The mechanism through which the second generation antipsychotic drug olanzapine affects viability and function of pancreatic beta cells

Madeleine Honey

A thesis submitted in partial fulfilment of the requirements of the University of Brighton for the degree of Doctor of Philosophy

September 2020

Abstract

Patients with schizophrenia have a reduced life expectancy compared to the general population. Second generation antipsychotics are commonly prescribed to patients, but treatment contributes to the increased mortality through adverse events, such as increased rates of diabetes. Olanzapine is considered to be one of the highest risk diabetogenic antipsychotics; it is known to promote insulin resistance and hyperglycaemia through pharmacological interaction with peripheral metabolic organs. Glucose stimulated insulin secretion (GSIS) from the pancreatic beta cells is fundamental to overall glucose homeostasis, but the impact on the function of the beta cells has not been thoroughly explored. This thesis aimed to test the hypothesis that olanzapine pharmacologically alters the viability or function of the pancreatic beta cells leading to reduced insulin secretion.

The mouse MIN6 beta cell line was exposed to olanzapine for 24-72 hour and analysed for viability using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, fluorescent staining and cell counts. Following 72 hour exposure, insulin secretion in response to 2 hour glucose stimulation was determined by ELISA. Cellular lysates or mRNA was extracted and analysed by real-time qPCR for mRNA expression, or Western blot for protein analysis. Statistical analysis was carried out using two-way ANOVA and Bonferroni's post-test.

There was no increase in cell death when exposed to 0.1–10 μM olanzapine, but these concentrations did cause a significant increase in proliferation in unstimulated conditions ($p<0.01$). Olanzapine treatment increased expression of cell cycle regulator cyclin D1 ($p<0.05$), and altered concentrations of components of the canonical Wnt/beta catenin signalling pathway (TCF7L2 and Axin2) indicating that olanzapine may disrupt activity in this pathway. Pre-treatment with 10 μM olanzapine significantly reduced GSIS ($p<0.01$) with no change in intracellular insulin concentration. There was significantly reduced cytoplasmic beta catenin ($p<0.05$) and increased axin2 ($p<0.05$), the central scaffold of the beta catenin destruction complex. Glycogen synthase kinase-3 (GSK3) is another component of the destruction complex, and addition of the GSK3 inhibitor 6-bromoindirubin-3'-oxime (BIO) reduced activity of the beta catenin destruction complex leading to

significant increases in cytoplasmic accumulation and significantly improved insulin secretion ($p<0.05$).

Beta catenin has been linked to insulin vesicle trafficking and these data imply that olanzapine alters beta catenin dynamics in MIN6 cells leading to a decline in function, reduced insulin secretion and hypertrophy. In patients treated with olanzapine, reduced beta cell function may add to hyperglycaemia and prevent adaptation to peripheral insulin resistance, advancing the development of diabetes and contributing to increased mortality.

Table of Contents

Abstract....1
Table of Contents....3
List of Tables....7
List of figures....8
Abbreviations....12
Acknowledgements....18
Author's declaration....19

Chapter 1: Introduction....20

1.1 Part 1: Severe mental illness and increased mortality....21
1.1.1 Severe mental illness....21
1.1.2 Antipsychotic treatment....22
1.1.3 SMI increases cardiovascular risk....25
1.2 Part 2: Current evidence and proposed mechanism for olanzapine induced hyperglycaemia....32
1.2.1 Weight Gain....33
1.2.2 Dyslipidaemia....37
1.2.3 Hyperglycaemia....39
1.2.4 Insulin resistance....49
1.2.5 Beta cell dysfunction....53
1.2.6 Insulin resistance and beta cell dysfunction....54
1.3 Part 3: Olanzapine and Beta cell function....56
1.3.1 Beta cell function....57
1.3.2 Modulation of beta cell function....60
1.4 Summary and aims....63

Chapter 2: Materials and Methods....65

2.1 General reagents and materials....66
2.2 Tissue culture....66
2.2.1 Cell lines....66
2.2.2 Maintenance of cell lines....66
2.2.3 Passaging of cells....67
2.2.4 Cryopreservation and thawing of cell lines....67
2.2.5 Treatment of cell lines....67
2.2.6 Experimental protocol....68
2.2.7 Small molecule inhibitors and agonists....68
2.3 Cell viability assays....70
2.3.1 The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) Assay....70
2.3.2 Acridine orange and DAPI staining....70
2.3.3 Cell count....71
2.4 Insulin assays....71
2.4.1 Insulin content analysis....71
2.4.2 Insulin secretion analysis....72
2.4.3 ELISA....73

2.4.4 Analysis and normalisation 73
2.5 Cell cycle analysis 73
2.5.1 Cell fixation and staining 73
2.5.2 Flow cytometry 74
2.6 Protein content assays 74
2.6.1 Protein extraction 74
2.7 Bradford Assay 76
2.8 Western Blot Analysis 76
2.8.1 SDS-PAGE 76
2.8.2 Gel preparation 76
2.8.3 Semi dry Transfer 78
2.8.4 Immunodetection 79
2.8.5 Densiometric analysis 79
2.9 Quantitative real Time Polymerase Chain Reaction (qRT- PCR) 80
2.9.1 RNA extraction 80
2.9.2 Reverse transcription 80
2.9.3 qRT-PCR 81
2.9.4 GeNorm analysis 82
2.9.5 ΔΔCt method 85
2.9.6 Agarose gel electrophoresis 85
2.9.7 Quantification and statistical analysis 85

Chapter 3: The effect of olanzapine on the viability and function of MIN6 beta cells 86

3.1 Introduction 87
3.1.1 Overview 87
3.1.2 Effect of olanzapine on beta cell viability 87
3.1.3 Effect of olanzapine on beta cell function 89
3.2 Results 94
3.2.1 Effect of olanzapine on MIN6 viability 94
3.2.2 Effect of olanzapine on beta cell function 97
3.2.3 Proliferation 99
3.2.4 The Cell Cycle 103
3.3 Discussion 105
3.3.1 Acute olanzapine exposure does not reduce beta cell viability at any concentration 105
3.3.2 Chronic exposure to lower concentration olanzapine (10 μM) results in reduced GSIS when stimulated with high glucose 106
3.3.3 Olanzapine can accelerate the cell cycle and result in increased proliferation of MIN6 in the absence of other stimulus 107
3.3.4 Conclusion 108

Chapter 4: The effect of olanzapine on cell cycle regulation and the canonical Wnt signalling pathway 109

4.1 Introduction 110
4.1.1 Proliferation 110
4.1.2 Cell cycle regulation 111
4.1.3 Mitogenic pathways 113

4.1.4 Canonical Wnt pathway 115
4.2 Results 118
4.2.1 Effect of olanzapine on regulators of the cell cycle 118
4.2.2 Effect of olanzapine on the canonical Wnt signalling pathway 120
4.2.3 Addition of inhibitors of the canonical Wnt signalling pathway 125
4.2.4 Effect of olanzapine on Axin2 over time 139
4.3 Discussion 142
4.3.1 Acute olanzapine exposure increases expression of cyclin D1 and c-myc 142
4.3.2 Acute olanzapine stimulates beta catenin signalling 143
4.3.3 XAV939 inhibits the effect of olanzapine on c-myc and cyclin D1 expression 145
4.3.4 Acute olanzapine exposure does not affect expression of cyclin D2 147
4.3.5 Chronic olanzapine alters the cytoplasmic/ nuclear distribution of beta catenin 148
4.3.6 Conclusion 150

Chapter 5: The mechanism through which chronic olanzapine reduces insulin secretion in MIN6 beta cells 152

5.1 Introduction 153
5.1.1 GSIS 153
5.1.2 Beta catenin involvement in insulin secretion 154
5.1.3 Mechanism for glucose induced changes in beta catenin 155
5.1.4 Beta catenin regulates vesicle organisation 157
5.2 Results 159
5.2.1 Effect of glucose stimulation on beta catenin in olanzapine treated cells 159
5.2.2 Effect of beta catenin modification on olanzapine induced changes in insulin secretion 163
5.2.3 Effect of glucose stimulation on proteins involved in beta catenin regulation in olanzapine treated cells 171
5.2.4 Effect of Wnt inhibition on chronic olanzapine effects 177
5.3 Discussion 179
5.3.1 Olanzapine reduces the concentration of cytoplasmic beta catenin during glucose stimulation 179
5.3.2 Reduced cytoplasmic beta catenin causes reduced GSIS 184
5.3.3 Olanzapine does not affect insulin secretion stimulated by membrane depolarisation 186
5.3.4 Acute Wnt stimulation is linked to the chronic effect of olanzapine to inhibit GSIS 187
5.3.5 Conclusion 188

Chapter 6: Conclusions and future work 189

6.1 Research aims 190
6.2 Summary of main findings 192
6.2.1 Cell viability 192
6.2.2 Beta cell proliferation and Compensation 192

6.2.3 Glucose stimulated insulin secretion 194
6.2.4 Growth vs. insulin secretion 196
6.2.5 Model 198
6.3 Further work 201
6.4 Impact of findings 203

References 206
Publications 225

List of Tables

Table 1 Receptor binding affinities of some commonly prescribed first and second-generation antipsychotic drugs. 24
Table 2 International Diabetes Federation metabolic syndrome definition .. 27
Table 3 ADA criteria for a diagnosis of diabetes 28
Table 4 Heat map of some commonly prescribed antipsychotic drugs ranked according to associated risk of change in weight, BMI, lipids and glucose 31
Table 5 Structure and pharmacological targets of drugs used in this study 69
Table 6 List of drugs used to stimulate or inhibit insulin secretion in the current study 72
Table 7 Composition of RIPA buffer used to prepare whole cell protein extracts 75
Table 8 composition of 10% hand cast gels 77
Table 9 Tris glycine SDS running buffer composition used for SDS PAGE .. 77
Table 10 Tris glycine transfer buffer composition used for semi-dry transfer 78
Table 11 Wash buffer composition 78
Table 12 Rotor gene Q cycling conditions for qRT-PCR 81
Table 13 qRT-PCR forward and reverse primer sequence for specified genes 81
Table 14 Genes tested in the GeNorm analysis to establish the most stable reference genes for qRT PCR 82
Table 15 Summary of *In vitro* insulin secretion studies: Olanzapine 91
Table 16 Summary of *In Vitro* insulin secretion studies: Clozapine 92

List of figures

Figure 1 Illustration depicting three side effects of olanzapine; weight gain, dyslipidaemia and hyperglycaemia and how these are linked 33

Figure 2 Illustration depicting the proposed mechanisms involved in olanzapine induced weight gain. 36

Figure 3 Illustration depicting olanzapine effect to increase SREBP and induce hepatic *de novo* lipogenesis and increase plasma triglycerides. 39

Figure 4 Illustration depicting the organs and hormones involved in glucose homeostasis. 48

Figure 5 Simplified illustration of insulin signalling in insulin sensitive cells. 51

Figure 6 Simplified illustration of insulin signalling at hepatocytes 52

Figure 7 Simplified illustration of Glucose stimulated insulin secretion from beta cells 58

Figure 8 Illustration depicting the 5 stages of beta cell compensation 59

Figure 9 Illustration depicting the known effects of olanzapine at metabolic organs and the unknown effects on pancreatic beta cell function 64

Figure 10 Infographic depicting the cell treatment times and extraction protocols. 68

Figure 11 GeNorm analysis of 9 reference genes to establish the most stable reference gene within the experimental model. 84

Figure 12 Illustration depicting the first hypothesis 88

Figure 13 Illustration depicting the second hypothesis that olanzapine inhibits beta cell compensation mechanisms 90

Figure 14 Illustration depicting the third hypothesis 93

Figure 15 Effect of 24 and 72 hours olanzapine exposure on MIN6 cell viability. 95

Figure 16 Effects of 72 hours treatment with olanzapine on MIN6 cell death 96

Figure 17 Effects of olanzapine on insulin secretion and content in MIN6 cells 98

Figure 18 Effects of olanzapine on proliferation in different culture conditions. 100

Figure 19 Effects of olanzapine on cell number after 72 hours incubation in different glucose concentrations 101
Figure 20 Effects of different concentrations of olanzapine on proliferation in unstimulated conditions 102
Figure 21 Effects of olanzapine on MIN6 cell cycle 104
Figure 22 Schematic diagram showing some key regulators of cell cycle progression through G1 112
Figure 23 schematic diagram of the canonical Wnt pathway with or without Wnt stimulation 117
Figure 24 Effect of 24 hour olanzapine on cell cycle gene expression 119
Figure 25 Effect of 48 hour olanzapine on gene expression 119
Figure 26 Schematic diagram showing Beta catenin nuclear translocation and formation of a bipartite transcription factor with TCF7L2 to upregulate Cyclin D1 and c-myc 120
Figure 27 Effect of 24 hour olanzapine on beta catenin cytoplasmic and nuclear concentration 122
Figure 28 Effect of 72 hour olanzapine on beta catenin cytoplasmic and nuclear localisation 123
Figure 29 Effect of 72 hour olanzapine on nuclear TCF7L2 protein concentration of MIN6 cells 124
Figure 30 Schematic diagram showing pharmacological inhibitors and activators of beta catenin signalling. 126
Figure 31 Effects of olanzapine and Wnt inhibitor KYA1797K on MTT absorbance in MIN6 cells 128
Figure 32 Effects of olanzapine and Wnt inhibitor XAV939 on MTT absorbance in MIN6 cells 130
Figure 33 Effects of olanzapine and metformin on MTT absorbance in MIN6 cells 132
Figure 34 Effect of Wnt signalling inhibitors on olanzapine induced changes in Cyclin D1 gene expression 134
Figure 35 Effect of Wnt inhibitor XAV939 and activator BIO on olanzapine induced changes in c-myc gene expression 135
Figure 36 Effect of Wnt signalling modulators and olanzapine on AXIN2 protein concentration 137

Figure 37 Effect of Wnt signalling modulators and olanzapine on AXIN2 mRNA expression 138
Figure 38 Effect of olanzapine on Tankyrase and AXIN2 protein concentration over time 140
Figure 39 Effect of olanzapine on Axin2 mRNA expression over time 141
Figure 40 Schematic diagram showing the possible mechanisms for olanzapine to increase beta catenin protein concentration 144
Figure 41 Schematic diagram of XAV939 effect to inhibit the canonical Wnt signalling pathway. 146
Figure 42 Schematic diagram demonstrating the regulation and function of beta catenin in beta cells. 156
Figure 43 investigating the effect of glucose on cytoplasmic beta catenin in in MIN6 cells pre- treated with olanzapine. 160
Figure 44 Illustration of the effect of BIO to inhibit the beta catenin destruction complex, reduce proteasomal degradation and increase concentration of beta catenin 161
Figure 45 investigating the effect of BIO on beta catenin concentration and location in MIN6 cells pre- treated with olanzapine 162
Figure 46 investigating the effect of BIO on glucose stimulated insulin secretion from MIN6 cells pre- treated with olanzapine for 72 hours..... 163
Figure 47 investigating the effect of stimulatory drugs on insulin secretion from MIN6 cells pre- treated with olanzapine for 72 hours..... 167
Figure 48 Illustration depicting the mechanism of action of Tolbutamide, KCl and nifedipine to stimulate and inhibit insulin secretion. 164
Figure 49 investigating the effect of inhibitory drugs on insulin secretion from MIN6 cells pre- treated with olanzapine for 72 hours 165
Figure 50 investigating the effect of GLP1 receptor agonist exenatide on insulin secretion from MIN6 cells pre- treated with olanzapine for 72 hours 169
Figure 51 investigating the effect of forskolin and PMA on insulin secretion from MIN6 cells pre- treated with olanzapine for 72 hours..... 170
Figure 52 Schematic diagram showing active tyrosine 216/279 phosphorylated GSK3 as a component of the beta catenin destruction complex and serine 9/21 phosphorylated inactive GSK3 that does not interact with beta catenin. 171

Figure 53 investigating the effect of glucose and BIO on active GSK3 in MIN6 cells pre- treated with olanzapine for 72 hours 173
Figure 54 Investigating the effect of glucose and BIO on Axin2 concentration in MIN6 cells pre- treated with olanzapine for 72 hours 175
Figure 55 Investigating the effect of glucose on PKA-C concentration in MIN6 cells pre- treated with olanzapine for 72 hours 176
Figure 56 investigating the addition of Wnt antagonist XAV939 to olanzapine incubation for 72 hours and the effect on insulin secretion 178
Figure 57 Illustration depicting the effect of olanzapine to increase axin2 concentration and reduce free bet catenin 183
Figure 58 Illustration depicting the observed effects of olanzapine 197
Figure 59 Illustration of the proposed mechanism through which olanzapine exerts its effects on beta cell function 200

Abbreviations

α	Alpha (receptor)
β-TrCP E3	Beta-transducin repeats-containing protein E3
μM	Micromolar
3t3-L1	Adipose cell line derived from 3T3 cells
5HT	5- hydroxytryptamine, serotonin
ADP	Adenosine diphosphate
AKT	Protein Kinase B
AMPK	5' adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
AO	Acridine orange
APC	Adenomatous polyposis coli
APS	Ammonium persulphate
ATP	Adenosine triphosphate
AUC	Area under the curve
BIO	6-bromoindirubin-3'-oxime
BMI	Body mass index
BSA	Bovine serum albumin
C/EBPβ	CCAAT-enhancer binding protein β
C57BL/6	Inbred mouse strain (C57 black 6)
CA^{2+}	Calcium ion
cAMP	3',5'-cyclic adenosine monophosphate
CATIE	Clinical Antipsychotic Trials of Intervention Effectiveness
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
CHO	Chinese hamster ovary cells
CK1α	Casein kinase 1α
c-myc	Cellular - Myelocytomatosis

CNS	Central Nervous system
CO_2	Carbon dioxide
CREB	cAMP response element-binding protein
Ct	Cycle Threshold
CVD	Cardiovascular disease
D	Dopamine (receptor)
DAPI	4′,6-diamidino-2-phenylindole
DKA	Diabetic ketoacidosis
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribose nucleoside triphosphate
DTT	Dithiothreitol
Dvl	Dishevelled
E2F	E2 promoter binding Factor
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
eIF2α	Eukaryotic Initiation Factor 2α
ELISA	Enzyme linked immunosorbent assay
EPSE	Extrapyramidal side effects
ER	Endoplasmic reticulum
ERK	Extracellular signal–regulated kinase
EUFEST	European First-episode schizophrenia trial
FBS	Foetal bovine serum
FFA	Free fatty acid
FGA	First generation antipsychotic
FOXO-1	Forkhead box protein O1
G0	Gap 0 phase
G1	Gap 1 phase

G2	Gap 2 phase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCk	Glucokinase
GLP-1	Glucagon-like peptide 1
GLUT2	Glucose transporter 2
GLUT4	Glucose transporter 4
GPCR	G protein-coupled receptors
GSIS	Glucose stimulated insulin secretion
GSK3	Glycogen synthase kinase 3
GSLC	Glioblastoma multiforme stem-like cells
H	Histamine (receptor)
H_20	Water
HbA1C	Glycated haemoglobin
HCl	Hydrochloric acid
HDL	High-density lipoprotein (cholesterol)
HGO	Hepatic glucose output
HOMA-IR	Homeostatic model assessment for insulin resistance
Hr	Hour
HRP	Horseradish Peroxidase
IDF	International Diabetes Federation
IGF-1	Insulin-like growth factor 1
INS-1	Rat insulinoma 1 cell line
IR	Insulin receptors
IRS	Insulin receptor substrate
IV	Intravenous
IVGTT	IV glucose tolerance test
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
K_{ATP}	ATP-sensitive potassium channel

KCl	Potassium Chloride
kg	Kilogram
L6 myotube	Rat skeletal muscle cell line
LAI	Long acting injections
LDL	Low-density lipoprotein
LEF-1	Lymphoid enhancer-binding factor 1
LiCl	Lithium Chloride
LIRKO	Liver-specific insulin receptor knockout
LoxP	Locus of X-over P1
LRP5/6	Low-density lipoprotein receptor-related protein 5/6
M	Muscarinic (receptor)
MAPK	Mitogen-activated protein kinase
MDD	Major depressive disorder
MetS	Metabolic syndrome
MIN6	Mouse insulinoma 6 cell line
MIRKO	Muscle- specific insulin receptor knockout
ml	Millilitres
mM	Millimolar
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium Chloride
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NEFA	Non-esterified fatty acids
nM	Nanomolar
OGTT	Oral glucose tolerance test
OLZ	Olanzapine
PAGE	Polyacrylamide gel electrophoresis

PBS	Phosphate-buffered saline
PC12	Adrenal pheochromocytoma cell line
PCR	Polymerase chain reaction
PERK	Protein kinase RNA–like endoplasmic reticulum kinase.
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PIC	Protease inhibitor cocktail
PitX	Paired-like homeodomain
PKA	protein kinase A
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PPAR-γ	Peroxisome proliferator-activated receptor-γ
PTSD	Post-traumatic Stress disorder
qRT-PCR	Quantitative real-time polymerase chain reaction
Raf	Rapidly Accelerated Fibrosarcoma
Ras	Rat sarcoma
Rb	Retinoblastoma protein
RCF	Relative centrifugal force
RGS	Regulators of G protein signalling
RIPA	Radioimmunoprecipitation assay buffer
RIP-Cre	Rat insulin promoter - Cre
RNA	Ribonucleic acid
RRP	Readily releasable pool
S	S phase
SDS	Sodium dodecyl sulphate
SEM	Standard error of the Mean
Ser	Serine
SERT	Serotonin transporter
SGA	Second generation antipsychotic

siRNA	Small interfering RNA
SMI	Severe mental illness
SNARE	Soluble NSF attachment proteins receptor
SNP	Single-nucleotide polymorphism
SREBP	Sterol regulatory element binding protein
SV40	Simian vacuolating virus 40
TCA	Tricarboxylic acid
TCF-4	Transcription factor 4 (also known as TCF7L2)
TCF7L2	Transcription factor 7-like 2 (also known as TCF4)
TEMED	Tetramethylethylenediamine
Tg	Triglyceride
TMB	3,3′,5,5′-Tetramethylbenzidine
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
TWEEN	Polysorbate 20
Tyr	Tyrosine
UK	United Kingdom
UPR	Unfolded Protein response
VDCC	Voltage dependent calcium channel
VLDL	Very low density lipoprotein
WHO	World Health organisation
Wnt	Wingless-related integration site

Acknowledgements

I would like to express my deepest gratitude toward my supervisors and mentors. Thank you for all your help guiding me through the PhD wilderness, keeping me on track, and offering encouragement and support along the way.

Dr. Greg Scutt

Dr. Wendy Macfarlane

Professor Adrian Bone

Dr. Mark Yeoman

Dr. Sandeep Kumar

I would like to pay special regards to Wendy and all the members of the Diabetes Research group. Thank you for always offering support, motivation, and a good sense of humour, I would have been lost without our Monday meetings.

I would also like to thank my amazing husband Liam for his never ending confidence in me. Thank you for always knowing when I needed a phone call to keep me going during long days in the lab, a glass of wine in the evening, a swim in the sea, or a night away in the campervan. Without your support this thesis would not have been possible.

Author’s declaration

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. The thesis has not been previously submitted to this or any other university for a degree, and does not incorporate any material already submitted for a degree.

Signed

Dated

Chapter 1: Introduction

1.1 Part 1: Severe mental illness and increased mortality

1.1.1 Severe mental illness

Patients with severe mental illness (SMI) are at high risk of poor physical health, and have a significantly reduced life expectancy [1]. The lifetime mortality risk for SMI is worse than that for heavy smoking, and patients with SMI die on average 15-20 years earlier than the general population [2]. Lifestyle, healthcare, genetics, and medication all contribute to premature mortality through development of long term conditions such as diabetes and cardiovascular disease (CVD) [3].

SMI is a term used to describe disorders that produce psychotic symptoms, and usually involves a diagnosis of schizophrenia, bipolar disorder, or major depressive disorder [4]. Almost all patients with a diagnosis of SMI are prescribed medication to manage symptoms, and the prevalence of type 2 diabetes is significantly higher in patients taking antipsychotic drugs [5]. It is thought that antipsychotic treatment is a central factor in the overall development of diabetes and CVD among patients, and there is a growing evidence base describing how some of the most commonly prescribed antipsychotic drugs cause rapid increases in weight, and hyperglycaemia [6, 7]. But there have been very few recent advances in treatment options, and in the absence of new antipsychotic agents, a thorough understanding of the drugs used presently might expose new approaches to improve current practice.

1.1.1.1 Schizophrenia

Symptoms of schizophrenia include positive symptoms of hallucinations, delusions and disordered thoughts, as well as negative symptoms such as social withdrawal and lack of motivation. While the lifetime prevalence is approximately 1%, the World Health Organisation (WHO) has highlighted the substantial burden of disease due to the young age of onset, low rates of remission and significant disability to patients [8]. WHO also emphasise that incidence is increasing worldwide [8], but with no cure available the aim of treatment is to reduce the frequency and severity of symptoms.

First line treatment involves oral antipsychotic medication in conjunction with psychological intervention [9].

1.1.1.2 Bipolar disorder

Bipolar disorder is characterised by recurrent periods of mania, involving abnormally elevated mood, disinhibited behaviour or irritability, and periods of depressed mood. The lifetime prevalence is approximately 1.4% [10], and associated disability is ranked higher than many common diseases such as asthma in people 15-44 years of age [11]. For the treatment of mania, first line options include haloperidol, olanzapine, quetiapine or risperidone [12]. And for the treatment of bipolar depression, a combination of fluoxetine and olanzapine, or quetiapine monotherapy is recommended [12].

1.1.1.3 Major depressive disorder

Major depressive disorder (MDD) is also classified under the SMI umbrella, and comprises symptoms of severe depression such as changes in sleep, appetite, concentration and motivation, often accompanying suicidal thoughts. Prevalence has been estimated to be between 2% and 10%, and MDD is a major public health issue due to impaired quality of life and burden on the community through loss of productivity [13]. Treatment recommendation for mild to moderate depression is monotherapy with an antidepressant drug in conjunction with psychotherapy. First line treatment options for MDD often includes the addition of an antipsychotic drug if psychotic symptoms are also present [14].

1.1.2 Antipsychotic treatment

1.1.2.1 History

The first antipsychotic medication, chlorpromazine, was developed in the 1950s for the treatment of acute and chronic psychosis. The discovery of chlorpromazine transformed psychiatric care [15] leading to further research and rapid development of many more antipsychotic drugs. Since then, pharmacological agents have

become dominant in the management of schizophrenia and today there are numerous different antipsychotic medications in use around the world. In the UK there are currently 24 antipsychotic drugs licensed for use [16]; this includes first generation agents such as haloperidol and flupentixol, and the newer second generation agents such as olanzapine, quetiapine and risperidone. More recently antipsychotic drugs have been found to be effective at managing conditions such as bipolar disorder [17], psychotic depression [18] and obsessive compulsive disorder [19], consequently prescribing has increased markedly over the past few decades [20]. However, there are numerous side effects associated with antipsychotic treatment, ranging from neurological, metabolic, cardiovascular, gastrointestinal, haematological, or musculoskeletal [21]. Hence treatment can significantly impact the physical health of patients, and the choice of drug is balanced against acceptable side effects.

1.1.2.2 First generation antipsychotics

The pharmacological mechanisms of action of the first-generation antipsychotics (FGA) helped to formulate concepts regarding the physiology of schizophrenia. The D_2 dopamine receptor was identified as a key target as antipsychotic potency directly corresponded to activity at these receptors [22]. Based on this observation the dopamine hypothesis of schizophrenia was established [23], which advocated that psychosis is caused by over-activity within the dopamine signal transduction pathways, and further FGA drugs were developed based on this theory [24]. However D_2 receptors in the nigrostriatal pathway are involved in control of movement, and it was soon clear that D_2 antagonism can cause debilitating extrapyramidal side effects (EPSE) such as Parkinsonism and tardive dyskinesia, which are common side effects of the FGA [25].

1.1.2.3 Second generation antipsychotics

Second generation antipsychotics (SGA) were developed to help reduce the problematic EPSE associated with FGA. Through simultaneous antagonism at serotonin 5-HT_{2A} receptors SGA can increase dopaminergic transmission in the nigrostriatal pathway which reduces EPSE associated with D_2 antagonism. Most

SGA possess more potent binding affinities for 5-HT receptors than for D_2 receptors. In the presence of dopamine, SGA also quickly dissociate from the D_2 receptors resulting in overall fewer dopaminergic side effects.

As demonstrated in **Table 1** antipsychotics commonly possess other complex binding profiles with activity at a variety of additional receptors including dopamine (D_1, D_3 and D_4) and 5-HT ($5\text{-}HT_{1A}$, $5\text{-}HT_2$, $5\text{-}HT_6$ and $5\text{-}HT_7$) as well as at histamine (H_1), muscarinic (M_1, M_2, M_3, M_4 and M_5) and adrenergic (α_1 and α_2) [25-27]. Despite the improvements regarding EPSE, the complex binding profile of SGA have been linked to many other adverse events such as weight gain, metabolic dysfunction, hyperprolactinemia, hyperlipidaemia, diabetes, cardiac arrhythmias, agranulocytosis, and sexual dysfunction [28].

	Receptor Ki (nM)							
Drug	**D_1**	**D_2**	**$5HT_{2A}$**	**$5HT_{2C}$**	**H_1**	**M (1-5)**	**α_1**	**α_2**
Haloperidol	270	1.4	25	>5000	727	4669	19	>5000
Aripiprazole	1960	0.74	8.7	76	25	6780	26	74
Olanzapine	250	17	1.9	7.1	3.5	26	60	170
Clozapine	540	150	3.3	13	2.1	34	23	160
Quetiapine	4240	310	120	3820	19	1020	58	1000
Risperidone	620	3.3	0.16	63	2.6	>5000	2.3	7.5

Table 1 Receptor binding affinities of some commonly prescribed first and second-generation antipsychotic drugs. Data are in Ki (nM), the lower the Ki, the greater the affinity for the receptor. Modified from Kusumi et al. (2015) [27]

1.1.2.4 Antipsychotic prescribing statistics

The prescribing of antipsychotic drugs increased markedly over the last decade, although it is not clear if this is due to reduction in more harmful classes of drugs such as benzodiazepines, an increase in SMI diagnosis, or increasing population size [20].

The Clinical Antipsychotic Trials of Intervention Effectiveness (CATIE) was a large multicentre double blind trial comparing some second generation antipsychotic drugs to a first generation drug, perphenazine, aiming to establish relative effectiveness. It is a widely regarded trial which enhanced the available knowledge of antipsychotic medication to help guide future treatment choices. The trial indicated that, with the exception of clozapine, no drug is superior to others in treatment of schizophrenia, and there is no clinical difference between second and first generation agents [29, 30]. Despite this; the prescribing of second generation antipsychotic medication prevails, and the most commonly prescribed antipsychotics in 2010 were second generation drugs olanzapine (24%), quetiapine (23%) and risperidone (17%) [18]. Clozapine is often regarded as the "gold standard" antipsychotic, utilised at times of treatment resistant schizophrenia when other antipsychotic drugs have failed. It is highly efficacious but carries high risks of severe side effects such as agranulocytosis that requires intensive clinical monitoring [29, 31].

Since the advent of second generation antipsychotic drugs, there has also been increased "off label" prescribing of these agents in the treatment of non-psychotic disorders such as sleep disorders, PTSD and dementia, as well as increased use in children and adolescents [32]. When evaluating antipsychotic prescribing trends in primary care, it was estimated that more than half of those treated with SGA have no record of SMI [18]. Considering the potential side effects and risks of these drugs; widespread prescribing is a growing cause for concern, and may have harmful consequences for the health of the overall population, and not only those affected by SMI [33].

1.1.3 SMI increases cardiovascular risk

The reduced life expectancy of patients with SMI is chiefly attributed to cardiovascular disease (CVD). There is also increased risk of other medical illnesses such as respiratory tract disease or viral infection, as well as increased self-harm and suicide, yet CVD accounts for approximately 75% of deaths [34]. There are numerous complex and conflicting factors that increase the risk of CVD in patients, hence investigating the correlation between SMI and CVD is challenging.

The most prominent modifiable risk factors for CVD are hypertension, high cholesterol, glucose intolerance, obesity, smoking, and inactivity. Unhealthy diet and lifestyle behaviours such as smoking and high alcohol consumption often accompany SMI [35] and obesity and glucose intolerance are also very common [36] suggesting that lifestyle factors associated with SMI may significantly increase CVD risk. There is also a possible underlying genetic predisposition associated with SMI that promotes a state of metabolic dysregulation [37]. Furthermore, there is evidence that many second generation antipsychotics can directly increase incidence of several risk factors for CVD such as obesity, dyslipidaemia and glucose intolerance [38], therefore medication is likely to also play a dominant role.

1.1.3.1 Metabolic dysfunction

The strong link between SMI and CVD has led to increased focus on metabolic health of psychiatric patients. To aid clinicians in identifying patients who are at high risk of CVD, the concept of the metabolic syndrome (MetS) was introduced [3]. MetS is a term used to describe a cluster of metabolic abnormalities; abdominal obesity, hypertension, elevated triglycerides, and hyperglycaemia. MetS has also been termed the “insulin resistance syndrome”, as insulin resistance is central to the pathogenesis of MetS and the resultant associated CVD risks [39].

1.1.3.1.1 Definition

The definition of MetS as defined by the International diabetes federation (IDF) is listed in Table 2 [40]. Key features of the MetS include increased abdominal fat distribution, insulin resistance, dyslipidaemia, and hypertension. A diagnosis is made if waist circumference is above the designated threshold (based on sex and ethnicity) alongside two other abnormal results.

Central obesity **(intra-abdominal fat deposition)**	Waist circumference (dependent on ethnicity) Male ≥94 cm female ≥80 cm plus any two of the following
Raised triglycerides	≥1.7 mmol/l
Low HDL cholesterol	Male <1.03 mmol/l Female <1.29 mmol/l
Raised blood pressure	Systolic ≥130 mmHg or diastolic ≥85 mmHg
Raised fasting plasma glucose	≥5.6 mmol/l

Table 2 International Diabetes Federation metabolic syndrome definition [40]

Sedentary lifestyle, unhealthy diet, excessive alcohol, and smoking are all associated with development of MetS, usually through initiation of obesity and inflammation. Weight gain, specifically visceral fat accumulation, leads to expanded adipose tissue mass which increases circulating free fatty acids (FFA). Increased circulating FFA inhibits insulin mediated glucose uptake into muscle tissue, leading to elevated plasma glucose and hyperinsulaemia [41]. Lipolysis of visceral fat supplies more FFA to the liver than subcutaneous fat, through the splanchnic circulation[42] [43]. At the liver, FFA increases hepatic glucose output, and increase secretion of very low density lipoproteins (VLDL) and triglycerides. Adiponectin is an anti-inflammatory and anti-atherogenic hormone, and increased adipose tissue mass reduces circulating adiponectin and increases leptin which is also directly correlated to CVD risk [41].

It is estimated that approximately 1 in 3 of patients with established SMI suffer from MetS, and as an initial step in the ensuing metabolic decline, this is a major concern in the field of psychiatric medicine [36]. There is growing evidence that commonly prescribed second generation antipsychotic drugs cause side effects that instigate or add to the development of metabolic dysfunction and MetS, which increases CVD risk [36, 38, 44].

1.1.3.2 Diabetes

The MetS is also a significant risk factor in the development of type 2 diabetes, which occurs when the pancreatic beta cells fail to secrete sufficient insulin to maintain euglycaemia. Hyperglycaemia is a hallmark of emerging diabetes, and is intricately correlated to MetS, but can also occur independently of MetS through failure of beta cells secondary to obesity, Insulin resistance, unhealthy diet, or beta cell toxicity. In the case of insulin resistance, the development of diabetes is a slow progression. Initially an increase in plasma glucose puts added pressure on the pancreatic beta cells to secrete extra insulin. Beta cell compensation occurs through increased beta cell mass, and increased insulin output [45], but if hyperglycaemia persists, beta cell compensatory mechanisms are eventually exhausted and diabetes ensues upon beta cell dysfunction [46, 47]. Dyslipidaemia and increased FFA, which are consistent features of the MetS, also have a negative impact on beta cell function, and LDL cholesterol has cytotoxic effects to beta cells [48], consequently reducing insulin secretion [49].

1.1.3.2.1 Definition

The definition of diabetes as defined by the American Diabetes Association [50] is listed in **Table 3**. Diabetes is characterised by chronic hyperglycaemia and if a patient meets any of the criteria a diagnosis of diabetes is made.

HbA1c	≥6.5%
Fasting plasma glucose	≥7 mmol/l
Oral glucose tolerance test	≥11.1 mmol/l
Random plasma glucose	≥11.1 mmol/l

Table 3 ADA criteria for a diagnosis of diabetes [50]

In a recent meta-analysis investigating rates of diabetes among patients with established SMI it was found that approximately 1 in 10 patients had a diagnosis of type 2 diabetes [5], at least 1 in 5 have significant hyperglycaemia [36], and the

overall risk of developing type 2 diabetes was at least double that of the general population [5]. Moreover, the rates of undiagnosed diabetes or hyperglycaemia are thought to be higher than this statistic [51]. Chronic hyperglycaemia leads to many microvascular and macrovascular complications. Endothelial damage can occur in the kidneys, retina and small blood vessels causing diabetic nephropathy, neuropathy and retinopathy. Hyperglycaemia and insulin resistance promote coronary artery disease, peripheral arterial disease and stroke. Indeed, diabetes is an independent risk factor in the development of CVD [52], and CVD is one of the most common causes of death among those with a diagnosis of diabetes [53].

It is suggested that second generation antipsychotic drugs increase risk of diabetes through increasing insulin resistance [54] or through beta cell toxicity [55]. Severe hyperglycaemia has been observed in patients treated with antipsychotics, often rapidly at the onset of treatment [56, 57], suggesting a pharmacological influence of antipsychotics on glucose regulation. The pathophysiology of SMI and lifestyle factors may also have a role in the underlying glucose dysregulation, but the incipient view is that antipsychotic medication has a substantial effect on glucose homeostasis, and this may be independent of other effects on metabolic parameters [58-60]. Even small changes in glucose homeostasis can have long term consequences to cardiovascular health, thus addressing the root cause of glucose dysregulation in SMI is of high importance.

1.1.3.3 Clinical evidence

Assessing the involvement of antipsychotics in the development of MetS and diabetes is challenging as there are very few placebo controlled trials available. The pathophysiology of SMI may increase risk of metabolic dysfunction over time [61] irrespective of treatment, and differentiating between an underlying risk associated with SMI, and a true side effect of treatment is difficult. Nonetheless, a growing evidence base including case reports, prospective trials, and retrospective and cohort studies implicates antipsychotic drugs as a major risk factor in the development of MetS and diabetes [7, 36, 57, 62, 63].

In large scale meta-analyses, first episode and drug naïve patients have been demonstrated to have approximately 10% risk of MetS [44, 64]. But the rate of MetS reported in meta-analyses for those patients established on an antipsychotic drug is

approximately 35%, which is substantially higher than the reported baseline statistics [36]. Rates of MetS have been proven to be higher for those on long term treatment [3, 36], and the leading predictor of risk is the choice of medication, as those taking certain antipsychotics, such as clozapine or olanzapine have significantly greater risk than other antipsychotics [36, 44]. This evidence indicates that the cumulative effect of antipsychotic treatment alongside SMI pathophysiology and unhealthy lifestyle can increase metabolic dysfunction over time.

In large scale meta analyses rates of hyperglycaemia and diabetes in first episode and unmedicated SMI patients are reported to be 6.4% and 2.1% respectively [44, 64]. Whereas rates of hyperglycaemia and diabetes in patients established on antipsychotic treatment are 27.8% and 12.8% respectively [64]. In addition to numerous case reports of treatment emergent hyperglycaemia, worsening diabetes, and in severe cases, diabetic ketoacidosis (DKA) [65-68] this convincing evidence suggests that antipsychotic treatment has a significant impact on glucose homeostasis and diabetes. However, whether this is secondary to increases in rates of MetS, or a direct causation is not clear.

Different antipsychotic medications have been shown to have markedly different effects on metabolic parameters [44]. In a recent network meta-analysis comparing 18 antipsychotic drugs, it was shown that antipsychotics have a wide range of effects, and that olanzapine and clozapine are associated with the greatest decline in metabolic function [38]. Reports of hyperglycaemia, lipid dysregulation, and ketoacidosis are more common with olanzapine and clozapine than other antipsychotics, and olanzapine and clozapine are also most strongly associated with the highest rates of diabetes [5].

1.1.3.3.1 Antipsychotic risk table

Table 4 shows the relative risk of changes in different metabolic parameters associated with some common antipsychotics [38]. Results from clinical studies and animal studies most frequently pinpoint olanzapine and clozapine as the antipsychotics with the highest risk for MetS and diabetes.

Drug	Weight	BMI	Triglycerides	HDL cholesterol	Glucose
Haloperidol	0.1	0.08	0.63		0.59
Aripiprazole	0.26	0.11	0.33	0.26	0.55
Flupentixol	0.44				
Risperidone	0.58	0.56	0.39	0.51	0.46
Quetiapine	0.65	0.68	0.71	0.59	0.47
Sertindole	0.81	0.72	0.29		0.36
Clozapine	0.90	0.85	0.97		0.97
Olanzapine	0.92	0.93	0.83	0.76	0.67

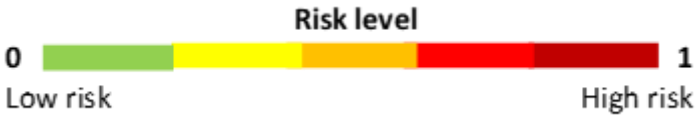


Table 4 Heat map of some commonly prescribed antipsychotic drugs ranked according to associated risk of change in weight, BMI, lipids and glucose. Data are P-score which ranks the antipsychotic on the basis of degree of metabolic dysregulation. P-score ranges from 0 -1 where 0 is low risk and a higher P-score indicates higher risk. Modified from Pillinger at al. (2020) [38]

1.1.3.4 Animal models

There are many conflicting factors that can affect the results of clinical studies investigating antipsychotic induced metabolic dysfunction and diabetes, especially in patients with a long history of mental illness or those receiving a combination of drugs. High rates of MetS in patients probably transpire as a result of numerous mechanisms, and in the event of severe metabolic side effects treatment may be stopped or changed. Thus it is difficult to make assumptions regarding comparable risk between antipsychotics based on clinical studies. The use of animal studies to investigate the effects of antipsychotic drugs on different metabolic parameters has been utilised to ensure other contributing factors can be controlled.

There have been many rodent studies investigating antipsychotic induced changes in weight, insulin resistance, glucose and dyslipidaemia. While the effects of antipsychotic drugs on glucose regulation in rodents is similar to that observed in humans, the effect on weight and lipids has proven to be inconsistent [58]. Despite the suggestion that olanzapine and clozapine are associated with the greatest

weight gain, many rat models do not show any weight gain associated with olanzapine or clozapine [58]. There are distinct differences between female and male rats, which are not analogous to the clinical studies which consistently show increased body weight regardless of sex. Several studies have suggested that the changes in glucose regulation instigated by antipsychotics in rodents occur independently of weight gain [58, 69, 70]. This suggests that there may be a direct effect of antipsychotics to directly increase risk of hyperglycaemia and diabetes, and not just a secondary effect of increased obesity which has been often assumed.

1.2 Part 2: Current evidence and proposed mechanism for olanzapine induced hyperglycaemia

Olanzapine is one of the most frequently prescribed antipsychotic drugs for treatment of SMI [18], yet it is more commonly associated with diabetes than other first line drugs [38]. Clozapine has also been highlighted to be high risk for metabolic complications, but clozapine is used in specialist treatment programmes with intensive monitoring, and is reserved for treatment resistant schizophrenia where the risk: benefit ratio may be skewed. On the other hand olanzapine is commonly prescribed as a first line treatment option in SMI, where there may be less frequent patient contact, and hyperglycaemia can go unnoticed for longer.

The most prominent side effects of olanzapine include weight gain, dyslipidaemia and hyperglycaemia [38]. While weight gain and dyslipidaemia are primary risk factors for MetS and CVD, here we reflect on the diabetogenic risk associated with these side effects. Both increased body weight and elevated circulating triglycerides promote a state of insulin resistance and hyperglycaemia (**Figure 1**). There is also evidence that olanzapine can pharmacologically alter peripheral insulin sensitivity, to increase risk of hyperglycaemia and diabetes. However, diabetes is characterised by a deficiency of insulin, yet the pharmacological effect of olanzapine at the pancreatic beta cells is less clear. In the face of increasing rates of diabetes in treated patients, we suggest that more research into the effect of olanzapine on beta

cell function is warranted. The next section will examine the current evidence describing the effect of olanzapine in organ systems involved in metabolic homeostasis, with a focus on propensity to cause hyperglycaemia.

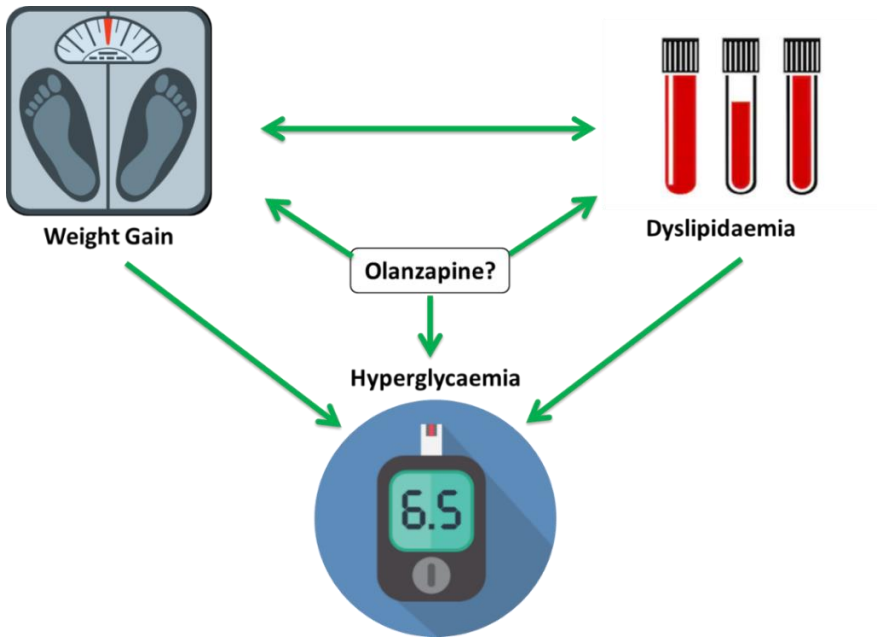


Figure 1 Illustration depicting three side effects of olanzapine; weight gain, dyslipidaemia and hyperglycaemia and how these are linked

1.2.1 Weight Gain

1.2.1.1 Clinical evidence

Central obesity is a key feature of the MetS, essential to meet the IDF diagnostic criteria [40]. Obesity is also a primary risk factor for insulin resistance and type 2 diabetes. Compared with the general population, early stage schizophrenia and bipolar disorder significantly increase risk of overweight or obesity [7, 34] and approximately 1 in 2 patients with SMI are overweight [36]. Weight gain is a well-known side effect of many antipsychotic drugs, and in a large network meta-analysis comparing 9 antipsychotics; Clozapine, olanzapine, sertindole, quetiapine, paliperidone, risperidone and lurasidone were all shown to significantly increase BMI. The meta-analysis found no evidence for BMI changes with haloperidol, or aripiprazole, when compared to placebo [38]. Although other studies have pointed out that no antipsychotic is body weight neutral, and all treatment is associated with

some increase in body weight over time [71]. Olanzapine is associated with the highest risk of severe weight gain, while haloperidol was considered the lowest risk [38].

The Clinical Antipsychotic Trials of Intervention Effectiveness (CATIE) trial found that 30% of patients treated with olanzapine gained over 7% of their baseline weight [72]. And weight gain in patients treated with olanzapine has been suggested to average approximately 4.15kg in the first 10 weeks of treatment, and up to 12kg in a year [73, 74]. There is also often an increase in subcutaneous and intra-abdominal fat deposition, which has been specifically linked to increased insulin resistance [75].

1.2.1.2 Animal models

Although olanzapine has been repeatedly shown to induce weight gain in clinical studies, in animal models the effect on weight is varying. One review of 29 studies only found evidence of weight gain in 16 studies, while nine showed no weight gain and four showed weight loss [58]. The difference possibly arises due to clear differences between the sexes, as in female rats olanzapine has been demonstrated to instigate rapid weight gain, which is reversible upon cessation of treatment [76], but male rats appear resistant to the side effect [77]. It is not clear why this discrepancy arises, and it is not reflective of clinical effects, where weight gain affects both males and females.

It is suggested that olanzapine induces changes in food preference, thus the diet administered by different groups could have a significant effect on results. Smith *et al*. (2011) demonstrated that olanzapine treated rats had a strong preference for high fat, high sugar diet, and rats administered high fat diet had significantly higher body fat percentage at the end of the 42 day study compared to vehicle, while rats fed a normal chow diet had significantly lower than control [78]. Many groups allow *ad libitum* feeding, while others control the diet, which may explain some of the disparity.

1.2.1.3 Mechanism

Many first generation antipsychotics that act primarily at D_2 receptors, such as haloperidol, are associated with very little weight gain, and it is the extensive binding profile of second generation antipsychotics that is thought to cause this side effect. The histamine 1 (H_1) receptor has most commonly been associated with olanzapine induced weight gain, as high H_1 affinity of an antipsychotic is closely correlated to weight gain propensity [79]. As shown in **Table 1** clozapine and olanzapine share a high affinity for the H_1 receptor [80] and they also cause the most extensive weight gain. The hypothalamic region of the central nervous system (CNS) has a high density of H_1 receptors, where histamine signalling is involved in food intake, and energy metabolism [81]. Several studies have determined that H_1 antagonism by olanzapine and clozapine is linked to increased food intake in the early stages of treatment resulting in acute weight gain, and reduced energy expenditure is responsible the long term effects on weight gain [82]. (**Figure 2**)

Olanzapine and clozapine also bind with high affinity to the serotonin 2C ($5HT_{2C}$) receptor (**Table 1**) and it has been suggested that the $5HT_{2C}$ receptor is also involved in antipsychotic induced weight gain [83]. Central $5HT_{2C}$ receptors are involved in appetite regulation [84], and Kirk *et al.* (2009) put forwards that $5HT_{2C}$ receptors may be more important than H_1 in the initial stages of olanzapine induced weight gain [85]. In a study by Lord *et al.* (2017) olanzapine was administered to female C57BL/6 mice for 6 weeks, which resulted in weight gain, increased fat mass, and glucose intolerance [83]. When investigating the food intake and energy expenditure, it was observed that hyperphagia was required for olanzapine induced weight gain [83]. Mice lacking the $5HT_{2C}$ receptor did not develop hyperphagia, weight gain or glucose intolerance, suggesting that it is through activity at this receptor that olanzapine increases appetite to increase weight gain [83]. (**Figure 2**)

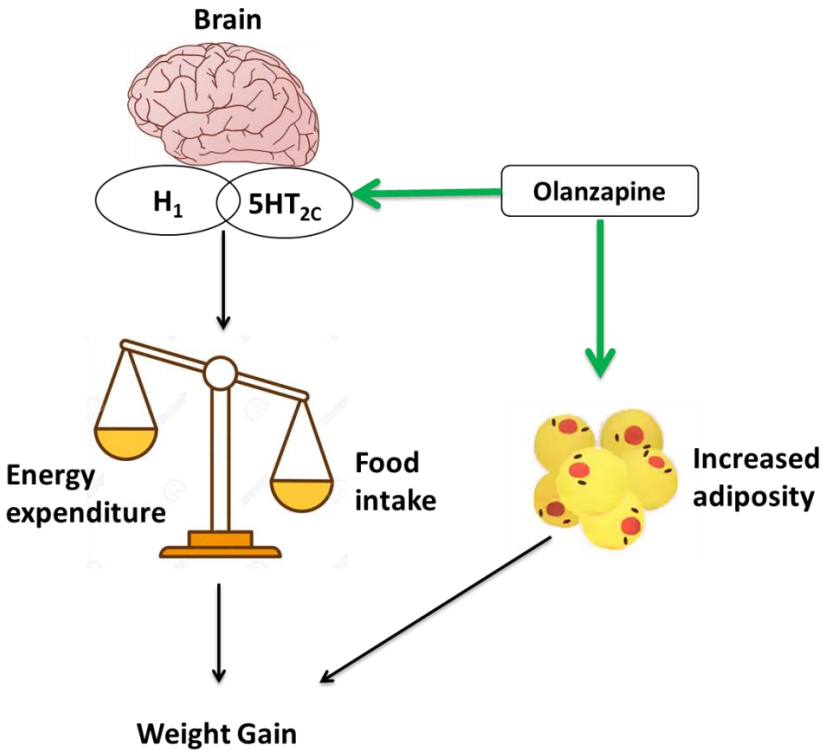


Figure 2 Illustration depicting the proposed mechanisms involved in olanzapine induced weight gain. Olanzapine acts at H_1 and 5-HT_{2C} receptors to disturb the balance between food intake and energy expenditure, and also acts to directly increase adiposity.

Adipose tissue is also a possible pharmacological target of olanzapine where the drug may directly increase adipose mass independently of increased food intake [77]. Lipids are stored (lipogenesis) or utilised (lipolysis) in adipocytes, and increased adiposity results from reduced lipolysis and increased lipogenesis [86]. It has been shown that adipocyte cell lines cultured with olanzapine have increased lipogenesis [87], and the lipolytic capacity is reduced [88]. Hence, olanzapine can increase adiposity and stimulate an increase in size of adipocytes [89]. Furthermore, olanzapine has been shown to upregulate peroxisome proliferator-activated receptor-γ (PPAR-γ) and CCAAT-enhancer binding protein β (C/EBPβ) in human and rodent pre-adipocytes which stimulates the progression from pre-adipocyte to adipocyte [88, 90] therefore increasing the overall adipose mass, and consequently increasing overall body mass.

1.2.2 Dyslipidaemia

1.2.2.1 Clinical evidence

Dyslipidaemia is a major risk factor for diabetes, but also may be a symptom of diabetes, and a treatable risk factor for ensuing CVD [91]. At least 2 in 5 patients with schizophrenia have been shown to have lipid abnormalities. Dyslipidaemia is often not present at onset of treatment, so it is thought that it is a direct effect of antipsychotic use [36].

Triglyceride production is usually regulated by non-esterified fatty acids (NEFA) which are secreted by adipose tissue into the circulation. At the liver NEFA stimulates triglyceride production as a source of energy. Insulin usually inhibits adipocyte NEFA secretion, and consequently inhibits hepatic secretion of triglycerides and VLDL, thus a lack of insulin or reduced insulin sensitivity associated with insulin resistance or diabetes can result in increased triglycerides [92].

Antipsychotic use has been shown to stimulate an increase in triglycerides. In a large network meta-analysis comparing 15 antipsychotic drugs, triglyceride concentration was shown to be increased with olanzapine, quetiapine, and clozapine [38]. Total cholesterol has also been shown to be increased with quetiapine, olanzapine and clozapine, with clozapine ranked as the worst on both counts [38].

There are numerous studies that highlight the extent that triglyceride concentration is elevated in patients treated with olanzapine [93-95]. In a large cohort analysis, patients prescribed olanzapine have been shown to have more than 3 fold risk of hyperlipidaemia compared with other antipsychotic agents, and over 5 fold increase in risk compared to untreated patients [96]. The effect on triglycerides is more pronounced than cholesterol, and in one study, following 16 months olanzapine treatment, total cholesterol, LDL and HDL were not shown to be altered, but average triglyceride concentration was shown to be significantly elevated (2.7 mmol/l) [93].

There is some disagreement whether hypertriglyceridaemia follows weight gain, or is an independent side effect of olanzapine. High triglycerides and low HDL cholesterol has been shown to be significantly more prevalent in olanzapine treated patients than unmedicated patients [97], or risperidone treated patients [98], but in

these studies, this was not correlated to a change in BMI. Conversely, in another study when correcting for changes in weight, it has been shown that increases in triglycerides are dependent on weight gain [95]. However there is a lack of quantitative data to confirm the link.

1.2.2.2 Animal models

The effect of olanzapine on lipids in animal models remains inconclusive, as some studies have detected no change in serum lipids, while others describe significant changes. The effect of olanzapine on body weight in female rats, and not male rats, has previously been demonstrated to involve increased appetite [76], hence the sex and diet of rodents used in the studies may delineate the differing results. In a study investigating if weight gain is the origin of dyslipidaemia, restricted feeding was used to reduce the rate of weight gain associated with treatment. Olanzapine was shown to cause a significant increase in triglycerides in both rats that gained weight, and control fed rats that did not [99]. Other serum lipids such as cholesterol were found to be unaffected by olanzapine. This suggests that olanzapine has a direct pharmacological effect to increase triglycerides unrelated to increases in body weight.

1.2.2.3 Mechanism

Plasma triglycerides are lipids that have been ingested in the diet, or secreted from hepatocytes in the form of VLDL particles. Adipose and muscle tissue containing lipoprotein lipase enzymes hydrolyse circulating triglycerides to generate FFA to be utilised for energy in the process of lipolysis, or to be stored in the process of lipogenesis. An increase in plasma triglycerides is likely to originate from increased hepatic secretion or reduced peripheral utilisation [100].

Using cultured cell lines [87, 88, 90, 101-103], and *in vivo* animal models [104-107] numerous studies have found that antipsychotic drugs directly interfere with lipid homeostasis to influence changes in triglycerides, through activation of sterol regulatory element binding protein (SREBP) transcription factors. Olanzapine upregulates activity of SREBP1c which is central to the regulation of fatty acid synthesis, and SREBP2 which regulates cholesterol metabolism [58]. SREBP

activation induces hepatic *de novo* lipogenesis and cholesterogenesis [108], and in female rats, olanzapine and other antipsychotics have been shown to upregulate SREBP target genes in both liver and adipose tissue to increase circulating triglycerides [106, 109]. (**Figure 3**)

It has been put forwards that the effect of olanzapine to increase SREBP activity may be downstream of AMP-activated protein kinase (AMPK). AMPK has central roles to regulate hepatic energy metabolism, and also has roles in glucose trafficking in the peripheral tissues. Olanzapine has been shown to reduce phosphorylation of AMPK in hepatocytes and 3t3-L1 adipocyte cells, resulting in enhanced SREBP dependent lipid synthesis [110, 111]. This suggests that the effect of olanzapine to increase triglycerides may be mediated through inhibition of AMPK.

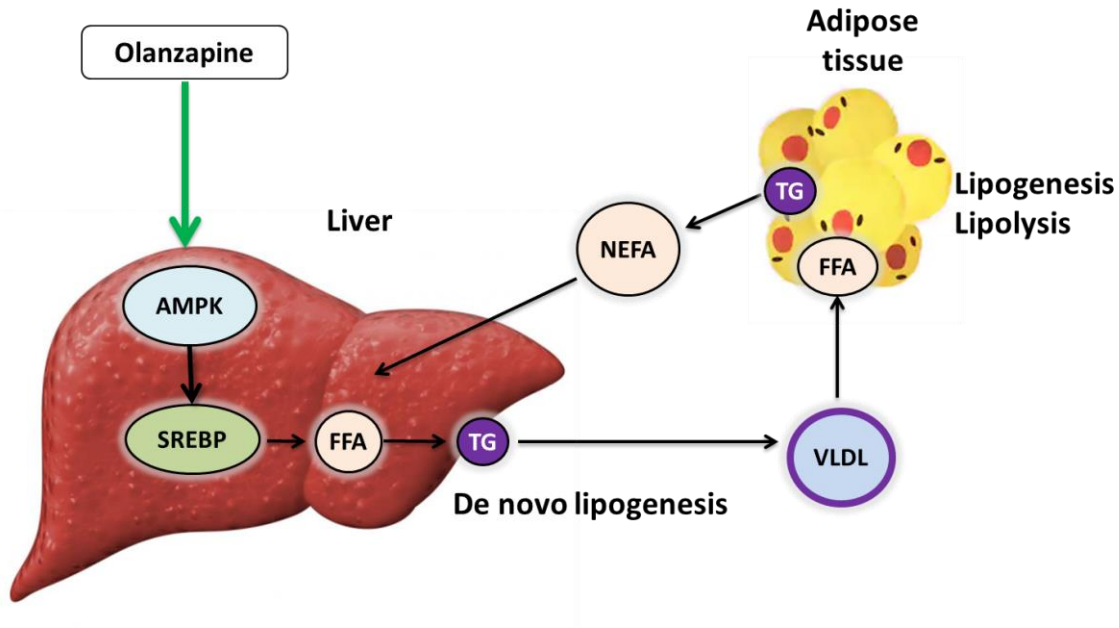


Figure 3 Illustration depicting olanzapine effect to increase SREBP and induce hepatic *de novo* lipogenesis and increase plasma triglycerides.

1.2.3 Hyperglycaemia

1.2.3.1 Clinical studies

At least 1 in 5 patients with SMI experience hyperglycaemia [36], and rates of hyperglycaemia are significantly higher in patients established on antipsychotic

drugs than first episode and unmedicated patients which implies that it is a side effect associated with treatment [64]. The presence of hyperglycaemia indicates that there is a dysregulation in the neuro-hormonal regulation of glucose, this could be due to insulin resistance, beta cell insufficiency, or a combination of effects [112].

Plasma glucose is usually maintained at normal fasting levels between 3.9 - 5.5 mmol/l. Hyperglycaemia is defined as a fasting plasma glucose ≥5.6 mmol/l and diabetes as fasting glucose ≥7 mmol/l [50]. In a large scale network meta-analysis comparing 16 different antipsychotics, elevated fasting glucose concentration has been consistently evident in patients treated with olanzapine and clozapine, while the evidence is weak for glucose changes with amisulpiride, sertindole, quetiapine, risperidone, paliperidone, aripiprazole, or haloperidol [38]. Olanzapine treatment is associated with a considerable elevation of glucose levels [6], and in the European First-episode schizophrenia trial (EUFEST) following one year of olanzapine treatment, 21% of patients had shifted from normal fasting glucose levels to hyperglycaemia ≥5.6mmol/l [113].

Hyperglycaemia has a complex aetiology with many possible causes. Different research groups have utilised different experimental techniques to investigate the hyperglycaemic liability of olanzapine. Oral glucose tolerance test (OGTT) or IV glucose tolerance test (IVGTT) measures blood glucose following ingestion or infusion of glucose, and is a measure of overall glucose homeostasis. Hyperinsulinemic euglycemic clamp involves an infusion of insulin and glucose to measure peripheral insulin sensitivity [114], and the hyperglycaemic clamp involves a continuous infusion of glucose as a measure of insulin secretion response from beta cells [115]. However, intensive glucose monitoring techniques are often impractical in acutely unwell patients, and many clinical studies lack sufficient power to make conclusions regarding the root cause of olanzapine induced hyperglycaemia.

1.2.3.2 Glucose tolerance

Newcomer *et al.* (2002) investigated fasting glucose, OGTT, and peripheral insulin resistance using the homeostatic model assessment for insulin resistance (HOMA-IR) calculations, in patients who were stable on antipsychotic treatment [57]. Patients treated with olanzapine had significantly higher fasting glucose, and

elevated glucose levels at all time points following an OGTT compared to untreated matched healthy controls, suggestive of dysregulated glucose homeostasis. There was also a modest increase in HOMA-IR suggesting that at least part of the hyperglycaemia was related to insulin resistance, possibly secondary to obesity [57]. However patients included in this small study had a range of treatment durations, disease history and polypharmacy, which limits its usefulness.

1.2.3.3 Insulin sensitivity

A report by Sacher *et al.* (2008) added to the evidence that olanzapine increases insulin resistance. The hyperinsulinemic euglycemic clamp procedure was used to investigate peripheral insulin sensitivity of healthy volunteers administered olanzapine for 10 days [116]. Olanzapine treatment resulted in significantly reduced insulin sensitivity, and while there was a small but significant increase in mean weight during the short 10 day exposure, reduced insulin sensitivity was not attributed to the weight gain [116]. This suggests that olanzapine may have a direct effect to reduce insulin action in the periphery independently of weight gain. This theory is supported by other studies where olanzapine increased fasting glucose, unrelated to weight gain [98, 117]. However, the techniques used in this this study do not allow for quantification of beta cell insulin response.

