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Differential effects of short- and long-term antipsychotic treatment on the expression of neuregulin-1 and ErbB4 receptors in the rat brain

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Keywords

Antipsychotic, aripiprazole, haloperidol, olanzapine, neuregulin-1, ErbB4 receptor

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Title: Differential effects of short- and long-term antipsychotic treatment on the expression of neuregulin-1 and ErbB4 receptors in the rat brain

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Abstract

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Key Word: Antipsychotic, aripiprazole, haloperidol, olanzapine, neuregulin-1, ErbB4 receptor.

1. Introduction

Neuregulin-1 (NRG1) is a family of membrane-anchored proteins containing an epidermal growth factor (EGF)-like domain that signals by stimulating membrane-associated tyrosine kinases, including ErbB4 receptors (Harrison and Law, 2006; Mei and Xiong, 2008). NRG1-induced stimulation of the ErbB4 receptor activates several signalling pathways that are involved in multiple biological functions in neurodevelopment, including neuronal specification, neuronal migration, neuronal development, and plasticity of the adult brain (Geddes et al., 2011; Mei and Xiong, 2008; Rico and Marin, 2011). The *NRG1* and *ERBB4* genes were identified as major susceptibility genes for schizophrenia by many association studies in several ethnic groups (Liu et al., 2005; Nicodemus et al., 2010; Stefanis et al., 2013; Stefansson et al., 2002). Furthermore, impaired NRG1-ErbB4 signalling is associated with cortical dysfunction, cognitive deficits and schizophrenia symptoms (Harrison and Law, 2006; Iwakura and Nawa, 2013; Law et al., 2012; Mei and Xiong, 2008; Rico and Marin, 2011).

Abnormal NRG1-ErbB4 signalling in various brain regions has been linked with schizophrenia in many studies. One study reported that NRG1 has been found to be involved in altering the size of the superior temporal gyrus in schizophrenia (Tosato et al., 2012). A number of post-mortem studies have examined the expression of NRG1 and ErbB4 in the brain of schizophrenia patients (Pan et al., 2011). For example, Hashimoto et al. reported an increase in the mRNA expression of the NRG1-type I isoform, and a decrease in the NRG1-type II isoform in the prefrontal cortex (PFC) (Hashimoto et al., 2004). A study of elderly schizophrenia patients indicated that gene expression of the NRG1-type I isoform decreased in the PFC (Brodmann's area 10, BA10), while expression of the NRG1-type II isoform increased in BA10 (Parlapani et al., 2010). Expression of NRG1-type I mRNA is also increased in the hippocampus of schizophrenia patients (Law et al., 2006). In addition, the

protein levels of the NRG1 intracellular part increased in the PFC of schizophrenia patients (Chong et al., 2008). Furthermore, post-mortem studies have shown that mRNA and protein expression of ErbB4 receptors is increased in the PFC of schizophrenia patients (Chong et al., 2008; Law et al., 2007; Silberberg et al., 2006). Importantly, an elevated phosphorylation of ErbB4 receptors and enhanced activity of downstream signalling pathways have been reported in the PFC of schizophrenia patients (Hahn et al., 2006). Therefore, although the results from post-mortem studies are not completely consistent, evidence from the majority of studies implied an elevation in NRG1-ErbB4 signalling in schizophrenia (Hahn, 2011; Pan et al., 2011). In addition, the cingulate cortex (Cg) is also involved in the pathophysiology of schizophrenia (Natesan et al., 2006; Newell et al., 2006; Newell et al., 2005). A recent animal study indicated that mutation in the NRG1 transmembrane domain altered the expression of glutamatergic receptors in the Cg (Newell et al., 2013).

To date, antipsychotic drugs have been widely used in the clinic to treat schizophrenia symptoms. It is interesting that schizophrenia patients with different NRG genotypes have been found to be able to respond differently to typical antipsychotics (Kampman et al., 2004). Haloperidol and clozapine have also been reported to improve the schizophrenia-like behaviour observed in *Nrg1*^{-/+} or *ErbB4*^{-/+} knockout mice (Dejaegere et al., 2008; Rimer et al., 2005; Savonenko et al., 2008). These reports suggest that antipsychotic drugs may exert their therapeutic effects partially through the NRG1-ErbB4 signalling pathway. Several studies have investigated the effects of particular antipsychotics (haloperidol, clozapine and risperidone) on the expression of NRG1 and ErbB4 receptors in the brain, however, the results are inconsistent (Chana et al., 2009; Hahn et al., 2006; Wang et al., 2008). A key issue is that schizophrenia patients often undergo chronic, even life-time, antipsychotic treatment; therefore, this study investigated the effects of chronic (12 weeks) antipsychotic treatment on

the expression of NRG1 and ErbB4 receptors, particularly treatment with olanzapine and aripiprazole, whose effects have not previously been examined. To summarise, the present study has investigated the short- and long-term effects of three antipsychotics (haloperidol, olanzapine and aripiprazole) on the expression of NRG1 and ErbB4 receptors in the PFC, Cg, and hippocampus of rats.

