The in vivo effects of aripiprazole on the PKA- and GSK3β-dependent signalling pathways in rat brains

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The in vivo effects of aripiprazole on the PKA- and GSK3β-dependent signalling pathways in rat brains

Bo Pan

This thesis is presented as part of the requirements for the award of the Degree of Doctor of Philosophy of the University of Wollongong

March, 2016
ABSTRACT

Aripiprazole is a unique antipsychotic drug with favourable therapeutic effects and improved extrapyramidal side-effects. Its partial agonism for the dopamine D_2 receptors (D_2R) is considered to contribute to the clinical effects of aripiprazole. However, how aripiprazole regulates D_2R-mediated cellular signalling pathways, as well as their downstream substrates, is not clear. Therefore, this thesis investigated the cellular mechanisms of aripiprazole on these signalling pathways and regulators in an animal model, by comparing aripiprazole with a D_2R antagonist – haloperidol and a D_2R partial agonist – bifeprunox.

The study in Chapter 3 investigated the acute effects of aripiprazole (a single injection) on the D_2R downstream cyclic adenosine monophosphate (cAMP)-protein kinase A (PKA) and protein kinase B (Akt)-glycogen synthase kinase 3 beta (GSK3β) signalling pathways. Aripiprazole affected PKA-C expression similarly to haloperidol, but not bifeprunox, in the caudate putamen (CPu) and ventral tegmental area (VTA). In addition, aripiprazole increased phosphorylation of GSK3β in the prefrontal cortex (PFC), nucleus accumbens (NAc), CPu and substantia nigra (SN). Haloperidol also increased phosph-GSK3β in the NAc. These results suggest acute administration of aripiprazole affected the cAMP-PKA and Akt-GSK3β signalling pathways differentially from haloperidol and bifeprunox in various brain areas. It also indicated that its relatively low intrinsic activity for D_2Rs might contribute to the effects of aripiprazole.

The study in Chapter 4 continued to examine the effects of 1-week aripiprazole treatment by oral administration. In addition to PKA signalling, γ-aminobutyric acid (GABA)_A receptors and cAMP-responsive element-binding protein (CREB) were
examined. The data have shown that 1-week administration of aripiprazole elevated PKA activity by increasing the phosphorylation levels of PKA in the NAc. Aripiprazole also increased the expression of the GABA\(_A\) (β-1) receptor and CREB in the NAc. Furthermore, haloperidol elevated PKA activity in both the NAc and CPu, while haloperidol increased GABA\(_A\) (β-1) receptor expression and CREB1 expression (not significantly) in the NAc. These findings suggest that aripiprazole might exert its clinical effects via the regulation of GABA\(_A\) receptors and CREB1 in the NAc, possibly via the D\(_2\)R-mediated PKA signalling pathway.

In the study in Chapter 5, the effects of 1-week administration of aripiprazole on the Akt-GSK3β and Dvl-GSK3β-β-catenin signalling pathways were examined. The results demonstrated that aripiprazole increased GSK3β phosphorylation in the PFC, NAc and CPu, while haloperidol and bifeprunox had such effects only in the NAc and CPu, respectively. Additionally, both aripiprazole and haloperidol increased the expression of Dvl-3 and β-catenin in the NAc. The studies in Chapters 4 and 5 suggest that up-regulation of GABA\(_A\) (β-1) receptors and CREB1 in the NAc, and GSK3β phosphorylation in the PFC and NAc may be involved in the clinical effects of aripiprazole; they further indicate that the relatively low intrinsic activity for D\(_2\)Rs might be associated with the actions of aripiprazole.

In the clinic, in order to control symptoms of schizophrenia, patients usually experience chronic antipsychotic treatment. The chronic effects of aripiprazole on the GSK3β-dependent signalling pathways, GABA\(_A\) receptor and CREB1 are unknown. Therefore, the effects of 10-week administration of aripiprazole on these cellular signalling pathways and regulators were examined in Chapter 6. Administration of both
aripiprazole and bifeprunox activated the Akt-GSK3β signalling in the PFC. In the NAc, chronic administration of all three drugs increased Akt-GSK3β signalling; while both aripiprazole and haloperidol up-regulated Dvl-3, β-catenin and GABA_A receptors, as well as CREB1 activity. This study has confirmed the involvement of Dvl-GSK3β-β-catenin, GABA_A receptors and CREB1 activity in the long-term actions of aripiprazole.

In brief, this thesis provided in vivo evidence that aripiprazole was able to activate the PKA, Akt-GSK3β and Dvl-GSK3β-β-catenin signalling pathways, and also up-regulate GABA_A receptor expression, as well as CREB1 activity. Moreover, by comparing aripiprazole with haloperidol and bifeprunox, particularly their effects in the NAc, these studies further suggest that a relatively low intrinsic activity for D2Rs might be the key factor for aripiprazole to exert its unique clinical effects, and for other potential D2R partial agonists to achieve meaningful therapeutic effects.
I dedicate this thesis to my family, for their unfaltering love and support.
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I wish to express my sincere appreciation to many people who have provided guidance and assistance throughout my PhD studies.

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STATEMENT FOR THE STYLE OF THE THESIS

In accordance with the University of Wollongong thesis committee “Guidelines for Preparation and Submission of HDR theses” (2014) and “Higher Degree Research (HDR) Thesis by Compilation Rules” (2014), this PhD thesis is presented in “Journal Article Compilation Style Format”. This comprises a series of four original studies, three published in peer-reviewed journals, including *PLoS One, Journal of Molecular Neuroscience*, and *International Journal of Molecular Sciences*, as well as one manuscript submitted to *Scientific Reports*. I am the first author of these four publications. I hereby declare that I am the primary designer of these studies, and have carried out all experiments, data analysis and manuscript preparation.

Bo Pan

2016

I consent to the presentation of the PhD in ‘Journal Article Style’ and I acknowledge the above statement pertaining to student contribution to be correct.

Prof. Chao Deng, Principal Supervisor

2016
LIST OF PUBLICATIONS AS PART OF THIS THESIS

The following four refereed journal papers are included as part of this thesis:


Other publications and presentations related to this thesis:

Published Abstract


Conference Proceedings


Additional publications from other projects that I have been involved in during my doctoral studies:

Publications in Refereed Journals


STATEMENT OF CONTRIBUTION OF OTHERS

I, Bo Pan, declare that this thesis, submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Medicine, University of Wollongong, is entirely my own work unless otherwise referenced or acknowledged. Two co-authors (Chao Deng and Xu-Feng Huang) of the four journal articles included in the thesis are my PhD supervisors, who have provided comments on experimental design, data analysis, results interpretation, and revision of manuscripts. Two co-authors (Jiezhong Chen and Jiamei Lian) of two of the journal articles included in the thesis are my colleagues, who have provided comments on experimental operations, data analysis, results interpretation, and revision of manuscripts.

Bo Pan

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LIST OF ABBREVIATIONS

Akt, protein kinase B

AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

BRET, bioluminescence resonance energy transfer

cAMP, cyclic adenosine monophosphate

CPu, caudate putamen

CREB, cAMP-responsive element-binding protein

D₂R, dopamine D₂ receptor

Dvl, dishevelled

ELISA, enzyme-linked immunosorbent assay

EPS, extrapyramidal side-effects

ERK, extracellular signal-regulated kinase

GABA, γ-aminobutyric acid

GAD, glutamic acid decarboxylase

GPCR, G protein-coupled receptor

GSK3β, glycogen synthase kinase 3-beta

NAc, nucleus accumbens

NMDA, N-methyl-D-aspartate
NMDAR, NMDA receptor,

PET, positron emission tomography

PFC, prefrontal cortex

PI3K, phosphoinositide 3-kinase

PKA, protein kinase A

PKC, protein kinase C

PLC, phospholipase C

PP2A, protein phosphatase 2A

SN, substantia nigra

TH, tyrosine hydroxylase

VTA, ventral tegmental area
Schizophrenia is a devastating mental disorder, affecting approximately 1% of the general population world-wide (van Os and Kapur, 2009). Various antipsychotics are used to control the symptoms of schizophrenia. Their action at dopamine D_2-like receptors (D_2Rs) contributes to the therapeutic effects of all available antipsychotics (Kapur and Mamo, 2003). However, blockade of D_2Rs by antipsychotics, especially the typical antipsychotics (e.g. haloperidol), induces unexpected side-effects, such as extrapyramidal side-effects (EPS) (Pierre, 2005). Aripiprazole is a unique antipsychotic drug that possesses favourable therapeutic effects, but with a very low rate of EPS (Di Sciascio and Riva, 2015). Aripiprazole’s partial agonism for the D_2Rs is considered to be associated with its unique clinical profile of (Di Sciascio and Riva, 2015). However, all other D_2R partial agonists that aimed to mimic aripiprazole have failed to achieve meaningful therapeutic effects in clinic trials. It was then proposed that the unique clinical profile of aripiprazole might be attributed to its functional selectivity for the D_2R, which is supported by previous *in vitro* and *in vivo* evidence (Han et al., 2009; Lawler et al., 1999; Mailman, 2007; Shapiro et al., 2003). However, how aripiprazole regulates D_2R-mediated cellular signalling pathways, as well as their downstream substrates, is not clear. Therefore, this thesis investigated the cellular mechanisms of aripiprazole in modulating the PKA- and GSK3β-dependent signalling pathways, by comparing it with a D_2R antagonist – haloperidol – and a D_2R partial agonist – bifeprunox.

In summary, this thesis systematically investigated the *in vivo* effects of aripiprazole on several signalling pathways that are related to the pathophysiology of schizophrenia and antipsychotic therapeutics in various brain regions. Overall, the data have shown that
antipsychotics have stronger effects in the NAc than in other brain regions. Specifically, antipsychotics were able to affect the Dvl-GSK3β-β-catenin pathway, GABA_A receptor expression and CREB in the NAc, but not in the other brain regions. Additionally, antipsychotics were able to alter the activity of GSK3β in both the NAc and PFC. Furthermore, aripiprazole displayed similar effects to haloperidol, but not bifeprunox, in the NAc, suggesting that aripiprazole is likely to exert antagonism for D2Rs due to its relatively low intrinsic activity for D2Rs; however, their effects in the PFC were different, probably indicating the therapeutic effects of aripiprazole on the negative and cognitive symptoms of schizophrenia. The results of this thesis provide in vivo evidence to develop further clues and strategies in the development of new antipsychotic drugs.

The study in Chapter 3 showed the acute effects of aripiprazole on the D2R downstream cyclic adenosine monophosphate (cAMP)-protein kinase A (PKA) and protein kinase B (Akt)-glycogen synthase kinase 3-beta (GSK3β) signalling pathways. Rats were intraperitoneally injected once with aripiprazole (0.75 mg/kg), bifeprunox (0.8 mg/kg), haloperidol (0.1 mg/kg) or vehicle. The protein kinases PKA-C, Akt, phospho-Akt, GSK3β and phospho-GSK3β were examined in five brain regions – the prefrontal cortex (PFC), nucleus accumbens (NAc), caudate putamen (CPu), ventral tegmental area (VTA) and substantia nigra (SN). Aripiprazole presented similar effects on PKA-C expression to haloperidol, but not bifeprunox, in the CPu and VTA. Aripiprazole, but not bifeprunox and haloperidol, increased the phosphorylation levels of GSK3β in the PFC, NAc, CPu and SN. These results suggested acute administration of aripiprazole affected the cAMP-PKA and Akt-GSK3β signalling pathways differentially from haloperidol and bifeprunox in various brain areas; it also indicated that the unique
pharmacological profile of aripiprazole may be attributed to its relatively lower intrinsic activity for D₂Rs.

In Chapter 3, acute administration of aripiprazole displayed effects on PKA signalling, so this chapter continued to investigate the short-term effects of aripiprazole. Additionally, in the clinic, patients usually take drugs orally to treat the symptoms. Therefore, in Chapter 4, we orally administered animals with aripiprazole (0.250 mg/kg), bifeprunox (0.267 mg/kg), haloperidol (0.033 mg/kg) or vehicle three times per day for 1 week. In addition to the PKA signalling pathway, γ-aminobutyric acid (GABA)\(_A\) receptor and cAMP-responsive element-binding protein 1 (CREB\(_1\)) were examined due to their involvement in the pathophysiology of schizophrenia. The results showed that aripiprazole elevated PKA activity by increasing the levels of p-PKA and the ratio of p-PKA/PKA only in the NAc. Correlated with this enhanced PKA activity, aripiprazole also increased expression of the GABA\(_A\) (β-1) receptor and CREB\(_1\) in the NAc. Haloperidol elevated PKA activity by increasing p-PKA levels and the ratio of p-PKA/PKA in both the NAc and CPu; while haloperidol increased the GABA\(_A\) (β-1) receptor and CREB\(_1\) in the NAc, but not significantly. Lastly, bifeprunox had no effects on PKA signalling in these brain regions. This study suggested that aripiprazole increased the GABA\(_A\) (β-1) receptor and CREB\(_1\) in the NAc, probably via activating PKA signalling, which cannot be achieved by haloperidol and bifeprunox.

The results of Chapter 3 showed that acute administration of aripiprazole affected GSK3\(β\), but not Akt. Since GSK3\(β\) is a multi-targeted kinase, antipsychotics may affect other GSK3\(β\)-dependent signalling pathways. The Dvl-GSK3\(β\)-β-catenin signalling pathway is involved in schizophrenia and the actions of antipsychotics. Therefore, the
effects of 1-week administration of antipsychotics on the Dvl-GSK3β-β-catenin
signalling pathway, along with the Akt-GSK3β signalling pathway were examined in
Chapter 5. The results showed that aripiprazole increased GSK3β phosphorylation in
the PFC, NAc and CPu, while haloperidol and bifeprunox had such effects only in the
NAc and CPu, respectively. Additionally, both aripiprazole and haloperidol, but not
bifeprunox, increased Dvl-3 and β-catenin expression in the NAc. This study suggested
that activation of GSK3β phosphorylation in the PFC and NAc may be involved in the
clinical effects of aripiprazole; it further indicated that the relatively low intrinsic
activity for D2Rs might contribute to the effects of aripiprazole on the up-regulation of
Dvl and β-catenin expression.

It should be noted that in order to control the symptoms of schizophrenia, patients
usually experience chronic antipsychotic treatment in clinics. Therefore, we investigated
the chronic in vivo effects of aripiprazole in Chapter 6. In this study, the animals were
orally administered aripiprazole (0.250 mg/kg), bifeprunox (0.267 mg/kg), haloperidol
(0.033 mg/kg) or vehicle three times per day for 10 weeks. The activity of PKA, Akt,
GSK3β and CREB was examined by Western blots; the expression of Dvl-3, β-catenin
and GABA_A (containing β-1 subunit) receptor was also measured. After administration
of aripiprazole and bifeprunox, activation of the Akt-GSK3β pathway was observed in
the PFC. In the NAc, all three drugs induced Akt-GSK3β signalling; while both
aripiprazole and haloperidol increased the expression of Dvl-3, β-catenin and GABA_A
receptors, as well as the phosphorylation levels of CREB. This study confirmed the
involvement of Dvl-GSK3β-β-catenin, GABA_A receptor and CREB activity in the NAc
in the long-term actions of aripiprazole and haloperidol, suggesting that chronic
antipsychotic drug administration might alleviate the positive symptoms of
schizophrenia via these cellular signalling pathways. It is also suggested that the Akt-
GSK3β signalling pathway in the PFC is a possible route through which aripiprazole
treats negative and cognitive symptoms of schizophrenia. Lastly, in comparison with the
results of acute and short-term antipsychotic drug administration, antipsychotics might
affect PKA and Akt-GSK3β signalling pathways in a time-dependent manner.
2.1 Characteristics of schizophrenia

Schizophrenia is a group of persistent, chronic, severe and disabling mental disorders, affecting approximately 1% of the world’s general population (van Os and Kapur, 2009). The term “schizophrenia” was first used by Swiss psychiatrist Eugen Bleuler in 1911 from the Greek roots schizein (“to split”) and phrēn (“mind”), roughly meaning "splitting of the mind", because patients who developed schizophrenia seemed to vacillate between normal and abnormal states (Picchioni and Murray, 2007). Studies of schizophrenia have been carried out for decades since it was first characterised; however, the exact pathophysiology of schizophrenia still remains unclear.

2.1.1 Symptoms of schizophrenia

Schizophrenia symptoms are clinically characterised by positive symptoms, negative symptoms and cognitive deficits (Lisman et al., 2008). Positive symptoms represent an excess or distortion of normal functions and behaviours. Patients who develop positive symptoms usually manifest abnormal thoughts and behaviours, including hallucinations (auditory, visual, olfactory, etc.), delusions, disorganised thinking and speech and disorganised or catatonic behaviours (Bennett, 2008).

Negative symptoms represent the loss or absence of normal traits or abilities. Patients with negative symptoms might experience social withdrawal, a reduction of emotional responsiveness, apathy, poverty of speech, inability to experience pleasure, memory impairment, lack of motivation and defects in attention control (Bodkin et al., 1996).
Cognitive deficits mainly include memory deficits, attenuated attention processes, and executive functioning impairment. Patients with cognitive deficits might experience delayed and impaired working memory, slowed cognitive speed, and impaired sequencing, organisation and flexibility (Gopal and Variend, 2005).

2.2 Pathophysiological mechanisms of schizophrenia

The exact pathophysiology of schizophrenia remains poorly understood. A variety of theories have been proposed to be associated with its pathophysiology. Several neuronal transmission systems are associated with the pathophysiology of schizophrenia, including the dopaminergic, GABA(γ-Aminobutyric acid)ergic and glutamatergic transmission systems (Elert, 2014; Insel, 2010). Among them, the present thesis focuses on the dopaminergic and GABAergic systems.

2.2.1 The role of dopaminergic neurotransmission in the pathophysiology of schizophrenia

2.2.1.1 Dopamine synthesis, release and reuptake

Dopamine is produced in several areas of the brain, largely in the VTA and SN. Dopamine is biosynthesised by the hydroxylation of the amino acid tyrosine to 3,4-dihydroxyphenylalanine (DOPA) via the enzyme tyrosine hydroxylase (TH) and then by the decarboxylation of DOPA by aromatic L-amino acid decarboxylase (AADC). After synthesis, dopamine is transported by vesicular monoamine transporter 2 (VMAT2) into vesicles, which are then released into the synapse in response to a presynaptic action potential (Eiden et al., 2004). After signalling transmission, the
remaining dopamine in the cleft is re-absorbed via the dopamine transporter (DAT) for recycling (Masson et al., 1999).

2.2.1.2 Dopamine receptors

The dopamine receptor is a class of G protein-coupled receptors (GPCRs). Five subtypes of dopamine receptors have been identified: D₁, D₂, D₃, D₄, and D₅. The D₁ and D₅ receptors are members of the D₁-like family of dopamine receptors; the D₂, D₃ and D₄ receptors belong to the D₂-like family (Missale et al., 1998). The structure of a dopamine receptor is illustrated in Fig. 2-1. Compared with D₁-like receptors, D₂Rs have a shorter COOH-terminal tail and a bigger third intracellular loop (I3). D₁-like family receptors are coupled to the G protein Gₐ(s) (G stimulatory), which can stimulate adenylyl cyclase (AC), increasing the formation of the second messenger cyclic adenosine monophosphate (cAMP). D₂-like family receptors interact with the G protein Gₐ(i) (G inhibitory), which can reduce AC activity, inhibiting the synthesis of cAMP.

Although both D₁- and D₂-like receptors are involved in the pathophysiology of schizophrenia, the antipsychotics (aripiprazole, haloperidol and bifeprunox) examined in this thesis have strong affinity for D₂-like, but not D₁-like, receptors (Correll, 2010; Wadenberg, 2007). Therefore, we focused on D₂Rs. Two isoforms of the D₂R have been identified: the short-form D₂ receptor (D₂S_R) and the long-form D₂ receptor (D₂L_R) (Missale et al., 1998). The D₂S_R is pre-synaptically located, regulating neurotransmission via feedback mechanisms, being involved in the regulation of synthesis, storage, and release of dopamine (Girault and Greengard, 2004). The D₂L_R is a classical post-synaptic receptor, transmitting extracellular information into cells (Girault and Greengard, 2004).
The structure of a dopamine receptor.

Potential phosphorylation sites are represented on 3rd intracellular loop (I3) and on COOH terminus. Potential glycosylation sites are represented on NH₂ terminal.

**Abbreviations:** E1–E3, extracellular loops; 1–7, transmembrane domains; I2–I3, intracellular loops; P, potential phosphorylation sites.

2.2.1.3  Dopaminergic neurotransmission pathways in the brain

There are three major dopaminergic neurotransmission pathways – the nigrostriatal pathway (Fig. 2-2A), mesolimbic pathway (Fig. 2-2B) and mesocortical pathway (Fig. 2-2C); it has been proposed that these pathways are associated with the pathophysiology of schizophrenia and the action of antipsychotics. In the mesolimbic pathway (Fig. 2-2B), dopaminergic neurons project from the VTA to the nucleus accumbens (NAc) in the striatum. In the mesocortical pathway (Fig. 2-2C), dopaminergic neurons project
from the VTA to the prefrontal cortex (PFC). In the nigrostriatal dopaminergic pathway, dopaminergic neurons project from the SN to the caudate putamen (CPu) in the striatum.

Fig. 2-2 The three major dopaminergic neurotransmission pathways in the human brain.

A. Nigrostriatal pathway. B. Mesolimbic pathway.
C. Mesocortical pathway.

Abbreviations: PFC = prefrontal cortex; CPu = caudate putamen; NAc = nucleus accumbens; SN = substantia nigra; VTA = ventral tegmental area.

2.2.1.4 Dopamine hypothesis of schizophrenia

The dopamine hypothesis of schizophrenia initially focused on excess neurotransmission at dopamine receptors (especially D$_2$Rs) and blockade of these receptors to treat the psychosis, which was based on the observations that psychotic symptoms similar to those seen in schizophrenia were induced by administration of amphetamine and other compounds that increase extracellular concentrations of dopamine; and drugs that deplete dopamine levels reduced psychotic symptoms (Creese et al., 1976; Howes and Kapur, 2009; Lieberman et al., 1987; Seeman et al., 1976).
Later, new evidence showed that impaired dopamine activity in the PFC results in increased levels of dopamine, dopamine metabolite and D₂R density in the striatum (Howes and Kapur, 2009; Pycock et al., 1980). Regional specificity and cortical-subcortical interactions of dopamine neurotransmission have been added into the hypothesis, which further proposed that schizophrenia is characterized by hypodopaminergia in the PFC resulting in hyper-dopaminergia in the NAc (Howes et al., 2015; Howes and Kapur, 2009). More specifically, it is hypothesised that hypodopaminergia in the PFC results in the cognitive deficits of schizophrenia and striatal hyper-dopaminergia in the NAc results in the positive symptoms (Howes et al., 2015; Howes and Kapur, 2009). Nowadays, abnormal dopamine synthesis and dopamine release have been taken into account. Radiolabelled L-DOPA was used in positron emission tomography (PET) studies and increased dopamine synthesis from presynaptic terminals has been found in schizophrenic subjects compared with healthy controls in these studies (Demjaha et al., 2012; Hietala et al., 1999; Howes et al., 2009; Howes et al., 2013; Lindstrom et al., 1999; McGowan et al., 2004; Meyer-Lindenberg et al., 2002; Reith et al., 1994). Moreover, studies using radiotracers [¹¹C]-raclopride and [¹²³I]-IBZM (they can compete with dopamine to bind with D₂Rs) have revealed that dopamine release is increased in schizophrenic patients (Abi-Dargham et al., 1998; Abi-Dargham et al., 2009; Breier et al., 1998; Egerton et al., 2010; Laruelle et al., 1999; Mizrahi et al., 2012; Pogarell et al., 2012). Therefore, it is suggested that presynaptic dopamine dysfunction, which leads to elevated levels of synaptic dopamine, is associated with the symptoms of schizophrenia; in addition, higher striatal dopamine synthesis capacity and dopamine release result in cortical dysfunctions (Fusar-Poli et al., 2011; Howes et al., 2015).
2.2.2 The role of GABAergic neurotransmission in the pathophysiology of schizophrenia

2.2.2.1 GABA synthesis, release and reuptake

GABA is the chief inhibitory neurotransmitter in the mammalian central nervous system. GABA is synthesised from glutamate using L-glutamic acid decarboxylase (GAD) and pyridoxal phosphate as a cofactor (Petroff, 2002) and then transported and stored in the vesicles through vesicular GABA transporter. Free GABA in the synaptic cleft is reuptaken by GABA transporter into the pre-synapse and stored in the vesicles; it is also transported into glial cells and converted into succinic semialdehyde and glutamate by GABA transaminase (Petroff, 2002) (Fig. 2-3).

