The Mechanism of D2R Overactivation in Neurite Impairment and Oxidative Stress

Peng Zheng

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The Mechanism of D2R Overactivation in Neurite Impairment and Oxidative Stress

PENG ZHENG

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This thesis is presented as part of the requirement for the conferral of the degree:
DOCTOR OF PHILOSOPHY

University of Wollongong
School of Medicine

August 2020
Abstract

Schizophrenia patients have altered neuronal connectivity, while the causal factor is not fully understood. Most antipsychotic drugs possess dopamine D2 receptor (D2R) antagonist property as a therapeutic target for reducing dopamine hyperactivity in schizophrenia. It is, however, not known whether the blockage of D2R is beneficial to neural connectivity. This study aimed to investigate the mechanisms of neurite lesion induced by D2R overactivation and prevention of such lesion by changing the D2R downstream signaling.

Disrupted in schizophrenia 1 (DISC1) is a genetic risk factor for a wide range of psychiatric illnesses, including schizophrenia. DISC1 is a multifunctional scaffold protein that regulates neurogenesis and neural development in the adult brain. The excessive D2R-DISC1 complex is observed in the post-mortem brain of schizophrenia patients. However, the role of D2R-DISC1 complex in neurite outgrowth is unknown. The aim of Chapter 2 was to study whether neurite lesion induced by D2R overactivation is through the D2R-DISC1 complex. This study applied fluorescence resonance energy transfer (FRET) technique to quantify the interaction between D2R and DISC1 in primary cortical neurons. D2R specific agonist quinpirole increased the interaction of D2R and DISC1 by over activating D2R in primary cortical neurons. Furthermore, the excessive D2R-DISC1 complex reduced glycogen synthase kinase β (GSK-3β) phosphorylation. The increased D2R-DISC1 complex formation in conjunction with the decreased GSK-3β phosphorylation resulted in neurite impairment of cortical neurons. The antipsychotics haloperidol and aripiprazole disrupted the excessive formation of the D2R-DISC1 complex caused by D2R overactivation. However, only aripiprazole
could reverse the downregulation of phosphorylated GSK3β caused by quinpirole. Aripiprazole displayed better preventative effect than haloperidol on neurite lesion induced by quinpirole, suggesting that aripiprazole and haloperidol may affect neuroplasticity via different signaling pathways. Also, both haloperidol and aripiprazole failed to rescue neurite lesion of primary cortical neurons from DISC1 mutant mice. In summary, the normal D2R-DISC1 complex signaling is critical for neurite outgrowth.

Based on the results that the excessive D2R-DISC1 complex inhibited neurite outgrowth, Chapter 3 aimed to determine whether and how the disruption of the D2R-DISC1 complex affect the dendritic spines. This part of study applied a cell-penetrating peptide (TAT-D2pep), which could specifically disrupt the interaction of D2R and DISC1 without blocking D2R itself. The super-resolution images showed that the D2R-DISC1 complex existed in dendritic spines, neurites, and soma of cultured striatal neurons. TAT-D2pep restored neurite length and spine density to the normal level by separating the excessive D2R-DISC1 complex in neurite and dendritic spines. In addition, this study revealed that uncoupling excessive D2R-DISC1 complex could prevent neurite impairment via increasing the expression of Neuropeptide-Y (NPY) in striatal interneurons. Taken together, this study indicates that TAT-D2pep prevents the synaptic loss caused by the D2R overactivation, suggesting that this cell-penetrating peptide is effective in treating cognitive deficits in schizophrenia.

Oxidative stress is reported in the first episode of drug-naïve schizophrenia patients. Although D2R hyperfunction is recognized in schizophrenia pathology, the relationship between D2R overactivation and oxidative stress is unknown. Therefore, Chapter 4 aimed to investigate whether and how D2R overactivation
affects mitochondrial reactive oxygen species (ROS) and ROS-mediated mitophagy. Firstly, cryo-electron tomography (Cryo-ET) demonstrated that D2R overactivation induced by quinpirole damaged cristae in mitochondria of cultured striatal neurons. Notably, this study for the first time identifies D2R located on neuronal mitochondria. Furthermore, overactivation of the membrane and mitochondrial D2R exacerbated oxidative stress with increased ROS and malondialdehyde (MDA). In addition, D2R overactivation caused incomplete mitophagy. This study revealed that D2R overactivation could induce oxidative stress and incomplete mitophagy.

In conclusion, these studies suggest that the neurite lesion induced D2R overactivation is associated with excessive D2R-DISC1 complex formation and oxidative stress. Thus, this study provides potential therapeutic targets for alleviating neurite deficits in schizophrenia.
Acknowledgments

I would like to express my heartfelt gratitude to Professor Xu-Feng Huang for giving me the opportunity to pursue what I am interested in. His patient guidance helped me to shape critical thinking skills, a rigorous attitude towards science, and academic writing skills. His constant support and encouragement also gave me the courage to overcome difficulties during my Ph.D. I would also like to thank my co-supervisor Dr. Yinghua Yu, who has dedicated her time to provide me detailed experimental design. She gave me great support on animal study and Cryo-EM project, which brought my research to a new level.

Furthermore, I would like to thank the China Scholarship Council and the University of Wollongong for years of financial support, travel grants.

I would like to thank Professor Dayong Jin and his fellow Dr. Qian Peter Su in School of Mathematical and Physical Sciences Core Member in the University of Technology Sydney, for their contribution to resolve protein interaction within spines by super-resolution microscopy. I would also like to thank Dr. Juanfang Ruan in the Electron Microscope Unit of University of New Sales Wales gave me great support in Cryo-EM data analysis.

My special appreciation to our lab manager Mrs. Hongqin Wang for your great supports and guidance not only on my laboratory work but also campus life during my candidature. I also hope to thank Ms. Maria Catacouzinos, Ms. Carley Mottley, and Mr. Josh Snow for your advice and help through my animal maintenance and care; Dr. Qingsheng Zhang and Dr. Tiantian Jin and Dr. Jeremy Lum for your great advice for my laboratory techniques.

Lastly, I would like to thank my father, mother, my wife Zhizhen and my son Jihang. Each one of you has played such a crucial role in my life by loving me,
taking care of me and encouraging me. You give me hope in my times of trial, joy in my difficult periods. I will always be grateful that I have you in my life.
Certification

I, PENG ZHENG, declare that this thesis submitted in fulfilment of the requirements for the conferral of the degree Doctor of Philosophy, from the University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. This document has not been submitted for qualifications at any other academic institution.

PENG ZHENG

<<4th August 2020>>
Statements

In accordance with the University of Wollongong thesis committee ‘Guideline for Preparation and Submission of HDR Thesis (June 2020), this Ph.D. thesis is presented in ‘Journal Article Style’. It is comprised of a series of original studies published in peer-reviewed journals, of which I am the first author. I hereby declare that I am the primary designer of these studies, have carried out experimental procedures, data analysis, and manuscript preparation.

Peng Zheng
2020

I consent to the preparation of this Ph.D. in ‘Journal Article Style’ and I acknowledge the above statement pertaining to student contribution to be correct.

Prof. Xu-Feng Huang, Supervisor

Dr. Yinghua Yu, Supervisor

2020

2020
Publication

Research Articles:


**Peng Zheng** and Xu-Feng Huang “Activation of Mitochondrial Dopamine D2 Receptor Inhibits Mitophagy and Causes Schizophrenia-like Behaviors in Mice” (Ready for Submission).


**Conference Papers:**


List of Abbreviations

**D2R**: dopamine D2 receptor  
**DISC1**: disrupted in schizophrenia 1  
**Akt**: protein kinase B  
**GSK3β**: glycogen synthase kinase 3 beta  
**FRET**: fluorescence resonance energy transfer  
**WT**: wild type  
**DISC1-LI**: DISC1 locus impairment  
**MAP-2**: microtubule-associated protein 2  
**PSD-95**: postsynaptic density protein 95  
**CaMKII**: Ca$^{2+}$/calmodulin-dependent protein kinase II  
**PBS**: phosphate buffered saline  
**SDS-PAGE**: sodium dodecyl sulphate polyacrylamide gel  
**STORM**: stochastic optical reconstruction microscopy  
**NPY**: Neuropeptide Y  
**TAT**: trans-activating transcriptional activator  
**ROS**: reactive oxygen species  
**Cryo-EM**: cryogenic electron microscopy  
**MDA**: malondialdehyde  
**GPCR**: G-protein coupled receptors  
**ANOVA**: one-way analysis of variance
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Chapter One

1.1 General Introduction

Schizophrenia is a devastating mental illness that influences thought and behavior (Howes et al., 2016). Neuroimaging findings have revealed that neurite deficits are associated with cognitive symptoms in schizophrenia (Rae et al., 2017). The hyperactivity of striatal D2R is predominantly responsible for psychosis in schizophrenia, which leads the current antipsychotics to block D2R (Howes and Kapur, 2009). However, the clinicians and researchers raise concerns about the potential risks of antipsychotic side-effects due to the complete blockade of D2R. Thus, there is a high urgency to understand the mechanism of D2R overactivation induced neurite deficits. Although cAMP-dependent and β-arrestin independent signaling pathways of D2R have been acknowledged as the principal downstream, the exact mechanism is still largely unknown, especially for several identified D2R-heterocomplex. Furthermore, oxidative stress has recently been determined in the first episode of drug-naïve schizophrenia patients with cognitive deficits (Solberg et al., 2019, Xie et al., 2019). Therefore, D2R overactivation may regulate different signaling pathways to affect neuroplasticity in the brain. The present series of Ph.D. studies are designed to elucidate the mechanism of D2R overactivation in neurite impairment and then discover proper atypical antipsychotics and cell-penetrating peptides to prevent neurite lesions. A better understanding of these mechanisms will help the development of new antipsychotic drugs to ameliorate neurite impairment and oxidative stress with fewer side-effects for schizophrenia patients.

1.2 Literature Review
1.2.1 Schizophrenia

Schizophrenia is a severe brain disorder that significantly impairs normal brain function and the patients' response to reality (Karl and Arnold, 2014). There are approximately 20 million people worldwide suffering schizophrenia at present (Disease et al., 2018). Schizophrenia usually occurs in adolescent period and lasts for a lifetime (Gomes et al., 2016). The symptoms of schizophrenia consisting of positive symptoms such as delusions, hallucinations, negative symptoms such as emotional flattening, social isolation, and cognitive symptoms such as learning difficulties (Mueser et al., 2013). The actual pathology of schizophrenia remains to be elucidated. Antipsychotic drugs are primary medications for treating schizophrenia and other psychiatric disorders. If the patients suffer the first episode of schizophrenia, the antipsychotics might treat them effectively (Girgis et al., 2011). However, the absence of response to antipsychotics in certain patients and their various side-effects, urging researchers to search for a better treatment for schizophrenia.

1.2.2 D2R and Schizophrenia

The onset of psychotic symptoms is strongly associated with alterations in dopamine function (Kesby et al., 2018). For healthy people, psychotic symptoms can be induced by dopamine stimulants such as amphetamine. Positron emission tomography findings report that elevated dopamine synthesis capacity and release are observed in the striatum of schizophrenia patients compared with healthy subjects (Howes et al., 2009). Alterations in dopaminergic function within the brain are considered as a critical factor of psychotic symptoms (Winton-Brown et al., 2014). Thus, striatal D2R antagonism is a general pharmacodynamic property of all current antipsychotics, which further supports that D2R hyperactivity is a core
target for schizophrenia treatment (Urs et al., 2017). It is revealed that most antipsychotics bind to D2R, but not D1R, and that blocking D2R is enough to inhibit hyperdopaminergic activity (Beaulieu and Gainetdinov, 2011). Clinical studies have confirmed that hyperdopaminergic activity occurs before the onset of psychosis in schizophrenia, suggesting that the dysfunction of the dopaminergic system is not the consequence of antipsychotic exposure or psychosis (Brisch et al., 2014). Further, the occupancy of D2R above 80% could not only increase the risk of cognitive decline but also extrapyramidal side effects (Sakurai et al., 2013). Antipsychotic drug can cause extrapyramidal syndrome (EPS) including dystonia, parkinsonism, and tardive dyskinesia. Thus, the evidence strongly supports that D2R activity plays an essential role in psychosis.

1.2.3 D2R and Its Intracellular Signaling

The neurotransmitter dopamine belongs to the catecholamine family, having various critical functional roles in humans, such as the regulation of voluntary motor activity, cognition, and reward pathways (Kapur and Marques, 2016). The effects of dopamine are mediated through five subtypes of dopamine receptors, D1 to D5. The dopamine D1-like receptor subtypes include the dopamine D1 and D5 receptors, whereas the D2-like receptor subtypes consist of dopamine D2, D3, and D4 receptors (Rangel-Barajas et al., 2015). Generally, the D1R and D2R distribute more widely than the D3R, D4R, and D5R in the brain (Hurley and Jenner, 2006, De Mei et al., 2009). There are two major D2R alternatively spliced isoforms, D2R-long isoform (D2RL) and D2R-short isoform (D2RS), which are distinguished by the additional 29 amino acids of the third intracellular loop (ICL3) (Giros et al., 1989). It has been reported that D2RL majorly represents in postsynapse, while D2RS expresses in pre-synapse and is associated with auto-receptor functions (De
Mei et al., 2009). The D2R is richly expressed in the striatum, nucleus accumbens, and frontal cortex. Further, D2R is also shown in the ventral tegmental area, hypothalamus, and hippocampus (Seeman, 2006).

D2R is a G protein-coupled receptor (GPCR) that activates both G protein-dependent signaling and non-G protein cascades (Beaulieu et al., 2009). Activation of the D2R can inhibit the activity of adenylyl cyclase after it couples to Gαi proteins (Bergson et al., 2003). D2R is also demonstrated to modulate other second messengers, including inositol phospholipid, arachidonic acid, potassium, and calcium currents (Missale et al., 1998). Notably, D2R can function not only through cAMP levels but also through the noncanonical Akt-GSK3 signaling cascade triggering by recruiting β-arrestin proteins (Beaulieu et al., 2005, Beaulieu et al., 2007). Thus, it is crucial to study which one of D2R signaling pathways is targeted by antipsychotic drugs.

1.2.4 Dual Role of D2R in Neurite Outgrowth

Neuronal development involves morphological alterations of neurite to become functional axons and dendrites (Ronan et al., 2013). Dopamine and its D2R are associated with neuronal development. Researchers started to investigate the effects of D2R activation on neurite outgrowth from dopamine. Decades ago, dopamine was firstly reported to elongate neurite through activating D2R in embryonic rat cortical neurons (Reinoso et al., 1996). Later, some researchers performed studies to investigate the outcomes of modulating D2R activity on neuronal morphology in vitro and in vivo. Cortical neuroplasticity is found to be another target of antipsychotics by blocking D2R activity (Rangel-Barajas et al., 2015). Todd et al. firstly reported treatment with 10 to 1000 nM quinpirole resulted in small increases in the average length of neurites in primary cortical neurons. The effects on
branching did not reach statistical significance. These two morphogenic effects were blocked by D2R antagonists eticlopride and spiperone, suggesting a low concentration of quinpirole promotes neurite growth via D2R (Todd, 1992). Following this critical finding, Todd's group transfected D2-like receptors (D2, D3 and D4) into the dopaminergic mesencephalic cell line MN9D, which confirmed the role of D2R overexpression in inhibiting neurite outgrowth (Swarzenski et al., 1994). At that time, psychiatric disorders are considered that the dopaminergic pathways are overactive (Seeman, 1987). Thus, researchers are interested in whether D2R overactivation will alter the neuronal morphology. Reinoso et al. reported that quinpirole at 1 µM promotes neurite outgrowth, which is consistent with previous findings. However, they also firstly demonstrated that quinpirole at 100 µM significantly inhibited neurite length and branch of cultured rat cortical neurons (Reinoso et al., 1996). Previous researches suggest that D2R plays a role in neuronal differentiation and neurogenesis. Combined with previous clinical research, this finding links the D2R overactivation to neuronal impairment and cognitive decline in psychiatric disorders. Therefore, this thesis aimed to investigate the mechanism underlying neuronal impairment caused by D2R overactivation.

1.2.5 Overactivation of D2R Causes Synaptic Loss

The synaptic reduction is the critical pathology in schizophrenia. The two well-established observations support this opinion. First, the progressive loss of gray matter was observed in patients at the time of disease onset (Vita et al., 2012). Second, significant reductions in the density of dendritic spines on pyramidal neurons of schizophrenia patients were observed (Moyer et al., 2015). A recent study revealed that dendritic spines with smaller volumes were significantly lost in
the post-mortem brain of schizophrenia patients, suggesting the inhibition of synaptogenesis exists in schizophrenia (MacDonald et al., 2017). Importantly, previous findings demonstrated that the synaptic loss in the prefrontal cortex is not the consequence of treatment with antipsychotic medications (Glantz and Lewis, 2000). Animal studies offer \textit{in vivo} evidence to reveal the importance of D2R in regulating dendritic spines. Chronic treatment with a selective D2R agonist PPHT decreased the spine density of cortical pyramidal neurons without affecting GABAergic interneurons. D2R may directly mediate the inhibition of dendritic spines by PPHT existed in pyramidal neurons, but also indirectly through D2R of interneurons (Castillo-Gomez et al., 2016). However, another study shows that quinpirole increased the dendritic spine density in hippocampal neurons, suggesting that the effects of D2R on the dendritic spine could also be concentration-dependent. Jia et al. thoroughly investigated the function of D2R in dendritic spine morphogenesis by using pharmacologic and genetic approaches (Jia et al., 2013). The dendritic spine density of hippocampal neurons was significantly reduced after male mice were intraperitoneally injected with D2R agonists quinpirole and bromocriptine. Continuous injection of eticlopride remarkably enhanced the spine density, whereas the single injection failed to do so. Interestingly, D1R is not involved in regulating hippocampal spine development. Besides, overexpression of D2R reduced the spine density of CA1 hippocampal neurons, whereas knockdown of D2R increased it (Jia et al., 2013).

It is believed that the abnormal neurodevelopment in schizophrenia can lead to improper function of synaptic communication (Harrison and Weinberger, 2005). A variety of proteins exists in synapses, which have been used as biomarkers of synaptic connection. Synaptophysin is widely used as the marker of presynapse,
which can also indicate neuronal synaptic density (Okabe et al., 2001). The expression of synaptophysin in the cortex is significantly declined in patients with schizophrenia (Osimo et al., 2019). PSD-95 is abundant in the brain and concentrated in the postsynaptic density. In human post-mortem studies, PSD-95 expressed at a relatively low level in brain regions, which may be associated with cognitive decline in schizophrenia (de Bartolomeis et al., 2014). Another post-mortem study reported that adolescent schizophrenia patients had reduced expression of PSD-95 protein in the thalamus and hippocampus (Clinton and Meador-Woodruff, 2004, Toro and Deakin, 2005). PSD-95 was reported to interact with other synaptic proteins, such as DISC1, in the development of excitatory synapses as well as in regulating synaptic strength and plasticity (Seshadri et al., 2015). Taken together, it is important to investigate the mechanisms underlying the inhibitory effects of D2R overactivation on synapse in this thesis.

1.2.6 Overactivation of D2R Causes Cognitive Decline

The neurocognitive decline is a core feature in schizophrenia patients (Simpson et al., 2010). Accumulating evidence has shown that the dopaminergic system in the central nervous is profoundly associated with cognition. Using single-photon computerized emission tomography, Abi-Dargham et al. found that the increased occupancy of striatal D2 receptors by dopamine occurs in first-episode drug-naive patients. Notably, the occupancy of striatal D2R is not statistically different between drug-naïve and previously treated patients (Kegeles et al., 2008). Thus, the increased activation of D2 receptors by dopamine observed in schizophrenia patients is present at the onset of illness and not a consequence of antipsychotic treatment. A recent study reported that decreased D2R occupancy by aripiprazole intervention contributed to cognitive improvement in schizophrenia patients (Shin
et al., 2018). To further investigate the role of D2R in specific aspects of cognition, a murine model of schizophrenia was generated featuring with D2R overexpression. Mice overexpressing D2R in the striatum displayed a significant deficit in working memory instead of a general cognitive deficit (Kellendonk et al., 2006). However, whether or not there is an impaired working memory caused by D2R overactivation in adolescence is still unknown.

1.2.7 DISC1 Regulates Neurodevelopment

Among all potential candidate genes, DISC1 is considered as one of the most convincing risk factors for major mental disorders. Initial evidence for the involvement of the DISC1 gene in schizophrenia is mainly based on a large Scottish pedigree where the gene is disrupted by a chromosomal translocation (Millar et al., 2000). There are two remarkable expression peaks of DISC1 during neurogenesis in the developing mouse brain, suggesting that its crucial role in neurodevelopment (Schurov et al., 2004). The pathology of DISC1-related psychiatric disorders is because of an altered interaction between DISC1 and other proteins. Therefore, identifying the novel interactors with DISC1 will be of great importance in understanding the therapeutic target for treating schizophrenia.

The researchers have demonstrated that DISC1 plays an essential role in neuronal development and maturation by utilizing the DISC1 mutation mice model. In the DISC1 transgenic model, mice have shown schizophrenia-like phenotypes, including deliberated neuronal morphology, lowered neuron density, diminished neurogenesis, and reduced striatal volumes (Clapcote et al., 2007, Lee et al., 2011). In human brains, DISC1 has a robust modulatory effect on cortical development and increases the thickness of the prefrontal cortex (Carless et al., 2011). Seeman proposed that D2R is in a high-affinity state (D2R^{high}) in schizophrenia, suggesting
the activity of D2R could be the target for treatment instead of blocking the D2R (Seeman et al., 2006). Results from the animal research exhibited that DISC1 can increase the proportion of D2R\textsuperscript{high} in the striatum, which led to the status of dopamine hyperactivity (Jaaro-Peled et al., 2013). In addition, it was reported that DISC1 dysfunction increased dopamine receptors in the striatum without altering the dopamine level in the DISC1-L100P mutant (Clapcote et al., 2007).

DISC1 contributes to the proliferation of neural progenitors through the interaction with GSK3 and, in turn, regulation of β-catenin activity (Mao et al., 2009, Singh et al., 2011). DISC1 directly inhibited GSK3β activity, which prevented the phosphorylation and degradation of β-catenin (Mao et al., 2009). Furthermore, knockdown of DISC1 inhibited progenitor proliferation, which is likely through exiting the premature cell cycle. These effects could be restored by overexpression of β-catenin or reducing GSK3β activity (Mao et al., 2009, Singh et al., 2011).

DISC1 is mainly located in dendritic spines in which relates to the synaptic architecture (Hayashi-Takagi et al., 2010). In the mature mouse brain, DISC1 determines the development of functional synapses and the rate of transduction of intrinsic excitability (Kim et al., 2012). Besides, DISC1 mice with point mutant on Gln31Leu and Leu100Pro showed a significantly decreased spine density (Lipina et al., 2013), indicating that synapse alternation may link to their behavioral phenotypes. This study aimed to reveal how the DISC1 would interact with its associated proteins in the synapse to regulate dendritic spine formation.

