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Letter to the Editor

Difficulty identifying spinocerebellar ataxia 17 from preceding psychiatric symptoms

SPINOCEREBELLAR ATAXIA (SCA) with preceding psychiatric symptoms has only ever been reported for SCA17.[1,2] SCA17, a rare autosomal dominant disorder, is characterized by various neurological symptoms and/or psychiatric symptoms. SCA17 with psychiatric symptoms is often diagnosed as a variety of psychiatric disorders such as schizophrenia and bipolar disorder before the onset of neurological symptoms.[1] SCA17 is a polyglutamine disease caused by expanded CAG/CAA repeats in the TATA box-binding protein (*TBP*) gene.[1] Here, we report two rare cases of undefined hereditary SCA other than SCA17 with preceding psychiatric symptoms.

A 65-year-old man had presented with hypomanic and depressive episodes on more than 10 occasions since the age of 18. He was diagnosed with bipolar disorder at the age of 45. The patient noticed progressive unsteadiness of gait from 58 years of age, with double vision and difficulties in fastening a button.

A 54-year-old man had exhibited social withdrawal since he was in high school. He complained of cenesthopathy and persecutory delusion since his 30s, and was diagnosed with schizophrenia. He complained of feeling vertigo since 47 years of age, and subsequently reported further symptoms, such as double vision, gait disturbance, dysphagia and slurred speech. There were past histories of paralytic ileus and aspiration pneumonia at 53 years of age. He had autonomic symptoms, such as mild orthostatic hypotension and severe constipation.

Both patients had limb and gait ataxia, intention tremor, saccades during slow pursuit, gaze nystagmus and convergent disturbance, and had mild rigidity only in the former patient. They had no other neurological symptoms. They had several biological relatives with SCA. The latter had relatives with progressive muscular dystrophy and schizophrenia. Brain magnetic resonance imaging indicated atrophy of the cerebellum (hemisphere and vermis) in both cases; additionally lower pons in the latter case. They were diagnosed with SCA in their late 50s. Mutation analysis failed to identify any mutations in well-known causative genes reported in Japan for SCA1, SCA2, DRPLA, SCA3 and SCA6 in the former patient. No abnormally expanded triplet repeats were identified in the *TBP* gene (SCA17) in both patients. Their mental state is relatively stable with medication (patient 1, paroxetine 40 mg/day and taltirelin hydrate 10 mg/day; patient 2, aripiprazole 12 mg/day), while their neurological symptoms and cerebral atrophy are gradually progressing. These patients met the diagnostic criteria for schizophrenia or bipolar disorder from DSM-IV and matched the criteria of SCA from the Japanese Ministry of Health, Labor and Welfare. Informed consent was obtained from both patients.

Because the presence of pre-existing psychiatric symptoms was consistent with SCA17,[1] we suspected that these symptoms were referable to SCA17, but SCA17 was excluded in both cases on genetic analysis. SCA is a heterogeneous syndrome with similar clinical phenotypes, such as ataxia and cerebellar atrophy, and is caused by different genetic abnormalities at different chromosomal loci. Some characteristic symptoms are considered to be useful to distinguish several subtypes of SCA. This approach failed to identify the subtype of SCA in the present patients. We suggest that it is difficult to identify SCA17 on the basis of preceding psychiatric symptoms.

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Brief Research Communication

No Association Between Tagging SNPs of SNARE Complex Genes (STX1A, VAMP2 and SNAP25) and Schizophrenia in a Japanese Population

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Abnormalities in neural connections and the neurotransmitter system appear to be involved in the pathophysiology of schizophrenia. The soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex, which consists of Syntaxin1A, vesicle-associated membrane protein 2 (VAMP2) and synaptosomal-associated protein 25 kDa (SNAP25), plays an important role in the neurotransmitter system, and is therefore an attractive place to search for candidate genes for schizophrenia. We conducted a two-stage genetic association analysis of Syntaxin1A (*STX1A*), *VAMP2* and *SNAP25* genes with schizophrenia (first-set screening samples: 377 cases and 377 controls, second-set confirmation samples: 657 cases and 527 controls). Based on the linkage disequilibrium, 40 SNPs (*STX1A*, 8 SNPs; *VAMP2*, 3 SNPs; *SNAP25*, 29 SNPs) were selected as 'tagging SNPs'. Only nominally significant associations of an SNP (rs12626080) and haplotype (rs363014 and rs12626080) in *SNAP25* were detected in the first-set screening scan. To validate this significance, we carried out a replication analysis of these SNP and haplotype associations in second-set samples with a denser set of markers (including five additional SNPs). However, these associations could not be confirmed in the second-set analysis. These results suggest that the SNARE complex-related genes do not play a major role in susceptibility to schizophrenia in the Japanese population.

KEY WORDS: Schizophrenia; SNARE complex; Syntaxin; VAMP; SNAP25

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There is growing evidence that the presynapse is involved with the pathophysiology of schizophrenia. Within the presynaptic area, neurotransmitters are released by synaptic vesicle exocytosis, and the regulation of this release is critical for neural function. The machinery for this release consists of several groups of proteins that work together as a functional unit, the soluble *N*-ethylmaleimide sensitive factor attachments receptor (SNARE) complex [Montecucco et al., 2005].

The SNARE complex consists of Syntaxin1A, vesicle-associated membrane protein 2 (VAMP2) and synaptosomal-associated protein 25 kDa (SNAP25) [Marz and Hanson, 2002], and it has been reported that alterations in the components in the SNARE complex may underlie the pathophysiology of schizophrenia. First, postmortem studies measuring the level of SNARE complex protein or its mRNA revealed specific brain region alternations in schizophrenia [Gabriel et al., 1997; Thompson et al., 1998; Young et al., 1998; Karson et al., 1999; Sokolov et al., 2000; Hemby et al., 2002; Honer et al., 2002; Halim et al., 2003; Thompson et al., 2003]. Second, genetic association studies showed a significant association between SNPs in the Syntaxin1A gene (*STX1A*) and schizophrenic patients from Portugal and Toronto [Wong et al., 2004]. In addition, a very recent report showed that SNPs in *SNAP25* were associated with schizophrenia in Irish high-density families [Fanous et al., 2007].

In this study, we investigated whether genetic polymorphisms within *STX1A* (7p11.23: OMIM *186590), *VAMP2* (17p13.1: OMIM *185881) and *SNAP25* (20p12-p11.2: OMIM *600322) were associated with schizophrenia in a Japanese population.

A first-set screening analysis was conducted with 377 schizophrenic patients (196 males and 181 females; mean age ± standard deviation (SD) 42.4 ± 14.8 years) and 377 healthy controls (212 males and 172 females; 35.9 ± 14.7 years). In a

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confirmation analysis a different panel of samples was used, consisting of 657 patients with schizophrenia (350 male and 307 female; 50.1 ± 14.4 years) and 527 controls (303 male and 224 female; 40.8 ± 15.3 years).

The patients were diagnosed according to DSM-IV criteria with the consensus of at least two experienced psychiatrists on the basis of unstructured interviews and a review of medical records. All healthy control subjects were also psychiatrically screened based on unstructured interviews. None of the subjects was known to be related to each other, and all were ethnically Japanese.

Written informed consent was obtained from each subject. This study was approved by the ethics committees at Fujita Health University, Nagoya University Graduate School of Medicine, Osaka University Graduate School of Medicine and Teikyo University School of Medicine.

After consulting the HapMap database (release#16.c.1, June 2005, www.hapmap.org, population: Japanese Tokyo: minor allele frequencies (MAFs) of more than 0.05 for *STX1A* and *VAMP2*, and 0.1 for *SNAP25*), 39 SNPs (*STX1A*, 7 SNPs; *VAMP2*, 3 SNPs; *SNAP25*, 29 SNPs) were selected as 'tagging SNPs' based on the criterion of an r^2 threshold greater than 0.8 in 'pair-wise tagging only' mode using the 'Tagger' program (Paul de Bakker, http://www/broad.mit.edu/mpg/tagger). For *STX1A*, since a previous report showed the positive association of an SNP in intron 7 [Wong et al., 2004], we included this SNP with the aforementioned 'tagging SNPs' for the association analysis. Overall, 40 SNPs were examined in this study (Supplementary Figures 1–3).

For denser mapping in the confirmation analysis, we added five SNPs around nominally significant SNPs or haplotypes detected in the first-set screening scan (rs610457, rs363013, rs363015, rs6039792 and rs363050).

