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Increased Nigral *SLC6A3* Activity in Schizophrenia Patients: Findings From the Toronto–McLean Cohorts

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***SLC6A3*, which encodes the primary regulator of extracellular dopamine (DA) concentration, the DA transporter, has been implicated in schizophrenia (SCZ). However, the details of its genetic effect on risk remain largely unknown. The purpose of this candidate gene study was to identify a specific *SLC6A3* activity associated with SCZ by using functional genetic approaches. We first examined gene activity in DA neurons isolated from case-control postmortem nigral tissue and found that the average *SLC6A3* mRNA level in controls was only 0.37-fold of that in cases (*P* = .0034). To understand this expression difference, we examined the association of 10 genetic markers, mostly located in the promoter region, with SCZ in 1717 subjects collected from Toronto and McLean cohorts, including 881 controls and 836 cases and identified the 5′ promoter SNP rs1478435 as having a significant association signal (uncorrected *P* value: .00462; adjusted *P* value: .0319) in unrelated Caucasians. Allele T was over-represented in controls (OR = .75); T-carrier controls had decreased mRNA levels in nigral DA neurons, contributing to the reduced activity in the controls. In vitro functional analysis confirmed that T carriers displayed attenuated enhancement of promoter activity. These findings collectively suggest that increased nigral *SLC6A3* activity may be a risk factor for SCZ, and may help to explain high rates of comorbidity with substance abuse.**

Key words: dopamine neurons/functional genetics/gene expression/human postmortem midbrain/promoter function

Introduction

Both clinical and preclinical findings implicate presynaptic dysregulation of dopamine (DA) transmission in the pathophysiology of schizophrenia (SCZ).[1–5] Amelioration of positive symptoms (ie, hallucinations, delusions, thought disorder) with DA receptor blockers further implicates dysregulation of DA transmission.[6,7] One potential source of DA dysregulation is *SLC6A3*, the gene that encodes the presynaptic, principal regulator (the DA transporter or hDAT) of DA transmission in the brain. The hDAT protein is expressed in brain regions (eg, prefrontal cortex and limbic) implicated in SCZ.[8–14] DNA sequence variation at *SLC6A3* regulatory sites may cause dysregulation of DA transmission in these brain regions by altering *SLC6A3* activity and protein levels.[15–18]

The promoter, not the 3′ end, appears to carry *SLC6A3*-related genetic risk for SCZ. Previous association studies used a 40-bp variable number tandem repeat (VNTR) marker located in the last exon (50 kb downstream of the promoter region) of *SLC6A3*, but none of these studies showed a significant association with SCZ (www.schizophreniaforum.org). Although this negative finding may seem inconsistent with the notion that dysregulation of DA transmission contributes to SCZ symptoms, such a conclusion would be premature, because 3′VNTR genotypes and in vivo hDAT protein levels are only weakly associated. In contrast, all 3 studies of the *SLC6A3* promoter region showed positive and significant associations between single nucleotide polymorphisms (SNPs) and SCZ in 4 populations[19–21] with different ancestral

backgrounds. However, there is currently no direct evidence regarding whether *SLC6A3* activity in DA neurons is altered in SCZ and which functionally distinct *SLC6A3* promoter variants confer risks for SCZ. In this study, we characterize *SLC6A3* activity in SCZ using 4 complementary approaches: (1) by measuring *SLC6A3* mRNA levels in DA neurons isolated from control and case postmortem brain tissue; (2) by typing functional polymorphisms located mainly in the 5′ promoter regions in Caucasian controls and SCZ patients; (3) by correlating SCZ-associated promoter variants with *SLC6A3* mRNA levels in DA neurons; and (4) by in vitro functional verification of significantly correlated variants (see supplementary figure 1 for study design).

