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Perinatal phencyclidine treatment alters neuregulin 1/erbB4 expression and activation in later life

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Abstract
Schizophrenia is a complex and devastating mental disorder of unknown etiology. Hypofunction of N-methyl-d-aspartate (NMDA) receptors are implicated in the disorder, since phencyclidine (PCP) and other NMDA receptor antagonists mimic schizophrenia-like symptoms in humans and animals so well. Moreover, genetic linkage and post mortem studies strongly suggest a role for altered neuregulin 1 (Nrg1)/erbB4 signaling in schizophrenia pathology. This study investigated the relationship between the NMDA receptor and Nrg1 signaling pathways using the perinatal PCP animal model. Rats (n = 5/group) were treated with PCP (10 mg/kg) or saline on postnatal days (PN) 7, 9 and 11 and were sacrificed on PN12, 5 weeks and 20 weeks for biochemical analyses. Western blotting was used to determine total and phosphorylated levels of proteins involved in NMDA receptor/Nrg1 signaling in the prefrontal cortex and hippocampus. In the cortex, PCP treatment altered Nrg1/erbB4 expression levels throughout development, including decreased Nrg1 and erbB4 at PN12 (− 25–30%; $p < 0.05$); increased erbB4 and p-erbB4 (+ 18–27%; $p < 0.01$) at 5 weeks; and decreased erbB4 and p-erbB4 (− 16–18%; $p < 0.05$) along with increased Nrg1 (+ 33%; $p < 0.01$) at 20 weeks. In the hippocampus, levels of Nrg1/erbB4 were largely unaffected apart from a significant decrease in p-erbB4 at 20 weeks (− 13%; $p < 0.001$); however NMDA receptor subunits and PSD-95 showed increases at PN12 and 5 weeks (+ 20–32%; $p < 0.05$), and decreases at 20 weeks (− 22–29%; $p < 0.05$). This study shows that NMDA receptor antagonism early in development can have long term effects on Nrg1/erbB4 expression which could be important in understanding pathological processes which might be involved in schizophrenia.

Keywords
activation, phencyclidine, perinatal, expression, later, erbb4, 1, neuregulin, alters, life, treatment

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Title: Perinatal NMDA receptor blockade induces alterations in neuregulin1/erbB4 expression and signaling in later life: implications for schizophrenia

Running title: Perinatal PCP affects Nrg1/erbB4 expression

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Abstract and keywords

Schizophrenia is a complex and devastating mental disorder of unknown etiology. Hypofunction of N-methyl-D-aspartate (NMDA) receptors are implicated in the disorder, since phencyclidine (PCP) and other NMDA receptor antagonists mimic schizophrenia-like symptoms in humans and animals so well. Moreover, genetic linkage and post mortem studies strongly suggest a role for altered neuregulin1 (Nrg1)/erbB4 signaling in schizophrenia pathology. This study investigated the relationship between the NMDA receptor and Nrg1 signaling pathways using the perinatal PCP animal model. Rats (n=5/group) were treated with PCP (10 mg/kg) or saline on postnatal days (PN) 7, 9 & 11 and were sacrificed on PN12, 5 weeks and 20 weeks for biochemical analyses. Western blotting was used to determine total and phosphorylated levels of proteins involved in NMDA receptor/Nrg1 signaling in the prefrontal cortex and hippocampus. In the cortex, PCP treatment altered Nrg1/erbB4 expression levels throughout development, including decreased Nrg1 and erbB4 at PN12 (-25−30%; $p<0.05$); increased erbB4 and p-erbB4 (+18−27%; $p<0.01$) at 5 weeks; and decreased erbB4 and p-erbB4 (-16−18%; $p<0.05$) along with increased Nrg1 (+33%; $p<0.01$) at 20 weeks. In the hippocampus, levels of Nrg1/erbB4 were largely unaffected apart from a small decrease in p-erbB4 at 20 weeks (-13%; $p<0.001$); however NMDA receptor subunits and PSD-95 showed increases at PN12 and 5 weeks (+20−32%; $p<0.05$), and decreases at 20 weeks (-22−29%; $p<0.05$). This study shows that NMDA receptor blockade early in development can have long term effects on Nrg1/erbB4 expression which could be important in understanding pathological processes which might be involved in schizophrenia.