2.2.2.2 The GABA_A receptor

Three general classes of GABA receptor have been identified: the GABA_A, GABA_B and GABA_C receptor. The GABA_A and GABA_C receptor are ionotrophic receptors, which are a ligand-gated ion channel complex; the GABA_B metabotropic receptors are GPCRs that open or close ion channels via G proteins (Hendry et al., 1989). Among these three classes of GABA receptor, the GABA_A receptor is an important therapeutic target in the brain because of its strong connection with schizophrenia (see section 2.2.2.3). The GABA_A receptor is a pentameric transmembrane receptor that consists of five subunits: two α-, two β-, and one γ-subunits arranged around a central pore (Braat and Kooy, 2015; Fritschy and Panzanelli, 2014). The major types of subunits that have been identified are the α_1-6, β_1-3, and γ_1-3 subunits, among which the α_1, α_2 and α_3 subunits, along with the β subunit variants and the γ_2 subunit are highly expressed in postsynaptic receptors (Fritschy and Panzanelli, 2014).
**Fig. 2-3** The synthesis, release, reuptake of glutamate.

**Abbreviations:** GABA = γ-aminobutyric acid; GAT = GABA transporter; vGAT = vesicular GABA transporter

Two GABA molecules bind with the receptor at the interface between an α and a β subunit (Fritschy and Panzanelli, 2014). Once bound to GABA, the protein receptor changes conformation within the membrane, opening the pore in order to allow Cl⁻ to pass down an electrochemical gradient, resulting in hyperpolarisation of the neuron (Braat and Kooy, 2015) (Fig. 2-4).
Fig. 2-4 Schematic diagram of the GABA_\text{A} receptor.

Left: The receptor consists of five subunits, which are symmetrically arranged, forming a central Cl\textsuperscript{−} ion channel pore.

Right: Five subunits form the receptor, the Cl\textsuperscript{−} ion channel pore and the two GABA active binding sites at the α and β interfaces.

2.2.2.3 Abnormal GABA receptors in schizophrenia

Of the three classes of GABA receptors, GABA_\text{A} receptors are the most complex. The abundant allosteric binding sites on these receptors could be targeted by various drugs and treatments, making GABA_\text{A} receptors an important therapeutic target (Wassef et al., 2003). The relationship between abnormalities in GABA_\text{A} receptors and schizophrenia is obvious. Post-mortem studies reported increased binding density of [\textsuperscript{3}H]-muscimol or [\textsuperscript{3}H]-GABA to the total population of GABA_\text{A} receptors in various brain regions, including the dorsolateral prefrontal cortex, caudate nucleus, posterior and anterior cingulate cortex, superior temporal gyrus and hippocampal formation in patients with schizophrenia (Benes et al., 1992; Dean et al., 1999; Deng and Huang, 2006; Ishikawa et al., 2004; Nakazawa et al., 2012; Newell et al., 2007; Woo et al., 2004). The increased binding might indicate a compensatory up-regulation of GABA_\text{A} receptors in
response to defective inhibitory modulation, probably caused by a decreased number of inhibitory interneurons, or impaired release and/or transport of GABA (Nakazawa et al., 2012). These changes in GABA<sub>A</sub> receptors in the schizophrenic brain may represent a primary pathogenesis, which might be associated with the symptoms of schizophrenia (Nakazawa et al., 2012). Since GABA plays an inhibitory role in counter-balancing glutamate-mediated neuronal excitation, abnormalities in the communication between them might result in a loss of inhibitory tone and consequent disinhibition of excitatory pyramidal cells, leading to neuronal dysfunctions in other brain regions, which finally may cause the impaired cognitive functions observed in schizophrenia (Looijestijn et al., 2015; Nakazawa et al., 2012).

2.3 Antipsychotics

2.3.1 Typical antipsychotics

Typical antipsychotics (also known as first-generation antipsychotics), discovered in the 1950s, were the first effective antipsychotic drugs to treat some of the symptoms of schizophrenia. Typical antipsychotics are effective to ameliorate the positive symptoms in schizophrenia, such as hallucinations, delusions and thought disorder. So far, a number of antipsychotics have been developed and approved for use clinically to treat psychosis, especially schizophrenia, including fluphenazine (Prolixin), haloperidol (Haldol), thioridazine (Mellaril), trifluoperazine (Stelazine), pipotiazine (Piportil) and perphenazine (Trilafon). Typical antipsychotics have high affinity specifically for D<sub>2</sub>Rs (e.g. haloperidol Ki value: 2.6 nM for D<sub>2</sub> receptors, 2 nM for D<sub>3</sub> receptors and 3 nM for D<sub>4</sub> receptors) (Table 2-1) and are potent antagonists for them (Ginovart and Kapur,
Typical antipsychotics achieve their clinical therapeutic effects mainly by potently blocking D₂Rs (Correll, 2010).

Although typical antipsychotics are effective in treating the positive symptoms in schizophrenia, they have few effects in treating negative symptoms or cognitive deficits (Agid et al., 2008). Due to blockade of the D₂R in the nigrostriatal dopaminergic pathway, typical antipsychotics can cause EPS (e.g. tardive dyskinesia and akathisia) and hyperprolactinemia, as well as body weight gain to some extent (Bishara and Taylor, 2008; Pierre, 2005).

Table 2-1 Receptor affinities of main typical and atypical antipsychotic drugs, including aripiprazole.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>HAL</th>
<th>OLAN</th>
<th>CLOZ</th>
<th>ARI</th>
<th>BIF</th>
</tr>
</thead>
<tbody>
<tr>
<td>D₁</td>
<td>210</td>
<td>31</td>
<td>85</td>
<td>265</td>
<td>—</td>
</tr>
<tr>
<td>D₂</td>
<td>2.6</td>
<td>20</td>
<td>210</td>
<td>0.66</td>
<td>8.5</td>
</tr>
<tr>
<td>D₃</td>
<td>2</td>
<td>49</td>
<td>473</td>
<td>0.8</td>
<td>9.1</td>
</tr>
<tr>
<td>D₄</td>
<td>3</td>
<td>27</td>
<td>35</td>
<td>44</td>
<td>8</td>
</tr>
<tr>
<td>5-HT₁ₐ</td>
<td>1,800</td>
<td>610</td>
<td>160</td>
<td>5.5</td>
<td>8.2</td>
</tr>
<tr>
<td>5-HT₂ₐ</td>
<td>61</td>
<td>1.5</td>
<td>2.59</td>
<td>8.7</td>
<td>—</td>
</tr>
<tr>
<td>5-HT₂c</td>
<td>4,700</td>
<td>4.1</td>
<td>4.8</td>
<td>26</td>
<td>—</td>
</tr>
<tr>
<td>a₁</td>
<td>17</td>
<td>44</td>
<td>6.8</td>
<td>74</td>
<td>—</td>
</tr>
<tr>
<td>a₂</td>
<td>600</td>
<td>280</td>
<td>158</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>H₁</td>
<td>260</td>
<td>0.08</td>
<td>3.1</td>
<td>30</td>
<td>—</td>
</tr>
<tr>
<td>M₁</td>
<td>&gt;10,000</td>
<td>2.5</td>
<td>1.4</td>
<td>6,780</td>
<td>—</td>
</tr>
</tbody>
</table>

Note: All values are reported as Ki (nM).

**Abbreviations:** HAL = haloperidol; OLAN = olanzapine; CLOZ = clozapine; ARI = aripiprazole; BIF = bifeprunox; D₁ = dopamine₁; D₂ = dopamine₂; D₃ = dopamine₃; D₄ = dopamine₄; 5-HT₁ₐ = serotonin₁ₐ; 5-HT₂ₐ = serotonin₂ₐ; 5-HT₂c = serotonin₂c; H₁ = histamine₁; M₁ = muscarinic₁.

(Modified from Correll, 2010; Wadenberg, 2007)

2.3.2 Atypical antipsychotics

In the 1960s, the first atypical antipsychotic drug (also known as the second-generation antipsychotic) – clozapine (Clozaril) was developed, and was used in the clinic in the
1970s. After clozapine, other atypical antipsychotic drugs, including risperidone (Risperdal), olanzapine (Zyprexa), quetiapine (Seroquel) and ziprasidone (Geodon), were developed and used clinically in treating symptoms in schizophrenia. Atypical antipsychotic drugs are effective to some degree in alleviating positive and negative symptoms, as well as cognitive deficits of schizophrenia, with reduced EPS (Kane and Correll, 2010; Meltzer, 2013).

The atypical antipsychotics have a lower affinity for D2Rs than the typical antipsychotics (Meltzer, 2013), but they can bind with various other receptors (Table 2-1). Atypical antipsychotics are predominantly antagonists for serotonin [5-hydroxytryptamine (5-HT)]_2A receptors (e.g. olanzapine Ki value: 1.5 nM; clozapine Ki value: 2.59 nM; aripiprazole Ki value: 8.7 nM) (Correll, 2010). In addition, atypical antipsychotics have affinity for 5-HT1A receptors (e.g. aripiprazole Ki value: 5.5 nM), 5-HT2C receptors (e.g. olanzapine Ki value: 4.1 nM; clozapine Ki value: 4.8 nM), α adrenergic receptors (e.g. clozapine Ki value: 6.8 nM), histamine H1 receptors (e.g. olanzapine Ki value: 0.08 nM; clozapine Ki value: 3.1 nM), as well as muscarinic M receptors (e.g. olanzapine Ki value: 2.5 nM; clozapine Ki value: 1.4 nM) (Correll, 2010).

Because of their reduced blockade of the D2R, atypical antipsychotics cause EPS at a much lower rate; however, they (e.g. olanzapine, clozapine) are associated with severe weight gain/obesity side-effects, and other metabolic diseases such as type II diabetes, dyslipidaemia and insulin resistance (Milano et al., 2013). Accumulated evidence has shown that these side-effects induced by olanzapine and clozapine may be partly due to the blockade of H1 receptors, 5-HT2C receptors and M3 receptors (Deng et al., 2010; Harris et al., 2013; He et al., 2013; Lian et al., 2016; Weston-Green et al., 2013).
2.3.3 Aripiprazole

Aripiprazole [7-[4-[4-(2,3-dichlorophenyl)-1-piperazinyl]-butyloxy]-3,4-dihydro-2(1H)-quinolinone], a dihydrocarbostyril derivative (Fig. 2-5), is an atypical antipsychotic drug. Aripiprazole showed efficacy and well tolerability in clinical trials over different periods for the treatment of schizophrenia and schizoaffective disorder (Stip and Tourjman, 2010). It was also shown to be effective and well tolerated in the manic and maintenance phases of bipolar disorder (Aitchison et al., 2009; Dratcu et al., 2012).

Fig. 2-5 The chemical structure of aripiprazole

Aripiprazole has a novel receptor action profile, which is different from those of all other available first- and second-generation antipsychotic drugs. For this reason, it is also called “the third-generation antipsychotic drug” by some researchers (Mailman and Murthy, 2010). Aripiprazole was first developed as a D$_2$R partial agonist (Burris, 2002; Wood and Reavill, 2007). It exhibits high affinity for the dopaminergic receptors (Ki value: 0.45 nM for D$_2$ receptors and 0.8 nM for D$_3$ receptors) (Table 2-1) (Correll, 2010) and possesses high occupancy for the D$_2$R, ranging between approximately 71% at 2 mg/day to 96% at 40 mg/day (Kegeles et al., 2008; Yokoi et al., 2002). Furthermore, aripiprazole displays partial agonism for the serotonin 5-HT$_{1A}$ receptor and antagonism for the 5-HT$_{2A}$ receptor (Table 2-1) (Correll, 2010; Mamo et al., 2007). Aripiprazole
also has moderate affinity for histamine, α-adrenergic, and D₄ receptors (Di Sciascio and Riva, 2015).

Despite of its high affinity for and occupancy of the D₂Rs (Correll, 2010; Kegeles et al., 2008; Yokoi et al., 2002), aripiprazole, compared with typical antipsychotics (e.g. haloperidol), induces a much lower rate of hyperprolactinemia and EPS which are associated with excessive blockade of dopamine receptors (Poyurovsky et al., 2008). It is due to its partial agonism for the D₂R that it produces a much lower level of functional antagonism of D₂R-mediated neurotransmission than that induced by full D₂R antagonists (Kegeles et al., 2008; Mamo et al., 2007; Shapiro et al., 2003; Strange, 2008).

2.3.4 Pharmacological mechanisms of aripiprazole

2.3.4.1 Comparison between aripiprazole and other D₂ receptor partial agonists

As mentioned above, aripiprazole was the first D₂R partial agonist, approved for use in the clinic as an antipsychotic drug. However, to date, with the exception of aripiprazole, other D₂R partial agonists were not able to achieve meaningful clinical efficacy or were abandoned due to tolerability and/or safety issues. The relative intrinsic activity of aripiprazole and other D₂R partial agonists tested previously compared with that of dopamine (shown as 100%) is listed in Table 2-2. Clinical trials indicated that these D₂R partial agonists possessed various therapeutic effects and/or side-effects. For example, OPC-4392 [7-[3-(4-[2,3-Dimethylphenyl]piperazinyl)propoxy]-2(1H)-quinolinone] exhibited therapeutic effects on negative symptoms, but not positive symptoms of schizophrenia (Benkert et al., 1995); terguride [1,1-diethyl-3-(6-methyl-
8α-ergolinyl)urea] failed for a reason similar to OPC-4392 (Benkert et al., 1995) and it is primarily used as a serotonin antagonist (Janssen et al., 2015); SDZ 208-912 [N-[8α-2-chloro-6-methylergoline-8-yl]-2,2-dimethylpropanamide], with the lowest intrinsic activity for D2R in the list (Table 2-2), could improve the psychiatric rating scale for the assessment of both positive and negative symptoms in clinical trials, but also induced side-effects similar to those observed after haloperidol treatment (Benkert et al., 1995); (-)-3PPP (preclamol) [(-)-3-(3-hydroxyphenyl)-N-n-propylpiperidine] displayed apparent antipsychotic effects at the beginning of treatment in the clinical test, but its antipsychotic action was not sustained for longer than 1 week (Lahti et al., 1998).

Bifeprunox [7-[4-(biphenyl-3-ylmethyl)piperazin-1-yl]-1,3-benzoxazol-2(3H)-one] shares a similar pharmacological profile with aripiprazole. Both of them are partial agonists for D2Rs and serotonin 5-HT1A receptors (Newman-Tancredi et al., 2007). They also present similar affinity with these receptors (Table 2-1). In vivo studies have shown that both bifeprunox and aripiprazole decreased the firing activity and bursting activity of dopamine neurons in the rat VTA (Dahan et al., 2009; Etievant et al., 2009). Bifeprunox possesses potent effects in animal models of psychosis, without inducing cataleptogenic activity (Bardin et al., 2006; Hesselink et al., 2005). In a 6-week, double-blind, placebo-controlled study, bifeprunox was efficacious in reducing symptoms in patients with an acute exacerbation of schizophrenia, with a safe EPS liability profile (Casey et al., 2008; Wadenberg, 2007). However, it was still disapproved and cancelled due to lack of meaningful effects on non-acute patients with schizophrenia; it also induced severe side effects (e.g. nausea) and safety issues (Lundbeck, 2009). Although it was abandoned, bifeprunox has been chosen as a reference drug to compare with aripiprazole in this thesis.
Table 2-2 Relative intrinsic activity of dopamine, aripiprazole and other D₂R partial agonists at D₂L and D₂S receptors in the CHO cell lines

<table>
<thead>
<tr>
<th>Drugs</th>
<th>D₂L receptors</th>
<th>D₂S receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dopamine</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Bifeprunox</td>
<td>95.1%</td>
<td>99.1%</td>
</tr>
<tr>
<td>Terguride</td>
<td>94%</td>
<td>101%</td>
</tr>
<tr>
<td>OPC-4392</td>
<td>93.3%</td>
<td>98.6%</td>
</tr>
<tr>
<td>(-)-3PPP</td>
<td>93%</td>
<td>100%</td>
</tr>
<tr>
<td>Aripiprazole</td>
<td>86%</td>
<td>94.5%</td>
</tr>
<tr>
<td>SDZ 208-912</td>
<td>57.3%</td>
<td>85%</td>
</tr>
</tbody>
</table>

(Modified from Tadori et al., 2007; Tadori et al., 2005)

2.3.4.2 Evidence of functional selectivity of aripiprazole

As the study of aripiprazole progressed, some evidence indicated that partial agonism for D₂Rs with moderate intrinsic activity might not be the only reason for the unique effects of aripiprazole. Some researchers proposed that the unique pharmaceutical property of aripiprazole is also attributed to its functional selectivity for D₂Rs (Lawler et al., 1999; Mailman, 2007; Shapiro et al., 2003).

The concept of functional selectivity is different from traditional pharmacology. Traditional pharmacology posits that a ligand can be classified either as an agonist (full or partial), antagonist or an inverse agonist through a single receptor isoform, and that this characteristic will be consistent with all effector (second messenger) systems coupled to that receptor. In contrast, functional selectivity posits that a ligand can produce functional multiple effects for certain signal transduction pathways when interacting with a single receptor isoform (Mailman and Murthy, 2010). This can be presented when a single receptor has several possible signal transduction pathways. A ligand that possesses the property of functional selectivity might induce atypical
A functionally selective ligand acting at a single receptor might cause an altered balance of therapeutic and side effects, whereas a typical ligand induces therapeutic and side effects equally.

(Modified from Mailman and Murthy, 2010)

Conformation changes of G-proteins (compared to the endogenous ligand), which in turn can induce unique activation of downstream pathways. The degree to which each pathway is activated depends on both the intracellular (the type of cell) and extracellular (the location in the cell) environment (Mailman and Murthy, 2010). For example, when the “typical” ligand (Fig. 2-6, left panel) acts at a single receptor, two intracellular signalling pathways are activated, one associated with the therapeutic effect, the other with the side effect. In contrast, the functionally selective ligand (Fig. 2-6, right panel) preferentially activates the intracellular pathway that is linked to the therapeutic effect, rather than the one associated with the side effect, thereby minimising the unexpected
side effect induced by this single receptor. Therefore, compared with traditional ligands, a ligand with functional selectivity cannot be classified simply as an agonist or antagonist for a single receptor, because it can be both, depending on its preferred signal transduction pathways.

There are several lines of the evidence that support the functionally selective effects of aripiprazole. In vitro studies have indicated that aripiprazole elicited agonism for presynaptic D₂ autoreceptors, but antagonised postsynaptic D₂Rs (Kikuchi et al., 1995; Shapiro et al., 2003). Another in vitro study suggested that aripiprazole acted as a potent partial agonist to the D₂R-mediated signalling responses (such as potentiation of arachidonic acid release), and as a weak partial agonist using MAPK phosphorylation, but lacked agonist activity on receptor internalisation (Urban et al., 2007). Koener et al. (2012) suggested that aripiprazole displayed functionally selective effects on D₂Rs, depending not only on receptor density but also on the surrounding cellular environment. Furthermore, an in vivo study demonstrated that aripiprazole had selective effects on the mesolimbic vs. the nigrostriatal dopaminergic pathways (Han et al., 2009). This study found that aripiprazole significantly increased D₂R mRNA expression and decreased TH mRNA expression in the VTA after both 1- and 12-week treatment, but had no effect on the SN; in contrast, haloperidol displayed non-selective influences on both the mesolimbic and nigrostriatal dopaminergic pathways (Han et al., 2009). It is worth noting that aripiprazole had no effect on D₂R mRNA expression and D₂R binding in the NAc and CPu, suggesting that the regulation of dopamine synthesis and release through the D₂ autoreceptors in the mesolimbic pathway may contribute to the therapeutic effects of aripiprazole (Han et al., 2009). Additionally, the absence of influence on D₂Rs in the nigrostriatal pathway may also explain the lower rate of EPS of aripiprazole.
Findings from these studies cannot be explained simply by its partial agonism for D₂Rs. Taken together, it seems likely that aripiprazole does not affect the signalling pathways mediated by D₂Rs globally; instead, aripiprazole could interact with signalling pathways in a brain-region-selective manner. However, further studies, in particular in vivo studies, are still required to validate the functional selective property of aripiprazole.

2.4 Effect of antipsychotics on various receptors and cellular signalling pathways

2.4.1 Effect of antipsychotics on D₂R downstream signalling pathways

As described in section 2.3, nearly all antipsychotics can act at D₂Rs, and the dopaminergic neurotransmission pathways are major pathways through which antipsychotics elicit their therapeutic effects and side-effects (e.g. EPS); additionally, aripiprazole might selectively affect the D₂R-mediated downstream signalling pathways. The dopamine D₂ receptor mediates two major downstream signalling pathways: the G-protein-dependent cAMP-PKA signalling pathway and Akt-GSK3β signalling pathway.

2.4.1.1 Effect of antipsychotics on cAMP-PKA signalling pathway

The cAMP-PKA signalling pathway

The D₂R is a kind of G-protein-coupled receptor, transmitting signals via coupled G-proteins, which consists of Gₐ, Gₛ and Gγ subunits. Without a ligand agonist, the Gₐ subunit is bound to guanosine diphosphate (GDP) and the complex of Gₛ and Gγ subunits (Gₛγ complex). Upon stimulus of the ligand, GDP will be released and guanosine triphosphate (GTP) will be bound with the Gₐ subunit instead of GDP. The Gₐ subunit and Gₛγ complex will then be released and separated. The Gₐ subunit can
stimulate AC, restraining the production of cAMP derived from adenosine triphosphate (ATP) and further inhibiting the activity of protein kinase A (PKA) (Fig. 2-7).

**Abbreviations:** AC = adenylate cyclase; ATP = adenosine triphosphate; cAMP = cyclic adenosine monophosphate; GDP = guanosine diphosphate; GTP = guanosine triphosphate; PKA = protein kinase A.

One previous study reported that the short-form D₂ autoreceptor interacts preferentially with the cAMP pathway to control synthesis, release and uptake of dopamine (Meyer-Lindenberg et al., 2002). Additionally, TH was found to be phosphorylated by the cAMP-dependent protein kinase (Sura et al., 2004) (Fig. 2-8).

**Fig. 2-7** The cAMP-PKA signalling pathway of the dopamine D₂ receptor.
Fig. 2-8 Modulation of D\textsubscript{2} autoreceptor on the synthesis, release and re-uptake of dopamine.

**Abbreviations:** AADC = Aromatic L-amino acid decarboxylase; DA = dopamine; DAT = dopamine transporter; DOPA = 3,4-dihydroxyphenylalanine; PKA = protein kinase A; MAPK = mitogen-activated protein kinase; TH = Tyrosine hydroxylase; VMAT2 = vesicular monoamine transporter 2.

