgical procedures were performed as previously published [46].

One hundred rats were randomized to 5 groups of 20 animals each. Anaesthesia was achieved with 3% isoflurane/97% oxygen in a tidal volume of 1 ml 100g⁻¹ body weight. The control group (n = 20) underwent sham surgery consisting of laparotomy and manipulation of both kidneys before wound closure. The other 80 rats underwent STNx performed by right
subcapsular nephrectomy and infarction of approximately 2/3 of the left kidney by selective ligation of two out of the three extrarenal branches of the left renal artery [47]. Any pain experienced as a result of the surgical procedure performed on these animals was minimized with the use of buprenorphine (0.03mg/kg) directly following surgery as routine. In the event of unrelieveable pain, the rats were euthanased with an overdose of Lethabarb (Sodium Pentobarbitone, 120 mg/kg). Two weeks post-surgery, STNx animals were then randomly assigned to 4 groups to receive treatment with either PPG (30 mg kg⁻¹ day⁻¹ gavaged) or Irb (10 mg kg⁻¹ day⁻¹, in drinking water) or combination of Irb (10 mg kg⁻¹ day⁻¹, in drinking water) and PPG (30 mg kg⁻¹ day⁻¹ gavaged), or vehicle (1% CMC) for 12 weeks. Every 4 weeks, rats were weighed and systolic blood pressure (SBP) was determined in preheated conscious rats via tail-cuff plethysmography using a non-invasive blood pressure (NIBP) controller and Powerlab (AD instruments, Bella Vista, Australia). Urine was collected over 24 hours at the end of the study for subsequent urinary biochemistry analysis.

Proteinuria
Proteinuria was determined from an aliquot of urine collected during the 24 hour period in the metabolic cages. In brief, once thawed, 20μl of urine was added to a clinical uristix (Bayer Diagnostics Manufacturing, Sudbury, England) to obtain an approximate urine protein concentration. Dilutions were made with 0.9% saline and samples were assayed on a Cobas Integra 400 employing the Tina-Quant-Albu2 assay as previously described [48]. The method was modified by calibrating the analyser with rat albumin standards (Sigma-Aldrich, Missouri, USA). Proteinuria was expressed as mg day⁻¹ using the total volume of urine collected over the 24 hours.

Glomerular filtration rate (GFR)
GFR was determined by injecting a single shot of 99Tc-DTPA into the tail vein of the rats. Blood was sampled after 43 min as previously described [49] and expressed as ml min⁻¹.

Histopathology
At the end of the study, rats were anaesthetised (Nembutal 60 mg kg⁻¹ body wt i.p.; Boehringer-Ingelheim, North Ryde, Australia). Kidneys were excised, de-capsulated and sliced transversely. Half of the kidney was snap-frozen for molecular biology and the other half was immersion fixed with formalin and paraffin-embedded for subsequent light microscopic evaluation. Histopathological changes such as glomerulosclerosis and tubulointerstitial fibrosis in the kidney were assessed in a masked protocol. Sections were stained with either periodic acid Schiff’s stain (PAS) for glomerulosclerosis or Masson’s modified trichrome to demonstrate collagenous matrix [46].

Immunohistochemistry
Immunohistochemical staining was performed on 4 μm tissue sections as previously described [50]. Sections were dewaxed in histolene, hydrated through graded ethanols, and then immersed in tap water. The antigen retrieval involved heating sections in a pressure cooker in 10mM sodium citrate buffer (pH 6) for 4 min, and allowing them to cool at room temperature for 30 min. To block non-specific staining due to endogenous peroxidase activity, all sections were incubated with 3% hydrogen peroxide for 10 min at room temperature, followed by 3 times of 5 min wash with Phosphate Buffered Saline (PBS) before being incubated for 20 min with normal goat serum (NGS) diluted 1:10 with PBS, pH 7.4 as protein block. Sections were
then incubated with rabbit anti-WT-1 antibody (Santa Cruz, 1:400 diluted with PBS) or mouse anti rat ED-1 (serotec, 1:300 diluted with PBS) at 4°C for 18 hours. The following day, sections were thoroughly washed in PBS (3 x 5 min), and then incubated with goat anti-rabbit or anti-mouse HRP (DAKO, CA) for 30 min at room temperature. Localization of the peroxidase conjugates was achieved using 3,3′-diaminobenzidine tetrahydrochloride (DAB; DAKO, CA) as a chromogen, for 1-3 min (development time assessed with light microscope), slides were then rinsed in tap water for 5 min to stop the development, counterstained in Mayer’s haemotoxylin, differentiated in Scott’s tap water, dehydrated, cleared and mounted in DPX. Sections incubated with 1:10 NGS, instead of the primary antiserum, served as negative controls.

Glomerulosclerotic index
In 4 μm kidney sections stained with PAS, 50 glomeruli from each rat were examined in a masked protocol. The extent of sclerosis in each glomerulus was subjectively graded on a scale of 0 to 4, as previously described [51] with Grade 0, normal; Grade 1, sclerotic area up to 25% (minimal); Grade 2, sclerotic area 25–50% (moderate); Grade 3, sclerotic area 50–75% (moderate to severe) and Grade 4, sclerotic area 75–100% (severe). A glomerulosclerotic index (GSI) was then calculated using the formula:

\[
GSI = \sum_{i=0}^{4} F(i)
\]

where Fi is the % of glomeruli in the rat with a given score (i).

Quantitation of matrix deposition
To measure interstitial fibrosis in the kidney, 10 random non-overlapping fields from 10 rats per group were captured and digitised using a Carl Zeiss microscope attached to Axio-CamMRc5 digital camera (Carl Zeiss, North Ryde, Australia) under 200x magnification. Digital images were then loaded onto a Pentium D Dell computer. An area of blue in the cortex of the kidney was selected for its colour range and the proportional area of the selected colour range was then quantified using image analysis (AxioVision Release 4.8.1; Carl Zeiss, North Ryde, Australia) based on the method adapted from Lehr et al [52]. Data were expressed as percentage change per area [46].

Quantitation of podocytes and macrophages
Quantitation of podocytes was assessed by examining approximately 20–30 hilar glomeruli per animal with a light microscope at x400, expressed as numbers per glomerular cross section (gcs). Macrophages were counted by examining 5 fields per section with a light microscope at x200, expressed as numbers per area.

Data analysis and statistical procedures
All in vitro data were analysed using Prism software (GraphPad, San Diego, CA, USA). Dose-response curves were fitted using nonlinear regression and statistical significance was determined by ANOVA with Bonferroni post-test or unpaired t-test where appropriate. For in vivo data, analysis was performed using Statview II + Graphics package (Abacus Concepts, Berkeley, CA). Statistical significance was determined by one-way ANOVA with Fisher’s post-hoc comparison. Where data were not normally distributed, statistical analysis was carried out following logarithmic transformation. A p-value < 0.05 was regarded as statistically significant.
Results

GPCR-HIT assay—AT₁ receptor activation negatively modulates CCR2-Gαᵢ₁ coupling

CCR2 primarily signals via coupling to the inhibitory Gαᵢ₁ protein. We therefore utilized the BRET assay to monitor agonist-promoted conformational changes within the complex formed by Rluc8-tagged Gαᵢ₁ (Gαᵢ₁-Rluc8) and YFP-tagged CCR2 (Fig. 1A) indicative of G protein activation as shown previously [39,41,42,44]. For the putative functional interaction between AT₁ receptor and CCR2, we used the GPCR-HIT assay on the BRET platform (Fig. 1B). This is an assay configuration whereby one receptor (e.g. CCR2) is labelled with one component (e.g. YFP) of a proximity-based reporter system (e.g. BRET), the complementary component of which (e.g. Rluc8) is fused to a receptor interacting partner (e.g. Gαᵢ₁). Treatment with a ligand (e.g. AngII) selective for the untagged receptor (e.g. AT₁ receptor) results in modulation of the proximity of the tagged receptor and the interacting partner, resulting in a change in BRET signal that is indicative of functional interaction between the two receptors [4,40].

