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Regulation of Akt and Wnt signalling by the dopamine D2 receptor and metabotropic glutamate receptor 2/3

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Graduate Program in Anatomy and Cell Biology
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy
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REGULATION OF AKT AND WNT SIGNALLING BY THE DOPAMINE D2
RECEPTOR AND METABOTROPIC GLUTAMATE RECEPTOR 2/3

(Spine title: Regulation of Akt and Wnt signalling by D₂DR and mGluR2/3)

(Thesis Format: Integrated Article)

By

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Graduate Program in Anatomy and Cell Biology

A thesis in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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THE UNIVERSITY OF WESTERN ONTARIO
SCHOOL OF GRADUATE AND POSTDOCTORAL STUDIES

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entitled:

**Regulation of Akt and Wnt signalling by the dopamine D2 receptor and
metabotropic glutamate receptor 2/3**

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ABSTRACT

Akt and the Wnt pathway, two cascades that regulate GSK-3, have been implicated in schizophrenia and antipsychotic drug action. Although it is known that antipsychotic drugs alleviate psychosis by blocking the dopamine D₂ receptor (D₂DR) and that metabotropic glutamate receptor 2/3 (mGluR2/3) agonists may improve some of the symptoms of schizophrenia, it is unclear if both classes of drugs exert their effects through Akt, GSK-3 and/or the Wnt pathway or if changes in these pathways are mediated through the D₂DR and mGluR2/3 respectively. In addition to antipsychotics, mood stabilizers and antidepressants also target GSK-3, suggesting that there must be something unique in the way GSK-3 is targeted by antipsychotics since neither mood stabilizers nor antidepressants alleviate psychosis. The current study examined whether Akt and the Wnt pathway are regulated by the D₂DR and mGluR2/3 and investigated the role of Akt and Dvl-3, a key activator in the Wnt pathway, in regulating GSK-3 in the rat brain. The study also compared the effects of antipsychotic, mood stabilizers and antidepressants on Akt and Wnt pathway proteins to determine if antipsychotics have unique effects on these signalling proteins. Results showed that raclopride (D₂DR antagonist) regulated Akt and the Wnt pathway via Dvl-3 and the response was identical to antipsychotic treatment. Administration of the mGluR2/3 agonist, LY379268 also targeted Akt and the Wnt pathway and induced a similar response as antipsychotics. In addition, repeated amphetamine treatment, an established animal model for the positive symptoms of schizophrenia, quinpirole (D₂DR agonist) and LY341495 (mGluR2/3 antagonist) induced similar changes in Akt and Wnt signalling that parallel alterations reported in schizophrenia. Furthermore, systemic inhibition of GSK-3 was able to

attenuate the increase in locomotion induced by LY341495, a behavioural measure that models the positive symptoms of schizophrenia. The study also showed that clozapine and haloperidol (antipsychotics) induced a common Wnt response that was not mimicked by the mood stabilizers or antidepressants tested but that all neuropsychiatric drugs tested induced changes in Akt. Collectively the data shows that the Wnt pathway is regulated specifically by drugs with antipsychotic properties and may represent a novel target for pharmaceutical intervention.

Keywords: Antipsychotic drugs, schizophrenia, dopamine D2 receptor, metabotropic glutamate receptor 2/3, Wnt pathway, Akt pathway, Glycogen synthase kinase-3

CO-AUTHORSHIP

Chapter 2 entitled “Alterations in GSK-3 and Akt signalling induced by the dopamine D2 receptor are mediated by Dvl-3” was written by L. Sutton with input from Dr. Rushlow and all studies were performed by L. Sutton. Chapter 3 entitled “The effect of neuropsychiatric drugs and amphetamine on GSK- 3 related signalling” was written by L. Sutton with input from Dr. Rushlow and all studies were performed by L. Sutton. Chapter 4 entitled “Regulation of Akt and Wnt signalling by the group II metabotropic glutamate receptor antagonist LY341495 and agonist LY379268” was written by L. Sutton with input from Dr. Rushlow and all studies were performed by L. Sutton.

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TABLE OF CONTENTS

TITLE ii
ABSTRACT..... iii
CO-AUTHORSHIP v
ACKNOWLEDGEMENTS..... vi
TABLE OF CONTENTS..... vii
TABLE OF FIGURES..... xi
LIST OF APPENDICES..... xiii
LIST OF ABBREVIATIONS..... xiv

CHAPTER ONE: Introduction 1

1.1 SCHIZOPHRENIA..... 2

 1.1.1 Epidemiology and clinical description of schizophrenia 2

 1.1.2 Etiology of Schizophrenia..... 3

1.2 THEORIES OF SCHIZOPHRENIA 3

 1.2.1 Dopamine Theory 4

 1.2.2 Dopaminergic Animal Models of Schizophrenia 6

 1.2.3 Glutamate Theory 7

 1.2.4 Glutamatergic Animal Models..... 9

 1.2.5 Connection between dopamine and glutamate 10

1.3 PREFRONTAL CORTEX..... 11

 1.3.1 Anatomy of the PFC 11

 1.3.2 Abnormalities in the PFC..... 14

1.4 STRIATUM..... 17

 1.4.1 Anatomy of the striatum 17

 1.4.2 Abnormalities in the STR 18

1.5 ANTIPSYCHOTIC DRUGS 19

 1.5.1 Clinical outcome of antipsychotic drugs..... 19

 1.5.2 Mechanism of action..... 20

1.6 GSK-3 SIGNALLING 21

 1.6.1 Regulation of GSK-3 21

1.6.2 Akt-GSK-3 Pathway	22
1.6.3 The Wnt Pathway.....	25
1.7 GSK-3, AKT AND THE WNT PATHWAY IN SCHIZOPHRENIA	29
1.7.1 GSK-3 and Schizophrenia.....	29
1.7.2 Akt and Schizophrenia.....	29
1.7.3 Wnt and Schizophrenia.....	31
1.8 mGLUR2/3 AGONISTS.....	32
1.9 OVERVIEW	33
1.10 OBJECTIVES AND RATIONALE	34
1.10.1 Alterations in GSK-3 and Akt signalling induced by the D ₂ DR are mediated by Dvl-3.....	34
1.10.2 The effect of neuropsychiatric drugs and amphetamine on GSK-3 related signalling.....	35
1.10.3 Regulation of Akt and Wnt signalling by the group II metabotropic glutamate receptor antagonist LY341495 and agonist LY379268	36
1.11 REFERENCE LIST	38
CHAPTER TWO: Alterations in GSK-3 and Akt signalling induced by the D₂DR are mediated by Dvl-3.....	57
2.1 INTRODUCTION	58
2.2 MATERIAL AND METHODS.....	59
2.2.1 Animal treatment	59
2.2.2 Cell culture.....	60
2.2.3 Retroviral infections and transfections	60
2.2.4 PI3-K inhibitor treatments of cell cultures.....	61
2.2.5 Protein isolation and western blotting.....	61
2.2.6 Co-Immunoprecipitations	62
2.3 RESULTS	63
2.3.1 Regulation of Akt and Wnt pathway proteins by D ₁ DR, D ₂ DR and D ₃ DR.....	63
2.3.2 The effects of Akt on GSK-3 and β-catenin	71
2.3.3 The effects of Dvl-3 on Akt, GSK-3 and β-catenin levels	74
2.3.4 D ₂ DR association with GSK-3.....	80

2.4 DISCUSSION	83
2.5 REFERENCES	87
CHAPTER THREE: The effect of neuropsychiatric drugs and amphetamine on GSK-3 related signalling	92
3.1 INTRODUCTION	93
3.2 MATERIAL AND METHODS	95
3.2.1 Drug Paradigm	95
3.2.2 Western blotting.....	96
3.2.3 Co-Immunoprecipitations	97
3.3 RESULTS	97
3.3.1 Differential effects of haloperidol and clozapine in the PFC and STR	97
3.3.2 The effects of mood stabilizers and antidepressants on Akt and the Wnt pathway	102
3.3.3 The association between the D ₂ DR and GSK-3 α/β	107
3.3.4 The effects of amphetamine on Akt and the Wnt pathway.....	110
3.4 DISCUSSION	113
3.5 REFERENCE LIST	120
CHAPTER FOUR: Regulation of Akt and Wnt signalling by the group II metabotropic glutamate receptor antagonist LY341495 and agonist LY379268	124
4.1 INTRODUCTION	125
4.2 MATERIALS AND METHODS.....	126
4.2.1 Drug Paradigm	126
4.2.2 Western blotting.....	127
4.2.3 Co-Immunoprecipitations	127
4.2.4 Locomotor activity.....	128
4.3 RESULTS	129
4.3.1 The effects of mGluR2/3 agonism (LY379268) on Akt and Wnt pathway proteins	129
4.3.2 The effects of mGluR2/3 antagonism (LY341495) on Akt and Wnt pathway proteins	134

4.3.3 Interactions between mGluR2/3 and Akt and Wnt pathway proteins.....	134
4.3.4 Effects of LY341495 on locomotion	142
4.4 DISCUSSION	145
4.4.1 mGluR2/3 mediates changes in Wnt and Akt signalling.....	145
4.4.2 LY341495 and Behavior.....	148
4.4.3 Comparison of LY379268 with antipsychotics	149
4.5 REFERENCE LIST	151
CHAPTER FIVE: GENERAL DISCUSSION.....	155
5.1 Summary of Results and Conclusions	156
5.1.1 Alterations in GSK-3 and Akt signalling induced by the dopamine D2 receptor are mediated by Dvl-3.....	156
5.1.2 The effects of neuropsychiatric drugs and amphetamine on GSK- 3 signalling.....	157
5.1.3 Regulation of Akt and Wnt signalling by the group II metabotropic glutamate receptor antagonist LY341495 and agonist LY379268	158
5.2 Discussion	158
5.3 Limitations of the Model and future studies	164
5.4 REFERENCE LIST	168
APPENDIX A.....	172
CURRICULUM VITA	174

TABLE OF FIGURES

Figure 1.1: Prefrontal cortex of the human and rat.....	12
Figure 1.2: The cytoarchitectural features in the PFC of schizophrenia.....	15
Figure 1.3: Schematic representation of Akt/GSK3 signalling	23
Figure 1.4: Schematic representation of canonical Wnt signalling	26
Figure 2.1: The effects of D ₁ DR, D ₂ DR and D ₃ DR antagonism on total protein levels and/or phosphorylation state of Akt and Wnt pathway proteins.....	64
Figure 2.2: The effects of D ₂ DR agonism on total protein levels and/or phosphorylation state of Akt and Wnt pathway proteins.....	67
Figure 2.3: Co-IP experiments showing the association between D ₂ DR and Akt or GSK-3 α/β in drug naïve rats, raclopride or quinpirole treated rats.....	69
Figure 2.4: The effect of a constitutively active Akt construct or Akt inhibitors, wortmannin and LY294002 on GSK-3 and β -catenin in SH-SY5Y cells	72
Figure 2.5: The effects of reducing Akt-1 protein levels <i>in vitro</i> and <i>in vivo</i> on Akt, Dvl-3, GSK-3 and β -catenin using siRNA or transgenic mice, respectively..	75
Figure 2.6: Changes in Akt, Dvl, GSK-3 and β -catenin following the overexpression or knockdown of Dvl-3 in SH-SY5Y cells.....	78
Figure 2.7: Co-IP experiments examining the association between D ₂ DR and GSK-3 α/β in native SH-SY5Y cells or following viral overexpression of Dvl-3 or transfection with a constitutively active Akt construct.....	81
Figure 3.1: Changes in the total protein levels and/or phosphorylation state of Wnt pathway proteins and Akt in the PFC of rats treated with haloperidol or clozapine.....	98
Figure 3.2: Changes in the total protein levels and/or phosphorylation state of Wnt pathway proteins and Akt in the STR of rats treated with haloperidol or clozapine.....	100
Figure 3.3: Changes in the total protein levels and/or phosphorylation state of Wnt pathway proteins and Akt in the PFC and STR of rats treated with lithium or valproic acid.....	103

Figure 3.4: Changes in the total protein levels and/or phosphorylation state of Wnt pathway proteins and Akt in the STR of rats treated with fluoxetine or imipramine..	105
Figure 3.5: Co-IP experiments showing the association between D ₂ DR and Akt, GSK-3 and/or Dvl-3 in the PFC of haloperidol, clozapine, lithium and valproic acid treated rats	108
Figure 3.6: Changes in the total protein levels and/or phosphorylation state of Wnt pathway proteins and Akt in the PFC and STR of rats treated with amphetamine	111
Figure 3.7: Co-IP experiments showing the association of between D ₂ DR and Akt and GSK-3 in the PFC of rats treated with amphetamine	114
Figure 4.1: The effects of repeated administration of LY379268 on Wnt pathway proteins and Akt	130
Figure 4.2: The effects of acute administration of LY379268 on Wnt pathway proteins and Akt	132
Figure 4.3: The effects of repeated administration of LY341495 on Wnt pathway proteins and Akt	135
Figure 4.4: The effects of acute administration of LY341495 on Wnt pathway proteins and Akt	137
Figure 4.5: Co-Immunoprecipitation experiments showing association between mGluR2/3 and Wnt pathway proteins and/or Akt in drug naïve rats or following LY379268 treatment	140
Figure 4.6: Total horizontal locomotor activity for rats treated acutely or repeatedly with LY341495 or LY341495 and SB216763	143
Figure 5.1: Working model of antipsychotics and mGluR2/3 agonist regulation of Akt and Wnt signalling	160

LIST OF APPENDICES

APPENDIX A: UWO Council of Animal Care - Animal Protocol Approval..... 170

LIST OF ABBREVIATIONS

ACd - dorsal anterior cingulated cortex

ACv - ventral anterior cingulated cortex

AId - dorsal agranular insular cortex

AIV - ventral agranular insular cortex

APC - adenomatous polyposis coli

CCAC - Canadian Council on Animal Care

Co-IP - co-immunoprecipitation

COMT - catechol-O-methyl transferase

D₁DR - dopamine D1 receptor

D₂DR - dopamine D2 receptor

D₃DR - dopamine D3 receptor

D₄DR - dopamine D4 receptor

D₅DR - dopamine D5 receptor

DA - dopamine

DAT - dopamine transporter

DAT-KO - dopamine transporter knockout mice

DISC1 - Disrupted-in-schizophrenia 1

DKK - Dickkopf

DMEM - Dulbecco's Modified Eagle Medium

DSM-IV - Diagnostic and Statistical Manual of Mental Disorders volume IV

Dvl - dishevelled

EPS - extrapyramidal symptoms

FBS - fetal bovine serum

FR2 - frontal area 2

FST - force swim test

Fz - Frizzled receptor

GABA - γ -aminobutyric acid

GPCR - G-protein coupled receptor

GSK-3 - glycogen synthase kinase-3

HZ - heterozygous

i.m. - intermuscular

IL - infralimbic cortex

IP - immunoprecipitation

KO - knockout

LEF - lymphoid enhancer factor

LO - lateral orbital cortex

LRP5/6 - low-density lipoprotein receptor-related protein 5/6

mGluR - metabotropic glutamate receptor

MAPK - mitogen-activated protein kinase

MO - medial orbital cortex

mTOR - mammalian target of rapamycin

NMDA - N-methyl d-aspartate

p - phosphorylated

PCP - phencyclidine

PCP Pathway - planar cell polarity pathway

PDK1 - 3-phosphoinositol-dependent kinase 1

PFC - prefrontal cortex

PI3-K - phosphoinositide 3-kinase

PIP₂ - phosphatidylinositol-4,5-biphosphate

PIP₃ - phosphatidylinositol-3, 4, 5-triphosphate

PKA - protein kinase A

PKB - protein kinase B

PKC - protein kinase C

PL - prelimbic cortex

PP2A - protein phosphatase 2

PPI - prepulse inhibition

RGS4 - regulator of G-protein signalling 4

s.c. - subcutaneous

Ser - serine

SNAP-25 - synaptosomal-associated protein 25

STR - striatum

TCF - T-cell factor

Thr - Threonine

VTA - ventral tegmental area

WB - western blotting

WM – white matter

WT - wild type

CHAPTER ONE
INTRODUCTION

1.1 SCHIZOPHRENIA

1.1.1 Epidemiology and clinical description of schizophrenia

Schizophrenia is a chronic debilitating mental disorder afflicting 1% of the population worldwide¹. The incidence rate of schizophrenia is similar throughout the world and independent of race, gender and social status^{1, 2}. Schizophrenia tends to emerge during late adolescence or early adulthood and usually persists throughout life³. There are currently no biological markers for schizophrenia and diagnosis relies on the presentation of clinical symptoms as defined by the Diagnostic and Statistical Manual of Mental Disorders volume IV (DSM-IV). According to the DSM-IV, schizophrenia is characterized by three broad symptom clusters; positive symptoms, negative symptoms and cognitive impairments. Positive symptoms are behaviours apparent in schizophrenia patients but are absent in healthy unaffected individuals and include hallucinations and delusions. Negative symptoms are deficit states in which basic emotional and behavioural processes are diminished or absent. Negative symptoms include blunted affect, anhedonia (lack of pleasure), avolition (lack of motivation) or social withdrawal. Cognitive impairments include deficits in attention, memory, learning and executive functioning.

Given the diversity of symptoms, schizophrenia impacts almost every aspect of life, ranging from difficulties in obtaining and holding competitive full-time employment to meeting basic living needs. Thus, there is a substantial financial burden associated with caring for patients with schizophrenia (i.e. health care cost, lost of productivity etc.). In 1996, it was reported that the estimated total financial burden for schizophrenia in

Canada was \$3 billion dollars⁴. Therefore, improving functioning remains the critical challenge for the management of schizophrenia.

1.1.2 Etiology of Schizophrenia

Schizophrenia is a complex illness and both genetic and environmental factors play a role in the etiology of schizophrenia. Rates of schizophrenia are higher among relatives of patients than in the general population suggesting there is a heritable component to schizophrenia⁵. Genetic risk increases with each affected relative, reaching nearly 50% when both parents are affected⁶ and 70% when a monozygotic twin is affected^{7, 8}. However, a single gene is not responsible for schizophrenia and schizophrenia does not follow a single gene inheritance pattern. Rather, several genes or clusters of genes contribute to the genetic vulnerability of an individual⁹. In addition, some environmental factors, have been identified that can contribute to an increased risk of schizophrenia¹⁰. For example, prenatal exposure to the influenza virus, low prenatal vitamin D, obstetrical complications (hypoxia/ischemia), exposure to adverse intrauterine events, cannabis use and repeated psychological stress have been identified as risk factors for the development of schizophrenia¹¹⁻¹⁷.

1.2 THEORIES OF SCHIZOPHRENIA

Although the etiology of schizophrenia is unknown, there are numerous theories that have been proposed to explain the cause and progression of schizophrenia. However, based on biological evidence, the dopamine (DA) and glutamate theories of schizophrenia remain the most dominant theories.

1.2.1 Dopamine Theory

Dopamine

DA is the predominant catecholamine neurotransmitter in the mammalian brain, where it regulates a variety of functions including locomotor activity, cognition, emotion and endocrine function¹⁸. At the cellular level, two classes of 7-transmembrane receptors that couple to heterotrimeric G binding proteins mediate the various physiological functions regulated by DA. The D1-class DA receptors are composed of D₁DR and D₅DR and are mostly coupled to G_{sα}, whereas D2-class DA receptors, D₂DR, D₃DR and D₄DR are coupled to Gi/Go¹⁸. DA receptors regulate a variety of signalling pathways, including protein kinase A (PKA), protein kinase C (PKC), mitogen-activated protein kinase (MAPK) and calcium channels.

There are four major dopaminergic pathways in the brain: mesolimbic, mesocortical, nigrostriatal and tuberoinfundibular pathways. The mesolimbic and mesocortical pathways have been associated with the positive and negative symptoms of schizophrenia. Dopaminergic neurons of the mesolimbic system originate in the ventral tegmental area (VTA) and project to the components of the limbic system such as the nucleus accumbens, amygdala, hippocampus and the medial prefrontal cortex. The mesocortical dopaminergic system also originates in the VTA and project to the frontal cortex, in particular to the prefrontal cortex.

Dopamine hypothesis

The DA hypothesis proposes that symptoms of schizophrenia are due to abnormal dopaminergic functioning¹⁹. The hypothesis originated with the discovery that all

antipsychotics, drugs used for the treatment of schizophrenia, are D₂DR antagonists²⁰⁻²². Reserpine, a drug that leads to the depletion of DA from the synapse was also found to be effective for treating psychosis²³. Further evidence was provided by studies using amphetamine, an indirect DA agonist, showing that amphetamine induces psychotic symptoms indistinguishable from the positive symptoms of schizophrenia in otherwise normal humans²⁴. Furthermore, use of amphetamine by schizophrenia patients often causes a relapse of psychosis and/or exacerbates the positive symptoms²⁵. Numerous studies have also investigated DA functioning in patients with schizophrenia and elevated presynaptic striatal DA has been consistently reported²⁶⁻²⁸. Meta-analyses have shown there is a slight elevation in striatal D₂DR/D₃DR density in schizophrenic patients independent of the effects of antipsychotic drugs²⁹⁻³¹. However, it has been suggested that a change in the functional state of the D₂DR may be more important for the symptoms of schizophrenia than changes in receptor density³². Recent studies have shown that the D₂DR can exist in a state of low (D₂^{low}) or high (D₂^{high}) affinity for DA, with D₂^{high} being the active state^{33, 34}. Patients with schizophrenia are supersensitive to dopamine^{24, 35}, which correlates with elevated levels of D₂ receptors in the D₂^{high} state³⁶.

Given the importance of DA and D₂DR in schizophrenia and antipsychotic drug action, numerous genetic studies have investigated elements of the dopamine system, including the DA transporter (DAT) and DA receptors, as possible susceptibility genes. Several studies have shown that DAT gene variants do not contribute to the etiology of schizophrenia³⁷⁻³⁹. This finding is consistent with imaging studies that show a normal distribution of DAT in the striatum^{40, 41}. Data for the D₂DR is conflicting but there appears to be no strong association between D₂DR gene variants and schizophrenia^{42, 43}.

Studies involving other dopamine receptors have also been conducted but do not appear to be associated with schizophrenia⁴⁴⁻⁴⁷. Given the lack of association between dopamine receptors and schizophrenia, studies have now begun to explore abnormalities in dopaminergic intracellular signalling pathways. Genetic studies have identified genes involved in dopaminergic pathways such as Akt-1⁴⁸⁻⁵³, disrupted-in-schizophrenia 1 (DISC1)⁵⁴, catechol-O-methyl transferase (COMT)⁵⁵⁻⁵⁸ and regulator of G-protein signalling 4 (RGS4)⁵⁹⁻⁶² as possible susceptibility genes for schizophrenia.

1.2.2 Dopaminergic Animal Models of Schizophrenia

Based upon the dopamine theory of schizophrenia several hyperdopaminergic animal models have been developed including the amphetamine-sensitization model and the dopamine transporter knockout (DAT-KO) mouse. The amphetamine-sensitization model is a well-established hyperdopaminergic animal model of schizophrenia⁶³. Amphetamine-sensitization is the consequence of repeated exposure to amphetamine, resulting in enhanced neurochemical and behavioural responses to a subsequent amphetamine challenge. More importantly, amphetamine-sensitization shares several key neurochemical and behavioural similarities to schizophrenia⁶⁴. For example, amphetamine treated rats show increased striatal dopamine release and have heightened displacement of raclopride binding to D₂DR following an amphetamine challenge, suggesting that a greater portion of D₂DR are occupied by DA^{65, 66}. Amphetamine-sensitization leads to dopamine supersensitivity and increase in D₂^{high} receptors⁶⁷. In addition, amphetamine-sensitized animals exhibit hyperlocomotor activity, a behaviour representative of the positive symptoms of schizophrenia in humans^{66, 68, 69}. In addition,

reductions in prepulse inhibition (PPI) are observed in amphetamine-sensitized animals^{66, 68, 69}. PPI is a phenomenon whereby a weaker prestimulus attenuates a subsequent startling stimulus and is a measure of sensorimotor gating⁷⁰. Deficits in PPI and sensorimotor gating have been reported in patients with schizophrenia⁷⁰⁻⁷². Treatment with antipsychotic drugs attenuates hyperlocomotor activity and reverses the deficits in PPI in amphetamine-sensitized rats^{69, 73}. Thus, the amphetamine-sensitization model also has predictive validity for antipsychotic efficacy. In addition, amphetamine sensitized rats show impairments in tasks that require sustained attention such as the operant signal detection task⁷⁴ and the 5 choice serial reaction time task⁷⁵, consistent with the attention deficits observed in schizophrenic patients. However, it appears working memory and long-term memory are not affected by amphetamine sensitization, even though impairments in memory are observed in patients with schizophrenia^{76, 77}. Therefore, amphetamine sensitization does not model all aspects of schizophrenia but is a good model for investigating the positive symptoms of schizophrenia.

1.2.3 Glutamate Theory

Glutamate Receptors

L-glutamate is the major excitatory neurotransmitter in the mammalian central nervous system binding to both ligand-gated ion channels and G-protein coupled receptors (GPCR)⁷⁸. N-methyl d-aspartate (NMDA) receptors are heteromeric ionotropic glutamate receptors composed of a NR1 subunit in combination with NR2 and/or NR3 subunits. There are eight different NR1 subunits generated by alternative splicing, four NR2 subunits (A, B, C and D) and two NR3 subunits (A and B). NMDA receptors

permit the movement of calcium across the post-synaptic membrane, thereby activating second messenger systems. Metabotropic glutamate receptors (mGluR) are GPCR and are divided into 3 groups based on sequence, pharmacology and G-protein coupling: Group I (mGluR1 and mGluR5), Group II (mGluR2 and mGluR3) and Group III (mGluR4, mGluR6, mGluR7 and mGluR8)⁷⁹. mGluR can regulate several different signalling pathways, including PKA and MAPK.

Glutamate theory

The glutamate theory postulates that abnormalities in glutamatergic neurotransmission are responsible for the symptoms of schizophrenia⁸⁰. The glutamate hypothesis of schizophrenia originated with the clinical observation that phencyclidine (PCP) and ketamine, non-competitive NMDA receptor antagonists, induce schizophrenia-like psychosis, emotional blunting and working memory disturbances in humans⁸¹⁻⁸⁴. Furthermore, PCP can exacerbate psychosis and cognitive impairments in patients with schizophrenia⁸⁵⁻⁸⁷. Genetic studies have investigated NMDA subunits as possible susceptibility genes for schizophrenia and have implicated NR2A (GRIN2A) and NR2B (GRIN2B) as candidate genes for schizophrenia^{88, 89}. Postmortem studies have revealed regional and subunit specific alterations in the expression of NMDA receptor subunits. For example, increases in the NR2A subunit were found in the occipital cortex⁹⁰, whereas no change in expression was found in the hippocampus or thalamus of schizophrenic patients⁹¹. For NR2B, increases in the mRNA levels were reported in the hippocampus and thalamus^{92, 93} but not in the prefrontal or occipital cortex of schizophrenic patients⁹⁰.

In addition to NMDA, evidence implicating mGluR in schizophrenia has emerged. For example, mGlu3R and mGlu5R have been identified as possible schizophrenia susceptibility genes⁹⁴⁻⁹⁶. However, there appears to be no significant change in the expression of mGluR5 mRNA in the prefrontal cortex, hippocampus or striatum of schizophrenic patients⁹⁷⁻⁹⁹. Several post-mortem studies in schizophrenic patients have also reported no significant changes in mGluR3 mRNA and protein levels or in the immunoreactivity of mGluR2/3^{98, 100-102}. However, a decrease in the dimer form of mGluR3 has been reported in the prefrontal cortex of schizophrenic patients¹⁰³. Furthermore, it has been shown that mGluR2/3 agonists have antipsychotic-like properties. The mGluR2/3 agonist, LY354740 normalized cognitive deficits induced by ketamine in humans¹⁰⁴. A preclinical study has shown that the mGluR2/3 agonist, LY2140023 (prodrug of LY404039) led to improvements in both positive and negative symptoms of schizophrenia compared to placebo¹⁰⁵.

1.2.4 Glutamatergic Animal Models

Based upon the schizophrenic-like symptoms NMDA receptor antagonists induce in humans, PCP and ketamine animal models have been developed. The PCP model is the most well-studied and accepted glutamatergic animal model of schizophrenia¹⁰⁶. It has been shown that PCP treated rats exhibit hyperlocomotion and deficits in PPI^{107, 108} and that a variety of different antipsychotics can attenuate these behaviours¹⁰⁹. PCP treated rats also exhibit deficits in social interaction^{110, 111} and impairments in working memory⁷⁶. Overall, PCP treated rats are thought to model various negative symptoms and cognitive deficits, as well as the positive symptoms of schizophrenia. However,

targeting NMDA receptors for pharmaceutical purposes is problematic. Attempts to develop therapeutic agents directed at NMDA receptors have been unsuccessful due to adverse effects including memory impairments and neurotoxicity^{112, 113}. A more gentle approach was to target the glycine modulation site on the NMDA receptor. Glycine is a co-agonist necessary for NMDA receptor activation. However, clinical trials of glycine or glycine derivatives failed to consistently demonstrate therapeutic benefits¹¹⁴⁻¹¹⁶.

Animal models targeting the mGluR2/3 have also been created, given recent findings concerning mGlu3R in schizophrenia. Although data is still limited, treatment with mGluR2/3 antagonists, LY341495 or LY366457 causes increases in locomotor activity that can be attenuated by mGluR2/3 agonists and antipsychotic drugs¹¹⁵. However, unlike amphetamine, increases in locomotor activity are thought to be due to impairments in habituation^{117, 118}, a process that is impaired in patients with schizophrenia¹¹⁹. Thus, targeting mGluR2/3 may provide valuable information about the role of mGluR2/3 in the manifestation of symptoms associated with schizophrenia.

1.2.5 Connection between dopamine and glutamate

Although, most descriptions of the dopamine and glutamate theories of schizophrenia centre solely around DA or glutamate respectively, it is important to note that these neurotransmitter systems are in fact extensively interconnected. Both the DA and glutamate systems interact in several brain regions relevant to schizophrenia, including the prefrontal cortex (PFC), midbrain and striatum¹²⁰ and work together to regulate normal cortical activity related to perception and cognition^{121, 122}. In the PFC, different types of DA and glutamate receptors are localized on the pyramidal neurons and

dopaminergic neurons from the VTA project to the glutamate-containing pyramidal cells in the PFC. Activation of DA receptors in the PFC are known to modulate glutamatergic neuronal activity by providing either an inhibitory^{123, 124} or excitatory¹²⁵ input to pyramidal neurons. More specifically, activation of D₂DR has been reported to inhibit pyramidal cells¹²⁴, while activation of D₁DR has been shown to excite pyramidal cells¹²⁶. Thus, it is important to keep in mind when discussing either the DA or glutamate theories of schizophrenia, that one neurotransmitter system directly affects the other.