2. Methods

2.1 Animals and drug treatment

Female Sprague Dawley rats (220-250g) were obtained from the Animal Resource Centre (Perth, Australia). Rats were housed in individual cages under environmentally controlled conditions (temperature 22°C, light cycle from 07:00 to 19:00 h), with *ad libitum* access to water and a standard laboratory chow diet. After 1-week acclimatisation to the new surroundings, rats were randomly assigned to one of the following treatment groups: aripiprazole (0.75mg/kg, 3 times/day; Bristol-Meyers Squibb, USA), olanzapine (0.5mg/kg, 3 times/day; Eli Lilly, USA), haloperidol (0.1mg/kg, 3 times/day; Sigma Aldrich, USA), or vehicle (control). Each drug group was randomly subdivided into short-term (1 week, $n = 6$ per group) and long-term (12 weeks, $n = 6$ per group) treatment groups. It is worthy of note that antipsychotics have a much shorter half-life in rats than in humans. For example, the half-life of olanzapine is 24.2 hours in plasma and 72 hours in the brain of humans (Tauscher et al., 2002), compared with 2.5 hours and 5.1 hours in the plasma and brain of rats, respectively (Aravagiri et al., 1999). Haloperidol's plasma half-life is 14.5-36.7 hours in humans (de Leon et al., 2004), but 1.5 hours in rats (Cheng and Paalzow, 1992). Aripiprazole has a long plasma elimination half-life (60-70 hours) in humans (Grunder et al., 2008), while in rats aripiprazole reached the maximal plasma concentration (C_{max}) 2 hours after oral administration (10mg/kg) with an elimination half-life of 2.2 hours (Shimokawa et al., 2005).

Therefore, all rats were treated three times per day, at 06:00, 14:00, and 22:00 h, orally by administering specially prepared sweet cookie dough pellets (0.3g) to ensure a consistently high concentration to better mirror the human scenario of oral administration once per day (Deng et al., 2012; Han et al., 2008; Weston-Green et al., 2011). The rats were sacrificed using carbon dioxide asphyxiation 48 hours after the last drug treatment. All experimental procedures were approved by the Animal Ethics Committee, University of Wollongong, and complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (Seventh Edition, 2004).

2.2 Western blotting

All rats were sacrificed between 07:00 and 09:00 hours to minimise possible circadian-induced variation of protein expression. Brains were immediately removed, frozen in liquid nitrogen and stored at -80°C. Three sites of the brain (the PFC, Cg, and hippocampus) involved in the pathophysiology of schizophrenia and antipsychotic therapeutics (Ginovart and Kapur, 2012; Lewis and Gonzalez-Burgos, 2006; Newell et al., 2006; Newell et al., 2005; Volk et al., 2010) were dissected to detect NRG1 and ErbB4 protein expression. In addition, abnormal expression of NRG1 and ErbB4 has been identified in the PFC and hippocampus of schizophrenia patients (Chong et al., 2008; Law et al., 2006). In brief, 500µm thickness fresh frozen brain was cut at -14°C and collected on glass slides (Lian et al., 2014a, c; Zhang et al., 2014). Identified using a standard rat brain atlas (Paxinos and Watson, 1997), the PFC (Bregma 4.68mm to 2.76mm), Cg (Bregma 2.28mm to -0.36mm) and hippocampus (Bregma -2.28mm to -5.40mm) were collected bilaterally using a microdissection puncher. Tissue obtained from individual rats was homogenised in ice-cold homogenising buffer [9.8ml NP-40 cell lysis buffer (Invitrogen, Camarillo, CA, USA), 100µl β-Glycerophosphate (50mM; Invitrogen), 33.3µl PMSF (0.3M; Sigma-Aldrich, St Louis, MO, USA), and 100µl Protease

Inhibitor Cocktail (Sigma-Aldrich)]. The samples were centrifuged, and the supernatants were collected and stored at -80°C until required.

Total protein concentrations were quantified spectrophotometrically using the Bio-Rad DC Protein Assay (500-0116, Bio-Rad, Hercules, CA, USA) at A750nm. A range of sample proteins (5, 10, 15, 20µg) were pre-tested, and 10µg of protein was selected because it best fitted the linear range of signal detection. Homogenised brain samples containing 10µg of protein were firstly heated at 95 °C in the loading buffer [950µl laemmli buffer (Bio-Rad) and 50µl β-mercaptoethanol (Sigma-Aldrich)] for five minutes to denature the protein. The samples were loaded into CRTGEL4-12% Bis-Tris Polyacrylamide Gels (Bio-Rad) and subjected to electrophoresis in 1x XT-MOPS running buffer [50ml 20x XT-MOPS running buffer (Bio-Rad) and 950ml distilled water] at 200V for 50 minutes. Proteins on the gels were then transferred electrophoretically onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) in the ice cold transfer buffer [150ml 10x Tris/Glycine Buffer (Bio-Rad), 300ml cold methanol and 1050ml distilled water] at 100V for one hour. To detect the proteins of interest, PVDF membranes were incubated in the Tris-Buffered Saline-Tween (TBST) (Sigma-Aldrich) solution containing 5% BSA for one hour at room temperature, and then incubated overnight at 4°C with primary antibodies for NRG1 (1:200; SC-348 Santa Cruz Biotechnology, Santa Cruz, CA, USA) and ErbB4 (1:500; SC-283 Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted in TBST buffer containing 1% BSA. Membranes were washed three times with TBST for five minutes and incubated for one hour at room temperature with horseradish peroxidase-conjugated goat anti-rabbit (1:3000; Millipore, Temecula, CA, USA) secondary antibodies diluted in TBST buffer containing 1% BSA, and following 3 TBST washes, proteins of interest were visualised using an ECL system (GE Life Sciences, Piscataway, NJ, USA) and Kodak BioMax film (Sigma-Aldrich). Membranes were then re-

probed with mouse anti-actin primary polyclonal antibody (1:10000; Millipore, Temecula, CA) and horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody (1:3000; Millipore, Temecula, CA).