**Effect of antipsychotics on the cAMP-PKA signalling pathway**

There is significant evidence that antipsychotic drug administration has various effects on the cAMP-PKA signalling pathway. Experiments using bioluminescence resonance energy transfer (BRET) have suggested that all classes of antipsychotics (including haloperidol, aripiprazole, clozapine, chlorpromazine, quetiapine, olanzapine, risperidone and ziprasidone) can differentially affect the cAMP-PKA pathway through the long dopamine (D\textsubscript{2L}) receptor mediated G\textsubscript{a(ii/o)} protein (Klewe et al., 2008; Masri et al., 2008). Additionally, a previous study has found that chronic treatment (21 days) with clozapine
inhibited cAMP binding to the subunit of PKA, reducing its protein levels of RIIα, RIIβ, and Catβ-subunit isoforms, as well as their respective mRNA expression; however, chronic treatment (21 days) with haloperidol significantly increased the activity of cAMP-PKA pathway in the striatum, as well as mRNA and protein levels of the RIIα and RIIβ subunit isoforms in the striatum (Dwivedi et al., 2002). Lastly, it has been reported that aripiprazole inhibited AC activity and cAMP accumulation in vitro (Burris, 2002; Lawler et al., 1999; Shapiro et al., 2003).

It has also been reported that antipsychotic treatment is able to affect dopamine autoreceptors, regulating dopamine synthesis and re-absorption through cAMP-PKA signalling. For example, Onali and colleagues (1992) have reported that activation of D₂ autoreceptors induced a decrease in the cAMP-dependent activation of TH both in vivo and in vitro. Aripiprazole may elicit its therapeutic effects in animals through causing the D₂ autoreceptors to regulate dopamine synthesis, release and reuptake (Han et al., 2009).

2.4.1.2 Effect of antipsychotics on Akt-GSK3β signalling pathway

The Akt-GSK3β signalling pathway

In addition to the G-protein-dependent cAMP-PKA signalling pathway, the dopamine D₂ receptor also transmits signals via the G-protein-independent β-arrestin2-Akt-Glycogen synthase kinase 3 (GSK3) signalling pathway. GSK3 is a serine/threonine protein kinase, having two isoforms, GSK3α and GSK3β, ubiquitously expressed in all mammalian tissue and subcellular organelles (Hur and Zhou, 2010). GSK3 is different from many other kinases in cells; it is active under basal conditions and loses its kinase
activity when phosphorylated by Akt at an N-terminal serine residue (Hur and Zhou, 2010).

**Fig. 2-9** The dopamine D₂ receptor-mediated β-arrestin2-Akt-GSK3β signalling pathway

**Abbreviations:** Akt = protein kinase B; GSK3β = glycogen synthase kinase 3-beta.

Accumulated evidence indicates that in addition to the canonical signalling pathways via G proteins, the recruitment of β-arrestin2 to the D₂R can also activate cellular signalling in a G protein-independent manner by inducing the formation of a signalling complex that comprises at least β-arrestin2, Akt and protein phosphatase 2A (PP2A) (Beaulieu et al., 2005; Lefkowitz and Shenoy, 2005) (Fig. 2-9). A previous study suggested that β-arrestin2-knockout mice displayed decreased responses to
apomorphine, a non-selective dopamine receptor agonist (Beaulieu et al., 2005) and impaired locomotor responses to the dopamine-dependent actions of amphetamine and morphine (Beaulieu et al., 2005; Bohn et al., 2003). Taken together with the results of the study on the D\(_2\)R antagonist haloperidol in non-transgenic animals (Emamian et al., 2004), a β-arrestin2-mediated cAMP-independent D\(_2\)R-regulated signalling pathway that involves Akt and GSK3β has been considered to be related to the above abnormal responses to the D\(_2\)R ligands in the β-arrestin2-knockout animals (Beaulieu et al., 2007; Beaulieu et al., 2006; Beaulieu et al., 2005; Beaulieu et al., 2004). It was also reported that activation of D\(_2\)R signalling via the β-arrestin2-PP2A-Akt complex deactivates Akt, followed by inhibition of GSK3β phosphorylation (stimulation of GSK3-mediated signalling) (Beaulieu et al., 2007, 2009; Beaulieu et al., 2004).

**Effect of antipsychotics on the Akt-GSK3β signalling pathway**

Both *in vitro* and *in vivo* studies have demonstrated the effects of antipsychotics on the Akt-GSK3β pathway, although the results are, to some degree, inconsistent. Among these studies, antipsychotics with antagonism for the D\(_2\)R (e.g. haloperidol) were shown to elevate Akt phosphorylation and increase concomitant GSK3β phosphorylation (Beaulieu et al., 2004; Emamian et al., 2004). In addition, several studies have reported that both acute and chronic treatment of first-generation (e.g. haloperidol) and second-generation (e.g. clozapine, olanzapine, risperidone, quetiapine, and ziprasidone) antipsychotics increased the levels of both GSK3β and phosphor-Ser-GSK3β in several brain regions of rodents, including the striatum, cortex and hippocampus (Alimohamad et al., 2005a; Beaulieu et al., 2009; Li et al., 2007). Furthermore, *in vitro* studies using BRET have demonstrated that antipsychotics (except aripiprazole), independent of class, can potently facilitate the D\(_{2L}\) receptor-mediated β-arrestin2-Akt-GSK3β signalling
pathway, but have various effects on the $D_{2L}$ receptor-mediated cAMP-PKA signalling pathway (Klewe et al., 2008; Masri et al., 2008). Lastly, a BRET study has indicated that aripiprazole antagonised β-arrestin2 recruitment to the $D_{2L}$ receptor (Masri et al., 2008); however, another BRET experiment has found that aripiprazole (as a partial agonist) was incapable of stimulating recruitment of β-arrestin2 to the $D_{2L}$ receptor in vitro (Klewe et al., 2008). These inconsistent results require further validation.

In summary, several in vitro studies have examined the effects of aripiprazole on the $D_2$-coupled intracellular pathways; however, the in vivo effects of aripiprazole on the $D_2$-coupled intracellular pathways are not clear and need to be investigated; in particular, no study has investigated whether aripiprazole affects the cellular dopaminergic pathways differently in different brain regions.

### 2.4.2 Effects of antipsychotics on Dvl-GSK3β-β-catenin signalling pathway

Wnts (Wingless/Int-1) are secreted glycolipoproteins that can activate canonical (β-catenin dependent) and non-canonical Wnt signalling pathways (Panaccione et al., 2013). The canonical Wnt-Dvl-GSK3β-β-catenin signalling pathway plays a critical role in diverse neuronal processes including cell proliferation, migration, differentiation and cell fate during the development of the central nervous system (Davis and Ghosh, 2007; Lu and Van Vactor, 2007; McMahon and Bradley, 1990; Ohigashi et al., 2005), and has been considered to be related to the pathophysiology of schizophrenia and can be affected by antipsychotic administration (Freyberg et al., 2010; Panaccione et al., 2013).
The Dvl-GSK3β-β-catenin signalling pathway is illustrated in Fig. 2-10. Without the binding of Wnt to the seven-pass transmembrane Frizzled family receptor, a “destruction complex” containing Axin, adenomatosis polyposis coli (APC), casein kinase 1 (CK1) and GSK3 functions (Hart et al., 1998; Ikeda et al., 1998; Minde et al., 2011; Zeng et al., 1997). Within this protein complex, CK1 phosphorylates β-catenin at Ser45, which generates a priming site for subsequent GSK3β phosphorylation at Thr41 (Amit et al., 2002; Hagen et al., 2002; Hagen and Vidal-Puig, 2002; Liu et al., 2002; Sakanaka, 2002; Yanagawa et al., 2002) and subsequently at Ser37 and Ser33. This results in the recognition of β-catenin by β-TrCP (an E3 ubiquitin ligase subunit), and subsequent ubiquitination-mediated digestion by proteasomal in the proteasome (Aberle et al., 1997; Amit et al., 2002; He et al., 2004; Liu et al., 2002). In addition, both Axin and APC are phosphorylated by GSK3β. Phosphorylated Axin and APC increase their stability and affinity to β-catenin (Ikeda et al., 1998; Jho et al., 1999; Rubinfeld et al., 1996; Yamamoto et al., 1999), which promotes β-catenin phosphorylation and degradation complex stability. Once the Wnt binds to Frizzled receptors, Dvl is phosphorylated, followed by the phosphorylation of GSK3β and inhibition of its functions. The inhibition of GSK3β leads to interruption of the phosphorylation and degradation of β-catenin. Axin and APC also become de-phosphorylated due to the phosphorylation of GSK3β and their stability and affinity to β-catenin decreases. All these processes induced by Wnt signalling result in the accumulation of β-catenin in the cytoplasm. Accumulated β-catenin is then translocated into the nucleus to act as a transcriptional coactivator of transcription factors that belong to the T-cell factor and lymphoidenhancing factor (TCF/LEF) family, which activates the transcription of several genes (Clevers and Nusse, 2012; Zimmerman et al., 2012).
The effects of antipsychotic administration on the Dvl-GSK3β-β-catenin signalling pathway varied in previous studies. Sutton et al. (2007) have reported that treatment with haloperidol and clozapine induced over-expression of Dvl-3 in pheochromocytoma (PC12) and neuroblastoma (SH-SY5Y) cells. Sutton et al. (2007) have also found that daily injection with haloperidol or clozapine (but not aripiprazole) for 2 weeks resulted in increases in the levels of Dvl-3, total and phosphorylated GSK3β and β-catenin in the rat PFC. Significant increases in the levels of β-catenin and GSK3β total protein were induced by sub-chronic and chronic administration of haloperidol in the PFC, by risperidone and clozapine in the PFC and NAc, and by all three antipsychotics in the ventral midbrain and hippocampus (Alimohamad et al., 2005a; Alimohamad et al.,

**Fig. 2-10** The Dvl-GSK3β-β-catenin signalling pathway.

**Abbreviations:** APC = adenomatous polyposis coli; CK1 = casein kinase 1; Dvl = dishevelled; GSK3β = glycogen synthase kinase 3-beta.
2005b). Similarly, another in vivo study has found that 2-week administration of haloperidol and clozapine increased the protein levels of Dvl-3, total and phosphorylated GSK3β and β-catenin in the PFC; haloperidol (but not clozapine) administration also had similar effects in the striatum (Sutton and Rushlow, 2011). Moreover, Park et al. (2011a) and Seo et al. (2015) have indicated that systematic administration of aripiprazole and olanzapine for 3 weeks was able to restore the reduced phosphorylation levels of GSK3β and decreased β-catenin protein levels in the PFC and hippocampus caused by immobilisation stress. It is worth noting that antipsychotics do not have affinity with Dvl upstream Frizzled receptors. Therefore, it is important to reveal the pathways through which antipsychotics affect Dvl-GSK3β-β-catenin signalling.

2.4.3 Effect of antipsychotics on GABA<sub>A</sub> receptors

As described in Section 2.2.2, abnormal GABA transmission is implicated in the pathophysiology of schizophrenia. Previous studies have reported increased GABA<sub>A</sub> receptor binding density in various brain regions in post-mortem tissue of schizophrenic subjects (Benes et al., 1996a; Benes et al., 1992; Benes et al., 1996b; Benes et al., 1997; Dean et al., 1999; Ishikawa et al., 2004; Newell et al., 2007; Woo et al., 2004). Antipsychotic drug administration showed influences on GABA<sub>A</sub> receptors in a brain region-dependent manner. Zink and colleagues (2004) have indicated that administration of haloperidol for 6 months increased binding density of [³H]-muscimol to GABA<sub>A</sub> receptors in the CPu, the core of the NAc, while reducing it in the PFC; in addition, both haloperidol and clozapine administration reduced the binding density of GABA<sub>A</sub> receptors, in the anterior cingulate and infralimbic cortex, respectively. Skilbeck and colleagues (2007) have found that the binding of [³H]-muscimol to the
total population of GABA<sub>A</sub> receptors and [³H]-flunitrazepam to the benzodiazepine-sensitive GABA<sub>A</sub> receptors was increased by administration of haloperidol and olanzapine in the PFC; however, longer treatment with these antipsychotics did not have significant effects. It has also been revealed that administration of haloperidol decreased GABA binding sites ([³H]-muscimol) in the thalamus, but increased binding sites in the hypothalamus; additionally, haloperidol administration induced a widespread decrease in the number of benzodiazepine binding sites ([³H]-flumazenil) (McLeod et al., 2008). Furthermore, a PET study employing [¹⁸F]-FFMZ has demonstrated that GABA<sub>A</sub> receptor binding potential was lower in the aripiprazole group than the risperidone group, but not controls, in the right medial PFC and right dorsolateral PFC; GABA<sub>A</sub> receptor binding potential was lower in the aripiprazole group than the risperidone and control groups in the left frontopolar cortex and right premotor cortex (Lee et al., 2013).

However, since antipsychotics have very low affinity for GABA receptors and cannot directly affect GABA receptors, it is necessary to identify the cellular signalling pathways through which antipsychotics indirectly modulate GABA<sub>A</sub> receptors. This thesis will investigate some of these signalling pathways. For example, it is interesting that it has been reported that GABA<sub>A</sub> receptors can be regulated by PKA (Connelly et al., 2013; Poisbeau et al., 1999). Therefore, it is necessary to investigate whether aripiprazole regulates GABA<sub>A</sub> receptors, and whether PKA is involved in this regulation.

### 2.4.4 Effect of antipsychotics on CREB

CREB is a regulator targeted by several signalling pathways, such as the PKA signalling pathway, Akt-GSK3β signalling pathway, the Ras/extracellular signal
regulated kinase (ERK)1/2 signalling pathways, PT3K (phosphoinositide 3-kinase)/Akt pathway, and stress induced signalling cascades (Chao and Nestler, 2004; Snyder and Gao, 2013; Yuan et al., 2010). A clinical study has identified novel variants in the CREB gene in schizophrenic patients who experienced the positive symptoms of schizophrenia (Kawanishi et al., 1999). CREB is a member of the basic leucine zipper domain (bZIP) super family of transcription factors. It has a C-terminal zipper domain that is responsible for binding to 11 DNA and a leucine zipper domain that controls dimerisation with itself or transcription factors in the CREB family (Chao and Nestler, 2004). CREB dimers bind to consensus cAMP response element (CREs), regulating transcription of downstream genes. Many genes have CREs in their promoters including genes coding for neuropeptides, neurotransmitter synthesizing enzymes, neurotransmitter receptors, signalling proteins, and other transcription factors. These CRE-mediated transcription responses require phosphorylation of CREB.

CREB activity is reported to have been altered by various antipsychotic ligands in both in vitro and in vivo studies. An in vitro study has demonstrated that amisulpride, but not haloperidol, induced phosphorylation of CREB via the Akt-GSK3β pathway in the SH-SY5Y cells (Park et al., 2011b). In addition, both amisulpride and clozapine increased CREB phosphorylation in both SH-SY5Y and U87 cells (Jeon et al., 2015). It has also been reported that a 25-day administration of haloperidol and risperidone increased phosphorylation of CREB in hippocampal neurons (Yang et al., 2004). In animals, both amphetamine and haloperidol were able to activate CREB in the rat striatum (Hsieh et al., 2002; Konradi et al., 1994; Konradi and Heckers, 1995). Pozzi et al. (2003) have found that haloperidol and a D2R antagonist – eticlopride – stimulated phosphorylation of CREB in the mouse dorsal striatum; however, clozapine reduced it. Furthermore,
acute administration of olanzapine produced dose-dependent decreases in the phosphorylation of CREB in a rat PFC, but not in the striatum, while haloperidol did not have such an effect in either brain region (Turalba et al., 2004). Lastly, a 3-week injection of aripiprazole induced increased CREB phosphorylation in the PFC and striatum of rats, probably through glutamatergic NMDA receptors (Mavrikaki et al., 2014). However, this study examined CREB in the whole striatum, not differentiating between the CPu or NAc. Since CPu and NAc play different roles in the pathophysiology of schizophrenia and actions of antipsychotics, it is important to study the different effects of antipsychotics on CREB in the CPu and NAc. It is also worth investigating the effects of long-term administration of antipsychotics on CREB in these brain regions.

2.5 Rationales, aims and hypotheses

2.5.1 Rationales of this thesis

As already reviewed, aripiprazole has therapeutic effects on both positive and negative symptoms of schizophrenia, but induces EPS at a lower rate compared with typical antipsychotics. The D₂R partial agonism has been attributed as the unique clinical profile of aripiprazole by many researchers. However, except aripiprazole, all other D₂R partial agonists have been abandoned in clinical trials. They either did not achieve meaningful therapeutic effects or induced severe side-effects. Therefore, the contribution of D₂R partial agonism to the molecular mechanism of aripiprazole requires further validation.
The dopamine systems play a key role in the pathophysiology of schizophrenia. There are three major dopaminergic pathways that are involved in schizophrenia and the action of antipsychotics. A previous study of our group demonstrated that aripiprazole differentially affected mesolimbic and nigrostriatal dopaminergic transmission by examining dopamine synthesis and D2R binding (Han et al., 2009). However, how aripiprazole affects the downstream signalling of D2R in a brain-regional dependent manner is yet to be revealed.

There are two major downstream signalling pathways of D2R – the PKA signalling pathway and Akt-GSK3 signalling pathway. These two signalling pathways are also involved in the regulation of several other substrates and receptors (i.e. β-catenin, GABA_A receptors and CREB1). A range of previous studies have shown that PKA and Akt-GSK3 signalling as well as their downstream regulators can be affected by administration of various antipsychotic drugs, as reviewed before. However, the effects of aripiprazole on these cellular signalling pathways has not been systematically examined and compared with other antipsychotics.

As stated above, it is, therefore, important to determine the molecular mechanisms underlying the actions of aripiprazole using our established rat model (De Santis et al., 2014; Han et al., 2009). Bifeprunox (a D2R partial agonist) and haloperidol (a D2R antagonist) will be used as reference drugs for comparison. Additionally, it has been proposed that positive, negative and cognitive symptoms of schizophrenia are associated with dysfunction of different brain regions (Buckley, 2005). Therefore, it is necessary to investigate any brain regional differences between the actions of aripiprazole and haloperidol to explain the differences in their clinical effects. Lastly,
under clinical conditions, patients always experience long-term antipsychotic treatment. Therefore, it is critical to investigate the effects of not only acute/short-term, but also chronic, administration of these antipsychotics on the cellular signalling pathways.

### 2.5.2 Aims

The **general aim** of this study is to understand the molecular mechanism of aripiprazole in the key brain regions that are related to the actions of antipsychotics by investigating the effects of aripiprazole on various cellular signalling pathways in an established animal model.

The **specific aims** of this research were to:

1. Investigate whether acute administration of aripiprazole differentially affects D$_2$R-mediated downstream signalling pathways in the key brain regions that are related to the action of antipsychotics, in comparison with bifeprunox and haloperidol.

2. Examine the effects of aripiprazole on the D$_2$R-related signalling pathways, as well as their potential regulation of GABA$_A$ receptors and CREB, in comparison with bifeprunox and haloperidol.

3. Reveal the effects of long-term administration of aripiprazole on the D$_2$R-related signalling pathways, as well as their potential regulation on GABA$_A$ receptors and CREB, in comparison with bifeprunox and haloperidol.
2.5.3 Hypotheses

1. Acute administration of aripiprazole differentially affects PKA and GSK3β activity, in comparison with bifeprunox and haloperidol. (Chapter 3)

2. Short-term administration of aripiprazole has different effects on PKA, GABA_A (containing β-1 subunit) receptors and CREB in the NAc, in comparison with bifeprunox and haloperidol. (Chapter 4)

3. Short-term 1-week administration of aripiprazole differentially affects the Akt-GSK3β and Dvl-3-GSK3β-β-catenin signalling pathways in various brain regions, in comparison with bifeprunox and haloperidol. (Chapter 5)

4. Long-term 10-week administration of aripiprazole has different effects on PKA signalling, Akt-GSK3β signalling, Dvl-GSK3β-β-catenin signalling, GABA_A (containing β-1 subunit) receptors and CREB activity in the PFC, CPu and NAc, in comparison with bifeprunox and haloperidol. (Chapter 6)

Overall, in the thesis, all the above hypotheses were tested in the established animal model for the oral administration of antipsychotics (De Santis et al., 2014; Han et al., 2009).
CHAPTER 3

UNIQUE EFFECTS OF ACUTE ARIPIPRAZOLE TREATMENT ON THE DOPAMINE D2 RECEPTOR DOWNSTREAM CAMP-PKA AND AKT-GSK3B SIGNALLING PATHWAYS IN RATS

RESEARCH ARTICLE

Unique Effects of Acute Aripiprazole Treatment on the Dopamine D2 Receptor Downstream cAMP-PKA and Akt-GSK3β Signalling Pathways in Rats

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Abstract

Aripiprazole is a wide-used antipsychotic drug with therapeutic effects on both positive and negative symptoms of schizophrenia, and reduced side-effects. Although aripiprazole was developed as a dopamine D2 receptor (D2R) partial agonist, all other D2R partial agonists that aimed to mimic aripiprazole failed to exert therapeutic effects in clinic. The present in vivo study aimed to investigate the effects of aripiprazole on the D2R downstream cAMP-PKA and Akt-GSK3β signalling pathways in comparison with a D2R antagonist – haloperidol and a D2R partial agonist – bifeprunox. Rats were injected once with aripiprazole (0.75mg/kg, i.p.), bifeprunox (0.8mg/kg, i.p.), haloperidol (0.1mg/kg, i.p.) or vehicle. Five brain regions – the prefrontal cortex (PFC), nucleus accumbens (NAc), caudate putamen (CPu), ventral tegmental area (VTA) and substantia nigra (SN) were collected. The protein levels of PKA, Akt and GSK3β were measured by Western Blotting; the cAMP levels were examined by ELISA tests. The results showed that aripiprazole presented similar acute effects on PKA expression to haloperidol, but not bifeprunox, in the CPu and VTA. Additionally, aripiprazole was able to increase the phosphorylation of GSK3β in the PFC, NAc, CPu and SN, respectively, which cannot be achieved by bifeprunox and haloperidol. These results suggested that acute treatment of aripiprazole had differential effects on the cAMP-PKA and Akt-GSK3β signalling pathways from haloperidol and bifeprunox in these brain areas. This study further indicated that, by comparison with bifeprunox, the unique pharmacological profile of aripiprazole may be attributed to the relatively lower intrinsic activity at D2R.

Introduction

Aripiprazole has therapeutic effects on both positive and negative symptoms of schizophrenia, with improved extrapyramidal side-effects (EPS) compared with first-generation antipsychotic
drugs (e.g. haloperidol) and reduced metabolic side-effects compared with second-generation antipsychotic drugs (e.g. olanzapine); aripiprazole is regarded as the third-generation antipsychotic drug [1, 2]. The exact mechanisms of aripiprazole remain unclear. Several studies suggested that the potent partial agonism of aripiprazole for the dopamine D₂ receptor (D₂R) stabilises the dopamine D₂ system, playing a critical role in its unique clinical actions [3, 4]. However, this hypothesis has been questioned because there are no other D₂R partial agonists that are widely accepted and used after aripiprazole. On the other hand, a theory called functional selectivity has been postulated to explain the pharmacological profile of aripiprazole [1]. The theory of functional selectivity suggests that depending on the cellular location and environment of the target G protein-coupled receptors (GPCRs), some ligands (drugs) can induce distinct conformations of GPCRs, resulting in differential regulation of canonical and non-canonical signal transduction pathways associated with these GPCRs [5–7]. Accumulated evidence from in vitro studies suggested that aripiprazole displays functional selectivity on D₂R; it may act as a potent partial agonist, weak agonist, or antagonist depending on the targeted D₂R [1, 4, 6, 8–10]. An in vivo study from our group found selective effects of aripiprazole on the mesolimbic vs. the nigrostriatal dopaminergic pathways compared with haloperidol, which could be explained by the functional selectivity of aripiprazole [11]. However, how aripiprazole differentially affects the downstream signalling pathways of the D₂R in various dopaminergic pathways in vivo has not been well studied.