Methods

Postmortem midbrain tissue was provided by the Harvard Brain Tissue Resource Center at McLean Hospital. Single nigral DA neurons were isolated randomly from nigral sections by laser capture microdissection (LCM). All subjects were recruited at both the Neurogenetics Laboratory at the Centre for Addiction and Mental Health (CAMH) in Toronto, Ontario, Canada and the Psychology Research Laboratory at McLean Hospital. All mRNA levels were assessed by standard quantitative reverse-transcription polymerase chain reaction (qRT-PCR) [17] where "β-actin" was used as an internal control because cases and controls did not differ in its expression and this internal control could address postmortem tissue-associated RNA quality issue in qRT-PCR.[22,23] Extraction of DNA from blood, genotyping, ancestry ascertainment, quality controls, imputation, association and permutation analyses used standard protocols[24–32] (see supplementary table 1 for primers and probes). Luciferase activity-based functional assay of rs1478435 allelic regulation of 2.5-kb *SLC6A3* promoter activity followed a previous in vitro procedure.[18] Association, ANOVA or Student's *t* test results with *P* values of $< .05$ were considered as statistically significant, either uncorrected or adjusted (detailed methods are provided in supplementary information).

Results

Significant Reduction in SLC6A3 *mRNA Levels in Controls Compared With Cases*

To measure *SLC6A3* mRNA levels, we used LCM and DA neurons isolated randomly from human postmortem brain nigral blocks in 20 controls and 20 age- and gender-matched cases (18/20 cases had medical records available showing 29.6 ± 4.5 years of exposure to antipsychotic (and often other psychotropics as well) medications with an average of 2.94 ± 0.50 antipsychotic drugs per case; see table 2 for demographic information; see figures 1A–D for LCM), followed by qRT-PCR analysis of *SLC6A3* mRNA levels in the isolated DA neurons. The average *SLC6A3* mRNA level in controls was only 0.37-fold of that in cases ($P = .0034$ by Student's *t* tests; figure 1E). Because it has been reported that haloperidol treatment reduced hDAT expression levels in SCZ patients,[33] our data suggest that significantly elevated *SLC6A3* activity was associated with SCZ and was not a medication effect. Supporting this conclusion, neither antipsychotic medication dose ($R^2 = .0052$, $P = .7899$) nor years of medication exposure ($R^2 = .048$, $P = .3979$) was correlated with *SLC6A3* mRNA levels, ruling out medication effects. To identify the cause of the *SLC6A3* elevation in cases, we took a genetic approach to delineate whether DNA sequence variation in the *SLC6A3* gene contributed to altered *SLC6A3* activity.

Selection of 10 Genetic Markers in SLC6A3

We selected 10 markers to type, including 6 that were previously reported to be associated with various mental disorders such as attention deficit hyperactivity disorder (ADHD), drug addiction, depression and SCZ (see table 1[17,21,34–44]). Of these 6 markers, Int8VNTR and rs3756450 are functional based on in vitro assays and 3 (rs2455391, rs67175440 and rs3756450) were previously implicated in SCZ.[21] The 5′VNTR site was included, because our previous study suggested that this marker was significantly correlated with mRNA levels in control midbrain.[17] Three novel SNPs were included: rs11564751 (a core promoter SNP), rs12652860 and rs6860992; the latter 2 are located near the distal functional region of the *SLC6A3* promoter.

Ancestry-Informative Marker-Defined vs Self-Reported Caucasians

Ancestry-informative marker (AIM) was used to genetically verify Caucasians of European ancestry for inclusion in the data analyses. As a result of the AIM analysis, 15.2% of the total sample was excluded from the AIM-defined Caucasian group (supplementary figure 2). The AIM-defined Caucasian group overlapped with the self-reported Caucasian group (supplementary table 2). This was especially evident in the Toronto case sample, perhaps due to its great admixture. Out of 610 patients, 433 (71.0%) were AIM-defined Caucasians and 427 (70.0%) were self-reported Caucasians. However, only 388 (63.6%) subjects met both AIM and self-report criteria for being "Caucasians," displaying a discordance of 10.4% in this subsample. The overall discordance rate was 3.9%. In this study, we classified Caucasians by AIM for the association analyses because it is more objective. Therefore, although association data on "Self-report" or "AIM+Self report" ethnicity were available (supplementary figure 4), we restricted our analyses to AIM-defined Caucasians to reduce ancestral heterogeneity and minimize false positive findings.

Linkage Disequilibrium Difference Between Controls and Cases

Among controls, rs11564751 (−1675 bp) had lower linkage disequilibrium (LD) scores with 2 upstream SNPs, rs11564750 (−2214 bp) and rs3756450 (−2600 bp), compared with the other SNP pairs (supplementary figure 3, upper panel). In cases, the low levels of LD displayed by rs11564750 and rs3756450 were even weaker and extended to rs12652860 (−8224 bp) (supplementary figure 3, lower panel). These LD data indicate a loss of LD in the *SLC6A3* promoter of SCZ patients.