1.2.8 A Novel Antipsychotic Target: D2R-DISC1 Complex

Accumulating data indicate that D2R is a hub receptor that interacts not only with many G protein-coupled receptors but also with scaffolding proteins (Bradshaw
and Porteous, 2012). One emerging new concept in neuropsychopharmacology is a
dysfunction of D2R complexes contributing to the development of schizophrenia.
The role of D2R in schizophrenia pathology has altered with the discovery of
various D2R complexes in the brain. These D2R complexes may offer novel targets
for antipsychotic drugs and provide better therapeutic outcomes.

Recent research showed the hyperdopaminergic status induces an increase of D2R-
DISC1 complex, and this complex is also found in the post-mortem brain from
schizophrenia patients and mouse models of schizophrenia (Su et al., 2014). Based
on these findings, a new compound targeted on interfering D2R-DISC1 interaction
with less antipsychotic-like side effects is synthesized (Su et al., 2014). The
achievement of antipsychotic-like outcomes only needs interrupting the interaction
between D2R and DISC1 without blocking D2R completely. The D2R-DISC1
complex is an important finding because it narrows down the targets of
antipsychotics, which may avoid side-effects. However, whether the D2R-DISC1
complex plays a role in neuroplasticity is unclear, which will be investigated in this
study. Furthermore, it will be essential to identify the location of the D2R-DISC1
complex in neurons by using high-resolution microscopy. Dysfunction of the
scaffolding protein can cause alternations of protein complexes. Thus, this study
also utilizes DISC1-L1 mice, which diminishes the D2R-DISC1 binding site, to
elucidate the role of DISC1 in neuroprotection.
Figure 1.1 Diagram of the human D2R sequence. Sequence highlighted with red represents D2R-DISC1 interacting site (211aa-225aa). TAT-D2pep will bind to the D2R-DISC1 interacting site to separate the D2R-DISC1 complex. Sequence highlighted with blue represents the difference between D2R long isoform and D2R short isoform. It illustrates that both D2R long isoform and D2R short isoform can interact with DISC1. Abbreviation: ECL: extracellular loop; ICL: intracellular loop; TM: transmembrane.
1.2.9 GSK-3 Converges D2R and DISC1 Intracellular Signaling Pathway

Several studies have implicated that GSK-3β is associated with the symptoms of neuropsychiatric disorders like schizophrenia (Lovestone et al., 2007). The phosphorylation of GSK-3β on serine 9 indicates the inhibited activity of GSK-3 (Grimes and Jope, 2001). Recent studies have shown that not only atypical antipsychotics but also typical antipsychotics increases the phosphorylation of GSK-3α and GSK-3β by either activating Akt or mimic Akt activity (Li and Xu, 2007, Park et al., 2011a). It is indicated that cAMP levels in neurons do not regulate the effect of D2R on GSK-3β activity. Instead, it is modulated by a multifunctional scaffolding protein β-arrestin, which is known to be responsible for G protein-coupled receptor internalization (Beaulieu et al., 2007). The D1R agonist SKF38393 does not alter GSK-3β phosphorylation at serine 9, suggesting that GSK-3β phosphorylation may be regulated explicitly by D2R (Lebel et al., 2009).

The regulation of D2R on GSK-3 is further determined by using D2R knockout mice. The absence of hippocampal D2R leads to neuronal apoptosis by increasing the activity of GSK-3β in the mice (Tripathi et al., 2010). D2R knockout mice showed delays in movement initiation and inhibition in total locomotor activity, implicating that the abnormal behavior induced by D2R may arise through GSK-3 activation (Kelly et al., 1998). There were no significant increases in phosphorylated CaMKIIα in neurons of nucleus accumbens shell and caudate nucleus of D2 knockout mice, suggesting that calcium signaling cascade was not involved in D2R signaling pathway (Rashid et al., 2007).

DISC1 is shown to determine the proliferation and fate of neural progenitors through interaction with GSK-3 and regulation of β-catenin activity (Mao et al.,
DISC1 directly inhibits GSK-3β activity, which prevents the phosphorylation and degradation of β-catenin (Mao et al., 2009). Knocking down DISC1 gene expression inhibits neuronal progenitor proliferation while over-expression of DISC1 rescues neurodevelopment in the developing cortex (Niwa et al., 2010). The inhibited neuronal proliferation can also be restored by over-expression of β-catenin or inhibiting GSK-3β activity (Mao et al., 2009, Singh et al., 2011). Furthermore, reduced progenitor proliferation is possibly due to exiting the premature cell cycle. In addition, the enhanced occupancy of D2R was observed in the striatum of DISC1 mutant (dominant-negative) mice, suggesting that the activation of GSK-3β induced by dysfunctional DISC1 may be through D2R overactivation (Jaaro-Peled et al., 2013).

Although GSK-3β knockout mice could die in utero, GSK-3β heterozygous knockout mice are viable and display decreases in exploratory activity and reduced locomotor activity to amphetamine (Urs et al., 2012). GSK-3α knockout mice are viable and exhibited a reduction in locomotion to a new environment and impaired sensorimotor gating (Kaidanovich-Beilin et al., 2009). Because GSK3β signaling is vital in promoting neurite outgrowth and activated GSK3β is associated with the development of psychosis, it is assumed that the GSK3β signaling pathway is involved in the D2R-DISC1 complex intracellular signaling (Beurel et al., 2015).

1.2.10 NPY Modulates Neurite Outgrowth

Neuropeptide Y (NPY) is a vital neuromodulator which is richly existed in the central nervous system (Gotzsche and Woldbye, 2016). More recently, we addressed the question of whether NPY is involved in the haloperidol-induced neuronal impairment. In our laboratory, it was found that the downregulation of NPY caused by haloperidol results in dendritic spine loss and neuronal damage in
primary striatal neurons. The short-chain fatty acid propionate protected against neuronal lesions by upregulating the NPY level (Hu et al., 2018). Treatment with quinpirole or amphetamine decreased NPY expression by overactivation of D2R in mice (Kuo, 2003). Previously, Hansel et al. revealed that NPY is identified in the immature neurons and precursor cells of the rat brain, suggesting its function in neurogenesis (Hansel et al., 2001). NPY-deficient mice contain about 25% fewer neurons than wild type mice, indicating loss of NPY in vivo inhibits the development of neuronal progenitors (Hansel et al., 2001). Accumulating pieces of evidence regarding the study of striatal NPY indicated that the existence of a possible reciprocal relationship between D2R and NPY. This study aimed to investigate the effect of D2R-DISC1 complexes on NPY.

1.2.11 Mechanism of Haloperidol on Neurite Impairment

Antipsychotic drug modulates dysfunction in neurotransmission, which plays an important role in reducing the symptoms of schizophrenia (Huang and Song, 2019). Substantial evidence has suggested that neuronal plasticity exhibited by neurite outgrowth and neuroprotection underlies the therapeutic effects of atypical antipsychotic drugs (Lieberman et al., 2008, Molteni et al., 2009). As a representative first-generation antipsychotic drug (FGA), haloperidol antagonizes D2R to reduce positive symptoms with little effect on cognitive improvement (Harvey et al., 2005). Further, the neurotoxic effects of haloperidol have been reported to cause side effects, including extrapyramidal symptoms and tardive dyskinesia (Rasmussen et al., 2017). The blockade of striatal D2R is believed to be responsible for haloperidol drawbacks (Kapur et al., 2000). So far, the effect of neurotoxicity of haloperidol has been compared with several second-generation antipsychotic drugs. Haloperidol has neurotoxicity in SH-SY5Y cells by increasing
caspase-3 activity (Gasso et al., 2012). Our group recently reported that haloperidol impaired neurite growth of human SH-SY5Y neurons and primary striatal neurons through down-regulating NPY expression (Hu et al., 2018). Notably, we found that haloperidol prevented the reduction of neurite length induced by D2R overactivation but no change in the number of branching in primary cortical neurons. Haloperidol had no significant effects on reversing synaptophysin and PSD-95 expression in the cortical neurons treated with quinpirole (Zheng et al., 2018). It has been demonstrated that haloperidol had no effects on promoting neurite growth and upregulating synaptic proteins compared with amisulpride, which is an atypical antipsychotic drug. They demonstrated that haloperidol failed to protect neuronal morphology mainly because it does alter BDNF expression and GSK-3β phosphorylation in human neuroblastoma cells (Park et al., 2011b). Other researchers found that haloperidol decreases the synaptophysin expression in the rat hippocampus (Lieberman et al., 2008). However, the expression of synaptophysin was not significantly changed in the primate cerebral cortex (Lidow et al., 2001). Thus, it is imperative to investigate why haloperidol cannot protect neurite and improve cognition in this thesis.

1.2.12 Mechanism of Aripiprazole on Neuronal Protection

Binding to D2R is considered as a necessary action for current antipsychotic drugs (Kapur et al., 2000). However, there is a clear distinction in the D2R binding affinity between FGAs and second-generation antipsychotics (SGAs). FGAs majorly antagonize D2R, while SGAs can bind to other membrane receptors with less D2R occupancy (Lieberman et al., 2008). Moreover, the pharmacological findings of aripiprazole have changed the understanding of antipsychotic action on dopamine receptors. Research on aripiprazole provides a clinically relevant
mechanism according to D2R occupancy instead of the D2R blockade. Depending on endogenous dopamine levels, aripiprazole may act as a full antagonist or a partial agonist on D2R (de Bartolomeis et al., 2015). Unlike FGAs, aripiprazole is effective in reducing positive symptoms and improving cognitive function (Kumar et al., 2017). More importantly, aripiprazole may pose a lower risk of extrapyramidal symptoms and relapse (Kane et al., 2007, Kane et al., 2002). Compared with haloperidol, aripiprazole is clinically proven to have superior efficacy and safety in the treatment of early-stage schizophrenia (Girgis et al., 2011). Besides, several pharmacological studies have revealed that aripiprazole possesses neuroprotective features. We report that aripiprazole ameliorates neurite impairment caused by D2R overactivation in cultured cortical neurons (Zheng et al., 2018). Ishima et al. also found that aripiprazole potentiates NGF-induced neurite outgrowth of PC 12 cells (Ishima et al., 2012). Similar to aripiprazole, another D2R partial agonist brexpiprazole is also reported to promote neurite outgrowth in conjunction with NGF in PC 12 cells, suggesting that the property of agonism could contribute to neuroplasticity (Ishima et al., 2015). Aripiprazole protects dopaminergic neurons against glutamate cytotoxicity, which can be blocked by D2R antagonist sulpiride (Matsuo et al., 2010). Aripiprazole is also reported to prevent synaptic loss, which is associated with the treatment of pathology of schizophrenia (Glausier and Lewis 2013). Compared with haloperidol treatment, the administration of aripiprazole significantly increases dendritic spine density with a rise in postsynaptic puncta (Takaki et al., 2018). Several pieces of research have contemporarily highlighted that the neuroprotective effect of aripiprazole is mediated by Akt-GSK3 signaling cascade (Pan et al., 2016, Takaki et al., 2018, Park et al., 2009). Although some atypical antipsychotic drugs, like
olanzapine or ziprasidone, have been revealed to promote neurite outgrowth through upregulating synaptophysin and PSD-95 expression, the investigation of aripiprazole on these synaptic proteins has not yet been performed. Since aripiprazole acts as a partial agonist of D2R, it is necessary to investigate whether the D2R-DISC1 complex and its downstream signaling pathway are affected by aripiprazole.

1.2.13 Super-resolution Technique to Reveal Synaptic Composition

Stochastic Optical Reconstruction Microscopy (STORM) is one of the most commonly used types of super-resolution microscopy. STORM has made it possible to break the diffraction barrier and reveal objects as small as 10-20 nm (Bates et al., 2013). Particularly exciting is the possibility of imaging the very early stages of spine formation and subsequent maturation, which has not been possible to study with conventional light microscopy. Additionally, the molecular composition and organization of synapses are essential for synaptic plasticity. The enhanced resolution of STORM permits a more precise analysis of protein localization and interaction within individual spines and synapses (Rust et al., 2006, Hruska et al., 2018). Previous research using the STORM technique has reported that clozapine may restore dendritic spine density by changing the number of D2R nanoclusters in the striatum of the mice model mimicking schizophrenia (Onishi et al., 2018). Our preliminary research showed that D2R activity is vital to strengthen synaptic plasticity (Hu et al., 2018, Zheng et al., 2018). However, we were unable to observe and quantify D2R within synapse due to a lack of nanoscale microscopy. Interestingly, a recent study reported that as the size of a dendritic spine increases, there is a net increase in the amount of PSD-95 and synaptophysin found in a single dendritic spine (Hruska et al., 2018). So far, it has not been revealed how the D2R
interacts with DISC1 in a single synapse. By applying STORM to observe dendritic spines of cultured neurons, this study aimed to study the interaction of D2R and DISC1 and its effects on synaptic connections from a nanoscale perspective.

1.2.14 Schizophrenia and Oxidative Stress

The brain is particularly vulnerable to oxidative damage (Gandhi and Abramov, 2012). Clinical data indicated that the risk factors converge upon oxidative stress in schizophrenia patients (Koga et al., 2016). The occurrence of oxidative stress adversely impacts the developmental brain, which is relevant to the development of schizophrenia. Oxidative stress occurs when there is an overproduction of reactive oxygen species. The oxidative stress in schizophrenia leads to mitochondrial damage, which drives mitochondria to produce more oxidants (Rajasekaran et al., 2015). Several studies have demonstrated that mitochondrial malfunction can lead to cellular degeneration (Dias et al., 2013). The degeneration is because the peroxidation of membrane lipids produces toxic aldehydes, which can impair critical mitochondrial enzymes (Murphy, 2009). Notably, a study using a combined transcriptomic, proteomic, metabolic approach found that altered proteins in schizophrenia patients are associated with mitochondrial function and oxidative stress responses (Prabakaran et al., 2004). Abnormal mitochondrial morphology, size, and density have been reported in the brains of schizophrenia individuals (Ben-Shachar, 2002). An increased level of oxidative stress was reported in the first episode of drug-naïve schizophrenia patients with cognitive deficits (Solberg et al., 2019, Xie et al., 2019). Clinical study also showed an increased lipid peroxidation, particularly in first-episode drug naïve patients with schizophrenia (Jordan et al., 2018). Ketamine and phencyclidine are often used to induce psychotic symptoms. These psychostimulants increase the ROS as part of
their mechanism of action (Kovacic and Cooksy, 2005). When the ROS inhibitor NAC was given to schizophrenia patients, it ameliorated the symptoms even in treatment-resistant patients (Zhang and Yao, 2013). Thus, ample evidence suggests a vital role for oxidative stress in the pathogenesis and progression of schizophrenia.

1.2.15 Neurite Lesion Induced by D2R Overactivation via Oxidative Stress

Neurons are well-known to be vulnerable to ROS-induced damage. ROS causes neurite lesions before the induction of cell death (Fukui, 2016). Neuron produces ROS at multiple organelles. Among these sites, mitochondria contribute to the most substantial amount of ROS. The production of mitochondrial ROS is primarily through the release of superoxide at complexes I and III of the mitochondrial respiratory chain (Murphy, 2009). Thus, mitochondrial integrity is essential for maintaining normal ROS level and neuronal morphology. Mitochondrial injury has been documented to result in neurite impairment. Firstly, mitochondrial integrity is fundamental to the formation of the neural network. Fragmented mitochondria and distorted cristae enhance mitochondrial ROS formation, reduce ATP levels, and show a severely disrupted neural network (Ganjam et al., 2019). Permeabilization of the mitochondrial membrane significantly upsurges oxidative stress, followed by the loss of hippocampal neurons in mice (Merkwirth et al., 2012). Secondly, the amount of mitochondrial is associated with neurite outgrowth. Immunocytochemistry results showed that the growing neurite of cortical neurons contains more active mitochondria, especially at the terminal of neurite (Trigo et al., 2019). Recent research revealed that excessive mitochondrial ROS production decreases mitochondrial number and motility, followed by neurite impairment in M17 human neuroblastoma cells (Parrado-Fernandez et al., 2018). Although
conventional electron microscopy provides us strong evidence about the alternation of mitochondria in neuronal impairment, the sample preparation is redundant, and sometimes it can cause structural damage. Using cryo-electron tomography (cryo-ET), this study aimed to examine whether D2R overactivation would cause neurite lesion through affecting mitochondrial in native environments within axons and synaptic vesicles of primary neurons.

A wide range of studies has reported that ROS inhibits neurite outgrowth. Excessive ROS production significantly decreases the percentage of cells with neurite outgrowth to 5% within 24 hours in N2a cells (Wang et al., 2010). Damaged neurites of N2a cells tend to become bead-shaped or fragmented (Fukui, 2016). The ROS specific inhibitor NAC blocks the axonal lesion caused by oxidative stress (Hervera et al., 2018). NAC can inhibit nerve growth factor-induced neurite outgrowth of PC12 cells by decreasing ROS amount, suggesting the dual role of ROS on neurites (Zhou and Too, 2011, Suzukawa et al., 2000). In addition, a high level of ROS significantly decreases the density of the dendritic spine, which has been documented in patients, animal models and cell models (Massaad and Klann, 2011). Thus, the effect of ROS on neurite outgrowth still requires further investigation.

The role of D2R in regulating ROS is debatable. Accumulating evidence showed that excessive endogenous or exogenous dopamine increased ROS production in the brain (Delcambre et al., 2016). The excessive ROS production induced by dopamine could cause cell death in SH-SY5Y cell line (Gomez-Santos et al., 2003). The early study revealed that primary cortical neurons treated with dopamine had significantly increased ROS production, which was blocked by D2R antagonists (Grima, 2003, Acquier et al., 2013). Blocking ROS production by D2R antagonist
also alleviates huntingtin-induced death of striatal neurons (Charvin et al., 2005).
Therefore, this study aimed to determine whether oxidative stress is involved in the reduced neurite outgrowth induced by D2R overactivation.

1.2.16 Targeting Mitophagy as Novel Strategy for Treating Neurite Deficits
Basal autophagy activity is critical to the maintenance of neuronal homeostasis and viability (Klionsky and Emr, 2000). Human cells have sophisticated regulatory pathways to maintain mitochondrial homeostasis through balanced mitochondrial biogenesis and clearance of damaged mitochondria. Mitophagy, a form of autophagy, mediates the removal of defective mitochondria (Lou et al., 2019). Neuronal autophagy also plays essential roles during neuronal development, for example, axon outgrowth and synapse formation (Tomoda et al., 2019). Mitophagy is crucial for protecting cells against the deleterious effects of damaged mitochondria (Yamada et al., 2019, Fang et al., 2019). In postmitotic neurons, the autophagosomes are continuously formed at the distal end of the axon and undergo transportation along microtubules toward the soma, which is enriched with lysosomes to clear unnecessary materials (e.g., amino acids, lipids) (Ashrafi et al., 2014). A recent study further showed that macroautophagic activity within the learning and memory center is responsible for synaptic plasticity by maintaining the expression of NPY in drosophila (Bhukel et al., 2019).

Disruptions in neuronal autophagy contribute to the pathophysiology of neuropsychiatric disorders (Tomoda et al., 2019). The SQSTM1 (also known as p62) is an autophagy substrate that is used as a reporter of autophagy activity (Liu et al., 2016). SQSTM1 is decreased when autophagy is induced while it is accumulated when autophagy is inhibited; therefore, SQSTM1 may be used as a biomarker to study the autophagic process (Bjørkøy et al., 2009). As elevated
SQSTM1 expression is found in the prefrontal cortex of schizophrenia patients, the autophagy-deficiency caused by hyperdopaminergic may be associate with SQSTM1 expression. One study reported that D2R knockdown inhibited autophagic influx, suggesting D2R is a positive regulator of autophagy (Wang et al., 2018). Recently, Aveleira and colleagues have shown that NPY can initiate autophagy in the hypothalamic neuronal cell line, as shown by the analysis of LC3B-II turnover, the decrease of SQSTM1 and the increase in the number of autophagosomes and autolysosomes (Aveleira et al., 2015). The research on D2R induced neuronal autophagy will also help us to have a greater level of understanding in the signaling pathway involving D2R-NPY-autophagy. Interestingly, quinpirole decreases cell viability without initiating neuronal autophagy (Leng et al., 2017). Thus, whether and how D2R activation regulates mitophagy is still largely unknown. A better understanding of D2R and mitophagy might provide new therapeutic targets for the treatment of schizophrenia.
1.3 Aims and Hypothesis

1.3.1 Specific Aims

The aims of this research were to:

1. Study whether and how the excessive D2R-DISC1 complex formation affects neurite outgrowth, D2R intracellular signaling, and synaptic proteins in the primary neurons.

2. Evaluate the effects of haloperidol and aripiprazole on D2R-DISC1 complex formation and neuroprotection.

3. Determine the neuroprotective effects of novel antipsychotic peptide TAT-D2pep and mechanisms underlying its protection in primary the striatal neurons.

4. Investigate the effects of D2R overactivation on oxidative stress and mitophagy in the primary striatal neurons.
1.3.2 Hypothesis

1. The excessive D2R-DISC1 complex caused by D2R specific agonist quinpirole will decrease the phosphorylation of Akt-GSK3β, which will lead to the reduction of neurite growth and dendritic spine density. Furthermore, quinpirole will decrease the expression of synaptic proteins, indicating that the increased interaction between D2R and DISC1 will also cause synaptic loss.

2. Aripiprazole may have better protection on neuronal morphology than haloperidol.

3. TAT-D2pep will protect neurite outgrowth and dendritic spine formation by specifically decreasing D2R-DISC1 complex formation.

4. Overactivation of intracellular and mitochondrial D2R will cause oxidative stress and incomplete mitophagy.
1.3.3 Significance

Schizophrenia influences approximately 20 million people throughout the world. Antipsychotic drugs are the primary medication for the treatment of schizophrenia as well as other psychiatric disorders. Nevertheless, the absence of response to antipsychotics in certain patients, along with their various side-effects, driving researchers to find a better treatment for schizophrenia. D2R antagonism is a general pharmacodynamic property of all current antipsychotics, which further supports that D2R hyperactivity is a core target for schizophrenia treatment. In addition, clinical and pre-clinical findings have indicated that neurite deficits are associated with schizophrenia featured by cognitive decline. Synaptic loss is considered as the critical phenotype of schizophrenia. Previous findings demonstrate that the reduction of dendritic spines in the brain is not the consequence of treatment with antipsychotic medications. Thus, investigating the effects of D2R overactivation on neuronal impairment, and antipsychotic drugs target which signaling pathway of D2R, will contribute to understanding the mechanisms of D2R overactivation induced neurite lesion from several interrelated perspectives. Comparing the effects of haloperidol and aripiprazole on neuroprotection will help us have a better understanding of the features of atypical and typical antipsychotic drugs.

The dysfunction of D2R complexes in schizophrenia subjects is a promising target in neuropsychopharmacology. These D2R complexes could offer novel targets for antipsychotic drugs and provide better therapeutic outcomes. The excessive D2R-DISC1 complex was recently identified in the post-mortem brain from schizophrenia patients and mouse models of schizophrenia. However, the role of the D2R-DISC1 complex in neuroplasticity is still unclear. The findings in this
thesis will provide evidence of whether the cell-penetrating peptide TAT-D2pep targeting on the D2R-DISC1 complex has neuroprotective effects. In addition, oxidative stress has been recently determined in the first episode of drug-naïve schizophrenia patients with cognitive deficits. Elucidating the effects of D2R overactivation caused oxidative stress on neurite outgrowth will provide another important mechanism underlying neurite deficits. Results from the current study may offer evidence for developing new antipsychotic drugs targeting on ameliorating cognitive decline for schizophrenia patients.
1.4 General Methods

1.4.1 Antibodies and Chemicals

The reagents used in this thesis are summarized in Table 1.4.1.