In cells co-expressing Gαᵢ₁-Rluc8 and CCR2-YFP, CCL2 resulted in dose-dependent Gαᵢ₁ activation as expected, reaching maximal activation by 100 nM CCL2 (Fig. 1C). Furthermore, increasing doses of AngII had no effect on the change in BRET signal induced by 100 nM CCL2 in the absence of AT₁ receptor (Fig. 1C). These findings indicate that AngII is not acting directly on CCR2 to mediate its effects, as it requires the presence of the AT₁ receptor. In cells co-expressing Gαᵢ₁-Rluc8, CCR2-YFP and AT₁ receptor, a similar dose-dependent change in BRET signal was induced by CCL2. However in contrast, the Gαᵢ₁ protein activation induced by 100 nM CCL2 was inhibited by AngII in a dose-dependent manner (Fig. 1D). This AngII-dependent modulation provides evidence for a functional interaction between CCR2 and AT₁ receptor.

Lack of synergy with respect to inositol phosphate signalling

AT₁ receptor primarily signals through Gα₉/₁₁, leading to increases in inositol phosphate signalling. Furthermore, CCR2 has been shown to couple to Ca²⁺ signalling through both pertussis toxin sensitive [39,53] and insensitive [39] mechanisms. We observed strong and potent AngII-induced IP₁ production with AT₁ receptor (Fig. 2A and B; Table 1), as well as weaker and less potent CCL2-induced IP₁ production with CCR2 (Fig. 2C and D; Table 1). However, no discernible synergistic effect was observed as a consequence of AT₁ receptor and CCR2 co-expression (Fig. 2E and F; Table 1).

GPCR-HIT assay—Evidence for potentiation of β-arrestin2 recruitment as a consequence of AT₁ receptor-CCR2 heteromerization

GPCR-HIT assays were performed in real-time on HEK293FT cells co-expressing CCR2-Rluc8 and β-arrestin2-Venus in the absence (Fig. 3A and C) or presence (Fig. 3B and D) of AT₁ receptor. Treatment with CCL2 caused recruitment of β-arrestin2-Venus to CCR2-Rluc8 in a manner that was not affected by AngII co-treatment in the absence of AT₁ receptor (Fig. 3C). Furthermore, no AngII-induced BRET signal was observed. This again indicates that AngII is not acting directly on CCR2 to mediate its effects, as it requires the presence of the AT₁ receptor.

In contrast, in the presence of AT₁ receptor, AngII induced a BRET signal indicative of recruiting β-arrestin2-Venus proximal to CCR2-Rluc8 (Fig. 3D). This effect is not observed simply because β-arrestin2-Venus is translocated to the plasma membrane upon activation of AT₁ receptor. If this was the case, a similar response would be expected if CCR2 was substituted
with any GPCR located in the plasma membrane. We have previously published that AngII treatment of HEK293FT cells expressing bradykinin receptor 2-Rluc8, β-arrestin2-Venus and AT1 receptor did not result in an increase in BRET signal, even though AT1 receptor was expressed functionally at the plasma membrane and addition of bradykinin resulted in a very robust BRET signal [4].

Remarkably, co-treatment with CCL2 and AngII resulted in a signal that was more than additive (Fig. 3D). This effect was also illustrated by the dose-response data, where co-stimulation...
with AngII (10 nM or 1 μM) increased the maximal CCL2-induced BRET signal to an extent that was more than additive (Fig. 3E) without significantly altering potency (Table 2). Similarly, AngII induced a dose-dependent BRET increase and the co-stimulation with CCL2 (10 nM or 1 μM) increased the maximal response to an extent that was more than additive (Fig. 3F), and again without altering potency (Table 2).

Fig 2. Measurement of inositol-1-phosphate (IP1) in cells co-expressing AT1 receptor and CCR2. HEK293FT cells expressing AT1 receptor (A and B), CCR2 (C and D) or both AT1 receptor and CCR2 (E and F) were used to measure agonist-induced IP1 production after 30 min at 37°C with increasing doses of CCL2 in the presence of 0, 10 or 1000 nM AngII (A, C and E) or with increasing doses of AngII in the presence of 0, 10 or 1000 nM CCL2 (B, D and F). Data are shown as a percentage of AngII-induced IP1 production in cells expressing AT1 receptor alone. Data are presented as mean ± SEM of five independent experiments. LogEC50 values are shown in Table 1.

doi:10.1371/journal.pone.0119803.g002
Effect of the combined AT1 receptor and CCR2 antagonists on β-arrestin2 recruitment

We used AT1 receptor-selective (Irb) and CCR2-selective (RS504393) antagonists to assess the impact of combined receptor blockade on β-arrestin2 interaction with AT1 receptor-CCR2 complexes (Fig. 4). In vehicle pre-treated cells, AngII and CCL2 induced BRET increases with a more than additive effect when applied together (Fig. 4A), consistent with Fig. 3D, E and F. Pre-treatment with 10 μM RS504393 decreased the CCL2-dependent signal and the CCL2 component of the co-stimulation (Fig. 4B), indicative of partial blockade of CCL2-induced β-arrestin2 recruitment by this compound. In contrast, pre-treatment with 10 μM Irb totally abolished both the AngII-dependent signal and the AngII component of the co-stimulation (Fig. 4C). When both antagonists were simultaneously applied, there was a dramatic inhibition of β-arrestin2 recruitment mediated by either individual or simultaneous AngII and CCL2 stimulation (Fig. 4D). These data confirm the specificity of the CCL2 and AngII effects, and provide justification for testing a combination of inhibitors in vivo.

CKD Animal characteristics

We performed in vivo studies using the STNx model of progressive kidney disease where animals were treated or not with either PPG, Irb or both combined (PPG+Irb). In comparison with sham animals, STNx rats developed hypertension (Table 3) and dysregulation of renal functions characterized by a decline in GFR (Table 3) and an increase in proteinuria (Fig. 5). Although not resulting in a significant reduction in blood pressure (Table 3), combined treatment (PPG+Irb) was associated with a significant reduction in proteinuria (Fig. 5) compared to vehicle, in contrast to Irb or PPG monotherapies. Furthermore, proteinuria with PPG+Irb was significantly lower than with Irb treatment alone (Fig. 5).

Macrophage Infiltration

The influx of infiltrating macrophages has been consistently implicated in cell apoptosis, proteinuria and interstitial fibrosis in CKD [54]. As shown in Fig. 6B and F, immunostaining with

<table>
<thead>
<tr>
<th>Table 1. LogEC50 data for IP1 production shown in Fig. 2.</th>
</tr>
</thead>
<tbody>
<tr>
<td>[AngII] (nM) CCL2 LogEC50 [CCL2] (nM) AngII LogEC50</td>
</tr>
<tr>
<td>AT1 receptor</td>
</tr>
<tr>
<td>CCR2</td>
</tr>
<tr>
<td>AT1 receptor + CCR2</td>
</tr>
<tr>
<td>10</td>
</tr>
<tr>
<td>1000</td>
</tr>
<tr>
<td>Data are mean ± SEM, n = 5. Note: co-treatment with 10 or 1000 nM AngII did not significantly alter the CCL2 logEC50 with cells expressing CCR2 and likewise, co-treatment with 10 or 1000 nM CCL2 did not significantly alter the AngII logEC50 with cells expressing either AT1 receptor or both AT1 receptor and CCR2 (P &gt; 0.05). In cells expressing AT1 receptor and CCR2, a significant difference was observed between the CCL2 logEC50 and AngII logEC50 in the absence of the other ligand (P &lt; 0.05). ND, not determined.</td>
</tr>
</tbody>
</table>

doi:10.1371/journal.pone.0119803.t001
Appendix II – Publications and Abstracts

Fig 3. GPCR-HIT analysis to show effect of AT1 receptor activation on CCR2/β-arrestin2 proximity. CCL2-induced activation of CCR2-Rluc8 leads to recruitment of β-arrestin2-Venus, resulting in a BRET signal (A). In the presence of AT1 receptor, AngII induces recruitment of β-arrestin2-Venus to the AT1-CCR2 heteromer, again resulting in a BRET signal (B) and thereby providing evidence for receptor heteromerization. Live HEK293FT cells co-expressing CCR2-Rluc8 and β-arrestin2-Venus in the absence (C) or presence (D) of AT1 receptor were used to measure the increase in agonist-induced BRET signal in real-time at 37°C before and after stimulation with 100 nM CCL2, AngII or both simultaneously. Data in (C) and (D) are representative of three independent
a macrophage marker ED-1 in STNx rats demonstrated a significant increase in the number of macrophages when compared with sham (Fig. 6A and F). Combined treatment (PPG+Irb) of STNx rats was associated with a further reduction in macrophage infiltration (Fig. 6E and F) when compared to PPG (Fig. 6C and F) or Irb (Fig. 6D and F) monotherapy.

