



Modulation by chronic antipsychotic administration of PKA- and GSK3 β -mediated pathways and the NMDA receptor in rat ventral midbrain

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Abstract

Rationale Antipsychotics exert therapeutic effects by modulating various cellular signalling pathways and several types of receptors, including PKA- and GSK3 β -mediated signalling pathways, and NMDA receptors. The ventral midbrain, mainly containing the ventral tegmental area (VTA) and substantia nigra (SN), are the nuclei with dopamine origins in the brain, which are also involved in the actions of antipsychotics. Whether antipsychotics can modulate these cellular pathways in the ventral midbrain is unknown.

Objective This study aims to investigate the effects of antipsychotics, including aripiprazole (a dopamine D₂ receptor (D₂R) partial agonist), bifeprunox (a D₂R partial agonist), and haloperidol (a D₂R antagonist) on the PKA- and GSK3 β -mediated pathways and NMDA receptors in the ventral midbrain.

Methods Male rats were orally administered aripiprazole (0.75 mg/kg, t.i.d. (*ter in die*)), bifeprunox (0.8 mg/kg, t.i.d.), haloperidol (0.1 mg/kg, t.i.d.) or vehicle for either 1 week or 10 weeks. The levels of PKA, p-PKA, Akt, p-Akt, GSK3 β , p-GSK3 β , Dvl-3, β -catenin, and NMDA receptor subunits in the ventral midbrain were assessed by Western Blots.

Results The results showed that chronic antipsychotic treatment with aripiprazole selectively increased PKA activity in the VTA. Additionally, all three drugs elevated the activity of the Akt–GSK3 β signalling pathway in a time-dependent manner, while only aripiprazole stimulated the Dvl-3–GSK3 β – β -catenin signalling pathway in the SN. Furthermore, chronic administration with both aripiprazole and haloperidol decreased the expression of NMDA receptors.

Conclusion This study suggests that activating PKA- and GSK3 β -mediated pathways and downregulating NMDA receptor expression in the ventral midbrain might contribute to the clinical effects of antipsychotics.

Keywords Antipsychotics · Ventral midbrain · PKA · GSK3 β · NMDA receptor

Introduction

Schizophrenia is a devastating mental disorder. Currently, all antipsychotics control symptoms of schizophrenia mainly by targeting the dopamine D₂ receptor (D₂R) (Ginovart and Kapur 2012). The ventral midbrain, including ventral tegmental area (VTA) and substantia nigra (SN), is a centre of dopamine that contains the cell bodies of a large number of

dopamine neurons projecting their axons to many other brain regions including the nucleus accumbens (NAc) and caudate putamen (CPu), forming mesolimbic (VTA–NAc) and nigrostriatal (SN–CPu) dopaminergic transmission pathways.

Dopamine neurons in the VTA receive excitatory glutamatergic inputs from various brain regions, including the prefrontal cortex, laterodorsal tegmental nucleus, and pedunculo-pontine tegmental nucleus (Morikawa and

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Paladini 2011). When these glutamatergic neurons are activated, the firing rates of dopamine neurons in the VTA are increased. Dopamine neurons in the SN also receive excitatory glutamatergic inputs from various brain areas. For example, a glutamatergic projection is sent to the pars reticulata from the subthalamic nucleus, exerting an excitatory effect on SN neurons (Morikawa and Paladini 2011). The ventral midbrain may be playing a pivotal role in the treatment of schizophrenia by antipsychotics either directly or indirectly; however, little attention has been paid to this brain area, and as a result, the ventral midbrain has generally not been studied as extensively as other brain areas such as the striatum.

Alternative mRNA splicing generates two isoforms of the dopamine D₂ receptor: the long and short isoforms (Dal Toso et al. 1989). In the ventral midbrain, short-form dopamine D₂ autoreceptors are largely presented at the pre-synapses, which generally provide a feedback mechanism that adjusts dopamine synthesis capacity and release (Carlsson and Lindqvist 1963; Sibley 1999; Wolf and Roth 1990). The G protein-dependent PKA (protein kinase A) pathway is a canonical D₂R-downstream signalling pathway, and it has been shown that long-term activation of D₂ autoreceptors decreases phosphorylation of tyrosine hydroxylase via PKA inducing reduced dopamine synthesis, packaging, and release (Ford 2014).

The Akt (protein kinase B)–GSK (glycogen synthase kinase)3 β signalling pathway is a G protein-independent pathway of the D₂R. It has been reported by many studies that various antipsychotics exerted their therapeutic effects via activating the Akt–GSK3 β signalling pathway (Alimohamad et al. 2005a, b; Allen et al. 2011; Beaulieu et al. 2009; Li et al. 2007; Pan et al. 2016a, b). Additionally, a previous study reported that activating the short isoform of D₂Rs leads to stimulation of Akt–GSK3 β signalling in HEK (human embryonic kidney)-293/rD2S cells. However, whether short isoforms of D₂Rs regulate Akt–GSK3 β signalling in vivo remains unclear. Furthermore, the GSK3 β -mediated Dvl-3– β -catenin signalling pathway is another GSK3 β -mediated signalling pathway that is related to the effects of antipsychotics. Previous evidence indicates that many antipsychotics (including typical and atypical antipsychotics) are able to alter the expression of Dvl-3 and β -catenin in various brain regions (Alimohamad et al. 2005a, b; Pan et al. 2016a, b; Seo et al. 2015; Sutton and Rushlow 2011). However, whether antipsychotics modulate GSK3 β -mediated Dvl-3– β -catenin signalling via short isoforms of D₂Rs is unknown.

Therefore, the present study also investigated the effects of these antipsychotics on PKA, Akt–GSK3 β , and Dvl-3– β -catenin signalling in the ventral midbrain, in order to understand the possible actions of D₂R short isoforms in the ventral midbrain under the influences of antipsychotics. Since dopamine neurons in the ventral midbrain receive glutamatergic inputs, the present study also investigated antipsychotic modulation

of the NMDA (*N*-methyl-D-aspartic acid) receptor in the ventral midbrain. Moreover, since patients always experience long-term antipsychotic treatment under clinical conditions, this project investigated the effects of not only short-term (1 week), but also chronic (10 weeks), administration of these antipsychotics on these signalling pathways and NMDA receptors.

