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Abstract

Postnatal administration of phencyclidine (PCP) in rodents causes major disturbances to neurological processes resulting in severe modifications to normal behavioral traits into adulthood. It is routinely used to model psychiatric disorders such as schizophrenia, producing many of the dysfunctional processes in the brain that are present in this devastating disorder, including elevated levels of apoptosis during neurodevelopment and disruptions to myelin and plasticity processes. Lingo-1 (or Leucine-rich repeat and immunoglobulin domain-containing protein) is responsible for negatively regulating neurite outgrowth and the myelination of axons. Recent findings using a postmortem human brain cohort showed that Lingo-1 signaling partners in the Nogo receptor (NgR)/p75/TNF receptor orphan Y (TROY) signaling complex, and downstream signaling partners With No Lysine (K) (WNK1) and Myelin transcription factor 1 (Myt1), play a significant part in schizophrenia pathophysiology. Here we have examined the implication of Lingo-1 and its signaling partners in a neurodevelopmental model of schizophrenia using PCP to determine if these pathways are altered in the hippocampus throughout different stages of neurodevelopment. Male Sprague-Dawley rats were injected subcutaneously with PCP (10 mg/kg) or saline solution on postnatal days (PN) 7, 9, and 11. Rats (n = 6/group) were sacrificed at PN12, 5 weeks, or 14 weeks. Relative expression levels of Lingo-1 signaling proteins were examined in the hippocampus of the treated rats. p75 and Myt1 were decreased ($0.001 \leq p \leq 0.011$) in the PCP treated rats at PN12. There were no significant changes in any of the tested proteins at 5 weeks ($p > 0.05$). At 14 weeks, p75, TROY, and Myt1 were increased in the PCP treated rats ($0.014 \leq p \leq 0.022$). This is the first report of an alteration in Lingo-1 signaling proteins in the rat hippocampus, both directly after PCP treatment in early development and in adulthood. Based on our results, we propose that components of the Lingo-1 signaling pathways may be involved in the acute neurotoxicity induced by perinatal administration of PCP in rats early in development and suggest that this may have implications for the hippocampal deficits seen in schizophrenia.

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Alterations of p75 neurotrophin receptor and Myelin transcription factor 1 in the hippocampus of perinatal phencyclidine treated rats

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Keywords: Schizophrenia; Lingo-1 signaling; neurodevelopment; apoptosis; phencyclidine animal model; hippocampus

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Abstract

Postnatal administration of phencyclidine (PCP) in rodents causes major disturbances to neurological processes resulting in severe modifications to normal behavioural traits into adulthood. It is routinely used to model psychiatric disorders such as schizophrenia, producing many of the dysfunctional processes in the brain that are present in this devastating disorder; including elevated levels of apoptosis during neurodevelopment, and disruptions to myelin and plasticity processes. Lingo-1 (or Leucine-rich repeat and immunoglobulin domain-containing protein), is responsible for negatively regulating neurite outgrowth and the myelination of axons. Recent findings using a postmortem human brain cohort showed that Lingo-1 signaling partners in the Nogo receptor (NgR)/p75/TNF receptor orphan Y (TROY) signaling complex, and downstream signaling partners With No Lysine (K) (WNK1) and Myelin transcription factor 1 (Myt1), play a significant part in schizophrenia pathophysiology. Here we have examined the implication of Lingo-1 and its signaling partners in a neurodevelopmental model of schizophrenia using PCP to determine if these pathways are altered in the hippocampus throughout different stages of neurodevelopment. Male Sprague Dawley rats were injected subcutaneously with PCP (10mg/kg) or saline solution on postnatal days (PN)7, 9 and 11. Rats (n=6/group) were sacrificed at PN12, 5 weeks or 14 weeks. Relative expression levels of Lingo-1 signaling proteins were examined in the hippocampus of the treated rats. p75 and Myt1 were decreased ($0.001 \leq p \leq 0.011$) in the PCP treated rats at PN12. There were no significant changes in any of the tested proteins at 5 weeks ($p > 0.05$). At 14 weeks, p75, TROY, and Myt1 were increased in the PCP treated rats ($0.014 \leq p \leq 0.022$). This is the first report of an alteration in Lingo-1 signaling proteins in the rat hippocampus; both directly after PCP treatment in early development, and in adulthood. Based on our results we propose that components of the Lingo-1 signaling pathways may be involved in the acute neurotoxicity induced by perinatal administration of PCP in rats early in development and suggest that this may have implications for the hippocampal deficits seen in schizophrenia.

Keywords: schizophrenia; Lingo-1 signaling; neurodevelopment; phencyclidine animal model; hippocampus

1. Introduction

Phencyclidine, also known as angel dust or PCP, is both a potent non-competitive *N*-methyl-D-aspartate (NMDA) receptor antagonist and an agonist for the dopamine D2 receptors¹⁻³, but also binds with a lower affinity to opiate, nicotinic, and muscarinic cholinergic receptors⁴⁻⁷. Due to its effects on a multitude of important receptor targets known to be implicated in the schizophrenia pathology, PCP treatment in rodents has been used to model the glutamate hypofunction hypotheses and dopamine hyperfunction hypotheses of schizophrenia^{2,8}. The use of PCP administration at postnatal days (PN)7, 9 and 11 has consistently been shown to induce hyperlocomotion, reduced prepulse inhibition and impaired social interactions in rodents⁹⁻¹¹. The behaviors it induces in rodents in addition to the psychomimetic effects it has in humans, makes it a valid model for studying the development of schizophrenia¹².

The administration of PCP to rodents both induces neurotoxicity and causes major damage to all parts of the brain^{13,14}. PCP administration at PN7, 9 and 11 has been repeatedly shown to result in an increase in neuronal degeneration in the frontal and cingulate cortex of rats^{15,16}. Similarly, pro-apoptotic factors have been shown to be upregulated, and anti-apoptotic factors have been shown to be downregulated in rats treated perinatally with PCP compared to controls¹⁵. It is thought that elevated neuronal death during this perinatal period may be directly responsible for deficits in brain development, neuronal cytoarchitecture and plasticity; and thus may contribute to the appearance of some of the schizophrenia-like symptoms seen in these rats in adulthood. Furthermore, neurons are not the only brain cells that are affected by disruptions to NMDA receptor antagonists. Oligodendrocytes, like neurons are sensitive to PCP toxicity during development¹⁴, and considering their significant role in axonal connectivity and conduction, disruption to these processes during early neurodevelopment can have significant negative consequences, impacting on normal brain

development. Furthermore, oligodendrocytes play a major role in myelination processes, which have been shown to be altered after PCP treatment in rats^{13,14}. We have previously shown in this rat model that myelin basic protein (MBP), a marker of mature oligodendrocytes is significantly reduced in early development by perinatal administration of PCP¹⁷.