For genotyping of these SNPs, a TaqMan assay (Applied Biosystems, CA), PCR-RFLP assay, and direct sequencing techniques were used. Detailed information is available in Supplementary Table 1. Genotype deviation from the Hardy–Weinberg equilibrium (HWE) was evaluated by chi-square test (SAS/Genetics, release 8.2, SAS Japan Inc., Tokyo, Japan). Marker-trait association was evaluated by a likelihood ratio test (allele-wise and haplotype-wise analyses) and χ^2-test (genotype-wise analysis). For exhaustive screening, we tested all one-marker (by conventional allele-wise analysis), two-marker, and three-marker haplotypes (and seven-marker haplotypes for second-set confirmation analysis) using the COCAPHASE 2.403 program [Dudbridge, 2003].

The power and sample size calculations were performed with a statistical program (http://biostat.mc.vanderbilt.edu/twiki/bin/view/main/powersamplesize). This significance threshold for all statistical tests was 0.05.

All genotype frequencies of each group were in HWE (data not shown). The LD structures examined in our control samples were almost the same as the one shown in HapMap database (Supplementary Figure 1–3).

The SNP (rs12626080: SNAP25-M8: $P = 0.0236$, uncorrected) in *SNAP25* and the haplotype constructed by M7 (rs363014) and M8 in *SNAP25* showed a nominally significant association with schizophrenia in the first-set screening samples (global $P = 0.0215$, uncorrected), although no association was detected with any tagging SNP in *STX1A* and *VAMP2*, including the SNP reported to be associated with schizophrenia in Caucasian samples [Wong et al., 2003] (Tables I and II and Supplementary Table 2).

To validate this nominal significance, we carried out a replication analysis using an independent set of samples. In this analysis, five additional SNPs were further included for denser mapping around M7 and M8 (rs610457, rs363013, rs363015, rs6039792 and rs363050: Supplementary Figure 4). However, this second-set confirmation analysis showed no

TABLE I. First-Set Association Analysis of Tagging SNPs in STX1A and VAMP2

Genes	Marker IDs		Distance to next SNP (bp)	N[a]		MAF[b]		*P*-Values			
				SCZ	CON	SCZ	CON	Genotype	(1) Window[c]	(2) Window	(3) Window
STX1A (minus strand)	SNP1	rs867500	0	375	375	0.204	0.217	0.754	0.527	0.947	
	SNP2	Intron7 SNP	3592	373	372	0.260	0.261	0.767	0.978	0.859	0.946
	SNP3	rs4363087	164	373	370	0.326	0.311	0.424	0.537	0.592	0.896
	SNP4	rs3793243	3151	375	377	0.419	0.386	0.304	0.196	0.553	0.775
	SNP5	rs875342	5751	375	370	0.228	0.238	0.211	0.653	0.781	0.698
	SNP6	rs6951030	6143	373	372	0.0563	0.0565	0.826	0.594	0.593	0.924
	SNP7	rs9654749	7145	376	374	0.483	0.469	0.460	0.602	0.869	0.872
	SNP8	rs2030921	785	376	377	0.249	0.240	0.728	0.696		
VAMP2 (minus strand)	m1	rs2278637	0	372	369	0.430	0.436	0.348	0.724	0.732	0.802
	m2	rs1061032	1981	377	377	0.399	0.403	0.750	0.975	0.694	
	m3	rs8067606	2800	375	371	0.425	0.431	0.490	0.765		

[a]N, number; SCZ, schizophrenia; CON, control.
[b]MAF, minor allele frequency.
[c]Identical as conventional allele-wise analysis.

TABLE II. First-Set Association Analysis of Tagging SNPs in SNAP25

Genes	Marker IDs		Distance to next SNP (bp)	N[a]		MAF[b]		P-Values			
				SCZ	CON	SCZ	CON	Genotype	(1) Window[c]	(2) Window	(3) Window
SNAP25	M1	rs6104567	0	377	370	0.263	0.242	0.298	0.341		
	M2	rs1889189	1,653	377	370	0.236	0.223	0.675	0.550	0.493	
	M3	rs3787303	11,662	377	376	0.312	0.300	0.306	0.630	0.830	0.825
	M4	rs2423487	4,347	374	368	0.171	0.171	0.146	0.978	0.874	0.721
	M5	rs363012	6,704	377	377	0.308	0.295	0.364	0.612	0.403	0.795
	M6	rs363039	697	375	368	0.432	0.397	0.128	0.173	0.552	0.364
	M7	rs363014	8,198	377	377	0.460	0.453	0.197	0.767	0.328	0.660
	M8	rs12626080	4,358	375	367	0.208	0.162	0.073	**0.0236**[d]	**0.0215**[d]	0.126
	M9	rs363052	2,374	375	369	0.163	0.159	0.177	0.866	0.0747	0.0882
	M10	rs363053	159	374	370	0.298	0.291	0.918	0.748	0.774	0.411
	M11	rs4813024	2,231	377	377	0.222	0.235	0.650	0.544	0.719	0.865
	M12	rs6074113	4,195	372	369	0.337	0.341	0.615	0.868	0.690	0.855
	M13	rs363022	383	374	369	0.394	0.413	0.950	0.457	0.509	0.643
	M14	rs362564	2,232	376	372	0.455	0.425	0.725	0.265	0.579	0.739
	M15	rs362547	513	372	376	0.222	0.184	0.380	0.0712	0.254	0.524
	M16	rs362567	952	377	376	0.144	0.129	0.259	0.379	0.0883	0.375
	M17	rs362570	773	375	368	0.351	0.331	0.572	0.414	0.615	0.134
	M18	rs362584	7,611	372	370	0.209	0.212	0.716	0.877	0.489	0.617
	M19	rs16991334	7,442	372	367	0.0970	0.0989	0.574	0.903	0.999	0.716
	M20	rs6039807	1,659	372	367	0.451	0.434	0.580	0.544	0.401	0.737
	M21	rs362995	13,463	377	377	0.269	0.248	0.764	0.361	0.601	0.891
	M22	rs363006	3,044	373	370	0.0134	0.0082	0.873	1	0.208	0.587
	M23	rs6108463	422	374	368	0.182	0.192	0.893	0.647	0.732	0.510
	M24	rs362988	865	374	370	0.379	0.401	0.325	0.391	0.679	0.689
	M25	rs6039820	657	377	376	0.400	0.373	0.253	0.286	0.526	0.788
	M26	rs6108464	1,923	377	376	0.401	0.405	0.892	889	0.612	0.807
	M27	rs3787283	468	376	375	0.460	0.465	0.614	0.850	0.942	0.548
	M28	rs3746544	2,666	372	367	0.260	0.249	0.746	0.634	0.642	0.862
	M29	rs6133852	3,876	377	377	0.237	0.206	0.176	0.156	0.437	0.676

[a]N, number; SCZ, schizophrenia; CON, control.
[b]MAF, minor allele frequency.
[c]Identical as conventional allele-wise analysis.
[d]Bold numbers represent significant *P*-values.

evidence of the significance of these markers (*P*-values for M7-M8 combination: 0.541: Supplementary Table 3). To increase the power, the genotypes of these five new SNPs in the first-set samples were determined and we then combined the samples (first-set and second-set samples), but again we could not detect an association in this explorative analysis (*P*-values for M7–M8 combination; 0.280: Table III and Supplementary Table 4).

This genetic two-stage case–control association study revealed no association between SNARE complex-related genes (*STX1A*, *VAMP2* and *SNAP25*) and schizophrenia in the Japanese population. Because postmortem studies showed a change in expression of SNARE complex genes (see Introduction), the most interesting variants of these genes are SNPs located in the promoter regions that might affect gene expression. To cover such regions, particularly the 5′ region of each gene, we applied the recently recommended 'gene-based' approach [Neale and Sham, 2004], in which it is important to include both the exon region and the flanking region. There is also emphasis on selecting genetic variants that adequately reflect the LD background in the targeted population (e.g., tagging SNPs). Our selection of tagging SNPs represented the all regions of these genes in the Japanese population, significantly reducing genotyping effort without much loss of power.

Moreover, we included confirmation analysis using an independent set of samples to check for Type I error, after significance was obtained in the screening samples. For *SNAP25*, an SNP and a two-marker haplotype were associated with schizophrenia in the first-set screening samples, but no significance could be seen in the larger second set, suggesting that the significance in the screening samples may have resulted from Type I error due to multiple testing or small sample size. We carried out power calculations and determined that our sample had sufficient power in the second-set analysis to detect association of 0.999 at $P < 0.05$, assuming an odds ratio of 1.69, which was shown in the first-set analysis of SNAP25-M8.