Keywords: Neuregulin 1; erbB4; NMDA receptor; schizophrenia; rat; brain

Introduction

Schizophrenia is a complex and devastating mental disorder with no known cause. Glutamatergic NMDA receptors are implicated in the disorder, primarily since NMDA receptor antagonists such as phencyclidine (PCP) mimic the full range of schizophrenia-like symptoms in both humans and animals, and exacerbate symptoms in schizophrenia patients (Kantrowitz and Javitt, 2010). Studies have also demonstrated changes in NMDA receptor binding, transcription and subunit expression in different brain regions of schizophrenia subjects (Newell et al., 2005; Zavitsanou et al., 2002). In addition, many candidate susceptibility genes for schizophrenia such as DAAO, G72, dybindin-1, DISC-1 and neuregulin1 (Nrg1) can directly or indirectly affect NMDA receptor signaling (Banerjee et al., 2010).

Nrg1 is a growth factor known to be important in many bodily systems, including the nervous system. Nrg1 functions are largely mediated by the receptor tyrosine kinases ErbB2, 3 and 4. ErbB4 has been indicated as the predominant receptor for Nrg1 (Stefansson et al., 2004). Recently, Nrg1/erbB4 signaling has been highlighted to have a crucial role in the control of cortical development of GABA circuitry, and erbB4 has been specifically localised to the postsynaptic density of glutamatergic synapses contacting the dendrites of inhibitory neurons (Fazzari et al., 2010). Deficits in GABAergic interneurons, particularly parvalbumin-containing basket and chanderlier cells, is one of the most salient features of schizophrenia.

Linkage studies have identified both Nrg1 and the erbB4 receptor as susceptibility genes for schizophrenia and there is evidence of altered mRNA expression of Nrg1 and ErbB4 receptors in post mortem human schizophrenia brain tissue in an isoform specific manner

(See Banerjee et al 2010 for review). In terms of changes at the protein level in schizophrenia, Bertram et al (2007) found decreases in levels of Nrg1α protein in the prefrontal cortex. Hahn et al (2006) found no change in Nrg1 or erbB4 protein levels in an aged population, but they demonstrated increased Nrg1-induced erbB4 activation in schizophrenia tissue. In another study, Chong et al (2008) found increases in Nrg1 and erbB4 protein in the prefrontal cortex.

It is not known whether changes in expression levels of Nrg1 and ErbB4 in the schizophrenia brain are primary causal factors or secondary to other molecular changes related to the disease. ErbB4 receptors are anchored at PSD-95 (at the postsynaptic density of glutamatergic synapses contacting inhibitory interneurons) along with NMDA receptor 2A and 2B subunits (Fazzari et al., 2010; Garcia et al., 2000; Huang et al., 2000), which places the glutamatergic NMDA receptor and Nrg1 signaling pathways together at the synapse, allowing for crosstalk between the systems. While studies have shown that Nrg1 can influence NMDA receptor function (Bjarnadottir et al., 2007; Gu et al., 2005; Li et al., 2007), there is a lack of studies examining how NMDA receptor function can modulate Nrg1/erbB4. The present study investigated whether disrupting the NMDA receptor system through perinatal PCP treatment could lead to long term changes in Nrg1 and erbB4 expression in the rat brain.

