The role of GPR55 in neuronal and immune cell signalling and function

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B.A. (Mod.), M.Sc., Ph.D.

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Supervisors: Professor Veronica Campbell & Dr. Andrew Irving (University College Dublin)

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Dublin 2
Ireland
Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work.

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Signed,

Orla Haugh

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Summary

The orphan G-protein coupled receptor, GPR55, is widely expressed throughout the body and is responsive to cannabinoids. To date research on GPR55 has been largely carried out using overexpressing cell line models, which do not accurately reflect the function of the receptor physiologically. GPR55 is expressed in peripheral immune cells, as well as in the neurons and glia of the brain. Its function in these cells remains little understood. GPR55 has been suggested to have a regulatory role in immune processes both peripherally and centrally and it is believed to regulate synaptic plasticity and neuronal growth. Research into its function in neuropathological conditions is also limited, although the suggested endogenous ligand for GPR55, L-α-lysophosphatidylinositol (LPI), exerts microglia-dependent neuroprotection after excitotoxic lesion, suggesting that GPR55 may have a regulatory role in neuroinflammation and neurodegeneration. This makes GPR55 an attractive therapeutic target for the treatment of neuropathological conditions where inflammation is a characteristic feature. Alzheimer’s disease (AD) for instance is a neurodegenerative disease associated with neuroinflammation, neuronal loss and cognitive decline. The present study aims to examine the role of GPR55 in central signalling and immune processes using a rat cortical neuron-enriched model, the BV2 microglial cell line and in peripheral immune cells.

Cells obtained from neonatal cortical neuron-enriched cultures were immunolabelled for GPR55. They were treated with the suggested endogenous agonist for GPR55, LPI and the novel GPR55 agonist N-((4-(N-Phenylsulfamoyl)phenyl)carbamothioyl)-[1,1′-biphenyl]-4-carboxamide (referred to as 17g in this study). 17g (1 μM) and LPI (10 μM)-induced signalling effects were assessed using ratiometric Fura-2 imaging of intracellular calcium ([Ca^{2+}]i) responses and phospho-cAMP element binding protein (pCREB) immunocytochemical staining and confocal microscopy. LPI- and 17g-induced effects on
neuroimmune processes were assessed using a BV2 microglial migration assay, TNF-α cytokine enzyme-linked immunosorbent assay and a phagocytosis assay. Cell apoptosis in response to the pathological hallmark of AD, Aβ, was assessed using active caspase-3 immunocytochemistry. The expression of GPR55 and its role in peripheral immune cell function was assessed during an Industrial PhD placement in GlaxoSmithKline, UK.

It was found that GPR55 was expressed in microglia and neurons from cortical neuron-enriched cultures. Both LPI and 17g modulated [Ca^{2+}]i activity in neurons and glia. In certain neuronal populations, basal Ca^{2+} activity exhibited a synchronous and spontaneous pattern, indicative of network activity and 17g appeared to increase this activity in a GPR55-dependent manner. LPI (10 μM) diminished the frequency of spontaneous [Ca^{2+}]i events in some neuronal populations and potentiated this activity in other populations. LPI induced these effects independently of GPR55. Both LPI and 17g significantly increased the stimulation of CREB phosphorylation. 17g-induced CREB phosphorylation was Gαq-dependent and partially inhibited by the selective GPR55 antagonist, CID16020046 (20 μM), Gαq partially contributed to LPI-induced CREB phosphorylation, but this effect was not GPR55-mediated. LPI and CID16020046 (20 μM) attenuated BV2 microglial migration in response to Aβ-primed neuronal medium. 17g induced a trend towards increased BV2 cell migration under basal conditions. LPI and 17g protected against Aβ-evoked active caspase-3 induction but significantly increased the number of capsase-3-positive cells when applied alone. In the peripheral system, GPR55 expression became upregulated over the course of monocyte differentiation into monocyte-derived dendritic cells. Preliminary data indicated that CID16020046 attenuates monocyte phagocytosis of bacterial bioparticles.

This study suggests that GPR55 plays a possible role in the regulation of [Ca^{2+}]i activity, gene expression, immune cell migration and neuronal apoptosis. This implicates GPR55 in having a regulatory role in processes relevant to pathological conditions such as AD. This makes it an attractive therapeutic target for intervention in future.
Acknowledgments

I would first and foremost like to express my utmost gratitude to my supervisors, Professor Veronica Campbell and Dr. Andrew Irving. They have both offered me invaluable guidance, expertise and encouragement throughout this project and as a result I feel that I have developed into an independent and confident researcher. I feel very lucky to have had the opportunity to work with them and I am sure their teachings will persist during the next phase of my career. I wish them the best of luck in future.

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Lastly, I wish to thank my family for always encouraging my love for science and supporting me throughout my academic career.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>μM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>β-HB</td>
<td>β-hydroxybutyrate</td>
</tr>
<tr>
<td>Δ⁹-THC</td>
<td>Δ⁹- Tetrahydrocannabinol</td>
</tr>
<tr>
<td>2-AG</td>
<td>2-Arachidonoylglycerol</td>
</tr>
<tr>
<td>17g</td>
<td>N-((4-(N-Phenylsulfamoyl)phenyl)carbamothioyl)-[1,1′-biphenyl]-4-carboxamide</td>
</tr>
<tr>
<td>Aβ</td>
<td>β-amyloid</td>
</tr>
<tr>
<td>Abn-CBD</td>
<td>Abnormal-cannabidiol</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
</tr>
<tr>
<td>AEA</td>
<td>N-arachidonoyl ethanolamine /Anandamide</td>
</tr>
<tr>
<td>AM251</td>
<td>1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APOE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
</tr>
<tr>
<td>ARA-S</td>
<td>N-arachidonoyl-L-serine</td>
</tr>
<tr>
<td>ATF</td>
<td>Activating transcription factor</td>
</tr>
<tr>
<td>BK&lt;sub&gt;ca&lt;/sub&gt;</td>
<td>Large conductance calcium-activated potassium channels</td>
</tr>
<tr>
<td>BV2</td>
<td>Murine microglial cell line</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Calcium</td>
</tr>
<tr>
<td>[Ca&lt;sup&gt;2+&lt;/sup&gt;]e</td>
<td>Extracellular calcium</td>
</tr>
<tr>
<td>[Ca&lt;sup&gt;2+&lt;/sup&gt;]i</td>
<td>Intracellular calcium</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
</tbody>
</table>
CaMK: Ca$^{2+}$/calmodulin-dependent protein kinase

CB$_{1/2}$: Cannabinoid receptor type 1/2

CBD: Cannabidiol

CD: Cluster of differentiation

CDK5: Cyclin-dependent kinase 5

CID16020046: 4-(4-(3-hydroxyphenyl)-3-(4-methylphenyl)-6-oxo-1H,4H,5H,6H-pyrrolo(3,4-c)pyrazol-5-yl)benzoic acid

COX: Cyclooxygenase

CO$_2$: Carbon dioxide

CNS: Central nervous system

CP 55,940: (-)-cis-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol

CRAC: Ca$^{2+}$ release-activated channel

CREB: cAMP response element-binding protein

CTNF: Corrected total nuclear fluorescence

DAG: Diacylglycerol

ddH$_2$O: Double deionised water

DAB: 3,3'-Diaminobenzidine

DC: Dendritic cell

DIV: Days in vitro

DMEM: Dulbecco's modified eagle medium

DMEM/F12: Dulbecco's modified eagle medium: Nutrient mixture F-12

DMF: Dimethyl fumarate

DMSO: Dimethyl sulfoxide

DRG: Dorsal root ganglion

EAE: Experimental autoimmune encephalomyelitis
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ECS</td>
<td>Endocannabinoid system</td>
</tr>
<tr>
<td>EC Space</td>
<td>Extracellular space</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EPSC</td>
<td>Excitatory post-synaptic current</td>
</tr>
<tr>
<td>EPSP</td>
<td>Excitatory post-synaptic potential</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FAAH</td>
<td>Fatty acid amide hydrolase</td>
</tr>
<tr>
<td>FCA</td>
<td>Freund's complete adjuvant</td>
</tr>
<tr>
<td>FFA</td>
<td>Free-fatty acid</td>
</tr>
<tr>
<td>fMLP</td>
<td>N-Formylmethionyl-leucyl-phenylalanine</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-Aminobutyric acid</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>GPR18</td>
<td>G-protein coupled receptor 18</td>
</tr>
<tr>
<td>GPR55</td>
<td>G-protein coupled receptor 55</td>
</tr>
<tr>
<td>GPR119</td>
<td>G-protein coupled receptor 119</td>
</tr>
<tr>
<td>G-protein</td>
<td>Guanine nucleotide-binding protein</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GTPγS</td>
<td>Guanosine 5'-O-[gamma-thio] triphosphate</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HA</td>
<td>Haemagglutinin</td>
</tr>
</tbody>
</table>
HBS  HEPES buffered saline  
HCA2  Hydroxycarboxylic acid receptor 2  
HEK293  Human embryonic kidney cell line 293  
hGPR55  Human GPR55  
HEPES  2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid  
hr(s)  Hour(s)  
HRP  Horseradish peroxidase  
HU-210  1,1-dimethylheptyl-11-hydroxytetrahydrocannabinol  
IC Space  Intracellular space  
IFNγ  Interferon gamma  
IKca  Intermediate conductance calcium-activated potassium channels  
IL  Interleukin  
IP3  Inositol trisphosphate  
IMDM  Iscove's Modified Dulbecco's medium  
IPSC  Inhibitory post-synaptic current  
JWH-015  2-Methyl-1-propyl-1H-indol-3-yl)-1-naphthalenylmethanone  
JNK  c-Jun N-terminal kinase  
K+  Potassium  
KCl  Potassium chloride  
LPA  Lysophosphatidic acid  
LPC  Lysophosphatidylcholine  
LPI  L-α-lysophosphatidylinositol  
LPS  Lipopolysaccharide  
LTD  Long-term depression  
LTP  Long-term potentiation  
LysoPtdGlc  Lyso-phosphatidyl-β-D-glucoside
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tbody>
<tr>
<td>MAGL</td>
<td>Monoacylglycerol lipase</td>
</tr>
<tr>
<td>MAP2</td>
<td>Microtubule-associated protein 2</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>ME</td>
<td>2-Mercaptoethanol</td>
</tr>
<tr>
<td>MEA</td>
<td>Methanandamide</td>
</tr>
<tr>
<td>MEM-NEAA</td>
<td>Minimum Essential Medium Non-Essential Amino Acids</td>
</tr>
<tr>
<td>min</td>
<td>Minute(s)</td>
</tr>
<tr>
<td>ML193</td>
<td>N-[4-[[3,4-Dimethyl-5-isoxazolyl]amino]sulfonyl]phenyl]-6,8-dimethyl-2-(2-pyridinyl)-4-quinolinecarboxamide</td>
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<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MMF</td>
<td>Monomethylfumarate</td>
</tr>
<tr>
<td>moDC</td>
<td>Monocyte-derived dendritic cell</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple Sclerosis</td>
</tr>
<tr>
<td>Na⁺</td>
<td>Sodium</td>
</tr>
<tr>
<td>NAGly</td>
<td>N-arachidonoylglycine</td>
</tr>
<tr>
<td>NCX</td>
<td>Na⁺-Ca²⁺ exchanger</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T-cells</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor κ of activated B cells</td>
</tr>
<tr>
<td>NFT</td>
<td>Neurofibrillary tangle</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate receptor</td>
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<td>O-1602</td>
<td>5-Methyl-4-[(1R,6R)-3-methyl-6-(1-cyclohexen-1-yl]-1,3-benzenediol</td>
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<td>O-1918</td>
<td>1,3-Dimethoxy-5-methyl-2-[(1R,6R)-3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]benzene</td>
</tr>
<tr>
<td>OEA</td>
<td>N-oleylethanolamide</td>
</tr>
<tr>
<td>OHSC</td>
<td>Organotypic hippocampal slice culture</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC12</td>
<td>Cell line derived from a phaeochromocytoma of the rat adrenal medulla</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PEA</td>
<td>Palmitoylethanolamide</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PGE&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Prostaglandin E&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>PIP&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 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>PLA</td>
<td>Phospholipase A</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PMCA</td>
<td>Plasma membrane Ca&lt;sup&gt;2+&lt;/sup&gt; ATPase</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>PSEN</td>
<td>Presenilin</td>
</tr>
<tr>
<td>Rho</td>
<td>Ras homolog gene</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-associated protein kinase</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SIP</td>
<td>Sphingosine-1-phosphate</td>
</tr>
<tr>
<td>S.E.M</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SERCA</td>
<td>Sarco/endoplasmic reticulum ATPase</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering ribonucleic acid</td>
</tr>
<tr>
<td>SOCE</td>
<td>Store-operated Ca&lt;sup&gt;2+&lt;/sup&gt; entry</td>
</tr>
<tr>
<td>SR141617A</td>
<td>N-(Piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride</td>
</tr>
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(a.k.a. Rimonabant)

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Term</th>
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<tbody>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-α</td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-Tetradecanoylphorbol-13-acetate</td>
</tr>
<tr>
<td>TritonTMx-100</td>
<td>4-(1, 1, 3, 3-Tetramethylbutyl) phenyl-polyethylene glycol</td>
</tr>
<tr>
<td>TRP</td>
<td>Transient receptor potential</td>
</tr>
<tr>
<td>TRPV</td>
<td>TRP channel vanilloid receptor</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labelling</td>
</tr>
<tr>
<td>VGCC</td>
<td>Voltage gated Ca(^{2+}) channel</td>
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<td>WIN 55, 212-2</td>
<td>(R)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-(\alpha)][1,2,3-(\alpha)]1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate</td>
</tr>
<tr>
<td>YM-254890</td>
<td>G(\alpha)_q inhibitor</td>
</tr>
</tbody>
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Chapter 1

Introduction
1.1. Lipid modulators

Lipids are naturally occurring macromolecules that include fats, sterols, waxes, phospholipids, mono-, di- and triglycerides and fat-soluble vitamins e.g. A, D, E and K. Lipids may either be hydrophobic or amphiphilic (Berg et al. 2013) and they have a range of biological functions. Triglycerides are stored in adipose tissue and are a major form of energy for organisms. Phospholipids are amphiphilic molecules and are integral constituents of the cell membrane. They contain a hydrophobic glycerol core component linked by esters to two fatty acid tails and a hydrophilic phosphate head region. Other types of lipids found in the cell membrane include sphingomyelin and sterols (e.g. cholesterol). Lipids are also a vital part of the cell signalling process. They can act via G-protein coupled receptors (GPCRs) or nuclear receptors. Some established lipid signalling molecules and messengers include lysophosphatidic acid (LPA), sphingosine-1-phosphate (S1P), diglycerides, prostaglandins and steroids. Some downstream effects that these lipid signalling molecules induce include 
Ca\(^{2+}\) mobilisation, protein kinase activation and gene transcription (Berg et al., 2013; Brindley, 2004; Tigyi & Parrill, 2003). Endogenously produced cannabinoids – or endocannabinoids – are a class of lipids that are produced naturally within the body. They are integral constituents of cell membrane bilayers and also act as lipid messengers to activate cannabinoid receptors.

1.1.1. Cannabis and cannabinoid receptors

The plant Cannabis sativa – also popularly known as marijuana – has been used for recreational and medicinal purposes for several centuries, with hashish preparations even being found alongside ancient Egyptian mummies (Sharma et al., 2012). The principal psychoactive constituent of the plant is \(\Delta^9\)-tetrahydrocannabinol (\(\Delta^9\)-THC), which was first
isolated from *C. sativa* by Gaoni & Mechoulam in 1964. Initially it was thought that $\Delta^9$-THC did not signal via a specific receptor owing to its lipophilicity and ability to cross cell membranes (Makriyannis, 1995). However, the synthesis of a highly selective cannabinoid ligand, CP 55,940, allowed for the identification of a true cannabinoid receptor in the brain (Devane *et al.*, 1988). This cannabinoid receptor was cloned and characterised in rat brain not long after by Matsuda *et al.* (1990) and named CB$_1$. A second cannabinoid receptor, CB$_2$, was cloned and characterised in macrophages in the marginal zone of spleen by Munro *et al.* in 1993. Its expression is mainly localised in peripheral tissue and immune cells (Leleu-Chavain *et al.*, 2013), but it is also expressed by microglia in the brain (Núñez *et al.*, 2004). Both receptors belong to the GPCR family. Upon ligand binding, both of these receptors mediate a $G_{i/o}$-dependent signalling system that results in the inhibition of adenylate cyclase and a $G_{i/o}$-independent activation of mitogen-activated protein kinase (MAPK) via the recruitment of $\beta$-arrestin (Ibsen, Connor & Glass, 2017). It is now believed that cannabinoid receptors mediate the effects of $\Delta^9$-THC in the brain and periphery (Di Marzo *et al.*, 1998).

### 1.1.2. The endocannabinoid system

The widespread expression of cannabinoid receptors (central expression of CB$_1$ and peripheral expression of CB$_2$) warranted the question as to whether $\Delta^9$-THC was the only cannabinoid capable of binding to these receptors, particularly because it is a plant-derived molecule and not produced endogenously in humans. This led to the discovery of the endogenously produced lipid molecule arachidonoyl ethanolamine (a.k.a. anandamide (AEA)) in the porcine brain (Devane *et al.*, 1992). The name anandamide comes from the Sanskrit word “Ananda” meaning “bliss” and –amide. AEA has a similar pharmacology to $\Delta^9$-THC and binds to CB$_1$ and to a lesser extent CB$_2$. Although different in structure, AEA
can reproduce the same effects as Δ⁹-THC in rodents, such as the inhibition of locomotor activity and analgesia (Mechoulam & Fride, 1995). Soon after, another lipid called 2-arachidonoyl-glycerol (2-AG) was shown to have affinity for cannabinoid receptors (Mechoulam et al., 1995; Sugiura et al., 1995). Unlike AEA, concentrations of 2-AG are produced at significantly high levels in the brain (Stella et al., 1997), which helped in the identification of cannabinoids as retrograde neuromodulatory compounds (Di Marzo, 2004). AEA and 2-AG are metabolised by two enzymes, fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) respectively (Di Marzo, 2008). Cannabinoid receptors, their ligands and enzymes are collectively termed the endocannabinoid system (ECS).

The ECS functions in a wide range of physiological processes. It maintains energy balance and metabolism and modulates the immune system (Grotenhermen & Müller-Vahl, 2012; Pertwee, 2006). It also directly modulates central nervous system (CNS) processes such as memory formation due to its regulatory role in synaptic plasticity and long-term potentiation, the stress and neuropathic pain response, motor coordination and neuroinflammation (Berghuis et al., 2007). Coupled with its widespread presence within the brain, studies have linked cannabinoids and the ECS with having a neuroprotective function.

1.1.3. Non-CB₁/CB₂ cannabinoid targets and ligands

Despite the apparent abundance with which cannabinoid receptors are expressed in various tissues and cell types, there is evidence that these receptors are not the only ones responsible for mediating the physiological effects of cannabinoid ligands and other lipids. Breivogel et al. (2001) demonstrated that AEA and WIN 55,212-2 were found to stimulate guanosine 5'-O-[gamma-thio]triphosphate (GTPγS) binding in brain homogenates prepared from CB₁⁻/⁻ mice. However, GTPγS binding was not stimulated by other cannabinoids such as CP
55,940, HU-210, or Δ9-THC. Furthermore, AEA-stimulated GTPγS binding only partially overlapped with regional cannabinoid receptor expression (Di Marzo et al., 2000). WIN 55,212-2 and CP 55,940 inhibited excitatory neurotransmission with equal efficacy in wild-type (WT) and CB1\(^{-/-}\) mice. Interestingly, this inhibitory response was abolished by the CB\(_1\) inverse agonist SR141716 and remained unaffected by its analogue AM251 (Hájos et al., 2001). The endocannabinoid, virodhamine and the atypical cannabinoid ligand, O-1602, both inhibit N-formyl-met-leu-phe (fMLP)-induced migration of neutrophils in double knockout CB\(_1\)^{-/-}/CB\(_2\)^{-/-} mice (Schicho et al., 2011). These data suggest a distinct pharmacological target for these cannabinoids.

Palmitoylethanolamide (PEA) is a fatty acid amide analogue of AEA and has previously been shown to downregulate inflammatory parameters in a CB\(_2\)-dependent manner (Conti et al., 2002; Facci et al., 1995). For this reason it is thought to be a putative cannabinoid ligand. However, other studies contradict these findings by showing that PEA binds to CB\(_1\) and CB\(_2\) with low affinity and stimulates GTPγS binding in the cerebellum and CB\(_2\)-expressing cell lines with low potency (Griffin et al., 2000). PEA inhibits nerve growth factor release (NGF) from human mast cells stimulated with 12-O-Tetradecanoylphorbol-13-acetate (TPA), but these cells do not express CB\(_1\) and CB\(_2\) (Cantarella et al., 2011). Due to these discrepancies, it has been suggested that PEA mediates its effects via one of two ways: PEA might either directly activate an unidentified CB\(_2\)-like receptor, or act indirectly by potentiating the effect of endocannabinoids like AEA. The latter scenario is termed the “entourage effect” (Ben-Shabat et al., 1998; Mackie & Stella, 2006). However, PEA may utilise both scenarios in certain circumstances. It has recently been shown that PEA acts via an orphan receptor, G-protein coupled receptor 55 (GPR55), to modulate inhibitory synaptic transmission and concurrently modulate the release of 2-AG, in what the authors deemed an “entourage effect” (Musella et al., 2017).
The transient receptor potential (TRP) channel family are responsive to cannabinoid ligands and are believed to act as ionotropic cannabinoid receptors. TRP channels are ubiquitously expressed and recognise exogenous signals such as danger- or pathogen-associated molecular patterns (DAMPs and PAMPs respectively) from the environment (e.g. heat, acidity, chemicals) and endogenous danger signals released during trauma or tissue injury (e.g. reactive oxygen species). For this reason they are suggested to have a modulatory role in neuropathic pain, inflammation, chronic visceral pain, neurodegeneration and tumour suppression (Santoni et al., 2015; Wu et al., 2010). The TRP channel vanilloid receptor 1 (TRPV1) is thermo- and capsaicin-sensitive, TRP channel melastatin 8 (TRPM8) is activated by cooling agents such as methanol and TRP channel subfamily A member 1 (TRPA1) is a chemoreceptor for noxious stimuli such as by isothiocyanates and thiosulfinates that constitute pungent agents from mustard, wasabi and garlic. TRP channels are localised on afferent nociceptor neurons. Once activated, these nerve fibers transmit noxious signals to secondary neurons in the dorsal horn of the spinal cord and subsequently the brain, eliciting a sensation of discomfort or pain (Julius, 2013). Microglia are the resident macrophage-like cells of the brain and spinal cord (Yang et al., 2010). The phytocannabinoid cannabidiol (CBD) increases TRPV1 and TRPV2 expression in the BV2 microglial cell line and inhibition of these channels leads to a decline in CBD-induced microglial phagocytosis, suggesting a role for these receptors in neuroinflammation (Hassan et al., 2014). AEA has been shown mediate activity through TRPV1. In fact, AEA is now frequently referred to as an “endovanilloid” (Tóth et al., 2009). There are also structural similarities between AEA and synthetic vanilloid receptor agonists such as N-Vanillyloleamide (olvanil). Di Marzo and colleagues (1998) extensively investigated this link by testing AEA and olvanil on TRPV1 and cannabinoid receptors, concluding that the analgesic properties of olvanil may be due to its interactions with the ECS. This suggests a possible overlap between the ECS and the vanilloid system.
The natural ligands for the peroxisome-proliferator-activated receptor (PPAR) family (PPARα, PPARβ and PPARγ) are fatty acids and eicosanoid derivatives. These receptors have important roles in the regulation of lipid metabolism, insulin sensitivity, glucose homeostasis, expression of hepatic peroxisomal enzymes and in inflammation (Sun & Bennett, 2007). Early work into PPAR responsiveness to cannabinoids was performed by Fu et al. (2003, 2005) in order to investigate the effects of oleoylethanolamide (OEA; a monounsaturated analogue of AEA) on regulating feeding behaviour in rats. OEA induced satiety and decreased food-seeking behaviour in rats via a PPARα-dependent mechanism. OEA binds with low affinity to cannabinoid receptors, but could potentially enhance the effects of endocannabinoids via an “entourage effect” (Ben-Shabat et al., 1998; Sun & Bennett, 2007). Δ9-THC was found to activate PPARγ in a concentration-dependent manner in transactivation assays in the human embryonic kidney (HEK293) cell line (O’Sullivan et al., 2005). PPARs have more recently been implicated in regulating neuroinflammatory disease such as AD. CBD reduced Aβ-induced astrogliosis, an effect that was inhibited by a PPARγ antagonist (Esposito et al., 2011). Similarly, WIN 55,212-2 treatment of control and Aβ-treated astrocytes led to an increase in PPARγ expression (Aguirre-Rueda et al., 2015). Studies such as these give support to the proposal that PPARs mediate the effects of some cannabinoids, showing that this family of receptors may be a potential therapeutic target.

Evidence for other targets of cannabinoid ligands continues to emerge. Splenocytes derived from CB1−/−/CB2−/− double knockout mice treated with Δ9-THC, cannabinol and HU-210 showed increases in [Ca2+]i compared to WT mice (Rao & Kaminski, 2006). This suggests a novel target for these cannabinoid ligands. Also, the putative cannabinoid ligand PEA may signal via a novel CB2-like receptor (Mackie & Stella, 2006). Patents were initially lodged by GlaxoSmithKline (Brown & Wise, 2002) and AstraZeneca (Drmota et al., 2004) demonstrating that a range of endocannabinoid and synthetic cannabinoid ligands were
capable of activating GPR55. Cannabinoids were also found to stimulate GTPγS binding in a GPR55-dependent manner (Ryberg et al., 2007). For this reason, GPR55 was penned as the putative “CB3” cannabinoid receptor.

1.1.4. The putative cannabinoid receptor, GPR55

GPR55 was first identified by Sawzdargo et al. (1999) using cloning experiments and its mRNA was found to be expressed at high levels in the human striatum. Its structural characteristics were found to be consistent with other GPCRs, including its possession of seven hydrophobic regions corresponding to seven putative transmembrane regions and conserved first and second extracellular (EC) loop cysteines. However, instead of a conserved DRY motif, GPR55 contains a DRF motif immediately following transmembrane (TM) loop 3 (Sawzdargo et al., 1999). Studies that utilised mutations in the positively charged residue, K2.60(80), in GPR55 has shown that this residue is crucial for ligand binding (Kotsikorou et al., 2011a). The structure of human GPR55 can be viewed in Figure 1.1. It has 319 amino acids and has been categorised into the Class A or Rhodopsin-like family of seven-transmembrane spanning GPCRs (Henstridge et al., 2011; Müller et al., 2012). The GPR55 gene is located on chromosome 2 in mice and chromosome 6 in humans (Sawzdargo et al., 1999). Interestingly, GPR55 has low homology with the classical cannabinoid receptors, CB1 (13.5%) and CB2 (14.4%) (Henstridge et al., 2011; Sharir & Abood, 2010). Although several cannabinoid ligands can activate GPR55, it lacks the classical ‘cannabinoid binding pocket’ present in both CB1 and CB2. Kotsikorou et al. (2011a) constructed a homology model of GPR55 in both its active and inactive state. The active conformation model of GPR55 revealed a deep, vertical and highly hydrated binding pocket consisting of many hydrophilic residues. In contrast, the CB1 and CB2 receptor binding pockets are highly
hydrophobic. The third EC loop of GPR55 is significantly longer than CB₁ and CB₂ and contains many charged amino acids. Indeed, GPR55 shares greater sequence homology with other receptors such as the platelet activating factor (PAF) purinergic P2Y⁹, GPR35 (27%), GPR92/LPA5 (30%), P2Y5 (29%), the purinergic receptor-like orphan receptor GPR23 (30%) and the chemokine receptor CCR4 (21%) (Elbegdorj et al., 2013) than the CB receptors. These discrepancies have led to speculation over whether GPR55 is a suitable candidate for being a putative novel cannabinoid receptor.

GPR55 is expressed ubiquitously around the body including in adipose tissue, adrenal glands, parts of the gastrointestinal (GI) tract, bone, blood, cardiovasculature and pancreatic tissue (Henstridge et al., 2011). It is also expressed in cancer cells and is thought to regulate cancer cell proliferation and metastasis (Andradas et al., 2011; Ford et al., 2010; Piñeiro et al., 2011).

Figure 1.1. A helix net representation for GPR55.
Figure obtained from Shore & Reggio, 2015.
GPR55 is highly expressed by immune cells, tissues such as the spleen and thymus (Henstridge et al., 2011) and in many regions of the CNS including the hippocampus, striatum, caudate, putamen, hypothalamus, cerebellum, thalamus and pons (Ross, 2011; Sawzdargo et al., 1999).

1.1.5. LPI, the putative endogenous agonist for GPR55

Despite the vast amount of research carried out surrounding the pharmacology of GPR55, the issue of whether or not it is a true cannabinoid receptor has not been resolved. Numerous reports demonstrate that GPR55 is sensitive to cannabinoid ligands (Brown & Wise, 2002; Drmota et al., 2004; Ryberg et al., 2007). Other research groups contest these findings. Using HEK293 cells that overexpressed GPR55, Johns et al. (2007) reported that only atypical cannabinoid ligands activate GPR55, while Oka et al. (2007) reported that no cannabinoid ligands could activate GPR55. Furthermore GPR55 only has limited homology with the classical CB receptors (<15%; Henstridge et al., 2011). This ambiguity surrounding the pharmacology of GPR55 is not helped by the fact that selective ligands for GPR55 have only been established in recent years (Kotsikorou et al., 2011a, 2013; Yrjölä et al., 2016).

Various studies have found that a different class of endogenous lyso-phospholipid interacts more effectively with GPR55. L-α-lysophosphatidylinositol (LPI) has previously been suggested to be the endogenous ligand for GPR55 (Nevalainen & Irving, 2010; Oka et al., 2007; Piñeiro & Falasca, 2012), with the 2-arachidonyl species of LPI being the most potent based on structure-activity relationships (Yamashita et al., 2013). LPI has a strong interaction with the K2.60(80) and E3.29A residues in GPR55. These residues form a salt bridge, allowing for interaction with LPI. Indeed, mutations in these residues leads to a loss of function for LPI (Lingerfelt et al., 2017). An extensive study was carried out by Yamashita et
al. (2013) to characterise the actions and metabolism of LPI. LPI is a product of phosphatidylinositol (PI) and is generated through catalysis by acyltransferases and phospholipase A₁/₂. The rat brain contains a significant amount of LPI (37.5 nmol/g tissue of LPI). Using an ERK phosphorylation assay, 2-arachidonoyl-LPI was found to be the most active at GPR55 (EC₅₀; 30 nM). 1-stearoyl-LPI is a less efficacious agonist (EC₅₀; 450 nM) yet is the most abundant species in the rat brain (8.3 nmol/g tissue, 22.1% vs. 18.9 nmol/g tissue, 50.5% respectively). See Figure 1.2. for the chemical structures of LPI species. The tissue levels of LPI in the rat brain are estimated to be 10 micromolar (µM) for 2-arachidonoyl-LPI and 25 µM for 1-stearoyl-LPI (Oka et al., 2008; Yamashita et al., 2013).

![Chemical structures of different LPI species.](image1.png)

**Figure 1.2. Chemical structures of different LPI species.**

2-arachidonoyl-LPI is the most active species of LPI at GPR55 (EC₅₀; 30 nM). 1-stearoyl-LPI is less efficacious (EC₅₀; 450 nM) yet is the most abundant species of LPI in the rat brain (2-arachidonoyl-LPI = 8.3 nmol/g tissue (10 µM), 22.1% vs. 1-stearoyl-LPI = 18.9 nmol/g tissue (25 µM), 50.5%). Images of structures obtained from Yamashita et al. (2013).

LPI has been reported to activate other orphan GPCRs. For instance, it was found that LPI significantly induced intracellular cyclic AMP (cAMP) accumulation via the orphan GPCR, GPR119, in a dose-dependent manner in rat hepatoma cells stably expressing human
GPR119 (Soga et al., 2005a). In contrast, Piñeiro et al. (2011) found that knockdown of GPR119 had no effect on LPI-induced extracellular signal-regulated kinase (ERK) activation or the release of Ca\(^{2+}\) from intracellular stores in prostate and ovarian cancer cell lines, whereas downregulation of GPR55 inhibited LPI-dependent ERK activation in prostate and ovarian cancer cells and prevented Ca\(^{2+}\) mobilisation in the prostate cancer cell line. The endogenous agonist for GPR119 is believed to be lysophosphatidylcholine (LPC; Soga et al., 2005b), yet LPC was recently shown to elicit [Ca\(^{2+}\)]\(_i\) mobilisation in GPR55-expressing PC-3 human prostate carcinoma cells (Drzazga et al., 2017a). Similarly phosphorothioate LPC analogues evoked GPR40-, GPR55- and GPR119-dependent [Ca\(^{2+}\)]\(_i\) signalling as well as glucose-stimulated insulin secretion in the MIN6 pancreatic β-cell line and murine islets of Langerhans (Drzazga et al., 2017b). LPC and LPI both inhibit acetylcholine (ACh)- and histamine-induced hyperpolarisation of endothelial cells (Bodarenko et al., 2017). Both ligands are lyso-phospholipids so these data may allude to the possibility that both lipids signal via similar targets. Other suggested lipid agonists at GPR55 include N-arachidonoylserine (ARA-S; Zhang et al., 2010), PEA (Mackie & Stella, 2006; Musella et al., 2017), N-arachidonoylglycine (NAGly; Console-Bram et al., 2017) and the hydrophilic glycerophospholipid, lyso-phosphatidyl-β-D-glucoside (LysoPtdGlc) (Guy et al., 2015).

Ligand binding to GPR55 induces a signalling cascade that leads to the induction of differential downstream effects, a phenomenon known as ligand-biased signalling (Sharir & Abood, 2010; Rankovic, Brust & Bohn, 2016). This process involves ligands binding to GPCRs and inducing either G-protein- or β-arrestin-mediated signalling pathways, depending on their selectivity (Rankovic, Brust & Bohn, 2016). Although it is now widely accepted that GPR55 can transduce signals via a G-protein coupled mechanism, there is still speculation over which type of heterotrimeric G-protein this receptor couples to. Initial experiments using specific peptides to block G\(_{\alpha}\), and G\(_{\alpha}\), proteins, as well as inhibitors of
each G-protein subtype, were conducted in order to determine which G-protein coupled to GPR55. This led to the characterisation of Gα₁₃ as a potential mediator of GPR55 signalling (Ryberg et al., 2007). Other studies such as the one conducted by Lauckner et al. (2008) suggested that GPR55 coupled to Gα₁₂ and Gα₉ proteins. Stimulation of both Gα₁₃ and Gα₉ signalling pathways occurred after LPI-induced GPR55 activation in sensory neurons (Gangadharan et al., 2013).

The Irving research group has extensively studied GPR55-mediated signalling events using a HEK293 cell line stably overexpressing human GPR55 (hGPR55-HEK293). Henstridge et al. (2009) demonstrated that LPI stimulates a Gα₁₃-RhoA-Rho-associated protein kinase (ROCK) signalling cascade in a GPR55-dependent manner. This cascade induces phospholipase-C-ε (PLCε) activation, which leads to the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP₂) into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP₃). IP₃ induces the release of Ca²⁺ from internal endoplasmic reticulum (ER) stores. This Ca²⁺ increase was shown to trigger the activation of nuclear factor of activated T-cells (NFAT) proteins. Once activated, NFAT translocates to the nucleus and causes gene transcription. They have also demonstrated that LPI induces phosphorylation of cAMP response element-binding protein (pCREB) and ERK stimulation. Interestingly, the putative GPR55 agonists, AM251 and SR141716A, induced more potent phospho-CREB and ERK induction than LPI, whereas LPI induced a more potent Ca²⁺ signalling response (Henstridge et al., 2010). These findings suggest that these agonists exhibit ligand bias at GPR55 (Sharir & Abood, 2010). The GPR55-mediated signalling mechanism that leads to the activation of pCREB and ERK is not yet characterised, however some studies have suggested that cross-talk occurs between MAPK and Rho GTPase signalling pathways. Indeed, Anavi-Goffer et al. (2012) demonstrated that LPI-induced ERK activation in hGPR55-HEK293 cells was
abrogated by a ROCK inhibitor. The suggested signalling mechanisms mediated by GPR55 when activated by LPI in hGPR55-HEK293 cells can be viewed in Figure 1.3.

Figure 1.3. GPR55 signalling cascade following LPI stimulation in hGPR55-HEK293 cells.

Upon activation of GPR55, Gz13 protein couples to GPR55 and stimulates RhoA GTPase activity. RhoA then activates ROCK which in turn induces PLCa to hydrolyse PIP2 to IP3 and DAG. IP3 causes the release of Ca2+ from intracellular stores. This increase in cytoplasmic Ca2+ levels leads to the activation and translocation of NFAT to the cell nucleus. pCREB and ERK can also be stimulated through GPR55 activation. pCREB translocates to the cell nucleus and induces gene transcription. GPR55-mediated ERK activation was suggested to occur via a ROCK-dependent pathway. ERK and other kinases induce gene transcription by activating transcription factors within the cell nucleus. Figure modified from template.
It has been suggested that there are other downstream effectors of GPR55 activated by the G\textsubscript{\alpha\textsubscript{13}}-RhoA-ROCK cascade, including protein kinase B/Akt, p38 MAPK and activating transcription factor-2 (ATF-2) (Oka et al., 2010; Piñeiro et al., 2011). The functionality of these effectors appears to differ from cell type to cell type (Gasperi et al., 2013). Agonist stimulation of GPR55 and subsequent signalling induces receptor trafficking. GPR55 normally resides on the cell membrane but agonist stimulation leads to its internalisation, most likely due to \(\beta\)-arrestin recruitment. \(\beta\)-arrestins can be recruited to activated GPCRs to form stable receptor-arrestin complexes. They regulate an array of cellular processes such as GPCR internalisation and desensitisation, MAPK signalling, receptor transactivation, receptor trafficking and regulation of transcription factors. \(\beta\)-arrestins desensitise GPCRs by sterically inhibiting G-protein interactions with the GPCR (Smith & Rajagopal, 2016).

Methods used to observe GPR55 internalisation include \(\beta\)-arrestin detector assays, which monitor \(\beta\)-arrestins binding to GPR55 following agonist stimulation (Kapur et al., 2009; Yin et al., 2009). Other methods used to observe this internalisation include the use of haemagglutinin (HA) antibodies, which bind to GPR55 tagged with the HA epitope (Henstridge et al., 2010).

1.1.6. Regulation of intracellular Ca\(^{2+}\)

Ca\(^{2+}\) is a ubiquitous intracellular messenger that controls diverse cellular functions. [Ca\(^{2+}\)]\textsubscript{i} is strictly regulated, with free Ca\(^{2+}\) in the cytosol typically maintained at 100 nM and extracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\textsubscript{e}) maintained at 1.2 mM (Bronner, 2001). Cellular Ca\(^{2+}\) homeostasis is maintained through the action of the plasma membrane Ca\(^{2+}\) transport ATPase (PMCA) and the Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) in a resting cell, however upon increased [Ca\(^{2+}\)]\textsubscript{i} the sarcoendoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) is activated and allows the accumulation
of Ca\textsuperscript{2+} into the ER. GPR55 stimulation induces the IP\textsubscript{3}R-mediated downstream release of Ca\textsuperscript{2+} from intracellular stores, which can accumulate 0.1 mM of Ca\textsuperscript{2+} (Henstridge \textit{et al.}, 2010; Bagur & Hajnóczky, 2017). Mitochondria can also accumulate Ca\textsuperscript{2+} via the mitochondrial Ca\textsuperscript{2+} uniporter (mtCU; Bagur & Hajnóczky, 2017). These cellular proteins sense changes in [Ca\textsuperscript{2+}]\textsubscript{i} and react accordingly. ATPase pumps maintain low cytoplasmic [Ca\textsuperscript{2+}]\textsubscript{i} by pumping Ca\textsuperscript{2+} into the ER via SERCA pumps or out of the cell via PMCA pumps. IP\textsubscript{3}R-mediated release of Ca\textsuperscript{2+} from the ER in response to receptor stimulation empties the ER as PMCA\texttextsubscript{s} pump Ca\textsuperscript{2+} out of the cell faster than it can be repleted. Following store depletion, a Ca\textsuperscript{2+} entry mechanism is slowly activated called store-operated Ca\textsuperscript{2+} entry (SOCE; Clapham, 2007). Ca\textsuperscript{2+} release–activated Ca\textsuperscript{2+} (CRAC) channels and TRP channels mediate SOCE. These CRAC channels are comprised of three Orai protein subunits and are gated by STIM1 protein. Upon depletion of the Ca\textsuperscript{2+} stores, STIM1 forms oligomers that translocate to ER–plasma membrane junctions and bind to Orai channels at the plasma membrane. Ca\textsuperscript{2+} influx occurs at this STIM-Orai complex (Majewski & Kuznicki, 2015), thereby allowing [Ca\textsuperscript{2+}]\textsubscript{i} levels to remain at an acceptable level. To view this Ca\textsuperscript{2+} regulation mechanism, see Figure 1.4.

Ca\textsuperscript{2+} modulates many cell functions including gene expression, cell signalling, muscle contraction, secretion and metabolism. Unsurprisingly, dysregulation of Ca\textsuperscript{2+} homeostasis can therefore lead to disruption in cellular function and in extreme cases, cell death (Bagur & Hajnóczky, 2017). Many pathological conditions are exacerbated by the dysfunction of Ca\textsuperscript{2+} regulation, including neurodegenerative disorders such as Alzheimer’s disease (AD), amyotrophic lateral sclerosis (ALS), Huntington’s disease (HD), fragile X syndrome (FXS) and Parkinson’s disease (PD; Rajagopal & Ponnusamy, 2017). Appropriate regulation of Ca\textsuperscript{2+} homeostasis is therefore of paramount importance in order to maintain cellular viability.
Figure 1.4. Store-operated Ca\textsuperscript{2+} entry (SOCE) mechanism.

Upon activation of a GPCR by a ligand, its cognate G\textsubscript{z} protein couples to it and stimulates PLC to hydrolyse PIP\textsubscript{2} to IP\textsubscript{3} and DAG. IP\textsubscript{3} binds to the IP\textsubscript{3}R which causes the release of Ca\textsuperscript{2+} from intracellular stores. This increase in [Ca\textsuperscript{2+}]\textsubscript{i} leads to increased Ca\textsuperscript{2+} influx into the ER lumen via SERCA pumps and increased Ca\textsuperscript{2+} efflux from the cytosol into the extracellular (EC) space via PMCA pumps. Stores become depleted because of Ca\textsuperscript{2+} efflux via IP\textsubscript{3}R and PMCAs pumping Ca\textsuperscript{2+} out of the cell before the stores can be replenished by SERCA pumps. This triggers SOCE, whereby STIM1 proteins on the ER membrane oligomerise, translocate and bind to Orai channels on the plasma membrane, forming a complex. Ca\textsuperscript{2+} influxes back into the cytosol via these complexes, called CRAC channels, thereby maintaining adequate [Ca\textsuperscript{2+}]\textsubscript{i} levels. Some elements of figure adapted from Servier Medical Art images.
1.1.7. Advances in GPR55 pharmacological characterisation

As previously discussed, a lack of selective ligands for GPR55 has further hindered its pharmacological characterisation. Thankfully selective agonists and antagonists have been developed in recent years. Studies conducted in order to produce next-generation agonists for GPR55 have attempted to model these agonists on LPI because LPI has been found to possess a conformation and binding mode that is suitable for interaction with GPR55 (Kotsikorou et al., 2011a, 2011b). Heynen-Genel et al. (2010a) identified several potent and selective agonists for GPR55. ML184 (CID2440433) has 263 nM potency for GPR55, ML185 (CID1374043) has 658 nM potency for GPR55 and ML186 (CID15945391) has 305 nM potency for GPR55. The agonists were all found to cause downstream ERK phosphorylation and PKC-β-II translocation. Brown et al. (2011) identified the benzoylpiperazines, GSK494581A and GSK575594A, as GPR55 agonists, which were originally patented as inhibitors of glycine transporter subtype 1 (GlyT1). GSK575594A was 60-fold selective for GPR55 (pEC$_{50}$ = 6.8 fold change units) over GlyT1 (pIC$_{50}$ = 5.0 fold change units). However these agonists only showed activity at human GPR55, whereas endogenous agonists such as LPI show specificity for both human and rodent GPR55 (Henstridge et al., 2010; Whyte et al., 2009). Yrjölä et al. (2016) evaluated three compounds that were part of a high-throughput screen (HTS) of selective GPR55 agonists: CID1792579, CID1252842 and CID1011163. They were used as a starting point to create a series of nanomolar potency GPR55 agonists with a N-((4-sulfamoylphenyl)thiourea scaffold. From this study, one compound was created, N-(((4-(N-phenylsulfamoyl)phenyl)carbamothioyl)-[1,1'-biphenyl]-4-carboxamide (previously patented as CAS 651297-31-3, named 17g in the screen) which was found to be a selective GPR55 agonist containing an N-phenyl group (see Figure 1.5). They reported that 17g potently induced the release of Ca$^{2+}$ in hGPR55-HEK293 cells (EC$_{50}$ = 7.0 nM).
Although there appears to be no shortage of potential agonist ligands for GPR55, only a small number of putative antagonists have been identified. Some research groups have suggested that certain cannabinoid ligands act as GPR55 antagonists. The cannabinoid ligand CP 55,940 was found to antagonise LPI-induced release of Ca\(^{2+}\) from ER stores in hGPR55-HEK293 cells (Henstridge et al., 2009). Lauckner et al. (2008) reported that low micromolar (µM) concentrations of CB\(_1\) antagonist SR141716A (rimonabant) caused antagonism of GPR55 and a decline in Ca\(^{2+}\) when co-perfused with various agonists (Δ\(^9\)-THC, methanandamide (MEA) and JWH-015) in hGPR55-HEK293 cells and dorsal root ganglion (DRG) neurons. JWH-015 and Δ\(^9\)-THC did antagonise GPR55 and cause a reduction in Ca\(^{2+}\) release in DRG neurons when co-perfused with SR141716A, but MEA did not. In contrast, Kapur et al. (2009) and Henstridge et al. (2010) demonstrated that varying concentrations of SR141716A caused agonist-induced effects to occur via GPR55 stimulation in human osteosarcoma and hGPR55-HEK293 cells respectively. The differing effects of SR141716A previously reported may have been related to the doses used, efficacy and even cell type (Sharir & Abood, 2010). However, it is also worth noting that SR141716A is structurally
similar to the CB₁ inverse agonist AM251, which has previously been reported to have agonistic effects at GPR55 (Henstridge et al., 2010; Ryberg et al., 2007).

CBD was suggested to act as a GPR55 antagonist in a GTPγS-binding assay (Ryberg et al., 2007). Similarly, the cannabidiol analogue O-1918 has been identified to act as a GPR55 antagonist in several studies. Schmuhl et al. (2014) demonstrated that treatment with the putative GPR55 antagonists, O-1918 or CBD, induced mesenchymal stem cell migration. Interestingly, this pro-migratory effect was associated with concentration-dependent activation of p42/44 MAPK and was inhibited by pre-treating cells with the putative GPR55 agonist, O-1602 (Schmuhl et al., 2014). O-1918 is also a recognised antagonist of GPR18 (McHugh et al., 2010) and O-1602 is a recognised agonist of both GPR55 and GPR18 (Ashton, 2012). O-1918 inhibited the firing of nociceptive C fibres induced by O-1602 in a rat model of acute joint inflammation (Schuelert & McDougall, 2011). O-1918 inhibited the effects on intraocular pressure (IOP) induced by the GPR18 agonists Abnormal-CBD (Abn-CBD) and NAGly in a murine model (Caldwell et al., 2013) and it also inhibited the Abn-CBD-, NAGly- and O-1602-induced cellular migration of both BV2 microglia and GPR18-HEK293 transfected cells (McHugh et al., 2010) These studies provide evidence for potential GPR55 cross-talk mechanisms with GPR18. Indeed, Malek et al. (2015) reported that GPR55/GPR18 antagonism suppressed nitric oxide (NO) production in cultured rat microglial cells. The antagonist they used however, CID16020046, has to date been solely characterised as a potent and selective GPR55 antagonist (Kargl et al., 2013), so it is unclear where this group is obtaining its assumptions regarding its efficacy at GPR18. Furthermore, there has been no study to date that has explicitly elucidated potential cross-talk mechanisms between or dimerisation of GPR55 and GPR18.

Rempel et al. (2013) used β-arrestin assays to identify 3-substituted coumarins as GPR55 antagonists. Structure-activity relationships were performed and compared to CB₁, CB₂ and
GPR18 receptors. The study showed that when there was no lipophilic residue in position 7 of the coumarin scaffold, this resulted in the antagonism of LPI-induced β-arrestin translocation. In contrast, these compounds had little or no affinity for CB receptors or GPR18. Coumarin derivatives with an enhanced alkyl moiety possessed the greatest antagonistic potency in the series of compounds tested. 7-(1,1-Dimethyloctyl)-5-hydroxy-3-(2-hydroxybenzyl)-2H-chromen-2-one (PSB-SB-487, IC$_{50}$ = 0.113 μM, KB = 0.561 μM) and 7-(1,1-dimethylheptyl)-5-hydroxy-3-(2-hydroxybenzyl)-2H-chromen-2-one (PSB-SB-1203, IC$_{50}$ = 0.261 μM) were the two most potent GPR55 antagonists tested. Interestingly, these two compounds also acted as potent CB$_2$ receptor agonists in this study. The compounds could therefore be useful therapeutic tools for the study of cross-talk mechanisms and dual functionality between CB$_2$ and GPR55 (Rempel et al., 2013). Indeed, heteromers were shown to form in HEK293 cells expressing GPR55 and CB$_2$. Signalling by agonists of either receptor was regulated by the presence or absence of the partner receptors (with the consequent formation of heteromers) and by the activation state of the partner receptor (Balenga et al., 2014).

Previous studies such as the ones described in this section have identified putative GPR55 antagonists. However not all of these antagonists are completely selective for GPR55 and this lack of selectivity considerably limits their suitability as pharmacological tools. This makes the need for pharmacologically selective GPR55 antagonists all the more essential so that the (patho)physiological roles of GPR55 can be properly elucidated. A study conducted by HeynenGenel et al. (2010b) screened for selective GPR55 antagonists. Three potent antagonists were reported on in that study: ML193 (CID1261822), ML192 (CID1434953) and ML191 (CID23612552) with IC$_{50}$ values determined in the range of 0-16-2.72 μM using a β-arrestin HTS screen. Kotsikorou et al. (2013) reported that these antagonists possess a head region that occupies a horizontal binding pocket extending into the EC loop region of
GPR55, a central ligand segment that occupies a vertical binding region of GPR55. The most electronegative region of each ligand is located close to the far end of this central section and an aromatic ring extends out from its central region which is located deepest in the horizontal binding pocket of GPR55. The pendant aromatic or heterocyclic ring binds deep enough in the binding pocket to block M3.36(104), thus preventing the M3.36(104)/F6.48(239) “toggle switch” from undergoing the conformational change that is believed to be associated with GPR55 activation. However, because the primary interaction site for the most electronegative region of each of these ligands is K2.60(80) (located two turns down from the EC loops), a portion of each of these antagonists extends horizontally into the EC loop region of GPR55. This is significant because GPR55 agonists such as LPI and ML184 also interact with K2.60(80) (Lingerfelt et al., 2017), which could explain the antagonistic action of these screened compounds. Kargl et al. (2013) utilised another antagonist from that original screen, CID16020046 ((4-[4-(3-hydroxyphenyl)-3-(4-methylphenyl)-6-oxo-1H,4H,5H,6H-pyrrolo[3,4-c]pyrazol-5-yl] benzoic acid; see Figure 1.6). They reported that CID16020046 (CID) antagonised agonist-induced receptor trafficking in yeast cells expressing hGPR55 and LPI-mediated effects in the hGPR55-HEK293 cell line e.g. Ca²⁺ release, stimulation of ERK, NFAT and Nuclear factor κ of activated B cells (NF-κB). Kargl et al. (2013) also showed that CID prevented LPI-induced stimulation of wound healing in human lung microvascular endothelial cells (HMVEC-L). The potency of CID at GPR55 and the inhibition of LPI-mediated effects was estimated to be between 0.1–1 µM in that study.

CID is thought to antagonise GPR55 in a similar manner to ML191-ML193. It has a methylphenyl ring that penetrates deepest into the GPR55 binding site and prevents M3.36(104) from undergoing a conformational change and forming a GPR55 “toggle switch” with F6.48(239). The 6-oxo group of the central heterocyclic ring has the highest
electrostatic potential and hydrogen bonds with K2.60(80) (Kotsikorou et al., 2013). CID and ML191-ML193 are different in structure to the synthetic GPR55 agonists, ML184-ML186 (Heynen-Genel et al., 2010a). GPR55 agonist structures lack the aromatic ring found in the antagonist structures after the central portion. Instead, agonist structures including LPI, have inverted-L or T shapes with a long, thin vertical profile that extends deep into the GPR55 binding pocket (Kotsikorou et al., 2011a). Perhaps the aromatic rings of the antagonists prevent conformational changes in the toggle switch residues. GPR55 antagonists also bind higher to the K2.60(80) residue in GPR55 than agonists, which in turn may cause them to interact directly with and block movement of the EC-2 loop. This EC-2 conformational change is believed to be crucial for GPCR signal transduction. The availability of a selective GPR55 antagonist such as CID will be beneficial in future because it will allow for the delineation of GPR55 as a potential therapeutic target.
1.1.8. Differences in reported GPR55-mediated effects

To date the majority of studies investigating GPR55 have used overexpressing cell lines such as the hGPR55-HEK293 cell line (Henstridge et al., 2009; Johns et al., 2007; Kapur et al., 2009; Lauckner et al., 2008; Oka et al., 2007). Inconsistencies in findings have been presented by various research groups using this cell line. This could partially be explained by the clonal background of HEK293 cells, which can differ from laboratory to laboratory. Furthermore, different types of HEK293 cells were used between groups. Ryberg et al (2007) used a HEK293s (suspension) cell line, Johns et al (2007) used a HEK293T (highly transfectable) cell line and Henstridge et al. (2009) used a HEK293-AD (adherent) cell line. The use of HEK293 cells themselves may also be problematic. Although referred to as “human embryonic kidney” cells, an investigation in to their origin indicates that they may instead be derived from the adenoviral transformation of a neuronal precursor present in human embryonic kidney cell cultures (Shaw et al., 2002). GPCRs are mediators of ligand-bias
signalling (Rankovic & Bohn, 2016), which can cause differential receptor-mediated responses depending on ligand efficacy. Regardless of any ligand-bias signalling effects at GPR55, having inconsistent cellular models between research groups has not aided in its pharmacological delineation because in many reports the same ligand is producing completely opposite effects. For instance, Lauckner et al. (2008) reported SR141716A to be a GPR55 antagonist whereas Kapur et al. (2009) and Henstridge et al. (2010) reported that this ligand induced agonistic effects at GPR55.

Another disadvantage of using overexpressing cell models to delineate receptor pharmacology is the fact that receptor density can alter ligand behaviour. For instance, a low affinity agonist may be able to induce a large response in cells with a high receptor density e.g. in overexpressing cells. However the same agonist may generate little to no response if the receptor expression levels are low e.g. in endogenous models. Furthermore, constitutive receptor activity itself can alter the effects induced by ligands (Kenakin 1996, 2001). Indeed, Chinese hamster ovary (CHO)-K1 cells transiently expressing GPR55 were found to exhibit constitutive inhibition of both baseline and forskolin-stimulated CRE-mediated gene expression (Martin, Steurer & Aronstam, 2015). Other mitigating factors include protein-protein interactions such as receptor dimerisation and accessory protein binding, which may alter receptor conformation. The CB₁ receptor has been shown to form homomers and heteromers with other GPCRs, with this interaction modifying the processes downstream of the receptor (Mackie, 2005). For instance, GPR55 forms heteromers with CB₁ in the rat and monkey striatum (Martínez-Pinilla et al., 2014).

It is now pertinent to elucidate the function of GPR55 in endogenous systems in order to delineate its role in more physiologically appropriate models. Studies have been carried out to investigate endogenously-expressed GPR55 and its function using human neutrophils (Balenga et al., 2011), endothelial cells (Waldeck-Weiermair et al., 2008), osteoclasts and
osteoblasts (Whyte et al., 2009) and certain cancer cells (Andradas et al., 2011; Piñeiro et al., 2011). In the nervous system, the endogenous expression of GPR55 has been investigated in DRG neurons. GPR55 is highly expressed in large DRG and the GPR55 endogenous agonist LPI (3 µM) induced increases in [Ca\(^{2+}\)]\(_i\) in these cells (Lauckner et al., 2008). Neurite and axon elongation were examined in the neuronal PC12 cell line (Obara et al., 2011) and in retinal ganglion cells (RGC; Cherif et al., 2015). Treatment with LPI (10 µM) caused projection retraction in both studies but interestingly LPI concentrations of 1-3 µM increased projection growth. These findings are novel compared to the effects reported by groups using overexpressing GPR55 cell lines, whereby non-specific effects at higher ligand concentrations were not reported. These examples show that endogenously expressed GPR55 most likely does not signal in the same fashion as it does in overexpressing cells. Indeed, the insufficient evidence surrounding GPR55 pharmacology and function in endogenous and \textit{in vivo} models undoubtedly contributes to its continued orphan receptor status (Alexander et al., 2017).