The immunoreactive signals were quantified by densitometry and the values were corrected based on their corresponding actin levels. For NRG1, the band of ~135kDa was quantified, as a likely full-length version of the NRG1 molecule (Kalinowski et al., 2010; Li et al., 2013). Bands of ~70kDa and ~40kDa were also quantified, which are most likely a reflection of altered processing / turnover of the NRG1 protein (Benvegnu et al., 2011; Lemmens et al., 2011; Li et al., 2013). It is worth noting that similar isoforms have been identified in both humans and rodents (Steinthorsdottir et al., 2004). For ErbB4 the band of ~185kDa was detected, representing the full-length molecule of ErbB4 (du Bois et al., 2012; Li et al., 2013; Wang et al., 2008). To determine the specificity of the manufacturer's antibody, membranes were pre-absorbed with corresponding antigen peptide, i.e. NRG1- β (SC-348, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or ErbB4 (SC-283, Santa Cruz Biotechnology, Santa Cruz, CA, USA), prior to the Western blot experiments. These antibodies are recommended to detect NRG1 and ErbB4 of both humans and rodents. All bands that were analysed in the present study were eliminated, confirming that the bands quantified with the antibody represent NRG1 isoforms or ErbB4. The β -actin protein was quantified at 46kDa. All results were normalised by taking the value of the vehicle group as 100%. Each sample from all groups ($n = 6$ per group) has been run in quadruplicate for Western blotting assay to confirm the reliability of the results.

2.3 Statistical analysis

All data was analysed using the SPSS 19.0 program (SPSS, Chicago, IL, USA) and expressed as mean \pm S.E.M. The protein expression in each brain region was analysed by Kruskal-Wallis H-test, followed by post-hoc comparisons between the control and drug treatment groups by a Mann-Whiney U-test. Statistical significance was accepted when $p \leq 0.05$.

3. Results

3.1 Effects of short- and long-term antipsychotic treatment on the protein expression of NRG1 and ErbB4 receptors in the prefrontal cortex

Figure 1 shows the changes in the protein levels of ErbB4 receptors (Figure 1B) and NRG1 isoforms (Figures 1C and 1D) in the PFC after treatment with the three antipsychotics. One-week treatment with haloperidol tended to up-regulate the NRG1-70kDa protein levels, but not reach significance (+40%, $p = 0.076$; Figure 1A and 1C), whereas 12-week treatment with haloperidol significantly decreased the level of NRG1-135kDa (40%, $p < 0.05$) and NRG1-40kDa (36%, $p < 0.05$) (Figure 1D). In addition, decreased protein levels of NRG1-135kDa (58%, $p < 0.01$; Fig 1D), NRG1-70kDa (47%, $p < 0.01$) and NRG1-40kDa (42%, $p < 0.05$) were observed in the 12-week aripiprazole group; however, no significant effects on NRG1 levels were revealed after short-term aripiprazole treatment (Figure 1C). Furthermore, 12-week treatment with olanzapine significantly decreased the level of NRG1-40kDa (35%, $p < 0.05$; Figure 1D).

The protein expression of ErbB4 in the PFC was significantly increased by 1-week treatment with haloperidol (+113%, $p < 0.05$; Figure 1B), while 1-week treatment with aripiprazole also increased ErbB4 receptors expression, but not significantly (+66%, $p > 0.1$). In addition, the expression of ErbB4 receptors was down-regulated by chronic treatment with aripiprazole (-58%, $p < 0.05$) and haloperidol (-61%, $p < 0.05$), respectively. Twelve-week olanzapine

treatment also induced a lower ErbB4 expression than the control group, although it did not reach significance (-33%, $p > 0.1$).

3.2 Effects of short- and long-term antipsychotic treatment on the protein expression of NRG1 and ErbB4 receptors in the cingulate cortex

Figure 2 shows the changes in the protein levels of ErbB4 receptors (Figure 2B) and NRG1 isoforms (Figure 2C and 2D) in the Cg after treatment with the three antipsychotics. The results indicate that 12-week treatment with aripiprazole decreased protein levels of all three NRG1 isoforms (NRG1-70kDa: -45%, $p < 0.05$; NRG1-40kDa: -48%, $p < 0.01$; NRG1-135kDa: -36%, $p = 0.07$, not significant; Figure 2D). Long-term treatment with both haloperidol and olanzapine significantly decreased the protein levels of the NRG1-40kDa (-48%, $p < 0.05$ for haloperidol; -35%, $p < 0.05$ for olanzapine). However, no significant alteration in the expression of NRG1 isoforms was observed after 1-week treatment with all three antipsychotics (all $p > 0.05$; Figure 2C). In addition, neither 1-week nor 12-week treatment with all three antipsychotics had any significant effects ($p > 0.1$) on the expression of ErbB4 receptors in the Cg (Figure 2B).

3.3 Effects of short- and long-term antipsychotic treatment on the protein expression of NRG1 and ErbB4 receptors in the hippocampus

Figure 3 shows the changes in the expression of ErbB4 receptors (Figure 3B) and NRG1 isoforms (Figure 3C and 3D) in the hippocampus after treatment with the three antipsychotics. It was revealed that 1-week treatment with haloperidol positively affected the protein levels of the NRG1-70kDa (+32%; $p < 0.05$) (Figure 3A and 3C). In addition, 1-week treatment with olanzapine significantly increased the protein levels of NRG1-70kDa (+67%, $p = 0.05$) (Figure 3A and 3C). On the other hand, 12-week treatment with aripiprazole induced a

significant decrease in the protein levels of NRG1-135kDa (-34%, $p < 0.05$), - 70kDa (-29%, $p < 0.05$) and a non-significant decrease in NRG1-40kDa (-32%, $p = 0.06$). Moreover, 12-week treatment with haloperidol down-regulated the expression of NRG1-40kDa (-36%, $p < 0.05$), whereas 12-week treatment with olanzapine showed no significant effect on the protein levels of all NRG1 isoforms.