Association Analysis of Genotypes

Among 836 typed patients, we excluded 44 with DSM-IV diagnoses of nonschizophrenic psychotic disorders who would not meet a "narrow" definition of SCZ: 19 patients with a diagnosis of bipolar disorder, 1 with a personality disorder, and 24 with a diagnosis of psychosis not otherwise specified. Considering the 792 patients who met the "narrow" phenotype definition (excluding schizoaffective disorders), 1222 subjects met AIM criteria; the male:female ratios were 1:1.30 on average (1:2.95 for controls and 1:0.30 for cases, table 2). The average recruitment age was approximately

Table 1. Ten Markers Selected for Genotyping in This Study

Marker	chr5 bp[a]	Type	*SLC6A3* Location[b]	Supporting Evidence	Reference
rs3836790	1411855-6	int8VNTR[d]	Intron 8	ADHD, Drug addiction	Guindalini et al[35]; Laucht et al[36]; O'Gara et al[37]; Franke et al[38]; Maitra et al[34]
				Correlation with mRNA levels	Brookes et al[39]
				In vitro functional	Guindalini et al[35]; Hill et al[40]
rs2455391	1443498	SNP	Intron 1 (2051)	SCZ	Zheng et al[41]
rs67175440	1443603[c]	SNP	Intron 1 (1945)	SCZ	Zheng et al[41]
rs11564751	1447223	SNP	−1675	(novel core promoter region)	
rs11564750	1447762	SNP	−2217	ADHD	Doyle et al[42]
rs3756450	1448148	SNP	−2590	SCZ	Talkowski et al[21]
				Protein binding in vitro	Bamne et al[43]
rs2550948	1450444	SNP	−4896	Depression	Huang et al[44]
rs12652860	1453772	SNP	−8223	(novel)	
rs6860992	1455946	SNP	−10 397	(novel)	
rs70957367	1456666	5′VNTR[e]	−11 115	Correlation with mRNA levels	Zhou et al[17]

Note: ADHD, attention deficit hyperactivity disorder; SCZ, schizophrenia; VNTR, variable number tandem repeat.
[a]Per GRCh37.p13.
[b]Assuming that TSS = 1.
[c]Together with its adjacent SNP rs2975223 form a dinucleotide polymorphism (DNP, LD = 1), see Zhou et al[17].
[d]30 bp repeat, $n = 5–7$.
[e]60 bp repeats $n = 6–8$.

Table 2. Demographic Information on Subjects Used in This Study

Phenotype	Number of Subjects	Male	Female	Unknown	Average Age	AAO
American postmortem sample used in mRNA level analysis						
Controls	20[a]	11	9	0	58.75 ± 3.58	
Cases	20	11	9	0	58.30 ± 3.62	
Total	40	22	18	0		
AIM-defined Caucasians of European ancestry used in association study						
Controls	796	201	592	3	55.28 ± 0.40	
Cases	426	328	98	0	40.53 ± 0.61[c]	21.14 ± 0.30
Total	1222[b]	529	690	3		

Note: AAO, age-at-onset; AIM, ancestry-informative marker.
[a]One of the 20 cases was self-reported African American and all other 39 as self-reported US Caucasians.
[b]Out of 1717 subjects studied.
[c]Recruitment age.

55.28 years old for controls and 40.53 years old for cases; average age-at-onset in cases was about 21.14 years old.

Significant association signals were found for 3 of the 10 typed markers: rs67175440, rs12652860, and 5′VNTR (independent uncorrected *P* values = .00830–.02272). Among the 2 typed VNTRs, 5′VNTR had the 509 bp variant underrepresented in cases (OR = 0.7868), whereas Intron 8 VNTR did not differ at a statistically significant level in either allelic or genotypic frequency in controls and cases. rs12652860 survived correction for multiple testing with an adjusted *P* value of .0477 (table 3). For rs12652860, located in the distal promoter region (−8224 bp), the A allele was under-represented in SCZ (OR = 0.7799). None of the other markers was significantly associated with SCZ.