1.3 PREFRONTAL CORTEX

1.3.1 Anatomy of the PFC

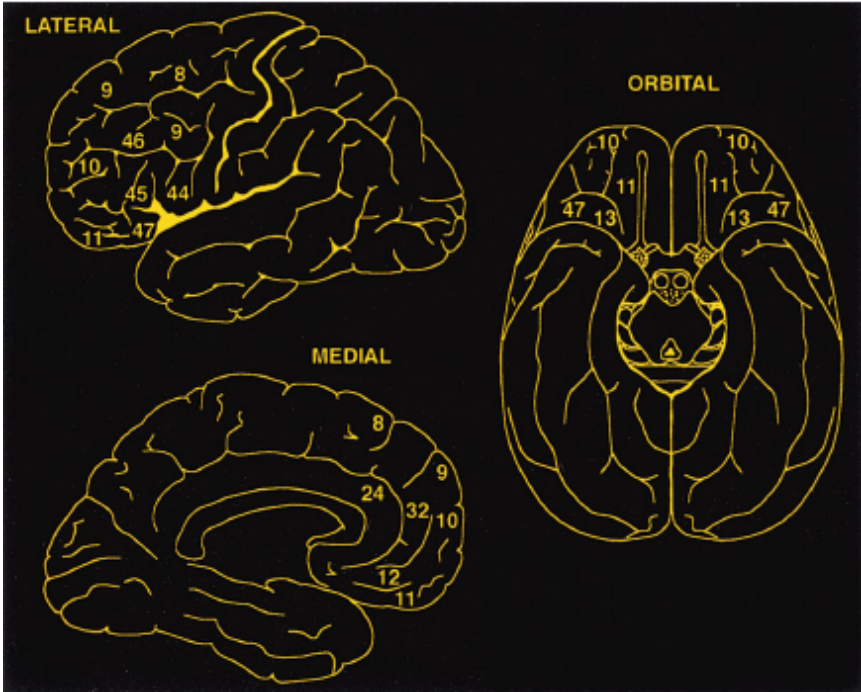
The prefrontal cortex (PFC) is involved in several higher brain functions, such as perception, attention, and memory¹²⁷. The PFC is the association cortex of the frontal lobe and is located anterior to the motor and premotor regions. The anatomical boundary of the PFC is not easily delineated but is defined by its connectivity with the mediodorsal nucleus of the thalamus¹²⁷. According to the cytoarchitectonic map of Brodmann it consists of areas 8-13, 24, 32, 46 and 47 (Figure 1.1a). Cortical neurons of the PFC are arranged in 6 layers, numbering from the pial surface of the cortex to the underlying white matter. Distributed across these layers are different types of glutamatergic pyramidal cells representing ~75% of the cortex. The remaining ~25% of neurons are interneurons and utilize the neurotransmitter γ -aminobutyric acid (GABA).

Anatomical and functional characteristics have been used to identify the PFC in different species. The PFC circuitry in rats, primates and humans share a number of connections, including connections with the thalamus (i.e. mediodorsal nucleus), VTA,

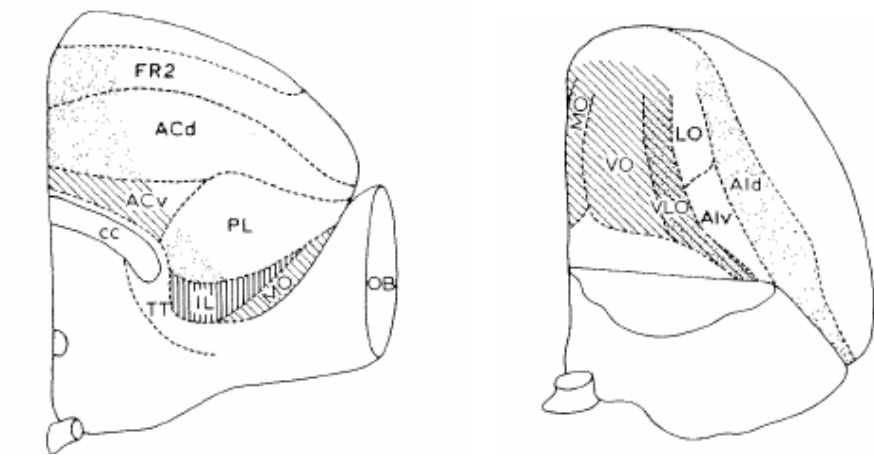
Figure 1.1: Prefrontal cortex of the human and rat.

- A) Lateral, medial and orbital view of the human prefrontal cortex are numbered according to the Brodmann's cytoarchitectonic map. (Adapted from Fuster *et al.*, 1997)
- B) Medial views of the rat prefrontal cortex composed of the frontal area 2 (FR2), dorsal and ventral anterior cingulated cortices (ACd and ACv, respectively), prelimbic cortex (PL) and the infralimbic cortex (IL), medial orbital cortex (MO) dorsal and ventral agranular insular cortices (AId and AIv, respectively) and the lateral orbital cortex (LO). (Adapted from Uylings *et al.*, 1990).

A) Human PFC



B) Rat PFC



basal ganglia and with other cortices¹²⁸. In the rat, the PFC is composed of the medial and lateral PFC (Figure 1.1b)¹²⁸. The medial PFC includes frontal area 2 (FR2), dorsal and ventral anterior cingulate cortices (ACd and ACv, respectively), prelimbic cortex (PL) and the infralimbic cortex (IL) and medial orbital cortex (MO). The lateral PFC is composed of the dorsal and ventral agranular insular cortices (AId and AIV, respectively) and the lateral orbital cortex (LO).

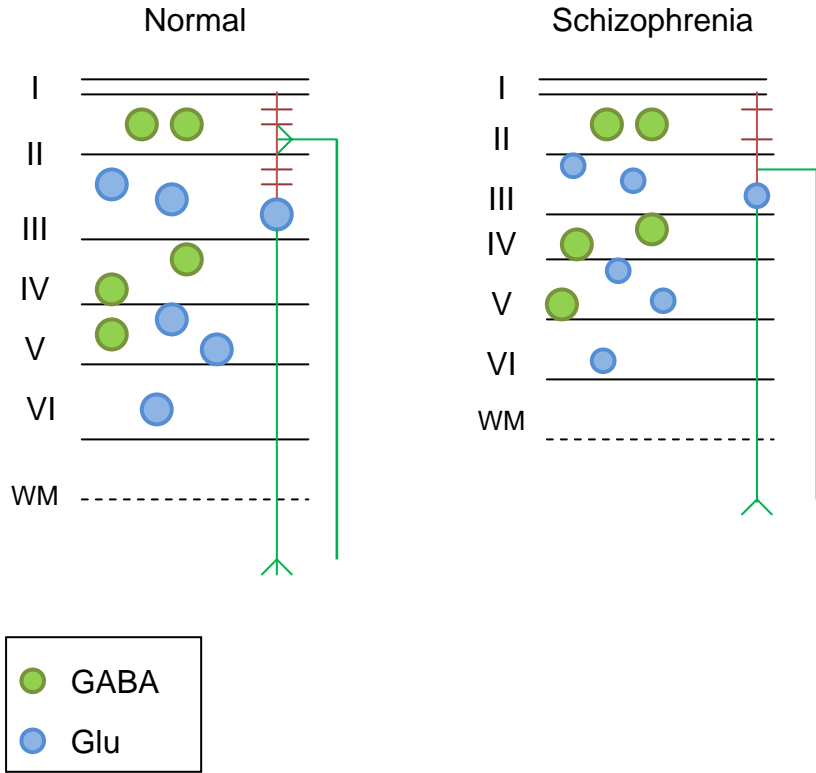
The PFC receives both dopaminergic and glutamatergic afferents. The PFC receives dopaminergic projections from the VTA, whereas glutamatergic afferents from the thalamus and hippocampus also project to the pyramidal neurons. Reciprocal glutamatergic projections from the pyramidal neurons project onto the thalamus and hippocampus. The striatum also receives glutamatergic projections from the PFC.

1.3.2 Abnormalities in the PFC

The PFC is a site of abnormal functioning in schizophrenia¹²⁹ and is also implicated in the action of antipsychotic drugs¹³⁰. Reduced blood flow in the PFC of schizophrenia patients, particularly during working memory tasks has been consistently reported¹³¹⁻¹³³. Several abnormalities in the cytoarchitecture of the PFC in schizophrenic patients have also been described (Figure 1.2). Postmortem studies of the PFC show smaller somal size of pyramidal cells, reduced neuropil and an increase in neuronal density but no change in the cell number¹³⁴. It has also been found that pyramidal neurons, particular in layers II and III have shorter basilar dendrites, lower dendritic spine density and a reduced axonal arbor in schizophrenia¹³⁵⁻¹³⁹. Reduction in the expression of presynaptic markers, including synaptophysin, complexin I and synaptosomal-associated

Figure 1.2: The cytoarchitectural features in the PFC of schizophrenia.

Schematic cartoon of the cytoarchitectural features in the PFC of schizophrenia. There is no change in the number of pyramidal neurons but the pyramidal neurons are smaller and are densely packed. The reduced neuronal size and increase in neuronal density correlates with a reduced neurophil. The cortex is thinner, especially in laminae II and III. There are abnormalities in the axonal (green) and dendritic (red) arborization. Pyramidal neurons have shorter dendritic spines and lower spine density and a reduced axonal arbor.



protein 25 (SNAP-25) in the PFC have been reported¹⁴⁰⁻¹⁴². Abnormalities in the constituents of pre- and postsynaptic markers are also suggestive of impairments in PFC connectivity. Changes in the PFC are not the only finding in schizophrenia, abnormalities have also been found in areas that are anatomically connected to the PFC, including the thalamus, striatum and hippocampus¹⁴³. For example, postmortem studies revealed a reduction in cell number in the mediodorsal thalamic nucleus, the principle source of thalamic projections to the PFC¹⁴⁴. Overall, abnormalities found in the PFC and the general function of the PFC indicates the importance of this brain region in studying schizophrenia.

1.4 STRIATUM

1.4.1 Anatomy of the striatum

The striatum (STR) is involved in motor, cognitive and emotional behaviours. The STR is composed of the caudate and putamen and is the primary input structure of the basal ganglia. The principle neuronal cell type of the STR is the GABAergic medium spiny projection neuron accounting for 90-95% of the neuronal population. Interneurons make up the remaining population of neurons. Interneurons have been identified as large aspiny neurons that utilize the neurotransmitter acetylcholine or medium aspiny neurons, which include neurons that contain somatostatin, neuropeptide Y or calcium-binding protein parvalbumin.

The STR receives both glutamatergic and dopaminergic projections. The glutamatergic inputs arise from all over the cerebral cortex and are topographically organized, with sensory and motor cortices projecting to the putamen and associative

cortex projecting to the caudate and rostral putamen. In addition, the STR receives glutamatergic projections from the amygdala and thalamus. The STR also receives dopaminergic input from the substantia nigra pars compacta (SNc). Projections from the STR participate in both the “direct” and “indirect” pathways of the basal ganglia. In the direct pathway the STR sends projections to the substantia nigra pars reticulata and globus pallidus internal segment, which in turn sends projections to the thalamus. In the indirect pathway, projections from the STR are received by the globus pallidus external segment, which sends projections to the subthalamic nucleus and in turn projects to the globus pallidus internal segment. The globus pallidus internal segments send projections to the thalamus. The thalamus in these circuitries projects back onto the cortex and it is the balance of these opposing pathways that regulates the cortical activity.

1.4.2 Abnormalities in the STR

Several studies have shown alterations in striatal synaptic contacts in schizophrenia. Smaller spines and an increase in the volume of postsynaptic densities have been reported^{145, 146}. In addition, differences in synaptic types have been observed, such as fewer symmetrical synapses in striatal tissue of schizophrenia patients have been documented¹⁴⁷. The STR has often been examined for antipsychotic effects and in animal models of schizophrenia. The STR has an abundance of DA terminals and D₂DR and as such this region has been the focus of several studies involving the dopaminergic animal models amphetamine-sensitization and DAT-KO mice^{63, 148}. In addition, numerous antipsychotic drugs studies have also focused on the STR, as antipsychotic drugs are D₂DR antagonists^{149, 150}.

1.5 ANTIPSYCHOTIC DRUGS

1.5.1 Clinical outcome of antipsychotic drugs

Antipsychotic drugs alleviate the positive symptoms of schizophrenia but have no or a limited effect on the negative or cognitive symptoms of the disease^{151, 152}. There are serious side effects associated with antipsychotic treatment, including sedation, cardiovascular complications, seizures and weight gain which can lead to type II diabetes¹⁵³. Other common side-effects, collectively referred to as extrapyramidal symptoms (EPS), include parkinsonism and tardive dyskinesia. Side effects are often serious and represent the main reason why patients stop treatment¹⁵³.

Patients with schizophrenia show a wide-range of outcomes when treated with antipsychotics. It is estimated that 12-30% of all patients with schizophrenia do not respond adequately to antipsychotic treatments and are classified as treatment-resistant¹⁵⁴. Treatment-resistant is defined as having tried at least three different antipsychotic medications for a 6-8 week period of time with persistent positive symptoms¹⁵⁵. Another problem is that patients can become unresponsive to antipsychotic medication resulting in a relapse of a psychotic episode¹⁵⁶. It is estimated that following a relapse, one in six patients will fail to achieve remission and experience psychotic episodes¹⁵⁷. Given the issues of side-effects, treatment-resistance and unresponsiveness to antipsychotics, there is a clear need to find more effective and safer treatment options for schizophrenia.

Antipsychotic drugs are categorized as either typical (first generation) or atypical (second generation) based on their binding profiles. In general, typical antipsychotics have a higher binding affinity than atypical antipsychotics for the D₂DR¹⁵⁸ and most atypical antipsychotics also bind to serotonin 5-HT₂ receptors^{159, 160}. Initially, it was

thought that atypical antipsychotics (i.e. clozapine, risperidone) would alleviate psychosis and improve negative symptoms and cognitive impairments, making them superior to typical antipsychotics (i.e. haloperidol). However, it has been shown that atypicals have a comparable efficacy to typicals when it comes to alleviating psychosis and also have a limited effect on negative symptoms and cognitive impairments¹⁵². The main difference between these classes of drugs is their side-effect profiles. Atypicals cause less EPS than typicals, whereas atypicals induce metabolic side effects (weight gain, increased triglycerides and cholesterol)^{152, 161}.

1.5.2 Mechanism of action

Although the binding profiles of antipsychotic drugs differ, they all antagonize D₂DR²⁰⁻²² and D₂DR antagonism is essential for the alleviation of psychosis¹⁶². However, beyond the blockade of the D₂DR, the molecular mechanism responsible for the therapeutic effects of antipsychotic drugs remains elusive. There are several intracellular signalling pathways known to be regulated by the D₂DR including protein kinase C (PKC), protein kinase A (PKA) and mitogen-activated protein kinase (MAPK)¹⁶³. However, there is currently no persuasive evidence linking PKC, PKA or MAPK to schizophrenia or antipsychotic drug action¹⁶⁴⁻¹⁶⁶. Recently, it has been shown that the D₂DR regulates glycogen synthase kinase-3 (GSK-3)¹⁶⁷. Furthermore, a variety of different antipsychotics can effect GSK-3¹⁵⁰ and GSK-3 has been implicated in schizophrenia¹⁶⁸.

1.6 GSK-3 SIGNALLING

1.6.1 Regulation of GSK-3

GSK-3 is a ubiquitously expressed serine/threonine protein kinase. There are two isoforms of GSK-3 that are encoded by different genes; GSK-3 α and GSK-3 β ¹⁶⁹. These two isoforms are structurally similar (84% homology) except for an additional glycine-rich extension on the N terminus of GSK-3 α ¹⁷⁰. GSK-3 has over 40 putative substrates that are generally divided into metabolic proteins, signalling proteins, structural proteins and transcription factors¹⁷¹. GSK-3 is involved in numerous signalling cascades and is regulated by distinct mechanisms to avoid indiscriminate phosphorylation of its many substrates. Mechanisms responsible for regulating GSK-3 include phosphorylation, binding with protein complexes and sub-cellular localization. With respect to phosphorylation, GSK-3 is constitutively active and its activity is inhibited by phosphorylation on serine residues (Ser9 of GSK-3 β ; Ser21 of GSK-3 α)¹⁷². GSK-3 is also autophosphorylated on tyrosine residues (Y216 of GSK-3 α ; Y279 of GSK-3 β) resulting in an increase in kinase activity¹⁷³. The ability of GSK-3 to phosphorylate select substrates is also regulated by the protein complexes it forms. For example, GSK-3 in complex with the scaffolding proteins Axin and adenomatous polyposis coli (APC) enables GSK-3 to phosphorylate the substrate, β -catenin¹⁷⁴. If this complex is disrupted, GSK-3 is unable to phosphorylate β -catenin. Lastly, the sub-cellular localization of GSK-3 regulates the availability of potential substrates. GSK-3 is located in the cytoplasm, plasma membrane and nuclear fractions of a cell¹⁷⁵. Activation of particular GSK-3 signalling pathway can result in the translocation of GSK-3 within the cell. For example, activation of the Wnt pathway translocates GSK-3 from the cytoplasm to the

plasma membrane¹⁷⁵. This change in the sub-cellular localization of GSK-3 determines what substrates are available to GSK-3.

Although GSK-3 is involved in several important signalling pathways, participation in the Wnt and Akt signalling cascades is of particular interest, given that these pathways have been implicated in schizophrenia and antipsychotic drug action¹⁷⁶.

1.6.2 Akt-GSK-3 Pathway

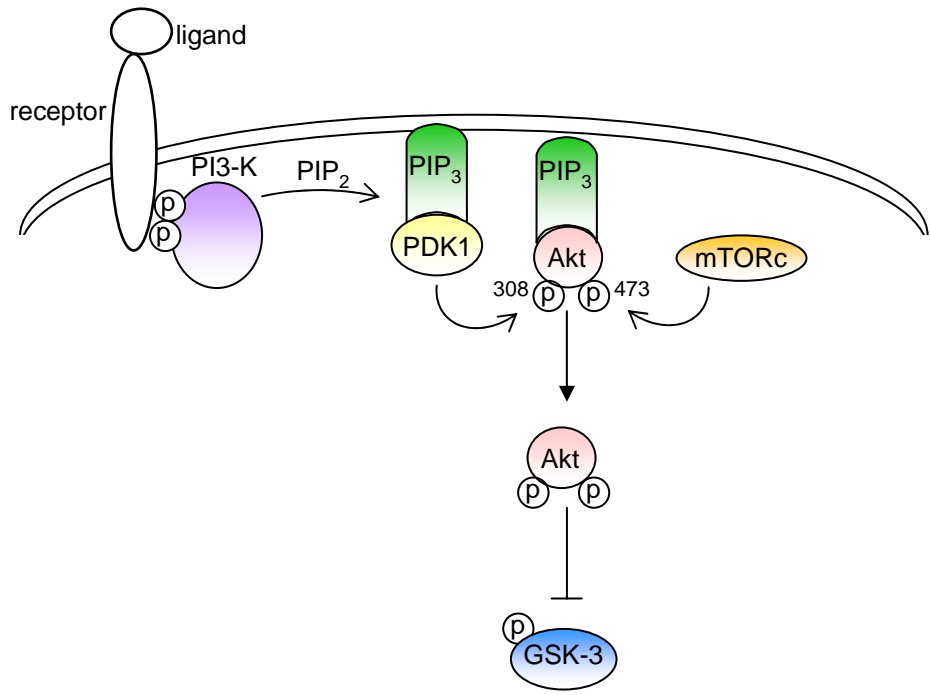
Akt, also known as protein kinase B (PKB) is a serine/threonine protein kinase. There are 3 isoforms of Akt denoted Akt-1, Akt-2 and Akt-3. Akt-1 is ubiquitously expressed. Akt-2 is predominantly expressed in insulin target tissues and Akt-3 is the predominant isoform in the brain.

All three isoforms of Akt are regulated by phosphorylation on Thr308 and Ser473 residues and phosphorylation of these sites results in increased kinase activity. Figure 1.3 depicts the Akt-GSK-3 pathway. Akt is activated in a phosphoinositide 3-kinase (PI3-K) dependent manner through the generation of membrane bound phosphatidylinositol-3, 4, 5-triphosphate (PIP₃). Akt binds to PIP₃ and is then phosphorylated on Thr308 by 3-phosphoinositol-dependent kinase1 (PDK1)^{177, 178} and on Ser473 by mammalian target of rapamycin complex (mTORc)¹⁷⁹. Akt then phosphorylates GSK-3 α/β on its serine residues, inhibiting GSK-3 activity¹⁷².

Recently, it has been shown that dopamine can regulate Akt activity. Administration of apomorphine (non-selective dopamine agonist) or amphetamine decreases phosphorylated Akt and consequently results in a decrease in phosphorylated

Figure 1.3: Schematic representation of Akt-GSK-3 signalling.

Akt is activated in response to phosphoinositide 3-kinase (PI3-K) mediated signalling. PI3-K is phosphorylated and subsequently activated by tyrosine kinase receptors. PI3-K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP₂) generating phosphatidylinositol-3,4,5 triphosphate (PIP₃). Akt is recruited to the plasma membrane by binding to the docking protein PIP₃ and is phosphorylated on two critical residues Thr308 and Ser473. Akt is phosphorylated on the Thr308 by 3-phosphoinositide-dependent kinase (PDK1) and on the Ser473 by mammalian target of rapamycin complex (mTORc). Activated Akt phosphorylates GSK-3.



GSK-3 in the striatum of rats^{148, 180}. The dopamine transporter knockout (DAT-KO) mice exhibit a increase in striatal DA levels resulting in a decrease in phosphorylated Akt and GSK-3 levels^{148, 180}. Furthermore, D₂DR and D₃DR knockout mice show increased phosphorylation of Akt and GSK-3 but this change was not observed in D₁DR knockout mice or mice treated with an antagonist for the D₄DR¹⁶⁷. Thus, Akt-GSK-3 signalling is regulated by dopamine and more specifically D₂DR and D₃DR.

1.6.3 The Wnt Pathway

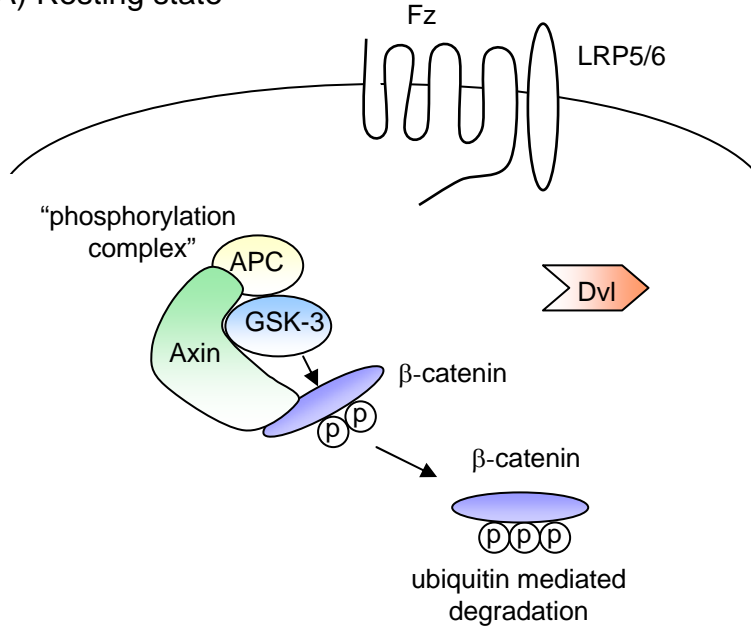
Wnt proteins are secreted glycoproteins that bind to Frizzled (Fz) receptors and the co-receptor low-density lipoprotein receptor-related protein 5/6 (LRP5/6). This event can activate one or more of the 3 distinct branches of the Wnt pathway: (1) the Wnt/Ca²⁺ pathway, (2) the planar cell polarity (PCP) pathway and/or (3) the canonical Wnt pathway. The precise combination of Wnt ligands (19 mammalian members), Fz receptors (10 mammalian members) and the LRP5/6 determines which branch is activated in a manner that is currently not well understood¹⁸¹. The Wnt/Ca²⁺ pathway results in the elevation of intracellular Ca²⁺ levels and subsequent activation of calcium-dependent kinases and phosphatases¹⁸². The PCP pathway involves the JNK cascade and serves to regulate the actin cytoskeleton¹⁸³. The canonical Wnt pathway involves the regulation of GSK-3 and it is the canonical Wnt pathway that has been implicated in schizophrenia and antipsychotic drug action.

For simplicity the canonical Wnt pathway will be referred to as the Wnt pathway throughout the remainder of the thesis. Figure 1.4 depicts the Wnt pathway in its resting state and upon activation. Briefly, when the Wnt pathway is not stimulated (resting state) GSK-3 forms a phosphorylation complex with the cytoplasmic proteins Axin and

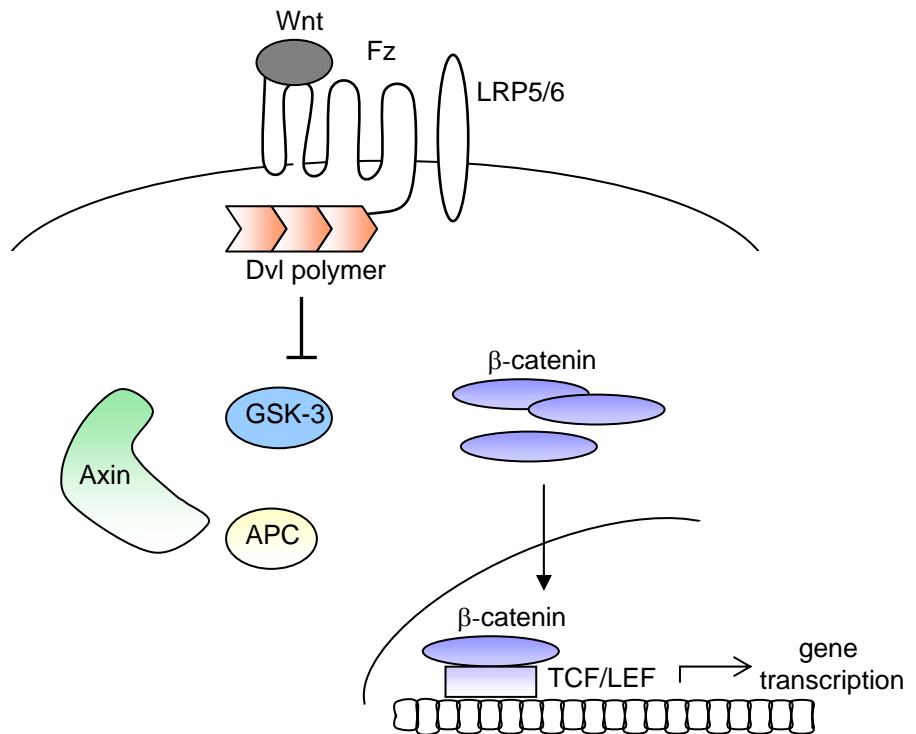
Figure 1.4: Schematic representation of canonical Wnt signalling

- a) Resting State: Under non-stimulated conditions, glycogen synthase kinase-3 (GSK-3), Axin and adenomatous polyposis coli (APC) forms the “phosphorylation complex” facilitating the phosphorylation of β -catenin by GSK-3. Phosphorylated β -catenin is targeted for ubiquitin-mediated degradation by a proteasome.
- b) Activation of the canonical Wnt pathway: The binding of a Wnt ligand to its cognate frizzled receptor (Fz) and low-density lipoprotein receptor-related protein 5/6 (LRP) activates the transducer protein, dishevelled (Dvl). Dvl is recruited to the Fz receptor and self-associates to form a long chain of Dvl proteins referred to as a Dvl polymer. Activation of Dvl leads to the distribution of the “phosphorylation complex”, allowing for the stabilization of β -catenin. Unphosphorylated β -catenin accumulates in the cytoplasm and then translocates to the nucleus where it associates with T-cell factor/lymphocyte enhancer factor (TCF/LEF) to mediate transcription.

A) Resting state



B) Activation of the canonical Wnt pathway



APC^{174, 184}. When complexed with Axin and APC, GSK-3 is able to phosphorylate the transcription factor, β -catenin. Phosphorylation of β -catenin targets it for ubiquitinated degradation by the proteasome¹⁸⁵. Binding of a Wnt ligand to its cognate Fz receptor and LRP activates the Wnt pathway leading to phosphorylation of the signal transducer protein, dishevelled (Dvl). Dvl inhibits GSK-3 by disrupting the GSK-3/Axin/APC phosphorylation complex resulting in the stabilization of β -catenin^{186, 187}. Subsequently, β -catenin translocates to the nucleus, where it interacts with the transcription factors, T-cell factor/lymphoid enhancer factor (TCF/LEF) to regulate the expression of target genes^{188, 189}.

There are three mammalian isoforms of Dvl denoted Dvl-1, Dvl-2 and Dvl-3. All three isoforms are expressed in the brain and all three isoforms regulate GSK-3. The exact mechanism concerning how Dvl interferes with the phosphorylation complex is poorly understood but Dvl possesses three highly conserved binding domains (DIX, DEP and PDZ) allowing for interactions with diverse proteins, including kinases, phosphatases and adaptor proteins^{190, 191}. Upon activation, Dvl is recruited to the plasma membrane¹⁹², which allows Dvl to self-associate via its DIX domain forming a long Dvl polymer chain¹⁹³. These Dvl polymers serve as scaffolds for the recruitment of other proteins and is necessary for further signalling¹⁹³. It is known that upon activation, Dvl recruits Axin and GSK-3 to the plasma membrane¹⁷⁵. It is thought that this series of events lead to the inhibition of GSK-3 and ultimately results in the stabilization of β -catenin.

1.7 GSK-3, AKT AND THE WNT PATHWAY IN SCHIZOPHRENIA

1.7.1 GSK-3 and Schizophrenia

Post-mortem studies have found a decrease in GSK-3 β mRNA and protein levels in the frontal cortex of schizophrenic patients^{168, 194}. In a separate study, reductions in phosphorylated GSK-3 β in the frontal cortex were noted but no change in total GSK-3 β protein levels were reported⁴⁹. GSK-3 β has also been identified as a possible schizophrenia susceptibility gene^{195, 196}. Although most studies have focused on GSK-3 β , reductions in GSK-3 α in lymphocytes of schizophrenic patients have also been found¹⁹⁷. Partial animal models of schizophrenia have also supported the involvement of GSK-3 in schizophrenia. Reductions in phosphorylated GSK-3 levels have been reported in several animal models, including the amphetamine, DAT-KO mouse, PCP and ventral hippocampal lesion models^{148, 198-200}. Furthermore, administration of GSK-3 inhibitors normalized spontaneous hyperlocomotion in DAT-KO mice¹⁴⁸, a behavioural measure that correlates to positive symptoms. Other animal studies have demonstrated the possible involvement of GSK-3 in schizophrenia. Although GSK-3 β knockout mice die *in utero*, GSK-3 β heterozygous knockout mice are viable and display decreases in exploratory activity and reduced locomotor activity to amphetamine^{148, 201, 202}. GSK-3 α knockout mice are viable and exhibited a decrease in locomotion to a novel environment and impaired sensorimotor gating²⁰³.

1.7.2 Akt and Schizophrenia

An association between schizophrenia and Akt-1 genetic variants has been reported in several independent cohorts of schizophrenic patients⁴⁸⁻⁵³. Reduced Akt-1

protein levels have been reported in the frontal cortex of schizophrenic patients but no difference in Akt-2 or Akt-3 protein levels were observed⁴⁹. Furthermore, an Akt-1 gene variant found in some schizophrenic patients has been linked to cognitive functioning including IQ, processing speed and executive cognitive control processes²⁰⁴. These cognitive measures have been associated with dopaminergic functioning and impairments in these cognitive measures are observed in patients with schizophrenia. This same genotype predicted reduced gray matter volume in the right PFC and bilateral caudate and was correlated to dopaminergic functioning²⁰⁴. Reduction in gray matter volume is a common feature in schizophrenia¹⁴³.

In animal studies, Akt-1 knockout mice do not display abnormalities in PPI, spatial working memory or locomotor activity compared with wild-type littermates^{49, 205}. However, Akt-1 knockout mice exhibit disruptions in these behavioural tests when challenged with dopamine receptor agonists, suggesting that Akt-1 modulation of these behaviours is influenced by the dopaminergic environment^{49, 205}. It is known that stress influences dopamine levels and is often a trigger for psychosis or a relapse²⁰⁶. Thus, an Akt-1-environment interaction may affect the expression and severity of functioning in patients with schizophrenia. Furthermore, Akt-1 knockout mice show changes in the dendritic architecture of pyramidal neuron in the PFC (layer V neurons), the output neurons of the cortex²⁰⁵. Abnormalities in the cytoarchitecture of the pyramidal neurons is a common feature in schizophrenia¹⁴³. Taken together, the human and animal studies implicate Akt-1 in modulating prefrontal-striatal structures and function and thus may be relevant in certain symptoms of schizophrenia.

1.7.3 Wnt and Schizophrenia

The Wnt pathway is essential for proper development of the central nervous system in general and dopaminergic neuron development in particular. Wnt-3a promotes the proliferation of ventral midbrain DA neurons, whereas Wnt-1 and Wnt-5a are involved in the differentiation of DA neurons²⁰⁷⁻²⁰⁹. Other studies have demonstrated that GSK-3- β -catenin signalling is involved in proliferation and differentiation of ventral midbrain DA neurons^{210, 211}. These results suggest that the canonical Wnt pathway regulates DA neuron development. Thus, dysregulation of the canonical Wnt pathway may lead to improper DA neuron development, a neurotransmitter system strongly implicated in schizophrenia.

