



THE UNIVERSITY *of* EDINBURGH

This thesis has been submitted in fulfilment of the requirements for a postgraduate degree (e.g. PhD, MPhil, DClinPsychol) at the University of Edinburgh. Please note the following terms and conditions of use:

- This work is protected by copyright and other intellectual property rights, which are retained by the thesis author, unless otherwise stated.
- A copy can be downloaded for personal non-commercial research or study, without prior permission or charge.
- This thesis cannot be reproduced or quoted extensively from without first obtaining permission in writing from the author.
- The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author.
- When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given.

Characterising the role of GPR50 in neurodevelopment and lipid metabolism

Nneka Ulunma Anyanwu

A thesis submitted for the degree of Doctor of Philosophy

The University of Edinburgh

2014

To Jesus, Allah and Vishnu

Declaration

I hereby declare that this thesis has been composed by me and that the work presented in this here is my own, unless otherwise stated, and has not been submitted for any other degree or profession qualification.

Nneka Anyanwu

Acknowledgments

First and foremost I need to give a massive thanks to my supervisor Dr. Pippa Thomson, who has always gone above and beyond. I will always appreciate the independence she gave me and the fact that she was never too busy; I know many students aren't lucky enough to have this. I'd also like to extend my gratitude towards my second supervisor, Dr. Aymen Idris, his enthusiasm and positivity always made things seem ok. I also have to thank members of my thesis committee, who helped guide this PhD; Prof. Cathy Abbott, who absolutely embodies Athena Swan, and Prof. Jeremy Hall, especially for his offer of a light box 10 weeks into my PhD. Also, I want to give a huge thanks to everyone who had a look at my thesis – Rob, Dr Allison, “Ra Ra” Radhika, Laura, Ade, K-dawg, and especially Nidhi-Nidhi Chub-Chubs, who has been my strength, my inspiration and my rock.

Of course I need to thank everyone in MedGen, especially Shaun Mackie, Fumiaki Ogawa and Ellen Grünewald, who have been my scientific encyclopaedias for a good three years, and Prof. David Porteous, for allowing me to be part of this section. I'd also like to acknowledge all the PhD students at the MMC/CGEM for making my time here so much jokes; a particular shout out to all my year (Yan, Darragh and Michelle), I heart hip hop cru (Kate, Nnidhi and Laura) and also Evi (who didn't fit into a group, unless rejecting my ring is a group). Finally, I'd like to thank my fellow GPR50-devotee (Qian “Qiantastic” Li); we've gone through a lot together and survived it - just about. I'm sure your thesis will be almost as spectacular as my performance at your wedding. As great as this whole experience was, thank fudge it's over!

Lastly I need to thank my family. My Pops; for his incredible passion for education, which led him from a village boy in Owerri to a professor in London. My Mum; for her refusal to let me give up my PhD in pursuit of what would have been an

extraordinary career in Jazz dance, and for being silly. Also, my siblings (Chill San, K-pac, Unis and J-dot) who have mainly been a financial burden (that's not a typo or a joke!).

And to everyone else that I haven't named – thanks a bunch.

Table of contents

Declaration	iii
Acknowledgments.....	iv
Table of contents	vi
List of figures	xiii
List of tables.....	xv
Abbreviations	xvi
Units	xx
Abstract	xxi
Chapter 1: Introduction	1
1.1 The study of G-protein coupled receptor 50	2
1.2 GPR50 – a G-protein coupled receptor	2
1.2.1 Signal transduction.....	3
1.2.2 GPCR interacting proteins (GIPs).....	4
1.2.3 Ligand independent functions of GPCRs.....	6
1.2.4 GPCRs and mental health	7
1.3 GPR50 – part of the melatonin receptor family	7
1.3.1 Melatonin receptors.....	8
1.3.1.1 Melatonin receptors structure and signalling.....	8
1.3.1.2 The function of melatonin receptors.....	9
1.3.2 The structure of GPR50	11
1.3.3 Subcellular localisation of GPR50	14
1.3.4 The distribution of GPR50	16
1.3.5 GPR50 signal transduction.....	17
1.3.6 GPR50 functions	18

1.3.6.1	Modulating the activity of interacting proteins.....	18
1.3.6.2	Neurodevelopment.....	19
1.3.6.3	Energy metabolism	23
1.3.6.4	Mental illness.....	25
1.4	Investigating the functions of GPR50	26
1.4.1	Y2H screen.....	28
1.4.1.1	Neuronal differentiation/development.....	32
1.4.1.2	Sterol/cholesterol metabolism.....	35
1.4.1.3	What does the Y2H screen tell us about GPR50?	38
1.5	Conclusion.....	38
1.5.1	Aim.....	39
Chapter 2: Materials and methods.....		41
2.1	Buffers and solutions.....	42
2.1.1	Bacterial culture	42
2.1.2	Cell culture	42
2.1.3	Molecular biology	43
2.2	Expression constructs	44
2.3	Antibodies	45
2.3.1	Primary antibodies	45
2.3.2	Secondary antibodies	47
2.4	Cell culture	47
2.4.1	Maintenance of cell lines	47
2.4.1.1	Cell culture conditions	47
2.4.1.2	Cell lines	48
2.4.1.3	Cell counting.....	48
2.4.1.4	Handling cell stocks.....	49

2.4.2	Preparation and maintenance of primary neurons.....	49
2.4.2.1	Preparation of plates for ICC	49
2.4.2.2	Dissections	50
2.4.3	Transient transfections	50
2.5	Cell treatments.....	52
2.5.1	U18666A treatment.....	52
2.5.2	Serum deprivation	53
2.5.3	Lipoprotein depletion.....	53
2.6	Protein analysis.....	54
2.6.1	Cell lysate preparation and quantification.....	54
2.6.2	Co-immunoprecipitation	55
2.6.3	Western blotting	55
2.6.3.1	Sample preparation and SDS-PAGE electrophoresis	55
2.6.3.2	Western blot transfer.....	56
2.6.3.3	Immunostaining and detection.....	56
2.6.3.4	Semi-quantitative analysis	57
2.6.4	Immunocytochemistry.....	57
2.6.4.1	Nile red staining.....	58
2.6.4.2	Microscopy	58
2.7	Image analysis	59
2.7.1	Measurements of fluorescence intensity	59
2.7.1.1	Total cellular fluorescence.....	59
2.7.1.2	Intensity profile plots	59
2.7.2	Colocalisation analysis.....	60
2.7.2.1	Colocalisation plots.....	60
2.7.2.2	Colocalisation coefficients.....	60

2.7.3	Neurite outgrowth	61
2.7.4	Sholl analysis	61
2.7.5	Spine morphology	63
2.7.6	Lipid droplet quantification.....	64
2.8	Statistical analysis	64
Chapter 3: GPR50 expression promotes dendritic branching and spine formation..		65
3.1	Introduction	66
3.2	Aim.....	68
3.3	Results	69
3.3.1	Overexpression of GPR50 increases the number of neurites on SH-SY5Y cells	69
3.3.2	GPR50 overexpression increases dendritic complexity of mature primary neurons	70
3.3.3	GPR50 transfection does not affect the morphology of immature cortical neurons	78
3.3.4	The density of postsynaptic spines is increased by GPR50 in hippocampal neurons	81
3.3.5	GPR50 transfection does not impact the morphology of dendritic spines.....	88
3.4	Discussion	91
3.4.1	GPR50 induces neuronal differentiation.....	92
3.4.1.1	Promotion of neurite outgrowth in cell lines	92
3.4.1.2	Differential effects of GPR50 on hippocampal and cortical neurons.....	92
3.4.1.3	How does GPR50 modulate neuronal differentiation?	94
3.4.2	Does GPR50 impact synaptic function?	94
3.4.3	Correlating the role of GPR50 to its developmental profile	97

3.4.4	The importance of neuronal development in psychiatric illness.....	98
3.4.5	Conclusion	99
Chapter 4: GPR50 localises at lamellipodia-like structures and activates the RAC1-WAVE pathway		
		101
4.1	Introduction	102
4.2	Aims	105
4.3	Results	105
4.3.1	GPR50 has no effect on the amount or localisation of the major components of the cytoskeleton	105
4.3.2	Enrichment of GPR50 at lamellipodia-like structures in SH-SY5Y cells.....	108
4.3.3	GPR50 is enriched in dendritic spines of hippocampal neurons	111
4.3.4	GPR50 modulates key components of the actin remodelling pathway.....	113
4.3.5	Increased mTORC2 activity upon overexpression of GPR50	117
4.3.6	GPR50 alters the cellular distribution of α -catenin in SH-SY5Y cells.....	119
4.3.7	In HEK293 cells GPR50 decreases α -catenin levels but does not alter its localisation	123
4.4	Discussion	125
4.4.1	GPR50 promotes the RAC1-WAVE pathway	125
4.4.2	Regulation of the RAC1-WAVE pathway may be mediated by mTORC2	129
4.4.3	The modulation of α -catenin by GPR50	131
4.4.4	Conclusion	132
Chapter 5: Validation of the putative GPR50-interacting proteins SREBF2 and ABCA2.....		
		133
5.1	Introduction	134

5.2	Aims	135
5.3	Results	136
5.3.1	GPR50 does not colocalise with SREBF2	136
5.3.2	GPR50 does not promote the translocation of SREBF2	139
5.3.3	GPR50 colocalises with ABCA2 in intracellular compartments	141
5.3.4	ABCA2 colocalises with GPR50 in additional cell lines.....	146
5.3.5	GPR50 does not interact with ABCA2	148
5.4	Discussion	151
5.4.1	GPR50 and SREBF2 are unlikely to interact.....	151
5.4.2	ABCA2 overexpression leads to the intracellular trafficking of GPR50.....	153
5.4.3	Conclusion	155
Chapter 6: GPR50 expression is associated with lipid metabolism.....		157
6.1	Introduction	158
6.2	Aim.....	160
6.3	Results	161
6.3.1	Investigating the relationship between GPR50 expression and lipid levels.....	161
6.3.1.1	GPR50 expression is responsive to the depletion of exogenous lipids.....	161
6.3.1.2	GPR50 reduces the accumulation of intracellular lipid droplets	164
6.3.2	The regulation of GPR50 localisation by lipids.....	166
6.3.2.1	U18666A promotes the intracellular localisation of GPR50.....	166
6.3.2.2	Inhibition of cholesterol transport attenuates the promotion of neurite outgrowth by GPR50	169
6.3.2.3	GPR50 transfection induces a depletion of lipid droplets in the presence of U18666A.....	171

6.4	Discussion	172
6.4.1	A role in lipid homeostasis.....	174
6.4.1.1	GPR50 expression is responsive to extracellular lipid levels	174
6.4.1.2	Increase of GPR50 during serum deprivation.....	175
6.4.1.3	GPR50 promotes lipolysis in SH-SY5Y cells	176
6.4.2	The intracellular localisation of GPR50.....	177
6.4.2.1	Defects in cholesterol transport leads to the cytoplasmic trafficking of GPR50.....	177
6.4.2.2	Functional consequence of GPR50 internalisation.....	178
6.4.2.3	Is GPR50 specifically internalised in response to U18666A?	178
6.4.3	Conclusion	179
Chapter 7:	Discussion	181
7.1	Investigating the role of GPR50	182
7.2	The role of GPR50 in neurodevelopment.....	183
7.3	GPR50 regulates actin remodelling.....	184
7.4	GPR50 as a lipid sensor.....	185
7.5	GPR50 promotes lipolysis.....	187
7.6	Does GPR50 signal via mTORC2?	188
7.7	Future work	190
References		193
Appendix A:	Antibody validation.....	233
A.1	Introduction	234
A.2	GPR50 is localised to the plasma membrane	234
A.3	ABCA2 is found in discrete intracellular vesicles	238
A.4	Staining of active and inactive SREBF2	240
A.5	Conclusion.....	243

List of figures

Figure 1.1: G-protein coupled receptor signalling pathways	4
Figure 1.2: Signalling pathways of MT ₁ and MT ₂	9
Figure 1.3: The structure of GPR50.....	13
Figure 1.4: Trafficking of transmembrane proteins	15
Figure 1.5: Actin dynamics at the lamellipodia	22
Figure 3.1: Neuronal development of cortical neurons.....	67
Figure 3.2 : GPR50 induces neurite formation in SH-SY5Y cells	70
Figure 3.3: GPR50 alters the morphology of hippocampal neurons.....	73
Figure 3.4: GPR50 increases dendritic complexity in hippocampal neurons	74
Figure 3.5: GPR50 alters the morphology of cortical neurons	76
Figure 3.6: GPR50 increases dendritic complexity and primary dendrites in cortical neurons	77
Figure 3.7: GPR50 does not affect the morphology of younger cortical neurons (DIV 7)	79
Figure 3.8: Transfection of GPR50 in immature cortical neurons, DIV 7, does not increase dendritic complexity	80
Figure 3.9: The effect of GPR50 overexpression on dendritic spines	83
Figure 3.10: Effects of GPR50 overexpression on the number of postsynaptic spines in hippocampal neurons	84
Figure 3.11: Effects of GPR50 overexpression on the number of postsynaptic spines in cortical neurons	87
Figure 3.12: Overexpression of GPR50 does not impact the morphology of spines.	90
Figure 4.1: Regulation of actin organisation.....	103
Figure 4.2: GPR50 overexpression has no effect on the expression of the major cytoskeletal elements in SH-SY5Y cells.....	106
Figure 4.3: GPR50 overexpression does not alter the cytoskeleton in SH-SY5Y cells	107
Figure 4.4: GPR50 colocalises with β -actin at the lamellipodia of SH-SY5Y cells	109
Figure 4.5: GPR50 is enriched at the lamellipodia of SH-SY5Y cells	110

Figure 4.6: GPR50 is expressed in the dendritic spines of hippocampal neurons ...	112
Figure 4.7: Expression of GPR50 is enriched in postsynaptic spines.....	113
Figure 4.8: Regulators of actin remodelling are activated by GPR50	114
Figure 4.9: GPR50 colocalises with RAC1 at lamellipodia in SH-SY5Y cells.....	116
Figure 4.10: Phosphorylation pattern of AKT and GSK-3 β	117
Figure 4.11: GPR50 transfection increases phosphorylation of AKT at S473	119
Figure 4.12: Levels of α and β -catenin are not affected by GPR50 in SH-SY5Y cells	120
Figure 4.13: GPR50 alters the cellular distribution of α -catenin in SH-SY5Y cells	122
Figure 4.14: Decrease in α -catenin levels upon GPR50 transfection in HEK293 cells	123
Figure 4.15: GPR50 overexpression does not alter the localisation of α -catenin in HEK293 cells	124
Figure 4.16: Regulation of the mTOR pathway.....	130
Figure 5.1: GPR50 does not colocalise with SREBF2 in SH-SY5Y cells.....	139
Figure 5.2: ABCA2 and GPR50 show some colocalisation within the cytoplasm of SH-SY5Y cells.....	143
Figure 5.3: The colocalisation between ABCA2 and GPR50 is positively correlated with the ABCA2 signal intensity	145
Figure 5.4: Colocalisation of ABCA2 and GPR50 is conserved in different cell lines	147
Figure 5.5: GPR50 and ABCA2 do not co-immunoprecipitate in HEK293 cells ...	149
Figure 5.6: GPR50 does not co-immunoprecipitate with ABCA2	150
Figure 6.1: Endogenous GPR50 is responsive to serum and lipoprotein levels in HEK293 cells	163
Figure 6.2: GPR50 overexpression decreases the accumulation of lipid droplets in SH-SY5Y cells.....	165
Figure 6.3: GPR50 transfection reduces the perinuclear distribution of lipid droplets in SH-SY5Y cells.....	166
Figure 6.4: U18666A treatment leads to a rise in intracellular GPR50 in transfected SH-SY5Y cells.....	168

Figure 6.5: Treatment with U18666A attenuates the GPR50-mediated increase in neurite outgrowth in SH-SY5Y cells	170
Figure 6.6: Overexpression of GPR50 in SH-SY5Y cells significantly reduced lipid droplets in the presence of U18666A.....	172
Figure 6.7: Relationship between GPR50 and lipid metabolism	173
Figure A.1: A 67 kDa protein is detected by both GPR50 antibodies	235
Figure A.2: The plasma membrane localisation of GPR50	237
Figure A.3: Characterisation of the ABCA2 antibodies	239
Figure A.4: Characterisation of the goat anti-SREBF2 antibody (SCBT).....	241
Figure A.5: Nuclear translocation of SREBF2 in COS-7 cells is detected by the goat anti-SREBF2 antibody	243

List of tables

Table 1.1: Results from the Y2H study with the CTD of GPR50 screened against a human brain cDNA library.....	30
Table 2.1: List of solutions required for bacterial work	42
Table 2.2: Buffers and solutions required for cell culture work	43
Table 2.3: Buffers and solutions required to perform western blotting and immunocytochemistry.....	44
Table 2.4: Expression constructs used in this thesis	44
Table 2.5: Primary antibodies used in this thesis.....	46
Table 2.6: Secondary antibodies used in this thesis.....	47
Table 2.7: Lipofectamine 2000 transfection	51
Table 4.1: The functions of the modulators of actin dynamics tested in this study	115
Table 5.1: Colocalisation coefficients between GPR50 and SREBF2.....	139
Table 5.2: Colocalisation coefficients between SREBF2 and calreticulin in SH-SY5Y cells expressing SREBF2 alone or with GPR50	140
Table 5.3: Colocalisation coefficients between GPR50 and ABCA2 in SH-SY5Y cells	144

Abbreviations

ABCA2	ATP-binding cassette sub-family A member 2
AD	Alzheimer's disease
ANOVA	Analysis of variance
ARC	Arcuate nucleus
ARP2/3	Actin related protein 2/3
BD	Bipolar disorder
BSA	Bovine serum albumin
C57BL/6	C57 black 6 mouse strain
Ca ²⁺	Calcium
cAMP	Cyclic Adenosine monophosphate
CDC42	Cell division cycle 42
CDH8	Cadherin 8
cDNA	Complementary DNA
CMV	Cytomegalovirus
CNS	Central nervous system
co-IP	Co-immunoprecipitation
CREB	cAMP response element binding protein
CTD	C-terminal domain
DABCO	1,4-diazabicyclo[2.2.2]octane
DAG	Diacylglycerol
DAPI	4'6-diamidino-2-phenylindole
dH ₂ O	Deionised water
DISC1	Disrupted-in-Schizophrenia 1
DIV	Days <i>in vitro</i>
DMEM	Dulbecco's modified eagle medium
DMH	Dorsomedial hypothalamic nucleus
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E(13)	Embryonic day (13)

ER	Endoplasmic reticulum
FA	Fatty acid
F-actin	Filamentous actin
FBS	Fetal bovine serum
<i>Fkbp5</i>	FK506 binding protein 5
G-actin	Globular actin
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDP	Guanosine diphosphate
GEF	Rho guanine nucleotide exchange factor
GFP	Green fluorescence protein
GIP	GPCR interacting protein
GO	Gene ontology
GPCR	G-protein coupled receptor
GPR50	G-protein coupled receptor 50
G-protein	Guanine nucleotide-binding protein
GR	Glucocorticoid receptor
GSK-3	Glycogen synthase kinase
GST	Glutathione S-transferase
GTP	Guanosine triphosphate
HBSS	Hank's Buffered Salt Solution
HDL	High-density lipoprotein
HEK293	Human embryonic kidney 293
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
ICA	Intensity correlation analysis
ICC	Immunocytochemistry
ICQ	Intensity correlation quotient
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IP	Inositol phosphate
IP ₂	Inositol bisphosphate
IP ₃	Inositol trisphosphate

K-S test	Kolmogorov–Smirnov test
L-Agar	Luria Agar
LAMP1	Lysosomal-associated membrane protein 1
LB	Lysogeny broth
LDL	Low-density lipoprotein
LH	Lateral hypothalamus
LPDS	Lipoprotein deficient serum
LSM 510	Laser Scanning Microscope 510
MAPK	Mitogen activated protein kinase
MDD	Major depressive disorder
Mel1a	Melatonin receptor 1a (non-mammalian)
Mel1c	Melatonin receptor 1c (non-mammalian)
MT ₁	Melatonin receptor type 1
MT ₂	Melatonin receptor type 2
mTOR	Mammalian target of rapamycin
mTORC	mTOR complex
N	Sample size
NCAD	Neural Cadherin
NHERF	Na ⁺ /H ⁺ exchanger regulatory factor
NOGO-A	Neurite outgrowth inhibitor A
NP-40	Nonidet P-40
NRG1	Neuregulin 1
NS-1	Neuroscreen 1
N-WASP	Neural Wiskott–Aldrich syndrome protein
P13K	Phosphoinositide-3-kinase
PAK	p21 activated kinase
PBS (buffer)	Phosphate buffered saline
PBS (score)	Predicted biological score
PCR	Polymerase chain reaction
PDK1	Phosphoinositide-dependent kinase-1
PFA	Paraformaldehyde
PIP	Phosphatidylinositol phosphate

PIP ₂	Phosphatidylinositol bisphosphate
PIP ₃	Phosphatidylinositol trisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC β	Phospholipase C β
PSD	Postsynaptic density
R	Manders' coefficient
RAC1	Ras-related C3 botulinum toxin substrate 1
RHOA	Ras homolog family member A
RIPA	Radio-immunoprecipitation assay
ROCK	Rho-associated protein kinase
ROI	Region of interest
Rr	Pearson's correlation coefficient
rt-qPCR	Reverse transcription quantitative PCR
S	Serine
SCN	Suprachiasmatic nucleus
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
SNX5	Sorting nexin 5
SRE	Sterol regulatory element
SREBF2	Sterol regulatory element binding transcription factor 2
SREBP2	Sterol regulatory element binding protein 2
T	Threonine
TBS	Tris buffered saline
TIP60	HIV-1 tat interactive protein
TM	Transmembrane domain
U18666A	3 β -(2-Diethylaminoethoxy)androst-5-en-17-one
VLA-2 α	Very late antigen 2 α
WAVE-2	WASP-family verprolin-homologous protein-2
WB	Western blot
Y	Tyrosine

Y2H Yeast-2-hybrid

Units

°C	Degrees centigrade
bp	Base pair
Da	Dalton
g	Gram
hrs	Hours
l	Litre
m	Metre
M	Molar
mins	Minutes
rpm	Revolutions per minute
V	Volt

Abstract

G-protein coupled receptor 50 (*GPR50*) is a genetic risk factor for psychiatric illness. It is a member of the melatonin receptor family, which includes the well characterised melatonin receptors 1 and 2 (MT₁ and MT₂). However, the ligand for *GPR50* remains elusive and little is known about *GPR50* signalling pathways. Despite this, *GPR50* is known to enhance neurite outgrowth and inhibit the actions of the neurite outgrowth inhibitor NOGO-A. Existing evidence also indicates a role in lipid metabolism; *GPR50* knockout mice displayed abnormalities in energy homeostasis and weight control, whilst sequence variants are associated with altered lipid levels in humans. Further, a yeast-2-hybrid screen identified SREBF2 and ABCA2, regulators of lipid homeostasis, as *GPR50* interactors. This thesis explores the role of *GPR50* in neuronal development and lipid metabolism.

The work presented in this thesis shows that *GPR50* promotes neuronal differentiation. Overexpression significantly increased the number of neurites per cell in SH-SY5Y cells. Further, dendritic branching was enhanced by *GPR50* transfection in hippocampal and cortical neurons (DIV 14). In hippocampal neurons, *GPR50* transfection also lead to a shift towards spine maturity although it had no effect on spine morphology, suggesting *GPR50* enhances spine development but may not alter synaptic strength. The effect of *GPR50* on neuronal morphology may be driven by actin remodelling. Immunocytochemistry showed an enrichment of *GPR50* in highly dynamic regions of the membrane, i.e. the lamellipodia and dendritic spines. Overexpression in SH-SY5Y cells also resulted in an increase in WAVE-2 and phosphorylated RAC1/CDC42, key modulators of actin dynamics. Additionally, *GPR50* transfection altered the protein level and localisation of α -catenin, another regulator of actin organisation, in HEK293 and SH-SY5Y cells respectively.

An involvement of GPR50 in lipid metabolism has also been demonstrated in this thesis. Verification of the Y2H study suggested GPR50 does not physically interact with SREBF2 or ABCA2. However, ABCA2 appears to induce the intracellular localisation of GPR50 in several cell lines. In SH-SY5Y cells, this was mimicked by the inhibition of cholesterol trafficking, suggesting the translocation of GPR50 to the plasma membrane is dependent on cholesterol transport. Further, the depletion of lipoproteins resulted in the downregulation of GPR50, indicating a responsiveness to lipid levels. Finally, GPR50 increased lipid metabolism, as seen by a decrease in intracellular lipid droplets upon GPR50 overexpression.

The data presented here extends previous work indicating a role of GPR50 in neurodevelopment. It also highlights a potential mechanism by which GPR50 regulates neuronal morphology, i.e. via actin remodelling. Reports that GPR50 is involved in energy homeostasis is also supported in this thesis, further, results presented here suggest GPR50 is specifically involved in lipid metabolism. These processes are often disrupted in mental illness, thus this work may provide a functional link between GPR50 and psychiatric disorders.

Chapter 1: Introduction

1.1 *The study of G-protein coupled receptor 50*

Interest in G-protein coupled receptor 50 (GPR50) stems from studies addressing the biological basis of bipolar disorder (BD). BD is amongst the most debilitating common disorders known, affecting a staggering 1% of the population and costing the UK approximately £4.59 billion a year (estimated 2007 value) (Fajutrao et al, 2009). Although there are reasonably effective treatments available, they are often accompanied with disturbing side effects and are ineffective in some patients. In an attempt to improve this, studies have been performed to isolate the genetic basis of BD, with the belief that identifying the contributing genes will provide valuable information about the pathogenesis of the disorder. This may also reveal novel drug targets. The identification of GPR50 as a candidate gene came from such an attempt.

The goal of this PhD was to improve our understanding of the role of GPR50 on a molecular level. This introduction aims to provide a comprehensive overview of what is known about GPR50 and how this has shaped this PhD project. Specifically, this chapter will discuss GPR50 as a G-protein coupled receptor, its position in the melatonin receptor family, its structure and function and how this knowledge leads to the overarching aims of this thesis.

1.2 *GPR50 – a G-protein coupled receptor*

With the characteristic seven transmembrane domains, GPR50 forms part of the G-protein coupled receptor (GPCR) family. GPCRs are a group of receptors, with over 800 members (Dorsam & Gutkind, 2007; Ritter & Hall, 2009). Their primary function is to mediate the intracellular response to external stimuli, i.e. ligands, such as neurotransmitters, lipids and hormones. GPCRs govern a vast array of cellular processes and appear to have important roles in numerous medical disorders. This is

demonstrated by the fact that GPCRs are targeted by over 50% of the therapeutic drugs currently available (Dorsam & Gutkind, 2007). This raises the possibility that GPR50 could also be a therapeutic target. The full characterisation of GPR50 would be a prerequisite for this; understanding how GPCRs work is the first step towards this characterisation process. This section summarises the basic features of GPCR signalling.

1.2.1 Signal transduction

GPCRs have been studied extensively for many decades, as a result there is a comprehensive understanding of their signalling pathways (Dorsam & Gutkind, 2007; Thomsen et al, 2005). Classically, the binding of an extracellular ligand to a GPCR induces a conformational change that favours the binding and activation of a G-protein (guanine nucleotide binding protein). This is a heteromeric protein consisting of an α -, β - and γ -subunit. In its unstimulated state the $G\alpha$ subunit is bound to GDP, this is exchanged with GTP upon activation. In the active state, $G\alpha$ dissociates from the heteromer to inhibit or stimulate downstream effectors such as adenylyl cyclase and phospholipase C β (PLC β), this in turn regulates secondary messengers such as cyclic adenosine monophosphate (cAMP) and phosphatidylinositol biphosphate (PIP₂), figure 1.1. G-proteins can be split into four subfamilies (G_s , $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$), which initiate different signalling cascades (figure 1.1). This adds a level of specificity to the signalling cascade governed by a particular GPCR. The $G\beta\gamma$ complex also regulates several effector molecules such as PLC β and mitogen-activated protein kinase (MAPK). The ultimate consequence of these signalling cascades is the regulation of genes involved in various biological responses.

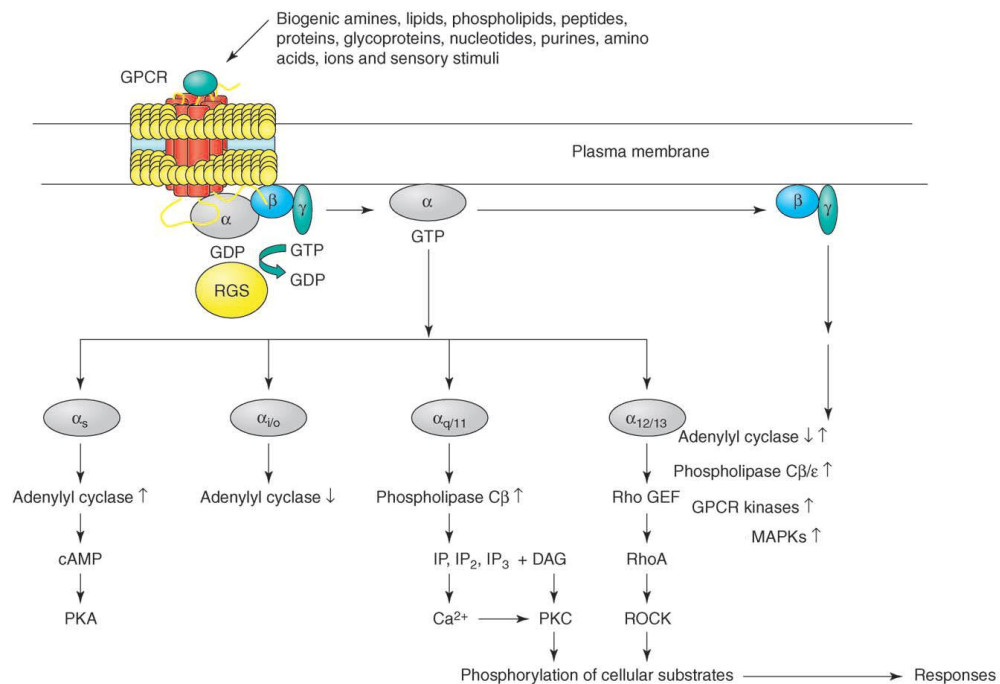


Figure 1.1: G-protein coupled receptor signalling pathways. Upon ligand binding, GPCRs undergo conformational changes which catalyse the exchange of GDP, bound to the Gα subunit, for GTP. In this active state the Gα subunit dissociates from the Gβγ dimer and initiates a signalling cascade depending on its subfamily. Gα_s typically stimulates adenylyl cyclase to increase cAMP levels, whereas Gα_{i/o} acts in an inhibitory fashion. Members of the Gα_{q/11} subfamily activate PLCβ, resulting in the generation of secondary messengers such as inositol phosphate (IP, IP₂ and IP₃) and DAG. The Gα_{12/13} subfamily regulates the activity of effectors such as Rho GEF. The Gβγ complex is also capable of signalling via various molecules including PLCβ and MAPKs. Abbreviations: cAMP, cyclic adenosine monophosphate; DAG, diacylglycerol; GEF, guanine nucleotide exchange factor; MAPK, mitogen activated protein kinase; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PLCβ, phospholipase Cβ; ROCK, rho-associated protein kinase; RGS, regulator of G-protein signalling. Figure modified from Thomsen et al, 2005.

1.2.2 GPCR interacting proteins (GIPs)

In addition to G-proteins, GPCRs interact with a diverse range of proteins which play an essential role in regulating their activity. The most well established examples are GPCR kinases (GRKs) and arrestins (Ritter & Hall, 2009). Binding of β-arrestin to GPCRs arises following their phosphorylation by GRKs. β-Arrestin blocks G-

protein coupling to GPCRs via steric hindrance, leading to desensitisation. β -Arrestin also drives the internalisation of GPCRs via clathrin-mediated endocytosis. Once internalised, GPCRs can be delivered to lysosomes for degradation or endosomes for recycling. These mechanisms regulate the extent of G-protein mediated signalling. Arrestins also act as scaffold proteins to bring signalling molecules together in order to initiate signal transduction independently of G-protein binding. MAPK, AKT and anti-apoptotic pathways can be activated in this manner (Ahn et al, 2009; Rajagopal et al, 2010; Shenoy et al, 2006).

Other GIPs are responsible for modulating the signalling cascade controlled by a given receptor. For instance, Na⁺/H⁺ exchange regulatory factor (NHERF) 1 and 2 binds to the lysophosphatidic acid receptor and parathyroid hormone 1 receptor (PTH1R), and by doing so, enhances their ability to activate PLC β in response to agonist binding (Mahon et al, 2002; Mahon & Segre, 2004; Oh et al, 2004). Interestingly, PTH1R signalling is predominately through adenylyl cyclase in the absence of NHERF, thus this GIP switches signalling from the adenylyl cyclase to the PLC β pathway. This is likely to be due to an altered affinity for G-protein subtypes. Under control conditions PTH1R shows a similar affinity towards G α_s , G α_q , and G α_i , however an association with NHERF1 shifts this towards G α_q . Further, NHERF2 favours G α_q and G α_i binding, whilst inhibiting G α_s stimulation (Wang et al, 2010). Thus, knowledge of the interactors of a particular GPCR provides insight into the signalling output.

Control of receptor trafficking is also essential to the regulation of GPCR signalling; again GIPs play a critical role here. Overexpression of dopamine receptor interacting protein 78 (DRiP78) increases the ER retention of the D1 dopamine receptor, A1 adenosine receptor and the CCR5 chemokine receptor (Bermak et al, 2001; Kuang et al, 2012; Malaga-Diequez et al, 2010). As a result, the signalling capacity of these receptors is attenuated. Another well characterised example of GIP-mediated trafficking is by GPCR associated sorting protein 1 (GASP-1), which promotes

lysosomal targeting and the subsequent degradation of internalised receptors. This is true for a number of GPCRs including the D2 dopamine receptor, cannabinoid 1 receptor and the US28 chemokine receptor (Bartlett et al, 2005; Martini et al, 2007; Tschische et al, 2010). Disrupting the interaction between these receptors and GASP-1 promotes their recycling. Other GIPs, such as NHERF, have the converse function and promote receptor recycling rather than degradation (Cao et al, 1999; Li et al, 2002).

These studies exemplify how dynamic the localisation of GPCRs can be. They also highlight the fundamental importance of modulating receptor trafficking in order to regulate its activity; GIPs are critical for this process. Moreover, these molecules play an important role in modulating GPCR signalling. In the absence of a ligand, the identification of GIPs can provide key insight into the function and regulation of a GPCR. Hence, this strategy was employed in the context of GPR50; this will be discussed in length later in this chapter.

1.2.3 Ligand independent functions of GPCRs

The activity of a GPCR is not necessarily dependent on ligand binding. Some receptors, such as somatostatin receptor type 2, possess constitutive activity whereby signalling occurs in the absence of ligand binding (Bousquet et al, 2006). The somatostatin receptor activates a phosphoinositide-3-kinase (PI3K) mediated pathway leading to cell survival; ligand binding, however, inhibits this activity. In addition, some orphan GPCRs modulate signalling of other receptors upon heterodimerisation. The orphan mas-related gene E receptor (MRGE) exemplifies this. The activation of MRGD by β -alanine was enhanced when co-expressed with MRGE (Milasta et al, 2006). MRGE also impedes agonist-induced internalisation of MRGD, therefore, potentiates MRGD signalling. These results demonstrate the

complexity of GPCR signalling, but also show prior knowledge of a ligand is not always a prerequisite to study the actions of a GPCR.

1.2.4 GPCRs and mental health

Most commonly administered drug interventions for psychiatric illness target GPCRs, or molecules within the associated signalling pathway (Berton & Nestler, 2006). For instance, the serotonin receptor is involved in the efficacy of a number of antidepressants (Launay et al, 2006; Pejchal et al, 2002). Clinical data shows ligand binding to various serotonin receptor subtypes is diminished in individuals with depression (Lewis et al, 1999; Yatham et al, 1999), highlighting the importance of this GPCR in the disorder. In addition, polymorphisms within serotonin receptor genes are associated with depression and schizophrenia (Lemondé et al, 2003; Lorenzo et al, 2006). As well as the serotonin receptor, signalling via dopamine and adrenergic receptors is routinely modified by drug treatment (Berton & Nestler, 2006; Catapano & Manji, 2007). Genetic studies have also linked these receptors to psychiatric disorders (Kaalund et al, 2011; Lochman et al, 2013; Wakeno et al, 2008). Thus, both genetic and pharmacological evidence link GPCRs to mental illness. The search for novel treatments may result in the targeting of another GPCR, understanding how GPR50 functions may be important in this advancement.

1.3 GPR50 – part of the melatonin receptor family

GPR50, also known as melatonin-related receptor, is a member of the melatonin receptor family. In mammals, this consists of melatonin receptors 1 and 2 (MT₁ and MT₂), which share a 45% sequence homology with GPR50 (Reppert et al, 1996). How does GPR50 fit in with other members of this family? This question will be explored in this section by looking at the structure and function of the well

characterised melatonin receptors. Information regarding GPR50's structure, anatomical distribution and function will also be collated here.

1.3.1 Melatonin receptors

As the name implies, the melatonin receptors bind to melatonin, a neurohormone predominately produced in the pineal gland during the night (Hardeland et al, 2011; Perreau-Lenz et al, 2003). Melatonin mediates a large number of processes but is best known for its involvement in the circadian cycle, the 24 hr cycle of activity which drives the rhythmic nature of events such as feeding, the sleep/wake cycle and energy homeostasis. Unlike MT₁ and MT₂, GPR50 does not bind to melatonin (Levoye et al, 2006). However, it does interact with both MT₁ and MT₂, and impacts the functionality of MT₁ (Levoye et al, 2006). This means the function of the three receptors may be linked, thus a solid understanding of these receptors may reveal some information about GPR50.

1.3.1.1 Melatonin receptors structure and signalling

In humans, MT₁ and MT₂ share a 55% sequence homology on the amino acid level; this increases to 70% when only including the transmembrane domains (Jockers et al, 2008). The signalling pathways of the two receptors have been well characterised, as outlined in figure 1.2 (Masana & Dubocovich, 2001). Both MT₁ and MT₂ mediate the inhibition of adenylyl cyclase and the activation of PLC β (Jockers et al, 2008; MacKenzie et al, 2002). Activation of these receptors also leads to the phosphorylation of MAPKs and c-Jun N-terminal kinase (Chan et al, 2002; Witt-Enderby et al, 2000). In addition, MT₂ inhibits the guanylyl cyclase pathway (Petit et al, 1999). There is some overlap between the actions of the two receptors, as may be expected from the similar signalling cascades. However, there are also some clear distinctions. This shall be discussed next.

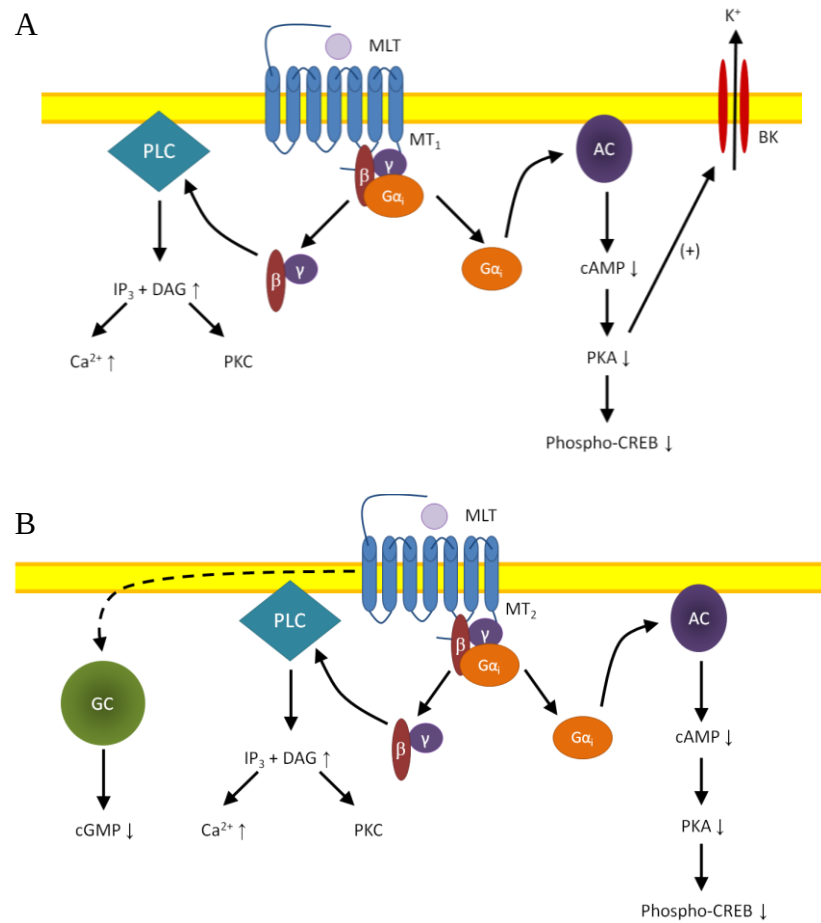


Figure 1.2: Signalling pathways of MT₁ and MT₂. (A) MT₁ signals via the G α_i pathway to inhibit adenylate cyclase. This leads to the reduction of cAMP, which in turn prevents the activation of PKA. G $\beta\gamma$ also mediates cell signalling via PLC to promote Ca²⁺ release. (B) Upon melatonin binding, MT₂ also activates cAMP and PLC signalling via G α_i and G $\beta\gamma$ respectively. In addition, MT₂ activation leads to the inhibition of guanylate cyclase, although this mechanism is poorly understood. Abbreviations: AC, adenylate cyclase; BK, big potassium channel; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; CREB, cAMP response element binding protein; DAG, diacylglycerol; GC, guanylate cyclase; IP₃, inositol trisphosphate; MLT, melatonin; PKA/C, protein kinase A/C; PLC, phospholipase C. Figure adapted from Masana & Dubocovich, 2001.

1.3.1.2 The function of melatonin receptors

Studies employing mRNA hybridisation and autoradiography have shown MT₁ is particularly enriched in the hypothalamus, especially in the suprachiasmatic nucleus (SCN) (Reppert et al, 1994). MT₂ is also found here, although at lower

levels. The two receptors appear to have slightly different functions in this region of the brain. MT₁, and not MT₂, has been shown to mediate the melatonin-induced inhibition to neuronal activity in the SCN (Jin et al, 2003; Liu et al, 1997).

Conversely, MT₂, rather than MT₁, has been reported to mediate the ability of melatonin to induce phase shifts, i.e. changes to the timings of the circadian cycle (Liu et al, 1997). However, in a more recent study, MT₁ depletion prevented melatonin-induced phase shift (Dubocovich et al, 2005). Thus, both receptors may be involved in phase shifting of the circadian rhythm but only MT₁ appears to modulate neuronal activity.

In animal studies, the administration of melatonin leads to a reduction in body weight, a resistance to weight gain and a decrease in circulating levels of insulin, glucose and triglycerides (Hussein et al, 2007; Rios-Lugo et al, 2010). Evidence suggests that the melatonin receptors mediate this effect. Association studies have linked MT₂ to type-2-diabetes and abnormal fasting glucose levels (Bonnetfond et al, 2012; Bouatia-Naji et al, 2009). Although this association is a matter of debate as MT₁ knockout mice were found to be insulin resistant and unable to metabolise glucose effectively, whilst the deletion of MT₂ had no effect on these parameters (Contreras-Alcantara et al, 2010). These inconsistencies may reflect the difference between humans and rodents or the alternative outcomes of gene deletion vs. polymorphisms. Whatever the case may be, these results show melatonin and its receptors have the ability to modulate metabolism.

A neurodevelopmental role of melatonin and its receptors is also evident. Melatonin has been shown to promote neurogenesis, neuronal survival and neuronal differentiation (Kim et al, 2004; Moriya et al, 2007; Ramirez-Rodriguez et al, 2009; Ramirez-Rodriguez et al, 2011). Treating neurons with luzindole, a melatonin receptor antagonist, almost completely abolished the neuronal differentiation induced by melatonin (Ramirez-Rodriguez et al, 2009), indicating the receptors are largely responsible for this outcome. In addition, researchers have demonstrated a

neuroprotective effect of melatonin in rodent models of ischemic stroke. This neuroprotection was associated with the upregulation of MT₂ and was blocked by treatment with MT₂ antagonists (Chern et al, 2012; Lee et al, 2010), indicating a dependency on MT₂. However, this has been contested as an independent study showed melatonin's neuroprotective effect was more pronounced in MT_{1/2} knockout mice compared to wild-type controls (Kilic et al, 2011). Thus, the role of the melatonin receptors in neuroprotection is currently ambiguous.

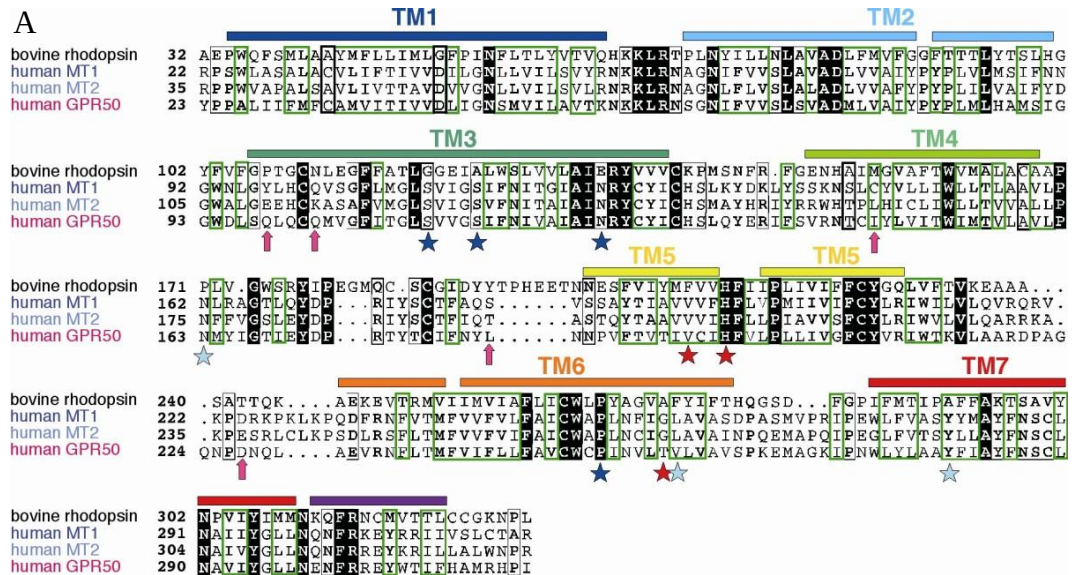
1.3.2 The structure of GPR50

In a recent evolutionary study it was established that GPR50 is an orthologue of the non-mammalian Mel1c receptor (Dufourny et al, 2008). During the evolutionary process, it has undergone extensive changes in the form of amino acid mutations and the addition of a long C-terminal tail. These changes have had a dramatic impact on the protein. An obvious example is the lost ability of GPR50 to bind to melatonin (Levoye et al, 2006; Reppert et al, 1996). Transmembrane domains 4 and 6 (TM4 and TM6) appear to be of great importance here. Replacing TM4 and TM6 of MT₁ with the corresponding regions of GPR50 led to the loss of ligand binding and signal transduction (Conway et al, 2000; Gubitz & Reppert, 2000). The reciprocal chimera, i.e. GPR50 with TM4 and TM6 of MT₁, gained the ability to bind to melatonin. In addition, melatonin dependent cAMP signalling was detected in this chimera. TM6 appears to be especially critical in ligand binding as the loss of this domain alone was sufficient to prevent melatonin binding to MT₁ (Gubitz & Reppert, 2000).

The importance of TM6 may be due to the presence of several amino acids which are essential for melatonin binding. For example, glycine 258 (G258) of MT₁ is conserved in MT₂, aligning to G271, but in GPR50 this is replaced with threonine, aligning to T257 (Dufourny et al, 2008). The substitution of this glycine residue with threonine in MT₁ and MT₂ significantly reduced melatonin binding (Gubitz &

Reppert, 2000; Mazna et al, 2004). Thus, this amino acid change in GPR50 may largely contribute to its inability to bind melatonin. Further, the substitution of leucine 271 in MT₂, also found within TM6, abolished melatonin binding (Mazna et al, 2004). Again this residue is conserved in MT₁ but not GPR50 (figure 1.3 A). Interestingly, these residues are located in the ligand binding pocket of the melatonin receptors (figure 1.3 B, star); the substitution of amino acids here may sterically hinder ligand binding (Barrett et al, 2003).

The C-terminal domain (CTD) of GPR50 shares high homology with RNA polymerase II (RNAPII) (Dufourny et al, 2008). In RNAPII, this region acts as a scaffold mediating the binding of proteins involved in transcriptional regulation. The CTD of RNAPII contains a particularly interesting structure - a seven amino acid repeat harbouring two serine residues. These residues are phosphorylated to regulate protein binding. As highlighted by Dufourny et al, this structure is conserved in GPR50 suggesting that the GPR50 CTD also functions as a scaffold structure modulated by phosphorylation. Whether or not this is true remains to be seen, however, the CTD of GPR50 is responsible for at least one of its interactions, i.e. with the HIV-1 tat interactive protein, TIP60 (Li et al, 2011). Coincidentally, GPR50 sequence variants associated with mental illness and lipid levels are located within the CTD (Bhattacharyya et al, 2006; Delavest et al, 2012; MacIntyre et al, 2010; Thomson et al, 2005). Together, these results suggest the CTD has an important functional role, perhaps this importance is as a scaffold domain.



B

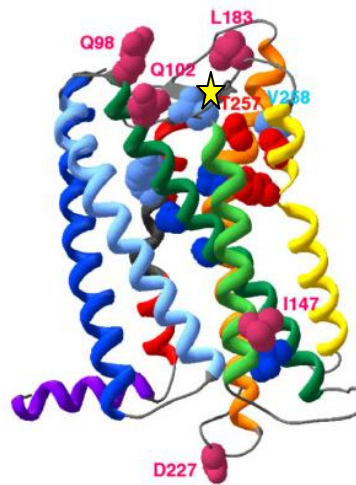


Figure 1.3: The structure of GPR50. (A) Sequence alignment between GPR50, bovine rhodopsin, MT₁ and MT₂. Transmembrane domains (TM) are labelled above; stars denote amino acid residues shown to be essential for ligand binding in MT₁ (dark blue), MT₂ (light blue) and both (red). Arrows indicate residues in GPR50 that have evolved under positive selection. (B) 3D representation of GPR50 with coloured helices corresponding to the TM domains labelled in (A). Amino acids essential in melatonin binding are also labelled, along with those that have evolved under positive selection. Note that T257 (star) is found within the ligand binding pocket. This amino acid corresponds to G258 in MT₁ and G271 in MT₂, which is crucial for melatonin binding. Figure taken from Dufourny et al, 2008.

1.3.3 Subcellular localisation of GPR50

On the cellular level GPR50 resides on the plasma membrane, as first demonstrated by overexpression experiments in HEK293 cells (Hamouda et al, 2007; Levoe et al, 2006). This has subsequently been demonstrated on an endogenous level in HEK293 cells and primary cortical neurons (Grünwald et al, 2009). Interestingly, the subcellular localisation of GPR50 appears to be dynamic. Upon co-transfection with TIP60, the full length GPR50 took up a perinuclear localisation, whilst the CTD adopted a nuclear distribution (Li et al, 2011). A truncated form of the receptor has been reported in both humans and mice (Li et al, 2011; Slominski et al, 2003). This arises from a 151 bp deletion within exon two, resulting in a premature stop codon which creates a protein lacking the cytoplasmic tail and two TM domains. It has been demonstrated that the murine form of this variant is unable to insert into plasma membrane and takes up a cytoplasmic localisation (Li et al, 2011).

Trafficking to the plasma membrane

The trafficking of proteins to the plasma membrane involves the intracellular shuttling of vesicles between various organelles. During protein translation, transmembrane proteins like GPCRs are targeted to the endoplasmic reticulum (ER) by the signal recognition particle (SRP) (Huang et al, 2010; Kochl et al, 2002). For most GPCRs, SRP interacts with a transmembrane domain, although some GPCRs contain a specific signal peptide sequence (Kochl et al, 2002). This interaction pauses translation and directs the nascent protein to the ER via the ER-bound SRP receptor. At the ER translation proceeds and the hydrophobic transmembrane regions of the protein are inserted into the lipid bilayer of the ER. Following translation the protein will undergo proper folding, post-translational modification, such as glycosylation, as well as dimerisation (Drake et al, 2006). The full length protein is then targeted to the Golgi complex in coatamer protein complex II (COPII)-coated vesicles (figure 1.4), which bud off the ER and fuse onto the Golgi via SNARE proteins (Bonifacino & Glick, 2004; Mellman & Nelson, 2008). The

nascent transmembrane protein can then traverse the Golgi complex via COPI-coated vesicles, again by the process of membrane budding and fusion. In the Golgi, proteins will undergo additional post-translational modifications resulting in the fully mature protein. This is then sorted into clathrin-coated transport vesicles for delivery to the plasma membrane.

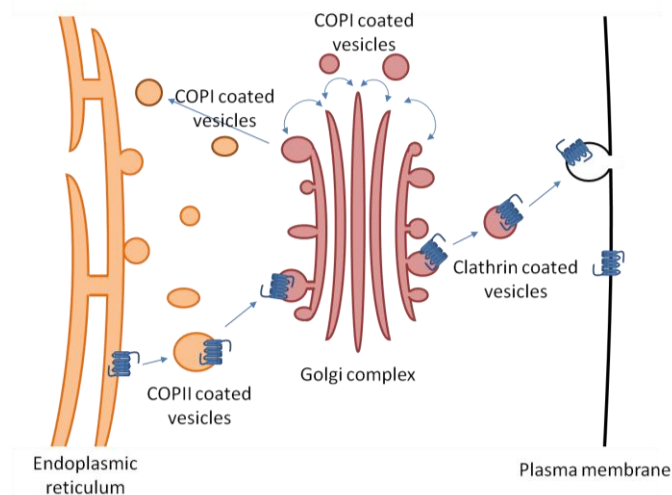


Figure 1.4: Trafficking of transmembrane proteins. Following synthesis in the endoplasmic reticulum, proteins are transported to the Golgi complex via coatamer protein complex (COP) II-coated vesicles. These vesicles tether and fuse onto the cis-face of the Golgi complex with the aid of SNARE proteins. Within the Golgi complex, proteins can be transported via COPI-coated vesicle until they reach the trans-face. At this stage, proteins are fully mature and are transported to the plasma membrane in clathrin-coated vesicles, along the cytoskeleton. Diagram authors own.

The intracellular trafficking of receptors through this series of organelles is a highly regulated process reliant on a number of factors. This includes conserved sequence motifs that mediate the exit of transmembrane proteins from the ER or Golgi complex. In GPCRs, many of these motifs are found within the C-terminus. A triple phenylalanine motif has been shown to be important in the cell surface expression of several GPCRs. Mutations within this site resulted in the ER accumulation of the dopamine D1 receptor and adiponectin receptors (Bermak et al, 2001; Juhl et al, 2012). Similarly, disruption of a di-leucine motif has been shown to cause the ER

retention of multiple GPCRs (Duvernay et al, 2009; Juhl et al, 2012). Recent studies have also demonstrated the existence of motifs regulating the exit of GPCRs from the Golgi; these are present in both the C- and N-terminal regions (Dong & Wu, 2006; Dong et al, 2010; Wu, 2012). In addition to these motifs, protein folding, glycosylation and interacting proteins mediate trafficking to the plasma membrane (Deslauriers et al, 1999; Dong et al, 2007; Kuang et al, 2012). Thus, the deletion of the CTD of GPR50, and its interaction with TIP60, may disrupt the regulation of protein trafficking, resulting in the intracellular localisation of GPR50 described above (Li et al, 2011; Slominski et al, 2003).

1.3.4 The distribution of GPR50

In the brain, GPR50 is particularly enriched in the hypothalamus. This was initially shown by *in situ* hybridisation studies in a range of species; namely mice, rats, sheep and humans (Drew et al, 2001; Drew et al, 1998; Sidibe et al, 2010). In these studies the expression of GPR50 was largely restricted to the dorsomedial hypothalamic nucleus (DMH) and the ependymal layer of the third ventricle. These regions have prominent roles in energy metabolism, the endocrine system and the circadian rhythm (Chao et al, 2011; Chou et al, 2003; Evans et al, 2004). The enrichment of GPR50 here indicates the receptor may be involved in these processes. GPR50 is also found in the pituitary and adrenal glands (Drew et al, 2001; Drew et al, 1998; Hamouda et al, 2007); this may denote an involvement in the hypothalamic-pituitary-adrenal (HPA) axis, a major part of the neuroendocrine system. This region of the brain is known to regulate processes such as metabolism, immunity and neurogenesis (Pariante & Lightman, 2008; Pasquali et al, 2006). The abundance of GPR50 in these areas may point towards a neuroendocrine function.

The development of more specific antibodies has meant the localisation of GPR50 could be ascertained with greater precision. This has been exploited with

immunohistochemistry to provide confirmation that GPR50 is found in the third ventricle and the DMH (Batailler et al, 2012; Grünewald et al, 2012; Sidibe et al, 2010). These studies have also identified novel sites of GPR50 expression in the brain, such as the hippocampus, arcuate nucleus (ARC), lateral hypothalamus (LH), cortex and the brainstem. The diverse distribution of GPR50 expands the number of putative functions to consider. For example, in the brainstem, GPR50 was found in noradrenergic, dopaminergic, and serotonergic neurons, suggesting an involvement in neurotransmission (Grünewald et al, 2012). In addition, the ARC and LH accommodates some of the major nutrient sensing neurons (Blouet & Schwartz, 2010), thus GPR50 may play a role in nutrient sensing and energy expenditure.

Aside from the brain, mammalian GPR50 is widely distributed, e.g. in the lung, testes, heart, kidneys and ovaries (Drew et al, 2001). This is in contrast to the peripheral distribution of Mel1c in fish, which is largely restricted to the skin (Sauzet et al, 2008). This may reflect the functional divergence of the two proteins. However, there are some similarities in the distribution of Mel1c and mammalian GPR50; they are both expressed in the retina, optic nerve and optic chiasm (Drew et al, 2001; Reppert et al, 1995). The preserved expression pattern here may indicate that the function of Mel1c and GPR50 crossover in these regions. Therefore, this observation may outline an involvement of GPR50 in light sensing and the governance of the circadian rhythm, as true for Mel1c and other members of the melatonin receptor family (Dubocovich et al, 2005; Park et al, 2007; Wiechmann et al, 2009).

1.3.5 GPR50 signal transduction

There is little known about the signalling pathways regulated by GPR50. However, interactions with $G\alpha_i$, indicate GPR50 has the capacity to initiate a signalling cascade (Levoye et al, 2006). This was recently replicated and extended to reveal that

GPR50 initiated signal transduction via $G\alpha_{i1}$ to inhibit the forskolin-stimulated rise in cAMP (Hand, 2011), see figure 1.1 and 1.2. Forskolin-dependent phosphorylation of the cAMP response element binding protein (CREB) was also inhibited by GPR50 transfection. In these experiments the addition of a ligand was not necessary to initiate signalling, suggesting GPR50 is constitutively active. However, GPR50 was overexpressed in these studies; this is known to shift the GPCR equilibrium towards an active state despite the absence of a ligand (Ango et al, 2001). Further, it cannot be ruled out that a ligand was present in the culture media and the increase in GPR50 provided additional binding sites, and therefore, resulted in the increase in signalling.

1.3.6 GPR50 functions

The purpose of this PhD was to develop our understanding of GPR50's function, which is relatively unclear. However, research currently available does provide strong clues as to what this might be. This is discussed below.

1.3.6.1 Modulating the activity of interacting proteins

As discussed in section 1.2.3, GPCRs can have ligand independent functions through binding to other proteins and modifying their activity. GPR50 appears to bear this ability. Through interacting with GPR50, the high affinity of MT_1 for melatonin is diminished (Levoye et al, 2006). Further, heterodimerisation prevented the coupling of $G\alpha_i$ and β -arrestin to MT_1 , thus MT_1 signalling is diminished. Interestingly, when the CTD of GPR50 was deleted the MT_1 -GPR50 heterodimer formed but the inhibitory effects were ablated. Thus the inhibition may be attributed to steric hindrance from the CTD. This work was carried out *in vitro*, using the overexpressed proteins; therefore, the physiological relevance may be questioned. However, there are similarities between the distribution of GPR50 and the MT_1 , especially in the cortex, hippocampus, DMH and retina (Jockers et al, 2008; Wu et al, 2006). This means the two proteins may interact *in vivo*. This is strengthened by

the observation that Mel1a and Mel1c, orthologues of MT₁ and GPR50 respectively, colocalise in the corneal epithelium of *Xenopus laevis* during the day, suggesting heterodimerisation may be a mechanism to modulate receptor sensitivity/activity during the active period of the day (Wiechmann et al, 2009). However, the lack of colocalisation in other areas of the brain indicates that the modulation of MT₁ signalling cannot be the only action of GPR50.

In contrast, the interaction between GPR50 and the transcriptional co-activator TIP60 was reported to potentiate TIP60 activity (Li et al, 2011). Co-transfection with GPR50 significantly heightened the ability of TIP60 to increase glucocorticoid receptor (GR) activity. Interestingly, GPR50 increased GR activity when transfected alone. However, this increase was markedly lower than that observed in cells transfected with TIP60 alone or co-transfected with TIP60 and GPR50. Further, the transfection of GPR50 alone did not affect the expression of *Fkbp5*, a GR-regulated gene. Whilst in co-transfected cells, GPR50 enhanced the TIP60-dependent increase in this gene. This suggests that GPR50 acts via TIP60 to modulate GR activity, and is largely unable to do this independently. This study also showed the transfection of the CTD alone was sufficient to enhance TIP60 activity. Conversely, the truncated form of GPR50, devoid of the CTD and unable to bind to TIP60, did not elicit any effect on GR signalling. This emphasises the importance of the CTD and indicates the interaction between the two proteins was vital for the observed results.

1.3.6.2 Neurodevelopment

The extensive expression of GPR50 within the brain suggests that it may be important for the development or maintenance of the central nervous system (CNS). Recent papers have shed some light on what this role may be. In Neuroscreen-1 cells, a neuron-like cell line, the overexpression of GPR50 increased neurite outgrowth by 40% (Grünewald et al, 2009). In the mouse brain, GPR50 was found to colocalise with the neuronal marker NeuN (Grünewald et al, 2012), thus the effect on neurite outgrowth may also occur *in vivo*. A developmental profile of *Gpr50* in

the mouse brain was also carried out. This showed *Gpr50* expression dramatically increased at embryonic day 18 (E18), particularly in the hypothalamus. This observation is very intriguing as this stage of development is characterised by intensive dendritic and axonal outgrowth and branching (Polleux & Snider, 2010). The increase in *Gpr50*, therefore, may promote the outgrowth and development associated with E18. At week 5, *Gpr50* increased again (Grünewald et al, 2012), suggesting it is also required at later developmental time points.

The role of actin dynamics in neuronal maturation

Actin plays a vital role in neuronal morphogenesis and maturation. In the cell, actin is found in two states: monomeric globular (G)-actin and polymeric filamentous (F)-actin, a double stranded helical filament consisting of G-actin (Miller, 2002). When bound to ATP, G-actin is readily incorporated into existing F-actin, preferentially at the plus (growing) end of the filament, via weak non-covalent bonds (figure 1.5). Upon hydrolysis of ATP to ADP the actin polymer destabilises, which results in the dissociation of G-actin at the minus end. This is then free to bind to another ATP molecule and re-enter the cycle of actin polymerisation. Due to the highly dynamic nature of F-actin, it is essential for various cellular processes involved in motility. This includes cell migration, cell spreading and growth cone extension (Heasman & Ridley, 2008; Sit & Manser, 2011). In neurons, F-actin is an essential component for the development and dynamics of dendritic spines, dendrites and axons. For example, drugs such as jasplakinolide, which interferes with actin dynamics, inhibit dendritic outgrowth (Tan et al, 2010). Likewise, latrunculin A, an inhibitor of actin polymerisation, attenuates spine development and plasticity (Ramachandran & Frey, 2009; Rex et al, 2007), whilst the promotion of polymerisation favours plasticity and increases spine density (Halpain et al, 1998; Huang et al, 2013).

The dynamic nature of actin organisation is regulated by an array of actin binding proteins (ABPs), some of which are shown in figure 1.5. Actin nucleators are an

important group of ABPs, which promote the polymerisation of actin filaments *de novo*. Formins and ARP2/3 are well studied examples of these; the former promotes nucleation and polymerisation of unbranched filaments and the latter favours branched filaments (Dos Remedios et al, 2003; Heasman & Ridley, 2008). Depolymerisation proteins, such as cofilin, promote the disassembly of actin filaments by altering the structure of ADP-bound actin (Dos Remedios et al, 2003). Other proteins bind to G-actin to regulate its incorporation or dissociation from F-actin. For instance, profilins binds to monomeric actin and promotes the exchange of ADP for ATP and enhances polymerisation at the plus end of F-actin (Wen et al, 2008). On the contrary, Thymosin β 4 sequesters G-actin and inhibits polymerisation (Dos Remedios et al, 2003). As can be expected, these regulators of actin dynamics are also critical in neuronal development. For instance, the loss of ARP2/3 led to a reduction in dendritic complexity, altered spine morphology and a progressive loss of synapses (Kim et al, 2013; Tan et al, 2010). Similarly, the silencing of profilin reduced dendritic length, complexity and spine density (Michaelson et al, 2010). Conversely, promoting actin depolymerisation by increasing the activity of cofilin resulted in the reduction of spine maturity (Pontrello et al, 2012; Shi et al, 2009).

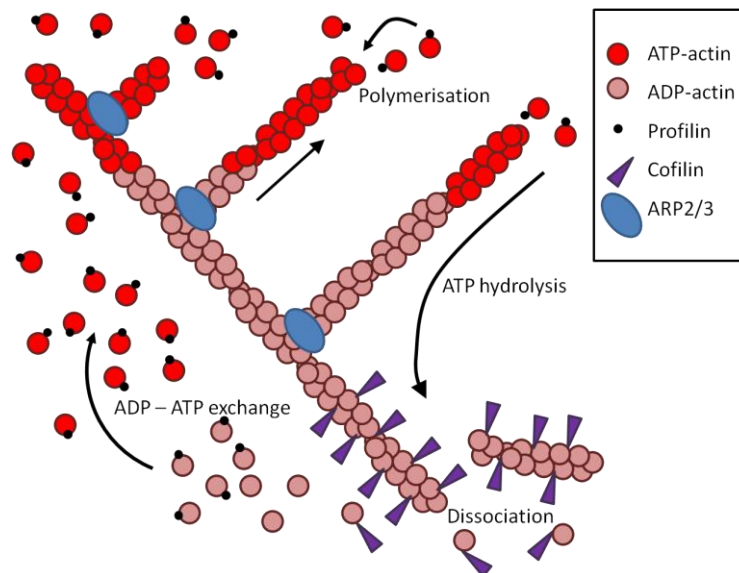


Figure 1.5: Actin dynamics at the lamellipodia. ATP-bound G-actin binds to the plus end of F-actin, resulting in F-actin elongation. Following ATP hydrolysis, ADP-bound actin can dissociate from the minus end of the actin filament. This process is supported by severing factors such as cofilin. Once in its monomeric form, profilin can bind to G-actin and promote the exchange of ADP for ATP, which can once again be incorporated into the growing actin filament. New filaments can be initiated by ARP2/3, which promote the nucleation of actin on the sides of existing filaments, resulting in a branched network of F-actin. Figure adapted from (Miller, 2002).

