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Sub-chronic administration of phencyclidine produces hypermethylation in the parvalbumin gene promoter in rat brain

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Abstract

Aims: A deficit in parvalbumin (PV) neurons is found in schizophrenia and several animal models of the disease. In this preliminary study, we determined whether one such model, phencyclidine (PCP) administration, results in changes in DNA methylation in the rat PV gene (*Pvalb*) promoter. Methods: DNA from hippocampus and prefrontal cortex (PFC) from rats, which 6 weeks previously received either 2mg/kg PCP or vehicle for 7 days, underwent bisulphite pyrosequencing to determine methylation. Results: PCP administration induced significantly greater methylation at one of two *Pvalb* CpG sites in both PFC and hippocampus, while no significant difference was found in Long Interspersed Nucleotide Element-1, a global measure of DNA methylation. Conclusion: Subchronic PCP administration results in a specific hypermethylation in the *Pvalb* promoter which may contribute to PV deficits in this animal model of psychosis.

Keywords: DNA methylation, parvalbumin, phencyclidine, schizophrenia, novel object recognition, rat.

1. Introduction

The NMDA receptor antagonist phencyclidine (PCP) has been widely used in the investigation and modelling of psychotic illness. Sub-chronic administration of PCP to animals can induce brain metabolic and neurochemical changes [1] as well as behaviours [2] that mimic aspects of schizophrenia. Furthermore, this regime can also produce enduring deficits in several neurochemical markers that are also diminished in the brain in schizophrenia. These include the calcium binding protein parvalbumin (PV), which is expressed in a subgroup of GABAergic neurons, and in schizophrenia demonstrates reductions in frontal cortical regions [3] and, particularly profoundly, in the hippocampus [4]. Equivalent losses, typically greater than 50%, of PV-immunoreactive (PV-IR) cells, are also seen in the rat hippocampus following sub-chronic PCP [2].

Deficits in immunostaining for PV-positive neurons have also been seen in other animal models that mimic some of the behavioural and also, in some models, the aetiological characteristics of schizophrenia. These include isolation rearing [5], administration of neonatal endotoxin [6] and prenatal methylazoxymethanol (MAM) [7] in rats, as well as following other psychotogenic drugs including ketamine [8,9] and amphetamine [10].

The pathogenic mechanisms underlying these deficits in PV are unclear, although PCP can also induce decreases in PV gene (*Pvalb*) expression [1,11]. We have speculated whether the PV deficit might relate to epigenetic changes induced by such pharmacological and environmental influences. One epigenetic factor is that of DNA methylation occurring at cytosine residues in CpG sequences; within promoter sequences this methylation can have major effects on gene expression [12]. There is evidence for dynamic effects on methylation of the mouse PV gene promoter sequence associated with manganese-induced neurotoxic damage [13]; we hypothesised that changes in methylation of this sequence might relate to PV deficits in schizophrenia and its animal models. In this preliminary study we have determined

the methylation status of CpG methylation sites within the equivalent sequence in frontal cortical (PFC) and hippocampal tissue from rats that had received subchronic PCP. The results were compared with a global measure of DNA methylation, that of Long Interspersed Nucleotide Element-1 (LINE-1) [14,15]. We used the novel object recognition (NOR) paradigm to assess the efficacy of the treatment regime to induce cognitive deficits prior to the post-mortem analysis.

2. Material and Methods

Female Lister-hooded rats (200-230g) were housed in groups of 4-5 under standard laboratory conditions under a 12hr light: dark cycle, lights on at 0700hr. Rats were randomly assigned to two groups and either received vehicle, (distilled water, i.p.) or phencyclidine hydrochloride (PCP, 2 mg/kg, i.p. twice daily for 7-days) dissolved in distilled water. Behavioural testing was carried out in the light phase. Rats were tested in the novel object recognition (NOR) paradigm and for locomotor activity 6 weeks after the last dose of PCP. Following the behavioural testing the brains were removed, flash frozen in isopentane and stored at -70%. These studies were carried out in accordance with the Animals Scientific Procedures Act (UK, 1986) and were approved by the University of Manchester ethical review panel.