Abnormalities in Wnt signalling have also been demonstrated in schizophrenia. Genetic studies have shown an association between the Frizzled-3 receptor gene and schizophrenia²¹²⁻²¹⁴. Two members of the dickkopf (DKK) family, negative regulators of the Wnt pathway, have been implicated in schizophrenia. DKK4 has been identified as a possible schizophrenia susceptibility gene²¹⁵ and reductions in DKK3 mRNA levels have been observed in the temporal cortex of schizophrenics²¹⁶. Alterations in Wnt-1 and β -catenin staining have also been found in the hippocampus^{217, 218}. In addition, animal studies have shown that disruptions in Wnt signalling leads to abnormalities in social interaction and hyperlocomotion, common behavioural measures used in schizophrenia research. Dvl-1 deficient mice exhibited abnormal social interactions²¹⁹. Transgenic mice overexpressing β -catenin inhibited amphetamine-induced hyperlocomotion²²⁰. Taken together the data suggests a potential role for the Wnt pathway in schizophrenia.

1.8 mGLUR2/3 AGONISTS

Several attempts have been made to find alternative drug treatments for schizophrenia that do not target the D₂DR in the hopes of obtaining pharmaceuticals with improved clinical efficacy and fewer side-effects. For example, drugs that specifically target serotonin (5-HT₂) receptors, D₄DR, D₁DR and 5-HT₂/D₄DR have been developed but have failed to show any clinical antipsychotic activity. However, there is recent evidence to suggest that mGluR2/3 agonists may be effective for the treatment of schizophrenia. Initial animal studies showed that mGluR2/3 agonists, LY379268 and LY404039 attenuated hyperlocomotion in amphetamine-sensitized and PCP treated rats²²¹. Studies in healthy human subjects reported that the mGluR2/3 agonist, LY354740 improved working memory in ketamine treated subjects¹⁰⁴. In addition, data from an early clinical trial suggests that the group II metabotropic glutamate receptor agonist LY2140023 may have antipsychotic properties¹⁰⁵. Treatment with LY2140023 was reported to improve the positive symptoms of schizophrenia with comparable efficacy to olanzapine, an atypical antipsychotic and also improved negative symptoms in patients¹⁰⁵. Thus, mGluR2/3 agonists may represent an alternative, non-dopaminergic treatment for schizophrenia. However, the molecular mechanism responsible for the ability of mGluR2/3 agonists to alleviate psychosis is unknown.

1.9 OVERVIEW

GSK-3 has been implicated in the manifestation of schizophrenia and the alleviation of the positive symptoms of schizophrenia. Postmortem studies have reported reductions in total¹⁹⁴ and phosphorylated GSK-3 levels⁴⁹, whereas increases in total and phosphorylated GSK-3 levels in the PFC of rats have been observed following antipsychotic treatment²²². There is also evidence that Akt and the Wnt pathway, two cascades that regulate GSK-3 are disrupted in schizophrenia and targeted by antipsychotics. Akt-1 and members of the Wnt pathway have been identified as susceptibility genes for schizophrenia and/or altered protein, mRNA and/or phosphorylation levels have been reported¹⁷⁶. Repeated treatment with haloperidol or clozapine increases Dvl-3 and β -catenin but has no effect on Dvl-1 or Dvl-2 protein levels in the PFC of rats²²². In addition, haloperidol treatment has been shown to increase pAkt at Thr308 and Ser473 in the frontal cortex of rats⁴⁹. However, it is unknown if there is a convergence of Akt and Wnt and how the coordinated action of Akt and Wnt may affect GSK-3. Thus, in chapter two, the role of Akt and Dvl-3 in regulating GSK-3 and the transcription factor β -catenin in the PFC was investigated. One of the major concerns with linking GSK-3 signalling to the ability of antipsychotics to alleviate psychosis is specificity. Several other neuropsychiatric drugs that are unable to alleviate psychosis, such as mood stabilizers and antidepressants, also regulate GSK-3. In the third chapter, the study compared the effects of antipsychotic, mood stabilizers and antidepressants on Akt and Wnt pathway proteins to determine if antipsychotics have a specific effect(s) on these pathways. Finally, the fourth chapter addressed whether Akt

and/or the Wnt pathway are affected by mGluR2/3 signalling, given the recent promise of mGluR2/3 in the treatment of schizophrenia.

1.10 OBJECTIVES AND RATIONALE

1.10.1 Alterations in GSK-3 and Akt signalling induced by the D₂DR are mediated by Dvl-3

Rationale:

Previous studies have shown that antipsychotic treatments induce a common and specific pattern of change on Akt and the Wnt pathway, two signalling cascades that regulate GSK-3. Antipsychotics, such as haloperidol, clozapine and/or risperidone increase pAkt, Dvl-3, GSK-3, pGSK-3 and β -catenin in the PFC^{150, 222}. All antipsychotics are D₂DR antagonists and it is thought that blockade of the D₂DR is involved in the ability of antipsychotics to alleviate psychosis¹⁶². Subsequently, it has been suggested that antipsychotics mediate their effects on Akt and the Wnt through the blockade of the D₂DR. Although it is known that the D₂DR can regulate Akt¹⁶⁷, it is unclear if changes in the Wnt pathway are mediated by the D₂DR or by other dopamine receptors. Furthermore, given that antipsychotics induce changes in Akt and Dvl-3, suggest a possible convergence of these cascades on GSK-3 and consequently β -catenin.

Hypothesis:

D₂DR regulates GSK-3 by targeting Dvl-3 and Akt.

Objectives:

- To examine whether the Wnt pathway is regulated by the D₁DR, D₂DR and/or D₃DR.
- To identify and then elucidate how the pattern of change in GSK-3, Akt and select Wnt proteins were induced by the D₂DR.

1.10.2 The effect of neuropsychiatric drugs and amphetamine on GSK-3 related signalling

Rationale:

GSK-3 has been implicated in schizophrenia, mood disorders and depression and in their treatment options, antipsychotic drugs, mood stabilizers and antidepressants, respectively. Reduction in total and phosphorylated GSK-3 protein levels have been reported in the frontal cortex of schizophrenic patients^{194, 223}. In contrast, several antipsychotics have increased total and phosphorylated GSK-3 protein levels in the prefrontal cortex of rats^{150, 224}. Mood stabilizers and antidepressants have also been shown to increase phosphorylated GSK-3 protein levels *in vivo*²²⁵⁻²²⁹. However, the clinical profile of antipsychotics, mood stabilizers and antidepressants are different, and only antipsychotics are able to alleviate psychosis. Thus, it has been suggested that if GSK-3 signalling is important for the therapeutic effects of antipsychotics, then there may be differences in the regulation of GSK-3 compared to other neuropsychiatric drugs. Antipsychotics have been shown to activate Akt and the Wnt pathway *in vivo*, two cascades that inhibit GSK-3^{49, 222}. The effects of mood stabilizers and antidepressants on Akt and/or the Wnt pathway may provide key differences in GSK-3 signalling between these neuropsychiatric drugs and antipsychotics.

Hypothesis:

Haloperidol and clozapine treatments induce a specific response on Akt and the Wnt pathway leading to the stabilization of β -catenin, which is not mimicked by lithium, valproic acid, imipramine or fluoxetine.

Objectives:

- To compare the effects of antipsychotics (haloperidol and clozapine), mood stabilizers (lithium and valproic acid) and antidepressant (fluoxetine and imipramine) on Akt, Dvl, GSK-3 and β -catenin in the PFC and STR of rats.
- To investigate the association between GSK-3 and Akt and the D₂DR complex following haloperidol, clozapine, lithium and valproic acid.
- To examine changes in Akt, Dvl-3, GSK-3 and β -catenin in the PFC and STR of the amphetamine model.

1.10.3 Regulation of Akt and Wnt signalling by the group II metabotropic glutamate receptor antagonist LY341495 and agonist LY379268

Rationale:

Metabotropic glutamate receptors 2/3 (mGluR2/3) have been implicated in schizophrenia and as a potential treatment option for certain symptoms of schizophrenia. Genetic studies have identified the mGluR3 as a possible susceptibility gene for schizophrenia⁹⁴,⁹⁵ and have shown that these influence certain aspects of cognition (i.e. attention and verbal learning)⁹⁴. A preclinical trial has shown that treatment of the mGluR2/3 agonist, LY2140023 (prodrug of LY404039) led to an improvement of positive and negative symptoms, compared to placebo¹⁰⁵. Animal studies have reported treatment with

mGluR2/3 agonists (LY379268, LY404039 or LY354740) attenuated amphetamine and PCP induced hyperlocomotion^{221, 230-233}. Although there is strong evidence suggesting mGluR2/3 agonists have antipsychotic-like properties, the molecular mechanism(s) that explain how activation of mGluR2/3 alleviates psychosis is unknown. Given that Akt and the Wnt pathway are implicated in traditional antipsychotic drug action, Akt and Wnt may represent potential targets for mGluR2/3 agonists.

Hypothesis:

Metabotropic glutamate receptors 2/3 regulates Akt, GSK-3 and the Wnt pathway leading to functional changes at the metabotropic glutamate receptor 2/3 complex.

Objectives:

- To investigate the effects of the mGluR2/3 agonist LY379268 and the mGluR2/3 antagonist LY341495 on Akt, Dvl, GSK-3 and β -catenin in the PFC and STR.
- To examine the association between the mGluR2/3 and Akt, Dvl and GSK-3 in the PFC and/or STR.
- To examine the effects of the mGluR2/3 antagonist, LY341495 on locomotion.

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CHAPTER TWO

Alterations in GSK-3 and Akt signalling induced by the D₂DR are mediated by Dvl-3¹

¹ Sections 2.1-2.5 have been submitted to Biological Psychiatry.

2.1 INTRODUCTION

Converging lines of research have implicated Akt and the Wnt pathway in schizophrenia and antipsychotic drug action. Reductions in Akt-1 protein levels in the frontal cortex of some schizophrenic patients has been reported and Akt-1 has been identified as a potential schizophrenia susceptibility gene in several independent studies¹⁻⁶. Postmortem studies have shown a decrease in GSK-3 β protein and mRNA levels in the frontal cortex of schizophrenic patients^{7, 8} and GSK-3 β has been linked genetically to schizophrenia^{9, 10}. Proteins involved in Wnt signalling such as the Frizzled-3 receptor, Wnt-1, Dickkopf 3 and Dickkopf 4 have been linked to schizophrenia or were shown to have altered protein levels in postmortem studies¹¹⁻¹⁵. In contrast, antipsychotics have been shown to increase phosphorylated Akt, Dvl-3 and GSK-3 protein levels in the PFC of mice or rats^{2, 16, 17}.

The Wnt pathway and Akt both negatively regulate GSK-3 (GSK-3 α and GSK-3 β), although the mechanisms by which these pathways inhibit GSK-3 differs. Traditional models of Wnt signalling suggest that GSK-3 forms a complex with other proteins allowing for the phosphorylation of the transcription factor β -catenin, thereby targeting it for degradation^{18, 19}. Stimulation of the Wnt pathway activates the signal transducer protein Dvl. Dvl interferes with the GSK-3 protein complex responsible for phosphorylating β -catenin^{20, 21}. Unphosphorylated β -catenin accumulates in the cytoplasm and translocates to the nucleus where it regulates TCF/LEF gene transcription^{22, 23}. Activation of Akt results in the subsequent phosphorylation of GSK-3 (ser21/9, GSK-3 α /GSK-3 β) and a reduction in GSK-3 kinase activity^{24, 25}. Since both Dvl and Akt can regulate GSK-3, there may be a convergence of these two signalling

cascades at GSK-3 leading to the regulation of β -catenin. Similar to Akt and GSK-3, β -catenin is of interest since it has also been implicated in schizophrenia²⁶ and antipsychotic drug action¹⁶.

Antipsychotics have been shown to target Akt, GSK-3 and β -catenin, presumably through the D₂DR, since all antipsychotics are D₂DR antagonists. This is of particular interest given that D₂DR antagonism is thought to be essential for alleviating psychosis²⁷,²⁸. Recent studies have shown that the D₂DR can regulate Akt signalling in the brain^{29, 30} but it is unclear if the D₂DR can also control the Wnt pathway. The current study investigated whether D₂DR antagonism can regulate the Wnt pathway leading to increased levels of β -catenin and compared these results with D₁DR and D₃DR antagonism. Furthermore, the role of Akt and Dvl-3 in the regulation of GSK-3 and β -catenin was investigated using both *in vivo* and *in vitro* approaches.

2.2 MATERIAL AND METHODS

2.2.1 Animal treatment

Adult male Sprague-Dawley rats 14+ weeks of age were housed in pairs with free access to food and water on a 12 hr light/dark cycle. Rats were injected with SCH23390 (D₁DR antagonist, 0.5mg/kg, s.c., Sigma-Aldrich), raclopride (D₂DR antagonist, 3mg/kg, intramuscular, Sigma-Aldrich), nafadotride (D₃DR antagonist, 0.5mg/kg, s.c., Sigma-Aldrich), quinpirole (D₂DR agonist, 3mg/kg, intramuscular, Tocris) or appropriate vehicles for 7 days (n=8/treatment). The doses selected are routinely used in the literature and/or based on behavioural studies involving prepulse inhibition³¹ and *in vivo* dopamine receptor occupancy studies³²⁻³⁴. Rats were sacrificed 2 hours following the

final injection of SCH23390, raclopride or nafadotride and 4 hours following the final treatment with quinpirole. Post-injection times were selected based on empirical data from the laboratory showing robust and consistent changes in Wnt/Akt proteins at these time points. Brains from Akt-1 knockout, heterozygous and wild type mice were generously provided by Dr. F. Beier (University of Western Ontario, Canada) although originally obtained from Jackson Laboratories. All experiments were conducted in accordance with the Canadian Council on Animal Care (CCAC) in a CCAC approved facility at the University of Western Ontario. Every effort was made to reduce the number of animals used in the study and to minimize suffering.

2.2.2 Cell culture

SH-SY5Y (neuroblastoma) cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and penicillin (100U/ml)/streptomycin (100µg/ml) and were maintained in a humidified 37°C incubator with 5% CO₂.

2.2.3 Retroviral infections and transfections

For Dvl-3 overexpression, SH-SY5Y cells were infected with Dvl-3 or control (nAP2) retrovirus as previously described¹⁷. Overexpression of Dvl-3 alone was shown to be sufficient to initiate Wnt signalling and mimic the affects of antipsychotics on the Wnt pathway *in vivo*¹⁷. To increase Akt activity, SH-SY5Y cells were transfected with a constitutively active Akt (myrAkt Δ4-129) or control construct (A2myrAkt Δ4-129) that does not alter Akt activity³⁵ using Lipofectamine 2000 (Invitogen) according to the manufacturer's instructions. The Akt vectors were generously provided by Dr. R. Roth

(Stanford University School of Medicine, California). To knockdown Akt-1 and Dvl-3 protein levels, SH-SY5Y cells were transfected with siRNA specific for Akt-1 or Dvl-3. Briefly, cells were plated in a 6-well plate (50,000cells/well) and allowed to adhere overnight. The cells were transfected with siRNA for Akt-1 (Ambion, s659), Dvl-3 (Ambion, s674) or a negative control siRNA (Ambion) at a concentration of 30pmol using Lipofectamine RNAiMAX (Invitrogen) and Optimum I media according to the manufacturer's instructions.

2.2.4 PI3-K inhibitor treatments of cell cultures

SH-SY5Y cells were treated with varying concentrations of the PI3-K inhibitors wortmannin (Calbiochem), LY294002 (Calbiochem) or vehicle (control) in serum reduced (1% FBS in DMEM) media. The PI3-K inhibitors wortmannin and LY294002 were selected since they are routinely used in the literature to inhibit PI3-K-Akt phosphorylation. Briefly, cells were plated on 60mm plates (300,000 cells) and allowed to adhere overnight. The next day media was replaced with fresh serum reduced media and treated with wortmannin (0.1 μ M, 1 μ M or 10 μ M), LY294002 (2 μ M, 20 μ M or 40 μ M) or vehicle (controls) for 1 hour (n = 6/treatment). In a separate series of experiments, SH-SY5Y were infected with Dvl-3 or nAP2 and treated with wortmannin (10 μ M), LY294002 (40 μ M) or vehicle alone (n = 6/treatment).

2.2.5 Protein isolation and western blotting

Protein isolation, quantification and western blotting were conducted as previously outlined for the PFC tissue¹⁶ or for cells¹⁷. Densitometry values were obtained from X-

ray film using a grey scale calibrated scanner (Epson) and Kodak Molecular Imaging software. Densitometry values were corrected for α -tubulin and expressed as a percentage of control. The data was analyzed using an ANOVA followed by Tukey's multiple comparison test or Student's *t*-test as appropriate. The source and dilution of the antibodies used in the study were as follows: Akt (Cell Signalling Technology, 1:1250), Akt-1 (Cell Signalling Technology, 1:1000), Akt-2 (Cell Signalling Technology, 1:1000), Akt-3 (Cell Signalling Technology, 1:1000), phospho-Akt Ser473 (Cell Signalling Technology, 1:1000), phospho-Akt Thr308 (Upstate, 1:1000), Dvl-1 (Santa Cruz Biotechnology, 1:100), Dvl-2 (Chemicon, 1:1000), Dvl-3 (Santa Cruz Biotechnology, 1:100), GSK-3 (Santa Cruz Biotechnology, 1:300), phospho-GSK-3 (Ser21/9, Cell Signalling Technology, 1:1000), β -catenin (Sigma-Aldrich, 1:20000), D₂DR (Santa Cruz Biotechnology, 1:100 rabbit and goat polyclonal) and α -tubulin (Sigma-Aldrich, 1:120000).

2.2.6 Co-Immunoprecipitations

Protein was isolated from the PFC of drug naïve rats or untreated SH-SY5Y cells using a non-denaturing lysis buffer with the addition of protease (Complete Mini, Roche Diagnostic) and phosphatase inhibitors (Cocktail Set 2, Sigma-Aldrich; Cocktail Set IV, Calbiochem) as outlined previously¹⁷. Immunoprecipitations (IPs) were conducted using the ExtraCruz IP system (Santa Cruz Biotechnology) with antibodies for D₂DR (2 μ g/IP, Santa Cruz Biotechnology, goat and rabbit polyclonal) or GSK-3 α/β (3 μ g/IP, Santa Cruz Biotechnology) and 500 μ g of protein. As a control, protein samples (500 μ g) were immunoprecipitated with IgG (Santa Cruz Biotechnology) from the same species as the

IP antibody. IPs were also conducted with protein (500 μ g) isolated from the PFC of raclopride, quinpirole or vehicle treated rats or SH-SY5Y cells infected with Dvl-3 or transfected with a constitutively active Akt construct using the ExtraCruz system and antibodies specific for D₂DR (2 μ g/IP, Santa Cruz Biotechnology, goat and rabbit polyclonal). The IP samples were subjected to western blotting (WB) and probed for the proteins of interest; D₂DR (Santa Cruz Biotechnology, 1:100), GSK-3 (Santa Cruz Biotechnology, 1:300) or Akt (Cell Signalling Technology, 1:1250).

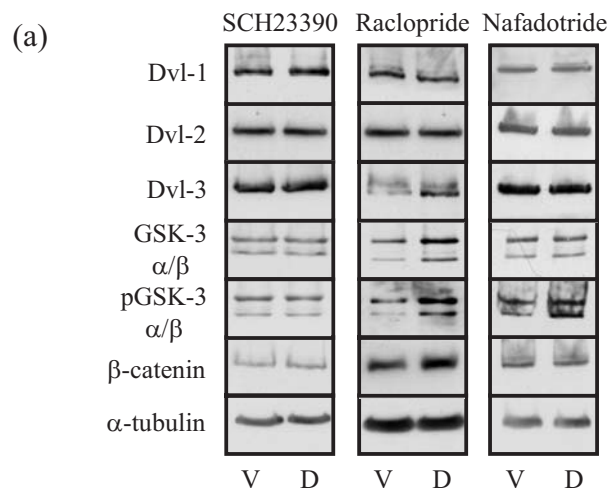
2.3 RESULTS

2.3.1 Regulation of Akt and Wnt pathway proteins by D₁DR, D₂DR and D₃DR

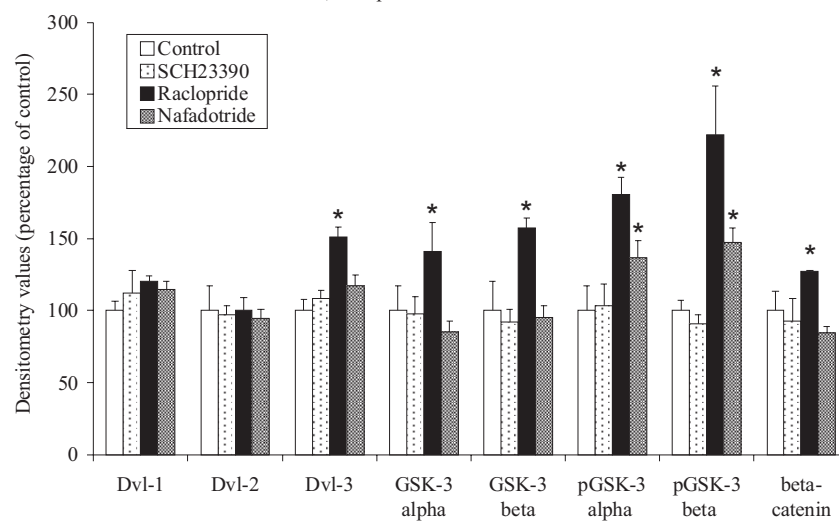
Raclopride was used to determine whether D₂DR antagonism is able to mediate changes in Akt and the Wnt pathway in the PFC. The results showed that raclopride increased Dvl-3, GSK-3 α/β , pGSK-3 α/β and β -catenin protein levels but had no effect on Dvl-1 or Dvl-2 (Figure 2.1a and b). Raclopride also increased the phosphorylation state of Akt Thr308 and Ser473 but did not affect total Akt levels (Figure 2.1c and d). To determine if Akt and/or Wnt pathway proteins are regulated by other dopamine receptors, D₁DR and D₃DR antagonists were also used. SCH23390 (D₁DR antagonist) had no effect on any of the Wnt pathway proteins tested (Figure 2.1a and b) or on the total and phosphorylated levels of Akt (Figure 2.1c and d). Nafadotride (D₃DR antagonist) increased pGSK-3 α/β but had no effect on Dvl-1, Dvl-2, Dvl-3, GSK-3 α/β or β -catenin (Figure 2.1a and b). Nafadotride also increased pAkt Ser473 but did not alter total Akt or pAkt Thr308 levels (Figure 2.1 c and d).

Figure 2.1: The effects of D₁DR, D₂DR and D₃DR antagonism on total protein levels and/or phosphorylation state of Akt and Wnt pathway proteins.

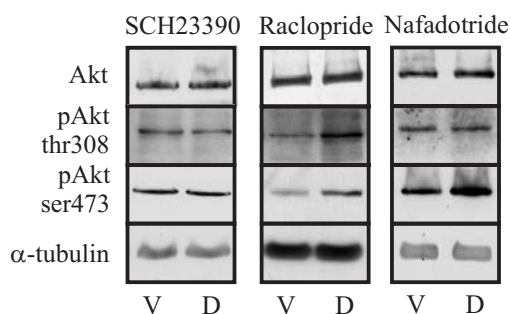
(a) Representative blots and (b) graph of densitometry values for Dvl, GSK-3 α/β and β -catenin in the PFC of rats treated with SCH23390 (D₁DR antagonist), raclopride (D₂DR antagonist) or nafadotride (D₃DR antagonist). (c) Representative blots and (d) graph of densitometry values for total and phosphorylated Akt in the PFC of rats treated with SCH23390, raclopride or nafadotride. For each representative western blot, the left band represents the vehicle (V) while the right band shows the drug treatment (D). The graphs compare densitometry values obtained from the western blots, with control values set at 100%. For simplicity, the graphs are shown using a single control. Statically significant changes are denoted by the asterisk ($p < 0.05$, $n=8$). Error bars represent the standard error of the mean.



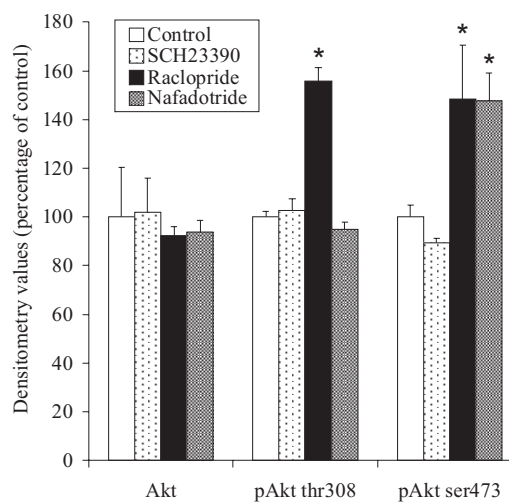
(b) Changes in Wnt pathway proteins in the PFC following repeated administration of SCH23390, raclopride or nafadotride treatment



(c)



(d) Changes in total and phosphorylated Akt in the PFC following repeated administration of SCH23390, raclopride or nafadotride



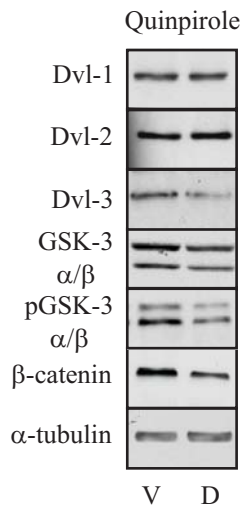
Hyperdopaminergic animal models, such as the DAT-KO mice and amphetamine-sensitization have been shown to inhibit Akt and/or Wnt signalling^{29, 36}. To determine if the D₂DR may be responsible for these changes, the D₂DR agonist quinpirole was used. Quinpirole caused decreases in Dvl-3, GSK-3 α/β , pGSK-3 α/β and β -catenin but had no effect on Dvl-1 or Dvl-2 in the PFC (Figure 2.2a and b). Decreases in pAkt Ser473 were also found but no changes in total Akt or pAkt Thr308 were detected (Figure 2.2c and d).

Previous studies showed an association between the D₂DR complex and Akt and Dvl-3¹⁷ and an association between Akt and GSK-3³⁷. Consequently, co-IP's were conducted to determine if there is an association between D₂DR and GSK-3. IPs conducted using an antibody for D₂DR followed by a WB for GSK-3 α/β showed an association between the D₂DR complex and GSK-3 α/β (Figure 2.3a). A similar result was observed when IPs were conducted using a GSK-3 α/β specific antibody followed by a WB for D₂DR (Figure 2.3b). The results indicate that GSK-3 α/β is associated with the D₂DR complex in naive PFC tissue. In addition, since D₂DR antagonists (raclopride and antipsychotics) activate the Wnt pathway and a previous study showed that activation of the Wnt pathway causes translocation of GSK-3 to the membrane³⁸, co-IPs were performed using protein isolated from the PFC of raclopride treated rats to determine if the association between the D₂DR complex and Akt and/or GSK-3 is altered. The results showed that the association between D₂DR and Akt is unaltered ($102.1\% \pm 7.9$, n=4) and as expected the total Akt levels in the lysate samples were unaffected ($90.5\% \pm 6.7$, n=4) (Figure 2.3c). D₂DR levels were also not significantly altered following the raclopride treatment (98.9 ± 3.0 , n=4) (Figure 2.3c). However, there is an increased association between D₂DR and GSK-3 α/β following raclopride treatment (Figure 2.3c and d). There

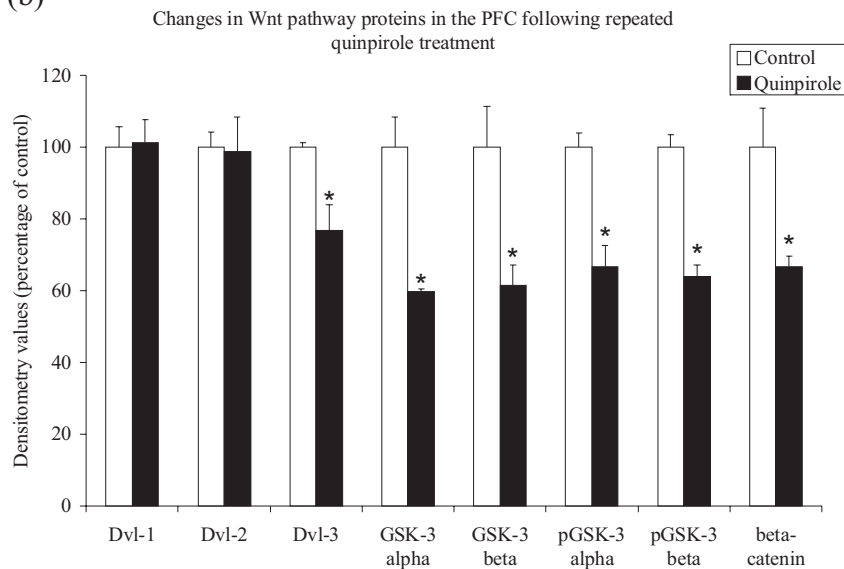
Figure 2.2: The effects of D₂DR agonism on total protein levels and/or phosphorylation state of Akt and Wnt pathway proteins.

(a) Representative blots and (b) graph of densitometry values for Dvl, GSK-3 α/β and β -catenin in the PFC of rats treated with quinpirole. (c) Representative blots and (d) graph of densitometry values for total and phosphorylated Akt in the PFC of rats treated with quinpirole. For each representative western blot, the left band represents the vehicle (V) while the right band shows the drug treatment (D). The graphs compared the densitometry values obtained from the western blot. Densitometry values are expressed as a percentage of control \pm SEM and statistically significant differences are indicated by the asterisks ($p < 0.05$, $n=8$).

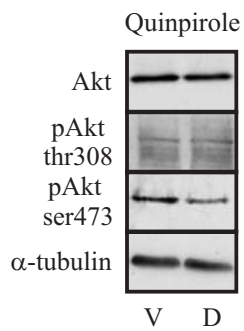
(a)



(b)



(c)



(d)

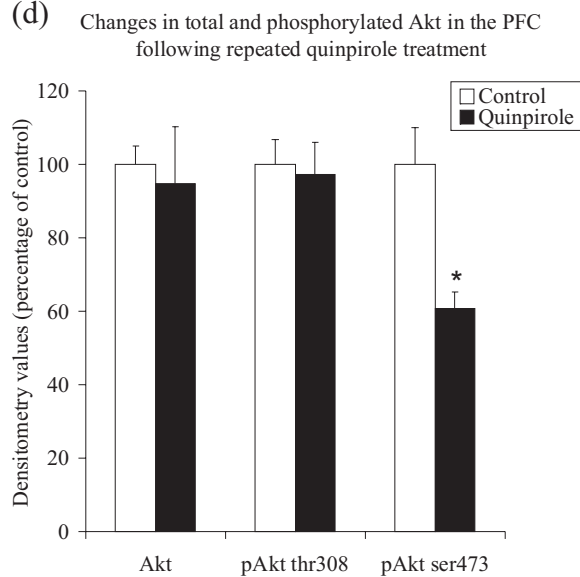
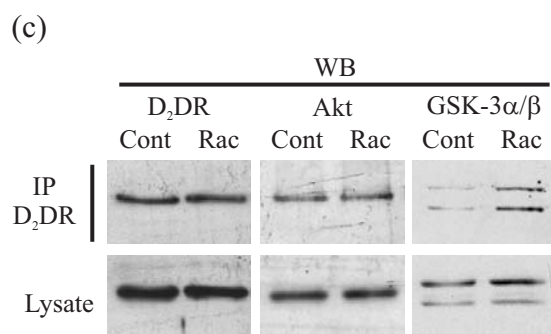
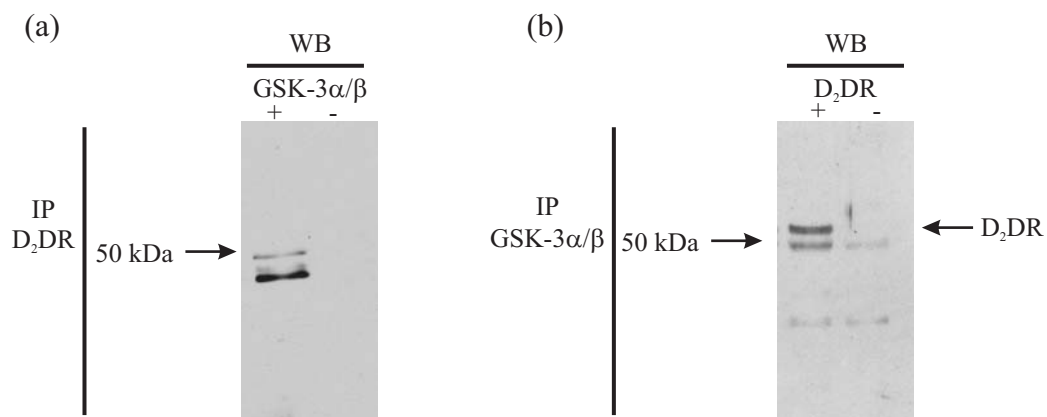


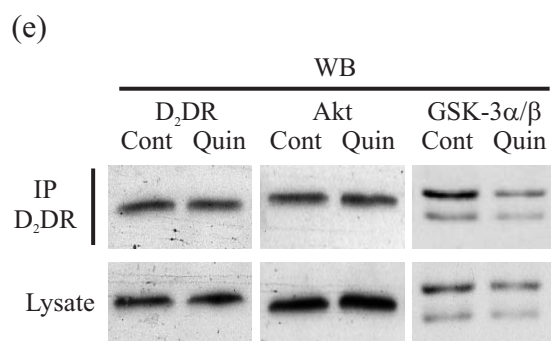
Figure 2.3: Co-IP experiments showing the association between D₂DR and Akt or GSK-3 α / β in drug naïve rats, raclopride or quinpirole treated rats.