Two major D₂R downstream cellular signalling pathways have been identified. The canonical transduction pathway is the G protein-dependent cyclic adenosine monophosphate (cAMP)-protein kinase A (PKA) pathway, which mediates various cellular responses such as proliferation, metabolism and gene transcription [12–14]. Blockade of D₂R in the brain is associated with the pharmacological properties of all antipsychotic drugs, including the therapeutic effects and some side-effects (e.g. EPS side-effects) [15]. However, whether and how D₂R-mediated PKA signalling is involved in these pharmacological properties is not clear. Therefore, unveiling the effects of aripiprazole on the PKA signalling in various brain regions might elucidate the mechanism of aripiprazole and provide a new route for the development of improved antipsychotics. Furthermore, previous evidence suggested that D₂-like dopamine autoreceptors have a preferential coupling to the PKA pathway to control dopamine synthesis [16, 17], release [18, 19] and uptake [20, 21]. However, previous studies showed discrepant influences of antipsychotic drugs on dopamine synthesis capacity. Acute treatment with haloperidol and aripiprazole both increased dopamine synthesis while quinpirole (a D₂R agonist) reduced it in the caudate putamen (CPu) in rodents [22]. Additionally, acute administration with haloperidol in healthy human subjects induced a significant increase in dopamine synthesis capacity in various brain regions, including the striatum, mesencephalon, and medial prefrontal cortex (PFC) [23]. An in vivo study from our group demonstrated that aripiprazole down-regulated tyrosine hydroxylase (TH) mRNA expression in the ventral tegmental area (VTA), but not in the substantia nigra (SN), indicating reduced dopamine synthesis capacity after 1-week and 12-week treatment [11]. Therefore, the present study further investigated the relationship between the PKA activity and the TH activity after acute antipsychotic treatment.

Besides the canonical G protein-dependent cAMP-PKA signalling pathway, the non-canonical D₂R transduction pathway is the G protein-independent protein kinase B (PKB/Akt)-glycogen synthase kinase 3 (GSK3) pathway. Studies implicated Akt and GSK3β signalling in the pathophysiology of neuropsychiatric disorders such as schizophrenia, bipolar disorder, and depression [24–26]. Studies on patients with schizophrenia showed decreased phosphorylation levels and GSK3 protein levels in the PFC [27–29]. In animal studies, GSK3β activity was elevated in the striatum, in a D₂R- and β-arrestin2-dependent manner, under hyperdopaminergic conditions [24, 30–32]. An animal behaviour study comparing
Aripiprazole with β-arrestin2-biased D₂R ligands also suggested that the β-arrestin2 signalling cascade can be simultaneously a significant contributor to antipsychotic therapeutic effects and a protective effect against motor side-effects [33]. Based on these findings, we proposed that aripiprazole might exert its therapeutic effects and reduced EPS side-effects via D₂R-dependent Akt-GSK3β signalling.

Therefore, the present study examined the in vivo effects of aripiprazole on the cAMP-PKA and Akt-GSK3β signalling pathways in the mesolimbic, mesocortical and nigrostriatal dopaminergic pathways in comparison with a potent D₂R antagonist—haloperidol and a D₂R partial agonist—bifeprunox.

Methods

Animals and drug treatment

Male Sprague Dawley rats (aged 8 weeks) were obtained from the Animal Resource Centre (Perth, Australia). After arrival, all rats were divided into four treatment groups (n = 6/group). Rats were housed in individual cages under environmentally controlled conditions (temperature 22°C, light cycle from 07:00 to 19:00 h), with ad libitum access to water and standard laboratory chow diet. After 1-week acclimatisation to the new surroundings, all rats were injected with aripiprazole (0.75mg/kg, intraperitoneal (i.p.)), haloperidol (0.1mg/kg, i.p.), bifeprunox (0.8mg/kg, i.p.) or vehicle, respectively. All drugs were suspended in a 10% hydroxypropyl-β-cyclodextrin (Sigma, St. Louis, MO) solution. The dosages used in the present study were equivalent to the recommended dosage for treating schizophrenia patients, and calculated based on body surface area according to the FDA guidelines for clinical trials [34, 35]. Rats were euthanised by using carbon dioxide 2 hours after injection. This time-pointed was determined because all of the three drugs were rapidly absorbed after administration. For example, the plasma levels of haloperidol reached the maximum at 1 hour after oral and intramuscular administration in rats [36], while the aripiprazole concentration reached 80% of peak level in plasma and brain of rats at 1 hour after oral administration [37]. Although no pharmacokinetic data of bifeprunox were found in rats, a human study reported that bifeprunox reached peak plasma concentration within 1.5 hours after oral administration [38]. Brains were collected and then stored at -80°C. All rats were sacrificed between 10:00 A.M. and 12:00 A.M. to minimise possible circadian-induced variation of protein expression.

Ethics Statement

All experimental procedures were approved by the Animal Ethics Committee (Application #: AE11/02), University of Wollongong, and complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004). All animals were euthanised by using carbon dioxide. All efforts were made to minimise animal distress and prevent suffering.

Microdissection

Following a standard procedure used in our lab, discrete brain regions were collected by using brain microdissection puncture [39–41]. Briefly, 500μm thickness fresh frozen brains were cut at -14°C and collected on glass slides. Based on the rat brain atlas [42], three sections through the forebrain (Bregma 3.30 to 4.20mm) were collected for microdissection of the PFC. Three sections through the striatum (Bregma 1.00 to 2.20mm) were collected for microdissection of the nucleus accumbens (NAc) and CPu. Three sections through the midbrain (Bregma -5.40 to -6.30mm) were collected for microdissection of the VTA and SN. For the PFC, NAc and CPu, bilateral punches (1.2mm) were collected from each of the three sections. For the VTA, each of
the bilateral punches (0.8mm) was taken at a region medial to the medial lemniscus and dorsal to the interpeduncular nucleus. For the SN, each of the bilateral punches (0.5mm) was directed to the lateral border of the medial lemniscus. Tissue punches from each region were collected in microfuge tubes chilled on dry ice and kept frozen for future use.

Western blot analyses

Tissue obtained from individual rat was homogenised and the supernatants were collected and stored at -80°C until required. Protein concentrations were determined spectrophotometrically at A750nm using the DC Protein Assay (Bio-Rad, #500–0111). Samples containing 10 μg of protein were resolved by 10% SDS–PAGE gels, and then transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane by using Bio-Rad Midi Format 1-D Electrophoresis Systems. The PVDF membranes were blocked for 1 hour at room temperature in Tris-buffered saline-Tween (TBST) containing 5% BSA, and incubated overnight at 4°C in primary antibodies diluted in TBST containing 1% BSA. Luminata Western horseradish peroxidase (HRP) Substrates (Millipore) and Kodak XBT-1 film were used to examine the membrane to visualise the immunoreactive bands. The immunoreactive signals were quantified using Bio-Rad Quantity One software. The data were then corrected based on their corresponding actin levels. All results were normalised by taking the value of the vehicle group as 100%. Experiments were performed in duplicate.

Four regulatory (RIα, RIIα, RIβ, and RIIβ) and two catalytic (Cα and Cβ) isoform genes have been identified previously [43]. PKA-RII expression is highest in brain tissues; PKA-Cα and PKA-Cβ are closely related (93% amino acid sequence similarity), PKA-Cα is ubiquitously expressed, and expression of PKA-Cβ is also highest in the brain [44–47]. Four antibodies for PKA subunits were chosen in the present study: PKA-RIIα (1:1000; Santa Cruz, #SC-908), PKA-Cα (1:1000; Cell Signalling, #5842), PKA-Cβ (1:1000; Santa Cruz, #SC-904) and phospho-PKA-C (Thr197) (1:1000; Cell Signalling, #5661). For the Akt-GSK3 pathway, the primary antibodies include: Akt (1:2000; Cell Signalling, #4691), phospho-Akt (Thr308) (1:1000; Cell Signalling, #13038), GSK3β (1:2000; Cell Signalling, #5676), and phospho-GSK3β (Ser9) (1:1000; Cell Signalling, #9322). We also examined tyrosine hydroxylase (1:1000; Millipore, #AB9983) and phospho-tyrosine hydroxylase (Ser40) (1:1000; Millipore #AB5935) in the VTA and SN to test their influence on dopamine synthesis. Mouse anti-actin primary polyclonal antibody (1:10000; Millipore, #MAB1501) and HRP-conjugated rabbit anti-mouse secondary antibody (1:3000; Cell Signalling, #7076) were used to determine the actin levels.

cAMP measurement

The brain tissue was collected by microdissection described before, and assayed using a cAMP Direct Immunoassay Kit (Abcam, #ab65355) according to the manufacturer’s instructions. Briefly, the brain tissue was homogenised with 0.1M HCl, and centrifuged at 10,000g for 15 minutes at 4°C to obtain the supernatant. Then 100 μL of the diluted cAMP standard solution (0.039, 0.078, 0.156, 0.3125, 0.625, 1.25, 2.5, 5, and 10pmol/μL) or 50μL of the supernatant (added with 50μL 0.1M HCl) was mixed with kit reaction solution accordingly, and loaded to 96-well plates. All standards and samples were run in duplicate to ensure consistency of the reading. The plates were then treated with cAMP antibody, cAMP- HRP and HRP Developer, and measured spectrophotometrically at 450nm. The cAMP values obtained from the luminescence measurements were converted by the total protein amount determined using the DC Protein Assay (Bio-Rad, #500–0111) accordingly, expressed as picomole of cAMP per nanogram of protein.
Statistics
All data was analysed using the SPSS Statistics v19.0 program. The data of both western blot analyses and cAMP measurement was normalised by taking the value of the control group as 100% and expressed as mean ± S.E.M. All phosphor proteins were also normalised to total protein levels. For example, p-PKA was normalised by the average levels of PKA-Cα and PKA-Cβ; p-TH, p-Akt and p-GSK3β were normalised by the levels of total TH, Akt and GSK3β, respectively. The Kolmogorov-Smirnov test was performed to test the normality of the data. One-way ANOVA was used if the data was normally distributed, followed by Post Hoc Tukey test to compare the control and drug treatment groups. Nonparametric Mann-Whiney U-test was applied when data was abnormal distributed. Statistical significance was accepted when \( p < 0.05 \).

Results and Statistical Analyses

The effects on the cAMP-PKA signalling pathway

**PFC.** ANOVA test did not identify any significant effects in the protein levels of PKA-Cα, PKA-Cβ, PKA-RII and p-PKA or the ratio of p-PKA/PKA after all three drug treatment in the PFC (Fig 1A and Fig 2A).

**NAc.** The levels of p-PKA in the NAc were shown to be significantly affected by drug treatment (\( F_{3, 22} = 11.157, \ p < 0.001 \)); however, the ratio of p-PKA/PKA was also not significantly affected by drug treatment (\( F_{3, 22} = 1.390, \ p > 0.05 \)). The protein levels of PKA-Cα, -Cβ, and-RII subunits did not change after drug treatment. Post Hoc analysis identified that only treatment with bifeprunox, not aripiprazole and haloperidol, enhanced the activity of the p-PKA subunit (+66.3%, \( p < 0.05 \)) (Fig 1B).

**CPu.** ANOVA test revealed significant effects of drug treatment on the expression of PKA-Cα (\( F_{3, 23} = 11.806, \ p < 0.001 \)), PKA-Cβ (\( F_{3, 23} = 4.985, \ p = 0.011 \)) and PKA-RII (\( F_{3, 23} = 7.041, \ p = 0.002 \)), but not p-PKA; however, the ratio of p-PKA/PKA was significantly changed by drug treatment (\( F_{3, 23} = 13.687, \ p < 0.001 \)). Post Hoc analysis showed that both aripiprazole (\( p < 0.01 \)) and haloperidol (\( p < 0.01 \)) significantly elevated the expression of PKA-Cα, by 33.1% and 34.6%, respectively; only treatment with bifeprunox significantly increased the expression of the PKA-Cβ subunit by 31.9% (\( p < 0.05 \)) (Fig 1C). Additionally, haloperidol treatment negatively affected expression of the PKA-RII subunit (-34.0%, \( p < 0.01 \)). Moreover, both aripiprazole and haloperidol had no effects on the protein levels of PKA-Cβ; and bifeprunox did not alter the protein levels of PKA-Cα, and-RII subunits. The ratio of p-PKA/PKA was observed to be decreased by treatment with bifeprunox (\( p < 0.01 \)), but increased by haloperidol treatment (\( p < 0.05 \)); aripiprazole did not affect the ratio of p-PKA/PKA (Fig 2C).

**VTA.** The expression of PKA-Cα (\( F_{3, 23} = 3.757, \ p = 0.027 \)), PKA-Cβ (\( F_{3, 22} = 5.079, \ p = 0.009 \)) and PKA-RII (\( F_{3, 23} = 3.698, \ p = 0.029 \)) has been significantly affected by drug treatment in the VTA. The ratio of p-PKA/PKA was also changed by drug treatment (\( F_{3, 23} = 3.197, \ p = 0.046 \), although the levels of p-PKA was not significantly altered (all \( p > 0.05 \). Post Hoc analysis demonstrated that the expression of both PKA-Cα and PKA-Cβ subunit was significantly increased by 20.9% and 49.4% by aripiprazole (\( p < 0.05 \)) and bifeprunox (\( p < 0.01 \)) treatment, respectively (Fig 3A). We also observed that bifeprunox treatment significantly reduced the ratio of p-PKA/PKA (\( p < 0.05 \)) (Fig 4A). Additionally, treatment with haloperidol significantly elevated the expression of the PKA-RII subunit by 41% (\( p < 0.05 \)). Furthermore, the levels of both TH and p-TH were showed to be increased by bifeprunox treatment, but not significantly (TH, +50.7%, \( p > 0.1 \); p-TH, +34.0%, \( p > 0.1 \)); aripiprazole and haloperidol did not cause any noticeable change in the levels of TH and p-TH.
Fig 1. The alterations in the PKA signalling in the prefrontal cortex, nucleus accumbens and caudate putamen. The acute effects of three chemicals (aripiprazole, bifeprunox and haloperidol) on the protein levels of three PKA subunits (PKA-Cα, -Cβ, and -RII) and phospho-PKA in the prefrontal cortex (A), nucleus accumbens (B) and caudate putamen (C). (* p < 0.05, ** p < 0.01 vs. the control)

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ANOVA analysis identified significant effects of drug treatment on the levels of p-PKA (\(F_{3, 19} = 6.956, p = 0.003\)) drug treatment; however, Post Hoc analysis did not reveal any significant effects induced by any drug treatment (all \(p > 0.1\)) (Fig 3B). Additionally, the levels of PKA subunits, TH and p-TH were not altered by any drug treatment in the SN. The ratio of either p-PKA/PKA or p-TH/TH was not altered by any drug treatment (Fig 4B).

Measurement of cAMP. ANOVA analysis indicated significant effects on cAMP levels only in the PFC (\(F_{3, 21} = 3.529, p = 0.036\)). Post Hoc analysis identified that only bifeprunox...
significantly reduced the cAMP levels in the PFC (-53.3%, $p < 0.05$). There were no other notable changes in the cAMP levels in other four brain regions (all $p > 0.05$, Table 1).

**The effects on the Akt-GSK3β pathway**

**PFC.** In the Akt-GSK3β signalling pathway, the levels of p-GSK3β was significantly influenced by drug treatment ($F_{3, 21} = 7.504$, $p = 0.002$) in the PFC; the ratio of p-GSK3β/GSK3β has also been significantly affected ($F_{3, 21} = 10.435$, $p < 0.001$). Post Hoc analysis indicated that only aripiprazole treatment, not haloperidol or bifeprunox treatment, significantly promoted the levels of p-GSK3β (+31.3%, $p < 0.05$) (Fig 5A). Aripiprazole also increased the ratio of p-GSK3β/GSK3β ($p < 0.01$) (Fig 2A).

**NAc.** ANOVA analysis showed that the levels of p-GSK3β were significantly affected by drug treatment ($F_{3, 23} = 9.161$, $p = 0.001$) in the NAc; ANOVA tests also revealed that drug treatment tended to significantly changed the ratio of p-GSK3β/GSK3β ($F_{3, 23} = 2.585$, $p = 0.082$). Post Hoc test revealed that the levels of p-GSK3β were significantly increased by both aripiprazole ($p < 0.05$) and haloperidol ($p < 0.05$), by 41.5% and 43.5%, respectively, whereas bifeprunox had no such effect (Fig 5B). In addition, the ratio of p-GSK3β/GSK3β was...
significantly increased by treatment with aripiprazole ($p < 0.05$); haloperidol treatment also tended to increase the ratio of p-GSK3B/GSK3β ($p = 0.062$) (Fig 2B).

**CPu.** Only the levels of p-GSK3β were shown to be significantly influenced ($F_{3, 22} = 19.320$, $p < 0.001$) by drug treatment in the CPu; the ratio of p-GSK3β/GSK3β was also significantly affected ($F_{3, 22} = 17.103$, $p < 0.001$). Post Hoc test identified significant enhancement on the levels of p-GSK3β induced by aripiprazole (+58.3%, $p < 0.05$) and bifeprunox (+60.6%, $p < 0.05$) treatment, respectively (Fig 5B). No drug treatment significantly influenced the levels of p-Akt.

**Unique Effects of Aripiprazole on PKA and Akt-GSK3β Signalling**

![Graph](image)

**Table 1.** The cAMP levels (pmol/ng protein; mean values ± SEM; n = 6/group) in five brain regions after acute antipsychotic treatment.

<table>
<thead>
<tr>
<th></th>
<th>PFC</th>
<th>NAc</th>
<th>CPu</th>
<th>VTA</th>
<th>SN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aripiprazole</td>
<td>75.0±16.8</td>
<td>55.0±7.9</td>
<td>34.3±7.5</td>
<td>166.7±32.7</td>
<td>85.0±13.3</td>
</tr>
<tr>
<td>Bifeprunox</td>
<td>49.0±7.4*</td>
<td>54.2±4.4</td>
<td>41.4±8.1</td>
<td>106.0±14.4</td>
<td>90.3±18.3</td>
</tr>
<tr>
<td>Haloperidol</td>
<td>74.8±15.2</td>
<td>58.9±3.2</td>
<td>40.7±3.5</td>
<td>115.8±8.3</td>
<td>132.5±22.5</td>
</tr>
<tr>
<td>Control</td>
<td>105.0±5.7</td>
<td>60.4±5.4</td>
<td>34.5±5.5</td>
<td>123.9±14.8</td>
<td>99.2±13.2</td>
</tr>
</tbody>
</table>

* $p < 0.05$

**Abbreviations:** CPu, caudate putamen; NAc, nucleus accumbens; PFC, prefrontal cortex; SN, substantia nigra; VTA, ventral tegmental area.

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Fig 5. The alterations in the Akt-GSK3β in the prefrontal cortex, nucleus accumbens and caudate putamen. The acute effects of three chemicals (aripiprazole, bifeprunox and haloperidol) on the levels of Akt, phospho-Akt, GSK3β and phospho-GSK3β in the prefrontal cortex (A), nucleus accumbens (B) and caudate putamen (C). (* p < 0.05 vs. the control)

doi:10.1371/journal.pone.0132722.g005
total Akt or total GSK3β. Furthermore, both aripiprazole and bifeprunox treatment significantly increased the ratio of p-GSK3β/GSK3β (aripiprazole, p < 0.01; bifeprunox, p < 0.05) (Fig 2C).

**VTA.** ANOVA analysis identified that only the levels of p-GSK3β in the Akt-GSK3β signalling pathway were significantly affected (F_{3, 22} = 7.124, p = 0.002) by drug treatment in the VTA; the ratio of p-GSK3β/GSK3β was also significantly changed (F_{3, 22} = 7.015, p = 0.002). Post Hoc test indicated a significant elevation in the expression of p-GSK3β after bifeprunox treatment (+47.8%, p < 0.05) (Fig 2C); the ratio of p-GSK3β/GSK3β was also significantly increased by bifeprunox (p < 0.01) (Fig 2C); no other significant result was observed.

**SN.** ANOVA test identified significant effects of drug treatment on the levels of p-Akt (F_{3, 21} = 4.243, p = 0.020) and the ratio of p-Akt/Akt (F_{3, 21} = 4.243, p = 0.020) in the SN; ANOVA analysis also indicated a trend towards significance on the levels of p-GSK3β (F_{3, 20} = 2.946, p = 0.061). Post Hoc analysis revealed that all three drugs significantly increased the levels of p-Akt (aripiprazole, +23.9%, p < 0.05; bifeprunox, +37.6%, p < 0.05; haloperidol, +40.3%, p < 0.05) (Fig 2B); they all elevated the ratio of p-Akt/Akt (aripiprazole, p < 0.05; bifeprunox, p < 0.01; haloperidol, p < 0.05) (Fig 2B). Additionally, only aripiprazole treatment significantly increased the ratio of p-Akt/Akt (p < 0.05) (Fig 2B).

![Fig 6. The alterations in the Akt-GSK3β in the ventral tegmental area and substantia nigra.](image)

The acute effects of three chemicals (aripiprazole, bifeprunox and haloperidol) on the levels of Akt, phospho-Akt, GSK3β and phospho-GSK3β in the ventral tegmental area (A) and substantia nigra (B). (*) p < 0.05 vs. the control.

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raised the levels of p-GSK3β by 33.0% ($p < 0.05$), but it did not significantly influence the ratio of p-GSK3β/GSK3β ($p > 0.05$).

**Discussion**

The present study demonstrated the *in vivo* effects of aripiprazole on the downstream pathways of the D2R, by comparing it with haloperidol and bifeprunox. The results of the current study showed that aripiprazole had similar effects on PKA subunits to haloperidol, but not bifeprunox in the CPu and VTA, indicating that relatively lower intrinsic activity of aripiprazole on D2R might be the mechanism of aripiprazole to exert its therapeutic effects on treating positive symptoms of schizophrenia; on the other hand, aripiprazole displayed a very different action mode on the GSK3β activity from the other two chemicals, probably explaining its therapeutic effects on both positive and negative symptoms of schizophrenia, with reduced EPS. Together, these *in vivo* findings suggest that the relatively low intrinsic activity at D2R might be the reason that aripiprazole possesses unique pharmacological profiles and clinical effects from the other antipsychotic drugs.

**Antipsychotic effects on PKA signalling and TH activity**

Dopamine is largely synthesised in the VTA and SN, and transported to the synapses in the PFC, CPu and NAc [48–51]. In the VTA and SN, short-form dopamine D2 autoreceptors are largely presented at pre-synapses, which generally provide a feedback mechanism that adjusts neuronal firing rate, dopamine synthesis capacity, and dopamine release in response to changes in extracellular dopamine level [52–54]. The G protein-dependent PKA signalling pathway is a canonical D2R downstream signalling pathway which mediates diverse cellular responses to external responses by activating the cAMP-dependent protein kinase including PKA kinase [12–14]. By reaction with cAMP, the inactive PKA could release a dimer of regulatory subunits, and two free monomeric catalytic subunits that can further phosphorylate other protein substrates [55]. It is suggested that the inhibition of the PKA transduction pathway induced by activation of D2 autoreceptors leads to reduced TH activity and decreased firing of dopamine neurons both *in vivo* and *in vitro* [56, 57]. In the present study, we observed that all three antipsychotics were able to differentially affect the expression of PKA subunits in various brain regions, as well as TH levels (although some results did not reach significance). For example, aripiprazole significantly increased the expression of PKA-Cα and PKA-RII subunits, and bifeprunox and haloperidol also significantly elevated the levels of PKA-Cβ and PKA-RII, respectively. In view of acute (2 hours) treatment, it is possible that these drugs may modulate protein expression of PKA subunits through affecting the actively translatable pool of mRNA, although further studies are necessary to confirm it. Moreover, aripiprazole reduced (not reaching significance) the expression of TH, while bifeprunox increased it (not significantly) in the VTA, which suggests that aripiprazole might potentially exert a different effect from that of bifeprunox and haloperidol on dopamine synthesis capacity. However, there were inconsistent reports about the effect of antipsychotics on dopamine synthesis capacity. For example, acute treatment with haloperidol and aripiprazole both increased dopamine synthesis while quinpirole (a D2R agonist) reduced it in rats CPu [22], although aripiprazole with agonistic activity at pre-synaptic D2 autoreceptors might reduce dopamine synthesis through feedback regulation [10, 58]. Moreover, acute administration with haloperidol in healthy human subjects induced a significant increase in dopamine synthesis capacity in various brain regions, including the striatum, mesencephalon, and medial PFC [23]. Our group using the same dosages of aripiprazole found that both 1-week and 12-week treatment with aripiprazole reduced the dopamine synthesis capacity in the VTA, but not in the SN [11]. The exact reason for the discrepancies

**Unique Effects of Aripiprazole on PKA and Akt-GSK3β Signalling**
between these studies remains unknown. One possible reason might be that the brain regions examined in the literature are not completely concordant with those in this study, which indicates that the effects of antipsychotics are quite brain-regional related; another possible reason might be the treatment dosages and treatment periods. For example, Der-Ghazarian’s study used 10mg/kg for aripiprazole and 1mg/kg for haloperidol; these doses are considerably larger than those used in this study and might be a non-physiological response. It is worth noting that aripiprazole, in the present study, displayed different effects on the PKA pathway and TH activity from those of bifeprunox and haloperidol in the VTA and SN (although some changes did not reach significance), probably implying that D₂R partial agonism or D₂R antagonism cannot fully explain the unique pharmacological profile of aripiprazole. It is also worth pointing out that since the mesolimbic dopaminergic pathway is hyper-activated in schizophrenia, the increased dopamine synthesis capacity of bifeprunox in the VTA might partly explain why it failed to exert therapeutic effects in treating schizophrenia. The present study used only acute treatment, and we observed only some trends in the effects of antipsychotics (especially on the TH activity), possibly because the treatment period was too short. Therefore, experiments with longer treatment periods are required to confirm these findings.