In addition, our sample size in the first-set screening analysis was large enough to deny Type II error in replicating the previous positive association of an SNP in *STX1A* intron 7 with schizophrenia in Caucasian samples [Wong et al., 2004]. The power was more than 0.997 at $P < 0.05$ when the odds ratio was set at 2.1, which is the estimated odds ratio of TDT in Wong's report [Wong et al., 2004]. One explanation for the different outcomes may be that *STX1A* susceptibility alleles were present in the Caucasian samples, but not in the Japanese population.

Although our sample size was large enough for replication of Wong's study, in general the odds ratios of common variants found to be associated with schizophrenia so far are less than 1.5. In this regard, a larger sample size might be required for conclusive results, since our sample size showed power surpassing 0.8 only when we set the odds ratio at more than 1.62.

With this statistical methodology, it is generally accepted that gene–gene interactions should be examined when a number of related genes are analyzed. We included explorative analysis to evaluate the interaction among these genes by multiple dimensionality reduction (MDR) [Hahn et al., 2003], but no interaction was detected (data not shown). In addition, we conducted MDR analysis for other genes related to SNARE complex genes, Complexin I and II (*CPLX I* and *CPLXII*), for which we previously found no association to schizophrenia [Kishi et al., 2006]. Again, no interaction could be detected in this analysis (data not shown).

There are numerous molecules related to the SNARE complex besides *CPLX* genes [Wang and Tang, 2006]. The most interesting molecule is dysbindin (*DTNBP1*: dustrobrevin-binding protein 1), for which there is evidence of an association

TABLE III. Confirmation Analysis Around the Nominally Significant SNPs Detected in First-Set Analysis

			N[a]		MAF[b]		*P*-Values						
Marker IDs		Distance to next SNP (bp)	SCZ	CON	SCZ	CON	Genotype	(1) Window[c]	(2) Window	(3) Window	(4) Window	(5) Window	(6) Window	(7) Window
	rs6104671	0	1,031 (656)	892 (527)	0.216 (0.196)	0.213 (0.217)	0.970 (0.468)	0.804 (0.200)	00.992 (0.956)					
	rs363013	1,661	1,031 (656)	892 (527)	0.011 (0.010)	0.011 (0.011)	0.962 (0.502)	0.872 (0.696)	0.657 (0.523)	0.146 (0.648)	0.183 (0.704)			
M7	rs363014	2,283	1,033 (656)	914 (537)	0.449 (0.444)	0.455 (0.422)	0.724 (0.155)	0.710 (0.280)	0.789 (0.659)	0.769 (0.493)	0.194 (0.706)	0.136 (0.691)	0.107 (0.525)	
	rs363015	5	1,031 (656)	892 (527)	0.060 (0.060)	0.062 (0.063)	0.540 (0.552)	0.733 (0.808)	0.192 (0.962)	0.195 (0.662)	0.142 (0.783)	0.142 (0.698)	0.107 (0.553)	0.076 (0.473)
	rs12826080	4,353	1,032 (657)	890 (523)	0.177 (0.160)	0.166 (0.168)	0.617 (0.861)	0.362 (0.582)	0.144 (0.925)	0.144 (0.925)	0.107 (0.560)			
M8	rs6039792	1,040	1,031 (656)	892 (527)	0.212 (0.191)	0.209 (0.212)	0.953 (0.468)	0.768 (0.204)	0.119 (0.884)	0.136 (0.761)	0.136 (0.761)			
	rs363050	165	1,031 (656)	892 (527)	0.225 (0.207)	0.209 (0.205)	0.511 (0.993)	0.246 (0.923)	0.056 (0.492)					

[a]N, number; SCZ, schizophrenia; CON, control.
[b]MAF, minor allele frequency.
[c]Identical as conventional allele-wise analysis.
[d]Numbers in parentheses indicate results from second set samples.

with schizophrenia, since recent studies showed that dysbindin regulates the expression of *SNAP25* [Numakawa et al., 2004]. Therefore, it will be essential to evaluate the other candidate genes related to SNARE complex genes for conclusive results.

With regard to interpretation of the results from this study, several limitations should be mentioned. Firstly, we did not perform mutation screening of these genes. Secondly, our samples were un-matched for age and gender between cases and controls, and were not assessed with the use of a standard structured interview. Therefore, detailed association analysis with mutation search in well-phenotyped samples will be essential in future study.

To conclude, our results provide no evidence that SNARE complex genes play a major role in susceptibility for schizophrenia in the Japanese population. Our results also imply that caution is needed in drawing conclusions about positive associations from small-sample case–control studies. We strongly suggest that two-stage genetic association analysis be conducted when positive results are found in screening samples.

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Behavioral abnormalities and dopamine reductions in *sdy* mutant mice with a deletion in *Dtnbp1*, a susceptibility gene for schizophrenia

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ABSTRACT

Genetic susceptibility plays an important role in the pathogenesis of schizophrenia. Genetic evidence for an association between the dysbindin-1 gene (*DTNBP1*: dystrobrevin binding protein 1) and schizophrenia has been repeatedly reported in various populations worldwide. Thus, we performed behavioral analyses on homozygous *sandy* (*sdy*) mice, which lack dysbindin-1 owing to a deletion in the *Dtnbp1* gene. Our results showed that *sdy* mice were less active and spent less time in the center of an open field apparatus. Consistent with the latter observation, *sdy* mice also displayed evidence of heightened anxiety-like response and deficits in social interaction. Compared to wild-type mice, *sdy* mice displayed lower levels of dopamine, but not glutamate, in the cerebral cortex, hippocampus, and hypothalamus. These findings indicate that *sdy* mice display a number of behavioral abnormalities associated with schizophrenia and suggest that these abnormalities may be mediated by reductions in forebrain dopamine transmission.

Schizophrenia is characterized by psychosis and profound disturbances of cognition, emotion, and social functioning. The dysbindin-1 gene (*DTNBP1*: dystrobrevin binding protein 1), have recently been identified as a susceptibility gene for schizophrenia [1,2]. In studies on postmortem brain tissue, decreased expression levels of dysbindin-1 protein [3] and mRNA [4] have been shown in patients with schizophrenia compared with controls. Chronic treatment of mice with antipsychotics did not affect the expression levels of dysbindin-1 protein and mRNA in their brains [3,5], suggesting that prior evidence of lower levels of dysbindin-1 protein and mRNA in the postmortem brains of schizophrenics is not likely to be a simple artifact of antemortem drug treatment. These data indicate that the dysbindin-1 gene may confer susceptibility to schizophrenia through reduced expression.

Dysbindin-1 is relatively ubiquitously expressed in neuronal cell bodies in most parts of the brain and in primary dendrites of those neurons and is concentrated in axon terminals of some areas such as the hippocampal formation, striatum, substantia nigra, and cerebellum [3,6]. Previous studies reported that down-regulation of endogenous dysbindin-1 by small interfering RNA (siRNA) resulted in a reduction in the release of glutamate from primary cultured neurons [7] and an increase in the release of dopamine from PC12 cells [8]. These results suggest possible roles for the dysbindin-1 gene in glutamatergic and dopaminergic systems related to the pathophysiology of schizophrenia [9].

To investigate the functions of dysbindin-1 *in vivo*, we analyzed *sandy* (*sdy*) mutant mice, which express no dysbindin-1 protein owing to a deletion in the dysbindin-1 gene [10]. *Sdy* is a mutant mouse with diluted pigmentation that arose spontaneously in the DBA/2J inbred mouse strain and has simultaneous defects in melanosomes, lysosomes and platelet dense granules [11]. Thus, we first performed several behavioral analyses and measured dopamine and glutamate contents in several brain regions in *sdy* mice.

Materials and methods

Animals. We obtained *sdy* mice from the Jackson Laboratory (Bar Harbor, ME). *Sdy* mice have an autosomal recessive coat color

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mutation that arose spontaneously in the inbred DBA/2J strain. *Sdy* mice have a large deletion in the dysbindin-1 gene, from nucleotide 3701 of intron 5 to nucleotide 12377 of intron 7, and this deletion results in essentially total loss of dysbindin-1 [10]. Both *sdy* mice and wild-type mice derived from heterozygote crossings were used in all experiments. To minimize the influence of cage environment, they were bred under the same conditions after weaning at 3 weeks of age. The genotypes of mice were identified by their coat color and genomic PCR. Primers i6_f (5′-GCACTCAGGA GACCATGACA-3′) and i6_r (5′-GGTTGACACTCTTGCGGAAT-3′) amplified a region in intron 6, and produced 305 bp PCR products from normal DNA. Primers i5, designed for intron 5 (5′-CCTAGCCCC TCAGGAATTGT-3′), and i7, designed for intron 7 (5′-GGGAATGGG GTCTTAATGGT-3′), amplified 733 bp PCR products from mutant DNA. The genomic sequences of these PCR products were confirmed by sequence analysis. The experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of the National Institute of Neuroscience, Japan.