(a) Protein samples were obtained from the PFC of drug naïve rats and immunoprecipitated (IP) with a D₂DR antibody. IP samples were subjected to western blotting (WB) and probed GSK-3 α / β (+ lanes). (b) Protein samples from the PFC of drug naïve rats were IP with a GSK-3 α / β antibody and IP samples were then subjected to WB and probed for D₂DR (+ lanes). For the negative control lanes, co-IPs were conducted using a non-specific IgG in place of the IP antibody (- lanes). Protein samples obtained from the PFC of (c) raclopride or (e) quinpirole treated rats were IP with a D₂DR antibody. IP samples were subjected to WB and probed for D₂DR, Akt and GSK-3 α / β (upper panels). WB were also conducted using the same lysate as the IPs to compare changes in total protein levels in the samples (lower panel). Table of densitometry values obtained from the (d) raclopride or (f) quinpirole co-IPs for D₂DR/GSK-3 α / β and total GSK-3 α / β protein levels. Statistically significant changes are represented by an asterisk ($p < 0.05$, mean \pm SEM, $n=6$).



(d) Quantification of Western blot (WB) analysis of D₂DR, Akt, and GSK-3 α/β levels in IP D₂DR and Lysate. The table shows the relative levels of GSK-3 α and GSK-3 β in IP D₂DR and Lysate, normalized to Control (100 ± 7.2).

		Control	Raclopride
IP	GSK-3 α	100 ± 11.6	215.0 ± 14.6*
	GSK-3 β	100 ± 15.1	257.6 ± 28.3*
Lysate	GSK-3 α	100 ± 4.2	142.0 ± 7.8*
	GSK-3 β	100 ± 7.2	131.1 ± 5.7*



(f) Quantification of Western blot (WB) analysis of D₂DR, Akt, and GSK-3 α/β levels in IP D₂DR and Lysate. The table shows the relative levels of GSK-3 α and GSK-3 β in IP D₂DR and Lysate, normalized to Control (100 ± 5.1).

		Control	Quinpirole
IP	GSK-3 α	100 ± 7.4	60.2 ± 9.4*
	GSK-3 β	100 ± 5.2	67.2 ± 5.6*
Lysate	GSK-3 α	100 ± 1.4	63.3 ± 4.9*
	GSK-3 β	100 ± 5.1	65.2 ± 6.3*

was also an increase in total GSK-3 α/β protein levels (Figure 2.3c and d) consistent with the previous findings. The results show that the increase in the association between D₂DR and GSK-3 α/β is greater than the increase in total GSK-3 α/β protein levels (Figure 2.3d). Co-IP's were also conducted using protein isolated from quinpirole treated rats. Similar to raclopride, quinpirole had no effect on the association between D₂DR and Akt (97.3 ± 5.5 , n=4) and no change in total Akt levels in the lysate samples (97.7 ± 5.3 , n=4) (Figure 2.3e). No change in the D₂DR protein levels were detected in the lysate samples (93.0 ± 7.1 , n=4) (Figure 2.3e). A decrease in the association between D₂DR and GSK-3 α/β and a reduction in total GSK-3 α/β protein levels in the lysate samples were observed following quinpirole treatment (Figure 2.3e and f). The change in association of GSK-3 α/β with the D₂DR complex, however, paralleled reductions in total GSK-3 α/β protein levels in the lysate (Figure 2.3f).

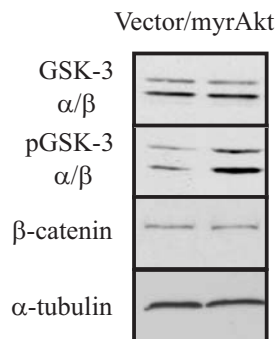
2.3.2 The effects of Akt on GSK-3 and β -catenin

To understand the role Akt may play in the pattern of change observed *in vivo*, Akt activity was either increased or inhibited in SH-SY5Y cells to parallel the changes observed following raclopride or quinpirole treatment, respectively. To increase Akt activity, SH-SY5Y cells were transfected with a constitutively activate Akt construct. Overexpression of constitutively active Akt resulted in increased pGSK-3 α/β but had no effect on total GSK-3 α/β or β -catenin protein levels compared to control (Figure 2.4a and b). To inhibit Akt activity, the PI3-K inhibitors wortmannin or LY294002 were used.

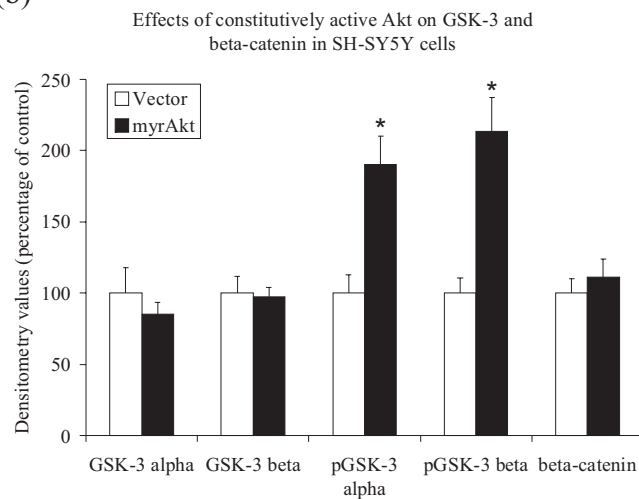
Figure 2.4: The effect of a constitutively active Akt construct or Akt inhibitors, wortmannin and LY294002 on GSK-3 and β -catenin in SH-SY5Y cells.

(a) Representative western blots and (b) graph comparing densitometry values showing the effects of constitutively active Akt (myrAkt) or control vector (vector) for GSK-3 α/β , pGSK-3 α/β and β -catenin. For each western blot the left band represents the control vector and the right band represents the constitutively active Akt. (c) Representative western blots and (d) graph showing the densitometry values obtained from western blots following wortmannin treatment (0.1 μ M, 1 μ M and 10 μ M) for Akt, pAkt Thr308, pAkt Ser473, Dvl-3, GSK-3 α/β , pGSK-3 α/β and β -catenin. (e) Graph showing the densitometry values obtained from western blots following LY294002 treatment (2 μ M, 20 μ M and 40 μ M). Densitometry values are expressed as a percentage of control \pm SEM and statistically significant differences are indicated by the asterisks ($p < 0.05$, $n=8$).

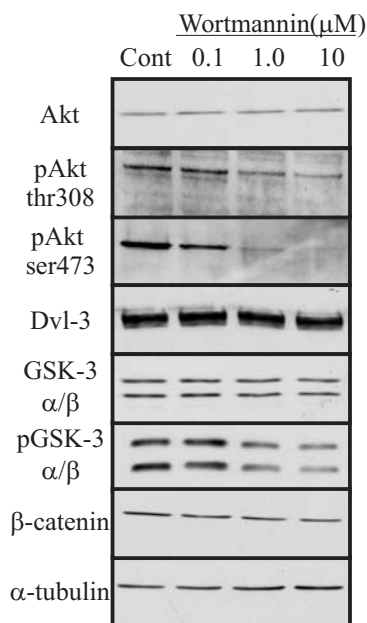
(a)



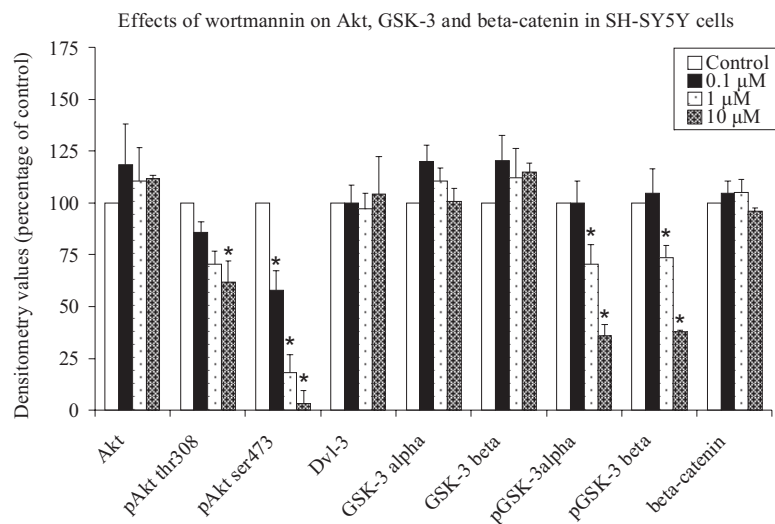
(b)



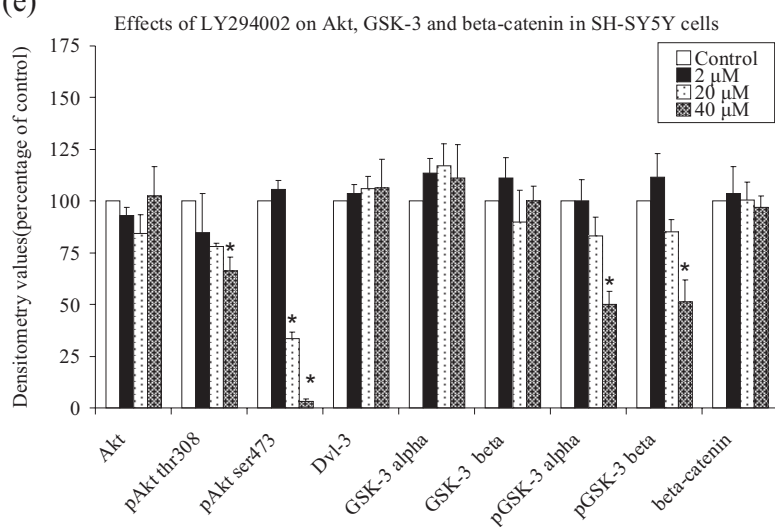
(c)



(d)



(e)



Wortmannin (Figure 2.4c and d) and LY294002 (Figure 2.4e) treatments caused significant reductions in pAkt Thr308, pAkt Ser473 and pGSK-3 α/β at 10 μ M and 40 μ M respectively but did not affect Dvl-3, GSK-3 α/β or β -catenin at any dose tested.

The initial overexpression and pharmacological experiments targeted total Akt activity and suggested that Akt regulates the phosphorylation of GSK-3 alone. Further analysis was conducted using Akt-1 siRNA and an Akt-1 deficient mice since Akt-1 protein levels were found to be altered in schizophrenia post-mortem samples². Knockdown of Akt-1 by siRNA nearly abolished Akt-1 protein expression but also caused a compensatory increase in Akt-3 protein levels (174.2% \pm 5.9, n=6) (Figure 2.5a). Akt-2 was not readily detectable in SH-SY5Y cells and was not examined further in this cell line. Despite the increase in Akt-3, knockdown of Akt-1 resulted in decreased pGSK-3 α/β but had no effect on Dvl-3, GSK-3 α/β or β -catenin protein levels (Figure 2.5a and b). To determine if similar results are obtained *in vivo*, Akt-1 transgenic mice were used. Compared to wild-type littermates, a decrease in pGSK-3 α/β was observed in the PFC of Akt-1 heterozygous (+/-) and Akt-1 knockout (-/-) mice (Figure 2.5c and d). However, no significant change in Akt-2, Akt-3, Dvl-3, GSK-3 α/β or β -catenin proteins levels were detected in either Akt-1 heterozygous or Akt-1 knockout mice (Figure 2.5c and d).

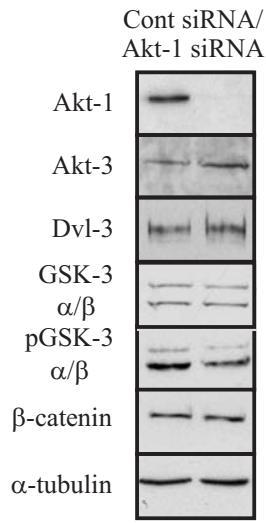
2.3.3 The effects of Dvl-3 on Akt, GSK-3 and β -catenin levels

Dvl-3 protein levels were increased following raclopride treatment and increased levels of Dvl/Dvl-3 have been shown to be sufficient to activate the Wnt pathway¹⁷. Consequently, Dvl-3 was overexpressed in SH-SY5Y cells to examine the effects of

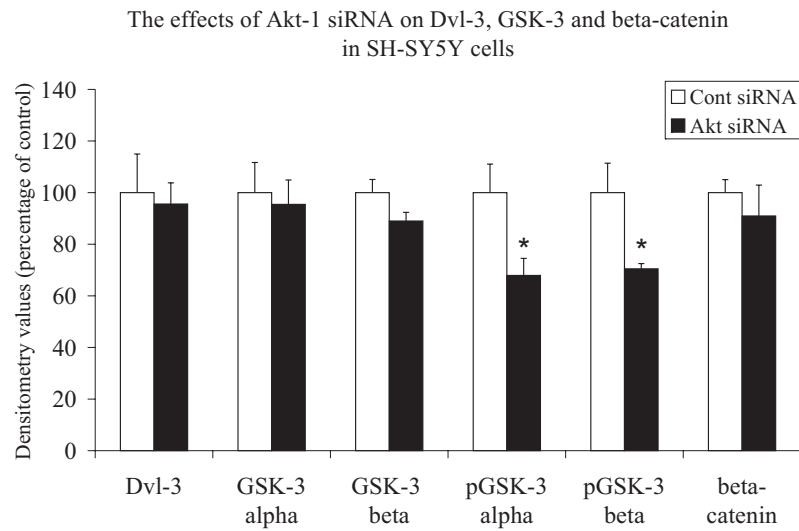
Figure 2.5: The effects of reducing Akt-1 protein levels *in vitro* and *in vivo* on Akt, Dvl-3, GSK-3 and β -catenin using siRNA or transgenic mice, respectively.

(a) Representative western blots and (b) graph showing the densitometry values obtained from western blots of protein isolated from SH-SY5Y cells transfected with Akt-1 siRNA or control siRNA. The band on the left represents the control siRNA and the band on the right represents the Akt-1 siRNA. (c) Representative western blots and (d) graph showing the densitometry values from western blots of protein isolated from the PFC of Akt-1 knockout (KO, n = 5), Akt-1 heterozygous (HZ, n = 4) or wild type (WT, n = 5) mice. The densitometry values are expressed as a percentage of control with a statistically significance difference denoted by an asterisk ($p < 0.05$, mean \pm SEM).

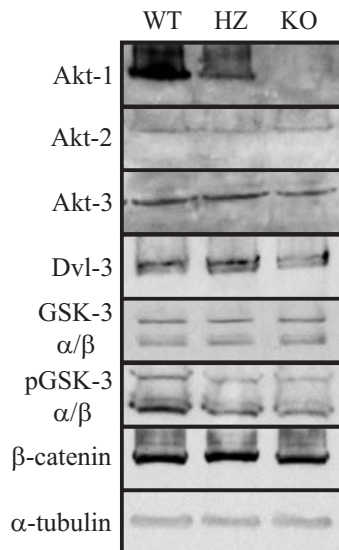
(a)



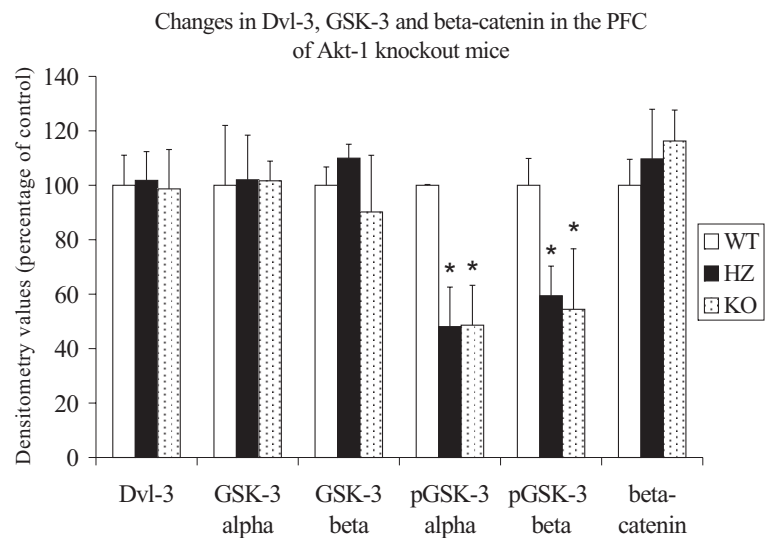
(b)



(c)



(d)



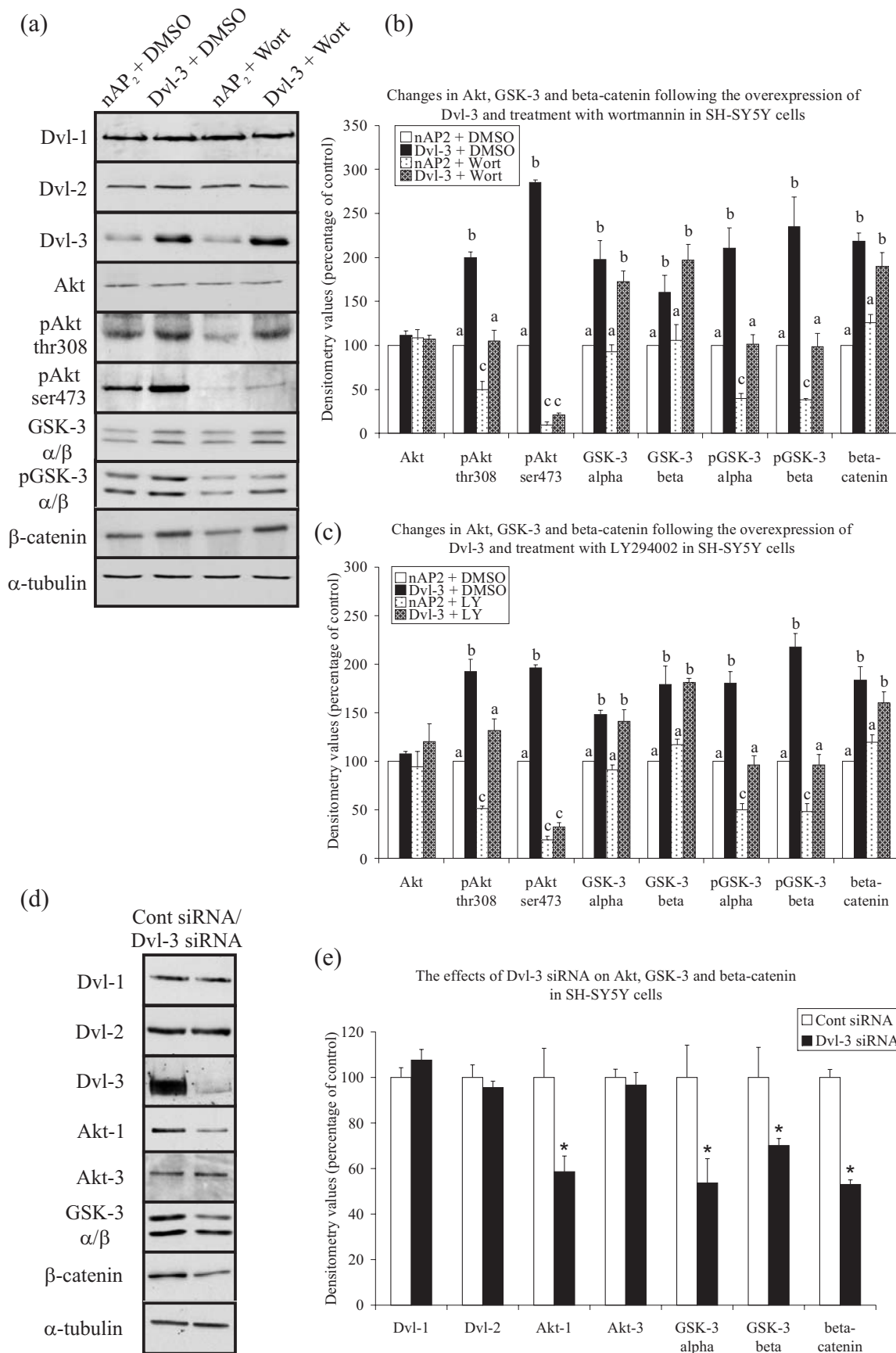
Dvl-3 on Akt, GSK-3 and β -catenin. Overexpression of Dvl-3, resulted in increased total GSK-3, pGSK-3 α/β and β -catenin protein levels but did not effect Dvl-1 or Dvl-2 levels as previously reported (Figure 2.6a-c)¹⁷. In addition, Dvl-3 overexpression also increased pAkt Thr308 and Ser473 without affecting total Akt protein levels (Figure 2.6a-c).

Although manipulations of Akt/Akt-1 activity/protein levels *in vitro* and *in vivo* did not affect β -catenin levels, recent reports have suggested that Wnt activation of Akt enhances the inhibition of GSK-3^{39, 40}. Therefore, it is possible that activation of Akt may contribute to increased β -catenin levels in Dvl-3 overexpressing cells. To test this possibility, SH-SY5Y cells infected with Dvl-3 were treated with either wortmannin or LY294002 to determine if the inhibition of PI3-K-Akt can block the increase in β -catenin and/or GSK-3 following Dvl-3 overexpression alone. Results indicate that the wortmannin (Figure 2.6a and b) and LY294002 (Figure 2.6c) treatments caused significant reductions in pAkt Thr308, pAkt Ser473 and pGSK-3 α/β levels. However, the PI3-K inhibitors had no effect on β -catenin or GSK-3 α/β in cells overexpressing Dvl-3 (Figure 2.6a-c). Collectively, the results suggest that Akt regulates the phosphorylation of GSK-3 whereas Dvl-3 regulates β -catenin and GSK-3 protein levels and is involved in Akt activation.

To further examine the role of Dvl-3 in Akt, GSK-3 and β -catenin regulation, SH-SY5Y cells were transfected with Dvl-3 siRNA. The knockdown of Dvl-3 nearly eliminated Dvl-3 protein expression but had no effect on either Dvl-1 or Dvl-2 (Figure 2.6d and e). Both GSK-3 α/β and β -catenin protein levels were also reduced consistent with the changes observed in quinpirole treated rats (Figure 2.6d and e). Interestingly,

Figure 2.6: Changes in Akt, Dvl, GSK-3 and β -catenin following the overexpression or knockdown of Dvl-3 in SH-SY5Y cells.

(a) Representative western blots and (b) graph showing the densitometry values obtained from western blots following the overexpression of Dvl-3 or nAP2 treated with wortmannin (10 μ M). (c) Graph showing the densitometry values obtained from western blots following the overexpression of Dvl-3 or nAP2 treated with LY294002 (40 μ M). (d) Representative western blots and (e) graph of densitometry values obtained from western blots following the transfection of Dvl-3 or control siRNA. The left lane shows the protein levels obtained from control siRNA while right lane shows the protein levels from Dvl-3 siRNA transfected cells. The values are shown as a percentage of control \pm SEM and a statistical significance is denoted by (b, c) a difference in lettering or e) by an asterisk (n=8, p<0.05).



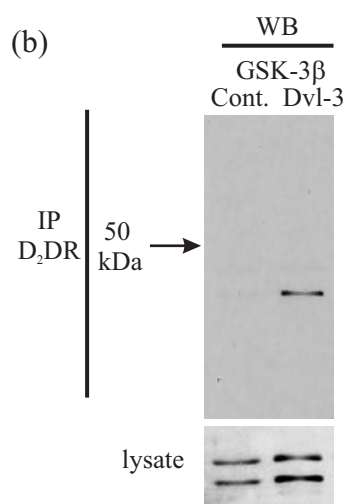
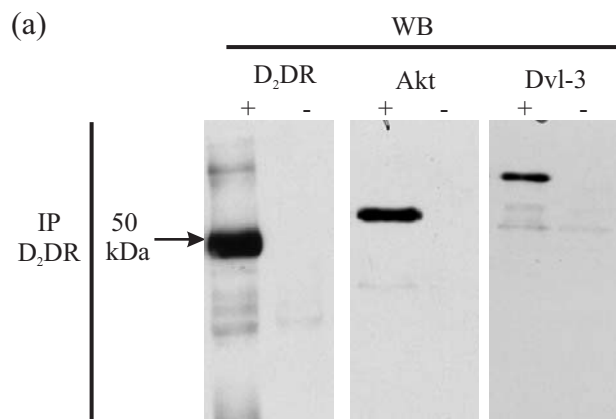
knockdown of Dvl-3 also caused a significant down regulation in Akt-1 but not Akt-3 (Figure 2.6d and e), similar to what was reported in postmortem tissue of schizophrenic patients². However, knockdown of Dvl-3 with siRNA did not significantly decrease pAkt levels as anticipated (Thr308 $98.28\% \pm 6.04$; Ser473 $84.27\% \pm 8.12$, n=6).

2.3.4 D₂DR association with GSK-3

The *in vitro* results suggest that Dvl-3 may be responsible for activation of Akt and the Wnt pathway following raclopride treatment. Therefore, it is plausible that upregulation of Dvl-3 may also be responsible for the translocation of GSK-3 to the D₂DR complex observed following raclopride treatment. To test this hypothesis, co-IPs were performed using protein isolated from Dvl-3 overexpressing SH-SY5Y cells. The D₂DR is endogenously expressed in SH-SY5Y cells and initial experiments indicated that Akt and Dvl-3 are associated with the D₂DR complex in untreated cells (Figure 2.7a) similar to what was seen in the PFC of drug naïve rats⁴¹. Overexpression of Dvl-3 led to an increased association between the D₂DR complex and GSK-3 β (Figure 2.7b and c). An increase in total GSK-3 β protein levels were also observed in the lysate samples (Figure 2.7b and c). The results show that the increase in GSK-3 β associated with the D₂DR complex was greater than the increase in total GSK-3 β protein levels induced by Dvl-3 overexpression (Figure 2.7c) consistent with raclopride treatment *in vivo*. Cells transfected with constitutively active Akt did not alter the relationship between the D₂DR complex and GSK-3 α/β nor was there a change in GSK-3 α/β protein levels in the lysate samples (Figure 2.7d and e).

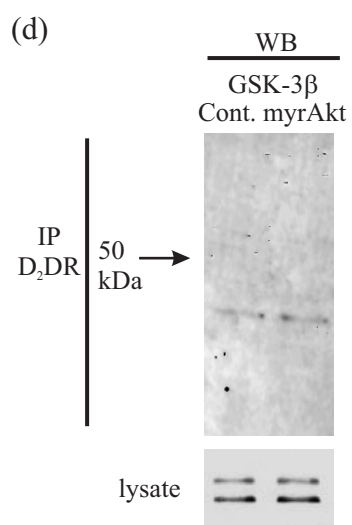
Figure 2.7: Co-IP experiments examining the association between D₂DR and GSK-3 α / β in native SH-SY5Y cells or following viral overexpression of Dvl-3 or transfection with a constitutively active Akt construct.

(a) Protein from treatment naïve SH-SY5Y cells were IP with a D₂DR antibody. IP sample were subjected to WB and probed using D₂DR, Akt or Dvl-3 antibodies (+ lane). For the negative control, co-IP's were conducted using a non-specific IgG in place of the IP antibody (- lane). (b) Protein samples obtained from SH-SY5Y cells infected with Dvl-3 or nAP2 (Cont) or (d) transfected with Akt construct (myrAkt) or empty vector (Cont) were IP with D₂DR antibody. IP samples were subjected to WB and probed using GSK-3 α / β antibody (upper panel). WB were also conducted using the same lysate as the IPs and probed using GSK-3 α / β antibody to demonstrate changes in total protein levels in the lysate sample (lower panel). Table of the densitometry values for co-IPs and total GSK-3 β protein levels for (c) the overexpression of Dvl-3 or (e) constitutively active Akt. Statistically significant changes are represented by an asterisk ($p < 0.05$, mean \pm SEM, n=6).



(c)

		Control	Dvl-3
IP	GSK-3 β	100 \pm 3.2	279.1 \pm 11.4*
Lysate	GSK-3 β	100 \pm 9.7	167.1 \pm 18.1*



(e)

		Control	MyrAkt
IP	GSK-3 β	100 \pm 6.3	96.3 \pm 10.5
Lysate	GSK-3 β	100 \pm 3.1	100.5 \pm 2.7

2.4 DISCUSSION

The *in vivo* results of the current study show that D₁DR and D₃DR do not affect the Wnt pathway, although D₃DR can regulate pAkt. D₂DR, in contrast, can regulate both the Akt and Wnt pathways. These results are consistent with a previous study showing altered Akt signalling in D₂DR and D₃DR but not D₁DR knockout mice³⁰. Antagonism of the D₂DR by raclopride results in reduced levels of Dvl-3, GSK-3 α/β , β -catenin and pAkt. The findings are consistent with previous studies showing that antipsychotics, haloperidol and/or clozapine increase Dvl-3, GSK-3 α/β and β -catenin and pAkt levels and that the D₂DR is associated with Akt^{2, 16, 17}. The results also suggest that the increases in the phosphorylation state of Akt and in levels of Wnt pathway proteins observed following antipsychotic treatment are mediated by the D₂DR, an important finding given that the D₂DR is thought to be essential for the therapeutic effects of antipsychotics^{27, 28}. In contrast, agonism of the D₂DR by quinpirole resulted in decreased levels of pAkt, Dvl-3, β -catenin and GSK-3 α/β . These findings compliment previous studies showing decreased pAkt levels in the STR of dopamine transporter knockout mice and following amphetamine treatment²⁹.

Raclopride and quinpirole led to increased or decreased pGSK-3 α/β levels, respectively. However, total GSK-3 α/β levels were also altered. These findings are consistent with previous results with antipsychotics and raclopride but complicate interpretation of the data¹⁶. At this time it is not clear why total GSK-3 α/β levels are altered, although the *in vitro* results indicate it may be a consequence of Dvl-3¹⁷.

The *in vitro* and Akt-1 knockout results suggest that Akt does not directly affect Wnt signalling and parallels previous *in vitro* findings^{39, 40}. Overexpression of activated

Akt, pharmacological inhibition of PI3-K/Akt, knockdown of Akt-1 by siRNA and Akt-1 knockout mice all had no effect on β -catenin although changes in pGSK-3 were consistently observed. However, recent studies have suggested that Akt may contribute to TCF/LEF activation in Dvl-1 or Wnt-1 stimulated cells^{40, 42}. Although the current study found no synergistic effect of PI3-K/Akt and Dvl-3 on overall β -catenin levels, differences in TCF/LEF activation cannot be excluded. Collectively, the data suggests that changes in pAkt are responsible for the alterations in pGSK-3 but Akt does not regulate β -catenin.