Here we study the effects of PCP treatment on Leucine-rich repeat and immunoglobulin domain-containing protein (Lingo-1) pathways, which are highly involved in the regulation of myelination and neurite outgrowth processes, both integral processes involved in the pathophysiology of schizophrenia. Membrane bound signal-transducing protein Lingo-1 is expressed on both neurons and oligodendrocytes¹⁸. It signals alongside the Nogo receptor (NgR) co-receptor and either the p75 neurotrophin receptor or its functional homolog, TNF receptor orphan Y (TROY)^{19–21}, both of which also play significant roles in apoptosis and cell survival pathways^{22,23}. Altogether, the activation of this trimolecular receptor complex sets up a signaling cascade leading to growth cone collapse, preventing further axonal growth and inhibiting myelination^{18,24}. Additional signaling co-factors such as With No Lysine K (WNK1)²⁵, Myelin transcription factor 1 (Myt1) and its homolog Myt1-like (Myt1l) are known to be associated with Lingo-1 signaling due to a lack of p75 and TROY receptors on Lingo-1 expressing neurons^{26,27}. Genetic associations with schizophrenia have been previously reported for the *Myt1l* gene in different populations^{28,29}, and the knockdown of *Myt1l* has been shown to induce apoptosis³⁰, strengthening the relevance of studying this Lingo-1 cofactor in a neurodevelopmental PCP induced model for schizophrenia.

We have recently reported the first evidence of an alteration in Lingo-1 signaling pathways in a postmortem hippocampal human brain cohort for schizophrenia³¹. Considering the role of Lingo-1 signaling proteins in myelin related processes, the present study specifically focuses on Lingo-1 signaling protein alterations in the hippocampus due to activity-mediated myelin

growth being higher early in human life in hippocampal regions compared to cortical regions³². Considering that the perinatal administration of PCP to rodents is a well-established developmental animal model for schizophrenia, we sought to investigate the effects of developmental PCP administration on levels of expression of Lingo-1 signaling proteins in the hippocampus, a critical region for early brain development.

2. Methods

2.1 Animals

Timed pregnant Sprague Dawley rats were obtained at gestation day 14 from the Animal Resource Centre (Perth, WA, Australia). Rats were housed in environmentally controlled conditions at 22°C in a 12 hours on, 12 hours off light dark cycle with food and water access *ad libitum*. The day of birth was denoted postnatal day (PN)0, and the pups were sexed on PN7 when the litters were subsequently randomly assigned to PCP or saline groups. The female pups remained in the litters until weaning, however only male rats were used in this study. The pups were weaned at PN24-28, and were housed in pairs according to treatment. This study was approved by the Animal Ethics Committee at The University of Wollongong (AE13/01), and was conducted according to the guidelines of the Australian code of Practice for the Care and Use of Animals for Scientific Purposes, conforming to the International Guiding Principles for Biomedical Research Involving Animals. All efforts were made to minimize numbers of animals used and their suffering.

2.2 Perinatal PCP Treatment

Male Sprague Dawley rat pups were given a subcutaneous injection on PN7, 9 and 11; of PCP (10 mg/kg/day; Sigma, Castle Hill, NSW, Australia) or saline (0.9% NaCl at a volume of 1 ml/kg). Six rats from each treatment group (PCP and control) were sacrificed at three

different time-points, PN12 days, 5 weeks or 14 weeks of age, representing perinatal, adolescent and adult developmental stages respectively as described previously^{33,34}.

2.3 Rat Brain Tissue Preparation

Rats were euthanized by carbon dioxide asphyxiation and decapitation at PN12, 5 weeks and 14 weeks. Brains were extracted and the hippocampus was regionally dissected on ice with the aid of a standard rat brain atlas³⁵. Immediately following dissection samples were snap frozen in liquid nitrogen and then stored at -80°C. Tissue was gently homogenized in a buffer (50 mM Tris pH 7.5, 50% glycerol), containing protease inhibitors (Sigma). Protein concentrations were determined by a spectrophotometer. All samples were diluted to a concentration of 2 µg/µL and stored at -80°C until required for immunoblotting, as previously detailed³¹.

2.4 Immunoblotting

Relative protein levels for all proteins of interest were determined by immunoblotting as previously described³¹. In short, proteins were resolved by SDS-PAGE, with a total of 10 µg protein loaded into each well of 4-12% pre-cast bis-Tris polyacrylamide gels (Bio-Rad). Proteins were subsequently transferred to polyvinylidene fluoride membranes (Bio-Rad). The polyclonal and monoclonal antibodies and their respective concentrations used to probe the membranes were as follows: anti-Lingo-1 (1:500; ab23631 Abcam), anti-NgR (1:500; ab26291 Abcam), anti-p75 (1:500; ab8874 Abcam), anti-TROY (1:200; ab12126 Abcam), anti-WNK1 (1:500; ab128858 Abcam), and anti-Myt1 (1:500; ab82844 Abcam). The Gel Logic 2200 Pro (Carestream Molecular Imaging; Rochester, NY, USA) was used to visualize and quantify the bands of interest. All samples were run in duplicate or triplicate, and were loaded in a randomized order with even numbers of PCP and control samples per time-point per gel to minimize the effects of gel-to-gel variability on the results. A pooled sample

combining aliquots from all 36 rats, was used as a positive control and was loaded onto each gel within the experiment to account for any gel-to-gel variability. Samples from each gel were then normalized to their respective pooled sample, and all bands were normalized to a β -actin (1:5000; MAB1501 Millipore) same lane loading control. Mean β -actin expression levels did not differ between PCP and control groups ($p>0.05$). All experiments and quantifications were performed blind to treatment and age group.

2.5 Statistics

As all data were normally distributed (Kolmogorov–Smirnov $p>0.10$), parametric testing was implemented. Two-way multivariate analyses of variance (MANOVA) followed by Tukey's HSD post-hoc tests, were performed to assess interactions between treatment (PCP or control) and time-point (PN12, 5 weeks or 14 weeks). One-way analyses of variance (ANOVA) followed by Tukey's HSD post-hoc tests, were used to assess for differences in protein expression across successive developmental time points within each treatment group. Unpaired two-tailed t-tests were also used at individual time-points to assess for differences in protein expression between PCP and control groups. The significance for all statistical tests was set to $p<0.05$. All data are expressed as mean \pm SD.

3. Results

3.1 Protein detection

Lingo-1, NgR, p75, TROY, WNK1 and Myt1 proteins were abundantly expressed and detectable in the rat hippocampus. Western blot analyses of all proteins examined resulted in a single distinct band at the expected, previously reported molecular weights for each protein: Lingo-1 (83 kDa)³⁶, NgR (51 kDa)³⁷, p75 (75 kDa)³⁸, TROY (46 kDa)³⁹, WNK1 (250 kDa)³¹, and Myt1 (135 kDa)³¹ (Figure 1).