Methods

Animals and drug administration

Male Sprague-Dawley rats (aged 8 weeks) were obtained from the Animal Resource Centre (Perth, Australia). After arrival, all rats were housed in individual cages under environmentally controlled conditions (temperature 22 °C, light cycle from 07:00 AM to 07:00 PM), with ad libitum access to water and standard laboratory chow diet. All experimental procedures were approved by the Animal Ethics Committee (AE11/02) of the University of Wollongong and complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes 2004. All efforts were made to minimise animal distress and prevent suffering.

The rats were randomly assigned to one of the following drug treatment groups ($n = 6$ /group): aripiprazole (0.75 mg/kg, t.i.d. (*ter in die*); Otsuka, Japan), bifeprunox (0.8 mg/kg, t.i.d.; Otava, Ukraine), haloperidol (0.1 mg/kg, t.i.d.; Sigma, Australia), or vehicle. Drug powder mixed with the cookie dough pellets was delivered orally 3 times per day at 07:00 AM, 03:00 PM, and 11:00 PM for either 1 week or 10 weeks. This drug administration method aims to mimic the clinical scenario of oral administration and has been well-established by our laboratory (Pan et al. 2016a, b; Pan et al. 2016c). These dosages were transferred from the recommended dosages in clinic based on body surface area, according to the FDA guidelines for clinical trials ((Administration) 2005; Reagan-Shaw et al. 2008). Specifically, 2.25 mg/kg aripiprazole, 2.4 mg/kg bifeprunox, and 0.3 mg/kg haloperidol daily dosages in rats are equivalent to ~22 mg, ~24 mg, and ~3 mg in humans (60 kg body weight), respectively. The selected dosages are within the recommended clinical dosages (Casey et al. 2008; Gardner et al. 2010; Mace and Taylor 2009), which have physiological and behavioural effects in rodents, but do not induce motoric side-effects (Assie et al. 2006; Han et al. 2009; Natesan et al. 2006; Wadenberg 2007). On the sacrifice days, 2 h after the last drug administration, all animals were euthanised with CO₂, followed by immediately collecting the brains and freezing in liquid nitrogen. All animals were sacrificed between 09:00 AM and 11:00 AM to minimise circadian-induced variation of protein expression.

Brain dissection

The brain microdissection puncture technique was employed as described previously (Pan et al. 2015; Pan et al. 2016c). According to the brain atlas (Paxinos and Watson 2005), the brain tissues through the ventral mid-brain (bregma – 5.40 to – 6.30 mm) were dissected for the VTA and SN. Tissues collected were chilled on dry ice and kept at – 80 °C for future use.

Western Blots

Western Blot experiments were performed as described previously (Pan et al. 2015; Pan et al. 2016c). Briefly, frozen brain samples were homogenised in homogenising buffer (NP-40 cell lysis buffer (Invitrogen, #FNN0021) mixed with Protease Inhibitor Cocktail (Sigma-Aldrich, #P8340), β -glycerophosphate (Sigma-Aldrich, #G9422), and phenylmethylsulfonyl fluoride (Sigma-Aldrich, #P7626)). The protein concentration of each sample was determined by the DC Protein Assay (Bio-Rad, #500-0111). Each sample containing 10 μ g of protein was loaded into 4–20% Criterion™ TGX™ Precast Gels (Bio-Rad, #5671095). The gels were run vertically using Criterion™ Vertical Electrophoresis Cells (Bio-Rad, #1656001), and then the proteins were electrophoretically transferred to polyvinylidene difluoride membranes using Criterion™ Blotters (Bio-Rad, #1704071). All membranes were blocked with 5% skim milk powder and incubated with primary antibodies. To visualise the immunoreactive bands, Amersham Hyperfilm ECL (GE Healthcare, #28-9068-36) and Luminata Classico Western HRP substrate (Millipore, #WBLUC0500) were used. All Western Blot experiments were performed in duplicate to ensure consistency.

The antibodies and their dilutions used in the present study included anti-PKA-C (1:2000; Santa Cruz, #SC-903), anti-phosphor-PKA-C (1:1000; Thr197) (Cell Signalling, #5661), anti-Akt (1:2000; Cell Signalling, #4691), anti-phosphor-Akt (Thr308) (1:1000; Cell Signalling, #13038), anti-GSK3 β (1:2000; Cell Signalling, #5676), anti-phospho-GSK3 β (Ser9) (1:1000; Cell Signalling, #9322), anti-Dvl-3 (1:1000; Santa Cruz Biotechnology, #SC-8027), anti- β -catenin (1:1000; Santa Cruz Biotechnology, #SC-7963), anti-CREB1 (1:2000, Abcam, #ab32515), anti-phospho-CREB1 (1:2000, Abcam, #ab32096), anti-NMDA NR1 (1:2000, Abcam, #ab109182), and anti-NMDA NR2A (1:500, Abcam, #ab124913). Mouse anti-actin primary polyclonal antibody (1:10000; Millipore, #MAB1501) was used to determine the actin levels. Secondary antibodies included HRP-conjugated anti-rabbit IgG antibody (1:3000; Cell Signalling, #7074) and HRP-conjugated anti-mouse IgG antibody (1:3000; Cell Signalling, #7076).

Statistics

All data were analysed using SPSS Statistics (Version 24). The immunoreactive signals were quantified using Bio-Rad Image Lab (Version 6). The data of every protein obtained by Bio-Rad Image Lab were corrected with their corresponding actin levels. Then, data normal distribution was examined using histograms and Kolmogorov-Smirnov Z tests, followed by one-way analysis of variance (ANOVA) if the data were normally distributed. The post hoc Dunnett's test was selected to compare each drug administration group with their corresponding control group. The results were normalised and expressed by taking the value of the control group as 100%. Statistical significance was accepted when $p \leq 0.05$.

Results

Antipsychotic modulation of PKA activity

In the VTA, after 1-week antipsychotic administration, it was shown that the expression of PKA-C ($F_{3, 20} = 10.097$, $p < 0.01$) and the ratio of p-PKA-C/PKA-C ($F_{3, 20} = 2.357$, $p < 0.05$) was significantly affected. Post hoc tests indicated that all three chemicals reduced the protein levels of total PKA-C (aripiprazole, – 20.0%, $p < 0.05$; bifeprunox, – 16.6%, $p < 0.05$; haloperidol, – 39.0%, $p < 0.01$); however, only haloperidol significantly affected the ratio of p-PKA-C/PKA-C ($p < 0.05$) (Fig. 1A, B, C).