For the protein expression of the ErbB4 receptor, Figure 3B shows that there was a significant decline after 12-week treatment with both aripiprazole (-43%, $p < 0.05$) and haloperidol (-33%, $p < 0.05$). There were no significant changes in the expression of ErbB4 in the hippocampus in the 1-week treatment groups, although there was a non-significant increase in the 1-week olanzapine treatment group (+54%, $p > 0.1$; Figure 3B).

4. Discussion

The present study is the first study that investigated the short- and long-term effects of olanzapine and aripiprazole treatment on the expression of NRG1 isoforms and ErbB4 receptors. The present study demonstrated that the protein expression of NRG1 and ErbB4 receptors was differentially affected by short- (1-week) and long-term (12-week) treatment with various antipsychotic drugs (Summarized in Table 1). Short-term treatment tended to increase the protein levels of NRG1 and ErbB4 receptors; in particular, short-term treatment with haloperidol increased the expression of NRG1-70kDa (not significant) and ErbB4 receptors in the PFC, and NRG1-70kDa in the hippocampus, respectively. In addition, short-term treatment with olanzapine induced a significant increase in the protein levels of NRG1-70kDa in the hippocampus. In contrast, long-term treatment with these antipsychotics was able to decrease the protein levels of NRG1 isoforms and ErbB4 receptors. Long-term treatment with aripiprazole induced a significant reduction in NRG1-135kDa, -70kDa and -

40kDa isoforms in the PFC, in NRG1-70kDa and -40kDa isoforms in the Cg, in NRG1-135kDa and -70kDa isoforms in the hippocampus and in ErbB4 receptors in the PFC and the hippocampus. Long-term treatment with haloperidol significantly decreased the expression of NRG1-135kDa, -40kDa and ErbB4 receptors in the PFC, and NRG1-40kDa in the Cg and hippocampus. Long-term treatment with olanzapine did not show as many effects as the other two antipsychotics, only reducing the expression of NRG1-40kDa in the PFC and Cg. Together, these results indicated differential effects of antipsychotics on the expression of NRG1 and ErbB4 receptors in brain regions involved in the pathophysiology of schizophrenia.

Several studies have investigated the effects of some antipsychotics on the expression of NRG1 and ErbB4 receptors, however, those results were discrepant (Chana et al., 2009; Shibuya et al., 2010; Wang et al., 2008; Zhang et al., 2008). A previous study found that 4-week treatment with clozapine (10mg/kg, once/day, i.p.) reduced the protein expression of NRG1 β in the rat PFC, and increased NRG1 and ErbB4 expression in the hippocampus (Wang et al., 2008), while 4-week treatment with risperidone (1mg/kg, once/day, i.p.) up-regulated NRG1 and ErbB4 expression in the hippocampus, but had no effect on expression in the PFC of rats (Wang et al., 2008). An *in vitro* study demonstrated that exposure to clozapine (but not haloperidol) for three weeks promoted NRG1 protein expression in human fetal brain aggregates (Chana et al., 2009). Differential effects of antipsychotic treatment in the expression of NRG1 have also been observed in blood samples. For example, treatment with risperidone and quetiapine for 2 weeks caused an increase in NRG1 mRNA expression in peripheral blood lymphocytes obtained from first-onset antipsychotic-naïve schizophrenia patients compared to baseline levels prior to antipsychotic therapies (Zhang et al., 2008). However, treatment with haloperidol for 8 weeks did not cause any significant changes in

serum NRG1 protein levels in cynomolgus monkeys (Shibuya et al., 2010). The exact reasons for these discrepancies are unknown; however, considering the different treatment duration between those studies and the present study, a plausible explanation could be a time-dependent effect of antipsychotic treatment on NRG1-ErbB4 signalling.

An *in vivo* study has reported that 4-week treatment with haloperidol (1mg/kg, once/day; i.p.) increased the protein expression of NRG1 β in the PFC, and both NRG1 β and ErbB4 in the hippocampus of rats (Wang et al., 2008). In contrast, 12-week treatment with haloperidol (2mg/kg/day, Medisorb polymer implant) was reported to significantly reduce NRG1-induced ErbB4 activation in the PFC of mouse brains (Hahn et al., 2006). Consistent with these findings, the present study shows that 1-week haloperidol treatment increased protein expression of NRG1 and ErbB4 receptors, but generally reduced their expression after 12-week treatment (although some data does not reach significance). Time-dependent effects have also been observed in NRG1-ErbB4 responses to olanzapine and aripiprazole treatment. For example, 1-week treatment with olanzapine increased the protein levels of NRG1 in the hippocampus, but had no effects in other brain regions; however, 12-week olanzapine treatment negatively affected NRG1-40kDa expression in the Cg, but did not reveal any other effects. On the other hand, although the expression of NRG1 and ErbB4 did not significantly changed after 1-week treatment with aripiprazole, their expression was decreased by 12-week aripiprazole treatment in all three brain areas to varying degrees. Together, the results of the present study suggest a general trend where short-term treatment with antipsychotics (up to 4 weeks) may increase the expression of NRG1 and ErbB4 receptors, whereas prolonged antipsychotic treatment could decrease their expression. Although the mechanisms underlying the time-dependent effects of antipsychotics are unknown, an adaptive change caused by repeated and chronic drug administration has been suggested (Chen and Chen,

2005). In view of the important roles of NRG1-ErbB4 signalling in neurotransmission/synaptic plasticity and the pathophysiology of schizophrenia (i.e. generally up-regulated NRG1-ErbB4 signalling in schizophrenia), these adaptations might contribute to the therapeutic effects of antipsychotics (Hyman and Nestler, 1996).