1.1.9. GPR55 regulation of CNS and neuro-immune processes

Due to its fairly ubiquitous expression, GPR55 has been implicated in numerous physiological processes such as bone metabolism, appetite and hormone release and regulation (Henstridge et al., 2011) and has been increasingly linked to having a regulatory role in cancer (Andradas et al., 2011; Ford et al., 2010). GPR55 is highly expressed in the immune system by organs such as the thymus and spleen (Balenga et al., 2011; Ross, 2011) and by immune cells (Henstridge et al., 2011; Haugh et al., 2016). GPR55 characterisation in the brain has proved more difficult to ascertain. Marichal-Cancino et al. (2017) recently performed a bibliographic search of GPR55 expression and function in the CNS and
determined from 79 papers that very few reported on GPR55 expression (mRNA/protein) in the CNS. Only some groups carried out in vitro and in vivo studies that allowed for speculation into the potential activity of GPR55 in the different brain areas e.g. frontal cortex, hypothalamus, striatum, amygdala and cerebellar granule cells (Chiba et al., 2011; Kerr et al., 2013; Ryberg et al., 2007; Sawzdargo et al., 1999). Regardless, GPR55 activity in the brain has been associated with a variety of functions. For instance GPR55 was suggested to have a modulatory effect on synaptic plasticity and neurotransmission. Sylyantev et al. (2013) demonstrated that GPR55 co-localised with the pre-synaptic vesicular glutamate transporter 1 (VGLUT1) in stratum radiatum. Using two-photon excitation Ca^{2+} imaging in pre-synaptic axonal boutons in rat hippocampal slices, they found that the GPR55 agonists, LPI and O-1602, induced transient increases in glutamate release probability at individual CA3-CA1 synapses by elevating pre-synaptic Ca^{2+} through the activation of local Ca^{2+} stores. This effect was not seen in GPR55/- mice, indicating that the effects were GPR55-dependent. Furthermore, GPR55 agonists failed to affect post-synaptic stores activated by two-photon spot-uncaging of IP_{3} in the dendritic spines of CA1 pyramid cells, indicating that GPR55 was not localised post-synaptically (Sylantyev et al., 2013). Musella et al. (2017) recently found that the putative GPR55 agonists, PEA and O-1602, most likely signalled via pre-synaptic GPR55 to enhance gamma-Aminobutyric acid (GABA) spontaneous inhibitory post-synaptic currents (sIPSCs) in the mouse striatum. The effect of PEA was inhibited by the GPR55 antagonist, CID16020046 (CID), indicating its effects were GPR55-dependent. PEA was also suggested to trigger the synthesis of 2-AG at the post-synaptic site that in turn acted in a retrograde manner to inhibit GABA release through the stimulation of pre-synaptic CB_{1} receptors. This effect is indicative of depolarisation-induced suppression of inhibition (DSI; Diana & Marty, 2014). This was due to PEA inducing a delayed rundown effect on sIPSC frequency which was abolished by co-incubating the slices with orlistat, a DAGL inhibitor that blocks 2-AG synthesis. Indeed, Uchigashima et al. (2007) demonstrated that CB_{1} was
highly expressed on the GABAergic axon terminals of medium spiny neurons and parvalbumin-positive interneurons in mouse brain slices. Electrophysiological recordings of IPSCs were carried out and DSI was induced by a depolarising pulse. DSI was abolished by the DAGL inhibitor, tetrahydrolipstatin, indicating that 2-AG is the major endocannabinoid mediating retrograde suppression at inhibitory synapses of medium neurons. These findings suggest that PEA mediated its previously speculated “entourage effect” on 2-AG in the model used by Musella et al. (Ben-Shabat et al., 1998; Mackie & Stella, 2006).

PEA was also recently shown to produce a GPR55-mediated hyper-dopaminergic state in the rodent mesolimbic system. Using *in vivo* electrophysiological recordings in rats, Kramar et al. (2017) demonstrated that ventral hippocampal (vHipp) infusion of PEA enhanced ventral tegmental area (VTA) dopaminergic neuron firing frequency. The effect of PEA was inhibited by the selective GPR55 antagonist, CID and the NMDA antagonist, MK-801, indicating that the effects were dependent on GPR55 and NMDA. They also tested the effect of PEA on rat behaviour. Using a conditioned place preference procedure, it was shown that intra-vHipp infusion of PEA did not modulate morphine-related associative reward memory formation. This was in contrast to intra-vHipp CB1 stimulation which caused a potent increase in the reward salience of morphine, a mechanism in the Nucleus Accumbens that was dopamine-dependent (Loureiro et al., 2015). There was also no effect of PEA intra-vHipp infusion on rat anxiety levels, locomotion or context fear conditioning, which were tested using a light/dark box anxiety test protocol, an open-field test and a contextual fear conditioning procedure respectively. Interestingly, vHipp infusion of PEA significantly decreased their performance in an object location recognition task, in social interaction and social memory tasks and in olfactory fear conditioning tasks in a GPR55-dependent manner. Furthermore, inhibition of NMDA with MK-801 rescued these impairments in all tested behaviors, with the exception of olfactory-associated fear memory
The authors suggested that GPR55 activation increases glutamatergic release in the hippocampus, resulting in enhanced excitatory outputs from the vHipp to mesolimbic regions such as the VTA which may have caused the altered behaviours observed.

Hurst et al. (2017) reported that GPR55 mRNA was expressed in the hippocampi of rodents. Furthermore, immunohistochemistry and single cell polymerase chain reaction (PCR) demonstrated that GPR55 protein was expressed in pyramidal cells of CA1 and CA3 layers in the hippocampus. Using electrophysiological recordings, excitatory post-synaptic potentials (EPSPs) from hippocampal CA1 stratum radiatum in mouse slices were measured while using a theta-burst protocol to induce long-term potentiation (LTP). LPI significantly enhanced CA1 LTP in hippocampal brain slices, an effect not seen when the selective GPR55 antagonist, CID, was co-applied or when LPI was applied to GPR55−/− mice. This was not a disinhibition mechanism because whole-cell patch clamp recordings showed that LPI did not depress Schaeffer Collateral (CA3) excitatory glutamatergic inputs to CA1 stratum radiatum interneurons. GPR55 localisation was suggested to be post-synaptic because it was expressed in CA1 pyramidal cells and LPI did not enhance EPSPs at CA3-CA1 pyramidal cell synapses in hippocampal slices, indicating that LPI-induced LTP is not likely occurring by enhancing glutamate neurotransmission at the CA3-CA1 pyramidal cell synapse. To further examine GPR55 localisation however, paired pulse ratios (PPRs) were applied as a measure of presynaptic probability of transmitter release at CA3-CA1 synapses in both GPR55+/+ and GPR55−/− mice. LPI significantly enhanced PPRs in GPR55+/+ mice but not in GPR55−/- mice, suggesting that GPR55 may play a role in spontaneous neurotransmitter release rather than evoked release. The authors therefore suggested that LPI is most likely mediating its effects via post-synaptic GPR55, but they could not rule out a pre-synaptic role. However, it was found that neither phenotype differed in performance in a novel object recognition task and Morris Water Maze. LPI was not applied to either
phenotype though, so it remains to be seen if GPR55 activation has an effect on memory. Indeed, another report showed that when the GPR55 antagonist, CID, was injected into striatum, it impaired procedural memory using a T-maze (Marichal-Cancino et al., 2016). However, GPR55\(^{-/-}\) mice displayed greater levels of immobile behaviour in a radial arm maze, but because both mouse phenotypes spent similar amounts of time in open and closed arms of the maze, the authors linked this immobility to general inactivity rather than increased anxiety (Hurst et al., 2017).

GPR55 was suggested to have a role in neurogenesis. Hill et al. (2018) demonstrated that GPR55 mRNA is expressed in neural stem cells (NSC) and that activation of GPR55 by LPI and synthetic agonists (ML184, O-1602) increased proliferation in a low-proliferating culture of human NSCs. The selective GPR55 antagonist, ML193, inhibited these effects, indicating they were GPR55-dependent. Furthermore, continuous O-1602 administration into the hippocampus of C57BL/6 mice increased immature neuron generation compared to vehicle-treated mice. In line with this, GPR55\(^{-/-}\) mice showed reduced rates of proliferation and hippocampal neurogenesis and O-1602 had no neurogenic effect in those mice. Given that GPR55 activation by LPI or O-1602 transiently increases the frequency of CA1 EPSPs and ESP currents (ESPCs) enhances hippocampal CA1 LTP (Hurst et al., 2017; Sylantyev et al., 2013), Hill et al. postulated that GPR55 ligands may strengthen the synaptic connections of newly generated progenitors into the hippocampal circuitry. These findings suggest that GPR55 could be a therapeutic target for the regulation of neurogenesis.

GPR55 has been associated with having a minor role in motor function. Wu et al., (2013) demonstrated that GPR55\(^{-/-}\) mice displayed significant impairments in motor control during an accelerating rotarod test, where they performing far worse than their WT littermates. The knockout mice did improve in their performance over time, although they still did not perform as well as their WT littermates. These impairments in performance were not linked
to increased anxiety because both phenotypes spent equal numbers of time in open arms in the elevated plus maze. This is in line with Hurst et al. (2017), who found that both mouse phenotypes spent equal amounts of time in open arms in a radial arm maze. GPR55<sup>-/-</sup> mice showed a significantly greater number of footslips or errors compared to WT during a parallel rod footslip assay, which is indicative of ataxia. However, their distance travelled during this test was similar to WT. These impairments in motor function were most likely not due to deficits in vestibular sensory-motor integration, because both phenotypes did not show abnormal swimming patterns in a forced swim test. Furthermore, response to prepulse inhibition (PPI) did not differ between phenotypes. The PPI test works by presenting a weak non-startling sound in order to suppress the startle response to a strong acoustic startle stimulus presented immediately after the pre-stimulus. As the prepulse level increases, there will be greater suppression of the startle response (Paylor & Crawley, 1997). In contrast, both phenotypes showed no significant difference in grip strength or their ability to stay on a narrow beam. These findings suggest that the presence of GPR55 does not alter normal gross motor skills and muscle strength, but may have a minor role in certain aspects of motor coordination.

GPR55 expression was localised to the growth cones of neurites in a neuron-like cell line (PC12 cells, from the pheochromocytoma of the rat adrenal medulla). LPI induced neurite retraction in differentiated PC12 cells in a GPR55-dependent manner via a G<sub>α<sub>13</sub> and RhoA signalling pathway (Obara et al., 2011). GPR55 was shown to mediate the guidance of nociceptive afferent axons in the developing spinal cord (Guy et al., 2015) and retinal ganglion cell (RGC) projections toward targets in the visual thalamus (Cherif et al., 2015). These studies highlight a novel role for GPR55 in regulating the processes involved in axon and neurite guidance.
GPR55 expression has been reported in brain regions associated with emotion and anxiety such as the cortex, hippocampus and limbic system (Ryberg et al., 2007). Intracerebroventricular (i.c.v.) administration of the putative GPR55 agonist, O-1602, into rat brain led to an increase in time spent in open arms of an elevated plus maze, which is indicative of an anxiolytic response. This response was significantly inhibited by the selective GPR55 antagonist, ML193 (Rahimi et al., 2015a). Similarly, Shi et al., (2017) performed a GPR55 knockdown in the mouse medial orbital cortex, an area that is important in cognitive function, mood control and decision-making (Kringelbach et al., 2005). GPR55 protein expression was significantly decreased in the medial orbital cortex of mice that underwent chronic 21-day restraint compared to control. This is in contrast to Marco et al. (2014), who found that GPR55 expression increased in the frontal cortex of male rats and in the hippocampus of female rats following exposure to early-life stress. Mice were then subjected to acute stress tests such as chronic restraint and forced swimming (Shi et al., 2017). Mice were intraperitoneally (i.p.) injected with O-1602 following exposure to acute stress, which caused acutely stressed mice to spend increased time in the open compared to untreated stressed mice in subsequent elevated plus maze and open field tests. This effect of O-1602 was inhibited by the selective antagonist, CID and abolished by GPR55 knockdown. This suggests that GPR55 activation has an anxiolytic effect. Interestingly, glutamate receptor subunit (GluA1 and GluN2A) expression in the medial orbital cortex was significantly increased in acutely stressed animals and i.p. O-1602 treatment induced a reduction in this GluA1 and GluN2A increase. Furthermore, this effect of O-1602 was not seen in knockdown mice. They therefore linked GluA1 and GluN2A expression in the medial orbital cortex to the development of anxiogenic behaviours and suggested that GPR55 activation may reverse these effects. In a rat model of autism induced by prenatal exposition to valproic acid, a decrease in GPR55 mRNA expression in the frontal cortex and hippocampus was
reported (Kerr et al., 2013). These studies highlight the potential role of GPR55 in higher cognitive functions such as in behaviour and anxiety as well as in conditions such as autism.

GPR55 mRNA is expressed by peripheral immune cells such as neutrophils (Balenga et al., 2011), lymphocytes (Oka et al., 2010), monocytes and natural killer (NK) cells (Chiurchiù et al., 2015). It is also highly expressed in the CNS by microglia (Pietr et al., 2009). For this reason GPR55 has been associated with having a modulatory role in inflammation and neuropathic pain (Haugh et al., 2016; Staton et al., 2008). Pietr et al. (2009) demonstrated that GPR55 mRNA is downregulated in primary microglial cells and BV2 microglial cells treated with lipopolysaccharide (LPS). In contrast, GPR55 mRNA is slightly upregulated in BV2 cells treated with interferon (IFN)-γ but downregulated in primary microglia. This suggests that cellular composition (i.e. primary or cell line) and activation state are potentially important for GPR55 function in microglia. IFN-γ-stimulated BV2 cells also exhibited significantly increased levels of ERK phosphorylation in response to LPI compared to control cells (Pietr et al., 2009). LPI was also found to evoke an elevation of intracellular Ca^{2+} in BV2 cells (Eldeeb et al., 2009) and prolonged LPI treatment was found to selectively downregulate GPR55 mRNA in primary rat microglia (Kallendrusch et al., 2013), which is indicative of ligand-induced desensitisation or internalisation of GPR55 (Kapur et al., 2009).

The putative GPR55 agonist O-1602 promotes BV2 microglial migration, but it was claimed that this response was mediated by GPR18 (McHugh et al., 2010) even though this ligand has affinity for both receptors (Ashton, 2012). GPR18 is an orphan receptor and like GPR55 shows sensitivity to cannabinoid ligands (Alexander, 2012; McHugh, 2012; Penumarti & Abdel-Rahman, 2014; Schicho et al., 2011), although evidence increasingly suggests that its endogenous agonist is NAGly (Burstein et al., 2011; McHugh et al., 2010; Penumarti & Abdel-Rahman, 2014). GPR55 shares a complex and overlapping pharmacology with GPR18. For instance, antagonism of GPR55/GPR18 suppressed NO release from LPS-
activated rat microglial cells (Malek et al., 2015), highlighting a potential cross-talk mechanism between these two orphan receptors. However, the antagonist CID was used in that study, which has to date been solely characterised as a GPR55 antagonist (Kargl et al., 2013).

GPR55 also has a suggested regulatory role in neuropathic pain. Staton et al. (2008) initially presented evidence that inflammatory mechanical hyperalgesia (i.e. nociceptive sensitisation) in response to Freud’s Complete Adjuvant (FCA) was completely absent in GPR55−/− mice compared to wild-type (WT) littermates (GPR55+/+). This was coupled with an increase in anti-inflammatory cytokine production (interleukin (IL)-4, IL-10, interferon-γ (IFNγ) and granulocyte-macrophage colony-stimulating factor (GM-CSF)) at 14 days post-FCA injection in GPR55−/− mice compared to WT. These findings suggest that GPR55 signalling can influence the regulation of certain cytokines and this may contribute to the lack of inflammatory mechanical hyperalgesia in the GPR55−/− mice. Additionally, they utilised a partial nerve ligation model of neuropathic hypersensitivity and demonstrated that GPR55−/− mice failed to develop mechanical hyperalgesia up to 28 days post-ligation, which also eludes to a modulatory role of GPR55 in the development of neuropathic pain. LPI was suggested to regulate sensitisation of peripheral sensory neurons in mice in vivo in Gα13 and Gαq/11-dependent mechanisms, which manifested as an exacerbated sensitivity to painful and innocuous pressure (Gangadharan et al., 2013). The overlapping pharmacology of GPR55 and GPR18 can be observed in models of neuropathic pain. Chronic constriction injury (CCI) is a model of peripheral neuropathy and GPR55 and GPR18 mRNA were both upregulated in the DRG and lumbar spinal cord following CCI (Malek et al., 2016). Furthermore the putative GPR55/GPR18 ligand, O-1602, reversed the desensitising effects of ethanol in rats that had undergone CCI (Breen, Brownjohn & Ashton, 2012). This evidence supports the theory that GPR55 has a pronociceptive role in neuropathic pain. However, this role may be age-dependent. Kwok et al. (2017) demonstrated that LPI and the
putative GPR55 agonist, AM251, induced analgesia in the early life of rats when injected into the periaqueductal gray (PAG) and rostral ventromedial medulla (RVM) by reducing spinal-reflex excitability and increasing mechanical withdrawal threshold. These effects were measured through the electromyographic (EMG) activity of flexor hind limb muscle in response to mechanical stimulation of the plantar hind paw using von Frey hairs. The inhibitory effect of AM251 on spinal-reflex excitability and mechanical withdrawal threshold only occurred in young rats in both the PAG and RVM and was inhibited by a selective GPR55 antagonist, ML193. Interestingly, although the effects of LPI were similar to AM251 in young rats, intra-RVM LPI significantly increased spinal reflex excitability at postnatal day (P)40 when compared with vehicle controls. This suggests that GPR55 stimulation has an antinociceptive effect in young rats and a pronociceptive effect in adult rats. Indeed, they also showed that GPR55 mRNA levels did not alter over time in the PAG, but became significantly upregulated in the RVM at P21, only to decrease again by P40. They suggested that P21 is a crucial time point when developmentally regulated changes occur, eluding to a potential switch in GPR55-mediated actions throughout postnatal development. Munawar et al. (2017) recently reported that AM251 did not alter the reaction latency of mice to a hot-plate test in a model of 2′,3′-Dideoxycytidine (ddC)-induced thermal hyperalgesia. However, it did significantly antagonise the antihyperalgesic effect of AEA and 2-AG. Furthermore, the selective GPR55 antagonists, ML193 and CID, both antagonised 2-AG-induced thermal hyperalgesia. These findings suggest that 2-AG may mediate its antinociceptive effects via GPR55. However, Carey et al. (2017) recently carried out an extensive study examining the responses of GPR55−/− and WT mice to mechanical and thermal stimulation in multiple models of inflammatory and neuropathic pain. They showed that genetic deletion of GPR55 did not alter the development of pathological pain in adult mice in any chronic pain model evaluated. Clearly the true function of GPR55 in the regulation of neuropathic pain remains elusive and further studies are required to truly characterise this function.
It is unclear exactly what the function of GPR55 is in the CNS, but due to its high expression levels in certain areas of the brain and in microglia, it is becoming more apparent that it may have an important role in modulating CNS and neuroinflammatory responses. A summary of the potential functions of GPR55 in the neuro- and peripheral immune systems can be observed in Figure 1.7.

**Figure 1.7. GPR55 receptor actions on peripheral immune cells and glia.**

GPR55 receptor activation can modulate a variety of processes associated with peripheral immune cells and glia including activated phenotype, proliferation and migration. Figure adapted from Haugh et al. (2016).
1.1.10. The role of GPR55 in neurodegeneration

Evidence for the role of GPR55 in neurodegenerative processes is limited, but research is beginning to emerge on a potential modulatory role. Janefjord et al. (2014) employed the use of an *in vitro* model of AD and reported that the putative ligand for GPR55, O-1602, reduced the formation of aggregated Aβ fibrils in cell-free conditions, as assessed using a thioflavin T (ThT) assay to confirm fibril formation. O-1602 also reduced activation of the microglial BV2 cell line in response to LPS. When LPS-conditioned media was removed from BV2 cells and applied to neuronal cells, it was found that the application of O-1602 led to decreased neurotoxicity compared to control treatments where no agonists were applied.

GPR55 promotes neuroprotection in a rat organotypic hippocampal slice culture (OHSC) model of excitotoxicity. Microglia were proposed to mediate this neuroprotective effect because GPR55 siRNA and microglial depletion led to amelioration in the protective effects of LPI following NMDA-induced lesion of OHSC (Kallendrusch et al., 2013). LPI has also been shown to be neuroprotective in a model of global cerebral ischemia. Blondeau et al. (2002) reported that LPI prevented neuronal death in an *in vitro* model of excitotoxicity using primary cultures of cerebellar granule cells exposed to high extracellular concentrations of glutamate. In an *in vivo* rat model of transient global ischemia (achieved by four-vessel occlusion), intravenous (i.v.) injection of LPI both before and after ischemic insult protected against CA3 and CA1 pyramidal cell loss.

Due to the high expression levels of GPR55 in the basal ganglia (Sawzdargo et al., 1999), it could be speculated that GPR55 may have a modulatory role in Parkinson’s disease (PD) models. Celorrio et al. (2017) demonstrated that striatal expression of GPR55 mRNA was downregulated in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and probenecid (MPTPp) murine model of PD, but not in the substantia nigra. This suggests that GPR55 might be
involved in the pathology of PD. Chronic intra-i.p. administration of the putative GPR55 agonist, Abn-CBD, caused greater preservation of striatal tyrosine-hydroxylase (TH; a catecholamine marker) positive neurons in MPTPp mice. Levels of dopamine and its metabolites were tested in MPTPp mice in response to Abn-CBD treatment. Abn-CBD failed to alter the levels of dopamine or its metabolites. However, Abn-CBD-treated MPTPp mice performed better in a pole test and rotarod test (both measures of motor ability) than untreated MPTPp mice. The improved motor performance induced by Abn-CBD was therefore not linked to dopamine levels or the density of dopaminergic neurons in the nigrostriatal pathway. CBD (putative GPR55 antagonist) and Abn-CBD administration to MPTPp animals also produced a significant decrease in microglial cell density. Abn-CBD also displayed anti-cataleptic effects in the haloperidol-induced catalepsy mouse model. Haloperidol is a dopamine D2 receptor antagonist and its use in mice allows for the manifestation of rigidity and catalepsy, symptoms that are reflective of the motor difficulties experienced by PD patients. In a bar test, Abn-CBD significantly decreased the time mice stayed on the bar relative to the haloperidol-treated mice that received the vehicle alone. This effect was inhibited by CBD and PSB1216, a newly synthesized GPR55 antagonist. Furthermore, two other novel GPR55 agonists (CID1792197 and CID2440433) replicated the anti-cataleptic effects of Abn.-CBD. These results implicate GPR55 in having a potential therapeutic role in PD. However, in an exploratory randomised, double-blind, placebo-controlled study, the putative GPR55 agonist, SR141716/rimonabant, was well tolerated but could not improve parkinsonian motor disability in human patients (Mesnage et al., 2004).

A role for GPR55 in Multiple Sclerosis (MS) has also emerged. MS is an inflammatory autoimmune disease that is characterised by demyelination and chronic neurodegeneration (Perry et al., 2003). Rahimi et al. (2015b) demonstrated that the peripheral administration of the putative GPR55 ligand, PEA and CBD were found to attenuate inflammation,
demyelination, axonal damage and inflammatory cytokine expression in the myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE) mouse model of MS. Treatment with either CBD or PEA during disease onset also reduced the severity of the neurobehavioral scores of EAE. Interestingly, these individual ligand effects on neurobehavioural scores did not occur upon their co-administration, reflecting a potential antagonistic effect of CBD on PEA. However, their administration both individually and together reduced pro-inflammatory cytokine and factor expression (TNF-α, IL-17, IFN-γ), indicating potential ligand bias depending on the mechanism. In contrast, Sisay et al. (2013) found that GPR55 knockout mice cross-bred onto the C57BL/6 background did not develop EAE as severely as their control counterparts. Furthermore, female GPR55−/− mice were notably more resistant to EAE induction than male mice, indicating a gender difference in disease onset. Interestingly, CB2 receptor knockout mice exhibited increased disease severity. This alludes to a possible GPR55-CB2 cross-talk mechanism that may alter EAE disease progression and implicate GPR55 in potentiating neurodegenerative disease progression. However, these effects of GPR55 and CB2 deletion were not apparent in mice with an ABH background, indicating that strain background may influence the effect of these receptors on EAE severity. Indeed, the authors noted that C57BL/6 are less susceptible to EAE than ABH mice. These two studies are the only ones to date that, to the best of my knowledge, attempt to elucidate the role of GPR55 in models of MS. However, their conflicting results make it difficult to discern the true function of GPR55 in models of MS, so additional studies are required.

Due to the suggested modulatory role GPR55 has in neuroimmune and neurodegeneration conditions (Haugh et al., 2016), it is worth exploring its therapeutic potential in these conditions. This will be far more achievable now that selective pharmacological tools for GPR55 have been established.
1.2. Alzheimer’s disease

There are an estimated 46 million people worldwide suffering with dementia and this number is set to triple by 2050 due to an increasingly aged population (Prince et al., 2015). Dementia is a general term used to describe a range of chronic neurodegenerative disorders which lead to symptoms such as memory loss, cognitive impairment and a decline in physical capacity over time. There are different types of dementia, including vascular dementia, dementia with Lewy bodies (DLB), frontotemporal lobar dementia (FTLD), mixed dementia, Creutzfeldt-Jakob disease (CJD) and Parkinson’s disease (PD), to name a few (Stefaniak & O’Brien, 2015). The most common type of dementia is Alzheimer’s disease (AD), accounting for 60-70% of cases. Alzheimer’s disease is characterised by the deterioration of memory and cognitive function. The cause of AD is currently unknown, but the number of studies investigating the associated pathological hallmarks and risk factors associated with this disease has increased in recent years.

Alzheimer’s disease was first characterised in 1906 by the Bavarian psychiatrist and neuropathologist Alois Alzheimer. He initially described the condition the following year in 1907 in a short paper entitled “A characteristic serious disease of the cerebral cortex”. In this paper Alzheimer described the behavioural and pathological presentations of one of his patients, Auguste D. Alzheimer first encountered Auguste D. in 1901, where she had been admitted for mental asylum after showing signs of progressive memory loss, personality changes and psychosocial impairment over a few short years. She died shortly before her 56th birthday. Alzheimer requested her records and brain following her death and discovered an extensive loss of neurons and the presence of “fibrils” that showed “characteristic thickness and peculiar impregnability” in her brain. He also noted “the storage of a peculiar substance in the cerebral cortex”. These features would later be recognised as tangles and plaques
respectively, the characteristic pathological hallmarks of AD. The condition would only be named after Alzheimer in 1910, when his mentor Emil Kraepelin named it after him in a chapter on "Presenile and Senile Dementia" in the 8th edition of his *Handbook of Psychiatry*. Alzheimer's initial pathological characterisation of the disease is generally still used today (Cipriani *et al.*, 2011). AD is known to begin manifesting in the entorhinal cortex before spreading to other regions. The lateral entorhinal cortex (LEC) is thought to be a gateway to the hippocampus, an area that plays a crucial role in memory consolidation and learning (Wilson *et al.*, 2013). Khan *et al.* (2014) demonstrated using cerebral blood volume (CBV)-functional magnetic resonance imaging (fMRI) that the entorhinal cortex and other cortical regions such as the parietal cortex show dysfunction in pre-clinical human AD. Interestingly, the parietal cortex is believed to be involved in cognitive functions such as attention, naming or goal-orientated behaviour, which deteriorate during AD progression (Jacobs *et al.*, 2012). Khan *et al.* (2014) then investigated the features responsible for this spread from the LEC. Using three mouse models of AD that expressed abnormal tau or abnormal amyloid precursor protein (APP), or both, they determined how tau and APP expression are linked to LEC dysfunction. Using CBV-fMRI, their findings implicated that tau alone, but not APP alone, can ultimately cause LEC dysfunction and that APP expression acts to exacerbate tau toxicity in driving LEC dysfunction and subsequent spread to the perirhinal cortex and posterior parietal cortex. In addition, the cerebral cortex and ventricles of the brain shrink as AD progresses. A schematic representing the progression of AD in the brain can be viewed in Figure 1.8.
1.2.1. Subtypes of Alzheimer’s disease

There are two types of AD, early-onset AD (~5% of patients) and late-onset AD (~95% of patients). Familial AD (FAD) is typically early-onset and accounts for only 1% of Alzheimer’s cases and has a strong genetic component. Sporadic AD (SAD) in contrast is typically late onset and is far more common than FAD. FAD is believed to be caused by mutations in three genes – amyloid precursor protein (APP), presenilin 1 and presenilin 2.
(PSEN1, PSEN2) genes (Calero et al., 2015). APP encodes a ubiquitously expressed transmembrane glycoprotein known as amyloid beta (Aβ) A4 protein. It is believed that this protein serves as a cell surface receptor and performs a variety of physiological functions on the cell surface of neurons in order to facilitate neurite outgrowth, neuronal adhesion and axon guidance (Nicolas & Hassan, 2014). The 37–43 amino acid Aβ is generated by proteolytic processing from its precursor, APP, in a physiologically normal pathway. APP processing can be amyloidogenic or non-amyloidogenic. The non-amyloidogenic APP processing pathway involves cleavages by α- and γ-secretases resulting in the generation of a long secreted form of APP (sAPPα) and C-terminal fragments (CTF 83, p3 and APP intracellular fragment domain-50 (AICD50)). The amyloidogenic APP processing pathway involves cleavages by β- and γ-secretases into the 40- to 42-amino-acid fragment of Aβ. APP is first cleaved by β-secretase to form the amino terminus of Aβ. The aspartic protease β-site APP-cleaving enzyme 1 (BACE1) is believed to be the authentic β-secretase enzyme (Vassar, 2004). APP is first cleaved by BACE1 into a soluble extracellular component called sAPPβ and an intracellular C-terminal end called C99. C99 is then cleaved by γ-secretase to produce Aβ fragments of varying lengths. Aβ fragments oligomerise and fibrillise leading to AD pathology (Tabaton & Tamagno, 2007). The integral membrane proteins PSEN1 and PSEN2 are structurally similar to APP and are part of the γ-secretase complex responsible for the cleavage of APP into Aβ. Up to 50% of early-onset AD cases have been reported to carry mutations in APP, PSEN1 and PSEN2 (Kowalska, 2004). These mutations are suggested to be associated with the accumulation of pathogenic Aβ peptide in the brain and it is this peptide that aggregates to form oligomers and ultimately the macroscopic plaques that form one of the hallmarks of AD pathology. Indeed, enhancing α-secretase activity in animal models of AD or in cultured cells can significantly lower Aβ generation and even amyloid plaque formation (Haass et al., 2012; Postina et al., 2004). The generation of Aβ from APP is detailed in Figure 1.9.
The vast majority of AD cases are late-onset or sporadic. Unlike in FAD, there is not as much of a genetic propensity to inherit SAD (Tanzi, 2012). However, there are heritable genetic risk factors that increase the likelihood of a person developing the disease. Polymorphisms in the apolipoprotein E gene (APOE) lead to the production of ε4 variants (as opposed to the more common ε3 isotype) which increase susceptibility to developing SAD by four-fold if one copy is inherited and ten-fold if two copies are inherited (Tambini et al., 2015; Tanzi, 2012). In contrast, the rarer ε2 allele confers neuroprotective effects by

Figure 1.9. Proteolytic processing of APP into pathological fibrillar Aβ.
Genetic risk factors such as mutations in APP and PSEN1/2 can result in the generation of pathological fibrillar species of Aβ. Figure adapted from a template.
lowering the risk of disease and increasing the age of onset. A longitudinal study that followed newly diagnosed AD patients over the course of a 1-6 year period found that APOE4/4 patients had a faster rate of cognitive decline, whereas the presence of the APOE2 allele slowed disease progression (Craft et al., 1998). It is unclear exactly why polymorphisms in APOE result in an increased likelihood to develop SAD. APOE has a role in lipid metabolism and clearance in the normal brain and in Aβ clearance in AD. Perhaps the APOE4 isoform somehow causes Aβ aggregation and its improper clearance in the brain (Holtzman, Herz, & Bu, 2012).

1.2.2. The pathological hallmarks of Alzheimer’s disease

Aβ peptides are 39-43 amino acid residue peptides and proteolytic cleavage of APP generates soluble Aβ1-40 and hydrophobic Aβ1-42 isoforms (Figure 1.9). Both isoforms circulate in the cerebrospinal fluid (CSF) and interstitial fluid (IF) of the brain, with Aβ1-40 being produced more-so than Aβ1-42 (Barage & Sonawane, 2015). Normal levels of Aβ production do not induce AD pathology. However, when increased levels of Aβ are produced this leads to their aggregation into insoluble oligomers and protofibrils. These aggregations make up the senile plaques characteristic of AD. The production of increased levels of Aβ and subsequent aggregation into senile Aβ plaques is described under the “Amyloid Cascade Hypothesis”. This hypothesis was first proposed in the early 1990s with the idea that amyloid deposits are the driving force in both familial and sporadic AD (Hardy & Higgins, 1992). Plaques contain both Aβ1-40 and Aβ1-42, but the latter aggregates at a faster rate and its biochemical properties favour aggregation (Barage & Sonawane, 2015; De-Paula et al., 2012).

Despite the common association between Aβ plaque levels and AD pathogenesis, it has been argued that it is not Aβ aggregations themselves that induce the neurotoxicity observed in
AD. For instance, the degree of dementia does not correlate with the number of plaques (Terry, 1996). Deposition of senile Aβ plaques leads to increased microglial activation and astrogliosis and increased cytokine and reactive oxygen species (ROS) production (Barage & Sonawane, 2015). ROS are normal by-products of mitochondrial metabolism of oxygen, but excessive ROS production can lead to oxidative stress in neurons that can ultimately lead to neurotoxicity (Atlante et al., 2001). It is these responses, along with reduced clearance of Aβ from the brain, that are suggested to cause synapse loss, neuronal loss and cerebral atrophy in AD (De-Paula et al., 2012; McGeer & McGeer, 1995).

Another main pathological feature of AD is the formation of intraneuronal fibrillary protein tangles. These tangles are made up of a microtubule protein known as tau. Tau is a proteolytic degradation product of the human microtubule-associated protein tau (MAPT) gene. It is a heat stable protein that is crucial for microtubule assembly (Andreadis, Brown, & Kosik, 1992) and it promotes the polymerisation of tubulin protein into microtubules under normal conditions (Herzog & Weber, 1978).

Under normal physiological conditions tau protein is phosphorylated at six to eight phosphorylation sites on the shorter isoforms of tau in the immature brain and at two to three sites in all six isoforms of tau in the adult brain. This is a developmental process that is regulated from foetal to adult stages (Guo, Noble & Hanger, 2017). Tau is hyperphosphorylated at the microtubule binding domain (consisting of four sequence repeats of serine, threonine and proline) by glycogen synthase kinase 3β (GSK3β), cyclin dependent kinase 5 (CDK5) and ERK2/MAPK. Hyperphosphorylation of tau reduces binding ability to microtubules and subsequent microtubule assembly (Mazanetz & Fischer, 2007). Increased levels of tau hyperphosphorylation are associated with AD pathogenesis (Gong & Iqbal, 2008) and abnormal levels of phosphorylation are found in the brains of AD patients and other tauopathies (Johnson & Stoothoff, 2004). Tauopathies are a class of neurodegenerative
disorders characterised by neuronal and/or glial inclusions composed of abnormal tau (Irwin, 2016). Aberrant tau phosphorylation along with a reduction in tau dephosphorylation can result in microtubule-assembly disruption and subsequent decreased microtubule binding (Abraha et al., 2000), tau filament formation and aggregation (Lu & Wood, 1993) and an increase in neuronal cell death (Fath et al., 2002). Aggregates of phosphorylated tau are known as neurofibrillary tangles (NFT) and first appear in the entorhinal cortex prior to any signs or symptoms of AD. NFTs then progress in a hierarchical pattern, distributing to the hippocampus and neocortex (Braak & Braak, 1991). Early changes in NFT development involve granular deposits of abnormally phosphorylated tau present in the neuronal cytoplasm. These deposits become progressively more fibrillar and fill the entire neuron with dense bundles of tau until ultimately the neuron dies and the filaments are left in the neuropil. However, NFTs cannot be fully associated with neurodegeneration in AD because the CA1 pyramidal neuron loss (~60%) identified in a stereological study of AD patients showed that NFTs accounted for only a small proportion of this loss (2.2–17.2%, mean 8.1%; Kril et al., 2002). Other non-NFT-mediated mechanisms of neurotoxicity may therefore be responsible for the neurodegeneration that occurs in AD progression.

1.2.3. Neuroinflammation in Alzheimer’s disease

Prior to the 1990s, the brain was thought to be an immune-privileged site in the body, with structures such as the blood-brain barrier (BBB) and blood-cerebrospinal fluid (CSF) barrier protecting it from peripheral immune responses. Evidence has since emerged showing that the CNS is not immune-privileged and forms a bi-directional communication network with the peripheral immune system, meaning that it is susceptible to damage and infection (Galea et al., 2007). In lieu of such threats, the immune system as well as cells in the nervous system
such as oligodendrocytes, astrocytes, the endothelial cells of the cerebrovasculature and even neurons, release inflammatory mediators such as cytokines and chemokines. Cytokines are small proteins that act as signalling molecules to regulate inflammatory responses and modulate cellular activities including growth, cell survival, proliferation and differentiation. Cytokines can be either pro- or anti-inflammatory and bind to corresponding cytokine receptors. Chemokines are a type of cytokine that primarily function in inducing cell migration to sites of injury (Ramesh et al., 2013). Microglia mediate the central innate immune response. Microglia are the resident macrophage-like cells of the brain and spinal cord. Under normal physiological circumstances they remain inactivated and function by surveying their surroundings for pathogens or damaged cells. They are immunologically quiescent under these conditions, expressing low levels of major histocompatibility complex (MHC) class I and II molecules as well as co-stimulatory molecules such as Cluster of differentiation (CD)-40 and CD80 (Yang et al., 2010). Microglia are activated in response to CNS insult or injury. They develop an amoeboid morphology and become increasingly phagocytic, motile (Gehrmann et al., 1995) and proliferative (Ponomarev et al., 2005). The BBB is formed by microvascular endothelial cells, which are surrounded by basement membranes, pericytes and astrocytes which function to protect the CNS from outside threats. Through the release of cytokines, chemokines and ROS, microglia mobilise the adaptive immune response and can induce cell chemotaxis, allowing for peripheral immune cells such as leukocytes to transmigrate across the endothelia of the BBB to the site of insult or injury (Ramesh et al., 2013). There are several routes that leukocytes can use to enter into CNS, including migration from the microvessels into parenchymal perivascular space, migration via the choroid plexus into the CSF and migration through post-capillary venules at the pial surface into subarachnoid and Virchow-Robin perivascular spaces (Takeshita & Ransohoff, 2012).
Neuroinflammatory processes are crucial in maintaining normal brain health and function. A key pathological feature of AD is the development of neuroinflammatory changes mediated by microglia. This phenomenon was described by Alois Alzheimer himself a century earlier (Town, 2010). Activated microglia attempt to confer neuroprotection by clustering around fibrillary Aβ plaques and releasing pro-inflammatory cytokines e.g. interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) in the AD brain (Lynch, 2014). However, neurodegeneration is associated with microgliosis, astrogliosis and cerebrovasculature disturbances. During normal aging, microglia develop dystrophic processes and abnormally cluster around innocuous threats (Solito & Sastre, 2012). They also show impaired phagocytic activity in a mouse model of AD (Krabbe et al., 2013). This implies that microglial mechanisms of Aβ clearance decline in effectiveness as AD progresses. This alteration in microglial phenotype during aging and disease leads to the development of an inherent inflammatory phenotype (Norden & Godbout, 2013). Prolonged pro-inflammatory cytokine and ROS release by activated microglia leads to oxidative stress and neurotoxicity. This chronic stimulation of neuroinflammation in response to Aβ and NFT accumulation in AD can therefore exacerbate neurodegeneration.

Astrocytes are the most abundant glial cell in the brain and under normal conditions they function in providing neurotrophic support and structure (Sofroniew & Vinters, 2010). Astrogliosis is a predominant pathological feature of AD and it has been suggested that astrocytes may bridge the gap between Aβ deposition and cerebrovascular disturbances. Likewise, astrocyte dysfunction may induce pathological features of AD such as excitotoxicity (Avila-Muñoz & Arias, 2014). This evidence shows that the neuroinflammatory response in AD is a double-edged sword. Glial cells in the brain mediate neuroprotection by helping to maintain homeostasis and protect the brain against insult and injury. However, their chronic activation in response to Aβ plaques and NFTs appear to exacerbate
neurodegeneration processes. To combat AD progression, a critical balance between repair and pro-inflammatory factors must therefore be maintained.

1.2.4. Neurodegeneration mechanisms in Alzheimer's disease

Aβ plaques contribute to the induction of oxidative stress and apoptosis in neurons, subsequently leading to their death. Extensive research has been carried out by the Campbell research group to investigate an in vitro rat cortical neuron model of Aβ toxicity. A key event in AD pathogenesis is the release of pro-inflammatory cytokines by glia and neurons and the induction of neuronal apoptosis in response to increases in intracellular calcium ([Ca$^{2+}$]$_i$). Ca$^{2+}$ increases are suggested to contribute to dysregulation of cell homeostasis (Mattson, 1994; Peng & Jou, 2010). MacManus et al. (2000) demonstrated that Aβ triggered a significant enhancement of Ca$^{2+}$ influx into rat cortical synaptosomes and primary cortical neurons. Aβ-evoked increases in [Ca$^{2+}$]$_i$ stimulate Ca$^{2+}$-dependent calpain proteases. Calpains are suggested to contribute to neuronal cell death by cleaving DNA repair enzymes, thereby promoting DNA fragmentation (Boland & Campbell, 2003). However, increases in Ca$^{2+}$ influx were ameliorated by IL-1β, suggesting that IL-1β may confer a protective effect against Ca$^{2+}$-induced apoptosis in an in vitro model of Aβ toxicity (MacManus et al., 2000). The lysosomal system is also important in AD as its dysfunction is thought to contribute to AD pathology (Nixon et al., 2000). Lysosomes are membrane-bound organelles that are responsible for the degradation of macromolecules. Lysosomes contain a variety of proteolytic enzymes. Cathepsin proteases are one such example and are divided in to subgroups depending on the amino acids present at their catalytic sites: cysteine (cathepsins B, C, F, H, K, L, N, O, S, T, U, W and X), aspartyl (cathepsins D and E) and serine cathepsins (cathepsins A and G) (Zhang et al., 2009). Aβ increases the expression activity of cathepsin-L in primary rat cortical neurons. Apoptotic events such as capsase-3 activation
and DNA fragmentation in response to Aβ were inhibited by a cathepsin-L inhibitor (Boland & Campbell, 2004). The tumour suppressor protein p53 is a mediator of apoptosis and an increase in p53 expression has been reported in AD patients (Kitamura et al., 1997). Aβ causes phosphorylated p53 to associate with the lysosomal compartment. This leads to lysosomal membrane destabilisation and an increase in cytosolic cathepsin-L activity (Fogarty et al., 2010). c-Jun N-terminal kinase (JNK)-1 is vital for the stabilisation of its substrate p53 during Aβ-mediated induction of apoptotic processes such as caspase-3 activation and DNA fragmentation (Fogarty et al., 2003). These studies demonstrate the range of downstream factors that mediate Aβ-induced toxicity.

The Campbell group have also shown that cannabinoids alter apoptotic processes in rat cortical neurons. Δ9-THC signalled via CB1 to evoke lysosomal instability via p53 as an early event in the apoptotic cascade (Downer et al., 2007; Gowran & Campbell, 2008), which is a downstream effect that Aβ also induces (Fogarty et al., 2010). Interestingly, while the phytocannabinoid Δ9-THC promotes neurodegenerative processes, endocannabinoids confer neuroprotection. Direct applications of 2-AG and AEA, along with pharmacological enhancement of AEA tone with the FAAH inhibitor URB597, all protect against Aβ-induced neurotoxicity. AEA mediated its effects via CB1 and a CB2 receptor antagonist failed to inhibit either AEA or 2-AG (Noonan et al., 2010). AEA and URB597 also augment signalling of the pro-survival Notch-1 pathway in neurons exposed to Aβ, with AEA mediating its effects via CB1 (Tanveer et al., 2012). These studies highlight the therapeutic potential of modulating the ECS in models of Aβ toxicity.

1.2.5. Treatments against Alzheimer’s disease
Despite an increasingly aged world population and higher prevalence rates, there is no cure for AD at present. Treatments have largely focused on treating symptoms rather than potential causes and underlying pathology. There is therefore an unmet clinical need to develop new therapies that can resolve the disease pathology. The first initial move towards therapy development came in the 1970s, where it was discovered that cholinergic neurons underwent severe degeneration during this condition. The activity and level of ACh synthesising (choline acetyltransferases) and degrading (acetylcholinesterases; AChE) enzymes in the limbic and cerebral cortices is altered in AD and is associated with a loss of cholinergic cell bodies in these regions. Following this discovery, efforts were maximised to develop pharmaceuticals capable of preventing or reversing this problem through the augmentation of ACh levels. This led to the development of AChE inhibitors. The first to be introduced in 1993 was a drug called tacrine. However it had very serious adverse effects such as hepatotoxicity in clinical trials so was withdrawn. There are currently three AChE inhibitors approved for use to treat mild to moderate AD called rivastagmine, donepezil and galantamine (Godyń et al., 2015; Selkoe, 2001). AChE inhibitors have proven efficacy in 3 major domains in AD patients: activities of daily living, behavior and cognitive function (Small & Bullock, 2010). On full assessment and diagnosis of AD, initiation of an AChE inhibitor treatment is recommended as early as possible, since not all patients respond to treatment (30–40%; McGlennon, Dynan & Passmore, 1999). Therefore, implementing AChE inhibitors during mild stages of AD may preserve higher cognitive function and help to preserve quality of life and independence (Small & Bullock, 2010), although it has been shown that patients with severe AD tend to respond better to AChE inhibitor therapy than those with mild disease (Schneider & Farlow, 1996).

Another approved drug used to treat moderate to severe AD is memantine. Glutamate is the main excitatory neurotransmitter in the CNS and overstimulation of NMDA receptors by
glutamate leads to excitotoxicity, which is a common feature of neurodegenerative disorders. Since memantine shows moderate affinity as a voltage-dependent and uncompetitive antagonist of the NMDA receptor it maintains the normal physiological function of the receptor at synapses. Memantine is believed to contribute to symptomatic improvement in moderate to severe AD patients by maintaining the neurological, behavioural and memory consolidation functions that normal NMDA receptor activation induces, while simultaneously preventing the excessive influx of Ca\textsuperscript{2+} associated with excitotoxicity (Rogawski & Wenk, 2006). Memantine has been considered for the treatment of mild to moderate AD but it has not yet been approved for this. Randomised controlled trials of memantine in the treatment of mild to moderate AD have demonstrated small treatment effects in measures of cognition, global assessment and behaviour. However, these effects were not significant compared to placebo (Cosman, Boyle & Porsteinsson, 2007).

AChE inhibitors and memantine work by improving the symptomology of AD but have little effect on the underlying pathology and progression of the disease. New treatments that halt or delay the onset of early pathological processes are therefore attractive options as they will help to combat the manifestation of AD symptoms. However, AD cannot be formally diagnosed until post-mortem examination. Identification of biomarkers that have diagnostic and prognostic value in the early stages of AD are therefore crucial. A method of quantification of amyloid deposition in the brain using neutrally charged derivatives of thioflavin-T were developed by Klunk et al. (2001, 2004) at the University of Pittsburgh, Pennsylvania. The resultant Pittsburgh Compound B (PiB) is used in conjunction with positron emission tomography (PET) for \textit{in vivo} identification of cortical amyloid burden. This technique is widely known as PiB PET imaging. This technique can be used in conjunction with anti-\textit{A}\textsubscript{\textbeta} therapies to document treatment efficacy. Indeed, an active immunisation strategy was initially developed, whereby transgenic mouse models of AD were
immunised with Aβ. This led to a reduction in Aβ accumulation in the brain (Schenk et al., 1999) and associated cognitive impairment (Wilcock et al., 2009). This active immunisation strategy was carried into Phase I human clinical trials, whereby a small number of people with no health conditions were administered with the antibody to determine its safety and pharmacokinetic profile (Norfleet & Cox Gad, 2009). However, the use of the immunisation vaccine against Aβ (called AN-1792) was halted in Phase IIA, which tests the safety and efficacy of the vaccine in a small cohort of patients (Friedman, Furberg & DeMets, 2010), following the diagnosis of aseptic meningoencephalitis in 6% of immunised subjects (Ferrer et al., 2004; Gilman et al., 2005; Nicoll et al., 2003; Orgogozo et al., 2003). Despite this negative outcome, the neuropathological data obtained showed that the vaccine did in fact succeed in reducing plaque burden in AD patients. Efforts were then re-directed towards developing a passive immunisation therapy. Passive immunisation involves the peripheral administration of antibodies that target the corresponding antigen (Jicha, 2009). One antibody called bapineuzumab was first developed by the pharmaceutical companies Élan and Wyeth. Bapineuzumab is a humanised monoclonal antibody that targets the Aβ peptide and it reached Phase III clinical trials (whereby its therapeutic effect was determined in a large cohort of patients) in 2007. However, no benefit on cognitive or functional effects were observed for bapineuzumab over placebo in humans with mild to moderate AD. Due to these disappointing results, both phase III i.v.and phase II subcutaneous trials of bapineuzumab were halted (Kerchner & Boxer, 2010). Trials for a range of different anti-Aβ antibodies have also failed, including the humanised monoclonal antibodies Solanezumab (Eli Lilly & Co.) and Gantenerumab (Roche) in Phase III. Interestingly, Roche initiated a new Phase III program for Gantenerumab in July 2018 for patients with prodromal to mild AD. Phase III trials for Crenezumab (AC Immune SA, Genentech, Hoffmann-La Roche), BAN2401 (Biogen, Eisai Co., Ltd) and Aducanumab (Biogen) are currently ongoing (“Therapeutics”, ALZFORUM, n.d.).
Immunisation strategies against neurofibrillary tau aggregates are currently ongoing. Since tau lesions correlate better with the degree of dementia than Aβ plaques, their clearance may be more efficacious than targeting Aβ in AD. Pedersen & Sigurdsson (2015) reviewed the active and passive tau-based company immunotherapy programs that have progressed to clinical development. They noted however that total tau may never be completely eliminated because there is a heterogeneous mix of phosphorylated and unphosphorylated tau as well as various tau fragments in the aging brain. There is also a risk of potential toxicity associated with antibody-mediated clearance of normal tau protein. However, researchers have been refining their approach to tau immunotherapy trials. UCB Biopharma and Janssen reported that their therapeutic antibodies against the mid-region of tau halt the spread of abnormal tau species in cell and mouse models. These antibodies, UCB00107 and JNJ-63733657, entered Phase I testing in 2018 (“2018—A Year in Research”, ALZFORUM, n.d.).

Aβ oligomers also bind to receptor for advanced glycation end products (RAGE), which is a multi-ligand member of the Ig superfamily of cell surface molecules found on neurons, microglia, astrocytes and cells of vessel wall. Its expression increases in regions of rich Aβ expression. The use of soluble RAGE to intercept the endogenous RAGE-Aβ interaction was found to improve learning, memory and synaptic function in a transgenic mouse model of AD (Yan et al., 2009). Indeed, vTv Therapeutics developed a RAGE antagonist, Azeliragon, which progressed to Phase III trials in 2015. However, this trial was terminated in 2018 (“Therapeutics”, ALZFORUM, n.d.).

A key pathological hallmark of AD is the stimulation of neuroinflammation in response to Aβ deposition and NFT accumulation. These neuroinflammatory responses are neuroprotective because they contribute to aggregate clearance. However, chronic stimulation of this response has been shown to exacerbate neurodegeneration and AD progression (Krause & Müller, 2010). The enzymes cyclooxygenase (COX)-1 and COX-2 are
expressed in the brain and they function to generate pro-inflammatory prostaglandins from membrane-derived arachidonic acid. These enzymes themselves are also upregulated by pro-inflammatory factors (Krause & Müller, 2010). The peroxidase activity that results from COX-mediated catalysis of prostaglandins results in the generation of ROS and free radicals, which further contribute to oxidative stress and neurotoxicity. COX inhibition has been shown to induce neuroprotective effects (Willard et al., 2000). Non-steroidal anti-inflammatory drugs (NSAIDs) e.g. ibuprofen, function by inhibiting COX enzyme expression. Anti-inflammatory therapy using NSAIDs may therefore be a suitable for treatment of AD. Pioneering epidemiological evidence was presented in the 1990s showing that arthritic patients who chronically treated their condition with NSAIDs reduced the incidence or severity of AD (Rogers et al., 1993; McGeer et al., 1996). These findings were confirmed by later epidemiological studies (Stewart et al., 1997; Etminan, 2003). Sadly, trials investigating the effect of COX-2 inhibitors in the progression of AD have to date not had encouraging outcomes. Rofecoxib (Reines et al., 2004) and celecoxib (Martin et al., 2008) are selective COX-2 inhibitors and both failed to slow AD progression over placebo in late-stage AD patients. Interestingly, another COX isoform, COX-3, is enriched in the mammalian CNS (Cui et al., 2004) and inhibited by the analgesic, paracetemol (acetaminophen; Botting & Ayoub, 2005). This is encouraging because paracetamol can readily cross the BBB (Chandrasekharan et al., 2002), unlike NSAIDs, indicating it may have therapeutic potential in the neuroinflammatory aspects of conditions such as AD.

Based on the accumulated findings of previous studies, anti-inflammatory therapies show potential as an early-intervention strategy in the treatment of AD. Identification of inflammatory AD biomarkers could therefore be vital in the early initiation of treatment. For instance, s100B is secreted by astrocytes and its levels are increased during inflammation (Galasko & Montine, 2010). Indeed, increased levels of s100B were identified in the CSF of
mild-to-moderate AD patients, but not in severe AD, compared to healthy controls (Peskind et al., 2001). Certain cytokines and chemokines were increased in the CSF of patients with mild cognitive impairment (MCI) compared to healthy controls, which indicates that activation of their signalling pathways occurs early in the clinical presentation of AD. Examples include IL-8, IL-1 receptor type II and IL-18 (Galasko & Montine, 2010). Concrete candidates for potential AD biomarkers will need to be tested in larger studies, particularly in people at risk of AD by virtue of age or genetic risk factors.

1.2.6. Limitations of Alzheimer's disease research

With an increasingly aged global population, the incidences of AD are on the rise. Despite this, there have been no new treatments approved for market in the past 15 years. This is because many recent therapies, such as the ones discussed in the previous section, have failed upon reaching clinical trials. Indeed, clinical trials for AD therapies have the highest failure rate in the biomedical research field (99.6%). This can partly be blamed on the use of animal models for pre-clinical research. These models run the risk of not effectively translating into therapeutic success in human patients (Pistollato et al., 2016).

Animals models of AD include transgenic (Tg) and inbred mice, which are generated in order to express the pathological features of AD at abnormal levels. A triple Tg mouse model was generated to replicate neuroanatomical aspects of AD. These mice express human Aβ, PSEN and tau protein. However, despite presenting with known pathological features of AD e.g. Aβ formation and plaques, NFT, gliosis and neurodegeneration, these animals do not develop the cognitive and pathological complexities of human AD. In fact, overexpression of mutant protein may induce cognitive defects even before the onset of AD pathology (Dodart et al., 2002). Furthermore, these animals were developed to replicate the
pathology of FAD. This itself is problematic because the majority of AD cases are sporadic, meaning these models are not reflective of the dominant form of human AD.

An example of this mismatch in rodent-to-human translation involves the initial development of Aβ antibodies. Anti-Aβ antibodies were found to inhibit Aβ peptide fibrillation and oligomerisation and prevent cell culture-based neurotoxicity. Furthermore, vaccination of Tg AD mice with Aβ₁₋₄₂ or Aβ homologous peptides co-injected with FCA, demonstrated striking reduction in Aβ deposition and, concomitantly, a reduction in behavioral deficits (Wisniewski & Sigurdsson, 2010). However, when the Aβ vaccine, AN-1792, was tested in clinical trials, it led to termination in Phase IIA when 6% of vaccinated patients manifested symptoms of acute meningoencephalitis (Ferrer et al., 2004). Although Aβ load was reduced in AD patients (like it was in the Tg animal models), the clinical cognitive benefits were very modest compared to the placebo group (Gilman et al., 2005). This failure could be linked to the fact that the root cause of AD remains under debate to this day. No one factor is thought to be responsible for its onset, rather a number of contributing factors e.g. Aβ, NFT, inflammation, are thought to lead the neurodegeneration and cognitive deficits observed in the disease. Targeting more than one of these hallmarks at a time may therefore prove to be more effective than targeting a singular hallmark.