Also, in the VTA, after 10-week antipsychotic administration, ANOVA tests indicated that the levels of p-PKA-C ($F_{3, 20} = 3.134$, $p < 0.05$) and the ratio of p-PKA-C/PKA-C ($F_{3, 20} = 5.536$, $p < 0.01$) were significantly affected. In comparison with the controls, aripiprazole decreased the levels of p-PKA-C (– 17.6%, $p < 0.05$) as well as the ratio of p-PKA-C/PKA-C ($p < 0.01$) (Fig. 1B, C, E).

In the SN, after 1-week antipsychotic administration, the results showed that the levels of PKA-C ($F_{3, 20} = 10.090$, $p < 0.01$) and p-PKA-C ($F_{3, 20} = 4.821$, $p < 0.05$) were significantly altered. Post hoc tests revealed that both aripiprazole and haloperidol significantly elevated the levels of total PKA-C (aripiprazole, + 20.6%, $p < 0.01$; haloperidol, + 27.2%, $p < 0.01$), and haloperidol also increased p-PKA-C (+ 41.7%, $p < 0.01$) (Fig. 1C, E). However, no drug changed the ratio of p-PKA-C/PKA-C (all $p > 0.05$). After 10-week antipsychotic administration, no antipsychotic drug significantly altered PKA activity in the SN (Fig. 1D, E, F).

Antipsychotic modulation of Akt–GSK3 β signalling pathway

In the VTA, after 1-week antipsychotic administration, it was revealed that the levels of total Akt, p-Akt, and the ratio of p-

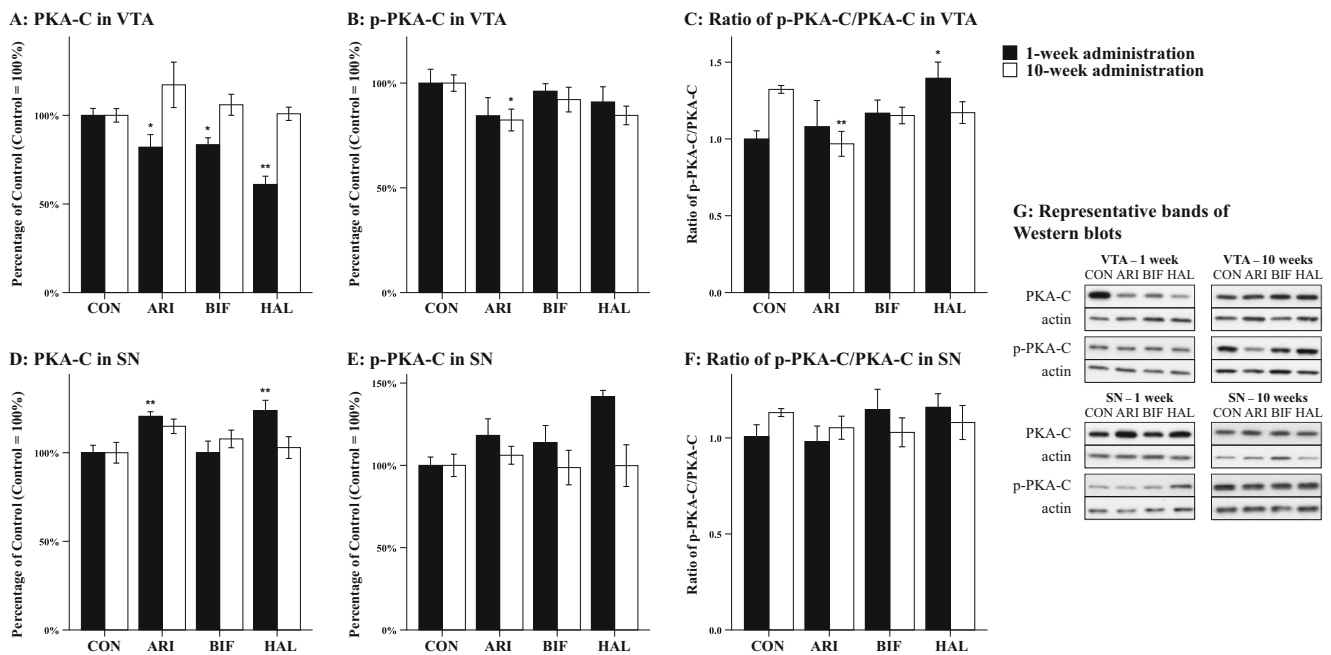


Fig. 1 The modulations by three antipsychotics of PKA activity. The effects of aripiprazole (ARI), bifeprunox (BIF), and haloperidol (HAL) on PKA activity were measured in the ventral tegmental area (VTA) and substantia nigra (SN): VTA 1 week (A), VTA 10 weeks (B), SN 1 week (C), and SN 10 weeks (D). The representative bands of Western Blot are

shown in (E). PKA was quantified at 42 kDa; p-PKA was quantified at 42 kDa. The sample size is six per group. The data were normalised by taking the average value of the control group as 100% and expressed as mean \pm S.E.M. (* $p \leq 0.05$, ** $p < 0.01$ vs the control)

Akt/Akt was significantly affected (Akt, $F_{3, 20} = 4.622$, $p < 0.05$; p-Akt, $F_{3, 20} = 2.958$, $p < 0.05$; p-Akt/Akt, $F_{3, 20} = 3.056$, $p < 0.05$). In addition, short-term antipsychotic administration significantly influenced the levels of total GSK3 β ($F_{3, 20} = 25.330$, $p < 0.01$), p-GSK3 β ($F_{3, 20} = 8.692$, $p < 0.01$), and the ratio of p-GSK3 β /GSK3 β ($F_{3, 20} = 11.154$, $p < 0.01$). Post hoc analyses demonstrated that both aripiprazole and haloperidol significantly inhibited Akt expression (aripiprazole, -55.1% , $p < 0.01$; haloperidol, -56.9% , $p < 0.01$), respectively, and short-term administration with bifeprunox increased p-Akt protein levels by $+21.4\%$ ($p < 0.05$); in addition, the ratio of p-Akt/Akt was increased by all three drugs (aripiprazole, $p < 0.05$; bifeprunox, $p < 0.05$; haloperidol, $p < 0.05$) (Fig. 2A, E). Furthermore, GSK3 β activity was shown to be affected in a similar manner as Akt activity. Specifically, aripiprazole and bifeprunox significantly changed the levels of GSK3 β (-40.8% , $p < 0.01$) and p-GSK3 β ($+42.9\%$, $p < 0.01$), respectively; and the ratio of p-GSK3 β /GSK3 β was elevated by all three drugs (aripiprazole, $p < 0.01$; bifeprunox, $p < 0.05$; haloperidol, $p < 0.05$) (Fig. 3A, B, C, E).