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| **Chemicals and Peptides**           |                   |            |
| Quinpirole hydrochloride             | Sigma             | Q102       |
| Haloperidol                          | MP Biomedical     | 11403062   |
| Aripiprazole                         | Sigma             | SML0935    |
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Experimental Models:

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Abbreviations: ABR: Australian BioResources; JHU: Johns Hopkins University.

*The design of TAT-D2pep was based on the region from K211 to T225 (KIYIVLRRRRKRVNT) of D2R, which was known to directly interact with DISC1 (Su et al., 2014, Lipina et al., 2018). The molecular weight and purity of TAT-D2pep were analyzed by high-performance liquid chromatography (HPLC) and mass spectrometry (MS) before it was applied in experiments.

1.4.2 Cortical and Striatal Neuron Culture
All experimental procedures were approved by the Animal Ethics Committee, University of Wollongong, Australia, and complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Dissociated mouse cortical (Chapter 2) and striatal cultures (Chapter 3 and Chapter 4) were prepared as previously described from postnatal 0 to 3-day-old C57BL/6J, or DISC1-LI mice described previously (kindly provided by Prof. A. Sawa, Johns Hopkins University School of Medicine) (Shahani et al., 2015, Seshadri et al., 2015). To generate DISC1-LI mice, the exon1-3 and Δ25bp on Exon 6 of DISC1 are deleted. Heterozygous DISC1-LI male and wild-type C57BL/6J female mice were mated to obtain heterozygous DISC1-LI and wild type pups. Genotypes were carried out by tail snipping and polymerase chain reaction prior to tissue collection at PN0. Briefly, cortical or striatal cells were gently dissociated with a plastic pipette after digestion with 0.5% trypsin (GIBCO, Los Angeles, USA) at 37°C for 30 min. Cells were cultured in Neurobasal medium (GIBCO) containing B27 supplement (GIBCO), 1M glucose (only for culturing cortical neurons) and 20mM glutamine (Sigma Aldrich). After 24 hours of culture, Cultures were maintained at 37°C in a humidified 5% CO₂ incubator and used for experiments at seven days in vitro. The half volume of the culture medium was changed twice a week. The final concentration of quinpirole used in this study was optimized based on its effect on the neurite length of cortical or striatal neurons, and the data was acquired by a Lionheart FX Automated Microscope (BioTek Instruments, Winooski, Vermont, USA).

1.4.3 HEK-293 Cell Culture and Transfection

HEK-293 cells were grown in DMEM (Life Technologies, Carlsbad, USA) containing 10% fetal bovine serum and 1% penicillin-streptomycin (Thermo
Fisher, Waltham, USA) at 37°C in 5% CO2. On the day before transfection, the culture medium was changed to DMEM containing 10% fetal bovine serum without penicillin-streptomycin. The D2R-EGFP, D2R mutant-EGFP, and DISC1-mCherry coding sequences were synthesized by GenScript (GenScript, Hong Kong Ltd) and sub-cloned into the pcDNA3 vector. To obtain the D2R mutant-EGFP, the sequence coding the DISC1 binding site, KIYIVLRRRRKRVNT, was deleted from the full sequence of the D2R long isoform. The D2R-Flag plasmid is from Sino Biological Incorporation (Beijing, China). Transfection was performed using Lipofectamine 2000 (Life Technologies) according to the manufacturer’s instructions. After 24 hours of transfection, cells were treated with different drugs.

1.4.4 Immunofluorescence

Cells for immunofluorescence were plated on 13 mm coverslips coated with 0.1 μg/ml poly-d-lysine (Sigma Aldrich) at a final concentration of $1.0 \times 10^5$ cells/well. Following treatment, cells were fixed in 4% freshly-made formaldehyde, permeabilized with 0.3% Triton X-100/phosphate buffered saline (PBS), and blocked with 5% normal donkey serum (Sigma Aldrich) in PBS. Cells were first immunostained with primary antibodies overnight and then with secondary antibodies for 2 hr. Cells were viewed using a 63× oil immersion objective on a DMI6500B confocal microscope or DMi8 fluorescence microscope (Leica, Mannheim, Germany). The neurite length and branches were measured by manually tracing the neurites using ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2016).
1.4.5 Fluorescence Resonance Energy Transfer (FRET)

Confocal FRET analysis was performed as described previously (Hasbi et al., 2009). In the cortical neurons, anti-D2R-Alexa Fluor 488 was used as a donor dipole, while anti-DISC1-Alexa Fluor 568 was used as an acceptor dipole. In HEK-293 cells, D2R-GFP was used as a donor dipole, while DISC1-mCherry was used as an acceptor dipole. The donor was excited with an argon laser at 488 nm, while the acceptor was excited with a DPSS laser at 568 nm. Samples were analyzed using the Leica application wizard for FRET sensitized emission.

1.4.6 Western Blot

After treatment, cells were immediately collected in lysis buffer containing NP40 (Sigma Aldrich), protease inhibitor cocktail (Sigma Aldrich), 1 mM phenylmethylsulfonyl fluoride (Sigma Aldrich) and 0.5 mM β-glycerophosphate (Sigma Aldrich). Total protein concentrations were determined by the DC-Assay (BioRad, Hercules, USA), and detected using a SpectraMax Plus384 absorbance microplate reader (Molecular Devices, Sunnyvale, USA). The sample protein was separated on SDS-PAGE gels and then transferred onto polyvinylidene fluoride (PVDF) membranes. Incubation with primary antibodies was performed overnight at 4 °C. The blots were then washed and incubated with secondary antibodies for 2 hours at room temperature. For visualization, immunoreactivity was detected using enhanced chemiluminescence detection reagents. The blots were scanned with an Amersham Imager 600 RGB (GE Health, Chicago, USA), and densitometry analysis was performed with ImageQuant TL 8.1 Software (GE Health, Chicago, USA).
1.4.7 Super-resolution Imaging of D2R and DISC1 in Dendritic Spines

Primary striatal neurons for STORM imaging were plated on μ-slide 8 well glass bottom plates (Ibidi, Martinsried, Germany). Endogenous D2R and DISC1 were fixed, cells were permeabilized and immunolabelled by primary and secondary antibodies conjugated with Cy3B-NHS and Alexa 647-NHS, respectively, as previously described (Du et al., 2016). An imaging buffer (100 mM Tris/HCl pH 8.0, 20 mM NaCl and 10% glucose, all from Sigma-Aldrich) and an oxygen scavenger system (60 mg/ml glucose oxidase and 6 mg/ml catalase, both from Sigma-Aldrich) were used for STORM imaging (Wang et al., 2015) and 140 mM β-mercaptoethanol was added to promote photo-switching. Two-color STORM imaging was sequentially acquired for up to 50,000 frames under the excitation of 647 nm and 561 nm lasers at a power density of 3~5 kW/cm2 and under the photo-activation of a 405 nm laser (Coherent Inc.) with a power density of 0.5 kW/cm2 at the sample. STORM image analysis, nearest neighbor distances calculation, drift correction, image rendering, protein nanocluster identification, quantification and image presentation were performed using Insight3 (a gift from Prof. Bo Huang at UCSF), custom-written Matlab (2012a, MathWorks) codes, SR-Tesseler (IINS, Interdisciplinary Institute for Neuroscience), and Image J (Image Processing and Analysis in Java).

1.4.8 Cell preparation for cryo-EM

Carbon-coated gold TEM grids (Quantifoil NH2A R2/2) were performed plasma cleaning at 2 mA for 10 seconds. Grids were sterilized under UV three times for 30 min at room temperature. Grids were carefully washed twice in PBS and coated with 0.1 μg/ml poly-d-lysine for 1 hour at 37 °C. After three PBS washes, striatal
neurons were plated on a final concentration of $3.0 \times 10^5$ cells/grid. For cell vitrification, live striatal neurons were blotted from the backside of the grid for 9 s and rapidly frozen in liquid nitrogen using a Leica EMGP system.

1.4.9 Image acquisition by Cryo-electron microscopy

Cryo-ET was performed on a Tecnai 20 equipped with a field emission gun and operated at 200 kV (Thermo Fisher company). Images were captured using either Explore 3D or SerialEM software on a 4k x 4k camera (Ultrascan from Gatan) and a Falcon II (FEI, Thermo Fisher) direct electron detector, with a 14 μm pixel size. Tilt series of mitochondria were acquired covering an angular range of -60° to +60° with a 2 to 4 degrees increment. All tilt series were acquired at magnifications of 25,000× or 29,000×, binning 2. Tomograms were manually segmented with the program AMIRA (FEI).

1.4.10 Statistical analysis

GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA) was used to calculate the significance between groups. Shapiro-Wilk test was used to examine if the data were normal distribution. Levene’s test of equality of error variances was performed to check the data for homogeneity of variance. The $t$-test was used to analyze the significant difference between the two groups. One-way ANOVA was performed for comparing the mean values of multiple groups, followed by Tukey’s post hoc test. Data were expressed as means ± SEM and $p < 0.05$ was considered statistically different.
1.5 Overview of the Thesis

Schizophrenia is a severe mental disorder featured with impaired neuronal connections in the brain. All the current antipsychotic drugs target on D2R to counterbalance its hyperactivity associated with psychotic symptoms. However, the neurite deficits in the majority of schizophrenia are not ameliorated by current antipsychotic drugs. Thus, it is of great importance to understand the mechanism of D2R overactivation caused neurite deficits, which will provide evidence in developing the novel antipsychotic drugs. The phenotypes of the primary cortical and striatal neurons have been demonstrated to be relevant to the cortex and striatum \textit{in vivo}. This study applied the primary neurons to investigate the mechanism of neurite impairment and evaluate the effects of antipsychotic drugs. Furthermore, the super-resolution technique facilitates us to observe the pattern of existence of interested proteins and the interaction of proteins within a single dendritic spine of neurons. This study highlights the significance of the D2R-DISC1 complex as well as oxidative stress in neuroplasticity. These findings contribute to developing novel antipsychotic drugs to treat neurite deficits and decrease oxidative stress with fewer side effects.
1.5.1 Aripiprazole and haloperidol protect neurite lesions via reducing excessive D2R-DISC1 complex formation

D2R hyperactivity causes altered brain development and later produces onset of symptoms mimicking schizophrenia. It is known that D2R interacts with DISC1; however, the effect of D2R-DISC1 interaction in intracellular signaling and neurite growth has not been studied. This study investigated the effect of D2R over-activation on Akt-GSK3β signaling and neurite morphology in cortical neurons. Over-activation of D2Rs caused neurite lesions, which were associated with decreased Akt and GSK3β phosphorylation in cortical neurons. The antipsychotic drug aripiprazole was more effective in the prevention of neurite lesions than haloperidol. Unlike haloperidol, aripiprazole prevented downregulation of pAkt-pGSK3β induced by D2R hyperactivity, indicating the involvement of different pathways. D2Rs were hyperactive in cortical neurons of mice with DISC1 mutation, which caused more severe neurite lesions in cortical neurons treated with quinpirole. Immunofluorescent staining for CaMKII confirmed that cortical pyramidal neurons were involved in the D2R hyperactivity-induced neurite lesions. Using the FRET technique, we provide direct evidence that D2R hyperactivity led to D2R-DISC1 complex formation, which altered pGSK3β signaling. This study showed that D2R hyperactivity-induced D2R-DISC1 complex formation is associated with decreased pAkt-pGSK3β signaling and, in turn, caused neurite impairment. Aripiprazole and haloperidol prevented the impairment of neurite growth but appeared to do so via different intracellular signaling pathways.
1.5.2 Prevention of Neurite Spine Loss Induced by Dopamine D2 Receptor Overactivation in Striatal Neurons

Psychosis has been considered a disorder of impaired neuronal connectivity. Evidence for excessive formation of D2R-DISC1 complexes has led to a new perspective on molecular mechanisms involved in psychotic symptoms. Here, we investigated how excessive D2R-DISC1 complex formation induced by D2R agonist quinpirole affects neurite growth and dendritic spines in striatal neurons. Fluorescence resonance energy transfer, stochastic optical reconstruction microscopy, and cell penetrating-peptide delivery were used to study the cultured striatal neurons from mouse pups. Using these striatal neurons, our study showed that: (1) D2R interacted with DISC1 in dendritic spines, neurites and soma of cultured striatal neurons; (2) D2R and DISC1 complex accumulated in clusters in dendritic spines of striatal neurons and the number of the complex were reduced after application of TAT-D2pep; (3) uncoupling D2R–DISC1 complexes by TAT-D2pep protected neuronal morphology and dendritic spines; and (4) TAT-D2pep prevented neurite and dendritic spine loss, which was associated with restoration of expression levels of synaptophysin and PSD-95. In addition, we found that NPY and GSK3β were involved in the protective effects of TAT-D2pep on the neurite spines of striatal spiny projection neurons. Thus, our results may offer a new strategy for precisely treating neurite spine deficits associated with schizophrenia.
1.5.3 Overactivation of Mitochondrial Dopamine D2 Receptor Inhibits Mitophagy

Oxidative stress has been recently observed in the first episode of drug-naïve schizophrenia patients. Although D2R dysfunction has long been recognized in schizophrenia pathology, whether it can affect mitochondrial ultrastructure and ROS-mediated mitophagy are largely unidentified. Here, we investigated how D2R over-activation inhibits ROS-initiated mitophagy and working memory in mice. Cryogenic electron microscopy (Cryo-EM) and schizophrenia mice model were used in this study. Our findings showed that (1) D2R overactivation caused by quinpirole reduced number and length of cristae in mitochondria of cultured striatal neurons; (2) D2R located on neuronal mitochondria; (3) D2R specific agonist quinpirole exacerbated oxidative stress with increased ROS and malondialdehyde (MDA) in primary striatal neurons; (4) SQSTM1 expression was up-regulated by quinpirole suggesting the mitophagy was inhibited; (5) Administration of quinpirole caused working memory impairment and stereotyped behavior in adult mice. Therefore, these findings may provide a new strategy for alleviating oxidative stress associated with schizophrenia.
1.5.4 Summary

In summary, D2R overactivation caused neurite lesion is associated with excessive D2R-DISC1 complex formation and incomplete mitophagy. This study for the first time discovers that the unbalanced interaction between D2R and DISC1 result in reductions in neurite outgrowth and dendritic spine formation. This study also examined the alternation of the D2R intracellular signaling pathway, which is associated with the neuroplasticity. The excessive formation of the D2R-DISC1 complex decreased the phosphorylation of GSK-3β in cortical and striatal neurons. D2R overactivation also downregulated the NPY expression level, while disrupting D2R-DISC1 complex caused by D2R overactivation restored the NPY level to normal. Besides the impairment in neuronal morphology, this study also reported the inhibited synaptic connections caused by increased interaction of D2R and DISC1.

By comparing the effects of haloperidol and aripiprazole on neuroplasticity, the findings showed that aripiprazole had better protection on neurite length than haloperidol. Aripiprazole could increase the expression level of phosphorylated GSK3β, whereas haloperidol had no effects on GSK3β phosphorylation. Using DISC1 mutant mice, this study also demonstrated that haloperidol and aripiprazole prevented neurite impairment through DISC1. In addition, this study first identified that both haloperidol and aripiprazole could block the quinpirole-induced interaction of D2R and DISC1 in cultured cortical and striatal neurons. This thesis has also firstly reported that TAT-D2pep disrupted the interaction between D2R and DISC1 to prevent neurite lesions, suggesting this complex would be a potential therapeutic approach for treating neurite deficits.
Protein-protein interactions exist in the synapse to affect synaptic architecture and neurotransmission. Here, STORM images revealed that both D2R and DISC1 existed within synapse in a nanocluster pattern instead of a single molecular. The cell-penetrating peptide TAT-D2pep separated the complex of D2R and DISC1 in synapses, suggesting that this peptide with antipsychotic-like effects may be capable of strengthening synaptic plasticity. Interestingly, the diameter of D2R and DISC1 nanoclusters was not changed by quinpirole or TAT-D2pep. This study for the first time reports the relationship between the actual distance of proteins and FRET occurrence, which is the first time that FRET has been elucidated from a nanoscale perspective.

Finally, this study reported that oxidative stress and incomplete mitophagy were involved in the neurite lesion caused by D2R overactivation. Cryo-EM has been the dominant technique to observe fine structures, like mitochondrion, inside the neuronal synapse. The current study observed that D2R overactivation damaged mitochondrial cristae, suggesting that the importance of D2R in controlling mitochondrial integrity. Furthermore, this study for the first time reveals the existence of D2R on the mitochondrial membrane. Overactivation of mitochondrial D2R remarkably stimulates mitochondrial ROS production. Understanding D2R subcellular localization can actively contribute to receptor function and even disease pathology. This study also demonstrated that mitophagy inhibition might be another mechanism underlying membrane and mitochondrial D2R overactivation.

Taken together, these findings have provided extensive new knowledge on the mechanism of D2R in regulating neuroplasticity. The TAT-D2pep could be a new drug for treating schizophrenia, especially with neurite deficits.
Chapter Two

Aripiprazole and Haloperidol Protect Neurite Lesions via Reducing Excessive D2R-DISC1 Complex Formation


Available at: https://doi.org/10.1016/j.pnpbp.2018.12.007
Aripiprazole and haloperidol protect neurite lesions via reducing excessive D2R-DISC1 complex formation

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ARTICLE INFO

Keywords:
Antipsychotic drug
D2 receptor
D2R-DISC1 complex
GSK3β
Neurite growth

ABSTRACT

Depenamine D2 receptor (D2R) hyperactivity causes altered brain development and later produces onset of symptoms mimicking schizophrenia. It is known that D2R interacts with disrupted in schizophrenia 1 (DISC1); however, the effect of D2R-DISC1 interaction on intracellular signalling and neurite growth has not been studied. This study investigated the effect of D2R over-activation on Akt-GSK3β signalling and neurite morphology in cortical neurons. Over-activation of D2R caused neurite lesions, which were associated with decreased protein kinase B (Akt) and glycogen synthase kinase 3 beta (GSK3β) phosphorylation in cortical neurons. The antipsychotic drug aripiprazole was more effective in the preservation of neurite length than haloperidol. Unlike haloperidol, aripiprazole prevented downregulation of phospho-(Ser) Akt (p-Akt) and p-GSK3β induced by D2R hyperactivity, indicating involvement of different pathways. D2R were hyperactive in cortical neurons of mice with DISC1 mutation, which caused more severe neurite lesions in cortical neurons treated with quinpirole. Immunofluorescence staining for p-Ca2+/calmodulin-dependent protein kinase II (DAB1) confirmed that cortical pseudonodal neurons were involved in the D2R hyperactivity-induced neurite lesions. Using the fluorescence resonance energy transfer (FRET) technique, we provide direct evidence that D2R hyperactivity led to D2R-DISC1 complex formation, which altered p-GSK3β signalling. This study showed that D2R hyperactivity-induced D2R-DISC1 complex formation is associated with decreased p-Akt-p-GSK3β signalling and, in turn, causes neurite impairment. Aripiprazole and haloperidol prevented the impairment of neurite growth but appeared to do so via different intracellular signalling pathways.

1. Introduction

Dopamine is a major neurotransmitter that regulates brain functions, neuronal connectivity and cognition (Asbury et al., 2015). Over-activation of the dopamine D2 receptor during the perinatal period induces D2R hyper-activity, which is an animal model of schizophrenia (Kostrova et al., 2016). Neurite outgrowth, a critical process in neurodevelopment, is highly associated with cognitive function (Bocchio et al., 2013). The D2R regulates cortical development and is also a common target for antipsychotic drug action (Rangel-Barradas et al., 2015). Administration of the D3R against quinpirole affects memory performance and induces "hallucination-like" behaviors in monkeys (Ammati et al., 1995). Over-activation of D2R by quinpirole dramatically inhibits neurodevelopment of cerebral cortical and hippocampal neurons (Bocchio et al., 1996; Jin et al., 2012).

Previous study showed that DISC1 has two significant peaks of expression corresponding to critical stages of neurogenesis periods (E13.5 and P35) in the developing brain in mice (Chen et al., 2004). As a scaffold protein, DISC1 interacts with multiple molecules including GSK3β, to facilitate neurite outgrowth (Mao et al., 2009; Dalmau et al., 2013). Knocking down DISC1 gene expression inhibits neuronal precursors proliferation while over-expression of DISC1 rescues neurodevelopment in the developing cortex (Niwa et al., 2016). Also, DISC1 mutant (dominant negative) mice have increased D2R binding sites in the striatum (Chiu et al., 2013). A recent study showed that hyperdopaminergic status increases the formation of D2R-DISC1 complex.
complexes that contribute to psychiatric-like symptoms (Su et al., 2014); however, the mechanism is not known. Given that GSK3β signaling is important in regulating neurtides and that altered GSK3β signaling is involved in the development of psychosis, this intracellular signaling pathway may be involved (Bennett et al., 2015).

Antipsychotic drugs are used to treat various mental illnesses, including schizophrenia, Alzheimer's disease and severe depression. Virtually all antipsychotic drugs used in the clinic have D2R antagonist properties. However, very little is known about whether/how the antipsychotic effects of D2R antagonism affect neurtides and the intracellular molecular signaling pathways regulating neurtides. This study investigated the effect of D2R hypersensitivity through the DISC1-Akt-GSK3β signaling pathway and the response to antipsychotic drug treatment on neurtides of cortical neurons.

2. Materials and methods

2.1. Antibodies and chemicals

Quinuprine hydrochloride (Sigma Aldrich, St Louis, Missouri, USA), haloperidol (MP Biomedicals, Solon, OH, USA), aprikopine (Sigma Aldrich), anti-MAP2 (1:1000; Sigma Aldrich), anti-PSD-95 (1:1000; Abcam, Cambridge, USA), anti-synaptophysin (1:2000; Sigma Aldrich), anti-phospho-Akt (Ser473) (1:1000; Cell Signaling Technology, Danvers, USA), anti-phospho-Akt (Thr308) (1:1000; Cell Signaling Technology), anti-GSK3β (1:1000; Cell Signaling Technology), anti-phospho-GSK3β (Ser9) (1:1000; Cell Signaling Technology), anti-β-actin (1:5000; Millipore, Bedford, USA), anti-GalKbR (1:800; Cell Signaling Technology), Cell Light™ Acridine Orange/BPP, BAUMAN 2.0 (Invitrogen, Waltham, USA), Alexa Fluor 488-conjugated goat anti-mouse IgG secondary antibody (1:400; Invitrogen) and Alexa Fluor 594-conjugated goat anti-rabbit IgG secondary antibody (1:400; Invitrogen).