Evidence from the present and previous studies indicates that antipsychotics might regulate NRG1-ErbB4 expression in a brain region-specific manner (Wang et al., 2008). The expression of NRG1-ErbB4 in the three brain regions examined in the present study displayed different responses following antipsychotic treatment, which might be due to the distinct neural circuits in these brain regions. The altered expression of NRG-ErbB4 in the PFC was more remarkable than in other brain regions in the present study. The functions of the PFC are intricately regulated by GABAergic and glutamatergic transmission that is affected by NRG-ErbB4 signalling (Woo et al., 2007). An *in vivo* study suggested that enhanced interaction among ErbB4, postsynaptic density-95 (PSD95) and N-methyl-D-aspartate receptor (NMDAR) is associated with impairment of working memory in rats, inducing behavioural abnormalities (Li et al., 2013). A clinical study has reported switching to aripiprazole improved working memory in schizophrenia patients (Schlagenhauf et al., 2010). In the present study, chronic treatment with aripiprazole displayed the strongest effects in reducing the expression of NRG1 isoforms and ErbB4 receptors among the three antipsychotics, which probably indicates that decreasing the interaction among ErbB4, PSD95 and NMDAR is one possible route for aripiprazole to improve the working memory. Therefore, the present study might add a new line of evidence that schizophrenia symptoms may be alleviated by aripiprazole by regulating NRG1-ErbB4 signalling in the PFC through antipsychotic treatment. Furthermore, clozapine and haloperidol could improve PPI (prepulse inhibition) deficits in NRG1 and ErbB4 knock-out mice (Barros et al., 2009; Dejaegere et al.,

2008; Savonenko et al., 2008); therefore the effects of haloperidol on the expression of NRG1 and ErbB4 observed in this study may provide a mechanism for this behavioural effect. However, further studies are important to investigate whether olanzapine and aripiprazole could improve PPI deficits in NRG1 and ErbB4 knock-out mice.

Several post-mortem studies indicated that both protein and mRNA levels of NRG1 isoforms and ErbB4 receptors are over-expressed in various brain regions of schizophrenia patients (although discrepancies exist) (Chong et al., 2008; Hashimoto et al., 2004; Law et al., 2007; Parlapani et al., 2010; Silberberg et al., 2006; Weickert et al., 2012). Weickert and colleagues (2012) also stated that HapICE risk alleles induced an earlier age of onset by increasing the mRNA expression of NRG1-type III, which indicates that the increased expression of NRG1 isoforms from post-mortem tissue is not a consequence of the disease or the drug treatment. In the present study, we found that long-term treatment with all three antipsychotics (especially aripiprazole and haloperidol) was able to reduce the protein expression of all three NRG1 isoforms and ErbB4 receptors, suggesting that inhibiting NRG1-ErbB4 expression is a possible route for antipsychotics to elicit their therapeutic effects although further studies are needed.

Evidence from the present and previous studies reveals that various antipsychotics with various pharmacological binding profiles regulate NRG1-ErbB4 expression in distinct ways (Chana et al., 2009; Wang et al., 2008; Zhang et al., 2008). There is no evidence that antipsychotics could directly bind with ErbB4 receptors, although they do possess affinities for several G-protein coupled receptors (GPCRs), particularly dopamine D₂ receptors and serotonin 5-HT receptors (Correll, 2010; Kapur and Mamo, 2003). For example, haloperidol (a first-generation antipsychotic) and olanzapine (a second-generation antipsychotic) are

potent dopamine D₂ receptor antagonists, while aripiprazole acts as a partial agonist (Correll, 2010) or a functionally selective ligand for the dopamine D₂ receptor (Mailman and Murthy, 2010). Additionally, olanzapine displays antagonism for serotonin 5-HT_{2A,2C} receptors, which interacts with dopamine D₂ receptor antagonism to achieve its clinical efficacy (Mathews and Muzina, 2007; Meltzer and Massey, 2011), while aripiprazole is a partial agonist of 5-HT_{1A} receptors and an antagonist of 5-HT_{2A} receptors (Jordan et al., 2002; Zhang et al., 2006). Therefore, the differential effects of various antipsychotic drugs on NRG1-ErbB4 expression might partly be related to their distinct pharmacological binding profiles (Deng et al., 2013). Furthermore, in view of the recent evidence that NRG1-ErbB4 signalling suppresses the Src tyrosine kinase-mediated increase in synaptic NMDA transmission (Pitcher et al., 2011), and that NRG1-ErbB4 signalling can activate Fyn and Pyk2 kinases leading to increased NMDA NR_{2B} phosphorylation (Bjarnadottir et al., 2007), NMDA hypofunction observed in schizophrenia could be caused by either increased or decreased NRG1-ErbB4 signalling (Deng et al., 2013). Therefore, antipsychotic effects on NRG1-ErbB4 expression may contribute to schizophrenia therapy by modulating NMDA transmission via the Src or Fyn/Pyk2 pathways (Deng et al., 2013).