ABPs are modulated by various signalling pathways typically involving members of the Rho-family of GTPases, such as RAC1, CDC42 and RHOA. In response to various stimuli, Rho GTPases are activated by guanine nucleotide-exchange factors (GEFs), which promote the dissociation of GDP and the binding of GTP (Heasman & Ridley, 2008; Sit & Manser, 2011). GTPase activating proteins (GAPs), on the other hand, inactivate Rho GTPases by promoting the hydrolysis of GTP. When active, Rho GTPases will interact with a variety of downstream effectors to modulate the polymerisation of actin. RHOA, for example, directly binds and activates the formin mDia, promoting actin nucleation and polymerisation (Heasman & Ridley, 2008; Sit & Manser, 2011). Whereas RAC1 promotes the formation of actin branches by activating the WAVE complex, which in turn activates ARP2/3 (Heasman & Ridley, 2008; Sit & Manser, 2011). Given the role of Rho GTPases in actin polymerisation, it is not surprising that modulating these molecules has a profound effect on neuronal morphology. The suppression of RAC1 and CDC42

results in the elimination of dendritic spines and the reduction of dendritic complexity (Nakayama et al, 2000; Scott et al, 2003). Conversely, RHOA negatively regulates dendritic complexity (Nakayama et al, 2000). Thus, the modulation of actin dynamics, at different points within the regulatory pathway, is a crucial aspect of neuronal development/maturation.

If GPR50 regulates neuronal development and neurite outgrowth, as evidence suggests, it may act on modulators of actin polymerisation such as ABPs or Rho GTPases. If true, GPR50 could influence neurodevelopment in numerous ways, in addition to its promotion of neurite outgrowth (Grünewald et al, 2009). This may correlate with the receptors developmental profile (Grünewald et al, 2012). Thus, it would be of great interest to look at how GPR50 affects various features of neuronal morphology and development.

1.3.6.3 Energy metabolism

As described above, GPR50 is enriched in areas of the brain which play a major part in energy homeostasis and nutrient sensing. Furthermore, GPR50 propagates GR signalling (Li et al, 2011), which is also known to regulate metabolism (Kadmiel & Cidlowski, 2013). This indicates that GPR50 has a role in energy metabolism, which seems to be supported by experimental data. GPR50 expression in the DMH and ependymal layer of the third ventricle decreased in wild-type mice placed on a high energy diet and those fasted (Ivanova et al, 2008). A similar reduction in GPR50 was found in these areas of the brain in Siberian hamsters placed on short-day photoperiods (Barrett et al, 2006), periods associated with decreased food intake (Mercer et al, 2000). In addition, leptin-deficient mice, characterised by weight gain and increased food intake, also had significantly lower levels of GPR50 in the same hypothalamic regions (Bechtold et al, 2012). These results imply GPR50 is sensitive to changes in energy/food intake. It has been suggested that fatty acids regulate the reduction of GPR50 as these are increased in conditions of fasting and high energy diets (Frier et al, 2011). The expression of GPR50 in the nutrient sensing regions of

the brain, i.e. ARC and LH (Grünewald et al, 2012), is very much in keeping with this possibility.

The deletion of *Gpr50* in mice also indicates that GPR50 regulates energy metabolism. These mice displayed attenuated weight gain and were resistant to diet-induced obesity (Ivanova et al, 2008). They were also resistant to weight loss upon fasting, suggesting a general disruption to the metabolic response to food. Interestingly, these changes to body weight are very similar to the outcome of melatonin administration in mice (Hussein et al, 2007). The absence of GPR50 may alleviate the inhibition to MT₁ signalling (Levoye et al, 2006), resulting in an increased responsiveness to melatonin. In a recent study, GPR50 was shown to mediate torpor, a response to cope with harsh environments by minimising energy expenditure (Bechtold et al, 2012). *Gpr50* knockout mice readily entered a state of torpor upon fasting, as characterised by a pronounced decline in body temperature and the metabolic rate. The administration of leptin, a potent inhibitor of torpor, did not attenuate this, indicating GPR50 acts downstream of leptin to increase metabolic expenditure (Bechtold et al, 2012).

In addition, GPR50 appears to modulate lipid levels. Sequence variants have been found to associate with increased fasting triglyceride levels and a reduction in circulating high-density lipoprotein (HDL)-cholesterol levels (Bhattacharyya et al, 2006). This may signify that GPR50 specifically acts on lipid metabolism. This has recently been supported by work showing GPR50 is expressed in adipocytes (Hand, 2011), cells specialised for lipid storage (Cristancho & Lazar, 2011). Further, the overexpression of GPR50 in adipogenic 3T3-L1 cells disrupted lipid homeostasis, resulting in the accumulation of intracellular lipids (Hand, 2011). GPR50 silencing had the converse effect and resulted in the reduction of lipid storage. This coincides with the reduced body fat observed in *Gpr50*^{-/-} mice (Ivanova et al, 2008). Thus, the previously reported relationship between GPR50 and energy metabolism may be due to its ability to modulate lipid homeostasis.

1.3.6.4 Mental illness

The first indication that GPR50 may have a role in BD came about through linkage analysis which revealed the genomic region harbouring GPR50, Xq28, was a candidate locus (Hayden & Nurnberger, 2006; Massat et al, 2002). The identification of GPR50 as a potential contributor for this linkage came in the form of a four amino acid deletion, $\Delta 502-505$, that was significantly associated with BD in women, $P=0.00023$ (Thomson et al, 2005). This study also demonstrated that the association was not limited to BD, $\Delta 502-505$ also associated with major depressive disorder (MDD) in females, $P=0.0064$. Further, two single nucleotide polymorphisms (SNPs) within *GPR50*, rs13440581 and rs2072621, were found to associate with MDD ($P=0.0096$) and schizophrenia ($P=0.0014$) respectively, again in a female specific manner. Therefore, GPR50 is not only implicated in the development of BD but also related disorders, possibly reflecting a shared aetiology.

Replicating these key findings has not been straightforward. In a smaller Swedish cohort, the $\Delta 502-505$ association was not reproduced (Alaerts et al, 2006). Similarly, a Hungarian study found no association between GPR50 SNPs and childhood-onset mood disorder, which pools MDD and BD together (Feng et al, 2007). This includes rs561077, which is in complete linkage disequilibrium with $\Delta 502-505$. This lack of replication may be attributable to a number of factors, such as genetic heterogeneity, the lack of power or differences in phenotypic definitions. Alternatively, the primary study may have reported a false positive.

Recent studies, however, support the link between GPR50 and mental illness, although do not fully replicate the initial study. An independent Scottish cohort found a female specific association between rs1202874 and BD ($P=0.0035$), but not with $\Delta 502-505$ (MacIntyre et al, 2010). Interestingly, $\Delta 502-505$ did associate with indices of severity in early onset MDD patients, e.g. age of onset, number of

episodes and hypomanic symptoms in women and a familial history in men. This suggests carriers of the deletion have a more severe form of MDD with a greater chance of developing BD later in life. More recently, rs2072621, the SNP linked to schizophrenia in the primary study (Thomson et al, 2005), was shown to be significantly associated with seasonal affective disorder (SAD) in women ($P=0.049$) (Delavest et al, 2012). However, an association with other GPR50 variants, including $\Delta 502-505$, were not detected in this study.

There are clear inconsistencies between these reports, possibly because the causal variant has not been typed in these studies or the result of the small sample sizes used. Despite this, these results seem to suggest GPR50 is a susceptibility gene for an array of psychiatric disorders. Understanding the function of GPR50 will provide insight into the disrupted pathways contributing to such disorders. Better treatment may in turn come from this knowledge.

1.4 Investigating the functions of GPR50

A number of methods can be used to deduce the functions of a GPCR; these usually revolve around G-protein activation. A commonly used assay involves measuring cAMP, the secondary messenger associated with $G\alpha_s$ and $G\alpha_{i/o}$ activation (figure 1.1). Other secondary messengers routinely assessed are inositol phosphate and Ca^{2+} , the functional screens for $G\alpha_q$ and $G\alpha_{i/o}$ -coupled receptors. The guanine nucleotide binding assay is also widely used. This records the binding of a labelled GTP analogue to the $G\alpha$ subunit, which provides a measure of its activity level. Knowledge of the G-protein that associates with the receptor is not necessary for this method, which makes it especially useful when the signalling pathway is unknown. However, the exposure to a ligand is often required for these assays. This means they are of limited use in the study of orphan GPCRs.

Attempts have been made to deorphanise GPR50 using the methods described above, but with little success. Recently, however, the biopharmaceutical company Omeros announced that they have identified compounds that selectively bind to GPR50 in a large scale deorphanisation program (Omeros-Corporation, 2012). They have yet to release any data on this, thus, for all intents and purposes, the ligand remains unknown. Despite this setback, it has been established that GPR50 is capable of inducing a signalling cascade via $G\alpha_{i1}$ (Hand, 2011). Further, the overexpression of GPR50 in various cell lines has an observable consequence (Grünewald et al, 2009; Hand, 2011; Levoye et al, 2006; Li et al, 2011), presumably through the signalling cascade induced by overexpression. Thus, the discovery of a ligand does not preclude the study of GPR50's functions; overexpression and knockdown strategies are likely to be an effective tool to investigate the actions of GPR50.

Determining the G-proteins that interact with GPR50 is crucial to identify the exact signalling cascades initiated. However, this provides limited information in regards to the cellular events that occur further downstream and the ultimate cellular response. For example, $G\alpha_i$ inhibits cAMP production which is required for PKA activation. This regulates a diverse range of cellular responses including proliferation, glucogenesis and cell survival (Kim et al, 2008; Li et al, 2000; Liu et al, 2004). Alone, knowledge that GPR50 acts through $G\alpha_i$ does not tell us what the activation of GPR50 leads to. This depends on factors such as the ligand, tissue and cell type. In addition, GIPs play an important role here. Thus, knowledge of GPR50-interacting proteins may provide much needed illumination on the function and activity of GPR50, if much is known about the associated GIP. With this in mind, a yeast-2-hybrid (Y2H) screen was performed prior to the start of this PhD (Grünewald et al, 2009).

1.4.1 Y2H screen

The Y2H screen is an assay used to identify protein interactions. It involves fusing the DNA-binding domain of a transcription factor onto a protein of interest; this is known as the bait. The prey is generated from a cDNA library of potential interactors expressed as a fusion product with the activation domain of the same transcription factor. These vectors are transformed into yeast, where the reaction occurs. Interactions between the prey and bait proteins place the DNA-binding domain and the activation domain in close proximity, which reconstitutes the transcription factor allowing it to activate the transcription of a reporter gene. The CTD of GPR50, amino acids residues 292-617, was used as the bait given the apparent functional importance of this domain. The screen was also performed with the $\Delta 502-505$ deletion variant; again this was limited to the CTD. Both constructs were screened against an adult human brain cDNA library by Hybrigenics (Grünewald et al, 2009).

Over 23 interactors were identified in the GPR50 Y2H screen (results shown in table 1) (Grünewald et al, 2009). Each interactor was assigned a predicted biological score (PBS) reflecting the likelihood of the interaction being specific. This is based on technical parameters such as the number of independent prey fragments found for a given interaction and the frequency of a prey protein appearing in independent screens using the same cDNA library (Blandin et al, 2013; Formstecher et al, 2005). A PBS of A-C are assigned to the more technically reliable associations and considered to be probable true positives, whereas D represents interactions detected from a single experimental clone, thus is less reliable. PBS E flags up prey with interacting domains that have frequently appeared in independent screens with unrelated bait proteins, therefore, may represent false positives. To decipher how these results reflect the function of GPR50, gene ontology (GO) analysis was performed. This revealed that two biological processes were significantly enriched

in the Y2H screen – neuronal differentiation/development ($P=0.004$) and sterol/cholesterol metabolism ($P=0.001$).

Global PBS	Gene symbol	Isoform	Gene name
A	RTN4 (NOGO-A)	Variant 5	Reticulon 4
C	CDH8	Type 2	Cadherin 8
C	SFRS18		Splicing factor, arginine/serine-rich 18
D	SHOT1		Shootin-1
D	RTN3	Variant 2	Reticulon 3
D	ABCA2	Variant 2	ATP-binding cassette, sub-family A, member 2
D	LRRC37A3		Leucine-rich repeat containing 37 member A3
D	OSBP2	Variant 1	Oxysterol binding protein 2
D	RTN4 (NOGO-C)	Variant 3	Reticulon 4
D	PSMA1	Variant 1	Proteasome (prosome, macropain) subunit, alpha type, 1
D	MADD	Variant 4	MAP-kinase activating death domain
D	PCDH9	Variant 2	Protocadherin 9
D	SATB1		Special AT-rich sequence binding protein 1
D	SLC12A5		Solute carrier family 12 (potassium-chloride transporter) member 5
D	SLC4A10		Solute carrier family 4 (sodium bicarbonate transporter-like) member 10
D	SNX6	Variant 2	Sorting nexin 6
E	SREBF2		Sterol regulatory element binding transcription factor 2
E	SNX5	Variant 2	Sorting nexin 5
E	DDX24		DEAD (Asp-Glu-Ala-Asp) box polypeptide 24
E	ST13		Suppression of tumorigenicity 13
E	PAX6	Variant 2	Paired box gene 6
E	SPTAN1		Spectrin, alpha, non-erythrocytic 1
E	CNST		Consortin
E	PICK1	Variant 1	Protein interacting with PRKCA 1

Table 1.1: Results from the Y2H study with the CTD of GPR50 screened against a human brain cDNA library. The gene symbol, variant and full gene name are presented along with the predicted biological scores (PBS) generated for the given interaction (Grünewald et al, 2009).

Alone, Y2H screens provide insufficient evidence to prove an interaction. This is due to a number of important limitations (Brueckner et al, 2009; Van Crielinge & Beyaert, 1999). For example, the screen takes place in yeast, a system in which proteins may not be expressed, folded or modified correctly, which may have a marked effect on interactions. Further, fusion proteins must be targeted to the nucleus to allow the transcription of the reporter gene. Proteins shown to interact in a Y2H screen may normally be found in different subcellular compartments in mammalian cells, thus would not be able to interact. Alternatively, the nuclear targeting of proteins that do interact may be unsuccessful; as a result, the reporter gene will not be transcribed despite the presence of a genuine association. Hence, Y2H screens are prone to false positive and false negative results. Therefore, it is essential to follow up Y2H results with more robust assays like co-immunoprecipitation (co-IP) in conjunction with immunocytochemistry (ICC), focusing on the most compelling associations.

The interaction between the neurite outgrowth inhibitor NOGO-A, the highest scoring protein in the screen (table 1), and GPR50 has been confirmed using co-IP and ICC (Grünewald et al, 2009). Upon investigating the outcome of this interaction it was discovered that GPR50 attenuated the inhibitory actions of NOGO-A. Moreover, GPR50 itself positively modulated neurite outgrowth in Neuroscreen-1 cells. In addition, the interactions with CDH8 and ABCA2 were tested; GFP-tagged GPR50 was found to co-immunoprecipitate with GFP-CDH8 and untagged ABCA2 (Grünewald et al, 2012). However, these results should be treated with caution as GFP-GPR50 took up a more internal localisation (Grünewald, 2011). Moreover, if both proteins have GFP tags, as was the case for GPR50 and CDH8, there is a risk of GFP dimerisation, resulting in a false positive result. Co-IP with the untagged constructs was attempted, however, due to technical issues results were inconclusive (Grünewald, 2011).

An independent group also used the Y2H method to elucidate the functions of GPR50 (Li et al, 2011). This group identified TIP60 as a potential GPR50-interactor, which they confirmed with a co-IP assay. Through the interaction with TIP60, a novel function for GPR50 in GR signalling was unveiled, as described earlier. Interestingly, only one common interactor was found in the two screens, from a total of 34 putative interactors (23 identified in Grünewald's paper, 2009, and 11 by Li et al, 2011). This was sorting nexin 5 (SNX5), a protein involved in intracellular trafficking (Wassmer et al, 2007). However, in the Y2H screen presented by Grünewald et al the GPR50-SNX5 interaction was assigned a PBS of E, thus, there is a strong possibility that this interaction is non-specific. The lack of crossover in the two papers may be due to the different libraries used, one utilised a human brain cDNA library (Grünewald et al, 2009), while the other used a mouse testes library (Li et al, 2011). These libraries would be expected to consist of a different subset of genes expressed at different levels, and therefore, likely to produce different results.

Whilst different proteins were identified in the two Y2H screens, both studies highlight how useful the discovery of GPR50-interacting proteins can be. They led to the identification of previously unknown roles of GPR50 in neurite outgrowth and GR signalling. For this reason, this PhD focused on, and attempted to extend, the results generated in the Y2H study performed by this group (Grünewald et al, 2009) by exploring the role of GPR50 in neurodevelopment and lipid metabolism. In this section the Y2H results pertinent to these processes will be discussed in more detail.

1.4.1.1 Neuronal differentiation/development

The proteins involved in neuronal development/differentiation enriched in the Y2H study included ABCA2, CDH8, PCDH9, PAX6, PICK1, RTN3, NOGO-A and SHOT1 (table 1). These have varied contributions to neurodevelopment including the development and maintenance of the myelin sheath, neuronal polarisation and synaptic transmission (Mack et al, 2007; Nakamura et al, 2011; Petrinovic et al,

2013; Toriyama et al, 2006). Of these, NOGO-A and CDH8 had the strongest likelihood to interact with GPR50, PBS of A and C respectively, making them the most compelling proteins to study. These interactions have now been confirmed with co-IP, although the GPR50-CDH8 interaction requires further experimentation (Grünewald, 2011; Grünewald et al, 2009). In addition to the PBS scores, the roles of NOGO-A and cadherins in neurodevelopment have been studied extensively. Thus, of all the interactors identified, it seems that these proteins are most likely to expand our current understanding of GPR50.

NOGO-A

In the Y2H screen the short form of NOGO-A (RTN4 variant 5) was found to bind to GPR50. NOGO-A is predominately expressed in the CNS, particularly in oligodendrocytes but also in neurons during development (Huber et al, 2002). There is also some peripheral distribution, although this appears to be limited to the heart and testes (Huber et al, 2002). On the plasma membrane, NOGO-A consists of two membrane spanning domains with two extracellular regions. The extracellular domains bind to receptors on adjacent neurons leading to neurite outgrowth inhibition, the prevention of cell spreading and growth cone collapse (Niederost et al, 2002; Oertle et al, 2003; Petrinovic et al, 2010). Rho GTPases seem to mediate these processes. Treating retinal ganglion cells with NOGO-A fragments activates RHOA and suppresses RAC1 (Niederost et al, 2002). Further, the inhibition of RHOA signalling abolished the inhibitory effects of NOGO-A. GPR50 may also alter RHO GTPase signalling pathways to attenuate the actions of NOGO-A, promoting neurite outgrowth by doing so.

More recently additional functions for NOGO-A have been noted, especially in regards to synaptic function. In neurons, both NOGO-A and its receptor localise to the synapse, at pre- and postsynaptic sites, and have a negative impact on neuronal maturity and dendritic complexity (Lee et al, 2008; Petrinovic et al, 2013). To add to

this, synaptic plasticity is negatively regulated by NOGO-A (Delekate et al, 2011; Petrinovic et al, 2013); this also appears to be true for its receptor (Lee et al, 2008). Further, increased expression of the Nogo receptor has been shown to significantly impair long-term memory in mice (Karlen et al, 2009). Thus, the inhibitory actions of NOGO-A are accompanied by physiological changes *in vivo*. As with neurite outgrowth, the association with GPR50 may dampen the inhibitory effect of NOGO-A on dendritic maturity and memory.

Cadherin 8

CDH8 is from a large family of transmembrane proteins which mediate cell-cell adhesions (Cavallaro & Dejana, 2011; Redies et al, 2012). In the CNS, CDH8 is found in the dorsal root ganglion, the dorsal horn, subthalamic nucleus and the cerebellum (Hertel et al, 2008; Korematsu et al, 1998; Suzuki et al, 2007). The function of this cadherin in the CNS has not been well defined, however, studies have demonstrated that CDH8 has a role in the migration of precerebellar neurons (Taniguchi et al, 2006). It appears to act in a similar way in facial branchiomotor neurons, where its expression is switched on at the final phase of migration (Garel et al, 2000). An interaction with CDH8 suggests GPR50 may regulate neuronal migration during brain development. However, this process occurs early in development; for example, the migratory pathway of the facial branchiomotor neuron terminates around E14 (Garel et al, 2000). *Gpr50* expression, on the other hand, is strongest at E18 and moderate in adolescence (Grünewald et al, 2012). At earlier ages, expression of *Gpr50* is low, thus it is hard to imagine that GPR50 has a significant contribution to neural migration.

Cadherins also act on the actin cytoskeleton through interactions with α/β -catenin (Desai et al, 2013). These are known to regulate actin remodelling, spine development and synaptic transmission (Abe et al, 2004; Desai et al, 2013; Drees et al, 2005; Murase et al, 2002). Neural cadherin (NCAD), the classical cadherin

typically studied in relation to neuronal development, promotes spine development and maturity and is also involved in synaptic transmission (Juengling et al, 2006; Mendez et al, 2010). In addition, NCAD has been shown to mediate the stability and maintenance of activity-dependent dendritic outgrowth and branching (Tan et al, 2010). Importantly, this appears to be reliant on NCAD mediated neuron-neuron interactions and its association with actin filaments via catenins. Further, disruption to actin dynamics completely abolished the dendrite-promoting effect of NCAD. As dendritic outgrowth, branching and synapse formation typically occur later in development, from approximately E17 in rodents (Polleux & Snider, 2010), an involvement of GPR50 here may be more likely.

1.4.1.2 Sterol/cholesterol metabolism

The cholesterol metabolism group of interactors included ABCA2, OSBP2 and SREBF2. These proteins were assigned a low PBS (ABCA2, D; OSBP2, D; SREBF2, E; table 1); however, they should not immediately be discounted. Biologically relevant interactions have been found within low confidence level groups (Formstecher et al, 2005). Further, their involvement in lipid metabolism makes them compelling proteins to study in light of the possible role GPR50 plays here (Bhattacharyya et al, 2006; Davis et al, 2004; Goldstein & Brown, 1997; Ivanova et al, 2008; Wang et al, 2002). Importantly, ABCA2 and SREBF2 have both been linked to mental health (Le Hellard et al, 2010; Wollmer et al, 2006). An interaction with these proteins may provide the link between GPR50 and its association with psychiatric illness and abnormal lipid levels, thus focus was placed on these two proteins.

ABCA2

ABCA2 is the largest member of the ATP-binding cassette transporter family (Vulevic et al, 2001a). The protein is predominately found in the brain, especially in oligodendrocytes (Broccardo et al, 2006b; Vulevic et al, 2001b; Zhou et al, 2001). It

is also notably expressed in the dentate gyrus and the subventricular zone of the lateral ventricle (Broccardo et al, 2006b), sites of adult neurogenesis. On the cellular level, ABCA2 shows partial overlap with lysosomal markers, suggesting it resides in lysosome-related organelles (Broccardo et al, 2006b; Vulevic et al, 2001b).

ABCA2 appears to be involved in the trafficking of sterol-related compounds. In CHO cells, overexpression led to the accumulation of cholesterol in lysosomes rather than the delivery to the ER (Davis et al, 2004) and therefore, prevents the subsequent storage in lipid droplets or packaging in lipoproteins (Guo et al, 2009).

Overexpression also altered the expression of genes involved in sterol biosynthesis (Davis, 2011; Davis et al, 2004). In mice, the deletion of *Abca2* resulted in abnormal myelin structure in the spinal cord and cerebrum (Mack et al, 2007). These mice also displayed a marked reduction in body weight and hyperactivity, similar to the phenotype observed in *Gpr50* knockout mice (Ivanova et al, 2008). In an independent study abnormalities in myelin structure were not detected, however, differences in the levels of sphingolipids were observed (Sakai et al, 2007). This is of note as sphingolipids, along with cholesterol, are major components of myelin (Obrien & Sampson, 1965). An interaction with ABCA2 may reflect the involvement of GPR50 in the regulation of these lipids, which could have an impact on myelination and neuronal transmission.

From a neurodevelopmental perspective an interaction between GPR50 and ABCA2 is interesting. A synonymous SNP in exon 14 has shown a strong association with early onset Alzheimer's disease (AD) (Macé et al, 2005; Wollmer et al, 2006). Interestingly, this SNP was also associated with cholesterol levels in the cerebrospinal fluid (Wollmer et al, 2006). The link between ABCA2 and AD has also been demonstrated on a molecular level. The overexpression of ABCA2 resulted in increased levels of intracellular APP and secreted A β , key determinants of AD (Chen et al, 2004). Intriguingly, there has been mounting evidence implicating lipids, especially cholesterol, to AD (Di Paolo & Kim, 2011). Thus the association

between ABCA2 and AD may be due to the ability of ABCA2 to modulate cholesterol homeostasis. These results are of high interest in the context of GPR50, which has been linked to cholesterol levels (Bhattacharyya et al, 2006). Further, an increase in GPR50-immunoreactive cells undergoing degeneration has been observed in AD patients (Hamouda et al, 2007), possibly underlining a role of GPR50 in neurodegeneration. An interaction with ABCA2, either physical or functional, may provide some explanation for these observations.

SREBF2

SREBF2 is a ubiquitously expressed transcription factor involved in the regulation of cholesterol levels (Goldstein & Brown, 1997). In its inactive form the protein resides in the ER where it is bound to SREBF2 cleavage activating protein, Scap. Under low intracellular cholesterol levels, Scap undergoes a conformational change which ultimately leads to the cleavage of SREBF2. This releases the functionally active N-terminal domain, which is translocated into the nucleus as a homodimer where it binds to sterol regulatory elements (SRE) to activate genes involved in cholesterol biosynthesis and uptake, e.g. 3-hydroxy-3-methylglutaryl-CoA reductase and the low-density lipoprotein receptor (Horton et al, 1998). If GPR50 binds to SREBF2 and alters its transcriptional activity, the modulation of cholesterol homeostasis would be expected.

Much of the work on SREBF2 has been carried out in liver derived cells, thus, little is known about its effects in the CNS specifically. However, it is particularly enriched in the hippocampus, especially in pyramidal neurons of the CA1 and CA3 regions (Kim & Ong, 2009). It is also highly expressed in neurons of the cerebral cortex and other regions of the brain at lower levels. This indicates that SREBF2 is likely to have a role in the cholesterol homeostasis of neurons. As cholesterol plays an essential role in neuronal processes such as synapse formation and function, myelination and axonal growth (Dietschy, 2009), SREBF2 may contribute to these

process. This may explain why SREBF2 has been implicated in mental health. Polymorphisms in SREBF2 and SREBF1, a member of the same family, were significantly associated with schizophrenia in two cohorts (Le Hellard et al, 2010). This is strengthened by *in vitro* work demonstrating SREBF2, and SREBF2-regulated genes, are upregulated and activated in response to antipsychotics and antidepressants (Ferno et al, 2005; Polymeropoulos et al, 2009; Raeder et al, 2006). This indicates that SREBF2 may contribute to the efficacy of drug treatment, possibly through the restoration of cholesterol homeostasis. Thus, validating the Y2H results may link GPR50 to drug efficacy.

1.4.1.3 What does the Y2H screen tell us about GPR50?

An interaction between GPR50 and any of these proteins could reveal novel functions of GPR50. The NOGO-A interaction, has already led to the knowledge that GPR50 enhances neurite outgrowth. This may extend to the promotion of dendritic complexity and synaptic transmission. Further, an interaction with CDH8 may reflect a role in actin remodelling or neuronal migration. An interaction with SREBF2 or ABCA2 could highlight a role of GPR50 in cholesterol homeostasis. In addition, this would provide mechanistic information into how GPR50 is involved in cholesterol metabolism, i.e. by modulating the transcriptional activity of SREBF2 or altering cholesterol trafficking by ABCA2. Thus, as a direct consequence of the Y2H screen several possible pathways that GPR50 may act on have been unveiled. With all the information that could be gleaned from these interactions, it is of high interest to validate the Y2H study.

1.5 Conclusion

From the data presented here, potential roles for GPR50 clearly emerge in neurodevelopment and lipid metabolism. However, there is a whole host of questions that must be addressed. In regards to neurodevelopment, the main question

is what is its contribution? Given the effects of GPR50 on neurite outgrowth and its peak expression at E18, GPR50 may promote dendritic and axonal outgrowth and branching. The interaction with NOGO-A and CDH8 also indicate GPR50 may act on synaptic development and actin organisation; further, this function may be governed by Rho GTPases. The involvement of GPR50 in metabolism is fairly evident; however, knowledge is far from complete. Does GPR50 directly sense energy/nutrient levels and relay this information to the appropriate effector? Or is it fed this information to modulate the required metabolic response? Current data depicts GPR50 as an energy sensor; however, it also seems to modify the metabolic rate, especially in conditions of fasting. Thus, its exact role is unclear. In addition, in what aspect of metabolism is GPR50 involved and how is this governed? Evidence from association studies and mouse work points toward lipid metabolism, which is supported by the putative interaction with ABCA2 and SREBF2, but this evidence is largely circumstantial.

Considering all that has been presented in this chapter, it is clear that our knowledge surrounding GPR50 has vastly improved since its initial identification. However, there are still large gaps in this understanding. The goal of this thesis is to fill in some of these gaps.

1.5.1 Aim

The aim of this PhD is to extend our understanding of the function of GPR50. The key aspects this thesis will focus on are neurodevelopment and lipid metabolism by addressing the following:

1. The role of GPR50 in neurodevelopment
 - a. Determining whether GPR50 affects neuronal maturation, namely dendritic complexity and spine formation (chapter 3).

- b. Elucidating a mechanism by which GPR50 acts on neurodevelopment, focusing on cytoskeletal organisation (chapter 4).
- 2. Involvement of GPR50 in lipid metabolism
 - a. Verify results from the Y2H screen in regards to ABCA2 and SREBF2 (chapter 5).
 - b. Determining whether GPR50 acts on lipid homeostasis, focusing on the relationship between GPR50 expression and lipid levels (chapter 6).

Addressing these points will provide a more rounded insight into the role of GPR50, which can be extended to understand why the gene is associated with mental illness and abnormal lipid levels. Potentially, this can be exploited to develop novel therapeutic interventions in the future.

Chapter 2: Materials and methods

2.1 Buffers and solutions

This section details the buffers and solutions routinely made up to perform the experiments described in this thesis.

2.1.1 Bacterial culture

Name	Protocol
L-Agar	50 g Tryptone (Sigma) 25 g Yeast extract (Sigma) 50 g NaCl (Fisher) dH ₂ O up to 5 L, pH 7.2, Autoclaved & stored at 4°C
LB broth	50 g Tryptone (Sigma) 25 g Yeast extract (Sigma) 25 g NaCl (Fisher) dH ₂ O up to 5 L, pH 7.2, Autoclaved & stored at 4°C

Table 2.1: List of solutions required for bacterial work.

2.1.2 Cell culture

Name	Protocol
Borate buffer	50 mM Boric Acid (Sigma) in dH ₂ O, pH 8.5 with NaOH
Dissection buffer	500 ml Hank's Balanced Salt Solution, HBSS, with CaCl ₂ and MgCl ₂ 3.5 ml 1M HEPES 5 ml GlutaMax

Freezing media	10% dimethylsulfoxide, DMSO 90% FBS
Poly-D-lysine	5 mg Poly-D-lysine (Sigma) 50 ml dH ₂ O 50 ml Borate buffer

Table 2.2: Buffers and solutions required for cell culture work. Unless otherwise stated reagents were purchased from Invitrogen.

2.1.3 Molecular biology

Name	Protocol
Blocking Buffer	1x Tris buffered saline, TBS 0.1% Tween-20 (Sigma) 5% w/v powdered milk (Marvel)
Laemmli protein sample buffer (2x)	125 mM Tris-HCl, pH 6.8 20% Glycerol (Promega) 4% SDS (Fisher) 0.02% Bromophenol blue (Sigma) 100 mM Dithiothreitol, DTT (Sigma) Made up in dH ₂ O, stored at -20°C
Mowiol	7.5 g Mowiol (Sigma) 10 ml Glycerol (Promega) 25 ml dH ₂ O; this was incubated overnight at room temperature 50 ml 0.2 M Tris-HCl (Fisher); heated for 20 minutes at 100°C to dissolve 1.75 g DABCO (Sigma), stored at -20°C 50 µg/ml DAPI (Vector Laboratories) was added to working aliquots, stored at 4°C
Phosphate buffered saline (PBS)	From tablets (Invitrogen) containing: 10 mM Phosphate 150 mM NaCl Made up in dH ₂ O, pH 7.3-7.5

Ponceau S stain	1 g Ponceau S (Sigma) 4 ml Acetic acid (Fisher) dH ₂ O up to 200 ml
Primary antibody dilution buffer	1x TBS 0.1% Tween-20 (Sigma) 5% Bovine serum albumin, BSA (Sigma)
Radio-immunoprecipitation assay (RIPA) lysis buffer (stringent)	1x PBS (Sigma) 1% NP-40 (Sigma) 0.1% SDS (Fisher); omitted for a mild lysis buffer 0.5% Sodium deoxycholate (Fisher) Made up in dH ₂ O 1 protease inhibitor cocktail tablet (Roche) per 50 ml if this solution
TBS (10x)	500 mM Tris base (Fisher) 1.5 M NaCl (Fisher) Made up in dH ₂ O, pH 7.6 with HCL
Wash Buffer	1x TBS 0.1% Tween-20 (Sigma)

Table 2.3: Buffers and solutions required to perform western blotting and immunocytochemistry.

2.2 Expression constructs

Gene	Vector	Promoter	Resistance	Company/source
ABCA2	pcDNA3.1/Hygro [®] (Invitrogen)	CMV	Ampicillin	Prof. Kenneth Tew ¹
GFP	pmaxCloning	CMV	Kanamycin	Amara
GPR50	pDEST40	CMV	Ampicillin	Invitrogen
SREBF2	pCMV6-XL5	CMV	Ampicillin	Origene
Empty vector	pDEST40	CMV	Ampicillin	Invitrogen

Table 2.4: Expression constructs used in this thesis. ¹ Gift from Prof. Tew, Medical University of South Carolina (Davis et al, 2004).

For amplification of expression constructs, competent *E. coli* (One Shot Omnimax 2-Ti, Invitrogen) were transformed with 1 µl of the relevant plasmid by electroporation. Transformed cells were streaked onto agar plates with the appropriate antibiotic (100 µg/ml ampicillin or 50 µg/ml kanamycin) and grown overnight at 37°C. A single colony was picked and expanded in LB broth, with the appropriate antibiotic, in a shaking incubator at 37°C, 200 revolutions per minute (rpm). Plasmids were extracted from the expanded bacterial colony using a Qiagen miniprep or maxiprep kit, following the manufacturer's instructions. For the *ABCA2* construct, an endofree maxiprep kit was used (Qiagen). The concentration of the purified plasmid was determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific) and the plasmid was subsequently stored at -20°C. Aliquots from the bacterial colony were also reserved to make a glycerol stock (20% glycerol) for long-term storage at -80%.

2.3 Antibodies

2.3.1 Primary antibodies

Name/target	Company/source	Species	Application/dilution
ABCA2	Prof. Tew ¹	Rabbit	ICC 1:2000 WB 1:5000
ABCA2	Santa Cruz Biotechnology (SCBT)	Goat	ICC 1:1000 WB 1:1000
Total AKT	Sigma	Rabbit	WB 1:1000
Phospho-AKT (S473)	Cell Signaling	Rabbit	WB 1:5000
ARP2	Cell Signaling	Rabbit	WB 1:500
β-Actin	SCBT	Mouse	ICC 1:2000 WB 1:1000
Calreticulin	Abcam	Mouse	ICC 1:500
α-Catenin	Sigma	Rabbit	ICC 1:1000 WB 1:500

β-Catenin	Sigma	Mouse	ICC 1:1000 WB 1:500
GAPDH	Millipore	Mouse	WB 1:100,000
GPR50	Proteintech	Rabbit	ICC 1:1000 WB 1:5000
GPR50	SCBT	Goat	ICC 1:1000 WB 1:2000
Total GSK-3β	BD Biosciences	Mouse	WB 1:1000
Phospho-GSK-3β (S9)	Cell signaling	Rabbit	WB 1:1000
Phospho-GSK-3β (Y216)	Millipore	Mouse	WB 1:5000
LAMP1	Abcam	Mouse	ICC 1:50
N-WASP	Cell Signaling	Rabbit	WB 1:5000
Profilin-1	Cell Signaling	Rabbit	WB 1:2000
PSD-95	Affinity BioReagents	Mouse	ICC 1:300
SREBP2	SCBT	Goat	ICC 1:500 WB 1:1000
Total RAC1	Millipore	Mouse	ICC 1:500 WB 1:500
Phospho-RAC1/CDC42 (S71)	Cell Signaling	Rabbit	WB 1:500
β-Tubulin III	Covance	Mouse	ICC 1:2000 WB 1:1000
Vimentin	Abcam	Mouse	ICC 1:1000 WB 1:500
WAVE-2	Cell Signaling	Rabbit	WB 1:10,000

Table 2.5: Primary antibodies used in this thesis. Presented with the dilutions for the relevant application. ICC, immunocytochemistry; WB, western blotting. ¹Gift from Prof. Tew, Medical University of South Carolina (Davis et al, 2004).

2.3.2 Secondary antibodies

Name/target	Company/source	Application/dilution
Alexa Fluor 488 donkey anti-goat	Invitrogen	ICC 1:1000
Alexa Fluor 488 donkey anti-rabbit	Invitrogen	ICC 1:1000
Alexa Fluor 594 donkey anti-goat	Invitrogen	ICC 1:2000
Alexa Fluor 594 donkey anti-rabbit	Invitrogen	ICC 1:2000
Alexa Fluor 647 chicken anti-mouse	Invitrogen	ICC 1:1000
HRP goat anti-rabbit	Cell Signaling	WB 1:2000
HRP rabbit anti-goat	DAKO	WB 1:2000
HRP swine anti-rabbit	DAKO	WB 1:3000

Table 2.6: Secondary antibodies used in this thesis. Given with the dilutions for the relevant application. ICC, immunocytochemistry; WB, western blotting.

2.4 Cell culture

2.4.1 Maintenance of cell lines

2.4.1.1 Cell culture conditions

Cells were maintained at 37°C in a humidified environment of 5% CO₂ and 95% air, in a Galaxy 170S or Galaxy 170 incubator (Scientific Laboratory Supplies). As standard, cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). FBS and all other cell culture reagents used were purchase from Invitrogen, unless otherwise stated. These were pre-heated to 37°C in a sterilised water bath before use. All plasticware were purchased pre-sterilised and glassware were autoclaved prior to use. Cultures were assessed for mycoplasma infection on a monthly basis and were only used if mycoplasma-free. In addition, cell morphology was routine checked using a light

microscope (Leica) to ensure cellular health. All cell culture work was carried out in a sterile laminar flow hood.

2.4.1.2 Cell lines

HEK293, SH-SY5Y and N1E-115 cells were purchased from the American Type Culture Collection (ATCC). Cells were cultured in 75 cm² or 175 cm² flasks and passaged every 3-5 days, when confluency reached ~90%. To do this culture media was aspirated from flasks and cells were washed with tissue culture grade PBS. Cells were then detached by incubating with TrypLE Express for 5 mins at 37°C, then gently tapping the sides of the flask. This reaction was quenched by the addition of FBS-supplemented DMEM and cells were resuspended by pipetting several times. Finally, cells were added to a new flask containing supplemented DMEM at dilutions of 1:5 – 1:20, depending on the cell line and future use. For immunocytochemistry (ICC), SH-SY5Y cells were plated onto glass coverslips in a 12-well dish at a density of 7.5×10^4 cells per well; HEK293 and N1E-115 cells were seeded at 5×10^4 cells per well. When co-immunoprecipitation (co-IP) experiments were planned, cells were grown in 10 cm plates at a density of 1×10^6 cells per plate. For western blotting, cells were maintained in 6-well dishes at 5×10^5 cells per well.

2.4.1.3 Cell counting

Cells were counted using a haemocytometer. This was first prepared by cleaning with 70% ethanol and affixing a glass coverslip onto the counting chamber. As described above, cells were dissociated from culture flasks and resuspended in supplemented DMEM. The cell suspension was then diluted at 1:5, and slowly applied to the counting chamber via capillary action. This was then visualised under the light microscope and the number of cells in the four large corner squares of the counting chamber were counted. The cell concentration (cells per ml) was calculated using the following equation: *Average count per square $\times$ dilution factor $\times 10^4$ (conversion factor)*.

2.4.1.4 Handling cell stocks

For long term storage of cell lines, cells were detached from culture flasks as described above and precipitated in a 15 ml falcon tube by centrifugation at 500 rpm for 5 mins. The cell media was then removed and cells were washed twice with PBS. Cells were resuspended in freezing media and aliquoted into 1 ml cryovials at a concentration of $1-2 \times 10^6$ cells/ml. These were placed in a Mr Frosty freezing container (Thermo Scientific) filled with isopropanol and stored overnight at -70°C . Cells were then transferred to liquid nitrogen for long term storage. To use frozen stocks, cells were thawed at 37°C for 1-2 mins. Cells were then centrifuged at 500 rpm for 5 mins and freezing media was aspirated. Precipitated cells were washed twice with PBS, to ensure the removal of DMSO, and then resuspended in supplemented DMEM. This was added to culture flasks, at an appropriate dilution, and maintained as described above.

2.4.2 Preparation and maintenance of primary neurons

2.4.2.1 Preparation of plates for ICC

Glass coverslips were thoroughly sterilised by washing in 100% ethanol, followed by autoclaving. Once dry, coverslips were added to 12-well plates and incubated overnight in poly-D-lysine (Sigma) at 37°C with 5% CO_2 . Plates were then washed three times with dH_2O and ready for use. This, along with all work involving primary cultures, was carried out in the tissue culture hood designated for primary mouse work. Prepared plates could be stored for up to 1 month at 4°C prior to use.

2.4.2.2 Dissections

Primary neurons were prepared from embryonic day 17-18 (E17-18) C57BL/6 mouse embryos as previously described (Bradshaw et al, 2008). Mothers were killed under the schedule 1 procedure by trained staff at the animal facility. Embryos were obtained from the mother and killed by immersion in anaesthetic. The brains were removed and placed into ice cold dissection buffer. Using a Leica MZ6 microscope with a Fiber-Lite MI-150 High Intensity Illuminator, hippocampi and cortices were isolated and placed into fresh dissection buffer on ice; cortices and hippocampi were collected in separate tubes. Once the hippocampi and cortices were isolated from each embryo they were placed into 0.1% TrypLE Express (diluted in HBSS) and incubated in a water bath at 37°C for 45 mins. TrypLE Express was then removed using a Pasteur pipette and the cortices and hippocampi were resuspended with DMEM supplemented with 10% FBS. Neurons were dissociated by pipetting the suspension repeatedly, ~ 30 times, with a Pasteur pipette until a homogenous solution was obtained. The suspension was expelled against the side of the tube to minimise foaming. The hippocampal and cortical suspensions were then passed through a 40 µM cell strainer and then centrifuged at 1500 rpm for 5 mins. Supernatant was discarded and cells were resuspended in Neurobasal media supplemented with 2% B27 and 2 mM GlutaMAX. The dissociated neurons were plated onto the prepared 12-well plates, with 1ml supplemented Neurobasal media in each well, at a density of 2.5×10^5 cells per well. Cells were maintained at 37°C with 5% CO₂. Every seven days, half of the media was replaced with fresh Neurobasal media (with 2% B27 and 2 mM GlutaMAX). When transfection was performed within a week of plating, the media was not changed.

2.4.3 Transient transfections

Transfections were carried out using the Lipofectamine 2000 protocol. Briefly, DNA constructs and Lipofectamine 2000 were separately diluted in Opti-MEM and incubated at room temperature for 5 mins. The two solutions were then combined

and incubated for a further 20 minutes at room temperature. Meanwhile, cells were briefly washed and maintained in Opti-MEM. Table 2.7 shows the exact amounts of the transfection reagents required for the different culture dishes used. The DNA-Lipofectamine mix was then added to the cells and incubated for 4-6 hrs at 37°C, 5% CO₂. After this incubation period, media was changed back to DMEM with 10% FBS and returned to 37°C overnight. Western blot analysis or ICC could then be performed the following day. For mock transfections, this protocol was followed without the addition of DNA.

Component	10 cm	6-well	12-well
Expression construct	12 µg	2.5 µg	1 µg
Lipofectamine 2000	40 µl	5 µl	2 µl
Plating volume	10 ml	1 ml	500 µl
Dilution volume	1 ml	500 µl	100 µl

Table 2.7: Lipofectamine 2000 transfection. The amount of plasmid DNA, lipofectamine and Opti-MEM required for transfections in the different types of culture dishes used; values are given per well. When cells were dual transfected, the same amount of DNA was used for each construct, e.g. in a 12-well plate 1 µg of each construct was used.

For neurons the same protocol was followed with a few modifications. Neurobasal media, supplemented with 2 mM GlutaMAX alone, was used instead of Opti-MEM. Further, in a 12-well dish, 2 µg of each expression construct and 4 µl of Lipofectamine 2000 was used per well. Finally, cells were used for further analysis two days after transfection.

2.5 Cell treatments

2.5.1 U18666A treatment

HEK293 cells were used to examine the effects of U18666A on endogenous GPR50 levels. Cells were seeded in 6-well culture dishes as previously described (day 0). On day 1, or when cells reached approximately 70% confluency, the culture media was replaced with DMEM supplemented with 10% FBS and U18666A (Cayman) at 1, 2 or 5 µg/ml. U18666A was taken from a 20 mg/ml stock solution made up with the solvent ethanol. To ensure that the amount of ethanol was not responsible for any of the observed differences to GPR50 expression, the level of ethanol was maintained at 0.025% in all conditions, i.e. the percentage present at the highest U18666A concentration used. For the vehicle control, supplemented DMEM containing 0.025% ethanol was used. Treated cells were incubated at 37°C, with 5% CO₂, for 18 hrs and cell lysates were collected for western blotting the following day (day 2).

In the experiments assessing the effect of U18666A on GPR50 localisation, SH-SY5Y cells were seeded onto 12-well plates on day 0 and transfected on day 1. On day 2, cells were treated with 1 µg/ml U18666A for 5 mins, 30 mins, 1 hr, 4 hrs or 18 hrs, at 37°C with 5% CO₂. For the vehicle control, cells were maintained in supplemented media containing 0.005% ethanol, i.e. the percentage present in 1 µg/ml U18666A. Cells were fixed and prepared for ICC after the incubation period.

2.5.2 Serum deprivation

In experiments assessing the effects of serum deprivation on endogenous GPR50 levels, HEK293 cells were seeded onto 6-well culture dishes. On day 1, or when cells reached ~70% confluency, the culture media was removed and exchanged with DMEM containing different levels of FBS; 10%, 5%, 2.5%, 1.25%, 0.625% and 0%. This was incubated at 37°C, 5% CO₂ for 18 hrs, followed by the collection of cell lysate on day 2 and the subsequent western blot analysis.

In the ICC experiments looking at the effects of serum starvation on GPR50 localisation, SH-SY5Y cells were seeded onto 12-well dishes (day 0). On day 1, cells were transfected with GPR50 and the following day (day 2) media was removed from all wells and replaced with fresh DMEM supplemented with 10% FBS (control) or DMEM without FBS. Cells were incubated at 37°C, 5% CO₂, for 5 mins, 1 hr, 4 hrs or 18 hrs. Cells were fixed on day 2 or 3, depending on the incubation time, and ICC was performed.

2.5.3 Lipoprotein depletion

On day 0, HEK293 cells were seeded in 6-well dishes containing DMEM supplemented with 10% FBS. The following day (day 1) the culture media was replaced with DMEM containing with 3 mg/ml lipoprotein deficient serum (LPDS) (Millipore), equivalent to 10% FBS according to the manufacturer's instructions. Cells were treated for 18 hrs in the presence or absence of low density lipoprotein (Millipore), at 100 µg/ml or 250 µg/ml, at 37°C with 5% CO₂. After the incubation period (day 2) cell lysate was collected.

2.6 Protein analysis

2.6.1 Cell lysate preparation and quantification

Following the relevant transfection/treatment, cells were washed with ice cold PBS containing protease inhibitors (Roche) and the appropriate amount of RIPA lysis buffer was then added; 1 ml for a 10 cm dish or 250 µl per well for a 6-well plate. RIPA lysis buffer was thoroughly dispersed throughout the culture dish and cells were fully dissociated using a cell scraper. Cells were then collected into separate eppendorf tubes and incubated at 4°C for 30 minutes with gentle agitation on a rotary wheel (20-25 rpm). Lysates were then centrifuged at 13,000 rpm for 10 mins and the supernatant was collected. This was maintained on ice or at -20°C, after snap freezing on dry ice, until ready for use.

The protein concentration of each cell lysate was measured using the Bio-Rad protein concentration assay. Briefly, five dilutions of bovine serum albumin (BSA) were prepared to create a protein standard (0.2, 0.4, 0.6, 0.8 and 1 mg/ml). Cell lysates were also diluted at 1:10. To each of these preparations, 125 µl of Reagent A and 1 ml of Reagent B were added. This was mixed by inverting the eppendorf tube several times and then incubated at room temperature for 20 mins. The absorbances of these dilutions were then measured at 720 nm, using the Ultrospec 3000 Optical Reader (Pharmacia Biotech). Using Excel, a standard curve was generated and the equation of the line was used to calculate the protein concentration of the cell lysates.

2.6.2 Co-immunoprecipitation

Cell lysates were collected from HEK293 cells the day after transfection. Lysates (1 mg total protein) were incubated with 3 µg of the primary antibody, or IgG (Sigma) for the control, overnight at 4°C under constant agitation on a rotary wheel. G-sepharose beads (Sigma) were added to each lysate and incubated at 4°C for 1 hr under gentle agitation. Beads were spun down at 10,000 rpm, and washed three times with RIPA lysis buffer. After the final wash, the supernatant was discarded and beads were resuspended in 40 µl laemmli sample buffer. The samples were then incubated at 40°C for 10 mins and centrifuged at 10,000 rpm. The supernatant was removed and reserved for western blotting.

2.6.3 Western blotting

2.6.3.1 Sample preparation and SDS-PAGE electrophoresis

Cell lysates (10-15 µg total protein) were diluted in laemmli buffer (1:1) and boiled for 5 mins to denature proteins. This was then cooled on ice. When GPR50 was being detected, samples were incubated at 40°C for 10 mins to avoid protein aggregation. Immunoprecipitated samples, already containing laemmli buffer, were also heated in the same manner. Samples, along with a pre-stained Precision Plus protein standard (Bio-Rad), were loaded onto a NuPage SDS-PAGE gel (Invitrogen). This was run at 150V for 60-90 mins at 4°C using the XCell Surelock Mini cell (Invitrogen). As standard, a 7% Tris-Acetate gel was used with the corresponding 1x Tris-Acetate running buffer (Invitrogen). When the separation of larger proteins was required the 3-8% Tris-Acetate gel was used. For proteins with a low molecular weight the 4-12% Tris-Bis gel was used with the 1x MOPS running buffer (Invitrogen).

2.6.3.2 Western blot transfer

When electrophoresis was complete proteins were transferred from the gel onto a polyvinylidene difluoride (PVDF) membrane (GE healthcare) using the XCell II Blot Module (Invitrogen). Briefly, PVDF membranes were pre-wet in methanol then soaked in 1x transfer buffer (Invitrogen) for several minutes. Five blotting pads and two pieces of filter paper were also soaked in transfer buffer. The gel was placed on top of a filter paper and the PVDF membrane was then layered on top of the gel, this was followed by another pre-soaked piece of filter paper. Care was taken to remove any bubbles from this “sandwich” by gently rolling a falcon tube over it. This was then placed into the XCell II Blot Module (Invitrogen), between transfer buffer soaked blotting pads. The Blot Module was then loaded into the XCell Surelock Mini cell. The inner chamber of this was filled with 1x transfer buffer, whilst the outer compartment was cooled with ice cold water. This was run at 30V for 60-90 mins at 4°C. When finished, transfer was verified by staining the membrane with Ponceau S.

2.6.3.3 Immunostaining and detection

After Ponceau S staining, membranes were washed in distilled water and incubated with blocking buffer overnight at 4°C, or for 1 hr at room temperature, on a rocker/shaker at 25 rpm. After blocking, membranes were incubated with the diluted primary antibody overnight at 4°C on the rocker/shaker. In cases where a western blot was performed to verify that transfection was successful, or for co-IP experiments, the primary antibody was incubated for 1 hr at room temperature. Membranes were then washed in wash buffer (3 x 5 mins, on a rocker at 40 rpm). This was followed by incubation with the appropriate HRP-conjugated secondary antibody, diluted in blocking buffer, for 30 mins at room temperature with gentle agitation. Membranes were washed again, followed by visualisation with the ECL western blotting detection kit (Pierce), according to the manufacturer’s instructions. The membrane was then exposed to an X-ray film (Scientific Laboratory Supplies) in

a dark room, which was developed using the Curix 60 film processor (AGFA healthcare).

Where membranes were probed with multiple antibodies, membranes were washed with washing buffer (3x 5 mins) and then incubated in stripping buffer (Thermo Scientific) for up to 1 hr, at room temperature, on a rocker (25 rpm). The membrane was then washed and blocked in blocking buffer for 1 hr. Membranes could then be re-stained with the relevant antibodies. In experiments looking at the effects of GPR50 transfection on the expression of other proteins, membranes were first probed for GPR50 to confirm transfection and then reprobed for the proteins of interest, following this stripping procedure.

2.6.3.4 Semi-quantitative analysis

Western blots were scanned into the computer and the open source imaging software Fiji (<http://fiji.sc/Fiji> (Schindelin et al, 2012)) was used to measure the densities of the protein bands (Liang et al, 2013). The band corresponding to the target protein was selected using the rectangular selection tool and a profile plot was generated. The area under the curve for each plot was measured to give the band density. This was normalised against the density of the internal loading control, GAPDH, for the respective sample. These results were then presented relative to the control condition. For statistical analysis, experiments were repeated a minimum of three times and a paired Student's t-test was performed between the replicates.

2.6.4 Immunocytochemistry

Following the appropriate cell treatment, media was aspirated from each well and cells were washed in ice cold PBS. Unless stated otherwise, cells were fixed by

incubating with 4% paraformaldehyde (PFA, Sigma) at room temperature for 10 mins and then permeabilised with 0.2% triton (Sigma) for 10 mins. Alternatively, cells were fixed and permeabilised with ice cold methanol for 10 mins. Cells were then washed three times in PBS and blocked with 10% donkey serum (Sigma), diluted in PBS, for 30 mins on a rocker (25 rpm). The blocking solution was then removed and the appropriate antibody, diluted in 1% BSA (Calbiochem)/PBS, was added to the fixed cells for 1 hr at room temperature, or overnight at 4°C, on a rocker/shaker. After incubation, cells were washed three times in PBS and the secondary antibody, diluted in 10% donkey serum, was added. Secondary antibodies were centrifuged at 10,000 rpm for 10 mins before use and cells were incubated for 1 hr at room temperature, wrapped in foil, under gentle agitation. After a further three washes with PBS, coverslips were mounted onto glass slides using mowiol (Sigma) + DAPI (Vector Laboratories). Slides were stored at 4°C, in darkness, for at least 1 day before visualisation.

2.6.4.1 Nile red staining

Nile red, an intracellular lipid stain (Greenspan et al, 1985; Keembiyehetty et al, 2011; Martin et al, 2005), was used to visualise lipid droplets in ICC. Following the removal of the secondary antibody, cells were washed and then incubated with 1 µg/ml Nile red (Sigma) for 10 mins at room temperature on a rocker. Cells were then washed three times in PBS and mounted onto glass slides as described above.

2.6.4.2 Microscopy

Unless otherwise stated, the fluorescent images presented in this thesis were collected using the Zeiss LSM 510 confocal scanning laser microscope with a Plan Apochromat 63x objective (NA 1.40, oil immersion). For primary neurons, optical sections (Z-stacks) were taken with 0.5 µm intervals. The optical sections were then merged into a single 2D image using the “Z-projection” function of Fiji (Schindelin et al, 2012). Morphological analysis was performed using these projections. Other

fluorescent images were taken with the Zeiss Axioskop 2 fluorescent microscope with a PlanNeofluar 40x objective (NA 1.30, oil immersion); this was mainly used before confocal microscopy to ensure transfection and ICC was successful.

2.7 Image analysis

2.7.1 Measurements of fluorescence intensity

2.7.1.1 Total cellular fluorescence

For measurements of cellular fluorescence the imaging software ImageJ was used (Schneider et al, 2012). First the “Subtract Background” tool was used to remove uneven background illumination within an image. An outline of the cell of interest was then drawn manually using the β -tubulin signal as a guide and the fluorescence intensity within this region of interest (ROI) was measured for the desired protein. To correct for non-specific fluorescence, the average background fluorescence was subtracted from the cellular fluorescence; the background intensity was averaged from three regions within the same image with no apparent signal. The corrected cellular fluorescence was calculated using the following equation: *Corrected total cell fluorescence = integrated density – (area of selected cell x mean fluorescence of background)* (Komitova et al, 2013; Rahman et al, 2014).

2.7.1.2 Intensity profile plots

Intensity profile plots were generated to visualise the areas in which colocalisation could be found. To do this, a line was drawn across a selected region of the cell and the fluorescence intensity was quantified along the length of this line using Fiji (Schindelin et al, 2012). These values were then plotted against the position along

the line (μm) using Microsoft Excel. In this thesis, intensity profiles were produced to assess the distribution of GPR50 on the plasma membrane, thus an effort was made to ensure multiple structures were captured along the line drawn.

2.7.2 Colocalisation analysis

2.7.2.1 Colocalisation plots

Colocalisation was assessed using the Intensity Correlation Analysis (ICA) plugin in ImageJ (Liu et al, 2004). After subtraction of the background, β -tubulin was used to trace the cell of interest and the ICA plugin was launched. For a visual representation of colocalisation, intensity scatter plots and ICA plots were generated. In the scatter plot, the signal intensity of each pixel in one channel is plotted against the intensity of the same pixel in another channel, within the selected ROI; pixels being the discrete points that make up an image. A positive correlation denotes the dependency of the two signals. The ICA plot shows the product of the differences from the mean (PDM) for each pixel in a given channel. If staining is dependent the pixel intensities will vary together around their respective means, i.e. if a pixel intensity is above average in the red channel it would also be above average in the green channel. Therefore the PDM would be positive; this is seen as a positive skew on the ICA plot. When two proteins are found in different subcellular compartments the ICA plot will have a negative skew; the pixel intensity may be above average in one channel but below average in the other, resulting in a negative PDM. Where there is no obvious skew, signal co-variation is random.

2.7.2.2 Colocalisation coefficients

The ICA plugin was also used to generate a number of colocalisation coefficients, including the Pearson's coefficient (R_r), Manders' coefficient (R) and the Intensity Correlation Quotient (ICQ). The Pearson's coefficient measures the correlation

between the signal intensity in two channels on a pixel-by-pixel basis. It varies between -1 and 1, with values over 0.5 indicative of colocalisation. The Manders' coefficient denotes the fraction of overlap between two signals. It ranges between 0 and 1, where 1 indicates complete colocalisation. Finally, the ICQ is a measurement of whether two signals vary in synchrony. Values range from -0.5 to 0.5; where a positive value shows dependent staining, a negative value denotes segregated staining and a value of 0 means co-staining is random. Changes to the colocalisation coefficients between two proteins under different experimental conditions were assessed using an unpaired Student's t-test.

2.7.3 Neurite outgrowth

Images of SH-SY5Y cells were randomly taken, with the conditions known to the experimenter. Fiji (Schindelin et al, 2012) was used for measurements of neurite outgrowth, where neurites were defined as projections from the cell soma equal to or greater than the soma width. The β -tubulin signal was used to visualise neurites and the average length and number of neurites per cell were measured manually. For the statistical analysis, the data from three experiments were pooled and a Mann-Whitney U test was performed. Where multiple comparisons were made, a Kruskal-Wallis test was carried out prior to the Mann-Whitney U test.

2.7.4 Sholl analysis

Neurons were transfected with GFP alone or doubly transfected with GFP and GPR50 after seven or 14 days *in vitro* (DIV 7 or DIV 14); ICC was performed 48 hrs later. Images of neurons with a pyramidal morphology were randomly taken, using the GFP signal, over three independent experiments. In the initial experiment, the experimenter was aware of the conditions, i.e. whether cells were transfected with

GFP alone or co-transfected with GFP and GPR50. The subsequent two replicates were performed blind, i.e. the conditions were not known. To do this, slides were labelled by another member of the laboratory, images were then taken using the GFP signal, and the generated images were re-labelled by another group member. Once analysis was complete, the transfection of GPR50 was confirmed before data was included in statistical analysis.

Measurements of dendritic complexity were made using Fiji (Schindelin et al, 2012). Dendrites were classified as stout tapering processes emerging from the neuronal soma, morphologically distinct from axons which are longer and thinner with a uniform diameter (Bartlett & Banker, 1984; Kaech & Banker, 2006). The Simple Neurite Tracer plugin of Fiji (Longair et al, 2011) was used to trace dendrites using the GFP signal as a guide. This plugin was also used to perform Sholl analysis (Sholl, 1953). This generates concentric circles radiating from the centre of the cell soma, at intervals of 10 μm , and measures the number of times dendrites intersect these virtual circles. This was done for a total of 15-20 cells for each condition and the average number of dendritic intersection was plotted against the distance from the soma. The number of primary dendrites was also counted, i.e. dendrites directly projecting off the neuronal soma (N= 17-25 cells per condition).

Statistical analysis was performed on the unblinded and blinded datasets individually and also on the combined data. An unpaired Student's t-test was used to assess the effects of GPR50 overexpression on the number of dendritic intersection for each Sholl interval. The dataset as a whole was analysed using a repeated measures ANOVA (transfection x Sholl interval). Only a small proportion of neurons had dendritic processes extending to the larger Sholl intervals. This was independent of GPR50 transfection and more likely to reflect neuronal age, thus could bias results. To prevent this, Sholl intervals in which less than 10% of the neurons were detected were excluded from the repeated measures analysis.

2.7.5 Spine morphology

The spine density was also measured in the neurons transfected at DIV 14. Spines were defined as protrusion emerging from dendrites with a definable head (Ciani et al, 2011). Using Fiji, spines were counted manually on 1-2 stretches of secondary dendrites per neuron and normalised against the dendritic length examined. Again, analysis was performed unblinded for the initial experiment and blinded for the subsequent two experiments, with a total of 15-21 cells analysed for each condition. For hippocampal neurons, a total dendritic length of 2141.4 μm was measured in the GPR50 + GFP transfected group; 601.1 μm in the unblinded and 1540.3 μm in the blinded experiments. In the GFP group, a total dendritic length of 1851.3 μm was measured (unblinded: 437.7 μm ; blinded: 1413.6 μm). For GPR50 + GFP transfected cortical neurons, a total dendritic length of 1324.5 μm was measured (unblinded: 803.7 μm ; blinded: 520.8 μm). In the GFP control condition, a total length of 1586.7 μm was measured (unblinded: 819.0 μm ; blinded: 767.7 μm). For statistical analysis, the average spine density was compared using an unpaired Student's t-test. As a measure of spine maturity, the density of spines that stained positively for PSD-95 was measured, as these are more likely to form part of a functional synapse (Teodoro et al, 2013); this was analysed in the same way.

Morphological analysis of spines was performed as previously described (Chen et al, 2011; Teodoro et al, 2013). Spine length was manually measured from the tip of the spine head to the base of the spine neck using Fiji (Schindelin et al, 2012). The width of the spine was taken at the widest part of the spine head, perpendicular to the spine length. For each spine, the length-to-head width ratio was also calculated. Comparisons of the cumulative frequency distribution were assessed using the Kolmogorov-Smirnov test, whilst comparisons of the average length, width and the length-to-head width ratio were evaluated with an unpaired Student's t-test. A total

of 257 spines were measured for GPR50 + GFP transfected neurons, and 211 spines for GFP-transfected neurons, from three independent experiments.

2.7.6 Lipid droplet quantification

Using Fiji (Schindelin et al, 2012), an outline of the cell of interest was generated with β -tubulin as a guide. The image was then converted into a binary image using the “Threshold” function of Fiji to remove unwanted background. The number of lipid droplets, within the ROI outlined with β -tubulin, was then automatically measured using the “Analyse particle” function. This also calculated the “Area fraction”, which in this case represents the area of the cell containing lipid droplets. Statistical significance was assessed by combining the data of three independent experiments and performing a Mann-Whitney U test.

2.8 *Statistical analysis*

Statistical analysis was performed using SPSS or Microsoft Excel. When statistical tests were carried out, as indicated above, experiments were repeated a minimum of three times. When a trend was observed, further replicates were often sought. Throughout this thesis, results are presented as mean $\pm$ standard error of the mean (SEM) and $P < 0.05$ was considered to be statistically significant, $P < 0.01$ was very significant and $P < 0.001$ was regarded as highly significant.

Chapter 3: GPR50 expression promotes dendritic branching and spine formation

3.1 Introduction

Multiple lines of evidence indicate that GPR50 has a role in neuronal development. Here, this refers to the process by which neurons acquire their distinctive polarised structure, typically consisting of multiple dendrites and a single axon. Analysis of cultured neurons has revealed that this process occurs in five stages, figure 3.1 A (Dotti et al, 1988; Polleux & Snider, 2010). Upon plating, neurons extend lamellipodia and filopodial protrusions (stage 1); these protrusions extend to form multiple immature neurites (stage 2). Several days later neuronal symmetry breaks and one of these neurites rapidly elongates to form an axon (stage 3). At stage 4, the remaining neurites elongate and develop into dendrites, with the development of dendritic branches. Likewise, axonal outgrowth and branching occurs at this stage. In the final stage of maturation (stage 5), spinogenesis occurs, i.e. dendrites develop spines. These are specialised protrusions capable of receiving synaptic input from axon terminals. These stages also occur *in vivo*, where neuronal development is coupled with migration. Figure 3.1 B shows this process for neocortical pyramidal neurons, which originate from the ventricular zone as neuronal precursors and migrate to their final position in the cortex, where neurons reside in their fully differentiated form (Hatanaka & Murakami, 2002; Nadarajah et al, 2003).

