January 2016

Aripiprazole increases the PKA signalling and expression of the $GABA_A$ receptor and CREB1 in the nucleus accumbens of rats

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Abstract

The GABAA receptor is implicated in the pathophysiology of schizophrenia and regulated by PKA signalling. Current antipsychotics bind with D2-like receptors, but not the GABAA receptor. The cAMP-responsive element-binding protein 1 (CREB1) is also associated with PKA signalling and may be related to the positive symptoms of schizophrenia. This study investigated the effects of antipsychotics in modulating D2-mediated PKA signalling and its downstream GABAA receptors and CREB1. Rats were treated orally with aripiprazole (0.75 mg/kg, ter in die (t.i.d.)), bifeprunox (0.8 mg/kg, t.i.d.), haloperidol (0.1 mg/kg, t.i.d.) or vehicle for 1 week. The levels of PKA-Cα and p-PKA in the prefrontal cortex (PFC), nucleus accumbens (NAc) and caudate putamen (CPu) were detected by Western blots. The mRNA levels of Gabrb1, Gabrb2, Gabrb3 and Creb1, and their protein expression were measured by qRT-PCR and Western blots, respectively. Aripiprazole elevated the levels of p-PKA and the ratio of p-PKA/PKA in the NAc, but not the PFC and CPu. Correlated with this elevated PKA signalling, aripiprazole elevated the mRNA and protein expression of the GABAA (β-1) receptor and CREB1 in the NAc. While haloperidol elevated the levels of p-PKA and the ratio of p-PKA/PKA in both NAc and CPu, it only tended to increase the expression of the GABAA (β-1) receptor and CREB1 in the NAc, but not the CPu. Bifeprunox had no effects on PKA signalling in these brain regions. These results suggest that aripiprazole has selective effects on upregulating the GABAA (β-1) receptor and CREB1 in the NAc, probably via activating PKA signalling.

Disciplines

Medicine and Health Sciences

Publication Details

Pan, B., Lian, J., Huang, X. & Deng, C. (2016). Aripiprazole increases the PKA signalling and expression of the $GABA_A$ receptor and CREB1 in the nucleus accumbens of rats. Journal of Molecular Neuroscience, 59 (1), 36-47.

Aripiprazole increases the PKA signalling and expression of the $GABA_A$ receptor and CREB1 in the nucleus accumbens of rats

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Keywords: antipsychotics, aripiprazole, bifeprunox, PKA, CREB1, haloperidol, $GABA_A$ receptors.

Abstract

The $GABA_A$ receptor is implicated in the pathophysiology of schizophrenia and regulated by PKA signalling. Current antipsychotics bind with D_2-like receptors, but not the $GABA_A$ receptor. The cAMP-responsive element-binding protein 1 (CREB1) is also associated with PKA signalling and may be related to the positive symptoms of schizophrenia. This study investigated the effects of antipsychotics in modulating D_2-mediated PKA signalling and its downstream $GABA_A$ receptors and CREB1. Rats were treated orally with aripiprazole (0.75mg/kg, t.i.d. (*ter in die*)), bifeprunox (0.8mg/kg, t.i.d.), haloperidol (0.1mg/kg, t.i.d.) or vehicle for 1 week. The levels of PKA-Cα and p-PKA in the prefrontal cortex (PFC), nucleus accumbens (NAc) and caudate putamen (CPu) were detected by Western Blots. The mRNA levels of Gabrb1, Gabrb2, Gabrb3 and Creb1, and their protein expression were measured by qRT-PCR and Western Blots, respectively. Aripiprazole elevated the levels of p-PKA and the ratio of p-PKA/PKA in the NAc, but not the PFC and CPu. Correlated with this elevated PKA signalling, aripiprazole elevated the mRNA and protein expression of the $GABA_A$ (β-1) receptor and CREB1 in the NAc. While haloperidol elevated the levels of p-PKA and the ratio of p-PKA/PKA in both NAc and CPu, it only tended to increase the expression of the $GABA_A$ (β-1) receptor and CREB1 in the NAc, but not the CPu. Bifeprunox had no effects on PKA signalling in these brain regions. These results suggest that aripiprazole has selective effects on up-regulating the $GABA_A$ (β-1) receptor and CREB1 in the NAc, probably via activating PKA signalling.

Introduction

Gamma-aminobutyric acid (GABA) is one of the major inhibitory neurotransmitters, and has been reported to be involved in the pathophysiology of schizophrenia (see review Benes, 2015). The GABA receptor family consists of three classes of receptors – $GABA_A$, $GABA_B$ and $GABA_C$; the $GABA_A$ receptor is widely distributed in the brain (Hendry et al., 1989). Previous studies reported abnormal $GABA_A$ receptor density in various brain regions of schizophrenia subjects, including the prefrontal cortex (PFC) (Dean et al., 1999, Ishikawa et al., 2004), cingulate cortex (Benes et al., 1992), superior temporal gyrus (Deng and Huang, 2006) and hippocampus (Benes et al., 1997). Antipsychotic drug administration also affects $GABA_A$ receptors. For example, a 6-month treatment with haloperidol increased the density of $GABA_A$ receptor binding of [^{3}H]-muscimol in the caudate putamen (CPu), the core of the nucleus accumbens (NAc), while reducing it in the parietal and temporal cortex; both haloperidol and clozapine increased the bindings of $GABA_A$ receptors in the anterior cingulate and infralimbic cortex, respectively (Zink et al., 2004). Skilbeck and colleagues (2007) reported that the total population of $GABA_A$ receptors was increased by 1-week treatment with haloperidol and olanzapine; however, antipsychotic effects diminished in the longer treatment groups. Additionally, McLeod et al. (2008) found that administration with haloperidol decreased the $GABA_A$ binding site in the thalamus but increased binding sites in the hypothalamus. It is worth noting that none of the antipsychotics directly binds with $GABA_A$ receptors, which raises a critical question: which pathway(s) do antipsychotics act on to affect the $GABA_A$ receptors?