Experimental Procedures

Animals

Timed, pregnant female Sprague–Dawley rats were obtained from the Animal Resource Centre (Perth, Australia) at day 14 of gestation. The dams were housed individually with a regular 12 hr light-dark cycle (lights on 07:00 hrs, off at 19:00 hrs) with food and water ad libitum. Day of birth was considered postnatal day (PN)0. Pups were sexed on PN7 and litters were assigned to PCP or saline groups. Each litter consisted of ten to twelve pups. This study was approved by the Animal Ethics Committee of the University of Wollongong, and procedures complied with the Australian Code of Practice for the Care and Use of Animal for Scientific Purposes, which conforms to International Guiding Principles for Biomedical Research Involving Animals. All efforts were made to minimize numbers of animals used and their suffering.

Drugs

Phencyclidine hydrochloride was synthesised and dissolved in 0.9% NaCl. Injections were administered s.c. at a volume of 1 ml/kg.

Experimental design

Male rat pups were treated on PN 7, 9 and 11 with PCP (10mg/kg) or saline. Pups were sacrificed by decapitation on PN12, or by CO2 asphyxiation at 5 weeks and 20 weeks. The frontal cortex and hippocampus were dissected on ice, snap frozen in liquid nitrogen and stored at -80°C.

Tissue was homogenised in NP-40 lysis buffer (Invitrogen Australia Pty Ltd, Mulgrave, Australia), containing protease inhibitor cocktail, beta-glycerophoshate and PMSF (Sigma, Castle Hill, Australia). Homogenates were spun at 20 000 x g for 10min at 4°C. The

supernatants were collected and equal amounts of protein were separated on 4-12% Bis-Tris gels (Bio-Rad Laboratories, Gladesville, Australia) using SDS-PAGE. Following electrophoresis (100 V for 50 min), proteins were transferred to polyvinylidene difluoride membranes (200 V for 60 min). Membranes were blocked in 5% BSA, followed by incubation with the primary antibody in 1% BSA overnight at 4°C. Following washes (3 × 5 min) in Tris Buffered Saline +0.1% Tween 20 (TBST), membranes were incubated with horseradish peroxidase conjugated secondary antibodies for 1 h at 25°C. Blots were visualised using ECL chemiluminescence detection reagents (Ge Healthcare, Rydalmere, Australia). The bands corresponding to the various proteins of interest were scanned and densitometrically analyzed using an automatic imaging analysis system (Quantity One, BioRad). All quantitative analyses were normalized to β-actin.

Antibodies

Polyclonal antibodies for NR1 (sc-1467), p-NR1 (ser896/897; sc-12890), NR2A (sc-1468), NR2B (sc-1469), Akt1,2,3 (sc-8312), p-Akt1,2,3 (ser473; sc-7985-R) Nrg1 (sc-348), erbB4 (sc-283) and p-erbB4 (Tyr1056; sc-33040) antibodies were purchased from Santa Cruz Biotechnology Inc (Scoresby, Victoria, Australia). Polyclonal antibodies for PSD-95 (ab18258), p-PSD-95 (ser295; ab16495), p-NR2A (Y1325; ab16646) and p-NR2B (ser1480; ab18533) antibodies were purchased from Abcam (Waterloo, Australia). Primary antibody dilution was 1:250 – 1:3000. Secondary antibodies were purchased from Millipore (AP307P, AP308P; North Ryde, Australia) and were used at a concentration of 1:3000. β-actin was purchased from Millipore (MAB1501) and used at a concentration of 1:250000.

Statistical analyses

The relative amounts of each protein of interest were determined based on the mean of 2-4 experiments using 5 animals/group/age. Differences between treatment and control groups were analysed at individual time-points and brain regions using a student's t-test. Statistically significance was set at an alpha level of $p<0.05$.

Results

Single bands were identified at approximately corresponding molecular weights for PSD-95 (80kDa), Akt (60kDa), NR1 (115kDa), NR2A (177kDa) and NR2B (178kDa). Nrg1 and erbB4 displayed multiple bands. For this study, only the 85kDa band of Nrg1 and the 185kDa band of erbB4 were quantified, which represent the Nrg1 type III isoform and erbB4 full length protein. These bands were eliminated following preabsorbtion of Nrg1 or erbB4 antibodies with their epitope peptides, indicating that the bands were specific for Nrg1 and erbB4.