2.2. Cortical neuron culture

All experimental procedures were approved by the Animal Ethics Committee, University of Wollongong, Australia, and complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. The phenotypic composition of primary cortical neurons has previously been shown to correspond closely to that of the cortex in vivo (Roy et al., 2009). Dissociated mouse cortical cultures were prepared, as previously described (Ilgenmehl and Smith, 2007), from postnatal 0 to 3-day-old C57BL/6J or DISC1-IIB mice described previously (kindly provided by Prof. A. Son, Johns Hopkins University School of Medicine) (Shah et al., 2015; Sehhati et al., 2015). Genetic background of DISC1-IIB is displayed in additional file (Fig. S2). Heterozygous DISC1-IIB male and wild-type C57BL/6J female mice were mated to obtain heteryozygous DISC1-IIB and WT pups. Genotypes were verified by tail snipping and polymerase chain reaction prior to tissue collection at P0. Briefly, cortical cells were gently dissociated with a plastic pipette after digestion with 0.5% trypsin (GIBCO, Los Angeles, USA) at 37°C for 20 min. Cells were cultured in Neurobasal medium (GIBCO) containing B27 supplement (GIBCO), 1 mg glucose and 20 mM glutamine (Sigma Aldrich). After 24 h of culture, 5-Bromo-2′-deoxyuridine (BrdU) was added to a final concentration of 10 μM to repres the growth of glial cells. Cultures were maintained at 37°C in a humidified 5% CO2 incubator and used for experiments at 7 days in vitro. The final concentration of quinuprine used in this study was selected based on its effect on the neurite length of cortical neurons and the data was acquired by Limheart FX Automated Microscope (BioTek Instruments, Winooski, Vermont) (Fig. S2). HEPES buffer or aprikopine was added to cultures 30 min prior to quinuprine addition for 48 h.

2.3. Cell culture and transfection

HEK-293 cells were grown in DMEM (Life Technologies, Carlsbad, USA) containing 10% fetal bovine serum and 1% penicillin-streptomycin (Thermo Fisher, Waltham, USA) at 37°C in 5% CO2. On the day before transfection, the culture medium was changed to DMEM containing 10% fetal bovine serum without penicillin-streptomycin. The D2R-EGFP, D2R mutant-EGFP and DISC1-ncCherry coding sequences were synthesized by GenScript (GenScript, Hang Kong Ltd) and cloned into the pdIAG vector. To obtain the D2R mutant-EGFP, the sequence coding the DISC1 binding site, KTVYHRRRRRRHPRRT, was deleted from the full sequence of the D2R long isoform. D2R-Flag plasmid is from Sino Biological Incorporation (Beijing, China). Transfection was performed using Lipofectamine 2000 (Life Technologies) according to the manufacturer’s instructions. After 24 h transfection, cells were treated with quinuprine and haloperidol.

2.4. Immunofluorescence

Cells for immunofluorescence were placed on 12-mm coverslips coated with 0.1 μg/ml poly-l-lysine (Sigma Aldrich) at a final concentration of 1.0 × 106 cells/well. Following treatment, cells were fixed in 4% freshly-made formaldehyde, permeabilized with 0.3% Triton X-100/phosphate buffered saline (PBS), and blocked with 5% normal donkey serum (Sigma Aldrich) in PBS. Cells were first immunostained with primary antibodies overnight and then with secondary antibodies for 2 h. Cells were viewed using a 63× oil immersion objective on a DMB8500 confocal microscope or DMI88 fluorescence microscope (Leica, Mannheim, Germany). The neurite length and branches were measured by manually tracing the neurites using ImageJ software (Rasband, W.S., Image, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2016).

2.5. Fluorescence resonance energy transfer (FRET)

Confocal FRET analysis was performed as described previously (Eliash et al., 2009). In the cortical neurons, anti-D2R-Alexa Fluor 488 was used as a donor dye, while DISC1-ncCherry was used as an acceptor dye. In HEK-293 cells, D2R-EGFP was used as a donor dye, while DISC1-ncCherry was used as an acceptor dye. The donor was excited with an argon laser at 488 nm, while the acceptor dye was excited with a DPSS laser at 568 nm. Samples were analysed using the Leica application window for FRET sensitized emission.

2.6. Western blot

After treatment, cells were immediately collected with lysis buffer containing NP40 (Sigma Aldrich), Protease Inhibitor Cocktail (Sigma Aldrich), 1 mM phenylmethylsulfonyl fluoride (Sigma Aldrich) and 0.5 mM β-glycerophosphate (Sigma Aldrich). Total protein concentrations were determined by the DC-Assay (Bio-Rad, Hercules, USA), and detected using a SpectrumMax Plus384 absorbance microplate reader (Molecular Devices, Sunnyvale, USA). Loading buffer was added to the samples, which were loaded onto 10% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) (Bio-Rad, Hercules, USA). Transfers to nitrocellulose membranes (GE Health, Chicago, USA), and incubated with antibodies overnight at 4°C. The blots were then washed and incubated with secondary antibodies for 2 h at room temperature. For visualization, immunoreactivity was detected using an ECL detection reagent. The blots were scanned with the Amersham Image 600 RGB (GE Health, Chicago, USA) and densitometry analysis was performed with ImageQuant TL 8.1 Software.

2.7. Statistical analysis

GraphPad Prism 5 (GraphPad Software, Inc, La Jolla, CA, USA) was used to calculate the significance between groups. For analyses of two groups with equal variances, P-values were determined by t-test. For analyses of three or more groups, P-values were determined by one-way
3. Results

3.1. A D2R agonist inhibits neurite growth and PSD95 in cortical neurons

To study the effects of D2R hyperactivity on neurite growth and synapse-associated proteins, we treated primary cortical neurons isolated from wild type (WT) and DISC1 LI mice with the D2R-specific agonist quinpirole. DISC1 gene is dysfunctional in DISC1 LI model (Shahabi et al., 2015; Shahabi et al., 2015). Neurite length and branching are two major features of neurite morphology. Here, we performed a dose-response analysis with quinpirole concentrations ranging from 0 to 300 μM (Fig. S1). Quinpirole at a concentration of 100 μM induced a 50% decrease in neurite length without neuronal death and was used in this study. Quinpirole (100 μM) significantly reduced the length of microtubule-associated protein 2 (MAP-2) immunostained cortical neurites compared with controls in both WT and DISC1 LI mice (Fig. 1a). This decline was significantly greater in DISC1 LI cortical neurites compared with WT group, suggesting that DISC1 LI enhanced neurite losses (Fig. 1b). The mean number of neurite branches was decreased by D2R hyperactivity, but no obvious difference was found between WT and DISC1 LI neurites treated with quinpirole (Fig. 1c). The alteration of neuronal morphology were
confirmed by western blot analyses for synaptophysin and post-synaptic density protein 95 (PSD-95) (Fig. 1d). We found that the level of PSD-95 and synaptophysin was significantly decreased by quinpirole treatment in WT neurons (Fig. 1e, f). Synaptophysin was significantly lower in DISC1-LI neurons compared with WT neurons even without quinpirole treatment (Fig. 1f). Therefore, our observation of the neurite lesion is in fact mediated by dysfunction of endogenous DISC1, but not an off-target effect. These results also confirmed that D2R hyperactivity reduced neurite growth and decreased synapse-associated protein expression.

As the Akt-GSK3β signalling pathway regulates neurite growth, including length and branching (Kim et al. 2011), we investigated whether D2R hyperactivity affects Akt-GSK3β signalling in WT and DISC1-LI neuronal model (Fig. 2a). Since Akt phosphorylation is a critical step in D2R-mediated GSK3β phosphorylation, we firstly tested whether over-activation of D2R would alter pAkt Ser473 and Thr308 in cortical neurons. Quinpirole significantly downregulated pAkt-Thr308 and pAkt-Ser473 in WT neurons (Fig. 2b, c). DISC1-LI resulted in a further decrease of quinpirole-suppressed pAkt-Thr308 compared with WT neurons, whereas no obvious change was observed in pAkt-Ser473 (Fig. 2b, c). Administration of 100 μM quinpirole for 48 h significantly reduced GSK3β-Ser9 phosphorylation in both WT and DISC1-LI neurons. The decline was significantly greater in DISC1-LI cortical neurons compared with WT group, suggesting that DISC1 plays an important role in regulating Akt-GSK3β signalling (Fig. 2d). Therefore, these results indicated that activity of the D2R affected neurite growth by at least partially regulating the pAkt-pGSK3β signalling pathway.

3.2. Haloperidol and antipsychotic ameliorate neurite impairment induced by D2R agonist

To test the effects of the antipsychotic drug haloperidol and the D2R partial agonist antipsychotic, cortical neurons were pre-treated with antipsychotic (10 μM, 30 min) or haloperidol (10 μM, 30 min) followed by 100 μM quinpirole for 48 h (Volzanka et al., 1995; Massi et al., 2008; Su et al., 2014). Both haloperidol and antipsychotic markedly ameliorated the neurite impairment induced by D2R over-activation (Fig. 2a). Furthermore, haloperidol and antipsychotic prevented the reduction of synaptophysin induced by D2R hyperactivity (Fig. 2b). Antipsychotic but not haloperidol prevented the neurite impairment induced by quinpirole (Fig. 2c). To examine Akt-GSK3β signalling, we measured pAktSer473, pAkt-Thr308, and pGSK3β-Ser9 in cortical neurons after co-treatment of haloperidol or antipsychotic with quinpirole. We found that antipsychotic had a different effect compared with haloperidol. Antipsychotic prevented the reduction of pAktSer473, pAkt-Thr308, and pGSK3β-Ser9 induced by quinpirole, but haloperidol did not (Fig. 4a-d). Therefore, these results suggested that haloperidol and antipsychotic affected neurite growth through different pathways.

3.3. Haloperidol and antipsychotic regulate neurite growth through DISC1

We examined the role of DISC1 in response to haloperidol and antipsychotic treatment. We found that antipsychotic increased neurite branches reduced by quinpirole, however neither haloperidol nor antipsychotic were able to reverse the neurite length decreased by quinpirole in cortical neurons derived from DISC1-LI mice (Fig. 3a-c) and had no protective effects on PSD-95 and synaptophysin expression (Fig. 3d-e). These results revealed that DISC1 mediates the effects of haloperidol and antipsychotic on neurite integrity and synapse-associated protein expression.

Since haloperidol or antipsychotic co-treatment was unable to prevent neurite length from decreasing when D2R was over-activated in the DISC1-LI cortical neurons, we hypothesised that DISC1 may play an important role in mediating the effects of haloperidol and antipsychotic on pAkt-pGSK3β signalling (Fig. 4a). The pAkt-Thr308 increased by 32.7% when DISC1-LI cortical neurons were pre-treated with antipsychotic (Fig. 6a, c). Furthermore, neither of these antipsychotic drugs blocked the action of quinpirole on pGSK3β (Fig. 4d). These results revealed that DISC1 is necessary for protective effects of haloperidol and antipsychotic.
3.4. D2R hyperactivity induced D2R-DISC1 complex formation then decreased pGSK3β

Since we have found that D2R hyperactivity decreased pGSK3β mediated by DISC1, we investigated D2R and DISC1 interaction which may influence pGSK3β. It is previously reported that D2R hyperactivity induced by quinpirole significantly increases D2R-DISC1 complex formation (Fu et al., 2014). To further demonstrate the specificity of D2R-DISC1 complex formation and effects on pGSK3β, we used HEK-293 cells transfected with D2R-EYFP or D2R-Flag and DISC1-mCherry plasmids. HEK-293 cells do not express D2R and DISC1 (Fudal et al., 2017). To examine the specific interaction, we constructed D2R mutant-EYFP and DISC1-mCherry fusion proteins and transfected them into HEK-293 cells. We confirmed that D2R hyperactivity increased D2R-DISC1 complex formation more than three-fold. This effect was blocked by the D2R antagonist haloperidol. Furthermore, we showed that D2R hyperactivity-induced D2R-DISC1 complex formation was abolished when the specific DISC1 binding site of D2R, KIYPYLRKRRKKKRVNT, which lies in the third intracellular loop, was deleted (Fig. 7a, b). This proved that this segment of D2R binds to DISC1 to form D2R-DISC1; however, we could not exclude possible changes of quinpirole binding affinity to D2R. To determine whether increased D2R-DISC1 complex formation functionally regulates pGSK3β, we expressed D2R-Flag and DISC1-mCherry in HEK-293 cells. As expected, pGSK3β was significantly reduced when the D2R-DISC1 complex was induced in HEK-293 cells. D2R antagonist haloperidol was unable to remarkably reverse pGSK3β level (Fig. 7c, d). We further demonstrate that D2R overstimulation decreased DISC1 and GSK3β interaction, which was prevented by D2R antagonist, haloperidol or aripiprazole (Fig. 8). These findings indicated that D2R-DISC1 formation functionally regulates pGSK3β level.
3.5. **D2R hyperactivity alters GSK3β phosphorylation via DISC1 in cortical pyramidal neurons**

DISC1 and GSK3β are highly expressed in cortical pyramidal neurons (Kaidanovich-Bellin et al., 2012; Koyama et al., 2015). Based on these findings in HEK-293 cells, we hypothesized that neurexin growth and its regulatory signalling pathway could be affected by D2R-DISC1 complex formation in cortical neurons. To test this, we used cortical FRET sensitized emission for detection and analysis. We observed that cortical neurons expressed D2R and DISC1 on both the cell body and neurites and that the levels of D2R-DISC1 complexes were increased when cortical neurons were treated with quinpirole. Haloperidol and a-pyridyl blocked D2R-DISC1 complex formation induced by quinpirole in the cortical neurons (Fig. 5a, b). We examined whether the D2R hyperactivity-induced neurite impairment was mediated by DISC1 in pyramidal neurons of the cortical. We used Calbiochem as a marker for the pyramidal neurons (Díaz et al., 2016; Salas et al., 2016) and confirmed that quinpirole decreased pSer933 associated with DISC1 (Fig. 5c). The cortical neurons were transfected on the day of plating with a fusion construct of human actin and FlagGFP packaged in baculovirus, and examined after drug treatment. We found that the immunoactivity of pGSK3β was significantly reduced by quinpirole treatment (100 µM, 48 h) compared with pyramidal neurons without quinpirole treatment in both WT and DISC1-LJ neurons. Furthermore, the decrease of pGSK3β expression was significantly greater in the DISC1-LJ pyramidal neurons than WT pyramidal neurons, suggesting that DISC1 dysfunction further inhibited pyramidal neurons (pGSK3β) under conditions of D2R hyperactivity (Fig. 5d).

4. **Discussion**

The dopamine D2R plays an important role in early brain development. Alteration of D2R signalling affects brain development and contributes to the development of severe mental illnesses. Also, the D2R is the putative site of antipsychotic drug action. The present study showed that an over-activation of the D2R leads to neurite impairment and alters Akt-GSK3β signalling in cortical neurons, which are essential for cognitive function. Importantly, D2R and DISC1 complex formation is involved in the pathological mechanism contributing to neurite impairment in cortical neurons.

4.1. **D2R hyperactivity inhibits neurite growth**

Although a number of studies have investigated D2R regulation of neuronal morphology, the mechanisms are still largely unknown. Over-activation of D2R has a negative effect on neurite growth. A quinpirole mouse model has been used to investigate the morphological and obsessive-compulsive disorder (Kostopoulos et al., 2016; Smith et al., 2016). An early study showed that the D2R agonist quinpirole induces D2R hyperactivity when it is administered to...
neonatal rats (Kontrova and Reus, 1993) and 100 µM quinpirole decreases neurite outgrowth in cortical neurons (Biello et al., 1999). In addition, D2R hyperactivity induced by quinpirole also potentiates neurite retraction in striatal and hippocampal neurons (Deyn et al., 2009; Liu et al., 2013). In line with these reports, we found that D2R hyperactivity strongly affects neurite outgrowth. In addition, it disregulated expression of PSD-95 and synaptophysin in cortical neurons, synapse-associated proteins that regulate neurite length and connectivity.

4.2. D2R hyperactivity-induced neurite impairment is aggravated in cortical neurons with DISC1 dysfunction

Dysregulation of DISC1 in the brain causes abnormal neurite growth (Ishida et al., 2006). Although both in vivo and in vitro studies have suggested DISC1 is connected to dopamine neurotransmission, the role of DISC1 in modulating D2R signalling has not been fully elucidated. DISC1 mutant mice were used to investigate altered dopaminergic systems relevant to psychiatric disorders (Yamada et al., 2016) and other studies have shown that mice with a dominant-negative isoform of DISC1 have increased D2R expression in the cortex and striatum (Vilwa et al., 2015; Juaro-Peled et al., 2013). In fact, altered DISC1
significantly affects dopamine homeostasis and animal behaviour (Yamasaki et al., 2016). It is DISC1 missense produced by its polymorphism that remarkably increases cyscognitive, which in turn causes perturbed DISC1 aggregates. In accordance with these observations, our study revealed that DISC1 LI mice had D2R hypoactivity that inhibited neurite outgrowth of cortical neurons. As a scaffolding protein, DISC1 is required to interact with other signalling molecules and facilitate neuronal development. An increased interaction between D2R and DISC1 was found not only in DISC1 LI mice but also in wild type mice treated with quinpirole and non-immunoprecipitation showed that D2R DISC1 complex formation was higher in DISC1 LI mouse compared with WT mice (Liu et al., 2019). In this study, we found that neurite length and branch number decreased when D2R DISC1 complex formation was induced by quinpirole in cortical neurons. One possible explanation is that DISC1 lost its ability to interact with other signalling molecules after it was coupled with D2R when D2R is over-activated. Adding to previous studies, we have provided new evidence that inactivation of the functional domain of DISC1 or increased D2R DISC1 complex formation leads to neurite impairment.

4.3. D2R hyper-activation affects Akt-GSK3β signalling in cortical neurons

The role of GSK3β in D2R-associated signalling is intriguing but whether and how it regulates neurites is not yet clear. The Akt-GSK3β signalling pathway has been demonstrated to control dendrite elongation and branching (Frenandez et al., 2013; Sridhar et al., 2013). In this study, we have provided the first evidence that D2R hyperactivity decreased pAkt-GSK3β signalling in cortical neurites. D2R hyper-activation induced by amphetamine decreases pGSK3β through inhibiting pAkt activation (Gonzalez et al., 2003). Oral administration of quinpirole or cocaine reduced pGSK3β levels, which could be reversed by GSK3β activity inhibitors (Gonzalez et al., 2006; Miller et al., 2014). In schizophrenia and experimental animal models, when the D2R and DISC1 complex was formed, there was reduced pGSK3β in the striatum, which are correlated with impaired cognitive behaviours (Sha et al., 2018). Similarly, we found that quinpirole dramatically increased the interaction between D2R and DISC1 to form a protein complex that led to decreased pAkt pGSK3β signalling and reduced neurite length in the cortical neurons.

4.4. Antipsychotics may protect against D2R hyperactivity-induced neurite loss via DISC1

Both typical and atypical antipsychotic drugs have therapeutic effects by preventing neurite loss during brain development (Lieberman et al., 2008). Clinical studies have reported that antipsychotics have better therapeutic efficacy for negative symptoms and cognitive deficits than haloperidol in the early stage of schizophrenia (Cheong et al., 2011). Analyses increased pAkt pGSK3β signalling and protected neurites from lesions by over-activation of D2R. Although haloperidol also protected against neurite lesions but to lesser degree than aripiprazole; haloperidol had no effect on pAkt pGSK3β signalling, suggesting that haloperidol may act through a different pathway to protect neurites. Aripiprazole unlike haloperidol is more selective to targeting on D2R/β-arrestin2 scaffold signalling pathway, which can in turn influence GSK3β phosphorylation (Mazet et al., 2008; Uns et al., 2017). The mechanism by which aripiprazole protects neurites may be related to promotion of neurite growth (de Bartolomeis et al., 2015).
Fig. 7. FRET analysis of D2R interaction with DISC1 and its regulation on pS935 in HEK-293 cells. (a) FRET image of HEK-293 cells transfected with wild type or mutant D2R-EGFP and DISC1-mCherry. (b) Quinpirole (Quin)-induced D2R hyperactivity increased D2R-DISC1 complex formation and was blocked by the D2R antagonist haloperidol (Hal). D2R hyperactivity-induced D2R-DISC1 complex formation was also abolished by transfection of mutant D2R-EGFP, in which the specific DISC1 binding site of D2R (RKKYVLRREIRRRT) was deleted. Scale bar = 50 μm. Data are shown means ± SEM and normalized; ***p < 0.001; n > 15 cells per group. (c, d) Representative immunofluorescent images and quantification of pS935 mice from HEK-293 cells transfected for indicated channels below. The increasing D2R-DISC1 complex desensitization pS935 level in HEK-293 cells. D2R antagonist haloperidol was unable to reverse this desensitization. Data are shown means ± SEM and normalized; ***p < 0.001; n > 15 cells per group. Scale bar = 5 μm.

We showed that neither antipsychotics nor haloperidol could rescue quinpirole-induced neurite length inhibition of cortical neurons from DISC1-L1 mice. Our results provide an explanation for why haloperidol does not have a strong therapeutic effect in DISC1 mutant mice (Clapcote et al., 2007), indicating antipsychotic drug resistance and possible D2R hyperactivity in these mice.

5. Conclusion

In summary, our results showed that D2R hyper-activation induced neurite impairment that may be caused by pS935-pS935 complex formation, followed by altered pAkt-pS935 signaling in cortical neurons. Antipsychotics increased neurite branches reduced by quinpirole, however neither haloperidol nor antipsychotics were able to reverse the neurite length decreased by quinpirole in cortical neurons derived from DISC1-L1 mice. Further, antipsychotics were more effective at treating D2R hyperactivity-induced neurite lesions than haloperidol in WT neurons, supporting the observation that antipsychotic was superior to haloperidol on the negative and general psychological symptoms for treatment of schizophrenia (Kong et al., 2011).

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

The authors acknowledge Illawarra Health and Medical Research Institute and School of Medicine for providing partnership funds to support this research. We would like to thank Prof. A. Saw from Johns Hopkins University School of Medicine for giving us the DISC1-L1 mice and A/Prof H. Eroroy from IIMB for helping us to design D2R-EGFP.
Fig. 8. Increasing DISC1-DCSC1 complexes reduces GSK3β in cortical pyramidal neurons in vivo. (a, b) PRT1 analysis showed that DISC1-DCSC1 complexes were increased when cortical neurons were treated with quinpirole, Haloperidol (Hal), and apomorphine (Apo). (c) Blocking the DISC1-DCSC1 complex formation induced by quinpirole. Data are shown as means ± SEM and normalized to the corresponding GSK3β of Wild Type. (d) Quantification of GSK3β phosphorylation of CAMKKII-positive pyramidal neurons showed that quinpirole induced a decrease in GSK3β phosphorylation that was greater in DISC1-DCSC1 neurons than in Wild Type cells. *p < 0.05; **p < 0.01; ***p < 0.001; n = 15 cells per group. Scale bar = 15 μm.

and DISC1-ehCherry fusion proteins for cell transfection.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yphspp.2018.12.007.