Experimental design for behavioral tests. All behavioral tests were carried out as described previously [12] using male mice that were 6–9 weeks old (*sdy* mice: $n = 119$; wild-type mice: $n = 120$). We used different batches of mice for each behavioral test. Mice were housed four per cage in a temperature-controlled room under a 12 h light–dark cycle (light on at 8:00 a.m.) with ad libitum access to food and water. All behavioral tests were performed between 10:00 a.m. and 7:00 p.m. After each trial, all apparatus were cleaned with water to prevent a bias based on olfactory cues.

Open field test. Locomotor activity was measured using an open field test. Activity was recorded during the first exposure to the open field apparatus (50 × 50 × 40 cm; O'Hara & Co., Tokyo, Japan). The illumination level was 40 lux at the floor of the open field. The field was divided by software (see below) into 16 equal-sized squares containing 4 central areas and 12 peripheral areas. Time spent in the central area defined as [stay time in center (%) = time spent in central areas/objective time for analysis (15 min or 30 min)] and the number of fecal boli were recorded. Data were collected for 30 min. Data acquisition and analysis were performed automatically, using Image OF software (see Behavioral data analysis).

Elevated plus maze test. The elevated plus maze consisted of two black plastic walkways (25 × 5 cm) 34 cm above the floor intersecting at right angles with one of the walkways having 15 cm high wall. To prevent animals from falling off the apparatus, 3-mm-high ledges were provided on the open arms (O'Hara & Co., Tokyo, Japan). A mouse was placed in the central square of the maze (5 × 5 cm), facing one of the enclosed arms. The behavior was recorded during a 20 min test session, because mice entered into the open arms a few times (*sdy* mice; 2.1 ± 0.7, wild-type mice; 3.6 ± 0.8) for a 10 min test session. The illumination level was 40 lux at the central square of the maze. For data analysis, we used the following four measures: the number of entries into open arms, the total number of arm entries, the time spent on the open arms and the total distance traveled. Data acquisition and analysis were performed automatically, using Image EP software (see Behavioral data analysis).

Social interaction test. A pair of mice was placed simultaneously at opposing corners in the open field apparatus (50 × 50 × 40 cm; O'Hara & Co., Tokyo, Japan) and allowed to explore freely for 30 min. The pair of mice tested was composed of the same genotype (*sdy–sdy* pair or wild–wild pair) and had been housed in the same environmental conditions, but in different cages. The illumination level was 40 lux at the floor of the open field. Mice were familiar with the test arena by placing them singly in the apparatus, under the same light level (40 lux), for a 30 min period at least 2 days preceding the test. Total duration of contacts, the number of contacts and total distance traveled were measured. Analysis was performed automatically using Image SI software (see Behavioral data analysis).

Behavioral data analysis. Behavioral data from the open field tests, elevated plus maze tests and social interaction tests were automatically analyzed as described previously [12]. Briefly, behaviors were monitored by a color charged-coupled device camera (Watec Co., Ltd., Yamagata, Japan) that was connected to a Macintosh computer. We used apparatuses with black-colored floors to detect behaviors of mice, because coat colors of mice were whitish (*sdy*) and dilute brown (wild-type). Images were captured at one or two frames per second. The applications used for the behavioral studies (Image OF, Image EP, Image SI, O'Hara & Co., Tokyo, Japan) were run using a Macintosh computer. These modified applications were based on the public domain NIH Image program developed at the U.S. National Institute of Mental Health.

Neurotransmitter measurements by HPLC-fluorometry. We measured the dopamine and glutamate levels in intact brain with a different batch of mice used for behavioral tests. Mice (male: 8–12 weeks old) were sacrificed by decapitation, and the decapitated heads were dropped directly into ice-cold water for 1 s to prevent degradation of neurotransmitters. Brains were removed from the calvarium and put on a chilled aluminum board. The brain was dissected into ten regions (olfactory bulb, OB; frontal pole cortex, FPC; non-frontal cerebral cortex, NF CX; cerebellum, CB; hippocampal formation, HF; striatum, ST; midbrain, MB; lower brainstem, LB; thalamus, TH; hypothalamus, HT) according to a previously reported method [13] with slight modification. Each block of brain tissue was put into a pre-weighed sampling tube. Brain tissues were homogenized in 9 volumes of 2% perchloric acid (PCA) solution (Katayama Chemical Industries Co., Ltd., Japan) including 1 mM EDTA-Na_2 and 1 mM $Na_2S_2O_5$ using a sonicator for 5–10 s. Homogenates were centrifuged at 10,000*g* for 30 min. The level of dopamine in the supernatant was determined by a fully automated HPLC system (Model HLC-725CA Catecholamine analyzer, Tosoh, Tokyo, Japan) using a diphenylethylenediamine condensation method [14]; glutamate levels were measured by a pre-label HPLC-fluorometric method [15].

Statistical analysis. Statistical analysis was conducted using SPSS 11.0J for Windows (SPSS Japan Inc., Tokyo, Japan). Data were analyzed by a two-tailed *t*-test unless otherwise noted. Fisher's exact test was used to compare *sdy* mice with wild-type mice for general health (physical characteristics, sensory/motor reflexes and the motor test). A repeated measures analysis of variance (ANOVA) was used to analyze differences in the time course of distance traveled in the open field test. For measurements of neurotransmitters, statistical significance was analyzed using the Student's *t*-test. All *p*-values reported are two-tailed. Statistical significance was defined as $p < 0.05$.

Results

General characteristics

There was no significant difference in body weight, physical characteristics (whiskers and fur), sensory-motor reflexes (eye blink, ear twitch, whisker response and righting reflex) or neuromuscular strength between *sdy* mice and wild-type mice (Supplementary Table S1). *Sdy* mice were more sensitive to 120DB auditory stimulation than wild-type mice, however, there was no significant difference in PPI between *sdy* and wild-type mice (data not shown).

Locomotor activity in a novel environment

Sdy mice showed a pronounced decrease in locomotor activity in the open field test compared with wild-type mice (Fig. 1A, geno-

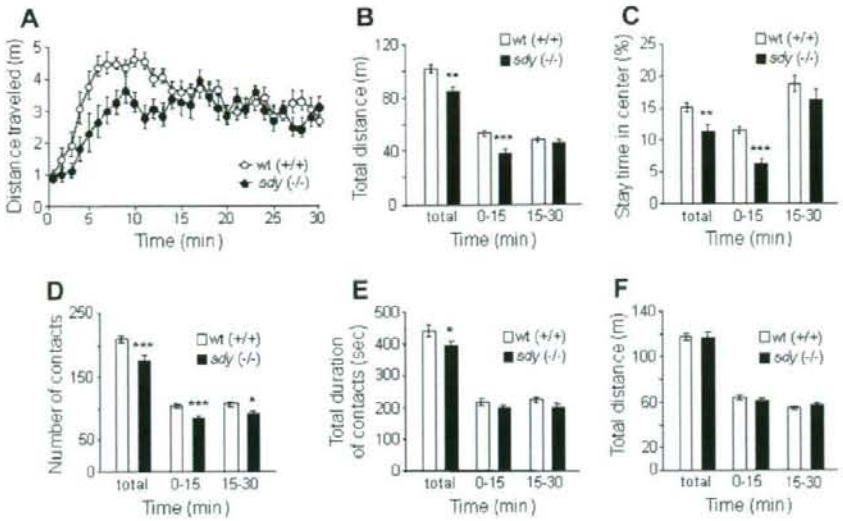

Fig. 1. Open field test with *sdy* mice (A–C). Time course of distance traveled (A), total distance traveled (B) and time spent in the central area (C), are shown (*sdy* mice: $n = 18$, wild-type mice: $n = 21$). Social interaction test in *sdy* mice (D–F). The total number of contacts (D), total duration of contacts (E) and distance traveled (F), in the social interaction test, are shown (pairs of *sdy* mice: $n = 22$, pairs of wild-type mice: $n = 21$). Data represent means ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared with wild-type mice.

type effect, $F(1,37) = 12.3$, $p = 0.001$). This hypoactivity phenotype was notable during the first half of the test period (Fig. 1B, total: $t(37) = 3.51$, $p = 0.001$, 0–15 min: $t(37) = 3.82$, $p < 0.001$, 15–30 min: $t(37) = 0.68$, $p = 0.50$). We then compared time spent in the center of the open field apparatus between *sdy* and wild-type mice. As shown in Fig. 1C, *sdy* mice spent significantly less time in the center (total: $t(37) = 2.99$, $p = 0.005$, 0–15 min: $t(37) = 5.26$, $p < 0.001$, 15–30 min: $t(37) = 1.19$, $p = 0.24$). There was no significant difference in the number of fecal boli during the open field test between *sdy* and wild-type mice (*sdy* mice: 8.0 ± 0.6, wild-type mice: 9.3 ± 1.0, $t(37) = 1.15$, $p = 0.26$).

