

ORIGINAL ARTICLE

DNA methylation as a putative mechanism for reduced dendritic spine density in the superior temporal gyrus of subjects with schizophrenia

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Reduced dendritic spine density (DSD) in cortical layer 3 of the superior temporal gyrus (STG), and multiple other brain regions, is consistently observed in postmortem studies of schizophrenia (SZ). Elucidating the molecular mechanisms of this intermediate phenotype holds promise for understanding SZ pathophysiology, identifying SZ treatment targets and developing animal models. DNA methylation (DNAm), the addition of a methyl group to a cytosine nucleotide, regulates gene transcription and is a strong candidate for such a mechanism. We tested the hypothesis that DNAm correlates with DSD in the human STG and that this relationship is disrupted in SZ. We used the Illumina Infinium HumanMethylation450 Beadchip Array to quantify DNAm on a genome-wide scale in the postmortem STG from 22 SZ subjects and matched non-psychiatric control (NPC) subjects; DSD measures were available for 17 of the 22 subject pairs. We found DNAm to correlate with DSD at more sites than expected by chance in NPC, but not SZ, subjects. In addition, we show that the slopes of the linear DNAm-DSD correlations differed between SZ and NPC subjects at more sites than expected by chance. From these data, we identified 2 candidate genes for mediating DSD abnormalities in SZ: brain-specific angiogenesis inhibitor 1-associated protein 2 (*BAIAP2*) and discs large, *Drosophila*, homolog of, 1 (*DLG1*). Together, these data suggest that altered DNAm in SZ may be a mechanism for SZ-related DSD reductions.

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INTRODUCTION

Schizophrenia (SZ) is thought to be a disorder of cerebral cortical circuitry disruption. Particular SZ symptoms are associated with dysfunction of certain cortical circuits.¹ For example, cortical circuit abnormalities in the superior temporal gyrus (STG), a brain region critical for auditory processing, are associated with auditory verbal hallucinations and impaired auditory sensory processing. Impaired auditory processing further contributes to phonologic dyslexia and difficulty recognizing and expressing spoken emotional tone (prosody) in SZ.²

Reduced dendritic spine density (DSD) in cortical STG layer 3, and other brain regions, is observed in postmortem studies of SZ.^{3–6} We have previously demonstrated reduced DSD in STG layer 3 of SZ subjects in multiple cohorts.^{5,6} We have also shown that the DSD reduction in SZ is of a similar magnitude in both the Heschl's Gyrus and planum temporale of the STG.⁶

Reduced DSD has several features indicating it is an intermediate phenotype for SZ. An intermediate phenotype is a heritable quantitative biological trait that is correlated with a disorder due, in part, to shared genetic architecture.⁷ A number of genes contribute to regulation of dendritic spine features including DSD⁸ and several of these are also SZ risk genes.^{9–22}

The most useful intermediate phenotypes are functionally associated with aspects of the core clinical deficits of the disorder. DSD is intimately linked to neuronal function and changes in DSD are essential for normal cognition and sensory processing.^{8,23,24}

Many disorders characterized, in part, by impaired cognition are also characterized by DSD abnormalities,^{8,25} thus suggesting that reduced DSD likely contributes to cognitive deficits in SZ. For example, in the auditory cortex, dendritic spines on layer 2/3 neurons segregate frequency inputs to the neurons.²⁶ Thus reduced DSD on these neurons would likely lead to impaired frequency discrimination, a deficit that has been observed in SZ.²

Elucidating the mechanisms of this intermediate phenotype is important for understanding SZ pathophysiology, identifying SZ treatment targets and developing animal models. DNA methylation (DNAm), the addition of a methyl group to a cytosine nucleotide, regulates gene transcription and is a strong candidate for such a mechanism. DNAm is altered in the brain^{27–34} of SZ subjects and DNAm alterations are present in other contexts characterized by DSD abnormalities including neurodevelopmental disorders, models of addiction, and activity-dependent plasticity.⁸ In this study, we evaluated the hypothesis that DNAm correlates with DSD in the human STG and that this relationship is disrupted in SZ.

MATERIALS AND METHODS

Postmortem brains

Brains were recovered and processed as described previously.³⁵ Briefly, brains were recovered during routine autopsies at the Allegheny County Medical Examiner's Office, Pittsburgh, PA, USA following informed consent

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Table 1. Cohort characteristics

Cohort	DNAm		DNAm and DSD	
	NPC	SZ	NPC	SZ
Number	22	22	17	17
Sex	17 M, 5 F	17 M, 5 F	12M, 5F	12M, 5F
Race	16 W, 6 B	16 W, 6 B	11W, 5B, 1O	11W, 6B
Age (years)	45.14 ± 2.30	47.14 ± 2.91	44.82 ± 2.76	47.53 ± 3.29
PMI (h)	17.58 ± 1.39	18.23 ± 1.79	16.27 ± 1.67	15.76 ± 1.89

Abbreviations: B, black, DNAm, DNA methylation; DSD, dendritic spine density; F, female; M, male; NPC, non-psychiatric control; O, Other (Asian Indian); PMI, postmortem interval; SZ, schizophrenia; W, white. Data for continuous variables are presented as group average ± s.e.m.

from next-of-kin. DSM-IV diagnoses were made based on clinical records and structured interviews with surviving relatives. The right hemisphere was blocked coronally and the resultant slabs snap frozen and stored at -80°C . Slabs containing the STG were identified and the STG was removed as a single block. Samples containing all six cortical layers of STG (planum temporale), but excluding the adjacent white matter, were harvested. All procedures were approved by the University of Pittsburgh Committee for the Oversight of Research and Clinical Training Involving the Dead and the Institutional Review Board for Biomedical Research.