In the PFC, CPu and NAc, long-form dopamine D₂ receptors are highly expressed, regulating post-synaptic functions. Similar to the situation in the VTA and SN, these three drugs influenced the expression of PKA subunits in different manners in the PFC, CPu and NAc. Previous studies also suggested that aripiprazole inhibited cAMP accumulation and reduced PKA pathway signalling due to its partial agonism for the D₂R, while haloperidol increased cAMP levels and facilitated its signalling. Additionally, PKA-C levels were reported to be elevated in the CPu 15 minutes after acute treatment with haloperidol, but not the PFC and NAc; the activity of the cAMP-PKA pathway in the striatum and protein levels of PKA-R1 subunit in the striatum were also significantly increased after chronic treatment (21 days) with haloperidol due to its antagonism for the D₂R.

In the present study, we observed that aripiprazole decreased PKA-Cα levels in the PFC, inhibiting PKA signalling, which is consistent with previous studies. Interestingly, our study exhibited that both aripiprazole and haloperidol affected PKA protein levels in a very similar pattern in the CPu and VTA; thereby, it is possible that both aripiprazole and haloperidol display similar antagonising effects on post-synaptic D₂R in the CPu and VTA after acute treatment. Since aripiprazole is a D₂R partial agonist, its intrinsic activity of aripiprazole for D₂R is lower than that of endogenous dopamine, which might result in antagonising effects on D₂R signalling; and this relatively low intrinsic activity might be the reason why not all D₂R partial agonists could have meaningful therapeutic effects as aripiprazole. Lastly, only bifeprunox, a potent D₂R partial agonist, was able to significantly inhibit the cAMP levels in the PFC, indicating that agonism for the D₂R in the PFC might not contribute to the therapeutic effects of aripiprazole; hence, bifeprunox also displayed different effects on the PKA activity from both aripiprazole and haloperidol in the present study, which probably explains the failure of bifeprunox in clinic trials. The intrinsic activity might also play a critical role in the different effects between bifeprunox and aripiprazole. The intrinsic activity of bifeprunox for D₂R is higher than that of aripiprazole. At current dosage, the intrinsic activity of bifeprunox might not be low enough to induce antagonising effects as aripiprazole, although it is possible that the dosage of bifeprunox was insufficient to compete against endogenous dopamine to exhibit antagonising effects. This point can be verified by their different alterations in the ratio of p-PKA/PKA in the CPu. The intrinsic activity of aripiprazole is neither high enough to positively affect the ratio of p-PKA/PKA as bifeprunox, nor low enough to negatively affect it as haloperidol. Since some D₂R partial agonist with very low intrinsic activity (e.g. SDZ 208-912) could induce side-effects like EPS, a moderate intrinsic activity for the D₂R might be a critical factor to develop optimum antipsychotics.
Antipsychotic effects on the Akt-GSK3β signalling

In addition to the canonical G protein-dependent cAMP-PKA signalling pathway, the D₂R signalling is also mediated by the Akt-GSK3β via β-arrestin2; many studies have indicated that Akt-GSK3β signalling plays a critical role in the pathophysiology of schizophrenia [24, 25, 27, 64]. Post-mortem studies on schizophrenic subjects demonstrated reduced phosphorylation levels and GSK3β protein levels in the PFC [28, 29]. In rodents, reduction of D₂R- and β-arrestin2-dependent locomotor behaviours was observed in the situation of hyperactivity of GSK3β [24, 30–32].

The effects of antipsychotic drugs on Akt-GSK3β signalling have also been confirmed by a range of studies. In vivo studies indicated enhanced GSK3β phosphorylation in various brain areas of rodents after various first- and second-generation antipsychotic treatment [65–68]. A human study also suggested haloperidol treatment compensated for the decreased levels of endogenous Akt in the PFC in schizophrenic subjects, phosphorylating GSK3β, and leading to inhibition of its activity [69]. Collectively, antipsychotic drugs are capable of increasing the phosphorylation of GSK3β, thus inducing inhibition of the GSK3β kinase. The present study demonstrated that acute treatment with aripiprazole increased the levels of p-GSK3β, as well as the ratio of p-GSK3β/GSK3β in the PFC, CPu, NAc, respectively, which indicates the inhibition of the functions of GSK3β in these brain areas. On the other hand, bifeprunox elevated the levels of p-Akt in the SN and p-GSK3β in the CPu and VTA, as well as the ratio, simultaneously. Haloperidol reduced the activity of GSK3β by elevating the levels of p-GSK3β and the ratio of p-GSK3β/GSK3β in the NAc as well as increased the phosphorylation of Akt in the SN, but haloperidol did not affect GSK3β levels.

Obviously, the three compounds displayed distinct effects on Akt-GSK3β in the current study. Aripiprazole, in the present study, elevated the phosphorylation of GSK3β in the PFC, NAc, CPu and SN, thus resulting in inhibition of the activity of GSK3β in these brain regions. These findings suggested that aripiprazole probably exerted its therapeutic effects and reduced EPS by affecting GSK3β. A previous study found that acute treatment with various second-generation antipsychotics (including olanzapine, risperidone, clozapine) facilitated the phosphorylation of GSK3β in the cortex, striatum and hippocampus [60]. Thus, together with our findings, aripiprazole probably shares common mechanisms with other second-generation antipsychotics to act on the GSK3β signalling to exert therapeutic effects. On the other hand, bifeprunox had fewer effects on GSK3β than aripiprazole, especially in the PFC and NAc. The reasons that aripiprazole and bifeprunox showed different effects on GSK3β signalling could be their different intrinsic activities at D₂R, however we could not completely exclude the possibility that the dosage of bifeprunox used in this study was insufficient to act on GSK3β signalling. Haloperidol here displayed very limited effects on GSK3β, which might indicate that haloperidol does not exert therapeutic effects via GSK3β signalling. Moreover, a previous study testing animal behaviour after treating β-arrestin2-biased D₂R ligands along with aripiprazole indicated that activation of β-arrestin2 contributed to the protection against motor side-effects [33]. Whether inhibition of GSK3β followed by activation of β-arrestin2 is directly linked to this protective effect is not clear. However, our finding that aripiprazole, but not haloperidol, reduced the activity of GSK3β in the CPu might provide an explanation. Lastly, it is worth noting that the total levels of GSK3β did not change after acute treatment with all three compounds, which is consistent with the findings of Alimohamad’s studies [65, 66].

It is clearly seen that the phosphorylation of GSK3β did not change completely in the same manner as that of Akt in the present study. Two phosphorylating sites of Akt have been identified: Thr308 and Ser473, both of which can be affected by antipsychotics [24, 31, 32, 70]. In the current study, the Thr308 site was examined since phospho-Thr308-Akt was indicated to be...
involved in the β-arrestin2/Akt/PP2A complex associated with D_{2}R [31, 32], but not phospho-Ser473-Akt. However, Akt with either of the sites phosphorylating can induce phosphorylation of GSK3β at Ser9 that was examined in this study. Thus, it is very possible that the increased levels of p-GSK3β in the present study might be induced by either phospho-Thr308- or phospho-Ser473-Akt from different signalling pathways. Although an animal behavioural study revealed that β-arrestin2-associated GSK3β signalling in D2R-expressing neurons is essential for the antipsychotic effects of aripiprazole [71], the results of the present study suggested that these drugs might affect the activities of GSK3β via multiple signalling pathways. In addition to the modulation of the β-arrestin2-mediated signalling pathway, GSK3β is also involved in the wingless (Wnt)-dishevelled-3 (Dvl3)-β-catenin signalling pathway [72]. Kang and Sutton found that both haloperidol and clozapine regulate the activity of GSK3β through Wnt signal pathways involving Dvl upstream in SH-SY5Y cells [73, 74]. In vivo studies indicated that haloperidol treatment significantly increased the phosphorylation of GSK3β at Ser9 in various brain regions through Wnt-Dvl3-β-catenin signalling [65, 66]. Another report found that aripiprazole was also able to increase the phosphorylated GSK3β along with elevated β-catenin levels in vivo [75]. Moreover, aripiprazole can also act at 5-HT_{1A} receptors which also targets GSK3β [76]. It has been reported that activation of 5-HT_{1A} receptors led to increased phosphorylation of GSK3β in vivo [77]. Evidence also indicated that aripiprazole, at the same dose as the present study, significantly increased 5-HT_{1A} receptor binding density in vivo [78]. However, further studies are required to investigate whether 5-HT-regulated GSK3β signalling is involved in the pharmacological properties of aripiprazole. Taken together, the results of the present study suggested that regulation via β-arrestin2/Akt/PP2A signalling pathway by antipsychotics might partly contribute to the alterations in the activities of GSK3β; and the alterations in the activities of GSK3β we observed in the present study might be an integrated consequence of multiple signalling pathways regulated by antipsychotics.

**Comparison between aripiprazole and haloperidol and bifeprunox**

**Aripiprazole vs. haloperidol.** Haloperidol is a potent D_{2}R antagonist [76], treating positive symptoms of schizophrenia effectively, but also inducing severe EPS. In the present study, aripiprazole and haloperidol displayed, to some extent, similar effects on PKA signalling in the mesolimbic dopaminergic pathway, indicating that aripiprazole might perform as an antagonist in the mesolimbic dopaminergic pathway within, at least, a few hours after treatment. In contrast, aripiprazole showed much stronger effects on the levels of p-GSK3β in several brain regions (e.g. PFC, NAc), probably elucidating haloperidol’s inability to treat negative symptoms of schizophrenia, since acute treatment with some other second-generation antipsychotics were able to widely affect GSK3β [68]. Furthermore, since activation of β-arrestin2 contributes to the protective effects against motor side-effects [33], as discussed before, the effects of aripiprazole in the CPus, compared with haloperidol, might reveal a connection between the inhibition of GSK3β activity and reduced EPS.

**Aripiprazole vs. bifeprunox.** Bifeprunox is a D_{2}R partial agonist that failed in clinic trials. Some researchers have suggested that the therapeutic effects of aripiprazole are attributable to its partial agonism at the D_{2}R [79, 80]. In the present in vivo study, it is obvious that bifeprunox affected both PKA and Akt-GSK3β transduction pathways and TH activity in a very different manner from aripiprazole. The action mode of aripiprazole on GSK3β is more like that of the second-generation antipsychotic drugs, not bifeprunox, probably due to a relatively lower intrinsic activity of aripiprazole at D_{2}R. However, we could not completely exclude the possibility that the dosage of bifeprunox used in the present study was insufficient to induce antagonising effects on D_{2} receptors.
In the present study, by comparison with haloperidol and bifeprunox, neither antagonism nor partial agonism for the D$_2$R could completely explain the unique activities of aripiprazole on the D$_2$R downstream signalling pathways. It is worth noting that functional selectivity has been proposed to explain the unique pharmacological properties of aripiprazole [1]. Unfortunately, no evidence in the present study can directly support the theory of functional selectivity for aripiprazole. It is probably because the treatment in the present study was too short to take effect; and the in vivo cellular environment is very complex and the activities of neurons in the living brain are regulated by multiple and convergent factors. Therefore, long-term study and more research methods are required to study this issue.

It is worth pointing out that acute antipsychotic treatment exerted very few or no effects on the levels of p-PKA and cAMP levels in this study. Beaulieu et al. stated that within the first 30min after activation of D$_2$R, G protein-coupled signalling induced a rapid and transient change in the PKA transduction pathway, resulting in a short-term response; after 30min, the Akt-GSK3 transduction pathway was activated, resulting in a more progressive and longer-lasting response [81]. In this study, we sacrificed animals 2 hours after drug administration. Therefore, it is possible that the activation of the PKA signalling pathway had already diminished when we sacrificed animals and collected brains.

There were also some limitations in the present study. It is worthy to note that the signalling pathways examined in the present study were multi-targeted, and the drugs could react with other dopamine receptors other than D$_2$R. For example, the three compounds used in this study have affinities with dopamine D$_3$ receptors. Therefore, we cannot completely exclude the effects of other dopamine receptors; further experiments are important to investigate the effects of antipsychotics on these signalling pathways through specific subtypes of dopamine receptors. Another limitation of the present study is that only the changes in the protein levels of the signalling pathways were examined, further studies are necessary to examine the functional and behavioural changes followed treatment of these antipsychotic drugs.

Conclusions

The present study demonstrated the various effects of acute treatment with aripiprazole, bifeprunox and haloperidol on the two downstream signalling pathways of the D$_2$R in five brain regions. Our study revealed that acute treatment of aripiprazole had differential effects on both cAMP-PKA and Akt-GSK3β signalling pathways from haloperidol and bifeprunox in various brain areas. Furthermore, the differential acute effects of aripiprazole (compared with haloperidol) on GSK3β signalling were also observed in the current study. Further studies in the effects of chronic aripiprazole treatment on these signalling pathways in a schizophrenia animal model might help to explain why aripiprazole has therapeutic effects on negative symptoms of schizophrenia, along with reduced EPS. Our study also suggested that the unique pharmacological profile of aripiprazole might contribute to the relatively low intrinsic activity at the dopamine D$_2$ receptor. Further studies are required to explore the involvement of functional selectivity theory in the mechanisms of aripiprazole.

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Author Contributions

Conceived and designed the experiments: CD BP. Performed the experiments: BP. Analyzed the data: BP JL CD. Contributed reagents/materials/analysis tools: CD XH. Wrote the paper:
Unique Effects of Aripiprazole on PKA and Akt-GSK3β Signalling

BP CD. Manuscript revision and data interpretation: BP JC JL XH CD. Final approval of the version to be published: BP JC JL XH CD.

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CHAPTER 4  ARIPIPRAZOLE INCREASES THE PKA SIGNALLING AND EXPRESSION OF THE GABA RECEPTOR AND CREB1 IN THE NUCLEUS ACCUMBENS OF RATS

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CHAPTER 5  
ARIPIPRAZOLE DIFFERENTIALLY ACTIVATES GSK3B-DEPENDENT SIGNALLING PATHWAYS IN VARIOUS BRAIN REGIONS OF RATS

Aripiprazole and Haloperidol Activate GSK3β-Dependent Signalling Pathway Differentially in Various Brain Regions of Rats

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Abstract: Aripiprazole, a dopamine D2 receptor (D2R) partial agonist, possesses a unique clinical profile. Glycogen synthase kinase 3β (GSK3β)-dependent signalling pathways have been implicated in the pathophysiology of schizophrenia and antipsychotic drug actions. The present study examined whether aripiprazole differentially affects the GSK3β-dependent signalling pathways in the prefrontal cortex (PFC), nucleus accumbens (NAc), and caudate putamen (CPu), in comparison with haloperidol (a D2R antagonist) and bifeprunox (a D2R partial agonist). Rats were orally administrated aripiprazole (0.75 mg/kg), bifeprunox (0.8 mg/kg), haloperidol (0.1 mg/kg) or vehicle three times per day for one week. The levels of protein kinase B (Akt), p-Akt, GSK3β, p-GSK3β, dishevelled (Dvl)-3, and β-catenin were measured by Western Blots. Aripiprazole increased GSK3β phosphorylation in the PFC and NAc, respectively, while haloperidol elevated it in the NAc only. However, Akt activity was not changed by any of these drugs. Additionally, both aripiprazole and haloperidol, but not bifeprunox, increased the expression of Dvl-3 and β-catenin in the NAc. The present study suggests that activation of GSK3β phosphorylation in the PFC and NAc, respectively, while haloperidol elevated it in the NAc only. However, Akt activity was not changed by any of these drugs. Additionally, both aripiprazole and haloperidol, but not bifeprunox, increased the expression of Dvl-3 and β-catenin in the NAc. The present study suggests that activation of GSK3β phosphorylation in the PFC and NAc may be involved in the clinical profile of aripiprazole; additionally, aripiprazole can increase GSK3β phosphorylation via the Dvl-GSK3β-β-catenin signalling pathway in the NAc, probably due to its relatively low intrinsic activity at D2Rs.

Keywords: antipsychotics; aripiprazole; β-catenin; bifeprunox; Dvl-3; GSK3β; haloperidol

1. Introduction

Aripiprazole is an atypical antipsychotic drug with therapeutic effects on both positive and negative symptoms of schizophrenia, but reduced extrapyramidal side-effects (EPS) compared with typical antipsychotics (e.g., haloperidol) [1]. The exact mechanisms of aripiprazole remain unclear. Glycogen synthase kinase 3β (GSK3β) has been implicated in the pathophysiology of schizophrenia and the actions of antipsychotic drugs [2]. GSK3β is a major downstream regulator of dopamine D2 receptors (D2Rs), which is targeted by most antipsychotics (including aripiprazole) [3]. Activation of D2Rs facilitates the formation of the β-arrestin2-protein phosphatase 2A-protein kinase B (PKB or Akt) complex, resulting in dephosphorylation of Akt (inactivation), followed by dephosphorylation (activation) of GSK3β [4–6]. Aripiprazole has been shown to have effects on regulating the Akt-GSK3β signalling pathway [2]. For example, Seo et al. [7] have revealed that aripiprazole altered GSK3β activity in the frontal cortex. However, whether aripiprazole can affect GSK3β activity in other schizophrenia-related brain regions has not yet been studied. Our previous acute study [8] has found that acute administration of aripiprazole increased the phosphorylation levels of GSK3β in...
various brain regions, including the prefrontal cortex (PFC), caudate putamen (CPu), and nucleus accumbens (NAc). However, it is interesting that Akt did not show parallel changes with GSK3β after acute administration [8]. One possibility is that aripiprazole might affect GSK3β activity via alternative pathway(s) that is independent of Akt. One candidate pathway is the dishevelled (Dvl)-GSK3β-β-catenin signalling pathway. In vitro evidence has suggested that various antipsychotics (e.g., clozapine, haloperidol) increase the cellular levels of Dvl and β-catenin via affecting D2Rs [9,10]. In vivo studies have reported that antipsychotic drug administration (including aripiprazole and haloperidol) promoted phosphorylation of GSK3β and expression of Dvl and β-catenin in various brain regions [10–14]. It has been also revealed that administration of aripiprazole attenuated the decreased phosphorylation of GSK3β and reduced expression of β-catenin in the frontal cortex and hippocampus caused by immobilisation stress [7,15]. It should be noted that all these previous studies used intramuscular or subcutaneous injections to deliver aripiprazole. The effects of oral administration that mimic the clinical situation is of importance. Therefore, in this study we examined the Dvl-GSK3β-β-catenin signalling pathway after sub-chronal oral administration of aripiprazole.

Aripiprazole is a D2R partial agonist. Researchers have attributed the unique clinical profile of aripiprazole to its partial agonism at D2Rs [16,17]. However, the role that D2R partial agonism plays in the regulation of the Dvl-GSK3β-β-catenin signalling pathway by aripiprazole is not clear. To investigate this issue, we chose a potent D2R partial agonist—bifeprunox [18] to compare with aripiprazole. Therefore, the present study examined the different effects of one-week oral administration of aripiprazole on the Akt-GSK3β and Dvl-GSK3β-β-catenin signalling pathways in three schizophrenia-related brain regions in comparison with a D2R antagonist—haloperidol and a D2R partial agonist—bifeprunox.

2. Results

2.1. Effects of Antipsychotics in the Prefrontal Cortex

Antipsychotic drug administration had significant effects on the expression of total GSK3β ($F_{3,20} = 3.656, p < 0.05$), p-GSK3β ($F_{3,20} = 3.722, p < 0.05$) and the ratio of p-GSK3β/GSK3β ($F_{3,20} = 9.207, p < 0.01$) in the PFC, but had no effect on Akt, p-Akt, or the ratio of p-Akt/Akt (Figure 1A,D). Post hoc tests demonstrated that administration of aripiprazole significantly increased the protein levels of p-GSK3β by 47.7% ± 6.4% ($p < 0.05$), but reduced total GSK3β expression by 24.9% ± 4.7% ($p < 0.05$) compared with the control; the ratio of p-GSK3β/GSK3β was also increased by administration of aripiprazole ($p < 0.01$) (Figure 1B,D). Furthermore, the protein levels of Dvl-3 and β-catenin in the PFC were not significantly altered by any antipsychotic drug administration (Figure 1C,D).

![Figure 1. Cont.](image-url)
2.2. Effects of Antipsychotics in the Caudate Putamen

One-way analysis of variance (ANOVA) tests indicated significant effects of antipsychotics on the protein levels of total Akt ($F_{3,20} = 9.707, p < 0.01$) in the CPu. Post hoc tests showed that the levels of total Akt were significantly increased by administration of bifeprunox (+18.7% ± 4.8%, $p < 0.05$) and haloperidol (+37.0% ± 4.0%, $p < 0.01$) in the CPu (Figure 2A,D); however, they did not affect the levels of $p$-Akt, nor the ratio of $p$-Akt/Akt. Additionally, the protein levels of Dvl-3 and $β$-catenin were not significantly affected by any antipsychotic drug administration in the CPu (Figure 2B–D).

Figure 1. Effects of three antipsychotics in the prefrontal cortex. The effects of aripiprazole (ARI), bifeprunox (BIF), haloperidol (HAL), and control (CON) on the activity of protein kinase B (Akt) (A); glycogen synthase kinase 3β (GSK3β) (B); and the expression of dishevelled (Dvl)-3 and $β$-catenin (C) were measured in the prefrontal cortex (*$p \leq 0.05$, **$p < 0.01$ vs. the control). All data were expressed as mean ± S.E.M. The representative bands of Western blot are shown in (D).