3.2 Hippocampal levels of p75, TROY and Myt1 protein expression are altered by perinatal administration of PCP in rats

There was a highly significant age x treatment interaction on levels of both p75 and TROY protein expression in the treated rats ($F_{2,30}=9.39$; $p<0.001$; and $F_{2,30}=7.69$; $p=0.002$ respectively). Post-hoc analyses reveal that at PN12, a significant decrease in p75 protein expression (-16%, $p=0.011$; Figure 1) was observed in perinatal PCP treated rats in comparison to control rats. Despite a decrease in levels of TROY, the functional homolog of p75, it was found not to be significantly different in PCP treated versus control rats at the PN12 time-point (-11%, $p=0.061$; Figure 1). Furthermore, a significant elevation in p75 protein expression was observed in PCP treated compared to control rats at the 14 week time-point (+22%, $p=0.022$; Figure 1). Similarly, levels of the p75 homolog TROY were also found to be significantly elevated in PCP treated compared to control rats at the 14 week time-point (+14.5%, $p=0.021$; Figure 1). While there was no considerable main effect of treatment on expression levels of p75 or its homolog TROY ($F_{1,30}=0.23$; $p=0.633$; and $F_{1,30}=0.15$; $p=0.705$ respectively), an extremely significant age effect on both p75 and TROY expression levels in the hippocampus of the treated rats was observed ($F_{2,30}=26.95$; $p<0.001$; and $F_{2,30}=34.27$; $p<0.001$ respectively; Supplementary Figures (SF) 1 and 2). Post-hoc analyses revealed that the age effect resulted in a significant increase in p75 (control: +25%, $p=0.001$, SF1; PCP: +47.5%, $p<0.001$, SF2) and TROY (control: +22.5%, $p<0.001$, SF1; PCP: +36%, $p<0.001$, SF2) when comparing the 5 week age groups to the PN12 age group. Furthermore, a significant elevation in both p75 (+49%, $p<0.001$, SF2) and TROY (+37%, $p<0.001$, SF2) expression was observed in the 14 week PCP treated rats compared to the PN12 PCP treated rats, however there was no significant alteration observed in the control rats when comparing the same age groups (SF1). Finally, there was a significant increase in

both p75 (+22%, $p=0.008$, SF1) and TROY (+15%, $p=0.037$, SF1) protein expression in control rats when comparing the 5 week to the 14 week group.

Similar to the results of p75 and TROY, there was an extremely significant interaction between age and treatment on Myt1 levels in the hippocampus of the treated rats ($F_{2,30}=25.52$; $p<0.001$). Post-hoc testing revealed that this interaction again results in a decrease at the PN12 (-18.5%, $p<0.001$; Figure 1) and an increase at the 14 week (+16%, $p=0.014$; Figure 1) time-points between the PCP treated rats and the controls. Furthermore, while there is no main treatment effect ($F_{1,30}=0.96$; $p=0.333$), there was a considerable age effect on levels of Myt1 protein expression ($F_{2,30}=103.13$; $p<0.001$). Post-hoc analyses showed that Myt1 was significantly altered when comparing the 5 week group to the PN12 group (control: -15.5%, $p=0.001$, SF1; PCP +9%, $p=0.016$, SF2), when comparing the 14 week group to the 5 week group (control: -43.5%, $p<0.001$, SF1; PCP -30%, $p<0.001$, SF2), as well as when comparing the 14 week group to the PN12 group (control: -69%, $p<0.001$, SF1; PCP -16%, $p=0.001$, SF2).

3.3 Hippocampal levels of Lingo-1, NgR and WNK1 protein expression are unaltered by perinatal PCP treatment in rats

There were no significant main effects of age ($F_{2,30}=3.01$; $p=0.064$) or treatment ($F_{1,30}=0.36$; $p=0.555$) on levels of Lingo-1 in the hippocampus of the treated rats; nor were there any significant interactions between the two factors ($F_{2,30}=1.41$; $p=0.259$; Figure 1). Furthermore there were no significant interactions between age and treatment on levels of NgR in the treated rats ($F_{2,30}=0.54$; $p=0.589$; Figure 1), nor an effect of treatment on NgR levels in the hippocampus ($F_{1,30}=0.14$; $p=0.710$). However, an extremely significant age effect was observed in relation to NgR protein levels in the hippocampus of the treated rats ($F_{2,30}=18.69$; $p<0.001$). Post-hoc analyses demonstrated that this age effect resulted in a considerable

increase in NgR expression levels in both control and PCP treated rats in both the 5 week (control: +55%, $p=0.002$, SF1; PCP: +48%, $p=0.001$, SF2) and 14 week (control: +37%, $p=0.019$, SF1; PCP: +49%, $p=0.005$, SF2) age groups compared to the PN12 age group, with the age effect being more highly significant in the PCP treated rats.

As with Lingo-1 and NgR, there were no notable age x treatment interactions on the expression of hippocampal WNK1 protein levels in the treated rats ($F_{2,30}=0.39$; $p=0.683$; Figure 1), nor an effect of treatment on WNK1 levels in the treated rats ($F_{1,30}=0.24$; $p=0.627$). There was however a considerable age effect on WNK1 protein expression levels ($F_{2,30}=12.60$; $p<0.001$). Similar to the expression pattern of NgR, post-hoc analyses revealed that the age effect resulted in a significant elevation of WNK1 expression levels in both control and PCP treated rats in both the 5 week (control: +29.5%, $p=0.007$, SF1; PCP: +46.5%, $p=0.003$, SF2) and 14 week (control: +34.5%, $p=0.014$, SF1; PCP: +49%, $p=0.003$, SF2) age groups compared to the PN12 age group, with the age effect again being more highly significant in the PCP treated rats.

4. Discussion

Here we provide the first study to investigate the developmental expression profile of hippocampal Lingo-1 signaling pathway proteins using a neurodevelopmental PCP model of schizophrenia. This study offers insight into the expression patterns of these proteins following the administration of an NMDA receptor antagonist at a critical period for brain development which is highly relevant in the context of the pathophysiology of schizophrenia. We have shown that the hippocampal expression of Lingo-1, NgR and WNK1 were not significantly altered by PCP treatment across the 3 tested developmental time-points, however the downstream signaling partners p75, TROY and Myt1 were decreased at PN12 and increased at 14 weeks in PCP treated rats compared to their controls. Considering current

literature and the role that these three proteins play in apoptotic processes, our results suggest that these proteins may have implications in acute PCP induced neurotoxicity and thus may contribute to deficits in brain development, neuronal cytoarchitecture and plasticity of the brain early in the development of schizophrenia.

4.1 Alterations of p75, TROY and Myt1 in a PCP induced neurodevelopmental animal model for schizophrenia

Perinatal PCP treatment was found to significantly reduce p75 expression in the juvenile rats, and increase the expression of p75 in the adult PCP treated rats, with no change in p75 expression in the adolescent rats (Figure 1). Reports on the expression of p75 neurotrophin receptor throughout development as well as in relation to schizophrenia in human postmortem brains, are limited and conflicting⁴⁰, despite the p75 binding protein, brain-derived neurotrophic factor (BDNF) being extensively studied^{41–43}. In support of our findings, levels of p75 expression have been previously found to be significantly reduced in neonatal pups in a developmental rodent model used to study environmental influences in disorders such as schizophrenia⁴⁴. It has been suggested that a reduction of p75 in early brain development could be quite detrimental due to the inability for neurotrophins, responsible for neurite outgrowth, to bind in a p75 dependent fashion, leading to an inability of axons to elongate throughout development⁴⁵. Even though p75 expression is known to be associated with various apoptotic processes^{46,47}, p75 neurotrophic factor also contributes to neuronal survival during development^{40,46}. Tropomyosin receptor kinases (Trk), involved in p75-induced neuronal survival processes⁴⁰, are also implicated in NMDA receptor activation and regulation⁴¹. With regards to the non-specific antagonistic effects of PCP on the NMDA receptor, a decrease in the activity of TrkB receptor signaling has been shown in postnatal PCP treated rats⁴⁸ (Figure 2). Due to the cross talk between p75 and Trk receptors, it was not surprising to see a significant reduction in the levels of p75 in the hippocampus of the

juvenile PCP treated rats compared to the controls in our study, which would lead to decreased survival of neurons early in development. As a follow-up to our study and to test this hypothesis, the levels of microtubule-associated protein 2 (MAP2; a commonly used neuronal marker, selectively labeling dendritic trees throughout the brain) were examined in the hippocampus of our perinatal treated rats (see supplementary methods, results and figure SF3). MAP2 levels were significantly decreased by 26.5% in the hippocampus of the juvenile PCP treated rats ($p=0.008$), supporting our hypothesis that a significant reduction in p75 in PCP treated rats would lead to an acute reduction in survival of neurons in the hippocampus of juvenile treated rats.