**Podocyte loss**

Podocyte loss has been implicated in the pathogenesis of proteinuria in CKD. WT-1 (podocyte marker) immunostaining in STNx rats demonstrated a significant reduction in the number of podocytes (Fig. 7B and F) when compared with sham (Fig. 7A and F). Treatment of STNx rats with PPG in combination with Irb significantly attenuated podocyte loss (Fig. 7E and F) when compared to vehicle (Fig. 7B and F), in contrast to PPG (Fig. 7C and F) or Irb (Fig. 7D and F) monotherapy.

**Fibrosis**

Glomerulosclerosis and tubulointerstitial fibrosis are prominent features in CKD. STNx rats developed severe glomerulosclerosis (Fig. 8B) and tubulointerstitial fibrosis (Fig. 9B) compared to sham (Fig. 8A and 9A). Irb but not PPG significantly attenuated glomerulosclerosis (Fig. 8C, D and F) and tubulointerstitial fibrosis (Fig. 9C, D and F) when compared to vehicle treated STNx rats (Fig. 8F and 9F). The combination treatment (PPG+Irb) had a similar effect to Irb alone (Fig. 8E, 8F, 9E and 9F).

**Discussion and Conclusions**

Through the application of the GPCR-HIT assay [4], configured on the BRET platform and utilising both G\(\alpha_{i1}\) protein and \(\beta\)-arrestin2 as interacting partners, we have generated evidence consistent with a functional interaction between AT1 receptor and CCR2 at the receptor level in HEK293FT cells. Very interestingly, AT1 receptor activation appears to inhibit the conformational change associated with CCR2-G\(\alpha_{i1}\) coupling. We suggest two potential explanations for this effect: There may be a direct allosteric modulation of CCR2 by the activated (AngII-bound) AT1 receptor through a macromolecular complex, resulting in modulation of the receptor-G\(\alpha_{i1}\) conformational change. Alternatively, co-activation of both receptors may result in an increase in \(\beta\)-arrestin recruitment that switches off CCR2-G\(\alpha_{i1}\) signalling more robustly. Furthermore, there could be a combination of both of these effects as they are not mutually-exclusive.

**Table 2. LogEC50 data for CCR2/\(\beta\)-arrestin2 proximity shown in Fig. 3.**

<table>
<thead>
<tr>
<th>[AngII] (nM)</th>
<th>CCL2 LogEC50</th>
<th>[CCL2] (nM)</th>
<th>AngII LogEC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR2-Rluc8 +</td>
<td>0</td>
<td>–8.26 ± 0.11</td>
<td>0</td>
</tr>
<tr>
<td>(\beta)-arrestin2-Venus +</td>
<td>10</td>
<td>–8.35 ± 0.10</td>
<td>10</td>
</tr>
<tr>
<td>AT1 receptor</td>
<td>1000</td>
<td>–8.40 ± 0.08</td>
<td>1000</td>
</tr>
</tbody>
</table>

Data are mean ± SEM, n = 5. Note: no significant difference was observed between values (\(P > 0.05\)).

doi:10.1371/journal.pone.0119803.t002
Appendix II – Publications and Abstracts

Fig 4. Effect of combined AT<sub>1</sub> receptor and CCR2 blockade on GPCR-HIT with β-arrestin2. Real-time kinetic profiles were generated with live HEK293FT cells co-expressing CCR2-Rluc8, β-arrestin2-Venus and AT<sub>1</sub> receptor with 30 min preincubation at 37°C with vehicle (A), RS504393 (RS; 10 μM; B), Irbesartan (Irb; 10 μM; C), or both combined (D). Cells were then stimulated with AngII and/or CCL2 (100 nM) and BRET signals measured. 100% is defined as the mean increase in BRET signal observed 20 min after addition of CCL2 and following preincubation with vehicle. Data are presented as mean ± SEM of five independent experiments.

doi:10.1371/journal.pone.0119803.g004

Table 3. Animal characteristics.

<table>
<thead>
<tr>
<th>Group</th>
<th>BW (g)</th>
<th>SBP (mmHg)</th>
<th>GFR (ml min&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>532 ± 17</td>
<td>145 ± 6&lt;sup&gt;†&lt;/sup&gt;</td>
<td>5.09 ± 0.26&lt;sup&gt;‡&lt;/sup&gt;</td>
</tr>
<tr>
<td>STNx</td>
<td>509 ± 13</td>
<td>220 ± 7&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>0.43 ± 0.14&lt;sup&gt;‡&lt;/sup&gt;</td>
</tr>
<tr>
<td>STNx + PPG</td>
<td>490 ± 30</td>
<td>247 ± 13&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>0.49 ± 0.31&lt;sup&gt;‡&lt;/sup&gt;</td>
</tr>
<tr>
<td>STNx + Irb</td>
<td>493 ± 15</td>
<td>180 ± 6&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>0.92 ± 0.13&lt;sup&gt;‡&lt;/sup&gt;</td>
</tr>
<tr>
<td>STNx + PPG + Irb</td>
<td>478 ± 7</td>
<td>213 ± 9&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>0.90 ± 0.13&lt;sup&gt;‡&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>†</sup>,<sup>‡</sup>, P < 0.05 vs Sham; STNx, Subtotal nephrectomized; Irb, Irbesartan; PPG, Propagermanium

Animal numbers: Sham = 20, STNx = 19, STNx+PPG = 17, STNx+Irb = 19 and STNx+PPG+Irb = 16. Data are mean ± SEM.

doi:10.1371/journal.pone.0119803.t003
The functional interaction of these receptors is also supported by the BRET signals observed when monitoring β-arrestin2 recruitment proximal to CCR2 in the presence of AT1 receptor, where combined treatment with CCL2 and AngII induced a substantially higher BRET signal than observed with CCL2 or AngII alone (more than additive). Indeed this is consistent with our previous findings for CCR2-CCR5 and CCR2-CXCR4 heteromers [4]. An interesting question raised by our results is whether the conformation of the Gαi1 in the complex prior to addition of CCL2 somehow causes impaired recruitment of β-arrestin2 to the AT1 receptor, perhaps as a consequence of the heterotrimeric G protein complex straddling the intracellular surface of both receptors such that it sterically hinders arrestin binding. This would be consistent with a CCL2-induced conformational change altering the position of the Gαi1 (and presumably the rest of the heterotrimeric complex as well), therefore potentially enabling increased recruitment of β-arrestin2, not only to CCR2, but also to AT1 receptor. This would be consistent with the synergistic increase in receptor-arrestin BRET signal that we observe upon co-activation. At this stage, this is merely speculation, however, we believe it to be an intriguing hypothesis.

Furthermore, it is interesting that the superior effect of combined antagonist treatment observed in HEK293FT cells is consistent with our in vivo findings. The potential for the allosteric of heteromerization impacting receptor function is now very well established [1], as is the concept of biased signalling whereby one pathway is influenced differently to another [2]. The current study is certainly consistent with this, considering the aforementioned effects on Gαi1 and β-arrestin2, but little apparent effect on IP1 signalling. Our observations with β-arrestin2 are of particular interest as this critical intracellular scaffolding protein is not only involved in GPCR desensitization/internalization, but also promotes G protein-independent signalling pathways via many GPCRs including AT1 receptor [5]. Therefore, these findings provide further evidence for the potential importance of receptor heteromerization involving AT1.
Received for the α₁D adrenoceptor [56], CB1 cannabinoid receptor [57] and angiotensin II receptor type 2 [36].