Social interaction test

In the social interaction test, *sdy* mice showed a significant decrease in the number of social contacts compared with wild-type mice (Fig. 1D, total: $t(41) = 3.57$, $p < 0.001$, 0–15 min: $t(41) = 3.87$, $p < 0.001$, 15–30 min: $t(41) = 2.65$, $p = 0.012$). The total duration of contacts was also decreased in *sdy* mice during a 30 min test session (Fig. 1E, total: $t(41) = 2.05$, $p = 0.047$, 0–15 min: $t(41) = 1.49$, $p = 0.14$, 15–30 min: $t(41) = 1.71$, $p = 0.095$). The total distance traveled was not significantly different between the two genotypes (Fig. 1F, total: $t(41) = 0.24$, $p = 0.81$, 0–15 min: $t(41) = 1.01$, $p = 0.32$, 15–30 min: $t(41) = 0.62$, $p = 0.54$).

Elevated plus maze test

In the elevated plus maze test, *sdy* mice exhibited a trend toward a reduced number of entries into the open arms during a 20 min test session compared with wild-type mice (Fig. 2A, total: $t(32) = 2.00$, $p = 0.054$). During the second half of the test period, the number of entries into the open arms was significantly lower in *sdy* mice than wild-type mice (Fig. 2A), 0–10 min: $t(32) = 1.41$, $p = 0.17$, 10–20 min: $t(32) = 2.11$, $p = 0.042$). *Sdy* mice also showed a significant decrease in the total number of arm entries during the second half of the test period and across the entire test period compared with wild-type mice (Fig. 2B, total: $t(32) = 2.35$, $p = 0.025$, 0–10 min: $t(32) = 2.00$, $p = 0.054$, 10–20 min: $t(32) = 2.19$, $p = 0.036$).

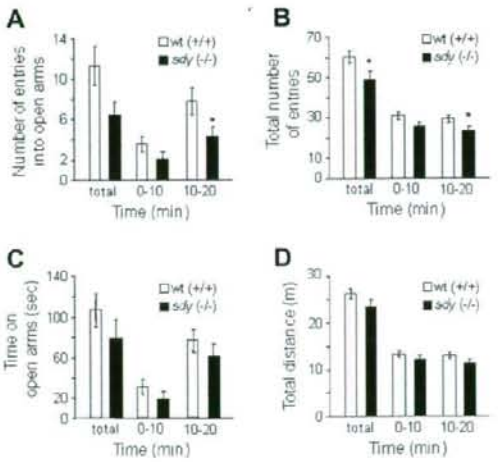

Fig. 2. Elevated plus maze test in *sdy* mice. The number of open arm entries (A), total number of arm entries (B), time on open arms (C), and distance traveled (D), are shown (*sdy* mice: $n = 16$, wild-type mice: $n = 18$). Data represent means ± SEM. $^{*}p < 0.05$, compared with wild-type mice.

There were no significant differences in the amount of time spent on the open arms (Fig. 2C, total: $t(32) = 1.13$, $p = 0.27$, 0–10 min: $t(32) = 1.18$, $p = 0.25$, 10–20 min: $t(32) = 0.94$, $p = 0.35$) or in the total distance traveled (Fig. 2D, total: $t(32) = 1.56$, $p = 0.13$, 0–10 min: $t(32) = 1.09$, $p = 0.28$, 10–20 min: $t(32) = 1.69$, $p = 0.10$) between *sdy* mice and wild-type mice.

Dopamine and glutamate contents in the brain

Dopamine content was significantly reduced in three brain regions of *sdy* mice compared with wild-type mice: non-frontal cerebral cortex (*sdy* mice: 0.114 nmol/g, wild-type mice: 0.222 nmol/g,

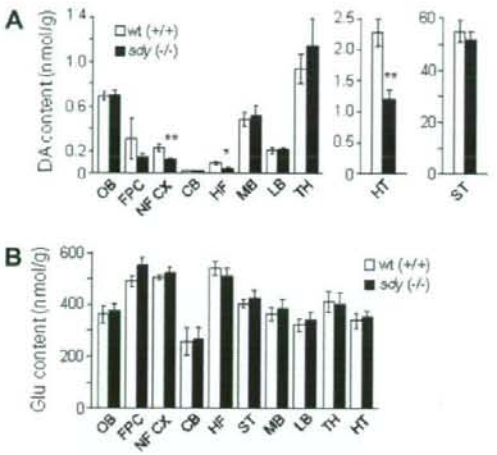

Fig. 3. Dopamine and glutamate content in the brains of *sdy* mice. Dopamine (DA) content (A) and glutamate (Glu) content (B) are shown (dopamine: $n = 4$–8, glutamate: $n = 11$). Olfactory bulb, OB; frontal pole cortex, FPC; non-frontal cerebral cortex, NF CX; cerebellum, CB; hippocampal formation, HF; striatum, ST; midbrain, MB; lower brainstem, LB; thalamus, TH; hypothalamus, HT. Data represent means ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with wild-type mice.

$p = 0.002$), hippocampal formation (*sdy* mice: 0.0392 nmol/g, wild-type mice: 0.0822 nmol/g, $p = 0.03$), and hypothalamus (*sdy* mice: 1.17 nmol/g, wild-type mice: 2.26 nmol/g, $p = 0.007$) (Fig. 3A). However, no significant difference in glutamate content was detected between *sdy* and wild-type mice in the brain areas examined (Fig. 3B).

Discussion

Several schizophrenia-related behaviors in rodents, such as hyperactivity, deficits in PPI, locomotor response to antipsychotics, disturbance in social interactions, and cognitive deficits, have commonly been observed in previous animal models for schizophrenia [16]. We report here that *sdy* mice, which express no dysbindin-1, show some, but not all, of these abnormalities.

In the open field test, *sdy* mice exhibited decreased locomotor activity during the first half of the test period compared with wild-type mice, and did not show habituation in a novel environment (Fig. 1A and B). It is unlikely that these abnormalities are due to a loss of motor function or general activity, which could be detected by the open field test, because of no differences in locomotor activity during the second half of the test or in neuromuscular strength between the two groups of animals. It could be caused by reduced adaptation, motivation to explore, and/or enhanced anxiety-like response in a novel environment. Indeed, *sdy* mice spent significantly less time in the center of the open field apparatus than wild-type mice (Fig. 1C), which is associated with anxiety-like response [17]. In addition, *sdy* mice showed a decrease in the number of entries into open arms and in the total number of arm entries in the elevated plus maze test (Fig. 2A and B), suggesting enhanced anxiety-related behavior in *sdy* mice. In the social interaction test, *sdy* mice showed reductions in the number of contacts and in the total duration of contacts without hypoactivity (Fig. 1D–F). The decreased social interactions may be caused largely by the proposed anxiogenic-like phenotype of the *sdy* mice and possibly by the reduced exploration. In rodents, reduced contacts with unfamiliar partners are indicative of social withdrawal, a clinical aspect of schizophrenia [16], suggesting a social withdrawal phenotype in *sdy* mice. Previous study demonstrated that retinal melanosomes were deficient in *sdy* mice [10]. As we did not examine the visual acuity in *sdy* mice, there is a possibility that reduced visual acuity resulting from retinal defects affects multiple behavioral parameters in this study. Increased locomotor activity, which is observed in most animal models of schizophrenia, is considered to be a positive symptom of schizophrenia, like delusions and hallucinations. However, *sdy* mice showed less activity, which is unique in an animal model for schizophrenia. This phenotype could be due to a decreased motivation to explore, which might be related to the negative symptoms of schizophrenia (blunted affect, decreased motivation, and social withdrawal). Other phenotypes such as less time in the center of the open field apparatus, abnormal response in elevated plus maze, and decreased social interactions could also be related to reduced exploratory tendencies. Indeed, two recent studies show a significant association between risk haplotypes of the dysbindin-1 gene and negative symptoms in patients with schizophrenia [18,19]. These data imply that *sdy* mice, which exhibit reduced exploratory activity, heightened anxiety-like response and deficits in social interaction, could be a potential genetic model for negative symptom endophenotypes of schizophrenia.