Cohort membership

The cohort was comprised of 22 subjects with either schizophrenia ($N=16$) or schizoaffective disorder.⁵ Schizophrenia and schizoaffective disorder were considered together because studies of DSD have not found differences between them.^{5,6} Each SZ subject was matched with a non-psychiatric control (NPC) subject for sex, hemisphere, and as closely as possible for postmortem interval (PMI), age and other characteristics (Table 1 and Supplementary Table 1). DSD measures from STG layer 3 were available for 17 SZ-NPC pairs (NPC = 0.036 ± 0.0019 spines per μm^3 , SZ = 0.028 ± 0.0021 spines per μm^3 ; $t=2.8$, $P=0.084$; Supplementary Figure 6). The 17 SZ-NPC pairs are a subset of the cohort studied in Shelton *et al.* (2015)⁵ and there is no overlap between the 17 SZ-NPC pairs and subjects studied in Sweet *et al.*⁶ Each pair was processed together to minimize experimental variability, and experimenter was blinded to subject's diagnosis throughout.

DNA preparation and bisulfite conversion

DNA (~10 μg) was isolated from STG gray matter (~20 mg) using AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Valencia, CA, USA) and bisulfite converted using EZ-96 DNA Methylation Kit (Zymo Research, Irvine, CA, USA), both as per manufacturer's protocol.

DNAm arrays

DNAm is the addition of a methyl group to a cytosine nucleotide within the context of a cytosine-phosphate-guanine (CpG) dinucleotide, usually, but also within the context of a cytosine-phosphate-H dinucleotide (CpH; H=adenine, cytosine or thymine).³⁶ CpGs and CpHs are referred to as 'DNAm sites' or 'sites' in this manuscript. DNAm was measured at 485 577 sites (482 421 CpG dinucleotides, 3091 CpH dinucleotides and 65 SNPs) using Infinium HumanMethylation450 Beadchip Array (HM450; Illumina, San Diego, CA, USA) as per manufacturer's protocol. β -values, the ratio of signal from a methylated probe relative to the sum of both methylated and unmethylated probes, were calculated. A β -value corresponds to the proportion of a particular site that is methylated in a sample.

Data preprocessing and filtering

Data analyses were performed using the R software environment (www.r-project.org). Color adjustment and background correction were performed using the bgAdjust2C method.³⁷ Normalization was performed using the β -mixture quantile normalization method.³⁸

Multidimensional scaling (MDS)³⁹ was used to visualize the degree of similarity among subjects using HM450 data. Prior to data filtering,

samples from four subjects were run in replicate and replicate samples from each of the four subjects clustered together (Supplementary Figure 1A). The β -values for each replicate pair were averaged for the remaining analyses. Samples also segregated by sex (Supplementary Figure 1A) and this segregation remained after filtering out data from SNP probes ($N=65$) and probes with detection P -values >0.01 in any sample ($N=3390$; Supplementary Figure 1B). After filtering out probe data from sex chromosomes ($N=11\,648$), samples no longer segregated by sex (Supplementary Figure 1C), but segregation by race became evident (Supplementary Figure 1D). Filtering data from invariable probes (s.d. <5 th percentile; $N=23\,547$), did not alter similarity among samples (Supplementary Figure 1E). Data from 447 392 probes remained for downstream analysis.

Because samples did not segregate by sex on MDS analysis after filtering out probe data from sex chromosomes, sex was not considered a covariate in downstream analyses. Others have shown that DNAm sex differences on autosomal chromosomes are often small in magnitude and inconsistently reproduced.^{40,41} Given segregation by race on the MDS analysis, race was included as a covariate in downstream analyses. One subject in this study was of Asian Indian ancestry. This subject, consistent with known genetic architecture,⁴² clustered with the subjects of European ancestry (Supplementary Figure 1D) and was thus combined with this group for analyses. Although the samples did not segregate by age on the MDS analyses (Supplementary Figure 1F), age was considered as a covariate in downstream analyses given the overwhelming evidence that DNAm varies extensively by age.⁴³⁻⁴⁵ Similarly, the samples did not segregate by PMI (data not shown) on the MDS analysis but because many factors that may have an impact on DSD in postmortem brain have been found to be particularly sensitive to PMI,^{46,47} PMI was considered as a covariate for downstream analysis. All analyses presented in the body of this paper adjust for race, age and PMI. Results of analyses adjusting only for race and age can be found in Supplementary Tables 3-6.

Cell population estimation

DNAm differs markedly between neurons and glia.⁴⁸ The proportion of neurons to glia in samples was estimated using a model based on β -values from cell-type-specific sites.⁴⁹ Neuronal proportion did not differ between SZ and NPC subjects (Supplementary Figure 2A).

Site-specific DNAm-DSD correlations

Pearson correlations between DNAm (normalized β -values) at each site and DSD (spines per μm^3) were calculated for all subjects. Examination of the linear DNAm-DSD correlations was performed for each of 3 groups (NPC and SZ subjects, NPC subjects and SZ subjects) using linear regression models with race, age and PMI adjusted.