Weak rs12652860 Correlation With mRNA Levels in DA Neurons

Based on these association findings (supplementary figure 4a), we next examined whether rs12652860 had any functional significance. To do so, we used qRT-PCR to measure *SLC6A3* mRNA levels in DA neurons from controls and SCZ as described above. These postmortem analyses showed that the A allele was associated with reduced mRNA levels in controls, but this reduction was not statistically significant (0.80-fold, *P* = .3009) (supplementary figure 5). By genotype, A-carriers showed reduced mRNA levels in controls as well, but this reduction also did not reach statistical significance (0.69-fold, *P* = .1779). No statistically significant differences were found in cases or in case/control ratios either for alleles or for genotypes (supplementary figure 5). The lack of significant association between mRNA level and allele or genotype suggested that examining other markers using imputation might be more informative.

Association Analysis of Imputed Genotypes

Imputation with the 1000 Genome Project multiple population reference panel increased the number of SNPs from 8 to 17. This additional association analysis revealed 7 markers showing statistically significant association signals (table 4). Among the 7, four significant associations survived multiple testing, all residing beyond rs12652860 in distal promoter regions. The most significant association was with rs1478435, which is 840 bp upstream of rs12652860. The T allele of rs1478435 was under-represented in SCZ, with an OR of 0.7500 (uncorrected *P* = .00444; adjusted *P* = .0356).

Strong rs1478435 Correlation With mRNA Levels in DA Neurons

Based on these association findings (supplementary figure 4b), we next examined whether rs1478435 had any functional consequences. A comparison of *SLC6A3* mRNA levels in isolated DA neurons between 20 controls and 20 cases carrying the T and C alleles was used for a variant-expression correlation analysis.

The T allele of rs1478435 was marginally associated with reduced mRNA levels in controls (0.61-fold, *P* = .0617 by *t* tests). Heterozygotes were present in both allelic groups. The genotypic comparisons, which

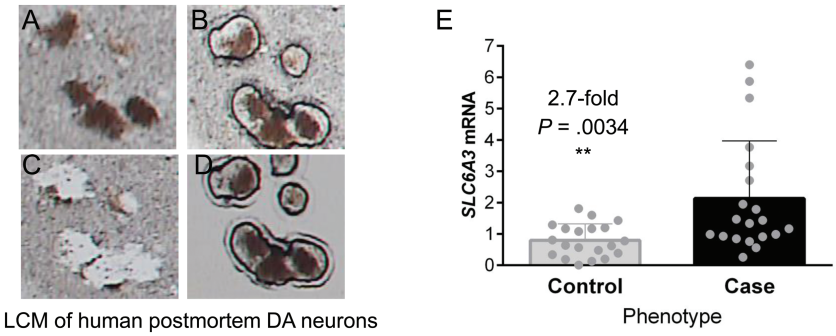

Fig. 1. *SLC6A3* mRNA levels in isolated postmortem dopamine (DA) neurons of controls vs patients with schizophrenia (SCZ; case). (A) TH-positive DA neurons on section before LCM. (B) Laser-capturing of DA neurons. (C) Section after DA neuron capture. (D) Captured DA neurons for RNA isolation and quantitative polymerase chain reaction. (E) Increased expression levels in SCZ than in controls.

Table 3. Association of Genotyped *SLC6A3* Markers With SCZ in Caucasians

Marker	BP	*SLC6A3* Location	A1	Case	Control	ChiSq	OR (95% CI)	*P*-Value	Adjusted *P*[a]
rs67175440	1443602	Intron 1	G	0.3873	0.4351	5.190	0.8209 (0.6927–0.9729)	.02272	.1201
rs12652860	1453772	5′ promoter	A	0.2176	0.3190	6.969	0.7799 (0.6483–0.9382)	.00830	**.0477**
rs70957367 (5′VNTR)[a]	1456666	5′ promoter	509	0.2700	0.3197	6.520	0.7868 (0.6543–0.9460)	.0107	

Note: [a]Both EMP2 and SNPSpD agreed; bold, *P*-value is less than .05 and considered statistically significant.

