2018

Chronic antipsychotic treatment differentially modulates protein kinase A- and glycogen synthase kinase 3 beta-dependent signaling pathways, N-methyl-D-aspartate receptor and γ-aminobutyric acid A receptors in nucleus accumbens of juvenile rats

Bo Pan
University of Wollongong, Yangzhou University, bp355@uowmail.edu.au

Jiamei Lian
University of Wollongong, jlian@uow.edu.au

Chao Deng
University of Wollongong, chao@uow.edu.au

Publication Details
Chronic antipsychotic treatment differentially modulates protein kinase A- and glycogen synthase kinase 3 beta-dependent signaling pathways, N-methyl-D-aspartate receptor and γ-aminobutyric acid A receptors in nucleus accumbens of juvenile rats

Abstract

**Background**: Antipsychotics are developed to treat mental disorders in adults; however, the prescription (mostly "off-label") of antipsychotics for children/adolescents has been constantly increasing over years. The influences of antipsychotics on juveniles requires investigation to validate their clinic use. Antipsychotics mainly exert their effects via several receptors and signaling pathways.

**Aims**: This study examined the effects of aripiprazole, olanzapine, and risperidone on selected signaling pathways, N-methyl-D-aspartate, and γ-aminobutyric acid A receptors in juveniles.

**Methods**: Rats were orally administered aripiprazole (1 mg/kg), olanzapine (1 mg/kg), risperidone (0.3 mg/kg), or vehicle three times/day from postnatal day 23 (±1 day) for three weeks. The effects of antipsychotics in the nucleus accumbens and caudate putamen were measured by Western blots.

**Results**: In the nucleus accumbens, all three drugs differentially increased N-methyl-D-aspartate and γ-aminobutyric acid A receptor expression. Additionally, all three antipsychotics differentially elevated the phosphorylation of glycogen synthase kinase 3 beta, β-catenin, and cAMP-responsive element-binding protein 1. In the caudate putamen, olanzapine increased β-catenin phosphorylation; and aripiprazole and olanzapine elevated γ-aminobutyric acid A receptor levels. Correlation analysis indicated that antipsychotics might modulate N-methyl-D-aspartate receptors via glycogen synthase kinase 3 beta-β-catenin signaling and/or cAMP-responsive element-binding protein 1 activation.

**Conclusions**: These findings suggest that antipsychotics can affect protein kinase A- and glycogen synthase kinase 3 beta-dependent signaling pathways in juveniles; and their modulation on N-methyl-D-aspartate and γ-aminobutyric acid A receptors is probably through glycogen synthase kinase 3 beta-β-catenin signaling and/or cAMP-responsive element-binding protein 1 activation.

**Disciplines**

Medicine and Health Sciences

**Publication Details**


This journal article is available at Research Online: https://ro.uow.edu.au/ihmri/1324
Chronic antipsychotic treatment differentially modulates protein kinase A- and glycogen synthase kinase 3 beta-dependent signalling pathways, N-methyl-D-aspartate receptor and \( \gamma \)-aminobutyric acid A receptors in nucleus accumbens of juvenile rats

Bo Pan\(^{1,4}\), Jiamei Lian\(^{3,4}\), *Chao Deng\(^{3,4}\)

\(^{1}\) The Key Laboratory of Syndrome Differentiation and Treatment of Gastric Cancer of the State Administration of Traditional Chinese Medicine, Yangzhou University Medical College, Yangzhou, China

\(^{2}\) Department of Pharmacy, Yangzhou University Medical College, Yangzhou, China

\(^{3}\) Antipsychotic Research Laboratory, Illawarra Health and Medical Research Institute, Australia

\(^{4}\) School of Medicine, University of Wollongong, Australia

**Corresponding Author:**
Prof. Chao Deng, Illawarra Health and Medical Research Institute, University of Wollongong, Wollongong, 2522, NSW, Australia. E-mail: chao@uow.edu.au
Abstract

Background: Antipsychotics are developed to treat mental disorders in adults; however, the prescription (mostly “off-label”) of antipsychotics for children/adolescents has been constantly increasing over years. The influences of antipsychotics on juveniles requires investigation to validate their clinic use. Antipsychotics mainly exert their effects via several receptors and signalling pathways.

Aims: This study examined the effects of aripiprazole, olanzapine, and risperidone on selected signalling pathways, N-methyl-D-aspartate, and $\gamma$-aminobutyric acid A receptors in juveniles.

Methods: Rats were orally administered aripiprazole (1mg/kg), olanzapine (1mg/kg), risperidone (0.3mg/kg), or vehicle 3 times/day from postnatal day 23 (±1 day) for 3 weeks. The effects of antipsychotics in the nucleus accumbens and caudate putamen were measured by Western Blots.

Results: In the nucleus accumbens, all three drugs differentially increased N-methyl-D-aspartate and $\gamma$-aminobutyric acid A receptor expression. Additionally, all three antipsychotics differentially elevated the phosphorylation of glycogen synthase kinase 3 beta, $\beta$-catenin, and cAMP-responsive element-binding protein 1. In the caudate putamen, olanzapine increased $\beta$-catenin phosphorylation; and aripiprazole and olanzapine elevated $\gamma$-aminobutyric acid A receptor levels. Correlation analysis indicated that antipsychotics might modulate N-methyl-D-aspartate receptors via glycogen synthase kinase 3 beta -$\beta$-catenin signalling
Conclusions: These findings suggest that antipsychotics can affect protein kinase A- and glycogen synthase kinase 3 beta-dependent signalling pathways in juveniles; and their modulation on N-methyl-D-aspartate and γ-aminobutyric acid A receptors is probably through glycogen synthase kinase 3 beta-β-catenin signalling and/or cAMP-responsive element-binding protein 1 activation.