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Progress in Neuro-psychopharmacology & Biological Psychiatry
Supplementary Information for

Aripiprazole and haloperidol protect neurite lesions via reducing excessive D2R-DISC1 complex formation

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**Fig. S1** The effects of different concentrations of quinpirole on cortical neurons’ neurite length. After quinpirole treatment, cortical neurons were put into Lionheart FX Automated Microscope to record neurite length in real-time for 48 hours. Values are shown as means ± SEM, expressed as the percentage of the values of control (Ctrl) cells; ***p < 0.001 vs Ctrl.
**Fig. S2** Genetic background of DISC1-LI mice. To generate DISC1-LI mice, the exon1-3 and Δ25bp on Exon 6 of DISC1 are deleted. aa: amino acid.
**Fig. S3** DISC1 and GSK3β interaction is reduced after quinpirole (Quin) treatment, which is prevented by haloperidol (Halo) and aripiprazole (Ari). a. Immunofluorescence images of cortical neurons double stained for GSK3β and DISC1. b. FRET analysis showed that GSK3β-DISC1 interaction was significantly reduced after quinpirole treatment. Halo and Ari blocked the reduction of GSK3β-DISC1 interaction. Data are shown as means ± SEM; ***p < 0.001. n >15 cells per group. Scale bar = 15 μm.
Chapter Three

Prevention of Neurite Spine Loss Induced by Dopamine D2 Receptor Overactivation in Striatal Neurons

Reprinted from Frontiers in Neuroscience, 14, Peng Zheng, Qian Peter Su, Dayong Jin, Yinghua Yu, and Xu-Feng Huang. Prevention of Neurite Spine Loss Induced by Dopamine D2 Receptor Overactivation in Striatal Neurons, 642, Copyright (2020), with permission from Frontiers.

Available at:
https://doi.org/10.3389/fnins.2020.00642
Prevention of Neurite Spine Loss Induced by Dopamine D2 Receptor Overactivation in Striatal Neurons

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Psychosis has been considered a disorder of impaired neuronal connectivity. Evidence for excessive formation of dopamine D2 receptor (D2R)– disrupted in schizophrenia 1 (DISC1) complexes has led to a new perspective on molecular mechanisms involved in psychotic symptoms. Here, we investigated how excessive D2R–DISC1 complex formation is induced by D2R agonist quinpirole affecting neurite growth and dendritic spines in striatal neurons. Fluorescence resonance energy transfer (FRET), stochastic optical reconstruction microscopy (STORM), and cell penetrating-peptide delivery were used to study the cultured striatal neurons from mouse pups. Using these striatal neurons, our study showed that: (1) D2R interacted with DISC1 in dendritic spines, neurites and soma of cultured striatal neurons; (2) D2R and DISC1 complex accumulated in clusters in dendritic spines of striatal neurons and the number of the complex were reduced after application of TAT-D2p-pep; (3) uncoupling D2R–DISC1 complexes by TAT-D2p-pep protected neuronal morphology and dendritic spines; and (4) TAT-D2p-pep prevented neurite and dendritic spine loss, which was associated with restoration of expression levels of synaptofysin and PSD-95. In addition, we found that Neurotensin Y (nPY) and GSK3β were involved in the protective effects of TAT-D2p-pep on the neurite spines of striatal spiny projection neurons. Thus, our results may offer a new strategy for precisely treating neurite spine deficits associated with schizophrenia.

Keywords: schizophrenia, cell penetrating-peptide, D2R–DISC1 complex, synaptic spine, SABA, Neuropeptide Y

INTRODUCTION

Schizophrenia is a severe mental disorder that affects thinking and behavior. Neuroimaging studies have revealed neurological deficits in schizophrenia patients and psychosis has been considered as a disorder of impaired neuronal connections (Fair et al., 2017). Striatal dopamine D2 receptor (D2R) hyperactivity is predominantly responsible for psychosis in schizophrenia (Hoyer and Kapur, 2009). Administration of the D2R-specific agonist quinpirole can induce hallucinatory-like behaviors and cognitive decline in monkeys (Arvanitaki et al., 1993) and dramatically inhibit neurodevelopment of cortical and hippocampal neurons (Reinoso et al., 1996; Jin et al., 2013). Although these studies suggest that striatal D2R hyperactivity accounts for the neuro-pathogenesis of psychosis, the mechanism that underlies increased D2R activity remains to be elucidated.

Antipsychotic drugs antagonizing D2R have been a cornerstone of psychotic pharmacotherapy for more than half a century. D2R is highly expressed in the striatum and striatal D2 receptor
blockade is considered the most effective mechanism to alleviate psychotic symptoms in schizophrenia. However, blockade of striatal D2R by antipsychotic drugs is likely to cause side effects, including tardive dyskinesia and obesity (Lally and MacCabe, 2015; Huang et al., 2018). We previously reported that both aripiprazole and haloperidol decrease excessive D2R-disrupted in schizophrenia (1-DisC1) complex formation (Zheng et al., 2018). Further, aripiprazole shows better protection of neurite growth than haloperidol in cortical neurons (Zheng et al., 2016). However, the role of D2R-DisC1 complexes in spine density and neuronal morphology of striatal neurons is as yet unknown.

The molecular composition and organization of synapses are important for synaptic plasticity (Yim et al., 2016). Stochastic optical reconstruction microscopy (STORM) allows molecules to be assessed at 20 nm resolution, which is sufficient to observe and detect a single molecular interaction (Joo et al., 2006). Recently, STORM imaging revealed that clathrin reverses the changes in D2R assembly in the stratum of DisC1-deficient mice (Ohishi et al., 2015). To date, the formation of D2R-DisC1 complexes has not been analyzed in a single synapse. By using STORM and primary striatal neurons, we aimed to reveal the relationship between D2R-DisC1 complexes and dendritic spines from a nanoscale perspective.

Neurotrophin Y (NY) is one of the most abundant peptides in the central nervous system, with a wide range of physiological functions (Gotzsche and Woldbye, 2016). Most findings regarding the regulation of striatal NPY indicate that there is reciprocal interaction between the D2R and NPY. In fact, D2R activation by administration of quinpirole or amphetamine decreases NPY expression in mice (Xiao, 2003) but the effect of D2R-DisC1 complexes on NPY has not yet been reported.

Collapsingpedal peptide delivery system is highly effective for small peptide delivery in both in vitro and in vivo studies (Guilleti et al., 2017). It is the trans-activating transcriptional activator (TAT) from human immunodeficiency virus 1 could be efficiently taken up by neurons in culture. This technique is used in this present study to deliver the interfering peptide TAT-D2ep to inhibit D2R and DisC1 complex formation.

In the current study, we show that D2R interacts with DisC1 in dendritic spines and is associated with neurite growth and spine density of striatal neurons. TAT-D2ep reduced excessive D2R-DisC1 complex formation in the spines, soma, and neurites and prevented dendritic spine loss of mouse striatal neurons. Furthermore, we observed that NPY and CCK-R were involved in the protective effects of TAT-D2ep in the neurites of striatal spiny projection neurons.

MATERIALS AND METHODS

Antibodies and Chemicals

The following reagents were purchased: Quinpirole hydrochloride (Sigma Aldrich, St Louis, MO, United States), anti-MAP2 (1:1,000; Sigma Aldrich), anti-PSD-95 (1:1,000; Abcam, Cambridge, United Kingdom), anti-synaptophysin (1:1,000; Sigma Aldrich), anti-GSK3β (1:1,000; Cell Signaling Technology, Danvers, MA, United States), anti-phospho-GSK3β (Ser9) (1:1,000; Cell Signaling Technology), anti-β-actin (1:500; Millipore, Bedford, MA, United States), anti-GAD67 (1:400; Cell Signaling Technology), anti-D2R (1:400; Santa Cruz Biotechnology, CA, United States), anti-AP1 (1:400; Santa Cruz Biotechnology), Alexa Fluor 568 Phalloidin (Invitrogen, Waltham, MA, United States), Alexa Fluor 488-conjugated goat anti-mouse IgG secondary antibody (1:400; Invitrogen) and Alexa Fluor 568-conjugated goat anti-rabbit IgG secondary antibody (1:400; Invitrogen), Cy3-NHS (GE Healthcare, NSW, Australia), and Alexa Fluor 647-NHS (Thermo Fisher, Waltham, MA, United States). The region from Kd3 to T23 (KTFFLVRKKKKVRNT) of D2R is known to directly interact with DisC1 (Tao et al., 2016; Lipina et al., 2018), which is used for designing TAT-D2eppeps. TAT-D2eppeps (YGKKEKKQRRE-KTYFLLRRKKVRNNT) and TAT-D2eppeps-negative control (NC): YGKEKKQUKQRE were synthesized through GeneScript (GeneScript, Hong Kong Ltd.), and dissolved in ultrapure water to a stock concentration of 10 μM. The molecular weight and purity of TAT-D2eppeps were analyzed by high performance liquid chromatography (HPLC) and mass spectrometry (MS) before it was applied in experiments (Supplementary Data, Supplementary Figures S1, S2).

Striatal Neuron Culture

The experimental procedures were approved by the Animal Ethics Committee, University of Wollongong, Australia for the Care and Use of Animals for Scientific Purposes. Dissociated mouse striatal cultures were prepared as previously described (Xiao and Wang, 2003). Briefly, striatal cells of postnatal day 0 of C57Bl/6 mice were gently dissociated with a plastic pipette after digestion with 0.3% trypsin (GIBCO, Los Angeles, CA, United States) at 37°C for 30 min. Cells were cultured in Neurobasal medium (GIBCO) containing B27 supplement (GIBCO) and 20 μM phenyltoin (Sigma Aldrich). After 24 h of culture, 0-thiorea-2-doxycycline (Sigma) was added to a final concentration of 10 μM to suppress the growth of gli cells. Cultures were maintained at 37°C in a humidified 5% CO2 incubator. Half volume of the culture medium was changed twice a week. After 14 days in culture, cells were treated with quinpirole for 2 h to induce neurite loss. The final concentration of quinpirole used in this study was optimized based on its effect on the neurite length of striatal neurons and the data was acquired by a LineScan X Automated Microscope (BioTek Instruments, Winooski, VT, United States). Addition of 10 μM TAT-D2eppeps and TAT-D2eppeps-NC to cultures was performed 30 min prior to quinpirole addition. We did not observe toxic effect as reported previously (Lee et al., 2016).

HEK-293 Cell Culture and Transfection

As transcriptomics analysis has shown that D2R and DisC1 are barely expressed in HEK-293 cells (Tao et al., 2017), HEK-293 cells were transfected with D2R and DisC1 plasmids to study the effect of D2R on DisC1 and their downstream signaling cascades. HEK-293 cells were grown in Dulbecco's
FIGURE 1 | Interaction of D2R-DISC1 in single dendritic spines by STORM analysis. (A) Two-color STORM combined with bright field images shows the interaction between D2R (green) and DISC1 (red) in single spines. Upper panels show representative images of D2R-DISC1 complexes formation in single dendritic spines. Scale bar = 1 μm. Lower panels show magnified representative images of single D2R-DISC1 complexes. Scale bar = 200 nm. (B) Measurement of D2R and DISC1 nanoclusters. (C) Quantification of the distance between D2R and DISC1 nanoclusters in dendritic spines by the nearest neighbor algorithm. (D,E) Number of D2R and DISC1 nanoclusters in single dendritic spines. *p < 0.05 versus control; **p < 0.01 versus control; ***p < 0.001 versus control; ****p < 0.0001 versus control, quinpirole, by one-way analysis of variance (ANOVA) with post hoc Tukey test.

Modified Eagle's medium (DMEM; Life Technologies, Carlsbad, CA, United States) containing 10% fetal bovine serum and 1% penicillin-streptomycin (Thermo Fisher) at 37°C in 5% CO2. On the day before transfection, the culture medium was changed to DMEM containing 10% fetal bovine serum without penicillin-streptomycin. The D2R-BFP and DISC1-actinCherry coding sequences were synthesized by GenScript (GenScript, Hong Kong Ltd.) and sub-cloned into the pcDNA3 vector. Transfection was performed using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions.
and after 24 h cells were treated with different drugs, as indicated below.

**Immunofluorescence**
Cells for immunofluorescence were plated on 13 mm coverslips coated with 0.1 µg/ml poly-d-lysine (Sigma-Aldrich) at a final concentration 1.0 × 10⁶ cells/well. Following treatment, cells were fixed in 4% formaldehyde for 10 minutes, permeabilized with 0.3% Triton X-100 in phosphate-buffered saline (PBS), and blocked with 5% normal donkey serum (Sigma-Aldrich) in PBS. Cells were subsequently immunostained with primary antibodies overnight at 4°C. Cells were then washed with PBS and incubated with secondary antibodies for 2 h. Cells were observed using a Leica TCS SP8 confocal microscope (Leica, Mannheim, Germany). The neurite length was measured by manually tracing the neurites using Simple Neurite Tracer in ImageJ software (Research, W.S., National Institutes of Health, Bethesda, MD, United States).

**Fluorescence Resonance Energy Transfer (FRET)**
Confocal fluorescent resonance energy transfer (FRET) analysis was performed as described previously (Zheng et al. 2018). In the present study, anti-D2R-Alexa Fluor 568 was used as a donor dipole, while anti-DISC1-Alpha Fluor 488 was used as an acceptor dipole. A 11LK-293 cell line, D2R-GFP was used as a donor dipole, while DISC1-mCherry was used as an acceptor dipole. The donor was excited with an argon laser at 488 nm, while the acceptor was excited with a Diodo-pumped solid-state (DPSS) laser at 568 nm. Sensitized emission is one of the most used methods for evaluation of FRET efficiencies. Samples were analyzed using the Leica application wizard for FRET sensitized emission (Walter et al. 2018). Super-Resolution Imaging of D2R and DISC1 in Dendritic Spines
Primary striatal neurons for STORM imaging were plated on µ-slide 8 well glass bottom plates (Ibidi, Martinsried, Germany). Endogenous D2R and DISC1 were fixed, cells were permeabilized and immunolabeled by primary and secondary antibodies conjugated with Cy5-IRG and Alexa 647-NHS, respectively, as previously described (Du et al. 2018). An imaging buffer (100 mM Tris/HC1 pH 8.0, 20 mM NaCl and 10% glucose, adjusted to pH 8.0), and an oxygen scavenger system (60 mg/ml glucose oxidase and 6 mg/ml catalase, both from Sigma-Aldrich) were used for STORM imaging (Wang et al. 2015) and 140 mM [Co-mercaptoethanol] was added to promote photo-switching. Two-color STORM imaging was sequentially acquired for up to 30,000 frames under the excitation of 647 nm and 561 nm lasers at a power density of 5–5 kW/cm² and the power density of 0.5 kW/cm² at the sample. STORM image analysis, nearest neighbor distances calculation, drift correction, image rendering, protein nanocluster identification, quantification and image presentation were performed using Insight3 (a gift from Prof. Po Huang at UCSF), custom-written Matlab (2012u, MathWorks) codes, SR-Tesseler (INS, Interdisciplinary Institute for Neuroscience), and ImageJ (Image Processing and Analysis in Java).

**Western Blot**
After treatment, cells were immediately collected in lysis buffer containing NP-40 (Sigma-Aldrich), protease inhibitor cocktail (Sigma-Aldrich), 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich), and 0.5 mM EDTA (Sigma-Aldrich). Total protein concentrations were determined by the DC Assay (BioRad, Hercules, CA, United States), and detected using a SpectraMax Plus384 absorbance microplate reader (Molecular Devices, Sunnyvale, CA, United States). Loading buffer was added to the samples, which were boiled under 10% sodium dodecyl sulfate polyacrylamide gels (Bio-Rad, Hercules, CA, United States), transferred to nitrocellulose membranes (GE Health, Chicago, IL, United States), and incubated with antibodies overnight at 4°C. The blots were then washed and incubated with secondary antibodies for 2 h at room temperature. For visualization, immunoreactivity was detected using enhanced chemiluminescence detection reagents. The blots were scanned with an Amersham Imager 600 RGB (GE Health, Chicago, IL, United States) and densitometry analysis was performed with ImageQuant TL 8.1 Software (GE Health, Chicago, IL, United States).

**Statistical Analysis**
GraphPad Prism 7 (GraphPad Software, Inc. La Jolla, CA, United States) was used to calculate the significance between groups. For significance analysis of four groups, p-values were determined by one-way analysis of variance (ANOVA) followed by Tukey's post hoc corrections. Data were expressed as mean ± SEM and p < 0.05 was considered statistically different.

**RESULTS**

**D2R Interacts With DISC1 in Dendritic Spines**
As dendritic spines contain interacting nanomodules, we hypothesized that D2R may interact with DISC1 in dendritic spines. To test this possibility, we firstly immunostained D2R and DISC1 with their respective antibodies and then assessed them in dendritic spines by use of STORM. STORM images revealed that D2R and DISC1 molecules exist in dendritic spines and assemble to form nanoclusters with an approximate diameter of 87.6 ± 7.3 nm and 97.1 ± 9.6 nm, respectively (Figures 1A,B). To examine whether D2R-DISC1 complexes were disrupted by TAT-D2Pep in spines, we incubated striatal neurons with TAT-D2Pep and then analyzed the distance between complexes by applying the nearest neighbor distance algorithm. The nearest neighbor distance between D2R and DISC1 nanoclusters in single dendritic spines was significantly reduced after striatal neurons were treated with quinpirole.
(control: 227.5 ± 137.5 nm, quinpirole: 69.64 ± 49.86 nm), suggesting the majority of D2R was coupled with DISC1 after treatment. Pre-incubation with TAT-D2pep but not the control TAT-D2pep-NC significantly enhanced the distance between D2R and DISC1 nanoclusters in the presence of quinpirole (TAT-D2pep: 232.3 ± 118 nm, TAT-D2pep-NC: 95.3 ± 56.9 nm), suggesting that their interaction in dendritic spines was blocked by the interfering peptide (Figure 1C). Notably, when excessive D2R-DISC1 complexes were formed, the number of D2R and DISC1 nanoclusters per dendritic spine were significantly decreased compared with control. The numbers of both D2R and DISC1 nanoclusters were restored after D2R-DISC1 complexes were disrupted by TAT-D2pep, suggesting that D2R-DISC1 complexes affect the D2R and DISC1 densities in dendritic spines (Figures 1D, E). Together, these nanocluster findings demonstrated that TAT-D2pep inhibits excessive D2R-DISC1 complex formation caused by D2R over-activation in dendritic spines.

**TAT-D2pep Inhibits Neurite Loss Caused by Excessive D2R-DISC1 Complexes**

We recently reported that apiravirone, an atypical antipsychotic drug, protects the neurite length of cortical neurons by blocking D2R signaling and upregulating D2R-DISC1 complexes (Zheng et al., 2018). To determine whether decreasing abnormal D2R-DISC1 complexes protects neurites, we pre-treated striatal neurons with TAT-D2pep or TAT-D2pep-NC prior to addition of quinpirole, followed by immunostaining for MAP-2 to visualize the effect of D2R-DISC1 complexes in neurites. The concentration of quinpirole required to induce neurite impairment was screened in primary striatal neurons prior to this experiment (Supplementary Figure S3). D2R over-activation induced by quinpirole significantly decreased neurite length compared with control. TAT-D2pep pre-treatment, but not TAT-D2pep-NC, prevented impairment of neurites in striatal neurons (Figures 2A, B). To evaluate the effects of TAT-D2pep on morphology of dendrites in whole neurons, we performed
Sholl analysis in striatal neurons treated with or without TAT-D2pep. Intersections of dendrites arising from the soma were determined within concentric shells of increasing diameter (5 μm) starting from the cell body (Figure 2C). There was a significant increase of dendritic intersections in the TAT-D2pep treatment group compared with quinpirole group (Figure 2D). To investigate whether TAT-D2pep inhibits denticiform spine loss, we labeled cultured striatal neurons with phalloidin, a marker for spine analysis. Quinpirole significantly reduced synaptic spine density of striatal neurons, suggesting that excessive D2R–DISC1 complexes may cause denticiform spine loss (Figures 2E,F). TAT-D2pep blocked the negative effect of quinpirole on denticiform spine density, suggesting that unclamping the abnormal formation of D2R–DISC1 complexes may protect spine density of striatal neurons (Figures 2E,F). Taken together, our results suggest that preventing D2R–DISC1 complex formation can block D2R overactivation-induced impairment of dendritic morphology and synaptic spine density in striatal neurons.

TAT-D2pep Uncouples Excessive D2R–DISC1 Complexes Caused by D2R Hyperactivity

We previously demonstrated that blockade of D2R by its antagonist prevents excessive D2R–DISC1 complex formation induced by quinpirole in mouse cortical neurons (Cheng et al., 2018). As both D2R and DISC1 are expressed in neurons and TAT-D2pep has neuroprotective effects, we next assessed whether TAT-D2pep decreases interaction of D2R and DISC1 in neurons and soma of primary striatal neurons by using FRET. Neurons preincubated with TAT-D2pep, but not TAT-D2pep–NC, showed decreased FRET efficiency in soma and neurites, suggesting that D2R–DISC1 complexes...
were uncoupled by the interfering peptide (Figures 3A-C). To further determine whether synthetic TAT-D2ppc peptide interferes with complex formation, we initially examined D2R-DISC1 interaction using a FRET assay in HEK-293 cells expressing D2R-EGFP and DISC1-mCherry. In this experiment, the existence of FRET between D2R-EGFP and DISC1-mCherry was used as an index of the interaction of the two fusion proteins. After addition of quinpirole the FRET efficiency increased more than three-fold compared with HEK-293 cells without quinpirole treatment. The FRET increase was markedly diminished by TAT-D2ppc. However, TAT-D2ppc-NC, a peptide without the binding domain of D2R and DISC1, failed to decrease FRET efficiency, suggesting the specificity of the designed peptide in uncoupling the D2R-DISC1 interaction (Figures 3D,E). The relationship between the FRET signal and the distance between D2R and DISC1 in dendritic spines, combined with the above super-resolution analysis, is illustrated in Figure 3F. When D2R was over-activated, the nearest neighbor distance between D2R and DISC1 became less than 70 nm, forming a complex in a single dendritic spine and resulting in a FRET signal. Those results indicated that TAT-D2ppc can specifically inhibit excessive D2R-DISC1 complex formation caused by D2R hyperactivity.

**TAT-D2ppc Protects Neurites and Increases Synaptic Protein Expression**

As synaptic proteins like synaptophysin and PSD-95 elicit changes in dendritic morphology (Zheng et al., 2016), we hypothesized that TAT-D2ppc protects neurites by regulating synaptic proteins. To determine this possibility, we initially performed immunoblot analysis of synaptophysin and PSD-95 in HEK-293 cells co-expressing D2R-EGFP and DISC1-mCherry in the presence of TAT-D2ppc or TAT-D2ppc-NC. Quinpirole treatment significantly downregulated synaptophysin and PSD-95 expression, suggesting that excessive D2R-DISC1 interaction could directly affect synaptic proteins. Blocking by TAT-D2ppc, but not the TAT-D2ppc-NC, inhibited the ability of D2R-DISC1
completes to decrease synaptophysin and PSD-95 expression in HEK-293 cells (Figures 4A–G). We next examined whether the TAT-D2pep peptide could also alter synaptic proteins in primary striatal neurons co-immunostained with MAP-2 and synaptophysin or PSD-95. The immunocytochemical analysis showed that both synaptophysin (presynaptic marker) and PSD-95 (postsynaptic marker) were obviously decreased, which was accompanied by neurite shortening in striatal neurons treated with 10 µM quinpirole. When striatal neurons were pre-treated with TAT-D2pep followed by quinpirole, the synaptophysin and PSD-95 immunofluorescence signals remained at control levels and were accompanied by normal neurites (Figures 4D–G).

