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Parvalbumin promoter hypermethylation in post-mortem brain in schizophrenia

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1 **Abstract**

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3 Deficits of brain parvalbumin (PV) are a consistent finding in schizophrenia and
4 models of psychosis. We investigated whether this is associated with abnormal PV
5 gene (PVALB) methylation in the brain in schizophrenia. Bisulfite pyrosequencing
6 was used to determine cytosine (CpG) methylation in a PVALB promoter sequence.
7 Greater PVALB methylation was found in schizophrenia hippocampus, while no
8 differences were observed in prefrontal cortex. LINE-1 methylation, a measure of
9 global methylation, was also elevated in both regions in schizophrenia, although the
10 PVALB change was independent of this effect. These results provide the first
11 evidence that PVALB promoter methylation is abnormal in schizophrenia and suggest
12 that this epigenetic finding may relate to the reduction of PV expression seen in the
13 disease.

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15 **Keywords:** Schizophrenia, parvalbumin, DNA methylation, post-mortem brain,

16 LINE-1.

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38 1. Introduction

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40 It is now well established that there is a dysfunction of GABAergic systems in
41 the brain in schizophrenia. Early post-mortem studies have shown deficits in
42 interneurons in the neocortex and hippocampus [1] reflected by lower density of
43 hippocampal GABA uptake sites [2]. Subsequent confirmation has come from
44 observations of deficits in the GABAergic marker glutamic acid decarboxylase
45 (GAD)-1 mRNA and GAD-67 protein throughout the cortex [3]. These deficits
46 appear to be selective for subtypes of GABAergic neurons, most notably those
47 containing parvalbumin (PV), immunostaining for which is reduced in frontal cortex
48 and hippocampus in schizophrenia [4,5]. It seems likely that these deficits contribute
49 to the cognitive disturbances in schizophrenia [6], although it is conceivable that
50 hippocampal parvalbumin/GABA deficits may result in dopaminergic hyperfunction
51 [2] and thereby contribute to positive symptoms.

52 The pathogenic mechanisms underlying the PV deficit in schizophrenia are
53 also unclear, although it has been suggested that the GABAergic cells are intact but
54 hypofunctioning [7]. This is consistent with the fact that the PV deficit in certain
55 animal models of the disease appears to be related to a reversible effect of oxidative
56 stress [8]. We have speculated whether the PV deficit might relate to epigenetic
57 changes that could be induced by such environmental influences. One epigenetic
58 factor is that of DNA methylation occurring at cytosine residues in CpG sequences;
59 within promoter sequences this methylation can have major effects on gene
60 expression [9]. There is some evidence for dynamic effects on methylation of the PV
61 gene (PVALB) promoter sequence associated with manganese-induced neurotoxic
62 damage in the mouse hippocampus [10]; also, we recently found a PVALB
63 hypermethylation in the hippocampus of rats undergoing subchronic phencyclidine
64 administration [11], in which a PV immunostaining and mRNA deficits are well-
65 established [12–16]. Additionally, a specific association between elevated PVALB
66 methylation and methamphetamine (METH)-induced psychosis was reported in
67 METH-dependent subjects compared to controls with no history of drug abuse or
68 psychiatric diagnosis [17].

69 We hypothesise that changes in methylation of the PVALB promoter might
70 relate to PV deficits in schizophrenia. Thus we have determined the methylation
71 status of several CpG methylation sites within this sequence in frontal cortical and

72 hippocampal tissue taken post-mortem from patients with schizophrenia and control
73 subjects. The results were compared with a global measure of DNA methylation, that
74 of LINE-1.

75

76 2. Material and Methods

77

78 2.1. *Post-mortem human brain tissue*

79 A post-mortem brain tissue sample from 15 schizophrenia subjects and 16
80 age-matched controls was collected at the University of Nottingham; this sample was
81 previously investigated for glutamatergic and GABAergic markers (e.g. Reynolds et
82 al., 1990). Tissues were taken and stored at -70°C in compliance with the UK Human
83 Tissue Act. Details of the sample subjects are provided in Table 1.