The STNx rat model of progressive CKD resembles the major hallmarks of chronic kidney injury in humans, developing secondary hypertension, persistent proteinuria and declining GFR in conjunction with interstitial macrophage infiltration, depletion of podocytes, glomerulosclerosis and tubulointerstitial fibrosis. Proteinuria associated with glomerular podocyte loss has long been accepted as the clinical hallmark of progressive CKD [58], with a correlation between proteinuria and extent of podocyte loss being observed in patients and animal intervention studies [59,60]. In the present study, we demonstrated that the combination of Irb and PPG is superior to Irb monotherapy in attenuating podocyte loss and proteinuria. These outcomes appear to be independent of the blood pressure lowering effect of Irb. Previous studies have shown that CCR2 expression is greatly enhanced in glomerular podocytes of patients with CKD, with a correlation between CCR2 expression and extent of proteinuria [28]. Furthermore, in cultured podocytes, recombinant CCL2 induces apoptosis and conversely, inhibition of CCR2 is associated with a significant decrease in podocyte apoptosis [17]. AngII has

Fig 6. ED-1 (macrophage) staining from STNx rats. As illustrated by representative photomicrographs, in sham rats (A), only occasional macrophages were observed in the interstitium, while STNx rats (B) were associated with numerous macrophages. When compared to PPG (C) and Irb (D) mono-therapy, treatment of STNx animals with PPG+Irb (E) was associated with a further reduction in the number of macrophages. Magnification x200. Quantitative data (F) are expressed as mean ± SEM. *: P < 0.05 vs sham; #: P < 0.05 vs vehicle-treated; †: P < 0.05 vs Irb-treated STNx rats. Animal numbers: Sham = 20, STNx = 19, STNx+PPG = 17, STNx+Irb = 19 and STNx+PPG+Irb = 16.

doi:10.1371/journal.pone.0119803.g006
been shown to cause podocyte apoptosis via AT1 receptor both in vitro and in vivo [18,61]. AngII, on the other hand, has also been demonstrated to induce CCL2 expression in renal tissues [62]. We speculate that AT1 receptor and CCR2 may act synergistically in mediating podocyte apoptosis, potentially as a receptor heteromer, and therefore blockade of both receptors is superior to inhibiting AT1 receptor or CCR2 alone.

In both inflammatory and non-inflammatory renal disease, macrophage infiltration is a prominent feature [47,63,64] and indeed, the number of interstitial mononuclear cells also correlates closely with declining renal function in a range of renal diseases [65]. These inflammatory cells, which contain reactive oxygen intermediates, proteases and inflammatory cytokines including CCL2, are viewed as playing a significant role in mediating cell apoptosis, proteinuria and fibrosis [63]. In the present study, interstitial macrophage infiltration was a prominent feature in the STNx rats. Treatment with PPG in combination with Irb was associated with a further reduction in macrophage accumulation when compared to PPG and Irb monotherapy. Thus, the further reduction in macrophage accumulation by blockade of both AT1 receptor and CCR2 may potentially contribute to the observed attenuation of podocyte loss and proteinuria.
In conclusion, our in vivo findings have demonstrated that combined inhibition of AT1 receptor and CCR2 signalling significantly reduces proteinuria, macrophage infiltration and podocyte loss, all of which are implicated in the pathogenesis of CKD. Furthermore, our novel GPCR-HIT assay approach has provided new insights into potential mechanisms of action that may contribute to this beneficial synergistic effect.

Fig 8. Glomerulosclerosis in STNx rats. As illustrated by representative photomicrographs, in sham rats (A) there was minimal glomerulosclerosis as determined by PAS stain, while STNx rats (B) demonstrated severe glomerulosclerosis. Intervention with PPG alone in STNx rats had no effect on reducing glomerulosclerosis (C). Treatment of STNx rats with Irb (D) or a combination of PPG+Irb (E) was associated with a significant reduction in glomerulosclerosis when compared to vehicle-treated STNx rats (B). Magnification x400. Quantitative data (F) are expressed as mean ± SEM. *, P < 0.05 vs sham; #, P < 0.05 vs vehicle-treated STNx rats. Animal numbers: Sham = 20, STNx = 19, STNx+PPG = 17, STNx+Irb = 19 and STNx+PPG+Irb = 16.

doi:10.1371/journal.pone.0119803.g008
Acknowledgments

The authors are grateful to Walter Thomas, Aron Chakera, Andreas Loening, Sanjiv Gambhir, Atsushi Miyawaki and Jean-Philippe Pin for providing cDNA constructs. The authors also thank Mariana Pacheco for excellent animal husbandry.

Author Contributions

Conceived and designed the experiments: MAA YZ EAM JHW DJK KDGP. Performed the experiments: MAA YZ RSK HBS EKMJ. Analyzed the data: MAA YZ RSK HBS EKMJ DJK KDGP. Wrote the paper: MAA YZ EKMJ DJK KDGP.
Appendix II – Publications and Abstracts

References


Appendix II – Publications and Abstracts
Appendix II – Publications and Abstracts


Chapter 13

Bioluminescence Resonance Energy Transfer Approaches to Discover Bias in GPCR Signaling

Elizabeth K.M. Johnstone and Kevin D.G. Pfleger

Abstract

Bioluminescence resonance energy transfer (BRET) is a well-established technique for investigating G protein-coupled receptor (GPCR) pharmacology. BRET enables the monitoring of molecular proximity through the use of heterologously expressed proteins of interest and/or fluorophore-labeled ligands. Fusion to a donor luciferase enzyme or an acceptor fluorophore and subsequent detection of resonance energy transfer indicate the close proximity of the molecules of interest. As BRET is readily applied to the study of numerous GPCR signaling and regulatory paths, it is an ideal technique for investigating the pharmacology of biased ligands and receptors.

Key words Bioluminescence resonance energy transfer, BRET, G protein-coupled receptor, GPCR, Rluc8, NanoLuc, Nluc, Venus, Ligand bias, Biased signaling

1 Introduction

Biophysical techniques are used extensively in the study of G protein-coupled receptor (GPCR) pharmacology. Bioluminescence resonance energy transfer (BRET) is an increasingly popular biophysical technique that involves the nonradiative transfer of energy from a donor luciferase enzyme to an acceptor fluorophore molecule [1–3]. Oxidation of the luciferase’s substrate results in the production of light; however if a suitable acceptor fluorophore is within 10 nM of the donor [4], less light is emitted and some energy is transferred as resonance energy. Light of a different wavelength is then emitted by the fluorophore. BRET is used to monitor protein–protein, and very recently protein–ligand, proximity through heterologous expression of proteins of interest fused to the donor or acceptor and/or fluorescent acceptor-labeled ligands. Comparison of the relative levels of acceptor fluorescence and donor luminescence (“BRET signal”) provides information on the proximity of the two molecules of interest.
There are several generations of BRET that utilize various donors, acceptors, and substrates with differing advantages and disadvantages. Most traditional forms of BRET use analogues of *Renilla* luciferase (Rluc) for the donor and variants of green fluorescent protein (GFP) for the acceptor. The first generation of BRET, BRET\(^1\), uses the original substrate coelenterazine \(h\) and produces strong BRET signals \([5,6]\). BRET\(^2\) uses bisdeoxycoelenterazine, also known as coelenterazine \(400a\) (previously marketed as “DeepBlueC”), and has greater spectral resolution but lower quantum yield and rapid decay kinetics in comparison with BRET\(^1\) \([1,7,8]\). Extended BRET (eBRET) uses a caged form of coelenterazine \(h\) called EnduRen which enables detection of BRET over many hours \([5]\). Most recently, a new form of BRET has been developed called NanoBRET. NanoBRET utilizes a new luciferase called NanoLuc (Nluc) \([9]\) which in conjunction with its substrate furimazine produces much brighter luminescence than previous generations of BRET \([3]\).

The ability of different ligands (or receptor mutations) to stabilize a variety of receptor states that produce unique cellular response patterns is known as biased signaling or functional selectivity \([10]\). Furthermore, the concept can be extended to bias as a consequence of heteromerization \([11]\). Biased ligands provide great therapeutic promise, with the potential development of pharmaceuticals that selectively activate beneficial signaling pathways without affecting pathways that regulate adverse effects. A major advantage of BRET to study ligand bias is that the one technique can be readily applied to monitor multiple different signaling or regulatory pathways \([3]\). Fusion of the BRET donor or acceptor to GPCR interacting proteins can enable quantification of bias towards numerous paths including G protein signaling \([12,13]\), GPCR kinase \([14,15]\) and \(\beta\)-arrestin recruitment \([16,17]\), trafficking and internalization \([18,19]\), and ubiquitination \([20–22]\).