One limitation of this study was that the plasma concentration of drugs was not measured during the experimental period. Antipsychotic dosages used in this study were chosen according to dosage translation between species based on body surface area following the FDA guideline (FDA, 2005; Reagan-Shaw et al., 2008). A 0.75mg/kg aripiprazole dosage in rats is equivalent to ~7.5mg in humans (60 kg body weight), while 0.5mg/kg olanzapine and 0.1mg/kg haloperidol is equivalent to ~5mg and ~1mg respectively; all of which are within the used/recommended clinical dosages (Emsley, 2009). It is important that the drug dosages used in this study have been used previously and have been shown to be pharmacologically

and behaviourally effective (Assié et al., 2006; Deng et al., 2007; Han et al., 2009a; Weston-Green et al., 2011). It should also be noted that female rats were used in this study, because our previous studies have shown that female rats are a suitable model for investigating pharmacological effects of antipsychotics (Han et al., 2009a; Han et al., 2009b; Han et al., 2008; Lian et al., 2013, 2014b; Matosin et al., 2013). Furthermore, rats were closely housed in a room occupied by only female rats in this study. Our pre-experiments have shown that, under this rearing condition, the estrus cycles of all female rats are synchronized (Lian et al., 2013). It is also interesting that the NRG1-40kDa isoform was consistently affected by haloperidol and olanzapine, but not aripiprazole. This is mostly due to their different pharmacological profiles as discussed above, however the possibility of changes in stability of this peptide could not be completely excluded. Another limitation of this study is that only protein levels of NRG1 and ErbB4 were measured; it is important to confirm these results by examining mRNA expression using qPCR.

In conclusion, the results from the present study demonstrated the time-dependent and region-specific effects of antipsychotics on NRG1 and ErbB4 receptor expression and signalling in rats. These changes might contribute to the efficacy of antipsychotics to treat schizophrenia. In future studies, it will be important to investigate whether antipsychotics have similar effects on NRG1-ErbB4 signalling and correlating behavioural responses in animal models for schizophrenia. In light of the fact that schizophrenia patients with different NRG genotypes respond differently to typical antipsychotics (Kampman et al., 2004), it is worth investigating whether schizophrenia patients with different NRG genotypes also respond differently to other classes of antipsychotics.

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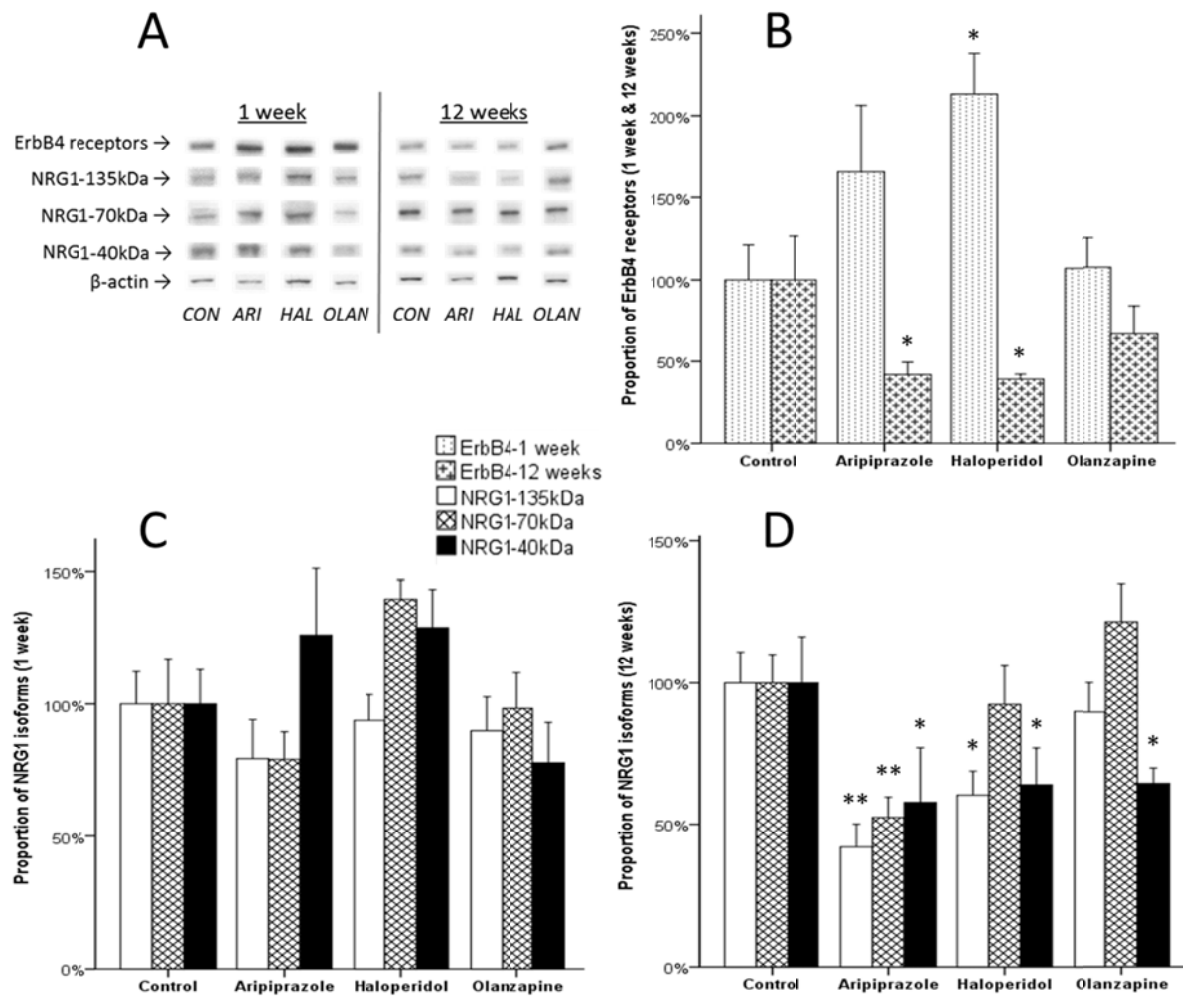