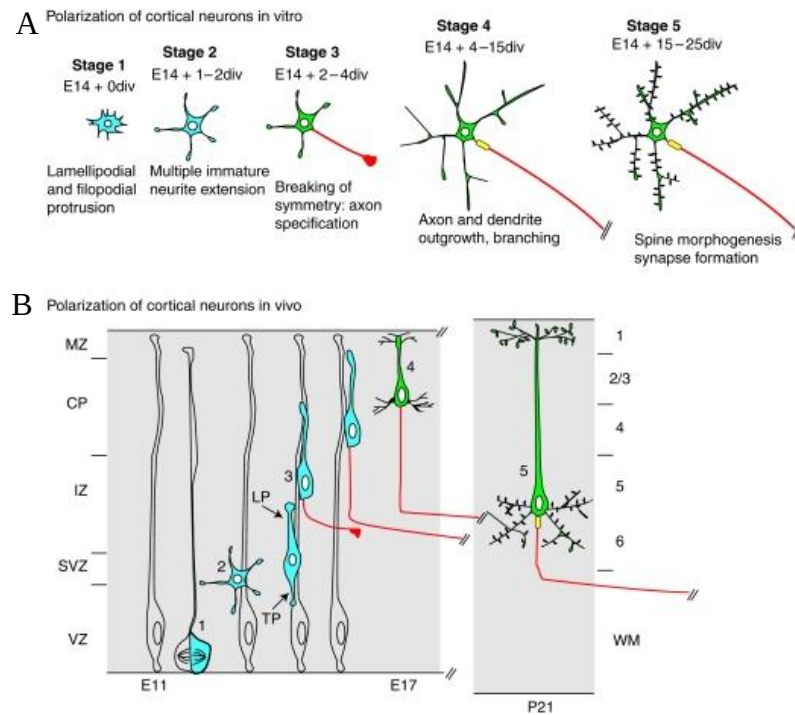


Figure 3.1: Neuronal development of cortical neurons. (A) The key stages of neuronal development and polarisation following dissociation at embryonic day 14 (E14). At stage 1, immature neurons are rich in lamellipodia and filopodia. Multiple neurites emerge by stage 2 and at stage 3 neuronal symmetry breaks and a single neurite becomes an axon. Extensive axonal and dendritic outgrowth and branching occurs at stage 4. Finally, at stage 5 neurons are terminally differentiated with dendritic spines and synapse formation. (B) The corresponding stages of development *in vivo*. As neurons migrate to their final position within the brain, axons and dendrites arise from the trailing (TP) and leading (LP) processes respectively. Key features of maturation, e.g. spine formation, arise within the first postnatal week. Abbreviations: CP, cortical plate; DIV, days *in vitro*; IZ, intermediate zone; MZ, marginal zone; P21, postnatal day 21; SVZ, subventricular zone; VZ, ventricular zone; WM, white matter. Figure taken from Polleux & Snider, 2010.

A link between GPR50 and neuronal development comes from the yeast-2-hybrid study which identified several potential GPR50 interactors involved in neuronal development (Grünewald et al, 2009). Amongst these were shootin-1, PICK1 and NOGO-A, proteins known to regulate neurite elongation and dendritic development (GrandPre et al, 2000; Rocca et al, 2008; Toriyama et al, 2006). The interaction with NOGO-A was confirmed through colocalisation and co-immunoprecipitation experiments (Grünewald et al, 2009). Furthermore, GPR50 was shown to block the

inhibitory actions of NOGO-A; when co-transfected with GPR50, NOGO-A did not lead to the anticipated attenuation of neurite outgrowth in Neuroscreen-1 (NS-1) cells. In addition, the transfection of GPR50 alone was sufficient to drive an increase in neurite length (Grünewald et al, 2009). These results depict GPR50 as a positive regulator of neurite outgrowth; whether this relies on the interaction with NOGO-A has yet to be investigated.

The expression pattern of GPR50 in the mouse brain is also indicative of a neurodevelopmental role. A number of studies have reported that GPR50 is highly expressed in multiple regions of the brain, most notably the hypothalamus (Batailler et al, 2012; Drew et al, 2001). Recently, *Gpr50* expression in the mouse brain was shown to be developmentally regulated (Grünewald et al, 2012). *Gpr50* was detected at low levels from embryonic day 13 (E13) and at E18 a dramatic rise was observed, particularly in the thalamus and hypothalamus. This is particularly suggestive as neurons enter stage four of maturation by E18, which is characterised by rapid axonal and dendritic outgrowth, figure 3.1 (Polleux & Snider, 2010). Given GPR50's neuronal expression and ability to promote of neurite outgrowth (Grünewald et al, 2009; Grünewald et al, 2012), its upregulation may serve to induce a more fully developed neuron. Further, *Gpr50* expression rises again in the adolescent mouse, i.e. 5 weeks of age (Grünewald et al, 2012), thus GPR50 may also have a role in later stages of neuronal development.

3.2 Aim

The aim of this chapter was to determine whether GPR50 contributes to neuronal development and, if so, characterise its involvement. This was investigated by examining the effects of GPR50 overexpression on the morphology of SH-SY5Y cells. The effect of GPR50 overexpression on primary neurons was subsequently assessed. Focus was placed on hippocampal and cortical neurons as abnormalities in

these areas are often reported in psychiatric disorders (Kolluri et al, 2005; Malberg et al, 2000). Furthermore, endogenous GPR50 has previously been found in these regions (Grünewald et al, 2012). Attention was placed on the phenotypic changes associated with late stages of neuronal development, namely dendritic branching and spine formation; neurons are most likely to be at this stage of development when endogenous *Gpr50* expression peaks in the mouse brain. The effect of GPR50 on the size of dendritic spines was also investigated as there is a strong correlation between spine morphology and synaptic function (Kasai et al, 2010; Kasai et al, 2003).

3.3 Results

3.3.1 Overexpression of GPR50 increases the number of neurites on SH-SY5Y cells

Immunocytochemistry (ICC) was used to examine the effects of GPR50 on the morphology of SH-SY5Y cells. Cells were either transfected with the previously validated GPR50 expression construct (Grünewald et al, 2009, and appendix A), or left untransfected. The following day, cells were fixed and stained with antibodies against GPR50 and β -tubulin. The image analysis software Fiji (Schindelin et al, 2012) was used to determine the number of neurites per cell and the average neurite length; neurites being defined as projections from the cell soma with a length equal to or greater than the soma width. As demonstrated in figure 3.2, GPR50-transfected cells had almost twice as many neurites per cell (2.7 ± 0.08 ; N= 419 cells) compared to untransfected cells (1.6 ± 0.05 ; N= 473 cells), $P < 0.001$, Mann-Whitney U test, three replicates. However, GPR50 did not affect the average length of neurites; this was $28.9 \pm 1.78 \mu\text{m}$ in untransfected cells (N= 184 cells) and $26.9 \pm 0.64 \mu\text{m}$ in GPR50-transfected cells (N= 207 cells); $P = 0.55$, Mann-Whitney U test, three independent experiments.

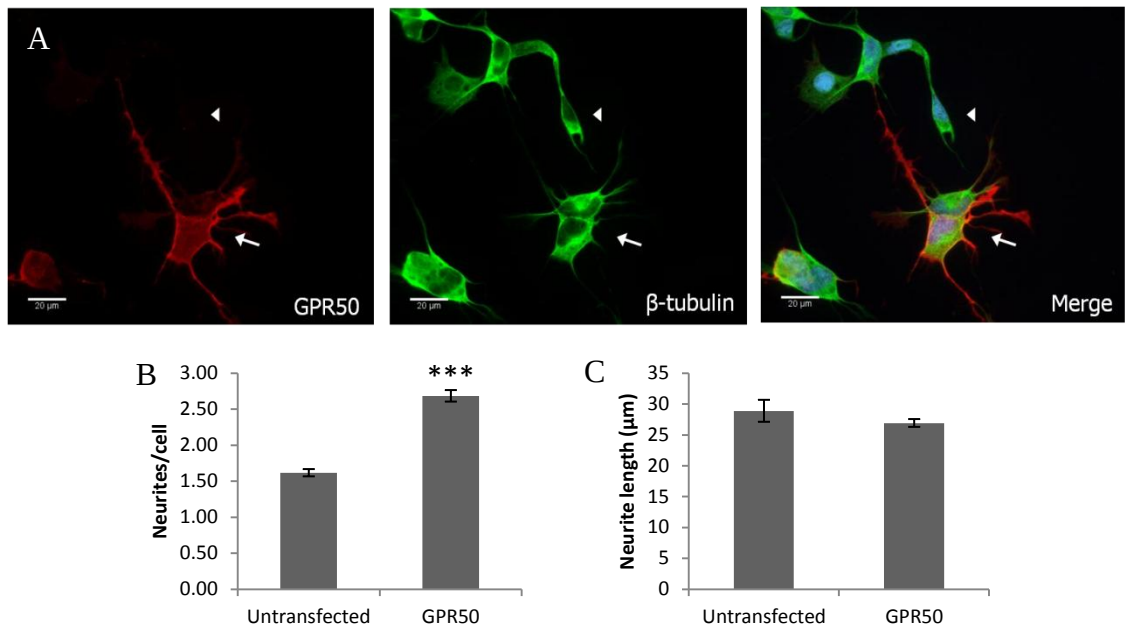


Figure 3.2 : GPR50 induces neurite formation in SH-SY5Y cells. SH-SY5Y cells were transfected with GPR50 or left untransfected. After 24 hrs, cells were fixed and immunostained for GPR50 and β -tubulin. (A) A representative fluorescent image showing a GPR50-transfected cell (arrow) with more neurites than an untransfected cell (arrowhead). Scale bar, 20 μ m. (B) GPR50 transfection resulted in a highly significant increase in the number of neurites per cell. N= 473 untransfected and 419 GPR50-transfected cells. (C) The average neurite length, measured using Fiji software, was not affected by GPR50. N= 184 untransfected and 207 GPR50-transfected cells. Results shown as mean $\pm$ SEM; averaged from three independent experiments; ***P<0.001, Mann-Whitney U test.

3.3.2 GPR50 overexpression increases dendritic complexity of mature primary neurons

To investigate the effect of GPR50 on neuronal morphology, hippocampal and cortical neurons were doubly transfected with GPR50 and GFP or transfected with GFP alone (control) after 14 days *in vitro* (DIV 14). ICC was performed at DIV 16 and dendritic complexity was measured by performing Sholl analysis (Sholl, 1953), using the Simple Neurite Tracer plugin on Fiji (Longair et al, 2011). Unless otherwise stated, these experiments were carried out in triplicate. The first

experiment was analysed unblinded, i.e. with the transfection conditions known, whilst the subsequent two experiments were blinded. Statistical analysis of the blinded dataset, comprising of two replicates, and unblinded dataset was initially carried out separately; this was to determine whether blinding had any impact on the results. The data was then pooled to increase the sample size (N).

Hippocampal neurons

In the unblinded experiment, a repeated measures ANOVA revealed a significant interaction between transfection (with or without GPR50) and the Sholl interval ($F_{3,37}= 3.1$, $P= 0.04$, $N=7$ neurons per condition). This indicates that GPR50 overexpression alters the pattern of dendritic branching. Comparisons of each Sholl interval showed GPR50 overexpression significantly increased the number of dendritic intersections 80-90 μm from the neuronal soma, compared to the GFP-transfected control cells (figure 3.3 and 3.4 A; $P<0.05$ for both Sholl intervals, Student's t-test). In contrast, the number of primary dendrites was unchanged (GFP: 4 ± 0.53 ; GPR50 + GFP: 4.14 ± 0.40 ; $P=0.83$, Student's t-test). Thus, the increase in the number of dendritic intersections was not due to an increase in newly formed dendrites. This indicates that GPR50 promotes dendritic branching.

Results from the blinded experiments also showed GPR50 overexpression effects dendritic complexity ($F_{3,64}= 3.2$, $P= 0.03$, repeated measures analysis, $N= 11-13$ neurons per condition, two independent experiment). Upon comparisons of individual Sholl intervals, the overexpression of GPR50 significantly increased the number of dendritic intersections 60-100 μm from the cell soma (figure 3.4 B; $P<0.05$, Student's t-test). Again, the number of primary dendrites was not affected by GPR50 (GFP: 5.71 ± 0.45 ; GPR50 + GFP: 6.67 ± 0.52 ; $P=0.17$, Student's t-test). The similarities between these results and those obtained in the unblinded experiment suggest there was little experimenter bias.

Combining the unblinded and blinded datasets led to the same results. Repeated measures analysis of the pooled data showed GPR50 significantly altered dendritic complexity ($F_{3,98} = 3.9$, $P = 0.01$; $N = 18-20$ neurons per condition). Further, neurons transfected with GPR50 and GFP displayed significantly more dendritic intersections 60-100 μm from the neuronal soma ($P < 0.05$ for each Sholl interval, Student's t-test; figure 3.4 C). In addition, GPR50 transfection had no effect on the number of primary dendrites (GFP: 5.14 ± 0.39 ; GPR50 + GFP: 5.74 ± 0.45 ; $P = 0.32$, Student's t-test). Together, these results suggest GPR50 increases dendritic arborisation.

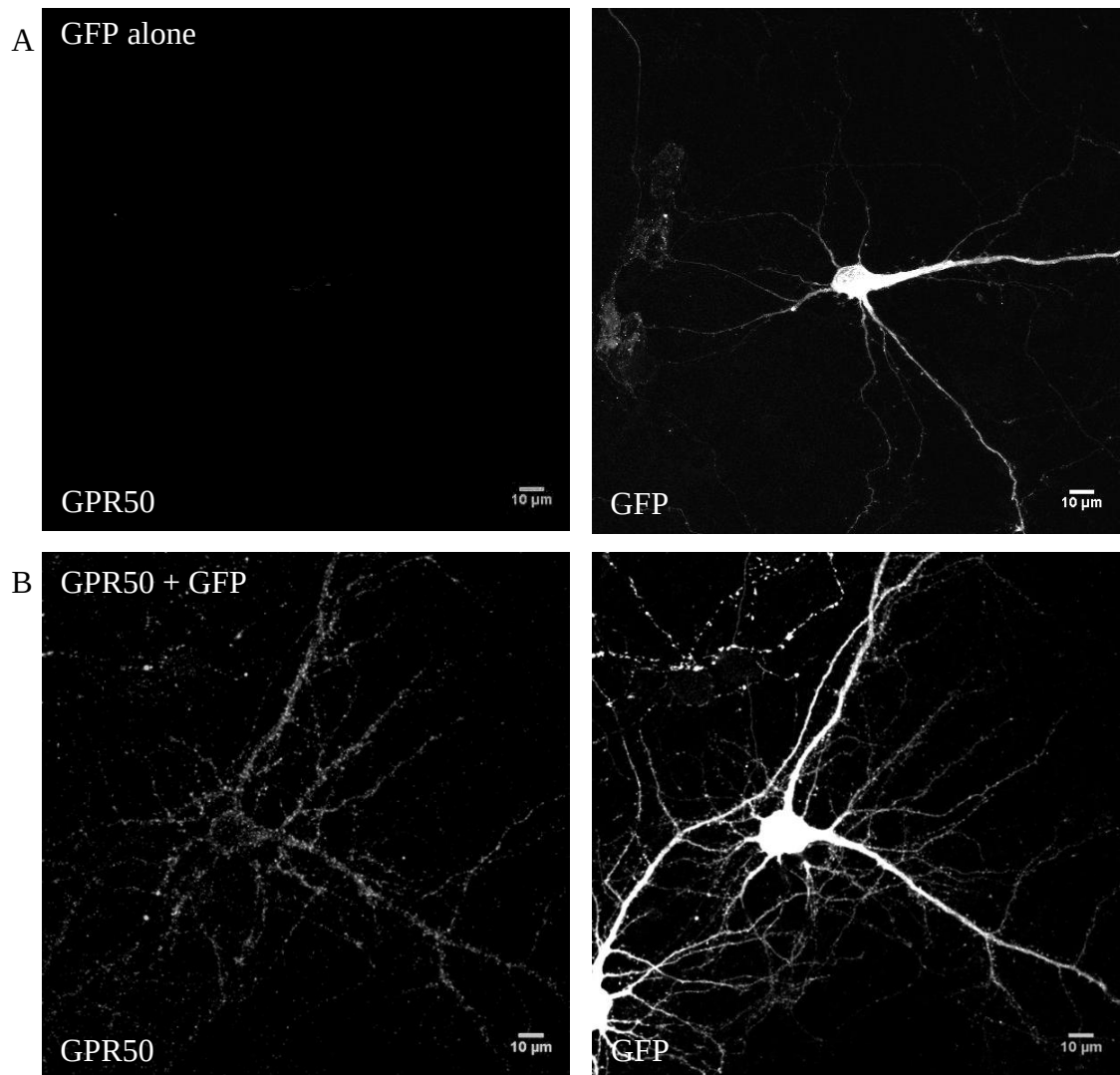


Figure 3.3: GPR50 alters the morphology of hippocampal neurons. Primary neurons were transfected on DIV 14 and ICC was performed 48 hrs later, staining for GPR50. (A) A representative image of a GFP-transfected neuron with a classical pyramidal morphology. (B) Neurons transfected with GPR50 and GFP typically displayed a more complex morphology. Scale bar, 10 μ m.

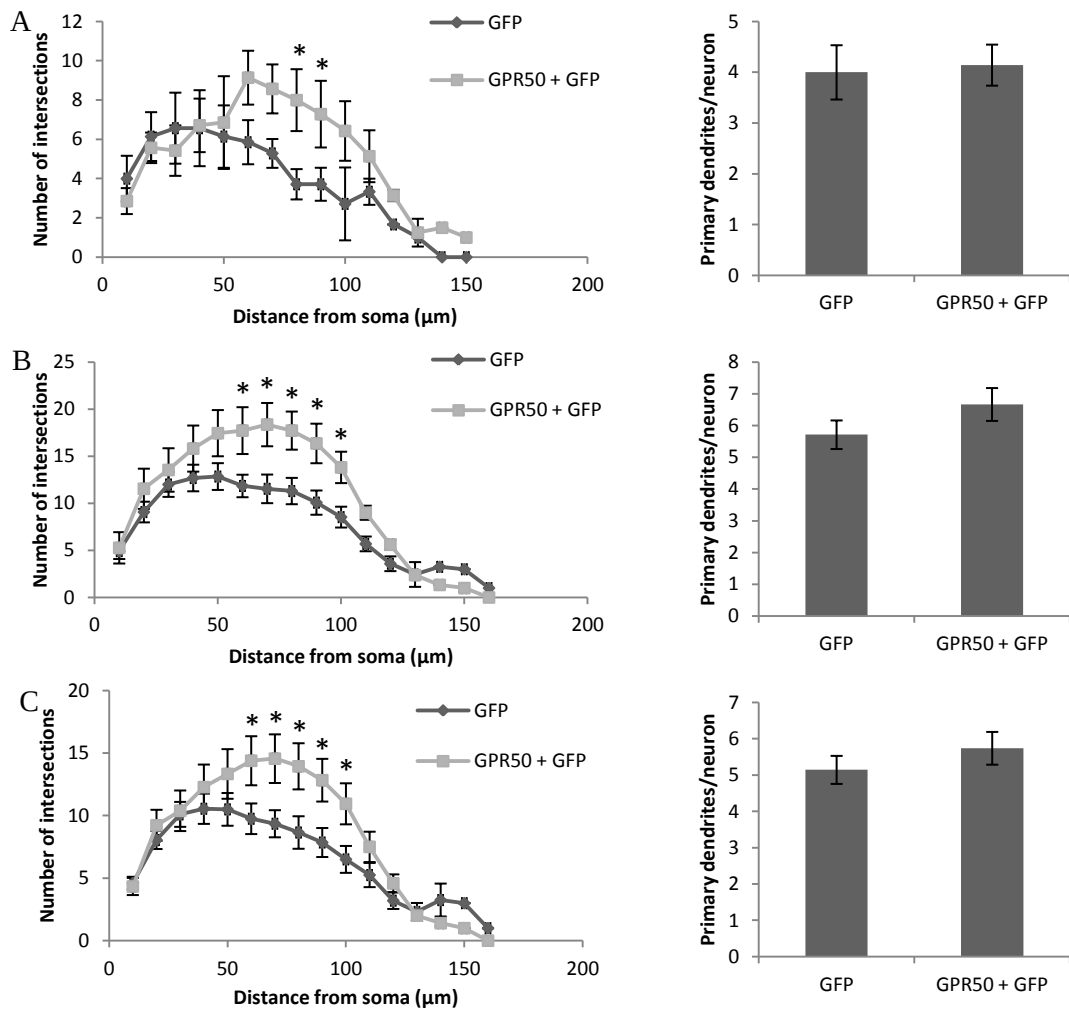


Figure 3.4: GPR50 increases dendritic complexity in hippocampal neurons. Cultured hippocampal neurons were transfected with GFP alone or co-transfected with GPR50 and GFP (DIV 14) and fixed on DIV 16. Dendritic complexity was assessed using Sholl analysis on Fiji. (A) In the unblinded experiment, GPR50 overexpression increased the number of dendritic intersections at Sholl intervals 80-90 μm (left). No changes were observed in the number of primary dendrites, right. N= 7 GFP and 7 GPR50 + GFP transfected cells. (B) Blinded experiments show similar results, i.e. a significant increase in dendritic branching (at Sholl intervals 60-100 μm) and no effect on primary dendrites. N= 13 GFP and 11 GPR50 + GFP transfected cells, two independent experiments. (C) Combined results from the three experiments show GPR50 transfection significantly increased the number of dendritic intersections 60-100 μm from the cell soma, but had no effect on the number of primary dendrites per neuron. N= 20 GFP and 18 GPR50 + GFP transfected cells. Results presented as mean $\pm$ SEM; *P<0.05, unpaired Student's t-test.

Cortical neurons

In the initial unblinded experiment, the overexpression of GPR50 in cortical neurons did not significantly affect dendritic complexity (3.5 and 3.6 A; $F_{3,49} = 0.9$, $P = 0.46$, transfection x Sholl interval, repeated measures analysis, $N = 8-9$ neurons per condition). Further, the comparison of individual Sholl intervals did not reveal any significant differences in neurons transfected with GPR50 and GFP compared to the control ($P > 0.05$ for each Sholl interval, Student's t-test). The number of primary dendrites was also unaffected in this experiment (GFP: 4.78 ± 0.47 ; GPR50 + GFP: 5.5 ± 0.80 ; $P = 0.45$, Student's t-test). This suggests that the morphology of cortical neurons is not affected by GPR50 overexpression.

The blinded experiments, however, yielded different results. GPR50 overexpression did not significantly affect the number of dendritic intersections when a repeated measures test was performed ($F_{3,49} = 1.9$, $P = 0.144$, 8-9 neurons per condition, from two experiments). However, an increase in dendritic intersections at Sholl intervals 20 μm and 30 μm was observed in neurons transfected with GPR50 (figure 3.6 B; $P < 0.05$, Student's t-test). This is likely to reflect an increase in the number of primary dendrites which increased from 4 ± 0.37 , in GFP-transfected neurons, to 6.86 ± 0.59 in GPR50 + GFP transfected neurons ($P = 0.002$, Student's t-test, from two experiments; figure 3.6 B). The inconsistency may reflect over-conservative measurements in the unblinded experiment or an inadequate sample size. An additional replicate may help determine which of the datasets, blinded or unblinded, most accurately reflects the actions of GPR50 in cortical neurons.

Again, the repeated measures analysis of the pooled dataset did not reveal any differences in dendritic complexity between neurons transfected with GFP alone or GFP with GPR50 ($F_{4,115} = 1.3$, $P = 0.27$, $N = 17$ neurons per condition). Comparisons of the individual Sholl intervals revealed an increase in dendritic intersections 20-40 μm from the cell soma in neurons transfected with GPR50 (figure 3.6 C; $P < 0.05$ for

each Sholl interval, Student's t-test). Again, GPR50-transfected cells had a greater number of primary dendrites (GFP: 5.1 ± 0.31 ; GPR50 + GFP: 6.1 ± 0.52 ; $P=0.008$, Student's t-test). As an additional Sholl interval was found to be significant when the two datasets were combined, i.e. $40 \mu\text{m}$, it is likely that an additional replicate to increase N would strengthen these results. However, as they stand, these results suggest that GPR50 does not promote dendritic branching but enhances the formation of primary dendrites in cortical neurons.

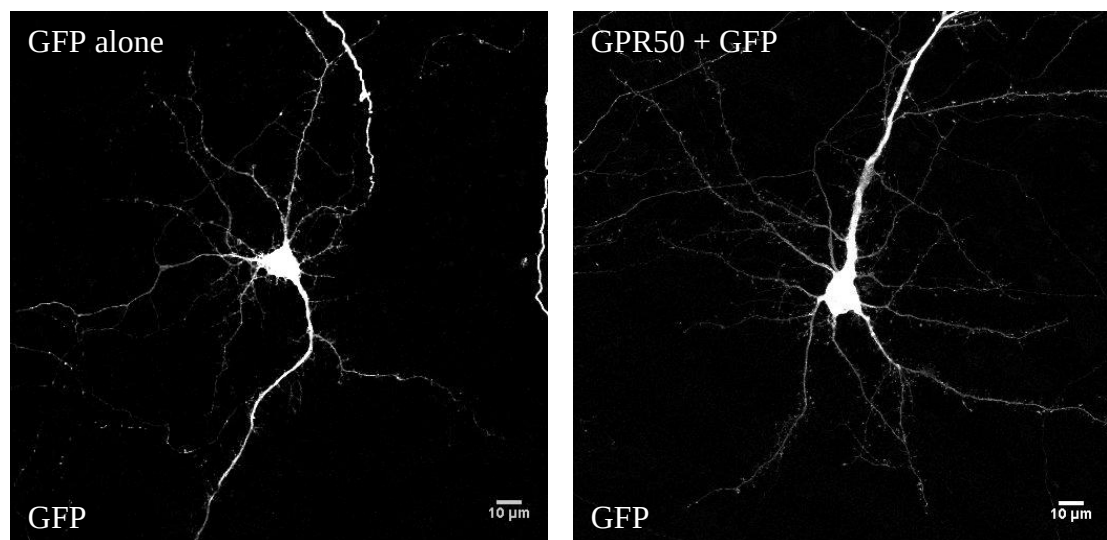


Figure 3.5: GPR50 alters the morphology of cortical neurons. ICC results of cortical neurons transfected on DIV 14 with GFP alone (left) or GPR50 and GFP (right), only the GFP signal is shown here. GPR50 overexpressing neurons typically displayed a more developed morphology. Scale bar, $10 \mu\text{m}$.

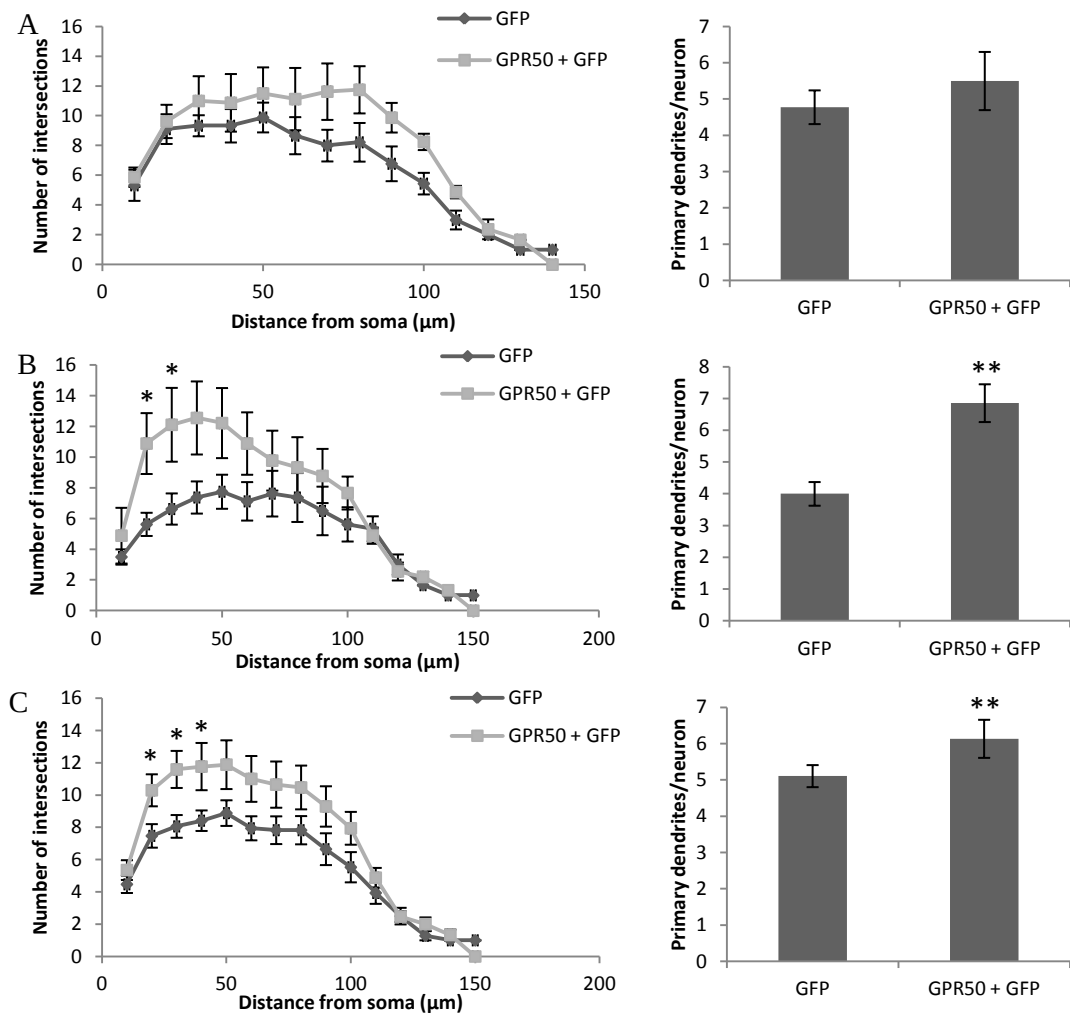


Figure 3.6: GPR50 increases dendritic complexity and primary dendrites in cortical neurons. ICC was performed on cortical neurons transfected (DIV 14) with GFP with or without GPR50. Sholl analysis was performed on Fiji to measure dendritic complexity. (A) In the unblinded experiment, no significant change to dendritic complexity was observed upon GPR50 overexpression, left. Likewise, GPR50 did not significantly affect the number of primary dendrites (right). N=9 GFP and 8 GPR50 + GFP transfected cells. (B) Blinded results show GPR50 increased dendritic complexity near the cell soma, right. The number of primary dendrites, left, also showed a significant increase. N=8 GFP and 9 GPR50 + GFP transfected cells, two independent experiments. (C) Pooled results show GPR50 overexpression significantly increased dendritic intersections 20-40 μm from the cell soma and the number of primary dendrites (N=17 GFP and 17 GPR50 + GFP transfected cells, three independent experiments).

Results presented as mean ± SEM; *P<0.05, **P<0.01, unpaired Student's t-test.

3.3.3 GPR50 transfection does not affect the morphology of immature cortical neurons

To ascertain whether GPR50 has a role at earlier stages of development, its effect on the morphology of immature neurons (transfected on DIV 7) was investigated. Due to very poor transfection efficiencies this experiment was only conducted on cortical neurons. A larger number of cortical neurons could be harvested compared to hippocampal neurons, therefore, the experiment could be attempted a greater number of times.

The number of dendritic intersections did not vary greatly in neurons co-transfected with GPR50 and GFP compared to the GFP control (figure 3.7 and 3.8). No significant differences were found using a Student's t-test or a repeated measures analysis in any of the datasets analysed, i.e. the unblinded, blinded and pooled datasets ($P > 0.1$, figure 3.8 A – C). As could be expected, the number of primary dendrites was also unaffected by GPR50 (unblinded experiment: GFP: 4.08 ± 0.58 ; GPR50 + GFP: 4.11 ± 0.54 ; $P = 0.97$. Blinded data: GFP: 4 ± 0.2 ; GPR50 + GFP: 4.44 ± 0.10 ; $P = 0.53$. Pooled data: GFP: 4.04 ± 0.10 ; GPR50 + GFP: 4.28 ± 0.2 ; $P = 0.63$). Thus, GPR50 expression does not appear to influence the complexity of cortical neurons at this stage of development.

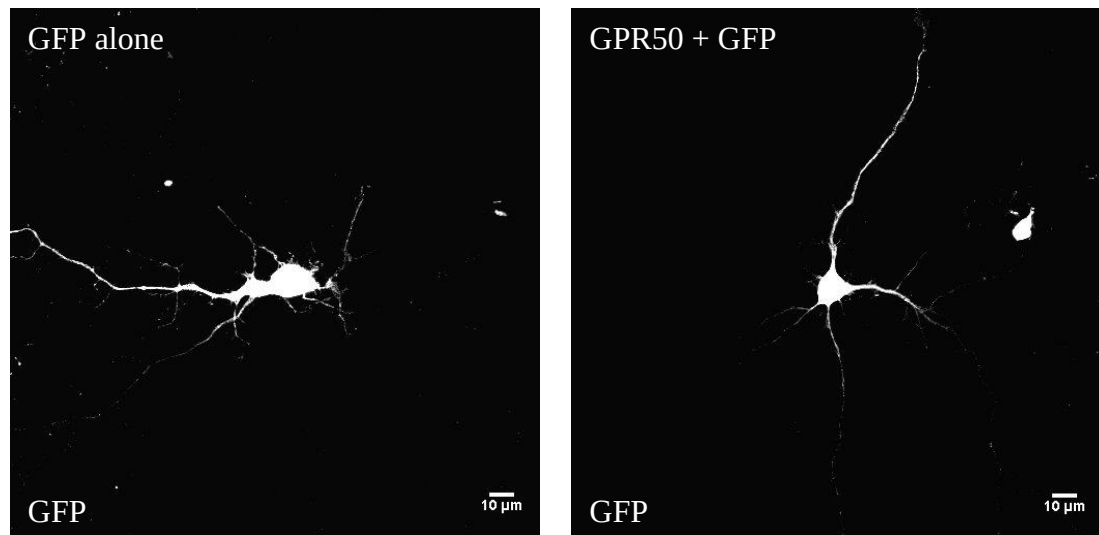


Figure 3.7: GPR50 does not affect the morphology of younger cortical neurons (DIV 7). ICC images taken from cortical neurons transfected for 48 hrs with GFP alone (left) or with GPR50 (right), the GFP signal is shown here. Obvious difference in neuronal morphology was not observed. Scale bar, 10 μ m.

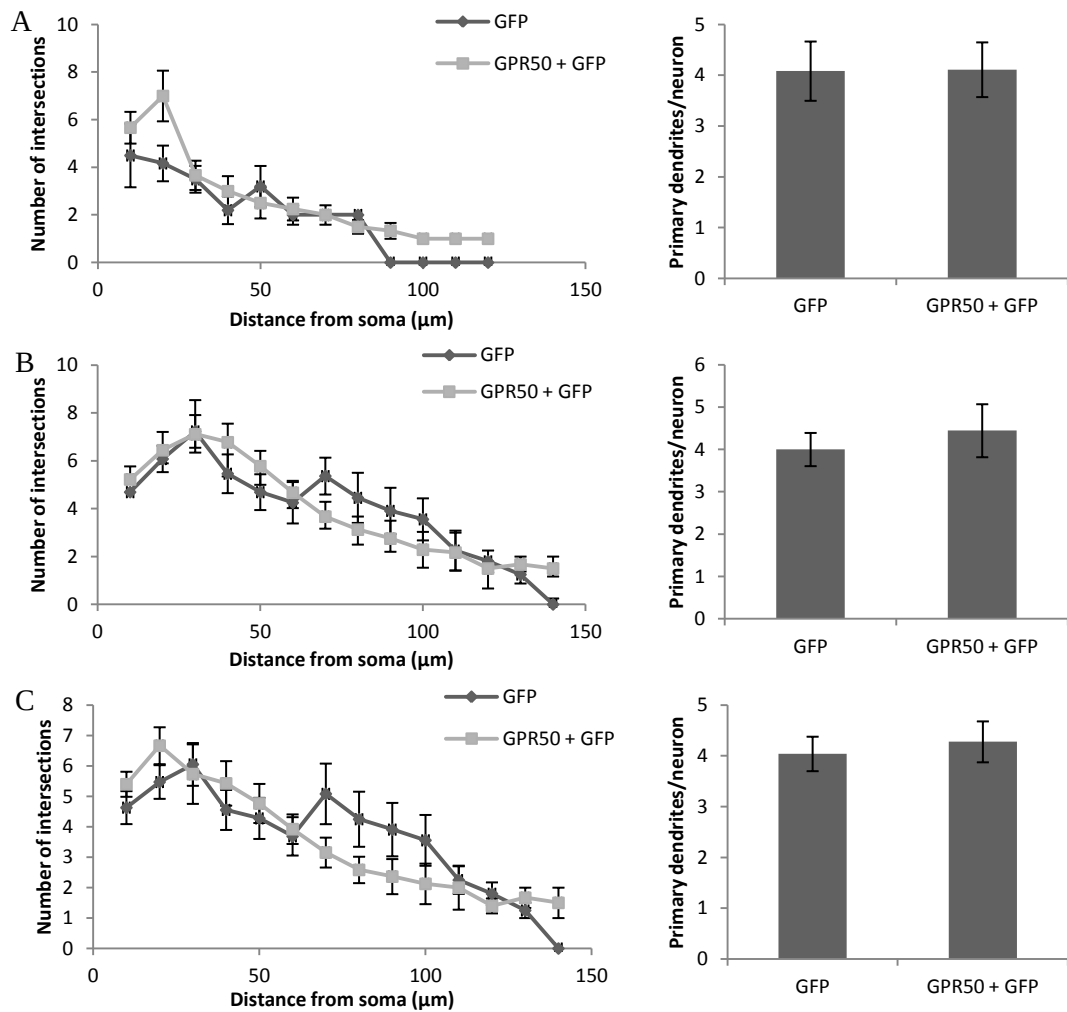


Figure 3.8: Transfection of GPR50 in immature cortical neurons, DIV 7, does not increase dendritic complexity. Cortical neurons were transfected with GFP alone or co-transfected with GPR50 and GFP. Cells were fixed 48 hrs later and ICC was performed. Sholl analysis was used to assess dendritic complexity. (A) The unblinded experiment detected no changes to dendritic branching upon GPR50 overexpression (left; N=6 GFP and 6 GPR50 + GFP transfected cells). Likewise, the number of primary dendrites was not altered (right; N= 12 GFP and 9 GPR50 + GFP transfected cells). (B) The blinded data show similar results; no significant changes to dendritic complexity (left) or the number of primary dendrites (right). N=13 GFP and N=9 GPR50 + GFP transfected cells, two independent experiments. (C) Combined results from the three experiments show GPR50 transfection had no effect on dendritic complexity (left); N=19 GFP and 15 GPR50 + GFP transfected cells. The number of primary dendrites was also unaffected (right); N=25 GFP and 18 GPR50 + GFP transfected cells. Results presented as mean $\pm$ SEM; statistical tests, unpaired Student's t-test.

3.3.4 The density of postsynaptic spines is increased by GPR50 in hippocampal neurons

The influence of GPR50 on dendritic spines was assessed by looking at changes in spine density. Cortical and hippocampal neurons were transfected (DIV 14) with GFP with or without GPR50. ICC was carried out at DIV 16, staining for GPR50 and postsynaptic density protein 95 (PSD-95). PSD-95 is enriched in mature excitatory spines (El-Hussein et al, 2000), thus quantifying spines containing PSD-95 would give insight into whether GPR50 affects spine maturity. Fiji was used to measure the density of dendritic spines, i.e. protrusion from dendrites with a definable head (Ciani et al, 2011). Again, three replicates were carried out, with two of these analysed blind. Pooling the data meant that in hippocampal neurons a total dendritic length of approximately 2000 μm was analysed for each condition; for cortical neurons this was approximately 1500 μm , see materials and methods for more detail.

Hippocampal neurons

Hippocampal neurons overexpressing GPR50 displayed more spines compared to the GFP controls (figure 3.9 A). Upon quantification of the unblinded set of results it was found that GPR50 transfection led to an increase in the average spine density, figure 3.10 A (GFP: 0.47 ± 0.16 spines per 10 μm ; GPR50 + GFP: 1.14 ± 0.18 spines per 10 μm , $P=0.03$, Student's t-test, $N=6$ neurons for each condition). The density of spines containing PSD-95 also increased upon GPR50 overexpression (GFP: 0.21 ± 0.10 spines per 10 μm ; GPR50 + GFP: 0.77 ± 0.04 spines per 10 μm ; $P<0.001$, Student's t-test). The proportion of spines containing PSD-95 was also assessed relative to the total number of spines. This was 2-fold greater in neurons overexpressing GPR50 (GFP: $33 \pm 11\%$; GPR50 + GFP: $70 \pm 4\%$; $P=0.002$, Student's t-test). This suggests GPR50 promotes the formation, or stabilisation, of dendritic spines and induces a shift towards maturity.

The blinded dataset generated consistent results. The overall spine density increased from 0.89 ± 0.12 spines per $10 \mu\text{m}$, in GFP-transfected cells, to 1.79 ± 0.15 spines per $10 \mu\text{m}$, in GPR50 + GFP transfected cells (figure 3.10 B; $P < 0.001$, Student's t-test, $N = 15$ cells per conditions, from two experiments). Likewise, the overexpression of GPR50 resulted in a marked increase in the density of PSD-95 positive spines (GFP: 0.07 ± 0.02 spines per $10 \mu\text{m}$; GPR50 + GFP: 0.57 ± 0.12 spines per $10 \mu\text{m}$; $P < 0.001$, Student's t-test, $N = 15$ cells per condition). This also corresponds to a greater proportion of PSD-95 positive spines (GFP: $9 \pm 2\%$; GPR50 + GFP: $35 \pm 6\%$; $P = 0.002$, Student's t-test).

Pooling the unblinded and blinded data together strengthened these results (figure 3.10 C). The average spine density was 2-fold greater in neurons transfected with GPR50 and GFP (1.61 ± 0.11 spines per $10 \mu\text{m}$) compared to GFP-transfected controls (0.79 ± 0.12 spines per $10 \mu\text{m}$); $P < 0.001$, Student's t-test, $N = 21$ cells per condition. The density of PSD-95 positive spines was 6-fold greater in GPR50 + GFP transfected cells compared to the control (0.63 ± 0.09 spines per $10 \mu\text{m}$ and 0.11 ± 0.03 spines per $10 \mu\text{m}$ respectively; $P < 0.001$, Student's t-test). Finally, the proportion of PSD-95 positive spines increased from $16 \pm 4\%$, in control cells, to $43 \pm 6\%$, in neurons transfected with GPR50 + GFP ($P < 0.001$, Student's t-test). These results indicate GPR50 is a positive regulator of spinogenesis and promotes spine maturity.

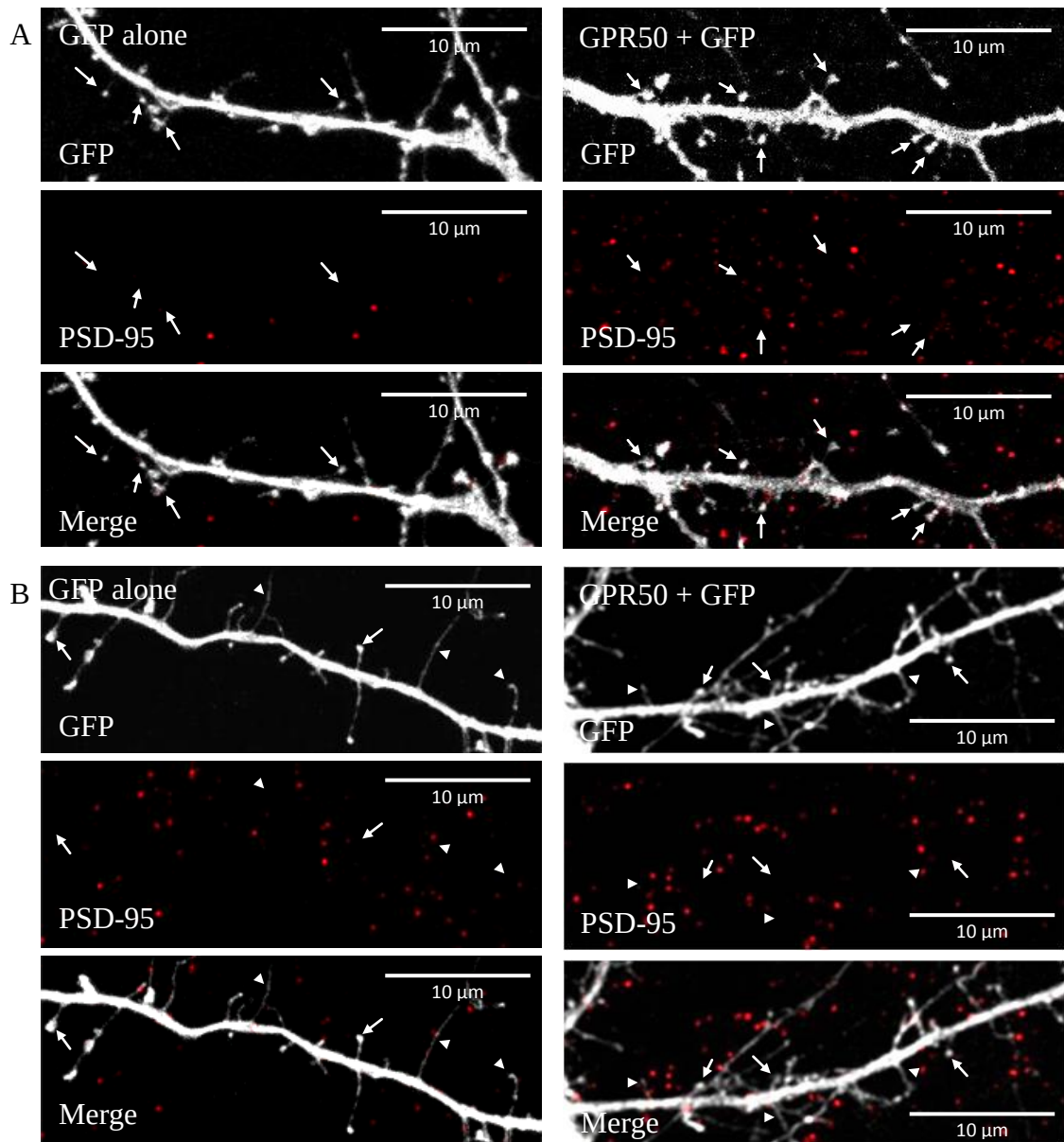


Figure 3.9: The effect of GPR50 overexpression on dendritic spines. Representative ICC results for neurons transfected on DIV 14 with GFP alone or GFP together with GPR50. Neurons were fixed after 48 hrs and stained for PSD-95. (A) Hippocampal neurons transfected with GPR50 and GFP, right, displayed more dendritic spines (arrows) compared to GFP-transfected neurons (left). (B) Few spines were visible in cortical neurons in the GFP (left) or GPR50 + GFP (right) transfected conditions; instead numerous filopodia was seen in both conditions (arrowheads). Scale bar, 10 μ m.

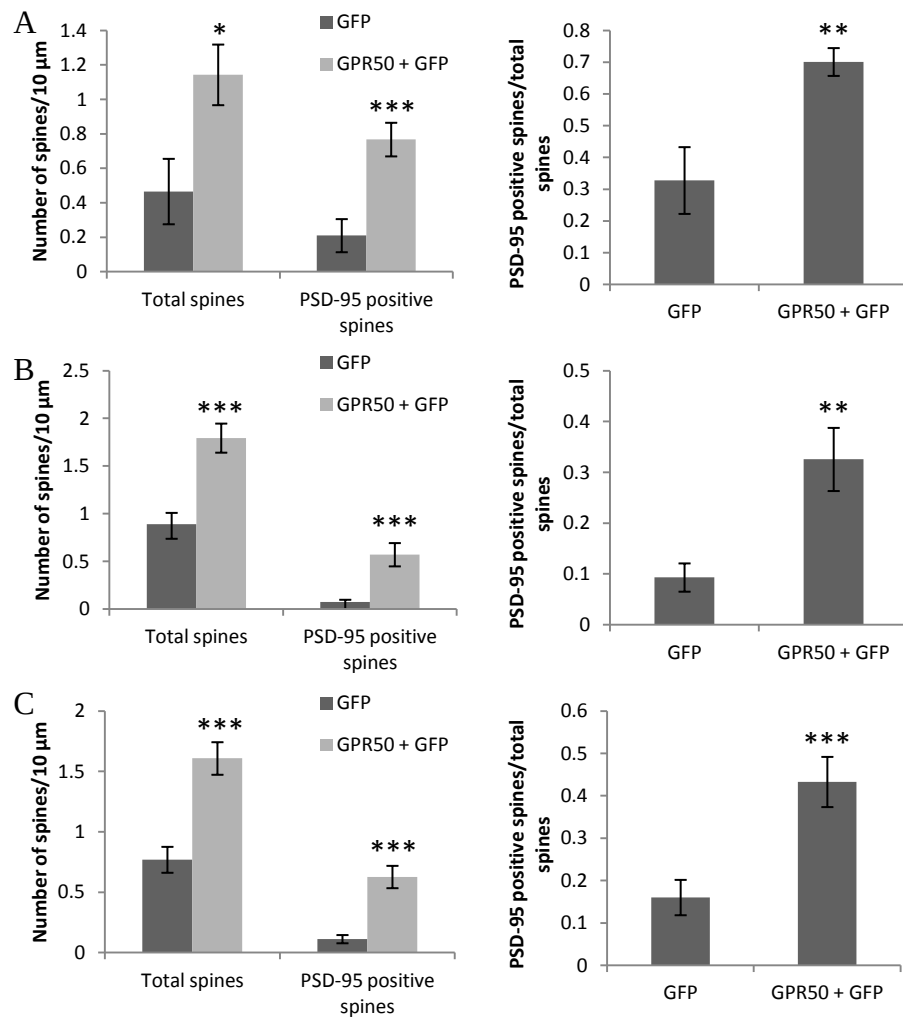


Figure 3.10: Effects of GPR50 overexpression on the number of postsynaptic spines in hippocampal neurons. Hippocampal neurons (DIV14) were transfected with GFP alone or co-transfected with GPR50 and GFP. On DIV 16, neurons were fixed and ICC was performed. Using Fiji, spine density was quantified. (A) In the unblinded experiment, GPR50 overexpression led to a significant increase in the overall number of spines and the number of PSD-95 immunoreactive spines (left). GPR50 transfection also increased the portion of PSD-95 positive spines relative to the total number of spines (right). N=6 GFP and 6 GPR50 + GFP transfected neurons. (B) In the blinded replicates, GPR50 transfection led to an increase in spine density (left) and a greater portion of PSD-95 positive spines (right). N= 15 GFP and 15 GPR50 + GFP transfected cells, two independent experiments. (C) Pooling the datasets generated similar results but with a higher degree of significance. N=21 GFP and 21 GPR50 + GFP transfected neurons. Results presented as mean $\pm$ SEM; *P<0.05, **P<0.01, ***P<0.001, Student's t-test.

Cortical neurons

Whilst spines were clearly visible, and relatively abundant, in hippocampal neurons (transfected on DIV 14) this was not the case for cortical neurons (figure 3.9). The average spine density in the control GFP-transfected conditions was two-fold greater in hippocampal neurons compared to cortical neurons (0.79 ± 0.12 spines per $10 \mu\text{m}$ and 0.34 ± 0.08 spines per $10 \mu\text{m}$ respectively; $P=0.002$, Student's t-test). Instead, there were numerous filopodia emerging from dendrites (figure 3.9 B, arrowhead), which may later develop into dendritic spines (Yuste & Bonhoeffer, 2004; Zuo et al, 2005). Thus, it is possible that cortical neurons were at an earlier developmental stage by the time ICC was carried out. Despite this, it was still possible to quantify dendritic spines on cortical neurons, thus spine density was assessed.

In the unblinded experiment, the spine density of cortical neurons expressing both GFP and GPR50 did not significantly differ from the GFP controls (figure 3.11 A; GFP: 0.28 ± 0.09 spines per $10 \mu\text{m}$; GPR50 + GFP: 0.42 ± 0.10 spines per $10 \mu\text{m}$; $P=0.33$, Student's t-test, $N= 8-9$ cells per condition). This was also the case for the number and proportion of PSD-95 positive spines, ($P=0.75$ and $P=0.64$ respectively, Student's t-test). From these results it appears that GPR50 does not have the same effect on cortical neurons as it does on hippocampal neurons.

The blinded experiments led to a different result; GPR50 overexpression caused an increase in the density of dendritic spines (figure 3.11 B). GFP-transfected cortical neurons displayed 0.39 ± 0.11 spines per $10 \mu\text{m}$ ($N= 10$ neurons), whilst neurons co-transfected with GPR50 and GFP had a spine density of 1.12 ± 0.22 spines per $10 \mu\text{m}$ ($N= 7$ cells); $P=0.006$, Student's t-test. However, the number and ratio of PSD-95 positive was not significantly altered by GPR50 overexpression ($P=0.19$ and 0.16 respectively, Student's t-test). Thus, according to these results GPR50 increases the number of spines, but does not promote spine maturation in cortical neurons. This observation is similar to what was seen when assessing the effects of GPR50 on

dendritic complexity, i.e. significance was only achieved in the blinded experiments. Thus an additional replicate should be sought.

Although there were differences in the blinded and unblinded results, the data was pooled together to increase N. In this combined dataset, the significant increase in the overall spine density was retained (figure 3.11 C). GFP-transfected neurons had a density of 0.34 ± 0.08 spines per $10 \mu\text{m}$ and neurons transfected with GPR50 + GFP had a density of 0.76 ± 0.15 spines per $10 \mu\text{m}$ ($P=0.01$, Student's t-test, $N= 15-19$). Again, the density and ratio of PSD-95 positive spines were not significantly affected by GPR50 transfection ($P=0.35$ and 0.37 respectively). These results indicate that GPR50 may increase spine density in cortical neurons but does not appear to affect spine maturity. However, the inconsistency between the blinded and unblinded datasets means that these results should be treated tentatively.

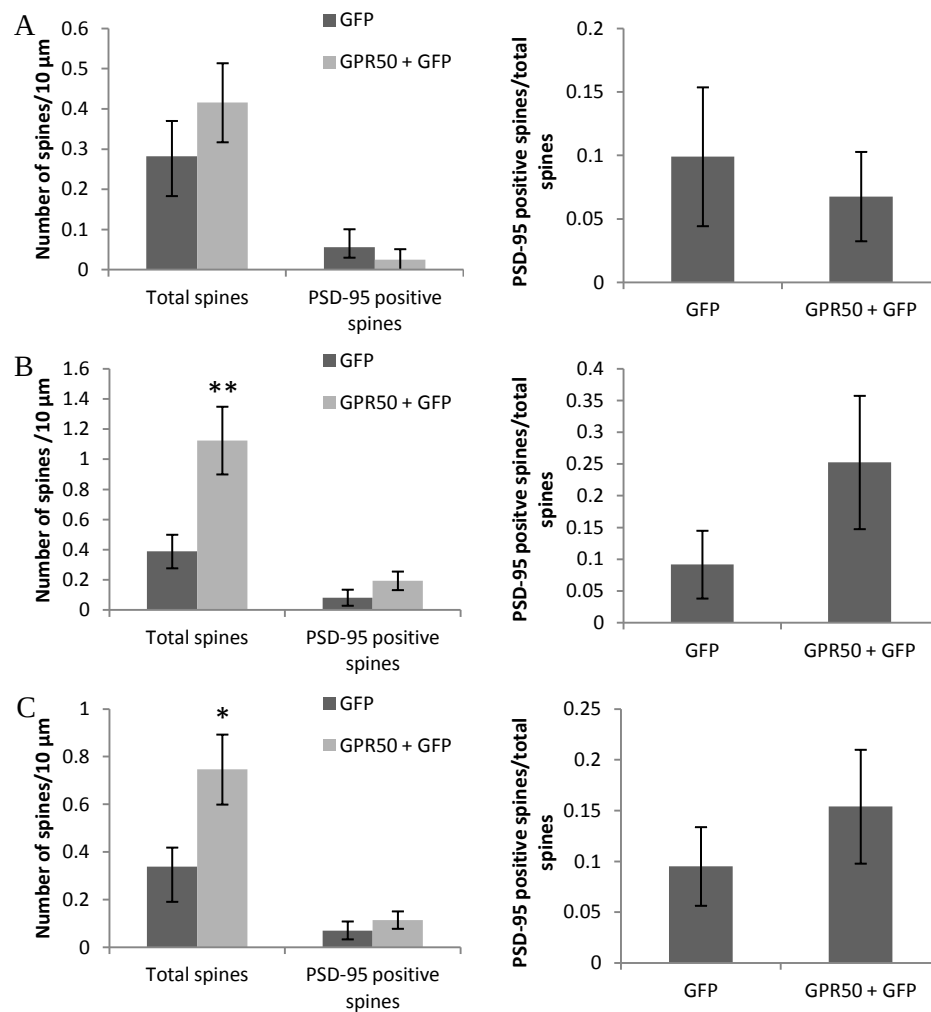


Figure 3.11: Effects of GPR50 overexpression on the number of postsynaptic spines in cortical neurons. Cortical neurons, DIV 14, were transfected with GFP with or without GPR50. ICC was performed on DIV 16 and dendritic spines were quantified using Fiji. (A) Unblinded results showed no significant changes to the overall spine density or the density of PSD-95 positive spines (left). Likewise the portion of PSD-95 positive spines was unaffected by GPR50, right. N= 9 GFP and 8 GPR50 + GFP transfected neurons. (B) In the blinded experiments, GPR50 transfection resulted in an increase in the total number of spines but not PSD-95 positive spines, left. The portion of PSD-95 positive spines was not significantly altered either, right. N= 10 GFP and 7 GPR50 + GFP transfected neurons, two independent experiments. (C) When the unblinded and blinded data were combined, a significant increase was seen in the total number of spines upon GPR50 overexpression (left). The number and proportion of PSD-95 immunoreactive spines were not significantly affected. N= 19 GFP and 15 GPR50 + GFP transfected neurons, from three experiments. Results presented as mean $\pm$ SEM; *P<0.05, **P<0.01, unpaired Student's t-test.

3.3.5 GPR50 transfection does not impact the morphology of dendritic spines

To further determine the effects of GPR50 on spine development, the length and head width of dendritic spines were measured using Fiji. Only hippocampal neurons were used here, as these had a greater spine density and were markedly affected by GPR50 transfection. It should be noted that this analysis was not carried out blind; hence, only the average results from the three replicates are presented. Further, due to the limitations of confocal microscopy these results may not provide precise measurements but highlight any relative changes to spine morphology.

Neurons that were doubly transfected (DIV 14) with GPR50 and GFP had an average spine length of $1.19 \pm 0.12 \mu\text{m}$ and head width of $0.60 \pm 0.03 \mu\text{m}$, whilst neurons transfected with GFP alone had an average length and width of 1.30 ± 0.18 and $0.58 \pm 0.07 \mu\text{m}$ respectively (figure 3.12 A and B). Comparisons of the mean spine length and head width for each condition did not yield any significant results (length: $P=0.23$, head width: $P=0.73$, Student's t-test, $N=21$ cells per condition, three independent experiments). A Kolmogorov–Smirnov (K-S) test was also used to compare the cumulative distributions of the datasets, again results were not significant (length, $P=0.19$; head width, $P=0.36$). Thus, spine morphology does not appear to be affected by GPR50.

By measuring the length or head width of spines separately it is possible to miss information about the morphological class of spines (Yuste & Bonhoeffer, 2004). For example, measurements of spine length would not differentiate between two spines of the same length but one with a small head diameter (“thin”) and the other one large (“mushroom”). Likewise, if the head width were the same but one was short (“stubby”) and the other was long, measuring the width alone would mask this. To avoid such confounds, the ratio of the spine length to head width for each spine was calculated (figure 3.12 C). Comparisons of this ratio revealed no statistical

differences between GPR50 + GFP transfected neurons and GFP controls (2.12 ± 0.21 and $2.34 \pm 0.12 \mu\text{m}$ respectively; $P=0.13$, Student's t-test; $P=0.20$, K-S test). These results provide further evidence that GPR50 does not impact spine morphology.

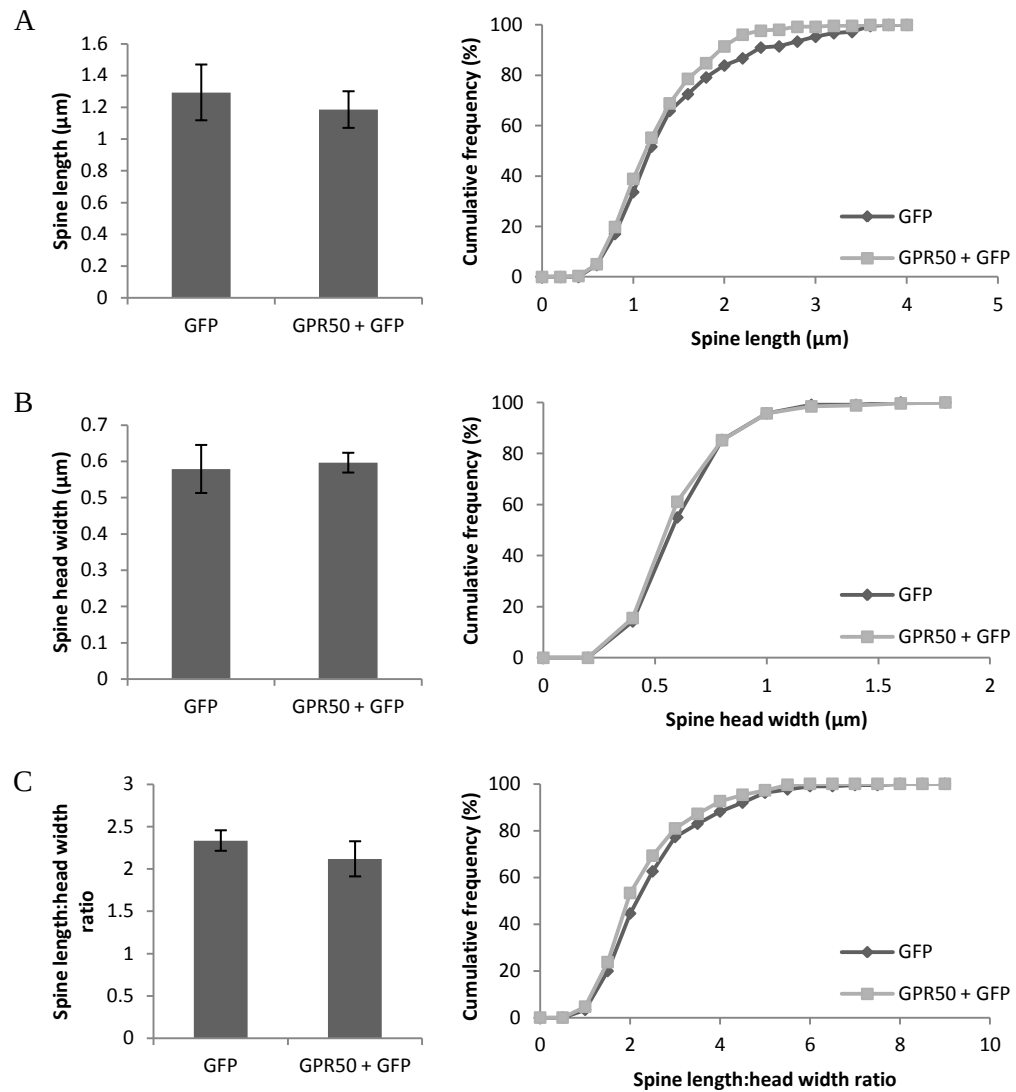


Figure 3.12: Overexpression of GPR50 does not impact the morphology of spines. Cultured hippocampal neurons (DIV 14) were transfected with GPR50 and GFP or GFP alone. On DIV 16, ICC was performed and dendritic spines were measured with Fiji. (A) The average length of spines did not significantly differ in neurons transfected with GPR50 and GFP compared to the GFP control (left). The cumulative distribution of spine length was not significantly altered either (right). (B) The average width of the spine head domain was not affected by GPR50 (left), nor were there any differences in the cumulative distribution (right). (C) No statistical differences were observed in the spine length to head width ratio.

Results presented as mean $\pm$ SEM; N= 21 neurons per conditions, from three independent experiment; Statistical tests, unpaired Student's t-test and Kolmogorov-Smirnov test.

3.4 Discussion

This chapter demonstrates that GPR50 promotes key aspects of neuronal differentiation and maturation. In SH-SY5Y cells, GPR50 expression led to an increase in the number of neurites per cell but not neurite length. Whilst in hippocampal neurons, transfection of GPR50 at DIV 14 significantly increased dendritic complexity. This increase appears to be the result of greater dendritic branching as GPR50 overexpression did not affect the number of primary dendrites. Conversely, an increase in primary dendrites was observed in cortical neurons upon GPR50 transfection. Interestingly, transfection of immature cortical neurons (DIV 7) with GPR50 had no effect on dendritic complexity or the number of primary dendrites, indicating the effects of GPR50 is temporally modulated.

GPR50 was also demonstrated to be a regulator of dendritic spines. Hippocampal neurons overexpressing GPR50 had significantly more spines compared to GFP transfected neurons. This suggests that GPR50 promotes the formation of spines or inhibits spine elimination. Further, in hippocampal neurons overexpressing GPR50, a greater proportion of spines contained PSD-95 clusters. This suggests a shift towards spine maturation. The morphology of these spines, however, was unaffected by GPR50, indicating that although GPR50 modulates spine density, it is unlikely to alter the strength of synaptic transmission. The effect of GPR50 transfection in cortical neurons of the same age was less dramatic. A significant increase in spine density was observed when transfected with GPR50, however, the amount of PSD-95 positive spines was not significantly affected. It should be noted that results generated using cortical neurons were inconsistent, thus should be regarded cautiously.

3.4.1 GPR50 induces neuronal differentiation

3.4.1.1 Promotion of neurite outgrowth in cell lines

The results presented here are somewhat contradictory to previously published work. In NS-1 cells, the transfection of GPR50 was reported to increase the length of neurites but not the number (Grünewald et al, 2009). Here, the number of neurites increased upon GPR50 transfection, whilst the length of neurites was unaffected (figure 3.2). This discrepancy may reflect the different cell types used. SH-SY5Y cells, a human derived neuroblastoma cell line, were used in this investigation rather than NS-1 cells, a rat cell line derived from an adrenal gland tumour. NS-1 cells are commonly used as quantifiable neurites can be readily generated; however, it is possible that the non-neuronal origin means GPR50 affects these cells in a different manner. However, an increase in neurite formation and elongation are both characteristics of neuronal differentiation (Polleux & Snider, 2010). Therefore, the work presented here, along with Grünewald's report, implicates GPR50 in neurite outgrowth and differentiation.

3.4.1.2 Differential effects of GPR50 on hippocampal and cortical neurons

The results obtained from the overexpression of GPR50 in primary neurons further supports a role in outgrowth and differentiation. In hippocampal neurons, the transfection of GPR50 increased dendritic branching, spine density and led to a shift towards spine maturity, features characteristic of a more fully developed neuron. GPR50 had a weaker effect on spine density in cortical neurons and did not significantly alter the number of PSD-95 positive spines. Further, GPR50 appeared to increase the number of primary dendrites in cortical neurons, rather than dendritic branching. These changes are also elements of neuronal differentiation, although characteristic of an earlier stage of the maturation process (figure 3.1). The difference between the results generated from cortical neurons and hippocampal neurons may imply GPR50 has a distinct role in these neurons.