Figure 2. Effects of three antipsychotics in the caudate putamen. The effects of aripiprazole (ARI), bifeprunox (BIF), haloperidol (HAL), and control (CON) on the activity of Akt (A); GSK3β (B); and the expression of Dvl-3 and $β$-catenin (C) were measured in the caudate putamen (*$p \leq 0.05$, **$p < 0.01$ vs. the control). All data were expressed as mean ± S.E.M. The representative bands of Western blot are shown in (D).
2.3. Effects of Antipsychotics in the Nucleus Accumbens

ANOVA tests revealed that antipsychotic drug administration had significant effects on the protein levels of Akt (F(3,20) = 6.792, p < 0.01), GSK3β (F(3,20) = 25.381, p < 0.01), p-GSK3β (F(3,20) = 11.817, p < 0.01), the ratio of p-GSK3β/GSK3β (F(3,20) = 42.603, p < 0.01), Dvl-3 (F(3,20) = 4.121, p < 0.01), and β-catenin (F(3,20) = 10.718, p < 0.01) in the NAc. Post hoc tests indicated that administration of all three chemicals was shown to be able to reduce the protein levels of total Akt (aripiprazole, −25.9% ± 5.9%, p < 0.01; bifeprunox, −16.5% ± 5.0%, p < 0.05; haloperidol, −23.4% ± 3.2%, p < 0.01) in the NAc; however, no antipsychotic drug administration significantly affected the protein levels of p-Akt, nor the ratios of p-Akt/ Akt (Figure 3A,D). Additionally, the expression of total GSK3β was reduced by both aripiprazole and haloperidol administration (aripiprazole, −34.5% ± 1.2%, p < 0.01; haloperidol, −15.3% ± 7.8%, p < 0.05). Moreover, both aripiprazole and haloperidol administration was able to elevate the levels of p-GSK3β (aripiprazole, +64.4% ± 11.0%, p < 0.05; haloperidol, +92.4% ± 16.7%, p < 0.01) and the ratios of p-GSK3β/GSK3β (aripiprazole, p < 0.01; haloperidol, p < 0.01) (Figure 3B,D). Furthermore, it was shown that administration of aripiprazole was able to promote the expression of both Dvl-3 (+64.1% ± 11.5%, p < 0.01) and β-catenin (+46.5% ± 10.7%, p < 0.01); haloperidol administration also had a positive effect on the protein levels of both Dvl-3 (+54.8% ± 9.4%, p < 0.05) and β-catenin (+59.9% ± 6.6%, p < 0.01) (Figure 3C,D). Lastly, we found that the ratio of p-GSK3β/GSK3β is positively correlated with the expression of Dvl-3 in the NAc (r = 0.245, p < 0.01) (Figure 4A); the ratio of p-GSK3β/GSK3β is also positively correlated with the expression of β-catenin (r = 0.294, p < 0.01) (Figure 4B).

![Figure 3](image-url)

**Figure 3.** Effects of three antipsychotics in the nucleus accumbens. The effects of aripiprazole (ARI), bifeprunox (BIF), haloperidol (HAL), and control (CON) on the activity of Akt (A); GSK3β (B); and the expression of Dvl-3 and β-catenin (C) were measured in the nucleus accumbens ( * p < 0.05, ** p < 0.01 vs. the control). All data were expressed as mean ± S.E.M. The representative bands of Western blot are shown in (D).
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Figure 4. Correlations between the ratio of p-GSK3β/GSK3β and the expression of Dvl-3 and β-catenin in the NAc. The ratio of p-GSK3β/GSK3β is positively correlated with the expression of Dvl-3 (A); and with the expression of β-catenin in the NAc (B).

3. Discussion

The present study has examined the effects of aripiprazole on the Akt-GSK3β and Dvl-GSK3β-β-catenin signalling pathways in three key brain regions that are related to the pathophysiology of schizophrenia, in comparison with bifeprunox and haloperidol. Our findings have provided in vivo evidence that aripiprazole is able to alter the activity of GSK3β in the PFC and NAc. We also found that both aripiprazole and haloperidol, but not bifeprunox, activated the Dvl-GSK3β-β-catenin signalling pathway in the NAc.

A wide range of evidence has identified reduced phosphorylation levels and elevated GSK3β protein levels in the brains of schizophrenic patients, indicating hyper-activity of GSK3β in schizophrenia [19,20]. In addition, antipsychotics, including aripiprazole and haloperidol, have been shown to be able to induce inhibition of GSK3β function in various brain regions [8,11–13,21]. In the present study, both aripiprazole and haloperidol were able to increase the phosphorylation levels of GSK3β (the ratio of p-GSK3β/GSK3β) in the NAc and PFC (only for aripiprazole), which is not completely consistent with the findings in previous studies [7,8,11–13,15,21]. It should be noted that the present study used oral administration to deliver the drugs (for one week) to mimic the clinical situations, which is different from the methods of other previous studies (e.g., intraperitoneal and subcutaneous injection); the dosages of antipsychotics used in this study are transferred from recommended clinical dosages, which are lower than those in previous studies [7,11–13,15,21]. Therefore, the results of the present study might be of more significance for clinic. However, whether these discrepancies are caused by different drug delivering methods requires further investigations.

However, the effects of aripiprazole and haloperidol on GSK3β were not completely consistent in every brain region in the present study. Therefore, by comparing the effects of aripiprazole with those of haloperidol, we may further understand the mechanisms of aripiprazole and elucidate its unique clinical profile. The present study has demonstrated that aripiprazole, but not haloperidol, increased the phosphorylation levels of GSK3β in the PFC. This effect is consistent with the result of our previous acute study [8] and another chronic in vivo study [7]. Since prefrontal dysfunction is linked to the negative symptoms of schizophrenia [22,23], it is suggested that suppression of GSK3β function in the PFC is very likely to contribute to the effects of aripiprazole on the negative symptoms of schizophrenia, which cannot be achieved by haloperidol [7,8]. Moreover, we have observed that aripiprazole increased GSK3β phosphorylation levels in the NAc in the present and previous acute study [8]; haloperidol also showed similar effects in the NAc presently and previously [8,12,21]. It is suggested that dysfunction of the NAc is related to the positive symptoms of schizophrenia [24]. Therefore, our finding further
indicates that inhibition of GSK3\(\beta\) function in the NAc may contribute to the effects of antipsychotics on the positive symptoms of schizophrenia.

It is worth noting that Akt did not change in parallel with GSK3\(\beta\), which is not consistent with previous reports \cite{12,21,25}. This might be explained by following reasons. First, Roh et al. \cite{25} have reported that the phosphorylation of Akt induced by antipsychotics were much shorter in duration than those of GSK3\(\beta\). In the present study, the animals were sacrificed several hours after the last administration. Therefore, the phosphorylation levels of Akt might have already decreased to undetectable levels. This might be the major reason that we only observed the altered p-GSK3\(\beta\) levels, but not p-Akt. Second, there are two phosphorylating sites of Akt—Thr308 and Ser473, both could be affected by antipsychotic drug administration \cite{4,21,25-27}. The present study has examined the Thr308 site of Akt only, since phospho-Thr308-Akt was involved in the D2Rs-mediated Akt-GSK3\(\beta\) signalling \cite{4,27}. However, Akt phosphorylated with either site induces phosphorylation of GSK3\(\beta\) at Ser9 that was examined in the current study. Thereby, it is possible that the elevated p-GSK3\(\beta\) levels in this study might be induced by phospho-Ser473-Akt from other signalling pathway(s), and further investigations are needed to study this issue. Lastly, GSK3\(\beta\) is a multi-targeted regulator. Antipsychotics might affect GSK3\(\beta\) via alternative pathway(s) rather than the D2Rs-mediated signalling pathway, such as the Dvl-GSK3\(\beta\)-\(\beta\)-catenin signalling pathway.

We have examined the effects of antipsychotics on the Dvl-GSK3\(\beta\)-\(\beta\)-catenin signalling pathway. It was observed that both aripiprazole and haloperidol administration increased the expression of Dvl-3 and \(\beta\)-catenin in accordance with the enhanced phosphorylation of GSK3\(\beta\) in the NAc, suggesting that antipsychotics is very likely to affect GSK3\(\beta\) activity via Dvl-GSK3\(\beta\)-\(\beta\)-catenin pathway in this study. However, further studies (e.g., pharmacological or genetic intervention) are required to confirm this suggestion. In addition, it has been reported that antipsychotics (e.g., aripiprazole, haloperidol, clozapine, and risperidone) increased the expression of Dvl-3 and/or \(\beta\)-catenin in various brain regions, including the PFC and striatum \cite{10,12,14}. It is worth noting that the studies by Alimohamad et al. \cite{12} and Sutton et al. \cite{14} have mixed NAc and CPu together, thus preventing identification of the sub-region(s) in which the levels of Dvl-3 and \(\beta\)-catenin were increased by antipsychotic drug administration. This study has separated NAc and CPu, and demonstrated that antipsychotics affect Dvl-GSK3\(\beta\)-\(\beta\)-catenin signalling specifically in the NAc. Taken together, it suggested that activation of Dvl-GSK3\(\beta\)-\(\beta\)-catenin signalling in the NAc is a common route, through which different classes of antipsychotics exert their effects. Lastly, our results do not show any alteration in the expression of Dvl-3 and \(\beta\)-catenin in the PFC, which is inconsistent with the findings of previous studies \cite{10,12,14}. The exact reason remains unclear. This may be because the previous studies \cite{10,12,14} used intramuscular or subcutaneous injection to deliver the drugs, whereas the current study used oral treatment with different dosages to mimic the clinical situation. Therefore, whether the effect of antipsychotics on Dvl-GSK3\(\beta\)-\(\beta\)-catenin signalling is treatment method-dependent requires further validation. Furthermore, one limitation of this study is that the samples have been investigated by only Western blots method, it is also worthy to further validate these findings using other methods such as qPCR and immunohistochemistry.

Previously, Min and colleagues \cite{9} have investigated the interaction between the dopaminergic nervous system and Dvl-GSK3\(\beta\)-\(\beta\)-catenin signalling, and found that only D2Rs directly affected \(\beta\)-catenin distribution in the cell nucleus. The present study used aripiprazole, haloperidol and bifeprunox, all of which have high affinity with D2Rs \cite{18,28}. Haloperidol is a potent D2R antagonist, whereas aripiprazole and bifeprunox are D2R partial agonists. Previous studies have revealed that the intrinsic activity of aripiprazole at D2Rs is weaker than that of bifeprunox (intrinsic activity at D2Rs: aripiprazole vs. bifeprunox vs. dopamine = 86.0% vs. 95.1% vs. 100%) \cite{22,29}. Our results have demonstrated that administration of both aripiprazole and haloperidol, but not bifeprunox, had significant effects on altering the expression of Dvl-3 and \(\beta\)-catenin in the NAc. Therefore, first, blockade of D2Rs is (indirectly) linked to the activation of the Dvl-GSK3\(\beta\)-\(\beta\)-catenin signalling pathway. Second, it is very possible that aripiprazole competes with endogenous dopamine in the normal brain.
due to its relatively low intrinsic activity to reduce significantly the activity of endogenous dopamine, displaying an overall antagonising effect like haloperidol. In contrast, bifeprunox cannot achieve such effects, probably because of its relatively stronger intrinsic activity at D₂Rs. Taken together, our study suggests that a relatively low intrinsic activity at D₂Rs might be essential for a D₂R partial agonist to achieve meaningful effects via affecting the Dvl-GSK3β-β-catenin signalling pathway.

4. Materials and Methods

4.1. Animals and Drug Administration

Male Sprague–Dawley rats (aged eight weeks) were obtained from the Animal Resource Centre (Perth, Australia). After arrival, all rats were housed in individual cages under environmentally controlled conditions (temperature 22 °C, light cycle from 07:00 a.m. to 07:00 p.m.), with ad libitum access to water and a standard laboratory chow diet. All experimental procedures were approved by the Animal Ethics Committee (Application #AE11/02, 02/2011), University of Wollongong, and complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004). All efforts were made to minimise animal distress and prevent suffering.

Before drug administration commenced, the rats were trained for self-administration of the cookie dough pellets without drugs. After 1-week training, rats were randomly assigned into one of the following four groups (n = 6/group): aripiprazole (0.75 mg/kg, t.i.d. (ter in die), Otsuka, Tokyo, Japan); bifeprunox (0.8 mg/kg, t.i.d., Otava, Kiev, Ukraine); haloperidol (0.1 mg/kg, t.i.d., Sigma, Castle Hill, Australia); or vehicle for one week. Rats were offered cookies with drugs three times a day (at 06:00 a.m., 02:00 p.m. and 10:00 p.m.) and observed to ensure complete consumption of each pellet. The dosages were translated from recommended clinical dosages based on body surface area according to the FDA guidelines [30,31]. This drug administration method has been well established in our laboratory [32,33]. Specifically, a 0.75 mg/kg aripiprazole, 0.8 mg/kg bifeprunox and 0.1 mg/kg haloperidol dosage in rats is equivalent to ~7.5, ~8, and ~1 mg in humans (60 kg body weight), respectively, all of which are within the used/recommended clinical dosages [34–36]. It is worth noting that aripiprazole and bifeprunox induced over 90% D₂ receptor occupancy in rat brains at these dosages [18], and haloperidol reached approximately 70% occupancy [37], all of which can display physiological and behavioural effects in rodents, without inducing EPS side-effects [18,38–40]. After one-week drug administration, all rats were sacrificed between 10:00 a.m. and 12:00 p.m. to minimise possible circadian-induced variation of protein expression. All animals were euthanised by using carbon dioxide. Brains were immediately dissected, frozen in liquid nitrogen and stored at −80 °C until further use.

4.2. Micro-Dissection of Brain Samples

Following a standard procedure used in our group [8], discrete brain regions were collected using brain microdissection puncture according to the brain atlas [41]. Briefly, three sections through the forebrain (Bregma 3.30 to 4.20 mm) were collected for the PFC; and three sections through the striatum (Bregma 1.00 to 2.20 mm) were collected for the CPu and NAc, respectively. Tissue dissected was kept at −80 °C.

4.3. Western Blots

The Western blot experiments were performed following standard procedures repeated in our previous studies [8,33]. Briefly, frozen tissue was homogenised with 9.8 mL NP-40 cell lysis buffer (Invitrogen, Camarillo, CA, USA) containing 100 µL Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO, USA), 100 µL β-Glycerophosphate (Invitrogen) and 33.3 µL phenylmethylsulfonylfluoride (Sigma-Aldrich). The homogenised samples were centrifuged, and the supernatants were collected. Protein concentration of each homogenising solution was measured by using the DC Protein Assay (Bio-Rad, #500-0111). After denaturing proteins, samples containing
10 µg of protein were loaded into 4%-20% Criterion™ TGX™ Precast Gels (Bio-Rad, Hercules, CA, USA, #5671095) in a Criterion™ Vertical Electrophoresis Cell (Bio-rad, #1656001) at 200 V voltage for 50 min, and then transferred electrophoretically to a polyvinylidene difluoride membrane in a Criterion™ Blotter (Bio-rad, #1704071) at 100 V voltage for 60 min. All membranes were blocked by 5% bovine serum albumin (BSA) for 60 min and incubated in primary antibodies (diluted in 1% BSA) overnight. Amersham Hyperfilm ECL (GE Healthcare, Chicago, IL, USA, #28-9068-36) and Luminata Classico Western HRP substrate (Millipore, Billerica, MA, USA, #WBLUC0500) were used to visualise the immunoreactive bands. The immunoreactive signals were quantified using Bio-Rad Quantity One software. The data of each targeted protein were then corrected based on their corresponding actin levels. Experiments were performed in duplicate to ensure consistency.

The antibodies used in the present study to examine the GSK3β-involved pathways were anti-Akt (1:2000; Cell Signalling, Danvers, MA, USA, #4691), anti-phosphor-Akt (Thr308) (1:1000; Cell Signalling, #13038), anti-GSK3β (1:2000; Cell Signalling, #5676), anti-phospho-GSK3β (Ser9) (1:1000; Cell Signalling, #9322), anti-Dvl-3 (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA, #SC-8027) and anti-β-catenin (1:1000; Santa Cruz Biotechnology, #SC-7963). Mouse anti-actin primary polyclonal antibody (1:10000; Millipore, #MAB1501) was used to determine the actin levels. The secondary antibodies were HRP-conjugated anti-rabbit IgG antibody (1:3000; Cell Signalling, #7074) and HRP-conjugated anti-mouse IgG antibody (1:3000; Cell Signalling, #7076).

4.4. Statistics

All data was analysed using SPSS Statistics V22.0 program (IBM, New York, NY, USA). Data normality was tested using histograms and a Kolmogorov–Smirnov Z test. For statistical evaluation, one-way analysis of variance (ANOVA) was performed if the data was normally distributed. The post hoc Dunnett t test was then conducted to compare each drug treatment group with the control group. The results of Western blots were normalised by taking the average value of the control group as 100%. The phosphorylation to total signal was calculated using the data from the same blot. Pearson’s correlation test was used to analyse the relationships. A p-value of less than 0.05 was considered as statistically significant.

5. Conclusions

The present study explored the in vivo effects of one-week oral administration of aripiprazole on the GSK3β-dependent signalling pathways in three brain regions that are associated with schizophrenia and the actions of antipsychotics, in comparison with haloperidol and bifeprunox. The current study provides in vivo evidence that inhibition of GSK3β activity in the PFC and NAc might be linked to the clinical profile of aripiprazole. This study further suggests that, like haloperidol, aripiprazole can activate Dvl-GSK3β-β-catenin signalling in the NAc, which is probably due to the relatively low intrinsic activity at D₂Rs.

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Author Contributions: Chao Deng and Bo Pan designed the study. Bo Pan performed the animal treatment. Bo Pan conducted experiments and analysed data. Bo Pan prepared the initial draft of the manuscript. Bo Pan, Chao Deng and Xu-Feng Huang revised the manuscript and interpreted the data. All of the authors approved the final manuscript.

Conflicts of Interest: The authors declare no conflict of interest.
Abbreviations

Akt: protein kinase B
CPu: caudate putamen
D2R: dopamine D2 receptor
Dvl: dishevelled
EPS: extrapyramidal side-effects
GSK3β: glycogen synthase kinase 3β
NAc: nucleus accumbens
PFC: prefrontal cortex

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CHAPTER 6  
CHRONIC ADMINISTRATION OF ARIPIPRAZOLE ACTIVATES THE GSK3β-DEPENDENT SIGNALLING PATHWAYS, AND UP-REGULATES GABA_A RECEPTOR EXPRESSION AND CREB1 ACTIVITY IN RATS

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Chronic administration of aripiprazole activates GSK3β-dependent signalling pathways, and up-regulates GABA_A receptor expression and CREB1 activity in rats

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Aripiprazole is a D2-like receptor (D2R) partial agonist with a favourable clinical profile. Previous investigations indicated that acute and short-term administration of aripiprazole had effects on PKA activity, GSK3β-dependent pathways, GABA_A receptors, NMDA receptor and CREB1 in the brain. Since antipsychotics are used chronically in clinics, the present study investigated the long-term effects of chronic oral aripiprazole treatment on these cellular signalling pathways, in comparison with haloperidol (a D2R antagonist) and bifeprunox (a potent D2R partial agonist). We found that the Akt-GSK3β pathway was activated by aripiprazole and bifeprunox in the prefrontal cortex; NMDA NR2A levels were reduced by aripiprazole and haloperidol. In the nucleus accumbens, all three drugs increased Akt-GSK3β signalling; in addition, both aripiprazole and haloperidol, but not bifeprunox, increased the expression of Dvl-3, β-catenin and GABA_A receptors, NMDA receptor subunits, as well as CREB1 phosphorylation levels. The results suggest that chronic oral administration of aripiprazole affects schizophrenia-related cellular signalling pathways and markers (including Akt-GSK3β signalling, Dvl-GSK3β-β-catenin signalling, GABA_A receptor, NMDA receptor and CREB1) in a brain-region-dependent manner; the selective effects of aripiprazole on these signalling pathways might be associated with its unique clinical effects.

Aripiprazole is a unique antipsychotic drug with a pharmacological profile different from other available antipsychotics, and this difference has been attributed to its partial agonism for the dopamine D2 receptor (D2R). A large body of evidence has shown that most antipsychotics (including aripiprazole and haloperidol) have a potent affinity at the D2Rs, regulating the D2R downstream protein kinase B (Akt)-glycogen synthase kinase 3 beta (GSK3β) and protein kinase A (PKA) signalling pathways. In addition, these two signalling pathways are also linked to several other pathways or substrates, such as the dishevelled(Dvl)-GSK3β-β-catenin pathway, γ-aminobutyric acid (GABA) receptor and cAMP-responsive element-binding protein 1 (CREB1).

GSK3β-dependent signalling pathways are involved in the pathophysiology of schizophrenia and the actions of antipsychotics. First, GSK3β is a major downstream regulator of D2-like receptors that is targeted by most antipsychotics. It has been reported that chronic haloperidol treatment phosphorylates GSK3β, and inhibits its activity, which is associated with increased phosphorylation levels of Akt in the frontal cortex. Second, antipsychotic administration can also influence the Dvl-GSK3β-β-catenin signalling pathway. Several studies have reported that various antipsychotics (including clozapine, haloperidol, risperidone, olanzapine and aripiprazole) were able to increase phosphorylation of GSK3β and expression of Dvl and β-catenin in the frontal cortex and striatum.

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The G protein-dependent PKA pathway is another downstream signalling pathway of D_{2} like receptors. PKA signalling has been shown to be related to the pathophysiology of schizophrenia by a post-mortem study\textsuperscript{34}. An in vivo study has indicated that acute administration of haloperidol and olanzapine increased the expression of PKA catalytic subunits in the rat caudate putamen (CPu)\textsuperscript{25,26}. PKA signalling has also been elevated by acute administration of haloperidol in the striatum\textsuperscript{17}; and furthermore, the activity of the PKA pathway and expression of PKA regulatory subunits in the striatum were elevated after a 3-week administration of haloperidol in various brain regions, but decreased by clozapine administration\textsuperscript{19}. A recent study has shown that 1-week administration of aripiprazole increased PKA phosphorylation in the nucleus accumbens (NAc), but reduced it in the CPu, while haloperidol decreased it in both the NAc and CPu\textsuperscript{25}. The GABA\_ receptors have been reported to be involved in the pathophysiology of schizophrenia\textsuperscript{35}. Increased binding density of GABA\_ receptors have been found in the prefrontal cortex (PFC)\textsuperscript{27–30}, cingulate cortex\textsuperscript{27}, superior temporal gyrus\textsuperscript{31} and hippocampus\textsuperscript{28} of schizophrenic subjects. Antipsychotic administration has been shown to have various effects on GABA\_ receptors. It has been reported that 1-week treatment with both haloperidol and olanzapine increased the binding density of [\H^{3}]Muscimol labelled GABA\_ receptors\textsuperscript{28} in the PFC. Zink and colleagues\textsuperscript{29} have found that haloperidol administration for 6 months increased the binding density of GABA\_ receptors in the CPu and core of the NAc, but reduced it in the PFC, anterior cingulate and infralimbic cortex; and 6-month clozapine administration reduced the bindings of GABA\_ receptors in the anterior cingulate and infralimbic cortex. Recent data suggested that expression of GABA\_ receptors in the rat NAc was elevated by 1-week aripiprazole administration, probably by activation of the PKA pathway\textsuperscript{25}. CREB1 is also a downstream substrate of the PKA pathway\textsuperscript{31}. Novel variants in the CREB1 gene have been identified in schizophrenic subjects, and a relationship between CREB1 and the positive symptoms of schizophrenia has been proposed\textsuperscript{32}. Previous in vivo and in vitro studies have shown that haloperidol increased phosphorylation levels of CREB1\textsuperscript{19,20}. Additionally, amisulpride, clozapine and olanzapine also elevated phosphorylation levels of CREB1 in vitro\textsuperscript{17,20}. Furthermore, a 3-week treatment of aripiprazole increased the expression of CREB1 in the PFC and striatum of rats, probably through NMDA receptors\textsuperscript{5}. A recent short-term study has reported that aripiprazole increased the gene and protein expression of CREB1, probably through the PKA pathway, in the NAc of rats\textsuperscript{25}. N-methyl-D-aspartate receptors (NMDARs) have been shown to be involved in the pathophysiology of schizophrenia in healthy subjects\textsuperscript{33,34}. Recent studies showed that acute and short-term administration of aripiprazole – a potent D_{2}R partial agonist – displayed different effects that cannot be achieved by haloperidol (a typical antipsychotic and a potential D_{2}R antagonist) and bifeprunox (a potent D_{2}R partial agonist), providing preliminary in vivo evidence that neither D_{2}R partial agonism nor D_{2}R antagonism could be solely explain the pharmacological mechanism and unique clinical effects of aripiprazole\textsuperscript{25–29}. It should also be noted that in clinics, antipsychotics require a long treatment period to reach maximum therapeutic effect, and thus are often used chronically\textsuperscript{35}. All previous chronic studies administered antipsychotics by various methods (e.g. mixing in drinking water, daily injection), rather than the oral administration that mimics the clinical situation. The chronic effects of aripiprazole by such oral administration are not clear. Therefore, the present study investigated the effects of 10-week oral administration of aripiprazole by examining PKA signalling, Akt- GSK3\beta and Dvl- catenin pathways, GABA\_ receptors and CREB1 activity; in comparison with haloperidol and bifeprunox.