All current antipsychotics act on D_2-like receptors (including D_2, D_3 and D_4) to achieve their therapeutic effects (Kapur and Mamo, 2003). Typical antipsychotic drugs (e.g. haloperidol) can potently block D_2-like receptors, being effective in alleviating the positive symptoms of

schizophrenia (such as delusions, hallucination, disordered speech and behaviours etc.), but inducing severe extrapyramidal side-effects (EPS; such as acute dyskinesias and dystonic reactions, tardive dyskinesia, and Parkinsonism etc.) (Kapur and Mamo, 2003). Furthermore, although binding to multiple receptors (including serotonin 5-HT_{2A}, 5-HT_{2C}, muscarinic receptors), the majority of the atypical antipsychotics (e.g. clozapine, olanzapine and risperidone) also antagonise D_2Rs (Kapur and Mamo, 2003). In recent years, partial agonists for D_2-like receptors were the focus of the new drug development. Aripiprazole is a successful D_2 partial agonist that possesses therapeutic effects in schizophrenia, with much lower EPS side-effects (Mailman and Murthy, 2010). However, to date, all other partial agonists, except aripiprazole, failed to achieve meaningful clinical efficacy for schizophrenia or were discontinued due to tolerability and/or safety issues (Benkert et al., 1995, Lahti et al., 1998). Recently, bifeprunox, a potent partial agonist for the dopamine D_2 receptor, exhibited therapeutic effects in clinical trials (Casey et al., 2008), but was still disapproved and cancelled due to severe side effects (e.g. nausea) and unstable long-term therapeutic effects (Casey et al., 2008, Lundbeck, 2009). Therefore, it is necessary to investigate the pharmacological mechanism of aripiprazole by comparing it with other D_2 partial agonists (e.g. bifeprunox).

The G protein-dependent cAMP-PKA pathway is a major downstream signalling pathway of D_2-like receptors, in which activation of D_2-like receptors inhibits the activity of adenylate cyclase and synthesis of cAMP via the G_i protein, followed by the inhibition of PKA signalling (Beebe, 1994). A post-mortem study found reduced PKA regulatory subunits in schizophrenia patients (Tardito et al., 2000), indicating that PKA signalling may be related to the pathophysiology of schizophrenia. *In vivo* studies have suggested that PKA signalling may be involved in the clinical effects of antipsychotics. For example, acute haloperidol and

olanzapine treatment can increase the expression of PKA catalytic subunits in the CPu in rats (Turalba et al., 2004); another study indicated that haloperidol facilitated this signalling (Kaneko et al., 1992); furthermore, the activity of the cAMP-PKA pathway and expression of PKA regulatory subunits in the striatum were elevated after 3-week treatment with haloperidol, whereas clozapine displayed opposite effects in various brain areas (Dwivedi et al., 2002).

PKA kinase regulates several downstream substrates that are involved in the pathophysiology of schizophrenia. The $GABA_A$ receptor can be regulated by D_2-like receptor-mediated PKA signalling (Connelly et al., 2013). In addition, cAMP-responsive element-binding protein (CREB) is another PKA downstream substrate (Shaywitz and Greenberg, 1999). Novel variants in the *CREB* gene have been identified in schizophrenic patients who have experienced the positive symptoms of schizophrenia (Kawanishi et al., 1999). Previous studies revealed that haloperidol increased phosphorylation levels of CREB in both *in vivo* and *in vitro* studies (Konradi and Heckers, 1995, Pozzi et al., 2003, Yang et al., 2004). Both amisulpride and clozapine can also induce the phosphorylation of CREB *in vitro* (Jeon et al., 2015, Park et al., 2011). Therefore, we proposed that antipsychotic drugs may modulate $GABA_A$ receptors and CREB1 activity through the PKA signalling pathway. The present study investigated the effects of antipsychotics on the PKA downstream $GABA_A$ receptors, and CREB1.

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

Before the drug administration commenced, the rats were fed with cookie dough (containing sucrose 30.9%, corn starch 30.9%, casein 15.5%, minerals 8.4%, fibre 6.4%, gelatine 6.3% and vitamins 1.6%) without drugs for 1 week to train them to self-administrate the drug. Then rats were randomly assigned into one of the following drug groups (n = 6 / group) and orally treated with cookie dough mixed with the drug powder: 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 for 1 week. Water was added to achieve a dry-dough consistency immediately prior to administration. Rats were offered cookies with drugs by a metal spoon three times a day (at 06:00 AM, 02:00 PM and 10:00 PM) and observed to ensure complete consumption of each pellet. These dosages were equivalent to the recommended dosage in the clinic, and they were translated based on body surface area according to the FDA guidelines for clinical trials (FDA, 2005, Reagan-Shaw et al., 2008). This drug administration method has been well established in our laboratory (De Santis et al., 2014, Deng et al., 2015). A 0.75 mg/kg aripiprazole dosage in rats is equivalent to ~7.5 mg in humans (60 kg body weight), while 0.8 mg/kg bifeprunox and 0.1 mg/kg haloperidol is equivalent to ~8 mg and ~1 mg respectively, all of which are within the used/recommended clinical dosages (Casey et al., 2008, Emsley, 2009, Mace and Taylor, 2009). It was reported

that aripiprazole and bifeprunox, at these dosages, had over 90% D_2 receptor occupancy in rat brains (Wadenberg, 2007), while haloperidol reached approximately 70% D_2R occupancy (Kapur et al., 2003, Naiker et al., 2006, Natesan et al., 2006). Furthermore, the dosages used in this study have been shown to be physiologically and behaviourally effective in rodents (Assie et al., 2006, De Santis et al., 2014, Han et al., 2009, Kesby et al., 2006, Wadenberg, 2007), whilst not causing EPS side-effects (Natesan et al., 2006, Wadenberg, 2007). After 1-week administration, all rats were sacrificed between 10:00 AM and 12:00 PM to minimise possible circadian-induced variation of protein expression. Brains were immediately removed, frozen in liquid nitrogen and stored at -80°C until required.

Microdissection

Following a standard procedure used in our lab, discrete brain regions were collected using brain microdissection puncture, which has been performed successfully (Pan et al., 2015). Based on the brain atlas (Paxinos and Watson, 2005), three sections through the forebrain (Bregma 3.30 to 4.20 mm) were dissected for the PFC; three sections through the striatum (Bregma 1.00 to 2.20 mm) were dissected for the NAc and CPu, respectively. The three brain regions were chosen because they are key brain areas involved in the effects of antipsychotics. Tissue obtained was stored at -80°C until use.

Western Blots

The Western Blots procedures were described previously (Lian et al., 2014, Pan et al., 2015). Tissue was homogenised with NP-40 cell lysis buffer (Invitrogen, Camarillo, CA, USA) mixed with Protease Inhibitor Cocktail (Sigma-Aldrich, St Louis, MO, USA), β-Glycerophosphate (Invitrogen) and phenylmethylsulfonylfluoride (Sigma-Aldrich). The homogenised samples were centrifuged, and the supernatants were collected. Protein

concentrations of the supernatants were determined spectrophotometrically using the *DC* Protein Assay (Bio-Rad, #500-0111). Samples containing 10 μg of protein were resolved by 10% SDS-PAGE gels, and then transferred electrophoretically to a polyvinylidene difluoride membrane using Bio-Rad Midi Format 1-D Electrophoresis Systems. The membranes were blocked by 5% skim milk and incubated in primary antibodies. Amersham Hyperfilm ECL (GE Healthcare, #28-9068-36) was used to visualise the immunoreactive bands. The immunoreactive signals were quantified using Bio-Rad Quantity One software. The data were normalised to the corresponding actin levels. Experiments of Western Blots were performed in duplicate to ensure consistency.