The contrast between the hippocampal and cortical results may also reflect differences in neuronal maturity. Neurite outgrowth in cortical neurons has been reported to be slower than in hippocampal neurons (Kim & Lee, 2012). Furthermore, the results presented in this chapter showed significantly fewer spines developed in cortical neurons compared to hippocampal neurons under the GFP-transfected condition, following transfection at DIV 14 (figure 3.7). Cortical neurons also displayed an abundance of filopodia, which have been shown to be precursors of dendritic spines (Yuste & Bonhoeffer, 2004; Zuo et al, 2005). These results suggest that cortical neurons were less mature than hippocampal neurons when ICC was performed. If this experiment was repeated in older cortical neurons, the results generated may have been more comparable to the hippocampal neurons. This is a pertinent matter to investigate as GPR50 is found in various regions of the brain, especially in the hypothalamus, so being able to extrapolate these findings to different types of neurons would provide a wealth of information.

The lack of consistency between the experiments using cortical neurons should also be addressed. Morphological changes upon GPR50 expression were observed in the blinded and pooled datasets, but not the initial unblinded experiment. It is possible that the unblinded procedure introduced experimenter bias; in this case it may be appropriate to discard the unblinded results. However, the impact of neuronal diversity should also be considered. Different neuronal subtypes can have very different structures in regards to the extent of dendritic branching and spine density (Kawaguchi et al, 2006; Voelgyi et al, 2009). Analysis that combines these different subtypes is likely to lead to a high degree of variation, especially if a particular subtype is disproportionately distributed amongst the different experiments. This could mask the true effects of GPR50. To minimise this confounding factor, only neurons with a pyramidal morphology were analysed in this chapter. However, cortical pyramidal neurons are also highly heterogeneous in regards to their function and structure (Morishima & Kawaguchi, 2006; van Aerde & Feldmeyer, 2013), apparently more so than hippocampal neurons (Mizuseki et al, 2011). This may

account for the inconsistent results generated when using cortical neurons and the differences compared to the hippocampal results. Increasing the number of cortical neurons analysed may help compensate for this.

3.4.1.3 How does GPR50 modulate neuronal differentiation?

Despite the issues discussed above, GPR50 has clear effects on hippocampal neurons and SH-SY5Y cells. A potential explanation for this may be the attenuation of NOGO-A. The overexpression of NOGO-A in NS-1 cells significantly reduced neurite outgrowth compared to GFP controls, however, co-expression with GPR50 prevented this (Grünewald et al, 2009). NOGO-A is also an important modulator of dendritic development. Mouse knockdown and overexpression experiments have shown NOGO-A negatively regulates the size and complexity of Purkinje neurons (Petrinovic et al, 2013). Furthermore, silencing *Nogo-A* in rats reduced spine density in pyramidal neurons and promoted a shift towards immature spines (Pradhan et al, 2010). As GPR50 had the opposing effect on dendritic morphology, i.e. it promoted dendritic complexity and spine formation, GPR50 may act by attenuating the actions of NOGO-A in primary neurons. This may occur via the interaction between the two proteins (Grünewald et al, 2009). Alternatively, NOGO-A and GPR50 may act on the same pathway but in opposing directions. This could be investigated by looking at the effects of GPR50 on neuronal morphology when *Nogo-A* is silenced.

3.4.2 Does GPR50 impact synaptic function?

It is tempting to propose GPR50 has a positive impact on neuronal function as genes inducing similar neuronal phenotypes promote synaptic transmission. An example of this is Neuregulin 1 (NRG1), a gene strongly associated with schizophrenia (Dutt et al, 2009). Like GPR50, NRG1 overexpression in cultured neurons increased spine density and, in addition, increased spine size (Barros et al, 2009; Li et al, 2007). The

inhibition of NRG1 signalling has been reported to suppress synaptic transmission, indicating that NRG1 is a positive modulator of both spine development and function (Li et al, 2007). Conversely, NOGO-A negatively regulated dendritic complexity and synaptic transmission size (Petrinovic et al, 2013). This negative regulation coincides with the negative impact of NOGO-A on spine density (Pradhan et al, 2010) and spine size (Petrinovic et al, 2013). Given the enhancement of dendritic development upon GPR50 transfection, it would be valuable to perform electrophysiology experiments to investigate whether GPR50 has a role in synaptic transmission.

In the absence of data regarding the impact of GPR50 on synaptic function, the effect on spine maturity was assessed. This was investigated by looking at the effect of GPR50 overexpression on the number of PSD-95 positive spines. PSD-95 is a major component of the postsynaptic density which is found in the head of spines that form mature excitatory synapses (El-Hussein et al, 2000). PSD-95 is essential for the stability of spines and the clustering of the glutamate receptors required for synaptic transmission and plasticity (Ehrlich et al, 2007; El-Hussein et al, 2000; Migaud et al, 1998). The number and proportion of spines expressing PSD-95 increased in hippocampal neurons transfected with GPR50, indicating an increase in maturity. This may also lead to a greater number of synaptic contacts, and therefore an overall increase in synaptic transmission. In cortical neurons, however, GPR50 did not significantly affect spine maturity, thus it is unlikely to alter synaptic transmission.

Another proxy to synaptic function is spine morphology. Larger spines are associated with stronger synaptic connections and memory formation, while smaller spines are linked to weaker connections and learning (Hung et al, 2008; Kasai et al, 2003). Thus, any changes to the size of dendritic spines in neurons overexpressing GPR50 could reflect the modulation of synaptic strength. However, GPR50 did not alter spine morphology as assessed by measurements of spine length and head width (figure 3.10). This indicates that GPR50 does not change the function of dendritic

spines. However, knockdown experiments may yield different results. Several studies have employed both overexpression and silencing techniques to find that whilst the knockdown of the protein of interest modulates the size (Choi et al, 2005) or density (Petrinovic et al, 2013) of dendritic spines, overexpression had no effect. Therefore, silencing experiments should be performed to confidently assess the effects of GPR50 on spine morphology. This would also strengthen the results reported in regards to dendritic complexity. However, spine size is only an indicator of synaptic strength; direct measurements using techniques such as electrophysiology are essential to determine whether GPR50 contributes to synaptic transmission.

There are additional issues to address in regards to the methods used to analyse dendritic spines in this chapter. Firstly, limitations to the resolution of confocal microscopy means measurements of such small structures, i.e. spines, would not be precise. Further, measurements were taken manually and are therefore subject to experimenter error. This may be improved by using a neuron modelling software, such as Neurolucida, which allows 3D reconstructions and automated measurements of dendritic spines (Halavi et al, 2012). Despite the technical limitations, however, the techniques employed in this chapter are routinely used for morphological assessments. Although measurements are not exact, changes to spine morphology relative to a control can be detected. Thus, insight into the function of a protein, in this case GPR50, can still be gained. Another matter to consider is that ICC was performed on DIV 16. Neurons at this age undergo spinogenesis, however, it may be at an early stage, figure 3.1 (Polleux & Snider, 2010). Potentially, this may be too early for fully developed spines to appear at a high density. GPR50 may accelerate spinogenesis so that larger PSD-95 positive spines appear earlier than in control conditions, however, analysing spine morphology too early could miss this. This is especially problematic in regards to cortical neurons, which appeared to lag behind hippocampal neurons in terms of development. Assessing spine morphology at a later point may be more informative.

3.4.3 Correlating the role of GPR50 to its developmental profile

The role GPR50 plays in neuronal differentiation sheds some light on its developmental profile. Dendritic outgrowth and branching are key features of the fourth stage of maturation, which neurons enter late in embryonic development (figure 3.1, Polleux & Snider, 2010). Coincidentally, this is the period in which *Gpr50* expression in the mouse brain dramatically rises, i.e. E18 (Grünewald et al, 2012). This may significantly contribute to stage four of neuronal development by inducing the increase in dendritic arborisation characteristic of this phase. Spinogenesis tends to occur later, from the first postnatal week and peaks in adolescence (Markus & Petit, 1987; Polleux & Snider, 2010). This is noteworthy as *Gpr50* expression is maintained postnatally and rises again in adolescence, although to a lesser degree than at E18 (Grünewald et al, 2012). This postnatal expression of *Gpr50* may support for the formation, or stabilisation, of dendritic spines, as well as further dendritic branching.

It is intriguing that GPR50 appeared to have no impact on immature cortical neurons (transfected at DIV 7). This may be because GPR50 requires the presence of additional factors that are absent in immature neurons in order to exert its effect. Alternatively, factors expressed in younger neurons may inhibit the actions of GPR50. Interestingly, GPR50 transfection at DIV 7 was particularly difficult, despite the fact that younger neurons tend to be more amenable to transfection (Buerli et al, 2007). It may be that GPR50 expression is inhibited in young neurons to prevent premature differentiation. This may account for the low mRNA levels at early points of development (Grünewald et al, 2012). To fully understand the different outcomes of GPR50 transfection in immature and mature neurons, work must be done to reveal the mechanisms behind its temporal regulation. However, as the actions of GPR50 on cortical neurons transfected on DIV 14 was less apparent than in hippocampal neurons, it is possible that the lack of a morphological change in immature cortical neurons was due to the type of neuron tested. Repeating this

experiment in hippocampal neurons may yield different results, and therefore, is necessary to conclude that GPR50 transfection has no impact on the structure of immature neurons.

It should be noted that the cultured neurons used in this chapter were harvested from mice at E17-18. This means that most neurons are likely to be fairly mature prior to dissociation. Therefore, these experiments may be recording the actions of GPR50 overexpression on previously polarised neurons, which may have retained some of their differentiated characteristics. This makes it harder to correlate the number of days *in vitro* to the developmental stages *in vivo* (as shown in figure 3.1). It may also mask the full extent of GPR50's influence on neuronal differentiation. To overcome this, dissections can be performed earlier, although, this would be restricted to time points where the hippocampus and cortex are sufficiently developed. Techniques such as *in utero* electroporation could also be used. Here, GPR50 would be prenatally introduced into neurons at selected regions of the brain. Dissections and cell culturing can then be performed as described in this chapter and changes to neuronal morphology can be assessed (Kwon et al, 2012). Alternatively, neurons can be visualised in organotypic (brain) slices, which maintains many aspects of the cellular environment found *in vivo* (Cho et al, 2007; Kwon et al, 2012).

3.4.4 The importance of neuronal development in psychiatric illness

The role of GPR50 in neuronal maturation fits in well with the growing body of evidence linking abnormal neurodevelopment to psychiatric disorders. For example, post-mortem studies have reported decreases in spine density in the prefrontal cortex of individuals with schizophrenia (Glantz & Lewis, 2000; Kolluri et al, 2005). Similar results were recently found in patients with major depressive disorder (MDD) (Kang et al, 2012). Microarray analysis was also performed using the samples derived from MDD patients; this revealed a significant reduction in the

expression of several genes which regulate the structure and function of synapses. Interestingly, Kang and colleagues also found the transcriptional repressor *GATA1* was upregulated in MDD patients. The overexpression of this gene in cortical neurons significantly decreased dendritic complexity, spine density and the expression of synapse-related genes. Furthermore, increased *GATA1* expression led to depression-like behaviour in rats. Thus, this study links neuronal atrophy to mental illness, and demonstrates how genes contributing to these structural changes may be responsible for the associated behavioural deficits.

There are also numerous studies that have shown antidepressants can promote neuronal development. Chronic treatment with fluoxetine, an antidepressant, increased the dendritic complexity of neurons in the dentate gyrus of mice (Wang et al, 2008). Antidepressants have also been reported to increase adult neurogenesis (Malberg et al, 2000). Furthermore, the inhibition of adult neurogenesis in mice was found to attenuate the beneficial effects of several antidepressants (Santarelli et al, 2003; Snyder et al, 2011). This indicates that the promotion of neurogenesis is central to the efficacy of drug treatments. However, adult neurogenesis is not required for all the therapeutic benefits associated with antidepressants (David et al, 2009), thus, drug efficacy can only be partly attributed to neurogenesis. These results show that to some extent neurodevelopment in the adult brain plays a role in psychiatric illness and is an important aspect of drug treatment. Thus, the involvement of GPR50 in neurodevelopment may contribute to its association with mental illness.

3.4.5 Conclusion

The data presented in this chapter suggests GPR50 promotes aspects of neuronal differentiation/maturation. GPR50 overexpression increased the number of neurites per cell in SH-SY5Y cells. In primary neurons, GPR50 increased dendritic

complexity and spine development. To increase confidence in these results, GPR50 knockdown experiments should be performed. This would not be applicable to SH-SY5Y cells, as this cell line does not express GPR50 endogenously. However, endogenous expression has been reported in cortical and hippocampal neurons (Grünwald et al, 2009; Grünwald et al, 2012). It would also be beneficial to assess the effects of GPR50 transfection at different time points, especially in regards to cortical neurons. This would help determine whether the phenotypic manifestation of GPR50 overexpression is directed by the cell type or the developmental stage. To build on the results presented in this chapter, it is also necessary to determine the mechanism by which GPR50 modulates cell morphology. One possibility is that GPR50 alters the organisation of the cytoskeleton. Cytoskeletal rearrangement is crucial for neurite outgrowth, dendritic branching and spine formation (Hotulainen & Hoogenraad, 2010; Koleske, 2013). Furthermore, various risk factors associated with psychiatric disorders, such as NRG1, are involved in cytoskeleton dynamics (Kamiya et al, 2005; Sparrow et al, 2012). The involvement of GPR50 in this process shall be explored in following chapter.

Chapter 4: GPR50 localises at lamellipodia-like structures and activates the RAC1-WAVE pathway

4.1 Introduction

In chapter three, GPR50 was demonstrated to be a positive regulator of neurite outgrowth, dendritic complexity and spine density. How GPR50 regulates this is the next question to address. There are numerous modulators of neuronal morphology, many of which act through members of the Rho family of GTPases, in particular ras-related C3 botulinum toxin substrate 1 (RAC1), cell division cycle 42 (CDC42) and ras homolog family member A (RHOA). These act on a diverse range of effector molecules to regulate a variety of cellular responses (some of these are shown in figure 4.1). RAC1 and CDC42 are regarded as promoters of dendritic outgrowth and spine formation. The suppression of these molecules dramatically reduces spine density and causes the simplification of the dendritic tree (Nakayama et al, 2000; Scott et al, 2003). Conversely, RHOA is a negative regulator; its constitutive activation causes the reduction of dendritic complexity (Nakayama et al, 2000). Modulation of Rho GTPases by GPR50 may provide some explanation for the results seen the previous chapter.

Once in the active GTP-bound state, Rho GTPases initiate signalling cascades which ultimately modulate actin dynamics. As shown in figure 4.1, CDC42 and RAC1 promote actin polymerisation via Wiskott-Aldrich syndrome protein (WASP) and WASP-family verprolin-homologous protein (WAVE) respectively (Heasman & Ridley, 2008). These factors are instrumental in the development of dendritic spines (Soderling et al, 2007; Wegner et al, 2008). RHOA acts via Rho-associated protein kinase (ROCK) to regulate actin organisation (Draber et al, 2012; Sit & Manser, 2011). This effector appears to have a critical role in the negative effects of RHOA on dendritic morphology; ROCK activation alone is sufficient to reduce dendritic branching in pyramidal neurons (Nakayama et al, 2000). GPR50 may act on these actin remodelling pathways to modulate dendritic morphogenesis. This may be through the regulation of Rho GTPases or their effectors, thus the effects of GPR50 on various components of the actin remodelling pathway should be investigated.

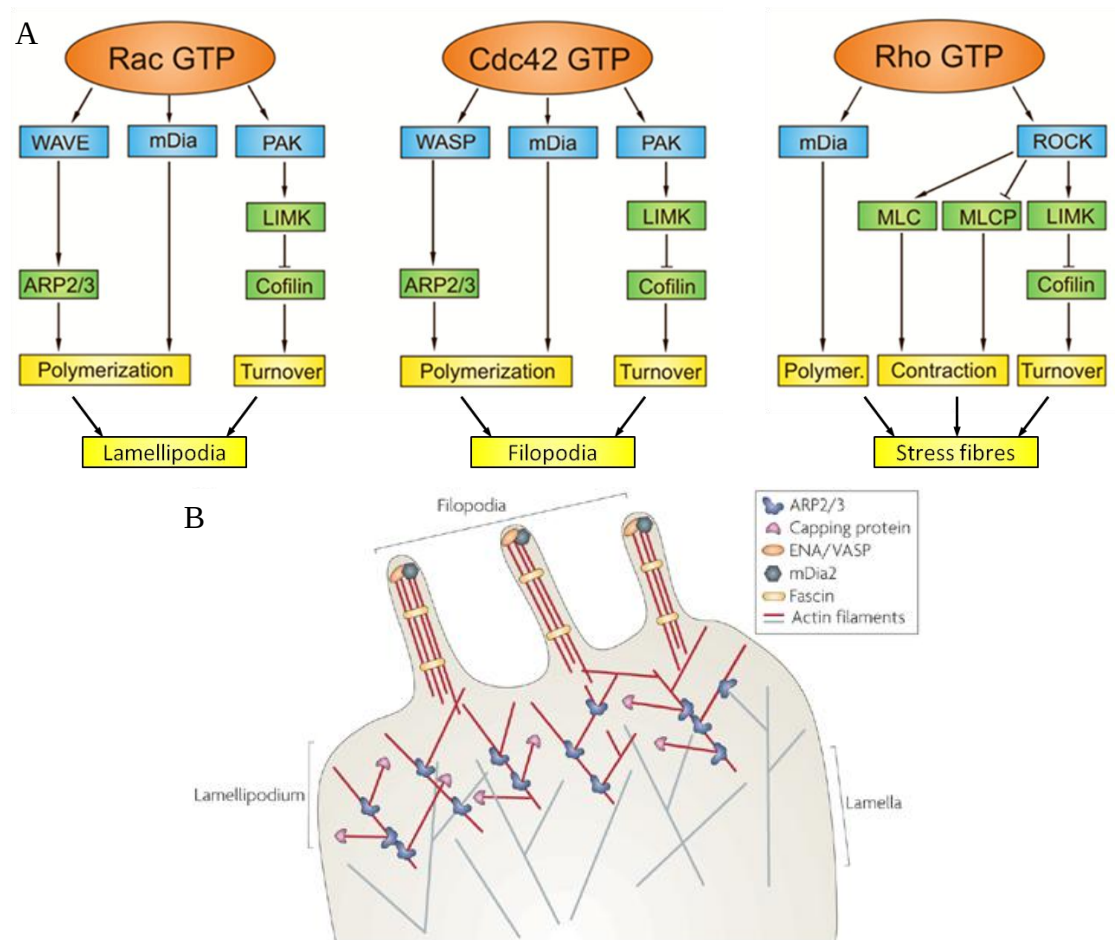


Figure 4.1: Regulation of actin organisation. (A) Highlights some of the key functions of Rho GTPases. RAC promotes the formation of lamellipodia through the activation of the WAVE complex, which mediates the formation of branched actin filaments via ARP2/3. CDC42 induces the activation of a number of effectors including WASP, which ultimately leads to filopodia formation. RHO has an important role in the formation of stress fibres, through the activation of ROCK and mDia, required for the retraction of the trailing edge of motile cells. Figure adapted from Draber et al, 2012. (B) Shows the structure of actin at the leading edge of a migrating cell. Actin is found in highly branched networks in the lamellipodia, whilst in filopodia actin is arranged in parallel bundles. Figure taken from Heasman & Ridley, 2008. Abbreviations: ARP2/3, actin-related protein-2/3; ENA/VASP, enabled/vasodilator-stimulated phosphoprotein; LIMK, LIM kinase; mDia, mammalian diaphanous; MLC, myosin regulatory light chain; MLCP, MLC phosphatase; PAK, p21-activated kinase; ROCK, RHO-associated protein kinase; WASP, Wiskott–Aldrich syndrome protein; WAVE, WASP-family verprolin-homologous protein complex.

A link between GPR50 and Rho GTPases may already exist. Neurite outgrowth inhibitor A (NOGO-A), the GPR50-interacting protein, activates RHOA and inhibits RAC1 (Kilic et al, 2010; Niederost et al, 2002). This would explain the inhibitory effects of NOGO-A on dendritic outgrowth and spine formation (Petrinovic et al, 2013; Pradhan et al, 2010). Furthermore, the inhibition of dendritic complexity and synapse formation by NOGO-66 receptor 1 is reliant on RHOA activation (Wills et al, 2012). GPR50 may counteract the inhibition of neurite outgrowth via NOGO-A (Grünewald et al, 2009) by attenuating its ability to modulate Rho GTPase signalling. Alternatively, GPR50 may modulate Rho GTPase activity of its own accord, leading to the changes in neuronal morphology induced by GPR50 overexpression (chapter three).

GPR50 may also modulate cytoskeletal organisation via α -catenin. This is the protein that links cadherin adhesion molecules to the actin cytoskeleton, via β -catenin (Miller et al, 2013). This linker activity occurs when α -catenin is in its monomeric form (Desai et al, 2013; Hansen et al, 2013); as a homodimer it inhibits actin branching via ARP2/3 (Drees et al, 2005; Hansen et al, 2013). It is the monomeric properties of α -catenin that are of greatest interest in the context of GPR50, as evidence suggests GPR50 binds to cadherin 8 (Grünewald et al, 2012). This may disrupt the α -catenin-mediated bridge between the cadherin/ β -catenin complex and filamentous actin (F-actin). This linker property is crucial for the regulation of dendritic growth and arborisation by neural cadherin (Tan et al, 2010). Further, silencing and overexpression studies have demonstrated that α -catenin promotes the stability of dendritic spines and branches (Abe et al, 2004; Tan et al, 2010). If GPR50 impacts α -catenin, changes to actin organisation and dendritic morphology would be expected.

4.2 Aims

This chapter set out to investigate whether GPR50 has a role in the modulation of the cytoskeleton, in particular actin organisation. To do this, the effect of GPR50 transfection on the localisation and cellular levels of the major cytoskeleton molecules, i.e. microtubules, intermediate filaments and microfilaments, was examined. A more targeted approach was used to investigate whether GPR50 alters the levels of key regulators in actin nucleation and polymerisation. Finally, another link to actin remodelling was explored by determining whether GPR50 acts on α -catenin.

4.3 Results

4.3.1 GPR50 has no effect on the amount or localisation of the major components of the cytoskeleton

The effect of GPR50 on the cytoskeleton was investigated in the neuronal SH-SY5Y cell line. Cells were transfected with GPR50 or an empty vector (pDEST40 vector), transfection rate ~ 40%, and a western blot was performed the following day. Western blot analysis confirmed the transfection of GPR50, with a doublet band clearly visible at the expected size of 67 kDa (figure 4.2; see appendix A for antibody characterisation). Blots were then re-stained for β -actin, β -tubulin or vimentin (representing microfilaments, microtubules and intermediate filaments respectively). Using the imaging software Fiji (Schindelin et al, 2012), band densities were quantified to show there were no differences between the levels of these cytoskeletal proteins in GPR50-transfected cells compared to the empty vector controls (figure 4.2; β -actin, $P=0.43$; β -tubulin, $P=0.79$; vimentin, $P=0.55$; Student's t-test, $N= 3$ replicates).

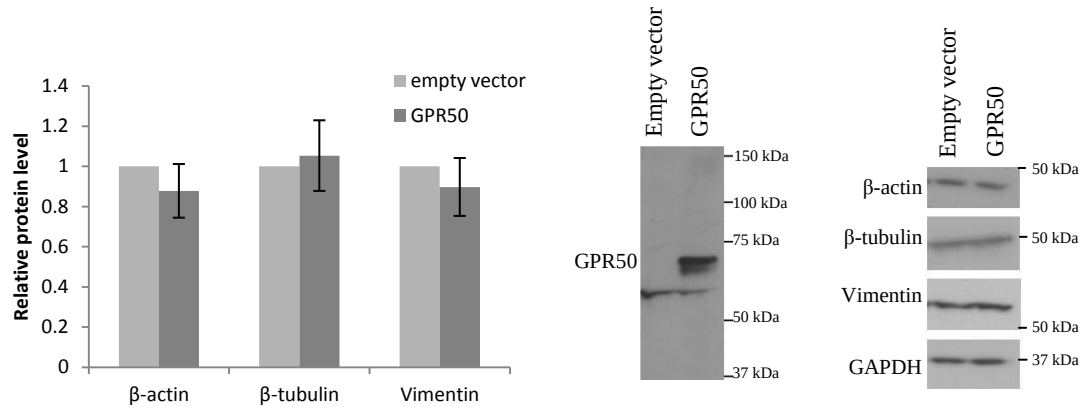


Figure 4.2: GPR50 overexpression has no effect on the expression of the major cytoskeletal elements in SH-SY5Y cells. Cells were transfected with GPR50, or an empty vector, and 24 hours later a western blot was performed. Protein levels were quantified by measuring the density of the appropriate band using Fiji, this was normalised with GAPDH and the data is presented relative to the empty vector control. The average levels of β -actin, β -tubulin and vimentin were not altered by GPR50 transfection, left. Representative western blots are shown on the right, including the confirmation blot showing GPR50 transfection was successful. Results shown as mean $\pm$ SEM; N= three independent experiments; Statistical test: paired Student's t-test.

Immunocytochemistry (ICC) was carried out to assess the cellular distribution of these filaments following GPR50 transfection (figure 4.3). β -Actin was distributed throughout the cell and characteristic structures such as stress fibres, cortical actin and lamellipodia were clearly visible (figure 4.3 A). β -Tubulin was also found throughout the cell in a filamentous form. It was mainly concentrated at the centre of the cell but also extended to the plasma membrane (figure 4.3 B). Finally, vimentin was arranged in intracellular filaments surrounding the cell nucleus (figure 4.3 C). These distribution patterns were observed regardless of GPR50 expression.

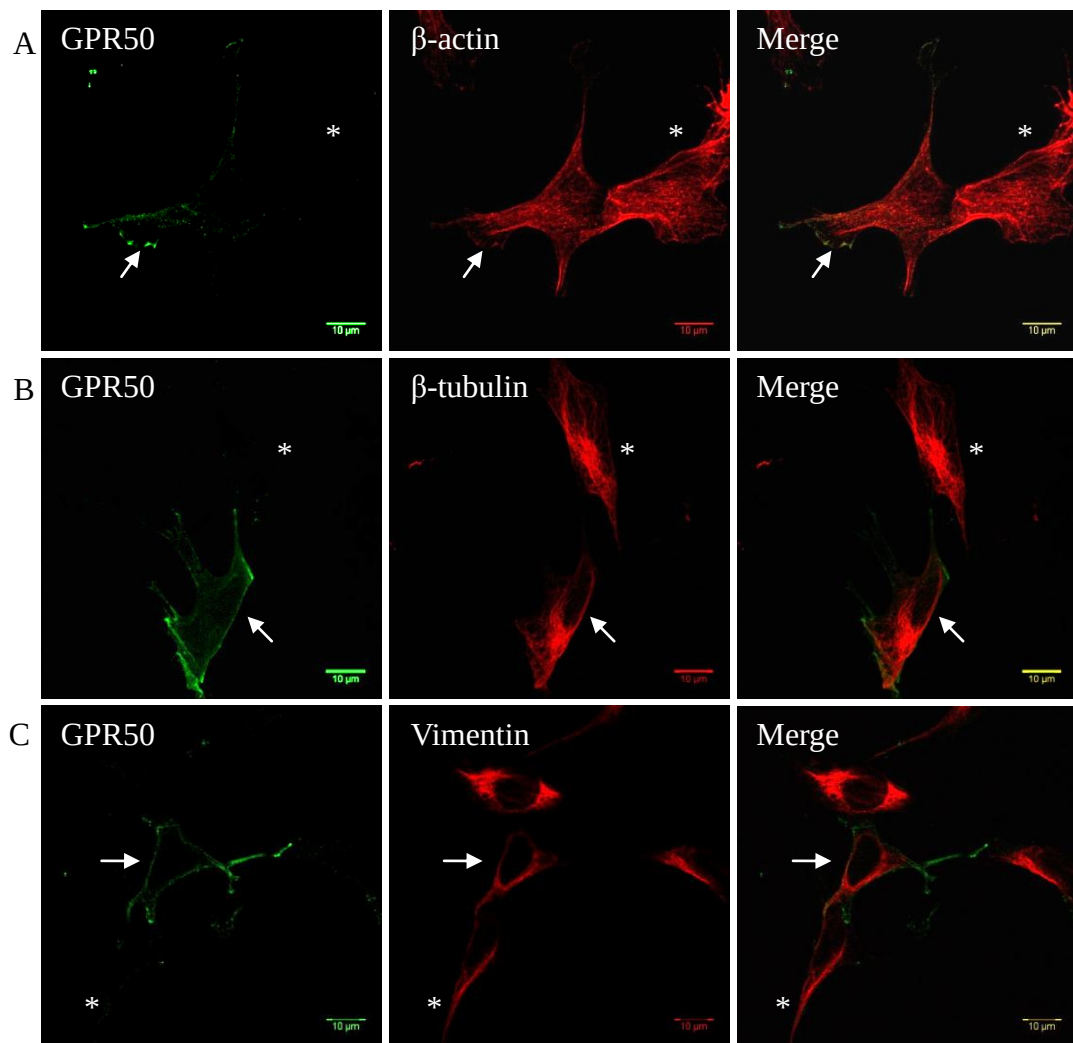


Figure 4.3: GPR50 overexpression does not alter the cytoskeleton in SH-SY5Y cells. Cells were transfected with GPR50 and ICC was performed the following day staining for GPR50 and the appropriate cytoskeletal filament. Representative results from over three independent experiments are shown. (A) No observable differences were detected in the organisation of β -actin in GPR50-expressing (arrow) and non-expressing (*) cells. (B) GPR50 overexpression did not affect the organisation of filamentous β -tubulin. (C) Intracellular localisation of vimentin was maintained in GPR50-expressing cells (arrow).

Scale bar, 10 μ m.

4.3.2 Enrichment of GPR50 at lamellipodia-like structures in SH-SY5Y cells

Upon investigating the effects of GPR50 on the cytoskeleton, it was noted that GPR50 was enriched at lamellipodia-like structures, i.e. flat veil-like protrusions of the plasma membrane. Thus, ICC was performed on SH-SY5Y cells transfected with GPR50 to assess the colocalisation between GPR50 and lamellipodia-associated proteins; namely β -actin and β -catenin (Etienne-Manneville & Hall, 2003; Suraneni et al, 2012).

The imaging software ImageJ (Schneider et al, 2012) was used to measure the colocalisation between GPR50 and β -actin. Colocalisation analysis, as assessed by the Pearson's correlation coefficient (R_r), revealed a moderate positive correlation between the GPR50 and β -actin fluorescence signals; $R_r = 0.40 \pm 0.01$ ($N = 31$ cells, three independent experiments), where 1 reflects a perfect positive correlation and 0 represents no relationship. The average Manders' coefficient (R) also indicated moderate colocalisation; $R = 0.63 \pm 0.01$, where 1 represents complete overlap between two signals and 0 represents none. This increased to 0.71 ± 0.02 when measuring the extent to which GPR50 overlaps with β -actin, indicating GPR50 largely colocalised with β -actin. Finally, the intensity correlation quotient (ICQ) also indicated a modest colocalisation between β -actin and GPR50; $ICQ = 0.20 \pm 0.01$, where +0.5 indicates that the intensities of two proteins vary in synchrony and 0 represents an absence of co-variation. Together, these results indicate the colocalisation between GPR50 and β -actin was moderate.

Interestingly, colocalisation between GPR50 and β -actin appeared to be restricted to lamellipodia and neurite tips (figure 4.4 A). Other areas of the membrane were largely devoid of GPR50. From the intensity profile generated for this image, figure 4.4 B, it can be seen that GPR50 expression peaked with β -actin at the neurite tip (peak 1) and lamellipodia (peak 3). In contrast, where cortical actin was present

(peak 2), β -actin was abundant but GPR50 was limited. This indicates that GPR50 preferentially localised to the lamellipodia and growth cones.

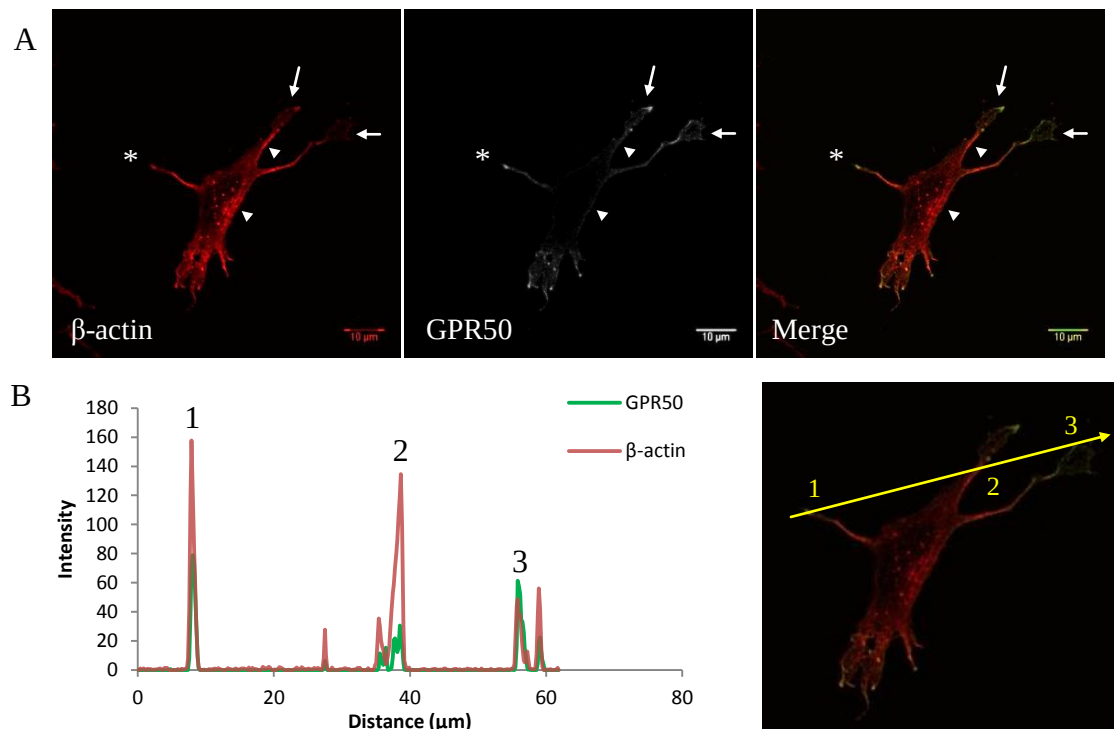


Figure 4.4: GPR50 colocalises with β -actin at the lamellipodia of SH-SY5Y cells. ICC was performed on GPR50-transfected cells staining for GPR50 and β -actin. (A) A representative image from three independent experiments shows exogenous GPR50 mainly aggregated at the lamellipodia (arrow) where it colocalised with β -actin. Areas rich in cortical actin (arrowhead) were largely deficient in GPR50. Scale bar, 10 μ m. (B) The intensity profile of this image (left) shows the expression of GPR50 peaked with β -actin at the neurite tip and lamellipodia (peaks 1 and 3 respectively). GPR50 was weaker where cortical actin was seen (peak 2). The line used to generate this profile is shown on the right, with the corresponding points labelled.

To focus on the localisation of GPR50 at the lamellipodia, ICC was performed, staining for GPR50 and β -catenin, which has been shown to localise at the lamellipodia of migrating cells (Etienne-Manneville & Hall, 2003), although it is not exclusively located at these sites. Results show GPR50 colocalised with β -catenin ($R_r = 0.52 \pm 0.02$; $R = 0.78 \pm 0.01$; $ICQ = 0.19 \pm 0.01$; $N = 27$ cells, three independent experiments). Again colocalisation was most abundant at the lamellipodia (figure

4.5 A). This can clearly be seen in the intensity profile, which shows the expression of GPR50 peaked with β -catenin at the leading edge of the cell (figure 4.5 B; peak 1).

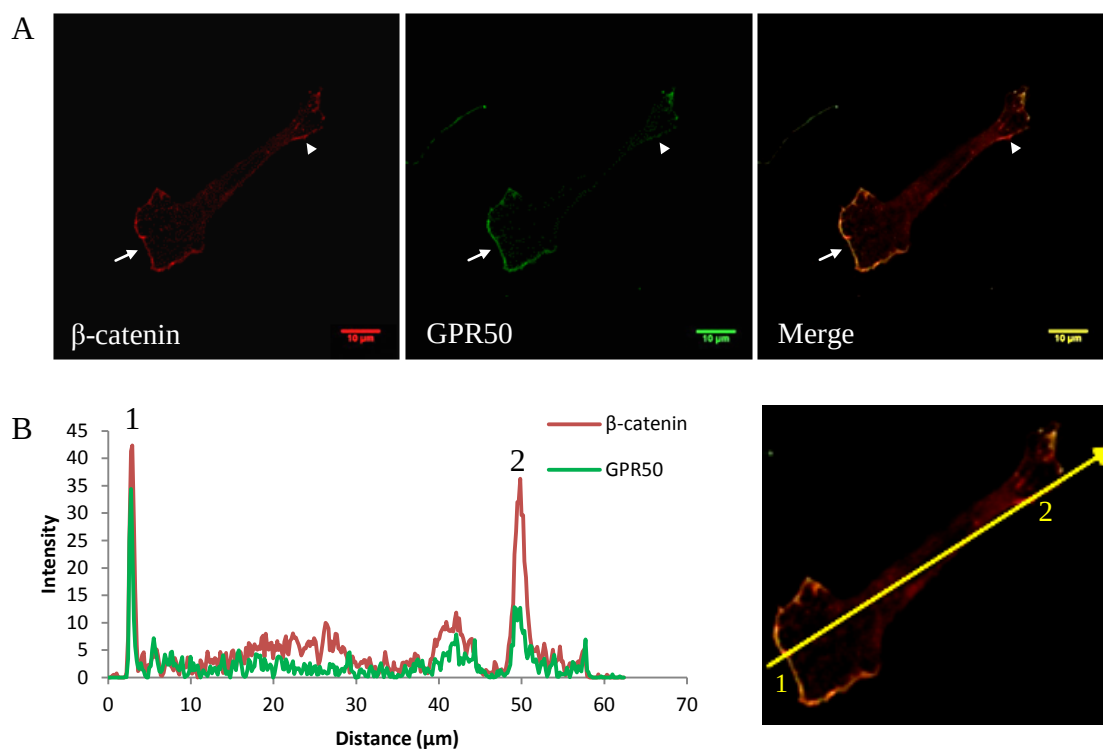


Figure 4.5: GPR50 is enriched at the lamellipodia of SH-SY5Y cells. GPR50 was transfected into SH-SY5Y cells and ICC was performed the following day staining for GPR50 and β -catenin. (A) A representative image, from three independent experiments, showing GPR50 colocalised with β -catenin at the leading edge (arrows). Other areas with high levels of β -catenin (arrowhead) displayed lower amounts of GPR50. Scale bar, 10 μ m. (B) The intensity of GPR50 peaked with β -catenin at the leading edge of the cell (peak 1). Peak 2 shows a non-lamellipodial region of the plasma membrane with high levels of β -catenin but little GPR50. The corresponding line used to generate this profile is shown on the right.

4.3.3 GPR50 is enriched in dendritic spines of hippocampal neurons

Dendritic spines share a very similar structure to lamellipodia. Both consist of highly branched networks of F-actin (dendritic actin) and are regulated by many of the same actin remodelling factors, including ARP2/3 and the WAVE complex (Hotulainen & Hoogenraad, 2010). Thus, in neurons, GPR50 may be enriched in dendritic spines. This was shown to be true in a subcellular fractionation experiment (Grünewald et al, 2012). In this chapter, ICC was performed to provide further support. Hippocampal neurons were transfected with GPR50 at DIV 19 and fixed 48 hrs later. Neurons were stained with antibodies against GPR50 and postsynaptic density 95 (PSD-95), β -actin or β -catenin, proteins known to accumulate in dendritic spines (Hotulainen & Hoogenraad, 2010; Togashi et al, 2002).

In hippocampal neurons, transfected GPR50 was found on the plasma membrane of dendrites (figure 4.6 A). The intensity of GPR50 appeared to be strong at dendritic spines, where a high degree of colocalisation with PSD-95 was observed (figure 4.6 A, asterisk). The intensity profile generated from this image shows GPR50 expression was strongest in dendritic spines, where it peaked with PSD-95 (figure 4.7, peaks 4, 7 and 9). Although GPR50 is not exclusively located at dendritic spines as high expression can be seen in other areas of dendrites (figure 4.7). Colocalisation between GPR50 and β -actin also occurred in dendritic spines (figure 4.6 B, asterisk). In contrast, GPR50 and β -catenin did not appear to colocalise (figure 4.6 C). β -Catenin was found within the dendritic shaft, i.e. the segment of the dendrite carrying spines, rather than in the dendritic spines. This may be consistent with reports showing β -catenin resides in the dendritic shaft and moves into spines upon neuronal stimulation or the formation of mature synaptic contacts (Murase et al, 2002; Togashi et al, 2002).

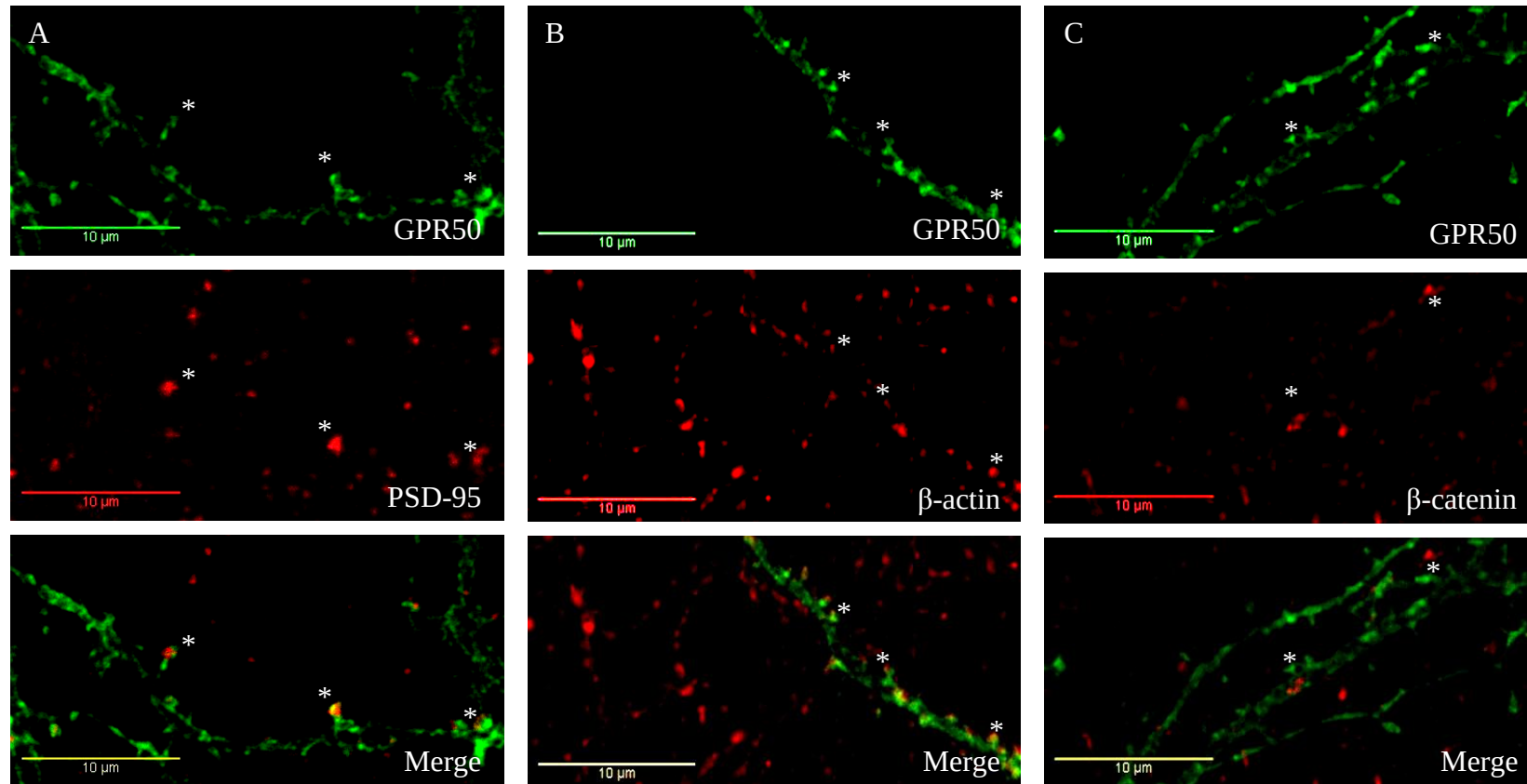


Figure 4.6: GPR50 is expressed in the dendritic spines of hippocampal neurons. Primary hippocampal neurons (DIV 19) were transfected with GPR50 and its dendritic localisation was assessed by ICC staining for GPR50 and the relevant dendritic protein. (A) GPR50 was found on the plasma membrane of dendrites, especially at dendritic spines where it colocalised with PSD-95 (*). (B) GPR50 also colocalised with β -actin at dendritic spines (*). (C) GPR50 and β -catenin did not colocalise; GPR50 was found in spines (*) and other areas of the plasma membrane, whilst β -catenin was located within the dendritic shaft (arrow). Scale bar, 10 μ m.

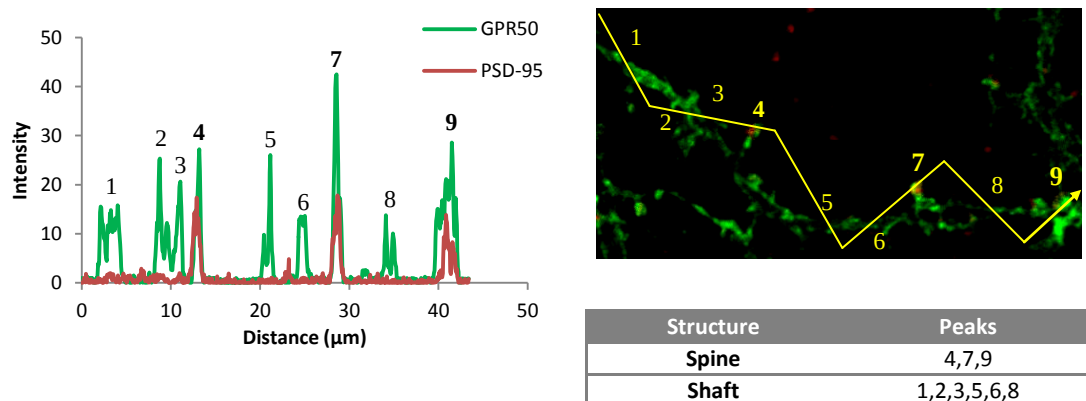


Figure 4.7: Expression of GPR50 is enriched in postsynaptic spines. Intensity profile (left) of a GPR50-transfected hippocampal neuron (DIV 19) stained for GPR50 and PSD-95; the image used to generate the profile is shown on the right, with the corresponding points labelled. Results show colocalisation between PSD-95 and GPR50, indicating GPR50 is found in postsynaptic spines (peaks 4, 7 and 9). GPR50 was also found at the dendritic shaft (peaks 1-3, 5, 6 and 8).

4.3.4 GPR50 modulates key components of the actin remodelling pathway

The aggregation of GPR50 in areas of the membrane containing dendritic actin may have important functional implications. GPR50 may localise here because it is directed to these sites, possibly via interactions with factors enriched in these areas. Alternatively, GPR50 itself may induce the formation of dendritic actin. To explore this possibility, SH-SY5Y cells were transfected with GPR50 or an empty vector and a western blot was performed the following day. After confirming the transfection of GPR50, the blot was probed for key regulators of actin remodelling, namely RAC1/CDC42, WAVE-2, N-WASP, ARP2 and profilin-1. The overexpression of GPR50 significantly increased the levels of phosphorylated RAC1/CDC42 and WAVE-2 by $45 \pm 8\%$ and $64 \pm 4\%$ respectively (phospho-RAC1/CDC42, $P=0.03$; WAVE-2, $P=0.004$; Student's t-test, $N=3$ replicates; figure 4.8). Total RAC1, N-WASP, ARP2 and profilin-1 were not significantly affected by GPR50 ($P<0.1$, $N=3-4$ replicates). Table 4.1 summarises these results and highlights the roles of these proteins in actin organisation.

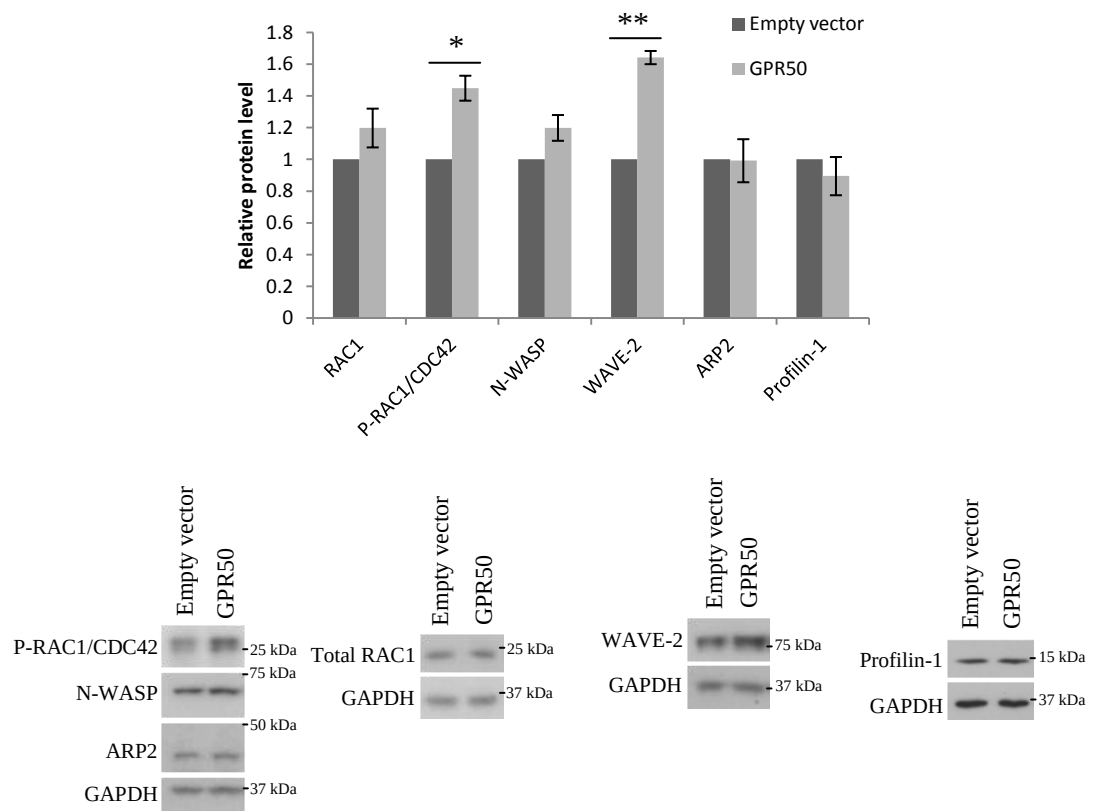


Figure 4.8: Regulators of actin remodelling are activated by GPR50. Western blotting results from SH-SY5Y cells transfected with GPR50 or an empty vector. Band densities were measured using Fiji and GAPDH served as the loading control. Results showed the transfection of GPR50 significantly increased the levels of phospho-RAC1/CDC42 and WAVE-2 compared to the empty vector control (top panel). The other proteins tested were not significantly affected by GPR50 overexpression. Representative western blots are shown below. Results shown as mean $\pm$ SEM; N= three independent experiments (N= four for total RAC1); *P<0.05, **P<0.01, paired Student's t-test.

Protein	Function	Effect of GPR50 overexpression
RAC1/CDC42	Promote lamellipodia and filopodia production respectively, through the regulation of downstream actin modulators such as WAVE-2, N-WASP and mammalian Diaphanous-related formins (Peng et al, 2003; Snapper et al, 2001; Steffen et al, 2013)	↑ phosphorylated form
WAVE-2	Activates the ARP2/3 complex to promote lamellipodia formation and cell spreading (Eto et al, 2007; Mendoza et al, 2011; Yamazaki et al, 2005).	↑
N-WASP	Activated by CDC42 to induce ARP2/3-mediated actin nucleation, which promotes filopodia formation (Martinez-Quiles et al, 2001; Miki et al, 1998; Snapper et al, 2001)	NS
ARP2	Forms a complex with ARP3 to induce actin polymerisation and branching (Rouiller et al, 2008; Smith et al, 2013). This supports lamellipodia and filopodia formation, cell spreading and cell migration (Korobova & Svitkina, 2008; Suraneni et al, 2012).	NS
Profilin-1	Modulates actin dynamics by binding to monomeric globular-actin (G-actin) and promoting the exchange of G-actin-bound ADP for ATP (Wen et al, 2008; Wolven et al, 2000). This enhances lamellipodia formation and cell migration (Mouneimne et al, 2012; Syriani et al, 2008).	NS

Table 4.1: The functions of the modulators of actin dynamics tested in this study. Key: ↑, significant increase in protein level; NS, no significant effect.

Activation of RAC1 is associated with the translocation to the plasma membrane (Bustelo et al, 2012; Even-Ram et al, 2007). It can then recruit and activate the WAVE complex leading to the subsequent induction of lamellipodia formation (Tahirovic et al, 2010). ICC was used to determine whether RAC1 localised to the plasma membrane of GPR50-transfected SH-SY5Y cells, giving additional insight to its level of activity. As can be seen in figure 4.9, RAC1 was found throughout the cell, especially at the lamellipodia and other areas of the plasma membrane. Interestingly, RAC1 colocalised with GPR50 ($R_r = 0.53 \pm 0.04$; $R = 0.81 \pm 0.02$;

ICQ= 0.20 ± 0.02 ; N= 10 cells, from one experiment). Again, colocalisation was found at lamellipodia-like structures (figure 4.9 A, arrows). This was also demonstrated in the corresponding intensity profile (figure 4.9 B), in which the fluorescence intensities of GPR50 and RAC1 peaked at lamellipodia-like regions, i.e. peaks 1 and 4. Conversely, little colocalisation was seen in peaks 2 and 3, which correlate to more stable areas of the membrane, likely to contain cortical actin. RAC1 was present at these sites, whereas GPR50 was largely absent. Thus, GPR50 may modulate RAC1 at specific areas of the plasma membrane leading to localised changes in actin organisation.

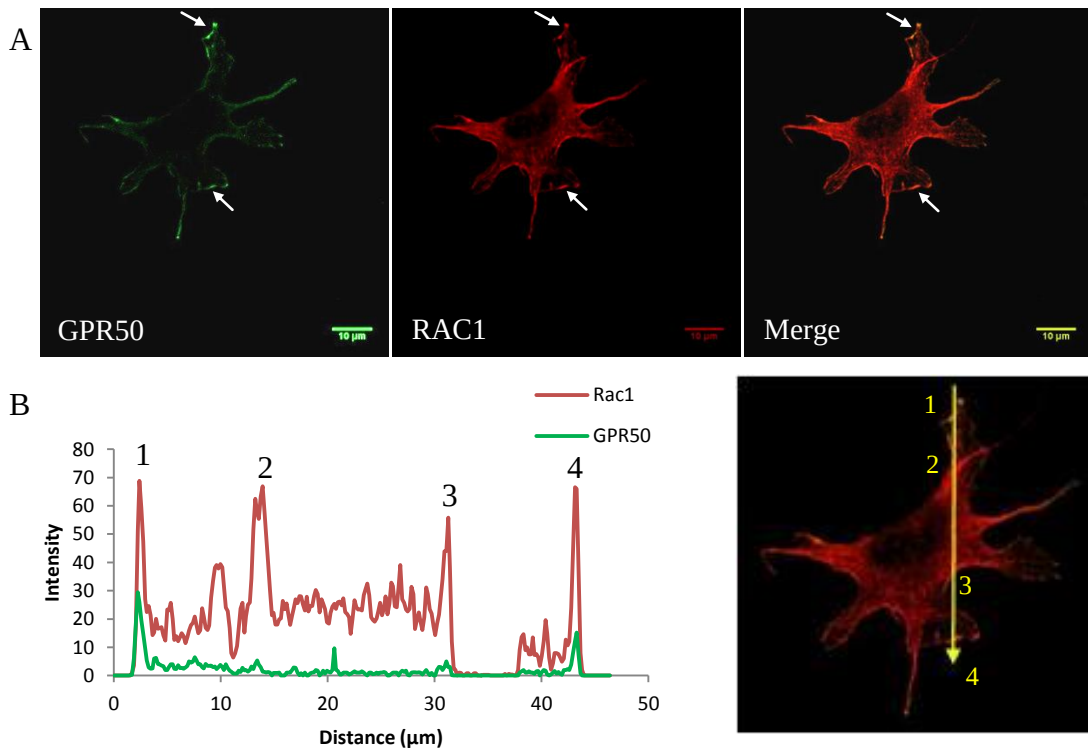


Figure 4.9: GPR50 colocalises with RAC1 at lamellipodia in SH-SY5Y cells. ICC was performed on GPR50 transfected SH-SY5Y cells, staining for GPR50 and RAC1. (A) A representative image shows GPR50 colocalised with RAC1 at lamellipodia-like structures (arrow). Scale bar, 10 μ m. (B) The intensity profile (left), with the corresponding image displayed on the right, shows GPR50 levels peaked with RAC1 at lamellipodia-like structures (peaks 1 and 4) and not other areas of the membrane (peaks 2 and 3).

4.3.5 Increased mTORC2 activity upon overexpression of GPR50

The mammalian target of rapamycin complex 2 (mTORC2) was investigated as a potential mediator of the increased phosphorylation of RAC1 by GPR50 transfection. It has been well recognized that mTORC2 modulates actin dynamics and functions via RAC1 (Huang et al, 2013; Jacinto et al, 2004), making it a promising protein to study in relation to GPR50. To investigate the involvement of mTORC2, western blot analysis was performed to assess the effect of GPR50 transfection on the levels of AKT phosphorylated at serine (S) 473, a well established readout for mTORC2 activity (Huang et al, 2013). The level of GSK-3 β , a substrate of AKT, was also assessed. GSK-3 β has two phosphorylation sites; one at S9, which is phosphorylated by AKT leading to its inhibition (Feng et al, 2004; Sarbassov et al, 2005). The other site is at tyrosine (Y) 216, which is auto-phosphorylated to increase GSK-3 β activity and is independent of mTORC2/AKT activity. The effect of GPR50 overexpression on both GSK-3 β phosphorylation sites was investigated. This phosphorylation/activation pathway is summarised in figure 4.10.

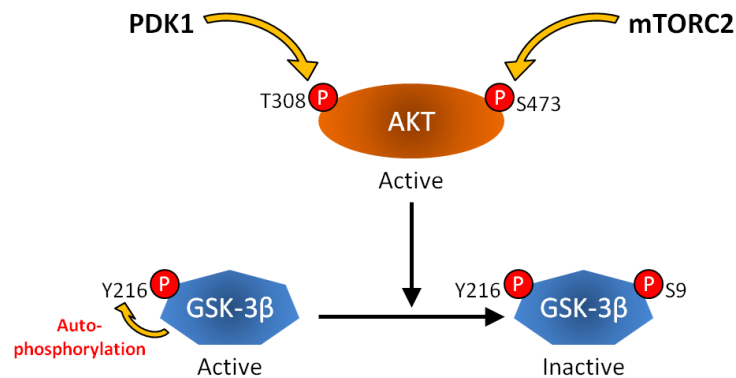


Figure 4.10: Phosphorylation pattern of AKT and GSK-3 β . AKT is phosphorylated at serine (S) 473 by mTORC2 and threonine (T) 308 by phosphoinositide-dependent kinase 1 (PDK1). This leads to the activation of AKT, which phosphorylates GSK-3 β at S9, leading to its inactivation. GSK-3 β is additionally auto-phosphorylated at tyrosine (Y) 216, which increases its activity levels. Schematic by the author.

SH-SY5Y cells were transfected with GPR50 or the empty vector and the following day cell lysates were subjected to western blot analysis. First, the effect of GPR50 on the absolute levels of AKT and GSK-3 β was assessed. GPR50 had no effect on the total protein levels (figure 4.11 A; $P > 0.1$, Student's t-test, $N = 3-6$ experiments). In an independent series of experiments, the effect of GPR50 overexpression on the phosphorylated forms of these proteins was investigated. Transfection of GPR50 increased the levels of phospho-AKT (S473) by $33 \pm 6.4\%$, (figure 4.11 B, $P = 0.02$, Student's t-test, $N =$ four replicates), indicating an increase in AKT activity. However, GPR50 did not increase the levels of phospho-GSK-3 β (S9) (figure 4.11 B, $P = 0.25$, Student's t-test, $N =$ four replicates), thus, the increased phosphorylation of AKT (S473) may not have been sufficient to increase its kinase activity. Interestingly, there was an apparent increase in phospho-GSK-3 β (Y216), however, this did not reach significance (figure 4.11 B; $P = 0.08$, Student's t-test, $N =$ four experiments). This may reflect a trend, thus further replicates may be necessary to confirm this observation. Together, these results indicate that phospho-AKT (S473) is specifically increased, which is likely to reflect an enhancement of mTORC2 activity.

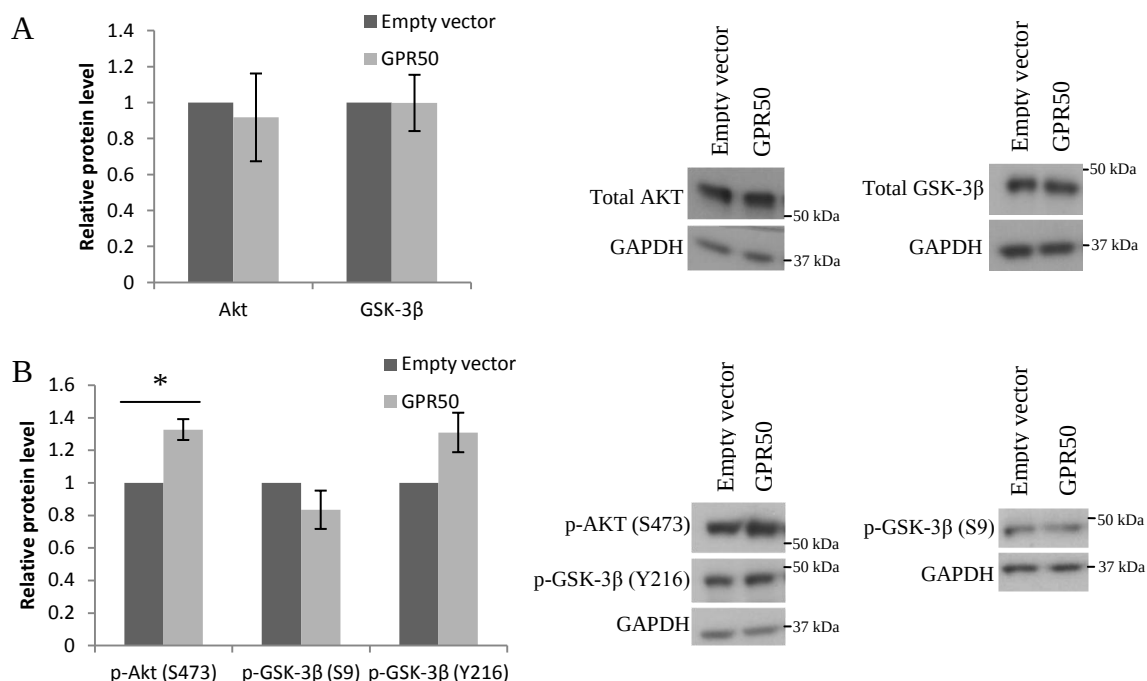


Figure 4.11: GPR50 transfection increases phosphorylation of AKT at S473. Western blot analysis of SH-SY5Y cells following transfection with GPR50 or an empty vector. Band densities were measured using Fiji, with GAPDH as the loading control. Results are presented relative to the empty vector control. (A) GPR50 transfection had no effect on the total levels of AKT and GSK-3β. N= three replicates in the case of AKT and six for GSK-3β; western blots representative of these results are shown on the right. (B) The overexpression of GPR50 led to a significant increase in the amount of phospho-AKT (S473). Phospho-GSK-3β (S9 and Y216) was not significantly affected. N= four independent experiments, with representative blots shown on the right. Results shown as mean ± SEM; *P<0.05, paired Student's t-test.

4.3.6 GPR50 alters the cellular distribution of α-catenin in SH-SY5Y cells

The interest in actin remodelling was also explored by examining the effects of GPR50 on α-catenin, the linker molecule that bridges cadherins to the actin cytoskeleton (Desai et al, 2013). To investigate this, SH-SY5Y cells were transfected with GPR50 or an empty vector and α-catenin protein levels were assessed by western blot analysis. As shown in figure 4.12, GPR50 expression had no effect on the amount of α-catenin compared to the empty vector control (P=0.17,

Student's t-test, N= six experiments). In addition, β -catenin, which mediates the interaction between cadherin and α -catenin, was unaffected by GPR50 (figure 4.9, P=0.53, Student's t-test, N= six experiments). This indicates that GPR50 does not regulate actin polymerisation via α - or β -catenin.

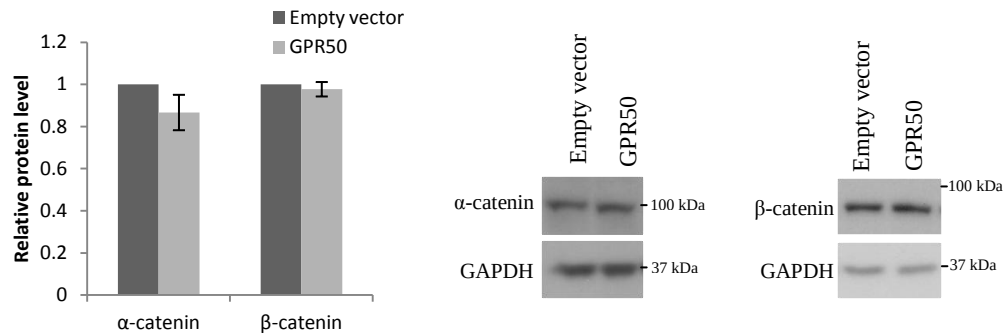


Figure 4.12: Levels of α and β -catenin are not affected by GPR50 in SH-SY5Y cells. Western blot analysis of SH-SY5Y cells transfected with GPR50 or an empty vector. Protein levels were measured using Fiji to quantify the band densities, which were normalised to GAPDH. Results are presented relative to the control. No significant changes to the amount of α -catenin or β -catenin were detected in response to GPR50 transfection (left). Representative western blots can be seen on the right. Results shown as mean $\pm$ SEM; N= six independent experiments; statistical test, paired Student's t-test.