1.2.3.4 Insulin secretion

The hyperglycaemic clamp is a technique used to investigate beta cell response to a steady infusion of glucose. Sowell *et al*. (2002) investigated the effect of short term treatment with olanzapine compared to placebo on insulin secretion in healthy volunteers [118]. There were significant increases in first phase, second phase and total insulin secretion compared to baseline for volunteers treated with olanzapine for 15 – 17 days. But it was concluded that increases in insulin secretion were correlated with significant increases in BMI, and using multivariate regression analyses, when data were corrected for weight gain there was no evident change in insulin secretion [118]. Indeed, increased body mass can increase beta cell mass through compensation mechanisms to significantly increase insulin output [45], which could explain the increased insulin secretion compared to baseline. But

without a control group with equal weight gain, it is unclear from this if the increase in insulin secretion is optimal for glucose control. In these small trials duration has been short, and healthy volunteers have often been tested, which does not replicate clinical practice. Ongoing pathophysiology and unhealthy lifestyle may also be involved in the metabolic dysregulation observed in patients, which may predispose patients to the increased side effects.

A further study by Chiu *et al*. (2010) used the IVGTT to investigate glucose and insulin response in drug naïve schizophrenic patients treated with olanzapine for 8 weeks [119]. In contrast to the report by Sowell *et al*. (2002)[118] compared to baseline there was a significant reduction in insulin secretion following 2 weeks of treatment, followed by increases at 4 and 8 weeks [119]. Mean body weight increased during the study, reaching significance at 4 weeks; so the increase in insulin response at 4 and 8 weeks is likely an adaptive beta cell response. However, the insulin response at 4 weeks was equal to baseline, despite the significant weight gain; therefore the beta cell response may indeed be insufficient. In the absence of an adequate control group with equal weight gain or insulin resistance, an effect on the beta cell response cannot be concluded from these studies.

The clinical data indicated that olanzapine induces weight gain, and leads to insulin resistance, and there is evidence to suggest that insulin resistance arises via a separate mechanism to weight gain. Hyperglycaemia is usually countered by beta cell adaptations, and it is very difficult to determine if there are changes to the normal insulin response in humans due to the conflicting effects of insulin resistance on beta cell function. Nonetheless, during the past decade reports have emerged that suggest olanzapine causes severe hyperglycaemic states that are feasible to involve beta cell dysfunction [120]. There are a number of reports linking olanzapine to exacerbation of existing diabetes [121], new onset diabetes [122], and diabetic ketoacidosis (DKA) [123, 124]. Indeed patients treated with any SGA have approximately 10 times the risk of developing DKA compared to the general population [125], but of all the SGA there have been more reports of DKA associated with initiation of olanzapine, usually within 5 months of treatment [126]. DKA is a symptom of severe hyperglycaemia that signifies an overt lack of insulin, which infers that in addition to increased insulin resistance, olanzapine treatment may reduce insulin secretion from beta cells.

1.2.3.5 Animal models

Clinical studies investigating hyperglycaemia associated with olanzapine are restricted by the conflicting effects of weight gain, previous antipsychotic use, and duration of illness that can all also considerably impact glucose homeostasis. Clinical studies are also limited by the ability to regularly test blood glucose levels at times of acute illness, and the intensive OGTT and hyperglycaemic clamp procedures that are reliable measures of glucose dysregulation, are costly and impractical to carry out regularly in practice. Using animal models, glucose dysregulation can be more thoroughly investigated than is practical in patients, although different responses between female and male rats, and the different metabolic rates of rodents leads us to approach the results cautiously.

1.2.3.6 Acute effects

Acute olanzapine has been shown to induce a state of hyperglycaemia in rats and mice. There are observed elevations of plasma glucose, insulin [127], glucagon [78], and corticosterone [128] shortly after an acute olanzapine infusion. It has also been shown that acute increases in glucose are correlated to the dose of olanzapine administered, as higher doses lead to higher glucose concentration [129]. Chintoh *et al*. (2008) carried out a series of comprehensive investigations following acute olanzapine exposure [130] and demonstrated that peripheral and hepatic insulin sensitivity was significantly decreased, there was acutely increased glucose levels, as well as impaired pancreatic beta cell response [130].

It is suggested that hyperglycaemia associated with acute administration predominantly occurs through an increase in hepatic glucose output (HGO) [78]. Houseknecht *et al*. (2007) and Chintoh *et al*. (2008) confirmed using radiolabelled glucose that HGO is significantly increased by acute olanzapine [130, 131]. Insulin usually inhibits HGO, but acute olanzapine was shown to prevent the inhibitory effect of insulin on HGO, suggesting there is a pharmacological interaction at the liver to increase plasma glucose levels [131]. Peripheral insulin resistance is also identified as a major contributor to hyperglycaemia following olanzapine administration. HOMA-IR is increased by acute olanzapine [132], and hyperinsulaemic euglycaemic clamp equally shows significantly recued insulin sensitivity in the periphery [130, 131].

Following acute administration, Chintoh *et al*. (2008) used the hyperglycaemic clamp technique and revealed a significant reduction in insulin secretion from the pancreatic beta cells [130]. Plasma Insulin levels following olanzapine administration were approximately 50% of control, and there was a diminished biphasic insulin response to prolonged glucose stimulation [130]. Where other studies have reported an increase in plasma insulin following acute olanzapine [69], this has been alongside elevated glucose; therefore it is likely glucose stimulated secretory response rather than a stimulatory drug effect. The use of the hyperglycaemic clamp has the advantage of controlling the plasma glucose to more thoroughly investigate insulin secretory response compared to a control.

1.2.3.7 Chronic effects

1.2.3.7.1 Fasting hyperglycaemia

The effect of an acute dose of olanzapine is not reflective of clinical practice, where treatment is usually ongoing. Chronic olanzapine has also been widely investigated in rodents, and has shown that the acute effects of peripheral and hepatic insulin resistance continue into chronic effects [70, 133-135]. Conversely, the effect of chronic olanzapine on fasting glucose is discordant as several studies suggest there is no change whereas other reports propose that there is a significant elevation. Some studies have shown that fasting glucose is unchanged by chronic olanzapine treatment for 21 days [133], 28 days [70] or 8 weeks [135, 136]. While other reports suggest there are small but insignificant elevations in fasting glucose following chronic exposure for 8 weeks [134], and others list significant elevations in fasting glucose [137] and significant elevations in random (unfasted) glucose [136, 138]. In a recent study by Ersland *et al*. (2019) female rats were administered long acting injections (LAI) of olanzapine for a period of 13 months, it was demonstrated that following 1 month of treatment fasting glucose was elevated, but over time it decreased [139].

There are several possible reasons for the disparity between results. Rodents have a high metabolic rate and feed during the dark cycle, hence an overnight fasting duration is analogous to starvation, causing significant depletion of glycogen reserves and lipolysis [140]. 5 hours is considered sufficient fasting time to assess fasting glucose in mice, however different groups have utilised different fasting times

such as 2 hours [139], 6 hours [138], 7 hours [133], or overnight [70, 134]. Furthermore, due to their high metabolic rate, olanzapine has a much shorter half-life in rodents; 2.5 hours compared to approximately 30 hours in humans [141]. Therefore the mode of drug administration is significant as fluctuating plasma drug levels could significantly influence the effect. Different groups have utilised different modes of administration, with some using osmotic mini pumps [70, 133, 138], others using oral gavage [134, 135], and other using LAI [139]. Osmotic mini-pumps have been utilised by several groups to administer a continuous infusion that bypasses the issues relating to the short half-life [60, 70, 133], however it has been pointed out that drug degradation in the mini-pump reservoir results in decreasing concentration over time, thus reducing reliability of results [142]. LAI has been shown to produce stable plasma olanzapine concentrations [143], but long term studies using LAI olanzapine have not reported changes in blood glucose as a primary outcome [139, 144].

1.2.3.7.2 Glucose tolerance

The OGTT is a measure of glucose tolerance following ingestion of glucose, but reports of plasma glucose following an OGTT in rodents are similarly varying. Some studies show that up to 8 weeks olanzapine treatment results in no difference in area under the curve (AUC), but suggest there is a significant difference in plasma glucose at the early time points following the glucose challenge [135, 138]. A similar pattern was observed following a year of LAI olanzapine [139] which suggests there may be alterations in first phase insulin response instigated by olanzapine. Interestingly there was concurrent elevated plasma insulin at the late stages of the OGTT highlighting a potential derangement of both first and second phase insulin secretion [139]. Other reports suggest there is a significant increase in plasma glucose following OGTT, up to 2 fold compared to control [133, 134, 137], although this has been shown to be dose dependent, as there was no significant effect at a lower olanzapine concentration.

1.2.3.7.3 Insulin Resistance

Chronic olanzapine has frequently been associated with increased fasting insulin which is a hallmark of insulin resistance [134, 135]. Likewise, HOMA-IR has been

shown to be significantly increased in several studies [133-135], implying that olanzapine increases insulin resistance. Chintoh *et al*. (2008) used the euglycaemic clamp to investigate insulin resistance in female rats administered olanzapine for 4 weeks, and showed there was a significant reduction in insulin sensitivity and an increase in hepatic glucose production. Added to the previous evidence that acute olanzapine inhibits the action of insulin to suppress hepatic glucose production and increase peripheral glucose uptake, this strengthens the theory that olanzapine has a direct pharmacological action to increase insulin resistance [70].

1.2.3.7.4 Insulin secretion

Insulin secretion following chronic olanzapine treatment has not been as thoroughly investigated. Shah *et al*. (2016) raised an interesting observation regarding the histopathological changes to the pancreas in male rats administered olanzapine. There was derangement of beta cells, and an increase in amylase and lipase [136]. It has also been noted that pancreatic tissue of female rats administered olanzapine for 21 days had significantly higher insulin content compared to control [133]. Although, this is likely to be correlated to increases in weight gain as the beta cell mass can dynamically expand in response to increased requirements through beta cell compensation [45].

Chintoh *et al*. (2008) utilised the hyperglycaemic clamp to investigate insulin secretion following 4 weeks of chronic olanzapine, but found no change in insulin secretion at any time point compared to control [70]. However, compared to control, olanzapine treated rats had significantly higher visceral fat and reduced insulin sensitivity, thus we conjecture that beta cell compensation would have resulted in an increase in insulin secretion compared to control, but this was not evident. Chintoh *et al*. (2008) also tested the insulin secretory response in the final experimental stages, after 300 minutes of previous tests including hyperinsulinaemic clamp and glucose infusion which may have had residual effects to influence insulin secretion [70].

Ader *et al.* (2005) also proposed that chronic olanzapine administration in dogs dramatically impaired the normal beta cell compensatory response to insulin resistance [56]. Dogs were administered olanzapine, risperidone or high fat diet for 4-6 weeks, and all conditions resulted in increased adiposity and insulin resistance.

But olanzapine treated dogs did not respond with any upregulation of beta cell output, compared to risperidone or high fat diet which elicited an adequate beta cell compensatory response to insulin resistance [56]. Interestingly, insulin secretion was not impaired, only beta cell adaptations to insulin resistance were impaired. This exposes weakness in several other clinical and animal studies that do not adequately control for weight gain or insulin resistance, and are thus unable to determine if the beta cell response is appropriate for the degree of insulin resistance or adiposity caused by olanzapine.

1.2.3.8 Mechanism

As depicted in **Figure 4** blood glucose homeostasis is regulated through a complex hormonal network between pancreatic beta cells, alpha cells, liver, muscle, adipose and the central nervous system (CNS) [145]. Due to the complex multifactorial regulatory system, it is challenging to determine the full mechanism through which olanzapine induces hyperglycaemia. The *in vivo* data suggest that olanzapine primarily causes hyperglycaemia through an increase in hepatic and peripheral insulin resistance [130]. But due to the additional influence that weight gain and insulin resistance have to concurrently modify beta cell function the *in vivo* data are less strong regarding beta cell insulin secretory function.

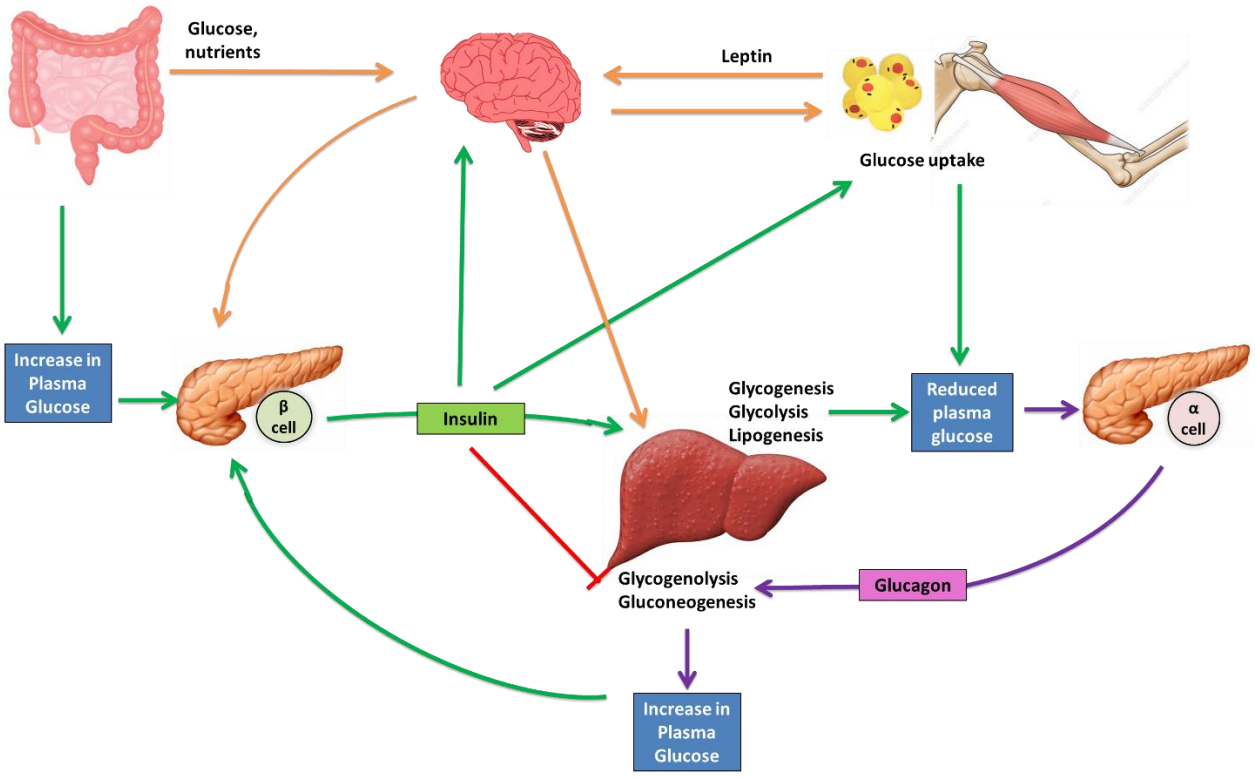


Figure 4 Simplified illustration depicting the organs and hormones involved in glucose homeostasis. An increase is plasma glucose stimulates the pancreatic beta cells to secrete insulin, which stimulates glucose uptake in muscle, adipose, and liver. In the absence of glucose, alpha cells secrete glucagon which stimulates glycogenolysis and gluconeogenesis from the liver to increase plasma glucose concentration. The Central nervous system senses changes in glucose, nutrients, insulin and leptin and has a regulating role in hepatic glucose output and peripheral glucose uptake.

1.2.3.8.1 Glucose homeostasis

Glucose homeostasis is regulated through complex interaction between the major metabolic organs. As portrayed in **Figure 4** an increase in plasma glucose following a meal stimulates insulin secretion from beta cells. Skeletal muscle cells and adipocytes are highly sensitive to insulin, and insulin facilitates glucose transport by binding to membrane bound insulin receptors (IR) to activate an intracellular cascade of events. Consecutively there is translocation of GLUT4 glucose transporters to the cell membrane which allows glucose to rapidly enter the cell [146]. At the liver, insulin inhibits gluconeogenesis and stimulates hepatic glycogen synthesis to store excess glucose. When circulating glucose levels decrease, insulin secretion from the beta cells stops, and the pancreatic alpha cells secrete glucagon to stimulate hepatic glucose production. Glucose production occurs in the form of glycogenolysis or gluconeogenesis and this hepatic glucose output prevents blood

glucose levels from dropping too low and ensures that vital organs such as the brain have a constant supply of energy [145, 147].

The central nervous system also has a regulatory role in the process of glucose homeostasis. The brain detects changes in hormones and nutrients and through neuronal networks it can modulate various aspects of metabolism [148]. Insulin or leptin signify long term energy stores, while nutrients, glucose, and circulating free fatty acids indicate short term energy availability. In response to variations in energy balance, the brain stimulates changes in appetite, food intake and energy expenditure [149]. The CNS can also regulate hepatic glucose output via autonomic efferent vagal nerves, where sympathetic innervation can increase hepatic output and parasympathetic innervation can potentiate insulin mediated inhibition of gluconeogenesis [150]. Insulin action in the hypothalamus has been shown to potently supress hepatic glucose output [151], suggesting the hypothalamus is an important component of glucose homeostasis.

1.2.4 Insulin resistance

1.2.4.1 Secondary to obesity

Due to the high rates of overweight and obesity in patients treated with olanzapine, it has been put forward that insulin resistance and hyperglycaemia occurs secondary to obesity. In obesity and states of inflammation there are increased levels of circulating hormones, cytokines and NEFAs which can all impact the normal insulin stimulated glucose uptake pathways, as well as triggering inappropriate stimulation of hepatic gluconeogenesis which can result in hyperglycaemia [152, 153]. However, several *in vivo* studies have shown that glucose intolerance is present in the absence of weight gain [60, 131], and other reports have demonstrated a direct pharmacological effect of olanzapine at skeletal muscle cells and hepatocytes. There may be a contribution of increased adiposity on insulin resistance and hyperglycaemia, but it is likely to be amplified by other pharmacological effects.

1.2.4.2 Adipose and muscle

In vivo studies have established using hyperinsulaemic clamps and HOMA-IR assessments that there is a reduction in peripheral insulin sensitivity following olanzapine treatment [70, 133, 154]. As depicted in **Figure 5** at the skeletal muscle and adipose tissue, insulin acts at the insulin receptors to stimulate downstream phosphorylation of insulin receptor substrates (IRS) that in turn stimulate multiple downstream signalling pathways [155]. Activation of the IRS/ phosphatidylinositol 3-kinase (PI3K)/ Akt (protein kinase B) pathway is involved in the translocation of GLUT4 receptors to the membrane for glucose uptake into the cell [156]. Peripheral insulin resistance is associated with reduced signalling in the IRS/PI3K pathway, which results in reduced glucose uptake and hyperglycaemia [157].

In vitro studies have shown that olanzapine can directly inhibit insulin signalling in muscle and adipose cells which may be linked to altered glucose uptake. The L6 myotube cell line is a model of rat skeletal muscle, and 72 hour olanzapine incubation was shown to reduce IRS1 tyrosine phosphorylation in response to insulin, and reduce downstream PI3K signalling [158]. There was consecutive reduction in phosphorylation of AKT and GSK3 and reduced concentration of glycogen within the cells, signifying that olanzapine can induce insulin resistance through direct inhibition of insulin signalling in skeletal muscle [158]. In fibroblast cells olanzapine also reduced phosphorylation of IRS1 [159], and in 3T2-L1 adipose cells olanzapine dose and time dependently inhibited insulin stimulated glucose uptake through a reduction in AKT phosphorylation and reduced GLUT4 translocation to the membrane [160, 161].

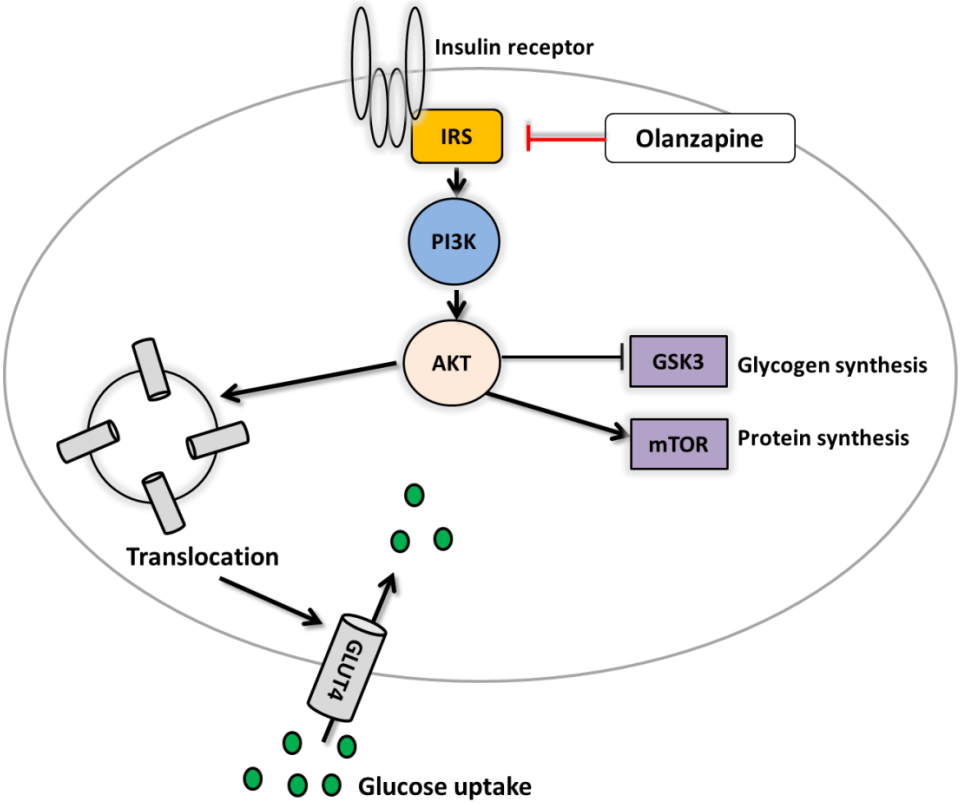


Figure 5 Simplified illustration of insulin signalling in insulin sensitive cells. Membrane bound insulin receptors initiate an intracellular signalling pathway leading to translocation of GLUT4 receptors to the membrane for glucose uptake.

1.2.4.3 Hepatic insulin resistance

The liver is fundamental to overall glucose homeostasis, in the fed state glycogen is synthesised and accumulates in hepatocytes, and in the fasted state glycogen is hydrolysed to produce glucose in the process of glycolysis. The liver is a major target of insulin, and downstream insulin signalling is also initiated by activation of IRS1 and IRS2 to regulate hepatic lipid and glucose metabolism [162]. Similarly to the skeletal muscle and adipose, olanzapine has been demonstrated to interfere with insulin/ IRS/ PI3K signalling in hepatocytes [134, 163]. Dysregulated IRS signalling is central to development of hepatic insulin resistance.

Tyrosine phosphorylation of IRS initiates downstream signalling, but it is put forward that serine phosphorylation has a negative regulatory function [164]. Phosphorylation of IRS1 at ser307 and IRS2 at ser731 has been shown to be significantly increased in the liver of rats administered olanzapine for 8 weeks [134], and downstream PI3K signalling was also significantly reduced.

Downstream of IRS/ PI3K activation, is activation of AKT, which has numerous intracellular targets such as glycogen synthase kinase 3 (GSK3) which is involved in glycogen synthesis, FOXO-1 which is involved in regulating enzymes involved in gluconeogenesis, and mTOR which is involved in protein synthesis. Following four weeks of olanzapine administration, there was upregulated expression of hepatic GSK3β, as well as significant increases in expression of the enzyme glucokinase (GCk) [138]. There was also observed activation of mammalian target of rapamycin (mTOR) and AMPK and it was suggested that olanzapine induces anaerobic glycolysis which depletes hepatic glycogen reserves [138]. These data strongly suggest that olanzapine disrupts hepatic glucose metabolism through interaction with the IRS/ PI3K/AKT pathway. (**Figure 6**)

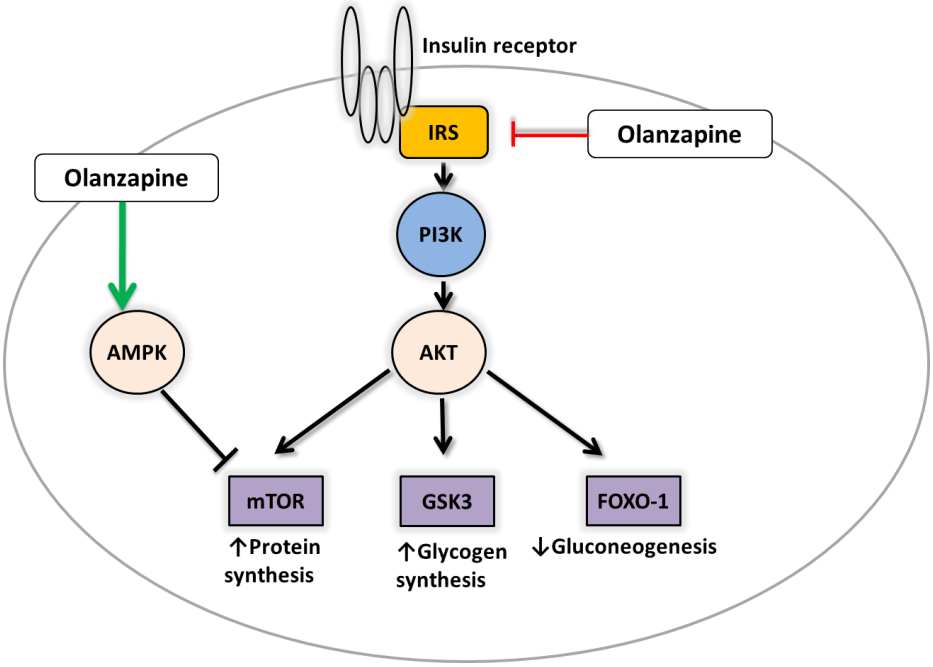


Figure 6 Simplified illustration of insulin signalling at hepatocytes. Membrane bound insulin receptors initiate an intracellular signalling pathway leading to increased protein and glycogen synthesis, and decreased gluconeogenesis

In vivo studies also indicate that olanzapine increases hepatic glucose output. Glycolysis and gluconeogenesis are the two key processes involved in glucose synthesis, which are usually regulated by insulin, glucagon and adrenaline [165], as well as hypothalamic stimulation [166]. A further potential mechanism for increased glucose output is increased glucagon or adrenaline stimulation. *In vivo* studies have demonstrated that olanzapine can increase glucagon secretion [78] which acts at

the liver to stimulate glucose production. Acute increases in plasma glucose have also been correlated to serum adrenaline which has been increased in response to high dose olanzapine administered to rats [127]. Acute glucose elevations have been demonstrated to be inhibited by propranolol, a β receptor inhibitor [127] as well as an α2 receptor antagonist, suggesting there may be involvement of the sympathetic nervous system [129].

Treatment with olanzapine for 8 weeks has also been shown to increase expression of TCF7L2 in liver, as well as skeletal muscle and adipose tissue [135]. Using multiple linear regression analysis, hepatic TCF7L2 protein level was significantly correlated to changes in body weight, HOMA-IR and glucose tolerance. TCF7L2 is a member of the TCF family that forms a bipartite transcription factor with beta catenin to influence transcription of many genes, and is a key effector of the Wnt signalling pathway [167]. TCF7L2 has recently gained noteworthy attention as a susceptibility gene for type 2 diabetes [168]; hence a documented interaction with this transcription factor is an interesting development. In a model of liver specific over expression of TCF7L2 there has been a measurable increase in hepatic glucose production, and resulting hyperglycaemia [169], thus pharmacological interaction with TCF7L2 may also be involved.

1.2.5 Beta cell dysfunction

The pancreatic beta cells synthesise, store, and secrete insulin to maintain plasma glucose concentration within a narrow range [170]. Insulin is secreted from the pancreatic beta cells in response to fluctuations in plasma glucose, and the beta cell mass is adaptive to fluctuating insulin requirements and can increase mass and secretion at times of increased demands[45]. Insulin resistance itself does not cause diabetes, but if there is additional beta cell dysfunction, diabetes follows. But while the evidence for peripheral and hepatic insulin resistance is strong, there is presently insufficient evidence to make conclusions relating to olanzapine influence on beta cell function, and any potential pharmacological mechanisms involved.

The *in vivo* results have suggested that acute olanzapine has a significant effect to reduce insulin secretion, but the *in vitro* evidence for olanzapine induced changes in insulin secretion is insufficient. Melkersson *et al*. (2001, 2004) investigated insulin secretion in primary rat islets and INS-1 beta cell lines in response to antipsychotic

treatment [171, 172], and suggested that up to 4 hour exposure to olanzapine increased basal insulin secretion but had no significant effect on glucose stimulated insulin secretion (GSIS). Conversely, a recent study by Nagata *et al*. (2019) demonstrated that acute exposure to therapeutic concentrations of olanzapine for one hour significantly reduced insulin secretion from hamster HIT-T15 cells to approximately 80% of control [173]. Several studies have also suggested that the structurally related antipsychotic, clozapine, induces a dose and time dependent reduction in GSIS [174, 175] thus olanzapine might have a similar effect during longer incubation times or at higher concentrations.

The most common mechanism proposed for reduced insulin secretion involves interaction with the muscarinic M_3 receptor, as both olanzapine and clozapine share high affinity for M_3 receptors and also have the highest risk of development of diabetes [176]. It is put forward that parasympathetic acetylcholine can regulate insulin secretion through beta cell M_3 receptors, although the evidence to support olanzapine interaction with beta cell M_3 receptors to reduce insulin secretion is mostly theoretical. Olanzapine was shown to inhibit insulin stimulation by the cholinergic agonist carbachol in primary rat islets, suggesting it may inhibit acetylcholine stimulated secretion [177]. But there was no effect on GSIS during the short 60 minute drug exposure, thus it is unclear if this relates to changes in normal insulin secretory function. It has further been postulated that it is interaction with central M_3 receptors that is involved in regulation of insulin secretion through cholinergic innervation [178], but there is little evidence to back this up.

It has also been proposed that olanzapine is toxic to beta cells, and causes an increase in apoptosis and a subsequent reduction in beta cell mass [55]. In the paper by Ozasa *et al*. (2013) olanzapine incubation resulted in significantly increased ER stress and apoptosis, and reduced insulin secretion In the HIT-T15 beta cell line [55]. However, the tested concentration (100 µM) was many times higher than plasma concentrations of olanzapine which limits the usefulness of these data.

1.2.6 Insulin resistance and beta cell dysfunction

It is acknowledged that olanzapine increases peripheral insulin resistance through disruption of the IRS/PI3K/AKT pathway [134, 158]. In previous studies investigating the importance of IRS in glucose homeostasis, it has been highlighted that the beta

cells have a critical responsive role, and the hyperglycaemic outcome of IRS modulation is dependent on the extent of beta cell involvement. There have been previous studies investigating the amount insulin resistance contributes to development of diabetes. The double heterozygote IR/IRS-1 (+/-) mouse is a model of insulin resistance, in which beta cell hyperplasia and hypersecretion almost completely compensates for the initial severe insulin resistance [179]. Conversely IRS2 deficient mice display a more rapid progression to diabetes due to peripheral insulin resistance and simultaneous beta cell dysfunction [180]. IRS2 has been shown to be involved in beta cell proliferation and regulation of beta cell mass [181], hence where there is an absence of beta cell compensation to counteract insulin resistance, there is a much more severe effect on glucose [180].

In a study by Michael *et al*. (2000), liver specific insulin receptor (IR) knockout (LIRKO) mice were used to investigate glucose tolerance [182]. An interesting pattern of glucose tolerance was observed, in fasting states there was only modest increases in glucose, while fasting insulin was up to 7 fold higher than wild type. Following glucose challenge blood glucose was significantly elevated, alongside up to 20 fold increase in plasma insulin [182]. Beta cell mass and insulin content was also shown to be significantly increased, which is indicative of compensatory hypertrophy and hypersecretion. In the classic model of beta cell compensation extra demands on the beta cells usually leads to irreversible dysfunction and diabetes, but in LIRKO mice the beta cells maintained hypersecretion for over a year without failure, and fasting glucose did not become uncontrolled during the course of the experiment [182]. This suggests that hepatic insulin resistance alone is not enough to instigate early onset, or severe hyperglycaemia, and there is likely a combination of effects involved. Furthermore, in muscle specific IR knockout mice (MIRKO) blood glucose and insulin were surprisingly not affected [183].

Patients treated with olanzapine experience rapid changes in plasma glucose, often including an increase in fasting glucose and hyperinsulinemia following a glucose challenge. In the IR knockout insulin resistance studies, hyperinsulinaemia has been reported up to 20-50 fold compared to wild type [182], as a result of beta cell compensation. However, it is unclear if the hyperinsulinaemia observed following olanzapine is maximal, as there may indeed be a reduction in secretion with resulting uncontrolled glucose.

The beta cell function is central to the development of type 2 diabetes, and there is no ongoing hyperglycaemia without the presence of beta cell dysfunction. The progression from insulin resistance to loss of beta cell functional mass usually takes several years. It is possible that the severity of the insulin resistance results in accelerated beta cell decline. Alternatively, we hypothesise that olanzapine simultaneously alters beta cell function to reduce insulin secretion and increase risk of hyperglycaemia and diabetes. Investigating the effect of olanzapine on beta cell function is therefore warranted in the face of high rates of hyperglycaemia and diabetes in treated patients.

1.3 Part 3: Olanzapine and Beta cell function

The evidence that olanzapine causes peripheral and hepatic insulin resistance through interaction with IRS/PI3K signalling is supported by clinical reports [57, 116], *in vivo,* and *in vitro* studies [131, 158]. But the evidence for any influence on beta cell function is less strong. While peripheral insulin sensitivity is important for glucose uptake, the total body homeostasis of glucose is also crucially dependent on the beta cells. In the absence of functional beta cells, hyperglycaemia ultimately leads to diabetes. The clinical and animal models point to a possible action of olanzapine to reduce insulin secretion [119, 130], but as weight gain and insulin resistance also have independent effects on beta cells, it has proven difficult to assess if this is through a direct pharmacological action.

As hyperglycaemia affects a high proportion of patients, and a large number of patients proceed to develop diabetes, it is conceivable that insulin secretion is simultaneously impaired, but parallel studies of beta cell function are lamentably absent. In the clinical study by Chiu *et al*. (2006) [184] there was a reduction in GSIS following 2 weeks of olanzapine treatment, and in work by Chintoh *et al*. (2008), and Nagata *et al*. (2019) a reduction in insulin secretion has also been observed following an acute olanzapine administration [130, 173]. Together, this suggests that there might be a possible pharmacological effect to prevent adequate insulin secretion, and this may be masked by apparent increases in plasma insulin due to beta cell compensation or reduced peripheral insulin disposal. *In Vitro* studies by Melkersson *et al*. (2001, 2004) have suggested there may be an interaction

between some antipsychotics and the insulin secretory pathway but assessments of olanzapine have proven inconclusive [171, 172].

The common clinical picture of olanzapine treated patients involves elevated insulin, however, it is likely that elevated insulin is secondary to elevated glucose, as beta cell compensatory mechanisms increase insulin secretion at times of increased demand [185]. Nonetheless, Ader *et al.* (2005) proposed that olanzapine reduces the beta cell compensatory response to insulin resistance [56], which would suggest there is a reduction in beta cell function instigated by olanzapine.

1.3.1 Beta cell function

1.3.1.1 Glucose stimulated insulin secretion

Beta cells carry out two key functions in the regulation of glucose homeostasis; they secrete insulin in response to elevations in plasma glucose, and they can dynamically regulate capacity for insulin secretion through increasing the overall beta cell mass. It is failure of the beta cells to perform these functions that denotes the onset of diabetes.

The process by which beta cells detect and respond to extracellular glucose concentration involves several steps as depicted in **Figure 7**. Glucose is primarily transported into the cell through membrane bound glucose transporters (GLUT2). GLUT2 is a relatively low affinity, high capacity transporter which allows glucose concentration to equillibrate across the membrane [186]. Glucose is phosphorylated by glucokinase to generate pyruvate, which subsequently enters the tricarboxylic acid (TCA) cycle in the mitochondria and leads to increased generation of ATP. ATP sensitive potassium channels (K_{ATP}) are open at times of low metabolic activity, allowing potassium (K^+) to move out of the cell, resulting in a negative resting membrane potential. An increase in the ATP/ADP ratio causes K_{ATP} channels to close and resultant cell depolarisation. L-type voltage dependent calcium channels detect the increased cell depolarisation and open to allow influx of calcium (Ca^{2+}) into the cell. Increased Ca^{2+} triggers secretory granule fusion with the membrane to release insulin and other secretory products into the extracellular space to be transported around the body [170].

Sustained glucose stimulation of beta cells results in a biphasic pattern of insulin secretion. In the first phase, a sharp increase in insulin secretion corresponds to the rapid influx of Ca^{2+} leading to release of insulin granules docked at the membrane, known as the readily releasable pool (RRP). The second, sustained phase of insulin secretion is slower, as insulin granules are mobilised from reserve pools to the membrane for secretion [187]. The first phase of secretion lasts approximately 10 minutes whereas the second phase can be sustained at a rate of for several hours if blood glucose levels persist [188].

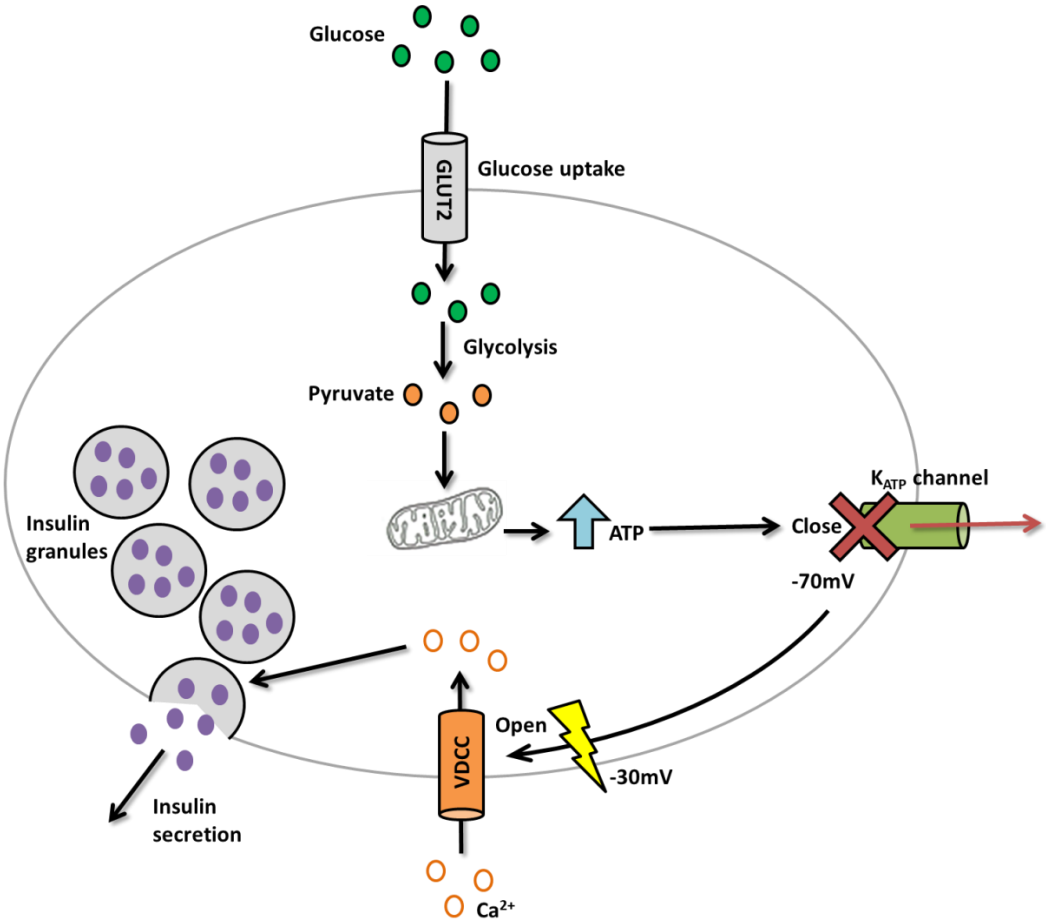


Figure 7 Simplified illustration of Glucose stimulated insulin secretion from beta cells. Glucose enters the cells through membrane bound GLUT2 receptors which causes an increase in ATP, closure of K_{ATP} channels and opening of voltage dependent calcium channels which stimulates insulin granules to move to the membrane for secretion.

1.3.1.2 Beta cell compensation

In cases of increased insulin demands such as during hyperglycaemia, increased body mass, pregnancy, or insulin resistance, the increased burden on the beta cells leads to functional adaptations to increase secretion. When exposed to ongoing hyperglycaemia the beta cell mass characteristically fluctuates through 5 key stages as illustrated in **Figure 8** [45]. Initially hyperglycaemia stimulates beta cell compensation, where the total cell mass increases to expand capacity for insulin production and secretion, resulting in hypertrophy, hyperplasia and overall increased GSIS. Beta cell compensation can significantly increase the beta cell mass, for example in patients with obesity, mass has been found to increase by 50-90% [189], and in pregnancy there is a 3-4 fold increase in beta cell mass [190].

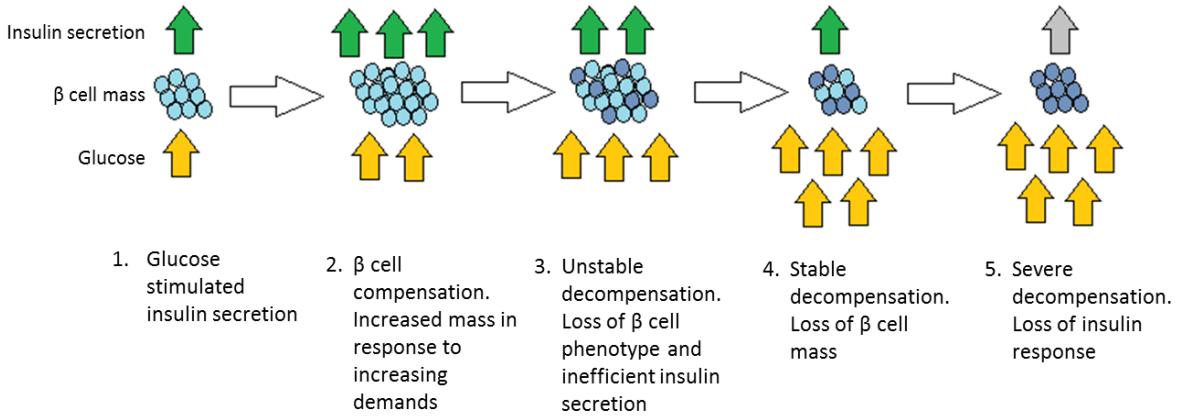


Figure 8 Illustration depicting the 5 stages of beta cell compensation, decompensation, stable decompensation and severe decompensation that lead to loss of beta cell function in response to ongoing hyperglycaemia

Through beta cell compensation, glucose homeostasis can be maintained, but it is a failure of compensation that leads to type 2 diabetes. As demonstrated in **Figure 8** ongoing hyperglycaemia initiates further changes in the beta cell phenotype [46] and instigates impairment of first phase insulin release [191]. The beta cell mass soon becomes unable to secrete sufficient insulin to maintain normal glucose levels and there is a subsequent decline in beta cell mass by up to 50% [47]. The final stage of severe decompensation occurs when the beta cell loss is extensive and there is insufficient insulin to prevent ketosis. The process towards severe decompensation may take several years [185]. The speed and severity of

hyperglycaemia associated with olanzapine treatment, and the high rates of patients that proceed to develop diabetes leads to the hypothesis that the beta cell compensatory mechanism is somehow impaired and does not counteract developing hyperglycaemia.

1.3.2 Modulation of beta cell function

Although glucose is the most potent stimulus for insulin secretion, numerous hormones, neurotransmitters and nutrients can also increase or decrease the glucose stimulated insulin response through action at membrane bound G protein coupled receptors (GPCR) [192]. Hormones and neurotransmitters are also involved in stimulating proliferation during beta cell compensation [193]. Olanzapine has a diverse binding prolife which could be linked to altered beta cell function; acting most prominently at dopamine and serotonin receptors, as well as muscarinic, adrenergic, and histamine receptors [194]. Olanzapine is also known to modulate downstream insulin receptor signalling [158], and interact with intracellular pathways such as mTOR [138] and Wnt [135] which have also been associated with insulin secretion and proliferation mechanisms.

1.3.2.1 Dopamine and serotonin receptors

Olanzapine antagonism at dopamine and serotonin receptors within the brain is central to the reduction in symptoms of psychosis; however the same receptors have been identified at beta cells with varying effects on function [195, 196]. Pharmacologically targeting beta cell dopamine receptors with dopamine and D_2 receptor agonist quinpirole was shown to inhibit GSIS in INS-1E and primary human and rodent islets [195], and knockdown of D_2 receptor in INS-1 cells resulted in enhancement of GSIS [197]. Additionally, D_2 antagonist domperidone increased beta cell proliferation and reduced cell death through increased intracellular cAMP [198]. As a D_2 receptor antagonist, olanzapine may also influence insulin secretion or proliferation of beta cells. First generation antipsychotic haloperidol is a more potent D_2 receptor antagonist that has been shown to significantly increase insulin

secretion [173], however olanzapine also acts at several other receptors which may have competing effects.

Beta cells not only express several different serotonin receptors, but they also express the serotonin transporter (SERT) and have the capacity for *de novo* serotonin synthesis from the amino acid tryptophan [199]. Serotonin is secreted in vesicles alongside insulin to have autocrine and paracrine effects at beta cells as well as alpha cells, where serotonin is involved in regulation of glucagon secretion [200]. Serotonin has been shown to both increase and decrease insulin secretion in different models, possibly due to the large number of different serotonin receptors with different downstream effects. Generally, stimulation of the 5-HT_1 family inhibits insulin secretion, and stimulation of the 5-HT_2 receptor family augments GSIS [199, 201, 202]. Although prolonged activation of 5-HT_{2B} receptors has also been shown to impair GSIS [203]. When specific 5-HT receptor antagonists were tested for effect on insulin secretion, the 5-HT_{2C} receptor antagonist was the only one associated with a significant reduction in insulin secretion, although this was a relatively minor reduction [173]. Additionally, serotonin has been implicated in the increase in beta cell mass during pregnancy, where there is an observed increase in 5-HT_{2B} receptor expression. Antagonism of 5-HT_{2B} receptors inhibited beta cell expansion during pregnancy [196]. Olanzapine is a potent antagonist at 5-$HT_{2A,}$ 5-$HT_{2B,}$ and 5HT_{2c} receptors [194], therefore based on the present observations, there may be a pharmacological modulation of insulin secretion and proliferation in beta cells.

1.3.2.2 Muscarinic, Histamine and adrenergic receptors

Olanzapine additionally has high affinity for several other receptors including adrenergic α_1, and histamine H_1 receptors [80, 194]. Beta cells likewise express GPCR that are targeted by adrenaline and histamine which can augment GSIS. Activation of alpha adrenergic receptors by adrenaline or clonidine has been shown to inhibit GSIS, effects which were reversed by α_2 antagonist idazoxan [204], suggesting these effects are through the α_2 receptor. Whereas olanzapine binds more strongly at α_1 receptors. Beta cells also contain histamine H_3 receptors and the H_3 receptor agonist imetit inhibited GSIS, whereas the H_3 inverse agonist amplified GSIS [205]. However, olanzapine is more potent at H_1 receptors [194].

Olanzapine also has antagonist properties at all five subtypes of muscarinic receptor, although with a weaker affinity than other receptors [194]. Acetylcholine

acts at muscarinic M_1 and M_3 receptors on beta cells to increase GSIS [206] and in M_3 receptor knockout islets, GSIS in inhibited [207]. Olanzapine has specifically been shown to inhibit insulin secretion stimulated by the muscarinic agonist carbachol, suggesting there may be an interaction with muscarinic receptors to inhibit insulin secretion [177]. However with an extensive binding profile at many receptors that can possibly inhibit or augment insulin secretion it is difficult to predict the accumulative pharmacological effect of olanzapine on glucose stimulated insulin secretion.

1.3.2.3 Intracellular pathways

As discussed above, olanzapine has been shown to interact with intracellular signalling pathways in adipose and skeletal muscle (**Figure 5**), and hepatocytes (**Figure 6**). There is evidence for olanzapine to interact with the IRS/PI3K/AKT signalling pathway [193], mTOR pathway [138] and the Wnt pathway [135] in various cell types, which prompts consideration of similar pathways within beta cells that could influence function.

Olanzapine can inhibit insulin signalling in hepatocytes through disruption of the IRS/PI3K/AKT pathway (**Figure 6**). IRS signalling is similarly involved in regulation of beta cell mass and function. IRS2 specifically is an important protein linking activation of membrane bound insulin and insulin like growth factor (IGF-1) receptors to numerous downstream signalling pathways to regulate cell cycle progression and physiological proliferation of beta cells [208]. Therefore, if there is interaction with IRS proteins in beta cells, olanzapine may also disturb function.

AKT phosphorylates a number of proteins to activate numerous downstream pathways, including mTOR and GSK3. GSK3 is a constitutively active kinase involved in several different cellular processes, and mTOR integrates signals relating to the nutrition status of the cell to influence growth. These signalling pathways meet at the G1/S checkpoint of the cell cycle, through activation of cyclins, thus interaction with these pathways could also influence beta cell proliferation.

GSK3 additionally plays an important role in the Wnt signalling pathway by regulating beta catenin concentration [209]. In beta cells, beta catenin has recently been shown to be involved in glucose induced localisation of insulin containing vesicles to the plasma membrane [210], as well as influencing the cell cycle through

upregulation of cell cycle related genes such as cyclin D1 and c-myc [211]. Recent work by Li *et al* (2018) [135] suggests that the Wnt pathway is involved in olanzapine induced metabolic side effects. Therefore investigation into the interaction with the Wnt signalling pathway is justified.

1.4 Summary and aims

Tight regulation of the plasma glucose concentration is maintained by insulin, secreted from the pancreatic beta cells, and acting at peripheral metabolic organs to stimulate glucose uptake. There are many pathways involved in total metabolic regulation, but insulin secretion is the crucial first stage of the process. Insulin secretion from beta cells is stimulated in response to rising and falling glucose concentrations, and a deficiency of insulin limits all other metabolic processes. Beta cell failure, and insufficient insulin results in hyperglycaemia irrespective of peripheral insulin resistance, and hyperglycaemia has many microvascular and macrovascular complications which are detrimental to health.

As we have discussed, there is evidence detailing how olanzapine can target peripheral organs to inhibit the actions of insulin, but there is limited evidence regarding its effects on insulin secretion. We know that olanzapine acts at the liver to inhibit glucose uptake and increase hepatic glucose output [134, 163] and at adipose and muscle to inhibit glucose uptake [158]. There is also evidence that olanzapine modifies areas of the brain that regulate food intake and energy expenditure which can lead to increased food intake and weight gain [82, 83]. But despite research showing the effect of olanzapine on insulin action, the effects of olanzapine on insulin secretion from the pancreatic beta cells remains unclear.

The lack of evidence relating to an olanzapine effect on beta cell function means that we only know half of the story. We know that the action of insulin is somewhat inhibited by olanzapine, and that olanzapine can increase weight gain, contributing to an overall increase plasma glucose. Yet we still do not know if the beta cells are functionally able to detect and respond to the increased glucose through secretion of insulin. This study aimed to complete the picture through establishing the pharmacological effect of olanzapine at the level of the pancreatic beta cells (**Figure 9**). We hypothesise that olanzapine damages beta cell function and that this is

directly linked to the increased hyperglycaemia and diabetes associated with treatment.

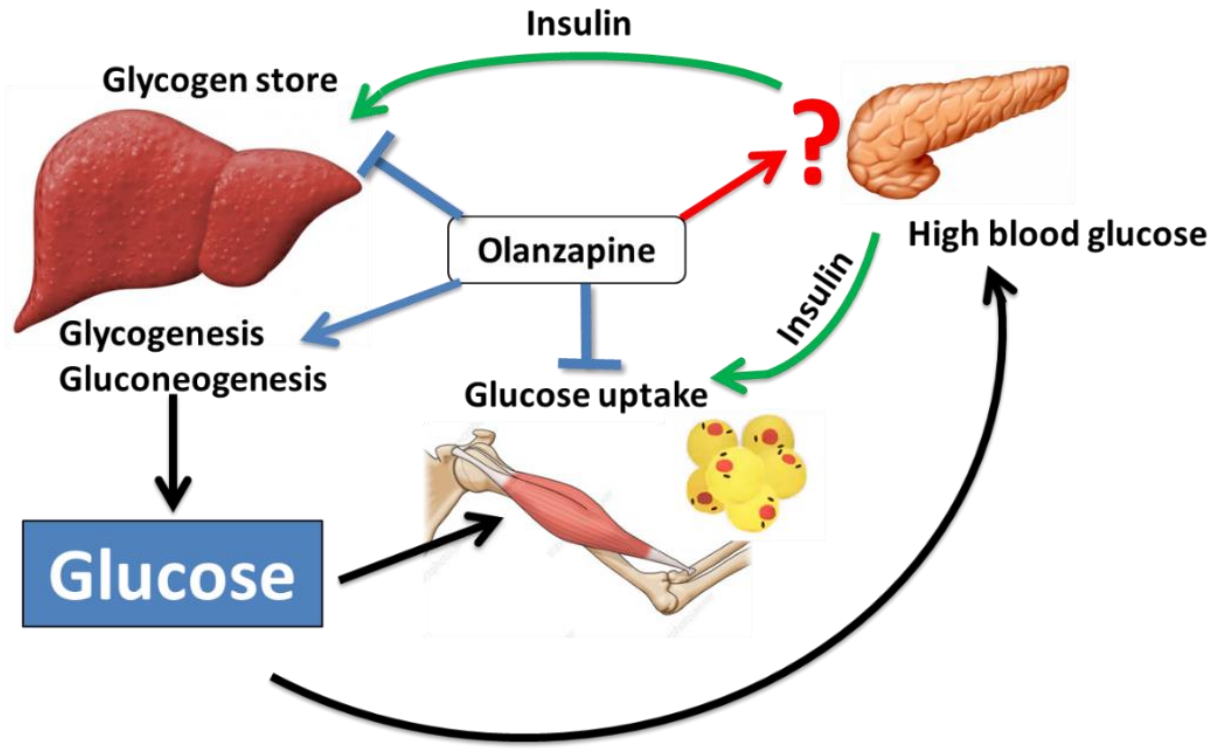


Figure 9 Illustration depicting the known effects of olanzapine at metabolic organs and the unknown effects on pancreatic beta cell function

This thesis aimed to test three initial hypotheses; that olanzapine causes beta cell death, that olanzapine inhibits beta cell compensation; or that olanzapine has a pharmacological action on the beta cell to modify the mechanism of GSIS. We also aimed to investigate the mechanisms involved in observed changes in function, with an emphasis on the role of the canonical Wnt signalling pathway. We propose that a detailed understanding of how olanzapine affects beta cell function and the mechanisms involved could lead to new strategies to manage the risk and reduce excess mortality in severe mental illness.

Chapter 2: Materials and Methods

2.1 General reagents and materials

Unless otherwise stated, all materials were of analytical grade. Chemicals were obtained from Sigma-Aldrich (UK), Stratech (UK) or MedChemExpress (USA). qRT-PCR reagents were supplied by Qiagen (UK) and primers were from Primer Design (Southampton, UK) or Sigma Aldrich (UK). Antibodies were supplied by Abcam (UK) or Santa Cruz Biotechnology (USA). Cell culture reagents and consumables were obtained from Thermo Fisher Scientific (UK).

2.2 Tissue culture

2.2.1 Cell lines

The cell line used in this study was the mouse insulinoma 6 (MIN6) [212] which was kindly donated by Professor Noel Morgan at Exeter University (UK). MIN6 cells are derived from transgenic mice expressing large T-antigen of SV40 in pancreatic beta cells. MIN6 cells are studied extensively in diabetes research due to maintained characteristics such as glucose stimulated insulin release, glucose transport and glucokinase activity which closely compares to those of isolated islets [212].

2.2.2 Maintenance of cell lines

MIN6 cells were cultured in Dulbecco's modified eagle medium (DMEM) (Gibco, UK) containing 5 mmol/l glucose and 2mM L-glutamine supplemented with 10% heat inactivated foetal bovine serum (FBS) and 100 U/ml penicillin and 100 mg/ml streptomycin. Cells were used at approximately 80% confluence and all present studies were performed using cells passage number (28-42). Cells were incubated in T75 cell culture flasks in 5% CO_2, 95% air at 37ºC, the medium was changed every 2-3 days and cells passaged every 6 - 8 days.

2.2.3 Passaging of cells

When cells reached approximately 80% confluence, media was removed and the cells washed once with 10 ml phosphate buffered saline (PBS). 1 ml trypsin/ EDTA 0.25% (Gibco UK) was added to the flask and incubated for 2 - 5 minutes at 37 ^{o}C until cells detached from the flask. 10 ml of complete DMEM was added to neutralise the trypsin, and the cell suspension was transferred to a 25 ml universal tube and centrifuged at relative centrifuge force (RCF) of 500 x g for 5 minutes. The media was removed and the cell pellet was resuspended in complete culture media and split 1:3 for maintenance, or as required for experiments.

2.2.4 Cryopreservation and thawing of cell lines

For long term storage, cell lines were kept in liquid nitrogen. Cells in T75 flasks at 80 - 90% confluence were passaged as above, and were resuspended in 3 ml freezing medium (70% DMEM, 20% FBS, 10% dimethyl sulphoxide (DMSO)). Cell suspension was split 1:3 and transferred into cryovials (Nalgene UK) then stored in cell freezing containers at -80^{o}C for 2-5 days to allow slow freezing of the cells. Cryovials were then transferred to liquid nitrogen until required.

To thaw cells, cryovials were removed from liquid nitrogen and immediately warmed to 37^{o}C. The cell suspension was added to 10ml complete culture media then centrifuged at RCF of 500 x g for 5 minutes. The cell pellet was resuspended in 15 ml complete culture media and transferred to a T75 flask and incubated at 37 ^{o}C in 5% CO_2, 95% air.

2.2.5 Treatment of cell lines

Olanzapine (OLZ) (Stratech UK) was dissolved in DMSO to a concentration of 64 mM then diluted in culture medium, containing 2 % FBS, to 1 mM then diluted to concentrations ranging from 0.1 µM – 100 µM depending on the experimental protocol. For all experiments the control wells included cells which were incubated in complete culture medium containing a percentage of DMSO equivalent to that of the treatment group (0.0015% - 0.15%)

2.2.6 Experimental protocol

Figure 10 shows the experimental treatment and extraction protocol used in all experiments. Monolayer cells were transferred to T25 flasks or plates and allowed to adhere overnight. Serum starvation for 24 hours was used to synchronise the cell population and then cells were stimulated with drug treatment for periods between 24 - 72 hours as indicated. Cells were extracted or assayed at different time points, or after 72 hour exposure, glucose stimulated insulin secretion was tested using the GSIS protocol.

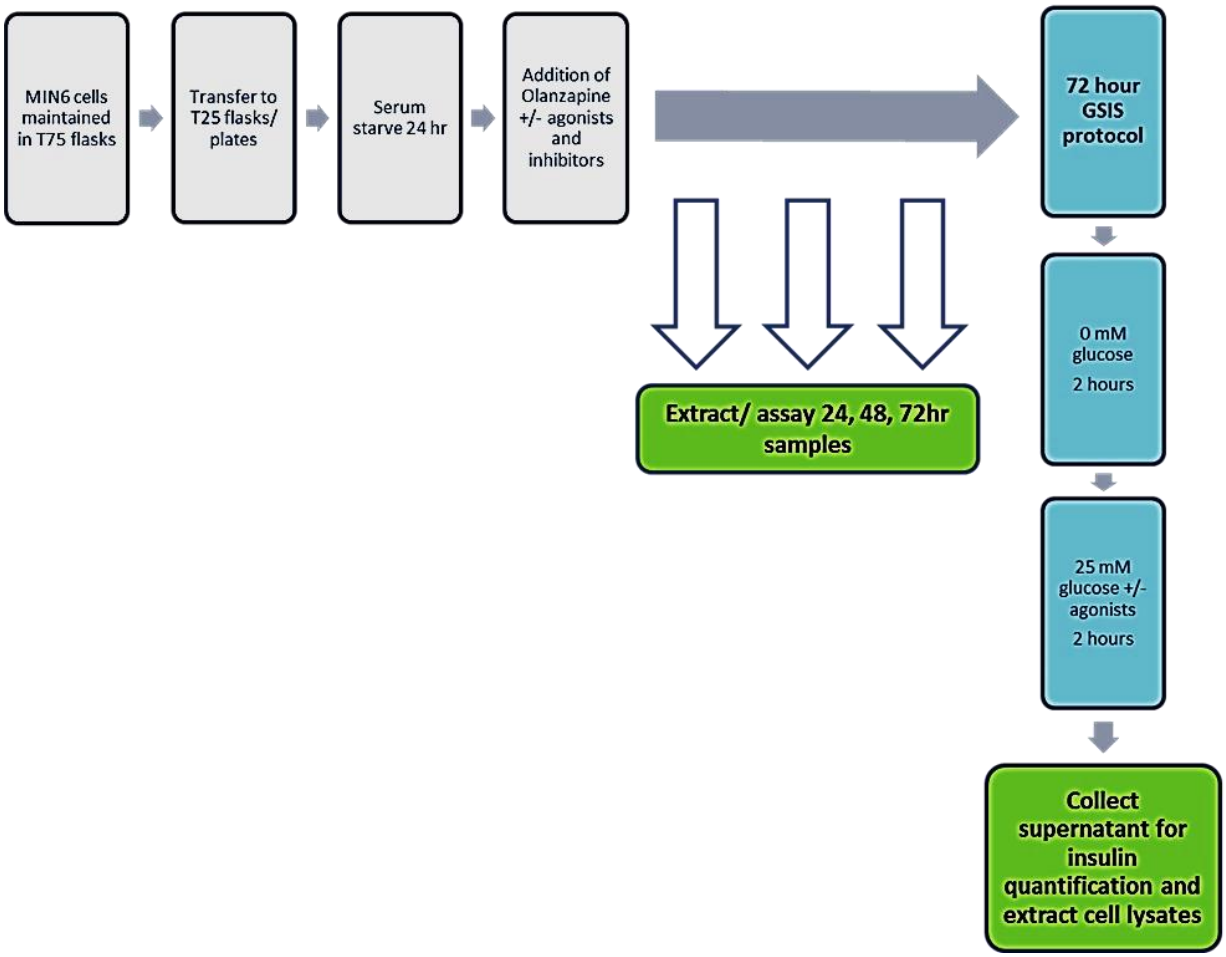


Figure 10 Infographic depicting the cell treatment times and extraction protocols.

2.2.7 Small molecule inhibitors and agonists

Small molecule inhibitors and agonists were used to investigate the mechanism behind olanzapine induced effects. **Table 5** lists the different drugs used in the following experiments.