Figure 1. Expression of NRG1 isoforms and ErbB4 receptors in the prefrontal cortex of rats following short- and long-term antipsychotic drug treatment. (A) Representative Western blots of two sub-types of NRG1 proteins after rats received 1-week and 12-week treatment of aripiprazole (0.75mg/kg, 3 times/day), haloperidol (0.1mg/kg, 3 times/day), olanzapine (0.5mg/kg, 3 times/day), or vehicle (control). Quantitative analysis of ErbB4 receptors (B) and NRG1-135kDa, -70kDa and -40kDa (C and D) immunoblots. The data were normalised by taking the value of the control group as 100% and expressed as mean \pm S.E.M. * $p \leq 0.05$, ** $p < 0.01$ compared to control group.

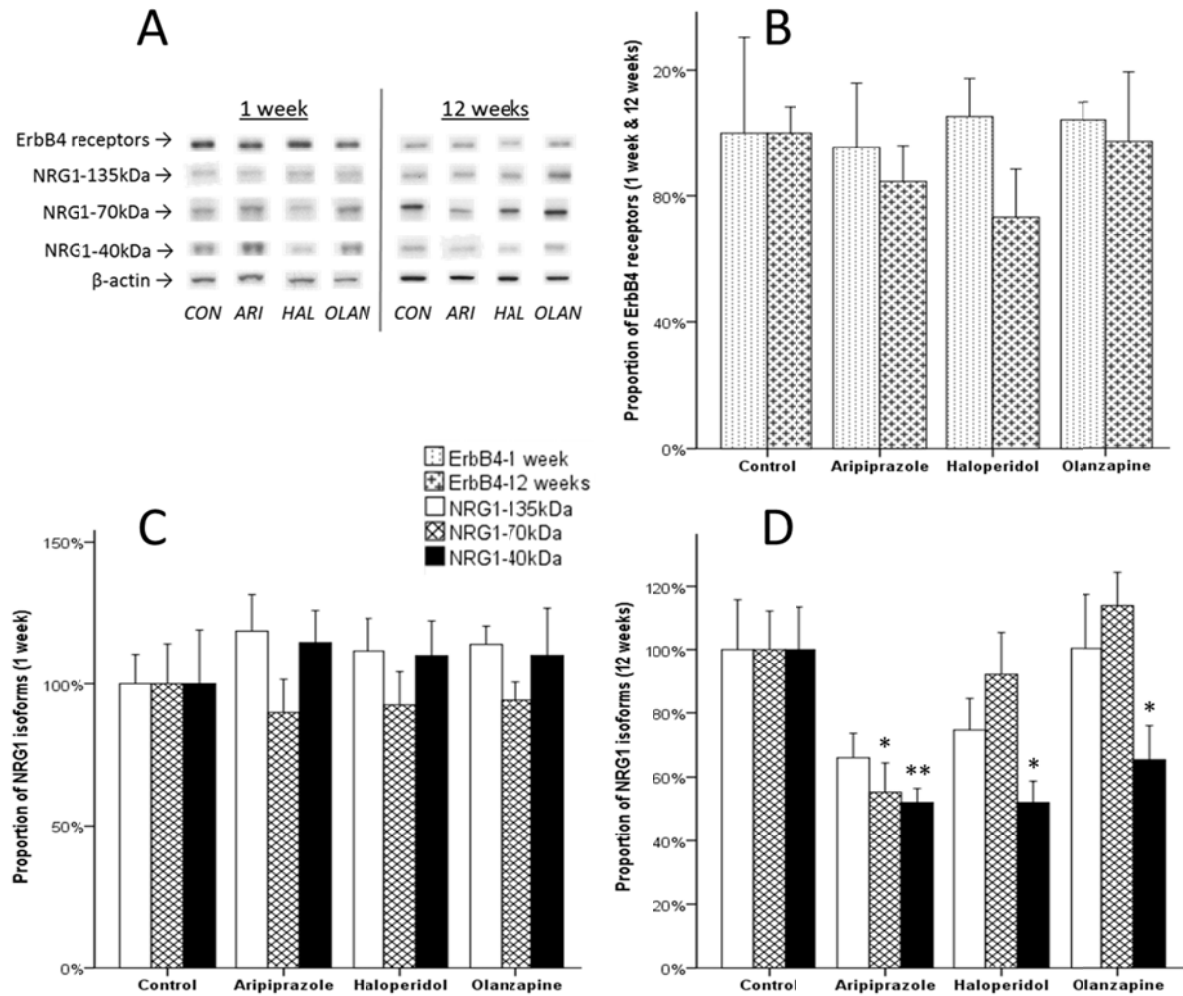


Figure 2. Expression of NRG1 isoforms and ErbB4 receptors in the cingulate cortex of rats following short- and long-term antipsychotic treatment. (A) Representative Western blots of two sub-types of NRG1 proteins after rats received 1-week and 12-week treatment of aripiprazole (0.75mg/kg, 3 times/day), haloperidol (0.1mg/kg, 3 times/day), olanzapine (0.5mg/kg, 3 times/day), or vehicle (control). Quantitative analysis of ErbB4 receptors (B) and NRG1-135kDa, -70kDa and -40kDa (C and D) immunoblots. The data were normalised by taking the value of the control group as 100% and expressed as mean \pm S.E.M. * $p \leq 0.05$, ** $p < 0.01$ compared to control group.