Prefrontal Cortex

PN12: In the prefrontal cortex at PN12, PCP treatment caused a reduction in expression of Nrg1 (-30%, $p<0.001$) and erbB4 (-25%, $p<0.05$) (Fig. 1A). Levels of p-Akt were also significantly reduced (-19%, $p<0.05$). When looking at the NMDA receptor system, treatment also tended to decrease phosphorylated levels of NR2A (-30%, $p=0.078$; Fig.1A), as well as phosphorylated levels of the anchoring molecule, PSD-95 (-32%, $p=0.065$; Fig. 1A).

5 weeks: At 5 weeks in the prefrontal cortex, Nrg1 expression remained slightly decreased (-13%, $p<0.01$), while significant increases were seen in total erbB4 (+27%, $p<0.01$), and phosphorylated levels oferbB4 (+18%, $p<0.001$; Fig. 1B). When looking at the effects on the

NMDA receptor system, there was a significant increase in phosphorylated levels of NR2A (+32%, $p<0.01$), and a trend for an increase in NR2B (+19%, $p=0.064$; Fig. 1B). Total levels of the anchoring molecule, PSD-95, were increased (+11%, $p<0.05$), along with a tendency for increased levels of phosphorylated PSD-95 (+20%, $p=0.08$; Fig. 1B).

20 weeks: Conversely, in the prefrontal cortex at 20 weeks, Nrg1 expression levels were significantly increased in the PCP group (+33%, $p<0.01$), while erbB4 (-18%, $p<0.01$) and phosphorylated erbB4 (-16%, $p<0.05$) levels were significantly decreased, along with a trend for decreased levels of p-Akt (-21%, $p=0.076$; Fig. 1C). Expression levels of NMDA receptor subunits and PSD-95 returned to control level (Fig. 1C).

Hippocampus

PN12: In the hippocampus at PN12, PCP treatment did not alter Nrg1 or erbB4 expression levels (Fig. 2A). However, in terms of the NMDA receptor system, treatment significantly increased phosphorylated levels of NR1 (+20%, $p<0.05$; Fig. 2A). PSD-95 also showed an increase in expression (+24%, $p<0.05$) and there was a trend for an increase in phosphorylated levels of Akt (+19%, $p=0.065$; Fig. 2A).

5 weeks: At 5 weeks in the hippocampus, PCP treatment did not alter Nrg1 or erbB4 expression levels (Fig. 2B). When looking at the NMDA receptor system, treatment significantly increased NR2A (+32%, $p<0.05$) and NR1 (+24%, $p<0.05$) expression levels (Fig. 2B). Additionally, levels of PSD-95 (+27%, $p<0.05$) and phosphorylated Akt (+22%, $p<0.01$) were significantly increased (Fig. 2B).

20 weeks: In the hippocampus at 20 weeks, there was a slight but significant reduction in expression of phosphorylated erbB4 levels (-13%, $p<0.001$; Fig. 2C). In terms of treatment effects on the NMDA receptor system, PCP-treated rats showed a significant reduction in total NR2A (-22%, $p<0.001$) and phosphorylated NR2A (-26%, $p<0.05$), as well as NR2B (-29%, $p<0.05$) levels (Fig. 2C). Total levels of NR1 and phosphorylated levels of NR2B also tended to be decreased though not significantly (*p's*>0.05). In addition, PSD-95 expression was significantly decreased at this time-point (-23%, $p<0.05$; Fig. 2C).

Discussion

Both Nrg1 and the NMDA receptor are highly implicated in schizophrenia etiology and pathology, though the relationship between NMDA receptor functioning and Nrg1 is unclear. This study for the first time has examined the effects of perinatal PCP treatment on expression of Nrg1/erbB4 during development in the rat brain in an effort to see whether altering NMDA function has long term effects on Nrg1/erbB4 signaling. In the cortex, PCP altered Nrg1 and total and phosphorylated levels of erbB4 throughout development while in the hippocampus, PCP did not alter total Nrg1/erbb4 levels, although levels of phosphorylated erbb4 were reduced at adulthood. Results are discussed in the context of parallel changes to NMDA receptor subunits, PSD-95 and Akt.