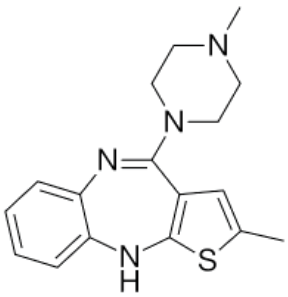
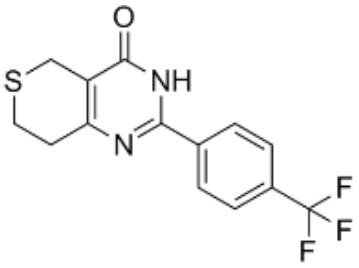
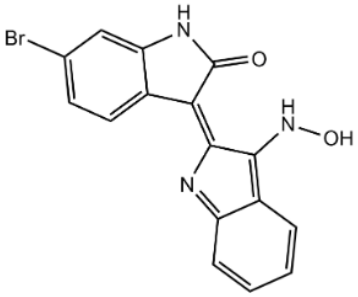
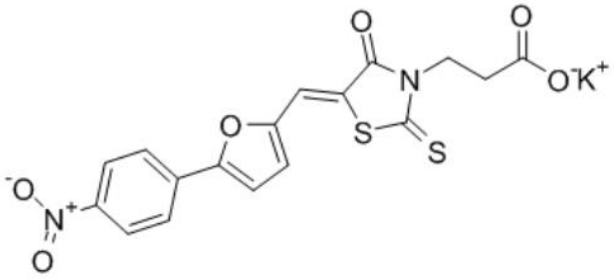
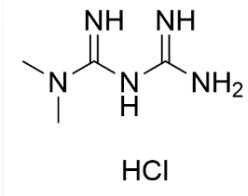
Drug	Structure	Pharmacological targets
Olanzapine		• 5-HT_{2A} antagonist • D_2 antagonist • H_1 antagonist • Complex activity at other 5-HT and D receptors, α_1, and Muscarinic [194]
XAV939		• Tankyrase inhibitor • Regulates Axin protein concentration [213]
BIO		• GSK3 inhibitor • Reduces beta catenin phosphorylation and destruction [214] • JAK inhibitor [215]
KYA1797K		• Binds to RGS domain of Axin to stabilise the beta catenin destruction complex [216]
Metformin hydrochloride		• Activates AMPK • Numerous other targets [217]

Table 5 Structure and pharmacological targets of drugs used in this study

2.3 Cell viability assays

2.3.1 The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) Assay

The MTT assay is a colorimetric assay used to assess metabolic activity within the cell as an indication of drug cytotoxic or cytostatic activity, and a measure of cell proliferation [218]. MTT is a tetrazolium salt that readily penetrates viable eukaryotic cells, where it is reduced by mitochondrial enzymes into an insoluble formazan in a NADPH and NADH dependent reaction.

For initial assays investigating cytotoxicity cells were cultured in DMEM (5 mmol/l glucose) containing 10% FBS in 6 well plates at a seeding density of 2 $x10^5$ cells/well. For further experiments investigating proliferation, cells were seeded at a lower density of 2 $x10^4$ in 24 well plates and cultured in DMEM containing 2% FBS. Cells were treated with serum free media overnight before drug treatment for the stated periods. To perform the MTT assay, media was removed and cells washed with PBS. 1 ml MTT solution (0.5 mg/ml in PBS) was added to each well in the 6 well plates and 250 µl was added to each well of a 24 well plate; the plates were protected from light and incubated for 1 hour at 37ºC to allow insoluble purple formazan crystals to develop.

MTT solution was removed and DMSO added to dissolve the crystals while the plate was agitated on an orbital shaker for 5 minutes. 200 µl of each sample was transferred to a 96 well plate in triplicate, alongside a blank DMSO control. Absorbance was read at 540 nm using ASYS UVM340 Microplate Photometer.

2.3.2 Acridine orange and DAPI staining

Monolayer MIN6 cells were cultured in 6 well plates and exposed to olanzapine or control for 72 hours. To determine total live and dead cells in the sample supernatant was collected and adherent cells were harvested by trypsinisation and resuspended. The resuspended cells were combined with the supernatant and centrifuged to form a pellet. This was resuspended with PBS to form a single cell suspension and loaded

into a Via1-Cassette™ and inserted into the automatic Nucleocounter® NC 3000™ (Chemometec, Denmark).

The cassette incorporates acridine orange (AO) dye for cell detection and DAPI dye for detecting non-viable cells. AO and DAPI are fluorescent dyes which can be used for cell counting and assessment of proportion of live and dead cells. AO easily passes through the cell membrane of viable cells and binds to cellular DNA resulting in a green fluorescence [219]. DAPI is also a nuclear dye which was used here to stain the non-viable cells blue [220]. The NC-3000™ uses fluorescence to automatically determine cell characteristics across the sample. Cell count of live and dead cells was produced automatically based on images from 4 separate fields within the cassette analysing a total volume of 1.4 µl of sample.

2.3.3 Cell count

Cells were seeded in 6 well plates at a density of 2 $x10^5$ and incubated with olanzapine for 72 hours in different culture conditions. Following incubation, media was removed and cells harvested by trypsinisation. The cell pellet was resuspended in 1ml of media and 10 µl transferred to a haemocytometer. Cells were counted manually in duplicate using a Zeiss Primo Vert microscope.

2.4 Insulin assays

2.4.1 Insulin content analysis

MIN6 cells were seeded into 6 well plates at a density of 2 $x10^5$ and incubated with olanzapine for 72 hours. Following olanzapine incubation, media was removed and the cells harvested by trypsinisation. Pellets were incubated with 1 ml acid ethanol solution (15 ml of 12 M HCl / L 70% ethanol) at 4^{o}C overnight to extract the insulin. Samples were centrifuged and the supernatant collected for quantification of insulin concentration.

2.4.2 Insulin secretion analysis

MIN6 cells were seeded into 12 well plates at a density of 1 x10^5 and incubated with olanzapine for 72 hours. Following olanzapine exposure, media was removed and cells were standardised with 0mM glucose for 2 hours. The secretory response of the cells was tested by adding low glucose (5 mM) or high glucose (25 mM) with or without addition of drugs to stimulate or inhibit secretion. **Table 6** lists the different tested drugs, concentration and the expected pharmacology. After 2 hours static incubation the supernatant was collected and quantified for total insulin concentration (first and second phase secretion). Samples were frozen at -20ºC until the ELISA was carried out.

Drug	Concentration	Pharmacology
Tolbutamide	200 µM	• K_{ATP} channel blocker • Causes membrane depolarisation, calcium influx and insulin secretion [221]
KCl	40 mM	• Membrane depolarisation leading to calcium influx and insulin secretion [222]
Exendin-4 (Exenatide)	20 nM	• Glucagon-like peptide 1 (GLP-1) receptor agonist, augments glucose stimulated insulin secretion [223]
Nifedipine	5 µM	• Calcium channel blocker, • inhibits calcium influx and insulin secretion [224]
Phorbol 12-myristate 13-acetate (PMA)	500 nM	• PKC activator • Augments glucose stimulated insulin secretion [225]
Forskolin	10 µM	• cAMP activator • Augments glucose stimulated insulin secretion [226]

Table 6 List of drugs used to stimulate or inhibit insulin secretion in the current study

2.4.3 ELISA

The mouse insulin ELISA (Mercodia, Sweden) was used to quantify the total amount of insulin in a sample. All samples were diluted to a pre-determined appropriate dilution factor prior to assay to ensure concentration was within readable range.

The manufacturer directions were followed. Briefly, 10 µl of calibrator or diluted test sample was added to each well of the pre-coated plate and 100 µl enzyme conjugate buffer was added to each well. The plate was incubated at room temperature for 2 hours on a plate shaker. The wells were manually washed 6 times with 350 µl wash buffer. 200 µl substrate TMB (Tetramethylbenzidine) was added to each well and incubated at room temperature for 15 minutes. The reaction was then stopped using 50 µl stop solution. Prior to reading the optical density the plate was shaken for 5 seconds to ensure thorough mixing, and then read at 450 nm on an ASYS UVM340 Microplate Photometer.

2.4.4 Analysis and normalisation

The concentration of insulin in each sample was obtained from a calibration plot of the 5 known calibrator samples plotted against absorbance using cubic spline regression. The results were normalised to the protein content of the lysed cell sample. After supernatant was collected for insulin quantification, the cells were harvested by trypsinisation and lysed using RIPA buffer. Protein concentration was determined using the Bradford assay.

2.5 Cell cycle analysis

2.5.1 Cell fixation and staining

DNA content of a cell was measured using DNA selective dye that is proportional to DNA concentration and can be used to determine stages of the cell cycle. MIN6 cells were serum starved for 24 hours to synchronise the cells at G_0 and then

exposed to olanzapine or control for 24 and 48 hours. Samples were collected at each stage of the exposure to determine how the cell cycle progresses. Cells were harvested by trypsinisation and washed with PBS. Cold 70% ethanol was slowly added to the cell pellet while vortexing the sample to avoid clumping of cells. Cells were fixed at -20^{o}C in ethanol for 1 week. Following fixation, the sample was centrifuged and ethanol removed. The pellet was washed twice with cold PBS while on ice then 50 µl of 100 µg/ml RNAse solution was added to the cell pellet to remove contaminating RNA. 150 µl of 50 µg/ml propidium iodide solution was added and the sample protected from light. The samples were incubated at 4^{o}C for 4 hours to ensure uniform staining.

2.5.2 Flow cytometry

Fluorescence intensity was measured using a BD Accuri™ C6 flow cytometer (BD Biosciences, USA). Propidium iodide fluorescence intensity is proportional to DNA content, used to determine stage of the cell cycle. Data analysis was carried out using Flowjo™ software (BD Biosciences, USA).

2.6 Protein content assays

2.6.1 Protein extraction

2.6.1.1 Whole cell

Experiments were performed to determine the whole cell protein concentration of proteins of interest; beta catenin, TCF7L2, Axin2, GSK3, and PKC. Cells were cultured in 6 well plates or T25 flasks according to specific experimental conditions and harvested by trypsinisation. The cell pellet was washed twice with PBS and transferred to a 1.5 ml Eppendorf tube®. The composition of RIPA buffer (Radioimmunoprecipitation Assay buffer) (Sigma, UK) is listed in **Table 7**. The buffer was prepared with added protease and phosphatase inhibitors (MS. Safe, Sigma UK). 50 µl RIPA buffer was added to the cell pellet and rigorously pipetted and vortexed and incubated on ice for 15 minutes. Samples were then centrifuged at

12000 x g for 13 minutes at 4 ºC. The supernatant contains the extracted protein and the pellet was discarded.

Reagents	Concentration
NaCl	150 mM
Tris HCl pH 8	50mM
NP-40	1%
Sodium deoxycholate	0.5%
SDS	0.1%

Table 7 Composition of RIPA buffer used to prepare whole cell protein extracts

2.6.1.2 Nuclear and cytoplasmic extract

Nuclear and cytoplasmic extracts were used to investigate localisation of beta catenin. To prepare extracts, cells were seeded in T25 flasks and cultured with olanzapine according to the experimental protocol. Cells were washed with PBS and harvested by trypsinisation, and then the cell pellet was washed again with cold PBS. The ab113474 nuclear extraction kit (abcam UK) was used to prepare nuclear and cytoplasmic extracts following the manufacturer directions and provided buffers. Briefly, 10x “pre-extraction buffer” was diluted according to manufacturer’s instructions with distilled water, and 150 µl was used to resuspend the cell pellet. The sample was incubated on ice for 10 minutes then vortexed for 10 seconds before centrifuging at 12000 x g for 1 minute. The supernatant containing the cytoplasmic extract was carefully removed and kept at -20ºC until required for experiments.

Following the manufacturer’s instructions, 1,4-Dithiothreitol (DTT) and protease inhibitor cocktail (PIC) solution (provided) were added to the ENE2 Extraction buffer at a 1:1000 ratio. The nuclear cell pellet was resuspended in 30 µl extraction buffer and incubated on ice for 15 minutes, vortexing for 10 seconds every 3 minutes. The sample was centrifuged at 12 000 x g for 10 minutes at 4ºC. The supernatant containing the nuclear extract was removed and kept at -20ºC until required for experiments.

2.7 Bradford Assay

Bio-Rad protein assay dye reagent concentrate (Bio-Rad UK) is a colorimetric assay to determine protein concentration within the isolated extract. Following the manufacturer instructions, the reagent concentrate was diluted 1:4 with distilled water and mixed thoroughly to make the stock solution. In a disposable cuvette 1 ml of stock solution was added to 5 µl of cell extract and a series of known concentrations of bovine serum albumin (BSA) protein standard. Cuvettes were mixed by inversion and incubated for at least 5 minutes at room temperature. Absorbance was measured at 595 nm using spectrophotometer (Eppendorf BioPhotometer No. 6131 22336).

Protein concentration was determined using a standard curve of known BSA protein concentrations against absorbance.

2.8 Western Blot Analysis

2.8.1 SDS-PAGE

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins with different molecular weights in a sample. When an electrical current is applied, proteins migrate through the polyacrylamide gel matrix at different rates depending on their size. Downstream immunoblotting allows for quantification of the proteins.

2.8.2 Gel preparation

Gels were hand cast using 1 mm gel thickness mini-protean tetra cell casting module (Bio-Rad UK). Resolving gels contained 10% polyacrylamide, and stacking gel 6%. **Table 8** shows the composition of one gel. The resolving gel was added first and isopropanol added to cover the gel to ensure there were no bubbles. When polymerised, isopropanol was removed and the gel rinsed with distilled water. The

stacking gel was added and a 10 well comb placed gently in the top of the module. Gels were used immediately, or stored wet at 4ºC for up to 5 days.

Resolving gel 10%	Volume	Stacking gel 6%	Volume
H_20	3.2 ml	**H_20**	2.6 ml
30% acrylamide	2.67 ml	**30% acrylamide**	1 ml
1.5 M Tris pH 8.8	2 ml	**0.5 M Tris pH 6.8**	1.25 ml
10% SDS	80 µl	**10% SDS**	50 µl
10% APS	80 µl	**10% APS**	50 µl
TEMED	8 µl	**TEMED**	5 µl

Table 8 composition of 10% hand cast gels

2.8.2.1 Gel Electrophoresis

25 µg of protein extract was mixed 1:1 with SDS sample buffer Laemmli 2x concentrate (Sigma UK). Proteins were denatured by heating the samples to 90 ºC for 10 minutes then cooling on ice for 5 minutes. 20 µl of sample was loaded to each well, alongside full range rainbow protein marker. Any empty wells were loaded with SDS sample buffer. The gel was run at 100 V for 2 hours in tris-glycine SDS running buffer, the composition of which is listed in **Table 9**.

Reagents	Concentration
Tris Base	3g /l
Glycine	14.4g /l
SDS	1g /l

Table 9 Tris glycine SDS running buffer composition used for SDS PAGE

2.8.3 Semi dry Transfer

Transferring of proteins from gel to membrane was carried out using the TRANS-BLOT semi-dry blotting apparatus (Bio-Rad UK). Following SDS page, the gel was carefully removed from the glass casing using an opening lever and allowed to equilibrate in tris- glycine transfer buffer for 10 minutes, the composition of which is listed in **Table 10**. The transfer sandwich was prepared by first soaking thick filter paper and the nitrocellulose membrane in transfer buffer. The sandwich was assembled from anode to cathode with filter paper, nitrocellulose membrane, gel and filter paper. Bubbles were removed by adding pressure to the sandwich and the transfer process was run at 15 V for 45 minutes.

Reagents	Concentration
Tris Base	1.51g/ l
Glycine	7.2g /l

Table 10 Tris glycine transfer buffer composition used for semi-dry transfer

Following transfer the membrane was blocked for 2 hours at room temperature, or overnight at 4ºC on an orbital shaker in a blocking buffer containing 10% dried milk powder diluted in wash buffer the composition of which is listed in **Table 11**. The membrane was then washed 3 times for 5 minutes using wash buffer on an orbital shaker.

Reagents	Concentration
NaCl	29.2g /l
1M Tris HCl pH 7.6	10 ml /l
Tween 20	100 µl /l

Table 11 Wash buffer composition

2.8.4 Immunodetection

Primary antibodies against proteins of interest were diluted in wash buffer to the optimised dilutions (1:2000 – 1:5000). Membranes were incubated with the primary antibody overnight at 4^{o}C with rotation. Following incubation, the membranes were washed thoroughly for 45 minutes with 3 x quick washes, followed by 3 x 10 minute and 3 x 5 minute washes on the orbital shaker.

HRP-linked secondary antibodies against the target species of the primary antibody (rabbit or mouse) were diluted 1:5000 in wash buffer. The membrane was incubated in the secondary antibody with rotation for 1 hour at room temperature. Followed by thorough washing for 45 minutes as described above.

Pierce™ ECL-plus solution (Thermo Scientific, UK) is a chemiluminescent substrate used to react with HRP for detection using X ray film. The ECL solution was prepared according to manufacturer instructions by mixing solution A and B together at a ratio of 1:1. The membrane was removed from the wash buffer and 1ml of the ECL solution was added directly to the membrane and incubated for 5 minutes at room temperature. The membrane was wrapped in saran wrap and placed inside a film cassette. In the Dark room, X-ray film (Amersham, Bioscience UK) was exposed to the membrane and developed using a Konika SRX101A automatic developer.

2.8.5 Densiometric analysis

Densiometric analysis of the resulting Western blot was carried out using Li-Cor Image Studio™ (Li-cor bioscience UK) software. The signal from each well was normalised by dividing the detected signal of the protein of interest by the detected signal of the loading control. Results were calculated relative to the control in each repeat.

2.9 Quantitative real Time Polymerase Chain Reaction (qRT- PCR)

2.9.1 RNA extraction

MIN6 cells were stimulated as indicated and RNA was extracted from the cells using the RNeasy Mini Kit (Qiagen UK) following the manufacturer's directions. Briefly, cells were harvested by trypsinisation and centrifuged to form a cell pellet. To lyse the cells 350 µl of Buffer RLT lysis solution was added to the cell pellet and mixed by pipetting and vortexing, then 350 µl 70% ethanol was added to the lysate and mixed by pipetting. The entire 700 µl solution was added to a RNeasy spin column supplied with a 2 ml collection tube and centrifuged with supplied buffers for the prescribed times to produce a precipitated RNA product on the spin column membrane. This RNA was then eluted into 30 µl RNase free water and quantified using a nanodrop at 260 nm (Thermo scientific nanodrop lite spectrophotometer). Purity of RNA was assessed using the nanodrop using the 260/280 ratio. Samples were excluded if the sample ratio was outside the range 1.8 - 2.1 as this indicates that there are impurities in the sample such as residual phenol, guanidine, or other chemical contamination.

2.9.2 Reverse transcription

In order to prevent any genomic DNA contamination, RNA samples were treated with DNase enzyme. Extracted pure RNA was then reverse transcribed using the Precision nanoScript2 reverse transcription kit (Primerdesign UK); a process which converts single stranded RNA into double stranded cDNA for further analysis. The manufacturer directions were followed; Oligo-dT primers were added to the RNA template and heated to 65 ^{O}C for 5 minutes in the annealing step. Samples were rapidly cooled on ice prior to the extension step. Buffer, dNTP mix, and nanoScript2 enzyme were added, mixed and incubated at 42 ^{O}C for 20 minutes. The reaction was heat inactivated at 75 ^{O}C for 10 minutes. cDNA samples were stored at -20 ^{O}C until use.

2.9.3 qRT-PCR

Amplification of DNA by quantitative real-time polymerase chain reaction (qRT-PCR) was used to quantify the total amount of cDNA in a sample. A Sybr green probe was incorporated into a Precision®FAST qPCR Master Mix (Primerdesign UK) which was added to forward and reverse primers and distributed into PCR strip tubes. 25 ng of each cDNA template was added to tubes in duplicate to a final reaction volume of 25 µl. Reactions were run on the rotor gene Q instrument (Qiagen UK) using cycling conditions specified in **Table 12**. Target genes were c-myc, cyclin D1, Cyclin D2 and Axin2. Primer sequences are given in **Table 13**. Control samples with no cDNA, and no reverse transcription were used to ensure there was no contamination, and melt curve analysis was used to analyse for primer dimer and contamination products.

	Step	**Time**	**Temperature**
	Enzyme activation	2 min	95^{O}C
Cycle x40	Denaturing	10 sec	95^{O}C
	Annealing/ extension	60 sec	60^{O}C
	Melt curve		

Table 12 Rotor gene Q cycling conditions for qRT-PCR

Gene	**5' Mouse forward primer**	**3' Mouse reverse primer**
c-myc	TTTTGTCTATTTGGGGACAG	CATAGTTCCTGTTGGTGAAG
Cyclin D1	AACACTTCCTCTCCAAAATG	GAACTTCACATCTGTGGC
Cyclin D2	CTTCATTGAGCACATCCTTC	GAGTGTGTTCACTTCATCATC
Axin2	AAGATCACAAAGAGCCAAAG	GAAAAAGTAGGTGACAACCAG
B2m	TGCTACTCGGCGCTTCAGTC	AGGCGGGTGGAACTGTGTTAC
UBC	AGGTCAAACAGGAAGACAGACGTA	TCACACCCAAGAACAAGCACA

Table 13 qRT-PCR forward and reverse primer sequence for specified genes

2.9.4 GeNorm analysis

For accurate quantification of gene expression GeNorm analysis was used to determine the most stable reference gene in the experimental system, as a quality control to ensure that results were highly accurate and sensitive. GeNorm analysis has been used widely to increase accuracy in qRT-PCR data [227].

Using the GeNorm kit and provided qbase+ analysis software (Primerdesign UK), the expression of 9 common reference genes was determined using qRT-PCR. cDNA samples that had been exposed to different experimental conditions were used to establish the most stable reference gene in all conditions. The Ct values were input into qbase+ software which generated some graphs to determine the most stable genes. The genes tested are listed in **Table 14**

ACTB	Beta actin
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
UBC	ubiquitin C
B2M	β-2 microglobulin
YWHAZ	phospholipase A2
RPL13A	ribosomal protein L13a
CANX	calnexin
CYC1	cytochrome c-1
18S	18S

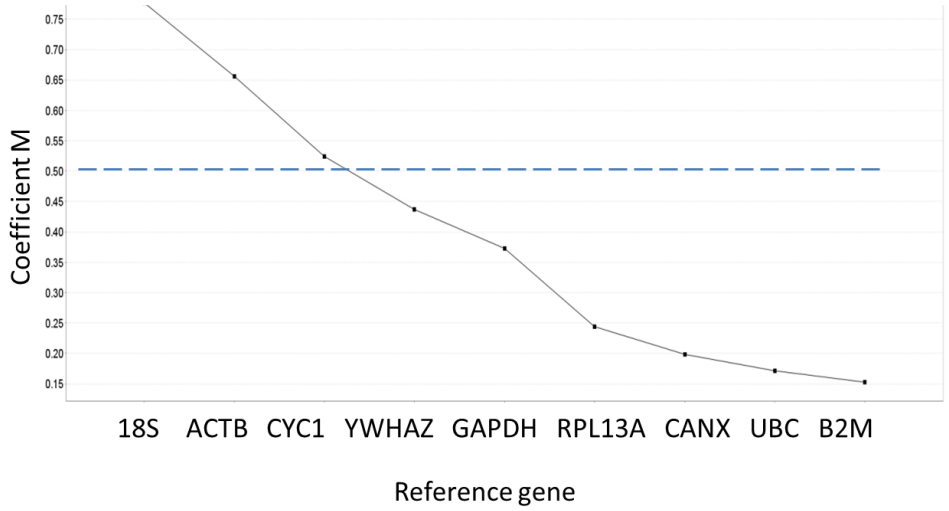
Table 14 Genes tested in the GeNorm analysis to establish the most stable reference genes for qRT PCR

The stability of each reference gene within the experimental system was termed “coefficient M”. Coefficient M was automatically calculated using qbase+ software by determining average pairwise variation between a specific candidate reference gene and all the reference genes tested. Low coefficient M values below the stability

threshold of 0.5 correspond to increased gene stability within the experimental model. Genes with the lowest coefficient M value are appropriate to be used for normalisation of data. From the results obtained in **Figure 11**A it was found that UBC and B2M were the most stable reference genes in this experimental model. Ubiquitin C (UBC) is a source of ubiquitin, a regulatory protein which is highly expressed in all cells and β2 microglobulin (B2M) is found on the surface of all nucleated cells. qRT-PCR data was henceforth normalised to UBC and B2M mRNA expression.

The optimal number of reference genes to be used for highly accurate qRT-PCR data was also determined using qbase+ software which automatically calculated pairwise variation to establish the "coefficient V". Coefficient V values below 0.15 indicate no additional reference genes are required [228]. **Figure 11**B shows that 2 reference genes have a coefficient V value less than 0.15 so no further reference genes were required, and all qRT-PCR data was normalised to two reference genes UBC and B2M.

A.



B.

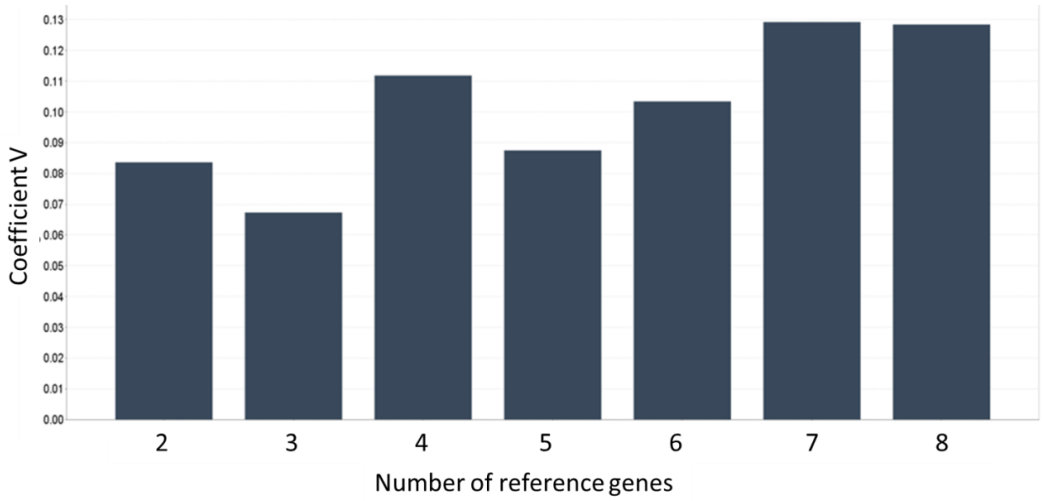


Figure 11 GeNorm analysis of 9 reference genes to establish the most stable reference gene within the experimental model. qBase+ software was used to determine the most stable reference gene within the experimental model using automatic mathematical algorithms. All samples were analysed for 9 reference genes; ACTB, YWHAZ, RPL13A, UBC, B2M, GAPDH, 18S, CANX, CYC1 and the Ct values input into qBase+. A target coefficient M value of <0.5 indicates a stable reference gene. UBC and B2M were the most stable (A). qBase+ analysis to determine the optimal number of reference genes to include in each experiment. Coefficient V value of <0.15 indicates a high level of stability. The results indicate that using 2 reference genes is sufficient (B).

2.9.5 ΔΔCt method

qRT-PCR data can be analysed using 2 methods; the ΔΔCt method or the Phaffl method. Efficiency was determined by performing qRT-PCR on a series of dilutions of cDNA sample to produce a standard curve. Efficiency (E) was calculated from the slope of the standard curve (s).

$$\mathrm{E} = (10^{(-\frac{1}{s})} - 1) \times 100$$

All primers used presently were determined to have efficiency greater than 90% therefore the ΔΔCt method was used for data analysis, and no mathematical correction was required to account for differing primer efficiency.

The $2^{-\Delta\Delta Ct}$ calculation is used widely in qRT-PCR analysis to determine relative gene expression, and assumes equal primer efficiencies [229]. Briefly, the Ct threshold value was determined, and the difference between gene of interest and reference gene was calculated (ΔCt). The difference between the treated sample and the control sample was then calculated (ΔΔCt). Fold change in gene expression was then calculated using the equation.

$$\text{Fold change gene expression} = 2^{-\Delta\Delta Ct}$$

2.9.6 Agarose gel electrophoresis

To check for primer specificity, qRT-PCR products were added to an agarose loading dye and were run on a 1% w/v agarose gel in TEA buffer for 30 minutes at 100V. Gels were visualised and images captured using the UV gel setting on the FlourChem instrument (AlphaInnotech MultiImage TM light Cabinet).

2.9.7 Quantification and statistical analysis

Statistical and graphical analysis was performed using GraphPad Prism software. One way ANOVA, 2 way ANOVA with Bonferroni post hoc test was used to analyse data unless otherwise stated. Significance was detected at a p value <0.05 and denoted with *, P values < 0.01 denoted with ** and p values <0.001 with ***

Chapter 3: The effect of olanzapine on the viability and function of MIN6 beta cells

3.1 Introduction

3.1.1 Overview

Blood glucose is tightly regulated through a complex network of hormones, and the pancreatic beta cells are central to glucose homeostasis. Beta cells respond to increases in glucose by secreting insulin, and at times of increased demand such as insulin resistance, obesity, or pregnancy, the overall functional beta cell mass can adapt by increasing in number to increase insulin output [45].

Hyperglycaemia is a common side effect of the second generation antipsychotic drug olanzapine [60], but it remains unknown if the drug has an influence on beta cell function to prevent sufficient insulin to be secreted. In a recent letter, Takahiko Nagamine (2018) [123] appealed for further research into the direct effects of olanzapine on beta cell function. They highlight the abundant case reports of acute onset hyperglycaemia and diabetic ketoacidosis (DKA) and rapid decline in metabolic function in patients commencing treatment [65-68], effects which are unlikely to be completely attributed to insulin resistance. In the present study we aimed to investigate if olanzapine affected the viability or function of beta cells using the MIN6 beta cell line. We evaluated if olanzapine caused beta cell death, had a pharmacological effect to alter insulin secretion, or had an effect to inhibit beta cell compensation.

3.1.2 Effect of olanzapine on beta cell viability

Homeostatic control of overall beta cell mass is regulated through equilibrium between cell regeneration (neogenesis, proliferation, and hypertrophy) and cell loss (apoptosis and atrophy). In healthy adults, beta cell renewal is slow, with steady rates of proliferation and apoptosis between 0.5-2% per day [230]. While rapid loss of beta cell mass is common in the development of type 1 diabetes, type 2 diabetes is usually characterised by fluctuating overall mass and eventual loss of function and mass over many years [45]. As illustrated in **Figure 12** pharmacological toxicity to beta cells can cause apoptosis, atrophy or autophagy to reduce the viable beta cell mass. Without a commensurate increase in proliferation or neogenesis to maintain the balance this may lead to decreased capacity for insulin secretion. We

hypothesise that olanzapine causes beta cell toxicity to cause acute hyperglycaemic effects associated with treatment.

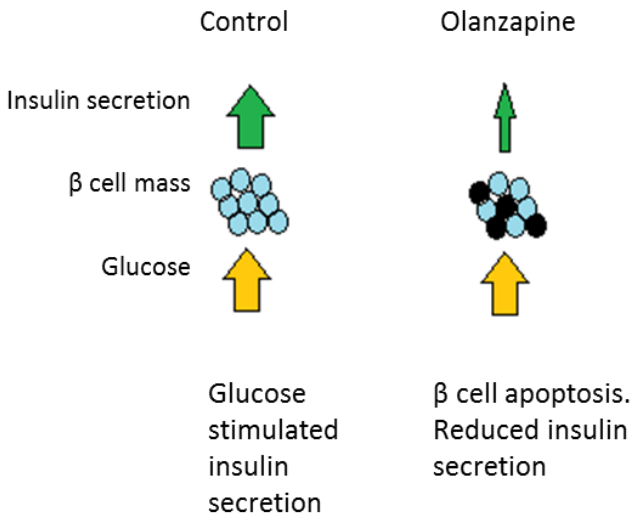


Figure 12 Illustration depicting the first hypothesis that olanzapine causes beta cell death and reduces insulin secretion into the circulation

Previous work carried out by Ozasa *et al*. (2013) advocated that olanzapine is toxic to beta cells. It was demonstrated that treatment of an insulin secreting beta cell line with 100 µM olanzapine caused significant apoptosis compared to control cells, an effect not replicated in other non- insulin secreting cell lines, implying that olanzapine is toxic to beta cells specifically [55]. However, 100 µM olanzapine may not reflect a true therapeutic concentration. In male rats following a single IV olanzapine infusion of 2.5 -10 mg/kg, plasma olanzapine concentration was established to peak at 1.3 -8.8 µM [127]. In humans, mean plasma concentration varies widely depending on the dose, compliance, polypharmacy, and sex of the patient. Plasma concentration of olanzapine usually ranges from 5 nM – 1 µM, with a linear correlation between concentration and dose, and significant differences among smokers and non-smokers [231, 232]. While the pancreatic tissue concentration of olanzapine has not been previously reported, the drug is widely distributed and the concentration in various tissues such as liver, spleen, lung and kidneys have been found to be 10-25 fold higher than that in the plasma [141]. It is possible that pancreatic concentrations will be higher than plasma, yet unlikely to be as high as 100 µM as tested by Ozasa *et al*. (2013). Loss of cell viability at this concentration may not be reflective of a therapeutic dose therefore we cannot yet

conclude or rule out that beta cell toxicity is a component of olanzapine induced hyperglycaemia. The present investigation firstly aimed to explore the effect of a range of therapeutic concentrations of olanzapine on beta cell viability.

3.1.3 Effect of olanzapine on beta cell function

3.1.3.1 Beta cell compensation

In numerous *in vivo* and *in vitro* models it has been demonstrated that olanzapine promotes a state of insulin resistance through interaction with peripheral metabolic organs. Olanzapine has been shown to increase hepatic glucose production [60, 69, 131], reduce glucose uptake into cultured adipocytes [161] and reduce glycogen synthesis in skeletal muscle cells [158]. This constellation of effects to promote hyperglycaemia should be counteracted with beta cell compensation and increased insulin secretion; but it is not yet known if olanzapine also prevents the pancreatic beta cells to fulfil this requirement.

The speed and severity of hyperglycaemia associated with olanzapine treatment, and the high rates of patients that proceed to develop diabetes leads to the hypothesis that the beta cell compensatory mechanism is insufficient to counteract developing hyperglycaemia, whether triggered by the antipsychotic or through alternate lifestyle factors.

Ader *et al*. (2005) investigated the effect of olanzapine and risperidone on insulin resistance and beta cell compensation in dogs, endeavouring to use fat fed dogs as a comparative control group for insulin resistance. The fat fed group had a threefold compensatory increase in insulin secretion but it was found that olanzapine significantly impaired the compensation response compared to control fat fed dogs [56] which suggested that there may indeed be an inhibitory effect by olanzapine. As illustrated in **Figure 13** the present study secondly aimed to investigate if olanzapine altered beta cell proliferation to determine if there is an inhibitory action to prevent beta cell compensation to hyperglycaemia.

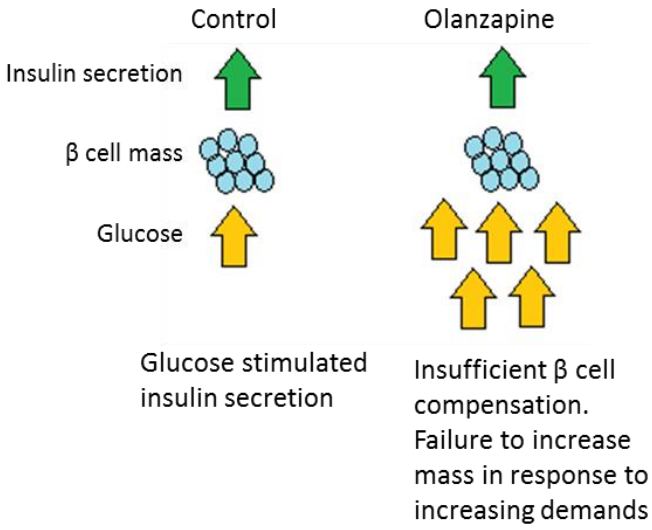


Figure 13 Illustration depicting the second hypothesis that olanzapine inhibits beta cell compensation mechanisms to prevent an increase in insulin secretion at times of hyperglycaemia

3.1.3.2 Insulin secretion

Ascertaining the pharmacological effect of olanzapine on insulin secretion in human and animal models is difficult due to numerous other side effects such as weight gain and insulin resistance [60], which may concomitantly influence beta cell function [45]. Cell studies involving primary beta cells and cultured cell lines allow for investigation into beta cell specific pharmacological effects aside from any external influence on beta cell function.

There have been few previous *in vitro* studies investigating the effect of olanzapine on insulin secretion, these are summarised in **Table 15** [167-169, 229]. Conversely, the effect of the related antipsychotic, clozapine, on insulin secretion has been demonstrated in several reports that suggest that there are concentration and time dependent changes in insulin secretion as summarised in **Table 16** [171, 172, 174, 175, 233, 234].

Olanzapine concentration	Model	Exposure time	Basal secretion	GSIS	Authors
1 – 1000 nM	HIT-T15 cell line	1 hour	Decrease (10mM glucose)	-	Nagata 2019 [173]
1 µM	Rat primary islets INS-1 cell line	4 hour	Increase	No change	Melkersson 2001 [172] Melkersson 2004 [171]
5 µM	Human islets	1 hour	-	Increase (15mM glucose) Very high variability between donors	Simpson 2012 [235]

Table 15 Summary of *In vitro* insulin secretion studies: Olanzapine. Glucose stimulated insulin secretion (GSIS)

The studies performed by Melkersson *et al*. (2001, 2004) (**Table 15**) suggest that 1 µM olanzapine can increase basal secretion with no change in GSIS [167,168], whereas Nagata *et al*. (2019) proposed that at concentrations up to 1 µM olanzapine there is reduced insulin secretion during a 1 hour exposure to 10 mM glucose [173]. Conversely, Simpson *et al.* (2012) reported that human donor islets secreted more insulin than control islets when incubated with 5 µM olanzapine in 15 mM glucose for 1 hour, although there was very high variability between donors [235]. However these short exposure times are insufficient to make conclusions. An increase in basal secretion and increase in GSIS does not fit the observed clinical effects and should be probed further. Olanzapine is a drug that is administered for long periods of time and reaches steady state, thus additional work investigating olanzapine induced changes to insulin secretion following chronic exposure is highly necessary.

While olanzapine and clozapine are distinctly individual drugs with different side effects, both share a high risk of metabolic adverse events and diabetes [62]. There are several structural and pharmacological similarities between the drugs which are unique compared to other second generation antipsychotic drugs. Each has been associated with a specific binding profile at a select number of GPCRs and both show antagonist activity at a complement of dopamine, 5-HT, α, muscarinic and histamine receptors [75]. As shown in **Table 16** clozapine has been found to increase basal secretion (<4mM glucose) and decrease glucose stimulated (>20mM

glucose) secretion in primary islets and cultured beta cell lines, which appears to be dose and time dependent [171, 172, 174, 175, 233, 234]. It remains unclear if olanzapine has the same effects on insulin secretion.

Clozapine concentration	Model	Exposure time	Basal secretion	GSIS	Authors
1 μM	Rat primary islets	1 hour	No change	No change	Melkerrson 2001 [172] Melkersson 2004 [171]
	INS-1 cell line	4 hour	Increase	-	Melkersson 2007 [174]
	Rat primary islets	7 days	-	Decrease	Sasaki 2006 [233]
5 μM	Rat primary islets	1 hour	No change	Decrease	Best 2005 [175]
	Rat primary islets	7 days	-	Decrease	Sasaki 2006 [233]
	Human islets	1 hour	-	Increase (15mM glucose)	Simpson 2012 [235]
10 μM	Rat primary islets	1 hour 4 hour	- Increase	Decrease Decrease	Melkersson 2007 [174]
	Rat primary islets	7 days	-	Decrease	Sasaki 2006 [233]
25 μM	INS-1 cell line	24 hour	Increase	Decrease	Menga 2013 [234]
100 μM	Rat primary islets	4 hour	Increase	Decrease	Melkersson 2007 [174]

Table 16 Summary of *In Vitro* insulin secretion studies: Clozapine. Glucose stimulated insulin secretion (GSIS)

An important clinical difference between clozapine and olanzapine is the high risk of agranulocytosis associated with clozapine [236], hence clozapine is initiated under specialist supervision for severe cases of treatment resistant SMI alongside intensive and ongoing monitoring. Conversely, olanzapine is frequently prescribed in primary and secondary care as a first line treatment for SMI to patients who are

monitored less frequently. For that reason, it is imperative to further investigate if olanzapine impacts GSIS to the same extent as clozapine to help shape future medical care of patients. This research aimed to investigate any changes in insulin secretion from the MIN6 cell line in response to chronic olanzapine exposure (**Figure 14**).

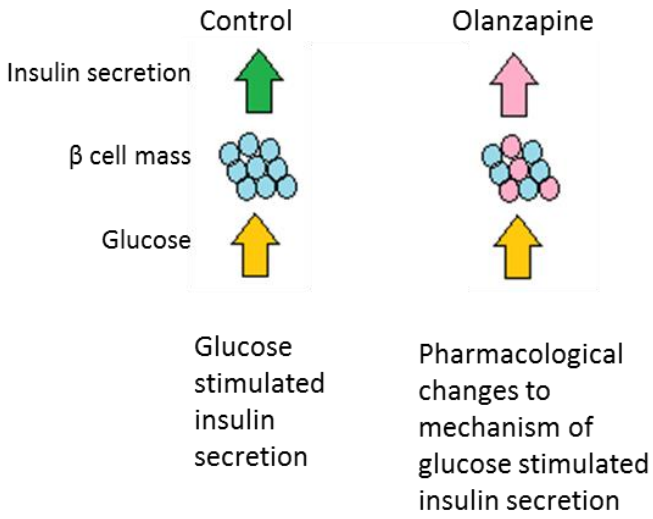


Figure 14 Illustration depicting the third hypothesis that olanzapine has a pharmacological action on the beta cell to modify the mechanism of glucose stimulated insulin secretion

To summarise, the currently reported *in vitro* effects of olanzapine on beta cell viability and function are limited, however the clinical data suggest that olanzapine does have a detrimental effect to prevent adequate insulin secretion to prevent hyperglycaemia. There have been few studies carried out investigating beta cell response to therapeutic concentrations of olanzapine for prolonged periods of time, and it is presently difficult to make conclusions about how olanzapine alters the functioning of beta cells independently of the accompanying insulin resistance. The first aims of the study were to investigate the effect of olanzapine on the viability and function of a beta cell line. Using the mouse insulinoma cell line MIN6, we aimed to investigate if olanzapine would produce similar effects on insulin secretion as have been demonstrated in different cell lines exposed to clozapine.

3.2 Results

3.2.1 Effect of olanzapine on MIN6 viability

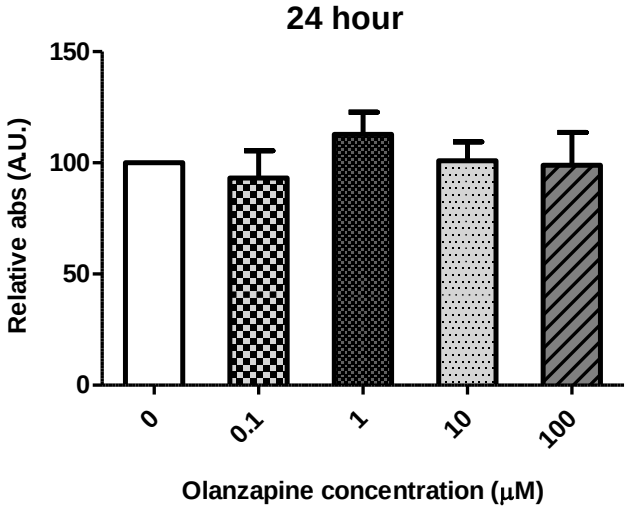
3.2.1.1 MTT assay

The first aim of this study was to investigate if olanzapine is toxic to MIN6 beta cells. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay was used to determine if the viability of a sample of MIN6 cells was reduced by acute or chronic treatment with olanzapine. Mitochondrial dehydrogenase enzymes in viable, metabolically active cells reduce MTT to an insoluble purple formazan product that can be detected using a spectrophotometer. The resulting absorbance is proportional to the metabolic activity of the cell sample and this can be used to determine cell viability.

A range of concentrations of olanzapine from 0.1 µM to 100 µM was tested to investigate any concentration dependent effects. As shown in **Figure 15**A acute treatment with olanzapine for 24 hours produced no significant changes in cell viability at any concentration.

To investigate chronic effects of olanzapine, the duration of exposure was extended to 72 hours. **Figure 15**B show that exposure to therapeutic concentrations of olanzapine from 0.1 µM – 10 µM did not produce any changes in MTT absorbance compared to control. There was a significant reduction in viable cells in the sample that was exposed to 100 µM compared to control (44.47 ± 2.65 %, $p < 0.001$). This suggests there is a significant reduction in viability, but this may also be attributable to decreases in mitochondrial metabolism.

A.



B.

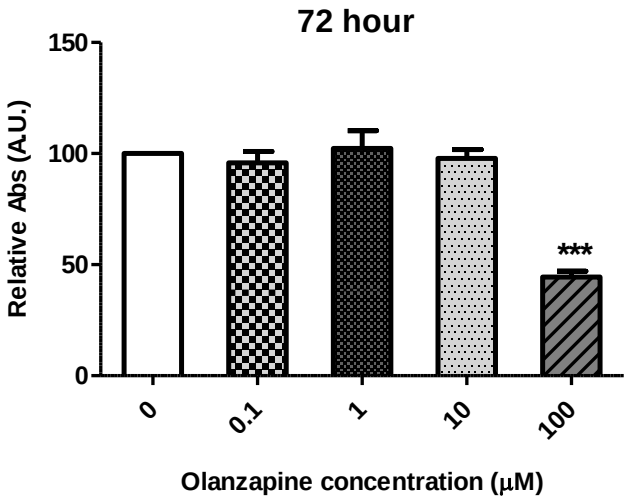


Figure 15 Effect of 24 and 72 hours olanzapine exposure on MIN6 cell viability. MIN6 cells were treated with olanzapine concentrations ranging from 0.1 – 100 μM as indicated for 24 hours (A) and 72 hours (B). Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Results shown are of 4 independent experiments. Data are displayed as mean ± SEM, ***p<0.001 versus control using one-way ANOVA and Bonferroni's Post-test.

3.2.1.2 Fluorescent staining

The MTT results suggested that chronic treatment with olanzapine 0.1- 10 µM caused no changes in cell viability, and 100 µM olanzapine significantly reduced cell viability. The MTT assay does not differentiate changes in metabolic activity of the sample from changes in cell number, and results could be influenced by drug induced metabolic fluctuations. To further investigate the effects of olanzapine on cell viability, and provide a more specific measure of cell death associated with chronic treatment with olanzapine, fluorescent staining with acridine orange and DAPI was used to measure total live and dead cells.

Figure 16 shows that there was no significant difference in total dead cells detected at any concentration of olanzapine following 72 hours of incubation. Thus it can be reasoned that olanzapine does not increase cell death at concentrations <100 µM for 72 hours. Treatment with 100 µM olanzapine resulted in significantly fewer live cells compared to control ($p<0.01$) however, as the dead cells did not change, the reduction in live cells may be attributed to cytostatic effects rather than an increase in cell death. There were no significant differences in total live cells at any lower concentrations of olanzapine.

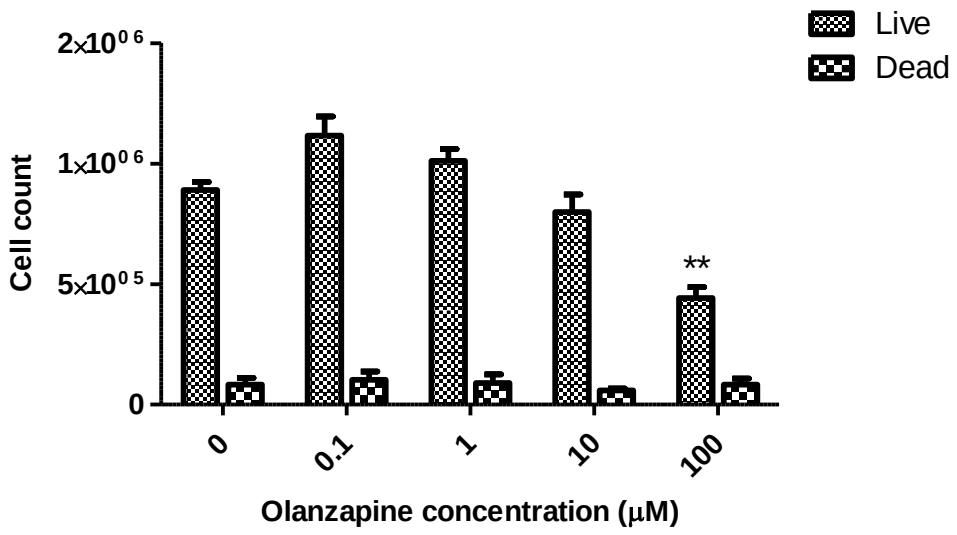


Figure 16 Effects of 72 hours treatment with olanzapine on MIN6 cell death. MIN6 cells were treated with olanzapine concentrations ranging from 0.1 – 100 µM as indicated for 72 hours. Cell death was assessed by fluorescent staining with Acridine orange and DAPI. Results shown are of 3 independent experiments. Data are displayed as mean ± SEM, **$p<0.01$ versus control using one-way ANOVA and Bonferroni's Post-test

3.2.2 Effect of olanzapine on beta cell function

3.2.2.1 Glucose stimulated insulin secretion

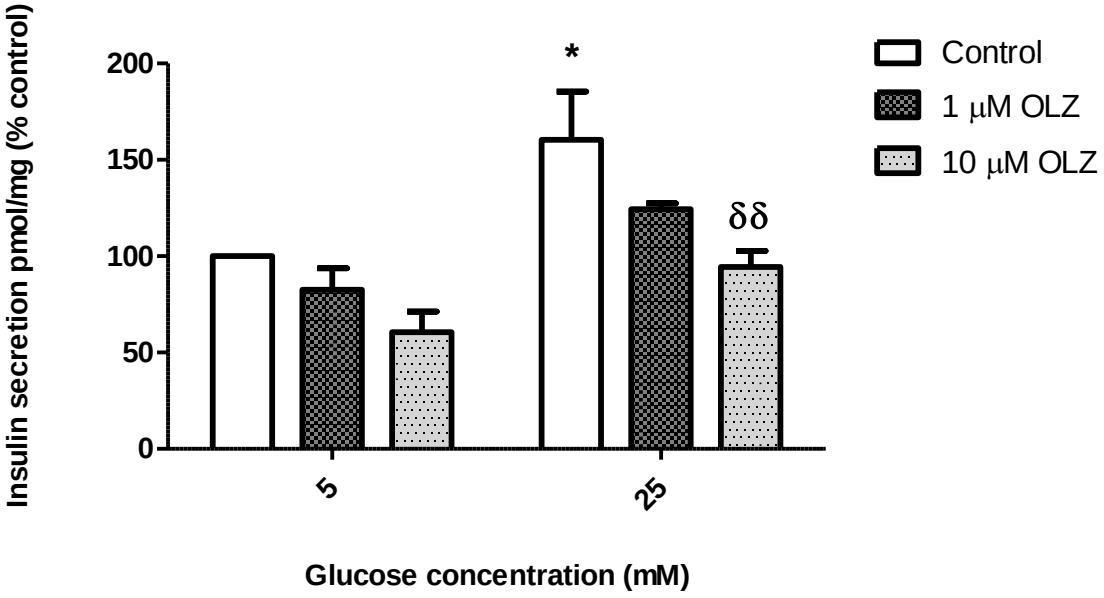
Treatment with therapeutic concentrations of olanzapine ≤10 µM did not reduce cell viability; the next aim of this study was to investigate if olanzapine produced changes to beta cell function. The primary function of beta cells is to secrete insulin in response to increases in extracellular glucose. The hypothesis that chronic olanzapine treatment would reduce glucose stimulated insulin secretion (GSIS) from MIN6 cells was tested by measuring insulin secretion.

Following chronic exposure to 1 µM or 10 µM olanzapine for 72 hours, MIN6 were glucose-starved to standardise the sample, followed by exposure to low (5 mM) or high (25 mM) glucose for 2 hours. As shown in **Figure 17**A MIN6 cells secreted insulin in response to 5 mM and 25 mM glucose, and the amount of insulin secreted in response to 25 mM glucose was significantly more than that at 5 mM ($p<0.05$). This confirmed that MIN6 cells were glucose responsive and a suitable model for GSIS studies.

Figure 17A shows that there was a trend for cells that had been incubated with 1 µM olanzapine for 72 hours to secrete slightly less insulin than control cells in response to glucose but this was not significant. Cells that had been incubated with 10 µM olanzapine for 72 hours secreted moderately less insulin in response to low glucose but this was not significant. In response to high glucose there was significantly less insulin secretion compared to control ($p<0.01$) suggesting that chronic exposure to 10 µM olanzapine may result in cellular changes that interfere with the glucose stimulated insulin response.

To further explore if the observed reduction in insulin was due to a deficiency in insulin synthesis or a defect in the mechanism of insulin secretion, cell lysates were assayed for total intracellular insulin content. There was no observed change in intracellular insulin content at any concentration as shown in **Figure 17**B This suggests that the effects of olanzapine on insulin secretion are associated with the mechanics of secretion.

A.



B.

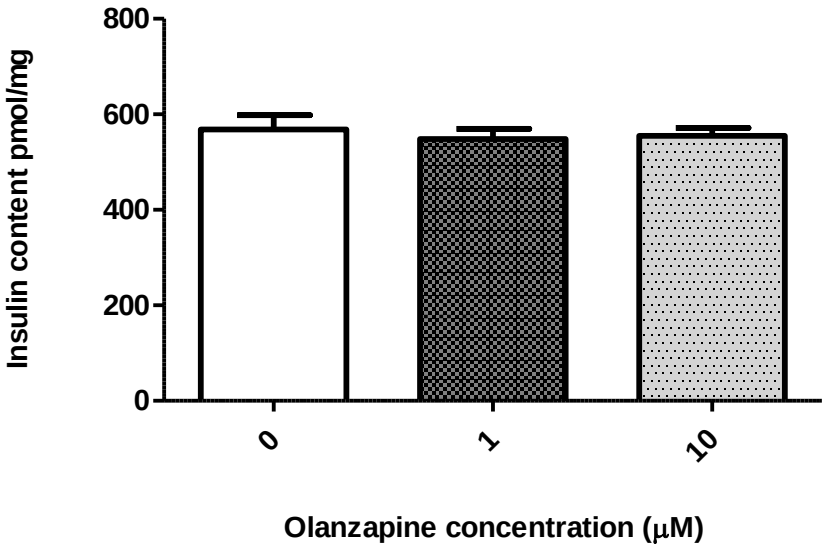


Figure 17 Effects of olanzapine on insulin secretion and content in MIN6 cells. MIN6 cells were incubated with 1 μM or 10 μM olanzapine as indicated for 72 hours prior to GSIS investigation or intracellular insulin quantification. Following treatment cells were incubated with glucose free culture media followed by exposure to low (5 mM) or high (25 mM) glucose. Supernatant was collected and insulin concentration determined with ELISA and normalised to protein content (A). Cells were lysed in an acid ethanol solution and insulin content assayed by ELISA (B). Results shown are of 3 independent experiments. Data are displayed as mean ± SEM, *$p<0.05$ versus control (5 mM glucose) δδ $p<0.01$ versus control (25 mM glucose) using two-way ANOVA and Bonferroni's Post-test

3.2.3 Proliferation

Another feature of the beta cell is the ability to increase in number at times of increased demands such as during insulin resistance or obesity. During beta cell compensation stimuli such as glucose or growth factors initiates cell proliferation to increase the cell mass [193]. Serotonin (5-HT) has also previously been associated with increases in beta cell mass during pregnancy through interaction with 5-HT2B receptors [196]. Olanzapine is an antagonist at 5-HT2 receptors [194] so we hypothesised that there may be an inhibition of proliferation. The next aim of the study was to test the hypotheses that olanzapine inhibits an increase in cell proliferation in response to proliferative stimuli.

To minimise simultaneous effects between growth factors and other stimuli, the FBS concentration in the culture media was reduced to 2%. Cells were incubated with 10 µM and 100 µM olanzapine for 72 hours in low glucose high FBS (5 mM glucose 10% FBS), low glucose low FBS (5 mM glucose 2% FBS), high glucose (25 mM glucose 2% FBS), and serotonin (5 µM 5-HT, 5 mM glucose, 2% FBS). The MTT assay was carried out at different time points and MTT absorbance was interpreted to represent the size of the cell population and show the increase in mass during the 72 hour exposure. For these studies cell confluency was reduced to minimise contact inhibition of growth during the 72 hour experiment.

As shown in **Figure 18**A-B Reducing the FBS concentration from 10% to 2% significantly reduced the proliferation of control MIN6 cells, and the MTT absorbance of the 2% control group was significantly lower at 48 hours ($p<0.05$) and 72 hours ($p<0.001$) than the 10% control group (2 way ANOVA with Bonferroni post-test, n=4). As shown in **Figure 18**B-D culturing with high glucose ($p<0.01$) and 5-HT ($p<0.05$) resulted in a significantly elevated MTT absorbance at 72 hours compared to low glucose (2 way ANOVA with Bonferroni post-test, n=4), indicating that these culture conditions independently stimulated proliferation.

The influence of 100 µM olanzapine to inhibit proliferation was evident in all stimulated culture conditions. MTT absorbance of cells treated with 100 µM olanzapine at 72 hours was significantly lower compared to control in high glucose conditions ($p<0.001$) (**Figure 18**C) and 5-HT ($p<0.001$) (**Figure 18**D). However, 10 µM olanzapine did not significantly inhibit proliferation in response to FBS, glucose or 5-HT, which does not support the initial hypothesis.

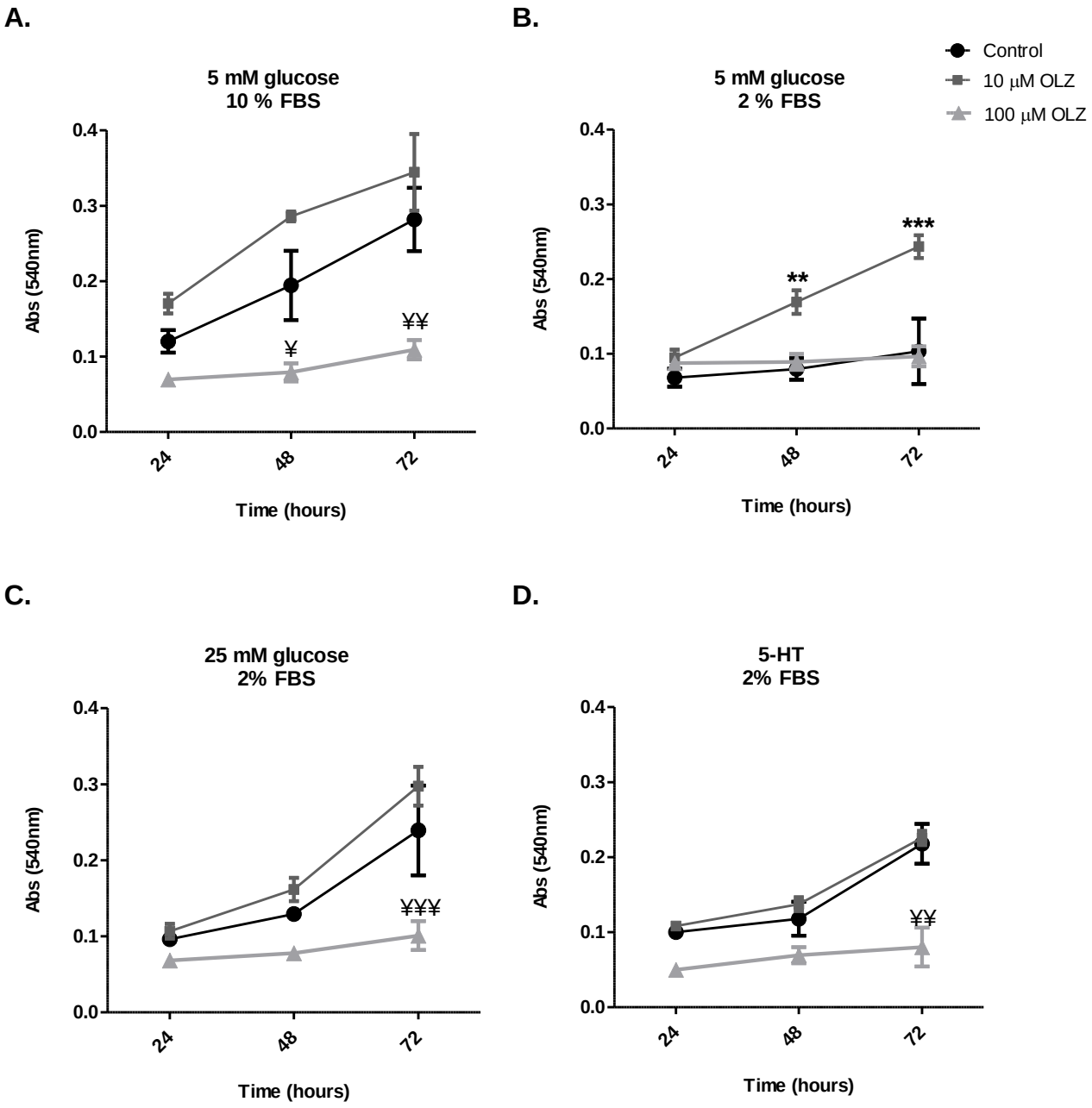


Figure 18 Effects of olanzapine on proliferation in different culture conditions. MIN6 cells were incubated with 10 µM or 100 µM olanzapine as indicated for 24, 48 and 72 hours in different stimulation conditions. Low glucose 10% FBS (A) low glucose 2% FBS (B), high glucose 2 % FBS (C) or low glucose + 5 µM 5-HT 2% FBS (D). MTT assay was performed and absorbance read at 540 nm at each time point. Results shown are of 4 independent experiments. Data are displayed as mean ± SEM, **p<0.01 *** p<0.001 10 µM olanzapine versus control, ¥ p<0.05, ¥¥ p>0.01, ¥¥¥ p<0.001 100 µM olanzapine versus control using two-way ANOVA and Bonferroni's Post-test

As shown in **Figure 18**B, a surprising observation was made that in unstimulated conditions, 10 µM olanzapine was able to independently stimulate proliferation of MIN6 cells. There was significantly higher MTT absorbance detected at 48 hours ($p<0.01$) and 72 hours ($p<0.001$) in cells treated with10 µM olanzapine compared to control. To confirm if this observation was a true increase in total cells rather than an increase in mitochondrial metabolic activity stimulated by olanzapine, cell counts were performed after 72 hour incubation in low and high glucose (2% FBS). **Figure 19** confirmed that incubation with 10 µM olanzapine resulted in a significant increase in total cells compared to control in low glucose, low FBS unstimulated conditions ($p<0.01$).

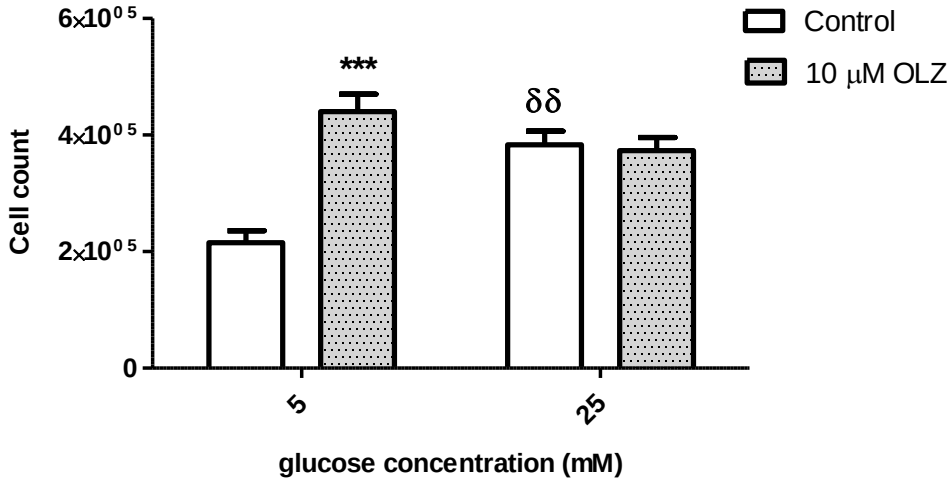


Figure 19 Effects of olanzapine on cell number after 72 hours incubation in different glucose concentrations. MIN6 cells were incubated with 10 µM olanzapine as indicated for 72 hours in low glucose (5 mM) and high glucose (25 mM) 2% FBS conditions. Cells were detached and counted using a haemocytometer. Results shown are of 3 independent experiments. Data are displayed as mean ± SEM, **$p<0.01$ versus control (5 mM glucose) δδ $p<0.01$ versus control (25 mM glucose) using two-way ANOVA and Bonferroni's Post-test

These results imply that in the absence of a stimulus 10 µM olanzapine can increase numbers of viable MIN6 cells over time. To further test the effect of a range of therapeutic concentrations on proliferation, the experiment was repeated with 0.1 µM, 1 µM and 10 µM olanzapine. **Figure 20** shows that all concentrations tested were able to produce a concentration dependent stimulation of proliferation. 0.1 µM had the weakest response, and at 48 hours there was no significant difference in MTT absorbance compared to control, however at 72 hours there was a significant difference ($p<0.01$) indicating that there was stimulated proliferation. 1 µM olanzapine produced a similar response to 10 µM olanzapine and there was significantly increased MTT absorbance at 48 ($p<0.01$) and 72 hours ($p<0.01$).