Diagnosis-dependent differences in the DNAm-DSD correlations

Differences in the slopes DNAm-DSD correlations were assessed using linear regression models. For each site, two models were fitted: (1) $\text{DSD} \sim \beta_0 + \beta_1 \times \text{DNAm} + \beta_2 \times \text{diagnosis}$ and (2) $\text{DSD} \sim \beta_0 + \beta_1 \times \text{DNAm} + \beta_2 \times \text{diagnosis} + \beta_3 \times (\text{DNAm} \times \text{diagnosis})$. The likelihood-ratio test (LRT) was then used to test whether the DNAm-DSD correlation differed between SZ and NPC subjects.

Candidate genes for mediating reduced DSD in SZ

For each candidate gene, a permutation-based test was performed to assess whether the difference in slope of the DNAm-DSD correlation (SZ-NPC; across all sites in that gene) is significant. The diagnosis label (SZ or NPC) was permuted 1000 times for all 34 samples. Within each permutation, the differences in slopes of the DNAm-DSD correlation (SZ-NPC) for all the sites in that gene were computed, and then a one-sample t -test statistic was computed for these difference values. Finally, the permutation-based P -value was generated by comparing this one-sample t -test statistic under the true diagnosis to those under the permutation.

RESULTS

There are more DNAm-DSD correlations than would be expected by chance in NPC, but not SZ, subjects

When all subjects are combined for analysis, there are more DNAm-DSD correlations than would be expected by chance

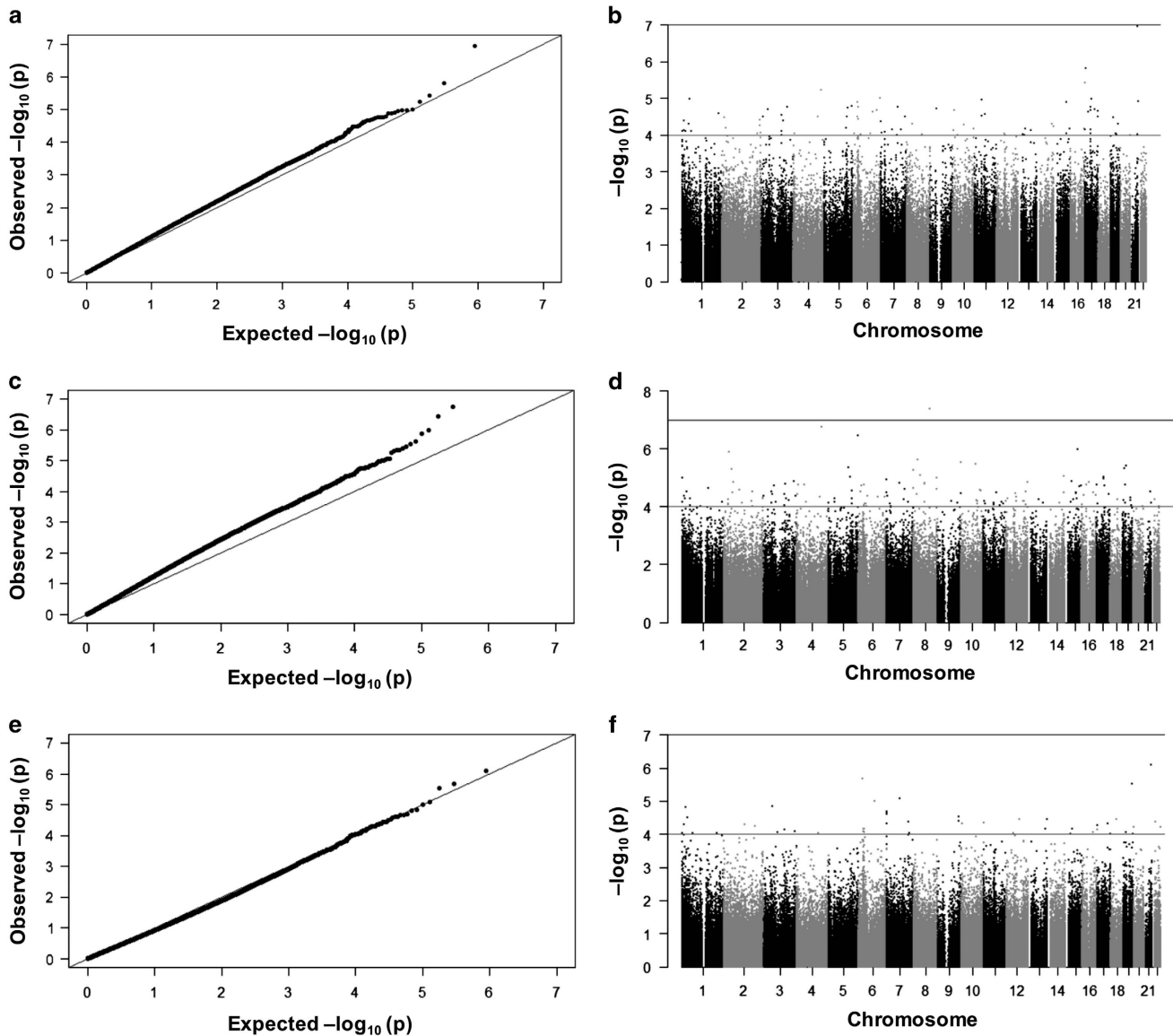


Figure 1. Q-Q plots showing that DNAm-DSD correlation analysis is enriched in small P -values for (a) the group comprised of NPC and SZ subjects and (c) the group comprised of NPC subjects only, but not (e) the group comprised of SZ subjects only. Manhattan plots showing that DNAm at many sites correlate with DSD at a suggestive level of significance ($P < 1 \times 10^{-4}$) in (d) NPC subjects and that the number of such sites is fewer in (f) SZ subjects and (b) when NPC and SZ subjects are considered together. DNAm, DNA methylation; DSD, dendritic spine density; NPC, non-psychiatric control; SZ, schizophrenia.