Traditionally, activation of the Wnt pathway has been described as binding of a Wnt ligand to an appropriate Frizzled receptor leading to the phosphorylation and thus activation of Dvl^{22, 43}. However, it has also been shown that increased levels of Dvl are sufficient to induce the formation of Dvl polymers and initiate the recruitment of Dvl to the plasma membrane leading to activation of the Wnt pathway^{44, 45}. Therefore, an increase or decrease in Dvl-3 protein levels may be sufficient for regulating the activity of the Wnt pathway consistent with the observations that Dvl-3 overexpression and knockdown of Dvl-3 with siRNA causes increases and decreases in β -catenin levels respectively. In addition to β -catenin, Dvl-3 overexpression led to elevated levels of GSK-3 α/β and pGSK-3 α/β as well as increased pAkt levels consistent to what was observed following raclopride treatment. It is unclear how increasing Dvl-3 protein levels cause an increase in Akt phosphorylation. However, the results are consistent with previous cell culture studies showing that activation of the Wnt pathway by Dvl-1 overexpression or the addition of Wnt-3a to the media can cause increased Akt activity in NIH 3T3 cells^{40, 46}. The knockdown of Dvl-3 *in vitro* resulted in a pattern of change that

was partially consistent with quinpirole treatment, suggesting that Dvl-3 may be responsible for changes in GSK-3 α/β and β -catenin following activation of the D₂DR. Surprisingly however, knockdown of Dvl-3 did not decrease pAkt levels, although increases were observed following overexpression of Dvl-3. The reason for this disparity is not clear. Reduction in Akt-1 protein levels caused by Dvl-3 knockdown was also surprising but intriguing. In non-neuronal cell lines it has been shown that Akt-1 mRNA and protein levels are regulated by β -catenin signalling^{47, 48} and Akt-1 levels may be reduced in schizophrenia². Collectively, the data suggests that the D₂DR regulates Wnt signalling via Dvl-3 and that Dvl-3 may also regulate Akt signalling.

Dvl has been shown to be responsible for the translocation of Wnt pathway proteins to the Frizzled receptor⁴⁴. The overexpression of Dvl-3 in SH-SY5Y cells caused increased association between GSK-3 β and the D₂DR complex. These results suggest that Dvl-3 may be involved in the increase or decrease association of GSK-3 α/β and the D₂DR complex following raclopride and quinpirole treatment, respectively. The consequences of the altered association between GSK-3 α/β and the D₂DR are not clear. However, it has been shown that activation of the serotonin 1B (5-HT1B) receptor leads to an increased association with GSK-3 and that GSK-3 mediated phosphorylation of the 5-HT1B receptor helped retain the receptor at the cell surface⁴⁹. Others have also demonstrated that GSK-3 effects the activity of the LPR6, the co-receptor for the Wnt pathway via phosphorylation^{50, 51}. Therefore, it is possible that the translocation of GSK-3 to the D₂DR complex may be involved in regulating D₂DR activity.

While there is an association between GSK-3 α/β and the D₂DR, it is unclear how these two proteins interact. It is possible that Dvl-3 mediates the interaction since Dvl-3

is bound to the D₂DR complex¹⁷ and can act as a scaffold for other proteins. The D₂DR is a well known G-coupled receptor and Dvl has recently been shown to possess a G protein binding domain^{52, 53}. In addition, β -arrestins may play a role in coupling the D₂DR to Dvl-3 and GSK-3. β -arrestin 2 is involved in mediating the effects of D₂DR activity³⁷ and an interaction between β -arrestin and Dvl has been reported^{54, 55}. Furthermore, β -arrestin 2 has been shown to be important for the regulation of Akt by the D₂DR *in vivo*³⁷. However, an *in vitro* study showed that antipsychotics block the translocation of β -arrestin 2 to the D₂DR⁵⁶ and Wnt activation would be expected to cause translocation of Dvl-3 and GSK-3 to the D₂DR. Finally, DISC-1 was recently shown to directly interact with GSK-3 and regulate β -catenin levels⁵⁷. Reduced levels of DISC-1 were shown to reduce β -catenin levels whereas increased levels of DISC-1 increased β -catenin levels. Disruption of DISC-1 in mice was also shown to cause schizophrenia-like behavioural deficits that could be corrected by inhibition of GSK-3⁵⁷. However, it is not clear if DISC-1 interacts with the D₂DR. Therefore, there are several potential mechanisms through which GSK-3 could interact with the D₂DR but additional studies will be needed to understand the precise nature of the relationship between D₂DR, Akt and Wnt proteins.

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CHAPTER THREE

The effect of neuropsychiatric drugs and amphetamine on GSK- 3 related signalling¹

¹ Sections 3.1-3.5 has been submitted to Journal of Neurochemistry.

3.1 INTRODUCTION

GSK-3 is a constitutively active protein kinase that has been implicated in schizophrenia and antipsychotic drug action. Decreases in total and phosphorylated GSK-3 β protein levels and a reduction in GSK-3 β mRNA levels have been reported in the brain of schizophrenia patients^{1,2}. GSK-3 protein levels and/or phosphorylation state were also found to be decreased in several animal models of schizophrenia including amphetamine-sensitization, the ventral hippocampal lesion model and the DAT-KO mice^{3,4}. Furthermore, hyperlocomotor activity in DAT-KO mice, is reduced to wild-type levels following treatment with inhibitors of GSK-3⁵. Antipsychotics, such as haloperidol, clozapine and risperidone, in contrast, have been shown to increase total and phosphorylated GSK-3 α/β (Ser21/9) protein levels in the PFC and/or STR of rats following repeated administration^{6,7}. Consequently, it has been suggested GSK-3 may play a role in the manifestation and amelioration of some of the symptoms of schizophrenia. However, in addition to schizophrenia, GSK-3 has also been implicated in mood disorders and depression and mood stabilizers and antidepressants have been shown to regulate the phosphorylation state of GSK-3 *in vivo*⁸⁻¹¹. Antipsychotics, mood stabilizers and antidepressants all have different clinical profiles and only antipsychotics alleviate the positive symptoms of schizophrenia. Consequently, it has been suggested that there may be something unique in the way GSK-3 is affected by antipsychotics compared to drugs used to treat other neuropsychiatric disorders¹².

GSK-3 function is regulated by several mechanisms including, phosphorylation, association of GSK-3 with specific protein complexes (i.e. β -catenin phosphorylation complex) and subcellular localization^{13,14}. Several signalling cascades are responsible

for controlling the characteristics of GSK-3 but the Akt and Wnt pathways are of particular interest given that they have both been linked to schizophrenia and antipsychotic drug action^{7, 15}. Akt and the Wnt pathway negatively regulate GSK-3, although via different mechanisms. Akt reduces GSK-3 kinase activity through phosphorylation of both isoforms of GSK-3 (ser21/9, GSK-3 α /GSK-3 β)¹⁶. The Wnt pathway regulates GSK-3 through its association with the β -catenin phosphorylation complex. GSK-3 associates with several proteins, including Axin and APC, forming a β -catenin phosphorylation complex that facilitates the phosphorylation and eventual degradation of the transcription factor, β -catenin. Activation of the Wnt pathway leads to the phosphorylation and/or increase in protein levels of the signal transducer protein Dishevelled (Dvl). Dvl negatively regulates GSK-3 by interfering with the β -catenin phosphorylation complex preventing the phosphorylation of β -catenin. The resultant accumulation of β -catenin in the cytoplasm followed by its translocation to the nucleus leads to modifications in TCF/LEF mediated gene transcription¹⁷.

Previous studies have shown that repeated treatment with the antipsychotics haloperidol and/or clozapine target Akt and Wnt proteins in the PFC/frontal cortex^{6, 7, 15}. However, given that other neuropsychiatric drugs may regulate GSK-3 signalling, the current study investigated whether the mood stabilizers, lithium and valproic acid and/or the antidepressants, fluoxetine and imipramine mimic the effects of antipsychotics on Akt and Wnt signalling or if there are key differences in the response of these drugs. In addition, Akt and Wnt pathway proteins were also examined following repeated treatment with the psychomimetic amphetamine.

3.2 MATERIAL AND METHODS

3.2.1 Drug Paradigm

Adult male Sprague Dawley rats 14+ weeks of age (Charles River) were housed in pairs in a 12 hour light/dark cycle with free access to food and water. Rats were injected once daily with haloperidol (0.5mg/kg, i.m.), clozapine (25mg/kg, s.c.) or appropriate vehicle for 14 days (n=8 rats/treatment). The doses of haloperidol and clozapine were based on a previous study showing changes in the proteins of interest in the PFC and/or STR⁶. To examine drug specificity, rats were injected with fluoxetine (10mg/kg, s.c., Sigma-Aldrich), imipramine (10mg/kg, s.c., Sigma-Aldrich) or appropriate vehicle for 14 days (n=8 rats/treatment). Fluoxetine and imipramine were chosen since they represent two different classes of antidepressants, and because they were found to alter phosphorylated GSK-3 levels following acute treatment¹⁰. In addition, rats were treated with the mood stabilizers, lithium or valproic acid via their chow or drinking water (n=8 rats/treatment). Rats were fed chow containing 2.0g/kg LiCl or chow lacking LiCl for 30 days ad libitum. Valproic acid (12g/L, Sigma Aldrich) was mixed in the rats' drinking water and supplemented with saccharin (300mg/L, Sigma Aldrich) for palatability, while the control rats received drinking water supplemented with saccharin alone for 30 days. The doses and delivery method for lithium and valproic acid were selected based on previous studies showing they achieve clinically relevant serum concentrations in Sprague-Dawley rats^{18, 19}. Finally, rats were injected with amphetamine (5mg/kg, i.p.) or saline (vehicle) once daily for 10 days (n=8 rats/treatment). The PFC and STR of rats were isolated 2 hours following final treatment of haloperidol, clozapine, fluoxetine and imipramine. The post-injection times were chosen as they were experimentally found to be optimal for

detecting changes in expression of Akt and Wnt pathway proteins following antipsychotics^{7, 15} and similar to an acute study showing changes in phosphorylated GSK-3 following antidepressant treatments¹⁰. For amphetamine, a 4-hour post-injection interval was selected based on a previous study showing reductions in β -catenin and GSK-3 in the ventral midbrain²⁰. Every effort was made to reduce the number of rats used in the experiments and to minimize pain and suffering. All experiments were reviewed and approved by the University of Western Ontario Animal research ethics committee in compliance with Canadian Council on Animal Care.

3.2.2 Western blotting

Isolation of total protein from the PFC and STR, protein quantification and western blotting was performed as previously outlined⁷. The PFC and STR were selected since both regions have been implicated in neuropsychiatric disorders and have been investigated with neuropsychiatric drugs. Densitometry values were obtained from X-ray film using a grey scale calibrated scanner (Epson) and Kodak 1-D Molecular Imaging software. Densitometry values were corrected for α -tubulin and expressed as a percentage of control. Data was analyzed using an ANOVA followed by Tukey's multiple comparison test or Student's *t*-test as appropriate. The source and dilution of the antibodies used in the study were as follows; Dvl-1 (Santa Cruz Biotechnology, 1:100), Dvl-2 (Chemicon, 1:1000), Dvl-3 (Santa Cruz Biotechnology, 1:100), GSK-3 (Santa Cruz Biotechnology, 1:300), phospho-GSK-3 (Cell Signalling Technology, 1:1000), β -catenin (Sigma-Aldrich, 1:20000), Akt (Cell Signalling Technology, 1:1250), phospho-

Akt Ser473 (Cell Signalling Technology, 1:1000), phospho-Akt Thr308 (Upstate, 1:1000) and α -tubulin (Sigma-Aldrich, 1:120000).

3.2.3 Co-Immunoprecipitations

Protein was isolated from the PFC of haloperidol, clozapine, lithium, valproic acid and amphetamine treated rats along with their appropriate vehicle controls using a non-denaturing lysis buffer containing protease (Complete Mini, Roche Diagnostic) and phosphatase inhibitors (cocktail Set 2, Sigma-Adrich; cocktail Set IV, Calbiochem). Co-immunoprecipitations (co-IPs) were performed with the ExactaCruz system (SantaCruz Biotechnology) according the manufacturers instructions using 500 μ g of protein and antibodies specific for the D₂DR (3 μ g/IP, Santa Cruz Biotechnology, rabbit and goat polyclonal) as outlined previously⁶. Co-IP samples and lysate used in the co-IP's were run on western blots and probed for Akt, GSK-3, Dvl-3 or the D₂DR. The blots were quantified using the densitometry values as described above.

3.3 RESULTS

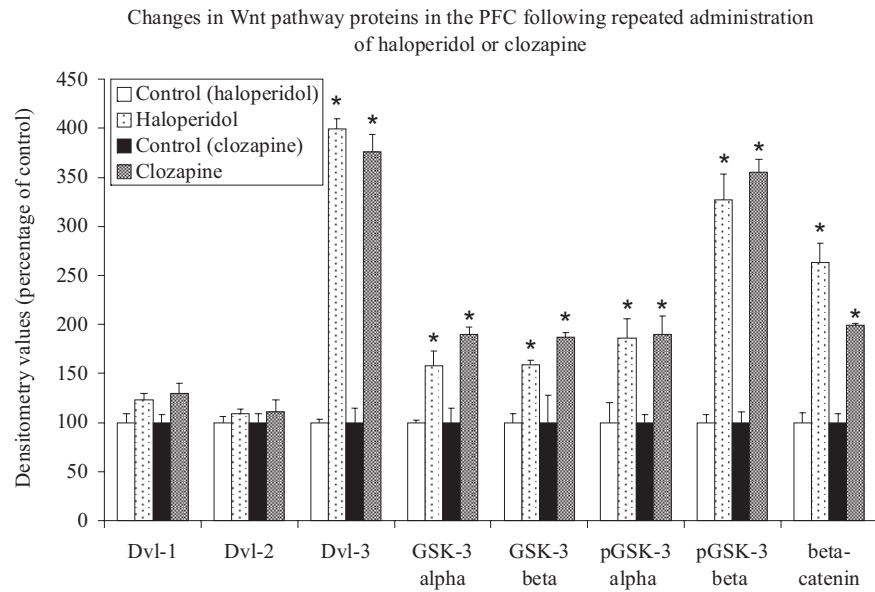
3.3.1 Differential effects of haloperidol and clozapine in the PFC and STR

In the PFC, repeated haloperidol or clozapine injections increased the protein levels of Dvl-3, GSK-3 α/β , pGSK-3 α/β and β -catenin but not Dvl-1 or Dvl-2 (Figure 3.1a) consistent with previous results⁶. Increases in pAkt Thr308 but not pAkt Ser473 or total Akt were also observed (Figure 3.1b and c). In the STR, repeated haloperidol administration increased Dvl-3, GSK-3 α/β , pGSK-3 α/β and β -catenin but did not affect Dvl-1 or Dvl-2 protein levels (Figure 3.2a and b). Clozapine treatment increased pGSK-3

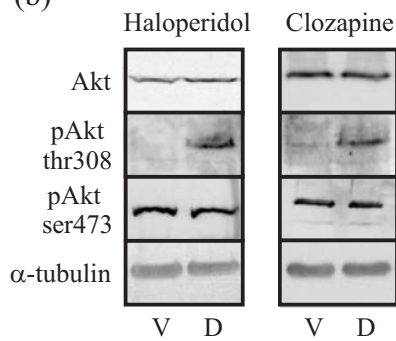
Figure 3.1: Changes in the total protein levels and/or phosphorylation state of Wnt pathway proteins and Akt in the PFC of rats treated with haloperidol or clozapine.

(a) Graph of densitometry values for Dvl-1, Dvl-2, Dvl-3, GSK-3 α/β , pGSK-3 α/β and β -catenin following the treatment of haloperidol, clozapine or an appropriate control. (b) Representative western blots and (c) graph of densitometry values for Akt, pAkt Ser473 and pAkt Thr308 following the treatment of haloperidol, clozapine or an appropriate control. For simplicity, the graphs for haloperidol and clozapine are shown using a single control. Statically significant changes are denoted by the asterisk ($p < 0.05$, $n=8$) and error bars denote the standard error of the mean. For each representative western blot, the left band represents the vehicle (V) while the right band shows the drug treatment (D).

(a)



(b)



(c)

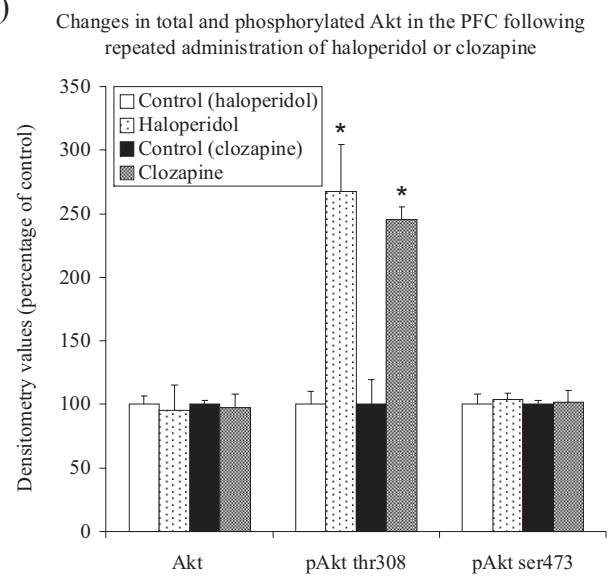
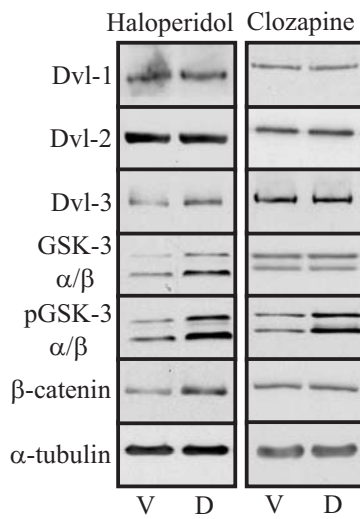


Figure 3.2: Changes in the total protein levels and/or phosphorylation state of Wnt pathway proteins and Akt in the STR of rats treated with haloperidol or clozapine.

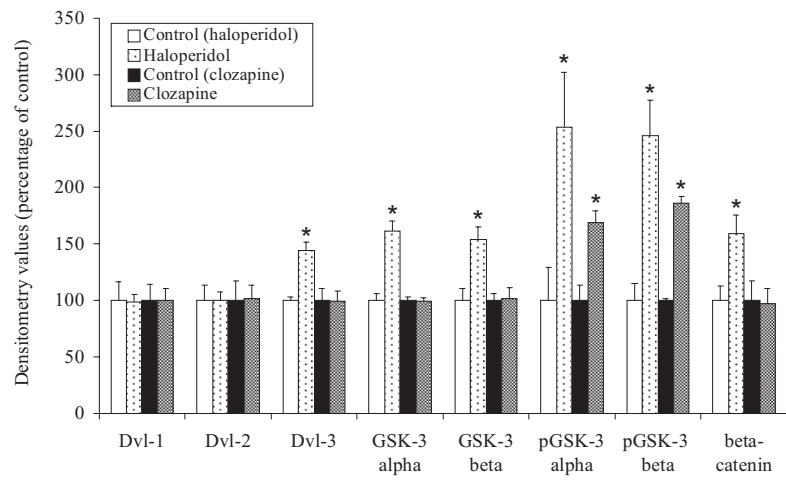
(a) Representative western blots and (b) graph of densitometry values for Wnt pathway proteins following the treatment of haloperidol, clozapine or an appropriate control. (c) Representative western blots and (d) graph of densitometry values for total and phosphorylated levels of Akt following the treatment of haloperidol, clozapine or an appropriate control. For each representative western blot, the left band represents the vehicle (V) while the right band shows the drug treatment (D). For simplicity, the graphs show a single control for haloperidol and clozapine. The densitometry values are expressed as a percentage of control with a statistically significance difference denoted by an asterisk ($p < 0.05$, mean \pm SEM, n=8).

(a)

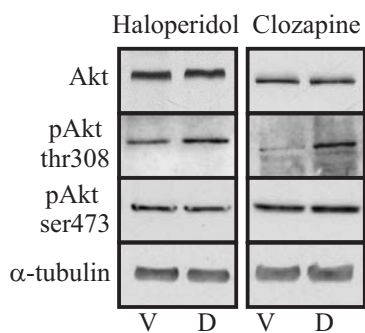


(b)

Changes in Wnt pathway proteins in the STR following repeated administration of haloperidol or clozapine

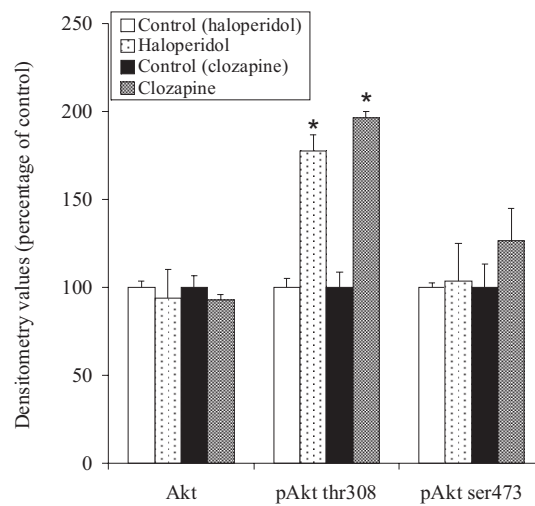


(c)



(d)

Changes in total and phosphorylated Akt in the STR following repeated administration of haloperidol or clozapine



protein levels but had no effect on Dvl-1, Dvl-2, Dvl-3, GSK-3 α/β or β -catenin (Figure 3.2a and b). Increases in pAkt Thr308 protein levels were observed for both antipsychotics but no change in pAkt Ser473 or total Akt levels were found following either treatment (Figure 3.2c and d). Collectively, the data revealed that haloperidol and clozapine had similar effects in the PFC but generated a distinct pattern of change in the STR.

3.3.2 The effects of mood stabilizers and antidepressants on Akt and the Wnt pathway

Western blotting revealed that lithium increased pGSK-3 α/β but had no effect on Dvl-1, Dvl-2, Dvl-3, GSK-3 α/β or β -catenin in the PFC (Figure 3.3a). In addition, an increase in pAkt Ser473 was also observed but no change in pAkt Thr308 or total Akt levels were found (Figure 3.3b). Valproic acid had no effect on Akt or any of the Wnt pathway proteins tested in the PFC (Figure 3.3a and b). In the STR, lithium upregulated GSK-3 α/β , pGSK-3 α/β and β -catenin but had no effect on any of the isoforms of Dvl (Figure 3.3c). In addition, an increase in pAkt Ser473 protein levels was observed but no change in Akt and pAkt Thr308 was found (Figure 3.3d). Valproic acid increased pGSK-3 α/β and pAkt Ser473 levels but had no effect on Dvl, GSK-3 α/β , β -catenin Akt or pAkt Thr308 in the STR (Figure 3.3c and d).

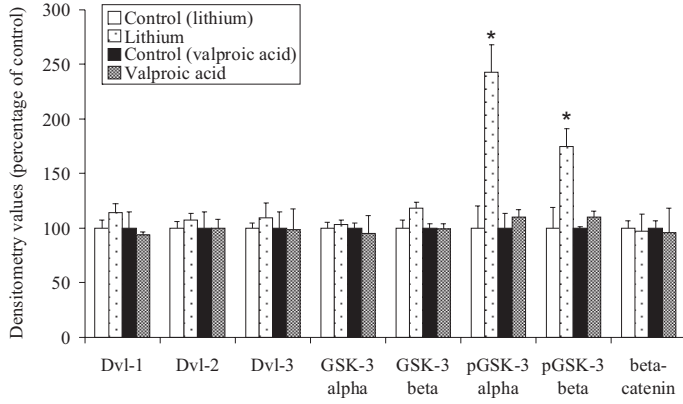
With respect to fluoxetine and imipramine, results from the PFC showed that fluoxetine and imipramine increased pGSK-3 α/β (Figure 3.4a) and pAkt Ser473 levels only (Figure 3.4b). Identical results were obtained in the STR (Figure 3.4c and d). Collectively, with the exception of lithium effect's on β -catenin in the STR, mood

FIGURE 3.3: Changes in the total protein levels and/or phosphorylation state of Wnt pathway proteins and Akt in the PFC and STR of rats treated with lithium or valproic acid.

Graphs of densitometry values for (a) Wnt pathway proteins and (b) total and phosphorylated Akt in the PFC following lithium and valproic acid treatment. Graphs of densitometry values for (c) Wnt pathway proteins and (d) total and phosphorylated Akt in the STR following lithium and valproic acid treatment. The densitometry values are shown as a percentage of control with a statistical significance denoted by an asterisk ($p < 0.05$, mean \pm SEM, $n=8$).

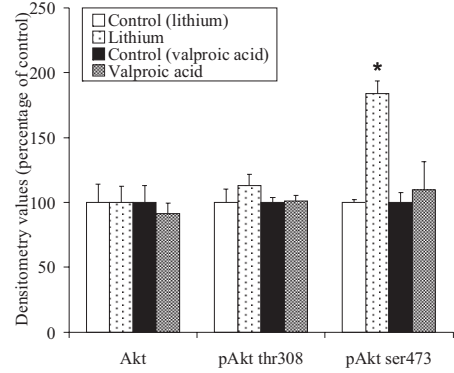
(a)

Changes in Wnt pathway proteins in the PFC following lithium or valproic acid treatment



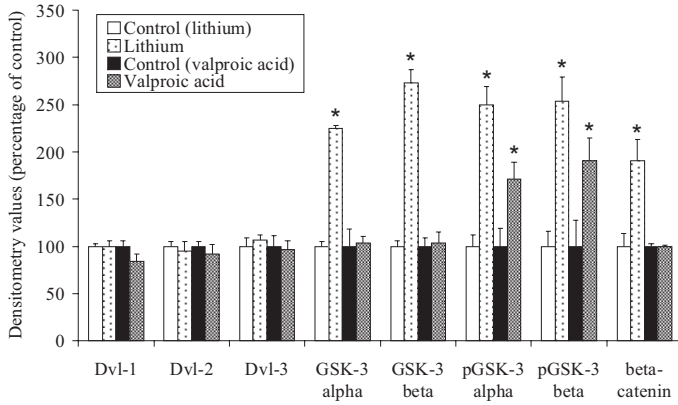
(b)

Changes in total and phosphorylated Akt in the PFC following lithium or valproic acid treatment



(c)

Changes in Wnt pathway proteins in the STR following lithium or valproic acid treatment



(d)

Changes in total and phosphorylated Akt in the STR following lithium or valproic acid treatment

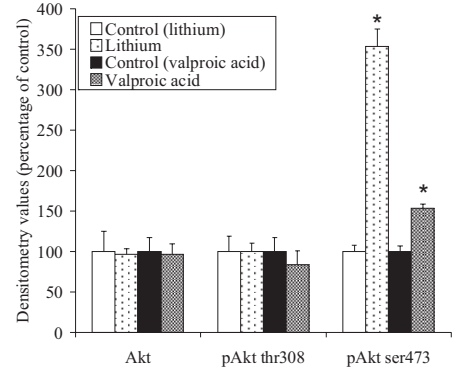
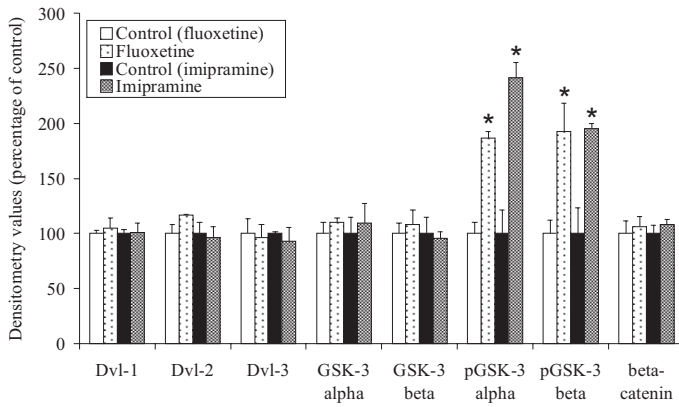


Figure 3.4: Changes in the total protein levels and/or phosphorylation state of Wnt pathway proteins and Akt in the STR of rats treated with fluoxetine or imipramine.

Graphs of densitometry values for (a) Wnt pathway proteins and (b) total and phosphorylated Akt in the PFC of fluoxetine or imipramine treated rats. Graphs of densitometry values for (c) Wnt pathway proteins and (d) total and phosphorylated Akt in the STR of fluoxetine or imipramine treated rats. The densitometry values are expressed as a percentage of control with a statistical significance denoted by the asterisk ($p < 0.05$, mean \pm SEM, $n=8$).

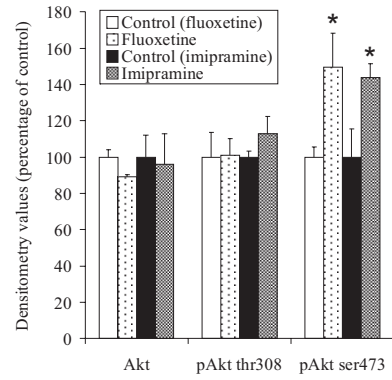
(a)

Changes in Wnt pathway proteins in the PFC following repeated administration of fluoxetine or imipramine



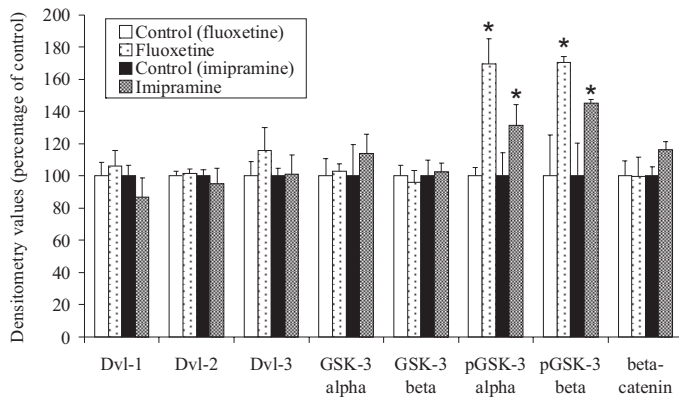
(b)

Changes in total and phosphorylated Akt in the PFC following repeated administration of fluoxetine or imipramine



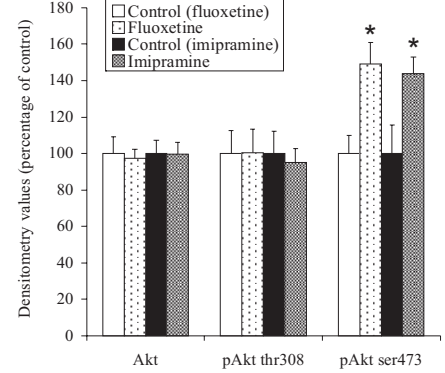
(c)

Changes in Wnt pathway proteins in the STR following repeated administration of fluoxetine or imipramine



(d)

Changes in total and phosphorylated Akt in the STR following repeated administration of fluoxetine or imipramine



stabilizers and antidepressants do not affect the Wnt pathway (i.e β -catenin) but have overlapping effects on pGSK-3 and pAkt Ser 473.