After 10-week antipsychotic administration, the chronic antipsychotic administration had no significant effect on the levels of Akt, p-Akt, GSK3 β , and p-GSK3 β , nor on the ratio of p-Akt/Akt and p-GSK3 β /GSK3 β in the VTA, compared with the controls (all $p > 0.05$) (Figs. 2A, B, C, G; 3A, B, C, G).

In the SN, after 1-week antipsychotic administration, it was found that antipsychotic administration significantly affected the levels of Akt ($F_{3, 20} = 19.949$, $p < 0.01$), p-Akt ($F_{3, 20} =$

12.136, $p < 0.01$), p-GSK3 β ($F_{3, 20} = 27.905$, $p < 0.01$), and the ratio of p-Akt/Akt ($F_{3, 20} = 2.819$, $p < 0.05$) and p-GSK3 β /GSK3 β ($F_{3, 20} = 10.030$, $p < 0.01$). Post hoc analyses of short-term administration revealed that aripiprazole reduced the expression of Akt (-41.8% , $p < 0.01$), while bifeprunox enhanced the levels of p-Akt ($+44.3\%$, $p < 0.01$); and, both drugs increased the ratio of p-Akt/Akt (aripiprazole, $p < 0.05$; bifeprunox, $p < 0.05$) (Fig. 2D, E, F, G). Furthermore, both aripiprazole and bifeprunox increased the levels of p-GSK3 β (aripiprazole, $+62.1\%$, $p < 0.01$; bifeprunox, $+47.7\%$, $p < 0.01$) and the ratio of p-GSK3 β /GSK3 β (aripiprazole, $p < 0.01$; bifeprunox, $p < 0.05$) (Fig. 3D, E, F, G).

In the SN after 10-week antipsychotic administration, it was observed that p-Akt ($F_{3, 20} = 10.074$, $p < 0.01$) and the ratio of p-Akt/Akt ($F_{3, 20} = 8.932$, $p < 0.01$) in the SN were changed by drug administration; the levels of p-GSK3 β ($F_{3, 20} = 3.101$, $p < 0.05$) and the ratio of p-GSK3 β /GSK3 β ($F_{3, 20} = 3.133$, $p < 0.05$) were also significantly altered by 10-week antipsychotic administration in the SN. Post hoc tests showed that only bifeprunox increased the levels of p-Akt ($+52.6\%$, $p < 0.01$) and the ratio of p-Akt/Akt ($p < 0.01$) (Fig. 2E, F); hence, aripiprazole and bifeprunox increased the levels of p-GSK3 β (aripiprazole, $+74.6\%$, $p < 0.05$; bifeprunox, $+64.7\%$, $p < 0.05$); and also, the ratio of p-GSK3 β /GSK3 β was elevated by these two chemicals (aripiprazole, $p < 0.05$; bifeprunox, $p < 0.05$) (Fig. 3E, F).

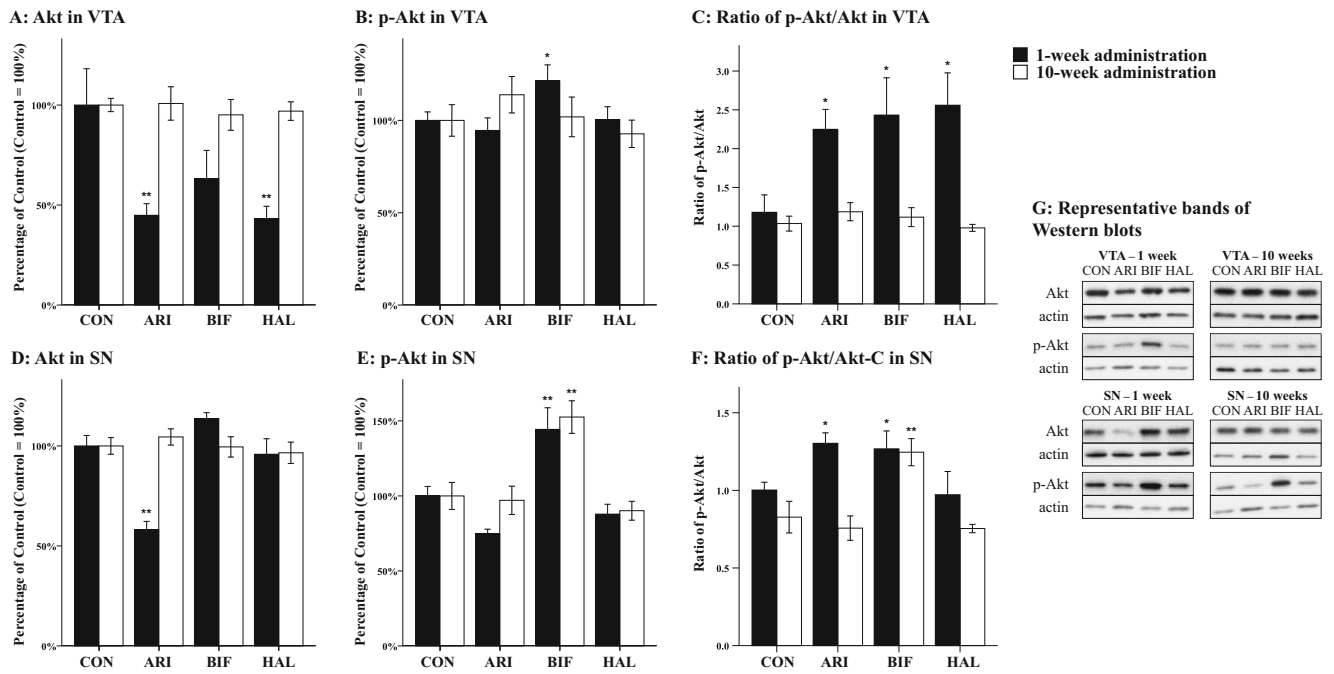


Fig. 2 The modulation by three antipsychotics of Akt activity. The effects of aripiprazole (ARI), bifeprunox (BIF), and haloperidol (HAL) on Akt activity were measured in the ventral tegmental area (VTA) and substantia nigra (SN): VTA 1 week (A), VTA 10 weeks (B), SN 1 week (C), and SN 10 weeks (D). The representative bands of Western Blot are shown in (E).