The NOR paradigm has been described in detail elsewhere [16]. Briefly, following habituation to the test box, the rats are given an initial acquisition trial during which the animals are allowed to explore two identical objects for 3 min. After a 1 min interval when the animals are removed, one object is replaced with a novel object and the animals allowed to explore the objects again for 3 min. The exploration times of each object in each trial are recorded on video for subsequent blind scoring. NOR was determined by the discrimination index (DI) in the retention trial, calculated as: $(t_n - t_f) / (t_n + t_f)$ where t_n and t_f are times spent exploring the novel and familiar objects respectively. Data are expressed as mean ± SEM. (n= 9 per group) and were analysed by a one way ANOVA.

Genomic DNA was extracted from PFC and hippocampal tissue using QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA), was bisulphite-modified to convert unmethylated cytosine residues to uracil using the EpiTec Fast DNA

Bisulphite Kit (Qiagen) with a calculated mean conversion of 99%. We identified a previously-studied [13] DNA sequence in the 5' region of the rat *Pvalb* gene (Rnor6.0, Chromosome 7, bases 119441848 - 119441803) that contained likely transcription factor binding sequences, and developed a pyrosequencing method for determination of methylation at the *Pvalb* CpG sites within this sequence following bisulphite reaction.

The region containing the sequence of interest in the *Pvalb* promoter was amplified to yield an amplicon of 149 bases using primers: 5'-TAAGGGGTTTTATTGGGGTAGA-3' (forward) and 5'[btn]-ATCTAAAATACCACCAACAAACACTA-3' (reverse) (Eurofins MWG Operon).

For LINE-1, a sequence of 4 CpGs was amplified [17] by PCR primers, as follows: 5'-TTGTTGTAAGAAAGTTGTTTGGTGAGTT-3' (forward) and 5'[btn]-ACCTCAAAAATACCCACCTAACC -3' (reverse) (Eurofins MWG Operon). Mean values for methylation were calculated and used as a measure of global methylation.

PCR reactions were carried out with 20 ng bisulphite-converted DNA using the PyroMark PCR kit in a final volume of 25 µl containing 12.5 µl 1x PyroMark PCR Master Mix, 2.5 µl 1x CoralLoad Concentrate, 1 µl of each primer in a final concentration of 0.05 µM, 7 µl RNase-free water. Amplification conditions were as follows: 95°C for 15 min, 45 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 30 s, finally, 72°C for 10 min. Methylation status of the *Pvalb* promoter sequence was determined with a PyroMark Q24 pyrosequencer (Qiagen UK) using 15‑20 µl PCR product and a sequencing primer, 5'- GTAGAGGTTGGTTAGTAT-3' (Eurofins MWG Operon). LINE-1 methylation was determined with a sequencing primer 5'-GGTGAGTTTGGGATA-3' (Eurofins MWG Operon). Pyrosequence setup and data reading were conducted by PyroMark Q24 2.0.6.20 software. Samples underwent PCR and pyrosequencing in duplicate; any inconsistencies between samples were resolved following further repetition. The extent of methylation at *Pvalb* CpG sites was analysed by repeated-measures ANOVA with region and treatment (PCP/vehicle) as factors; significant effects were further investigated by post-hoc t-test.

3. Results

During the acquisition trial in the NOR paradigm both groups spent an equal time exploring the left and right objects. In the retention trial the vehicle-treated, but not PCP-treated, animals spent more time exploring the novel versus the familiar object resulting in a significant difference in the discrimination index (vehicle: 0.35±0.04 vs PCP: 0.08±0.04; t-test $p<0.01$). There was no significant difference in locomotor activity between the groups (data not shown).