Apart from biological markers, other factors such as lifestyle also contribute to AD progression. Indeed, a Canadian Study of Health and Aging reported that regular and high levels of physical exercise are associated with a decreased risk of dementia and AD (Lindsay et al., 2002). A healthy diet is also linked to having a beneficial effect in reducing the risk factors associated with AD. This diet consists of a high intake of plant-based foods, antioxidants, soy beans, omega-3 polyunsaturated fatty acids (n-3 PUFA) and low intake of saturated fats and refined sugars (Pistollato et al., 2018). Changing diet and physical activity could therefore attenuate the progression of AD.
Going forward, it is clear that researchers will need to move away from rodent models to human-based approaches in order to develop effective therapies. The use of non-animal based techniques are becoming increasingly implemented in the study of AD. Neuroimaging studies e.g. MRI, PET, have been critical in the identification of similarities in neuroanatomical features shared by AD, type-2 diabetes and the metabolic syndrome (Pistollato et al., 2015). Patient sample analysis e.g. CSF, plasma, blood, is useful for the identification of potential biomarkers (Galasko & Montine, 2010). The collection of late-onset, sporadic AD patient-derived fibroblasts for the generation of AD patient induced-pluripotent stem cells (iPSCs) will be useful in elucidating late-onset AD pathology, which represents the majority of AD cases (~95%) compared to early-onset AD (~5%; Reitz, Brayne & Mayeux, 2011). However, all of these techniques come with their own limitations, including expense and data variability (Pistollato et al., 2016). Increasing funding towards the use of human-based studies would therefore be beneficial and hopefully lead to the identification of more therapeutic targets suitable for intervention, not discounting the ones already undergoing trial.

1.2.7. GPR55: a potential therapeutic target for Alzheimer's disease?

GPR55 is believed to have a modulatory role in neuroinflammation and neurodegeneration (Haugh et al., 2016; Yang et al., 2016). It is highly expressed in microglia (Pietr et al., 2009), the innate immune cells of the brain. It has a suggested regulatory role in nervous system disorders such as MS, excitotoxicity and cerebral ischemia and neuropathic pain (Blondeau et al., 2002; Gangadharan et al., 2013; Kallendrusch et al., 2013; Sisay et al., 2013; Staton et al., 2008). The putative GPR55 agonist, O-1602, reduced the formation of aggregated Aβ fibrils in cell-free conditions and reduced activation of the microglial BV2 cell line in response to
LPS (Janefjord et al., 2013). GPR55 is thought to have a regulatory role in promoting synaptic plasticity (Hurst et al., 2017; Sylantyev et al., 2013), a process that is severely compromised by the progression of AD. Indeed, degeneration of synapses appears to be an early event in AD pathogenesis, with synapse loss evident in patients with early AD and MCI (Masliah et al., 2001; Scheff et al., 2007). The Campbell research group has shown that stimulation of the ECS has a modulatory role in an in vitro cortical neuron model of Aβ toxicity (Gowran, Noonan & Campbell, 2011). Furthermore, the elevation of endocannabinoid levels (Van der Stelt et al., 2006), the administration of synthetic selective CB₁ or CB₂ receptor agonists (Haghani et al., 2009; Wu et al., 2013), non-specific CB₁ or CB₂ receptor agonists or CBD (Fakhfouri et al., 2012; Martín-Moreno et al., 2011) reduced memory impairment in Aβ-injected rodents. GPR55 is a putative cannabinoid receptor (Brown & Wise, 2002; Ryberg et al., 2007) and its stimulation has the potential to induce modulatory effects in this model of Aβ toxicity. Given that the present development of novel treatments for AD seem to focus more on tackling the early neuroinflammatory aspect of the condition, this makes GPR55 an ideal therapeutic candidate for treating the neuroinflammatory events present in AD.
HYPOTHESIS & OBJECTIVES

GPR55 responds to certain cannabinoid ligands along with other endogenous lipids. Its most potent endogenous ligand to date is believed to be LPI. Studies to date have largely utilised overexpressing cell systems to study GPR55 pharmacology and function, but the need for endogenous models is becoming increasingly vital in order to delineate its true physiological role. For instance, GPR55 appears to have a key regulatory role in the immune system and in inflammation. Evidence for its role in the brain has only recently begun to emerge and there are limited studies that have specifically investigated its role in neurodegeneration.

HYPOTHESIS

GPR55 modulates neuronal and immune cell signalling and function.

OBJECTIVES

The objectives of this research project were as follows:

1. To investigate the role of GPR55 in the regulation of intracellular Ca\(^{2+}\) and CREB phosphorylation in neurons and glia.
2. To investigate the role of GPR55 in microglial function and neuronal apoptosis.
3. To investigate GPR55 expression and function in human peripheral immune cells.
Chapter 2

Materials & Methods
2.1. Materials

2.1.1. Trinity College Dublin, Dublin, Ireland

*Cell culture: Plasticware and general reagents*

**Abcam, Cambridge, United Kingdom**

- Normal goat serum, Cat #ab7481
- Normal horse serum, Cat #ab7484

**Corning, Wiesbaden, Germany**

- Cell strainer, 40 μm, 50/pk, blue, Cat # CLS431750-50EA
- Falcon™ Easy-grip Bacteriological Petri Dishes with Lid (30 mm), Cat #353001
- Surfactant-free Cellulose Acetate (SFCA) Syringe Filters, 0.2 μm, 25mm, 50/pk, Cat #431219

**EZBiolab, Carmel, Indiana, USA**

- Aβ1-40 peptide, 5 mg, Cat #amp40-m5

**Merck Millipore Ltd., Watford, UK**

- Millicell® hanging cell culture insert, polyethylene terephthalate, 8.0 μm, 48/pk, Cat #MCEP24H48

**Sarstedt, Wexford, Ireland**

- Pipette tips (no filter): 0.1-10 μL (Cat #70.1130); 20-200 μL (Cat # 70.760.002); 200-1000 μL (Cat # 70.762).
TC plates, standard, welled: 6-well plates (Cat # 83.3920); 24-well plates (Cat #83.3922.005)

**Sigma-Aldrich, Dorset, UK**

- Calcium Chloride, 250 mL, Cat # 21115-250ML
- Carboxylate-modified polystyrene, fluorescent yellow-green beads, Cat #L4655
- Dimethyl Sulphoxide (DMSO), 250 mL, Cat #276855-250ML
- D-Glucose, 1 kg, Cat # G8270-1KG
- HEPES, Cat #90909C
- Magnesium Sulphate, 1 kg, Cat #M7506-1KG
- Paraformaldehyde (PFA), 500g, Cat #158127-500G
- Poly-D-lysine hydrobromide, 5mg, Cat # P6407-5MG
- Phosphate Buffered Saline, 10X, Cat # D1283-500ML
- Phorbol 12-myristate 13-acetate/12-O-Tetradecanoylphorbol-13-acetate (TPA), 1mg, Cat #P1585-1MG
- Penicillin (10,000 U)/streptomycin (10 mg) per mL, 100 mL, Cat #P4333-100ML
- Sodium Hydroxide, Cat #276855-250ML

**Sparks Lab Supplies, Dublin, Ireland**

- Abdos microcentrifuge tubes, PP, 1.5 mL, 500/pk, Cat #AB-P10202
- Abdos Falcon tubes: 15 mL (Cat # AB-P10401, 500/pk); 50 mL (Cat # AB-P10403, 500/pk)
- Autoclave tape, Cat #AT019
- Clarity microscope slides, 1.0/1.2mm,76 x 26mm, frosted, 50/pk, Cat #U-MS106
- Robust Blue Nitrile Gloves, Box of 100, Cat # SAF93897X10
Thermo Fisher Scientific, Waltham, MA, USA

- B27 supplement, 50X (Gibco®), 10 mL, Cat #17504044
- Dulbecco Modified Eagle Medium/F-12 (Gibco®), 500 mL, Cat #21331020
- Dulbecco Modified Eagle Medium (Gibco®), high glucose, 500 mL, Cat #11965092
- GlutaMAX™ (Gibco®), 100 mL, Cat # 35050061
- Heat inactivated foetal bovine serum, US origin (Gibco®), 500 mL, Cat #16140071
- Nalgene™ Cryo 1°C freezing container, Cat # 5100-0001
- Neurobasal Medium®-A (Gibco®), 500 mL, Cat #A2477501
- Trypsin-EDTA 1X (0.25%), 100 mL, Cat # 25200056

Tocris Bioscience, Bristol, UK

- Fura 2-acetoxymethyl (AM) ester, 1 mg, Cat #2220

Vector Laboratories, Peterborough, UK

- Biotinylated goat anti-rabbit secondary antibody, Cat #BA-1000
- Biotinylated rabbit anti-goat secondary antibody, Cat #BA-5000
- Biotinylated horse anti-mouse secondary antibody, Cat #BA-2000
- Vectashield mounting medium (no DAPI), 10 mL, Cat #H-1000

VWR International, Leicestershire, UK

- Coverglasses, round, 13 mm, No.1 thickness, Cat #631-0149
- Dow Corning® high-vacuum silicone grease, 50g, Cat #291-0039
- Potassium Chloride, 500g, Cat #0395-500G
- Magnesium Chloride, 100g, Cat #J364-100G
- Sodium Chloride, 500g, Cat #27810.262
2.1.2. GlaxoSmithKline, Stevenage, UK

Antibodies

Cayman Chemicals

- GPR55 polyclonal antibody, 500 µL, Cat #CAY10224-1 ea

Novus Biologicals

- Anti-human HM74A/GPR109A Mab (IgG2b; Allophycocyanin (APC)-conjugated) 500 µg, Cat #MAB2760
- Rat IgG2b Isotype Control (APC-conjugated), 500 µg, Cat #MAB0061

Culture materials

Gibco (Life Technologies), Paisley, UK

- β-Mercaptoethanol, Cat #21350010
- Dulbecco’s Phosphate Buffered Saline (-MgCl₂/-CaCl₂), Cat #14190094
- Isocove’s modified Dulbecco’s medium (IMDM), Cat #21980032
- L-Glutamine, Cat #25030081
- MEM Non-Essential Amino Acids (100x), Cat #1140035
- Penicillin/streptomycin (10,000 units/mL), Cat #15140122
- Recombinant Human IFNγ, 100 µg, Cat #PHC4031

Greiner Bio-One, Gloucestershire, UK

- Leucosep tubes, Cat #227290
Leo Laboratories Ltd, Berkshire, UK

- Heparin, Cat #PL0043/0149

Miltenyi Biotec, Surrey, UK

- Human granulocyte-macrophage colony-stimulating factor (GM-CSF), Cat #130-093-867
- Human interleukin-4 (IL-4), Cat #130-093-922

Sigma-Aldrich, Dorset, UK

- Bovine serum albumin (30%), Cat #A9576-50mL
- Ethylenediaminetetraacetic acid (EDTA), Cat #E7889-100mL

Thermofisher scientific, Waltham, MA, USA

- Heat-inactivated-foetal bovine serum (US origin), Cat #10082147

Equipment

- Hela Cell 204 cell incubator
- Olympus IX50 Microscope (Inverted)
- SAFE 2020 ThermoFisher Flowhood
2.2. Cell culture

2.2.1. Aseptic technique maintenance

In order to achieve successful cell cultures, a sterile work environment and proper adherence to anti-microbial techniques must be maintained at all times. This helps in the prevention of microbial infections of cell cultures and the areas that come into contact with cell culture. The following procedures were strictly adhered to for all cell culture procedures.

2.2.2. Sterilisation of glassware, plastics and dissection instruments

Pipette tips, microcentrifuge tubes and cryovials were placed into empty and lidded pipette tip boxes. Solutions such as phosphate-buffered saline (PBS) and double deionised water (ddH2O) were poured into glass bottles and sealed with a lid. The lids of both the boxes and bottles were further sealed with autoclave tape and autoclaved at 145°C for 30 minutes (min; Systec 3850 MIV, Unitech, Dublin, Ireland) in order to achieve sterilisation. Prior to use, all dissection instruments were sonicated (VWR International) for 15 min to break up blood residues from prior dissections and placed in a sterilising oven (Sanyo-Gallenkamp Hotbox Oven, Model #OHG050, Loughborough, UK) set at 180°C for overnight baking.

2.2.3. Sterilisation of work environment

All cell culture work was carried out in a laminar flow hood (Astec-Microflow laminar flow workstation, Florida, USA). The sterile environment is maintained in this hood due to a downward flow of air filtered through high efficiency particle air (HEPA) filters located at
the top of the hood. This airflow creates a barrier between the flowhood and the open and non-sterile environment in front of the hood, thus preventing the entry of airborne microbes and contaminants into the flowhood. The filters were allowed to run for 15 min before commencing any culture work and remained on throughout the procedure. The surface of the laminar flowhood was wiped with tissue paper doused with 70% absolute alcohol, along with accessible areas. Disposable nitrile powder-free gloves were worn at all times and sprayed with 70% absolute alcohol before placing the gloved hands inside the laminar flow hood. The gloves were changed at regular intervals to prevent contamination of materials within the hood. Movements were kept to a minimum while working to prevent disturbance of the protective airflow barrier provided by the laminar flowhood.

At the end of each day, all bench areas were wiped down with 70% absolute alcohol and the laminar flowhood was exposed to ultraviolet (UV) light overnight. The cell culture incubator used in this study (Binder CO₂ incubator, series CB, Binder GmbH, Tuttlingen, Germany) has a self-sterilisation function, which was utilised every 2 weeks. The entire culture room was thoroughly cleaned every week and the whole room was exposed to an overhead source of UV light for a minimum of 24 hrs.

2.2.4. Reagents and medium formation

All supplements used for cell culture were sterile filtered into plain, unsupplemented medium using 0.2 µm surfactant-free cellulose acetate (SFCA) syringe filters attached to a sterile syringe. Neurobasal-A culture medium (Gibco, ThermoFisher Scientific, MA, USA) was used for maintaining primary cortical neuron-enriched cultures. It was supplemented with penicillin/streptomycin (P/S; 100 units/mL; Sigma-Aldrich, Dorset, UK) and GlutaMAX (2 mM; Gibco, ThermoFisher Scientific, MA, USA) and B27 supplement (2%; Gibco,
ThermoFisher Scientific, MA, USA). Dulbecco’s Modified Eagle Medium (DMEM; high glucose, 4500 μg/mL) was used for the culture of BV2 microglial cells. It was supplemented with either 10% or 2% foetal bovine serum (FBS; Gibco, ThermoFisher Scientific, MA, USA), P/S (100 units/mL) and GlutaMAX (2 mM). DMEM F-12 was used to culture HEK293 cells. It was supplemented with 10% FBS, P/S (100 units/mL) and GlutaMAX (2 mM). FBS was used because it contains a complex mixture of different factors and contains a large number of constituents such as growth factors, proteins, vitamins, trace elements, hormones, etc. that are essential for the growth and maintenance of cells (Van der Walk et al., 2010). All types of medium containers were only opened within the laminar flow hood to ensure sterility.

2.2.5. Waste disposal

All hazardous material (lab plastic, sharps, gloves and carcasses) was separated into appropriate UN-approved primary packaging and sent to the hazardous material facility (Trinity College Dublin) where it was disposed of in accordance with Irish and EU legislative requirements (Annex III to Directive 2008/98/EC), which lists the properties of waste which render it hazardous.

2.3. Preparation of cortical neuron-enriched cultures

2.3.1. Coating coverslips with poly-D-lysine
Glass coverslips (13 mm; VWR international, Leceistershire, UK) were removed from their packaging, placed in tinfoil and baked in a sterilising oven at 180°C overnight. The next day, the tinfoil parcel containing the coverslips was placed in a laminar flowhood, unwrapped and the coverslips were individually laid out in tissue culture (TC-)-treated welled plates. Poly-D-lysine (50 µg/mL in sterile ddH$_2$O; Sigma-Aldrich, Dorset, UK) was decanted using a pipette onto each coverslip. The coverslips were incubated with poly-D-lysine at 37°C for a minimum of 1 hr. The poly-D-lysine was then aspirated off the coverslips and the coverslips were washed x3 with ddH$_2$O. The lid was left off the plates so that the coverslips could air-dry in the laminar flowhood for ≥2 hrs. The plates were then wrapped in parafilm and stored at 4°C until needed or for a maximum of 2 weeks.

2.3.2. Animals

Postnatal 0-2-day-old (P0-P2) male or female Wistar rats (specified-pathogen free) were born at the Comparative Medicine Animal Facility (Trinity College Dublin, Dublin 2, Ireland). Parent animals were maintained under a 12 hr light/dark cycle at an appropriate temperature of 22-23°C. On the mornings of neuronal cell culture preparations, the pups were removed from a sterilised litter cage and carefully placed in to a ventilated box containing clean bedding. The animals were then transported to the Trinity College Institute of Neuroscience and were kept warm until dissection.

2.3.3. Dissection procedure

Primary forebrain neurons were obtained from P0-P2 male or female Wistar rats. Sex hormones influence neurite outgrowth and synaptogenesis in certain areas of the rat brain.
during neonatal development. These alterations are suggested to induce changes in brain structure and function between the sexes (Lustig et al., 1993). P0-P2 rats were used in the current study because this is still an early age in rat development, thus attempting to ensure that the brains were not yet significantly altered by the different sex hormones. It has been reported however that dimorphic differences occur in rat cortical neurons, with the total number of surviving neurons obtained from neonatal rats being significantly higher in female-derived cultures after 14 days in vitro (DIV) than in male-derived cultures (Zhang et al., 2003).

The rats were decapitated using a large sterile scissors. The skull was exposed by carefully cutting through skin in a straight line from the neck to the bridge of the nose using a smaller sterile scissors and pulling the flaps of skin backwards. The skull was removed by cutting along either side of it using a small sterile scissors and using a sterile forceps to pull it back and away, thus exposing the brain. The meninges were removed from the cerebral hemispheres using a sterile curved forceps. The cerebral hemispheres (not the brainstem or cerebella) were removed using a sterile curved forceps and placed in a sterile petri dish (Särstedt Ltd., Wexford, Ireland) containing sterile 1x PBS (10 mM PO₄³⁻, 137 mM NaCl and 2.7 mM KCl; pH 7.4). The olfactory bulbs were removed along with any remaining meninges using a sterile curved forceps and the hemisphere tissue was chopped into smaller pieces of tissue using a sterile scalpel razor blade.

2.3.4. Dissociation procedure

Using a sterile Pasteur pipette (VWR International, Leicestershire, UK), the chopped cerebral hemisphere tissue was aspirated and transferred into a sterile 15 mL conical tube and incubated with trypsin-ethylenediaminetetraacetic acid (EDTA; 0.25% (v/v)) for 3 min at
37°C with 5% CO₂ and 95% O₂ in a humidified chamber (Binder CO₂ incubator, series CB, Binder GmbH, Tuttlingen, Germany). Following trypsin dissociation of the tissue, the tube was removed from the incubator and a solution DMEM F-12 with 10% FBS was applied to the tissue. The FBS in the culture medium contains protease inhibitors, such as α1-antitrypsin and α2-macroglobulin, which cease the trypsination process and act by inhibiting lysosomal peptidases that may be released (Melnick & Wallis, 1975). The tube was inverted repeatedly and centrifuged at 2000 rpm for 3 min (Centrifuge Model 2-16K, Sigma-Aldrich, Dorset, UK). The supernatant was discarded and DMEM-F12 (10% FBS) was applied again. The tissue was then triturated repeatedly using a sterile Pasteur pipette and passed through a sterile mesh filter (40 µm; ThermoFisher Scientific, MA, USA). The tissue was centrifuged at 2000 rpm for 3 min at 20°C. The supernatant was aspirated off and the pellet was re-suspended in warm (37°C) neurobasal culture medium supplemented with P/S (100 units/mL), GlutaMAX (2 mM) and B27 supplement (2%(v/v)). B27 supplement is a serum-free medium that has antioxidant properties and promotes neuronal survival, while also attenuating glial growth (Brewer et al., 1993; Huang et al., 2000). It is recommended for use at 2% concentration (Brewer at al., 1993).

2.3.5. Plating of resuspended cells and maintenance of culture

The resuspended neurons in culture medium (with 2% B27) were pipetted onto the centre of each poly-D-lysine-coated coverslip in each well of a 24-well plate at a density of 0.25 x 10⁶. Cell density was measured using a haemocytometer. From 1 mL of total cell suspension, 2 µL was retrieved and mixed gently using a pipette in a microcentrifuge tube with 8 µL of 0.4% Trypan Blue (final concentration 0.32%). Trypan blue does not penetrate live cells, so live cells should remain clear in colour (Strober, 1997). The trypan blue-cell suspension (10
μL) was then applied to the haemocytometer. The total live cells were counted from each corner containing 16 squares, then added together and multiplied by 5 (to account for the 1:5 trypan blue dilution), then multiplied by $10^6$. This calculation determines the viable cells/mL in the original cell suspension. The cells were allowed to settle and adhere to the coverslips for 3 hrs in a humidified atmosphere of 37°C with 5% CO$_2$ and 95% O$_2$. The cells were then flooded with 300 μL of culture medium supplemented with B27.

The cells were monitored daily. The original culture media was not replaced because cell culture protocols often recommend not removing all of the original culture medium when maintaining cell cultures (see Babu et al., 2011 for an example) in order to preserve the growth factors produced during cell development e.g. basic fibroblast growth factor (bFGF; Morrison et al., 1986; Ray et al., 1993). The culturing protocol in this current study was optimised to ensure the cells remained as healthy as possible during development. The addition of B27 helped to ensure a high density of neurons compared to glia (Brewer et al., 1993). Optimisation of the culturing process can be viewed in the Appendix (Supplementary Section S1).

Cultured neurons were monitored daily using a light microscope (Nikon TMS, Nikon Instech Co., Ltd., Kanagawa, Japan). Sample images of cultured neurons at (i-i) 1 DIV and (iii-iv) 7 DIV can be viewed at different magnifications in Figure 2.1. At 1 DIV, the cells are very rounded and it is difficult to identify cell types (see inset). By 7 DIV the cells have processes extending out from the oblong and almost pear-like cell bodies (see inset). It is assumed these are neurons because glia are incapable of forming synaptic networks like neurons do because they do not posses axons (LoTurco, 2000). These images were obtained using CellD software (Olympus, Tokyo, Japan).
Figure 2.1. Microscopic images of cells *in vitro* from cortical neuron-enriched cultures at different timepoints.

Images of cultured cells at 1 DIV and 7 DIV taken at (i, iii) 10x and (ii, iv) 20x magnification. Insets: close-up view of cells at different stages of development. Scale bar = 200 µm.
2.3.6. Cell types in forebrain culture.

Cells were isolated from intact cerebral hemispheres. Therefore they contained a mixture of neurons from all regions of the forebrain. These include the cerebral cortex, corpus callosum, basal ganglia, limbic structures (e.g. hippocampus, thalamus, hypothalamus) and pineal gland. The cerebrum is the largest part of the mammalian brain. Herculano-Houzel, Mota & Lent (2006) used an isotropic fractionator to determine the number of neurons in the brains of different rodent species. It was found that the rat brain contained 200 million neurons and the cerebral cortex contained roughly 17% of all neurons in the rat brain. There are 86 billion neurons in the average male human brain and of these there were 16 billion neurons located in the cerebral cortices (19%), 69 billion (80%) in the cerebella and roughly 1 billion (1%) in the collated remaining regions of the basal ganglia, diencephalon (thalamus, hypothalamus) and brainstem (Azevedo et al., 2009). The olfactory bulbs, cerebella and brainstem were removed during dissection in the present study, meaning that the cerebral cortices from the isolated hemisphere tissue contained the greatest number of neurons.

Although steps were taken to ensure neuronal purity during the dissociation of forebrain tissue, along with the culturing of cells with neurobasal medium containing B27 serum supplement, there was no guarantee that glial cells were not present in the cultures. To determine the ratio of neurons to glia, immunocytochemical staining was carried out for the neuronal dendrite marker MAP2 (red; Soltani et al., 2005), the astrocytic marker glial fibrillary acidic protein (GFAP, green; Hol & Pekny, 2015) and the nuclear marker Hoechst (blue; Crowley, Marfell & Waterhouse, 2016). An Axiovert 200M inverted confocal microscope (Zeiss, LSM-510-META, Carl Zeiss, Cambridge, UK) was used to examine the incorporated fluorophores (Figure 2.2).
Three representative images were taken from a culture and the number of MAP2-positive cells and GFAP-positive cells were counted. It was determined that of all the cells counted, MAP2-positive cells made up 80.29% and GFAP-positive cells made up the remaining 19.71%. The greatest number of neurons in our cultures were obtained from the cortex. Given that the cultures were also made up primarily of neurons, the cultures in the present study will be referred to as cortical neuron-enriched cultures. Cells of a microglial phenotype were not labelled, but previous findings suggest that mixed neuron-glial rat cortical cultures contain around 7% microglia, with the remaining 93% made up of neurons and astrocytes (Huang & Wang, 2015).
Figure 2.2. Molecular markers to differentiate cell types present in cortical neuron-enriched cultures.

Confocal images of cultured cells taken at 20x magnification. MAP2 = red (80% of cells), GFAP = green (20% of cells), Hoechst = blue. Scale bar = 20 µm
2.4. Primary culture of human monocyte-derived dendritic cells

2.4.1. Human blood samples

Fresh blood samples were donated by anonymous and healthy human donors on the morning of the culture. The required volume of blood had been requested prior to donation. Trained phlebotomists in the GlaxoSmithKline (Stevenage, UK) Blood Donation Unit (BDU) drew blood from the donors using venous puncture. Donors had to meet strict inclusion criteria in order to donate blood e.g. non-smokers and no medications permitted. Heparin (Leo Laboratories Ltd., Berkshire, UK) was added to the samples to thin the blood.

2.4.2. Peripheral blood mononuclear cell isolation

The samples were retrieved from the BDU and brought to the laboratory. There they were placed in a sterile laminar flowhood (SAFE 2020 Class II ThermoFisher Flowhood, ThermoFisher, Paisley, UK). The blood was poured in to a Leucosep tube (~30 mL per tube) and centrifuged at 400xg for 25 min at room temperature on the lowest brake setting (deceleration 3) to separate the blood by density gradient in to a plasma layer, a peripheral blood mononuclear cell (PBMC) layer and a red blood cell layer. The PBMC layers were carefully collected from each Leucosep tube using a Pasteur pipette and transferred to a fresh 50 mL Falcon tube.
2.4.3. CD14⁺ monocyte cell isolation

The PBMC layers were diluted 1:1 (equal volume) with PBS (no MgCl₂/CaCl₂) and the tubes were centrifuged at 400xg at room temperature for 10 min. The supernatants were discarded and the cells were washed by topping up the volume to 50 mL with PBS. A cell suspension was taken using a NucleoCounter Via1-Cassette™ and a cell count was performed using the NucleoCounter® NC-200™ high precision automated cell counter (ChemoMetec A/S, Allerod, Denmark). The cells were centrifuged again at 400xg for 10 min and the supernatants were discarded. The cell pellets were resuspended in cold MACS buffer (PBS, 0.5% BSA, 2 mM EDTA) at 80 µL per 1 x 10⁷ cells. CD14⁺ microbeads (Miltenyi Biotec, Surrey, UK) were added at 20 µL per 1x10⁷ cells. The cells were gently mixed with the beads using a pipette and incubated at 4°C for 15 min. After incubation, cold MACS buffer was added up to 50 mL and the suspension was centrifuged at 400xg for 10 min at room temperature. The supernatants were discarded and the cells were resuspended in 500 µL of cold MACS buffer per 1 x 10⁸ cells. LS separation columns were placed in magnetic separators on a magnetic stand (Miltenyi Biotec, Surrey, UK) and a 50 mL Falcon tube was placed underneath each column for the collection of CD14⁺ effluent. Each column was primed with 3 mL of cold MACS buffer. The cells were added to each separation column and allowed to pass through by gravity flow. The column was washed 3x3 mL with cold MACS buffer. The 50 mL Falcon tubes containing the CD14⁺ cells were removed and replaced with 15 mL Falcon tubes under each column. The columns were removed from the magnetic separators and each column nozzle was placed over the top of separate 15 mL Falcon tube openings. An additional 5 mL of cold MACS buffer was added to each column and the CD14⁺ cells were immediately flushed out of each column by firmly and quickly pushing a plunger into column. A cell suspension was retrieved using a Via1-Cassette™ and a cell count was obtained using the NucleoCounter. There were typically >10% CD14⁺
monocytes obtained from the original PBMC count e.g. \(1 \times 10^9\) PBMC \(\rightarrow\) \(\geq 1 \times 10^7\) CD14\(^+\) cells.

2.4.4. Human monocyte-derived dendritic cell (moDC) generation

Following the cell count, the CD14\(^+\) monocytes were centrifuged cells at 400xg for 10 min at room temperature. At this stage and if required, CD14\(^+\) cells could be plated out in 96-well plates for 24 hrs in order to proceed with a subsequent monocyte phagocytosis assay (see Section 2.12.2). However, in order to generate dendritic cells from the CD14\(^+\) monocytes, human interleukin (IL)-4 (final concentration 20 ng/mL) and granulocyte macrophage colony-stimulating factor (GM-CSF, final concentration 30 ng/mL; Miltenyi Biotec, Surrey, UK) were diluted in Iscove's Modified Dulbecco's culture medium (IMDM; supplemented with 10% FBS, 1% P/S, 2 mM L-glutamine, 1% MEM Non-Essential Amino Acids (MEM-NEAA) & 50 µM 2-Mercaptoethanol (ME)) and this IMDM was applied to the cell suspension. Both cytokines promote monocyte differentiation into dendritic cells (Sallusto & Lanzavecchia, 1994). The amount of cytokine-containing IMDM applied to the cells depended on an adjustment of the original CD14\(^+\) monocyte cell count, so that \(3 \times 10^6\) cells per well were ultimately seeded into the wells of a 6-well low-adherence plate (Corning, Flintshire, UK). For example, if the CD14\(^+\) cell count yielded \(9 \times 10^6\) cells/mL, then the cell suspension would be adjusted by diluting this suspension with 2 mL cytokine-containing IMDM in order to yield \(3 \times 10^6\) cells/mL (\(9 \times 10^6\) cells/3 mL). One mL of this adjusted suspension would then be seeded into 3 wells of the 6-well plate to yield \(3 \times 10^6\) cells per well.
The IMDM in the wells was topped up with more cytokine-containing IMDM to prevent the cells from drying out during the differentiation process.

The seeded cells were incubated for 3-5 days at 37°C and 5% CO₂ in a Hela Cell 204 cell incubator (ThermoFisher, Paisley, UK). The entire moDC isolation process and images of the cells *in vitro* can be viewed in Figure 2.3. Microscope images were obtained using an inverted Olympus IX50 Microscope (Olympus, Tokyo, Japan; Figure 2.3B).
Figure 2.3. Dendritic cell differentiation from human blood monocytes.

A: Process of isolating PBMC from human blood, selecting CD14⁺ monocytes and differentiating monocytes into moDC. Figure adapted from Hubo et al. (2013). B: Images of cultured cells in vitro taken at 20x magnification. (i) CD14⁺ cells in vitro 1 day after plating. (ii) By day 4, CD14⁺ cells should have differentiated into moDC. Inset: a potential dendritic cell. Note the dendritic processes extending from the cell body, as indicated by the red arrow. Scale bar = 100 µm.
2.4.5. moDC maturation

In order to mature moDC, a stimulating agent must be added to the culture medium. Dendritic cells are professional antigen-presenting cells (Banchereau & Steinman, 1998) and are semi-adherent in nature (Thurner et al., 1998). In the presence of IL-4 and GM-CSF, CD14+ monocytes should differentiate into moDC 2-5 days after seeding (Chapuis et al., 1997). After 3-5 days in vitro, the cells previously seeded into the 6-well plates were washed by gently aspirating and decanting the IMDM culture media in the wells to dislodge as many moDC as possible. Monocytes and macrophages are quite adherent in the presence of serum (Maoz et al., 1986) so should remain attached to the bottom of the wells. The dislodged cells were collected and decanted into a 15 mL Falcon tube. The cells were centrifuged at 400xg for 5 min at room temperature, the supernatant was discarded and the cells were resuspended in 10 mL of fresh culture medium. A cell count was taken on the NucleoCounter. Following cell differentiation into moDC, there were typically ~50% moDC from the original CD14+ monocyte count prior to seeding e.g. 1x10^7 CD14+ cells \( \rightarrow \geq 5 \times 10^6 \) moDC. The cells were centrifuged again for 5 min at 400xg at room temperature. The cells were adjusted to 4 x 10^6 cells/mL by resuspending in IMDM (without cytokines). The cell suspension (2 x 10^5 cells/50 µL) was pipetted into each well of a 96-well round low-adherence U-bottomed plate. More IMDM was added (50 µL) on top of this suspension containing x2 cytokines in order to create a 1:1 dilution of cytokines. Interferon-gamma (IFNγ) and LPS are capable of maturing moDC (Han et al., 2009). The moDC were incubated with IFNγ (75 ng/mL final concentration) and/or LPS (50 ng/mL final concentration) (either separately or together) at 37°C with 5% CO₂ in a humidified chamber for 24 hrs in order to induce maturation. The normal moDC differentiation factors, IL-4 (final concentration 20 ng/mL) and GM-CSF (final concentration 30 ng/mL), were applied
as well as the maturation agents. Following this period, the mature moDC were used for relevant assays such as the Prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) production assay.

2.5. Cell line culture

2.5.1. Human Embryonic Kidney 293 cell line

The Human Embryonic Kidney 293 (HEK293) cell line is derived from human embryonic kidney cells grown in tissue culture. This particular cell line was initiated by the transformation and culturing of normal HEK cells with sheared adenovirus 5 DNA. The transformation resulted in the incorporation of approximately 4.5 kilobases from the viral genome into human chromosome 19 of the HEK cells. Adherent HEK293 (HEK293-AD) cells are derived from the parental 293 cell line but are transfected with a gene that improves cell adherence (Graham et al., 1977). The health and confluence of adherent cells are easier to visually monitor than cells in suspension (“Adherent Cell Culture vs. Suspension Cell Culture”, ThermoFisher, n.d.).

Cryopreservation and resuscitation of HEK293 cell line

HEK293 cells used in this project were obtained from Life Technologies, Paisley, UK. They were cryopreserved at a concentration of 1 x 10\textsuperscript{6} cells/mL in Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 medium (DMEM/F-12; 10% heat-inactivated foetal bovine serum (FBS), 100 units/mL P/S, 2 mM GlutaMAX) with 5% dimethyl sulphoxide (v/v; DMSO; Sigma-Aldrich, Dorset, UK). Aliquots of the cell suspension were decanted into cryogenic vials and placed in to an isopropanol chamber (Nalgene™, Thermo Fisher Scientific, MA, USA). The chamber was placed into a -80°C freezer so that the temperature
of the chamber would drop -1°C per min overnight. The following day the cryovials were removed from the chamber and stored indefinitely at -80°C.

For HEK293 cell resuscitation, a cryovial containing frozen cell suspension was removed from the -80°C freezer. The outside of the vial was gently sprayed with ethanol and placed into an incubator set at 37°C until almost all of the solution inside had thawed. The vial was then removed from the incubator; the outside was again gently sprayed with ethanol and placed in a sterile laminar flowhood. The cap of the cryovial was opened and its contents were gently pipetted into a Falcon tube containing 10 mL of warm culture medium. The cell suspension was centrifuged at 2000 rpm for 5 min at 20°C. The supernatant was removed and the pellet was resuspended in 10 mL of fresh, warm DMEM/F-12 medium. The cell suspension was decanted into a 75cm²/T_75 flask (Corning, Wiesbaden, Germany). The flask was placed into a humidified incubator maintained at 37°C with 5% CO₂ and 95% O₂.

**Maintenance and subculture of HEK293-AD cell line**

HEK293-AD cells were cultured in DMEM/F-12 supplemented with 10% heat-inactivated FBS, 100 units/mL P/S and 2mM GlutaMAX™ and maintained at 37°C with 5% CO₂ and 95% O₂ in a humidified incubator. Cells were grown and maintained in T_75 flasks. The medium was removed (75% removal) and replaced with fresh DMEM/F-12 every few days until the cells had reached 80-90% confluency. To subculture cells, the culture medium in the flask was removed and the cells were washed with 1x PBS. The PBS was removed and pre-warmed 0.05% trypsin-EDTA solution was added to the confluent flask. The flask was gently tapped or rocked side to side to dislodge adherent cells. To aid the cells in lifting off, the flask was placed in an incubator set at 37°C for 1-2 min. Cells were checked under the microscope to examine rate of detachment. Once the cells had detached, pre-warmed culture
medium was added to the flask. The cell suspension inside was then poured in to a 50 mL Falcon tube. The cell suspension was centrifuged at 2000 rpm for 5 min at 20°C. The supernatant was discarded and the pellet was re-suspended in 1 mL of warm DMEM/F-12 medium. A 1:100 dilution of cell suspension was made. A cell count was carried out from this diluted cell suspension using a haemocytometer. Once the cell count was obtained, the remaining cell suspension was diluted with warm DMEM/F-12 medium to achieve a $1 \times 10^6$ concentration of cells. The cell suspension was then decanted in to new T$_{75}$ flasks and maintained at 37°C with 5% CO$_2$ and 95% O$_2$. For experimentation, HEK293 cells were seeded out at a desired concentration onto poly-D-lysine coated coverslips in culture plates or onto the bottom surface of culture plates. Cultured HEK293 cells were monitored daily using a light microscope (Nikon TMS, Nikon Instech Co., Ltd., Kanagawa, Japan).

**Stably expressing human GPR55 (hGPR55)-HEK293 cell line**

In this study, a HEK293 cell line that stably overexpresses human GPR55 was used (hGPR55-HEK293) to study the signalling effects of ligand activation at GPR55. These cells were first transfected and established by Henstridge *et al.* (2009, 2010) and were used in the current study. Briefly, human GPR55 cDNA was found to have a single amino acid substitution (R124C) with respect to the published sequence (European Molecular Biology Laboratory accession number BC032694). This mutation was corrected using site-directed mutagenesis (QuickChange; Stratagene) and subcloned into a plasmid construct DNA (pcDNA) 3.1 vector(+) with a neomycin selective marker. The receptor was tagged with a triple hemagglutinin epitope (HA) at the N terminus (3xHA-GPR55) preceded by an optimised artificial signal sequence from the human growth hormone (HGH; residues 1–33) to ensure efficient surface expression. The plasmid vector was transfected in to the HEK293
cells using Lipofectamine™ 2000 Reagent (Invitrogen, Life Technologies, Paisley, UK), which is a cationic liposome based reagent that confers high transfection efficiency and high levels of transgene expression in a range of mammalian cell types [*in vitro*] (Dalby *et al*., 2004).

hGPR55-HEK293 cells were cultured and maintained in the same manner as HEK293 cells. The main difference however involved the selection of hGPR55-expressing cells using G418 sulphate (400µg/mL G418 sulphate diluted in DMEM/F-12 medium). G418 sulfate is an aminoglycoside antibiotic produced by *Micromonospora rhodorangea*. G418 blocks polypeptide synthesis by inhibiting the elongation step in both prokaryotic and eukaryotic cells (Ursic *et al*., 1981). Resistance to G418 is conferred by the Neomycin resistance gene, which the pcDNA(+) 3.1 vector includes.

**Transiently-transfected mouse GPR55 (mGPR55)-HEK293 cell line**

To test GPR55 immunoreactivity in cells obtained from cortical neuron-enriched cultures, HEK293-AD cells were transiently transfected with x3-HA-mouse GPR55 cDNA to investigate comparative labelling. Due to the closeness of rodent genetic structure (Zhao *et al*., 2004), this will lead to similarities between antibody reactivities across both species. HEK293 cells were trypsinised at least one day before transfection and seeded onto poly-D-lysine-coated coverslips in a sterile petri dish. They were maintained in serum-containing DMEM/F-12 medium and incubated in a humidified incubator at 37°C with 5% CO₂. On the day of transfection, the medium was removed and replaced with serum-free medium. Separately, Lipofectamine™ 2000 (2 mg/ml) was diluted in OptiMEM® (1:50 dilution) in one tube. In a second tube cDNA (1 mg/ml) was diluted in OptiMEM (1:50 dilution). Both tubes were mixed by gently pipetting the mixture up and down several times and left for 5 min. The cDNA mix was then added to the Lipofectamine™ 2000 mix and again gently
pipetted up and down 4-5 times to mix well. The mixture was left for 20 min to allow time for cDNA-liposome complexes to form. The cDNA-liposome complexes were then gently applied to the cells. The cell/cDNA mix were then placed in a humidified environment and left overnight before the commencement of experiments.

2.5.2. Murine BV2 microglial cell line

The BV2 murine microglial cell line has extensively been used as an alternative to primary microglia in the study of neuroinflammatory and neurodegenerative processes (Stansley et al., 2012). This is an immortalised cell line that can be generated by infecting murine microglia with the v-raf/v-myc (J2) retrovirus (Blasi et al., 1990). Primary microglial cells responded to LPS stimulation with greater expression of pro-inflammatory cytokines in response to LPS than BV2 cells (Horvath et al., 2008). Similarly, primary microglia reacted stronger to LPS and therefore a much larger (10-fold) number of genes were significantly regulated. However, 90% of the genes induced in BV2 by LPS were also found in primary microglia (Henn et al., 2009). This indicates that BV2 cells appear to be a valid substitute for primary microglia in many experimental settings, but they both need to be compared to draw a conclusive result.

Cryopreservation and resuscitation of BV2 microglial cell line

BV2 murine microglial cells used in this project were gifted by the laboratory of Professor Marina Lynch, Trinity College Dublin. BV2 cells were cryopreserved at a concentration of 1 x 10⁶ cells in DMEM (10% FBS, 100 units/mL P/S, 2 mM GlutaMAX, 4500 mg/L glucose) and 5% dimethyl sulphoxide (v/v; DMSO; Sigma-Aldrich, Dorset, UK). Aliquots of the cell suspension were decanted into cryogenic vials and placed into an isopropanol chamber
(Nalgene™, Thermo Fisher Scientific, MA, USA). The chamber was placed into a -80°C freezer so that the temperature of the chamber would drop -1°C per min overnight. The following day the cryovials were removed from the chamber and stored indefinitely at -80°C.

For BV2 cell resuscitation, a cryovial containing frozen cell suspension was removed from the -80°C freezer. The outside of the vial was gently sprayed with ethanol and placed into an incubator set at 37°C until almost all of the solution inside had thawed. The vial was then removed from the incubator, the outside was again gently sprayed with ethanol and placed in a sterile laminar flowhood. The cap of the cryovial was opened and its contents were gently pipetted into Falcon tube containing 10 mL of warm DMEM medium. The cell-suspension was centrifuged at 2000 rpm for 5 min at 20°C. The supernatant was removed and the pellet was resuspended in 10 mL of fresh, warm DMEM medium. The cell suspension was decanted into a 75cm²/T75 flask (Corning, Wiesbaden, Germany). The flask was placed into a humidified incubator maintained at 37°C with 5% CO₂ and 95% O₂.

**Maintenance and subculture of BV2 microglial cell line**

BV2 cells were cultured in DMEM supplemented with 2% heat-inactivated FBS, 100 units/mL P/S, 2mM GlutaMAX and high glucose (4500 mg/mL) and maintained at 37°C with 5% CO₂ and 95% O₂ in a humidified incubator. Cells were grown and maintained in T_75 flasks. The medium was removed (75% removal) and replaced with fresh culture medium every few days until the cells had reached 80-90% confluency. To subculture cells, cells were dislodged from the bottom of the flask using a cell scraper. The suspension inside was then poured into a 50 mL Falcon tube. The cell suspension was centrifuged at 2000 rpm for 5 min at 20°C. The supernatant was discarded and the pellet was re-suspended in 1 mL of warm culture medium (with 2% FBS). A 2% dilution of FBS in DMEM was used in order to
minimise glial activation and maintain a quiescent cell phenotype (Kniss & Burry, 1988). Furthermore, serum-derived lipoproteins and other bioactive components such as sphingosine 1-phosphate (S1P) are capable of activating membrane surface proteins and proteases (Benaud et al., 2002) as well as Rho GTPases (Dupont et al., 2011). It was therefore crucial to attempt to eliminate the possibility of receptor activation and internalisation before experimentation. A 1:100 dilution of cell suspension was made. A cell count was carried out from this diluted cell suspension using a haemocytometer. Once the cell count was obtained, the remaining cell suspension was diluted with warm DMEM to achieve a $1 \times 10^6$ concentration of cells. The cell suspension was then decanted into new T-75 flasks and maintained at $37^\circ C$ with 5% CO$_2$ and 95% O$_2$. For experimentation, BV2 cells were seeded out at an appropriate concentration onto coverslips or into welled-plates. Cultured BV2 microglial cells were monitored daily using a light microscope (Nikon TMS, Nikon Instech Co., Ltd., Kanagawa, Japan).

### 2.6. Cell treatment

#### 2.6.1. Amyloid-beta 1-40 peptide

Amyloid-beta 1-40 ($A\beta_{1-40}$) peptide was obtained from EZBiolab Inc., (Carmel, IN, USA). The peptide was dissolved in 84% PBS and 16% double dH2O and allowed to aggregate for 48 hrs in a humidified chamber at $37^\circ C$, 5% CO$_2$ and 95% O$_2$. The peptide was made up as a 200 µM stock and stored at -20°C. Dr. Janis Noonan of our laboratory has previously carried out work to show that this method of aggregation results in primarily fibrillary $A\beta$ species, with some presence of oligomeric species (unpublished data).
Although in the literature it is more common that the Aβ$_{1-42}$ peptide is used to induce neurodegeneration in AD models, Aβ$_{1-40}$ peptide was used instead. Our lab have previously shown that Aβ$_{1-40}$ peptide induces neurotoxicity in cultured cortical neurons (Boland & Campbell, 2003). Similarly, aggregated Aβ$_{1-40}$ peptide results in a dramatic and rapid loss in cultured cortical neuron viability (Pillot et al., 1999) and also induces cortical lesions in both young and aged rats (Giordano et al., 1994).

For treatment of cells from cortical neuron-enriched cultures, Aβ was diluted to a final concentration of 10 µM in pre-warmed culture medium. For culture use, cells were initially exposed to ligands (diluted in medium) for 30 min without Aβ and following this 30 min period, this medium was removed and replaced with warm culture medium containing the same drug treatments and Aβ (10 µM). Control treatment groups were incubated in normal culture medium only.
2.6.2. CID16020046

CID16020046 (chemical name 4-(4-(3-hydroxyphenyl)-3-(4-methylphenyl)-6-oxo-1H,4H,5H,6H-pyrrolo(3,4-c)pyrazol-5-yl)benzoic acid) is a selective GPR55 antagonist (Kargl et al., 2013; Kotsikorou et al., 2011b) and was obtained from ChemDiv Inc., (San Diego, CA, USA). It is specific for both human and rodent GPR55 (Kargl et al., 2013; Stančić et al., 2015). The drug was dissolved in DMSO and stored as a 10 mM stock solution at -20°C. For culture use, the stock drug was diluted to a final concentration of 10 μM in pre-warmed culture medium. Cells were pre-treated with CID16020046 antagonist alone for 30 min prior to co-treatment with antagonist and agonist together. Control treatment groups were incubated in normal culture medium only. In calcium imaging experiments, the stock drug was diluted to a final concentration in HEPES-buffered saline (HBS).

2.6.3. L-α-lysophosphatidylinositol (LPI)

L-α-lysophosphatidylinositol (LPI) is the established endogenous agonist for GPR55 (Nevalainen & Irving, 2010; Yamashita et al., 2013) and was obtained as a sodium salt (from
soybean) from Sigma-Aldrich Company Ltd. (Dorset, UK). The most abundant fatty acid group species present in this salt are palmitic acid and stearic acid (58%), followed by linoleic acid (42%). Although the 2-arachidonyl species of LPI is the most efficacious at GPR55 (Oka et al., 2009) it is not available commercially so the soybean species was used. The salt was dissolved in double distilled water and stored as a 1 mM stock solution at -20°C. For culture use, the stock drug was diluted to a final concentration in pre-warmed culture medium. Control treatment groups were incubated in normal culture medium only. In calcium imaging experiments, the stock drug was diluted to a final concentration in HBS.

![Lipopolysaccharide (LPS) Structure](image)

2.6.4. Lipopolysaccharide (LPS)

LPS is a major component of the cell wall of gram negative bacteria. It activates Toll-like receptor 4 (TLR4) on the cell membrane of immune cells in order to stimulate immunogenic responses (Lu, Yeh & Ohashi, 2008). It was obtained as a ready-to-use aqueous solution of eBioscience™ LPS from *Escherichia coli* (*E. coli*; 2.5 mg/mL) 026:B6 (ThermoFisher, Paisley,
UK) and stored at -20°C. For culture use, a final concentration of 5 μg/mL was created in warm DMEM medium, as per the manufacturer’s instructions.

2.6.5. Nifedipine

Nifedipine is an L-type Ca\(^{2+}\) channel blocker and was obtained from the Merck Calbiochem® line of inhibitor products (Millipore, Watford, UK). The yellow solid was dissolved in DMSO and stored in darkness as a 1 mM stock solution at -20°C. For experimentation, cells were pre-treated with 10 μM nifedipine diluted in warm culture medium alone for 45 min prior to co-treatment with nifedipine and ligand together. Control treatment groups were incubated with nifedipine only.

2.6.6. Thapsigargin

Thapsigargin (TSG) is a plant-derived sesquiterpene lactone and is potent inhibitor of sarco-endoplasmic reticulum Ca\(^{2+}\)-ATPases (Thastrup, 1990). It was obtained from Tocris (Bristol, UK). The solid was dissolved in DMSO and stored as a 2 mM stock solution at -
20°C. In calcium imaging experiments, the stock drug was diluted to a final concentration of 5 μM in HBS.

2.6.7. 12-O-Tetradecanoylphorbol-13-acetate (TPA)

TPA is a phorbol ester and a protein kinase C (PKC) activator (Niedel et al., 1983). It was obtained from Sigma-Aldrich Company Ltd. (Dorset, UK). The ester was dissolved in PBS and stored as a 1 mM stock solution at -20°C. For culture use, the stock drug was diluted to a final concentration of 100 nM in pre-warmed culture medium. Control treatment groups were incubated in normal culture medium only.
2.6.8. 17g

N-((4-(N-Phenylsulfamoyl)phenyl)carbamothioyl)-[1,1′-biphenyl]-4-carboxamide (also known as 17g) is a selective GPR55 agonist with an N-phenyl group. It was gifted by the Nevalainen research group from the University of Eastern Finland (Yrjölä et al., 2016). Synthesised compound 17g (CAS 651297-31-3) is a known commercial compound that appears in the patent WO 2011022393. The compound will be referred to as 17g in this thesis. The drug was dissolved in DMSO and stored as a 1 mM stock solution at -20°C. For culture use, the stock drug was diluted to a final concentration in pre-warmed culture medium. Control treatment groups were incubated in normal culture medium only. In calcium imaging experiments, the stock drug was diluted to a final concentration in HBS.
2.7. Intracellular Ca\(^{2+}\) imaging of cells obtained from cortical neuron-enriched cultures

Intracellular calcium (Ca\(^{2+}\)) ions generate versatile intracellular signals that determine a large range of biological functions in almost all cell types in biological organisms, including neuronal cells (Grienberger & Konnerth, 2012). Calcium imaging employs the use of Ca\(^{2+}\) indicator dyes, such as the ratiometric dye bis-fura 2-acetoxymethyl (AM) ester (fura-2 AM), to detect ratiometric changes in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) at distinct wavelengths (Barreto-Chang & Dolmetsch, 2009). Fura-2 AM crosses cell membranes and once inside the cell it is converted to fura-2 by cellular esterases, which remove the AM group (Gryniewicz, Poenie & Tsien, 1985). Fura-2 that is unbound to Ca\(^{2+}\) is excited at 380 nm and fura-2 that is Ca\(^{2+}\)-bound is excited at 340/350 nm. The emitted light is measured at around 510 nm. By ratioing fluorescence intensities produced by excitation at two wavelengths, artifacts such as uneven dye distribution and cell number variation are minimised because they effect both measurements equally (Burger & Diehl, 2013). This is advantageous compared to other indicator dyes such as Fluo-4, which is non-ratiometric. However, due to it being a dual excitation dye, Fura-2 is not suitable for confocal microscopy (Paredes et al., 2008). Fura-2 Ca\(^{2+}\) imaging has previously been used by the Irving laboratory to investigate the GPR55-mediated release of Ca\(^{2+}\) ions from intracellular endoplasmic reticulum stores in a HEK293 cell line stably overexpressing GPR55 (Henstridge et al., 2009).
2.7.1. Preparation prior to Ca$^{2+}$ imaging experiments

On the day of experimentation, coverslips containing live cells from cortical neuron-enriched cultures aged between 7-10 DIV were washed once with 1x HBS (135 mM NaCl; 5 mM KCl; 1 mM MgCl$_2$; 2 mM CaCl$_2$; 10 mM HEPES; 10 mM Glucose; pH 7.4). The cells were loaded with fura-2 AM (Tocris, R&D Systems, Abingdon, UK) at 6 µM for 45 min-1 hr and were left to incubate in darkness at room temperature. The cells were washed x2 in HBS and left for 30 min to allow for the de-esterification of the AM groups.

2.7.2. Intracellular Ca$^{2+}$ imaging experimental procedure

In the current study, a digital epifluorescence imaging system mounted on an Olympus BX50WI microscope (Olympus, Tokyo, Japan) was used to measure changes in [Ca$^{2+}$]i signal. Prior to imaging an experiment, washing solution was perfused through the system using a perfusion pump. This was achieved by applying the solution to an open and empty petri dish mounted to a microscopic stage below the objective lens of the microscope. The tube opening was suspended above the dish, so the solution could decant gently onto the bottom of the open dish. The tubes were washed for 10 min with 70% ethanol, followed by a 10 min wash with distilled water. The solution was removed using another tube located on the other side of the objective lens that was attached to another perfusion pump. This tube removed the waste buffer and decanted it into a waste jar. The washed tubes were then perfused with 1x HBS for 10-15 min (to ensure any previous ethanol and water was washed out). The temperature of the buffer and apparatus was maintained at 30°C using a stage warmer connected by wire to a controlled heating system.
The empty petri dish was then removed and a single coverslip containing cells loaded with fura-2 AM was adhered to the bottom surface of another Falcon petri dish using high vacuum grease (Corning, ThermoFisher, Leicestershire, UK).

Cells were exposed to HBS for 10-15 min to obtain a baseline reading using MetaFluor software. They were excited at 350 and 380 nm simultaneously with emission detected above 500 nm. Cells were perfused with ligand solution typically for ~5 min at a rate of 2 mL/min. Recordings were made at 5 second intervals.

Experimental treatments were typically conducted as follows: firstly, cells were continuously perfused with 1x HBS for 10-15 min to obtain a baseline. Then (1) if only stimulant responses were being examined, stimulant solution was applied and allowed to perfuse over the cells to allow for an increase in Ca$^{2+}$ concentration, as visualised by a change in fluorescence ratio. Cells were then washed for 30 min with HBS to completely rid the tubing of stimulant solution before beginning another experiment with another coverslip; (2) if antagonist/inhibitor effectiveness was being examined, antagonist/inhibitor solution was applied and allowed to perfuse over the cells. Immediately after this, a solution was applied which contained both stimulant and antagonist/inhibitor. After this, a solution of inhibitor alone was again applied to inhibit any possible change in [Ca$^{2+}$]i that may have occurred during the prior period. The cells were washed for ≥30 min in HBS to rid the tubing of stimulant and inhibitor.

2.7.3. Differentiation of cell types using potassium chloride during live imaging

In this study primary cortical neuron-enriched cultures were used for Ca$^{2+}$ imaging. The serum-supplement B27 was used to promote neuronal purity in these cell cultures. However
B27 does not eradicate glial cells in culture, so low levels of glial contamination occur (<10%; Meberg & Miller, 2003). Potassium chloride (KCl) depolarises neurons which leads to the removal of Mg$^{2+}$ ions from the NMDA receptor channel and the subsequent activation of NMDA receptors (Macías et al., 2001). Ion influx through the NMDA receptor channel amplifies the depolarisation and causes the opening of L-type Ca$^{2+}$ channels along the dendrites which leads to an influx of Ca$^{2+}$ into the cell cytoplasm and subsequent depolarisation of neuronal cell membranes (Bading, Ginty & Greenberg, 1993). Glial cells outnumber neurons in the CNS but have traditionally been considered to be electrically inexcitable and play only a passive role in the electrical activity of the brain (Kuffler, 1967). Therefore KCl (50 mM) was applied at the end of a Ca$^{2+}$ imaging experiment using cortical neuron-enriched cell cultures to allow for the differentiation between neuronal and glial cells.