## 2 Materials

### 2.1 Generation and Validation of Fusion Constructs

1. cDNA for proteins of interest (e.g., GPCR and other proteins such as G protein subunit, \(\beta\)-arrestin, or Rab\(5\) early endosomal marker).
2. cDNA for BRET donor and acceptor in appropriate expression vectors (such as pcDNA3.1 from Invitrogen). The BRET donor and acceptor must be complementary to one another, for example Rluc\(^8\) (from S.S. Gambhir, Stanford University, CA \([23]\)) and the yellow fluorescent protein (YFP) Venus (from A. Miyawaki, RIKEN, Japan \([24]\)) when using BRET\(^2\) or eBRET.
3. Assay reagents and instruments required for validation of fusion protein expression and function, such as confocal microscopy and signaling assays.
Appendix II – Publications and Abstracts

2.2 Cell Culture

1. Cell culture plates: six-well clear plates and 96-well white plates.
2. Appropriate cell line for transfection, such as COS7 or HEK293.
3. Appropriate media for cell culture, such as Dulbecco’s modified Eagle’s medium (DMEM) containing 0.3 mg/ml glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin and 10 % fetal calf serum.
4. Transfection reagent, such as FuGENE (Promega).
5. Phenol red free DMEM containing 0.3 mg/ml glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin, 5 % fetal calf serum and 25 mM HEPES.
6. 0.05 % Trypsin-0.53 mM ethylenediamine tetraacetic acid (EDTA) in phosphate-buffered saline (PBS).

2.3 Detection of BRET

1. Appropriate luciferase substrate such as 60 mM EnduRen (Promega) dissolved in dimethyl sulfoxide (DMSO) for eBRET.
2. Appropriate buffer for dilution of luciferase substrate such as phenol red free DMEM containing 25 mM HEPES.
3. Microplate luminometer capable of measuring light through two filters. Examples include the PHERAstar FS, CLARIOstar, POLARstar Omega and LUMIstar (BMG LabTech), VICTOR Light (PerkinElmer), and Mithras LB 940 (Berthold Technologies).
4. If visualization of the BRET spectral shift is desired, then a scanning spectrometer is needed. Examples include the CLARIOstar (BMG LabTech), Spex fluorolog or fluoromax (Jobin Yvon), and Cary Eclipse (Varian).

3 Methods

Although there are now four generations of BRET assays, the basic methodology remains similar between the different variations. This protocol will illustrate the BRET technique using eBRET to monitor ligand-induced proximity over time. The method describes standard transfection techniques and uses an adherent assay protocol. Subheading 4 will describe variations to this protocol including the methodology for the different generations of BRET.

3.1 Generation and Validation of Fusion Constructs

1. Fusion constructs are generated by in-frame insertion of the cDNA of the protein of interest into a suitable expression vector such as pcDNA3.1 (Invitrogen) containing the cDNA for the donor or acceptor protein. The choice of donor-acceptor combination (see Note 1), placement position on the protein of interest (see Note 2), and optimization of donor–acceptor orientation (see Note 3) are dependent on several factors.
2. Using recombinant DNA techniques, the stop codon between the cDNA sequences is removed and replaced with a linker region if necessary (see Note 4).

3. The fusion proteins are tested to ensure appropriate levels of luminescence (using a luminometer following addition of coelenterazine substrate) or fluorescence (using a fluorometer following direct laser excitation) (see Note 5).

4. The fusion proteins are validated with respect to expression and function of the proteins of interest. GPCR function can be assessed using ligand binding and/or secondary signaling assays to ensure retention of ligand affinity/efficacy/potency. Confocal microscopy may be advisable to confirm correct cellular localization of proteins of interest (see Note 6).

3.2 Cell Culture

1. Cells are plated into 6-well clear cell culture plates so that they are 50–80% confluent after 24 h (or as appropriate for the selected transfection reagent).

2. Cells are maintained at 37 °C, 5% CO₂ in a humidified incubator.

3. After 24 h cells are transiently transfected (see Note 7) with an appropriate concentration and ratio of donor to acceptor cDNA (see Note 8).

4. For detection of BRET in an adherent layer (see Note 9), the cells are detached with trypsin-EDTA 24 h after transfection. The cells are resuspended in HEPES-buffered DMEM without phenol red and redistributed into a 96-well white cell culture plate (see Note 10).

5. Cells are maintained at 37 °C, 5% CO₂ in a humidified incubator for a further 24 h to allow attachment.

6. Measurement of the relative expression of fluorescent and luminescent fusion proteins can be assessed if required. This requires a separate aliquot of each sample to be excited directly by a laser followed by measurement of fluorescence. The appropriate coelenterazine substrate is then added to the same sample and luminescence is measured (see Note 11).

7. Scanning spectral analysis can also be performed with a separate aliquot of each sample.

3.3 Detection of BRET

1. The eBRET coelenterazine substrate EnduRen is reconstituted in tissue culture grade DMSO. To ensure complete resuspension, the substrate will likely require extensive vortexing for up to 10 min and warming to 37 °C. Aliquots are stored at -20 °C and protected from light (see Note 12).

2. Immediately prior to adding to cells, the EnduRen substrate is diluted in HEPES-buffered DMEM without phenol red at
Appendix II – Publications and Abstracts

37 °C to produce a final concentration of 30 μM. The substrate continues to be protected from light (see Note 13).

3. The media is aspirated from the cells and replaced with the diluted EnduRen. The cells are then incubated for at least 1.5 h at 37 °C, 5% CO₂ in a humidified incubator (see Note 14).

4. BRET is detected at 37 °C using a luminometer capable of measuring light through two filter windows. When using EnduRen as substrate and Rluc8 and Venus as donor and acceptor, suitable filters are 400–475 and 520–540 nm (see Note 15). Light from each well is measured through each filter (either simultaneously or sequentially) for 0.1–2 s before moving on to the next well (see Note 16).

5. Measurements are repeated as required, ideally using an instrument that automates this process using appropriate kinetics software.

6. When investigating real-time ligand-mediated changes in proximity, basal measurements are taken for a period prior to treatment. Ligand is then added (by injection if time points are required immediately after treatment) and repeated measurements are taken over time to evaluate the ligand-mediated effects. In parallel, duplicate samples are treated with vehicle only (see Note 17).

7. If desired, the BRET spectral shift can be visualized using scanning spectrometry. This shift appears as a peak or shoulder at the wavelengths characteristic of acceptor emission [6] (see Note 18).

1. The raw BRET ratio for each well is calculated by dividing the light emission from the long-wavelength emission filter (e.g., 520–540 nm for eBRET with YFP) by the light emission from the short-wavelength emission filter (e.g., 400–475 nm for eBRET). The raw BRET ratio is not the “BRET signal” as the background signal needs to be taken into account.

2. The ligand-induced BRET signal is calculated by subtracting the raw BRET ratio of the vehicle-treated sample from the raw BRET ratio of the ligand-treated sample [2, 5]. There are various instances where a negative ligand-induced BRET signal could be generated (see Note 19); however it is important to note that a lack of a ligand-induced BRET signal does not necessarily mean a lack of protein–protein interaction (see Note 20). With ligand-induced BRET signal calculations, the vehicle-treated sample represents the background and eliminates the requirement for measuring a donor-only control sample (see Note 21).

3. The data obtained by scanning spectrometry can also be used to quantify the BRET signal. This requires measurement of the...
area under the curve (AUC) within the wavelength windows that correspond to the filters in the luminometer. The BRET signal is then calculated in a similar manner to that described for dual filter luminometry (see Note 22).

4. Kinetic profiles can be generated by plotting the BRET signal against time, as seen in Figs. 1 and 2. These data illustrate the sensitivity of the BRET assay in detecting distinct kinetic profiles and how the real-time nature of BRET enables antagonist competition kinetics to be observed. Apparent association or dissociation rate constants can also be generated from such data [8].

5. BRET can also be used to investigate the pharmacology of receptor heteromers through the GPCR-heteromer identification technology (GPCR-HIT; Figs. 2 and 3), whereby one receptor is tagged with the donor or acceptor and the complementary tag is fused to an interacting partner such as a β-arrestin (Fig. 2) or G protein (Fig. 3). If coexpression and activation of the untagged second receptor results in a change in the BRET signal, this indicates the close proximity of the two receptors [3, 25, 26].

6. Concentration-response curves can be generated by plotting BRET data from one time point against the logarithm of ligand concentration, as seen in Fig. 3. Curve fitting by nonlinear regression analysis allows calculation of the concentration eliciting the maximal response ($E_{\text{max}}$), a measure of relative efficacy, and the half maximal effective concentration ($EC_{50}$), a measure of relative potency [8, 21] (see Note 23).