The sample size is six per group. Akt was quantified at 60 kDa; p-Akt was quantified at 60 kDa. The data were normalised by taking the average value of the control group as 100% and expressed as mean ± S.E.M. (* $p \leq 0.05$, ** $p < 0.01$ vs control)

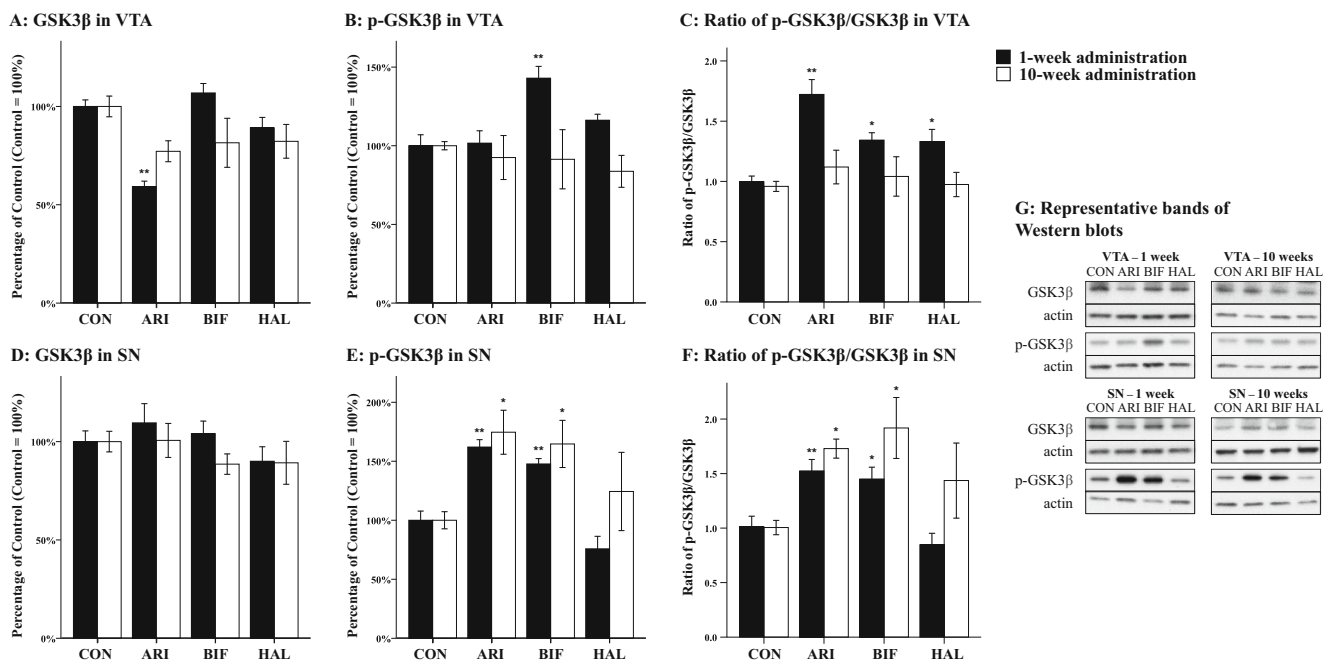


Fig. 3 The modulation by three antipsychotics of GSK3β activity. The effects of aripiprazole (ARI), bifeprunox (BIF), and haloperidol (HAL) on GSK3β activity were measured in the ventral tegmental area (VTA) and substantia nigra (SN): VTA 1 week (A), VTA 10 weeks (B), SN 1 week (C), and SN 10 weeks (D). The representative bands of Western

Blot are shown in (E). The sample size is six per group. GSK3β was quantified at 46 kDa; p-GSK3β was quantified at 46 kDa. The data were normalised by taking the average value of the control group as 100% and expressed as mean ± S.E.M. (* $p \leq 0.05$, ** $p < 0.01$ vs control)

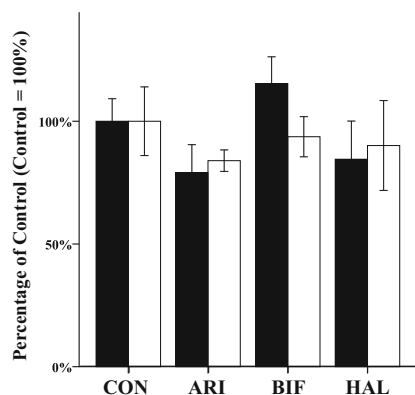
Antipsychotic modulation of Dvl-3 and β -catenin expression

Short-term antipsychotic administration had no observable effects on the expression of Dvl-3 ($F_{3, 20} = 1.858, p > 0.05$) and β -catenin ($F_{3, 20} = 2.313, p > 0.05$) in the VTA; however, the expression of Dvl-3 ($F_{3, 20} = 5.657, p < 0.01$) and β -catenin ($F_{3, 20} = 6.202, p < 0.01$) in the SN was significantly changed after 1-week antipsychotic administration. Similarly, after chronic administration, both Dvl-3 ($F_{3, 20} = 3.878, p < 0.05$) and β -catenin ($F_{3, 20} = 6.151, p < 0.01$) expressions were significantly altered in the SN, but not in the VTA (both $p > 0.05$). Post hoc tests indicated that 1-week administration with aripiprazole (but not bifeprunox and haloperidol) significantly increased the protein levels of Dvl-3 (+31.8%, $p < 0.05$) and β -catenin (+49.3%, $p < 0.01$) in the SN, respectively; in addition, 10-week aripiprazole administration also significantly elevated the expression of Dvl-3 (+42.2%, $p < 0.05$) and β -catenin (+24.4%, $p < 0.05$) in the SN (Fig. 4C, D, E). No drug significantly affected the expression of Dvl-3 and β -catenin in the VTA (Fig. 4A, B, E).

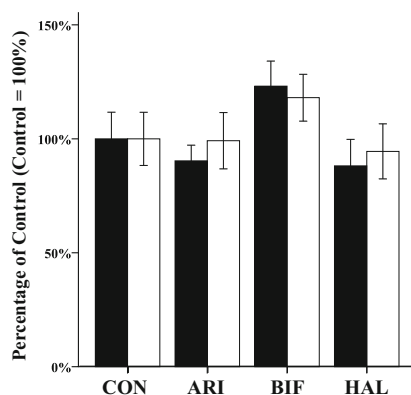
Antipsychotic modulation of the expression of NMDA receptor subunits

The present study revealed that the protein expression of NMDA receptor subunits NR1 and NR2A in both the VTA and SN was significantly affected by 10-week antipsychotic administration (NR1 in VTA, $F_{3, 20} = 10.301, p < 0.01$; NR2A in VTA, $F_{3, 20} = 5.226, p < 0.01$; NR1 in SN, $F_{3, 20} = 33.785, p < 0.01$; NR2A in SN, $F_{3, 20} = 15.356, p < 0.01$), whereas short-term administration exerted no influence on NMDA receptor expression in the two brain regions (all $p > 0.05$). In comparison with the controls, 10-week administration with aripiprazole and haloperidol reduced the expression of NMDA NR1 subunit in the VTA (aripiprazole, $-59.9\%, p < 0.01$; haloperidol, $-30.5\%, p < 0.05$); additionally, chronic aripiprazole administration also decreased NR2A expression in the VTA ($-31.9\%, p < 0.01$) (Fig. 5A, B, E). In the SN, the protein levels of both NMDA NR1 and NR2A were inhibited by aripiprazole (NR1, $-45.2\%, p < 0.01$; NR2A, $-56.9\%, p < 0.01$) and haloperidol (NR1, $-47.5\%, p < 0.01$; NR2A, $-38.3\%, p < 0.01$) (Fig. 5C, D, E).