**Results**

**The effect of antipsychotics on Akt and GSK3\beta activity.**  
**PFC.** It has been shown that the expression of total Akt and total GSK3\beta was significantly affected by 10-week antipsychotic drug administration in the PFC (Akt, F_{3,20} = 5.201, p = 0.004; GSK3\beta, F_{3,20} = 3.083, p = 0.026); however, the levels of p-Akt and p-GSK3\beta were not significantly affected (p-Akt, F_{3,20} = 1.554, p = 0.232; p-GSK3\beta, F_{3,20} = 1.208, p = 0.332). The ratio of p-Akt/ Akt (F_{3,20} = 4.523, p = 0.007) and p-GSK3\beta/GSK3\beta (F_{3,20} = 4.112, p = 0.010) was also significantly affected by antipsychotic drug administration. Post-hoc testing demonstrated that chronic administration of both aripiprazole and bifeprunox reduced expression of Akt (t-ariprazole, − 14.2%, p = 0.014; bifeprunox, − 13.6%, p = 0.008) in the PFC (Fig. 1A-D). Additionally, administration of aripiprazole significantly suppressed GSK3\beta expression (by 25.0%, p = 0.043) compared with controls (Fig. 2A-D). The levels of p-Akt and p-GSK3\beta were not significantly affected. In addition, aripiprazole and bifeprunox administration significantly increased the ratio of p-Akt/ Akt (ariprazole, p = 0.021; bifeprunox, p = 0.005) (Fig. 1A). The ratio of p-GSK3\beta/GSK3\beta was also increased by administration of aripiprazole (p = 0.012) and bifeprunox (p = 0.019) (Fig. 2A).

**CPu.** Chronic antipsychotic drug administration had no significant effects on the levels of Akt, p-Akt (Fig. 1B-D), GSK3\beta and p-GSK3\beta (Fig. 2B-D) in the CPu compared with controls (all p > 0.05).

**NAc.** Drug treatment was able to significantly change the levels of p-Akt (F_{3,20} = 4.315, p = 0.009), p-GSK3\beta (F_{3,20} = 9.798, p < 0.001), as well as the ratio of p-Akt/Akt (F_{3,20} = 5.268, p = 0.004) and p-GSK3\beta/GSK3\beta (F_{3,20} = 7.024, p = 0.001) in the NAc. Compared with the control group, both bifeprunox and haloperidol administration significantly elevated the levels of p-Akt in the NAc (bifeprunox, + 38.1%, p = 0.005; haloperidol, + 40.8%, p = 0.006); aripiprazole also tended to increase the levels of p-Akt (+25.3%, p = 0.072) (Fig. 1C-D). All three...
Figure 1. Effects of three antipsychotics on Akt activity. The effects of aripiprazole (ARI), bifeprunox (BIF) and haloperidol (HAL) on Akt activity were measured in the prefrontal cortex (A), caudate putamen (B) and nucleus accumbens (C). The representative bands of Western blot are shown in (D). Akt was quantified at 60 kDa; p-Akt was quantified at 60 kDa. The data were normalised by taking the average value of the control group as 100% and expressed as mean ± S.E.M. (*p ≤ 0.05, **p < 0.01 vs the control).

Figure 2. Effects of three antipsychotics on GSK3β activity. The effects of aripiprazole (ARI), bifeprunox (BIF) and haloperidol (HAL) on GSK3β activity were measured in the prefrontal cortex (A), caudate putamen (B) and nucleus accumbens (C). The representative bands of Western blot are shown in (D). GSK3β was quantified at 46 kDa; p-GSK3β was quantified at 46 kDa. The data were normalised by taking the average value of the control group as 100% and expressed as mean ± S.E.M. (*p ≤ 0.05, **p < 0.01 vs the control).
drugs increased the ratio of p-Akt/Akt (aripiprazole, \( p = 0.044 \); bifeprunox, \( p = 0.007 \); haloperidol, \( p = 0.002 \)) (Fig. 1C). Furthermore, administration of all three was able to elevate the levels of p-GSK3\( \beta \) (aripiprazole, +69.3%, \( p < 0.001 \); bifeprunox, +33.2%, \( p = 0.026 \); haloperidol, +38.4%, \( p = 0.010 \)) (Fig. 2C,D) and significantly increased the ratio of p-GSK3\( \beta \)/GSK3\( \beta \) (aripiprazole, \( p = 0.001 \); bifeprunox, \( p = 0.048 \); haloperidol, \( p = 0.005 \)) (Fig. 2C).

The effect of antipsychotics on Dvl-3 and \( \beta \)-catenin expression. Chronic antipsychotic administration had significant effects on the expression of Dvl-3 (\( F_{(2,20)} = 4.629, p = 0.007 \)) and \( \beta \)-catenin (\( F_{(2,20)} = 15.704, p < 0.001 \)) in the NAc. Post-hoc tests indicated that the protein levels of Dvl-3 were significantly elevated by administration of both aripiprazole (\( +40.8%, p = 0.011 \)) and haloperidol (\( +33.7%, p = 0.031 \)) in the NAc; they also significantly increased the expression of \( \beta \)-catenin (aripiprazole, \( +30.3%, p = 0.008 \); haloperidol, \(+49.6%, p = 0.001 \)) (Fig. 3C,D). Additionally, the ratio of p-GSK3\( \beta \)/GSK3\( \beta \) was positively correlated with the expression of \( \beta \)-catenin (\( r = 0.297, p = 0.039 \)) (Fig. 4A). On the other hand, no significant effect was observed in the other two brain areas (Fig. 3A,B,D).

The effect of antipsychotics on GABA\(_A\) receptor expression. GABA\(_A\) receptors containing \( \beta \)-1 subunit were examined in the present study. The expression of GABA\(_A\) \( \beta \)-1 receptors in the NAc was significantly affected by antipsychotic drug administration (\( F_{(2,20)} = 4.926, p = 0.005 \)). Both aripiprazole and haloperidol administration significantly increased GABA\(_A\) \( \beta \)-1 receptor expression in the NAc (aripiprazole, \( +19.8%, p = 0.008 \); haloperidol, \(+22.7%, p = 0.003 \)) (Fig. 3C,D) but not in the PFC and CPu (Fig. 3A,B,D).

The effects of antipsychotics on NMDAR subunits expression. PFC. The expression of NR2A was significantly altered by antipsychotic drug administration in the PFC (\( F_{(2,20)} = 4.976, p = 0.010 \)), but not NR1 (\( F_{(2,20)} = 1.067, p = 0.137 \)). Post-hoc tests revealed that NR2A levels were reduced by both aripiprazole (\(-19.9%, p = 0.020 \)) and haloperidol (\(-27.9%, p = 0.002 \)) (Fig. 5A,D).

CPu. The expression of NR1 and NR2A was not affected by drug administration in the CPu (NR1, \( F_{(2,20)} = 0.828, p = 0.969 \); NR2A, \( F_{(2,20)} = 0.909, p = 0.454 \)) (Fig. 5B,D).

NAc. Both NR1 and NR2A expression was significantly changed by antipsychotic drug administration (NR1, \( F_{(2,20)} = 4.653, p = 0.013 \); NR2A, \( F_{(2,20)} = 6.923, p = 0.002 \)). Post-hoc tests showed that aripiprazole increased the expression of both NR1 (\(+36.3%, p = 0.066 \)) and NR2A (\(+41.9%, p = 0.001 \)); haloperidol also elevated NR1 expression (\(+27.4%, p = 0.033 \)) in the NAc (Fig. 5C,D).
The effect of antipsychotics on CREB1 activity. The levels of CREB1 and p-CREB1 in the PFC and CPu were not altered by any of the three antipsychotic drug administration (Fig. 6A,B,D). In the NAc, antipsychotic drug administration had a significant effect on CREB1 ($F_{3,20} = 2.502, p = 0.045$), p-CREB1 ($F_{3,20} = 2.502, p = 0.045$), p-CREB1 ($F_{3,20} = 10.698, p < 0.001$), and the ratio of p-CREB1/CREB1 ($F_{3,20} = 5.972, p = 0.002$). Post-hoc tests showed that the expression of CREB1 was significantly elevated by administration of aripiprazole (+30.5%; $p = 0.020$). Additionally, the levels of p-CREB1 were significantly promoted by aripiprazole (+90.0%; $p < 0.001$) and haloperidol (+68.8%; $p = 0.002$) in the NAc (Fig. 6C,D); they also elevated the ratio of p-CREB1/CREB1 (aripiprazole, $p = 0.019$; haloperidol, $p = 0.004$) (Fig. 6C). Furthermore, the ratio of p-GSK3β/GSK3β was positively correlated with the ratio of p-CREB1/CREB1 ($r = 0.572, p = 0.012$) in the NAc (Fig. 4B).

The effect of antipsychotics on PKA activity. The levels of PKA-Cα and p-PKA-C were not changed in any brain regions after chronic antipsychotic administration in the present study (data not shown).
Aripiprazole and bifeprunox also decreased total protein levels of both Akt and GSK3β, which has been confirmed in this chronic study. It should also be noted that antipsychotics affect Akt/GSK3β signalling in a time-dependent manner. Roh et al.15 found that the duration of p-Akt signalling induced by antipsychotics (about 1 hour) was much shorter than that of p-GSK3β. The time interval between the last administration and sacrifice in the previous12–14 and the present studies was longer than 1 hour. The levels of p-Akt were undetectable in the acute and short-term studies12–14, but were observed in this chronic study, which suggests that chronic administration of antipsychotic drugs might be able to have prolonged effects on Akt activity and thereby on Akt-GSK3β signalling.

Although aripiprazole, bifeprunox and haloperidol were able to influence GSK3β activity in the present study, their effects on GSK3β were not identical in each brain region. In the PFC, administration of aripiprazole and bifeprunox, but not haloperidol, was able to suppress the activity of GSK3β via the Akt-GSK3β signalling pathway. Aripiprazole and bifeprunox also decreased total protein levels of both Akt and GSK3β in response to the chronic treatment of these two drugs. It has been suggested that inhibition of GSK3β contributes to the therapeutic effects of antipsychotics14 and the functional impairment in the PFC is related to the negative symptoms and cognitive deficits of schizophrenia15. Our results suggested that the therapeutic effects of aripiprazole on the negative symptoms and cognitive deficits of schizophrenia might be attributed to its inhibiting effects on GSK3β activity in the PFC. Furthermore, bifeprunox was able to improve the negative symptoms of schizophrenia in clinical trials13 and it inhibited the activity of GSK3β in the present study, which further confirms that suppression of GSK3β activity is very likely to be associated with the effects of antipsychotics on the negative and cognitive symptoms.
of schizophrenia. In contrast, haloperidol did not display any significant effects on GSK3β in the PFC in the present study and a previous acute study24, which may explain why haloperidol does not have therapeutic effects on the negative symptoms and cognitive deficits of schizophrenia. It should be noted that the present study did not find any changes of β-catenin, CREB1 and GABAA receptor in the PFC. Therefore, whether antipsychotics control the negative symptoms and cognitive deficits of schizophrenia via the enhancement of Akt-GSK3β signalling requires further validations. In the NAc, administration of all three drugs increased the phosphorylation of both Akt and GSK3β in the present study. Since abnormal function of the NAc is linked to the positive symptoms of schizophrenia25, our results indicated that the suppression of GSK3β functions via the Akt-GSK3β signalling pathway in the NAc is very likely to be involved in the therapeutic effects of antipsychotics (probably on the positive symptoms of schizophrenia) (Fig. 7A). In the CPu, chronic treatment with these antipsychotics had very limited effects on the Akt-GSK3β signalling pathway, which suggests that GSK3β activity in the CPu may not be involved in the long-term clinical effects of antipsychotics. Yager et al.17 have reviewed that the NAc and CPu are heterogeneous structures with different neural inputs and outputs connected with various brain regions. For example, the NAc receives dopaminergic inputs from the ventral tegmental area, while sending outputs to limbic areas and the PFC; the CPu receives dopaminergic inputs from the substantia nigra pars, while sending projections to neocortical areas. Therefore, chronic antipsychotic treatment might have different effects in the NAc and CPu. However, the brain-regional differences in the effects of antipsychotics require further clarification.

Previously, various classes of antipsychotics (e.g. aripiprazole, haloperidol, clozapine and risperidone) have been found to be able to increase the expression of Dvl-3 and/or β-catenin in various brain regions of animals, including the frontal cortex and striatum, indicating enhanced activity of β-catenin in these brain areas8–11. The current data has demonstrated that Dvl-3 and β-catenin expression was significantly increased by administration of both aripiprazole and haloperidol in the NAc only. These results are partly consistent with those of previous studies. Since dysfunction of the NAc is associated with the positive symptoms of schizophrenia and the main target of antipsychotics8 as mentioned above, the present study suggests that regulation of β-catenin via GSK3β might be a common mechanism by which antipsychotics exert their effects, even if they have different pharmacological profiles (Fig. 7A). It is further suggested that the increased expression of β-catenin in the NAc is very likely to be a route through which antipsychotics exert their therapeutic effects, especially on the positive symptoms of schizophrenia.

It has been reported that chronic haloperidol administration increased the binding of GABAA receptors in the NAc; and both haloperidol and clozapine administration also increased GABAA receptor binding in the limbic cortex13. A recent short-term study has demonstrated that both aripiprazole and haloperidol increased GABAA receptor expression in the NAc (although the increasing effect of haloperidol did not reach significance9), which is validated by the present study. In this study, we found that chronic administration of
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aripiprazole and haloperidol were able to significantly increase GABA<sub>B</sub> (containing 3-1) receptor expression in the NAc, but no effects were shown in the other two brain regions. Since dysfunction of the NAc is related to the positive symptoms of schizophrenia<sup>28–30</sup> as mentioned above, our findings together with other studies, suggest that increased GABA<sub>B</sub> receptor expression and probably enhanced GABA<sub>B</sub> signalling transmission in the NAc is very likely to be involved in the therapeutic effects of antipsychotics (possibly on the positive symptoms of schizophrenia) (Fig. 7B). It is worth noting that the GABA<sub>B</sub> receptor is regulated by D<sub>2</sub>-like receptor downstream PKA signalling<sup>31–33</sup>. A 1-week study has revealed that PKA phosphorylation levels in the NAc, paralleling the expression of GABA<sub>B</sub> receptors, was increased by aripiprazole and haloperidol<sup>17</sup>. In this chronic study, the effects of aripiprazole and haloperidol in increasing GABA<sub>B</sub> receptor expression persisted, whereas the changes in PKA activity were undetectable. The reason is unknown. It is possible that the up-regulated expression of GABA<sub>B</sub> receptors by short-term antipsychotic drug administration (through PKA) is an adaptive and prolonged change; even if there was nothing but an alteration in PKA activity after chronic administration (probably due to adaptive changes in D<sub>2</sub>Rs), the increased GABA<sub>B</sub> receptor expression could be maintained.

A variety of studies has indicated dysfunction of NMDARs in schizophrenia<sup>2</sup>. In the current study, both aripiprazole and haloperidol administration was able to increase the expression of NMDAR subunits in the NAc, but reduce it in the PFC. Specifically, aripiprazole increased the expression of both NR1 and NR2A in the NAc, while haloperidol increased NR1 expression; in the PFC, both aripiprazole and haloperidol decreased NR2A levels. Previously, Segnit et al.<sup>4</sup> have reported that NR2A mRNA levels were decreased in the PFC by aripiprazole treatment for 4 months, but not changed in the CPU. Schmitt et al.<sup>5</sup> have also found reduced NR2A mRNA in the PFC induced by both haloperidol and clozapine treatment. Furthermore, it was found that 4-week administration with haloperidol and D<sub>2</sub>-like receptor antagonist – receptor antagonist – receptor antagonist – receptor antagonist in the striatum<sup>2</sup>. Additionally, [H] MK-801 binding in the NAc was increased by haloperidol<sup>46</sup>. These previous findings are consistent with those of this study that both typical and atypical antipsychotics can increase NMDARs expression in the striatum (particularly in the NAc, but not in the CPU), and reduce NR2A expression in the PFC. It is worth noting that antipsychotics have a very low affinity with NMDARs. Therefore, it is necessary to further reveal through which pathway(s) antipsychotics regulate NMDARs.

Previously, haloperidol was able to increase phosphorylation levels of CREB1 in the striatum and hippocampal neuron culture<sup>28–30</sup>. In addition, amisulpride, clozapine and olanzapine were also able to induce CREB1 phosphorylation in in vitro studies<sup>31–33</sup>. A 1-week in vitro study has shown that a 1-week administration of aripiprazole increased the expression of CREB1 in the NAc<sup>17</sup>. In the present study, chronic administration of aripiprazole increased CREB1 expression in the NAc and both aripiprazole and haloperidol enhanced CREB1 activity via increasing the ratio of p-CREB1/CREB1. This confirmed that CREB1 is involved in the actions of antipsychotics. Moreover, extensive communication occurs between CREB1 and PKA, and the Akt-GSK3<beta> pathway<sup>47</sup>. In the present study, we did not observe any change in PKA activity, nor in the correlation between PKA and CREB1 activity in the NAc (indicated as a dashed arrow in Fig. 7C). However, GSK3<beta> activity is positively correlated with CREB1 activity. A previous study has indicated that CREB1 activity was increased by inhibition of GSK3<beta> in cultured rat cerebral cortical neurons<sup>48</sup>. Amsulpride also induced the phosphorylation of CREB1 via the Akt-GSK3<beta> pathway in SH-SY5Y cells<sup>49</sup>. Since patients with novel variants in the CREB1 gene experienced the positive symptoms of schizophrenia<sup>50</sup>, our data suggests that activation of CREB1 via the Akt-GSK3<beta> pathway in the NAc is very likely to be associated with the therapeutic effects of aripiprazole and haloperidol on the positive symptoms of schizophrenia (Fig. 7C). It is worth noting that Mavraki et al.<sup>51</sup> suggested that phosphorylation of CREB1 was increased by 3-week injection of aripiprazole in the PFC and striatum of rats, probably through NMDA receptors. The regulation of CREB1 via NMDARs has also been confirmed by other studies<sup>52–54</sup>. The present study has shown both aripiprazole and haloperidol were able to increase CREB1 phosphorylation and NMDAR expression in the NAc, simultaneously. Whether the increased expression of NMDARs partly contributes to the enhanced phosphorylation of CREB1 in the NAc requires further validation. Lastly, phosphorylation of histone H3S10, an epigenetic modification, has been reported to be significantly increased in schizophrenia patients<sup>55</sup>. Since histone H3 phosphorylation could be regulated by G protein-coupled receptor and NMDAR signalling<sup>56</sup>, it is important to further investigate how the modulations of antipsychotics on these signalling pathways contribute to their therapeutic effects.

In summary, the present study has demonstrated that chronic administration of aripiprazole had different effects on the Akt-GSK3<beta>, Dvl-GSK3<beta>-3-catenin, GABA<sub>B</sub> receptor and CREB1 activity in a brain region-dependent manner. Compared to the effects of haloperidol only in the NAc and bifeprunox mainly in the PFC, aripiprazole affected these cellular signalling pathways in both the PFC and NAc, which may explain its unique clinical effects. It is also worth noting that the present and previous studies mentioned above examined the effects of antipsychotic drugs in healthy animals. It is necessary to investigate the effects of antipsychotics in the animal models for schizophrenia and other mental disorders in future studies.

Methods

Animals and drug administration. Male Sprague-Dawley rats (aged 8 weeks) were obtained from the Animal Resource Centre (Perth, Australia). After arrival, all rats were housed in individual cages under environmentally controlled conditions (temperature 22°C, light cycle from 07:00AM to 07:00PM), with ad libitum access to water and standard laboratory Chow diet. All experimental procedures were approved by the Animal Ethics Committee (Application #. AEI1602), University of Wollongong, and complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004). All efforts were made to minimise animal distress and prevent suffering.

Before drug administration commenced, the rats were trained for self-administration of the sweet cookie dough pellets for a week. Then the rats were randomly assigned into one of the following drug treatment groups (n = 6/group): aripiprazole (0.75 mg/kg; Otsuka, Japan), bifeprunox (0.8 mg/kg; Otsuka, Ukraine), haloperidol
(0.1 mg/kg; Sigma, Australia), or vehicle. Drug powder mixed with the cookie dough pellets was delivered orally 3 times per day at 07:00, 13:00 and 19:00 for 10 weeks. This drug administration method aims to mirror the human scenario of oral administration and has been well-established in our laboratory(35,36). These dosages were transferred from the recommended dosages in humans based on body surface area, according to the FDA guidelines for clinical trials(37,38). A 0.75 mg/kg triazolam, 0.8 mg/kg biperiden and 0.1 mg/kg haloperidol dosage in rats is equivalent to ~7.5 mg, ~8 mg and ~1 mg in humans (60 kg body weight), respectively, all of which are within the recommended clinical dosages(35-38). Moreover, the dosages used in this study have been shown to be physiologically and behaviourally effective in rodents, without inducing EPS side-effects(35-37).

After 10-week administration, all animals were euthanised in a CO2-filled chamber. Brains were immediately removed and frozen in liquid nitrogen. All animals were sacrificed between 09:00 AM and 11:00 AM to minimise circadian-induced variation of protein expression.

Brain dissection. The discrete brain regions were collected using a brain microdissection puncture technique, which has been well-established(39). Specifically, based on the brain atlas(40), three sections through the forebrain (Bregma 3.30 to 4.02 mm) were dissected for the PFC; and three sections through the striatum (Bregma 1.00 to 2.20 mm) were dissected for the NAC and CPu, respectively. Dissected tissue was kept at ~80 °C for future use.

Western blots. Frozen brain samples were homogenised in homogenising buffer (9.8 ml NP-40 cell lysis buffer (Invitrogen, #FNN0021) mixed with 100 μl Protease Inhibitor Cocktail (Sigma-Aldrich, #P8340), 100 μl β-Glycerophosphate (Sigma-Aldrich, #G9422) and 33.3 μl phenylmethylsulfonyl fluoride (Sigma-Aldrich, #P7626). Protein concentration of each sample was measured by the DC Protein Assay (Bio-Rad, #500-0111). Each sample containing 10 μg of protein was denatured at 95 °C, and loaded into 4–20% Criterion™ TGX™ Precast Gels (Bio-rad, #5671095). The gels were run vertically in Criterion™ Vertical Electrophoresis Cell (Bio-rad, #1656001) until the proteins separated, followed by the electrophoretic transfer of the proteins to a polyvinylidene difluoride membrane in Criterion™™ Blotter (Bio-rad, #1704071). All membranes were then blocked by 5% skim milk powder and incubated in primary antibodies. Amersham HyperBlot ECL (GE Healthcare, #28-9068-36) and Luminata Classico Western HRP substrate (Millipore, #WBLUC0500) were used to visualise the immunoreactive bands. All Western blot experiments were performed in duplicate to ensure consistency.