Therapeutic concentrations of olanzapine do not influence stimulated proliferation; but these data suggest that in the absence of a proliferative stimulus olanzapine can independently stimulate proliferation.

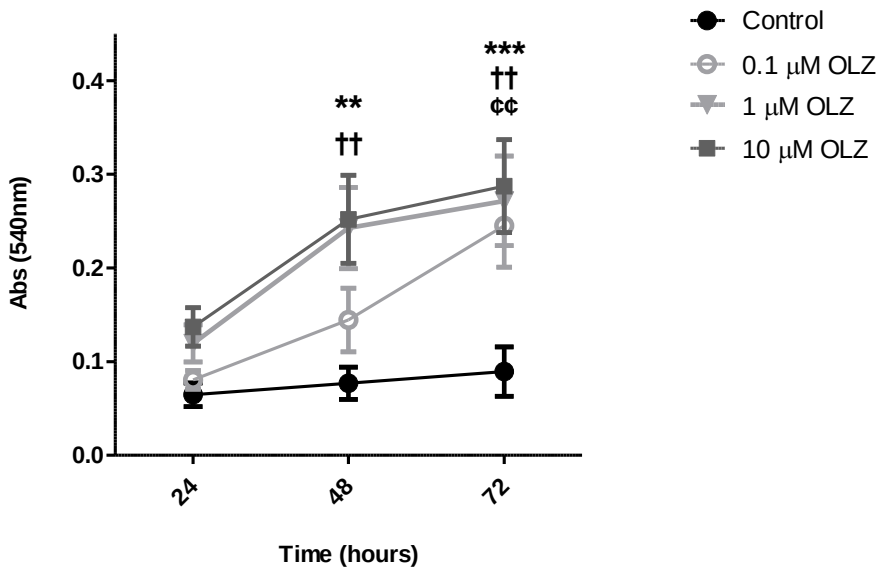


Figure 20 Effects of different concentrations of olanzapine on proliferation in unstimulated conditions MIN6 cells were incubated with 0.1, 1 or 10 µM olanzapine as indicated for 24, 48 and 72 hours in unstimulated conditions (low glucose 2 % FBS) MTT assay was performed and absorbance read at 540 nm. Results shown are of 4 independent experiments. Data are displayed as mean ± SEM, **p<0.01 *** p<0.001 10 µM olanzapine versus control, †† p>0.01 1 µM olanzapine versus control, ¢¢ p>0.01 0.1 µM olanzapine versus control using two-way ANOVA and Bonferroni's Post-test

3.2.4 The Cell Cycle

To further investigate the observation that 10 µM olanzapine increases proliferation and cell number during 72 hour incubation in unstimulated conditions (low glucose, low FBS) flow cytometry was used to establish the effect of olanzapine on the cell cycle.

MIN6 cells were cultured in serum free media for 24 hours to synchronise the cell population in G0 (baseline) then cells were treated with 10 µM olanzapine in culture media containing 5 mM glucose and 2% FBS for 24 and 48 hours. MIN6 cells have a doubling time of 48 hours [237] indicating that the cell population should go through one revolution of the cell cycle approximately each 48 hours.

Cells were fixed at each stage and stained with DNA dye, propidium iodide (PI). PI relative fluorescence intensity (RFU) was proportional to the total DNA in each individual cell, and can be extrapolated to determine stage of the cell cycle. Cells in G2 have approximately double the DNA concentration of those in G1 and when plotted on a histogram there are 2 peaks indicating the number of cells in each stage of the cell cycle. **Figure 21**A-B shows representative graphs of PI intensity after cell cycle synchronisation (baseline), and after 24 hours and 48 hours incubation with 10 µM olanzapine.

Figure 21 shows that following serum starvation, 24 hour incubation with FBS containing media increased the proportion of control cells in G2/M compared to the baseline serum starved group ($p<0.05$), which suggests that there was stimulation of the cell cycle. After 48 hours of incubation, the proportion of cells in the sample in stage G2 is higher than that at 24 hours, indicating cell cycle progression.

Compared to control cells 24 hour incubation with 10 µM olanzapine resulted in a significant reduction in the proportion of cells in stage G0/G1 ($p<0.05$) and significantly more cells in stage G2/M of the cell cycle ($p<0.05$) but there was no change to the proportion of cells at any stage after 48 hours of incubation (**Figure 21**D-E). This suggests that 10 µM olanzapine moderately accelerated the transition from G1-G2 in MIN6 cells.

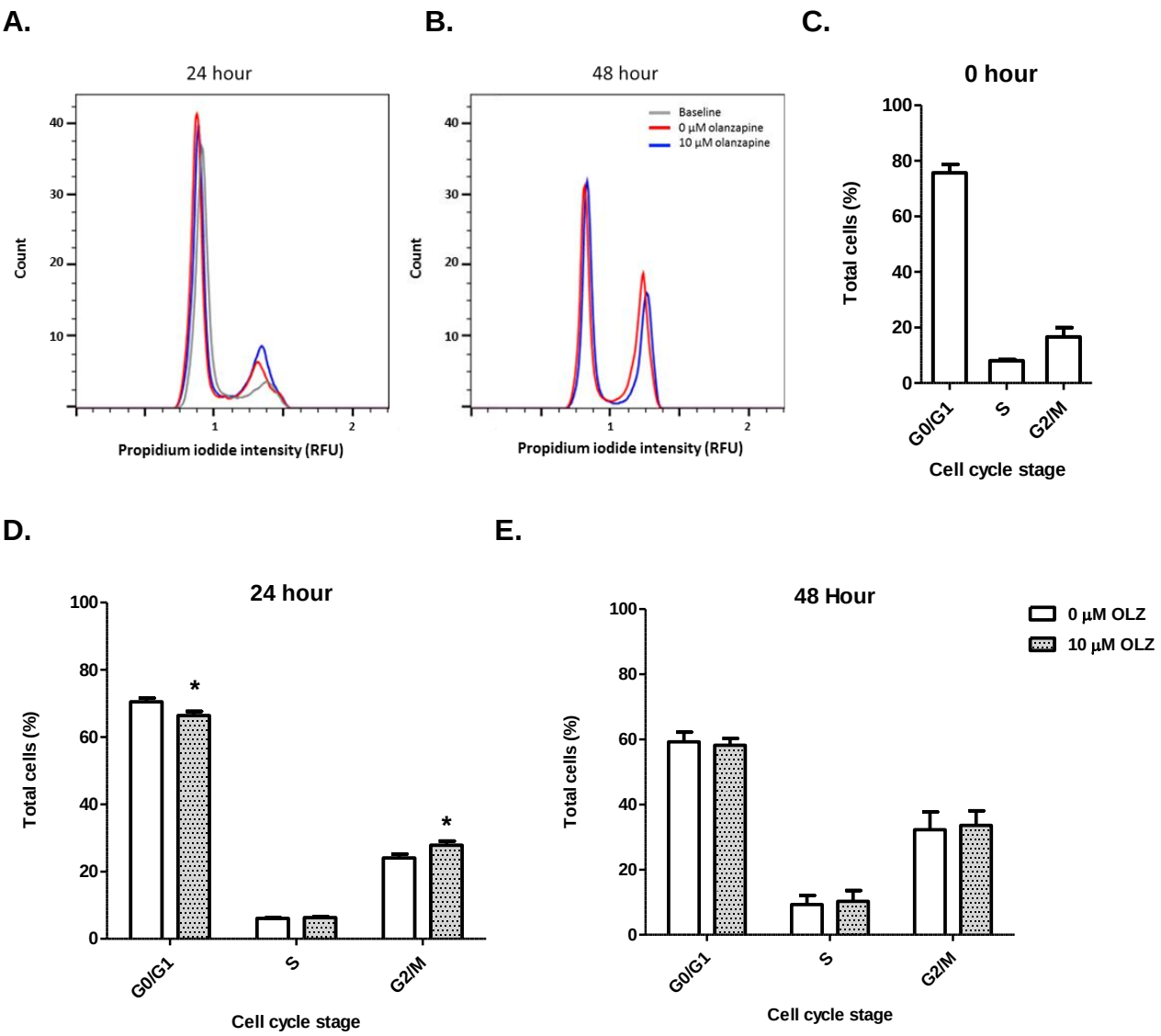


Figure 21 Effects of olanzapine on MIN6 cell cycle. MIN6 cells were serum starved for 24 hours to synchronise the population in G0 (baseline) (A, C) then incubated for 24 hours (A, D) or 48 hours (B, E) with 10 µM olanzapine. Following treatment cells were stained with propidium iodide and cell cycle stage measured by FL2-A on flow cytometry. The cells in G2 have approximately double the DNA content than those in G1. Representative graphs showing the DNA intensity at 24 and 48 hours (A, B). Calculated proportion of cells in each stage of the cell cycle from 4 independent experiments (C, D, E). Data are displayed as mean ± SEM, *p<0.05 versus control using two-way ANOVA and Bonferroni's Post-test

3.3 Discussion

This Chapter aimed to explore the effects of olanzapine on viability and function of MIN6 beta cells. There is a common link between olanzapine and development of hyperglycaemia, so we hypothesised that olanzapine has pharmacological effects at the pancreatic beta cells to cause cell death or to reduce function. The presented data show that 72 hour exposure to 10 µM olanzapine did not increase cell death, but resulted in a significant reduction in glucose stimulated insulin secretion. Proliferation of cells was also significantly altered by olanzapine, with stimulation at low concentrations, and inhibition at high concentrations. Taken together the results suggest there are pharmacological targets of olanzapine that can disrupt normal beta cell function.

3.3.1 Acute olanzapine exposure does not reduce beta cell viability at any concentration

The initial hypothesis that olanzapine is toxic to beta cells and will increase beta cell death was investigated using the MTT assay and fluorescent staining. There was no observed reduction in cell viability, but this is in contradiction to data presented by Ozasa *et al*. (2013) who proposed that olanzapine is toxic to insulin secreting beta cell lines [55]. Ozasa *et al.* (2013) used the Terminal deoxy transferase-mediated dUTP-X nick end labelling (TUNEL) assay and DNA fragmentation assay to determine DNA breakage as an assessment of apoptosis. They reported that 24 hours MIN6 incubation with 100 µM olanzapine caused 15% of the cells to become apoptotic, compared to 3% in the control group, which suggests that the remaining cell population is 85% viable. Conversely using the MTT assay to investigate cell viability **Figure 15**A showed that cells incubated with 100 µM olanzapine for 24 hours were 98.96% viable.

According to the DNA damage response theory, DNA damage does not always lead to apoptosis, and may alternatively initiate cell cycle arrest, programmed cell death, or activation of DNA repair processes [238]. The MTT assay and cell counts used in the present work do not measure DNA damage, and do not give information relating to apoptosis, only necrotic cell death. In another study, cells that tested

highly positive for TUNEL activity were also found to be 100% viable by MTT [239]. Thus it is impossible to make comparisons between these results, instead we offer that 100 µM olanzapine causes DNA damage to beta cells resulting in cell cycle arrest, and inhibition of proliferation as is shown in this study (**Figure 18**). The observation that olanzapine inhibits cell proliferation might be clinically relevant in terms of reduced beta cell compensation; however 100 µM olanzapine is most likely a supra-therapeutic concentration and lower, therapeutic concentrations were not found to have the same cytostatic effect or any reduction in cell viability.

3.3.2 Chronic exposure to lower concentration olanzapine (10 µM) results in reduced GSIS when stimulated with high glucose

As concentrations < 10 µM of olanzapine were not found to affect beta cell viability, next the impact on cellular function was investigated. The primary function of the beta cell is to secrete insulin in response to fluctuating glucose concentrations to prevent hyperglycaemia. Patients treated with olanzapine often suffer rapid hyperglycaemia leading to the hypothesis that insulin secretion may be compromised by the drug. Olanzapine was shown to decrease the insulin secretion response to high glucose stimulation in a dose dependent manner as shown in **Figure 17**A.

To our best knowledge this is the first *in vitro* study showing that chronic exposure to a therapeutic concentration of olanzapine, 10 µM, significantly reduces GSIS from beta cells. This is a noteworthy observation as it is difficult to make assumptions about olanzapine effect on beta cell function in *in vivo* models separately from the accompanying insulin resistance which also negatively affects beta cell function.

There have been several previous *in vitro* studies that have probed changes in GSIS from beta cells and primary islets incubated with clozapine, but the only previous *in vitro* studies involving olanzapine found no change in GSIS [171, 172]. In previous studies Melkersson *et al.* (2001) (2004) showed that 1-4 hour exposure to 1 µM olanzapine did not affect insulin secretion in response to 16 mM glucose in rodent

islets or INS1 cells [171, 172]. However, the low tested concentration and short incubation time might delineate the lack of response.

In further work exploring clozapine only, Melkersson *et al.* (2007)[174] investigated a wider concentration range (1- 100 μM) and found that clozapine concentrations above 10 μM significantly reduced GSIS after 1 and 4 hour incubation. These results closely matched those presented by Best *et al*. (2005)[175] which suggested that clozapine concentrations higher than 5 μM inhibited GSIS by approximately 40% compared to control. An additional report by Menga *et al*. (2013)[234] added to the evidence that clozapine induced changes in insulin secretion are dose dependent. However, olanzapine was not investigated beyond the initial studies.

In additional clozapine studies, Sasaki *et al*. (2006) [233] highlighted the influence of incubation time on deterioration of GSIS, and showed that up to 3 day exposure to 1 μM clozapine there was no significant effect on GSIS, however after 7 days there was a significant inhibition of insulin secretion to approximately 53% of control.

The present data supports the theory that olanzapine similarly reduces GSIS in a dose dependent manner. It was found that 72 hour incubation with 10 μM olanzapine significantly reduced secretion, but 1 μM only mildly reduced secretion. Considering the patterns observed with clozapine, we may speculate that longer incubation with lower concentrations of olanzapine would also significantly reduce secretion.

There was no change in intracellular insulin content detected so it is reasoned that the changes are associated with alterations in secretory machinery of the cell.

3.3.3 Olanzapine can accelerate the cell cycle and result in increased proliferation of MIN6 in the absence of other stimulus

The beta cell mass can dynamically adapt to increase the insulin output at times of increased demand. The third hypothesis that olanzapine reduces the adaptive response of MIN6 cells in response to hyperglycaemia was tested in various culture conditions. It was found that therapeutic concentrations of olanzapine do not inhibit the proliferation of MIN6 cells in stimulatory conditions. However as shown in **Figure**

20, in the absence of other stimulation, addition of concentrations ≤10 μM olanzapine lead to increased proliferation of MIN6 cells and accelerated transition between G0/1 and G2 in the cell cycle (**Figure 21**). Unlike primary cells, MIN6 cells continually replicate so may not be an appropriate model to make final conclusions regarding olanzapine induced increases in cell proliferation in humans. But by reducing concentration of FBS and growth factors, basal proliferation was significantly reduced and the observed stimulatory effect of olanzapine may be indicative of specific pathways associated with proliferation and metabolism that are targeted by olanzapine which may have significance related to loss of function.

The effects of 100 μM olanzapine on beta cells by Ozasa *et al*. (2013) were found to include continuous protein synthesis, accumulation of intracellular insulin, mild endoplasmic reticulum (ER) stress and PERK activation [55]. PERK is involved in the unfolded protein response (UPR), and during states of ER stress PERK phosphorylates eIF2α to halt protein synthesis and inhibit translation [240]. It was deduced that the toxicity stimulated by 100 μM olanzapine occurred due to a blunted UPR response and continual protein synthesis which increases ER stress and activates cell death pathways. Therefore considering the observed effect of lower concentrations of olanzapine to stimulate cell proliferation, we hypothesise that there may be a specific receptor or signalling pathway within beta cells that is targeted by olanzapine to stimulates protein synthesis and growth, which at high concentrations becomes excessive and triggers the unfolded protein response to cause cell cycle arrest.

3.3.4 Conclusion

To conclude, the effects of chronic olanzapine treatment lead to reduced insulin secretion, which relates to the known hyperglycaemic side effects. The increase in proliferation suggests that olanzapine can stimulate one or more pathways resulting in cell cycle acceleration. Numerous pathways are involved in the regulation of cell proliferation, and the following Chapters will aim to investigate the influence of olanzapine on signalling pathways linked to increased proliferation, and aim to elucidate if these pathways are linked to cellular changes and reduced GSIS.

Chapter 4: The effect of olanzapine on cell cycle regulation and the canonical Wnt signalling pathway

4.1 Introduction

The data presented in Chapter 3 revealed that olanzapine can adversely alter the function of MIN6 beta cells; to stimulate proliferation, disrupt the cell cycle, and reduce GSIS. The following Chapters aimed to probe the pharmacological mechanism behind the effects of olanzapine on MIN6 cell function. In this Chapter the effect of olanzapine to stimulate proliferation was investigated, with special focus on interaction with the canonical Wnt signalling pathway. Further work in Chapter 5 builds on suggested mechanisms and examines if the signalling pathways involved in olanzapine induced proliferation are linked to changes in insulin secretion.

4.1.1 Proliferation

In Chapter 3 we made the novel observation that, in the absence of other stimulation, olanzapine ≤10 μM is able to stimulate MIN6 cell proliferation and accelerate progression of the cell cycle. An increase in proliferation suggests there may be a subsequent increase in beta cell mass, which is in contradiction to the pathogenesis of type 2 diabetes in which there is usually a progressive reduction in beta cell mass [45]. However, an increase in cell proliferation alongside reduced GSIS points to a loss of normal beta cell functioning instigated by olanzapine.

Beta cells are principally a differentiated cell type, and during differentiation primary beta cells withdraw from the cell cycle [241]. The total beta cell mass is maintained through low level proliferation, and neogenesis from stem or progenitor cells [242] and a steady rate of proliferation balanced with cell death maintains the overall beta cell mass [243, 244].

The replicative capacity of mature beta cells has been extensively investigated, as researchers pursue methods to increase the beta cell mass as a treatment for diabetes [245]. However beta cells have been proven to be somewhat resistant to replication. Replicating beta cells have been shown to have reduced functional capacity compared to quiescent cells, as the cell bioenergetics favour cell growth over insulin processing and secretion [246]. When investigating the genetic makeup of isolated replicating mature beta cells, it was found that upregulation of genes associated with cell proliferation was linked to significantly reduced expression of genes involved in beta cell function and GSIS [247]. C-myc is an important cell cycle

regulator in beta cells, which is upregulated by glucose to activate cyclins and stimulate cell cycle progression [248]. In a study where c-myc expression was modified, the pool of actively replicating cells increased, alongside a reduction in GSIS and expression of genes relating to normal insulin secretion [246]. This suggests that stimulation of cell proliferation can lead to an immature beta cell phenotype, or in cell dedifferentiation. The overall balance between cell replication and cell function is usually maintained through very low numbers of replicating cells at any time, however, if olanzapine is able to stimulate cell cycle entry and increase the number of replicating cells then overall beta cell function may indeed be expected to decline. This leads to the hypothesis that through increased cell proliferation, olanzapine negatively influences beta cell function.

4.1.2 Cell cycle regulation

The cell cycle is a highly regulated process in which a cell divides to make two daughter cells. Numerous pathways and mitogenic stimuli are able to activate the cell cycle, by triggering the irreversible progression from phase G1 to S. As illustrated in **Figure 22** the cell cycle initially starts in the resting phase G0 where cells are quiescent and do not divide. Cells in the G1 phase of growth increase in size, then upon stimulation move into S phase where there is DNA synthesis and duplication. In the G2 phase of growth, the cell continues to increase in size in preparation for M phase in which mitosis occurs to produce two daughter cells [249]. It is at the restriction point during G1 phase that cells are responsive to mitogenic stimulus to either trigger the cycle of cell division, or to exit the cell cycle and become quiescent. Thus the G1 restriction point is a crucial regulator of cell division, and beyond this point cells will continue with the process irrespective of ongoing mitogenic stimulation [249].

The G1 phase restriction point is predominantly activated by cyclin and cyclin dependent kinase (Cdk) regulation of the Retinoblastoma protein (Rb) pathway. As shown in **Figure 22** when stimulated, Cyclin and Cdk complexes trigger the phosphorylation and inactivation of Rb, and then E2F is available to initiate processes involved in cell cycle entry. There are numerous phosphorylation sites on the Rb molecule, and it is coordinated phosphorylation by cyclins and Cdk complexes which result in ongoing inactivation and cell cycle progression. Cyclin D/

Cdk4/6 complexes phosphorylate Rb during early G1, cyclin E/ Cdk2 phosphorylate in late G1, and cyclin A/ Cdk2 maintain phosphorylation during S phase [250, 251].

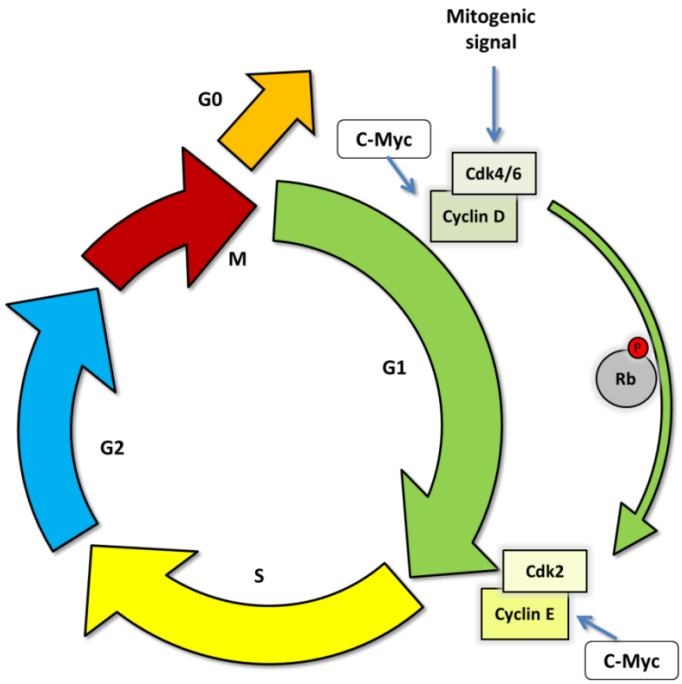


Figure 22 Schematic diagram showing some key regulators of cell cycle progression through G1

During the course of the cell cycle, cyclin concentrations fluctuate through regulated transcription, synthesis and destruction, and only during specific windows are cyclins available to form a complex with Cdks to initiate activity. The D type cyclins are crucial for entry into the cell cycle, and unlike other cyclins these are only expressed in the presence of mitogenic stimulation [252]. In mature human beta cells, the cell population is predominantly quiescent, but cells are able to respond to mitogenic stimulation through increased expression of D type cyclins. Different mitogenic stimuli include glucose, amino acids, free fatty acid (FFA), insulin, IGF-1, GLP-1, growth hormone, prolactin, and leptin [253]. All three cyclins; D1, D2, and D3 are expressed in islets with overlapping function, but cyclin D2 is expressed at 10 fold higher levels than cyclin D1, and cyclin D3 is expressed at very low levels compared to D1 and D2 suggesting its activity in beta cells is low [254].

Amplified expression of D cyclins results in early entry to S phase and consequently accelerates the cell cycle [255]. The observation that olanzapine was able to

stimulate proliferation in MIN6 cells and accelerate the transition between G1 and S leads to the first hypothesis that olanzapine can alter expression of D cyclins to accelerate entry to the cell cycle.

4.1.3 Mitogenic pathways

Different mitogenic stimuli exert diverse effects on beta cell proliferation by activating signal transduction pathways that increase expression or stability of transcription factors and cyclins. For example; Insulin/ IGF-1 can induce proliferation through activation of AKT and mitogen-activated protein kinase (MAPK) signalling pathways [256]. Canonical Wnt signalling can stimulate proliferation through upregulation of c-myc and cyclin D1 [257, 258]. Incretins stimulate multiple pathways such as mammalian target of rapamycin (mTOR) that impact proliferation [259]. Serotonin receptor activation also has a role to increase proliferation, which is thought to be associated with increased beta cell mass during pregnancy [196]. Olanzapine is pharmacologically active at several membrane bound receptors that are linked to cell proliferation, and there is evidence for interaction with numerous intracellular signalling pathways. We propose that in MIN6 beta cells, olanzapine interacts with one or more mitogenic signalling pathways to induce early entry to the cell cycle and adversely affect function.

Three significant pathways involved in beta cell proliferation include; Akt/ mTOR pathway, Ras/ extracellular-regulated kinase (ERK)/ MAPK pathway, or the canonical Wnt signalling pathway [257, 258]. Olanzapine has previously been linked to each of these pathways in various cell types, but due to varying functions of different tissue, some interactions and outcomes are likely to be cell type specific.

4.1.3.1 mTOR

The AKT/mTOR pathway is activated in response to growth factors and amino acids to stimulate protein synthesis, leading to accumulation of biomolecules required for cell division [260]. mTOR activation can also modulate expression and stability of cyclin D2, D3 and CDK4, critical factors in the G1 – S transition [261]. Olanzapine has previously been shown to activate AKT in PC12 cells [262] and mTOR in hepatocytes [138] so a potential interaction with mTOR in beta cells may be of

interest. However, in isolated islets and INS-1E beta cells mTOR activation has been shown to increase individual cell size, increase intracellular insulin synthesis and content, and increase GSIS [263]. These observations are in contrast to the presented data detailing the pharmacological effects of olanzapine on beta cell function to reduce insulin secretion, with no impact on intracellular content. This suggests if there is an interaction between olanzapine and mTOR in beta cells, it is minimal, or that there are several other converging pathways involved in the observed functional decline instigated by olanzapine.

4.1.3.2 MAPK

There are numerous MAPK pathways also involved in beta cell proliferation. The classic MAPK signalling cascade Ras-Raf-ERK leads to activation of several transcription factors including c-myc [264]. Through modification of activity of transcription factors, the MAPK pathway leads to increased expression of cell cycle regulator cyclin D1 [265]. In PC12 cells, olanzapine triggers ERK activation to stimulate proliferation [262] and in Chinese hamster ovary cells (CHO) transfected with 5-HT_{1A} receptors olanzapine has also been shown to moderately phosphorylate ERK and MAPK [266]. In beta cells, glucose can stimulate ERK activation to increase transcription of beta cell specific genes, but there is negligible effect on insulin secretion, and ERK inhibition has no effect on GSIS [267, 268]. We suggest that if olanzapine modifies ERK signalling to stimulate proliferation in MIN6 cells, this does not account for the GSIS decline, and there may multiple converging pathways involved in the pharmacological effects of olanzapine on beta cell function.

4.1.3.3 Wnt

Stimulation of the canonical Wnt signalling pathway directly activates transcription of cyclin D1 and c-myc, which are essential regulatory components of cell cycle stimulation [269, 270]. In beta cells, Wnt signalling activation has been associated with beta cell compensation mechanisms, leading to increased cell proliferation [271, 272]. In a model of type 2 diabetes, mice were fed a high fat diet, and key markers of Wnt signalling such as beta catenin nuclear translocation, and upregulation of cyclin D1 and c-myc was detected in pancreatic islets [273].

Furthermore, stimulation of the Wnt signalling pathway has been shown to have an important influence on insulin secretion [274], with specific predicted roles for beta catenin [210, 275] and TCF7L2 [276] within the insulin secretory mechanism. Olanzapine significantly reduces beta catenin concentration in osteoblasts [277] and has been shown to modulate Wnt and beta catenin signalling In glioma stem-like cells (GSLC) [278]. It has also recently been inferred that the Wnt signalling pathway effector TCF7L2 mediates some of the metabolic side effects of olanzapine such as weight gain and glucose intolerance [135]. The dual role of the Wnt pathway to modify proliferation as well as insulin secretion echoes the effects of olanzapine to influence both proliferation and insulin secretion, which highlights this as a pathway of interest. This leads to the next hypothesis that olanzapine interacts with the canonical Wnt pathway in MIN6 beta cells to influence function.

4.1.4 Canonical Wnt pathway

Beta catenin cytoplasmic concentration is central to the activity of the canonical Wnt pathway, illustrated in **Figure 23**. Beta catenin is a multifaceted protein that is involved in proliferation [273], insulin secretion [210], and is also a key component of cell adherens junctions [279].

As shown in **Figure 23**A in the absence of Wnt activation at membrane bound frizzled receptors, cytoplasmic beta catenin interacts with the 'destruction complex'. The destruction complex is comprised of glycogen synthase 3 (GSK3), casein kinase 1α (CK1α), Axin (Axin and Axin2), and adenomatous polyposis coli (APC). Axin, as the central scaffold protein, directly interacts with, and leads to coordinated phosphorylation of all components of the destruction complex resulting in a large complex that targets beta catenin for proteasomal degradation.

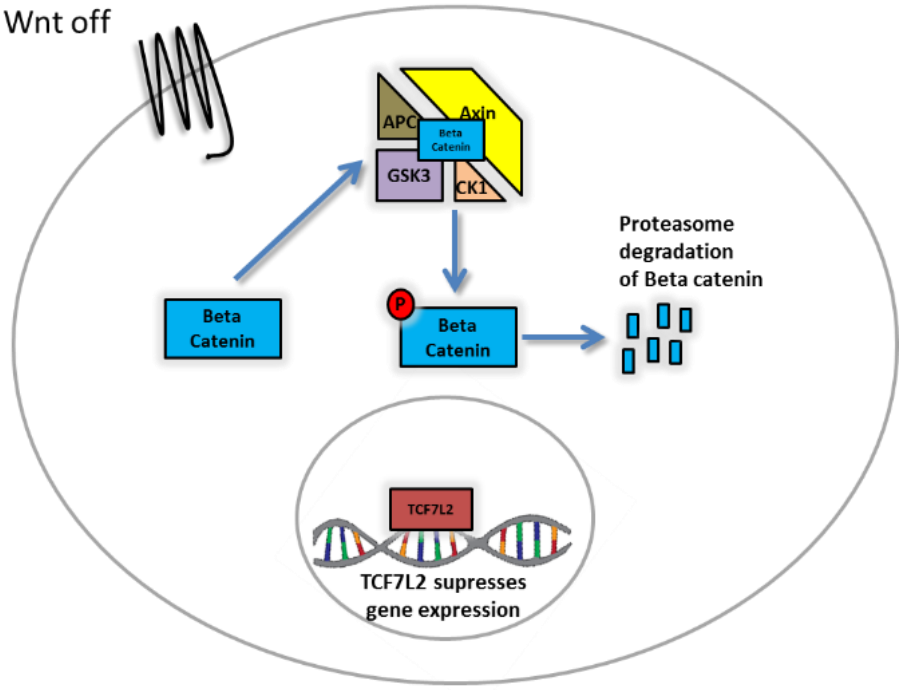
Briefly, the priming kinase CK1α first phosphorylates beta catenin at Ser 45, GSK3β subsequently phosphorylates beta catenin at Ser 33, Ser 37 and Ser 41, followed by phosphorylation of APC by CK1α and GSK3β which increases the bond between APC and beta catenin. The beta catenin is successively transferred from Axin to APC, which exposes the phosphorylated beta catenin to the ubiquitin ligase, leading to ubiquitinisation and degradation by the proteasome [280, 281]. Through continual degradation, beta catenin does not accumulate or translocate to the nucleus, thus Wnt signalling is switched off and target genes are not expressed [281, 282]. All

components of the destruction complex have the capacity to regulate the free beta catenin concentration, therefore altering concentration or phosphorylation of any protein can have a substantial impact on signalling.

Wnt ligands are secreted glycoproteins that bind to the G protein coupled frizzled receptors and low density lipoprotein receptor related protein 5 or 6 (LRP5/6) co-receptors [283]. As shown in **Figure 23**B Wnt ligand binding at the frizzled receptor activates the intracellular protein dishevelled (Dvl) which initiates intracellular disruption of the beta catenin destruction complex, and recruitment to the cell membrane. This leads to beta catenin cytoplasmic accumulation and translocation to the nucleus, where it forms a bipartite transcription factor with TCF7L2 and increases expression of Wnt downstream target genes, many of which are involved in cell proliferation [281].

As our results suggest that olanzapine can interfere with proliferation and insulin secretion, as well as evidence linking olanzapine metabolic side effects to increased expression of TCF7L2, we hypothesise that olanzapine stimulates the Wnt signalling pathway to accelerate the cell cycle, and the following Chapter aimed to probe this.

A.



B.

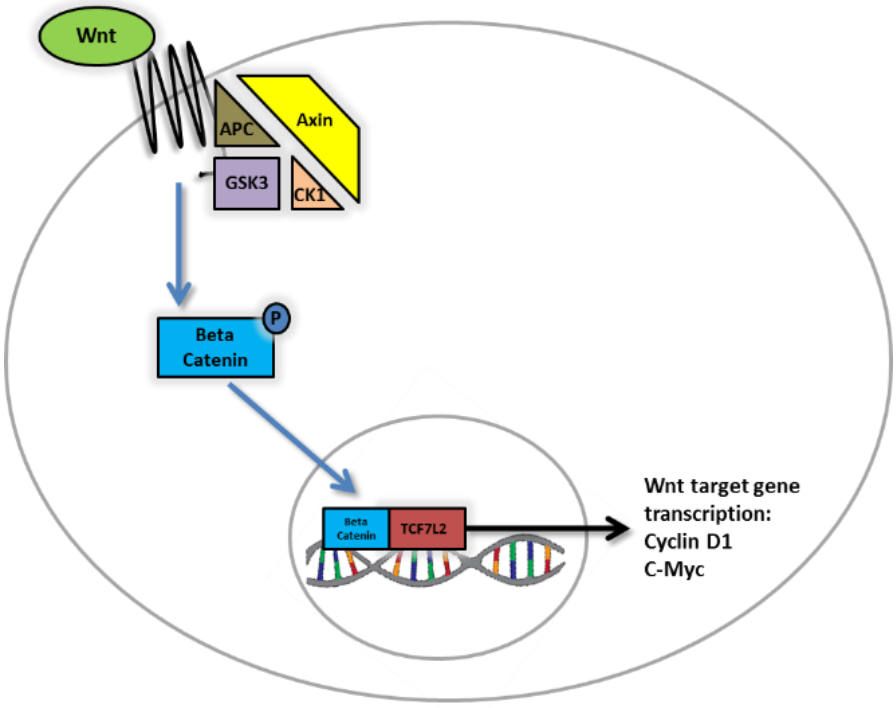


Figure 23 schematic diagram of the canonical Wnt pathway with or without Wnt stimulation [281] In the absence of Wnt stimulation, beta catenin is targeted for degradation by the destruction complex and TCF7L2 supresses downstream gene expression (A). Wnt stimulation at frizzled receptors recruits the components of the destruction complex to the membrane and liberates beta catenin to move to the nucleus and bind to TCF7L2. Forming a bipartite transcription factor, beta catenin and TCF7L2 upregulate target genes such as c-myc and cyclin D1 (B)

4.2 Results

4.2.1 Effect of olanzapine on regulators of the cell cycle

In Chapter 3 it was demonstrated that in the absence of other stimuli olanzapine was able to stimulate cell proliferation and accelerate cell cycle transition from G1 – G2. This suggested that olanzapine may interact with one or more signalling pathways that are involved in proliferation. We hypothesised that cell cycle acceleration by olanzapine is caused by upregulation of cell cycle regulating genes, and Initial experiments aimed to investigate the effect of olanzapine on the mRNA expression of various components of the cell cycle.

Cyclin D1, cyclin D2 and c-myc are all considered to be important regulators of beta cell proliferation that respond to mitogenic stimuli to initiate the process of cell division [284]. D cyclin expression fluctuates throughout the cell cycle, with maximal expression during G1 [285]. In Chapter 3 **Figure 21** showed that at 24 hours most cells are in G1, thus 24 hours is an appropriate incubation time to investigate increased expression of D cyclins. C-myc is a transcription factor that is also upregulated by mitogenic stimuli to increase expression of genes involved in cell proliferation. The effect of 24 hour olanzapine exposure on expression of these cell cycle regulating genes was investigated using qRT-PCR.

Figure 24 shows that after 24 hour exposure to 10 µM olanzapine, there was significantly increased expression both of c-myc and cyclin D1 compared to control ($p<0.05$). Cyclin D2 expression was not significantly different at 24 hours. Expression of the D cyclins fluctuates throughout the cell cycle, peaking during G1, and after 48 hours **Figure 25** shows that the expression of both cyclins had reduced. This is consistent with the theory that olanzapine disrupts the regulation of the cell cycle.

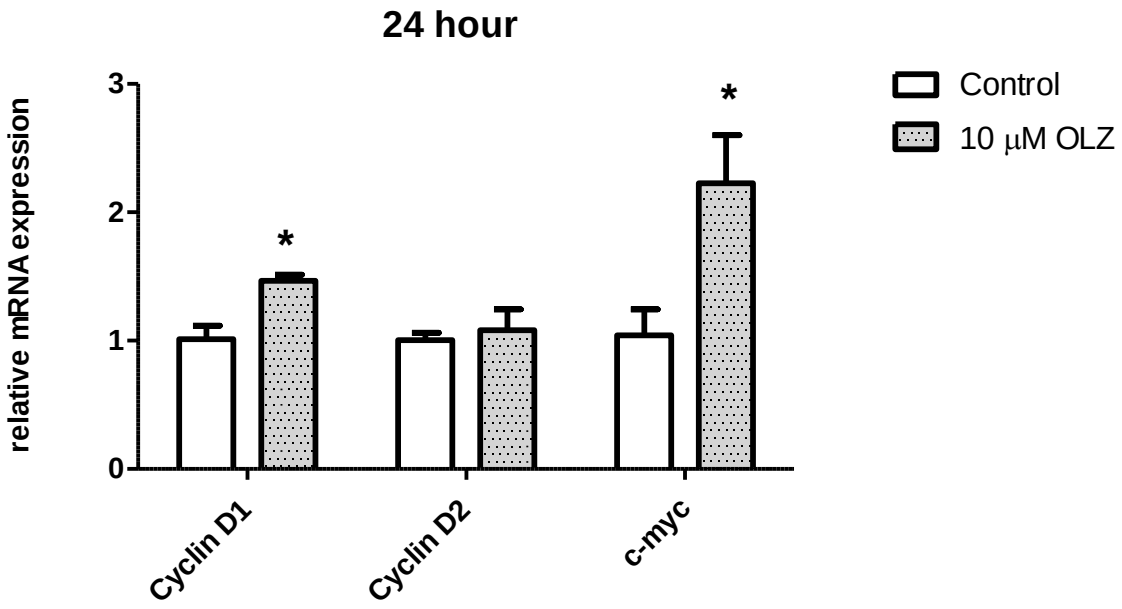


Figure 24 Effect of 24 hour olanzapine on cell cycle gene expression MIN6 cells were incubated with 10 μM olanzapine for 24 hours. qRT-PCR was performed and gene expression calculated relative to UBC and B2M reference gene expression. Results shown are of 3 independent experiments. Data are displayed as mean ± SEM, *p<0.05 versus 0 μM olanzapine using a student T test

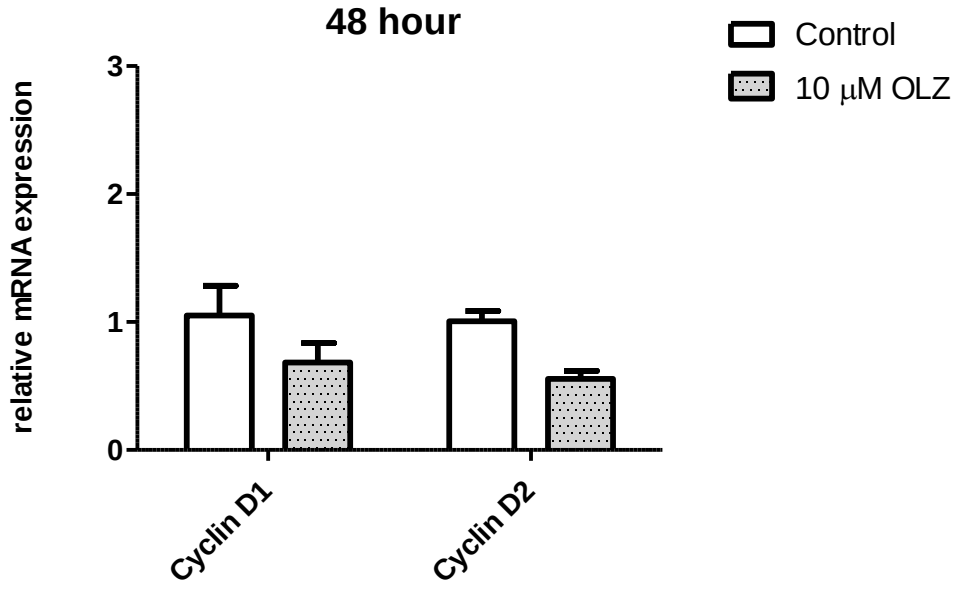


Figure 25 Effect of 48 hour olanzapine on gene expression MIN6 cells were incubated with 10 μM olanzapine for 48 hours. qRT-PCR was performed and gene expression calculated relative to UBC and B2M reference gene expression. Results shown are of 3 independent experiments. Data are displayed as mean ± SEM

4.2.2 Effect of olanzapine on the canonical Wnt signalling pathway

Numerous signalling pathways, growth factors, and cytokines are involved in stimulation of cyclins and c-myc to progress the cell cycle. Both cyclin D1 [286] and c-myc [269] are upregulated by canonical Wnt/ beta catenin signalling, while cyclin D2 is not a known direct target.

During activation of the canonical Wnt signalling pathway, the components of the beta catenin destruction complex are recruited to the membrane and there is reduction of cytoplasmic proteasomal degradation of beta catenin. As depicted in **Figure 26** this is followed by beta catenin cytoplasmic accumulation and nuclear translocation, where beta catenin forms a bipartite transcription factor with TCF7L2 to upregulate expression of Wnt target genes. Based on the observations that 10 µM olanzapine can stimulate proliferation and upregulate cyclin D1 and c-myc, alongside previous work linking the transcription factor TCF7L2 to the metabolic side effects of olanzapine [135], we hypothesised that olanzapine can alter the protein level and location of beta catenin and TCF7L2 in MIN6 cells.

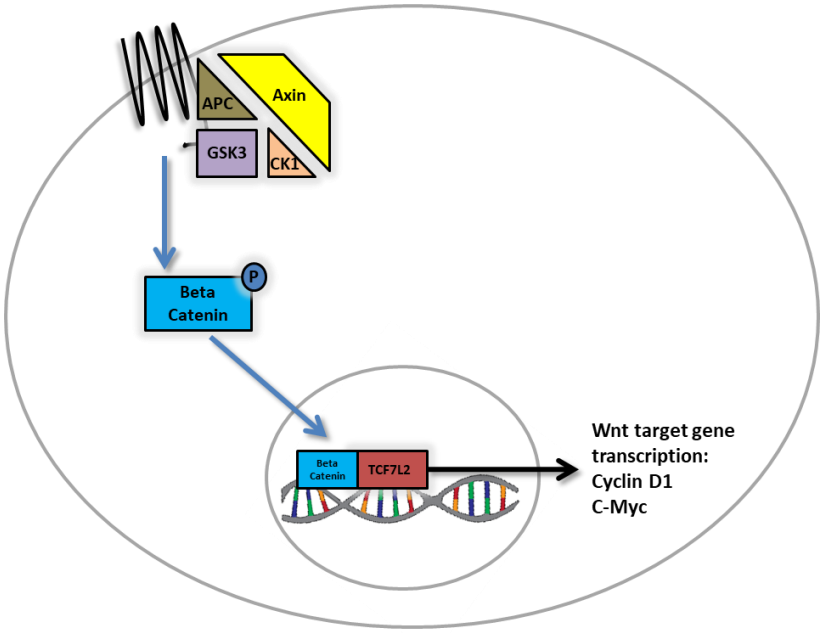


Figure 26 Schematic diagram showing Beta catenin nuclear translocation and formation of a bipartite transcription factor with TCF7L2 to upregulate Cyclin D1 and c-myc

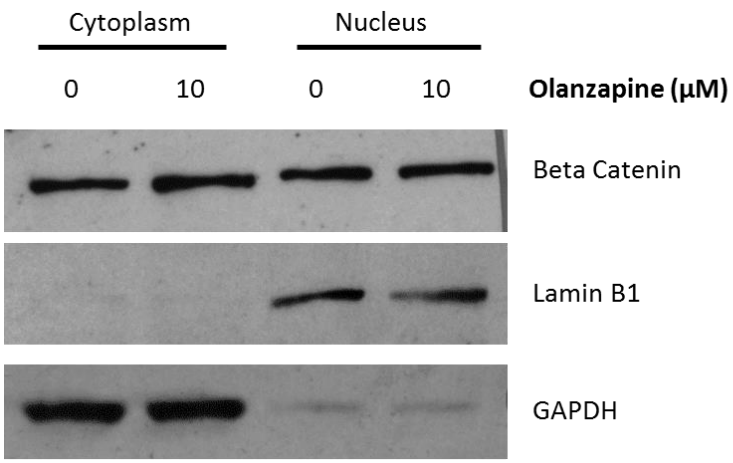
4.2.2.1 Beta catenin

Initially, the concentration and localisation of beta catenin was investigated using Western blot. MIN6 cells were incubated with 10 µM olanzapine for 24 (**Figure 27**) and 72 hours (**Figure 28**) in unstimulated growth conditions as discussed previously. Nuclear and cytoplasmic fractions were assessed for protein concentration of beta catenin relative to GAPDH (cytoplasmic reference) and Lamin B1 (nuclear reference).

Figure 27 shows that 24 hour treatment with 10 µM olanzapine had a significant effect on cytoplasmic beta catenin concentration ($p<0.05$) and no observed change in nuclear beta catenin concentration. Compared to control, olanzapine produced a mean increase in cytoplasmic beta catenin of 26.0 ±6.7%. As nuclear beta catenin was unchanged, this suggests there may be a reduction in cytoplasmic beta catenin proteasomal degradation leading to increased free concentration. This is suggestive of increased activity within the canonical Wnt signalling pathway.

Figure 28 shows that after 72 hours, there was a small but significant shift in localisation of beta catenin. Compared to control there was a significant reduction in mean cytoplasmic beta catenin, and an increase in nuclear beta catenin. This suggests that chronic exposure to olanzapine confers some effect on the cytoplasmic destruction of beta catenin, or alternatively promotes nuclear sequestration.

A.



B.

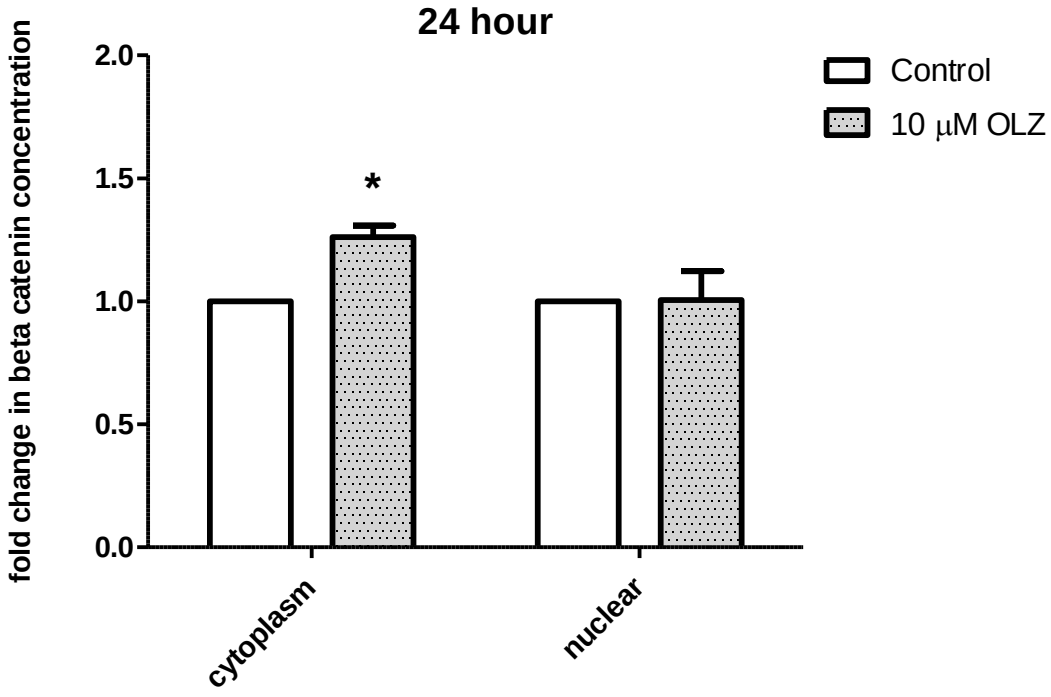
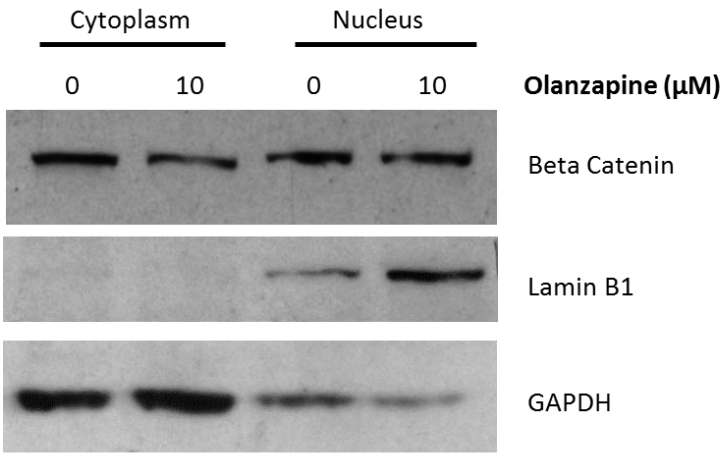


Figure 27 Effect of 24 hour olanzapine on beta catenin cytoplasmic and nuclear concentration MIN6 cells were incubated with 10 μM olanzapine for 24 hours. Nuclear and cytoplasmic fractions were extracted and lysates were resolved by SDS page and immunoblotted against beta catenin antibodies and loading control antibodies against GAPDH (cytoplasm) and Lamin B1 (nuclear). Representative western blot (A). Quantified densiometric data of cytoplasmic beta catenin relative to GAPDH and nuclear beta catenin relative to Lamin B1 from 3 independent experiments (B). Data are displayed as mean ± SEM, *p<0.05 10 μM versus 0 μM olanzapine using a one sample t test

A.



B.

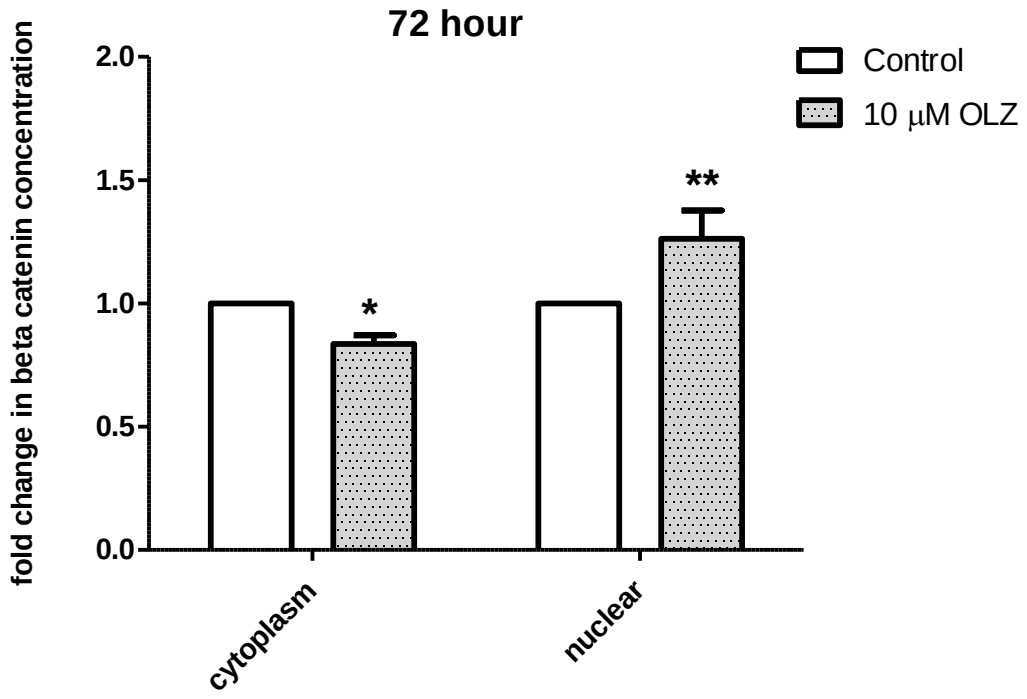
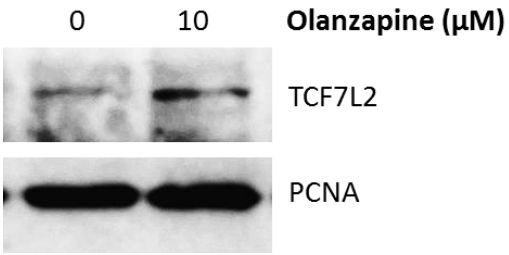


Figure 28 Effect of 72 hour olanzapine on beta catenin cytoplasmic and nuclear localisation MIN6 cells were incubated with 10 μM olanzapine for 72 hours. Nuclear and cytoplasmic fractions were extracted and lysates were resolved by SDS page and immunoblotted against beta catenin antibodies and loading control antibodies against GAPDH (cytoplasm) and Lamin B1 (nuclear). Representative western blot (A). Quantified densiometric data of cytoplasmic beta catenin relative to GAPDH and nuclear beta catenin relative to Lamin B1 from 4 independent experiments (B). Data are displayed as mean ± SEM, *$p<0.05$, **$p<0.01$ 10 μM versus 0 μM olanzapine using a one sample t test

4.2.2.2 TCF7L2

Within the nucleus beta catenin binds to and forms a bipartite transcription factor with TCF7L2 to upregulate downstream Wnt target genes. Olanzapine has previously been linked to increased expression of TCF7L2 [135] and TCF7L2 overexpression is known to increase beta catenin nuclear localisation [287]. As olanzapine increases nuclear beta catenin after 72 hours, we hypothesised that the concentration of nuclear TCF7L2 may also be altered by olanzapine. The nuclear fraction was assessed by Western blot for protein concentration of TCF7L2 relative to nuclear loading control PCNA. **Figure 29** shows that there was significantly higher TCF7L2 protein concentration in cells treated for 72 hours with 10 µM olanzapine compared to control ($p<0.05$).

A.



B.

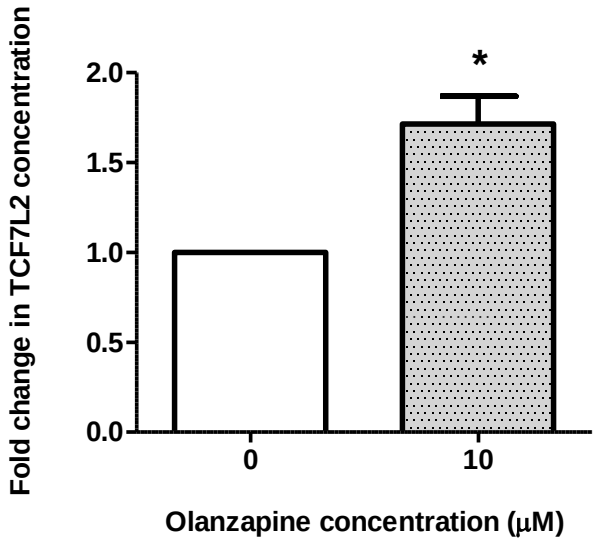


Figure 29 Effect of 72 hour olanzapine on nuclear TCF7L2 protein concentration of MIN6 cells MIN6 cells were incubated with 10 µM olanzapine for 72 hours. Nuclear fractions were extracted and Lysates were resolved by SDS page and immunoblotted with antibodies against TCF7L2 and loading control antibodies against PCNA. Representative western blot (A). Quantified densiometric data of TCF7L2 relative to PCNA from 3 independent experiments (B). Data are displayed as mean ± SEM. *$p<0.05$ versus 0 µM olanzapine using a one sample t test

4.2.3 Addition of inhibitors of the canonical Wnt signalling pathway

We have shown that olanzapine can alter MIN6 cell proliferation, beta catenin concentration and localisation, and Wnt target gene expression. These results are suggestive of interaction between olanzapine and the canonical Wnt signalling pathway. We hypothesised that olanzapine stimulates the canonical Wnt signalling pathway. To further test the hypothesis, known pharmacological inhibitors of Wnt signalling were added to the culture alongside olanzapine to assess the impact on olanzapine induced effects.

Activity of the beta catenin destruction complex is the principal regulator of the canonical Wnt signalling pathway. The complex comprises the central scaffold protein Axin (Axin1 and Axin2), APC, CK1α and GSK3β. Beta catenin concentration is usually tightly regulated by the destruction complex, which collectively phosphorylates beta catenin and targets it for ubiquitinisation and degradation. Wnt Inhibitors and activators target the destruction complex to alter free beta catenin concentration.

In the following assays KYA1797K, XAV939, and BIO have been utilised to stimulate or inhibit beta catenin destruction as illustrated in **Figure 30**. KYA1797K and XAV939 increase the activity of the beta catenin destruction complex to increase beta catenin destruction and inhibit signalling. We hypothesised that addition of these agents would inhibit the effects of olanzapine to stimulate proliferation. BIO is a GSK3 inhibitor that prevents its incorporation into the destruction complex to stimulate beta catenin signalling. BIO has previously been shown to reduce activity of the destruction complex, increase beta catenin accumulation, nuclear translocation, and increase Wnt target gene expression [214]. Here BIO is used as a positive control for beta catenin activation. Metformin is not known to interact with the Wnt signalling pathway, but it was also investigated as a recognised antiproliferative and clinically relevant antidiabetic drug.

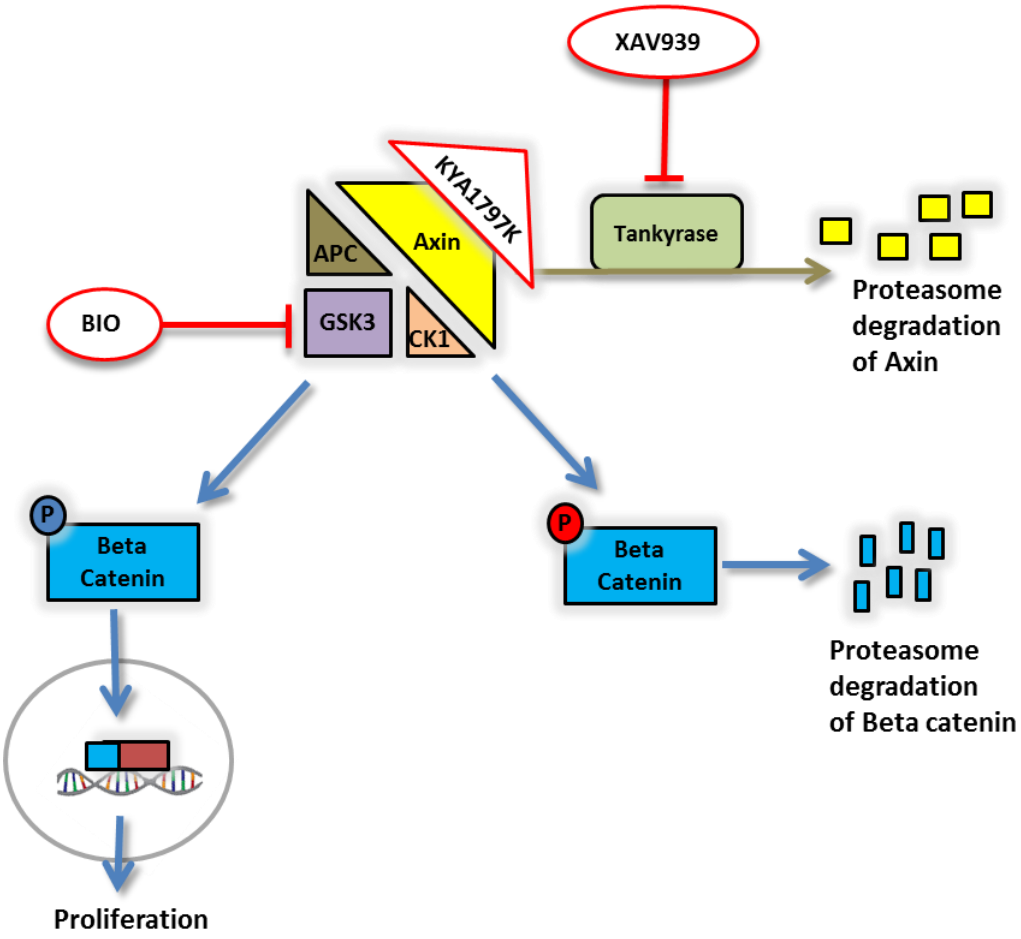


Figure 30 Schematic diagram showing pharmacological inhibitors and activators of beta catenin signalling. BIO is a GSK3 inhibitor which reduces beta catenin destruction and increases signalling. KYA1797K binds to the RGS domain of Axin to stabilise and increase functioning of the destruction complex. XAV939 is a tankyrase inhibitor which reduces Axin degradation and increases Axin concentration to increase beta catenin destruction and reduce signalling.

4.2.3.1 Proliferation

Initially the MTT assay was used to investigate the effect of additional Wnt activators/ inhibitors. As shown previously in Chapter 3, the accelerated proliferation of olanzapine treated cells results in significantly higher MTT absorbance after 72 hour incubation **Figure 18**). The following study aimed to investigate if the increase in proliferation was inhibited by pharmacological Wnt inhibitors.

4.2.3.1.1 KYA1797K

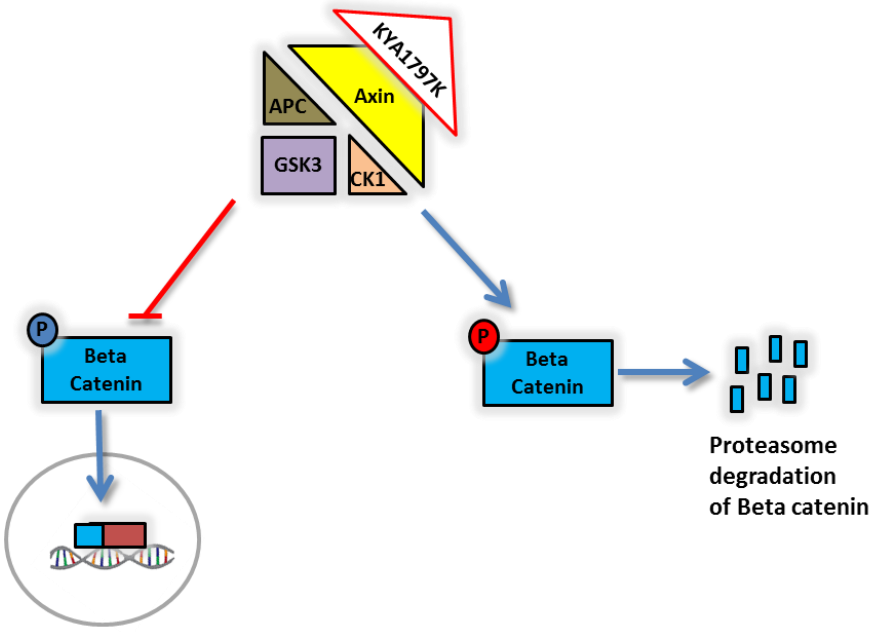
As depicted in **Figure 31**A KYA1797K binds to the regulator of the G protein signalling (RGS) domain of Axin. Through binding to Axin, KYA1797K stabilises and enhances the beta catenin destruction complex and leads to increased beta catenin degradation. KYA1797 has previously been shown to inhibit Wnt signalling in HEK293 cells with an IC50 of 0.75 µM [216].

Similarly to our previous results **Figure 31**B shows that there was a significant difference in relative absorbance between control and olanzapine treated cells after 72 hour incubation ($p<0.001$). Incubation with 1 µM KYA1797K did not have any significant effect on the MTT absorbance of olanzapine treated cells or control cells.