Declaration of interest/Finding: None of the authors has a conflict of interest. This work was supported by the National Health and Medical Research Council (APP1104184), Australia.

Keywords:
Antipsychotics, protein kinase A, glycogen synthase kinase 3 beta, N-methyl-D-aspartate receptor, γ-aminobutyric acid A receptor, juvenile animals, aripiprazole, olanzapine, risperidone
Introduction

Over the past decade, the prescription of antipsychotic drugs (mostly off-label use) in children and adolescents has increased markedly (Ronsley et al., 2013; Caccia, 2013). Particularly, risperidone (neuroscience-based nomenclature (NbN): dopamine D$_2$, serotonin 5-HT$_2$, noradrenaline NE α-2 receptor antagonist (Nutt and Blier, 2016)) is accounted for ~70% of total antipsychotic prescriptions (Olfson et al., 2010; Karanges et al., 2014). These antipsychotics are mostly prescribed to treat mental disorders, such as childhood-onset schizophrenia, depression, bipolar disorder, and autism (Schneider et al., 2014). Clinical studies have shown that children/adolescents are more likely to be affected by antipsychotics than adults, especially by the side-effects (Vitiello et al., 2009). Since the pharmacodynamic sensitivity to antipsychotics in children/adolescents is different from that in adults (Caccia, 2013), understanding the pharmacological mechanisms of antipsychotics in children/adolescents is required and might provide important evidence for the prescription of antipsychotics for children/adolescents in clinics.

Our previous evidence demonstrates that various antipsychotics influence G-protein dependent protein kinase A (PKA) signalling and G-protein independent protein kinase B (Akt)-glycogen synthase kinase 3 beta (GSK3β) signalling pathways to exert their effects in the striatum of adult animals (Pan et al., 2016b; Pan et al., 2016a; Pan et al., 2016c; Pan et al., 2015). However, to our knowledge, there is no study that investigates antipsychotic effects on these signalling pathways during the childhood-adolescent period. In addition, it has been widely accepted that antipsychotics are able to exert their effects on several other signalling pathways or substrates. For example, the
dishevelled(Dvl)-GSK3β-β-catenin pathway has been reported to be modulated by various antipsychotics (including aripiprazole (NbN: dopamine D2, serotonin 5-HT1A receptor partial agonist (Nutt and Blier, 2016)), olanzapine (NbN: dopamine D2, serotonin 5-HT2 receptor antagonist (Nutt and Blier, 2016)), risperidone, etc.) in adults (Alimohamad et al., 2005b; Alimohamad et al., 2005a; Sutton and Rushlow, 2011; Seo et al., 2015; Pan et al., 2016a; Pan et al., 2016b). Antipsychotics (e.g. aripiprazole) might also exert therapeutic effects via cAMP-responsive element-binding protein 1 (CREB1) in adult animals (Mavrikaki et al., 2014; Pan et al., 2016c; Pan et al., 2016b). Whether these pathways and substrates are involved in the regulation of antipsychotics in children/adolescents is not clear.

N-methyl-D-aspartate (NMDA) and GABA_A(γ-aminobutyric acid) receptor signalling play key roles in neurodevelopment and the formation of brain core functions, and deficits in these receptors have been considered to be associated with various mental disorders in children/adolescents (Panaccione et al., 2013; Schmidt and Mirnics, 2015; Mouri et al., 2007; Sakamoto et al., 2011). Previous studies have shown that the GABA_A receptor can be regulated by various antipsychotics in adults through dopamine D_2 receptor (D_2R)-downstream PKA signalling, which was also suggested by our recent studies (Skilbeck et al., 2007; Zink et al., 2004; Pan et al., 2016c; Pan et al., 2016b); similarly, modulation of antipsychotics on NMDA receptors (NMDARs) in adults has also been well documented (Schmitt et al., 2003; Segnitz et al., 2011; Pan et al., 2016b); antipsychotics might regulate NMDARs via D_2R-mediated GSK3β and CREB1 signalling in adult rat brains (Pan et al., 2016b). Both NMDA and GABA_A receptors are in an immature from during the postnatal developmental period which may cause animals
to be more sensitive to antipsychotic treatment (Fritschy et al., 1994; Lopez-Tellez et al., 2004; Sheng et al., 1994). Although these antipsychotics target multiple receptors such as dopamine D$_2$ and 5-HT$_{2A}$ receptors, D$_2$Rs play a critical role in their therapeutic effects (Ginovart and Kapur, 2012). Both risperidone and aripiprazole have very high affinity with D$_2$Rs (Correll, 2010). Unfortunately, there are very limited studies that have systematically examined the effects of early treatment with these antipsychotics on the D$_2$R-mediated signalling pathways and substrates during childhood-adolescence, which is the key issue that needs to be addressed in the present study. Furthermore, the striatum, which mainly contains the nucleus accumbens (NAc) and caudate putamen (CPu), is a key brain region that is associated with the pathophysiology of various mental disorders in children/adolescents, such as schizophrenia, autism, depression, and bipolar disorder (DelBello et al., 2006; James et al., 2016; Langen et al., 2009; Gabbay et al., 2013). Therefore, in this study, we investigated the effects of oral treatment (3 times per day) of aripiprazole, olanzapine, and risperidone at a clinical equivalent dosage (a better mimicking of the clinical treatment paradigm) on the above mentioned PKA- and GSK3β-dependent signalling pathways as well as GABA$_A$ and NMDA receptors in the NAc and CPu of juvenile rats.