7. To determine the bias of a ligand, the outputs of various BRET assays can be compared. For example, β-arrestin data can be compared with the results of BRET assays investigating G protein-mediated signaling, GPCR kinase and ubiquitin recruitment, receptor trafficking and internalization. The ligand bias can be quantified by comparing apparent rates of association or dissociation from kinetic data, and affinity or efficacy values from concentration-response curves (see [10] for review).

Fig. 1 (continued) receptor fusion proteins R181C V2R/Rluc8 (b, f), V266A V2R/Rluc8 (c, g) or M311V V2R/Rluc8 (d, h). Following a 2 h incubation with EnduRen, BRET was measured for 10 min pretreatment and then 2 h posttreatment with either vehicle or agonist. The data illustrate the arginine vasopressin dose-dependent recruitment of β-arrestin2 to the V2R mutants, highlighting the different kinetics for the M311V mutant, lack of recruitment to the R181C mutant, and suggesting the V266A mutant may be a polymorphism that does not modify receptor function. Data shown are mean ± SEM of three independent experiments. This research was originally published in [21].
Fig. 1 Detection of β-arrestin2 recruitment to vasopressin type 2 receptor (V2R) mutants using eBRET in real time. HEK293FT (a–d) and COS7 (e–h) cells were cotransfected with β-arrestin2/Venus and wild-type V2R/Rluc8 (a, e) or mutant
Notes

1. The generation of BRET being used (e.g., BRET\textsuperscript{1}, BRET\textsuperscript{2}, eBRET, or NanoBRET) determines the choice of donor-acceptor combination and the accompanying luciferase substrate. Rluc8 \cite{23} and Venus \cite{24} are arguably the best donor-acceptor combination when using BRET\textsuperscript{1} (coelenterazine \textit{h}) or eBRET (EnduRen) \cite{6}. GFP\textsuperscript{5} or GFP\textsuperscript{10} is the preferred acceptor with Rluc2 or Rluc8 as donor when using BRET\textsuperscript{2} (coelenterazine 400a) \cite{1, 2, 6}. For NanoBRET, the
Appendix II – Publications and Abstracts

314

199

BRET Approaches to Study GPCRs

Fig. 3 Detection of dose-dependent change in proximity of Gαi1 with the angiotensin II receptor type 1 (AT1)-chemokine (C-C motif) receptor 2 (CCR2) (AT1-CCR2 receptor) heteromer using GPCR-HIT. BRET was measured following addition of coelenterazine h to HEK293FT cells coexpressing Gαi1-Rluc8 and CCR2-YFP in the absence (a, c) or presence (b, d) of AT1 receptor. Cells were treated with increasing doses of CCL2 (CCR2 agonist) or 100 nM CCL2 with increasing doses of angiotensin II (AngII). The change in BRET signal was plotted against the logarithm of agonist concentration, enabling the calculation of EC50 and IC50 values as appropriate. As the BRET signal decreased as a consequence of the ligand-induced conformational change associated with Gαi1 activation in this case (a), the data are presented as change in BRET signal as a percentage of CCL2-induced change. 100 % is defined as the decrease in BRET signal observed upon addition of 1 μM CCL2 (for curves with black circles), or 100 nM CCL2 + 1 pM AngII (for curves with white triangles). Data shown are mean ± SEM of at least three independent experiments. Interestingly, the presence and activation of AT1 receptor resulted in a dose-dependent inhibition of the CCL2-induced change in BRET signal between Gαi1-Rluc8 and CCR2-YFP (d). This research was originally published in [33]

3. As the N-terminus of a GPCR is extracellular, BRET labels are more commonly fused to the receptor’s C-terminus to enable investigations of proximity to cytosolic or other membrane-

 donor Nluc can be paired with a more red-shifted acceptor [27]; however it also works well with YFPs and fluorophores with emission spectra in between.
localized proteins. N-terminally tagged GPCR fusion proteins can be used to investigate extracellular interactions (e.g., ligand binding), but it is necessary to confirm that they are still able to traffic to the membrane correctly (NanoBRET is preferable in this instance as the small size and evolved secretory nature of Nluc appear to assist in membrane trafficking [27]). With regard to the interacting protein, an N-terminal, C-terminal, or intramolecular tag may be appropriate, and this will likely depend on the nature and function of the protein itself. For example, G proteins are usually tagged in a suitable loop region, such as at position Glycine 60, Leucine 91, or Glutamate 122 in Gαi1 [28].

3. The relative orientation of the donor and acceptor dipoles is an important factor for achieving efficient resonance energy transfer. A potential consequence of this is that some fusion protein donor–acceptor combinations work more successfully than others. Therefore it may be beneficial to create both donor- and acceptor-fusion proteins with each protein of interest, enabling empirical determination of the best donor–acceptor-fusion pairing.

4. Due to the requirement of having appropriate orientation of the donor and acceptor dipoles, efficient resonance energy transfer can potentially be improved by incorporating a linker region between the protein of interest and the label. This can enable greater freedom of movement and potentially increase the probability of achieving optimal orientation.

5. Low relative luminescence or fluorescence counts could be attributable to various parameters including: substrate viability; presence of a reducing agent such as ascorbic acid; incorrect cDNA sequence of fusion proteins; inadequate transfection optimization with respect to amount and ratio of cDNAs; cell number; and instrument calibration.

6. If a protein of interest is not functioning correctly despite confirmation of the cDNA sequence, the donor or acceptor may be interfering with protein function. This may be alleviated by altering: which label is used (see Note 1); the position of each label (see Note 2); the configuration of the labels (see Note 3); or the length of linker regions (see Note 4).

7. Cells that are stably transfected with BRET donor- and/or acceptor-linked fusion proteins may also be used for BRET assays. Additionally, the transient transfection procedure can be adapted for different requirements. For example, simultaneous transfection and seeding of cells directly into a 96-well plate can be more efficient (particularly with numerous parallel transfections); however, there is the potential for increased sample variability.
8. To determine the optimal ratio of donor to acceptor cDNA, a titration should be carried out. For optimal BRET efficiency, a protein concentration of 1:3 or 1:4 (donor:acceptor) is often considered ideal; however, there is not necessarily a direct relationship between cDNA quantity and final concentration of functional protein. Thus the optimal ratio will depend on expression efficiency and needs to be empirically determined.

9. Cells can also be assessed in suspension by detaching, resuspending, and aliquoting into a 96-well plate immediately prior to detection of BRET.

10. The optimal quantity of cells to seed per well can also be empirically determined through titration of cell number. Typically between 30,000 and 100,000 cells in a volume of 40–100 μl will be appropriate.

11. Measurement of relative expression of fluorescent and luminescent fusion proteins is particularly important if constitutive interactions are to be assessed using BRET saturation assays [29, 30], but can still be beneficial when investigating ligand-induced interactions.

12. The other generations of BRET use different substrates as follows: BRET1, coelenterazine h (dissolved in methanol or ethanol; from Promega); BRET2, coelenterazine 400a (dissolved in anhydrous or absolute ethanol; from Biotium); and NanoBRET, furimazine (no reconstitution required; from Promega). All substrates should be stored at −20 °C and protected from light.

13. Coelenterazine substrates can be diluted in a variety of other solutions, such as Dulbecco's phosphate-buffered saline (D-PBS) or Hank's Balanced Salt Solution (HBSS). In our hands HBSS produces larger signals with less background noise; however for eBRET experiments with sustained kinetic measurements it may still be preferable to use HEPES-buffered phenol red free media.

14. When using coelenterazine h, coelenterazine 400a, or furimazine, the diluted substrate is added immediately prior to detection of BRET without the incubation period required for EnduRen.

15. BRET1 (coelenterazine h) with any Rluc variant as donor and any YFP as acceptor typically also uses the 400–475 and 520–540 nm filter combination [31]. For BRET2 (coelenterazine 400a) with any Rluc variant as donor and GFP or GFP10 as acceptor, a filter combination of 370–450 and 500–525 nm is favorable [17]. NanoBRET with furimazine typically uses a 410–490 nm filter for the Nluc donor emission while the acceptor emission filter depends upon the fluorophore being used. A 520–540 nm filter could be used for YFP, while a >610 nm
longpass filter would be suitable for a red-shifted acceptor depending on the emission spectral characteristics [27].