In preliminary experiments, however, the localisation of α -catenin appeared to be modulated by GPR50. Focus was placed on α -catenin rather than β -catenin as it is α -catenin that has the capacity to modulate actin organisation (Desai et al, 2013; Drees et al, 2005; Hansen et al, 2013). Further, any disruption to β -catenin at the plasma membrane would ultimately affect the distribution of α -catenin given their interaction (Drees et al, 2005). SH-SY5Y cells were transfected with GPR50 or GFP and ICC was performed the following day, staining for α -catenin, β -actin and GPR50. In GFP-transfected cells, the distribution of α -catenin was predominately found at lamellipodia (figure 4.13 A). However, upon transfection with GPR50, α -catenin was less apparent at the lamellipodia and often assumed a radial distribution (figure 4.13 B). This pattern was found in 38% of cells transfected with GPR50 and

15% of GFP-transfected cells. However, this was quantified from one experiment with an N of 20 GFP-transfected cells and 15 GPR50-transfected cells. Before any conclusions are drawn, further replicates are essential. Although it may be better to develop this by looking at whether GPR50 transfection alters the colocalisation between α -catenin and a robust lamellipodia marker such as ARP2/3 or abl-interactor 1 (Yang et al, 2007).

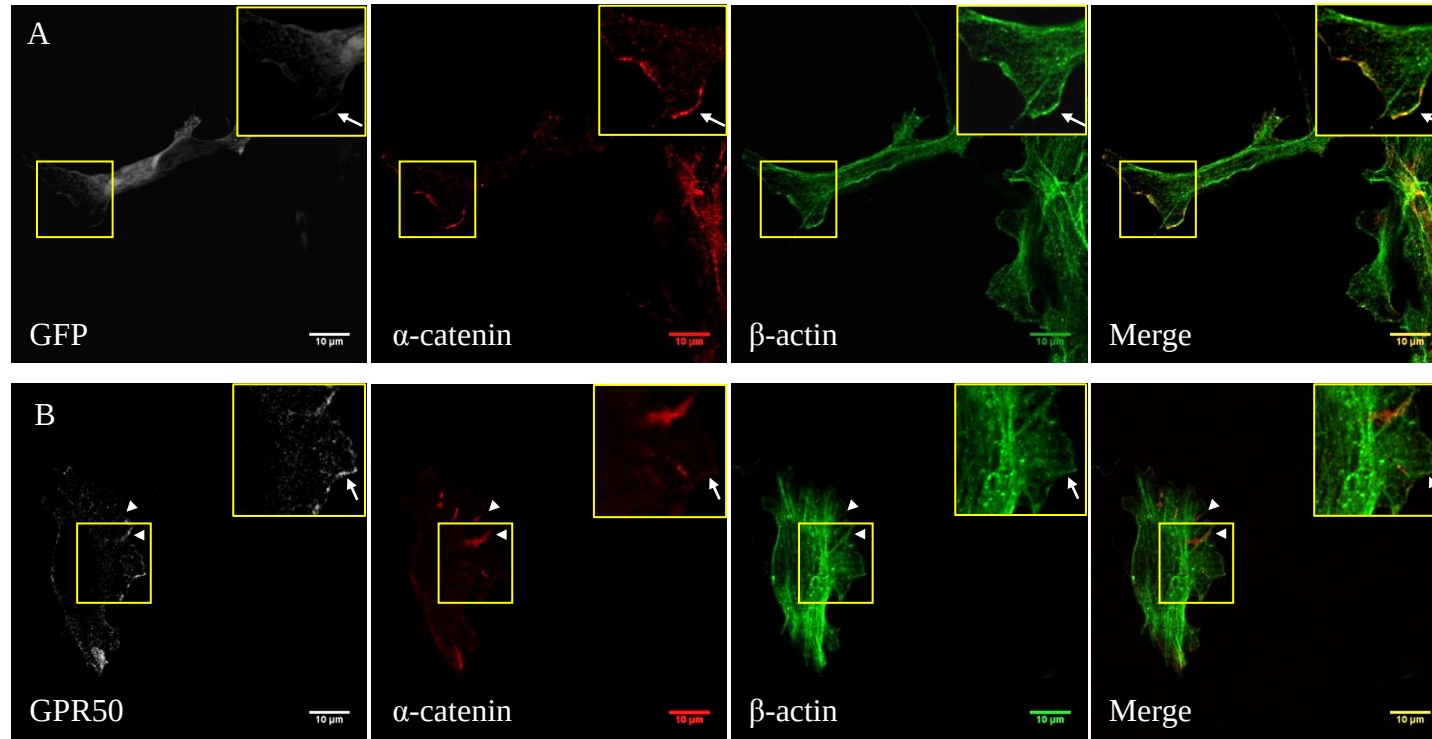


Figure 4.13: GPR50 alters the cellular distribution of α -catenin in SH-SY5Y cells. ICC results from SH-SY5Y cells transfected with GFP or GPR50 and stained for GPR50, α -catenin and β -actin. (A) In GFP-transfected cells, α -catenin typically had a plasma membrane distribution, this was particularly apparent at lamellipodia (arrow). (B) In GPR50-transfected cells, α -catenin was less abundant at lamellipodia (arrow). Further, there was an increased radial distribution of α -catenin (arrowhead).

Scale bar, 10 μ m.

4.3.7 In HEK293 cells GPR50 decreases α -catenin levels but does not alter its localisation

The cadherin/catenin interaction is essential for cell-to-cell adhesion. Such contacts are more readily detected in HEK293 cells than in SH-SY5Y cells, thus HEK293 cells were also tested. In this cell line, the level of α -catenin was significantly lower in GPR50-transfected cells compared to those transfected with an empty vector (figure 4.14; $25 \pm 3\%$ decrease, $P=0.005$, Student's t-test, $N=$ four replicates). On the other hand, β -catenin was not significantly affected by GPR50 overexpression ($P=0.10$, Student's t-test, $N=$ five replicates). This suggests that the decrease in α -catenin was not due to a limited availability of β -catenin. In terms of protein localisation, α -catenin was found at cell-to-cell junctions of GFP-transfected cells, as expected (figure 4.15 A, arrows). GPR50 transfection did not appear to alter this localisation (figure 4.15 B, arrows).

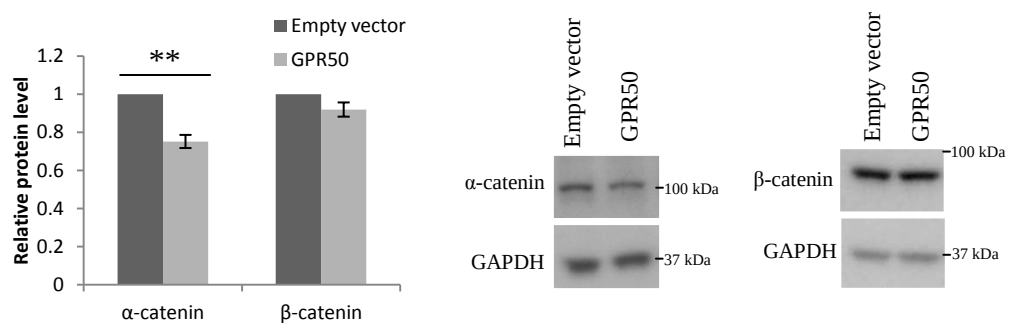


Figure 4.14: Decrease in α -catenin levels upon GPR50 transfection in HEK293 cells. Western blot analysis of HEK293 cells transfected with GPR50 or an empty vector. Band densities were quantified with Fiji and normalised with GAPDH; results are presented relative to the empty vector control. GPR50 overexpression significantly reduced levels of α -catenin but did not affect β -catenin levels (left). Representative western blot results are displayed on the right. Results shown as mean $\pm$ SEM; $N=$ four independent replicates for the α -catenin experiments and five for β -catenin; ** $P<0.01$, paired Student's t-test.

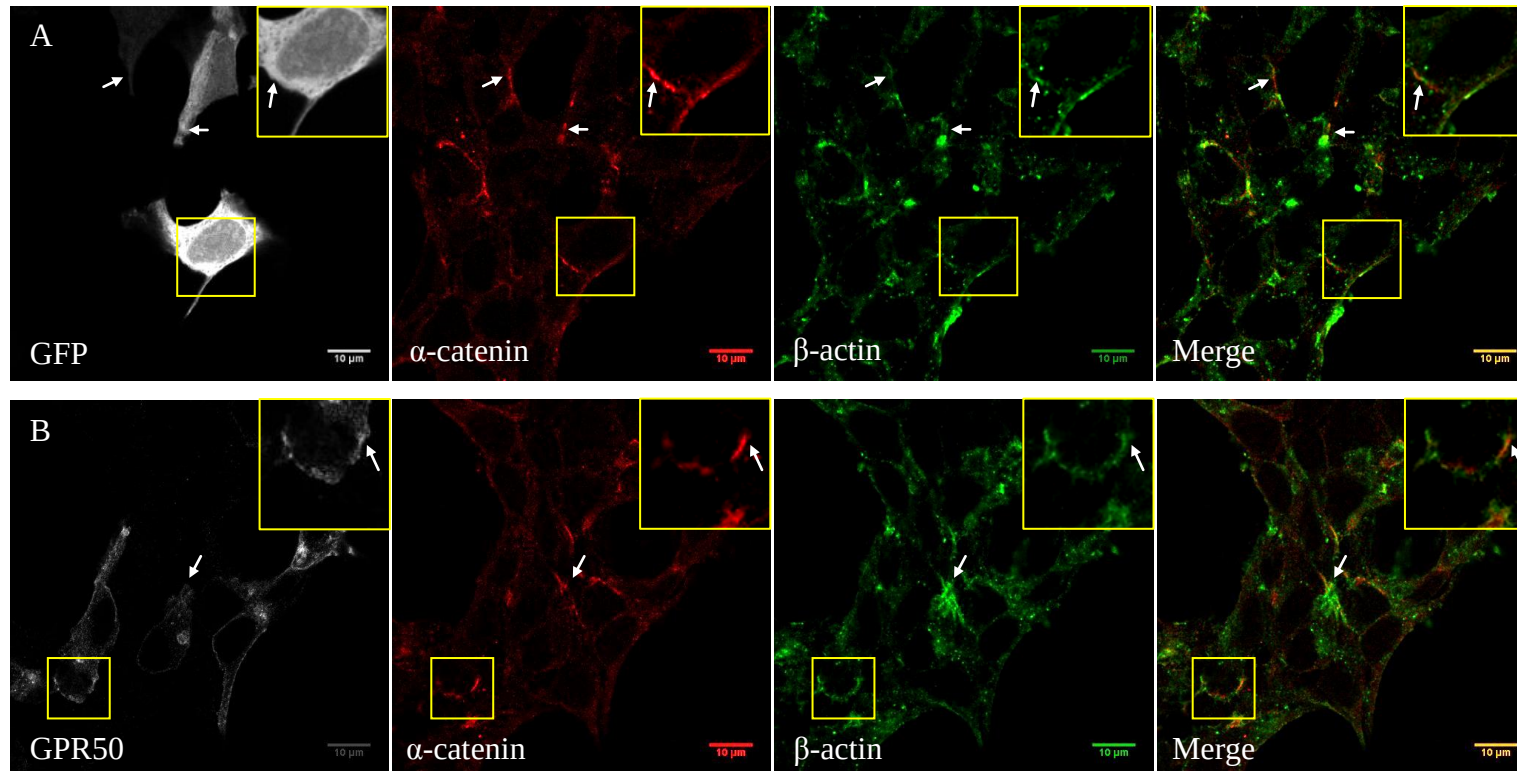


Figure 4.15: GPR50 overexpression does not alter the localisation of α -catenin in HEK293 cells. ICC was performed on HEK293 cells 24 hrs after transfection with GPR50 or GFP, staining for GPR50, α -catenin and β -actin. (A) Localisation of α -catenin was most predominant at cell-to-cell contact sites (arrows) in GFP-transfected cells. (B) Upon GPR50 transfection, α -catenin maintained its localisation at cell-to-cell junctions (arrows). Scale bar, 10 μ m.

4.4 Discussion

The aim of this chapter was to determine whether GPR50 affects cytoskeletal organisation. The overexpression of GPR50 in SH-SY5Y cells did not impact the levels of β -actin, β -tubulin or vimentin; the cellular organisation of these proteins was also unaffected. However, GPR50 did appear to localise with F-actin at the lamellipodia. This was supported by the colocalisation with β -catenin, a factor known to localise to the lamellipodia of migrating cells. In addition, GPR50 transfection increased levels of phospho-RAC1/CDC42 and WAVE-2, indicating the induction of actin remodelling. GPR50 overexpression also led to a significant increase in phospho-AKT (S473), suggesting GPR50 enhances mTORC2 activity. Finally, GPR50 overexpression led to changes in the localisation of α -catenin in SH-SY5Y cells and reduced its protein level in HEK293 cells. As α -catenin also modulates actin organisation, changes to its cellular distribution or levels may have an impact on the structure of actin. Thus, this chapter highlights two mechanisms by which GPR50 may influence actin organisation: by regulating the RAC1-WAVE pathway and by preventing the accumulation of α -catenin at the lamellipodia.

4.4.1 GPR50 promotes the RAC1-WAVE pathway

Although not explicitly shown, the results presented in this chapter indicate that GPR50 activates RAC1. The uncertainty stems from the antibody used in this investigation. This was specific for the phosphorylated form of RAC1/CDC42 (S71), which has been shown to exclusively localise at the plasma membrane and is predominately found in the active GTP-bound state (Schoentaube et al, 2009; Schwarz et al, 2012). Thus, this antibody is used as an indicator of active RAC1/CDC42 (Faraone et al, 2006; John et al, 2004). However, it is not possible to distinguish between phospho-RAC1 and phospho-CDC42. The differentiation between RAC1 and CDC42 is important as they activate different pathways; the

former promotes lamellipodia formation via the WAVE complex and the later favours filopodia via N-WASP (figure 4.1). Crucially, GPR50 had a different impact on the effector proteins; WAVE-2 was markedly increased by GPR50 transfection, whilst N-WASP was not significantly affected (figure 4.8). This suggests that WAVE-2 was activated; activation increases its stability, which would result in an apparent increase in protein levels (Dubielecka et al, 2011). As RAC1 activates the WAVE complex rather than WASP, GPR50 is likely to signal via RAC1. The activation of RAC1 would also be in keeping with the attenuation of NOGO-A by GPR50 (Grünewald et al, 2009). NOGO-A has been shown to suppress RAC1 activity (Niederost et al, 2002), thus GPR50 may antagonise NOGO-A via the enhancement of RAC1 activity.

Although these results are suggestive, it may be necessary to show GPR50 specifically activates RAC1, especially as the phosphorylation of RAC1/CDC42 does not always correlate with its functional activity. For example, the phosphomimetic form of RAC1/CDC42 (S71) appeared to promote nuclear factor- κ B-mediated signalling, suggesting an increase in activity (Schwarz et al, 2012). However, the phosphomimetic protein also had a reduced affinity to certain interactors including the Specifically Rac1-associated protein (Sra-1); this implies a reduced level of activity. Therefore, direct measurements of GTP-bound RAC1, or CDC42, should be taken following GPR50 transfection. This can be achieved with a pull-down activity assay. This involves precipitating active GTP-bound RAC1/CDC42 from cell lysate using a GST-fusion construct containing the CDC42/RAC interactive binding (CRIB) region of p21-activated protein kinase (PAK). Western blots can then be performed to assess levels of active RAC1 or CDC42 using specific antibodies.

The effect of GPR50 transfection on the interaction between RAC1 and WAVE-2 should also be assessed. This would help determine whether the change in RAC1 activity is responsible for the apparent activation of WAVE-2. Alternatively, the

effect of RAC1 depletion, using genetic or pharmacological interventions, on the GPR50-induced increase in WAVE-2 levels can be monitored. If GPR50 is unable to induce WAVE-2 activation under such conditions, it may be concluded that GPR50 acts via RAC1 to modulate WAVE-2. However, redundancy between RAC1 and CDC42 could complicate this experiment, as a CDC42-mediated signalling cascade may be switched on to compensate for the absence of RAC1 (Wheeler et al, 2006). If this occurs, the effect of GPR50 may be obscured.

Despite the ambiguity regarding the activity of RAC1/CDC42, the increase in WAVE-2 indicates that GPR50 is able to promote lamellipodia development. When WAVE-2, or its isoform WAVE-1, is silenced, lamellipodia formation and stability is severely disrupted (Yamazaki et al, 2005). In light of this, the increase of WAVE-2 in response to GPR50 overexpression may lead to the promotion of lamellipodia formation or stability. It would be of great interest to explore the exact role GPR50 has here. Its presence at the membrane may lead to a localised activation of RAC1, which in turn may drive WAVE-2 to promote actin branching and lamellipodia production. Alternatively, GPR50 may be recruited to the lamellipodia after the structure has formed. In this case, the activation of the RAC1-WAVE pathway by GPR50 may result in the extension of existing lamellipodia, which is an important aspect of growth cone development and cell migration (Polleux & Snider, 2010; Suraneni et al, 2012). Time-lapse confocal imaging could be used to determine whether the localisation of GPR50 precedes or follows lamellipodia formation.

To develop these results, it is important to determine whether the GPR50-induced activation of RAC1/CDC42 and WAVE-2 translates to structural changes. This can be achieved by using highly motile cells, such as fibroblasts, in which lamellipodia dynamics are readily observable. When plated onto an adhesive substrate these cells will flatten and spread with a uniform lamellipodia surrounding the cell. If GPR50 is a positive regulator of lamellipodia development, silencing would impede normal cell spreading. This technique has been used to show WAVE-2 and ARP2/3 are

essential for lamellipodia formation (Suraneni et al, 2012; Yamazaki et al, 2005). A wound heal assay can also be performed. Here, a gap is created in a layer of cultured cells and the time taken for the “wound” to heal, i.e. the closure of the gap by migrating cells, is monitored. The silencing of factors that induce actin remodelling, such as ARP2/3, increases this recovery time (Suraneni et al, 2012). If GPR50 is also involved in actin dynamics, its overexpression or knockdown will influence the recovery time. These experiments should be performed to demonstrate that GPR50 actually induces lamellipodia formation; as they stand, the results presented in this chapter are only suggestive.

It should be noted that GPR50 does appear to induce some of the structural changes associated with actin remodelling in primary neurons. In chapter 3, GPR50 was shown to increase spine density in hippocampal neurons. This is important as dendritic spines closely resemble lamellipodia and are formed in a similar manner (Hotulainen & Hoogenraad, 2010; Soderling et al, 2007). In addition, GPR50 was enriched in dendritic spines (figure 4.7), thus it would be reasonable to hypothesise that GPR50 is capable of inducing actin remodelling in primary neurons. However, the effects of GPR50 overexpression on modulators of actin organisation was not investigated in primary neurons, this should be followed up.

4.4.2 Regulation of the RAC1-WAVE pathway may be mediated by mTORC2

Having observed that GPR50 acts on the RAC1-WAVE pathway, it was of high interest to discover how this was mediated. To this end, preliminary experiments were carried out to investigate the involvement of mTORC2. This seemed particularly fitting as the key function of mTORC2 is the regulation of the actin cytoskeleton. Silencing mTORC2 in NIH/3T3 cells, a fibroblast cell line, resulted in a marked reduction of cell spreading and the absence of F-actin (Jacinto et al, 2004). *In vivo* work has also shown a reduced ratio of F-actin to G-actin, in mTORC2 deficient mice (Huang et al, 2013). These mice also had diminished levels of phospho-PAK and phospho-cofilin, modulators of actin polymerisation (figure 4.1). Importantly, mTORC2 appears to signal through RAC1. RAC1 activity was diminished in the absence of mTORC2, whereas CDC42 was not altered (Huang et al, 2013; Jacinto et al, 2004). Further, constitutively active RAC1 has been shown to reverse the actin cytoskeleton defects associated with the loss of mTORC2, indicating it acts via RAC1 to modulate actin organisation (Jacinto et al, 2004). Thus, the activation of mTORC2 by GPR50 could explain its effects on the RAC1-WAVE pathway.

The results presented in this chapter indicate that GPR50 does activate mTORC2. This can be inferred by the increase in phospho-AKT (S473) levels in cells overexpressing GPR50. The activation of AKT is mediated by mTORC2 and PDK1, figure 4.16 (Alessi et al, 1997; Costa-Mattioli & Monteggia, 2013; Jacinto et al, 2006). These phosphorylate AKT on S473 and T308 respectively. Although full activation requires phosphorylation at both sites, the phosphorylation of T308 is sufficient to increase AKT activity (Feng et al, 2004; Sarbassov et al, 2005). Phosphorylation of S473 enhances this activity but also appears to regulate substrate specificity, e.g. the phosphorylation of FOXO1/3 and Bad is dependent on AKT phosphorylation at S473 (Jacinto et al, 2006; Murata et al, 2011). Conversely, TSC2 and GSK-3 β phosphorylation is not affected by phospho-AKT (S473), indicating that

the phosphorylation at T308 alone is sufficient to drive the activation of these proteins. The results in this chapter showed the level of phospho-GSK-3 β (S9) was not significantly affected by GPR50 (figure 4.11). As phosphorylation of this substrate requires the activation of AKT via T308 phosphorylation, this result indicates that GPR50 is unlikely to modulate the activity of PDK1. These results also indicate that GPR50 may play a role in driving the substrate specificity of AKT.

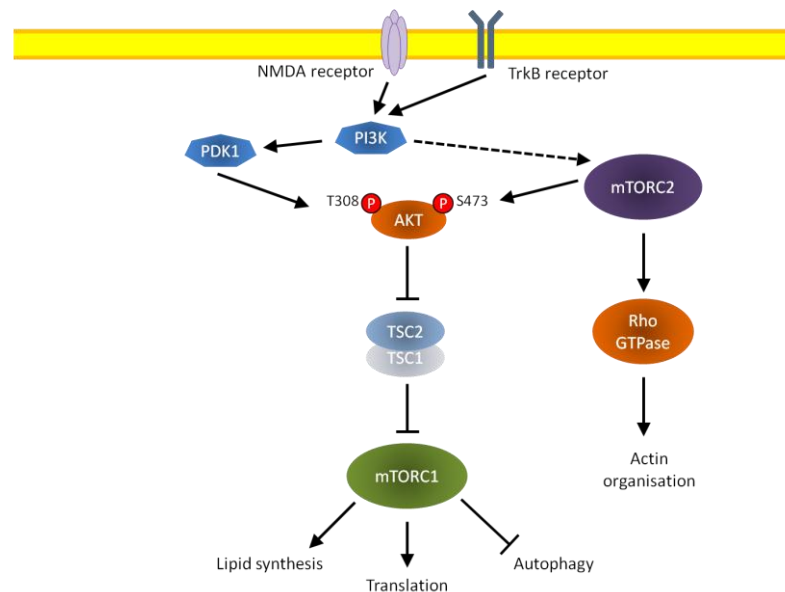


Figure 4.16: Regulation of the mTOR pathway. Upon activation by a variety of signals, including glutamate and neurotrophins, mTORC2 activates actin remodelling via RAC1 and phosphorylates AKT (at S473). This is also phosphorylated at T308, by PDK1, which leads to the full activation of AKT. Once active, AKT can phosphorylate various substrates including TSC2, preventing its inhibitory actions on mTORC1. Ultimately, this leads to the modulation of a number of cellular processes such as lipid synthesis, translation and autophagy. Abbreviations: mTORC, mammalian target of rapamycin complex; PDK1, phosphoinositide-dependent kinase 1; PI3K, phosphoinositide-3 kinase; TSC, tuberous sclerosis complex; TrkB, tyrosine receptor kinase B . Schematic authors own.

Additional evidence supporting a GPR50-mediated increase in mTORC2 activity comes from the increased levels of phospho-RAC1/CDC42 (S71), which is also a substrate of AKT (Schoentaube et al, 2009). However, these results must be expanded to understand the full nature of mTORC2 in GPR50 signalling. This can

be done by investigating the effects of GPR50 on substrates specifically targeted by phospho-AKT (S473), such as FOXO1/3 (Jacinto et al, 2006). Further, the effects of GPR50 on the level of mTORC2 itself and phospho-AKT (T308) should be determined. If the relationship between mTORC2 and GPR50 is confirmed, it should be established whether the increase in mTORC2 activity is responsible for the increase in phospho-RAC1/CDC42 and WAVE-2 that arises as a result of GPR50 transfection. To do this, the effects of mTORC2 depletion/inhibition on the ability of GPR50 to activate this pathway should be investigated.

4.4.3 The modulation of α -catenin by GPR50

This chapter also touches upon an additional mechanism GPR50 may employ to regulate actin organisation. This involves the modulation of α -catenin. The overexpression of GPR50 in HEK293 cells reduced α -catenin levels by 25%. This decrease was not seen in SH-SY5Y cells; instead α -catenin appeared to be redistributed away from the lamellipodia, although this requires replication. On the surface, these results appear to be different; however, the amount of α -catenin at the lamellipodia may be reduced in both situations. To begin to understand the functional consequence of these results it is important to consider the multiple actions of α -catenin. As a monomer it anchors F-actin to cadherins, stabilising F-actin at cell adhesion sites (Desai et al, 2013; Tan et al, 2010). While as a homodimer it appears to compete with ARP2/3 for F-actin, which prevents actin branching in favour of actin bundling (Drees et al, 2005; Hansen et al, 2013). Thus, a reduction in α -catenin could result in the destabilisation of F-actin at the plasma membrane and alleviate any inhibition to ARP2/3, therefore, allowing actin branching and lamellipodia formation to occur. The increased radial distribution of α -catenin noted in GPR50-transfected SH-SY5Y cells may support this hypothesis. This pattern is indicative of focal adhesion sites, which are associated with cell spreading and migration (Burks & Agazie, 2006; Gavard et al, 2004).

However, before the functional impact of these results is fully investigated, some more fundamental experiments are required. Firstly, experiments investigating the effects of GPR50 on the subcellular distribution of α -catenin should be repeated. This can be improved by assessing whether GPR50 transfection changes the colocalisation between α -catenin and markers of lamellipodia and focal adhesion sites. This can be supported with a subcellular fractionation experiment to determine whether GPR50 affects the level of α -catenin at the plasma membrane. In addition, co-immunoprecipitation could be performed to establish whether GPR50 impacts α -catenin's interactions with cadherin or F-actin. This information is essential to understand the effects of GPR50 on α -catenin.

4.4.4 Conclusion

The modulation of the RAC1-WAVE pathway and the regulation of α -catenin highlight the potential of GPR50 to initiate actin polymerisation and branching. This means GPR50 may promote lamellipodia and growth cone extension. However, this must be tested directly with assays measuring actin polymerisation, cell spreading and cell migration. In addition, the results reported here should be confirmed using a RAC1 activity assay. This can be expanded by looking at the activity of alternative Rho GTPases, e.g. RHOA or CDC42, as these are also important modulators of actin organisation. Finally, the information gathered from this should be used to investigate GPR50's contribution to actin remodelling in primary neurons. The results presented in this chapter show GPR50 localised to dendritic spines; the induction of actin remodelling here could explain the results obtained in chapter 3. To develop this investigation, it is essential to determine whether GPR50 also affects the levels actin remodelling factors in primary neurons.

Chapter 5: Validation of the putative GPR50-interacting proteins SREBF2 and ABCA2

5.1 Introduction

The focus of this chapter is the verification of the yeast-2-hybrid (Y2H) screen, suggesting that GPR50 interacts with sterol regulatory element binding transcription factor 2 (SREBF2) and ATP-binding cassette transporter 2 (ABCA2) (Grünewald et al, 2009). The Y2H assay is a useful tool in the identification of protein interactions; however, they tend to be prone to false positives. For this reason, it is essential to confirm the interactions reported in a Y2H screen using techniques such as immunocytochemistry (ICC) in conjunction with co-immunoprecipitation (co-IP). In the context of GPR50, this method has already proven successful; the highest scoring interaction in the Y2H screen, GPR50 with the neurite outgrowth inhibitor NOGO-A, was confirmed in this way (Grünewald et al, 2009). This association was explored further to show GPR50 positively regulates neurite outgrowth and attenuates the inhibitory actions of NOGO-A. In an independent Y2H study, GPR50 was shown to interact with the HIV-1 TAT-interactive protein, TIP60 (Li et al, 2011). Once this interaction was confirmed, a role for GPR50 in glucocorticoid receptor signalling emerged. In both Y2H studies, the identification of GPR50-interacting proteins revealed functions of the receptor that were previously unknown. Thus, these studies emphasise the value of uncovering GPR50 interactors.

In the Y2H screen performed by this laboratory (Grünewald et al, 2009), the interaction between GPR50 and NOGO-A was assigned a predicted biological score (PBS) of A (from A – E), indicating that the likelihood of this being a true-positive interaction was high. On the other hand, ABCA2 and SREBF2 were given low scores, D and E respectively, indicating a low probability of these interactions being specific (Grünewald et al, 2009). However, their potential as GPR50-interacting proteins should not be immediately discounted as they possess biological properties that make them compelling candidate interactors. Both proteins play a role in the regulation of cholesterol homeostasis; ABCA2 modulates cholesterol trafficking (Davis et al, 2004), while SREBF2 regulates genes essential for cholesterol synthesis (Horton et al, 1998). This is significant given the link between GPR50 and

lipid/cholesterol metabolism (Bechtold et al, 2012; Bhattacharyya et al, 2006; Ivanova et al, 2008). In addition, both proteins have been linked to mental illness (Le Hellard et al, 2010; Macé et al, 2005). As interactions assigned low PBS values have been confirmed with co-IP (Formstecher et al, 2005), investigating GPR50's putative interaction with ABCA2 and SREBF2 may be fruitful.

Interestingly, recent work indicates that there may be a functional relationship between GPR50 and these proteins. The developmental expression pattern of *Abca2* showed a significant correlation with *Gpr50* expression in the mouse brain, suggesting the two proteins may be co-regulated (Grünewald et al, 2012). This could reflect an involvement in the same biological pathway. In addition, co-IP was observed between ABCA2 and GFP-tagged GPR50, indicating that an interaction does exist (Grünewald et al, 2012). However, validation with the untagged protein is necessary to ensure that this was not an artefact caused by the presence of the GFP-tag. The expression of *Srebp2*, the mouse homologue of *SREBF2*, did not correlate with *Gpr50* (Grünewald, 2011). However, there was a strong correlation between the expression of *Srebp2* and *Abca2*, possibly signifying that the three proteins act in the same processes, e.g. cholesterol homeostasis. A physical interaction may be the mechanism by which GPR50 is involved in such processes.

5.2 Aims

The aim of this chapter was to test the interactions observed in the Y2H screen with regards to SREBF2 and ABCA2. First colocalisation between the proteins was assessed to determine whether they reside within the same subcellular compartment. The human neuroblastoma SH-SY5Y cell line was used to provide a neuronal model. If colocalisation was found, the experiment was repeated in additional cell lines for confirmation; namely, human embryonic kidney (HEK) 293 cells and the mouse neuroblastoma-derived N1E-115 cells. Colocalisation was followed up using co-IP

to determine whether a physical interaction exists. This was done in HEK293 cells, a cell line with high transfection rates. In addition, the effect of GPR50 on the localisation, and therefore activity, of SREBF2 was investigated by assessing changes to its localisation in the endoplasmic reticulum (ER) upon GPR50 transfection. Note, all the expression constructs and antibodies used in this chapter have been previously validated by independent researchers (Davis et al, 2004; Grünewald et al, 2009; Logette et al, 2005; Zhu et al, 2013). This was confirmed prior to these experiments, see appendix A.

5.3 Results

5.3.1 GPR50 does not colocalise with SREBF2

ICC was performed on SH-SY5Y cells to investigate whether GPR50 and SREBF2 were found at the same subcellular location. Cells were transfected with SREBF2 or GPR50 and ICC was performed the following day, staining for the relevant protein and subcellular marker. When transfected alone, SREBF2 strongly colocalised with calreticulin, indicating that it resides in the ER (figure 5.1 A), consistent with previous reports (Horton et al, 1998). In cells transfected with GPR50 alone, the receptor localised to the plasma membrane, as can be seen by the colocalisation with VLA-2 α , a plasma membrane marker (figure 5.1 B). Again, this is in agreement with published data (Grünewald et al, 2009; Hamouda et al, 2007). Upon co-transfection of GPR50 and SREBF2, GPR50 retained its plasma membrane localisation whilst SREBF2 remained intracellular (figure 5.1 C). The Intensity Correlation Analysis (ICA) plugin of the imaging software ImageJ (Li et al, 2004) was used to assess the degree of colocalisation within the cell in figure 5.1 C. ICA plots provide a visual representation of colocalisation; a positive or negative skew denotes the colocalisation or segregated staining of two proteins respectively. In the case of SREBF2 and GPR50, no obvious skew was seen in the corresponding ICA plots

(figure 5.1 D and E respectively), this indicates that the signal intensities of the two proteins do not vary together and any colocalisation seen is likely to be random. Similarly, no correlation was seen in the GPR50-SREBF2 signal intensity scatter plot (figure 5.1 F). Thus, there is little colocalisation between the two proteins in this cell.

ImageJ was also used to calculate colocalisation coefficients (see materials and methods for full descriptions). The average results indicate GPR50 does not colocalise with SREBF2 (N=13 cells; table 5.1). The average Pearson's coefficient (R_r) was 0.17 ± 0.03 , from a range of -1 to 1, indicating that the signal intensity of the two proteins do not correlate. The average Manders' (R) value of 0.59 ± 0.02 , from 0 to 1, indicates a modest overlap between the GPR50 and SREBF2 signals. Finally, the average ICQ was close to zero (0.11 ± 0.01 , from -0.5 to 0.5), indicating that there was little co-variation between the GPR50 and SREBF2 signals. These results show, the two proteins are not found within the same subcellular compartment, and therefore, it is unlikely that GPR50 and SREBF2 interact with each other under the tested conditions. For this reason, co-IP was not attempted.

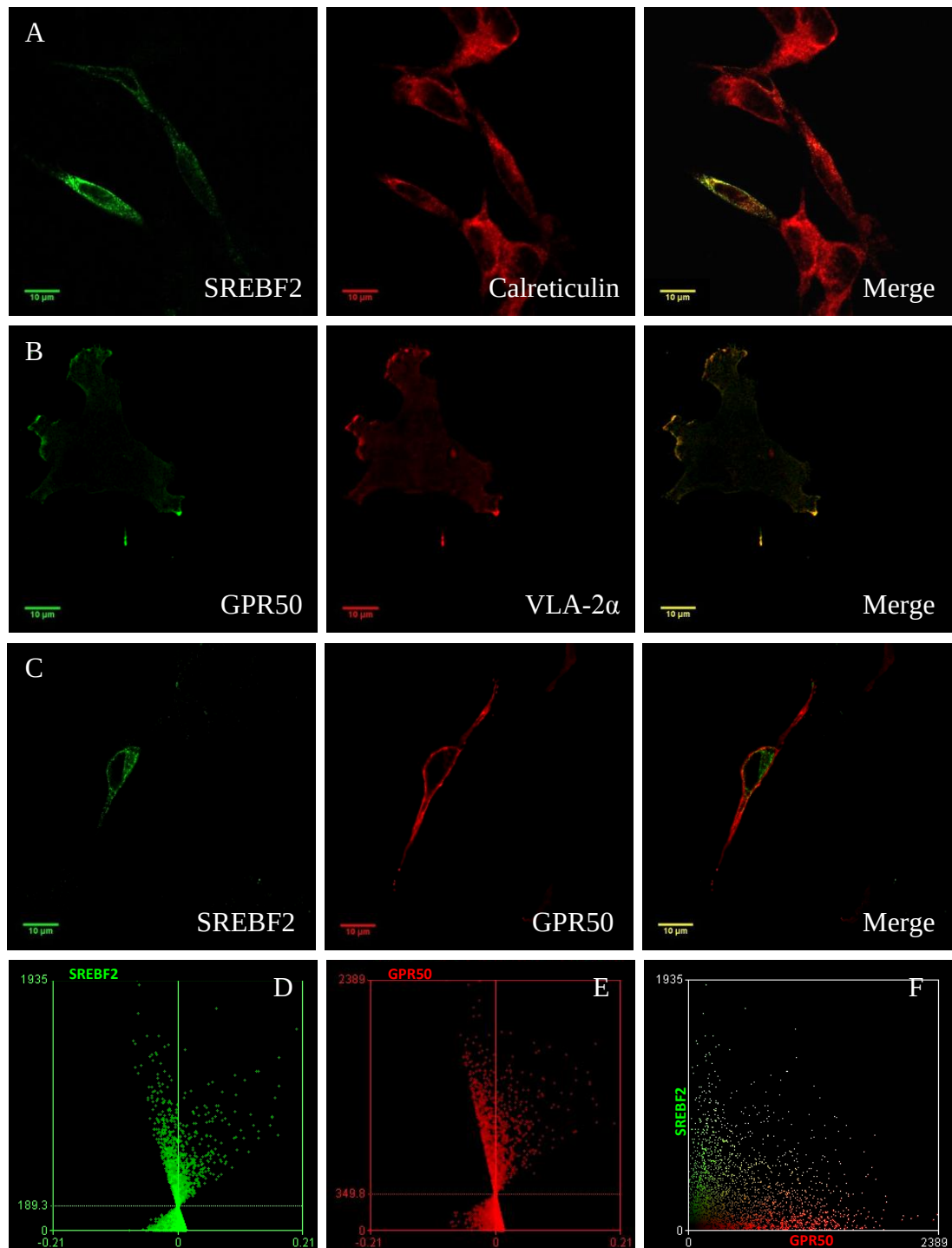


Figure 5.1: GPR50 does not colocalise with SREBF2 in SH-SY5Y cells. (See the following page for figure legend).

Figure 5.1: GPR50 does not colocalise with SREBF2 in SH-SY5Y cells. Cells were transiently transfected with SREBF2 and GPR50 individually or together and ICC was performed the following day to assess the subcellular localisation. (A) SREBF2 colocalised with calreticulin, an ER marker, when cells were transfected with SREBF2 alone. (B) Overexpressed GPR50 was found at the plasma membrane of singly transfected cells, as determined by the colocalisation with VLA-2 α . (C) Upon co-transfection, SREBF2 remained in the ER and GPR50 retained a plasma membrane staining. ICA plots of SREBF2 (D) and GPR50 (E) generated from the dual transfected cell (C) were not obviously skewed, indicating little co-variation. (F) The scatter plot of the fluorescence intensities of GPR50 (x-axis) vs. SREBF2 (y-axis), generated from the cell shown in image (C), shows little correlation between the signals.

Representative results from three experiments; scale bar, 10 μ m.

Coefficient	Average	SEM
Pearson's (Rr)	0.17	0.03
Manders' (R)	0.59	0.02
ICQ	0.11	0.01

Table 5.1: Colocalisation coefficients between GPR50 and SREBF2. SEM, standard error of mean; N=13 cells, from three independent experiments.

5.3.2 GPR50 does not promote the translocation of SREBF2

The effect of GPR50 on the ER localisation of SREBF2 was also examined. The inactive form of SREBF2 is found in the ER, whilst the active form resides in the nucleus; thus, assessing its localisation gives an indication of the level of activity. To investigate this, ImageJ was used to quantify the colocalisation between SREBF2 and calreticulin in SH-SY5Y cells transfected with SREBF2 individually or together with GPR50. As anticipated, a high degree of colocalisation was observed between SREBF2 and calreticulin in cells transfected with SREBF2 alone (table 5.2; Rr= 0.60 $\pm$ 0.03; R= 0.81 $\pm$ 0.02; ICQ= 0.25 $\pm$ 0.01; N=13 cells). This indicates SREBF2 was predominately found within the ER.

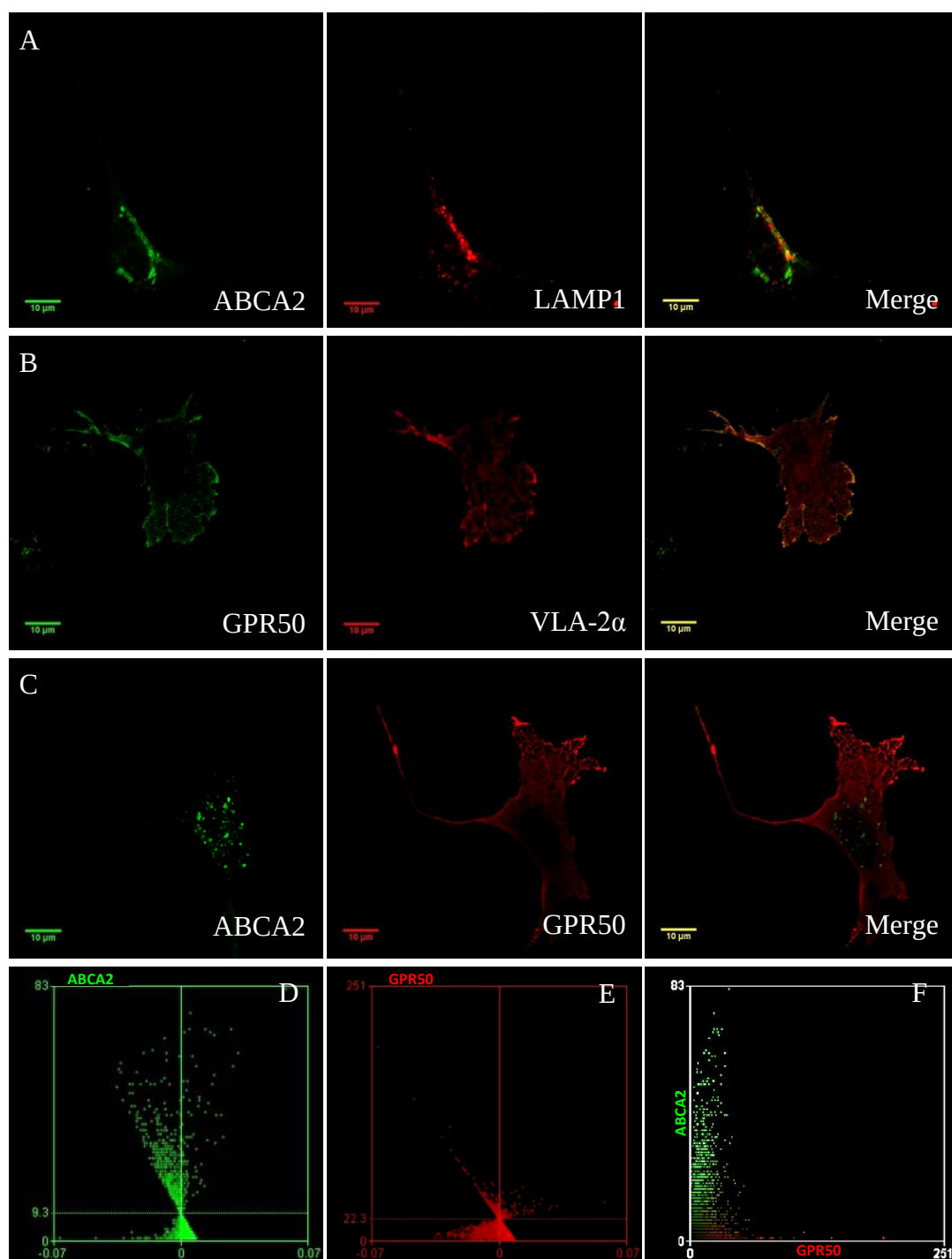
In the presence of GPR50, the SREBF2-calreticulin colocalisation remained strong (table 5.2; $R_r = 0.52 \pm 0.03$; $R = 0.78 \pm 0.01$; ICQ, 0.21 ± 0.01 ; $N = 12$ cells). Further, the Mander's coefficient (R) and ICQ were not significantly altered upon GPR50 overexpression ($P = 0.16$ and 0.09 respectively, Student's t-test), suggesting GPR50 had no effect on the localisation of SREBF2. However, the Pearson's coefficient (R_r) was significantly lower in dual transfected cells ($P = 0.046$, Student's t-test), which could suggest that SREBF2 was translocated from the ER. Whilst it is possible that this inconsistency was due to the differences between each coefficient (Dunn et al, 2011), technical issues are likely to be the cause. Co-transfection resulted in low SREBF2 signal intensities. This reduced the signal-to-noise ratio, which makes colocalisation measurements less reliable and can cause the underestimation of colocalisation (Dunn et al, 2011). Further, the Pearson's coefficient appears to be particularly sensitive to noise (Adler & Parmryd, 2010). With questions surrounding the accuracy of these measurements, it is difficult to draw any conclusions from these results. Further replicates may not improve this; instead, an alternative method to measure SREBF2 translocation should be considered.

Coefficient	SREBF2 alone		SREBF2 + GPR50		p-value
	Average	SEM	Average	SEM	
Pearson's (R_r)	0.60	0.03	0.52	0.03	0.05
Manders' (R)	0.81	0.02	0.78	0.01	0.16
ICQ	0.25	0.01	0.21	0.01	0.09

Table 5.2: Colocalisation coefficients between SREBF2 and calreticulin in SH-SY5Y cells expressing SREBF2 alone or with GPR50. SEM, standard error of mean; $N = 13$ SREBF2 and 12 SREBF2 + GPR50 transfected cells, three independent experiments; Statistical test, unpaired Student's t-test.

5.3.3 GPR50 colocalises with ABCA2 in intracellular compartments

ICC was also used to assess colocalisation between exogenous GPR50 and ABCA2 in SH-SY5Y cells. As previously reported (Broccardo et al, 2006a; Vulevic et al, 2001a), ABCA2 partially colocalised with the lysosomal marker LAMP1 when transfected alone (figure 5.2 A), suggesting it resides in lysosome-related organelles. As before, GPR50 was predominately plasma membrane bound in GPR50-transfected cells (figure 5.2 B). These distributions were maintained in doubly transfected cells when ABCA2 expression was relatively low (figure 5.2 C). The ICA plots for this cell were negatively skewed (figure 5.2 D and E), which indicates GPR50 and ABCA2 were found in different subcellular compartments. In addition, the scatter plot clearly demonstrates that there was no correlation between the signals of the two proteins (figure 5.2 F). However, intracellular colocalisation was seen in cells expressing high amounts of ABCA2 (figure 5.2 G). The corresponding ICA plots displayed positive skews indicating the staining of ABCA2 and GPR50 co-varied (figure 5.2 H and I). The ABCA2-GPR50 intensity scatter plot also had a positive trend (figure 5.2 J), further denoting colocalisation.



Continued on the following page

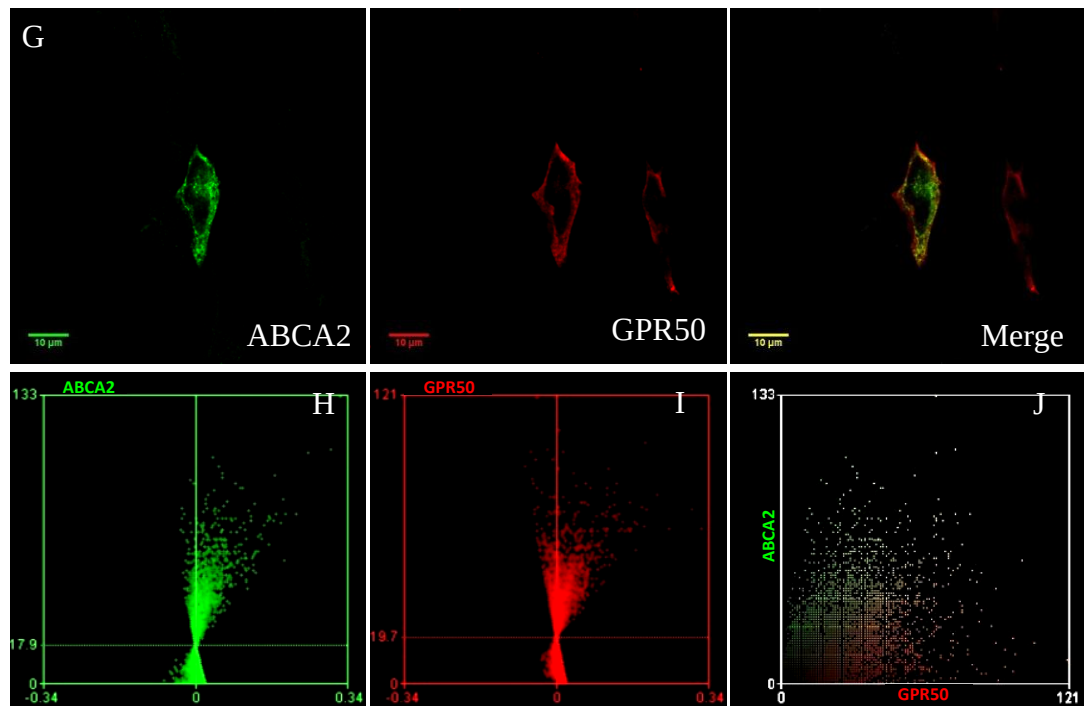


Figure 5.2: ABCA2 and GPR50 show some colocalisation within the cytoplasm of SH-SY5Y cells.

Cells were transfected with ABCA2 and/or GPR50 and ICC was carried out to determine the subcellular localisation. (A) When transfected alone ABCA2 partially colocalised with the lysosomal marker LAMP1. (B) GPR50 was predominately found on the plasma membrane, with VLA-2 α , when singly transfected. (C) In dual transfected cells expressing low levels of ABCA2, very little colocalisation was seen between GPR50 and ABCA2. The corresponding ICA plots for ABCA2 (D) and GPR50 (E) have a negative skew, denoting segregated staining. (F) The scatter plot for the signal intensity of GPR50 vs. ABCA2 shows no correlation between the two proteins in image (C). (G) A doubly transfected cell expressing high levels of ABCA2, showing intracellular colocalisation between ABCA2 and GPR50. Corresponding ICA plots (H and I) have a positive skew and a positive correlation can be seen in the scatter plot (J), indicating colocalisation. Representative results from three experiments; scale bar, 10 μ m.

Using ImageJ, colocalisation coefficients for ABCA2 and GPR50 were generated (table 5.3). These values suggested that overall colocalisation between ABCA2 and GPR50 was weak ($R_r = 0.28 \pm 0.07$; $R = 0.60 \pm 0.05$; ICQ, 0.16 ± 0.03 ; $N = 12$ cells, from three replicates). However, if colocalisation is dependent on ABCA2 expression, it is likely that only a small subset of doubly transfected cells would display any colocalisation, i.e. those expressing high levels of ABCA2. Averaging the data would mask this. To overcome this, ImageJ was used to measure the

ABCA2 fluorescence intensity in doubly transfected cells, correcting for background fluorescence, and this was plotted against the average Pearson's, Manders' and ICQ value (N=37; figure 5.3). Again, the average colocalisation coefficients for this dataset suggested that there was little colocalisation between the two proteins ($R_r = 0.16 \pm 0.04$; $R = 0.59 \pm 0.02$; ICQ, 0.11 ± 0.02). However, linear regression analysis revealed that there was a moderate positive correlation between ABCA2 fluorescence and colocalisation between GPR50 and ABCA2 ($r=0.4$ and $P<0.05$ for each coefficient; figure 5.3). These results indicate that colocalisation between ABCA2 and GPR50 is dependent on ABCA2 expression. Moreover, colocalisation indicates ABCA2 and GPR50 can be found in close proximity to each other, therefore an interaction between the two proteins may be possible.

Coefficient	Average	SEM
Pearson's (Rr)	0.28	0.07
Manders' (R)	0.60	0.05
ICQ	0.16	0.03

Table 5.3: Colocalisation coefficients between GPR50 and ABCA2 in SH-SY5Y cells. SEM, standard error of mean; N=12 cells from three independent experiments.

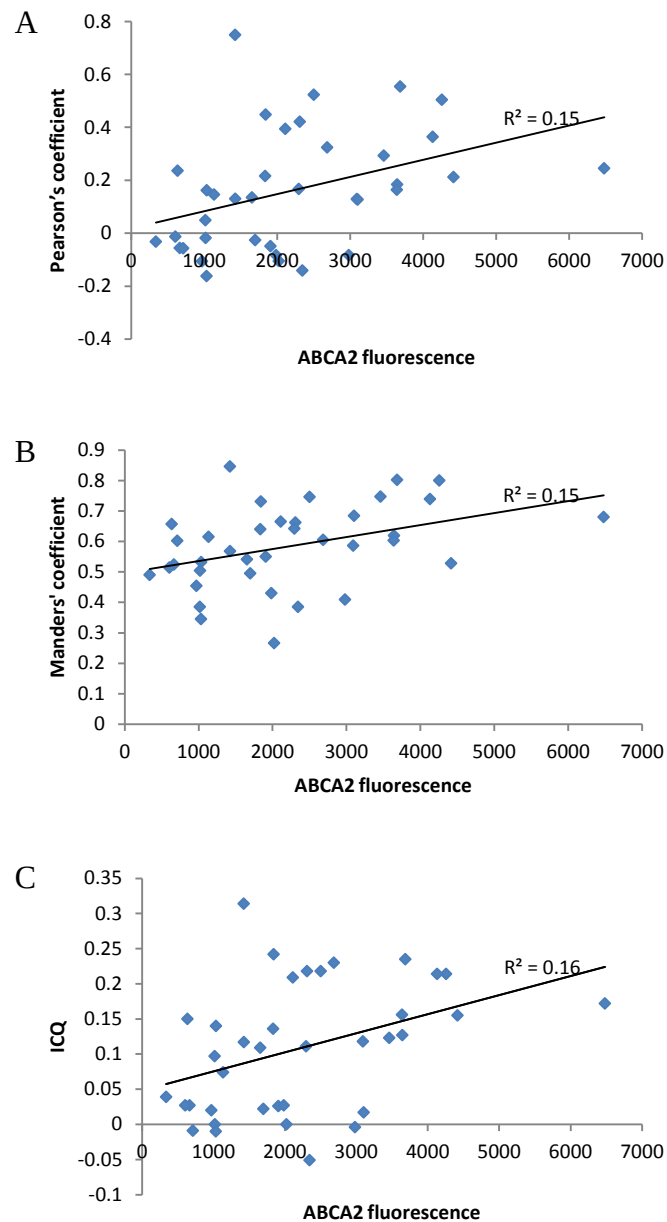


Figure 5.3: The colocalisation between ABCA2 and GPR50 is positively correlated with the ABCA2 signal intensity. ICC was performed on SH-SY5Y cells co-transfected with ABCA2 and GPR50. ImageJ was used to quantify colocalisation and measure the total cellular fluorescence of ABCA2. Linear regression analysis detected a significant correlation between ABCA2 fluorescence and the colocalisation between ABCA2 and GPR50, as measured by the Pearson's coefficient (A; $r=0.39$; $P=0.017$), Manders' coefficient (B; $r=0.39$; $P=0.016$), and ICQ (C; $r=0.40$; $P=0.015$). $N=37$ cells, from three independent experiments.

5.3.4 ABCA2 colocalises with GPR50 in additional cell lines

The colocalisation between ABCA2 and GPR50 was also tested in N1E-115 cells and HEK293 cells. The cytoplasmic colocalisation between transfected GPR50 and ABCA2 was reproduced in these cell lines (figure 5.4). This strengthens the results generated in SH-SY5Y cells, suggesting ABCA2 and GPR50 may interact. It also indicates that alternative cell lines could be used in a co-IP assay. However, caution must be taken when using N1E-115 cells as intracellular GPR50 was often observed when cells were transfected with GPR50 alone (figure 5.4, asterisk). This may reflect the mislocalisation of the transfected protein, thus the colocalisation observed with this cell line may be an artefact.

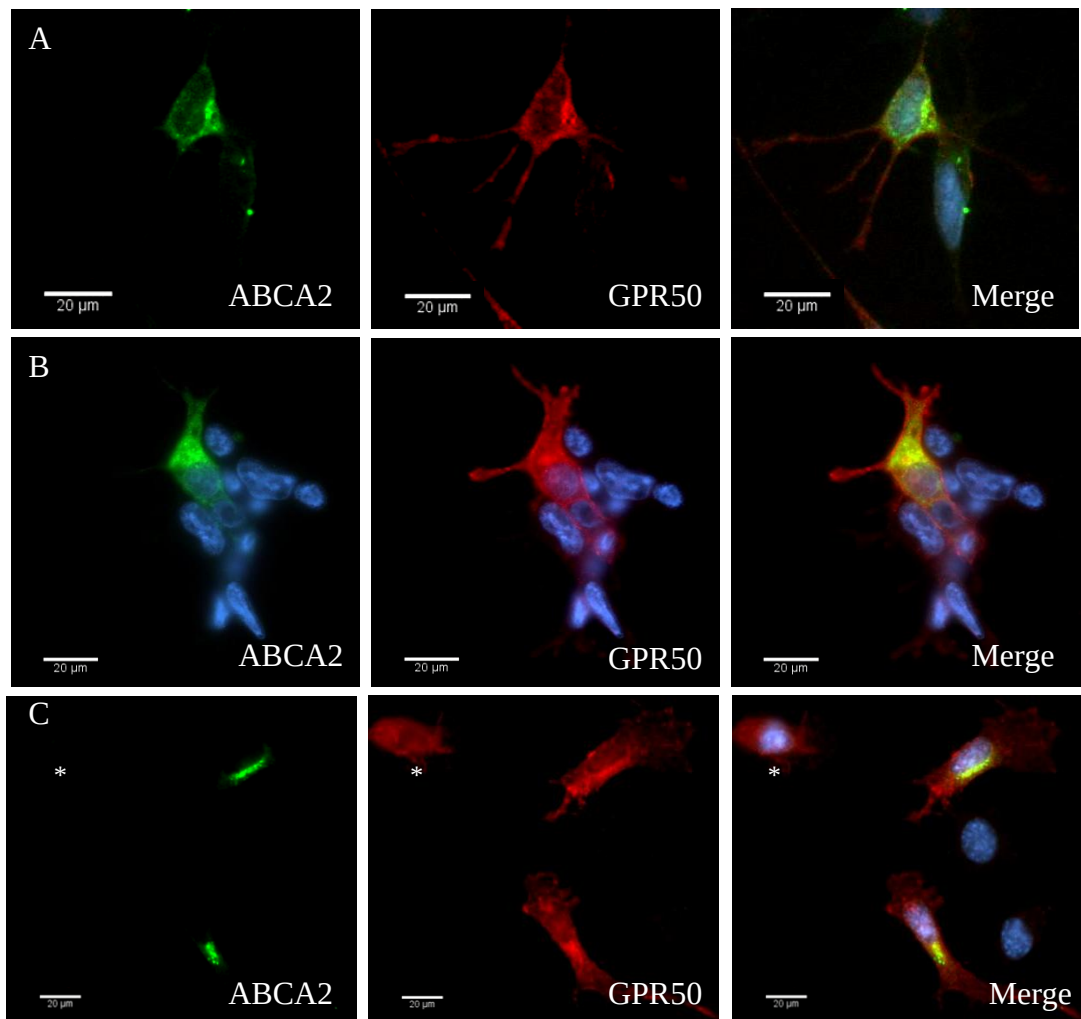


Figure 5.4: Colocalisation of ABCA2 and GPR50 is conserved in different cell lines. Both proteins were overexpressed in various cell lines and ICC was performed, staining for ABCA2, GPR50 and counterstained with DAPI for nuclear staining (blue). (A) In SH-SY5Y cells, colocalisation of exogenous ABCA2 and GPR50 occurred within the cytoplasm. Similar patterns of colocalisation occurred in HEK293 cells (B) and N1E-115 cells (C). Note, intracellular GPR50 was often seen in N1E-115 cells in the absence of ABCA2 overexpression (C, *).

Scale bar, 20 µm. Note, these images were taken with a fluorescence microscope and cells were fixed with methanol rather than the standard 4% paraformaldehyde.

5.3.5 GPR50 does not interact with ABCA2

Co-IP was performed to determine whether GPR50 and ABCA2 physically interact. This was done in HEK293 cells as colocalisation was observed in this cell line and transfection rates were high, ~70% compared to 40% in SH-SY5Y cells. Thus, an interaction would be more readily detectable in this cell line. Cells were transfected with ABCA2 and GPR50, individually or together, and GPR50 was immunoprecipitated from the cell lysates. Western blotting of the immunoprecipitated samples showed the enrichment of GPR50, however, ABCA2 was not co-immunoprecipitated (figure 5.5 A). This experiment was performed using a stringent lysis buffer, containing the ionic detergent SDS as well as the non-ionic NP-40. Stringent buffers benefit from a reduced chance of reporting false positives as they are more efficient in disrupting non-specific associations. However, interactions that are weak, but genuine, may also be disrupted. To circumvent this, the experiment was repeated with a mild lysis buffer, without SDS. Again co-IP was not detected (figure 5.5 B). This experiment was repeated in triplicate with consistently negative results. Thus, GPR50 and ABCA2 do not appear to interact with each other.

Figure 5.5 also shows that the extraction of endogenous GPR50 was greater when using the stringent lysis buffer compared to the mild buffer. It is widely known that stringent RIPA buffer is more effective in solubilising transmembrane proteins and those that form complexes, which may account for this observation. Alternatively the level of endogenous GPR50 may have differed between the two experiments. It may be necessary to investigate this further as if the former is true, the ease of extracting exogenous GPR50 with the mild lysis buffer could signify that the overexpressed protein is not properly anchored to the plasma membrane or does not form multimers. This is important to note as it could mean that the functional properties of the exogenous protein are not equivalent to that of the endogenous one.

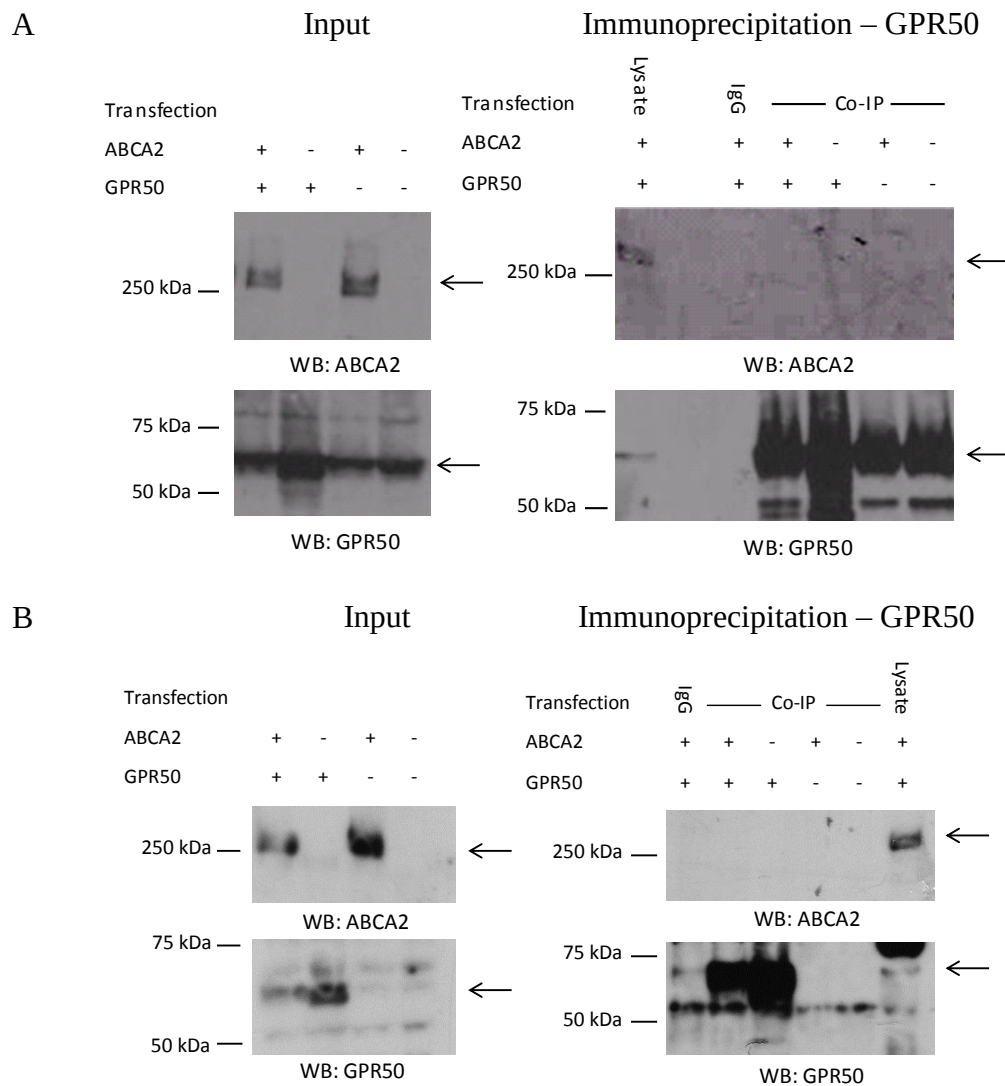


Figure 5.5: GPR50 and ABCA2 do not co-immunoprecipitate in HEK293 cells. Cells were transfected with ABCA2 and/or GPR50 and cell lysates were subjected to immunoprecipitation with an anti-GPR50 antibody. (A) Co-IP results using a stringent lysis buffer containing SDS. Both GPR50 and ABCA2 were detected in the input lysate, arrow (left), however, when GPR50 was immunoprecipitated ABCA2 was not pulled down (right). Note there was a high level of endogenous GPR50 expression in these cells, which was also immunoprecipitated; ABCA2 did not co-immunoprecipitate with endogenous GPR50. (B) The same negative results were generated using a mild lysis buffer. ABCA2 was not co-immunoprecipitated with GPR50 (right). Again, the input lysate showed both proteins were successfully transfected (left), although endogenous GPR50 expression was low in these cells. Results shown are representative of three independent experiments.

The negative results may have been due to insufficient levels of ABCA2 expression. Colocalisation was only found in a small subset of cells, thus, if an interaction exists it would be at relatively low levels; low amounts of ABCA2 in the input lysate would lessen the chance of the interaction being detected. For this reason the reciprocal experiment was also performed, i.e. ABCA2 was immunoprecipitated and GPR50 was probed for in the western blot (figure 5.6). Again, co-IP results were negative; GPR50 was not immunoprecipitated with ABCA2. This experiment was performed only once, and therefore could benefit from further replicates. However, it does agree the previous results indicating ABCA2 and GPR50 do not physically interact, thus additional experiments may not be necessary.

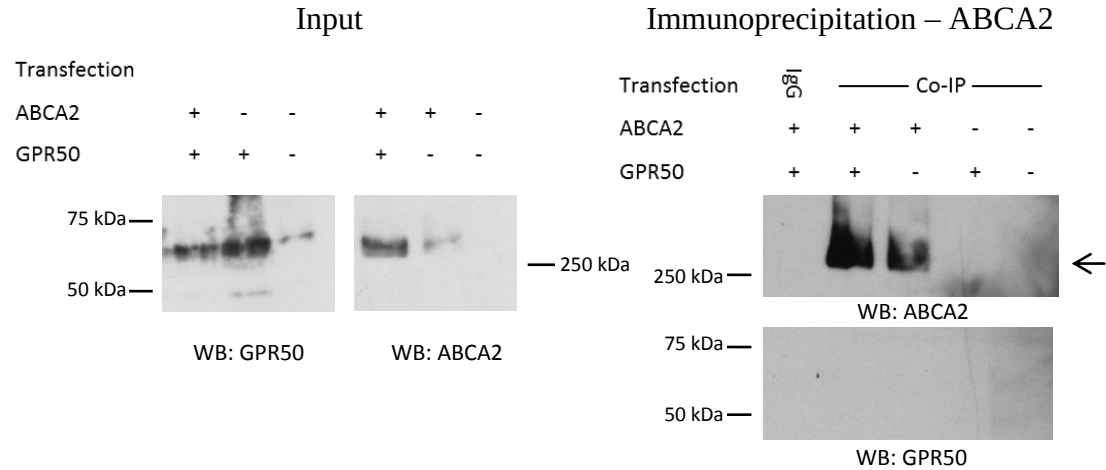


Figure 5.6: GPR50 does not co-immunoprecipitate with ABCA2. HEK293 cells were transfected with ABCA2 and/or GPR50. Cell lysate was collected the following day and subjected to immunoprecipitation with an anti-ABCA2 antibody. GPR50 and ABCA2 were detected in the input lysate of transfected HEK293 cells (left) and ABCA2 was successfully immunoprecipitated, arrow (right). However, GPR50 was not precipitated with ABCA2 (right).