BIO treatment significantly increased MTT absorbance ($p<0.001$), indicating that increased beta catenin accumulation by BIO is sufficient to stimulate cell proliferation during 72 hour incubation. KYA1797K combined with BIO was significantly higher than KYA1797K alone ($p<0.001$) highlighting that the balance of the different components of the beta catenin destruction complex is important related to cell proliferation.

There was no difference between 0 µM and 10 µM olanzapine with the addition of BIO, this may be due to the pharmacology of BIO to stimulate proliferation downstream of the effects of olanzapine. Alternatively, MTT absorbance may reach a maximal response when the cell population is confluent, and we were unable to detect the additional proliferative response of olanzapine plus BIO.

A.



B.

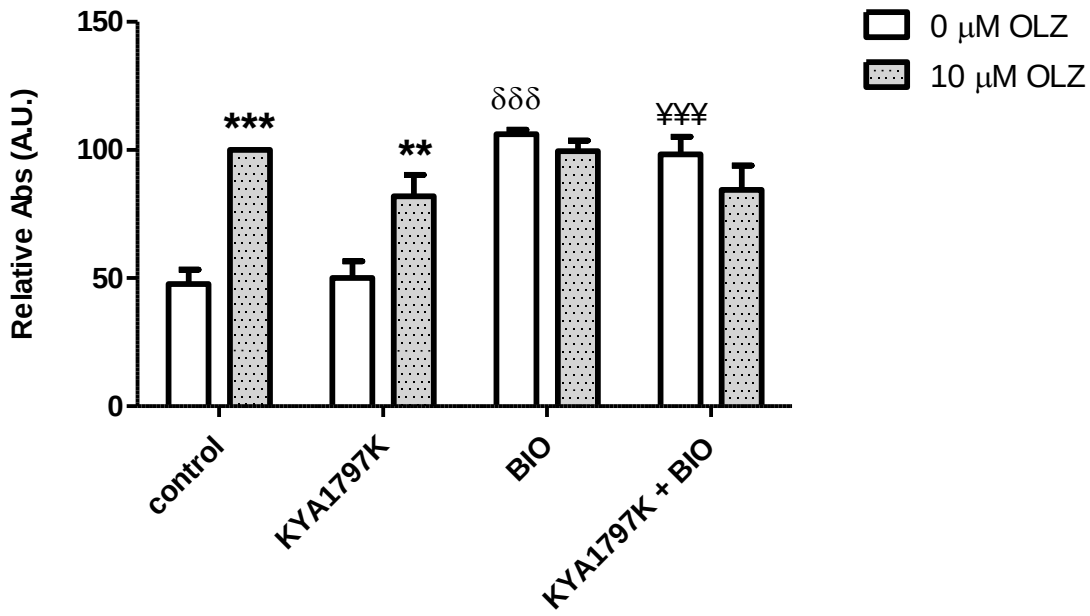


Figure 31 Effects of olanzapine and Wnt inhibitor KYA1797K on MTT absorbance in MIN6 cells MIN6 cells were incubated with 10 µM olanzapine with 1 µM KYA1797K and 0.1 µM BIO as indicated for 72 hours. MTT assay was performed and absorbance read at 540 nm. Results shown are of 3 independent experiments. Data are displayed as mean ± SEM, **p<0.05 versus 0 µM olanzapine, δδδ p<0.001 versus control, ¥¥¥ p<0.001 versus KYA1797K using two-way ANOVA and Bonferroni's Post-test

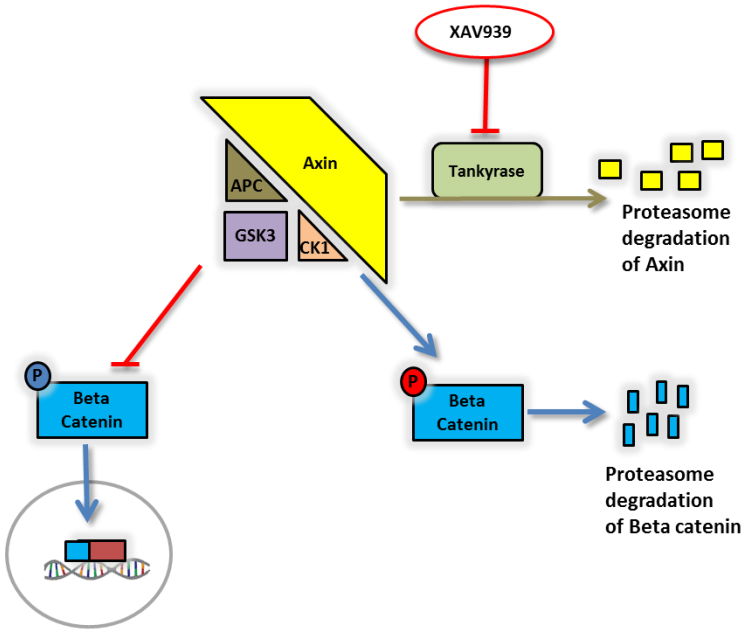
4.2.3.1.2 XAV939

Tankyrase (TNKS) enzymes target Axin for proteasomal degradation. XAV939 is a TNKS inhibitor that leads to reduced Axin degradation and increases in cellular concentration. Biochemical activity assays describe the IC50 as 0.01 µM for TNKS1 and 0.004 µM for TNKS2 enzyme activity [213]. Axin2 concentration is the rate limiting factor in the beta catenin destruction complex thus by increasing Axin2 concentration, there is subsequent increased capacity for beta catenin degradation and downregulation of signalling, as depicted in **Figure 32**A.

As before, **Figure 32**B shows that there was a significant difference in relative absorbance between control and olanzapine treated cells after 72 hour incubation ($p<0.05$). Incubation with 0.1 µM XAV939 did not significantly alter the MTT absorbance compared to control, but did prevent the significant effect of 10 µM olanzapine compared to 0 µM olanzapine.

Again, BIO treatment significantly increased MTT absorbance compared to control ($p<0.001$) and XAV939 combined with BIO was significantly higher than XAV939 alone ($p<0.05$). This suggests that, despite increasing the concentration of Axin, in the absence of GSK3β the destruction complex cannot function adequately and beta catenin accumulates to stimulate proliferation.

A.



B.

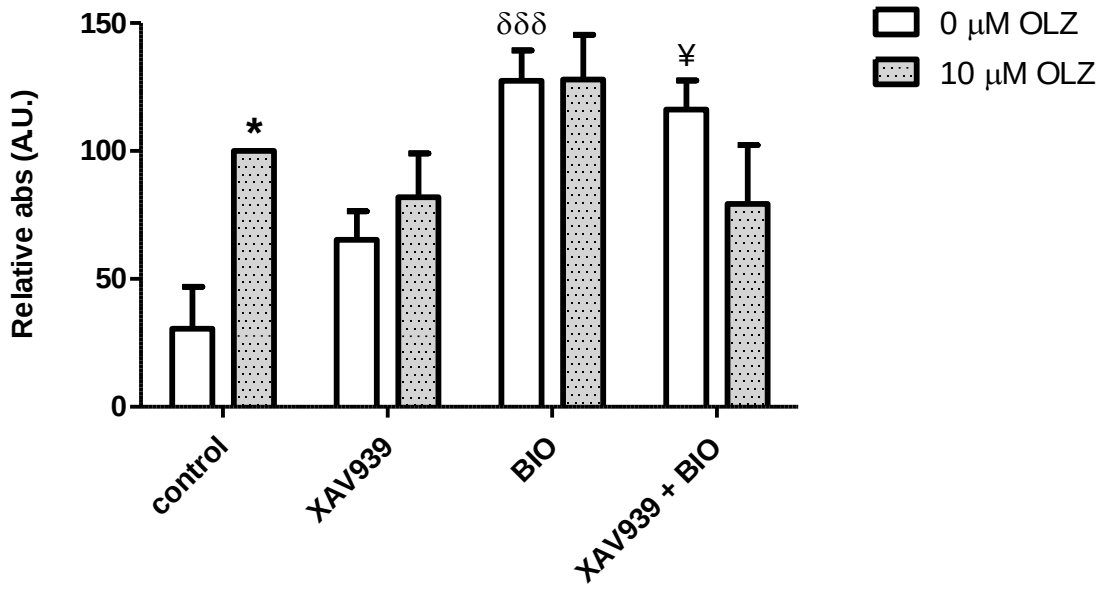


Figure 32 Effects of olanzapine and Wnt inhibitor XAV939 on MTT absorbance in MIN6 cells MIN6 cells were incubated with 10 µM olanzapine with 0.1 µM XAV939 and 0.1 µM BIO as indicated for 72 hours. MTT assay was performed and absorbance read at 540 nm. Results shown are of 4 independent experiments. Data are displayed as mean ± SEM, *p<0.05 versus 0 µM olanzapine, δδδ p<0.001 versus control, ¥ p<0.05 versus XAV939 using two-way ANOVA and Bonferroni's Post-test

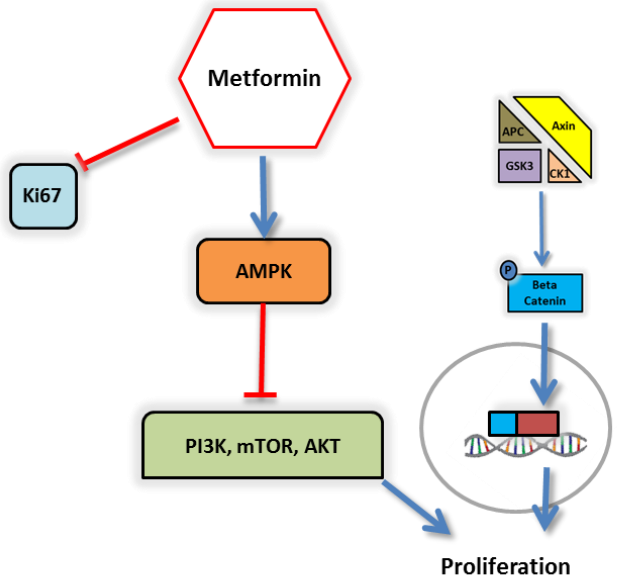
4.2.3.1.3 Metformin

Metformin is a common antidiabetic drug that has been proposed to be a beneficial addition to olanzapine to ameliorate the metabolic side effects [288, 289]. Metformin has also been shown to prevent olanzapine induced changes in TCF7L2 in liver, skeletal muscle and adipose tissue of mice [135]. When applied to MIN6 cells metformin has also been shown to have antiproliferative properties [290]. Hence, we hypothesised that addition of metformin to the culture would reduce the proliferative effect of olanzapine.

Metformin has numerous pharmacological targets, and can influence signalling in many different proliferative pathways. As shown in **Figure 33**A, activation of AMPK, leads to inhibition of PI3K, mTOR and AKT signalling which in turn can prevent cell growth and proliferation.

As shown in **Figure 33**B, 0.5 mM metformin significantly reduced MTT absorbance of cells incubated with 10 µM olanzapine for 72 hours ($p<0.01$) indicating that metformin is able to inhibit the proliferation induced by olanzapine. BIO again significantly stimulated proliferation in control cells ($p<0.001$), but the addition of BIO to metformin was unable to increase proliferation in both control and olanzapine groups. Metformin plus BIO resulted in significantly reduced MTT absorbance compared to BIO. This suggests that metformin inhibits proliferation of MIN6 cells through an alternate pathway that does not involve Wnt and beta catenin.

A.



B.

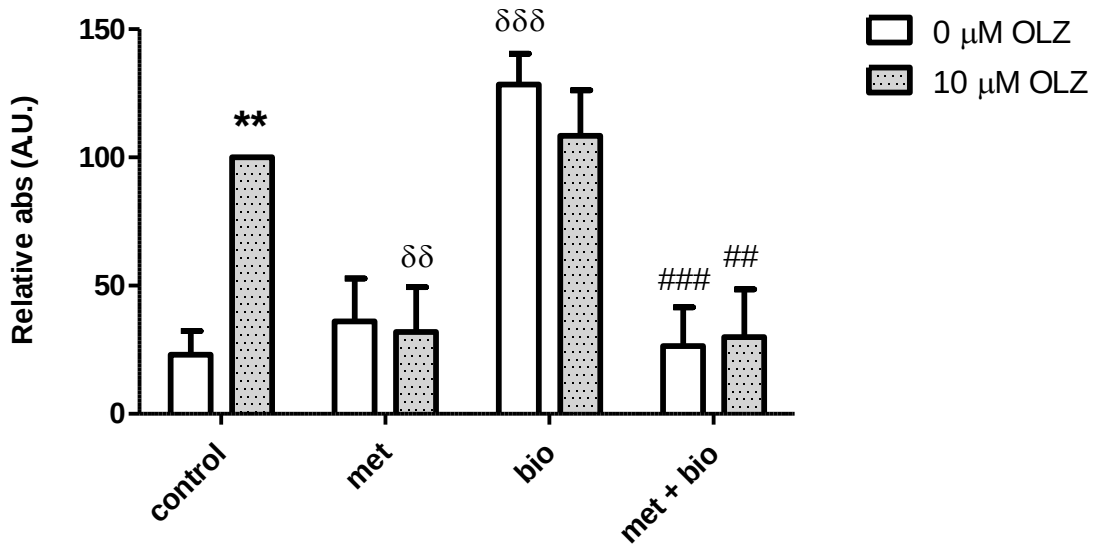


Figure 33 Effects of olanzapine and metformin on MTT absorbance in MIN6 cells MIN6 cells were incubated with 10 µM olanzapine with 0.5 mM metformin and 0.1 µM BIO as indicated for 72 hours. MTT assay was performed and absorbance read at 540 nm. Results shown are of 4 independent experiments. Data are displayed as mean ± SEM, **p<0.01 versus 0 µM olanzapine, δδ p< 0.01, δδδ p<0.001 versus control, ## p< 0.01, ### p<0.001 versus BIO using two-way ANOVA and Bonferroni's Post-test

4.2.3.2 Wnt target gene expression

4.2.3.2.1 Cyclin D1

The above MTT proliferation assays were inconclusive regarding the effect of Wnt inhibitors on olanzapine induced proliferation. XAV939 had some influence to reduce the significant effect of olanzapine, and BIO increased MTT absorbance in all experiments, but several factors can influence MTT results. Contact inhibition of growth, drug induced cellular metabolic changes, media pH and nutrient concentration can all significantly impact the proliferation and final MTT absorbance at 72 hours.

We have shown in **Figure 24** that the cell cycle regulator Cyclin D1 is upregulated by 24 hour incubation with 10 µM olanzapine. Cyclin D1 expression is stimulated by several different converging pathways, but it is also a downstream target gene of the Wnt signalling pathway. Next the effect of metformin and Wnt inhibitors KYA1797K and XAV939 on the expression of cyclin D1 was investigated by qRT-PCR to investigate olanzapine Wnt pathway activation in MIN6 cells.

Figure 34 shows that 24 hour incubation with 10 µM olanzapine significantly increased expression of cyclin D1 ($p<0.05$) which is consistent to the results presented in **Figure 24**.

XAV939 1 µM was the only Wnt inhibitor to reduce the olanzapine induced expression of cyclin D1. MIN6 cells treated with XAV939 + olanzapine had significantly reduced cyclin D1 expression compared to olanzapine alone ($p<0.001$). This suggests that XAV939 is able to inhibit the pathway involved in olanzapine induced expression of cyclin D1 (**Figure 34**).

KYA1797K and metformin in this model did not significantly affect olanzapine induced cyclin D1 expression, implying that olanzapine stimulates Wnt signalling through a pathway that is not affected by stabilisation of the beta catenin destruction complex by KYA1797K, or by a pathway targeted by metformin. XAV939 is the only tested inhibitor to act by altering Axin2 protein concentration; this suggests that Axin2 concentration is the rate limiting factor in Wnt/ beta catenin signalling in this model (**Figure 34**).

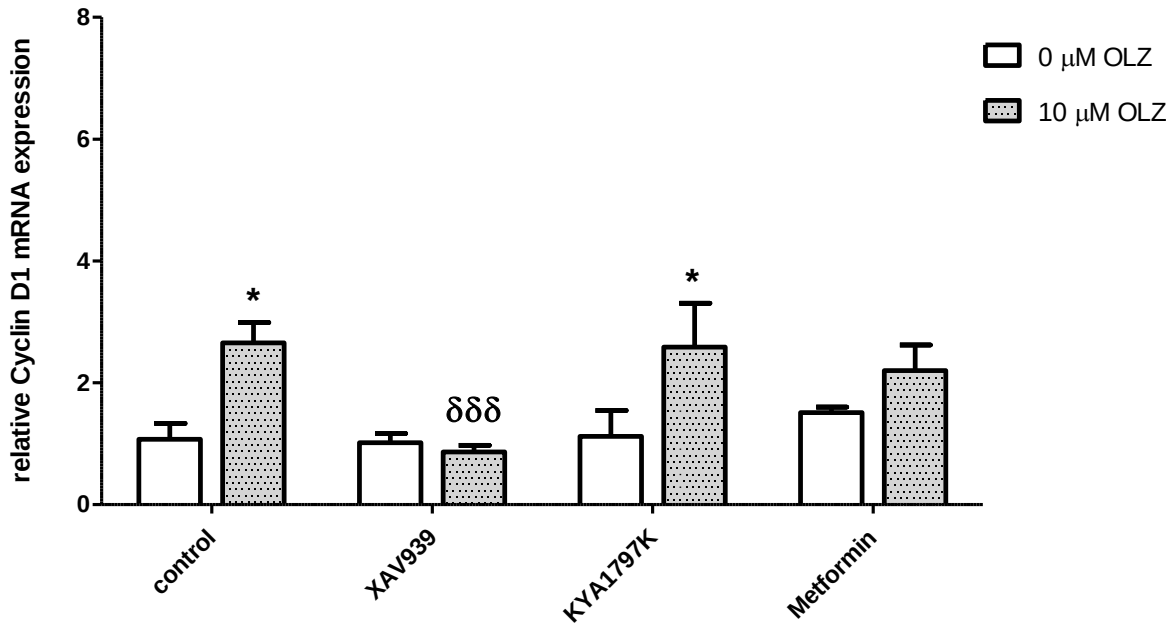


Figure 34 Effect of Wnt signalling inhibitors on olanzapine induced changes in Cyclin D1 gene expression MIN6 cells were incubated for 24 hours with 10 μM olanzapine and addition of 1 μM KYA1797K, 1μM XAV939, and 0.5 mM metformin. qRT-PCR was performed and cyclin D1 expression calculated relative to UBC and B2M reference gene expression. Results shown are of 4 independent experiments. Data are displayed as mean ± SEM, *p<0.05, versus 0 μM olanzapine, δδδ p<0.001 versus control using two-way ANOVA and Bonferroni's post-test

4.2.3.2.2 C-myc

C-myc is similarly regulated by numerous mitogenic stimuli, but also a recognised target of the canonical Wnt signalling pathway. We have shown that 24 hour olanzapine upregulates c-myc (**Figure 24**), next the role of XAV939 as an inhibitor of olanzapine induced signalling was probed further by investigating if it modified the olanzapine induced expression of c-myc. **Figure 35** shows that XAV939 reduced olanzapine induced expression of c-myc but this was not significant.

MIN6 cells were incubated with 10 μM olanzapine with or without inhibitor XAV939 1 μM and activator BIO. In this experimental model the effect of olanzapine on c-myc expression was not significant, although there was a trend for increased expression of c-myc which was reduced by XAV939. BIO significantly increased c-myc expression ($p<0.05$), confirming that c-myc is in part regulated by beta catenin signalling.

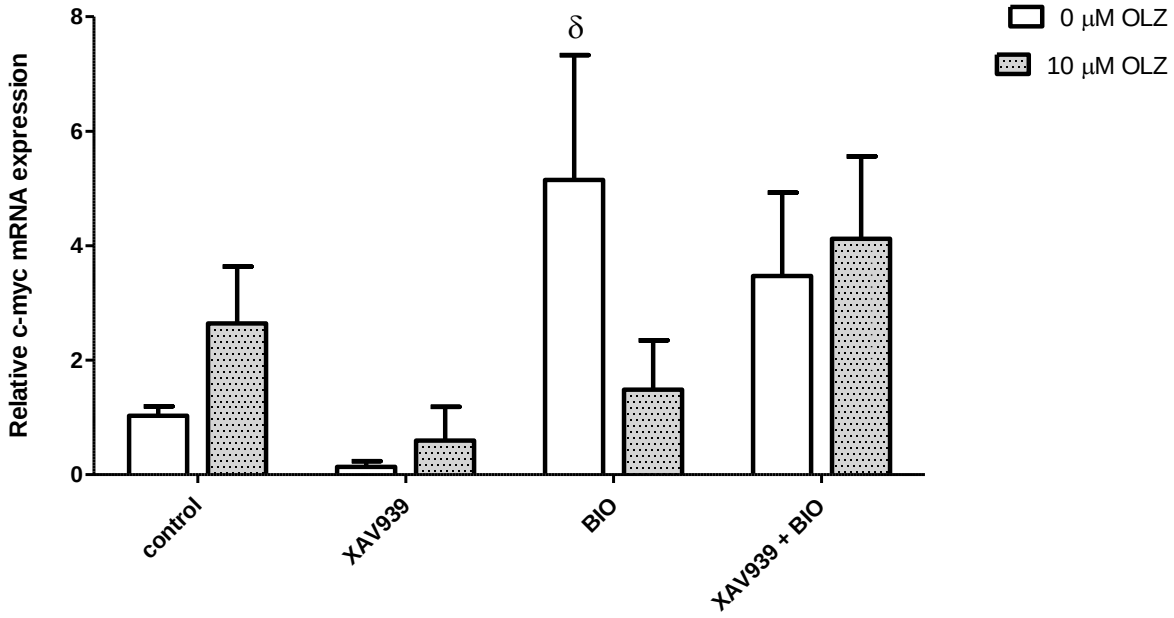


Figure 35 Effect of Wnt inhibitor XAV939 and activator BIO on olanzapine induced changes in c-myc gene expression MIN6 cells were incubated for 24 hours with 10 μM olanzapine and addition of 1 μM XAV939 and 0.1 mM BIO. qRT-PCR was performed and c-myc expression calculated relative to UBC and B2M reference gene expression. Results shown are of 3 independent experiments. Data are displayed as mean ± SEM δ $p<0.05$ versus control using two-way ANOVA and Bonferroni's post-test

4.2.3.3 Wnt signalling effectors

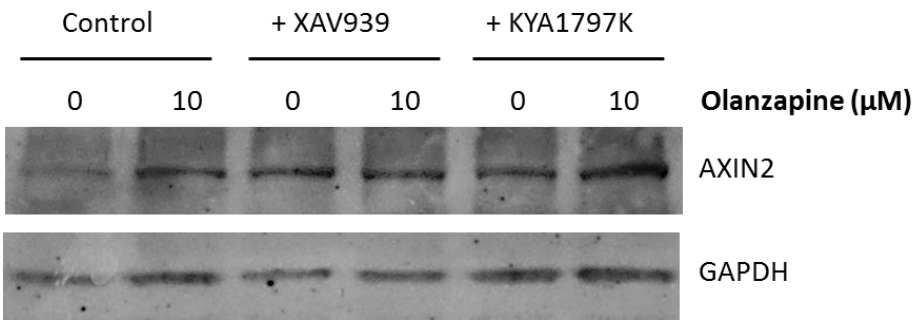
4.2.3.3.1 Axin2

As the two different Wnt inhibitors tested produced different effects on olanzapine induced cyclin D1 expression, further investigations aimed to probe the differing pharmacology and how this relates to olanzapine. XAV939 acts to pharmacologically alter protein concentration of Axin2 through tankyrase inhibition, while KYA1797K does not interact with tankyrase. We hypothesised that XAV939 would increase Axin2 concentration, and KYA1797K would not. Western blot was used to attempt to clarify the effect of 1 µM XAV939 and 1 µM KYA1797K on concentration of Axin2 following 72 hour incubation.

Figure 36 shows that there is a trend for 72 hour incubation with 10 µM olanzapine to increase Axin2 protein concentration, but this was not significant. XAV939 also moderately increased Axin2 concentration, in both control and olanzapine treated cells, an expected observation based on the pharmacology of XAV939. KYA1797K did not have a notable effect on Axin2 concentration, which also follows the known pharmacology to bind to Axin2 and not interfere with its enzymatic degradation.

To further investigate the potential interaction between XAV939 and Axin2 concentration related to olanzapine, qRT-PCR was used to investigate Axin2 mRNA expression following 72 hour incubation. **Figure 37** shows that olanzapine has no significant effect on Axin2 mRNA, but XAV939 has an overall significant effect to downregulate Axin2 mRNA expression when assessed by 2 way ANOVA ($p<0.05$). This highlights the pharmacology of XAV939 to post-translationally alter Axin2 concentration through interaction with tankyrase enzymes, and suggests that through Wnt inhibition, Axin2 expression is also inhibited.

A.



B.

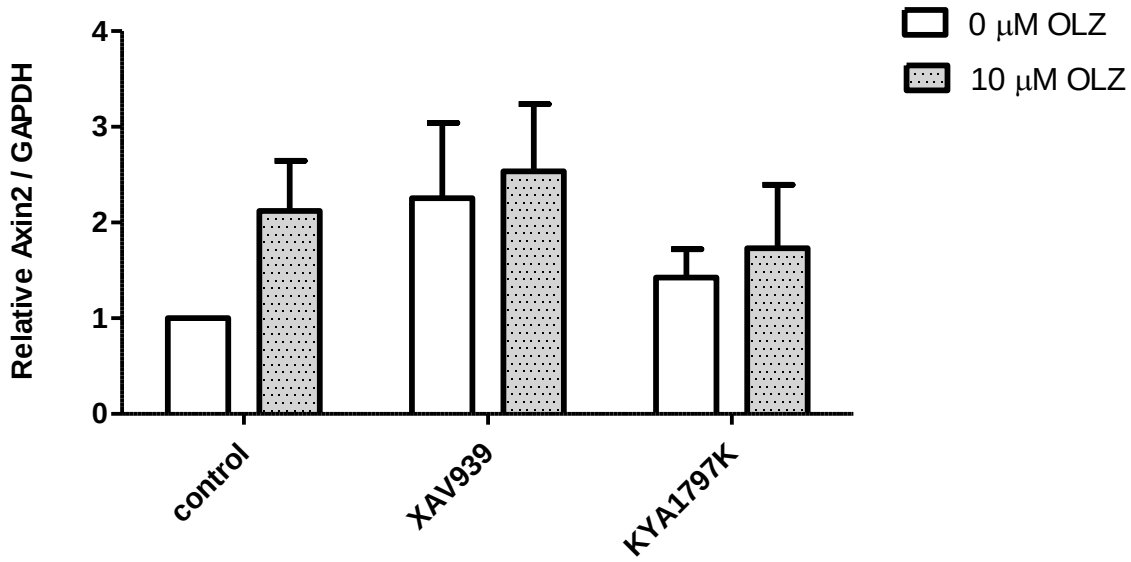


Figure 36 Effect of Wnt signalling modulators and olanzapine on AXIN2 protein concentration MIN6 cells were incubated for 72 hours with 10 µM olanzapine and 1µM XAV939 or 1 µM KYA1797K. Whole cell lysates were resolved by SDS page and immunoblotted with antibodies against AXIN2 loading control antibodies against GAPDH. Representative western blots (A). Quantified data of AXIN2 relative to GAPDH from 3 independent experiments (B). Data are displayed as mean ± SEM.

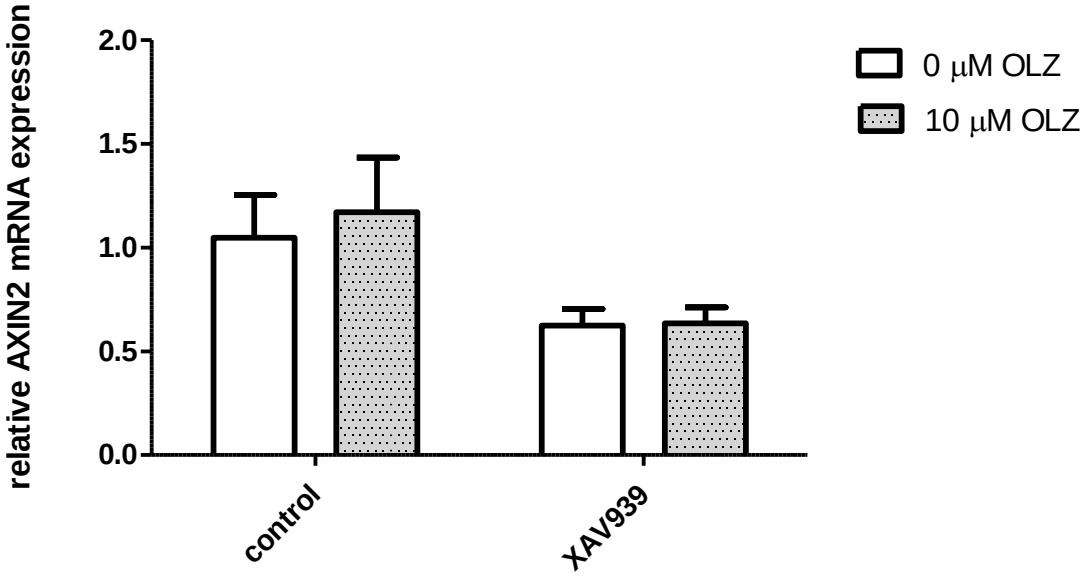


Figure 37 Effect of Wnt signalling modulators and olanzapine on AXIN2 mRNA expression MIN6 cells were incubated for 72 hours with 10 μM olanzapine and 1μM XAV939. qRT-PCR was performed and Axin2 expression calculated relative to UBC and B2M reference gene expression. Results shown are of 3 independent experiments. Data is displayed as mean ± SEM

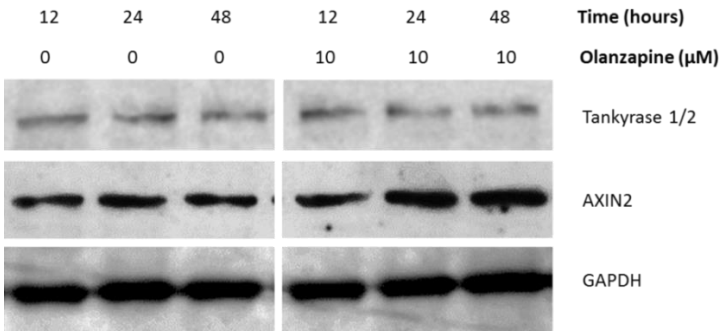
4.2.4 Effect of olanzapine on Axin2 over time

The presented results suggest that the Wnt inhibitor XAV939 can inhibit some of olanzapine induced cellular changes, but both olanzapine and XAV939 were found to moderately increase concentration of Axin2 after 72 hour incubation. Axin2 is the rate limiting component of the beta catenin destruction complex and increased Axin2 is considered to have an inhibitory effect on beta catenin signalling. Tankyrase inhibition by XAV939 triggers a rapid increase in Axin2 concentration and to inhibit Wnt signalling [291]. But **Figure 36** suggests that 72 hour olanzapine can also increase protein concentration of Axin2 which would suggest olanzapine has the capacity to inhibit beta catenin, and this is in contrast to the theory that olanzapine has an activating effect on beta catenin signalling.

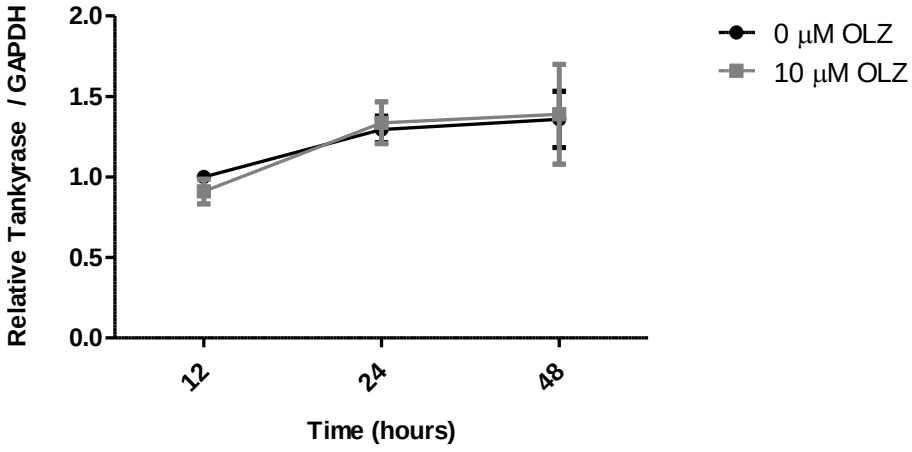
To further probe the effect of olanzapine on Axin2 protein concentration, and investigate if tankyrase activity is altered by olanzapine, cells were incubated with olanzapine and samples were extracted at different time points. Lysates were probed by Western blot for tankyrase and Axin2 relative to GAPDH. As shown in **Figure 38** olanzapine had no effect on tankyrase concentration, but there was a slow and significant increase in the concentration of Axin2 concentration over 48 hours ($p<0.05$). This confirms that chronic olanzapine does increase Axin2 steadily over time, but not through changes in tankyrase concentration, suggesting a different mechanism than XAV939.

To assess if the increase in Axin2 protein concentration over time is a result of increased Axin2 mRNA expression, qRT-PCR was used to investigate Axin2 mRNA expression at 24 and 48 hours. **Figure 39** shows that olanzapine has no significant effect on Axin2 mRNA at any tested time point.

A.



B.



C.

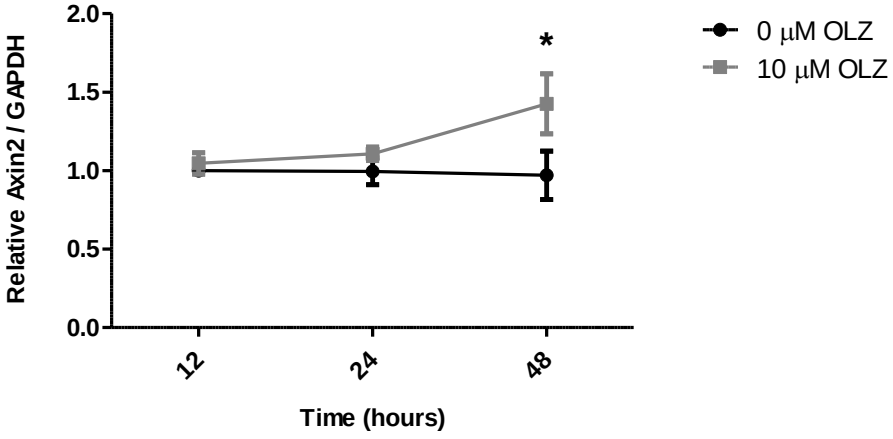


Figure 38 Effect of olanzapine on Tankyrase and AXIN2 protein concentration over time MIN6 cells were incubated for 12, 24 and 48 hours with 10 µM olanzapine. Whole cell lysates were resolved by SDS page and immunoblotted with antibodies against Tankyrase (A, B) and AXIN2 (A, C) and loading control antibodies against GAPDH. Representative western blots (A). Quantified data of tankyrase relative to GAPDH (B). Quantified data of Axin2 relative to GAPDH from 3 independent experiments (C) Data are displayed as mean ± SEM, *$p<0.05$, versus 0 µM olanzapine using two-way ANOVA and Bonferroni's post-test

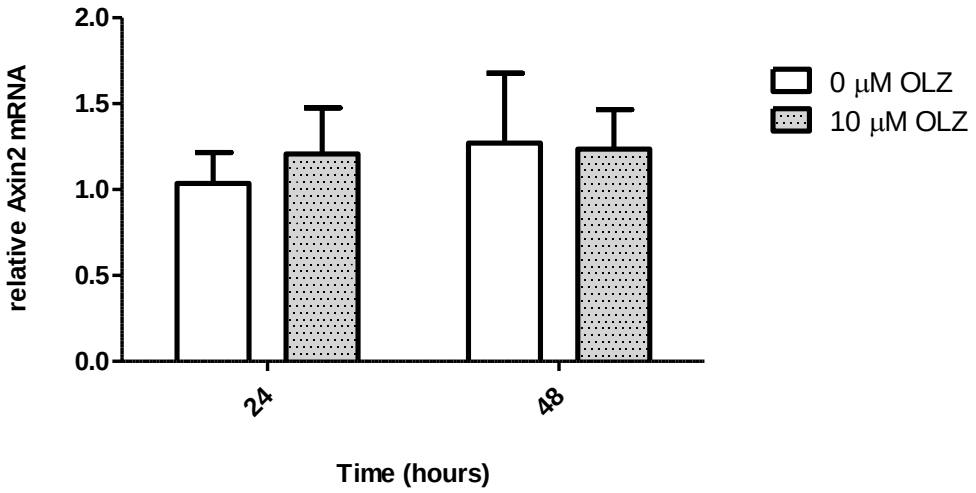


Figure 39 Effect of olanzapine on Axin2 mRNA expression over time MIN6 cells were incubated for 24 and 48 hours with 10 μM olanzapine. qRT-PCR was performed and Axin2 expression calculated relative to UBC and B2M reference gene expression. Results shown are of 3 independent experiments. Data is displayed as mean ± SEM

4.3 Discussion

This Chapter aimed to investigate the mechanism through which olanzapine stimulates proliferation of MIN6 beta cells. We hypothesised that olanzapine interacts with a mitogenic signalling pathway to accelerate the cell cycle. Based on evidence that the Wnt signalling effector TCF7L2 can mediate some of the metabolic side effects of olanzapine [135] and the dual role of the Wnt signalling pathway in beta cells to influence both proliferation and insulin secretion [210, 273], we conjectured that the canonical Wnt signalling pathway was involved. The presented data support the theory that olanzapine modulates Wnt/ beta catenin signalling in MIN6 beta cells. Acute olanzapine exposure increased the concentration of beta catenin, alongside increased expression of cell cycle regulating genes c-myc and cyclin D1; observations which closely resemble increased activity within the canonical Wnt pathway. Chronic exposure conversely leads to increased TCF7L2 and Axin2 and altered beta catenin distribution between the cytoplasm and nucleus, suggestive of altered beta catenin dynamics which may be related to loss of function. To the best of our knowledge this is first report of olanzapine induced changes in Wnt/ beta catenin signalling in a beta cell line.

4.3.1 Acute olanzapine exposure increases expression of cyclin D1 and c-myc

A significant finding was made in **Figure 24** that acute olanzapine can increase expression of cyclin D1 and c-myc in MIN6 cells. Cyclin D1 is central to cell cycle progression from G1 – S and can be upregulated by Wnt/ beta catenin signalling [211, 286]. The transcription factor c-myc is also positively regulated by Wnt/ Beta catenin signalling and regulates numerous cell cycle related genes [46, 269]. Wnt signalling stimulates beta catenin and TCF7L2 to form a bipartite transcription factor which binds to the gene promoter and directly upregulates expression of cyclin D1 and c-myc [283]. Hence, we suggest that increased cyclin D1 and c-myc is linked to the observed increased beta catenin (**Figure 27**). The increase in expression of cell cycle regulating genes induced by olanzapine also links directly to the results in Chapter 3 where it was demonstrated that olanzapine stimulates cell proliferation (**Figure 20**) and accelerates the transition from G1- S in the cell cycle (**Figure 21**).

There have been no previous studies investigating the effect of olanzapine on cell cycle regulation in beta cells, but we speculate that through increased expression of important cell cycle regulators, olanzapine also alters the normal functioning of the cell. Cyclin D1 expression can induce beta cell proliferation *in vitro* [292] and *in vivo* [293], and although there is resultant beta cell hyperplasia, there is little impact on insulin secretory function. Conversely, increased c-myc expression has been associated with both proliferation and beta cell dysfunction and diabetes. In mature beta cells expression of c-myc is low, but in models of hyperglycaemia, expression is induced [46] and is associated with loss of function. By increasing expression of c-myc, cells are poised for replication, with upregulation of genes associated with proliferation, while beta cell specific genes such as PDX1 are significantly reduced [246]. Pascal *et al*. (2008) showed that 2-3 day c-myc activation resulted in a decline in insulin secretion [294], and in a study using a transgenic mouse model as well as human beta cells Puri *et al*. (2018) showed that upregulation of c-myc stimulated beta cell proliferation, but also significantly altered insulin secretion, leading to increased basal secretion, but stunted secretion in response to glucose [246]. The increase in c-myc instigated by olanzapine as shown in **Figure 24** may therefore be linked to the reduced GSIS we observed in **Figure 17**A.

4.3.2 Acute olanzapine stimulates beta catenin signalling

Beta catenin is the primary signalling effector of the canonical Wnt signalling pathway and in **Figure 27** we showed that acute olanzapine caused a significant increase in cytoplasmic beta catenin concentration, which is a hallmark of activated Wnt signalling. Cytoplasmic beta catenin concentration is usually maintained at low concentrations through coordinated phosphorylation by the destruction complex which targets the protein for ubiquitinisation as illustrated in **Figure 40**. Wnt stimulation at frizzled receptors activates Dvl which consequently recruits Axin and GSK3 to the membrane and disrupts co-ordination of the destruction complex, to reduce beta catenin proteasomal degradation [282, 295]. Beta catenin accumulates and forms a bipartite transcription factor with TCF7L2 to upregulate target genes [281, 282]. We showed that olanzapine also increased expression of Wnt target genes cyclin D1 and c-myc [211, 269] (**Figure 24**) which further adds to the evidence that acute olanzapine is able to stimulate Wnt/ beta catenin signalling in MIN6 beta cells.

An increase in cytoplasmic beta catenin concentration by olanzapine indicates that there is reduced proteasomal degradation. Aside from frizzled receptor activation, possible mechanisms include direct inhibition of the destruction complex, or beta catenin protein stabilisation (**Figure 40**). Inhibition of any component of the beta catenin destruction complex would prevent incorporation into the complex to reduce overall activity, and olanzapine has previously been reported to directly inhibit GSK3β which may be linked to reduced beta catenin degradation [296]. Alternatively, interaction with some stimulatory pathways that involve AKT/ PKA can also phosphorylate beta catenin to increase its functional capacity [297].

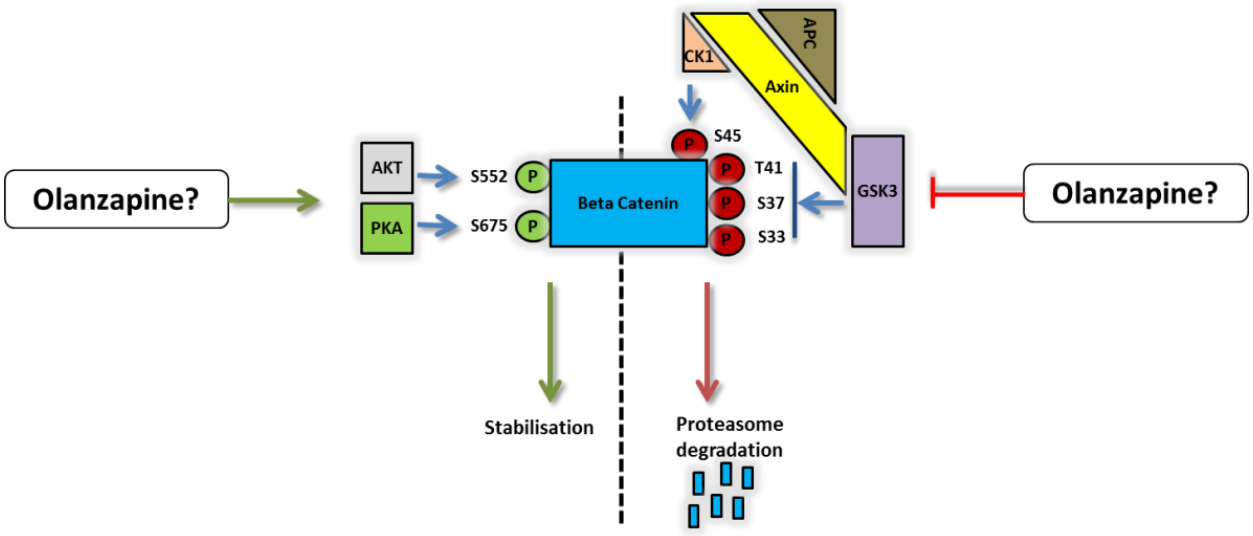


Figure 40 Schematic diagram showing the possible mechanisms for olanzapine to increase beta catenin protein concentration

The effect of olanzapine on beta catenin signalling has not previously been investigated in beta cells, but beta catenin has been shown to have a significant role to modulate proliferation of beta cells [273, 297, 298] as well as insulin secretion [210, 275]. In a study by Cognard *et al*. (2013) using glucose starved INS-1E beta cells it was found that glucose stimulated an increase in beta catenin concentration, alongside increased expression of cyclin D1 in a mechanism involving cAMP and PKA [297]. Using siRNA for beta catenin, the glucose induced increase in cyclin D1 expression was inhibited, confirming the regulatory role of beta catenin in this model. GLP-1 stimulation of beta cells also leads to increases in AKT and PKA which phosphorylates beta catenin at Ser 552 and Ser 675, to enhance its functional capacity [297-299] (**Figure 40**). Addition of the GLP-1 activator exenatide was also

shown to increase cyclin D1 and c-myc expression in beta cells through increased beta catenin concentration [298]. These effects are linked to beta cell compensation mechanisms and expansion of the beta cell mass at times of increased demand.

We did not investigate the effect of glucose on cyclin D1 or c-myc expression in MIN6 cells but in Chapter 3 we observed similar rates of proliferation in olanzapine treated cells and glucose treated cells (**Figure 18**). We suggest that the stimulatory effect of olanzapine on MIN6 cells to increase beta catenin, cyclin D1 and c-myc mimics the effect of a pathway such as glucose or GLP-1. We speculate that unregulated stimulation by olanzapine is comparable to chronic hyperglycaemia, which contributes to beta cell exhaustion and reduced function [300].

4.3.3 XAV939 inhibits the effect of olanzapine on c-myc and cyclin D1 expression

Addition of the Wnt inhibitor XAV939 prevented olanzapine induced changes in expression of cyclin D1 (**Figure 34**) and c-myc (**Figure 35**), adding to the evidence that the canonical Wnt signalling pathway is involved in increased expression. XAV939 has previously been shown to reduce cyclin D1 and c-myc in SHSY5Y cells [301] and H446 cells [302] and we hypothesise that it is through a reduction in free beta catenin concentration that expression of cyclin D1 and c-myc by olanzapine is inhibited by XAV939.

Axin is the central scaffold protein of the beta catenin destruction complex, and XAV939 inhibits Wnt signalling through increasing concentration of Axin [291]. Axin exists as two functionally equivalent isoforms, Axin1 and Axin2, Axin1 is ubiquitously expressed, whereas Axin2 transcription is transiently induced by canonical Wnt signalling [303]. The poly-ADP Ribose polymerase enzyme, tankyrase, targets Axin1/ Axin2 for ubiquitination and destruction [213], thus by inhibiting Tankyrase, XAV939 increases Axin1/Axin2 concentration. Axin2 is considered to be the rate limiting factor in beta catenin destruction, so increased Axin2 enhances beta catenin destruction complex activity and reduces free beta catenin [213]. We showed that XAV939 increased Axin2 protein concentration in MIN6 cells (**Figure 36**) which verified that there was tankyrase inhibition. Conversely we also showed a reduction in Axin2 mRNA by XAV939 (**Figure 37**) but as Axin2 expression is strongly induced

by the canonical Wnt signalling pathway [303], a reduction in mRNA expression by XAV939 is indicative of inhibited signalling. A theorised mechanism for XAV939 interaction is illustrated in **Figure 41**.

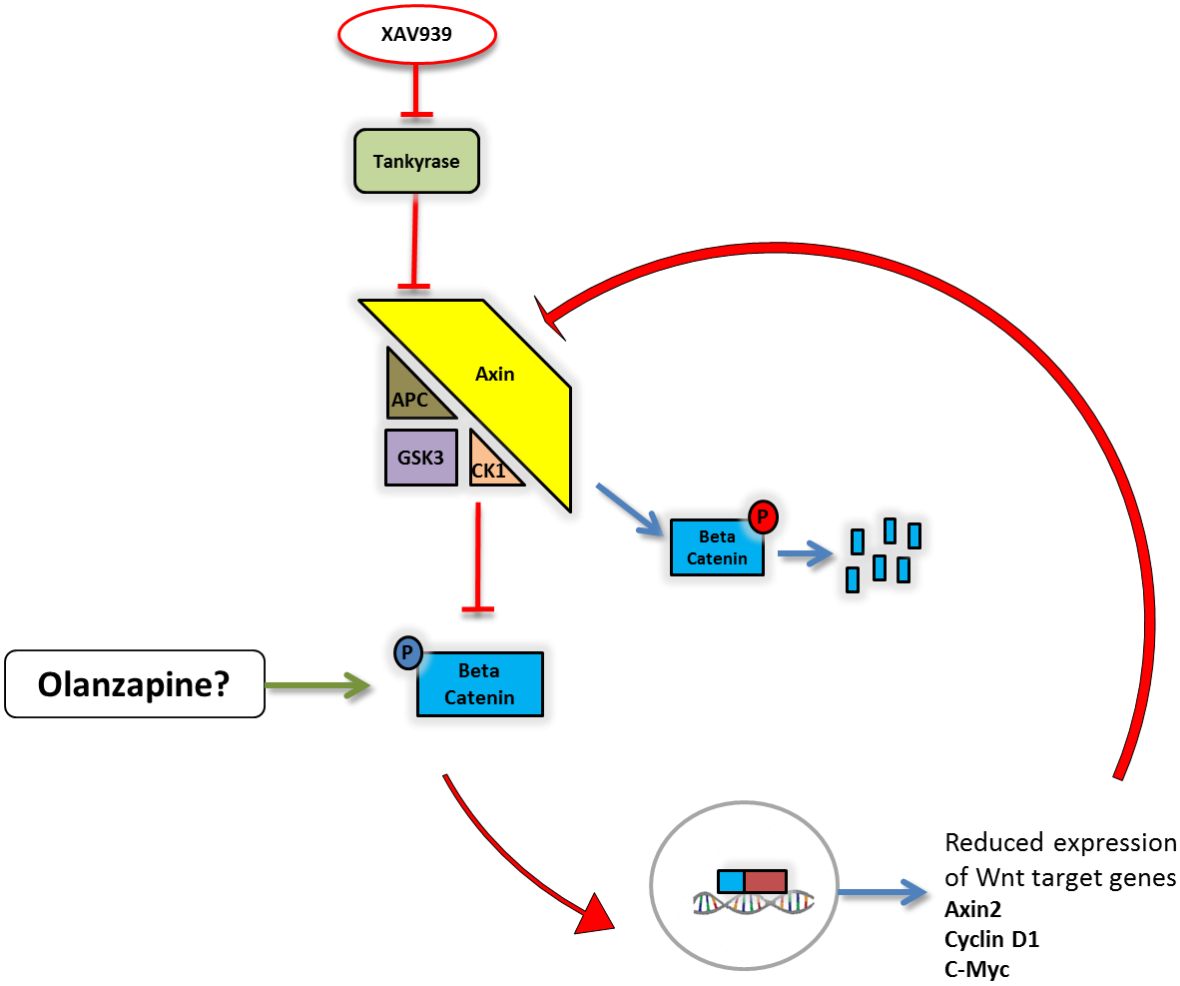


Figure 41 Schematic diagram of XAV939 effect to inhibit the canonical Wnt signalling pathway XAV939 inhibits Tankyrase enzymes which increase concentration of Axin/ Axin2. Axin/Axin2 increases activity of the beta catenin destruction complex and accelerates beta catenin destruction. This reduces beta catenin signalling and reduces beta catenin/ Wnt target gene expression.

The GSK3β inhibitor BIO acts as a Wnt signalling activator by preventing GSK3β incorporation into the beta catenin destruction complex [214]. We showed that BIO increased MIN6 cell proliferation (**Figure 32**) as well as c-myc expression (**Figure 35**), which verifies the important role of GSK3β and beta catenin in these processes. Furthermore, BIO also increased the expression of c-myc in cells incubated with

XAV939, which highlights that despite increased Axin2 concentration, in the absence of GSK3β, the destruction complex does not function and beta catenin concentration is able to increase, and upregulate target gene expression. In contrast metformin was shown to reduce olanzapine induced proliferation, but this was proven to be autonomous to Wnt signalling as BIO was unable to surmount the inhibition of proliferation (**Figure 33**), and Wnt target gene cyclin D1 was unchanged (**Figure 34**).

4.3.4 Acute olanzapine exposure does not affect expression of cyclin D2

It is generally accepted that cyclin D1 and c-myc are direct targets of the canonical Wnt signalling pathway, but the effect of Wnt stimulation on cyclin D2 expression is more complex. In a study by Rulifson *et al*. (2007) MIN6 cells were incubated with purified Wnt3A protein for 24 hours, and proliferation was increased alongside increased expression of cyclin D1, as well as cyclin D2, PitX and Cdk4 [272]. This suggests that Wnt frizzled receptor activation increases expression of both cyclin D1 and D2, but **Figure 24** shows that 24 hour olanzapine exposure only increases expression of cyclin D1 is and not cyclin D2.

Several possible explanations exist to explain the disparity. Cyclin expression is a moving target that fluctuates during the cell cycle. After an increase during the G1 phase, D cyclin expression is suppressed in S phase [304], **Figure 25** shows that the expression of both cyclin D1 and D2 at 48 hour olanzapine exposure is reduced compared to control, which is indicative of advanced progression of the cell cycle linked to the increased proliferation. Thus we cannot determine if at 24 hours cyclin D2 expression has already peaked and is beginning to decline. Additionally, cyclin D2 is expressed at a higher rate than cyclin D1 in beta cells [254], and the high basal expression might make it difficult to detect small drug induced changes in expression.

Many pathways are involved in expression of cyclin D1 and cyclin D2, as the promoters contain numerous binding sites for transcription activators. As well as Wnt, MAPK pathways and the Janus activated kinase (Jak)/ signal transducers and activators of transcription (STAT) pathway are well known activators of D cyclin

expression [305]. There is also evidence for cross talk between different pathways, and Frizzled receptor activation by Wnt3A has been shown to stimulate ERK/MAPK and c-Jun N-terminal kinases (JNK) as well as canonical Wnt pathways [306]. An increase in activity of an alternate pathway may therefore be involved in increased cyclin D2 by Wnt3A reported by Rulifson *et al*. (2007). Equally, in studies where only beta catenin or TCF7L2 are modified independently of Wnt frizzled receptor activation, cyclin D1 expression has been shown to be altered while cyclin D2 remained unchanged [286]. In HEK cells transfected with constitutively active beta catenin there was direct binding of beta catenin/TCF to the cyclin D1 promoter leading to increased expression [211], but Cyclin D2 expression was unaffected, potentially due to the lack of a specific Beta catenin/TCF binding site on the cyclin D2 promoter [286].

Beta catenin can be independently affected by changes to the destruction complex, or pathways such as AKT/ PKA which phosphorylate the protein and have differential effects to membrane activation (**Figure 40**) [298]. We suggest that olanzapine modulates beta catenin through a mechanism that does not involve frizzled receptor activation, and that this leads to increased expression of cyclin D1 and c-myc and not cyclin D2.

4.3.5 Chronic olanzapine alters the cytoplasmic/ nuclear distribution of beta catenin

An interesting finding was shown in **Figure 28** that beta catenin cellular distribution is further altered when MIN6 cells are exposed to olanzapine for a longer time period. After olanzapine treatment for 72 hours, the cytoplasmic beta catenin was significantly reduced, and the nuclear beta catenin was significantly increased. Alongside altered beta catenin cellular distribution, there was increased Axin2 concentration over time (**Figure 38**) and increased nuclear TCF7L2 (**Figure 29**). As the rate limiting scaffold protein of the beta catenin destruction complex, an increase in Axin2 may be directly involved in increased activity of the beta catenin destruction and ensuing reduction in cytoplasmic concentration. Alternatively, TCF7L2 can sequester beta catenin in the nucleus [275], thus an increase in nuclear TCF7L2 may be linked to the altered cellular distribution. It is cytoplasmic beta catenin that

is thought to be involved in important beta cell functions such as insulin secretion [210, 275, 297], therefore the chronic effects to alter cellular distribution may be linked to the previously observed olanzapine induced changes in GSIS as shown in **Figure 17**A.

Beta catenin has cellular roles in both the cytoplasm and the nucleus, and the nucleocytoplasmic distribution is a tightly regulated process. Adenomatous polyposis coli (APC) is a component of the beta catenin destruction complex, but it also acts as a chaperone to beta catenin, and is involved in shuttling the protein between the cytoplasm and nucleus [307]. During increases in nuclear beta catenin, APC exports the protein to the cytoplasm for degradation. However, nuclear beta catenin can bind to specific T-cell factor/lymphocyte growth factor (TCF/LEF) co-transcription factors such as LEF-1 [308], and TCF-4 (also referred to as TCF7L2) [309] which can anchor beta catenin within the nucleus. It is the association with LEF-1 that is most commonly associated with nuclear retention, and once bound to LEF-1 beta catenin is not able to be exported to the cytoplasm [308]. Antipsychotic interaction with LEF-1 has not been reported, but TCF-4, also known as TCF7L2 has been shown to be altered by olanzapine in MIN6 beta cells (**Figure 29**). TCF7L2 has also been shown to enrich nuclear beta catenin, and reduce nucleo/cytoplasmic shuttling through recruitment to the chromatin [309]. In beta cells, Sorrenson *et al*. (2016) showed that increased expression of TCF7L2 resulted in reduced insulin secretion in response to glucose and exenatide, which was linked to increased nuclear beta catenin retention [210].

Li *et al*. (2018) showed that olanzapine increased concentration of TCF7L2 in the liver, skeletal muscle, and adipose of mice, but due to high variability they found no significant differences in the pancreas [135]. We offer that olanzapine can also increase nuclear TCF7L2 concentration in pancreatic beta cells and hypothesise that this is related to loss of normal beta cell function.

Axin2 concentration is the rate limiting factor in cytoplasmic beta catenin destruction [310]. An increase in Axin2 concentration over time by olanzapine as shown in **Figure 38** suggests there is increased destruction complex activity to target a larger proportion of the free cytoplasmic beta catenin for destruction, resulting in reduced cytoplasmic beta catenin concentration as shown in **Figure 28**. A slow increase in Axin2 suggests that the mechanism involved is separate to the acute effects of olanzapine to stimulate beta catenin signalling, or alternatively, the increase in Axin2

could occur as a consequence of stimulated signalling. It is postulated that a negative feedback loop exists to prevent aberrant Wnt signalling where Axin2 is a target gene of Wnt signalling, as well as a negative regulator of beta catenin [311]. Although we did not find any changes in Axin2 mRNA, previous studies have demonstrated increased Axin2 expression upon Wnt stimulation [311]. In a Wnt transfected mammary gland cell line, 12 hour exposure to GSK3β inhibitor LiCl, increased both mRNA and protein concentration of Axin2 [311], and in kidney mesenchymal cells 4 hour LiCl also increased Axin2 mRNA [312], and this was postulated to be due to increased beta catenin/TCF7L2 binding to the Axin2 promoter [311].

Despite increases in protein concentration, we found no significant difference in Axin2 mRNA after olanzapine exposure (**Figure 39**). This suggests that olanzapine instead induces post-translational modifications to Axin2 to increase protein concentration, or that the mRNA expression peaked at an earlier untested time point. For example; in endometrial stromal cells, Wnt3A stimulated signalling which triggered a significant increase in Axin2 mRNA that peaked at 4 hours, but returned to basal levels of expression at 12 hours and was no longer significantly different to control from 12 – 72 hours [313]. We cannot rule out that a similar pattern in expression would be evident in our model prior to 24 hours, although an earlier increase in Axin2 protein concentration may have been expected.

Axin2 has been identified in beta cells, and has been shown to be a negative regulator of Wnt signalling [272], but its expression and regulation have not been widely explored in this model. Nonetheless, by increasing Axin2 concentration, the stimulatory effect of olanzapine on beta catenin signalling is converted into an inhibitory effect on signalling, and further expression of Wnt target genes would be expected to be supressed.

4.3.6 Conclusion

To conclude, this Chapter has presented evidence that acute olanzapine stimulates beta catenin signalling in MIN6 cells, resulting in an increase in expression of cell cycle regulating genes cyclin D1 and C-myc. Chronic exposure to olanzapine results in secondary dysregulation of beta catenin dynamics. There is increased nuclear beta catenin which is linked to nuclear retention by TCF7L2, and reduced

cytoplasmic beta catenin which is linked to increase Axin2 and augmented destruction complex activity. Cytoplasmic beta catenin is postulated to be involved in insulin secretion therefore altered cellular distribution may be deleterious to function; the following Chapter aimed to investigate the impact of deranged beta catenin distribution on insulin secretion.

Chapter 5: The mechanism through which chronic olanzapine reduces insulin secretion in MIN6 beta cells

5.1 Introduction

The data presented in Chapter 4 revealed that olanzapine can influence signalling in the canonical Wnt signalling pathway. It was established that there was acute stimulation of beta catenin signalling by olanzapine, followed by chronic effects to deregulate cellular distribution of the protein. Concentration and nucleocytoplasmic distribution of beta catenin has been postulated to be significant in the regulation of insulin secretion. In the following Chapter we aimed to test the hypothesis that it is through shifting beta catenin cellular dynamics that olanzapine reduces GSIS from MIN6 cells.

5.1.1 GSIS

Diabetes is a disease characterised by beta cell dysfunction and insufficient insulin secretion, which results in glucose accumulation and ongoing hyperglycaemia. There is abundant evidence linking olanzapine treatment to hyperglycaemia and diabetes [60, 70, 120, 123, 314] but, despite evidence linking the drug to peripheral insulin resistance [54], the effect on beta cells remains unclear. We have shown in Chapter 3 that chronic olanzapine can cause a significant reduction in GSIS with no change in intracellular insulin content. Thus we hypothesise that the effect of olanzapine on insulin secretion is through a mechanism involved in the regulation of secretion rather than a change in insulin transcription or processing.

The process of glucose stimulated insulin secretion starts with glucose transport into the beta cell through GLUT2 transporters. Glucose is phosphorylated by glucokinase which leads to increased mitochondrial generation of ATP and closure of K_{ATP} channels, which results in membrane depolarisation. L-type voltage dependent calcium channels detect the increased cell depolarisation and open to allow influx of calcium (Ca^{2+}) into the cell [170]. Insulin secretory vesicles are strictly organised within the cell by microtubules and the actin cytoskeleton which allow movement of vesicles along intracellular structures, but prevent contact with the membrane during unstimulated conditions [315, 316]. An increase in intracellular Ca^{2+} triggers changes in microtubule structures [316] and activates synaptotagmins to allow secretory granule fusion with the membrane in a mechanism involving Soluble NSF attachment proteins receptor (SNARE) proteins [317]. Subsequently,

insulin and other secretory products are released into the extracellular space to be transported around the body [170]. A functional role for beta catenin in the process of insulin secretion has recently been recognised, and altering beta catenin concentration can modify insulin vesicle trafficking and secretion [210, 297]. The data presented in Chapter 4 highlights that olanzapine can stimulate changes in beta catenin signalling, thus we aimed to establish if changes in Wnt/beta catenin signalling by olanzapine are linked to the reduced GSIS.

5.1.2 Beta catenin involvement in insulin secretion

While beta catenin is a well-known effector of canonical Wnt signalling, and an important regulator of beta cell proliferation [273, 298], the role of beta catenin as a modulator of insulin secretion is a relatively recent and evolving concept. Genetic studies revealed that single nucleotide polymorphisms (SNPs) in TCF7L2 are frequently associated with development of diabetes [276]. TCF7L2 and beta catenin form a nuclear bipartite transcription factor, and subsequent research aimed to establish what roles TCF7L2 and beta catenin play in beta cell function. Early work by Hodgkin *et al*. (2007) investigated the role of adherens junctions within beta cells and showed that free beta catenin was largely co-localised with insulin secretory granules [318]. This suggested there was a role for beta catenin in insulin secretion. Schinner *et al*. (2008) added to the suggestion of a role for Wnt signalling in insulin secretion. The addition of adipocyte derived fat cell-conditioned medium (FCCM) to beta cells was shown to increase proliferation, TCF specific transcription, and cyclin D1 expression, which was indicative of Wnt stimulation as discussed in Chapter 4. Furthermore, there was also a significant increase in insulin secretion which was inhibited by a frizzled receptor antagonist, implying the effect was stimulated by Wnt activation [319]. This prompted further research investigating a possible role for Wnt/beta catenin to regulate insulin secretion.