**Methods**

*Animals and drug administration*

Fourteen timed, pregnant Sprague-Dawley rats were obtained at gestation day 14 from the Animal Resource Centre (Perth, WA, Australia). They were housed individually at 22°C, on a 12h light-dark cycle (lights on: 07:00 AM and light off: 7:00 PM), and allowed *ad libitum* access to water and standard laboratory chow diet throughout the
experiment (Lian et al., 2016). To avoid variations from potential interactions with sexual hormones, twenty-four male pups born from these mother rats were used for this study. The day of birth was considered as postnatal day (PN) 0. On PN21, young male rats ($n = 6$/group) were randomly assigned to one of the following treatments: aripiprazole (1.0 mg/kg, t.i.d., Bristol-Myers Squibb, New York, USA), olanzapine (1.0 mg/kg, t.i.d., Eli Lilly, Indianapolis, IN, USA), risperidone (0.3 mg/kg, t.i.d., Apotex, Macquarie Park, NSW, Australia), or vehicle. Drug powders mixed with the cookie dough pellets was delivered orally 3 times per day at 07:00 AM, 03:00 PM and 11:00 PM (Pan et al., 2016b; Lian et al., 2016) from PN23 (±1 day). The treatment period was 3 weeks, which corresponds to childhood-adolescence in humans (Brenhouse and Andersen, 2011). Controls received equivalent pellets without drugs. All rats were sacrificed and the brains were obtained two hours after the final dose of antipsychotics. The brains were immediately frozen in liquid nitrogen and then stored under -80°C for future use.

The rats were administered antipsychotic drugs three times/day to ensure consistently high concentrations to better mirror the human scenario of oral administration once per day (Lian et al., 2016; Pan et al., 2016b). The dosages were based on the recommended dosages in humans based on body surface area, according to the FDA guidelines for clinical trials (FDA, 2005; Reagan-Shaw et al., 2008), all of which are within the recommended dosage ranges for the psychiatric treatment of children/adolescents (Fraguas et al., 2011; Greenaway and Elbe, 2009). It has been previously reported that, at these used dosages, all these drug reaches 60-80% D$_2$ receptor occupancy rates in the rat brain (Kapur et al., 2003; Natesan et al., 2006). All experimental procedures were approved by the Animal Ethics Committee, University of Wollongong (AE12/20), and
complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (National Health and Medical Research Council, Australia, 2004).

**Brain dissection**

The discrete brain regions were collected using a brain microdissection puncture technique as described previously (Pan et al., 2015; Pan et al., 2016c; Pan et al., 2016b). Specifically, based on the brain atlas (Paxinos and Watson, 2005), three sections through the striatum (Bregma 1.00 to 2.20mm) were dissected for the NAc and CPu, respectively. Dissected tissue was kept at -80°C for future use.

**Western blots**

Frozen brain samples were homogenised in homogenising buffer containing NP-40 cell lysis buffer (Invitrogen, #FNN0021), Protease Inhibitor Cocktail (Sigma-Aldrich, #P8340), β-Glycerophosphate (Sigma-Aldrich, #G9422), and PMSF (Sigma-Aldrich, #P7626). Protein concentration of each sample was measured by the DC Protein Assay (Bio-Rad, #500-0111). Western blot experiments were performed as described previously (Pan et al., 2016b; Pan et al., 2016a; Pan et al., 2016c). Briefly, each sample containing 10μg of protein was denatured, and loaded into Criterion™ TGX™ Precast Gels (Bio-rad, #5671095). The proteins were separated in Criterion™ Vertical Electrophoresis Cells (Bio-rad, #1656001), and then electrophoretically transferred to a polyvinylidene difluoride membrane in Criterion™ Blotters (Bio-rad, #1704071). All membranes were then blocked by 5% skim milk powder, and incubated in primary antibodies and secondary antibodies, respectively. The immunoreactive bands were visualised using Amersham Hyperfilm ECL (GE Healthcare, #28-9068-36) and Luminata Classico.
Western HRP substrate (Millipore, #WBLUC0500). All Western blot experiments were performed in duplicate to ensure consistency.

The following antibodies were used to detect corresponding proteins: anti-PKA-Cα (1:1000; Santa Cruz Biotechnology, #SC-903), anti-phosphor-PKA-C (Thr197) (1:1000; Cell Signaling Technology, #5661), anti-Akt (1:2000; Cell Signaling Technology, #4691), anti-phosphor-Akt (Thr308) (1:1000; Cell Signaling Technology, #13038), anti-GSK3β (1:2000; Cell Signaling Technology, #5676), anti-phospho-GSK3β (Ser9) (1:1000; Cell Signaling Technology, #9322), anti-Dvl-3 (1:1000; Santa Cruz Biotechnology, #SC-8027), anti-β-catenin (1:1000; Santa Cruz Biotechnology, #SC-7963), anti-GABA_β (1:1000; Abcam, #ab154822), anti-CREB1 (1:2000, Abcam, #ab32515), and anti-phospho-CREB1 (1:2000, Abcam, #ab32096). Mouse anti-actin primary polyclonal antibody (1:10000; Millipore, #MAB1501) was used to determine the actin levels. The secondary antibodies were HRP-conjugated anti-rabbit IgG antibody (1:3000; Cell Signaling Technology, #7074) and HRP-conjugated anti-mouse IgG antibody (1:3000; Cell Signaling Technology, #7076).