5.4 Discussion

The aim of this chapter was to verify the findings of the GPR50 Y2H screen carried out by this group (Grünewald et al, 2009), focusing on the proposed interaction with SREBF2 and ABCA2. The results described here do not support the Y2H study. When SH-SY5Y cells were transfected with GPR50 and SREBF2, colocalisation was not found; GPR50 was found on the plasma membrane whilst SREBF2 had an ER localisation. As the two proteins are not found within the same cellular compartment they are unlikely to interact. In addition, GPR50 transfection did not appear to significantly alter the retention of SREBF2 in the ER, suggesting it does not affect SREBF2 activity. On the other hand, colocalisation was observed between ABCA2 and GPR50 in dual transfected cells, although this was only seen in cells expressing high levels of ABCA2. This was followed up with co-IP, which showed GPR50 and ABCA2 did not physically interact. This suggests ABCA2 overexpression alters the localisation of GPR50 by a mechanism that does not involve an interaction.

5.4.1 GPR50 and SREBF2 are unlikely to interact

The results described above show GPR50 and SREBF2 are unlikely to interact as they are found in different cellular locations. The positive result in the Y2H study is likely to be due to the tendency of SREBF2 to form non-specific interaction, which is reflected in the low confidence level assigned to this interaction in the Y2H screen, i.e. PBS E (Grünewald et al, 2009). However, the results of this chapter also indicate that the localisation of GPR50 is not static; in the presence of ABCA2 it can take up an intracellular localisation. GPR50 has also been reported to adopt a cytoplasmic localisation when co-transfected with TIP60 (Li et al, 2011). Moreover, when the C-terminal domain (CTD) of GPR50 was transfected with TIP60 it was translocated from the cytoplasm to the nucleus. Thus, under conditions where GPR50 is not plasma membrane bound, especially if it is found within the ER or nucleus, an

interaction with SREBF2 may be possible. Hence, investigating the conditions that alter GPR50 localisation would be beneficial to fully investigate the potential SREBF2-GPR50 interaction.

The effect of GPR50 on the localisation of SREBF2 was unclear. The Manders' coefficients and ICQ indicated that there were no changes to the localisation of SREBF2 when transfected with GPR50. SREBF2 showed strong colocalisation with calreticulin in the presence and absence of GPR50, thus remained in the ER in both condition. The Pearson's coefficient between SREBF2 and calreticulin contradicts this; there was a significant reduction in doubly transfected cells ($R_r = 0.60$ in SREBF2-transfected cells and $R = 0.52$ in dual transfected cells; $P = 0.046$). This would indicate a reduction in ER-bound SREBF2 upon GPR50 transfection. However, no obvious nuclear staining was observed; this is unlikely to be due to the antibody used, which has been shown to detect the active nuclear form of the protein (Kannenberg et al, 2013; Vecchini et al, 2003), also see appendix A. The discrepancy between the colocalisation coefficients may have arisen as a result of technical issues. In doubly transfected cells, the signal intensity for SREBF2 and GPR50 was weaker, which made quantification difficult and possibly led to inaccurate measurements (Adler & Parmryd, 2010; Dunn et al, 2011). Thus, an alternative approach should be taken to investigate the effect of GPR50 on SREBF2 activity. For example, a subcellular fractionation experiment could be performed to determine whether nuclear SREBF2 levels changes upon GPR50 transfection. Alternatively, rt-qPCR or western blotting could be performed to investigate whether GPR50 alters the transcription of SREBF2 target genes.

5.4.2 ABCA2 overexpression leads to the intracellular trafficking of GPR50

Unlike SREBF2, co-localisation was observed between GPR50 and ABCA2. This was largely restricted to cells expressing high levels of ABCA2, with the extent of colocalisation being positively correlated with the level of ABCA2 (figure 5.3). However, this colocalisation did not translate to a physical interaction as co-IP results were negative. This contradicts a previous report showing ABCA2 interacts with GFP-tagged GPR50 (Grünewald et al, 2012). This difference is unlikely to be due to methodology as the same protocol was used initially, i.e. cell lysis with a stringent buffer. This experiment was then repeated with a mild lysis buffer; however, the same negative outcome was obtained (figure 5.5). Instead, the contradictory results may be due to the constructs used. In this thesis the previously validated untagged-GPR50 construct was used, this correctly localised to the plasma membrane (Grünewald, 2011; Grünewald et al, 2009). The GFP-tagged protein, however, often assumed an intracellular localisation which raised concerns about mislocalisation (Grünewald, 2011). Thus, results generated using the untagged protein may be more physiologically accurate than the GFP-tagged construct. However, the low levels of ABCA2 expression in the doubly transfected cells should not be ignored as this may have prevented the detection of an interaction. Further optimisation of ABCA2 transfection rates or a more sensitive technique may be necessary to confidently assess the putative ABCA2-GPR50 interaction.

If the colocalisation between ABCA2 and GPR50 was not due to a physical interaction between the two proteins, the next question to address is: how does ABCA2 induce the intracellular trafficking of GPR50? To begin to understand the process behind this, it is essential to consider the actions of ABCA2. The overexpression of ABCA2 in Chinese hamster ovary (CHO) cells has been demonstrated to cause an accumulation of cholesterol in lysosomal vesicles and a reduction of cholesterol within the plasma membrane (Davis et al, 2004). Further, in N2a cells, a mouse neuroblastoma cell line, ABCA2 expression decreased total and

membrane cholesterol levels (Davis, 2011). In addition, constitutive expression of ABCA2 altered the expression of genes involved in cholesterol homeostasis. Thus, ABCA2 appears to have a role in regulating intracellular cholesterol levels. The regulatory ability of ABCA2 does not appear to be restricted to cholesterol; abnormal sphingolipid levels have been observed in ABCA2 deficient mice (Sakai et al, 2007). The ability of ABCA2 to modulate lipid levels may be responsible for GPR50 internalisation; this shall be addressed in chapter six.

The internalisation of GPR50 may have important functional implications. This was recently demonstrated by Li et al, 2011, who found that co-transfection with TIP60 resulted in the cytoplasmic trafficking of GPR50, where the two proteins strongly colocalised. Colocalisation also occurred between TIP60 and the CTD of GPR50, within the nucleus. Expanding these results, the authors showed that TIP60 interacted with full length GPR50 and its CTD, further, both forms of GPR50 enhanced the co-activation of glucocorticoid receptor signalling by TIP60. Thus, in the study by Li and colleagues, the translocation of GPR50 reflected the enhancement of TIP60 activity. Likewise, the ABCA2-driven intracellular trafficking of GPR50 may signify the modulation of GPR50's functionality.

Internalisation is also a mechanism to attenuate GPCR signalling (Ritter & Hall, 2009). Once internalised, GPCRs can be recycled back to the plasma membrane via recycling endosomes or delivered to lysosomes for degradation. As ABCA2 is largely found in endo-lysosomal vesicles (Broccardo et al, 2006a), it is possible that colocalisation occurs here. This would support the idea that the internal trafficking of GPR50 attenuates its signalling properties. However, intracellular GPR50 did not appear to take up the vesicular pattern usually associated with endo-lysosomal proteins (figure 5.2 G and 5.4). Rather, colocalisation between GPR50 and ABCA2 was largely perinuclear. This suggests that colocalisation occurred in other compartments such as the ER or trans-golgi network, where ABCA2 has also been detected (Broccardo et al, 2006a). Interestingly, the trans-golgi network has also

been implicated in GPCR degradation (Cheng & Filardo, 2012; Lelouvier et al, 2008). Thus, if colocalisation occurs here, ABCA2 may contribute to GPR50 downregulation rather than increasing/modulating its functionality. To build upon this observation it is important to identify the exact compartment in which colocalisation occurs. This is of high interest as it may dictate the outcome of GPR50 translocation, which would provide insight into how the receptor is regulated.

It should be noted that the lipids modulated by ABCA2, i.e. sphingolipids and cholesterol, are essential components of the plasma membrane and lipid rafts (LRs) in particular. LRs are specialised microdomains of the plasma membrane which act as platforms for the assembly of signalling molecules (Allen et al, 2007). These structures are known to modulate receptor trafficking to the plasma membrane; moreover, the disruption of LRs leads to the internalisation of LR-associated proteins (Borroni & Barrantes, 2011; Xu et al, 2006). If ABCA2 overexpression compromises the integrity of LRs, the internalisation of GPR50 may not reflect a specific mode of regulation. Rather, it may signify a non-selective disruption to the localisation of various plasma membrane bound proteins, including GPR50. Therefore, it would be useful to ascertain the effect of ABCA2 overexpression on the integrity of LRs by assessing how its expression affects the distribution of flotillin-1, an LR marker (Nothdurfter et al, 2010). Following this, the impact of LR disruption on GPR50 localisation could be tested (Allen et al, 2007; Nothdurfter et al, 2010).

5.4.3 Conclusion

The aim of this chapter was to verify Y2H results showing GPR50 interacts with SREBF2 and ABCA2. Evidence presented here indicates that GPR50 does not interact with these proteins; GPR50 and SREBF2 were found in separate subcellular compartments, whilst ABCA2 did not immunoprecipitate with GPR50. Despite the lack of a physical interaction, ABCA2 overexpression appeared to modulate the

subcellular distribution of GPR50. Finding the driving force behind the internalisation of GPR50 is an important step in understanding its function, as it may provide information about its regulation. To do this, the aspect of ABCA2 overexpression causing the intracellular trafficking of GPR50 must be isolated. For example, GPR50 may take up an internal localisation in response to the disruption of cholesterol trafficking and homeostasis caused by ABCA2 overexpression (Davis et al, 2004). Investigating how lipid levels, especially cholesterol, regulate GPR50 localisation and protein levels may provide some explanation to the results presented in this chapter. This chapter also indicated that the localisation of GPR50 is dynamic. Fully characterising the subcellular localisations in which GPR50 may be found, and the functional impact of alternative GPR50 trafficking, may reveal significant information about the role of the protein.

Chapter 6: GPR50 expression is associated with lipid metabolism

6.1 Introduction

The potential role of GPR50 in lipid metabolism first emerged from its anatomical distribution; GPR50 is predominately found within the hypothalamus, in particular the dorsomedial hypothalamic nucleus (DMH) and the ependymal layer of the third ventricle (Drew et al, 2001). These are regions with an established role in feeding, body weight and energy expenditure (Chao et al, 2011). In mice, the expression of GPR50 in these areas appears to be modulated by energy/food intake, suggesting it may have a role in the metabolic response to feeding (Barrett et al, 2006; Ivanova et al, 2008). Support for this comes from knockout studies showing GPR50 modulates weight control, fat storage and energy expenditure (Bechtold et al, 2012; Ivanova et al, 2008). In humans, *GPR50* polymorphisms have been linked to elevated levels of circulating triglycerides and lower levels of high density lipoprotein (HDL)-cholesterol (Bhattacharyya et al, 2006). Together these results indicate GPR50 is involved in lipid homeostasis.

The yeast-2-hybrid screen carried out by this group has also contributed to this growing body of evidence. This screen identified several potential GPR50-interactors with strong links to lipid metabolism, i.e. ATP-binding cassette transporter 2 (ABCA2) and sterol regulatory element binding transcription factor 2 (SREBF2) (Grünwald et al, 2009). In chapter five, these interactions could not be verified; however, it was observed that ABCA2 promoted the intracellular localisation of GPR50. As this altered trafficking does not appear to be the consequence of a physical interaction between the two proteins, it may be induced by the modulation of cholesterol homeostasis by ABCA2. Stable transfection of ABCA2 has been demonstrated to cause an accumulation of intracellular cholesterol, a reduction in cholesterol esterification and a decrease in the low-density lipoprotein (LDL)-receptor (Davis, 2011; Davis et al, 2004). These characteristics are similar to U18666A treatment, an inhibitor of cholesterol trafficking (Sparrow et al, 1999; Underwood et al, 1996). U18666A also inhibits *de novo* cholesterol synthesis

through the inhibition of oxidosqualene cyclase, a key enzyme in cholesterol biosynthesis (Issandou et al, 2004; Sexton et al, 1983). Together, this creates a sterol deprived intracellular environment, even when extracellular cholesterol is present (Issandou et al, 2004; Sparrow et al, 1999; Zhang et al, 2004). If the ABCA2-mediated change to the intracellular cholesterol environment was responsible for the cytoplasmic distribution of GPR50, U18666A would be expected to have the same effect.

In this chapter, particular interest was placed on the role of GPR50 in the regulation of intracellular lipid levels. In most eukaryotic cells, excess lipid, such as triglycerides and cholesterol esters, are stored in lipid droplets (Guo et al, 2009). This provides a reservoir of lipids for membrane synthesis and metabolic energy. When lipid demand is high, lipids are broken down (lipolysis) and released in a process known as mobilisation (Duncan et al, 2007; Guo et al, 2009). Thus, investigating the effect of GPR50 on lipid droplets would provide insight into whether cellular lipid use is altered by the receptor. Coincidentally, this was explored by an independent group who found GPR50 overexpression increased lipid accumulation in adipocyte cells (Hand, 2011); supporting mouse work showing *Gpr50* deletion reduced fat accumulation (Ivanova et al, 2008). Interestingly, this group also found GPR50 increased basal lipolysis (Hand, 2011), indicating the balance between lipogenesis and lipolysis was disrupted upon GPR50 overexpression. As GPR50 expression is abundant in neurons (Grünewald et al, 2012), it would be of interest to investigate how its expression affects lipid levels in neuronal cells. The GPR50-induced neurite outgrowth and dendrite maturation (chapter three) is likely to increase the cellular requirement for lipids, in order to support membrane synthesis. Thus, GPR50 may promote lipid mobilisation and lipolysis.

6.2 *Aim*

This chapter set out to characterise the role of GPR50 in lipid homeostasis, with particular interest in neuronal cells. To this end, the relationship between GPR50 expression and lipid levels was investigated. This was done by assessing whether lipids regulate GPR50 expression in human embryonic kidney (HEK) 293 cells. Although not of a neuronal origin, HEK293 cells provide an endogenous system for GPR50 expression (Atwood et al, 2011). HEK293 cells have also been suggested to possess neuronal characteristics due to the expression of multiple neuronal genes such as neurofilament, glutamate receptors and functional sodium channels (He & Soderlund, 2010; Shaw et al, 2002). The ability of GPR50 to regulate intracellular lipid levels was also investigated in this chapter. This was done by investigating the effect of GPR50 on lipid droplets in SH-SY5Y cells, a neuroblastoma-derived cell line.

The second section of this chapter aimed to build upon results from chapter five and determine whether the disruption of cholesterol trafficking modified the localisation of GPR50, again in SH-SY5Y cells. The functional implication of the cytoplasmic trafficking of GPR50 was also investigated, focusing on how this affects the ability of GPR50 to promote neurite outgrowth. Finally, the relationship between GPR50-induced neurite development and the modulation of intracellular lipid levels was investigated.

6.3 Results

6.3.1 Investigating the relationship between GPR50 expression and lipid levels

6.3.1.1 GPR50 expression is responsive to the depletion of exogenous lipids

The first strategy used to assess whether GPR50 is regulated by lipids was serum deprivation. This is known to alter the expression of key regulators of lipid metabolism, such as SREBF1, SREBF2 and the LDL-receptor (Horie et al, 2010; Zeng et al, 2004). If GPR50 is involved in this process it may also be affected by serum starvation. Thus, HEK293 cells were cultured in media containing reduced levels of fetal bovine serum (FBS) for 18 hrs followed by western blot analysis to measure endogenous GPR50 levels. As can be seen in figure 6.1 A, the expression of GPR50 was increased upon serum starvation; incubation with 0.625% and 0% FBS resulted in a respective increase of $26 \pm 9.31\%$ and $56 \pm 17.43\%$ compared to cells maintained in 10% FBS (control) ($P=0.03$ and 0.02 respectively, Students t-test, $N=$ six replicates). Significance was not detected using a linear regression model ($P=0.15$), thus GPR50 expression did not have a linear relationship with the level of FBS. Instead it appears to be raised in conditions of extreme deprivation.

As lipoproteins are the major source of lipids in FBS, lipoprotein deficient serum (LPDS) was used to determine whether the increase in GPR50 expression was due to lipid depletion. Cells were maintained in media containing 3 mg/ml LPDS or 10% FBS (control) for 18 hrs and GPR50 levels were measured. Surprisingly, lipoprotein depletion resulted in a reduction of GPR50 compared to the control condition (figure 6.1 B; $26 \pm 7\%$ decrease, $P=0.04$, Students t-test, $N=$ four replicates). The addition of LDL, at 100 $\mu\text{g/ml}$ or 250 $\mu\text{g/ml}$, restored GPR50 expression to control levels ($P=0.84$ and 0.44 respectively). This confirms that the reduction of GPR50 was due to the absence of lipoproteins. When assessing cells maintained in LPDS, it was found that the increase in GPR50 upon the addition of 250 $\mu\text{g/ml}$ LDL, compared to

cells maintained in LPDS without LDL, reached near significance ($P=0.054$). However, the apparent increase following the addition of 100 $\mu\text{g/ml}$ LDL did not reach significance ($P=0.15$). This may indicate that the effect of LDL on GPR50 expression was dose dependent; further replicates with additional LDL concentrations are required to determine this. Together, these results indicate that GPR50 is positively regulated by lipids/lipoproteins and the effect of serum starvation on GPR50 expression was not due to a lack of lipids. It should be noted that in this set of experiments 0% FBS was used as a positive control for the upregulation of GPR50. An apparent increase in GPR50 was observed, however, this was not significant ($P=0.26$, Student's t-test, figure 6.1 B). Thus, further experiments may be required to validate this finding.

As LDL is mainly responsible for the delivery of cellular cholesterol (Ikonen, 2008), the restoration of GPR50 levels in response to LDL application suggests that cholesterol may be the primary modulator of GPR50 expression. To investigate this, a cholesterol-deprived environment was induced by treating HEK293 cells with U18666A for 18 hrs. Western blot analysis showed GPR50 levels were not significantly affected by U18666A (figure 6.1 C); incubation with U18666A at 1, 2 and 5 $\mu\text{g/ml}$ generated P-values of 0.31, 0.3 and 0.25 respectively (Students t-test, $N=$ four replicates). This implies that GPR50 expression is not affected by intracellular cholesterol levels. It was noted that there was unequal variance between the different U18666A concentrations (figure 6.1 C), which may be indicative of an increase in cell death or stress. Based on cell morphology this was not evident, however, a cell viability assay may be necessary to ensure cellular health and survival following U18666A treatment.

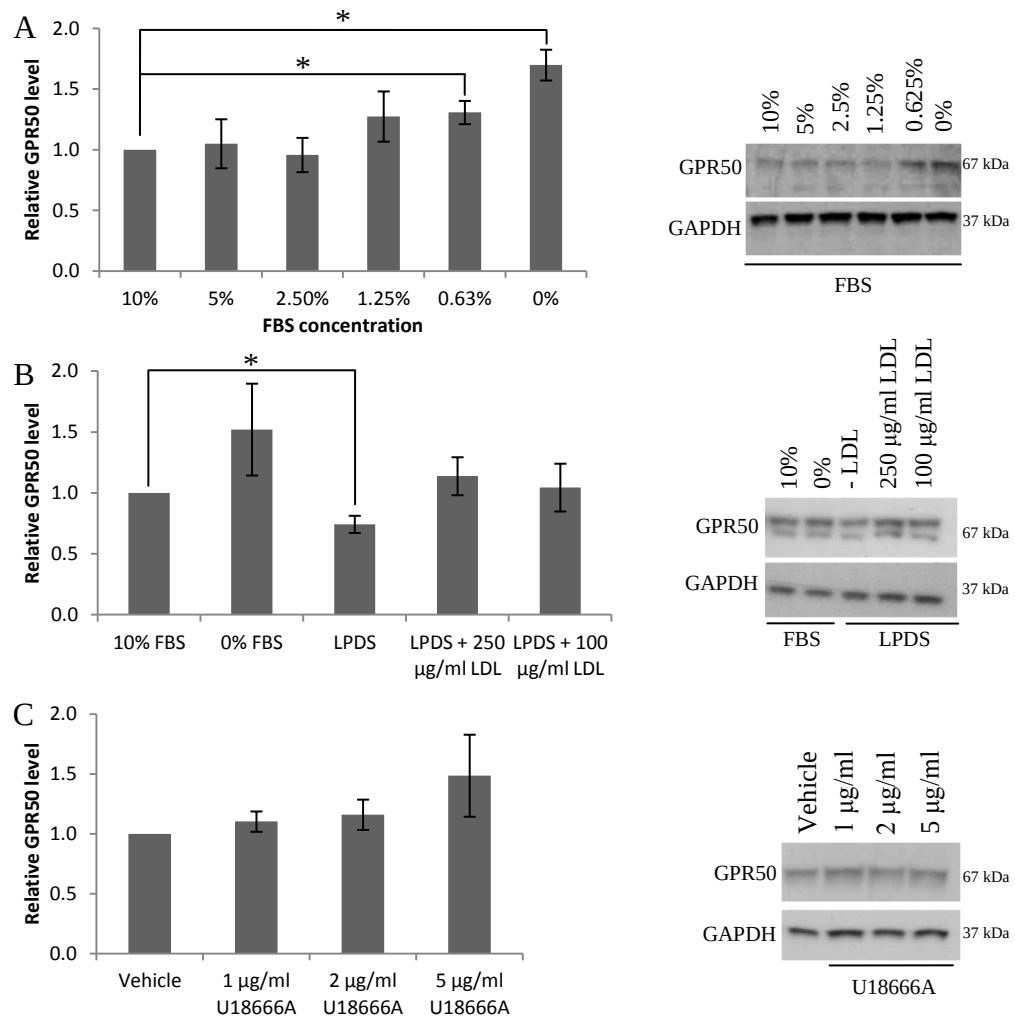


Figure 6.1: Endogenous GPR50 is responsive to serum and lipoprotein levels in HEK293 cells.

Western blot analysis was carried out on cell lysates taken from HEK293 cells subjected to various 18 hr treatments to alter lipid levels. GPR50 levels were quantified by densitometric analysis using Fiji, with GAPDH as the loading control. Representative blots are shown on the right. (A) GPR50 levels were significantly greater in cells maintained in 0.625% or 0% FBS compared to 10% FBS (left). N= six replicates. (B) Lipoprotein depletion reduced GPR50 expression compared to the control. The addition of LDL restored GPR50 to control levels. N= four experiments. Note, the rabbit anti-GPR50 antibody (Proteintech) was used here, opposed to the goat anti-GPR50 antibody (SCBT) routinely used elsewhere, this detects endogenous GPR50 as a doublet; see appendix A for comparisons. (C) Treatment with U18666A did not significantly alter the level of GPR50 compared to vehicle treated cells (left). N= four experiments.

Results presented as mean $\pm$ SEM; *P<0.05, paired Students t-test.

6.3.1.2 GPR50 reduces the accumulation of intracellular lipid droplets

To investigate whether GPR50 regulates intracellular lipid levels, the effect of GPR50 transfection on intracellular lipid droplets was assessed in SH-SY5Y cells. These were visualised using the lipophilic dye Nile red, which stains neutral lipids such as cholesterol esters and triglyceride (Greenspan et al, 1985; Keembiyehetty et al, 2011; Martin et al, 2005). Following immunocytochemistry (ICC), lipid droplets were quantified using Fiji. GPR50 transfection significantly decreased the number of lipid droplets in SH-SY5Y cells ($P < 0.001$, Mann-Whitney U test, $N =$ three experiments), figure 6.2 A and B. To account for any changes in cell size/morphology caused by GPR50 transfection, the percentage of the cell occupied by lipid droplets was also measured with Fiji. Again, this was significantly lower in GPR50-transfected cells ($P < 0.001$, figure 6.2 C). The reduction of lipid droplets indicates that GPR50 induces the breakdown of lipid reserves.

Lipid droplets are believed to form at the periphery of cells and are transported to the perinuclear region for storage (Wolins et al, 2005), thus the distribution of lipid droplets provides some information about lipid metabolism. In mock-transfected cells lipid droplets accumulated at the perinuclear region, whereas in GPR50-transfected cells lipid droplets were largely absent from the perinuclear region and mostly found at the cell periphery (figure 6.2 A). A total of $51 \pm 4.73\%$ of GPR50-transfected cells displayed a lack of perinuclear lipid droplets, whilst in mock-transfected cells this was only $17 \pm 2.61\%$ (figure 6.3; $P = 0.03$, Student's t-test, $N =$ 110-155 cells per condition). These results provide supporting evidence that the overexpression of GPR50 caused the depletion of lipid stores.

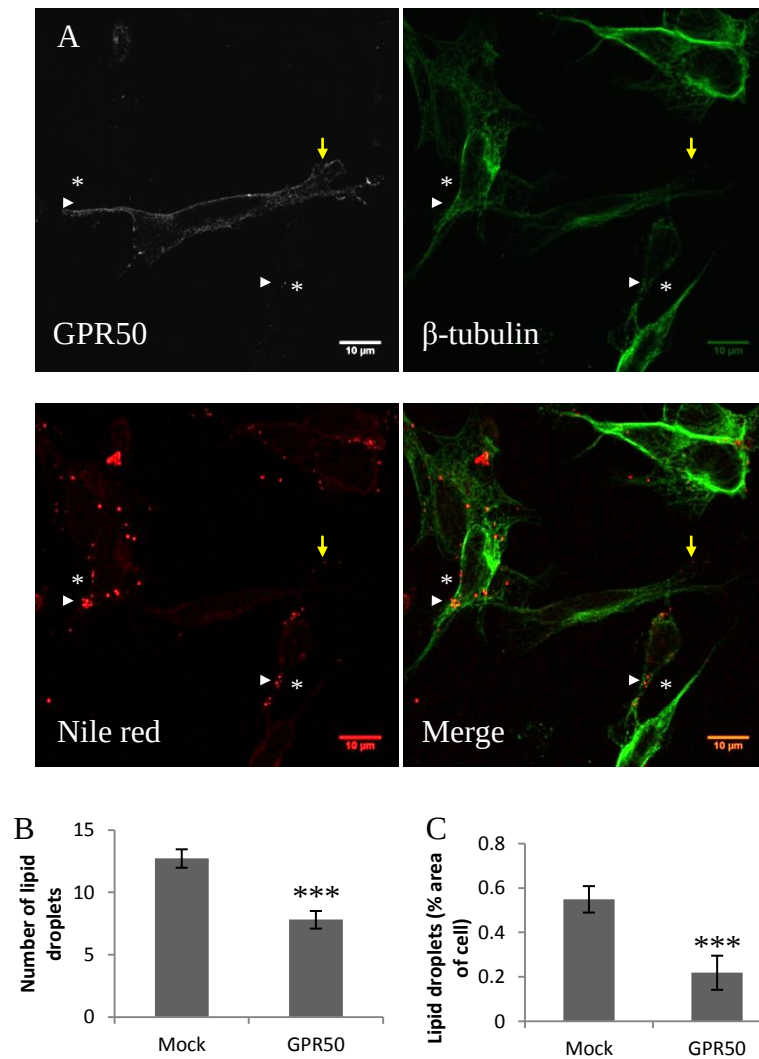


Figure 6.2: GPR50 overexpression decreases the accumulation of lipid droplets in SH-SY5Y cells.

GPR50 or mock-transfected cells were subjected to ICC, staining for GPR50, β -tubulin and lipid droplets, using Nile red. (A) Cells overexpressing GPR50 (arrow) displayed fewer lipid droplets compared to neighbouring cells which did not express the protein (*). Lipid droplets typically took up a perinuclear localisation in cells which did not express GPR50 (arrowhead). This distribution was largely absent in GPR50-transfected cells (arrow). (B) Quantification of lipid droplets using Fiji showed the average number of lipid droplets was significantly reduced upon GPR50 transfection. (C) The area of the cell occupied by lipid droplets was also significantly lower in cells transfected with GPR50 compared to mock-transfected cells.

Results presented as mean $\pm$ SEM; N=57 mock and 66 GPR50-transfected cells, from three independent experiments; ***P<0.001, Mann-Whitney U test.

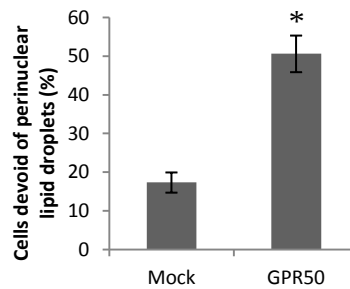


Figure 6.3: GPR50 transfection reduces the perinuclear distribution of lipid droplets in SH-SY5Y cells. ICC was used to assess the localisation of lipid droplets in GPR50 or mock-transfected cells. The overexpression of GPR50 resulted in a greater proportion of cells devoid of lipid droplets in the perinuclear region of the cell and solely at the periphery. Results presented as mean $\pm$ SEM; N=155 mock and 110 GPR50-transfected cells, from three experiments; *P<0.05, paired Students t-test.

6.3.2 The regulation of GPR50 localisation by lipids

6.3.2.1 U18666A promotes the intracellular localisation of GPR50

In the previous chapter, ABCA2 was shown to cause the intracellular trafficking of GPR50. As ABCA2 has been shown to inhibit cholesterol transport (Davis et al, 2004), the localisation of GPR50 was assessed in response to the cholesterol trafficking inhibitor U18666A. SH-SY5Y cells were transfected with GPR50 and treated 24 hrs later with vehicle or 1 μ g/ml U18666A, a concentration in which cholesterol accumulation is observed with no affect on cell viability (Appelqvist et al, 2011; Cheung et al, 2004). Transfection rates were not affected by U18666A; this was approximately 35% for each condition, including untreated cells. The localisation of GPR50 was assessed using ICC after varying incubation times. As shown in figure 6.4 A – C, when cells were incubated with U18666A for a short duration, i.e. 5 mins, 30 mins or 1 hr, the proportion of cells with intracellular GPR50 was significantly greater than the vehicle-treated cells (control), in which GPR50 was predominately located at the plasma membrane (5 mins, P=0.004; 30 mins, P=0.017; 1 hr, P=0.005, Students t-test, N=118-180 cells per condition, from three replicates). Thus, the inhibition of cholesterol trafficking, by ABCA2 or U18666A, appears to alter the localisation of GPR50. However, this effect was

transient as U18666A treatment had no effect on GPR50 localisation after incubation for 4 and 18 hrs ($P=0.47$ and 0.67 respectively).

As U18666A is known to create a sterol deprived environment it was necessary to determine whether the cytoplasmic trafficking of GPR50 was due to the inhibition of cholesterol transport or cholesterol deprivation. To look at this, the effect of serum starvation on GPR50 localisation was also investigated; serum being a rich source of exogenous cholesterol. The absence of serum had no effect on the localisation of GPR50 compared to cells maintained in 10% FBS; for each time point tested $P>0.1$, Students t-test, $N=114-161$ cells per condition, three replicates (figure 6.4 D). This suggests that the increase in intracellular GPR50 in cells treated with U18666A was due to deficiencies in cholesterol trafficking rather than the depletion of cholesterol.

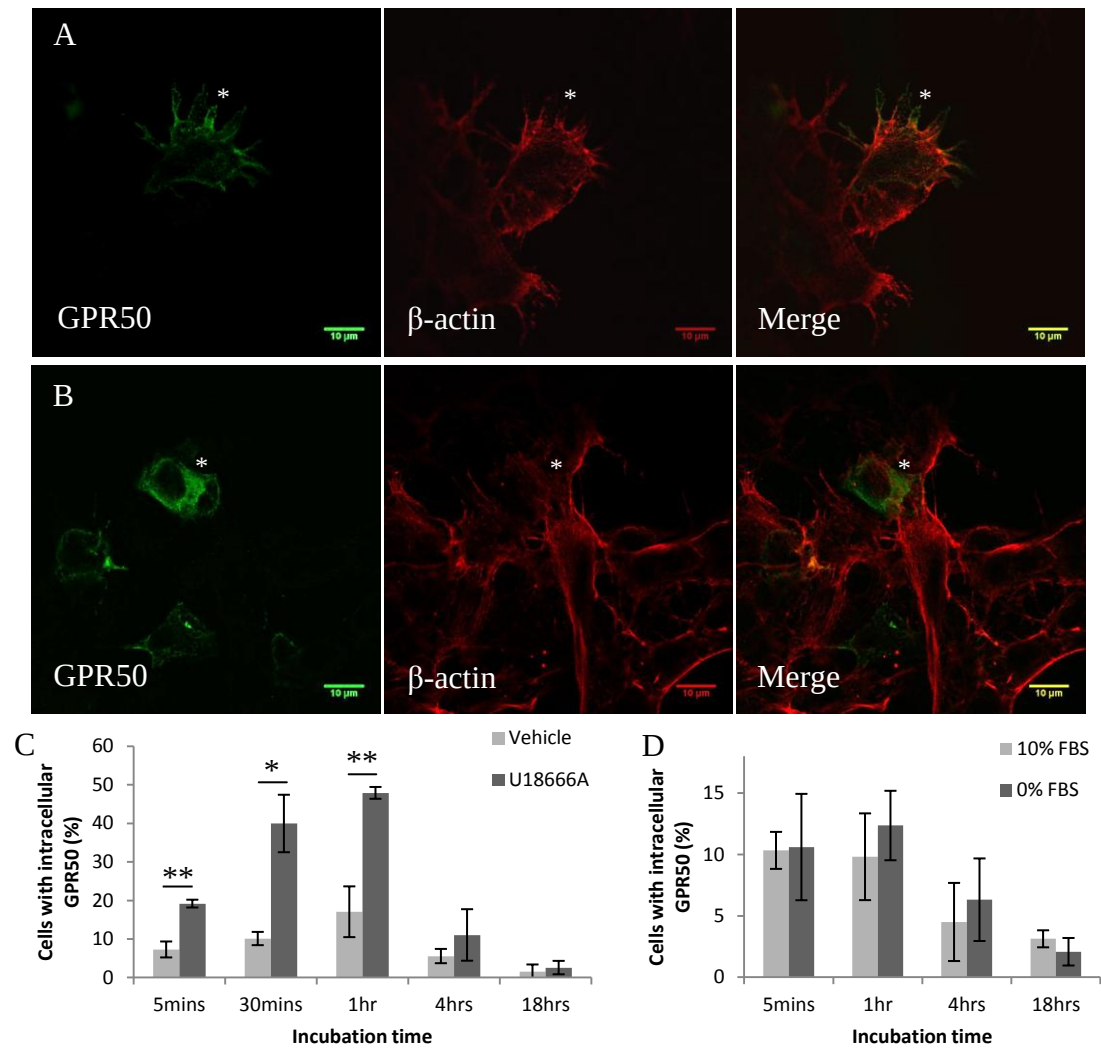


Figure 6.4: U18666A treatment leads to a rise in intracellular GPR50 in transfected SH-SY5Y cells.

Following transfection with GPR50, cells were treated with U18666A, or vehicle, for various incubation periods. The localisation of GPR50 was then assessed with ICC. (A) Overexpressed GPR50 in vehicle treated cells (1 hr) was predominately found at the plasma membrane (*). (B) In cells treated with U18666A (1 hr), GPR50 localisation was often intracellular (*). (C) U18666A treatment, for 5 mins, 30 mins or 1 hr, significantly increased the amount of cells with intracellular GPR50 compared to vehicle treated cells. For each condition, N=118 – 180 cells. (D) The effect of serum deprivation was also assessed in GPR50-transfected cells. This had no effect on the subcellular distribution of GPR50 compared to cells maintained in 10% FBS. N=114 – 161 cells. Results presented as mean $\pm$ SEM; N= three independent experiments; *P<0.05, **P<0.01, paired Students t-test.

6.3.2.2 Inhibition of cholesterol transport attenuates the promotion of neurite outgrowth by GPR50

The plasma membrane trafficking of G-protein coupled receptors (GPCRs) is often essential for their activity. This means the internalisation of GPR50 may have a negative impact on the receptors role in neurite outgrowth. To explore this hypothesis, SH-SY5Y cells transfected with GPR50 were treated with 1 $\mu\text{g/ml}$ U18666A for 18 hrs and neurites were quantified following ICC, using the imaging software Fiji. In mock transfected cells, U18666A had no effect on the number or length of neurites per cell compared to the vehicle control (figure 6.5; $P=0.74$ and 0.86 respectively). This indicates that alone, U18666A has no effect on neurite development in SH-SY5Y cells.

However, the stimulation of neurite outgrowth by GPR50 was significantly impeded by U18666A. GPR50 transfection led to a 2.8-fold increase in the number of neurites per cell in the vehicle control condition (figure 6.5 A; GPR50: 1.63 ± 0.14 ; mock: 0.59 ± 0.1 , $P<0.001$, Mann-Whitney U test, $N= 88-96$ cells per condition, three experiments). This dropped to a 1.7-fold increase when U18666A was present ($P=0.01$, Mann-Whitney U test, $N=87-91$ cells per condition). Further, the number of neurites on GPR50-transfected cells was two-fold lower when grown in the presence of U18666A compared to vehicle ($P<0.001$, Mann-Whitney U test). The increase in neurite length was also attenuated (figure 6.5 B). In the vehicle treated condition, GPR50 significantly increased neurite length when compared to mock transfected cells (GPR50: $29.68 \pm 1.63 \mu\text{m}$; mock: $23.03 \pm 1.24 \mu\text{m}$, $P=0.002$, Mann-Whitney U test, $N= 45-58$ cells per condition, from three replicates). The application of U18666A completely blocked this effect ($P=0.86$, Mann-Whitney U test, $N=35-51$ cells per condition). Collectively, these results illustrate U18666A impedes the promotion of neurite development by GPR50. This means the mobility of cholesterol, and the subsequent maintenance of GPR50 at the plasma membrane, is essential for GPR50 to exert its full effects on neurite outgrowth.

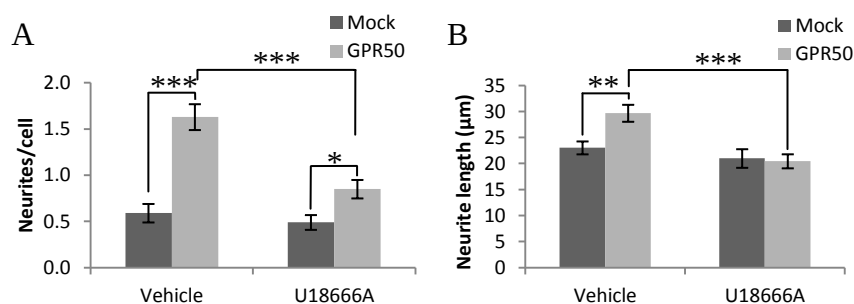


Figure 6.5: Treatment with U18666A attenuates the GPR50-mediated increase in neurite

outgrowth in SH-SY5Y cells. SH-SY5Y cells were transfected with GPR50, or mock transfected, then subjected to U18666A/vehicle treatment for 18 hrs. Following ICC, neurites were quantified with Fiji. (A) GPR50 overexpression led to a significant increase in the number of neurites per cell in vehicle and U18666A (1 μg/ml)-treated cells. However, this increase was significantly lower in the presence of U18666A. N= 96 mock and 88 GPR50-transfected cells in the vehicle condition. In the U18666A condition, N= 91 mock and 87 GPR50-transfected cells. (B) The average neurite length was significantly enhanced by GPR50 transfection when cells were treated with vehicle. This increase was completely abolished upon treatment with U18666A. For the vehicle treated condition, N= 45 mock and 58 GPR50-transfected cells. For U18666A treatment, N= 33 mock and 51 GPR50-transfected cells.

Results presented as mean ± SEM; N= three independent experiments; *P<0.05, **P<0.01, ***P<0.001, Mann-Whitney U test after a one-way Kruskal–Wallis test.

It should be noted that GPR50 transfection did not appear to have an effect on neurite length in chapter three (figure 3. 1 C). This discrepancy is likely to lie in the differences between the protocols used. Previously, cells were transfected on day 1 and ICC was performed on the following day (day 2). However, in this chapter, cells were incubated for an additional 18 hrs (in the presence of U18666A or vehicle) on day 2 and ICC was carried out on day 3. This means ICC was performed 48 hrs after transfection, compared to the 24 hrs in chapter three. This extra period may have been sufficient for GPR50 to drive neurite extension under the control condition. This shows some similarities to the work by Grünwald et al, 2009, who found an increase in neurite length in Neuroscreen-1 cells 48-72 hrs after GPR50 transfection. However, an increase in the number of neurites per cell was not observed.

6.3.2.3 GPR50 transfection induces a depletion of lipid droplets in the presence of U18666A

The depletion of lipid droplets upon GPR50 transfection may reflect an increased requirement for lipids to support the enhanced neurite outgrowth. If true, the GPR50-induced reduction of lipid droplets should be attenuated in the presence of U18666A, as true for neurite outgrowth (figure 6.5). To investigate this, transfected SH-SY5Y cells were incubated with 1 µg/ml U18666A, or vehicle, for 18 hrs and lipid droplets were quantified using Fiji. U18666A treatment led to an increase in Nile red staining, which meant it was difficult to accurately count individual lipid droplets. For this reason, lipid droplets were only measured as a percentage of the cell in this experiment.

As expected, GPR50 transfection resulted in a highly significant reduction of lipid droplets in the vehicle-treated condition (figure 6.6; mock: $0.26 \pm 0.03\%$; GPR50: $0.11 \pm 0.02\%$; $P < 0.001$, Mann-Whitney U test, $N = 75-86$ cells per condition, three replicates). This reduction was maintained in the presence of U18666A (mock: $1.2 \pm 0.07\%$; GPR50: $0.8 \pm 0.06\%$; $P < 0.001$, Mann-Whitney U test, $N = 78-84$ cells per condition, three replicates). The magnitude of the GPR50-induced decrease in lipid droplets appeared to be lower in the presence of U18666A, with a $31 \pm 2.05\%$ decrease detected in the presence of U18666A and $55 \pm 5.68\%$ in the absence (figure 6.6). However, this difference did not reach significance ($P = 0.08$, Student's t-test). This indicates that GPR50 promotes lipid turnover despite the presence of U18666A, suggesting that GPR50 internalisation does not prevent the ability of GPR50 to promote lipid mobilisation. However, these results may be indicative of a trend, thus additional replicates should be considered.

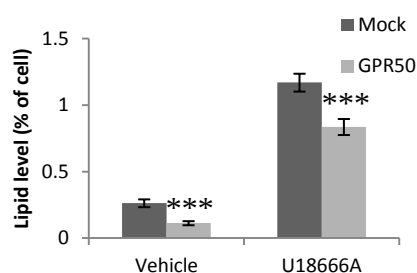


Figure 6.6: Overexpression of GPR50 in SH-SY5Y cells significantly reduced lipid droplets in the presence of U18666A. Following mock or GPR50 transfection, SH-SY5Y cells were treated with vehicle or U18666A for 18 hrs. ICC was then performed and lipid droplets were visualised using Nile red. U18666A did not appear to affect the depletion of lipid droplets; a highly significant reduction was seen in response to GPR50 overexpression when cells were cultured with either 1 μ g/ml U18666A or vehicle. Results presented as mean $\pm$ SEM; N=86 mock and 75 GPR50-transfected cells in the vehicle condition; N=84 mock and 78 GPR50-transfected cells in the U18666A condition; results taken from three replicates. ***P<0.001, Mann-Whitney U test.

6.4 Discussion

This chapter investigated the involvement of GPR50 in lipid homeostasis, the key findings are summarised in figure 6.7. To initiate this study, serum deprivation experiments were performed, which revealed an increase in GPR50 expression upon serum starvation. This does not appear to be driven by a lack of lipids as lipoprotein depletion resulted in the reduction of GPR50 expression. These findings suggest GPR50 is responsive to both lipoprotein and non-lipoprotein components of FBS. In addition, GPR50 was demonstrated to be a regulator of intracellular lipid levels. Its overexpression in SH-SY5Y cells led to the depletion of lipid droplets. Thus, GPR50 appears to be both responsive to lipid availability and involved in their utilisation.

An additional aim of this chapter was to elucidate why ABCA2 transfection promoted the intracellular localisation of GPR50 (chapter 5) and determine the functional significance of this. Results presented in this chapter suggest that the

ability of ABCA2 to inhibit cholesterol transport may be responsible for this effect. In SH-SY5Y cells, treatment with the cholesterol transport inhibitor U18666A resulted in the transient cytoplasmic distribution of exogenous GPR50. Interestingly, U18666A also blocked the ability of GPR50 to induce neurite outgrowth, suggesting the plasma membrane localisation of the receptor is required for this function. Conversely, U18666A did not alter the ability of GPR50 to induce the depletion of lipid droplets, which may suggest the two processes are independent.

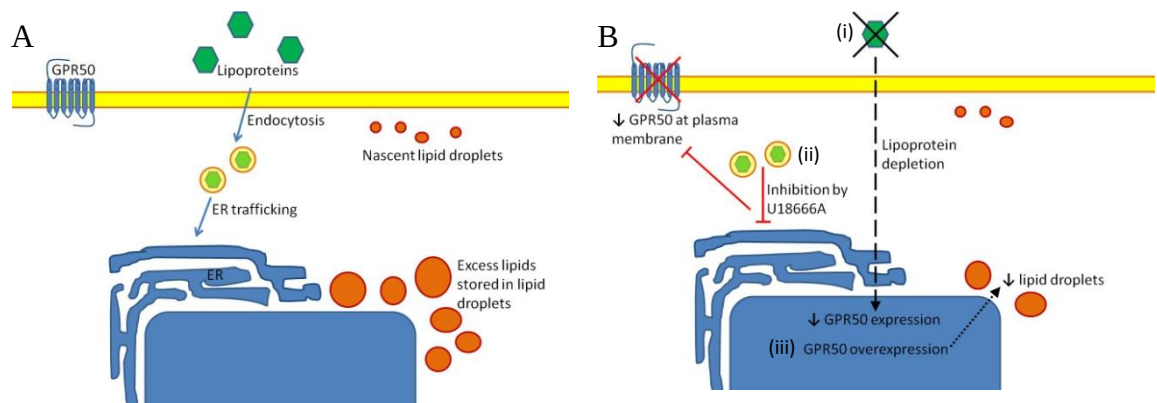


Figure 6.7: Relationship between GPR50 and lipid metabolism. (A) Under standard conditions exogenous GPR50 was predominately located at the plasma membrane and lipid droplets could be found throughout the cell. (B) When cells were maintained in lipoprotein-depleted culture media, GPR50 expression significantly decreased (i). The inhibition of intracellular cholesterol trafficking and synthesis with U18666A did not alter the levels of GPR50, however, it did reduce the localisation of GPR50 at the plasma membrane (ii). GPR50 expression also appeared to increase lipid utilisation as the overexpression of the receptor reduced the level of intracellular lipid droplets, especially at the perinuclear region (iii). Diagram authors own.

6.4.1 A role in lipid homeostasis

6.4.1.1 GPR50 expression is responsive to extracellular lipid levels

Results presented in this chapter suggest that lipids positively regulate GPR50 expression. In HEK293 cells, the depletion of lipoproteins, a rich source of lipids, caused a significant decrease in the expression of endogenous GPR50. This may support previous work showing *Gpr50* was downregulated in the DMH and the third ventricle of the hypothalamus in response to decreased food intake (Barrett et al, 2006; Ivanova et al, 2008). Potentially, this reduction in GPR50 was due to a reduction in dietary lipids. An attempt was made to isolate the component of lipoproteins responsible for the modulation of GPR50 levels. Given that the addition of LDL reversed the effects of lipoprotein depletion, focus was placed on cholesterol, as LDL is a major cholesterol-carrier (Ikonen, 2008). Incubation with U18666A, which has been shown to create a cholesterol deprived intracellular environment (Issandou et al, 2004; Sparrow et al, 1999; Zhang et al, 2004), had no effect on GPR50 levels. This suggests that the downregulation of GPR50 was due to another lipoprotein component. As well as cholesterol, lipoproteins provide a source of phospholipids, triglycerides, and a variety of proteins (Ikonen, 2008); any of which may modulate GPR50 expression. Alternatively, GPR50 is responsive to the presence of extracellular lipoproteins, which have the capacity to act as signalling molecules (Rubina et al, 2005).

Another possible explanation for the results described above is that GPR50 is responsive fatty acid (FA) levels. Lipoprotein depletion induces lipid mobilisation (Masuda et al, 2006; Ozeki et al, 2005), which in turn raises FA release (Zimmermann et al, 2004). This is in keeping with the hypothesis presented by Ivanova et al, 2008, who found GPR50 expression decreased in response to high energy diets, as well decreased food intake. This is suggestive as FA levels are raised in both conditions (Frier et al, 2011). A role of GPR50 in FA detection is especially intriguing given the metabolic dysfunction associated with the disruption

of known FA receptors. For example, like *Gpr50* deletion, *Gpr43* knockdown led to a reduced body weight and fat accumulation in mice placed on a high fat diet (Bjursell et al, 2011). Conversely, GPR120 dysregulation appears to promote obesity in both humans and mice (Ichimura et al, 2012). Thus, studying GPR50 as a FA receptor is a compelling hypothesis to explore. The first step to investigating this is establishing whether FAs alter GPR50 expression.

6.4.1.2 Increase of GPR50 during serum deprivation

As serum deprivation caused an increase in GPR50, rather than a decrease, it is unlikely that the lack of lipids/lipoprotein was responsible for this effect. Instead, this increase may be due to the cellular changes invoked by serum deprivation. For example, the depletion of serum is known to reduce cell viability (Wang et al, 2009) and activate pro-survival pathways, such as the mitogen-associated kinase (MAPK) and AKT pathways (Katsuma et al, 2005; Wang et al, 2009). Therefore, the upregulation of GPR50 may denote a role in pro-survival or pro-apoptosis pathways. Interestingly, GPR50 appears to mediate leptin signalling (Bechtold et al, 2012), which has been shown to increase cell survival under serum deprivation (Russo et al, 2004). In addition, GPR50 was shown to promote AKT activation in chapter four; this may implicate GPR50 in a pro-survival pathway. Taking these findings into account, it would be useful to investigate GPR50's effect on cell viability, especially in the absence of serum. Further insight can be gained by assessing whether GPR50 initiates pro-survival or pro-apoptosis signalling cascades. However, it is important to bear in mind that the upregulation of GPR50 may not have any functional significance, rather it may reflect the transcriptional dysregulation often associated with apoptosis (Kerr et al, 2013).

In addition to cell death, there are other factors to consider in regards to the serum deprivation experiment. Firstly, FBS is a source of a multitude of factors required for cell growth and propagation, such as growth factors, hormones and adhesion molecules (Brunner et al, 2010). The upregulation of GPR50 may be the response to

the absence of any of these factors, thus, it is difficult to isolate the cause of this upregulation. Further, the serum deprivation experiment was performed on two occasions, with different anti-GPR50 antibodies (results shown in figure 6.1 A and B). Both sets of experiments showed an upregulation of GPR50, although, significance was only achieved in one (figure 6.1 A). Thus, further replicates are necessary to ascertain which of the two sets of results accurately reflect GPR50's response to serum starvation. However, this may not be particularly informative as numerous variables are altered upon serum deprivation; this makes it difficult to isolate the driving force behind the upregulation of GPR50.

6.4.1.3 GPR50 promotes lipolysis in SH-SY5Y cells

The reduction of lipid droplets in response to GPR50 transfection suggests there is an increase in lipid metabolism, leading to the depletion of lipid reserves. This is strengthened by the observation that GPR50-transfected cells were largely devoid of lipid droplets in the perinuclear region of the cell, where they are believed to be stored (Wolins et al, 2005). As lipolysis is coupled with the reduction of lipid droplets (Garciafigueroa et al, 2013; O'Rourke & Ruvkun, 2013), these results imply that GPR50 promotes lipid mobilisation and lipolysis. Existing evidence appears to support this. Stable transfection of GPR50 in 3T3-L1 cells increased levels of glycerol and FAs, i.e. products of lipolysis, providing more direct evidence that GPR50 promotes basal lipolysis (Hand, 2011). Indirect associations between GPR50 and lipolysis have also been established. GPR50 has been reported to increase glucocorticoid receptor (GR) activity (Li et al, 2011), which is of note as GR activation has been shown to enhance basal lipolysis in 3T3-L1 cells (Campbell et al, 2011). Further, GPR50 appears to mediate leptin signalling (Bechtold et al, 2012), which is also known to induce lipolysis in white adipose tissue (Siegrist-Kaiser et al, 1997). The GPR50-mediated depletion of lipid droplets, seen in this chapter, complements these studies. However, this should be backed up with direct measurements of triglyceride levels, along with measurements of glycerol and FAs.

6.4.2 The intracellular localisation of GPR50

6.4.2.1 Defects in cholesterol transport leads to the cytoplasmic trafficking of GPR50

In chapter 5, ABCA2 overexpression was found to induce a cytoplasmic distribution of GPR50. As ABCA2 transfection has been shown to cause defects in cholesterol trafficking (Davis, 2011; Davis et al, 2004), it was hypothesised that the altered localisation of GPR50 was due to abnormalities in cholesterol transport. Thus, the effect of U18666A on GPR50 localisation was assessed. As shown in figure 6.4, U18666A increased the intracellular localisation of GPR50. This was only true for short incubation times (5 mins – 1 hr), at the longer periods tested (4 hrs and 18 hrs) GPR50 resumed a plasma membrane distribution. Importantly, these results were not obtained following serum deprivation, indicating that defects in cholesterol trafficking, rather than cholesterol depletion, mediates the internalisation of GPR50. Therefore, the results obtained here supports the hypothesis established in chapter five, that the intracellular localisation of GPR50 was due to the inhibition of cholesterol transport. This can be further confirmed by using an alternative cholesterol trafficking inhibitor, such as imipramine (Xu et al, 2010). In addition, the localisation of GPR50 could be assessed in Niemann-Pick type C1 (NPC1) deficient cells, which are characterised by the accumulation of cholesterol in endo-lysosomal vesicles (Appelqvist et al, 2011).

The transient nature of GPR50's cytoplasmic trafficking may suggest the receptor was recycled rather than degraded. This appears to be supported by western blotting results which showed U18666A did not lead to the reduction of GPR50 in HEK293 cells (figure 6.1), suggesting GPR50 was not downregulated. However, this experiment is not entirely comparable to the ICC experiment described above. In the ICC experiment, exogenous GPR50 was assessed in SH-SY5Y cells and internalisation was observed between 5 mins – 1 hr of U18666A application. When assessing protein levels, endogenous GPR50 was measured in HEK293 cells treated

with U18666A for 18 hrs. Additional work is required to determine the outcome of GPR50 internalisation. This can be done by using HEK293 cells to investigate the effects of U18666A on the localisation of endogenous GPR50. This must be done in conjunction with western blot analysis performed under an appropriate range of U18666A incubation times. In addition, live cell imaging can be used to track the changes to GPR50 trafficking following U18666A treatment.

6.4.2.2 Functional consequence of GPR50 internalisation

Regardless of whether GPR50 is ultimately degraded or recycled, internalisation may be a mechanism to regulate its actions, which is often the case for GPCRs (Kuang et al, 2012; Ritter & Hall, 2009). Interestingly, U18666A treatment, and therefore the internalisation of GPR50, attenuated GPR50's enhancement of neurite outgrowth. Thus the internalisation of GPR50 may reflect a mechanism to regulate the receptor's activities. Interestingly, the intracellular distribution of GPR50 did not appear to affect its metabolic properties; U18666A did not prevent the reduction of lipid droplets upon GPR50 transfection (figure 6.6). This suggests that the induction of lipid mobilisation by GPR50 may not be dependent on a G-protein mediated pathway. An alternative route may be through the modulation of GR activity, in which the intracellular trafficking of GPR50, and its C-terminal domain (CTD), appears to be essential (Li et al, 2011). The importance of the altered trafficking of GPR50 can be investigated further by transfecting cells with a cytoplasmic GPR50 construct, or the CTD of GPR50, and assessing the effects on lipid mobilisation and neurite outgrowth.

6.4.2.3 Is GPR50 specifically internalised in response to U18666A?

It is important to note that U18666A may not specifically promote the cytoplasmic localisation of GPR50. U18666A is known to inhibit cholesterol synthesis and cause the depletion of cholesterol from the plasma membrane (Davis et al, 2004; Sexton et al, 1983), both of which have been reported to lead to receptor instability at the plasma membrane (Hering et al, 2003; Nothdurfter et al, 2010). Therefore, GPR50

internalisation may be a non-specific side effect to the disruption to cholesterol maintenance, especially at the plasma membrane. Further, U18666A and similar inhibitors are known to induce autophagy (Ishibashi et al, 2009; Pacheco et al, 2007; Xu et al, 2010), a process in which cellular components, such as proteins, are internalised and broken down in a non-specific manner. The initiation of this process could also explain the increase in cytoplasmic GPR50. However, serum deprivation is also known to induce autophagy (Wei et al, 2008), yet had no effect on GPR50 localisation (figure 6.4 D). This indicates that GPR50 internalisation was unlikely to be a consequence of non-selective autophagy, thus is more likely to reflect a genuine regulatory mechanism. However, stronger evidence should be obtained by establishing whether the internalisation of GPR50 occurs without an upregulation of autophagy markers, e.g. the microtubule-associated protein 1 light chain (LC3). Additionally, colocalisation between intracellular GPR50 and autophagic vesicles should be assessed.

6.4.3 Conclusion

From the data reported in this chapter it is evident that GPR50 plays a role in lipid homeostasis. GPR50 expression and localisation appeared to be regulated by lipids, moreover, GPR50 was found to be a modulator of intracellular lipid levels. To extend the findings of this chapter, it is important to determine how lipoproteins modulate GPR50 expression, possibly by focusing on FAs. The internalisation of GPR50 should also be investigated further, especially in terms of isolating the mechanism behind it. This would provide insight into how GPR50 is regulated and why it is necessary to modulate GPR50's localisation and expression when lipids are limited. Finally, whether GPR50 directly senses the changes to lipid levels should be explored. To do this it is pertinent to look at whether GPR50 acts on signalling pathways involved in nutrient sensing, such as mammalian target of rapamycin (mTOR) and AMP-activated protein kinase (AMPK) (Kume et al, 2012). Chapter four touched upon this; however, a more detailed investigation is necessary. By

doing so, a better understanding of the role GPR50 plays in energy metabolism could be gained.

Chapter 7: Discussion

7.1 Investigating the role of GPR50

In the last decade there have been major advances in the understanding of GPR50. Links to lipid metabolism have been established from expression studies (Batailler et al, 2012; Drew et al, 2001; Grünewald et al, 2012), *Gpr50* knockout mice (Bechtold et al, 2012; Ivanova et al, 2008) and a genetic association study (Bhattacharyya et al, 2006). Association studies have also implicated GPR50 in neurodevelopment. Sequence variants are associated with a range of psychiatric disorders (Delavest et al, 2012; MacIntyre et al, 2010; Thomson et al, 2005); which are increasingly being considered as disorders of a neurodevelopmental origin (Ansorge et al, 2007; Owen et al, 2011). However, information regarding how GPR50 is involved in these processes is missing.

A number of routes have been taken to unravel the function of GPR50. This includes attempts to identify the receptor's ligand using high-throughput screening assays and homology-based approaches (Levoye et al, 2006). These attempts were largely unsuccessful, however, Omeros have recently reported the identification of a ligand, although they have yet to reveal its identity (Omeros-Corporation, 2012). On the whole, deorphanisation strategies are hindered by the limited pool of known ligands (Chung et al, 2008). The lack of success in regards to GPR50 suggests the receptor binds to an unknown agent, making deorphanisation especially complicated. Hence an alternative strategy was used by this group to ascertain the function of GPR50; the identification of interacting proteins. G-protein coupled receptor (GPCR)-interacting proteins (GIPs) are known to modulate numerous aspects of GPCR activity (Ritter & Hall, 2009), thus can provide a remarkable functional insight into the actions of a given receptor.

To this end a yeast-2-hybrid (Y2H) assay was performed using the C-terminal domain of GPR50. This identified two classes of interactors; proteins involved in

neurodevelopment and those involved in cholesterol metabolism (Grünewald et al, 2009), reinforcing previous reports implicating GPR50 in these processes. Results from this assay were expanded to reveal GPR50 is a positive regulator of neurite outgrowth (Grünewald et al, 2009), providing insight into the neurodevelopmental role of the receptor. The aim of this thesis was to build upon the findings of this Y2H screen and characterise the role of GPR50 in neurodevelopment, focusing on neuronal maturation. The function of GPR50 in lipid metabolism was also investigated in this thesis. This was done by verifying the Y2H interactions within the “cholesterol metabolism” group of interactors and looking at the relationship between GPR50 expression and lipid levels.

7.2 The role of GPR50 in neurodevelopment

In chapter three, the overexpression of GPR50 in mouse hippocampal and cortical neurons led to a significant increase in dendritic branching. In addition, an increase in spine density and maturity was also seen in hippocampal neurons. These morphological changes are well matched with the peak expression of *Gpr50* at embryonic day 18 (E18) (Grünewald et al, 2012), a developmental stage characterised by rapid dendritic outgrowth and the initiation of spinogenesis (Koleske, 2013; Polleux & Snider, 2010). Notably, GPR50 overexpression had no effect on the morphology of dendritic spines, which implies it has no effect on synaptic strength (Kasai et al, 2003), although this has yet to be tested. Importantly, dendritic abnormalities have been implicated in psychiatric disorders through animal models (Barros et al, 2009; Hajszan et al, 2009; Hayashi-Takagi et al, 2010) and post-mortem studies in humans (Glantz & Lewis, 2000; Kang et al, 2012). Thus the ability of GPR50 to regulate dendritic morphology may be the root of its association with mental illness.

7.3 GPR50 regulates actin remodelling

A potential signalling pathway governed by GPR50 was explored in chapter four. Here, it was shown that in SH-SY5Y cells, GPR50 increased the levels of phospho-RAC1/CDC42 and Wiskott–Aldrich syndrome protein (WASP)-family verprolin homologous protein 2 (WAVE-2), promoters of actin branching. In addition, GPR50 was enriched in highly dynamic areas of the membrane, such as dendritic spines and lamellipodia, where colocalisation with RAC1 was observed. Thus, GPR50 may induce remodelling of the plasma membrane through a localised activation of RAC1 and the subsequent activation of the WAVE complex, leading to the induction of actin branching via actin-related protein (ARP) 2/3 (Tahirovic et al, 2010). The induction of the RAC1/WAVE pathway is known to regulate dendritic structure. Loss of WAVE-1, the isoform enriched in the brain, reduced the density of mature dendritic spines and attenuated neurite outgrowth (Kim et al, 2006; Soderling et al, 2007). This is mimicked by the knockdown of other components of the WAVE complex and its downstream effectors (Choi et al, 2005; Kim et al, 2013; Proepper et al, 2007). Thus, the GPR50-induced changes to dendritic morphology may be due to the activation of this actin remodelling pathway.

Accumulating evidence suggests actin remodelling plays a role in the pathology of psychiatric disorders. The well known schizophrenia risk factor, Disrupted-in-Schizophrenia 1 (DISC1), has been shown to negatively regulate spine maturation through the interaction with kalirin-7, attenuating the ability of kalirin-7 to activate RAC1 (Hayashi-Takagi et al, 2010). Similarly, dysbindin-1, another schizophrenia susceptibility factor, has been shown to promote the formation of the WAVE-2/Abl interactor 1 complex (Ito et al, 2010). Further, Ito and colleagues found *Dysbindin-1* deletion prevented the maturation of dendritic spines, presumably a result of the reduction in WAVE-2/ Abl interactor 1 and inefficient actin polymerisation. In addition, ARP2/3 has been linked to the cognitive deficits, social disturbances and manic behaviour that is characteristic of a range of psychiatric disorders (Han et al,

2013; Kim et al, 2013). Considering these studies, the involvement of GPR50 in actin remodelling seems very fitting.

Preliminary work in chapter four also suggests that GPR50 may modulate actin dynamics via α -catenin. Overexpression led to the reduction of α -catenin in HEK293 cells and the altered localisation in SH-SY5Y cells, although this requires replication. In neurons, α -catenin is essential for the long-term stability of dendrites and synapses but not their formation (Abe et al, 2004; Tan et al, 2010). Therefore, the reduction of α -catenin by GPR50 may promote structural plasticity. This could be beneficial at E18, when *Gpr50* expression peaks (Grünewald et al, 2012). At this age dendrites and spines are highly dynamic, undergoing rapid formation and elimination (Koleske, 2013). The loss of α -catenin may favour this. Dendrites gradually stabilise throughout adolescence and adulthood, where the level of *Gpr50* is considerably lower compared to E18 (Grünewald et al, 2012). This may alleviate the inhibition to dendrite stability caused by the reduction in α -catenin and ensure stabilisation occurs once appropriate synaptic contacts are made. Much work is required to substantiate this theory, starting with the relevant replications; however, this is an interesting hypothesis to explore.

7.4 GPR50 as a lipid sensor

The Y2H screen performed by this group suggested GPR50 was involved in lipid regulation through interactions with ATP-binding cassette transporter 2 (ABCA2) and sterol regulatory element binding transcription factor 2 (SREBF2) (Grünewald et al, 2009). However, results in chapter five indicated that a direct interaction between GPR50 and these proteins is unlikely. Nonetheless, GPR50 does appear to be involved in lipid metabolism (chapter six). In HEK293 cells, lipoprotein deficiency led to a decrease in endogenous GPR50, supporting reports of decreased *Gpr50* expression in regions of the hypothalamus in response to fasting (Barrett et al, 2006;

Ivanova et al, 2008). Interestingly, excess food consumption also reduced *Gpr50* in these areas (Bechtold et al, 2012; Ivanova et al, 2008). It has been suggested that *Gpr50* is responsive to fatty acids as these are increased upon fasting and high energy diets (Frier et al, 2011). Although this hypothesis is compatible with the results from chapter six, the effects of fatty acids on GPR50 expression should be directly tested. The cellular distribution of GPR50 also appears to be regulated by lipids. An internal localisation was observed upon co-transfection with ABCA2 (chapter five) and U18666A treatment (chapter six), conditions in which cholesterol trafficking and synthesis are inhibited (Davis et al, 2004; Sexton et al, 1983). This indicates that GPR50 is sensitive to changes in the intracellular cholesterol environment.

The responsiveness of GPR50 to lipid levels may denote a role in lipid sensing. This is compelling as GPR50 is found in areas of the brain harbouring key nutrient sensing neurons, such as the arcuate nucleus and lateral hypothalamus (Grünewald et al, 2012). These neurons detect nutrient levels and use this information to appropriately modulate effectors such as feeding, glucose production and energy expenditure (Blouet & Schwartz, 2010; Migrenne et al, 2011). Interestingly, *Gpr50* deletion in mice led to increased food intake (Ivanova et al, 2008) and lower fasted glucose levels compared to wild-type mice (Bechtold et al, 2012). In addition, the energy expenditure of *Gpr50* knockout mice were markedly higher than wild-type mice in control conditions (Ivanova et al, 2008) and drastically lower in fasted conditions (Bechtold et al, 2012). Together these results, along with those presented in this thesis, implicate GPR50 in lipid sensing pathways. Whether GPR50 directly binds to lipid mediators or is indirectly regulated by lipids should be investigated in the future.