As fluctuating extracellular glucose concentration is fundamental for initiation of insulin secretion Anagnostou and Shepherd (2008) aimed to clarify if beta catenin can be modified through changes in glucose [299]. Most cell types, including beta cells, have a high concentration of highly stable cadherin bound beta catenin, which can restrict the ability to detect small fluctuations in free beta catenin, but macrophage cell lines have low levels of cadherins [299]. Glucose addition was

found to significantly increase beta catenin concentration in the two macrophage cell lines tested, suggesting that beta catenin can indeed be modulated by glucose [299]. The glucose stimulatory effect on beta catenin was subsequently confirmed in a beta cell line by Cognard *et al*. (2013) who showed that 4 hour glucose exposure increased both cytoplasmic and nuclear beta catenin in glucose starved INS-1E cells [297].

The role of free beta catenin in beta cells was further substantiated by Sorrenson *et al*. (2016) who showed that modulating concentration of free beta catenin resulted in changes in insulin secretion. SiRNA and pyrvinium were added to reduce the beta catenin concentration, which was found to reduce GSIS, while BIO increased beta catenin concentration and accordingly increased GSIS [210]. Total internal reflection fluorescence (TIRF) microscopy was used to show that beta catenin depletion disturbs the insulin vesicle density at the cell membrane and it was concluded that beta catenin is involved in insulin secretion [210].

5.1.3 Mechanism for glucose induced changes in beta catenin

As discussed in Chapter 4, cytoplasmic beta catenin concentration is usually maintained at low levels through interaction with the beta catenin destruction complex. But it is suggested that the glucose stimulatory effect on beta catenin is independent of the destruction complex, and instead involves increasing stabilisation of the protein through an alternate pathway. The destruction complex comprises GSK3, CK1α, APC and Axin (Axin1 and Axin2), which constitutively phosphorylate beta catenin at the N-terminal to target the protein for proteasomal degradation [280, 281]. Although the stability of beta catenin is more commonly discussed in terms of activity of the destruction complex, there is also a noteworthy influence of phosphorylation at the C- terminus which stabilises the protein, and increases functional activity [320]. (**Figure 42**)

GSK3β is essential for activity of the beta catenin destruction complex, and GSK3β inhibitors are commonly used as small molecule activators of beta catenin [321], but in both macrophages [299] and beta cells [297], the glucose stimulatory effect on beta catenin was shown to be independent of GSK3β. GSK3β activity was not found

to be significantly affected by glucose, but the amount of beta catenin phosphorylated by the destruction complex to be targeted for ubiquitination was reduced [299], and instead, phosphorylation at Ser 552 was increased [297]. Ser 552 phosphorylation of beta catenin has previously been shown to be linked to changes in cAMP, CREB and PKA [297]. Therefore the evidence suggests that beta catenin exists in a balance between stable, functional beta catenin, and beta catenin that is unstable and inescapable from proteasomal degradation.

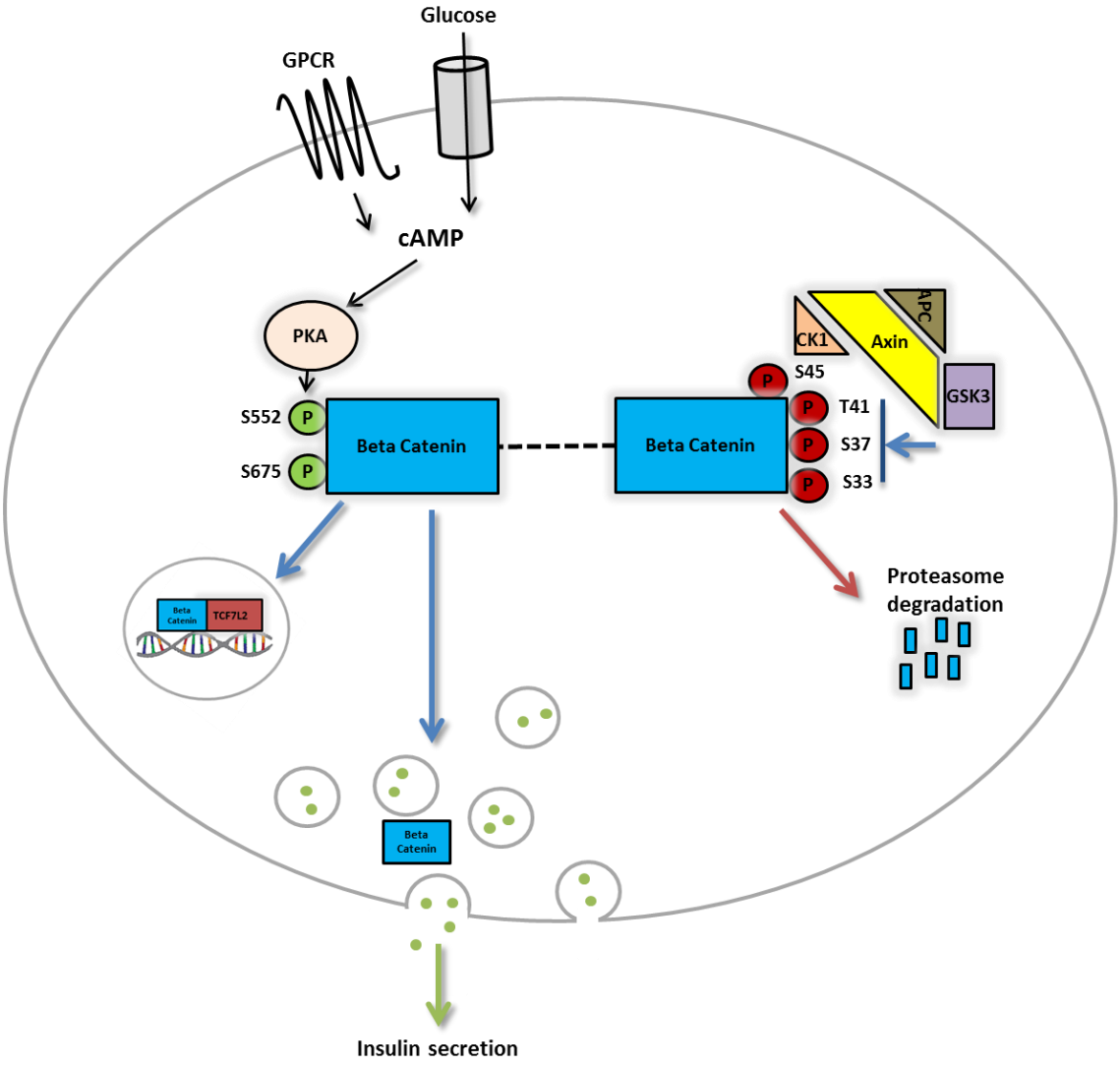


Figure 42 Schematic diagram demonstrating the regulation and function of beta catenin in beta cells.

Results from Chapter 4 suggest that chronic olanzapine can alter the concentration and distribution of beta catenin in following culture in low glucose, unstimulated conditions. Under such conditions beta catenin is already tightly regulated through activity of the destruction complex, and the functional role is negligible. But considering the effect of glucose to increase beta catenin stability, and the possible functional requirement of increased beta catenin stability for insulin secretory mechanisms, we do not yet know the consequence of olanzapine induced imbalance of beta catenin on GSIS. We hypothesise that it is through disturbing the balance of beta catenin and other components of the Wnt signalling pathway that olanzapine has a negative impact on insulin secretion.

5.1.4 Beta catenin regulates vesicle organisation

At this time the mechanism through which beta catenin modifies insulin secretion and vesicle trafficking has not been extensively explored in beta cells. But in neurons, beta catenin has been shown to have a functional role to regulate the localisation of the reserved pool of synaptic vesicles [322].

Neurons and beta cells share many similar functional characteristics; for example in neurons an action potential triggers calcium influx through voltage gated calcium channels, which triggers exocytosis of synaptic vesicles [323]. The process of insulin secretion equally involves membrane depolarisation triggering calcium influx [324]. An increase in intracellular calcium is detected by SNARE proteins which initiate fusion of the vesicles with the membrane and exocytosis of insulin or neurotransmitters [325].

Regulation of secretory vesicles in neurons and beta cells is also closely comparable. Synaptic vesicles exist in different pools; the readily releasable pool (RRP) which contains a small number of vesicles docked at the cell membrane ready for quick release when stimulated, and the undocked pool which is referred to as the reserve/ resting pool. Following rapid release of the RRP, the reserve pool maintains secretion at a lower rate [326]. In beta cells, there are similarly different pools of insulin secretory granules. The RRP comprises insulin containing vesicles docked at the membrane, which are in a complex with SNARE and other proteins that are calcium sensitive to allow rapid secretion upon glucose stimulated calcium influx [327]. The RRP is responsible for first phase peak in insulin secretion from

beta cells, and the reserve pool moves at a slower rate to the membrane upon ongoing stimulation and elicits the prolonged and sustained second phase of insulin secretion [328]. Therefore we suggest that the role of neuronal beta catenin in the regulation of synaptic vesicles may certainly be comparable to beta cells.

The role of beta catenin in pre-synaptic vesicle assembly has been described in several studies. For example; in hippocampal neurons deletion of beta catenin was shown to modify the reserve pool of vesicles, resulting in fewer, more diffuse vesicles, and impaired response to repetitive or prolonged stimulation [322, 329]. Wnt and Dvl deficiency *in vivo* was also shown to reduce the number of synaptic vesicles close to the membrane and impaired formation of the SNARE complex [330]. This suggests that beta catenin has a role in vesicle organisation and localisation at the pre-synaptic site. The effect is thought to be mediated through interaction with the C-terminal PDZ domain of beta catenin and the protein scribble, which clusters with the vesicles at the synapse [331].

In beta cells, the role of beta catenin in cellular vesicle distribution has also been suggested to be involved in its role in insulin secretion [210]. Beta catenin deletion has been shown to cause redistribution of insulin vesicles in INS-1E cells, causing restriction of insulin vesicle mobilisation upon glucose stimulation [210, 275]. This results in inhibited insulin secretion and implicates beta catenin in insulin vesicle movement to the membrane for the final stages of the process of GSIS.

If chronic olanzapine treatment causes a reduction in cytoplasmic beta catenin we hypothesise that this leads to a reduction in available beta catenin for vesicle trafficking and insulin secretion. The following Chapter aimed to explore the impact of chronic olanzapine and resultant changes in beta catenin dynamics on subsequent insulin secretion.

5.2 Results

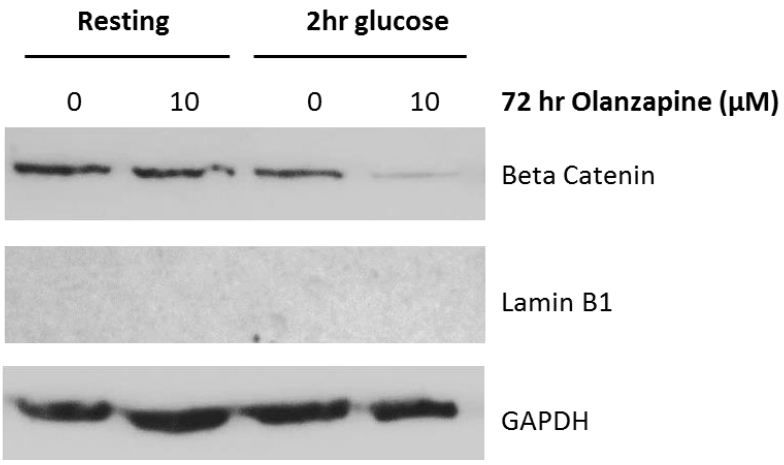
5.2.1 Effect of glucose stimulation on beta catenin in olanzapine treated cells

In Chapter 4 it was demonstrated that olanzapine interacts with components of the canonical Wnt signalling pathway to upregulate Wnt target gene expression and alter the concentration and distribution of beta catenin within the cell. Beta catenin is central to the canonical Wnt signalling pathway, but it has also recently been linked to mechanisms involved in insulin secretion from beta cells [210, 297]. In Chapter 3 it was shown that olanzapine can reduce GSIS from MIN6 cells with no change in intracellular insulin content, suggesting that there may be interference in the mechanics of secretion. We now hypothesise this is due to alterations in beta catenin dynamics. Initial experiments aimed to test the hypothesis that fluctuations in beta catenin induced by chronic olanzapine can influence GSIS from MIN6 cells.

To investigate if chronic olanzapine has an effect on beta catenin during glucose stimulation Western blot was used to determine cytoplasmic concentration before and after a 4 hour GSIS protocol. **Figure 43** shows that prior to glucose stimulation, chronic treatment with 10 μM olanzapine (resting) caused a small reduction in cytoplasmic beta catenin concentration.

Beta catenin is a glucose sensitive protein, and glucose concentration has previously been shown to relate to beta catenin concentration in beta cells, an effect which is linked to insulin secretion mechanisms [297, 299]. Following treatment with olanzapine, cells were glucose starved, which has previously been shown to reduce and normalise beta catenin in beta cells [297]. High glucose (25 mM) was then added to stimulate GSIS pathways for 2 hours. **Figure 43** demonstrates that following the GSIS protocol, there was a pronounced difference in cytoplasmic beta catenin in the olanzapine group compared to control ($p<0.05$). This suggests that the effects of chronic olanzapine exposure result in a reduced cytoplasmic beta catenin concentration during glucose stimulation. Possibly by preventing glucose induced increase in concentration following glucose starvation.

A.



B.

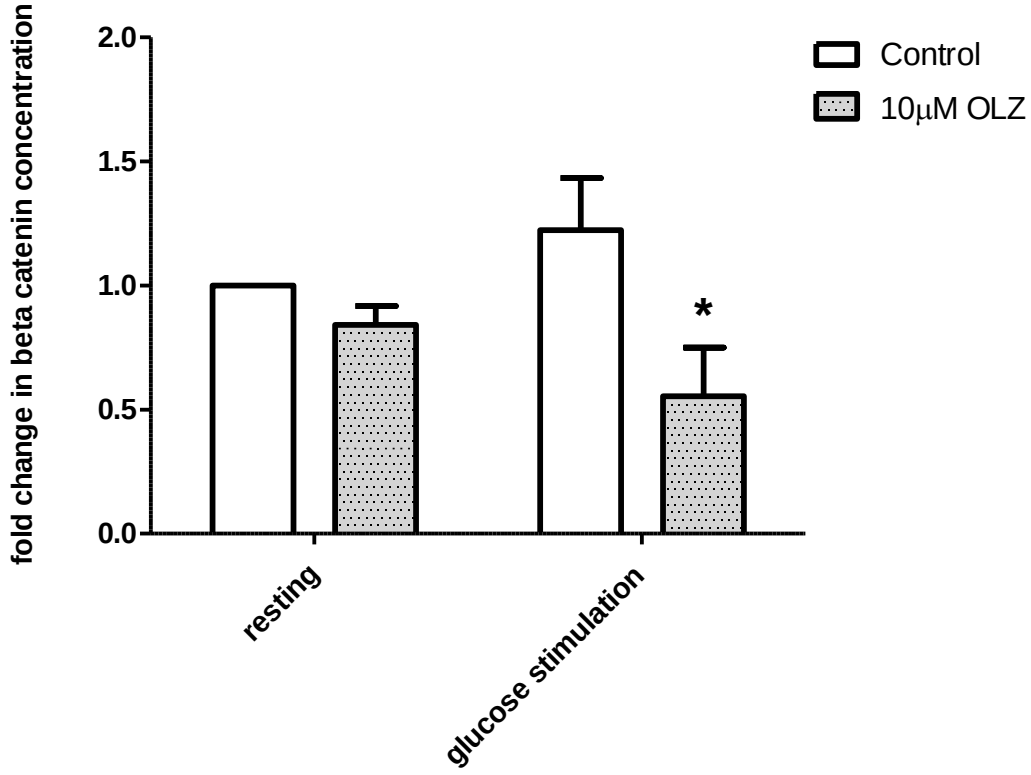


Figure 43 investigating the effect of glucose on cytoplasmic beta catenin in in MIN6 cells pre- treated with olanzapine. MIN6 cells were incubated with 10 µM olanzapine for 72 hours (resting) then standardised with 0 mM glucose for 2 hours followed by 2 hour exposure to 25 mM glucose (glucose stimulation). The cytoplasmic fraction was extracted and lysates were resolved by SDS page and immunoblotted against beta catenin antibodies and loading control antibodies against GAPDH (cytoplasm) and Lamin B1 (nuclear). Representative western blot (A). Quantified data of cytoplasmic beta catenin relative to GAPDH from 3 independent experiments (B). Data are displayed as mean ± SEM * $p<0.05$ versus 0 µM using a two-way ANOVA and Bonferroni's post-test

5.2.1.1 Addition of small molecule inhibitor BIO

As beta catenin has recently been shown to be involved in the process of insulin secretion [297, 299] we next tested the hypothesis that it is through reduced beta catenin that olanzapine reduces insulin secretion. Firstly we aimed to investigate if addition of a small molecule inhibitor of the beta catenin destruction complex could increase beta catenin levels during glucose stimulation.

GSK3β is a component of the destruction complex, and as depicted in **Figure 44**, through inhibition of GSK3β, BIO can reduce the activity of the beta catenin destruction complex and reduce proteasomal degradation of beta catenin.

In support of the result obtained above (**Figure 43**), **Figure 45** adds to the evidence that following 2 hour glucose stimulation there was a significant reduction in cytoplasmic beta catenin in olanzapine treated cells compared to untreated cells ($p<0.05$). Addition of 1 μM BIO to the 2 hour glucose stimulation caused a significant increase in cytoplasmic beta catenin in olanzapine treated cells ($p<0.05$). In control cells BIO addition did not have any significant effect on cytoplasmic beta catenin. Nuclear beta catenin was not significantly affected by olanzapine or glucose/ BIO stimulation.

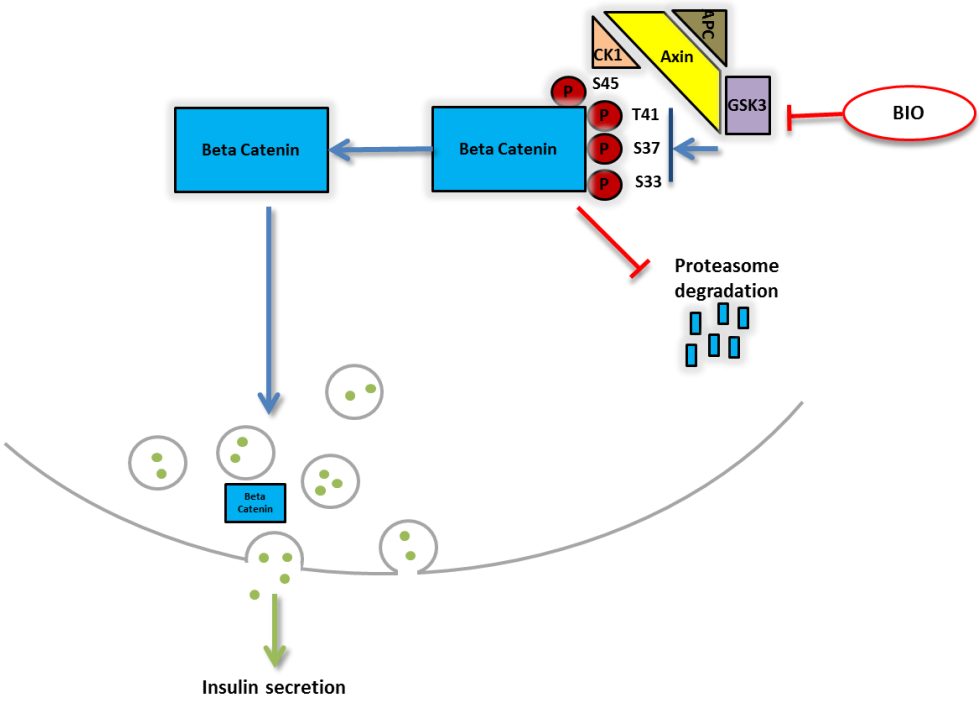


Figure 44 Illustration of the effect of BIO to inhibit the beta catenin destruction complex, reduce proteasomal degradation and increase concentration of beta catenin

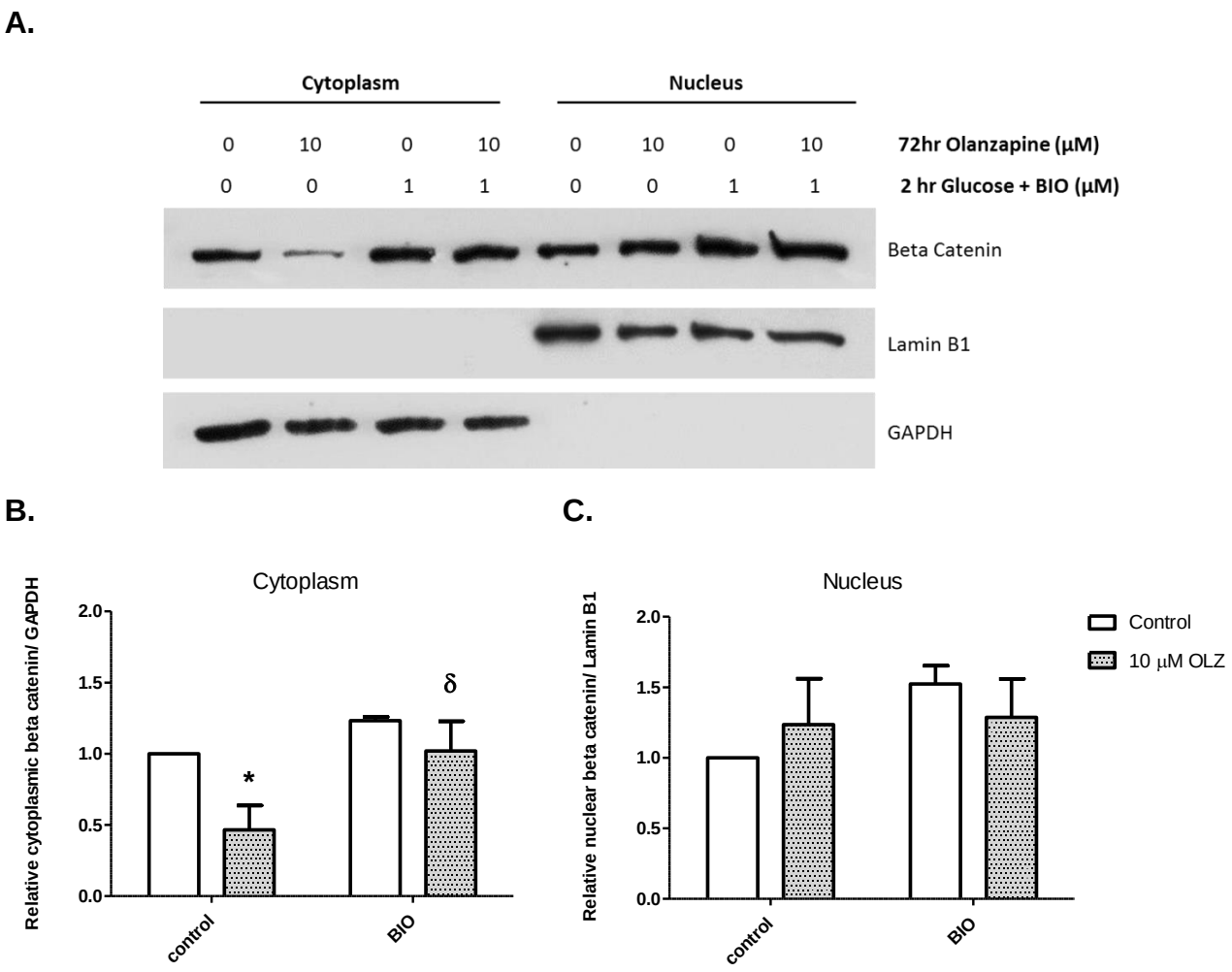


Figure 45 investigating the effect of BIO on beta catenin concentration and location in MIN6 cells pre- treated with olanzapine. MIN6 cells were incubated with 10 μM olanzapine for 72 hours then standardised with 0 mM glucose for 2 hours followed by 2 hour exposure to 25 mM glucose (control) or 25 mM glucose + 1μM GSK3 inhibitor BIO (BIO). Nuclear and cytoplasmic fractions were extracted and lysates were resolved by SDS page and immunoblotted against beta catenin antibodies and loading control antibodies GAPDH (cytoplasm) and Lamin B1 (nuclear). Representative western blot (A). Quantified data of cytoplasmic beta catenin relative to GAPDH C. Quantified data of nuclear beta catenin relative to Lamin B1 from 3 independent experiments (B). Data are displayed as mean ± SEM, *p<0.05 versus 0 μM olanzapine δ p<0.05 versus control using a two-way ANOVA and Bonferroni's Post-test

5.2.2 Effect of beta catenin modification on olanzapine induced changes in insulin secretion

5.2.2.1 Glucose

Insulin secretion assay was next used to establish if improving the cytoplasmic beta catenin concentration with BIO had an effect on glucose stimulated insulin secretion. In accordance with previous results shown in Chapter 3, **Figure 46** shows that 10 µM olanzapine caused a significant reduction in glucose stimulated insulin secretion compared to control ($p<0.05$). The addition of BIO to the 2 hour glucose stimulation significantly increased the insulin secretion in olanzapine treated cells ($p<0.05$), and the secreted insulin was similar to control cells. BIO alone had no impact on insulin secretion in this model. This suggests that the improvement in insulin secretion in olanzapine treated cells by BIO is linked to the increased cytoplasmic beta catenin by BIO observed in **Figure 45**. Taken together these results imply that beta catenin has a significant role in mediating the negative effects of olanzapine on insulin secretion.

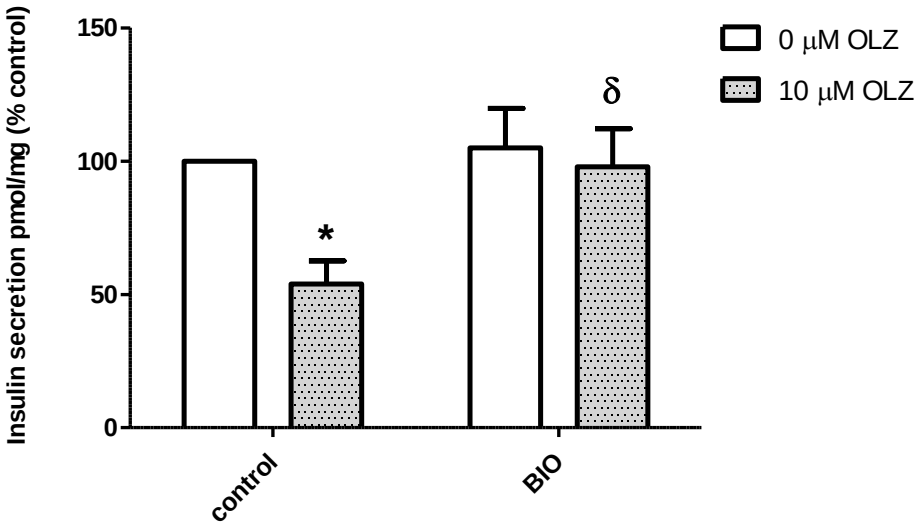


Figure 46 investigating the effect of BIO on glucose stimulated insulin secretion from MIN6 cells pre- treated with olanzapine for 72 hours. MIN6 cells were incubated with 10 µM olanzapine for 72 hours then standardised with 0 mM glucose followed by 2 hour exposure to 25 mM glucose (control) or 25 mM glucose + 1µM GSK3 inhibitor BIO (BIO). Following treatment supernatant was collected and insulin concentration determined with ELISA and normalised to protein content. Results shown are of 4 independent experiments. Data are displayed as mean ± SEM, *$p<0.05$ versus 0 µM olanzapine δ $p<0.05$ versus control using a two-way ANOVA and Bonferroni's Post-test

5.2.2.2 Membrane depolarisation and calcium influx

We have shown that by restoring cytoplasmic beta catenin concentration, BIO can recover glucose stimulated insulin secretion in olanzapine treated cells, highlighting the significance of beta catenin to mediate the effect of olanzapine. It has been suggested that beta catenin is involved in insulin vesicle trafficking to the membrane, and that a reduction in beta catenin concentration prevents normal vesicle movement and insulin secretion [210, 275]. We hypothesised that it is through changes in vesicle trafficking downstream of glucose stimulation that olanzapine exerts its effects on insulin secretion.

To test if olanzapine acts through changes in vesicle movement independently of glucose, calcium channel blockers and membrane depolarising agents were used to inhibit and stimulate secretion in the absence of glucose stimulation. As illustrated in **Figure 47** tolbutamide and KCl depolarise the cell membrane in a process that bypasses the requirement of glucose stimulation. Depolarisation leads to opening of voltage gated calcium channels, resulting in calcium influx. Nifedipine is a calcium channel blocker that inhibits insulin secretion by preventing calcium influx into the beta cell. Calcium influx is the final stage in the process of insulin secretion, where calcium triggers vesicle fusion with the membrane and exocytosis of insulin.

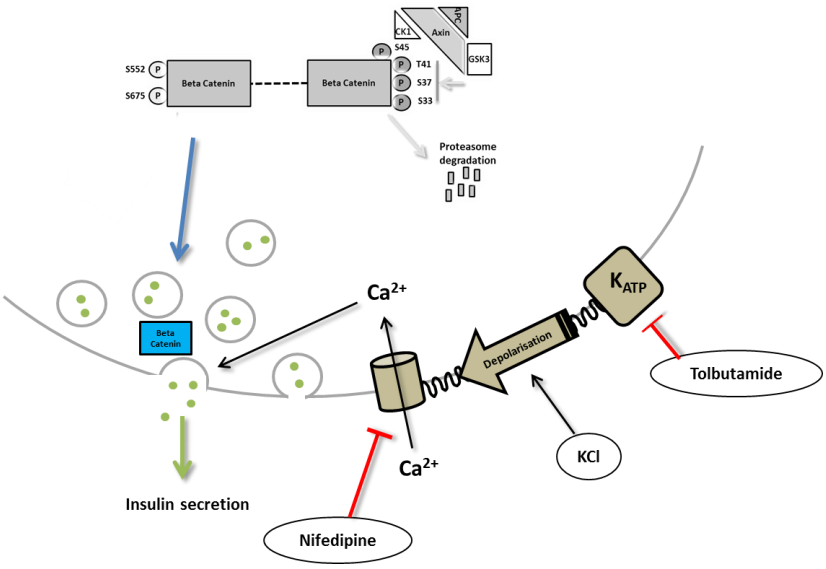


Figure 47 Illustration depicting the mechanism of action of Tolbutamide, KCl and nifedipine to stimulate and inhibit insulin secretion. Tolbutamide is a K_{ATP} channel blocker which inhibits K^+ transport out of the cell and causes membrane depolarisation. KCl directly depolarises the membrane which causes opening of voltage gated calcium channels and influx of calcium which trigger insulin exocytosis. Nifedipine blocks calcium channels and prevents influx of calcium and insulin secretion.

5.2.2.2.1 Nifedipine

In agreement with our previous results, **Figure 48** shows that olanzapine caused a significant reduction in insulin secretion in response to glucose stimulation ($p<0.05$) which was reversed by addition of BIO to the glucose challenge ($p<0.05$). 5 µM Nifedipine addition significantly reduced insulin secretion in all conditions, confirming that MIN6 cells are functioning beta cells that secrete insulin in a calcium dependent mechanism. Beta catenin is postulated to be involved in vesicle trafficking, but an increase in beta catenin concentration stimulated by BIO was unable to stimulate insulin secretion in the absence of calcium influx. This suggests that olanzapine acts through a different pathway to reduce insulin secretion.

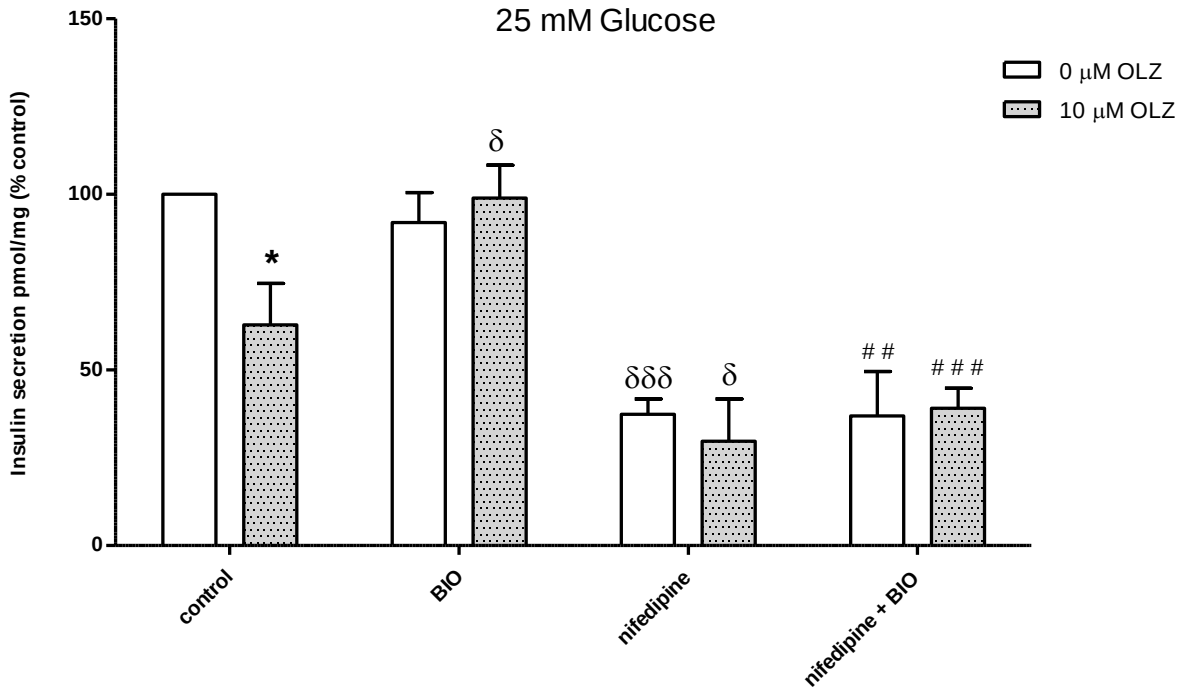


Figure 48 investigating the effect of inhibitory drugs on insulin secretion from MIN6 cells pre- treated with olanzapine for 72 hours. MIN6 cells were incubated with 10 µM olanzapine for 72 hours then standardised with 0 mM glucose followed by 2 hour exposure to 5 µM nifedipine in 25 mM glucose with and without 1µM GSK3β inhibitor BIO. Following treatment supernatant was collected and insulin concentration determined with ELISA and normalised to protein content. Results shown are of 3 independent experiments. Data are displayed as mean ± SEM, *$p<0.05$ versus 0 µM olanzapine, δ $p<0.05$, δδδ $p<0.001$ versus control # # $p<0.01$, # # # $p<0.001$ versus BIO using a two-way ANOVA and Bonferroni's Post-test

5.2.2.2.2 Tolbutamide and KCl

To test if olanzapine reduces insulin secretion downstream of membrane depolarisation, addition of depolarising agents tolbutamide and KCl was used to stimulate insulin secretion with or without the addition of BIO.

As shown in **Figure 49** in low glucose stimulation, olanzapine caused a minor but insignificant reduction in insulin secretion. 200 µM Tolbutamide significantly stimulated insulin secretion from untreated cells compared to control ($p<0.05$), and 40 mM KCl more profoundly stimulated insulin secretion from untreated cells compared to control ($p<0.01$). Tolbutamide and KCl also significantly increased insulin secretion from olanzapine treated cells ($p<0.05$). The effect of stimulation was not significantly different between olanzapine treated and untreated cells suggesting that the mechanism through which Tolbutamide and KCl stimulate insulin secretion is not affected by olanzapine.

This suggests that olanzapine acts through a glucose dependent pathway upstream of membrane depolarisation, possibly through interference with glucose induced changes in beta catenin concentration.

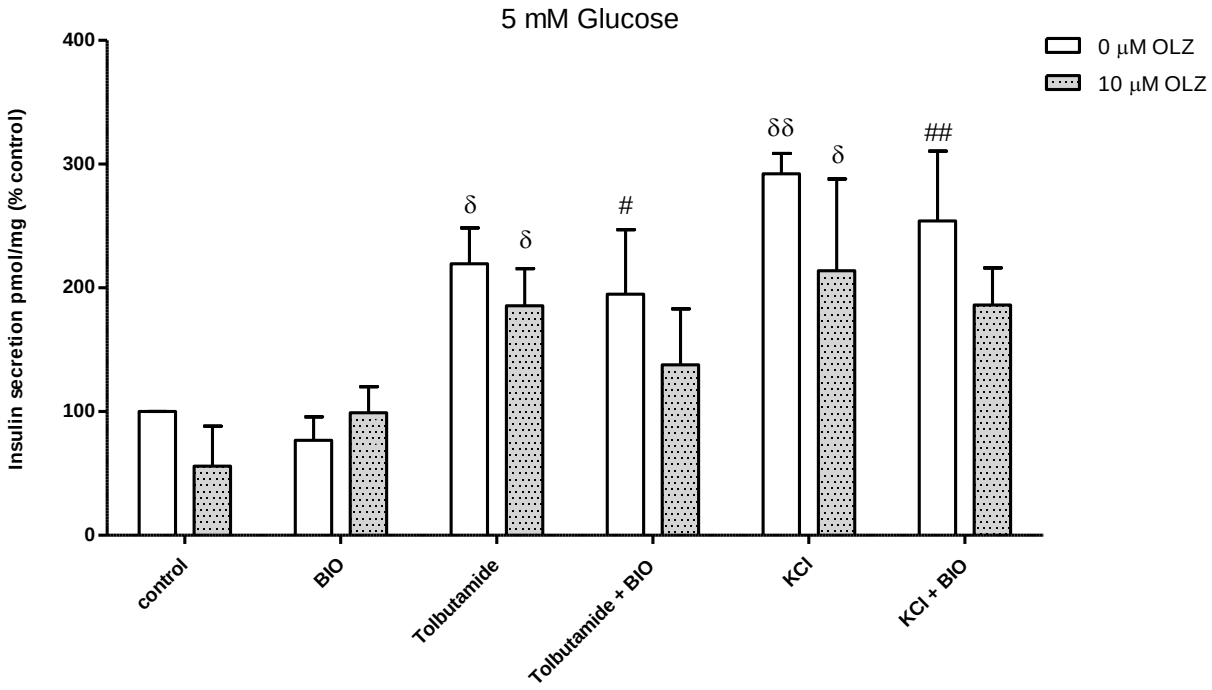


Figure 49 investigating the effect of stimulatory drugs on insulin secretion from MIN6 cells pre- treated with olanzapine for 72 hours. MIN6 cells were incubated with 10 μM olanzapine for 72 hours then standardised with 0 mM glucose followed by 2 hour exposure to 5 mM glucose (control) with or without BIO, Tolbutamide or KCl. Following treatment supernatant was collected and insulin concentration determined with ELISA and normalised to protein content. Results shown are of 3 independent experiments. Data are displayed as mean ± SEM, δ $p<0.05$, δδ $p<0.01$ versus control, # $p<0.05$, ## $p<0.01$ versus BIO using a two-way ANOVA and Bonferroni's Post-test

5.2.2.3 Exenatide

Olanzapine had no significant effect on insulin secretion stimulated by membrane depolarisation independently of glucose, which suggests that olanzapine induced cellular changes are associated with a stimulatory pathway upstream of membrane depolarisation. Possibly by inhibiting glucose stimulated increases in beta catenin. It has been suggested that beta catenin concentration can be modified by both glucose and GLP-1, with subsequent involvement in insulin secretion [210, 297]. To test the hypothesis that olanzapine reduces insulin secretion in response to GLP-1 receptor activation, exenatide was added to the low glucose stimulation with or without 1 µM BIO.

As shown in **Figure 50** olanzapine treated cells secreted significantly less insulin when exposed to 20 nM exenatide than untreated cells ($p<0.05$). Addition of BIO to the exenatide stimulation was able to significantly increase the insulin secretion in olanzapine treated cells ($p<0.001$), which was a similar trend to the glucose stimulated results shown in **Figure 46** where BIO also improved glucose stimulated insulin secretion. This implies that olanzapine induced changes in insulin secretion are through a pathway that can be stimulated by glucose or GLP-1, and the effect of BIO to improve insulin secretion is also associated with interaction with this pathway. Glucose and GLP-1 agonists have previously been shown to phosphorylate and stabilise beta catenin to influence insulin secretion [210], the present data suggest that olanzapine inhibits this effect to reduce beta catenin concentration and reduce insulin secretion.

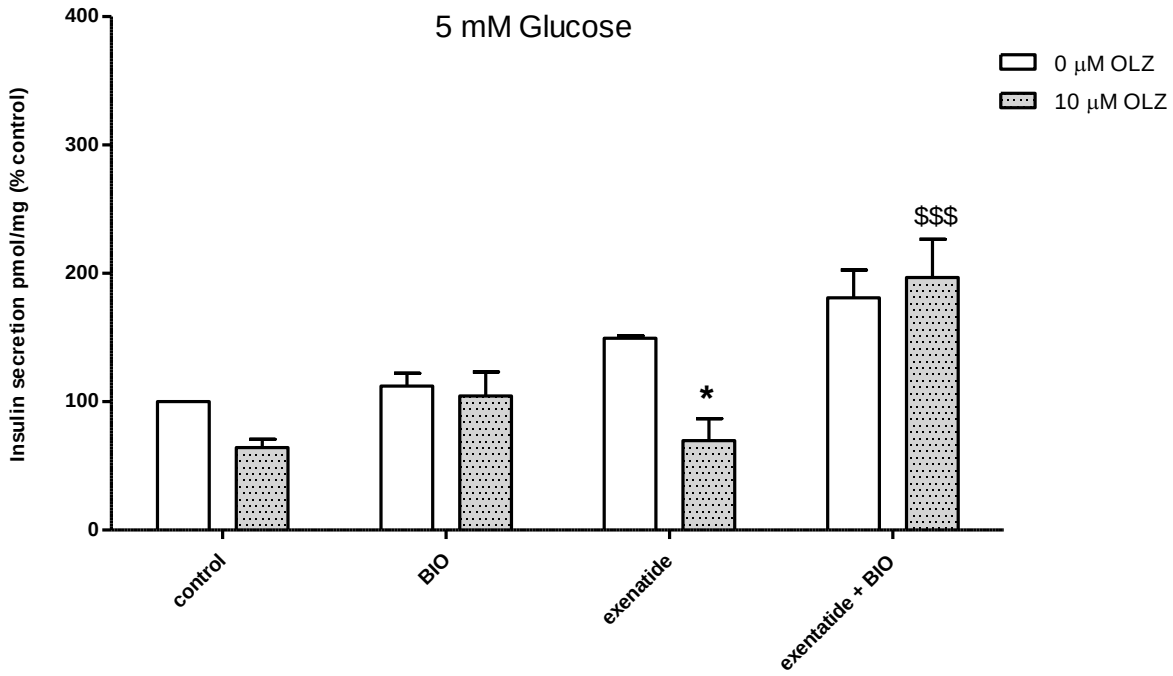


Figure 50 investigating the effect of GLP1 receptor agonist exenatide on insulin secretion from MIN6 cells pre- treated with olanzapine for 72 hours. MIN6 cells were incubated with 10 μM olanzapine for 72 hours then standardised with 0 mM glucose followed by 2 hour exposure to 5 mM glucose (control) with or without 20 nM exenatide and 1 μM BIO. Following treatment supernatant was collected and insulin concentration determined with ELISA and normalised to protein content. Results shown are of 3 independent experiments. Data are displayed as mean ± SEM, *p<0.05 versus 0 μM olanzapine, $$$ p<0.001 versus exenatide using a two-way ANOVA and Bonferroni's Post-test

5.2.2.4 Forskolin and PMA

It is suggested that glucose and GLP-1 agonists modify beta catenin through changes in cAMP and PKA [210]. To further examine if olanzapine inhibits the pathway involved in glucose stimulated beta catenin stabilisation, forskolin was added to the stimulation. Forskolin is an activator of adenylyl cyclase which increases cAMP levels [332]. PMA was also investigated as an activator of PKC, which is a downstream effector of several GPCR which may also be targeted by olanzapine [333].

As shown in **Figure 51** both 10 µM forskolin and 0.5 µM PMA significantly stimulated insulin secretion compared to control ($p<0.01$), and in both stimulation conditions, cells pre-treated with 10 µM olanzapine secreted significantly less insulin compared to 0 µM. This suggests that olanzapine influence on insulin secretion is downstream of changes in cAMP and GPCR interaction.

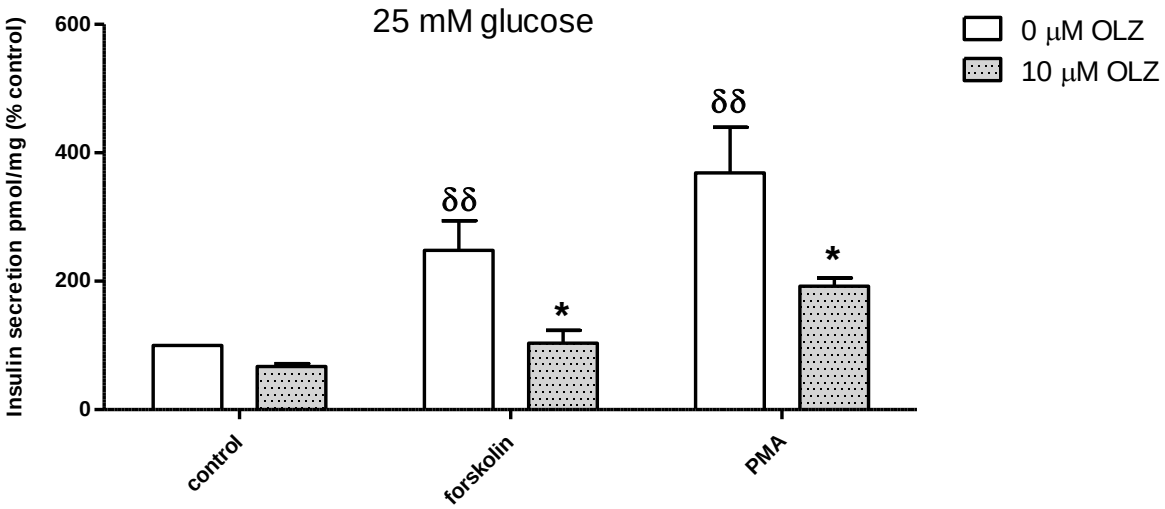


Figure 51 investigating the effect of forskolin and PMA on insulin secretion from MIN6 cells pre- treated with olanzapine for 72 hours. MIN6 cells were incubated with 10 µM olanzapine for 72 hours then standardised with 0 mM glucose followed by 2 hour exposure to 25 mM glucose (control) with or without 10 µM forskolin or 0.5 µM PMA. Following treatment supernatant was collected and insulin concentration determined with ELISA and normalised to protein content. Results shown are of 3 independent experiments. Data are displayed as mean ± SEM, *$p<0.05$ versus 0 µM olanzapine, δδ $p<0.01$ versus control using a two-way ANOVA and Bonferroni's Post-test

5.2.3 Effect of glucose stimulation on proteins involved in beta catenin regulation in olanzapine treated cells

5.2.3.1 GSK3β

Olanzapine treatment has been shown to reduce cytoplasmic beta catenin during glucose stimulation, and BIO addition has been demonstrated to improve beta catenin (**Figure 45**). Further experiments were undertaken to investigate the mechanism through which olanzapine treatment leads to reduced beta catenin in stimulated cells, and to establish how BIO improves the olanzapine induced changes beta catenin and insulin secretion.

BIO is a pharmacological inhibitor of GSK3 that leads to inhibited action of the beta catenin destruction complex. GSK3 is constitutively active protein that is a component of numerous signalling pathways, and several different enzymes are able to inactivate GSK3 through phosphorylation at different sites. The active form of GSK3α is auto phosphorylated at Tyr 216 and GSK3β is auto phosphorylated at Try 279[334]. Tyr 216/279 phosphorylated GSK3 is a component of the beta catenin destruction complex as illustrated in **Figure 52**.

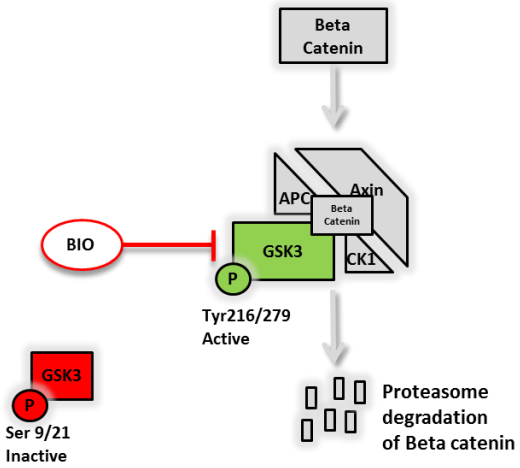


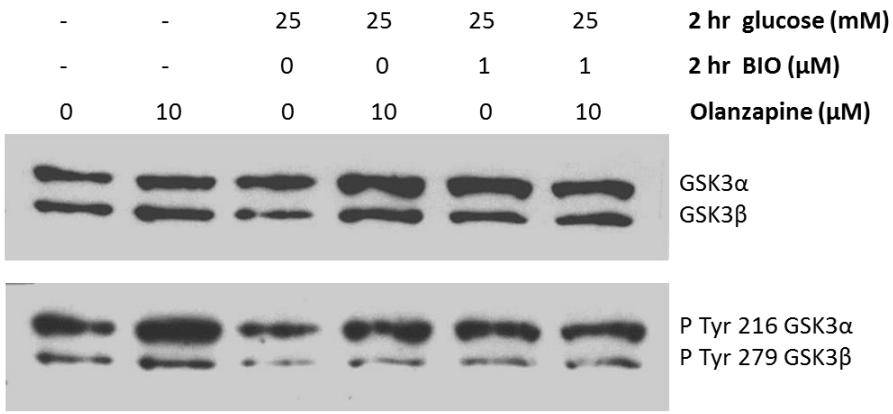
Figure 52 Schematic diagram showing active tyrosine 216/279 phosphorylated GSK3 as a component of the beta catenin destruction complex and serine 9/21 phosphorylated inactive GSK3 that does not interact with beta catenin.

Figure 53 shows that olanzapine did not have any significant effect on active GSK3 compared to control. There was no difference in active GSK3 following 72 hour treatment (resting), or following glucose and BIO stimulation. This suggests that olanzapine does not alter Tyr 216/279 phosphorylated active GSK3 activity in this model.

Glucose and BIO had an overall significant effect on active GSK3 when assessed by 2 way ANOVA ($p<0.05$). There was a trend for active GSK3 to reduce following glucose challenge, and further reduce on addition of BIO. This relates to the known pharmacology of BIO as a GSK3 inhibitor.

The action of BIO to inhibit GSK3 disrupts the beta catenin destruction complex, but the complex comprises many components which are all involved in the process of targeting beta catenin for destruction. It is possible that olanzapine modifies the activity of the complex to reduce cytoplasmic beta catenin through an alternate member of the destruction complex, or an alternate pathway.

A.



B.

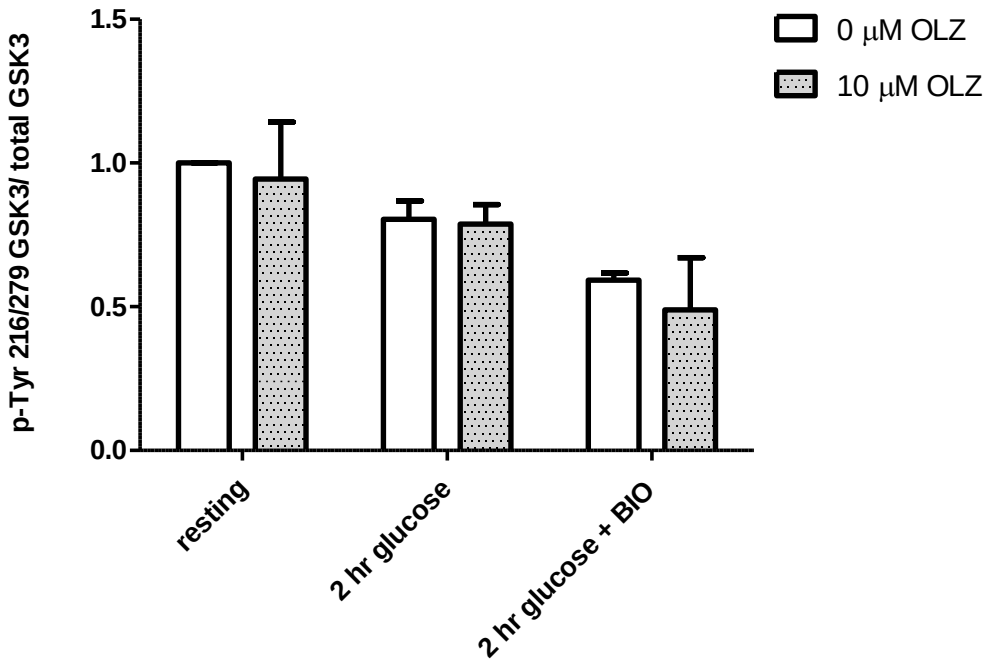


Figure 53 investigating the effect of glucose and BIO on active GSK3 in MIN6 cells pre- treated with olanzapine for 72 hours. MIN6 cells were incubated with 10 µM olanzapine for 72 hours then standardised with 0 mM glucose for 2 hours followed by 2 hour exposure to 25 mM glucose or 25 mM glucose + 1µM GSK3 inhibitor BIO. Whole cell lysates were extracted and resolved by SDS page then immunoblotted against phospho T216/279 GSK3 and total GSK3 antibodies. Representative Western blot (A). Quantified data of phospho GSK3 relative to total GSK3 from 3 independent experiments (B). Data are displayed as mean ± SEM

5.2.3.2 Axin2

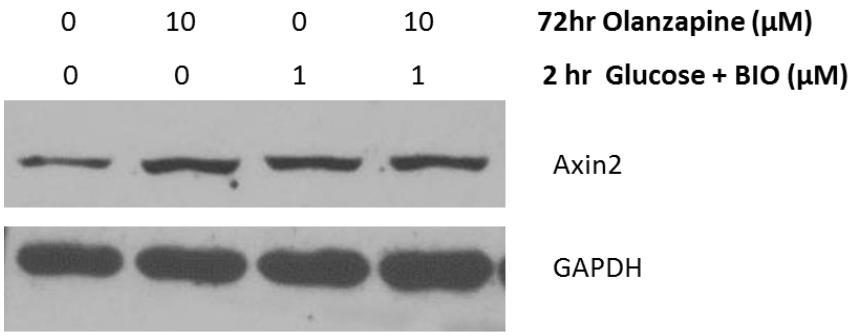
As well as GSK3, the beta catenin destruction complex comprises other proteins; Axin, CK1α and APC, that synchronise as a complex to phosphorylate beta catenin and target it for proteasomal destruction. Inhibition of one component is sufficient to disable the entire complex from interacting with beta catenin. Inhibition of GSK3 by BIO improves beta catenin concentration through inhibition of the entire destruction complex, but the effect of olanzapine to reduce beta catenin has been shown to be independent of GSK3 activity (**Figure 53**).

In Chapter 4 it was shown that olanzapine increases Axin2 concentration slowly over time. Axin2 is the central scaffold of the beta catenin destruction complex; it promotes phosphorylation of beta catenin by GSK3 and has been considered the rate limiting factor in beta catenin destruction complex stability. We hypothesised that an increase in Axin2 concentration is linked to reduced beta catenin. Further experiments aimed to investigate if Axin2 is involved in olanzapine effects on beta catenin concentration following glucose stimulation.

As shown in **Figure 54** following glucose stimulation, Axin2 concentration was significantly higher in the olanzapine group compared to control ($p<0.05$). BIO addition did not have a significant effect, but it reduced the significant effect of olanzapine on Axin2.

This suggests that the mechanism through which olanzapine reduces beta catenin concentration, and insulin secretion may be linked to increases in Axin2 and amplified activity of the beta catenin destruction complex. The effect of BIO to inhibit GSK3 prevents co-ordination of the complex to improve beta catenin and insulin secretion.

A.



B.

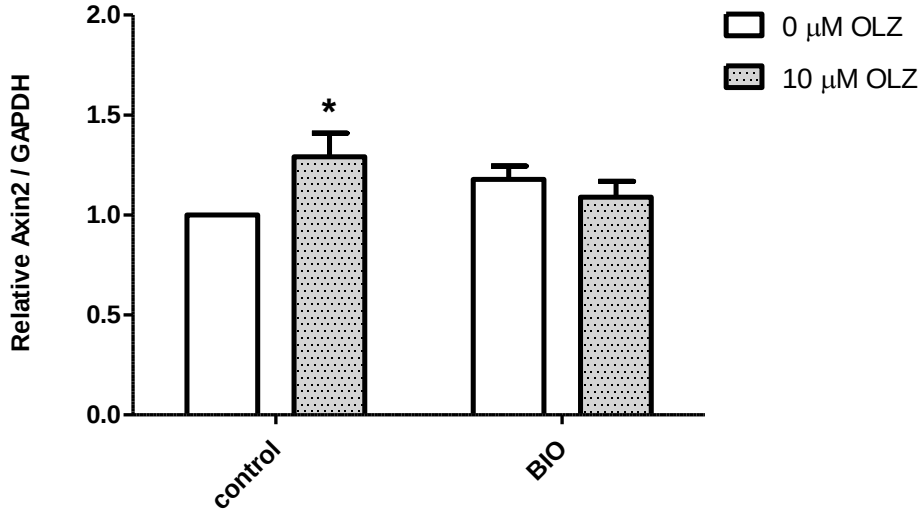


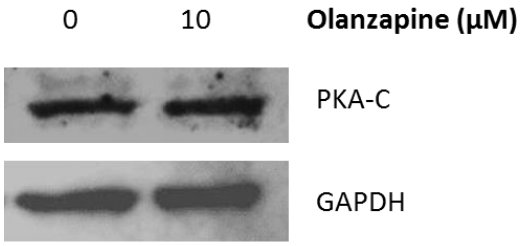
Figure 54 Investigating the effect of glucose and BIO on Axin2 concentration in MIN6 cells pre- treated with olanzapine for 72 hours. MIN6 cells were incubated with 10 μM olanzapine for 72 hours then standardised with 0 mM glucose for 2 hours followed by 2 hour exposure to 25 mM glucose or 25 mM glucose + 1μM GSK3 inhibitor BIO. Whole cell lysates were extracted and resolved by SDS page then immunoblotted against Axin2 antibody and loading control antibody against GAPDH. Representative Western blot (A). Quantified data of Axin2 relative to GAPDH from 4 independent experiments (B). Data are displayed as mean ± SEM, $p<0.05$ versus 0 μM olanzapine

5.2.3.3 PKA

The destruction complex negatively regulates protein levels of beta catenin, but beta catenin also interacts with PKA which positively regulates concentration. PKA phosphorylates and stabilises the protein to prevent incorporation into the destruction complex [335] and it is thought that glucose and exenatide increase beta catenin concentrations through increased cAMP and PKA [210, 297].

As shown in **Figure 55** following glucose stimulation there was no significant difference in PKA concentration between olanzapine treated and untreated cells. This suggests that changes in PKA may not be involved in the changes in beta catenin by olanzapine.

A.



B.

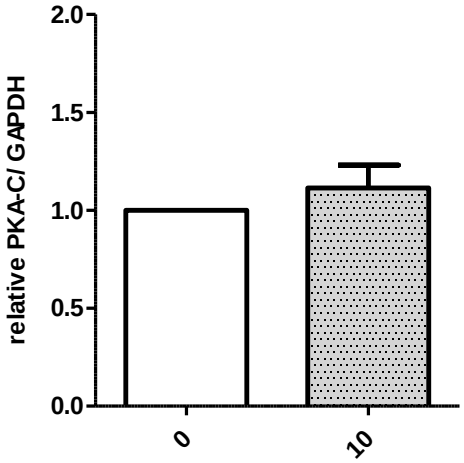


Figure 55 Investigating the effect of glucose on PKA-C concentration in MIN6 cells pre- treated with olanzapine for 72 hours. MIN6 cells were incubated with 10 µM olanzapine for 72 hours then standardised with 0 mM glucose for 2 hours followed by 2 hour exposure to 25 mM glucose. Whole cell lysates were extracted and resolved by SDS page then immunoblotted against PKA-C antibody and loading control antibody against GAPDH. Representative Western blot (A). Quantified data of PKA-C relative to GAPDH from 3 independent experiments (B). Data are displayed as mean ± SEM

5.2.4 Effect of Wnt inhibition on chronic olanzapine effects

5.2.4.1 XAV939

We have shown that olanzapine treated cells have a higher concentration of Axin2 which we suggest is related to the significant difference in cytoplasmic beta catenin and insulin secretion compared to untreated cells. In Chapter 4 we showed that olanzapine caused a slow increase in Axin2 concentration over time, and suggested that this may be linked to increased signalling in the canonical Wnt signalling pathway. XAV939 was shown to inhibit some of the effects of olanzapine to stimulate Wnt signalling. To investigate if increased Wnt signalling is linked to the cellular changes that result in reduced insulin secretion, Wnt inhibitor XAV939 was added to the 72 hour olanzapine treatment.

Figure 56 shows that a concentration of 1 µM, XAV939 addition to olanzapine was able to improve the insulin secretion. This concentration was also shown in Chapter 4 to inhibit olanzapine's influence on downstream Wnt target genes cyclin D1 and c-myc; therefore we suggest that at this concentration XAV939 inhibits olanzapine stimulated Wnt signalling. In agreement with our previous results, chronic olanzapine significantly reduced insulin secretion in response to a 2 hour high glucose stimulation ($p<0.01$). Adding 0.1 µM XAV939 to the olanzapine treatment moderately improved secretion, and 1 µM XAV939 significantly improved the insulin secretion in response to glucose ($p<0.05$). This suggests that it is through Wnt signalling that olanzapine induces cellular changes that subsequently inhibits normal GSIS.