84

85 2.2. *DNA extraction, Bisulphite Conversion and Pyrosequencing*

86 Genomic DNA from human samples was extracted from PFC and
87 hippocampus, using QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA), and was
88 bisulphite-modified to convert unmethylated cytosine residues to uracil using the
89 EpiTec Fast DNA Bisulphite Kit (Qiagen) with a calculated mean conversion of 99%.
90 We identified an equivalent DNA sequence to that chosen previously in an animal
91 study [11], in the 5' regions of the human PVALB gene and developed a
92 pyrosequencing method for determination of methylation at each CpG sites within
93 this sequence following bisulphite reaction. The sequence was amplified by PCR
94 using primers, including a biotinylated reverse primer, as follows: 5'-
95 AGTGGAGAGAGAAAGGGAGTA-3' (forward) and 5'-
96 [btn]AACACCAAAAAAAAAACCACCTCTAAAATT-3' (reverse) (Eurofins MWG
97 Operon).

98 PyroMark Q24 CpG LINE-1 sequence-based pyrosequencing was used to quantify
99 methylation at four CpG sites in positions 331 to 318 of LINE-1 (GenBank accession
100 number X58075) (Qiagen).

101 PCR reactions, amplification conditions and the methylation profile were
102 carried out according to our previous study [11]. The sequencing primer used for
103 PVALB studies was as follows: 5'- ATTAGTTAAGGTTTTAGATTGA -3'
104 (Eurofins MWG Operon). Pyrosequence setup and data reading were conducted by
105 PyroMark Q24 2.0.6.20 software (UK). Samples underwent PCR and pyrosequencing

106 in duplicate; any inconsistencies between samples were resolved following further
107 repetition.

108

109 2.3. Statistical Analysis

110 Data obtained from the pyrosequencing were compared by unpaired t test and
111 were considered significantly relevant when $p \leq 0.05$. All the analysis was done using
112 SPSS 20.0 (IBM Corp: Armonk, NY, USA). Variance analysis was used to evaluate
113 possible associations of age and sex of the patients with the methylation levels found.

114

115 3. Results

116

117 A series of samples of both frontal cortex and hippocampus from 16 control
118 subjects and 15 schizophrenia subjects (Table 1) successfully underwent bisulphite
119 conversion, PCR and pyrosequencing to determine methylation in the LINE-1 and
120 PVALB sequences. All samples demonstrated single PCR bands with no evidence of
121 DNA degradation. A significant effect of diagnostic category on PVALB methylation
122 was found in the hippocampus ($F=3.465$; $p=0.021$) but not in the frontal cortex
123 ($F=0.715$; $p=0.591$). Figure 1 shows that the effect in the hippocampus reflected
124 increases in methylation in schizophrenia at CpG2 ($F=8.250$; $p=0.008$) and CpG4
125 ($F=12.195$; $p=0.002$).

126 The mean methylation of LINE-1 was highly significantly increased in both
127 frontal cortex ($t=2.995$; $p=0.006$) and hippocampus ($t=2.786$; $p=0.009$) in
128 schizophrenia (Figure 2). Including the respective LINE-1 methylation results as a
129 covariate in the PVALB analyses above, there were no qualitative differences in the
130 statistical results: methylation at CpG2 and CpG4 in the hippocampus remained
131 significantly elevated in schizophrenia.

132 Age was significantly different between the two groups but showed no
133 significant correlation with any methylation measure; including it as a covariate also
134 had no substantial influence on the results of the analyses above; differences in LINE-
135 1 methylation remained significant as did hippocampal PVALB methylation at CpG2
136 and CpG4.

137

138 4. Discussion

139 The major findings from our study indicate a specific increase in PVALB
140 promoter methylation in the hippocampus in schizophrenia which is independent of
141 increases in a measure of global methylation in the brain.

142 To the best of our knowledge, this is the first study reporting hypermethylation
143 in PVALB promoter in schizophrenia patients; these data are supported by previously
144 identified evidence suggesting that hyperfunctional DNA methylation may be
145 responsible for deficiencies in GABAergic neurotransmission [18,19]. As DNA
146 promoter hypermethylation can contribute to reduced gene expression, we suggest
147 that the well-established reduction in PV expression in the brain in schizophrenia may
148 be related to increased methylation of CpG sites within the gene promoter region.
149 The deficit in PV expression is much greater in the hippocampus [5] than in the cortex
150 [4], which may relate to the fact that a statistically significant hypermethylation was
151 only observed in the hippocampus. We have reported elevated methylation of an
152 equivalent sequence in the PVALB promoter of the hippocampus of rats which have
153 undergone a sub-chronic phencyclidine (PCP) regime, modelling some symptoms of
154 schizophrenia [11] and also find an elevation of CpG 2 methylation in blood-derived
155 DNA in subjects with methamphetamine-induced psychosis [17].