completely separated the individuals into 2 groups on the basis of genotypes, showed that T-carrier controls had a significantly reduced mean mRNA level (0.53-fold, $P = .0246$) (figure 2A). This genotypic correlation was not found in cases (figure 2A, *Insert*), resulting significant phenotypic difference in genotypic expression ($P = .0062$). Furthermore, the case/control ratio for mRNA levels was 2.04-fold higher in T-carriers than in the non-T carriers ($F_{(11,7)} = 6.493$, $P = .0204$; figure 2B), suggesting that the T-associated variant was associated with significantly upregulated *SLC6A3* activity in cases. Consistent with this finding, the T allele was also associated with reduced gene activity in lung tissue from 123 subjects based on eQTL results in the GTEx database (P-value = 7×10^{-10}, www.gtexportal.com). On the basis of these findings, we carried out an in vitro functional analysis of this SNP.

rs1478435: Allelic Regulation of SLC6A3 *Promoter Activity in Cultured DA Cells*

On the basis of the strong association between rs1478435 genotypes and mRNA levels in isolated DA neurons and lung tissue, we examined whether the observed correlation depended on linkage with another underlying functional polymorphism or whether rs1478435 was the underlying functional polymorphism; in the latter case, the 2 alleles of rs1478435 could differentially regulate promoter activity. To distinguish between these 2 possibilities, we carried out an in vitro functional analysis of the 2 alleles in 2 different DA cell lines, SK-N-AS derived from human and SN4741 from mouse substantia nigra DA neurons. Using exogenous DNAs for the expression analysis allowed well-defined conditions for better control of allelic activity. By Luc reporting of allelic regulations of the *SLC6A3* promoter, we have observed that the C allele conferred higher promoter

Table 4. Association of Imputed *SLC6A3* Markers With SCZ in Caucasians

Marker	BP	*SLC6A3* Location	Prot. Allele	Case	Control	ChiSq	OR (95% CI)	*P*-Value	Adjusted P^b
rs2975223	1443603	Intron 1	A	0.3873	0.4351	5.190	0.8209 (0.6927–0.9729)	.02272	.4401
rs67175440	1443604	Intron 1	G	0.3849	0.4332	5.199	0.8187 (0.6893–0.9724)	.02260	.1445
rs12652860	1453772	5′ promoter	A	0.2676	0.3190	6.969	0.7799 (0.6483–0.9382)	.00830	.0622
rs1478435[a]	1454612	5′ promoter	T	0.2124	0.2645	8.094	0.7500 (0.6150–0.9147)	.00441	**.0356**
rs10061889	1456803	5′ promoter	A	0.2222	0.2741	7.458	0.7566 (0.6192–0.9246)	.00632	**.0477**
rs748209	1457554	5′ promoter	A	0.2175	0.2714	8.308	0.7463 (0.6114–0.9109)	.00395	**.0319**
rs2937650	1458018	5′ promoter	A	0.2175	0.2714	8.308	0.7463 (0.6114–0.9109)	.00395	**.0319**

Note: [a]Selected for functional assessments.
[a]Bold, *P*-value is less than .05 and considered statistically significant.