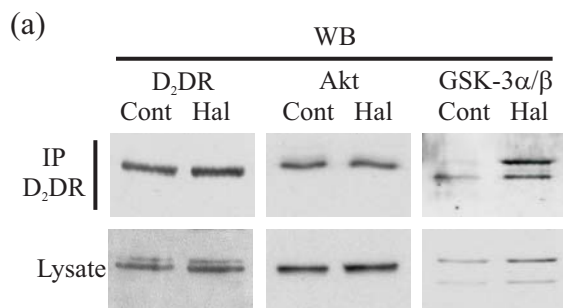
3.3.3 The association between the D₂DR and GSK-3 α/β

A recent study showed that Akt and GSK-3 α/β are associated with the D₂DR in the PFC of untreated rats⁶ and that there is an increase in the amount of GSK-3 α/β bound to the D₂DR following treatment with the D₂DR antagonist, raclopride (chapter 2). Consequently, co-IP's were performed to determine if haloperidol or clozapine treatment alters the association between the D₂DR and Akt or D₂DR and GSK-3. There were no alterations in the association between the D₂DR complex and Akt following repeated haloperidol (103.6% \pm 14.0, n=6) or clozapine (110.5% \pm 8.9, n=6) treatment (Figure 5a and c, upper panel). Furthermore, the antipsychotic treatments did not affect the total protein levels of Akt (haloperidol, 120.8% \pm 11.1; clozapine, 104.5% \pm 8.9, n=6) or D₂DR (haloperidol, 108.5% \pm 8.1; clozapine, 115.0% \pm 6.4, n=6) (Figure 3.5a and c, lower panel). However, both antipsychotics caused an increase in the association between GSK-3 α/β and the D₂DR complex, as well as total GSK-3 α/β protein levels (Figure 3.5a and c). The densitometry values showed the increased association between D₂DR and GSK-3 α/β was greater than the increase in total GSK-3 α/β protein levels, suggesting there may be an enrichment of GSK-3 α/β at the D₂DR following haloperidol (Figure 3.5b) or clozapine (Figure 3.5d).

Given that an association between the D₂DR complex and Dvl-3 has been reported⁶ and it has been shown that activation of Dvl leads to its translocation to receptor complexes,^{21, 22} co-IPs were also performed to determine if the association

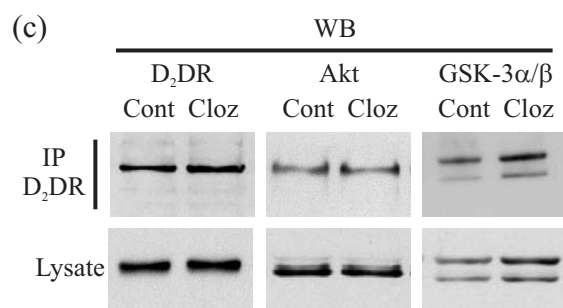
Figure 3.5: Co-IP experiments showing the association between D₂DR and Akt, GSK-3 and/or Dvl-3 in the PFC of haloperidol, clozapine, lithium and valproic acid treated rats.

Protein samples isolated from the PFC of (a,e) haloperidol (Hal), (b,e) clozapine (Cloz), (g) lithium (Li) and (i) valproic acid (Val) were immunoprecipitated (IP) with a D₂DR antibody. IP samples were subjected to western blotting (WB) and probed for D₂DR, Akt GSK-3 and/or Dvl-3 (upper panel). WB was performed using the same lysate as the co-IPs and probed for D₂DR, Akt, GSK-3 α/β and/or Dvl-3 (lower panel). Table of the densitometry values from the co-IPs for D₂DR/GSK-3 α/β and total GSK-3 α/β protein levels for the (b) haloperidol, (d) clozapine, (h) lithium and (j) valproic acid samples. (f) Table of the densitometry values from the co-IPs for D₂DR/Dvl-3 and total Dvl-3 protein levels for the haloperidol and clozapine samples. Statistically significant changes are represented by an asterisk ($p < 0.05$, mean \pm SEM, n=6).



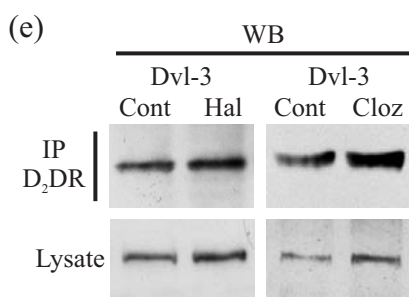
(b)

		Control	Haloperidol
IP	GSK-3 α	100 \pm 13.8	246.6 \pm 20.0*
	GSK-3 β	100 \pm 5.6	209.7 \pm 10.5*
Lysate	GSK-3 α	100 \pm 8.0	153.2 \pm 10.8*
	GSK-3 β	100 \pm 4.0	146.0 \pm 6.2*



(d)

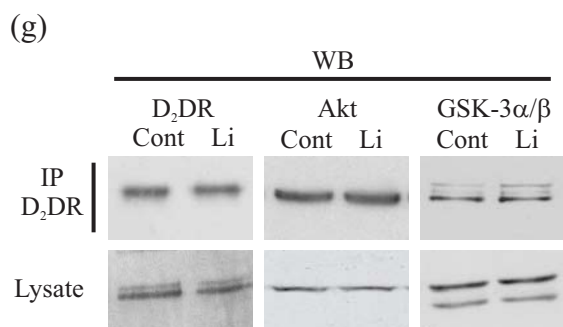
		Control	Clozapine
IP	GSK-3 α	100 \pm 9.6	214.5 \pm 13.0*
	GSK-3 β	100 \pm 6.5	171.9 \pm 6.3*
Lysate	GSK-3 α	100 \pm 6.7	140.5 \pm 9.0*
	GSK-3 β	100 \pm 6.2	142.7 \pm 7.9*



(f)

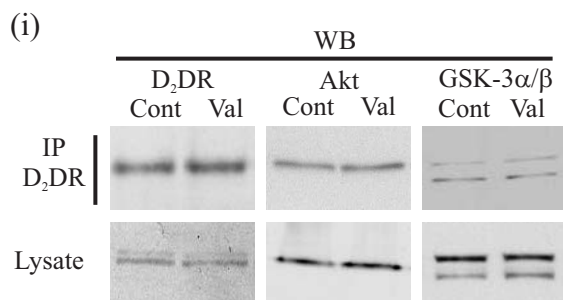
		Control	Haloperidol
IP	Dvl-3	100 \pm 7.7	157.2 \pm 6.6*
Lysate	Dvl-3	100 \pm 5.4	143.7 \pm 3.6*

		Control	Clozapine
IP	Dvl-3	100 \pm 7.3	159.0 \pm 7.5*
Lysate	Dvl-3	100 \pm 4.9	151.2 \pm 6.4*



(h)

		Control	Lithium
IP	GSK-3 α	100 \pm 20.2	114.0 \pm 22.0
	GSK-3 β	100 \pm 13.1	85.7 \pm 12.4
Lysate	GSK-3 α	100 \pm 9.0	107.0 \pm 4.1
	GSK-3 β	100 \pm 5.6	112.2 \pm 5.4



(j)

		Control	Valproic acid
IP	GSK-3 α	100 \pm 7.7	103.9 \pm 9.9
	GSK-3 β	100 \pm 13.3	93.7 \pm 4.9
Lysate	GSK-3 α	100 \pm 6.9	84.7 \pm 7.2
	GSK-3 β	100 \pm 12.8	112.3 \pm 3.5

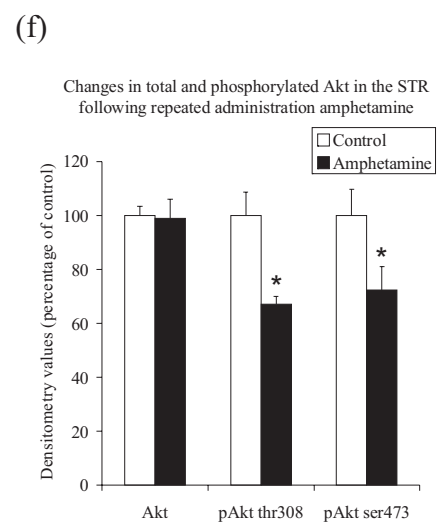
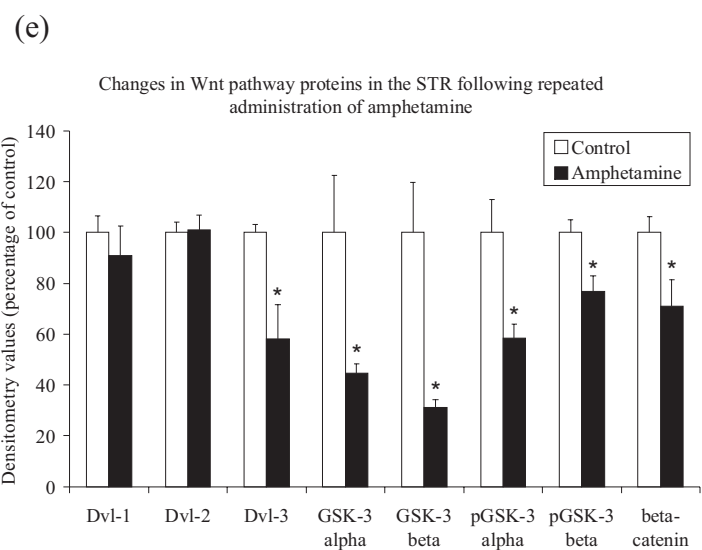
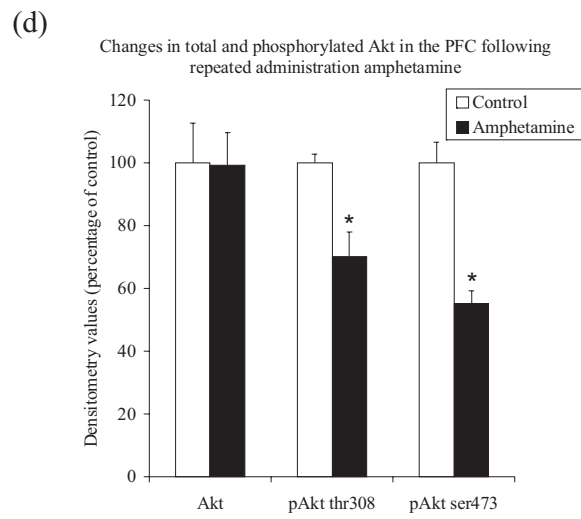
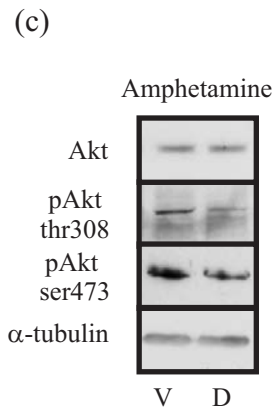
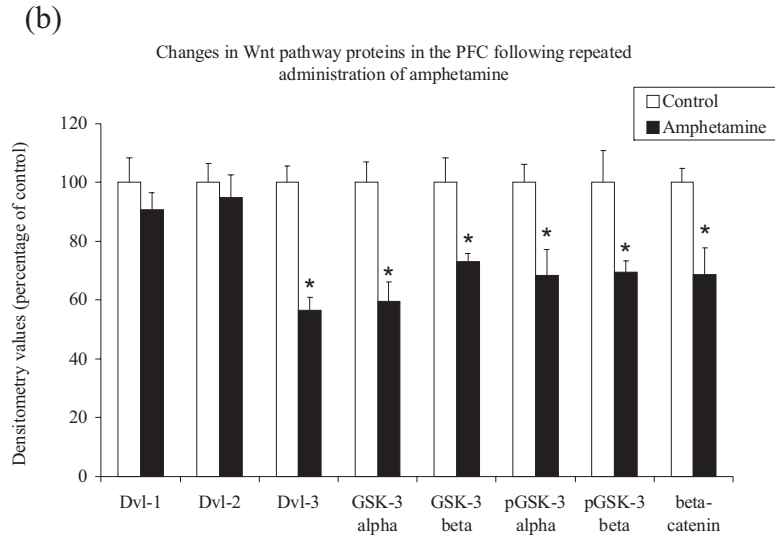
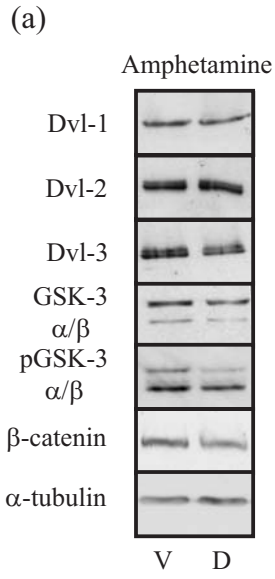
between the D₂DR and Dvl-3 were altered by antipsychotics. The co-IPs revealed an increased association between Dvl-3 and the D₂DR complex that is comparable to the increase in Dvl-3 protein levels following haloperidol and clozapine treatments (Figure 3.5e and f). Both lithium and valproic acid were also examined. There were no significant changes in the association between the D₂DR complex and Akt following lithium (99.5% ± 8.7, n=6) or valproic acid treatment (96.8% ± 4.8, n=6) (Figure 3.5g and i, upper panel). No significant changes were detected for total D₂DR (lithium, 105.6% ± 10.2; valproic acid 103.4% ± 9.8, n=6) or Akt (lithium, 99.5% ± 8.7; valproic acid 96.8% ± 4.8, n=6) protein levels following either drug treatments (Figure 3.5g and i, lower panel). Furthermore, the association between the D₂DR complex and GSK-3 α/β and total GSK-3 α/β protein levels were unaffected by lithium (Figure 3.5g and h) or valproic acid in the PFC (Figure 3.5i and j). Collectively, the results suggest that changes in the association between GSK-3 α/β and the D₂DR may be specific to antipsychotics.

3.3.4 The effects of amphetamine on Akt and the Wnt pathway

Repeated amphetamine administration in rodents is frequently used as a partial model of schizophrenia (Featherstone 2007) and was used in the current study to determine if changes in Akt and Wnt signalling paralleled those observed in schizophrenia. Repeated amphetamine treatment caused a decrease in Dvl-3, GSK-3 α/β , pGSK-3 α/β and β -catenin but did not affect Dvl-1 or Dvl-2 in the PFC (Figure 3.6a and b) and STR (Figure 3.6e). Decreased levels of pAkt Thr308 and pAkt Ser473 were also observed but no change in total Akt was detected in the PFC (Figure 3.6c and d) and STR (Figure 3.6f).

Figure 3.6: Changes in the total protein levels and/or phosphorylation state of Wnt pathway proteins and Akt in the PFC and STR of rats treated with amphetamine.

(a) Representative western blots and (b) graph of densitometry values of Wnt pathway proteins following treatment with amphetamine. (c) Representative western blots and (d) graph of densitometry values of total and phosphorylated Akt in the PFC of amphetamine treated rats. Graphs of densitometry values of (e) Wnt pathway proteins and (f) total and phosphorylated Akt in the STR of amphetamine treated rats. For each representative western blot, the left band represents the vehicle (V) while the right band shows the drug treatment (D). The densitometry values are expressed as a percentage of control with a statistically significance difference denoted by an asterisk ($p < 0.05$, mean \pm SEM, $n=8$).



Co-IPs revealed that repeated amphetamine treatment had no effect on the association between D₂DR and Akt or on total D₂DR or Akt protein levels (Figure 3.7a). A significant decrease in the association between the D₂DR and GSK-3 α/β and in total GSK-3 α/β protein levels was observed (Figure 3.7a). The decrease in the association between D₂DR and GSK-3 α/β was greater than the reduction in total GSK-3 α/β protein levels, suggesting the change in the amount of GSK-3 α/β at the D₂DR complex is not due to the reduced GSK-3 α/β protein levels alone (Figure 3.7b). Collectively, the results show that changes in Akt and Wnt signalling in the amphetamine model are functionally opposite to antipsychotic treatment.

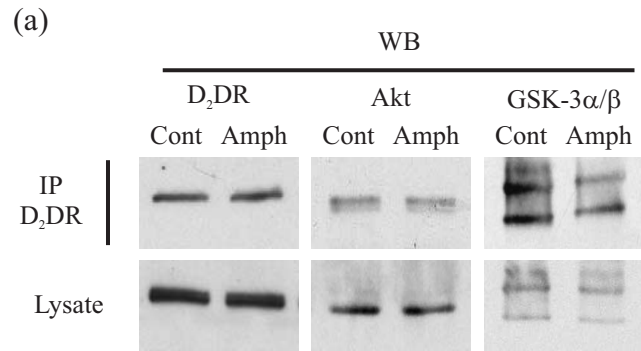
3.4 DISCUSSION

The current study suggests that there may be some important similarities and differences in GSK-3 signalling between antipsychotics, mood stabilizers and antidepressants. Only the antipsychotic treatments increased the protein levels of Dvl-3, β -catenin and pAkt Thr308 in the PFC and/or STR suggesting that antipsychotics activate Akt and the Wnt pathway in these regions of the brain. In contrast to antipsychotics, the mood stabilizers and antidepressants increased pAkt Ser473 in the PFC and/or STR. However, all of the neuropsychiatric drugs induced a similar changes pGSK-3 α/β .

The changes on Akt and Wnt signalling induced by the antipsychotic treatments are consistent with previous studies showing increased pAkt Thr308 levels in haloperidol treated mice¹⁵ and increases in β -catenin, GSK-3 α/β and Dvl-3 in the PFC and/or STR of haloperidol and clozapine treated rats^{6, 7}. Changes in pGSK-3 α/β are likely due to the activation of Akt since it is well established that GSK-3 α/β is phosphorylated by Akt¹⁶.

Figure 3.7: Co-IP experiments showing the association of between D₂DR and Akt and GSK-3 in the PFC of rats treated with amphetamine.

(a) Immunoprecipitations (IP) were performed using protein obtained from the PFC of amphetamine or control treated rats with a D₂DR antibody. IP samples were subjected to western blotting (WB) and probed using an antibody specific for D₂DR, Akt or GSK-3 α / β (upper panels). WB were conducted using the same lysate as the co-IPs and probed for D₂DR, Akt and GSK-3 α / β (lower panel). (b) Table of densitometry values from the co-IPs for D₂DR/GSK-3 α / β and total GSK-3 α / β protein levels. Statistically significant changes are represented by an asterisk ($p < 0.05$, mean \pm SEM, $n=6$).



(b)

		Control	Amphetamine
IP	GSK-3 α	100 \pm 7.4	59.3 \pm 9.1*
	GSK-3 β	100 \pm 25.3	45.8 \pm 6.9*
Lysate	GSK-3 α	100 \pm 4.2	77.5 \pm 3.4*
	GSK-3 β	100 \pm 5.2	69.5 \pm 10.8*

The state of GSK-3 α/β activity was unclear since antipsychotics altered both total and pGSK-3 α/β levels. The reason for the change in total GSK-3 α/β protein levels is unknown, although it is a consistent finding following repeated antipsychotic treatment^{6, 7}. It is interesting to note, however, that increases in total GSK-3 α/β levels parallel increases in β -catenin. Further experiments will need to be conducted to determine if there is a link between the two. In addition to changes in Wnt signalling and pAkt Thr308, antipsychotics also increased the levels of Dvl-3 and GSK-3 α/β that are associated with the D₂DR complex. Interestingly, previous studies have shown that overexpression of Dvl/Dvl-3 is sufficient to activate the Wnt pathway and that following activation, Dvl translocates to the membrane along with other Wnt signalling proteins²¹ providing a possible mechanism explaining how GSK-3 α/β may be translocated to the D₂DR complex. At this time, the consequence associated with increased levels of GSK-3 or Dvl-3 at the D₂DR complex are unclear. However, a recent study showed that GSK-3 interacts with serotonin 1B (5-HT_{1B}) receptor and this interaction could be increased by activation of the receptor leading to the retention of the receptor at the cell surface²³. In addition, it has been shown that GSK-3 can phosphorylate LRP6, co-receptor for the Wnt pathway leading to an increase in receptor activation²⁴. Since antipsychotics increase the translocation of GSK-3 to the D₂DR, GSK-3 may induce a functional change at the receptor. Additional experiments will be required to determine if this is indeed the case.

Several studies including the current one has shown that antipsychotic increase β -catenin levels^{6, 7, 20}. Increased β -catenin levels were also induced by lithium treatment in the STR, suggesting that β -catenin may not be linked to the clinical effectiveness of antipsychotics since lithium is not an effective antipsychotic. However, several

behavioural studies have suggested that β -catenin may be involved in schizophrenia. Disrupted in schizophrenia-1 (DISC-1), a potential schizophrenia susceptibility gene²⁹, negatively regulates GSK-3 and stabilizes β -catenin levels³⁰. Mice with DISC-1 loss of function in the hippocampus showed hyperlocomotion in response to a novel environment that was normalized by pharmacological inhibition of GSK-3 suggesting that changes in β -catenin may have rescued the behaviour³⁰. In addition, amphetamine-induced hyperlocomotion is attenuated in β -catenin overexpressing mice³¹. It has also been shown that lithium can regulate dopamine-dependent behaviours in mice suggesting there may be some functional overlap between lithium and D₂DR antagonists such as antipsychotics. Additional experiments will be necessary to understand the role of β -catenin in schizophrenia and antipsychotic drug efficacy.

Mood stabilizers and antidepressants, with the exception of lithium's effect on β -catenin in the STR, only targeted pGSK-3 α/β and pAkt Ser473 in the PFC and/or STR suggesting these drugs increase Akt activity leading to increased GSK-3 α/β phosphorylation and decreased GSK-3 activity. Increased pGSK-3 β and/or pAkt Ser473 have similarly been reported in the frontal cortex of rats or SH-SY5Y cells treated with mood stabilizers and antidepressants^{8-11, 25, 26}. Interestingly, antipsychotics increased pAkt Thr308 whereas mood stabilizers and antidepressants increased pAkt Ser473. However, it should be noted that a previous study found increased pAkt Ser473 levels in addition to pAkt Thr308 following haloperidol treatment in mice using a double-dosing treatment paradigm¹⁵. The difference in the phosphorylation state of Akt between antipsychotics and antidepressants and mood stabilizers may be due to distinct upstream signalling, since Thr308 and Ser473 are phosphorylated by PDK1 and mTOR,

respectively. However, the observation that antidepressants, mood stabilizers and antipsychotics all target Akt and GSK-3 is intriguing since atypical antipsychotics are also used to manage the symptoms of major depressive disorder²⁷ either as a monotherapy or in combination with antidepressants²⁸. Consequently, the findings of the current study are also consistent with the hypothesis that Akt/GSK-3 signalling may be involved in mood disorders and depression.

Haloperidol and clozapine are D₂DR antagonists although binding of clozapine to the D₂DR is much weaker than haloperidol. The D₂DR has been shown to regulate Akt signalling in mice³² and Dvl-3 is associated with the D₂DR⁶ suggesting that antipsychotics induce their effects on Akt and Wnt through the D₂DR. However, other receptors may cause or contribute to changes seen following antipsychotic treatment. For example, the D₃DR regulates Akt signalling³² and both haloperidol and clozapine antagonize the D₃DR. In addition, clozapine's action at the 5-HT_{2A} receptor may contribute to the upregulation of pGSK-3 since the blockade of the 5-HT₂ receptor has been shown to increase pGSK-3 β levels¹⁰. Differences in the receptor binding profiles between clozapine and haloperidol or the weaker binding of clozapine to the D₂DR may explain why clozapine does not activate the Wnt pathway in the STR but haloperidol does. Consistent with this notion, a previous study showed that the atypical antipsychotic risperidone also did not alter GSK-3 or β -catenin in the STR at an atypical dose (low D₂DR binding) but at a higher dose caused increases in β -catenin and GSK-3 similar to haloperidol⁷.

Although the antipsychotics, mood stabilizers and antidepressants that were tested caused an increase in pGSK-3 and pAkt, these different neuropsychiatric treatments

induce these changes by different mechanisms. In contrast to antipsychotics, fluoxetine and imipramine likely cause increases in pAkt and pGSK-3 through serotonin receptors¹⁰. Lithium has been shown to dose dependently inhibit GSK-3^{8, 33} directly and indirectly through activation of Akt^{34, 35}. Valproic acid also inhibits GSK-3 activity, although the precise mechanism is less clear. An *in vitro* study using SH-SY5Y cells attributed the increase in pAkt and pGSK-3 to the inhibition of histone deacetylase by valproic acid²⁶.

Changes in neurochemistry and behavior following repeated amphetamine treatment have been reported to parallel some of the abnormalities found in patients with schizophrenia³⁶. Consequently, Akt and Wnt were investigated in rats following repeated treatment with amphetamine. Reductions in pAkt, GSK-3, pGSK-3, Dvl-3 and β -catenin were noted, consistent with previous reports for β -catenin and GSK-3 in the ventral midbrain²⁰ and pAkt in the striatum^{37, 38}. The changes observed following amphetamine treatment are opposite to those observed following antipsychotic treatment. In addition, alterations in the Akt and Wnt pathways found in the amphetamine model parallels changes reported in schizophrenia. Reductions in GSK-3 β and pGSK-3 β protein levels and β -catenin staining has been reported in the frontal cortex and hippocampus of schizophrenia postmortem brains respectively^{1, 2, 15, 39}. Reductions in Akt-1 protein levels have also been reported in the frontal cortex of schizophrenic patients¹⁵. Although, amphetamine did not affect total Akt protein levels, the reduction in pAkt functionally parallels the changes observed in the postmortem study. Thus, amphetamine treatment may provide a useful model for further studying the role of Akt and Wnt in schizophrenia and the potential therapeutic value of drugs that target Akt and Wnt.

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CHAPTER FOUR

**Regulation of Akt and Wnt signalling by the group II metabotropic glutamate
receptor antagonist LY341495 and agonist LY379268**

4.1 INTRODUCTION

Converging lines of research have implicated Group II metabotropic glutamate receptors (mGluR2 and mGluR3) in schizophrenia and as a potential new therapeutic target to treat the symptoms of schizophrenia. mGluR2/3 are G-protein coupled receptors and are highly expressed in regions of the brain associated with schizophrenia including the prefrontal cortex and striatum^{1,2}. mGluR3 has been identified as a possible schizophrenia susceptibility gene^{3,4} and a decrease in the dimer form of mGluR3 has been reported in the prefrontal cortex of schizophrenic patients⁵. Animal studies have shown that mGluR2/3 antagonists LY341495 and LY366457 increase locomotor activity and that the increase in activity can be attenuated by treatment with antipsychotics^{6,7}. Animal studies have also demonstrated that mGluR2/3 agonists may be effective antipsychotics. Treatment with the mGluR2/3 agonists, LY379268 and LY354740 attenuated phencyclidine (PCP) and/or amphetamine induced hyperlocomotor activity^{8,9}. Finally, a phase 2 clinical trial has shown that the mGluR2/3 agonist, LY2140023 may be effective for alleviating the positive and negative symptoms of schizophrenia in human patients¹⁰. However, while there is evidence to suggest that mGluR2/3 may be involved in schizophrenia, the molecular mechanism responsible for mediating the effects of mGluR2/3 agonists/antagonists have not been established.

GSK-3 is regulated by Akt and the Wnt pathway and both signalling pathways have been implicated in schizophrenia and antipsychotic drug action. Akt-1, Frizzled-3, Wnt-1 and GSK-3 have all been identified as possible schizophrenia susceptibility genes and/or have been shown to have altered protein levels in postmortem studies¹¹⁻¹⁶. Animal studies using models such as amphetamine-sensitization, the DAT-KO mouse and PCP

show changes in GSK-3 and/or Akt phosphorylation¹⁶⁻²⁰. In addition, changes in total and/or phosphorylated Akt, GSK-3 and Wnt pathway proteins have also been reported following repeated treatment with typical or atypical antipsychotics^{16, 21-23}.

Since mGluR2/3 agonists may exhibit antipsychotic properties, it is possible that there is overlap in the signalling pathways targeted by traditional antipsychotics and mGluR2/3 agonists. Consequently, the purpose of the current study was to determine if mGluR2/3 regulates Akt and/or Wnt signalling in the PFC and STR following acute and repeated administration of the mGluR2/3 agonist LY379268 and mGluR2/3 antagonist LY341495. In addition, since inhibition of GSK-3 is implicated in antipsychotic drug action, locomotor studies were performed to examine the effects of GSK-3 inhibition on increased locomotor activity induced by LY341495.

4.2 MATERIALS AND METHODS

4.2.1 Drug Paradigm

Adult male Spraque Dawley rats 14+ weeks of age (Charles River) were housed in pairs in a 12 hour light/dark cycle with free access to food and water. Rats were treated with the mGluR2/3 agonist LY379268 (1.0 mg/kg or 3.0 mg/kg, i.p., Tocris Bioscience), mGluR2/3 receptor antagonist LY341495 (1.0 mg/kg or 3.0 mg/kg, i.p., Tocris Bioscience) or vehicle (n=8 rats/treatment) once or daily for 7 days. Doses were selected based on behavioural studies that assessed the ability of LY379268 or LY341495 to attenuate PCP induced hyperlocomotion⁸ or increase locomotor activity respectively⁷. Protein was isolated from the PFC and STR of rats following either acute or repeated treatment. Every effort was made to minimize pain and suffering and reduce the number

of rats used in the experiments. All experiments were conducted in accordance with the Canadian Council on Animal Care.

4.2.2 Western blotting

Protein isolation, quantification and western blotting were performed as previously described for the PFC and STR²². Densitometry values were obtained from X-ray film using a grey scale calibrated scanner (Epson) and Kodak Molecular Imaging software. Densitometry values were corrected for α -tubulin and expressed as a percentage of control. Data was analyzed for statistical significance using a Student's *t*-test. The source and dilution of the antibodies were as follows Akt (Cell Signalling Technology, 1:1250), phospho-Akt Ser473 (Cell Signalling Technology, 1:1000), phospho-Akt Thr308 (Millipore, 1:1000), Dvl-1 (Santa Cruz Biotechnology, 1:100), Dvl-2 (Chemicon, 1:1000), Dvl-3 (Santa Cruz Biotechnology, 1:100), GSK-3 (Santa Cruz Biotechnology, 1:300), phospho-GSK-3 (Cell Signalling Technology, 1:1000), β -catenin (Sigma-Aldrich, 1:20000) and α -tubulin (Sigma-Aldrich, 1:120000).

4.2.3 Co-Immunoprecipitations

Protein was isolated from the PFC of drug naïve rats or from the PFC and/or STR of LY379268 (3 mg/kg), raclopride (1 mg/kg) or clozapine (25 mg/kg) treated rats using a non-denaturing lysis buffer with the addition of protease (Complete Mini, Roche Diagnostic) and phosphatase (Cocktail Set 2, Sigma-Adrich; Cocktail Set IV, Calbiochem) inhibitors (n=6 rats/treatment). Immunoprecipitations (IPs) were performed using the ExactCruz system (SantaCruz Biotechnology), 500 μ g of protein and antibodies

for the mGluR2/3 (4 µg/IP, Millipore) or D₂DR (3 µg/IP, Santa Cruz Biotechnology) as outlined previously²³. Negative controls were performed using a non-specific IgG (Santa Cruz Biotechnology) from the same species in place of the IP antibody. The IP samples were subjected to western blotting (WB) and probed for the protein of interest; Dvl-2 (Santa Cruz Biotechnology, 1:100), Dvl-3 (Santa Cruz Biotechnology, 1:100), GSK-3 (Santa Cruz Biotechnology, 1:300) Akt (Cell Signalling Technology, 1:1250).

4.2.4 Locomotor activity

Horizontal locomotor activity was recorded from rats treated with LY341495 (1.0 mg/kg and 3.0 mg/kg, i.p.) or an appropriate vehicle following a single injection or following 7 consecutive daily injections (n=12 rats/treatment). In a separate set of experiments, rats were treated once or daily for 7 consecutive days with LY341495 (3.0 mg/kg, i.p.) or vehicle followed 5 minutes later by the administration of the GSK-3 inhibitor, SB216763 (3.0 mg/kg, i.p.) or vehicle (n=16 rats/treatment). Horizontal locomotor activity was recorded on day 1 and day 7. The dose of SB216763 was selected based on previous studies showing that it attenuated amphetamine-induced hyperlocomotion at 3.0mg/kg. Horizontal locomotor activity was recorded using Med Associates activity monitor chambers and software for 60 minutes. Data was analyzed using a one-way ANOVA or two-way ANOVA followed by the Newman-Keuls post hoc test.