156 The PVALB promoter region selected spans many transcription factor (TF)
157 binding sites including those for paired box domain gene 5 (PAX5) and cyclic AMP-
158 responsive element (CREB). At CpG2 there is a recognition site for PAX5, which has
159 an important role in regulate the mid-hindbrain organisation during neurodevelopment
160 [20–22], while at CpG4 is spanned by the binding site for CREB which possesses
161 intrinsic histone acetyltransferase activity [23,24] important for gene regulation. It has
162 been demonstrated that methylation can block this binding [25]. Additionally,
163 genome-wide association studies have demonstrated that this TF is associated with
164 schizophrenia [26] and interestingly, increases in DNA methylation of the CREB
165 binding protein gene following clozapine treatment were significantly correlated with
166 clinical improvements in treatment-resistant schizophrenia [23].

167 Our results reveal hypermethylation in LINE-1 in brain tissue of schizophrenia
168 patients compared to controls. These repetitive elements play an important role in
169 gene expression and may be involved in the regulation of diverse biological
170 processes, including DNA damage and repair, inflammation, immune function,
171 embryogenesis, cell differentiation, cell response to external stimuli and hormonal

172 responses [27], so epigenetic dysfunction in these elements in the brain might be
173 involved in neurodegenerative and psychiatric diseases [28].

174 The increase in LINE-1 methylation indicates that there may be a global
175 elevation in brain DNA methylation in schizophrenia. It has been reported that in the
176 brain in schizophrenia there is an upregulation of DNA-methyltransferases (DNMT)
177 [29,30], so a LINE-1 hypermethylation found in our study could well be a
178 consequence of this DNMT upregulation. Abnormalities in LINE-1 methylation are
179 seen in association with early life trauma in schizophrenia [31] and in PTSD [32],
180 although such studies inevitably rely on blood-derived DNA.

181 However, we found that the increase in PVALB methylation was unrelated to
182 the change in LINE-1 methylation, and thus it would appear that the finding in
183 PVALB is an independent effect, perhaps selective to this gene and potentially related
184 to the specific deficit in PV in the brain in schizophrenia. It was not possible to
185 determine PV expression in the samples used in the current study, and thus a direct
186 assessment of the correlation between DNA methylation and gene expression could
187 not be performed.

188 This study has some further limitations; the sample size was not large and, as
189 it is a post-mortem study, the patients were inevitably mostly elderly. There are other
190 variables associated with post-mortem studies that are difficult to control; these
191 include the post-mortem interval, although modelling this with rat brains at room
192 temperature over 96 hours has demonstrated no effect on DNA methylation [33].
193 Furthermore, the patients were not drug free and, as it is known that antipsychotic
194 drug administration may have effects on gene methylation [34], we cannot distinguish
195 relationships with disease from effects of drug treatment.

196

197 **5. Conclusions and Future Perspectives**

198 This is the first evidence for an elevation of DNA methylation in the promoter
199 sequence of PVALB in schizophrenia, consistent with recent findings in both drug-
200 induced psychosis and in an animal model of the disease. This epigenetic effect may
201 underlie the PV deficits seen in both the disease and the PCP model. The PVALB
202 hypermethylation occurs in conjunction with, but independent of, increases in a
203 measure of global DNA methylation in the brain in schizophrenia. Much more needs
204 to be investigated in order to determine the effects of PVALB promoter methylation
205 in schizophrenia and related diseases and animal models. It would be important to

206 determine if the hypermethylation seen in these specific CpG sites is directly related
207 to decreased PV expression in the disease.

208

209 **Executive Summary**

- 210 • A deficit of parvalbumin (PV) expression in GABAergic neurons of the
211 hippocampus and frontal cortex is a feature common to schizophrenia.
- 212 • Increased methylation of the promoter region of the PV gene (PVALB) is
213 associated with methamphetamine psychosis.
- 214 • Equivalent sequence in rat brain DNA also shows increased methylation in the
215 phencyclidine model of schizophrenia.
- 216 • We found greater PVALB promoter DNA methylation in hippocampus of
217 post-mortem schizophrenia patients compared to control subjects.
- 218 • This increase in methylation is specific to a site within a transcription factor
219 binding sequence.
- 220 • We found hypermethylation in LINE-1 in hippocampus and prefrontal cortex
221 of schizophrenia post-mortem brains.
- 222 • The changes in PVALB methylation were independent of those in LINE-1.
- 223 • This hypermethylation may, through effects on transcription, contribute to the
224 enduring reduction in PV in schizophrenia.