Decreased dopamine content in three brain regions of *sdy* mice measured by HPLC-fluorometry in the present study (Fig. 3A) was consistent with dopamine reduction measured by HPLC with an electrochemical detection in the previous study [20]. Recent studies reported that reduced dysbindin-1 protein by *DTNBP1* siRNA transfection increased surface expression of dopamine D2 receptor (DRD2) and blocked dopamine-induced internalization of DRD2 in SH-SY5Y cells [21], and that dopamine release was increased by siRNA-mediated silencing of dysbindin-1 protein in PC12 cells [8]. These results suggest that the lack of dysbindin-1 causes an imbalance of the dopaminergic system. As DRD2 mutant mice show decreased activity [22,23], decreased locomotor activity in *sdy* mice could be due to the abnormal regulation of dopaminergic system by lack of dysbindin-1 protein. As glutamate content in *sdy* mouse brain was not altered, behavioral abnormalities in *sdy* mice could be related to the dopaminergic system rather than the glutamatergic system.

This new genetic mouse model could shed light on the etiology of schizophrenia and lead us to new hypotheses, novel diagnostic tools, and more effective therapies for the disorder.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.06.016.

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Failure to replicate the association between *NRG1* and schizophrenia using Japanese large sample

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Abstract

Systematic linkage disequilibrium (LD) mapping of 8p12–21 in the Icelandic population identified neuregulin 1 (*NRG1*) as a prime candidate gene for schizophrenia. However, results of replication studies have been inconsistent, and no large sample analyses have been reported. Therefore, we designed this study with the aim of assessing this putative association between schizophrenia and *NRG1* (especially HAP_{ICE} region and exon region) using a gene-based association approach in the Japanese population.

This study was a two-stage association analysis with a different panel of samples, in which the significant association found in the first-set screening samples (1126 cases and 1022 controls) was further assessed in the confirmation samples (1262 cases and 1172 controls, and 166 trio samples). In the first-set scan, 60 SNPs (49 tagging SNPs from HapMap database, four SNPs from other papers, and seven SNPs detected in the mutation scan) were examined.

One haplotype showed a significant association in the first-set screening samples (Global *P*-value = 0.0244, uncorrected). However, we could not replicate this association in the following independent confirmation samples. Moreover, we could not find sufficient evidence for association of the haplotype identified as being significant in the first-set samples by imputing ungenotyped SNPs from HapMap database.

Abbreviations: NRG1, neuregulin 1; SNP, single nucleotide polymorphism; GGF2, glial growth factor 2; LD, linkage disequilibrium; dHPLC, denaturing high performance liquid chromatography; MAF, minor allele frequency; TDT, transmission disequilibrium test; UTR, untranslated region.

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These results indicate that the positionally and functionally attractive regions of *NRG1* are unlikely to contribute to susceptibility to schizophrenia in the Japanese population. Moreover, the nature of our results support that two-stage analysis with large sample size is appropriate to examine the susceptibility genes for common diseases.

Keywords: Schizophrenia; Neuregulin 1; Association study; False positive; Linkage disequilibrium

1. Introduction

Schizophrenia is a common psychiatric disorder with a lifetime prevalence of 1% worldwide. Family, twin and adoption studies show conclusive evidence of a substantial genetic component in this disorder. Progress towards detecting these genetic elements is now being made (Harrison and Weinberger, 2005).

The neuregulin 1 gene (*NRG1*) was first reported to be a prime candidate gene for schizophrenia in the Icelandic population (Stefansson et al., 2002). The significant association of a haplotype was detected in the 5′-region of glial growth factor 2 (GGF2) isoforms, and this at-risk haplotype, consisting of five single nucleotide polymorphisms (SNPs) and two microsatellites, was named as HAP_{ICE}. Several subsequent studies provided the following evidence to support this association with schizophrenia.

Firstly, the location of this gene corresponds to the linkage regions for schizophrenia (8p12–21, OMIM: SCZ5), which were identified by recent meta-analyses of genome-wide linkage studies (Badner and Gershon 2002; Lewis et al., 2003). Secondly, recent evidence suggests that mutation within the *NRG1* region might give rise to functional alterations that are in line with the neurodevelopmental hypothesis and glutamate/GABA hypothesis of schizophrenia (Corfas et al., 2004).

Thirdly, several independent association studies have replicated the original significant association found by Stefansson et al. (2002). However, the results of replication studies using the identical number or fewer sets of markers have been inconsistent. Thus, while some research groups did not report any association (Iwata et al., 2004), other studies showed a positive association but showed different 'at-risk' haplotypes to be associated with schizophrenia (Harrison and Law, 2006).

These inconsistent results could stem from the possibility that *NRG1* is not involved in the etiology of schizophrenia in all populations. However, this inconsistency could be a consequence of the unique structure of the human genome. In other words, differences in linkage disequilibrium (LD) among populations may also be responsible for the differences in the results, and the negative findings may only indicate a failure to reflect the actual predisposing variants due to the differences in populations.

Therefore, gene-wide (or region-wide) replication analysis based on LD pattern within the *NRG1* region is essential to detect an association in a certain population setting (Neale and Sham, 2004). In such analyses, particular attention should be paid to selection of genetic variants which adequately reflect the LD background in the targeted population (e.g. tagging SNPs).

Although the above-mentioned LD-based association analysis is based on the common disease–common variant hypothesis, one study reported an association between *NRG1* and schizophrenia from the standpoint of the common disease–rare variant hypothesis (Walss-Bass et al., 2006). The authors scanned the whole exon region, detected a non-synonymous SNP in exon 11, and showed a significant association of this SNP with schizophrenia. Detection of rare but potent functional variants relies on large mutation scan samples; however, such rare variants may also differ among populations (Pritchard, 2001).

Thus, in this study, we first focused on two attractive regions: the 5′ regions of GGF2, where the original study showed the association (henceforth referred to as 'HAP_{ICE} region') and the exon region (henceforth referred to as 'exon region'). In the exon region, prior to association analysis of tagging SNPs, we performed a mutation scan in order to detect the existence of possible potent functional variants in the ethnic samples. In addition, this study was a two-stage association analysis with a different panel of samples, in which the significant association in the first-set screening samples (1126 cases and 1022 controls) was further assessed in confirmation samples (1262 cases, 1172 controls, and 166 trio samples). This approach was adopted in order to avoid the possibility of type I or type II error.

2. Methods and materials

2.1. Subjects

Two independent sample sets were used in this study. For the first-set screening analysis, 1126 patients with schizophrenia (627 male and 499 female; mean age ± standard deviation (SD) 47.0 ± 15.3 years) and 1022

healthy controls (530 male and 492 female; 38.8± 14.5 years) were examined. Confirmation analysis was conducted with three samples consisting of: (a) 1262 patients with schizophrenia (662 male and 600 female; 49.1±14.5 years) (b) 1172 controls (576 male and 596 female; 41.7±14.3 years), and (c) 166 family trios samples (of the patients, 91 male and 75 female; 30.0± 8.3 years).

The subjects for mutation search were 96 patients with schizophrenia. These subjects were also included in the first-set samples. 385 cases and 336 controls in the first-set samples, and 349 cases (including 84 cases from family samples) and 424 controls in confirmation samples are identical to those in our previous report (Iwata et al., 2004) and Fukui et al.'s (2006) report, respectively.

Characterization details and psychiatric assessment of these subjects were as follows. The patients were diagnosed according to DSM-IV criteria consensus of at least two experienced psychiatrists on the basis of unstructured interviews and review of medical records. All healthy controls were also psychiatrically screened based on unstructured interviews. All subjects were ethnically Japanese.

After the study had been described to subjects, written informed consent was requested from each. This study was approved by the ethics committees at Fujita Health University, Teikyo University, Okayama University, Osaka University, Niigata University and Nagoya University Graduate School of Medicine.

2.2. Mutation scan

We performed denaturing high performance liquid chromatography (dHPLC) analysis, details of which can be seen in a previous paper (Ikeda et al., 2005). Primer sequences were designed in accordance with another report (Walss-Bass et al., 2006).

2.3. Tagging SNP selection

We included the three signal SNPs (SNP8NRG221533, SNP8NRG241930 and SNP8NRG243177) from the report of Stefansson et al. (2002) (we excluded SNP8NRG221132 and SNP8NRG433E1006 from the first-set analysis due to low minor allele frequencies (MAFs) in the Japanese population), one positive SNP from the report of Walss-Bass et al. (2006), and SNPs we detected in the mutation scan. Next we consulted the HapMap database (release#19, population: Japanese in Tokyo (JPT), MAF: more than 0.05). In this step, we determined the boundaries of the 'HAP_{ICE} regions' that cover 5′ regions including 19,425 bp and 155,564 bp downstream (3′) from the significant SNPs (SNP8NRG221132 and SNP8NRG433E1006, respectively) in Stefansson's report (Table 1 and Supplementary Fig. 1) (Stefansson et al., 2002), and of the 'exon regions' that cover 5′ regions including 120,576 bp from the first exon and 3510 bp downstream 3′ from the last exon (GenBank accession No. NT_007995: Table 2 and Supplementary Fig. 2). Then fifteen and thirty-four 'tagging SNPs' for the HAP_{ICE} regions and exon regions, respectively were selected with the criterion of an r^2 threshold greater than 0.8 in 'Aggressive tagging: use 2- and 3-markers haplotypes' mode of the 'Tagger' program (de Bakker et al., 2005), a function of HAPLOVIEW software (Barrett et al., 2005).