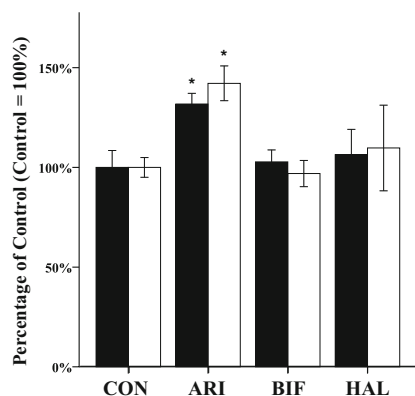
A: Dvl-3 in VTA



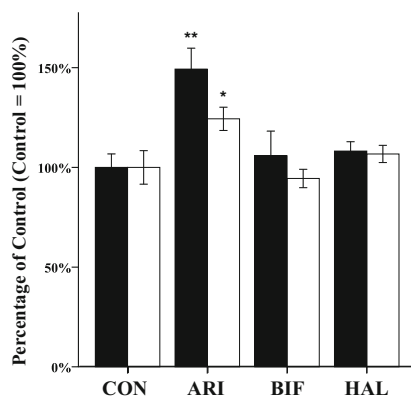
B: β -catenin in VTA



C: Dvl-3 in SN



D: β -catenin in SN



■ 1-week administration
□ 10-week administration

E: Representative bands of Western blots

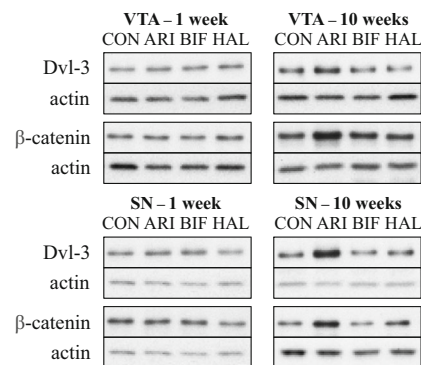


Fig. 4 The modulation by three antipsychotics of Dvl-3 and β -catenin expression. The effects of aripiprazole (ARI), bifeprunox (BIF), and haloperidol (HAL) on Dvl-3 and β -catenin expression were measured in the ventral tegmental area (VTA) and substantia nigra (SN): VTA 1 week (A), VTA 10 weeks (B), SN 1 week (C), and SN 10 weeks (D). The

representative bands of Western Blot are shown in (E). The sample size is six per group. Dvl-3 was quantified at 85 kDa; β -catenin was quantified at 92 kDa. The data were normalised by taking the average value of the control group as 100% and expressed as mean \pm S.E.M. (* $p \leq 0.05$, ** $p < 0.01$ vs control)