(Figures 1a and b). This is true when NPC subjects only (Figures 1c and d) are considered. The number of DNAm-DSD correlations in SZ subjects is no more than would be expected by chance (Figures 1e and f). In the combined group, no DNAm-DSD correlations reached significance ($P < 1 \times 10^{-7}$) and 84 reached a suggestive level of significance ($P < 1 \times 10^{-4}$; Figure 1b). In NPC subjects, one DNAm-DSD correlation reached significance and 150 reached a suggestive level of significance (Figure 1d). In SZ subjects, no DNAm-DSD correlations reached significance and 51 reached a suggestive level of significance (Figure 1f and Table 3). After adjusting for potential confounders, DNAm-DSD correlations were, in general, less statistically significant (Table 2).

DNAm-DSD correlations at multiple sites differ between NPC and SZ subjects

Not only were there many fewer strong DNAm-DSD correlations in SZ subjects, the slopes of the linear DNAm-DSD correlations

differed between NPC and SZ subjects at more sites than would be expected by chance (Figure 2a). The slopes of the DNAm-DSD correlations at two sites differed significantly ($P < 1 \times 10^{-7}$), and at 269 sites suggestively ($P < 1 \times 10^{-4}$), between SZ and NPC subjects (Table 3, Supplementary Table 4 and Figure 2b).

Candidate genes for mediating reduced DSD in SZ

We selected for more detailed follow-up genes meeting three criteria: (1) it was a gene for which there is evidence that one of its variants, either rare or common, genetically associates with SZ; (2) it was a gene in which a role in regulation of dendritic spines is established; and (3) it was one of the genes (or closest genes) to a site with a DNAm-DSD correlation reaching at least the suggestive level of significance, $P < 1 \times 10^{-4}$. For this latter criterion the more liberal suggestive level of significance was chosen so as not to preclude detection of potentially causally related genes due to the limited power inherent in a study of the current sample size. Two

Table 2. DNAm sites of DNAm-DSD correlations

Probe	Marginal correlation		Linear regression			
	Pearson's R	P-value	Slope	P-value	Gene	Closest gene(s)
	(DNAm-DSD)		(DNAm-DSD)			
cg06741996	-0.77	1.09E-07	-0.17	8.30E-07	PDXK	PDXK
cg11828470	0.72	1.53E-06	0.23	2.04E-06	NLRP1	NLRP1
cg07596086	-0.70	3.74E-06	-1.04	1.84E-05	ZNF276	ZNF276, CHMP1A
cg26805882	-0.69	5.85E-06	-0.38	1.12E-05	HAND2-AS1	HAND2-AS1
cg15393304	-0.68	9.93E-06	-0.76	7.96E-05	SMOC2	SMOC2

Abbreviations: DNAm, DNA methylation; DSD, dendritic spine density; NPC, non-psychiatric control; PMI, postmortem interval; SZ, schizophrenia. The five DNAm sites at which DNAm correlates with DSD at a level of $P < 1 \times 10^{-5}$ in the combined group (both SZ and NPC subjects) are listed in the table. See Supplementary Table 3 for a list of all 84 DNAm sites significant at $P < 1 \times 10^{-4}$. The Pearson R and P -value for the marginal correlation between DNAm and DSD at each DNAm site as well as the slope and P -value for the regression equation between DNAm and adjusting for race, age and PMI are included in the table.