Statistics

The immunoreactive signals were quantified using Bio-Rad Image Lab (version 6.0). All data were analysed by using SPSS Statistics (version 24.0). The data of each targeted protein were then corrected based on their corresponding actin levels. Data normal distribution was tested using histograms and a Kolmogorov–Smirnov Z-test. One-way analysis of variance (ANOVA) was performed if the data were normally distributed, the post-hoc Dunnett t test was used to compare each drug treatment group with the control
group (using raw data). If the data were not normally distributed, the protein expression in each brain region was analysed by a Kruskal–Wallis H-test, followed by the post-hoc Mann–Whiney U-test. The results of each protein expression were expressed by taking the value of the control group as 100%. The ratios of each phosphorylated proteins were analysed by a Kruskal–Wallis H-test and the post-hoc Mann–Whiney U-test. Pearson’s correlation tests were employed to analyse the relationships among certain measurements. Statistical significance was accepted when \( p \leq 0.05 \).

Results

*The effect of antipsychotics on Akt and GSK3\( \beta \)*

In the NAc, antipsychotic administration did not exert any effects on Akt (Akt, \( F_{3, 20} = 2.486, p > 0.05 \); p-Akt (Thr308), \( F_{3, 20} = 1.182, p > 0.05 \)), whereas GSK3\( \beta \) was significantly affected by antipsychotic treatment (GSK3\( \beta \), \( F_{3, 20} = 4.003, p < 0.05 \); p-GSK3\( \beta \), \( F_{3, 20} = 4.696, p > 0.05 \)). Post-hoc tests have shown that risperidone down-regulated the expression of GSK3\( \beta \) in the NAc by 64.0% (\( p < 0.05 \)); in addition, both aripiprazole (\( p < 0.05 \)) and olanzapine (\( p < 0.01 \)) increased the expression of p-GSK3\( \beta \) (Ser9) by 59.4% and 90.0%, respectively (Figure 1(b), 1(d)). Furthermore, all three antipsychotics significantly elevated the ratio of p-GSK3\( \beta \)/GSK3\( \beta \) (aripiprazole, \( p < 0.01 \); olanzapine, \( p < 0.05 \); risperidone, \( p < 0.01 \)) (Figure 1(b)).

In the CPu, on the other hand, the protein levels of Akt, p-Akt, GSK3\( \beta \), and p-GSK3\( \beta \) were not significantly affected by any antipsychotic administration (Akt, \( F_{3, 20} = 0.910, p > 0.05 \); p-Akt (Thr308), \( F_{3, 20} = 2.159, p > 0.05 \); GSK3\( \beta \), \( F_{3, 20} = 1.671, p > 0.05 \); p-GSK3\( \beta \) (Ser9), \( F_{3, 20} = 0.091, p > 0.05 \)) (Figure 2), nor the ratio of p-Akt/Akt and p-
The effect of antipsychotics on Dvl-3 and β-catenin

No antipsychotic treatment showed significant effect on the protein expression of Dvl-3 in either the NAc or CPu (NAc, $F_{3, 20} = 1.170, p > 0.05$; CPu, $F_{3, 20} = 0.647, p > 0.05$). However, β-catenin was significantly altered in both regions. In the NAc, antipsychotics significantly affected the expression of β-catenin ($F_{3, 20} = 4.430, p < 0.05$); while in the CPu, the levels of p-β-catenin were changed (NAc, $F_{3, 20} = 5.698, p < 0.01$). Post-hoc tests have indicated that in the NAc, both aripiprazole and risperidone significantly reduced the expression of β-catenin by 34.9% ($p < 0.01$) and 24.5% ($p < 0.05$), respectively (Figure 1(c), 1(d)); they also significantly elevated the ratio of p-β-catenin/β-catenin (both $p < 0.05$) (Figure 1(c)). Olanzapine also tended to decrease the expression of β-catenin ($p = 0.63$, -20.6%) and increase the ratio of p-β-catenin/β-catenin in the NAc ($p = 0.092$). In the CPu, only risperidone was able to exert significant effects on the protein levels of p-β-catenin (+139.7%, $p < 0.01$) (Figure 2(c), 2(d)) as well as the ratio of p-β-catenin/β-catenin ($p < 0.01$) (Figure 2(c)).

The effects of antipsychotics on PKA

The protein levels of PKA-C (NAc, $F_{3, 20} = 1.196, p > 0.05$; CPu, $F_{3, 20} = 0.158, p > 0.05$) and p-PKA-C (Thr197) ($F_{3, 20} = 0.409, p > 0.05$; CPu, $F_{3, 20} = 0.644, p > 0.05$) were not significantly affected by any antipsychotic treatment in the two brain regions (Figure 3(a), 3(d), 4(a), 4(d)).

The effects of antipsychotics on CREB1

Significant changes in the protein levels of phosphorylated CREB1 were found in the
NAc (CREB1, $F_{3,20} = 0.449, p > 0.05$; p-CREB1, $F_{3,20} = 4.451, p < 0.05$), but no change was found in the CPu (CREB1, $F_{3,20} = 0.088, p > 0.05$; p-CREB1, $F_{3,20} = 1.439, p > 0.05$). Individual comparisons have shown that in the NAc, aripiprazole and risperidone significantly elevated the levels of p-CREB1 by 34.9% ($p < 0.05$) and 59.1% ($p < 0.01$), as well as the ratios of p-CREB1/CREB1 (both $p < 0.01$) (Figure 3(b), 3(d)). Moreover, the ratio of p-CREB1/CREB1 was shown to be positively correlated with the ratio of p-GSK3β/GSK3β ($p < 0.01$, $r = 0.493$) (Figure 5(a)).