Therefore, these results indicated that synaptophysin and PSD-95 were associated with the protective effects of TAT-D2pep on neurite and synaptic spine density.

**NPY Positive Inteurneurs as a Novel Target for Neuroprotection**

Neuropeptide Y is mainly expressed in GABAergic neurons and plays an important role in neuritic protection (Hohlich et al., 2006). Thus, we first assessed whether the D2R-DISC1 complex formation induced by D2R over-activation affects NPY expression by performing immunoblot analysis of NPY in HEK-293 cells. When cells were treated with the D2R
agonist quinpirole. NPY levels were markedly decreased in lysates of HEK-293 cells co-expressing TAT-D2R and DSK33-NCCherry compared with untreated cells. The decreased NPY levels were inhibited by TAT-D2pep (Figures S5A,B). This result indicates that excessive D2R-DSK33 complex formation caused by D2R over-activation may down-regulate NPY expression. Next, we asked whether NPY is associated with the effects of D2R-DSK33 complexes on neurite growth of striatal spiny projection neurons. Striatal neurons were triple-immunostained for phalloidin-actin, GAD-67 (GABAergic marker) and NPY after quinpirole treatment. Quinpirole significantly decreased NPY expression in striatal GABAergic interneurons, which was accompanied by neurite impairment. The reduction in NPY expression in striatal spiny neurons was significantly inhibited by pre-incubating with TAT-D2pep, but not with the control TAT-D2pep-NC peptide (Figures S5C,D). Thus, these data demonstrated that the decreased excessive formation of D2R-DSK33 complex upregulated NPY in striatal spiny neurons.

**TAT-D2pep Protects Neurites Through Regulating pGSK3β**

As an important downstream signaling molecule of D2R, GSK-3β is critically involved in neuronal morphological development.
including neurite outgrowth (Kim and Snyder, 2011). To determine whether GSK-3β phosphorylation is regulated by D2R-DISC1 complexes, we first performed immunoblot analysis in HEK-293 cells. As shown in Figures 6A,B, GSK-3β Ser-9 phosphorylation was significantly reduced in quinpirole-treated cells. The ability of quinpirole to decrease GSK-3β Ser-9 phosphorylation was significantly inhibited by TAT-D2lep. Next, we investigated whether GSK-3β was involved in mediating the protective effects of TAT-D2lep on neurites. To address this hypothesis, striatal neurons were triple-immunostained with phosphorylated GSK-3β, phosphorylated AKT, and GSK-3β protein after quinpirole treatment. Pre-incubating with TAT-D2lep prevented the inhibitory effect of quinpirole on GSK-3β phosphorylation and neurite growth in striatal spine protection neurons, suggesting that specifically disrupting D2R-DISC1 complex formation could upregulate GSK-3β phosphorylation at Ser-9 (Figures 6C,D). Taken together, these data indicate that GSK-3β Ser-9 phosphorylation is associated with the effects of D2R-DISC1 complexes on neurite growth.

**DISCUSSION**

The present study showed that excessive formation of D2R and DISC1 complexes alters intracellular signaling pathways and dendritic spine morphology of striatal neurons. We found that D2R formed a protein complex with DISC1 not only in some and neurites but also in dendritic spines and caused neurite impairment following D2R over-stimulation. We also showed that TAT-D2lep prevented neurite spine impairment caused by D2R over-activation and was associated with increased NPY and pGSK-3β in striatal GABAergic neurons.

In the current study, we firstly identified that D2R interacts with DISC1 in dendritic spines. Hrnia et al. recently proposed an important theory that a single synaptic spine is composed of nanomolecules, the formation of which scales with the size of the dendritic spines (Hrnia et al., 2018). It has been observed that D2R excitates in higher order oligomers in mouse brain slices (Onishi et al., 2018). Super-resolution images indicated that D2R-DISC1 complexes are potential nanomolecules existing in a single spine of striatal neurons. By uncoupling D2R-DISC1 complexes in dendritic spines, we linked the actual nanochip scale to DISC1 occurrence, which is the first time to be elucidated a nanoscale perspective. Our study further revealed that TAT-D2lep may restore dendritic spine density by reducing synaptic D2R-DISC1 complexes. Since dendritic spines have several phenotypes associated with learning and memory (Bourque and Harris, 2007), further research is required to define the effects of D2R-DISC1 complexes on spine morphology. Overall, reducing synaptic D2R-DISC1 interaction by TAT-D2lep protected synaptic spine density.

We also found that TAT-D2lep protects neuronal morphology by precisely uncoupling D2R-DISC1 complexes. Notably, although both haloperidol and antipsychotic are able to diminish D2R-DISC1 complexes, haloperidol shows no effect on neuronal protection (Zheng et al., 2018). It suggests that complete blockade of D2R by antipsychotic drugs reduces D2R and DISC1 interaction, but may not protect neurites, which reflects their insufficiency for cognitive improvement (Huang and Song, 2019). It has been reported that there is increased D2R-DISC1 complex formation in the striatum of post-mortem schizophrenia brains (Su et al., 2014). Here, we demonstrated that TAT-D2lep afforded neuronal protection by decreasing D2R-DISC1 complexes instead of blocking D2R. We found that excessive D2R-DISC1 complexes caused dendritic spine loss in striatal neurons, indicating that the loss of spine density in striatal neurons may be a phenotype of psychosis caused by D2R hypereactivity. In fact, reduction of spine density of cortical and striatal neurons in a schizophrenia-like animal model has been reported previously (Tolis et al., 2009). We further found that TAT-D2lep restored the reduced spine density of over-activated striatal neurons, indicating that the dendritic spines of the striatum could be therapeutic targets of this cell-penetrating peptide. In line with our results, increasing D2R-DISC1 complex formations caused by D2R over-activation has been recently demonstrated to decrease long term potentials in mouse hippocampus, indicating a role in synaptic plasticity (Upma et al., 2018). To our knowledge, this is the first time that TAT-D2lep has been shown to reverse neurite impairment in vivo. Further experiments are required to confirm our findings in vivo.

We also found that the excessive formation of D2R-DISC1 complexes caused by D2R over-activation remarkably downregulated synaptophysin and PSD-95 in both HEK-293 cells and primary striatal neurons. By transiently expressing D2R and DISC1 in HEK-293 cells, we provided direct evidence that excessive D2R-DISC1 complexes significantly reduced synaptic protein expression. Synaptophysin and PSD-95 act as important regulators of neurodevelopment and synaptic structure (Park et al., 2013; Zhang et al., 2016). It is possible that PSD-95 may indirectly interact with D2R and affect its downstream signaling pathways. For example, DISC1 binds to PSD-95 to facilitate spine enlargement, and knockdown or loss of DISC1 function decreases spine size (Hayashi-Takagi et al., 2014). When more DISC1 binds to D2R, caused by D2R over-activation, less DISC1 may interact with PSD-95, resulting in the reduction of dendritic spine density. We also showed that TAT-D2lep pre-treatment was able to reverse the decreased synaptophysin expression in striatal neurons. Thus, the improvement of neurite and spine density may be a consequence of the enhancement of PSD-95 and synaptophysin expression.

In the current study, we firstly demonstrated that excessive D2R-DISC1 complexes induced by quinpirole directly decreased NPY expression in HEK-293 cells, which was blocked by TAT-D2lep. In the brain, NPY is mainly expressed in GABAergic interneurons and acts as a modulator of neuroplasticity and synaptic transmission (Goeders and Wadiche, 2016). Over-activation of D2R by psychostimulants, such as amphetamine,
significantly decreases NPY expression (Xiao, 2003). We recently reported that reduced NPY expression is associated with decreased striatal neurite growth and dendritic spine number (Fu et al., 2010). Here, we found that blocking D2R-DISC1 complexes by TAT-D2pep upregulated NPY immunoreactivity in striatal GABAergic neurons. Our evidence suggests that NPY is involved in the protective effects of TAT-D2pep on acetyl and spine density. NPY treatments up-regulate gGSK3β, which is inhibited in the presence of a Y1 receptor antagonist (Wu et al., 2017). We further reported that TAT-D2pep increased GSK3β phosphorylation, likely since NPY up-regulation in striatal GABAergic interneurons. Several pharmacological studies from our group previously demonstrated that atypical antipsychotic drugs, such as olanzapine, significantly increased both NPY mRNA and protein expression levels in a schizophrenia mouse model (Huang et al., 2006; Lin et al., 2014). Thus, TAT-D2pep produces an antipsychotic-like response on GABAergic NPY and GSK3β to protect neuronal morphology.

In the striatum, NPY can affect other neuronal populations via NPY receptors. In particular, abundant NPY-Y1 receptor is reported in the human striatum (Casela-Rocha et al., 1997). NPY-Y1 receptor is shown to mediate the inhibition of locomotor activity after the administration of NPY into rat brain (Heilig et al., 1990, 1993). Therefore, it is speculated that NPY-Y1 receptor may participate in NPY neurotransmission regulating other striatal neurons and locomotor activity.

Although quinpirole is widely used as D2R agonist for many in vivo and in vitro studies, it should be noted that the quinpirole at 10 μM concentration could act as a partial agonist on alpha 2A adrenoceptor (Sánchez-Soto et al., 2018). A reduction of alpha 2A adrenoceptor activity could increase the length and density of dendritic spines (Hu et al., 2008). Also, we cannot exclude the possibility that other receptors may interact with D2R as striatal neurons, which requires the verification of a series of follow-up pharmacological experiments.

In summary, uncoupling D2R-DISC1 complexes by TATD2pep prevents neurodegeneration and upregulates NPY and GSK3β signaling in striatal neurons. Thus, the D2R-DISC1 complex might be a novel therapeutic target for treating neurite deficits in patients suffering psychosis.

DATA AVAILABILITY STATEMENT
All datasets generated for this study are included in the article/Supplementary Material.

REFERENCES

ETHICS STATEMENT
The animal study was reviewed and approved by the Animal Ethical Committee, University of Wollongong, Australia.

AUTHOR CONTRIBUTIONS
PZ and X-FH: conceptualization, PZ, QS, YY, and D8; data curation, X-FH; funding acquisition, writing – review and editing, and project administration, PZ and QS; methodology, PZ; writing – original draft. All authors contributed to the article and approved the submitted version.

FUNDING
This work was supported by the National Health and Medical Research Council of Australia (Grant IDs: APP1176503 and APP1175747).

ACKNOWLEDGMENTS
The authors acknowledge Illawarra Health and Medical Research Institute and School of Medicine for providing partnership funds to support this research.

SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2020.00542/full#supplementary-material

FIGURE S1

FIGURE S2

FIGURE S3

FIGURE S4

FIGURE S5

Supplementary Figure 1: (A) Chemical structural formulas of TAT D2pep; (B) TAT D2pep was intraperitoneally administered into multiple-dose positive control. To calculate the mass molar weight (MW) of TAT D2pep, the formula was MW = z × 154.156 – 154.156/MW = 879.21 [8]; z = 4 = 2013.20. z represents charge number of the ion.

Quantification of the average neurite length with different concentrations of quinpirole. **P = 0.001 versus control, n = 12 per group.

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Figure S1. Purity of TAI-D2pep by reversed-phase HPLC. The area under the major peak corresponds to the percentage purity. For the TAI-D2pep described by the mass spectrum trace above, TAI-D2pep accounts for 98.835% of the peptide species in the sample. Thus, the peptide is almost 99% pure. A UV spectrometer is used in RP-HPLC to detect a peptide as it elutes from the column.
Figure S2. Molecular weight of TAT-D2pep by electrospray ionization mass spectrometry. (a) Chemical structural formula of TAT-D2pep. (b) TAT-D2pep was ionized into multiple-charged states positively. To calculate the measured molecular weight (MW) of TAT-D2pep, the formula is $\text{MW} = (m/z)^{+}z^{-}$. TAT-D2pep $\text{MW} = 879.3^{+}4\text{-}4\equiv 3513.20$. $z$ represents charge number of the ion.
Figure S3. Quinpirole inhibits neurite growth of striatal neurons in concentration-dependent manner. (a) Immunofluorescence with MAP-2 demonstrated that 10 µM quinpirole induced D2R hyperfunction reduced neurite length of primary striatal neurons. Scale bar= 25 µm. Photos are taken under 63x oil objective with zoom in 3.5X (Upper lane) and 1X (Bottom lane). (b) Quantification of average neurite length affected by different concentrations of quinpirole. ***P < 0.001 versus control. N= 12 per group.
Chapter Four

Overactivation of Dopamine D2 Receptor Inhibits Mitophagy

Abstract

Oxidative stress has been recently observed in the first episode of drug-naïve schizophrenia patients. Although D2R dysfunction has long been recognized in schizophrenia pathology, whether it can affect mitochondrial ultrastructure and reactive oxygen species (ROS)-mediated mitophagy are unknown. Here, this study determined if D2R overactivation by quinpirole affects mitochondrial ultrastructure in cultured primary striatal neurons by cryo-electron tomography (Cryo-ET). The oxidative stress, mitophagy, and neurite outgrowth were further examined in quinpirole-treated neurons. The present study showed that (1) quinpirole decreased number and length of cristae in mitochondria of cultured striatal neurons; (2) D2R located on neuronal mitochondria; (3) D2R specific agonist quinpirole exacerbated oxidative stress with increased ROS and malondialdehyde (MDA) in primary striatal neurons; and (4) SQSTM1 expression was up-regulated by quinpirole suggesting the mitophagy process was inhibited. Altogether, this study indicates that D2R overactivation inhibits oxidative stress-induced mitophagy deficit, which may be involved in neurite impairment. The identification of mitochondrial D2R provides a new target for treating neurite lesions.
4.1 Introduction

Schizophrenia is a complex mental disorder influenced by genetic and environmental factors; however, the underlying pathological mechanisms are mostly unknown (Howes et al., 2016). Oxidative stress has recently been identified in the first episode of drug-naïve patients with schizophrenia and cognitive deficits (Solberg et al., 2019, Xie et al., 2019). Another study also observed increased lipid peroxidation, particularly in first-episode drug naïve patients with schizophrenia. These findings suggest that oxidative stress is involved in the pathogenesis and progression of schizophrenia (Jordan et al., 2018).

D2R dysfunction has long been reported in schizophrenia and psychiatric disorders (Kostrzewa et al., 2018). Striatal D2R upregulation is frequently documented in schizophrenia patients without antipsychotic treatment history (Kubota et al., 2017). Recent pharmacological studies involving antipsychotics highlighted how D2R signaling could regulate mitochondrial ROS production (Anglin et al., 2012). A growing list of mitochondrial G-protein coupled receptors (GPCR) have also been linked with mitochondrial ROS production and include the 5-HT4 receptor (Wang et al., 2016), cannabinoid CB1 receptors (Benard et al., 2012), and melatonin MT1 receptors (Ahluwalia et al., 2018). The existence of D2R in neuronal mitochondria has not yet been confirmed, although past studies showed that administration of the D2R agonists, quinpirole and dopamine, could directly affect mitochondrial functions (Brenner-Lavie et al., 2008). Besides the well-known functions of D2R in the cell membrane, it is hypothesized here that D2R is located on the mitochondria and regulates mitochondrial ROS production.
The selective removal of damaged mitochondria by autophagy (mitophagy) is the primary approach to degrade damaged or unwanted mitochondria (Lou et al., 2019). Mitophagy is crucial for protecting cells against the deleterious effects of damaged mitochondria (Yamada et al., 2019, Fang et al., 2019). Nevertheless, how D2R overactivation regulates mitophagy is still unclear. Subsequently, a better understanding of D2R and mitophagy might provide new therapeutic targets for the treatment of schizophrenia.

The present study firstly showed that D2R overactivation caused mitochondrial ultrastructure damage. This observation prompted investigations into the existence of D2R in neuronal mitochondria. Furthermore, over activating D2R led to oxidative stress, incomplete mitophagy, and neurite impairment in cultured striatal neurons.

4.2 Materials and Methods

4.2.1 Antibodies and chemicals

The following reagents were purchased: Quinpirole hydrochloride (Sigma Aldrich, St Louis, Missouri, USA), anti-MAP2 (1:1,000; Sigma Aldrich), anti-β-actin (1:5000; Millipore, Bedford, USA), anti-D2R (1:400, Santa Cruz Biotechnology, CA, USA), and Alexa Fluor 568-conjugated goat anti-rabbit IgG secondary antibody (1:400; Invitrogen). D2R shRNA was synthesized by GenScript (GenScript, Hong Kong Ltd).

4.2.2 Striatal neuron culture

The experimental procedures were approved by the Animal Ethics Committee, the University of Wollongong, Australia (AE18/07), and comply with the Australian
Striatal cells were gently dissociated with a plastic pipette after digestion with 0.5% trypsin (GIBCO, Los Angeles, USA) at 37°C for 30 min. Cells were cultured in Neurobasal medium (GIBCO) containing B27 supplement (GIBCO) and 20 mM glutamine (Sigma Aldrich). Cultures were maintained at 37°C in a humidified 5% CO₂ incubator.

4.2.3 Cell preparation for cryo-EM

Plasma cleaning of carbon-coated gold TEM grids (Quantifoil NH2A R2/2) was performed at 2 mA for 10 seconds. Grids were sterilized under UV for 30 min at room temperature. Grids were carefully washed twice in PBS and coated with 0.1 μg/ml poly-d-lysine for 1 hour at 37°C. After three PBS washes, striatal neurons were plated on a final concentration of 3.0 x 10⁵ cells/grid. For cell vitrification, live striatal neurons were blotted from the backside of the grid for 9 s and rapidly frozen in liquid nitrogen using a Leica EMGP system.

4.2.4 Image acquisition by Cryo-electron microscopy

Cryo-ET was performed on a Tecnai 20 equipped with a field emission gun and operated at 200 kV (Thermo Fisher company). Images were captured using either Explore 3D or SerialEM software on a 4k x 4k camera (Ultrascan from Gatan) and a Falcon II (FEI, Thermo Fisher) direct electron detector, with a 14 μm pixel size. Tilt series of mitochondria were acquired covering an angular range of -60° to +60° with a 2 to 4 degrees increment. All tilt series were acquired at magnifications of 25,000× or 29,000 ×, binning 2. Tomograms were manually segmented with the program AMIRA (FEI).
4.2.5 HEK-293 cell culture and transfection

HEK-293 cells had no expression of D2R (Thul et al., 2017). In order to study the effect of over-activation by quinpirole on mitochondria, D2R plasmid was transfected into HEK-293 cells. HEK-293 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Carlsbad, USA) containing 10% fetal bovine serum and 1% penicillin-streptomycin (Thermo Fisher) at 37°C in 5% CO₂. On the day before transfection, the culture medium was changed to DMEM containing 10% fetal bovine serum without penicillin-streptomycin. The D2R-EGFP coding sequences were synthesized by GenScript (GenScript, Hong Kong Ltd) and sub-cloned into the pcDNA3 vector. Transfection was performed using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions.

4.2.6 Immunofluorescence

Cells for immunofluorescence were plated on 13 mm coverslips coated with 0.1 μg/ml poly-d-lysine (Sigma Aldrich) at a final concentration of 1.0 × 10⁵ cells/well. Following treatment, cells were fixed in 4% freshly made formaldehyde, permeabilized with 0.3% Triton X-100/phosphate-buffered saline (PBS), and blocked with 5% normal donkey serum (Sigma Aldrich) in PBS. Cells were firstly immunostained with primary antibodies overnight and then with secondary antibodies for 2 hours. Cells were viewed using a 63× oil immersion objective on a TCS SP8 confocal microscope (Leica, Mannheim, Germany).

4.2.7 Western blot
After treatment, cells were immediately collected in lysis buffer containing NP40 (Sigma Aldrich), protease inhibitor cocktail (Sigma Aldrich), one mM phenylmethylsulfonyl fluoride (Sigma Aldrich) and 0.5 mM β-glycerophosphate (Sigma Aldrich). Total protein concentrations were determined by the DC-Assay (BioRad, Hercules, USA) and detected using a SpectraMax Plus384 absorbance microplate reader (Molecular Devices, Sunnyvale, USA). The sample protein was separated on SDS-PAGE gels and then transferred onto polyvinylidene fluoride (PVDF) membranes. Incubation with primary antibodies was performed overnight at 4 °C. The blots were then washed and incubated with secondary antibodies for 2 hours at room temperature. For visualization, immunoreactivity was detected using enhanced chemiluminescence detection reagents. The blots were scanned with an Amersham Imager 600 RGB (GE Health, Chicago, USA), and densitometry analysis was performed with ImageQuant TL 8.1 Software (GE Health, Chicago, USA).

4.2.8 Statistical analysis

GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA) was used to calculate the significance between groups. For significance analyses of multi-groups, p-values were determined by one-way analysis of variance (ANOVA) followed by Tukey's post hoc corrections. Data were expressed as means ± SEM, and p < 0.05 was considered statistically different.