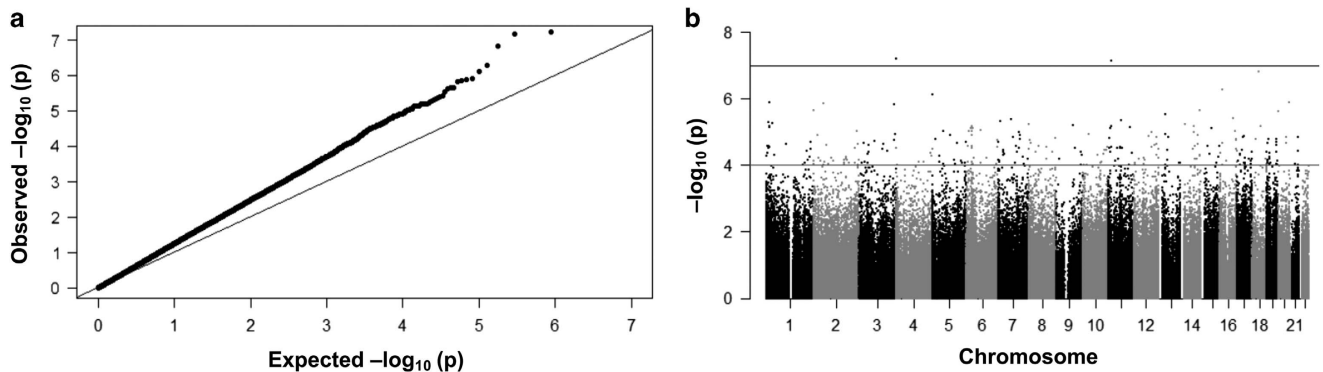


Figure 2. (a) Q-Q plot showing that the differential DNAm-DSD correlation analysis is enriched in small P -values. (b) Manhattan Plot showing that the slopes of DNAm-DSD correlation at two sites significantly differ ($P < 1 \times 10^{-7}$) between NPC and SZ subjects and that the slopes of DNAm-DSD correlation differ at an additional 269 DNAm sites at a level of suggestive genome-wide significance ($P < 1 \times 10^{-4}$). DNAm, DNA methylation; DSD, dendritic spine density; NPC, non-psychiatric control; SZ, schizophrenia.

genes met all three criteria: Brain-specific angiogenesis inhibitor 1-associated protein 2 (*BAIAP2*) and Discs Large, *Drosophila*, Homolog of, 1 (*DLG1*).

BAIAP2

DNAm-DSD correlations at two *BAIAP2* sites (cg01276536 and cg23261327) reached a suggestive level of significance (Supplementary Table 3; criterion 1). Multiple rare *BAIAP2* mutations have been associated with SZ^{16,18} (criterion 2). *BAIAP2* encodes a scaffolding and adaptor protein that regulates membrane and actin dynamics in dendritic spines and *Baiap2* null mice exhibit reduced DSD¹⁷ (criterion 3).

DNAm (normalized β -values) at 120 of 176, or 68.2%, of the *BAIAP2* sites were relatively hypomethylated in SZ subjects (Figure 3a), significantly more than the proportion of such sites observed among all sites analyzed (43.2%, Pearson χ^2 -test, $P = 0.00013$).

DNAm was correlated with DSD at many sites across *BAIAP2* in both SZ and NPC subjects but the direction and magnitude of correlation often differed by diagnosis (Figure 3b). The slopes of the linear DNAm-DSD correlations differed between NPC and SZ subjects using the LRT (Figure 3c). Further, the difference in slope of the DNAm-DSD correlation (SZ-NPC; across all 176 *BAIAP2* sites) is significant (permutation-based $P = 0.011$).

The *BAIAP2-AS1* gene is an antisense-oriented long non-coding RNA with a head-to-head orientation with respect to the 5' region of *BAIAP2*. Like *BAIAP2*, *BAIAP2-AS1* is characterized by DNAm-DSD

correlations at multiple sites, which differ between NPC and SZ subjects (Figure 3b). The LRT performed to assess whether the correlations differ by diagnosis showed an excess of small P -values compared to what would be expected by chance (data not shown). The difference in slopes of the DNAm-DSD correlation (SZ-NPC; across 13 *BAIAP2-AS1* sites) is significant (permutation-based $P < 0.001$). Notably, the slope of the correlation was negative at all sites in NPC subjects and positive in 11 of 13 of the sites in SZ subjects (Supplementary Table 5 and Figure 3b).

DLG1

One site for which the DNAm-DSD correlation differed significantly between SZ and NPC subjects (cg07756562) is located in the region 5' to *DLG1* (Table 3; criterion 1). Studies have found common *DLG1* variants to be associated with SZ.^{20,21} Further, studies of copy-number variation have found a significant excess of deletions at the chromosomal position 3q29, which includes the *DLG1* gene, in SZ^{50,51} (criterion 2). *DLG1* encodes a scaffolding protein that participates in the localization of glutamate receptors to the post-synaptic membrane and overexpression of *DLG1* in organotypic slice cultures alters dendritic spine morphology^{19,52} (criterion 3).

DNAm sites in *DLG1* and the genomic region immediately 5' to *DLG1* were characterized by a wide range of DNAm levels (Supplementary Figure 3B). DNAm levels did not exhibit any discernible pattern with respect to *DLG1* gene features, though such an assessment is limited by the fact that no data for DNAm

Table 3. DNAm sites at which slope of DNAm-DSD correlations differed most between NPC and SZ subjects