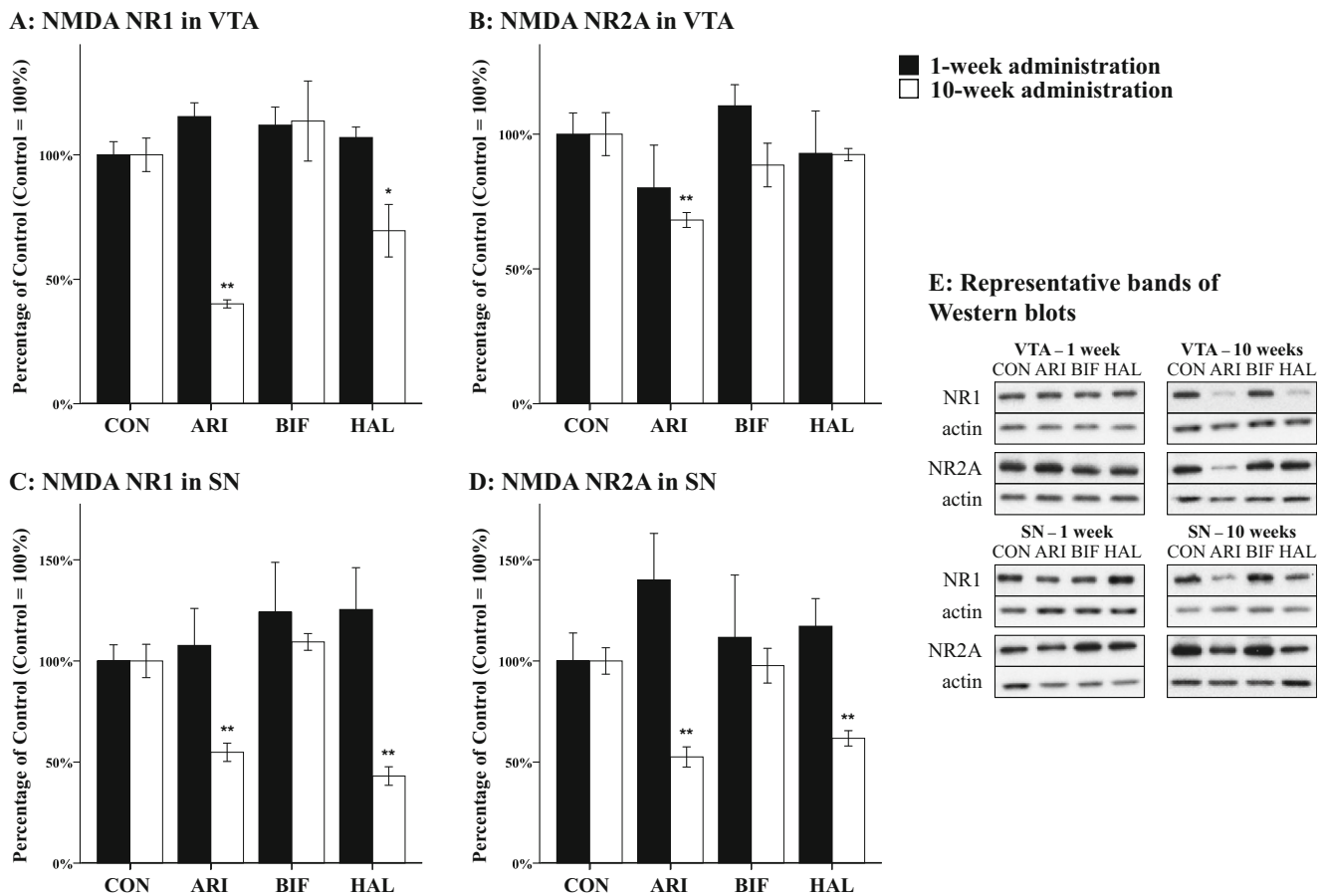


Fig. 5 The modulation by three antipsychotics of expression of NMDA receptor subunits. The effects of aripiprazole (ARI), bifeprunox (BIF), and haloperidol (HAL) on the expression of NMDA receptor subunits were measured in the ventral tegmental area (VTA) and substantia nigra (SN): VTA 1 week (A), VTA 10 weeks (B), SN 1 week (C), and SN

10 weeks (D). The representative bands of Western Blot are shown in (E). The sample size is six per group. NR1 was quantified at 105 kDa; NR2A was quantified at 165 kDa. The data were normalised by taking the average value of the control group as 100% and expressed as mean \pm S.E.M. (* $p \leq 0.05$, ** $p < 0.01$ vs control)

Discussion

The present study investigated the effects of 1-week and 10-week treatment with three antipsychotics on PKA, Akt–GSK3 β , Dvl-3– β -catenin signalling, as well as the expression of the NMDA receptor in the ventral midbrain (e.g., the VTA and SN). To the best of our knowledge, this is the first study to examine the in vivo effects of these antipsychotic drugs on cellular signalling and receptor expression in the ventral midbrain. The results have shown that antipsychotics could alter the signalling activity of PKA, Akt–GSK3 β , Dvl-3– β -catenin, and the expression of the NMDA receptor in a time-dependent manner (Table 1), revealing possible patterns of how antipsychotics regulate dopamine neurons in the ventral midbrain.

The selective effects of aripiprazole on PKA activity in the VTA

The PKA signalling pathway is a canonical D₂R-downstream signalling pathway which mediates diverse cellular responses

via activating the cAMP-associated PKA kinase (Beaulieu and Gainetdinov 2011). Previous studies indicated that enhancement of PKA activity via antagonising D₂R is associated with the effects of antipsychotics in various brain regions, regardless of the type of antipsychotic drug (Dwivedi et al. 2002; Pan et al. 2015; Turalba et al. 2004). In the present study, we found that 1-week administration with haloperidol increased PKA activity in the VTA, whereas 10-week administration with aripiprazole reduced it.

Short-form dopamine D₂ autoreceptors are largely expressed at the pre-synapses of the ventral midbrain. These autoreceptors are located on the soma and dendrites of midbrain dopamine neurons (Ford 2014), and negatively regulate the phosphorylation/activation (Ser40) of tyrosine hydroxylase (TH), a rate-limiting enzyme in dopamine synthesis in the pre-synaptic nerve terminals, through acting on the PKA pathway (Harada et al. 1996; Lindgren et al. 2003; Roskoski Jr and Roskoski 1987) and release (el Mestikawy and Hamon 1986; Strait and Kuczenski 1986). Han et al. (2009) reported that 12-week administration with aripiprazole reduced TH mRNA

Table 1 Summary of the effects of aripiprazole, bifeprunox, and haloperidol in the ventral tegmental area and substantia nigra of the rats

		Effects in the ventral tegmental area	
		1 week	10 weeks
Aripiprazole	Akt activity↑, GSK3β activity↑		PKA activity↓, NMDA NR1 & NR2A expression↓
Bifeprunox	Akt activity↑, GSK3β activity↑		–
Haloperidol	PKA activity↑, Akt activity↑, GSK3β activity↑		NMDA NR1 expression↓
		Effects in the substantia nigra	
		1 week	10 weeks
Aripiprazole	Akt activity↑, GSK3β activity↑, Dvl-3 & β-catenin expression↑		GSK3β activity↑, Dvl-3 & β-catenin expression↑, NMDA NR1 & NR2A expression↓
Bifeprunox	Akt activity↑, GSK3β activity↑		Akt activity↑, GSK3β activity↑
Haloperidol	–		NMDA NR1 & NR2A expression↓

expression in the VTA, but not in the SN, and haloperidol had no effects on TH mRNA expression. Taken together, our findings suggest that dopamine synthesis capacity in the VTA can be suppressed by chronic aripiprazole administration via inhibiting the PKA pathway and these results further confirm that chronic aripiprazole administration is very likely to have selective effects on dopaminergic activity in the VTA.

As a partial agonist for D₂Rs, bifeprunox did not show the selective effects on PKA activity that aripiprazole showed. Thus, D₂R partial agonism cannot fully explain the mechanism of aripiprazole. Also, in comparison with haloperidol, a potent D₂R antagonist, it seems that potently antagonising D₂Rs does not contribute to modulation of PKA activity in this study, either. Therefore, this unique activity of aripiprazole requires a novel explanation. It has been proposed that aripiprazole possesses functional selectivity at D₂Rs (Mailman and Murthy 2010); however, this notion has not been completely validated. Our findings may possibly add new evidence of the functionally selective effects of aripiprazole.

Antipsychotics modulated the GSK3β-mediated signalling pathways in the ventral midbrain

GSK3β-mediated signalling pathways (including Akt–GSK3β and Dvl-3–GSK3β–β-catenin signalling pathways) have been implicated in schizophrenia (Emamian 2012; Singh 2013). Previous studies including our own studies indicated that GSK3β-mediated signalling pathways can be modulated by various antipsychotics (including aripiprazole and haloperidol) in a wide range of brain regions (Alimohamad et al. 2005a; Alimohamad et al. 2005b; Beaulieu et al. 2009; Emamian 2012; Li et al. 2007; Pan et al. 2016a, b; Park et al. 2011; Seo et al. 2015; Sutton and Rushlow 2011). However, the effects of antipsychotics on GSK3β-mediated signalling pathways in the ventral midbrain have not been examined before the present study.