4.3 Results

4.3.1 Cryo-ET reveals the abnormal architecture of mitochondrial cristae in striatal neurons induced by D2R agonist
The Cryo-ET is an advanced technique to observe the ultrastructure of mitochondria within its native environment (Tao et al., 2018). To investigate the effect of D2R overactivation on mitochondrial ultrastructure, this study applied cryo-ET to analyze the alterations of mitochondria treated with or without D2R specific agonist quinpirole. Primary striatal neurons were cultured on carbon-coated gold grids and vitrified before loading to Cryo-EM. Each tilt series were reconstructed into a three-dimensional tomogram representing an intact mitochondrion within its native environment (Figure 1A). In the control group, the mitochondria showed normal morphology with the intact mitochondrial inner and outer membrane, as well as canonical cristae folding. However, the mitochondria of the quinpirole group exhibited remarkable ultrastructural defects compared with the control group, including a decreased number of folded cristae and length of cristae (Figure 1B and C). These results suggest that overactivation of D2R could cause the damage of mitochondrial ultrastructure in primary striatal neurons.
Figure 4.1 Cryo-ET of mice striatal mitochondria from neurons treated with or without quinpirole. (A) Tomographic slice from a reconstructed stack of images taken at different tilt angles of one striatal mitochondrion. The tilt degree is ± 70. Scale bar = 200 nm. (B) Corresponding three-dimensional segmentation of the mitochondrion from the control and quinpirole group. Quinpirole damaged striatal mitochondrial ultrastructure with fewer cristae. Scale bar = 20 nm. (C) The tomographic volume of the mitochondrion from the control and quinpirole group.
4.3.2 Identification of D2R on neuronal mitochondria

Since this study found that D2R agonist quinpirole impaired the mitochondria ultrastructure of neurons, I investigated if D2R existed on the neuronal mitochondria. The primary striatal neurons were co-stained with D2R-Alexa 568 antibody and mitochondrial marker MitoTracker Green. The fluorescent images showed that the co-localization of D2R and mitochondria distributed not only in soma but also on the neurite (Figure 2A). The co-localization of D2R with mitochondria was quantified by measuring the overlapped fluorescence intensities along neurite. The overlapped signal peaks in fluorescent intensities of D2R and mitotracker revealed that D2R was co-localized with mitochondria on the neurite of primary striatal neurons (Figure 2B). To further confirm D2R expresses on neuronal mitochondria, primary striatal neurons were incubated with D2R shRNA followed by immunoblot analysis. D2R shRNA transfected into cultured neurons significantly down-regulated D2R protein level of striatal neurons. (Figure 2C and D). The expression of D2R was detected in the isolated mitochondria from cultured striatal neurons. Moreover, D2R shRNA significantly decreased the D2R expression level in isolated mitochondria (Figure 2E). Taken together, these data reveal for the first time that D2R is localized in the mitochondria of striatal neurons.
A

Mitotracker
D2R

B

Mitotracker
D2R

Plot Profile (µm)

Fluorescent Intensity

0 1 2 3 4 5 6 7 8 9 10

C

SCL WT SCL D2R KD Mito WT Mito D2R KD

D2R

β-actin

-51 kDa

-45 kDa

D

E

Striatal Cell Lysate

Mitochondria

D2R Expression (Percent of WT)

D2R Expression (Percent of WT)

WT D2R-KD

WT D2R-KD

*
**Figure 4.2 D2R exists in neuronal mitochondria.** (A-B) Fluorescent detection of D2Rs on mitochondria of primary striatal neurons in the wild-type mice. (B) Co-localization analysis of D2R with mitochondria in cultured striatal neurons. The graph shows the overlap of fluorescence intensity peaks of D2R and mitochondria along neurite, as indicated in the merged micrograph. Scale bar = 5 µm. Arrows, specific co-localization of D2R with mitochondria. (C) Representative images of D2R expression in striatal cell lysate (SCL) and purified mitochondria treated with or without D2R shRNA. (D-E) Quantification of D2R expression in cultured striatal cell lysates and isolated mitochondria. N=3, experiments were performed in triplicate. Data were shown as means ± SEM. * p < 0.05.

4.3.3 D2R overactivation elevates ROS and oxidative stress in striatal neurons

It is reported that the impairment of mitochondrial ultrastructure can lead to the accumulation of ROS (Jezek et al., 2018). To determine whether quinpirole increases intracellular ROS level, the primary striatal neurons were firstly treated with different concentrations of quinpirole followed by staining with DCFDA, an intracellular ROS marker. Quinpirole increased intracellular ROS level in U shaped dose-response curves (Figure 3A). Quinpirole at 20, 40, and 60µM increased ROS with the maximum effects at 20µM, while quinpirole at 10 and 100 µM did not significantly affect intracellular ROS production (Figure 3B). I further confirmed that quinpirole at 20 µM increased ROS production in striatal neurons by visualizing the increased intensity of DCFDA (Figure 3C). The flow cytometric analysis revealed that the increment of mitochondrial ROS was significant at 20 µM quinpirole, which was similar to the alternation of intracellular ROS (Figure 3D and E). The immunofluorescent images also displayed that quinpirole at 20 µM increased the intensity of MitoSox Red in the cultured striatal neurons (Figure 3F).
To investigate the role of mitochondrial D2R in ROS increment, isolated mitochondria from D2R transfected HEK-293 cells were incubated with 20 µM quinpirole. Quinpirole treatment displayed the higher fluorescent intensity of MitoSox compared with the control mitochondria (Figure 3G). MDA, an indicator of oxidative stress, is produced from the degradation of the cell membrane by excessive ROS (Massaad and Klann, 2011). This study further found that quinpirole significantly increased the MDA level (Figure 3H). Thus, these findings demonstrate that D2R overactivation induced ROS production and oxidative stress in primary striatal neurons.
A

Intracellular ROS (A.U.)

Quinpirole Concentration (μM)

0.0
0.5
1.0
1.5

0 10 20 40 60 100

B

Ctrl

Quin

Normalized To Mode

Intracellular ROS (DCFDA)

10^3 10^4 10^5

C

Control

Quinpirole

DCFDA

Striatal Neurons

10 µm

D

Mitochondrial ROS (A.U.)

Quinpirole Concentration (μM)

0.0
0.5
1.0
1.5

0 10 20 40 60 100

E

Normalized To Mode

Mitochondrial ROS (MitoSOX Red)

10^3 10^4 10^5

F

Control

Quinpirole

MitoSox

Striatal Neurons

10 µm
Figure 3 continued

Figure 4.3 Quinpirole increases reactive oxygen species (ROS) generation in cultured striatal neurons. (A) The effects of quinpirole on intracellular ROS of primary striatal neurons. The striatal neurons were treated with quinpirole at different concentrations, including 0, 10, 20, 40, 60, and 100 μM. Intracellular ROS was assessed using the DCFDA probe after treatment with quinpirole for 24 h and quantified by flow cytometry. (B) Flow cytometry of intracellular ROS marked by DCFDA after primary striatal neurons treated with 20 μM quinpirole. (C) Representative fluorescent images of intracellular ROS in cultured striatal neurons treated with 20 μM quinpirole (D) The effects of quinpirole on mitochondrial ROS.
The neurons were incubated for 24 h with different quinpirole concentrations. Mitochondrial ROS generation was detected using the MitoSox Red and quantified by flow cytometry. (E) Flow cytometry of mitochondrial ROS after cultured striatal neurons treated with 20 μM quinpirole. (F) Representative fluorescent images of mitochondrial ROS marked by MitoSox in primary striatal neurons treated with 20 μM quinpirole. (G) Quinpirole increases ROS production in isolated mitochondria from HEK-293 transfected with D2R plasmid. (H) Overactivation of D2R by quinpirole at 20 μM increases the MDA level in primary striatal neurons. N=3, experiments were performed in triplicate. Data were shown as means ± SEM. *** p < 0.001, ** p < 0.01 or * p < 0.05 compared with the controls. Each column represents the means ± SEM.

4.4.4 D2R overactivation increases autophagosome, but not autolysosomes

Excessive mitochondrial ROS contributes to initiating mitophagy, which eliminates damaged mitochondria (Schofield and Schafer, 2020). Since I found that quinpirole treatment increased ROS production, I examined if quinpirole initiates the autophagy process to clear impaired mitochondria in striatal neurons. During the process of autophagy, an increase in the conversion rate of LC3-I to LC3-II indicates enhanced autophagosome formation (Liu et al., 2016). In the quinpirole-treated neurons, not only the LC3-II protein level, but also LC3-II/LC3-I ratio was significantly increased compared with control group (Figure 4A and B), suggesting that D2R overactivation led to autophagosome formation. Furthermore, the colocalization of LC3-II with mitochondria marker, Mitotracker Green, was significantly increased in quinpirole-treated striatal neurons (Figure 4C and D), confirming abnormal mitochondria are started for autophagic degradation. The SQSTM1 is degraded with autolysosome formation, indicating autophagy
completion (Larsen et al., 2010). However, the present study found that SQSTM1 protein expression and immunofluorescent level significantly increased in quinpirole-treated primary striatal neurons (Figure 4E-G), suggesting inhibition of autolysosome formation by D2R over-activation. Overall, these results indicated that D2R overactivation failed to remove damaged mitochondria by mitophagy. Quinpirole could initiate the formation of autophagosome, but not process to autolysosome, indicating D2R overactivation results in an incompletion of mitophagy.
A

LC3-I  

-16 kDa  

LC3-II  

-14 kDa  

β-actin  

-45 kDa

B

![Graph showing LC3-I/Actin and LC3-II/Actin levels for Ctrl and Quin.](image)

C

![Immunofluorescence images showing Mitotracker and LC3 II for Control and Quinpirole treatments.](image)

D

![Bar graph showing Pearson's coefficient for Mitotracker/LC3 for Ctrl and Quin.](image)
Figure 4.4 Overactivation of D2R inhibits mitophagy. (A-B) Immunoblot analysis of LC3-I and LC3-II expression after quinpirole treatment. (C-D) Representative images and quantification of the co-localization of LC3-II with mitochondria. Cultured striatal neurons after quinpirole treatment were co-stained with LC3 II and Mitotracker Green. (E-F) Immunoblot analysis of SQSTM1 expression after quinpirole treatment. (G) Representative immunofluorescent images of the up-regulation of SQSTM1 in primary striatal neurons. N=3, experiments were performed in triplicate. Data were shown as means ± SEM. ** $p < 0.01$ or * $p < 0.05$ compared with the controls.
4.5 D2R overactivation impairs neurite in cultured striatal neurons

It has been reported that dysfunctional mitophagy inhibits neurite growth (Chakravorty et al., 2019). Since the above study showed that the mitophagy process was inhibited by D2R overactivation, a further experiment was designed to determine whether this incomplete mitophagy affects neurite growth. The cultured striatal neurons were incubated with 20 µM quinpirole for 24 hours, which was demonstrated to inhibit mitophagy (Figure 4). The result showed that quinpirole treatment significantly shortened the average length of neurite compared with the control group (Figure 5 A and B). Moreover, the number of branches was also significantly decreased in primary striatal neurons treated with quinpirole (Figure 5C). These findings suggest that incomplete mitophagy induced by D2R overactivation may impair neurite.
Figure 4.5 D2R overactivation suppresses neurite outgrowth in the primary striatal neurons. (A) Representative immunofluorescent images for cultured striatal neurons treated with or without quinpirole at 20 μM for 24 hours. Scale bar=100 μm. (B) Quantification of neurite length of striatal neurons treated with or without quinpirole. (C) Quantification of neurite branches of striatal neurons treated with or without quinpirole. N=3, experiments were performed in triplicate. Values were shown as means±SEM. *** p < 0.001; **** p < 0.0001.
Discussion

Over-stimulation of D2R damages the ultrastructure of neuronal mitochondria with fewer cristae observed in Cryo-ET. D2R specific agonist quinpirole causes elevation of ROS production and MDA level in cultured striatal neurons. Notably, SQSTM1 is unable to be degraded after quinpirole treatment, indicating the mitophagy cannot be completed due to D2R overactivation.

In the current study, our cryo-ET analysis firstly provided strong evidence that overactivation of D2R damages mitochondrial ultrastructure in primary striatal neurons. Conventional EM observations have shaped much of our current knowledge about mitochondrial alterations in schizophrenia (Sorra and Harris, 2000; Harris and Weinberg, 2012). For example, a reduced density of mitochondria is reported in the striatum and prefrontal cortex of post-mortem brains in schizophrenia subjects (Uranova et al., 2001). However, ultrastructural studies of mitochondria are only possible with well-preserved brain tissue within short post-mortem intervals. Furthermore, the sample preparation of conventional EM requires the chemical fixation and embedding process, which are highly possible to cause damage and shrinkage of mitochondrial structures (Siegmund et al., 2018). In this study, the live striatal neurons were applied plunge-freezing within seconds, which significantly improves structural preservation. Frozen striatal neurons on the grid were directly observed in the Cryo-EM chamber without sectioning. This study was the first to report that mitochondrial cristae were damaged after D2R was over-stimulated in striatal neurons, indicating the critical role of D2R in controlling mitochondrial integrity. A similar result was also reported that more than 90% of mitochondria show damaged cristae in dopamine treated human neuroblastoma cells (Zilocchi et al., 2018). Although D2R was linked to mitochondria function
before, its role in mitochondrial ultrastructure has not been revealed so far. Further experiments may need to study the effects of D2R activation on mitochondrial function, such as ATP synthesis.

This study is the first to demonstrate that D2R was located on the neuronal mitochondria. Accumulating evidence demonstrates that mitochondria are essential targets for the actions of dopamine (Burbulla et al., 2017, Chen et al., 2008, Devine and Kittler, 2018), suggesting that the potential existence of D2R on mitochondria. This study reported that the existence of D2R immunoblotting signal in isolated mitochondria of striatal neurons. Importantly, images revealed that D2R is co-localized with mitochondria on the neurite of striatal neurons, suggesting mitochondrial D2R may have a role in regulating neurite growth. One study showed that D2R is located around the mitochondrial membrane by transmission electrical microscopy, although the author did not mention the existence of mitochondrial D2R (Lane et al., 2012). Taken together, understanding D2R subcellular localization can actively contribute to receptor function and even disease pathology. As the new roles of mitochondrial D2R need to be defined, the development of antipsychotics to both mitochondrial and membrane D2R would produce better therapeutic outcomes for patients.

The current study provided evidence that the overactivation of D2R by quinpirole increases cellular ROS production in primary striatal neurons. Although many studies have already revealed that dopamine causes oxidative stress through dopamine receptors, the role of D2R in ROS production is not fully explained. A previous study demonstrated that dopamine shows no effect on ROS production and huntingtin protein aggregation in striatal neurons cultured from D2R knockout mice, suggesting that D2R mediates oxidative stress and neurotoxicity (Charvin et
The mitochondrion is a significant site for ROS production. The uncontrolled ROS accumulation can further damage mitochondria (Itoh et al., 2013). The findings confirmed that overactivation of D2R by its specific agonist increases mitochondrial ROS production in neurons and isolated mitochondria.

Further, this study demonstrated that the excess of mitochondrial ROS induced by D2R overactivation evoked membrane lipoxidation, indicated by increased MDA. To my knowledge, this study was the first to reveal the role of D2R in regulating oxidative stress in striatal neurons. Interestingly, neuronal cultures exposed to dopamine show oxidative stress as well as a significant decrease in dendritic spines (Beckhauser et al., 2016). Overactivation of D2R by quinpirole impairs neurite and synaptic spines in cortical and striatal neurons (Zheng et al., 2018). Consistent with previous findings, the current study showed that quinpirole at 20 µM decreased neurite length and branches. The current study implies that excessive ROS generation may underly the mechanism of neuronal impairment caused by D2R. Further experiments are required to explore the effects of D2R on the antioxidant system in the brain.

Mitophagy belonging to a type of autophagy, which aims to eliminate damaged mitochondria in schizophrenia (Tomoda et al., 2019). Although several published findings reported that mitophagy is inhibited by antagonizing or down-regulating D2R, whether over activating D2R will inhibit the mitophagy process is not well understood (Klein et al., 2019). In the present study, quinpirole un-regulated LC3-II expression, suggesting that promoted autophagosome formation at the initial stage of mitophagy. The increased expression of SQSTM1 suggests that autophagic clearance or transportation is impaired (Su et al., 2018). In this study, although autophagosome formation was initiated by D2R overactivation with quinpirole, the
SQSTM1 was unable to be degraded, suggesting that autophagic clearance was inhibited in primary striatal neurons. The findings in the present study are supported by the previous study in which dopamine treatment can also increase the amount of LC3-II and SQSTM1 in N2a cells leading to the failure of autophagosome degradation (da Luz et al., 2015). Another study also reported that overactivation of D2R inhibits autophagy, causing the misfolding and aggregation of the mutated huntingtin protein in neuroblastoma cells (Vidoni et al., 2016). A recent study reported that the developing schizophrenia rodent model exhibits an elevated expression of SQSTM1 in the prefrontal cortex, suggesting that autophagy is deficient in schizophrenic subjects (Sumitomo et al., 2018). Akira Sawa et. al. also proposed that elevated SQSTM1 may be a novel mechanism underlying synaptic functions and cognitive impairment in neurological and neuropsychiatric disorders (Tomoda et al., 2019). The present study reported that D2R overactivation damaged neurite growth concomitantly with incompleteness of mitophagy. Taken together, our findings indicate that the inhibited autophagic clearance may play a role in neurite impairment caused by D2R overactivation.

In conclusion, this study elucidates that D2R overactivation damages mitochondria and inhibits ROS-mediated mitophagy. The identification of mitochondrial D2R may provide a new target for treating neurite deficits, and oxidative stress occurred in schizophrenia.

References


5.1 Overall Discussion and Conclusions

This thesis demonstrates that neurite impairment caused by D2R overactivation is associated with the increased D2R-DISC1 complex formation and incomplete mitophagy. Prevention of neurite damage by antipsychotic drugs and cell-penetrating peptide of the D2R-DISC1 complex is also examined in this thesis. This chapter will begin by reviewing the significant findings from each chapter and then provide a general discussion of the key findings in my Ph.D. project.

This study firstly discovered the unrecognized signaling pathway where neuronal impairment caused by D2R overactivation is through D2R-DISC1 complex formation. The excessive formation of the D2R-DISC1 complex decreased the phosphorylation of Akt and GSK-3β in cortical neurons. This study further showed that the downregulation of GSK-3β phosphorylation occurs in cortical pyramidal neurons. Although both atypical antipsychotic drug aripiprazole and typical antipsychotic drug haloperidol prevented neurite lesion, aripiprazole displayed better protection on the neurite length than haloperidol. Using DISC1 mutation mice, this study also demonstrated that haloperidol and aripiprazole prevented neurite impairment through DISC1.

This thesis has also contributed with the first description that TAT-D2pep disrupted the interaction between D2R and DISC1 to prevent neurite lesion, suggesting this complex would be a potential therapeutic target in treating neurite deficits. Finally, for the first time, this study reported that mitochondrial D2R existed on the neurite of primary striatal neurons. This study also demonstrated that mitophagy inhibition
might be another mechanism underlying membrane and mitochondrial D2R over-
activation.

Overall, these findings have provided extensive new knowledge on the mechanism
of D2R in regulating neuroplasticity. We have also addressed several gaps in the
literature by identifying neuronal D2R-DISC1 complex and mitochondrial D2R
that can underlie D2R signaling in neuronal protection. The main findings of this
thesis are summarized in Table 1, Table 2, and Table 3.
Table 5.1: Summary of main findings in cortical neurons

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*NSC indicates no significant change.
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Table 5.3: Summary of main findings in HEK-293 cells (D2R and DISC1 plasmid transfected)

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Abbreviations: D2R: Dopamine D2 receptor; DISC1: Disrupted-in-Schizophrenia 1; LI: Locus impairment; GSK3β: Glycogen synthase kinase 3 beta; Akt: Protein kinase B (PKB); NPY: Neuropeptide Y; Cryo-ET: Cryo electron tomography; ROS: Reactive oxygen species; MDA: Malondialdehyde; LC3: Microtubule-associated proteins 1A/1B light chain 3; SQSTM1: Sequestosome 1; ↑: increase; ↓: decrease; NSC*: no significant change versus control group; NSC#: no significant change versus quinpirole group; *P< 0.05 versus control; **P< 0.01 versus control; ***P< 0.001 versus control; ****P< 0.0001 versus control; #P< 0.05 versus quinpirole; ##P< 0.01 versus quinpirole; ###P< 0.001 versus quinpirole; ####P< 0.0001 versus quinpirole;
5.1.1 Overall Discussion of the Findings

In Chapter 2, it is the first time to demonstrate that D2R overactivation damages neural networks through the excessive formation of the D2R-DISC1 complex. Quinpirole induced D2R overactivation has been widely used as a model to study the mechanisms of schizophrenia and other psychiatric disorders (Kostrzewa et al., 2016, Stuchlik et al., 2016). The current research reveals that quinpirole significantly reduces neurite length and branches by increasing the formation of the D2R-DISC1 complex. It has been previously reported that quinpirole at 100 µM inhibits neurite outgrowth, while quinpirole at 1 µM mainly promotes axonal outgrowth, suggesting that D2R activation affects neuronal morphology in a concentration-dependent manner (Reinoso et al., 1996). In addition, the excessive formation of the D2R-DISC1 complex decreased the expressions of synaptophysin and PSD-95, suggesting that the increased D2R-DISC1 complex could weaken synaptic connections. Chapter 2 also indicates that DISC1 is necessary for neurite outgrowth by using cultured cortical neurons from DISC1-LI mice. In DISC1-LI mice, the exons from 1 to 3 in the N-terminal of DISC1 are deleted, which makes DISC1 lose function and abolishes DISC1 binding with D2R. Thus, I did not observe significant FRET signals from the D2R-DISC1 complex in cortical neurons of DISC1-LI mice. It has been demonstrated that DISC1 mutation mice display enhanced dopamine function associated with psychiatric disorders (Tomoda et al., 2016). In fact, DISC1 mutation shows no effect on D2R density in the striatum, but a remarkable rise in the affinity of D2R is observed in rats (Trossbach et al., 2016). These crucial pieces of evidence explain my study that the Akt-GSK3 signaling could be suppressed by DISC1-LI induced D2R hyperfunction. In addition, DISC1, as a scaffolding protein, promotes neuronal development by interacting with other
signaling molecules, such as GSK3β (Ishizuka et al., 2006, Lipina et al., 2011). Interestingly, my study demonstrated that quinpirole treatment disassociates the interaction between DISC1 and GSK3β, leading to more DISC1 binds to D2R in cortical neurons. Cortical neurons of DISC1-LI mice showed decline in neurite length and branches, suggesting that the functional domain of DISC1 plays a critical role in maintaining the neuronal morphology. This study provided novel proof that dysfunction of the N-terminal of DISC1 or excessive D2R-DISC1 complex results in neurite impairment.

The findings of Chapter 2 further indicate that haloperidol and aripiprazole have different mechanisms to prevent neurite lesions caused by the excessive D2R-DISC1 complex formation. The typical and atypical antipsychotic drugs displayed different neuroprotective effects in the developing brain (Lieberman et al., 2008). Clinically, aripiprazole has better efficacy in treating cognitive symptoms than haloperidol in the onset of schizophrenia (Girgis et al., 2011). Further, haloperidol has been shown to be cytotoxic to neurons in a concentration-dependent manner via oxidative stress (Gasso et al., 2012). In the current study, I first identified that both haloperidol and aripiprazole could block the quinpirole-induced formation of the D2R-DISC1 complex in cultured cortical neurons. Haloperidol and aripiprazole restored the neurite length and branches to normal by disassociating the interaction between D2R and DISC1, suggesting the D2R-DISC1 complex mediates the neuroprotection by haloperidol and aripiprazole. However, haloperidol showed no effects on the Akt-GSK3 signaling pathway compared with aripiprazole, suggesting that haloperidol and aripiprazole affect neuroplasticity via different pathways. It has been previously reported that aripiprazole but not haloperidol prefers to target on β-arrestin2 dependent signaling cascades, which leads to primarily affecting the
activity of GSK3β (Masri et al., 2008, Urs et al., 2017). Importantly, for cultured cortical neurons from DISC1-LI mice, neither haloperidol nor aripiprazole could prevent neurite lesion induced by quinpirole. This result indicates that DISC1 is necessary for haloperidol and aripiprazole to protect neurite, which may provide a novel target when designing antipsychotic drugs.