Brain DNA extracts gave consistent results for methylation at the two CpG sites in the *Pvalb* sequence of interest. Methylation status of the *Pvalb* promoter sequence was significantly different between the two treatment groups ($F=14.82$, $p=0.001$) and showed a significant interaction with CpG site ($F=11.05$, $p=0.002$) but did not significantly differ between or interact with brain region. These findings reflected significantly greater methylation in CpG2 from the hippocampus ($t=2.778$; $p=0.015$) and the PFC ($t=2.635$; $p=0.022$) in the PCP group, with mean increases of 16% and 21% respectively (Fig 1). Mean methylation in the LINE-1 sequence showed no statistically significant effect of drug group or region with results as follows for PFC (PCP: 73.59±5.01%; vehicle: 77.06±7.91%) and hippocampus (PCP: 77.90±2.86%; vehicle: 77.45±4.81%)

4. Discussion

In this preliminary study we have demonstrated a highly significant elevation in DNA methylation in one of two CpG sites in the promoter sequence of the PV gene in brain tissue taken from rats previously receiving a sub-chronic regime of PCP. It is well-established that such PCP administration can induce an enduring deficit of PV-IR neurons and a reduction in PV gene expression [18], as well as producing cognitive deficits, demonstrated here by the reduction in NOR. The increase in *Pvalb* methylation is found not to reflect a general, global increase in DNA methylation, being seen in the absence of a significant change in LINE-1 methylation, and therefore appears to be a relatively gene-specific consequence of PCP administration. The specific increase seen is of a similar magnitude to changes seen in other schizophrenia-associated genes in both human [19] and rat brain tissue [20]. Generally, DNA methylation is inversely related to gene expression, as promoter sequence methylation could directly interfere with transcription factor binding sites

and also indirectly cause gene silencing through methylated DNA binding proteins that recruit histone deacetylases, leading to chromatin condensation [21].

The promoter activity of the sequence we have studied has been demonstrated [22,23], and the methylation of this sequence has previously been shown to be increased following challenge by another neurotoxic factor, that of manganese [13]. These authors showed effects of maternal manganese exposure on epigenetic gene regulation, with *Pvalb* promoter hypermethylation and transcript downregulation at the weaning stage in the offspring.

The CpG sites studied here are at the 5' boundary of a large CpG island with a high density of both CpG sites and transcription factor recognition sequences. These include multiple binding sites for Nrf2, several close to CpG2 and further CpG sites within the CpG island. Thus methylation in this sequence could conceivably modify Nrf2 binding and its associated transcriptional activity. Nrf2 has been identified as a transcription factor important in antioxidant effects on gene expression [24]. Given that the disruptive effects of repeated administration of another NMDA antagonist, ketamine, on PV neurons are mediated through oxidative damage [8] and that a natural antioxidant and potent activator of Nrf2 can protect against PCP-induced cognitive dysfunction [25], these findings provide an intriguing mechanistic link between oxidative damage and PV deficits due to PCP administration.

Reduced expression of PV is a well-established consequence of multiple dosing with NMDA antagonists such as PCP [1, 11]. Thus we suggest that this specific *Pvalb* hypermethylation may contribute to the decreased expression of PV mRNA and protein in the hippocampus and frontal cortex that is associated with sub-chronic PCP administration, although we have not reported corresponding expression data in this preliminary communication. However, measures of PV mRNA and protein expression do not always correspond [26] and it is not possible to demonstrate a causal effect of PCP-induced changes in methylation on PV expression with this model. Thus we cannot rule out the possibility that DNA hypermethylation and reduced expression of PV are independent consequences of subchronic NMDA receptor antagonism by PCP.

5. Conclusion and Future Perspectives

This preliminary study demonstrates that sub-chronic PCP administration to rats 6 weeks previously, and resulting in a cognitive deficit, can also have a specific and enduring effect on DNA methylation in the promoter region of *Pvalb*. Much more needs to be done to fully understand the process and effects of this *Pvalb* promoter hypermethylation. It would be important to determine whether *Pvalb* methylation parallels the time course of PV deficits and the protective effects of antioxidant or other pharmacological intervention. We have studied what is likely to be a functionally important sequence within the promoter region of *Pvalb*, but further sequences important in transcriptional activity need to be investigated. Although the finding is independent of one measure of global DNA methylation, it may well be that other specific genes may also demonstrate changes in DNA methylation, particularly those implicated in the GABAergic deficits following PCP administration such as those of GAD67 expression [27] and calbindin [28]. Most important would be to determine whether hypermethylation of the PV gene occurs in psychotic illness including schizophrenia, for which sub-chronic PCP administration can model both PV deficits and certain symptoms [29].