Considering that p75 and TROY are functional homologs of one another, it seems fitting that we found a decrease of a similar magnitude in both p75 and TROY expression in the hippocampus of perinatal PCP treated rats, despite the reduction in TROY protein levels not reaching significance ($p=0.061$). We suggest that the very similar transient expression pattern of the homologous receptors p75 and TROY in PCP treated rats compared to controls throughout development may play a significant role in PCP induced neurotoxicity in juvenile rats in our neurodevelopmental animal model of schizophrenia. In support of this, it has been shown that postnatal PCP injections resulted in lower levels of phosphorylated ERK1/2 in the hippocampus of juvenile rats compared to controls^{49,50}. Furthermore PCP treatment in organotypic brain slice culture from rats showed decreased phosphorylation levels of MEK1/2 and ERK1/2, both downstream signaling partners of p75 cell survival pathways, suggesting that PCP may induce cell death through the inhibition of the MEK/ERK/1/2 pro-survival pathways⁵¹ (Figure 2). The expression pattern of p75 and TROY seems to recover over time, normalizing around adolescence as there are no significant differences in either p75 or TROY expression in the 5 week old rats ($p>0.05$). Furthermore, there are no significant alterations in MAP2 levels to indicate any significant alterations in neuronal

survival during adolescence (SF3). The levels of p75 and TROY are raised above control levels in the hippocampus of PCP treated rats by the time they reach adulthood at 14 weeks. This is supported by our previous finding in a postmortem human cohort showing an increase of both of these proteins in the hippocampus of adult schizophrenia brains compared to controls, despite p75 levels not reaching significance in our human study³¹. Considering there are no significant alterations in MAP2 levels between PCP treated and control rats at adulthood in the present study (SF3), this suggests that additional mechanisms may be involved in the regulation of these proteins in the adult schizophrenia brain which will need to be further investigated.

In addition, Myt1 expression in our study was found to be significantly decreased by PCP administration in the hippocampus of the juvenile PN12 rats in comparison to control rats, but increased in the adult rats treated perinatally with PCP compared to controls. Knowing that Lingo-1 can directly suppress EGFR signaling leading to the inhibition and blockade of PI3-K/Akt signaling pathways³¹, and since we found Lingo-1 to be unaltered in the hippocampus in the perinatal PN12 PCP treated rats in the present study, we hypothesize that Akt signaling pathway inhibition by Lingo-1 would be reduced, thus resulting in increased levels of phosphorylated Akt signaling pathway proteins (Figure 2). Elevated Akt activation levels would lead to increased negative regulation of Myt1 intracellularly (Figure 2), thus resulting in lower Myt1 levels in the hippocampus of the juvenile PCP treated rats³¹. While we were unable to examine levels of phosphorylated Akt levels to support this hypothesis in the present study (due to an alternate method of sample preparation being required for the procedure), we were able to examine levels of total Akt protein (see supplementary methods, results and figure SF3). We found that there were no significant alterations in total Akt levels in any of the tested age groups in PCP rats compared to controls ($p>0.05$). This result has been previously shown by our research group³⁴; furthermore in the same cohort of rats our

group has demonstrated that perinatal PCP treatment at PN7, 9 and 11 results in increased levels of phosphorylated Akt at both juvenile and adolescent stages of life in the hippocampus³⁴, supporting our hypothesis for increased levels of activated Akt in this model leading to the reduced levels of Myt1 protein observed in juvenile rats in the present study. In further support of this, Xia and Johnson (2009) have also demonstrated that phosphorylated Akt levels were increased in adolescent rats that were treated with PCP on postnatal days 7, 9 and 11 compared to rats treated with saline^{49,50}. Along with the decreased levels of Myt1 protein in PCP treated rats at the juvenile period compared to their controls, we have reported in a previous study¹⁷ the levels of MBP as a marker of mature oligodendrocytes in this animal model, and have shown that MBP is significantly reduced in PN12 animals following perinatal PCP administration, suggesting an acute alteration of glia by PCP treatment.

In addition, elevated levels of activated Akt may contribute to an increased expression of the apoptotic marker p53, responsible for regulating another apoptotic marker bcl-2-associated X protein (or BAX) which has previously been reported to be increased after PCP administration at PN7, 9 and 11⁵². To test this hypothesis as a follow-up to our study, we examined levels of BAX protein in our rats (see supplementary methods, results and figure SF3) and found a significant 26% increase in BAX expression in the juvenile PCP treated rats compared to controls ($p=0.03$). Since p53 and BAX are regulated by Mouse double minute 2 homolog (MDM2)^{53,54}, we would also expect to see an increase in MDM2 expression in the juvenile rats due to a rise in the levels of p53 and following perinatal PCP administration requiring a large recruitment of MDM2 for its ubiquitination (Figure 2).

4.2 Hippocampal expression of Lingo-1 and its signaling proteins NgR, and WNK1 are unaltered following perinatal PCP treatment

Hippocampal levels of Lingo-1 and NgR both presented with similar transient expression patterns throughout development in both the control and PCP treated rats, despite neither of the proteins being significantly altered by PCP treatment at any of the three developmental time-points (Figure 1); both proteins were increased during adolescence (SF1 and SF2). Considering the extensive synaptic changes that occur during adolescence (reductions in dendritic arborization, and pruning of synapses)⁵⁵, it was not surprising to detect higher levels of these important negative regulators of neurite outgrowth at this critical period of development in the control rats. Although slightly reduced following PCP treatment, levels of both proteins were still elevated in the adolescent rats above that which is seen in the juvenile rats, and remained elevated into adulthood (SF2).

Similarly, levels of WNK1 expression were gradually increased with age in the hippocampus of both the control and PCP treated rats. Our finding is consistent with a recent observation demonstrating elevated WNK1 expression in the hippocampus of adult compared to juvenile PN10 mouse brains⁵⁶. Furthermore, WNK1 levels were also found to be unaltered in all age groups of the present study when comparing PCP treated rats to control rats; similar to the expression patterns of Lingo-1 and NgR in this study. Considering that WNK1 is a direct binding partner of Lingo-1 and since WNK1 directly regulates NgR³¹, it seems appropriate that we observed these similar profiles of expression for Lingo-1, NgR and WNK1 following the perinatal PCP treatment.

5. Conclusion

In review, we report for the first time alterations in Lingo-1 signaling pathway proteins, particularly those which have significant roles in neuronal survival and apoptotic processes,

in the hippocampus of rats from a neurodevelopmental PCP model of schizophrenia. We have shown an altered developmental trajectory of a number of these signaling proteins, in particular at perinatal and adult stages of life. Our results in concert with current literature, suggest that components of the Lingo-1 signaling pathways may be involved in the acute neurotoxicity induced by perinatal administration of PCP in rats and suggest that this may have implications for the hippocampal deficits seen in schizophrenia, however further studies will be required to fully elucidate the molecular mechanisms involved.

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The funding sources had no role in the study design, in the collection, analysis and interpretation of data; in the writing of the report or in the decision to submit the paper for publication.