7.5 GPR50 promotes lipolysis

In chapter six the effects of GPR50 on intracellular lipid levels was also assessed. GPR50 overexpression in SH-SY5Y cells led to a decrease in lipid droplets, suggesting an increased requirement for lipids. This agrees with the increase in basal lipolysis seen in adipocytes overexpressing GPR50 (Hand, 2011). Lipids have a crucial role in neurite outgrowth and dendritic morphogenesis (Hering et al, 2003; Hozumi et al, 2009; Kim et al, 2009b; Ko et al, 2005). For this reason, it was hypothesised that the increased use of lipids served to support the neurite outgrowth seen in SH-SY5Y cells following GPR50 transfection. However, this does not appear to be the case as U18666A treatment inhibited GPR50-induced neurite outgrowth but not lipid mobilisation (chapter six). Thus, GPR50's role in lipid metabolism may be unrelated to its role in neurite outgrowth.

It should be noted that lipids have been shown to regulate the stability of dendrites and spines. Depletion of the LDL-receptor related protein 1 (LRP1), a major lipoprotein receptor in the brain, resulted in the progressive loss of spines in adult mice (Liu et al, 2010). After 18 months *Lrp1*^{-/-} mice had fewer synapses than control mice, whereas at earlier time points *Lrp1* deletion had no effect. Diacylglycerol (DAG), a product of triglyceride hydrolysis, has also been implicated in spine stability. Depletion of DAG kinase ζ (DGK ζ) resulted in a dramatic increase in spine elimination but had no effect on spine formation (Kim et al, 2009b). Further, cholesterol depletion has been demonstrated to reduce spine density and cause the reduction of filamentous actin (F-actin) in spines (Hering et al, 2003). This decrease in spine density was reversed by Jasplakinolide, an F-actin stabilising drug, suggesting the effects of cholesterol depletion on spine stability was mediated by the actin cytoskeleton. Importantly, these studies show dendritic development occurs despite lipid dysregulation, suggesting that although lipid metabolism is an important aspect of neuronal morphogenesis the two processes are not inherently coupled. In view of this, GPR50 may induce dendritic outgrowth and spinogenesis via actin

remodelling pathways, while its role in lipid mobilisation may support the retention of these structures through an independent pathway.

There appears to be an intricate relationship between lipid levels and cognition. Deletion of the lipid regulators *Lrp1* and *Dgk β* caused cognitive deficits in addition to changes to dendritic structure (Liu et al, 2010; Shirai et al, 2010). This supports genetic and molecular studies which have linked LRP1 to Alzheimer's disease (Fuentealba et al, 2010; Kang et al, 2000) and DGK to bipolar disorder and schizophrenia (Baum et al, 2008; Zeng et al, 2011). Thus, the modulation of lipid levels by GPR50 may be central to its association with mental illness (Delavest et al, 2012; MacIntyre et al, 2010; Thomson et al, 2005). This is an intriguing prospect as the *GPR50* polymorphisms linked to mental illness are also associated with high triglyceride and low high-density lipoprotein (HDL)-cholesterol levels (Bhattacharyya et al, 2006). Coincidentally, this lipid profile has repeatedly been reported in individuals with psychiatric disorders (Lehto et al, 2008; Lehto et al, 2010; Saari et al, 2004). Given this, it is of high interest to characterise the full effects of GPR50 on neuronal lipid metabolism.

7.6 Does GPR50 signal via mTORC2?

GPR50 transfection was shown to increase levels of phospho-AKT (S473) in SH-SY5Y cells (chapter four), suggesting the mammalian target of rapamycin (mTOR) complex 2 (mTORC2)/AKT pathway may be activated. At this stage, this finding is speculative; more work is required to confirm this pathway is activated by GPR50. However, this link may be highly significant as mTOR signalling is known to regulate multiple aspects of dendritic morphogenesis (Huang et al, 2013; Kumar et al, 2005; Lee et al, 2011). Further, the promotion of spine density by mTORC2 appears to be dependent on RAC1-mediated actin polymerisation (Huang et al, 2013). Huang et al also showed long-term synaptic plasticity and long-term memory

were impaired by mTORC2 ablation, which is supported by an independent study showing synaptic deficits upon the depletion of mTORC2 (Thomanetz et al, 2013). This coincides with the reported link between mTORC2 signalling and disorders impairing cognition and memory (Humbert et al, 2002; Malagelada et al, 2008). Interestingly, the defects to synaptic plasticity and memory were largely restored through the promotion of actin polymerisation by Jasplakinolide (Huang et al, 2013). In light of these studies, it is reasonable to hypothesise that the activation of mTORC2/AKT by GPR50 may account for the induction of the RAC1/WAVE pathway and the changes to dendritic morphology. By doing so, GPR50 may also impact synaptic plasticity and memory formation.

The effects of GPR50 on intracellular lipids may also require mTORC2. TORC2 disruption in *Caenorhabditis elegans* led to an accumulation of fat (Jones et al, 2009; Soukas et al, 2009). Likewise, the depletion of mTORC2 in mice caused lipid accumulation in the liver and skeletal muscle (Kumar et al, 2010). This would suggest that the activation of mTORC2 by GPR50 would cause a reduction in lipid droplets, which was seen in chapter six. However, mTORC2 deficient mice also displayed increased levels of lipolysis (Kumar et al, 2010) and a reduction in hepatic lipogenesis (Hagiwara et al, 2012; Yuan et al, 2012). This implies mTORC2 activation would increase lipid stores through the production of lipids and the reduction of its breakdown. Thus, mTORC2 appears to be involved lipid homeostasis, although its exact role is unclear. Until more is known about the role of mTORC2 in lipid metabolism it will be difficult to understand how/if activation by GPR50 contributes to lipid mobilisation.

Interest in mTORC2 signalling also comes from its link to neurodevelopmental disorders. Schizophrenia risk factors, DISC1 and neuregulin 1, have been shown to signal through mTORC2/AKT (Kim et al, 2009a; Schulz et al, 2013). This is supported by genetic studies which show *AKT1* is associated with schizophrenia and bipolar disorder (Karege et al, 2012; Mathur et al, 2010; Norton et al, 2007).

Interestingly, the inhibition of mTOR signalling has been shown to ameliorate the cognitive defects in models of Alzheimer's disease and schizophrenia (Spilman et al, 2010; Zhou et al, 2013). Conversely, the antidepressant properties of ketamine appear to be dependent on mTOR activity (Li et al, 2010). These results highlight the importance of mTOR signalling in the pathogenesis and treatment of psychiatric illness. For this reason, along with the role in dendritic morphogenesis and lipid metabolism, further investigation into the involvement of mTORC2 in GPR50 signalling is of great interest.

7.7 Future work

This thesis presents a role for GPR50 in the regulation of dendritic morphology and lipid metabolism. It also highlights signalling pathways GPR50 may use to carry out these functions. Although these results are compelling there are important gaps to fill. In addition, new questions have arisen. The most pertinent points are addressed below.

- 1. Characterising the role of GPR50 in actin remodelling.** Results from chapter four suggest GPR50 is capable of inducing actin remodelling via the activation of the RAC1/WAVE pathway. However, this must be formally tested by measuring the effects of GPR50 on actin polymerisation and cell migration/spreading. It must also be established whether the activation of this pathway caused the changes to dendritic morphology observed following GPR50 transfection. Finally, the effect of GPR50 on alternative actin remodelling pathways should be considered.
- 2. Does GPR50 affect synaptic function?** It is important to determine whether the morphological changes to dendrites are coupled with changes to synaptic transmission, as this may translate to abnormalities in memory, cognition and

behaviour. Electrophysiological measurements can be taken from GPR50 transfected or silenced neurons to study this.

3. **Characterisation of GPR50's involvement in lipid metabolism.** Although the depletion of lipid droplets suggests that GPR50 increases lipolysis, this should be accurately assessed by measuring the levels of the relevant lipids, e.g. triglyceride and DAG. The role of GPR50 as a lipid sensor should also be explored by uncovering the lipoprotein component modulating the expression of GPR50. Further, whether this lipid element acts as a ligand should be investigated.
4. **Does GPR50 function through mTORC2?** There are marked similarities between the actions of GPR50 and mTORC2 signalling; suggesting GPR50 may activate the latter. However, the only tangible piece of evidence comes from the phosphorylation and activation of AKT, via S473, and RAC1 via S71. This must be extended by looking at additional AKT substrates. It should also be established whether the effects of GPR50 on dendritic morphology and lipid mobilisation are dependent on mTORC2 signalling using the relevant inhibitors.
5. **Does GPR50 act alone or does it modulate the function of interacting proteins?** Much of the results described in this thesis may be attributed to the ability of GPR50 to modulate the activity of its interactors, such as neurite outgrowth inhibitor A (NOGO-A) and HIV-1 tat interactive protein (TIP60), rather than the direct effect of the receptor. To determine whether this is the case experiments can be repeated in the absence of the GPR50-interactors using knockdown strategies.
6. **Characterisation of *Gpr50* knockout mice.** Loss-of-function studies have assessed the impact of GPR50 on metabolism, but the effects on neurodevelopment have been overlooked. Although this group does not have access to these mice, it would be of great interest to examine this. As well as investigating structural changes within the brain, behavioural data should be collected.

7. How do polymorphisms within *GPR50* impact its function? As this PhD stemmed from an association study linking *GPR50* to mental illness, it is important to assess how the identified sequence variants impact the actions of *GPR50* revealed in this thesis. Mechanistic insight into how *GPR50* is involved in the pathology of mental illness can be ascertained from this data.

References

References

- Abe K, Chisaka O, van Roy F, Takeichi M (2004) Stability of dendritic spines and synaptic contacts is controlled by alpha N-catenin. *Nature Neuroscience* **7**: 357-363
- Adler J, Parmryd I (2010) Quantifying Colocalization by Correlation: The Pearson Correlation Coefficient is Superior to the Mander's Overlap Coefficient. *Cytometry Part A* **77A**: 733-742
- Ahn S, Kim J, Hara MR, Ren X-R, Lefkowitz RJ (2009) {beta}-Arrestin-2 Mediates Anti-apoptotic Signaling through Regulation of BAD Phosphorylation. *The Journal of biological chemistry* **284**: 8855-8865
- Alaerts M, Venken T, Lenaerts AS, De Zutter S, Norrback KF, Adolfsson R, Del-Favero J (2006) Lack of association of an insertion/deletion polymorphism in the G protein-coupled receptor 50 with bipolar disorder in a Northern Swedish population. *Psychiatric Genetics* **16**: 235-236
- Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PRJ, Reese CB, Cohen P (1997) Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase B alpha. *Current Biology* **7**: 261-269
- Allen JA, Halverson-Tamboli RA, Rasenick MM (2007) Lipid raft microdomains and neurotransmitter signalling. *Nature Reviews Neuroscience* **8**: 128-140
- Ango F, Prezeau L, Muller T, Tu JC, Xiao B, Worley PF, Pin JP, Bockeaert J, Fagni L (2001) Agonist-independent activation of metabotropic glutamate receptors by the intracellular protein Homer. *Nature* **411**: 962-965
- Ansorge MS, Hen R, Gingrich JA (2007) Neurodevelopmental origins of depressive disorders. *Current Opinion in Pharmacology* **7**: 8-17
- Appelqvist H, Nilsson C, Garner B, Brown AJ, Kagedal K, Ollinger K (2011) Attenuation of the Lysosomal Death Pathway by Lysosomal Cholesterol Accumulation. *American Journal of Pathology* **178**: 629-639
- Atwood BK, Lopez J, Wager-Miller J, Mackie K, Straiker A (2011) Expression of G protein-coupled receptors and related proteins in HEK293, AtT20, BV2, and N18 cell lines as revealed by microarray analysis. *BMC genomics* **12**: 14-14

Barrett P, Conway S, Morgan PJ (2003) Digging deep - structure-function relationships in the melatonin receptor family. *Journal of Pineal Research* **35**: 221-230

Barrett P, Ivanova E, Graham ES, Ross AW, Wilson D, Ple H, Mercer JG, Ebling FJ, Schuhler S, Dupre SM, Loudon A, Morgan PJ (2006) Photoperiodic regulation of cellular retinoic acid-binding protein 1, GPR50 and nestin in tanycytes of the third ventricle ependymal layer of the Siberian hamster. *Journal of Endocrinology* **191**: 687-698

Barros CS, Calabrese B, Chamero P, Roberts AJ, Korzus E, Lloyd K, Stowers L, Mayford M, Halpain S, Mueller U (2009) Impaired maturation of dendritic spines without disorganization of cortical cell layers in mice lacking NRG1/ErbB signaling in the central nervous system. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 4507-4512

Bartlett SE, Enquist J, Hopf FW, Lee JH, Gladher F, Kharazia V, Waldhoer M, Mailliard WS, Armstrong R, Bonci A, Whistler JL (2005) Dopamine responsiveness is regulated by targeted sorting of D2 receptors. *Proceedings of the National Academy of Sciences of the United States of America* **102**: 11521-11526

Bartlett WP, Banker GA (1984) AN ELECTRON-MICROSCOPIC STUDY OF THE DEVELOPMENT OF AXONS AND DENDRITES BY HIPPOCAMPAL-NEURONS IN CULTURE .1. CELLS WHICH DEVELOP WITHOUT INTERCELLULAR CONTACTS. *Journal of Neuroscience* **4**: 1944-1953

Batailler M, Mullier A, Sidibe A, Delagrangre P, Prevot V, Jockers R, Migaud M (2012) Neuroanatomical distribution of the orphan GPR50 receptor in adult sheep and rodent brains. *Journal of Neuroendocrinology* **24**: 798-808

Baum AE, Akula N, Cabanero M, Cardona I, Corona W, Klemens B, Schulze TG, Cichon S, Rietschel M, Noethen MM, Georgi A, Schumacher J, Schwarz M, Abou Jamra R, Hoefels S, Propping P, Satagopan J, Detera-Wadleigh SD, Hardy J, McMahon FJ, Bipolar NGI (2008) A genome-wide association study implicates diacylglycerol kinase eta (DGKH) and several other genes in the etiology of bipolar disorder. *Molecular Psychiatry* **13**: 197-207

Bechtold DA, Sidibe A, Saer BRC, Li J, Hand LE, Ivanova EA, Darras VM, Dam J, Jockers R, Luckman SM, Loudon ASI (2012) A Role for the Melatonin-Related Receptor GPR50 in Leptin Signaling, Adaptive Thermogenesis, and Torpor. *Current Biology* **22**: 70-77

Bermak JC, Li M, Bullock C, Zhou QY (2001) Regulation of transport of the dopamine D1 receptor by a new membrane-associated ER protein. *Nature Cell Biology* **3**: 492-498

Berton O, Nestler EJ (2006) New approaches to antidepressant drug discovery: beyond monoamines. *Nature Reviews Neuroscience* **7**: 137-151

Bhattacharyya S, Luan J, Challis B, Keogh J, Montague C, Brennand J, Morten J, Lowenbeim S, Jenkins S, Farooqi IS, Wareham NJ, O'Rahilly S (2006) Sequence variants in the melatonin-related receptor gene (GPR50) associate with circulating triglyceride and HDL levels. *Journal of Lipid Research* **47**: 761-766

Bjursell M, Admyre T, Goransson M, Marley AE, Smith DM, Oscarsson J, Bohlooly-Y M (2011) Improved glucose control and reduced body fat mass in free fatty acid receptor 2-deficient mice fed a high-fat diet. *American Journal of Physiology-Endocrinology and Metabolism* **300**: E211-E220

Blandin G, Marchand S, Charton K, Daniele N, Gicquel E, Boucheteil J-B, Bentaib A, Barrault L, Stockholm D, Bartoli M, Richard I (2013) A human skeletal muscle interactome centered on proteins involved in muscular dystrophies: LGMD interactome. *Skeletal muscle* **3**: 3-3

Blouet C, Schwartz GJ (2010) Hypothalamic nutrient sensing in the control of energy homeostasis. *Behavioural Brain Research* **209**: 1-12

Bonnefond A, Clement N, Fawcett K, Yengo L, Vaillant E, Guillaume J-L, Dechaume A, Payne F, Roussel R, Czernichow S, Hercberg S, Hadjadj S, Balkau B, Marre M, Lantieri O, Langenberg C, Bouatia-Naji N, Charpentier G, Vaxillaire M, Rocheleau G, Wareham NJ, Sladek R, McCarthy MI, Dina C, Barroso I, Jockers R, Froguel P, Meta-Anal Glucose I-R (2012) Rare MTNR1B variants impairing melatonin receptor 1B function contribute to type 2 diabetes. *Nature Genetics* **44**: 297-U298

Borroni V, Barrantes FJ (2011) Cholesterol Modulates the Rate and Mechanism of Acetylcholine Receptor Internalization. *Journal of Biological Chemistry* **286**: 17122-17132

Bouatia-Naji N, Bonnefond A, Cavalcanti-Proenca C, Sparso T, Holmkvist J, Marchand M, Delplanque J, Lobbens S, Rocheleau G, Durand E, De Graeve F, Chevre J-C, Borch-Johnsen K, Hartikainen A-L, Ruukonen A, Tichet J, Marre M,

Weill J, Heude B, Tauber M, Lemaire K, Schuit F, Elliott P, Jorgensen T, Charpentier G, Hadjadj S, Cauchi S, Vaxillaire M, Sladek R, Visvikis-Siest S, Balkau B, Levy-Marchal C, Pattou F, Meyre D, Blakemore AIF, Jarvelin M-R, Walley AJ, Hansen T, Dina C, Pedersen O, Froguel P (2009) A variant near MTNR1B is associated with increased fasting plasma glucose levels and type 2 diabetes risk. *Nature Genetics* **41**: 89-94

Bousquet C, Guillermet-Guibert J, Saint-Laurent N, Archer-Lahlou E, Lopez F, Fanjul M, Ferrand A, Fourmy D, Pichereaux C, Monsarrat B, Pradayrol L, Esteve J-P, Susini C (2006) Direct binding of p85 to sst2 somatostatin receptor reveals a novel mechanism for inhibiting PI3K pathway. *Embo Journal* **25**: 3943-3954

Bradshaw NJ, Ogawa F, Antolin-Fontes B, Chubb JE, Carlyle BC, Christie S, Claessens A, Porteous DJ, Millar JK (2008) DISC1, PDE4B, and NDE1 at the centrosome and synapse. *Biochemical and Biophysical Research Communications* **377**: 1091-1096

Broccardo C, Nieoullon V, Amin R, Masméjean F, Carta S, Tassi S, Pophillat M, Rubartelli A, Pierres M, Rougon G, Nieoullon A, Chazal G, Chimini G (2006a) ABCA2 is a marker of neural progenitors and neuronal subsets in the adult rodent brain. *Journal of Neurochemistry* **97**: 345-355

Broccardo C, Nieoullon V, Amin R, Masméjean F, Carta S, Tassi S, Pophillat M, Rubartelli A, Pierres M, Rougon G, Nieoullon A, Chazal G, Chimini G (2006b) ABCA2 is a marker of neural progenitors and neuronal subsets in the adult rodent brain. *J Neurochem* **97**: 345-355

Brueckner A, Polge C, Lentze N, Auerbach D, Schlattner U (2009) Yeast Two-Hybrid, a Powerful Tool for Systems Biology. *International Journal of Molecular Sciences* **10**: 2763-2788

Brunner D, Frank J, Appl H, Schoeffl H, Pfaller W, Gstraunthaler G (2010) Serum-free Cell Culture: The Serum-free Media Interactive Online Database. *Altex-Alternatives to Animal Experimentation* **27**: 53-62

Buerli T, Pellegrino C, Baer K, Lardi-Studler B, Chudotvorova I, Fritschy J-M, Medina I, Fuhrer C (2007) Efficient transfection of DNA or shRNA vectors into neurons using magnetofection. *Nature Protocols* **2**: 3090-3101

Burks J, Agazie YM (2006) Modulation of alpha-catenin Tyr phosphorylation by SHP2 positively effects cell transformation induced by the constitutively active FGFR3. *Oncogene* **25**: 7166-7179

Bustelo XR, Ojeda V, Barreira M, Sauzeau V, Castro-Castro A (2012) Rac-ing to the plasma membrane: the long and complex work commute of Rac1 during cell signaling. *Small GTPases* **3**: 60-66

Campbell JE, Peckett AJ, D'Souza AM, Hawke TJ, Riddell MC (2011) Adipogenic and lipolytic effects of chronic glucocorticoid exposure. *American Journal of Physiology-Cell Physiology* **300**: C198-C209

Cao TT, Deacon HW, Reczek D, Bretscher A, von Zastrow M (1999) A kinase-regulated PDZ-domain interaction controls endocytic sorting of the beta 2-adrenergic receptor. *Nature* **401**: 286-290

Catapano LA, Manji HK (2007) G protein-coupled receptors in major psychiatric disorders. *Biochimica Et Biophysica Acta-Biomembranes* **1768**: 976-993

Cavallaro U, Dejana E (2011) Adhesion molecule signalling: not always a sticky business. *Nature Reviews Molecular Cell Biology* **12**: 189-197

Chan ASL, Lai FPL, Lo RKH, Voyno-Yasenetskaya TA, Stanbridge EJ, Wong YH (2002) Melatonin mt1 and MT2 receptors stimulate c-Jun N-terminal kinase via pertussis toxin-sensitive and -insensitive G proteins. *Cellular Signalling* **14**: 249-257

Chao P-T, Yang L, Aja S, Moran TH, Bi S (2011) Knockdown of NPY Expression in the Dorsomedial Hypothalamus Promotes Development of Brown Adipocytes and Prevents Diet-Induced Obesity. *Cell Metabolism* **13**: 573-583

Chen B-S, Thomas EV, Sanz-Clemente A, Roche KW (2011) NMDA Receptor-Dependent Regulation of Dendritic Spine Morphology by SAP102 Splice Variants. *Journal of Neuroscience* **31**: 89-96

Chen Z, Vulevic B, Ile K, Soulika A, Davis WJ, Reiner P, Connop B, Nathwani P, Trojanowski J, Tew K (2004) Association of ABCA2 expression with determinants of Alzheimer's disease. *FASEB J* **18**: 1129-1131

Cheng S-B, Filardo EJ (2012) Trans-Golgi Network (TGN) as a Regulatory Node for beta 1-Adrenergic Receptor (beta 1AR) Down-modulation and Recycling. *Journal of Biological Chemistry* **287**: 14178-14191

Chern C-M, Liao J-F, Wang Y-H, Shen Y-C (2012) Melatonin ameliorates neural function by promoting endogenous neurogenesis through the MT2 melatonin receptor in ischemic-stroke mice. *Free Radical Biology and Medicine* **52**: 1634-1647

Cheung NS, Koh CHV, Bay BH, Qi RZ, Choy MS, Li QT, Wong KP, Whiteman M (2004) Chronic exposure to U18666A induces apoptosis in cultured murine cortical neurons. *Biochemical and Biophysical Research Communications* **315**: 408-417

Cho S, Wood A, Bowby MR (2007) Brain slices as models for neurodegenerative disease and screening platforms to identify novel therapeutics. *Current Neuropharmacology* **5**: 19-33

Choi J, Ko J, Racz B, Burette A, Lee JR, Kim S, Na M, Lee HW, Kim K, Weinberg RJ, Kim E (2005) Regulation of dendritic spine morphogenesis by insulin receptor substrate 53, a downstream effector of Rac1 and Cdc42 small GTPases. *Journal of Neuroscience* **25**: 869-879

Chou TC, Scammell TE, Gooley JJ, Gaus SE, Saper CB, Lu J (2003) Critical role of dorsomedial hypothalamic nucleus in a wide range of behavioral circadian rhythms. *Journal of Neuroscience* **23**: 10691-10702

Chung S, Funakoshi T, Civelli O (2008) Orphan GPCR research. *British Journal of Pharmacology* **153**: S339-S346

Ciani L, Boyle KA, Dickins E, Sahores M, Anane D, Lopes DM, Gibb AJ, Salinas PC (2011) Wnt7a signaling promotes dendritic spine growth and synaptic strength through Ca²⁺/Calmodulin-dependent protein kinase II. *Proceedings of the National Academy of Sciences of the United States of America* **108**: 10732-10737

Contreras-Alcantara S, Baba K, Tosini G (2010) Removal of Melatonin Receptor Type 1 Induces Insulin Resistance in the Mouse. *Obesity* **18**: 1861-1863

Conway S, Drew JE, Mowat ES, Barrett P, Delagrangre P, Morgan PJ (2000) Chimeric melatonin mt(1) and melatonin-related receptors - Identification of domains and residues participating in ligand binding and receptor activation of the melatonin mt(1) receptor. *Journal of Biological Chemistry* **275**: 20602-20609

Costa-Mattioli M, Monteggia LM (2013) mTOR complexes in neurodevelopmental and neuropsychiatric disorders. *Nature neuroscience* **16**: 1537-1543

Cristancho AG, Lazar MA (2011) Forming functional fat: a growing understanding of adipocyte differentiation. *Nature Reviews Molecular Cell Biology* **12**: 722-734

David DJ, Samuels BA, Rainer Q, Wang J-W, Marsteller D, Mendez I, Drew M, Craig DA, Guiard BP, Guilloux J-P, Artymyshyn RP, Gardier AM, Gerald C, Antonijevic IA, Leonardo ED, Hen R (2009) Neurogenesis-Dependent and -Independent Effects of Fluoxetine in an Animal Model of Anxiety/Depression. *Neuron* **62**: 479-493

Davis W, Jr. (2011) The ATP-binding cassette transporter-2 (ABCA2) regulates cholesterol homeostasis and low-density lipoprotein receptor metabolism in N2a neuroblastoma cells. *Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids* **1811**: 1152-1164

Davis WJ, Boyd J, Ile K, Tew K (2004) Human ATP-binding cassette transporter-2 (ABCA2) positively regulates low-density lipoprotein receptor expression and negatively regulates cholesterol esterification in Chinese hamster ovary cells. *Biochim Biophys Acta* **1683**: 89-100

Delavest M, Even C, Benjemaa N, Poirier MF, Jockers R, Krebs MO (2012) Association of the intronic rs2072621 polymorphism of the X-linked GPR50 gene with affective disorder with seasonal pattern. *European Psychiatry* **27**: 369-371

Delekate A, Zagrebelsky M, Kramer S, Schwab ME, Korte M (2011) NogoA restricts synaptic plasticity in the adult hippocampus on a fast time scale. *Proceedings of the National Academy of Sciences of the United States of America* **108**: 2569-2574

Desai R, Sarpal R, Ishiyama N, Pellikka M, Ikura M, Tepass U (2013) Monomeric alpha-catenin links cadherin to the actin cytoskeleton. *Nature Cell Biology* **15**: 261-273

Di Paolo G, Kim T-W (2011) Linking lipids to Alzheimer's disease: cholesterol and beyond. *Nature Reviews Neuroscience* **12**: 284-296

Dietschy JM (2009) Central nervous system: cholesterol turnover, brain development and neurodegeneration. *Biological Chemistry* **390**: 287-293

Dorsam RT, Gutkind JS (2007) G-protein-coupled receptors and cancer. *Nature Reviews Cancer* **7**: 79-94

Dotti CG, Sullivan CA, Banker GA (1988) THE ESTABLISHMENT OF POLARITY BY HIPPOCAMPAL-NEURONS IN CULTURE. *Journal of Neuroscience* **8**: 1454-1468

Draber P, Sulimenko V, Draberova E (2012) Cytoskeleton in mast cell signaling. *Frontiers in immunology* **3**: 130-130

Drees F, Pokutta S, Yamada S, Nelson WJ, Weis WI (2005) alpha-catenin is a molecular switch that binds E-cadherin-beta-catenin and regulates actin-filament assembly. *Cell* **123**: 903-915

Drew J, Barrett P, Mercer J, Moar K, Canet E, Delagrang P, Morgan P (2001) Localization of the melatonin-related receptor in the rodent brain and peripheral tissues. *J Neuroendocrinol* **13**: 453-458

Drew JE, Barrett P, Williams LM, Conway S, Morgan PJ (1998) The ovine melatonin-related receptor: Cloning and preliminary distribution and binding studies. *Journal of Neuroendocrinology* **10**: 651-661

Dubielecka PM, Ladwein KI, Xiong X, Migeotte I, Chorzalska A, Anderson KV, Sawicki JA, Rottner K, Stradal TE, Kotula L (2011) Essential role for Abi1 in embryonic survival and WAVE2 complex integrity. *Proceedings of the National Academy of Sciences of the United States of America* **108**: 7022-7027

Dubocovich ML, Hudson RL, Sumaya IC, Masana MI, Manna E (2005) Effect of MT1 melatonin receptor deletion on melatonin-mediated phase shift of circadian rhythms in the C57BL/6 mouse. *Journal of Pineal Research* **39**: 113-120

Dufourny L, Levasseur A, Migaud M, Callebaut I, Pontarotti P, Malpoux B, Monget P (2008) GPR50 is the mammalian ortholog of Mel1c: Evidence of rapid evolution in mammals. *Bmc Evolutionary Biology* **8**

Duncan RE, Ahmadian M, Jaworski K, Sarkadi-Nagy E, Sul HS (2007) Regulation of lipolysis in adipocytes. *Annual Review of Nutrition* **27**: 79-101

Dunn KW, Kamocka MM, McDonald JH (2011) A practical guide to evaluating colocalization in biological microscopy. *American Journal of Physiology-Cell Physiology* **300**: C723-C742

Dutt A, McDonald C, Dempster E, Prata D, Shaikh M, Williams I, Schulze K, Marshall N, Walshe M, Allin M, Collier D, Murray R, Bramon E (2009) The effect of COMT, BDNF, 5-HTT, NRG1 and DTNBP1 genes on hippocampal and lateral ventricular volume in psychosis. *Psychological Medicine* **39**: 1783-1797

Ehrlich I, Klein M, Rumpel S, Malinow R (2007) PSD-95 is required for activity-driven synapse stabilization. *Proceedings of the National Academy of Sciences of the United States of America* **104**: 4176-4181

El-Hussein AE, Schnell E, Chetkovich DM, Nicoll RA, Brecht DS (2000) PSD-95 involvement in maturation of excitatory synapses. *Science* **290**: 1364-1368

Etienne-Manneville S, Hall A (2003) Cdc42 regulates GSK-3 beta and adenomatous polyposis coli to control cell polarity. *Nature* **421**: 753-756

Eto K, Nishikii H, Ogaeri T, Suetsugu S, Kamiya A, Kobayashi T, Yamazaki D, Oda A, Takenawa T, Nakauchi H (2007) The WAVE2/Abi 1 complex differentially regulates megakaryocyte development and spreading: implications for platelet biogenesis and spreading machinery. *Blood* **110**: 3637-3647

Evans SB, Wilkinson CW, Gronbeck P, Bennett JL, Zavosh A, Taborsky GJ, Figlewicz DP (2004) Inactivation of the DMH selectively inhibits the ACTH and corticosterone responses to hypoglycemia. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology* **286**: R123-R128

Even-Ram S, Doyle AD, Conti MA, Matsumoto K, Adelstein RS, Yamada KM (2007) Myosin IIA regulates cell motility and actomyosin microtubule crosstalk. *Nature Cell Biology* **9**: 299-U104

Fajutrao L, Locklear J, Prialux J, Heyes A (2009) A systematic review of the evidence of the burden of bipolar disorder in Europe. *Clinical practice and epidemiology in mental health : CP & EMH* **5**: 3-3

Faraone D, Aguzzi MS, Ragone G, Russo K, Capogrossi MC, Facchiano A (2006) Heterodimerization of FGF-receptor 1 and PDGF-receptor-alpha: a novel mechanism underlying the inhibitory effect of PDGF-BB on FGF-2 in human cells. *Blood* **107**: 1896-1902

Feng JH, Park J, Cron P, Hess D, Hemmings BA (2004) Identification of a PKB/Akt hydrophobic motif Ser-473 kinase as DNA-dependent protein kinase. *Journal of Biological Chemistry* **279**: 41189-41196

Feng Y, Wigg K, King N, Vetro A, Kiss E, Kapornai K, Mayer L, Gadoros J, Kennedy JL, Kovacs M, Barr CL, Int Consortium Childhood-Onset M (2007) GPR50 is not associated with childhood-onset mood disorders in a large sample of Hungarian families. *Psychiatric Genetics* **17**: 347-350

Ferno J, Raeder MB, Vik-Mo AO, Skrede S, Glambek M, Tronstad KJ, Breilid H, Lovlie R, Berge RK, Stansberg C, Steen VM (2005) Antipsychotic drugs activate SREBP-regulated expression of lipid biosynthetic genes in cultured human glioma cells: a novel mechanism of action? *Pharmacogenomics Journal* **5**: 298-304

Formstecher E, Aresta S, Collura V, Hamburger A, Meil A, Trehin A, Reverdy C, Betin V, Maire S, Brun C, Jacq B, Arpin M, Bellaiche Y, Bellusci S, Benaroch P, Bornens M, Chanet R, Chavier P, Delattre O, Doye V, Fehon R, Faye G, Galli T, Girault JA, Goud B, de Gunzburg J, Johannes L, Junier MP, Mirouse V, Mukherjee A, Papadopoulo D, Perez F, Plessis A, Rosse C, Saule S, Stoppa-Lyonnet D, Vincent A, White M, Legrain P, Wojcik J, Camonis J, Daviet L (2005) Protein interaction mapping: A Drosophila case study. *Genome Research* **15**: 376-384

Frier BC, Jacobs RL, Wright DC (2011) Interactions between the consumption of a high-fat diet and fasting in the regulation of fatty acid oxidation enzyme gene expression: an evaluation of potential mechanisms. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology* **300**: R212-R221

Fuentealba RA, Liu Q, Zhang J, Kanekiyo T, Hu X, Lee J-M, LaDu MJ, Bu G (2010) Low-Density Lipoprotein Receptor-Related Protein 1 (LRP1) Mediates Neuronal A beta 42 Uptake and Lysosomal Trafficking. *Plos One* **5**

Garciafigueroa DY, Klei LR, Ambrosio F, Barchowsky A (2013) Arsenic-Stimulated Lipolysis and Adipose Remodeling Is Mediated by G-Protein-Coupled Receptors. *Toxicological Sciences* **134**: 335-344

Garel S, Garcia-Dominguez M, Charnay P (2000) Control of the migratory pathway of facial branchiomotor neurones. *Development* **127**: 5297-5307

Gavard J, Lambert M, Grosheva I, Marthiens V, Irinopoulou T, Riou JF, Bershadsky A, Mege RM (2004) Lamellipodium extension and cadherin adhesion: two cell responses to cadherin activation relying on distinct signalling pathways. *Journal of Cell Science* **117**: 257-270

Glantz LA, Lewis DA (2000) Decreased dendritic spine density on prefrontal cortical pyramidal neurons in schizophrenia. *Archives of General Psychiatry* **57**: 65-73

Goldstein JL, Brown MS (1997) The SREBP pathway: Regulation of cholesterol and fatty acid metabolism by proteolysis of a membrane-bound transcription factor. *Faseb Journal* **11**: 4

GrandPre T, Nakamura F, Vartanian T, Strittmatter SM (2000) Identification of the Nogo inhibitor of axon regeneration as a Reticulon protein. *Nature* **403**: 439-444

Greenspan P, Mayer EP, Fowler SD (1985) NILE RED - A SELECTIVE FLUORESCENT STAIN FOR INTRACELLULAR LIPID DROPLETS. *Journal of Cell Biology* **100**

Grünewald E (2011) Investigating the role of orphan GPR50 in normal brain function and mental illness PhD Thesis, The University of Edinburgh, Edinburgh

Grünewald E, Kinnell HL, Porteous DJ, Thomson PA (2009) GPR50 interacts with neuronal NOGO-A and affects neurite outgrowth. *Molecular and Cellular Neuroscience* **42**: 363-371

Grünewald E, Tew KD, Porteous DJ, Thomson PA (2012) Developmental Expression of Orphan G Protein-Coupled Receptor 50 in the Mouse Brain. *Acs Chemical Neuroscience* **3**: 459-472

Gubitza AK, Reppert SM (2000) Chimeric and point-mutated receptors reveal that a single glycine residue in transmembrane domain 6 is critical for high affinity melatonin binding. *Endocrinology* **141**: 1236-1244

Guo Y, Cordes KR, Farese RV, Jr., Walther TC (2009) Lipid droplets at a glance. *Journal of Cell Science* **122**: 749-752

Hagiwara A, Cornu M, Cybulski N, Polak P, Betz C, Trapani F, Terracciano L, Heim MH, Rueegg MA, Hall MN (2012) Hepatic mTORC2 Activates Glycolysis and Lipogenesis through Akt, Glucokinase, and SREBP1c. *Cell Metabolism* **15**: 725-738

Hajszan T, Dow A, Warner-Schmidt JL, Szigeti-Buck K, Sallam NL, Parducz A, Leranath C, Duman RS (2009) Remodeling of Hippocampal Spine Synapses in the Rat Learned Helplessness Model of Depression. *Biological Psychiatry* **65**: 392-400

Halavi M, Hamilton KA, Parekh R, Ascoli GA (2012) Digital reconstructions of neuronal morphology: three decades of research trends. *Frontiers in neuroscience* **6**: 49-49

Hamouda HO, Chen P, Levoye A, Sozer-Topcular N, Daulat AM, Guillaume JL, Ravid R, Savaskan E, Ferry G, Boutin JA, Delagrang P, Jockers R, Maurice P (2007) Detection of the human GPR50 orphan seven transmembrane protein by polyclonal antibodies mapping different epitopes. *Journal of Pineal Research* **43**: 10-15

Han K, Holder JL, Jr., Schaaf CP, Lu H, Chen H, Kang H, Tang J, Wu Z, Hao S, Cheung SW, Yu P, Sun H, Breman AM, Patel A, Lu H-C, Zoghbi HY (2013) SHANK3 overexpression causes manic-like behaviour with unique pharmacogenetic properties. *Nature* **503**: 72-+

Hand L (2011) The Biology of GPR50, the Melatonin-Related Receptor. PhD Thesis, Faculty of Life Sciences, University of Manchester, Manchester

Hansen SD, Kwiatkowski AV, Ouyang C-Y, Liu H, Pokutta S, Watkins SC, Volkmann N, Hanein D, Weis WI, Mullins RD, Nelson WJ (2013) alpha E-catenin actin-binding domain alters actin filament conformation and regulates binding of nucleation and disassembly factors. *Molecular Biology of the Cell* **24**: 3710-3720

Hardeland R, Cardinali DP, Srinivasan V, Spence DW, Brown GM, Pandi-Perumal SR (2011) Melatonin-A pleiotropic, orchestrating regulator molecule. *Progress in Neurobiology* **93**: 350-384

Hatanaka Y, Murakami F (2002) In vitro analysis of the origin, migratory behavior, and maturation of cortical pyramidal cells. *Journal of Comparative Neurology* **454**: 1-14

Hayashi-Takagi A, Takaki M, Graziane N, Seshadri S, Murdoch H, Dunlop AJ, Makino Y, Seshadri AJ, Ishizuka K, Srivastava DP, Xie Z, Baraban JM, Houslay MD, Tomoda T, Brandon NJ, Kamiya A, Yan Z, Penzes P, Sawa A (2010) Disrupted-in-Schizophrenia 1 (DISC1) regulates spines of the glutamate synapse via Rac1. *Nature Neuroscience* **13**: 327-U312

Hayden E, Nurnberger JJ (2006) Molecular genetics of bipolar disorder. *Genes Brain Behav* **5**: 85-95

Heasman SJ, Ridley AJ (2008) Mammalian Rho GTPases: new insights into their functions from in vivo studies. *Nature Reviews Molecular Cell Biology* **9**: 690-701

Hering H, Lin CC, Sheng M (2003) Lipid rafts in the maintenance of synapses, dendritic spines, and surface AMPA receptor stability. *Journal of Neuroscience* **23**: 3262-3271

Hertel N, Krishna K, Nuernberger M, Redies C (2008) A cadherin-based code for the divisions of the mouse basal ganglia. *Journal of Comparative Neurology* **508**: 511-528

Horie T, Ono K, Horiguchi M, Nishi H, Nakamura T, Nagao K, Kinoshita M, Kuwabara Y, Marusawa H, Iwanaga Y, Hasegawa K, Yokode M, Kimura T, Kita T (2010) MicroRNA-33 encoded by an intron of sterol regulatory element-binding protein 2 (Srebp2) regulates HDL in vivo. *Proceedings of the National Academy of Sciences of the United States of America* **107**: 17321-17326

Horton JD, Shimomura I, Brown MS, Hammer RE, Goldstein JL, Shimano H (1998) Activation of cholesterol synthesis in preference to fatty acid synthesis in liver and adipose tissue of transgenic mice overproducing sterol regulatory element-binding protein-2. *Journal of Clinical Investigation* **101**: 2331-2339

Hotulainen P, Hoogenraad CC (2010) Actin in dendritic spines: connecting dynamics to function. *Journal of Cell Biology* **189**: 619-629

Hozumi Y, Watanabe M, Otani K, Goto K (2009) Diacylglycerol kinase beta promotes dendritic outgrowth and spine maturation in developing hippocampal neurons. *Bmc Neuroscience* **10**

Huang W, Zhu PJ, Zhang S, Zhou H, Stoica L, Galiano M, Krnjevic K, Roman G, Costa-Mattioli M (2013) mTORC2 controls actin polymerization required for consolidation of long-term memory. *Nature Neuroscience* **16**: 441-U105

Huber AB, Weinmann O, Brosamle C, Oertle T, Schwab ME (2002) Patterns of Nogo mRNA and protein expression in the developing and adult rat and after CNS lesions. *Journal of Neuroscience* **22**: 3553-3567

Humbert S, Bryson EA, Cordelieres FP, Connors NC, Datta SR, Finkbeiner S, Greenberg ME, Saudou F (2002) The IGF-1/Akt pathway is neuroprotective in Huntington's disease and involves huntingtin phosphorylation by Akt. *Developmental Cell* **2**: 831-837

Hung AY, Futai K, Sala C, Valtschanoff JG, Ryu J, Woodworth MA, Kidd FL, Sung CC, Miyakawa T, Bear MF, Weinberg RJ, Sheng M (2008) Smaller dendritic spines, weaker synaptic transmission, but enhanced spatial learning in mice lacking Shank1. *Journal of Neuroscience* **28**: 1697-1708

Hussein MR, Ahmed OG, Hassan AF, Ahmed MA (2007) Intake of melatonin is associated with amelioration of physiological changes, both metabolic and morphological pathologies associated with obesity: an animal model. *International Journal of Experimental Pathology* **88**: 19-29

Ichimura A, Hirasawa A, Poulain-Godefroy O, Bonnefond A, Hara T, Yengo L, Kimura I, Leloire A, Liu N, Iida K, Choquet H, Besnard P, Lecoecur C, Vivequin S, Ayukawa K, Takeuchi M, Ozawa K, Tauber M, Maffeis C, Morandi A, Buzzetti R, Elliott P, Pouta A, Jarvelin M-R, Koerner A, Kiess W, Pigeyre M, Caiazzo R, Van Hul W, Van Gaal L, Horber F, Balkau B, Levy-Marchal C, Rouskas K, Kouvatsi A, Hebebrand J, Hinney A, Scherag A, Pattou F, Meyre D, Koshimizu T-a, Wolowczuk I, Tsujimoto G, Froguel P (2012) Dysfunction of lipid sensor GPR120 leads to obesity in both mouse and human. *Nature* **483**: 350-U149

Ikonen E (2008) Cellular cholesterol trafficking and compartmentalization. *Nature Reviews Molecular Cell Biology* **9**: 125-138

Ishibashi S, Yamazaki T, Okamoto K (2009) Association of autophagy with cholesterol-accumulated compartments in Niemann-Pick disease type C cells. *Journal of Clinical Neuroscience* **16**: 954-959

Issandou M, Guillard RL, Boullay AB, Linhart V, Lopez-Perez E (2004) Up-regulation of low-density lipoprotein receptor in human hepatocytes is induced by

sequestration of free cholesterol in the endosomal/lysosomal compartment. *Biochemical Pharmacology* **67**: 2281-2289

Ito H, Morishita R, Shinoda T, Iwamoto I, Sudo K, Okamoto K, Nagata K (2010) Dysbindin-1, WAVE2 and Abi-1 form a complex that regulates dendritic spine formation. *Molecular Psychiatry* **15**: 976-986

Ivanova EA, Bechtold DA, Sandrine MD, Brennand J, Barrett P, Luckman SM, Loudon ASI (2008) Altered metabolism in the melatonin-related receptor (GPR50) knockout mouse. *American Journal of Physiology-Endocrinology and Metabolism* **294**: E176-E182

Jacinto E, Facchinetti V, Liu D, Soto N, Wei S, Jung SY, Huang Q, Qin J, Su B (2006) SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. *Cell* **127**: 125-137

Jacinto E, Loewith R, Schmidt A, Lin S, Ruegg MA, Hall A, Hall MN (2004) Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. *Nature Cell Biology* **6**: 1122-U1130

Jin XW, von Gall C, Pieschl RL, Gribkoff VK, Stehle JH, Reppert SM, Weaver DR (2003) Targeted disruption of the mouse Mel(1b) melatonin receptor. *Molecular and Cellular Biology* **23**: 1054-1060

Jockers R, Maurice P, Boutin JA, Delagrange P (2008) Melatonin receptors, heterodimerization, signal transduction and binding sites: what's new? *British Journal of Pharmacology* **154**: 1182-1195

John GR, Chen LF, Rivieccio MA, Melendez-Vasquez CV, Hartley A, Brosnan CF (2004) Interleukin-1 beta induces a reactive astroglial phenotype via deactivation of the rho GTPase-rock axis. *Journal of Neuroscience* **24**: 2837-2845

Jones KT, Greer ER, Pearce D, Ashrafi K (2009) Rictor/TORC2 Regulates *Caenorhabditis elegans* Fat Storage, Body Size, and Development through sgk-1. *Plos Biology* **7**: 604-615

Juengling K, Eulenburg V, Moore R, Kemler R, Lessmann V, Gottmann K (2006) N-cadherin transsynaptically regulates short-term plasticity at glutamatergic synapses in embryonic stem cell-derived neurons. *Journal of Neuroscience* **26**: 6968-6978

Kaalund SS, Newburn EN, Tianzhang Y, Wang L, Tao R, Li C, Vakkalanka R, Deep-Soboslay A, Bigelow LB, Straub R, Hyde TM, Weinberger DR, Lipska BK, Joel KE (2011) Expression of DRD2 and DRD1 transcripts in the prefrontal cortex and hippocampus of patients with schizophrenia and affective disorders. *Society for Neuroscience Abstract Viewer and Itinerary Planner* **41**

Kadmiel M, Cidlowski JA (2013) Glucocorticoid receptor signaling in health and disease. *Trends in Pharmacological Sciences* **34**: 518-530

Kaech S, Banker G (2006) Culturing hippocampal neurons. *Nature Protocols* **1**: 2406-2415

Kamiya A, Kubo K, Tomoda T, Takaki M, Youn R, Ozeki Y, Sawamura N, Park U, Kudo C, Okawa M, Ross CA, Hatten ME, Nakajima K, Sawa A (2005) A schizophrenia-associated mutation of DISC1 perturbs cerebral cortex development. *Nature Cell Biology* **7**: 1167-1178

Kang DE, Pietrzik CU, Baum L, Chevallier N, Merriam DE, Kounnas MZ, Wagner SL, Troncoso JC, Kawas CH, Katzman R, Koo EH (2000) Modulation of amyloid beta-protein clearance and Alzheimer's disease susceptibility by the LDL receptor-related protein pathway. *Journal of Clinical Investigation* **106**: 1159-1166

Kang HJ, Voleti B, Hajszan T, Rajkowska G, Stockmeier CA, Licznarski P, Lepack A, Majik MS, Jeong LS, Banasr M, Son H, Duman RS (2012) Decreased expression of synapse-related genes and loss of synapses in major depressive disorder. *Nature Medicine* **18**: 1413-+

Kannenbergh F, Gorzelniak K, Jaeger K, Fobker M, Rust S, Repa J, Roth M, Bjorkhem I, Walter M (2013) Characterization of Cholesterol Homeostasis in Telomerase-immortalized Tangier Disease Fibroblasts Reveals Marked Phenotype Variability. *Journal of Biological Chemistry* **288**: 36936-36947

Karege F, Meary A, Perroud N, Jamain S, Leboyer M, Ballmann E, Fernandez R, Malafosse A, Schuerhoff F (2012) Genetic overlap between schizophrenia and bipolar disorder: A study with AKT1 gene variants and clinical phenotypes. *Schizophrenia Research* **135**: 8-14

Karlen A, Karlsson TE, Mattsson A, Lundstromer K, Codeluppi S, Pham TM, Backman CM, Ogren SO, Aberg E, Hoffman AF, Sherling MA, Lupica CR, Hoffer BJ, Spenger C, Josephson A, Brene S, Olson L (2009) Nogo receptor 1 regulates

formation of lasting memories. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 20476-20481

Kasai H, Fukuda M, Watanabe S, Hayashi-Takagi A, Noguchi J (2010) Structural dynamics of dendritic spines in memory and cognition. *Trends in Neurosciences* **33**: 121-129

Kasai H, Matsuzaki M, Noguchi J, Yasumatsu N, Nakahara H (2003) Structure-stability-function relationships of dendritic spines. *Trends in Neurosciences* **26**: 360-368

Katsuma S, Hatae N, Yano T, Ruike Y, Kimura M, Hirasawa A, Tsujimoto G (2005) Free fatty acids inhibit serum deprivation-induced apoptosis through GPR120 in a murine enteroendocrine cell line STC-1. *Journal of Biological Chemistry* **280**: 19507-19515

Kawaguchi Y, Karube F, Kubota Y (2006) Dendritic branch typing and spine expression patterns in cortical nonpyramidal cells. *Cerebral Cortex* **16**: 696-711

Keembiyehetty CN, Krzeslak A, Love DC, Hanover JA (2011) A lipid-droplet-targeted O-GlcNAcase isoform is a key regulator of the proteasome. *Journal of Cell Science* **124**: 2851-2860

Kerr CA, Hines BM, Shaw JM, Dunne R, Bragg LM, Clarke J, Lockett T, Head R (2013) Genomic homeostasis is dysregulated in favour of apoptosis in the colonic epithelium of the azoxymethane treated rat. *BMC Physiology* **13**: 2-Article No.: 2

Kilic E, ElAli A, Kilic U, Guo Z, Ugur M, Uslu U, Bassetti CL, Schwab ME, Hermann DM (2010) Role of Nogo-A in neuronal survival in the reperfused ischemic brain. *Journal of Cerebral Blood Flow and Metabolism* **30**: 969-984

Kilic U, Yilmaz B, Ugur M, Yüksel A, Reiter RJ, Hermann DM, Kilic E (2011) Evidence that membrane-bound G protein- ϵ coupled melatonin receptors MT1 and MT2 are not involved in the neuroprotective effects of melatonin in focal cerebral ischemia. *Journal of Pineal Research* **52**: 228-235

Kim IH, Racz B, Wang H, Burianek L, Weinberg R, Yasuda R, Wetsel WC, Soderling SH (2013) Disruption of Arp2/3 Results in Asymmetric Structural Plasticity of Dendritic Spines and Progressive Synaptic and Behavioral Abnormalities. *Journal of Neuroscience* **33**: 6081-6092

Kim J-H, Ong W-Y (2009) Localization of the transcription factor, sterol regulatory element binding protein-2 (SREBP-2) in the normal rat brain and changes after kainate-induced excitotoxic injury. *Journal of Chemical Neuroanatomy* **37**: 71-77

Kim JY, Duan X, Liu CY, Jang M-H, Guo JU, Pow-anpongkul N, Kang E, Song H, Ming G-l (2009a) DISC1 Regulates New Neuron Development in the Adult Brain via Modulation of AKT-mTOR Signaling through KIAA1212. *Neuron* **63**: 761-773

Kim K, Yang J, Zhong X-P, Kim M-H, Kim YS, Lee HW, Han S, Choi J, Han K, Seo J, Prescott SM, Topham MK, Bae YC, Koretzky G, Choi S-Y, Kim E (2009b) Synaptic removal of diacylglycerol by DGK zeta and PSD-95 regulates dendritic spine maintenance. *Embo Journal* **28**: 1170-1179

Kim MJ, Kim HK, Kim BS, Yim SV (2004) Melatonin increases cell proliferation in the dentate gyrus of maternally separated rats. *Journal of Pineal Research* **37**: 193-197

Kim Y, Sung JY, Ceglia I, Lee K-W, Ahn J-H, Halford JM, Kim AM, Kwak SP, Park JB, Ryu SH, Schenck A, Bardoni B, Scott JD, Nairn AC, Greengard P (2006) Phosphorylation of WAVE1 regulates actin polymerization and dendritic spine morphology. *Nature* **442**: 814-817

Kim YD, Park K-G, Lee Y-S, Park Y-Y, Kim D-K, Nedumaran B, Jang WG, Cho W-J, Ha J, Lee I-K, Lee C-H, Choi H-S (2008) Metformin inhibits hepatic gluconeogenesis through AMP-activated protein kinase-dependent regulation of the orphan nuclear receptor SHP. *Diabetes* **57**: 306-314

Kim YG, Lee YI (2012) Differential Expressions of Synaptogenic Markers between Primary Cultured Cortical and Hippocampal Neurons. *Exp Neurobiol* **21**: 61-67

Ko M, Zou K, Minagawa H, Yu WX, Gong JS, Yanagisawa K, Michikawa M (2005) Cholesterol-mediated neurite outgrowth is differently regulated between cortical and hippocampal neurons. *Journal of Biological Chemistry* **280**: 42759-42765

Koleske AJ (2013) Molecular mechanisms of dendrite stability. *Nature Reviews Neuroscience* **14**: 536-550

Kolluri N, Sun ZX, Sampson AR, Lewis DA (2005) Lamina-specific reductions in dendritic spine density in the prefrontal cortex of subjects with schizophrenia. *American Journal of Psychiatry* **162**: 1200-1202

Komitova M, Xenos D, Salmaso N, Kathy May T, Brand T, Schwartz ML, Ment L, Vaccarino FM (2013) Hypoxia-Induced Developmental Delays of Inhibitory Interneurons Are Reversed by Environmental Enrichment in the Postnatal Mouse Forebrain. *Journal of Neuroscience* **33**: 13375-13387

Korematsu K, Nishi T, Okamura A, Goto S, Morioka M, Hamada J, Ushio Y (1998) Cadherin-8 protein expression in gray matter structures and nerve fibers of the neonatal and adult mouse brain. *Neuroscience* **87**: 303-315

Korobova F, Svitkina T (2008) Arp2/3 complex is important for filopodia formation, growth cone motility, and neuritogenesis in neuronal cells. *Molecular Biology of the Cell* **19**: 1561-1574

Kuang Y-Q, Charette N, Frazer J, Holland PJ, Attwood KM, Dellaire G, Dupre DJ (2012) Dopamine Receptor-Interacting Protein 78 Acts as a Molecular Chaperone for CCR5 Chemokine Receptor Signaling Complex Organization. *Plos One* **7**

Kumar A, Lawrence JC, Jr., Jung DY, Ko HJ, Keller SR, Kim JK, Magnuson MA, Harris TE (2010) Fat Cell-Specific Ablation of Rictor in Mice Impairs Insulin-Regulated Fat Cell and Whole-Body Glucose and Lipid Metabolism. *Diabetes* **59**: 1397-1406

Kumar V, Zhang MX, Swank MW, Kunz J, Wu GY (2005) Regulation of dendritic morphogenesis by Ras-PI3K-Akt mTOR and Ras-MAPK signaling pathways. *Journal of Neuroscience* **25**: 11288-11299

Kume S, Thomas MC, Koya D (2012) Nutrient Sensing, Autophagy, and Diabetic Nephropathy. *Diabetes* **61**: 23-29

Kwon H-B, Kozorovitskiy Y, Oh W-J, Peixoto RT, Akhtar N, Saulnier JL, Gu C, Sabatini BL (2012) Neuroligin-1-dependent competition regulates cortical synaptogenesis and synapse number. *Nature Neuroscience* **15**: 1667-+

Launay J-M, Schneider B, Loric S, Da Prada M, Kellermann O (2006) Serotonin transport and serotonin transporter-mediated antidepressant recognition are

controlled by 5-HT_{2B} receptor signaling in serotonergic neuronal cells. *FASEB Journal* **20**: 1843-1854

Le Hellard S, Muhleisen TW, Djurovic S, Ferno J, Ouriaghi Z, Mattheisen M, Vasilescu C, Raeder MB, Hansen T, Strohmaier J, Georgi A, Brockschmidt FF, Melle I, Nenadic I, Sauer H, Rietschel M, Nothen MM, Werge T, Andreassen OA, Cichon S, Steen VM (2010) Polymorphisms in SREBF1 and SREBF2, two antipsychotic-activated transcription factors controlling cellular lipogenesis, are associated with schizophrenia in German and Scandinavian samples. *Molecular Psychiatry* **15**: 463-472

Lee C-C, Huang C-C, Hsu K-S (2011) Insulin promotes dendritic spine and synapse formation by the PI3K/Akt/mTOR and Rac1 signaling pathways. *Neuropharmacology* **61**: 867-879

Lee CH, Yoo K-Y, Choi JH, Park OK, Hwang IK, Kwon Y-G, Kim Y-M, Won M-H (2010) Melatonin's Protective Action Against Ischemic Neuronal Damage Is Associated With Up-Regulation of the MT₂ Melatonin Receptor. *Journal of Neuroscience Research* **88**: 2630-2640

Lee H, Raiker SJ, Venkatesh K, Geary R, Robak LA, Zhang Y, Yeh HH, Shrager P, Giger RJ (2008) Synaptic function for the Nogo-66 receptor NgR1: Regulation of dendritic spine morphology and activity-dependent synaptic strength. *Journal of Neuroscience* **28**: 2753-2765

Lehto SM, Hintikka J, Niskanen L, Tolmunen T, Koivumaa-Honkanen H, Honkalampi K, Viinamaki H (2008) Low HDL cholesterol associates with major depression in a sample with a 7-year history of depressive symptoms. *Progress in Neuro-Psychopharmacology & Biological Psychiatry* **32**: 1557-1561

Lehto SM, Niskanen L, Tolmunen T, Hintikka J, Viinamaki H, Heiskanen T, Honkalampi K, Kokkonen M, Koivumaa-Honkanen H (2010) Low serum HDL-cholesterol levels are associated with long symptom duration in patients with major depressive disorder. *Psychiatry and Clinical Neurosciences* **64**: 279-283

Lelouvier B, Tamagno G, Kaindl AM, Roland A, Lelievre V, Le Verche V, Loudes C, Gressens P, Faivre-Baumann A, Lenkei Z, Dournaud P (2008) Dynamics of somatostatin type 2A receptor cargoes in living hippocampal neurons. *Journal of Neuroscience* **28**: 4336-4349

Lemonde S, Turecki G, Bakish D, Du LS, Hrdina PD, Bown CD, Sequeira A, Kushwaha N, Morris SJ, Basak A, Ou XM, Albert PR (2003) Impaired repression at a 5-hydroxytryptamine 1A receptor gene polymorphism associated with major depression and suicide. *Journal of Neuroscience* **23**: 8788-8799

Levoye A, Dam J, Ayoub MA, Guillaume JL, Couturier C, Delagrangé P, Jockers R (2006) The orphan GPR50 receptor specifically inhibits MT1 melatonin receptor function through heterodimerization. *Embo Journal* **25**: 3012-3023

Lewis R, Kapur S, Jones C, DaSilva J, Brown GM, Wilson AA, Houle S, Zipursky RB (1999) Serotonin 5-HT₂ receptors in schizophrenia: A PET study using F-18 setoperone in neuroleptic-naïve patients and normal subjects. *American Journal of Psychiatry* **156**: 72-78

Li B, Woo R-S, Mei L, Malinow R (2007) The neuregulin-1 receptor ErbB4 controls Glutamatergic synapse maturation and plasticity. *Neuron* **54**: 583-597

Li J, Hand LE, Meng Q-J, Loudon ASI, Bechtold DA (2011) GPR50 Interacts with TIP60 to Modulate Glucocorticoid Receptor Signalling. *Plos One* **6**

Li JG, Chen CG, Liu-Chen LY (2002) Ezrin-radixin-moesin-binding phosphoprotein-50/Na⁺/H⁺ exchanger regulatory factor (EBP50/NHERF) blocks U50,488H-induced down-regulation of the human kappa opioid receptor by enhancing its recycling rate. *Journal of Biological Chemistry* **277**: 27545-27552

Li MT, Wang XM, Meintzer MK, Laessig T, Birnbaum MJ, Heidenreich KA (2000) Cyclic AMP promotes neuronal survival by phosphorylation of glycogen synthase kinase 3 beta. *Molecular and Cellular Biology* **20**: 9356-9363

Li N, Lee B, Liu R-J, Banasr M, Dwyer JM, Iwata M, Li X-Y, Aghajanian G, Duman RS (2010) mTOR-Dependent Synapse Formation Underlies the Rapid Antidepressant Effects of NMDA Antagonists. *Science* **329**: 959-964

Li Q, Lau A, Morris TJ, Guo L, Fordyce CB, Stanley EF (2004) A syntaxin 1, G_α(o), and N-type calcium channel complex at a presynaptic nerve terminal: Analysis by quantitative immunocolocalization. *Journal of Neuroscience* **24**: 4070-4081

Liang W, Mason AJ, Lam JKW (2013) Western blot evaluation of siRNA delivery by pH-responsive peptides. *Methods in molecular biology (Clifton, NJ)* **986**: 73-87

Liu C, Weaver DR, Jin XW, Shearman LP, Pieschl RL, Gribkoff VK, Reppert SM (1997) Molecular dissection of two distinct actions of melatonin on the suprachiasmatic circadian clock. *Neuron* **19**: 91-102

Liu J, Li LD, Ora A, Heikkila P, Vaheri A, Voutilainen R (2004) cAMP-dependent protein kinase activation inhibits proliferation and enhances apoptotic effect of tumor necrosis factor-alpha in NCI-H295R adrenocortical cells. *Journal of Molecular Endocrinology* **33**: 511-522

Liu Q, Trotter J, Zhang J, Peters MM, Cheng H, Bao J, Han X, Weeber EJ, Bu G (2010) Neuronal LRP1 Knockout in Adult Mice Leads to Impaired Brain Lipid Metabolism and Progressive, Age-Dependent Synapse Loss and Neurodegeneration. *Journal of Neuroscience* **30**: 17068-17078

Lochman J, Balcar VJ, Stastny F, Serny O (2013) Preliminary evidence for association between schizophrenia and polymorphisms in the regulatory Regions of the ADRA2A, DRD3 and SNAP-25 Genes. *Psychiatry Research* **205**: 7-12

Logette E, Le Jossic-Corcus C, Masson D, Solier S, Sequeira-Legrand A, Dugail I, Lemaire-Ewing S, Desoche L, Solary E, Corcos L (2005) Caspase-2, a novel lipid sensor under the control of sterol regulatory element binding protein 2. *Molecular and Cellular Biology* **25**: 9621-9631

Longair MH, Baker DA, Armstrong JD (2011) Simple Neurite Tracer: open source software for reconstruction, visualization and analysis of neuronal processes. *Bioinformatics* **27**: 2453-2454

Lorenzo CV, Baca-Garcia E, Diaz-Hernandez M, Botillo-Martin C, Perez-Rodriguez MM, Fernandez-Ramos C, Saiz-Gonzalez MD, Quintero-Gutierrez FJ, Saiz-Ruiz J, Piqueras JF, de Rivera JLG, de Leon J (2006) Association between the T102C polymorphism of the serotonin-2A receptor gene and schizophrenia. *Progress in Neuro-Psychopharmacology & Biological Psychiatry* **30**: 1136-1138

MacIntyre DJ, McGhee KA, MacLean AW, Afzal M, Briffa K, Henry B, Thomson PA, Muir WJ, Blackwood DHR (2010) Association of GPR50, an X-linked orphan G protein-coupled receptor, and affective disorder in an independent sample of the Scottish population. *Neuroscience Letters* **475**: 169-173

Mack J, Beljanski V, Soulika A, Townsend D, Brown C, Davis W, Tew K (2007) "Skittish" Abca2 knockout mice display tremor, hyperactivity, and abnormal myelin ultrastructure in the central nervous system. *Mol Cell Biol* **27**: 44-53

MacKenzie RS, Melan MA, Passey DK, Witt-Enderby PA (2002) Dual coupling of MT1 and MT2 melatonin receptors to cyclic AMP and phosphoinositide signal transduction cascades and their regulation following melatonin exposure. *Biochemical Pharmacology* **63**: 587-595

Macé S, Cousin E, Ricard S, Génin E, Spanakis E, Lafargue-Soubigou C, Génin B, Fournel R, Roche S, Haussy G, Massey F, Soubigou S, Bréfort G, Benoit P, Brice A, Champion D, Hollis M, Pradier L, Benavides J, Deleuze J (2005) ABCA2 is a strong genetic risk factor for early-onset Alzheimer's disease. *Neurobiol Dis* **18**: 119-125

Mahon MJ, Donowitz M, Yun CC, Segre GV (2002) Na⁺/H⁺ exchanger regulatory factor 2 directs parathyroid hormone 1 receptor signalling. *Nature* **417**: 858-861

Mahon MJ, Segre GV (2004) Stimulation by parathyroid hormone of a NHERF-1-assembled complex consisting of the parathyroid hormone I receptor, phospholipase C beta, and actin increases intracellular calcium in opossum kidney cells. *Journal of Biological Chemistry* **279**: 23550-23558

Malaga-Dieguez L, Yang Q, Bauer J, Pankevych H, Freissmuth M, Nanoff C (2010) Pharmacochaperoning of the A(1) Adenosine Receptor Is Contingent on the Endoplasmic Reticulum. *Molecular Pharmacology* **77**: 940-952

Malagelada C, Jin ZH, Greene LA (2008) RTP801 Is Induced in Parkinson's Disease and Mediates Neuron Death by Inhibiting Akt Phosphorylation/Activation. *Journal of Neuroscience* **28**: 14363-14371

Malberg JE, Eisch AJ, Nestler EJ, Duman RS (2000) Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. *Journal of Neuroscience* **20**: 9104-9110

Markus EJ, Petit TL (1987) NEOCORTICAL SYNAPTOGENESIS, AGING, AND BEHAVIOR - LIFE-SPAN DEVELOPMENT IN THE MOTOR SENSORY SYSTEM OF THE RAT. *Experimental Neurology* **96**: 262-278

Martin S, Driessen K, Nixon SJ, Zerial M, Parton RG (2005) Regulated localization of rab18 to lipid droplets - Effects of lipolytic stimulation and inhibition of lipid droplet catabolism. *Journal of Biological Chemistry* **280**: 42325-42335

Martinez-Quiles N, Rohatgi R, Anton IM, Medina M, Saville SP, Miki H, Yamaguchi H, Takenawa T, Hartwig JH, Geha RS, Ramesh N (2001) WIP regulates N-WASP-mediated actin polymerization and filopodium formation. *Nature Cell Biology* **3**: 484-491

Martini L, Waldhoer M, Pusch M, Kharazia V, Fong J, Lee JH, Freissmuth C, Whistler JL (2007) Ligand-induced down-regulation of the cannabinoid 1 receptor is mediated by the G-protein-coupled receptor-associated sorting protein GASP1. *Faseb Journal* **21**: 802-811

Masana MI, Dubocovich ML (2001) Melatonin receptor signaling: finding the path through the dark. *Science's STKE : signal transduction knowledge environment* **2001**: pe39-pe39

Massat I, Souery D, Del-Favero J, Oruc L, Noethen M, Blackwood D, Thomson M, Muir W, Papadimitriou G, Dikeos D, Kaneva R, Serretti A, Lilli R, Smeraldi E, Jakovljevic M, Folnegovic V, Rietschel M, Milanova V, Valente F, Van Broeckhoven C, Mendlewicz J (2002) Excess of allele1 for alpha3 subunit GABA receptor gene (GABRA3) in bipolar patients: a multicentric association study. *Mol Psychiatry* **7**: 201-207

Masuda Y, Itabe H, Odaki M, Hama K, Fujimoto Y, Mori M, Sasabe N, Aoki J, Arai H, Takano T (2006) ADRP/adipophilin is degraded through the proteasome-dependent pathway during regression of lipid-storing cells. *Journal of Lipid Research* **47**: 87-98

Mathur A, Law MH, Megson IL, Shaw DJ, Wei J (2010) Genetic association of the AKT1 gene with schizophrenia in a British population. *Psychiatric Genetics* **20**: 118-122

Mazna P, Obsilova V, Jelinkova I, Balik A, Berka K, Sovova Z, Ettrich R, Svoboda P, Obsil T, Teisinger J (2004) Molecular modeling of human MT2 melatonin receptor: the role of Val204, Leu272 and Tyr298 in ligand binding. *Journal of Neurochemistry* **91**: 836-842

- Mendez P, De Roo M, Poglia L, Klauser P, Muller D (2010) N-cadherin mediates plasticity-induced long-term spine stabilization. *Journal of Cell Biology* **189**: 589-600
- Mendoza MC, Er EE, Zhang W, Ballif BA, Elliott HL, Danuser G, Blenis J (2011) ERK-MAPK Drives Lamellipodia Protrusion by Activating the WAVE2 Regulatory Complex. *Molecular Cell* **41**: 661-671
- Mercer JG, Moar KM, Ross AW, Hoggard N, Morgan PJ (2000) Photoperiod regulates arcuate nucleus POMC, AGRP, and leptin receptor mRNA in Siberian hamster hypothalamus. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology* **278**: R271-R281
- Migaud M, Charlesworth P, Dempster M, Webster LC, Watabe AM, Makhinson M, He Y, Ramsay MF, Morris RGM, Morrison JH, O'Dell TJ, Grant SGN (1998) Enhanced long-term potentiation and impaired learning in mice with mutant postsynaptic density-95 protein. *Nature* **396**: 433-439
- Migrenne S, Le Foll C, Levin BE, Magnan C (2011) Brain lipid sensing and nervous control of energy balance. *Diabetes & Metabolism* **37**: 83-88
- Miki H, Sasaki T, Takai Y, Takenawa T (1998) Induction of filopodium formation by a WASP-related actin-depolymerizing protein N-WASP. *Nature* **391**: 93-96
- Milasta S, Padiani J, Appelbe S, Trim S, Wyatt M, Cox P, Fidock M, Milligan G (2006) Interactions between the Mas-related receptors MrgD and MrgE alter signalling and trafficking of MrgD. *Molecular Pharmacology* **69**: 479-491
- Miller PW, Pokutta S, Ghosh A, Almo SC, Weis WI, Nelson WJ, Kwiatkowski AV (2013) Danio rerio alphaE-catenin is a monomeric F-actin binding protein with distinct properties from Mus musculus alphaE-catenin. *The Journal of biological chemistry* **288**: 22324-22332
- Mizuseki K, Diba K, Pastalkova E, Buzsaki G (2011) Hippocampal CA1 pyramidal cells form functionally distinct sublayers. *Nature Neuroscience* **14**: 1174-U1235
- Morishima M, Kawaguchi Y (2006) Recurrent connection patterns of corticostriatal pyramidal cells in frontal cortex. *Journal of Neuroscience* **26**: 4394-4405

Moriya T, Horie N, Mitome M, Shinohara K (2007) Melatonin influences the proliferative and differentiative activity of neural stem cells. *Journal of Pineal Research* **42**: 411-418

Mouneimne G, Hansen SD, Selfors LM, Petrak L, Hickey MM, Gallegos LL, Simpson KJ, Lim J, Gertler FB, Hartwig JH, Mullins RD, Brugge JS (2012) Differential Remodeling of Actin Cytoskeleton Architecture by Profilin Isoforms Leads to Distinct Effects on Cell Migration and Invasion. *Cancer Cell* **22**: 615-630

Murase S, Mosser E, Schuman EM (2002) Depolarization drives beta-catenin into neuronal spines promoting changes in synaptic structure and function. *Neuron* **35**: 91-105

Murata H, Sakaguchi M, Jin Y, Sakaguchi Y, Futami J-i, Yamada H, Kataoka K, Huh N-h (2011) A New Cytosolic Pathway from a Parkinson Disease-associated Kinase, BRPK/PINK1 ACTIVATION OF AKT VIA MTORC2. *Journal of Biological Chemistry* **286**: 7182-7189

Nadarajah B, Alifragis P, Wong ROL, Parnavelas JG (2003) Neuronal migration in the developing cerebral cortex: Observations based on real-time imaging. *Cerebral Cortex* **13**: 607-611

Nakamura Y, Wood CL, Patton AP, Jaafari N, Henley JM, Mellor JR, Hanley JG (2011) PICK1 inhibition of the Arp2/3 complex controls dendritic spine size and synaptic plasticity. *Embo Journal* **30**: 719-730

Nakayama AY, Harms MB, Luo LQ (2000) Small GTPases Rac and Rho in the maintenance of dendritic spines and branches in hippocampal pyramidal neurons. *Journal of Neuroscience* **20**: 5329-5338

Niederost B, Oertle T, Fritsche J, McKinney RA, Bandtlow CE (2002) Nogo-A and myelin-associated glycoprotein mediate neurite growth inhibition by antagonistic regulation of RhoA and Rac1. *Journal of Neuroscience* **22**: 10368-10376

Norton N, Williams HJ, Dwyer S, Carroll L, Peirce T, Moskvina V, Segurado R, Nikolov I, Williams NM, Ikeda M, Iwata N, Owen MJ, O'Donovan MC (2007) Association analysis of AKT1 and schizophrenia in a UK case control sample. *Schizophrenia Research* **93**: 58-65

Nothdurfter C, Tanasic S, Di Benedetto B, Rammes G, Wagner E-M, Kirmeier T, Ganai V, Kessler JS, Rein T, Holsboer F, Rupprecht R (2010) Impact of Lipid Raft Integrity on 5-HT₃ Receptor Function and its Modulation by Antidepressants. *Neuropsychopharmacology* **35**: 1510-1519

O'Rourke EJ, Ruvkun G (2013) MXL-3 and HLH-30 transcriptionally link lipolysis and autophagy to nutrient availability. *Nature Cell Biology* **15**: 668-+

O'Brien JS, Sampson EL (1965) LIPID COMPOSITION OF NORMAL HUMAN BRAIN - GRAY MATTER WHITE MATTER AND MYELIN. *Journal of Lipid Research* **6**: 537-&

Oertle T, van der Haar ME, Bandtlow CE, Robeva A, Burfeind P, Buss A, Huber AB, Simonen M, Schnell L, Brosamle C, Kaupmann K, Vallon R, Schwab ME (2003) Nogo-A inhibits neurite outgrowth and cell spreading with three discrete regions. *Journal of Neuroscience* **23**: 5393-5406

Oh YS, Jo NW, Choi JW, Kim HS, Seo SW, Kang KO, Hwang JI, Heo K, Kim SH, Kim YH, Kim LH, Kim JH, Banno Y, Ryu SH, Suh PG (2004) NHERF2 specifically interacts with LPA(2) receptor and defines the specificity and efficiency of receptor-mediated phospholipase C-beta 3 activation. *Molecular and Cellular Biology* **24**: 5069-5079

Omeros-Corporation. (2012) Omeros Announces It Has Unlocked 35 Percent of Class A Orphan GPCRs. PRNewswire, Seattle.