The effects of antipsychotics on NMDA receptor subunits

In the NAc, both NMDA NR1 and NR2A expression were significantly altered by antipsychotic treatment (NR1, $F_{3,20} = 5.099, p < 0.01$; NR2A, $F_{3,20} = 10.903, p < 0.01$). Post-hoc comparisons have indicated that all three antipsychotics up-regulated the protein expression of the NDMA NR1 subunit (aripiprazole, +35.0%, $p < 0.05$; olanzapine, +47.2%, $p < 0.01$; risperidone, +53.1%, $p < 0.01$); additionally, both aripiprazole and olanzapine significantly elevated the expression of the NMDA NR2A subunit (aripiprazole, +67.1%, $p < 0.01$; olanzapine, +106.2%, $p < 0.01$) (Figure 2(c), 2(d)). In the CPu, no drug was able to alter the expression of NMDA receptor subunits (NR1, $F_{3,20} = 1.127, p > 0.05$; NR2A, $F_{3,20} = 0.404, p > 0.05$) (Figure 4(c), 4(d)). Furthermore, correlation tests have demonstrated that the expression of NMDA NR1 subunit was positively correlated with the phosphorylation ratio of β-catenin ($p < 0.01$, $r = 0.705$) and CREB1 ($p < 0.01$, $r = 0.475$) (Figure 5(b), 5(c)).

The effect of antipsychotics on GABA_A receptor

In the NAc, the expression of GABA_A (β-1) receptor was significantly altered by
antipsychotic administration ($F_{3, 20} = 3.363, p < 0.05$). Elevated expression of $\text{GABA}_A$ ($\beta$-1) receptor induced by aripiprazole ($+52.5\%, p < 0.05$) has been observed; additionally, risperidone tended to increase $\text{GABA}_A$ (β-1) receptor expression ($p = 0.1, +37.4\%$) (Figure 3(c), 3(d)). In the CPu, $\text{GABA}_A$ (β-1) receptor was also significantly influenced by antipsychotic administration ($F_{3, 20} = 9.732, p < 0.01$), and its expression was promoted by the administration with both aripiprazole and olanzapine (aripiprazole, $+90.0\%, p < 0.01$; olanzapine, $+85.6\%, p < 0.01$) (Figure 4(c), 4(d)).

**Discussion**

The present study has examined the antipsychotic modulations on PKA- and GSK3$\beta$-dependent signalling pathways, as well as NMDA and $\text{GABA}_A$ receptors, in the NAc and CPu of juvenile male rats. Our results indicated that aripiprazole, olanzapine, and risperidone differentially affected these signalling pathways and receptors; and their effects are also brain-regionally dependent (Table 1).

**Modulations of antipsychotics on the GSK3$\beta$-associated signalling pathways**

Abnormal GSK3$\beta$ signalling has been reported in a number of mental disorders, including schizophrenia, autism, bipolar disorders, and depression (Hur and Zhou, 2010). The present study has revealed that all three antipsychotics were able to significantly increase the ratio of phosphorylated GSK3$\beta$ in the NAc of the juvenile rats, indicating that the function of GSK3$\beta$ in the juvenile rats was inhibited by these antipsychotic drugs. These findings are generally consistent with those of various previous studies in adult rats (Emamian et al., 2004; Alimohamad et al., 2005a; Alimohamad et al., 2005b; Beaulieu et al., 2009; Li et al., 2007). Furthermore, our previous studies that examined...
the effects of antipsychotics on adult rats have also shown that acute, short-term, and chronic administration with aripiprazole elevated the phosphorylation levels of GSK3β in the NAc (Pan et al., 2015; Pan et al., 2016a; Pan et al., 2016b). Taken together, it is very likely that antipsychotics (at least aripiprazole) modulate GSK3β activity in juvenile rats in a similar manner as in adults.

A number of previous studies, including two studies from our group, have demonstrated that various classes of antipsychotics (e.g. aripiprazole, olanzapine, and risperidone) can increase the signalling of β-catenin in the striatum of adult animals (Alimohamad et al., 2005b; Alimohamad et al., 2005a; Sutton and Rushlow, 2011; Seo et al., 2015; Pan et al., 2016a; Pan et al., 2016b; Park et al., 2011). Consistent with these previous studies, the current study has also found up-regulation of the phosphorylation levels of β-catenin in the NAc of juvenile rats by all three antipsychotics (although the effect of olanzapine did not reach significance). Therefore, it could be concluded that β-catenin-mediated signalling in the NAc is very likely to be one of the major targets of antipsychotics in both youths and adults.

Modulations of antipsychotics on CREB1

Novel variants in the CREB1 gene have been identified in schizophrenic subjects (Kawanishi et al., 1999), and a number of in vivo studies reported that antipsychotics can increase the phosphorylation levels of CREB1 in adult animals (Pozzi et al., 2003; Konradi and Heckers, 1995; Mavrikaki et al., 2014; Pan et al., 2016c; Pan et al., 2016b; Rogoz et al., 2017; Einoch et al., 2017). CREB1 has also been found to be associated with neurodevelopment (Sakamoto et al., 2011) and involved in childhood-onset mood
disorders (Burcescu et al., 2010). In the present study in juvenile rats, both aripiprazole
and risperidone, but not olanzapine, significantly elevated the phosphorylation levels of
CREB1 in the NAc. These data were consistent with those findings from adult animals or
the neurons from adult animals, suggesting that in both juveniles and adults, antipsychotics react with CREB1 in similar patterns.