Author Contributions

JLA and FFE designed the study. JLA, KAN, NM and FFE performed the animal experiments. JLA performed the biochemical experiments, acquired data, performed analyses and interpreted the data. JLA wrote the first draft of the manuscript. FFE, KAN, NM and XFH critically reviewed and contributed to the manuscript. All authors have read and approved the final version of the manuscript.

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Figure Legends

Figure 1: Relative levels of protein expression in the hippocampus of phencyclidine (PCP) treated rats compared to controls (**n=6 per treatment per time-point**). * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

Figure 2: Proposed schematic representation of the acute effects of phencyclidine (PCP) administration resulting in activation of cell death pathways. A reduction in p75 protein expression was observed in the present study, and TrkB receptor signaling activity has previously been shown to be reduced in postnatal PCP treated rats. Together these proteins are normally involved in p75-induced neuronal survival processes¹; a reduction in protein or phosphorylation levels can lead to a reduction in MEK1/2 and ERK1/2 activation. Reduced phosphorylation of the MEK1/2 ERK1/2 pathways has been shown to result from PCP treatment². Lingo-1 can directly inhibit EGFR leading to the inhibition of PI3-K/Akt signaling pathways³; since Lingo-1 was unaltered in the present study, we suggest that Lingo-1 induced inhibition of Akt signaling pathways would be reduced and result in increased Akt signaling, causing increased negative regulation of Myt1 intracellularly³. Our group has previously shown that perinatal administration of PCP results in an acute increase in phosphorylated Akt levels in the hippocampus of rats⁴. Furthermore, activation of apoptotic signaling cascades via Akt leads to an increase in p53 and Bax⁵ (also shown by the present study), mainly regulated by MDM2^{6,7}. An immediate rise in the levels of p53 following perinatal PCP administration would require a large recruitment of MDM2 for its ubiquitination.

References for Figure 2 (also included in main reference list)

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Figure 1

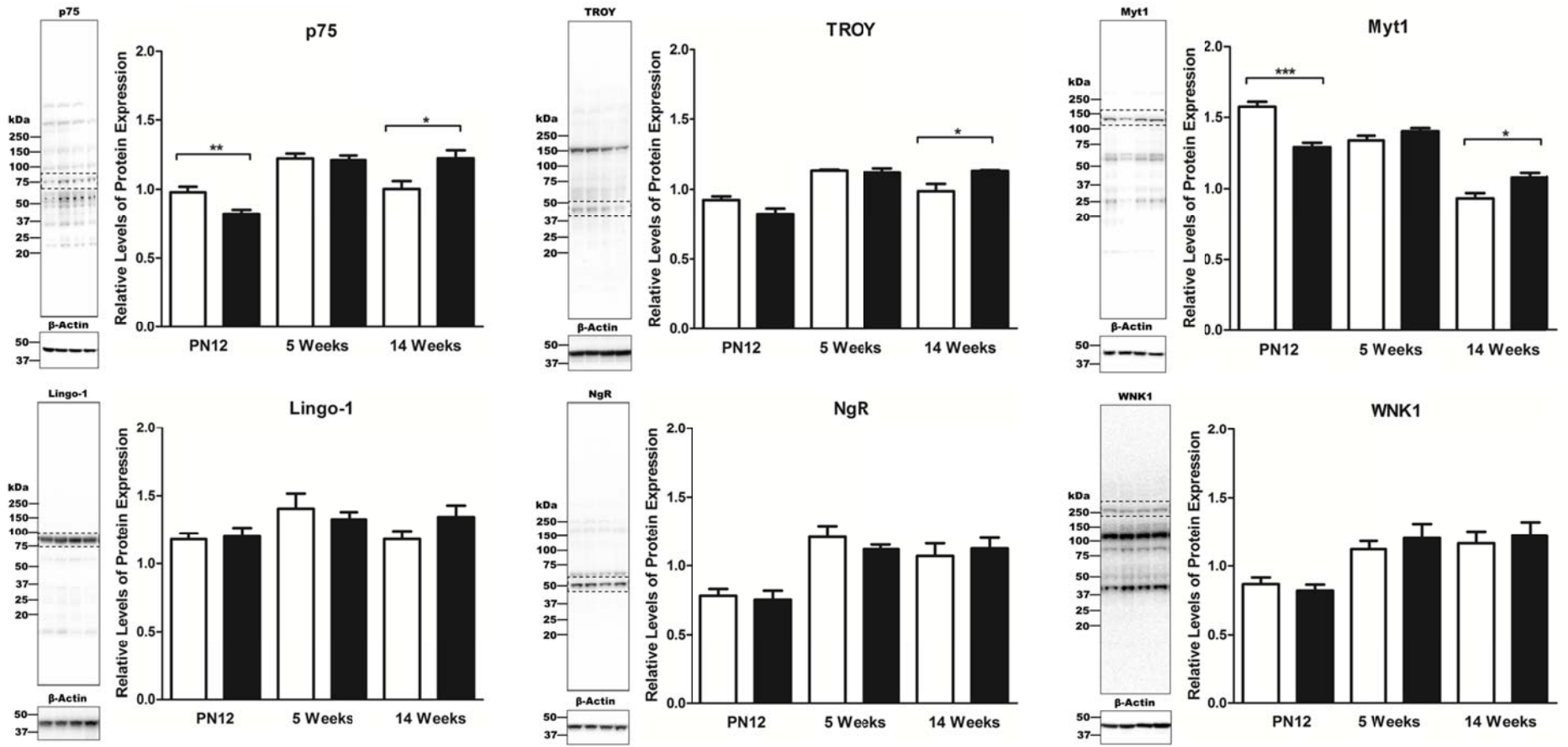
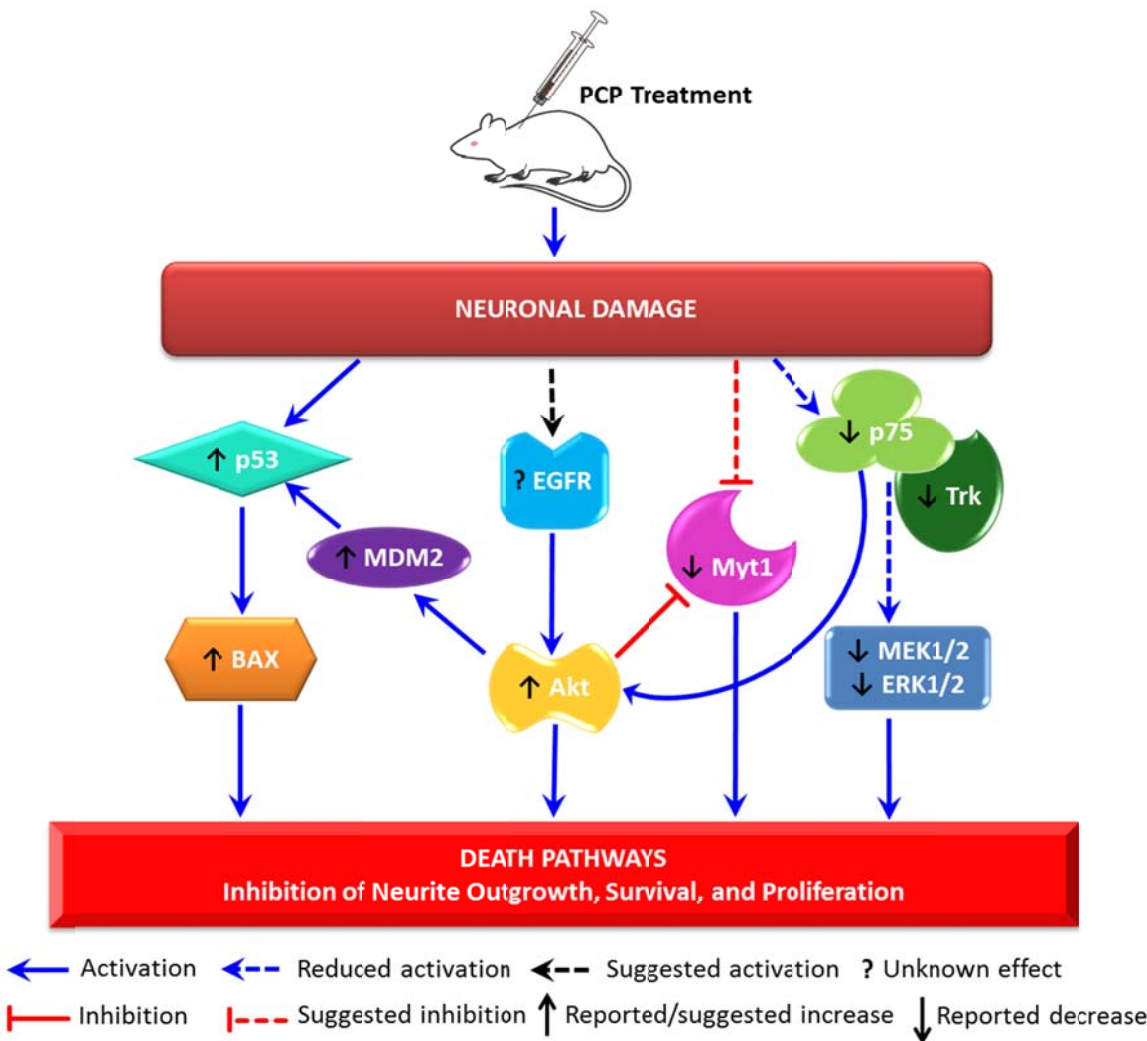