2.4. SNP genotyping

All SNPs were genotyped by TaqMan assay (Applied Biosystems Japan Ltd, Tokyo).

The genotyping of C#5, C#6, C#7 (which were positive SNPs in the first-set screening analysis) was done with 768 randomly selected samples (384 cases and 384 control subjects) with direct sequencing to check for genotyping error. Detailed information including primer sequences of custom TaqMan SNP genotyping assays can be seen in Supplementary Tables 1 and 2.

Table 1
First-set case control analysis of HAP_{ICE} region

Markers	SNP ID	*P*-values		
		1-window	2-windows	3-windows
HAP_{ICE}#1	rs12674974	.0794		
HAP_{ICE}#2	rs4513929	.846	.181	
HAP_{ICE}#3	SNP8NRG221533	.188	.384	.196
HAP_{ICE}#4	rs10096573	.200	.397	.620
HAP_{ICE}#5	rs4733263	.310	.414	.462
HAP_{ICE}#6	rs4733263	.274	.616	.267
HAP_{ICE}#7	SNP8NRG241930	.724	.399	.578
HAP_{ICE}#8	SNP8NRG243177	.288	.113	.326
HAP_{ICE}#9	rs4733267	.769	.520	.492
HAP_{ICE}#10	rs13277456	.862	.889	.190
HAP_{ICE}#11	rs13274954	.457	.736	.847
HAP_{ICE}#12	rs12677942	.312	.670	.255
HAP_{ICE}#13	rs4403369	.0803	.271	.128
HAP_{ICE}#14	rs4566990	.625	.268	.548
HAP_{ICE}#15	rs13270788	.541	.628	.525
HAP_{ICE}#16	rs1503491	.813	.730	.699
HAP_{ICE}#17	rs2202262	.704	.866	.0960
HAP_{ICE}#18	rs10087212	.682	.324	.0653
HAP_{ICE}#4–#5		.414		
HAP_{ICE}#14–#16		.247		
HAP_{ICE}#15–#16		.730		

Table 2
First-set case control analysis of exon region

Markers	SNP ID	*P*-values[a]		
		1-window	2-windows	3-windows
C#1	rs10503915	.116		
			.0603	
C#2	rs7016691	.231		.349
			.371	
C#3	rs11782671	.472		.296
			.474	
C#4	rs10103930	.168		.508
			.322	
C#5	rs10503917	.699		.0935
			.628	
C#6	rs10107065	.765		**.0244**
			.138	
C#7	rs6468118	.138		.174
			.154	
C#8	rs7000590	.0939		.158
			.107	
MS1	rs7820838	.110		.181
			.142	
MS2	rs7834206	.149		.145
			.0879	
C#9	rs4236709	.0786		.352
			.187	
C#10	rs13260545	.0994		.403
			.248	
C#11	rs4316112	.948		.0984
			.144	
C#12	rs2439305	.196		.132
			.130	
C#13	rs7826814	.715		.129
			.851	
C#14	rs2466064	.690		.436
			.313	
MS3	rs3924999	.162		.0699
			.113	
C#15	rs10954864	.803		.602
			.969	
C#16	rs2439281	.965		.301
			.0725	
C#17	rs9642729	.0680		.137
			.0988	
C#18	rs12547858	.0801		.523
			.457	
C#19	rs10098373	.801		.654
			.835	
C#20	rs10095694	.380		.872
			.727	
MS4	rs3735774	.762		.718
			.727	
C#21	rs2466058	.372		.587
			.526	
C#22	rs2466052	.379		.509
			.286	
C#23	rs2466046	.187		.431
			.372	
C#24	rs10503923	.546		.203
			.473	
C#25	rs2466084	.310		.197
			.551	
C#26	rs2976515	.253		.563
			.654	
C#27	rs4445183	.702		.500
			.484	
C#28	rs2919377	.151		.455
			.341	
C#29	rs2919375	.819		.182
			.222	
MS5	rs3735776	.740		.129
			.758	
C#30	rs7007436	.711		.866
			.815	
C#31	rs3757934	.758		.562
			.421	
MS7	rs4733376	.379		.357
			.336	
C#32	rs4360253	.357		.789
			.893	
C#33	rs7005288	.864		.738
			.812	
C#34	rs6992642	.569		
MS6 (C#24–#30)[b]	rs17731664	.772		
C#5–#11–#14		1.00		
C#5–#14		.180		
C#16–#27		.751		
C#23–#26–#28		.245		

[a] Bold number represents significant *P*-value.
[b] MS6 could be represented by the haplotypes constructed by C#24–30.

2.5. Statistical methods for conventional association analysis

In the case–control samples, the marker–trait association was evaluated with the χ^2 test in allele- and genotype-wise analyses. Haplotype frequencies were estimated in a 2- to 3-marker sliding window fashion by EM algorithm and Log likelihood ratio tests were performed for Global *P*-values with COCAPHASE program version 3.06 (Dudbridge, 2003). In the family samples, the transmission disequilibrium test (TDT) and 3-marker haplotype analyses were performed with the TDTPHASE program version 3.06 (Dudbridge, 2003). In these haplotype-wise analyses, rare haplotypes (less than 0.05) of cases and controls were excluded from the association analysis in order to provide greater sensitivity and accuracy.

The significance level was set at $P<0.05$.

2.6. Imputation of ungenotyped SNPs

Our conventional haplotype-wise analysis was done in a sliding window fashion, since our selection for tagging SNPs was not based on the haplotype block concept. Although this type of haplotype-wise analysis does not adapt to the degree of LD, so that it is unclear which markers should be considered jointly, it results in a higher level of statistical power since it can reflect unknown SNPs that were not included in the analysis. Considering this, we included a recently developed method, imputation, to test for any SNPs that reflect the significant haplotypes (Marchini et al., 2007). The IMPUTE program imputes the genotypic distribution of un-observed SNPs using observed SNP information (60 SNPs used in the screening scan) and the HapMap database (fine-scale recombination map, haplotype for JPT/CHP).

The targeted region for imputation was limited to within known recombination hot spots, because our data targeted only the HAP_{ICE} and exon regions.

After imputation, we applied a Bayesian test with an additive model to assess the association using SNPTEST software (Marchini et al., 2007). Default values were used in all settings needed in IMPUTE and SNPTEST (e.g. effective population size for JPT/CHP, buffer, call threshold for calling genotyped SNPs and number of samples of genotypes that should be used for Bayesian tests).

Table 3
Individual haplotype analyses from significant Global *P*-values in first-set samples

	haplotypes	Case Freq (%)	Con Freq (%)	*P*-value	Global *P*-value
C#5–	1–1–1	9.36	11.8	.0104	.0244
6–7	1–1–2	15.6	13.6	.0896	
	1–2–2	65.8	65.5	.886	
	2–1–1	7.21	6.27	.300	

2.7. Power calculation

Power calculation was performed with a web-based statistical program, Genetic Power Calculator (Purcell et al., 2003). Power was estimated under a multiplicative model of inheritance, assuming the disease prevalence to be 1% and the population susceptibility allele frequencies to be the values observed in control samples.

3. Results

3.1. Mutation scan and first-set association analysis

We detected seven SNPs through dHPLC analysis of the exon region (MS1–7: Table 2). One of them, MS3 (rs3924999), is a non-synonymous SNP (Gly38Arg) and had shown a significant association in the Chinese population (Yang et al., 2003). The other SNPs were located in an untranslated region (UTR) or branch site, and may therefore have a functional effect (Table 2).

Next, 49 SNPs and 7 haplotypes were selected as Tagging SNPs from the HapMap database. These SNPs are located in the HAP_{ICE}- and coding regions based on the HapMap database (Tables 1 and 2).

Consequently, by involving 11 SNPs (the 7 SNPs we detected and 4 SNPs reported in other papers (Stefansson et al., 2002; Walss-Bass et al., 2006)), a total of 60 SNPs were genotyped in the first-set screening samples (however, since we were unable to design a genotyping method for one SNP that we detected (MS6) by TaqMan Assay by Design (Applied Biosystems), we determined the genotype distribution of some samples (192 cases and 192 controls) using a direct sequencing method. With these samples we confirmed that MS6 could be represented by the haplotypes constructed by C#24–30 in LD evaluation.).