It is interesting that similar to the influences of aripiprazole, risperidone, and olanzapine
on the phosphorylation levels of GSK3β and β-catenin, aripiprazole and risperidone also
induced larger alterations in the phosphorylation of CREB1 than that induced by
olanzapine in the NAc. It has been reported that the affinity of aripiprazole and
risperidone for D2Rs is higher than that of olanzapine (Correll, 2010). Therefore, the
stronger influences induced by aripiprazole and risperidone on CREB1 is very likely to
be caused by their higher affinity for D2Rs. However, the signalling pathway(s) through
which D2Rs regulate CREB1 requires further exploration.

It has been revealed that extensive communication occurs between CREB1 and GSK3β
(Lonze and Ginty, 2002). Consistent with the findings of our previous study in adult rats
(Pan et al., 2016b), the data of the present study revealed a positive correlation between
the phosphorylation level of GSK3β and that of CREB1 in the NAc. The phenomenon
that CREB1 activity can be enhanced by inhibition of GSK3β was observed in both in
vitro and in vivo studies (Liang and Chuang, 2006; Park et al., 2011). Moreover, it has
been revealed that patients with novel variants in the CREB1 gene experienced positive
symptoms of schizophrenia (Kawanishi et al., 1999). Therefore, taken together, it is
suggested that activation of CREB1 via inhibition of the function of GSK3β in the NAc is
very likely to be associated with the actions of antipsychotics in both juveniles and
adults. Considering the extent of the alterations in GSK3β and CREB1 caused by these antipsychotics, it is also suggested that the activation of CREB1 via inhibiting GSK3β functions is likely to be associated with the levels of the binding affinity for D₂Rs of antipsychotics.

*Antipsychotics might modulate NMDA receptor subunits via GSK3β-β-catenin and/or CREB1 signalling*

It has been widely accepted that abnormal NMDAR neurotransmission is associated with many types of mental disorders, including schizophrenia, depression, bipolar disorder, and autism (Yamamoto et al., 2015). Previous studies showed that antipsychotic drug administration (e.g. clozapine and aripiprazole) elevated the NMDAR binding density and expression of protein and mRNA of NMDAR subunits in various brain regions of adult rats, including the NAc, hippocampus, and cortex (Pan et al., 2016b; Schmitt et al., 2003). The present study demonstrated that both aripiprazole and olanzapine administration for 20 days were able to raise the expression levels of NMDA NR1 and NR2A subunits in the NAc of juvenile rats. Therefore, elevating NMDAR expression is very likely to be a shared action of antipsychotics in both juvenile and adult rats.

It should be noted that antipsychotics do not directly bind with NMDARs. Thus, it is possible that antipsychotics modulate NMDARs via D₂R-mediated signalling pathways. Previous evidence has revealed the association between GSK3β-β-catenin signalling and the activity of NMDARs (Saiepour et al., 2017; Singh et al., 2017; Wu et al., 2016; Wan et al., 2012; Mills et al., 2014; Sanges et al., 2013). In the current study, we found that the expression of NMDA NR1 subunit was positively correlated with the phosphorylation
level of β-catenin in the NAc after antipsychotic treatment (Figure 5(c)). Thus, taken together with previous studies, our finding further proposes a potential regulation by antipsychotics of NMDARs via GSK3β-β-catenin signalling through the D2R (Figure 6). It is also worth noting that antipsychotics might regulate NMDARs via CREB1, as has been reported by several previous studies (Mavrikaki et al., 2014; Yuan et al., 2010; Snyder and Gao, 2013). The present study has shown that the NMDA NR1 expression was positively correlated with the CREB1 phosphorylation in the NAc, further confirming the relationship between CREB1 and NMDARs. Taken together with previous evidence (Lonze and Ginty, 2002), it has been suggested that antipsychotics might modulation NMDARs via PKA-CREB1 signalling (Figure 6). However, exact evidence is still required.

**Modulations of antipsychotics on the GABA_A (β-1) receptor**

The GABA_A receptor has also been widely reported to be involved in various mental disorders in children/adolescents, such as schizophrenia, depression, bipolar disorder, and autism (Rudolph and Mohler, 2014; Chiapponi et al., 2016), while antipsychotics can regulate GABA_A receptors. For example, 1-week treatment with both haloperidol and olanzapine increased the binding density of GABA_A receptors in the prefrontal cortex of adult rats (Skilbeck et al., 2007). A 6-month clozapine administration reduced the bindings of GABA_A receptors in the anterior cingulate and infralimbic cortex of adult rats (Zink et al., 2004). Our previous studies have found that the expression of GABA_A receptors was elevated by both 1-week and 10-week aripiprazole administration in the NAc of adult rats (Pan et al., 2016c; Pan et al., 2016b). In this study on juvenile rats, both
Aripiprazole and olanzapine administration were able to elevate GABA$_A$ receptor expression, which were generally consistent with the results of previous studies (Skilbeck et al., 2007; Zink et al., 2004; Pan et al., 2016c; Pan et al., 2016b), suggesting that the modulation of antipsychotics (at least aripiprazole) on GABA$_A$ (β-1) receptors are similar in both youths and adults.

Like NMDARs, although antipsychotics can impact GABA$_A$ receptors, they do not directly interact with these receptors. Previous studies pointed out that GABA$_A$ receptors can be regulated by the D$_2$R-downstream PKA signalling pathway (Poisbeau et al., 1999; Connelly et al., 2013). Our previous studies revealed that 1-week antipsychotic treatment modulated both the PKA phosphorylation levels and the expression of GABA$_A$ receptors in the NAc (Pan et al., 2016c), whereas 10-week antipsychotic treatment affected GABA$_A$ receptor expression only (Pan et al., 2016b). The results of the current study in which animals were treated for 3 weeks were similar as those of the 10-week in vivo study in adult rats (Pan et al., 2016b). It seems that antipsychotics alter PKA in a time-dependent manner (Figure 6), probably due to adaptive changes in dopamine D$_2$ receptors after a relatively long period (more than 1 week) of treatment, which however needs further validation.