Figure 2



Alterations of p75 neurotrophin receptor and Myelin transcription factor 1 in the hippocampus of perinatal phencyclidine treated rats

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Supplementary Methods

Immunoblotting of neuronal marker microtubule-associated protein 2 (MAP2), total Akt, and apoptotic marker Bcl-2-associated X protein (BAX) in the hippocampus of PCP treated and control rats

Relative protein expression for MAP2, total Akt and BAX levels were determined by immunoblotting conducted according to the protocol described in the main methods of this study; the only modification from the protocol described in manuscript being the primary antibodies used. The primary antibodies and concentrations used to perform these additional experiments were as follows: anti MAP2 (1:1000; M4403; Sigma), total Akt (1:500; sc-8312; Santa Cruz) and BAX (1:500; ab7977; Abcam).

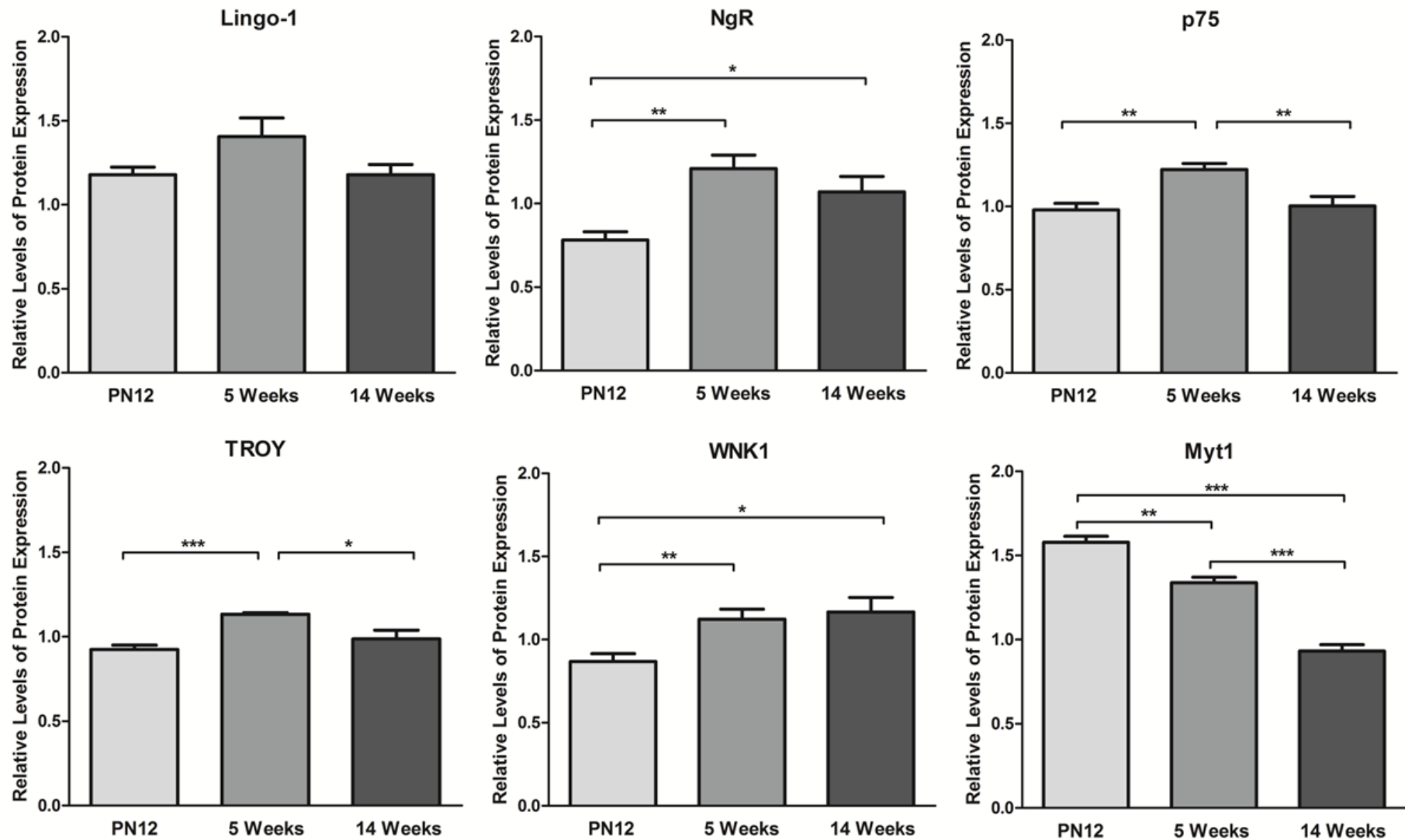
Supplementary Results

MAP2. There was a significant age x treatment interaction on MAP2 protein expression in the treated rats ($F_{2,29}=3.87$; $p<0.001$). Post-hoc analyses reveal that there was a 26.5% decrease in MAP2 protein expression in the PN12 PCP treated rats compared to control rats ($p=0.008$; SF3). While there was no main effect of treatment on expression levels of MAP2 ($F_{1,29}=0.07$; $p=0.789$), there was an extremely significant age effect on MAP2 expression levels in the hippocampus of the treated rats ($F_{2,29}=35.23$; $p<0.001$). Post-hoc analyses showed that MAP2 protein expression was 41% and 44% higher in the 5 week and 14 week control groups respectively compared to the PN12 group, similarly MAP2 protein expression was increased by 57.5% and 61.5% respectively in the 5 week and 14 week PCP treated groups compared to the PN12 group ($0.001<p<0.05$; SF4).