Probe	P-value	Gene	Closest gene(s)
cg07756562	6.22E-08		<i>DLG1, BDH1</i>
cg02546690	6.99E-08	<i>SERGEF</i>	<i>SERGEF</i>
cg21278787	1.49E-07		<i>TPGS2</i>
cg04616529	5.14E-07	<i>CLEC16A</i>	<i>CLEC16A</i>
cg04021706	7.66E-07	<i>AHRR</i>	<i>AHRR</i>
cg03699749	1.26E-06	<i>OSBPL2</i>	<i>OSBPL2</i>
cg10758286	1.32E-06	<i>TMEM51</i>	<i>TMEM51</i>
cg26106316	1.39E-06		<i>CCDC85A</i>
cg02260885	1.48E-06	<i>IGF2BP2</i>	<i>IGF2BP2</i>
cg01755336	2.20E-06	<i>WDR20</i>	<i>WDR20</i>
cg11495377	2.30E-06		<i>TMEM18</i>
cg23462402	2.35E-06		<i>VPS16</i>
cg00946491	3.00E-06	<i>STARD13</i>	<i>STARD13</i>
cg07139162	3.83E-06	<i>ST3GAL2</i>	<i>ST3GAL2</i>
cg09191732	4.10E-06		<i>RABGEF1</i>
cg02584610	4.39E-06	<i>TPCN2</i>	<i>TPCN2</i>
cg25269432	4.78E-06	<i>NDUFA4</i>	<i>NDUFA4</i>
cg12177942	5.05E-06	<i>SRM</i>	<i>SRM</i>
cg07110043	5.33E-06	<i>FABP3</i>	<i>FABP3</i>
cg09229797	5.82E-06		<i>DHRS1</i>
cg09020384	5.98E-06		<i>ERICH1-AS1</i>
cg14304674	6.39E-06		<i>C9orf47</i>
cg01646639	6.56E-06	<i>SLC8A3</i>	<i>SLC8A3</i>
cg23264776	6.59E-06	<i>IGSF21</i>	<i>IGSF21</i>
cg26306636	6.59E-06		<i>RPL17-C18orf32</i>
cg01674361	6.59E-06		<i>MRGPRX4</i>
cg10503635	6.72E-06		<i>MOG</i>
cg17277729	7.15E-06		<i>TMEM51</i>
cg25574849	7.22E-06	<i>UBE4A</i>	<i>UBE4A</i>
cg02293354	7.52E-06	<i>HLA-DQA2</i>	<i>HLA-DQA2</i>
cg16706631	7.53E-06	<i>HIST1H4E</i>	<i>HIST1H4E</i>
cg12645247	7.59E-06		<i>ADM</i>
cg19433697	7.69E-06		<i>FOXB1</i>
cg02447304	8.69E-06		<i>NA</i>
cg12960782	9.13E-06	<i>DEPDC1B</i>	<i>DEPDC1B</i>
cg16248435	9.23E-06	<i>JARID2</i>	<i>JARID2</i>
cg17134302	9.38E-06	<i>FBXO36</i>	<i>FBXO36</i>
cg00509921	9.48E-06	<i>MAF</i>	<i>MAF</i>
cg04018738	9.54E-06	<i>VAR5</i>	<i>VAR5</i>
cg14628708	9.77E-06	<i>COG5</i>	<i>COG5</i>

Abbreviations: DNAm, DNA methylation; DSD, dendritic spine density; NPC, non-psychiatric control; PMI, postmortem interval; SZ, schizophrenia. The 40 DNAm sites at which the DNAm-DSD correlation differed between NPC and SZ subjects at a level of $P < 1 \times 10^{-5}$ (adjusted for age, race and PMI) are listed in the table, see Supplementary Table 4 for list of sites at which the association differed at $P < 1 \times 10^{-4}$ level.

sites at the 3' end of *DLG1* were available in the data set. No overall hypo- or hypermethylation in SZ is evident in *DLG1* (Pearson χ^2 -test, $P=1$).

The linear DNAm-DSD correlation at site cg07756562 differed between SZ and NPC subjects ($P=6.22 \times 10^{-8}$). At this site, DNAm correlates positively with DSD in SZ subjects and negatively with DSD in NPC subjects (Supplementary Table 6, Figure 4b).

DISCUSSION

To our knowledge, this is the first postmortem brain study of the relationship of DNAm to DSD in SZ subjects. We evaluated the hypothesis that DNAm correlates with DSD in the human STG and that this relationship is disrupted in SZ subjects. Consistent with our hypothesis, we found DNAm to correlate with DSD at more sites than expected by chance in NPC, but not SZ, subjects. We also found that the slopes of DNAm-DSD correlations often differed between NPC and SZ subjects. We identified *BAIAP2* and *DLG1* as candidate genes for mediating DSD abnormalities in SZ.

DNAm-DSD correlations

Our findings suggest that DNAm is an important upstream mechanism for generating normal DSD and that this mechanism is disrupted in SZ subjects. Although, to our knowledge, this is the first time a DNAm-DSD relationship has been demonstrated in SZ, DNAm is altered in a number of contexts characterized by abnormal DSD.⁵³ Perhaps the most convincing evidence for a causal effect of DNAm on DSD comes from the study of addiction models where overexpression of a DNA methyltransferase, and downstream DNAm, alone, is sufficient to alter DSD.⁵⁴

The DNAm alterations observed in SZ, and thus the disrupted DNAm-DSD relationships, are likely to result from a combination of both genetic and environmental factors.³⁶ Notably, common risk variants for SZ have been shown to regulate local DNAm,^{31,32} but none of the sites in Tables 2 and 3 have been identified as targets of methylation quantitative trait loci (mQTLs) that overlap with SZ risk loci³² or neurodevelopmental mQTLs (<http://epigenetics.essex.ac.uk/mQTL/>).³¹ A number of environmental factors have been implicated in the pathogenesis of SZ,⁵⁵ and many of them have been shown to alter DNAm.⁵⁶ It is also important to consider that alterations in DNAm and DNAm-DSD correlations observed in SZ subjects may be the result of treatment-induced changes in the brain. We have previously found that antipsychotic treatment does not alter STG DSD in an animal model⁶ but accumulating evidence suggests that antipsychotics do alter DNAm.⁵⁷ However, DNAm alterations are observed in peripheral blood from early SZ subjects with only brief (< 16 weeks) antipsychotic treatment⁵⁸ thus suggesting that not all DNAm alterations in SZ are explained by antipsychotic treatment. In some cases, SZ-associated DNAm alterations are normalized by antipsychotic drugs,⁵⁹ perhaps suggesting that the therapeutic effect of antipsychotics are mediated, in part, by affecting DNAm.