Cells were separated according to their KCl-responsiveness (i.e. neuron or glial cell; Figure 2.4). Each individual population was analysed for a statistically significant change in [Ca$^{2+}$]i in response to earlier experimental ligand application during the Ca$^{2+}$ imaging experiment. For the purpose of clarity, cell traces throughout the results section in the present study will have the KCl response omitted in order to give focus to ligand-induced responses.
2.7.4. Basal [Ca$^{2+}$]i profiles of neurons from cortical neuron-enriched cultures

In the current study, neurons (7-10 days old) exhibited one of two basal [Ca$^{2+}$]i profiles. Some neurons exhibited spontaneous [Ca$^{2+}$]i activity during baseline vehicle application. These spontaneous Ca$^{2+}$ events were abolished by the Na$^+$-channel blocker, tetrodotoxin (TTX, 500 nM; Lee & Ruben, 2008), but not irreversibly because the events restarted following TTX washout (See Figure 2.5(1)). Other neurons did not show spontaneous Ca$^{2+}$

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**Figure 2.4. Example traces of cells exposed to 50 mM KCl.**

Cells were separated into groups depending on their responsiveness to KCl i.e. responsive = neuron, unresponsive = glial cell. This allowed for statistical analyses of the responsiveness of each group to earlier experimental ligand application.
activity and were categorised as quiescent (Figure 2.5(2)). Both types of neurons were grouped and analysed separately for GPR55 ligand-responsiveness.

![Figure 2.5. Basal [Ca²⁺]i profiles of neurons from cortical neuron-enriched cultures](image)

Neurons were categorised depending on their basal [Ca²⁺]i activity: (1) neurons exhibiting TTX-sensitive spontaneous Ca²⁺ activity or (2) quiescent neurons. Red arrows signify spontaneous Ca²⁺ events. Scale: x-axis = time (min), y-axis = ratio units.

### 2.7.5. Processing and analysis of intracellular Ca²⁺ imaging data

To analyse the extent of cellular [Ca²⁺]i changes during an experiment, raw data were extracted from cellular recordings using MetaFluor® offline software (Molecular Devices Corporation, CA, USA). The background was subtracted from these recordings. The raw data were transferred to Origin 7 software (OriginLab Corporation, Stoke Mandeville, UK) where cell traces were created.
For neurons exhibiting spontaneous Ca\textsuperscript{2+} events: the frequency counts of events were measured. This was performed in Origin 7 by measuring the raw data from each cell 10 min immediately prior to ligand application (i.e. the baseline vehicle reading) and 10 min from the point of ligand application (see Figure 2.6A). The latter 10 min included part of the subsequent washout period to account for any latent ligand-induced responses. Events were counted by determining the minimum ratio unit reading of an event and applying this minimum value to all cellular raw data collected. Therefore any reading that was above this minimum value was counted as a spontaneous event and collated as the total number of events per cell during that time period. The baseline event frequency was then compared to event frequency during ligand application.

For quiescent neurons/glia: ratio changes were measured in Origin 7 by measuring the raw data from each cell 10 min immediately prior to ligand application (i.e. the baseline vehicle reading) and 10 min from the point of ligand application (see Figure 2.6B). The latter 10 min included part of the subsequent washout period to account for any latent ligand-induced responses. Changes in fluorescence ratio during ligand application were compared with baseline fluorescence ratio readings. An average max response height was calculated from cells from independent cultures.

The oscillation frequency and peak response height data from the Ca\textsuperscript{2+} imaging experiments were transferred to GraphPad Prism 5 software (GraphPad Software, Inc., CA, USA) in order to construct graphs and histograms and to perform statistical analyses.
Figure 2.6. Processing of response data for analysis.

**A:** Neurons exhibiting spontaneous Ca\(^{2+}\) activity are measured for total event frequency in response to ligands. The minimum threshold value that constitutes an event is calculated (see black box, Y = ratio unit) and any unit above this value is considered an event. This value is applied to all cellular ratio unit data during vehicle application and compared with event frequency during ligand application.

**B:** The peak/max response height during ligand application (see blackbox, Y = ratio unit) is compared with peak/max response height during vehicle application in order to detect the max [Ca\(^{2+}\)] increase induced by the ligand.
2.7.6. Statistical analysis of intracellular Ca$^{2+}$ imaging data

Statistical analyses were performed using GraphPad Prism 5 software to measure changes in [Ca$^{2+}$]$_i$ in response to vehicle and subsequent ligand application. For ratio changes that were compared in the same cellular population, a Paired two-tailed Student’s $t$ test was applied for two groups. For more than two groups, a Repeated measures ANOVA was applied. A Student Newman-Keuls post hoc test was subsequently applied if there was significance detected among the groups. P<0.05 was considered a significant result.
# 2.8. Immunocytochemistry

## Table 1. Antibodies, probes and dyes: Trinity College Dublin

<table>
<thead>
<tr>
<th>ANTIBODY/DYE</th>
<th>EPITOPES RECOGNISED</th>
<th>SUPPLIER</th>
<th>DILUTION</th>
<th>SECONDARY ANTIBODY</th>
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</thead>
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<tr>
<td>Mouse polyclonal anti-hemagglutinin (HA) antibody</td>
<td>Synthetic peptide from influenza HA epitope</td>
<td>Abcam, Cambridge, UK</td>
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<td>Donkey anti-mouse, Alexa Fluor® 555 conjugated</td>
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<td>Rabbit polyclonal anti-GPR55 antibody</td>
<td>Entire C terminus of mouse GPR55</td>
<td>Gifted by Professor Ken Mackie from Indiana University, IN, USA</td>
<td>1:500</td>
<td>Donkey anti-rabbit, Alexa Fluor® 488 conjugated</td>
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<tr>
<td>Mouse monoclonal anti-phospho-CREB (serine 133) clone 10E9</td>
<td>Amino acids 125-135 of human CREB</td>
<td>Millipore, Watford, Hertfordshire, UK</td>
<td>1:250</td>
<td>Donkey anti-mouse, Alexa Fluor® 488 conjugated</td>
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<td>Chicken polyclonal anti-MAP2 antibody</td>
<td>MAP2 isoforms (MAP2A, MAP2B, MAP2C)</td>
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<td>Abcam, Cambridge, UK</td>
<td>1:500</td>
<td>Biotinylated rabbit anti-goat conjugated to Alexa Fluor® 633 fluorophore</td>
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2.8.1. GPR55 and HA-tag antibody labelling in fixed cells

HEK293 cells transiently transfected with mouse 3xHA-GPR55 cDNA and cells from cortical neuron-enriched cultures were fixed with 4% paraformaldehyde (PFA + 200 mM sucrose diluted in PBS) for 15 min at room temperature. Following fixation, the cells were washed 3x5 min in PBS and permeabilised using 0.2% Triton X-100 for 10 min at room temperature. The cells were washed 3x5 min and blocked for 30 min with 5% milk (diluted in PBS) at room temperature. After washing 3x5 min in PBS, the cells were incubated with primary rabbit anti-GPR55 polyclonal antibody (1:500 dilution, no milk) for 1 hr at room temperature. The antibody was gifted by Professor Ken Mackie (Indiana University, IN, USA). The cells were washed 3x5 min in PBS and a goat anti-rabbit secondary antibody conjugated with AlexaFluor® 488 dye (1:500 dilution) was applied to the coverslips and they were left to incubate at room temperature in darkness for 40 min. Cells were washed 3x5 min with PBS. mGPR55-HEK293 cells were then stained with mouse polyclonal anti-hemagglutinin (HA; 1:1000 dilution) antibody for 1 hr at room temperature. The cells were washed 3x5 min in PBS and a donkey anti-mouse secondary antibody conjugated with AlexaFluor® 555 dye (1:500 dilution) was applied to the coverslips and they were left to incubate at room temperature in darkness for 40 min. The cells were washed 3x5 min in PBS. The coverslips were affixed to glass slides using Vectashield mounting medium (Vector Laboratories, Peterborough, UK) and allowed to set for ≥1 hr at 4°C in darkness. Nail polish was applied around the coverslips to ensure they remained adherent to the microscope slides. An Axiovert 200M inverted confocal microscope (Zeiss, LSM-510-META, Carl Zeiss, Cambridge, UK) was used to examine the incorporated fluorophores.
Live hGPR55-HEK293 cells were labelled with anti-HA antibody (1:1000 in culture medium; Abcam, Cambridge, UK) for 30-45 min in a humidified chamber at 37°C with 5% CO₂. The antibody was removed and ligand was applied for 60 min at 37°C with 5% CO₂. Following ligand treatment, the cells were fixed using paraformaldehyde (PFA; 4%) (diluted in PBS) supplemented with 200 mM sucrose at room temperature for 15 min. Sucrose was added to the fixative to preserve the integrity of the cell membrane and to enhance processes during cross-linking (Al-Ali et al., 2014; Hare et al., 2014). Sucrose can also augment the density of PFA, which causes it to drop to the bottom of the wells in the culture plate and ensure rapid cell fixation (Al-Ali et al., 2014). Following fixation the cells were washed 3x5 min in PBS. A donkey anti-mouse secondary antibody conjugated with AlexaFluor® 488 dye (1:500 dilution; in 10% normal horse serum) was applied to the coverslips and they were left to incubate at room temperature in darkness for 40 min. Horse serum was used as a blocking agent in order to prevent cross-reactivity of the donkey anti-mouse secondary antibody. Protein patterns in the serum of horses and donkeys demonstrate their phylogenetic similarity. However, it is worth noting protein profiling does also reveal that despite the phylogenetic similarity, the genetic differences account for significant differences in the serum proteome of these species (Henze et al., 2011). Application of the secondary antibody after fixation allowed for visualisation of receptors localised on the cell membrane (Arancibia-Cárcamo et al., 2006). Cells were washed 3x5 min with PBS. The cells were then permeabilised with 0.2% Triton X-100 for 15 min at room temperature. A donkey anti-mouse secondary antibody conjugated with AlexaFluor® 488 dye (1:500 dilution; in 10% normal horse serum) was applied again and the cells were left to incubate at room temperature in darkness for 40 min. By permeabilising the cells and applying secondary
antibody again, the visualisation of internalised receptors could be achieved (Arancibia-Cárdenas et al., 2006).

The coverslips were affixed to glass slides using Vectashield mounting medium (Vector Laboratories, Peterborough, UK) and allowed to set for ≥1 hr at 4°C in darkness. Nail polish was applied around the coverslips to ensure they remained adherent to the microscope slides. An Axiovert 200M inverted confocal microscope (Zeiss, LSM-510-META, Carl Zeiss, Cambridge, UK) was used to examine the incorporated fluorophores.

2.8.3. Phospho-CREB

Reduction of basal phospho-CREB (pCREB) tone

In the present study, measures were introduced to reduce tonic CREB phosphorylation prior to stimulating cells with ligands. Constitutive CREB phosphorylation can occur if cells are exposed to serum-containing medium due to membrane surface protein activation (Herzig et al., 2000). 7-10 day old cells were incubated in culture medium containing a serum-free supplement (B27) and pre-treated with a Ca$^{2+}$ channel blocker prior to ligand treatment in order to minimise tonic phosphorylation. L-type Ca$^{2+}$ channels localise to the soma and dendrites in neurons (Hell et al., 1993) and are thought to be recruited under physiological conditions that promote prolonged membrane depolarisation (Helton et al., 2005). Ca$^{2+}$ influx into neurons via L-type Ca$^{2+}$ channels induces the phosphorylation of CREB at site Ser133 (Bito et al., 2001; Kornhauser et al., 2002; Weick et al., 2003; Zhang et al., 2006). Nifedipine is a dihydropyridine (DHP) L-type Ca$^{2+}$ channel blocker (Yonemochi et al., 1990; Nguemo et al., 2013). Nifedipine (10 µM) was diluted in warm culture medium and applied to the cells from cortical neuron-enriched cultures for 45 min prior to ligand treatment. Nifedipine was co-incubated with ligands during subsequent cell treatment in order to
prevent tonic CREB phosphorylation and allow for ligand-mediated phospho-CREB activation only. Control cells were incubated with 10 μM nifedipine only.

Positive control for pCREB immunocytochemistry

Following nifedipine pre-treatment, the 7-10 day old cells were treated with culture medium containing both nifedipine and ligands together. TPA (100 nM for 15 min) was used as a positive control stimulus in the detection of phospho-CREB activation. TPA is a specific activator of PKC and has previously been found to induce the transcription of CREB in mouse embryonic fibroblast cells (Johannessen et al., 2004).

pCREB staining procedure

Following ligand treatment, cells were fixed and permeabilised in ice-cold methanol for 5 min at -20°C. Methanol was used because fixatives such as PFA can mask antigenic epitopes from recognition by specific antibodies (Al-Ali et al., 2014). Methanol fixes and permeabilises in one step because it dissolves lipids in cell membranes and is used ice-cold to slow down the reaction. In room temperature conditions the cellular structures of interest would be rapidly destroyed (Omary & Liem, 2016).

Following fixation, the cells were washed 3x5 min in PBS and stored at 4°C until the commencement of staining. The cells were blocked for 30 min in 10% normal horse serum (diluted in PBS) at room temperature. After washing 3x5 min in PBS, the cells were incubated with primary mouse anti-phospho-CREB monoclonal antibody (1:250 dilution in 10% normal horse serum blocking buffer) for 1 hr at room temperature. The cells were washed 3x5 min in PBS. A donkey anti-mouse secondary antibody conjugated with AlexaFluor® 488 dye (1:500 dilution in 10% normal horse serum) was applied to the
coverslips and they were left to incubate at room temperature in darkness for 40 min. Cells were washed 3x5 min with PBS.

For this staining protocol, cells were double-stained to visualise both pCREB and MAP2. MAP2 is a neuronal somato-dendritic marker (Soltani et al., 2005) so therefore allows for the identification of neurons in culture. After washing 3x5 min in PBS, the cells were incubated with primary chicken anti-MAP2 antibody (1:2500 dilution in PBS) for 1 hr at room temperature. The cells were washed 3x5 min in PBS. A goat anti-chicken secondary antibody conjugated with AlexaFluor® 555 dye (1:500 dilution in 10% normal goat serum) was applied to the coverslips and they were left to incubate at room temperature in darkness for 40 min. The cells were washed 3x5 min in PBS. The coverslips were affixed to glass slides using Vectashield mounting medium (Vector Laboratories, Peterborough, UK) and allowed to set for ≥1 hr at 4°C in darkness. Nail polish was applied around the coverslips to ensure they remained adherent to the microscope slides. An Axiovert 200M inverted confocal microscope (Zeiss, LSM-510-META, Carl Zeiss, Cambridge, UK) was used to examine the incorporated fluorophores.

**Processing and analysis of pCREB fluorescence data**

pCREB fluorescence measurements from the nuclei of individual cells were transferred from Image-J (National Institutes of Health, MD, USA) to an excel file. An average background fluorescence value was obtained from background areas measured from negative control images. The negative controls were cells that were not labelled for pCREB but were labelled with secondary antibody. This ensured that there was no non-specific staining in pCREB-labelled cells. The corrected total nuclear fluorescence (CTNF) was then obtained from the cell fluorescence measurements using the following formula:
CTNF = Integrated Density – (Area of nucleus x Mean of background)

This formula ensured that the size of the area of the nucleus in number of pixels, the integrated intensity of all pixels within the nuclear area and the average intensity per pixel were all corrected for background. Essentially, CTNF is a measurement of fluorescence intensity within a cell nucleus minus the background fluorescent intensity (Bülow et al., 2014; Nederlof et al., 1992; Potapova et al., 2011). CTNF values were normalised in an experiment against the mean control value of that experiment. The data were expressed as a fold change in pCREB fluorescence intensity from control.

Statistical analysis of phospho-CREB fluorescence data

Statistical analysis of pCREB fluorescence data were performed using GraphPad Prism 5 software (GraphPad Software, Inc., CA, USA). A One-Way ANOVA was applied and if the results were significant, a Student Newman-Keuls post hoc test was applied. P<0.05 was considered a significant result. Fluorescence data were obtained from 15 neurons from each treatment group per experiment. This equalled 30 neurons total per treatment group from 2 independent pCREB experiments.

2.8.4. Active Caspase-3

Following ligand treatment, 7-10 day old cells from cortical neuron-enriched cultures were fixed with 4% PFA (diluted in PBS) supplemented with 200 mM sucrose. To begin, the cells were permeabilised using 0.2% Triton X-100 (diluted in 1x PBS) for 5-10 min at room temperature. The cells were washed 2x5 min with PBS. The cells were washed 3x5 min in 1x PBS and endogenous cellular peroxidases were blocked using 0.3% H₂O₂. The cells were
washed 3x5 min in PBS. The neurons were blocked with normal goat serum (10%) for 2 hrs at room temperature. The cells were washed 3x5 min in PBS. The cells were incubated for 1 hr at room temperature with polyclonal anti-active caspase-3 primary rabbit antibody (1:250 dilution in 10% normal goat serum blocking buffer). The antibody specifically recognises the mature and active p17 subunit of the caspase-3 enzyme (Kavanagh et al., 2014). The cells were washed 3x5 min with PBS. They were incubated with secondary biotinylated goat anti-rabbit antibody (1:500 dilution in 20% normal goat serum blocking buffer) for 40 min at room temperature. The cells were washed 3x5 min with PBS. The cells were incubated in streptavidin-horseradish peroxidase (HRP) (1:500 dilution in PBS) for 30 min at room temperature. The cells were washed 3x5 min in 1x PBS. To facilitate the colorimetric detection of the HRP-bound fragmented DNA, 3,3'-Diaminobenzidine (DAB) buffer was applied for 10 min at room temperature. The DAB solution consisted of 4.5% DAB substrate buffer (20x), 4.5% DAB Chromogen (20x) and 4.5% H2O2 (20x) dH2O. The cells were washed 3x5 min in dH2O to stop the reaction. The cells were counterstained with 10% methyl green (w/v) for 20 min at room temperature. The cells were dehydrated with increasing alcohol concentrations (70-100% alcohol) and xylene and affixed to glass microscope slides using DPX mounting medium (Sigma-Aldrich, Dorset, UK). The slides were left to dry overnight at room temperature and stored indefinitely at room temperature.

Images of caspase-3-stained cells were obtained using an Olympus BX51 light microscope and Cell^P software. Cells were counted blind by first covering the microscope labels (corresponding to each treatment group) with autoclave tape prior to the commencement of imaging. The slides were then all scattered and mixed around so the author was unable to differentiate them and random numbers were written on the autoclave tape that corresponded to each coverslip on a slide. Images were then taken from each numbered coverslip using Image-J software and assigned to folders with those numbers. Cells were counted from the images. Only after the cells had been counted were the treatment groups
revealed by removing the autoclave tape from the slides. Caspase-3 positive cells were expressed as a percentage of total neurons counted (~500 cells per treatment group).

**Statistical analysis of the percentage of caspase-3 positive neurons**

Statistical analysis of the percentage of caspase-3 positive neurons was performed using GraphPad Prism 5 software. A One-Way ANOVA was applied to the data. If the results were significant, a Student Newman-Keuls post-hoc test was applied to determine which groups were significantly different from one another. P<0.05 was considered a significant result.

**2.9. Microglial migration assay**

**2.9.1. Chemotactic BV2 cell migration assay**

Cultured primary rat cells from cortical neuron-enriched cultures (7 DIV) were primed with neurobasal medium in the presence or absence of Aβ (10 µM) for 24 hrs. The media was then removed and the neurons were incubated with fresh untreated culture medium for a further 48 hrs. This was to ensure that Aβ would not come into direct contact with the BV2 cells during the migration assay. Therefore any BV2 migration that occurred would be in response to the primed neuronal media (PNM) only. After 24 hr priming of the neurons, BV2 microglial cells were subcultured in DMEM with 2% FBS and the cells in suspension (7.5 x 10^3 cells) were seeded into Boyden chambers with polyethylene terephthalate (PET) membranes (8.0 µm pore size; Millipore, Watford, UK) suspended above empty wells in a sterile 24-well plate and the BV2 cells were cultured for 48 hrs.
After separate incubation of the two cell types, GPR55 ligand treatments (diluted in DMEM with 2% FBS) were applied to the BV2 cells. Concurrently, the untreated neuronal media (UT-NM) and the Aβ-PNM (from the now 10 day old neurons) were removed and centrifuged at 1500 rpm for 3 min to separate out any cellular debris from the supernatants. The supernatants (900 μL) were then applied to the wells beneath the Boyden chambers containing the BV2 cells. Chemotactic migration of BV2 cells occurred in response to ligand treatments and PNM for 3 hrs. Control treatments were (1) BV2 cells exposed to neurobasal medium that had not been incubated with neurons and (2) BV2 cells exposed to UT-NM. The positive control used was neurobasal medium supplemented with 30% FBS. This was because the neurons were originally grown in media not containing FBS and FBS itself is a chemotactic agent (Mishima & Lotz, 2008). See Figure 2.7. for a schematic of the experimental set up.
Figure 2.7. Schematic of BV2 cell migration assay preparation and experiment.

The blue arrows denote the chemotactic movement of the BV2 cells to the basolateral side of the membrane towards the conditioned neuronal medium. Some elements of figure adapted from Servier Medical Art images.
2.9.2 Rapi-diff staining of migrated cells

Following the migration assay, the BV2 cells that had migrated to the basolateral side of the membrane were histologically stained using Rapi-diff staining reagents (Atom Scientific, Cheshire, UK). Migrated cells were viewed using a light microscope at x40 magnification and were counted by hand from 5 regions of interest using a cell counter. Migration levels of the different treatment groups were recorded blindly by covering the microscope labels (which had the names of the treatment groups) with autoclave tape. The slides were then scattered and mixed around so the author was unable to differentiate them and random numbers were written on the autoclave tape that corresponded to each coverslip on a slide. Images were then taken from each numbered coverslip using Image-J software and assigned to folders with those numbers. Cells were counted from the images. Only after the cells had been counted were the treatment groups revealed by removing the autoclave tape from the slides. Images were obtained using an Olympus BX51 light microscope and Cell software.

2.9.3. Statistical analysis of the levels of chemotactic BV2 cell migration

Statistical analysis of cell migration levels was performed using GraphPad Prism 5 software. A One-Way ANOVA was applied to the data. If the results were significant, a Student Newman-Keuls post-hoc test was applied to determine which groups were significantly different from one another. P<0.05 was considered a significant result.
2.10. Microglial phagocytosis assay

2.10.1. BV2 cell phagocytosis assay

BV2 microglial cells were subcultured and seeded onto poly-D-lysine-coated (50 μg/mL) coverslips at 100,000 cells per well in a sterile 24-well plate and left to incubate at 37°C and 5% CO₂ for 24 hrs. Cells were then pre-treated with ligands (diluted in DMEM with 2% FBS) for 30 min before applying latex beads (w/v; carboxylate-modified polystyrene, fluorescent yellow-green; Cat #L4655, Sigma-Aldrich) directly into the wells (final concentration 0.025%). The cells were left to incubate in darkness with ligands and beads at 37°C and 5% CO₂ for 2 hrs. The beads are fluorescent, so all further steps were carried out in darkness. The cells were fixed with 4% PFA (with 200 mM sucrose in PBS) for 15 min at room temperature. The cells were washed 3x5 min in PBS.

2.10.2. IBA1 immunocytochemistry

The fixed cells were permeabilised using 0.2% Triton X-100 (in PBS) for 10 min at room temperature. The cells were blocked for 30 min in 1% BSA (diluted in PBS) at room temperature. After washing 3x5 min in PBS, the cells were incubated with primary goat anti-IBA1 polyclonal antibody (1:500 dilution in PBS) for 1 hr at room temperature. IBA1 is a microglia/macrophage-specific calcium-binding protein (Ohsawa et al., 2004). The cells were washed 3x5 min in PBS. A rabbit anti-goat biotinylated secondary antibody (1:500 dilution in PBS) was applied to the cells and they were left to incubate at room temperature for 40 min. Cells were washed 3x5 min with PBS. An AlexaFluor® 633 dye (1:500 dilution in PBS) was applied to the cells and they were left to incubate at room temperature for 40 min. The cells were washed 3x5 min in PBS. The cells were incubated with Hoechst dye (1:1000) for 20 min.
at room temperature. The cells were washed 3x5 min in PBS. The coverslips were affixed to
glass slides using Vectashield mounting medium (Vector Laboratories, Peterborough, UK)
and allowed to set for $\geq$1 hr at 4°C in darkness. Nail polish was applied around the
coverslips to ensure they remained adherent to the microscope slides. An Axiovert 200M
inverted confocal microscope (Zeiss, LSM-510-META, Carl Zeiss, Cambridge, UK) was
used to examine the incorporated fluorophores.

2.10.3. Quantification and analysis of phagocytosis

Images obtained using LSM software were analysed using ImageJ software. Regions of
interest (ROI; i.e. cells) were chosen and the number of phagocytosed beads per cell were
counted. Results were calculated as described by Caldeira et al. (2017). The number of beads
phagocytosed was expressed as a percentage of the total cell population. These populations
were divided into different categories: % cells that had ingested 0 beads, % cells that had
ingested 1-9 beads and % cells that had ingested 10+ beads. The number of cells counted per
independent culture was 30 cells per treatment group. Graphs displaying these data were
created using GraphPad Prism 5 software (GraphPad Software, Inc., CA, USA).

2.10.4. Statistical analysis of the levels of chemotactic BV2 cell migration.

Statistical analysis of the % phagocytic cells was performed using GraphPad Prism 5
software. A Two-Way ANOVA was applied to the data. If the results were significant, a
Bonferroni post-hoc test was applied to determine which groups were significantly different
from one another. P$<0.05$ was considered a significant result.
2.11. Enzyme-linked Immunosorbent Assay (ELISA)

2.11.1. Tumour Necrosis Factor-α (TNF-α)

Supernatants obtained from BV2 cells treated with GPR55 ligands for 24 hrs were processed for TNF-α production using the Biolegend Mouse TNF-α ELISA MAX™ Deluxe kit (Cat# 430901, Biolegend, CA, USA). The ELISA was carried out as per the manufacturer’s instructions. A mouse TNF-α specific hamster monoclonal antibody (1:200 dilution in 1x coating buffer) was first coated on a Nunc™MaxiSorp™ 96-well ELISA plate. The plate was sealed and stored overnight at 4°C. The next day the plate was washed 4x using PBS + 0.05% Tween-20 and blocked using 1x assay diluent for 1 hr at room temperature. The plate was sealed and placed on an orbital shaker during the blocking stage. The plate was washed 4x with PBS + 0.05% Tween-20. A stock of lyophilised Mouse TNF-α standard was prepared by diluting the standard in 1x assay diluent to create 45 ng/mL of stock. A two-fold serial dilution series was created using this stock to generate a standard range of 0-500 pg/mL mouse TNF-α. The standards and supernatant samples (diluted in assay diluent) were then added in duplicate to the appropriate wells. The plate was sealed, placed on an orbital shaker and incubated at room temperature for 2 hrs. TNF-α binds to immobilised capture antibody. The plate was washed 4x with PBS + 0.05% Tween-20 and a biotinylated goat polyclonal anti-mouse TNF-α detection antibody (1:200 dilution in assay diluent) was applied to each well. The addition of the detection antibody produces an antibody-antigen-antibody “sandwich”. The plate was sealed, placed on an orbital shaker and incubated at room temperature for 1 hr. The plate was washed 4x with PBS + 0.05% Tween-20 and avidin-horseradish peroxidase (HRP) solution (1:1000 dilution in assay diluent) was applied to each well. The plate was sealed, placed on an orbital shaker and incubated at room temperature for 30 min. The plate was washed 5x with PBS + 0.05% Tween-20. TMB substrate solution
was applied and the plate was sealed and incubated in the dark for 15 min. The TMB substrate solution produced a blue colour in proportion to the concentration of TNF-\(\alpha\) present in each sample. Stop solution (2M H\(\text{SO}_4\), sulphuric acid) was added, which changed the colour from blue to yellow and the optical densities of each sample were read at 450 nm on a plate reader. A standard curve was generated using the absorbance values obtained from the TNF-\(\alpha\) standard range. The 0 pg/mL reading acted as a blank and its absorbance value was subtracted from the absorbance values of the supernatant sample readings to correct for optical imperfections. The sample absorbance values were input into the standard curve equation to establish the amount of TNF-\(\alpha\) in the supernatants.

2.11.2. Prostaglandin E2 (PGE\(_2\))

Cultured moDC were incubated in serum-free IMDM for 90 min. They were then treated with HCA2 ligands for 45 min and the supernatants were processed for PGE\(_2\) production using the DetectX Prostaglandin E2 (PGE2) Multi-Format ELISA kit (Cat# K051, Arbor Assays, MI, USA). The ELISA was carried out as per the manufacturer’s instructions. A two-fold serial dilution series of PGE\(_2\) standard stock (20,000 pg/mL stock) was created by diluting the stock in 1x assay buffer to generate a standard range of 0-500 pg/mL mouse PGE\(_2\). The standards or supernatant samples (50 \(\mu\)L per well) were pipetted in duplicate into a clear microtiter plate coated with an antibody to capture mouse IgG. Assay buffer only (75 \(\mu\)L) was added to duplicate wells to detect for non-specific binding (NSB). Assay buffer only (50 \(\mu\)L) was added to two more wells to detect maximum binding (\(B_0\)). DetectX\(^\text{®}\) PGE\(_2\) conjugate (25 \(\mu\)L) was added to each well. DetectX\(^\text{®}\) PGE\(_2\) monoclonal antibody (25 \(\mu\)L) was added to each well, except the NSB wells. The plate was sealed, placed on an orbital shaker and incubated at room temperature for 2 hrs. The plate was washed 4x using 1x wash buffer. TMB substrate solution (100 \(\mu\)L) was applied and the plate was sealed and incubated in the
dark for 30 min. The TMB substrate solution produced a purple colour in proportion to the concentration of PGE₂ present in each sample. Stop solution (50 µL) was added to each well, which changed the colour from purple to yellow and the optical densities (OD) of each sample were read at 450 nm on a plate reader. A standard curve was generated using the absorbance values obtained from the PGE₂ standard range. The NSB mean OD was subtracted from the standard and sample values. The % sample bound/maximum binding (%B/B₀) for each standard was calculated and a standard curve was generated by plotting the %B/B₀ versus the log concentration of PGE₂ (pg/mL). Sample PGE₂ concentrations were calculated from this curve.

2.11.3. Statistical analysis of cytokine production by immune cells.

Statistical analysis of cell migration levels was performed using GraphPad Prism 5 software. For two groups, an Unpaired Student’s t test was applied. For more than two groups, a One-Way ANOVA was applied to the data. If the results were significant, a Student Newman-Keuls post-hoc test was applied to determine which groups were significantly different from one another. P<0.05 was considered a significant result.
2.12. Flow cytometric analysis of human peripheral immune cells

2.12.1. Staining procedure for moDC analysis

Cells were removed from culture and centrifuged at 400xg for 5 min at room temperature. The supernatant was discarded and the cells were washed with fluorescence-activated cell sorting buffer (FACS) buffer (PBS + 1% BSA). The cells were centrifuged again 400xg for 5 min at room temperature. The supernatant was discarded and the cells were stained with FcR blocking reagent (Miltenyi Biotec, Surrey, UK) in FACS buffer for 15 min at 4°C in darkness. FcR Blocking Reagent can be used to block the undesirable binding of antibodies to human Fc receptor-expressing cells such as B cells, monocytes and macrophages (Fridman, 1991). The supernatant was discarded and the cells were washed with FACS buffer. Relevant antibodies were applied (diluted in FACS buffer) and the cells were stored at 4°C for 20 min in darkness. The cells were centrifuged at 400xg for 5 min at room temperature. The cells were washed x2 with FACS buffer. If necessary, unconjugated primary antibodies were further tagged with a relevant fluorophore for 15 min at 4°C in darkness. The cells were centrifuged at 400xg for 5 min at room temperature and washed with FACS buffer. Samples were then analysed using flow cytometry.

Cell samples could also be double-stained with multiple antibodies, as long as the fluorophore emission wavelengths did not cause spectral overlap. This allowed for the observation of multiple biomarkers at once using flow cytometry. Samples could also be fixed prior or following staining to allow for later analysis of cells.
<table>
<thead>
<tr>
<th>ANTIBODY/DYE</th>
<th>EPITOPES RECOGNISED</th>
<th>SUPPLIER</th>
<th>DILUTION</th>
<th>SECONDARY ANTIBODY</th>
<th>ISOYPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit polyclonal anti-GPR55 antibody</td>
<td>Synthetic peptide from an internal region of human GPR55</td>
<td>Cayman Chemicals, Cambridge, UK</td>
<td>5 µL per 1x10^6 cells</td>
<td>Biotinylated goat anti-rabbit conjugated to Alexa Fluor® 647 fluorophore</td>
<td>Rabbit polyclonal IgG conjugated to Alexa Fluor® 647 fluorophore</td>
</tr>
<tr>
<td>Rabbit polyclonal IgG anti-G2A antibody (H-70)</td>
<td>Amino acids 311-380 mapping within a C-terminal cytoplasmic domain of G2A of human origin.</td>
<td>Santa Cruz Technologies, Texas, USA</td>
<td>5 µL per 1x10^6 cells</td>
<td>Biotinylated goat anti-rabbit conjugated to Alexa Fluor® 647 fluorophore</td>
<td>Rabbit polyclonal IgG conjugated to Alexa Fluor® 647 fluorophore</td>
</tr>
<tr>
<td>Rat Anti-human HM74A/GPR109A monoclonal antibody (IgG2b; APC-conjugated)</td>
<td>Detects human HM74A/GPR109A. Epitope not communicated by manufacturer.</td>
<td>Novus Biologicals, Abingdon, UK</td>
<td>5 µL per 1x10^6 cells</td>
<td>N/A</td>
<td>Rat IgG2b Isotype Control (APC-conjugated)</td>
</tr>
<tr>
<td>FITC anti-human CD11c Antibody</td>
<td>A 145-150 kD type I transmembrane glycoprotein also known as integrin αX and CR4.</td>
<td>Biolegend, London, UK</td>
<td>5 µL per 1x10^6 cells</td>
<td>N/A</td>
<td>FITC Mouse IgG1, α</td>
</tr>
</tbody>
</table>

Table 2. Antibodies and dyes: GSK
2.12.2. Staining procedure for *E.coli* pHrodo Green monocyte phagocytosis assay

CD14\(^+\) monocytes were positively selected from PBMCs. The cells were adjusted to 2 x 10\(^6\) cells (2 x 10\(^7\)/100 µL) using IMDM. Cells were plated out (100 µL cells per well) in a low adherence 96-well U-bottom plate. They were incubated for 24 hrs in a humidified chamber at 37\(^\circ\)C and 5% CO\(_2\).

The next day, a vial of reconstituted pHrodo® Green *E. coli* BioParticles® Conjugate (1 mg/mL stock; Cat# P35366, ThermoFisher, Paisley, UK) was removed from the -20\(^\circ\)C freezer. The particles were vortexed for 3 min and a sonicating bath was used for 15 min to homogenously disperse the particles. The particles were passed through a 21 g needle to further disperse the particles. Various titrations of particle concentrations diluted in assay buffer (Hanks’ balanced salt solution: 1x, HBSS; no Ca\(^{2+}\), no Mg\(^{2+}\), no phenol red + 20 mM HEPES; Gibco ThermoFisher, Paisley, UK) were tested. Two-fold particle titrations were tested between 50 µg/mL-0.4 mg/mL; eventually a concentration of 0.4 mg/mL particles was confirmed to be the most appropriate for assay purposes.

The 1-day old monocytes were centrifuged at 400xg for 8 min and the culture media supernatants were removed. The cell pellets were resuspended in assay buffer. The pHrodo Green particles were applied to the cells either alone or with GPR55 compounds. The cells were incubated at 37\(^\circ\)C in a humidified chamber in darkness for 3 hours (t=3 hrs). Control wells consisted of (1) cells with no particles, (2) wells with particles but no cells and (3) cells incubated with particles that were immediately washed (t=0). Following incubation, the cells were removed from culture and immediately washed x2 with ice-cold PBS. They were then resuspended in FACS buffer and levels of phagocytic cells were analysed using flow cytometry.
Stained samples were processed using a Beckman Coulter CyAn ADP flow cytometer (Beckman Coulter, High Wycombe, UK). The system was equipped with 3 lasers (488, 643 and 405 nm) and can detect up to 9 fluorescent parameters (FL1-9). Blue detectors measure light from the blue laser (488 nm), red detectors measure light from the red laser (635 nm) and violet detectors measure light from the violet laser (405 nm). Its acquisition software was Summit 4.4.

Before commencing with sample analyses, the sample acquisition settings were set in Summit software. “Events” represent the particles that flow through the flow cytometer during acquisition i.e. cells. Therefore the event threshold was set (i.e. max number of events recorded per sample) and the apparatus voltage and detector gains were applied. These settings remained fixed over the course of the analysis to ensure there was no variability in data acquisition.

Plots of desired parameters were created so that the correct sample fluorophores were detected. An unlabelled sample was set up on the CyAn cytometer. The cytometer was switched on and the sample began to flow through the cytometer. An unlabelled sample was used initially to allow for acquisition optimisation. Optimisation involved altering the rate of flow, detector gain and voltages. Events were gated using forward scatter and side scatter parameters. Forward scatter (FSC) detection allows for the discrimination of cells by size. FSC intensity is proportional to the diameter of the cell and is primarily due to light diffraction around the cell. Side scatter (SSC) detection provides information about the internal complexity (i.e. granularity) of a cell (Shapiro & Howard, 1985). Using these parameters is helpful in distinguishing between different classes of cell in the immune system. In Figure 2.8, one can see the different populations of cells present in a human PBMC sample. Monocytes are larger and more granular than lymphocytes so exhibit a higher
intensity on the FSC and SSC axes. Lymphocytes in comparison exhibit lower intensity on the FSC and SSC axes.

Once the cells were gated using FSC and SSC, the fluorescence of unlabelled sample events were detected. The event count was plotted against a fluorescent parameter of interest and the voltage and detector gain were modified to generate a basal level of fluorescence for that parameter. Henceforth, any labelled samples should show an increase in fluorescence provided they are labelled with that fluorophore. In Figure 2.9, an example event acquisition can be observed. An moDC sample obtained on Day 5 of culture was passed through the CyAn flow cytometer and the moDC were gated using FSC and SSC. The moDC sample had been labelled with the dendritic differentiation marker CD11c conjugated to Brilliant Violet

Figure 2.8. Scatter plot of PBMC sample.
Note the different FSC and SSC intensities of the monocyte and lymphocyte populations. Figure obtained using FlowJo LLC, Version 7 (Tree Star, Inc, Oregon, USA).
421, so the event count was measured against violet fluorescence to determine how many moDC in the sample expressed CD11c.

![Figure 2.9. Acquisition of moDC sample using Summit software.](image)

The first panel shows detection of cell intensity using FSC and SSC. The clustered population was gated and cell expression of CD11c was detected using a histogram. Event count was plotted against for Violet (log) intensity.

Once the sample events were acquired, the file was saved as a FACS file. It could then be further processed using other analysis software such as FlowJo LLC Version 7 (TreeStar, Inc., Oregon, USA) in order to generate histograms and record fluorescence data.
2.12.4. Processing of sample data using FlowJo software

Gating for singlets

A FACS file was opened using FlowJo Version 7 desktop software. This software has more advanced functions than Summit Software and allows for the generation of high quality histograms and the recording of fluorescence data. A sample of a FACS file open in FlowJo can be viewed in Figure 2.10. It shows an unlabelled PBMC sample (Figure 2.10i). Lymphocyte and monocyte populations are gated out. Cell populations were further gated into “singlets”. Singlets represent a single event or particle passing through the flow cytometer. These are most likely cells of interest. Singlets were determined by plotting the gated cell populations on a SSC Linear vs SSC Area plot. In the below panels, one can see in the left-hand panels of the gated lymphocyte and monocyte populations (Figure 2.10ii & iv respectively) that the majority of events remain tightly clustered together. However, some events drift to the right of the SSC Area scale. This indicates that the “area” (event height x width) of the events are larger. These events are most likely dead cells or “doublets”. Doublets are two particles or cells that have stuck together. Their width and area is double that of singlets. Including them in the population may therefore skew the interpretation of the data. For instance, if doublets were not excluded from the event population then the fluorescence reading is at risk of not being accurate. This is because doublets may contain particles/cells that are not labelled with the desired fluorophore, yet if they are attached to a particle/cell that does express the fluorophore, then they will both be counted. The populations analysed in this study were therefore gated for singlets only. Lymphocyte and monocyte singlets can be viewed in Figure 2.10iii & v respectively.
Dead cells can be more concisely detected using a viability dye such as Zombie Aqua™ (Biolegend, London, UK). This dye is an amine-reactive fluorescent dye that is non-permeant to live cells but permeant to cells with compromised membranes. It can therefore stain dead cells and these cells can be detected at the relevant fluorescence intensity and omitted from FACS analysis. For the purposes of this study a viability dye was not applied because we wished to utilise as many fluorophores as possible in order to test for cell marker expression.

Figure 2.10. Gating for singlets.
A PBMC sample (i) was gated into lymphocyte and monocyte populations. These populations were further gated into singlets (ii, iv). These singlets (iii, v, plotted SSC vs FSC) were analysed for relevant fluorophores.

**Measuring sample fluorescence**
Cell singlets were measured for fluorescence. A FACS file of an acquired sample was opened in FlowJo Version 7. In Figure 2.11, a sample of PBMC was gated into two different populations (i). In this case, lymphocyte singlets were gated out and examined (ii-iii). A FACS file was opened in FlowJo Version 7 and the relevant fluorescence parameters were selected. The fluorescence of the sample events were plotted as log values on histograms (Figure 2.15v-viii). In this lymphocyte population, CD4 conjugated to FITC was plotted on a histogram (v-vi). By comparing the rightward shift of the cell count on the FITC log scale compared to an unlabelled sample and a Mouse IgG isotype conjugated to FITC, it appears that this cell population does express CD4 (vi). These cells were also examined for human GPR55 expression (conjugated to Alexafluor 647; viii). Alexafluor 647 can be excited by the same laser as the APC fluorophore because they are excited at around 650 nm. By comparing the rightward shift of the cell count on the APC log scale compared to an unlabelled sample, a Rabbit IgG isotype conjugated to Alexafluor 647 and the Alexafluor 647 secondary antibody itself, it appears that this cell population does express GPR55. However there is some event overlap between the GPR55 measurement and the Rabbit isotype measurement, which may indicate some non-specific binding of Rabbit antibody in this cell sample. Fluorescence parameters could also be plotted against one another and depending on the position of the events on each scale, one could determine if the cells expressed one or both markers conjugated to the relevant fluorophore. In this lymphocyte population for instance, one can observe heterogeneous levels of marker expression. Some cells express CD4high and GPR55^high (CD4high, GPR55^high), some cells don’t express CD4 but do express GPR55 (CD4low, GPR55^high), some cells don’t express GPR55 but do express CD4 (CD4high, GPR55low) and some cells express neither (CD4low, GPR55low). This shows how flow cytometry is a useful technique for measuring heterogenous expression patterns in cell populations. Numerical fluorescence values reflecting the visual fluorescence data were obtained from FlowJo software and relevant statistical analyses were performed.
In the current study, if there was a distinctive separation between the marker of interest and isotype (i.e. marker expression was greater than isotype), the isotype was not included in the resulting histograms and graphs for clarity.

The lymphocyte population was examined (ii-iii) and their fluorescence was measured and plotted on histograms (v-viii) and scatter plots (iv) using FlowJo software.
2.12.5. Statistical analysis of flow cytometric studies.

Statistical analysis of cell migration levels was performed using GraphPad Prism 5 software. For two groups, an Unpaired two-tailed Student’s $t$ test was applied. For more than two groups, a One-Way ANOVA was applied to the data. $P<0.05$ was considered a significant result.
Chapter 3

GPR55 regulation of intracellular Ca\(^{2+}\) and CREB phosphorylation in neurons and glia
3.1. Introduction

GPR55 is a novel GPCR that mediates a variety of downstream signalling effects. Although initially touted as a putative cannabinoid receptor (Ryberg et al., 2007), various signalling assays have been implemented in recent years and subsequently the bioactive lipid LPI has emerged as the primary endogenous ligand for GPR55 (Oka et al., 2007; Nevalainen & Irving, 2010).

One of the most well-characterised downstream signalling effects of GPR55 stimulation is the modulation of \([\text{Ca}^{2+}]_i\). The Irving group have previously shown that GPR55 activation induces \(G_\alpha_{13}\)-RhoA-ROCK-mediated release of \(\text{Ca}^{2+}\) from intracellular ER stores in an overexpressing HEK293 cell line model (hGPR55-HEK293; Henstridge et al., 2010). The signalling effects of GPR55 activation are less well characterised in the CNS. GPR55 was first identified by Sawzdargo et al. (1999) using cloning experiments and high levels of GPR55 mRNA have been reported in the human striatum and in the frontal cortex (Marichal-Cancino et al., 2017). GPR55 is also highly expressed in both primary mouse microglia and the BV2 mouse microglial cell line (Pietr et al., 2009).

Reports of intracellular \(\text{Ca}^{2+}\) regulation by GPR55 in CNS models have emerged in recent years. Neurons utilise \(\text{Ca}^{2+}\) signalling for a variety of important functions including control of membrane excitability, neurotransmitter release and regulation of neuronal growth (Berridge, 1998). Disruption of \(\text{Ca}^{2+}\) regulation is therefore suggested to contribute to dysregulation of cell homeostasis (Mattson, 1994; Peng & Jou, 2010). GPR55 agonists were found to cause a slow release of \(\text{Ca}^{2+}\) from presynaptic stores at individual CA3-CA1 synapses, which enabled glutamate transmission to occur. This effect was not seen in GPR55\(^{-/-}\) mice (Sylantyev et al., 2013). GPR55 immunoreactivity was identified in \textit{in vitro} mouse dorsal root ganglia (DRG), specifically large diameter neurons and stimulation with LPI induced \(\text{Ca}^{2+}\) responses in these cells that were mediated by both \(G_\alpha_4\) and \(G_\alpha_{12}\) (Lauckner et al., 2008). Understanding the role
of GPR55 in the regulation of Ca\(^{2+}\) in the nervous system is therefore crucial in order to
determine its impact on cell homeostasis and plasticity between neurons.

GPR55 stimulation also leads to the activation of downstream effectors and transcription
factors. CREB is a transcription factor that is responsive to a vast array of stimuli including
hormones, cytokines and growth factors. These stimuli activate a complex array of kinases
such as PKA, PKC, ERK and CaMKs which culminate in the phosphorylation of CREB,
thus altering the transcription of its target genes (Shaywitz and Greenberg, 1999). LPI has
previously been shown to induce pCREB activation in hGPR55-HEK293 cells. Henstridge
et al. (2010) demonstrated that LPI (1 µM) induces a robust concentration dependent increase
in pCREB. This is significant because CREB is believed to play an important role in synaptic
plasticity, memory consolidation and learning (Carlezon Jr et al., 2005; Josselyn & Nguyen,
2005), yet GPR55-mediated regulation of CREB has not yet been explored in other studies.

The limited evidence into the effects mediated by GPR55 stimulation in the CNS is not aided
by its contentious pharmacological classification. A lack of selective ligands has further
hindered the pharmacological characterisation of GPR55. In recent years selective agonists
and antagonists have been developed. Yrjölä et al. (2016) recently evaluated compounds that
were part of a high-throughput screen (HTS) of selective GPR55 agonists. Nanomolar
potency agonists were generated during this study, one of which – 17g – was found to be a
selective GPR55 agonist containing an N-phenyl group. They reported that 17g potently
induced the release of Ca\(^{2+}\) in hGPR55-HEK293 cells (EC\(_{50}\) = 7.0 nM).

Given that GPR55 has such a capricious pharmacological classification and its expression
and signalling mechanisms are little characterised in the CNS, the aim of this chapter was to
characterise the expression of GPR55 by probing for GPR55 immunoreactivity and to
investigate GPR55 signalling effects by treating \textit{in vitro} cells from cortical neuron-enriched
cultures with selective GPR55 ligands and employing assays such as ratiometric Ca\textsuperscript{2+} imaging and labelling for phospho-CREB immunoreactivity to study the effects of these ligands.
3.2. Results

3.2.1. GPR55 is expressed in neurons and glia obtained from cortical neuron-enriched cultures

In this study it was of interest to delineate the pharmacology and function of GPR55 in native rat cortical neuron-enriched cultures. It was therefore vital to examine the levels of GPR55 expression in these cells and compare them to overexpressing cell lines. There are limited GPR55 antibodies available commercially and several were tested to elucidate immunoreactivity in cortical cells, with little success (See Appendix, Supplementary Figure S3). A rabbit anti-GPR55 polyclonal antibody was gifted by Professor Ken Mackie for use in this study because it has been successfully used to test for GPR55 immunoreactivity in mouse salivary glands (Korchynska et al. 2019, in press). However, Korchynska et al. were unable to detect credible GPR55 signal in healthy adult mouse hippocampus, striatum and cortical tissue, so it was pertinent to test the antibody at cellular level.

To confirm the specificity of the antibody, immunocytochemistry was carried out in transiently transfected HEK293 cells expressing triple hemagglutinin (HA)-tagged mouse GPR55 (mGPR55; Figure 3.1i-iii). The cells were double-labelled for GPR55 and HA to ensure that both markers were co-localised. It was observed that mGPR55-HEK293 cells showed high expression of GPR55 in the membrane of some cells; GPR55 and HA were co-localised in these cells.

Next, the antibody was tested in native central cells. In 7 day-old rat cortical neuron-enriched cultures, it was observed that GPR55 was highly expressed in ionised calcium binding adaptor molecule 1 (IBA1)-positive microglia (Figure 3.1iv-vi). However, consideration was given to the fact that immune cells like microglia express Fc receptors (Vedeler et al., 1994). These are antibody-binding proteins found on the surface of immune cells such as...
neutrophils, macrophages, B cells, NK cells and some T cells. Fc regions of many antibodies readily bind to the Fc-receptors at different levels (Buchwalow et al., 2011), so it is possible that the GPR55 antibody used herein bound to some Fc-receptors in cortical microglia.

Neurons in comparison appeared to show punctate localisation of GPR55 along the processes of neurons (Figure 3.2i-iv) and in some cases localisation in the membrane of the neuronal soma (Figure 3.2vi). If Figure 3.2iv in particular, one can observe several cells in close proximity to one another and punctate GPR55 immunoreactivity evident along the processes of these cells. However, these images of neurons are based on n = 1 culture, so immunolabelling will need to be repeated to validate these results.
Figure 3.1. GPR55 is expressed in transfected HEK293 cells and in microglia obtained from cortical neuron-enriched cultures.

Representative confocal images of HEK293 cells transiently expressing 3xHA-mGPR55 (i-iii); and cortical microglia (iv-vi). Transfected HEK293 cells were double-labelled with GPR55 (green) and HA (red) to ensure successful transfection and antibody specificity. Microglia were double-labelled with GPR55 (green) and IBA1 (microglial marker, red). White arrows denote GPR55 expression. Scale bar = 20 µm. n = 2 independent cultures.
Figure 3.2. Somal and punctate localisation of GPR55 in neurons obtained from cortical-neuron enriched cultures.

Representative confocal images of neurons labelled for GPR55 (green). (i-iv) White arrows denote punctate localisation of GPR55. (v) White arrow denotes membrane expression of GPR55. Scale bar = 20 µm. n = 1 culture.
3.2.2. LPI (10 μM) induces heterogeneous changes in \([\text{Ca}^{2+}]_i\) in neurons.

GPR55 stimulation using the endogenous agonist LPI has previously been shown to induce downstream release of Ca\(^{2+}\) from internal stores in hGPR55-HEK293 cells and PC12 neuronal cells in a G\(_{\alpha_{13}}\)-RhoA-ROCK signalling pathway (Henstridge \textit{et al.}, 2009; Obara \textit{et al.}, 2011). Cells from cortical neuron-enriched cultures were treated with LPI to assess its impact on \([\text{Ca}^{2+}]_i\) changes.

Representative traces of cells obtained from independent cortical neuron-enriched cultures and their exposure to the endogenous GPR55 agonist LPI (10 μM can be observed in Figure 3.3i-iii. It was observed that neurons exhibited one of two basal \([\text{Ca}^{2+}]_i\) profiles: (1) quiescent or (2) spontaneous Ca\(^{2+}\) activity. Both categories were examined for ligand-induced modulation of \([\text{Ca}^{2+}]_i\). LPI application induced heterogeneous changes in \([\text{Ca}^{2+}]_i\). These changes could be divided into separate categories: (1) Attenuation of \([\text{Ca}^{2+}]_i\) activity in neurons exhibiting spontaneous events (17 out of 55 total neurons, from \(n=5\) independent cultures; Figure 3.3i), (2) Increase in \([\text{Ca}^{2+}]_i\) activity in quiescent neurons (16 out of 55 total neurons, \(n=5\) independent cultures; Figure 3.3ii) or (3) inconclusive or no effect (22 out of 55 total neurons, \(n=5\) independent cultures; Figure 3.3Aiii).

The results here demonstrate that in the majority of the neuronal populations examined, LPI induced heterogeneous changes in neuronal \([\text{Ca}^{2+}]_i\).
Figure 3.3. LPI (10 μM) induces heterogeneous changes in [Ca$^{2+}$]i in neurons.

(i-iii) Representative traces of neuronal cell recordings from independent cortical cell cultures. Each trace represents one cell. Cells were exposed to LPI (10 μM) and later KCl (50 mM) to differentiate between neurons and glia, but the KCl response has been omitted for clarity. The traces represent LPI-induced modulation of [Ca$^{2+}$]i activity in (i) neurons exhibiting spontaneous activity and (ii) quiescent neurons and when (iii) there is an inconclusive or no effect of LPI.
3.2.3. The majority of LPI- and CCh-induced responses are not co-localised in neurons.

It was determined in Section 3.2.3 that LPI (10 µM) was capable of inducing changes in 
$[\text{Ca}^{2+}]_i$ in neurons. LPI is the endogenous agonist at GPR55, but it was important to include a GPCR comparator in Ca$^{2+}$ imaging experiments to compare ligand-induced responses. The muscarinic acetylcholine receptor (mAChR) is a GPCR expressed in neural tissue. These receptors are involved in signal transduction of cholinergic signals in the CNS, autonomic ganglia, smooth muscles and other parasympathetic organs (Aronstam & Patil, 2009). Carbachol (CCh; 10 µM) is a mAChR agonist (Shiroma & Costa, 2015) so was used in the present study to compare with LPI-induced responses. Representative traces of neurons treated with LPI and later CCh can be observed in Figure 3.4i-iv. It was found that only 5 out of 48 neurons exhibited responses to both LPI and CCh ($n=4$ independent cultures; Figure 3.4i). The other cells could be further subdivided into LPI-responsive only (7 out of 48 total neurons, $n=4$ independent cultures; Figure 3.4ii), CCh-responsive only (11 out of 48 total neurons, $n=4$ independent cultures; Figure 3.4iii) and inconclusive or no responses (25 out of 48 total neurons, $n=4$ independent cultures; Figure 3.4iv). Neurons responsive to LPI alone seemed to exhibit long lasting $[\text{Ca}^{2+}]_i$ activity following LPI application.

The results here demonstrate that only a minority of cortical neurons exhibit co-localised responses to LPI and the mAChR agonist, CCh. Other minority populations of neurons were responsive to only LPI or only CCh.
Figure 3.4. The majority of LPI- and CCh-induced responses are not co-localised in neurons.

(i) Representative traces of neurons responsive to (i) LPI (10 µM) and CCh (10 µM), (ii) LPI only, (iii) CCh only or (iv) showing an inconclusive or no response.
3.2.4. LPI modulates the frequency of spontaneous Ca²⁺ events.

In Section 3.2.2, it was shown that LPI diminishes neuronal spontaneous Ca²⁺ events. It was of interest to observe this effect in greater detail. Figure 3.5A shows representative traces of independent neurons and their exposure to LPI (10 μM). In certain neuronal populations, LPI application diminished the frequency of neuronal spontaneous Ca²⁺ events (18.44±1.68 events) compared to vehicle (32.22±2.72 events, [t=8.310 df=17]; p<0.001, Paired Student’s t test, 18 neurons obtained from n=4 independent cultures; Figure 3.5i-ii). However, in other neuronal populations, LPI appeared to potentiate spontaneous Ca²⁺ activity (11.20±3.34 events) when neurons exhibited a minimal level of basal [Ca²⁺]i activity (4.70±1.86 events; [t=3.993 df=9]; p<0.01, Paired Student’s t test, 10 neurons obtained from n=3 independent cultures; Figure 3.5iii-iv).

The results here demonstrate that LPI modulates neuronal spontaneous Ca²⁺ activity in a differential manner depending on their basal [Ca²⁺]i activity.
Figure 3.5. LPI modulates the frequency of spontaneous Ca\(^{2+}\) events.