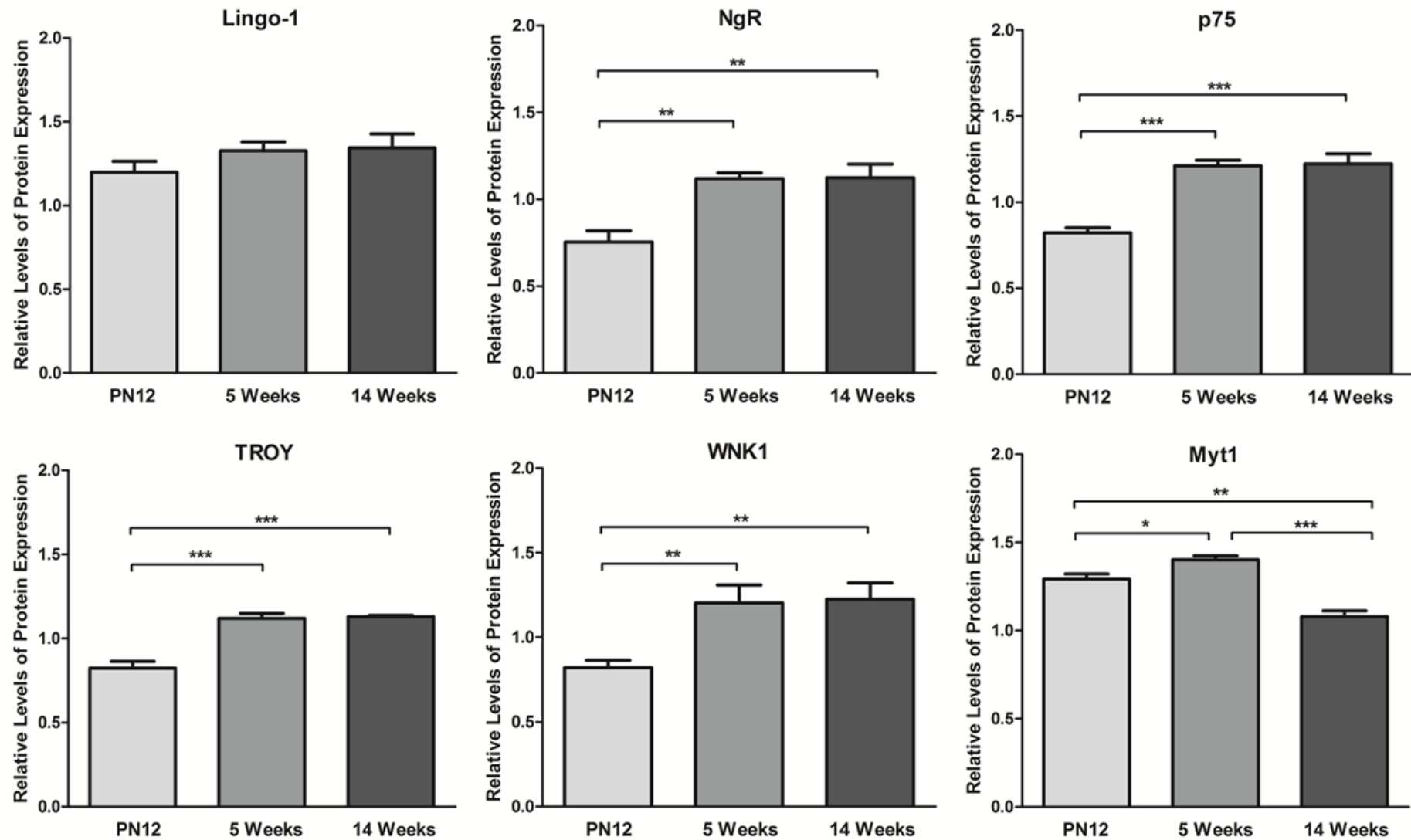
Akt. As expected, there were no significant interactions between age and treatment on total Akt levels in the hippocampus of the treated rats ($F_{2,30}=0.24$; $p=0.791$; SF3); nor was there a significant main effect of treatment ($F_{1,30}=2.08$; $p=0.159$). There was however a highly significant main effect of age on total Akt levels ($F_{2,30}=22.03$; $p<0.001$). Post-hoc analyses revealed that total Akt protein expression levels were 16% and 28.5% lower in the 5 week and 14 week control age groups respectively compared to the PN12 age group, and were 29.5% lower in the 14 week PCP treated group compared to the PN12 PCP group ($0.001<p<0.05$; SF4).

Bax. There were no significant interactions between age and treatment on levels of BAX protein in the treated rats ($F_{2,29}=1.042$; $p=0.366$); however there was a significant effect of treatment on BAX levels in the hippocampus ($F_{1,29}=6.706$; $p=0.014$), with post-hoc analyses revealing that the treatment effect was observed only in the PN12 age group, with a 26% increase in BAX protein in the hippocampus of PN12 PCP treated rats compared to controls ($p=0.03$; SF3). There was an extremely significant age effect in relation to BAX protein levels in the hippocampus of the treated rats ($F_{2,29}=39.71$; $p<0.001$). Post-hoc analyses revealed that BAX protein levels were 29% higher in 5 week old control rats, 42% higher in 14 week old control rats compared to PN12 rats, and 19% higher in 14 week old control rats compared to 5 week old rats ($0.001<p<0.01$). Furthermore BAX levels were found to be 30% higher in 14 week old PCP treated rats compared to PN12 PCP treated rats ($p<0.001$).