225

226 **Ethical Conduct of Research**

227

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

231

232 **Conflict of Interest**

233 The authors report no biomedical financial interests or potential conflicts of
234 interest.

235

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245

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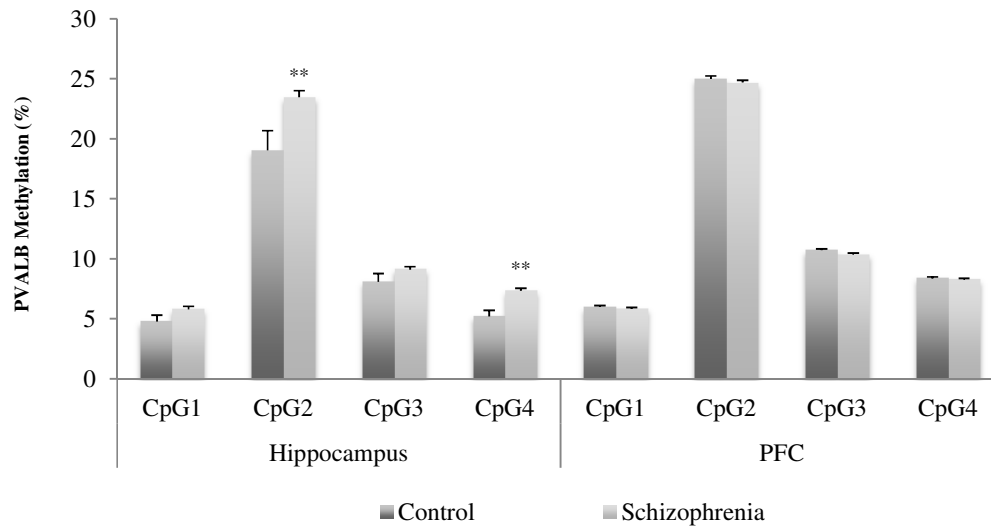
Figure 1.

Figure 1. Percentage methylation PVALB in the hippocampus and prefrontal cortex (PFC) in post mortem brains in schizophrenia and controls of Nottingham Series. Values are expressed as the mean \pm SEM. (Student's t test, n = 15 schizophrenia and n= 16 controls). **p<0.01.

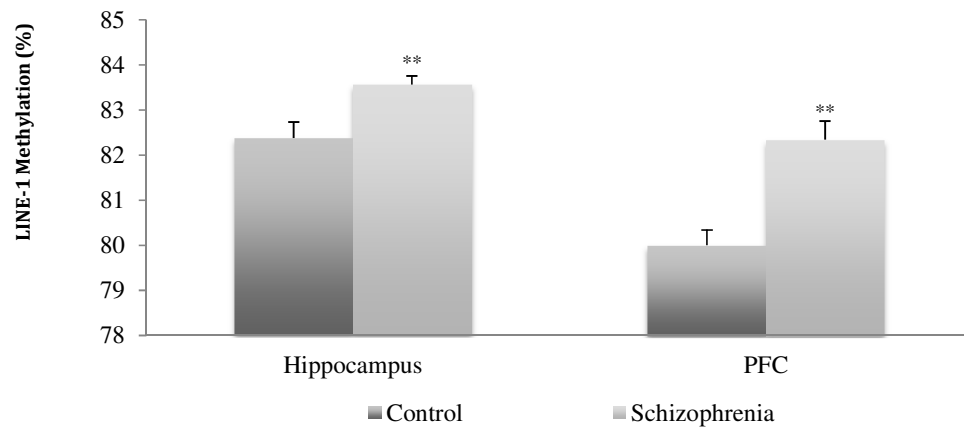
Figure 2.

Figure 2. LINE-1 global methylation in the hippocampus and prefrontal cortex (PFC) in post mortem brains in schizophrenia and controls of Nottingham Series. Values are expressed as the mean \pm SEM. (Student's t test, n = 15 schizophrenia and n = 16 controls). **p < 0.01.

Table 1. Description of demographic data of schizophrenia patients and controls

	Control (n = 16)	Schizophrenia (n = 15)	P value
Age (Mean ±SD)	67.25 ± 12.73	52.60 ± 18.18	0.016
Men (%)	11 (68%)	11 (73%)	
PM Hrs (Mean ±SD)	27.68 ± 11.42	27.08 ± 14.05	0.906

*PM Hrs: *Post-mortem* interval of collection of the samples in hours

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