PCP affects cortical expression of Nrg1/erbB4 throughout development

In the present study, PCP treatment reduced Nrg1/erbB4 signaling early during development in the cortex, as shown by decreases in levels of Nrg1 and its receptor erbB4 at PN12 as well as phosphorylated Akt, which is a downstream target of erbB4. This result is

not surprising given that NMDA antagonists block excitation of neurons, triggering decreased activation of cell survival pathways and ultimately widespread apoptosis. Lei et al (2008) also found that PCP treatment on PN7 reduced Akt (ser473) phosphorylation at 3, 6 and 9hrs after treatment, resulting in neurotoxicity. Interestingly, decreased Akt1 has been found in peripheral lymphocytes of schizophrenia patients, as well as an association between schizophrenia and an Akt1 haplotype linked to decreased Akt protein levels (Emamian et al., 2004).

Nrg1/erbB4 signaling plays a crucial role in the control of cortical GABA circuitry development (Fazzari et al., 2010). It has recently been confirmed that the ErbB4 receptor is specifically expressed on inhibitory interneurons, particularly parvalbumin-containing chanderlier and basket cells, where it localised to axon terminals and postsynaptic densities receiving glutamatergic input. Here, erbB4 promotes formation of axo-axonic inhibitory synapses over pyramidal neurons and regulates formation of excitatory synapses to interneurons. ErbB4 has also shown to be expressed exclusively on interneurons in the hippocampus (Buonanno, 2010). This is in contrast to the previous view implicating nrg1/erbb4 in excitatory synapses between pyramidal cells.

Reduced Nrg1/erbB4 signaling early in brain development caused by PCP treatment could impair the wiring of GABA-mediated circuits, via decreased erbB4-mediated excitatory inputs and reduced activity-dependent cell survival of interneurons. In this regard, Wang et al., (2008) have shown that perinatal PCP treatment causes a loss of parvalbumin positive interneurons in the cortex. Similar losses are seen after treatment with another NMDA receptor antagonist, MK-801 (Braun et al., 2007). Moreover, we have previously shown

increased $GABA_A$ receptor binding in several brain regions in adulthood following perinatal PCP treatment, which may represent a compensatory upregulation of receptors in response to deficits in the GABAergic system (du Bois et al., 2009).

When looking at Nrg1/erbb4 expression at adolescence, Nrg1 remained slightly lower in PCP-treated rats, while both erbB4 and phosphorylated levels of erbb4 were upregulated. This may represent a compensatory upregulation in response to reduced signaling earlier in development. Also occurring at this time-point was increased PSD-95 and phophorylated levels of the NR2A subunit, suggesting increased activation of NMDA receptors. PSD-95 may be facilitating enhanced erbB4 and NMDA receptor signaling at the synapse at adolescence in PCP-treated rats.

At the 20 week time-point, Nrg1 was increased, while total and phosphorylated erbb4 levels were decreased. NMDA receptor subunit and PSD-95 levels returned to control. The latter finding is consistent with our previous autoradiography data showing increased NMDA receptor binding at adolescence in cortical areas after perinatal PCP treatment, then a return to control level at adulthood (du Bois et al., 2009). The mechanisms underlying the switch in direction of change of Nrg1 are not completely understood at present. However it can be hypothesised that an increase in NMDA receptor activity (increased p-2A) at adolescence in the cortex is what drives the increase in Nrg1 at later stages, which acts to attenuate NMDA receptor activity in the normal state (Bjarnadottir et al., 2007; Gu et al., 2005; Hahn et al., 2006) . This is supported by the finding that soluble Nrg1 can be released from presynaptic nerve terminals in response to neuronal activity (Fernandez et al., 2000; Ozaki et al., 2004).