The antibodies used in the present study to examine PKA activity were anti-PKA-C (1:1000; Santa Cruz, #SC-903) and anti-phosphor-PKA-C (Thr388) (1:1000; Santa Cruz; Cell Signalling, #9322). The antibodies used to examine the GSK3β-involved pathways were anti-Akt (1:2000; Cell Signalling, #4691), anti-phospho-Akt (Thr388) (1:1000; Cell Signalling, #9322), anti-GSK3β (1:2000; Cell Signalling, #9601), anti-phospho-GSK3β (Ser9) (1:1000; Cell Signalling, #9601), anti-DEV-3 (1:1000; Santa Cruz Biotechnology, #5C-8027), anti-β-catenin (1:1000; Santa Cruz Biotechnology, #SC-71963). The antibodies used to detect subunits of GABA<sub>A</sub> receptors were: anti-GABA<sub>A</sub>β3 (1:1000; Abcam, #ab154222), anti-GABA<sub>A</sub>β3-2 (1:1000; Abcam, #ab156000), and anti-GABA<sub>A</sub>β3-3 (1:1000; Abcam, #ab98968). The antibodies used to examine NMDAR were anti-NMDARI (1:2000, Abcam, #ab199182) and anti-NMDAR2A (1:500, Abcam, #ab124913). In addition, anti-CREB1 (1:2000, Abcam, #ab32515) and anti-phospho-CREB1 (1:2000, Abcam, #ab2096) were used to measure the activity of CREB1. Mouse anti-actin primary polyclonal antibody (1:10000; Millipore, #MAB1501) was used to visualise the immunoreactive bands. All Western blot experiments were performed in duplicate to ensure consistency.

Statistics. All data were analysed using SPSS Statistics V22.0 program. The immunoreactive signals were quantified using Bio-Rad Quantity One software. The data of each targeted protein were then corrected based on their corresponding actin levels. Data normal distribution was tested using histograms and a Kolmogorov-Smirnov Z test. For statistical evaluation, one-way analysis of variance (ANOVA) was performed if the data was normally distributed. The post-hoc Dunnett’s test was used to compare each drug treatment group with the control group. The results were normalised by taking the value of the control group as 100%. Pearson’s correlation test was used to analyse the relationships among the measurements. Statistical significance was accepted when p<0.05.

References


Competing financial interests: The authors declare no competing financial interests.

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CHAPTER 7 GENERAL DISCUSSION

7.1 Overall discussion

As discussed in earlier chapters, aripiprazole possesses favourable clinical effects and reduced side-effects (e.g. EPS), which have been attributed to its partial agonism for D2Rs (Di Sciascio and Riva, 2015). However, since all other D2R partial agonists either fail to achieve meaningful therapeutic effects and/or induce severe side-effects, the contribution of the D2R partial agonism to the unique effects of aripiprazole has been questioned. Therefore, it is important to understand the cellular mechanism of aripiprazole to validate the role of the D2R partial agonism in the clinical action of aripiprazole. This thesis investigated the effects of aripiprazole on the D2R-mediated PKA and Akt-GSK3β signalling pathways, as well as potential regulators of these two pathways, in the key brain regions related to the pathophysiology of schizophrenia and actions of antipsychotics, in comparison with bifeprunox (a D2R partial agonist) and haloperidol (a D2R antagonist). The current thesis is also the first study to investigate the effects of aripiprazole on these cellular signalling pathways by employing oral administration to mimic clinical treatment situation of aripiprazole. The following key outcomes have been achieved.

7.1.1 Effects of aripiprazole on various cellular pathways and regulators

The present studies have demonstrated that aripiprazole is able to modulate both the PKA and Akt-GSK3β signalling pathways, as well as the Dvl-GSK3β-β-catenin pathways, the GABA_A receptor and CREB1. Acute administration of aripiprazole increased phosphorylation of GSK3β in the PFC, CPu and NAc, without altering PKA
phosphorylation (Chapter 3). As reported in Chapters 4 and 5, 1-week administration of aripiprazole significantly increased the phosphorylation of PKA in the NAc, but, interestingly, reduced it in the CPU; the phosphorylation levels of GSK3β were elevated by aripiprazole administration in both the PFC and NAc. It is worth noting that the phosphorylation of Akt did not change in the acute and short-term study. Furthermore, 1-week administration of aripiprazole increased the expression of Dvl-3, β-catenin, GABA_Α (containing β-1) receptors, as well as total CREB, in the NAc. In the 10-week study (Chapter 6), the increased phosphorylation of GSK3β in the PFC and NAc persisted after chronic administration of aripiprazole. Additionally, long-term aripiprazole administration also elevated the expression of Dvl-3, β-catenin and GABA_Α receptors in the NAc.

7.1.1.1 Aripiprazole activates GSK3β-dependent signalling pathways

This thesis has shown that aripiprazole increased the phosphorylation of GSK3β in all acute, short- and long-term studies, but the phosphorylation of Akt was observed only in the chronic study (Chapter 6). Previous evidence has revealed that various antipsychotics with antagonism for the D_{2}R increase the phosphorylation of Akt with a concomitant increase in phosphorylated GSK3β (Beaulieu et al., 2004; Emamian et al., 2004) (aripiprazole was not examined). In addition, *in vitro* studies have reported that aripiprazole, unlike other antipsychotics, did not facilitate the D_{2L} receptor-mediated β-arrestin2-Akt-GSK3β signalling pathway (Klewe et al., 2008; Masri et al., 2008). Our study (Chapter 6) is the first study to provide *in vivo* evidence that aripiprazole is able to activate Akt-GSK3β signalling in the PFC and NAc. This finding suggests that activation of GSK3β signalling is associated with the actions of aripiprazole; and aripiprazole might display antagonism for the D_{2}R, even though it’s a D_{2}R partial
agonist. It is worth noting that only the 10-week study (Chapter 6) revealed increased Akt phosphorylation, which suggests that oral administration of aripiprazole, on current dosage, might require long-term use to stabilise its increase in Akt-GSK3β signalling.

Previous studies have indicated that antipsychotics (including haloperidol and clozapine) have various effects on the Dvl-GSK3β-β-catenin signalling pathway in vitro and in vivo (Alimohamad et al., 2005a; Alimohamad et al., 2005b; Sutton et al., 2007; Sutton and Rushlow, 2011). In addition, it has been reported that systematic administration of aripiprazole for 3 weeks was able to elevate the phosphorylation levels of GSK3β and the expression of β-catenin in the PFC and hippocampus (Park et al., 2011a; Seo et al., 2015). The present studies (Chapter 5 and 6) have demonstrated that aripiprazole has similar effects on the Dvl-GSK3β-β-catenin signalling pathway in the NAc, which suggests that regulation of the Dvl-GSK3β-β-catenin signalling pathway is very likely to be involved in the clinical effects of antipsychotics. However, a brain regional difference emerged between the present and previous studies (Park et al., 2011a; Seo et al., 2015). The exact reason for this brain regional difference is not clear. It might be because of the different treatment methods and dosages. For example, animals in Park’s study (2011a) and Seo’s study (2015) received daily injections of aripiprazole (1.5 mg/kg/day), while our studies employed oral administration (0.75 mg/kg/day). This issue needs to be further investigated.

7.1.1.2 The effects of aripiprazole on PKA signalling

This thesis has shown that aripiprazole had inconsistent effects on PKA signalling. In the acute study (Chapter 3), aripiprazole affected the expression of PKA, but not its phosphorylation levels; in the short-term study (Chapter 4), aripiprazole had significant
effects in altering the PKA phosphorylation in both NAc and CPu, whereas these effects were not observed in the chronic study (Chapter 6). These findings suggest that PKA signalling might be involved in the short-term actions of aripiprazole; however, PKA signalling, unlike Akt-GSK3β pathway, might not be associated with the long-term clinical effects of aripiprazole.

7.1.1.3 Aripiprazole up-regulates GABA_A receptors

Although none of the antipsychotics used in the present studies directly bind with GABA_A receptors, both short- and long-term administration of aripiprazole (Chapters 4 and 6) increased the expression of GABA_A (containing β-1) receptors in the NAc. Previously, only one image study showed that GABA_A receptor binding potential was reduced by aripiprazole administration in the left frontopolar cortex and right premotor cortex (Lee et al., 2013). Other antipsychotics in previous studies had discrepant effects on the binding density of GABA_A receptors (McLeod et al., 2008; Skilbeck et al., 2007; Zink et al., 2004). Our studies are the first studies to examine the effects of aripiprazole on the protein levels of GABA_A receptors in the brain. Both the current short- and long-term studies have shown similar effects of aripiprazole on GABA_A receptors, which confirms that up-regulation of GABA_A receptors is very like to be involved in the actions of aripiprazole.

It should be noted that aripiprazole has very low affinity for GABA_A receptors. Therefore, aripiprazole regulates GABA_A receptors via indirect pathway(s). One possible pathway is the D_2R downstream PKA signalling pathway (Connelly et al., 2013; Poisbeau et al., 1999). Our short-term study (Chapter 4) has revealed that the expression of GABA_A receptors in the NAc was increased by aripiprazole, paralleling
the increased phosphorylation of PKA. It is very interesting that in the chronic study (Chapter 6), the increasing effects of aripiprazole on GABA<sub>A</sub> receptors persisted, but PKA did not change. The exact reason is unknown. It might be because after long-term administration, the protein levels of GABA<sub>A</sub> receptors are stabilised, even without the alteration in PKA signalling. It is also possible that aripiprazole might regulate GABA<sub>A</sub> receptors in alternative manners. For example, aripiprazole can regulate the expression of glutamic acid decarboxylase 67 and GABA transporters (Peselmann et al., 2013), which might change the intercellular levels of GABA and subsequently affect GABA receptors.

7.1.1.4 Aripiprazole activates CREB1

The present data have demonstrated that aripiprazole affected CREB1 (Chapter 4 and 6). Previous studies have reported that various antipsychotics (including haloperidol, amisulpride, clozapine and olanzapine) were able to increase phosphorylation levels of CREB1 in vitro and in vivo (Jeon et al., 2015; Konradi and Heckers, 1995; Liang and Chuang, 2006; Park et al., 2011b; Pozzi et al., 2003; Yang et al., 2004). Therefore, the present study (Chapter 6) has confirmed the role of CREB1 in the actions of antipsychotics.

It should be noted that CREB1 can be regulated by both Akt-GSK3β and PKA signalling (Lonze and Ginty, 2002). Previous in vitro studies have indicated that CREB1 activity was increased by inhibition of GSK3β via the Akt-GSK3β pathway in cultured rat cerebral cortical neurons and SH-SY5Y cells (Liang and Chuang, 2006; Park et al., 2011b). As discussed in Chapters 4, 5 and 6, aripiprazole affects both GSK3β and CREB1 activity. In Chapter 6, the phosphorylation of GSK3β is positively
correlated with the phosphorylation of CREB1. Therefore, our studies support the proposition that antipsychotics might affect CREB1 via the Akt-GSK3β signalling pathway. On the other hand, the effects of antipsychotics on the PKA activity were observed only in the short-term study (Chapter 4). Further studies are necessary to validate whether aripiprazole affects CREB1 via PKA signalling (see Section 7.2 for recommended studies).

7.1.2 The time-dependent effects of aripiprazole

The short- and long-term studies have also shown that aripiprazole exerts its effects in a time-dependent manner. In the short-term study, aripiprazole and haloperidol increased phosphorylation of PKA (Chapter 4), but not Akt (Chapter 5), whereas, in the long-term study, aripiprazole induced Akt activation, but not PKA (Chapter 6). These findings suggest that PKA signalling is very likely to be involved in the short-term effects of aripiprazole, while Akt signalling is associated with the prolonged effects of aripiprazole. It should be noted that the up-regulation of Dvl-β-catenin, GABA_A receptors and CREB1 induced by aripiprazole appeared time-independent in both the short- and long-term studies. The exact reason is not clear. It is possible that antipsychotic drug administration not only induced the alterations in these pathways and regulators, it also caused adaptive changes in the synapses; therefore, even if aripiprazole changes its effects on Akt and PKA activity after chronic administration (probably due to adaptive changes in D_2Rs), the prolonged alterations in these pathways and regulators could be maintained due to the modified synapses.
7.1.3 A relatively low intrinsic activity for D₂Rs is essential for aripiprazole

The present studies have compared aripiprazole with bifeprunox and haloperidol. It has been demonstrated that aripiprazole had generally similar effects on Akt-GSK3β signalling, Dvl-GSK3β-β-catenin signalling, GABA_\text{A} receptors expression and CREB1 activity as haloperidol in the NAc in the short- and long-term studies. These findings suggest that aripiprazole is very likely to display similar antagonistic effects to inhibit D₂ receptor-downstream signalling in the NAc as haloperidol, even though it is a D₂R partial agonist. As discussed in the Section 2.3.4.1, among these three drugs, bifeprunox has the highest intrinsic activity for D₂Rs, higher than that of aripiprazole and haloperidol (intrinsic activity for long-form D₂Rs in vitro: dopamine vs. bifeprunox vs. aripiprazole vs. haloperidol = 100% vs. 95.1% vs. 86.0% vs. antagonist) (Tadori et al., 2007; Tadori et al., 2005) (Table 2-2). Since with the exception of aripiprazole, no other D₂R partial agonist has been successful in the treatment of schizophrenia, the present studies suggest that a relatively low intrinsic activity to achieve antagonistic effects on D₂Rs might be essential for a D₂R partial agonist to exert meaningful therapeutic effects in schizophrenia.

7.1.4 Proposed cellular mechanisms of aripiprazole in the nucleus accumbens

The present studies have indicated that aripiprazole has consistent effects on the PKA and Akt-GSK3β signalling pathways, as well as the Dvl-GSK3β-β-catenin signalling pathway, GABA_\text{A} receptors and CREB1 in the NAc. As discussed before, these cellular effects of aripiprazole might be attributed to its relatively low intrinsic activity for D₂Rs. A schematic diagram of proposed cellular mechanism of the actions of aripiprazole is
shown in Fig. 7-1. Due to its relatively low intrinsic activity, aripiprazole is able to antagonise D₂Rs (Fig. 7-1A), resulting in increased phosphorylation levels of Akt and Aripiprazole (relatively low intrinsic activity for D₂-like receptors) 

Fig. 7-1 A schematic diagram of the proposed cellular mechanisms of aripiprazole in the nucleus accumbens.

(A) aripiprazole antagonises D₂Rs due to its relatively low intrinsic activity for D₂Rs; (B) aripiprazole increases the phosphorylation of Akt and GSK3β (inhibiting GSK3β function), and the expression of Dvl-3 and β-catenin (elevating β-catenin function); (C) aripiprazole increases the expression of GABA_A (containing β-1) receptors probably via PKA signalling; (D) aripiprazole elevates the phosphorylation of CREB1 via Akt-GSK3β signalling and probably PKA signalling.

Abbreviations: Akt = protein kinase B; CREB1 = cAMP-responsive element-binding protein 1; Dvl = dishevelled; GABA = γ-aminobutyric acid; GSK3β = glycogen synthase kinase 3-beta; PKA = protein kinase A.

subsequent elevated phosphorylation levels of GSK3β, as well as up-regulation of the Dvl-GSK3β-β-catenin signalling pathway (Fig. 7-1B), which might contribute to the therapeutic effect of aripiprazole. In addition, by antagonising D₂Rs, aripiprazole increases the expression of GABA_A (containing β-1) receptors (Fig. 7-1C) probably via activating PKA signalling. Aripiprazole also elevates CREB1 activity probably via the
activation of GSK3β and PKA (Fig. 7-1D). Since dysfunction of the NAc is linked to
the positive symptoms of schizophrenia (Mikell et al., 2009), the current studies suggest
that the suppression of GSK3β functions (increased phosphorylation), activation of Dvl-
GSK3β-β-catenin, increased expression of GABA_A receptors and elevated activity of
CREB1 in the NAc are very likely to be involved in the therapeutic effects of
antipsychotics (probably on the positive symptoms of schizophrenia).

7.2 Recommendations for further research

The present studies have examined the effects of antipsychotics on various cellular
signalling pathways in the brain regions related to the pathophysiology of schizophrenia.
The results raised some important issues that need to be addressed. Suggested further
studies are as follows.

1. All of the current three antipsychotics have potent affinity for D_2Rs, however, it is
not clear whether these antipsychotics affected D_2Rs solely to exert these effects.
Therefore, further experiments are important to validate this issue, such as (1) using
specific D_2R antagonists (e.g. L-741,626 and raclopride) in combination with
antipsychotics to treat animals, and (2) using imaging technique (e.g. PET) to
investigate the binding properties of D_2Rs in various brain regions.

2. The transcription factor phosphorylation of relevant protein kinases (e.g. PKA, Akt,
GSK3β and CREB1) are also required to be evaluated in the future studies.

3. The present study (Chapter 4) suggests that antipsychotics might affect GABA_A
receptors via PKA signalling. To further support this finding, the pharmacological
PKA inhibitors (e.g. H-89 and KT-5720) may be used in the future studies to validate the role of PKA signalling in the actions of antipsychotics.

4. The current studies have used three drugs with different pharmacological profiles. The comparisons between these drugs were performed by evaluating the biomarkers only. Behavioural studies are required to validate whether these drugs are able to induce different behavioural changes corresponding to their pharmacological properties.

5. Knockout animals (e.g. GSK3β knockout, CREB Ser133Ala knockout animals) might also be used to confirm the effects of antipsychotics on the relevant signalling pathways and behavioural changes.

6. The current studies have employed only one dosage of aripiprazole. According to the FDA guidelines for clinical trials (FDA, 2005; Reagan-Shaw et al., 2008), a 0.75 mg/kg/day aripiprazole dosage in rats is equivalent to ~7.5 mg/day in humans (60 kg body weight) based on body surface area. The effective dosage for aripiprazole to treat schizophrenia ranges from 10 to 30 mg/day (Di Sciascio and Riva, 2015). Therefore, whether other dosages of aripiprazole induce different cellular effects is worth further investigations.

7. It is worth noting that aripiprazole has different effects from bifeprunox and haloperidol in the PFC and CPu. For example, acute administration of both aripiprazole and bifeprunox, but not haloperidol, increased GSK3β phosphorylation in the CPu (Chapter 3); in addition, 1-week administration of aripiprazole decreased
PKA phosphorylation in the CPu, whereas haloperidol increased it (Chapter 4); moreover, 10-week administration of both aripiprazole and bifeprunox, but not haloperidol, elevated Akt and GSK3β phosphorylation in the PFC (Chapter 6). These different effects of aripiprazole could not be explained simply by its low intrinsic activity for D2Rs. Instead, these findings suggest that aripiprazole might have selective effects on the PKA and Akt-GSK3β signalling pathways in different brain regions. Since in vitro studies have demonstrated the functional selectivity of aripiprazole at D2Rs (Kikuchi et al., 1995; Koener et al., 2012; Shapiro et al., 2003; Urban et al., 2007), further studies (particularly in vivo studies) are necessary to investigate the mechanisms of functional selectivity of aripiprazole. For example, employing biosensors based on fluorescence and bioluminescence resonance energy transfer to monitor the conformation changes of relevant receptors induced by aripiprazole (Stallaert et al., 2011); using animal model to investigate the relationship between the functionally selective effects of aripiprazole and its unique therapeutic effects (Kenakin, 2011).

8. Aripiprazole has partial agonism for 5-HT1A receptors and antagonism for 5-HT2A receptors, as well as affinity for many other receptors (e.g. 5-HT2C, H1 receptors) (Correll, 2010). It is unknown whether these receptors are involved in the effects on the signalling pathways and regulators examined in the present studies, and in the therapeutic actions of aripiprazole. Therefore, more specific studies are required to investigate the roles of these receptors in the actions of aripiprazole.

9. Lastly, the studies included in this thesis have examined the effects of antipsychotic drugs in healthy animals only. Since schizophrenia can induce structural and...
neuronal changes in the brains (McCarley et al., 1999; McGuire et al., 2008; Wible et al., 2001; Zhuo et al., 2014), antipsychotics might act differentially and exert different effects in the diseased brains from the healthy ones. It is, therefore, necessary to investigate the effects of these drugs in the animal models for schizophrenia and other mental disorders in future studies.

7.3 Conclusion

The present studies have investigated the molecular mechanisms of aripiprazole on the PKA, Akt-GSK3β, Dvl-GSK3β-β-catenin signalling pathways, as well as GABA_A receptors and CREB1. Aripiprazole, bifeprunox and haloperidol were injected in an acute study, and orally administered for three different time periods in short- and long-term studies. The effects of these antipsychotics in the key brain regions that are related to the actions of antipsychotics have been examined. The results have provided in vivo evidence that aripiprazole has time- and brain regional-dependent effects on the PKA and Akt-GSK3β signalling pathways, as well as the relevant Dvl-GSK3β-β-catenin signalling, GABA_A receptors and CREB1. These findings suggest that aripiprazole might exert its clinical effects via integrated effects of several signalling pathways and regulators.

On the other hand, the present studies have found that aripiprazole and haloperidol, but not bifeprunox, had some similar effects on the Dvl-GSK3β-β-catenin signalling, GABA_A receptors and CREB1 in the NAc, which suggests that aripiprazole and haloperidol might share common mechanisms in their actions at these signalling pathways and regulators. Therefore, by comparing aripiprazole with haloperidol, the present studies further suggest that a relatively low intrinsic activity for D_2R might be
essential for aripiprazole to perform its functions, and for the development of other potential D₂R partial agonists to achieve meaningful clinical effects.

Last but not least, the long-term study has revealed the adaptive changes in Akt-GSK3β, Dvl-GSK3β-β-catenin signalling, GABA_A receptors and CREB1 induced by chronic antipsychotic drugs administration. Since these pathways and regulators are closely associated with the pathophysiology of schizophrenia, these findings indicate that potential antipsychotics and treatments of schizophrenia may focus on these pathways and regulators to more effectively control symptoms of schizophrenia.
Supplementary 1  Statement from co-authors

This is to attest that the PhD candidate, Bo Pan, contributed significantly to the investigation (Pan, B., Chen, J., Lian, J., Huang, X.F., Deng, C. (2015). Unique Effects of Acute Aripiprazole Treatment on the Dopamine D₂ Receptor Downstream cAMP-PKA and Akt-GSK3β Signalling Pathways in Rats. PLoS One 10, e0132722. DOI: 10.1371/journal.pone.0132722): designed and performed the experimental work, analysed the data, interpreted results, and wrote the manuscript. The four co-authors are my PhD supervisors or my colleagues, who have provided comments on experimental design, data analysis, results interpretation and revision of manuscript, and/or technical support on experimental procedures.

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Appendix B – Chapter 4 supplementary

Supplementary 1 Statement from co-authors

This is to attest that the PhD candidate, Bo Pan, contributed significantly to the investigation (Pan, B., Lian, J., Huang, X.F., and Deng, C. (2016). Aripiprazole Increases the PKA Signalling and Expression of the GABA Receptor and CREB1 in the Nucleus Accumbens of Rats. *Journal of Molecular Neuroscience* DOI: 10.1007/s12031-016-0730-y): designed and performed the experimental work, analysed the data, interpreted results, and wrote the manuscript. The three co-authors are my PhD supervisors or my colleague, who have provided comments on experimental design, data analysis, results interpretation and revision of manuscript, and/or technical support on experimental procedures.

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Appendix C – Chapter 5 supplementary

Supplementary 1 Statement from co-authors

This is to attest that the PhD candidate, Bo Pan, contributed significantly to the investigation (Pan, B., Huang, X.F., Deng, C. (2016). Aripiprazole and Haloperidol Activate GSK3β-dependent Signalling Pathway Differentially in Various Brain Regions of Rats. *International Journal of Molecular Sciences* 17, 459, 1-11. DOI: 10.3390/ijms17040459): designed and performed the experimental work, analysed the data, interpreted results, and wrote the manuscript. The co-authors are my PhD supervisors, who have provided technical support on experimental procedures, and/or comments on experimental design, data analysis, results interpretation, and revision of manuscript.

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Appendix D – Chapter 6 supplementary

Supplementary 1  Statement from co-authors

This is to attest that the PhD candidate, Bo Pan, contributed significantly to the investigation (Pan, B., Huang, X.F., Deng, C. (2016). Chronic Administration of Aripiprazole Activates the GSK3β-dependent Signalling Pathways, and Up-regulates GABA_A Receptor Expression and CREB1 Activity in Rats. Scientific Reports 6, 30040. DOI: 10.1038/srep30040): designed and performed the experimental work, analysed the data, interpreted results, and wrote the manuscript. The co-authors are my PhD supervisors, who have provided comments on experimental design, data analysis, results interpretation, and revision of manuscript.

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Bo Pan
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