Two catalytic (Cα and Cβ) isoform of PKA subunits have been previously identified (Cadd and McKnight, 1989). PKA-Cα expression was measured due to its highest expression in brain tissues (Soberg et al., 2013, Uhler et al., 1986). Therefore, two antibodies for PKA subunits were chosen in the present study: anti-PKA-Cα (1:1000; Santa Cruz, #SC-903) and anti-phosphor-PKA-C (Thr197) (1:1000; Cell Signalling, #5661). Subunits of $GABA_A$ receptors and CREB1 were examined using: anti-$GABA_A$ β-1 (1:1000; Abcam, #ab154822), anti-$GABA_A$ β-2 (1:1000; Abcam, #ab156000), anti-$GABA_A$ β-3 (1:1000; Abcam, #ab98968) and anti-CREB1 (1:1000; Abcam, #ab32515). Mouse anti-actin primary polyclonal antibody (1:10000; Millipore, #MAB1501) were used to determine the actin levels. The secondary antibodies used in this study were HRP-linked anti-rabbit IgG antibody (1:3000; Cell Signalling, #7074) and HRP-linked anti-mouse IgG antibody (1:3000; Cell Signalling, #7076).

RNA isolation and related quantitative real-time PCR

Quantitative real-time polymerase chain reaction (qRT-PCR) was employed to measure the mRNA expression levels of Gabrb1, Gabrb2, Gabrb3 and Creb1 in the brain regions where the PKA signalling was significantly affected by antipsychotic drug administration. The qRT-PCR procedures were described previously (Liu et al., 2015). Briefly, PureLink® RNA Mini Kit (Invitrogen Life Technologies, Carlsbad, CA, USA) was used to extract RNA. First-strand cDNA was synthesised from RNA with Superscript® VILO^{TM} cDNA Synthesis Kit (Life Technologies, NSW, Australia) by incubation at 42 °C for 60 min. Then, each sample cDNA was performed qRT-PCR in duplicate using TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, USA) on LightCycler® 480 (Roche, Penzberg, Germany). The assay (Life Technologies, NSW, Australia) identifications of the target genes were Gabrb1 (Rn00564146_m1), Gabrb2 (Rn00564149_m1), Gabrb3 (Rn00567029_m1) and Creb1 (Rn00578828_g1). All gene expression levels were normalized relative to two endogenous control genes glyceraldehyde-3-phosphatedehydrogenase (GAPDH) (Rn01775763_g1) and β-actin (Rn00667869_m1). The 2-ΔΔCT method was used to calculate the results.

Statistics

All data were analysed using the SPSS Statistics v22.0 program. The data of both Western Blots and qRT-PCR experiments were normalised by taking the average value of the control group as 100% and expressed as mean ± S.E.M. The individual control value was also normalised to the average of overall control experiments for statistical analysis. The phosphorylated protein p-PKA was normalised by the levels of total PKA-Cα. Normality test was performed to test data distribution. One-way analysis of variance (ANOVA) was performed if the data was normally distributed, followed by Post-hoc Dunnett t test to compare the control and each drug treatment group. Pearson's correlation test was used to

analyse the relationships among the measurements. Statistical significance was accepted when $p < 0.05$.

Results

Antipsychotic drug effects on PKA signalling

PFC. The expression of PKA-Cα subunits in the PFC was significantly affected by 1-week drug administration ($F_{3,\ 20} = 3.723$, $p < 0.05$). In comparison with the control group, administration with aripiprazole induced a significant elevation in the protein levels of PKA-Cα (123.6 ± 3.7%, $p < 0.05$) (Fig. 1A & 1D). However, no drug significantly affected the levels of p-PKA and the ratio of p-PKA/PKA (Fig. 1A).

NAc. It was shown that antipsychotic drug administration had a significant effect on the levels of p-PKA ($F_{3,\ 20} = 13.123$, $p < 0.01$) and the ratio of p-PKA/PKA ($F_{3,\ 20} = 3.216$, $p < 0.05$) in the NAc. It was further revealed that administration with both aripiprazole and haloperidol was able to significantly elevate the levels of p-PKA (aripiprazole: 134.3 ± 7.0%, $p < 0.01$; haloperidol: 140.6 ± 6.0%, $p < 0.01$) in the NAc (Fig. 1B & 1D). The ratio of p-PKA/PKA was also significantly increased by administration with aripiprazole (118.0 ± 7.5%, $p < 0.05$) and haloperidol (118.6 ± 3.8%, $p < 0.05$) (Fig. 1B).

CPu. In the CPu, drug administration significantly altered the protein levels of PKA-Cα ($F_{3,\ 20} = 24.183$, $p < 0.01$) and p-PKA ($F_{3,\ 20} = 16.281$, $p < 0.01$) and the ratio of p-PKA/PKA ($F_{3,\ 20} = 20.043$, $p < 0.01$). Post-hoc tests showed all three chemicals were able to significantly elevate the expression of PKA-Cα in the CPu (aripiprazole: 159.3 ± 3.5%, $p < 0.01$; bifeprunox: 126.7 ± 7.1%, $p < 0.01$; haloperidol: 125.0 ± 5.4%, $p < 0.01$). However, only haloperidol significantly elevated the levels of p-PKA (+88.1%, $p < 0.01$) (Fig. 1C & 1D).

The ratio of p-PKA/PKA was significantly reduced by aripiprazole administration (61.0 ± 1.6%, $p < 0.01$), but increased by haloperidol administration (148.5 ± 12.4%, $p < 0.01$) (Fig. 1C).