4.3 RESULTS

4.3.1 The effects of mGluR2/3 agonism (LY379268) on Akt and Wnt pathway proteins

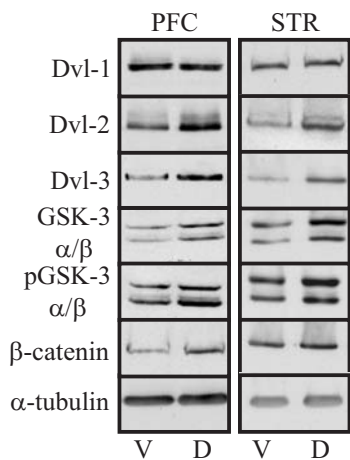
A preclinical animal study suggested that mGluR2/3 agonists possess antipsychotic-like properties¹⁰ and Wnt pathway proteins and Akt have been implicated in schizophrenia and antipsychotic drug action^{16, 24}. Consequently, Akt and proteins involved in Wnt signalling were assessed in the PFC and STR following repeated administration of the mGluR2/3 agonist, LY379268 to determine if LY379268 targets Wnt pathway proteins and/or Akt. At 3.0mg/kg, LY379268 increased Dvl-2, Dvl-3, GSK-3 α/β , pGSK-3 α/β and β -catenin protein levels in the PFC and STR (Figure 4.1a and b). Increases in pAkt Thr308 and pAkt Ser473 were also observed in both the STR and PFC following LY379268 treatment (Figure 4.1c and d). However, at a lower dose of LY379268 (1.0 mg/kg), no significant changes in any of the Wnt pathway proteins (Figure 4.1e) or in total and phosphorylated Akt levels were found in the PFC or STR (Figure 4.1f).

Previous studies have shown that changes in the Wnt pathway are observed following repeated but not acute administration of antipsychotics²² consistent with the clinical observation that antipsychotics requires repeated treatment to exert their beneficial effects. To determine if this is also true for LY379268, rats were treated acutely with LY379268 and the effects on Akt and the Wnt pathway were assessed. The higher dose was chosen since changes following repeated administration of LY379268 were only observed following 3.0 mg/kg. Acute administration of LY379268 resulted in increased phosphorylation of GSK-3 α/β but did not effect Dvl, GSK-3 α/β or β -catenin in the PFC and STR (Figure 4.2a and b). Increased pAkt Ser473 was also detected but no

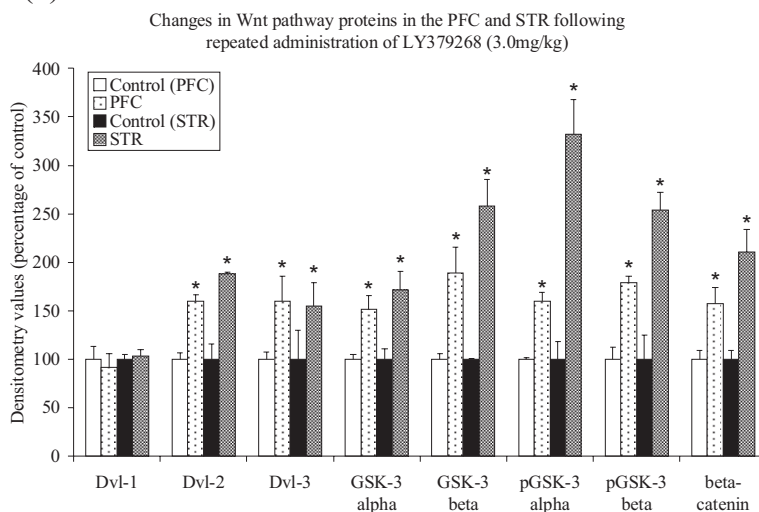
Figure 4.1: The effects of repeated administration of LY379268 on Wnt pathway proteins and Akt.

(a) Representative western blots and (b) graph of densitometry values for Wnt pathway proteins in the PFC and STR of rats treated with 3.0mg/kg LY379268. (c) Representative western blots and (d) graph of densitometry values for total and phosphorylated Akt in the PFC and STR of rats treated with 3.0mg/kg LY379268. Graphs of densitometry values for (e) Wnt pathway proteins and (f) total and phosphorylated Akt in the PFC and STR of rats treated with 1.0mg/kg LY379268. For each representative western blot, the left band represents the vehicle (V) while the right band shows the drug treatment (D). The densitometry values are expressed as a percentage of control with a statistically significance difference denoted by an asterisk ($p < 0.05$, mean \pm SEM, n=8).

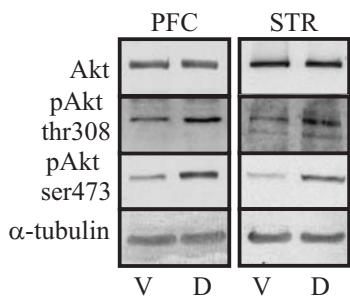
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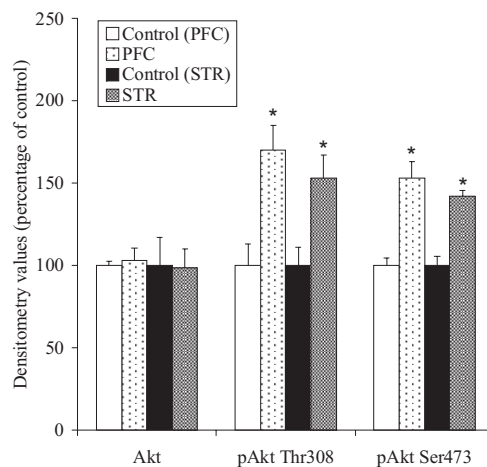


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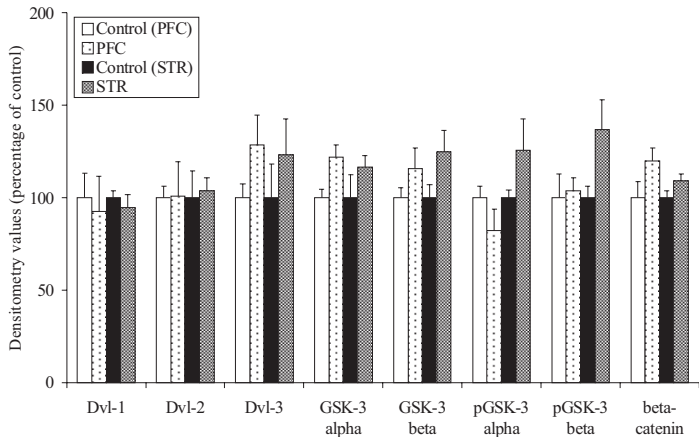
(d)

Changes in total and phosphorylated Akt in the PFC and STR following repeated administration of LY379268 (3.0mg/kg)



(e)

Changes in Wnt pathway proteins in the PFC and STR following repeated administration of LY379268 (1.0mg/kg)



(f)

Changes in total and phosphorylated Akt in the PFC and STR following repeated administration of LY379268 (1.0mg/kg)

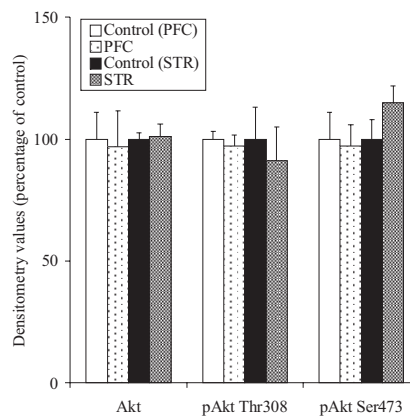
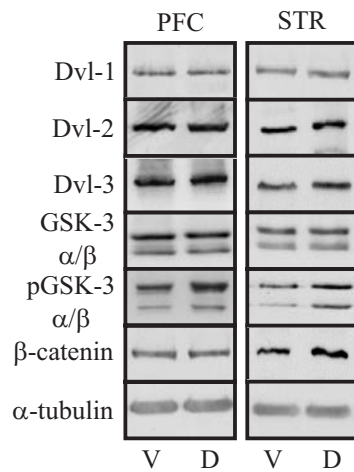


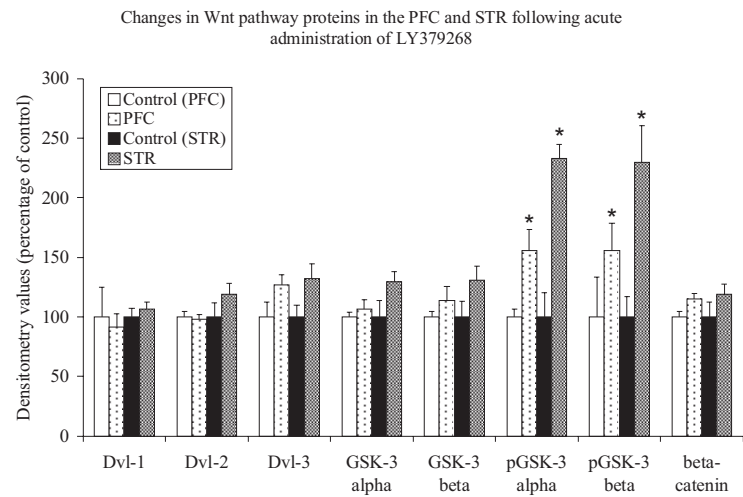
Figure 4.2: The effects of acute administration of LY379268 on Wnt pathway proteins and Akt.

(a) Representative western blots and (b) graph of densitometry values for Wnt pathway proteins in the PFC and STR of rats treated with 3.0mg/kg LY379268. (c) Representative western blots and (d) graph of densitometry values for total and phosphorylated Akt in the PFC and STR of rats treated with 3.0mg/kg LY379268. For each representative western blot, the left band represents the vehicle (V) while the right band shows the drug treatment (D). The densitometry values are expressed as a percentage of control with a statistically significance difference denoted by an asterisk ($p < 0.05$, mean \pm SEM, n=8).

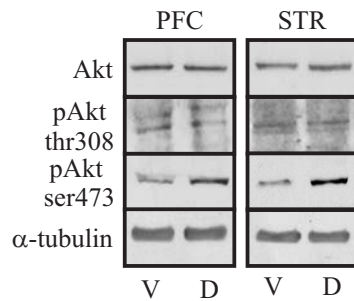
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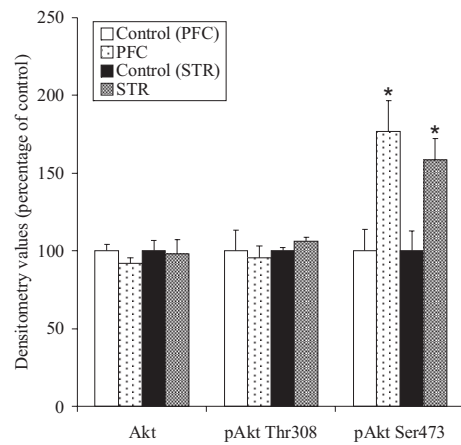


(c)



(d)

Changes in total and phosphorylated Akt in the PFC and STR following acute administration of LY379268



changes in pAkt Thr308 or total Akt levels were observed in the PFC and STR (Figure 4.2c and d). Therefore, acute administration altered the phosphorylation of Akt and GSK-3 α/β but only repeated administration of LY379268 had an effect on total protein levels including changes in β -catenin.

4.3.2 The effects of mGluR2/3 antagonism (LY341495) on Akt and Wnt pathway proteins

The mGluR2/3 antagonist LY341495 was used to examine the effects of blocking the mGluR2/3 on Akt and Wnt pathway proteins. Repeated treatment with 3.0mg/kg of LY341495 decreased Dvl-2, pGSK-3 α/β and β -catenin protein levels but Dvl-1, Dvl-3 and GSK-3 α/β were unaffected in both the PFC and STR (Figure 4.3a and b). In addition, to changes in the Wnt proteins, a reduction in pAkt Ser473 but not in total Akt or pAkt Thr308 were observed in the PFC and STR (Figure 4.3c and d). A lower dose of repeated LY341495 (1.0 mg/kg) was also used and had no effect on any of the proteins of interest in the PFC or STR (Figure 4.3e). Finally, changes in Akt and Wnt pathway protein were assessed following acute administration of LY341495. Decreases in pGSK-3 α/β (Figure 4.4a and b) and pAkt Ser473 (Figure 4.4c and d) were observed in the PFC and STR following acute administration of LY341495 (3.0 mg/kg). Therefore, acute administration of LY341495 decreased pAkt and pGSK-3 levels but repeated treatment (3.0mg/kg) is needed to reduce β -catenin levels. Furthermore, LY341495 had generally the opposite effect following acute and chronic administration compared to mGluR2/3 agonist, LY379268.

Figure 4.3: The effects of repeated administration of LY341495 on Wnt pathway proteins and Akt.

(a) Representative western blots and (b) graph of densitometry values for Wnt pathway proteins in the PFC and STR of rats treated with 3.0mg/kg LY341495. (c) Representative western blots and (d) graph of densitometry values for total and phosphorylated Akt in the PFC and STR of rats treated with 3.0mg/kg LY341495. (e) Graph of densitometry values for Wnt pathway proteins in the PFC and STR of rats treated with 1.0mg/kg LY341495. For each representative western blot, the left band represents the vehicle (V) while the right band shows the drug treatment (D). The densitometry values are expressed as a percentage of control with a statistically significance difference denoted by an asterisk ($p < 0.05$, mean \pm SEM, n=8).

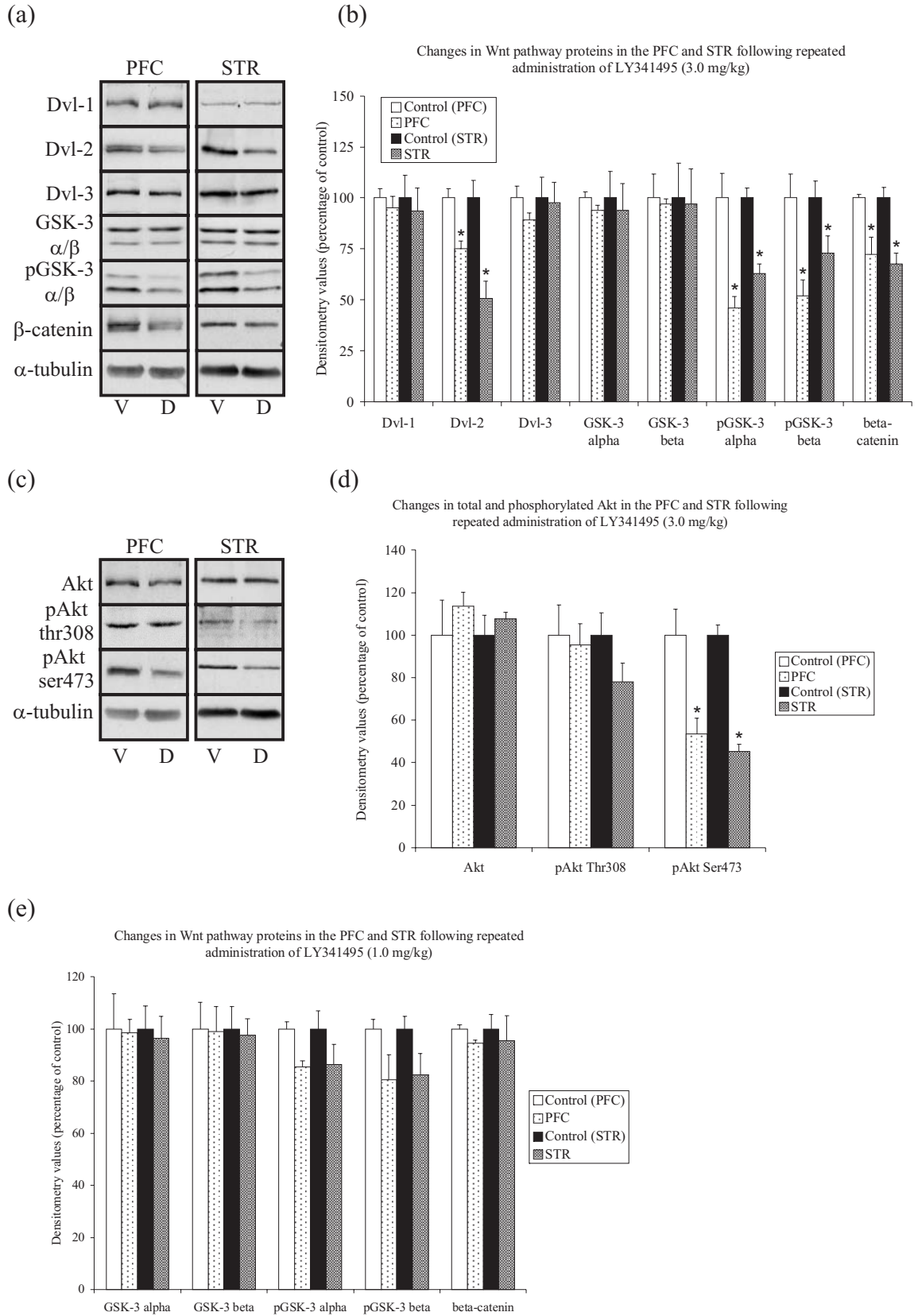
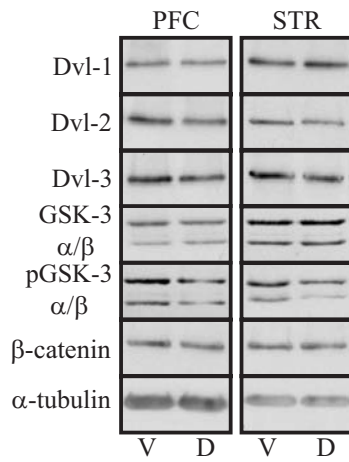


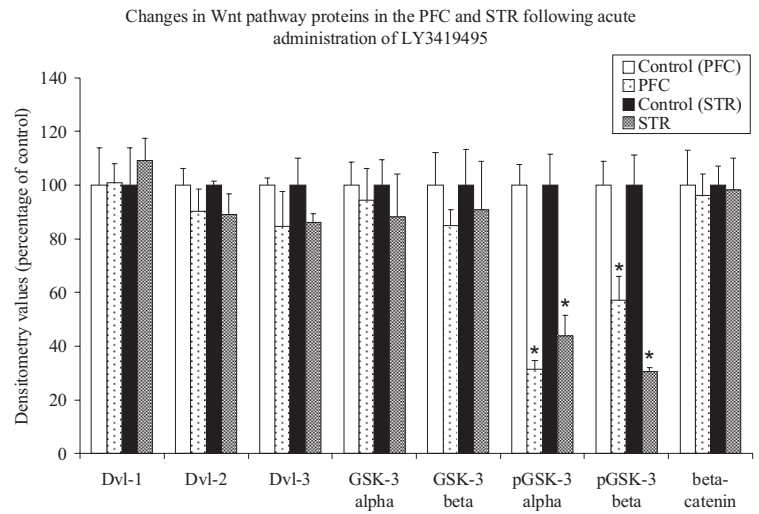
Figure 4.4: The effects of acute administration of LY341495 on Wnt pathway proteins and Akt.

(a) Representative blots and (b) graph of densitometry values for Wnt pathway proteins in the PFC and STR of rats treated acutely with 3.0mg/kg LY341495. (c) Representative blots and (d) graph of densitometry values for total and phosphorylated Akt in the PFC and STR of rats treated acutely with 3.0 mg/kg LY341495. For each representative western blot, the left band represents the vehicle (V) while the right band shows the drug treatment (D). The densitometry values are expressed as a percentage of control with a statistically significance difference denoted by an asterisk ($p < 0.05$, mean \pm SEM, n=8).

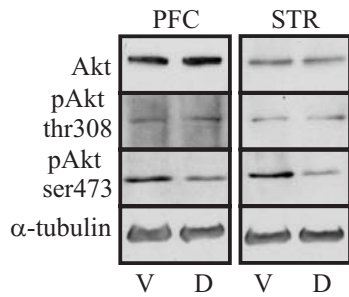
(a)



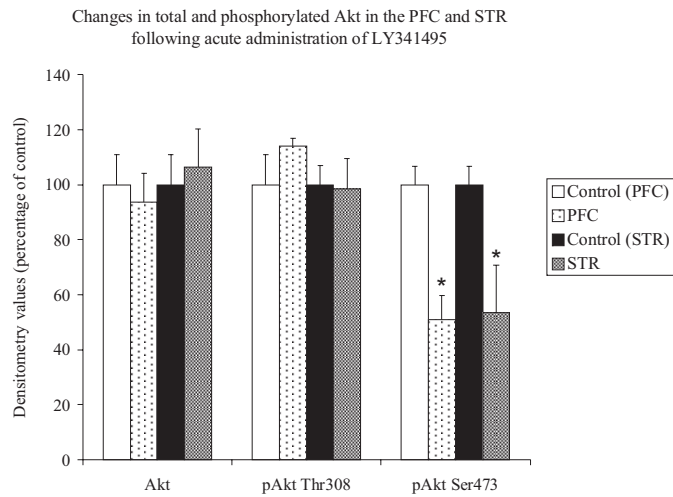
(b)



(c)



(d)



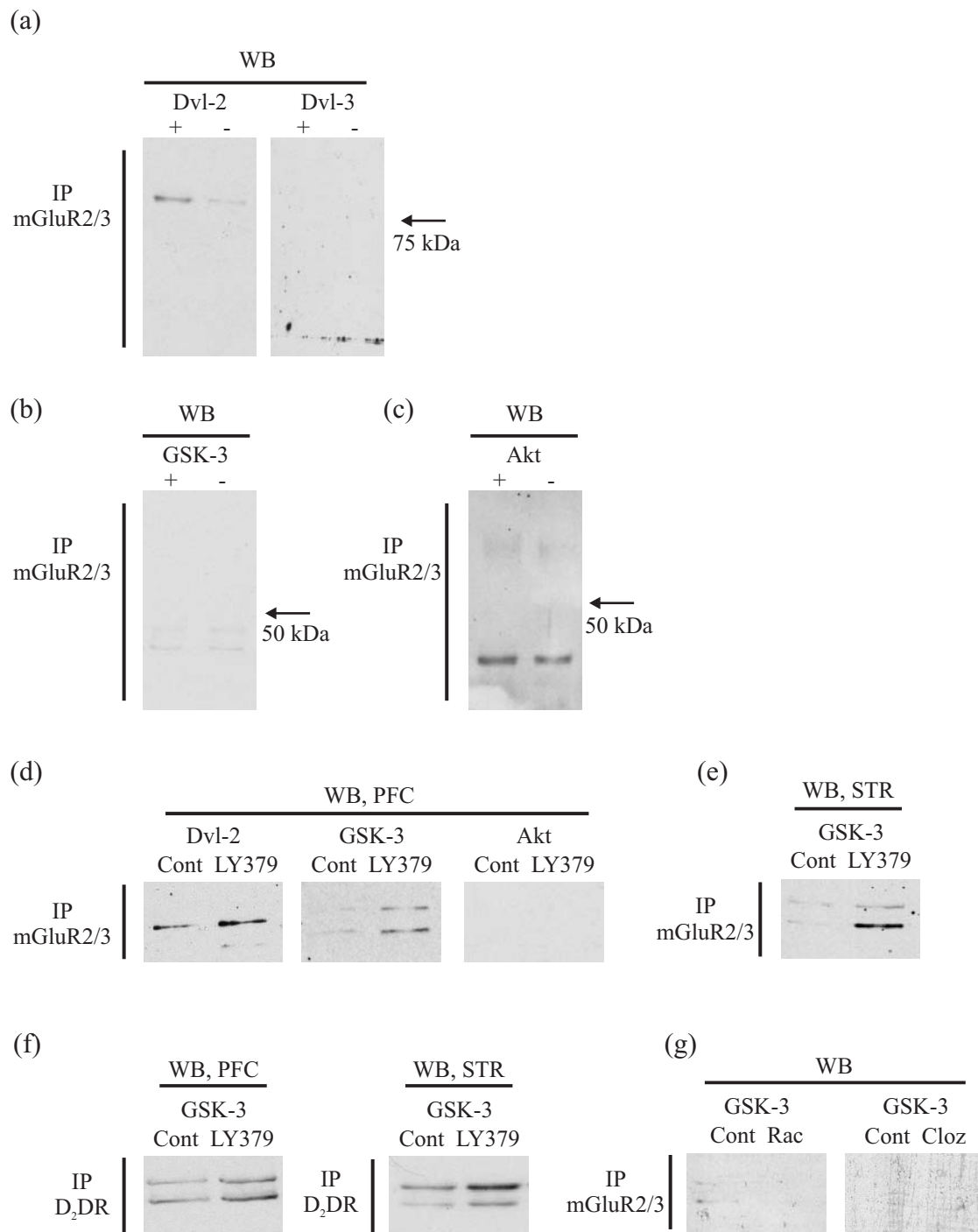
4.3.3 Interactions between mGluR2/3 and Akt and Wnt pathway proteins

Treatment with LY341495 or LY379268 affected both Akt and Wnt pathway proteins but how these events occur is unclear. Associations between Akt and Dvl-3 and the D₂DR complex have previously been reported²³ and thus co-IP experiments were conducted to determine if there is also an association between the mGluR2/3 complex and Dvl, GSK-3 and/or Akt. Co-IPs revealed a weak association between mGluR2/3 and Dvl, GSK-3 and/or Akt. Co-IPs revealed a weak association between mGluR2/3 and Dvl-2 in the PFC of drug naïve rats (Figure 4.5a), whereas no association was found between the mGluR2/3 and Dvl-3 (Figure 4.5b). Furthermore, the co-IPs suggest there is no association between mGluR2/3 and GSK-3 α/β (Figure 4.5b) or Akt (Figure 4.5c).

Activation of the Wnt pathway has been shown to cause the translocation of Dvl and GSK-3 to receptor complexes. Since repeated LY379268 treatment activates the Wnt pathway, co-IP's were performed using protein isolated from the PFC of LY379268 treated rats, to determine if there is a translocation of Dvl-2, GSK-3 and/or Akt to the mGluR2/3 complex. In the PFC, co-IPs showed an increased association between the mGluR2/3 and Dvl-2 and GSK-3 α/β but not with Akt following repeated LY379268 treatment (Figure 4.5d). Since activation of the Wnt pathway was also seen in the STR, the association between mGluR2/3 and GSK-3 was also assessed in the STR. A translocation of GSK-3 α/β to the mGluR2/3 complex in the STR was observed following LY379268 treatment (Figure 4.5e). The results show that LY379268 can induce changes in Akt and Wnt proteins as well as alter the association between the mGluR2/3 complex and Dvl-2 and GSK-3 α/β .

Figure 4.5: Co-Immunoprecipitation experiments showing association between mGluR2/3 and Wnt pathway proteins and/or Akt in drug naïve rats or following LY379268 treatment.

Protein samples obtained from the PFC of drug naïve rats were immunoprecipitated (IP) with a mGluR2/3 antibody. IP samples were subjected to western blotting (WB) and probed for (a) Dvl-2, Dvl-3, (b) GSK-3 and (c) Akt (+ lanes). For the negative control lanes, co-IPs were conducted using a non-specific IgG in place of the IP antibody (- lanes). Protein samples obtained from the (d) PFC and (e) STR of LY379268 treated rats were IP with a mGluR2/3 antibody. IP samples were subjected to WB and probed for Dvl-2, GSK-3 and/or Akt. Protein samples obtained from (f) the PFC and STR of LY379268 treated rats were IP with a D₂DR antibody. IP samples were subjected to WB and probed for GSK-3. (g) IP samples isolated from the PFC of raclopride and clozapine treated rats were IP with a mGluR2/3 antibody. IP samples were subjected to WB and probed for GSK-3.



Increased association of GSK-3 α/β and the D₂DR complex has been observed in the PFC following repeated treatment with the antipsychotics haloperidol and clozapine (chapter 3) and with D₂DR antagonist raclopride (chapter 3). Given that mGluR2/3 agonists may affect extracellular dopamine levels^{25, 26}, the study investigated whether LY379268 altered the association between the D₂DR and GSK-3 α/β . The co-IPs revealed that LY379268 increased the association of GSK-3 α/β and D₂DR complex in the PFC and STR (Figure 4.5f) in addition to the mGluR2/3. In addition, since D₂DR antagonists can affect the translocation of GSK-3 (chapter 2 and 3), raclopride (D₂DR antagonist) and clozapine (atypical antipsychotic) were examined to determine if they are able to alter the association of GSK-3 α/β at the mGluR2/3 complex. Neither raclopride nor clozapine altered the association between mGluR2/3 and GSK-3 α/β in the PFC (Figure 4.5g). The results suggest that changes at the mGluR2/3 induced by LY379268 were due to mGluR2/3 signalling and not the indirect effects of dopamine/D₂DR signalling. Finally, LY379268, clozapine and raclopride increased GSK-3 protein levels, as previously reported but did not effect mGluR2/3 protein levels (data not shown).

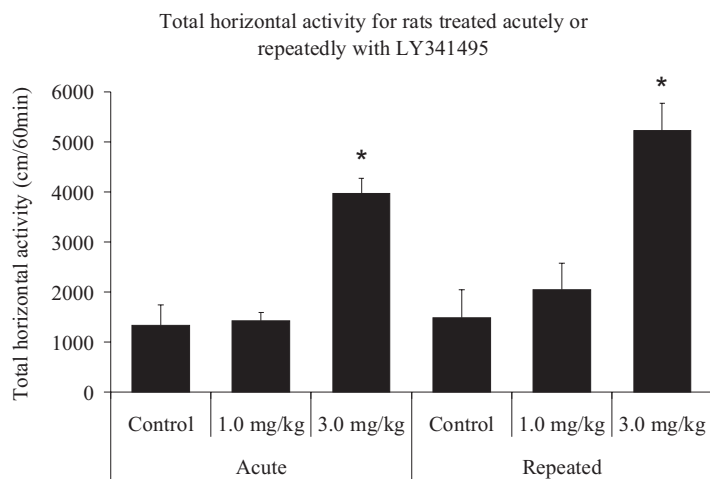
4.3.4 Effects of LY341495 on locomotion

Previous studies have demonstrated that acute administration of LY341495 dose dependently increased locomotor activity in mice⁷. Given that acute and repeated LY341495 treatment had different effects on Wnt signalling, we assessed horizontal locomotor activity following both acute and repeated administration of LY341495. Consistent with previous studies, Figure 4.6a shows increased horizontal activity was

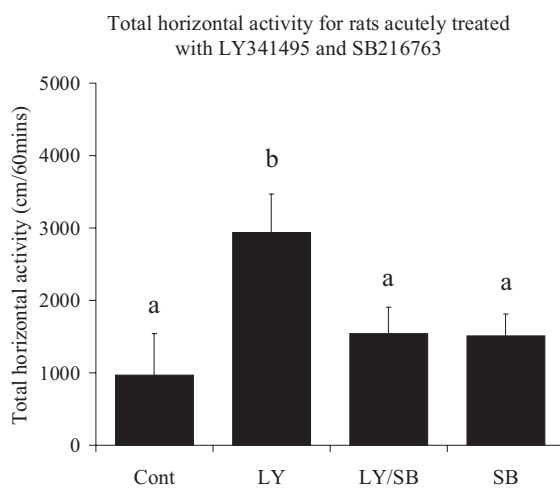
Figure 4.6: Total horizontal locomotor activity for rats treated acutely or repeatedly with LY341495 or LY341495 and SB216763.

(a) Total horizontal locomotor activity for rats acutely and repeatedly treated with 1.0mg/kg and 3.0 mg/kg LY341495. A statistical significance is denoted by an asterisk as compared to the control (n=12, mean \pm SEM, $p < 0.05$). Total horizontal locomotor activity for rats (b) acutely and (c) repeatedly treated with LY341495 (3.0mg/kg) and administered SB216763 (3.0 mg/kg). Statistical significance is denoted by a difference in lettering (n=16, mean \pm SEM, $p < 0.05$). (Control, Cont; LY341495, LY; SB216763, SB)

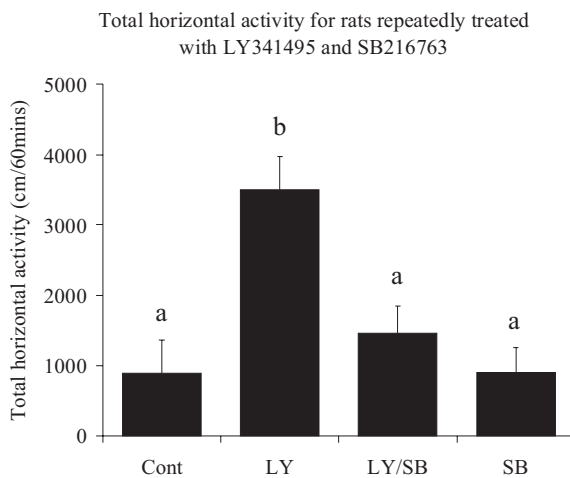
(a)



(b)



(c)



observed following acute treatment with 3.0 mg/kg of LY341495 but not at a lower dose of 1.0 mg/kg. Repeated treatment with LY341495 also increased horizontal activity at a dose of 3.0 mg/kg but not at 1.0 mg/kg (Figure 4.6a). Overall, similar changes in total horizontal activity were observed following acute and repeated LY341495 treatment (3.0mg/kg).