The brain-regional differences of the modulations of antipsychotics

In the present study, the antipsychotics had very limited affections in the CPu in comparison with those in the NAc, indicating brain-regional differences of the modulations of antipsychotics in juvenile rats. This phenomenon is generally consistent with that in adult animals in our previous studies (Pan et al., 2016a; Pan et al., 2016b).
The exact reason for these differences in these two brain regions (NAc and CPu) remains unclear. It is possible that these differences might be caused by the heterogeneous structures of these brain regions that possess different neural inputs and outputs connected with various brain regions (Yager et al., 2015). For example, the NAc and CPu receive dopaminergic inputs from different brain areas – the ventral tegmental area and substantia nigra pars, respectively; in addition, outputs of NAc connect with the limbic areas and prefrontal cortex, while neurons in the CPu project to neocortical areas (Yager et al., 2015).

**Notes of intrinsic activity of the three antipsychotics for D\textsubscript{2}Rs**

In the present study, the three agents have different intrinsic activities for D\textsubscript{2}Rs. As a D\textsubscript{2}R partial agonist, the intrinsic activity of aripiprazole for D\textsubscript{2}R is lower than that of endogenous dopamine. Thus, when aripiprazole competes with endogenous dopamine to bind with D\textsubscript{2}Rs in normal animals, the overall activation of D\textsubscript{2}Rs could be weaker than that caused by endogenous dopamine solely, thereby showing antagonistic effects on D\textsubscript{2}Rs. Therefore, in the present study, aripiprazole displayed antagonistic effects on D\textsubscript{2}Rs as haloperidol.

In our previous studies (Pan et al., 2016; Pan et al., 2016b; Pan et al., 2016c), we found that bifeprunox, a potent D\textsubscript{2}R partial agonist, also exerted certain antagonistic effects on D\textsubscript{2}Rs instead of agonistic effects in healthy animals. However, the intrinsic activity of bifeprunox is higher than that of aripiprazole, thereby, the observed antagonistic effects of bifeprunox were relatively weaker.
Conclusion

In conclusion, the present study investigated the modulations of aripiprazole, olanzapine, and risperidone on various signalling pathways in the NAc and CPu of juvenile rats, revealing that these antipsychotics share some common effects on these signalling pathways, but differential modulations of these antipsychotics also existed. Furthermore, this study found that NMDA and GABA\textsubscript{A} receptors can be modulated by these antipsychotics and revealed possible involvement of GSK3\textbeta-\textbeta-catenin and/or CREB1 pathways in these modulations. Overall, in view of the involvement of NDMA and GABA\textsubscript{A} receptors in the pathophysiology of various mental disorders, this study suggests that antipsychotics might exert their therapeutic effects in treating mental disorders by modulating NMDA and GABA\textsubscript{A} receptors via PKA- and GSK3\textbeta-dependent signalling pathways in childhood-adolescence. The current study has provided \textit{in vivo} evidence at the molecular level that could be a reference for clinical prescription of childhood schizophrenia, further studies, however, are still necessary by using juvenile animal disease models (such as bipolar disorder, autism, schizophrenia, etc.) to investigate how antipsychotics impact behaviours and reverse deficits of animals via these signalling pathways, as well as to examine direct regulations of antipsychotics on genes and protein expression of downstream targets.
Funding

This work was supported by the National Health and Medical Research Council (APP1104184), Australia to Chao Deng and Jiamei Lian. Bo Pan was supported by the Natural Science Foundation of the Higher Education Institutions of Jiangsu Province, China (17KJB310018), the China Postdoctoral Science Foundation (2018M632401), and the Natural Science Foundation of Jiangsu Province of China (BK20171290). Jiamei Lian was also supported by a National Health and Medical Research Council Early Career Fellowship (APP1125937). The funding organisation did not play a role in the design and conduct of the study, in data interpretation or paper writing.

Declaration of conflicting interests

None of the authors has a conflict of interest.
References


Wu HF, Chen PS, Chen YJ, et al. (2016) Alleviation of N-Methyl-D-Aspartate Receptor-Dependent Long-Term Depression via Regulation of the Glycogen Synthase Kinase-3beta Pathway in the Amygdala of a Valproic Acid-Induced Animal Model


<table>
<thead>
<tr>
<th>Table 1. Summary of the effects of three antipsychotics in the nucleus accumbens (NAc) and caudate putamen (CPu) of juvenile rats.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Effects of antipsychotics in NAc</strong></td>
</tr>
<tr>
<td><strong>Aripiprazole</strong></td>
</tr>
<tr>
<td>p-GSK3β↑, Ratio of GSK3β↑; β-catenin↓, Ratio of β-catenin↑; p-CREB↑, Ratio of CREB↑; NMDA NR1↑, NMDA NR2A↑; GABA_A p-1↑</td>
</tr>
<tr>
<td><strong>Olanzapine</strong></td>
</tr>
<tr>
<td>p-GSK3β↑, Ratio of GSK3β↑; NMDA NR1↑, NMDA NR2A↑</td>
</tr>
<tr>
<td><strong>Risperidone</strong></td>
</tr>
<tr>
<td>GSK3β↓, Ratio of GSK3β↑; β-catenin↓, Ratio of β-catenin↑; p-CRF↑, Ratio of CRF↑; NMDA NR1↑</td>
</tr>
<tr>
<td><strong>Effects of antipsychotics in CPu</strong></td>
</tr>
<tr>
<td><strong>Aripiprazole</strong></td>
</tr>
<tr>
<td>CABA_α p 1↑</td>
</tr>
<tr>
<td><strong>Olanzapine</strong></td>
</tr>
<tr>
<td>GABA_α β-1↑</td>
</tr>
<tr>
<td><strong>Risperidone</strong></td>
</tr>
<tr>
<td>β-catenin↑, Ratio of β-catenin↑</td>
</tr>
</tbody>
</table>