The SNP for which significance was shown in the report of Walss-Bass et al. (2006) was not polymorphic in our samples.

Allele- and genotype-wise analyses did not show association either the HAP_{ICE} region or the exon region. In this haplotype-wise analysis, 3-marker haplotypes of C#5–6–7 were associated with schizophrenia (Global P-value=0.0244, uncorrected: Tables 1, 2 and 3, Supplementary Tables 3 and 4). The genotyping of C#5, C#6, C#7 in a subset of the screening samples was re-confirmed by direct sequencing, and the results were perfectly identical to those shown by TaqMan assay. Hence, we speculate that it was unlikely that genotyping error had occurred.

3.2. Imputation of ungenotyped SNP for first-set samples

Data for ungenotyped SNPs could not provide sufficient evidence for association in either region (Fig. 1). In particular, the weights of evidence for the regions near the significant haplotypes in first-set samples were less than one. Since weights of evidence of at least four are required for evidence for association

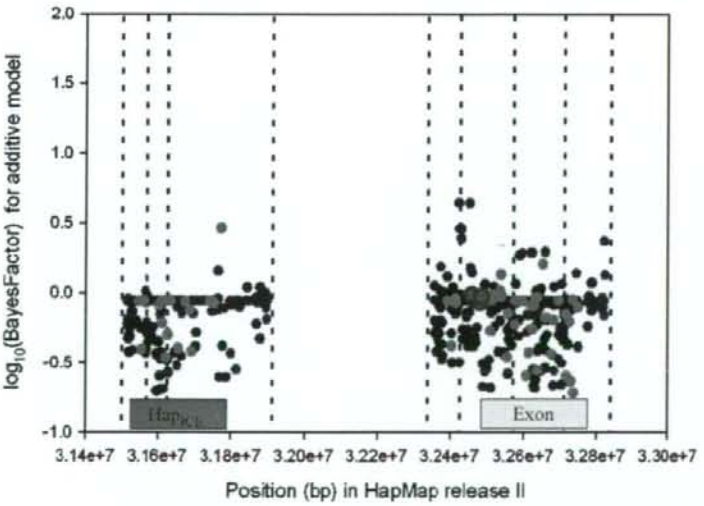

Fig. 1. Results of imputing SNP in the *NRG1* gene. The weights of evidence were calculated using imputed genotypes (red circles) and observed genotypes (black circles). Data from SNPs that constructed the significant haplotype in the first-set samples are shown in blue circles. Dotted lines indicate the estimated hot spots from the HapMap database. The SNP position from the HapMap release II database is plotted on the X axis.

Table 4
Confirmation analysis of significant haplotypes from first-set analysis

Samples	SNPID	1-window	2-windows	3-windows
Case–control	C#5	.408		
	C#6	.362	.101	.120
	C#7	.371	.601	
Family samples	C#5	.107		
	C#6	.964	.323	.505
	C#7	.499	.846	
Combined samples	C#5	.976		
	C#6	.389	.591	.478
	C#7	.801	.303	

(if 1000 SNPs of 10,000,000 common human SNPs might be associated with a disease, we may assign a prior odds of association of 1/10,000. Therefore, a Bayes factor more than 10,000 (or $\log_{10}$ [Bayes factor] more than 4) is required (Balding, 2006)). Thus, these results indicate a low probability for association in our sample.

3.3. *Confirmation analysis of the positive haplotypes using different case–control samples and family samples*

To confirm the significance of exon region C#5–6–7 in the first-set samples, we conducted a confirmation analysis using independent case–control samples and family samples. In these analyses, we could not replicate this association. To increase the power, we combined samples (first-set and confirmation samples) but again we could not detect an association in this explorative analysis (Table 4).

4. Discussion

In the present study, using three large and independent samples, our data did not provide sufficient evidence for associations between tagging SNPs in the HAP_{ICE} and exon regions of *NRG1* and schizophrenia in the Japanese population.

We could not replicate previous reports for the HAP_{ICE} region (Stefansson et al., 2002; Stefansson et al., 2003); however, the results of this study are in concordance with our previous replication study in the Japanese population (schizophrenia=607, controls=515) (Iwata et al., 2004). Another study (Fukui et al., 2006), however, examined independent Japanese samples (belonging to one-third of confirmation case–control samples) and reported a positive association. Specifically, that study reported a significant association of haplotypes constructed by three core SNPs from Stefansson et al. (SNP8NRG221533 (HAP_{ICE}#3), SNP8NRG241930 (HAP_{ICE}#7) and SNP8NRG243177 (HAP_{ICE}#8)), and one more intronic SNP (rs1081062), as well as a trend for association of rs1081062. Since our tagging SNPs could not involve this SNP (rs1081062), we found by consulting the latest HapMap database (release#21a) that rs1081062 is tagged by rs13274954 (HAP_{ICE}#11); moreover, neither HAP_{ICE}#10 nor its haplotypes (HAP_{ICE}#3–7–8–11) were associated with schizophrenia (Global *P*-value= 0.540). Therefore, the aforementioned positive report could have been the result of type I error due to inadequate sample size (schizophrenia=349, controls=424) (Fukui et al., 2006). Or, as the authors speculated (Fukui et al., 2006), the different clinical backgrounds (e.g. genetic loading) in each sample could have led to inconsistent results. In this regard, a recent study reported that *DAOA/G30*, which is also a strong candidate gene for schizophrenia, influences susceptibility to the symptomatology of psychiatric disorders including schizophrenia and bipolar disorder, but not to diagnosis itself (Williams et al., 2006).

In the coding region, our results indicated the importance of controlling inflation of the type I error rate due to multiple testing, when a significant association is obtained in an analysis that involves several markers. In this study we found significant associations only from haplotype-wise analysis, not from allele- or genotype-wise analysis. It is generally accepted that a haplotype-wise analysis gives high power. At the same time, haplotype-wise analysis, especially multi-marker analysis or sliding-window analysis, tends to increase the chance of false positive results, since numerous hypotheses are examined. Bonferroni correction is typically used for solving multiple testing problems; however, since markers are not independent due to the existence of LD, Bonferroni correction is thought to be too conservative.

Therefore, we adopted two methods to validate the observed association; firstly, we imputed ungenotyped SNPs that might reflect a significant haplotype based on observations including our genotypic distribution of tagging SNPs and LD structure from the HapMap database. However, our simulation suggests that results for ungenotyped SNPs do not provide sufficient evidence for association. In other words, there was no SNP which could reflect a significant haplotype in the current data in HapMap release II. Secondly, we examined independent sets of samples for which a significant association was obtained in the initial screening analysis. We considered this to be the best strategy at present; however, the former significance of the exon region haplotype could not be replicated though independent case–control and family trios samples.

It is unlikely that negative results are due to type II error since a large sample size was used in this study; moreover, power analyses showed that the power was more than 80% when genotype relative risk (GRR) was set at 1.2–1.65 and 1.6–3.1 for confirmation case–

control samples and family samples, respectively (MAF=2.4% and 47%), under a multiplicative model of inheritance in first-set screening samples.

Regarding interpretation of the results from this study, several limitations should be mentioned: Firstly, we did not screen the entire region of *NRG1*. On that point, Corvin et al. showed an independent 'at-risk' haplotype close to an EST cluster of unknown function (*Hs*.97362) within intron 1 of *NRG1* (Corvin et al., 2004). Secondly, our samples were not assessed with the use of the standard structured interview, and therefore have the possibility of false negatives due to misdiagnosis or sampling bias. Detailed association analyses with dense markers in the entire region of *NRG1* in well-phenotyped samples, including symptomatology, are essential in future study.

In conclusion, these results indicate that the positionally and functionally attractive regions of *NRG1* are unlikely to contribute to susceptibility to schizophrenia in the Japanese population. Moreover the nature of our results support that two-stage analysis with large sample size is appropriate to examine the susceptibility genes for common diseases; independent samples for examination of significance found in screening results should be an integral part of experimental design in genetic association analysis. Imputation methods should also be used when only haplotype association shows significance, in order to check for possible causal SNPs that can reflect the haplotype.

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Contributors

MI and NT designed the study, wrote the protocol and drafted the manuscript. MI, NT, SS, BA, YW, AN, YY, TK, YK, TK, and KK performed laboratory assays and the data-analysis. RH, HU, TI, TS, and MT advised on data-analysis. NO and NI participated in the design of the study, interpretation of the data, and drafting of the manuscript. All authors contributed to and have approved the final manuscript.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.schres.2008.01.010.

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