A previous study has implicated GSK-3 signalling in attenuating hyperlocomotion in dopamine-transporter knockout mice²⁷. Therefore, the GSK-3 inhibitor SB216763 was used to determine if inhibiting GSK-3 could attenuate the increase in locomotion observed following acute and repeated treatment of LY341495. Results showed that SB216763 significantly attenuated the increase in horizontal activity following acute administration of LY341495 (Figure 4.6b). In addition, the effects of repeated SB216763 decreased horizontal activity induced by daily administration of LY341495 (Figure 4.6c). Overall, acute and repeated co-administration of SB216763 in LY341495 had similar effects on locomotion.

4.4 DISCUSSION

4.4.1 mGluR2/3 mediates changes in Wnt and Akt signalling

The current study demonstrated that the mGluR2/3 causes changes in Akt and the Wnt pathway. Repeated administration of LY379268 (mGluR2/3 agonist) activated the Wnt pathway leading to the stabilization of β -catenin and also activated Akt. Repeated LY341495 (mGluR2/3 antagonist) treatment induced a similar but inverse response compared to LY379268, including a decrease in β -catenin protein levels and a reduction of pAkt. Acute treatment of LY379268 or LY341495, in contrast, induced changes in the

phosphorylation state of Akt and GSK-3 but had no effect on total protein levels or the transcription factor β -catenin.

The link between mGluR2/3 and Akt and Wnt signalling is unclear, although the association between Dvl-2 and the mGluR2/3 complex provides a potential mechanism to explain how mGluR2/3 exerts its affect on the Wnt pathway. Consistent with this hypothesis, a previous study has shown that increased Dvl-2 levels results in translocation of Dvl-2 to the plasma membrane, a necessary step for β -catenin stabilization²⁸. Thus, upregulation of Dvl-2 may induce the translocation of Dvl-2 to the mGluR2/3 observed following repeated LY379268 treatment. In addition, Dvl-2 may be responsible for the recruitment of GSK-3 to the mGluR2/3 since previous studies have shown that Dvl-2 recruits Wnt pathway proteins such as Axin to the plasma membrane²⁸. Finally, overexpression of Dvl-2 in Neuro 2A cells has been shown to be sufficient to stabilize β -catenin and increase TCF mediated gene transcription, consistent with the increased levels of β -catenin observed after LY379268 treatment²⁹. Collectively, the results of the Dvl-2 studies parallel the changes observed following LY379268 treatment and suggest that changes in Dvl-2 levels induced by mGluR2/3 agonism or antagonism may be responsible for changes in Wnt signalling observed following repeated treatment of LY379268 and LY341495.

GSK-3 is a major target of Akt^{30, 31} and changes in the phosphorylation state of Akt following LY379268 or LY341495 likely account for the change in GSK-3 phosphorylation. However, it is unknown how mGluR2/3 signalling regulates the Akt-GSK-3 cascade given there is no association between Akt and the mGluR2/3 complex in either the drug naïve rats or following LY379268 treatment. This suggests Akt signalling

may not be affected by the mGluR2/3 directly. Several studies have shown that the phosphorylation state of Akt and consequently GSK-3 is regulated by the D₂DR/D₃DR³² and mGluR2/3 have been shown to modify dopamine release^{25, 33, 34}. Alternatively, it has been suggested that LY379268 has a significant affinity for the D₂DR and may function as a partial dopamine agonist^{35, 36}, although a separate study failed to show a direct interaction between D₂DR and LY379268³⁷. Therefore, changes in D₂DR/D₃DR and not mGluR2/3 signalling may be responsible for the regulation of the Akt-GSK-3 cascade following treatment with LY379268 and LY341495. In support of this hypothesis, LY379268 also caused an increased association between GSK-3 and the D₂DR complex. Increased association between GSK-3 and the D₂DR complex has been observed following raclopride, haloperidol and clozapine treatment (chapter 2 and 3), suggesting that GSK-3 and D₂DR interactions are regulated by D₂DR signalling. In addition, alterations in D₂DR signalling may explain the increase in Dvl-3 protein levels observed following repeated LY379268 treatment since it has been shown that D₂DR may regulate Dvl-3 levels (chapter 2).

While D₂DR signalling may be responsible for pAkt, pGSK-3 and Dvl-3 levels, the changes in Dvl-2 protein levels and at the mGluR2/3 complex can't be attributed to dopamine signalling. Changes in Dvl-2 protein levels were induced by LY379268 and LY341495 but not by raclopride, antipsychotics or amphetamine^{22, 23}. In addition, translocation of GSK-3 to the mGluR2/3 was induced by LY379268 but not by repeated raclopride or clozapine treatments. Overall, LY379268 and LY341495 may mediate changes associated with Dvl-2 via the mGluR2/3, whereas changes in Akt and Dvl-3 may be mediated by D₂DR.

4.4.2 LY341495 and Behavior

The increase in horizontal activity observed following LY341495 administration is consistent with previous studies showing that acute treatment of LY341495 dose dependently increased locomotor activity^{7, 38}. The changes in the locomotor activity correlated to the alterations in the phosphorylation state of Akt and GSK-3 α/β . Decreases in pGSK-3 α/β and pAkt Ser473 and increased locomotor activity were observed following 3.0mg/kg and not following 1.0mg/kg of LY341495 treatment.

It has been suggested that increased locomotion induced by LY341495 is due to impairments in the ability of the rat to habituate to its surrounding and may be relevant to the cognitive impairments of schizophrenia⁶. It is important to note that the effects of LY341495 on locomotor activity differs from amphetamine, since LY341495 does not induce hyperlocomotion⁶. Thus, LY341495 and amphetamine induced locomotion involve different mechanisms. Both LY341495 and amphetamine treatments mediate changes in Akt and Wnt signalling. However, there are differences in Wnt signalling as amphetamine decreases Dvl-3 and GSK-3 protein levels (chapter three) and LY341495 decreased Dvl-2 and had no effect on GSK-3. Both repeated administration of LY341495 and amphetamine induce similar changes, including reductions in pAkt, pGSK-3 and β -catenin protein levels. Overall, LY341495 and amphetamine induce distinct but overlapping changes in Akt and Wnt signalling.

Studies have shown that the inhibition of GSK-3 normalizes hyperlocomotion in amphetamine-sensitized rats and hyperdopaminergic DAT-KO mice²⁷ suggesting that GSK-3 plays an important role in hyperlocomotion. Administration of LY379268, clozapine, haloperidol and risperidone, have all been shown to reverse LY341495

induced increases in locomotor activity⁶. All of these compounds have similar effects on GSK-3^{21, 22}, suggesting that GSK-3 may also be involved in mGluR2/3 induced locomotor activity. Consistent with this hypothesis, inhibition of GSK-3 with SB216763 attenuated LY341495 induced increases in locomotion. Interestingly, attenuation of locomotion by SB216763 was observed following both acute and repeated treatment. Acute administration of LY341495 only affected pAkt and pGSK-3 suggesting that changes in total protein levels of GSK-3, Dvl and β -catenin may not be important for mGluR2/3 mediated locomotor activity. However, other animal models have suggested that β -catenin may be important for hyperlocomotor activity. For example, amphetamine-induced hyperlocomotion is inhibited in transgenic mice overexpressing β -catenin³⁹. Disrupted-in-schizophrenia-1 (DISC-1) interacts directly with GSK-3 and regulates β -catenin stabilization. DISC-1 knockout mice exhibit hyperlocomotion that is normalized by SB216763 treatment⁴⁰. Thus, the data suggests that different downstream targets of GSK-3 may regulate LY341495-induced versus amphetamine-induced increases in locomotor activity.

4.4.3 Comparison of LY379268 with antipsychotics

A phase 2 clinical trial suggested that the mGluR2/3 agonist, LY2140023 can alleviate the positive symptoms of schizophrenia and may represent an alternative to traditional antipsychotic drugs for the treatment of schizophrenia¹⁰. Interestingly, the mGluR2/3 agonist, LY379268 and antipsychotic drugs both target Akt and Wnt signalling. In addition, translocation of GSK-3 to the D₂DR complex has also been observed following LY379268, haloperidol and clozapine treatments²³. However, there

are also important differences. The D₂DR is associated with Dvl-3, Akt and GSK-3 whereas the mGluR2/3 is associated with Dvl-2 and GSK-3 following treatment with LY379268. In addition, LY379268 altered levels of Dvl-2, whereas antipsychotic treatment affected Dvl-3²³. Dvl-2 may activate different signalling pathways compared to Dvl-3. For example previous *in vitro* studies have shown that the overexpression of Dvl-2 activates JNK⁴¹. Overexpression of Dvl-3 in PC12 or SH-SY5Y cells *in vitro* or upregulation of Dvl-3 by antipsychotics *in vivo* does not effect JNK signalling²³. Although there are differences in Wnt signalling, the overall pattern of change generated by LY379268 and antipsychotics is similar. Therefore, Akt and/or the Wnt pathway may be important for some of the therapeutic benefits of antipsychotics and mGluR2/3 agonists and may represent new targets for the development of drugs to treat schizophrenia.

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CHAPTER FIVE

GENERAL DISCUSSION

5.1 SUMMARY OF RESULTS AND CONCLUSIONS

5.1.1 Alterations in GSK-3 and Akt signalling induced by the dopamine D₂ receptor are mediated by Dvl-3

Chapter two demonstrated that D₂DR regulation of Akt and Wnt signalling is mediated by Dvl-3. The results showed that raclopride (D₂DR antagonist) increased the levels of specific proteins associated with Akt and the Wnt pathway in a pattern identical to what we previously reported following antipsychotic treatment¹⁻³. In addition, raclopride induced the translocation of GSK-3 to the D₂DR complex. *In vitro* studies showed that overexpression of Dvl-3 induced changes in Akt and Wnt signalling identical to what was observed *in vivo* following raclopride and antipsychotic treatments suggesting that raclopride and antipsychotics regulate Akt and the Wnt pathway via Dvl-3. Neither SCH23390 (D₁DR antagonist) nor nafadotride (D₃DR antagonist) affected Wnt pathway proteins, although nafadotride altered the phosphorylation state of Akt and GSK-3. This key difference in Wnt signalling may explain why drugs that target D₁DR and D₃DR fail to alleviate psychosis but D₂DR antagonism is critical. The study also showed that alterations in Akt and Wnt signalling induced by quinpirole (D₂DR agonist) were consistent with findings from genetic and post-mortem schizophrenia studies and from animal models of schizophrenia such as the DAT-KO mouse and amphetamine-sensitization. Given the potential involvement of Akt and Wnt pathways in the manifestation of schizophrenia and the findings of the current study concerning Dvl-3 as the trigger for the observed cascade of changes, an investigation (post-mortem and genetic) of Dvl-3 in schizophrenia might be of particular interest.

5.1.2 The effects of neuropsychiatric drugs and amphetamine on GSK- 3 signalling

Chapter three identified key differences in GSK-3 signalling that were induced by antipsychotics but not by mood stabilizers or antidepressants. It has found that haloperidol and clozapine induced a common and specific response in Wnt signalling that was not mimicked by the mood stabilizers and antidepressants tested. The unique antipsychotic response provides additional evidence that the Wnt pathway may be important for antipsychotic drug action. In contrast to Wnt, similar changes in pAkt and pGSK-3 were observed following antipsychotic, mood stabilizer and antidepressant treatments. This is an interesting observation given the overlap between antipsychotics, mood stabilizers and antidepressants in treating bipolar disorder and major depressive disorder, respectively. In particular, atypical antipsychotics have been shown to be effective in the treatment of bipolar depression (depressive phase of bipolar disorder)⁴ and major depressive disorder⁵.

The study also found that amphetamine-sensitization, a putative model for the positive symptoms of schizophrenia induced the opposite response on Akt and Wnt signalling as the antipsychotic treatments. Furthermore, the changes in these pathways were functionally consistent with abnormalities observed in post-mortem studies using brains of schizophrenic patients. Thus, it is hypothesized that the regulation of the Wnt pathway by antipsychotics may compensate for abnormalities found in schizophrenia leading to behavioural changes. Therefore, targeting the Wnt pathway, Dvl-3 in particular, may be of interest for pharmaceutical intervention for the positive symptoms of schizophrenia.

5.1.3 Regulation of Akt and Wnt signalling by the group II metabotropic glutamate receptor agonist LY379268 and antagonist LY341495

Chapter four examined the relationship between mGluR2/3 and Akt and Wnt signalling, since mGluR3 have been associated with schizophrenia⁶ and mGluR2/3 agonists may have antipsychotic-like effects⁷. The study showed that administration of the mGluR2/3 agonist, LY379268 and mGluR2/3 antagonist, LY341495 induced opposite responses on Akt and Wnt signalling. The association between Dvl-2 and mGluR2/3 complex revealed a potential mechanism that may explain how LY379268/LY341495 regulates the Wnt pathway. In addition, the results also showed there was no association between Akt and mGluR2/3 suggesting that changes in Akt signalling induced by LY379268/LY341495 are not directly regulated by the mGluR2/3. Instead, the data suggest that LY379268/LY341495 direct or indirect effects on the D₂DR are responsible for the changes observed in Akt signalling. Overall, the study demonstrates that LY379268 and LY341495 likely mediate their changes on Wnt and Akt signalling through the mGluR2/3 and D₂DR.

In conclusion, one of the most striking findings of the thesis is that LY379268, haloperidol and clozapine mediate similar changes on Akt and Wnt signalling, including increases in pAkt and β -catenin protein levels. The results suggest these signalling cascades may be involved in the therapeutic effects of antipsychotics and mGluR2/3 agonists and may represent a potential target for the development of pharmaceuticals.

5.2 DISCUSSION

Although antipsychotic have been prescribed since the 1950s, the molecular mechanism(s) involved in their ability to alleviate psychosis remains elusive. It is known

that the blockade of the D₂DR is critical for the ability of antipsychotics to alleviate psychosis⁸ but beyond D₂DR antagonism the molecular mechanisms are unclear. Known D₂DR signalling pathways, such as MAPK, PKA and PKC have provided no persuasive evidence for their involvement in antipsychotic drug action⁹⁻¹¹. Recently, our laboratory and others have reported new signalling pathways associated with antipsychotics and schizophrenia involving Akt, GSK-3 and Wnt^{1-3, 12}. Given the possible involvement of Akt and Wnt signalling in schizophrenia, the thesis examined the role of Akt and Wnt signalling in drugs that alleviate psychosis and in animal models of schizophrenia. Collectively, the studies showed that drugs that alleviate psychosis induce a common and specific pattern of change in Wnt signalling. Thus, it is proposed that antipsychotics and mGluR2/3 agonists ability to alleviate psychosis may be mediated by the Wnt pathway.

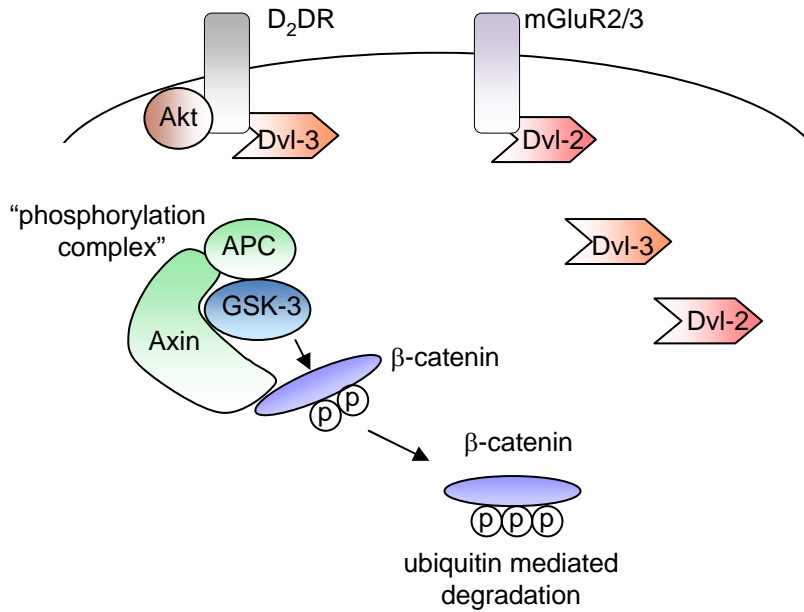
Based on the conclusions from this thesis and previous studies a working hypothesis has been developed to explain how antipsychotics and mGluR2/3 agonists may regulate Akt and Wnt signalling (Figure 5.1). Antipsychotic drugs and mGluR2/3 agonists cause an increase in Dvl protein levels leading to functional changes at the respective receptor complexes, activation of Akt and the stabilization of β -catenin levels. More specifically, antipsychotics cause an increase in Dvl-3 protein levels leading to the translocation of Dvl-3 and GSK-3 to the D₂DR complex. LY379268 activates the mGluR2/3 resulting in an upregulation of Dvl-2 protein levels, which leads to the recruitment of Dvl-2 and GSK-3 to the mGluR2/3 complex. In addition to its effects at the mGluR2/3, LY379268 also effects D₂DR signalling either directly by binding to the D₂DR¹³ or indirectly by influencing extracellular DA levels^{14, 15}. LY379268 effects on

Figure 5.1: Working model of antipsychotics and mGluR2/3 agonist regulation of Akt and Wnt signalling.

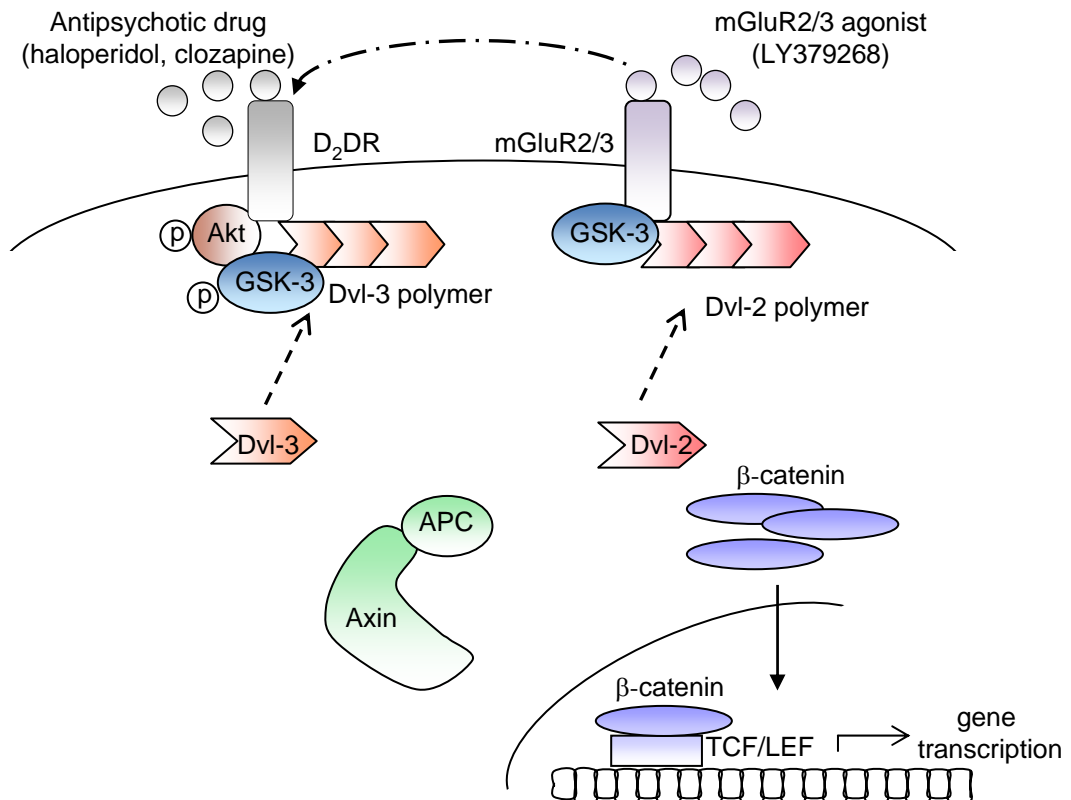
A) Resting State Resting State: In a native state, Dvl-3 and Akt are associated with the D₂DR complex but there is no association between Dvl or Akt and the mGluR2/3 complex. A “phosphorylation complex” involving glycogen synthase kinase-3 (GSK-3), Axin and adenomatous polyposis coli (APC) facilitates the phosphorylation of β -catenin by GSK-3. Phosphorylated β -catenin is targeted for ubiquitin-mediated degradation.

B) Antipsychotic or mGluR2/3 agonist treatment: Haloperidol or clozapine bind to and antagonize the D₂DR resulting in an upregulation of Dvl-3 protein levels. An increase in Dvl-3 levels leads to the translocation of itself and GSK-3 to the D₂DR complex. LY379268, mGluR2/3 agonist activates the mGluR2/3 resulting in an upregulation of Dvl-2. The increase in Dvl-2 leads to recruitment of itself and GSK-3 to the mGluR2/3 complex. LY379268 also effects D₂DR signalling leading to an increase in Dvl-3 protein levels. The increase in Dvl-3 levels facilitates the translocation of GSK-3 to the D₂DR complex. Dvl-3 is also involved in the increase in phosphorylated Akt levels, resulting in the phosphorylation of GSK-3. Dvl-3 and/or Dvl-2 interference of the “phosphorylation complex” inhibits GSK-3 ability to phosphorylate β -catenin resulting in an increase in β -catenin protein levels. The stabilization of β -catenin leads to its translocation into the nucleus, where β -catenin interacts with TCF/LEF to initiate gene transcription.

A) Resting state



B) Antipsychotic or mGluR2/3 agonist treatment



D₂DR signalling leads to the upregulation of Dvl-3 protein levels, resulting in the recruitment of GSK-3 to the D₂DR complex. Dvl-3 is also involved in the increase in phosphorylated Akt levels, resulting in the phosphorylation of GSK-3. Dvl-3 and/or Dvl-2 interference of the “ β -catenin phosphorylation complex” inhibits GSK-3 from phosphorylating β -catenin, ultimately leading to the accumulation of β -catenin. β -catenin translocates to the nucleus and binds to TCF/LEF to alter gene transcription.

Regulation of Dvl-3/Dvl-2 at the protein level and its association with receptor complexes appears to mediate the downstream effects on both the Wnt and Akt pathway following antipsychotic/LY379268 treatments. Our results are consistent with a recent study showing that altered Dvl protein levels, Dvl polymer formation and the translocation of Dvl to receptor complexes is responsible for changes in downstream signalling events in the Wnt pathway¹. *In vitro* studies have also shown that increases in Dvl-3/Dvl-2 protein levels lead to the stabilization of β -catenin and consequently the activation of TCF/LEF transcription^{2, 3}. The translocation of Dvl to the plasma membrane and the formation of Dvl polymers at the plasma membrane correlates with Dvl ability to activate β -catenin signalling^{4, 5}. Dvl-2 overexpression studies have shown that an increase in Dvl-2 protein levels is sufficient to cause the translocation and polymerization of Dvl-2⁵. Based on our studies, it is anticipated that the increase in Dvl-3/Dvl-2 and its translocation to receptor complexes (D₂DR and mGluR2/3 complexes respectively) induced by antipsychotics/LY379268 leads to the formation of Dvl polymers, which is necessary for further signalling. Further studies will be needed to validate this hypothesis.

The model also shows that antipsychotic/LY379268 treatment recruits Dvl-3/Dvl-2 and GSK-3 to the D₂DR/mGluR2/3 complex, leading to the stabilization of β -catenin levels. Others have reported that recruitment of Dvl to Fz/LRP6 is responsible for the translocation of Axin-GSK-3 to the receptor complex following stimulation of the Wnt pathway by a Wnt ligand⁶⁻⁸. Although the Fz receptor is known to activate the Wnt pathway, several studies have shown that other GPCR may also regulate Wnt signalling and that the stabilization of β -catenin and subsequently activation of TCF/LEF transcription can occur through GPCRs independent of the Fz receptor. For example, signalling through the gonadotrophin releasing hormone receptor⁹, parathyroid receptor¹⁰ and the prostaglandin receptor¹¹ have been shown to regulate β -catenin. Therefore, activation of the Wnt pathway following antipsychotic/LY379268 may occur through D₂DR and mGluR2/3 independent of Fz.

Understanding how antipsychotics or mGluR2/3 agonists improve the positive symptoms of schizophrenia could provide alternative therapeutic targets that are more effective and have fewer severe side-effects than traditional antipsychotics. Furthermore, understanding how antipsychotic drugs or mGluR2/3 agonist alleviate psychosis may provide insight into the manifestation of the positive symptoms of schizophrenia. One of the most interesting findings of the study was the common and specific (i.e. not mimicked by other drug classes) response haloperidol, clozapine and LY379268 induced on Dvl-3 protein levels. Thus, Dvl-3 may represent a potential therapeutic target for alleviating psychosis. Furthermore, Dvl-3 may be a more appropriate target than GSK-3 since GSK-3 is involved in multiple pathways throughout the body¹². For example, GSK-3 plays an important role in glycogen metabolism by phosphorylating glycogen

synthase. Thus targeting GSK-3 may lead to metabolic disorders. In contrast, Wnt signalling (i.e. Dvl) does not effect glycogen synthase activity¹³. Pathway specificity is another issue since drugs that target GSK-3 must effect the pool of GSK-3 that participates in Wnt signalling. Lithium and valproic acid are inhibitors of GSK-3 that do not effect β -catenin signalling in the PFC and have no effect on the positive symptoms of schizophrenia. Therefore targeting Dvl-3 may provide more selectivity and specificity than GSK-3. Unfortunately, there are currently no commercially available inhibitors of Dvl-3 to test in animal models of schizophrenia and Dvl-3 knockout mice do not survive past weaning.

5.3 LIMITATIONS OF THE MODEL AND FUTURE STUDIES

One of the main findings of the thesis is that D₂DR signalling regulates Akt and the Wnt pathway. However, the current and previous studies have shown that D₃DR also regulates the phosphorylation state of Akt¹⁴. In the proposed model it was shown that antipsychotics regulate Akt through the D₂DR and that D₃DR either has no effect or contributes to D₂DR regulation of Akt as previously described¹⁴. Antipsychotics have some affinity for the D₃DR¹⁵ and thus it can not be ruled out the D₃DR does not effect Akt signalling. However, the data does suggest that activation of Akt by antipsychotics is mediated by Dvl-3. In Dr. Rushlow's laboratory it has recently been observed that Dvl-3 is associated with D₂DR³ and not with the D₃DR (data not shown) suggesting that antagonism of the D₂DR by antipsychotics regulates Dvl-3 leading to changes in the phosphorylation state of Akt. One of the limitations of the model is the presumption that Akt and Dvl-3 are localized at the same D₂DR complex. Future experiments will have to

be performed to determine if Dvl-3 and Akt co-localize to the same D₂DR complex. Unpublished co-IP experiments that were performed showed an association between Dvl-3 and Akt suggesting that both are indeed co-localized. However, the antibodies available for Dvl-3 and D₂DR are not adequate for immunohistochemistry and thus we were not able to confirm co-localization using confocal microscopy or by other techniques. Efforts are being made to develop new Dvl-3 antibodies that can be used for immunohistochemistry to address this problem. Furthermore, co-IPs experiments can also be performed to determine if the association between Dvl-3 and Akt is affected by antipsychotic treatment.

Mechanistically, the model depicts Akt phosphorylating GSK-3 at the D₂DR complex following antipsychotics/LY379268 treatment. Although from the protein extracts there is an increase in pAkt and pGSK-3 it has yet to be shown that this change in phosphorylation occurs at the D₂DR complex. Future studies examining a change in pAkt and/or pGSK-3 at the D₂DR complex will need be conducted. If there is indeed a change in pAkt and pGSK-3 at the D₂DR complex, this change in phosphorylation state at the D₂DR may have functional consequences for D₂DR activity. GSK-3 has been shown to effect the activity of the co-receptor for the Wnt pathway, low density lipoprotein receptor-related protein 6 (LRP6) via phosphorylation^{16, 17}. Thus, the association between GSK-3 and the D₂DR suggests that GSK-3 may influence the phosphorylation state and hence activity of the D₂DR. Additional experiments will need to be conducted to determine if this is the case.

Although the study showed that antipsychotic/LY379268 treatments increased Dvl-3 protein levels it is unclear how antipsychotics/LY379268 cause an upregulation of

Dvl-3 levels in the first place. There are several mechanisms that could explain how an increase in Dvl-3 levels occur including the stabilization/degradation of Dvl-3 or changes at the transcriptional level of the Dvl-3 gene. Degradation of Dvl-3 through an ubiquitination pathway is facilitated by prickle-1¹⁸. It has been shown that the downregulation of prickle-1 correlates with the upregulation of Dvl-3 and the accumulation of β -catenin in hepatocellular carcinoma cells¹⁸. Alternatively, changes at the transcriptional levels could result in an increase in Dvl-3 levels. Unfortunately, the Dvl-3 promoter has not been well characterized and its unclear what elements regulate Dvl-3 transcription.

The consequence of increasing Dvl-3/Dvl-2 by antipsychotics/LY379268 treatment is the stabilization of β -catenin levels. Within the Wnt pathway, β -catenin is a co-transcription factor that interacts with TCF proteins to regulate Wnt-mediated gene transcription^{19,20}. In addition to its role in the Wnt pathway, β -catenin also participates in the formation and maintenance of synaptic junctions²¹. Changes in β -catenin levels at synaptic sites can influence both the size and strength of synapses²² and alternations in synaptic structure have been observed in patients with schizophrenia²³. Thus, it is possible that the increase in β -catenin levels following antipsychotic/LY379268 treatment may also effect synaptic functioning. Although, the thesis did not directly address the consequence of β -catenin stabilization, results from our laboratory do suggest that increases in β -catenin following antipsychotics/LY379268 treatment likely lead to alterations in TCF-mediated transcription. For example, increases in Dvl-3/Dvl-2 protein levels, as observed following antipsychotics/LY379268 treatment, has previous been shown to be sufficient to activate TCF-transcription^{2, 3}. Furthermore, antipsychotic

treatment induces the translocation of β -catenin to the nucleus, an event tied to changes in TCF-mediated transcription²⁴. To confirm that antipsychotics and LY379268 activate TCF transcription, TCF-reporter mice may be used in the future.

Some target genes of β -catenin have been identified but most are associated with cancer or development, two major fields of investigation in Wnt signalling, and consequently little is known about TCF target genes that may be relevant to schizophrenia. However, it has been shown that connexin 30 and 43 transcription are regulated by β -catenin and connexins have been associated with schizophrenia. Connexins are transmembrane proteins that assemble to form gap junctions and play an important role in intercellular communication. Although there have been no reports on connexin 30 or 43 and schizophrenia, connexin 50 has been identified as a susceptibility gene for schizophrenia²⁵. Thus, studying the effects on connexins following antipsychotics and in animal models of schizophrenia may be of interest. Identifying genes mediated by β -catenin following antipsychotic and LY379268 may help to further understand the role of the Wnt pathway in antipsychotic drug action.

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APPENDIX A



November 1, 2009

***This is the 3rd Renewal of this protocol**
*A Full Protocol submission will be required in 2010

Dear Dr. **Rushlow**

Your Animal Use Protocol form entitled:

Characterization of Signaling Molecules Potentially Responsible for the Manifestation and Amelioration of Schizophrenic Symptoms

has had its yearly renewal approved by the Animal Use Subcommittee.

This approval is valid from **November 1, 2009 to October 31, 2010**

The protocol number for this project remains as **2006-074**

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

REQUIREMENTS/COMMENTS

Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

c.c. Approved Protocol - W. Rushlow, W. Lagerwerf
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