16. The various protein–protein and ligand–protein interactions that can be investigated using BRET occur over different timescales, so it may be necessary to alter measurement reading time. For example, some G protein interactions occur rapidly, and shorter reading intervals would be more appropriate [32] than those used for arrestin recruitment or receptor internalization.

17. A second population of cells expressing only the donor-linked fusion protein may be assayed in parallel with those containing both donor- and acceptor-linked proteins. While this may not be necessary when investigating ligand bias, it is particularly important when assessing non-ligand-mediated proximity. When investigating receptor mutations, it is also important to have the wild-type receptor control assessed in parallel and the BRET ratio can even be calculated relative to the wild-type baseline if the mutations result in constitutive BRET signals [17].

18. Scanning spectrometry can be used to visualize eBRET, BRET\(^1\), BRET\(^2\), and NanoBRET. Rluc8 is far more amenable than native or codon-humanized Rluc to BRET\(^2\) detection by this method [6]. Scanning spectrometry is useful to ensure correct filter selection; however, once appropriate filters have been established, full spectral scans generally become unnecessary.

19. A negative ligand-induced BRET signal indicates that the treatment has reduced the donor–acceptor proximity and/or caused the donor and acceptor to be less optimally oriented relative to each other. This could occur when an inverse agonist reduces an interaction dependent upon active receptor conformation (see Fig. 2; [16]). It also occurs in BRET trafficking assays as the receptor moves away from the plasma membrane BRET marker [18]. Alternatively the BRET signal may be reduced as a consequence of a conformational change within a complex (see Fig. 3; [33]).

20. The high proximity and orientation dependence of resonance energy transfer means that it is possible for donor- and acceptor-linked fusion proteins to interact without a signal being detected.

21. Calculation of a non-ligand-induced BRET signal necessitates using the donor-only control to account for the background signal. This calculation requires subtraction of the raw BRET ratio for the donor-only sample from the raw BRET ratio for the sample containing both donor and acceptor. When assessing ligand bias, this will not usually be required.
22. Calculation of the BRET signal from scanning spectrometry data requires normalization of the emission spectra to the donor luciferase peak as an intensity of 1. The area under the curve (AUC) between 500 and 550 nm (for eBRET) is used to calculate the BRET signal by subtracting the AUC from the vehicle-treated sample from the AUC from the ligand-treated sample.

23. When plotting concentration-response curves, it is advisable to graph data from several time points. This is because the apparent affinity and efficacy of a ligand may change over time, and this should be taken into account when determining ligand bias.

References


Receptor-Heteromer Investigation Technology and its application using BRET

Elizabeth K. M. Johnstone and Kevin D. G. Pfleger

INTRODUCTION
There are many types of membrane receptors that can be broadly classified into three families based on distinct mechanisms of signal transduction, namely G protein-coupled receptors (GPCRs), receptor tyrosine kinases (RTKs), and ionotropic receptors, which are channels that directly allow flux of ions upon activation. Additionally, there are intracellular receptors such as those binding steroids. It is well established that many of these receptors exist as oligomeric species consisting of two or more receptor subunits (Neubig et al., 2001). In many cases, dimerization or oligomerization is required for the formation of a functional receptor unit. These receptors are known as “homomeric receptors” if the constituents are the same and “heteromeric receptors” if the constituents differ (Ferré et al., 2009). The concepts of GPCR homomerization and heteromerization have been described for different receptor systems currently providing the major method to study heteromers. The first step is the identification of a heteromer, and subsequent characterization of its biochemical fingerprint. In time, this biochemical fingerprint will ideally be used to demonstrate the presence of the heteromer in native tissue. To achieve this end, it is critical that the initial cell-based assays employed are able to...
robusly differentiate heteromer-specific pharmacology from that of the component receptors.

**RECEPTOR-HETEROMER INVESTIGATION TECHNOLOGY**

A novel technique recently developed to enable identification and pharmacological profiling of heteromers is the Receptor-Heteromer Investigation Technology (Receptor-HIT). This provides information on ligand-dependent functional responses specific to the heteromer. Receptor-HIT uses a proximity-based reporter system comprising four elements, three of which are labeled Receptor A, untagged Receptor B, and a labeled interacting Protein C that is recruited to the heteromer in a ligand-dependent manner (see et al., 2011). This configuration is illustrated in Figure 1 using bioluminescence resonance energy transfer (BRET), however, the approach can be applied using a variety of reporter systems including fluorescence resonance energy transfer (FRET), bimolecular fluorescence complementation (BFC), bimolecular luminescence complementation (BLIC), enzyme fragment complementation (EFC), and the protease-cleaved transcription factor assay system known as Tango™ (Mustafa et al., 2010; Mustafa and Pfleger, 2011). Co-expression of the aforementioned elements in cells enables the signal between the label of choice on Receptor A and complementary label on Protein C to be monitored. The fourth element in the system is a ligand that, upon binding to untagged Receptor B or the heteromer, selectively modulates the recruitment of Protein C to Receptor B and/or the heteromer (see et al., 2011; Mustafa et al., 2012). Receptor-HIT is unsuitable for investigating homomers due to this receptor-selectivity requirement, but heteromers of closely related receptor subtypes where a selective agonist may be unavailable can still be assessed. This issue is overcome by additional use of an antagonist selective for Receptor A, thereby meaning that Receptor B

---

![Figure 1](image-url)

**FIGURE 1** Utilization of Receptor-HIT on the BRET platform to investigate receptor heteromerization. BRET is a biophysical phenomenon involving the non-radiative transfer of energy from a donor enzyme to an acceptor fluorophore. The donor enzyme is a variant of Renilla luciferase (Rluc), and energy emission results from the oxidation of its substrate coelenterazine h to coelenteramide (Pfleger and Eidne, 2006; Pfleger et al., 2006). If the acceptor fluorophore (such as yellow fluorescent protein; YFP) is correctly oriented and within 10 nm, it will be excited by the energy transferred from the donor resulting in the emission of light at a characteristic wavelength (Gao et al., 2010). BRET is used to study protein-protein interactions through tagging one of the proteins of interest with the donor enzyme and the other protein with the acceptor fluorophore (Pfleger and Eidne, 2006). If the two proteins are in close proximity, the energy generated by the donor enzyme will be transferred to the acceptor fluorophore. The resulting BRET signal provides evidence for the two fusion proteins being in the same complex. In contrast to BRET between Rluc and YFP fused to each receptor, a specific receptor heteromer can be monitored by ligand-induced BRET due to proximity of a tagged receptor and a tagged intracellular protein used as a reporter partner (such as β-arrestin or G protein). In this system, energy transfer is measured between Receptor A-Rluc and a Protein C-YFP that interacts with the heteromer complex after selective activation of Receptor B or the heteromer itself. In addition to the induction of BRET by the Receptor B or heteromer-selective ligand providing evidence for Receptor AB heteromerization, it also identifies a biological function of the heteromer. Reprinted from Ayoub and Pfleger (2010). Copyright © 2010, with permission from Elsevier.
and/or the heteromer are still activated selectively. Alternatively, it is possible to use a non-selective ligand if it does not modulate recruitment of Protein C to Receptor A in the absence of Receptor B (Porrello et al., 2011). Whichever approach is used, generation of a signal upon application of the ligand indicates that Protein C has been recruited to the heteromer, thereby bringing the label on Receptor A into close proximity with the label on Protein C. The signal obtained is not only indicative of the receptors being in a heteromeric complex, it also reveals an aspect of the heteromer’s pharmacology through generation of ligand-dependent functional responses.

Receptor-HIT is an excellent assay for identifying and profiling heteromers as signals do not result from the homomeric or functional responses.

The signal obtained is not only indicative of the receptors being in a heteromeric complex, it also reveals an aspect of the heteromer’s pharmacology through generation of ligand-dependent functional responses.

The Receptor-HIT assay has largely been published with respect to GPCRs in the form of the GPCR-Heteromer Identification Technology (GPCR-HIT; Ayoub and Pfleger, 2010; Mustafa et al., 2010, 2012; Mustafa and Pfleger, 2011; Porrello et al., 2011; See et al., 2011), however it can also be applied to other receptors, including RTKs (Pfleger, 2011; Story et al., 2011), ionotropic receptors and steroid receptors. Consequently, there is also an extensive number of interacting partners that can be used. For example, GPCR-HIT studies can utilize G proteins or β-arrestins, whereas we have found Grb2 to be particularly amenable to Receptor-HIT assays investigating RTKs (Pfleger, 2011; Story et al., 2011).