In this study, we found that short-term administration with all three drugs increased the phosphorylation levels of Akt in parallel with the elevation in GSK3β phosphorylation in the

VTA; in addition, 1-week administration with both aripiprazole and bifeprunox, but not haloperidol, significantly increased the phosphorylation of Akt and GSK3β in the SN; furthermore, after 10-week administration, the influences of all three drugs on Akt and GSK3β phosphorylation in the VTA disappeared; in contrast, the effects of aripiprazole and bifeprunox on GSK3β phosphorylation in the SN persisted, although their effect on Akt in the SN cannot be observed after 10-week administration; moreover, both 1-week and 10-week administration with aripiprazole increased Dvl-3 and β-catenin expression in the SN only. These findings together suggest that GSK3β-mediated signalling pathways in the ventral midbrain can be affected by antipsychotic administration; these data are generally consistent with the results of most previous studies that activation of these GSK3β-mediated signalling pathways contributes to the effects of various types of antipsychotics. It is interesting that aripiprazole also displayed selective effects on the Dvl-3–GSK3β–β-catenin signalling pathway in the SN. Whether this selective modulation of the Dvl-3–GSK3β–β-catenin signalling pathway is related to the functionally selective profile of aripiprazole still needs to be verified.

It is worth noting that although GSK3β-mediated signalling pathways can be regulated by D₂Rs, these D₂Rs in previous reports were primarily long-form D₂ receptors and there is no evidence that D₂ autoreceptors also function via these GSK3β-mediated pathways. Therefore, whether these GSK3β-mediated signalling pathways are regulated by short-form dopamine D₂ receptors, and the exact role of GSK3β-mediated signalling pathways in the actions of antipsychotics in the ventral midbrain, require further exploration.

Antipsychotics suppressed the expression of NMDA receptors in the ventral midbrain

The cell body of dopaminergic neurons in the ventral midbrain expresses NMDA receptors, receiving glutamatergic afferents from various brain areas (Beier et al. 2015; Lerner et al. 2015). Glutamate is an excitatory neurotransmitter that stimulates

neuron activity. In the present study, decreased NMDA receptor expression in the ventral midbrain might indicate inhibited activity of dopaminergic neurons, suggesting that in addition to blocking D₂Rs, inhibiting dopaminergic neuron activity in the ventral midbrain might also be a route through which antipsychotics exert their therapeutic effects. However, whether this inhibition could affect dopamine synthesis and release requires further investigation.

The present study demonstrated that aripiprazole and haloperidol, but not bifeprunox, decreased the expression of NMDA NR1 and/or NR2A subunits in the ventral midbrain after 10-week administration only, indicating a time-dependent manner in which antipsychotics modulate NMDA receptors. It is worth noting that antipsychotics have very low affinity with NMDA receptors (Roth and Driscoll 2018). Thus, these modulations of NMDA receptors are primarily indirect, which probably require long-term treatment to achieve.

In comparison with aripiprazole and haloperidol, bifeprunox did not modulate NMDA receptor expression in the ventral midbrain in the current study. The cause of this discrepancy is unclear. Due to the fact that the modulation by these antipsychotics of NMDA receptors is indirect and that the three chemicals used in the present study share a common mechanism—interacting with D₂Rs, the alteration of NMDA receptor expression is very likely to be associated with their actions on D₂Rs. Haloperidol is a potent D₂R antagonist, whereas aripiprazole and bifeprunox are D₂R partial agonists. Previous studies revealed that the intrinsic activity of aripiprazole at D₂Rs is weaker than that of bifeprunox (intrinsic activity at D₂Rs: aripiprazole vs. bifeprunox vs. dopamine = 86.0% vs. 95.1% vs. 100%) (Tadori et al. 2007; Tadori et al. 2005). It is possible that by competing with endogenous dopamine in the normal brain, aripiprazole would exert an overall antagonistic effect like haloperidol due to its relatively low intrinsic activity at D₂Rs. Therefore, a low intrinsic activity at D₂Rs might be associated with antipsychotic modulation of NMDA receptors. However, this hypothesis needs further validation.

It should be noted that antipsychotics exerted time-dependent effects on these signalling pathways and NMDA receptor expression in the VTA. For example, both aripiprazole and haloperidol elevated the activity of Akt–GSK3 β signalling in the VTA after 1-week but not 10-week administration, while these two drugs downregulated the NMDA receptor expression after 10-week administration only (Table 1). The possible reason could be that the regulation of aripiprazole and haloperidol on the Akt–GSK3 β signalling pathway through D₂Rs was compromised due to the adaptive changes on D₂Rs followed chronic antipsychotic treatment which has been well reported in both clinical and preclinical studies (El Hage et al. 2015; Li 2016; Moran-Gates et al. 2006). On the other hand, since these antipsychotics do not bind directly with NMDA receptors (Correll 2010), it might require a longer time for antipsychotics to modulate the expression of NMDA receptors via indirect signalling pathways (Beaulieu and Gainetdinov 2011; Pan et al. 2016b). It

is also worthy to note that several other cellular pathways might become targets of current and/or potential antipsychotic drugs. For example, Long et al. (2017) reported that overexpression of Mash1 to activate the Mash1-dependent Notch signalling pathway in stem cells through genetic engineering could generate GABAergic neuron-like cells to treat neurological disorders including schizophrenia. Furthermore, the Sonic hedgehog (Shh) signalling pathway is associated with the pathophysiology of schizophrenia (Boyd et al. 2015), and abnormal tectonic 3 disrupts the Shh signalling (Wang et al. 2018), which enables the Shh pathways to be a potential target of future antipsychotic treatment.

In conclusion, the present study examined the short-term and long-term effects of antipsychotics on the activity of PKA- and GSK3 β -mediated signalling pathways, as well as NMDA receptor expression in the ventral midbrain of rats. The findings suggest that aripiprazole might have selective effects on the PKA activity and Dvl-3–GSK3 β – β -catenin signalling pathway in the ventral brain and that upregulation of the Akt–GSK3 β signalling pathway and suppression of the NMDA receptor in the ventral midbrain might be the major routes through which antipsychotics exert their clinical effects. It is worth noting that the current study was conducted in healthy rats without any affection of the disease, showing the *in vivo* effects of aripiprazole and haloperidol on these pathways in the ventral midbrain; therefore, future studies are important to further validate these findings using animal models (such as Poly I:C models) for schizophrenia.

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Compliance with ethical standards

All experimental procedures were approved by the Animal Ethics Committee (AE11/02) of the University of Wollongong and complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004).

Conflict of interest The authors declare that they have no conflict of interest.

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