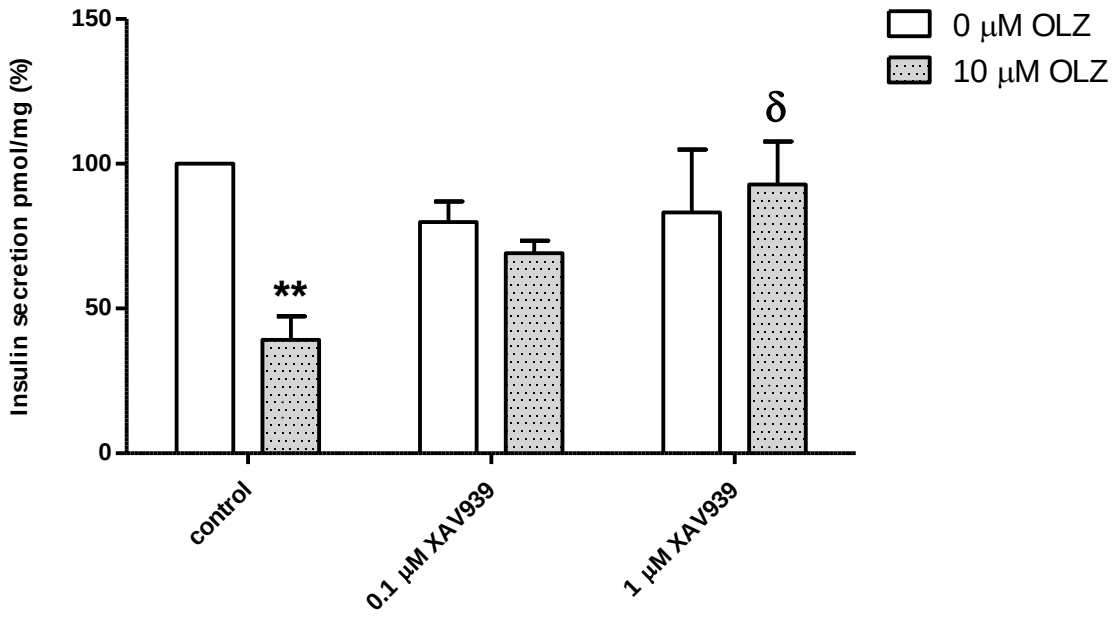


Figure 56 investigating the addition of Wnt antagonist XAV939 to olanzapine incubation for 72 hours and the effect on insulin secretion. MIN6 cells were incubated with 10 μM olanzapine with or without 0.1 μM or 1 μM XAV939 for 72 hours then standardised with 0 mM glucose followed by 2 hour exposure to 25 mM glucose. Following stimulation supernatant was collected and insulin concentration determined with ELISA and normalised to protein content. Results shown are of 3 independent experiments. Data are displayed as mean ± SEM, **p<0.01 versus 0 μM olanzapine, δ p<0.05 versus control using a two-way ANOVA and Bonferroni's Post-test

5.3 Discussion

In Chapter 4 we established that chronic olanzapine caused an imbalance of nuclear/ cytoplasmic beta catenin, and this Chapter aimed to investigate how these chronic effects impact GSIS from MIN6 beta cells. We hypothesised that following on from acute stimulation of Wnt signalling, chronic olanzapine would cause changes to the concentration and cellular distribution of beta catenin and other Wnt pathway effectors, which would result in reduced available beta catenin upon glucose stimulation. Recent evidence has linked free beta catenin concentration to insulin secretory mechanisms [210, 275] thus we speculated that by disturbing beta catenin dynamics, olanzapine would also alter insulin secretion. The presented data support the theory that chronic olanzapine reduces GSIS from MIN6 cells through modification of cytoplasmic beta catenin concentration. The results suggest that in olanzapine treated cells, beta catenin stability cannot be induced by glucose and there is a resultant significant difference in cytoplasmic beta catenin concentration. Through correction of beta catenin concentration, BIO also improves insulin secretion; strongly implying that reduced beta catenin is closely allied to reduced insulin secretion in olanzapine treated cells. Through testing different stimulatory drugs, and assessing concentration of various interacting proteins, we have presented data suggesting that the effect of olanzapine on beta catenin is through shifting the balance towards beta catenin proteasomal degradation upon glucose stimulation. To the best of our knowledge this is the first study detailing how olanzapine can alter beta catenin dynamics and how this is related to inhibition of GSIS from beta cells.

5.3.1 Olanzapine reduces the concentration of cytoplasmic beta catenin during glucose stimulation

Following on from the chronic effects of olanzapine to deregulate beta catenin and cause a small but significant reduction in cytoplasmic beta catenin (**Figure 28**), **Figure 43** demonstrated that after glucose stimulation there was further reduction and a more profound difference in concentration compared to control. We speculate that olanzapine inhibits the glucose stimulatory effect on beta catenin, increases proteasomal degradation, or promotes nuclear retention of the protein.

We have presented data in Chapter 4 that olanzapine increases protein concentration of Axin2, and following glucose stimulation, Axin2 concentration remains amplified (**Figure 54**). We suggest a possible mechanism that increased Axin2 increases the functional volume of the beta catenin destruction complex, and leads to increased proteasomal degradation of the beta catenin. However, as Axin2 is similarly increased in unstimulated conditions (**Figure 38**), it is not yet clear why the reduction in beta catenin concentration is more profound following glucose stimulation. The interplay between the activity of the beta catenin destruction complex, and the stimulatory effect of glucose on beta catenin has not been previously documented, hence this result raises interesting questions regarding the cross talk between canonical Wnt signalling and glucose signalling.

Increasing glucose concentration has been previously shown to increase protein concentration of beta catenin in macrophage cell lines [299], human islets [336] and the rat INS-1E beta cell line [297]. Anagnostou and Shepherd (2008) demonstrated in macrophage cell lines that following overnight serum starvation in 0.5 mM glucose, 2 hour stimulation with different glucose concentrations up to 20 mM glucose stimulated a concentration dependent increase in beta catenin protein concentration [299]. Liu *et al* (2009) investigated human islets, and showed that increasing glucose concentration from 5 mM to 8 mM also increased beta catenin cytoplasmic accumulation [336]. Further investigation using INS-1E beta cells by Cognard *et al*. (2013) also showed that following a 4 hour glucose starvation period, active (Ser 552 phosphorylated) and total beta catenin concentration was low, and upon stimulation with 10 mM glucose slowly increased, plateauing at 1 hour [297]. In our model we reduced the glucose concentration to 0 mM for 2 hours prior to glucose stimulation; therefore the beta catenin concentration would have reduced during the starvation period, followed by an increase when stimulated with 25 mM glucose. But in olanzapine treated cells, glucose addition failed to stimulate an increase in cytoplasmic concentration.

Reports defining the mechanism involved in glucose induced changes in beta catenin are limited, but Cognard *et al*. (2013) proposed that in beta cells, glucose increases beta catenin concentration through an increase in cAMP, and activation of PKA which phosphorylates beta catenin at Ser 552 [297]. But phosphorylation at Ser 552 of the C-terminus is incongruent to the activity of the destruction complex, which targets the N terminus of the protein. At the N terminal of beta catenin Ser 45

is phosphorylated by the priming kinase CK1α, followed by phosphorylation at Ser 37, Ser 33, Thour41 by GSK3β. Axin (Axin1 and Axin2) and APC form the scaffold of the destruction complex, with binding pockets for beta catenin, GSK3 and CK1α to bring all the components to close proximity. Phosphorylation at Ser 37 and Ser 33 is detected by β-TrCP E3 ubiquitin ligase which ubiquitylates the protein for rapid degradation by the proteasome [337].

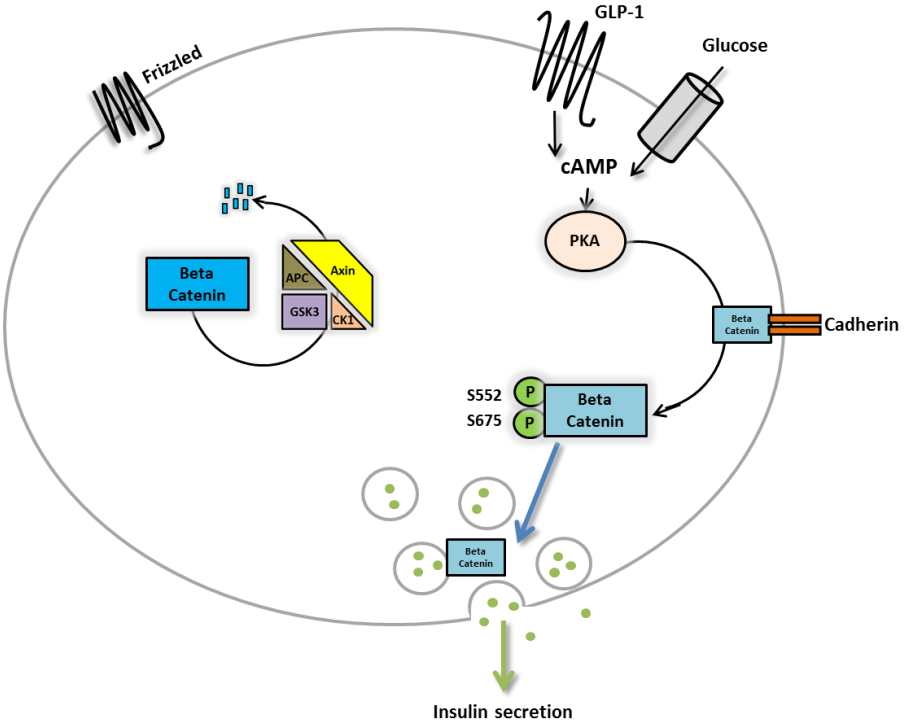
Conversely, AKT and PKA phosphorylate beta catenin at Ser 552 and Ser 675 at the C terminal, which promotes interaction with transcriptional co-factors and enhances beta catenin functional activity [320, 335, 338]. In beta cells glucose has been shown to increase PKA, resulting in beta catenin phosphorylation at Ser 552 [297], and the GLP-1 agonist exenatide has also been shown to increase beta catenin through phosphorylation at Ser 675 in a mechanism involving cAMP and PKA [298]. Phosphorylation at these residues is linked to the role of beta catenin in insulin secretion [210, 297]. Olanzapine inhibited insulin secretion stimulated by forskolin but did not cause any change to PKA-C concentration following glucose stimulation (**Figure 55**), which suggests the mechanism for olanzapine induced effects is independent of PKA activation.

Beta catenin has numerous further binding sites and diverse roles at the membrane, cytoplasm and nucleus; hence it is not yet clear which pool of beta catenin is modulated by glucose signalling. The beta catenin involved in canonical Wnt signalling is tightly regulated in the cytoplasm/ nucleus through interaction with the destruction complex and transcription factors. Cadherin bound beta catenin is firmly sequestered at the membrane to regulate cell-cell contact, with minimal interaction with the destruction complex [339]. Previous studies investigating carcinoma cell lines suggested that beta catenin phosphorylated at Ser 552 by AKT was not recruited from the Canonical Wnt pool, but from the cadherin bound pool [320]. Ser 552 phosphorylated beta catenin was shown to dissociate form the cadherin to the cytosol. But notwithstanding increased availability for other functions, the new pool of liberated beta catenin was shown to be vulnerable to the destruction complex. Cytoplasmic Ser 552 phosphorylated beta catenin had the same rate of interaction with the destruction complex and the same half-life as non-phosphorylated beta catenin [320]. In another example, phosphorylation at Ser 552 and Ser 675 was induced by forskolin, an adenylyl cyclase activator which increases cAMP and PKA. PKA increased cytoplasmic and nuclear beta catenin [335] and promoted beta

catenin activity in non-canonical pathways, but again, Ser 552/675 phosphorylation did not protect beta catenin from the destruction complex or proteasomal degradation [338].

This leads to the suggestion that activity of the destruction complex is the rate limiting factor in regulating the concentration of free beta catenin. Olanzapine increases concentration of Axin2 and therefore increases activity of the destruction complex. As depicted in **Figure 57**A the destruction complex usually has limited capacity, and if glucose stimulates AKT/PKA to trigger beta catenin dissociation from cadherins to increase the functional cytoplasmic pool, the destruction complex quickly becomes saturated. Further increases in liberated beta catenin result in an increased pool of active protein available to increase target gene expression, or as proposed, to be involved in insulin secretion pathways. Moreover, Axin (Axin1/ Axin2) concentration is the rate limiting constituent of the destruction complex, and addition of 5 – 10 nM Axin protein has previously been shown to increase rate of beta catenin degradation 5- 10 fold [310]. We speculate that by increasing Axin2 concentration, olanzapine increases the size of the destruction complex aggregation and causes more of the newly dissociated beta catenin to be targeted for destruction, thus leading to a significantly reduced cytoplasmic concentration following glucose stimulation (**Figure 57**B).

A. Control



B. Olanzapine

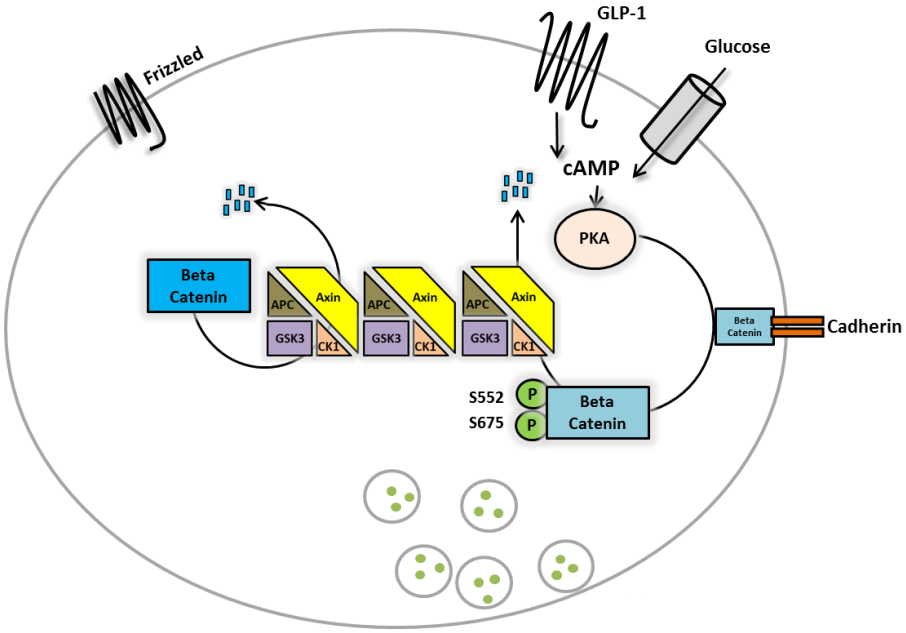


Figure 57 Illustration depicting the effect of olanzapine to increase axin2 concentration and reduce free beta catenin. A. Glucose stimulated beta catenin is recruited from the cadherin bound pool, phosphorylated at Ser552/675 and available for functional involvement in insulin secretion. **B.** In olanzapine treated cells there is higher concentration of Axin2, so the liberated beta catenin is also targeted for proteasomal degradation. There is less active beta catenin available for functional roles.

5.3.2 Reduced cytoplasmic beta catenin causes reduced GSIS

Addition of the GSK3 inhibitor BIO to the glucose stimulation significantly increased the final cytoplasmic beta catenin concentration of olanzapine treated cells (**Figure 45**). BIO addition also significantly improved insulin secretion (**Figure 46**) which strongly suggests that it is through reduction of cytoplasmic beta catenin that olanzapine causes a reduction in GSIS. BIO is a GSK3 inhibitor, which prevents its incorporation into the destruction complex, leading to reduced beta catenin proteasomal degradation. There was no difference in GSK3 activity in olanzapine treated cells compared to control (**Figure 53**) thus we suggest that olanzapine increases beta catenin destruction complex activity through an alternate mechanism, such as increased Axin2 concentration as discussed above.

Similarly to our result, experiments where beta catenin was knocked down using siRNA showed that GSIS was likewise inhibited [210, 297]. Sorrenson *et al*. (2016) showed that knockdown of beta catenin in INS832/3 and MIN6 cells, resulted in significantly reduced insulin secretion in response to glucose stimulation, alongside deranged insulin vesicle dynamics at the cell periphery [210]. Using TIRF microscopy it was demonstrated that following glucose stimulation there was minimal movement of insulin vesicles in the region 100 nM adjacent to the cell membrane in cells transfected with siRNA for beta catenin. In comparison, control cells have depleted insulin vesicle density at the membrane following glucose stimulation, signifying that the readily releasable pool of vesicles had been released. This supports the suggestion that beta catenin is required for vesicle fusion with the membrane for regulated exocytosis. Furthermore, this convincing evidence that beta catenin is involved in the regulation of GSIS supports our theory that it is through reduction in beta catenin that olanzapine treatment leads to reduced insulin secretion.

Addition of pyrvinium to glucose stimulation has also been shown to inhibit glucose stimulated increases in insulin secretion in INS1E [297], MIN6 cells, and isolated mouse islets [210]. Pyrvinium binds to and potentiates activity of CK1α, the priming kinase of the beta catenin destruction complex, which accordingly increases beta catenin proteasomal destruction [340]. This corroborates the presented theory that modulation of the beta catenin destruction complex can subsequently influence

insulin secretion. As shown in **Figure 57**B we suggest that olanzapine modulates activity of the destruction complex by increasing Axin2 concentration, to decrease beta catenin concentration and reduce GSIS, in a similar mechanism to pyrvinium which also augments capacity of the destruction complex to reduce GSIS.

The effect of decreasing beta catenin concentration appears to have a more substantial influence on GSIS than increasing beta catenin concentration, suggesting that the influence that beta catenin has on insulin secretion has an upper limit that cannot be surpassed by further stimulation. In transgenic RIP-Cre, β-Cat^{active} mice which have loxP-flanked constitutively active beta catenin, there was no difference in insulin secretion in response to glucose stimulation compared to control [272]. RIP-Cre, β-Cat^{active} mice express beta catenin which is truncated at the N-terminal [341], and cannot be phosphorylated by the destruction complex. However, glucose signalling results in phosphorylation at the C-terminus, and this is unaffected by the genetic mutation, so we suggest that this is why there is no difference.

Addition of 5 µM BIO which increases beta catenin concentration through disruption of the destruction complex, has been shown to have a positive effect on insulin secretion only during 15 minute stimulation [210]. But on extending the duration to 2 hours, there was no difference compared to control. We similarly did not find any significant amplifying effect of 1 µM BIO on insulin secretion following 2 hour stimulation (**Figure 46**). This adds to the proposition that glucose stimulated beta catenin is phosphorylated at the C-terminal, and does not interact with the Wnt regulated pool which is phosphorylated at the N-terminal. By increasing the concentration of the Wnt regulated pool through N terminal truncation, or targeting the destruction complex with BIO, this does not have the same effect as increasing glucose stimulated beta catenin. Liu *et al*. (2009) showed that glucose can stimulate an increase in cytoplasmic beta catenin, but the protein remains in the cytoplasm for functional roles, and there is little translocation to the nucleus. Conversely, modification of Wnt regulated beta catenin with addition of a GSK3 inhibitor resulted in beta catenin accumulation and translocation to the nucleus [336], highlighting the differing pathways involved in glucose and Wnt stimulation of beta catenin.

5.3.3 Olanzapine does not affect insulin secretion stimulated by membrane depolarisation

We have put forward a theory that olanzapine reduces beta catenin by interfering with the process of glucose stimulation. However, prior to glucose stimulation there is already evidence of dysregulated beta catenin signalling in olanzapine treated cells. In Chapter 4 we showed that chronic olanzapine can shift the cellular distribution towards nuclear localisation of beta catenin (**Figure 28**), and increase the nuclear concentration of TCF7L2 (**Figure 29**) which is suggested to tether the beta catenin to the nucleus. It is cytoplasmic beta catenin that is posited to be involved with insulin vesicle trafficking and fusion with the cell membrane [210, 275]. TCF7L2 over expression has previously been shown to reduce glucose and exenatide stimulated insulin secretion with little influence on total beta catenin concentration [210, 342] suggesting that by sequestering beta catenin to the nucleus, GSIS might be moderated.

By testing the insulin secretion response to secretagogues that act downstream of glucose, we aimed to verify that the effect of olanzapine to reduce insulin secretion was associated with the process of glucose stimulation and not the result of chronic cellular distribution changes only. In **Figure 49** we showed that both tolbutamide and KCl significantly stimulated insulin secretion in olanzapine treated cells, which substantiates the theory that that olanzapine impedes glucose stimulation.

Sorrenson *et al*. (2016) showed that in cells transfected with siRNA for beta catenin, KCl was unable to stimulate an increase in insulin secretion [210], which highlights the role of beta catenin in the process of insulin vesicle exocytosis. But the fact that olanzapine does not interact with secretion verifies that the chronic effects to redistribute beta catenin to the nucleus and moderately decrease cytoplasmic concentration, are insufficient to inhibit secretion. It is during the glucose induced stimulation of beta catenin that olanzapine produces the most profound effect on beta catenin concentration, with resultant effect on GSIS.

In addition to this, olanzapine significantly inhibited insulin secretion in response to exenatide (**Figure 50**). Exenatide is a GLP-1 receptor agonist that has also been shown to stimulate beta catenin to influence insulin secretion [210]. Addition of BIO similarly improved exenatide stimulated insulin secretion in olanzapine treated cells,

confirming the negative regulation of beta catenin within this mechanism. Furthermore, olanzapine also inhibited insulin secretion in response to forskolin (**Figure 51**), which targets the PKA pathway and is known to potently increase insulin secretion [343]. Forskolin activates adenylate cyclase to increase cAMP, increasing PKA which is a known signalling effector of the glucose and GLP-1 induced activation pathway to phosphorylate beta catenin at Ser 552/675 [335]. The observation that olanzapine also inhibited forskolin stimulated insulin secretion supports the proposed mechanism that the effects of olanzapine to reduce beta catenin occur downstream of PKA phosphorylation. It is through PKA stimulation that glucose leads to beta catenin dissociation from the membrane, resulting in increased access to the larger destruction complex aggregation induced by olanzapine (**Figure 57**B).

Olanzapine also inhibited insulin secretion in response to PMA (**Figure 51**). PMA activates PKC, which has not been previously linked to beta catenin function in beta cells, but as olanzapine also reduced insulin secretion in response to PMA, there may be an as yet undefined interaction. Forskolin and PMA have both previously been shown to modify the RRP and the sensitivity of insulin vesicles to calcium, with no additive effect suggesting that there is a shared mechanism [344]. From our understanding of beta catenin and the regulation of vesicle dynamics in beta cells [210, 275] as well as in neurons [322, 329], we conjecture that in addition to PKA, PKC may also be able to modify beta catenin, but further research is required to support this.

5.3.4 Acute Wnt stimulation is linked to the chronic effect of olanzapine to inhibit GSIS

At the centre of the proposed mechanism through which olanzapine inhibits GSIS, is Axin2; the scaffold protein of the beta catenin destruction complex which we advocated in Chapter 4 increased over time in a mechanism allied to an increase in canonical Wnt signalling (**Figure 38**). The small molecule inhibitor XAV939 was utilised to inhibit Wnt signalling, and in **Figure 56** XAV939 also prevented the effect of olanzapine to inhibit GSIS. At 1 μM XAV939 inhibited olanzapine induced expression of Wnt target genes cyclin D1 (**Figure 34**) and c-myc (**Figure 35**),

suggesting that there is inhibition of the Wnt signalling pathway. 1 μM XAV939 also prevented olanzapine induced changes in insulin secretion. This strongly suggests that it is through the stimulation of Wnt signalling that olanzapine induces cellular changes that reduce GSIS.

5.3.5 Conclusion

To conclude, this Chapter has presented evidence showing that chronic olanzapine exposure causes cellular changes to MIN6 cells that result in a reduction in cytoplasmic beta catenin concentration upon glucose stimulation. Correction of cytoplasmic beta catenin with addition of BIO was shown to improve insulin secretion, leading to the conclusion that it is through alterations in beta catenin dynamics that olanzapine inhibits insulin secretion. Axin2 is a key component of the beta catenin destruction complex which we have shown can be increased by chronic olanzapine; hence we suggest that it is through an increase in concentration of beta catenin degradasomes that olanzapine has an effect on beta catenin concentration.

Chapter 6: Conclusions and future work

6.1 Research aims

Olanzapine is associated with considerably higher rates of hyperglycaemia compared to other first line antipsychotics [38]. However as olanzapine is effective at managing symptoms of psychosis, it is a therapy often favoured by psychiatrists and remains one of the most frequently prescribed antipsychotic drugs. Uncontrolled hyperglycaemia has extensive risks to health and is a significant risk factor for the development of diabetes and CVD. Yet despite the awareness that hyperglycaemia is commonly associated with treatment, the current strategy for dealing with metabolic side effects falls short; resulting in poor physical health and increased mortality that could be avoided [3, 44].

In practice, blood sugars are monitored closely in patients with pre-existing diabetes or pre-diabetes, but less often in patients with no preceding physical health problems. However the presented data in this study adds to the growing evidence that olanzapine is able to forthrightly cause hyperglycaemia and increase risk of developing diabetes in previously healthy patients, as a direct consequence of treatment.

As yet, the evidence that olanzapine pharmacologically increases insulin resistance [134, 158] has not clarified why severe and persistent hyperglycaemia develops. Usually the beta cells respond to insulin resistance through beta cell compensation and increased insulin secretion, so we questioned if there was also deterioration in beta cell function. Insulin resistance can lead to diabetes if beta cells simultaneously fail to secrete adequate insulin [182, 183, 185]. Our findings confirmed that olanzapine significantly altered the function of MIN6 beta cells to secrete insulin.

Previous research into the effect of olanzapine on beta cell function has been inconclusive, with suggestions of apoptosis [55], increased basal insulin secretion [171, 172], and varying effects on insulin secretion in response to glucose [173, 235]. However no study has previously investigated the chronic effect of olanzapine on beta cell function beyond 4 hour exposure at therapeutic concentrations. In this study we sought to thoroughly investigate for the first time, if there were any concurrent pharmacological effects of chronic olanzapine exposure in pancreatic beta cells to influence function. We present good evidence that beta cell dysfunction is a central part of olanzapine induced hyperglycaemia and diabetes.

This research contributes to the existing literature in the following ways:

I. We have examined a range of therapeutic concentrations of olanzapine (≤ 10 μM) in different culture conditions and demonstrated that cells remain viable during 72 hour culture, suggesting that olanzapine is not toxic to beta cells. This is the first time that concentrations below 100 μM have been tested for viability, and this result transcends the previously accepted belief that olanzapine is toxic to beta cells.

II. We have established for the first time a link between olanzapine and the canonical Wnt/ beta catenin signalling pathway in beta cells. In the MIN6 cell line activation of beta catenin signalling by olanzapine resulted in three novel observations; increased expression of cell cycle regulating genes cyclin D1 and c-myc, increased rate of proliferation, and accelerated cell cycle. We postulate that through altering beta catenin signalling, olanzapine exposure results in a deterioration of beta cell function.

III. We have shown that 72 hour culture with olanzapine results in altered cell biology and redistribution of cellular components. The result of such cellular changes is a significant reduction in glucose stimulated insulin secretion, and reduced insulin secretion upon GLP-1 receptor stimulation. This is the first time that the influence of olanzapine on insulin secretion has been investigated beyond 4 hours exposure *in vitro,* and the first time a mechanism for reduced insulin secretion has been proposed.

IV. By demonstrating that a reduction in cytoplasmic beta catenin is related to reduced insulin secretion from MIN6 cells, we have added to the evolving theory that beta catenin is an important component in the process of insulin secretion.

V. This research is the first to present a potential model whereby increased Axin2 concentration can influence insulin secretion from beta cells through increased beta catenin degradation upon glucose stimulation.

6.2 Summary of main findings

This thesis aimed to test three initial hypotheses; that olanzapine causes beta cell death, that olanzapine inhibits beta cell compensation; or that olanzapine has a pharmacological action on the beta cell to modify the mechanism of GSIS. We identified that olanzapine can negatively influence beta cell function and modify GSIS through pharmacological interaction with the canonical Wnt signalling pathway.

We have demonstrated that it is through acute activation of beta catenin that olanzapine stimulates proliferation of MIN6 cells and causes cellular changes that result in reduced glucose stimulated insulin secretion. We speculate that, alongside peripheral pharmacological effects, a pharmacological reduction in beta cell function can accelerate the development of hyperglycaemia and diabetes associated with treatment.

6.2.1 Cell viability

We initially postulated that olanzapine was toxic to beta cells, as has been previously suggested by Ozasa *et al.* (2013) [55]. We showed that olanzapine did not reduce cell viability of MIN6 cells treated with olanzapine ≤ 10 μM for 24 or 72 hours. 100 μM olanzapine significantly reduced cell viability, however it was established that this was due to an inhibition of growth during 72 hour incubation caused by cell cycle arrest rather than an increase in cell death. However, we considered that 100 μM olanzapine is many times higher than any reported plasma concentration, and further experiments were undertaken using lower, more therapeutic concentrations ≤ 10 μM at which we found no change in cell viability.

6.2.2 Beta cell proliferation and Compensation

As olanzapine is known to increase insulin resistance through pharmacological action at the liver, adipose, and muscle tissue, we reasoned that beta cells would usually mount a compensatory response to increase mass and insulin secretion. Ader *et al.* (2005) proposed that in dogs olanzapine can inhibit beta cell

compensatory mechanisms [56] thus we hypothesised that olanzapine would pharmacologically inhibit proliferation of MIN6 cells in response to different stimulus. We tested the proliferation of MIN6 cells in different culture conditions, but found that concentrations ≤10 μM had no significant effect on proliferation in high glucose, high growth factor (FBS), or serotonin (5-HT). Instead we offered a contrasting model where in unstimulated conditions, activated Wnt/ beta catenin signalling stimulated proliferation and upregulation of cell cycle regulating genes c-myc and cyclin D1. Albeit the increased number of cells was further revealed to have reduced functional capacity; hence we propose a model where activating a pathway to increase proliferation yields a negative overall influence on beta cell function.

We might approach an observed increase in proliferation with caution as MIN6 cells are an immortalised cell line that are genetically engineered to continually replicate. Primary beta cells proliferate at very low rates, but the MIN6 cell line is derived from a transgenic mouse expressing the large T antigen of SV40 [212] which inhibits tumour suppressors Rb and p53 [345]. P53 and Rb are involved in regulating the cell cycle, cell senescence, and apoptosis; hence cell cycle progression is dependent on these functional proteins. Therefore, in primary beta cells the effect of beta catenin stimulation on the cell cycle could be different to that which is observed in an immortalised cell line.

Nonetheless, beta catenin signalling, and c-myc expression are known to be involved in beta cell proliferation in response to proliferative stimulus *in vivo,* such as during prolonged hyperglycaemia [300] or a high fat diet [346]. For example; mice fed a high fat diet for 60 days developed insulin resistance and the pancreatic islets expanded in mass, alongside beta catenin nuclear translocation and upregulation of cyclin D1 and c-myc [346]. Such effects were not seen at 30 days, at which point insulin resistance had not yet become established, indicating that beta catenin activation was involved in the mechanism of compensation. C-myc has also independently been shown to drive proliferation of beta cells in adult mice, although, comparable to what we have observed in olanzapine treated cells, the daughter beta cells had an immature phenotype with compromised function [246]. It has also previously been suggested that when there is sustained c-myc activation within beta cells in adult mice, apoptosis eventually predominates over proliferation, resulting in cell loss [347].

As there is a recognised mechanism for beta catenin, cyclin D1 and c-myc to stimulate beta cell proliferation *in vivo*, our observation of olanzapine induced beta catenin signalling to stimulate proliferation in a cell line may indeed also translate *in vivo*. We conclude that olanzapine shifts the balance of the cell to a more proliferative phenotype. We suggest that the effects of olanzapine to increase proliferation but reduce secretory function mirrors what has been previously demonstrated in beta cell models of upregulated c-myc in glucotoxicity [246, 347]. We also postulate that through interaction with a pathway that is involved in compensation, olanzapine may hinder the dynamic nature of beta cells to appropriately respond to fluctuating requirements.

We have taken care to ensure that MIN6 cells were used at low passage number and in culture conditions with low concentration of growth factors in effort to maintain beta cell characteristics and minimise basal proliferation. However, further work should be also carried out to fully ascertain if there is a similar influence on beta catenin signalling and proliferation in primary beta cells exposed to olanzapine.

6.2.3 Glucose stimulated insulin secretion

Beta cells respond to elevations in extracellular glucose concentrations by secreting insulin into the circulation where it travels to peripheral tissues to facilitate glucose uptake. The beta cell mass is dynamic and responsive to numerous external stimuli, such as hormones, amino acids, and glucose. Hence, due to the complex side effect prolife, and known pharmacological effects of olanzapine at peripheral organs, a pharmacological effect at pancreatic beta cells has thus far been difficult to differentiate from other external influences. Some *in vivo* studies have pointed to a possible alteration in insulin secretion [118, 119, 130] so we hypothesised that olanzapine might pharmacologically interfere with the normal glucose stimulated insulin response to elevated glucose.

We measured the total insulin secreted during 2 hour glucose stimulation from cells that had been exposed to olanzapine for 72 hours, and found that there was a significant reduction in insulin secretion compared to control. We have also presented a model by which 72 hour olanzapine exposure pharmacologically altered the concentration and localisation of cellular components of the Wnt/ beta catenin

signalling pathway. We suggest that it is through increased Axin2 concentration and decreased cytoplasmic beta catenin that olanzapine exerts its effects on GSIS.

To the best of our knowledge this is the first time chronic olanzapine has been shown to have an effect on GSIS *in vitro*. Previous work by Melkersson *et al.* (2001, 2004) investigated 1- 4 hour olanzapine exposure in media containing different concentrations of glucose, and measured the total insulin secreted during the drug/glucose exposure [171, 172]. Similarly, Nagata *et al.* (2019) investigated insulin secretion during 1 hour drug exposure, although media glucose concentration was not elevated [173]. While Melkersson *et al*. (2001, 2004) report no change in GSIS; Nagata *et al*. (2019) reported a significant difference, approximately 80% of control. Investigations beyond 4 hour acute exposure have not been carried out previously. We considered that in humans olanzapine treatment results in steady state plasma concentrations, thus our results detailing the effects of 72 hour exposure allows for a precise illustration of cellular changes initiated by chronic olanzapine and the functional outcomes of these. Acute reductions in GSIS reported by Nagata *et.al* (2019) [173] may be the result of initial activity at receptors, but we suggest that it is chronic adaptive changes in the cell over time that have a bigger impact on beta cell function. And we demonstrate that GSIS is reduced to approximately 55% of control in cells treated with 10 µM olanzapine. We also demonstrated that treatment with lower concentration 1 µM also mildly reduced secretion, although this was not significant due to low power.

Recent work by Sorrenson *et al.* (2016) [210] has linked cytoplasmic beta catenin to insulin vesicular release. Following on from our observations of acute interaction with Wnt/ beta catenin signalling we were interested whether the observed interactions were also linked to inhibited GSIS. We observed that, compared to control, olanzapine treated cells had significantly reduced cytoplasmic beta catenin concentration following a 2 hour glucose stimulation. Alongside other observations that addition of the GSK3 inhibitor BIO improved cytoplasmic beta catenin and also improved insulin secretion, we assert that it is through shifting beta catenin dynamics that olanzapine interferes with the process of insulin secretion. We present a model where olanzapine increases the activity of the destruction complex through increased Axin2 concentration, and upon glucose stimulation, activated beta catenin is quickly targeted for degradation and not available for insulin secretory roles.

Olanzapine treated cells also secreted less insulin compared to control in response to the GLP-1 agonist exenatide, but there was no difference in secretion in response to membrane depolarising agents KCl or tolbutamide. Similarly to the glucose stimulation, addition of BIO was able to improve the exenatide stimulated secretion suggesting that overactivity of the destruction complex may again be involved. As described by Sorrenson *et al.* (2016) [210] in beta cells, glucose and GLP-1 act activate beta catenin through a unified pathway involving cAMP and PKA. Based on our observations, we suggest that the chronic effects of olanzapine to increase Axin2 result in interference with the process of beta catenin activation by both glucose and GLP-1.

6.2.4 Growth vs. insulin secretion

The presented results demonstrate that olanzapine induces MIN6 proliferation, and reduces insulin secretion, and as we identified changes in beta catenin suggestive of Wnt involvement in both instances we deduce that the two effects are linked. The Wnt inhibitor XAV939 inhibited upregulation of Wnt target genes cyclin D1 and c-myc, and also improved insulin secretion in olanzapine treated cells. Cyclin D1 and c-myc are key regulators of the cell cycle, thus if inhibition of cell cycle progression also improves insulin secretion this supports the concept of a link between increased proliferation, and reduced secretion, involving Wnt activation. Previous work has also linked the Wnt target gene c-myc with increased beta cell proliferation and reduced insulin secretion [246, 347], which points to a common pathway.

We initially postulated that olanzapine inhibited beta cell compensation mechanisms based on observations by Ader *et al.* (2005) that olanzapine administration in dogs impaired the normal beta cell response to insulin resistance [56], and Chintoh *et al.* (2008) who found no increase in insulin secretion following chronic olanzapine exposure, despite a significant increase in body weight and insulin resistance [70].

Pancreatic beta cells in adult humans have very low replicative capacity, but proliferation can be stimulated in response to increased metabolic demands in the process of compensation. Compensation is characterised by increased cell mass, but maintained secretory function [45] which increases the overall insulin output to counteract increased demand. But in the event of ongoing and chronic

hyperglycaemia, the function of the beta cell mass begins to become compromised, and decompensation occurs [185]. In decompensation, beta cells become dedifferentiated and insulin secretion is reduced. C-myc expression is thought to be central to the process of beta cell hypertrophy and dedifferentiation [185], and its expression is considered to be a typical characteristic of glucotoxicity and reduced beta cell function [348].

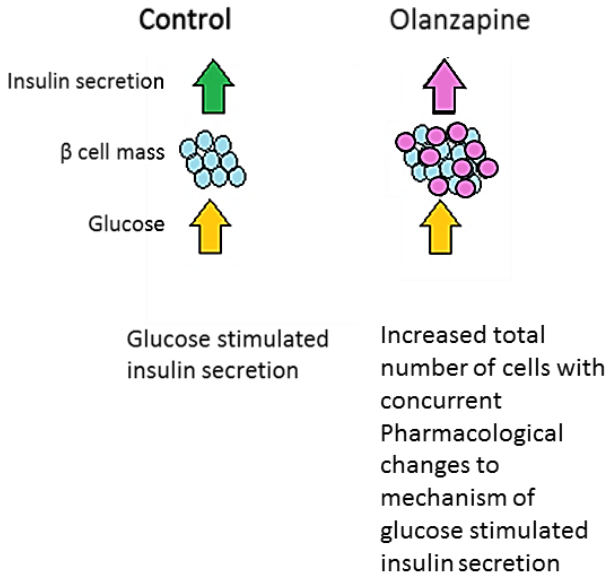


Figure 58 Illustration depicting the observed effects of olanzapine to increase total number of beta cells but to pharmacologically reduce insulin secretion in response to glucose

Fundamentally, the pharmacological effects of olanzapine that we have observed would interfere with the normal increase in insulin output in response to increased demands. If the beta cell mass increases, but the secretory capacity declines, then this means that the insulin output is likely to still be insufficient to meet increased demands. This could explain why Ader *et al.* (2005) [56] and Chintoh *et al.* (2008) [70] detected no change in insulin secretion despite overt insulin resistance (**Figure 58**).

Alongside its influence on beta cell function, olanzapine also pharmacologically increases insulin resistance [134, 158, 160, 161, 163] which exerts independent effects to initiate beta cell compensation. It is likely that the effect of insulin

resistance is augmented by the concurrent direct pharmacological impact of olanzapine on the beta cell to stimulate proliferation and reduce GSIS. We submit that olanzapine effectively fast-tracks the transition of the beta cell mass from compensation to decompensation where there is increased mass but inefficient insulin secretion.

C-myc has been linked to the mechanism involved in beta cell hyperplasia, glucotoxicity, and reduced insulin secretion [246, 347]. In response to prolonged hyperglycaemia or high fat diet, beta catenin nuclear translocation and elevated c-myc expression has been detected in beta cells [346]. Axin2 is also a key downstream target gene of activated beta catenin [311], but it has received much less attention in terms of beta cell function. Axin exists as two functionally equivalent isoforms, Axin1 and Axin2 [303], which form the structural backbone of the beta catenin destruction complex. Axin is the rate limiting factor in the beta catenin destruction complex, and Axin1 is tightly expressed, while Axin2 is upregulated by Wnt signalling and forms part of negative feedback mechanism to increase the volume of beta catenin degradasomes and reduce Wnt signalling.

We have inferred that it is through increased Axin2 that olanzapine reduces insulin secretion from MIN6 beta cells. Yet there has been very little previous research into the impact Axin or Axin2 have on insulin secretion. As there is evidence that Axin2 is a target gene of beta catenin [311], it would be co-expressed with c-myc, therefore it is conceivable that increased Axin2 expression could be involved in the mechanism through which c-myc overexpression leads to a reduction in insulin secretion.

6.2.5 Model

Figure 59 illustrates the proposed mechanism through which we theorise olanzapine exerts its effects on beta cell function. The effects are separated into acute effects on beta catenin activation (1-4), and successive chronic effects on insulin secretion (5-6) as discussed below.

1. Olanzapine acutely stimulates beta catenin, as evidenced by increased cytoplasmic beta catenin concentration following 24 hour exposure. Nuclear beta catenin is unaffected, thus increased concentration is the result of cytoplasmic protein stabilisation.
2. Beta catenin activation leads to up regulation of target genes cyclin D1 and c-myc which cause advancement of the cell cycle and increased proliferation in the absence of other stimulation. Expression of cyclin D1 and c-myc is inhibited by Wnt inhibitor XAV939 further verifying that the pathway involves Wnt/beta catenin signalling.
3. Axin2 is another reported target gene of Wnt/beta catenin signalling, but although there was no evidence of an increase in mRNA expression of Axin2, there was a significant increase in protein concentration over time. There may have been experimental reasons why a small change in mRNA was not detected, or olanzapine may directly increase Axin2 protein through posttranslational modifications.
4. Increased Axin2 protein concentration increases the volume of beta catenin degradasomes within the cell, and increases capacity for beta catenin cytoplasmic degradation. This is evidenced by reduced cytoplasmic beta catenin following 72 hour exposure.
5. Upon glucose stimulation, the process of beta catenin recruitment from the cadherin bound pool for insulin secretion is inhibited. The increased volume of degradasomes causes the liberated beta catenin to be targeted for degradation by the proteasome, and the cytoplasmic concentration is significantly reduced. Addition of GSK3 inhibitor BIO inhibits the augmented destruction complex aggregation and beta catenin does not become reduced upon glucose stimulation.
6. Beta catenin is involved in insulin vesicle trafficking to the membrane. Reduced cytoplasmic beta catenin leads to reduced insulin secretion. Addition of BIO to inhibit changes in beta catenin, improved insulin secretion.

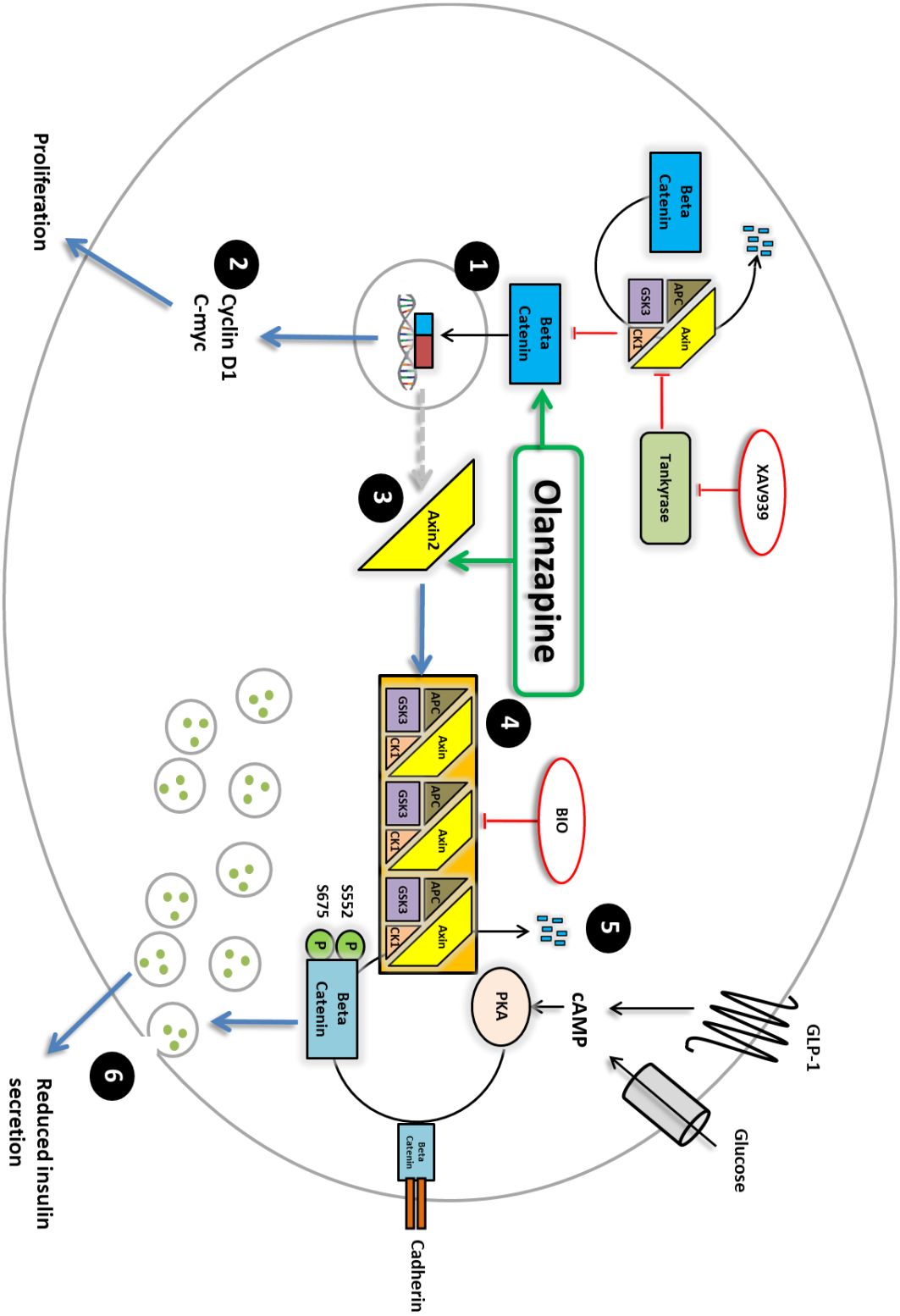


Figure 59 Illustration of the proposed mechanism through which olanzapine exerts its effects on beta cell function 1. Olanzapine stimulates beta catenin protein stabilisation 2. Beta catenin and TCF7L2 bipartite transcription factor upregulates target genes cyclin D1 and c-myc 3. Axin2 protein concentration increases 4. Increased Axin2 increases the volume of beta catenin degradasomes 5. On glucose stimulation, beta catenin is recruited from the cadherin bound pool to be involved in insulin secretory mechanisms, but the enlarged degradasome targets more beta catenin for destruction 6. less beta catenin is available for insulin vesicle trafficking and insulin secretion is reduced.

6.3 Further work

This study has established several novel pharmacological effects of olanzapine within the MIN6 beta cell line, but also raises several further questions. Above we have presented a model through which olanzapine initially stimulates beta catenin protein stabilisation, but we have not yet established how olanzapine does this. There are several possible mechanisms through which beta catenin protein stabilisation could transpire, such as receptor activation, or inhibition of the beta catenin destruction complex and further work should aim to establish the source of beta catenin activation.

A suggested mechanism involves an interaction with D_2 receptors to increase levels of Dvl-3. It has previously been postulated that some antipsychotics can increase the concentration of the protein Dvl-3 which is involved in frizzled receptor activation [349-351]. In the prefrontal cortex and striatum increased protein concentration of Dvl-3 has been shown to positively stimulate the canonical Wnt signalling pathway [351] and stimulate increased beta catenin concentration [349, 350]. Clozapine and haloperidol have been previously shown in PC12 and SH-SY5Y neuronal cells to increase Dvl-3, alongside increased beta catenin concentration, effects which were found to be linked to D_2 receptor antagonism [350]. The effects of olanzapine on Dvl-3 have not been previously investigated but olanzapine is also an antagonist at D_2 receptors [80] so may be expected to have a similar effect on Dvl-3 to activate Wnt signalling. Further investigation into Dvl-3 protein concentration, and repeated experiments with the addition of dopamine agonists and antagonists should be considered.

Equally, olanzapine may increase beta catenin concentration through direct inhibition of proteins involved in the destruction complex. GSK3β is an essential component of the destruction complex and there is evidence that olanzapine can reduce GSK3β activity. Olanzapine acts at numerous GPCR that are known to have regulatory effects on GSK3 such as 5-HT receptors. 5-HT_{1A} receptor activation leads to inhibited ser-9 phosphorylated GSK3β, whereas stimulation of 5-HT_{2A} increases the active dephosphorylated form of GSK3β [352]. Olanzapine is a potent antagonist of 5-HT_{2A} receptors [80], suggesting it may lead to a reduction in the concentration of active GSK3β, but it is unknown if such changes would intersect the Wnt regulated functional pool of GSK3β. Moreover, olanzapine is also

postulated to directly fit into the binding pocket to inhibit GSK3β [296], an effect likely to reduce incorporation into the beta catenin destruction complex. Investigations including 5-HT agonists and antagonists and quantification of phosphorylated active GSK3β should also be considered.

Nonetheless, the chronic cellular changes induced by olanzapine clearly result in a reduction in insulin secretion. Although we have presented convincing evidence that it is through depletion in cytoplasmic beta catenin concentration that olanzapine inhibits insulin secretion, further investigation into the precise mechanics of this process is warranted. The role of beta catenin in insulin secretion is a new area if research, and a deeper understanding of the mechanism involved in glucose activation of beta catenin would be of great interest to beta cell biologists. We have put forward a mechanism through which glucose recruits beta catenin from the cadherin bound pool to be involved in insulin secretion mechanisms. We suggest that it is through increased Axin2 that olanzapine inhibits an increase in beta catenin concentration, but further work is required reinforce the projected mechanism. For example, beta catenin is phosphorylated at numerous different residues by the destruction complex and cadherins, as well as AKT and PKA, hence a concise picture of the phosphorylation status of beta catenin at different stages of glucose stimulation would be valuable.

We have demonstrated that XAV939 can inhibit upregulation of cell cycle genes, and also dose dependently improve insulin secretion in olanzapine treated cells. However, further investigations are required to fully establish if these two effects are linked. Investigating beta catenin protein concentration and localisation at 24 hour, 72 hours, and following glucose stimulation in cells cultured with both olanzapine and XAV939 is required to investigate if the same pathway is involved. And other Wnt inhibitors should also be utilised to establish if there are any off-target effects of XAV939 that we are not accounting for.

A portion of the study is investigating the effect of olanzapine on cell growth, and investigating an immortalised cell line may not truly reflect growth pathways that are present in primary beta cells. Where we have observed an increase in proliferation, beta catenin activation may manifest as a different effect in primary cells with very little capacity for growth. Therefore duplicating the experimental techniques in primary beta cells should commence to determine if cyclin D1 and c-myc are equally

upregulated, and if there is a similar change in proliferation or downstream effects on beta catenin and insulin secretion.

Further work should also aim to investigate a range of other antipsychotics, and a range of different concentrations, to establish if the effects to activate Wnt/ beta catenin are unique to olanzapine, or reveal that this is a class effect.

6.4 Impact of findings

The overarching conclusion of this study is that olanzapine is detrimental to beta cell function. Appropriate insulin secretion is critical for glucose homeostasis, so a decline in beta cell function is a major side effect which will have a dramatic influence on health. Our research using the MIN6 cell line indicates that beta cell dysfunction is unrelated to other peripheral effects, demonstrating that olanzapine could independently initiate hyperglycaemia and diabetes through beta cell targets. Considering this alongside other known metabolic effects of olanzapine, we feel there should be a prompt re-evaluation of olanzapine as a first line antipsychotic. There is now a great deal of evidence demonstrating that olanzapine can cause hyperglycaemia, and there are other treatment options with less life-shortening side effects. We advocate that lower risk antipsychotics should be used first line, with olanzapine reserved as a second or third line option, or reserved for more severe illness.

We acknowledge that psychosis can be a challenge to treat and all antipsychotic drugs are associated with side effects which can affect quality of life. Olanzapine is a treatment often favoured by psychiatrists as it is very effective at managing symptoms, and side effects are not instantly visible when compared to other options. But in the event of such far reaching side effects on metabolic organs, the likelihood of developing metabolic syndrome or diabetes is almost certain when on long term treatment. Prescribing olanzapine will lead to serious metabolic complications and physical health problems that will ultimately shorten a patient’s life. Going forward, a balance needs to be struck between treating acute psychosis, and effectively managing the known side effects long term. The aim should be to prevent patients from developing long term chronic conditions such as diabetes.

First generation antipsychotics can cause extrapyramidal side effects which are easily identified and treatment modified, yet comparatively, close monitoring of blood sugars of patients prescribed second generation antipsychotics happens rarely. For olanzapine treated patients the guidelines advise monitoring of blood glucose at 12 weeks, then annually, however little advice is given regarding continuation of treatment based on results. Physicians are cautious to make changes to treatment regimens that are effectively controlling the symptoms of psychosis. In the event of hyperglycaemia, usually metformin or another antidiabetic medication is started alongside the antipsychotic, but metabolic dysfunction worsens and a diagnosis of diabetes is inevitable. Considering the evidence detailing the pharmacological effects of olanzapine to cause hyperglycaemia, we consider the lack of a more rigorous monitoring and management programme to be unethical. It is incongruous to treat one chronic disease, by creating another chronic disease, with minimal attempt to reduce the risk. Henceforth we suggest that more needs to be done to improve the care of patients with an aim to reduce the hyperglycaemic effect of treatment and reduce rates of diabetes. We suggest there needs to be further research into management strategies, and changes to clinical monitoring guidelines so that hyperglycaemia is picked up before irreversible beta cell damage occurs and prompt changes to treatment can be made.

We have identified that the Wnt/ beta catenin pathway is involved in beta cell dysfunction associated with olanzapine. With more detailed knowledge of the pharmacological effects of olanzapine to cause metabolic dysfunction and hyperglycaemia, there may be potential methods to reduce this side effect through additional pharmacological agents or supplements, diet, or exercise. Further research is needed to investigate the use of specific supplementation, but our research reveals a pathway which should provoke new targeted approaches to management. For example; Vitamin D has been shown to supress Wnt/beta catenin signalling and reduce c-myc expression in some cell types [353], and has previously been noted to reduce the risk of diabetes in olanzapine treated patients [354]. A starting point could include studies to investigate if optimising vitamin D levels of treated patients lessens hyperglycaemia.

Finally we advocate that patients and next of kin should be made fully aware of the health risks of treatment. Psychosis requires prompt treatment, and many patients lack capacity at the time of acute treatment, but consent should be obtained before

long term continuation of a drug that can cause diabetes. There are programmes in place to mitigate risks associated with other mental health treatment such as the clozapine monitoring service, or Valproate pregnancy prevention programme. We suggest a similar programme should be in place for patients taking olanzapine, so patients are made explicitly aware of the risks of long term treatment. Patients should be educated about the diabetogenic risks, and given an information pack and a patient card. A card detailing how the treatment can affect metabolic health could be shown to other healthcare professionals which could help bridge the gap between mental healthcare and physical healthcare and help patients take personal ownership of the physical health risks of treatment.

In conclusion, the side effects of olanzapine are wide ranging. We present good evidence that olanzapine causes beta cell dysfunction. This adds to the existing evidence pool showing that olanzapine has pharmacological targets at the liver, adipose, and muscle to cause derangement of glucose homeostasis and hyperglycaemia. In the face of increased mortality among psychiatric patients, the side effects of first line antipsychotic treatment urgently needs reviewing, and steps taken to improve physical healthcare of patients. We hope that by persistently drawing attention to the issues with the current treatment options for severe mental illness, common practice may begin to change. If olanzapine is to be upheld as a first line antipsychotic then closer monitoring of glucose and increased attention to the physical health of patients is essential.

References

1. Brown, S., *Excess mortality of schizophrenia. A meta-analysis.* The British Journal of Psychiatry, 1997. **171**(6): p. 502-508.
2. Chesney, E., G.M. Goodwin, and S. Fazel, *Risks of all-cause and suicide mortality in mental disorders: a meta-review.* World Psychiatry, 2014. **13**(2): p. 153-160.
3. Vancampfort, D., et al., *A meta-analysis of cardio-metabolic abnormalities in drug naïve, first-episode and multi-episode patients with schizophrenia versus general population controls.* World Psychiatry, 2013. **12**(3): p. 240-250.
4. Scharf, D.M., et al., *Improving the Physical Health of Adults with Serious Mental Illness.* 2014.
5. Vancampfort, D., et al., *Diabetes mellitus in people with schizophrenia, bipolar disorder and major depressive disorder: a systematic review and large scale meta-analysis.* World Psychiatry, 2016. **15**(2): p. 166-174.
6. Rummel-Kluge, C., et al., *Head-to-head comparisons of metabolic side effects of second generation antipsychotics in the treatment of schizophrenia: A systematic review and meta-analysis.* Schizophrenia Research, 2010. **123**(2–3): p. 225-233.
7. De Hert, M., et al., *Metabolic and cardiovascular adverse effects associated with antipsychotic drugs.* Nat Rev Endocrinol, 2012. **8**(2): p. 114-126.
8. Charlson, F.J., et al., *Global Epidemiology and Burden of Schizophrenia: Findings From the Global Burden of Disease Study 2016.* Schizophr Bull, 2018. **44**(6): p. 1195-1203.
9. Kuipers, E., et al., *Management of psychosis and schizophrenia in adults: summary of updated NICE guidance.* BMJ : British Medical Journal, 2014. **348**: p. g1173.
10. Merikangas, K.R., et al., *Lifetime and 12-month prevalence of bipolar spectrum disorder in the National Comorbidity Survey replication.* Arch Gen Psychiatry, 2007. **64**(5): p. 543-52.
11. Murray, C.J.L. and A.D. Lopez, *Global mortality, disability, and the contribution of risk factors: Global Burden of Disease Study.* The Lancet, 1997. **349**(9063): p. 1436-1442.
12. Kendall, T., et al., *Assessment and management of bipolar disorder: summary of updated NICE guidance.* BMJ : British Medical Journal, 2014. **349**: p. g5673.
13. Lepine, J.P., et al., *Depression in the community: the first pan-European study DEPRES (Depression Research in European Society).* Int Clin Psychopharmacol, 1997. **12**(1): p. 19-29.
14. Gelenberg, A., et al., *American Psychiatric Association practice guidelines for the treatment of patients with major depressive disorder.* Am J Psychiatry, 2010. **167**(Suppl. 10): p. 9-118.
15. Ban, T.A., *Fifty years chlorpromazine: a historical perspective.* Neuropsychiatric disease and treatment, 2007. **3**(4): p. 495-500.
16. Committee, J.F., *British National Formulary.* 77 ed, ed. L.B.G.a.P. Press. 2018.
17. Yatham, L.N., *Atypical antipsychotics for bipolar disorder.* Psychiatr Clin North Am, 2005. **28**(2): p. 325-47.
18. Marston, L., et al., *Prescribing of antipsychotics in UK primary care: a cohort study.* BMJ Open, 2014. **4**(12).

19. Baldwin, D.S., et al., *Evidence-based guidelines for the pharmacological treatment of anxiety disorders: recommendations from the British Association for Psychopharmacology.* Journal of Psychopharmacology, 2005. **19**(6): p. 567-596.
20. Ilyas, S. and J. Moncrieff, *Trends in prescriptions and costs of drugs for mental disorders in England, 1998–2010.* The British Journal of Psychiatry, 2012. **200**(5): p. 393-398.
21. Stroup, T.S. and N. Gray, *Management of common adverse effects of antipsychotic medications.* World psychiatry : official journal of the World Psychiatric Association (WPA), 2018. **17**(3): p. 341-356.
22. Creese, I., D. Burt, and S. Snyder, *Dopamine receptor binding predicts clinical and pharmacological potencies of antischizophrenic drugs.* Science, 1976. **192**(4238): p. 481-483.
23. Snyder, S.H., *The dopamine hypothesis of schizophrenia: focus on the dopamine receptor.* Am J Psychiatry, 1976. **133**(2): p. 197-202.
24. Kapur, S. and D. Mamo, *Half a century of antipsychotics and still a central role for dopamine D2 receptors.* Progress in Neuro-Psychopharmacology and Biological Psychiatry, 2003. **27**(7): p. 1081-1090.
25. Farinde, F.G.A. *First-Generation Antipsychotics: An Introduction*. 2016 Nov 9 2016; Available from: http://psychopharmacologyinstitute.com/antipsychotics/first-generation-antipsychotics/#Mechanism_of_Action.
26. Nasrallah, H.A., *Atypical antipsychotic-induced metabolic side effects: insights from receptor-binding profiles.* Mol Psychiatry, 2007. **13**(1): p. 27-35.
27. Kusumi, I., S. Boku, and Y. Takahashi, *Psychopharmacology of atypical antipsychotic drugs: From the receptor binding profile to neuroprotection and neurogenesis.* Psychiatry and Clinical Neurosciences, 2015. **69**(5): p. 243-258.
28. Muench, J. and A.M. Hamer, *Adverse effects of antipsychotic medications.* Am Fam Physician, 2010. **81**(5): p. 617-622.
29. Meyer, J.M., et al., *Change in Metabolic Syndrome Parameters With Antipsychotic Treatment in the CATIE Schizophrenia Trial: Prospective Data From Phase 1.* Schizophrenia research, 2008. **101**(0): p. 273-286.
30. Swartz, M.S., et al., *What CATIE Found: Results From the Schizophrenia Trial.* Psychiatric services (Washington, D.C.), 2008. **59**(5): p. 500-506.
31. Kane, J., et al., *Clozapine for the treatment-resistant schizophrenic. A double-blind comparison with chlorpromazine.* Arch Gen Psychiatry, 1988. **45**(9): p. 789-96.
32. Maglione, M., et al., *AHRQ Comparative Effectiveness Reviews*, in *Off-Label Use of Atypical Antipsychotics: An Update*. 2011, Agency for Healthcare Research and Quality (US): Rockville (MD).
33. Verdoux, H., M. Tournier, and B. Bégaud, *Antipsychotic prescribing trends: a review of pharmaco-epidemiological studies.* Acta Psychiatrica Scandinavica, 2010. **121**(1): p. 4-10.
34. De Hert, M., et al., *Physical illness in patients with severe mental disorders. I. Prevalence, impact of medications and disparities in health care.* World Psychiatry, 2011. **10**(1): p. 52-77.
35. Marder, S.R., et al., *Physical Health Monitoring of Patients With Schizophrenia.* American Journal of Psychiatry, 2004. **161**(8): p. 1334-1349.
36. Mitchell, A.J., et al., *Prevalence of Metabolic Syndrome and Metabolic Abnormalities in Schizophrenia and Related Disorders—A Systematic Review and Meta-Analysis.* Schizophrenia Bulletin, 2013. **39**(2): p. 306-318.
37. Correll, C.U., et al., *Prevalence, incidence and mortality from cardiovascular disease in patients with pooled and specific severe mental illness: a large-scale meta-*

analysis of 3,211,768 patients and 113,383,368 controls. World psychiatry : official journal of the World Psychiatric Association (WPA), 2017. **16**(2): p. 163-180.
38. Pillinger, T., et al., *Comparative effects of 18 antipsychotics on metabolic function in patients with schizophrenia, predictors of metabolic dysregulation, and association with psychopathology: a systematic review and network meta-analysis.* The Lancet Psychiatry, 2020. **7**(1): p. 64-77.
39. Haffner, S.M., et al., *Prospective Analysis of The Insulin-Resistance Syndrome (Syndrome X).* Diabetes, 1992. **41**(6): p. 715-722.
40. Alberti, K.G., P. Zimmet, and J. Shaw, *Metabolic syndrome--a new world-wide definition. A Consensus Statement from the International Diabetes Federation.* Diabet Med, 2006. **23**(5): p. 469-80.
41. Rochlani, Y., et al., *Metabolic syndrome: pathophysiology, management, and modulation by natural compounds.* Therapeutic advances in cardiovascular disease, 2017. **11**(8): p. 215-225.
42. Lewis, G.F. and G. Steiner, *Acute effects of insulin in the control of VLDL production in humans. Implications for the insulin-resistant state.* Diabetes Care, 1996. **19**(4): p. 390-3.
43. Aganović, I. and T. Dušek, *Pathophysiology of Metabolic Syndrome.* EJIFCC, 2007. **18**(1): p. 3-6.
44. Vancampfort, D., et al., *Risk of metabolic syndrome and its components in people with schizophrenia and related psychotic disorders, bipolar disorder and major depressive disorder: a systematic review and meta-analysis.* World psychiatry : official journal of the World Psychiatric Association (WPA), 2015. **14**(3): p. 339-347.
45. Weir, G.C. and S. Bonner-Weir, *Five Stages of Evolving Beta-Cell Dysfunction During Progression to Diabetes.* Diabetes, 2004. **53**(suppl 3): p. S16.
46. Jonas, J.-C., et al., *Chronic hyperglycemia triggers loss of pancreatic β cell differentiation in an animal model of diabetes.* Journal of Biological Chemistry, 1999. **274**(20): p. 14112-14121.
47. Butler, A.E., et al., *β-cell deficit and increased β-cell apoptosis in humans with type 2 diabetes.* Diabetes, 2003. **52**(1): p. 102-110.
48. Lu, X., et al., *Cholesterol induces pancreatic β cell apoptosis through oxidative stress pathway.* Cell stress & chaperones, 2011. **16**(5): p. 539-548.
49. Bardini, G., C.M. Rotella, and S. Giannini, *Dyslipidemia and diabetes: reciprocal impact of impaired lipid metabolism and Beta-cell dysfunction on micro- and macrovascular complications.* The review of diabetic studies : RDS, 2012. **9**(2-3): p. 82-93.
50. American Diabetes, A., *Diagnosis and classification of diabetes mellitus.* Diabetes care, 2010. **33 Suppl 1**(Suppl 1): p. S62-S69.
51. Holt, R.I.G. and A.J. Mitchell, *Diabetes mellitus and severe mental illness: mechanisms and clinical implications.* Nature Reviews. Endocrinology, 2015. **11**(2): p. 79-89.
52. Brownlee, M., *The Pathobiology of Diabetic Complications.* A Unifying Mechanism, 2005. **54**(6): p. 1615-1625.
53. Leon, B.M. and T.M. Maddox, *Diabetes and cardiovascular disease: Epidemiology, biological mechanisms, treatment recommendations and future research.* World journal of diabetes, 2015. **6**(13): p. 1246-1258.
54. Ballon, J.S., et al., *Molecular pathophysiology of metabolic effects of antipsychotic medications.* Trends in Endocrinology & Metabolism, 2014. **25**(11): p. 593-600.