BRET is our preferred platform for Receptor-HIT (Figure 1) because it can monitor protein proximity in live cells in real time at 37°C without the need for cell lysis, the assay does not rely upon proteins refolding in a complementation event to produce a read-out, and no alteration of receptor function is required (Mustafa et al., 2010). The traditional configuration for studying receptor heteromers using BRET involves tagging one receptor with the Renilla enzyme, while the second receptor is tagged with the acceptor fluorophore. A particular limitation to this approach is that overcrowding of receptors in the endoplasmic reticulum or degradative compartments can lead to non-specific “bystander” BRET (Pfleger and Edson, 2006). This is commonly addressed by employing BRET saturation assays (Mercier et al., 2002), however these are rather laborious. The ligand dependency of Receptor-HIT addresses this issue as it requires Receptor B or the heteromer to be capable of binding ligand (Figure 1), either because it is sufficiently mature and/or because it is appropriately localized to provide the ligand access for binding. Furthermore, although providing evidence of proximity of the two receptors, no functional information about the heteromer is revealed by saturation assays (Mustafa et al., 2012). In contrast, the use of an interacting protein also enables functional responses to be assessed, with the potential to uncover novel heteromer-specific pharmacology (Mustafa et al., 2012).

While there are advantages to using BRET as outlined above, there are also advantages to using other platforms in certain situations. For example, although EFC is not a real-time assay and requires cell lysis for signal detection, it is probably capable of achieving higher levels of screening throughput than BRET. Furthermore, assay systems like FRET and BiFC are more amenable to assessing subcellular localization if combined with confocal microscopy. However, because FRET uses a fluorophore as donor, there are issues arising from the need for external excitation. These include autofluorescence, photobleaching, cell damage, and direct acceptor excitation. Some of these issues can be addressed using time-resolved FRET (TR-FRET; Cottet et al., 2012). BiFC enables specific visualization of complemented fluorophores, and therefore the fused proteins of interest, but this is not a real-time assay due to a time delay while refolding occurs and once complemented, the proteins remain associated (Porrello et al., 2011). BRET is very sensitive to distance and relative donor-acceptor orientation. This is advantageous in terms of proximity specificity, however, it means that receptors could potentially form a heteromer without this being detected by BRET, and a lack of signal should be interpreted with caution (Pfleger et al., 2006). Other platforms may have a lower false-negative rate than BRET, however, the potential for higher false-positives may then need to be considered.

**APPLICATION OF RECEPTOR-HIT USING BRET**

Receptor-HIT has been used effectively on the BRET platform to investigate multiple established and novel heteromers. The CCR2-CCR5 and CCR2-CXCR4 heteromers that have been described by a number of studies (Mellado et al., 2001; Rodriguez-Prado et al., 2004; El-Asmar et al., 2005; Percherancier et al., 2005; Springael et al., 2006; Sohy et al., 2007, 2009) have recently been profiled in terms of dose-response curves, kinetics and Z’ data using GPCR-HIT (See et al., 2011). In particular note were the findings with the combination of CXCR4/Rh8, β-arrestin2/Venus and CCR2. Treatment with CXCL12 (CXCR4 agonist) resulted in a relatively transient BRET signal that returned to baseline before 40 min, whereas addition of CCL2 (CCR2 agonist) resulted in a more prolonged BRET kinetic profile, indicative of CCR2 forming a complex with CXCR4. Intriguingly, treatment with a combination of CXCL12 and CCL2 resulted in a prolonged and substantially higher BRET signal than observed with either agonist alone. Possible explanations for this include β-arrestin2 recruitment being facilitated by both types of receptor complex being in active receptor conformations, or proximity of the donor and acceptor being sufficiently close to enable detection of changes in donor-acceptor distance and/or relative orientation. Either way, this observation provides good evidence for specific reporting of β-arrestin2 recruitment to the heteromer complex (See et al., 2011).

When generating dose-response curves with Receptor-HIT data, the Hill slope has been seen to alter for particular combinations depending upon whether the tagged or untagged receptor is activated, consistent with stabilization of distinct complex conformations with the different ligands. For example, with the CCR5/Rh8, β-arrestin2/Venus and CCR2 combination, the dose-response curve with CCL2 was significantly steeper than with CCL4 (CCR5 agonist; See et al., 2011). As discussed previously, the reason for this difference is currently unclear, however, as the protein expression profile is identical in both cases and the only difference is the agonist treatment, this observation may help to shed light on the mechanism of GPCR heteromerization and/or allosterism across the complex in the future (See et al., 2011).
Receptor-HIT (in the form of GPCR-HIT) has also been used to investigate the heteromer between the angiotensin II (AngII) type 1 receptor (AT1R) and the AngII type 2 receptor (AT2R; Porrello et al., 2011). A number of studies have shown that the AT1R does not couple to arrestins and does not internalize following treatment with AngII (Pucell et al., 1991; Hunyady et al., 1994; Turu et al., 2006). Our BRET data indicating a lack of β-arrestin2/Rluc8 recruitment to AT2R/Venus are also consistent with these findings (Porrello et al., 2011). Therefore, upon co-expression of untagged AT1R with β-arrestin2/Rluc8 and AT2R/Venus, even though AngII can bind to both receptors, the ligand is strictly selective in terms of recruiting β-arrestin2 to only the untagged receptor. Therefore, the observation that a ligand-induced BRET signal results upon addition of AngII is indicative of AT1R-AT2R heteromerization (Porrello et al., 2011).

Receptor-HIT on the BRET platform has recently been used to characterize the novel heteromer between the α1A-adrenoceptor (α1AAR) and the CXC chemokine receptor 2 (CXCR2) that may play a role in prostate stroma (Mustafa et al., 2012). The Receptor-HIT studies showed that the heteromer recruits β-arrestin2 in a norepinephrine (NE)-dependent manner that can be blocked by both the α1AR antagonist Terazosin and the CXCR2-specific allosteric inverse agonist SB265610 (Figure 2). This specificity of this change in α1AR pharmacology with co-expression of CXCR2 was demonstrated by the lack of effect upon co-expression of C. chemokine receptor 2, vasopressin receptor 2 (V2R), or orexin receptor 1 (Mustafa et al., 2012).

The ligand-dependent nature of Receptor-HIT enables it to report on, albeit without differentiating between, constitutive and dynamic heteromers (Mustafa and Pfleger, 2011). The α1AAR-CXCR2 complex is an example of a constitutive heteromer that exhibits novel pharmacology revealed by the ligand dependency of Receptor-HIT (Mustafa et al., 2012). Indeed, BRET

---

**FIGURE 2** Use of Terazosin (α1AR antagonist) and SB265610 (CXCR2 inverse agonist) to interrogate β-arrestin2 recruitment to the α1AAR-CXCR2 heteromer. Extended BRET kinetic profiles were generated for the CXCR2/Rluc8, β-arrestin2/Venus and α1AAR combination in HEK293FT cells by treating with CXCL8 or vehicle (A) or NE or vehicle (B) ∼30 min before a second treatment with vehicle, 10 µM Terazosin, and/or 10 µM SB265610. Data are representative of three independent experiments. This research was originally published in Mustafa et al. (2012). Copyright © 2012 the American Society for Biochemistry and Molecular Biology.

**REFERENCES**


Johnstone and Pfleger Application of receptor-HIT using BRET technolo


Johnstone and Pfleger Application of receptor-HIT using BRET


Conflict of Interest Statement: In addition to being Head of the Laboratory for Molecular Endocrinology — GPCRs, Western Australian Institute for Medical Research and Centre for Medical Research, The University of Western Australia, Associate Professor Kevin D. G. Pfleger is Chief Scientific Officer of Dimerix Bioscience, a spin-out company of The University of Western Australia that has been assigned the rights to the "Receptor-HIT" technology. Associate Professor Kevin D. G. Pfleger has a minor shareholding in Dimerix.

Received: 30 April 2012; accepted: 04 August 2012; published online: 22 August 2012.


This article was submitted to Frontiers in Molecular and Structural Endocrinology, a specialty of Frontiers in Endocrinology. Copyright © 2012 Johnstone and Pfleger. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.