*CREB: cAMP-responsive element-binding protein; GABA: γ-aminobutyric acid; GSK3β: glycogen synthase kinase 3 beta; NMDA: N-methyl-D-aspartate.*
Figure 1. Effects of three antipsychotics on Akt, GSK3β, Dvl-3 and β-catenin in the nucleus accumbens. The effects of aripiprazole (ARI), olanzapine (OLA) and risperidone (RIS) on Akt (a), GSK3β (b), Dvl-3 and β-catenin (c) were measured in the nucleus accumbens. The representative bands of Western blot are shown in (d). Akt was quantified at 60kDa; p-Akt (Thr308) was quantified at 60kDa; GSK3β was quantified at 46kDa; p-GSK3β (Ser9) was quantified at 46kDa; Dvl-3 was quantified at 85kDa; β-catenin was quantified at 92kDa; p-β-catenin was quantified at 92kDa. The data were normalised by taking the average value of the control group as 100% and expressed as mean ± S.E.M. (* p ≤ 0.05, ** p < 0.01 versus the control)
Figure 2. Effects of three antipsychotics on Akt, GSK3β, Dvl-3 and β-catenin in the caudate putamen. The effects of aripiprazole (ARI), olanzapine (OLA) and risperidone (RIS) on Akt (a), GSK3β (b), Dvl-3 and β-catenin (c) were measured in the caudate putamen. The representative bands of Western blot are shown in (d). Akt was quantified at 60kDa; p-Akt (Thr308) was quantified at 60kDa; GSK3β was quantified at 46kDa; p-GSK3β (Ser9) was quantified at 46kDa; Dvl-3 was quantified at 85kDa; β-catenin was quantified at 92kDa; p-β-catenin was quantified at 92kDa. The data were normalised by taking the average value of the control group as 100% and expressed as mean ± S.E.M. (* p ≤ 0.05, ** p < 0.01 versus the control)
Figure 3. Effects of three antipsychotics on PKA-C, CREB1, NMDA NR1, NR2A and GABA_A β-1 receptors in the nucleus accumbens. The effects of aripiprazole (ARI), olanzapine (OLA) and risperidone (RIS) on PKA-C (a), CREB1 (b), NMDA NR1 and NR2A (c) and GABA_A β-1 receptor (c) were measured in the nucleus accumbens. The representative bands of Western blot are shown in (d). PKA-C was quantified at 42kDa; p-PKA-C (Thr197) was quantified at 42kDa; CREB1 was quantified at 40kDa; p-CREB1 was quantified at 37kDa; NMDA NR1 subunit was quantified at 105kDa; NMDA NR2A subunit was quantified at 165kDa; and GABA_A β-1 receptors were quantified at 54kDa. The data were normalised by taking the average value of the control group as 100% and expressed as mean ± S.E.M. (* p ≤ 0.05, ** p < 0.01 versus the control).
**Figure 4. Effects of three antipsychotics on PKA-C, CREB1, NMDA NR1, NR2A and GABA\textsubscript{A} \(\beta\)-1 receptors in the caudate putamen.** The effects of aripiprazole (ARI), olanzapine (OLA) and risperidone (RIS) on PKA-C (a), CREB1 (b), NMDA NR1 and NR2A (c) and GABA\textsubscript{A} \(\beta\)-1 receptor (c) were measured in the caudate putamen. The representative bands of Western blot are shown in (d). PKA-C was quantified at 42kDa; p-PKA-C (Thr197) was quantified at 42kDa; CREB1 was quantified at 40kDa; p-CREB1 was quantified at 37kDa; NMDA NR1 subunit was quantified at 105kDa; NMDA NR2A subunit was quantified at 165kDa; and GABA\textsubscript{A} \(\beta\)-1 receptors were quantified at 54kDa. The data were normalised by taking the average value of the control group as 100% and expressed as mean ± S.E.M. (* \(p \leq 0.05\), ** \(p < 0.01\) versus the control)
Figure 5. Correlations between the ratio of p-GSK3β/GSK3β and the ratio of p-CREB1/CREB1, the ratio of p-β-catenin/β-catenin and the expression of NMDA NR1 subunit, and the ratio of p-CREB1/CREB1 and the expression of NMDA NR1 subunit in the nucleus accumbens. The ratio of p-CREB1/CREB1 was positively correlated with the ratio of p-GSK3β/GSK3β (a); the expression of NMDA NR1 subunit was positively correlated with the ratio of p-β-catenin/β-catenin (b), as well as the ratio of p-CREB1/CREB1 (c).
**Figure 6.** A proposed schematic diagram illustrating the possible signalling pathways through which antipsychotics affect NMDA and GABA_A receptors in the nucleus accumbens. Antipsychotics bind with the dopamine D_2-like receptor, probably resulting in the phosphorylation of GSK3β and β-catenin, which finally induces the elevation in the expression of NMDA receptor subunits (a). Reaction with D_2-like receptors by antipsychotics results in the increase in the expression of NMDA receptors probably via the PKA-CREB1 signalling pathway (b). Antipsychotics might modulate GABA_A receptors via PKA signalling in juveniles in a time-dependent manner (c). (The dashed arrows indicate our speculative ideas.)