(i) Representative traces of neurons with diminished spontaneous Ca\(^{2+}\) events after exposure to LPI (10 µM). Each coloured trace represents one cell. Note the synchronous activity pattern. (ii) LPI diminishes neuronal spontaneous Ca\(^{2+}\) event frequency. Results are expressed as mean±S.E.M, ***p<0.001, Paired Student’s t test, 18 neurons obtained from n=4 independent cultures. (iii) Representative traces of neurons with amplified spontaneous Ca\(^{2+}\) events after exposure to LPI (10 µM). Each coloured trace represents one cell. Note the synchronous activity pattern. (iv) LPI amplifies neuronal spontaneous Ca\(^{2+}\) event frequency. Results are expressed as mean±S.E.M, **p<0.01, Paired Student’s t test, 10 neurons obtained from n=3 independent cultures.
3.2.5. The effect of LPI on tetrodotoxin-sensitive network activity in neurons.

As shown in Section 2.4.7, the Na$^+$-channel blocker, tetrodotoxin (TTX; 500 nM), abolished basal spontaneous Ca$^{2+}$ activity in neurons in a reversible manner. It was of interest to observe if LPI (10 µM) had a modulatory effect on TTX-sensitive network activity. In Figure 3.6i, one can observe representative traces of neurons exposed to TTX and LPI. In some neurons, TTX fails to completely abrogate spontaneous Ca$^{2+}$ activity, but upon TTX washout, the Ca$^{2+}$ events appear to be greater in amplitude and frequency. This may be due to LPI application during co-exposure to TTX, leading to amplification of intrinsic neuronal activity and potentiation of event amplitude and frequency following TTX washout. In Figure 3.6ii however, events do not appear to be effected by earlier LPI application following TTX washout. These are preliminary findings from n=2 independent cultures, so further experiments are required to validate the potential stimulatory effects of LPI on TTX-sensitive network activity.
Figure 3.6. The effect of LPI on tetrodotoxin-sensitive network activity in neurons.

Representative traces of cortical neurons exhibiting spontaneous $\text{Ca}^{2+}$ activity and their exposure to TTX (500 nM) and LPI (10 µM). Each trace represents one cell. (i) Spontaneous $\text{Ca}^{2+}$ activity following TTX washout appears to be greater in amplitude and frequency, potentially due to earlier LPI stimulation. (ii) No effect of LPI on oscillatory activity following TTX washout. Traces representative of 11 neurons obtained from $n=2$ independent cultures.
3.2.6. CID does not inhibit LPI-induced changes in \([\text{Ca}^{2+}]_i\) in neurons exhibiting spontaneous \(\text{Ca}^{2+}\) activity.

It was determined in Section 3.2.4 that LPI (10 µM) was capable of attenuating spontaneous \(\text{Ca}^{2+}\) activity in neurons. The next step was to elucidate if this effect was GPR55-mediated. A control experiment was initially performed by applying LPI (10 µM) alone to ensure the cells in that culture were responsive to LPI (Figure 3.7Ai). The next experiment was performed using a separate coverslip from the same culture and cells were treated with LPI in the presence of the selective GPR55 antagonist, CID16020046 (CID; 20 µM). Figure 3.7Aii shows a representative trace of a neuron obtained from a cortical neuron-enriched culture co-treated with CID and LPI. There were significant effects of treatment ([F(4, 32) = 2.210]; p<0.001, Repeated measures ANOVA, n=9 neurons obtained from n=2 independent cultures). Even in the presence of CID, neurons exhibited an attenuated \(\text{Ca}^{2+}\) event frequency upon exposure to LPI (18.78±4.41 events; Figure 3.7Aii, B) compared to vehicle (36.00±6.11 events, p<0.05) and CID application alone (42.11±4.70 events; p<0.01, Student Newman-Keuls). LPI was applied again without CID following a washout period in order to replicate the response, but produced no significant diminishment of events (18.11±2.83 events; p>0.05).

The results here demonstrate that LPI diminishes neuronal \(\text{Ca}^{2+}\) event frequency and that this effect is independent of GPR55.
Figure 3.7. CID does not inhibit LPI-induced changes in $[\text{Ca}^{2+}]_i$ in neurons exhibiting spontaneous Ca$^{2+}$ activity.

**A:** Representative traces of neurons exhibiting spontaneous Ca$^{2+}$ activity when they are exposed to (i) LPI (10 µM) only (control experiment) and (ii) CID (20 µM) and LPI (10 µM). Each trace represents one cell. **B:** The effect of CID and LPI on neuronal Ca$^{2+}$ event frequency. Results are expressed as mean±S.E.M, *p<0.05 vs vehicle, ++p<0.01 vs CID, Repeated measures ANOVA & Student Newman-Keuls, 9 neurons obtained from n=2 independent cultures.
3.2.7. LPI increases $\left[\text{Ca}^{2+}\right]_i$ in some quiescent neuron populations.

In Section 3.2.2, it was shown that LPI has a modulatory effect on quiescent neurons. It was of interest to observe this effect in greater detail. One can observe that LPI (10 μM) application induced heterogeneous changes in $\left[\text{Ca}^{2+}\right]_i$ in quiescent neurons. A representative neuronal trace of LPI inducing a latent and sustained increase in $\left[\text{Ca}^{2+}\right]_i$ can be observed in Figure 3.8i. A representative neuronal trace of LPI inducing repetitive and long-lasting $\text{Ca}^{2+}$ transients can be observed in Figure 3.8ii. A representative trace of neurons that were unresponsive to LPI or exhibited an inconclusive response can be observed in Figure 3.8iii. LPI-responsive cells were pooled together and Figure 3.8iv shows the maximum $\left[\text{Ca}^{2+}\right]_i$ response induced by LPI. LPI induced a significant increase in $\left[\text{Ca}^{2+}\right]_i$ (0.27± 0.03 units) compared to vehicle (0.16± 0.01 units; $t=3.756 \ df=14$; $p<0.01$, Paired Student’s $t$ test, 15 quiescent neurons obtained from n=3 independent cultures).

The results here demonstrate that LPI induced heterogeneous changes in $\left[\text{Ca}^{2+}\right]_i$ in quiescent neurons. Neurons that were responsive to LPI exhibited a significant increase in $\left[\text{Ca}^{2+}\right]_i$ compared to vehicle.
Figure 3.8. LPI increases [Ca^{2+}]_i in some quiescent neuron populations.

(i-iii) Representative traces of quiescent neurons exposed to LPI (10 µM). Each trace represents one cell. (i) Trace showing LPI-induced latent and sustained [Ca^{2+}]_i increase. (ii) Trace showing LPI-induced Ca^{2+} transients. (iii) Trace representing inconclusive or no effect. (iv) LPI significantly increases [Ca^{2+}]_i responses. Results are expressed as mean±S.E.M, **p<0.01, Paired Student’s t test, 15 quiescent neurons obtained from n=3 independent cultures.
3.2.8. LPI fails to induce an effect on $[\text{Ca}^{2+}]_i$ in the presence of CID in quiescent neurons.

It was determined in Section 3.2.7 that LPI (10 µM) induces an increase in $[\text{Ca}^{2+}]_i$ in quiescent neurons. The next step was to elucidate if these responses were GPR55-mediated. Figure 3.9i depicts a control experiment whereby LPI induces an increase in $[\text{Ca}^{2+}]_i$. The following experiment was then performed in the presence of the selective GPR55 antagonist CID16020046 (CID (20 µM); Figure 3.9ii). It was found that quiescent neurons failed to respond in the presence of CID alone or with co-application of LPI. To ensure that LPI was capable of inducing responses, it was applied alone after a washout period. However, LPI failed to induce any responses in quiescent neurons (Figure 3.9ii). It is difficult to accurately determine from these findings if CID has an effect on cell responsiveness to LPI – this can be attributed to the heterogeneity of culture responsiveness, with only 16 quiescent neurons out of 55 neurons total (29%; n=5 independent cultures) responding to LPI (see Section 3.2.3).
Figure 3.9. LPI fails to induce an effect on \([\text{Ca}^{2+}]_i\) in the presence of CID in quiescent neurons.

(i-ii) Representative traces of quiescent neurons recorded from independent cortical neuron-enriched cell cultures. Each trace represents one cell. (i) Control experiment with LPI (10 µM) treatment only. (ii) CID (20 µM) and LPI co-application. Neither ligand induced responses. 14 quiescent neurons obtained from n=3 independent cultures.
3.2.9. The effect of LPI on neuronal \([\text{Ca}^{2+}]_i\) following intracellular store depletion.

Thapsigargin (TSG) has previously been shown to irreversibly deplete \(\text{Ca}^{2+}\) stores in hGPR55-HEK293 cells (Henstridge et al., 2009). It was therefore of interest to investigate the effects of TSG in neurons derived from primary rat cortical neuron-enriched cell cultures.

In control cells, LPI (10 μM) induced an increase in \([\text{Ca}^{2+}]_i\) (Figure 3.10i). In separate experiments using separate coverslips from control, but from the same culture, KCl (30 mM) was applied initially to differentiate between neurons and glia. KCl was applied at the beginning of experiments that intentionally interfered with \([\text{Ca}^{2+}]_i\) levels (i.e. store depletion) because there was no guarantee KCl would prove effective after direct \([\text{Ca}^{2+}]_i\) manipulation.

After KCl application and washout, cells were treated with LPI to determine if they were responsive to it. The neurons from the cultures tested in this cohort did not show LPI-responsiveness on initial application. Nevertheless, TSG (5 μM) was applied following a washout period. TSG induced differential responses in neurons and LPI modulated these responses in distinctive ways. LPI induced a transient increase in \([\text{Ca}^{2+}]_i\) activity following TSG washout in 3 out of 25 total neurons (n=3 independent cultures; Figure 3.10ii). However, in the majority of neurons, LPI failed to modulate \([\text{Ca}^{2+}]_i\) before or after TSG application. TSG induced an elevation in \([\text{Ca}^{2+}]_i\) followed by a slow decrease during the washout period. \(\text{Ca}^{2+}\) levels eventually stabilised but did not return to baseline levels (22 out of 25 total neurons from n=3 independent cultures; Figure 3.10iii,).

The results here demonstrate that LPI does not induce a change in \(\text{Ca}^{2+}\) activity following store depletion in the majority of neurons. This indicates that LPI-induced changes in \([\text{Ca}^{2+}]_i\) under normal circumstances requires \(\text{Ca}^{2+}\)-containing stores. However, in a small minority of neurons, LPI has a modulatory effect on neuronal \([\text{Ca}^{2+}]_i\) even after the depletion of ER
stores, suggesting that in rare circumstances the effects of LPI may not be wholly store-mediated.

Figure 3.10. The effect of LPI on neuronal [Ca\(^{2+}\)]\(_i\) following intracellular store depletion.

(i-iv) Representative traces of neurons recorded from independent cortical neuron-enriched cell cultures. Each trace represents one cell. (i) Control experiment with LPI (10 µM) treatment only. (ii) LPI induces a transient increase in [Ca\(^{2+}\)]\(_i\) following TSG (5 µM) application (red). (iii) TSG depletes stores and LPI has no modulatory effect. KCl (30 mM) was applied at the beginning of these experiments to differentiate the cell types, but the responses have been omitted for clarity. Cell traces (ii-iii) representative of neurons obtained from n=3 independent cultures (25 total neurons).
3.2.10. The effect of LPI on neuronal \([Ca^{2+}]_i\) in the absence of extracellular Ca\(^{2+}\).

LPI has previously been shown to induce a singular \([Ca^{2+}]_i\) transient in hGPR55-HEK293 cells in the absence of extracellular Ca\(^{2+}\) ([Ca\(^{2+}\)_e]), indicating that the internal ER stores were responsible for the \([Ca^{2+}]_i\) increase (Henstridge et al., 2009). It was therefore of interest to investigate if LPI requires [Ca\(^{2+}\)_e] in order to induce changes in neuronal [Ca\(^{2+}\)_i].

Extracellular \(Ca^{2+}\) was depleted by perfusing cells from cortical neuron-enriched cultures with Ca\(^{2+}\)-free HBS. In control cells, LPI (10 \(\mu M\) in Ca\(^{2+}\)-containing HBS) induced an increase in \([Ca^{2+}]_i\) (Figure 3.11i). In a separate experiments using separate coverslips from control, but from the same culture, KCl (30 mM in Ca\(^{2+}\)-containing HBS) was initially applied to differentiate between neurons and glia. KCl was applied at the beginning of experiments that intentionally interfered with [Ca\(^{2+}\)_i] levels (i.e. [Ca\(^{2+}\)_e] depletion) because there was no guarantee KCl would prove effective after direct [Ca\(^{2+}\)_i] manipulation.

After KCl application and washout, neurons were perfused with Ca\(^{2+}\)-free HBS. LPI (10 \(\mu M\)) in Ca\(^{2+}\)-free HBS was applied. The majority of neurons exhibited no response to LPI in the presence or absence of Ca\(^{2+}\) (23 out of 30 total neurons obtained from \(n=3\) independent cultures; Figure 3.11iii). In a small minority of neurons, LPI did not induce an increase in \([Ca^{2+}]_i\) in the presence of Ca\(^{2+}\)-free HBS but subsequently stimulated a \([Ca^{2+}]_i\) increase when [Ca\(^{2+}\)_e] was re-introduced (7 out of 30 total neurons obtained from \(n=3\) independent cultures; Figure 3.11ii). This indicates that in this small minority, LPI was only capable of inducing a response in the presence of [Ca\(^{2+}\)_e].

The results here demonstrate that LPI does not induce a response in the majority of neurons in the presence or absence of [Ca\(^{2+}\)_e]. However, it is potentially capable of stimulating Ca\(^{2+}\) in a small population upon the re-introduction of [Ca\(^{2+}\)_e].
Figure 3.1. The effect of LPI on neuronal [Ca\textsuperscript{2+}]i in the absence of extracellular Ca\textsuperscript{2+}.

(i-iv) Representative traces of neurons recorded from independent cortical neuron-enriched cell cultures. Each trace represents one cell. (i) Control experiment with LPI (10 µM) treatment only. (ii) LPI induces a latent increase in [Ca\textsuperscript{2+}]i in the presence of [Ca\textsuperscript{2+}]e. (iii) LPI has no effect on [Ca\textsuperscript{2+}]i in the presence or absence of [Ca\textsuperscript{2+}]e. KCl (30 mM) was applied at the beginning of these experiments to differentiate the cell types, but the responses have been omitted for clarity. Cell traces (ii-iii) representative of neurons obtained from n=3 independent cultures (30 total neurons).
3.2.11. LPI (10 μM) induces heterogeneous changes in [Ca$^{2+}$]i in glia.

KCl was applied during ratiometric Ca$^{2+}$ imaging experiments in order to differentiate between neurons and glia. Glia are non-excitable cells (Kuffler, 1967), so therefore do not respond to KCl. GPR55 is expressed in primary microglia (Pietr et al., 2009) so it was of interest to examine glia obtained from cortical neuron-enriched cultures using ratiometric Ca$^{2+}$ imaging in order to determine the effect of GPR55 ligands on changes in glial [Ca$^{2+}$]i.

Representative glial cell traces obtained from independent cortical neuron-enriched cultures stimulated with the endogenous GPR55 agonist LPI (10 μM) can be observed in Figure 3.12Ai-ii. LPI application induced heterogeneous changes in [Ca$^{2+}$]i. These changes could be divided into separate categories: (1) Increase in [Ca$^{2+}$]i activity (23 out of 56 total glia from n=6 independent cultures; Figure 3.12Ai, B) or (2) inconclusive or no effect (33 out of 56 total glia from n=6 independent cultures; Figure 3.12Aii, iii).

LPI-responsive glia were further analysed to determine the maximum [Ca$^{2+}$]i response induced. LPI typically induced a latent and sustained increase in [Ca$^{2+}$]i (Figure 3.23Ai). LPI-responsive glia were pooled together and Figure 3.12iii shows the maximum [Ca$^{2+}$]i response induced by LPI. LPI induced a significant increase in [Ca$^{2+}$]i (0.24±0.01 units) compared to vehicle (0.17±0.01 units; [t=4.275 df=23]; p<0.001, Paired Student’s t test, 19 glia obtained from n=3 independent cultures).

The results here demonstrate that LPI induces increases in [Ca$^{2+}$]i in certain populations of glial cells.
(i) Representative traces of glial cell recordings from independent cortical neuron-enriched cell cultures. Each trace represents one cell. Cells were exposed to LPI (10 µM) and later KCl (50 mM) to differentiate between neurons and glia, but the KCl application has been omitted for clarity. The traces represent (i) LPI-induced increase in \([\text{Ca}^{2+}]_i\) activity and when (ii) there is an inconclusive or no effect of LPI. (iii) The maximum response height induced by LPI. Results are expressed as mean±SEM, ***p<0.001, Paired Student’s t test, 19 glia obtained from n=3 independent cultures.

Figure 3.12. LPI (10 µM) induces increases in \([\text{Ca}^{2+}]_i\) in glia.
3.2.12. The majority of LPI- and CCh-induced responses are not co-localised in glia.

It was determined in Section 3.2.11 that LPI (10 µM) was capable of inducing changes in 
$[\text{Ca}^{2+}]_i$ in glia. LPI is the endogenous agonist at GPR55, but it was important to include a 
GPCR comparator in Ca$^{2+}$ imaging experiments to compare ligand-induced responses. The 
muscarinic acetylcholine receptor (mACHR) is a GPCR that is expressed by astroglia in the 
cortex of young and aged rats (Van der Zee et al., 1993). Carbachol (CCh; 10 µM) is a 
mACHR agonist so was used in the present study to compare to LPI-induced responses. 
Representative traces of glial cells treated with LPI and CCh can be observed in Figure 3.13- 
iv. It was found that only 3 out of 30 total glia showed responses to both LPI and CCh 
(Figure 3.13i, n=4 independent cultures). The other cells could be further subdivided into 
LPI-responsive only (5 out of 30 total glia, n=4 independent cultures; Figure 3.13ii); CCh- 
responsive only (7 out of 30 total glia, n=4 independent cultures; Figure 3.13iii) or 
inconclusive or no response (15 out of 30 total glia, n=4 independent cultures; Figure 
3.13iv).

The results here demonstrate that only a small minority of glial cells exhibit responses to LPI 
and the mACHR agonist, CCh. However, responsiveness to LPI only or CCh only was 
similar (17% vs 23% respectively), indicating that the distribution of the target for LPI and 
mACHRs in glia varies across populations in cortical neuron-enriched cultures.
Figure 3.13. The majority of LPI- and CCh-induced responses are not co-localised in glia.

(i) Representative traces of glial cells responsive to (i) LPI (10 µM) and CCh (10 µM); (ii) LPI only, (iii) CCh only, or (iii) showing an inconclusive or no response. 30 glial cells obtained from n=4 independent cultures.
3.2.13. The effect of LPI in the presence of CID in glia.

It was determined in Section 3.2.11 that LPI (10 µM) induces an increase in [Ca$^{2+}$]$_i$ in a subset of glia. The next step was to elucidate if these responses were GPR55-mediated. Figure 3.14i depicts a control experiment whereby LPI induces a latent increase in [Ca$^{2+}$]$_i$. In separate experiments using coverslips from the same culture, cells were exposed to the selective GPR55 antagonist, CID16020046 (CID; 20 µM; Figure 3.14ii-iii). It was found that the majority of glia analysed failed to respond to CID alone or with co-application of LPI (22 out of 24 total glia from n=3 independent cultures; Figure 3.14ii).

In 2 out of 24 total glia (n=3 independent cultures), LPI caused a sharp decrease in [Ca$^{2+}$]$_i$ which eventually increased and overshot the baseline (Figure 3.14iii). This response was not GPR55-mediated because this decrease occurred both in the presence of CID and again following a washout period when LPI was applied alone. In the inset, the red arrow signifies the cell that generated the ratiometric trace (Figure 3.14iii).

The results here demonstrate that LPI failed to induce a response in the majority of glial cells, thereby making it difficult to determine if CID is an effective inhibitor. However, in a small minority of glia, CID did not inhibit LPI-induced responses.
Figure 3.14. The effect of LPI in the presence of CID in glia.

(i-iii) Representative traces of glia recorded from independent cortical neuron-enriched cell cultures. Each trace represents one cell. (i) Control experiment with LPI (10 μM) treatment only. (ii-iii) CID (20 μM) and LPI co-application. (ii) CID and LPI do not modulate \([\text{Ca}^{2+}]_i\) activity. (iii) CID does not inhibit LPI-induced decreases in \([\text{Ca}^{2+}]_i\) in 2 out of 24 total glia. The red arrow in the inset indicates the cell that produced the trace. Cell traces (ii-iii) representative of glia obtained from n=3 independent cultures (25 total glia).
3.2.14. The effect of LPI on glial \([Ca^{2+}]_i\) following intracellular store depletion.

Thapsigargin (TSG) has previously been shown to induce a gradual increase in \([Ca^{2+}]_i\) in glial cells that is consistent with SERCA inhibition and \(Ca^{2+}\) leakage from the ER into the cytoplasm (Charles et al., 1993). It was therefore of interest to investigate the effects of TSG in glial cells derived from primary rat cortical neuron-enriched cell cultures and to determine if GPR55 ligands could stimulate a response following store depletion.

In control cells, LPI (10 \(\mu\text{M}\)) induced a latent and sustained increase in glial \([Ca^{2+}]_i\) (Figure 3.15i). In a separate experiment using separate coverslips from control, but from the same culture, KCl (30 mM) was applied initially to differentiate between neurons and glia. KCl was applied at the beginning of experiments that intentionally interfered with \([Ca^{2+}]_i\) levels (i.e. store depletion) because there was no guarantee KCl would prove effective after direct \([Ca^{2+}]_i\) manipulation.

After KCl application, cells were treated with LPI to determine if they were responsive to it. The glia from the cultures tested in this cohort did not show LPI-responsiveness on first application. Nevertheless, TSG (5 \(\mu\text{M}\)) was applied following a washout period. TSG predominantly induced rapid and sustained release of \(Ca^{2+}\) that gradually stabilised. LPI modulated the TSG-induced release of \(Ca^{2+}\) in distinctive ways. LPI induced a transient increase in \([Ca^{2+}]_i\) activity after TSG was washed out and \([Ca^{2+}]_i\) levels had stabilised (5 out of 17 total glia from \(n=3\) independent cultures; Figure 3.15ii). In the majority of glia however, LPI failed to modulate \([Ca^{2+}]_i\) before or after TSG application (12 out of 17 total glia from \(n=3\) independent cultures; Figure 3.15iii).

The results here demonstrate that in the majority of glia, LPI fails to induce a \(Ca^{2+}\) response following store depletion, indicating that stores are required for responses. However, in a small minority of glia, LPI has a modulatory effect on glial \([Ca^{2+}]_i\) even after the depletion of
ER stores. This suggests that the effects of LPI may not be wholly store-mediated in these cells.
Figure 3.15. The effect of LPI on glial \([\text{Ca}^{2+}]_i\) following intracellular store depletion.

(i-ii) Representative traces of glia recorded from independent cortical neuron-enriched cell cultures. Each trace represents one cell. (i) Control experiment with LPI (10 \(\mu\text{M}\)) treatment only. (ii) LPI modulates \([\text{Ca}^{2+}]_i\) following TSG (5 \(\mu\text{M}\)) application, stabilising \([\text{Ca}^{2+}]_i\) levels and causing a transient increase (red). (iii) TSG depletes stores and LPI induces no modulatory effect. KCl (30 mM) was applied at the beginning of these experiments to differentiate the cell types but the application has been omitted for clarity. Cell traces (ii-iii) representative of neurons obtained from \(n=3\) independent cultures (17 total neurons).
3.2.15. The effect of LPI on glial [Ca\(^{2+}\)]\(_i\) in the absence of extracellular Ca\(^{2+}\).

Extracellular [Ca\(^{2+}\)] was depleted by perfusing cells from cortical neuron-enriched cultures with Ca\(^{2+}\)-free HBS. In control cells, LPI (10 μM in Ca\(^{2+}\)-containing HBS) induced an increase in [Ca\(^{2+}\)]\(_i\) (Figure 3.16i). In a separate experiments using separate coverslips from control, but from the same culture, KCl (30 mM in Ca\(^{2+}\)-containing HBS) was initially applied to differentiate between neurons and glia. KCl was applied at the beginning of experiments that intentionally interfered with [Ca\(^{2+}\)]\(_i\) levels (i.e. [Ca\(^{2+}\)]\(_e\) depletion) because there was no guarantee KCl would prove effective after direct [Ca\(^{2+}\)]\(_i\) manipulation.

After KCl application and washout, glia were perfused with Ca\(^{2+}\)-free HBS. LPI (10 μM) in Ca\(^{2+}\)-free HBS was applied. In some glia, LPI did not induce an increase in [Ca\(^{2+}\)]\(_i\) in the presence of Ca\(^{2+}\)-free HBS. However, LPI did stimulate an [Ca\(^{2+}\)]\(_i\) increase when [Ca\(^{2+}\)]\(_e\) was re-introduced (5 out of 31 total glia obtained from n=3 independent cultures; Figure 3.16ii). Other glia exhibited no response to LPI in the presence or absence of Ca\(^{2+}\) (26 glia out of 31 total glia obtained from n=3 independent cultures; Figure 3.16iii).

The results here demonstrate that LPI does not induce a response in the majority of glia in the presence or absence of [Ca\(^{2+}\)]\(_e\). However, it is potentially capable of stimulating Ca\(^{2+}\) in a small population of glia upon the re-introduction of [Ca\(^{2+}\)]\(_e\).
Figure 3.16. The effect of LPI on glial [Ca\(^{2+}\)]\(_i\) in the absence of extracellular Ca\(^{2+}\).

(i-iv) Representative traces of glia recorded from independent cortical neuron-enriched cell cultures. Each trace represents one cell. (i) Control experiment with LPI (10 µM) treatment only. (ii) LPI induces a latent increase in [Ca\(^{2+}\)]\(_i\) in the presence of [Ca\(^{2+}\)]\(_e\). (iii) LPI has no effect on [Ca\(^{2+}\)]\(_i\) in the presence or absence of [Ca\(^{2+}\)]\(_e\). KCl (30 mM) was applied at the beginning of these experiments to differentiate the cell types, but the application has been omitted for clarity. Cell traces (ii-iii) representative of glia obtained from n=3 independent cultures (31 total glia).
The selective GPR55 ligand 17g causes internalisation of GPR55 in the hGPR55-HEK293 cell line.

Previous data obtained by the Irving laboratory has shown that GPR55 is localised to the membrane of unstimulated HEK293 cells overexpressing 3xHA-hGPR55 (hGPR55-HEK293 cell line). LPI (1 µM) stimulation for 60 min causes internalisation of GPR55 in these cells. Control HEK293 cells do not express GPR55 (Henstridge et al., 2010). To determine the specificity of the novel and selective GPR55 agonist, 17g (Yrjölä et al., 2016), hGPR55-HEK293 cells were pre-labelled for 30 min with anti-hemagglutinin (HA) antibody and then stimulated with 1 µM 17g for 60 min. The cells were then fixed with 4% PFA and processed for surface and intracellular HA-immunoreactivity.

In Figure 3.17, one can observe that HA-tagged GPR55 localises to the cell membrane of unstimulated hGPR55-HEK293 cells, which is consistent with previous data. Stimulation with 17g causes internalisation of GPR55, which is consistent with the effects of GPCR stimulation (von Zastrow & Kobilka, 1992).

The results here demonstrate that 17g induces the internalisation of GPR55 in overexpressing HEK293 cells.
Figure 3.17. 17g stimulates GPR55 internalisation in hGPR55-HEK293 cells.

Representative confocal images of HEK293 cells stably expressing 3xHA-GPR55 and stained for HA (green). Scale bar = 20 µm.
3.2.17. 17g induces heterogeneous changes in $[\text{Ca}^{2+}]_i$ in neurons obtained from cortical neuron-enriched cultures.

17g (1 µM) application induced heterogeneous changes in neuronal $[\text{Ca}^{2+}]_i$ (Figure 3.18i-iii). These changes could be divided into separate categories: (1) Amplification of $[\text{Ca}^{2+}]_i$ events in neurons exhibiting spontaneous $\text{Ca}^{2+}$ activity (12 out of 58 total neurons from $n=5$ independent cultures; Figure 3.18i), (2) Increase in $[\text{Ca}^{2+}]_i$ activity in quiescent neurons (13 out of 58 total neurons from $n=5$ independent cultures; Figure 3.18ii) or (3) an inconclusive effect i.e. any changes in $[\text{Ca}^{2+}]_i$ could not be definitively associated with 17g application, or no effect (33 out of 58 total neurons from $n=5$ independent cultures; Figure 3.18iii). In the former two categories, it was interesting to note that 17g increased $[\text{Ca}^{2+}]_i$ responses.
Figure 3.18. 17g induces heterogeneous changes in [Ca^{2+}]_i in neurons obtained from cortical neuron-enriched cultures.

(i-iii) Representative traces of neuronal cell recordings from independent cortical neuron-enriched cell cultures. Each trace represents one cell. Cells were exposed to 17g (1 µM) and later KCl (50 mM) to differentiate between neurons and glia, but the KCl response has been omitted for clarity. The traces represent 17g-induced increases in [Ca^{2+}]_i activity in (i) neurons exhibiting spontaneous Ca^{2+} activity and (ii) quiescent neurons and when (iii) there is an inconclusive or no effect of 17g. Cell traces representative of neurons obtained from n=5 independent cultures (58 total neurons).
3.2.18. 17g- and CCh-induced responses are not co-localised in neurons.

It was determined in Section 3.2.17 that 17g (1 µM) was capable of inducing changes in $[Ca^{2+}]_i$ in neurons. Carbachol (CCh; 10 µM) is a mAChr agonist so was used in the present study to compare with 17g-induced responses. Figure 3.19i-iii shows representative traces of neurons treated with 17g and CCh. It was found that in the cohort of neurons examined, no cells exhibited responses to both 17g and CCh. The other cells did respond to 17g-responsive only (2 out of 42 total neurons Figure 3.19i), CCh-responsive only (21 out of 42 total neurons; Figure 3.19ii), or inconclusive or no response (19 out of 42 total neurons; Figure 3.19iii). The low responsiveness to 17g only in this cohort is substantially different to the cohort of neurons in Section 3.2.17 (44% responsiveness). This demonstrates the variability in responsiveness to 17g among independent cortical neuron-enriched cultures.

The results here demonstrate that in this cohort of neurons from cortical neuron-enriched cultures, there were no co-localised responses to 17g and the mAChr agonist, CCh. However, the majority of cells exhibited responsiveness to CCh alone.
Figure 3.19. 17g- and CCh-induced responses are not co-localised in neurons.

(i) Representative traces of neurons responsive to (i) 17g (1 µM) only, (ii) CCh (10 µM) only (iii) or showing an inconclusive or no response. Cell traces representative of neurons obtained from n=4 independent cultures (42 total neurons).
3.2.19. 17g amplifies spontaneous Ca\textsuperscript{2+} activity.

In Section 3.2.17, it was shown that 17g has a modulatory effect on neurons exhibiting spontaneous Ca\textsuperscript{2+} events. It was of interest to observe this effect in greater detail. Representative cell traces of two individual neurons exhibiting spontaneous Ca\textsuperscript{2+} activity when treated with 17g (1 μM) can be observed in Figure 3.20A. In Figure 3.20B, one can observe that 17g application significantly increased the frequency of spontaneous Ca\textsuperscript{2+} activity (25.64±4.34 events) compared to vehicle (15.18±4.27 events; [t=6.475 df=10]; ***p<0.001, Paired Student’s t test, 11 neurons obtained from n=3 independent cultures).

The results here demonstrate that 17g amplifies the frequency of spontaneous Ca\textsuperscript{2+} events.
Figure 3.20. 17g amplifies spontaneous Ca$^{2+}$ activity.

A: Representative traces of neurons with amplified spontaneous Ca$^{2+}$ events after exposure to 17g (1 µM). Each coloured trace represents one cell. Note the synchronous activity pattern. B: The effect of 17g on neuronal spontaneous Ca$^{2+}$ event frequency. Results are expressed as mean±S.E.M, **p<0.01, Paired Student’s t test, 11 neurons obtained from n=3 independent cultures.
3.2.20. **The effect of 17g on \([\text{Ca}^{2+}]_i\) in quiescent neurons.**

In Section 3.2.18, it was shown that 17g has a modulatory effect on quiescent neurons. It was of interest to observe this effect in greater detail. Representative cell traces of quiescent neurons obtained from cortical neuron-enriched cultures stimulated with 17g (1 μM) can be observed in Figure 3.21. One can observe that 17g application induced heterogeneous changes in \([\text{Ca}^{2+}]_i\) in quiescent neurons. 17g induced long-lasting and repetitive \(\text{Ca}^{2+}\) transients in some neurons (Figure 3.21i). 17g was also capable of inducing a latent and sustained increase in \([\text{Ca}^{2+}]_i\) (Figure 3.21ii). Other cells were unresponsive to 17g or exhibited an inconclusive response (Figure 3.21iii).

Each trace shown in Figure 3.21 was obtained from separate cultures. The traces show the variability of responses induced by 17g from culture to culture. For this reason, responses were not pooled together for further statistical analyses.
Figure 3.1. 17g modulates \([\text{Ca}^{2+}]_i\) in quiescent neurons.

(i-iv) Representative traces of quiescent neurons from cortical neuron-enriched cultures exposed to 17g (1 µM). Each coloured trace represents one cell. (i) Independent traces showing repetitive 17g-induced \([\text{Ca}^{2+}]_i\) transients. Note the synchronisation in activity.
(ii) Trace showing 17g-induced latent and sustained \([\text{Ca}^{2+}]_i\) increase.
(iii) Trace representing inconclusive or no effect.
3.2.21. CID inhibits 17g-induced [Ca\(^{2+}\)]i in certain neuronal populations.

It was determined in Section 3.2.17 that 17g (1 µM) induced heterogeneous changes in neuronal [Ca\(^{2+}\)]i. The next step was to elucidate if these effects were GPR55-mediated.

A control experiment was performed first using 17g (1 µM) alone to ensure cells in that culture were responsive to 17g (Figure 3.22i). In separate experiments using separate coverslips from control, but from the same culture, representative traces of neurons were obtained from cortical neuron-enriched cultures co-treated with the selective GPR55 antagonist CID16020046 (CID; 20 µM) and 17g (1 µM; Figure 3.22ii-ii). It was found that CID inhibited 17g-induced changes in [Ca\(^{2+}\)]i in certain populations of neurons (8 out of 22 neurons total obtained from n=3 independent cultures; Figure 3.22).

Figure 3.22ii-iii represents neuronal traces obtained from each individual culture analysed in this study to demonstrate the variability of responses induced in the cultures. In Figure 3.22ii, basal neuronal [Ca\(^{2+}\)]i was spontaneous. 17g did not modulate [Ca\(^{2+}\)]i activity in the presence of CID, but 17g did interfere with the pattern of event frequency when applied again alone following a washout period. In Figure 3.22iii, basal neuronal [Ca\(^{2+}\)]i events were also spontaneous but CID alone appeared to diminish event frequency. There appeared to be small spikes of activity in the presence of 17g and CID but the neurons did not exhibit the typical amplification in activity seen with 17g alone. Furthermore, subsequent application of 17g alone following a washout period robustly increased Ca\(^{2+}\) event activity, suggesting that CID effectively attenuated 17g-induced [Ca\(^{2+}\)]i activity in the neurons from this culture. The other neurons from these cultures showed an inconclusive effect or no effect upon co-exposure to CID and 17g (14 out of 22 neurons total obtained from n=3 independent cultures). Since the profiles of Ca\(^{2+}\) activity varied from culture to culture, statistical analyses that accurately reflected these data could not be performed.
The results here demonstrate the 17g modulates [Ca$^{2+}$]i activity in a GPR55-dependent manner in certain neuronal populations.
Figure 3.22. CID inhibits 17g-induced \([\text{Ca}^{2+}]_i\) in certain neuronal populations.

(i-iv) Representative traces of neurons recorded from independent cortical neuron-enriched cell cultures. Each trace represents one cell.

(i) Control experiment with 17g (1 \(\mu\text{M}\)) treatment only. (ii-iii) CID (20 \(\mu\text{M}\)) and 17g co-application. Each trace represents one cell from a separate culture to demonstrate the variability of effects induced. Cell traces (ii-iii) representative of 8 neurons obtained from \(n=3\) independent cultures (22 total neurons).
3.2.22. The effect of 17g on neuronal [Ca^{2+}]i following intracellular store depletion.

It was demonstrated in Section 3.2.9 that LPI in some neuronal populations had a modulatory effect on neuronal [Ca^{2+}]i even following store depletion with thapsigargin (TSG). It was of interest to determine if the novel and selective agonist, 17g, also had a modulatory effect on neurons derived from primary rat cortical cell cultures following store depletion with TSG.

In control cells, 17g (1 μM) induced an increase in [Ca^{2+}]i (Figure 3.23i). In separate experiments using separate coverslips from control, but from the same culture, KCl (30 mM) was applied initially to differentiate between neurons and glia. KCl was applied at the beginning of experiments that intentionally interfered with [Ca^{2+}]i levels (i.e. store depletion) because there was no guarantee KCl would prove effective after direct [Ca^{2+}]i manipulation.

After KCl application and washout, cells were treated with 17g to determine if they were responsive to it. Neurons did not show responsiveness with initial 17g application (Figure 3.23i-iv). TSG (5 μM) was applied following a washout period and induced a sustained increase in [Ca^{2+}]i that did not return to baseline (Figure 3.23ii-iv). This is indicative of store depletion. 17g modulated [Ca^{2+}]i following store depletion in distinctive ways. Some neurons exhibited an increase in [Ca^{2+}]i when treated with 17g, even following store depletion (4 out of 20 neurons obtained from n=3 independent cultures; Figure 3.23ii). In the majority of neurons however, 17g failed to modulate [Ca^{2+}]i after TSG application (16 out of 20 neurons from n=3 independent cultures; Figure 3.23iii-iv). There were two basal [Ca^{2+}]i profiles in the latter instance: quiescent neurons, whereby TSG depleted stores and subsequent 17g application did not notably modulate [Ca^{2+}]i levels (Figure 3.23iii) or neurons exhibiting spontaneous Ca^{2+} activity, whereby TSG and subsequent 17g application did not modulate the frequency of Ca^{2+} events (Figure 3.23iv).
The results here demonstrate that in the majority of neurons, 17g cannot induce changes in 
$[\text{Ca}^{2+}]_i$ activity following store depletion. However, in a small minority of neurons, 17g has a 
modulatory effect on neuronal $[\text{Ca}^{2+}]_i$ even after the depletion of ER stores. This suggests 
that the effects of 17g may not be wholly store-mediated in certain cells.
Figure 3.23. The effect of 17g on neuronal [Ca\textsuperscript{2+}]\textsubscript{i} following intracellular store depletion.

(i-iv) Representative traces of neurons recorded from independent cortical neuron-enriched cell cultures. Each trace represents one cell. (i) Control experiment with 17g (1 µM) treatment only. (ii) 17g induces transient [Ca\textsuperscript{2+}]\textsubscript{i} increases following TSG application (red). (iii) TSG depletes stores and subsequent 17g application does not modulate [Ca\textsuperscript{2+}]\textsubscript{i} levels. (iv) TSG and subsequent 17g application does not modulate neuronal spontaneous Ca\textsuperscript{2+} activity. KCl (30 mM) was applied at the beginning of these experiments to differentiate the cell types, but the responses have been omitted for clarity. Cell traces (ii-iv) representative of neurons obtained from n=3 independent cultures (20 total neurons).
3.2.23. The effect of 17g on neuronal [Ca\textsuperscript{2+}]i in the absence of extracellular Ca\textsuperscript{2+}.

Extracellular [Ca\textsuperscript{2+}] was depleted by perfusing cortical cells with Ca\textsuperscript{2+}-free HBS. In control cells, 17g (1 μM in Ca\textsuperscript{2+}-containing HBS) induced an increase in [Ca\textsuperscript{2+}]i (Figure 3.24i). In separate experiments using separate coverslips from control, but from the same culture, KCl (30 mM in Ca\textsuperscript{2+}-containing HBS) was initially applied to differentiate between neurons and glia. KCl was applied at the beginning of experiments that intentionally interfered with [Ca\textsuperscript{2+}]i levels (i.e. [Ca\textsuperscript{2+}]e depletion) because there was no guarantee KCl would prove effective after direct [Ca\textsuperscript{2+}]i manipulation.

After KCl application and washout, neurons were perfused with Ca\textsuperscript{2+}-free HBS. 17g (1 μM) in Ca\textsuperscript{2+}-free HBS was applied. 17g induced a change in [Ca\textsuperscript{2+}]i only when [Ca\textsuperscript{2+}]e was re-introduced in some neurons (Figure 3.24ii). In the majority of neurons however, 17g did not induce an increase in [Ca\textsuperscript{2+}]i in the presence of Ca\textsuperscript{2+}-free HBS or later when [Ca\textsuperscript{2+}]e was re-introduced (Figure 3.24iii).

These preliminary results demonstrate that 17g does not induce changes in [Ca\textsuperscript{2+}]i in both the presence and absence of [Ca\textsuperscript{2+}]e in the majority of neurons. However, in a small minority of neurons, 17g induced Ca\textsuperscript{2+} increases following store depletion. More replicate experiments are required in order to validate this result.
(i) Control experiment with 17g (1 µM) treatment only. (ii) 17g induces a latent modulatory change in \([\text{Ca}^{2+}]_i\) in the presence of \([\text{Ca}^{2+}]_e\). (iii) 17g has no effect on \([\text{Ca}^{2+}]_i\) in the presence or absence of \([\text{Ca}^{2+}]_e\). KCl (30 mM) was applied at the beginning of these experiments to differentiate the cell types, but the responses have been omitted for clarity. Cell traces (ii-iii) representative of neurons obtained from \(n=2\) independent cultures (18 total neurons).
3.2.24. 17g (1 μM) does not stimulate [Ca^{2+}]i activity in glial cells.

Representative glial cell traces obtained from independent cortical neuron-enriched cultures stimulated with 17g (1 μM) can be observed in Figure 3.25. Cells exhibited no effect (37 glia from n=5 independent cultures).

The results here demonstrate that 17g does not stimulate [Ca^{2+}]i activity in glial cells.
Figure 3.25. 17g (1 μM) does not stimulate [Ca^{2+}]i activity in glial cells.

Representative trace of a glial cell recording from a cortical cell culture. Cells were exposed to 17g (1 μM) and later KCl (50 mM) to differentiate between neurons and glia, but the KCl application has been omitted for clarity. The trace represents no effect of 17g. 37 glia obtained from n=5 independent cultures.
3.2.25. Neuronal phospho-CREB is activated by the phorbol ester, TPA.

Stimulation of hGPR55-HEK293 cells using the endogenous GPR55 agonist LPI has previously been shown to induce CREB phosphorylation (Henstridge et al., 2010). Phospho-CREB activation in primary cortical neurons was assessed in the present study using immunocytochemistry and confocal microscopy.

Cells were pre-treated with 10 μM nifedipine to reduce basal pCREB expression before stimulating with ligands. Cells were double-labelled for pCREB and the neuronal somatodendritic marker MAP2 (Figure 3.26A). Stimulation of pCREB is denoted by bright green nuclear staining, whereas lack of pCREB stimulation is indicated by dark green nuclei. To ensure neurons were responsive, they were treated with the PKC activator TPA (100 nM) for 15 min as a positive control. TPA induced significant pCREB activation (2.68±0.35 units vs control, 1.00±0.13 units; p<0.001, Unpaired Student t test, 30 total neurons per treatment group from n=2 independent cultures; Figure 3.26B).

The results here demonstrate that the PKC activator, TPA, robustly stimulates pCREB. All subsequent experiments performed with GPR55 ligands also included a separate TPA treatment as a positive control.
Figure 3.26. Neuronal phospho-CREB is activated by the phorbol ester, TPA.

A: Representative confocal microscopy images taken at 60x magnification of cells from cortical neuron-enriched cultures treated with control and 100 nM TPA. pCREB = green, MAP2 = red. White arrows indicate phosphorylated CREB. Scale bar = 20 μm. 

B: The effects of TPA on nuclear pCREB expression in neurons. All data are represented as a fold change in pCREB fluorescence units from control. Results are expressed as mean±S.E.M, ***p<0.001 vs control, Unpaired Student’s t test, 30 neurons obtained from n=2 independent cultures.
3.2.26 LPI (10 μM) stimulates CREB phosphorylation in neurons.

Cells were pre-treated with 10 μM nifedipine for 45 min to reduce basal pCREB expression before stimulating with ligands. Cells were then treated with the GPR55 agonist, LPI (1 & 10 μM), for 5, 15, 30 min and 1 hr to determine at which time and concentration that CREB is the most robustly phosphorylated. Nifedipine remained present throughout and control was 10 μM nifedipine only. Cells were double-labelled for pCREB and the neuronal somatodendritic marker MAP2 (Figure 3.27A). Out of all timepoints tested, 15 min treatment (2.12±0.24 units, p<0.01) with LPI induced the most robust stimulation of pCREB (One-Way ANOVA & Student Newman-Keuls, 30 total neurons per treatment group from n=2 independent cultures; Figure 3.27B). LPI (10 μM) did not induce significant pCREB expression at 5 min treatment (1.50±0.23 units, p>0.05) or later timepoints (30 min = 0.98±0.12 units, 1 hr = 0.84±0.08 units, p>0.05) and LPI (1 μM) did not induce pCREB at all (5 min = 0.80±0.08 units, 15 min = 1.33±0.27 units, 30 min = 0.95±0.19 units, 1 hr = 0.94±0.12 units, p>0.05).

The results here demonstrate that LPI (10 μM) stimulates neuronal pCREB.
Figure 3.2. LPI (10 μM) stimulates CREB phosphorylation in neurons.

A: Representative confocal microscopy images taken at 60x magnification of neurons from cortical neuron-enriched cultures treated for increasing amounts of time with the endogenous GPR55 agonist, LPI. (i) control, (ii-v) 5 min-1 hr 1 μM LPI or (vi-ix) 5 min-1 hr 10 μM LPI. TPA was used as a positive control (data not shown). pCREB = green, MAP2 = red. White arrows indicate phosphorylated CREB. Scale bar = 20 μm.

B: The effect of LPI on nuclear pCREB expression in neurons. All data are represented as a fold change in pCREB fluorescence units from control (control level is represented by the dashed line). Results are expressed as mean±S.E.M, **p<0.01 vs control, One-way ANOVA & Student Newman-Keuls, 30 neurons from n=2 independent cultures.
3.2.27. LPI (10 µM) stimulates phospho-CREB in a GPR55-independent manner.

Cells from cortical neuron-enriched cultures were pre-treated with 10 µM nifedipine for 45 min to reduce basal pCREB expression before applying ligands. Cells were then treated with the selective GPR55 antagonist, CID (20 µM), alone for 30 min before co-incubating them with CID and LPI (10 µM) for a further 15 min. Nifedipine remained present throughout. Cells were double-labelled for pCREB and the neuronal somato-dendritic marker MAP2 (Figure 3.28A). There was a significant effect of agonist ([F (3, 116) = 13.55]; p<0.01, One-way ANOVA, 30 total neurons per treatment group from n=2 independent cultures). A post hoc test subsequently showed that LPI significantly stimulated pCREB (1.82±0.15 units) compared to control (1.00±0.13 units; p<0.01; Figure 3.28B) and this response was not inhibited by CID (2.19±0.25 units; p>0.05, Student Newman Keuls). CID failed to induce pCREB alone (0.94±0.08 units; p>0.05).

This result demonstrates that LPI induces CREB phosphorylation independently of GPR55.
Figure 3.28. LPI (10 µM) induces phospho-CREB in a GPR55-independent manner.

A: Representative confocal microscopy images taken at 60x magnification of neurons from cortical neuron-enriched cultures treated with (i) control, (ii) 10 µM LPI, (iii) 20 µM CID or (iv) CID+LPI. pCREB = green, MAP2 = red. TPA was used as a positive control (data not shown). White arrows indicate phosphorylated CREB. Scale bar = 20 µm. B: The effects of LPI and CID on nuclear pCREB expression in neurons. All data are represented as a fold change in pCREB fluorescence units from control. Results are expressed as mean±S.E.M, ***p<0.001 vs control, $$$p<0.001 vs CID, One-Way ANOVA & Student Newman-Keuls, 30 neurons from n=2 independent cultures.
3.2.28. $G_{\alpha_q}$ contributes to LPI (10 µM) stimulation of CREB phosphorylation pathway.

GPR55 stimulation in transiently-expressing hGPR55-HEK293 cells led to $Ca^{2+}$ release from intracellular stores in $G_q$-PLC- and $G_{12}$-RhoA-mediated pathways (Lauckner et al., 2008). The potential role of $G_{\alpha_q}$ in CREB phosphorylation was of interest to elucidate, particularly because stimulation of $G_{q/11}$-coupled GPCRs such as M3 muscarinic acetylcholine (mACh) and B2 bradykinin receptors can promote CREB phosphorylation in human SH-SY5Y neuroblastoma cells (Rosethorne, Nahorski & Challiss, 2008).

Cells from cortical neuron-enriched cultures were pre-treated with 10 µM nifedipine for 45 min to reduce basal pCREB expression before applying ligands. Cells were then treated with the $G_{\alpha_q}$ inhibitor, YM-254890 (1 µM), alone for 30 min before co-incubating them with YM-254890 and LPI (10 µM) for a further 15 min. Nifedipine remained present throughout. Cells were double-labelled for pCREB and the neuronal somato-dendritic marker MAP2 (Figure 3.29A). There was a significant interaction between inhibitor and agonist ($[F (3, 116) = 7.669]$; $p<0.001$, One-way ANOVA, 30 total neurons per treatment group from $n=2$ independent cultures). A post hoc test subsequently showed that LPI significantly stimulated pCREB (1.82±0.15 units) compared to control (1.00±0.13 units; $p<0.001$, Student-Newman Keuls, Figure 3.29B). YM-254890 attenuated LPI-induced pCREB but (1.61±0.16 units; $p<0.01$) but did not completely abolish it. YM-254890 failed to induce pCREB alone (1.36±0.12 units; $p>0.05$).

This result demonstrates that a $G_{\alpha_q}$ inhibitor reduced LPI-induced pCREB but did not completely abolish it, indicating that $G_{\alpha_q}$ contributes to this LPI-induced mechanism.
Figure 3.29. G\(\alpha_q\) contributes to LPI (10 \(\mu\)M) stimulation of CREB phosphorylation pathway.

A: Representative confocal microscopy images taken at 60x magnification of neurons from cortical neuron-enriched cultures treated with (i) control, (ii) 10 \(\mu\)M LPI, (iii) 1 \(\mu\)M YM-254890 (YM) or (iv) YM+LPI. pCREB = green, MAP2 = red. TPA was used as a positive control (data not shown). White arrows indicate phosphorylated CREB. Scale bar = 20 \(\mu\)m.

B: The effects of LPI and YM-254890 on nuclear pCREB expression in neurons. All data are represented as a fold change in pCREB fluorescence units from control. Results are expressed as mean\(\pm\)S.E.M, ***p<0.001 vs control, +++p<0.001 vs LPI, One-Way ANOVA & Student Newman-Keuls, 30 neurons from n=2 independent cultures.
3.2.29. The selective GPR55 agonist, 17g, stimulates CREB phosphorylation in a time-dependent manner in neurons.

Cells from cortical neuron-enriched cultures were pre-treated with 10 μM nifedipine for 45 min to reduce basal pCREB expression before applying ligands. Neurons from cortical were then treated with 17g (1 μM) for 5, 15, 30 min and 1 hr to determine at which time CREB is most robustly phosphorylated. Nifedipine remained present throughout and control was 10 μM nifedipine only. Cells were double-labelled for pCREB and the neuronal somatodendritic marker MAP2 (Figure 3.30A). Out of all timepoints tested, the later 30 min (2.07± 0.26 units) and 1 hr treatments (2.32± 0.20 units) with 17g (1 μM) induced the most robust stimulation of pCREB (Figure 3.30B, p<0.01, p<0.001 vs control, One-Way ANOVA & Student Newman-Keuls, 30 total neurons per treatment group from n=2 independent cultures), but an earlier 5 min timepoint also significantly induced pCREB (1.95± 0.24 units, p<0.05). Only 15 min treatment (1.50± 0.18 units, p>0.05) did not induce pCREB.

The results here demonstrate that 17g stimulates neuronal pCREB in a time-dependent manner.
Figure 3.30. 17g stimulates CREB phosphorylation in a time-dependent manner.

A: Representative confocal microscopy images taken at 60x magnification of neurons from cortical neuron-enriched cultures treated for increasing amounts of time the selective GPR55 agonist, 17g. (i) control or (ii-v) 5 min-1 hr 1 µM 17g. TPA was used as a positive control (data not shown). pCREB = green, MAP2 = red. White arrows indicate phosphorylated CREB. Scale bar = 20 µm. B: All data are represented as a fold change in pCREB fluorescence units from control (control level is represented by the dashed line). Results are expressed as mean±S.E.M, *p<0.05, **p<0.01, ***p<0.001 vs control, One-way ANOVA & Student Newman-Keuls, 30 neurons from n=2 independent cultures.
3.2.30. CID partially inhibits 17g-induced phospho-CREB

Given that the endogenous agonist LPI failed to stimulate pCREB via GPR55 (see Section 3.2.10), it was of interest to investigate the effect of the selective and novel GPR55 agonist 17g on pCREB stimulation.

Cells from cortical neuron-enriched cultures were pre-treated with 10 µM nifedipine for 45 min to reduce basal pCREB expression before applying ligands. The cells were then treated with the selective GPR55 antagonist CID16020046 (CID; 20 µM) for 30 min before co-incubating them with CID and 17g (1 µM) for a further 30 min. Nifedipine remained present throughout. Cells were double-labelled for pCREB and the neuronal somato-dendritic marker MAP2 (Figure 3.31A). There was a significant interaction between antagonist and agonist (F (3, 116) = 21.81; p<0.01, One-way ANOVA, 30 total neurons per treatment group from n=2 independent cultures). A post hoc test subsequently showed that 17g significantly stimulated pCREB (2.86±0.25 units) compared to control (1.00±0.13 units; p<0.001) and this response was partially inhibited by CID (1.74±0.24 units; p<0.001, Student Newman-Keuls, 30 neurons from n=2 independent cultures; Figure 3.31B). CID failed to induce pCREB alone (0.94±0.08 units; p>0.05, 30 neurons from n=2 independent cultures).

The results here demonstrate that CID partially inhibits 17g-induced neuronal CREB phosphorylation.
Figure 3.31. CID partially inhibits 17g-induced phospho-CREB.

A: Representative confocal microscopy images taken at 60x magnification of neurons from cortical neuron-enriched cultures treated with (i) control, (ii) 1 µM 17g, (iii) 20 µM CID or (iv) CID+17g. pCREB = green, MAP2 = red. TPA was used as a positive control (data not shown). White arrows indicate phosphorylated CREB. Scale bar = 20 µm. B: The effects of 17g and CID on nuclear pCREB expression in neurons. All data are represented as a fold change in pCREB fluorescence units from control. Results are expressed as mean±S.E.M, **p<0.01, ***p<0.001 vs control, +++p<0.001 vs 17g, $p<0.05$ vs CID, One-Way ANOVA & Student Newman-Keuls, 30 neurons from n=2 independent cultures.
3.2.31. 17g induces phospho-CREB via a G\textsubscript{q}-dependent pathway.

Cells were pre-treated with 10 μM nifedipine for 45 min to reduce basal pCREB expression before applying ligands. The cells were then treated with the G\textsubscript{q} inhibitor YM-254890 (1 μM) for 30 min before co-incubating them with YM-254890 and 17g (1 μM) for a further 30 min. Nifedipine remained present throughout. Cells were double-labelled for pCREB and the neuronal somato-dendritic marker MAP2 (Figure 3.32A). A Two-Way ANOVA showed a significant interaction between inhibitor and agonist ([F (3, 116) = 24.29]; p<0.001 One-way ANOVA, 30 neurons from n=2 independent cultures). A post hoc test subsequently showed that 17g significantly stimulated pCREB (2.86±0.25 units) compared to control (1.00±0.13 units; p<0.001) and this response was inhibited by YM-254890 (1.33±0.17 units; p<0.001, Student Newman-Keuls, 30 neurons from n=2 independent cultures; Figure 3.32B). YM-254890 also failed to induce pCREB alone (1.36±0.12 units; p>0.05, 30 neurons from n=2 independent cultures).

The results here demonstrate that 17g induces CREB phosphorylation in a G\textsubscript{q}-dependent manner.
Figure 3.17. 17g stimulates phospho-CREB via a Gαq-dependent pathway.