Hippocampal Nrg1/erbb4 expression is largely unaffected altered by perinatal PCP treatment

PCP treatment affected the NMDA system throughout development in the hippocampus, while changes to the Nrg1 system were limited to a small decrease in phosphorylated erbb4 levels at adulthood. At PN12, activation of NR1 was increased, along with increased PSD-95 expression. By the adolescent time-point, more changes were evident in the NMDA receptor system, including increases in NR2A and NR1, as well as increases in phosphorylated Akt and PSD-95. The p-NR1 antibody used for this study only recognises the NR1 subunit when both Ser 896 and 897 residues are phosphorylated. Both residues are required to be phosphorylated at the same time to increase NMDA receptor surface expression (Scott et al., 2001). In this study, we found increased levels of p-NR1 in the hippocampus at PN12 and NR1 and NR2A were increased at 5 weeks. At PN12 and 5 weeks, PSD-95 is possibly increasing trafficking, clustering and stabilization of the NMDA receptor at the postsynaptic density. Increased NR1 activity could then lead to increase in phosphorylated Akt as NMDA receptor potentiation regulates phosphorylation of Akt via ser473 (Soriano et al., 2006). Other studies also report an upregulation of NMDA receptor levels after PCP treatment (Sircar, 2003; Wang et al., 2001).

The upregulation of NMDA receptors at adolescence may have allowed for the accumulation of toxic levels of intracellular free calcium, even more so if endogenous levels of glutamate are increased in this model through a loss of GABAergic inhibiton of pyramidal cell output. On its own, calcium overload acts to suppress NR1 transcription (Gascon et al., 2005). This may explain why at 20 weeks, there was a switch in the direction of change with

decreases in NMDA receptor subunits 2A, phosphorylated 2A and 2B (and trends for decreases in phosphorylated 2B and NR1). Phosphorylated levels of PSD-95 were also significantly decreased. A decrease in p-PSD-95 at ser295 could affect the ability of PSD-95 to accumulate and recruit receptors and decrease synaptic potentiation (Kim et al., 2007). Instability of the PSD-95/NR1+NR2A complex can lead to receptor internalization and ultimately downregulation of NMDA receptors (Dong et al., 2004) and as a consequence may impact on NMDA receptor-Nrg1 interactions by affecting NMDA/ErbB4 receptor cross-talk. This may be why a decrease in erbB4 activity appears without a change in Nrg1 or erbB4 levels. These deficits in hippocampal NMDA receptor function may contribute to the deficits in cognitive function in rodents in the long term after treatment with PCP (see du Bois and Huang, 2007).

Summary and Conclusion

This study has demonstrated that Nrg1/erbB4 expression and activation levels are altered throughout development as a consequence of NMDA receptor blockade during early brain development. This demonstrates that changes in Nrg1/erbB4 can be secondary to that of the NMDA receptor, which is relevant to both the NMDA receptor hypofunction and neurodevelopmental hypotheses of schizophrenia. While these findings could represent similar changes occurring in the schizophrenia brain it is difficult to directly compare Nrg1/erbB4 protein expression in human and animal studies due to various confounding variables such as age and medication. However in future studies it will still be useful to examine the effects of specific NMDA receptor blockers on Nrg1/erbB4 signaling and how

this relates to GABAergic system function as these three systems are closely linked and have a disrupted balance in schizophrenia.

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Titles and legends to figures:

Figure 1. Protein expression in the prefrontal cortex at A) PN12, B) 5 week and C) 20 week time-points. ^statistical trend; *p<0.05; **p<0.01; ***p<0.001 vs. controls.

Figure 1. Protein expression in the hippocampus at A) PN12, B) 5 week and C) 20 week time-points. ^statistical trend; *p<0.05; **p<0.01; ***p<0.001 vs. controls.