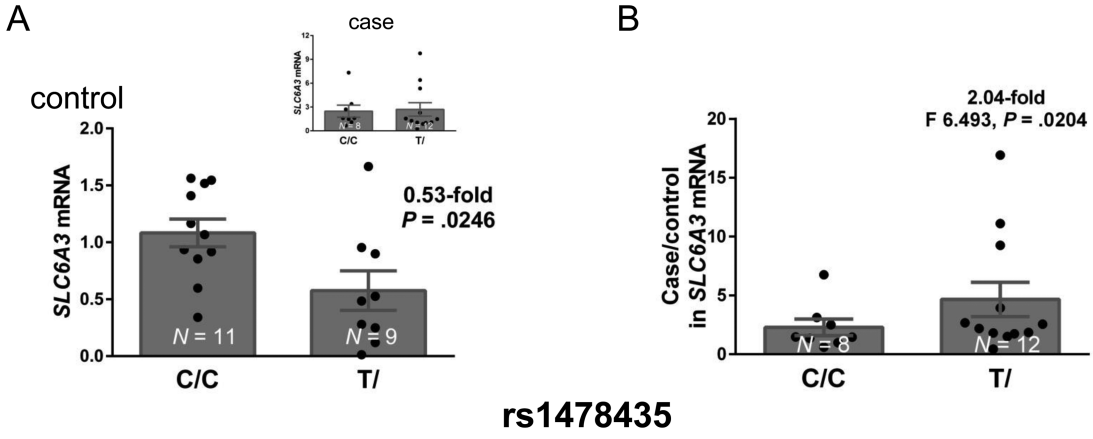

Fig. 2. Correlation of rs1478435 genotypes with mRNA levels in dopamine (DA) neurons. (A) Reduced mRNA expression levels in rs1478435 T-carrying controls. *Insert*, no genotypic difference in cases. Regression analysis showed a significant phenotype difference ($P = .0062$, with no interaction between genotype and phenotype). (B) Case/control ratio in expression levels increased in rs1478435 T-carriers. mRNA levels were normalized by actb mRNA levels; *$P < .05$; **$P < .01$ by Student's *t* tests ($N = 20$ for each phenotype).

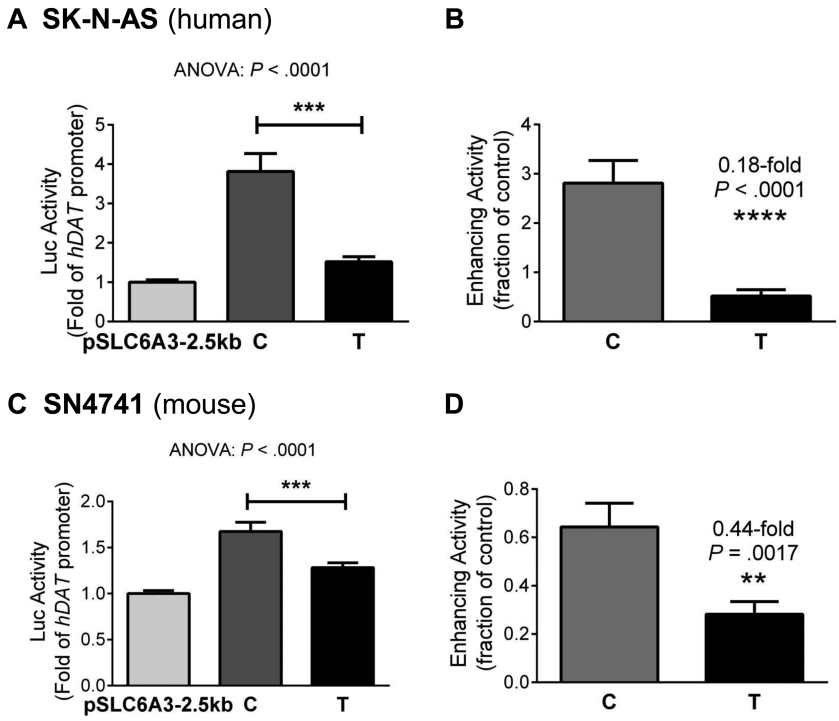

Fig. 3. In vitro functionality of rs1478435 in SK-N-AS (A, B) and SN4741 (C, D). (A, C) Lower 2.5-kb *SLC6A3* promoter activity conferred by T than by C allele (by ANOVA tests, $N = 3–5$). (B, D) Reduced enhancement by T. Student's *t* tests: $^{**}P < .01$; $^{****}P < .0001$ (data in A, C).

activity than the T allele ($P < .0001$ by ANOVA; figures 3A and 3C). This transcriptional enhancement was reduced consistently to 0.18- or 0.44-fold in the T allele compared with the C allele ($P < .0001$ by *t* tests, $F_{(35, 35)} = 12.72$, $P < .0001$ in SK-N-AS; $P = .0017$ by *t* tests, $F_{(42, 42)} = 3.449$, $P = .0001$ in SN4741; figures 3B and 3D). The consistency in the results between 2 independent cell lines validated the allelic difference in regulating promoter activity. Importantly, this allelic difference was consistent with the postmortem findings showing an association between the T allele and reduced *SLC6A3* activity (low mRNA levels prevented obtaining reliable allelic expression information in the postmortem samples). Together, these new functional data also complement current tissue homogenate-based *cis*-eQTL information (http://www.braineac.org).[45]

Discussion

In isolated nigral DA neurons, we found increased *SLC6A3* activity in schizophrenics compared to controls. This finding was not related to medication dose or years of exposure. There was no information on smoking history in the medical records so that we cannot rule out possible smoking effects. Furthermore, we identified a novel functional SNP, rs1478435, which is located −9064 bp in the *SLC6A3* distal promoter region and was associated with SCZ in our sample of unrelated Caucasians recruited in Toronto and at McLean Hospital. We also showed that the T allele of rs1478435, which is associated with reduced *SLC6A3* transcription activity, is consistently under-represented in cases.