A: Representative confocal microscopy images taken at 60x magnification of neurons from cortical neuron-enriched cultures treated with (i) control, (ii) 1 µM 17g, (iii) 1 µM YM-254890 (YM) or (iv) YM+17g. pCREB = green, MAP2 = red. TPA was used as a positive control (data not shown). White arrows indicate phosphorylated CREB. Scale bar = 20 µm. B: The effects of 17g and YM-254890 on nuclear pCREB expression in neurons. All data are represented as a fold change in pCREB fluorescence units from control. Results are expressed as mean ± S.E.M, **p<0.001 vs control, +++p<0.001 vs 17g. One-Way ANOVA & Student Newman-Keuls, 30 neurons from n=2 independent cultures.
3.3. Discussion

The aim of this chapter was to investigate the expression of GPR55 and its involvement in downstream signalling pathways. GPR55 expression and internalisation was investigated through receptor labelling and the subsequent immunoreactivity was analysed using confocal microscopy. The effects of GPR55 stimulation on changes in [Ca$^{2+}$]$_i$ were investigated using ratiometric Ca$^{2+}$ imaging with the Ca$^{2+}$ indicator dye Fura-2 AM. Phospho-CREB (pCREB) activation in response to GPR55 stimulation was investigated using immunocytochemistry and confocal microscopy. The results here demonstrate that in cells from cortical neuron-enriched cultures, GPR55 is expressed in glia, in the soma of neurons and in a punctate localisation pattern along neuronal processes. LPI (10 μM) and 17 (1 μM) induced changes in [Ca$^{2+}$]$_i$ in neurons but the types of changes induced were disparate in nature. For instance, LPI was capable of both diminishing and potentiating neuronal spontaneous Ca$^{2+}$ activity, whereas 17g only amplified this activity. LPI induced changes in [Ca$^{2+}$]$_i$ in both neurons and glia and these responses were independent of GPR55, whereas 17g mainly induced changes in [Ca$^{2+}$]$_i$ in neurons in a GPR55-dependent manner. Gα$_q$ contributed to CREB phosphorylation induced by both agonists. 17g-induced pCREB was partially inhibited by a selective GPR55 antagonist, but LPI–induced pCREB was not GPR55-dependent.

3.3.1. GPR55 expression in neurons and glia

G-protein coupled receptors (GPCR) are found in eukaryotes and are localised to cell membranes. GPR55 is a Class-A GPCR coupled to Gα$_q/11$ and Gα$_{12/13}$ proteins (Henstridge et al., 2010; Lauckner et al., 2008; Ryberg et al., 2007). Preliminary findings from this study demonstrate that GPR55 is expressed in primary neurons and glia obtained from cortical neuron-enriched cultures. The findings herein complement the data reported by Cherif et al.
(2015), who demonstrated that GPR55 is expressed in murine cortical neuron somas and neurites. Furthermore, Lauckner et al. (2008) demonstrated that large diameter DRG neurons express GPR55. Interestingly, the processes of neurons exhibited punctate localisation of GPR55 in the present study, indicating that it may be expressed in synapses. Sylantyev et al. (2013) suggested that GPR55 was expressed pre-synaptically and had an adaptive role in Ca\textsuperscript{2+}-store–dependent short-term potentiation of hippocampal CA3-CA1 glutamate neurotransmission. In contrast, Hurst et al., (2017) suggested that GPR55 was most likely expressed post-synaptically because GPR55 mediated CA1 pyramidal cell LTP. However, they could not rule out a pre-synaptic role for GPR55. Immunolabelling of cells from the cortical neuron-enriched cell culture model used herein for markers such synaptophysin (pre-synaptic) or PSD-95 (post-synaptic) may clarify where GPR55 is localised. GPR55 also plays a key regulatory role in the peripheral immune system (Chiurchiù et al., 2015) and is expressed by murine microglia in the brain (Pietr et al., 2009). In the present study, it was demonstrated that microglia from cortical neuron-enriched cultures and the murine BV2 microglial cell line express GPR55.

The novel and selective agonist for GPR55, 17g, was utilised in this study (Yrjölä et al., 2016). Agonist stimulation of GPCRs and subsequent signalling induces receptor trafficking. GPCRs normally reside on the cell membrane but agonist stimulation leads to receptor internalisation (Magalhaes, Dunn and Ferguson, 2012). Indeed, it was found that treatment of the hGPR55-HEK293 cell line with 17g (1 µM) induced GPR55 internalisation. Unstimulated cells showed receptor localisation on the cell membrane in comparison. Similarly, Henstridge et al. (2010) reported that HA-tagged GPR55 remained localised to the cell membrane in unstimulated cells and upon stimulation with certain cannabinoid compounds and the endogenous GPR55 agonist, LPI, there was a pronounced redistribution of GPR55 into intracellular vesicles. These findings corroborate what is already known about GPCR stimulation (Magalhaes, Dunn and Ferguson, 2012).
3.3.2. LPI regulation of [Ca\(^{2+}\)]\(_i\) spontaneous Ca\(^{2+}\) activity in neurons

Ca\(^{2+}\) plays a major role in normal cell functioning and homeostasis. Ca\(^{2+}\) ions play an important role as a second messenger in signal transduction pathways such as neurotransmitter release from neurons, in muscle cell contraction, in fertilisation and in bone formation (Brini et al., 2013). Under normal physiological conditions Ca\(^{2+}\) levels are tightly regulated. In neurons, cytosolic Ca\(^{2+}\) is maintained at a steep gradient between low intracellular free Ca\(^{2+}\) and high extracellular Ca\(^{2+}\). Ca\(^{2+}\) homeostasis is typically maintained by Ca\(^{2+}\) channels, Ca\(^{2+}\) binding proteins, Ca\(^{2+}\) release from internal stores and sequestration by intracellular organelles e.g. mitochondria and ER and by energy-dependent pumps that mediate Ca\(^{2+}\) efflux (Rapaka et al., 2014). In the present study the effects of GPR55 ligands on the regulation of [Ca\(^{2+}\)]\(_i\) were investigated in cells obtained from rat cortical neuron-enriched cultures.

It was found that the endogenous GPR55 agonist, LPI (10 μM), modulated [Ca\(^{2+}\)]\(_i\) activity in a differential manner depending on the [Ca\(^{2+}\)]\(_i\) profile of neurons in this current study. Basal neuronal [Ca\(^{2+}\)]\(_i\) activity manifested in one of 2 ways: (1) quiescent or (2) synchronous and spontaneous. Spontaneous [Ca\(^{2+}\)]\(_i\) oscillations have been described in a variety of cells. In some cases, [Ca\(^{2+}\)]\(_i\) oscillations reflect cycles of membrane depolarisation and voltage-dependent Ca\(^{2+}\) entry. In others, they are caused by periodic Ca\(^{2+}\) uptake and release by internal stores, with little immediate requirement for external Ca\(^{2+}\) (Friel, 1995). Synchronous [Ca\(^{2+}\)]\(_i\) transients have previously been shown to occur in the hippocampus (Meister et al. 1991), retina (Garaschuk et al. 1998), spinal cord (Wenner & O’Donovan, 2001) and neocortex (Garaschuk et al. 2000; Sisk & Moody, 2003). This spontaneous neuronal Ca\(^{2+}\) activity is believed to play a pivotal role in many aspects of neuronal development including neuronal migration, differentiation and connection patterning (Shatz, 1990). Corlew et al. (2004) demonstrated that spontaneous and synchronised [Ca\(^{2+}\)]\(_i\) transients occurred in
isolated cortical neurons obtained from both embryonic and neonatal mice. These transients were inhibited by the Na\(^+\)-channel blocker, tetrodotoxin (TTX) and the L-type VGCC blocker, nifedipine, indicating that they resulted from Ca\(^{2+}\) influx during neuronal electrical activity. The transients occurred independently of mobilisation of Ca\(^{2+}\) from intracellular stores (Wang & Gruenstein, 1997).

The basal spontaneous [Ca\(^{2+}\)]\(_i\) activity observed in neurons in the present study was also abolished by TTX (See Section 2.4.7), implicating that this activity is dependent on electrical activity. Preliminary findings in the current study showed that in some neurons, LPI potentiated TTX-sensitive oscillatory activity upon TTX washout. This indicates that LPI has a modulatory role in neuronal network activity. Indeed, LPI diminished the frequency of spontaneous Ca\(^{2+}\) events in neurons and potentiated event frequency in neurons that were showing minimal levels of basal [Ca\(^{2+}\)]\(_i\) activity. It was therefore important to determine if these modulatory effects were mediated via GPR55. Indeed, GPR55 activation leads to Ca\(^{2+}\) release from ER stores and SOCE in hGPR55-HEK293 cells (Henstridge et al., 2009).

SOCE occurs due to CRAC channel formation, which are composed of STIM and Orai proteins. However, STIM protein itself has been shown to suppress VGCC activity and Ca\(^{2+}\) influx (Harraz & Altier, 2014). The selective GPR55 antagonist, CID16020046 (CID), was therefore employed to determine if LPI mediated its effect via this STIM-dependent inhibition of ion channels. Kargl et al. (2013) characterised the effects of the selective GPR55 antagonist, CID16020046 (CID), in inhibiting [Ca\(^{2+}\)]\(_i\) responses to GPR55 ligands in overexpressing HEK293 cells. It was shown in that study that CID significantly abolished the [Ca\(^{2+}\)]\(_i\) response to LPI and the selective GPR55 agonist, GSK319197A, in a concentration dependent manner. In the present study however, it was found that CID did not inhibit the LPI-induced decrease neuronal Ca\(^{2+}\) event frequency, thus indicating that LPI induces its modulatory effects on [Ca\(^{2+}\)]\(_i\) independently of GPR55. This implicates another target in mediating these changes in [Ca\(^{2+}\)]\(_i\).
LPI is a bioactive lipid that is generated intracellularly from the cleavage of phosphatidylinositol (PI) by cytoplasmic phospholipase A$_{1/2}$. LPI is then transported into the extracellular space by ATP-binding cassette transporters from the sub-family C (ABCC) and is capable of signalling via GPR55 on the extracellular membrane of certain cells (Hamashita et al., 2013; Piñeiro et al., 2011). Since it is a lysosphospholipid, it can also alter the mechanical properties of cell membranes. LPI has an inverted cone shape compared to bilayer-forming phospholipids. This is due to its large head group relative to the smaller hydrophobic domain. When lysosphospholipids are mixed with phospholipids, they lead to alteration of the physical properties of the phospholipid bilayer by introducing lateral stress. Changes in membrane conformation can then result in changes in protein properties (e.g. ion channels) and therefore lead to cellular effects that are not mediated by direct binding of LPIs to a receptor such as GPR55 (Alhouayek, Masquelier & Muccioli, 2018). Indeed, LPIs can reversibly activate the K$^+$ channels TREK-1 and TRAAK (Maingret et al., 2000). LPI was also shown to inhibit Na$^+$/K$^+$-ATPase and to activate large- and intermediate-conductance Ca$^{2+}$-dependent potassium channels (BK$_{Ca}$ and IK$_{Ca}$ respectively) independently of GPR55 (Bondarenko et al., 2011a, 2011b). This direct alteration of cellular membranes may aid in explaining the GPR55-independent effects of LPI on neuronal spontaneous [Ca$^{2+}$]i activity. Indeed, LPI (20 and 30 µM) has previously been shown to attenuate, but not completely abolish, L-type VGCC Ca$^{2+}$ currents in pituitary cells. The authors postulated that perturbation in the lipid bilayer, produced by partition of cone-shaped lysosphospholipids such as LPI, alters the mechanical properties of the bilayer, thereby affecting the normal function of VGCCs in pituitary cells (Ben-Zeev, Telias & Nussinovitch, 2010). Furthermore, they found that the effects of LPI were slow onset, which complements the findings here because the attenuation in Ca$^{2+}$ event frequency by LPI was slow onset but long-lasting. Ben-Zeev, Telias & Nussinovitch reasoned that electrostatic repulsion between the negatively-charged LPI and negative surface membrane charges may have delayed the incorporation of
LPI into the outer leaflet of the bilayer. These findings suggest that neurons exhibiting spontaneous Ca\(^{2+}\) activity show altered Ca\(^{2+}\) event frequency in response to LPI via two potential mechanisms: (1) direct modulation of ion channels such as VGCCs through its mechanical influence on the membrane bilayer (Alhouayek, Masquelier & Muccioli, 2018; Ben-Zeev, Telias & Nussinovitch, 2010), leading to suppression of Ca\(^{2+}\) influx or (2) indirect modulation of ion channels via an unknown GPR55-independent target. LPI may bind to this target on the plasma membrane, induce Ca\(^{2+}\) release from ER stores and subsequently activate SOCE. During CRAC channel formation, STIM protein itself could suppress VGCC activity and Ca\(^{2+}\) influx (Harraz & Altier, 2014). Future work should investigate this LPI-mediated modulation of Ca\(^{2+}\) activity in more detail. For instance, a VGCC inhibitor such as nifedipine and/or STIM inhibitor could be used alongside LPI treatment to see which mechanism it favours. A suggested mechanism of LPI-induced effects on neuronal Ca\(^{2+}\) event frequency can be viewed in Figure 3.33.
Figure 3.33. Suggested mechanisms involved in the LPI-induced diminishment of neuronal [Ca\textsuperscript{2+}]i events.

PI is cleaved by PLA\textsubscript{1/2} to generate LPI intracellularly (Hamashita et al., 2013). LPI may then go on to mediate its inhibitory effect on spontaneous Ca\textsuperscript{2+} events via 2 ways: (1) direct modulation of the cell membrane, thus altering VGCC conformation and suppressing Ca\textsuperscript{2+} influx (Ben-Zeev, Telias & Nussinovitch, 2010) or (2) transport extracellularly via ABCC and bind to an unknown target on the plasma membrane. This will lead to downstream IP\textsubscript{3}R-mediated Ca\textsuperscript{2+} release from ER stores. Concomitantly, CRAC channel proteins will directly inhibit VGCC and prevent Ca\textsuperscript{2+} influx (Harraz & Altier, 2014). Elements of figure in colour were directly identified in the current study. Elements of figure in grey were not directly investigated in the current study but are instead referenced from the literature. Some elements of figure adapted from Servier Medical Art images.
3.3.3. LPI modulation of \([Ca^{2+}]_i\) in quiescent neurons and glia.

Interspersed within the excitatory cortical network are inhibitory interneurons, which play vital roles in the modulation of cortical activity (Neske, Patrick & Connors, 2015). The presence of these inhibitory neurons may explain why certain neurons imaged in this study exhibited a quiescent \([Ca^{2+}]_i\) profile. LPI induced an increase in \([Ca^{2+}]_i\) in quiescent neurons, as well as in glia. In line with this, LPI was also shown to induce increases in \([Ca^{2+}]_i\) in the PC12 neuron-like cell line and in DRG neurons (Obara et al., 2011; Lauckner et al., 2008). Neurons exhibited different profiles of \([Ca^{2+}]_i\) increases in response to LPI. LPI induced latent and sustained increases in some neurons (as well as in glia), while it induced an increase in \(Ca^{2+}\) event frequency in other neurons.

Although GPR55 is expressed in primary microglia (Pietr et al., 2009), Sylantyev et al. (2013) demonstrated that in hippocampal cultures, LPI evoked \(Ca^{2+}\) rises in axons exposed to bath medium, with no glia present nearby. GPR55 agonists also had no effect on astrocytic \(Ca^{2+}\). These data suggest little role of astroglia in the observed phenomena, which conflicts with the findings herein. However, LPI was found to activate a SOCE-like \(Ca^{2+}\) influx in Bergmann glia and granule cell layer astrocytes (Singaravelu, Lohr & Deitmer, 2006), which complements the findings here. Perhaps glial responsiveness to LPI may depend on their localisation in the brain. Nevertheless, it was of interest to determine if GPR55 was responsible for the LPI-induced increases in \([Ca^{2+}]_i\) seen in quiescent neurons and glia observed here.

The selective GPR55 antagonist, CID16020046 (CID), was co-applied with LPI in neurons and glia. Quiescent neurons failed to respond to LPI in the presence of CID and failed to respond following a washout period to LPI application alone. It is therefore unclear if CID had an effect on LPI-responsiveness in these neurons. However, in 2 glia, CID failed to inhibit a sharp decline induced by LPI. This decrease in \([Ca^{2+}]_i\) eventually stabilised and LPI
later induced the same effect alone following a washout period, indicating it occurred independently of GPR55. It is difficult to determine why LPI induced a decrease in \([\text{Ca}^{2+}]_i\) in these instances as opposed to an increase as expected. The cells that generated the traces were believed to be astrocytes due to their morphology and proximity to neurons. Perhaps the LPI-induced decrease in \([\text{Ca}^{2+}]_i\) observed in these glial cells could have been as a result of modulation of ion channel activity. Indeed, antisense knockdown of TRPC1 led to the reduction of \([\text{Ca}^{2+}]_i\) transients induced by ATP in astrocytes \textit{in vitro} (Golovina, 2005). Bearing this in mind, it can be postulated that LPI inhibited TRP-mediated SOCE in the glial cells observed herein, leading to extrusion of cytoplasmic \(\text{Ca}^{2+}\) to the extracellular space through PMCs. This would lead to the \([\text{Ca}^{2+}]_i\) decrease observed herein, because stores cannot be replenished following LPI-mediated store depletion if SOCE is blocked. However, because this decrease in \([\text{Ca}^{2+}]_i\) occurred in only 2 glia, then future work should validate if these effects are consistent. If so, then measures should be taken to identify the cause of this \([\text{Ca}^{2+}]_i\) decrease.

CRAC channels are activated by receptors that deplete the internal \(\text{Ca}^{2+}\) stores. Non-excitable cells such as lymphocytes and microglia lack voltage-gated \(\text{Ca}^{2+}\) influx but do express CRAC channels, whereas neurons express both. Thapsigargin (TSG) irreversibly inhibits SERCA which leads to depletion of \(\text{Ca}^{2+}\) from the ER and a resultant increase in cytoplasmic \(\text{Ca}^{2+}\) levels (Lytton, Westlin & Hanley, 1991). TSG itself can therefore stimulate SOCE through passive ER store depletion. TSG induced store depletion in both quiescent neurons and glia in the current study. However, LPI induced a transient increase in \([\text{Ca}^{2+}]_i\) even after store depletion in a minority of quiescent neurons and glia. This indicates that LPI can induce \([\text{Ca}^{2+}]_i\) changes even after store depletion in these cells, meaning another target may be involved. Indeed, in the absence of \([\text{Ca}^{2+}]_e\), LPI did not induce a response. However, upon re-introduction of \([\text{Ca}^{2+}]_e\), LPI induced a robust and transient increase in \([\text{Ca}^{2+}]_i\) in neurons and glia. This indicates that LPI potentially mediates its effect on \([\text{Ca}^{2+}]_i\) via ion
channels as opposed to ER stores. TRPV2 is a store-independent Ca\(^{2+}\) channel which can be stimulated without the release of Ca\(^{2+}\) from stores, leading to Ca\(^{2+}\) influx into the cell. It is expressed in the brain, but its physiological role is little understood (Ramsey, Delling & Clapham, 2006). LPI was found to induce TRPV2 translocation to the plasma membrane of enteroendocrine L cells which resulted in increased Ca\(^{2+}\) influx (Harada et al., 2017). These effects were significantly inhibited by the putative GPR55 antagonist, O-1918, suggesting that TRPV2 translocation takes place in response to the activation of GPR55. This suggests that the actions of LPI on L cells are at least partially independent of GPR55 (Harada et al., 2017). Furthermore, Monet et al. (2009) demonstrated that activation of TRPV2 by LPI and another lysophospholipid, LPC and subsequent Ca\(^{2+}\) entry were mediated by the P13,4K pathway in the PC3 prostate cancer cell line. However, Harada et al. (2017) used another putative GPR55 agonist, O-1602, as a comparator in their study and, unlike LPI, it did not induce \([\text{Ca}^{2+}]_i\) release via TRPV2. However, both O-1918 and O-1602 have shown to be efficacious at GPR18 (Ashton, 2012), so more selective GPR55 ligands should be used to validate the effect of GPR55 on TRPV2 recruitment. LPI was also shown to inhibit Na\(^{+}/K^{+}\)-ATPase and to activate large- and intermediate-conductance Ca\(^{2+}\)-dependent potassium channels (BK\(_{ca}\) and IK\(_{ca}\) respectively) independently of GPR55 (Bodarenko et al., 2011a, 2011b). It can therefore be theorised that LPI may be mediating its effects on \([\text{Ca}^{2+}]_i\) in quiescent neurons and glia via one of two ways: (1) direct modulation of ion channels through its mechanical influence on the membrane bilayer (Alhouayek, Masquelier & Muccioli, 2018), leading to alteration in the levels of Ca\(^{2+}\) influx or (2) indirect modulation of ion channels via an unknown target (perhaps GPR55), whereby its activation leads to the recruitment of ion channels e.g. TRPV2, to the plasma membrane and resultant Ca\(^{2+}\) influx (Harada et al., 2017). This may help to explain the \([\text{Ca}^{2+}]_i\) changes occurring following store depletion in the current study. Future work should seek to further expand on our knowledge of the GPR55-independent effects of LPI in order to truly understand its effects and
potential targets. A suggested mechanism of LPI-induced effects on \([\text{Ca}^{2+}]_i\) in quiescent neurons and glia can be viewed in Figure 3.34.
Figure 3.34. Suggested mechanisms involved in the LPI-induced modulation of \([Ca^{2+}]_i\) after TSG-induced store depletion in quiescent neurons and glia.

TSG inhibits SERCA which leads to the depletion of \(Ca^{2+}\) from ER stores. The increases in \([Ca^{2+}]_i\) following store depletion observed in some populations of neurons and glia may have been caused by two potential mechanisms: (1) Direct binding of LPI to the cell membrane, leading to alteration of the membrane and ion channel conformations, leading to \(Ca^{2+}\) influx (Alhouayek, Masquelier & Muccioli, 2018) or (2) LPI stimulation of an unknown target (potentially GPR55) which leads to recruitment of ion channels such as TRPV2 to the plasma membrane (large dashed grey arrow; Harada et al., 2017) and subsequent \(Ca^{2+}\) influx. \(BK_{ca}\) and \(IK_{ca}\) = large- and intermediate-conductance \(Ca^{2+}\)-dependent \(K^+\) channels respectively.

Elements of figure in colour were directly identified in the current study. Elements of figure in grey were not directly investigated in the current study but are instead referenced from the literature. Some elements of figure adapted from Servier Medical Art images.
3.3.4. 17g regulation of spontaneous [Ca$^{2+}$]$_i$ events in neurons.

It was shown in this chapter that the novel and selective GPR55 agonist, 17g, was effective in stimulating GPR55 internalisation in an overexpressing cell line model and it was also previously shown to induce an increase in [Ca$^{2+}$]$_i$ in those cells (Yrjölä et al., 2016). 17g induced an increase in neuronal [Ca$^{2+}$]$_i$ event frequency in neurons obtained from cortical neuron-enriched cultures and this amplifying effect of 17g was blocked by the selective GPR55 antagonist, CID16020046 (CID). In contrast, the attenuative effect of LPI on spontaneous Ca$^{2+}$ events was not blocked by CID, indicating that LPI mediates its modulatory effect on neuronal Ca$^{2+}$ events independently of GPR55. 17g most likely induces increases in Ca$^{2+}$ event frequency via GPR55-mediated release of Ca$^{2+}$ from ER stores. Indeed, LPI-induced activation of GPR55 induced oscillatory release of Ca$^{2+}$ from stores in hGPR55-HEK293 cells (Henstridge et al., 2009). Furthermore, when TSG was applied in the current study in order to deplete ER stores, it failed to drastically alter the frequency of Ca$^{2+}$ events in these neurons. 17g did not alter event frequency following TSG-induced store depletion, indicating it needs replenished stores in order to stimulate Ca$^{2+}$ release. This further supports the claim that 17g induces an increase in neuronal Ca$^{2+}$ event frequency through GPR55-mediated stimulation of IP$_3$-sensitive stores. Future work should involve determining the G-protein responsible for coupling to GPR55. Indeed, Lauckner et al. (2008) has reported that DRG neurons couple to both G$\alpha_q$ and G$\alpha_{12}$ proteins in order to stimulate Ca$^{2+}$ release. A suggested mechanism for GPR55-mediated increase in neuronal spontaneous [Ca$^{2+}$]$_i$ events can be viewed in Figure 3.35.
Figure 3.35. Suggested mechanism involved in the 17g-induced amplification of neuronal spontaneous [Ca\textsuperscript{2+}]i activity.

17g binds to GPR55, leading to IP\textsubscript{3}-mediated release of Ca\textsuperscript{2+} from stores (1: Henstridge et al., 2009). This process is inhibited by the selective GPR55 antagonist, CID16020046 (CID). TSG inhibits this GPR55-mediated Ca\textsuperscript{2+} release process in neurons exhibiting spontaneous Ca\textsuperscript{2+} events. Elements of figure in colour were directly identified in the current study. Elements of figure in grey were not directly investigated in the current study but are instead referenced from the literature. Some elements of figure adapted from Servier Medical Art images.
3.3.5. 17g modulation of [Ca\(^{2+}\)]_{i} in quiescent neurons.

Although 17g was effective at amplifying neuronal Ca\(^{2+}\) event frequency, it had differential effects on [Ca\(^{2+}\)]_{i} in quiescent neurons. In some neurons it induced an increase in spontaneous Ca\(^{2+}\) activity. LPI-induced activation of GPR55 has been shown to induce oscillatory release of Ca\(^{2+}\) from stores in hGPR55-HEK293 cells (Henstridge et al., 2009). In other neurons it induced a latent increase in [Ca\(^{2+}\)]_{i}. 17g was previously shown to induce a single elevated and sustained increase in [Ca\(^{2+}\)]_{i} in hGPR55-HEK293 cells. These data suggest that GPR55-induced stimulation of Ca\(^{2+}\) release from IP\(_3\)-sensitive stores can lead to different Ca\(^{2+}\) mobilisation profiles. The selective GPR55 antagonist, CID16020046 (CID), inhibited 17g-induced increases in [Ca\(^{2+}\)]_{i}.

In some quiescent neurons, TSG-induced store depletion did not abolish 17g-induced modulation of [Ca\(^{2+}\)]_{i}. Some neurons showed transient increases [Ca\(^{2+}\)]_{i} after store depletion. Furthermore, preliminary findings indicate that 17g only induced responses in the presence of [Ca\(^{2+}\)]_{e} in some neurons. Indeed, PLC-mediated cleavage of PIP\(_2\) into IP\(_3\) and DAG is suggested to have a direct modulatory role on TRP ion channel modulation. PIP\(_2\) is believed to inhibit TRP channels, so depletion of PIP\(_2\) pools through cleavage alleviates this inhibitory effect. Furthermore, DAG itself is suggested to activate TRP channels, leading to Ca\(^{2+}\) influx (Hardie, 2004). Therefore, 17g binding to GPR55 may indirectly modulate ion channel activation by inducing downstream PIP\(_2\) cleavage into DAG, thus activating TRP channels. This may explain how [Ca\(^{2+}\)]_{i} is increased upon 17g application even after ER store depletion. Indeed, Harada et al. (2017) demonstrated that LPI accelerated TRPV2 translocation to the plasma membrane of enteroendocrine L cells and induced Ca\(^{2+}\) influx into the cytoplasm via TRPV2. These effects were significantly inhibited by the putative GPR55 antagonist, O-1918, suggesting that TRPV2 translocation takes place in response to the activation of GPR55. This would complement the findings herein, whereby 17g mediates
its effects via GPR55 yet can still induce \([\text{Ca}^{2+}]_i\) increases after store depletion. However, another GPR55 agonist, O-1602, was used as a comparator by Harada et al. (2017) and unlike LPI, did not induce \(\text{Ca}^{2+}\) influx via TRPV2 in that study. This conflicts with the findings herein and suggests that the actions of LPI on L cells are therefore at least partially independent of GPR55. However, O-1918 and O-1602 are both reported to be putative ligands at the orphan GPCR, GPR18 (Ashton, 2012). This in theory implicates GPR18 in mediating at least part of the actions observed in that study, potentially through a cross-talk mechanism with GPR55. Indeed, GPR55 has been shown to dimerise with the classic cannabinoid receptors (Martínez-Pinilla et al., 2014; Moreno et al., 2014). Perhaps the potential off-target effects of O-1602 and O-1918 at GRP18 prevented TRPV2 recruitment. Since 17g is an agonist at GPR55 (Yrjölä et al., 2016), perhaps it is capable of recruiting additional ER-independent \(\text{Ca}^{2+}\) mediators such as TRPV2 to maintain \([\text{Ca}^{2+}]_i\) homeostasis following store depletion. The suggested mechanism for 17g-induced increases in \([\text{Ca}^{2+}]_i\) can be viewed in Figure 3.36.
Figure 3.36. Suggested mechanisms involved in the 17g-induced modulation of \([Ca^{2+}]_i\) after TSG-induced store depletion in quiescent neurons.

Under normal conditions, 17g stimulates GPR55 which leads to the release of \(Ca^{2+}\) from ER stores. TSG inhibits SERCA which leads to store depletion. 17g-induced stimulation of GPR55 may lead to the recruitment of additional \(Ca^{2+}\) channels such as TRPV2 (large dashed grey arrow), leading to \(Ca^{2+}\) influx and a rise in \([Ca^{2+}]_i\) (1: Harada et al., 2017). Furthermore, GPR55-stimulated PIP\(_2\) cleavage into DAG and IP\(_3\) may lead to direct DAG-mediated activation of TRP channels (2; Hardie, 2004). 17g effects on \([Ca^{2+}]_i\) are GPR55-dependent because they are inhibited by the selective GPR55 antagonist, CID16020046 (CID).

Elements of figure in colour were directly identified in the current study. Elements of figure in grey were not directly investigated in the current study but are instead referenced from the literature. Some elements of figure adapted from Servier Medical Art images.
3.3.6. The effect of 17g on [Ca$^{2+}$]i in glia.

In comparison to neurons, glia showed no responsiveness to 17g. This is surprising given that GPR55 is expressed in primary microglia (Malek et al., 2015; Pietr et al., 2009). There is little evidence confirming whether GPR55 expression occurs in astrocytes however. Sylantyev et al. (2013) demonstrated that in hippocampal cultures, LPI still evoked Ca$^{2+}$ rises in axons exposed to bath medium, even with no glia present nearby. GPR55 agonists also had no effect on astrocytic Ca$^{2+}$. However, LPI was found to activate a SOCE-like Ca$^{2+}$ influx in Bergmann glia and granule cell layer astrocytes (Singaravelu, Lohr & Deitmer, 2006). As discussed, LPI is a lysophospholipid that is capable of modulating multiple targets (Alhouayek, Masquelier & Muccioli, 2018; Harada et al., 2017). Its effects in those studies may therefore have been independent of GPR55. In contrast, 17g is an agonist for GPR55 (Yrjölä et al., 2016).

There are potential reasons behind the poor responsiveness of glia to 17g in the current study. It is likely that the majority of glial cells analysed using ratiometric Ca$^{2+}$ imaging were astrocytes. Microglia typically make up 7% of mixed cortical neuron-glia cultures (Huang & Wang, 2015). KCl differentiated between neurons and glia, but specific markers for astrocytes and microglia were not used in the current study. This meant that cells were chosen blindly in the fields of view using MetaFluor imaging software. Future work should seek to specifically include microglia in the field of view while imaging and observe their responsiveness to 17g. Since they express GPR55, the proportion of cells responsive to 17g should be expected to increase.
It was interesting that LPI-induced and 17g-induced changes in $[\text{Ca}^{2+}]_i$ varied across cortical neuron-enriched cultures. This may be due to the density of GPR55 expression. Carbachol (CCh) is a non-selective muscarinic- and nicotinic-acetylcholine receptor (AChR) agonist. It was used as a positive control in this study because it is known to increase $[\text{Ca}^{2+}]_i$ in a similar mechanistic fashion to GPR55 (Mayerhofer et al., 1992). CCh leads to robust $[\text{Ca}^{2+}]_i$ increases via the formation of IP$_3$, which induces the release of Ca$^{2+}$ from ER stores (VanDeMark et al., 2009), as one would expect with GPR55-mediated intracellular signalling (Henstridge et al., 2009). The putative GPR55 agonist, O-1602, was shown to partially inhibit exogenous ACh-induced contractions in the mouse intestine (Ross et al., 2012). ACh is the endogenous agonist of AChRs. However, AChR and GPR55 co-localisation in the cortical neuron-enriched cultures has not previously been examined.

There was a relatively large percentage of both neurons and glia that were responsive to only CCh in the current study. The rat cortex displays a significant increase in the number of m2 and m4 mAChRs in the adult rat relative to the juvenile rat. The hippocampus in contrast displays consistent expression levels of muscarinic receptor subtypes (Tice, Taylor & McQuade, 1995). This widespread mAChR expression in the cortex may explain the greater percentage of CCh-responsive neurons observed here. mAChRs are also expressed by astroglia in the cortex of young and aged rats (Van der Zee et al., 1993) and a large percentage of glia also showed responsiveness to CCh in the present study. Only a small percentage of both neurons and glia showed co-localised responses to LPI and CCh (neurons and glia = 10% each) in the present study. In contrast, no neurons or glia showed co-localised 17g and CCh responses. It was interesting that the percentages of LPI- and CCh-only responsive neurons and glia were not hugely divergent in number (Neurons: LPI = 15%, CCh = 23%; Glia: LPI = 17%, CCh = 23%). This indicates the differences in target distribution across
cortical cultures and further substantiates the theory that LPI, unlike 17g, may not mediate its effects on $[\text{Ca}^{2+}]_i$ via GPR55.

A concentration of 10 µM LPI was used in the current study for Ca$^{2+}$ imaging experiments, which is well below critical micelle concentration of approximately 30 µM (Corda, Iurisci & Berrie, 2002). This suggests that the concentration of LPI was not too high to be inhibited by the selective antagonist for GPR55, CID16020046 (CID), if LPI was selective for GPR55. However, it is worth noting that the species of LPI used in this study is derived from soybean salt. The most abundant fatty acid group species present in this salt are palmitic acid and stearic acid (58%), followed by linoleic acid (42%). Although the 2-arachidonyl species of LPI is the most efficacious at GPR55 (Oka et al., 2009), it is not available commercially, so the soybean species (Sigma-Aldrich, Dorset, UK) was used in the present study. This may explain why CID did not antagonise this species of LPI, since it may not be wholly GPR55-specific. Indeed, Oka et al. (2009) compared 2-arachidonyl-LPI with other species and found that 2-arachidonyl-LPI makes up 22.1% of rat brain tissue, while 1-stearoyl-LPI was the most abundant species present in this tissue (50.5%). Interestingly, even though 2-arachidonyl-LPI was not the most abundant LPI species in the rat brain, its EC$_{50}$ in an ERK phosphorylation assay using hGPR55-HEK293 cells was 30 nM, which was 15 times more efficacious than 1-stearoyl-LPI. The LPI species may help to explain the GPR55-independent effects observed in the present study.

These findings show that GPR55 and mAChRs are not co-localised in populations of cortical neurons and glia. mAChR expression seems far more abundant in these cultures compared to GPR55. Cells were more sensitive to LPI compared to the selective GPR55 agonist, 17g, indicating that LPI most likely mediates its effects via a different target. These data further contribute to our knowledge of GPR55 expression in the cortex.
3.3.8. GPR55 regulation of CREB

CREB is a transcription factor that is widely expressed in the body, with particularly high levels of expression found in the brain (Carlezon Jr et al., 2005). It was initially observed that cAMP induced the transcription of somatostatin gene via the phosphorylation of CREB at Serine 133 (Gonzalez & Montminy, 1989). CREB phosphorylation occurs via different effectors depending on cell type, with some mediators including protein kinase A (PKA), MAPKs (Carlezon Jr et al., 2005; Gonzalez & Montminy, 1989; Sheng et al., 1991) and PKC (Johannessen et al., 2004). Phosphorylation of CREB leads to the translocation of phospho-CREB (pCREB) into the cell nucleus and the activation of CREB-mediated gene transcription (Stevenson et al., 2001).

In the current study, it was found that the endogenous GPR55 agonist, LPI and the selective GPR55 agonist, 17g, induced CREB phosphorylation in neurons from cortical neuron-enriched cultures in a time-dependent manner. The selective GPR55 antagonist, CID16020046 (CID), partially antagonised 17g-induced pCREB but did not antagonise LPI-induced pCREB. These findings indicate that GPR55 partially mediates 17g-induced pCREB but does not mediate LPI-induced pCREB. In contrast, Henstridge et al. (2010) demonstrated that LPI (1 µM) induced a robust increase in pCREB in hGPR55-HEK293 cells. Both agonists induced pCREB via a Gαq-dependent mechanism in the current study, although Gαq only partially contributed to LPI-induced pCREB. GPR55 stimulation in transiently-expressing hGPR55-HEK293 cells was previously shown to induce Ca²⁺ release from intracellular stores in Gq-PLC- and G12-RhoA-mediated pathways (Lauckner et al., 2008). Although LPI-induced pCREB occurred independently of GPR55, other Gq/11-coupled GPCRs such as M₃ mAChRs and B₂ bradykinin receptors can promote CREB phosphorylation in human SH-SY5Y neuroblastoma cells (Rosethorne, Nahorski & Challiss, 2008). It is therefore possible that LPI is inducing pCREB via another Gq-coupled receptor.
CREB is $\text{Ca}^{2+}$-sensitive and pCREB can be stimulated by $\text{Ca}^{2+}$-calmodulin-dependent protein kinases (CaM kinases), thereby promoting CREB-dependent gene transcription (West et al., 2001). Kornhauser et al. (2002) demonstrated that $\text{Ca}^{2+}$ influx induced CREB phosphorylation at Ser133 and two additional sites, Ser142 and Ser143 in primary rat neurons from cortical neuron-enriched cultures. Indeed, pre-treatment of cortical neurons with the L-type VGCC blocker, nifedipine, was required to reduce basal pCREB in the current study, indicating that network activity alone can induce pCREB (Hu, Chrivia, & Ghosh, 1999).

Finkbeiner et al. (1997) reported that a CaMK-dependent mechanism stimulated neurotrophins such as brain-derived neurotrophic factor (BDNF) and in turn these neurotrophins stimulated CREB in cortical neurons. CREB was thereby suggested to play a central role in mediating neurotrophin responses in neurons. Neuronal activity was reported to increase the frequency of CREB binding to CRE sites on DNA, thereby implicating neuronal activity in CREB-dependent gene transcription (Kitagawa et al., 2016). These findings corroborate the suggestions that due to its high levels of expression and activity within the brain, CREB is believed to play an important role in synaptic plasticity, memory consolidation and learning (Carlezon Jr et al., 2005; Josselyn & Nguyen, 2005). The results herein therefore implicate GPR55 in promoting these beneficial effects in the CNS.

LPI-induced activation of GPR55 has been shown to result in the recruitment of multiple nuclear transcription factors including ERK1/2, NFAT and NFκB (Henstridge et al., 2009, 2010). ERK activation is the most well characterised downstream signalling effect of GPR55 stimulation. LPI induced the sustained activation of ERK phosphorylation in hGPR55-HEK293 cells, with maximal stimulation occurring after 20 min ($\text{EC}_{50} = 0.27 \ \mu\text{M};$ Henstridge et al., 2010). Kargl et al. (2013) demonstrated that CID significantly inhibited LPI-induced activation of ERK1/2 in hGPR55-HEK293 cells. CID had no significant effect on ERK1/2 activation in CB$_1$-HEK293 and CB$_2$-HEK293 cells treated with the non-selective cannabinoid agonist, WIN55,212-2, demonstrating selectivity of the antagonism to GPR55.
This is important because activated ERK translocates from the cytosol into the nucleus and in turn phosphorylates CREB at Ser133. Phosphorylation of CREB and subsequent binding of pCREB to CRE on the target gene regulates gene expression and potentiates the function of ERK. Some studies on CNS models demonstrate that ERK-mediated CREB phosphorylation is required for the induction of stable, late-phase LTP and long-term memory (Kelleher et al., 2004; Morozoy et al., 2003; Thomas & Huganir, 2004). Further study into the role of GPR55 in the activation of CREB and other transcription factors could therefore prove to be hugely encouraging.

3.3.10. Conclusion

The aim of this chapter was to investigate the expression of GPR55 and the effects of its stimulation on \([\text{Ca}^{2+}]_i\) changes and CREB phosphorylation in primary cells obtained from cortical neuron-enriched cultures. The findings herein demonstrate that GPR55 is expressed in neurons and microglia. The novel and selective GPR55 agonist, 17g, induces GPR55 internalisation in overexpressing hGPR55-HEK293 cells. It was found that both the endogenous GPR55 agonist, LPI and 17g regulate changes in \([\text{Ca}^{2+}]_i\) and CREB phosphorylation in cortical cells. LPI regulated these signalling processes in a GPR55-independent manner, whereas 17g did so in a GPR55-dependent manner. Furthermore, these findings implicate both agonists in modulating ion channel activity in order to regulate \([\text{Ca}^{2+}]_i\). \(G_{q}\) contributed to pCREB stimulation by both agonists. These findings further our knowledge of the role of GPR55 in downstream signalling mechanisms in the CNS.
Chapter 4

The role of GPR55 in modulating microglial function and neuronal apoptosis
4.1. Introduction

The putative cannabinoid receptor GPR55 is expressed widely throughout the body, particularly in the immune system. It is expressed by organs such as the thymus and spleen (Balenga et al., 2011; Ross, 2011) and by immune cells such as neutrophils, monocytes, natural killer cells and mast cells (Cantarella et al., 2011; Chiurchiù et al., 2015; Henstridge et al., 2011; Yang et al., 2015). GPR55 has therefore been suggested to have a modulatory role in peripheral inflammatory processes. Indeed, production levels of endocannabinoids and the endogenous GPR55 agonist, LPI, are enhanced during inflammation (Di Marzo et al., 1999; Klein, 2005; Zoeller et al., 1987). Immune cell migration during inflammatory conditions is crucial for the delivery of protective immune responses to tissues (Luster, Alon & von Adrian, 2005). Balenga et al. (2011) reported that LPI and AM251 induced a directional migration of human peripheral blood neutrophils and enhanced their migratory capacity. Furthermore, these ligands inhibited neutrophil degranulation and reactive oxygen species (ROS) production, thus suggesting that GPR55 may limit tissue-injuring inflammatory responses.

GPR55 is also highly expressed centrally in primary mouse microglia and the microglial cell line, BV2 (Pietr et al., 2009). Microglia are the resident innate immune cells in the brain and are responsible for inducing neuroinflammatory responses to combat potential threats (Streit, Mrak & Griffin, 2004). Administration of the gram-negative bacterial endotoxin lipopolysaccharide (LPS) causes downregulation of GPR55 mRNA in both primary mouse microglia and BV2 cells (Pietr et al., 2009). LPS signals via toll-like receptor-4 (TLR4) expressed by microglia and causes them to induce an inflammatory response (Lehnardt et al., 2003). Interestingly the putative GPR55 agonist O-1602 protects against LPS-induced neurotoxicity in vitro (Janefjord et al., 2014). This implicates GPR55 in having an immunoregulatory effect in response to infection. GPR55 may also have a role in injury.
Following NMDA-induced excitotoxic lesion, the GPR55 endogenous agonist LPI (1 µM) protected neurons in rat organotypic hippocampal slice cultures (OHSC) and in parallel reduced the number of activated microglia at sites of neuronal injury in a GPR55-dependent manner (Kallendrusch et al., 2013). These studies implicate GPR55 in having a modulatory role in neuroinflammatory conditions (Haugh et al., 2016).

Neuroinflammation is also a key pathological feature of many neurodegenerative conditions such as AD. AD is characterised by neuronal loss and cognitive decline due to the deposition of Aβ plaques in the brain. Previous work from the Campbell research group has shown that Aβ induces apoptotic events such as lysosomal instability, caspase-3 activation and DNA fragmentation in a rat cortical neuron model of AD (Boland & Campbell, 2004; Fogarty et al., 2010). Endocannabinoids confer protection against these apoptotic events (Noonan et al., 2010; Tanveer et al., 2012). GPR55 mRNA is expressed in many areas of the brain e.g. frontal cortex, hypothalamus, striatum, amygdala and cerebellar granule cells (Chiba et al., 2011; Kerr et al., 2013; Ryberg et al., 2007; Sawzdargo et al., 1999), yet little is known of its role in regulating cellular apoptosis. The only study to date investigating the potential role of GPR55 in an AD model was performed by Janefjord et al. (2014). They showed that the putative GPR55 agonist O-1602 reduced the formation of aggregated Aβ fibrils in cell-free conditions, however it failed to protect against Aβ-induced toxicity in the SH-SY5Y neuroblastoma cell line.

Due to the limited studies performed to date that aim to elucidate the role of GPR55 in central immune processes and in neurodegeneration, the aim of this chapter was to investigate the role of GPR55 in regulating microglial migration and phagocytosis in response to stimuli; and to investigate the role of GPR55 in regulating neuronal apoptosis.
4.2. Results

4.2.1. GPR55 is expressed in BV2 microglial cells.

As previously shown in Section 3.2.1, GPR55 is expressed in microglia obtained from cortical neuron-enriched cultures. GPR55 was also reported to be expressed by the BV2 microglial cell line (Pietr et al., 2009). The present study confirmed this, with GPR55 expression observed in cultured BV2 cells (Figure 4.1). The BV2 cultures showed heterogeneous GPR55 expression levels, with some cells robustly expressing GPR55 (Figure 4.1, white arrows) and other cells showing low levels of expression (blue arrows). GPR55 was localised mainly at the membrane of the BV2 cells. However, there was punctate localisation evident in the cytosol. The results here demonstrate that GPR55 is expressed in BV2 microglia.
Figure 4.1. GPR55 is expressed in BV2 microglial cells.

Representative confocal images of BV2 cells labelled for GPR55. White arrows indicate high GPR55 expression. Note that some cells show low GPR55 expression (blue arrows). Scale bar = 20 µm. n=1.
4.2.2. BV2 microglial cell migration in response to untreated- and Aβ primed-neuronal medium.

Murine BV2 microglia are capable of migrating and forming clusters in response to fibrillar Aβ (Huang et al., 2010). GPR55 mRNA is expressed in both primary microglia and in the BV2 microglial cell line (see Section 3.2.1 and Pietr et al., 2009) and the endogenous GPR55 agonist LPI (1 µM) was previously found to induce the significant chemotaxis of primary microglia compared to untreated control cells (Kallendrusch et al., 2013). There are no findings published to date that elucidate the effect of GPR55 in regulating BV2 migration in response to Aβ, so an investigation was carried out in the current study.

The migration of the BV2 microglial cell line in response to untreated- or Aβ (10 µM)-primed neuronal medium (UT-NM and Aβ-PNM respectively) was assessed in the present study using a chemotactic Boyden chamber assay. Control groups involved exposing BV2 cells to neurobasal culture medium only beneath the chamber i.e. the medium had never come into contact with neurons. Foetal bovine serum diluted in neurobasal medium (30% FBS) was decanted underneath a separate chamber and acted as a positive control because FBS is a recognised chemoattractant (Mishima & Lotz, 2008).

Representative images and counts of migrated BV2 cells are displayed in Figures 4.2A. There was a significant difference between the treatment groups ([F (2, 9) = 7.299]; p<0.05, One-way ANOVA, Figure 4.2B). Further investigation indicated that BV2 cells exposed to Aβ-PNM (124.00±8.40) and 30% FBS (140.50±15.31) showed a significant increase in migration compared to control (87.25±8.47; p<0.05) and UT-NM (88.50±8.57; p<0.05, p<0.01, Student Newman Keuls, n=4 independent cultures).

The results here demonstrate that Aβ-PNM and FBS induce migration of BV2 microglial cells.
Figure 4.2. Aβ-primed neuronal medium induces significant migration of BV2 microglia.

A: Representative images of Rapi-diff stained membranes taken at 40x magnification in order to elucidate the effect of untreated-neuronal medium (UT-NM) and Aβ-primed NM (Aβ-PNM) on BV2 cell migration. BV2 cells were also exposed to neurobasal medium only (no cells; control) or foetal bovine serum diluted in neurobasal medium (30% FBS) as comparators. Scale bar = 100 µm. B: The effect of neurobasal medium (control), untreated-neuronal medium (UT-NM), Aβ-primed NM (Aβ-PNM) and 30% FBS on BV2 cell migration. Results are expressed as mean±S.E.M, *p<0.05 vs control, +p<0.05, ++p<0.01 vs UT-NM, One-way ANOVA & Student Newman Keuls, n=4 independent cultures. Data partially contributed to by Christian Thomas Gabrielsen and Sean Cassidy.
4.2.3. The GPR55 endogenous agonist LPI and selective antagonist CID significantly attenuate Aβ-PNM-induced BV2 microglial migration.

It has previously been shown that the endogenous GPR55 agonist LPI (1 µM) induced significant chemotaxis of primary microglia compared to untreated control cells (Kallendrusch et al., 2013). In the present study, BV2 cells seeded in Boyden chambers were exposed to LPI (10 µM) and/or the selective GPR55 antagonist CID16020046 (CID; 20 µM). Concomitantly, the cells were exposed to primed neuronal medium (PNM) below the chamber. Rapi-diff staining was subsequently carried out on the membranes and BV2 migration counted (Figure 4.3A). It was found that PNM almost induced a significant effect among the groups (\(F (1, 24) = 3.66; p=0.07\), Two-way ANOVA, n=4 independent cultures, data not shown). It was therefore of interest to elucidate the effect of LPI and CID on each type of PNM individually. There was a significant difference between treatment groups (\(F (4, 15) = 4.708\); p<0.05, One-way ANOVA). A post hoc test then indicated that LPI significantly attenuated Aβ-PNM-induced BV2 migration (Figure 4.3Bii; Aβ-PNM = 124.00±8.40; LPI+Aβ-PNM= 83.00±15.93; p<0.05, Student Newman Keuls, n=4 independent cultures). CID (20 µM) also attenuated BV2 migratory activity in cells exposed to Aβ-PNM (90.00±7.22; p<0.05) but did not inhibit LPI-induced attenuation of Aβ-evoked migration (101.50±12.69; p>0.05). Both LPI and CID did not modulate BV2 migration activity in response to UT-NM (Figure 4.5Bi; UT-NM = 80.50±10.14, LPI+UT-NM = 82.25±13.63; CID+UT-NM = 80.50±12.91; CID+LPI+UT-NM = 90.00±13.01; [F (4, 15) = 1.019]; p>0.05, One-Way ANOVA, n=4 independent cultures).

The results here demonstrate that LPI and CID attenuate Aβ-PNM-induced BV2 microglial migration. CID did not attenuate the effects of LPI, indicating the effects were independent of GPR55.
Figure 4.3. LPI and CID significantly attenuate Aβ-PNM-induced BV2 microglial migration.

A: Representative images of Rapi-diff stained membranes taken at 40x magnification in order to elucidate the effect of LPI (10 µM) and/or CID (20 µM) on modulating the BV2 migratory response to UT-NM and Aβ-PNM. Scale bar = 100 µm. B: (i) The effect of LPI (10 µM) and/or CID (20 µM) on BV2 cell migratory activity when co-exposed to UT-NM. BV2 cells were also exposed to neurobasal medium only (no neuronal exposure = control). Results are expressed as mean±S.E.M, p>0.05, One-way ANOVA, n=4 independent cultures. (ii) The effect of LPI (10 µM) and/or CID (20 µM) on BV2 cell migratory activity when co-exposed to Aβ-PNM. Results are expressed as mean±S.E.M, **p<0.01, *p<0.05 vs control, +p<0.05 vs Aβ-PNM, One-way ANOVA & Student Newman-Keuls, n=4 independent cultures. Data partially contributed to by Sean Cassidy.
4.2.4. LPS induces a significant increase in BV2 microglia phagocytosing latex beads.

BV2 cells were incubated with a 0.025% latex bead solution in presence or absence of LPS (5 µg/mL). They were left to incubate for 3 hrs, before being fixed and stained. Images of the cells were taken with a confocal microscope and analysed as outlined in the methods section (Figure 4.4A). LPS-treated cells phagocytose more beads than control cells. These observations are reflected in Figure 4.6B, where it was found that fewer LPS-treated cells phagocytosed 0 beads compared to control (33.33±9.81% vs 69.17±2.50% respectively; [F (2, 18) = 14.32]; p<0.01, Two-way ANOVA & Bonferroni post-hoc test, 120 total cells per treatment group obtained from n=4 independent cultures). Beads not ingested by cells that were not washed away during the fixation process were sometimes observed in control groups (Figure 4.4Ai).

The results here demonstrate that LPS enhances the ability of BV2 microglia to phagocytose beads. LPS was henceforth used as a positive control in all phagocytosis experiments to ensure they were viable.
Figure 4.4. LPS induces a significant increase in BV2 microglia phagocytosing latex beads.

A: Representative confocal images of BV2 microglia taken at 40x magnification. Cells were treated with control or LPS (5 µg/mL). Cells were co-labelled with IBA1 (microglial marker, red) and hoechst (nuclear marker, blue). White arrows indicate phagocytosed beads. Blue arrows indicate beads that have not been ingested. B: The effect of LPS on the % of BV2 cells phagocytosing latex beads. Results are expressed as mean±S.E.M, **p<0.01 vs control, Two-way ANOVA & Bonferroni post-hoc test, 120 total cells obtained from n=4 independent cultures. Data partially contributed to by Christian Thomas Gabrielsen and Sean Cassidy.
4.2.5. LPI does not induce BV2 phagocytosis.

BV2 cells were incubated with a 0.025 % latex bead solution in presence or absence of LPI (10 µM). They were left to incubate for 3 hrs, before being fixed and stained. LPI did not modulate bead phagocytosis compared to control (1-9 beads: control = 25.54±6.20% vs LPI =31.1±7.76%; 10+ beads: control = 2.23±2.23% vs LPI = 5.88±3.77%, p>0.05, Two-way ANOVA, 90 total cells per treatment group obtained from n=3 independent cultures; Figure 4.5).

The results here demonstrate that LPI does not modulate the ability of BV2 microglia to phagocytose beads.
Figure 4.5. LPI does not induce BV2 phagocytosis.

A: Representative confocal images of BV2 microglia taken at 40x magnification. Cells were treated with (i) control or (ii) LPI (10 µM). Cells were co-labelled with IBA1 (microglial marker, red) and hoechst (nuclear marker, blue). White arrows indicate phagocytosed beads. B: The effect of LPI on the % of BV2 cells phagocytosing latex beads. Results are expressed as mean±S.E.M, Two-way ANOVA, 90 total cells obtained from n=3 independent cultures. Data partially contributed to by Sean Cassidy.
4.2.6. LPS induces a significant increase in TNF-α production by BV2 microglia.

It was previously shown that LPS or Aβ_{1-42} treatment resulted in increased levels of tumour-necrosis factor (TNF)-α secretion by BV2 cells (Bussi et al., 2017; Jana, Palencia & Pahan, 2009). In the present study, a suitable stimulator of TNF-α production was confirmed by incubating BV2 cells for 24 hrs in the presence or absence of Aβ (10 µM) or LPS (50 ng/mL). The supernatants were collected and an enzyme-linked immunosorbent assay (ELISA) was performed to measure TNF-α production. There was a significant difference between each treatment ([F (2, 12) = 12.31]; p<0.01, One-way ANOVA, n=5 independent cultures). In Figure 4.6, it can be seen that more LPS-treated cells produced significant levels of TNF-α (322.70±55.51 pg/mL) compared to control (93.38±21.27 pg/mL; p<0.01, Student Newman-Keuls, n=5 independent cultures). Aβ-treated cells did not induce an increase in TNFα production (107.70±22.07 pg/mL; p>0.05, Student Newman-Keuls, n=5 independent cultures).

The results here demonstrate that LPS increases TNF-α production by BV2 microglia. LPS was henceforth used as a positive control when measuring for TNF-α production.
Figure 4.6. LPS induces a significant increase in TNFα production by BV2 cells.

The effect of Aβ (10 µM) or LPS (50 ng/mL). Results are expressed as mean±S.E.M, **p<0.01 vs control, One-way ANOVA & Student Newman-Keuls, n=5 independent cultures.
4.2.7. LPI does not stimulate TNF-α production by BV2 microglia.

Chiurchiù et al. (2015) reported that LPI enhanced production of the pro-inflammatory cytokines interleukin (IL)-12 and TNF-α by LPS-activated monocytes. It was therefore of interest to investigate if GPR55 ligands induced pro-inflammatory cytokine production in the BV2 microglial cell line. BV2 cells were incubated for 24 hrs in the presence or absence of LPI (1 µM, 10 µM). The supernatants were collected and an ELISA was performed to measure TNF-α production (Figure 4.7). There was no significant difference between each treatment group (LPI 1 µM = 112.80±24.29 pg/mL; LPI 10 µM = 124.10±26.18 pg/mL; [F (2, 12) = 0.4191]; p>0.05, One-way ANOVA, n=5 independent cultures).

The results here demonstrate that LPI does not stimulate TNF-α production by BV2 microglia.
Figure 4.7. LPI does not stimulate TNF-α production by BV2 cells.

The effect of LPI (1, 10 μM). Results are expressed as mean±S.E.M, One-way ANOVA, n=5 independent cultures.
4.2.8. CID attenuates LPI (10 µM)-induced active caspase-3.

Caspase-3 is an executioner caspase and forms part of the intrinsic apoptotic pathway. It functions in apoptotic processes in a cell- and tissue-specific manner (Porter & Jänicke, 1999). GPR55 has in recent years been suggested to have a regulatory function in apoptosis in pathological conditions such as cancer. In cancer, caspase-3 activity and apoptosis are downregulated (Devarajan et al., 2002) and in cancer cells transfected with GPR55 siRNA, a decrease in capase-3 activity and an absence of apoptosis induction was observed (Piñeiro et al., 2011). This suggests that GPR55 has a regulatory role in cancer cell proliferation. However, there has been no investigation into active caspase-3 induction by GPR55 ligands in the CNS. It was of interest to determine if GPR55 had a role in regulating active caspase-3 induction in cells obtained from cortical neuron-enriched cultures.