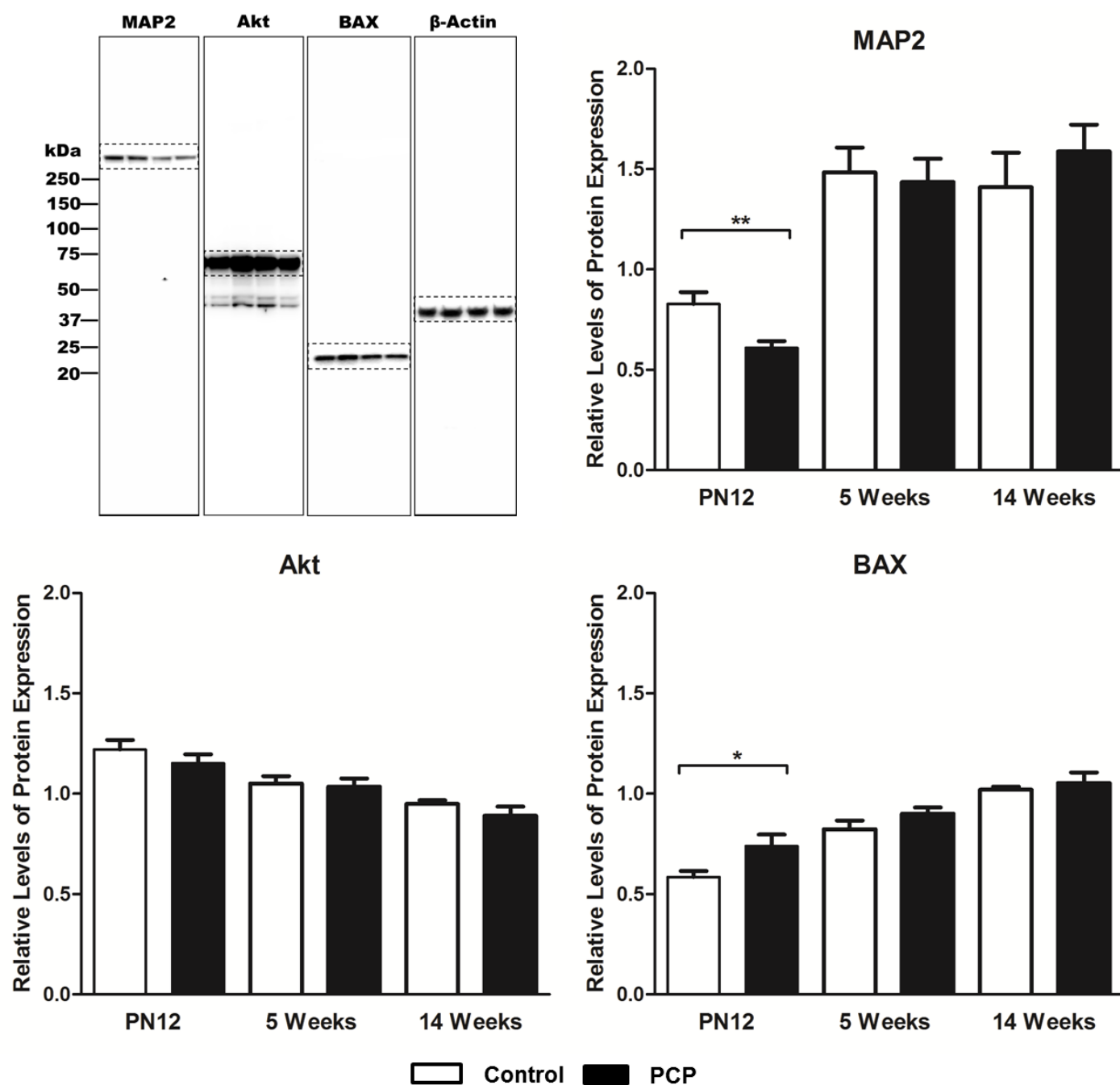
Supplementary Figures



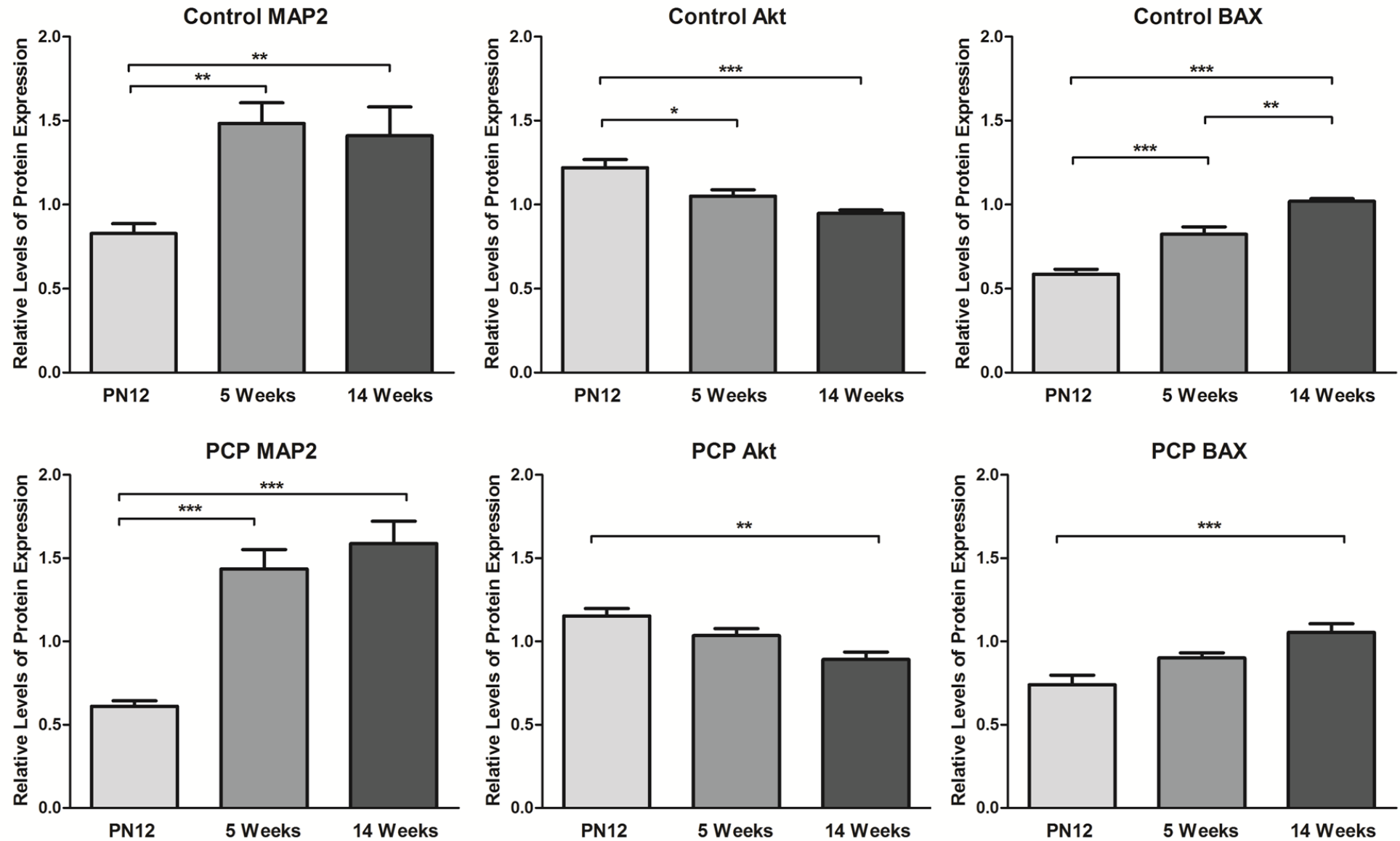
SF1: Developmental profile of Lingo-1, NgR, p75, TROY, WNK1 and Myt1 protein expression in the hippocampus of control rats at PN12, 5 week, and 14 week time-points (n = 6 per time-point). *p<0.05, **p<0.01, and ***p<0.001.



SF2: Developmental profile of Lingo-1, NgR, p75, TROY, WNK1 and Myt1 protein expression in the hippocampus of phencyclidine-treated rats at PN12, 5 week, and 14 week time-points (n = 6 per time-point). *p<0.05, **p<0.01 and ***p<0.001.



SF3: Representative immunoblots and relative levels of MAP2, total Akt and BAX protein expression in the hippocampus of phencyclidine (PCP) treated rats compared to controls (n = 6 per treatment per time-point). *p<0.05, and **p<0.01.



SF4: Developmental profile of MAP2, total Akt and BAX protein expression in the hippocampus of control and phencyclidine (PCP) treated rats at PN12, 5 week, and 14 week time-points (n = 6 per treatment per time-point). *p<0.05, **p<0.01, and ***p<0.001.