Antipsychotic drug effects on mRNA and protein expression of $GABA_A$ subunits and CREB1

Since significant antipsychotic drug effects on the activation of PKA signalling were observed in the NAc and CPu, $GABA_A$ receptors and CREB1 were examined in these two brain regions. The results showed that the mRNA expression levels of Gabrb1 ($F_{3, 20} = 7.898$, $p < 0.01$) were significantly altered by antipsychotic drug administration in the NAc. Furthermore, both aripiprazole and haloperidol administration up-regulated the mRNA levels of Gabrb1 (aripiprazole: 125.7 ± 4.7%, $p < 0.05$; haloperidol: 135.2 ± 4.2%, $p < 0.01$), whereas the mRNA levels of Gabrb2 and Gabrb3 were not significantly altered by any drug administration (all $p > 0.05$) (Fig. 2A). In Western Blots, it was revealed that drug administration significantly changed the levels of $GABA_A$ β-1 ($F_{3, 20} = 7.627$, $p < 0.01$) in the NAc. Post-hoc tests demonstrated that administration with aripiprazole was able to elevate significantly the levels of $GABA_A$ β-1 (164.3 ± 13.1%, $p < 0.01$) (Fig. 3A & 3C) in the NAc; in addition, haloperidol showed trends to increase the expression of $GABA_A$ β-1 (126.1 ± 7.3%, $p < 0.1$) in the NAc. It is worth noting that the protein levels of $GABA_A$ β-1 was also positively correlated with the levels of p-PKA ($r = 0.691$, $p < 0.05$) (Fig. 4A) and the ratio of p-PKA/PKA ($r = 0.583$, $p < 0.05$) in the NAc (Fig. 4B). On the other hand, in the CPu, neither the mRNA levels (Fig. 2B) nor the protein expression of the subunits of $GABA_A$ receptors (Fig. 3B & 3D) were significantly altered by any antipsychotic drug.

It was also observed that the mRNA expression levels of Creb1 ($F_{3, 20} = 2.952$, $p < 0.05$) were significantly altered by antipsychotic drug administration in the NAc. Post-hoc tests indicated that both aripiprazole and haloperidol administration significantly elevated the mRNA levels of Creb1 (aripiprazole: 124.1 ± 5.3%, $p < 0.05$; haloperidol: 125.7 ± 7.7%, $p < 0.01$) in the NAc (Fig. 2A). Western Blots indicated that drug administration significantly changed the levels of CREB1 ($F_{3, 20} = 3.656$, $p < 0.05$) in the NAc. Post-hoc tests showed that administration with aripiprazole was able to significantly promote the expression of CREB1 (127.4 ± 5.9%, $p < 0.05$) (Fig. 3A & 3C) in the NAc; additionally, haloperidol also elevated the protein levels of CREB1, but did not reach significance (119.1 ± 6.7%, $p < 0.1$). Moreover, the protein expression of CREB1 was positively correlated with the levels of p-PKA ($r = 0.371$, $p < 0.05$) (Fig. 4C), as well as the ratio of p-PKA/PKA ($r = 0.750$, $p < 0.01$) in the NAc (Fig. 4D). On the other hand, in the CPu, no drug administration significantly changed either the mRNA levels of Creb1 (all $p > 0.1$) (Fig. 2B) or its protein expression (Fig. 3B & 3D).

Discussion

The present study measured the *in vivo* effects of antipsychotics on PKA signalling in the PFC, NAc and CPu. We observed significant effects on PKA signalling induced by aripiprazole and haloperidol in the NAc and CPu. Aripiprazole and haloperidol, but not bifeprunox, activated PKA phosphorylation in the NAc. However, different effects were observed in the CPu – aripiprazole, unlike haloperidol, inhibited PKA activity in the CPu. Further analysis showed that mRNA expression and protein levels of $GABA_A$ receptors (containing β-1 subunit) and CERB1 were up-regulated by both aripiprazole and haloperidol administration, and were also significantly correlated with p-PKA levels and/or the ratios of p-PKA/PKA in the NAc. This indicates that aripiprazole and haloperidol might up-regulate

the expression of $GABA_A$ and CREB1 through PKA signalling in the NAc. In the CPu, on the other hand, unexpectedly, $GABA_A$ receptors and CREB1 were not significantly altered by aripiprazole and haloperidol, although they changed the ratios of p-PKA/PKA.

The present study demonstrated that haloperidol enhanced PKA activity in the NAc and CPu by increasing the levels of p-PKA and/or the ratio of p-PKA/PKA. Previously, a study showed the protein levels of PKA-Cα were elevated in the CPu by acute administration with haloperidol, but not in the PFC and NAc (Turalba et al., 2004). A long-term study revealed that the activity of the cAMP-PKA pathway in the striatum was significantly increased after administration with haloperidol for 3 weeks (Dwivedi et al., 2002). Our results are consistent with those of previous studies, suggesting that haloperidol is able to antagonise D_2-like receptors to increase PKA signalling. Furthermore, our study is the first study to examine the effects of aripiprazole; we found increased PKA phosphorylation levels and an increased ratio of p-PKA/PKA in the NAc, which is similar to the effects of haloperidol in this brain region, suggesting its antagonistic effects on D_2-like receptors. It is interesting that aripiprazole reduced the ratio of p-PKA/PKA in the CPu in the present study, whereas haloperidol increased it. This reduction may indicate that aripiprazole exerts agonistic effects on D_2-like receptors together with endogenous dopamine in this brain region. Moreover, aripiprazole is a functionally selective ligand for the dopamine D_2 receptor (Han et al., 2009, Mailman and Murthy, 2010). These opposite effects of aripiprazole on PKA signalling in the NAc and CPu might be (at least partly) attributed to its functional selectivity for the D_2 receptor. This may also explain why aripiprazole has fewer EPS side-effects than haloperidol, since haloperidol can increase PKA signalling in both the NAc and CPu. Lastly, bifeprunox had no effect on PKA activity in all brain regions in this study, probably because bifeprunox

possesses a high level of intrinsic activity (Tadori et al., 2007), exhibiting overall agonistic effects with endogenous dopamine.

We further observed that haloperidol enhanced PKA activity in the CPu. However, aripiprazole reduced it by decreasing the ratio of p-PKA/PKA, probably due to its functional selectivity for the D_2 receptor, as discussed earlier. It was also revealed that aripiprazole may act as a potent partial agonist, weak agonist, or antagonist depending upon the cellular environments of the targeted D_2 receptors (Burris et al., 2002, Kikuchi et al., 1995, Lawler et al., 1999, Mailman and Murthy, 2010, Shapiro et al., 2003, Urban et al., 2007). It should be noted that the NAc and CPu are heterogeneous structures with different connections with various brain regions (reviewed by Yager et al., 2015). For example, the NAc receives dopaminergic inputs from the ventral tegmental area (VTA) and links with limbic areas and the PFC, while the CPu receives dopaminergic inputs from the substantia nigra pars (SN) and links with neocortical areas, particularly the motor areas (Yager et al., 2015). Blockade of dopamine D_2 receptor activity in the mesolimbic (VTA–NAc) pathway is the common mechanism of antipsychotic actions, particularly in the control of positive symptoms of schizophrenia (Ginovart and Kapur, 2012); in addition, EPS side-effects induced by antipsychotics are related to the blockade of D_2 receptors in the nigrostriatal (SN–CPu) pathway (Tauscher et al., 2002). Since the cellular environments are distinct between the NAc and CPu, this theory of functional selectivity may be applied to the present study to explain the regional differences of aripiprazole on PKA signalling. However, the previous evidence was primarily based on *in vitro* studies (Burris et al., 2002, Kikuchi et al., 1995, Lawler et al., 1999, Mailman and Murthy, 2010, Shapiro et al., 2003, Tadori et al., 2011, Tadori et al., 2007, Tadori et al., 2005, Urban et al., 2007), and the *in vivo* effects of aripiprazole require further investigation. Moreover, in view that the pathological changes in

various mental disorders, the extra- and intra-cellular environments should be different in the patients' brains from those of the normal subjects. Therefore, it could be reasoned that psychoactive drugs may affect the PKA signalling differently in patients or rodent models for schizophrenia (such as the amphetamine and phencyclidine rat models, neuregulin-1 and ErbB4 knock-out models for schizophrenia, etc.).