Cells from cortical neuron-enriched cultures were treated for 72 hrs, immunolabelled for active caspase-3 and imaged using light microscopy. The cells were counted and caspase-3 positive cells were expressed as a percentage of total cells (Figure 4.8A). There was a significant interaction between antagonist and agonist (F (3, 8) = 4.577; p<0.05, One-Way ANOVA, n=3 independent cultures). In Figure 4.8B, it can be observed that LPI (10 µM) significantly stimulated active caspase-3 (33.04±6.60%) compared to control (11.74±3.75%; p<0.05, Student Newman-Keuls, n=3 independent cultures). It is noteworthy that the selective GPR55 antagonist CID16020046 (CID; 20 µM) attenuated this LPI-induced caspase-3 stimulation (22.79±2.76%). CID also did not stimulate caspase-3 alone (20.58±1.31%; p>0.05).

The results here demonstrate that LPI 10 µM induced caspase-3 stimulation and this stimulation was attenuated by CID, indicating it was GPR55-dependent.
Figure 4.8. CID attenuates LPI (10 µM)-induced active caspase-3.

A: Representative images taken at 40x magnification of cells obtained from cortical neuron-enriched cultures labelled for active caspase-3. The cells were treated with (i) control, (ii) 10 µM LPI, (iii) 20 µM CID or (iv) CID+LPI. Cells are counterstained with methyl green. Insets represent caspase-3 negative (green, top left) and positive cells (dark brown, top right). Scale bar = 100 µm. B: The effects of 10 µM LPI and CID on cellular caspase-3 activity. Results are expressed as mean±S.E.M, *p<0.05, One-way ANOVA & Student Newman-Keuls, n=3 independent cultures.
4.2.9. LPI (10 µM) protects against Aβ-evoked caspase-3 activation.

Previous findings have suggested that GPR55 may have a regulatory role in neurodegenerative processes. For instance, the putative GPR55 agonist, O-1602, reduced Aβ fibril formation in cell-free conditions, but failed to prevent Aβ-induced neurotoxicity in the SH-SY5Y cell line (Janefjord et al., 2014). LPI has also been suggested to be neuroprotective in rat organotypic hippocampal slice cultures (OHSC) and mediated these neuroprotective effects via microglial cells (Kallendrusch et al., 2013). Due to the limited studies published to date on the role of GPR55 in neurodegeneration, it was therefore of interest to elucidate the effect of GPR55 ligands on Aβ-evoked active caspase-3 stimulation in the present study.

Cells from cortical neuron-enriched cultures were treated with LPI (10 µM) in the presence or absence of Aβ for 72 hrs, immunolabelled for active caspase-3 and imaged using light microscopy (Figure 4.9A). There was a significant interaction between agonist and challenge ([F (3, 12) = 9.067]; p<0.001, One-way ANOVA, n=4 independent cultures). In Figure 4.9B, it can be observed that Aβ (10 µM) induced significant caspase-3 activation (40.05± 5.27%) compared to control (11.28± 2.69%; p<0.01) and LPI (10 µM) significantly attenuated this Aβ-evoked caspase-3 activation (21.91± 3.79%; p<0.05, Student Newman-Keuls, n=4 independent cultures).

The results here demonstrate that 10 µM LPI protects against Aβ-evoked caspase-3 activation.
Figure 4.9. LPI (10 µM) protects against Aβ-evoked caspase-3 activation.

A: Representative images taken at 40x magnification of cells obtained from cortical neuron-enriched cultures stained for active caspase-3. The cells were treated with (i) control, (ii) 10 µM Aβ, (iii) 10 µM LPI or (iv) LPI+Aβ. Cells are counterstained with methyl green. Insets represent caspase-3 negative (green nuclei; top left) and positive neurons (dark brown; top right). Scale bar = 100 µm. B: The effects of 10 µM LPI and Aβ on cellular caspase-3 activity. Results are expressed as mean±S.E.M, **p<0.01 vs control, +p<0.05 vs Aβ, One-way ANOVA & Student Newman-Keuls, n=4 independent cultures.
4.2.10. Co-treatment of CID and LPI (1 µM) increases active caspase-3.

In section 4.2.9, it was shown that 10 µM LPI protected against Aβ-evoked caspase-3 stimulation. It has been previously shown that at different concentrations, LPI promotes opposing effects. For instance, 1 µM LPI acted as a chemoattractant to retinal ganglion cells whereas 10 µM LPI acted as a chemorepulsive (Cherif et al., 2015). It was therefore of interest to investigate a lower concentration of LPI to observe if it also modulated caspase-3 activation in cells obtained from cortical neuron-enriched cultures.

Cells from cortical neuron-enriched cultures were treated for 72 hrs, immunolabelled for active caspase-3 and imaged using light microscopy (Figure 4.10A). The cells were counted and caspase-3 positive cells were expressed as a percentage of total cells. There was a significant effect between treatment groups (F (3, 8) = 5.931; p<0.05, One-way ANOVA, n=3 independent cultures). In Figure 4.10B, it can be observed that LPI (1 µM) alone does not induce active caspase-3 (18.80 ± 0.52%; Student Newman-Keuls, n=3 independent cultures). However, co-treatment with the selective GPR55 antagonist CID16020046 (CID, 20 µM) resulted in increased caspase-3 (26.33±2.91%) compared to control (11.74±3.75%; p<0.05, Student Newman-Keuls, n=3 independent cultures).

The results here demonstrate that co-treatment of LPI (1 µM) and CID leads to an increase in caspase-3.
Figure 4.10. Co-treatment of CID and LPI (1 µM) increases active caspase-3.

A: Representative images taken at 40x magnification of cells obtained from cortical neuron-enriched cultures labelled for active caspase-3. The cells were treated with (i) control, (ii) 1 µM LPI, (iii) 20 µM CID or (iv) CID+LPI. Cells are counterstained with methyl green. Insets represent caspase-3 negative (green, top left) and positive cells (dark brown, top right). Scale bar = 100 µm.

B: The effects of 1 µM LPI and CID on cellular caspase-3 activity. Results are expressed as mean±S.E.M, *p<0.05 vs control, One-way ANOVA & Student Newman-Keuls, n=3 independent cultures.
4.2.11. LPI (1 µM) does not protect against Aβ-evoked caspase-3 activation.

Cells from cortical neuron-enriched cultures were treated with LPI (1 µM) in the presence or absence of Aβ for 72 hrs, immunolabelled for active caspase-3 and imaged using light microscopy (Figure 4.11A). The cells were counted and caspase-3 positive cells were expressed as a percentage of total cells. There was a significant effect between treatment groups ([F (3, 12) = 8.127]; p<0.01, One-way ANOVA, n=4 independent cultures). In Figure 4.11B, it can be observed that Aβ (10 µM) induced significant caspase-3 activation (40.05±5.27%) compared to control (11.28±2.69%). LPI (1 µM) did not attenuate this Aβ-evoked caspase-3 increase (29.51±4.47%) and did not increase capsase-3 alone (22.84±4.69%; p>0.05, Student Newman-Keuls, n=4 independent cultures).

The results here demonstrate that 1 µM LPI does not alter Aβ-evoked caspase-3 activation.
Figure 4.11. LPI (1 µM) does not protect against Aβ-evoked caspase-3 activation.

A: Representative images taken at 40x magnification of cells obtained from cortical neuron-enriched cultures stained for active caspase-3. The cells are treated with (i) control, (ii) 10 µM Aβ, (iii) 1 µM LPI or (iv) LPI+Aβ. Cells are counterstained with methyl green. Insets represent caspase-3 negative (green nuclei; top left) and positive neurons (dark brown; top right). Scale bar = 100 µm. B: The effects of 1 µM LPI and Aβ on cellular caspase-3 activity. Results are expressed as mean±S.E.M, **p<0.01, *p<0.05 vs control, One-way ANOVA & Student Newman-Keuls, n=4 independent cultures.
4.2.12. The selective GPR55 agonist 17g increases BV2 migration when exposed to UT-NM.

BV2 cells seeded in Boyden chambers were exposed to the selective GPR55 agonist 17g (1 µM) and/or the selective antagonist CID16020046 (20 µM). Concomitantly, the cells were exposed to primed neuronal medium (PNM) below the chamber. Rapi-diff staining was subsequently carried out on the membranes and BV2 migration was counted (Figure 4.12A). It was found that PNM and ligand treatment induced a significant effect among the groups (ligand = [F (3, 24) = 3.919], p<0.05; PNM = [F (1, 24) = 9.443]; p<0.01, Two-way ANOVA, n=4 independent cultures, data not shown). However, only Aβ-PNM induced a significant effect compared to UT-NM (p<0.05). It was therefore of interest to elucidate the effect of 17g and CID on each type of PNM individually (Figure 4.12B). There was a significant difference between treatment groups (UT-NM = [F (4, 15) = 3.782]; p<0.05; Aβ-PNM = [F (4, 15) = 8.68]; p<0.01, One-way ANOVA). A post hoc test indicated that 17g significantly enhanced BV2 migration in response to UT-NM (Figure 4.12Bi; UT-NM = 80.50±10.14; 17g+UT-NM = 106.00±6.50; p<0.01, Student Newman-Keuls, n=4 independent cultures). In contrast, 17g did not modulate Aβ-PNM-evoked BV2 migration (Figure 4.12Bii; Aβ-PNM = 124.00±8.40; 17g+Aβ-PNM= 130.30±12.32; p>0.05). CID (20 µM) did not inhibit 17g-induced BV2 migratory activity in cells exposed to either types of PNM (CID+17g+UT-NM = 107.50±12.84; CID+17g+Aβ-PNM = 124.3±13.59; p>0.05) but, as previously discussed in Section 4.2.3, it did attenuate Aβ-PNM-evoked migration when applied alone (CID+Aβ-PNM = 90.00±7.22; p<0.05).

The results here demonstrate that 17g enhances BV2 microglial migration in response to UT-NM. This effect was not inhibited by CID.
Figure 4.12. 17g increases BV2 migration when exposed to UT-NM.
A: Representative images of Rapi-diff stained membranes taken at 40x magnification in order to elucidate the effect of 17g (1 µM) and/or CID (20 µM) on modulating the BV2 migratory response to UT-NM and Aβ-PNM. Scale bar = 100 µm. B: (i) The effect of 17g (1 µM) and/or CID (20 µM) on BV2 cell migratory activity when co-exposed to UT-NM. BV2 cells were also exposed to neurobasal medium only (no neuronal exposure = control). Results are expressed as mean±S.E.M, **p<0.01, *p<0.05 vs control, p>0.05, One-way ANOVA & Student Newman-Keuls, n=4 independent cultures. (ii) The effect of 17g (1 µM) and/or CID (20 µM) on BV2 cell migratory activity when co-exposed to Aβ-PNM. Results are expressed as mean±S.E.M, **p<0.01, *p<0.05 vs control, +p<0.05 vs Aβ-PNM, One-way ANOVA & Student Newman-Keuls, n=4 independent cultures. Data partially contributed to by Christian Thomas Gabrielsen.
4.2.13. 17g does not induce BV2 phagocytosis.

BV2 cells were incubated with a 0.025 % latex bead solution in presence or absence of 17g (1 µM). They were left to incubate for 3 hrs, before being fixed and stained. There was significant interaction between ligand treatment and bead number ([F (2, 18) = 4.672]; p<0.05, Two-way ANOVA, 120 total cells per treatment group obtained from n=4 independent cultures). In Figure 4.13A-B, it can been seen that 17g induces a trend towards an increase in bead phagocytosis, with more cells phagocytosing 1-9 beads and 10+ beads than control cells (1-9 beads: control = 25.00±5.18% vs 17g =45±12.95%; 10+ beads: control = 5.83±2.85% vs 17g = 8.33±5.53%). However a Bonferroni post-hoc test failed to show any significant effect of 17g (0 beads = p = 0.10).

The results here demonstrate that 17g does not induce an increase in BV2 phagocytic activity.
**Figure 4.13.** 17g does not induce BV2 phagocytosis.

**A:** Representative confocal images of BV2 microglia taken at 40× magnification. Cells were treated with (i) control or (ii) 17g (1 µM). Cells were co-labelled with IBA1 (microglial marker, red) and hoechst (nuclear marker, blue). White arrows indicate phagocytosed beads. **B:** The effect of 17g on the % of BV2 cells phagocytosing latex beads. Results are expressed as mean±S.E.M, 120 cells obtained from n=4 independent cultures. Data partially contributed to by Christian Thomas Gabrielsen.
4.2.14. 17g does not stimulate TNF-α production by BV2 cells.

BV2 cells were incubated for 24 hrs in the presence or absence of 17g (1 μM). The supernatants were collected and an ELISA was performed to measure TNF-α production (Figure 4.14). An unpaired two-tailed t test was performed and indicated that there was no significant difference between 17g and control (17g 1 μM = 112.8±24.29 pg/mL vs control = 93.38±21.27 pg/mL; [t=0.6026 df=8]; p>0.05, n=5 independent cultures).
Figure 4.14. 17g does not stimulate TNF-α production by BV2 cells.

The effect of 17g (1 μM). Results are expressed as mean±S.E.M, Unpaired Student’s $t$ test, n=5 independent cultures.
4.2.15. CID attenuates 17g-induced caspase-3 activation.

Cells from cortical neuron-enriched cultures were treated for 72 hrs, immunolabelled for active caspase-3 and imaged using light microscopy. The cells were counted and caspase-3 positive cells were expressed as a percentage of total cells (Figure 4.15A). There was a significant effect between treatment groups (\[F (3, 8) = 4.740]; p<0.05, One-Way ANOVA, n=3 independent cultures). In Figure 4.15B, it can be observed that 17g (1 µM) significantly stimulated active caspase-3 (32.49±7.07%) compared to control (11.74±3.75%; p<0.05, Student Newman-Keuls, n=3 independent cultures). It is noteworthy that the selective GPR55 antagonist CID16020046 (CID; 20 µM) attenuated this 17g-induced increase (22.36±4.13%) and did not stimulate caspase-3 alone (20.58±1.31%; p>0.05).

The results here demonstrate that CID attenuates 17g-induced caspase-3 activation.
Figure 4.15. CID attenuates 17g-induced active caspase-3.

A: Representative images taken at 40x magnification of cells obtained from cortical neuron-enriched cultures labelled for active caspase-3. The cells were treated with (i) control, (ii) 1µM 17g, (iii) 20 µM CID or (iv) CID+17g. Cells are counterstained with methyl green. Insets represent caspase-3 negative (green, top left) and positive cells (dark brown, top right). Scale bar = 100 µm. B: The effects of 17g and CID on cellular caspase-3 activity. Results are expressed as mean±S.E.M, *p<0.05 vs control, Two-way ANOVA & Tukey’s post hoc test; n=3 independent cultures.
4.2.16. 17g (1 µM) protects against Aβ-evoked caspase-3 activation.

Cells from cortical neuron-enriched cultures were treated with 17g (1 µM) in the presence or absence of Aβ for 72 hrs, immunolabelled for active caspase-3 and imaged using light microscopy (Figure 4.16A). There was a significant interaction between treatment groups (\(F(2, 6) = 12.24\); p<0.01, One-way ANOVA, n=3 independent cultures). In Figure 4.16B, it can be observed that Aβ (10 µM) induced significant caspase-3 activation (42.74±6.40%) compared to control (11.74±3.75%) and 17g (1 µM) significantly attenuated this Aβ-evoked caspase-3 activation (20.93±3.84%; p<0.05, Student Newman-Keuls, n=3 independent cultures).

The results here demonstrate that 17g protects against Aβ-evoked caspase-3 activation.
Figure 4.16. 17g (1 µM) protects against Aβ-evoked caspase-3 activation.

A: Representative images taken at 40x magnification of cells obtained from cortical neuron-enriched cultures stained for active caspase-3. Cells were treated with (i) control, (ii) 10 µM Aβ, (iii) 1 µM 17g or (iv) 17g+Aβ. Cells are counterstained with methyl green. Insets represent caspase-3 negative (green nuclei; top left) and positive neurons (dark brown; top right). Scale bar = 100 µm. B: The effects of 17g and Aβ on cellular caspase-3 activity. Results are expressed as mean±S.E.M, **p<0.01, *p<0.05 vs control, +p<0.01 vs Aβ, One-way ANOVA & Student Newman Keuls, n=3 independent cultures.
4.2.17. CID does not induce BV2 phagocytosis.

BV2 cells were incubated with a 0.025 % latex bead solution in presence or absence of CID16020046 (20 µM). They were left to incubate for 3 hrs, before being fixed and stained. There was no significant effect of CID (0 beads: control = 72.22±7.30% vs CID = 70±7.58%; [F (6, 36) = 2.70]; p>0.05, Two-way ANOVA, 120 total cells per treatment group obtained from n=4 independent cultures; Figure 4.17A-B).

The results here demonstrate that CID does not stimulate BV2 phagocytic ability.
Figure 4.17. CID does not induce BV2 phagocytosis.
A: Representative confocal images of BV2 microglia taken at 40x magnification. Cells were treated with (i) control or (ii) CID (20 µM). Cells were co-labelled with IBA1 (microglial marker, red) and hoechst (nuclear marker, blue). B: The effect of CID on the % of BV2 cells phagocytosing latex beads. Results are expressed as mean±S.E.M, Two-way ANOVA, 120 cells obtained from n=4 independent cultures. Data partially contributed to by Christian Thomas Gabrielsen & Sean Cassidy.
4.2.18. CID does not stimulate TNF-α production by BV2 cells.

BV2 cells were incubated for 24 hrs in the presence or absence of the selective GPR55 antagonist CID16020046 (CID; 20 µM). The supernatants were collected and an ELISA was performed to measure TNF-α production (Figure 4.18). An unpaired two-tailed t test was performed and indicated that there was no significant difference between each treatment group (CID 20 µM = 114.9±28.13 pg/mL vs control = 93.38±21.27 pg/mL; [t=0.6093 df=8]; p>0.05, n=5 independent cultures).

The results here demonstrate that CID does not stimulate TNF-α production by BV2 microglia.
Figure 4.18. CID does not stimulate TNF-α production by BV2 cells.

The effect of CID (20 µM). Results are expressed as mean±S.E.M, Unpaired Student's t test, n=5 independent cultures.
Cells from cortical neuron-enriched cultures were treated with CID16020046 (CID; 20 µM) in the presence or absence of Aβ for 72 hrs, labelled for active caspase-3 and imaged using light microscopy (Figure 4.19A). There was a significant interaction between agonist and challenge ([F (3, 8) = 12.32]; p<0.01, One-way ANOVA, n=3 independent cultures). In Figure 4.19B, it can be observed that Aβ (10 µM) induced significant caspase-3 activation (42.74±6.40%) compared to control (11.74±3.75%) and CID (20 µM) attenuated this Aβ-evoked caspase-3 activation (27.02±1.09%; p<0.05, Student Newman-Keuls, n=3 independent cultures).

The results here demonstrate that CID attenuates Aβ-evoked caspase-3 activation.
Figure 4.19. CID attenuates Aβ-evoked caspase-3 activation.

A: Representative images taken at 40x magnification of cells obtained from cortical neuron-enriched cultures stained for active caspase-3. Cells were treated with (i) control, (ii) 10 µM Aβ, (iii) 20 µM CID or (iv) CID+Aβ. Cells are counterstained with methyl green. Insets represent caspase-3 negative (green nuclei; top left) and positive neurons (dark brown; top right). Scale bar = 100 µm. B: The effects of CID and Aβ on cellular caspase-3 activity. Results are expressed as mean±S.E.M, *p<0.05, **p<0.01 vs control, +p<0.05 vs Aβ, One-way ANOVA & Student Newman Keuls, n=3 independent cultures.
4.3. Discussion

The aim of this chapter was to investigate the role of GPR55 in regulating microglial cytokine production, migration and phagocytosis in response to stimuli using the BV2 microglial cell line and its role in apoptosis using active caspase-3 immunocytochemical labelling of cells from cortical neuron-enriched cultures. It was found that GPR55 is expressed in BV2 microglial cells. The endogenous GPR55 agonist, LPI and the selective antagonist, CID16020046, attenuate Aβ-PNM-evoked BV2 cell migration. In contrast, the selective GPR55 agonist, 17g, promotes BV2 cell migration under basal conditions. GPR55 ligands do not stimulate BV2 phagocytosis or TNF-α production. LPI and 17g protect against Aβ-evoked active capsase-3 stimulation in cells from cortical neuron-enriched cultures and CID16020046 attenuated this effect.

4.3.1. GPR55 regulation of cell migration and phagocytosis.

GPR55 plays a key regulatory role in the immune system and is expressed by immune organs such as the spleen as well as by peripheral immune cells e.g. neutrophils (Balenga et al., 2011), lymphocytes (Oka et al., 2010), monocytes and NK cells (Chiurchiù et al., 2014). It is also expressed by microglia in the brain (Pietr et al., 2009). In the present study it was confirmed that microglia from cortical neuron-enriched cultures (see Section 3.2.1) and the BV2 microglial cell line express GPR55. Cell migration is an important part of the inflammatory response. Inflammation due to tissue damage or infection induces the release of cytokines and chemokines from distressed stromal cells, oligodendrocytes, astrocytes and even neurons. In response to these signals, innate immune cells migrate to the site of injury (Luster, Alon & von Andrian, 2005; Ramesh et al., 2013). The results here demonstrate that the endogenous agonist for GPR55, LPI and the selective agonist, 17g, both modulated BV2
microglial migration in response to external stimuli. LPI attenuated BV2 migration in response to Aβ-primed neuronal medium (Aβ-PNM). 17g in contrast enhanced BV2 migration under basal conditions, but did not modulate Aβ-PNM-evoked migration. However, both LPI and 17g did not stimulate BV2 phagocytosis. The selective GPR55 antagonist CID16020046 (CID) did not inhibit LPI- and 17g-induced migration, thus suggesting that the effects of these agonists were independent of GPR55. These data suggest that these GPR55 agonists modulate BV2 migration but may not be acting through the same target and/or mechanism. See Figure 4.20. for a summary of these effects.

To date there are limited studies elucidating the role of GPR55 in regulating inflammation in the CNS. The putative GPR55 agonist, O-1602, was previously shown to promote BV2 microglial migration, but it was claimed that this response was mediated by the orphan receptor, GPR18 (McHugh et al., 2010). In the present study, all suggested ligands to act at GPR55 exhibited individual effects on BV2 migration. Kallendrusch et al. (2013) showed that 1 μM LPI attenuated ATP-induced microglial migration in a chemotactic Boyden chamber assay and reduced the number of activated microglia after N-methyl-D-asparate (NMDA)-induced lesions in rat OHSC. The release of ATP from dying cells is thought to contribute to ischemia-induced neurodegeneration (Le Feuvre et al., 2003). Kallendrusch et al. postulated that LPI may have induced a beneficial phenotypic switch in the microglia, particularly because they showed that LPI alone promoted microglial migration in the Boyden chamber assay. This may help to explain the results herein. AD is associated with the accumulation of pathogenic Aβ aggregates in the brain and results in mitochondrial dysfunction and the progressive degeneration of synapses and neuronal networks (Clayton, Enoo & Ikezu, 2017; Palop & Mucke, 2010). Stress signals such as reactive oxygen species (ROS) are released by the neurons as a result. Indeed, soluble Aβ species were suggested to induce an increase in hydrogen peroxide (H₂O₂) production and decreased cytochrome C oxidase activity in a mouse model of AD (Manzchak et al., 2004). Furthermore, microglia can become activated in
response to neuronal ROS as well as Aβ itself. They produce a catalogue of factors as a result such as ROS and cytokines that are toxic to neurons (Block, 2007). Neuronal ROS were likely present in the Aβ-PNM, which may have caused the resultant increase in BV2 migration. LPI however attenuated this migration. Perhaps this indicates that LPI may induce a less inflammatory phenotype in response to Aβ-PNM. In contrast, the selective GPR55 agonist, 17g, promoted migration under basal conditions but did not modulate Aβ-PNM-induced migration. Interestingly, CID failed to inhibit LPI- and 17g-induced BV2 migration, but attenuated Aβ-PNM-evoked migration when applied alone. This reflects findings reported by Stančić et al. (2015), whereby treatment with CID alone decreased the migration of J774A.1 mouse macrophages. CID also attenuated nitric oxide (NO) production in cultured rat microglial cells activated by LPS (Malek et al., 2015). This suggests that inhibiting GPR55 may have a therapeutic effect in models of inflammation or AD.

Cell migration involves alteration of the cell cytoskeleton, cell-substrate adhesions and the extracellular matrix. It is mediated by the Rho family of proteins. At least 20 Rho family proteins have been identified so far in humans and of these RhoA/B, Rac1/2 and Cdc42 have been the most widely studied for their effects on cell migration (Ridley et al., 2011). GPR55 was shown to couple to Gα13-RhoA-ROCK which resulted in the cytoskeletal modification of HEK293 cells and human neutrophils (Balenga et al., 2011). Rho GTPases are also responsible for mediating cellular phagocytosis. Activation of Rho GTPases induces F-actin reorganisation which allows for the formation of phagocyte filopodia and membrane ruffles. Foreign particles or pathogens can then be taken up by the phagocyte and eliminated (Mao & Finnemann, 2015). The results herein demonstrate that LPI and 17g do not stimulate phagocytosis. Chiurchiù et al. (2015) demonstrated that LPS-activated monocytes showed a significant increase in phagocytosis of fluorescein isothiocyanate (FITC)-dextran particles coupled with an increase in IL-12 and TNF-α production compared to unstimulated cells. Interestingly, treatment with the GPR55 agonist, O-1602, attenuated this phagocytic
increase but exacerbated IL-12 and TNF-α production, thus indicating that GPR55 stimulation promoted a pro-inflammatory phenotype in LPS-activated monocytes. LPI also promoted IL-12 and TNF-α production in LPS-activated monocytes in that study. Without LPS activation however, O-1602 failed to stimulate phagocytosis. This complements the findings herein, whereby LPS stimulation of BV2 cells significantly increased phagocytosis of latex beads but GPR55 ligands alone did not. Perhaps activation of BV2 cells with LPS and/or Aβ would reveal any regulatory effects of LPI and 17g on phagocytic ability and such an experiment should be included in any continuation of this work.
4.3.2. GPR55 regulation of microglial cytokine production.

GPR55 mRNA has previously been shown to be expressed in primary microglial cells and BV2 microglial cells. There is conflicting evidence regarding the expression levels of GPR55 in activated microglia. Treatment with LPS induced a downregulation of GPR55 mRNA expression in these cells (Pietr et al., 2009). The results here confirm that GPR55 is expressed by microglia from cortical neuron-enriched cultures and BV2 cells. However, the GPR55 agonists, LPI or 17g, or the selective antagonist CID16020046 (CID), do not stimulate the production of the pro-inflammatory cytokine TNF-α. Malek et al. (2015) have previously

Figure 4.20. The suggested effect of GPR55 ligands in Aβ-evoked microglial migration.

Aβ-PNM evokes an increase in microglial migration. LPI (agonist, blue) and CID (antagonist, red) attenuate this migration in response to Aβ-PNM. However, LPI may be acting via a separate target to GPR55 because it is not inhibited by CID.
shown that GPR55 antagonism with CID significantly increased GPR55 mRNA levels and attenuated LPS-induced NO release and M1 phenotypic markers in cultured primary microglia. Furthermore, AEA administration alone also attenuated LPS-induced NO release and M1 phenotypic markers in cultured primary microglia and CID pre-treatment had no effect on this. They postulated that GPR55 participates in cross-talk mechanisms with CB receptors and acts to dampen excessive CB receptor activation. The putative GPR55 agonist, O-1602 and LPI exacerbated IL-12 and TNF-α production in LPS-activated monocytes but failed to stimulate cytokine production in the absence of LPS (Chiurchiù et al., 2015). Similarly, foam cells (macrophages which have abnormally taken up cholesterol-rich modified low density lipoproteins) expressed higher levels of IL-12 and TNF-α compared to normal macrophages. When these cells were treated with O-1602, TNF-α mRNA levels were further increased (Lanuti et al., 2015). These data, coupled with my own findings, suggest that the GPR55 ligands used in the current study may have a modulatory effect on cytokine production in BV2 microglial cells only following activation by an inflammatory challenge. Indeed, a preliminary ELISA was subsequently carried out using supernatants exposed to both GPR55 ligands and LPS. However, the resultant effect of these challenges on TNF-α production was inconclusive because of variability between replicates. These data were therefore not included in this thesis. Future work should involve increasing replicate numbers in order to obtain consistent findings.

Aβ failed to induce TNF-α production by BV2 cells in the current study. In line with this, Janefjord et al. (2014) reported that Aβ1-42 evoked a loss of cell viability in SH-SY5Y neuroblastoma cells but negligible TNF-α and nitrite production in BV2 cells compared to albumin or LPS. In mouse models of AD, the level of pro-inflammatory cytokine production is compromised compared with that observed in other inflammatory models, such as acute microbial challenge. This is not unusual as it is believed that Aβ stimulates a phagocytic phenotype in microglia. Indeed, at sites of Aβ deposition, a wide range of studies suggest that
microglia phagocytose Aβ fibrils and several ultrastructural studies have reported that microglia in the AD cortex contain intracytoplasmic Aβ fibrils (Frackowiak et al., 1999; Rogers et al., 2002). Perhaps future experiments to assess GPR55 regulation of microglial phenotype could involve stimulating BV2 cells with Aβ or LPS and co-treating them with GPR55 ligands using the phagocytosis assay discussed in this chapter.

4.3.3. GPR55 regulation of apoptosis.

Under physiological conditions cellular homeostasis in eukaryotes is tightly maintained through a balance of cell mitosis and programmed cell death, the latter also termed apoptosis (Alberts et al., 2002). However in certain pathological conditions aberrant regulation of apoptosis is a predominant abnormality. Apoptotic mechanisms have previously been associated with neurodegenerative conditions (Mattson, 2000) and the induction of caspase-mediated apoptosis is thought to be implicated in neurodegeneration and aging (Bredesen, 2009; D’Amelio et al., 2011; Friedlander, 2003; Zhang et al., 2003). Apoptosis is a distinguishing feature in the brains of patients suffering from neurodegenerative diseases such as AD (Shimohama, 2000). The results herein demonstrate that LPI (10 µM) and 17g (1 µM) induce an increase in cellular active caspase-3 when applied alone. These effects were attenuated by the selective GPR55 antagonist, CID16020046 (CID). Moreover, LPI (10 µM) and 17g protected against Aβ-evoked caspase-3. See Figure 4.21. for a summary of these effects. These data are in line with findings reported by Janefjord et al. (2014), whereby the putative GPR55 agonist, O-1602, protected against LPS-induced neurotoxicity in the SH-SY5Y neuroblastoma cell line. Kallendrusch et al. (2013) also demonstrated that LPI (1 µM) protected dentate gyrus granule cells in rat OHSC after excitotoxic lesion in a GPR55-dependent manner. GPR55 stimulation also induces the
activation of pro-survival downstream effectors such as CREB and ERK (Henstridge et al., 2011), which may have contributed to downstream neuroprotective functions. Interestingly, the selective GPR55 antagonist CID attenuated Aβ-evoked microglial migration and caspase-3 induction. Similarly, Malek et al. (2015) reported that GPR55 antagonism with CID alone suppressed LPS-induced NO production in cultured rat microglial cells. Perhaps GPR55 antagonism can be of therapeutic benefit in combating neuroinflammation and apoptosis. However, while 1 µM LPI alone did not induce caspase-3, co-treatment with CID significantly promoted caspase-3 stimulation. It is worth noting that replicate numbers were n=3 for the caspase-3 assay when cells were co-treated with agonist and CID, so additional replicates would validate these data in order to discern the definitive effects of these co-treatments.

It was surprising that LPI and 17g agonists induced an increase in caspase-3 activation in the absence of Aβ. Cells from these cortical neuron-enriched cultures were treated with these agonists for 72 hrs, so it is possible that chronic stimulation led to downstream caspase-3 activation. GPR55 is a lipid-sensing receptor and with prolonged or severe lipid overload, cellular responses can become maladaptive and contribute to cellular demise (Schaffer et al., 2016). Furthermore, overstimulation of GPCRs can lead to receptor desensitisation and subsequent cellular toxicity (Rajagopal & Shenoy, 2017). However, caspase-3 also modulates synaptic plasticity in the brain without inducing cell death. In neurons, there is evidence that caspases can be activated in dendrites, synaptosomes and growth cones (Campbell & Holt, 2003; Gilman and Mattson, 2002; Kuo et al., 2006; Williams et al., 2006). LTP and long-term depression (LTD) are long-lasting modifications of synapses. LTD is NMDA-receptor-dependent in CA1 and requires Ca^{2+} influx and the serine/threonine phosphatases calcineurin/PP2B and PP1. Sheng and colleagues reported that LTD and AMPA receptor internalisation in hippocampal neurons require the activity of caspase-9 and caspase-3/7. They also found that caspase-3 can be transiently activated via the mitochondrial pathway by
stimulating NMDA receptor-dependent LTD, without causing cell death. They suggested that activation of caspase-3 induced by short-duration NMDA-receptor stimulation was not associated with increased cell death, implying that caspase-3 activation does not inevitably lead to apoptosis (Li et al., 2010). Furthermore, Sheng and colleagues also discovered that caspase-3 deficiency in mice interferes with specific aspects of cognition and behaviour, particularly attention and inhibitory control. These attention processes were tested using a five-choice serial reaction time task and it was found that caspase-3−/− mice exhibited more premature and impulsive responses and higher locomotor activity than WT. In the Morris Water Maze, these deficient mice also showed poor reversal learning compared to WT when their platform was moved, suggesting they have impaired cognitive flexibility. They linked these changes in attention behaviour and control to caspase-3-dependent homeostatic synaptic scaling, whereby caspase-3 deficiency led to chronic elevation of EPSPs and increased network activity. They postulated that caspase-3 was involved in synaptic suppression in the hippocampus and the regulation of attention behaviour (Lo et al., 2015). These findings could help to explain the results herein, whereby LPI or 17g alone promoted caspase-3 induction. Perhaps treatment with these agonists could promote caspase-3-dependent LTD without inducing neuronal cell death. To determine if these ligands are cytotoxic, assays could be performed in future such as a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay to measure cell viability, cytotoxicity and cell proliferation (Gerlier & Thomasset, 1986) or terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) to measure levels of DNA fragmentation in cells (Noonan et al., 2010).
There are certain possibilities that may explain why both LPI and 17g did not mediate the same effects and, in some assays, were not antagonised by CID16020046 (CID). GPR55 expression is altered under certain pathological conditions. For instance, GPR55 mRNA is downregulated in primary microglial cells and BV2 microglial cells treated with LPS. In
contrast, GPR55 mRNA is slightly upregulated in BV2 cells treated with interferon (IFN)-γ but downregulated in primary microglia. This suggests that cellular composition (i.e. primary cell or cell line) and activation state are potentially important for GPR55 function in microglia (Pietr et al., 2009). Celorrio et al. (2017) demonstrated that striatal expression of GPR55 mRNA was downregulated in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and probenecid (MPTPp) murine model of Parkinson’s disease (PD). These studies implicate that the addition of Aβ may have altered GPR55 expression in the BV2 cells and cortical cultures utilised herein, suggesting that ligand efficacy may have been consequentially altered. A measure of GPR55 protein could be performed in future using Western Blotting in order to determine the effects of Aβ on GPR55 protein expression.

LPI and 17g were not inhibited by CID in the BV2 migration assay used herein. The ligands may therefore have been inducing effects independent of GPR55. Indeed, Bondarenko et al. (2010) reported that LPI elicited a biphasic response in endothelial cells. The immediate Ca^{2+} signalling depended on GPR55 while the subsequent depolarisation was associated with Na^{+} loading via non-selective cation channels and an inhibition of the Na^{+}/K^{+}-ATPase. LPI also acts on TRP channels such as TRPV2 and increases cell migration of the prostate cancer cell line PC3 (Monet et al., 2009). The orphan receptor GPR119 has also been implicated in mediating the effects of LPI in RH7777 rat hepatoma cells (Soga et al., 2005). The orphan receptor GPR18 also shares pharmacology with GPR55, with the agonist O-1602 reported to mediate effects at both GPR55 and GPR18. Indeed, O-1602 induced cellular migration of BV2 microglia and GPR18-HEK293 transfected cells (McHugh et al., 2010). In contrast, O-1602 administration into the hippocampi of mice increased immature neuron generation, whereas in GPR55-/- animals, there were reduced rates of proliferation and neurogenesis within the hippocampus. O-1602 had no effect in these animals compared to vehicle controls. It is therefore possible that the effects of LPI and 17g on BV2 migration in the current study are not dependent on GPR55 but instead on related targets like GPR18. Using
siRNA or animal knockouts for GPR55 or other potential targets like GPR18 could aid in determining which target these ligands exhibit efficacy.

Another possibility is the pharmacological interaction of the ligands at GPR55. For instance, CID is a competitive antagonist at GPR55. The concentration of 1 µM 17g was selected in the present study because previous findings demonstrated that 17g-induced increases in \([\text{Ca}^{2+}]_i\) at picomolar concentrations in the hGPR55-HEK293 cell line (EC\(_{50}\) = 7 nM; Yrjölä \textit{et al.}, 2016). A higher concentration of agonist would be needed to induce effects in the endogenous central model used herein because receptor density is lower in endogenous cells (Kenakin, 1986). Indeed, 1 µM 17g appeared to be the optimal concentration to use in the ratiometric \([\text{Ca}^{2+}]_i\) imaging assay used herein in Chapter 3. The authors of a recently accepted study used 1 µM 17g in order to evaluate its effect on \([\text{Ca}^{2+}]_i\) in secretory granulated ductal cells in adult rats (Korchynska \textit{et al.}, 2019, in press). However, at 1 µM, 17g may have displaced CID at their binding site at GPR55, meaning that CID failed to antagonise 17g in the present model (Tallarida, Cowan & Adler, 1979). However, CID is believed to be an effective antagonist because it interacts with the primary ligand interaction site, K2.60(80) and its methylphenyl ring penetrates deepest into the binding site and prevents the M3.36(104) / F6.48(239) toggle switch from undergoing the conformational change that accompanies GPR55 activation (Kotsikorou \textit{et al.}, 2013). A docking model of how 17g interacts with GPR55 will need to be generated in order to discern how this agonist interacts with GPR55 and if it is blocked by CID. Furthermore, a dose-response series will need to be performed, either using lower concentrations of 17g or a higher concentration of CID. However, a recent study reported that the selective GPR55 antagonist, ML193, showed constitutive effects at high concentrations. ML193 inhibited thapsigargin-evoked \([\text{Ca}^{2+}]_i\) release in mouse cortical neurons when applied alone and also prevented the mobilisation of \([\text{Ca}^{2+}]_i\) and \([\text{Zn}^{2+}]_i\) by \(N\)-arachidonoylglycine, an endocannabinoid believed to be an endogenous agonist for GPR18 (Bouron, 2018). Increasing the concentration of CID may therefore
induce off-target effects. The results shown here suggest that these ligands may be useful therapeutic agents in the treatment of AD, so determining their target and efficacy is of crucial importance going forward.

4.3.5. Conclusion

The aim of this chapter was to investigate the role of GPR55 in regulating cytokine production, microglial migration and phagocytosis in response to stimuli using the BV2 microglial cell line and its role in apoptosis using caspase-3 immunocytochemistry. LPI and CID attenuated Aβ-PNM-evoked microglial migration. Moreover, LPI and 17g protected against Aβ-evoked apoptosis in a GPR55-dependent manner. Suggested mechanisms for these effects can be viewed in Figures 4.20 and 4.21. These findings demonstrate that GPR55 ligands appear to be potential therapeutic agents for the treatment of AD.
Chapter 5

GPCR expression and function in human peripheral immune cells
The following chapter describes the data obtained during a 5-month Industrial PhD Placement in 2016 that took place in the Research & Development sector of GlaxoSmithKline, Stevenage, UK.

The human biological samples were sourced ethically and their research use was in accord with the terms of the informed consents under an IRB/EC approved protocol.
5.1. Introduction

The current study is concerned with the pharmacology of novel GPCR receptors, particularly the putative cannabinoid receptor, GPR55. While this is an interesting research area, the field is limited by the availability of novel pharmacological tools and assays. An industrial placement was carried out during the current study in GlaxoSmithKline (GSK), Stevenage, UK, in their Research & Development sector, which allowed for access to novel compounds and assays that complemented the research performed in Trinity College Dublin (TCD) and University College Dublin (UCD).

During the industrial placement, assays were utilised in order to investigate the expression and function of GPR55 and another novel GPCR, hydroxycarboxylic acid receptor 2 (HCA2)/HM74A/GPR109A, in peripheral immune cells. HCA2 was investigated because ligands for this receptor were previously tested using monocyte-derived dendritic cells (moDC) by James, C. (unpublished data). Using a PGE$_2$ ELISA, James reported that the endogenous agonist for HCA2, niacin, stimulated PGE$_2$ production by these cells. The initial premise of the placement involved the replication of this experiment. If I could replicate the findings reported by James, I would then test GPR55 ligands using this moDC model.

5.1.1. Hydroxycarboxylic acid receptor 2

HCA2 is a Class A rhodopsin-like 7 transmembrane GPCR that is expressed on human chromosome 12q24.31 (see Figure 5.1). It couples to G$\alpha_i$ (Lee et al., 2001) and was first identified in macrophages stimulated by IFN$\gamma$ in mice. For this reason its mouse ortholog was named Protein Upregulated in Macrophages by IFN-gamma (PUMA-G), but its human ortholog pseudonyms include GPR109A, Niacin receptor 1 (NIACR1) and HCA2/HM74A (Offermanns, 2006). HCA2 is expressed in immune cells such as macrophages and dendritic
cells and is also highly expressed in adipose tissue. HCA2 is responsive to hydroxycarboxylic acids which are intermediates of energy metabolism. One such example is β-hydroxybutyrate (β-HB) which is synthesised during ketosis from acetoacetate. β-HB can be used as an energy source by the brain when glucose levels in the blood are low. β-HB levels increase in the liver, heart, muscle and brain during exercise, calorie restriction, fasting, periods of reduced carbohydrate intake and ketogenic diets. Once the concentration of β-HB reaches millimolar concentrations it activates HCA2, leading to reduced lipolysis and free-fatty acid (FFA) formation in adipocytes (Offermanns, 2017).

Another established ligand for HCA2 is niacin/nicotinic acid (hence the receptor pseudonym NIACR1). Niacin is also known as vitamin B3 and can be synthesised endogenously from tryptophan via the kynureine pathway. Like β-HB, niacin has anti-
lipolytic effects. Altschul et al. (1955) initially discovered that the administration of relatively high doses of niacin (3x1 g per day) decreased total plasma levels of cholesterol by ~10% in healthy volunteers and by >20% in hypercholesterolemic patients. This eventually led to the introduction of niacin to clinical practice as the first anti-dyslipidemic drug. Unfortunately niacin induces unwanted adverse effects. It causes cutaneous vasodilatation of the skin (flushing), particularly in the upper body and face and gastrointestinal problems when administered at relatively low doses (50-100 mg). These effects have a negative effect on patient compliance (Andersson et al., 1971). Other ligands of HCA2 include dimethyl fumarate (DMF) and its active metabolite monomethylfumarate (MMF; Chen et al., 2014; Hanson, Gille & Offermanns, 2014). Fumarates also induce flushing (Phillips et al., 2014). The chemical structures of HCA2 ligands can be viewed in Figure 5.2.
It is now believed that the flushing effect of niacin is mediated via HCA2 expressed by Langerhans cells in the epidermis of the skin. Langerhans cells are a type of dendritic cell (Romani, Björn & Stoitzner, 2010). Upon niacin administration there is an increase \([\text{Ca}^{2+}]_i\) (Benyó et al., 2006), which triggers phospholipases such as phospholipase \(A_2\) (PLA\(_2\)) to release arachidonic acid from lipid stores in the cell. This free arachidonic acid acts as a precursor to the production of eicosanoids, including lipoxygenases, thromboxanes and prostaglandins (Lin, Lin & Knopf, 1992). Prostaglandins (e.g. PGD\(_2\), PGE\(_2\)) released by Langerhans cells cause vasodilation of small capillaries under the skin which leads to flushing (Benyó et al., 2006; Kamanna, Ganji & Kashyap, 2009; Maciejewski-Lenoir et al., 2006).
These data are substantiated by the fact that depletion of Langerhans cells in murine models abolishes the niacin-induced flushing response (Benyó et al., 2006; Figure 5.3).

Due to the adverse skin flushing effects of niacin, measures have been taken in recent years to establish a drug that replicates the anti-lipolytic effects of niacin but does not induce flushing. One such drug is GSK256073 (GSK073), a selective agonist of HCA2 (Figure 5.2). Only 3 studies have been published to date using this drug and evidence indicates that this agonist induces the same anti-lipolytic effects as niacin but does not induce flushing in preclinical rodent models and in healthy human male subjects. Furthermore GSK073 exhibited a longer half-life than niacin (Sprecher et al., 2015). GSK073 also improved glucose control and significantly decreased serum non-esterified fatty acid (NEFA) concentrations in

Figure 5.3. The mechanism of niacin-induced PG synthesis via HCA2 in Langerhans cells.
Schematic obtained from Gille et al. (2008).
patients with type II diabetes mellitus. Glucose improvements correlated with decreased insulin concentrations and measures of enhanced insulin sensitivity (Dobbins et al., 2013). However GSK073 failed to sustain its beneficial effects because its ability to suppress plasma NEFA concentrations, its modulatory effects on glucose oxidation and insulin sensitivity diminished over time (Dobbins et al., 2015). Nevertheless these data demonstrate that HCA2 stimulation has beneficial effects in conditions such as obesity, which is characterised by exaggerated adipose tissue lipolysis and increased NEFAs (Wanders et al., 2013) and in conditions compounded by diabetes (e.g. diabetes retinopathy; Gambhir et al. 2012). Compounds that do not induce skin flushing but promote anti-lipolysis (e.g. GSK073) will be crucial for patient compliance going forward.

HCA2 shares some similarities with GPR55. Both are Class A rhodopsin-like lipid-sensing GPCRs. Both GPR55 (Henstridge et al., 2011) and HCA2 (Offermanns, 2006) are expressed in adipose tissue and regulate metabolic effects. Both are expressed in immune tissue and mediate immunomodulatory effects (Feingold et al., 2014; Haugh et al., 2016). Both are expressed by microglia (Pietr et al., 2009; Parodi et al., 2015), by monocytes/macrophages (Chiurchiù et al., 2015; Feingold et al., 2014) and dendritic cells (Chiurchiù et al., 2015; Benyó et al., 2006). However there is no evidence to date that shows that HCA2 is expressed in central neurons, whereas GPR55 is expressed in neurons (Henstridge et al., 2011).

5.1.2. Evidence into GPR55 and HCA2 expression in peripheral immune cells and their roles in functional activity

Previous work in this thesis sought to investigate GPR55 expression, signalling effects and functional activity in a rat cortical neuron-enriched culture model and in BV2 microglia. GPR55 and HCA2 overlap in expression localisations and suggested functions, yet there are
very few studies investigating these receptors in peripheral immune cells. Chiurchiù et al. (2015) were the first group to characterise GPR55 expression and functional activity in distinct populations of human mononuclear cells of both innate and adaptive immunity using polychromatic flow cytometry. They found that the highest levels of GPR55 expression occurred in innate immune cells such as natural killer (NK) cells and monocytes with modest levels observed in myeloid DCs (mDC). They then went on to investigate the functional activity of GPR55 in these cells. Stimulation of GPR55 with its putative ligand, O-1602, decreased the phagocytic ability of activated monocytes while concomitantly increasing the production of the pro-inflammatory cytokines IL-12 and TNF-α. Furthermore, GPR55 stimulation with O-1602 enhanced cytolytic activity and pro-inflammatory IFNγ and TNF-α production in activated NK cells. These responses were inhibited by treatment with the putative GPR55 antagonist cannabidiol (CBD). However, O-1602 and CBD are not selective for GPR55, so these effects warrant further investigation.

Benyó et al. (2005) investigated the expression of the mouse ortholog of HCA2, PUMA-G, in immune cells and reverse transcriptase-polymerase chain reaction (RT-PCR) revealed its mRNA expression in MHC class II–positive skin cells and dendritic cells as well as in peritoneal macrophages. No expression was observed in peripheral monocytes. Niacin-induced flushing was later investigated by Benyó et al. (2006). Depletion of dermal macrophages and dendritic cells using diphtheria toxin failed to diminish niacin-induced flushing, whereas depletion of epidermal Langerhans dendritic cells using diphtheria toxin successfully inhibited niacin-induced flushing, as determined by cutaneous vasodilation in the mouse ear using laser-Doppler flowmetry. Furthermore niacin failed to induce an increase in [Ca²⁺]i in Langerhans cells from HCA2-deficient mice. They also investigated the link between niacin and prostaglandin production by testing for PGD₂ synthase and PGE₂ synthase expression in epidermal Langerhans cells. They detected expression of PGD₂ synthase and the constitutive type 2 PGE₂ synthase (mPGES-2; Benyó et al. 2006) and have
previously shown that deletion of PGD$_2$ and PGE$_2$ receptors (DP and EP$_2$ respectively) diminish but do not fully abrogate the niacin-induced flushing response (Benyó et al., 2005). Flow cytometry has been used to show that mature neutrophils also express HCA2, but immature neutrophils and eosinophils do not. Niacin accelerated apoptosis in cultured mature neutrophils in a concentration-dependent manner (Kotsylina et al., 2007).

Given that GPR55 and HCA2 are expressed in innate immune cells and appear to have modulatory roles on the function of these cells, their expression and their effect on cell functional activity was elucidated in peripheral monocytes and differentiated moDC. Polychromatic flow cytometry was used to determine changes in cellular marker expression profiles as monocytes differentiated into moDC. Functional activity of GPR55 was investigated using flow cytometric measurement of monocyte phagocytosis in response to ligands. Functional activity of HCA2 was investigated using ELISA to determine levels of PGE$_2$ production by mature moDC in response to ligand stimulation. If HCA2 stimulation promoted PGE$_2$ production, then GPR55 ligands would subsequently be tested using this assay.
5.2. Results

5.2.1. Expression of the dendritic cell differentiation marker CD11c increases during monocyte differentiation in to dendritic cells.

To investigate the expression of the novel GPCRs in myeloid cells, it was at first vital to establish a common marker to differentiate between monocytes and monocytes that had differentiated into DCs (moDC). GPR55 is expressed in monocytes and mDC (Chiurchiù et al., 2015) and the HCA2 mouse ortholog PUMA-G is expressed in mouse peritoneal macrophages and epidermal Langerhans cells (Benyó et al., 2005). Neither study reported on changes in GPCR expression levels during differentiation, so it was therefore of interest to elucidate if GPR55 or HCA2 expression altered as monocytes differentiated into moDC in the present study. CD11c is expressed in monocytes and its expression increases as monocytes differentiate in to moDC (Ito et al., 1999; Wang et al., 2017). CD11c was therefore used as a marker of dendritic cell differentiation in the current study.

CD14⁺ monocytes were positively selected from peripheral blood mononuclear cells (PBMC) and incubated with GM-CSF and IL-4 in order to differentiate them in to moDC. Cells were fixed either at 0 min (t=0) or 72 hrs (t=72 hrs) and stained for fluorescein isothiocyanate (FITC)-conjugated human CD11c (Figure 5.4A-B). In Figure 5.4A, it can be observed in the scatter plots that the population of the fixed cells measured at the two different timepoints (i, t=0; ii, t=72 hrs) differ in size (as measured by forward scatter, FSC) and complexity (as measured by side scatter, SSC). The gates applied in each scatter plot allowed for differentiation of the population into singlets. CD11c shifted right on the FITC log scale, indicating CD11c\textsuperscript{high} fluorescence. A high or low position on the log scale (referring to high or low fluorescence respectively) shall be referred to as marker\textsuperscript{high} or marker\textsuperscript{low} going forward. The geometric mean fluorescence intensity (MFI) values of the
singlets were analysed for levels of CD11c fluorescence (Figure 5.4Bi-ii). It was found that CD11c expression significantly increased after 72 hr cell incubation with GM-CSF and IL-4 (t=0, 38.97±3.24 units vs t=72 hrs, 83.87±8.79 units; **p<0.01 vs t=0, unpaired two-tailed Student’s t test, cells isolated from n=3 independent donors).

The results here demonstrate that expression of the dendritic cell differentiation marker CD11c increases over time as monocytes differentiate into moDC.
Figure 5.4. Expression of the dendritic cell differentiation marker CD11c increases during monocyte differentiation into dendritic cells.

A: (i, ii) Representative scatter plots of FSC (linear) and SSC (linear) showing the size and complexity respectively of ungated cells fixed at \( t=0 \) (i) and after differentiation for 72 hours (\( t=72 \) hrs. (ii). B: Representative histogram of the log fluorescence intensity of cellular CD11c expression of cell singlets at \( t=0 \) (red) and \( t=72 \) hrs (blue) versus singlet event count (normalised to mode). (ii) The log geometric mean fluorescence intensity (MFI) of CD11c expression in \( t=0 \) cells vs. \( t=72 \) hrs. Results are expressed as log mean±S.E.M; **\( p<0.01 \), unpaired two-tailed Student’s \( t \)-test, cells isolated from \( n=3 \) independent donors.
5.2.2. GPR55 expression significantly increases in CD11c+ cells over time.

Previous work in this thesis sought to elucidate GPR55 expression and functional activity in a rat cortical neuron-enriched culture model and the BV2 microglial cell line. Attention turned to investigating the expression of this receptor in peripheral immune cells after Chiurchiù et al. (2015) found that innate immune cells such as NK cells and monocytes highly express GPR55 and mDC express GPR55 at modest levels. However, they did not attempt to differentiate monocytes into moDC so therefore did not measure any changes in GPR55 expression during differentiation. The present study sought to expand on the data obtained by Chiurchiù et al. by determining if GPR55 expression altered during the moDC differentiation process.

CD14+ monocytes were positively selected from PBMC and incubated with GM-CSF and IL-4 for 72 hrs in order to differentiate them in to moDC. Cells were fixed either at 0 min (t=0) or 72 hrs (t=72 hrs) and stained for the detection of human CD11c (FITC-conjugated) and rabbit polyclonal GPR55 (specific to human; subsequently conjugated to goat anti-rabbit AlexaFluor 647 secondary antibody). Alexafluor 647 is excited at 651 nm which is in the same excitation wavelength spectrum as allophycocyanin (APC). Therefore, Alexafluor 647 signal was read by the APC channel. The cells were not permeabilised following fixation.

In Figure 5.5Ai-vi, cell sample populations obtained from 3 independent donors were compared to show that variability that can occur in marker expression between donors. Each donor shows variation in the levels of GPR55 expressed, particularly at t=0. Cells fixed at t=0 from the first two donors (in red; Figure 5.5Ai-iv) show particularly heterogenous levels of GPR55 expression in both the scatter plots and histograms, with some cells being GPR55high and other cells GPR55low. In contrast, at t=72 hrs CD11c+ cells express largely as a GPR55high single population (blue), except donor 2, whereby there seems to be a GPR55high population of cells that are CD11clow. The third donor shows a much lower expression of
GPR55 at t=0 (Figure 5.5Av, vi), with only a small proportion of the population of cells (red) overlapping with the t=72 hr sample population (blue), although GPR55 expression increases over time.

Quantitative values of the levels of GPR55 expression were obtained by measuring the geometric MFI values of the entire cell populations in the samples (Figure 5.5B). It was found that GPR55 expression significantly increased in CD11c⁺ cells after 72 hr incubation with GM-CSF and IL-4 compared to cells fixed at t=0 (t=0, 13.82±5.73 units vs t=72 hrs, 48.31±4.18 units; **p<0.01, unpaired two-tailed Student’s t-test, cells isolated from n=3 independent donors).

The results here demonstrate that GPR55 expression increases and becomes more homogeneous as monocytes differentiate into moDC. These findings conflict with the findings reported by Chiurchiù et al. (2015).
Figure 5.5. GPR55 expression significantly increases in CD11c+ cells over time.

Cells fixed at t=0 are in red and t=72 hrs are in blue. (i, iii, v) Representative scatter plots displaying the log fluorescence intensities of CD11c (FITC, x-axis) and GPR55 (APC, y-axis). (ii, iv, vi) Representative histograms of the log fluorescence intensity of GPR55 expression versus event count (normalised to mode). B: The log geometric mean fluorescence intensity (MFI) of GPR55 expression in t=0 cells vs. t=72 hrs. Results are expressed as log mean±S.E.M; **p<0.01, unpaired two-tailed Student’s t-test, cells isolated from n=3 independent donors.
5.2.3. G2A expression significantly increases in CD11c+ cells over time.