Executive Summary

Background

- Subchronic phencyclidine (PCP) administration to rats can produce enduring deficits in the calcium binding protein parvalbumin (PV) in GABAergic neurons; these resemble equivalent deficits in schizophrenia.

- Effects on DNA methylation may contribute to these PV deficits.

Results

- We found increased brain DNA methylation at one site in the PV gene promoter following subchronic PCP administration to rats.

- This increase in methylation is specific to a site within a transcription factor binding sequence.

- No differences were found in a global measure of DNA methylation.

Conclusion

- PCP sub-chronic administration to rats results in hypermethylation of a specific site in the promoter sequence of the PV gene.

- This hypermethylation may, through effects on transcription, contribute to the enduring reduction in PV following PCP administration.

6. Financial Disclosure and Acknowledgements

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7. Ethical Conduct of Research

The authors state that they have obtained appropriate institutional review board approval and have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations.

8. References

1. Cochran SM, Kennedy M, McKerchar CE, Steward LJ, Pratt JA, Morris BJ. Induction of metabolic hypofunction and neurochemical deficits after chronic intermittent exposure to phencyclidine: differential modulation by antipsychotic drugs. *Neuropsychopharmacology*. 28(2), 265–275 (2003).

2. Abdul-Monim Z, Neill JC, Reynolds GP. Sub-chronic psychotomimetic phencyclidine induces deficits in reversal learning and alterations in parvalbumin-immunoreactive expression in the rat. *J. Psychopharmacol.* 21(2), 198–205 (2007).

3. Beasley CL, Reynolds GP. Parvalbumin-immunoreactive neurons are reduced in the prefrontal cortex of schizophrenics. *Schiz. Res.* 24(3), 349–355 (1997).

   * This was the first of many reports indicating losses of PV in the brain in schizophrenia

4. Zhang ZJ, Reynolds GP. A selective decrease in the relative density of parvalbumin-immunoreactive neurons in the hippocampus in schizophrenia. *Schiz. Res.* 55, 1-10 (2002).

5. Harte MK, Powell SB, Swerdlow NR, Geyer MA, Reynolds GP. Deficits in

parvalbumin and calbindin immunoreactive cells in the hippocampus of isolation reared rats. *J. Neural Transm.* 114(7), 893–8 (2007).

6. Jenkins TA, Harte MK, Stenson G, Reynolds GP. Neonatal lipopolysaccharide induces pathological changes in parvalbumin immunoreactivity in the hippocampus of the rat. *Behav. Brain Res.* 205(2), 355–359 (2009).

7. Penschuck S, Flagstad P, Didriksen M, Leist M, Michael-Titus AT. Decrease in parvalbumin-expressing neurons in the hippocampus and increased phencyclidine-induced locomotor activity in the rat methylazoxymethanol (MAM) model of schizophrenia. *Eur. J. Neurosci.* 23(1), 279–84 (2006).

8. Behrens MM, Ali SS, Dao DN, *et al.* Ketamine-induced loss of phenotype of fast-spiking interneurons is mediated by NADPH-oxidase. *Science*. 318(5856), 1645–7 (2007).

*An interesting finding demonstrating the role of reactive oxygen species in the toxic effects on NMDA antagonists on PV-containing neurons.

9. Sabbagh JJ, Murtishaw AS, Bolton MM, Heaney CF, Langhardt M, Kinney JW. Chronic ketamine produces altered distribution of parvalbumin-positive cells in the hippocampus of adult rats. *Neurosci. Lett.* 550, 69–74 (2013).

10. Morshedi MM, Meredith GE. Differential laminar effects of amphetamine on prefrontal parvalbumin interneurons. *Neuroscience*. 149(3), 617–624 (2007).

11. Pratt JA, Winchester C, Egerton A, Cochran SM, Morris BJ. Modelling prefrontal cortex deficits in schizophrenia: implications for treatment. *Br. J. Pharmacol.* 153 Suppl , S465–S470 (2008).

12. Grayson DR, Guidotti A. The dynamics of DNA methylation in schizophrenia and related psychiatric disorders. *Neuropsychopharmacology* 38(1), 138–66 (2013).

13. Wang L, Shiraki A, Itahashi M, *et al.* Aberration in epigenetic gene regulation in hippocampal neurogenesis by developmental exposure to manganese chloride in mice. *Toxicol. Sci.* 136(1), 154–165 (2013).