Putative mechanisms underlying DNAm-DSD correlation

The study of DNAm function has historically focused on its role in promoter regions. In this context, DNAm usually blocks transcription. It is now recognized that DNAm function is context dependent⁶⁰ and that intragenic and intergenic DNAm affects transcription. Notably, DNAm affects alternative promoter usage, regulation of short and long non-coding RNAs, alternative splicing and enhancer activity.^{61,62}

DNAm-DSD correlations in *BAIAP2* and *DLG1* were distributed throughout intragenic, associated non-coding RNAs, and promoter regions, suggesting that DNAm may alter DSD in SZ by affecting transcription via both canonical and non-canonical mechanisms. DNAm at two *BAIAP2* sites within intron 7–8 are strongly and positively correlated with DSD. These two sites are in a CCCTC-binding factor (CTCF) binding site. CTCF binds unmethylated DNA and, in intragenic contexts, promotes exon inclusion into the mature transcript.⁶² We predict that DNAm at these sites leads to an increase in *BAIAP2* transcript variants with exclusion of exons local to intron 7–8 and that these transcript variants positively regulate DSD. Consistent with this prediction, multiple *BAIAP2* transcript variants have been identified which differ with respect to the composition of their 3' end (Miyahara *et al.*, 2003) and primary data including mRNA and EST alignments suggest that there are transcripts that do not contain exon 7 and/or 8.⁶³

Most of the *BAIAP2* DNAm-DSD correlations in NPC subjects, however, were negative ones in which lower DNAm was correlated with higher DSD. DNAm at a site 5' to *DLG1* (cg07756562) is similarly correlated with DSD in NPC subjects. Decreased DNAm in 5' regions is usually associated with increased total transcription.⁶⁴ We suggest that lower DNAm at these sites allows for increased *BAIAP2* and *DLG1* transcription, promoting dendritic spine formation. Supporting this interpretation is evidence that *BAIAP2* overexpression promotes DSD¹⁷ and *DLG1* overexpression promotes dendrite growth and complexity.^{65,66}

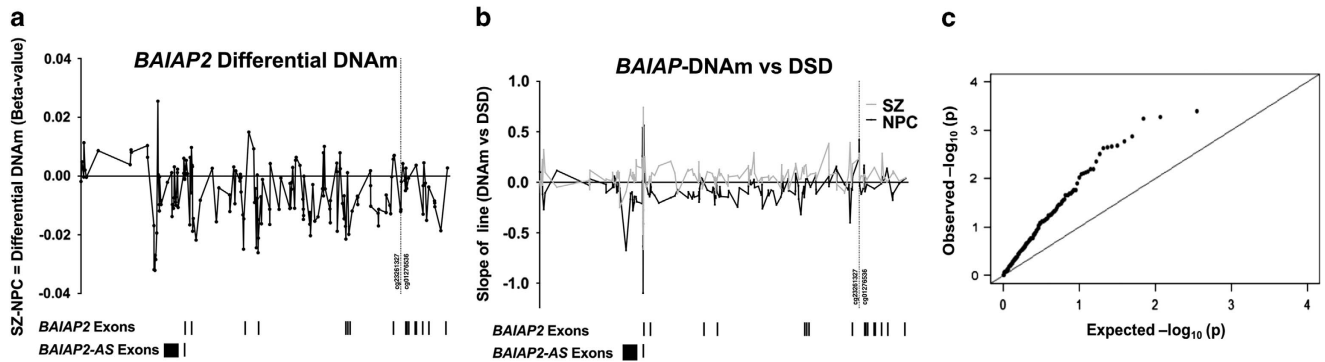


Figure 3. (a) *BAIAP2* is hypomethylated in SZ subjects relative to NPC subjects and (b) the slopes of the DNAm-DSD correlations at most *BAIAP2* sites analyzed differ between SZ and NPC subjects but DNAm at the sites associated with both cg01276536 and cg23261327 positively correlates with DSD independent of diagnosis. (c) Q-Q plot showing that the differential DNAm-DSD correlation analysis is enriched in small *P*-values compared to what would be expected by chance for the DNAm sites analyzed in *BAIAP2*. DNAm, DNA methylation; DSD, dendritic spine density; NPC, non-psychiatric control; SZ, schizophrenia.