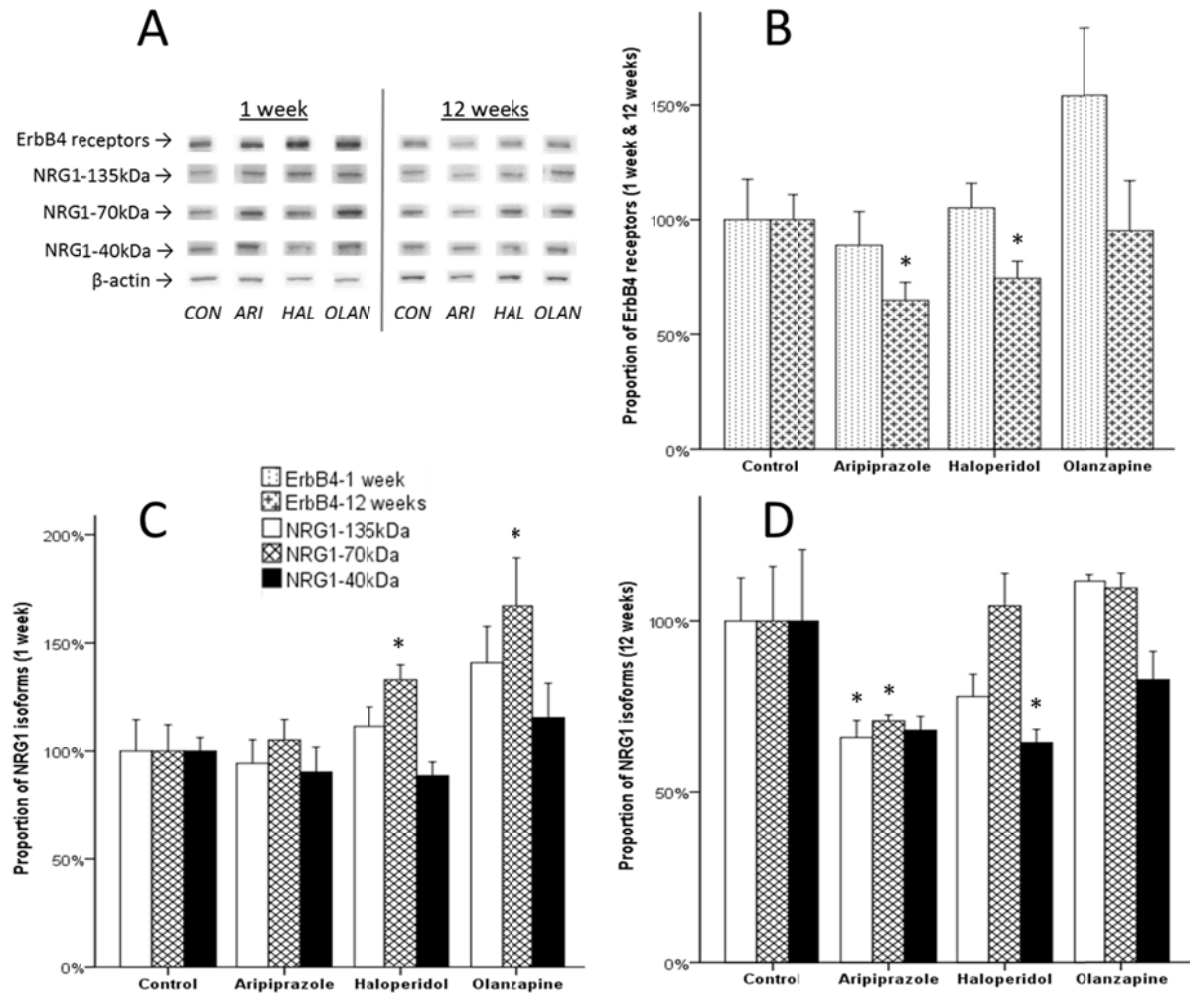


Figure 3. Expression of NRG1 isoforms and ErbB4 receptors in the hippocampus of rats following short- and long-term antipsychotic treatment. (A) Representative Western blots of two sub-types of NRG1 proteins after rats received 1-week and 12-week treatment of aripiprazole (0.75mg/kg, 3 times/day), haloperidol (0.1mg/kg, 3 times/day), olanzapine (0.5mg/kg, 3 times/day), or vehicle (control). Quantitative analysis of ErbB4 receptors (B) and NRG1-135kDa, -70kDa and -40kDa (C and D) immunoblots. The data were normalised by taking the value of the control group as 100% and expressed as mean \pm S.E.M. * $p \leq 0.05$, ** $p < 0.01$ compared to control group.

Table 1. Summary of alterations in the expression of three NRG1 isoforms and the ErbB4 receptor in the prefrontal cortex, cingulate cortex and hippocampus after treatment with aripiprazole, haloperidol and olanzapine for either 1 week or 12 weeks.

		<i>Prefrontal Cortex</i>	<i>Cingulate Cortex</i>	<i>Hippocampus</i>
Aripiprazole	1 week	--	--	--
	12 weeks	NRG1-135kDa↓, -70kDa↓, -40kDa↓, ErbB4↓	NRG1-70kDa↓, -40kDa↓	NRG1-135kDa↓, -70kDa↓, ErbB4↓
Haloperidol	1 week	ErbB4↑	--	NRG1-70kDa↑
	12 weeks	NRG1-135kDa↓, -40kDa↓, ErbB4↓	NRG1-40kDa↓	NRG1-40kDa↓, ErbB4↓
Olanzapine	1 week	--	--	NRG1-70kDa↑
	12 weeks	NRG1-40kDa↓	NRG1-40kDa↓	--