Hypofunction of dopamine signalling in the PFC has been found in schizophrenia subjects, and it is believed that this hypofunction of dopamine signalling is connected with the negative symptoms of schizophrenia (Howes and Kapur, 2009). Therefore, it can be assumed that aripiprazole can exert agonistic effects on D_2-like receptors under hypo-dopaminergic conditions. Unexpectedly, we did not observe this agonistic effect in the PFC in the current study. This study used healthy animals with normal dopamine signalling; in this situation, the intrinsic activity of aripiprazole may not be potent enough to increase dopamine signalling when it acts together with endogenous dopamine, so that we could not observe the agonistic effect of aripiprazole while it happens under hypo-dopaminergic condition.

Previous evidence suggests that dysfunction in the GABA system is implicated in the pathophysiology of schizophrenia (Lewis et al., 2004); and the $GABA_A$ receptor can be regulated by D_2-like receptor-mediated PKA signalling (Connelly et al., 2013, Poisbeau et al., 1999). In this study, we found that both aripiprazole and haloperidol administration, but not bifeprunox, were able to significantly increase mRNA expression of Gabrb1 in the NAc after 1-week administration, whereas in the CPu no alterations in the $GABA_A$ receptor were induced by antipsychotic drug administration. In addition, both aripiprazole and haloperidol elevated the protein expression of $GABA_A$ (containing β-1) receptor (although the increasing effect of haloperidol did not reach significance). Previously, it is reported that chronic

haloperidol administration increased the binding density of $GABA_A$ receptors in the NAc (Zink et al., 2004), which is consistent with the finding of the present study. However, the effects of antipsychotics on the $GABA_A$ receptor are quite brain region-dependent. Zink et al. (2004) also revealed that haloperidol reduced $GABA_A$ receptor binding in the temporal and parietal cortex, and both haloperidol and clozapine reduced it in the anterior cingulate and infralimbic cortex. Additionally, 1-week administration with both haloperidol and olanzapine increased the binding of $GABA_A$ receptors in the PFC (Skilbeck et al., 2007). Tanahashi et al. (2012) and Peselmann et al. (2013) also suggested that aripiprazole affected GABA signalling in a brain region-dependent manner.

In this study, both aripiprazole and haloperidol have the ability to facilitate PKA activity and increase the levels of the $GABA_A$ receptor; we also revealed that the mRNA levels of $GABA_A$ receptors are positively correlated with both the levels of p-PKA and the ratio of p-PKA/PKA. It is therefore suggested that the regulation of $GABA_A$ receptors by antipsychotics in the NAc might occur through regulating PKA signalling via D_2-like receptors. In addition, since dysfunction of the NAc is involved in the positive symptoms of schizophrenia (Mikell et al., 2009) and the positive symptoms could be controlled by blocking D_2 receptors in NAc, our finding further suggests that PKA-$GABA_A$ signalling transmission is very likely to be involved in the therapeutic effects of antipsychotics (possibly in the positive symptoms of schizophrenia) (Fig. 5). On the other hand, antipsychotic administration did not exert any effects on the $GABA_A$ receptor in the CPu, whereas significant effects were revealed in the previous study after chronic haloperidol treatment (Zink et al., 2004). This discrepancy may be due to the treatment period. It seems likely that antipsychotic administration needs longer to exert a delayed effect on the $GABA_A$ receptor in the CPu, but this requires further chronic study to validate.

It should be noted that $GABA_A$ signalling was increased in various brain regions in schizophrenia subjects in post-mortem studies (Benes et al., 1992, Benes et al., 1997, Dean et al., 1999, Deng and Huang, 2006, Hanada et al., 1987, Ishikawa et al., 2004, Woo et al., 2004) (although conflicting data existed (Pandey et al., 1997, Squires et al., 1993)). However, our study exhibited increasing effects of antipsychotics on $GABA_A$ signalling in the NAc, which was also reported by Zink et al., (2004). These findings conflict with the situation observed in schizophrenia patients. Since most schizophrenia subjects have been exposed to chronic antipsychotic treatment, the increased $GABA_A$ signalling observed in post-mortem tissues might be at least partially a secondary effect of chronic antipsychotic treatment. Additionally, there is no post-mortem data that directly describes the changes in $GABA_A$ signalling in the NAc of schizophrenia patients, and further related studies are required to address this issue.

Novel variants in the CREB gene have been identified in schizophrenic subjects (Kawanishi et al., 1999, Shaywitz and Greenberg, 1999). CREB can be phosphorylated by PKA and affected by antipsychotic administration (Dash et al., 1991). Previous studies reported that haloperidol was able to increase phosphorylation levels of CREB both *in vivo* and *in vitro* (Konradi and Heckers, 1995, Pozzi et al., 2003, Yang et al., 2004). In addition, atypical antipsychotics amisulpride and clozapine can also induce phosphorylation of CREB *in vitro* (Jeon et al., 2015, Park et al., 2011). The above antipsychotics are all D_2R antagonists, which may indicate that dis-inhibition of cAMP and activation of PKA via antagonising D_2-like receptors leads to increased activity of CREB. In the present study, administration with haloperidol significantly increased mRNA expression of CREB1, as well as its protein expression (tended to be significant), in the NAc, which is consistent with those previous

findings (Konradi and Heckers, 1995, Pozzi et al., 2003, Yang et al., 2004). Our study is the first study to examine the *in vivo* effects of D_2 partial agonists (e.g. aripiprazole) on CREB so far. We observed that aripiprazole had positive effects on elevating the protein expression and mRNA levels of CREB1 in the NAc, whereas bifeprunox had no such effects. Since patients with novel variants in the CREB gene experienced the positive symptoms of schizophrenia (Kawanishi et al., 1999), up-regulation of CREB1 in the NAc is very likely to be associated with the therapeutic effects of antipsychotics on the positive symptoms of schizophrenia. Fig. 5 depicts a proposed mechanism indicating that antipsychotics activate PKA signalling via antagonising D_2-like receptors, and might link to the increase in the expression of CREB1 to exert therapeutic effects on the positive symptoms of schizophrenia.