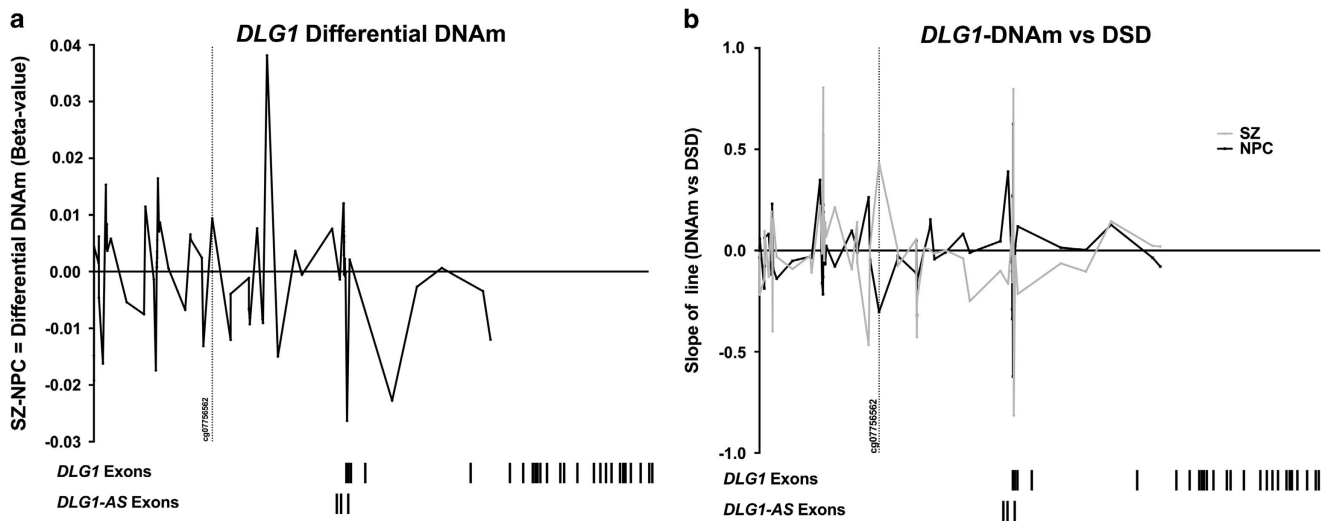


Figure 4. (a) *DLG1* DNAm does not differ between subjects with SZ and NPC subjects. (b) The DNAm site cg0775662 is 5' of *DLG1* and is one of two DNAm sites in which the differential correlation between DNAm and DSD reached significance. DNAm, DNA methylation; DSD, dendritic spine density; NPC, non-psychiatric control; SZ, schizophrenia.

Other DNAm-DSD correlations are annotated to *BAIAP2-AS1* and are also relatively hypomethylated in SZ subjects. We anticipate increased *BAIAP2-AS1* transcription in SZ as a result of this hypomethylation. It is difficult to know how higher levels of *BAIAP2-AS1* might affect expression of *BAIAP2*. Antisense long non-coding RNAs, like *BAIAP2-AS1*, often regulate local gene transcription at multiple levels⁶⁷ but *BAIAP2-AS1* has not been studied.

DNAm differences between SZ and NPC subjects at particular sites is one mechanism by which the DNAm-DSD correlations may be disrupted in SZ. Indeed, our data suggest that there are many sites where DNAm differs between SZ and NPC subjects (Supplementary Table 2 and Supplementary Figure 4). Global DNAm, however, does not appear to differ between SZ and NPC subjects (Supplementary Figure 5). Disruptions of DNAm-DSD correlations in SZ that do not result from a change in DNAm may reflect an abnormality downstream of DNAm (e.g., disrupted binding of a DNAm-dependent transcription factor and so on).

Limitations

Despite plausible relationships between DNAm in multiple genes (including *BAIAP2* and *DLG1*) and DSD, the findings, like those of any postmortem brain study, are only correlative and cannot establish a mechanistic relationship. Our use of SZ risk gene and

DSD regulator criteria to define candidate genes limits the ability to identify novel genes important in SZ pathophysiology or dendritic spine regulation. However, because of the large number of sites tested, there is a likelihood that some DNAm-DSD correlations are spurious and not relevant to the DSD phenotype in SZ. We chose to use these criteria to increase the probability of identifying DNAm alterations that may contribute causally to the DSD phenotype in SZ. Another potential technical limitation is that the sites studied were constrained by the use of the HM450 array. It only measures a fraction of the >28 million DNAm sites in the human genome and coverage is biased toward CpG islands, promoters and genic regions.

Conclusions and future directions

The study of reduced DSD as an intermediate phenotype in SZ across different levels of analysis including genetics,⁶⁸ transcriptomics⁶⁹ and proteomics³⁵ has provided valuable insights into SZ. This study suggests that epigenetic alterations, specifically disrupted DNAm-DSD correlations, in SZ may be a mechanism for SZ-related reductions in DSD and justify future studies probing this relationship.

Studies to confirm the DNAm-DSD relationship and the effect of DNAm on candidate gene transcription in additional, larger

cohorts will be a critical next step. Also, DNAm varies widely between cell types in the human cerebral cortex, with studies indicating that DNAm in GABA neurons is more extensive by several fold than in glutamatergic neurons.⁷⁰ Thus, future cell-type-specific studies, using laser capture microdissection,⁷¹ fluorescent-activated nuclei sorting⁷² or similar methods, may increase the likelihood of detecting diagnosis-specific DNAm alterations by decreasing variability and revealing findings that were masked by opposing DNAm changes in different cell types. Studies in model systems to evaluate the correlative versus causal nature of the DNAm-DSD relationship will be also important. Understanding the DNAm-DSD relationship may facilitate the development of new, and/or the repurposing of existing, DNAm modifying drugs for SZ treatment.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

DAL currently receives investigator-initiated research support from Pfizer and in 2012–2014 served as a consultant in the areas of target identification and validation and new compound development to Autifony, Bristol-Myers Squibb, Concert Pharmaceuticals and Sunovion. The remaining authors declare no conflict of interest.

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