Reduced SLC6A3 *Activity Might Confer Protection Against SCZ*

Davis et al[46] proposed a model of DA contributions to the pathophysiology of SCZ, in which low DA activity contributes to negative symptoms (ie, blunted affect, lack of initiative, social withdrawal) whereas high DA concentration is associated with positive symptoms. Our findings provide direct genetic evidence to support the view that DA deficiency secondary to elevated *SLC6A3* expression or excessive reuptake activity, may contribute to the pathophysiology of SCZ.[47,48] Interestingly, these findings also support the possibility that antipsychotic medications may exert their therapeutic effects partly by attenuating elevated *SLC6A3* activity. This possibility is consistent with a recent brain imaging finding that SCZ patients after 2 weeks of treatment with haloperidol displayed significantly reduced hDAT protein density compared with nontreated SCZ patients.[33] The T allele of rs1478435 may provide a protective effect by reducing hDAT expression levels to permit sufficient DA signaling. This interpretation is consistent with human genetic studies on the DA-catabolizing *COMT* gene that have reported reduced DA signaling in the prefrontal cortex in SCZ[49] and with imaging studies showing higher hDAT protein density in drug-naïve SCZ patients than in healthy controls.[50]

Increased DAT mRNA levels were recently found in the peripheral blood leukocytes (PBLs) of medicated SCZ patients (25 acute and 27 chronic, compared to 30

controls),[51] consistent with our nigral results. However, other studies of protein levels in different tissue yielded inconsistent results. Imaging studies found either increased DAT binding in basal ganglia of schizophrenics, no difference in 1 cohort or reduced striatal DAT density in medicated patients in another cohort.[52–54] Other postmortem studies reported reduced DAT density in cortex or amygdala in SCZ.[55,56] The discrepancy with the protein findings reported here might be attributable to tissue dependence of protein expression or inhibitory effects of medication.[33]

Comparison With Previous Association Findings

Many genetic studies of *SLC6A3* in SCZ used the 3′VNTR marker, located in the last exon of the gene, and none of them found a significant association with SCZ.[57–67] These negative findings were inconsistent with a principal role for hDAT in DA transmission or dysregulation of DA activity in the pathophysiology of SCZ. However, these negative findings are likely attributable to the weak LD between 3′VNTR and the 5′ promoter, limiting the ability of the 3′VNTR to capture promoter association signals.[17] This 3′ marker was only implicated in SCZ in 1 imaging study when considered in interaction with the *COMT* gene.[15]

More recently, promoter markers have been examined in association studies of SCZ. One core promoter SNP, rs2975226 (−68 bp), was found to affect risk for SCZ in 2 independent Asian populations,[19,68] but these results have not yet been replicated in other ethnic groups. SNP rs2652511 (−841 bp) was associated with SCZ in Chinese, but not in Iranian samples.[69] They are approximately 8 kb downstream of rs1478435, but display high LD with each other (see SNPs # 7 and 8 vs #22 in supplementary figure 6), suggesting that the Chinese findings are likely to be LD-based signals. In another study, rs3756450 (−2600 bp) was found to be significantly associated with SCZ,[21] but that finding has not yet been replicated in other cohorts. In our entire cohort of mixed ancestries (1717 subjects), rs3756450 also displayed significant association signals (uncorrected P = .0006833; adjusted P = .0050, where G was the risk allele with an OR of 1.388) and was the smallest P-value among the examined markers.