It is worth noting that CREB activity can also be affected by the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinases (ERK) signalling cascade, and this regulation plays a role in schizophrenia (Kyosseva et al., 1999). Previously, both ERK and CREB activity were reported to be up-regulated by both haloperidol and risperidone (Yang et al., 2004). Therefore, MAPK/ERK signalling cascade via D_2-associated $G_{\beta\gamma}$ protein is another possible (probably indirect) signalling pathway by which aripiprazole and haloperidol regulate CREB1 to achieve their therapeutic effects on the positive symptoms of schizophrenia. Therefore, the role of MAPK/ERK-mediated CREB1 activity in the antipsychotic treatment of schizophrenia is worthy exploring in the future.

In summary, the present study demonstrated that aripiprazole was able to increase the expression of $GABA_A$ (containing β-1 subunit) receptor and CREB1 in the NAc, which were significantly correlated with the enhanced PKA signalling. However, further studies are important to validate whether aripiprazole affects $GABA_A$ receptors and CREB1 via D_2R-

mediated PKA pathway by (1) using specific D_2R antagonists, such as L-741,626 in combination with the antipsychotic drugs, (2) evaluating transcription factor phosphorylation, and (3) inhibiting PKA signalling. This study also suggested that aripiprazole may have functionally selective effects on the dopamine D_2 receptor to differentially regulate PKA signalling in the NAc and CPu. Further studies are required to explore what roles the up-regulation of $GABA_A$ receptors and CREB1 induced by antipsychotics plays in the treatment of schizophrenia. It is worthy noting that the present and previous studies mentioned above examined the effects of antipsychotic drugs on the PKA-related signalling pathways only in healthy animals. It is, therefore, necessary to investigate whether these drugs have similar effects in the animal models for schizophrenia and other mental disorders in future studies.

Conflicts of interest: All authors declare that no competing interests exist.

Funding: This study was supported by the Australian National Health and Medical Research Council project grant (APP1008473) to Chao Deng. These funding sources had no role in study design; in data analysis and interpretation; in writing of the report; or in the decision to submit the manuscript for publication.

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

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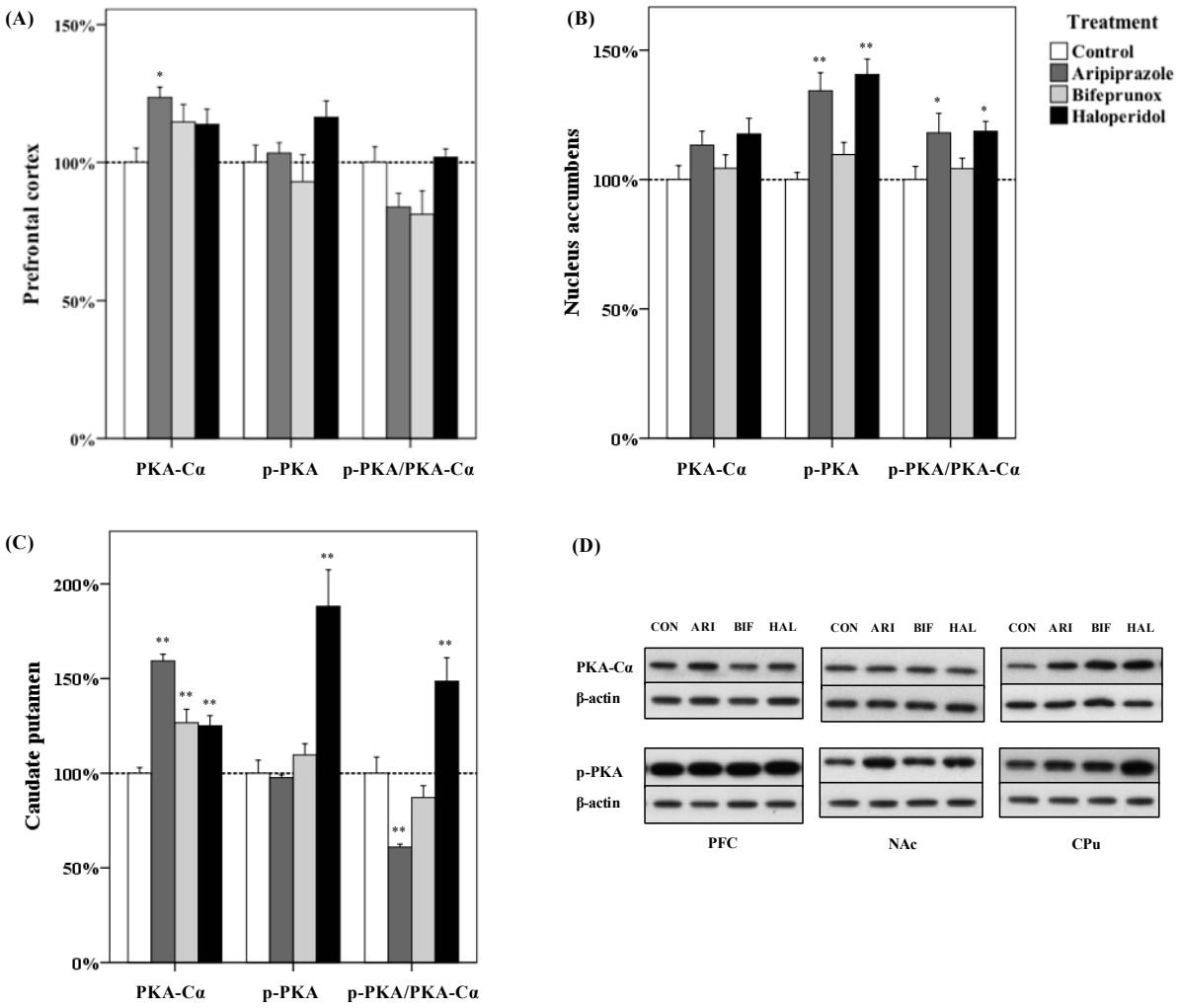
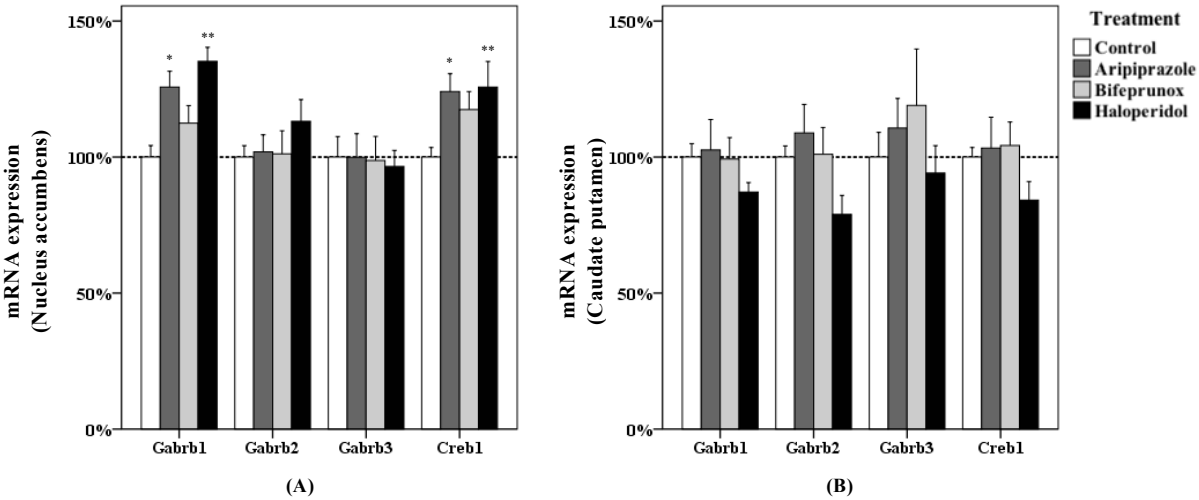
Figures:

Figure 1. Effects of three antipsychotics on PKA signalling.



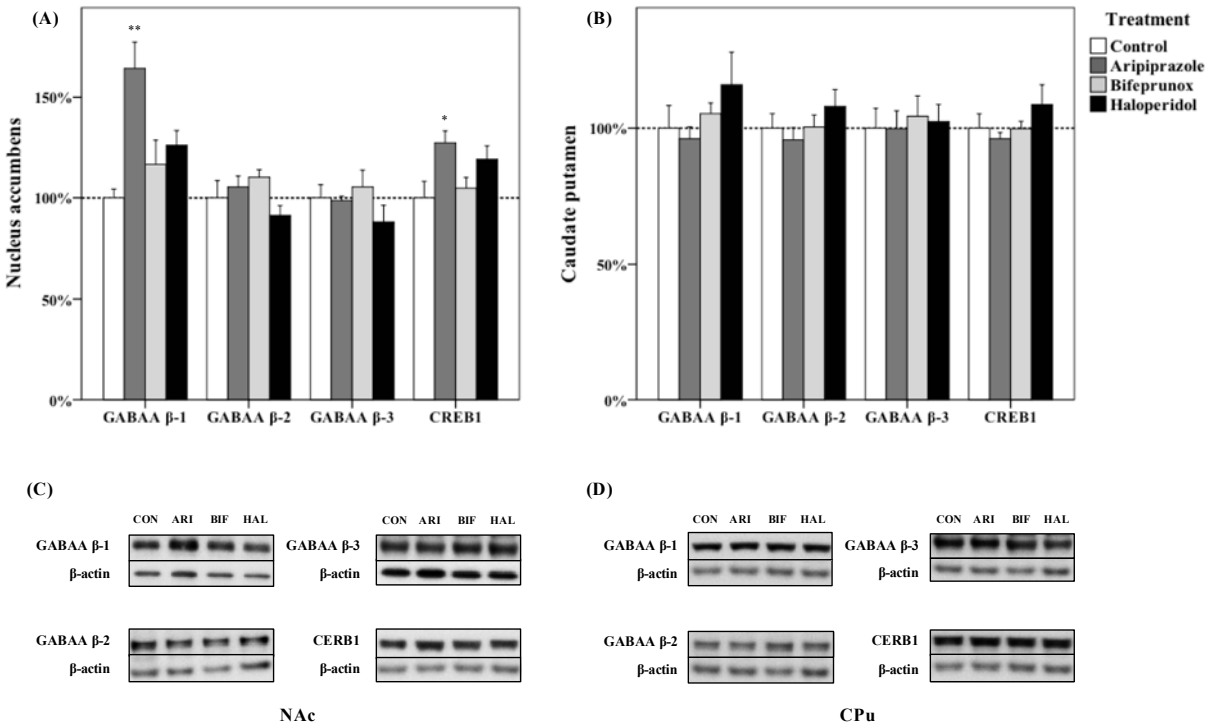
The effects of control (CON), aripiprazole (ARI), bifeprunox (BIF) and haloperidol (HAL) on PKA signalling were measured in the prefrontal cortex (A), nucleus accumbens (B) and caudate putamen (C). The representative bands of Western blot are shown in (D). PKA-Cα was quantified at 42kDa; p-PKA was quantified at 42kDa. 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* the control)

Figure 2. Effects of three antipsychotics on the mRNA levels of PKA downstream targets.