Owen MJ, O'Donovan MC, Thapar A, Craddock N (2011) Neurodevelopmental hypothesis of schizophrenia. *British Journal of Psychiatry* **198**: 173-175

Ozeki S, Cheng JL, Tauchi-Sato K, Hatano N, Taniguchi H, Fujimoto T (2005) Rab18 localizes to lipid droplets and induces their close apposition to the endoplasmic reticulum-derived membrane. *Journal of Cell Science* **118**: 2601-2611

Pacheco CD, Kunkel R, Lieberman AP (2007) Autophagy in Niemann-Pick C disease is dependent upon Beclin-1 and responsive to lipid trafficking defects. *Human Molecular Genetics* **16**: 1495-1503

Pariante CM, Lightman SL (2008) The HPA axis in major depression: classical theories and new developments. *Trends in Neurosciences* **31**: 464-468

Park Y-J, Park J-G, Jeong H-B, Takeuchi Y, Kim S-J, Lee Y-D, Takemura A (2007) Expression of the melatonin receptor Mel(1c) in neural tissues of the reef fish *Siganus guttatus*. *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology* **147**: 103-111

Pasquali R, Vicennati V, Cacciari M, Pagotto U (2006) The hypothalamic-pituitary-adrenal axis activity in obesity and the metabolic syndrome. *Stress, Obesity, and Metabolic Syndrome* **1083**: 111-128

Pejchal T, Foley MA, Kosofsky BE, Waeber C (2002) Chronic fluoxetine treatment selectively uncouples raphe 5-HT_{1A} receptors as measured by S-35 -GTP gamma S autoradiography. *British Journal of Pharmacology* **135**: 1115-1122

Peng J, Wallar BJ, Flanders A, Swiatek PJ, Alberts AS (2003) Disruption of the diaphanous-related formin Drf1 gene encoding mDia1 reveals a role for Drf3 as an effector for Cdc42. *Current Biology* **13**: 534-545

Perreau-Lenz S, Kalsbeek A, Garidou ML, Wortel J, van der Vliet J, van Heijningen C, Simonneaux V, Pevet P, Buijs RM (2003) Suprachiasmatic control of melatonin synthesis in rats: inhibitory and stimulatory mechanisms. *European Journal of Neuroscience* **17**: 221-228

Petit L, Lacroix I, de Coppet P, Strosberg AD, Jockers R (1999) Differential signaling of human Mel1a and Mel1b melatonin receptors through the cyclic guanosine 3'-5'-monophosphate pathway. *Biochemical Pharmacology* **58**: 633-639

Petrinovic MM, Duncan CS, Bourikas D, Weinman O, Montani L, Schroeter A, Maerki D, Sommer L, Stoeckli ET, Schwab ME (2010) Neuronal Nogo-A regulates neurite fasciculation, branching and extension in the developing nervous system. *Development* **137**: 2539-2550

Petrinovic MM, Hourez R, Aloy EM, Dewarrat G, Gall D, Weinmann O, Gaudias J, Bachmann LC, Schiffmann SN, Vogt KE, Schwab ME (2013) Neuronal Nogo-A negatively regulates dendritic morphology and synaptic transmission in the cerebellum. *Proceedings of the National Academy of Sciences of the United States of America* **110**: 1083-1088

Polleux F, Snider W (2010) Initiating and Growing an Axon. *Cold Spring Harbor Perspectives in Biology* **2**

Polymeropoulos MH, Licamele L, Volpi S, Mack K, Mitkus SN, Carstea ED, Getoor L, Thompson A, Lavedan C (2009) Common effect of antipsychotics on the biosynthesis and regulation of fatty acids and cholesterol supports a key role of lipid homeostasis in schizophrenia. *Schizophrenia Research* **108**: 134-142

Pradhan AD, Case AM, Farrer RG, Tsai SY, Cheatwood JL, Martin JL, Kartje GL (2010) Dendritic Spine Alterations in Neocortical Pyramidal Neurons following Postnatal Neuronal Nogo-A Knockdown. *Developmental Neuroscience* **32**: 313-320

Proepper C, Johannsen S, Liebau S, Dahl J, Vaida B, Bockmann J, Kreutz MR, Gundelfinger ED, Boeckers TM (2007) Abelson interacting protein 1 (Abi-1) is essential for dendrite morphogenesis and synapse formation. *Embo Journal* **26**: 1397-1409

Raeder MB, Ferno J, Glambek M, Stansberg C, Steen VM (2006) Antidepressant drugs activate SREBP and up-regulate cholesterol and fatty acid biosynthesis in human glial cells. *Neuroscience Letters* **395**: 185-190

Rahman MM, Rosu S, Joseph-Strauss D, Cohen-Fix O (2014) Down-regulation of tricarboxylic acid (TCA) cycle genes blocks progression through the first mitotic division in *Caenorhabditis elegans* embryos. *Proceedings of the National Academy of Sciences of the United States of America* **111**: 2602-2607

Rajagopal S, Rajagopal K, Lefkowitz RJ (2010) Teaching old receptors new tricks: biasing seven-transmembrane receptors. *Nature Reviews Drug Discovery* **9**: 373-386

Ramirez-Rodriguez G, Klempin F, Babu H, Benitez-King G, Kempermann G (2009) Melatonin Modulates Cell Survival of New Neurons in the Hippocampus of Adult Mice. *Neuropsychopharmacology* **34**: 2180-2191

Ramirez-Rodriguez G, Ortiz-Lopez L, Dominguez-Alonso A, Benitez-King GA, Kempermann G (2011) Chronic treatment with melatonin stimulates dendrite maturation and complexity in adult hippocampal neurogenesis of mice. *Journal of Pineal Research* **50**: 29-37

Redies C, Hertel N, Huebner CA (2012) Cadherins and neuropsychiatric disorders. *Brain Research* **1470**: 130-144

Reppert SM, Weaver DR, Cassone VM, Godson C, Kolakowski LF (1995) MELATONIN RECEPTORS ARE FOR THE BIRDS - MOLECULAR ANALYSIS OF 2 RECEPTOR SUBTYPES DIFFERENTIALLY EXPRESSED IN CHICK BRAIN. *Neuron* **15**: 1003-1015

Reppert SM, Weaver DR, Ebisawa T (1994) CLONING AND CHARACTERIZATION OF A MAMMALIAN MELATONIN RECEPTOR THAT MEDIATES REPRODUCTIVE AND CIRCADIAN RESPONSES. *Neuron* **13**: 1177-1185

Reppert SM, Weaver DR, Ebisawa T, Mahle CD, Kolakowski LF (1996) Cloning of a melatonin-related receptor from human pituitary. *Febs Letters* **386**: 219-224

Rios-Lugo MJ, Cano P, Jimenez-Ortega V, Fernandez-Mateos MP, Scacchi PA, Cardinali DP, Esquifino AI (2010) Melatonin effect on plasma adiponectin, leptin, insulin, glucose, triglycerides and cholesterol in normal and high fat-fed rats. *Journal of Pineal Research* **49**: 342-348

Ritter SL, Hall RA (2009) Fine-tuning of GPCR activity by receptor-interacting proteins. *Nature Reviews Molecular Cell Biology* **10**: 819-830

Rocca DL, Martin S, Jenkins EL, Hanley JG (2008) Inhibition of Arp2/3-mediated actin polymerization by PICK1 regulates neuronal morphology and AMPA receptor endocytosis. *Nature Cell Biology* **10**: 259-U257

Rouiller I, Xu X-P, Amann KJ, Egile C, Nickell S, Nicastro D, Li R, Pollard TD, Volkmann N, Hanein D (2008) The structural basis of actin filament branching by the Arp2/3 complex. *Journal of Cell Biology* **180**: 887-895

Rubina K, Talovskaya E, Cherenkov V, Ivanov D, Stambolsky D, Storozhevych T, Pinelis V, Shevelev A, Parfyonova Y, Resink T, Erne P, Tkachuk V (2005) LDL induces intracellular signalling and cell migration via atypical LDL-binding protein T-cadherin. *Molecular and Cellular Biochemistry* **273**: 33-41

Russo VC, Metaxas S, Kobayashi K, Harris M, Werther GA (2004) Antiapoptotic effects of leptin in human neuroblastoma cells. *Endocrinology* **145**: 4103-4112

Saari K, Jokelainen J, Veijola J, Koponen H, Jones PB, Savolainen M, Jarvelin MR, Lauren L, Isohanni M, Lindeman S (2004) Serum lipids in schizophrenia and other

functional psychoses: a general population northern Finland 1966 birth cohort survey. *Acta Psychiatrica Scandinavica* **110**: 279-285

Sakai H, Tanaka Y, Tanaka M, Ban N, Yamada K, Matsumura Y, Watanabe D, Sasaki M, Kita T, Inagaki N (2007) ABCA2 deficiency results in abnormal sphingolipid metabolism in mouse brain. *J Biol Chem* **282**: 19692-19699

Santarelli L, Saxe M, Gross C, Surget A, Battaglia F, Dulawa S, Weisstaub N, Lee J, Duman R, Arancio O, Belzung C, Hen R (2003) Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. *Science* **301**: 805-809

Sarbassov DD, Guertin DA, Ali SM, Sabatini DM (2005) Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* **307**: 1098-1101

Sauzet S, Besseau L, Perez PH, Coves D, Chatain B, Peyric E, Boeuf G, Munoz-Cueto JA, Falcon J (2008) Cloning and retinal expression of melatonin receptors in the European sea bass, *Dicentrarchus labrax*. *General and Comparative Endocrinology* **157**: 186-195

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A (2012) Fiji: an open-source platform for biological-image analysis. *Nature Methods* **9**: 676-682

Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. *Nature Methods* **9**: 671-675

Schoentaube J, Olling A, Tatge H, Just I, Gerhard R (2009) Serine-71 phosphorylation of Rac1/Cdc42 diminishes the pathogenic effect of *Clostridium difficile* toxin A. *Cellular Microbiology* **11**: 1816-1826

Schulz A, Kyselyova A, Baader SL, Jung MJ, Zoch A, Mautner V-F, Hagel C, Morrison H (2013) Neuronal merlin influences ERBB2 receptor expression on Schwann cells through neuregulin 1 type III signalling.

Schwarz J, Proff J, Haevemeier A, Ladwein M, Rottner K, Barlag B, Pich A, Tatge H, Just I, Gerhard R (2012) Serine-71 Phosphorylation of Rac1 Modulates Downstream Signaling. *Plos One* **7**

- Scott EK, Reuter JE, Luo LQ (2003) Small GTPase Cdc42 is required for multiple aspects of dendritic morphogenesis. *Journal of Neuroscience* **23**: 3118-3123
- Sexton RC, Panini SR, Azran F, Rudney H (1983) EFFECTS OF 3BETA- 2-(DIETHYLAMINO)ETHOXY ANDROST-5-EN-17-ONE ON THE SYNTHESIS OF CHOLESTEROL AND UBIQUINONE IN RAT INTESTINAL EPITHELIAL-CELL CULTURES. *Biochemistry* **22**: 5687-5692
- Shaw G, Morse S, Ararat M, Graham FL (2002) Preferential transformation of human neuronal cells by human adenoviruses and the origin of HEK 293 cells. *Faseb Journal* **16**: 869-+
- Shenoy SK, Drake MT, Nelson CD, Houtz DA, Xiao KH, Madabushi S, Reiter E, Premont RT, Lichtarge O, Lefkowitz RJ (2006) beta-arrestin-dependent, G protein-independent ERK1/2 activation by the beta 2 adrenergic receptor. *Journal of Biological Chemistry* **281**: 1261-1273
- Shirai Y, Kouzuki T, Kakefuda K, Moriguchi S, Oyagi A, Horie K, Morita S-y, Shimazawa M, Fukunaga K, Takeda J, Saito N, Hara H (2010) Essential Role of Neuron-Enriched Diacylglycerol Kinase (DGK), DGK beta in Neurite Spine Formation, Contributing to Cognitive Function. *Plos One* **5**
- Sholl DA (1953) DENDRITIC ORGANIZATION IN THE NEURONS OF THE VISUAL AND MOTOR CORTICES OF THE CAT. *Journal of Anatomy* **87**: 387-&
- Sidibe A, Mullier A, Chen P, Baroncini M, Boutin JA, Delagrang P, Prevot V, Jockers R (2010) Expression of the orphan GPR50 protein in rodent and human dorsomedial hypothalamus, tanycytes and median eminence. *Journal of Pineal Research* **48**: 263-269
- Siegrist-Kaiser CA, Pauli V, Juge-Aubry CE, Boss O, Pernin A, Chin WW, Cusin I, Rohner-Jeanrenaud F, Burger AG, Zapf J, Meier CA (1997) Direct effects of leptin on brown and white adipose tissue. *Journal of Clinical Investigation* **100**: 2858-2864
- Sit S-T, Manser E (2011) Rho GTPases and their role in organizing the actin cytoskeleton. *Journal of Cell Science* **124**: 679-683
- Slominski A, Pisarchik A, Zbytek B, Tobin DJ, Kauser S, Wortsman J (2003) Functional activity of serotonergic and melatonergic systems expressed in the skin. *Journal of Cellular Physiology* **196**

Smith BA, Daugherty-Clarke K, Goode BL, Gelles J (2013) Pathway of actin filament branch formation by Arp2/3 complex revealed by single-molecule imaging. *Proceedings of the National Academy of Sciences of the United States of America* **110**: 1285-1290

Snapper SB, Takeshima F, Anton I, Liu CH, Thomas SM, Nguyen D, Dudley D, Fraser H, Purich D, Lopez-Ilasaca M, Klein C, Davidson L, Bronson R, Mulligan RC, Southwick F, Geha R, Goldberg MB, Rosen FS, Hartwig JH, Alt FW (2001) N-WASP deficiency reveals distinct pathways for cell surface projections and microbial actin-based motility. *Nature Cell Biology* **3**: 897-904

Snyder JS, Soumier A, Brewer M, Pickel J, Cameron HA (2011) Adult hippocampal neurogenesis buffers stress responses and depressive behaviour. *Nature* **476**: 458-U112

Soderling SH, Guire ES, Kaech S, White J, Zhang F, Schutz K, Langeberg LK, Banker G, Raber J, Scott JD (2007) A WAVE-1 and WRP signaling complex regulates spine density, synaptic plasticity, and memory. *Journal of Neuroscience* **27**: 355-365

Soukas AA, Kane EA, Carr CE, Melo JA, Ruvkun G (2009) Rictor/TORC2 regulates fat metabolism, feeding, growth, and life span in *Caenorhabditis elegans*. *Genes & Development* **23**: 496-511

Sparrow N, Manetti ME, Bott M, Fabianac T, Petrilli A, Bates ML, Bunge MB, Lambert S, Fernandez-Valle C (2012) The Actin-Severing Protein Cofilin Is Downstream of Neuregulin Signaling and Is Essential For Schwann Cell Myelination. *Journal of Neuroscience* **32**: 5284-5297

Sparrow SM, Carter JM, Ridgway ND, Cook HW, Byers DM (1999) U18666A inhibits intracellular cholesterol transport and neurotransmitter release in human neuroblastoma cells. *Neurochemical Research* **24**: 69-77

Spilman P, Podlitskaya N, Hart MJ, Debnath J, Gorostiza O, Bredesen D, Richardson A, Strong R, Galvan V (2010) Inhibition of mTOR by Rapamycin Abolishes Cognitive Deficits and Reduces Amyloid-beta Levels in a Mouse Model of Alzheimer's Disease. *Plos One* **5**

Steffen A, Ladwein M, Dimchev GA, Hein A, Schwenkmezger L, Arens S, Ladwein KI, Holleboom JM, Schur F, Small JV, Schwarz J, Gerhard R, Faix J, Stradal TEB,

Brakebusch C, Rottner K (2013) Rac function is crucial for cell migration but is not required for spreading and focal adhesion formation. *Journal of Cell Science* **126**: 4572-4588

Suraneni P, Rubinstein B, Unruh JR, Durnin M, Hanein D, Li R (2012) The Arp2/3 complex is required for lamellipodia extension and directional fibroblast cell migration. *Journal of Cell Biology* **197**: 239-251

Suzuki SC, Furue H, Koga K, Jiang N, Nohmi M, Shimazaki Y, Katoh-Fukui Y, Yokoyama M, Yoshimura M, Takeichi M (2007) Cadherin-8 is required for the first relay synapses to receive functional inputs from primary sensory afferents for cold sensation. *Journal of Neuroscience* **27**: 3466-3476

Syriani E, Gomez-Cabrero A, Bosch M, Moya A, Abad E, Gual A, Gasull X, Morales M (2008) Profilin induces lamellipodia by growth factor-independent mechanism. *Faseb Journal* **22**: 1581-1596

Tahirovic S, Hellal F, Neukirchen D, Hindges R, Garvalov BK, Flynn KC, Stradal TE, Chrostek-Grashoff A, Brakebusch C, Bradke F (2010) Rac1 Regulates Neuronal Polarization through the WAVE Complex. *Journal of Neuroscience* **30**: 6930-6943

Tan Z-J, Peng Y, Song H-L, Zheng J-J, Yu X (2010) N-cadherin-dependent neuron-neuron interaction is required for the maintenance of activity-induced dendrite growth. *Proceedings of the National Academy of Sciences of the United States of America* **107**: 9873-9878

Taniguchi H, Kawauchi D, Nishida K, Murakami F (2006) Classic cadherins regulate tangential migration of precerebellar neurons in the caudal hindbrain. *Development* **133**: 1923-1931

Teodoro RO, Pekkurnaz G, Nasser A, Higashi-Kovtun ME, Balakireva M, McLachlan IG, Camonis J, Schwarz TL (2013) Ral mediates activity-dependent growth of postsynaptic membranes via recruitment of the exocyst. *Embo Journal* **32**: 2039-2055

Thomanetz V, Angliker N, Cloetta D, Lustenberger RM, Schweighauser M, Oliveri F, Suzuki N, Rueegg MA (2013) Ablation of the mTORC2 component rictor in brain or Purkinje cells affects size and neuron morphology. *Journal of Cell Biology* **201**: 293-308

Thomsen W, Frazer J, Unett D (2005) Functional assays for screening GPCR targets. *Current Opinion in Biotechnology* **16**: 655-665

Thomson PA, Wray NR, Thomson AM, Dunbar DR, Grassie MA, Condie A, Walker MT, Smith DJ, Pulford DJ, Muir W, Blackwood DHR, Porteous DJ (2005) Sex-specific association between bipolar affective disorder in women and GPR50, an X-linked orphan G protein-coupled receptor. *Molecular Psychiatry* **10**: 470-478

Togashi H, Abe K, Mizoguchi A, Takaoka K, Chisaka O, Takeichi M (2002) Cadherin regulates dendritic spine morphogenesis. *Neuron* **35**: 77-89

Toriyama M, Shimada T, Kim KB, Mitsuba M, Nomura E, Katsuta K, Sakumura Y, Roepstorff P, Inagaki N (2006) Shootin1: a protein involved in the organization of an asymmetric signal for neuronal polarization. *Journal of Cell Biology* **175**: 147-157

Tschische P, Moser E, Thompson D, Vischer HF, Parzmair GP, Pommer V, Platzer W, Schwarzbraun T, Schaidler H, Smit MJ, Martini L, Whistler JL, Waldhoer M (2010) The G-protein Coupled Receptor Associated Sorting Protein GASP-1 Regulates the Signalling and Trafficking of the Viral Chemokine Receptor US28. *Traffic* **11**: 660-674

Underwood KW, Andemariam B, McWilliams GL, Liscum L (1996) Quantitative analysis of hydrophobic amine inhibition of intracellular cholesterol transport. *Journal of Lipid Research* **37**: 1556-1568

van Aerde KI, Feldmeyer D (2013) Morphological and Physiological Characterization of Pyramidal Neuron Subtypes in Rat Medial Prefrontal Cortex.

Van Crielinge W, Beyaert R (1999) Yeast two-hybrid: State of the art. *Biological Procedures Online* **2**: 1-41

Vecchini A, Ceccarelli V, Orvietani P, Caligiana P, Susta F, Binaglia L, Nocentini G, Riccardi C, Di Nardo P (2003) Enhanced expression of hepatic lipogenic enzymes in an animal model of sedentariness. *Journal of Lipid Research* **44**: 696-704

Voelgyi B, Chheda S, Bloomfield SA (2009) Tracer Coupling Patterns of the Ganglion Cell Subtypes in the Mouse Retina. *Journal of Comparative Neurology* **512**: 664-687

Vulevic B, Chen ZJ, Boyd JT, Davis W, Walsh ES, Belinsky MG, Tew KD (2001a) Cloning and characterization of human adenosine 5'-triphosphate-binding cassette, sub-family A, transporter 2 (ABCA2). *Cancer Research* **61**: 3339-3347

Vulevic BI, Boyd JT, Tew KD (2001b) Subcellular localization and tissue distribution of ABCA2. *Proceedings of the American Association for Cancer Research Annual Meeting* **42**: 279-280

Wakeno M, Kato M, Okugawa G, Fukuda T, Hosoi Y, Takekita Y, Yamashita M, Nonen S, Azuma J, Kinoshita T (2008) The alpha 2A-adrenergic receptor gene polymorphism modifies antidepressant responses to milnacipran. *Journal of Clinical Psychopharmacology* **28**: 518-524

Wang B, Ardura JA, Romero G, Yang Y, Hall RA, Friedman PA (2010) Na/H Exchanger Regulatory Factors Control Parathyroid Hormone Receptor Signaling by Facilitating Differential Activation of G alpha Protein Subunits. *Journal of Biological Chemistry* **285**: 26976-26986

Wang C, JeBailey L, Ridgway ND (2002) Oxysterol-binding-protein (OSBP)-related protein 4 binds 25-hydroxycholesterol and interacts with vimentin intermediate filaments. *Biochemical Journal* **361**: 461-472

Wang J, Whiteman MW, Lian H, Wang G, Singh A, Huang D, Denmark T (2009) A Non-canonical MEK/ERK Signaling Pathway Regulates Autophagy via Regulating Beclin 1. *Journal of Biological Chemistry* **284**: 21412-21424

Wang J-W, David DJ, Monckton JE, Battaglia F, Hen R (2008) Chronic fluoxetine stimulates maturation and synaptic plasticity of adult-born hippocampal granule cells. *Journal of Neuroscience* **28**: 1374-1384

Wassmer T, Attar N, Bujny MV, Oakley J, Traer CJ, Cullen PJ (2007) A loss-of-function screen reveals SNX5 and SNX6 as potential components of the mammalian retromer. *Journal of Cell Science* **120**: 45-54

Wegner AM, Nebhan CA, Hu L, Majumdar D, Meier KM, Weaver AM, Webb DJ (2008) N-WASP and the Arp2/3 complex are critical regulators of actin in the development of dendritic spines and synapses. *Journal of Biological Chemistry* **283**: 15912-15920

Wei Y, Pattingre S, Sinha S, Bassik M, Levine B (2008) JNK1-mediated phosphorylation of Bcl-2 regulates starvation-induced autophagy. *Molecular Cell* **30**: 678-688

Wen K-K, McKane M, Houtman JCD, Rubenstein PA (2008) Control of the ability of profilin to bind and facilitate nucleotide exchange from G-actin. *Journal of Biological Chemistry* **283**: 9444-9453

Wheeler AP, Wells CM, Smith SD, Vega FM, Henderson RB, Tybulewicz VL, Ridley AJ (2006) Rac1 and Rac2 regulate macrophage morphology but are not essential for migration. *Journal of Cell Science* **119**: 2749-2757

Wiechmann AF, Hollaway LR, Rada JAS (2009) Melatonin receptor expression in *Xenopus laevis* surface corneal epithelium: Diurnal rhythm of lateral membrane localization. *Molecular Vision* **15**: 2384-2403

Wills ZP, Mandel-Brehm C, Mardinly AR, McCord AE, Giger RJ, Greenberg ME (2012) The Nogo Receptor Family Restricts Synapse Number in the Developing Hippocampus. *Neuron* **73**: 466-481

Witt-Enderby PA, MacKenzie RS, McKeon RM, Carroll EA, Bordt SL, Melan MA (2000) Melatonin induction of filamentous structures in non-neuronal cells that is dependent on expression of the human mt1 melatonin receptor. *Cell Motility and the Cytoskeleton* **46**: 28-42

Wolins NE, Quaynor BK, Skinner JR, Schoenfish MJ, Tzekov A, Bickel P (2005) S3-12, adipophilin, and TIP47 package lipid in adipocytes. *Journal of Biological Chemistry* **280**: 19146-19155

Wollmer M, Kapaki E, Hersberger M, Muntwyler J, Brunner F, Tsolaki M, Akatsu H, Kosaka K, Michikawa M, Molyva D, Paraskevas G, Lütjohann D, von Eckardstein A, Hock C, Nitsch R, Papassotiropoulos A (2006) Ethnicity-dependent genetic association of ABCA2 with sporadic Alzheimer's disease. *Am J Med Genet B Neuropsychiatr Genet* **141B**: 534-536

Wolven AK, Belmont LD, Mahoney NM, Almo SC, Drubin DG (2000) In vivo importance of actin nucleotide exchange catalyzed by profilin. *Journal of Cell Biology* **150**: 895-903

Wu Y-H, Zhou J-N, Balesar R, Unmehopa U, Bao A, Jockers R, Van Heerikhuize J, Swaab DF (2006) Distribution of MT1 melatonin receptor immunoreactivity in the human hypothalamus and pituitary gland: Colocalization of MT1 with vasopressin, oxytocin, and corticotropin-releasing hormone. *Journal of Comparative Neurology* **499**: 897-910

Xu J, Dang Y, Ren YR, Liu JO (2010) Cholesterol trafficking is required for mTOR activation in endothelial cells. *Proceedings of the National Academy of Sciences of the United States of America* **107**: 4764-4769

Xu W, Yoon SI, Huang P, Wang YL, Chen CG, Chong PLG, Liu-Chen LY (2006) Localization of the kappa opioid receptor in lipid rafts. *Journal of Pharmacology and Experimental Therapeutics* **317**: 1295-1306

Yamazaki D, Fujiwara T, Suetsugu S, Takenawa T (2005) A novel function of WAVE in lamellipodia: WAVE1 is required for stabilization of lamellipodial protrusions during cell spreading. *Genes to Cells* **10**: 381-392

Yang C, Czech L, Gerboth S, Kojima S-I, Scita G, Svitkina T (2007) Novel roles of formin mDia2 in lamellipodia and filopodia formation in motile cells. *Plos Biology* **5**: 2624-2645

Yatham LN, Liddle PF, Dennie J, Shiah IS, Adam MJ, Lane CJ, Lam RW, Ruth TJ (1999) Decrease in brain serotonin 2 receptor binding in patients with major depression following desipramine treatment - A positron emission tomography study with fluorine-18-labeled setoperone. *Archives of General Psychiatry* **56**: 705-711

Yuan M, Pino E, Wu L, Kacergis M, Soukas AA (2012) Identification of Akt-independent Regulation of Hepatic Lipogenesis by Mammalian Target of Rapamycin (mTOR) Complex 2. *Journal of Biological Chemistry* **287**: 29579-29588

Yuste R, Bonhoeffer T (2004) Genesis of dendritic spines: Insights from ultrastructural and imaging studies. *Nature Reviews Neuroscience* **5**: 24-34

Zeng LF, Liao HL, Liu Y, Lee TS, Zhu MJ, Wang X, Stemerman MB, Zhu Y, Shyy JYJ (2004) Sterol-responsive element-binding protein (SREBP) 2 down-regulates ATP-binding cassette transporter A1 in vascular endothelial cells - A novel role of SREBP in regulating cholesterol metabolism. *Journal of Biological Chemistry* **279**: 48801-48807

Zeng Z, Wang T, Li T, Li Y, Chen P, Zhao Q, Liu J, Li J, Feng G, He L, Shi Y (2011) Common SNPs and haplotypes in DGKH are associated with bipolar disorder and schizophrenia in the Chinese Han population. *Molecular Psychiatry* **16**: 473-475

Zhang J, Dudley-Rucker N, Crowley JR, Lopez-Perez E, Issandou M, Schaffer JE, Ory DS (2004) The steroidal analog GW707 activates the SREBP pathway through disruption of intracellular cholesterol trafficking. *Journal of Lipid Research* **45**: 223-231

Zhou C, Zhao L, Inagaki N, Guan J, Nakajo S, Hirabayashi T, Kikuyama S, Shioda S (2001) Atp-binding cassette transporter ABC2/ABCA2 in the rat brain: a novel mammalian lysosome-associated membrane protein and a specific marker for oligodendrocytes but not for myelin sheaths. *J Neurosci* **21**: 849-857

Zhou M, Li W, Huang S, Song J, Kim JY, Tian X, Kang E, Sano Y, Liu C, Balaji J, Wu S, Zhou Y, Zhou Y, Parivash SN, Ehninger D, He L, Song H, Ming G-l, Silva AJ (2013) mTOR Inhibition Ameliorates Cognitive and Affective Deficits Caused by Disc1 Knockdown in Adult-Born Dentate Granule Neurons. *Neuron* **77**: 647-654

Zhu J, Jiang X, Chehab FF (2013) FoxO4 Interacts with the Sterol Regulatory Factor SREBP2 and the Hypoxia Inducible Factor HIF2alpha at the CYP51 Promoter. *J Lipid Res*

Zimmermann R, Strauss JG, Haemmerle G, Schoiswohl G, Birner-Gruenberger R, Riederer M, Lass A, Neuberger G, Eisenhaber F, Hermetter A, Zechner R (2004) Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase. *Science* **306**: 1383-1386

Zuo Y, Lin A, Chang P, Gan WB (2005) Development of long-term dendritic spine stability in diverse regions of cerebral cortex. *Neuron* **46**: 181-189

Appendix A: Antibody validation

A.1 Introduction

Much of the work presented in this thesis is reliant on the integrity of antibodies and expression constructs. This is especially important in chapter five, where the localisation and trafficking of GPR50, ABCA2 and SREBF2 are monitored. The expression constructs and antibodies used for these proteins have been validated elsewhere (Davis et al, 2004; Grünewald et al, 2009; Logette et al, 2005; Zhu et al, 2013). However, they were also characterised here to ensure their integrity in the model systems used in this thesis. To do this, cells were transfected with the relevant protein, or left untransfected, and 24 hrs later immunocytochemistry (ICC) or western blot analysis was performed. Western blot analysis was used to determine whether the antibodies detected a specific band at the anticipated size, whilst ICC was used to determine whether the protein of interest assumed the correct subcellular localisation. Given the high transfection rates of HEK293 cells, this cell line was favoured in western blot analysis. For ICC, SH-SY5Y cells were predominately tested, as the colocalisation experiments presented in this thesis were mainly based on this cell line, although other cell lines were also tested.

A.2 GPR50 is localised to the plasma membrane

Two GPR50-specific antibodies were used in this thesis; the goat anti-GPR50 antibody (Santa Cruz Biotechnology, SCBT), which was primarily used, and the rabbit anti-GPR50 antibody (Proteintech). In HEK293 cells, the goat anti-GPR50 antibody detected a doublet band at the expected size of 67 kDa (figure A.1 a), as previously shown (Grünewald, 2011). A singlet band at 67 kDa was also found in untransfected HEK293 cells using this antibody. This is likely to correspond to endogenous GPR50, which has been demonstrated in this cell line (Atwood et al, 2011; Grünewald et al, 2009). In SH-SY5Y cells, the 67 kDa doublet band was also detected in GPR50-transfected cells using the goat antibody (figure A.1 b). This

band was not observed in untransfected cells, indicating the absence of endogenous GPR50 expression. The rabbit anti-GPR50 antibody generated similar results. In HEK293 cells, a strong doublet band at 67 kDa was observed in transfected and untransfected cells (figure A.1 c), with very little non-specific bands. This band was also detected in GPR50-transfected SH-SY5Y cells, in a highly specific manner; again, this was not observed in untransfected cells (figure A.1 d). These results show that both antibodies are capable of detecting GPR50. In addition, HEK293 cells provide an endogenous system to study GPR50 expression, whereas SH-SY5Y cells are only suitable for overexpression studies.

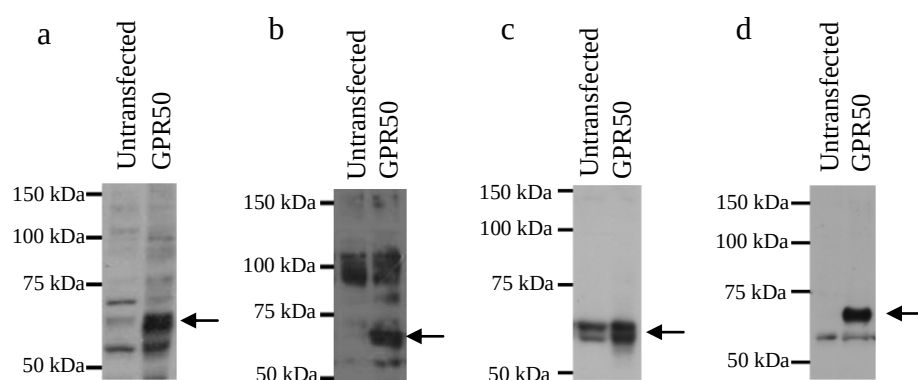


Figure A.1: A 67 kDa protein is detected by both GPR50 antibodies. Western blot analysis was carried out using cells that were transfected with GPR50 or left untransfected. (a) In HEK293 cells, a strong band was detected at 67 kDa (arrow) using the goat anti-GPR50 antibody (SCBT); in untransfected cells a 67 kDa band was also seen, suggesting the endogenous protein was detected. (b) The goat antibody also recognised a 67 kDa doublet band in transfected SH-SY5Y cells; this was not observed in untransfected cells, indicating SH-SY5Y cells do not endogenously express GPR50. (c) The rabbit anti-GPR50 antibody (Proteintech) clearly detected a 67 kDa doublet band in transfected and untransfected HEK293 cells. (d) In SH-SY5Y cells, the rabbit antibody also detected a band at 67 kDa in GPR50-transfected cells. Again, this band was not present in untransfected cells.

Full length GPR50 has repeatedly been shown to reside on the plasma membrane (Grünwald et al, 2009; Hamouda et al, 2007). Further, the commercially available goat anti-GPR50 antibody (SCBT) has been shown to detect this (Grünwald et al, 2009). For further confirmation, ICC was performed on GPR50-transfected SH-

SY5Y cells, staining for GPR50 and VLA-2 α , a plasma membrane marker. Using this antibody, GPR50 was found to strongly colocalise with VLA-2 α , indicating that it resides on the plasma membrane (figure A.2 a). This plasma membrane distribution was also detected in GPR50-transfected N1E-115 cells and HEK293 cells (figure A.2 b and c), although GPR50 often assumed a cytoplasmic distribution in N1E-115 cells. As this may reflect the mislocalisation of GPR50, the use of N1E-115 cells was limited in this thesis. The goat antibody also detected GPR50 on the plasma membrane of untransfected HEK293 cells, confirming the endogenous expression of GPR50 in this cell line (figure A.2 d). Endogenous GPR50 was not detected in untransfected SH-SY5Y cells or N1E-115 cells (results not shown). The goat anti-GPR50 antibody was then used to validate the rabbit antibody (Proteintech). Figure A.2 e shows strong colocalisation between the two antibodies at the plasma membrane, indicating they detect the same protein. Thus, both antibodies can be used for the detection of GPR50.

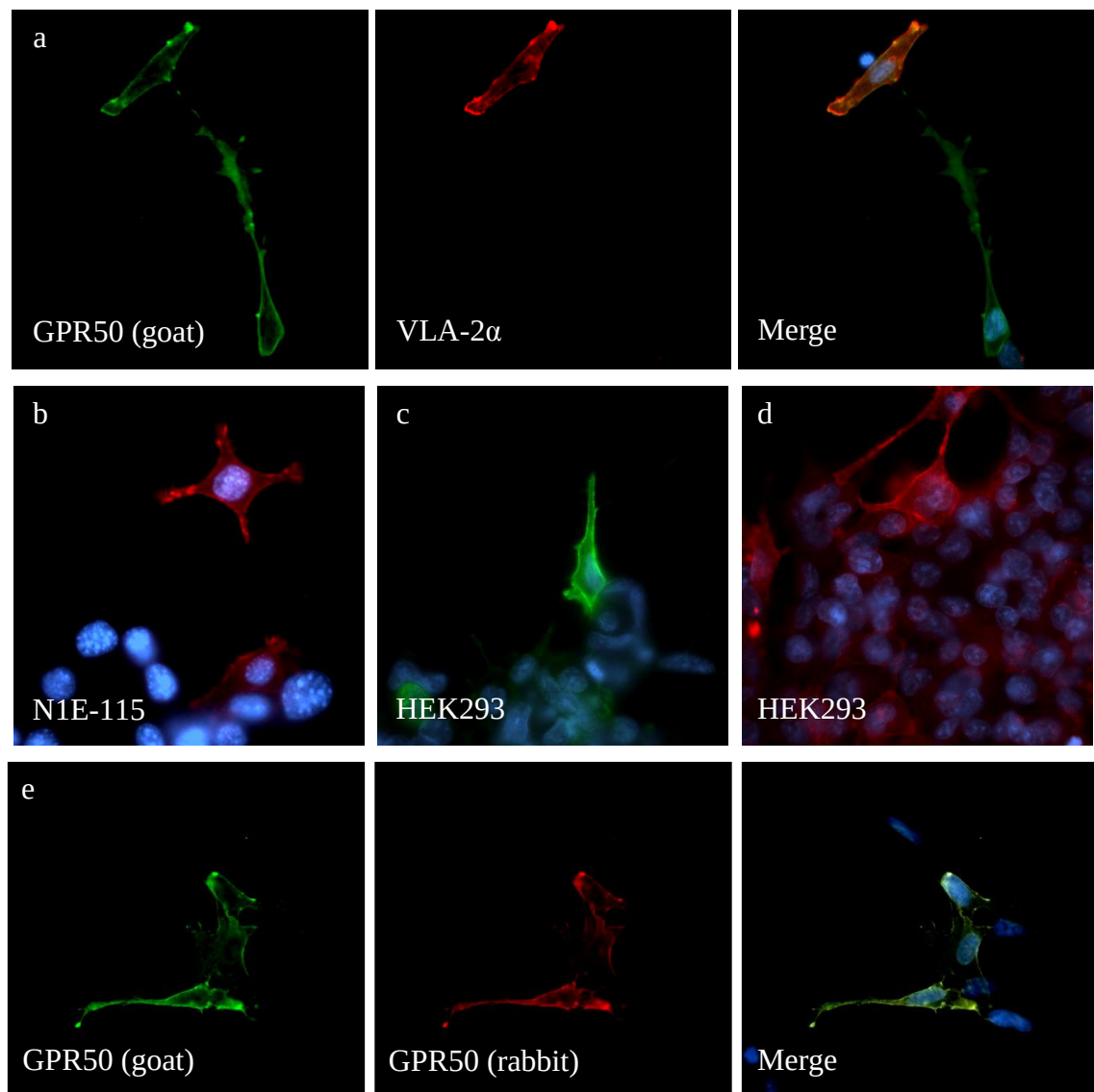


Figure A.2: The plasma membrane localisation of GPR50. ICC was performed on various cell lines following GPR50 transfection and fixation with methanol. Cells were stained for GPR50, the plasma membrane marker VLA-2 α and counterstained with DAPI (blue). Note these images were acquired using a fluorescent microscope. (a) In SH-SY5Y cells, GPR50 was detected with the goat anti-GPR50 antibody (SCBT). This was largely plasma membrane bound, as seen by colocalisation with VLA-2 α . The plasma membrane distribution was also detected in GPR50-transfected N1E-115 cells (b) and HEK293 cells (c), as detected by the goat antibody. (d) The goat antibody also detected endogenous GPR50 in untransfected HEK293 cells, which again showed a plasma membrane distribution. (e) In SH-SY5Y cells, a strong colocalisation was observed between the goat and the rabbit anti-GPR50 antibodies. Again, this was mostly seen at the plasma membrane.

A.3 ABCA2 is found in discrete intracellular vesicles

ABCA2 is a large 270 kDa protein which resides in lysosome-related vesicles (Broccardo et al, 2006b; Vulevic et al, 2001b). Two antibodies were used to detect this protein, the most extensively used was the rabbit anti-ABCA2 antibody (gifted by Prof. Tew), which has been previously validated (Davis et al, 2004; Grünwald, 2011). In addition, the commercially available goat anti-ABCA2 antibody (SCBT) was used in the ABCA2 immunoprecipitation experiment. These antibodies were first tested with western blot analysis on untransfected and ABCA2-transfected HEK293 cells. Both the goat and the rabbit antibodies detected a doublet band at the expected size of 270 kDa, figure A.3 a and b. Neither antibody detected endogenous ABCA2. This was reproduced in SH-SY5Y cells, although the band density was substantially weaker (results not shown). This is likely to reflect the lower transfection rates in SH-SY5Y cells.

These antibodies were also assessed with ICC. SH-SY5Y cells were transfected with ABCA2, or were left untransfected, and the following day cells were fixed and stained with the rabbit anti-ABCA2 antibody. In keeping with previous work, ABCA2 was found in discrete punctuate vesicles which partially colocalised with LAMP1 (figure A.3 c). Again, this was not detected in untransfected cells. This antibody was then used as a positive reference to validate the goat anti-ABCA2 antibody. As can be seen in figure A.3 d, colocalisation between the two antibodies was observed. This was also true in HEK293 cells (results not shown). Thus, both antibodies appear to successfully detect ABCA2.

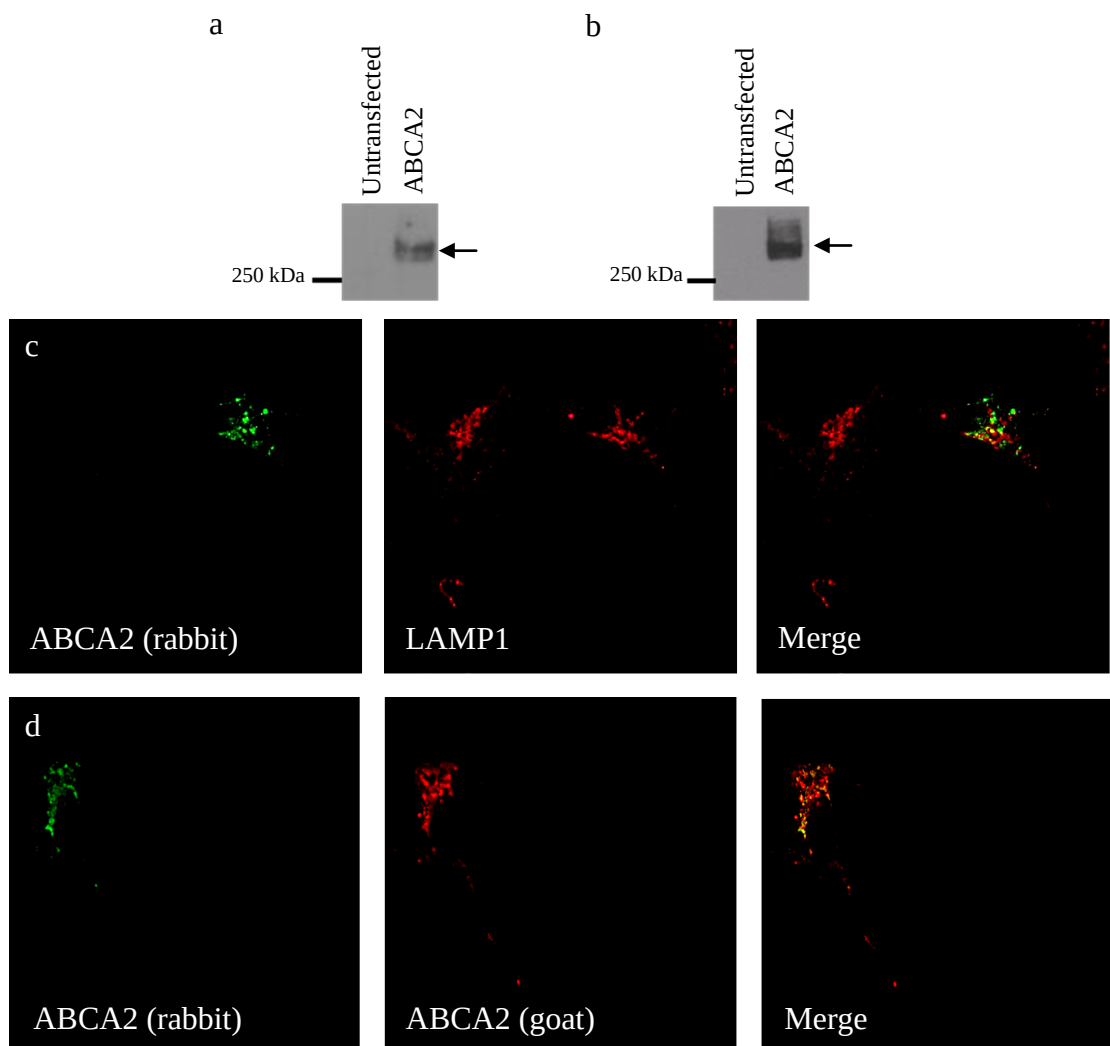


Figure A.3: Characterisation of the ABCA2 antibodies. HEK293 cells were transfected with ABCA2, or left untransfected, and cell lysates were subjected to western blot analysis. (a) Using the rabbit anti-ABCA2 antibody (Tew), a doublet band at 270 kDa (arrow) was detected in transfected HEK293 cells. This was not seen in untransfected cells. (b) The goat anti-ABCA2 antibody (SCBT) also detected a 270 kDa protein (arrow) in transfected HEK293 cells. Again, this was absent in untransfected cells. (c) ICC was performed in SH-SY5Y cells transfected with ABCA2, staining for ABCA2 and the lysosomal marker LAMP1. The rabbit antibody detected ABCA2 in a highly specific manner, which partially colocalised with LAMP1. (d) A high degree of overlap was observed when ABCA2-transfected SH-SY5Y cells were stained with the rabbit (green) and the goat (red) anti-ABCA2 antibodies.

A.4 Staining of active and inactive SREBF2

SREBF2 exists in an inactive or active form, at 120 kDa and 60 kDa respectively (Goldstein & Brown, 1997; Horton et al, 1998). In its inactive form, SREBF2 resides in the endoplasmic reticulum (ER). Upon stimulation, SREBF2 is cleaved to release the active N-terminal domain, which is translocated to the nucleus. In chapter five, the commercially available goat anti-SREBP2 antibody (SCBT) was used. This antibody is raised against SREBP2 (sterol regulatory element binding protein 2), i.e. the mouse homologue of SREBF2; however, it also detects human SREBF2. Importantly, this antibody targets the N-terminal region of the protein, therefore, should be able to detect both forms of SREBF2.

Western blot analysis was carried out in HEK293 cells to reveal a highly specific band at 120 kDa, when cells were transfected with SREBF2 (figure A.4 a). This band was also seen in transfected SH-SY5Y cells, although this was very weak (results not shown). The 120 kDa band was absent in untransfected cells, suggesting that the antibody does not detect endogenous SREBF2 in the tested cell lines. However, a weak band at 60 kDa was seen in untransfected and SREBF2-transfected HEK293 cells (figure A.4 a, arrowhead). This may represent the nuclear form of SREBF2, which has previously been detected using this antibody (Kannenbergh et al, 2013; Vecchini et al, 2003).

ICC was also used to validate the goat-anti SREBP2 antibody. As shown in figure A.4 b, a strong signal was detected in SREBF2-transfected SH-SY5Y cells, with very little background. SREBF2 showed a high degree of colocalisation with the ER marker calreticulin, confirming its presence in the ER. However, nuclear staining was not observed. In addition, the signal was not obtained in untransfected cells; again, this indicates that the endogenous protein was not detected with this antibody. These results suggest that at high levels of expression the inactive form of SREBF2

can be detected with this antibody. However, detection of the active nuclear form of SREBF2 was less evident.

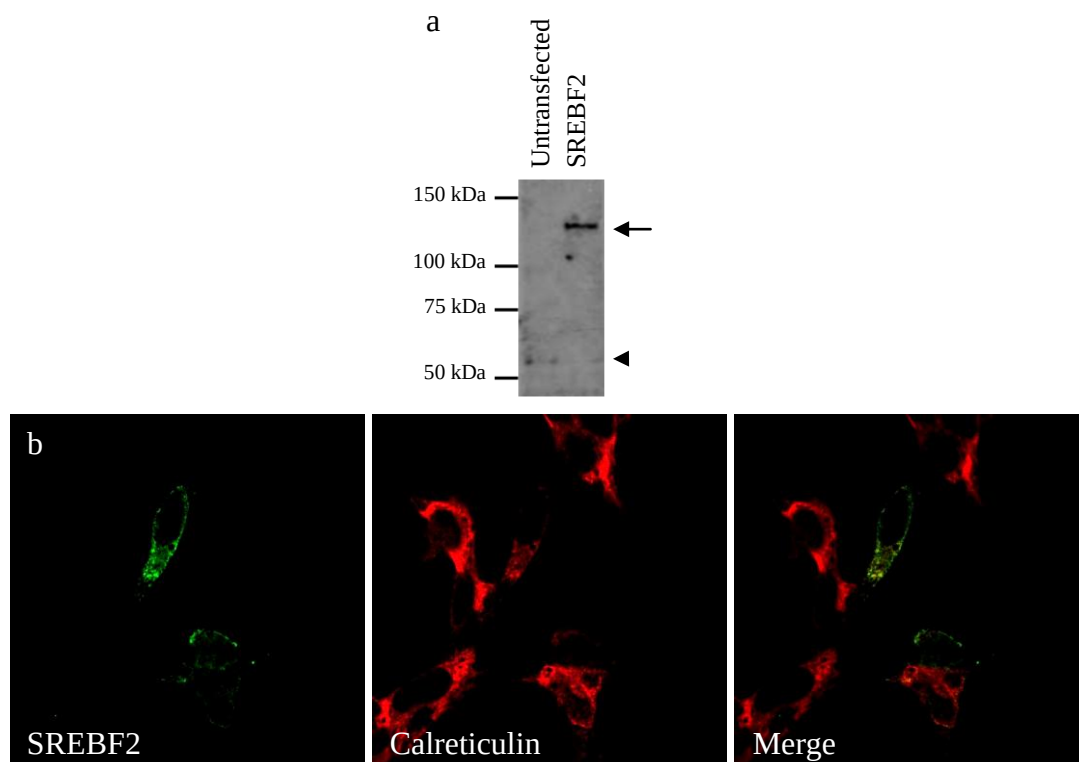


Figure A.4: Characterisation of the goat anti-SREBP2 antibody (SCBT). (a) Western blotting results from HEK293 cells transfected with SREBF2 or left untransfected. Using the goat anti-SREBP2 antibody (SCBT), a band at 120 kDa (arrow) was detected in transfected HEK293 cells; this was not detected in untransfected cells. A weak band at 60 kDa (arrowhead) was also seen, possibly representing the active form of SREBF2. (b) SH-SY5Y cells were transfected with SREBF2 and ICC was performed the following day, staining for the ER-marker calreticulin and SREBF2. Strong colocalisation was observed between SREBF2 and calreticulin, confirming the ER localisation of SREBF2.

It is possible that poor detection of nuclear SREBF2 was due to the lack of SREBF2 activation under the tested conditions. To explore this possibility, the localisation of endogenous SREBF2 was investigated under conditions of serum deprivation, which is known to activate the protein (Harrison et al, 2009; Horie et al, 2010). COS-7 cells were used here, a cell line derived from monkey kidney tissue, as endogenous SREBF2 was detected and the large cell size made SREBF2 translocation readily observable. Under standard conditions (DMEM supplemented with 10% FBS), SREBF2 was largely absent from the nucleus (figure A.5 a), displaying an ER-like distribution. Whilst in serum starved cells (DMEM with 0.5% FBS, 18 hrs), SREBF2 was predominately found within the nucleus of cells, as seen by colocalisation with the nuclear stain DAPI (figure A.5 b). Thus, the goat anti-SREBF2 antibody appears to detect active SREBF2, as well as the inactive form.

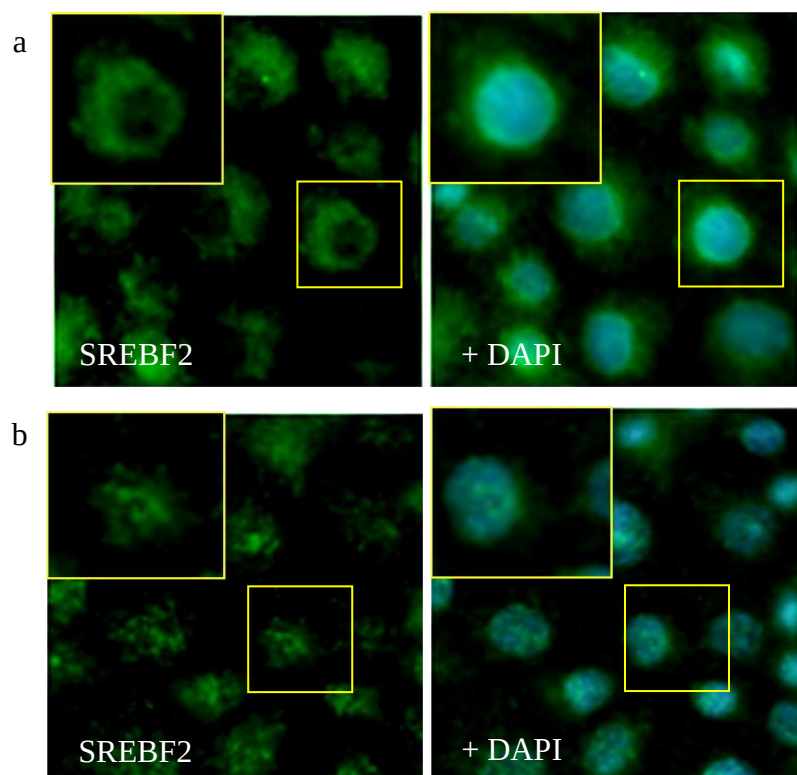


Figure A.5: Nuclear translocation of SREBF2 in COS-7 cells is detected by the goat anti-SREBF2 antibody. Untransfected COS-7 cells were grown in 10% or 0.5% FBS. After 18 hrs cells were fixed with methanol and ICC was performed, staining for SREBF2 and counterstaining with DAPI (blue). Images were obtained with fluorescence microscopy. (a) In cells maintained in media containing 10% FBS, SREBF2 took up an ER-like distribution. (b) When serum deprived, SREBF2 was predominately found in the nucleus where it colocalised with DAPI.

A.5 Conclusion

The work presented here confirms the integrity of the antibodies and expression constructions used in this thesis, particularly in chapter five. Western blot analysis with each antibody detected proteins of the expected size, with little non-specific signals. Further, the antibodies, and constructs, tested showed the same patterns of subcellular distribution that have been noted in previous reports. Thus, they can be confidently used in this thesis.

References

- Atwood BK, Lopez J, Wager-Miller J, Mackie K, Straiker A (2011) Expression of G protein-coupled receptors and related proteins in HEK293, AtT20, BV2, and N18 cell lines as revealed by microarray analysis. *BMC genomics* **12**: 14-14
- Broccardo C, Nieoullon V, Amin R, Masméjean F, Carta S, Tassi S, Pophillat M, Rubartelli A, Pierres M, Rougon G, Nieoullon A, Chazal G, Chimini G (2006) ABCA2 is a marker of neural progenitors and neuronal subsets in the adult rodent brain. *J Neurochem* **97**: 345-355
- Davis WJ, Boyd J, Ile K, Tew K (2004) Human ATP-binding cassette transporter-2 (ABCA2) positively regulates low-density lipoprotein receptor expression and negatively regulates cholesterol esterification in Chinese hamster ovary cells. *Biochim Biophys Acta* **1683**: 89-100
- Goldstein JL, Brown MS (1997) The SREBP pathway: Regulation of cholesterol and fatty acid metabolism by proteolysis of a membrane-bound transcription factor. *Faseb Journal* **11**: 4
- Grünewald E (2011) Investigating the role of orphan GPR50 in normal brain function and mental illness PhD Thesis, The University of Edinburgh, Edinburgh
- Grünewald E, Kinnell HL, Porteous DJ, Thomson PA (2009) GPR50 interacts with neuronal NOGO-A and affects neurite outgrowth. *Molecular and Cellular Neuroscience* **42**: 363-371
- Hamouda HO, Chen P, Levoye A, Sozer-Topcular N, Daulat AM, Guillaume JL, Ravid R, Savaskan E, Ferry G, Boutin JA, Delagrangé P, Jockers R, Maurice P (2007) Detection of the human GPR50 orphan seven transmembrane protein by polyclonal antibodies mapping different epitopes. *Journal of Pineal Research* **43**: 10-15
- Harrison KD, Miao RQ, Fernandez-Hernando C, Suarez Y, Davalos A, Sessa WC (2009) Nogo-B Receptor Stabilizes Niemann-Pick Type C2 Protein and Regulates Intracellular Cholesterol Trafficking. *Cell Metabolism* **10**: 208-218
- Horie T, Ono K, Horiguchi M, Nishi H, Nakamura T, Nagao K, Kinoshita M, Kuwabara Y, Marusawa H, Iwanaga Y, Hasegawa K, Yokode M, Kimura T, Kita T (2010) MicroRNA-33 encoded by an intron of sterol regulatory element-binding

protein 2 (Srebp2) regulates HDL in vivo. *Proceedings of the National Academy of Sciences of the United States of America* **107**: 17321-17326

Horton JD, Shimomura I, Brown MS, Hammer RE, Goldstein JL, Shimano H (1998) Activation of cholesterol synthesis in preference to fatty acid synthesis in liver and adipose tissue of transgenic mice overproducing sterol regulatory element-binding protein-2. *Journal of Clinical Investigation* **101**: 2331-2339

Kannenbergh F, Gorzelniak K, Jaeger K, Fobker M, Rust S, Repa J, Roth M, Bjorkhem I, Walter M (2013) Characterization of Cholesterol Homeostasis in Telomerase-immortalized Tangier Disease Fibroblasts Reveals Marked Phenotype Variability. *Journal of Biological Chemistry* **288**: 36936-36947

Logette E, Le Jossic-Corcus C, Masson D, Solier S, Sequeira-Legrand A, Dugail I, Lemaire-Ewing S, Desoche L, Solary E, Corcos L (2005) Caspase-2, a novel lipid sensor under the control of sterol regulatory element binding protein 2. *Molecular and Cellular Biology* **25**: 9621-9631

Vecchini A, Ceccarelli V, Orvietani P, Caligiana P, Susta F, Binaglia L, Nocentini G, Riccardi C, Di Nardo P (2003) Enhanced expression of hepatic lipogenic enzymes in an animal model of sedentariness. *Journal of Lipid Research* **44**: 696-704

Vulevic BI, Boyd JT, Tew KD (2001) Subcellular localization and tissue distribution of ABCA2. *Proceedings of the American Association for Cancer Research Annual Meeting* **42**: 279-280

Zhu J, Jiang X, Chehab FF (2013) FoxO4 Interacts with the Sterol Regulatory Factor SREBP2 and the Hypoxia Inducible Factor HIF2alpha at the CYP51 Promoter. *J Lipid Res*