For supporting information, we consulted available GWAS datasets in both dbGaP and the Psychiatric Genomics Consortium (PGC, http://www.med.unc.edu/pgc) databases. *SLC6A3* genotype information was available for ancestry control from 2 dbGaP GWAS datasets, phs000021.v3.p2 and phs000167.v1.p1, on American SCZ. Phenotype QC was also performed to remove schizoaffective depression cases for phs000021.v3.p2. Neither GWAS was able to infer rs2975223, and phs000167.v1.p1 did not allow imputation of rs1478435, but both allowed the imputation of rs3756450. Meta-analysis of these studies (a Caucasian and an African American cohort from phs000021.v3.p2 and Caucasians only in phs000167.v1.p1) resulted in nonsignificant P values of .06009 for rs1478435 and .1126 for rs3756450 (see supplementary table 3 for more information; no rigorous phenotype control was performed in these GWAS). Meta-analysis of our data and the African American datasets showed a P value of .006122 for rs1478435 (the smallest P-value among 11 imputed SNPs) and 0.0848 for rs3756450. These results from the meta-analyses suggest that rs1478435 may have a stronger effect than rs3756450. PGC had 4 GWAS results available on SCZ (not included in the meta-analysis here because neither genotype nor phenotype information was available for the quality control). Two of these 4 PGC GWAS were imputed to the 1000 Genome Project templates and had rs1478435 marker information, but neither study implicated this marker in SCZ risk. One possible reason for the difference between our results and those of the PGC is that our cases underwent rigorous diagnostic assessments using standardized instruments, whereas the diagnostic methods were more varied in the PGC, resulting in a potentially more heterogeneous clinical phenotype. Another possibility is that the SNP-SCZ association finding reported here was a false-positive; replications in independent homogeneous cohorts are required (*SLC6A3* was not reported in a more recent association study (Schizophrenia Working Group of the Psychiatric Genomics Consortium).[70] Nevertheless, dbGaP and PGC together provided a total of 7 GWAS datasets on SCZ and 4 of the 7 had information on rs1478435. Among these 4 rs1478435-informative datasets, 3 (75%) showed ORs <1 for rs1478435, consistent with our association findings. For comparison purposes, we found that rs1478435 was not significantly associated with either bipolar disorder (P = .3914) or substance abuse (meta-analysis P = .9238) (no information was obtained for ADHD and Parkinson's disease). Overall, these SNP-SCZ association findings from various populations implicate a genetic basis of our gene activity results.

Genetic Dissection of Comorbidity With Smoking

SCZ patients have significantly higher rates of cigarettes smoking than the general population,[71,72] suggesting that common genetic risks may contribute to both phenotypes. Nicotine injection or exposure to smoke can increase *SLC6A3* mRNA levels significantly in rat substantia nigra.[73] Clinically, early exposure to secondhand smoke increased smoking risks in adulthood.[74,75] These preclinical and clinical findings suggest that high *SLC6A3* gene activity is associated with risks for substance abuse, including smoking. The high SLC6A3 activity in SCZ reported here may provide a genetic basis, together with the previous findings, for the high comorbidity of SCZ and cigarette smoking.

Strengths and Weaknesses of the Current Study

Two key requirements for an association study of unrelated individuals are controls for ancestry and phenotypic homogeneities. Less strict requirements mean that larger

sample sizes would be needed to have adequate power to detect association signals.[76] We used 2 methods, AIM and self-report (referring to parental ethnicities as well), to define Caucasian ethnicity and we used a "narrow" definition of the SCZ phenotype, excluding schizoaffective and non-SCZ-related psychiatric conditions in order to reduce phenotypic heterogeneity. The main limitation of this study is the modest sample size, which may be the reason for the relatively marginal adjusted *P*-values. Therefore, validation in larger samples is warranted. Another limitation is the relatively low marker density in the 18 kb promoter regions. Because of this, the imputation inferred only a very small fraction (3.4%–6.4%) of known polymorphisms in the 18-kb promoter regions and was unable to capture a smoother signal curve (see supplementary figure 3b) to make sure that there were no additional signal peaks. Future studies would benefit from larger sample sizes and denser markers in the promoter regions.

In summary, we have identified reduced *SLC6A3* activity and the T allele of rs1478435 as potential protective factors in SCZ. These findings support the role of reduced DA transmission in the pathophysiology of SCZ, particularly with respect to negative symptoms, which are thought to reflect reduced DA signaling in some areas of the SCZ brain and may help to explain the well-known comorbidity between SCZ and substance abuse.

Supplementary Material

Supplementary material is available at http://schizophreniabulletin.oxfordjournals.org.

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