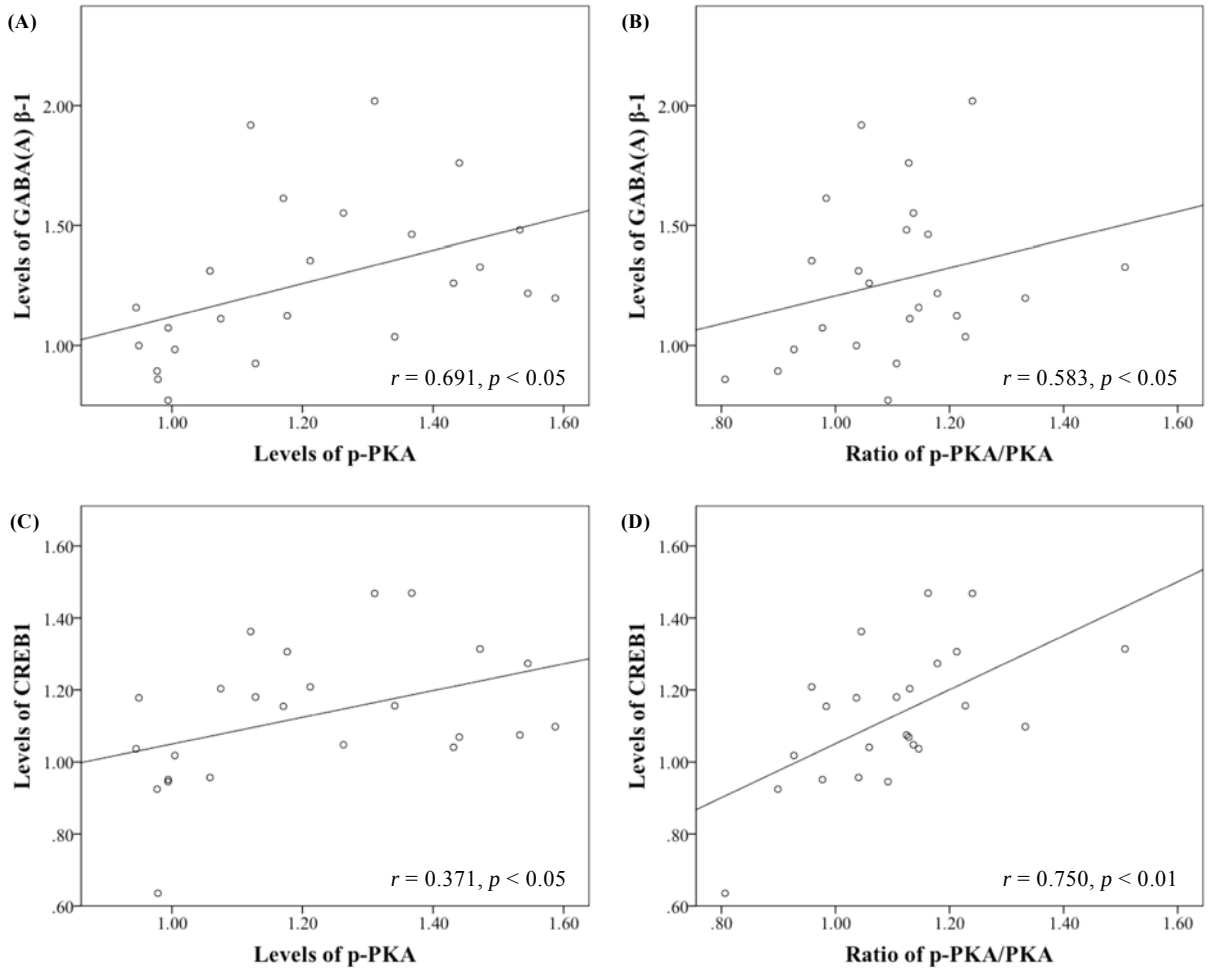
The effects of control (CON), aripiprazole (ARI), bifeprunox (BIF) and haloperidol (HAL) on the PKA downstream Gabrb1, Gabrb2, Gabrb3 and Creb1 were measured in the nucleus accumbens (A) and caudate putamen (B). (* $p \leq 0.05$, ** $p < 0.01$ *vs* the control)

Figure 3. Effects of three antipsychotics on protein expression of PKA downstream targets.



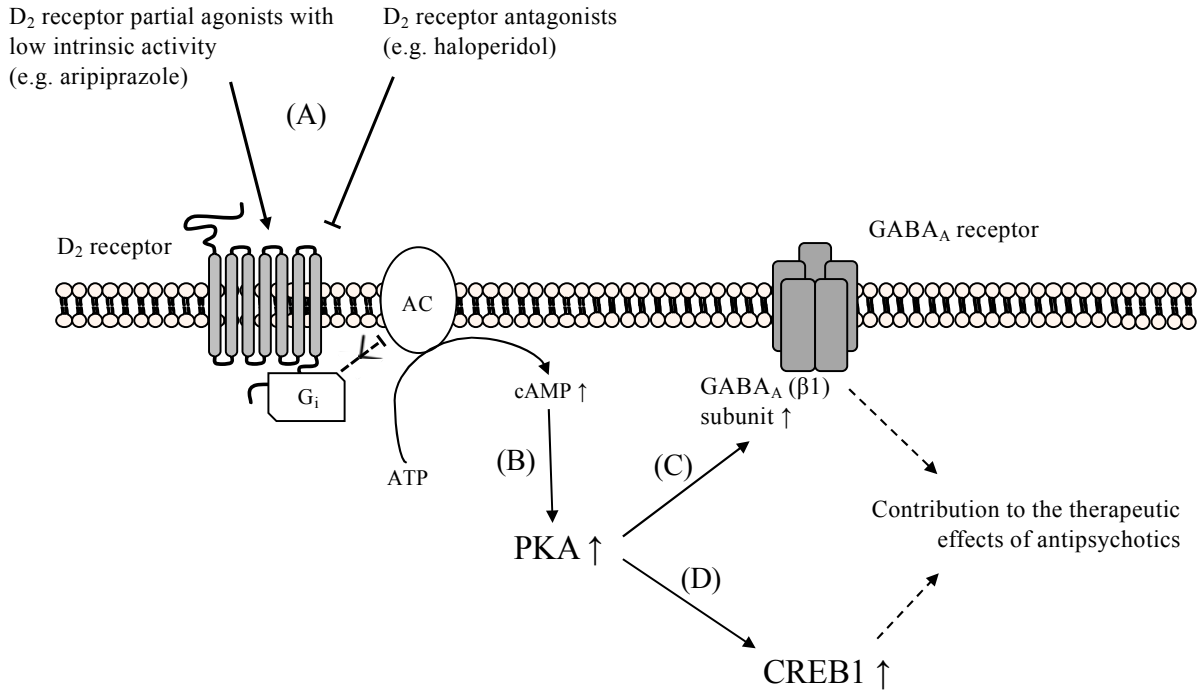
The effects of control (CON), aripiprazole (ARI), bifeprunox (BIF) and haloperidol (HAL) on the $GABA_A$ β-1, β-2 and β-3 subunits, as well as CREB1, were measured in the nucleus accumbens (NAc) (A) and caudate putamen (CPu) (B). The representative bands of Western blot of the NAc and CPu are shown in (C) and (D), respectively. $GABA_A$ β-1, β-2 and β-3 subunits were quantified at 54, 59, 55 kDa, respectively. CREB1 was quantified at 40k Da. (* $p \leq 0.05$, ** $p < 0.01$ *vs* the control)

Figure 4. Correlations between mRNA expression of Gabrb1 and Creb1 with the activation of PKA signalling in the NAc.



The mRNA expression of Gabrb1 is positively correlated with the levels of p-PKA (A) and the ratio of p-PKA/PKA (B) in the NAc. The mRNA expression of Creb1 is positively correlated with the levels of p-PKA (C) and the ratio of p-PKA/PKA (D).

Figure 5. Possible mechanisms of increased expression of $GABA_A$ and CREB1 by antipsychotic drugs through D_2-like receptor modulated PKA signalling in the nucleus accumbens.



D_2-like receptor activation can be reduced by D_2 receptor antagonists (e.g. haloperidol) and D_2 receptor partial agonists with low intrinsic activity (e.g. aripiprazole) (A), resulting in decreased inhibition (indicated by " X ") of adenylyl cyclase (AC), followed by an increased cAMP level and PKA activation (B). The activation of PKA signalling may lead to increased expression of $GABA_A$ (β-1) receptors (C) and enhanced CREB1-dependent gene expression (D), which might be involved in the therapeutic actions of antipsychotics.