*This paper first reports hypermethylation of the PV gene promoter following a neurotoxic challenge.

14. Thomas CA, Paquola ACM, Muotri AR. LINE-1 Retrotransposition in the Nervous System. *Annu. Rev. Cell Dev. Biol.* 28(1), 555–573 (2012).

15. Rosser JM. L1 expression and regulation in humans and rodents. *Front. Biosci.* E4(1), 2203 (2012).

16. Grayson B, Idris NF, Neill JC. Atypical antipsychotics attenuate a sub-chronic PCP-induced cognitive deficit in the novel object recognition task in the rat. *Behav. Brain Res.* 184(1), 31–8 (2007).

17. Tryndyak VP, Kovalchuk O, Muskhelishvili L, *et al.* Epigenetic reprogramming of liver cells in tamoxifen-induced rat hepatocarcinogenesis. *Mol. Carcinog.* 46(3), 187–197 (2007).

18. Cochran SM, Fujimura M, Morris BJ, Pratt JA. Acute and delayed effects of phencyclidine upon mRNA levels of markers of glutamatergic and GABAergic neurotransmitter function in the rat brain. *Synapse*. 46(3), 206–214 (2002).

* The first observation of diminished PV mRNA following sub-chronic PCP administration.

19. Ryan J, Saffery R. Crucial timing in schizophrenia: role of DNA methylation in early neurodevelopment. *Genome Biol.* 15(10), 495 (2014).

20. Kundakovic M, Gudsnuk K, Herbstman JB, Tang D, Perera FP, Champagne FA. DNA methylation of BDNF as a biomarker of early-life adversity. *Proc. Natl. Acad. Sci. U. S. A.* 112(22), 6807–13 (2015).

21. Jones PL, Veenstra GJ, Wade PA, *et al.* Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nat. Genet.* 19(2), 187–91 (1998).

22. Berchtold MW, Epstein P, Beaudet AL, Payne ME, Heizmann CW, Means AR. Structural organization and chromosomal assignment of the parvalbumin gene. *J. Biol. Chem.* 262(18), 8696–8701 (1987).

23. Berchtold MW. Parvalbumin genes from human and rat are identical in intron/exon organization and contain highly homologous regulatory elements and coding sequences. *J. Mol. Biol.* 210(3), 417–27 (1989).

24. Joshi G, A. Johnson J. The Nrf2-ARE Pathway: A Valuable Therapeutic Target for the Treatment of Neurodegenerative Diseases. *Recent Pat. CNS Drug Discov.* 7(3), 218–229 (2012).

25. Shirai Y, Fujita Y, Hashimoto R, *et al.* Dietary Intake of Sulforaphane-Rich Broccoli Sprout Extracts during Juvenile and Adolescence Can Prevent Phencyclidine-Induced Cognitive Deficits at Adulthood. *PLoS One* 10(6), e0127244 (2015).

26. Kaalund SS, Riise J, Broberg B V, *et al.* Differential expression of parvalbumin in neonatal phencyclidine-treated rats and socially isolated rats. *J. Neurochem.* 124(4), 548–57 (2013).

27. Amitai N, Kuczenski R, Behrens MM, Markou A. Repeated phencyclidine administration alters glutamate release and decreases GABA markers in the prefrontal cortex of rats. *Neuropharmacology*. (2012).

28. Beasley CL, Zhang ZJ, Patten I, Reynolds GP. Selective deficits in prefrontal cortical GABAergic neurons in schizophrenia defined by the presence of calcium-binding proteins. *Biol. Psychiatry*. 52(7), 708–715 (2002).

29. Neill JC, Barnes S, Cook S, *et al.* Animal models of cognitive dysfunction and negative symptoms of schizophrenia: focus on NMDA receptor antagonism. *Pharmacol. Ther.* 128(3), 419–32 (2010).

Figure Legend

Figure 1. Mean percentage of methylation in the *Pvalb* promoter sequence in hippocampal and prefrontal cortex tissue samples in rats undergoing PCP or vehicle. Bars represent SEM.*p <0.05.

Figure 1.