G2A/GPR132 is a proton-sensing Class A GPCR that is expressed in immune cells such as B and T cells, macrophages, neutrophils and dendritic cells (Kabrowski, 2009). Although originally classed in to the proton-sensing receptor family, G2A has been shown to respond to fatty acid metabolites such as 9-hydroxyoctadecadienoic acid (9-HODE; Obinata & Izumi, 2009) and lyso-phospholipids such as lysophosphatidylcholine (LPC; Lan et al., 2014). G2A was used as a comparator in this study because like GPR55 it is expressed by immune cells (Haugh et al., 2016; Kabrowski, 2009) and it responds to lyso-phospholipids such as LPC (Drzazga et al., 2017a, 2017b; Lan et al., 2014). Monocytes are thought to contribute to the inflammatory effect observed in the dextran sodium sulphate (DSS)-induced mouse model of colitis, with G2A−/− mice showing an exacerbated disease phenotype. Monocyte depletion from wild-type and G2A−/− mice was measured using flow cytometry and it was found that monocyte depletion led to a decrease in disease effects in both genotypes (Frasch et al., 2016). G2A expression itself was not measured in that study. It was therefore of interest in the present study to use flow cytometry to measure G2A expression in myeloid immune cells such as monocytes and moDC and to see if G2A expression altered during the course of monocyte differentiation into moDC.

CD14+ monocytes were positively selected from PBMC and incubated with GM-CSF and IL-4 for 72 hrs in order to differentiate them in to moDC. Cells were fixed either at t=0 or t=72 hrs and stained for the detection of human CD11c (FITC-conjugated) and rabbit polyclonal G2A (specific to human; conjugated to goat anti-rabbit AlexaFluor 647 secondary antibody). The cells were not permeabilised following fixation.

In Figure 5.6Ai-vi, cell sample populations obtained from 3 independent donors were compared to show the variability that can occur in marker expression between donors. Each
donor shows variation in the levels of G2A expressed, particularly at t=0. The cell populations fixed at t=0 from Donor 1 and Donor 3 (in red) show mainly G2A^dim populations (Figure 5.6Ai, ii, v, vi). Donor 1 also appears to be CD11c^low (Figure 5.6Ai). CD11c^+ cells fixed at t=0 from Donor 2 show greater G2A^high expression than Donors 1 and 3 (Figure 5.6Aiii-iv). However, CD11c^+ cells fixed at t=72 hrs in all 3 donors (blue) are G2A^high and G2A is expressed largely as a single population compared to t=0 cells.

Quantitative values of the levels of G2A expression were obtained by measuring the geometric MFI values of the entire cell populations in the samples (Figure 5.6B). It was found that G2A expression significantly increased in CD11c^+ cells after 72 hr incubation with GM-CSF and IL-4 compared to cells fixed at t=0 (t=0, 9.48±4.13 units vs t=72 hrs, 41.40±4.40 units; **p<0.01, unpaired two-tailed Student’s t-test, cells isolated from n=3 independent donors).

The results here demonstrate that G2A expression increases and becomes more homogeneous as monocytes differentiate into moDC.
Figure 5.6. G2A expression significantly increases in CD11c+ cells over time.

Cells fixed at t=0 are in red and t=72 hrs are in blue. (i, iii, v) Representative scatter plots displaying the log fluorescence intensities of CD11c (FITC, x-axis) and G2A (APC, y-axis). (ii, iv, vi) Representative histograms of the log fluorescence intensity of G2A expression versus event count (normalised to mode). B: The log geometric mean fluorescence intensity (MFI) of G2A expression in t=0 cells vs. t=72 hrs. Results are expressed as log mean±S.E.M; **p<0.01, unpaired two-tailed Student’s t-test, cells isolated from n=3 independent donors.
5.2.4. Validation of HCA2 expression in CD11c$^+$ cells.

HCA2 is expressed in innate immune cells such as mature neutrophils (Kotsyliina et al., 2007) and its mouse ortholog PUMA-G is expressed in mouse peritoneal macrophages and epidermal Langerhans cells (Benyó et al., 2005). Flow cytometry was used to measure levels of HCA2 expression in immature and mature neutrophils, with HCA2 levels increasing as neutrophils matured (Kotsyliina et al., 2007). To date, alterations in HCA2 levels have not been measured during monocyte differentiation into moDC, so flow cytometry was used in the present study to measure expression levels during the differentiation process.

CD14$^+$ monocytes were positively selected from PBMC and incubated with GM-CSF and IL-4 for 72 hrs in order to differentiate them into moDC. Cells were fixed either at t=0 or t=72 hrs and stained for the detection of human CD11c (FITC-conjugated) and rat monoclonal HCA2 (human-specific; conjugated to APC). The cells were not permeabilised following fixation.

CD11c$^+$ populations fixed at both timepoints were HCA2$^\text{dim}$ for all 3 donors (Figure 5.7A-B). Although cells at t=72 hrs appear to show a slightly higher fluorescence than t=0 cells, populations at both timepoints failed to show distinctive staining from isotype (data not shown). This was reflected in the geometric MFI values (Figure 5.7B; t=0, 1.86±0.35 units vs t=72 hrs, 2.72±0.56 units; p>0.05, unpaired two-tailed Student’s t-test, cells isolated from n=3 independent donors). The HCA2 antibody used in this experiment was an un-validated antibody, which may explain the lack of immunoreactivity. Further tests using different experimental methods are therefore required to validate the specificity of the antibody.

The results here demonstrate that further validation of HCA2 expression during monocyte to moDC differentiation is required in future.
Figure 5.7. Validation of HCA2 expression in CD11c+ cells.

Cells fixed at t=0 are in red and t=72 hrs are in blue. (i, iii, v) Representative scatter plots displaying the log fluorescence intensities of CD11c (FITC, x-axis) and HCA2 (APC, y-axis). (ii, iv, vi); Representative histograms of the log fluorescence intensity of HCA2 expression versus event count (normalised to mode). Cells isolated from n=3 independent donors. B: The log geometric mean fluorescence intensity (MFI) of HCA2 expression in t=0 cells vs. t=72 hrs. Results are expressed as log mean±S.E.M. Cells isolated from n=3 independent donors.
5.2.5. A proportion of human monocytes exhibit a time-dependent increase in fluorescence upon exposure to *E.coli* Green bioparticles.

To date the functional activity of GPR55 in immune cells has been largely unexplored. Previous work in this thesis demonstrated that the selective GPR55 agonist 17g induced a trend towards an increase in BV2 microglial cell phagocytosis of fluorescent latex beads. It was therefore of interest to elucidate if GPR55 stimulation regulated peripheral monocyte phagocytosis.

Previously in this thesis, fluorescent latex beads were used to examine BV2 phagocytic ability and Chiurchiù *et al.* used FITC dextran as a substrate for phagocytosis and measured the resulting fluorescence using flow cytometry. In line with this, *E.coli* pHrodo Green bioparticles were used in the present study and monocyte phagocytosis ability was analysed using flow cytometry. Monocytes (1-day old) were incubated with *E.coli* pHrodo Green bioparticles (ThermoFisher Warrington, UK; Catalog #P35366) for 3 hrs at 37°C. These bioparticles fluoresce when exposed to an acidic environment such as the phagosome (Kim *et al.*, 2015).

Control groups were processed using flow cytometry to account for any background signal caused by the bioparticles. Control samples containing (i) cells+no bioparticles and samples which contained (ii) bioparticles only, were processed using flow cytometry and plotted using FSC/SSC area parameters. In the plot, cells incubated with bioparticles for 3 hrs (t=3 hrs, Figure 5.8Ai iii) appear to represent the superimposition of event populations from both control group sample plots (i-ii). Using this information, a gate was applied corresponding to an area of the FSC/SSC area plot containing cells but not bioparticles (gate in red, 5.8A). Within these populations, a secondary gate was applied to isolate a population having high FITC fluorescence (Figure 5.8Bi), corresponding to monocytes that had phagocytosed bioparticles.

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It was found that the number of monocytes that phagocytosed bioparticles at t=3 hrs was significantly greater (Figure 5.8Bii; 268.5±53.83 events; monocytes isolated from n=4 independent donors) than the number of phagocytic monocytes at t=0 (2.38±1.19 events; monocytes isolated from n=4 independent donors) and control groups (no bioparticles = 0±0 events; bioparticles only = 0.5 events; ***p<0.001, One-way ANOVA & Student Newman-Keuls). Furthermore, this phagocytic response was significantly inhibited by the potent inhibitor of actin polymerisation, cytochalasin D (30 µg/mL; 5.63±1.63 events; monocytes isolated from n=4 independent donors, ***p<0.001, One-way ANOVA & Student Newman-Keuls).

The results here demonstrate that monocytes are capable of phagocytosing fluorescent bioparticles in a time-dependent manner.
Figure 5.8. A proportion of human monocytes exhibit time-dependent increase in fluorescence upon exposure to *E. coli* Green bioparticles.

A: (i-iii) FSC vs SSC area plots displaying the gate (in red) applied to differentiate cytometric events. (i) Cells only, (ii) bioparticles only or (iii) cells + bioparticles, t=3 hrs.

B: (i) Histogram of the log fluorescence intensity of *E. coli* pHrodo Green bioparticles. A secondary phagocytosis-positive gate was applied and it was assumed that any event within this gate were monocytes that had phagocytosed bioparticles. (ii) The effects of human monocytes exposed to *E. coli* pHrodo Green bioparticles. Several control groups were applied in order to compare the number of monocytes that phagocytosed bioparticles. These control groups were compared to cells + bioparticles t=3 hrs. Cells were also exposed to cytochalasin D treatment (t=3 hrs). Results are expressed as mean±S.E.M; ***p<0.001, One-way ANOVA & Student Newman-Keuls, monocytes isolated from n=4 independent donors.
5.2.6. The effect of GPR55 ligands on monocyte phagocytosis of *E.coli* pHrodo Green bioparticles.

As previously discussed, GPR55 stimulation increased monocyte production of pro-inflammatory cytokines and decreased phagocytic ability of FITC dextran (Chiurchiù *et al.*, 2015). It was of interest to see whether GPR55 ligands enhanced or decreased phagocytosis in our model. Monocytes (1-day old) were incubated with *E.coli* pHrodo Green bioparticles and GPR55 ligands for 3 hrs at 37°C. The event populations were then were processed using flow cytometry and plotted using FSC/SSC area parameters (Figure 5.9A). A gate was applied (in red, Figure 5.9Ai-iii) which corresponded to events that contained cells but not bioparticles. A secondary phagocytosis-positive gate was then applied to isolate a population that had high FITC fluorescence, corresponding to monocytes that had phagocytosed bioparticles. (Figure 5.9Bi).

It was found that monocyte phagocytosis of bioparticles was not significantly altered when the cells were exposed to the endogenous GPR55 agonist LPI (10 µM; Figure 5.9Aii, B; 290.5±33.06 events; p>0.05, One-way ANOVA, monocytes isolated from n=3 independent donors) or the selective GPR55 antagonist CID16020046 (CID; 10 µM; Figure 5.9Aiii, B; 194.25±43.04 events; p>0.05, One-way ANOVA, monocytes isolated from n=4 independent donors) for 3 hrs, compared to control (Figure 5.9Ai, B; 268.5±53.83 events, monocytes isolated from n=4 independent donors). Interestingly, it was observed that CID showed a trend towards a reduction in the number of phagocytosis positive cells by ~100 cells, although this trend was not significant compared to control. Co-treatment of LPI and CID showed similar levels of monocyte phagocytosis as control and LPI-treated levels, indicating that CID did not antagonise LPI (Figure 5.9Bii; 276.25±17.15 events; monocytes isolated from n=2 independent donors). However, statistical analysis of this dataset was not performed because it only included 2 donors.
The results here demonstrate that GPR55 ligands did not significantly modulate monocyte phagocytosis of bioparticles. However, GPR55 antagonism induced a trend towards a decrease in phagocytosis. These findings warrant further investigation in future.
Figure 5.9. The effect of GPR55 ligands on monocyte phagocytosis of *E. coli* pHrodo Green bioparticles.

**A:** (i-iii) FSC vs SSC area plots displaying the gate (in red) applied to differentiate events between treatment groups. (i) t=3 hrs, (ii) 10 µM LPI (t=3 hrs) or (iii) 10 µM CID (t=3 hrs).

**B:** Histogram of the log fluorescence intensity of *E. coli* pHrodo Green bioparticles. A secondary phagocytosis-positive gate was applied and it was assumed that any event within this gate were monocytes that had phagocytosed bioparticles. **B:** (i) The effects of human monocytes exposed to *E. coli* pHrodo Green bioparticles and GPR55 ligands. Cells were exposed to bioparticles for 3 hrs. The effect of GPR55 ligands (10 µM LPI, 10 µM CID, LPI+CID) on the number of phagocytosis-positive cells was analysed. Results are expressed as mean±S.E.M; p>0.05, One-Way ANOVA, monocytes isolated from n=3-4 independent donors. The LPI+CID group had n=2 donors so no statistical analyses were performed.
5.2.7. Mature moDC do not release PGE$_2$ in response to niacin stimulation.

Previous work shown in this thesis demonstrated that GPR55 ligands do not modulate pro-inflammatory TNF-$\alpha$ production in BV2 microglia. It was of interest to observe if GPR55 ligands regulated inflammatory mediator production in peripheral moDC. Indeed, Chiurchiu et al. (2015) demonstrated that activated monocytes and NK cells produce increased levels of pro-inflammatory cytokines and mediators (IL-12, IFN$\gamma$, TNF-$\alpha$) in response to GPR55 stimulation. However, they did not study pro-inflammatory mediator production by moDC. Previous unpublished data demonstrate that mature moDC release the pro-inflammatory mediator PGE$_2$ in response to niacin in a concentration-dependent manner (James, C., GSK, unpublished data). In the present study, these previous data were validated by treating moDC with increasing concentrations of niacin. If niacin induced PGE$_2$, then the effect of GPR55 ligands on PGE$_2$ release by moDC would be investigated.

moDC in this study were stimulated to maturity using IFN$\gamma$ or LPS or IFN$\gamma$+LPS for 24 hrs. They were then treated with increasing millimolar concentrations of the HCA2 agonist, niacin (3 mM-30 mM) for 45 min and levels of PGE$_2$ release were measured.

Cultured moDC release PGE$_2$ at significant levels in response to the calcium ionophore ionomycin (5 µM) compared to control, regardless of which maturation factor was applied (IFN$\gamma$ control vs ionomycin = 102.16±72.45 pg/mL vs 807.37±74.56 pg/mL; LPS control vs ionomycin = 44.32±16.34 pg/mL vs 707.05±128.67 pg/mL; IFN$\gamma$+LPS control vs ionomycin = 49.54±29.60 vs 657.48±115.40 pg/mL; **p<0.01, ***p<0.001 vs ionomycin, One-Way ANOVA & Student Newman-Keuls, n=5; Figure 5.10). Ionomycin has previously been shown to robustly induce PGE$_2$ (Leis & Windischhofer, 2016), so was used as a positive control in the current study. In comparison, niacin (3-30 mM) did not induce significant PGE$_2$ release in IFN$\gamma$, LPS- or IFN$\gamma$+LPS-stimulated cells compared to control cells (IFN$\gamma$ 3 mM niacin = 113.56±66.94 pg/mL, 10 mM niacin = 46.15±31.57pg/mL, 30
mM niacin = 78.49±33.08 pg/mL; LPS 3 mM niacin = 193.36±37.91 pg/mL, 10 mM = 29.46±7.52 pg/mL, 30 mM niacin = 81.89±15.46 pg/mL; INFγ+LPS 3 mM niacin = 159.72±94.73 pg/mL, 10 mM niacin = 67.93±15.94 pg/mL, 30 mM niacin = 279.99±178.75 pg/mL; p>0.05, One-Way ANOVA, n=3-5).

The results here demonstrate that niacin did not stimulate PGE₂ production by activated moDC.
Figure 5.10. Mature moDC do not release PGE₂ in response to niacin stimulation.
Levels of PGE₂ release produced by moDC in response to control, 5 µM ionomycin or 3-30 mM niacin. Results are expressed as mean±S.E.M; **p<0.01, ***p<0.001, One-Way ANOVA & Student Newman-Keuls, n=3-5.
5.2.8. Mature moDC do not release PGE$_2$ in response to GSK073 stimulation.

Due to the adverse skin flushing effects of niacin, measures have been taken in recent years to establish a drug that replicates the anti-lipolytic effects of niacin but does not induce flushing. GSK073 is a selective agonist of HCA2 that does not induce skin flushing (Sprecher et al., 2015). Differentiated and immature moDC were stimulated to maturity using IFN$_\gamma$ for 24 hrs. Mature moDC were then treated with GSK073 for 45 min and levels of PGE$_2$ release were measured.

GSK073 treatment of moDC did not stimulate PGE$_2$ release (control = 52.18 pg/mL, 300 nM GSK073 = 40.66 pg/mL, 1 µM GSK073 = 39.76 pg/mL, 3 µM GSK073 = 59.05 pg/mL, 10 µM GSK073 = 49.83 pg/mL, n=2; Figure 5.11).

There were several limitations regarding this dataset. Significance could not be drawn because only two sets of replicates were obtained. The positive control ionomycin was not applied to the moDC which meant that ionomycin- and GSK073-induced levels of PGE$_2$ release could not be compared. Niacin has previously been shown to induce PGE$_2$ release (Benyó et al., 2006). However, in the current study niacin did not induce significant PGE$_2$ release from moDC (see section 5.2.7), so it was not logical to compare the effects of GSK073- and niacin-treated moDC.

The results here demonstrate that the novel HCA2 compound GSK073 does not stimulate PGE$_2$ production by moDC.
Figure 5.1. Mature moDC do not release PGE$_2$ in response to GSK073 stimulation.

Levels of PGE$_2$ release produced by moDC in response to control or 300 nM-10 µM GSK073. Results are expressed as mean, n=2.
5.3. Discussion

The expression and functional activity of mononuclear innate immune cells was previously investigated by Chiurchiù et al. (2015). The aim of this chapter was to expand on these findings and to elucidate the expression and functional activity of novel GPCRs in human peripheral blood immune cells. Expression of the GPCRs, GPR55 and HCA2, in peripheral blood cells (monocytes and dendritic cells) and phagocytosis of *E. coli* pHrodo Green particles by monocytes were elucidated using flow cytometry. Previous data obtained by James, C. (GSK, unpublished data) demonstrated that niacin stimulated moDC to produce PGE$_2$. An ELISA was therefore used in the current study to investigate PGE$_2$ production by mature moDC in response to the HCA2 ligands, niacin and GSK073. GPR55 expression increased as monocytes differentiated into moDC. GPR55 ligands did not stimulate phagocytosis of *E. coli* pHrodo Green bioparticles. The findings reported by James, C. could not be replicated in the current study, so an ELISA to determine the effect of GPR55 ligands on PGE$_2$ production by mature moDC was not performed.

5.3.1. GPR55 expression and functionality in monocytes and moDC

GPR55 expression in human peripheral blood cells was reported in the ranking order monocytes/NK cells > myeloid DCs > B cells > Treg > T cells > plasmacytoid DCs (Chiurchiù et al., 2015). The results presented herein demonstrate that GPR55 is significantly expressed following the differentiation of CD11c$^+$ monocytes into CD11c$^+$ dendritic cells after 72 hr incubation with IL-4 and GM-CSF. CD11c is also known as Integrin, alpha X (complement component 3 receptor 4 subunit) (ITGAX) and is expressed in monocytes (Sándor et al., 2016). This expression increases as monocytes differentiate into moDC (Wang et al., 2017). Interestingly, in the present study, CD11c$^+$ monocytes (t=0) showed
heterogenous levels of GPR55 expression with some donor cell populations showing a much higher level of GPR55 expression compared to other donors. This contrasted to CD11c⁺ dendritic cells (t=72 hrs), which seemed to express GPR55 in a more homogenous manner. It is important to note however that the investigation into GPR55 and CD11c expression occurred in unpermeabilised cells. CD11c is expressed on the membrane (see Entrez gene entry: ITGAX integrin, alpha X (complement component 3 receptor 4 subunit)), so the CD11c antibody (Biolegend, UK) is likely to have bound to this target. The GPR55 antibody produced by Cayman Chemicals (Cambridge, UK) however targets an internal region of human GPR55. The antibody therefore may not have properly bound to GPR55 in the current study as a result. In fact, immunolabelling and imaging using confocal microscopy of permeabilised hGPR55-HEK293 cells showed that this antibody did not exhibit immunoreactivity for GPR55 (see Appendix, Supplemental Figure S3Aii). Nevertheless, there appeared to be immunoreactivity of the antibody that was distinctive from isotype in monocytes and moDC using the flow cytometric assay herein (data not shown), so perhaps cell fixation with PFA permeabilised the cell membrane to some extent to allow for the antibody to bind. These experiments should therefore be repeated in permeabilised cells to validate the data presented herein.

G2A was studied as a GPCR comparator in this study and has previously been shown to be expressed by macrophages, T- and B-lymphocytes, bone marrow-derived mast cells and dendritic cells (Parks et al., 2005). Similarly, G2A expression levels were observed in t=0 CD11c⁺ monocytes and t=72 hrs CD11c⁺ moDC in the present study and the pattern of expression was similar to GPR55 expression. Expression of G2A by CD11c⁺ cells varied from donor to donor at t=0, with one donor exhibiting greater G2A fluorescence than the other two. However, these expression levels became more homogenous at t=72 hrs and were found to be significantly different to t=0 cells. The G2A antibody used in this study targeted the amino acids 311-380 mapping within a C-terminal cytoplasmic domain of G2A.
Flow cytometric analysis of G2A occurred in fixed but unpermeabilised cells. Despite this there was immunoreactivity of the antibody that was distinctive from isotype (data not shown), so perhaps cell fixation with PFA permeabilised the cell membrane to some extent to allow for the antibody to bind.

GPR55 functional activity was investigated in 1-day old monocytes by elucidating the effect of GPR55 ligands on monocyte phagocytosis of fluorescent and pH-sensitive E. coli pHrodo Green bioparticles. The results here demonstrate that the GPR55 endogenous agonist, LPI, did not modify the levels of monocyte bioparticle phagocytosis compared to control conditions. This is in line with the findings reported by Zizza et al. (2012), whereby LPI (10-30 µM) did not modulate the uptake of IgG-opsonized beads by Raw 264.7 macrophage cells. Furthermore, at 5 µM, LPI did not have any effect on IL-4–induced phagocytosis of opsonised zymosan (from yeast cell wall) or E. coli by human macrophages (Rubio et al., 2015). Treatment with the selective GPR55 antagonist, CID16020046, did appear to induce a trend towards reduction in phagocytosis but these levels did not reach significance.

The role of GPR55 in phagocytosis remains largely unexplored. Stimulation of GPR55 using the putative but non-selective agonist, O-1602, led to decreased phagocytic activity of activated monocytes, whereas concomitantly pro-inflammatory cytokine release was promoted (Chiurchiù et al., 2015). Furthermore, GPR55 was shown to have pro-atherogenic effects in a macrophage model. The THP-1 monocyte cell line expresses GPR55 and was differentiated into macrophages using the phorbol ester, TPA. These macrophages were exposed to oxidised low-density lipoprotein in order to transform them into foam cells (ox-LDL-induced foam cells; Lanuti et al., 2015). Foam cells are lipid-loaded macrophages (Valledor et al., 2015). Treatment of the foam cells with O-1602 led to an increase in scavenger receptor-mediated lipid accumulation and impediment of cholesterol efflux through downregulation of ATP-binding cassette (ABC) transporters (Lanuti et al., 2015). O-1602 also induced increased pro-inflammatory cytokine and pro-metalloprotease-9 (pro-
MMP-9) production in these cells. CBD inhibited the pro-atherogenic and pro-inflammatory O-1602-mediated effects (Lanuti et al., 2015). However, these ligands are not selective for GPR55, so their findings should be validated using selective ligands. These data suggest that GPR55 antagonism or modulation of GPR55 tone may be effective in regulating monocyte phagocytosis in atherogenic or inflammatory conditions. Indeed, GPR55 antagonism with CID suppressed NO production in LPS-activated primary microglial cells (Malek et al., 2015).

5.3.2. HCA2 expression and functionality in monocytes and moDC

HCA2 is expressed in immune cells such as macrophages and dendritic cells and is also highly expressed in adipose tissue (Offermanns, 2017). In this study, an un-validated HCA2 antibody was used and the results obtained made it difficult to conclude whether (1) HCA2 was not expressed in CD11c+ monocytes or CD11c+ moDC, or (2) the antibody itself was not viable. This may be due to the permeability of the cells tested. Novus Biologicals (Abingdon, UK) produce the human-specific rat HCA2 antibody, but they do not communicate whether the epitope targeted is extra- or intracellular. Flow cytometric analysis occurred in fixed but unpermeabilised cells. These experiments should therefore be repeated in permeabilised cells to validate the specificity of the antibody. Other experimental methods should also be used to test the antibody. The results presented may also be attributed to the maturity of the cells because the cells assessed for receptor expression in this study were not stimulated with a maturation factor such as IFNγ or LPS (Han et al., 2009). Nastasi et al. (2015) showed that maturation of moDC with LPS led to a significant increase in the expression of DC maturation markers (e.g. CD83, CD86, Human Leukocyte Antigen – antigen D Related (HLA-DR)), however changes in HCA2 expression levels between immature and mature DCs were not investigated in that study. The authors did investigate
the effects of short-chain fatty acids, which are low potency ligands of HCA2, on mature moDC and they found that butyrate and propionate significantly reduced IL-6 expression and protein release as well as a decrease in LPS-induced gene expression and protein production of IL-12B. Activation of other immune cells such as macrophages by LPS increased HCA2 mRNA ~19-fold in cultured mouse peritoneal macrophages and ~30-80-fold in cultured Raw 264.7 murine macrophage cells (Feingold et al., 2014). These data coupled with data presented herein suggest that HCA2 expression may increase upon moDC maturation, so it would be of interest to investigate moDC maturation on HCA2 expression in future studies.

HCA2 functional activity was determined by using an ELISA to measure PGE₂ release from mature moDC. The findings presented in this study suggest that increasing concentrations of niacin (3-30 mM) and GSK073 (300 nM-10 µM) do not induce PGE₂ release from mature moDC. The cells were capable of PGE₂ release however because the Ca²⁺ ionophore, ionomycin, induced significant PGE₂ release compared to control moDC. These findings contradict previous data showing that niacin induces prostaglandin release from Langerhans cells (Maciejewski-Lenoir et al., 2006). Furthermore, mice lacking PGD₂ and PGE₂ receptors showed reduced flushing in response to niacin (Benyó et al., 2006). Previous unpublished data demonstrate that moDC release PGE₂ in response to niacin in a concentration-dependent manner (James, C., GSK). It is therefore unclear why the mature moDC in the present study failed to respond to niacin. As a result, GPR55 ligands were not tested using this assay. James, C. also tested a novel GSK compound that exhibited the same effects as GSK073 (i.e. no skin flushing) and found that it did not induce PGE₂ release and was also capable of antagonising niacin-induced PGE₂ release. GSK073 was applied in the present study but because niacin failed to induce PGE₂ release from moDC then the effects of the two compounds could not be accurately compared.
Due to the lack of niacin-induced PGE$_2$ production by moDC, possible explanations were postulated. Cell health was not implicated because cell counts during the culture process showed $\geq$90% live moDC. One theory is that culture medium was a factor. Previous experiments used moDC cultured in RPMI medium (James, C., GSK), whereas in the present study IMDM medium was used. This could have modified the moDC response. In the present study, optimisation of the culture protocol of moDC was carried out to compare the effects of RPMI and IMDM medium. RPMI-cultured moDC showed $\geq$80% live cells and also failed to produce PGE$_2$ in response to niacin (see Supplementary Figure S5). Furthermore, they produced less PGE$_2$ in response to ionomycin than IMDM-cultured moDC. For these reasons, moDC were cultured in IMDM going forward. RPMI medium will support the growth of a wide variety of cells in suspension (DeliveRed, 2012) and DCs are semi-adherent cells (Madaan et al., 2014). IMDM-cultured moDC were previously shown to have a different cytokine profile to RPMI-cultured moDC, with IMDM-cultured moDC exhibiting reduced CD1a dendritic cell marker expression and significant reduction in the stimulation of T-cell proliferation, as well as high expression of IL-6, IL-8 and IL-10 but low expression of IL-12. IMDM-cultured moDC were therefore associated with the induction of immune tolerance compared to RPMI-cultured moDC (Chen et al., 2011). Further investigation and optimisation of the culturing and experimental process using moDC is clearly necessary going forward.

5.3.3. Conclusion

The aim of this chapter was to elucidate the expression and functional activity of novel GPCRs in human peripheral blood immune cells. It was found that GPR55 expression increased as monocytes differentiated into moDC. Furthermore, expression patterns became
more homogenous in moDC compared to monocytes. A summary of these findings can be viewed in Figure 5.12.

Figure 5.12. GPR55 expression and function in monocytes and moDC.

Incubation of monocytes with GM-CSF and IL-4 for 3 days increases the expression of GPR55 upon their differentiation into moDC. Figure adapted from Hubo et al. (2013).
Chapter 6

Final Discussion
6.1. Introduction

GPR55 is an orphan GPCR that is widely expressed throughout the body, both peripherally and centrally (Henstridge et al., 2011). It is responsive to cannabinoids (Brown & Wise, 2002; Ryberg et al., 2007), but the lysospholipid, LPI, is believed to be its endogenous agonist (Oka et al., 2007). Studies investigating the pharmacology of GPR55 to date have predominantly used overexpressing cell lines, which do not accurately reflect the true physiological function of this receptor. For this reason, GPR55 is still classed as an orphan receptor because there is not enough evidence that explores the efficacy of LPI at this receptor in endogenous or in vivo systems (Alexander et al., 2017). Additionally, its function in the central nervous system is little understood (Marichal-Cancino et al., 2017). The aim of the current study was therefore to investigate the role of GPR55 an in vitro central model, specifically in cortical-neuron enriched cultures.

6.2. Summary of findings

6.2.1. Activation of downstream Ca\(^{2+}\) signalling and CREB phosphorylation by GPR55 ligands.

Preliminary findings from this study demonstrate that GPR55 is exhibits a punctate localisation along the processes of neurons from cortical neuron-enriched cultures and confirms the evidence that GPR55 is expressed in primary microglia and the BV2 microglial cell line (Malek et al., 2015; Pietr et al., 2009). It highlights for the first time that the endogenous GPR55 agonist, LPI and the novel and selective agonist, 17g, stimulate changes in [Ca\(^{2+}\)]i and CREB phosphorylation in primary rat cortical-neuron enriched cultures. In a ratiometric Ca\(^{2+}\) assay, LPI was found to modulate the frequency of spontaneous Ca\(^{2+}\) events.
in neurons in a GPR55-independent manner. In neurons exhibiting spontaneous Ca\textsuperscript{2+} activity, LPI diminished event activity. However, in quiescent neurons or neurons exhibiting minimal basal [Ca\textsuperscript{2+}]i activity, it increased event activity. LPI also induced robust and sustained increases in [Ca\textsuperscript{2+}]i in quiescent neurons and glia. LPI induced neuronal pCREB independently of GPR55, but this stimulation was partially contributed to by G\textsubscript{q}. Previous findings from the Irving group have shown that LPI induces Ca\textsuperscript{2+} mobilisation in a G\textsubscript{q13}-RhoA-ROCK-dependent manner (Henstridge \textit{et al.}, 2009) and pCREB stimulation (Henstridge \textit{et al.}, 2010) in hGPR55-HEK293 cells. In contrast, the findings here demonstrate that LPI-induced changes in [Ca\textsuperscript{2+}]i and stimulation of pCREB in cells from cortical neuron-enriched cultures were not dependent on GPR55. Evidence from the literature suggests that other ion channels may instead contribute to LPI-induced changes in [Ca\textsuperscript{2+}]i in neurons and glia e.g. VGCC, TRPV2 (Ben-Zeev, Telias & Nussinovitch, 2010; Harada \textit{et al.}, 2017). This study demonstrated that LPI-induced pCREB was partially dependent on G\textsubscript{q} not G\textsubscript{q13}. These findings are in line with evidence reported by Lauckner \textit{et al.} (2008), whereby they found that LPI induced Ca\textsuperscript{2+} mobilisation in DRG neurons via both G\textsubscript{q12} and G\textsubscript{q}. Furthermore, Andradas \textit{et al.} (2016) reported that LPI induced the stimulation of ERK (an upstream activator of CREB phosphorylation) through G\textsubscript{q/11} coupling in cancer cells. The potential ion channels and downstream mediators responsible for LPI-induced [Ca\textsuperscript{2+}]i changes in cortical cell-enriched cultures were not elucidated in the current study, so future work should seek to determine which mediators are stimulated by LPI.

The novel and selective agonist, 17g, was found to cause GPR55 internalisation in hGPR55-HEK293 cells in the current study. Yrjölä \textit{et al.} (2016) generated the compound 17g and reported that it induced Ca\textsuperscript{2+} mobilisation in hGPR55-HEK293 cells. 17g amplified the frequency of spontaneous Ca\textsuperscript{2+} events in neurons and modulated [Ca\textsuperscript{2+}]i in quiescent neurons in a GPR55-dependent manner. However, it is believed that GPR55 stimulation
may recruit other targets to maintain Ca\(^{2+}\) homeostasis after ER store depletion e.g. TRPV2 (Harada et al., 2017), particularly because 17g still induced changes in [Ca\(^{2+}\)]\textsubscript{i} following store depletion. 17g also stimulated pCREB in a G\(\alpha_q\)-dependent manner and the GPR55 antagonist, CID16020046, partially inhibited 17g-induced pCREB. Future work should seek to determine which G-protein(s) and effectors are stimulated by 17g upstream of Ca\(^{2+}\) mobilisation.

6.2.2. GPR55 ligands modulate microglial function and neuronal apoptotic mechanisms in response to Aβ.

This study demonstrates that the endogenous GPR55 agonist, LPI and the selective GPR55 antagonist, CID16020046 (CID), modulate BV2 microglial migration in response to Aβ-primed neuronal medium (PNM) and LPI and the novel and selective GPR55 agonist, 17g, protect against Aβ-evoked active caspase-3 in rat cortical neuron-enriched cultures. In contrast, Janefjord et al. (2014) reported that the putative GPR55 agonist, O-1602, failed to protect against Aβ-induced neurotoxicity, but did protect against LPS-induced neurotoxicity in a SH-SY5Y neuroblastoma cell line. However, O-1602 did reduce Aβ fibril formation in cell-free conditions. The results herein therefore show for the first time that putative GPR55 ligands are protective in an \textit{in vitro} model of Aβ toxicity. The current study is also the first to date to utilise a BV2 migration assay to elucidate the effect of GPR55 ligands on BV2 microglial migration in response to Aβ-PNM. LPI attenuated Aβ-PNM-evoked BV2 migration independently of GPR55. Furthermore, antagonism of Aβ-PNM-evoked with CID attenuated BV2 migration alone, indicating that inhibition of GPR55 tone may reduce microglial migration. These findings are in line with evidence reported by Malek et al. (2015), where they demonstrated that CID attenuated LPS-induced nitric oxide (NO) production in cultured rat microglial cells. Sisay et al. (2013) also showed that GPR55 knockout improved
mortality in a mouse model of experimental autoimmune encephalomyelitis (EAE). The results herein implicate GPR55 in being a potential therapeutic target in the treatment of neurodegenerative diseases such as AD.

Interestingly, 17g alone stimulated BV2 microglial migration under basal conditions and both LPI and 17g stimulated active caspase-3 when applied alone. 17g-induced BV2 migration was independent of GPR55. This contradicts findings by Yrjölä et al. (2016), who demonstrated that 17g stimulates Ca\(^{2+}\) mobilisation in hGPR55-HEK293 cells. However, the GPR55-independent effects of 17g observed herein may have been due to competitive antagonism. A 1 μM 17g concentration was employed as it appeared to be an optimal concentration to use in the ratiometric Ca\(^{2+}\) assay used in this study. This dose may have been too high for inhibition by CID and displaced CID at the cognate GPR55 binding site (Tallarida, Cowan & Adler, 1979; Heynen-Genel et al., 2011). Future work should utilise different doses of 17g with CID to ensure GPR55 selectivity. In contrast to 17g, LPI failed to stimulate migration under basal conditions in this study. This contradicts findings where LPI was found to promote microglial migration when applied alone (Kallendrusch et al., 2013). However, in that same study, LPI induced attenuated ATP-induced microglial migration and reduced the number of activated microglia after NMDA-induced lesions in rat OHSC in a GPR55-dependent manner. These findings, along with those presented herein, imply that LPI is most effective when disease is present. Indeed, 72 hr stimulation with 17g and LPI alone induced active caspase-3 in a GPR55-dependent manner. While GPCR signalling is essential, overstimulation can be deleterious and can result in cellular toxicity or uncontrolled cellular growth (Rajagopal & Shenoy, 2018). However, caspase-3 may also be involved in homeostatic synaptic scaling without the induction of apoptosis (Lo et al., 2015), suggesting that its activation by these ligands may be physiologically beneficial. Nevertheless, these findings illustrate that GPR55 is a credible target for therapeutic intervention in models of AD.
6.2.3. GPR55 is upregulated during the moDC differentiation process and may regulate phagocytosis.

A 5-month Industrial PhD placement was undertaken in the R&D sector of GlaxoSmithKline, Stevenage, UK. Using a flow cytometric assay, the findings here demonstrate that GPR55 is expressed in human monocytes in a heterogeneous pattern, with some populations of monocytes expressing GPR55 more robustly than others. Furthermore, donors also exhibited heterogeneity in expression, with some donors showing higher GPR55 expression than others. GPR55 expression was upregulated and became more uniform after 72 hr incubation with the cytokines, GM-CSF and IL-4, which differentiate monocytes into monocyte-derived dendritic cells (moDC; Sallusto & Lanzavecchia, 1994). Chiurchiù et al. (2015) were the first group to characterise GPR55 expression and functional activity in distinct populations of human mononuclear cells of both innate and adaptive immunity using polychromatic flow cytometry. Chiurchiù et al. (2015) demonstrated that GPR55 protein was highly expressed in monocytes, with modest levels present in myeloid dendritic cells. However, they did not elucidate changes in GPR55 expression during monocyte differentiation into moDC. These data herein therefore conflict with Chiurchiù et al. because the data herein show that GPR55 expression increases over the course of monocyte differentiation into moDC.

The findings here demonstrate that the selective GPR55 antagonist, CID16022046 (CID), induced a trend towards attenuation of the number of phagocytic monocytes. However, this attenuation was not significant. Stimulation of GPR55 using the putative agonist, O-1602, was shown to decrease the phagocytic activity of LPS-activated monocytes (Chiurchiù et al., 2015). These contradict the findings here because the endogenous GPR55 agonist, LPI, did not modulate the number of phagocytosis-positive monocytes in the current study. An increased number of replicates in the current study could aid in properly confirming this role.
6.2.4. The role of GPR55 in neuronal and immune cell function in AD.

The aim of the current study was to investigate the role of GPR55 in central and peripheral in vitro models. The findings here are encouraging as for the first time, the endogenous agonist at GPR55, LPI and the novel and selective agonist, 17g, were shown to regulate changes in \([\text{Ca}^{2+}]_i\), promote CREB phosphorylation and protect against Aβ- evoked microglial migration and neuronal apoptosis. These findings suggest that modulation of GPR55 activity has a therapeutic benefit in models of AD, only one of two to studies (Janefjord et al., 2014) to make this association.

The regulation of \(\text{Ca}^{2+}\) is vital for cellular homeostasis and dysregulation of \(\text{Ca}^{2+}\) homeostasis can occur in AD (Rapaka et al., 2014). The Campbell group have previously shown that Aβ evokes a significant increase in \([\text{Ca}^{2+}]_i\) in primary rat cortical neurons. This \(\text{Ca}^{2+}\) increase is believed to contribute to apoptosis (Boland & Campbell, 2003; MacManus et al., 2000). Phospho-CREB is a pro-survival transcription factor that induces gene transcription (Stevenson et al., 2001) and can be regulated by \(\text{Ca}^{2+}\). Indeed, Kornhauser et al. (2002) demonstrated that \(\text{Ca}^{2+}\) influx induced CREB phosphorylation in primary rat cortical neurons. Acute application of the GPR55 agonists, LPI and 17g, stimulated both \([\text{Ca}^{2+}]_i\) changes and pCREB activation. However, only 17g induced these effects in a GPR55-dependent manner. This implicates that LPI mediates its effects via another or several targets.

The stimulation of \(\text{Ca}^{2+}\) changes and pCREB by GPR55 agonists may have effects on downstream functional processes in neurons and glia. Indeed, it was reported that \(\text{Ca}^{2+}\)-mediated purinergic receptors regulate the migration and phagocytic ability of microglia during post-natal brain development (Sunkaria et al., 2016). Balenga et al. (2011) reported that LPI and AM251 induced a directional migration of human peripheral blood neutrophils and enhanced their migratory capacity. LPI and 17g diverged in effects in regulating microglial
migration in the current study, whereby LPI attenuated Aβ-primed neuronal medium (PNM)-evoked migration and 17g promoted migration under basal conditions. Both agonists were not antagonised by the selective antagonist, CID16022046 (CID), in that assay. However, CID itself attenuated Aβ-PNM-evoked microglial migration. GPR55 antagonism alone was shown to reduce LPS-induced NO production in primary rat microglia (Malek et al., 2015), suggesting that under certain conditions inhibiting GPR55 may be beneficial. Chronic stimulation of GPR55 may have deleterious effects and lead to apoptosis (Rajagopal & Shenoy, 2018), as seen in this study when treatment of cortical neuron-enriched cultures with LPI or 17g alone increased capase-3 induction. However, capase-3 stimulation may promote NMDA-dependent LTD without inducing cell death (Li et al., 2010). Interestingly, LPI and 17g attenuated Aβ-evoked capase-3 induction. All of these findings suggest that reported ligands at GPR55 have regulatory effects in this model of Aβ toxicity and promote downstream signalling and functional processes in order to protect against apoptotic mechanisms induced by Aβ. A summary schematic of these suggested events can be observed in Figure 6.1.
Figure 6.1. GPR55 ligands regulate downstream signalling and functional processes in neurons and glia. LPI (endogenous agonist) and 17g (selective agonist) stimulate changes in [Ca$^{2+}$]i and CREB phosphorylation in neurons and glia. Phospho-CREB is dependent on G$\alpha_q$-coupling. The stimulation of these signalling mediators may have functional effects downstream on processes such as microglial migration and neuronal apoptosis.
6.2.5. Limitations of study & future work.

Novel pharmacological agonists and antagonists for GPR55 were utilised in this study. However, in some assays these ligands exhibited unclear pharmacological effects, such as when CID16020046 (CID) failed to antagonise either LPI or 17g in the BV2 migration assay, yet attenuated Aβ-evoked migration by itself. Therefore, using techniques such as GPR55 siRNA knockdown will confirm GPR55-dependent effects.

There is a lack of effective GPR55 antibodies available commercially and two were tested here (Abcam and Cayman Chemicals; see Appendix, Supplementary Figure S3) without significant success. An antibody was gifted by Professor Ken Mackie (Indiana University, IN, USA) and immunolabelling showed encouraging immunoreactivity in overexpressing cells and central cells using confocal microscopic imaging. However, it was only tested twice in total, so future work should seek to stain with this antibody using multiple replicates. Confocal microscopic imaging should be coupled with western blotting to observe levels of GPR55 protein expression in these cells. Furthermore, quantitative polymerase chain reaction (qPCR) should be employed to determine mRNA expression of GPR55 in cortical neuron-enriched cultures.

The robustness of the [Ca\textsuperscript{2+}] data was variable due to the heterogeneity of physiological responses by cortical neuron-enriched cultures. Although this more accurately depicts the endogenous processes that occur in the CNS, it makes replicating experiments difficult. This was reflected in some of my findings. For instance, the effects of 17g-induced changes in [Ca\textsuperscript{2+}] in quiescent neurons could not be pooled together for statistical analyses because the induced [Ca\textsuperscript{2+}] profiles differed from culture to culture. Future work should attempt obtain more cultures in order to increase replicate numbers in experiments like these.
Time or dose-response courses were not carried out in all assays here. Some timepoints of ligand application or concentrations of ligands were chosen based on previous protocols from my supervisors’ laboratories or evidence from the literature. This may have accounted for discrepancies in some of my findings e.g. GPR55 agonists alone induced caspase-3 induction after 72 hr incubation, whereas a shorter time period of application may not have induced this effect. Future work should aim to thoroughly elucidate the best ligand concentrations and timepoints to use in these assays.

LPS was shown to be a robust stimulator of microglial phagocytosis and TNF-α cytokine production, whereas GPR55 ligands alone did not modulate TNF-α production. A preliminary ELISA was carried out using supernatants obtained from BV2 cells treated with both GPR55 ligands and LPS to observe if the ligands modulated LPS-induced TNF-α production. However, there was a lot of variability between the replicates, so an accurate effect could not be discerned. These experiments should be repeated in order to truly elucidate the effect of GPR55 ligands on LPS-induced TNF-α production.

Some of my findings had a low number of replicates, which may have accounted for variability in the final data. In future, replicates should be increased to fully confirm the results of experiments.

6.3. Conclusion

The physiological function of GPR55 is still little understood due to the shortage of studies that explore its role endogenously. The findings here further advance our knowledge of GPR55 and its potential functions in neurons and glia, as well as in peripheral immune cells. I have shown for the first time that agonists for GPR55, LPI and 17g and a selective
antagonist, CID16020046, have a regulatory role in a cortical neuron-enriched model of Aβ toxicity. This implicates GPR55 in being a therapeutic target for the treatment of AD.
Publications & Conference Proceedings

Publications


Conference Proceedings


  - **Donegan Medal Runner-Up, First time oral presenter competition.**


Personal Reflection

My PhD research project investigated the role of novel GPCRs in endogenous cellular models. The majority of the project took place at Trinity College Dublin (TCD) under the supervision of Professor Veronica Campbell, with some experiments performed at University College Dublin (UCD) under the supervision of Dr. Andrew Irving. This co-supervised project allowed me to gain experience in performing unique experimental techniques in the two top universities in Ireland. It also gave me the opportunity to exhibit my mobility as a researcher; I was quickly able to adapt to both laboratories in order complete necessary experiments. I became adept at optimising and performing new protocols. For instance, the techniques for pCREB immunocytochemical staining and ratiometric calcium imaging had never been performed in a primary cortical neuron model in either of my supervising labs. Throughout my career I have been eager to disseminate my research and network with peers and experts. Consequently I have published a review and I have received numerous travel awards to present my research at 10 conferences/symposia to date.

My leadership and managerial skills were greatly enhanced over the course of my PhD. From 2015 onwards, I was the sole research member of the Campbell laboratory. I was therefore in charge of supervising all the undergraduate and M.Sc students undertaking their research projects in the lab. I was in charge of budgeting for and ordering animals and lab supplies. I acted as a paid tutor to first-year medical students for Problem-Based Learning tutorials and I was a paid demonstrator for undergraduate and M.Sc laboratory practical classes. I joined the TCD Neuroscience Society committee as Secretary for 2015-2016 and later became President for 2016-2017. This role involved leading and managing the society and its committee; organising and hosting academic seminars presented by renowned international and national neuroscience-affiliated speakers; and organising social events throughout the
academic year. During my committee’s term we also established and published the first ever edition of "Discovering Neuroscience", a TCD magazine celebrating the TCD Neuroscience community.

I have always had a keen understanding of the need to bridge the gap between academia and industry. This is reflected in my involvement in the Innovation Academy from which I received a Postgraduate Certificate in Innovation & Entrepreneurship. The Innovation Academy is a joint venture between TCD and Queen’s University Belfast, Ireland. Its objective is to provide modules to PhD students to allow for transferrable skills-based learning that will enhance research competence and employability. The modules it provides include: Creative Thinking & Innovation, Opportunity Generation, Leadership Development, Planning Your Venture and Creative Capital.

My project is focussed on discovery-related research into novel pharmacological targets for disease therapies. The academic field is limited by the availability of novel pharmacological tools and assays so I proactively sought to engage with industry during my PhD in order to employ and enhance the skills that I gained from my laboratory research project and the Innovation Academy. I completed a 5-month industrial placement in the world-leading Research & Development (R&D) Platform Technology & Science department of GlaxoSmithKline (GSK), Stevenage, UK, under the supervision of Senior Scientific Investigator, Dr. Andrew Brown. I received external funding for this placement from both the TCD Association & Trust and from GSK. This was an enormous opportunity because Dr. Brown has an active interest in novel GPCRs such as GPR55. He was therefore able to provide me with invaluable guidance during my placement. When I arrived at the R&D department at GSK, the protocol for differentiating dendritic cells from human blood-derived monocytes had not yet been optimised. I aided in optimising this protocol, even though I had previously never worked with primary human cells before. Furthermore, I developed a monocyte phagocytosis assay that could be analysed using flow cytometry.
Phagocytosis was regularly analysed using fluorescent microscopy in the department, but flow cytometry had never been used to analyse phagocytosis. The data obtained during this placement complemented my data from TCD and UCD on the role of GPR55 in neuro-glial function.

My time as a PhD student has allowed me not only to develop into a confident and able researcher but also to develop invaluable life skills. I am now greatly confident in my leadership, management, presentation and organisational skills. These are skills I will take with me as I progress to the next stage of my career and throughout my life.
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Entrez Gene: ITGAX integrin, alpha X (complement component 3 receptor 4 subunit


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S.1. Optimisation of primary rat cortical neuron-enriched culture.

During earlier stages of the current study, primary cells from cortical neuron-enriched cultures were isolated and seeded as normal, as described in Section 2.2. However after 3-4 days DIV, 75% of the media was removed from the plates and replaced with warm neurobasal culture medium containing cytosine-arabinofuranoside (ARA-C; 5 µg/ml; Sigma-Aldrich, Dorset, UK) and 2% B27. ARA-C is an anti-mitotic agent that kills proliferating cells by inhibiting DNA synthesis (Verbin et al., 1972). Glial cells are capable of mitosis (Ge et al., 2009), whereas neurons are believed to be post-mitotic so are therefore not thought to be susceptible to the anti-mitotic effects of ARA-C (Herrup & Yang, 2007). It was therefore added in an attempt to maintain the neuronal purity of the cell cultures. The cells were incubated in this ARA-C-containing medium and maintained at 37°C with 5% CO$_2$ and 95% O$_2$ overnight. The next day (≤16 hours), this medium was removed and replaced with normal culture medium supplemented with 2% B27. Henceforth, 75% of the media was removed and replaced every 72 hours prior to treatment for experimental assays.

In contrast to the previous evidence from the literature, it has been suggested that ARA-C is capable of inducing degeneration in post-mitotic neurons in a mechanism that is similar to growth factor deprivation (Martin, Wallace & Johnson, 1990). In the current study it was ultimately identified that the addition of ARA-C to the cultures appeared to affect the healthiness of the cells. A stain for active caspase-3 (a marker of apoptosis) showed a high basal level of caspase-3 activation in cells exposed to ARA-C compared to cells not exposed to ARA-C (Supplementary Figure S1). Following this discovery, cells were no longer exposed to ARA-C during the culture process and instead only cultured in neurobasal medium containing 2% B27.
Supplementary Figure S1. ARA-C versus no ARA-C in cortical neuron-enriched cultures.

Active caspase-3 staining of cortical cells. Caspase-3 positive cells appear dark brown in colour, whereas caspase-3 negative cells are generally pale in colour. Note that ARA-C exposure causes an increase in basal active caspase-3 expression. Methyl green counterstain was used to visualise nuclei (bright green/blue). The difference in background colour was due to the length of DAB staining, but did not lead to non-specific labelling. Scale bar = 100 μm.
S.2. Antibody for GPR55 epitopes do not show immunoreactivity in hGPR55 HEK293 cells

HEK293 cells stably overexpressing 3xHA tagged to hGPR55 (hGPR55-HEK293) were stained with two different GPR55 antibodies. One rabbit polyclonal antibody from Abcam (Cambridge, UK) targets the amino acids 159-180 peptide within human GPR55. This epitope is described by the manufacturer to be extracellular (Supplementary Figure S2). The second GPR55 antibody is also rabbit polyclonal and is synthesised by Cayman Chemicals (Cambridge, UK). It is specific for an internal region of human GPR55 according to the manufacturer.

Supplementary Figure S2. A helix net representation for GPR55.

Amino acid epitope for Abcam GPR55 antibody is highlighted in red. Image obtained from Shore & Reggio, 2015.
Both antibodies failed to show immunoreactivity that was consistent with anti-HA staining in untreated hGPR55-HEK293 cells (Supplementary Figure S3A). The Abcam antibody caused non-specific staining, whereas the Cayman Chemicals antibody showed little immunoreactivity at all. In Figure S3B, one can observe a preliminary western blot of a rat cortical neuron-enriched cell lysate labelled for GPR55 (Abcam antibody). GPR55 labelling was compared to β-actin as a loading control. The lysate did show faint immunoreactivity for GPR55 using the Abcam antibody. However, it was difficult to replicate these results thereafter. An antibody gifted by Professor Ken Mackie was then used for the remainder of this research study because it was used successfully in another study (Korchynska et al., 2018, under review).
Supplementary Figure S3. Optimisation of GPR55 immunolabelling.

A: Representative confocal images of HEK293 cells stably expressing 3xHA-GPR55 stained for GPR55; (i) Abcam antibody, (ii) Cayman Chemicals antibody. Scale bar = 20 µm.

B: Western blot of rat cortical cell lysate labelled for GPR55 (Abcam antibody) and β-actin as a loading control.
S.3. Dose-response of changes in neuronal [Ca\textsuperscript{2+}]i induced by 17g.

GPR55 stimulation with LPI has previously been shown to induce downstream release of Ca\textsuperscript{2+} from internal stores in hGPR55-HEK293 cells and PC12 neuronal cells via a G\textsubscript{z13}-RhoA-ROCK signalling pathway (Henstridge et al., 2009; Obara et al., 2011). Previous findings by Yrjölä et al. (2016) demonstrated that the novel and selective GPR55 agonist, 17g, induced an increase in [Ca\textsuperscript{2+}]i in hGPR55-HEK293 cells (E\textsubscript{50} = 7 nM). This agonist has not been used in a rat neuronal cell model to date, so it was of interest to determine the most suitable concentration of 17g to use when investigating [Ca\textsuperscript{2+}]i changes in these cells.

Changes in [Ca\textsuperscript{2+}]i were measured using a digital epifluorescence imaging system and MetaFluor\textsuperscript{®} software. Cells were excited at 350 and 380 nm simultaneously and [Ca\textsuperscript{2+}]i changes were measured as a change in fura-2 fluorescence ratio. Supplementary Figure S4 depicts separate experiments whereby increasing concentrations of 17g were applied. One replicate culture was used to test increasing concentrations of 17g (100 nM, 1 μM, 10 μM) in order to determine which concentration induced the most consistent or robust responses. Each concentration was applied to a different population of cells from the same replicate culture. KCl (50 mM) was applied at the end of each experiment to differentiate between neurons and glia. Each cell population tested had different numbers of total neurons, so the number of neurons subsequently analysed for 17g-responsiveness was normalised to 5 neurons in order to accurately compare responsiveness to each concentration.

It was found that all 3 concentrations of 17g tested induced variable [Ca\textsuperscript{2+}]i responses. 17g (1 μM) appeared to stimulate an increase in [Ca\textsuperscript{2+}]i in some neurons (Supplemental Figure S4ii), whereas it did not induce responses in other neurons from the same culture (Supplemental Figure S4iv). It was difficult to interpret if [Ca\textsuperscript{2+}]i activity following 100 nM (Supplemental Figure S4i, iii) or 10 μM (Supplemental Figure S4v, vi) 17g application was definitively linked to its application, instead of occurring spontaneously. For this reason, and given that 1 μM
17g stimulated GPR55 internalisation in hGPR55-HEK293 cells (Figure 3.33), this concentration was used in experiments going forward.
Supplementary Figure S4. Dose-response of changes in neuronal 
$[\text{Ca}^{2+}]_i$ induced by 17g.

(i, iii, v) Representative traces of neurons potentially responding to increasing concentrations of 17g (100 nM-10 μM). (ii, iv, vi) Representative traces of neurons not responding to 17g. KCl (50 mM) was applied at the end of each experiment to differentiate between neurons and glia, but the KCl response has been omitted for clarity.
S.4. Differences in levels of PGE$_2$ release by mature moDC cultured in either RPMI- or IMDM-culture medium.

A preliminary experiment was carried out whereby moDC were cultured in either RPMI or IMDM culture medium. The cells were then stimulated to maturity using IFN$\gamma$ or LPS or IFN$\gamma$+LPS for 24 hrs. They were then treated with 5 µM ionomycin and increasing millimolar concentrations of the HCA2 agonist, niacin (3 mM-30 mM) for 45 min and levels of PGE2 release were subsequently measured using ELISA. The results in Supplementary Figure S5 show that IMDM-cultured moDC release greater levels of PGE$_2$ in response to ionomycin compared to control, regardless of which maturation factor was applied. RPMI-cultured moDC produce roughly $\geq$350 pg/mL less PGE$_2$ in comparison, regardless of the maturation factor. However, both types of cultured cells failed to respond to increasing concentrations of niacin.
Supplementary Figure S5. PGE₂ production by mature moDC cultured in RPMI medium or IMDM medium.

Results are expressed as mean±SEM, n=1.