55. Ozasa, R., et al., *The Antipsychotic Olanzapine Induces Apoptosis in Insulin-secreting Pancreatic β Cells by Blocking PERK-mediated Translational Attenuation.* Cell Structure and Function, 2013. **38**(2): p. 183-195.
56. Ader, M., et al., *Metabolic Dysregulation With Atypical Antipsychotics Occurs in the Absence of Underlying Disease A Placebo-Controlled Study of Olanzapine and Risperidone in Dogs.* Diabetes, 2005. **54**(3): p. 862-871.
57. Newcomer, J.W., et al., *Abnormalities in glucose regulation during antipsychotic treatment of schizophrenia.* Archives of General Psychiatry, 2002. **59**(4): p. 337-345.
58. Boyda, H.N., et al., *Preclinical models of antipsychotic drug-induced metabolic side effects.* Trends in Pharmacological Sciences, 2010. **31**(10): p. 484-497.
59. Smith, R.C., et al., *Effects of olanzapine and risperidone on glucose metabolism and insulin sensitivity in chronic schizophrenic patients with long-term antipsychotic treatment: a randomized 5-month study.* The Journal of clinical psychiatry, 2009. **70**(11): p. 1501-1513.
60. Chintoh, A.F., et al., *Insulin resistance and secretion in vivo: effects of different antipsychotics in an animal model.* Schizophr Res, 2009. **108**(1-3): p. 127-33.
61. Nousen, E.K., J.G. Franco, and E.L. Sullivan, *Unraveling the mechanisms responsible for the comorbidity between metabolic syndrome and mental health disorders.* Neuroendocrinology, 2013. **98**(4): p. 254-66.
62. Newcomer, J.W. and D.W. Haupt, *The metabolic effects of antipsychotic medications.* Can J Psychiatry, 2006. **51**(8): p. 480-91.
63. De Hert, M., et al., *Prevalence of diabetes, metabolic syndrome and metabolic abnormalities in schizophrenia over the course of the illness: a cross-sectional study.* Clinical Practice and Epidemiology in Mental Health : CP & EMH, 2006. **2**: p. 14-14.
64. Mitchell, A.J., et al., *Is the Prevalence of Metabolic Syndrome and Metabolic Abnormalities Increased in Early Schizophrenia? A Comparative Meta-Analysis of First Episode, Untreated and Treated Patients.* Schizophrenia Bulletin, 2012. **39**(2): p. 295-305.
65. Agrawal, Y., et al., *Antipsychotic therapy-induced new onset diabetic ketoacidosis.* American journal of therapeutics, 2016. **23**(6): p. e1944-e1945.
66. Abramowitz, J. and R. Schulman, *Severe Hyperglycemia, Diabetic Ketoacidosis, and Hyperosmolar State.* Handbook of Medicine in Psychiatry, 2015: p. 235.
67. Vuk, A., et al., *Diabetic ketoacidosis associated with antipsychotic drugs: case reports and a review of literature.* Psychiatria Danubina, 2017. **29**(2): p. 121-135.
68. Ragucci, K.R. and B.J. Wells, *Olanzapine-induced diabetic ketoacidosis.* Annals of Pharmacotherapy, 2001. **35**(12): p. 1556-1558.
69. Smith, G.C., et al., *Atypical antipsychotic drugs induce derangements in glucose homeostasis by acutely increasing glucagon secretion and hepatic glucose output in the rat.* Diabetologia, 2008. **51**(12): p. 2309-17.
70. Chintoh, A.F., et al., *Insulin resistance following continuous, chronic olanzapine treatment: An animal model.* Schizophrenia Research, 2008. **104**(1): p. 23-30.
71. Kahn, R.S., et al., *Effectiveness of antipsychotic drugs in first-episode schizophrenia and schizophreniform disorder: an open randomised clinical trial.* Lancet, 2008. **371**(9618): p. 1085-97.
72. Lieberman , J.A., et al., *Effectiveness of Antipsychotic Drugs in Patients with Chronic Schizophrenia.* New England Journal of Medicine, 2005. **353**(12): p. 1209-1223.
73. Allison, D.B., et al., *Antipsychotic-induced weight gain: a comprehensive research synthesis.* Am J Psychiatry, 1999. **156**(11): p. 1686-96.
74. Beasley, J.C., G.D. Tollefson, and P.V. Tran, *Safety of olanzapine.* J Clin Psychiatry, 1997. **58**: p. 13-7.

75. Zhang, Z.J., et al., *Effects of antipsychotics on fat deposition and changes in leptin and insulin levels. Magnetic resonance imaging study of previously untreated people with schizophrenia.* Br J Psychiatry, 2004. **184**: p. 58-62.
76. Goudie, A.J., J.A. Smith, and J.C. Halford, *Characterization of olanzapine-induced weight gain in rats.* J Psychopharmacol, 2002. **16**(4): p. 291-6.
77. Xu, H. and X. Zhuang, *Atypical antipsychotics-induced metabolic syndrome and nonalcoholic fatty liver disease: a critical review.* Neuropsychiatric disease and treatment, 2019. **15**: p. 2087-2099.
78. Smith, G.C., M.H. Vickers, and P.R. Shepherd, *Olanzapine effects on body composition, food preference, glucose metabolism and insulin sensitivity in the rat.* Archives of Physiology and Biochemistry, 2011. **117**(4): p. 241-249.
79. Kroeze, W.K., et al., *H1-Histamine Receptor Affinity Predicts Short-Term Weight Gain for Typical and Atypical Antipsychotic Drugs.* Neuropsychopharmacology, 2003. **28**(3): p. 519-526.
80. Bymaster, F.P., et al., *Radioreceptor Binding Profile of the Atypical Antipsychotic Olanzapine.* Neuropsychopharmacology, 1996. **14**: p. 87.
81. Haas, H.L., O.A. Sergeeva, and O. Selbach, *Histamine in the nervous system.* Physiol Rev, 2008. **88**(3): p. 1183-241.
82. He, M., C. Deng, and X.F. Huang, *The role of hypothalamic H1 receptor antagonism in antipsychotic-induced weight gain.* CNS Drugs, 2013. **27**(6): p. 423-34.
83. Lord, C.C., et al., *The atypical antipsychotic olanzapine causes weight gain by targeting serotonin receptor 2C.* The Journal of clinical investigation, 2017. **127**(9): p. 3402-3406.
84. Tecott, L.H., et al., *Eating disorder and epilepsy in mice lacking 5-HT2C serotonin receptors.* Nature, 1995. **374**(6522): p. 542-546.
85. Kirk, S.L., et al., *Olanzapine-induced weight gain in the rat: role of 5-HT2C and histamine H1 receptors.* Psychopharmacology (Berl), 2009. **207**(1): p. 119-25.
86. Saponaro, C., et al., *The Subtle Balance between Lipolysis and Lipogenesis: A Critical Point in Metabolic Homeostasis.* Nutrients, 2015. **7**(11): p. 9453-9474.
87. Yang, L.-H., et al., *Olanzapine induces SREBP-1-related adipogenesis in 3T3-L1 cells.* Pharmacological Research, 2007. **56**(3): p. 202-208.
88. Raeder, M.B., et al., *SREBP activation by antipsychotic- and antidepressant-drugs in cultured human liver cells: relevance for metabolic side-effects?* Mol Cell Biochem, 2006. **289**(1-2): p. 167-73.
89. Monteiro, S., et al., *Metabolic syndrome in dyslipidemia consultations.* Rev Port Cardiol, 2006. **25**(9): p. 821-31.
90. Ferno, J., et al., *Drug-induced activation of SREBP-controlled lipogenic gene expression in CNS-related cell lines: marked differences between various antipsychotic drugs.* BMC Neurosci, 2006. **7**: p. 69.
91. Goldberg, I.J., *Diabetic Dyslipidemia: Causes and Consequences.* The Journal of Clinical Endocrinology & Metabolism, 2001. **86**(3): p. 965-971.
92. Nikkila, E.A. and M. Kekki, *Plasma triglyceride transport kinetics in diabetes mellitus.* Metabolism, 1973. **22**(1): p. 1-22.
93. Sheitman, B., et al., *Olanzapine-Induced Elevation of Plasma Triglyceride Levels.* American Journal of Psychiatry, 1999. **156**(9): p. 1471-a-1472.
94. Melkersson, K.I. and M.L. Dahl, *Relationship between levels of insulin or triglycerides and serum concentrations of the atypical antipsychotics clozapine and olanzapine in patients on treatment with therapeutic doses.* Psychopharmacology, 2003. **170**(2): p. 157-166.

95. Osser, D.N., D.M. Najarian, and R.L. Dufresne, *Olanzapine increases weight and serum triglyceride levels.* The Journal of Clinical Psychiatry, 1999. **60**(11): p. 767-770.
96. Koro, C.E., et al., *An Assessment of the Independent Effects of Olanzapine and Risperidone Exposure on the Risk of Hyperlipidemia in Schizophrenic Patients.* Archives of General Psychiatry, 2002. **59**(11): p. 1021-1026.
97. Birkenaes, A.B., et al., *Dyslipidemia independent of body mass in antipsychotic-treated patients under real-life conditions.* J Clin Psychopharmacol, 2008. **28**(2): p. 132-7.
98. Meyer, J.M., *A retrospective comparison of weight, lipid, and glucose changes between risperidone- and olanzapine-treated inpatients: metabolic outcomes after 1 year.* J Clin Psychiatry, 2002. **63**(5): p. 425-33.
99. Skrede, S., et al., *Olanzapine, but not aripiprazole, weight-independently elevates serum triglycerides and activates lipogenic gene expression in female rats.* International Journal of Neuropsychopharmacology, 2012. **15**(2): p. 163-179.
100. Yuan, G., K.Z. Al-Shali, and R.A. Hegele, *Hypertriglyceridemia: its etiology, effects and treatment.* CMAJ : Canadian Medical Association journal = journal de l'Association medicale canadienne, 2007. **176**(8): p. 1113-1120.
101. Polymeropoulos, M.H., et al., *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, 2009. **108**(1-3): p. 134-142.
102. Kristiana, I., et al., *Antipsychotic drugs upregulate lipogenic gene expression by disrupting intracellular trafficking of lipoprotein-derived cholesterol.* The pharmacogenomics journal, 2010. **10**(5): p. 396.
103. Canfrán-Duque, A., et al., *Atypical antipsychotics alter cholesterol and fatty acid metabolism in vitro.* Journal of lipid research, 2013. **54**(2): p. 310-324.
104. Lauressergues, E., et al., *Overweight induced by chronic risperidone exposure is correlated with overexpression of the SREBP-1c and FAS genes in mouse liver.* Naunyn Schmiedebergs Arch Pharmacol, 2011. **383**(4): p. 423-36.
105. Lauressergues, E., et al., *Antipsychotic drug action on SREBPs-related lipogenesis and cholesterogenesis in primary rat hepatocytes.* Naunyn Schmiedebergs Arch Pharmacol, 2010. **381**(5): p. 427-39.
106. Liu, X., et al., *Acute effects of oral olanzapine treatment on the expression of fatty acid and cholesterol metabolism-related gene in rats.* Life sciences, 2015. **128**: p. 72-78.
107. Fernø, J., et al., *Olanzapine depot exposure in male rats: dose-dependent lipogenic effects without concomitant weight gain.* European Neuropsychopharmacology, 2015. **25**(6): p. 923-932.
108. Shimano, H. and R. Sato, *SREBP-regulated lipid metabolism: convergent physiology — divergent pathophysiology.* Nature Reviews Endocrinology, 2017. **13**(12): p. 710-730.
109. Minet-Ringuet, J., et al., *Alterations of lipid metabolism and gene expression in rat adipocytes during chronic olanzapine treatment.* Molecular psychiatry, 2007. **12**(6): p. 562.
110. Oh, K.-J., et al., *Atypical antipsychotic drugs perturb AMPK-dependent regulation of hepatic lipid metabolism.* American Journal of Physiology-Endocrinology and Metabolism, 2011. **300**(4): p. E624-E632.
111. Li, Y., et al., *Berberine Alleviates Olanzapine-Induced Adipogenesis via the AMPKα–SREBP Pathway in 3T3-L1 Cells.* International journal of molecular sciences, 2016. **17**(11): p. 1865.

112. Fathallah, N., et al., *Drug-Induced Hyperglycaemia and Diabetes.* Drug Safety, 2015. **38**(12): p. 1153-1168.
113. Fleischhacker, W.W., et al., *Metabolic risk factors in first-episode schizophrenia: baseline prevalence and course analysed from the European First-Episode Schizophrenia Trial.* International Journal of Neuropsychopharmacology, 2013. **16**(5): p. 987-995.
114. Shen, S.W., G.M. Reaven, and J.W. Farquhar, *Comparison of impedance to insulin-mediated glucose uptake in normal subjects and in subjects with latent diabetes.* J Clin Invest, 1970. **49**(12): p. 2151-60.
115. DeFronzo, R.A., J.D. Tobin, and R. Andres, *Glucose clamp technique: a method for quantifying insulin secretion and resistance.* Am J Physiol, 1979. **237**(3): p. E214-23.
116. Sacher, J., et al., *Effects of Olanzapine and Ziprasidone on Glucose Tolerance in Healthy Volunteers.* Neuropsychopharmacology, 2008. **33**(7): p. 1633-1641.
117. Jean-Pierre Lindenmayer, M.D. ,, et al., *Changes in Glucose and Cholesterol Levels in Patients With Schizophrenia Treated With Typical or Atypical Antipsychotics.* American Journal of Psychiatry, 2003. **160**(2): p. 290-296.
118. Sowell, M.O., et al., *Hyperglycemic Clamp Assessment of Insulin Secretory Responses in Normal Subjects Treated with Olanzapine, Risperidone, or Placebo.* The Journal of Clinical Endocrinology & Metabolism, 2002. **87**(6): p. 2918-2923.
119. Chiu, C.-C., et al., *The time-dependent change of insulin secretion in schizophrenic patients treated with olanzapine.* Progress in Neuro-Psychopharmacology and Biological Psychiatry, 2010. **34**(6): p. 866-870.
120. Seaburg, H.L., B.M. McLendon, and P.M. Doraiswamy, *Olanzapine-Associated Severe Hyperglycemia, Ketonuria, and Acidosis: Case Report and Review of Literature.* Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy, 2001. **21**(11): p. 1448-1454.
121. Beliard, S., R. Valero, and B. Vialettes, *Atypical neuroleptics and diabetes.* Diabetes & metabolism, 2003. **29**(3): p. 296-299.
122. Haddad, P.M., *Antipsychotics and diabetes: review of non-prospective data.* The British Journal of Psychiatry, 2004. **184**(S47): p. s80-s86.
123. Nagamine, T., *Olanzapine and diabetic ketacidosis: What is the underlying mechanism?* Innovations in Clinical Neuroscience, 2018. **15**(3-4): p. 11-11.
124. Vuk, A., et al., *Treatment of Diabetic Ketoacidosis Associated With Antipsychotic Medication: Literature Review.* Journal of clinical psychopharmacology, 2017. **37**(5): p. 584-589.
125. Cohen, D. and C.U. Correll, *Second-generation antipsychotic-associated diabetes mellitus and diabetic ketoacidosis: mechanisms, predictors, and screening need.* J Clin Psychiatry, 2009. **70**(5): p. 765-6.
126. Polcwiartek, C., et al., *Diabetic ketoacidosis in patients exposed to antipsychotics: a systematic literature review and analysis of Danish adverse drug event reports.* Psychopharmacology (Berl), 2016. **233**(21-22): p. 3663-3672.
127. Nagata, M., et al., *Mechanism Underlying Induction of Hyperglycemia in Rats by Single Administration of Olanzapine.* Biol Pharm Bull, 2016. **39**(5): p. 754-61.
128. Assié, M.-B., et al., *The antipsychotics clozapine and olanzapine increase plasma glucose and corticosterone levels in rats: Comparison with aripiprazole, ziprasidone, bifeprunox and F15063.* European Journal of Pharmacology, 2008. **592**(1): p. 160-166.
129. Savoy, Y.E., et al., *Differential Effects of Various Typical and Atypical Antipsychotics on Plasma Glucose and Insulin Levels in the Mouse: Evidence for the Involvement of Sympathetic Regulation.* Schizophrenia Bulletin, 2008. **36**(2): p. 410-418.

130. Chintoh, A.F., et al., *Insulin Resistance and Decreased Glucose-Stimulated Insulin Secretion After Acute Olanzapine Administration.* Journal of Clinical Psychopharmacology, 2008. **28**(5): p. 494-499.
131. Houseknecht, K.L., et al., *Acute effects of atypical antipsychotics on whole-body insulin resistance in rats: implications for adverse metabolic effects.* Neuropsychopharmacology, 2007. **32**(2): p. 289-97.
132. Boyda, H.N., et al., *A parametric study of the acute effects of antipsychotic drugs on glucose sensitivity in an animal model.* Progress in Neuro-Psychopharmacology and Biological Psychiatry, 2010. **34**(6): p. 945-954.
133. Coccurello, R., et al., *30 Days of Continuous Olanzapine Infusion Determines Energy Imbalance, Glucose Intolerance, Insulin Resistance, and Dyslipidemia in Mice.* Journal of Clinical Psychopharmacology, 2009. **29**(6): p. 576-583.
134. Ren, L., et al., *The IRS/PI3K/Akt signaling pathway mediates olanzapine-induced hepatic insulin resistance in male rats.* Life Sciences, 2019. **217**: p. 229-236.
135. Li, R., et al., *The Wnt Signaling Pathway Effector TCF7L2 Mediates Olanzapine-Induced Weight Gain and Insulin Resistance.* Frontiers in Pharmacology, 2018. **9**: p. 379.
136. Shah, R., et al., *Olanzapine induced biochemical and histopathological changes after its chronic administration in rats.* Saudi Pharmaceutical Journal, 2016. **24**(6): p. 698-704.
137. Albaugh, V.L., et al., *Olanzapine promotes fat accumulation in male rats by decreasing physical activity, repartitioning energy and increasing adipose tissue lipogenesis while impairing lipolysis.* Molecular psychiatry, 2011. **16**(5): p. 569-581.
138. Schmidt, R.H., et al., *Olanzapine Activates Hepatic Mammalian Target of Rapamycin: New Mechanistic Insight into Metabolic Dysregulation with Atypical Antipsychotic Drugs.* The Journal of Pharmacology and Experimental Therapeutics, 2013. **347**(1): p. 126-135.
139. Ersland, K.M., et al., *One-Year Treatment with Olanzapine Depot in Female Rats: Metabolic Effects.* The international journal of neuropsychopharmacology, 2019. **22**(5): p. 358-369.
140. Agouni, A., et al., *In vivo differential effects of fasting, re-feeding, insulin and insulin stimulation time course on insulin signaling pathway components in peripheral tissues.* Biochemical and Biophysical Research Communications, 2010. **401**(1): p. 104-111.
141. Aravagiri, M., Y. Teper, and S.R. Marder, *Pharmacokinetics and tissue distribution of olanzapine in rats.* Biopharm Drug Dispos, 1999. **20**(8): p. 369-77.
142. van der Zwaal, E.M., et al., *Olanzapine-induced weight gain: chronic infusion using osmotic minipumps does not result in stable plasma levels due to degradation of olanzapine in solution.* European journal of pharmacology, 2008. **585**(1): p. 130-136.
143. Skrede, S., et al., *Olanzapine depot formulation in rat: a step forward in modelling antipsychotic-induced metabolic adverse effects.* Int J Neuropsychopharmacol, 2014. **17**(1): p. 91-104.
144. Ersland, K.M., et al., *Antipsychotic-induced metabolic effects in the female rat: Direct comparison between long-acting injections of risperidone and olanzapine.* Journal of Psychopharmacology, 2015. **29**(12): p. 1280-1289.
145. Gerich, J.E., *Control of glycaemia.* Baillière's Clinical Endocrinology and Metabolism, 1993. **7**(3): p. 551-586.
146. Wilcox, G., *Insulin and Insulin Resistance.* Clinical Biochemist Reviews, 2005. **26**(2): p. 19-39.

147. Sharabi, K., et al., *Molecular pathophysiology of hepatic glucose production.* Molecular Aspects of Medicine, 2015. **46**(Supplement C): p. 21-33.
148. Roh, E., D.K. Song, and M.-S. Kim, *Emerging role of the brain in the homeostatic regulation of energy and glucose metabolism.* Experimental & Molecular Medicine, 2016. **48**(3): p. e216-e216.
149. Morton, G.J. and M.W. Schwartz, *Leptin and the Central Nervous System Control of Glucose Metabolism.* Physiological Reviews, 2011. **91**(2): p. 389-411.
150. Püschel, G.P., *Control of hepatocyte metabolism by sympathetic and parasympathetic hepatic nerves.* The Anatomical Record Part A: Discoveries in Molecular, Cellular, and Evolutionary Biology, 2004. **280A**(1): p. 854-867.
151. Schwartz, M.W., et al., *Cooperation between brain and islet in glucose homeostasis and diabetes.* Nature, 2013. **503**(7474): p. 59-66.
152. Boden, G. and G.I. Shulman, *Free fatty acids in obesity and type 2 diabetes: defining their role in the development of insulin resistance and β-cell dysfunction.* European Journal of Clinical Investigation, 2002. **32**: p. 14-23.
153. Bergman, R.N. and S.D. Mittelman, *Central role of the adipocyte in insulin resistance.* J Basic Clin Physiol Pharmacol, 1998. **9**(2-4): p. 205-21.
154. Henderson, D.C., et al., *Glucose metabolism in patients with schizophrenia treated with atypical antipsychotic agents: A frequently sampled intravenous glucose tolerance test and minimal model analysis.* Archives of General Psychiatry, 2005. **62**(1): p. 19-28.
155. Paz, K., et al., *Interaction between the Insulin Receptor and Its Downstream Effectors. Use of individually expressed receptor domains for structure/function analysis* Journal of Biological Chemistry, 1996. **271**(12): p. 6998-7003.
156. Cheatham, B., et al., *Phosphatidylinositol 3-kinase activation is required for insulin stimulation of pp70 S6 kinase, DNA synthesis, and glucose transporter translocation.* Molecular and cellular biology, 1994. **14**(7): p. 4902-4911.
157. Draznin, B., *Molecular Mechanisms of Insulin Resistance: Serine Phosphorylation of Insulin Receptor Substrate-1 and Increased Expression of p85α.* The Two Sides of a Coin, 2006. **55**(8): p. 2392-2397.
158. Engl, J., et al., *Olanzapine impairs glycogen synthesis and insulin signaling in L6 skeletal muscle cells.* Mol Psychiatry, 2005. **10**(12): p. 1089-96.
159. Alghamdi, F., et al., *A novel insulin receptor-signaling platform and its link to insulin resistance and type 2 diabetes.* Cellular Signalling, 2014. **26**(6): p. 1355-1368.
160. Li, H., et al., *Chronic olanzapine administration causes metabolic syndrome through inflammatory cytokines in rodent models of insulin resistance.* Scientific Reports, 2019. **9**(1): p. 1582.
161. Vestri, H.S., et al., *Atypical antipsychotic drugs directly impair insulin action in adipocytes: effects on glucose transport, lipogenesis, and antilipolysis.* Neuropsychopharmacology, 2007. **32**(4): p. 765-72.
162. Valverde, A.M., et al., *Molecular Mechanisms of Insulin Resistance in IRS-2-Deficient Hepatocytes.* Diabetes, 2003. **52**(9): p. 2239-2248.
163. Mondelli, V., et al., *Haloperidol and olanzapine mediate metabolic abnormalities through different molecular pathways.* Transl Psychiatry, 2013. **3**: p. e208.
164. Aguirre, V., et al., *Phosphorylation of Ser307 in insulin receptor substrate-1 blocks interactions with the insulin receptor and inhibits insulin action.* J Biol Chem, 2002. **277**(2): p. 1531-7.
165. Nordlie, R.C., J.D. Foster, and A.J. Lange, *Regulation of glucose production by the liver.* Annu Rev Nutr, 1999. **19**: p. 379-406.

166. Shimazu, T. and S. Ogasawara, *Effects of hypothalamic stimulation on gluconeogenesis and glycolysis in rat liver.* Am J Physiol, 1975. **228**(6): p. 1787-93.
167. Smith, U., *TCF7L2 and type 2 diabetes—we WNT to know.* Diabetologia, 2007. **50**(1): p. 5-7.
168. Duggirala, R., et al., *Linkage of type 2 diabetes mellitus and of age at onset to a genetic location on chromosome 10q in Mexican Americans.* American journal of human genetics, 1999. **64**(4): p. 1127-1140.
169. Boj, Sylvia F., et al., *Diabetes Risk Gene and Wnt Effector Tcf7l2/TCF4 Controls Hepatic Response to Perinatal and Adult Metabolic Demand.* Cell, 2012. **151**(7): p. 1595-1607.
170. Ashcroft, F.M. and P. Rorsman, *Diabetes mellitus and the beta cell: the last ten years.* Cell, 2012. **148**(6): p. 1160-71.
171. Melkersson, K., *Clozapine and olanzapine, but not conventional antipsychotics, increase insulin release in vitro.* European Neuropsychopharmacology, 2004. **14**(2): p. 115-119.
172. Melkersson, K., et al., *Different effects of antipsychotic drugs on insulin release in vitro.* European Neuropsychopharmacology, 2001. **11**(5): p. 327-332.
173. Nagata, M., et al., *Blockade of multiple monoamines receptors reduce insulin secretion from pancreatic β-cells.* Scientific Reports, 2019. **9**(1): p. 16438.
174. Melkersson, K. and E. Jansson, *Effects of the atypical antipsychotic clozapine on insulin release in vitro.* Neuro Endocrinol Lett, 2007. **28**(6): p. 854-60.
175. Best, L., A.P. Yates, and G.P. Reynolds, *Actions of antipsychotic drugs on pancreatic beta-cell function: contrasting effects of clozapine and haloperidol.* J Psychopharmacol, 2005. **19**(6): p. 597-601.
176. Silvestre, J.S. and J. Prous, *Research on adverse drug events. I. Muscarinic M3 receptor binding affinity could predict the risk of antipsychotics to induce type 2 diabetes.* Methods Find Exp Clin Pharmacol, 2005. **27**(5): p. 289-304.
177. Johnson, D.E., et al., *Inhibitory Effects of Antipsychotics on Carbachol-Enhanced Insulin Secretion From Perifused Rat Islets.* Role of Muscarinic Antagonism in Antipsychotic-Induced Diabetes and Hyperglycemia, 2005. **54**(5): p. 1552-1558.
178. Weston-Green, K., X.F. Huang, and C. Deng, *Alterations to melanocortinergic, GABAergic and cannabinoid neurotransmission associated with olanzapine-induced weight gain.* PLoS One, 2012. **7**(3): p. e33548.
179. Brüning, J.C., et al., *Development of a Novel Polygenic Model of NIDDM in Mice Heterozygous for IR and IRS-1 Null Alleles.* Cell, 1997. **88**(4): p. 561-572.
180. Withers, D.J., et al., *Disruption of IRS-2 causes type 2 diabetes in mice.* Nature, 1998. **391**(6670): p. 900-904.
181. Niessen, M., *On the role of IRS2 in the regulation of functional beta-cell mass.* Arch Physiol Biochem, 2006. **112**(2): p. 65-73.
182. Michael, M.D., et al., *Loss of insulin signaling in hepatocytes leads to severe insulin resistance and progressive hepatic dysfunction.* Mol Cell, 2000. **6**(1): p. 87-97.
183. Brüning, J.C., et al., *A muscle-specific insulin receptor knockout exhibits features of the metabolic syndrome of NIDDM without altering glucose tolerance.* Molecular cell, 1998. **2**(5): p. 559-569.
184. Chiu, C.C., et al., *The early effect of olanzapine and risperidone on insulin secretion in atypical-naive schizophrenic patients.* J Clin Psychopharmacol, 2006. **26**(5): p. 504-7.
185. Weir, G.C., et al., *Beta-cell adaptation and decompensation during the progression of diabetes.* Diabetes, 2001. **50**(suppl 1): p. S154.

186. Rutter, Guy A., et al., *Pancreatic β-cell identity, glucose sensing and the control of insulin secretion.* Biochemical Journal, 2015. **466**(2): p. 203-218.
187. Henquin, J.-C., et al., *Signals and Pools Underlying Biphasic Insulin Secretion.* Diabetes, 2002. **51**(suppl 1): p. S60-S67.
188. Wang, Z. and D.C. Thurmond, *Mechanisms of biphasic insulin-granule exocytosis - roles of the cytoskeleton, small GTPases and SNARE proteins.* Journal of cell science, 2009. **122**(Pt 7): p. 893-903.
189. Chen, C., et al., *Human beta cell mass and function in diabetes: Recent advances in knowledge and technologies to understand disease pathogenesis.* Molecular Metabolism, 2017. **6**(9): p. 943-957.
190. Rieck, S. and K.H. Kaestner, *Expansion of β-cell mass in response to pregnancy.* Trends in Endocrinology & Metabolism, 2010. **21**(3): p. 151-158.
191. Brunzell, J.D., et al., *Relationships between fasting plasma glucose levels and insulin secretion during intravenous glucose tolerance tests.* J Clin Endocrinol Metab, 1976. **42**(2): p. 222-9.
192. Fridlyand, L.E. and L.H. Philipson, *Pancreatic Beta Cell G-Protein Coupled Receptors and Second Messenger Interactions: A Systems Biology Computational Analysis.* PLoS ONE, 2016. **11**(5): p. e0152869.
193. Kulkarni, R.N., et al., *Human β-Cell Proliferation and Intracellular Signaling.* Driving in the Dark Without a Road Map, 2012. **61**(9): p. 2205-2213.
194. Bymaster, F.P., et al., *Antagonism by olanzapine of dopamine D1, serotonin2, muscarinic, histamine H1 and alpha 1-adrenergic receptors in vitro.* Schizophr Res, 1999. **37**(1): p. 107-22.
195. Rubí, B., et al., *Dopamine D2-like Receptors Are Expressed in Pancreatic Beta Cells and Mediate Inhibition of Insulin Secretion.* Journal of Biological Chemistry, 2005. **280**(44): p. 36824-36832.
196. Kim, H., et al., *Serotonin regulates pancreatic beta cell mass during pregnancy.* Nat Med, 2010. **16**(7): p. 804-808.
197. Wu, W., et al., *Identification of glucose-dependent insulin secretion targets in pancreatic β cells by combining defined-mechanism compound library screening and siRNA gene silencing.* Journal of biomolecular screening, 2008. **13**(2): p. 128-134.
198. Sakano, D., et al., *Dopamine D2 Receptor-Mediated Regulation of Pancreatic β Cell Mass.* Stem cell reports, 2016. **7**(1): p. 95-109.
199. Cataldo Bascunan, L.R., et al., *Serotonergic regulation of insulin secretion.* Acta Physiol (Oxf), 2019. **225**(1): p. e13101.
200. Almaça, J., et al., *Human Beta Cells Produce and Release Serotonin to Inhibit Glucagon Secretion from Alpha Cells.* Cell Reports, 2016. **17**(12): p. 3281-3291.
201. Bennet, H., et al., *Altered serotonin (5-HT) 1D and 2A receptor expression may contribute to defective insulin and glucagon secretion in human type 2 diabetes.* Peptides, 2015. **71**(Supplement C): p. 113-120.
202. Bennet, H., et al., *Serotonin (5-HT) receptor 2b activation augments glucose-stimulated insulin secretion in human and mouse islets of Langerhans.* Diabetologia, 2016. **59**(4): p. 744-754.
203. Cataldo, L.R., et al., *Prolonged Activation of the Htr2b Serotonin Receptor Impairs Glucose Stimulated Insulin Secretion and Mitochondrial Function in MIN6 Cells.* PLOS ONE, 2017. **12**(1): p. e0170213.
204. Hsu, W.H., et al., *Activation of alpha 2-adrenergic receptors decreases Ca2+ influx to inhibit insulin secretion in a hamster beta-cell line: an action mediated by a guanosine triphosphate-binding protein.* Endocrinology, 1991. **128**(2): p. 958-64.

205. Nakamura, T., et al., *The expression and function of histamine H_3 receptors in pancreatic beta cells.* British journal of pharmacology, 2014. **171**(1): p. 171-185.
206. Duttaroy, A., et al., *Muscarinic Stimulation of Pancreatic Insulin and Glucagon Release Is Abolished in M3 Muscarinic Acetylcholine Receptor–Deficient Mice.* Diabetes, 2004. **53**(7): p. 1714-1720.
207. Kong, K.C., et al., *M3-muscarinic receptor promotes insulin release via receptor phosphorylation/arrestin-dependent activation of protein kinase D1.* Proceedings of the National Academy of Sciences, 2010. **107**(49): p. 21181-21186.
208. Elghazi, L., et al., *Regulation of β-cell mass and function by the Akt/protein kinase B signalling pathway.* Diabetes, Obesity and Metabolism, 2007. **9**(s2): p. 147-157.
209. Zachary Freyberg, Stephen J. Ferrando, and Jonathan A. Javitch, *Roles of the Akt/GSK-3 and Wnt Signaling Pathways in Schizophrenia and Antipsychotic Drug Action.* American Journal of Psychiatry, 2010. **167**(4): p. 388-396.
210. Sorrenson, B., et al., *A Critical Role for β-catenin in Modulating Levels of Insulin Secretion from β-cells by Regulating Actin Cytoskeleton and Insulin Vesicle Localization.* Journal of Biological Chemistry, 2016.
211. Shtutman, M., et al., *The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway.* Proceedings of the National Academy of Sciences of the United States of America, 1999. **96**(10): p. 5522-5527.
212. Ishihara, H., et al., *Pancreatic beta cell line MIN6 exhibits characteristics of glucose metabolism and glucose-stimulated insulin secretion similar to those of normal islets.* Diabetologia, 1993. **36**(11): p. 1139-1145.
213. Huang, S.-M.A., et al., *Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling.* Nature, 2009. **461**(7264): p. 614-620.
214. Meijer, L., et al., *GSK-3-selective inhibitors derived from Tyrian purple indirubins.* Chem Biol, 2003. **10**(12): p. 1255-66.
215. Liu, L., et al., *6-Bromoindirubin-3'-oxime inhibits JAK/STAT3 signaling and induces apoptosis of human melanoma cells.* Cancer Res, 2011. **71**(11): p. 3972-9.
216. Cha, P.H., et al., *Small-molecule binding of the axin RGS domain promotes beta-catenin and Ras degradation.* Nat Chem Biol, 2016. **12**(8): p. 593-600.
217. Hawley, S.A., et al., *The Antidiabetic Drug Metformin Activates the AMP-Activated Protein Kinase Cascade via an Adenine Nucleotide-Independent Mechanism.* Diabetes, 2002. **51**(8): p. 2420-2425.
218. Mosmann, T., *Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays.* J Immunol Methods, 1983. **65**(1-2): p. 55-63.
219. Robbins, E. and P.I. Marcus, *Dynamics of acridine orange- cell interaction. Interrelationships of acridine orange particles and cytoplasmic reddening* The Journal of cell biology, 1963. **18**(2): p. 237-250.
220. Tarnowski, B.I., F.G. Spinale, and J.H. Nicholson, *DAPI as a useful stain for nuclear quantitation.* Biotech Histochem, 1991. **66**(6): p. 297-302.
221. Larsson, O., et al., *Oscillations in KATP channel activity promote oscillations in cytoplasmic free Ca2+ concentration in the pancreatic beta cell.* Proceedings of the National Academy of Sciences, 1996. **93**(10): p. 5161-5165.
222. Atwater, I., B. Ribalet, and E. Rojas, *Cyclic changes in potential and resistance of the beta-cell membrane induced by glucose in islets of Langerhans from mouse.* The Journal of Physiology, 1978. **278**(1): p. 117-139.
223. Göke, R., et al., *Exendin-4 is a high potency agonist and truncated exendin-(9-39)-amide an antagonist at the glucagon-like peptide 1-(7-36)-amide receptor of insulin-secreting beta-cells.* Journal of Biological Chemistry, 1993. **268**(26): p. 19650-19655.

224. Vasseur, M., A. Debuyser, and M. Joffre, *Sensitivity of pancreatic beta cell to calcium channel blockers. An electrophysiologic study of verapamil and nifedipine.* Fundam Clin Pharmacol, 1987. **1**(2): p. 95-113.
225. Yaney, G.C., et al., *Potentiation of insulin secretion by phorbol esters is mediated by PKC-alpha and nPKC isoforms.* Am J Physiol Endocrinol Metab, 2002. **283**(5): p. E880-8.
226. Wiedenkeller, D.E. and G.W. Sharp, *Effects of forskolin on insulin release and cyclic AMP content in rat pancreatic islets.* Endocrinology, 1983. **113**(6): p. 2311-3.
227. Chua, S.L., et al., *UBC and YWHAZ as suitable reference genes for accurate normalisation of gene expression using MCF7, HCT116 and HepG2 cell lines.* Cytotechnology, 2011. **63**(6): p. 645-654.
228. Vandesompele, J., et al., *Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes.* Genome Biol, 2002. **3**(7): p. Research0034.
229. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.
230. Swenne, I. and A. Andersson, *Effect of genetic background on the capacity for islet cell replication in mice.* Diabetologia, 1984. **27**(4): p. 464-467.
231. Bergemann, N., et al., *Olanzapine plasma concentration, average daily dose, and interaction with co-medication in schizophrenic patients.* Pharmacopsychiatry, 2004. **37**(2): p. 63-8.
232. Mauri, M.C., et al., *Clinical outcome and olanzapine plasma levels in acute schizophrenia.* European Psychiatry, 2005. **20**(1): p. 55-60.
233. Sasaki, N., et al., *The atypical antipsychotic clozapine impairs insulin secretion by inhibiting glucose metabolism and distal steps in rat pancreatic islets.* Diabetologia, 2006. **49**(12): p. 2930-8.
234. Menga, A., et al., *Insight into mechanism of in vitro insulin secretion increase induced by antipsychotic clozapine: Role of FOXA1 and mitochondrial citrate carrier.* European Neuropsychopharmacology, 2013. **23**(8): p. 978-987.
235. Simpson, N., et al., *Dopamine-Mediated Autocrine Inhibitory Circuit Regulating Human Insulin Secretion in Vitro.* Molecular Endocrinology, 2012. **26**(10): p. 1757-1772.
236. Wicinski, M. and M.M. Weclewicz, *Clozapine-induced agranulocytosis/granulocytopenia: mechanisms and monitoring.* Curr Opin Hematol, 2018. **25**(1): p. 22-28.
237. Barrow, J., et al., *Transcription factor cycling on the insulin promoter.* FEBS Letters, 2006. **580**(2): p. 711-715.
238. Zhou, B.-B.S. and S.J. Elledge, *The DNA damage response: putting checkpoints in perspective.* Nature, 2000. **408**(6811): p. 433-439.
239. Liu, X., et al., *Cigarette Smoke Extract Induces DNA Damage but Not Apoptosis in Human Bronchial Epithelial Cells.* American Journal of Respiratory Cell and Molecular Biology, 2005. **33**(2): p. 121-129.
240. Harding, H.P., et al., *Perk Is Essential for Translational Regulation and Cell Survival during the Unfolded Protein Response.* Molecular Cell, 2000. **5**(5): p. 897-904.
241. Wang, P., et al., *Diabetes mellitus--advances and challenges in human beta-cell proliferation.* Nat Rev Endocrinol, 2015. **11**(4): p. 201-12.
242. Khadra, A. and S. Schnell, *Development, growth and maintenance of β-cell mass: models are also part of the story.* Molecular aspects of medicine, 2015. **42**: p. 78-90.

243. Dor, Y., et al., *Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation.* Nature, 2004. **429**(6987): p. 41-6.
244. Teta, M., et al., *Growth and regeneration of adult beta cells does not involve specialized progenitors.* Dev Cell, 2007. **12**(5): p. 817-26.
245. Prentki, M. and C.J. Nolan, *Islet beta cell failure in type 2 diabetes.* J Clin Invest, 2006. **116**(7): p. 1802-12.
246. Puri, S., et al., *Replication confers β cell immaturity.* Nature Communications, 2018. **9**(1): p. 485.
247. Klochendler, A., et al., *The Genetic Program of Pancreatic β-Cell Replication In Vivo.* Diabetes, 2016. **65**(7): p. 2081-2093.
248. Metukuri, M.R., et al., *ChREBP Mediates Glucose-Stimulated Pancreatic β-Cell Proliferation.* Diabetes, 2012. **61**(8): p. 2004-2015.
249. Sherr, C.J., *The Pezcoller lecture: cancer cell cycles revisited.* Cancer research, 2000. **60**(14): p. 3689-3695.
250. Sherr, C.J., *Cancer cell cycles.* Science, 1996. **274**(5293): p. 1672-7.
251. Harbour, J.W. and D.C. Dean, *The Rb/E2F pathway: expanding roles and emerging paradigms.* Genes & Development, 2000. **14**(19): p. 2393-2409.
252. Vermeulen, K., D.R. Van Bockstaele, and Z.N. Berneman, *The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer.* Cell Proliferation, 2003. **36**(3): p. 131-149.
253. Nielsen, J.H., et al., *Regulation of beta-cell mass by hormones and growth factors.* Diabetes, 2001. **50**(suppl 1): p. S25.
254. Kushner, J.A., et al., *Cyclins D2 and D1 Are Essential for Postnatal Pancreatic β-Cell Growth.* Molecular and Cellular Biology, 2005. **25**(9): p. 3752-3762.
255. Ekholm, S.V. and S.I. Reed, *Regulation of G1 cyclin-dependent kinases in the mammalian cell cycle.* Current Opinion in Cell Biology, 2000. **12**(6): p. 676-684.
256. Beith, J.L., E.U. Alejandro, and J.D. Johnson, *Insulin stimulates primary beta-cell proliferation via Raf-1 kinase.* Endocrinology, 2008. **149**(5): p. 2251-2260.
257. Tarn, W.-Y. and M.-C. Lai, *Translational control of cyclins.* Cell Division, 2011. **6**(1): p. 5.
258. VADLAKONDA, L., M. Pasupuleti, and P. Reddanna, *Role of PI3K-AKT-mTOR and Wnt Signaling Pathways in Transition of G1-S Phase of Cell Cycle in Cancer Cells.* Frontiers in Oncology, 2013. **3**(85).
259. Miao, X.-Y., et al., *The human glucagon-like peptide-1 analogue liraglutide regulates pancreatic beta-cell proliferation and apoptosis via an AMPK/mTOR/P70S6K signaling pathway.* Peptides, 2013. **39**: p. 71-79.
260. Blandino-Rosano, M., et al., *mTORC1 signaling and regulation of pancreatic β-cell mass.* Cell Cycle, 2012. **11**(10): p. 1892-1902.
261. Balcazar, N., et al., *mTORC1 activation regulates beta-cell mass and proliferation by modulation of cyclin D2 synthesis and stability.* The Journal of biological chemistry, 2009. **284**(12): p. 7832-7842.
262. Lu, X.-H., R.J. Bradley, and D.S. Dwyer, *Olanzapine produces trophic effects in vitro and stimulates phosphorylation of Akt/PKB, ERK1/2, and the mitogen-activated protein kinase p38.* Brain Research, 2004. **1011**(1): p. 58-68.
263. Koyanagi, M., et al., *Ablation of TSC2 Enhances Insulin Secretion by Increasing the Number of Mitochondria through Activation of mTORC1.* PLOS ONE, 2011. **6**(8): p. e23238.
264. Adjei, A.A., *Blocking oncogenic Ras signaling for cancer therapy.* J Natl Cancer Inst, 2001. **93**(14): p. 1062-74.

265. MacCorkle, R.A. and T.-H. Tan, *Mitogen-activated protein kinases in cell-cycle control.* Cell Biochemistry and Biophysics, 2005. **43**(3): p. 451-461.
266. Cussac, D., et al., *Stimulation by antipsychotic agents of mitogen-activated protein kinase (MAPK) coupled to cloned, human (h)serotonin (5-HT)1A receptors.* Psychopharmacology, 2002. **162**(2): p. 168-177.
267. Khoo, S., et al., *MAP kinases and their roles in pancreatic β-cells.* Cell Biochemistry and Biophysics, 2004. **40**(3): p. 191-200.
268. Khoo, S. and M.H. Cobb, *Activation of mitogen-activating protein kinase by glucose is not required for insulin secretion.* Proceedings of the National Academy of Sciences of the United States of America, 1997. **94**(11): p. 5599-5604.
269. He, T.C., et al., *Identification of c-MYC as a target of the APC pathway.* Science, 1998. **281**(5382): p. 1509-12.
270. Wlodarchak, N. and Y. Xing, *PP2A as a master regulator of the cell cycle.* Crit Rev Biochem Mol Biol, 2016. **51**(3): p. 162-84.
271. Welters, H.J., et al., *The protein tyrosine phosphatase-BL, modulates pancreatic beta-cell proliferation by interaction with the Wnt signalling pathway.* J Endocrinol, 2008. **197**(3): p. 543-52.
272. Rulifson, I.C., et al., *Wnt signaling regulates pancreatic β cell proliferation.* Proceedings of the National Academy of Sciences, 2007. **104**(15): p. 6247-6252.
273. Maschio, D.A., et al., *Activation of the Wnt/β-catenin pathway in pancreatic beta cells during the compensatory islet hyperplasia in prediabetic mice.* Biochemical and Biophysical Research Communications, 2016. **478**(4): p. 1534-1540.
274. Gui, S., et al., *Wnt3a regulates proliferation, apoptosis and function of pancreatic NIT-1 beta cells via activation of IRS2/PI3K signaling.* Journal of Cellular Biochemistry, 2013. **114**(7): p. 1488-1497.
275. Dissanayake, Waruni C., B. Sorrenson, and Peter R. Shepherd, *The role of adherens junction proteins in the regulation of insulin secretion.* Bioscience Reports, 2018. **38**(2): p. BSR20170989.
276. da Silva Xavier, G., et al., *TCF7L2 Regulates Late Events in Insulin Secretion From Pancreatic Islet β-Cells.* Diabetes, 2009. **58**(4): p. 894-905.
277. Li, P., et al., *Atypical antipsychotics induce human osteoblasts apoptosis via Wnt/β-catenin signaling.* BMC pharmacology & toxicology, 2019. **20**(1): p. 10-10.
278. Guo, Q., H. Yang, and S. Wang, *Olanzapine inhibits the proliferation and induces the differentiation of glioma stem-like cells through modulating the Wnt signaling pathway in vitro.* Eur Rev Med Pharmacol Sci, 2015. **19**(13): p. 2406-2415.
279. Korswagen, H.C., M.A. Herman, and H.C. Clevers, *Distinct β-catenins mediate adhesion and signalling functions in C. elegans.* Nature, 2000. **406**(6795): p. 527.
280. Voronkov, A. and S. Krauss, *Wnt/beta-catenin signaling and small molecule inhibitors.* Current pharmaceutical design, 2013. **19**(4): p. 634-664.
281. Ip, W., Y.-t.A. Chiang, and T. Jin, *The involvement of the wnt signaling pathway and TCF7L2 in diabetes mellitus: The current understanding, dispute, and perspective.* Cell & Bioscience, 2012. **2**(1): p. 28.
282. MacDonald, B.T., K. Tamai, and X. He, *Wnt/beta-catenin signaling: components, mechanisms, and diseases.* Developmental cell, 2009. **17**(1): p. 9-26.
283. Huelsken, J. and J. Behrens, *The Wnt signalling pathway.* Journal of Cell Science, 2002. **115**(21): p. 3977.
284. Georgia, S. and A. Bhushan, *Beta cell replication is the primary mechanism for maintaining postnatal beta cell mass.* J Clin Invest, 2004. **114**(7): p. 963-8.
285. Baldin, V., et al., *Cyclin D1 is a nuclear protein required for cell cycle progression in G1.* Genes & Development, 1993. **7**(5): p. 812-821.

286. Tetsu, O. and F. McCormick, *β-Catenin regulates expression of cyclin D1 in colon carcinoma cells.* Nature, 1999. **398**(6726): p. 422-426.
287. Morgan, R.G., et al., *Factors Affecting the Nuclear Localization of β-Catenin in Normal and Malignant Tissue.* Journal of Cellular Biochemistry, 2014. **115**(8): p. 1351-1361.
288. Praharaj, S.K., et al., *Metformin for olanzapine-induced weight gain: a systematic review and meta-analysis.* British journal of clinical pharmacology, 2011. **71**(3): p. 377-382.
289. Remington, G.J., et al., *Metformin attenuates olanzapine-induced hepatic, but not peripheral insulin resistance.* J Endocrinol, 2015. **227**(2): p. 71-81.
290. Jiang, Y., et al., *Metformin plays a dual role in MIN6 pancreatic β cell function through AMPK-dependent autophagy.* International journal of biological sciences, 2014. **10**(3): p. 268-277.
291. Bao, R., et al., *Inhibition of Tankyrases Induces Axin Stabilization and Blocks Wnt Signalling in Breast Cancer Cells.* PLOS ONE, 2012. **7**(11): p. e48670.
292. Cozar-Castellano, I., et al., *Induction of beta-cell proliferation and retinoblastoma protein phosphorylation in rat and human islets using adenovirus-mediated transfer of cyclin-dependent kinase-4 and cyclin D1.* Diabetes, 2004. **53**(1): p. 149-59.
293. Zhang, X., et al., *Overexpression of Cyclin D1 in Pancreatic β-Cells In Vivo Results in Islet Hyperplasia Without Hypoglycemia.* Diabetes, 2005. **54**(3): p. 712-719.
294. Pascal, S.M.A., et al., *Effects of c-MYC activation on glucose stimulus-secretion coupling events in mouse pancreatic islets.* American Journal of Physiology-Endocrinology and Metabolism, 2008. **295**(1): p. E92-E102.
295. Liu, C., et al., *Control of β-Catenin Phosphorylation/Degradation by a Dual-Kinase Mechanism.* Cell, 2002. **108**(6): p. 837-847.
296. Mohammad, M.K., et al., *Olanzapine inhibits glycogen synthase kinase-3β: An investigation by docking simulation and experimental validation.* European Journal of Pharmacology, 2008. **584**(1): p. 185-191.
297. Cognard, E., et al., *Identification of a pathway by which glucose regulates β-catenin signalling via the cAMP/protein kinase A pathway in β-cell models.* Biochemical Journal, 2013. **449**(3): p. 803-811.
298. Liu, Z. and J.F. Habener, *Glucagon-like Peptide-1 Activation of TCF7L2-dependent Wnt Signaling Enhances Pancreatic Beta Cell Proliferation.* Journal of Biological Chemistry, 2008. **283**(13): p. 8723-8735.
299. Anagnostou, Sasha H. and Peter R. Shepherd, *Glucose induces an autocrine activation of the Wnt/β-catenin pathway in macrophage cell lines.* Biochemical Journal, 2008. **416**(2): p. 211-218.
300. Jonas, J.-C., et al., *High Glucose Stimulates Early Response Gene c-Myc Expression in Rat Pancreatic β Cells.* Journal of Biological Chemistry, 2001. **276**(38): p. 35375-35381.
301. Tian, X.-H., et al., *XAV939, a tankyrase 1 inhibitior, promotes cell apoptosis in neuroblastoma cell lines by inhibiting Wnt/β-catenin signaling pathway.* Journal of Experimental & Clinical Cancer Research, 2013. **32**(1): p. 100.
302. Pan, F., et al., *Inhibitory effects of XAV939 on the proliferation of small-cell lung cancer H446 cells and Wnt/beta-catenin signaling pathway in vitro.* Oncol Lett, 2018. **16**(2): p. 1953-1958.
303. Chia, I.V. and F. Costantini, *Mouse axin and axin2/conductin proteins are functionally equivalent in vivo.* Molecular and cellular biology, 2005. **25**(11): p. 4371-4376.

304. Yang, K., M. Hitomi, and D.W. Stacey, *Variations in cyclin D1 levels through the cell cycle determine the proliferative fate of a cell.* Cell Division, 2006. **1**(1): p. 32.
305. Klein, E.A. and R.K. Assoian, *Transcriptional regulation of the cyclin D1 gene at a glance.* Journal of cell science, 2008. **121**(Pt 23): p. 3853-3857.
306. Zhang, Y., T. Pizzute, and M. Pei, *A review of crosstalk between MAPK and Wnt signals and its impact on cartilage regeneration.* Cell and tissue research, 2014. **358**(3): p. 633-649.
307. Henderson, B.R., *Nuclear-cytoplasmic shuttling of APC regulates β-catenin subcellular localization and turnover.* Nature Cell Biology, 2000. **2**(9): p. 653-660.
308. Henderson, B.R., et al., *Lymphoid Enhancer Factor-1 Blocks Adenomatous Polyposis Coli-mediated Nuclear Export and Degradation of β-Catenin: Regulation by histone deacetylase 1.* Journal of Biological Chemistry, 2002. **277**(27): p. 24258-24264.
309. Krieghoff, E., J. Behrens, and B. Mayr, *Nucleo-cytoplasmic distribution of beta-catenin is regulated by retention.* J Cell Sci, 2006. **119**(Pt 7): p. 1453-63.
310. Salic, A., et al., *Control of β-Catenin Stability: Reconstitution of the Cytoplasmic Steps of the Wnt Pathway in Xenopus Egg Extracts.* Molecular Cell, 2000. **5**(3): p. 523-532.
311. Jho, E.-h., et al., *Wnt/β-Catenin/Tcf Signaling Induces the Transcription of Axin2, a Negative Regulator of the Signaling Pathway.* Molecular and Cellular Biology, 2002. **22**(4): p. 1172-1183.
312. Price, K., et al., *Lithium induces mesenchymal-epithelial differentiation during human kidney development by activation of the Wnt signalling system.* Cell Death Discovery, 2018. **4**.
313. Matsuzaki, S. and C. Darcha, *Involvement of the Wnt/β-Catenin Signaling Pathway in the Cellular and Molecular Mechanisms of Fibrosis in Endometriosis.* PLOS ONE, 2013. **8**(10): p. e76808.
314. Caro, J.J., et al., *The risk of diabetes during olanzapine use compared with risperidone use: a retrospective database analysis.* J Clin Psychiatry, 2002. **63**(12): p. 1135-9.
315. Arous, C. and P.A. Halban, *The skeleton in the closet: actin cytoskeletal remodeling in β-cell function.* American Journal of Physiology-Endocrinology and Metabolism, 2015. **309**(7): p. E611-E620.
316. Zhu, X., et al., *Microtubules Negatively Regulate Insulin Secretion in Pancreatic β Cells.* Developmental Cell, 2015. **34**(6): p. 656-668.
317. Röder, P.V., et al., *Molecular regulation of insulin granule biogenesis and exocytosis.* Biochemical Journal, 2016. **473**(18): p. 2737-2756.
318. Hodgkin, M.N., G.J. Rogers, and P.E. Squires, *Colocalization Between β-Catenin and Insulin Suggests a Novel Role for the Adherens Junction in β-Cell Function.* Pancreas, 2007. **34**(1): p. 170-171.
319. Schinner, S., et al., *Regulation of insulin secretion, glucokinase gene transcription and beta cell proliferation by adipocyte-derived Wnt signalling molecules.* Diabetologia, 2008. **51**(1): p. 147-154.
320. Fang, D., et al., *Phosphorylation of β-catenin by AKT promotes β-catenin transcriptional activity.* Journal of Biological Chemistry, 2007. **282**(15): p. 11221-11229.
321. Huang, J., et al., *Activation of Wnt/β-catenin signalling via GSK3 inhibitors direct differentiation of human adipose stem cells into functional hepatocytes.* Scientific Reports, 2017. **7**(1): p. 40716.
322. Bamji, S.X., et al., *Role of β-Catenin in Synaptic Vesicle Localization and Presynaptic Assembly.* Neuron, 2003. **40**(4): p. 719-731.

323. Schweizer, F.E. and T.A. Ryan, *The synaptic vesicle: cycle of exocytosis and endocytosis.* Current opinion in neurobiology, 2006. **16**(3): p. 298-304.
324. Satin, L. and D. Cook, *Voltage-gated Ca 2+ current in pancreatic B-cells.* Pflügers Archiv, 1985. **404**(4): p. 385-387.
325. Rorsman, P., et al., *The Cell Physiology of Biphasic Insulin Secretion.* Physiology, 2000. **15**(2): p. 72-77.
326. Rizzoli, S.O. and W.J. Betz, *Synaptic vesicle pools.* Nature Reviews Neuroscience, 2005. **6**(1): p. 57-69.
327. Daniel, S., et al., *Identification of the docked granule pool responsible for the first phase of glucose-stimulated insulin secretion.* Diabetes, 1999. **48**(9): p. 1686-90.
328. Hou, J.C., L. Min, and J.E. Pessin, *Insulin granule biogenesis, trafficking and exocytosis.* Vitamins and hormones, 2009. **80**: p. 473-506.
329. Bamji, S.X., et al., *BDNF mobilizes synaptic vesicles and enhances synapse formation by disrupting cadherin-beta-catenin interactions.* J Cell Biol, 2006. **174**(2): p. 289-99.
330. Ciani, L., et al., *Wnt signalling tunes neurotransmitter release by directly targeting Synaptotagmin-1.* Nature Communications, 2015. **6**(1): p. 8302.
331. Sun, Y., et al., *Scribble interacts with beta-catenin to localize synaptic vesicles to synapses.* Mol Biol Cell, 2009. **20**(14): p. 3390-400.
332. Alasbahi, R.H. and M.F. Melzig, *Forskolin and derivatives as tools for studying the role of cAMP.* Pharmazie, 2012. **67**(1): p. 5-13.
333. Boneh, A., S. Mandla, and H.S. Tenenhouse, *Phorbol myristate acetate activates protein kinase C, stimulates the phosphorylation of endogenous proteins and inhibits phosphate transport in mouse renal tubules.* Biochim Biophys Acta, 1989. **1012**(3): p. 308-16.
334. Beurel, E., S.F. Grieco, and R.S. Jope, *Glycogen synthase kinase-3 (GSK3): regulation, actions, and diseases.* Pharmacology & therapeutics, 2015. **148**: p. 114-131.
335. Hino, S.-i., et al., *Phosphorylation of β-Catenin by Cyclic AMP-Dependent Protein Kinase Stabilizes β-Catenin through Inhibition of Its Ubiquitination.* Molecular and Cellular Biology, 2005. **25**(20): p. 9063-9072.
336. Liu, H., et al., *Glycogen synthase kinase-3 and mammalian target of rapamycin pathways contribute to DNA synthesis, cell cycle progression, and proliferation in human islets.* Diabetes, 2009. **58**(3): p. 663-672.
337. Daugherty, R.L. and C.J. Gottardi, *Phospho-regulation of β-Catenin Adhesion and Signaling Functions.* Physiology, 2007. **22**(5): p. 303-309.
338. Taurin, S., et al., *Phosphorylation of β-Catenin by Cyclic AMP-dependent Protein Kinase.* Journal of Biological Chemistry, 2006. **281**(15): p. 9971-9976.
339. Nelson, W.J. and R. Nusse, *Convergence of Wnt, beta-catenin, and cadherin pathways.* Science (New York, N.Y.), 2004. **303**(5663): p. 1483-1487.
340. Thorne, C.A., et al., *Small-molecule inhibition of Wnt signaling through activation of casein kinase 1alpha.* Nat Chem Biol, 2010. **6**(11): p. 829-36.
341. Harada, N., et al., *Intestinal polyposis in mice with a dominant stable mutation of the β-catenin gene.* The EMBO Journal, 1999. **18**(21): p. 5931-5942.
342. Lyssenko, V., et al., *Mechanisms by which common variants in the TCF7L2 gene increase risk of type 2 diabetes.* The Journal of Clinical Investigation, 2007. **117**(8): p. 2155-2163.
343. McClenaghan, N., P. Flatt, and A. Ball, *Actions of glucagon-like peptide-1 on K-ATP channel-dependent and -independent effects of glucose, sulphonylureas and nateglinide.* The Journal of endocrinology, 2006. **190**: p. 889-96.

344. Wan, Q.-F., et al., *Protein kinase activation increases insulin secretion by sensitizing the secretory machinery to Ca2+.* The Journal of general physiology, 2004. **124**(6): p. 653-662.
345. Ahuja, D., M.T. Sáenz-Robles, and J.M. Pipas, *SV40 large T antigen targets multiple cellular pathways to elicit cellular transformation.* Oncogene, 2005. **24**(52): p. 7729-7745.
346. Maschio, D.A., et al., *Activation of the Wnt/beta-catenin pathway in pancreatic beta cells during the compensatory islet hyperplasia in prediabetic mice.* Biochem Biophys Res Commun, 2016. **478**(4): p. 1534-40.
347. Laybutt, D.R., et al., *Overexpression of c-Myc in β-Cells of Transgenic Mice Causes Proliferation and Apoptosis, Downregulation of Insulin Gene Expression, and Diabetes.* Diabetes, 2002. **51**(6): p. 1793-1804.
348. Cheung, L., et al., *c-Myc directly induces both impaired insulin secretion and loss of beta-cell mass, independently of hyperglycemia in vivo.* Islets, 2010. **2**(1): p. 37-45.
349. Alimohamad, H., et al., *The effects of antipsychotics on β-catenin, glycogen synthase kinase-3 and dishevelled in the ventral midbrain of rats.* Journal of Neurochemistry, 2005. **95**(2): p. 513-525.
350. Sutton, L.P., et al., *Activation of the canonical Wnt pathway by the antipsychotics haloperidol and clozapine involves dishevelled-3.* Journal of Neurochemistry, 2007. **102**(1): p. 153-169.
351. Sutton, L.P. and W.J. Rushlow, *The dopamine D2 receptor regulates Akt and GSK-3 via Dvl-3.* International Journal of Neuropsychopharmacology, 2012. **15**(7): p. 965-979.
352. Li, X., et al., *In vivo regulation of glycogen synthase kinase-3beta (GSK3beta) by serotonergic activity in mouse brain.* Neuropsychopharmacology, 2004. **29**(8): p. 1426-31.
353. Larriba, M.J., et al., *Vitamin D Is a Multilevel Repressor of Wnt/b-Catenin Signaling in Cancer Cells.* Cancers, 2013. **5**(4): p. 1242-1260.
354. Nagashima, T., et al., *Prevention of antipsychotic-induced hyperglycaemia by vitamin D: a data mining prediction followed by experimental exploration of the molecular mechanism.* Scientific Reports, 2016. **6**: p. 26375.

Publications

Honey M., Scutt G., Bone AJ., Macfarlane WM., Yeoman M., Olanzapine disrupts normal beta cell functioning in MIN6 cells [conference abstract]. *Diabetic Medicine, Volume 35(1),* P44, March 2018.

Honey M., Scutt G., Bone AJ., Macfarlane WM., Yeoman M., Investigating the interaction of the second generation antipsychotic drug olanzapine with the Wnt signalling pathway in MIN6 beta cells [conference abstract]. *Diabetic Medicine, Volume 36(1),* P42, March 2019.

Honey M., Scutt G., Bone AJ., Macfarlane WM., Yeoman M., Olanzapine alters β catenin dynamics in mouse MIN6 cells to reduce glucose stimulated insulin secretion [conference abstract]. British Journal of Pharmacology Volume 177 (11), P2639, Dec 2019