In Chapter 3, the study found that D2R interacts with DISC1 within dendritic spines, which is related to the dendritic spine density of cultured striatal neurons. A previous study observed a reduction of spine density in a schizophrenia-like animal model (Solis et al., 2009). Accumulating evidence has indicated that protein-protein interactions exist in the synapse to affect synaptic functions. It has been recently reported that the altered interaction between nanomodules within synapse could influence the size and density of dendritic spines (Hruska et al., 2018). Here, using STORM imaging, the existence of D2R and DISC1 is firstly revealed in a nanocluster type instead of a single molecule. Consistent with recent super-resolution findings, D2R forms oligomers in higher-order in the mouse brain (Onishi et al., 2018). In addition, the diameter of D2R and DISC1 nanoclusters was not changed by quinpirole. In the current study, I demonstrated that quinpirole decreased the distance between D2R and DISC1 nanoclusters to form D2R-DISC1 complex in a single dendritic spine. STORM imaging showed that the complex formed by D2R and DISC1 could be potential synaptic nanomodules. FRET is a widely used fluorescence technique to study protein-protein interaction within cells. In Chapter 2, I used FRET technique to quantify the formation of D2R and DISC1 complex after drugs treatment. This study reports the relationship between the actual distance of proteins and FRET occurrence, which is the first time that FRET has been elucidated from a nanoscale perspective. FRET has been proven to be an
invaluable technique to detect protein interactions in living cells. And most of the super-resolution microscopy methods are also suitable for living cells. Thus, it is very interesting to observe at high-resolution where and when the protein interactions take place by applying FRET in a super-resolution setting. In addition, this study further revealed that disassociating the D2R-DISC1 complex by TAT-D2pep restores the dendritic spine density of cultured neurons, indicating that synaptic D2R-DISC1 complex could be involved in the synaptic formation. Since dendritic spines have different morphologies (thin, stubby, and mushroom) associated with learning and memory (Bourne and Harris, 2007), it is interesting to investigate that the role of synaptic D2R-DISC1 complex in spine morphology. Similar to the findings in Chapter 2, TAT-D2pep also prevented neurite lesion induced by the excessive formation of D2R–DISC1 complexes by upregulating synaptophysin and PSD-95 level. During the process of neurodevelopment, synaptophysin and PSD-95 serve as essential regulators (Park et al., 2013, Zhang et al., 2016). Increasing interaction between PSD-95 and DISC1 facilitates dendritic spine growth (Hayashi-Takagi et al., 2010). As discussed in Chapter 2, the excessive D2R-DISC1 complex formation decreases the GSK3β-DISC1 interaction. Here, when more DISC1 binds to D2R by quinpirole, less DISC1 may interact with PSD-95, leading to the reduction of dendritic spine density in cultured striatal neurons. The results of synaptophysin and PSD-95 in Chapter 2 and Chapter 3 indicates that synaptophysin and PSD-95 mediates the effect of D2R-DISC1 complex on neuronal morphology. Striatal D2R hyperactivity is associated with the deficits of conditional learning, temporal precision, and working memory in mice selectively overexpress D2R in the striatum (Kellendonk et al., 2006). Although schizophrenia patients also have similar cognitive deficits (Gold et al., 2000,
Carroll et al., 2008, Tek et al., 2002), it may not be appropriate to superimpose the animal data to humans. More human research is required in this regard. In addition, findings of Chapter 3 originally reported that D2R overactivation directly decreased NPY expression by forming excessive D2R-DISC1 complexes, which were blocked by TAT-D2pep. Previously, it has been reported that D2R overactivation caused by amphetamine remarkably reduce NPY expression in rat, suggesting that NPY is regulated by D2R (Kuo, 2003). Here I provided further evidence that D2R regulates NPY expression through the D2R-DISC1 complex. NPY has been found mainly existed in GABAergic interneurons and contributes to neuroplasticity and synaptic communications in the brain (Gotzsche and Woldbye, 2016). Our lab recently reported that the reduction of NPY expression caused by D2R antagonist haloperidol in striatal GABAergic interneurons leads to neuronal damage (Hu et al., 2018). This finding further explains that why haloperidol is not good in protecting neurite compared with TAT-D2pep even though both haloperidol and TAT-D2pep can block D2R-DISC1 complex formation. Our lab has previously demonstrated that the administration of atypical antipsychotic drugs olanzapine remarkably upregulated mRNA and protein expression levels of NPY in the schizophrenia rat model (Huang et al., 2006, Lian et al., 2014). The findings of Chapter 3 suggest that NPY is associated with the neuroprotective effects of TAT-D2pep on neuronal morphology of cultured striatal neurons.

In Chapter 4, this study revealed that oxidative stress and incomplete mitophagy are involved in the neurite impairment caused by D2R overactivation in striatal neurons. This thesis has shown that over activating D2R significantly damaged neural networks in Chapter 2 and Chapter 3. Since neuronal growth is a highly energy-demanding process that majorly depends on dendritic mitochondria, I
assumed that D2R overactivation might result in mitochondrial damage. In the current study, our cryo-ET analysis firstly provides strong evidence that overactivation of D2R damaged mitochondrial ultrastructure in primary striatal neurons. This study was the first to report that mitochondrial cristae were damaged after D2R was over-stimulated in striatal neurons, indicating the important role of D2R in controlling mitochondrial integrity. A similar result was also reported that more than 90% of mitochondria showed damaged cristae in dopamine treated human neuroblastoma cells (Zilocchi et al., 2018). Further experiments needed to study the effects of D2R activation on mitochondrial function, like ATP synthesis. Although many studies have already revealed that dopamine causes oxidative stress through dopamine receptors, the role of D2R in ROS production is not fully explained. The mitochondrion is a major site for ROS production, and unrestrained ROS accumulation can further injure mitochondria. This study confirmed that overactivation of D2R by its specific agonist increased mitochondrial ROS production in neurons and isolated mitochondria, indicating hyperdopaminergia induced ROS generation is conceivably through D2R. To my knowledge, this is the first time to reveal the role of D2R in regulating oxidative stress in striatal neurons. Interestingly, neuronal cultures exposed to dopamine show oxidative stress as well as a significant decrease in dendritic spines (Beckhauser et al., 2016). The current study implied that excessive ROS generation might underly the mechanism of neuronal impairment caused by D2R. The results may be responsible for part of the symptoms of schizophrenia, particularly those involving cognitive functions (Bitanihirwe and Woo, 2011). At the initial stage of mitophagy, quinpirole promoted autophagosome formation through an un-regulating LC3-II level. p62 is the receptor protein that delivers the injured organelles and potentially toxic protein
aggregates to autophagosomes (Vidoni et al., 2016). The increased expression of p62 suggests that autophagic clearance or transportation is impaired (Su et al., 2018). Although the autophagosome formation was initiated, this study reported the expression of p62 was up regulated following quinpirole treatment, indicating that autophagic process was inhibited. Consistent with previous study, dopamine treatment can also increase amount of LC3-II and p62/SQSTM1 in N2a cells leading to the failure of autophagosome degradation (da Luz et al., 2015). Akira Sawa et. al. proposed that elevated p62 pathology may serve as a mechanism underlying synaptic functions and cognitive impairment in neurological and neuropsychiatric disorders (Tomoda et al., 2019). Taken together, this study revealed that the inhibited autophagic flux might be a potential consequence of D2 receptor overactivation. Notably, I originally demonstrated that D2R located on the neuronal mitochondrion and regulated mitochondrial ROS production. Activation or inhibition of mitochondrial GPCR appears to affect mitochondrial processes such as ROS production and cell apoptosis (Jong et al., 2018). Importantly, I visualized that D2R is co-localized with mitochondria on the neurite of striatal neurons, suggesting mitochondrial D2R may have a role in regulating neurite growth. Taken together, understanding D2R subcellular localization can strongly contribute to receptor function and even disease pathology.

5.1.2 Proposed mechanisms of D2R overactivation induced neurite impairment in different cell models

The main aim of this dissertation is to understand how D2 overactivation would affect neurite outgrowth and dendritic spine formation. The rationale for this work is based on evidence that D2R overactivation exists in psychiatric disorder accompanied by neuronal damage. The findings from the studies provide valuable
insights for the mechanism through different cell models. As discussed in Chapter 2, the excessive D2R-DISC1 complex formation was firstly reported to inhibit neurite outgrowth in cortical neurons. The cerebral pathology of schizophrenia has been studied extensively in recent decades. Deficits in neurite density are fundamental to the reduction in cerebral volume in early psychosis (Rae et al., 2017). Findings of Chapter 2 elucidate that the excessive D2R-DISC1 is involved in the neurite deficits of cortical neurons, suggesting that the D2R-DISC1 could be a pharmacological target to rescue neurons. Subsequently, the intracellular signaling pathway of D2R was inhibited by over activating D2R, including reduced expression of phosphorylated Akt and GSK3β. Interestingly, the reduced phosphorylation of GSK3β could further disassociate GSK3β-DISC1 complex leading to more DISC1 binds to D2R. Finally, the excessive D2R-DISC1 complex caused by D2R overactivation damaged neurite of cortical neurons. To prevent neurite impairment caused by D2R overactivation, this study further applied typical antipsychotic drug haloperidol and atypical antipsychotic drug aripiprazole, which are also D2R antagonists. Surprisingly, although haloperidol and aripiprazole blocked quinpirole induced D2R-DISC1 formation, they displayed different effects on Akt-GSK3β signaling and synaptic proteins. Haloperidol had no effects on the phosphorylation of Akt and GSK3β, suggesting that the D2R independent signaling pathway is not influenced by typical antipsychotic drug haloperidol. Furthermore, aripiprazole reversed the quinpirole-induced reduction of synaptophysin and PSD-95, while haloperidol had no effect on PSD-95 expression. Studies have shown that both haloperidol and aripiprazole can stimulate mTOR signaling (Bowling et al., 2014, Ishima et al., 2012). Activation of mTOR signaling is associated with neurite outgrowth, branching, and spine density (Kim et al., 2009, Speranza et al., 2015,
He et al., 2019). Conversely, mTOR inhibitor rapamycin prevents neurite outgrowth-induced by haloperidol and aripiprazole (Bowling et al., 2014, Ishima et al., 2012). This may explain the difference in PSD-95 expression between the two drugs, but the same neurite growth effect observed in this study.

As DISC1 is essential in brain neuroplasticity, this study also examined whether DISC1 would mediate the effects of haloperidol and aripiprazole on neuroplasticity. The binding site of D2R and DISC1 is abolished in DISC1-LI mice. Notably, neither haloperidol nor aripiprazole protected neurite of cultured DISC1-LI cortical neurons, displaying no alternations in Akt-GSK3β signaling and synaptic proteins compared with quinpirole treated group. This study for the first time demonstrates that haloperidol and aripiprazole protect neuroplasticity via DISC1, which may provide further evidence for designing novel antipsychotic drugs. Further, in vivo experiments need to be performed to study whether haloperidol and aripiprazole could ameliorate cognitive decline in the schizophrenia animal model by decreasing the D2R-DISC1 complex.
(Figure legend on next page)
Figure 5.1 Proposed mechanisms of D2R-DISC1 induced neurite impairment in cultured cortical neurons. In cortical neurons of wild type mice, ① D2R overactivation induced by quinpirole increased the D2R-DISC1 complex formation. Both haloperidol and aripiprazole blocked the D2R-DISC1 complex formation induced by quinpirole. Administration of 100 µM quinpirole for 48 hours significantly inhibited ② Akt phosphorylation on Thr308 and Ser473 and ③ GSK3β phosphorylation on Ser9. ④ The declined phosphorylation of GSK3β Ser9 then led to disassociation of GSK3β-DISC1 complex, which lets more DISC1 bind to D2R. Aripiprazole and haloperidol displayed different effects on Akt-GSK3 signaling. Aripiprazole prevented the reduction of ② Akt phosphorylation on Thr308 and Ser473 and ③ GSK3β phosphorylation on Ser9. The elevated formation of the D2R-DISC1 complex caused by quinpirole significantly inhibited the expression of ⑤ synaptophysin and ⑥ PSD-95. Aripiprazole reversed the quinpirole-induced reduction of ⑤ synaptophysin and ⑥ PSD-95, while haloperidol has no effect on PSD-95 expression. Finally, the excessive formation of the D2R-DISC1 complex caused by quinpirole significantly decreased ⑦ neurite length and ⑧ branches in cortical neurons. In cortical neurons of DISC1-LI mice, ⑨ the D2R-DISC1 complex formation is abolished because the binding site on DISC1 is deleted. Quinpirole exacerbates the decreased phosphorylation of ⑩ Akt on Thr308 and Ser473 and ⑪ GSK3β on Ser9 compared with wild type neurons. Aripiprazole and haloperidol were unable to fully reverse the phosphorylation of ⑩ Akt on Thr308 and Ser473 and ⑪ GSK3β on Ser9. Further, both haloperidol and aripiprazole failed to change the decreased expression of synaptic proteins ⑫ synaptophysin and ⑬ PSD-95 caused by quinpirole. Finally, haloperidol and aripiprazole failed to rescue ⑭ neurite impairment in cortical neurons of DISC1-LI
mice.

Abbreviations: Quin: quinpirole at 100 µM; Halo: haloperidol at 10 µM; Ari: aripiprazole at 10 µM. D2R: dopamine D2 receptor; SYN: synaptophysin; DISC-LI: DISC1 locus impairment. Blue arrow: quinpirole treatment; Red arrow: pretreatment of haloperidol in the presence of quinpirole at 10 µM. Green arrow: pretreatment of aripiprazole in the presence of quinpirole at 10 µM. Yellow arrow: cortical neurons of DISC1 locus impairment mice.

It has been demonstrated that D2R abundantly expresses in the striatum, which makes striatal D2R is the major target for antipsychotic drugs. Based on the findings in Chapter 1, the thesis further investigated the role of D2R-DISC1 in the synaptogenesis of striatal neurons by using super-resolution microscopy. Notably, this thesis firstly identified that the interaction between D2R and DISC1 exited in a single dendritic spine of striatal neurons, suggesting that the synaptic D2R-DISC1 complex could be a novel nanomodule consisting of synaptic architecture. As discussed in Chapter 2, I used FRET technology to demonstrate whether the D2R-DISC1 complex was formed, which showed the distance of D2R and DISC1 is less than 10 nm. In this study, the STORM images quantified the actual distance of D2R and DISC1 nanoclusters in synapses, which provides the possibility of using a super-resolution technique to quantify fluorescent-based FRET. The cell-penetrating peptide, TAT-D2pep, targeted explicitly on separating excessive synaptic D2R-DISC1 complex induced by quinpirole. The findings from STORM also expanded our understandings that D2R and DISC1 existed in dendritic spines with nanoclusters manner instead of single molecular. The density of D2R and DISC1 nanoclusters were decreased by quinpirole, which was prevented by cell-penetrating peptide TAT-D2pep. Further, this study reported that the excessive
synaptic D2R-DISC1 complex was accompanied by a reduction in dendritic spine density. TAT-D2pep protected dendritic spines, suggesting that the D2R-DISC1 complex plays an important role in regulating spine formation. In addition, this study for the first time reveals that the protective effects of TAT-D2pep on neurite is through GSK3β-NPY signaling in striatal interneurons. Accumulating evidence indicated that D2R is involved in regulating the mitophagy process in several diseases, like Parkinson’s disease. Here, the findings showed that D2R overactivation significantly increased intracellular and mitochondrial ROS levels. Although the excessive ROS production initiated autophagosome formation, the formation of autolysosome was inhibited due to over activating D2R. Interestingly, D2R was firstly identified on the mitochondrial membrane. Overactivation of mitochondrial D2R led to the decreased number of folded cristae and length of cristae, which provides novel targets for preventing mitochondrial damage.
(Figure legend on next page)
**Figure 5.2 Proposed mechanisms of D2R-DISC1 induced neurite impairment in cultured striatal neurons.** In striatal neurons, ① the D2R-DISC1 complex formation was significantly increased by quinpirole at 10 µM on neurite and soma. Preincubation with TAT-D2pep uncoupled excessive D2R-DISC1 complex induced by quinpirole. The excessive D2R-DISC1 complex formation induced by quinpirole then decreased the ② GSK3β phosphorylation on Ser9 and ③ NPY, while TAT-D2pep reversed the declined GSK3β phosphorylation and NPY in striatal interneurons. The inhibited GSK3β signaling pathway then led to a reduction in the intensity of ④ synaptophysin and ⑤ PSD-95 on neurite, which was reversed by TAT-D2pep. TAT-D2pep protected neurite growth by disrupting the D2R-DISC1 complex, increasing GSK3β signaling, and synaptic protein expression. In a single dendritic spine of cultured striatal neurons, the interaction between D2R and DISC1 was observed by using super-resolution microscopy (STORM). ⑥ STORM images revealed that the distance between D2R and DISC1 nanoclusters was reduced in dendritic spines after quinpirole treatment. TAT-D2pep increased the distance between D2R and DISC1 nanoclusters in the presence of quinpirole. In addition, the density of ⑦ D2R and ⑧ DISC1 nanoclusters were decreased after the D2R-DISC1 complex was excessively formed by quinpirole in dendritic spines. TAT-D2pep restored the density of D2R and DISC1 nanoclusters by decreasing the interaction between D2R and DISC1. Further, the excessive formation of the D2R-DISC1 complex in single dendritic spines significantly reduced the ⑨ spine density, which was reversed by TAT-D2pep. In mitochondria of cultured striatal neurons. In addition, D2R overactivation significantly increased ⑩ the intracellular ROS level. Although the excessive ROS production initiated ⑪ autophagosome formation (LC3 II / LC3 I), ⑫ the formation of autolysosome.
(SQSTM1) was inhibited due to over activating D2R. The incomplete mitophagy failed to eliminate ROS leading to MDA accumulation within striatal neurons. The excessive D2R-DISC1 complex formation and incomplete mitophagy impaired neurite outgrowth. D2R was firstly identified on the mitochondrial membrane. Overactivation of D2R led to mitochondrial ROS production, resulting in a decreased number of folded cristae and length of cristae. Thus, D2R-DISC1 complex induced oxidative stress may also involve in neurite lesion.

Abbreviation: Quin: quinpirole at 10 µM; TAT-D2: TAT-D2pep at 10 µM. ROS: reactive oxygen species. Blue arrow: quinpirole treatment; Orange arrow: Pretreatment of TAT-D2pep in the presence of quinpirole at 10 µM.

To specifically determine which signaling molecule is influenced by the D2R-DISC1 complex, this study constructed a HEK-293 cell model that was transfected with D2R and DISC1 plasmids. Consistent with the findings in cortical neurons and striatal neurons, D2R overactivation induced by quinpirole significantly increased the formation of the D2R-DISC1 complex. D2R antagonist haloperidol and cell-penetrating peptide TAT-D2pep disrupted the excessive formation of the D2R-DISC1 complex. Increased interaction of D2R and DISC1 induced by quinpirole further decreased the GSK3β phosphorylation on Ser 9. The decreased GSK3β phosphorylation induced by D2R-DISC1 interaction was reversed by TAT-D2pep but not D2R antagonist haloperidol. The expression of NPY was inhibited by quinpirole, which can be restored to the control level by TAT-D2pep. Finally, the expressions of synaptophysin and PSD-95 were reduced by the excessive D2R-DISC1 complex, which was restored to the control level by TAT-D2pep. In HEK-293 cells, I also reported that the excessive D2R-DISC1 complex resulted in
incomplete mitophagy by failing to degrade SQSTM1.

Figure 5.3 TAT-D2pep reverses the reductions of synaptic proteins and autophagy induced by D2R-DISC1 in HEK-293 cells. In HEK-293 cells, the D2R-eGFP and DISC1 mCherry constructs were transfected to generate a cell model for studying the interaction between D2R and DISC1. ①D2R overactivation induced by quinpirole increased the formation of D2R-DISC1 complex more than three folds. The excessive formation of the D2R-DISC1 complex was blocked by D2R antagonist haloperidol and cell-penetrating peptide TAT-D2pep. Further, quinpirole failed to induce D2R-DISC1 complex formation when the specific binding site on D2R was deleted. ②Increased D2R-DISC1 complex induced by quinpirole further decreased the GSK3β phosphorylation on Ser 9. The decreased GSK3β phosphorylation caused by the D2R-DISC1 complex was reversed by TAT-D2pep but not D2R antagonist haloperidol. ③The expression of NPY was inhibited by quinpirole, which can be restored to the control level by TAT-D2pep.
Finally, the expressions of ④synaptophysin and ⑤PSD-95 were reduced by the excessive D2R-DISC1 complex, which was restored to the control level by TAT-D2pep. ⑥ Although the excessive D2R-DISC1 initiated autophagosome formation (LC3 II / LC3 I), ⑦ the formation of autolysosome (SQSTM1) was inhibited due to the excessive D2R-DISC1 complex.

Abbreviation: Quin: quinpirole at 10 µM; TAT-D2: TAT-D2pep at 10 µM. Blue arrow: quinpirole treatment; Orange arrow: Pretreatment of TAT-D2pep in the presence of quinpirole at 10 µM.
5.1 Recommendations for Future Research

According to the findings, the recommendations are made as follows:

1. This study investigated the effect of TAT-D2pep on neuroprotection using cell-based models only. Although the results were promising, a preclinical trial will be necessary to verify the neuroprotective effect and study the consequence of behavioral changes in rodents after TAT-D2pep treatment. It is recommended to use the rodent models with dopamine hyperactivity relevant to schizophrenia together with appropriate controls.

2. Dendritic spines are highly dynamic, which are re-categorized by super-resolution microscopy into two major types: thin and mushroom. Thin spines are more flexible ‘learning spines,’ whereas mushroom spines are more stable ‘memory spines’ (Bourne and Harris, 2007). Although we were the first to link D2R-DISC1 complex to dendritic spine formation in this study, it is still unknown whether this complex will influence thin and mushroom spine dynamics.

3. It is interesting to observe D2R locates on mitochondrion. Due to the access to D2R knockout mice and time limitation, we were unable to clarify the existence of mitochondrial D2R in vivo. A future study could further investigate the D2R is located on the inner or outer membrane of mitochondrion by using the Cryo-EM technique.

4. Last, the measurements of neurite length in this study are from a two-dimensional surface, which may not fully reflect how neurons may grow in the actual environment. Thus, the three-dimensional culturing device may be applied in future research to confirm these neuroprotective effects.
5. It is well known that excessive dopamine activity can cause an onset of psychosis. This is reported in several psychiatric disorders, including schizophrenia, bipolar disorder at manic state, obsessive-compulsive disorder, and some drug abuses. Based on my study, it is likely that hyperdopaminergic activity will cause an abnormal D2R-DISC1 complex; however, such assumptions will require further studies to prove.
5.2 Conclusion

Neurite deficits result in the alternation in neural connection and psychotic symptoms in psychiatric disorders. Here the findings of the present thesis have demonstrated that the excessive D2R-DISC1 complex caused by D2R overactivation had remarkable effects on the neuroplasticity of cortical and striatal neurons. The abnormal D2R-DISC1 complex caused downregulation of phosphorylated Akt and GSK3β, synaptic proteins, and NPY in neurons. This study also provided evidence that haloperidol showed less protection on neurite than aripiprazole, mainly because haloperidol had no effects on the D2R intracellular signaling pathway. The DISC1-LI mutant made the neuroprotective effects of haloperidol and aripiprazole invalid. The new antipsychotic peptide TAT-D2pep was demonstrated to protect neuroplasticity and synapse. This study initially demonstrated that D2R existed on the mitochondrial membrane of neurons. Overactivation of cellular and mitochondrial D2R significantly increased ROS production, which resulted in incomplete mitophagy. Taken together, the D2R-DISC1 complex and mitochondrial D2R might be potential therapeutic targets for treating neurite deficits in schizophrenia patients.
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